Pertanika Journal of

TROPICAL AGRICULTURAL SCIENCE

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PERTANIKA JOURNALS

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Journal of Tropical Agricultural Science
Journal of Tropical Agricultural Science

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About the Journal

Overview

Pertanika Journal of Tropical Agricultural Science (JTAS) is the official journal of Universiti Putra Malaysia published by UPM Press. It is an open-access online scientific journal which is free of charge. It publishes the scientific outputs. It neither accepts nor commissions third party content.

Recognized internationally as the leading peer-reviewed interdisciplinary journal devoted to the publication of original papers, it serves as a forum for practical approaches to improving quality in issues pertaining to tropical agriculture and its related fields.

JTAS is a **quarterly** (*February, May, August and November*) periodical that considers for publication original articles as per its scope. The journal publishes in **English** and it is open to authors around the world regardless of the nationality.

The Journal is available world-wide.

Aims and scope

Pertanika Journal of Tropical Agricultural Science aims to provide a forum for high quality research related to tropical agricultural research. Areas relevant to the scope of the journal include: agricultural biotechnology, biochemistry, biology, ecology, fisheries, forestry, food sciences, genetics, microbiology, pathology and management, physiology, plant and animal sciences, production of plants and animals of economic importance, and veterinary medicine.

History

Pertanika was founded in 1978. A decision was made in 1992 to streamline Pertanika into three journals as Journal of Tropical Agricultural Science, Journal of Science & Technology, and Journal of Social Sciences & Humanities to meet the need for specialised journals in areas of study aligned with the interdisciplinary strengths of the university.

After 40 years, as an interdisciplinary journal of Agriculture, the revamped Journal, a leading agricultural journal in Malaysia now focuses on tropical agricultural research and its related fields.

Goal of *Pertanika*

Our goal is to bring the highest quality research to the widest possible audience.

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Pertanika is **40 years old**; this accumulated knowledge has resulted in Pertanika JSSH being abstracted and indexed in **SCOPUS** (Elsevier), **Web of Science™ Core Collection** Emerging Sources Citation Index (ESCI). Web of Knowledge [BIOSIS Previews], **EBSCO** and EBSCOhost, **Google Scholar**, **TIB**, **MyCite**, **ISC**, **NAL, Cabell's Directories** & Journal Guide.

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The abbreviation for Pertanika Journal of Tropical Agricultural Science is *Pertanika J. Trop. Agric. Sci.*

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International Standard Serial Number (ISSN)

An ISSN is an 8-digit code used to identify periodicals such as journals of all kinds and on all media–print and electronic. All Pertanika journals have ISSN as well as an e-ISSN.

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Authors are not permitted to add or remove any names from the authorship provided at the time of initial submission without the consent of the Journal's Chief Executive Editor.

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Most scientific papers are prepared according to a format called IMRAD. The term represents the first letters of the words **I**ntroduction, **M**aterials and Methods, **R**esults, **A**nd, **D**iscussion. IMRAD is simply a more 'defined' version of the "IBC" [Introduction, Body, Conclusion] format used for all academic writing. IMRAD indicates a pattern or format rather than a complete list of headings or components of research papers; the missing parts of a paper are: *Title, Authors, Keywords, Abstract, Conclusions*, and *References*. Additionally, some papers include Acknowledgments and Appendices.

The *Introduction* explains the scope and objective of the study in the light of current knowledge on the subject; the *Materials and Methods* describes how the study was conducted; the *Results* section reports what was found in the study; and the *Discussion* section explains meaning and significance of the results and provides suggestions for future directions of research. The manuscript must be prepared according to the Journal's **Instructions to Authors**.

Editorial process

Authors are notified with an acknowledgement containing a *Manuscript ID* on receipt of a manuscript, and upon the editorial decision regarding publication.

Pertanika follows a **double-blind peer-review** process. Manuscripts deemed suitable for publication are usually sent to reviewers. Authors are encouraged to suggest names of at least three potential reviewers at the time of submission of their manuscript to Pertanika, but the editors will make the final choice. The editors are not, however, bound by these suggestions.

Notification of the editorial decision is usually provided within ten to fourteen weeks from the receipt of manuscript. Publication of solicited manuscripts is not guaranteed. In most cases, manuscripts are accepted conditionally, pending an author's revision of the material.

As articles are double-blind reviewed, material that might identify authorship of the paper should be placed only on page 2 as described in the first-4 page format in Pertanika's **Instructions to Authors** given at the back of this journal.

The Journal's peer-review

In the peer-review process, three referees independently evaluate the scientific quality of the submitted manuscripts.

Peer reviewers are experts chosen by journal editors to provide written assessment of the **strengths** and **weaknesses** of written research, with the aim of improving the reporting of research and identifying the most appropriate and highest quality material for the journal.

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What happens to a manuscript once it is submitted to *Pertanika*? Typically, there are seven steps to the editorial review process:

1. The Journal's chief executive editor and the editorial board examine the paper to determine whether it is appropriate for the journal and should be reviewed. If not appropriate, the manuscript is rejected outright and the author is informed.

2. The chief executive editor sends the article-identifying information having been removed, to three reviewers. Typically, one of these is from the Journal's editorial board. Others are specialists in the subject matter represented by the article. The chief executive editor asks them to complete the review in three weeks.

   Comments to authors are about the appropriateness and adequacy of the theoretical or conceptual framework, literature review, method, results and discussion, and conclusions. Reviewers often include suggestions for strengthening of the manuscript. Comments to the editor are in the nature of the significance of the work and its potential contribution to the literature.

3. The chief executive editor, in consultation with the editor-in-chief, examines the reviews and decides whether to reject the manuscript, invite the author(s) to revise and resubmit the manuscript, or seek additional reviews. Final acceptance or rejection rests with the Edito-in-Chief, who reserves the right to refuse any material for publication. In rare instances, the manuscript is accepted with almost no revision. Almost without exception, reviewers' comments (to the author) are forwarded to the author. If a revision is indicated, the editor provides guidelines for attending to the reviewers' suggestions and perhaps additional advice about revising the manuscript.

4. The authors decide whether and how to address the reviewers' comments and criticisms and the editor's concerns. The authors return a revised version of the paper to the chief executive editor along with specific information describing how they have answered' the concerns of the reviewers and the editor, usually in a tabular form. The author(s) may also submit a rebuttal if there is a need especially when the author disagrees with certain comments provided by reviewer(s).

5. The chief executive editor sends the revised paper out for re-review. Typically, at least one of the original reviewers will be asked to examine the article.

6. When the reviewers have completed their work, the chief executive editor in consultation with the editorial board and the editor-in-chief examine their comments and decide whether the paper is ready to be published, needs another round of revisions, or should be rejected.

7. If the decision is to accept, an acceptance letter is sent to all the author(s), the paper is sent to the Press. The article should appear in print in approximately three months.

   The Publisher ensures that the paper adheres to the correct style (in-text citations, the reference list, and tables are typical areas of concern, clarity, and grammar). The authors are asked to respond to any minor queries by the Publisher. Following these corrections, page proofs are mailed to the corresponding authors for their final approval. At this point, **only essential changes are accepted**. Finally, the article appears in the pages of the Journal and is posted on-line.

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ABSTRACTING AND INDEXING OF PERTANIKA JOURNALS

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Contents

Foreword

Welcome to the Second Issue of 2019 for the Journal of Tropical Agricultural Science (JTAS)!

JTAS is an open-access journal for studies in Tropical Agricultural Science published by Universiti Putra Malaysia Press. It is independently owned and managed by the university for the benefit of the world-wide science community.

This issue contains 32 articles; 3 are review articles, 5 are short communications and the rest are regular articles. The authors of these articles come from different countries namely Algeria, Egypt, India, Indonesia, Japan, Malaysia, Nepal, Nigeria and Phillippines.

Articles submitted in this issue cover various scopes of Tropical Agricultural Science crop and pasture production, food and nutrition development, veterinary sciences, genetics and molecular biology, biotechnology, microbiology, soil and water sciences, animal production, botany, ecology, fisheries sciences, plant physiology and zoology.

Selected from the scope of crop and pasture production is a regular article entitled "Phosphorus Nutrition Provoked Improvement on the Growth and Yield of 'MD-2' Pineapple" by *Vences Cuyno Valleser* from College of Agriculture, Central Mindanao University, Philippines. The study focussed on response of fruit crops to phosphorus application. Hence, this study was conceptualized to evaluate the growth, yield and fruit quality of 'MD-2' pineapple in response to varying rates of P under Adtuyon clay soil in Bukidnon, Philippines. The experiment was laid out in a randomized complete block design (RCBD) with five amounts (0, 84, 127, 169 and 211 kg ha^{-1}, respectively) of P as treatments with three replications. Results revealed that 'MD-2' pineapple growth was significantly influenced by P application. Although higher dose showed negative influence, it was undeniable that higher doses of P (≥169 kg ha^{-1}) improved the fruit mass (≥1.42 kg) as well as yield (≥96.92 tons ha^{-1}) of 'MD-2' pineapple. In general, 169 kg P ha-1 was found as the optimal amount of P for 'MD-2' pineapple production in Adtuyon clay soil. The study suggested that these results could be used as guidance by commercial pineapple growers on optimum phosphorus fertilizer application. Details of the study is available on page 467.

Selected from the scope of biotechnology is an article entitled "Bioconversion of Solid Waste into Nutritional Rich Product for Plants by using *Eudrilus eugeniae*" by *Arun Karnwal* and *Ravi Kumar*, fellow researchers from Lovely Professional University and Bhojia Institute of Life Sciences, India. The study discussed on the use of fusion of eco-friendly efficient techniques for solid waste disposal. The tested the efficacy of Eudrilus eugeniae on food, medical and paper waste decomposition. They found out that vermicompost of food waste (VFW) resulted with organic carbon 21.67%, 1.98% nitrogen content, and phosphate 0.59 mg/ml. Vermicompost of medical waste (VMW) analysis resulted with organic carbon 15.3%, 1.17% nitrogen, and 0.54 mg/

ml phosphate. Whereas physico-chemical results of vermicompost of paper waste (VPW) showed 18.67% organic carbon, 1.39% nitrogen, and 0.79 mg/ml phosphate. When tested for nutritional values, all three were better than the normal soil. Hence they suggested that the decomposition of waste materials by earthworms was the preeminent concept of nutrient renewal from green waste. Details for the study is available on page 681.

Selected from the scope of genetics and molecular biology is a regular article entitled "Partial Purification and Model Structure of BPSL2774, a Hypothetical Protein from *Burkholderia pseudomallei* Predicted to be a Glycosyltransferase" by Siti Marhamah Drahaman, Hanisah Ujang, Nor Azurah Mat Akhir, Noraslinda Muhamad Bunnori and Aisyah Mohamed Rehan, fellow researchers from International Islamic University Malaysia and Malaysia Genome Institute, Malaysia. The study discussed on the identification of essential genes and drug targets in antimicrobial therapy of a disease named Melioidosis. The researchers selected a hypothetical genes predicted to be essential for B. pseudomallei by transposon-directed insertion site sequencing (TraDIS) technique. One target gene (*BPSL2774*) was successfully amplified and cloned from genomic DNA of *B. pseudomallei* and the target protein (BPSL2774 protein) was successfully expressed in soluble form. BPSL2774 protein have considerable homology to glycosyltransferase GTB type superfamily and RfaB superfamily. The study recommended functional annotation of BPSL2774 protein as a glycosyltransferase, though future validation from biochemical experiments are warranted. Details of the study is available on page 609.

We anticipate that you will find the evidence presented in this issue to be intriguing, thought-provoking and useful in reaching new milestones in your own research. Please recommend the journal to your colleagues and students to make this endeavour meaningful.

All the papers published in this edition underwent Pertanika's stringent peer-review process involving a minimum of two reviewers comprising internal as well as external referees. This was to ensure that the quality of the papers justified the high ranking of the journal, which is renowned as a heavily-cited journal not only by authors and researchers in Malaysia but by those in other countries around the world as well.

We would also like to express our gratitude to all the contributors, namely the authors, reviewers, Editor-in-Chief and Editorial Board Members of JTAS, who have made this issue possible.

JTAS is currently accepting manuscripts for upcoming issues based on original qualitative or quantitative research that opens new areas of inquiry and investigation.

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Review Article

Gynura procumbens: Agronomic Practices and Future Prospects in Malaysia

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ABSTRACT

Good agronomic practice is a key to a successful crop production aiming at high yield and quality in small or large scale planting. *Gynura procumbens* (Lour.) Merr. (Family Asteraceae) is a herbal plant rich in phytochemical compounds of wellbeing benefits to the consumers. Suitable and complete technology on agronomic practices such as cultivation technique, fertilization application, water requirement, weed control and pest and disease management of *G. procumbens* or locally known as Sambung Nyawa for the Malaysian environment can ensure quality product and high yield. This may attract local producers and smallholders to cultivate this medicinal plant commercially. Complete technology package documented for commercial planting of *G. procumbens* may encourage its planting which would ensure the sustainable supply of *G. procumbens* raw materials for the pharmaceutical and health industries. Suitable agronomic practices adopted contribute to sustainable commercial production of *G. procumbens*. It endeavours to support the country's Entry Point Project under The Agriculture National Key Economic Area (NKEA) to become a potential hub for the manufacturing of high-value *G. procumbens* based herbal products as well as other herbal species. Therefore, it is crucial to develop and to adopt the necessary agronomic practices for the commercial cultivation for this medicinal herb to support the demand for the industry while directly increase farmers' farm profit.

Keywords: Agronomic practices, bioactive compound, cultivation, *Gynura procumbens*, medicinal plant

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INTRODUCTION

Benefits of medicinal plants or herbs are acknowledged by the people nowadays due to the belief that natural remedies are reliable and more effective compared to conventional drugs (Alsarhan et al., 2014). For hundreds of years, nature has been the resource of medicine to treat ailments such as gout, high blood pressure, diarrhoea and skin infection (Shafaei et al., 2014), with large number of herbal preparations have been concocted from natural sources and used in traditional medicine. In Malaysia, more than 2000 species of medicinal plants were recorded which some have been used in herbal products manufacturing such as those with important components of hypoglycemic agents for the treatment of diabetes (Bukhori et al., 2015).

In fact, Malaysia has been acknowledged as one of the 12 mega- diverse countries by the United Nations Environment Programme (UNEP) as to harbour the majority of the earth's species (Nasir et al., 2015). In addition, since the Malaysian government had launched the Economic Transformation Programme (ETP) in 2010 through the implementation of National Key Economic Areas (NKEA) including in the agriculture sector, production of herbs and the downstream products in Malaysia has significantly increased. The importance and demand of medicinal plants in Malaysia can be discerned from the Herbs and Spices Statistics 2016 reported by Department of Agriculture Malaysia. In 2013, a total of 1,298 hectares of planted area were utilized to produce 8,428 metric tonnes of herbal raw materials. Herbs production in 2014 increased greatly by 37% with 13,567 metric tonnes production using 2,176 hectares of planted area. Looking at this progressive demand for herbs or medicinal plants, *G. procumbens* has been identified one of 18 selected herbs under the Agriculture NKEA Initiative for Herbal Subsector in 2010 (Ministry of Agriculture and Agro-Based Industry [MOA], 2018).

Origin and Distribution

Gynura procumbens is one of the most common medicinal plants belonging to the Asteraceae family which is widely distributed in Africa (Sukadeetad et al., 2018) and tropical regions of South East Asia including Malaysia, Indonesia, Thailand, Vietnam, Philippines, Myanmar and China (Mou & Dash, 2016; Nasir et al., 2015; Tan et al., 2016). The genus *Gynura* comprises 44 species and is widely distributed from tropical Africa to South East Asia through southern China, Japan, Southeast Asia and New Guinea into northern Australia. Ten species were enumerated in Thailand (Vanijajiva, 2009). In Malaysia this species has its distribution limited to the western part of Peninsular Malaysia (Keng et al., 2009).

Gynura procumbens is commonly known as Sambung Nyawa in Malay which means "prolongation of life", whereas in Chinese, it is named as "bai bing cao" which bears the meaning of "100 ailments" (Rohin et al., 2018). Other vernacular names for *G. procumbens* according to Global Information Hub on Integrated Medicine

(Global Information Hub on Integrated Medicine [Globinmed], n.d.-a, "*Gynura procumbens* (Lour.) Merr.", para. 3) are listed in Table 1.

Table 1
Vernacular names for G. procumbens

Country	Vernacular names
Malaysia	Daun dewa, dewa raja, akar sebiak, kelemai merah, kacham akar
Indonesia	Sambung Nyawa, daun dewa, kalingsir (Sundanese)
Cambodia	Chi angkam
Thailand	Pra-kham dee khwaai, ma kham dee khwaai (Pattani), mu maeng sang (Chumphon)

Morphological Characteristics and Environmental Requirement

Gynura procumbens is a shrub plant that can grow about 1-3 m in height and can easily be propagated from stem cuttings. The plant grows vertically, or sometimes the edge collapses and forms roots. The stem is purplish in colour and has fleshy characteristics. The stem is also angular and has many branches with length of node shortens from base to shoot. The leaves are ovate-elliptic or lanceolate shape that alternately arranged (Rahman & Asad, 2013). The upper leaf surface is yellowish green colour when matures and light green colour on the lower leaf surface. The size of petiole is between 0.5-1.5 cm long, leaf width between 1-5.5 cm and leaf blades measure up to 12.5 cm. In Malaysia, it is very rare for *G. procumbens* to produce flowers, but according to Wiart (2002), *G. procumbens* has purple tubular bisexual flowers. This plant can grow well in shady areas with 25-50% rate of light intensity. The suitable soil pH is around 5.5-7.0, whereas for the air temperature is between 20-30°C and preferably of medium humidity with annual rainfall between 1500-2500 mm.

Main Uses

Gynura procumbens is an annual evergreen shrub with the fresh leaves usually used as vegetables in cooking. In Malaysia, fresh leaves of *G. procumbens* are usually used as salad or *ulam* (Hew & Gam, 2011) and are eaten raw. Previous studies have shown that leaf contents did not have any toxic effect (Rohin et al., 2018). Apart from being consumed in the diet, *G. procumbens* also traditionally has been used to treat various ailments such as rash, constipation, hypertension, migraines, diabetes mellitus, urinary infection, cancer and as anti-inflammatory and anti-allergic agents (Perry, 1980). In Malaysia, *G. procumbens* is used by the traditional practitioners to control the blood glucose levels of diabetic patients in the form of decoction (Akowuah et al., 2002). They found that the benefits of consuming *G. procumbens* were related to the amount of bioactive compounds present, such as saponins, flavonoids and terpenoids. Whilst in Indonesia and Vietnam, it is used to relieve kidney discomfort and for the treatment of fever, respectively (Tan et al., 2016).

PRODUCTION AND CULTIVATION

Preparation of Cuttings

Propagation of *G. procumbens* is done through stem cuttings as planting materials. The middle part of cuttings with a total of 5 nodes are selected from 2 months old mother plants. The shoot part is not suitable to be used for propagation since it is still young, too sensitive to wet condition and can lead to infection of stem rot disease. Rooting hormone (Seradix 1) can be used to encourage root growth. Cuttings can be propagated in plastic cups containing sand or peat moss, rather than using trays to minimize the destruction of the root system during the transplanting process. During the propagation stage, suitable places to place the cuttings can be one of the reasons for better growth. Normally, a glass house or other shaded area is suitable. During the propagation stage also, *G. procumbens* cuttings are quite sensitive to wet condition since it can lead to stem rot problems. The cuttings need to be monitored and watering daily if using sand as the medium or every two days if peat moss is used. This is because these two media have their own characteristics in terms of absorption and water retention capacity. Sand medium is more porous that allows water to drain easily whereas peatmoss remains moist and retains considerable amount of water after being watered. The stem cuttings after reaching 6 to 8 leaves with 4 to 6 weeks old are ready to be transferred to the field or shade house. The growth process of cuttings until 6 weeks of propagation is shown in Figure 1.

Field Preparation

To achieve maximum crop growth in the field, good land preparation is crucial. The land is cleared before ploughing to remove shrubs and weeds. About 1-2 weeks before the transplanting process, the planting area is ploughed and rotovated to prepare for planting. Planting beds with size 1 m

(a) (b) (c)

Figure 1. (a) One week old of stem cuttings propagated in the peatmoss medium; (b) Rooted cuttings after three weeks of propagation; (c) *Gynura procumbens* plants at six weeks after propagation and ready to be transplanted

wide, 3 m long and 30 cm height raised from the ground are prepared according to the number of rooted cuttings to be transplanted. According to the Globinmed (n.d.-b, "Sambung Nyawa", para. 5) the recommended bed size is about 120 cm wide with a distance of about 30 cm between the beds.

(a)

(b)

(c)

Figure 2. Suitable planting methods for *G. procumbens* including on (a) planting beds; (b) pots; and (c) polybags

Planting

For successful planting of the *G. procumbens*, cuttings with 6 weeks old are most suitable to be transferred to the field. There are several methods of planting that can be applied such as planting on the bed, pot or polybag (Figure 2). Holes with 10 cm depth are prepared on planting beds for the planting process. Rooted cuttings are carefully removed from the plastic cups to avoid any disturbance of the root system. A planting distance of 20 to 35 cm between plants and 20 cm within rows equivalent to the density of 15 to 25 plants per m^2 is suitable for production of high yielding *G. procumbens*. Planting that is done using polybag can be arranged with the spacing 1 m x 1 m (Globinmed, n.d.-b, "Sambung Nyawa", para. 6).

Fertilizer Application

Gynura procumbens can be fertilized using organic and inorganic fertilizers for optimum crop growth. Chicken manure is one of the most suitable organic fertilizers that can be applied to *G. procumbens*. Chicken manure as a source of organic fertilizer is recommended to be applied with a rate of 300 kg N/ha. Half of the total fertilizer is applied 1-2 weeks before transplanting and the balance of the fertilizer applied after 2 weeks transplanting.

Irrigation

Gynura procumbens are water loving plants and optimum water content in soil is required for maximum growth (Globinmed,

n.d.-b, "Sambung Nyawa", para. 6). A drip irrigation and sprinkler systems are suitable to be used. Plants need to be watered at least twice a day, in the morning and afternoon, and no irrigation is needed during a rainy season.

Weed Control

Weeding process of the planting area is normally done manually every 1-2 weeks after planting. Plastic mulching using silver shine to cover the surface of the planting bed can also be used for weed control (Figure 3). The area between planting beds can also be covered using silver shine to control weed growth.

Figure 3. The use of plastic mulching of silver shine to cover the planting bed surface to control the growth of weeds in the planting area

COMPOSITION OF BIOACTIVE COMPOUNDS IN *Gynura procumbens*

Extracts from plant have been used as traditional remedies for certain diseases due to the presence of biologically active compounds that have medicinal properties which can contribute to the prospects for new drug leads. For example, *G. procumbens* is culturally acceptable to many medicinal purposes because of its efficacy in the traditional management of diabetes mellitus. Beneficial effects of *G. procumbens* were described for pharmacological properties such as anti-inflammatory, anticancer, antidiabetic, anti-herpes simplex virus activity, anti-ulcerogenic activity, vasorelaxant activity and antiplasmodial activity (Mou & Dash, 2016; Tan et al., 2016).

A significant work was carried out to identify and isolate the chemical constituents from different extracts of *G. procumbens*. Numerous studies have demonstrated that various extracts of *G. procumbens* contained several bioactive chemical constituents such as flavonoids, saponins, tannins, terpenoids and sterol glycosides (Akowuah et al., 2002; Kaewseejan et al., 2012; Zahra et al., 2011). Previous studies also reported that *G. procumbens* leaves extracts contained rutin, kaempferol and two potential antioxidant components which are kaempferol-3-O-rutinoside and astragalin (Rosidah et al., 2008). A comprehensive work done by Kaewseejan and Siriamornpun (2015) showed that the individual bioactive compounds in *G. procumbens*, were different, especially gallic, p-coumaric and ferulic acids for phenolic acids, and myricetin, quercetin and kaempferol for flavonoids (Figure 4).

Among phenolic compounds, flavonoids are widely distributed in the plant kingdom and they were reported to exhibit strong antioxidant activity (Galati & O' Brien, 2004; Havsteen, 2002; Lila, 2004). The antioxidative characteristics of phenolic compounds were related to the number of hydroxyl group position (Arora et al.,

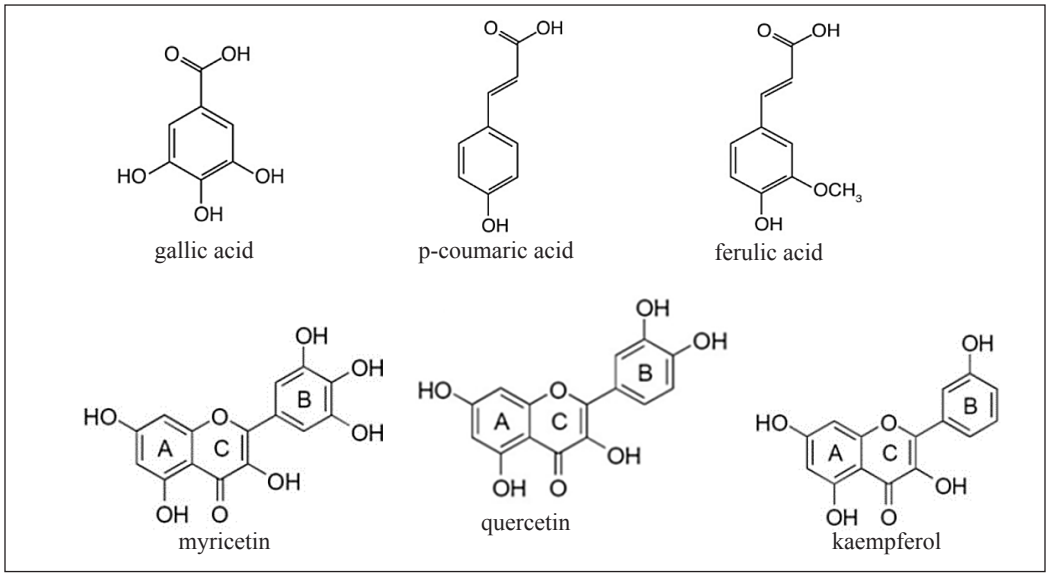

Figure 4. Structures of some phenolic acids and flavonoids found in the leaves of *G. procumbens*

1998). Due to reducing activities of phenolic hydroxyl groups, flavonoids are able to donate more hydrogen. On the other hand, through delocalization of phenoxy radical products, flavonoids can protect against various disease damages from reactive oxygen species (ROS) (Verma et al., 2012).

PESTS AND DISEASES

Plant protection is the practice of managing weather, weeds, pests and diseases that damage or inhibit the growth of fruits, vegetables and other horticultural crops. The practice includes either reducing the plant density that causes plants to suffer stunted growth and their death or causes lower production capacity by reducing the yield or quality of agricultural produce. Proper crop protection is important to produce higher quality crops with minimal wastage (Strickland, 1969). In general, the plants are known to be attacked by a number of pests which include mites, aphids, nematodes, rodents, birds, slugs and snails whereas plant pathogens include fungi, bacteria and viruses. Weeds have also affected agricultural production by competing for nutrients with the crops (Bohmfalk et al., 2011; Flint, 1998; James et al., 2010). Apart from the above pests, humans also suffer crop losses from other abiotic causes such as lack or excess of water during the crop's growth season, extreme temperatures (high or low) as well as improper nutrient supply. Biotic stresses have the ability to reduce production substantially in various ways which can either be qualitative and/or quantitative (Nagarajan & Nagarajan, 2009).

Gynura procumbens is relatively pest-free plant, however few instances of whiteflies and mites attacks during the hot seasons were recorded. Similarly, leaf miners, mealy bugs, aphids, caterpillars, snails and slugs were also partially reported.

However, *G. procumbens* seemingly do not appear to be susceptible to any pests or diseases in the tropical climate. Apart from insect pests, plant diseases and viruses have also been found to associate with this herbal plant.

Insect Pests

Small insects such as whiteflies and aphids are found on new stems on the underside of the leaves. They suck the fluids from the plant and leaving a honey dew substance behind. The leaves turn pale yellow and then black due to the promotion of sooty mold on leaves that further disturb the process of photosynthesis. Similarly, leaf miners create small mines inside leaf epidermis and green leaves become stunted in growth and yellow in color and drop on the soil. Small whitish maggots feed between the leaf surfaces. Damage appears as winding trails in leaf tissue. As mines enlarge, they may merge and from large, light-colored blotched areas. Feeding lasts 1 to 3 weeks. They may pupate in the leaf or in the soil at 0.6 cm depth and flies after emergence in 2 to 4 weeks (Murakami et al., 2000).

Moreover, various species belong to order Lepidoptera and green grasshopper (*Acrida turrita*) voraciously feed on leaves of *Gynura* and many of them are among the most serious of agricultural pests. In fact, many moth species are best known in their caterpillar stage because of the damage they cause to fruits and other agricultural produce, whereas the moths are obscure and do no direct harm. This insect pest causes much damage, mainly by eating the leaves (Figure 5). The propensity for damage is enhanced by mono- cultural farming practices, especially where the caterpillar is specifically adapted to the host plant under cultivation. Therefore, caterpillars are so critical of *G. procumbens* because the consumed part is the leaf and result in the decreased production of leaves and photosynthesis process.

(a)

(b)

Figure 5. (a) Caterpillars; (b) Green grasshopper (*Acrida turrita*)

Diseases

There are various classes of phytopathogenic bacteria such as *Xanthomonas* (Tudor-Nelson et al., 2003) and *Erwinia* (Echandi & Moyer, 1979), which were reported to attack on *G. procumbens* plants (Figure 6). The *Xanthomonas* species can cause bacterial spots and blights of leaves, stems and fruits on a wide variety of plant species

(Boch & Bonas, 2010) and is predominantly observed on citrus plants. Likewise, *Erwinia* spp. is also an imperative plant disease and its occurance were reported from soil and in different plants particularly potato (Burr & Schroth, 1977). These plant diseases do not only provide the quantitative loss in order to cause reduction in the photosynthetic area, but also responsible for qualitative loss in stunting the growth of plants and resulted in the depreciation of commercial value of the fruits (Bonini et al., 2007). The attack of such diseases on *Gynura* is of great concern to researchers as it requires the integrated plant disease management to supress its further expansion. Other than bacteria, virus disease e.g. broad bean wilt virus (BBWV) could also infect economically important horticultural and ornamental crops (Taylor & Stubbs, 1972). It is transmitted by aphids, mostly *Aphis gossypii* and *Myzus persicae.* In Korea, natural infections of BBWV on number of vegetables were reported and it increased gradually (Cho et al., 2007; Choi et al., 2001; Lee et al., 2000).

Wilt and root rot diseases also infect *G. procumbens* and they are caused by fungal pathogen. Soil-borne fungi are wide-spreading with more than ten different species are known to infect roots, causing wilt or root rot diseases in plants. This disease attacks during the early stage of propagation, which causes the stem is to be infected with stem rot disease (Figure 7). Symptoms of wilt are more noticeable under reduced moisture and hot conditions and are often mistakenly diagnosed. Infected plants have brown vascular tissue in the roots and stems and show wilting of the stem tips. Root rot is a disease that attacks the roots of plant growing in wet or damp soil. This decaying disease can cut the life short of just about any type of tree or plant and has symptoms like poor growth, wilted leaves, early leaf drop, branch dieback, and eventually death. Several fungal pathogens that cause wilt disease in plants are *Fusarium* spp., *Rhizoctonia* spp., *Pythium* spp. and *Phytophthora* spp.

Other species of *Gynura*, *Gynura bicolor* (Roxb. ex Willd.) DC. known as Okinawa spinach or hong-feng-cai can also be infected with blight and wilt symptoms in commercial vegetable farms in Changhua, Taiwan (Shen et al., 2011). Symptoms included light brown-to-black blight lesions developed from the top of the stems to the petioles and extended to the base of the

Figure 6. Bacterial infection on *Gynura procumbens*

(a)

(b)

(c)

Figure 7. (a) Root disease; (b) wilt disease; and (c) stem rot disease on cuttings of *G. procumbens* during propagation stage

leaves, with severely infected plants and they eventually caused the casualty to the plants. The disease incidence amounted to approximately 20% (Shen et al., 2011). *Gynura bicolor* is a perennial herbaceous plant in the family Asteraceae. It is an important Chinese vegetable and commonly used as Chinese herbal medicine. In 2010, a severe leaf spot disease was observed on *Gynura* grown in the main production areas in Tong Nan County, Chongqing City, China (Shen et al., 2011). Some farms experienced 60% disease incidence. Symptoms usually began on the lower leaves, as circular to elliptical or irregular spots with concentric rings. Individual spots were dark brown with grayish centers, sometimes coalescing and leading to extensive necrosis. The fungus associated with lesions was characterized as Conidiophores existing in single or in clusters, straight or flexuous, unbranched, percurrent, cylindrical, pale to dark brown.

Other Pests

Snails and Slugs. Few species of snail such as Giant snail (*Lissachatina fulica*) and slugs have also been found to affect the vegetative part of *Gynura* (Figure 8). They usually feed on decomposing vegetation however, when they were introduced to a new environment, they feed on a wide range of plants. All stages of plant development are eaten, leading to severe damage in those species that are most often attacked (Figure 9). However, cuttings and seedlings are the preferred source of food items, even of plants.

FUTURE PROSPECTS OF *Gynura*

Figure 8. Giant snail (*Lissachatina fulica*)

Figure 9. Plant condition attacked by Giant snail

procumbens

Gynura species are among the most important and thoroughly investigated plants with many applications in the food and pharmaceutical industries. In Malaysia, *G. procumbens*, which were recently listed under Agriculture NKEA under the Entry Point Project of Agriculture by the Malaysian Government have received particular attention in the pharmacological industry as an antidiabetic medicinal plant. Currently this herb is found growing naturally in the secondary forests. When there are demands, the indigenous people will search for *G. procumbens*, then collect and sun-dry them. After they are dried completely, the dried raw materials are sold to the buyers. In order to provide a regular and sustained supply of this plant materials, it is essential to domesticate and to develop a commercial cultivation method. A comprehensive understanding of their reproductive and growth physiology as well as ecological constraints, therefore, become necessary.

After completing the fundamental investigations on their physiological characteristics, one can embark on the commercialization of *G. procumbens*. Our local farmers should be educated with improved management practices for the production of *G. procumbens*. Therefore, there is critical need that the farmers should have adequate knowledge in the appropriate agronomic practices to maximize the yield and quality of this medicinal plant. Currently, little information is available on the cultivation of this medicinal plant. A manual of technical package of cultivation and post harvest technologies of *G. procumbens* is therefore of immediate need which may include in improving the quality of this herb.

CONCLUSION

In conclusion, based on current information, the middle part of stem from mother plants are suitable to be used as planting materials to be propagated and six weeks old rooted cutting are the most suitable to be transferred to the field for planting since it has high adaptability and stable to adapt with the new environment. While the mixture of peat moss and sand (1:3) is the most suitable growing media for propagation process. Chicken manure as a source of organic fertilizer is suggested to be applied with a rate of 300 kg N/ha. Whereas the information on the valuable phythochemical contents of *G. procumbens* shows that this herbal plant has a bright future to be planted commercially and chosen as a new source of

beneficial compounds which are very useful to be used in pharmaceutical industry.

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Review Article

Sago Palm (*Metroxylon sagu* Rottb.): Now and Beyond

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ABSTRACT

The sago palm (*Metroxylon sagu* Rottb.) is one of the underutilized food crops that has the promising potential to strengthen food security program especially in Sarawak, Malaysia. Thriving well in harsh environments such as the freshwater swampy area, the sago palm is also unique for its ability to store starch within its trunk, compared to other types of starch storage organs. With its superb high starch yield as compared to commonly found starch sources such as the corn, rice and wheat, it is deemed as the palm of many uses. Researches sprouting from this unique palm come from various fields of study, namely microbiology, food technology, polymer synthesis, bioprocess technology and most recently, computational biology. In this review, we presented a survey of recently published results from each field and further provided future recommendations and knowledge gaps to be filled. It is hoped that with the consolidation of research talents and funding from around the world, the sago palm industry will be matured in time to equip mankind with the solutions to combat the oncoming global food scarcity issues.

Keywords: Food crop, food security, industry, starch yield, underutilized

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INTRODUCTION

The sago palm (*Metroxylon sagu* Rottb.) is a true palm classified under the order Arecales, family Palmae and subfamily Calamideae. This palm can thrive well in low-land freshwater swamp and tropical rain forests and it is native to Southeast Asia including Papua New Guinea, Malaysia and

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Indonesia (Johnson, 1977). It is one-of-its-kind underutilized crop that stores starch in its trunk instead of legumes, cereals and tubers. With the capability to yield starch three- to four-fold to that of corn, rice and wheat as well as 17-fold to that of cassava (Karim et al., 2008), it has the potential to strengthen food security program (Husaini et al., 2016; Ehara, 2009).

The sago palm can grow up to the height of 10 to 15 metres with diameter of trunk reaching 35 to 75 centimetres (Kiew, 1977; Kueh, 1977). Photosynthesis are carried out throughout the seven to fifteen years of vegetation phase where nutrients generated from the leaves are channelled to the trunk for long term starch storage, filling the trunk with starch (Lim, 1991). Flowering occurs at palm's full maturity after eight to twelve years and the fruiting process follows. The palm death is foreshadowed by the event of the mature fruit falling off (Kueh et al., 1987). In Sarawak, the growth stages of sago palm are characterized into five stages: "*Plawei*" where the palm at maximum vegetative growth, "*Plawei Manit*" where the emergence of inflorescence occurs, "*Bubul*" where the development of inflorescence takes place, "Angau Muda" where flowering happens and "*Angau Tua*" where fruiting ensues (Lim, 1991) as depicted in Figure 1. While the "*Plawei*", "*Plawei Manit*" and "*Bubul*" stages show no significant differences in terms of starch yield (Lim, 1991), the "*Angau Muda*" stage is the only stage where the greatest starch yield per trunk can be found at 39% to 41% on dry weight basis (Lim, 1991; Pei-Lang et al., 2006) among all other stages, producing 216 to 219 kg of starch (Hamanishi et al., 1999).

The sago palm is a cash crop with high economic value as different parts such as frond, leaf petioles, sap, pith and even the "*hampas*" (fibrous residue) are valuable raw materials for various industries (Singhal et al., 2008). The sago starch is one of the highly utilized part of the palm in polymer, pharmaceutical, food and textile industries (Ishiaku et al., 2002; Nuttanan et al., 1995; Purwani et al., 2006; Radley, 1976).

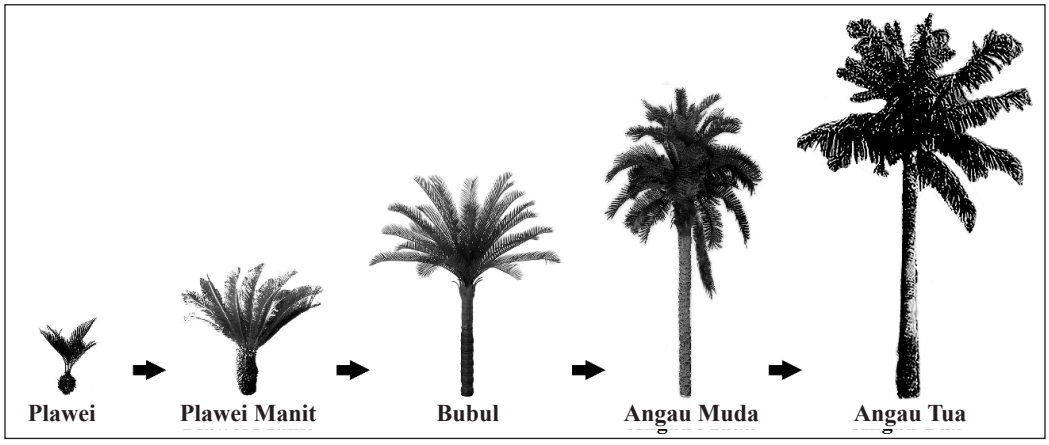

Figure 1. The five developmental stages of sago palm, namely: Plawei, Plawei Manit, Bubul, Angau Muda and Angau Tua

Besides, the papermaking and thatching are perfected with the use of sago fronds as raw materials due to its high durability (Jamaludin et al., 1995). The sago "*hampas*" is not wasted as it has been utilized widely as biosorbent, animal feed, fermentable sugar and microbial conversion agent via solid state fermentation (Awg-Adeni et al., 2010; Bujang et al., 1996; Kumaran et al., 1997; Vickineswary et al., 1994). With many more on-going researches in the fields of microbiology, genetics, bioprocess technology, population studies, food technology, sago waste management, computational biology and phenotypic variations, the undiscovered potential of the sago palm is limitless. In this review, we will highlight on the current sago palm research progress in the respective fields and further provide future directions and potentials.

Microbiology

The microbiotas exist within and surrounding the sago palm are getting into the limelight of sago-related research as the microbial activities within the palm affects the quality of the starch and the surrounding microbiota interactions affects the palm growth greatly (Toyota, 2018). The cultivation of the sago palm is generally under unfertilized conditions, the acquisition of nitrogen of this palm is greatly dependent on the nutrient-furnishing ability of soil which is driven by the decomposition of soil organic matter by animals and soil microbiota (Toyota, 2018).

The nitrogen-fixing microbiota exploration in sago palm has successfully isolated a myriad of nitrogen-fixing bacteria such as *Enterobactor cloacae*, *Klebsiella pneumoniae*, *Bacillus megaterium*, *Pantoea agglomerans* and *Klebsiella oxytoca* from various parts of the palm, namely root, trunk, bark, midrib and starch (Shrestha et al., 2006). Further investigation on the microbial interactions between these nitrogen-fixing bacteria and different indigenous bacteria that were both isolated from the same sago palm sources showed higher nitrogen-fixing activities in almost all amalgamations tested than single culture of these nitrogen-fixing bacteria (Shrestha et al., 2007). At the end of these study, they left an open window for isolation of anaerobic, uncultivated or slow-growing bacteria that are believed to have beneficial roles in nitrogen-fixing interactions (Shrestha et al., 2007).

Shipton et al. (2010) attempted to isolate nitrogen fixers particularly diazotrophic bacteria in pith and rhizosphere of the sago palm. Nitrogen fixers were found in moist starch compared to that in pith, suggesting that these bacteria might have been originated from the soil. They further suggested that fixed nitrogen was the key element in sustaining the growth of numerous toxigenic and pathogenic microbiota in sago. A study on bacterial pathogens and mycological screening of the traditionally prepared sago starch in Papua New Guinea found that three quarter of the sample was high in faecal coliforms and filamentous fungi from the genera *Scytalidium*, *Acremonium*, *Penicillium* and *Aspergillus* (Greenhill, 2006). It is believed that a more detailed study on the

haemolytic metabolites released by fungi found in sago starch can aid in garnering further understanding on the aetiology of sago haemolytic disease that has caused foodborne illness to the people of Papua New Guinea.

Genetics and Omics

There are not many molecular studies conducted on sago palm to date, and the ones being reported are in the field of genetics, transcriptomics and proteomics. In terms of genetics, a number of genes has been isolated from the genome of the sago palm, including the GA20-oxidase, ADP-glucose pyrophosphorylase, xylanase, fructose-1,6-bisphosphate, chloroplast petD and chitinase gene (Jamel et al., 2011; Roslan & Anji, 2011; Wee & Roslan, 2012b). With a total of 1465 nucleotide sequences (1053 nucleotide entries and 412 EST entries) being deposited to the GenBank database to date, this number is merely 0.028% of that available for the maize plant with a total of 5,200,499 nucleotide sequences deposited despite the huge potential of sago palm to emerge as future cash crop in future for its disease-free superior characteristics (Sasaoka et al., 2014).

In terms of the transcriptomics of the sago palm, Wee and Roslan (2012a) had performed transcriptome analysis via the construction of cDNA library to discover the expressed sequence tags (ESTs) from young leaves of sago palm. Upon functional annotation, there were 49 ESTs found to have stress tolerance functions in which reactive oxygen species (ROS)-combating enzymes such as glutathione S-transferase, alcohol dehydrogenase class III and ascorbate peroxidase were found (Wee & Roslan, 2012a). Besides, defence-related enzymes such as class I chitinase, cytosolic ascorbate peroxidase and cysteine protease are discovered, and they are believed to have contributed to the strong disease defence mechanism found in young sago palm allowing it to thrive in harsh conditions (Wee & Roslan, 2012a). Wee and Roslan (2012a) concluded that 5.38% of ESTs studied were novel to sago palm and that this palm was a great reservoir for the study of defence- and stress-related proteins.

The proteomics aspect of the sago palm has only been explored at the tip of an iceberg only as it is only used as a comparison tool to distinguish trunking from non-trunking sago palm to date (Hussain et al., 2012a). This aspect will be discussed in detail in the phenotypic variation studies section below. Apart from transcriptomics and proteomics, another approach is currently being employed in sago palm study is based on metabolomics analysis (Hussain et al., 2012b). The metabolites in leaf of sago palms are analysed using nuclear magnetic resonance spectroscopy (NMR) and gas chromatography-mass spectrometry (GC-MS) (Hussain et al., 2012b). These are to determine differential metabolites in non-trunking and trunking sago palm. Current study shows several metabolite groups such as haloalkanes, sulfite esters and phosphonates which are differently expressed between trunking and non-trunking sago palm (Hussain et al., 2012b).

The genomic research of the sago palm is on-going with the plans on the study of trunk formation and starch biosynthesis pathways (Roslan, 2012). The sequencing of the nuclear genome as well as the organellar genome (mitogenome and plastogenome) which would help us understand more on the mechanisms behind the ability of sago palm to adapt to harsh environment and stresses. Furthermore, genome-wide enhancer studies can also be conducted to aid in the understanding of the contributors towards the high adaptability of this palm at the molecular level, emulating researches that have been done onto some plant model organisms like *Zea mays* and *Arabidopsis thaliana* (Lim et al., 2018a; Lozano et al., 2018; Marand et al., 2017; Weber et al., 2016; Zhu et al., 2015; Zicola, 2017).

Bioprocess Technology

The sago starch is the one of the most utilized part of the palm in the field of bioprocess technology to produce bioethanol, lactic acid and sugar. The research involving the simultaneous saccharification and ethanol fermentation of sago starch started back in the year 1986 by Lee et al. (1986) using amyloglucosidase and immobilized *Zymomonas mobilis*. Since then, the goals in this field of research were always targeting at high ethanol yield in terms of both quantity and quality via the optimization of various parameters such as starch concentration, pH, enzyme and nitrogen concentration (Bujang et al., 1996; Mel et al., 2010; Niyong, 2012).

In 2010, two pre-production stages were introduced prior to the bioethanol synthesis, namely the hydrolysis and fermentation (Mel et al., 2010). The enzymes used are glucoamylase and α-amylase for the saccharification and liquefaction step respectively (Mel et al., 2010). This study has determined the optimal conditions for the hydrolysis step in bioethanol production in terms of glucoamylase enzyme concentration, sago starch concentration and time of saccharification (Mel et al., 2010). For the bioethanol fermentation step, factors such as agitation, pH, and inoculum concentration are optimized (Mel et al., 2010). Not long after, Niyong (2012) attempted the "Ishizaki Process" in the continuous fermentation of bioethanol and had determined the optimal nitrogen concentrations from three different sources: corn steep liquor, yeast extract and Mieki. In 2017, several strains of solvent-tolerant *Clostridium* sp. were employed for biobutanol synthesis directly from sago waste residues and the NSW strain was found to be the most suitable candidate that had the potential to replace *Bacillus coagulans* (Johnravindar et al., 2017).

The lactic acid fermentation is another prominent and indispensable role of sago starch in the bioprocess field, employing lactic acid bacteria such as *Lactococcus lactis IO-1* and *Enterococcus faecium* (Hipolito et al., 2000; Shibata et al., 2007). The amylolytic lactic acid bacterium, *E. faecium* that was identified and utilized by Shibata et al. (2007) on sago starch yielded productivity comparable to that of glucose and the lactic acid yield was the top among all other starches tested. Hipolito et al.

(2012) further improved the lactic acid yield from sago starch by using the repeated batch fermentation approach, which was proven to be much more effective than continuous fermentation and simple batch mode in terms of lactic acid concentration. Interestingly, Bujang et al. (2018) utilized abundantly found sago frond sugar (SFS) as a cheaper raw material alternative to produce lactate, achieving 85% fermentation efficiency in which the lactic acid amount produced was similar to that produced by the Standard Medium.

The current view is to produce sugars directly from both sago starch and sago "*hampas*", without the fermentation into bio-ethanol or lactic acid (Bujang, 2015). Production of sugars have been performed in 1 L lab-scale vessels for the hydrolysis of sago starch and the optimal parameters for enzymatic hydrolysis of sago starch has been comprehensively determined (Bujang et al., 2000a). The enzymatic hydrolysis of sago starch involves the utilization of Termamyl-12OL (a thermostable -amylase from *Bacillus licheniformis,* 120 KNU/g) for liquefaction (0.5µl/gram of starch), followed by incubation at 90^{o}C for 2 hours (Bujang, 2015). Next, Dextrozyme (a mixture of pullulanase from *Bacillus acidopullulyticus* and glucoamylase from *Aspergillus niger*, 225 AGU/ml) are employed for saccharification (0.6µl/gram of starch), before incubation at 60^{o}C for another four to six hours to yield hydrolysed sago sugars (Bujang et al., 2000b [as cited in Bujang, 2018, p. 300]; Bujang & Jobli, 2002) which generates a 100% glucose recovery from sago starch (Bujang, 2015). It has also been previously established that the optimum starch concentration is at 20% (w/v) sago starch (Bujang et al., 2000a; Bujang et al., 2004), harvested from best pH of 6.5 for liquefaction as well as pH 4.5 for saccharification (Bujang, 2015). Future research in this field will be focusing on improving the reusability of cells to withstand higher fermentation cycles (Hipolito et al., 2012) as well as establishing more cost-effective approaches to lower the production expenditures.

Pest Management

The cultivation of sago palm is affected by the invasion of 40 insect species known to have adverse effects on the health of the palm (Smith, 2015). For instance, the trunk of sago palm is the host for larvae of *Oryctes rhinoceros* and *Graphosoma rubrolineatum* (Kimura, 1979) whereas the leaves of sago palm are often infested by the *Psychidae* and *Monema flavescens* (crickets and locusts) (Jong, 2006; Kimura, 1979). Particularly, termites, sago beetles and hispid beetle larvae are pests vastly found in sago palm cultivation in Sarawak and Indonesia (Smith, 2015). Besides, fungal infections such as the white root diseases has been reported in young palms, but this is not lethal to the palm as there is no life-threatening disease reported to date on sago palm (Smith, 2015).

The widespread cultivation of the *Cycas* plant like the sago palm had also led to the rapid expansion of unwanted alien pests such as *Chilades pandava* on a global

scale (Donaldson, 2003). Wu et al. (2010) investigated the outbreak of this pest in 50 different localities in Taiwan and had identified the source of outbreak using COII gene as marker. A total of 29 haplotypes were discovered despite the record of high haplotype diversity and this study further highlighted the importance of monitoring within the horticultural plantation field to avoid the threatening of the native species (Wu et al., 2010). Future work will involve the investigation of other gene markers to further tap into the demographic backgrounds of the introduced alien species as well as their native counterparts and it is hoped that the pest management issues can be addressed in a sustainable manner.

Population Studies

The variations within the sago palm populations were studied using markers such as isozymes, DNA methylation profiles, random amplified polymorphism DNA (RAPD), amplified fragment length polymorphism (AFLP) and chloroplast DNA markers. Boonsermsuk et al. (1995) had succeeded in distinguishing sago palm harvested from 15 populations in three different provinces in southern Thailand from the other ten palm species via zymogram techniques. From the isozyme profiles (zymogram) of esterase, sorbitol dehydrogenase, peroxidase and acid phosphatase obtained via polyacrylamide vertical slab gel electrophoresis (PAGE), low isozyme variations were detected, depicting the closely associated genetic background and very limited ancestry (Boonsermsuk et al., 1995). The AFLP (Kjær et al., 2004) and RAPD (Ehara, 2009) analysis that follows, have all come to the same conclusion, which is in line with the observations done by Rauwerdink (1986): regardless of the spiny and non-spiny phenotypes, there is only one species of sago palm across Mindanao, Philippines, Indonesia and Malaysia.

The genetic diversity of the sago palm was also explored in Indonesia employing the chloroplast DNA markers (Abbas et al., 2010). In this study, a sum of 97 individuals have contributed to the discovery of ten haplotypes and eleven alleles across Sulawesi, Papua and Kalimantan (Abbas et al., 2010). With the individuals from Papua scoring the highest in both specific haplotypes and haplotype numbers, Papua was crowned as the origin centre of sago palm diversities in Indonesia (Abbas et al., 2010). Besides, DNA methylation profiling of sago palm was conducted by Novero et al. (2012) in an attempt to distinguish spiny from non-spiny plants. Using high performance liquid chromatography (HPLC), the methylated (5dmC) and non-methylated cytosine (dC) were used as benchmark for DNA methylation patterns investigation. As a result, the significant difference in methylation percentage between spiny and non-spiny palms suggested that spine formation was indeed an age-dependant epigenetic event, accounted by the environmental conditions (wet or dry) they thrive in.

In 2017, Abbas et al. (2017) studied the sago palm genetic variations in a whole new aspect: progenies produced from natural pollination. Interestingly, RAPD genetic variations as well as morphological dissimilarities were detected, indicating the natural cross-pollination event occurring among the sago palms. This phenomenon was believed to have resulted from the genetically differing seeds and fruits of the sago palm. While the RAPD approach lacks reproducibility, it is recommended to conduct DNA fingerprinting investigations such as the sequence characterized amplified regions (SCAR) analysis onto the sago palm as a more sensitive and reproducible way to identify polymorphisms undetected by other approaches (Bhagyawant, 2016).

Food Technology and Polymer Synthesis

The food technology that evolves around the sago palm is mainly focused on the sago starch as it has the potential to channel into food security program as products such as noodles, vermicelli and sohun (Ahmad, 2013). In fact, the quality of the noodles made from sago starch were examined under the effects of heat moisture treatment and as a result, excellent elasticity and firmness as well as less cooking loss were found to be strong points of this newly emerged sago noodle (Purwani et al., 2006).

Furthermore, the sago starch oligosaccharides were employed in the growth enhancement of lactic acid bacteria in homemade yogurt with a bright potential to be developed as prebiotic health food in the near future (Shima et al., 2012). Apparently, sago sugar (glucose) has numerous health benefits, compared to consumption of sugar (sucrose) from cane sugar (Bujang, 2015). Purified (white) sago sugar, which can be harvested from both sago starch and "*hampas*" (Janggu & Bujang, 2009; Monib, 2015), was found to encompass 94% glucose content and it is a much healthier (50% less in sweetness test) alternative to sugar cane sucrose (Bujang, 2015; Monib, 2015). Due to its high carbohydrate contents, Che Jusoh et al. (2016) foresaw the feasibility of the sago starch, in terms of its chemical and physical characteristics, as one of the underutilized supplement sources in the peri-exercise period for sustaining performance and enhancing recovery.

Besides, sago frond sugar (SFS) containing 16% to 18% cellobiose and 9% to 11% glucose, has been synthesised from enzymatic hydrolysis of sago frond (as dried powder) where these prebiotic cellobiose are employed in the fermentation of dairy products associated with bifidobacteria (Ahmad, 2015; Awg-Adeni et al., 2018). Gunawan et al. (2018) had recently succeeded in increasing the nutritive value of sago flour in terms of amylose and protein content by 20% to 33% and 1.4% to 4.1% respectively by optimizing bacterial count and fermentation time. The sago food technology field will be further placing major focus onto presenting the sago starch as a cheaper carbohydrate alternative to currently available starch products and shifting the consumer base paradigm towards the acceptance of this dark horse of the food industry.

In the field of polymer synthesis involving the sago palm, Muljana et al. (2016) had pioneered the transesterification approach of sago starch and waste palm cooking oil in the production of thermoplastics starch using densified carbon dioxide as a solvent. The end product was found to have improved thermal stability and hydrophobicity compared to native sago starch and this study serves as a green movement in plastics synthesis towards the utilization of biodegradable compounds, safer solvent as well as renewable feedstocks for the environment (Muljana et al., 2016).

Sago Waste Management

The sago waste comes in two forms: sago solid waste ("*hampas*") and sago wastewater. The sago "*hampas*" encompassing 60 to 70% starch on dry weight basis (Vickineswary et al., 1994) is very useful in various applications such as animal feed, production of laccase by solid state fermentation, biosorbent, particle board production, fermentable sugars and compost for mushroom cultivation (Awg-Adeni et al., 2010; Kumaran et al., 1997; Linggang et al., 2012).

Recent sago "*hampas*" research has been moving towards bioethanol synthesis via fermentation (Awg-Adeni et al., 2013; Bukhari et al., 2017). Awg-Adeni et al. (2013) attempted to hydrolyse sago "*hampas*" via three enzymatic treatment cycles and had achieved improved glucose yield and greater conversion yield in which these substrates will be fed into bioethanol synthesis process. Bukhari et al. (2017) further optimized the conditions for acid hydrolysis and enzymatic digestion to make sago "*hampas*" feasible for bioethanol production. They had also proven that 98% ethanol harvest was achievable in the absence of nutrients and nitrogen as higher yield and conversion rate was determined in acid hydrolysis compared to that of the enzymatic hydrolysis.

The sago wastewater is also a valuable resource for cultivation. The cultivation of *Rhodopseudomonas palustris* strain B1 in 50% sago wastewater had not only produced useful biomass supplement for prawn feed, but also reduced chemical oxygen demand (COD) by 77% (Vickineswary et al., 1997). Phang et al. (2000) had successfully cultivated *Spirulina platensis* (Arthrospira) in sago wastewater with an upflow packed bed digester. They had accomplished 98%, 99.4% and 99.9% reduction of COD, phosphate level and ammoniacal-nitrogen level in digested effluent respectively (Phang et al., 2000). Moreover, various treatments had been proposed to treat sago wastewater before disposal into the environment. For instance, Saravanane et al. (2001) had attempted a fluidized bed reactor approach and attained maximum efficiency of 82% with the digested sludge being vital for agricultural utilization. Banu et al. (2006) proposed to treat sago wastewater with Hybrid Upflow Anaerobic Sludge Blanket (HUASB) reactor and had resulted with the success of 87 to 91% COD reduction and 67 to 70% total solid removal. Priya et al. (2015) further improved the HUASB reactor with the addition of effective microorganisms and

the outcome was promising with 88% and 77% reduction in COD and total suspended solids respectively. Using response surface methodology, Sangeetha and Sivakumar (2016) converted sago wastewater into biogas, resulting in 91.61% BOD reduction and 81.85% COD reduction as well as 99.4 ml/day biogas yield. Further directions in this field will be in the discovery of conditions to increase product yield and the design of low-cost treatment system with short retention periods.

Phenotypic Variation Studies

The sago palm can usually be harvested upon the 6th to 8th years of growth under normal growth conditions, however some palms tend to remain in its stunted growth state even after 10 to 14 years (Hussain et al, 2012a). The non-trunking phenotype of the sago palm (Figure 2) is deemed to have depreciation economic value to the sago palm planting industry. There are several researches that aimed to study the differences between the trunking and non-trunking sago palms in terms of proteomics, starch granule sizes and gene profiling.

In terms of proteomics, Hussain et al. (2012a) had identified that there were indeed differential protein expression profiles between trunking and non-trunking sago palms. Kamal (2014) further employed two-dimensional gel electrophoresis and matrix-assisted laser desorption/ionization-time of light/time of flight mass spectrometry (MALDI-TOF/TOF MS) analysis to discover the differential proteins originating from five different protein families. These proteins identified were postulated to have contributed towards the phenotypic makeup (physical characteristics) differences across trunking and non-trunking sago.

Figure 2. The sago palm tree on the right is one of the many trees that expresses the non-trunking phenotype whereas the sago palm tree on the left is its counterpart that depicts the trunking phenotype

Alias (2012) had studied the phenotypic differences between trunking and non-trunking sago palms in terms of their starch quality. The starch content and starch granule sizes did not show any significant differences between trunking and non-trunking sago palms. The starch granule size of both trunking and non-trunking sago palms were reported to be in the range of 10 µm to 20 µm from the trunk as well as 1 µm to 5 µm from the leaves (Alias, 2012). Moreover, distinct transcript patterns were also observed in leaf samples harvested from trunking sago palm in comparison to that of its non-trunking counterpart (Edward, 2013). Future researches in this field of study will be placing the limelight onto the genetic markers that can act as a diagnosis tool to distinguish trunking progenies from non-trunking ones at early stages of the palms to help reduce the economic loss in the industry.

Computational Biology

The utilization of computational biology on the sago palm is a brand new field to be explored in the future. With only one research published recently, to the best of our knowledge, it opens up vast possibilities in the future digital world. Based on the needs for near-real-time geospatial information on sago palm stands, Hidayat et al. (2018) had developed a semi-automated classification system in the sago palm mapping by the means of employing object-based image analysis outline with Pleiades-1A imagery.

Hidayat et al. (2018) considered various parameters such as spectral, textural, geometric and arithmetic characteristics to further improve the support vector system (SVM) classification accuracy up to 85%. The sago palm tree classification remains a daunting task, with the scattered distributions, similar spectral features it shares with nipa and oil palm as well as its ununiformed spatial patterns of the cultivation (Hidayat et al., 2018). Future directions of this field will be driven by the improvement of sampling approaches for more accurate measurement of segmentation, atmospheric and geometric corrections via multi-strip images (Hidayat et al., 2018).

CONCLUSION

The sago palm is one of the most promising food crops and cash crops in the future to cater to the increasing world demand for food each year. It is hoped that with the addition of new knowledge from this palm species from various fields, the research database for the endemic flora and fauna in Sarawak will be further enriched (Kadir et al., 2013; Lim et al., 2018b, 2019, in press; Ministry of Natural Resources and Environment [MNRE], 2016; Phillips, 2016; Soepadmo & Wong, 1995), adding data to enable for the study of biodiversity ecosystem in whole. With its utilization in various important fields are being realized throughout the years, it is essential that we consolidate all talents and funding towards the research of this underutilized food crop in the light of providing food security to the needy in time of world food scarcity.

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Effects of Need-Based Nitrogen Management and Varieties on Growth and Yield of Dry Direct Seeded Rice

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ABSTRACT

Proper application of nitrogen fertilizer is vital to improve crop growth and development. A field experiment was conducted to evaluate the need based nitrogen management using leaf color chart (LCC) on crop growth and yield of dry direct seeded rice (DDSR) with five nitrogen management practices {0 kg N ha^{-1}, 120 kg N ha^{-1} in three equal split applications, 30 kg N ha^{-1} as basal + 30 kg N ha^{-1} top dressing based on LCC critical value four, 30 kg N ha^{-1} top dressing (without basal N dose) at 15 days after sowing (DAS) + LCC based N application and pure LCC (without basal N dose) based N application} tested on three rice varieties (Radha-4, US-312 and Sukhkhadhan-5) in split plot design. The results revealed that the highest grain yield was observed in hybrid US-312 (4,695 kg ha^{-1}) with higher plant height, leaf area index (LAI) and dry matter production than the inbred varieties i.e. Radha-4 and Sukhkhadhan-5. All the nitrogen management treatments including LCC were similar to each other in respect of grain yield formation (4,695-4,891 kg ha^{-1}), but remained significantly superior over three split applications (4,408 kg ha^{-1}). Likewise, values of growth parameters were higher in LCC based treatments than the recommended practice and grain yield being the highest in pure LCC (4,891 kg ha^{-1}). Thus, pure LCC based nitrogen management found to be the best practice for both inbred and hybrid rice varieties.

Keywords: Crop growth, direct seeded rice, leaf color chart, yield

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INTRODUCTION

Rice (*Oryza sativa* L.) is one of the most popular cereal crops in the world. It is the principal food for one third of the world's population. More than 90% of this rice is produced and consumed in Asia (Pathak et al., 2011). It provides some 700 calories per person, mostly residing in developing countries (Tari et al., 2009). Recently released statistics from the United States Department of Agriculture (USDA) (2018) reveal that total global rice production is 486.78 million mt from the area 160.82 million ha with average productivity of 4.52 mt ha^{-1} during 2016/17.

Puddled rice transplanting is the burdensome and time consuming crop establishment method with more labor and water requirement which are becoming scarce too. It destroys the soil physical properties by dismantling the soil aggregates and ultimately affects the growth and productivity of succeeding wheat crop (Bhurer et al., 2013). Due to climate change there may be the risks of early season drought and farmers are compelled for delayed transplanting by 1-3 weeks (Ladha et al., 2000; Pathak et al., 2011). Similarly, the methane emission is higher in flooded rice fields than in the non-flooded rice fields (Kakumanu et al., 2011). Therefore, DSR may be one of the best options to cope with the climate change.

Nitrogen is one of the most yield limiting nutrients in crop production in all agro-ecological regions of the world (Fageria & Baligar, 2005; Yoshida, 1981). Nitrogen management is considered as one of the most challenging parts of the direct seeded rice to achieve higher grain yield and nitrogen use efficiency (Ali et al., 2012). Shukla et al. (2004) stated that due to lack of synchronization between the nitrogen demand and nitrogen supply more than 60% of applied nitrogen was lost. Farmers generally apply nitrogen fertilizer in fixed time split doses without considering the plant's need for nitrogen at that time (Ladha et al., 2000). This does not consider the dynamic crop nitrogen requirement and soil nitrogen supply because recommendations are mainly derived from empirical testing of nitrogen response to few fixed doses (Cassman et al., 1998; Shukla et al., 2004). Therefore, it requires different amounts of nitrogen in different fields, depending on native nitrogen supply and crop demand.

The optimum use of nitrogen can be achieved by matching its supply with the crop demand. Synchronization of nitrogen fertilizer application with the crop demand following need-based nitrogen management is another approach for higher yields, reduced nitrogen losses and improved nitrogen use efficiency (Thind & Gupta, 2010). Need based nitrogen management include periodic assessment of nitrogen status in standing crop following its application according to the need of the crop (Witt et al., 2004). For this, chlorophyll meter or leaf color chart (LCC) can be used to assess the actual plant nitrogen status (Balasubramanian et al., 2003; Singh et al., 2002). Leaf color chart consists of panels (generally 4 or 6) that range from yellow-green to dark green sequentially starting from no 1 as yellow-green. It was jointly developed by the International Rice Research Institute (IRRI) and the Philippine

Rice Research Institute (PhilRice) from a Japanese prototype in late 1990s (Shukla et al., 2004). LCC provides the guideline for effective nitrogen management by giving the idea of when and how much nitrogen fertilizer to apply for maintaining and optimizing nitrogen status in rice plants (Sathiya & Ramesh, 2009).

Considering these facts, this experiment was conducted to evaluate growth and yield of dry direct seeded rice under LCC based nitrogen management.

MATERIALS AND METHODS

Study Site

A field experiment was conducted at Agronomy research block of Agriculture and Forestry University (AFU), Rampur, Chitwan, Nepal (27^{o} 37' North latitude and 84^{o} 25' East longitudes) from June to October 2014. The experimental soil was sandy loam having following characteristics in the top 20 cm profile; clay 5.1%, silt 22.8%, sand 72.1% and pH 5.4. Similarly, the soil had 3.18% soil organic matter, 0.16 % total N, 41.45 kg ha^{-1} available P and 98.40 kg ha^{-1} available K which were under the medium category based on rating chart (Jaishy, 2000).

Experimental Design. The experiment was arranged in Split plot design with four replications. There were 15 treatments including three rice varieties (Radha-4, US-312 and Sukhkhadhan-5) as main plot factor and five nitrogen management practices as sub plot factor (Treatments detail in Table 1). Among the three rice varieties, Radha-4 and Sukhkhadhan-5 are the Nepalese high yielding inbred varieties whereas US-312 is Indian hybrid rice registered for cultivation in Nepal. Both Radha-4 and Sukkhadhan-5 (drought tolerant) varieties were originated from International Rice Research Institute (IRRI), Philippines with their designations IR8423-156-2-2-1 (Khush & Virk, 2005) and IR 83388-B-B-B-108-3 (Khanal et al., 2017) respectively. These varieties were purposively selected as they were commonly grown by the farmers in the region. Rice seeds were sown continuously in line at the seed rate of 40 kg ha^{-1} with row spacing of 20 cm under dry condition. Individual plot size was 10.5 m^2.

Observations

Biometrical observations on plant height, leaf area index (LAI), above ground dry matter production were measured and recorded starting from 15 DAS to 90 DAS at 15 days interval. The leaf area was measured by digital leaf area meter (LiCor-3100). Then, leaf area index was calculated by following formula (Lan et al., 2009):

$$\text{Leaf area index (LAI)} = \frac{\text{Leaf area (cm}^2\text{)}}{\text{Crop geometry (cm}^2\text{)}}$$

In LCC based treatments, nitrogen was applied through urea based on average LCC readings taken from 21 days after sowing (DAS) to heading at every 10 days interval. In each reading, the plots with average LCC reading (taken from 10 randomly selected upper healthy leaves)

below the critical value i.e. 4, received nitrogen at the rate of 30 kg ha^{-1} through urea topdressing (Devkota et al., 2013). In pure LCC treatment, nitrogen was applied only based on the LCC readings without its basal application. The six-panel leaf color chart (Nitrogen Parameters, Chennai, India) was used for this experiment. For the above ground dry matter, destructive sampling was done following oven drying at temperature of 70°C for 72 hours until the constant weight. Likewise, grain and straw yields were calculated in kg ha^{-1}. The grain yield adjusted at 14% moisture level and harvest index (HI) were calculated by the following formula as described by Shahiddullah et al. (2009) and Ismail (1993) respectively:

$$\text{Grain yield (kg ha}^{-1}) = \frac{\text{Plot yield (kg)* (100 – grain moisture content \%)* 10000 m}^2}{\text{(100-14)* net plot area (m}^2)}$$

$$\text{Harvest index (HI)} = \frac{\text{Grain yield}}{\text{Biological yield}}$$

Data Analysis

The data were statistically analyzed with GEN-STAT statistical software programs. Analysis of variance (ANOVA) was done on every measured parameter to determine the significance of differences between means of treatments. Means for each parameter were separated by the Duncan's multiple

Table 1
Details of treatment used in the experiment

Treatment combinations	Treatment details
T_1	Radha-4 + N_0 (control)
T_2	Radha-4 + N_{120} (in three equal splits at basal, AT and PI)
T_3	Radha-4 + N_{30} (basal) + LCC at cv. 4 @30 kg ha^{-1} N
T_4	Radha-4 + N_{30} (15 DAS) + LCC at cv. 4 @30 kg ha^{-1} N
T_5	Radha-4 + N_0 (basal) + LCC at cv. 4 @30 kg ha^{-1} N
T_6	US-312 + N_0 (control)
T_7	US-312 + N_{120} (in three equal splits at basal, AT and PI)
T_8	US-312 + N_{30} (basal) + LCC at cv. 4 @30 kg ha^{-1} N
T_9	US-312 + N_{30} (15 DAS) + LCC at cv. 4 @30 kg ha^{-1} N
T_{10}	US-312 + N_0 (basal) + LCC at cv. 4 @30 kg ha^{-1} N
T_{11}	Sukhkhadhan-5 + N_0 (control)
T_{12}	Sukhkhadhan-5 + N_{120} (in three equal splits at basal, AT and PI)
T_{13}	Sukhkhadhan-5 + N_{30} (basal) + LCC at cv. 4 @30 kg ha^{-1} N
T_{14}	Sukhkhadhan-5 + N_{30} (15 DAS) + LCC at cv. 4 @30 kg ha^{-1} N
T_{15}	Sukhkhadhan-5 + N_0 (basal) + LCC at cv. 4 @30 N kg ha^{-1} N

Note: DAS = days after sowing, LCC = leaf color chart, AT = active tillering, PI = panicle initiation, cv = critical value, N_0 = control i.e. no nitrogen applied, N_{30} (basal) = N application @30 kg ha^{-1} at the time of transplanting, N_{30} (15 DAS) = N topdressing @30 kg ha^{-1} at 15 DAS, N_{120} = N application @120 kg ha^{-1} in three equal splits

range test (DMRT) at $P \leq 0.05$. Similarly, correlation-regression analysis was carried out to assess the relationship of grain yield with yield attributing characters and growth parameters.

RESULTS AND DISCUSSION

Growth Parameters

Effect of Varieties. The experimental results revealed that plant heights, leaf area index and dry matter production were found significantly influenced by the varieties. Significantly different plant heights were observed at 30 DAS and 75 DAS only (Table 2). The tallest variety was US-312 (77.95 cm) which was found statistically at par with Radha-4 (74.27 cm) whereas the lowest plant height was found in Sukhkhadhan-5 (70.19 cm) at 75 DAS. Regarding LAI, variety US-312 had significantly higher LAI as compared to Radha-4 and Sukhkhadhan-5 at 30, 60, 75 and 90 DAS (Table 3). The LAI of Radha-4 and Sukhkhadhan-5 did not differ significantly with each other. Similarly, significantly higher dry matter production of US-312 than other varieties was observed at 45 and 75 DAS only while it remained non-significantly higher than other varieties at 30, 60 and 90 DAS (Table 4). Varieties produced different plant heights based on their varietal characters and also different growth parameters. Sucharitha and Boopathi (2001) found notable superiority of hybrid over inbred varieties in terms of growth parameters and yield attributes. Similarly, variable plant heights among the rice varieties were observed (Hossain et al., 2008). Yamauchi (1994) reported that hybrid varieties had a vigorous growth rate during early vegetative stages due to rapid expansion of leaf area resulting higher dry matter production. Higher values of LAI in hybrid varieties than in inbred were also observed by Salem et al. (2011).

Effect of N Management Practices. Different N management practices had significant influence on plant height, LAI and dry matter production at all growth stages of the crop. In general, LCC based treatments showed greater plant heights as compared to the recommended practice of three split applications and control at 30, 60 and 75 DAS (Table 2). During the rapid growth at 75 DAS, LCC based treatments showed significantly higher plant heights as compared to recommended practice and control. Regarding the LAI, pure LCC resulted significantly higher LAI than recommended practice at all the dates of observation except at 30 DAS (Table 3). All the LCC based treatments were at par with each other all the dates of observations except in 30 DAS. In case of dry matter production, all the treatments receiving nitrogen fertilizer had significantly higher dry matter production than the control treatment (Table 4). More interestingly, LCC based N management produced higher dry matter than recommended practice at all the dates of observation. However, it was non- significant. The highest LAI (4.51) and dry matter production (596.1 g m^{-2}) were observed in pure LCC based treatment.

LCC based N management supplied the nitrogen according to the crop needs whereas in recommended practice N was applied at fixed time regardless the crop's need that promoted the higher N losses. Application of nitrogen in split according to the crop needs is the reason for better rice growth parameters (Sathiya & Ramesh, 2009). The reason behind higher LAI in LCC based treatments was the maintenance of nitrogen concentration at balance which enhanced the process of cell division and elongation and ultimately increased the leaf number and leaf area (Om et al., 1989). Brady (1999) reported that plant growth and development were influenced through several biochemical and physiological processes as affected by nitrogen application. Similarly, nitrogen fertilizer helps to increase the rate of leaf expansion leading to increased interception of solar radiation by the crop canopy (Squire et al., 1987). The decline in LAI after 75 DAS was mainly due to the senescence of lower leaves. Decrease of leaf area (due to senescence of early formed leaves) after initiation of panicle was associated with

Table 2
Effect of varieties and nitrogen management practices on plant height of dry direct seeded rice

Treatments	Plant height (cm)				
	30 DAS	45 DAS	60 DAS	75 DAS	90 DAS
Varieties					
Radha-4	34.44^{b}	45.99	57.05	74.27^{ab}	96.05
US-312	37.33^{a}	47.96	61.72	77.95^{a}	94.13
Sukhkhadhan-5	32.61^{b}	45.19	54.92	70.19^{b}	92.56
Nitrogen management					
N_0	31.43^{c}	40.89^{d}	51.29^{c}	67.18^{c}	83.18^{b}
N_{120}	31.94^{c}	50.46^{a}	57.43^{b}	72.38^{b}	96.69^{a}
N_{30} (basal) + LCC	38.25^{a}	46.66^{bc}	61.13^{a}	76.45^{a}	98.44^{a}
N_{30} (15DAS) + LCC	34.86^{b}	48.73^{ab}	59.02^{ab}	77.96^{a}	96.11^{a}
Pure LCC	37.49^{a}	45.15^{c}	60.61^{a}	76.71^{a}	96.81^{a}
CV, %	5.47	5.93	4.57	5.21	4.33
Grand mean	34.79	46.37	57.89	74.13	94.24

Note: Means followed by the same letter(s) in the same column are not significantly different at 5% probability level by Duncan's multiple range test

Table 3
Effect of varieties and nitrogen management practices on leaf area index of dry direct seeded rice

Treatments	LAI				
	30 DAS	45 DAS	60 DAS	75 DAS	90 DAS
Varieties					
Radha-4	0.55^{b}	1.42	2.26^{b}	3.62^{b}	2.64^{b}
US-312	0.71^{a}	1.87	2.99^{a}	4.51^{a}	3.70^{a}
Sukhkhadhan-5	0.49^{b}	1.44	2.21^{b}	3.32^{b}	2.77^{b}

Table 3 (*continue*)

Treatments	LAI				
	30 DAS	45 DAS	60 DAS	75 DAS	90 DAS
Nitrogen management					
N_0	0.36^{c}	0.91^{c}	1.26^{c}	2.28^{c}	1.57^{c}
N_{120}	0.52^{b}	1.49^{b}	2.37^{b}	3.65^{b}	2.94^{b}
N_{30} (basal) + LCC	0.82^{a}	1.81^{a}	2.74^{ab}	4.36^{a}	3.43^{ab}
N_{30} (15DAS) + LCC	0.63^{b}	1.85^{a}	3.03^{a}	4.30^{ab}	3.52^{ab}
Pure LCC	0.59^{b}	1.83^{a}	3.04^{a}	4.51^{a}	3.72^{a}
CV, %	31.20	23.70	26.60	21.00	22.70
Grand mean	0.59	1.58	2.49	3.82	3.04

Note: Means followed by the same letter(s) in the same column are not significantly different at 5% probability level by Duncan's multiple range test

Table 4
Effect of varieties and nitrogen management practices on dry matter production of dry direct seeded rice

Treatments	Dry matter production (g m^{-2})				
	30 DAS	45 DAS	60 DAS	75 DAS	90 DAS
Varieties					
Radha-4	91.35	137.30^{b}	256.70	505.30^{b}	617.40
US-312	103.61	188.40^{a}	297.10	582.30^{a}	725.00
Sukhkhadhan-5	89.91	131.10^{b}	274.50	497.00^{b}	700.00
Nitrogen management					
N_0	65.88^{c}	96.90^{c}	158.60^{c}	332.30^{b}	387.90^{b}
N_{120}	88.32^{b}	149.70^{b}	278.20^{b}	562.70^{a}	710.00^{a}
N_{30} (basal) + LCC	108.03^{a}	175.70^{a}	297.80^{ab}	576.50^{a}	760.00^{a}
N_{30} (15DAS) + LCC	104.64^{a}	167.70^{ab}	314.70^{ab}	573.50^{a}	764.30^{a}
Pure LCC	107.91^{a}	171.30^{a}	331.30^{a}	596.10^{a}	781.80^{a}
CV, %	16.40	14.50	19.60	19.30	15.40
Grand mean	95.00	152.20	276.10	528.00	681.00

Note: Means followed by the same letter(s) in the same column are not significantly different at 5% probability level by Duncan's multiple range test

the remobilization of stored metabolites from leaf sheath and stem to panicles (Chandrasekhar et al., 2001). Adhikari et al. (1999) stated the inefficient use of applied N in broad based blanket recommendation. This might be the reason for higher dry matter production in LCC based N application then in fixed time split application.

Grain Yield, Yield Attributes and Harvest Index

Effect of Varieties. Varieties as well as N management practices had significant effect on grain yield, yield attributes and HI (Tables 5 and 6). Among the varieties, hybrid US-312 gave significantly higher grain yield (4,695.23 kg ha^{-1}) than Radha-4 (4,089.39

kg ha^{-1}) and Sukhkhadhan-5 (4,315.43 kg ha^{-1}). The grain yields of Sukhkhadhan-5 and Radha-4 remained statistically similar with each other. Higher grain yield in US-312 was attributed to vigorous crop growth with significantly higher LAI (Table 3) and more dry matter production (Table 4) than Radha-4 and Sukhkhadhan-5. The variety Radha-4 was found significantly superior over hybrid US-312 and Sukhkhadhan-5 for panicle weight, thousand grains weight and sterility percentage (Table 6). However, panicle length and grains per panicle were significantly higher in hybrid US-312 (24.24 cm and 95.51 respectively) as compared to Radha-4 and Sukhkhadhan-5 that ultimately contributed for higher grain yield. Hybrid rice varieties have higher heterosis for grains per panicle than the improved rice varieties resulting higher grain yields (Salgotra et al., 2002). Hosain et al. (2014) reported higher yield of hybrid was ascribed due to more panicle length (3.4 %) and more spikelets per panicle (9.8 %) as compared to inbred variety. Regarding the harvest index, Sukhkhadhan-5 had significantly higher HI (0.48) as compared to Radha-4 (0.43) and US-312 (0.45). Similarly, HI of US-312 (hybrid) was statistically greater than Radha-4. The highest HI in Sukhkhadhan-5 might be attributed to its lowest straw yield (4987.12 kg ha^{-1}) in relation to its grain yield (4315.43 kg ha^{-1}). Higher grain yield and HI of hybrids than that of inbreed varieties were also reported by Yang et al. (2007).

Effect of N Management Practices. The highest grain yield (4891.23 kg ha^{-1}) was obtained in pure-LCC treatment (Table 5). In case of yield attributes, all LCC based treatments were found insignificantly different from recommended practice in effective tillers per m^2, thousand grain weight and sterility percentage (Table 7). But grains per panicle remained significantly higher in LCC based treatments as compared to the recommended practice. All the LCC based treatments had statistically similar grain yields with each other but statistically higher than recommended practice and control. The lowest grain yield (3,094.15 kg ha^{-1}) was observed in control. Grain yield in LCC based treatments ranged from 4,695.21 to 4,891.23 kg ha^{-1} whereas it was only 4408.06 kg ha^{-1} ha in recommended practice. Higher recovery efficiency of nitrogen in LCC based treatments contributed to increase in chlorophyll concentration in leaves which led to higher photosynthetic rate and ultimately plenty of photosynthates available during grain development (Manzoor et al., 2006). Straw production followed the trend as that of grain yield being the highest in pure LCC and the lowest in control treatment. Regarding the harvest index, all the nitrogen management practices including control had non-significant influence on HI. Non-significant influence on HI when applying nitrogen from 0 to 400 kg ha^{-1} in upland rice was also observed by Fageria et al. (2011).

Significantly higher grain yield in LCC based treatments was attributed to optimization of N supply and more efficient utilization by the crop which ultimately maintained higher LAI (Table

3) and higher dry matter production (Table 4). In recommended practice, fixed rate of nitrogen was applied in the fixed time irrespective to the crop demand which leads to loss of applied nitrogen through various processes occurring in the soil. Therefore, less efficient N utilization caused lower grain yield in recommended practice of fixed time three equal splits.

In an experiment, comparable or even higher grain yield was observed in LCC based nitrogen application with basal application of 30 kg ha^{-1} N and critical value 4 receiving 30 kg ha^{-1} N than in recommended practice (Singh et al., 2010). Similar results were also reported by Jayanthi et al. (2007). Similarly, grain yield increment of 400 kg ha^{-1} in Aman and 600

Table 5
Effect of varieties and nitrogen management practices on grain yield, straw yield and harvest index of dry direct seeded rice

Treatments	Parameters		
	Grain yield (kg ha^{-1})	Straw yield (kg ha^{-1})	Harvest Index (HI)
Varieties			
Radha-4	4089.39[b]	6619.35[a]	0.43[c]
US-312	4695.23[a]	6631.03[a]	0.45[b]
Sukhkhadhan-5	4315.43[b]	4987.12[b]	0.48[a]
Nitrogen management			
N_0	3094.15[c]	3982.23[c]	0.45
N_{120}	4408.06[b]	6216.11[b]	0.45
N_{30} (basal) + LCC	4695.21[a]	6352.04[b]	0.45
N_{30} (15DAS) + LCC	4746.08[a]	6923.50[a]	0.45
Pure LCC	4891.23[a]	6924.21[a]	0.46
CV, %	7.80	9.52	2.40
Grand mean	4367.35	6079.14	0.4533

Note: Means followed by the same letter(s) in the same column are not significantly different at 5% probability level by Duncan's multiple range test

Table 6
Effect of varieties and nitrogen management practices on yield attributing characters of dry direct seeded rice

Treatments	Yield attributing characters					
	ET m^{-2}	Sterility (%)	TGW (g)	Panicle length (cm)	Panicle weight (g)	Grains per panicle
Varieties						
Radha-4	322.40	13.42[a]	23.38[a]	20.89[b]	2.03[a]	77.94[b]
US-312	321.10	10.36[b]	18.12[c]	24.24[a]	1.83[b]	95.51[a]
Sukhkhadhan-5	319.70	9.72[c]	22.35[b]	20.60[b]	1.77[b]	76.01[b]
Nitrogen management						
N_0	248.3[b]	14.42[a]	20.00[b]	19.72[c]	1.38[c]	62.33[c]

Table 6 *(continue)*

Treatments	Yield attributing characters					
	ET m^{-2}	Sterility (%)	TGW (g)	Panicle length (cm)	Panicle weight (g)	Grains per panicle
Nitrogen management						
N_{120}	337.6[a]	10.67[b]	21.46[a]	21.98[b]	1.85[b]	81.81[b]
N_{30} (basal) + LCC	335.6[a]	9.84[b]	21.69[a]	22.74[a]	2.13[a]	89.43[a]
N_{30} (15DAS) +LCC	337.3[a]	10.52[b]	21.29[a]	22.43[ab]	1.99[ab]	90.26[a]
Pure LCC	346.4[a]	10.38[b]	21.97[a]	22.68[ab]	2.04[a]	91.96[a]
CV, %	10.27	9.40	3.70	3.70	9.50	9.30
Grand mean	321.03	11.17	21.28	21.91	1.88	83.20

Note: Means followed by the same letter(s) in the same column are not significantly different at 5% probability level by Duncan's multiple range test, ET = effective tillers, TGW = thousand grain weight

kg ha^{-1} in Boro rice were observed with LCC based nitrogen management over the recommended practice (Alam et al., 2005). Fixed time LCC based N application (120 kg ha^{-1}) produced significantly higher grain yield (4,940 kg ha^{-1}) than 120 kg ha^{-1} (4,170 kg ha^{-1}) applied in three equal splits in direct seeded Boro rice **(**Islam & Rahman, 2003).

Interaction between Varieties and Nitrogen Management

There was non-significant effect of interaction between rice varieties and nitrogen management practices on plant height, leaf area index, dry matter production), yield attributes, grain yield and harvest index. In case of grain yield, there was a quantitative difference among treatments. In all nitrogen management practice, the highest grain yield was observed in US-312. But among the varieties, US-312 and Radha-4 produced the maximum grain yield in Pure LCC treatment and Sukhkhadhan-5 produced the maximum in N_{30} (basal) + LCC treatment (Table 7).

Table 7
Interaction effect of varieties and nitrogen management on grain yield (kg ha^{-1}) of dry direct seeded rice

Treatments	Varieties		
	Radha-4	US-312	Sukkhadhan-5
N_0	3054.18	3358.71	2869.55
N_{120}	4032.69	4710.48	4480.51
N_{30} (basal) + LCC	4208.75	5058.67	4816.22
N_{30} (15DAS) + LCC	4433.79	5061.84	4741.51
Pure LCC	4717.52	5286.83	4668.70
LSD (0.05)		ns	
SEm (±)		179.70	
Grand mean		4367.35	

Note: 'ns' means non-significant

Regarding the correlation, yield attributing characters like panicle length, grains per panicle and effective tillers per meter square were found highly correlated with grain yield (i.e. highly significant). Similarly, same trend was observed in case of leaf area index at 75 DAS and dry matter production at 90 DAS. But, harvest index remained non-significantly correlated with grain yield (Table 8).

Table 8
Regression equation and correlation coefficient between different parameters and yield of dry direct seeded rice

Linear regressions	Equations (Y = a + bx)	'r' value
Yield (kg ha^{-1}) against LAI at 75 DAS	Y = 483.53x + 2520.306	0.739**
Yield (kg ha^{-1}) against dry matter (gm^{-2}) at 90 DAS	Y = 3.209x + 2182.115	0.755**
Yield (kg ha^{-1}) against grains per panicle	Y = 34.402x + 1505.9	0.708**
Yield (kg ha^{-1}) against panicle length	Y = 240.2x - 895.93	0.666**
Yield (kg ha^{-1}) against effective tillers per m^2	Y = 11.244x + 757.08	0.705**
Yield (kg ha^{-1}) against HI	Y = 4143.57x + 2488.524	0.129^{ns}

**means significant at 0.01 level of significance, 'ns' means non-significant

CONCLUSION

Better performance was observed in LCC based practices in terms of growth and yield parameters than in the recommended practice on nitrogen management in rice. Pure LCC based nitrogen management was the most efficient practice for both inbred and hybrid varieties of rice.

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Phosphorus Nutrition Provoked Improvement on the Growth and Yield of 'MD-2' Pineapple

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ABSTRACT

Phosphorus (P) is one of the essential nutrients needed by plants in large amount. However, response of fruit crops to phosphorus application may vary depending on soil and climatic conditions of the area. Hence, this study was conceptualized to evaluate the growth, yield and fruit quality of 'MD-2' pineapple in response to varying rates of P under Adtuyon clay soil in Bukidnon, Philippines. The experiment was laid out in a randomized complete block design (RCBD) with five amounts (0, 84, 127, 169 and 211 kg ha^{-1}, respectively) of P as treatments with three replications. Results revealed that 'MD-2' pineapple growth was significantly influenced by P application. However, highest dose (211 kg ha^{-1}) of P in Adtuyon clay soil did not provoked significant P uptake of 'MD-2' pineapple. Instead, P uptake declined beyond 169 kg P ha^{-1} application. Zn uptake of pineapple declined also as P applied in soil was beyond 169 kg P ha^{-1}. Unpredictably, 'MD-2' pineapple plants starved with P still produced fruits (mean fruit mass of 1.28 kg) with a yield of 76.80 tons ha^{-1}, however it could not be denied that higher doses of P (≥169 kg ha^{-1}) improved the fruit mass (≥1.42 kg) as well as yield (≥96.92 tons ha^{-1}) of 'MD-2' pineapple. Fruit physico-chemical properties of 'MD-2' pineapple was not dependent on the amount of P applied in the soil. In general, 169 kg P ha^{-1} was found as the optimal amount of P for 'MD-2' pineapple production in Adtuyon clay soil.

Keywords: Adtuyon clay, *Ananas comosus*, 'MD-2' pineapple, phosphorus, pineapple nutrition

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INTRODUCTION

Pineapple (*Ananas comosus* L.) ranks third in tropical fruit crops production next to banana and citrus. In the Philippines, pineapple ranks second produced major fruit crop next to banana (Philippine Statistics

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Authority [PSA], 2017). "Smooth Cayenne" is the predominant pineapple cultivar grown in the Philippines. The said cultivar is ideal for fresh consumption as well as processing. It was in the late 1990's when some local pineapple growers started to cultivate 'MD-2' pineapple. This pineapple cultivar has exceptional flavour making it ideal for fresh consumption than any other cultivars.

Pineapple is a perennial crop which demands high amount of nutrients. Hence, commercial pineapple production intended for export requires heavy fertilizer dosage. Moreover, it cannot be denied that different pineapple cultivars require varying amount of nutrients. Further, plant nutrition studies conducted to assess the growth and yield of crops focused mainly on the three primary nutrients (N, P and K). In pineapple, NPK fertilization researches were carried out in Smooth Cayenne (Spironello et al., 2004) and MD-2 (Sakimin et al., 2017) pineapple cultivars, respectively under varying soil types.

This study primarily focused only on phosphorus (P) fertilization level for 'MD-2' pineapple grown under Adtuyon clay soil in Bukidnon, Philippines. Adtuyon clay is the most extensive soil type covering almost exclusively the entire Bukidnon plateau (Philippine Council for Agriculture, Forestry and Natural Resources Research and Development [PCARRD], 2006). T. S. Castro (personal communication, November 8, 2013) stated that the physical and chemical properties of Adtuyon clay soil was ideal for pineapple production, except for P (4-10 ppm) which was below the critical level (20 ppm). Garcia et al. (2017) recommended only two rates of P (138 kg hectare^{-1} and 161 kg hectare^{-1}) depending on the cultivars grown and soil classifications under Mexico condition.

This study was conducted to evaluate the growth of 'MD-2' pineapple in response to varying rates of P; assess the nutrient uptake of 'MD-2' pineapple applied with varying amounts of P; evaluate the yield and fruit quality of 'MD-2' pineapple in response to varying rates of P; and determine the optimum P fertilization level for the production of 'MD-2' pineapple under Adtuyon clay soil in Bukidnon, Philippines.

MATERIALS AND METHODS

The research was carried out at Mt. Kitanglad Agricultural Development Corporation (MKADC), Lurugan, Valencia City, Bukidnon, Philippines with an elevation of 510 meters above sea level (masl). The soil was identified as Adtuyon clay. Based on laboratory analysis, soil texture was classified as clay. Soil pH was within the optimum range of growing pineapple. Extractable Ca and Mg were above the critical levels, whereas organic matter (OM), P and K of the experiment area were found below the critical levels (Table 1) for pineapple production.

Prior to the conduct of experiment, three months fallow period was employed in the particular research field. Harrowing of the experiment area was conducted twice at monthly interval. Deep plowing (mouldboard) then commenced one month

Table 1
Soil physico-chemical properties of the experiment area prior to land preparation

Depth (cm)	Texture	pH	OM (%)	P	K	Ca	Mg
				----------ppm----------			
0-30	clay	5.49	1.68	7.98	187	430	151
31-60	clay	5.11	0.70	8.97	160	370	96
Mean	clay	5.30	1.19	8.48	174	400	199
Critical level[1/]	---	≤4.50 & ≥5.20	≤3.00	≤20	≤300	≤100	≤50

[1/]- Source: Malézieux and Bartholomew (2003)

after the last harrow activity. Raised beds with a height of 25-30 cm were established using a tractor-drawn mound implement.

The experiment was laid out in a randomized complete block design (RCBD) with five levels of P (0, 84, 127, 169 and 211 kg ha^{-1}, respectively) as treatments as shown on Table 2. Each treatment was replicated into four with a total of 20 experiment units. Each experiment unit had an area of 26.8 m^2. A total of 200 medium sucker (300 to 350 grams) were planted per experiment unit. The 100 suckers planted within the inner two raised beds served as the data plants, whereas the other 100 suckers planted within the outer two raised beds served as border plants. In this way, fertilizer treatment contamination from one experiment unit to the other was prevented.

Similar cultural management practices such as rate/timing of fertilizer application, pest/disease control, and flower induction treatment were employed to all treatments after planting except for the P levels which served as treatments. The five levels of P was applied in the form of triple superphosphate (0-46-0) as side band to pineapple plants at two months old after planting (2 MAP) and at 4 MAP. Other essential plant nutrients (462 kg N, 523 kg K, 223 kg Ca, 205 kg Mg, 24 kg Fe, 4 kg Zn, 3 kg B and 560 kg S kg ha^{-1}, respectively) were applied through pre-plant application (dolomite), side band applications (ammonium sulphate, potassium sulphate and magnesium sulphate) and foliar applications (urea, iron sulphate, zinc sulphate, solubor, potassium sulphate and calcium boron) based on MKADC farm

Table 2
Varying amounts of P which served as treatments

Treatment description	Amount of applied P (kg ha^{-1})
0% P (Control treatment)	0
50% P of local pineapple grower practice (LPGP)	84
75% P of LPGP	127
100% P of LPGP	169
125% P of LPGP	211

Note: LPGP- MKADC commercial pineapple practice as of calendar year 2010 (T. S. Castro, personal communication, November 8, 2013)

fertilization program. Flower induction treatment (Ethrel + urea) was applied at 11.5 months after planting (MAP). As a standard practice in commercial pineapple production, degreening or fruit ripening solution (Ethrel + phosphoric acid) was applied 155 days after flower induction treatment. Fruits were harvested at shell color index 2-3. In this experiment, three harvest rounds were executed to clear all data fruits in all experiment units.

Data gathered includes:

1. Plant height - measurement of plant height was performed a day prior to flower induction treatment. Height (ground level to the tip of tallest leaf) of plant was measured using a measuring stick. Average plant height was computed using the formula (Valleser, 2019):

$$\text{Plant height} = \frac{\sum \text{Plant height}}{\text{Number of data plants}}$$

2. Plant mass - this data was taken a day prior to flower induction treatment. Three representative plants from the border rows were pulled-out and weighed excluding the stem apex (below ground level). The mass of three representative plants served as baseline data in estimating the plant mass of data plants through visual examination. Average plant mass was computed using the formula (Valleser, 2019):

$$\text{Plant mass} = \frac{\sum \text{Plant mass}}{\text{Number of data plants}}$$

3. D-leaf mass - D-leaf is the longest leaf of pineapple plant. 20 D-leaves from 20 randomly selected data plants were collected and weighed a day prior to flower induction treatment. Average D-leaf mass was computed using the formula (Valleser, 2018):

$$\text{D-leaf mass} = \frac{\sum \text{D-leaf mass}}{20}$$

4. Crop nutrient level - this parameter was taken at flower induction treatment. 20 D-leaves were collected from randomly selected data plants per experiment unit. As a standard practice of MKADC, the middle 2/3 part (5 cm length) of the D-leaf was cut-off for N, Fe and B analyses. Likewise, the basal white portion (5 cm length) of the D-leaf was cut-off for P, K, Ca, Mg analyses. Three pineapple stem apices were collected and brought to plant tissue laboratory for Zn analysis. Nutrient analyses were performed by the laboratory technicians of the Biotechnology Research Services laboratory at Alanib, Lantapan, Bukidnon, Philippines.

5. Fruit mass - fruits with peel color index 2-3 based on MKADC pineapple color index guide were harvested. A total of five harvest rounds (3 days interval) were conducted to clear all fruits inside the data rows. All harvested fruits were weighed using a pre-calibrated weighing scale. Mean fruit mass was computed using the formula:

$$\text{Fruit mass} = \frac{\sum \text{Fruit mass}}{\text{Number of data fruits}}$$

6. Yield - this parameter was expressed in tons hectare^{-1}. Yield was computed using the formula: Yield = [(fruit mass, kg x planting density hectare^{-1}) x (percent plant mortality ÷ 100)] x [1 ton ÷ 1000 kg]
7. Translucency rating - five samples per experiment unit per harvest round were utilized in this parameter. Fruits were cut vertically into halves. Translucency rating was determined using the hedonal rating scale as shown in Table 3 (Valleser, 2019):

Average translucency rating was computed using the formula:

$$\text{Average translucency rating} = \frac{\sum \text{Translucency rating}}{\text{Number of fruit samples}}$$

8. Total soluble solids (TSS) - five samples per experiment unit per harvest round were utilized in this parameter. 10 mL of pineapple fruit juice was extracted and brix was measured using an Atago handheld refractometer following the guidance on objective tests to determine quality of fruits and vegetables and dry and dried produce (Organization for Economic Co-operation and Development [OECD], 2005; Valleser, 2019).
9. Titratable acidity (TA) - five samples per experiment unit per harvest round were utilized in this parameter. 10 mL pineapple juice was placed inside a beaker, and 2 mL of phenolphthalein solution was added. Titration then follows by adding a basic solution (0.1 N sodium hydroxide, NaOH) to the fruit juice until the color turns to

Table 3
Determination of translucency rating using the hedonal rating scale

Translucency rating	Description	Remarks
1	No translucency	Good fruit for export
3	Translucence affecting ≤ 10 % of flesh	Tolerable for export
5	Translucence affecting ≤ 15 % of flesh	Tolerable for local
7	Fruits with translucence affecting > 15% of flesh	Reject

light red. The formula was then used to determine the TA in the form of citric acid (OECD, 2005; Valleser, 2019):

TA = [volume (mL) of NaOH added x 0.1 (NaOH concentration) x 0.064 x 100] ÷ volume of juice (mL)

10. TSS/TA- mean TSS and mean TA values of the fruits evaluated were used to determine this data. TSS/TA value was basically determined using the formula (OECD, 2005; Valleser, 2019):

TSS/TA= mean TSS value ÷ mean TA value

Statistical Analysis

All data gathered were subjected to analysis of variance (ANOVA). Post hoc comparison between means was performed through Duncan's Multiple Range Test (DMRT) using the Statistical Package for Social Sciences (SPSS) 14.0 for Windows Evaluation software.

RESULTS AND DISCUSSION

Growth

Growth of 'MD-2' pineapple plants prior to flower induction treatment were found to be significantly influenced by the amount of phosphorus applied in the soil. An increasing height of 'MD-2' pineapple plant was noticeable with the increasing amount of phosphorus application (Figure 1). Tallest plants (106.55 cm) were those applied with 211 kg of P ha^{-1}. In contrast, no application of P resulted to the shortest height of pineapple plants (80.22 cm) among treatments. This result implies that the dearth phosphorus availability for 'MD-2' pineapple plants would cause underdeveloped plant growth. According to Malézieux and Bartholomew (2003), growth of all plant parts is reduced as a result of phosphorus deficiency although pineapple requires only little amount of the said essential nutrient. Based on local pineapple grower's experience, T. S. Castro (personal communication, November 8, 2013) stated that 'MD-2' pineapple plants should reach a minimum height of 90 cm prior to flower induction treatment to produce marketable fruits.

On the other hand, the heaviest plant (2.23 kg) was obtained with the application of 211 kg P ha^{-1} treatment although comparable with the plant mass (2.16 kg) of 'MD-2' pineapple applied with 169 kg P ha^{-1} (Figure 2). On the contrary, inferior plant mass (1.74 kg) was exemplified by 'MD-2' pineapple starved with phosphorus. Plant mass at flower induction treatment can be used as baseline to predict fruit size of pineapple. Correlation between plant mass and fruit mass can be as low as 0.4 in areas with warm nights and fast growth rates and can exceed 1.0 where irradiance is high and night temperatures are cool (Hepton, 2003).

'MD2' pineapple showed an increasing D-leaf mass as amount of phosphorus applied increases (Figure 3). A D-leaf is the youngest among the adult leaves of pineapple as well as the most physiologically active. Its mass is reflective of the readiness of pineapple for flower forcing and bases

for forecasting fruit mass. In 'Flhoran 41' pineapple, a D-leaf of 70 g is sufficient to get exportable fruits, whereas 80 g is the standard for "MD2" and "Smooth Cayenne" (Fornier et al., 2007). Results of this study revealed that 'MD-2' pineapple not applied with phosphorus were the only plants having D-leaf mass (77.98 g) below 80 g.

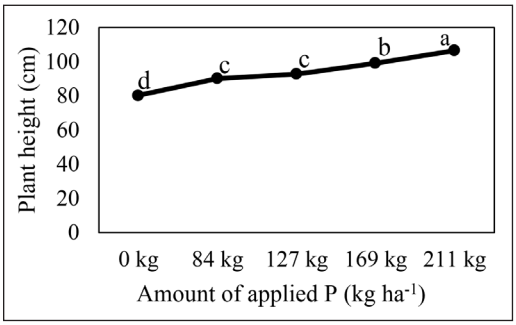

Figure 1. Plant height of 'MD-2' pineapple a day prior to flower induction treatment in response to varying amounts of applied P
Note: Data expressed in figures with same letter as superscript are not significantly different at 0.05 level DMRT

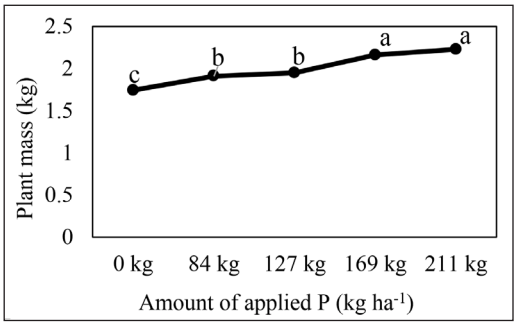

Figure 2. Plant mass of 'MD-2' pineapple a day prior to flower induction treatment in response to varying amounts of applied P
Note: Data expressed in figures with same letter as superscript are not significantly different at 0.05 level DMRT

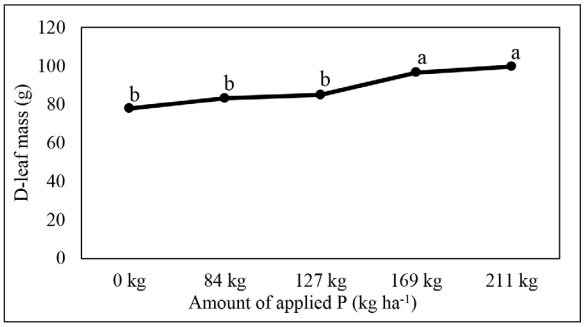

Figure 3. D-leaf mass of 'MD-2' pineapple plant a day prior to flower induction treatment in response to varying amounts of applied P
Note: Data expressed in figures with same letter as superscript are not significantly different at 0.05 level DMRT

Nutrient Levels

Visual deficiency symptoms and critical nutrient levels are used to check the nutritional status of plants. In pineapple, the D-leaf is used because it is the only leaf that can be consistently identified and, as the youngest almost physiologically mature leaf, it reflects current plant nutrient status with acceptable accuracy (Malézieux & Bartholomew, 2003).

Results indicate that all plants have sufficient nutrient levels at flower induction treatment except for nitrogen (Table 4). Nitrogen is very mobile in plants, hence

D-leaf tissue analysis is not preferred by pineapple growers to assess nitrogen level (T. S. Castro, personal communication, November 8, 2013). On the other hand, all treatment plants have comparable nutrient levels except for P and Zn.

Moreover, the highest P uptake (0.29%) was notable in plants applied with 169 kg P hectare^{-1}. However, this result was comparable to 84 kg P hectare^{-1} and 127 kg P hectare^{-1} treatments which resulted to 0.28% and 0.27% P uptakes of plants, respectively. In contrary, the highest amount of P (211 kg P hectare^{-1}) application resulted to least P uptake (0.21%) of 'MD-2' pineapple plants. Although P uptakes of all treatment plants were beyond the critical level (T. S. Castro, personal communication, November 8, 2013), results of this study revealed that plants not applied with P had higher P level at flower induction treatment compared to plants applied with the highest amount (211 kg hectare^{-1}) of P. There could be other factors involved in this condition. Mourichon (as cited in Malézieux & Bartholomew, 2003, p. 150) stated that mycorrhizal associations existed in pineapple roots. However, mycorrhizal fungi apparently do not contribute significantly to the P nutrition of pineapple, except where soil P is extremely low according to Aziz et al. (as cited in Malézieux & Bartholomew, 2003, p. 150). The soil analysis of the experiment area has low available P (8.48 ppm) based on soil analysis. Hence, it was hypothesized that the mychorrhizal fungi contributed to P nutrition of 'MD-2' pineapple plants. Conversely, excessive P (211 kg hectare^{-1}) application in Adtuyon clay soil does not warrants increase in P uptake of 'MD-2' pineapple plants. Spironello et al. (2004) reported also that the addition of P fertilizer had a little effect only on the concentration of leaf P. Moreover, Garcia et al. (2017) suggested only two rates (138 kg hectare^{-1} and 161 kg hectare^{-1}) of P depending on the type of cultivars grown and soil classifications of the area in Mexico.

Table 4
Nutrient levels of 'MD-2' pineapple plants a day prior to flower induction treatment as influenced by varying amounts of applied P

Amount of applied P (kg ha^{-1})	Nutrient levels							
	----------%----------					----------ppm----------		
	N	P	K	Ca	Mg	Fe	B	Zn
0	0.70	0.25^{b}	4.77	0.18	0.24	97.59	20.72	86.57^{a}
84	0.72	0.28^{ab}	4.79	0.16	0.24	86.11	18.78	90.56^{a}
127	0.73	0.27^{ab}	4.33	0.17	0.23	94.68	19.80	85.18^{ab}
169	0.76	0.29^{a}	4.52	0.15	0.22	102.4	22.10	97.83^{a}
211	0.74	0.21^{c}	4.64	0.17	0.21	76.96	22.38	71.76^{b}
Critical level$^{1/}$	1.00	0.20	3.50	0.10	0.20	80.00	10.00	36.00
Significance	ns	**	ns	ns	ns	ns	ns	*

$^{1/}$ critical nutrient levels adopted by MKADC farm as of calendar year 2010 (T. S. Castro, personal communication, November 8, 2013)
Mean(s) within same column with same superscript(s) is/are not significantly different at 0.05 level DMRT
ns- not significant; *- significant; **- highly significant

With regards to Zn, the highest uptake (97.83 ppm) was remarkable in plants applied with 169 kg P hectare^{-1}. This data was comparable to lower amounts (0, 84 and 127 kg hectare^{-1}, respectively) of P applications. On the other hand, plants applied with the highest amount (211 kg hectare^{-1}) of P have the lowest Zn level at flower induction treatment. Excessive application of P leads to Zn deficiency in pineapple (Malézieux & Bartholomew, 2003). However, results of stem apex analysis have shown that Zn uptakes of all treatment plants were beyond the critical level. Malézieux and Bartholomew (2003) suggested that a level of 36 ppm on a dry mass basis in the stem apex of pineapple would be associated with typical zinc-deficiency symptoms.

Fruit Mass and Yield per Hectare of 'MD-2' Pineapple Plants as Influenced by Varying Amounts of P Application

The heavier fruits (means of 1.42 kg and 1.44 kg) were obtained from plots applied with 169 and 211 kg hectare^{-1} P applications (Figure 4) although these fruit mass data were comparable to the mass of fruits harvested in plots applied with 0, 84 and 127 kg P hectare^{-1}, respectively. In terms of yield per hectare (Figure 5), application of 211 kg P hectare^{-1} resulted to the heaviest yield (98.28 tons hectare^{-1}) of 'MD-2' pineapple. This yield was followed and statistically comparable with 169 kg P hectare^{-1} treatment with 96.92 tons hectare^{-1}. The pineapple plants starved with P produced the lowest yield (76.80 tons hectare^{-1}) and was comparable to yields (80.91 and 83.16 tons hectare^{-1}) obtained from plots applied with 84 kg P hectare^{-1} and 127 kg P hectare^{-1} treatments, respectively. Results indicate that 'MD-2' pineapple still produce fruits even without P in the fertilizer program under Adtuyon clay soil. Spironello et al. (2004) reported that 'Smooth Cayenne' pineapple had slight response to P, despite its low availability in the soil. In this study, the yield differences of 21.48 tons hectare^{-1} (between 211 and 0 kg P hectare^{-1} treatments) as well as 20.12 tons hectare^{-1} (between 211 and 0 kg P hectare^{-1}

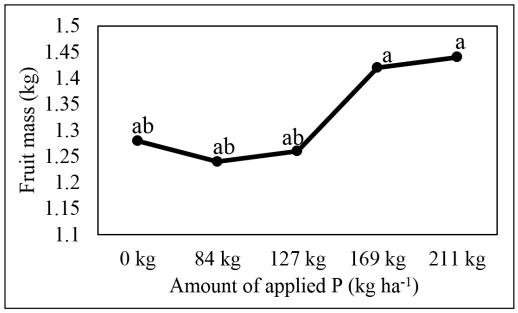

Figure 4. Fruit mass of 'MD-2' pineapple at harvest in response to varying amounts of applied P
Note: Data expressed in figures with same letter(s) as superscript are not significantly different at 0.05 level DMRT

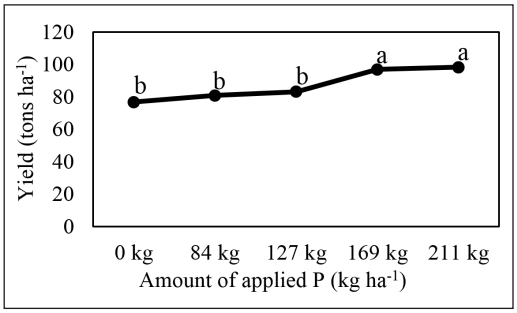

Figure 5. Yield per hectare of 'MD-2' pineapple in response to varying amounts of applied P
Note: Data expressed in figures with same letter as superscript are not significantly different at 0.05 level DMRT

treatments) would still be economical to commercial pineapple growers. Cost of P application can be compensated by the yield of 'MD-2' pineapple. In Jimma, Southwest Ethiopia condition, Tewodros et al. (2018) suggested the application of 134.8 kg hectare^{-1} as the economical dose of P application.

Fruit Physico-Chemical Properties of 'MD-2' Pineapple at Harvest as Influenced by Varying Amounts of P Application

Fruit physico-chemical properties of 'MD-2' pineapple at harvest in response to varying amounts of P revealed that translucency was not displayed by 'MD-2' pineapple fruits in response to varying amounts of P applications (Table 5). On the other hand, TSS values ranged from 16.96-17.50, whereas TA values ranged from 0.47-0.50 and TSS/TA values ranged from 36.46-39.18. These values surpassed the market standard which requires only a minimum TSS value of 13, TA range value of 0.5-0.7 and TSS/TA range value of 20-40 ("Fresh fruit varieties", 2006). Results indicate that P nutrition has no significant effects on the fruit physico-chemical properties of 'MD-2' pineapple grown in Adtuyon clay soil. This result was in line to the findings of Spironello et al. (2004) of which phosphorus had little importance in the fruit quality of 'Smooth Cayenne' pineapple.

Table 5
Fruit physico-chemical properties of pineapple at harvest in response to varying amounts of applied P

Amount of applied P (kg ha^{-1})	Physico-chemical properties			
	Translucency rating	Total soluble solids (TSS)	Titratable acidity (TA)	TSS/TA
0	1	17.18	0.48	37.50
84	1	17.46	0.50	36.46
127	1	17.73	0.47	39.18
169	1	17.50	0.48	37.94
211	1	16.96	0.48	37.36
Market requirement[1/]	1	14.00	0.5-0.7	20-40
Significance	ns	ns	ns	ns

[1/] Source: "Fresh fruit varieties" (2006)
ns- not significant

CONCLUSIONS

The application of 169 kg P hectare^{-1} would result in optimum growth and yield of 'MD-2' pineapple under Adtuyon clay soil in Bukidnon, Philippines. On the other hand, fruit physico-chemical properties of 'MD-2' pineapple is not dependent on the amount of P applied in the soil. These results can be used as guide to commercial pineapple growers in order to have a sound decision making with regards to phosphorus fertilizer application.

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Analysis of Weed Management Options on Weed Infestation and Cane Yield of Sugarcane

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ABSTRACT

A field study was conducted in a sugarcane field in the southern Guinea savanna ecology of Nigeria to document the influence of weed management methods on the pattern of weed seedlings emergence and cane yield of sugarcane across 3 crop cycles. The experiment which had 6 weed management strategies (weedy check; pre- terbutylazine at 2.0 kg a.i/ ha + supplementary hand hoeing (SHH) at 4, 10 and 16 weeks after planting (WAP); post-ametryn at 3.0 kg a.i/ha + SHH at 10 & 16 WAP; post-dicamba at 0.5 kg a.i/ha + SHH at 10 and 16 WAP; pre-terbutylazine at 2.0 kg a.i/ha + post- 2, 4-D at 3.0 kg a.i/ ha; and monthly hand hoeing) was laid out in a randomized complete block design and replicated three times during 2014, 2015 and 2016 growing seasons. Weed seedlings emergence was monitored in 0.5m^2 quadrats continuously at 1, 2, 4, 6, 8 and 12 months after planting (MAP). The result shows that, the weed spectrum comprised 57-62% grasses, 23-29% broadleaves and 13% were sedges. *Dactyloctenium aegyptium* had 12.05% relative abundance, *Digitaria horizontalis* (10.84%), *Cynodon dactylon* (8.0%), and *Tephrosia linearis* (8.80%) *Eclipta alba* (7.50%), *Echinochloa obstusiflora* (7.17) were the top dominant weed species identified in plant and ratoon crops, respectively. Weed seedling emergence peaks occurred at 1 and 4 MAP across weed control options and crop cycles. Pre-terbutylazine at 2.0 kg a.i/ha + post 2, 4-D at 3.0 kg a.i/ ha was adjourned to minimized weeds emergence which translated into higher cane yields across crop cycles. This study concludes that pre-emergence application of terbutylazine at 2.0 kg a.i/ha + post-emergence of 2,4-D at 3.0 kg a.i/ ha prior to peak periods of weed emergence is the most stable and ideal weed

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management option for sugarcane estates in the southern Guinea savanna ecology of Nigeria.

Keywords: 2, 4-D, biplot analysis, cane yield, dicamba, ratoon crops, sugarcane, terbutylazine, weed emergence

INTRODUCTION

Sugarcane is mostly grown in the savanna regions of the Nigerian ecology which is characterized by erratic rainfall pattern. These changes in climate have an indirect influence on weed emergence which is also directly influence by land use, land intensification and crop management. The slow growth at the beginning of sugarcane life cycle couple with wide plant spacing provides weeds the opportunity to strive well in such ecology (Singh & Kumar, 2013) and this lowers the competitive ability of the crop thus reducing cane and sucrose yields (Chattha et al., 2007).

Weed emergence occurs in flushes and weed species exhibits different patterns of emergence and these have negative effect for site-specific weed management. The above weed characteristics limit the ability to predict weed emergence while management decisions could be less efficient, less reliable, and might be prone to agronomic and financial risk (Yirefu et al., 2013). Therefore a better understanding of sugarcane-weed interactions and identification of emergence pattern of dominant weed species with respect to sugarcane crop cycles will assist in making informed decisions, timely operations, and better management.

Knezevic et al. (2003) reported a significant yield increase due to the application of split and lowered doses of herbicides while Takim et al. (2017b) obtained a significant high maize grain yield with application of pre-Primextra [proprietary mixture of metolachlor (290 g/L) and atrazine (370 g/L)] and post-Guardforce (Nicosulfuron 40 g/L) due to low level of weed interference. Kandil and Kordy (2013) believed that combining herbicides with supplementary weeding control would efficiently eradicate stunting weeds. Olatunji et al. (2016) reemphasized that herbicides with one or two hand weeding ensured a broad spectrum for weed control over a longer period of time. Takim et al. (2017b) concluded that, although herbicides and supplementary weeding might lead to a reasonable high yield, but the method was tedious, time-consuming and not appropriate for large scale production especially a long duration crop like sugarcane. This study therefore was conducted to evaluate weed management options on the pattern of weed seedlings emergence and sugarcane yield across three cropping cycles.

MATERIALS AND METHODS

Description of Study Area

The study was carried out during the 2014, 2015 and 2016 growing seasons at Josepdam Sugar Company Bacita, Nigeria. The site is located on longitude 9° 05' N; latitude 4° 57' E and 93.5 m above sea level. The average rainfall recorded during the study years was 1029.4 mm, bimodal rainfall distribution with peaks in June and September, the

temperature range between minimum of 28°C and maximum of 34°C. The area is characterized by well drained sandy loam soil with good soil nutritional status.

Experimental Layout and Field Establishment

The experiment which had 6 weed management strategies (weedy check; pre-terbutylazine at 2.0 kg a.i/ha + supplementary hand hoeing (SHH) at 4, 10 and 16 weeks after planting (WAP); post-ametryn at 3.0 kg a.i/ha + SHH at 10 and 16 WAP; post-dicamba at 0.5 kg a.i/ha + SHH at 10 and 16 WAP; pre-terbutylazine at 2.0 kg a.i/ha + post- 2, 4-D at 3.0 kg a.i/ ha; and monthly hand hoeing) was laid out in a randomized complete block design and replicated three times during the plant crop in 2014, first ratoon crop in 2015 and second ratoon crop during the 2016 growing seasons. Ratoon is new sugarcane stand which develops from the underground subterranean buds on stubble of harvested plant cane. Such first regrowth is called first ratoon crop and subsequent regrowth after the harvest of first ratoon is a second ratoon crop and so on.

Allotted weed control treatments, field establishment and maintenance are as detailed in Takim and Suleiman (2017).

Data Collection

Quadrat measuring 0.2 5 m × 0.25 m, was placed at four random locations within each plot at 1, 2, 4, 6, 8 and 12MAP. The emerged weed seedlings were observed, counted, pulled for identification to species level with the aid of relevant weed handbooks like West African weeds by Akobundu et al. (2016). The weeds were separated into broadleaves, grasses and sedges. Weed fresh biomass was determined using a weighing balance and the dry biomass was obtained by subjecting the sampled weeds to oven drying. Data on cane yield was estimated at harvest (12MAP).

Analysis of Variance

The composition of the weed flora was analyzed by calculating the relative abundance (RA) of each species within each experimental field as in Fadayomi and Takim (2009). Crop cycle – weed control interaction for each weed species and morphological group was determined at $p \leq 0.05$ while significant interactions were further analyzed using a biplot software to identify dominant weed species, morphological group and ideal weed control strategy for sugarcane cultivation in the Nigerian savanna.

RESULTS AND DISCUSSION

A total of forty-three (43) weed species were encountered across the trial sites (Table 1) which comprised 63% annual weed species, 28% perennials and 9% were either annuals or perennials depending on the environmental variation. Grasses made up of 66.7% of the 43 weed species identified, 20.0% were sedges and 13.3% were broadleaved. Based on the level of importance, 15 weed species were identified as most prevalent species: *Dactyloctenium aegyptium* (9.61%); *Digitaria horizontalis* (9.36%); *Echinochloa stagnina* (6.87);

Cynodon dactylon (6.12%); *Chroris pilosa* (5.74%); *Ludwigia decurrens* (5.62%); *Paspalum scrobiculatum* (5.24%); *Cyperus iria* (4.87%); *Tridax procumbens* (4.39%); *Cyperus haspan* (3.62%); *Sida acuta* (3.62%); *Senna occidentalis* (3.37%); *Andropogon gayanus* (3.49%); *Rottbeollia cochinchinensis* (2.76%); and *Sorghum arundinaceum* (2.62%).

Table 1
Relative abundance of weed species encountered on sugarcane ecology in Bacita, Nigeria

FAMILY	Weed species	MG	Relative Abundance (%)		
			PC	1RC	2RC
Amaranthaceae	*Amaranthus spinosus* Linn.	B	0.00	0.00	0.56
	Celosia Isertii C.C.Townsend	B	0.00	0.00	1.89
	Celosia leptostachya Benth.	B	0.00	0.00	1.61
Asteraceae	*Conyza sumatrensis* (Retz.) Walker	B	0.00	0.00	0.08
	Eclipta alba (L.) Hassk	B	1.23	2.93	4.39
	Malanthera scandens (Schum. & Thonn.)	B	0.28	0.00	0.00
	Tridax procumbens Linn.	B	3.68	4.29	5.27
	Vernonia cinerea (Linn.) Less.	B	0.42	0.00	0.31
	Vernonia perrottetti Sch. Bip.	B	0.28	0.98	5.73
Caesalpinioideae	*Anthonotha macrophylla* P. Beauv.	B	0.21	0.00	0.21
	Chamaecrista minosolides (L.) Greene	B	0.00	1.29	0.19
	Senna occidentalis (L.) Link	B	3.27	2.85	0.00
Cleomaceae	*Cleome viscosa* L.	B	2.34	3.07	3.10
Combretaceae	*Combretum zenkeri* Engl. & Diels	B	0.14	1.06	3.15
Commelinaceae	*Commelina benghalensis* L.	B	0.00	1.01	1.34
	Commelina diffusa Burm. f.	B	0.59	0.24	0.00
Convolvulaceae	*Evolvulus alsinoides* (Linn.) Linn.	B	2.08	2.76	5.55
	Ipomoea eriocarpa R. Br.	B	0.00	0.00	0.06
Cyperaceae	*Cyperus esculentus* Linn.	S	0.00	0.96	0.00
	Cyperus haspan Linn.	S	3.92	2.36	3.29
	Cyperus iria Linn.	S	4.53	4.70	5.89
	Cyperus rotundus Linn.	S	1.95	1.66	1.63
	Fimbristylis littoralis Gaudet	S	0.91	1.91	3.55
Euphorbiaceae	*Euphorbia hyssopifolia* Linn.	B	0.98	0.73	0.65
Laminaceae	*Solenostemon monostrochyus* (P. Beauv.) Brig	B	1.02	1.00	1.42
Malvaceae	*Malvastrum coromandelianum* (Linn.) Garcke	B	0.49	0.00	1.27
	Sida acuta Burn. f.	B	3.20	4.46	0.26
Onagraceae	*Lugwigia abyssinica* A. Rich	B	1.46	1.75	1.61
	Lugwigia decurrens Walt.	B	5.62	4.21	0.00
Papilionoideae	*Indigofera hirsuta* Linn.	B	0.49	1.22	4.50
	Trephrosia linearis (Willd.) Pers.	B	1.79	1.05	0.00
Poaceae	*Andropogon gayanus* Kunth	G	4.02	1.38	1.74
	Andropogon tectorum Schum. & Thonn.	G	0.53	1.59	2.03
	Brachiaria falcifera (Trin.) stapf	G	1.40	6.75	0.00

Table 1 *(continue)*

FAMILY	Weed species	MG	Relative Abundance (%)		
			PC	1RC	2RC
Poaceae	*Chloris pilosa* Schumach	G	6.07	6.29	0.00
	Cynodon dactylon (Linn.) Pers.	G	5.50	3.18	3.60
	Dactyloctenium aegyptium (Linn.) P. Beauv.	G	9.75	5.02	5.16
	Digitaria horizontalis Willd.	G	8.74	5.19	6.34
	Echinochloa obstusiflora Stapf	G	1.60	1.35	3.00
	Echinochloa stagnina (Retz.) P. Beauv.	G	5.95	5.55	0.00
	Oryza barthii A. Chev.	G	1.27	2.87	0.03
	Panicum laxum Sw.	G	1.75	1.79	0.00
	Pannicum repens Linn.	G	0.24	0.13	0.00
	Paspalum conjugatum Berg.	G	0.38	0.00	0.00
	Paspalum scrobiculatum Linn.	G	5.27	5.83	5.00
	Rottbellia cochinchinensis (Lour.) Clayton	G	2.01	1.43	4.82
	Setaria pumila (Poir.) Roem & Schult.	G	0.24	1.04	0.00
	Sorghum arundinaceum (Desv.) Stapf.	G	2.77	2.68	2.48
Rubiaceae	*Mitracorpus villosus*(Sw.) DC.	B	0.37	0.00	1.18
Solanaceae	*Physalis angulate* Linn.	B	0.68	0.90	2.74
Sterculiaceae	*Melochia corchorifolia* Linn.	B	0.02	0.00	0.00
Tiliaceae	*Corchorus tridens* Linn.	B	0.55	0.56	4.37

MG =Morphological group, PC=Plant crop, 1RC= First Ratoon crop, 2RC =Second Ratoon crop, B= Broadleaves, G= Grasses, S= Sedges

The results from this study agreed with Takim and Amodu (2013) who reported that broadleaves had higher diversity in species on the sugarcane fields but members of the Poaceae dominated the sugarcane weed community Takim et al. (2014). There is a gradual shift from the natural vegetation with predominant annual grasses and broadleaves to perennial weed species in the ratoon cycles. This call for the use of broad spectrum herbicides or mixture of herbicides during the plant crop while a schedule herbicide rotations plan across the ratoon cycles to avoid a decrease responses of weed species to herbicide as a result of its continuous application.

The pattern of weed emergence indicates that weed seedlings emerged throughout the trial period (Figure 1). The peak of weed 'seedling' emergence was at 1 and 4MAP then declined gradually to the lowest emergence at 8MAP and suddenly increased. This implies that, weed control should be targeted during the peak periods of weed seedlings emergence (Takim & Fadayomi, 2013).

Figure 2 is a polygon view biplot showing which weed control strategy had a better efficacy and at which cropping cycle. Guilly et al. (2017) defined the small circle on the polygon as the average-environmental axis (AEA) and the arrow

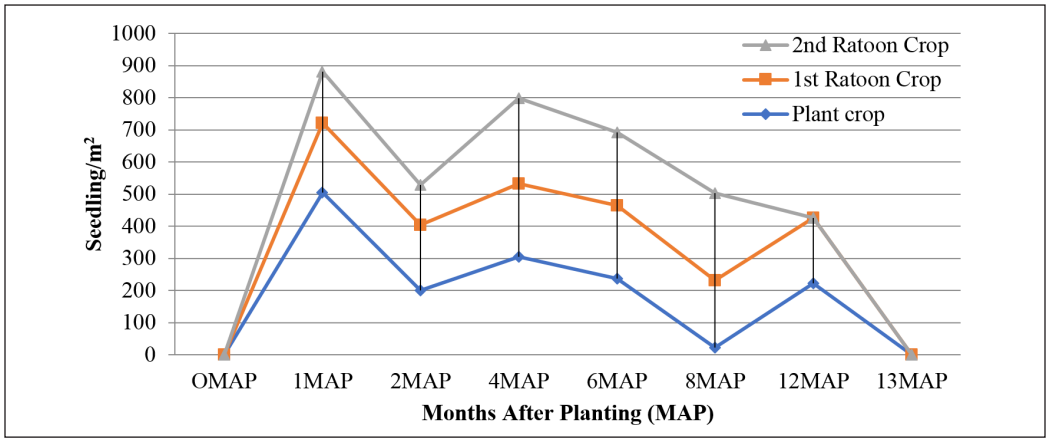

Figure 1. Pattern of weed seedling emergence in a sugarcane ecology

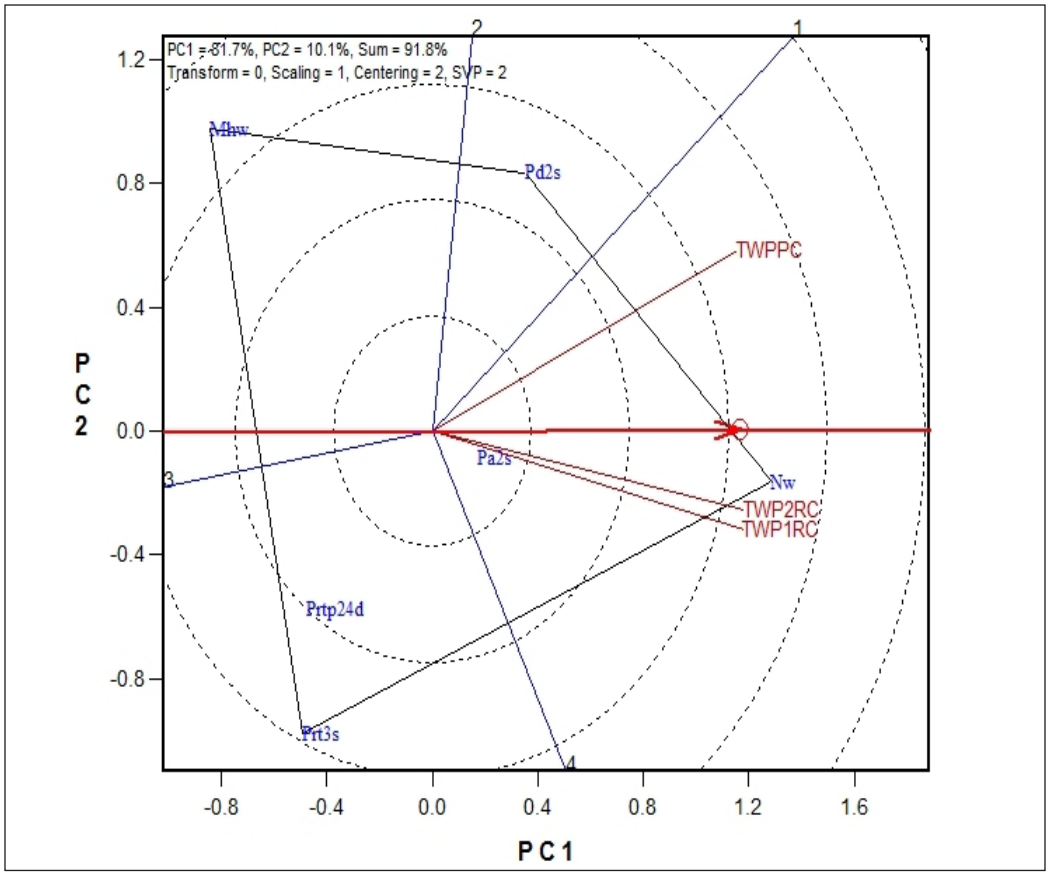

Figure 2. The polygon view of biplot showing which weed control strategy won in which sugarcane cropping cycle TWPPC = total weed population at plant crop, TWP1RC = total weed population at first ratoon crop, TWP2RC = total weed population at second ratoon crop, Pd2s = post dicamba + 2 hoeing, Pa2s = post-ametryn + 2 hoeing, Prtp24d = pre-terbutylazine and 2, 4-D, Prt3s = pre-terbutylazine + 3 hoeing, Mhw = monthly hand handing, Nw = no weeding (weeding check)

pointing to it indicated the direction of the AEA and weed control option. The line ranks the weed control strategy according to their mean efficacy that it approximates the contribution of each method to the cropping cycle. No Weeding (Nw) is the most stable weed control option while post-Ametryn and 2 supplementary hand hoeing (Pa2s) the most unstable weed control option. The monthly hand weeding (MHW) and pre-terbutylazine + post 2, 4-D (Prtp24d) plots had less weed population compared to no weeding plots across the crop cycles. The crop cycles also differed weed population while plant crop (TWPPC) had high weed population but unstable, first ratoon crop (TWP1RC) and second ratoon crop (TWP2RC) had high but stable weed population across the growing seasons.

Figure 3 showed that PC (plant crop) and 1RC (first ratoon crop) had high weeds population as compared to 2RC (second ratoon crop) while grasses were relatively high during PC and 1RC, broadleaves dominated 2RC. The line with the small circle defined by the average efficacy across the cycles and thus it expressed the weed control method contribution to cane yield. Ideal method should have high project towards the arrowed line and near zero projection (Yan, 2001). Pre-terbutylazine + post-2, 4-D was the most stable weed

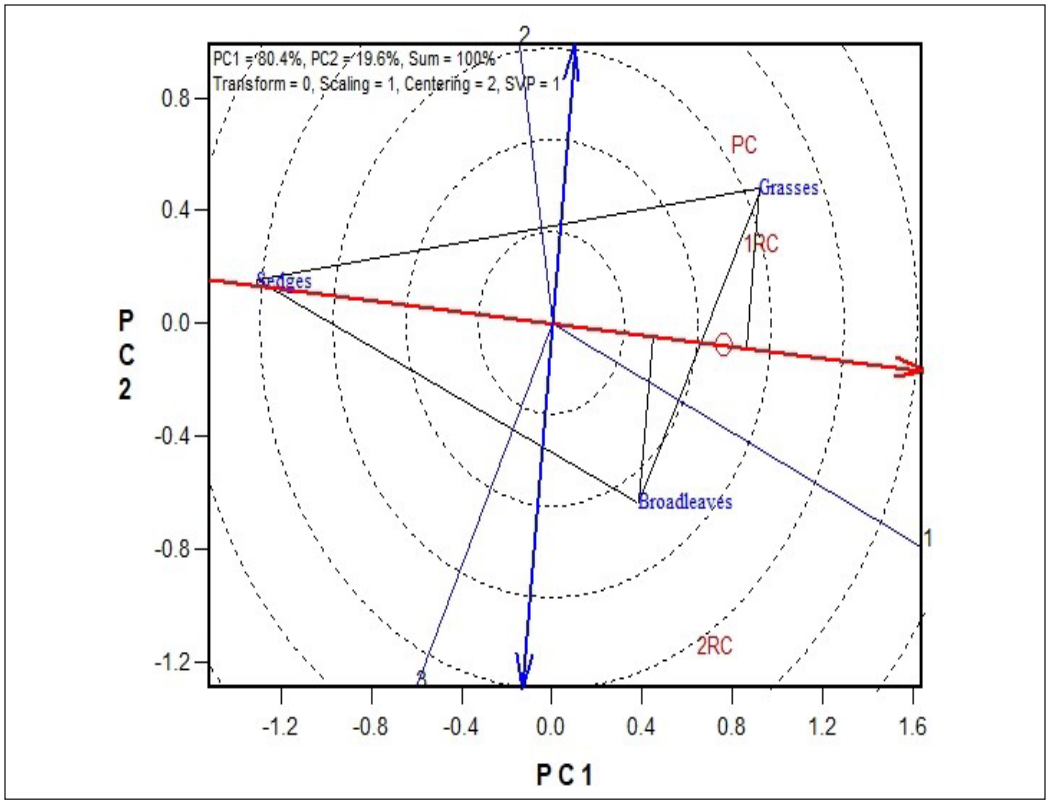

Figure 3. The polygon view of biplot showing which morphological group won in which sugarcane cropping cycle PC = plant crop, 1RC=first ratoon crop, 2RC = second ratoon crop

control method and it was most ideal for plant crop cycle (Figure 4). Other ideal methods but unstable are post-dicamba + 2 supplementary hoe weeding (pd2s) and monthly hoeing. Figure 4 also showed that cane yield of plant crop (CYPC) was more representative and stable while cane yields of other cycles were unstable (Olaoye et al., 2017).

The polygons are divided into several sectors and some of these sectors have weed management options within them suggesting the possibility of different mega-environments (Yan & Rajcan, 2002) existing for the crop cycles of sugarcane (Mattos et al., 2013). Figure 4 shows three mega-environments which included: PrTP2, 4-D, Hoeing and Pd2s as a mega-environment with CYPC, CY1RC and CY2RC. The PC was the best performing crop cycle and PrTP2, 4-D as the most ideal weed management option. Second mega environment had Weedy with no crop cycle and the third mega-environment had two weed management options (Prt3s and Pa2s). However, this mega-environment pattern needs verification through other multi-environment trials for this target region (Takim et al., 2017a). Regarding this pattern, PrTP2, 4-D was the most favourable weed management option having high efficacy across the crop cycles.

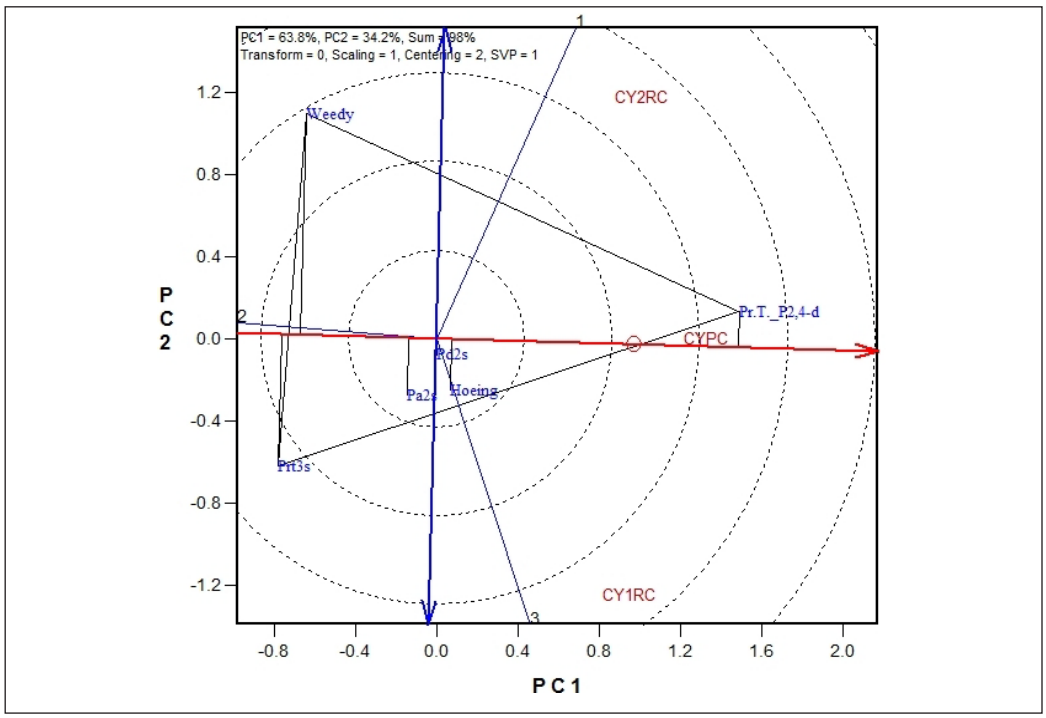

Figure 4. The polygon view of biplot showing which weed control strategy won in sugarcane yield across cropping cycle

CYPC = cane yield of plant crop, CY1RC = cane yield of first ratoon crop, CY2RC = cane yield of second ratoon crop, Pd2s = post dicamba + 2 hoeing, Pa2s = post-ametryn + 2 hoeing, Prtp24d = pre-terbutylazine + 2, 4-D, Prt3s = pre-terbutylazine + 3 hoeing

CONCLUSION

The study concluded that sugarcane ecology was dominated with grasses while broadleaved species were high in diversity; weed control should be targeted at 1 and 4 months after planting; and pre-terbutylazine at 2.0 kg a.i/ha + post-2, 4-D at 3.0 kg a.i/ ha was adjourned to minimized emergence of weed seedlings across crop cycles. This study therefore recommends that pre-emergence application of terbutylazine at 2.0 kg a.i/ha and post-emergence of 2,4-D at 3.0 kg a.i/ ha prior to weeds peak periods could be the appropriate weed control option to the advantage of sugarcane in the southern Guinea savanna of Nigeria.

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Profile of Phenolic Compounds, DPPH-Scavenging and Anti α-Amylase Activity of Black Rice Bran Fermented with *Rhizopus oligosporus*

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ABSTRACT

Fermentation by the solid state fermentation (SSF) technique is an alternative method that may increase bioactive compounds and their functionality due to enzymatic activities. This study evaluated the effect of the fermentation time of black rice bran on the bioactive compound profile and the antioxidant and anti-α-amylase activities of the compounds. Fermentation was performed by using *Rhizopus oligosporus* (*R. oligosporus*) for 0, 24, 48, 72 and 96 hours. Fermented rice bran samples were collected every 24 h. The results showed that the bioactive compounds in black rice bran significantly increased ($p < 0.05$) during fermentation. Further significant increases were found for DPPH free radical scavenging activity (76.91 ± 0.06%) and α-amylase inhibition (73.05 ± 0.25%) in fermented (96 h) black rice bran compared to those in non-fermented bran (0 h). It was confirmed that fermentation had a significant effect on increasing the amount and activity of bioactive compounds in black rice bran.

Keywords: Anti α-amylase, antioxidant, bioactive compound, fermented bran, *Rhizopus oligosporus*

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INTRODUCTION

Bran is a by-product of rice milling that contains high nutrient contents such as oil (12-22%), protein (11-17%), food fibre (6-14%), water (10-15%), and ash (8-17%). It also contains bioactive compounds such as

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phenolic acids, flavonoids, anthocyanins, γ-oryzanol, tocopherols, phytic acid, and others (Sharif et al., 2014). These bioactive compounds are known to have antioxidant activity that can scavenge free radicals, enhance the immune system of the body, prevent high blood pressure, hyperlipidaemia, and hyperglycaemia and reduce the risk of cancer (Nagendra et al., 2011; Zhang et al., 2010).

The content of rice bran bioactive compounds varies depending on the type of rice, climatic conditions and processing methods (Gul et al., 2015). The highest total amounts of phenolic and flavonoid compounds are found in black rice bran (Chen et al., 2017). According to Tuarita (2017), the IC_{50} value of the methanol extract of black rice variety Cempo Ireng (67.58 µg/ml) was lower than that of red and white rice (82.50 µg/mL and 410.02 µg/mL, respectively). In general, these compounds are intricately bonded to polysaccharides, lignocelluloses, fat or protein matrices via ester bonds (Cheng et al., 2016; Oliveira et al., 2012; Rashid et al., 2015). Phenolic compounds with ester bonds have a complex structure and a large molecular weight, there the compounds cannot be absorbed by humans; their utilisation in the body is also not optimal. Several methods can be used to increase the concentration of bioactive compounds in bran, one of which is fermentation. In fermentation, microorganisms produce enzymes that hydrolyse the complex of bioactive compounds to result in more active free bioactive compounds (Oliveira et al., 2012).

Fermentation with the solid state fermentation (SSF) method is one of the fermentation techniques that can produce better product characteristics, such as improving the nutritional value and bioactive compounds and their functionality which can increase the value of rice bran (Pourali et al., 2010; Razak et al., 2015). The SSF of rice bran using various microorganisms with different fermentation times has been reported, such as *Rhizopus oryzae* for 120 h and 96 h (Oliveira et al., 2012; Schmidt & Furlong, 2012, Schmidt et al., 2014), *R. oryzae* and *R. oligosporus* for 96 h (Zulfafamy et al., 2018), *R. oligosporus* and *Monascus purpureus* for 12 days (Razak et al., 2015), *Saccharomyces boulardii* (Ryan et al., 2011), *Aspergillus oryzae* and *R. oryzae* for 12 days (Razak et al., 2017) and *Saccharomyces cerevisiae* with 24 h of fermentation (Chaiyasut et al., 2017). These studies reported that fermentation time of 96 h can increase the bioactive compounds and bran fermentation activity.

The utilisation of fungi from the genus *Rhizopus* sp. is highly effective in the SSF process due to its capability of producing enzymes that can degrade lignocellulose and polysaccharide matrices, increase the chemical content and bioactivity, and increase the availability of bioactive compounds in the bran (Oliveira et al., 2012; Schmidt & Furlong, 2012). The utilisation of *Rhizopus* sp. also improves digestibility and specific catalytic activity without producing toxins or toxic substances in the environment during the controlled fermentation process (Oliveira et al., 2012; Schmidt et al., 2014; Razak et al., 2015).

The *R. oligosporus* is able to provide good results in the bran fermentation process for 12 days (Razak et al., 2015), 72 h and 96 h (Zulfafamy et al., 2018). To date, this study used *R. oligosporus* with fermentation time of 0, 24, 48, 72, and 96 hours, as it has not yet been extensively explored.

The condition of diabetes or hyperglycaemia encourages free radical formation by accelerating reactive oxygen species (ROS) formation that triggers a state of oxidative stress. Oxidative stress causes decreased insulin secretion, inhibits glucose uptake in muscle, and contributes to complications from diabetes, such as heart attacks and hypertension (Hasim et al., 2017). Therefore, antioxidants-rich food intake can be an alternative way to prevent complications from diabetes. The increased bioactivity in rice bran through fermentation is expected to be used in diabetes management. The α-amylase inhibitory activity is a measure that can be analysed as an approach to evaluate the antidiabetic potential of fermented bran. The purpose of this research is to determine the effect of the fermentation time of black rice bran with *R. oligosporus* mould using the SSF technique on the bioactive compound profile, antioxidant activity and α-amylase inhibition of the bran.

MATERIALS AND METHODS

Sample Preparation

The black rice bran sample from the Indonesian varieties of Cempo Ireng was prepared as follows: dry grains of black rice were first removed using a Rice Machine (Satake, Japan) to obtain black brown rice. The skin of the rice was broken and then the rice was milled with a Grain Testing Mill (Satake, Japan). The obtained bran was then sieved with a 40-mesh sieve to separate the chaff and groats. Next the bran samples were packaged in plastic bags of high-density polypropylene (HDPE) and stored in a freezer at -20^{o}C for further analysis.

Preparation of the Culture

The culture preparation was performed according to Razak et al. (2015). The *R. oligosporus* was obtained from the Indonesian Institute of Science Research Center. The *R. oligosporus* isolate was prepared in potato dextrose agar (PDA) medium and incubated for 7 days at 30°C. The mould inoculum was then suspended in 10 ml of sterile distilled water. The suspension was calculated as the number of spores up to 10^6 spores/mL. The spore suspension solution was ready to be inoculated into the rice bran for the fermentation process.

The Fermentation Process

The bran fermentation process was performed according to the procedure of Zulfafamy et al. (2018). The fermentation process was initiated with the addition of water to the substrate at as much as 50% of the weight of the substrate (100 g), which aimed to create a moist condition so that mould could subsist. Subsequently, the bran substrate was sterilised at 121°C for 15 min by autoclaving. After that, the sterile culture suspension 15% (v/w) of *R. oligosporus*

(0.15×10^6 spores/g bran) was added to the sterile substrate, mixed well. The plastic container used (22 x 20 cm^2) as a fermenter was then perforated (0.2 mm/1 cm^2) to create aerobic conditions. The fermentation process was carried out at a temperature of 30°C for 24, 48, 72, and 96 hours, hereafter referred to as fermented rice bran (FRB). Non-fermented rice bran (NFRB), which was sterilised under the same conditions served as control (Webber et al., 2014). Next, NFRB and FRB were dried at 50°C for 4 h and stored in a freezer at -18°C until analysed.

Sample Extraction

Each 5 g of sample was extracted with 50 mL of 70% ethanol at room temperature by using a shaker for 30 min and then centrifuged for 15 min at 1207 × *g* (3000 rpm). Then the extract was evaporated (50°C for 1 h) until all the solvents were removed and used for analysis (Cheng et al., 2016).

Total Phenol Analysis

The total phenol analysis was based on Slinkard and Singleton (1977) by the Folin-Ciocalteu method. Gallic acid was used as the standard. A 1 mL sample was mixed with 5 mL of Folin-Ciocalteu (5 min), and then 5% sodium carbonate (1 mL) was added. The sample solution was vortexed and incubated in a dark room for 2 h at room temperature. The sample absorbance was measured by using a UV spectrophotometer at 725 nm. The obtained results were expressed in milligrams of gallic acid equivalent per 100 gramme of dried sample (mg GAE/100 g db).

Total Flavonoid Analysis

The total flavonoid analysis was performed by the colorimetric method of aluminium chloride as described by Chang et al. (2002). The extract 3 mL was added to 7 mL of distilled water. After that it was reacted with 0.1 mL of $AlCl_3$ and 0.1 mL of potassium acetate. The sample absorbance was measured by using a UV-VIS spectrophotometer at 432 nm. Quercetin was used as the standard. The obtained results were expressed in milligrams of quercetin equivalent per 100 gramme of dried sample (mg QE/100 g db).

Total Anthocyanin Analysis

The total anthocyanin content in the fermented bran was measured by the pH difference based on the method described by Lee et al. (2005). The sample was dissolved in KCl buffer with a pH of 1.0 and sodium acetate buffer with a pH of 4.5, and the absorbance of the sample was measured at 510 and 700 nm. The absorbance value was calculated as A = [(A510-A700) at pH 1 - (A510-A700) at pH 4.5]. The results were expressed as mg anthocyanin (cyanidin-3-glucoside) per g of dried sample using 26.9 as the molar coefficient and the molecular weight (BM) of 449.

Analysis of γ-Oryzanol

The γ-oryzanol analysis was conducted by using high-performance liquid chromatography (HPLC) (Sabir et al., 2017). A mixture of methanol and acetonitrile (35:65) was used as the mobile phase with a C-18 column (Zorbax Eclipse XDB

C-18 column 4.6 X 150 nm) and 1 mL/min flow rate. The separation process was carried out with an HPLC system (Agilent Technologies) and UV-VIS detector at 325 nm wavelength. Quantification was done using the γ-oryzanol standard curve, and the results were expressed as milligrams per gramme of dried sample (mg/g db).

Analysis of α-Tocopherol

The analysis of α-tocopherol was performed following the official Association of Official Analytical Chemists (AOAC) (2007) method 940.28. The sample (20 µL) was injected into an HPLC (Agilent Technologies) system with a UV-VIS detector at a 280 nm wavelength, a C-18 column (Zorbax Eclipse XDB C-18 column 4.6 X 150 nm), methanol and isopropanol (98:2) as the mobile phase, and a 1 mL/min flow rate. The α-tocopherol standard was used for standard curve, and the results were expressed as milligrams per gramme of dried sample (mg/g db).

The Ability to Scavenge DPPH Free Radicals

The ability to scavenge DPPH was evaluated to assess antioxidant activity (Kubo et al., 2002). A mixture of 0.1 mL of DPPH, 1.87 mL of methanol and 1 mL of acetate buffer (pH 5.5) was vortexed. Then, 0.03 mL of the extracted sample was added. The sample was vortexed and then incubated for 20 min in the dark room. The sample absorbance was measured by using a UV-VIS spectrophotometer at a wavelength 517 nm. DPPH radical scavenging was calculated as (1-absorbance of sample/absorbance of blank) x 100. Ascorbic acid was used as the standard.

Analysis of α-Amylase Inhibition

The analysis of α-amylase inhibition was based on Thalapaneni et al. (2008). A sample of 125 µL was reacted with 125 µL of pancreatic amylase enzyme solution and was incubated for 10 min at 37°C. Then, 125 µL of starch solution (1%) was added to the sample solution and was incubated again for 10 min at 37°C. Next, 96 µL of 3.5-dinitrosalicylate (DNS) reagent was added and the sample was incubated for 5 min in boiling water. As much as 5 mL of distilled water was added to the sample, and its absorbance was measured using UV-VIS spectrophotometry at a wavelength of 540 nm.

Statistical Analysis

The data were analysed by one-way analysis of variance (ANOVA) using Statistical Package for the Social Science (SPSS) version 20. If a significant difference was obtained at the treatment level, the data were then analysed with Duncan's multiple range test at $\alpha = 0.05$. The data are displayed as the mean±SD (n=3).

RESULTS AND DISCUSSION

Total Phenols, Flavonoids and Anthocyanins

The phenolic content of NFRB was 193.47 mg GAE/100 g, significantly increased ($p < 0.05$) to 304.50 mg GAE/100 g after

four days of fermentation (Table 1). In this study, the total phenolics increased by as much as 57.39% after fermentation. This result was in accordance with that by Oliveira et al. (2012), who stated that the SSF technique improved the total phenolic content in rice bran. Rice bran (variety BR-IRGA 417) fermentation with the use of *R. oryzae* (Schmidt et al., 2014) doubled the total phenol content at 48 h. However, rice bran (Thailand varieties) fermentation with the use of *S. cerevisiae* did not significantly increase the amount of total phenolics. It was suspected that *S. cerevisiae* activity did not affect the phytochemical content in rice bran for 24 h (Chaiyasut et al., 2017). These results show that the phenolic profile changes through fermentation are strongly influenced by substrate types, microorganisms, and fermentation conditions (Martins et al., 2011).

Total flavonoid content at 0 h (NFRB) was about 168.08±0.22 mg QE/100 g, and slightly decreased at the 24 h of fermentation (165.95±1.59 mg QE/100 g). After 24 h of fermentation, the flavonoid levels significantly increased ($p < 0.05$) to 184.54±0.79 mg QE/100 g at the 96 h of fermentation (Table 1). Flavonoids are the most widespread group of phenolic compounds. Fungal genera such as *Rhizopus, Aspergillus, Monascus* and *Penicillium* are known to divide the heterocyclic C-ring flavonoids (Das & Rosazza, 2006).

The amount of anthocyanin obtained in this study significantly increased ($p < 0.05$) during fermentation, at the beginning (0 h) 26.43±0.03 mg/g sample increased to 96.38±0.42 mg/g sample on the fourth day (96 h) of fermentation. Anthocyanin is a glycoside compound and belongs to the class of flavonoids that are mostly contained in black rice bran (Zhang et al., 2010). The presence of a high amount of anthocyanins in FRB is particularly useful because anthocyanins have anticancer, antioxidant properties (Pengkumsri et al., 2015), and antidiabetic properties (Jayaprakasam et al., 2005).

The phenolic compounds can be classified into free and bound phenolic compounds. Fermentation process can

Table 1
Phenolic compounds of non-fermented and fermented rice bran (db)

Fermentation time (hours)	Total phenolics (mg/100g)	Total flavonoids (mg/100g)	Total anthocyanins (mg/g)	γ-Oryzanol (mg/g)	α-Tocopherol (mg/g)
0	193.47±0.21^{a}	168.08±0.22^{b}	26.43±0.03^{a}	12.58±0.49^{a}	0.04±0.00^{a}
24	258.57±0.93^{b}	165.94±1.59^{a}	51.55±0.18^{b}	17.29±0.61^{b}	0.72±0.01^{c}
48	298.39±1.16^{c}	170.99±0.86^{c}	58.74±0.36^{c}	20.03±0.32^{c}	0.56±0.03^{b}
72	300.44±2.04^{c}	174.87±1.59^{d}	69.48±0.26^{d}	22.44±0.46^{d}	0.77±0.03^{d}
96	304.50±1.75^{d}	184.54±0.79^{e}	96.38±0.42^{d}	23.48±0.65^{e}	1.51±0.03^{e}

Values are mean ± SD (n = 3). Different letters in the same column are significantly different by Duncan's Multiple Range Test ($p < 0.05$)

enhance the release of bound phenolic compounds to free phenolics were due to extracellular enzymatic hydrolytic activity of mould in the rice bran. Free phenolic aglycones that contribute to the high antioxidant activity. According to Dey and Kuhad (2014), the main enzyme produced by *R. oligosporus* is β-glucosidase. This enzyme may increase the availability of free hydroxyl groups in phenolic structures by hydrolysing glycosidic bonds between carbohydrates or between carbohydrates and non-carbohydrates and acting as a catalyst in the process of hydrolysing glycosidic bonds in alkyl and aryl β-D-glucoside groups. Compared to phenolic glycosides, phenolic aglycones have better bioactivity and bioavailability in the human digestive system because they are more easily absorbed by the small intestine (Celep et al., 2014; Huynh et al., 2014). The β-glucosidase enzyme is also capable of converting anthocyanins into aglycone forms that have better antioxidant properties than the glycoside forms (Kong et al., 2003). In addition to the β-glucosidase enzyme *R. oligosporus* also produces enzymes such as β-glucuronidase, α-amylase, xylanase, proteases, laccase, phenolic esterase, and lipases that help break down the phenolic compounds attached to cell wall compounds, such as cellulose and proteins in conjugated forms (Huynh et al., 2014; Razak et al., 2015). According to Schmidt et al. (2014), the laccase enzyme is able to degrade the phenyl ring during fermentation so that the phenolic compounds increase.

The Compounds of γ-Oryzanol and α-Tocopherol

The enzymatic hydrolysis process that occurs during fermentation also increased the contents of γ-oryzanol and α-tocopherol in the rice bran (Table 1). The NFRB content of γ-oryzanol (13.11 mg/g) increased to 23.78 mg/g on the 4th day of fermentation. The increase was approximately 81.39%. These results were similar to those reported by Massarolo et al. (2017) that showed that control rice bran had 13.54 mg/g γ-oryzanol which increased to 20.52 mg/g after the fermentation process with *R. oryzae*. During fermentation mould activity produces several enzymes that can degrade cell walls, producing intracellular compounds that may contribute to the increase in oryzanol (Massarolo et al., 2017).

The content of α-tocopherol also significantly increased ($p < 0.05$) during the fermentation from only 0.04 mg/g dried sample to 1.51 mg/g dried sample on the 4th fermentation day. This increase was due to the enzymatic reactions occur during fermentation that break the phytochemical bonds of the toxoid-bound forms to produce free forms with a high bioavailability (Belobrajdic & Bird, 2013; Gani et al., 2012). According to Chen and Bergman (2005) the content of γ-oryzanol was 10-20 times greater than that of total tocopherol in rice bran, which was similar to the results in this study.

Scavenging Activity of DPPH Free Radicals

Antioxidant activity is related to the activity of compounds that maintain or protect the biological system from oxidation reactions or processes involving ROS. The most common method to measure antioxidant activity is by using radical compounds, such as DPPH compounds to simulate ROS (Razak et al., 2017).

The DPPH free radical scavenging activity in NFRB was 26.28±4.16%, which significantly increased ($p < 0.05$) to 76.91±0.06% after 96 h of fermentation (Figure 1). The DPPH free radical scavenging activity increased approximately three times after 96 h of fermentation compared to that of NFRB. These results were different from those reported by Oliveira et al. (2012), in which rice bran fermented with *R. oryzae* for 96 h showed antioxidant activity of 59±1.7%. Razak et al. (2015) showed that rice bran fermented with *R. oligosporus* for 12 days had DPPH free radical scavenging activity of 90.19±0.77%. The differences in the results were due to different types of microbes, types of substrate and fermentation time used in the experiment.

According to Sompong et al. (2011), pigmented rice bran is rich in sources of phenolic antioxidants. An increase in the flavonoid content during fermentation may contribute to antioxidant activity (Cheng et al., 2016). But on this studied, increased amounts of phenols, flavonoids, and anthocyanins that contributed to the increased antioxidant activity of the fermented rice bran. This result supported by the correlation result obtained which showed that there was a positive correlation between the scavenging activity of DPPH and total phenols, flavonoids and anthocyanins, i.e $r = 0.95$, $r = 0.61$, and $r = 0.90$ (respectively). According to Heim et al. (2002), the phenolic aglycones obtained by fermentation have the higher antioxidant activity than the glycoside forms and are effectively absorbed by the gut. The hydroxyl group of phenolic compounds becomes a hydrogen donor for

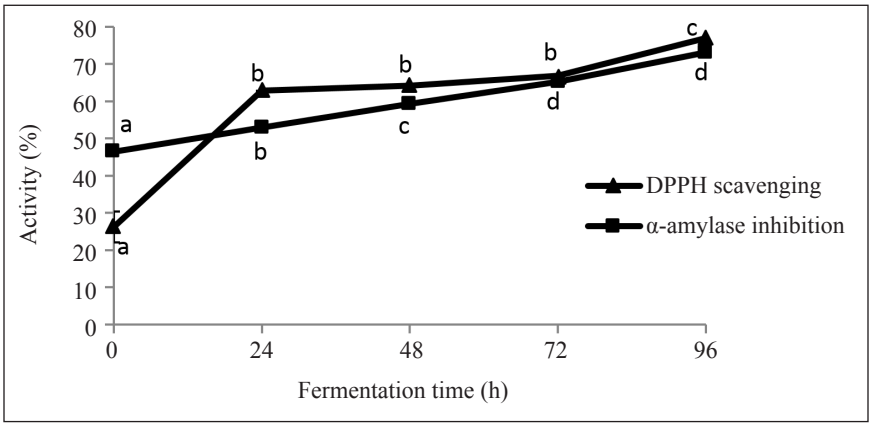

Figure 1. DPPH radical scavenging activity and inhibition of α-amylase by NFRB and FRB. The points in each line with different letters are significantly different at $p < 0.05$ by Duncan's Multiple Range Test

DPPH free radicals, thereby it increases the antioxidant activity of fermented rice bran (Dey & Kuhad, 2014; Razak et al., 2015). The processes of free radical scavenging, ion metal chelation and pro-oxidant enzymes are possible mechanisms of this change in antioxidant activity (Amorati et al., 2006). According to Kong et al. (2003), anthocyanins also have an antioxidant effect through several mechanisms: hydrogen donation, metal binding and protein binding. Its antioxidant activity is attributed to the glycosilated B-ring structure (Khoo et al., 2017).

Rashid et al. (2015) reported that the bioactive compounds in rice bran strongly contributed not only to the phenolic content but also to the antioxidant capacity. This statement is supported by the positive correlation between the scavenging activity of DPPH free radicals and γ-orizanol (r = 0.93) and α-tocopherol (r = 0.88). The γ-oryzanol compound is a mixture of ferulic acid esters of sterols (cholesterol, stigmasterol and beta-sitosterol) and triterpenes of alcohol (cycloartanol, 24-methylenecycloartanol and cyclobranol) (Nagendra et al., 2011). The ferulate in the molecular structure of γ-oryzanol is known to be a potent antioxidant that is stable at high temperatures. The γ-oryzanol has antioxidant activity that is four times more effective than that of the compounds of vitamin E (α-tocopherol, β-tocopherol, α-tocotrienol, and β-tocotrienol) in inhibiting oxidation (Nagendra et al., 2011). In addition to γ-oryzanol, the antioxidant ability of rice bran is also due to the availability of other antioxidant compounds, such as tocopherols, tocotrienols, and ferulic acids that can act as good radical scavengers against DPPH radicals (Arab et al., 2011; Gul et al., 2015; Razak et al., 2015).

α-Amylase Inhibition Activity

The inhibitions of α-amylase activity by NFRB and FRB extracts are presented in Figure 1. The percentage of rice bran inhibition against α-amylase significantly increased from 46.45% for NFRB to 73.05% after 96 h of fermentation. The inhibition of α-amylase is highly effective in delaying glucose absorption, thus preventing increased postprandial blood glucose, and may act as one of the therapeutic agents to prevent diabetes. According to Randhir and Shetty (2007), fermentation can increase the amount of free phenolics and antioxidant activity. The antioxidant in fermented rice bran contributes to the inhibition of α-amylase. Other researchers also reported that the phenolic phytochemicals foods had inhibitory activity against α-amylase (Yilmazer-Musa et al., 2012). In addition, it is known that flavonoids such as luteolin, quercetin, myricetin and cyanidin may act as α-amylase inhibitors and are claimed to be useful for regulating type 2 diabetes (McCue et al., 2004; Tadera et al., 2006). In this study, there was a positive correlation between the anti-amylase activity of the fermented rice bran and phenolic compounds (r = 0.87), total flavonoids (r = 0.92) and anthocyanins (r = 0.98). Jung et al. (2015) also reported that γ-oryzanol-containing granules might be anti-hyperglycaemic agents by

stimulating the activity of peroxisome proliferator-activated receptor-gamma (PPAR-γ), which was an important receptor in lipid metabolism and glucose balance. The γ-oryzanol also increased glucose digestion by stimulating the translocation of glucose transporter type 4 (GLUT-4) from the cytosol to the cell surface. A positive correlation was also obtained from the results of this study between the α-amylase inhibition activity and γ-oryzanol ($r = 0.96$) and α-tocopherol ($r = 0.91$).

CONCLUSIONS

The 96 h fermentation process with the use of the SSF method and *R. oligosporus* significantly increased total phenolics, total flavonoids, total anthocyanins, γ-oryzanol and α-tocopherol contents in black rice bran. The increases in the bioactive compounds in fermented bran contributed to the increased in DPPH free radical scavenging activity and α-amylase inhibition. It is suggested that the fermented rice bran contains bioactives and antioxidative activities, which could be potentially explored for the development of functional foods.

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Functional Properties, Antioxidant Activities and Storage Stability of Cookies from Germinated Brown Rice and Rice-Potato Starch Composite Flour

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ABSTRACT

Cookies are convenient food for people of all ages. Consumers' interest for gluten free cookies is increasing recently due to awareness on gluten allergy. This study evaluated the functional properties and antioxidant activities of germinated brown rice flour (GBRF) and non-germinated brown rice flour (NGBRF), and GBRF-potato starch blend (3:1), and NGBRF-potato starch blend (3:1). Storage stability and sensory acceptance of cookies produced from the various flour samples were also evaluated. The flours had significantly different values in most of the functional properties. However, the Housner's ratio (1.18-1.35), Carr index (14.94-25.67) and water absorption index (1.88-2.14 g/g) of the flours were not significantly different. GBRF and germinated brown rice flour cookies (GBRFC) had the highest antioxidant activities with DPPH values of 40.61 μM TE/g and 37.67 μM TE/g, respectively, and FRAP values of 39.84 μM TE/g and 38.29 μM TE/g, respectively. The GBRFC had higher total phenolic content (152.30 mg GAE/100g) than cookies from GBRF-starch blend (107.37 mg GAE/100 g). Sensory evaluation results showed that all the cookies were similarly rated for aroma, texture, mouth feel, crispiness and overall acceptance. However, cookies prepared from wheat flour, and germinated brown rice-potato starch (GBRPS) had the highest sensory scores for overall acceptance. The hardness of cookies produced from GBRPS was similar to that of the freshly produced cookies after 7 days of storage at room temperature.

Keywords: Antioxidant activities, brown rice cookies, functional properties, germinated brown rice flour, pasting properties, storage stability

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INTRODUCTION

Rice is a very popular and important cereal crop consumed as staple food in most part of the World. Rice flour is recently becoming an important ingredient in food formulation (because of its gluten-free properties) for patients allergic to gluten (Gujral et al., 2003). Rice flour has been used for making noodles (Tong et al., 2015), bread (Cornejo et al., 2015; Nakamura et al., 2010; Toufeili et al., 1994), cake (Orts et al., 2000), and cookies (Chung et al., 2014). However, in order to improve the quality of rice-based products, rice grains are subjected to germination process. Germinated rice has been reported to contain more vitamins, minerals, fibre, antioxidants and other bioactive compounds than raw rice (Usuki et al., 2007; Watanabe et al., 2004; Yodpitak et al., 2013). The process has been used to produce healthy foods (Kayahara, 2001) and enhance the quality of baked products from rice (Morita et al., 2007).

Antioxidant plays important role in protecting humans from degenerative diseases and aging (Arab et al., 2011). Phenolic compounds have been reported to exhibit free radical scavenging ability and antioxidant activity (Shashidi et al., 1992). In addition, it also helps in reducing cholesterol level through the prevention of lipid oxidative damage and LDL (low-density lipoprotein) (Morton et al., 2000), prevents the aggregation of platelet (Daniel et al., 1999), and reduces the risks of cancer and cardiovascular diseases (Martinez-Valverde et al., 2000; Newmark, 1996). Although common sources of phenolic compounds are vegetables and fruits, researches have shown that cereals are also excellent sources of phenolic compounds (Scalbert & Williamson, 2000).

Cookies consumption is becoming increasingly popular in all countries of the world due to its low cost, long shelf life and palatability. In order to increase the nutritive value of cookies, and provide healthy alternative to gluten based cookies many researchers are now focusing on either partial substitution (Kaur et al., 2017a; Noor Aziah et al., 2012) or total replacement of wheat flour (Bolarinwa et al., 2016; Inglett et al., 2015; Jan et al., 2016) with gluten-free flour in cookies preparation. Substitution of rice flour with buckwheat flour in cookies preparation improved the texture (hardness and fracturability) and overall acceptability of cookies (Hadnađev et al., 2013). In another study, cookies prepared from composite flour of wheat and pre-treated (heat-moisture treatment) germinated brown rice had better nutritional composition than 100% wheat cookies (Chung et al., 2014). Most studies on cookies preparation with germinated brown rice make use of composite flour (Chung et al., 2014).

Potato starch has unique physical and chemical properties that are essential for human nutrition. Its granules swell more readily, and form more viscous gel than other starches (Tomasik, 2009). Potato starch has been used to substitute wheat flour in bread making (Nemar et al., 2015). Previous report on cookies preparation from brown rice flour and potato starch composite flour only reported proximate composition

of the flour and physical properties of the cookies (Bolarinwa et al., 2018). The aim of the study was to determine the functional properties and antioxidant activity of GBRF and GBRF-potato starch composite flour (GBRPS), antioxidant activity, sensory characteristic and storage stability of cookies from GBRF and GBRPS. Data obtained in this study would provide useful information for functional food products formulation from GBRF and GBRPS. The use of germinated rice flour and potato starch as ingredient for cookies will create varieties in gluten-free cookies, and increase the nutritional composition of gluten-free cookies.

MATERIALS AND METHODS

Materials

Rice paddies (variety CL2) from Rakyat Sekinchan, Malaysia were used for the rice flour preparation. All other ingredients (butter, sugar, salt and baking soda) for cookies preparation including potato starch were obtained from IOI City Mall, Selangor, Malaysia.

Preparation of Germinated Brown Rice Flour

Germinated brown rice flour was prepared as previously described by Bolarinwa et al. (2018). Paddies (1 kg) were washed and cleaned thoroughly using distilled water. Floating paddies were decanted and the remaining grains were soaked in distilled water for 24 h at ambient temperature. The water was decanted and hydrated grains were placed on moistened Whatman filter paper (No. 7). The filter papers were placed on a stainless steel tray inside a water bath set at 30°C. The grains were separated with thickness of 1 cm and watered with 10 mL distilled water 3 times daily. The grains were allowed to germinate until the radicle reached approximately 0.5-1.0 mm in length (this was achieved in 48 h). The germinated grains were dried in a universal oven at 55°C for 2 h. The dried rice were dehusked using a dehusker (Motion Smith, Singapore), ground and sieved using a Haver EML digital plus test sieve shaker (Harver and Boecker, 59302, OELDE, Germany) to particle size of <200 μm.

Cookie Formulation and Preparation

Cookies were produced from (A) Non-germinated brown rice flour (NGBR); (B) Germinated brown rice flour (GBR); (C) NGBR-potato starch composite flour (3:1; rice:starch); (D) GBR-potato starch composite (3:1; rice:starch); and (E) Wheat flour. The composite blend ratio was chosen based on preliminary studies. Cookies were prepared according to standard analytical method of American Association of Cereal Chemists International (AACC) (2000) with some modifications. Butter (60 g) and sugar (40 g) were mixed in a heavy duty kitchen aid mixer (5K5SS, Michigan, USA) to form cream. Flour (100 g), sodium bicarbonate (1 g) and salt (1 g) were then mixed with the cream to form dough. The dough was moulded and baked at 170°C for 10 min in an electric oven, cooled at ambient condition for 2 h and packed in low-density polyethylene bags prior to analyses.

Determination of Functional Properties of the Flour

Bulk Density and Tapped Density. The bulk and tapped density of the flours were determined as described by Wang et al. (2017). Each flour sample (5 g) was poured into a 10 mL graduated measuring cylinder. The tapped density was estimated as mass of the flour divided by volume of flour in the measuring cylinder (after tapping). Bulk density was estimated as the mass of flours divided by the volume of the flour in the measuring cylinder. Bulk and tapped density were expressed as grams per mL of flour sample.

Hausner's Ratio and Carr Index. Hausner ratio (*HR*) is an indicator of flour compaction while Carr index (*CI*) measures flour cohesiveness. Hausner's ratio and Carr index of the flours were determined according to Wang et al. (2017) method. Hausner ratio was determined by dividing tapped density of the flour by their corresponding bulk density, while the Carr index was determined as stated:

Carr index = (tapped density - bulk density) × 100/ tapped density

Water Solubility Index. Water solubility index (WSI) of the flour samples was determined according to Jan et al. (2016) with a slight modification. The flour (2 g) was added to 25 mL water to form a suspension which was vortex for 2 min. The suspension was left to sediment at room temperature for 30 min, after which it was centrifuged (3500 × *g*, 15 min). The supernatant was decanted in a pre-weighed moisture can and evaporated. WSI was calculated as the amount of dry solid in the supernatant expressed as percentage of the original sample weight.

Water Absorption Index and Oil Absorption Capacity. Water absorption index (WAI) and Oil absorption capacity (OAC) were determined as described by Jan et al. (2016). WAI and OAC were expressed as grams of water and oil held per gram of flour sample, respectively.

Pasting Properties. The pasting properties of the flour samples were evaluated according to the method described by Limpisut and Jindal (2002), using a Rapid Visco Analyzer. RVA pasting curve and pasting properties values were obtained from the instrument after the runs.

Sample Preparation for Antioxidant Activity and Total Phenolics. The flour (0.5 g) and ground cookies (0.5 g) were separately mixed with 10 mL methanol (80%, v/v) and incubated at 40^{o}C for 24 h. The mixture was centrifuged (3500 × *g*, 15 min) and the resulting supernatant was used to quantify the amount of DPPH, FRAP and total phenolic in the flour and cookie samples.

Antioxidant Activity.

DPPH (2, 2-diphenyl-l-picrylhydrazyl) Radical Scavenging Activity. The DPPH assay was carried out according to the

method described by Thaipong et al. (2006) with some modifications. DPPH stock solution was prepared by dissolving DPPH chemical (2.3 mg) with 100 mL methanol (80% v/v) in amber bottle, and sonicated for 5 min. The flour and cookie samples (100 µL) were mixed with the DPPH stock solution (3.9 mL) in a capped glass tube, vortex (15 s) and kept in a dark place at room temperature for 2 h. The optical density of the mixture was recorded at 515 nm. Methanol solution (80%) was used as blank and trolox was used to construct standard curve. Results were presented in mM Trolox equivalent of dry weight of sample (TE/g).

FRAP (Ferric Reducing Antioxidant Power) Assay. The FRAP assay was conducted as described by Wong et al. (2006) with slight modifications. FRAP reagent consisted of acetate buffer (pH 3.6), TPTZ solution (10 mmol) in HCl (40 mmol) and iron (III) chloride solution (20 mmol) in proportions of 10:1:1 (v/v), respectively. The samples (100µL) were added to FRAP solution (2.85 mL) and allowed to react for 30 min in the dark. The colored products were vortex (1 min) and the optical density was recorded at 593 nm. Standard curve was constructed using Trolox. Results were presented in mM Trolox equivalent of dry weight of sample (TE/g).

Total Phenolic Compound Analysis. Total phenolic content of the germinated and non-germinated rice flour, the rice composite flours and cookies were determined by the Folin-Ciocalteu method described by Ragaee and Abdel-Aal (2006) with some modifications. The sample (250 µL) was allowed to react with Folin-Ciocalteu reagent (2.5 mL) and 2 mL of sodium carbonate (7.5% w/v). The mixture was kept in darkness for 2 h and vortex (2 min). Optical density was recorded at 765 nm. Standard curve was constructed using gallic acid. Results were presented as gallic acid equivalent of dry weight of sample (mg GAE/g).

Hardness and Moisture Content of Stored Cookies

Cookies hardness was measured at a distance of 5 mm using a cutting probe. The pre-test, test, and post-test speeds were 1.5, 2, and 10 mm/s, respectively. The maximum force required to break the cookies was recorded as the cookies hardness. Moisture content of the freshly baked and stored cookies was determined by drying ground cookie (2 g) at 105^{o}C to a constant weight, which was achieved at 16 h (Association of Official Analytical Chemists [AOAC], 2011).

Sensory Evaluation

The cookies were evaluated by thirty untrained panellists. The panellists were served with the cookies once and allowed to rinse their mouth with portable water after tasting each cookie. Scorecards were presented to the panellists for evaluating the following attributes of the cookies: appearance, color, texture, taste, mouthfeel, crispiness, aroma, and overall acceptability. The panellists were asked to rate the cookies

on a 9-point hedonic scale, where 9 is equivalent to like extremely and 1 is dislike extremely.

Statistical Analysis

Data were expressed as mean value of triplicate analysis. Results obtained were subjected to analysis of variance, using Minitab (version 16). Means were separated using Turkey's multiple comparison tests with significant differences at $P < 0.05$. Sensory data were subjected to analysis of variance (ANOVA), means were separated using Duncan's multiple range test.

RESULTS AND DISCUSSION

Functional Properties of Germinated Brown Rice Flour and Rice-Potato Starch Composite Flour

Results of the functional properties are presented in Table 1. Generally, there were significant differences ($p < 0.05$) in the functional properties of the flours, except for Housner's ratio, Carr index and water absorption index. The bulk density (flour heaviness) of the GBR flour (sample B) was similar to that of the NGBR (sample A), while addition of potato starch to rice flour only increased the bulk density of the NGBR-potato starch composite flour (sample C). Crude fibre content of flours is directly related to their bulk density (Jan et al., 2016). According to Hanis-Syazwani et al. (2018), rice bran contains up to 8.6% crude fibre. Thus, similar values obtained for the bulk density of GBR and NGBR flour could be due to the presence of bran in the brown rice flours. The bulk density of wheat flour was significantly lower than that of the rice flour samples (Table 1).

Tapped density is a useful parameter for determining packaging requirement of the flour samples. The tapped density of the GBR flour was 3.4% lower than that of the NGBR flour but 7% higher than that of wheat flour (sample E) (Table 1). Lower tapped density recorded for GBR compared to NGBR could be due to the smoother texture of GBR which made it more compact compared to NGBR. However, the tapped density of the composite rice flours was not significantly different ($P > 0.05$).

Housner's ratio and Carr index are important parameters for the determination of flour blends flow ability, which is significantly affected by particle properties (Wang et al., 2017). The Housner's ratio and Carr index of all the flour samples and the control sample (wheat flour) were not significantly different ($p > 0.05$) (Table 2). This indicates that NGBR and GBR mixed uniformly with the potato starch and their size, spread, shape and surface were in conformity with that of wheat flour.

Results of the water solubility index (WSI) showed that GBR flour had the highest WSI (5.8 g/100 g), followed by NGBR (4.1 g/100 g) and wheat flour (3.4 g/100g) (Table 1). Higher value of WSI for GBR flour was probably due to its finer particles, uniform particle size flour and soluble components such as reducing sugar and free sugar (Choi et al., 2006; Kadan et al., 2008). Water absorption index (WAI) affects product moistness and texture. The

Table 1
Functional properties of germinated brown rice, non-germinated brown rice and rice-potato starch composite flour

Sample	Bulk density (g/mL)	Tapped density (g/mL)	Housner's ratio	Carr index	Water solubility index (g/100g)	Water absorption index (g/g)	Oil absorption index (g/g)
A	0.45b ± 0.02	0.59a ± 0.02	1.32a ± 0.06	23.86a ± 3.23	4.09b ± 0.7	1.98b ± 0.05	1.92a ± 0.03
B	0.45b ± 0.04	0.57c ± 0.02	1.27a ± 0.15	23.83a ± 5.19	5.78a ± 0.25	2.14b ± 0.02	1.87b ± 0.03
C	0.49a ± 0.04	0.58b ± 0.03	1.18a ± 0.06	14.94a ± 3.98	3.05bc ± 0.07	1.88b ± 0.04	1.79c ± 0.05
D	0.45b ± 0.01	0.58b ± 0.02	1.27a ± 0.04	20.92a ± 2.43	2.20c ± 0.21	2.01b ± 0.05	1.76c ± 0.00
E	0.40c ± 0.01	0.53d ± 0.00	1.33a ± 0.02	25.03a ± 1.32	3.43bc ± 0.53	3.03a ± 0.25	1.93a ± 0.02

Values are mean ± standard deviation (n = 3). Mean value with different letter (s) along the same column are significantly different ($p < 0.05$). A - Non-germinated brown rice flour; B – Germinated brown rice flour; C - Non-germinated brown rice and potato starch composite flour; D - Germinated brown rice and potato starch composite flour; E – Wheat flour

WAI of most of the flour samples was not significantly different ($p > 0.05$) except for the control sample (wheat flour). Wheat flour had the highest WAI (3.03 g/g) followed by GBR (2.14 g/g) and GBR-starch composite flour (2.01 g/g). This result indicates that germination process slightly increased the WAI of the GBR flour (Table 1).

Oil absorption index (OAI) is an important property that affects product crispness and appearance (colour, gloss, shape) (Joshi et al., 2015). The OAI of wheat flour was 3% and 0.5% higher than the OAI of GBR and NGBR, respectively. This indicates that cookies produced from wheat flour will be similar to those of NGBR in terms of crispiness, shape, colour and appearance, but slightly different from GBR flour cookies. Lower OAI obtained in the composite flours indicates that cookies from the flour will be crispier and are likely to have better shape than cookies from GBR and NGBR flour.

Pasting Properties

Pasting properties of the rice flours and the composite flours are shown in Table 2. Interestingly, the pasting curve of the rice flour and the composite flour were distinctive from each other. The NGBR (sample A) and NGBR-potato composite flours (sample C) had higher peak viscosity (PV), trough viscosity (TV), final viscosity (FV) and breakdown viscosity (BV), while GBR (sample B) and GBR-starch composite (sample D) had much lower PV, FV, and BV. Thus, NGBR and NGBR-potato composite flours had higher modes than the other flours (Figure 1). This could be due to the presence of unmodified bran in the NGBR and

NGBR-potato composite flours, which took longer and required higher temperatures to gelatinize, and imparted higher viscosities than the GBR bran components, which had been modified during germination process. The pasting temperature of the GBR and the NGBR flours were similar.

On the other hand, the composite rice flours had lower pasting temperature than that of the rice flours but higher than that of the wheat flour (Table 2). The peak time of the rice flours and their composite flours were not significantly different, however, wheat flour required slightly longer time (6.27 min) to attain peak viscosity. This could be as a result of high WAI in wheat flour (Table 1) coupled with reduced absorption and swelling ability of wheat starch granules (Ragee & Abdel-Aal, 2006) compared to the other flours (Figure 1).

Table 2
Pasting properties of germinated brown rice, non-germinated brown rice and rice-potato starch composite flours

Sample	Peak viscosity (RVU)	Trough (RVU)	Breakdown (RVU)	Final viscosity (RVU)	Setback (RVU)	Peak time (RVU)	Pasting Temperature (°C)
A	3610	1813	1797	4038	428	5.6	80.65
B	874	256	618	703	-171	4.6	80.55
C	4315	1839	2476	3441	-874	5.20	70.4
D	655	235	420	486	-169	4.67	69.65
E	2999	1885	1114	3028	29	6.27	68.05

Values are mean ± standard deviation (n = 3). Mean value different letter(s) along the same column are significantly different ($p < 0.05$). A - Non-germinated brown rice flour; B – Germinated brown rice flour; C - Non-germinated brown rice and potato starch composite flour; D - Germinated brown rice and potato starch composite flour; E – Wheat flour

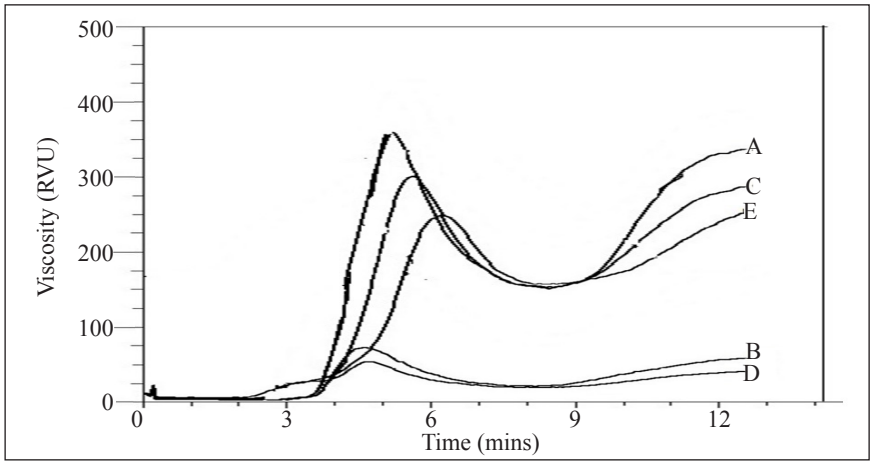


Figure 1. RVA pasting curves of germinated brown rice, non-germinated brown rice and rice-potato starch composite flour; A - Non-germinated brown rice flour; B – Germinated brown rice flour; C - Non-germinated brown rice and potato starch composite flour; D - Germinated brown rice and potato starch composite flour; E – Wheat flour

Antioxidant Activity of Germinated Brown Rice Flour and Rice-potato Starch Composite Flour

Numerous researchers have reported the antioxidants capacity of germinated brown rice from different rice varieties (Cho & Lim, 2016; Chung et al., 2016) and its health implications (Wu et al., 2013).

DPPH (2, 2-diphenyl-l-picrylhydrazyl) Radical Scavenging Activity

The DPPH-radical scavenging assays are used to estimate the capacity of an extract to donate electron/hydrogen to free radicals DPPH (Moon & Shibamoto, 2009). Germination significantly increased the DPPH, FRAP and Total phenolic of the flour and cookies. This could be because germination causes increased synthetic of high amount of powerful low-molecular-weight antioxidant molecules and accumulation of bioactive components such as antioxidants (Falcioni et al., 2002; Kaur et al., 2017b). There was significant difference ($p < 0.05$) in the DPPH value of both germinated and non-germinated flour and cookies (Table 3). Among all the samples, GBR and cookies (sample B) exhibited the highest DPPH radical scavenging activity. The radical scavenging activity was also significantly higher in germinated brown rice-starch composite flour and cookies (sample D) than that of non-germination brown rice (Table 3). This result is in congruent with previous study on antioxidant capacity of pigmented rice samples, where germinated rice samples were reported to contain significantly higher DPPH radical scavenging activity than non-germinated rice samples (Chung et al., 2016). Addition of potato starch to non-germinated rice flour and cookies increased the DPPH radical scavenging activity of the sample.

FRAP (Ferric Reducing Antioxidant Power) Assay

FRAP assay is a measure of the secondary antioxidant activity of sample extract (Chung et al., 2016; Vladimir-Knezevic et al., 2011). A significant high ferric reducing antioxidant power observed in germinated brown rice and germinated brown rice-potato starch composite flour and cookies, indicates that germinated samples had strong antioxidant potentials. These results are in agreement with previous study, which reported that germination process increased antioxidant activity in all germinated rough rice cultivars (Lee et al., 2007). Germination process has been reported to modify antioxidant activities of grains (López-Amorós et al., 2006). This could be because the colour of germinated rice grain is due to the accumulation of phenolics and flavonoid compounds which are responsible for increased in antioxidant activity in germinated grain (Gan et al, 2016). Generally, the FRAP antioxidant power of the rice flours (samples A and B) and the rice-starch composite flours (samples C and D) were significantly higher than that of the wheat flour (sample E). This could be due to relatively high antioxidant activity in brown rice (Esa et al., 2013).

Table 3
Antioxidant activity of germinated brown rice, non-germinated brown rice and composite flours and cookies

	DPPH (μM TE/g DM)		FRAP(μM TE/g DM)		Total phenolic(mg GAE/100 g)	
Sample	Flour	Cookies	Flour	Cookies	Flour	Cookies
A	35.61bc	34.78c	38.10b	35.33a	137.53a	138.67ab
B	40.61a	37.67a	39.84a	38.29a	151.80a	152.30a
C	36.42b	35.32b	36.57ab	33.90b	103.80b	107.37b
D	38.69ab	35.70ab	37.22ab	36.49a	113.17b	114.97ab
E	30.59c	30.07c	32.34c	31.10b	98.83b	119.80b

Values are mean ± standard deviation (n = 3). Mean value with different letter (s) along the same column are significantly different ($p < 0.05$). A - Non-germinated brown rice flour; B – Germinated brown rice flour; C - Non-germinated brown rice and potato starch composite flour; D - Germinated brown rice and potato starch composite flour; E – Wheat flour; DPPH – (2, 2-diphenyl-l-picrylhydrazyl) radical scavenging activity; FRAP - Ferric Reducing Antioxidant Power Assay

Total Phenolic Compound

The total phenolic contents in germinated and non-germinated brown rice flour, rice-composite flours and cookies are shown in Table 3. The results showed that total phenolic compound contained in germinated brown rice flour (151.80 mg GAE/100 g) and cookies (152.30 mg GAE/100 g) were higher than those of the non-germinated brown rice flour (137.50 mg GAE/100 g) and cookies (138.67 mg GAE/100 g). Similarly, the total phenolic contents of germinated composite flour and cookies were higher than that of the non-germinated composite flour and cookies. These results are in congruent with the findings of Tian et al. (2004), who reported between one and two fold increment in phenolic compounds in germinated brown rice when compared with brown rice. On the other hand, the total phenolic compounds in all the rice flours and rice composite flours and cookies were higher than that of the control sample (wheat flour and cookie). In addition, higher phenolic compounds recorded for GBRF and GBRF cookies might be as a result of cell wall dismantling which occured during germination process (Tian et al., 2004). Higher total phenolic compound in germinated brown rice flour and germinated brown rice composite cookie suggests that the flour and cookies have higher antioxidant potential.

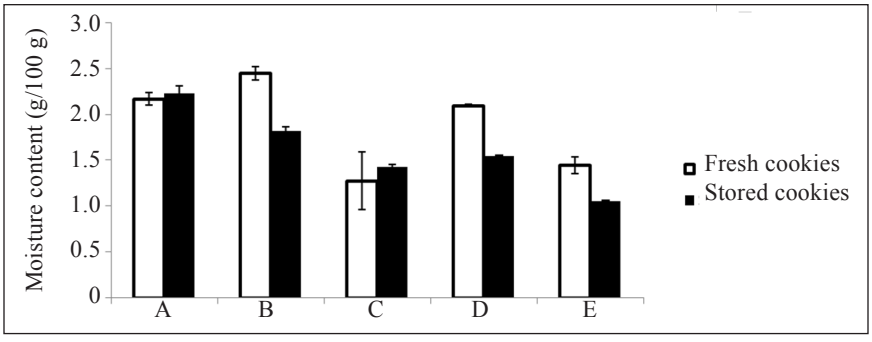
Generally, germinated brown rice flour and cookies exhibited higher antioxidant activities than the non-germinated samples as evidence in their DPPH, FRAP and total phenolic contents. This could be due to increase in the levels of rice phytochemicals as a result of germination (Chung et al., 2016).

Changes in Moisture Content and Hardness of Stored Cookies

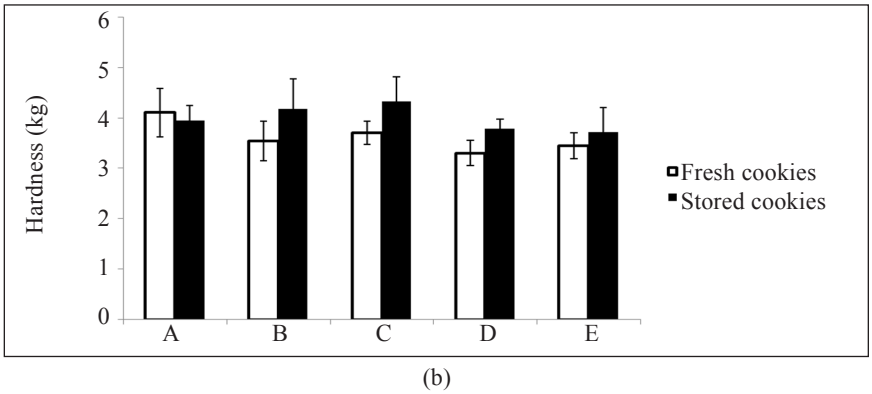
The storage stabilities of the cookies were evaluated after 7 days of storage at ambient condition. The moisture content of the non-germinated brown rice cookie (sample A) increased slightly from 2.17 g/100 g to 2.23 g/100 g while the moisture

contents of the control (sample E) and the other cookie samples reduced significantly (Figure 2). This indicates that only cookies from non-germinated brown rice cookies absorbed moisture from the surrounding air while cookies from the germinated rice flour and composite flour lost moisture to the surrounding. Increased in the moisture content of stored non-germinated brown rice cookies may shorten the shelf-life of the cookies. Lower moisture content recorded for germinated brown rice and composite cookies compared to cookies from non-germinated rice flour is an indication that the cookies have different interior structures. Cookies containing germinated brown rice and potato starch composite flours might have much higher contents of fibers and degraded sugars as compared to those from non-germinated brown rice cookies. The simple sugars in germinated brown rice flour could hold cookies contents better and improve the internal structure of the cookies. Furthermore, reduced sugars or starch dextrins produced by germination process may retard starch retrogradation, inhibit sugar crystallization and cause little water migration during storage (Chung et al., 2014).

Addition of potato starch to germinated brown rice flour significantly reduced the hardness of the cookies, but slightly



(a)



(b)

Figure 2. Effect of storage on (a) moisture content and (b) hardness of cookies. A - Non-germinated brown rice flour; B – Germinated brown rice flour; C - Non-germinated brown rice and potato starch composite flour; D - Germinated brown rice and potato starch composite flour; E – Wheat flour

increased the hardness of non-germinated brown rice composite cookies (Figure 2). The hardness of the germinated brown rice cookies significantly increased from 3.54 kg to 4.18 kg after 7 days of storage at room temperature. However, addition of potato starch to the germinated cookies reduced the hardness of the cookies from 4.18 kg to 3.78 kg after storage. Lower moisture contents recorded for germinated brown rice cookies and the composite cookies could be responsible for increased hardness of the cookies. In addition, starch retrogradation may facilitate network formation which will be responsible for increase rigidity of the cookies, leading to increased hardness of the cookies. Previous study also reported similar observation for the hardness of stored cookies (Chung et al., 2014).

Sensory Evaluation

The sensory acceptance of cookies prepared from germinated brown rice, non-germinated brown rice, and rice-potato starch composite flour are presented in Table 4. There were no significant differences ($p > 0.05$) in the aroma, taste, texture, mouth feel, crispiness and overall acceptance of all the cookie samples. However, the control (100% wheat cookie) was significantly different ($p<0.05$) from all the rice cookies in terms of appearance and colour. Cookies prepared from non-germinated brown rice had sensory scores that were close to that of the control sample (Table 4). On the other hand, cookies from 100% germinated brown rice had the lowest sensory scores among all the samples. This could be due to distinguishable taste and flavor of germinated rice. Lower sensory score recorded for the colour of the germinated brown rice cookies could be due to high phenolic content (Table 3) in the cookies, which made it darker in colour compared to other samples. This observation is in agreement with previous study by Jan et al. (2016), who also attributed reduced sensory score for colour of cookies prepared from *Chenopodium album* flour to high phenolic content in germinated *Chenopodium album*

Table 4
Sensory scores of cookies prepared from germinated and non-germinated brown rice and rice-potato starch composite flours

Sample	Appearance	Color	Aroma	Taste	Texture	Mouth feel	Crispiness	Overall acceptance
A	6.77ab	6.57ab	6.53a	6.43a	6.50a	6.53a	6.73a	6.80a
B	5.86b	5.80b	6.17a	5.63b	6.23a	5.97a	6.27a	5.85a
C	5.93b	5.87b	6.50a	6.20ab	6.10a	6.33a	6.03a	6.23a
D	6.40ab	6.00ab	6.37a	6.33ab	6.63a	6.50a	6.63a	6.87a
E	7.27a	7.07a	6.40a	6.47a	6.93a	6.83a	6.67a	6.90a

Values are mean ± standard deviation (n = 30). Mean value with the same letter(s) along the same column are not significantly different ($p > 0.05$). A - Non-germinated brown rice flour; B – Germinated brown rice flour; C - Non-germinated brown rice and potato starch composite flour; D - Germinated brown rice and potato starch composite flour; E – Wheat flour

flour. However, addition of potato starch to germinated brown rice flour increased the sensory scores of germinated brown rice-composite cookies.

CONCLUSION

Germination and addition of potato starch to brown rice flour improved the functional and pasting properties of the flour. Germination significantly increased the DPPH, FRAP and total phenolic compounds in germinated brown rice flour, germinated brown rice-composite flour and germinated brown rice cookies. Substituting germinated brown rice with potato starch for cookies preparation resulted in better storage stability of the cookies. Cookies prepared from germinated brown rice flour had darker colour, distinguished flavor and aftertaste, however, addition of potato starch to germinated brown rice for cookies preparation effectively improved the sensory attributes of the cookies. Germinated brown rice-potato starch composite flour has the potential of replacing wheat flour in confectioneries and baked products.

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CONFLICT OF INTEREST

None.

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Quality Characteristics and Sensory Profile of Stirred Yogurt Enriched with Papaya Peel Powder

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ABSTRACT

Dietary fibre enriched food is gaining more popularity due to their numerous health benefits. In this study, papaya peel powder was prepared as a source of dietary fibre to be fortified in yogurt at different concentrations (1.5 and 3.0 % w/w). Papaya peel was dried at three different temperatures (45, 55 and 65°C) and the results showed that drying at temperature of 55 and 65°C was able to retain higher total dietary fibre content of 40.21 and 48.00 g/100 g, respectively, and was used for enrichment in yogurt. The quality characteristics in terms of viscosity, pH and colour (L^*, a^*, b^*, chroma, hue angle and total colour difference) of stirred yogurt added with papaya peel powder stored at 4°C was investigated weekly up to 21 days. Sensory evaluation (9-point hedonic scale) was also conducted for the yogurt samples prepared. Results showed the viscosity of yogurt was higher when the amount of papaya peel powder added was increased. It also showed an increasing trend during 21 days of storage. The pH values of the samples prepared had no significant difference ($P > 0.05$) as the concentration of powder was raised. However, a notable reduction in pH was observed after storage. Colour parameters were also significantly affected ($P < 0.05$) by the addition of powder, with fortified samples exhibiting darker (lower L^* value) and more yellowish (higher b^* value) colour than the control sample.

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Nonetheless, the parameters remained unchanged during storage of 21 days. Sensory results revealed that the stirred yogurt with 1.5% concentration of papaya peel powder dried at 55°C received the highest sensory scores among other samples.

Keywords: Papaya peel, quality characteristics, sensory analysis, stirred yogurt, storage analysis

INTRODUCTION

Papaya or paw-paw (*Carica papaya*) is a tropical fruit which belongs to the family of Caricaceae. This fruit is originally native to Tropical America and is grown in many countries; Australia, Hawaii, India, Philippines and Malaysia to name a few (Anuar et al., 2008; Yogiraj et al., 2014). Papaya has gained popularity owing to its numerous health benefits and is often termed as a nutraceutical fruit. It is low in calories and is a source of antioxidants, minerals, fibre and vitamins (Prajapati et al., 2017). This has led the fruit to be consumed in large quantities globally and thus, resulting in a huge amount of by-products.

The by-products of papaya mainly constitute of peels and seeds, which make up 20 to 25% by fruit weight (Pavithra et al., 2017). Papaya peels have been found to be rich in fibre as they constitute total, soluble and insoluble dietary fibre (g/100) with 59.8 ± 0.5, 19.93 ± 0.01 and 39.9 ± 0.5, respectively (Calvache et al., 2016). Besides being rich in nutrients like protein, carbohydrates, ash, fat and minerals (phosphorous and potassium), they are also a potential source of antioxidants (Ang et al., 2012; Jamal et al., 2017). Large quantities of waste generated are disposed into the environment without subjecting to further treatment. This not only poses as a threat to the environment, but is also a loss of potential by-products which can be utilized and converted into value added products, materials for industries or even animal feed/fodder after proper treatments are performed. Recovery and re-utilization of these wastes will profit the agriculture sector economically and also be beneficial to the environment (Laufenberg et al., 2003).

Nowadays, dietary fibre rich-products are becoming more desirable in terms of health benefits. Consumers also prefer natural sources as to synthetics ones as they are feared to be toxic to health. Papaya peels present with a generous amount of fibre and can be utilized in the development of innovative products (Calvache et al., 2016). Fibre enriched food products are shown to have many health benefits. They reduce the chances of heart diseases and some forms of cancer, gastro- intestinal disorders, decrease blood cholesterol and help in controlling obesity. Since, they are not digested or absorbed in the small intestine of humans, they therefore increase faecal bulk and promote healthy bowl movement. Supplementation of fibre into food also enhances various functional properties including increase in water and oil holding capacity, emulsification and gel formation. Addition into bakery, dairy, jams and meat products leads to modification in the textural properties, prevention of syneresis, stabilization of high fat and emulsions and overall improvement in shelf life (Elleuch et al., 2011).

Yogurt is the most popular fermented dairy product which provides large health benefits. It is easy to digest, provides high nutrition and contains lactic probiotic cultures. Yogurt is known for relieving gut inflammations, infections, bowl problems and cholesterol levels (García-Pérez et al., 2005; Jaster et al., 2018). On the basis of flavour, yogurt can be classified into plain, fruit and flavoured yogurt. While on the basis of textural properties it can be grouped as set or stirred/drinking yogurt. Essentially, the only difference between set and stirred type yogurt is that the later involves stirring or agitation at the end of its processing. Nowadays, yogurt products are added with different food ingredients to make them more attractive to consumers in terms of flavour and nutrition (Tamime & Robinson, 2007).

Even though plain yogurts are tagged as healthy dairy products, they still lack when it comes to dietary fibre content (Tseng & Zhao, 2013). Yogurt can therefore act as a perfect candidate to be used in this study for addition with dietary fibre. Previous studies have also utilized this method where pineapple peel, passion fruit peel, pomegranate peel extracts and orange fibres were used to enrich yogurt (El-Said et al., 2014; García-Pérez et al., 2005; Sah et al., 2016; Vieira et al., 2015). Thus, the objective of this study is to enrich commercial yogurt with papaya peel powder as a dietary source. The quality characteristics such as viscosity, pH and colour of fortified yogurt during storage was studied. Also, a sensory analysis was conducted to investigate consumer acceptance on the papaya peel powder fortified yogurt.

MATERIALS AND METHODS

Preparation of Papaya Peel Powder

Carica papaya var. Sekaki were procured from a wet market in Seri Kembangan, Selangor, Malaysia with an approximate weight of 1.77 ± 0.28 kg. Ripe papaya fruits were selected as per ripeness index chart given by Ruslan and Roslan, (2016). The fruits were washed and sliced into peels, pulp and seeds using a stainless-steel knife. The peels were rinsed with water again to remove any adhering mucilages and then cut into pieces of 2x2 cm^2 and thickness of approximately 3.45 mm. The peels were not blanched as ripe papaya shows very low activity of polyphenol oxidase which is responsible for enzymatic browning (Othman, 2014). They were distributed uniformly on a 40 x 28 cm stainless-steel tray and dried using a convection oven (Memmert, Schwabach, Germany) at 45, 55 and 65^{o}C until constant weight was obtained after 8 to 48 hours. Then, the peels were ground using a hammer mill (Perten-120, Perten Instruments A.S. Sweden) and the powder obtained was passed through a sieve shaker (MINOR, Endecotts Limited, England) with a sieve size of 250µm. Sieving was done to standardize the particles for stirred yogurt enrichment. The powders were then were kept in a Ziplock bag and stored at 4^{o}C.

Determination of Total Dietary Fibre

Total dietary fibre content in papaya peel powders obtained by convection oven at three temperatures of 45, 55 and 65°C after drying for 8 - 48 hours till constant weight were established using Enzymatic-gravimetric method (AOAC 985.29). Gelatinization of dried samples was carried out with heat stable α-amylase. It was then digested enzymatically with protease and amyloglucosidase for removing protein and starch, respectively. Soluble dietary fibre was precipitated out by adding four volumes of ethyl alcohol. After filtering the residue, it was washed with ethyl alcohol and acetone and dried in an oven overnight. One test portion was used to determine protein content while the other was incinerated at 525°C to determine ash. The total dietary fibre was calculated using the formula given below:

$$\text{Total dietary fibre (\%)} = \frac{\text{weight residue} - \text{protein} - \text{ash} - \text{blank}}{\text{weight test portion}} \times 100 \quad [1]$$

The powder was selected on the basis of maximum retainment of total dietary fibres. The optimized levels (PP1 and PP2) obtained after drying were then further used as a source of dietary fibre in stirred yogurt.

Preparation of Fortified Stirred Yogurt

Commercial plain yogurt (Nestl*é*) of net weight 1400 g was purchased from a local supermarket in Seri Kembangan, Selangor, Malaysia. It was stored at 4°C and used to make papaya peel powder fortified stirred yogurt. Papaya peel powder PP1 and PP2 were the optimized levels which were added at 1.5 g and 3.0 g to make a 100 g of yogurt (w/w). Coagulation of yogurt was broken by manually stirring by the same person until the peel powder was uniformly incorporated. A control stirred yogurt was also prepared by stirring in the same manner. These concentrations were selected in order to avoid any unappealing lump formation and achieve good sensory acceptability. This amount is in compliance with what has been set by the USA regulations for fibre-fortified products. The maximum dose of powder used in this study accounts for only 10% of what is recommended for daily fibre consumption, if 200 mL of yogurt was taken (Fernández-García & McGregor, 1997). The Food and Drug Administration (FDA) recommends 25 g of dietary fibre to be taken daily (Santos et al., 2014). Five samples were obtained, namely, control stirred yogurt with no addition of powder, fortified stirred yogurt with addition of PP1 at 1.5% and 3.0% (1.5% PP1 and 3.0% PP1) and fortified stirred yogurt with PP2 at 1.5% and 3.0% (1.5% PP2 and 3.0% PP2). The mixed yogurt was placed in glass beakers covered with aluminium foil and stored in a refrigerator at 4°C. Quality analysis was conducted on storage Day 0, 7, 14 and 21.

Viscosity

Apparent viscosity of fortified stirred yogurt was measured using a Brookfiled DV-II + Pro Viscometer (Brookfield

Engineering Laboratory Inc., Stoughton, MA). Measurement was conducted at 30 rpm (4°C) with a Helipath spindle (no. D). This spindle is exclusively used for non-Newtonian fluids. Apparent viscosity was measured 10 seconds after the spindle had entered the stirred yogurt. The measurement was done in triplicates and recorded in centipoise as displayed on the screen.

pH Determination

pH determination of stirred yogurt samples was done using a benchtop pH meter (Sartorius PB-10, Germany). The pH meter was calibrated with buffer solution (pH 7 and 4) prior to commencement of analysis. The samples were first homogenized before recording any measurements. Readings of the samples were taken by immersing the electrode up to the immersion level at three different points in each sample container. This corresponded to three replicates and average values were then calculated (Vieira et al., 2015).

Colour Analysis

The colour of stirred yogurt samples was analysed using a Hunter lab UltraScan Pro with EasyMatch QC software (Hunter Associate Laboratory Inc., Reston, USA). CIE $L^*a^*b^*$ was used to determine the brightness parameter, L which denotes whiteness to darkness and chromacity coordinates, a^* and b^* which represents redness to greenness and yellowness to blueness, respectively (Abid et al., 2013). The equipment was first calibrated using light trap and white reference tile before proceeding with the experiment. The yogurt was poured into a glass cuvette and clamped onto the device. The analysis was run in triplicates and average values were taken.

Chroma was determined using the formula given below:

$$C^* = (a^{*2} + b^{*2})^{1/2} \quad [2]$$

Hue angle ranges from 0^0, 90^0, 180^0 to 270^0 and was obtained from a^* and b^* values:

$$H^0 = \tan^{-1}\frac{b^*}{a^*} \quad [3]$$

Total colour difference (ΔE) was determined as shown in the formula:

$$\Delta E = \sqrt{(L^*_i - L^*_t)^2 + (a^*_i - a_t)^2 + (b^*_i - b^*_t)^2} \quad [4]$$

where, L^*_i, a^*_i and b^*_i are the initial values while L^*_t, a^*_t and b^*_t are the corresponding values for stored yogurt samples.

Sensory Analysis

Hedonic test was conducted to evaluate the sensory attributes of the yogurt samples. Permission to conduct consumer acceptance was granted by Ethics Committee, Research Management Centre, Universiti Putra Malaysia with reference number JKEUPM-2018-117 and was conducted in accordance with the Declaration of Helsinki. A respondent's consent form was first signed by the panellist to confirm they met the inclusion criteria for the study and had no allergies to the ingredients in yogurt

samples. A 9-point hedonic scale was used for evaluation with 1 to 9 denoting dislike extremely, dislike very much, dislike moderately, dislike slightly, neither like or dislike, like slightly, like moderately, like very much and like extremely respectively (Hashim et al., 2009). Six attributes were evaluated for each sample, namely, colour, appearance, aroma, texture, taste and overall acceptability. Appearance and colour were analysed as two separate parameters as colour alone is not enough to determine the food appearance (MacDougall, 2003). Appearance was evaluated as the shape and size (geometric shape), surface texture (dullness/shininess) and clarity while colour indicates colour perception. The panellists consisted of 30 untrained students from Universiti Putra Malaysia. The analysis was conducted in an environmentally controlled room (25 ± 2 ºC) under white fluorescent light in Faculty of Engineering, Universiti Putra Malaysia. The samples were randomly presented in a transparent cup marked with a 3-digit code. A cup of water was also given for rinsing mouth between tasting.

Statistical Analysis

One-way analysis of variance (ANOVA) was performed to investigate the significant treatment effect of two independent factors: different papaya peel powder concentrations and storage time. Means of sensory acceptance results for each attribute namely, appearance, texture, odour, flavour, colour and overall impression for five samples were analysed by ANOVA and compared at the $P < 0.05$ level by Tukey test. Statistical Package for Social Sciences (SPSS, version 18.0) was used for analysis.

RESULTS AND DISCUSSION

Total Dietary Fibre of Papaya Peels

The total dietary fibre content of papaya peel powder dried at 45, 55 and 65^{o}C were 31.84, 40.21 and 48.00 g/100 g, respectively. These values were in agreement with previously reported total dietary fibre as 44.66% by Pavinthra et al., (2017), 33.05 ± 0.70 g/100 g for *Hawai* and 34.70 ± 0.54 g/100 g for *Calimosa* papaya cultivars by Santos et. al. (2014). As per the results, drying temperature at 55 and 65^{o}C presented with the highest retention of total dietary fibre and therefore were used for enrichment in yogurt. This trend is in agreement with previous studies by Quispe-Fuentes et. al. (2017) on native Chilean berries. It was found that an increase in dietary fibre content was observed with increase in drying temperature. Dried Cape gooseberry showed that the highest total dietary fibre content was present at 90^{o}C drying temperature while the lowest was at 50^{o}C drying temperature (Vega-Gálvez et al., 2015).

Viscosity

Figure 1 portrays apparent viscosity of samples at Day 0 versus time in seconds at a steady spindle rotation (30 rpm). This figure establishes that yogurt is a non-Newtonian fluid, which exhibits a thixotropic behaviour. The decreasing of yogurt viscosity is time-dependent and

was reported by Denin-Djurdjević et al. (2002). This behaviour observed provides justification for the use of Helipath spindle which is essentially ideal for determining viscosity in a non-Newtonian fluid. The T spindle attached to Helipath system moves up and down inside the sample in a vertical motion as it rotates, preventing any local syneresis and exceedingly small viscosity readings (Tamime & Robinson, 2007).

The apparent viscosity of stirred yogurt is illustrated in Table 1. The addition of papaya peel powder resulted in an increase in viscosity. This might be due to the absorption of some water from the yogurt by the added fibre and thus, resulting in a viscous product as compared to the control sample. Yogurt added with 3.0% of peel powder for both types of dietary fibre (3.0% PP1 and 3.0% PP2) exhibited significant higher viscosity than yogurt added with 1.5% peel powder (1.5% PP1 and 1.5% PP2) as seen in Table 1. Prior study reported by Vieira et al. (2015) on passion fruit peel flour enrichment in yogurt is in agreement with this. The resulting yogurt had higher viscosity values as the passion fruit peel flour concentration was increased.

In addition, the viscosity was increased significantly ($P < 0.05$) during 21 days storage for all samples. The lowest viscosity was observed on Day 0 and it rose until

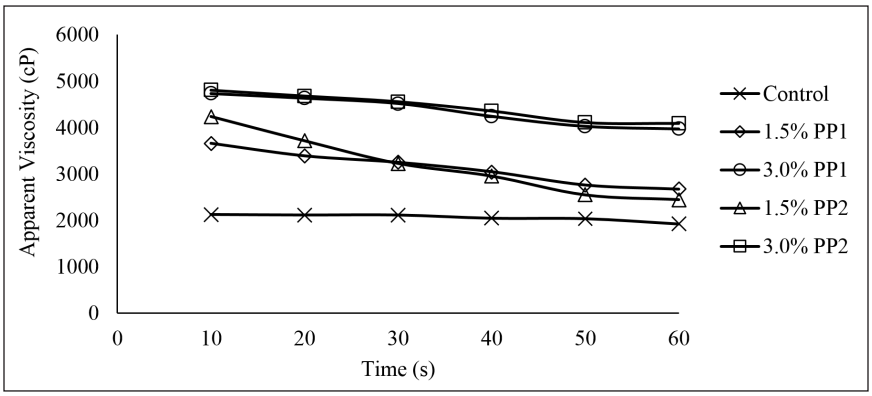

Figure 1. Apparent viscosity of stirred yogurt samples at 10 second interval on Day 0

Table 1
Apparent viscosity (centiPoise) of stirred yogurt samples with 21-Day storage

	Days	Stirred yogurt types at two treatments				
		Control	1.5% PP1	3.0% PP1	1.5% PP2	3.0% PP2
Viscosity (centiPoise)	0	2122 ± 19.05^{Aa}	3656 ± 19.63^{Ab}	4733 ± 33.50^{Ac}	4233 ± 33.50^{Ad}	4803 ± 50.08^{Ae}
	7	2385 ± 25.98^{Ba}	3639 ± 34.83^{Ab}	4911 ± 19.05^{Bc}	4823 ± 40.71^{Bd}	5648 ± 41.87^{Be}
	14	2841 ± 35.73^{Ca}	3919 ± 17.06^{Bb}	4974 ± 36.14^{Bc}	5407 ± 44.91^{Cd}	5818 ± 31.75^{Ce}
	21	2911 ± 19.05^{Da}	3956 ± 19.63^{Bb}	5348 ± 45.37^{Cc}	5607 ± 60.28^{Dd}	5933 ± 33.50^{De}

Note. Means followed by the same lowercase superscript letters (a–e) in the same row were not significantly different for stirred yogurt types ($P > 0.05$)
Means followed by the same uppercase superscript letters (A–D) in the same column were not significantly different for same type of sample at 0, 7, 14 and 21 day of refrigerated storage ($P > 0.05$)
Mean ± standard deviation of 3 replicates

it reached the highest value on 21st day. This increasing viscosity observed in yogurts can be attributed to the phenomenon of rebodying. In other words, there is a recovery of the structure in yogurt with passage of time as similarly found by Sah et al. (2016) for pineapple peel powder added to probiotic yogurt and by Tufeanu et al. (2017) for chia powder added to stirred yogurt.

Figure 2 displays the percentage change of viscosity during storage for each stirred yogurt sample type. Control and 1.5% PP1 sample showed the highest percentage change on Day 14 while for 1.5% PP2 and 3.0% PP2 sample, the highest change was observed on Day 7. For 3.0% PP1 sample, greatest change was observed on Day 21.

pH

Table 2 displays the pH values recorded for all the yogurt samples. Overall, the yogurt samples exhibited pH value between 3.99 to 4.22, indicating that the yogurt sample fortified with papaya peel was within the limit prescribed in Food and Drug Administration (FDA) (2017). As per FDA, pH of yogurt is defined as 4.6 and lower. However, the pH values may vary and the consumer may appreciate a lower pH of 4.2

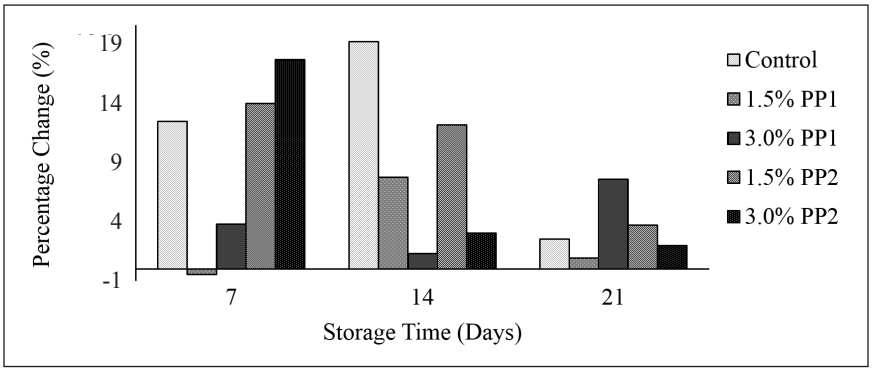

Figure 2. Percentage change in apparent viscosity of stirred yogurt samples with 21-Day storage

Table 2
pH values of stirred yogurt samples with 21-Day storage

	Days	Stirred yogurt types at two treatments				
		Control	1.5% PP1	3.0% PP1	1.5% PP2	3.0% PP2
pH	0	4.24 ± 0.01^{Aa}	4.22 ± 0.01^{Aa}	4.20 ± 0.02^{Aa}	4.20 ± 0.02^{Aa}	4.20 ± 0.01^{Aa}
	7	4.21 ± 0.01^{Aa}	4.21 ± 0.02^{Aa}	4.19 ± 0.02^{Aab}	4.17 ± 0.02^{Aab}	4.16 ± 0.01^{Bb}
	14	4.10 ± 0.02^{Ba}	4.11 ± 0.01^{Ba}	4.09 ± 0.01^{Ba}	4.03 ± 0.02^{Bb}	4.04 ± 0.01^{Cb}
	21	4.04 ± 0.01^{Ca}	4.03 ± 0.01^{Ca}	4.02 ± 0.01^{Ca}	4.02 ± 0.07^{Ba}	3.99 ± 0.01^{Da}

Note. Means followed by the same lowercase superscript letters (a–e) in the same row were not significantly different for stirred yogurt types ($P > 0.05$)
Means followed by the same uppercase superscript letters (A–D) in the same column were not significantly different for same type of sample at 0, 7, 14 and 21 day of refrigerated storage ($P > 0.05$)
Mean ± standard deviation of 3 replicates

(Chen et al., 2018). pH values between 4.2 and 4.4 are considered likable by consumers in fermented products (Benedetti et al., 2016).

Although the initial pH values for all samples were not significantly different ($P > 0.05$), it was noted that the control sample was 0.02 to 0.04 units higher than the other samples during storage. Also, 3.0% PP1 and 3.0% PP2 had slightly acidic pH that their respective lower concentration samples, 1.5% PP1 and 1.5% PP2. These findings are comparable to the effect of passion fruit peel powder dosage on yogurt as reported by Vieira et al. (2015). The final pH measured at 21st day for all the samples showed no significant difference among each other. The fortified samples had pH values 0.01 to 0.05 units lower than the control.

Going through the 21-Day storage, it was observed that the values of pH decreased significantly ($P < 0.05$). For each stirred yogurt samples, control and fortified, the reduction was by > 0.21 units. The main reason behind the decrease of pH value might be due to the production of lactic acid. During storage, the activity of lactic acid bacteria is increased which consume lactose from the yogurt and produce lactic acid. Thus, making the yogurt more acidic. This decreasing trend in yogurt during storage was also observed by Tseng and Zhao (2013) for wine grape pomace powder enriched yogurt and salad dressing and by Chen et al. (2018) for chickpea powder fortified yogurt. Both the studies concluded that the pH of samples reduced with storage period at 4^{o}C.

Figure 3 illustrates the percentage decrease in pH during storage. It can be seen that on Day 14 the percentage decrease was the highest, followed by Day 21 while the lowest was observed on Day 7 for all the samples.

Colour

Colour of yogurt plays an important role in consumer acceptance and marketability. Any functional yogurts prepared should not only provide essential nutrients but should also be attractive to the consumers (Ajila et al., 2008). The colour parameters measured for stirred yogurt samples were

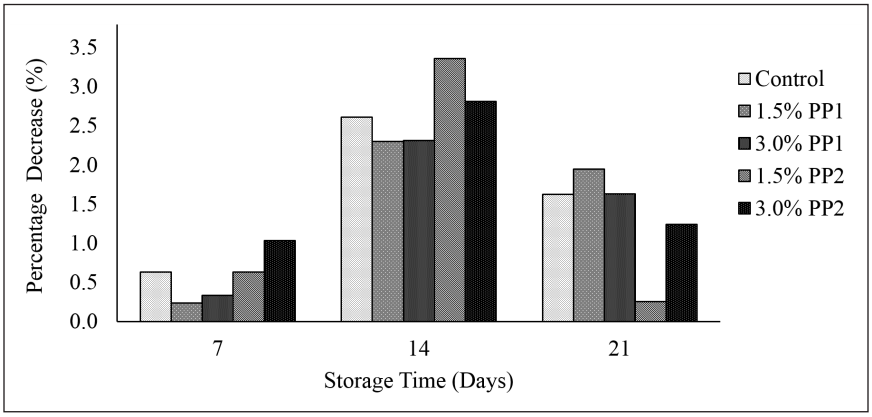

Figure 3. Percentage decrease in pH values of stirred yogurt samples with 21-Day storage

statistically different ($P < 0.05$) as presented in Table 3. The lightness parameter, *L** was reduced as the concentration of peel powder was increased in yogurt. In other words, the powder had a darkening effect in yogurt samples which may be due to fibres in powder absorbing water. *L** was the highest for control sample as compared to the fortified throughout the storage period. The results obtained in this study can favour consumers as they prefer lower brightness in dairy related products (García-Pérez et al., 2005). In control samples, since there was no addition of papaya peel powder, the redness factor, *a** was in negatives. While for fortified samples, it was noted that the increase in concentration of powder was accompanied by more redness. Similar

Table 3
Colour parameter of stirred yogurt samples with 21-Day storage

Colour parameters	Days	Stirred yogurt types at two treatments				
		Control	1.5% PP1	3.0% PP1	1.5% PP2	3.0% PP2
L*	0	89.73±0.07^{Aa}	77.49±0.01^{Ab}	72.13±0.00^{Ac}	73.51±0.02^{Ad}	67.30±0.01^{Ae}
	7	89.67±0.01^{Aa}	77.06±0.02^{Bb}	72.14±0.02^{Ac}	73.85±0.01^{Bd}	66.95±0.01^{Be}
	14	89.67±0.04^{Aa}	77.03±0.02^{Bb}	72.11±0.02^{Ac}	73.63±0.02^{Cd}	66.91±0.02^{Be}
	21	89.66±0.01^{Aa}	76.98±0.02^{Cb}	72.12±0.02^{Ac}	73.59±0.02^{Cd}	66.85±0.03^{Ce}
*a**	0	-1.87±0.02^{Aa}	3.53±0.01^{Ab}	5.56±0.02^{Ac}	5.21±0.04^{Ad}	7.33±0.01^{Ae}
	7	-1.86±0.03^{Aa}	3.51±0.02^{Ab}	5.20±0.03^{Bc}	4.77±0.02^{Bd}	6.93±0.02^{Be}
	14	-1.87±0.02^{Aa}	3.52±0.03^{Ab}	5.13±0.03^{Cc}	4.55±0.03^{Cd}	6.96±0.03^{Be}
	21	-1.83±0.03^{Aa}	3.53±0.11^{Ab}	5.11±0.02^{Cc}	4.47±0.02^{Dd}	6.46±0.05^{Ce}
*b**	0	13.10±0.02^{Aa}	20.41±0.05^{Ab}	24.66±0.04^{Ac}	24.01±0.06^{Ad}	27.47±0.05^{Ae}
	7	12.38±0.04^{Ba}	19.57±0.06^{Bb}	22.89±0.08^{Bc}	22.27±0.06^{Bd}	25.67±0.07^{Be}
	14	12.32±0.03^{Ba}	19.03±0.01^{Cb}	22.82±0.01^{Bc}	22.16±0.04^{Bd}	25.74±0.03^{Be}
	21	12.33±0.03^{Ba}	19.02±0.01^{Cb}	22.79±0.04^{Bc}	22.18±0.03^{Bd}	25.73±0.02^{Be}
Hue angle (H^o)	0	-81.86±0.05^{Aa}	80.18±0.04^{Ab}	77.29±0.06^{Ac}	77.76±0.11^{Ad}	75.05±0.04^{Ae}
	7	-81.46±0.13^{Ba}	79.84±0.07^{ABb}	77.21±0.05^{Ac}	77.90±0.05^{Ad}	74.90±0.02^{Be}
	14	-81.37±0.08^{Ba}	79.53±0.09^{Bb}	77.34±0.06^{Ac}	78.39±0.08^{Bd}	74.87±0.04^{Be}
	21	-81.58±0.16^{ABa}	79.48±0.31^{Bb}	77.36±0.06^{Ac}	78.60±0.06^{Cd}	75.90±0.10^{Ce}
Chroma (C^*)	0	13.24±0.02^{Aa}	20.72±0.05^{Ab}	25.28±0.03^{Ac}	24.57±0.05^{Ad}	28.44±0.04^{Ae}
	7	12.52±0.05^{Ba}	19.88±0.05^{Bb}	23.48±0.08^{Bc}	22.78±0.06^{Bd}	26.58±0.07^{BCe}
	14	12.46±0.03^{Ba}	19.35±0.01^{Cb}	23.39±0.01^{Bc}	22.62±0.04^{Cd}	26.66±0.03^{Be}
	21	12.47±0.02^{Ba}	19.34±0.02^{Cb}	23.36±0.03^{Bc}	22.63±0.03^{Cd}	26.53±0.00^{Ce}
ΔE	0	0	0	0	0	0
	7	0.73±0.06^{Aa}	0.94±0.02^{Ab}	1.80±0.11^{Ac}	1.83±0.02^{Ac}	1.88±0.05^{Ac}
	14	0.79±0.04^{Aa}	1.46±0.06^{Bb}	1.88±0.02^{Acd}	1.97±0.06^{Bc}	1.82±0.04^{Ad}
	21	0.78±0.02^{Aa}	1.49±0.05^{Bb}	1.92±0.07^{Ac}	1.97±0.02^{Bc}	2.00±0.04^{Bc}

Note Means followed by the same lowercase superscript letters (a–e) in the same row were not significantly different for stirred yogurt types ($P > 0.05$)
Means followed by the same uppercase superscript letters (A–D) in the same column were not significantly different for same type of sample at 0, 7, 14 and 21 day of refrigerated storage ($P > 0.05$)
Mean ± standard deviation of 3 replicates

observations were made for values of yellowness factor, *b**. Yogurts with higher levels of powder showed more yellowness. As for control sample, the values of *b** was much lower due to non-supplementation. Chroma and hue angle values were also greater in fortified samples, thus shifting towards yellow. Hue angle was especially in the negatives for control sample owing to the negative *a** value.

ΔE was determined by the taking colour parameters of the sample at Day 0 as a reference for each yogurt type and is illustrated in Figure 4. This was to determine if the difference in colour among the samples during storage which were measured using the device could be perceived by the human eye. Generally, if ΔE value is > 3 then the difference is evident to the human eye (Francis & Clydesdale, 1975). From Table 3, it is observed that all the values among each sample fall below 3. This indicates that the colour did not change much during storage ($P > 0.05$) and the human eye cannot differentiate it. Similar results were also seen from other colour parameters which did not show much significant difference ($P > 0.05$) throughout the storage at 4°C. The values were not modified during 21 days, although the difference in dosage of powder effected the colour coordinates. Similar findings of this unchanged behaviour in colour parameters during storage were also reported by García-Pérez et al. (2005) on orange fibre addition in yogurt and Sah et al. (2016) on pineapple peel powder enriched yogurt.

Sensory Properties

The scores from sensory analysis in terms of 9-point hedonic scale are summarized in Table 4. The sensory attributes of appearance, texture, colour and overall acceptability received scores which were statistically different ($P < 0.05$) excluding odour and flavour. Scores for fortified samples were slightly less than the control sample. 3.0% PP2 sample received the lowest scores for all sensory attributes while the control received the highest. It was noted that the liking scores were affected by the concentration of the papaya peel powder added. From the table it can be seen that 3.0% PP1 and 3.0% PP2 had

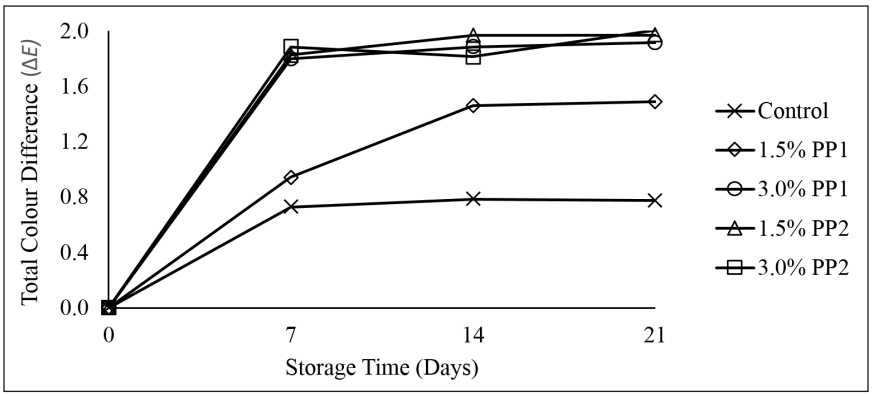

Figure 4. Total colour difference (ΔE) of stirred yogurt samples with 21-Day storage

Table 4
Consumer acceptance of stirred yogurt samples

Sensory attributes	Stirred yogurt types at two treatments				
	Control	1.5% PP1	3.0% PP1	1.5% PP2	3.0% PP2
Appearance	8.00 ± 1.29^{a}	7.33 ± 1.35^{ab}	6.53 ± 1.78^{bc}	6.73 ± 1.62^{bc}	5.93 ± 2.13^{c}
Texture	8.07 ± 1.11^{a}	7.60 ± 1.35^{ab}	7.03 ± 1.54^{bc}	6.97 ± 1.35^{bc}	6.53 ± 1.66^{c}
Odour	7.90 ± 1.42^{a}	7.40 ± 1.65^{a}	6.90 ± 1.84^{a}	6.97 ± 2.06^{a}	6.93 ± 1.60^{a}
Flavour	6.80 ± 1.61^{a}	6.37 ± 2.17^{a}	5.83 ± 2.07^{a}	6.13 ± 2.10^{a}	5.70 ± 2.49^{a}
Colour	7.93 ± 1.30^{a}	7.33 ± 1.30^{a}	6.87 ± 1.70^{ab}	7.00 ± 1.44^{ab}	6.13 ± 1.85^{b}
Overall Impression	7.60 ± 1.22^{a}	7.13 ± 1.46^{ab}	6.67 ± 1.40^{ab}	6.73 ± 1.55^{ab}	6.27 ± 1.84^{b}

Means followed by the same superscript letters (a–c) in the same row were not significantly different by the Tukey test ($P > 0.05$)
9-point hedonic scale used where 1= extremely dislike till 9 = extremely like; Mean ± standard deviation

lower ratings than their corresponding lower concentration powder yogurts (1.5% PP1 and 1.5% PP2). Consumers gave moderate scores for colour and appearance to all samples prepared, even though there was an evident light-yellow colour in fortified samples. Texture scores ranged from 6.53 to 8.07. Since, higher dosage of powder resulted in a more viscous sample, the stirred yogurt also received lower scores for 3.0% PP1 and 3.0% PP2. Addition of papaya peel powder in yogurt exhibited no significant change in odour and flavour. This means the taste was acceptable to consumers even if the concentration of powder was increased. Overall impression of the samples was moderately positive (6.27 to 7.60). In previous study, yogurts developed from skins of two varieties of grapes mostly met with lower sensory scores due to a grainy texture and sour taste (Marchiani et al., 2016). Tseng and Zhao, (2013) reported that yogurt with 1% concentration of wine grape pomace powder received favourable score for flavour and consistency. Some consumers commented on its fruit taste while few considered of it as chalky and medicine-like. From the current study it can be concluded that the stirred yogurt prepared by adding papaya peel powder dried at 55°C (1.5% PP1) was the most preferable to consumers as compared to other fortified samples. Although measurement by sensory analysis is subjective, the scores given by consumers correspond well with the food preferences (Córdova-Ramos et al., 2018).

CONCLUSIONS

Papaya peels have a significant amount of dietary fibre which can be utilized to develop new products and in turn help to reduce waste disposal. In the present study, it was used as a source of dietary fibre to enrich yogurt. The peels were dried at three temperatures (45, 55 and 65°C) and at drying temperature of 55 and 65°C, the highest dietary fibre content was obtained which was added to yogurt at concentration of 1.5

and 3.0% (w/w). Quality characteristics of the fortified stirred yogurt was analysed weekly for 21 days. The addition of peel powder resulted in an increase in viscosity of yogurt as compared to control. In addition, the viscosity also increased with the storage time. pH values showed no statistical difference ($P > 0.05$) among the samples prepared. Colour parameter remained unchanged with storage, even though it was significantly altered ($P < 0.05$) with concentration of papaya peel added. Sensory analysis showed that stirred yogurt enriched with 1.5% papaya peel powder dried at 55°C emerged as the best product in terms of highest sensory scores. Hence, this work can be proposed to the fruit industry that the fruit peels can be utilized as a functional ingredient to improve food quality.

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Effect of Storage Time on Ascorbic Acid and Total Phenolic Contents and Colour of Blanched, Boiled and Steamed Cauliflowers (*Brassica oleracea* L. ssp. *botrytis*)

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ABSTRACT

The effect of storage time on ascorbic acid content (AAC), total phenolic content (TPC) and colour of blanched, boiled and steamed cauliflowers (*Brassica oleracea* L. ssp. *botrytis*) was investigated. Blanching and steaming retained more AAC in cauliflower as compared to boiling. Storage time showed no significant changes in AAC measured at 30 min interval up to 2.5 h in all samples with the expection of boiled sample at 2.5 h in which significant reduction was observed. Blanching resulted in a higher TPC in cauliflower as compared to boiling or steaming. There were no significant differences between the TPC of boiled and steamed cauliflowers. Storage time showed no significant changes in TPC measured at 30 min interval up to 2.5 h in all samples. As for the colour measurement, there were significant reduction in the L*, a* and b* values of the blanched, boiled and steamed cauliflowers as compared to raw cauliflower but there were no significant changes in these colour values measured at 30 min interval up to 2.5 h. This study demonstrated that blanched, boiled or steamed cauliflower can be stored up to 2 h with no significant changes in the AAC, TPC, L*, a* and b* colour values.

Keywords: Blanching, boiling, cooking, room temperature storage, steaming, thermal processing, vitamin C

INTRODUCTION

The Brassicaceae is a plant family that contains about 340 genera and 3700 species and is of huge economic importance (Pedras & Yaya, 2010). Cauliflower (*Brassica oleracea* L. ssp. *botrytis*) belongs to this family. Examples of other plants under the Brassiceae family are cabbage, brussel

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sprouts and broccoli (Kadam et al., 2005; Pellegrini et al., 2010). Cauliflower has been mainly cultivated in the Northern Europe and England and has been extended to United States, India and China as well. Optimum temperatures for growth of cauliflower are 15-20^{o}C with maximum and minimum temperature of 25^{o}C and 8^{o}C, respectively (Tsao & Lo, 2003). The texture of fresh cauliflower is firm with slight spongy texture and minor bitter flavour contributed by the presence of glucosinolates. The enzymatic derivatives of glucosinolates are biologically active (Kapusta-Duch et al., 2016). Cauliflower contains a number of phytochemicals and vitamins such as vitamin C and K which are good sources of antioxidant associated in reducing risk of cardiovascular diseases and cancer (Byers & Perry, 1992; Singh et al., 2007). The total phenolic and ascorbic acid contents change during ripening process of fruits and vegetables; as ripening progresses, the total soluble solid (TSS) increases (Guleria, 2000). Broccoli is a highly perishable vegetable and colour is one of the main external quality attributes of broccoli (Schouten et al., 2009).

Exposure of most of the food to temperature above ambient conditions causes physical and chemical changes in texture, flavour, colour or nutrients due to oxidation and degradation of nutrients. Nonetheless, thermal processes such as blanching, boiling and steaming are important as these processes contribute in preserving and producing food that is safe for consumption by ensuring that most pathogenic and spoilage-causing microorganisms are destroyed. This would also lead to creating an environment that does not support the growth of spoilage microorganism (Ramaswamy & Chen, 2002). The losses of nutrients in cauliflower can be reduced by ensuring that thermal heat applied on food is at the optimum level and over processing of food is avoided. There is a relationship between the temperature and duration of exposure on the loss of nutrients. Low temperature with long duration time causes less damage as compared to high temperature with short duration (Goullieux & Pain, 2005). Common cooking processes treated on cauliflowers are blanching, boiling and steaming. The differences in each processes is the duration of time exposed to boiling water except for steaming which does not have a direct contact to boiling water but rather the steam from the boiling water is transferred as heat to the food. The advantage of steaming over the other two thermal processes is that it reduces the contact of water with food which decreases the loss of nutrients via leaching (Fellows, 2009). Blanching is a thermal process done by briefly immersing the samples into boiling water for approximately 3 to 5 min. The main purpose of this process is to inactivate the enzymes such as peroxides that affect the sensory quality and nutritional values during storage. Therefore, to ensure minimal losses, this process should be carried out really quick and at most 5 min (Parreño & Torres, 2011). Besides that, these thermal processes can cause browning in cauliflower due to the activity of polyphenol oxidase which catalyzes the oxidation of phenols to quinones that

produce brownish red pigments as seen on fruit or vegetables browning (Pellegrini et al., 2010; Roy et al., 2007; Vamos-Vigyazo, 1981). Vegetables are usually cooked and may be left at room temperature for a period of time before consumption. There is currently no reported study on the stability of ascorbic acids, phenolic compounds and colour of cauliflowers after thermal processing or cooking. The objective of this study was to determine the effect of storage time on ascorbic acid content, total phenolic content and colour of blanched, boiled and steamed cauliflowers (*Brassica oleracea* L. ssp. *botrytis*).

MATERIALS AND METHODS

Materials

White cauliflowers with total soluble solids of 7.4 ± 0.2° Brix measured using a refractometer (Atago, Minato-ku, Japan) were obtained from a local supermarket in Shah Alam, Selangor, Malaysia. All chemicals and solvents were of analytical grade and purchased either from Sigma-Alrich (St. Louis, U.S.A.) or Merck (Darmstadt, Germany).

Cooking Processes

Blanching. Blanching was carried out according to the method of Volden et al. (2009) with some modifications. Florets were immersed into water at 96ºC with a ratio of [weight of sample (g): volume of water (mL), 1: 10] for 3 min. Then, the samples were removed and left to drain for 1 min.

Boiling. Boiling was carried out according to the method of Volden et al. (2009) with some modifications. Florets were immersed into a pot of boiling water with a ratio of [weight of sample (g): volume of water (mL), 1: 5] for 10 min. Then, the samples were removed from the pot of boiling water and left to drain for 1 min.

Steaming. Steaming was carried out according to the method of Volden et al. (2009) with some modifications. Florets (500 g) were placed in a steamer above 1 L of boiling water for 10 min. Then, the samples were removed from the steamer and left to cool for 1 min.

Storage Studies

Single layer of blanched, boiled or steamed samples were left exposed at 25ºC for 2.5 h and samples were taken for analysis at 30-min interval time.

Sample Preparation

The sample preparation was carried out according to the method of Choo et al. (2014) with some modifications. For the ascorbic acid content, 5 g of florets were cut, crushed and mixed with 50 mL of 4% (w/v) metaphosphoric acid. It was mixed for 15 min followed by filtration of the mixture under vacuum and transferred into a 100 mL volumetric flask. For the total phenolic content, 70 g of florets were cut and crushed into paste-like state with addition of 100 mL of water using a Waring blender for 1 min (with intermittent stops to minimize heating at every 10 s interval between 30 s

of blending). The homogenized sample was transferred into a 250 mL volumetric flask and top up to the mark using 50% ethanol. The mixture was mixed for 15 min and then filtered under vacuum. All the filtered extracts obtained were stored at -20°C. The ascorbic acid content and colour were measured immediately after extraction.

Ascorbic Acid Content (AAC)

The AAC in cauliflower was measured using the iodine titration method of Suntornsuk et al. (2002). Firstly, starch solution was prepared by mixing 1g of starch into 200 mL of boiling water. The solution was immediately removed from heat and left to cool.

The iodine titration was performed by mixing 25 mL of juice extracted from cauliflower into a 250 mL Erlenmeyer flask. Then, 25 mL of 2N sulphuric acid, 50 mL of water and 3 mL of starch indicator were added into the flask and mixed well. The sample was titrated using 0.001N of iodine solution. Each 1 mL of 0.1N iodine used is equivalent to 8.806 mg of ascorbic acid. The AAC was expressed in terms of milligram of ascorbic acid in 100 g of sample.

Total Phenolic Content (TPC)

TPC in cauliflower was determined using Folin-Ciocalteu's reagent according to the method of Lim et al. (2007). Sample extracted (0.3 mL) was placed into test tubes followed by addition of 1.5 mL of Folin-Ciocalteu's reagent (10% v/v) and 1.2 mL of sodium carbonate (7.5% w/v). Then the test tubes were covered using parafilm, vortexed and left to stand for 30 min at room temperature before the absorbance was measured at 765 nm against a blank reagent. If the sample absorbance exceeded 1, appropriate dilution was required to give absorbance reading less than 1. TPC is expressed in terms of gallic acid equivalents in mg per 100 g of sample. As ascorbic acid contributes to the formation of blue molybdenum-tungsten complex, absorbance originating from it is corrected by measuring an ascorbic acid calibration curve.

Colour Measurement

Colour determination of cauliflower was carried out using a Hunter colorimeter (Hunter Associates Laboratory, Virginia, U.S.A). L* designates the lightness of the sample, 100=white, 0=black, a* indicates redness when positive, greenness when negative, b* indicates yellowness when positive, blueness when negative.

Statistical Analysis

All experiments were carried out in independent triplicates. The data obtained were analyzed using one-way analysis of variance (ANOVA) followed by Duncan's multiple range test using SAS software package (SAS Institute Inc, Cary, U.S.A.). The statistical significance was evaluated at $p<0.05$.

RESULTS AND DISCUSSION

Ascorbic Acid Content (AAC)

The AAC of the raw cauliflowers (Table 1) in this study is in accordance to the AAC

reported for raw cauliflowers (Korus, 2010; Mazzeo et al., 2011). Thermal processing caused significant reduction in AAC of cauliflowers as compared to that of raw cauliflower (Table 1). The decrease of AAC after processing was due to heat degradation as ascorbic acid is heat sensitive which is easily destroyed under high heat temperature. Among the thermal treatment, the AAC of boiled cauliflowers was the lowest (Table 1). This is in accordance to the study of Volden et al. (2009). Ascorbic acids are water soluble; therefore, ascorbic acids in the cauliflower can easily leach from the cauliflower into the boiling water. In addition, less water and longer duration are usually used for boiling of vegetables as more energy is required in the process compared to blanching (Ferracane et al., 2008; Miglio et al., 2008). There were no significant changes in the AAC of raw, blanched and steamed samples during storage for 2.5 h (Table 1). These indicate that the TPC of the raw, blanched and steamed cauliflowers remained stable during this period of time. However, there was a significant reduction ($p<0.05$) of the AAC of boiled sample stored at 150 min or 2.5 h as compared to immediately after boiling. This corresponds with the AAC of boiled sample was the lowest among the thermal treatment (Table 1) and after storage for 2.5 h, further reduction occurred. This also suggests that to avoid a significant loss in AAC, boiled cauliflower should be consumed before 2.5 h.

Total Phenolic Content(TPC)

The TPC of cauliflowers (Table 2) in this study was lower than that reported by Volden et al. (2009). The difference in TPC might be due to the different environmental growth conditions such as temperature, light intensity, humidity, water availability, wind strength and rainfall that would greatly affect the growth of plants (Solomon et al., 2002). Raja et al. (2011) reported the presence of gallic acid, ferulic acid, chlorogenic acid and catechin whereas Lee at al. (2011) reported the presence of caffeic acid, p-coumaric acid, ferulic acid and sinapic acid in cauliflowers. There was

Table 1
Ascorbic acid contents of raw, blanched, boiled and steamed cauliflowers

Storage time (min)	AAC (mg/100 g of fruits)			
	Raw	Blanched	Boiled	Steamed
0	59.4 ± 1.8^{Aa}	51.6 ± 1.1^{Ab}	39.3 ± 0.8^{Ac}	52.1 ± 1.4^{Ab}
30	59.4 ± 1.1^{Aa}	50.9 ± 0.4^{Ab}	38.5 ± 0.9^{ABc}	51.2 ± 0.8^{Ab}
60	59.7 ± 1.1^{Aa}	52.6 ± 1.5^{Ab}	38.5 ± 0.9^{ABc}	50.9 ± 0.8^{Ab}
90	59.0 ± 1.1^{Aa}	52.6 ± 1.5^{Ab}	37.8 ± 0.9^{ABc}	51.2 ± 1.1^{Ab}
120	60.4 ± 0.4^{Aa}	52.3 ± 0.4^{Ab}	38.1 ± 1.3^{ABc}	51.2 ± 1.5^{Ab}
150	59.9 ± 0.7^{Aa}	52.1 ± 0.7^{Ab}	37.3 ± 0.7^{Bc}	51.2 ± 0.4^{Ab}

Results were presented as mean ± standard deviation
^{AB}Values with different superscript letters within a column indicate significant differences at $p<0.05$
^{abc}Values with different superscript letters within a row indicate significant differences at $p<0.05$

no significant difference between the TPC of raw and blanched cauliflowers and the TPC of these cauliflowers were significantly higher ($p<0.05$) than boiled and steamed cauliflowers (Table 2). Furthermore, the TPC of steamed cauliflowers was not significantly different from that of boiled cauliflowers. Factors such as temperature, exposure time and volume of water used would affect the total loss of phenolic content in vegetables (Ismail et al., 2004; Natella et al., 2010; Turkmen et al., 2005). The reduction of TPC in boiled cauliflower is in accordance with the study of Mazzeo et al. (2011) on boiled frozen cauliflower and Watchel-Galor et al. (2008) on boiled cauliflower.

Steamed frozen cauliflowers (Mazzeo et al., 2011) and steamed cauliflowers (Watchel-Galor et al., 2008) were reported to have higher TPC compared to the raw samples. These authors suggested that steaming process caused tissue softening which enhanced the availability of the compounds to be extracted and led to the production of redox-active secondary plant metabolites products. These results are in contrast to that obtained in this study and by Pellegrini et al. (2010) on steamed frozen cauliflowers. The discrepancy between the TPC observed for cauliflower as a result of steaming was suggested by Mazzeo et al. (2011) to probably related to a different size of cauliflower. There were no significant changes in the TPC of raw, blanched, boiled and steamed samples during storage for 2.5 h (Table 1). These indicate that the TPC of the raw and thermal processed cauliflowers remained stable during this period of time.

Colour Measurement

The L*, a* and b* values of cauliflower (Tables 3-5) in this study is in accordance with those reported by Pellegrini et al. (2010). The L* value of frozen cauliflowers were lower than fresh cauliflowers (Pellegrini et al., 2010). The three thermal processes reduced the L* value of cauliflowers with steamed cauliflowers with the lowest L* value (Table 3). These thermal treatment may have induced the development of browning in cauliflowers. Low temperature

Table 2
Total phenolic contents of raw, blanched, boiled and steamed cauliflowers

Storage time (min)	TPC (mg GAE /100g of fruits)			
	Raw	Blanched	Boiled	Steamed
0	29.1 ± 3.2^{Aa}	36.0 ± 3.6^{Aa}	17.9 ± 5.9^{Ab}	18.8 ± 5.9^{Ab}
30	32.6 ± 1.8^{Aa}	27.8 ± 3.4^{Aa}	16.8 ± 5.8^{Ab}	15.5 ± 7.2^{Ab}
60	34.0 ± 5.9^{Aa}	28.9 ± 7.5^{Aab}	16.7 ± 5.9^{Ab}	16.6 ± 6.5^{Ab}
90	31.9 ± 4.9^{Aa}	31.1 ± 2.1^{Aa}	17.9 ± 5.9^{Ab}	17.0 ± 4.5^{Ab}
120	29.9 ± 5.0^{Aa}	28.6 ± 6.7^{Aa}	15.4 ± 5.9^{Ab}	16.7 ± 6.4^{Ab}
150	33.3 ± 6.0^{Aa}	27.8 ± 2.9^{Aab}	16.5 ± 6.6^{Ab}	16.2 ± 7.4^{Ab}

Results were presented in mean ± standard deviation
[A]Values with different superscript letters within a column indicate significant differences at $p<0.05$
[abc]Values with different superscript letters within a row indicate significant differences at $p<0.05$

storage of cauliflowers at 4ºC also resulted in decreased L* values (Berrang et al., 1990). These are attributed to the development of browning in cauliflowers (Nunes, 2008). Decrease in L* value indicates lower brightness intensity but at the same time, there was an increase in –a* value in the three thermal processed cauliflowers which indicates a shift towards green, moving away from the redness (Table 4). These results are in accordance to the studies of Mazzeo et al. (2011) on frozen cauliflowers and Pellegrini et al. (2010) on both fresh and frozen cauliflowers. Blanching and boiling reduced the b* values of cauliflowers but there was no significant difference between raw and steamed cauliflowers. As for the effect of storage, there were no significant changes in the L*, a* and b* values of the three thermal processed cauliflowers during storage for 2.5 h (Tables 3-5). These indicate that colour of the raw and thermal processed cauliflowers remained stable during this period of time.

Table 3
Colour (L) of raw, blanched, boiled, steamed cauliflowers*

Storage time (min)	Colour (L*)			
	Raw	Blanching	Boiling	Steaming
0	82.5 ± 0.4^{Aa}	79.3 ± 1.0^{Ab}	77.7 ± 0.8^{Abc}	77.3 ± 1.4^{Ac}
30	82.5 ± 0.8^{Aa}	77.5 ± 1.2^{Ab}	79.9 ± 3.5^{Aab}	77.5 ± 1.8^{Ab}
60	82.5 ± 0.2^{Aa}	79.4 ± 0.8^{Ab}	77.8 ± 0.9^{Abc}	77.2 ± 1.6^{Ab}
90	82.5 ± 0.6^{Aa}	77.7 ± 1.6^{Ab}	78.2 ± 0.6^{Ab}	77.4 ± 2.4^{Ab}
120	82.4 ± 0.2^{Aa}	78.9 ± 0.6^{Ab}	77.4 ± 0.9^{Ac}	77.3 ± 0.7^{Ac}
150	82.6 ± 0.2^{Aa}	77.6 ± 1.7^{Ab}	77.9 ± 0.6^{Ab}	77.1 ± 1.6^{Ab}

Results were presented as mean ± standard deviation
[A]Values with different superscript letters within a column indicate significant differences at $p<0.05$
[abc]Values with different superscript letters within a row indicate significant differences at $p<0.05$
L* designates the lightness of the sample; 100=white, 0=black

Table 4
Colour (a) of raw, blanched, boiled, steamed cauliflowers*

Storage time (min)	Colour (a*)			
	Raw	Blanched	Boiled	Steamed
0	0.6 ± 0.3^{Aa}	-0.4 ± 0.4^{Ab}	-1.9 ± 0.4^{Ab}	-1.8 ± 0.3^{Ab}
30	0.7 ± 0.6^{Aa}	-0.6 ± 0.3^{Ab}	-1.7 ± 0.4^{Ab}	-1.7 ± 0.9^{Ab}
60	0.5 ± 0.4^{Aa}	-0.7 ± 0.6^{Ab}	-1.7 ± 0.4^{Ab}	-1.7 ± 0.4^{Ab}
90	0.4 ± 0.4^{Aa}	-0.5 ± 0.4^{Ab}	-1.6 ± 0.4^{Ab}	-0.9 ± 0.1^{Ab}
120	0.8 ± 0.4^{Aa}	-0.4 ± 0.4^{Ab}	-1.8 ± 0.2^{Abc}	-1.9 ± 0.5^{Ac}
150	0.7 ± 0.4^{Aa}	-0.8 ± 0.4^{Ab}	-1.5 ± 0.2^{Ac}	-1.1 ± 0.5^{Ac}

Results were presented as mean ± standard deviation
[A]Values with different superscript letters within a column indicate significant differences at $p<0.05$
[abc]Values with different superscript letters within a row indicate significant differences at $p<0.05$
a* indicates redness when positive; greenness when negative

Table 5
Colour (b) of raw, blanched, boiled, steamed cauliflowers*

Storage time (min)	Colour (b*)			
	Raw	Blanching	Boiling	Steaming
0	21.0 ± 1.4^{Aab}	20.0 ± 1.6^{Ab}	19.2 ± 1.2^{Ab}	23.0 ± 1.4^{Aa}
30	22.1 ± 1.6^{Aa}	21.7 ± 0.5^{Aa}	19.2 ± 1.6^{Ab}	24.0 ± 1.1^{Aa}
60	21.4 ± 2.1^{Aab}	20.7 ± 1.0^{Aab}	19.7 ± 1.3^{Ab}	22.6 ± 1.0^{Aa}
90	21.1 ± 1.3^{Aa}	21.5 ± 0.7^{Aa}	19.4 ± 2.1^{Aa}	22.8 ± 3.2^{Aa}
120	22.1 ± 1.6^{Aab}	21.2 ± 1.2^{Aab}	19.6 ± 1.7^{Ab}	23.4 ± 0.3^{Aa}
150	21.5 ± 0.7^{Aa}	22.0 ± 1.5^{Aa}	19.3 ± 2.8^{Aa}	21.7 ± 1.5^{Aa}

Results were presented as mean ± standard deviation
[A]Values with different superscript letters within a column indicate significant differences at $p<0.05$
[ab]Values with different superscript letters within a row indicate significant differences at $p<0.05$
b* indicates yellowness when positive; blueness when negative

CONCLUSION

There were no significant changes in the AAC, TPC, L*, a* and b* colour values of blanched, boiled or steamed cauliflowers during storage up to 2 h. These results serve as a food preparation guide for consumers to avoid nutrient loss after food preparation. Longer storage time can be investigated in the future. Besides measuring AAC, TPC and colour values, other bioactive compound or antioxidant activity in cauliflower can be measured.

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Short Communication

Effect of Glucose and Ascorbic Acid on Total Phenolic Content Estimation of Green Tea and Commercial Fruit Juices by Using Folin Ciocalteu and Fast Blue BB Assays

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ABSTRACT

Folin Ciocalteu (FC) assay had been widely used in the estimation of total phenolics content (TPC) in foods. However, the main disadvantage of this assay is that the reagent reacts with reducing substances and measures the total reducing capacity of a sample, not just phenolic compounds. The Fast Blue BB (FBBB) assay is another assay that can be used to estimate the total phenolic content in foods instead. The aim of the study was to estimate and compare the total phenolic content of green tea and commercial fruit juices using FC and FBBB assays, and also the effects of glucose and ascorbic acid on both assays. Green tea had the highest ascorbic acid content and TPC value among the samples. TPC estimated using FC assay were significantly higher than FBBB assay in all the samples. FC and FBBB assays were not affected by glucose at different concentrations. However, FC assay was significantly affected by the presence of ascorbic acid compared to FBBB assay. In conclusion, FC assay overestimated the TPC value in the sample extracts and was significantly affected by the presence of ascorbic acid. The FBBB assay can be considered as an alternative for estimation of total phenolic content in ascorbic acid-rich foods.

Keywords: Ascorbic acid, Fast Blue BB assay, Folin Ciocalteu assay, green tea, total phenolic content

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INTRODUCTION

Folin Ciocalteu (FC) assay has been widely used in the estimation of total phenolics content in foods. The FC reagent, a mixture of phosphomolybdate and phosphotungstate, is used for the colorimetric *in vitro* assay of phenolic and polyphenolic antioxidants (Simoni et al., 2002). The assay was originally developed as an improvement to the Folin Denis assay, which was used to determine total protein concentration by measuring tyrosine and tryptophan contents in the sample (Folin & Denis, 1912; Sánchez-Rangel et al., 2013). Eventually, the FC assay was adapted further for determining total phenolic content in food samples. The assay was applied to a wide variety of food samples, from plants (Baba & Malik, 2015), and also beverages such as wine (Hosu et al., 2014; Singleton & Rossi, 1965) and tea (Alarcon et al., 2008).

However, a major disadvantage of the FC assay is that the reagent reacts with any reducing substance and measures the total reducing capacity of a sample, not just phenolic compounds. The reagent has been shown to be reactive towards thiols, vitamins, nucleotide bases (adenine, guanine, cytosine, uracil, and thymine), organic acids such as uric acid and oleic acid, and proteins (Prior et al., 2005). Also, some inorganic substances such as hydrazine, hydroxyammonium chloride, iron ammonium sulfate, iron sulfate, manganese sulfate, potassium nitrite, sodium cyanide, sodium metabisulfite, sodium phosphate, sodium sulfite, and tin chloride may also react with the FC reagent to give elevated apparent phenolic concentrations (Prior et al., 2005). Another disadvantage of the assay is that it is quite general, in a sense that it only measures the total phenolic content, without determining the specific phenolic compounds present in the sample. Hence, overestimation of the total phenolic content in food samples are more likely to occur when using FC assay due to the interference by the compounds stated earlier. As a result, food with low phenolic content may be falsely accepted as good source of phenolic compound due to overestimation.

The Fast Blue BB (FBBB) assay is another assay used in estimating the total phenolic content in food. It was developed by Medina (2011a) and utilizes FBBB diazonium salt where the diazonium group only reacts to reactive phenolic hydroxyl groups under alkaline conditions, forming stable azo complexes which can be measured at 420 nm (Lester et al., 2012). The advantage of this assay is that the diazonium salt only reacts with phenolic hydroxyl groups and leaving the non-phenolic compounds behind, thus providing a more accurate quantification of total phenolic content in the sample (Medina, 2011b). However, this assay is also quite general, where it can only quantify the total phenolic content of the samples, instead of determining every phenolic compounds present and their exact amount in the samples.

Tea is made from the leaves of the *Camellia sinensis* plant and is considered as one of the most commonly consumed beverage in the world (Hilal & Engelhardt, 2007). Green tea is made by inactivating the enzymes in the fresh leaves by drying or steaming for a short time, which prevents

the enzymatic oxidation of catechins (Wang et al., 2000). Green tea also contains considerable amount of antioxidant and ascorbic acid, albeit a low sugar content. Similarly, commercial fruit juices also contain considerable amount of ascorbic acid and sugars, similar to their fresh fruit counterparts. Since both ascorbic acid and sugars are commonly present inside fruits and vegetables, finding an assay that will not be affected by these compounds can be very useful in estimating the total phenolic content of plant samples accurately.

In this study, the effects of different concentrations of glucose and ascorbic acid on the total phenolic content of green tea and selected fruit juices estimated using FC assay and FBBB assay were determined. In addition, the ascorbic acid and total phenolic content of each samples estimated using both assays was also compared.

MATERIALS AND METHODS

Chemicals and Reagents

Absolute methanol (CH_3OH, 99.9%) was purchased from J. Kollin Chemicals (UK). Folin Ciocalteu (FC) reagent, metaphosporic acid (HPO_3) and sodium carbonate (Na_2CO_3) pellets were obtained from Merck (Darmstadt, Germany). Gallic acid, 2, 6-dichlorophenol indophenol (DCPIP) sodium salt, ethylenediaminetetraacetic acid (EDTA), and 4-benzoylamino-2, 5-dimethoxybenzene diazonium chloride hemi (-zinc chloride) salt (FBBB salt) were obtained from Sigma Aldrich (Missouri, USA). Glucose standard was obtained from Scharlau Chemicals (Barcelona, Spain).

Preparation of Sample

The tea leaves were harvested and processed into green tea at Kakuda Seicha Tea Plantation in Yame-shi (Fukuoka prefecture, Japan), before delivered to Kyushu Institute of Technology (KIT) (Fukuoka Prefecture, Japan). The green tea was placed in sealed plastic before shipped to Universiti Putra Malaysia. The leaves were stored at -20°C until used. Prior to the extraction process for estimation of total phenolic content, the leaves were ground into powder using a commercial blender from Waring Commercial (Connecticut, USA) and passed through a 0.025mm sieve.

Commercial orange & guava juices of a particular brand were purchased from several supermarkets around Serdang district (Selangor, Malaysia).The fruit juices were stored at 6°C until used. Prior to the extraction process for estimation of total phenolic content, the juices were freeze dried for 3 days and then stored at -20°C before being re-dissolved in 70% methanol.

Determination of Ascorbic Acid Content

Ascorbic acid content was determined using indophenol titration method (Official Method of Analysis [OMA], 2012). 6% and 3% HPO_3 – EDTA solutions were prepared by mixing 6% HPO_3 with 0.005M EDTA solution, and mixing 3% HPO_3 with 0.0025M EDTA solution, respectively. The sample (100g) was mixed with 100 mL of 6% HPO_3 – EDTA solution and blended. The homogenous mixture (10mL) was taken and diluted to 100mL with 3% HPO_3 – EDTA solution and then filtered. The

filtrate (10 mL) was taken and titrated with 0.025% DCPIP solution to a faint pink end point which lasted for 15 sec. Ascorbic acid equivalent value was determined by taking 5 mL of ascorbic acid standard (100 mg ascorbic acid + 400 mL of 3% HPO_3 – EDTA solution) and diluting it with 5 mL of 3% HPO_3 – EDTA solution before titration with DCPIP solution. The ascorbic acid content, in mg per 100 g of sample, was calculated using formulas (1) and (2):

$$\text{Ascorbic acid content (mg per 100 g)} = \frac{\text{Vsample (mL)} \times 100}{\text{Vstandard (mL)} \times \text{Ws (g)}} \quad (1)$$

Vsample = amount of dye used during titration of sample
Vstandard = Amount of dye used during titration of standard
Ws = Weight of sample in aliquot of filtrate of diluted sample used for titration

$$\text{Ws} = \text{Va(mL)} \times \frac{\text{W1 (g)}}{\text{Vb(mL)}} \times \frac{\text{Vc(mL)}}{100} \quad (2)$$

Va = Volume of aliquot of mixture diluted with 3% HPO_3
Vb = Final volume of mixture (sample + 6% HPO_3)
Vc = Volume of filtrate used for titration
W1 = Weight of sample used

Sample Extraction for Total Phenolic Content Determination

The method of extraction was adapted based on the method for analysis of phenolic compounds in grains and beverages described by Medina (2011a). One gram of dry, powdered sample was extracted with 20 mL of 70% methanol. The mixture was centrifuged at 2683 × g for 30 min and the filtered using a syringe filter. The collected supernatant was stored at 6°C before analysis.

Folin Ciocalteu (FC) Assay

FC assay was done according to Medina (2011a) with some modification (higher dilution factor). The pre-diluted (50 times dilution factor) sample extract (0.1 mL) was transferred into borosilicate tubes, followed by the addition of 1.7 mL of distilled water and 0.1 mL of 1N FC reagent. Then, 0.1 mL of saturated 20% Na_2CO_3 was added and mixed, and the solution was allowed to react for 90 min. The absorbance of the mixture was measured at 750 nm using UV-1800 UV spectrophotometer from Shimadzu Corporation (Japan). Gallic acid standard at varying concentrations (0.001M, 0.005M, 0.01M, 0.015M, 0.02M, and 0.025M) were prepared.

Fast Blue BB (FBBB) Assay

FBBB assay was done according to Medina (2011a) with some modification (higher dilution factor). The pre-diluted (50 times dilution factor) sample extract (0.1 mL) was transferred into borosilicate tubes followed by the addition of 1.7 mL of distilled water.

Next, 0.1 mL of sonicated 0.1% FBBB salt was added. The solution was mixed for 30 sec before 0.1 mL of 20% Na_2CO_3 was added and mixed for another 30 sec. The resulting solution was incubated in the dark for 90 min at room temperature. The absorbance was measured at 420 nm using UV-1800 UV spectrophotometer from Shimadzu Corporation (Japan). Gallic acid standard at varying concentrations (0.001M, 0.005M, 0.01M, 0.015M, 0.02M, and 0.025M) were prepared.

Effect of Glucose and Ascorbic Acid on Total Phenolic Content

Different concentrations of glucose (0.01M, 0.02M, 0.03M, 0.04M, and 0.05M) and ascorbic acid (0.001M, 0.002M, 0.003M, 0.004M, and 0.005M) were prepared in distilled water. Next, a mixture containing 50 µL of pre-diluted sample (50 times dilution factor) and 50 µL of the glucose or ascorbic acid solution was prepared and assayed according to the FC and FBBB assays stated earlier. The resulting total phenolic content (TPC) value was calculated. The assays were repeated using different concentrations of glucose and ascorbic acid.

Statistical Analysis

The results were analyzed using SPSS version 21. The experiments were done in triplicates and the average value was obtained. The total phenolic content obtained was expressed as gallic acid equivalent (GAE) per 100 g dry weight with standard deviation (SD). ANOVA (p-value set at $p<0.05$) was used to determine whether there were significant differences for ascorbic acid content and total phenolic content of each samples obtained using both assays. Independent sample t-test (p value set at $p<0.05$) was used to determine any significant difference in the TPC content obtained using the two assays in the presence of glucose and ascorbic acid.

RESULTS AND DISCUSSION

Sugar Composition and Ascorbic Acid Content

Total sugar content of green tea, orange juice and guava juice had been previously reported by several studies. Among the samples, orange juice showed the highest amount of sugar, followed by guava juice and green tea. Abel and Aidoo (2016) reported that the total sugar content of commercial orange juice was 10.72 g per 100 mL of sample, with the highest amount represented by sucrose (4.21 g per 100 mL), fructose (3.39g per 100mL), and lastly glucose (3.11 g per 100 mL). Sanz et al. (2004) reported that fructose is the main sugar (2.74 g per 100 mL) found inside fresh guava juice, followed by glucose (0.95g per 100mL) and sucrose (0.57 g per 100 mL). For green tea, the main sugar found in it was fructose (0.72 g per 100 g), glucose (0.68 g per 100 g), and sucrose (0.71 g per 100 g) according to Shanmugavelan et al. (2013).

Ascorbic acid content in the samples determined using indophenol titration method is tabulated in Table 1. Overall, significant difference ($p<0.05$) was observed between all the samples. The highest amount of ascorbic acid obtained was from green tea

(295 ± 9 mg per 100 g of sample), followed by commercial guava juice (68 ± 4 mg per 100 g of sample), and commercial orange juice (38 ± 5 mg per 100 g of sample). The value obtained by the samples in this study was almost similar to the values reported by previous studies. According to Somanchi et al. (2017), the amount of ascorbic acid available in green tea ranges between 250 to 280 mg per 100 g of dried leaves. Green tea usually have higher ascorbic acid content than processed tea such as black and oolong tea, possibly due to the lack of manufacturing and fermentation process during its production (Cabrera et al., 2006; Lee & Kader, 2000).

Table 1
Ascorbic acid content of the samples

Samples	Ascorbic acid content (mg/100 g) ± SD
Green Tea	295 ± 9 [a]
Commercial Guava Juice	68 ± 4 [a]
Commercial Orange Juice	38 ± 5 [a]

Note. The values were expressed as mean of mg per 100 g sample ± SD (n = 3).Same superscript shows significant difference ($p<0.05$) in ascorbic acid content between each samples

As for commercial orange juice, the value obtained in this study was slightly lower than the value reported by Bungau et al. (2011) which was 56.4 mg per 100 g of sample. Similarly, Dauda and Sadisu (2013) reported that the ascorbic acid content of guava fruit was 180 mg per 100 g of sample, which was higher than the value obtained from the commercial guava juice in this study. However, it should be noted that the value reported by both of these studies was obtained from fresh fruits, not manufactured juice. The reason for commercialised fruit juices having lower ascorbic acid content than fresh fruit is probably due to the packaging process during manufacture. Kaleem et al. (2015) stated that processes such as clarification, filtration and pasteurization could easily destroy vitamin C. In addition, temperature and oxygen also play a major role in the loss of vitamin-C during processing of the fruit juices (Kaleem et al., 2015).

Total Phenolic Content

The total phenolic contents (TPC) of green tea, commercial guava juice, and commercial orange juice extracts using Folin Ciocalteu (FC) and Fast Blue BB (FBBB) assays are tabulated in Table 2. The TPC of the green tea extract estimated using FC assay was 2744 ± 99 mg GAE per 100 g DW, and FBBB assay was 1504 ± 37 mg GAE per 100 g DW. In the guava juice extract, TPC estimated using FC assay was 2403 ± 74 mg GAE per 100 g DW, and FBBB assay was 1408 ± 55 mg GAE per 100 g DW. The orange juice extract's TPC estimated by FC and FBBB assays were 2428 ± 67 mg GAE per 100 g DW, and 1346 ± 51 mg GAE per 100 g DW, respectively. Statistical analysis showed significant difference ($p<0.05$) between the total phenolic content estimated using both assays in all of the samples.

In the FC assay, green tea extract had the highest TPC value, followed by orange juice and guava juice extracts. However, there were no significant differences ($p>0.05$) in

Table 2
Total phenolic content of the samples

Extract	Assay	Total phenolic content (mg GAE/100 g DW) ± S.D
Green tea	Folin Ciocalteu	2744 ± 99 [a]
	Fast Blue BB	1504 ± 37 [a]
Guava juice	Folin Ciocalteu	2403 ± 74 [b]
	Fast Blue BB	1408 ± 55 [b]
Orange juice	Folin Ciocalteu	2428 ± 67 [c]
	Fast Blue BB	1346 ± 51 [c]

Note. The values were expressed as mean of mg GAE per 100 g DW ± SD (n = 3).Same superscript shows significant difference ($p<0.05$) in TPC between assays in each sample extract

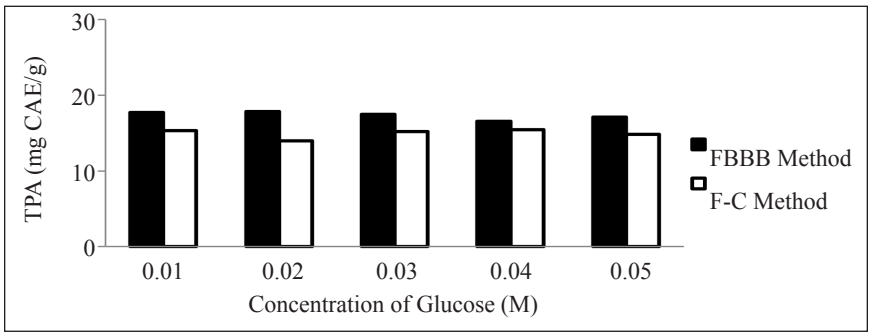
the TPC values between all three extracts for FC assay. In the FBBB assay, green tea extract also had the highest TPC value, followed by guava juice and orange juice extracts. Significant difference ($p<0.05$) was found between the green tea and orange juice extracts.

In general, FC assay estimated a higher level of TPC in all the sample extracts compared to FBBB assay. The differences in the estimated value of TPC using both assays can be attributed to several factors. The same sample extract can give out different values when analysed using different assays. As shown in the present study, FC assay showed a higher TPC in all the samples compared to FBBB assay. However, the TPC obtained using FC assay was probably affected by the presence of ascorbic acid in the samples. As stated earlier, the amount of ascorbic acid in the samples ranged from 38 mg to 295 mg per 100 g of sample. The presence of ascorbic acid in the samples could affect the TPC obtained using FC assay, resulting in a significantly higher TPC value than FBBB assay.

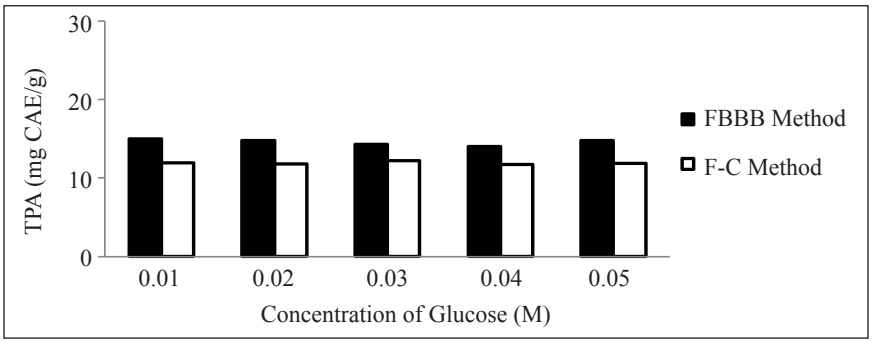
The TPC of a sample can also be influenced by the method of sample preparation, type of solvent used and its concentration, and the extraction temperature (Jahangiri et al., 2011). A study by Jahangiri et al. (2011) showed that higher incubation temperature might provide higher yield of total phenol recovery. A reason for this is that incubation in hot water may break down some pectic polysaccharides from the cell wall (Sun et al., 2002), and weaken the cell wall's integrity, allowing the solvent to get into contact with the phenolic compounds.

Effect of Glucose and Ascorbic Acid Concentration on TPC value

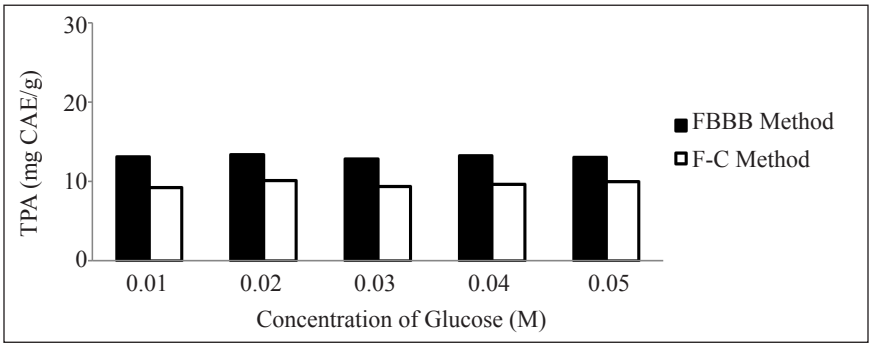
Figure 1 shows the effect of different glucose concentrations on the TPC value (mg GAE/g) obtained by FC and FBBB assays. Based on the results, FBBB assay showed higher TPC values at every glucose concentration compared to FC assay for all the samples. Statistical analysis showed significant difference ($p<0.05$) between the TPC values obtained by both assays. However, the TPC value did not increase linearly with the glucose concentration for both assays. It shows that glucose, or possibly other sugars, will not affect the absorbance value of both assays significantly,



(a)



(b)



(c)

Figure 1. Effect of different glucose concentrations on the TPC value of (a) Green tea, (b) Commercial guava juice and (c) Commercial orange juice using FC and FBBB assays. P-value was set at ($p<0.05$)

regardless of its concentration in the sample. It corresponds with a previous study done by (Lester et al., 2012) where neither FC nor FBBB gave a response to sugar (fructose, glucose and sucrose) standards; as sugars were reported to interfere with the FC assay only when heated (Slinkard & Singleton, 1977). As such, both assays can be used to estimate the total phenolic content of sugar-rich food samples without interference as long as it is done at room temperature.

Figure 2 shows the effect of different concentrations of ascorbic acid on the TPC value (mg GAE/g) obtained by both assays. In FC assay for all the samples, there was a significant difference ($p<0.05$) in the

TPC value as it increased linearly with the ascorbic acid concentration. On the other hand, the FBBB assay did not show any significant difference in the TPC value as the ascorbic acid concentration increased. This indicated that TPC value obtained by FC assay can be influenced by ascorbic acid amount in the samples and thus providing an overestimation of the sample's phenolic content. Hence, the total phenolic content of food samples with high ascorbic acid content, such as fruits, will be much higher than its actual value when assayed using FC assay. The result corresponded with

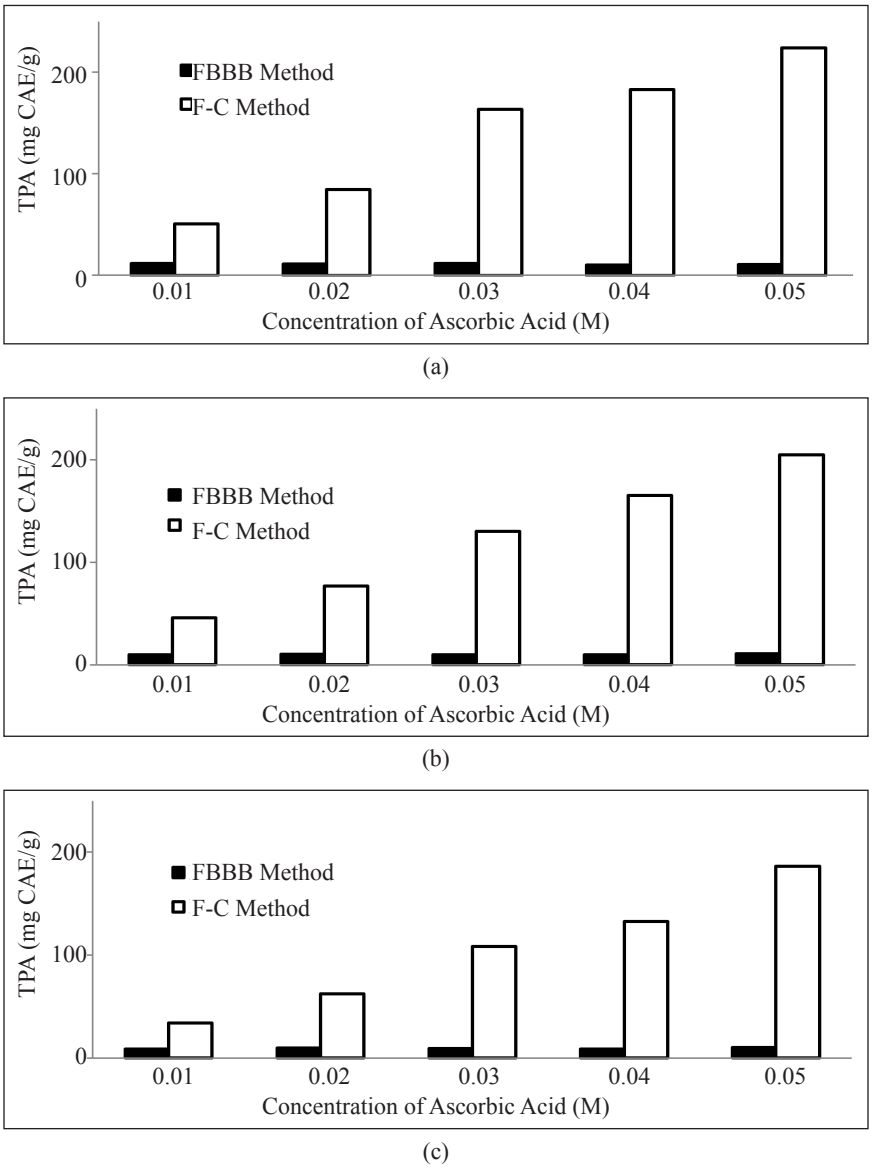


Figure 2. Effect of different ascorbic acid concentrations on the TPC value of (a) Green tea, (b) Commercial guava juice and (c) Commercial orange juice using FC and FBBB assays. P-value was set at ($p<0.05$)

previous studies that stated that the FC assay suffered from a number of interfering substance including ascorbic acid (Musa & Abdullah, 2009; Prior et al., 2005). The presence of ascorbic acid, most notably in fruit and its juices, provides a strong non-phenolic FC signal that can easily exceed the magnitude of that from actual phenolics (Singleton et al., 1999).

FC reagent is a yellow coloured solution consisting of a mixture of the heteropoly acids, phosphomolybdic and phosphotungstic acids in which the molybdenum and the tungsten are in the positively-charged oxidation state (Agbor et al., 2014). Under alkaline conditions, the phenolic compound dissociates a proton, leading to the formation of phenolate ion which reduces the FC reagent and forming the molybdenum blue and the tungsten blue which gives the mixture its blue colour (Agbor et al., 2014). The presence of ascorbic acid in the sample can interfere with the previous reaction by rapidly reacting with the FC reagent without requiring an alkaline condition, giving a blue colour right after mixing the plant extract and the reagent (Sánchez-Rangel et al., 2013).

On the contrary, FBBB assay uses the coupling property of a diazonium salt with phenolic compounds to form azo compounds in its reaction. The aromatic diazonium ion is coupled to electron-rich substrates such as phenols (Medina, 2011b). The aromatic diazonium ions ($-^{+}N{=}N-$) specifically couples with reactive phenolic hydroxyl (–OH) groups by releasing a proton (Medina, 2011a), resulting in an electrophilic aromatic substitution (Medina, 2011b). Under alkaline conditions, the coupling process results in azo compounds with wavelengths that can be read using spectrophotometer (Medina, 2011b). As a result, FBBB assay showed very minimal reaction towards ascorbic acid even at a higher concentration based on the absorbance value which is in correspondence with the results reported by Lester et al. (2012). It indicates that the absorbance value obtained by FBBB assay was not significantly influenced by the ascorbic acid in the sample. Therefore, FBBB assay is more suitable for estimating the total phenolic content of food samples with high ascorbic acid content as it will provide a reliable estimation compared to FC assay.

CONCLUSION

Based on the present study, green tea had the highest ascorbic acid content and TPC among the samples. TPC value obtained using FC assay was significantly higher than TPC value obtained using FBBB assay for all samples. Significant increase in the TPC value estimated using FC assay as the ascorbic acid concentration increased indicated that FC assay is severely affected by the presence of ascorbic acid. On the contrary, FBBB assay was not affected by the presence of glucose and ascorbic acid. Hence, Fast Blue BB assay can be considered as an alternative assay in estimating the total phenolic content in food samples with high ascorbic acid content more accurately. The present study only investigated the effects of two compounds on the estimation of TPC

using FC and FBBB assays. Future studies should include other types of compounds that might have the possibility to affect the TPC determination assays so that estimation of phenolic content of plant samples can be conducted more accurately.

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Detection of Potentially Zoonotic *Cryptosporidium* and *Giardia* among Livestock in Sariaya, Quezon, Philippines

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ABSTRACT

Livestock plays a great role in the economic development of smallholder farmers. However, the population of livestock has been affected by diseases such as cryptosporidiosis and giardiasis. Sariaya has a large production of livestock that is being distributed in Quezon Province. Thus, the current study aimed to identify the presence of *Cryptosporidium* sp. and *Giardia* sp. in livestock in selected farms of Sariaya, Quezon. Risk factors were also assessed in the present study. A total of 103 collected faecal samples from livestock were subjected to microscopic and molecular detection. Faecal samples were processed through Formalin-Ether Concentration Technique (FECT) and Polymerase Chain Reaction. Microscopy results revealed that 14 out of 103 (13.59%) samples were positive for *Giardia* with meant intensity of 13 cysts per gram (cpg) of faeces, while molecular detection confirmed that 13 out of 103 (12.62%) had amplified for the target gene of *Giardia*, *tpi*, with expected band size of 530 bp. Sequenced samples of *G. intestinalis* were characterised as assemblages A, B and E. Moreover, pigs showed the highest prevalence (15.91%), while cattle had the highest mean intensity (14 cpg) for *Giardia*. *Cryptosporidium* were absent in all the samples. Statistical analysis of the risk factors such as diet, feeding floor, habit, presence of illness and faecal consistency of the animals revealed no significant association ($p>0.05$) in the presence of *Giardia*. This study revealed the presence of zoonotic *Giardia* species in the

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area; however, future investigation in other possible risk factors such as the season and age is still recommended.

Keywords: *Cryptosporidium*, *Giardia*, livestock animals, zoonosis

INTRODUCTION

Livestock is one of the major sources of income in the Philippines, which contributes to the development of the country's agricultural and rural livelihood (PlantAsian Agro-forestry Management Systems Inc. [PlantAsian], 2017). The Department of Science and Technology (DOST) (2014) claimed that animal production in the Philippines has been increasing rapidly. In Sariaya, Quezon, where the lands are suited for grazing animals, the predominant livestock consists of carabaos, cattle and pigs. Carabaos are mostly used in land preparation and farm product transportation; cattle are raised for meat and dairy products; while pigs are used for home consumption and additional sources of income (Department of Agriculture-CALABARZON [DA- CALABARZON], 2017).

In 2016, a decline in livestock population was noted as a result of the lowering of inventory in backyards and diseases such as cholera and diarrhea, which raised death rate by 15.17% (Philippine Statistics Authority [PSA], 2016). As was observed, the increasing death rate could have been an effect of diseases such as parasitic infections, resulting in major economic problems. Livestock diseases and parasitic infections have direct (stunting growth, infertility and death) and indirect (additional costs on vaccines, drugs, labour cost, and profit losses) effects on productivity and could thereby result in major economic implications to the producer (Lamy et al., 2012; Rajakaruna & Warnakulasooriya, 2011).

Giardia and *Cryptosporidium* are two of the neglected parasites that cause parasitic infections. *Giardia intestinalis* assemblages A and B and *Cryptosporidium parvum* subtype IIa, IId and III are the recorded zoonotic strains of the two parasites (Feng & Xiao, 2011; Hunter & Thompson, 2005). Both parasites can be transmitted directly, from host to host, or indirectly, through ingestion of contaminated food or water (Lim et al., 2013). It has been found by Aloisio et al. (2006) that these parasites caused malabsorption, reduced feeding efficiency, and severe weight loss to the livestock which could lead to lower production and economic losses.

There are a number of studies that have reported the prevalence of *Giardia* and *Cryptosporidium* in some parts of Southeast Asia. In Thailand, 9.4% of 363 cows were found positive for *Cryptosporidium* (Jittapalapong et al., 2006). In Malaysia, *Cryptosporidium* and *Giardia* have been recorded to be present in different animals which may indicate that many Malaysian environments, particularly soil and water, are contaminated and may act as vehicles for the transmission of these parasites (Lim et al., 2008). In addition, cryptosporidiosis is frequently reported in both humans and animals from many countries such as Laos, Vietnam, Philippines, Myanmar,

Indonesia, Cambodia, Malaysia, and Thailand (Pumipuntu & Piratae, 2018). Meanwhile, the only reported case about *Cryptosporidium* relating to livestock animals from the Philippines was made by Laxer et al. (1988) in the rural areas of Palawan, where they obtained oocysts of *Cryptosporidium* from human, cattle, and carabao faeces. The *Cryptosporidium* was identified up to the genus level through microscopic examination, while *Giardia* showed negative result.

To date, there have been no updated published reports on the presence of *Cryptosporidium* and *Giardia* infection among livestock in the Philippines, particularly in Quezon. Moreover, there are also no recorded molecular characterisation and analysis of these zoonotic parasites from livestock animals in the Philippines. Hence, this study plays a significant role in the diagnosis of zoonotic parasites which may benefit the local agriculture and veterinary offices in the municipalities of Sariaya. Also, this study was designed to determine the prevalence of *Cryptosporidium* and *Giardia* in the livestock animals, specifically in cattle, pigs, and carabaos through molecular detection in selected farms of Sariaya, Quezon. This study also sought to correlate some risk factors that might be associated with the prevalence of these parasites.

MATERIALS AND METHODS

Study Design

This research was a cross-sectional study which determined the prevalence of *Cryptosporidium* and *Giardia* protozoan parasites up to its species level. The barangays were purposively selected based on the livestock animal population number.

The final population sampling size was computed using the formula [1] adopted from Cochran (1977):

$$n = z^2 \frac{p(1-p)}{a^2} \quad [1]$$

where z = 1.96 (95%), p = 7.2 % prevalence from a previous study of Wang et al. (2014) and a = 0.05%. Using formula [1], the final sampling number of 103 livestock individuals was obtained and divided into 47 individual cattle, 44 individual pigs and 12 individual carabaos using formula [2].

$$n = \frac{\text{Population of special livestock}}{\text{Total population of livestock}} \times \text{Final sampling number} \quad [2]$$

Sampling Method

The animals were randomly selected regardless of their sex and age. The collection of faecal samples was done on the 14th of October 2018. The inner portion of the fresh faecal samples from each individual animal was collected to minimise contamination. Stool cups were filled with faeces to serve as the master sample. From each master sample, 2 g were preserved in 10% formalin for FECT, while the remaining were preserved in a cooler (2-8°C) and used for DNA extraction. The samples were immediately processed for

faecalysis in the laboratory of Southern Luzon State University, Lucban, Quezon. Molecular characterisation was held in the Interactive Laboratory of the Department of Biological Sciences, College of Arts and Sciences, Central Luzon State University, Nueva Ecija, Philippines.

Recovery and Identification of *Cryptosporidium* and *Giardia* through Microscopy

Kinyoun acid fast staining was used to recover *Cryptosporidium* oocysts while Lugol's Iodine staining was used for *Giardia* cyst (Abeywardena et al., 2014; Paller et al., 2013). All slides were observed under compound light microscope with 400× and 1000× magnification. The oocysts/cysts were manually and carefully counted for each slide. Morphological characteristics of the parasites were identified through comparison from the literatures and validated by a parasitologist from the University of the Philippines, Los Baños, Laguna.

Identification of *Cryptosporidium* and *Giardia* spp. using Polymerase Chain Reaction

Different protocols and gene markers were used for each parasite. For PCR reactions, positive and negative controls (distilled water) were included.

DNA Extraction. DNA was extracted from each sample using *NucleoSpin*® Soil Kit (Macherey-Nagel, USA) following the manufacturer's manual and stored at 4°C until further use. All the DNA samples were subjected to Polymerase Chain Reaction (PCR) following specific protocol for *Giardia* and *Cryptosporidium*. The DNA was not quantified after extraction using spectrophotometric method. However, gel electrophoresis was performed to confirm the presence of the extracted DNA in the samples.

Nested PCR for the Detection of *Giardia* Species. The triosephosphate isomerase (*tpi*) gene was the target gene for the nested PCR protocol in detecting *Giardia* (Sulaiman et al., 2003). For the primary reaction, an amplicon of 605 bp was amplified using primers AL3543 [forward: 5′-AAATATGCCTGCTCGTCG-3′] and AL3546 [reverse: 5′-CAAACCTTTTCCGCAAACC-3′]. The primary reaction (20 µl) consisted of 10 µl of Premix (SolGent™ 2x h-Taq PCR Smart Mix), 1 µl for each primer, 5 µl of dH_2O and 3 µl of the DNA template. It was incubated in the MyCycler thermal cycler (Bio-Rad, Hercules, USA) having thermocycling condition of an initial hot start of 94°C for 5 min, followed by 35 cycles of 94°C for 45s denaturation, 50°C for 45s annealing, 72°C for 60s extension, and a final extension of 72°C for 10 min.

For the secondary reaction, an amplicon of 530 bp was amplified using primers AL354 [forward: 5′-CCCTTCATCGGTGGTAACTT-3′] and AL3545 [reverse: 5′-GTGGCCACCACICCCGTGCC-3′]. The secondary reaction (20 µl) consisted of 10 µl of Premix (SolGent™ 2x h-Taq

PCR Smart Mix), 1 µl for each primer, 5 µl of dH_2O and 3 µl of the DNA template. The thermocycling conditions were as follows: initial hot start of 94°C for 5 min, followed by 40 cycles of 94°C for 45 seconds denaturation, 50°C for 45 seconds annealing, 72°C for 60s extension, and a final extension of 72°C for 10 min.

Nested Detection of *Cryptosporidium* Species. The protocol of Nichols et al. (2003) was used to obtain the SSU rRNA gene, which was the target gene in detecting *Cryptosporidium*. For primary reaction, a 655 to 667 bp amplicon was amplified using a 26-mer primer N-DIAGF2 [forward: 5′-CAATTGGAGGGCAAGTCTGGTGC CAGC-3′] and N-DIAGR2 [reverse: 5′-CCTTCCTATGTCTGGACCTGGT GAGT-3′]. The primary reaction (20 µl) consisted of 10 µl of Premix (X-Prime Taq Premix 2x), 1 µl for each primer, 5 µl of dH_2O and 3 µl of the DNA template. PCR thermocycling was performed as follows: 95°C for 5 mins, followed by 35 cycles of denaturing for 30 seconds at 94°C, annealing for 1 minute at 68°C and extension for 30 sec at 72°C, followed by a final extension at 72°C for 10 minutes.

For the secondary reaction, a 435 bp amplicon was amplified using primers CPB-DIAGF [5′-AAGCTCGTAGTTGGATTTCTG-3′] and CPB-DIAGR [5′-TAAGGTGCTGA AGGAGTAAGG-3′] (Johnson et al., 1995). The secondary reaction (20 µl) consisted 10 µl of Premix (X-Prime Taq Premix 2x), 1 µl for each primer, 3 µl of dH_2O and 5 µl of the first PCR product. PCR thermocycling conditions were as follows: initial hot start of 80°C for 5 min, followed by 39 cycles of 98°C for 30s denaturation, 55°C for 30s annealing, 72°C for 60s extension and a final extension of 72°C for 10 min.

Sequencing and Analysis of Sequence Data

PCR products were visualized on 2% agarose gel-electrophoresis at 100V for 40 minutes for both *Cryptosporidium* and *Giardia* using Gel Documentation Systems (Uvitec Imaging Systems, United Kingdom). Positive bands were excised from the gel and purified using *NucleoSpin*® Gel and PCR Clean-up (Macherey-Nagel, USA), according to the manufacturer's instructions, and sent to 1st Base-Asia Gel, Ltd., Malaysia, for sequencing.

The sequence analysis of the DNA sequences obtained was assessed through BioEdit Sequence Alignment Editor (version 7.1.11) (Hall, 1999). Sequences from positive samples were aligned and ambiguous sites were removed prior to phylogenetic construction. The sequences were compared with known reference sequence using the Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1990).

The phylogenetic construction and analyses were conducted using MEGA version 6 software, with bootstrap values establishing 1000 replicates. The Phylogenetic tree was constructed using neighbour-joining tree [NJ] (Tamura et al., 2013).

Survey of Risk Factors Associated with Prevalence of Parasites

A survey questionnaire, validated by a statistician, was distributed to the farm owners to retrieve information regarding the diet, feeding floor, habit, presence of illness and faecal consistency of their livestock animals.

Statistical Analysis. The prevalence rate and mean intensity of *Giardia* from faecal samples were obtained using formulas [3] and [4] by Ordoñez et al. (2018), respectively:

$$\text{Prevalence} = \frac{\text{No. of positive samples}}{\text{Total number of samples}} \times 100 \quad [3]$$

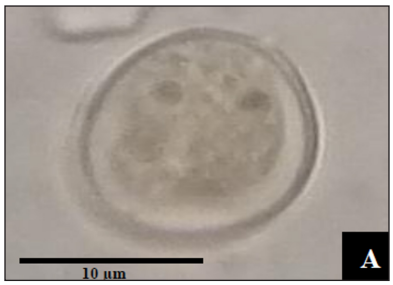
$$\text{Mean intensity} = \frac{\text{Individual count of parasites}}{\text{Total number of positive samples}} \times 10 \quad [4]$$

Chi-square test was used to determine the association between prevalence of *Giardia* and possible risk factors such as diet, feeding floor, habit, presence of illness and faecal consistency. Differences are considered statistically significant when $p < 0.05$. Statistical analysis was conducted using SPSS version 20.0 (SPSS Inc., Chicago, IL, USA).

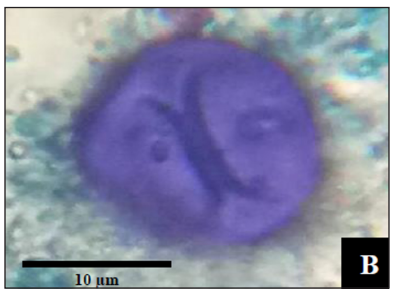
RESULTS AND DISCUSSION

Determination of Prevalence of *Cryptosporidium* and *Giardia*

In microscopy, *Giardia intestinalis* was detected in 14 out of 103 (13.59%) faecal samples from livestock animals, while no *Cryptosporidium* oocysts were observed in all the samples. The overall mean intensity of *Giardia* was 13 cpg wherein, cattle had the highest intensity (14 cpg) followed by pigs and carabao (10 cpg).



(a)



(b)

Figure 1. *Giardia intestinalis* recovered from faecal samples 1000× magnification. (a) Stained by Lugol's Iodine solution; (b) Stained by Methylene blue

The cysts of *Giardia* were observed to have oval shape, ranging between 7-11 µm in width and 12-16 µm in length. Most of them were with two visible nuclei and remnants of axonemes, although the axonemes were slightly disintegrated in some cysts (Figure 1).

Giardia can be found in environmental and faecal samples of livestock animals and has the potential to be zoonotic (Bawm et al., 2014). Sastry and Bhat (2014) described *Giardia* cysts to have 4 nuclei while Adam (1991) stated that it had 11–14 µm in length and 7–10 µm in width. Meanwhile, *Cryptosporidium* was found to be negative in all the faecal samples in the present study. Padilla and Ducusin (2015) studied the prevalence of endoparasites from smallholder farms in Sariaya, Quezon, and also did not recover *Cryptosporidium* in their samples. The absence of *Cryptosporidium* may have been caused by several factors such as ages of the livestock, shedding of oocysts/cysts in the faeces, long storage of samples before PCR methods and travel of DNA extracts (Abeywardena et al., 2014; Ghaffari & Kalantari, 2014; Olson et al., 2004; Qi et al., 2016).

All of the 103 faecal samples underwent PCR and only 13 out of 103 were amplified for the target gene of *Giardia*, *tpi*, with expected band size of 530 bp. However, only 5 out of 13 positive samples were subjected to DNA sequencing since the other bands have low DNA intensity when subjected under blue light during excision. BLAST results of the obtained sequences for the parasites in the five faecal samples showed 98-99% identity (Table 1).

Phylogenetic analysis by Neighbour-Joining tree was performed on the five *Giardia* isolates (Figure 2). It showed that 3G-9P and 4G-19P clustered with assemblage B, while 1G-1K and 5G-19B clustered with assemblage A, and 7G-40B clustered with assemblage E.

The recovered *Giardia intestinalis* in the present study falls under assemblages A and B, which are zoonotic, while assemblage E is considered as livestock specific without zoonotic capability. The presence of zoonotic parasites indicates risks for humans especially farmers in the nearby area since they can contract infection caused by the livestock animals.

Table 1
The BLAST results for Giardia intestinalis *recovered in positive faecal samples in molecular analysis*

From this study		Closest match		
Faecal samples	Accession number from Genbank	Accession number from Genbank	Locality	Hosts
1G-1K Carabao	MK330179	KR075936.1	Mongolia, China	Sheep
		KY320581.1	Tehran, India	Human
3G-9P Pig	MK330180	KR902357.1	Shanghai, China	Water samples
		GU564279.1	Henan, China	Human
4G-19P Pig	MK330180	KR902357.1	Shanghai, China	Water samples
		GU564279.1	Henan, China	Human
5G-19B Cattle	MK330179	KR075936.1	Mongolia, China	Sheep
		KY320581.1	Tehran, India	Human
7G-40B Cattle	MK330181	KT922260.1	Central Ethiopia	Cattle
		EF654692.1	Georgia, USA	Cattle

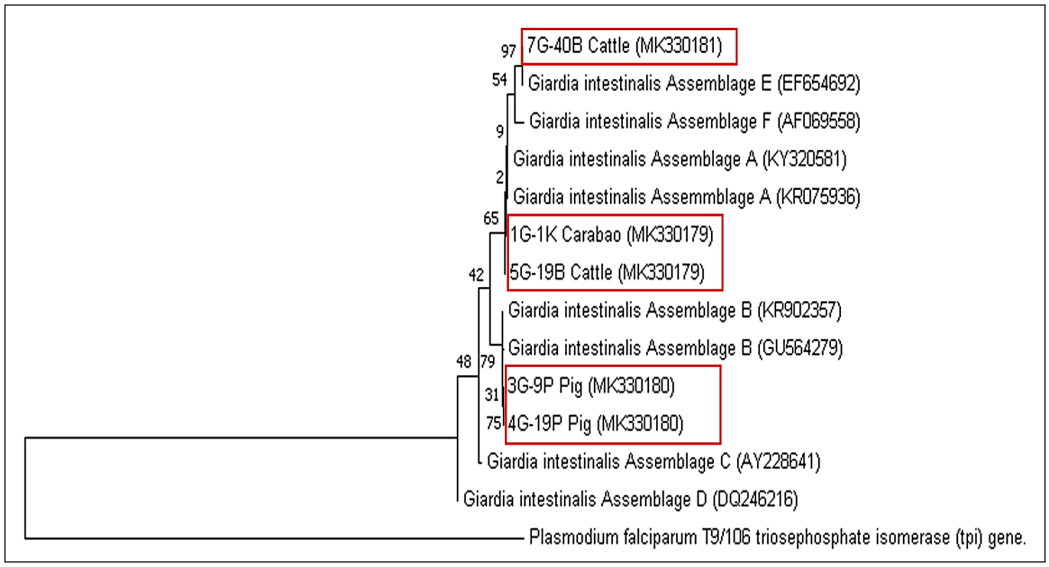


Figure 2. Phylogenetic relationship of sequences based on the TPI gene sequence by Neighbour-Joining Tree methods. Bootstrap: 1000 replicates

In the study of Abeywardena et al. (2013) in Australia, *Giardia* was detected under assemblage A among carabaos. In addition, the results in cattle are also the same with the study of Abeywardena et al. (2014) from Sri Lanka, where both assemblages A and E were found among the cattle. Although there are no reported cases of assemblage B among pigs in Asia, it is the most common assemblage among humans in Bangladesh (Ryan & Caccio, 2013). Additionally, in Asia, a significant number of infected cattle were found with assemblage A, but very few were infected with assemblage B. Meanwhile, Xiao and Fayer (2008) claimed that most pigs and ruminants from Western countries were infected with *Giardia* from assemblage E.

Possible Risk Factors among *Giardia*-positive Faecal Samples

The data showing the samples that were found positive with *Giardia* were used to see its association with the possible risk factors. However, the results of the current study showed that all the factors had no significant association with the prevalence of *Giardia* (Table 2). Several studies also revealed no association of the presence of *Giardia* with these factors such as feeding floor and diet (Kakandelwa, 2015), habit of livestock animals, whether it was freely roaming, tied or caged (Castro-Hermida et al., 2002), diarrhea and faecal consistency (Langkjaer et al., 2007; Maddox-Hyttel et al., 2006; Paz e Silva et al., 2012; Petersen et al., 2015; Toledo et al., 2016). Furthermore, Siwila (2017) stated that limited information was available on studies about *Giardia* infection since typically it came asymptomatically.

Table 2
Association of risk factors with Giardia-positive faecal samples of livestock animals

Risk Factors	Livestock Animals			Total	*P value
	Pig n=44	Carabao n=12	Cattle n=47		
Diet					
Commercial Feeds	6 (40)	-	-	6 (40)	
Grass	-	1 (12)	4 (40)	5 (52)	0.625
Milk	1 (4)	-	1 (7)	2 (11)	
Feeding floor					
Open field	-	1 (12)	5 (47)	6 (59)	0.386
Cemented floor	7 (44)	-	-	7 (44)	
Habit					
Free roaming in the field	-	-	5 (47)	5 (47)	0.671
Tied	-	1 (12)	-	1 (12)	
Caged Indoor	7 (44)	-	-	7 (44)	
Presence of Illness (Diarrhea)					
Yes	4 (20)	-	2 (32)	6 (52)	0.682
No	3 (24)	1 (12)	3 (15)	7 (51)	
Faecal consistency					
Liquid	-	-	2 (8)	2(8)	
Slightly Watery	2 (10)	1 (12)	2(31)	5 (53)	0.661
Firm	5 (34)	-	1 (8)	6 (42)	

*$p<0.05$= significant
*The number of livestock species for each factor were enclosed in the bracket; the number outside the bracket is the positive samples from molecular detection

CONCLUSION

This study revealed the presence of *Giardia intestinalis* among livestock of Sariaya, Quezon. It is categorised as a zoonotic parasite of public health importance. Thus, it is recommended that water from their facilities and human faecal samples from livestock farms be tested for the presence of *Giardia* to better understand the possible source of this parasite. Although *Cryptosporidium* was not detected in the present study, it should still be considered in future researches. It is suggested to assess other risk factors in livestock such as changes of season (dry/wet season) and age of the animals under longitudinal type of sampling method.

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Isolation and Molecular Characterization of Newcastle Disease Virus from Imported Birds at an Animal Quarantine Station in Malaysia

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ABSTRACT

Five Newcastle disease viruses (NDVs) were isolated from imported birds at an animal quarantine station in Malaysia from 2012 to 2017. Analysis of the deduced amino acid sequences of the fusion (F) protein cleavage site showed that all the Newcastle disease virus (NDV) isolates were virulent with the ^{112}RRQ/KKRF^{117} motif. Phylogenetic analysis of the F gene revealed that four isolates were grouped in genotype VIa while one was in genotype VIIi. Among the four VIa viruses, three were clustered together with the Belgium strain and one with the United States strain. Meanwhile, the VIIi virus was highly similar to the Pakistan strain. VIa viruses in Malaysia were mostly detected from imported avian and there are no currently reported outbreaks caused by this virus. Whereas NDV VIIi viruses caused outbreaks in poultry in Malaysia in 2011 to 2012. There were only slight differences between the F gene of the imported and local existing VIIi viruses. This study revealed the isolation of different genotypes of virulent NDV of different origin from imported birds. As captive birds can transmit NDV across international boundaries and viral transmission between birds and poultry may lead to outbreaks, hence, screening imported birds for diseases is the most crucial step in preventing the spread of this disease. NDV can cause significant economic losses to the poultry industries worldwide. With the increase in international trade of birds and poultry, strict quarantine systems and good laboratory diagnostic capabilities that can characterize and differentiate imported viral strains from existing circulating strains in

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the country are important in attempts to prevent the entry of foreign viral NDV strains.

Keywords: Genotype VI, genotype VII, imported birds, Newcastle disease

INTRODUCTION

Newcastle disease (ND) is a highly contagious disease in chicken where outbreaks can cause flock mortality up to 100% and has been one of the major causes of economic losses in the poultry industry (Aldous & Alexander, 2001). ND is caused by avian paramyxovirus serotype 1 (APMV–1) viruses, a member of the genus Rubulavirus, sub-family *Paramyxovirinae* and family *Paramyxoviridae* (Aldous & Alexander, 2001). NDV is an enveloped, non-segmented, negative-sense RNA virus. The genome encodes for the nucleocapsid protein (NP), phosphoprotein (P), matrix protein (M), fusion protein (F), haemagglutinin-neuraminidase (HN) and large protein (L) (Aldous & Alexander, 2001; Dortmans et al., 2011b). V and W proteins are expressed by RNA editing during P gene transcription (Dortmans et al., 2011b; Guo et al., 2013). NDV is divided into class I viruses that are mainly isolated from shorebirds and waterfowl; and class II is detected mainly in poultry and multiple wild birds where most viruses in this class are virulent (Diel et al., 2012). Based on the phylogenetic analysis of the partial or complete F gene, the NDV can be further classified into genotypes (Aldous et al., 2003; Diel et al., 2012; Kim et al., 2008).

The first panzootic of ND had started in Indonesia and England in the mid-1920s, the second was in the Far East and had spread to Europe in the 1960s (Lomniczi et al., 1998). In the 1980s, the APMV-1 infection had spread worldwide among racing, show and feral pigeons which caused the third ND panzootic (Aldous et al., 2004; Guo et al., 2013; Kim et al., 2008; Liu et al., 2013; Lomniczi et al., 1998). These APMV-1 viruses can be differentiated by monoclonal antibodies (mAb) and were termed as pigeon paramyxovirus type 1 (PPMV-1) and they also form a special sublineage (VIb / 4b) in the phylogenetic analysis (Aldous et al., 2004; Lomniczi et al., 1998).

Wild birds are a natural reservoir of NDV and different genotypes of NDV have been identified (Liu et al., 2013). Viral transmission can occur between wild birds and poultry that may lead to outbreaks and losses in poultry industries (Liu et al., 2013). Hence, screening of birds especially those for import/export purposes is very important. Therefore, the aim of this study is to molecularly characterize NDV strains isolated from imported birds at an animal quarantine station in Malaysia based on the partial F gene of the NDV.

MATERIALS AND METHODS

Virus Isolates

Samples such as cloacal swabs and tracheal swabs were collected from the birds upon arrival at the quarantine station. The samples were then sent to Veterinary Research Institute for Avian Influenza and ND

screening. From year 2012 to 2017, five NDVs were isolated from live imported birds at an animal quarantine station. The isolates were designated as VRI 286-2012, VRI 2344-2012, VRI 9681-2012, VRI 10951-2012 and VRI 2164-2016 and were used in the present study. The viruses were propagated in 9 to 11 days old Specific Pathogen Free (SPF) embryonated chicken eggs via the intra-allantoic route. The isolates were confirmed as NDV by the Hemagglutination-Inhibition (HI) test using a specific antiserum against ND (World Organisation for Animal Health [OIE], 2012).

RNA Extraction and Reverse Transcription - Polymerase Chain Reaction (RT-PCR)

The viral RNA was extracted from the infected allantoic fluid by the phenol chloroform method using TRIzol Reagent (Invitrogen) based on manufacturer's instruction. RT-PCR was carried out using the SuperScript III One-Step RT-PCR System with Platinum Taq (Invitrogen). A primer set, MV1: 5'- CCY RAA TCA YYR YGR YRC YRG ATA A -3' and B2: 5'- KCR GCR TTY TGK KTG GCT KGT AT -3' (Herczeg et al., 1999) was used to amplify 557 bp which covered the partial matrix and F gene of NDV. In brief, the RT was carried out at 48°C for 30 min. The reaction mix was then subjected to 94°C for 5 min for initial denaturation, followed by 40 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1 min and extension at 68°C for 1 min with a final extension for 10 min at 68°C. The amplicons were then analysed by electrophoresis on 1.5% agarose gel stained with SYBR Safe DNA gel stain (Invitrogen).

Sequencing and Phylogenetic Analysis

The amplified PCR products were cut from the gel and sent for sequencing (First Base Laboratories Sdn Bhd). Nucleotide sequences were assembled using SeqMan Pro software (DNAStar Lasergene, USA). Fifty nine NDVs retrieved from GenBank representing each genotype were included in the study (Table 1). The nucleotide and deduced amino acid sequences were aligned and compared using *BioEdit* Sequence Alignment Editor (version 7.1.9). Subsequent phylogenetic analysis was carried out using MEGA version 6.06 by neighbor-joining statistical method with Kimura 2-parameter model and setting bootstrap 1000 replicates. A segment of 372 bp from the start codon of the F gene and ending just downstream from the cleavage activation site (from nucleotide 47 to 418) was used in constructing the phylogenetic tree.

RESULTS

The details of the NDV isolates are shown in Table 2. All five isolates were positive for NDV by *HI test using specific antiserum against ND and generated* PCR product of 557bp. Analysis of the F protein cleavage site showed all isolates were virulent with the presence of multiple basic amino acid sequence at position 112 to 116 and phenylalanine (F) at residue 117. Four isolates had the RRQKRF and one had the

RRKKRF motifs respectively. Phylogenetic analysis based on the 372 nucleotides of the F gene showed that four isolates i.e., the VRI 286-2012, VRI 9681-2012, VRI 10951-2012 and VRI 2164-2016 belonged to genotype VIa while VRI 2344-2012 was grouped in genotype VIIi (Figures 1 and 2).

Table 1
Details of NDVs retrieved from GenBank representing each genotype

Strain	Year isolated	Country	Host	Genetic group	Accession number
chicken/Australia/I-2/2005	2005	Australia	Chicken	I	AY935499.2
chicken/N. Ireland/Ulster/67	1967	Northern Ireland	Chicken	I	AY562991.1
V4	1966	Australia	Chicken	I	JX524203.1
chicken/US/LaSota/1946	1946	USA	Chicken	II	AF077761.1
turkey/US/VG/GA/1987	1987	USA	Turkey	II	EU289028.1
B1	NA[a]	NA	NA	II	AF309418.1
chicken/JS/7/05	2005	China	Chicken	III	FJ430159.1
goose/JS/9/05	2005	China	Goose	III	FJ430160.1
India/Mukteswar	NA	China	NA	III	EF201805.1
fowl/UK/Herts/33	NA	UK	Chicken	IV	AY741404.1
Italien	1944	Italy	NA	IV	EU293914.1
anhinga/USA(FL)/44083/93	1993	USA	Anhinga	V	AY288989.1
cormorant/ Canada/95DC2345/1995	1995	Canada	Cormorant	V	FJ705461.1
Rosella/Belgium/4940/08	2008	USA	Rosella	VIa	JN872162.1
PPMV-1/Belgium/11-09620/2011	2011	Belgium	Pigeon	VIa	JX901124.1
pigeon/Italy/1166/00	2000	Italy	Pigeon	VIa	AY288996.1
pigeon/US/RI166/2000	2000	USA	Pigeon	VIa	EU477189.1
WX-10-07-Pi	2007	China	Pigeon	VIa	GQ281086.1
YZ-23-07-Pi	2007	China	Pigeon	VIa	GQ281088.1
W4	2005	China	White-breasted water hen	VIa	HM063423.1
GB 1168/84	1984	Great Britain	NA	VIb	AF109885.1
Pigeon/Argentina/Tigre 6/99	1999	Argentina	Pigeon	VIb	AY734535.1
IT-227/82 Italy	1982	Italy	Pigeon	VIb	AJ880277.1
PPMV-1/New York/1984	1984	USA	Pigeon	VIb	FJ410145.1
PPMV-1 s-1 China	2002	China	Pigeon	VIb	FJ865434.1
Japan/Ibaraki/85	1985	Japan	Chicken	VIc	AB465606.1
Sh-1/97	1997	China	Chicken	VIc	AF458018.1
ZhJ-3/97	1997	China	Chicken	VIc	FJ766529.1
DK-1/95	1995	Denmark	Fowl	VId	AF001129.1
DK-6/95	1995	Denmark	Ostrich	VId	AF001130.1

Table 1 *(continue)*

Strain	Year isolated	Country	Host	Genetic group	Accession number
S-1/95	1995	Sweden	Fowl	VId	AF001131.1
CH-1/95	1995	Switzerland	Fowl	VId	AF001132.1
A-24/96	NA	Austria	Fowl	VId	AF001133.1
NDV BBGPI95039 Bulgaria	1995	Bulgaria	Pigeon	VId	AY135742.1
JS/2/98/Go	1998	China	Goose	VIe	AF456439.1
PPMV-1 YN-P1	NA	NA	Pigeon	VIe	AY325798.1
pigeon STP96 China	NA	China	Pigeon	VIe	DQ417113.1
PG/CH/JS/1/05	2005	China	Pigeon	VIe	FJ480825.1
NDV05-029	2005	China	Pigeon	VIe	FJ766528.1
Pigeon/Indiana/18002/1991	1991	USA	Pigeon	VIf	JN872186.1
Chicken/Texas/309968/2004	2004	USA	Chicken	VIf	JN942022.1
dove/Nigeria/VRD07-163/2007	2007	Nigeria	Dove	VIg	JQ039385.1
pigeon/Nigeria/VRD07-173/2007	2007	Nigeria	Pigeon	VIg	JQ039395.1
Pigeon/Argentina/Capital 3/97	1997	Argentina	Pigeon	VIh	AY734536.1
pigeon/Nigeria/VRD08-37BRpe(7-9)/2008	2008	Nigeria	Pigeon	VIh	JQ039387.1
pigeon/Nigeria/VRD07-231/2007	2007	Nigeria	Pigeon	VIh	JQ039391.1
NDV/DOVE/IT/11RS98_102VIR/2011	2011	Italy	Dove	VIi	JN638234.1
NDV/DOVE/IT/11RS100_104VIR/2011	2011	Italy	Dove	VIi	JN638235.1
TW/2000	2000	Taiwan	Fowl	VII	AF358786.1
JS-3/00	2000	China	Chicken	VII	AF458010.1
chicken/Sukorejo/019/10	2010	Indonesia	Chicken	VII	HQ697255.1
MB128/04-Malaysia	2005	Malaysia	Chicken	VII	GQ901900.1
chicken/China/QH1/1979	1979	China	Chicken	VIII	FJ751918.1
chicken/China/QH4/1985	1985	China	Chicken	VIII	FJ751919.1
AF2240 Malaysia	1960s	Malaysia	NA[a]	VIII	AF048763.1
duck/JS/1/02	2002	China	Duck	IX	FJ436306.1
China/F48E9	NA	China	NA	IX	AF163440.1
DE-R49/99	NA	Germany	Duck	Class I	DQ097393.1
Canada goose/US(OH)/87-78/1987	1987	USA	Canada goose	Class I	EF564833.1

[a] Not Available

Table 2
Details of NDV isolates used in this study

Isolates	Year isolated	Host	Origin Country of Import	Specimen submitted	BLAST Analysis			F0 cleavage site motif[a]	Virulence[b]	Sub-genotype[c]
					Significant alignment	Query Covery (%)	Identity (%)			
VRI-286-2012	Jan 2012	Bird	*Philippines*	Cloacal swabs	NDV/Pigeon/PA/USA	100	97	RRKKRF	Virulent	VIa
VRI-2344-2012	Mac 2012	Bird	Pakistan	Cloacal swabs	chicken/Pakistan/ Sheikhupura/12A/994/2015	100	99	RRQKRF	Virulent	VIIi
VRI-9681-2012	Aug 2012	Bird	Belgium	Cloacal swabs	PPMV-1/ Belgium/11-09620/2011	100	99	RRQKRF	Virulent	VIa
VRI-10951-2012	Sep 2012	Pigeon	Bulgaria	Tracheal swabs	PPMV-1/ Belgium/11-09620/2011	99	97	RRQKRF	Virulent	VIa
VRI-2164-2016	Mac 2016	Avian	Belgium	Cloacal swabs	PPMV-1/ Belgium/11-09620/2011	99	97	RRQKRF	Virulent	VIa

[a]Cleavage site motif corresponds to amino acids 112 to 117 of the fusion protein
[b]OIE (2012) defines virulent ND viruses as those with a phenylalanine (F) residue at position 117 and at least three basic amino acids (R or K) between residues 113 and 116
[c]Genotypes within class II of NDV

Table 3
Comparison of fusion amino acid in this study using Lomniczi's study (1998) as reference

Genotype	4	5	9	10	13	16	17	19	23	28	63	93	101	104	106	107	109	112	113	114	115	116	117	118	121	124[a]
VIa[c]	K	P	I	P	L	I	T	I	L	L	V/I	T	R	G	V	S	S	R	R	Q	K	R	F	I	I	S[b]
VIb[c]	-[d]	-	-	-	-	-	-	T	-	P	V	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-
VRI-286-2012	-	-	-	L	-	-	-	-	-	-	V	-	-	-	-	-	P	-	-	K	-	-	-	-	-	-
VRI-9681-2012	-	-	-	-	-	-	-	-	-	S	V	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
VRI-10951-2012	-	-	-	-	-	-	-	-	-	S	V	-	-	-	-	-	-	-	-	-	-	-	-	V	-	-
VRI-2164-2016	-	-	-	-	-	-	-	-	-	S	V	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
PPMV-1/Belgium/11-09620/2011	-	-	-	-	-	-	-	-	-	S	V	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
VII[c] (Indonesia)	K	P	I	P	L	I	T	I	L	L	V	T	K	G	V	S	S	R	R	Q	K	R	F	I	V	S
VRI-2344-2012	-	L	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-

[a] Position of residues in the amino acids of the fusion protein
[b] Amino acid of the fusion protein at the corresponding position (Lomniczi et al., 1998)
[c] Amino acid of genotypes and sub-genotypes within class II of NDV (Lomniczi et al., 1998)
[d] (-) Indicate identical amino acid

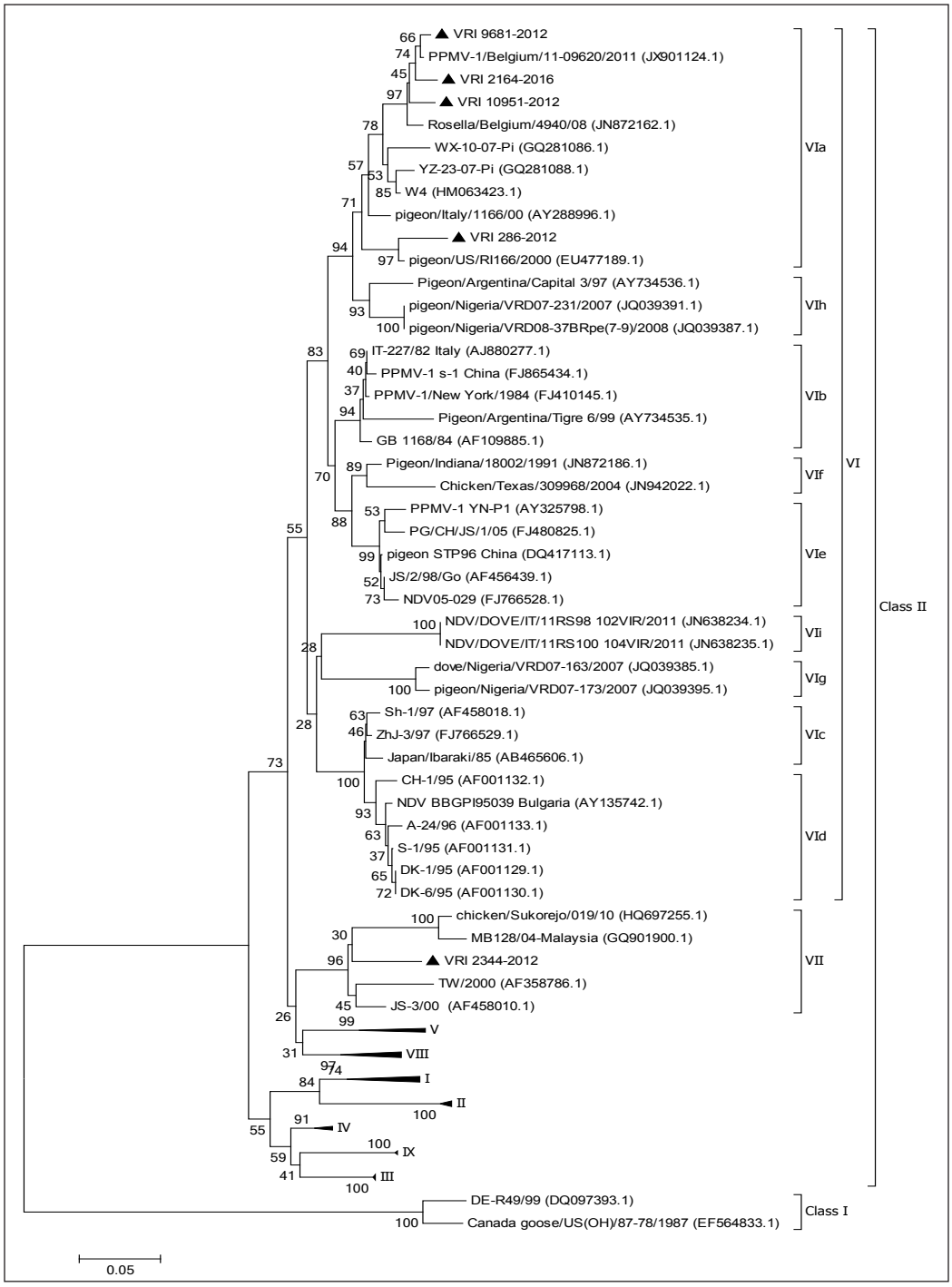

Figure 1. Phylogenetic tree of NDV isolates from quarantine station based on partial fusion gene (nuleotide 47-418) of NDV. Tree was constructed using MEGA version 6.06 by Neighbor-Joining statistical method with Kimura 2-parameter model and setting bootstrap 1000 replicates. GenBank accession numbers are shown in parenthesis

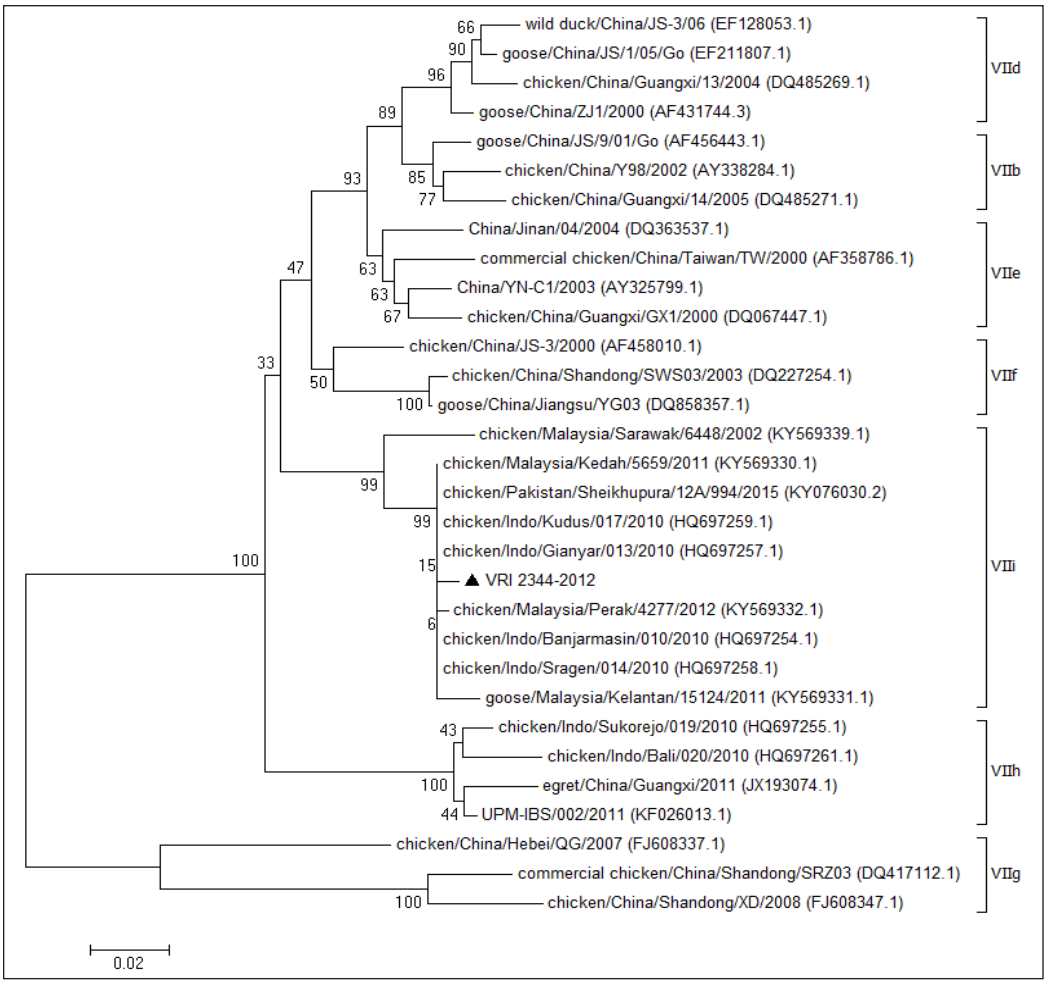

Figure 2. Phylogenetic tree of NDV isolate genotype VII isolated from birds in quarantine station based on partial fusion gene (nucleotide 47-418) of NDV. Tree was constructed using MEGA version 6.06 by Neighbor-Joining statistical method with Kimura 2-parameter model and setting bootstrap 1000 replicates. GenBank accession numbers areshown in parenthesis

DISCUSSION

Phylogenetic analysis of the F gene revealed that four NDVs isolated from imported birds belonged to genotype VIa and one was to genotype VIIi. Isolation of ND genotype VI and VII from imported birds at quarantine stations is common in Malaysia. Muhammad et al. (2012) had reported similar findings where ND genotype VIa, VIb and VIId were isolated from imported pigeons while genotype VIa was also retrieved from imported chicken.

Genotype VI had emerged since the 1960's (Lomnizci et al., 1998) and could be further divided into nine (a-i) subgenotypes (Liu et al., 2013). Genotype VI was often detected in the *Columbidae* species and had become a threat to the poultry industry (Diel et al., 2012). The genotype VIa includes mainly ancient isolates originating from

the Middle East (Aldous et al., 2003) and have been found mainly in *Columbidae* birds in Asia, Europe, the Middle East and the United States since the 1990s (Guo et al., 2013; Kim et al., 2008; Snoeck et al., 2013b). This is consistent with the finding in this study where the VIa viruses were retrieved from Asia and Europe (Table 2). To our knowledge, VIa viruses in Malaysia were mostly detected from imported avian (Mohamad et al., 2012) and to date there is no reported outbreak by this virus. However, the importance of VIa viruses cannot be neglected as two outbreaks caused by genotype VIa have been reported in South Africa (Snoeck et al., 2013a).

Genotype VII is a very large and genetically diverse group of viruses and always associated with ND outbreak in the Middle East and Asia currently (Diel et al., 2012). Genotype VII viruses can be subdivided into VIIb, VIId, VIIe, VIIf, VIIg, VIIh and VIIi (Miller et al., 2015). This virus is related to the fourth ND panzootic, where it started in Southeast Asia in the year 1985, and has been spreading from Asia, Europe, Africa and in South America (Miller et al., 2015) until today. In this study, the isolate VRI-2344-2012 was grouped together with the Pakistan and Indonesian strains in genotype VIIi. The Indonesian strains such as chicken/Indo/Kudus/017/2010, chicken/Indo/Gianyar/013/2010, chicken/Indo/Banjarmasin/010/2010 and chicken/Indo/Sragen/014/2010 were initially grouped as genotype VIIa by Choi et al. (2014). It was later re-classified by Miller et al., (2015) as genotype VIIi as Miller and colleagues found that this new sub-genotype VIIi is associated with the Indonesian NDV isolated from wild birds since the 1980s. The grouping of VRI-2344-2012 together with the Indonesian strains is in agreement with Miller et al. (2015) who stated that VIIi viruses isolated from poultry and infrequently from pet birds in Pakistan were associated with the current Indonesian strains. From the BLAST analysis, this isolate was 99% similar with the chicken/Pakistan/Sheikhupura/12A/994/2015 strain which corresponded to the origin of the import country, Pakistan. Again, Wajid et al. (2017) reported that sub-genotype VIIi was frequently isolated from poultry and various non-poultry avian species from year 2011 to 2016 in Pakistan. In Pakistan, the majority of ND outbreaks during the winter season from October 2011 to March 2012 was caused by VIIi viruses (Miller et al., 2015) which coincides with the isolation of VRI-2344-2012 in March 2012.

In Malaysia, genotype VII had caused several outbreaks from2000 to 2001 (Tan et al., 2010) and in 2010 (Shohaimi et al., 2015). Previously, genotype VIIa (it has been now reclassified as VIIi by Miller et al., 2015) had only been reported in East Malaysia (Sabah since 2004 and Sarawak since 2002) but it had not caused any outbreaks (Shohaimi et al., 2015). However, Shohaimi et al., (2015) found out that the same genotype had caused outbreaks not only in East Malaysia but also in Peninsular Malaysia from 2011 to 2012 and the same virus had also caused outbreaks in Pakistan and Indonesia. The rapid spreading of the VIIi virus and the ability of this

virus to infect various avian species may cause a panzootic if the disease is not kept under control (Miller et al., 2015). It is important for Malaysia and other countries in Southeast Asia which practise multiple farming practices such as commercial and backyard farming to control this virus as it may lead to the emergence of a virulent NDV as a new sub-genotype due to this production system (Miller et al., 2015).

The BLAST search showed that three VRI isolates, the VRI 9681-2012, VRI 10951-2012 and VRI 2164-2016 were 97-99% similar to the PPMV-1/Belgium/11-09620/2011 strain and were grouped in genotype VIa. Nevertheless, it was in contrast with the study conducted by Wang et al., (2015) who reported that the PPMV-1 Belgium strain belonged to genotype VIb. Lomniczi et al. (1998) reported that the deduced amino acid of the F gene at a certain position can be used to differentiate between a genetic group or sub-group. By comparing the amino acid we identified that the PPMV-1 Belgium strain has the same amino acid at all positions described by Lomniczi et al. (1998) for genotype VIa except at position 28 where Lomniczi et al. showed that Leucine (L) was specific for this position whereas the Belgium strain was observed to have Serine (S) (Table 3 and Figure 3). Lomniczi et al. (1998) also mentioned that the amino acid to differentiate genotype VIa and VIb was located at position 19, 28 and 112 where VIa had I^{19}, L^{28} and R^{112} while VIb has T^{19}, P^{28} and G^{112} (Table 3). Since the Belgium strain did not possess T^{19}, P^{28} and G^{112} as required for genotype VIb and more importantly, the strain was grouped under VIa in the phylogenetic analysis; therefore, we strongly suggest that this Belgium strain belongs to the genotype VIa group.

Although the four isolates belonged to genotype VIa, there were discrepancies among the isolates. As for VRI 286-2012, it did not fall in the same cluster as others. It is clustered with pigeon US strain which also corresponds with the BLAST search. The discrepancies among the four VIa isolates can be further explained from the aspect of amino acid sequences (Table 3).

Figure 3. Alignments of fusion amino acid sequences of imported genotype VIa NDVs compared with reference strain. A *dot* indicates an amino acid identical to that of PPMV-1 Belgium strain. The position of amino acids start with methionine that encodes the start codon of the F protein

Three isolates that were similar to the PPMV Belgium strain have the same amino acids when compared to the Lomniczi's study (1998) except for position 28 where the three isolates have S (serine) instead of L (leucine). Among these, VRI-10951-2012, has V (Valine) at position 118 which is different from others. On the other hand, VRI-286-2012 has more differences in amino acid as compared to the Lomniczi's study and the other three VIa isolates. It has L^{10}, P^{109} and K^{114} instead of P^{10}, S^{109} and Q^{114}. It is noteworthy that the pigeon US strain also has the same amino acid of L^{10} and K^{114} respectively as compared to the VRI-286-2012 isolate (Figure 3). Whereas the S at position 109 is in contrast with VRI-286-2012 and is in agreement with Lomniczi's study.

Meanwhile, the VIIi isolate, VRI 2344-2012 has the same amino acid as defined by Lomniczi for genotype VII in all positions except at positions 5 and 107 (Table 3). This VRI isolate has L^{5} and A^{107} instead of P^{5} and S^{107} respectively. In order to understand the difference between the imported VIIi virus and the existing local VIIi viruses in Malaysia, the F protein's amino acid is compared (Figure 4). All the local VIIi viruses have P (proline) at position 5 while the imported VIIi virus has L (leucine). In general, the five local isolates do not differ much as compared to the imported virus except the Sarawak isolate which has W^{18}, V^{19}, C^{25}, N^{30} and I^{52} that was totally dissimilar from others. Perhaps these unique amino acids categorized this Sarawak isolate into a distinct lineage in the VIIi sub-genotype (Figure 2).

All isolates in this study were virulent NDV belonging to the VI and VII genotypes. These findings which are in agreement with Diel et al., (2012) who reported that genotype V, VI, VII and VIII contained only virulent viruses and were the predominant genotypes circulating in the world. Pathogenicity of the NDV is correlated with the F protein (Meulemans et al., 2002) and the amino acid sequence at the F cleavage site is a major determinant of virulence (Dortmans

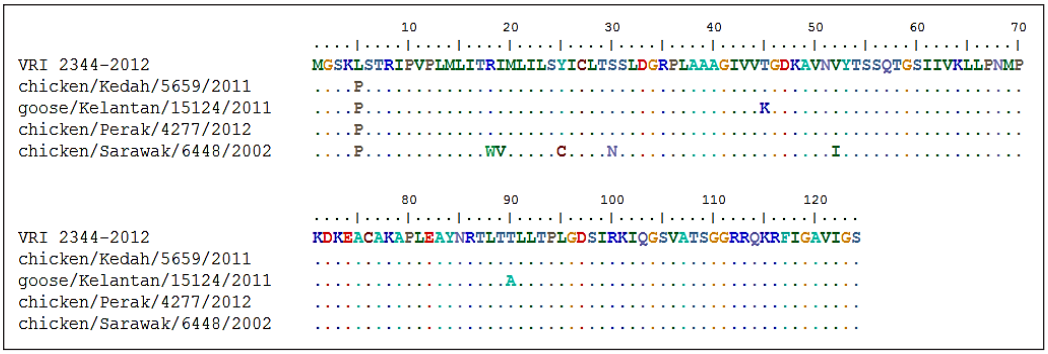

Figure 4. Alignments of fusion amino acid sequences of imported genotype VIIi NDVs compared with local VIIi isolates. A *dot* indicates an amino acid identical to that of VRI-2344-2012 strain. The position of amino acids start with methionine that encodes the start codon of the F protein

et al., 2011a). In this study, four isolates have the ^{112}RRQKRF^{117} motif at the F cleavage site while VRI-286-2012 has ^{112}RRKKRF^{117}. Tan et al. (2010) pointed out that most of the published NDV had a Q (glutamine) at position 114 as a dominant motif. However, the presence of R (arginine) or K (lysine) in the same position has also been reported. Motif ^{112}RRKKRF^{117} has been reported in Japan (Mase et al., 2002) from pigeon and in Taiwan from chicken (Lien et al., 2007). A similar motif has also been reported in European countries in PPMV-1 (Huovilainen et al., 2001; Meulemans et al., 2002; Terregino et al., 2003; Werner et al., 1999) and also in North America (Kim et al., 2008).

Kaleta and Baldauf (1988) explained that NDV outbreaks could occur in captive birds in quarantine stations, zoos and bird parks. Factors such as the high population density of one or more species in the captive environment, uncleaned droppings, uncontrolled flow of air and water and human traffic, limited water containers, and the constant flow of arriving and departing birds can contribute to the newly captive birds acquiring NDV infection when they transit in the quarantine station (Kaleta & Baldauf, 1988). Unsatisfactory environmental conditions and lack of social stimuli may suppress the inherited behaviour of the birds to just survive (Kaleta & Baldauf, 1988). This may increase the susceptibility of the birds to disease and eventually cause the high incidence of ND in captive birds (Kaleta & Baldauf, 1988). Kaleta and Baldauf (1988) also highlighted that in many countries, birds for export purposes were frequently infected with the NDV from backyard chicken when both birds and chicken were kept nearby. It is believed that a panzootic ND in Europe and North America during 1969/73 was associated with imported NDV from parrots from South American countries where these parrots were kept with infected backyard chickens in their local collection centre (Kaleta & Baldauf, 1988). Hence, NDV recovered from birds in quarantine stations in this study is not uncommon.

As mentioned earlier, NDV has been isolated from imported pigeons and chicken at quarantine stations in Malaysia. Similar cases have also been reported where velogenic NDV has been detected in pet birds that were imported to Canada and the United States (Clavijo et al., 2000). Therefore, strict enforcement of the regulations for the importation of live birds, and import risk analysis to assess the biosecurity risk associated with importing birds/animals into Malaysia are important. Preventive measures such as satisfactory hygiene in quarantine stations, placing different batches of birds arriving and departing in separate rooms, and controlled movement of water, animal feed and human traffic can prevent the chances of viral transmission between the birds. At the same time, screening the birds for diseases before release from the quarantine station is the most crucial step to prevent introducing viruses from other countries.

CONCLUSION

In conclusion, NDVs isolated from imported birds at a quarantine station were virulent NDVs as characterized by the presence of the ^{112}RRQ/KKRF^{117} motif at the F cleavage site. These viruses belonged to genotype VIa and VIIi respectively. Imported birds can therefore be a source of new strains of NDVs introduced into the country. With the increase in international trade in birds and poultry, efficient quarantine systems especially, the establishment of rapid and accurate diagnostic capabilities that can identify virulent NDVs are crucial in preventing the entry and exit of foreign NDV strains via the import and export trade respectively.

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Multiple Antibiotic Resistances in *Escherichia coli* Isolated from Cattle and Poultry Faeces in Abraka, South-South Nigeria

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ABSTRACT

Rise in antibiotic resistance among clinical and environmental isolates in Nigeria is becoming worrisome. The unprofessional sales and consumption of veterinary antibiotics together with lack of adequate sanitation and hygiene during slaughtering, processing and consumption of cattle and poultry products in Abraka, Delta State of Nigeria, could spread antibiotic resistant bacteria to the surrounding environments. A study was therefore conducted to isolate *Escherichia coli* from 200 poultry litters and 200 cattle feaces and screen them for beta-lactamases. Antimicrobial susceptibility of 412 *E. coli* strains isolated from 400 samples of fresh and dry faeces of cattle and poultry and 25 samples each of rectal and cloacae swabs were carried out. All the strains were screened phenotypically for extended spectrum beta-lactamase (ESBL), carbapenemase and metallo beta-lactamase (MBL). Results showed a high incidence of resistance to all the antibiotics except meropenem. Resistance to nitrofurantoin, amoxicillin and sulfamethoxazole-trimethoprim reached 80-90%, while resistance to other beta-lactams and fluoroquinolones ranges from 40-60%, and over 50% of them exhibited multidrug resistance. Strains of *E. coli* from poultry droppings are more resistant to antibiotics than their counterparts from cow dungs. ESBL, carbapenemase and MBL production was detected in 10.5, 5.26 and 7.89% of isolates from cow dungs and 27.2, 10.2 and 6.81% isolates from poultry droppings. High level of antibiotic resistance and incidence of ESBL, carbapenemase, and MBL have public health implication as poor sanitation,

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poor water supply and lack of personal hygiene among the handlers, processors and consumers are very high in the locality.

Keywords: Antibiotic, carbapenemase, cow, ESBL, faeces, poultry, resistance

INTRODUCTION

The overuse of antibiotics in animal agriculture both in developed and developing countries has been considered as an important factor that contributes to the evolution and spread of antibiotic-resistant pathogens (ARPs) in human and environments. Wastes from human, animals, domestic and hospital sources can serve as a major reservoir of these ARPs (Diallo et al., 2013; Dierikx et al., 2010; Egea et al., 2012). In addition, during animal farming, the animals themselves and their broader environment can also serve as important reservoirs of ARP genes which have the potentiality of being exchanged within or across bacterial species. During production or processing of animals or their products, excreta and other fluids with possibly large amount of ARPs can come into contact with a human food or water, from where serious infections with potential high mortality may occur (Costa et al., 2009; Korzeniewska et al., 2013).

Scientists in the field of antibiotic resistance research globally have called for the restriction of antibiotic use in animal husbandry. However, in developed countries, there is an increase in the level of compliance, but in the developing countries, the non-therapeutic use of antibiotics is increasing, mainly for economic gain, where growth promotion of chicken and cattles is heavily dependent on large use of antiobics (Braykov et al., 2016). A study in 3 Nigerian states has revealed that, tetracycline is the most widely used antibiotic for livestock production, followed by flouroquinolones and betalactams (Adesokan et al., 2015).

Generally in Nigeria, and specifically in Abraka, Delta State, poultry farming and cattle rearing are promoted for economic development and nutrition supplementation strategy by all levels of government. As a result of this sustained increase in poultry production and concurrent use of veterinary antibiotics in Nigeria, rapid rise in antibiotic consumption in animals was observed in some states from 2010 to 2013, with the majority of the antibiotics acquired without professional prescriptions and were wrongly used (Ojo et al., 2016). This practice of indiscriminate antibiotic use, may impose selection pressure on bacteria which can lead to emergence of multi-drug resistant bacteria in the animal and subsequent passage to human, since the antibiotics, their metabolites, and resistant bacteria can be excreted with urine and faeces to the surrounding environment especially where poor sanitation and lack of effective infection control management are in place (Diallo et al., 2013; Yusuf & Adam, 2014).

Extended-spectrum β-lactamase (ESBL) and carbapenemase-producing enteric bacteria such as *Escherichia coli* and *Salmonella* sp. are globally recognised ARPs, with high spreading rate and mortality (Dierikx et al., 2010). Several infections caused by ARP producing

ESBLs and carbapenemase such as urinary tract infections, bacteraemia, peritonitis, pneumonia, intra-abdominal infections, and meningitis have a record of resisting treatment with many antibiotics such as ceftazidime, cefotaxime, aztreonam, fluoroquinolones and even antibiotics that are rarely used in many hospitals (Motayo & Akinduti, 2014; Yusuf et al., 2014).

Given the sustained use of antibiotics indiscriminately in Abraka for cattle and poultry production, there is need for studies that will determine the antibiotic susceptibility pattern of *E. coli* isolated from cow rectum, poultry cloacae and faeces (dungs and droppings) obtained from farms and surrounding environment and also to examine the *E. coli* for ESBL and carbapenemase production.

MATERIALS AND METHODS

Study Site

This study was carried out in Abraka, a town in Delta State located in South-South geo-political zone of Nigeria. The town was selected due to their relatively high poultry and cattle production in the region. Fertile land and a long period of rainfall provide evergreen grass for Fulani's owned cattle to graze. Poultry and livestock activities in the area provide a source of income to a large number of both registered and unregistered pharmaceutical outlets that provide drugs including antibiotics to both poultry and cattle producers. While poultry farms are often reared in a cage system, cattle in the region are reared mainly in semi-intensive systems where the animals are taken out to graze and then returned to their pens later in the evening. Both poultry farmers and cattle rearers have unregulated access to veterinary drugs in the region.

Sample Collection, Processing, Isolation, and Characterization of *Escherichia coli*

A total of 200 poultry litter samples was collected from Delta State University, Abraka poultry farms and 200 faecal samples of cattle from cattle farms located in Abraka and environs. For each, four sampling sites were identified and coded as P1, P2, P3, P4 and C1, C2, C3, C4 for poultry and cattle farms respectively. The cattle and poultry farms were independent of each other epidemiologically and to the best of our ability, the animals in the farms were healthy and not suspected of having disease or infection. At each sampling site, 50 samples were collected during the months of April to June 2017 in triplicates in sterile bottles. Samples P1, P2, C1, and C2 were taken fresh while P3, P4, C3 C4 were taken from dry faeces of the respective animals. To collect fresh poultry droppings, a large sterile autoclavable polythene was spread on clean and disinfected cage at 7pm. The feet of 6 week old broiler chickens were immersed in mild disinfectant solution before putting them back to the cage. The chickens were fed overnight and samples of fresh droppings were collected in the morning (7am) while dried ones were collected in the evening (7pm). For cow dungs, middle portion of fresh and dry dungs that is not in contact with soil were

collected with the aid of sterile spatula. Rectal and cloacal swabs from 25 randomly selected cattle and poultry were collected aseptically with sterile swab sticks. Swabs were collected equally from all the sampling sites. All the samples were transported to the laboratory within 1 h for bacterial isolation. About 1 g of faecal samples were homogenized in 9 mL of buffered peptone water and 1ml were rediluted in another 9 mL of buffered peptone water to make a dilution of 1:10. Cloacal swabs were soaked in 10 mL of buffered peptone water.

Isolation of *E. coli* was carried out by inoculation of 0.1 mL of appropriate dilutions of each sample on MacConkey (Oxoid, UK) plates in triplicates and incubated at 37°C for 24 h (Egea et al., 2012). Distinct colonies of *E. coli* on the plates (pink color) were identified and purified by repeated culturing. Pure cultures of presumptive *E. coli* from all the samples were characterised and identified biochemically according to standard protocol and stored in glycerol stock at –20°C until use.

Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing was performed by the standard disk diffusion method on Mueller–Hinton agar medium (Oxoid, UK) according to the criteria established by the Clinical and Laboratory Standards Institute [CLSI], (2016). The antibiotics used were ceftazidime (CAZ-30µg), cefuroxime (CRX-30 µg), gentamycin (GEN-10 µg), cefixime (CXM-5.0 µg), cefotaxime (CTX-30 µg), ofloxacin (OFL-5.0 µg), amoxicillin-clavulanic acid (AUG-30 µg), nitrofurantoin (300 µg), ciprofloxacin (CPR-5 µg, sulfamethoxazole-trimethoprim (SXT-23.75/1.25 µg), meropenems (MEM-10 µg) (Oxoid UK). Isolates resistant to beta-lactams were selected and tested for ESBL production. The susceptibility breakpoints for all antimicrobials were those recommended by CLSI (2016). *Escherichia coli* that are resistant to three or more classes of antibiotics were classified as multi-drug resistant strain.

Detection of Extended-Spectrum Beta-Lactamases (ESBL) and Carbapenemase

ESBL production was detected phenotypically by the double disc synergy test (DDST) method according to the criteria established by the CLSI using CAZ and CXT discs alone and in combination with AUG (CLSI, 2011, 2016).

Screening for the production carbapenemase by the animal isolates was performed with the modified Hodge test using *E. coli* ATCC 25922 (a carbapenem susceptible strain) as the indicator strain and ertapenem (ETP) (10µg) disc (Birgy et al., 2012).

Isolates that showed reduced susceptibility to ETP (< 24mm in diameter) were suspected as carbapenemase producers. However, all the isolates (both suspected and non-suspected) were subjected to carbapenemase confirmatory test by the modified Hodge test. Carbapenemase producers were identified by the formation of a clover leaf type indentation at the

intersection of the test organism and *E. coli* ATCC 25922 within the zone of inhibition around the carbapenem

Phenotypic Differentiation of MBLs and KPC Carbapenemase Production

MBL production was detected according to modified combined-disc tests of ETP alone and with EDTA (Kim et al., 2015). Briefly, a 0.5 McFarland standard suspension of the test isolates were inoculated on MHA plates (Oxoid, UK). Two discs separated by a distance of 20-30mm were placed on the surface of the inoculated MHA using a sterile forceps. One of the discs was ETP and the other was a sterile cut filter paper disc impregnated with 292 µg EDTA. The plates were incubated at 37°C for 24 h. The diameter of a clear zone around the ETP+EDTA was compared with a clear zone around the plain ETP-EDTA disc. Enhancement of zone around plain ETP by at least 2mm was taken as positive for MBL production.

RESULTS AND DISCUSSION

Livestock and poultry production in developing countries like Nigeria has enormous environmental, economic and health implications. At one end they provide a source of living, protein, and manure for the immediate community, but at the other end serves as reservoirs for untreatable bacteria. Cross-transmission to human is very much easier since the level of environmental sanitation and personal hygiene among the handlers and consumers is very low.

Of the total 400 faecal samples, comprising cow dungs and poultry droppings, and 50 swabs, a total of 412 *E. coli* were isolated, 188 from poultry samples and 224 from cow samples. Sixty eight percent (68.0%) and 73.6% of the isolates were from fresh poultry and cattle faeces respectively, while the remaining comes from dry samples. Specifically, 56% and 76% of the total rectal and cloacal swabs of cow and poultry respectively, yielded positive culture of *E. coli*. Of the total *E. coli* isolates, 94 and 88 from poultry and cattle samples respectively were resistant to at least two antibiotics while 61 and 51 were multidrug resistance respectively (Table 1). Further, the *E. coli* isolated from rectal and cloacal swabs were 42.8% and 63.1% resistant to at least 2 commonly used veterinary antibiotics in the area. The result indicates that highly resistant *E. coli* was present in faecal samples from poultry and cattle reared in the study area, possibly due to a general increase in the unprofessional use of antibiotics in animal production in the entire region. This is serious; especially in such community where awareness level of effect and sources of ARPs even among the health care workers is very limited, so for members of the community where literacy level is very low (Yusuf et al., 2015). The presence of 28.5% and 10.5% multi drug resistant *E. coli* in the cloacal and rectal swabs of the apparently healthy cattle and poultry is also worrisome, and may be due to prolonged exposure of *E. coli* in the gastro intestinal tracts of the animals to antibiotics. These increased population of resistant

Table 1
Screening of isolates from various sampling sites of cattle and poultry farms for susceptibility to selected common antibiotics

Sample code	Nº of *E. coli* isolated	Nº of *E. coli* resistant to at least 2 antibiotics (%)	Nº of *E. coli* resistant to multiple antibiotics (%)	Nº of *E. coli* susceptible to all antibiotics (%)
P1	67	34 (50.7)	15 (22.3)	18 (26.8)
P2	58	33 (56.8)	20 (34.4)	5 (8.6)
P3	32	10 (31.2)	13 (40.6)	9 (28.1)
P4	28	11 (39.2)	9 (32.1)	7 (25.0)
C1	71	31 (43.6)	15 (21.1)	11 (15.4)
C2	64	19 (29.6)	14 (21.8)	2 (3.12)
C3	28	17 (60.7)	8 (28.5)	0 (0.00)
C4	31	9 (29.0)	12 (38.7)	10 (32.2)
PC	14	6 (42.8)	4 (28.5)	4 (28.5)
CR	19	12 (63.1)	2 (10.5)	5 (26.3)
Total	412	182(44.1)	112(27.1)	71(17.2)

Note: PC=poultry cloacal swab, CR=Cow rectal swab

strains of *E. coli* will remained in the environment and may later cause infection in the animals or human that consume them.

Of specific interest, 44.1% of the total isolates were resistant to at least two antibiotics commonly used for human treatment in hospitals while 27.1% were resistant to multiple antibiotics. Surprisingly, only 71 (17.2%) of the isolates were susceptible to tested antibiotics. Possibility of community acquirement of these bacteria in the study area is very easy and will be rapid, since cattle are allowed to move about freely in the environment, with their faeces scattered all over the environment. Even though, poultry in the study area are often caged in farms meant for commercial production, but the poultry manures are heaped with no appropriate disposal means. Studies in developed countries where cattle and poultry are caged or ranched and infection control system is functional, showed that certain amount of *E. coli* producing ESBL were found in meats of broilers, turkeys and other livestocks (Dierikx et al., 2010; Egea et al., 2012; Horton et al., 2011).

In this study, almost all the isolates were about 50% resistant to commonly used beta-lactam antibiotics such as ceftazidime, cefuroxime, cefixime and cefotaxime (Figure 1). Similarly, high resistance to fluoroquinolones was also observed. However, a higher resistance of strains of *E. coli* from both samples was observed with amoxicillin, nitrofurantoin, and sulfamethoxazole-trimethoprim. Overall resistance to meropenem was less but slightly higher in isolates from poultry wastes. This clearly reflected a high level of antibiotic abuse in poultry than in cattle in Nigeria, since most of the commercial poultry productions in the region are often carried out in the more sanitized environment due

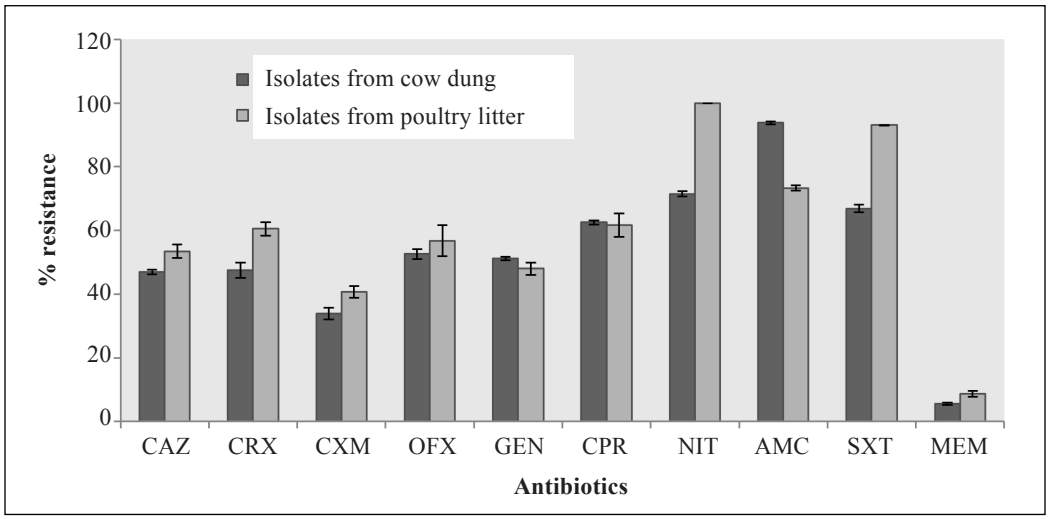

Figure 1. Antimicrobial resistance pattern of overall *E. coli* isolates from poultry litter and cow dung. Data are the means ± standard deviations of three independent replicates

to the fragile nature of birds, hence the birds received more attention and large volume of antibiotics than cattle which are often treated with antibiotics only when sick. The result obtained in this study is in consonant with previous reports where a high level of resistance in isolates from poultry litters was reported (Sayah et al., 2005; Smith et al., 2007). A remarkable observation is the high level of resistance to the β-lactam antibiotic, particularly the 3rd generation cephalosporins, ceftazidime, and cefotaxime even though they are rarely used in poultry and cattle treatment in the study area. Of high interest, is a low level of resistance to the meropenem, an antibiotic often reserved for treatment of life-threatening infections in human.

Screening the strains of *E. coli* isolates phenotypically for ESBLs showed that 10.5% and 27.27% of the isolates from cow dung and poultry litters respectively produces ESBL (Table 2). The prevalence of ESBL in this study, especially in poultry droppings is

Table 2
Prevalence of ESBLs, carbapenemase and MBL among E. coli from cow dung and poultry litter

Source	Nº of *E. coli* screened	Nº of ESBL producers	Nº of carbapenemase producers	Nº of MBL producers
Cow dung	76	8(10.5)	4(5.26)	6(7.89)
Cow rectal swab	12	2 (16.6)	2(16.6)	0(0.0)
Poultry litter	88	24(27.27)	9(10.2)	6(6.81)
Poultry cloacal swab	6	3(50.0)	2(33.3)	1(16.6)
Total	182	37 (20.3)	17 (9.34)	13 (7.14)

Note: Values in parenthesis are percentages

high when compared with the prevalence of ESBL in *E. coli* isolated from hospitalised and non-patients in neighbouring states in Nigeria as earlier reported (Aibinu et al., 2003; Iroha et al., 2009; Olowe et al., 2010; Yusuf et al., 2014). Further, carbapenemase production including MBL was also higher in *E. coli* strains from poultry samples (droppings=10.4%,cloacal swab=33.3%) when compared with isolates from cow (dungs=5.26%, rectal swab=16.6%). With no trace of carbapenem usage in Agriculture in Abraka or elsewhere in Nigeria, means that the observed resistance of the *E. coli* to carbapenem through production of carbapenemase and MBL, is not due to use of carbapenem usage in animal husbandry in the region. Molecular testing for the presence of resistance genes was not carried out for the isolates, and was therefore a limitation of the present study.

This observation has great public heath implication, since human and animals from some of the sampling area, especially in rural areas use the same source of water for drinking, washing, cooking and irrigation of crops. The fresh cattle faeces which contain MDR can contaminate water, fruits, and food and may became a source of environmental ESBL and carbapenemase-producing *E. coli* in the community. Isolates harbouring ESBL, carbapenemase, and other related genes can transfer the gene by the lateral and horizontal mechanism. This study can also provide a clue as to how patients with no previous treatment with carbapenem or travel to endemic area harbour isolates of *Klebsiella pneumoniae* and *E. coli* in some hospitals in another state in Nigeria as earlier reported.

CONCLUSION

The study showed that there were high sales of unregulated antibiotics and high prevalence of resistance of strains of *Escherichia coli* isolated from fresh and dry faecal samples of cattle and poultry in selected farms in Abraka, Delta state of Nigeria. The isolates produced ESBL, carbapenemase, and metallo beta-lactamase and were highly resistant to commonly used beta-lactam and non beta-lactam antibiotics used in the area. Poultry droppings harbour resistant *E. coli* more than cow dungs. The high level of free cattle range, lack of effective disposal of poultry wastes, indiscriminate use of antibiotics in rearing of both animals, poor personal hygiene of food handlers and lack of functional infection control in the area pose a high danger of transmission of these multi-drug resistant bacteria to human living in the surroundings and beyond.

CONFLICT OF INTEREST

The authors report no conflicts of interest.

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Colostrum and Milk Fatty Acids Profiles from Imported Prim'Holstein Cows

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ABSTRACT

Colostrum could provide significant fatty acids (FAs) essential for the development of living organisms. Yet to date, only a few works was performed to evaluate the composition of this important resource. In order to enrich data availability on the first milk, the objective of this work was to study bovine colostrum by comparing its composition in FAs with that of cow's mature milk. In this study, colostrum was sampled in warm season during the first day of postpartum from imported Prim'Holstein cows, and comparing it with mixed milk produced from a dairy-farm located in Tipaza region, northern Algeria. The proportion of saturated short-chain fatty acids was higher in mature milk than in colostrum ($P<0.05$). Medium and long chain saturated fatty acids present greater contents ($P<0.01$) in colostrum, with predominance ($P<0.01$) of myristic (C14:0) and palmitic (C16:0) acids. Among monounsaturated fatty acids, oleic acid (C18:1 n-9) revealed more elevated proportions ($P<0.05$) in milk. Polyunsaturated fatty acids were more marked ($P<0.01$) in colostrum, with high linoleic acid (C18:2 n-6) levels, while α-linolenic acid (C18:3 n-3) contents were weaker. The n-6/n-3 ratio as well as the atherogenicity index (AI) values confirmed that both were to the advantage of bovine colostrum. Even though the AI is significantly higher in colostrum, the value (1.89±0.01) obtained nevertheless remains lower than those previously reported.

Keywords: Bovine colostrum, fatty acids, lactation, mammary secretion, postpartum

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INTRODUCTION

The mammary secretion collected during the first 7 days of postpartum is commonly known as «colostrum». It has a reddish-yellow appearance, thick, viscous and is denser than milk. Colostrum is a part of the newborn calf's diet, as it contains immunoglobulins (IgG, IgA, IgM, IgE and IgD) and immune cells (leukocytes, epithelial cells) which, through their richness, provide protection against pathogens. Colostral volume of Holstein cows collected after calving is widely flexible, with reported values from 2.8 to 26.5 L (Morin et al., 2001). It is well established that colostrum fed to calves, has greatest importance on passive immunity, with 3-4 L volume required to deliver an adequate immunoglobulin mass (McGuirk & Collins, 2004). Nutrients (proteins, lipids, lactose, minerals), hormones, growth factors, enzymes such as lactoperoxidase and alkaline phosphatase are also found in abundance for calf development (McGrath et al., 2016; Penchev, 2008).

In these last few years, many industries have introduced colostrum-based food supplements, drinks and chewing gums to the market. Bovine colostrum based food is also used in gastrointestinal treatments and to improve immune system defense. Indeed, it contains components which are important for human organism with multiple applications in preventive purposes but also to reduce side effects during antibiotic therapy or chemotherapy (Borad & Singh, 2018; Dzik et al., 2017). In addition, bovine colostrum can provide an important source of fatty acids (FAs) in the human diet (Dzik et al., 2017; Yurchenko et al., 2016). However, it would be interesting to know the proportion of FAs that is beneficial for human healthcare and that would be included in its composition. Among constituents of udder secretions, fat is made up of more than 98% of triglycerides, source of more than 400 FAs, some of which would have a protective effect against cardiovascular diseases and various types of cancers (Lindmark-Månsson, 2008).

The FAs of mammary secretions take their origin either from the diet or from *de novo* synthesis in the gland. They can be released from body fat deposits. Several factors would influence the variation in the lipid fraction. We can quote the season, breed, dietary intake, health status, parity and lactation stage (Palmquist, 2006).

Few experiments have already been initiated in order to study the FAs fractions of bovine milk or other animal species such as camel (Meribai et al., 2018). However, despite the growing importance of bovine colostrum for human nutrition (Borad & Singh, 2018; Dzik et al., 2017), very scarcer data on its FAs composition are available (Parodi, 1983; Yurchenko et al., 2016).

In this context, this study contributes to the comparison by gas chromatography coupled with mass spectrometry (GC-MS) of the FAs fractions of colostrum and mature milk from Prim'Holstein cows imported into Algeria mainly from Netherlands. The results achieved enrich existing information on nutritional aspects and also characterize postpartum mammary secretion under real dairy-farming conditions.

MATERIALS AND METHODS

Study Location

The experiment was carried out on a private dairy farm located near the Ben Nassah region (36°36'25.2"N latitude and 2°42'38.8"E longitude), Attatba's municipality (Tipaza province), from central Northern Algeria. The region is characterized by a hot-dry climate in the period from June to August, with temperatures ranging from 31°C to 34°C.

Dairy cattle size in course of lactation was estimated at 133 Prim'Holstein cows, 49 among them in 4th lactation number. Animals from this farm, which is conducted in semi-intensive farming, had previously been imported, mainly from Europe.

Animal Management

The experiment was conducted under the real breeding conditions practiced by the farmer. Animals were selected on the basis of the same genetic type (Prim'Holstein breed), parity, a healthy status against mastitis after clinical exploration or by using the California Mastitis Test (CMT). Total mixed ration (TMR) consisting of alfalfa hay delivered *ad libitum*, and concentrate feed are as detailed in Table 1.

Table 1
Concentrate feed formulation

Concentrate composition	Percentage (%)
Corn grains	40
Wheat bran	30
Barley grains	28
Mineral-vitamin supplement	2
Total	100

Mature milk (n=12) is a mixture from the evening milking. It is intended for the manufacture of butter and fresh cream. On the other side, the mammary secretion collected during the first 24 h of postpartum is the colostrum (n=12) studied. Sampling was performed over the same period in warm season (from June to August); each batch was collected in sterile, sealed and labelled containers. These latter were stored at 4°C during transportation for the laboratory analysis.

Fatty Acids Composition of Colostrum and Mature Milk

Lipid from mammary secretions was extracted according to Hara and Radin (1978) procedure modified by Feng et al. (2004), then converted into fatty acid methyl esters (FAMEs) using a solution of boron trifluoride (BF_3) in methanol (14% w/v) in accordance with Morrison and Smith's (1964) method. The FAMEs were analyzed by an Agilent GC 6890 gas chromatograph coupled to a MSD 5973A mass spectrometer (Agilent Co. Ltd, USA), using a polyethylene glycol (PEG) fused silica capillary column (HP-Wax, 60 m x 0.25 mm, 0.25 µm film thickness, Agilent Co. Ltd, USA).

GC-MS spectra were obtained using the following conditions: carrier gas helium; flow rate 0.5 mL/min; 1 µl injection volume in 1:20 split mode. The injection temperature was set at 250°C. The initial oven temperature was 40°C for 4 mins, increased to 140°C at 10°C/min (held for 1 min) and then increased by 5°C/min to

a final temperature of 220°C (held for 40 mins). The electron impact mass spectral analysis was carried out at 70 eV ionization energy. Identification of common FAs was performed by comparing the retention time with those of standard compounds (Supelco® 37- Component FAME Mix certified reference material) and NIST'02 [US National Institute of Standards and Technology (NIST), Gaithersburg, MD, USA] mass spectral database.

Atherogenicity Index (AI)

The assessment of the risk to human health resulting from the FAs composition of mammary secretions (colostrum and milk) was investigated using the atherogenicity index (AI) according to the following formula proposed by Ulbricht and Southgate (1991):

$$AI = \frac{(aS12) + (bS14) + (cS16)}{(dP) + (eM) + (fM')}$$

where: S12 = C12:0, S14 = C14:0 and S16 = C16:0; P = sum of n-3 and n–6 PUFAs; M = oleic acid and M' = sum of other MUFAs. a-f are empirical constants: b = 4 and a, c, d, e, f = 1.

Therefore, the final calculation of the AI becomes:

$$AI = \frac{(C12:0) + (4 \times C14:0) + (C16:0)}{(C18:2 + C18:3) + (C18:1) + (C14:1 + C16:1 + C17:1)}$$

Statistical Analysis

Data were compiled using Microsoft Excel 2007 software. FAs profiles of colostrum and milk were statistically processed by Statistica® software version 6.1 (Statsoft, France) using the Student's t-test. Results are expressed as mean ± standard deviation, and considered significant at $p<0.05$.

RESULTS AND DISCUSSION

Changes in FAs composition of colostrum and milk samples are reported in Table 2 and also in Figures 1 and 2.

Saturated fatty acids (SFAs), which are predominant among FAs mammary secretion, are widely known to be harmful in excessive intake. However, they should no longer be considered in their whole but rather on an individual form because not all of them would have the same deleterious effects, some may, exert a hypocholesterolemic action (Ulbricht & Southgate, 1991).

Statistical analysis revealed no changes in SFAs contents, with higher values in colostrum than in mature milk. The proportion of short-chain saturated fatty acids (SCSFAs) in milk (6.21±0.13%) was greater ($P<0.05$) than in colostrum (4.32±0.25%). These results are consistent with those obtained from other studies (Bitman & Wood, 1990; Contarini et al., 2014). They could be explained by the weakness *de novo* synthesis ability in mammary gland during the first hours of postpartum, a period in which animals are in a negative energy balance situation with limited availability of volatile fatty acids

precursors (acetates and β-hydroxybutyrates) that are derived from ruminal fermentation. This situation would lead to a weakening in the ability to synthesize SCSFAs in udder (Palmquist et al., 1993). In addition, SCSFAs (C6:0, C8:0, C10:0, C10:0), mainly arising from *de novo* synthesis in mammary gland, displayed higher values in mature milk with respective levels of +0.38%, +0.51%, +0.88%. According to Ebringer et al. (2008), these FAs appear to be very relevant to be regarded, by the significant

Table 2
Fatty acids composition of bovine mammary secretions (%)

FAMEs		Colostrum	Milk	*P* value
Commun name	Formula			
Butyric	C4:0	0.39±0.06	0.51±0.00	0.114
Caproic	C6:0	1.02±0.07	1.40±0.04	0.023
Caprylic	C8:0	0.91±0.04	1.42±0.03	0.005
Capric	C10:0	2.00±0.07	2.88±0.07	0.006
Lauric	C12:0	3.34±0.08	3.40±0.00	0.377
Myristic	C14:0	10.96±0.14	9.06±0.12	0.005
Pentadecanoic	C15:0	1.50±0.00	1.75±0.00	<0.001
Palmitic	C16:0	25.10±0.30	18.77±0.07	0.001
Margaric	C17:0	1.13±0.00	1.12±0.06	0.796
Stearic	C18:0	14.93±0.20	18.96±0.75	0.018
Arachidic	C20:0	0.50±0.12	0.40±0.02	0.372
SFAs		61.78±0.08	59.67±0.89	0.079
SCSFAs[1]		4.32±0.25	6.21±0.13	0.011
MCSFAs[2]		15.81±0.22	14.21±0.12	0.012
LCSFAs[3]		41.65±0.38	39.24±0.64	0.045
Myristoleic	C14:1	0.79±0.02	1.34±0.02	0.001
Palmitoleic	C16:1	2.58±0.03	1.94±0.00	0.001
Heptadecenoic	C17:1	0.51±0.02	0.55±0.03	0.207
Oleic	C18:1 n-9	27.99±0.04	30.97±0.90	0.042
UFAs		38.22±0.08	40.33±0.89	0.079
MUFAs		31.85±0.07	34.80±0.84	0.039
Linoleic	C18:2 n-6	5.94±0.04	4.93±0.05	0.002
α-Linolenic	C18:3 n-3	0.43±0.03	0.59±0.00	0.015
PUFAs		6.36±0.01	5.53±0.05	0.002
n-6/n-3		13.99±1.05	8.31±0.09	0.017
AI		1.89±0.01	1.45±0.04	0.005

Saturated fatty acids (SFAs), unsaturated fatty acids (UFAs), monounsaturated fatty acids (MUFAs), polyunsaturated fatty acids (PUFAs)
[1] Short-chain saturated fatty acids C_4-C_{10}
[2] Medium-chain saturated fatty acids C_{12}-C_{15}
[3] Long-chain saturated fatty acids C_{16}-C_{20}
Atherogenicity index (AI)

part they would play in adiposity, as well as by their hypocholesterolemic action and multiple antiviral activities. However, butyric acid (C4:0), whose presence is specific to the cow's udder secretions (Ebringer et al., 2008), exhibited a constant level. Percentages recorded were close to those obtained from other previous experiments conducted in Algeria involving Prim'Holstein cows' imported from Europe (Meklati et al., 2017). Nevertheless, other researches (Nardone et al., 1997; Parodi, 1983), reported markedly higher percentages for this FA (8.9-9.5%) during the colostrum secretion period. Finally, *in vitro* studies demonstrated that butyric acid would be beneficial to human health by being useful in the prevention and treatment of ulcerative colitis, Crohn's disease and colon cancer (Di Sabatino et al., 2005; Pouillart, 1998).

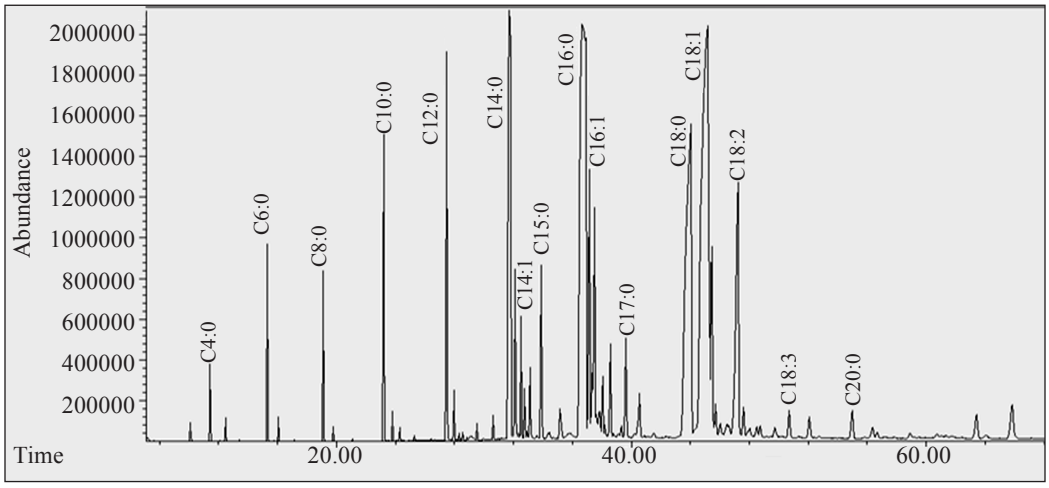

Figure 1. Example of a chromatogram (GC-MS) for a bovine colostrum sample

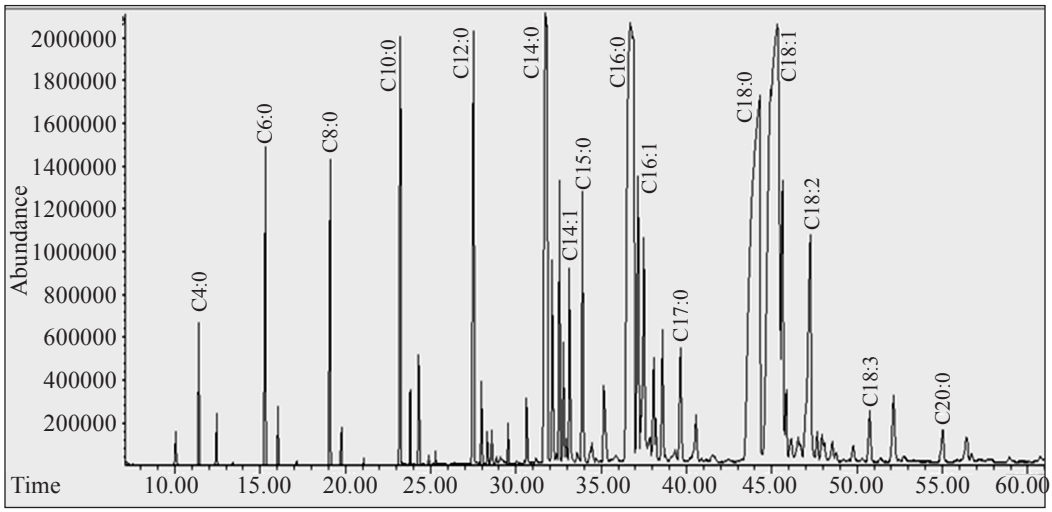

Figure 2. Example of a chromatogram (GC-MS) for a mature milk sample

Medium-chain saturated fatty acids (MCSFAs), displayed, for their part, values inversely proportional to SCSFAs with higher ($P<0.05$) levels in colostrum. Nevertheless, these results disagree with those of Contarini et al. (2014), in which no significant variation was reported between colostrum collected at 24 h and mature milk. In addition, myristic acid (C14:0), the most abundant FA among the MCSFAs of mammary secretions, revealed a greater content in colostrum. However, these values (10.96±0.14%) remain lesser than those recorded in colostrum (13.1%) by Nardone et al. (1997) on animals exposed to high air temperatures, thus similar to our experimental conditions in warm season, during the first 24 h of the postpartum period. The contribution of myristic acid to human nutrition is still a debate topic until now. Indeed, Zock et al. (1994) indicated that it would increase total plasma cholesterol and LDL cholesterol levels, whereas Rioux et al. (2002) had suggested that myristic acid played a major functional part in the cell, being involved in protein acylation processes. In colostrum, C12:0 and C15:0 FAs values (3.34±0.08%, 1.50±0.00% respectively) were similar to those obtained by Nardone et al. (1997). According to Craninx et al. (2008), C15:0 and C17:0 could be partially *de novo* synthesized in mammary gland from propionate.

Long-chain saturated fatty acids (LCSFAs) showed higher contents ($P<0.05$) in colostrum. These findings are in agreement with those of Contarini et al. (2014). This abundance in LCSFAs could be explained by the richness of colostrum in palmitic acid (C16:0). In addition, the analysis of FAMEs revealed an influence of the type of udder secretions on palmitic acid (C16:0) variation levels, with values in the order of 25.10±0.30% in colostrum. Several studies (Mi et al., 2016; Nardone et al., 1997; Parodi, 1983) have reported marked proportions of this FA during the first 24 h of postpartum in bovidae (within the range 28.7-30.9%).

In colostrum, myristic (C14:0) and palmitic (C16:0) acids appear to offset the reduced levels of *de novo* SCSFAs synthesized in the mammary gland. *De novo* synthesis is the source of more than half of C16, while the remaining palmitic acid is derived from blood circulatory or body lipids, which may explain the high contents of C16:0 recorded during the first 24 h postpartum (Bitman & Wood, 1990; Craninx et al., 2008). However, as reported by Williams (2000), this FA has hypercholesterolemic properties, raising both total and LDL-cholesterol levels. The LCSFAs of type C17:0 and C20:0, which are part of udder secretion, did not display any significant variation. Several studies highlighted that these FAs, considered as minority, varied slightly during the different lactation stages (Garnsworthy et al., 2006; Stoop et al., 2009). Stearic acid (C18:0) presented an opposite tendency to C14:0 and C16:0, with elevated proportions in mature milk (18.96±0.75%). These results are in accordance with literature, on which a lower C18:0 content on the first day (D1) of colostrum secretion was recorded (Leiber

et al., 2011; Mi et al., 2016). Nevertheless, these findings are contrary to those obtained from other sources (Bitman & Wood, 1990; Kay et al., 2005). Garnsworthy et al. (2006) revealed that there was no noticeable variation in the percentage of C18:0 from triggering to late lactation. The impact of stearic acid on human health is still controversial: According to Forouhi et al. (2014), the presence of this FA may be related to an increasing incidence of type 2 diabetes, while Hunter et al. (2010) had highlighted the protective effect of C18:0 against cardiovascular diseases with a favourable influence on plasma LDL-cholesterol concentrations.

The level of UFAs was not influenced by the type of udder secretion. However, it remains predominant in milk, sustained by high concentrations of MUFAs (C14:1, C17:1, C18:1), except C16:1. The latter displayed more marked values in colostrum, which could be explained by the action of the desaturase enzyme, responsible for the conversion of palmitic acid (C16:0) to palmitoleic acid (C16:1). Indeed, during the colostrum secretion period, the high concentrations of C16:1 would eventually come from C16:0 (Parodi, 1983). In addition, C16:1 proportions during the experiment are in line with those of Nardone et al. (1997). On the contrast, oleic acid contents (C18:1) are greater in mature milk. This result confirms those already obtained by Leiber et al. (2011), while beyond the first day of mammary secretion, several references have reported more elevated C18:1 values in colostrum (Bitman & Wood, 1990; Kay et al., 2005). These authors suggested that this FA derived from plasma circulatory and body fats, with strongly higher concentrations when animals at early lactation were in a negative energy balance. In addition, long-chain FAs exert an inhibition on *de novo* synthesis of short- and medium-chain FAs (Craninx et al., 2008).

Oleic acid is reputed to play a positive contribution to human healthcare (Ebringer et al., 2008). It reduces LDL cholesterol and plasma triglycerides, as well as raises plasma HDL cholesterol contents. Therefore, this FA could be of interest in reducing the cardiovascular risk factors and coronary heart disease in healthy subjects or those with moderate hypercholesterolemia (Lopez-Huertas, 2010).

Polyunsaturated fatty acids (PUFAs) include omega 3 (n-3) and omega 6 (n-6) families, each one of them possessing unique biochemical properties and divergent cardiovascular effects (Ramsden et al., 2013). The PUFAs displayed greater proportions ($P<0.01$) in colostrum, supported by high levels of linoleic acid (C18:2 n-6). Similar observations were reported by Meklati et al. (2017) and Meribai et al. (2018) on milk from cows farmed in Algeria with elevated amounts of this FA, within range from 3.43±0.02 to 5.50±0.02%. The proportions reported by Leiber et al. (2011) are in the same order, with maximum values recorded on the first day of udder secretion, then gradually declining even beyond the 10th day of lactation. Contarini et al. (2014)

revealed that the amount of this FA in the first 24 h of colostrum secretion did not differ from that of milk at 5 months. Values of α-linolenic acid (C18:3 n-3) recorded in colostrum are smaller, in contrast to those reported in literature (Contarini et al., 2014; Leiber et al., 2011). Increasing PUFAs is usually a desirable outcome to improve the dietary profile of milk. Indeed, this FA seems to provide some significant benefits, particularly in the prevention of cardiovascular disease, or being also essential to the brain development, retina, and immune system (Barceló-Coblijn & Murphy, 2009; De Caterina, 2011).

The n-6/n-3 ratio indicated that it is significantly higher in colostrum (14.0±1.0) than in mature milk (8.3±0.1). According to Yang et al. (2017), the n-6/n-3 ratio measured on cow's milk showed average values (10.3±0.8), close to those recorded in this study. The n-6/n-3 ratio for cow's milk was markedly higher than that of other animal species such as yak or camel (Yang et al., 2017). However, a targeted ratio of linoleic acid (n-6) to α-linolenic acid (n-3) would most likely be within a 2:1 ratio range (Connor, 2000), whereas it is only within an 8:1 ratio in our mature milk experiments. On the other side, the results recorded in mammary secretions are in contrast to those mentioned by Leiber et al. (2011). This difference could be explained by the nature of the diet studied by these authors, with a ration enhanced with oilseeds that increased the proportion of α-linolenic acid in milk, thus bringing the n-6/n-3 ratio to desirable levels. In addition, the n-6/n-3 ratio of milk varied significantly depending on the type of diet distributed, and the lactation stage with values in the range of 3.35-4.27 (Nantapo et al., 2014).

Atherogenicity index (AI) is used to assess the nutritional quality of milk by being strongly correlated with the occurrence of cardiovascular disease. The high value of the index increases the risk of developing coronary heart disease (Ulbricht & Southgate, 1991). The AI measured in colostrum was more elevated than in milk. This is in compliance with the large proportions of SFAs, and more specifically for myristic (C14:0) and palmitic (C16:0) acids. A typical value of AI for milk and dairy products was evaluated around 2 (Ulbricht & Southgate, 1991). Moreover, the scores (1.45-1.89) obtained in the present study are favourably inferior to those reported in the milk of Friesian and Jersey cows at different lactation stages by Nantapo et al. (2014) with values in the range of 4.08- 5.13. Breed and lactation stage would be among the factors that could explain the changes in the AI value of milk. As reported by Kuczyńska et al. (2012), the AI value (2.08) of Holstein cow's milk was higher than that of this study. In addition, according to Bobe et al. (2003), cows with a low milk AI index (1.51) had lower C14:0 and C16:0 proportions than those with a high milk AI (2.83). The AI variation of mammary secretions would therefore be closely correlated with *de novo* synthesis capacities in udder.

CONCLUSION

Colostrum released within 24 h of postpartum has a distinct fatty acids composition, very different from that of mature cow's milk. The AI and n-6/n-3 ratio reported higher values in colostrum. The latter has displayed a specific abundance of polyunsaturated fatty acids, especially linoleic acid (C18:2 n-6), which could enhance growing of the interest in colostrum as a promoting product with highly beneficial health properties. These data may be relevant to take into account in the formulation of colostrum, supplements and alternative products for human food intake. Further investigations will be required to assess the variation of the FAs composition during the remaining days of the colostral period.

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Partial Purification and Model Structure of BPSL2774, a Hypothetical Protein from *Burkholderia pseudomallei* Predicted to be a Glycosyltransferase

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ABSTRACT

Melioidosis is a disease that infects humans and animals, and can be detrimental in humans. Mortality rate from melioidosis septic shock due to infection from Gram negative *Burkholderia pseudomallei* (*B. pseudomallei*) in endemic regions of Malaysia and Thailand remains high despite available antimicrobial therapy. Multiple strategies are employed to identify essential genes and drug targets in this bacterium to improve current antimicrobial therapies. This is important as *B. pseudomallei* is intrinsically resistant to many commonly used antibiotics. In this study, hypothetical genes predicted to be essential for *B. pseudomallei* by transposon-directed insertion site sequencing (TraDIS) technique were selected. One target gene, *BPSL2774,* has been successfully amplified and cloned from genomic DNA of *B. pseudomallei* strain K96243. Glutathione S-transferase (GST) affinity tag chromatography was performed for partial protein purification. The target protein was successfully expressed in soluble form with satisfactory yield output. Mass spectrometry analysis of 60 kDa Coomassie-stained gel band confirmed the presence of the soluble expressed tagged-target protein, co-purified with *Escherichia coli* chaperonin proteins, possibly due to their interaction with the target protein. BPSL2774 protein have considerable homology to glycosyltransferase GTB type superfamily and RfaB superfamily. On the basis of this similarity, the three-

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dimensional structure of BPSL2774 has been modelled and assessed by protein model quality servers. Taking all the results into account, the functional annotation of BPSL2774 protein as a glycosyltransferase is recommended, though future validation from biochemical experiments will be needed to support this.

Keywords: *Burkholderia pseudomallei*, BPSL2774 hypothetical protein, glycosyltransferase

INTRODUCTION

Melioidosis is an infectious disease spread by *Burkholderia pseudomallei* (*B. pseudomallei*), a Gram negative bacterium which resides in contaminated water and soil. Direct contact with the contaminated source either through exposed skin abrasions, inhalation, or ingestion can spread the disease to human and animals. Soil, stagnant water and rice fields are the natural habitat for this bacterium and can be found in endemic regions including Southeast Asia and northern Australia (Chewapreecha et al., 2017; Limmathurotsakul et al., 2016).

Acute cases of melioidosis typically present within 1-21 days after infection, while chronic cases can persist for months (Wiersinga et al., 2006). Death can occur within the first 48 hours due to septic shock, even with optimal antimicrobial chemotherapy given (Holden et al., 2004). Examples of common clinical manifestations include localized abscess formation, metastatic pneumonia, hepatic and splenic abscesses, displaying evidence of bacterial dissemination to distant sites. Melioidosis often affects individuals with underlying medical conditions associated with an altered immune response. The major underlying risk factor for melioidosis are diabetes mellitus, followed by chronic renal disease (Nathan et al., 2018; Wiersinga et al., 2006).

In the past two decades, melioidosis was categorized as an important human infection in Malaysia, Singapore and across the north of Australia (Nathan et al., 2018; Schweizer, 2012; Sim et al., 2018). There are increasing awareness of this disease from other melioidosis-endemic tropical countries i.e. Indonesia (Tauran et al., 2018), Brunei (Pande et al., 2018), Sri Lanka (Corea et al., 2018), Laos (Dance et al., 2018) and Cambodia (Turner et al., 2016). In Malaysia, incidence of melioidosis varies between state, with recorded cases of melioidosis in Kedah, Kelantan, Pahang, Johor, Sabah and Sarawak (reviewed by Nathan et al., 2018). Case fatality varied between 33-54% in four Malaysian case series that included all cases irrespective of bacteraemic status. Cases of bacteraemic melioidosis have higher mortality rates, with up to 63% mortality recorded in Kelantan (Deris et al., 2010).

Burkholderia pseudomallei is naturally resistant to many commonly used antibiotics (Holden et al., 2004; Wiersinga et al., 2006). The intrinsic antibiotic resistance is due to the bacterium's physicochemical properties that exclude entry of drug molecules using its lipopolysaccharide component of the cell membrane, enzymatic inactivation, target mutation or efflux from the cell (Rhodes & Schweizer, 2016; Schweizer,

2012). Putative resistance mechanisms for this bacterium that have been reported include the action of seven Ambler class A, B and D β-lactamases, ten multidrug efflux systems and a putative aminoglycoside acetyl transferase (Holden et al., 2004). It secretes lecithinase, lipase, hemolysin and siderophore for its survival and maintenance (Stevens et al., 2002).

The genome of *B. pseudomallei* (strain K96243, a clinical isolate from Thailand was first to be fully sequenced) is known to be one of the largest and most complex genome (Holden et al., 2004). It comprised two chromosomes of 4.07 and 3.17 megabase pairs, respectively. The large chromosome is important for metabolism and growth, whereas the small chromosome encodes accessory functions associated with adaptation and survival (Holden et al., 2004).

Current research efforts include a focus on prevention of disease and finding ways to reduce mortality and the rate of relapse. A potential vaccination strategy has also been considered using the closely related avirulent *Burkholderia thailandensis* and other attenuated strains. However, this approach was not pursued due to the extensive exposure of both *B. thailandensis* and *B. pseudomallei* to the patients (Cheng & Currie, 2005). A current review on potential melioidosis vaccine candidates indicated that the vaccination strategy required more extensive development and evaluation to protect against multiple routes of disease acquisition, as well to consider risk factors for infection e.g. diabetes (Peacock et al., 2012).

The development of new antimicrobial therapies is emerging, with researchers utilizing different tools to identify essential genes and drug targets to combat melioidosis due to its persistence. Hence, identification of *B. pseudomallei* essential genes and its products may represent excellent targets for development of novel antimicrobial drugs. As an example, one study by Moule et al. (2016) on a transcription accessory protein in *B. pseudomallei*; Tex, had shown that deletion of the particular protein produced a highly attenuated *B. pseudomallei tex* mutant phenotypes. This indicates that identifying essential genes and their subsequent characterization can provide fundamental information on the bacterium survival strategy or pathogenicity.

Transposon library sequencing techniques known as transposon-directed insertion site sequencing (TraDIS) and transposon sequencing (Tn-seq) have been recently used to screen *B. pseudomallei* K96243 bacterial libraries and identify essential genes within the genome (Moule et al., 2014). In this study, hypothetical genes predicted to be essential for *B. pseudomallei* from the TraDIS technique were selected for protein expression and purification. Previously, one target gene; *BPSL2774*, had been successfully amplified and cloned from genomic DNA of *B. pseudomallei* strain K96243 (Drahaman et al., 2016). BPSL2774 protein was expressed in bacterial cells and the soluble phase was utilized for protein purification. Affinity binding tests were performed to confirm expression and solubility. The three-dimensional structure

of BPSL2774 protein was modelled and its active sites predicted to aid in future functional experiments to validate its function as a glycosyltransferase.

MATERIALS AND METHODS

Bacterial Cell Culture and Lysis

The cells were cultured using the auto-induction method described by Studier in his 2005 report (Studier, 2005). The gene of interest coding for the target protein had been obtained from genomic DNA of *B. pseudomallei* K96243, cloned into Gateway™ pDEST15 (GST-tagged *BPSL2774*) and transformed into *Escherichia coli* DH5α maintenance strain, which had been kept in glycerol stock at -80°C. Prior to protein expression and purification, the cloned gene was transformed into expression strain, *E. coli* BL21(DE3) competent cell (Life Technologies). The colony was inoculated in a minimal non-inducing medium MDG (25 mM Na_2HPO_4, 25 mM KH_2PO_4, 50 mM NH_4Cl, 5 mMNa_2SO_4, 2 mM $MgSO_4$, 0.1 × trace metals, 0.5% glucose, 0.25% aspartate) added with 100 µg/mL ampicillin before being incubated in 37°C at 200 rpm (approximately 24 *g*), overnight. MDG medium was further inoculated in complex auto-induction medium ZYM-5052 (1% N-Z-amine AS, 0.5% yeast extract, 25 mM Na_2HPO_4, 25 mM KH_2PO_4, 50 mM NH_4Cl, 5 mM Na_2SO_4, 2 mM $MgSO_4$, 1 × trace metals, 0.5% glycerol, 0.05% glucose, 0.2% α-lactose) added with 100 µg/mL ampicillin and incubated in 37°C at 200 rpm (approximately 24 × *g*) for 4 hours, followed by at 18°C at 200 rpm, overnight. Then, the cell was harvested by centrifugation at 900 × *g* for 30 minutes at 4°C. The pellet was re-suspended in cold lysis buffer (50 mM Tris-HCl pH8, 100 mM NaCl, 1 mM EDTA). The suspension was sonicated (Omni Sonic Ruptor 400) with sonication condition of 6 × 30 Seconds / 60 Seconds off, Amplitude 40%. After that, the lysed cell was centrifuged at 4500 × *g* for 60 minutes at 4°C.

Protein Purification

The soluble supernatant fraction was purified using GST Fusion Protein Purification Kit (Genscript). The supernatant was filtered through a 0.25 µm filter fitted to a syringe. 500 µL of settled glutathione resin (1 mL 50% resin slurry) was equilibrated four times with Buffer A (50 mM Tris-HCl pH8, 100 mM NaCl, 1 mM EDTA). The resin was then transferred to a new tube and mixed with the filtered supernatant. The supernatant-resin mixture was incubated for 30 minutes on ice. After 30 minutes, the mixture was transferred into a mini spin column 500 µL at a time and centrifuged at 500 × *g* for 5 seconds. All the flow-through was collected in a sterile tube. This step was repeated until all the supernatant-resin mixture was centrifuged, leaving behind the resin suspension on the spin column. The column containing the resin was then washed 20 times with 400 µL of Buffer A at a time by centrifugation at 500 × *g* for 5 seconds, with each elution fractionated at 800 µL in volume. Finally, the column was eluted 20 times with 400 µL of Buffer B

(50 mM Tris-HCL pH 8.08, 100 mM NaCl, 1 mM EDTA, 2 mM βME, 10 mM reduced glutathione) by centrifugation at 500 × *g* for 5 seconds (each elution fractionated at 800 µL in volume). All flow-through fractions were collected and stored in -20°C for SDS-PAGE analysis. Protein concentrations in each elution fractions were determined using Nanodrop 2000c (Thermo Fisher Scientific).

Bioinformatics – Homology Identification and Domain Analysis

BPSL2774 protein sequence was retrieved from UniProtKB server (UniProtKB ID Q63R99) (Apweiler et al., 2004). The searches for related protein sequences were conducted using the National Center for Biotechnology (NCBI) with Basic Local Alignment Search Tool (BLAST) against Protein Data Bank (PDB) to find regions of sequence similarity for the functional and evolutionary descriptions (Altschul et al., 1990). Subcellular localisation of BPSL2774 was determined using WoLF PSORT (Horton et al., 2007), Yloc (Briesemeister et al., 2010) and TargetP (Emanuelsson et al., 2000) prediction tools. A trans-membrane helixes Hidden Markov Model (HMM)-based prediction tool, TMHMM (Krogh et al., 2001), was used to predict the presence or absence of trans-membrane domains in BPSL2774 protein. VICMpred tool (Saha & Raghava, 2006) and MP3 tool (Gupta et al., 2014) were used to predict the virulence and pathogenicity of BPSL2774 protein.

Structure Prediction, Model Quality Assessment and Active Site Determination

Secondary structure and the three-dimensional structure of the protein were predicted by using I-TASSER (Roy et al., 2010; Yang et al., 2015) and SWISS-MODEL Workspace softwares (Waterhouse et al., 2018). The quality of the predicted structure was determined by the C-score calculation in I-TASSER server and QMEAN Z-score calculation in the QMEAN server (Benkert et al., 2010) available at SWISSMODEL Workspace, as well as verify3D of the UCLA-DOE Structure Evaluation server (Eisenberg et al., 1997). Model structure refinement was performed using ModRefiner algorithm tool (Xu & Zhang, 2011) from I-TASSER database. Ramachandran Plot assessment of the protein 3D model was performed using RAMPAGE server (Lovell et al., 2003). Active site and ligand binding site of BPSL2774 protein was predicted by using COFACTOR and COACH server based on the I-TASSER structure prediction from the I-TASSER website. Besides, metaPocket 2.0 server (Zhang et al., 2011) was used to determine the active site of BPSL2774 protein.

RESULTS AND DISCUSSION

Protein Purification and Expression

The transformed GST-tagged BPSL2774 construct was expressed from *E. coli* BL21(DE3) expression strain using auto-induction method for protein purification and expression screening. After lysis of

the sedimented cell pellet, the supernatant containing GST-tagged BPSL2774 protein was purified using GST fusion protein purification kit (Genscript) to isolate the GST-tagged protein. In this study, two cultures of 500 mL and 1 L respectively, of *E. coli* BL21(DE3) expression strain were induced in the auto-induction phase for expression of the target GST-tagged BPSL2774 protein and purified using GST-tagged affinity chromatography. All flow-through fractions, pellet, and resin from the purification step were retained for analysis with SDS-PAGE (Figures 1 and 2).

The combined molecular weight of the target protein (35.1 kDa) and the GST tag (25.5 kDa) is 60.6 kDa. Both SDS-PAGE gels showed the presence of intense band between 60 kDa and 80 kDa marker for Elution 1 and Elution 2 from the 500 mL culture preparation and for Elution 1 and Elution 5 from the 1 L culture preparation.

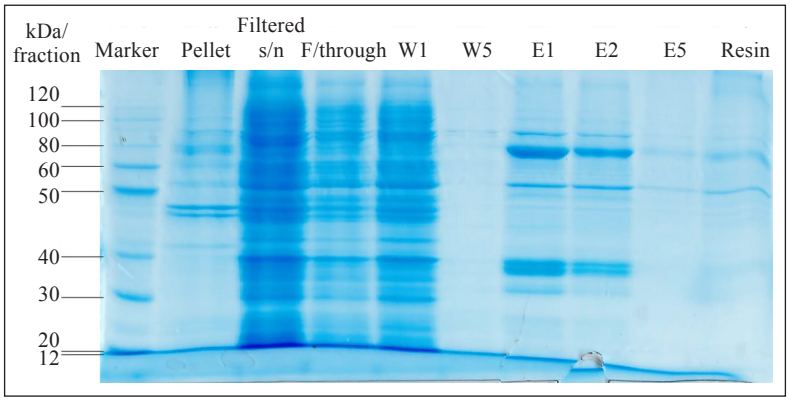

Figure 1. SDS-PAGE for fractions from 500 mL culture of *E. coli* BL21(DE3) expression strain. Marker: ProteinRuler® II (12-120 kDa) (Transgen Biotech Co., Ltd.), Pellet: sedimented lysed cells, Filtered s/n: filtered supernatant, F/through: flow-through, W1: first wash fraction, W5: final wash fraction, E1: first elution fraction, E2: elution fraction 2, E5: final elution fraction, Resin: resin sample

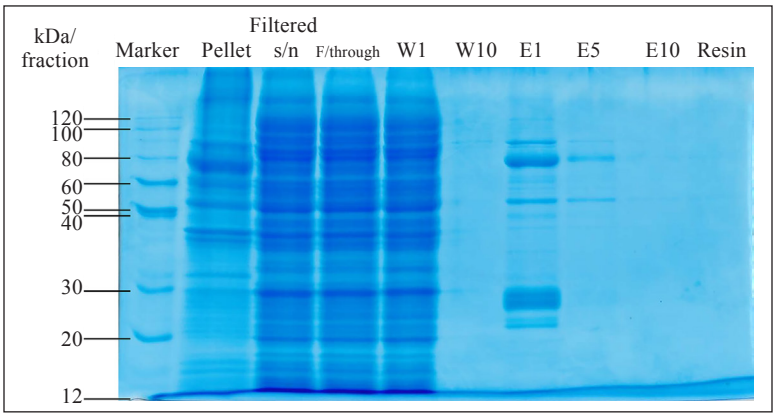

Figure 2. SDS-PAGE for fractions from 1 L culture of *E. coli* BL21(DE3) expression strain. Marker: ProteinRuler® II (12-120 kDa) (Transgen Biotech Co., Ltd.), Pellet: sedimented lysed cells, Filtered s/n: filtered supernatant, F/through: flow-through, W1: first wash fraction, W10: final wash fraction, E1: first elution fraction, E5: elution fraction 5, E10: final elution fraction, Resin: resin sample

This indicated a promising possibility that there was a relatively significant soluble overexpression of GST-tagged BPSL2774 protein. This band was then cut from the SDS-PAGE gel and sent for mass spectrometry analysis for confirmation. This band was also observed in the insoluble pellet fraction, markedly for the 1 L culture preparation (Figure 2). This may indicate that at a larger scale protein preparation, there is a tendency for the recombinant GST-tagged BPSL2774 to become insoluble or highly aggregated (otherwise known as inclusion bodies) in *E. coli* host. Future attempts to purify this insoluble fraction of the expressed protein can be made in the denatured form using detergent and refolding method (Yang et al., 2011).

Other protein bands were also observed in the SDS-PAGE gels. Two neighbouring bands at 50 kDa and 80 kDa, as well as two other distinct bands near the 30 kDa mark were observed. These contaminating bands can be deduced to have been eluted together with the target protein as they were not observed in the wash fractions wells. We suspect that these may be contaminating proteins from the host cells, *E. coli* BL21(DE3) that was co-purified with the target protein due to their interaction with BPSL2774. In the future, purification process needs to be followed with several more purification steps e.g. ion exchange chromatography followed by gel filtration to aid in the removal of these contaminants.

For both cultures, high concentrations of the target protein were obtained. The highest protein concentration was for the first 800 µL elution fraction (E1) at 1382 µg/mL from the 500 mL culture, and from the 1 L culture the highest concentration was obtained for the first 800 µL elution fraction (E1) at 910 µg/mL.

Mass Spectrometry of Purified Samples from SDS-Page Gel

The Coomassie-stained protein gel band near the 60 kDa mark was cut and sent to First BASE Laboratories Sdn. Bhd. for further analysis using mass spectrometry.

Mass spectrometry analysis of the purified samples in SDS-PAGE confirmed the presence of the target protein, BPSL2774 as the third hit with a score of 390 and 27% protein sequence coverage (Figures 3 and 4 respectively). By performing BLASTp search to non-redundant database using the mass spectrometry result, it showed 100% sequence identity to BPSL2774 hypothetical protein from *B. pseudomallei* K96243 (NCBI Reference Sequence: WP 004550046.1) (Figure not shown).

The two upper hits found to be present at 60 kDa were *E. coli* chaperonin GroL protein and *E. coli* chaperonin GroEL protein, with less protein sequence coverage, at 14% and 9% respectively. In protein synthesis, molecular chaperones are commonly present to interact with new proteins as they form their final structure (Rosano & Ceccarelli, 2014). As the target protein was purified from *E. coli* expression system, it could be expected that some protein chaperones from the host were co-purified together with the target protein. In order to obtain the target protein in high purity, further purification steps would be required (e.g. GST-tag removal, ion exchange chromatography followed by gel filtration).

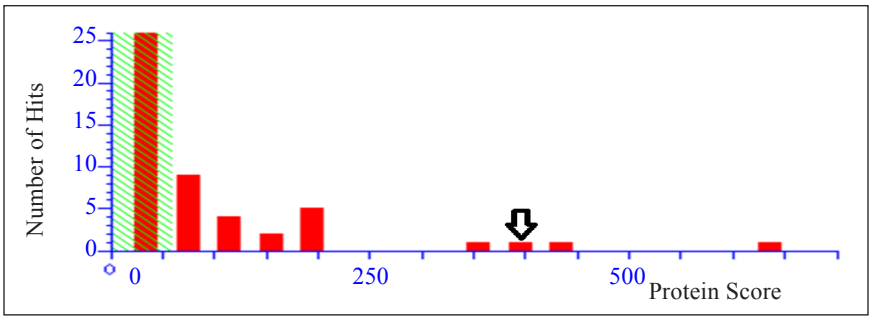

Figure 3. Mascot Score histogram of mass spectrometry analysis. Ions score is 10*Log(P), where P is the probability that the observed match is a random event. Individual ions scores > 59 indicate identity or extensive homology ($p<0.05$). Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits. The target protein BPSL2774 score at 390 (labeled with an arrow) is the third histogram from the right of graph

3. Q63R99 Mass: 35102 Score: 390 Matches: 7(3) Sequences: 7(3)
tr|Q63R99|BPSL2774 Q63R99_BURPS Uncharacterized protein n=3 Tax_Id=272560 [Burkholderia pseudomallei (strain K96243)]
Check to include this hit in error tolerant search or archive report

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Unique	Peptide
13	949.6718	948.6645	948.4818	0.1828	0	37	12	1	U	R.FVIHDYR.S
18	1026.7838	1025.7765	1025.5770	0.1995	0	27	96	1	U	R.AWNAKPALR.I
22	1112.8352	1111.8279	1111.6138	0.2141	0	62	0.03	1	U	R.HVEGLVFVGR.V
25	1140.7867	1139.7794	1139.5611	0.2183	0	59	0.065	1	U	K.ASYPEIAAYR.D
35	1480.9202	1479.9129	1479.6379	0.2750	0	99	5.2e-06	1	U	R.GFDVHDGDASAYAR.F
52	2037.4723	2036.4650	2036.1055	0.3596	0	61	0.0091	1	U	R.SHVTGAAIFDELLPLAQVR.A
54	2062.4114	2061.4041	2061.0419	0.3623	0	44	1	1	U	R.DSLVLVGQPEPEIYEAFR.H

Figure 4. Peptide summary report from mass spectrometry analysis for the third hit (labelled Q63R99) showing the presence of target protein BPSL2774

Homology Identification and Domain Analysis

Through BLASTP search of the PDB database for BPSL2774, the protein is shown to have conserved domains of glycosyltransferase GTB type superfamily and RfaB superfamily (glycosyltransferase involved in cell wall biosynthesis). Glycosyltransferases catalyse the transfer of sugar moieties from activated donor molecules to specific acceptor molecules, forming glycosidic bonds (Breton et al., 2006). It has 11% sequence similarity to two glycosyltransferase enzymes from *Bacillus anthracis* strains (PDB ID: 3MBO A and 2JJM A). Likewise, BPSL2774 has also been included and mapped into the Burkholderia Ortholog Group #BG016035 (downloadable listing of the group members is available at http://www.burkholderia.com/orthologs/list?id=374551) in which glycosyltransferases across *Burkholderia* species or strains are grouped together (Winsor et al., 2008). The target protein is shown to have conserved domains of glycosyltransferase GTB type superfamily (Figure 5).

From WoLF PSORT, Yloc, TargetP and TMHMM prediction tools, BPSL2774 protein was predicted to be located within the cytoplasm with no trans-membrane domain evident. It is also predicted to

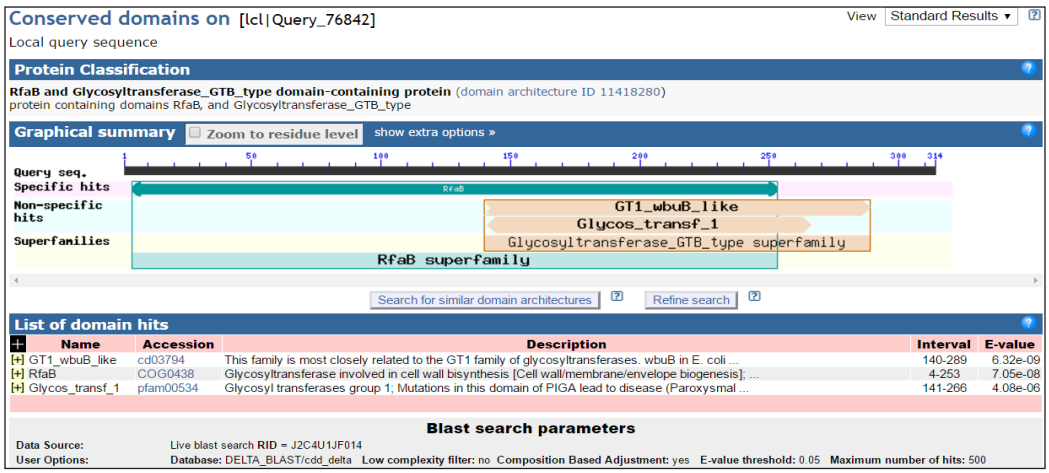

Figure 5. Conserved domain found on BPSL2774 through DELTA-BLAST search of the PDB database

be involved in metabolic processes by VICMpred and is not involved in the pathogenicity of *B. pseudomallei* by MP3 software (data not shown).

Three-Dimensional Model of BPSL2774

The initial model of BPSL2774 from I-TASSER server has a C-score of 0.13, with a relatively average confidence in the quality of the predicted model (Figure 6). Two ModRefiner refinement runs were performed to refine the initial model. Figure 7 showed the Ramachandran plot of the initial model and the final refined model. The final Ramachandran Plot Statistics from RAMPAGE server showed 92.3% residues in the most favoured regions, with two residues in the disallowed regions (ARG220 and GLY299). An estimated 89.8% of the amino acid had an averaged 3D-1D score of more than 0.2 using Verify3D Plot (figure not shown), indicating that the environment profile of the model was acceptable. The QMEAN (Qualitative Model Energy ANalysis) scoring function provided an estimate of the quality of the model with QMEAN6 score of 0.62 (Figure 8). The QMEAN6 score range from 0 to 1, with one being considered to be a model of good quality (Benkert et al., 2010).

The generated model of the BPSL2774 protein tertiary structure from I-TASSER was found to have two domains which is similar to GT-B type superfamily of glycosyltransferase. It consists of two β/α/β Rossmann fold domains with six parallel beta strands found in each domain and the two domains are less tightly associated (Lairson et al., 2008). Only two structural folds, GT-A and GT-B, have been identified for the nucleotide sugar-dependent enzymes, but other folds are now appearing for the soluble domains of lipid phosphosugar-dependent glycosyltransferases (Lairson et al., 2008). They play essential roles in biosynthesis pathways of oligo- and poly-saccharides, as well as protein glycosylation and formation of valuable natural products (Schmid et al., 2016).

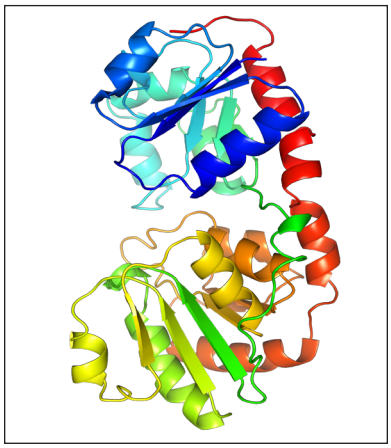

Figure 6. Predicted three-dimensional structure of BPSL2774, with N-terminal coloured blue and C-terminal coloured red

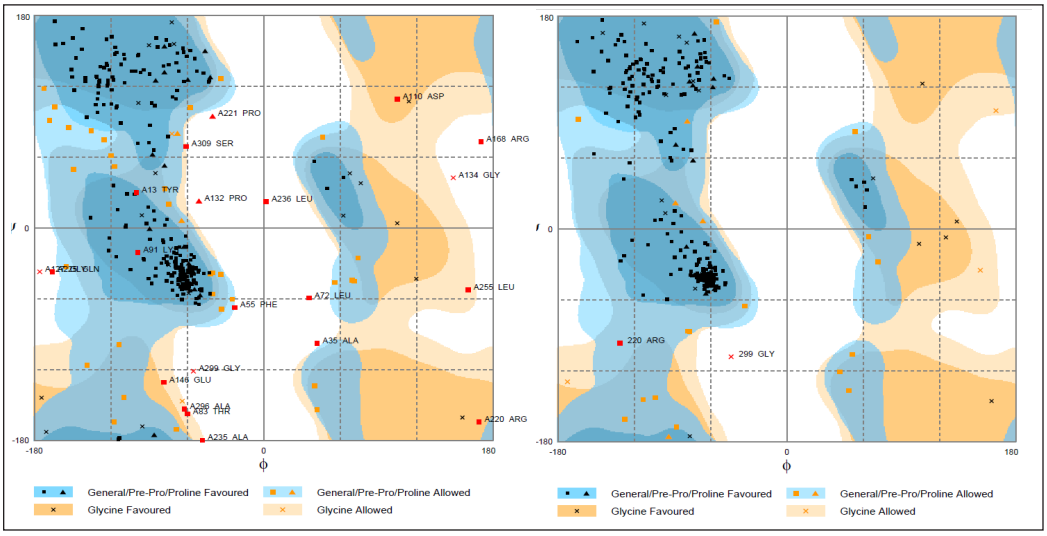

Figure 7. Ramachandran plot analysis of modeled structure of the original model (left) and the final refined model (right)

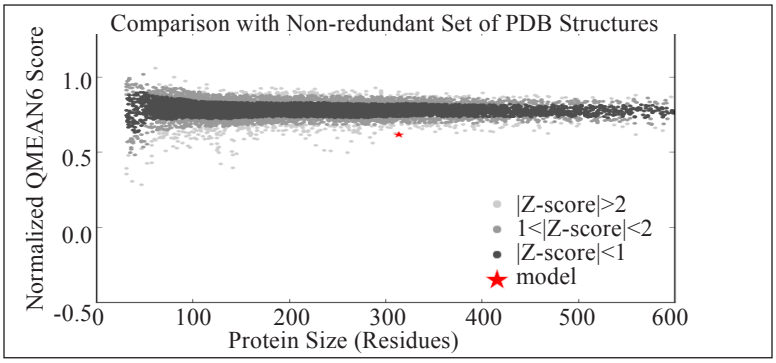

Figure 8. Graphical presentation of estimation of absolute quality of model with QMEAN

Active Site Prediction

The active site of the protein as predicted by metaPocket server is as shown in Figure 9. The top two metaPocket clusters were shown in Figure 9, with the first cluster having a Z-score of 23.82 of six pocket sites and the second cluster with two pocket sites and a Z-score of 1.35. I-TASSER server suite provides biological annotation of the target protein by COFACTOR and COACH programs. The two top predictions for BPSL2774 for ligand binding were uridine diphosphate (UDP) for a glycosyltransferase function (based on its PDB hit, 3mboE, a glycosyltransferase from *B. anthracis*) with the highest C-score of 0.33. This glycosyltransferase structure from *B. anthracis* is involved in bacillithiol (a novel low-molecular-weight thiol) biosynthetic pathway (Parsonage et al., 2010). The second highest C-score of 0.25 was N-acetylglucosamine (NAG) ligand, derived from clusters of PDB hit to 5e9uA, which is also a glycosyltransferase from *Streptococcus gordonii* (Figure 10, Table 1). This glycosyltransferase is involved in O-glycosylation reactions, which has a critical role for biogenesis and modification of adhesins in *streptococci* and *staphylococci* bacteria (Chen et al., 2016). In general,

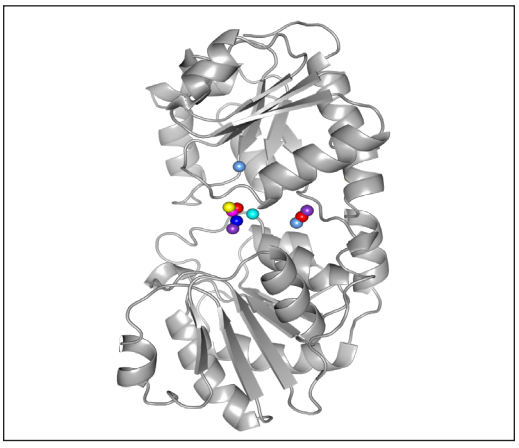

Figure 9. Active sites of the predicted 3D structure of the target protein as determined by metaPocket server. The color of the spheres indicated the active site predicted by MetaPocket method (red ball), PASS method (actinium ball), LIGSITE method (magenta ball), FPocket method (potassium ball), SURFNET method (wheat ball), GHECOM method (yellow ball) and ConCavity method (blue ball)

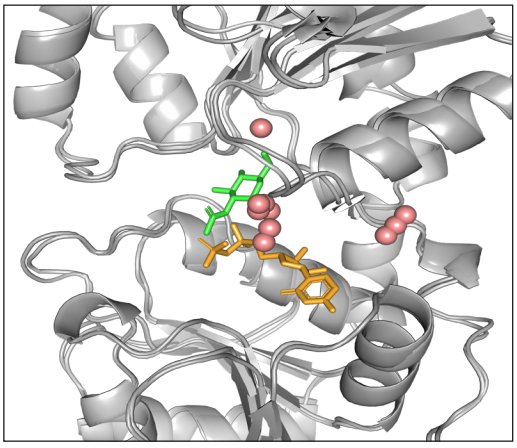

Figure 10. Predicted ligands that bind to the predicted active site by I-TASSER, and superimposed on active sites prediction spheres from metaPocket server (spheres colored pink). Model structure is colored gray, UDP ligand is colored orange, and NAG ligand is colored green

Table 1
Amino acid residues for predicted ligand binding sites from I-TASSER prediction suite

Ligand	PDB Hit	Ligand Binding Site Residues
UDP	3mboE	144,145,146,151,197,198,199,202,227,228,229,232
NAG	5e9uA	67,68,119,223,224,225,226,227,228

models with C-score > −1.5 have a correct fold (Roy et al., 2010). The results from both metaPocket and I-TASSER active sites prediction corresponds well and located in the crevice between the two Rossman fold domains.

Glycosyltransferase Function in Pathogenic Bacteria

Glycosyltransferases catalyze glycosidic bond formation using sugar donors containing a nucleoside phosphate or a lipid phosphate leaving group (Breton et al., 2006). Most commonly, the donor sugar substrate is activated in the form of nucleoside diphosphate sugars e.g. UDP galactose or GDP mannose. However, other forms such as nucleoside monophosphate sugars, lipid phosphates, and unsubstituted phosphates are also used. Glycosyl transfer frequently occurs to the nucleophilic oxygen of a hydroxyl substituent of the acceptor, but it can also occur to nitrogen nucleophiles, such as in formation of N-linked glycoproteins; sulfur nucleophiles, such as in formation of thioglycosides in plants; and also in carbon nucleophiles, such as in C-glycoside antibiotics (Lairson et al., 2008) The product of this reaction may be a growing oligosaccharide, a lipid, or a protein (Breton et al., 2006).

Some pathogenic *Streptococcus* and *Pasteurella* bacteria have capsules that contain nonimmunogenic hyaluronan, which protects them against a mammalian host's immune system. The mammalian body possesses an abundance of hyaluronan, which means that any significant response against the bacterial hyaluronan capsule could cause widespread autoimmune complications in the host. Hyaluronan is synthesized by the glycosyltransferase hyaluronan synthase (DeAngelis, 1999).

In Gram negative bacteria, glycosyltransferases are valuable in the formation of lipolysaccharides (LPS), i.e. the major cell-surface component protecting the bacterium from extracellular threats (Cote & Taylor, 2017). In Gram negative *Neisseria meningitidis* and *Haemophilus influenzae*, small molecular inhibitors of LgtC glycosyltransferase are being analysed as potential anti-virulence drug candidates (Xu et al., 2018). The *B. pseudomallei* genome carries four large polysaccharide loci, all of which have been demonstrated to play a role in virulence in vivo; these encode the type I O antigen polysaccharides (O-PS) capsule, the type II O-PS LPS, and two additional clusters defined as type III O-PS and type IV O-PS (Moule et al., 2016).

In other pathogenic bacteria such as *Escherichia coli*, *Salmonella enterica* and *Shigella dysenteriae*, glycosyltransferases along with other proteins play significant roles to ensure their survival in mammalian host, namely in the modification processes of bacteria's protein, enterobactin. This protein can successfully compete for iron, Fe (II) binding against the host, which is important to the pathogen's ultimate survival (Fishbach et al., 2006).

Three genes predicted to be glycosyltransferases in *B. pseudomallei; BPSS2167, BPSS2248* and *BPSL1444* have recently been identified as newly discovered

genes involved in in vivo virulence with roles in different stages of *B. pseudomallei* pathogenesis, including extracellular and intracellular survival (Moule et al., 2016). In the same 2016 paper, Moule suggested that "the role of polysaccharides in *B. pseudomallei* infections is even more complex than has been previously described", as these three genes identified in their screen did not belong to any of the four polysaccharide clusters (Moule et al., 2016).

CONCLUSION

The GST-tagged BPSL2774 target protein was able to be expressed in soluble form from high density cultures and has been partially purified using affinity chromatography. Higher protein purity can be achieved through further purification steps following the initial GST-tagged affinity chromatography. *Escherichia coli* chaperonin proteins from the *E. coli* host system was found to be co-purified along with the target protein. The purified protein however is at acceptable purity and at sufficient concentration for use as samples in functional assays, e.g. a fluorescence-based or bioluminescence-based glycosyltransferase assay in the near future. Due to the challenges to determine both the sugar donor and acceptor for a GT of unknown function, *in silico* approaches were performed to annotate the structure and function of BPSL2774 protein. The quality of the refined model was verified by using Ramachandran plot. Through preliminary docking runs using AutoDock 4.2 (Morris et al., 2009) and AutoDock Vina suite (Trott & Olson, 2010) on predicted ligands UDP and NAG to BPSL2774 structure model, satisfactory docking results were obtained (results not shown). It was worth to note that the bioinformatics structural and functional annotation predictions all pointed towards BPSL2774 functioning as a glycosyltransferase. Taking all the results into account, the functional annotation of BPSL2774 protein as a glycosyltransferase is recommended, though future validation from biochemical experiments or a more exhaustive docking simulation experiments will be needed to support this.

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Isolation and Characterization of Putative Liver-specific Enhancers in Proboscis Monkey (*Nasalis larvatus*)

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ABSTRACT

Enhancers are indispensable DNA elements responsible for elevation of gene transcriptional efficiency that regulates biological processes tightly at various developmental stages, linking them to numerous genetic diseases. Discovering the enhancer landscape of the genome will not only benefit mankind, but also aid in conservation researches involving endangered non-human primates such as the proboscis monkey. As one of the most ancient colobine endemic to Borneo Island, the proboscis monkey offers a wide spectrum of unique and exclusive characteristics that distinguish it from other primates. This study has successfully isolated 13 liver-specific enhancers from this primate and tested for their activities in HepG2 and A549 cell line. The TFBS-enriched regions such as pairs of AP-1, clusters of C/EBP-β and triplets of HNF-1 in enhancers contributed to enhancer activities whereas huge clusters of HNF-3β possess suppressing effects, but generally these regions contributed to the cell specificities of enhancers. It is hoped that this study serves as a stepping stone in knowledge enrichment on this primate and future conservation researches.

Keywords: Computational approach, conservation, enhancer, liver, proboscis monkey

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INTRODUCTION

Enhancers are DNA elements capable of elevating transcriptional efficiency of the genes they regulate regardless of their orientation and locality in the genome (Khoury & Gruss, 1983; Lim et al., 2018a). There are many roles that require the involvements of the enhancers for the determination of phenotypes especially

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in various biological processes and developmental stages (Kleftogiannis et al., 2015; Pennacchio et al., 2015). Enhancers (size varying from 50 bp to 1.5 kbp), when activated, can work in tandem with multiple counterparts of their own to activate gene transcriptions up to 1 Mbp away from their positions (Blackwood & Kadonaga, 1998; Pennacchio et al., 2015).

The first enhancer, SV40 enhancer, was discovered almost 37 years ago and it was found that deletion of this element led to at least 100-fold decrement of early gene expression of T antigen (Banerji et al., 1981; Benoist & Chambon, 1981; Gruss et al., 1981). Since then, the discovery of enhancers had progressed throughout the decades utilizing both experimental (reporter assays and high-throughput assays) as well as computational approaches (Cao & Yip, 2016). Experimental approaches such as enhancer trap and transient transgenesis involve reporter plasmids being expressed in the host cells for detection (Kvon, 2015). These approaches (although some that involve high-throughput technologies) can only be conducted on limited number of cellular conditions and cellular properties at one go (Shlyueva et al., 2014). Contrary to the experimental approaches, the computational approaches offer rapid, low-cost, no context restrictions and less labour-intensive alternatives to conduct genome-wide enhancer identification via various machine learning methods (Cao & Yip, 2016). A myriad of computational enhancer prediction tools which employed one or integration of the many enhancer features such as histone modifications, sequence feature, motif signal feature as well as open chromatin feature, had successfully achieved high predictive accuracies (>90%) (He & Jia, 2016, 2017; Kim et al., 2016; Lim et al., 2018a; Liu et al., 2015; Liu et al., 2016). Interestingly, Omar et al. (2017) had attempted to consolidate results from five enhancer predictor tools (namely LS-GKM, GMFR-CNN, CSI-ANN, DeepBind and iEnhancer-2L) employing different enhancer features (Alipanahi et al., 2015; Firpi et al., 2010; Ghandi et al., 2014; Liu et al., 2015; Wong et al., 2016) and had achieved improved accuracies.

The link between enhancer and diseases has been reported in many human-related diseases such as X-linked deafness type 3 (CFN3), coronary heart disease, prostate cancer, Hirschsprung disease and preaxial polydactyly (de Kok et al., 1996; Emison et al., 2005; Grice et al., 2005; Lettice et al., 2002; McPherson et al., 2007; Yegnasubramanian et al., 2011). Kim et al. (2011) took one step further by comparing how enhancer variations between different ethnic groups were correlated to drug responses, focusing on liver-specific enhancers found surrounding nine major liver membrane transporter genes. These enhancer researches are not only beneficial towards the wholesomeness of humankind, but it also channels towards a better understanding of the enhancer landscape of other non-human primates for conservation purposes. One of the non-human primates that is interesting to look upon is none other than one of the most ancient primate colobine of all, the proboscis monkey (*Nasalis larvatus*).

The proboscis monkey, a species endemic to the coastal regions of Borneo, is a reddish-brown skin-coated colobine with a very distinctive large nose (in males) and huge pot-shaped belly (Groves, 2001). There are various characteristics of this primate that are clearly distinguishable from its primate counterparts that spikes interest into the effort of bringing it into the limelight of primate enhancer research (Lim et al., 2019). For instance, its morphological characteristics and behaviours such as terrestrial movement, highly-distinctive sexual dimorphism, proportions of the extremities and skull structure suggest its ancient status that is associated to that of *Rhinopithecus* (Peng et al., 1993). Besides, the digestive physiology (in its four-chambered stomach) such as the regurgitation/remastication (R/R) of this foregut fermenter allows it to possess lysozymes that are believed to have adaptive convergence with that of the ruminants (Bigoni et al., 2003; Matsuda et al., 2011; Stewart et al., 1987). Despite that this primitive primate is being listed as endangered by the International Union for Conservation of Nature (IUCN) (Meijaard et al., 2008), the conservation research on this primate is still at its infancy and requires immediate attention. Furthermore, with the completion of the genome sequencing of the proboscis monkey (Abdullah et al., 2014; Tamrin, 2016), this study aimed to isolate genome-wide liver-specific enhancers from the proboscis monkey and further characterize them. As most enhancer studies to date involving non-human primates like the chimpanzee are focused on evolutionary studies (Boyd et al., 2015; Prescott et al., 2015), it is hoped that this study acts as a stepping stone towards the establishment of conservation research on the proboscis monkey (an ancient "long-neglected" endemic primate), similar to that of the toxicology gene expression studies on endemic *Rasbora* fish (Lim et al., 2018b), antibody production against elephant endotheliotropic herpesvirus (EEHV) (Kochagul et al., 2018) and pathogen battling in white-nose syndrome in bats (Palmer et al., 2018).

MATERIALS AND METHODS

Computational Enhancer Prediction Data Collection

The computational enhancer prediction data collection was conducted with three different approaches (Supplementary Figure 1). First, the chromosome 18 sequences of the proboscis monkey were retrieved from GenBank database (Accession number: CM003007.1). The strong enhancer regions (>300 bp, flanked by weak and non-enhancer regions) identified by iEnhancer-2L using default parameters (Liu et al., 2015) were selected. Second, the genome-wide human liver membrane transporter gene enhancer data was obtained from Kim et al. (2011). The enhancers are aligned with genome of rhesus macaque and chimpanzee using ECR browser (Ovcharenko et al., 2004) and only enhancers achieving above 70% conservation were selected. Third, the enhancers (>300 bp) identified by Omar et al. (2017) were obtained for the next step. All enhancers identified from the three different

approaches were pooled and ranked based on the liver-specific TFBS per nucleotide frequency enumerated by MATCH (Kel et al., 2003) using liver-specific profile and other default parameters. Thirteen top ranked proboscis monkey enhancers (pme) were selected for further procedures. A total of four, five and four enhancers were selected from the first, second and third approach respectively, and they were termed pme001 to pme004, pme101 to pme105 and pme201 to pme204 accordingly.

Isolation and Cloning of Enhancers

A total of 13 pairs of primers (each added with *Bam*H I and *Sal* I restriction sites at the 5' end of forward and reverse primers, respectively) were designed using Primer3 (Untergasser et al., 2012) to isolate all 13 candidate enhancers from the proboscis monkey genome (Table 1). The conditions of the primers were examined using OligoCalc (Kibbe, 2007) to ensure they are optimal in terms of GC content (35-60%), melting temperatures as well as absence of

Table 1
Gene-specific primers designed to isolate enhancers and their characteristics

Enhancer	Sequences (5'-3')	Length (bp)	GC Content (%)
pme001	F: **AA**GGATCCTGGGCCTCGTAGTTAAA	25	48.0
	R: **AT**GTCGACACACACACTTCTACGGT	25	48.0
pme002	F: **AT**GGATCCGACTGTGCTTTTCCCCTG	26	53.8
	R: **AA**GTCGACTTTTGTTGTTGATGCTGTTG	28	39.3
pme003	F: **AA**GGATCCTTTGACTCCATGTCTCAC	26	46.2
	R: **AA**GTCGACCAAGACAATGGGGAAAAT	26	42.3
pme004	F: **AT**GGATCCAGCACTGGGACTGATA	24	50.0
	R: **AT**GTCGACAGTGGGGACTTTTGTTGTT	27	44.4
pme101	F: **AT**GGATCCGGCAGGAGAATTGCTTGAA	27	48.1
	R: **AT**GTCGACAAAATTAGCTGGGCCTGGT	27	48.1
pme102	F: **AT**GGATCCTACCCAAATAGTGCTTGCTG	28	46.4
	R: **AA**GTCGACGCCACATTTCAAGTGCTCA	27	48.1
pme103	F: **AA**GGATCCTTTCCAATCTGACCAGGTG	27	48.1
	R: **AT**GTCGACTACCCTGAAACTTTGCTGA	27	44.4
pme104	F: **AT**GGATCCGTGGCTCTCAGTTTCCTG	26	53.8
	R: **AA**GTCGACAGGCATGAGCCACTACAT	26	50.0
pme105	F: **AT**GGATCCCTTCACAACCAACGTTCAT	27	44.4
	R: **AT**GTCGACTATGGAAGGAGCCTTTGG	26	50.0
pme201	F: **AA**GGATCCATGTGTCATGCGTGTGTA	26	46.2
	R: **AA**GTCGACTGACACGTCACATACGAAAA	28	42.9
pme202	F: **AA**GGATCCGGGTAGACAGCAAGGACA	26	53.8
	R: **AA**GTCGACGGCAAACTGCTTCAGGAT	26	50.0
pme203	F: **AA**GGATCCACATTTGGCAGACATAGT	26	42.3
	R: **AA**GTCGACGAGGTATGTGTCCAAAGCAA	28	46.4
pme204	F: **AA**GGATCCCAATCACCTCTCACCA	24	50.0
	R: **AA**GTCGACTCCCAAATCTTTCCTTGG	26	46.2

Restriction enzyme sites (underlined) where BamH I site was added to 5' end of each forward primer whereas Sal I site was added to 5' end of each reverse primer. Letters in bold represent additional base pair for optimal restriction digestion

secondary structures, self-complementary and hairpin. The chosen primer pairs were sent to Apical Scientific Sdn. Bhd. for synthesis. The stool of proboscis monkey was collected from Bako National Park, Sarawak, Malaysia (Permit number: NCCD.907.4.4 (JLD.11), park permit number: 537/2014 and 538/2014) and was frozen at -80°C before DNA extraction.

Stool DNA extraction was conducted using QIAamp DNA Stool Mini Kit (Qiagen, Germany) to isolate the genomic DNA of the proboscis monkey according to the manufacturer's protocol. Species verification was done via the polymerase chain reaction (PCR) isolation of cytochrome *b* gene using universal primers by Irwin et al. (1991) [forward primer L14724 (5′-GACTTGAAAAACCACCGTTG-3′) and reverse primer H15915R (5′-GGAATTCATCTCTCCGGTTTACAAGAC-3′)], sequenced and blasted to a 99% similarity to the entry KM889667.1 in the GenBank database. Gradient PCR was conducted using LA *Taq* (Takara Bio, USA) to achieve temperature optimization of each primer pair for enhancer isolation. The PCR products (enhancers) were purified using QIAquick PCR Purification Kit (Qiagen, Germany) before subjected to cloning. The enhancers were then inserted into pGL4.23 [*luc2*/minP] vector (Promega, USA) via restriction digestions of both *Bam*H I and *Sal* I, and this was followed by the cloning process. Colony selection was done via colony PCR and plasmid re-digestion using the same restriction enzymes before these plasmids each containing the enhancer insert were isolated using QIA Spin Miniprep Kit (Qiagen, Germany).

Luciferase Activity Detection

The cell lines used in this study were HepG2 (human liver carcinoma) and A549 (human lung carcinoma) (Riken, Japan). Both the cell lines were grown in MEM medium (Gibco, USA) supplemented with 10% fetal bovine serum (FBS) and 1% Pen-Strep (100 U/mL Penicilium and 100 µg/mL Streptomycin) under the 5% CO_2, pH 7.0 to 7.6 and 37°C conditions. Cell passaging was conducted when the cell reached 80-90% confluency before cell counting was conducted to allocate around 7500 to 10000 cells per well in the each of the 24-well plate. Transient transfection was performed using Lipofectamine 2000 (Thermo Fisher Scientific, USA). For each experimental plasmid, a total of nine replicates (three technical replicates for each of the three biological replicates), positive (pGL3-control plasmid) (Promega, USA) and negative control (empty pGL4.23 [*luc2*/minP] plasmid) (Promega, USA) sets were co-transfected alongside with pRL-TK plasmid (Promega, USA) according to manufacturer's protocol. The luciferase activity was detected using Dual-Luciferase® Reporter Assay (Promega, USA) and measured using Infinite M200 Pro luminometer (Tecan, Switzerland). The ratios for firefly luciferase: *Renilla* luciferase were determined and normalized to that of the non-insert control. Statistical analysis was carried out using one-way ANOVA ($p<0.05$) and post-hoc Tukey's test.

Deletion Analysis

The 13 enhancer candidates were subjected to transcription factor binding site (TFBS) analysis using MATCH liver-specific profile (Kel et al., 2003) to determine the relationship between TFBS and the resultant enhancer activities. A total of three aspects were investigated across all enhancers, namely overall TFBS abundance, TFBS composition and TFBS distribution pattern. A total of three enhancers were selected for their unique TFBS distribution pattern and luciferase activity levels: enhancer pme001, pme101 and pme103 (enhancer pme001 being the highest in activity, pme103 being the second highest and pme101 being one of the lowest in activity). Several internal primers (Table 2) were designed to exclude some key TFBS-enriched regions from the wildtype enhancer fragments to test for the effects of the deletions implied. The PCR reactions were conducted using the original forward or reverse primer pairing with the newly designed internal forward or reverse primer with flanking RE site (for new primers with suffix of "a", "b" and "c"). For new primers with suffix "D", the newly designed forward primer was

Table 2
The list of internal primers used for modified enhancer fragments amplification via PCR

Modified Enhancer Fragment	Sequences (5'-3')	Length (bp)	GC Content (%)
pme001a	F: **AA**GGATCCTGGGCCTCGTAGTTAAA	25	48
	R: **AA**GTCGACAGCGCAGTGCTTCTTCG	25	56
pme001b	F: **AA**GGATCCTGGGCCTCGTAGTTAAA	25	48
	R: **AA**GTCGACCAGCACAATGTCGCGAA	25	52
pme001c	F: **AA**GGATCCTGGGCCTCGTAGTTAAA	25	48
	R: **AA**GTCGACCATCCTGGCTGATTTTT	25	44
pme001D	F: CGCGACATTGTGCTGTGCTGTATCTATGCT	30	50
	R: AGCATAGATACAGCACAGCACAATGTCGCG	30	50
pme101D1	F: AGCGAGACTTTGACTATCTAGAGCGTTGTG	30	47
	R: CACAACGCTCTAGATAGTCAAAGTCTCGCT	30	47
pme101D2	F: ATGCTCTAACTGTAGGAACTGGCACACTGC	30	50
	R: GCAGTGTGCCAGTTCCTACAGTTAGAGCAT	30	50
pme101D3	F1: AGCGAGACTTTGACTATCTAGAGCGTTGTG	30	47
	R1: CACAACGCTCTAGATAGTCAAAGTCTCGCT	30	47
	F2: ATGCTCTAACTGTAGGAACTGGCACACTGC	30	50
	R2: GCAGTGTGCCAGTTCCTACAGTTAGAGCAT	30	50
pme103a	F: **AA**GGATCCTCACACCTACCCTTTCG	25	52
	R: **AT**GTCGACTACCCTGAAACTTTGCTGA	27	44
pme103D1	F: GGCTTCCCTTCCTACGTTACCAGCTATGCT	30	53
	R: AGCATAGCTGGTAACGTAGGAAGGGAAGCC	30	53
pme103D2	F: AATCGTAAATCCTAAAAAGTGTCTTTTAGT	30	27
	R: ACTAAAAGACACTTTTTAGGATTTACGATT	30	27

Restriction enzyme sites (underlined) where BamH I site was added to 5' end of each forward primer whereas Sal I site was added to 5' end of each reverse primer. Letters in bold represent additional base pair for optimal restriction digestion

matched with the original reverse primer and in a separate tube, while the newly designed reverse primer was matched with the original forward primer in another tube, and both tubes went through the first round of PCR reaction. Both the PCR products from the first round of PCR were pooled into one tube and diluted with dilution factor of 100 using double distilled water. This PCR product mixture was then used as template for the second round of PCR with the use of the original forward and reverse primer designed for the enhancer. Next, these modified enhancer fragments were inserted into pGL4.23 [*luc2*/minP] plasmid before subjected to cloning. Cloning and luciferase reporter assays were carried out according to the abovementioned protocols.

RESULTS AND DISCUSSION

Computational Enhancer Prediction Data Collection

The computational enhancer prediction data collection was carried out via three major approaches: functional conservation, sequence conservation and combinatorial approach. The functional conservation approach was performed on chromosome 18 of proboscis monkey using iEnhancer-2L (Liu et al., 2015) because this software not only has the capability to distinguish strong enhancers from non- and weak enhancers, also its benchmark datasets are constructed based on chromatin states of nine different cell lines (NHEK, HSMM, NHLF, HepG2, K562, HMEC, H1ES, GM12878 and HUVEC) (Liu et al., 2015). In this study, a total of 9,960,241 strong enhancers, 11,855,707 weak enhancers and 50,983,878 non-enhancers were identified from the chromosome 18 of the proboscis monkey genome.

The sequence conservation approach utilized the human liver membrane transporter gene enhancers identified by Kim et al. (2011) to search for the proboscis monkey orthologue based on sequence conservation across other non-human primate genomes available such as chimpanzee and rhesus macaque. Applying the methodology by Kim et al., (2011), a total of 105 sequence conserved enhancers surrounding the nine major liver membrane transporter genes (SLC47A1, SLC22A7, SLCO1B3, SLC22A1, SLCO2B1, ABCB11, SLCO1B1, ABCC2 and SLC10A1) were identified.

The third approach, which is the combinatorial approach of five different enhancer predictor tools (namely LS-GKM, GMFR-CNN, CSI-ANN, DeepBind and iEnhancer-2L) (Alipanahi et al., 2015; Firpi et al., 2010; Ghandi et al., 2014; Liu et al., 2015; Wong et al., 2016) utilized by Omar et al. (2017) had yielded a sum of 3861 enhancers. The enhancers obtained from all three approaches were subjected to MATCH liver-specific profile screening to enumerate TFBS per nucleotide frequency based on the six major liver-specific TFBS: AP-1, HNF-3β, C/EBP-β, GATA-3, NF-1 and HNF-1. The top 13 ranked proboscis monkey enhancers (pme) were chosen for enhancer assays: four from the first approach (pme001-pme004), five from the second approach (pme101-pme105), and four from the third approach (pme201-pme204).

Association of TFBS with Enhancer Activity

The activities of all 13 candidate enhancers were measured via the luciferase reporter assay and were displayed in normalized relative luciferase unit (RLU) bar graph as shown in Figure 1. The enhancer pme001 scored the highest activity level among all in HepG2 cell line, rocketing even above that of the positive control set. While all the others have moderate enhancer activities, the bottom three with the lowest luciferase score level are the enhancer pme003, pme101 and pme104 (all three of them are not significantly different from one another) in HepG2 cell line. No observable trends can be detected in the enhancer activities across three different approaches (no significant differences across groups).

The association of TFBS with enhancer activity was investigated based on three aspects: overall TFBS frequency per nucleotide, TFBS composition and TFBS distribution pattern. Based on Figure 2, the enhancer pme105 has the highest liver-specific TFBS per nucleotide frequency. The enhancer pme101 and pme203 are second and third in place whereas the enhancer pme001 is ranked among the bottom five in terms of TFBS frequency per nucleotide. Clearly, the correlation between the overall TFBS frequency per nucleotide and enhancer activity is weak, and in some cases, a negative correlation in enhancer pme001 and pme101 particularly. It is believed that other factors such as the TFBS composition and distribution pattern dictating the enhancer activities where compact TFBS within the enhancers is related to active enhancers (Bery et al., 2014). Therefore, TFBS abundance itself is inadequate to influence enhancer activity (Gotea et al., 2010; Hu et al., 2007; Lusk & Eisen, 2010).

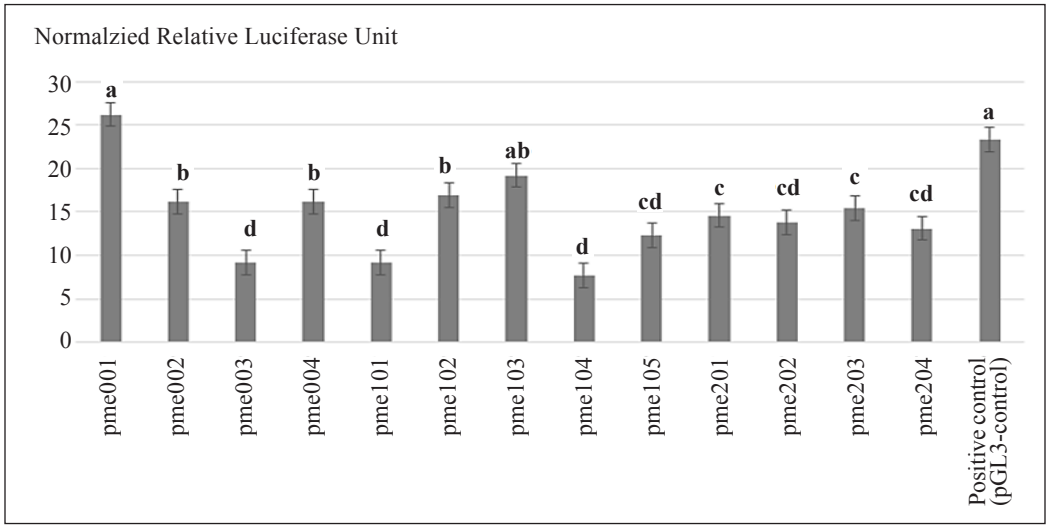

Figure 1. The measured normalized relative enhancer luciferase activity (relative to NIC=1) across 13 enhancer candidates. Statistical analysis was conducted using one-way ANOVA and post-hoc Tukey's Test with $p<0.05$. Significantly different data are represented by different alphabets

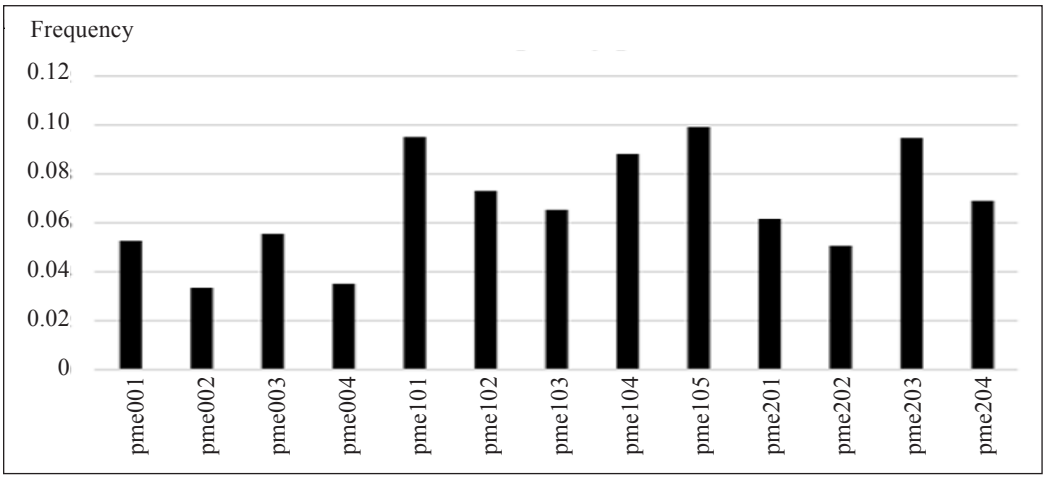

Figure 2. The predicted TFBS frequency per nucleotide across 13 enhancers

The TFBS composition of all 13 candidate enhancers were also examined for its association with the resultant enhancer activity (Figure 3). The enhancer pme001 has the greatest number of activator protein-1 (AP-1) TFBS within its sequences and this may be the reason for its high enhancer activity among all. In other study, the mutation of AP-1 and ATF (activating transcription protein) has resulted in at least two-fold reduction in expression of 87% of the enhancers tested (Grossman et al., 2017). The CCAAT enhancer binding protein beta (C/EBP-β) TFBS was most

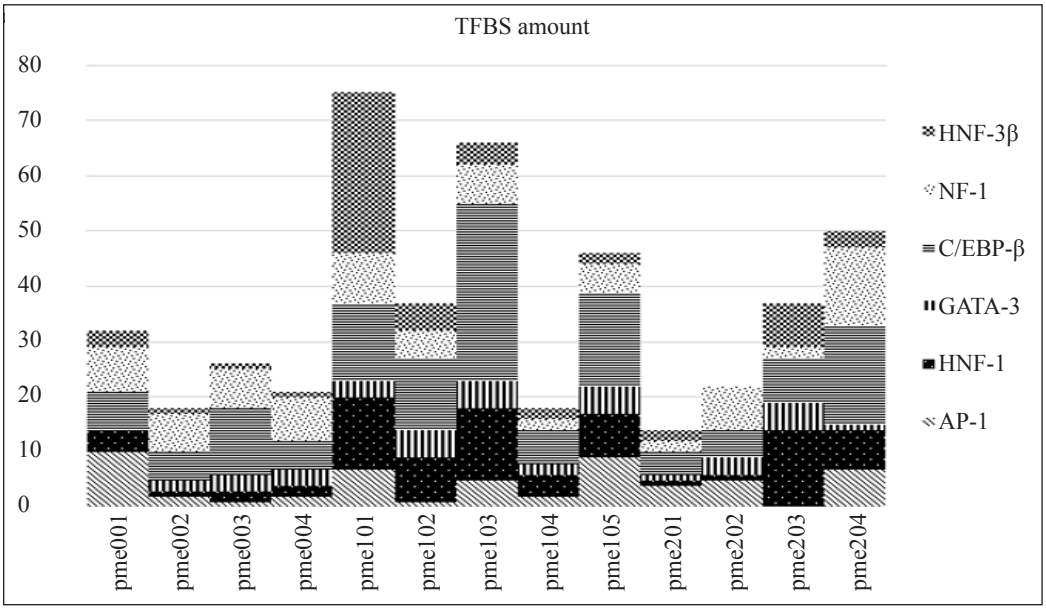

Figure 3. The predicted TFBS composition (based on six most abundant liver-specific transcription factors) across 13 enhancers

abundantly found in enhancer pme103 and it was shown in this study to be the main driver in placing this enhancer in the second place in terms of luciferase activity level among the 13 enhancers. As one the of vital players in the liver, the C/EBP-β is indispensable in various liver-specific processes such as adipose tissue differentiation, inflammation and metabolism (van der Krieken et al., 2015). The enhancer pme101 is one of the lowest activity enhancers but it was found to have high amount of hepatocyte nuclear factor 3 beta (HNF-3β) TFBS within its sequence. This phenomenon may suggest the supressing role of HNF-3β TFBS. However, in other literatures, the HNF-3β was found to contribute substantially towards liver-specific enhancers and human prothrombin gene enhancers (Ceelie et al., 2003; Kim et al., 2011). According to Chen et al. (2015), the contribution of TFBS-clustered regions towards cellular functions and enhancer activities exceeds that of individually scattered TFBS within the enhancer. Hence, this leads us to further investigate the TFBS distribution pattern of these proboscis monkey enhancers.

The TFBS distribution pattern of the 13 selected enhancers was inspected at every 100 bp interval to discover more on their associations with enhancer activity (Figure 4). Apart from having the most abundant AP-1 TFBS within its sequence, the enhancer pme001 had also four AP-1 adjacent pairs located at four different 100 bp intervals. This may explain for the two-fold difference in enhancer activity between enhancer pm001 and pme105 (containing two adjacent AP-1 pairs). According to Kerppola and Curran (1993), pairs of AP-1 TFBS which are capable of bending DNA can improve enhancer functioning more than that of individual one. The enhancer pme103 was found to encompass five adjacent C/EBP-β TFBS as well as two triplets of HNF-1TFBS which had unleashed the expression

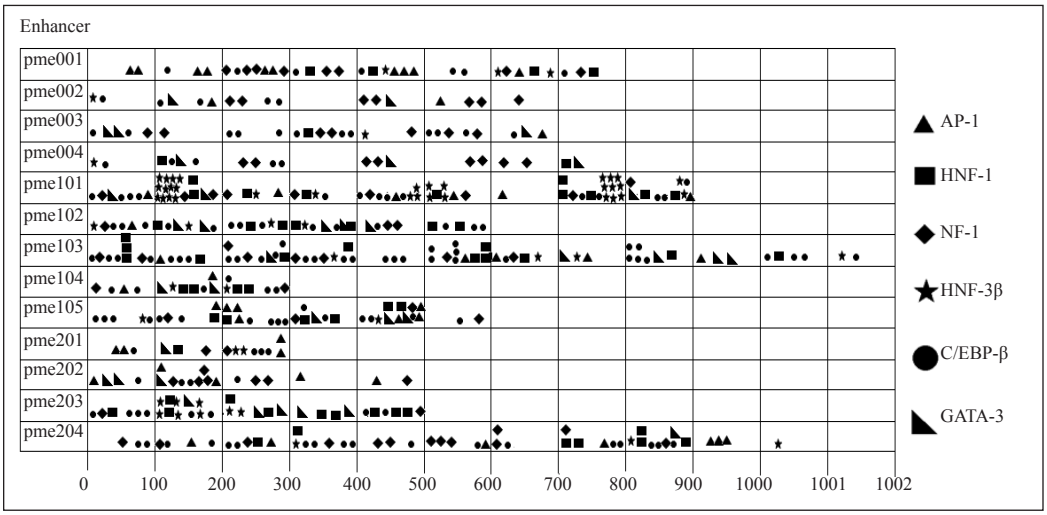

Figure 4. The TFBS distribution pattern per 100 bp interval of 13 enhancers. Stacking TFBS represents overlapping TFBS sites

capability of this enhancer in HepG2 cell line. The C/EBP-β has the ability to recruit other key transcription factors (including its counterparts) to orchestrate the proliferation, antiapoptotic responses as well as survivability of multiple myeloma (Pal et al., 2009) whereas the HNF-1 is essential regulator of various liver specific genes such as albumin, CYP2E1, HBV, Apolipoprotein B and Apolipoprotein AII gene (Maire et al., 1989). Both of these liver-specific TFBSs composite the strong enhancer activity in pme103. There are two huge clusters of HNF-3β TFBS found exclusively in the enhancer pme101 and we have proven in this study that this accounts for the low enhancer activity outcome in HepG2 cell line. Despite many lines of evidence pointing out on the enhancing effects of HNF-3β onto regulative activities in the liver (Ceelie et al., 2003; Rausa et al., 1997; Schrem et al., 2002), the consequence of deletion of large clusters of HNF-3β TFBS in proboscis monkey enhancers remains unproven until this study, to the best of our knowledge. Thus, deletion analysis was designed to elucidate the deletional effects of these TFBS regions in the selected proboscis monkey enhancers.

Deletion Analysis

The deletion analysis was performed onto selected enhancers (pme001, pme101 and pme103) to determine the contributions of the TFBS-enriched regions towards enhancer activities (Figures 5, 6 and 7). The internal primer pairs used for deletion analysis was listed in Table 2 and the deletion descriptions are shown in Table 3. In general, the deletion of each pair of AP-1 or the triple AP-1 region had resulted in reduction of enhancer activity as compared to the wildtype enhancer. However, the enhancer activity level of modified enhancer fragment pme001a and pme001D were the same even though the pme001a only contains the first two adjacent AP-1 pairs whereas the pme001D has all three adjacent AP-1 pairs with the tailing AP-1 TFBS. On the other hand, modified enhancer fragment pme001b has all three adjacent AP-1 TFBS pair sets (similar to pme001D but without the tailing AP-1 TFBS) and had scored a much higher expression level in HepG2 cell line compared to pme001D. On the side note, no new TFBS had been introduced following the deletion done onto the modified enhancer fragment pme001D. Fonseca et al. (2018) found that collaborative interactions between TFs could affect the binding affinity of all AP-1 monomers greatly in macrophage. Therefore, it is postulated that in the absence of the triple AP-1 region, the tailing AP-1 TFBS located nearest to the 3' end has disrupted DNA bending patterns and subsequently the collaborative interactions of all TFs within enhancer pme001.

The deletion analysis conducted onto enhancer pme101 had yielded modified enhancer fragments with activity level higher than that of the wildtype enhancer. The deletion of the first HNF-3β-enriched regions containing 12 HNF-3β TFBS from enhancer pme101 had elevated the enhancer activity to 1.7-fold (pme101D1) whereas the deletion of the second HNF-3β-enriched region containing 8 HNF-3β TFBS from the

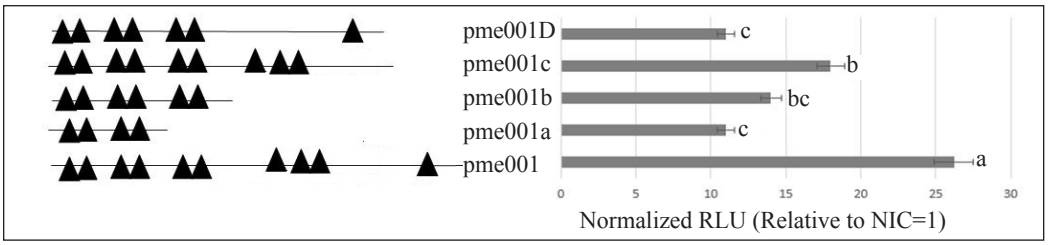

Figure 5. Overview of selected TFBS distribution and deletion analysis of enhancer pme001 in pGL4.23 [*luc*/minP], activities as measured in HepG2 cell line. Deletion constructs are shown on the left. AP-1 TFBS is represented by black triangle. Enhancer activities are shown on the right with values indicating normalized firefly luciferase: *Renilla* luciferase activities relative to no-insert control (NIC). Results are mean ± S.E. (n=3). Mean values were subjected to One-Way ANOVA and Tukey's test, $p<0.05$. Mean values represented by different alphabets are significantly different

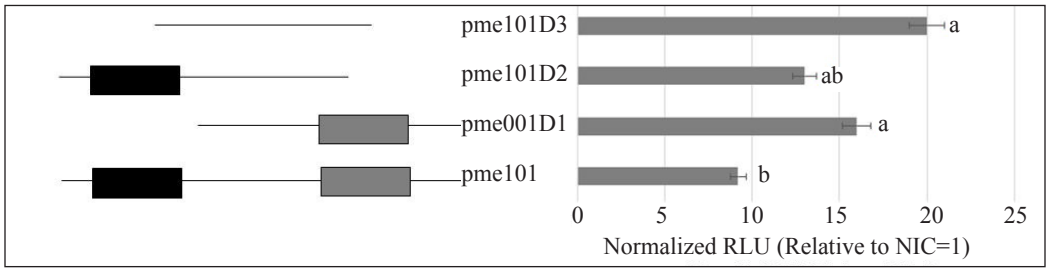

Figure 6. Overview of selected TFBS distribution and deletion analysis of enhancer pme101 in pGL4.23 [*luc*/minP], activities as measured in HepG2 cell line. Deletion constructs are shown on the left. The first HNF-3β TFBS-enriched region (12 HNF-3β TFBSs) is represented by black rectangle; The second HNF-3β TFBS-enriched region (8 HNF-3β TFBSs) is represented by grey rectangle. Enhancer activities are shown on the right with values indicating normalized firefly luciferase: *Renilla* luciferase activities relative to no-insert control (NIC). Results are mean ± S.E. (n=3). Mean values were subjected to One-Way ANOVA and Tukey's test, $p<0.05$. Mean values represented by different alphabets are significantly different

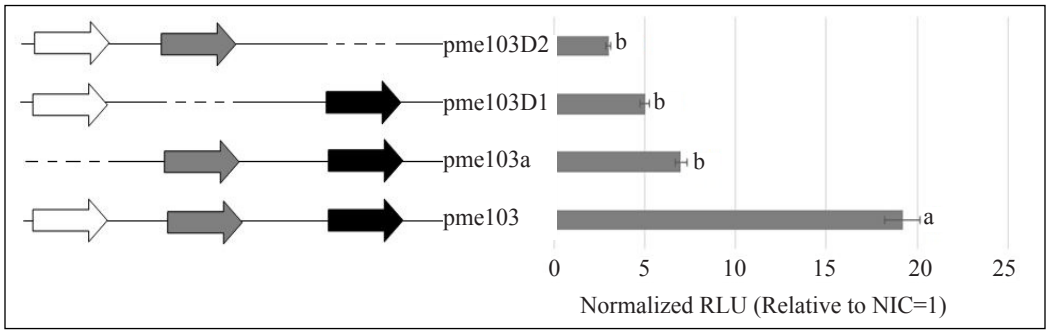

Figure 7. Overview of selected TFBS distribution and deletion analysis of enhancer pme103 in pGL4.23 [*luc*/minP], activities as measured in HepG2 cell line. Deletion constructs are shown on the left. The 1-100 bp region is represented by white arrow; The 500-599 bp are represented by grey arrow; The 800-899 bp are represented by black arrow; Deleted regions are represented by dotted lines. Enhancer activities are shown on the right with values indicating normalized firefly luciferase: *Renilla* luciferase activities relative to no-insert control (NIC). Results are mean ± S.E. (n=3). Mean values were subjected to One-Way ANOVA and Tukey's test, $p<0.05$. Mean values represented by different alphabets are significantly different

enhancer pme101 had yielded a 1.4-fold increase in activity (pme101D2). In the case of modified enhancer fragment pme101D3, the deletion of the both abovementioned HNF-3β-enriched regions had produced enhancer activity 2.2-fold higher than that of the wildtype enhancer pme101. Despite the repressing activity of the large HNF-3β clusters has never been reported previously. Li et al. (2002) however discovered that NKX2.1 could cooperate with HNF-3β to induce repression effects onto the transcription of surfactant protein B (*Sp-B*) gene. While the underlying mechanism of this phenomenon remains unknown, this study revealed the suppressing effects of huge clusters of the HNF-3β TFBS onto proboscis monkey enhancer.

The major players in regulating the enhancer activity of pme103 have been revealed following the deletion analysis: C/EBP-β and HNF-1 transcription factors. The deletion of the first C/EBP-β-HNF-1-enriched region (three C/EBP-β and three HNF-1 TFBSs overlapping in 1-100 bp region) had resulted in 2.7-fold activity reduction. A much higher decline (3.8-fold) in enhancer activity was observed in modified enhancer fragment pme103D1 with the second C/EBP-β-HNF-1-enriched region (five C/EBP-β and three HNF-1 TFBSs overlapping in 500-599 bp region) being deleted. The most significant decrease (6.4-fold) in enhancer activity was observed in modified enhancer fragment pme103D2 with the adjacently located five C/EBP-β-enriched region (800-899 bp region) being eliminated. The result from this study is consistent with that conducted by Plachetka et al. (2008) where they deduced that the opening of chromatin at *mim-1* enhancer could be activated by C/EBP-β when several binding sites were present within a close distance. Thus, it can be deduced that adjacently located five C/EBP-β-enriched region has the most significant influence towards pme103 enhancer activity in HepG2 cell line.

Comparison of Enhancer Activity across Two Cell Lines

In this study, the selected 13 candidate enhancers were tested for their activity in two cell lines, namely A549 (human lung carcinoma) and HepG2 (human liver carcinoma) and the comparison graphs are depicted in Figure 8. Interestingly, all studied enhancers have shown a significantly (unpaired student's t-test, $p<0.05$) lower luciferase activity level in A549 cell line compared to HepG2 cell line. This is a strong indication of high cell-type functional specificities of the liver-specific enhancers identified in this study. Similarly, Duan and Simpson-Haidaris (2006) had elucidated the liver tissue-specific role of IL-6-receptor-gp100-Stat3 signalling in Interleukin-6 (IL-6) initiation of γFBG promoter following the discovery that the promoter activity in HepG2 was 15-fold higher than that in A549.

As for the modified enhancer fragments generated from the deletion analysis, most of the modified enhancer fragments exhibited higher activity level than their wildtype enhancer in A549 cell line, with the

exception for modified enhancer fragments pme001a, pme001b and pme001c. The deletion of the TFBS-enriched regions in enhancer pme101 and pme103 had resulted in a significant (unpaired student's t-test, $p<0.05$) increment in enhancer activity in A549 cell line. This suggests that these TFBS-enriched regions are responsible for the maintenance of the cell-type functional specificities in the liver. In the case of the enhancer pme001, the modified enhancer fragments pme001a, pme001b and pme001c did not show higher enhancer activity in A549 cell line compared to their wildtype enhancer. This condition suggests that these three AP-1 TFBS pairs are not involved in the regulation of liver enhancer cell-specificity, probably due to their miniature length.

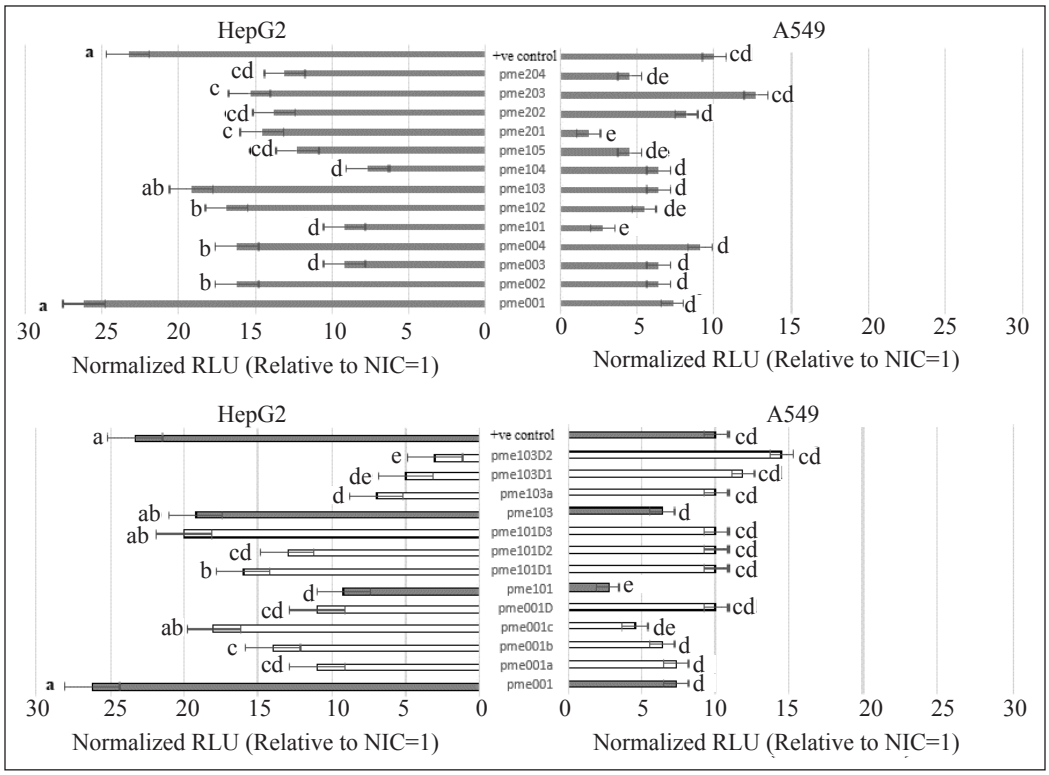

Figure 8. The normalized relative luciferase unit (relative to NIC=1) of wildtype enhancer and modified enhancer fragments in both HepG2 and A549 cell lines. Statistical analysis was conducted using one-way ANOVA and post-hoc Tukey's Test with $p<0.05$. Significantly different data are represented by different alphabets

CONCLUSION

In this study, a total of 13 liver-specific enhancers were isolated from the genome of the proboscis monkey, forming a population of enhancers identified via three different criterions: functional conservation, sequence conservation as well as combinatorial features. The luciferase reporter assay had

provided the information on the enhancer activities and the associations of these activities with TFBS are briefly investigated. The TFBS distribution pattern at every 100 bp interval had revealed a clearer picture on the possible contributors towards enhancer activities compared to the overall TFBS abundance and TFBS composition.

The deletion analysis was performed to further verify the influence of the TFBS-enriched regions exclusively identified in selected enhancers that accounted for their activities. Pairs of AP-1 TFBS and clusters of C/EBP-β TFBS were found to possess enhancing effects onto liver-specific enhancers of proboscis monkey whereas huge clusters of HNF-3β TFBS were found to have suppressing influences on enhancer activities in HepG2 cell line. Besides, these TFBS-enriched regions (except for AP-1 TFBS pairs) had substantial function in maintaining cell-type specificities in liver-specific enhancers of the proboscis monkey. This study serves as a preliminary exploration into the enhancer landscape of the proboscis monkey and as stepping stone for future conservation research for this endangered non-human primate. In future, *in vivo* studies using mice as model organism are essential for the verification of enhancer activities in the living organism context.

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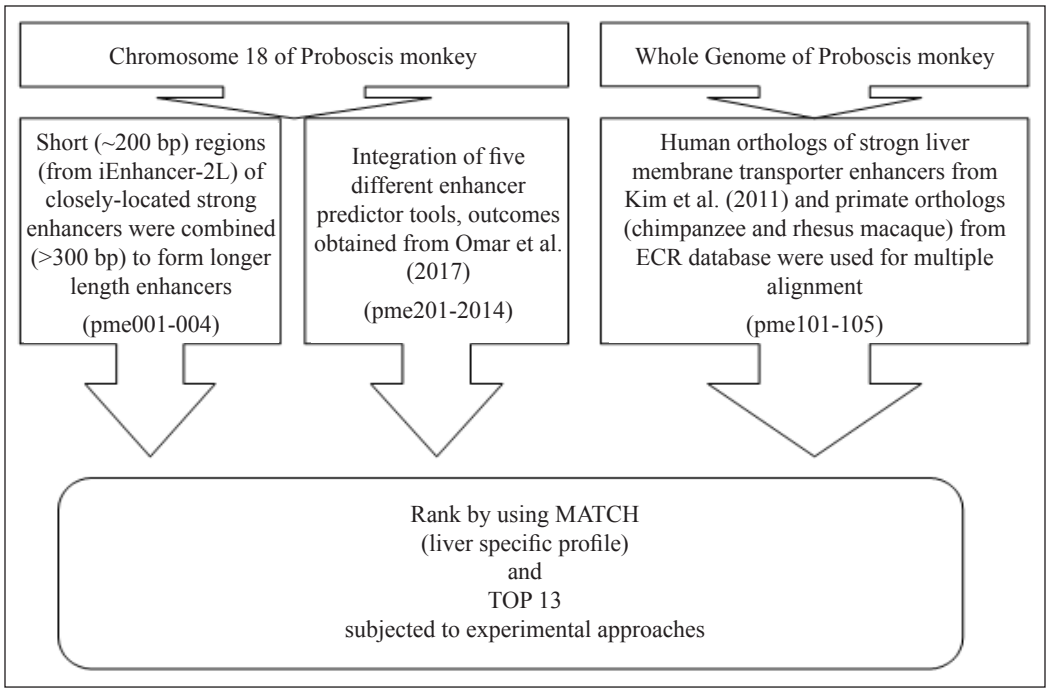
APPENDIX



Supplementary Figure 1. Flow chart of computational enhancer predictions

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Genetic Variation and Relationship of N'Dama Cattle Toll-like Receptor 5 with Other Bovine Species

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ABSTRACT

Toll-like receptor 5 is involved in innate immune responses that are initiated by host pattern recognition receptors (PRRs), which recognize molecular structures of conserved pathogen-associated molecular patterns (PAMPs) expressed by microorganisms especially bacteria. In this study, we sequenced 2577 bp bovine TLR5 in N'Dama cattle and discovered four synonymous mutations with one (C2127T) being shared between the N'Dama and the wild cattle. Sequences of other bovine species including *Bos taurus*, *Bos indicus* and *Bos javanicus* from public domain revealed higher number of non-synonymous mutations 19, 7 and 6 in wild cattle, *Bos indicus* subspecies and the *Bos taurus* respectively with a higher ratio of total number of non-synonymous mutations to that of synonymous mutations suggesting that the gene is evolving under adaptive evolution. The results of genetic diversity revealed a combination of high haplotype diversity and low nucleotide diversity which is an evidence of past and rapid demographic expansion from a small effective population size. Haplotype reconstructions, median-joining networks and phylogenetic analysis revealed haplotype sharing among *Bos taurus*, *Bos indicus* and their hybrid suggesting retention of conserved ancestral variation that predates subspecies divergence in this immune gene. There is no haplotype sharing between the wild and the domestic cattle, but a close relationship of the wild cattle clade with one of the N'Dama cluster suggesting

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little exchange of genetic material between these two groups of cattle. This results will facilitate effort towards understanding the relationship between mutations in different bovine species and their involvement in differential susceptibility and or tolerance to various diseases.

Keywords: Bovine phylogeny, genetic diversity, network analysis, Toll-like receptor 5

INTRODUCTION

Livestock has historically constituted one of Africa's major economic resources in terms of the livelihoods of its populations and this is because livestock has largely resisted to transfer from the traditional sector to modern production methods, especially in West-Central Africa. Indeed, throughout most of this region, the majority of the livestock, especially cattle is still being managed under traditional system of rearing (Blench & MacDonald, 2006). Cattle domestication (*Bos taurus* and *Bos indicus*) around 11,000 years ago from wild aurochsen (*Bos primigenius*) represents a major development in the Neolithic transition and was an important step in human history, leading to extensive modifications of the diet, the behavior, and the socioeconomic structure of many populations (Beja-Pereira et al., 2006; Bellwood, 2004; Clutton-Brock, 1999; Edwards et al., 2004). Recent mitochondrial DNA (mtDNA) studies indicated that *B. taurus* was introduced into Europe and Africa, where they interbred with local wild animals (Beja-Pereira et al., 2006). Hybridization of domestic bovine (*Bos indicus*) with their wild species (*Bos javanicus*) or banteng occurs worldwide either spontaneously or through organized cross breeding which usually lead to hybrid vigour or combination of desired attributes of the parental species (Nijman et al., 2003). The Indonesian Madura zebu breed is reputed to be hybrid of domestic and wild cattle as it carries mitochondrial DNA of either zebu or banteng origin. The banteng mitochondrial type was found in all animals of isle of Bali, Indonesia and the introgression was also found in 35% of the animals from a Malaysian Bali-cattle population (Nijman et al., 2003). In Africa, introgression of the Indian zebu in taurine breeds occurs, and has been hypothesized to improve the tolerance of the cattle (*Bos taurus*) to hot and dry environments (Bradley et al., 1994, 1996; Epstein, 1971; Hanotte et al., 2000; Loftus et al., 1994). The N'Dama taurine cattle breed from West Africa is trypanotolerant and have resistance to tick and tick-borne diseases as well as resistance to *Haemonchus contortus*. The cattle also possesses important attributes such as heat tolerance, adaptation to harsh environments and ability to survive on poor quality feeds and has been involved in various breeding progammes to exploit its adaptive potentials (Claxton & Leperre, 1991; Murray et al., 1991). The mammalian Toll-like receptors (TLRs) play an important role in the recognition of invading pathogens and the modulation of innate immune responses (Aderem & Ulevitch, 2000). Several studies have shown that mutations in the TLRs may reduce the ability of the protein to recognize PAMP, and hence

interfere with innate immune activation (Lin et al. 2012; Medmedev, 2013; Pandey & Agrawal, 2006). Characterizing genetic variation in these loci may be useful for guiding genetic selections for disease resistances. Toll-like receptor 5 (TLR5) is known to bind bacterial flagellin while these gene in *E. coli*, primates and other mammals have shown evidences of adaptive positive selection which suggest that interspecies competition between host and pathogen is likely to be driving the co-evolution of pathogen and host (Areal et al., 2011; Smith et al., 2012; Takeda & Akira, 2001). Genes that modulate innate immunity have often been considered as candidate loci for improving host resistance to disease in agricultural species (Plain et al., 2010). The ultimate goal of bovine genomic study is the identification of genetic variation that modulates corresponding variation in economically important production traits, differential susceptibility to disease, and favorable host response to vaccines, which is expected to enable the improvement of these phenotypes via informed genomic selections (VanRaden et al., 2009). The bovine genome sequence and first-generation HapMap projects (Elsik et al., 2009; Tellam, 2009) have directly enabled genome-assisted selective breeding (VanRaden et al., 2009), nascent investigations of non-traditional traits such as marker assisted vaccinations (as diagnostics for enhanced vaccine design or animal response), the development of a new class of anti-infectives known as innate immunologicals (Rosenthal, 2006), and the elucidation of loci that have evolved under strong selection, thus providing important computational evidences for genomic regions which may underlie economically important traits. Indiscriminate hybridization especially in natural population may have had a significant impact on the formation of domestic breeds and can also affect the genetic integrity and diversity of domestic and wild species. Evaluation and monitoring the species composition of these animals may be essential for future preservation of genetic diversity. Therefore the current study is to assess the relationship of N'Dama cattle with other bovine species using TLR5 sequence. To evaluate the naturally occurring variation and haplotype structure of Toll-like receptor 5 in bovine species and determine whether there is variation and haplotype sharing between the wild and the domestic cattle.

MATERIALS AND METHODS

Sampling, DNA Extraction and Purification

Genomic DNA was extracted from air-dried blood samples preserved on FTA classic cards (Whatman Biosciences) with the recommended manufacturer protocol, from 60 unrelated N'Dama cattle samples that were sampled from three different locations; Institute of Agricultural Research and Training (IAR and T) Moore plantation Apata, Ibadan, Fasola farms in Oyo, and the Cattle Production Venture (CPV) of the Federal University of Agriculture Abeokuta, Nigeria.

The extracted DNA was quantified for concentration and purity, A260/280

ratio between 1.8 and 2.0 were assessed using a Nanodrop 1000 spectrophotometer in congruent with protocol reported by Desjardins and Conklin (2010). The potential genomic DNA degradation was determined using 1% agarose gel electrophoresis. After quantification the samples were kept at -20˚C for further analyses. All procedures were approved by the Animal Experimentation local ethics board at Federal University of Agriculture, Abeokuta, Nigeria. To assess the relationship of N'Dama cattle with other bovine populations based on TLR5 gene, sequences of the gene of *Bos indicus* (n = 5), *Bos javanicus* (n = 1), crossbred (n = 3) and other *taurine* (n = 20) TLR5 were downloaded from the National Centre for Biotechnology Information (NCBI) and included in the analysis (Supplementary Table ST1). The downloaded sequences have not been previously analyzed for relationship among bovine species.

Table ST1

Sequence of cattle TLR5 retrieved from GENBANK and their accession numbers

Accession number	Cattle name	Cattle type
JQ805131.1	Red Angus	*Bos taurus*
JQ805126.1	Brown swiss	*Bos taurus*
JQ805132.1	Texas Longhorn	*Bos taurus*
JQ805135.1	Texas Longhorn	*Bos taurus*
JQ805125.1	Angus	*Bos taurus*
JQ805137.1	Angus	*Bos taurus*
EU006639.1	Angus	*Bos taurus*
JQ805128.1	Holstein	*Bos taurus*
JQ805129.1	Holstein	*Bos taurus*
EU006638.1	Holstein	*Bos taurus*
DQ335128.1	Holstein	*Bos taurus*
EU006640.1	Limousin	*Bos taurus*
JQ805127.1	Charolais	*Bos taurus*
EU006637.1	Charolais	*Bos taurus*
JQ805133.1	Hereford	*Bos taurus*
JQ805134.1	Hereford	*Bos taurus*
JQ805130	Maine Anjou	*Bos taurus*
JQ805136.1	Maine Anjou	*Bos taurus*
AY634631.2		*Bos taurus*
NM_001040501.1		*Bos taurus*
XM_019976811.1	Nelore	*Bos indicus*
EU006636.1	Brahman	*Bos indicus*
GQ248711.1	Sahiwal	*Bos indicus*
GQ866979.1		*Bos indicus*
EU006641.1	Nelore	*Bos indicus*
EU006643.1	Romagnola	*Bos taurus x Bos indicus*

Table ST1 *(Continued)*

Accession number	Cattle name	Cattle type
EU006642.1	Piedmontese	*Bos taurus x Bos indicus*
EU006635.1 JQ811841.1	Braford	Bos taurus x Bos indicus Bos javanicus

Toll–like Receptor 5 PCR Assay and DNA Sequencing

Published primer sets based on cattle TLR5 (Table 1) was used to amplify the target region from the extracted DNA with the amplification product length of 2577 bp exonic region. The PCR mixture consisted of 1 disc of cattle DNA on FTA card as template, 0.25µl of 10 µM of forward and reverse primers, 3.2 µl dNTP mixture, 0.2 µl Taq polymerase (Promega, USA), 2.0 µl 10X Buffer and ddH_20 to a final volume of 20 µl. After initial denaturation of 95°C for 3 min and final denaturation of 95°C for 1 min, the samples were subjected to 35 cycles of 60°C annealing temperature, 72°C for 90 sec initial elongation and followed by 75°C final elongation step for 5 min on Agilent Surecycler 8800. The PCR products were run on 1.2% agarose gels with ethidium bromide (0.05µL/ml) using 200 bp size standard ladder. After passing 100 v for 5 mins, the gels were viewed under UV light and photographed using Alpha mega® 2200version 5.5 gel documentation system. The PCR products were purified using a commercial kit (QIAquick®PCR purification kit, Canada). The purified products were subjected to sequencing in a 20 µL reaction mixture comprising approximately 20 ng of purified PCR products, 3.2 pmol of primer and 8 µl of BigDye® terminator cycle sequencing kit (Big Dye Terminator Ready Reaction Mix (mixture of dNTPs, ddNTPs, buffer, enzyme and $MgCl_2$), 8 µL of deionized water, 2 µL template DNA using a ABI 3730×1 (Applied Biosystems) capillary DNA analyzer with 25 cycles at 96°C for 10 s, 50°C for 5 s and 60°C for 4 min followed by a rapid thermal ramp to 4°C after the last cycle and holding until the purification of the sequencing product. This was carried out at genome Quebec facility situated in the McGill University Campus, Quebec, Canada.

Table 1

Cattle Toll-like receptor 5 sequence primers (Smith et al., 2012)

Sequence Primer	Primer Type	Direction	Sequence
TLR5 Primer Set 1	Forward	5' > 3'	GCTCAGTGCCTTGAGCTTAGA
	Reverse	3' > 5'	TCAAGGAATTCAGTTCCCG
TLR5 Primer Set 2	Forward	5' > 3'	CCGATGCTGTATTAAAAGATGG
	Reverse	3' > 5'	TTCAGCTCCTGGAGTGTCTC

Table 1 *(Continued)*

Sequence Primer	Primer Type	Direction	Sequence
TLR5 Primer Set 3	Forward	5' > 3'	CCAGGAGCTCGATGATACAG
	Reverse	3' > 5'	GGGCATGGTTTTGGTGAC
TLR5 Primer Set 4	Forward	5' > 3'	TTCCTTCTCCAGGTACCTCATC
	Reverse	3' > 5'	AAAGACTGTAAATGGAAACCCC
TLR5 Primer Set 5	Forward	5' > 3'	ATCACAATAGCTGGGTCTCCA
	Reverse	3' > 5'	CAGGCCACCTCAAGTACTGC
TLR5 Primer Set 6	Forward	5' > 3'	CCCAGAGTCTGCTGTTCAAG
	Reverse	3' > 5'	GGCTTGCGATAAGTGGAAAC

Data Analysis

Cattle TLR5 that had only one exon which was 2577-bp long was used for the analysis. Other sequences that were not complete were removed during quality check and preliminary analysis. Sequences viewing, trimming and editing were carried out using BioEdit software (Hall, 1999), sequence alignment with the reference sequence excluding all gaps was carried out using CLC Genomics Workbench 7 (http://www.clcbio.com/blog/clc-genomics-workbench-7-5/) and CLUSTAL W software (Thompson et al., 1994) implemented in MEGA 7 (Tamura et al., 2011). Polymorphism within the sequences of TLR5 of N'Dama and other downloaded cattle populations with their allele frequencies were determined using MEGA 7 (Tamura et al., 2011) and Codon code aligner (www.codoncode.com/aligner/down loade.htm). Hereford cattle sequence (accession number, JQ805134.1) was used as the reference sequence. Genetic diversity indices in terms of number of haplotypes, haplotype diversity, nucleotide diversity and average number of nucleotide differences were estimated using DnaSP V6 (Librado & Rozas, 2009). For phylogenetic and median-joining network analyses, a neighbor-joining with distance matrices generated according to the Kimura 2p model was used using MEGA7 (Tamura et al., 2011) and NETWORK 5.0.0.3 (Bandelt et al., 1999; Fluxus Technology [www.fluxus-engineering.com]) softwares were used to determine the evolutionary relationship between the N'Dama TLR5 gene and other downloaded taurine, zebu and the cattle hybrid populations. The reliability of the inferred phylogenetic tree was evaluated using bootstrap analysis of 1000 replications.

The Federal University of Agriculture, Abeokuta Animal Care and Use Committee approved the sampling procedures including the number of animals sampled. The license number for our sampling procedures was unaabACUC F003/1015. The samples involved no endangered or protected animal species while blood sample collection was carried out by veterinarian with no

tranquilizer nor short-acting anesthetics used on manually restrained animals.

RESULTS

Identification of Single Nucleotide Polymorphism within the Tlr5 Sequence of N'Dama, Other Taurine, Zebu, Crossbred and Banteng

Because of the length of the gene (2577 bp) and the use of four primer pairs, analysis of the N'Dama sequences showed only 28 samples that passed quality test and were complete out of the 60 animals sampled. These complete samples were used for the downstream analysis. Screening for polymorphism within the sequences of the N'Dama cattle used in the course of this study revealed four polymorphisms with two of them being fixed (T1761C, G2460A) in N'Dama population and were also detected in other taurine and zebu cattle, but not in banteng (Table 2). The two other polymorphisms were specific (G993A, C2127T) to our samples and were however still segregating as their frequencies in the population were less than 100. All the four polymorphisms are synonymous as seen in Table 2.

Table 2

Single nucleotide polymorphisms (SNPs) detected in NDAMA cattle TLR5

S/N	Alleles	Exonic position	Observed frequency	Amino acid position	Amino acid	Effect on protein function
1	G/A	993	14.29	331	A/A	Synonymous
2	T/C	1761	100	587	L/L	Synonymous
3	C/T	2127	14.29	709	N/N	Synonymous
4	G/A	2460	100	820	E/E	Synonymous

The exonic position is based on Hereford cattle sequence with accession number JQ805134.1

From the 20 sequences of TLR5 of taurine cattle available at NCBI, 11 polymorphisms were observed with four of these polymorphisms synonymous while the remaining seven were non-synonymous (Table 3).

The two synonymous polymorphisms at position 1761 and 2460 were shared with our N'Dama samples while seven of the polymorphism were being shared with the zebu cattle with four specific to taurine cattle as shown in Table 3.

Eleven polymorphisms were detected within this region of zebu cattle that were available in the public domain. Five of these polymorphisms were synonymous while the other six were non-synonymous (Table 4). Eight of these polymorphisms were shared between the zebu and taurine cattles while three were specific to the zebu including two non-synonymous (A541R, C2037S) and one synonymous (A504R) as shown in Table 4.

Only two synonymous polymorphisms that were shared between both taurine and

Table 3

Single nucleotide polymorphisms (SNPs) detected in taurine cattle TLR5

S/N	Alleles	Exonic position	Amino acid position	Amino acid	Effect on protein function	Breeds
1	C/Y	1131	377	D/D	Synonymous	Texas longhorn
2	T/Y/C	1761	587	L/L	Synonymous	Holstein, Angus, Charolais, N'Dama, Brown Swiss, Maine Anjou, Texas longhorn
3	C/Y	1938	646	V/V	Synonymous	Charolais
4	G/R/A	2460	820	E/E	Synonymous	Holstein, Angus, Charolais, N'Dama, Brown Swiss, Maine Anjou
5	T/Y	470	157	I/T	Non-Synonymous	Texas longhorn
6	A/R	1132	378	K/E	Non-Synonymous	Texas longhorn
7	T/Y	1324	442	Y/H	Non-Synonymous	Texas longhorn
8	G/R	1975	658	E/K	Non-Synonymous	Texas longhorn
9	G/S	2208	736	P/?	Non-Synonymous	Texas longhorn
10	G/R	2524	842	E/K	Non-Synonymous	Texas longhorn
11	G/R	2542	848	A/T	Non-Synonymous	Texas longhorn

The exonic position is based on Hereford cattle sequence with accession number JQ805134.1

Table 4

Single nucleotide polymorphisms (SNPs) detected in zebu cattle TLR5

S/N	Allele	Exonic position	Amino acid position	Amino acid	Effect on protein function	Breed
1	A/R	504	168	E/E	Synonymous	Sahiwal
2	C/Y	1131	377	D/D	Synonymous	Nelore, Brahman, Sahiwal, other indicus
3	T/Y/C	1761	587	L/L	Synonymous	Nelore, Brahman, other indicus
4	G/C/S	2208	736	P/P	Synonymous	Nelore, Brahman, other indicus

Table 4 *(Continued)*

S/N	Allele	Exonic position	Amino acid position	Amino acid	Effect on protein function	Breed
5	G/A/R	2460	820	E/E	Synonymous	Nelore, Brahman, other indicus
6	A/R	541	181	K/E	Non-Synonymous	Sahiwal, Nelore
7	A/R	1132	378	K/E	Non-Synonymous	Nelore, Brahman, Sahiwal, other indicus
8	T/Y/C	1324	442	Y/H	Non-Synonymous	Nelore, Brahman, other indicus
9	G/R	1975	659	A/T	Non-Synonymous	Nelore, Brahman, Sahiwal, other indicus
10	C/S	2037	679	F/L	Non-Synonymous	Nelore, Sahiwal
11	G/R	2524	842	E/K	Non-Synonymous	Nelore, Brahman, Sahiwal, other indicus

The exonic position is based on Hereford cattle sequence with accession number JQ805134.1

zebu cattles were detected in the sequence of crossbred cattle (Table 5). Twenty seven polymorphisms were observed within this region of banteng (*Bos javanicus)* sequence. Eight of these polymorphisms were synonymous while 19 were non-synonymous. *Bos javanicus* shared one non-synonymous polymorphism (A541R) with *Bos indicus,* one non- synonymous mutation (G2542R) together with taurine and two other polymorphisms, one synonymous (n.2208) and one non-synonymous (n.1975) with the taurine and zebu populations. *Bos javanicus* also shared one synonymous mutation (C2127T) with our locally adapted N'Dama cattle population (Table 6).

Table 5

*Single nucleotide polymorphisms (SNPs) detected in sequence of TLR5 of the crossbred (*Bos indicus *X* Bos taurus*) cattle*

S/N	Alleles	Exonic position	Amino acid position	Amino acid	Effect on protein function	Breeds
1	T/Y/C	1761	587	L/L	Synonymous	Braford, Romangnola, Piedmontese
2	G/A/R	2460	820	E/E	Synonymous	Braford

The exonic position is based on Hereford cattle sequence with accession number JQ805134.1

Table 6

Single nucleotide polymorphisms (SNPs) detected in banteng sequence of TLR5

S/N	Alleles	Exonic position	Amino acid position	Amino acid	Effect on protein function	Breeds
1	G/R	633	211	R/R	Synonymous	Bos javanicus
2	C/Y	1005	335	L/L	Synonymous	*Bos javanicus*
3	C/Y	1512	504	L/L	Synonymous	*Bos javanicus*
4	T/A	1983	661	T/T	Synonymous	*Bos javanicus*
5	G/A	2070	690	T/T	Synonymous	*Bos javanicus*
6	C/T	2127	709	N/N	Synonymous	*Bos javanicus*
7	G/C/S	2208	736	P/P	Synonymous	*Bos javanicus*
8	T/Y	2241	747	A/A	Synonymous	*Bos javanicus*
9	G/R	190	64	A/T	Non- Synonymous	*Bos javanicus*
10	A/R	322	108	N/D	Non- Synonymous	*Bos javanicus*
11	G/R	397	133	D/N	Non- Synonymous	*Bos javanicus*
12	G/R	500	167	R/Q	Non- Synonymous	*Bos javanicus*
13	A/R	541	181	K/E	Non- Synonymous	*Bos javanicus*
14	G/R	785	262	R/H	Non- Synonymous	*Bos javanicus*
15	G/A	1558	520	G/R	Non- Synonymous	*Bos javanicus*
16	C/T	1844	615	S/F	Non- Synonymous	*Bos javanicus*
17	G/A	1852	618	G/R	Non- Synonymous	*Bos javanicus*
18	A/G	1882	628	S/G	Non- Synonymous	*Bos javanicus*
19	T/C	1884	628	S/G	Non- Synonymous	*Bos javanicus*
20	T/C	1934	645	L/S	Non- Synonymous	*Bos javanicus*
21	G/A	1935	645	L/S	Non- Synonymous	*Bos javanicus*
22	A/G	1966	656	I/V	Non- Synonymous	*Bos javanicus*
23	C/T	1970	657	T/I	Non- Synonymous	*Bos javanicus*
24	G/A/R	1975	659	A/T	Non- Synonymous	*Bos javanicus*
25	A/M	2115	705	E/D	Non- Synonymous	*Bos javanicus*
26	C/A	2158	720	H/N	Non- Synonymous	*Bos javanicus*
27	G/R	2542	847	A/T	Non- Synonymous	Bos javanicus

Bovine Haplotype Networks, Haplotype Sharing and Genetic Diversity

The network analysis revealed 19 haplotypes that differed from each other by a small number of mutations within the sequence of TLR5 of domesticated and wild bovine with an overall high diversity of 0.7675 and low nucleotide diversity of 0.00093 (Table 7), among which two were specific to the wild cattle forming an haplogroup

with 27 polymorphic site, high hd, low pi, and high average number of nucleotide differences. The other haplogroup was for the domesticated bovine and which formed a star. The median-joining networks (Table ST1, ST2 and Figure 1) of TLR5 in the bovine species revealed no haplotype sharing between the wild and the domesticated species and predicted low level of haplotype sharing between the *Bos taurus* and *Bos indicus*. The recent formation of hybrid of *Bos taurus* and *Bos indicus* predicted haplotypes which fell essentially within the network node that was dominated by both *Bos taurus* and *Bos indicus*. Some of the predicted haplotypes of the hybrid cattle were shared only with *Bos taurus* cattle while the other stood out as a unique network node. Our N'Dama which is an African taurine had two unique haplotypes and shared one other haplotype with the other taurine breeds used in the study. The least haplotype, nucleotide diversity and average number of nucleotide differences were detected in the N'Dama cattle. In total, 19 haplotypes were discovered in the study with 8 found in *Bos taurus* among which one haplotype (Hap_1) was shared with *Bos indicus* and the hybrid while another one was shared with the hybrid only (Table ST3). In the *Bos indicus*, nine haplotypes were discovered with only being shared with the *Bos taurus* and the hybrid and which could be referred to as the domesticated haplotype while the other eight were not shared but were specific to the sub species. The zebu also had high haplotype diversity (0.909±0.047) with the highest number of haplotypes. The hybrid had only 3 haplotypes with only one being specific or found in that population alone, while one is shared between the hybrid and the *Bos taurus* and the other one found among the hybrid, the taurine and the zebu. The result of the genetic diversity revealed that the *Bos taurine* possessed the least diversity while the highest was observed in the *Bos javanicus* or the wild cattle (Table ST3).

Table 7

Genetic diversity of Toll-like receptor 5 in bovine species

Breed	N	S	h	hd	π	K
N'Dama	40	4	4	0.502±0.098	0.00024±0.00022	0.63±0.22
Taurine	23	11	4	0.686±0.040	0.00047±0.00028	1.21±0.34
Zebu	10	11	14	0.909±0.047	0.0015±0.00035	3.86±0.47
Bos indicus X *Bos taurus*	3	2	3	0.679±0.122	0.00030±0.00021	0.79±0.19
Bos javanicus	1	27	3	0.833±0.222	0.0062±0.0011	16.00±3.43
Mean	66	38	19	0.7675±0.027	0.00093±0.00044	2.41±0.75

N = number of sample, Number of polymorphic site = S, haplotype = h, haplotype diversity = hd, Nucleotide diversity = π, Average number of nucleotide differences = K

Table ST2

Toll-like receptor 5 haplotypes detected in the bovine species

	0190	0322	0397	0470	0500	0504	0541	0633	0785	0993	1005	1131	1132	1324	1512	1558	1761	1844	1852	1882	1884	1934	1935	1938	1966	1970	1975	1983	2037	2070	2115	2127	2158	2208	2460	2524	2542	2524
Haplotypes	G	A	G	T	G	A	A	G	G	G	C	C	A	T	C	G	T	C	G	A	T	T	G	C	A	C	G	T	C	G	A	C	C	G	T	G	G	G
Hap_1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Hap_2	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.
Hap_3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.
Hap_4	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	A	.	.
Hap_5	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Hap_6	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	C	.	.	.	.
Hap_7	.	.	.	C	.	.	.	.	.	.	.	T	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.
Hap_8	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	A	.	.
Hap_9	.	.	.	.	.	.	.	.	.	.	.	T	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.
Hap_10	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	C	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	A	A	.
Hap_11	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	C	.	A	.	.
Hap_12	.	.	.	.	.	.	.	.	.	.	.	T	G	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.
Hap_13	.	.	.	.	.	G	.	.	.	.	.	T	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	C	.	.	A	.
Hap_14	.	.	.	.	.	.	G	.	.	.	.	.	G	C	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	C	.	.	.	.
Hap_15	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	A	.	.
Hap_16	.	.	.	.	.	.	.	.	.	.	.	T	.	C	.	.	C	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	A	.
Hap_17	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.
Hap_18	A	G	.	.	A	.	.	.	.	.	.	.	.	.	.	A	.	T	A	G	C	C	A	.	G	T	A	A	.	A	.	T	A	C	.	.	.	A
Hap_19	.	.	A	.	.	.	G	A	A	.	T	.	.	.	T	A	.	T	A	G	C	C	A	.	G	T	A	A	.	A	C	T	A	C	C	.	.	.

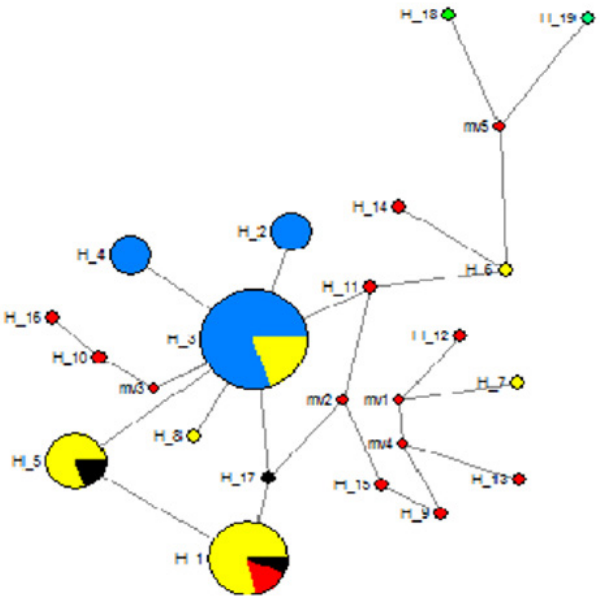

Figure 1. Toll-like receptor 5 haplotype network of bovine species constructed using median-joining method (Bandelt et al. 1999). The circles represent different haplotypes, and the areas of the circles are proportional to the frequency of each haplotype. The different species are distinguished by colour: Domesticated species: N'Dama (Blue), *Bos taurine* (Yellow), *Bos indicus* (Red), crossbred (Black), Wild Species: *Bos javanicus* (Green)

Table ST3

TLR5 haplotypes shared within and between the bovine species

Haplotype	Frequency	Species			
		Bos taurus	Bos indicus	Cross	Bos javanicus
Hap_1	28	22	4	2	-
Hap_2	8	8(N'Dama)	-	-	-
Hap_3	50	40(N'Dama) 10 (other taurine)	-	-	-
Hap_4	8	8(N'Dama)	-	-	-
Hap_5	16	13	-	3	-
Hap_6	1	1	-	-	-
Hap_7	1	1	-	-	-
Hap_8	1	1	-	-	-
Hap_9	1	-	1	-	-
Hap_10	1	-	1	-	-
Hap_11	1	-	1	-	-
Hap_12	1	-	1	-	-

Table ST3 *(Continued)*

Haplotype	Frequency	Species			
		Bos taurus	Bos indicus	Cross	Bos javanicus
Hap_13	1	-	1	-	-
Hap_14	1	-	1	-	-
Hap_15	1	-	1	-	-
Hap_16	1	-	1	-	-
Hap_17	1	-	-	1	-
Hap_18	1	-	-	-	1
Hap_19	1	-	-	-	1

Phylogenetic Relationships

Phylogenetic analyses were performed to estimate the genealogical relationships between the sequences and haplotypes that were detected in the study (Figures 2 and 3). Using the sequences, four clades were discovered with the major one being shared among *Bos taurus*, *Bos indicus* and their hybrid, two of clades were specific to our N'Dama cattle population while the last one belonged to the wild cattle. Although the topology of the consensus tree using the haplotypes (Figure 3) showed polytomies in the domesticated cattle in which the relationships could not be fully resolved to dichotomies, in general, we observed a clade that contained only the wild cattle which formed an haplogroup in the network (Figure 1), the other clade was regarded as the domesticated cattle haplogroup (Figure 1) with three subclades named C1, C2 and C3 (Figure 3). In this polytomy, subclades C3 comprises the *Bos taurus*, *Bos indicus* and their hybrid which indicate some levels of haplotype sharing between them. Subclade C2 is majorly dominated by *Bos taurus* while C1 was dominated by *Bos indicus*. Finally the three subclades correspond to expansions of haplotypes shared among the subspecies of cattle. The phylogenetic analyses further confirmed the close relationship between these two species.

DISCUSSION

Molecular information is crucial for preserving genetic diversity as well as preventing undesirable loss of alleles. The genomes of modern cattle basically reflect the history of animal movements by migratory farmers out of the ancient centers of the cattle domestication. Different cattle breeds therefore are expected to show genetic diversity consistent with their history of migration following domestication. TLR5 is among the evolutionary conserved pattern recognition receptors involved in the activation of the immune system in response to various pathogens and the innate defense against infection while polymorphisms within it have been shown to evolve differently under different evolutionary forces (Alcade & Edward, 2011; Seabury et al., 2007; Smith et al., 2012).

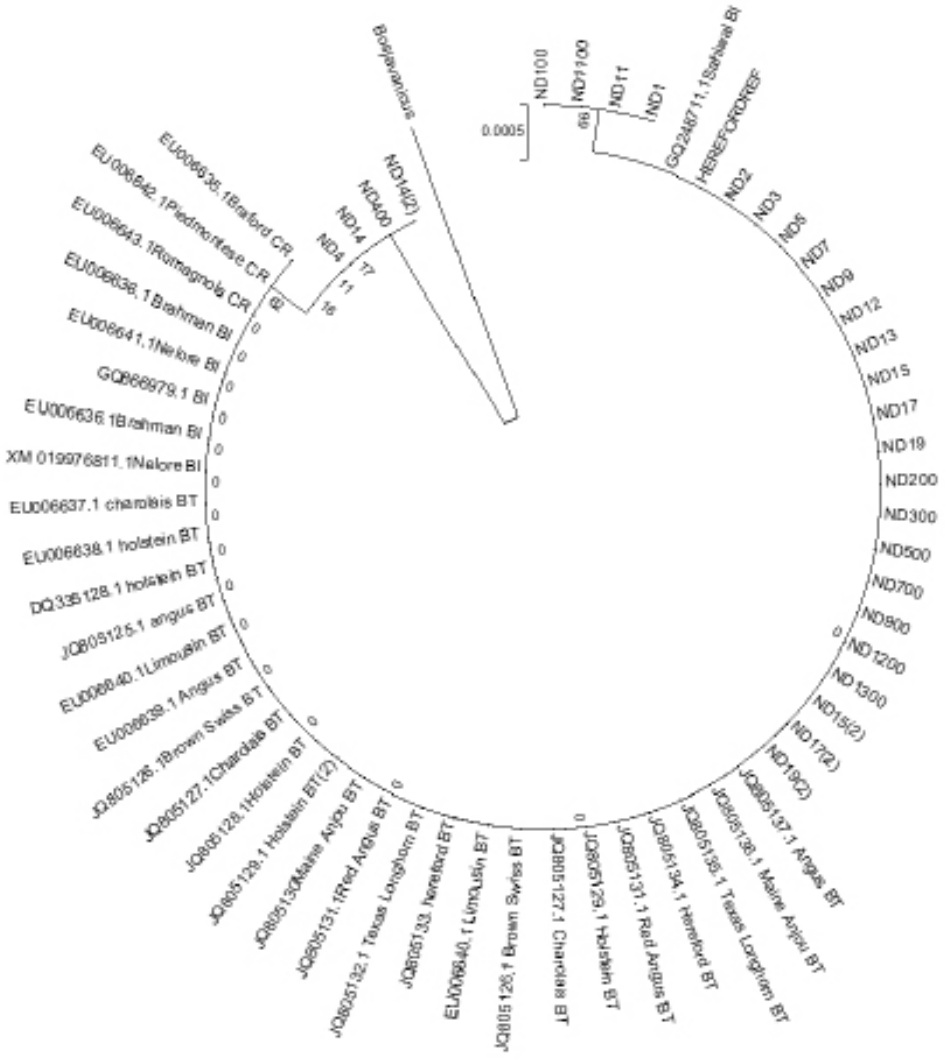

Figure 2. Neighbor-joining tree reconstructed from the sequence of Toll-like receptor 5 of our samples and other bovine species used in this study. The framework was based on Kimura 2p distances as grouped by the neighbor-joining method. The percentage bootstrap value is represented by the numbers at the node after 1000 replications

In this study four polymorphisms were detected in the TLR5 sequence of N'Dama cattle. The polymorphism rate in the animal is low suggesting that the gene is conserved. Although the polymorphism detected are synonymous, they may however be evolutionary relevant as evidence has suggested that supposed silent mutations have effect on protein expression, conformation and function of immune genes (Sauna & Kimchi-Sarfaty, 2011). On the basis of nucleotide sequence variation, two of the

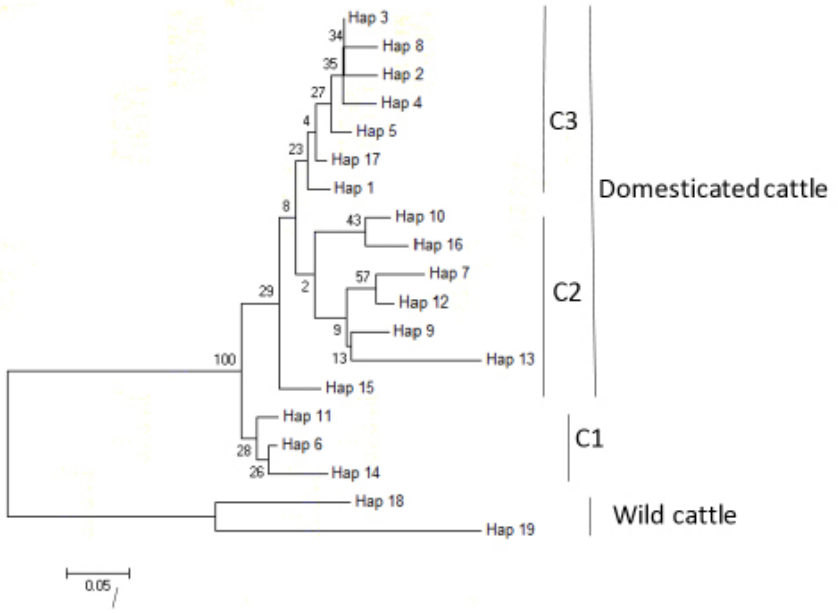

Figure 3. Neighbor-joining tree reconstructed from the 19 haplotypes identified in the TLR5 sequences of N'Dama and other bovine species in this study. The framework was based on p-distances as grouped by the neighbor-joining method. The percentage bootstrap value is represented by the numbers at the node after 1000 replications

mutations are fixed and are being shared between the sub-species of domestic cattle. These suggest that these two mutations must have been ancestral and in existence before the divergence of the *Bos taurus* and *Bos indicus* cattle some 250, 000 years ago (250 Kyr) (Bradley et al., 1996). Unlike other, the domesticated cattle, the N'Dama cattle shared one synonymous mutation with the wild cattle which supported an evidence of genetic introgression of wild cattle into the domestic cattle. It has been reported that hybridization between wild and zebu cattle sub-species occurs worldwide either spontaneously or by organized crossing and it is possible, especially if closely related species share an overlapping habitat or through human intervention during captive breeding (Nijman et al., 2003). The banteng (wild) mitochondrial type has been reported to be found in Bali cattle of Indonesia, while Madura animals also carry mitochondrial DNA of either zebu or banteng origin and which were confirmed using AFLP, SFLP and microsatellite markers (Nijman et al., 2003). In the sequence of TLR5 of bovine species used in this study, there is higher ratio of non-synonymous to synonymous mutations especially in the wild cattle which suggests adaptive evolution in this immune gene (Smith et al., 2012). Previous studies had found evidence of adaptive evolution

in mammalian TLR5 and also revealed that TLR5 genes of domestic livestock had a concentration of single nucleotide polymorphisms which suggests a specific signature of adaptation (Areal et al., 2011; Seabury et al., 2007; 2010; Smith et al., 2012; Wlasiuk et al., 2009). Smith et al. (2012) using codon models of evolution, detected concentration of rapidly evolving codons within the TLR5 extracellular domain which was a site of interaction between host and the bacterial surface protein flagellin.

The analysis of sequences generated for this study and from the sequences from the NCBI GenBank database revealed a high level of haplotype and nucleotide diversity suggesting the cattle population had remained stable with an old evolutionary history (Grant, 1998; Grant & Bowen, 1998). Domesticated cattle through analysis of metrics of genetic diversity by group revealed that domesticated cattle had moderate nucleotide and haplotype diversity which might suggest that they originated from small number of founders while the higher diversity observed in the wild cattle might indicate a bottleneck which was followed by a period of rapid expansion (Grant, 1998; Grant & Bowen, 1998). The combination of high haplotype diversity and low nucleotide diversity in this study can be evidence of past and rapid demographic expansion from a small effective population size (Avise, 2000). Recent studies have provided evidences of population bottlenecks at the time of domestication and breed formation in modern cattle (Bovine HAPMAP Consortium et al., 2009, Villa-Angulo et al., 2009). Apart from the wild cattle with the highest number of non-synonymous mutations, *Bos indicus* subspecies was found to contain a higher proportion of the total number of non-synonymous mutations compared to the total number of synonymous mutations suggesting that the mutations are evolutionarily recent events following the divergence of *Bos indicus* and *Bos taurus* which is estimated to have diverged as early as 200,000 years ago (Loftus et al., 1994). Future studies with more samples across the breeds which will give additional polymorphisms and haplotypes will be necessary to ascertain evolutionary history of this immune genes.

We found nineteen haplotypes that differ from each other by a small number of mutations with few of these haplotypes being shared among the *Bos taurus*, *Bos indicus* and their hybrid while majority of the haplotypes are specific to each subspecies which corroborate the assertion that the 250 Kyr divergence between these two subspecies has allowed genetic drift and or selection to drive different haplotypes to a high frequency between the subspecies (Bradley et al., 1996; Seabury et al., 2010). Haplotype sharing between these two subspecies lineages may suggest retention of conserved ancestral variation that predates subspecies divergence, or that both lineages have evolutionarily converged on a relatively small number of innate immune haplotypes at these loci (Seabury et al., 2010). Haplogoup C3

combines the two subspecies with their hybrid and can be regarded as the ancestral haplotype from which the other two radiate. The existence of this haplogroup with the other peripheral haplogroups as a star is an indication of population expansion from a small number of founders (Grant, 1998; Grant & Bowen, 1998). On the other hand, our results indicated that the dominant haplogroup C3 was the main founding haplotype of domesticated TLR5 since these haplogroup was detected in individuals of the *Bos taurus, Bos indicus* and their crossbred. The occurrence of no haplotype sharing between the domesticated and the wild cattle suggests that the evolution of this gene in the two groups is independent of each other. The presence of two unique haplotypes in N'Dama which is an African taurine suggests that the N'Dama although a taurine cattle, has been subjected to different evolutionary processes than the other taurines in the African sub-continent and might have contributed to its special adaptive features.

The phylogenetic relationship corroborated the result of the network analysis and revealed little or no introgression of the genetic material of the wild cattle in to the domestic cattle except for the sharing of mutation between the taurine N'Dama and the wild cattle and their grouping close together using sequences of TLR5. For the domestic cattle, a single maternal origin was predicted for the two subspecies before the recent sub speciation around 200,000 years ago. Our results also revealed introgression and exchange of genetic materials among the breeds of *Bos taurus* and *Bos indicus*. Despite the fact that our N'Dama cattle samples were from different locations, they were grouped into one cluster while some samples formed two other smaller clades. The phylogenetic relationship of the *Bos indicus* had shown that the breed Romangnola, Piedmontese, Braford were descendant from European cattle and they were from the same lineage with Brahman cattle, while *Bos indicus*, N'Dama and Sahiwal were closely related and are of African descent (Beja-Pereira et al., 2006). *Bos taurus* phylogenetic relationship also showed the relationship between all their breeds, indicating their descendant from the same ancestral lineage despite acquiring different polymorphisms and are slightly different from the N'Dama cattle because of their different evolutionary history having been subjected to different environmental conditions with different evolutionary processes (Beja-Pereira et al., 2006; Hanotte, 2000).

CONCLUSION

Our analysis of genetic variation, nucleotide diversity, haplotype network and phylogenetic relationship of bovine Toll-like receptor 5 immune gene will provide additional information in the study of the relationship between the mutations within this gene and the differential susceptibility and or tolerance or resistant to various diseases caused by bacteria, fungi and protozoans in future studies. Based on the use of TLR5 in bovine species, highest number of non-synonymous mutations was observed

in the wild cattle, followed by *Bos indicus* subspecies and the *Bos taurus* with a higher ratio of total number of non-synonymous mutations to that of synonymous mutations supporting the fact that the gene is evolving under adaptive evolution. The occurrence of sub specific haplotype sharing between the *Bos taurus* and *Bos indicus* with their hybrid led credence to the evidence of interbreeding between these subspecies. The separate clustering of the wild cattle from the domestic one except for sharing of mutation with N'Dama support the fact that the domestic cattle descends from aurochs with little or no introgression from the wild cattle.

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In vivo Fecundity Evaluation of *Phaleria macrocarpa* Extract Supplementation in Male Adult Rats

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ABSTRACT

"Mahkota Dewa" fruit (*Phaleria macrocarpa* [Scheff.] Boerl.), is a traditional Indonesian plant-based remedy that has been used traditionally for generations in treating multiple illness and diseases. This investigation intends to assess the fecundity effects of *P. macrocarpa* supplementation in adult male rats through hormonal, physical and histological changes. Sixty male Sprague Dawley rats were randomly distributed into two by five experimental design with two supplementation periods (3 and 7 weeks) allotted to 5 different doses of extract (0, 24, 48, 240 mg PM aqueous extract/ kg bw and 80 mg of commercial PM product/ kg bw). The mean sperm count (455 cells/ml), body weight (301 g), histological assessment of spermatogonia cells (87 cells), and thickness of seminiferous tubule layer (79 µm) significantly increased ($P < 0.05$) in rats treated with 240 mg/ kg dose. However, there were no changes in both physical appearances of testes (size and volume) and testosterone hormone levels among the treatment groups. Our findings indicated that supplementation of *P. macrocarpa* significantly increased the fecundity of rats and the effect was dose and time-dependent. The study suggested that *P. macrocarpa* offered an attractive and alternative potential for improving the fertility in men.

Keywords: Fertility, herbal remedy, male, *Phaleria macrocarpa*, spermatogenesis, testosterone

INTRODUCTION

Andropause is an age-related decline of sexual hormones in men. Sex hormones, especially testosterone, are responsible in basic sexual differentiation, brain development, regenerative organs and different frameworks in the perinatal period. Thus, hormonal confusion may trigger

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actuate irreversible changes in conceptive organs or capacity at developed ages. Testosterone level increases during puberty and is kept at high level during the adult age. Testosterone starts to reduce progressively with age starting at 35 (Oyebode et al., 2016) and if continued for a long period, results in andropause associated with slower sexual responses, fewer living sperm, low libido, age-related diseases such as atherosclerosis, high blood pressure, diabetes mellitus, loss of muscle or bone mass and prone to fatigue (Kaufman & Vermeulen, 2005). Five million men experience the ill effects from low testosterone levels but only 5% of them are being treated (Csatari, 2015). One-third of male Malaysians are facing impotency problems, while only a limited number of patients are currently being treated (Badarudin, 2017). Testosterone replacement therapy is the current treatment applied but causes various side effects to patients. Thus, plant-based medications are preferred as an alternative to treat andropause in a safer and more effective way.

A recent African government report shows that 80% of the world population utilizes plant-based remedies for treating various medical healthcare problems (World Health Organization [WHO], 2016). Herbal plants have been used traditionally for generations by the indigenous peoples although threatened by the introduction of synthetic medicines. Andropause caused by diseases or aging has increased dramatically and becoming a global concern. One of the famous medical plants which exhibits excellent prospects for development is *Phaleria macrocarpa* [Scheff.] Boerl.). Indonesian plant-based remedies with *P. macrocarpa* fruits have been used traditionally for generations in treating multiple lifestyle health problems (Benzie & Wachtel-Galor, 2011) and diseases (Altaf et al., 2013). They have been proven effective as anti-cancer (Trilaksana et al., 2017), and anti-cholesterol (Chong et al., 2011) and remedies in treating diabetes mellitus (Harmanto, 2003). They have anti-microbial, anti-inflammatory, and antioxidant properties (Randhir et al., 2004) as well as improving sexual libido (Parhizkar et al., 2013).

Phaleria macrocarpa is claimed to induce testosterone production in the body associated with sexual strength improvement and libido behaviour in men by the rural people. Unfortunately, there are very few published data regarding the potential and estimation of *P. macrocarpa* in improving fertility in males. There is a need for a series of research to exploit this potential medicinal plant as an alluring alternative option to the synthetic hormonal medications currently in use for improving infertility in male. Thus, a study was outlined to evaluate the medicinal effects of the fruits on male fecundity in adult rats.

MATERIALS AND METHODS

Aqueous Extraction of *Phaleria macrocarpa*

The red colour fruit of *P. macrocarpa* (diameter of 3 cm, Registration no. SK1929/11) was washed, seed disposed, cut into thin slices then oven dried for 24 hours at 65^{o}C. 250 ±1 g of the dried fruit cuts were drenched in 4 litres of water and heated until the water became half of the original

amount. Then, the mixture was sifted and the filtrate was centrifuged at 3000 rpm (503 × *g*) for 30 minutes to separate the particles. The supernatant was filtered again and freeze-dried to obtain the powder extract. The powder extract was weighed and frozen at -20°C for later use. The yield obtained was approximately 13%. The extraction process was repeated until about 3 kg of dried fruit slices were extracted.

Experimental Design

Sixty adult male Sprague Dawley rats (5 to 7 weeks, weighing 200-250 g) were randomly distributed into two by five experimental design with two supplementation periods (3 and 7 weeks) allotted to 5 different doses of extract (0, 24, 48, 240 mg PM aqueous extract/kg bw and 80 mg of commercial PM product/kg bw as standard positive control). The extracts were given using oral gavage at the volume of 0.2 mL daily. The rats were maintained under standard laboratory conditions in a well-ventilated room at room temperature of 28° ± 1°C with 70-80 % humidity and constant programmed 12 hours light-dark cycle inside the faculty animal house, Universiti Putra Malaysia, Serdang, Malaysia. All the rats were fed with commercial rat pellet once a day in the morning and water was given *ad libitum*. They were kept in 20 cages with three rats per cage and they were checked to be free from disease and malformation. The experimental rats were allowed to acclimatize for one week before the treatments started and the bedding was changed weekly. All animals were approved by the institutional animal care committee.

Body Weight

The body weights of the experimental rats were measured weekly by utilizing electronic balance (Mettler Toledo, Malaysia) throughout the time frame.

Cauda Epididymal Sperm Collection

The rats were sacrificed by using diethyl ether overdose. The sperm was collected from the cauda epididymis of the rats by excising the cauda epididymis and mincing it into pieces on petri-dishes in the 1 mL 0.1M phosphate buffered saline (PBS). The spermatozoa were allowed to flow into the buffer. After that, the sperm suspension was left at the room temperature for 30 ± 0.1 minutes to allow debris to settle down. 1 ml of the top portion of the suspension was collected into a new clean tube with cap and centrifuged at 500 rpm (14 × *g*) for 5 minutes. Then, the supernatant was thrown away. The sperm pellet was reconstituted with 1 ml of 0.1M Phosphate buffered saline (PBS) and subjected to sperm count analysis. A drop (10 µl) of sperm solution was loaded on the hemocytometer and viewed under ×400 magnification under the light microscope. The counting of sperm number was done by calculating in 4 × 4 squares (horizontally or vertically) (Figure 1). Sperm numbers were calculated by the formula in equation [1]:

$$\text{Sperm cell count} = \text{Total number of spermatozoa in 5 squares} \times 50{,}000 \times 100 \text{ (cells/ ml)} \quad [1]$$

Plasma Testosterone Analysis

Blood sample of 3 mL was collected from each rat via retro-orbital sinus/ peri-orbital bleeding procedures 24 hours after the last treatment for each treatment period. The plasma testosterone levels were evaluated through radioimmunoassay technique utilizing the kit TESTO-CTK (DiaSorin Diagnostics GmbH, USA). The analysis was carried out using COBRA II auto-gamma analyzer.

Testis Tissue Histology Study

The testes together with epididymis were pulled out gently, removed and encapsulated. The testes specimen was cleaned 3 times with 0.9% normal saline and fixed in 10% formalin for 2-3 days. The lateral and longitudinal section of testes were cut in order to observe the internal cell arrangement. The specimens were later fixed in 10% formalin solution for 72 hours. Then, they were dehydrated in various levels of ethanol, cleared with xylene, and embedded in paraffin wax for sectioning. The sections were cut (5 μm thick), mounted and stained with hematoxylin and eosin. The histopathological changes data were examined by counting the spermatogonia (SG) cell numbers and layer thickness of seminiferous tubules (ST) in testes.

Statistical Analysis

Data were subjected to two- way analysis of variance (ANOVA) using SPSS software version 21. All the mean differences were compared using Duncan multiple range test (DMR) after a significant F-test at $P < 0.05$ and $P < 0.01$.

RESULTS

Body Weight

The body weight in the study was significantly increased ($P < 0.05$) by the dose of *P. macrocarpa* supplementation treatment given, duration of the supplementation, and the experimental animals (Table 1).

Testosterone Concentration

The mean testosterone concentration was neither affected ($P > 0.05$) by the dose nor the period of *P. macrocarpa* supplementation (Table 2).

Table 1
Effects of various doses of P. macrocarpa *fruit extracts supplemented at different periods on body weight (mean ± SE) (g) of adult rats*

Period (weeks)	Dose of *P. macrocarpa* extract (mg/ kg bw)					Total mean
	0	24	48	240	80	
3	210 ± 7.78^{a} (2.83)	268 ± 10.86^{cd} (2.64)	258 ± 14.22^{bc} (1.64)	291 ± 10.44^{cd} (2.67)	261 ± 11.80^{bc} (2.01)	258 ± 2.02^{X} (4.98)
7	229 ± 8.60^{ab} (2.40)	300 ± 12.25^{d} (3.96)	305 ± 11.86^{d} (3.82)	305 ± 10.44^{d} (3.04)	271 ± 10.02^{cd} (3.22)	282 ± 1.32^{Y} (4.02)

( ) Average daily gain (g/ day)
± Standard Error of Mean
XY Means within the same column with different superscripts are significantly different ($p<0.05$)
abcde Means within the same row with different superscripts are significantly different ($p<0.05$)

Table 2
Effects of various doses of P. macrocarpa *fruit extracts supplemented at different periods on the testosterone concentration (mean ± SE) (ng/ ml) of adult rats*

Period (weeks)	Dose of *P. macrocarpa* extract (mg/ kg bw)					Total Mean
	0	24	48	240	80	
3	0.91 ± 0.07^{a}	1.52 ± 0.52^{a}	1.49 ± 0.40^{a}	1.21 ± 0.25^{a}	0.86 ± 0.27^{a}	1.20 ± 0.15^{X}
7	1.14 ± 0.48^{a}	0.75 ± 0.07^{a}	1.35 ± 0.49^{a}	1.18 ± 0.35^{a}	1.16 ± 0.38^{a}	1.12 ± 0.16^{X}

± Standard Error of Mean
XY Means within the same column with different superscripts are significantly different ($p<0.05$)
abcde Means within the same row with different superscripts are significantly different ($p<0.05$)

Epididymal Sperm Count

The sperm count in the study was significantly increased ($P < 0.05$) by the treatment given. The duration of the supplementation (Table 3) did not show significant effect. The total mean sperm count was the highest ($P < 0.05$) in rats treated with 240 mg/kg, followed by the group supplemented with 48 mg/kg, 24 mg/kg dose and untreated rats. In term of total mean sperm count, there was a positive change ($P < 0.05$) among the treatments but no obvious difference between treatments for the dosage of 48 and 80 mg/kg. The commercial product had an effect and the value was equivalent to 42.5 mg/kg PM extract.

Table 3
Effects of various doses of P.macrocarpa *fruit extracts supplemented at different periods on the sperm count (mean ± SE) (million cells/ ml) of adult rats*

Period (weeks)	Dose of *P. macrocarpa* extract (mg/ kg bw)					Total Mean
	0	24	48	240	80	
3	223 ± 33.81^{ab}	304 ± 13.13^{bc}	370 ± 13.42^{cd}	433 ± 16.72^{de}	353 ± 24.79^{cd}	336 ± 15.88^{X}
7	162 ± 30.13^{a}	308 ± 13.64^{bc}	417 ± 47.45^{de}	478 ± 22.57^{e}	398 ± 45.44^{de}	353 ± 24.91^{X}

± Standard Error of Mean
XY Means within the same column with different superscripts are significantly different ($p<0.05$)
abcde Means within the same row with different superscripts are significantly different ($p<0.05$)

Testis Histological Study

The mean of SG cells counts and the thickness of ST layer of the treatment groups were significantly higher compared to the control group (Tables 4 and 5). The total mean count of SG cells and the thickness of ST layer were higher ($P < 0.05$) with the increase in *P. macrocarpa* dose. The increase of SG cells was dose and time dependent, but the thickness of ST was only dose-dependent. The commercial product had an effect and the value was equivalent to 45 mg/kg PM extract. A substantial increase in SG cells and ST thickness was observed between control and treatment rats (Figures 1-3).

Table 4
Effects of various doses of P. macrocarpa *fruit extracts supplemented at different periods on the SG cell number (mean ± SE) (cells) of adult rats*

Period (weeks)	Dose of *P.macrocarpa* extract (mg/ kg bw)					Total Mean
	0	24	48	240	80	
3	36 ± 1.27^{a}	52 ± 0.47^{b}	69 ± 1.31^{c}	83 ± 1.93^{d}	55 ± 0.98^{b}	59 ± 3.02^{X}
7	33 ± 1.58^{a}	55 ± 0.93^{b}	71 ± 1.80^{c}	90 ± 3.08^{e}	83 ± 2.25^{d}	66 ± 3.92^{Y}

± Standard Error of Mean
XY Means within the same column with different superscripts are significantly different ($p<0.05$)
abcde Means within the same row with different superscripts are significantly different ($p<0.05$)

Table 5
Effects of various doses of P. macrocarpa *fruit extracts supplemented at different periods on the thickness of ST (mean ± SE) (µm) of adult rats*

Period (weeks)	Dose of *P. macrocarpa* extract (mg/ kg bw)					Total mean
	0	24	48	240	80	
3	34.22 ± 0.72^{a}	51.37 ± 1.57^{b}	60.17 ± 2.73^{c}	79.61 ± 2.26^{e}	58.11 ± 4.11^{c}	56.69 ± 2.92^{X}
7	36.88 ± 0.63^{a}	46.34 ± 0.68^{b}	62.60 ± 1.34^{c}	78.39 ± 2.69^{de}	72.66 ± 1.76^{d}	59.37 ± 2.98^{X}

± Standard Error of Mean
XY Means within the same column with different superscripts are significantly different ($p<0.05$)
abcde Means within the same row with different superscripts are significantly different ($p<0.05$)

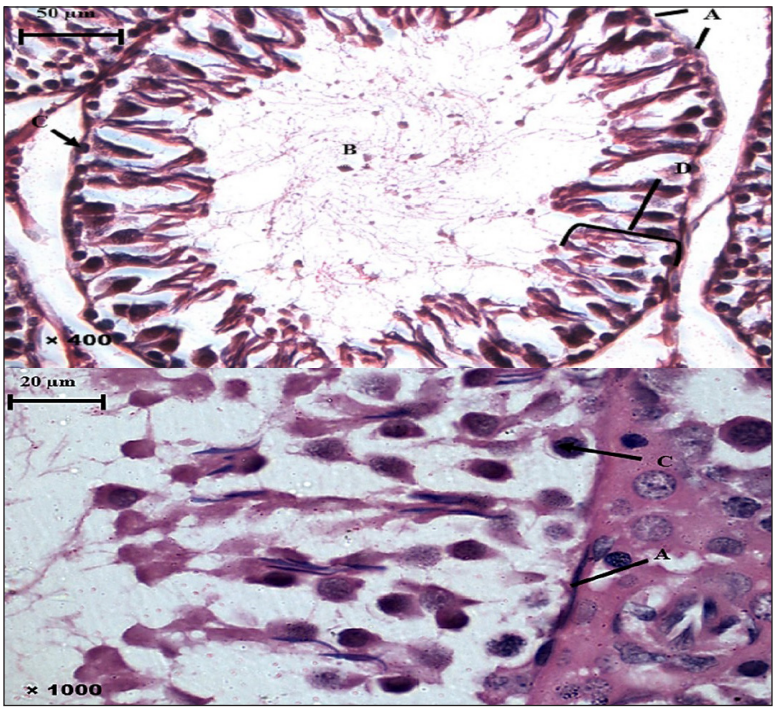

Figure 1. The thickness of ST layer and SG cells observed in control rats (untreated): (A) Basement membrane; (B) Lumen; (C) Spermatogonia cell; (D) Thickness of seminiferous tubule

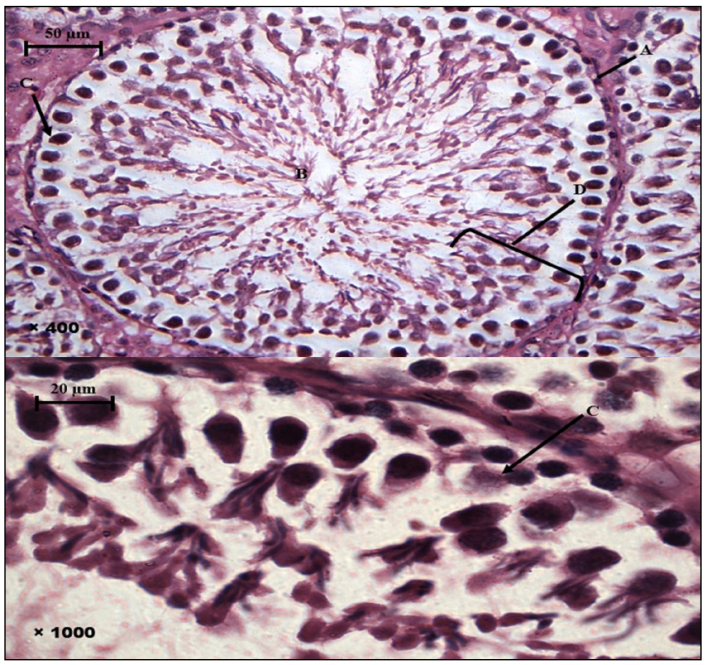

Figure 2. A substantial increase in SG cells number and thickness of ST layer was remarked in treated rats with PM extract (3 weeks): (A) Basement membrane; (B) Lumen; (C) Spermatogonia cell; (D) Thickness of seminiferous tubule

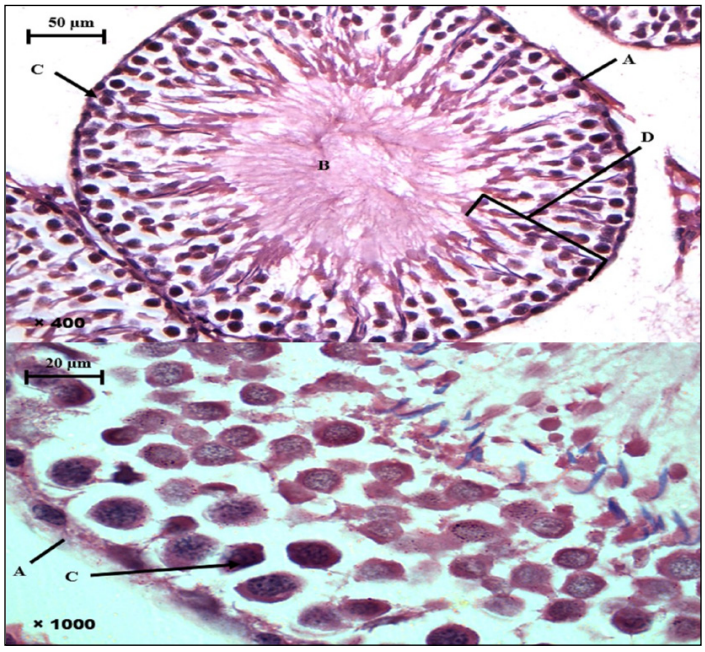

Figure 3. A substantial increase in SG cells number and thickness of ST layer was noticed in treated rats with PM extract (7 weeks): (A) Basement membrane; (B) Lumen; (C) Spermatogonia cell; (D) Thickness of seminiferous tubule

DISCUSSION

After the supplementation, the average daily gain of mature male rats increased at a positive slope and higher than the control group. The initial body weight of the rats was 200-250 g. Body weight changes were the first indication of the onset of the supplementation effect (Shapses et al., 2004). The increase in body weight of treated rats could be due to androgenic properties of *P. macrocarpa*. Testosterone has anabolic effects that mainly acts on the skeletal muscle which may account for part of the body weight (Carson & Manolagas, 2015). Testosterone also has a direct anabolic action on muscle, not to be confused with the skeletal muscle as it is not able to convert testosterone to DHT. This leads to the increase in nitrogen retention through the synthesis of protein resulting in the growth rate increase (Yoshioka et al., 2006). Previous studies by other researchers showed that testosterone levels corresponded positively with lean body mass, body weight and muscle mass (Gates et al., 2013). Thus, *P. macrocarpa* which contains saponin has the stimulatory effect on testosterone hormone (Djannah, 2009). Besides, body weight gain may be due to caloric intake because daily feed intake of the rats was not controlled in the present study. Hence this could increase body fat deposition. Previous research reported that a significant increase in body weight was mainly due to the increase of the percentage of body fat (Chantler et al., 2016). Thus, the increase of average daily gain was mostly caused by the increase of body fat.

Although the body weight of the treated rats increased significantly, the plasma testosterone levels tested were found in a pulsatile rhythm with doses. This may be due to insufficient supplementation duration to deliver any obvious effects in increasing the testosterone concentration in this study. Perhaps, longer period of supplementation would produce more significant anabolic effects. However, there was a slight increase in total mean testosterone concentration over the study periods. Testosterone hormone was released into the spermatic vein plasma in pulsatile pattern and demonstrated in these experimental rats. Besides, the duration of the testosterone secretion was about 15 to 90 minutes. Thus, the level of testosterone was fluctuating or varying among the rats during the blood collection causing the pulsatile pattern. Rats treated with 48 mg/kg produced the highest mean testosterone concentration as compared with all the other doses used. This may be caused by the fluctuation sequence of testosterone secretion. Previous research conducted on pre-pubertal and pubertal boys and girls (Mitamura et al., 2000) as well as removal of testosterone in men (Veldhuis et al., 2010) showed that serum testosterone peaked in the early morning and decreased to minimum value in the late evening. Thus, this diurnal rhythm of testosterone secretion may also apply to rats. Such inconsistency in plasma testosterone concentration has also been reported by most researchers. Besides, the formation and degradation of testosterone in the blood are constant. Luteinizing hormone (LH) and follicular

stimulating hormone increase on short days and decreases on long days, thus responsible for the cycle of plasma concentration of testosterone (Jones et al., 2012). Any decline in the testosterone levels in the blood stream is regulated by the LH hormone secreted by anterior pituitary as it initiates the synthesis and secretion of testosterone to elevate the level back to original (Jones et al., 2012). Other studies have shown that testosterone level is positively regulated by libido. The male rats' testosterone level may increase if they are together with female rats. Female rats would increase the male rats' libido and when they are sexually active, the hormone level increases (Parhizkar et al., 2013). Change in serum testosterone levels demonstrates either an immediate effect of the medication at Leydig cell level or an indirect effect by blocking the hormonal milieu at hypothalamo-pituitary axis (Jones et al., 2012). A different result stated that *P. macrocarpa* extract enhanced the serum testosterone level significantly and also increased frequency of mounting but not latency of tested rats (Parhizkar et al., 2013).

Fecundity is commonly linked to sperm production. The study focused on sperm count production as the quality of a functional testes. The quality of testes was measured using sperm count, SG cells count and ST layer thickness. Following *P. macrocarpa* supplementation, the mean sperm count was improved in mature male rats. *Phaleria macrocarpa* has been known as a sexual tonic among rural people and is used for the treatment of impotence and infertility. The researcher documented that *P. macrocarpa* fruit consisting of alkaloid, saponin and flavonoid (Noorehan & Piakong, 2016) can impact the microanatomic arrangement of testis cell in male mouse. Studies have shown that saponin in plants improves aphrodisiac properties since it has stimulatory impact in androgen secretion (Tang et al., 2017) which can upgrade sex charisma. Therefore, saponin has the potential to enhance the secretion of male primary testosterone hormone. The increase in the sperm production and quality is due to the expansion in testosterone hormone in the testicular tissue. Testosterone is critical in the development of sperms. In addition, it is the primary hormone in charge of spermatogenesis and spermiogenesis in ST (O'Donnell et al., 2017). A similar study done reported that supplementation *P. macrocarpa* increased sperm count (Abdul Razak et al., 2017).

Spermatogenesis cycle begins with the division of primary germ cells, producing a number of cells known as SG, follows by the production of primary spermatocytes. Primary spermatocytes are further divided into two secondary spermatocytes and young spermatozoa. The spermatozoa undergo maturation and develop into sperm cells. Thus, the increase of sperm production in this study was mainly linked to the histopathological changes. There was a quantitative increase in the SG cells number in testes for all treatment groups when compared to the normal control group as shown from the testicular histopathological study of the experimental rats (Tables 4 and 5). Testosterone is the basis of sex drive

in males and females. A higher dose of *P. macrocarpa* had resulted in an increased sperm cell (Table 3) and a slight increase in testosterone level (Table 2) which triggered some effects and increased the SG cell number in the testes. Testosterone's main function in males is to facilitate sperm production.

The histopathological study expressed an improvement in the thickness of ST layer in the testes of treated rats. Eighty percent of testicular mass comprised seminiferous tubules. Thus, the morphological measurements of these ST are imperative in the evaluation of testis tissues (Hsieh et al., 2009). The small differences between the mean standard errors indicated that differences in the diameter of these 50 different ST collected were constant. The data were adequate for the estimation of average ST in treatment animals which is in agreement with Parhizkar et al. (2014). They proved that testosterone was imperative for spermatogenesis. The higher the testosterone level, the larger the diameter thickness of the ST layer. The current study result was in agreement with a previous study that reported similar effects of hexane and aqueous extract of *P. macrocarpa* leading to obvious enhancement of male animals' fertility by expanding the wall of thickness in ST (Parhizkar et al., 2015).

The testicles of typical rodents are framed by ST encompassed by tunica albuginea. Every tubule is covered by external stratified adventitial cells and inner basement film. There are interstitial connective tissues in between the tubules. The ST are uniformly fit and lined by regularly arranged rows of SG cells of various phases of development. Following treatment with *P. macrocarpa*, both the quantities of SG cells and the thickness of ST layer were positively increased. The blood-testicles barrier is potentially a vital aspect while considering the conceptive and mutagenic impact of medications and environmental synthetics (Liu et al., 2009). Thus, the present study reported that aqueous extraction of *P. macrocarpa* was able to pass through the blood-testicles boundary and get access to the germ cells in the ST.

CONCLUSION

Phaleria macrocarpa possesses a potential value as an alternative for improving sexual strength by increasing the sperm count, SG cell count, ST layer thickness, and body weight in mature male rats. Thus, it is suggested that *P. macrocarpa* is able to improve the fecundity in men.

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Bioconversion of Solid Waste into Nutritional Rich Product for Plants by using *Eudrilus eugeniae*

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ABSTRACT

Rise in human population and financial growth complicated the solid waste disposal worldwide and increase the possibilities of dispersion of diseases. This issue can only be solved through fusion of eco-friendly efficient techniques. In the present study, the efficacy of *Eudrilus eugeniae* has been tested for the food, medical and paper waste decomposition. During vermicomposting, *Eudrilus eugeniae* development was recorded with significant increase in length, weight, cocoon production, and adult individuals in final compost. Results of 60 days study suggested positive impact of vermicomposting on waste decomposition. Vermicompost of food waste (VFW) resulted with organic carbon 21.67%, 1.98% nitrogen content, and phosphate 0.59 mg/ml. Vermicompost of medical waste (VMW) analysis resulted with organic carbon 15.3%, 1.17% nitrogen, and 0.54 mg/ml phosphate. Whereas physico-chemical results of vermicompost of paper waste (VPW) showed 18.67% organic carbon, 1.39% nitrogen, and 0.79 mg/ml phosphate. The nutritional values of produced vermicompost from different solid wastes were estimated. The VFW resulted with increased nutrient contents than the VMW and VPW. Therefore, decompositing of waste materials by earthworms is the preeminent concept of nutrient renewal from green waste.

Keywords: Cocoon, *Eudrilus eugeniae*, juvenile, medical waste, solid waste management, vermibed

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INTRODUCTION

Increase in population leads to creation of undesirable effect on environment i.e. variability in waste type generated from urban and rural background, food problem, cutting of forests for cultivation, industrialization, air pollution and

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global warming (Gupta et al., 2015). Waste produced due to urban and rural consumption are undesirable pollutants to the environment (Khajuria et al., 2010) and sometimes could even be a health menace (Boffa et al., 2010). The rate of waste generation is an index of socio-economic development and economic prosperity of the region (Sudkolai & Nourbakhsh, 2017). Solid waste is generated from households, offices, shops, markets, restaurants, public institutions, industrial installations, water works and sewage facilities, construction and demolition sites, and agricultural activities (Gupta et al., 2015). In the solid waste stream, waste is broadly classified into organic and inorganic.

It has been reported that India produces approximately 960 million tonnes solid waste every year in form of by-products produced by various sectors i.e. industry, municipal, mining, agriculture and other process in form of organic waste (roughly 350 million tonnes agricultural), inorganic waste (around 290 million tonnes from industry and mining) and hazardous waste (approximately 4.5 million tonnes) (Pappu et al., 2007). In India, per capita waste generated is ~0.4 kg per day having compostable matter more than 50-60% (Gupta et al., 2015).

Earthworms are ecological engineers (Suthar, 2010). These organisms impact the channelization of various nutrients in soil. They are responsible for construction of physical and chemical structures of soil that effect availability of nutrients and resources for other organisms habitat in soil (Gómez-Brandón & Domínguez, 2014). It is reported in literature that presence of earthworms in soil impart a positive effect on available number of bacteria (around hundred times higher bacteria reported) than non-worm based soil (Hussain et al., 2016). About 2,350 years ago Aristotle had said, "Earthworms are intestines of the earth" (Yadav & Mullah, 2017). Darwin also supported the Aristotle statement: "No other creature has contributed to building of earth as earthworm" (Feller et al., 2003). Vermiculture is a biological process belonging to breeding and increasing earthworms in natural system. It has the ability to reduce biological and non-biological waste, biofertilizer production and range of potential applications for future (Karimi et al., 2017).

Vermicomposting is a natural biological process that stabilize the organic energy-rich contents available in raw substrate into vermicompost through the mutual activity of microorganisms and earthworms (Bhardwaj & Sharma, 2015). It generally does not have any hazardous consequence on plant, animal and environment. Vermicompost has higher range of available nutrients in plant utilizable form i.e. carbon, nitrates, phosphates, potassium, calcium carbonate and magnesium derived from the wastes (Gupta et al., 2015). Availability of vermicompost in soil enhances aeration and texture of soil by increasing soil porosity. However, it also enhances water holding ability of soil due to higher organic content (Suthar, 2010). Vermicomposting process can be carried out by using several earthworm species

(Manyuchi & Whingiri, 2014) i.e. *Drawida willis*, *Megascolex mauritii*, *Eisenia andrei*, *Perionnyx excavatus*, *Lampito mauritii*, *Eudrilus eugeniae*, *Eisenia fetida* and *Lampito rubellus*. Vermicompost has large particulate surface areas that provide numerous micro sites for microbial activity and for the strong retention of nutrients (Pathma & Sakthivel, 2012). Vermicompost consistently improves biological functions in soil which inturn can trigger seeds germination, flowering,plant growth and yield, independent of nutrient availability (Pattnaik & Reddy, 2010). Gut activity of earthworms (Huang & Xia, 2018) changes the physical and chemical composition of soil. This chemical change can be notified in form of plant growth regulators, plant growth hormones, and humates. It appears that activity of earthworms on organic waste initiate the conversion of organic waste in to various plant hormones i.e. cytokinins and auxins and humic acid (Pathma & Sakthivel, 2012).

The attempt of the present study was to review the outcome of earthworm on various types of solid waste (food, medical, and paper) and nutritional value in final vermicompost.

MATERIALS AND METHODS

Vermibed preparation was done at Bhojia Institute of Life Sciences, Baddi, Himachal Pradesh, India (latitude 30° 57' 7.4592'' N and longitude 76° 46' 32.358'' E). In present study, earthworm (*Eudrilus eugeniae*) was collected from vermicomposting centre, located at CSK Himachal Pradesh Krishi Vishvavidyalaya, Palampur, H.P., India (latitude 32° 6' 0.9828'' N and longitude 76° 32' 48.3108'' E). During complete study, all vermibeds were regularly monitored for moisture content and fulfilment of water requirement was achieved continuously by water spraying.

Solid Waste

Collection of food waste for our research was done from nearby restaurants (30° 58' 1.7292'' N 76° 45' 39.5748'' E and 30° 56' 55.4208'' N 76° 48' 7.8732'' E) and student mess (latitude 30° 57' 7.4592'' N and longitude 76° 46' 32.358'' E) of Bhojia Institute of Life Sciences (BILS), whereas hospital/medical waste in form of used cotton and bandages was acquired from Bhojia General Hospital situated at Bhojia campus, Baddi, Himachal Pradesh (India). Hospital waste was properly sterilized in autoclave before application in vermibeds (Karnwal & Kumar, 2012). Paper waste for our study was collected and screened from municipal waste. The food, hospital and paper waste was shredded by waste shredder before application in vermibeds. The moisture amount of all vermibeds was kept at about 55%.-75% (Pattnaik & Reddy, 2010).

Experimental Design

Research was carried out in form of three different treatments of vermibeds (VFW, VMW and VPW), prepared by adding food, medical and paper waste separately with cow dung in 3:1 ratio. One standard/control treatment (SS) was also placed for comparing

effectiveness of vermicomposting. Plastic tubs of radius 33 cm and depth 25 cm was used for vermibed preparation.

Treatment VFW. Treatment VFW vermibed was prepared by the addition of soil, food waste in different levels. Base level was prepared with 2 cm depth of natural garden soil, next level (2 cm depth) was constructed with food waste slurry, third level with natural garden soil (8 cm of depth), second last level was again with food waste slurry (6 cm depth), and thin soil layer in upper most level.

Treatment VMW. Second vermibed was framed with used sterilized hospital or medical waste (used cotton and bandages) in 2nd level and 4th level (2 cm and 6 cm depth), while sterilized natural garden soil in third and uppermost level (8 cm and 2 cm depth) whereas base level was setup with natural garden soil having 2 cm thickness.

Treatment VPW. Treatment VPW vermibed was setup with five layer system. Base level was constructed with natural garden soil having 2 cm thickness, second and forth level with paper waste (2 cm and 6 cm depth) while third and uppermost layer with garden soil (8 cm and 2 cm of depth).

Treatment SS. One treatment was constructed without solid waste and earthworms, which worked as control (SS).

All four setups were left for 15 days for decomposition through natural micro flora. Moisture content in all vermibeds was preserved constantly by sprinkling sterilized water (Sim & Wu, 2010). After 15 days of Pre-decomposition, all three treatments (VFW, VMW and VPW) were inoculated with 50 healthy earthworms (*Eudrilus eugeniae*). This complete setup was retained for sixty days until fine granular vermicompost was ready. After 60 days of compositing, chemical estimation of vermicompost for each treatment against control was carried-out (Sharma & Garg, 2018). Quality of vermicompost was governed by various factors i.e. pre-processing time, pH, moisture content, organic carbon content, sulphate content, calcium carbonate content, chloride content, inorganic phosphate contents, temperature, and the maturation stage (Pattnaik & Reddy, 2010).

Physico-chemical Analysis of Samples

pH. The pH of the final compost was determined (Azarmi et al., 2008) using pH Meter (CD 510, WPA). The pH is defined as the negative log to the base 10 of the H^+ ion concentration (Sim & Wu, 2010). Air-dried compost (20 g), sieved through a 2mm filter, was transferred to a sterilized 200ml beaker to in which 50ml of sterilized distilled water [soil: water, 1:2.5 (w/v) ratio] was added (Sim & Wu, 2010). The contents were stirred intermittently and the sample suspension was again stirred just before taking the reading. After shaking the solution for 30 min pH was measured with pH Meter (CD 510, WPA) fitted with a glass electrode.

Electrical Conductivity. Electrical conductivity (EC) of the samples was determined by taking sample water suspension at 1:2 ratio and the electrical conductivity was measured in terms of the resistance offered to the flow of current using a conductivity bridge. Electrical conductivity was expressed as mS/cm (Hawkes, 2009).

Moisture % Content. The moisture value was calculated as a percentage for compost and vermicompost (Fudholi et al., 2012). Ten gram of fresh soil/compost was weighed (w1) and oven dried for 24 hours at 105°C. After 24 hours, final weight (w2) of dried vermicompost was estimated through digital weighing machine. Percent Moisture content was calculated by applying w1 and w2 values in following formula:

Moisture content (%) = (w1-w2)*100/w2

Total Organic Carbon (TOC) (Modified Walkley-Black Procedure, Sato et al., 2014). Five gram compost was ground up so as to sieve through 0.5 mm mesh sieve. This fine sieved compost (500 mg) was transferred in a 1000 ml beaker. In this beaker, 10 ml of 0.166 mol $K_2Cr_2O_7$ and 20 ml of concentrated H_2SO_4 were added by directing steam into the suspension. Mixing of all contents was done gently and carefully. After 30 minute of stand at room temperature, 30 ml distilled water with 0.2 ml o-phenanthroline indicator was added in the mixture. Titration of mixture was done by using $FeSO_4$ solution until solution colour changed to greenish and then changed to dark green or greenish blue occur (Initially the colour was dark brown). At the end point the color changed quickly from greenish blue to reddish brown (Walkley & Black, 1934).

% C = M x V1-V2 / weight of sample (g) × 0.39

where,
M = molarity of the $FeSO_4$ solution.
V1 = $FeSO_4$ volume (ml), required for blank
V2 = $FeSO_4$ volume (ml), required for sample
$0.39 = 3 \times 10^{-3} \times 100 \times 1.3$, where 3 is equivalent weight of C and 1.3 is the factor explained below.
The factor of 1.3 was based on the assumption that their was77% recovery.

Total Nitrogen (N) Content. Estimation of Total N value in prepared vermicompost was done by Kjeldahl method (Yadav et al., 2013). Final vermicompost was air dried and ground to obtain fine powder. One gram of fine powder vermicompost was transferred in Kjeldhal's digestion flask and enriched with 3 g of digestion mixture (Potassium Sulphate: Cupric Sulphate: Selenium in the ratio of 100:20:1) with 10-15 ml concentrated H_2SO_4. This mixture was digested for a period of time until a bluish-green residue was appeared, followed by cooling of flask contents by adding distilled water to make total volume up to 100ml.

After cooling, 10 ml of digested mixture was transferred in a micro-distillation flask with 10ml of distilled water. The outlet of the condenser was dipped in 25 ml of 4% boric acid solution in a 250 ml conical flask. Before starting the distillation process, 10 ml NaOH (40%) was added in distillation flask and the mixture were distilled. After distillation, boric acid was titrated through 0.05N H_2SO_4 till pink color obtained. A blank was also run in similar manner and final % of N in samples was calculated by using following formula:

$$\%N = \frac{\text{(Burette reading)} \times \text{(Normally of acid)} \times (0.014) \times \text{(Volume made of digested sample)} \times 100}{\text{(Weight of sample taken)} \times \text{(Aliquot taken for distillation)}}$$

Inorganic Phosphate Content. Phosphorus is one of the key nutrient required by all living beings (Dharni et al., 2014). In natural environment distribution of phosphorus is majorly found as phosphate form. It is the 11th utmost eco-rich component on the upper layer of the soil (Gallego et al., 2001). The increased amount of phosphate in soil/ compost plays the active role for the positive growth of plants.

In present study inorganic phosphate concentration in all treatments were measured by spectrophotometric method (Mahadevaiah et al., 2007) with some modification. Air dried compost was mixed with 0.002N H_2SO_4 in 500ml sterilized conical flask to make 10 ml 0.5% (w/v) homogenized suspension by using magnetic stirrer. After 30 min of proper mixing suspension was carefully sieved by using Whatman 1442-125 Ashless Grade 42 Quantitative Filter Paper. Filtered soil extract was used for Inorganic phosphate determination in all samples.

In a conical flask, 5 ml of soil filtrate was mixed with 0.2 ml $(NH_4)_2MoO_4$ solution and one drop of $SnCl_2$ solution (Mahadevaiah et al., 2007). Mixture was stand alone for 10 minutes for the development of blue colour and absorbance was recorded at 690 nm with UV-VIS Spectrophotometer. Deionised water blank was also run in similar manner. Inorganic phosphate estimation was carried out with following formula:

Inorganic phosphate contents in the all treatments was determined by comparing determined phosphate content against standard curve of potassium dihydrogen phosphate (P, 0-1 mg/l) at 690 nm wavelength.

Potassium Estimation. Potassium estimation in vermicompost was done through flame photometry method (Zebec et al., 2017). Ten gram of air-dried vermicompost was ground in fine particles and sieved with Whatman No. 40 filter paper. Filtered 5 g fine vermicompost was mixed in 25 ml 1N $NH_4CH_3CO_2$ for 10 minutes on a stirrer for uniform shaking and filtered with Whatman No. 40 filter paper. Final volume of filtrate was made up to 100ml by adding ammonium acetate solution. Photometeric analysis was performed by using 5 ml of

ammonium acetate supplemented sample. *Potassium* chloride solution was applied as standard for estimation of Potassium content.

Chloride Assay. Twenty gram of compost was added in 100 ml of distilled water to make 1:5 solutions in 250 ml conical flask. The contents of flask was stirred for one hour at regular intervals and filtered with *Whatman Grade 50 filter paper by using funnel in 250 ml beaker. Filtered suspension (50 ml) was mixed with 2 ml of* K_2CrO_4 solution and titrated with 0.02N $AgNO_3$ till a stable red circle observe (Karnwal & Kumar, 2012).

% Chloride = N of $AgNO_3$ (ml) × 1000 × 35.5/ soil solution (ml) × 2

To convert the values in mg/100g, multiply the values in % with 1000

Calcium Carbonate Test. Five gram compost was ground up so as to sieve through 0.5 mm mesh sieve. Sieved compost was added in a 500 ml conical flask with 100 ml of 1M HCl per litre of solution. The contents of flask were left overnight at room temperature. After overnight incubation, contents were centrifuged into 100 ml centrifuge tubes at 2000 x *g* for 10 minutes. Ten millilitre of supernatant was mixed with 25 ml distilled water and 0.1 ml phenolphthalein in a 250 ml conical flask. This mixture was titrated with 0.5M NaOH solution (Yadav et al., 2013).

CaCO3 % = 50N x a-b/S

where,
a = ml NaOH used for blank
b = ml NaOH used for soil samples
S = weight of dried soil samples
N = concentration of soda /solution mol/l

Sulphate Test. Sulphate is another important nutrients required by plants after nitrogen and phosphates. Requirement of sulphate content for plants is very low in amount. Twenty gram of compost was mixed in 100 ml distilled water in 250 ml conical flask. This mixture was regularly stirred over magnetic stirrer for one hour. Compost suspension was filtered with Whatman No.50 filter paper to get turbidity free solution (Mussa et al., 2009). Analysis of sulphate was carried out with 100 ml of filtered solution in a conical flask supplemented with 5 ml of conditioning reagent. This mixture was agitated over magnetic stirrer and during stirring, 0.2-0.3 g $BaCl_2$ crystals was added. After stirring, without any delay, the solution was poured in a 4 cm silica cell and absorbance was recorded at 420 nm through UV-VIS spectrophotometer.

Turbidimetric method: % SO_4 = SO_4 mg/l soil solution/2000

To convert values in mg/100 g, multiply the results in % with 1000

Standard curve of sulphate constructed by measuring absorbance of sodium sulfate ranging from 0.0-50.0 mg/l at 420 nm through UV-VIS spectrophotometer with

interval of 5 mg/l. This standard curve was used to estimate sulphur contents in the samples.

RESULTS AND DISCUSSION

Earthworm Development and Production Efficiency

The development characteristic of *Eudrilus eugeniae* in treatments VFW, VMW and VPW revealed that length was increased by 26% in VFW, 17% in VMW and 20% in VPW, whereas individual weight gained at the end of vermicomposting by *Eudrilus eugeniae* in each treatment was 428.0, 187.3, and 221.4% in VFW, VMW and VPW treatment respectively (Table 1). Cocoon production rate in VFW was more than that of VMW and VPW treatments. The earthworm production per cocoon was 68.2 in VFW and 18.0% VPW than that of VMW treatment. VMW treatment showed higher number of juveniles 219.4% compared to VFW and 47.4% compared to VPW treatment. Adult earthworm count was more in VFW compared to other two treatments. The production of cocoons, juveniles, and adults of *Eudrilus eugeniae* was higher in food waste vermicompost than that of medical and paper waste based vermicompost, these findings suggest that food waste worked as nutritious medium for the earthworm development (Lalander et al., 2015). The higher body mass of inoculated worms was observed in all three treatments during vermicomposting phases (Table 1), which resulted because of nutrients available in treatment mixture or environmental conditions (Yadav et al., 2013). Cocoon production was better in food waste, whereas paper waste treatment also showed good cocoon production rate in comparison to VMW treatment. Our results are also supported by other studies (Kuntz et al., 2013; Suthar, 2009). The higher numbers of cocoons, juveniles, and adults were collected from the vermicompost processed by using food waste plus cow dung.

Table 1
Development parameters of Eudrilus eugeniae *in treatments during vermicomposting*

Development parameters		Treatments supplemented with different solid waste		
		VFW	VMW	VPW
Mean Individual length	Initial (cm)	14.3 ± 0.02	14.3 ± 0.02	14.3 ± 0.02
	Final (cm)	18.0 ± 0.05	16.7 ± 0.04	17.2 ± 0.1
Mean Individual weight	Initial (g)	3.1 ± 0.02	3.1 ± 0.02	3.1 ± 0.02
	Final (g)	13.3 ± 0.01	5.8 ± 0.05	6.9 ± 0.04
Mean Total biomass	Initial (g)	155 ± 0.04	155 ± 0.04	155 ± 0.04
	Final (g)	2627.1 ± 0.06	841.9 ± 0.03	1070.7 ± 0.05
Mean Worm number/cocoon		2.4 ± 0.06	1.4 ± 0.01	1.7 ± 0.07
Mean Juvenile number at the end		36 ± 0.04	115 ± 0.04	78 ± 0.04
Mean Adult number at the end		198 ± 0.05	145 ± 0.07	156 ± 0.02

Values represent the mean of three replicates ±standard error of the mean

Physico-Chemical Analysis

After 60 days of vermicomposting, compost of all treatments was chemically analyzed for various physico-chemical analysis. Compost analysis revealed that change in waste material severely impacted on the nature and chemical composition of finally produced vermicompost (Sharma & Garg, 2018). Various parameter i.e. pH, Electric conductance, % moisture content, Temperature, Organic carbon content, Nitrogen content, Inorganic phosphate content, Potassium content, , Chloride content, Calcium carbonate, and sulphate content were analysed in final vermicompost of treated and non-treated samples.

pH. pH analysis of vermicompost showed a variation in final pH from 7.33 to maximum 8.86. Treatment VFW vermicompost showed pH 8.86 whereas VMW and VPW recorded with 8.68 and 8.79 pH, respectively (Figure 1). It was observed that control treatment had neutral pH. Change in pH, particularly happened due to the activity of earth worms, and deposition of nutrients increased in final vermicompost that changed the pH from neutral to basic (Pattnaik & Reddy, 2010).

EC Value. In our study, EC value was found to increase in comparison to control treatment (Figure 2). This rise in EC during compositing and vermicomposting happened due to decomposition of organic waste (Pattnaik & Reddy, 2010) and increased the availability of exchangeable cationinc minerals i.e. Ca, K, P and Mg in compost and vermicompost (Pérez-Godínez et al., 2017).

% Moisture Content. In present study, all treatments (instead of SS) had % moisture value between 55 to 85% (Table 2). Nigussie et al. (2016) reported that 70-85% moisture content played an important role for the good and luxuriant growth of *E. Eugeniae* during organic waste decomposition. Manyuchi and Whingiri (2014) also observed the effect of good moisture value on the growth of earthworm and waste decomposition rate. Earlier research (Jadia & Fulekar, 2008) also showed higher microbial activity during the availability of 60–70% moisture in samples, whereas decrement of moisture value negatively impacted the activity of bacteria and fungi during decomposition process. In

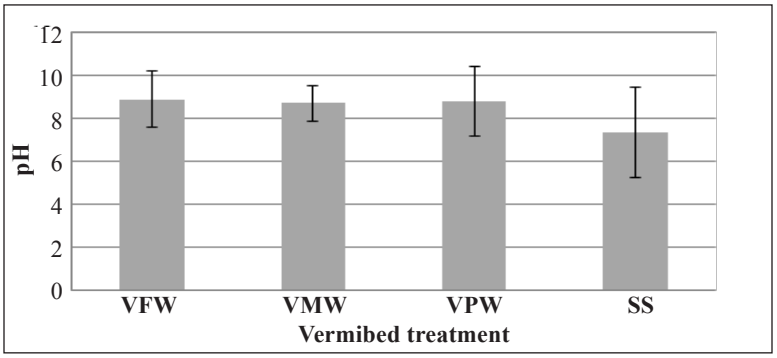

Figure 1. pH value in final vermicompost after 60 days of vermicomposting (VFW: vermicompost of food waste; VMW: vermicompost of medical waste; VPW: vermicompost of paper waste; SS: standard sample)

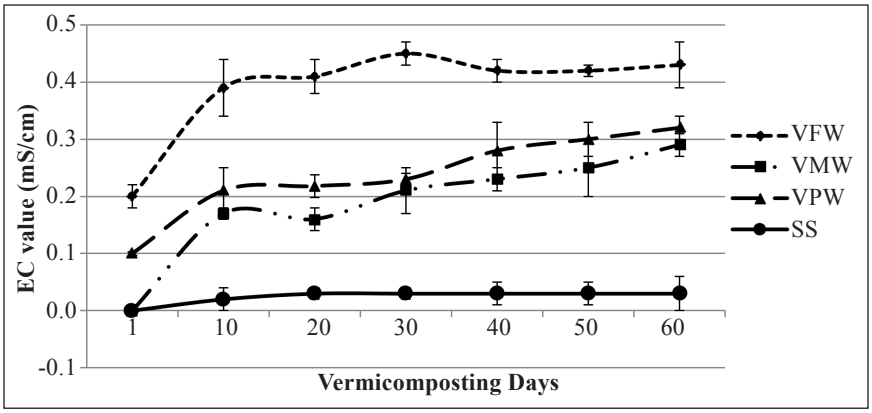

Figure 2. EC value in different vermicompost during vermicomposting

our study, vermicompost samples showed higher % moisture value due to their high absorption capacity than the compost and substrate (Table 2).

Temperature. It was observed during different experiment phases that all treatments showed a higher temperature at the start of experiment because of degrading activity of mesophilic bacteria and fungi during initial compositing process (Jadia & Fulekar, 2008), while at the end of experiment a decrease in temperature recorded (Singh et al., 2003). In first mesophilic phase temperature increased up to 40°C because of oxidative breakdown of organic matter available in all treatments (Pérez-Godínez et al., 2017) and then due to activity of thermophilic bacteria and fungi it elevated from 40°C to 60°C when most of the organic matter degradation and sharp depletion in oxygen occurred (Nigussie et al., 2016). Once anaerobic decomposition of waste completed, maturation phase of compost initiated with the decrement of compost temperature (cooling phase) (Tognetti et al., 2005) which happened due to less bacterial action and regular sprinkling of water.

Organic Carbon by Wet Digestion. Available carbon in substrate is mainly used in compositing and vermicomposting process by earthworms and bacteria as a

Table 2
% moisture value of final vermicompost

Vermicomposting treatments	Initial sample weight	Sample Dry Weight 105°C	Net moisture value	% moisture value
	(w1) g	(w_2) g	(w_1- w_2) g	(%)
VFW	10± 0.02	5.8± 0.03	4.2± 0.01	72.4138
VMW	10± 0.04	6.5± 0.02	3.5± 0.02	53.8462
VPW	10± 0.02	6.1± 0.05	3.9± 0.03	63.9344
SS	10± 0.02	8.1± 0.01	1.9± 0.01	23.4568

Values represent the mean of three replicates ±standard error of the mean

source of energy (Pérez-Godínez et al., 2017), resulting in decrement of total organic carbon of final vermicompost. In present study, it was observed that compositing and vermicomposting process caused reduction of total organic carbon during the passage of time. These results are favoured by other earlier studies (Ndegwa & Thompson, 2000; Pérez-Godínez et al., 2017). However, the TOC content was 21.67%, 15.33%, 18.67%, 12.33% in the VFW, VMW, VPW and SS after completion of vermicomposting, respectively (Figure 3). The organic carbon is converted in CO_2 due to microbial respiration and decomposition of organic contents available in raw material (Kharrazi et al., 2014). Some part of organic carbon is also assimilated in form of biological mass of microbes and earthworms (Pérez-Godínez et al., 2017).

Total N Content. Total nitrogen content consists two inorganic form of nitrogen NH_4–N and NO_3–N in final vermicompost (Adhikary, 2012). In present study, vermicompost prepared by in all treatments showed a considerable variation in N content. Total % nitrogen content was 1.98% in VFW followed by medical waste 1.17% and 1.39% in VPW vermicompost, respectively (Figure 4). Tripathi and Bhardwaj (2004) reported an increase in available nitrogen in final vermicompost in variety of biological forms i.e. mucus, nitrogenous biological

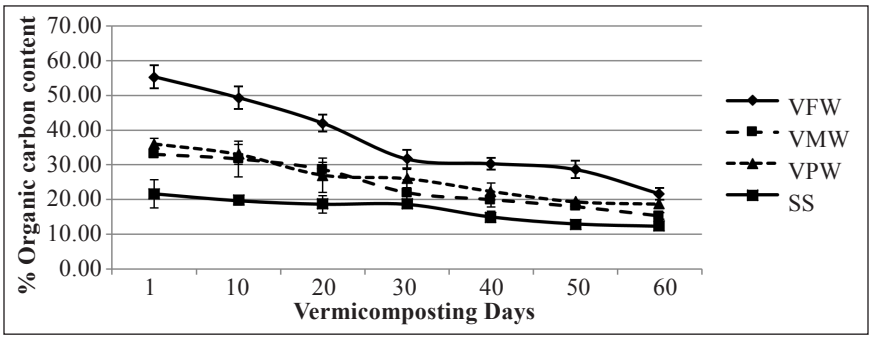

Figure 3. % carbon content in different treatments during vermicomposting process

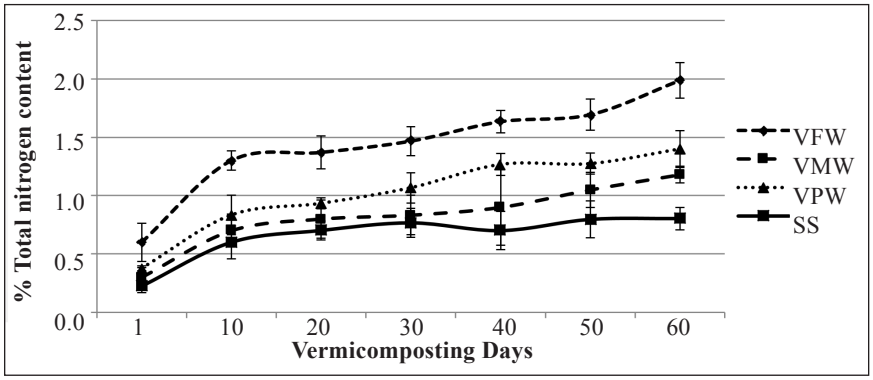

Figure 4. % Nitrogen content during vermicomposting process in different treatments

excretory compounds, decaying dead tissues of worms, hormones and enzymes secreted by earthworms. Increment of nitrogen content may also be possible because of mineralization of total organic carbon containing proteins (Hanc et al., 2017).

C/N Ratio

The C:N ratio was decreased in end product after 60 days. Initially value of C:N ration was higher due to availability of sufficient amount of carbon and nitrogen in pre-compost mixture (Yadav et al., 2013). Lower C:N ratio in final vermicompost reflects the range of changing in carbon and nitrogen content during vermicomposting process. It was observed that initial value of C:N ratio decreased to final value in all treatments (VFW from 92.22% to 10.91%, VMW from 110 % to 12.99%, VPW from 95.58% to 13.34% and SS from 97.01% to 15.35%) as shown in Figure 5. The reduction in C:N ratio throughout vermicomposting happens due to elevated oxidation of organic matter and may possibly be attributed to raise in the earthworm numbers which contributes towards quick reduction in organic C (Pattnaik & Reddy, 2010). The emission of portion from the carbon as CO_2 during the course of breathing, synthesis of mucus and N excrements, raises the amounts of N and decreases the C:N proportions (Adhikary, 2012).

Inorganic Phosphate Content. The total inorganic phosphate content in four treatments ranges from 0.51 to 0.79 mg/ml. It was found that total inorganic phosphate content in final vermicompost was higher compared to initial amount in various substrates (Adhikary, 2012). In present study, minimum inorganic phosphate value was reported 0.51 mg/ml in SS (control) whereas maximum amount was 0.79 mg/ml in VPW final vermicompost. Enhanced inorganic phosphate content in treatments indicates phosphorous solubilization/ mineralization during compositing process (VFW 0.59 mg/ml, VMW 0.54 mg/ml, VPW 0.79 mg/ml). It has been reported by various researchers that earthworms solubilise insoluble P in the presence of P-solubilizing

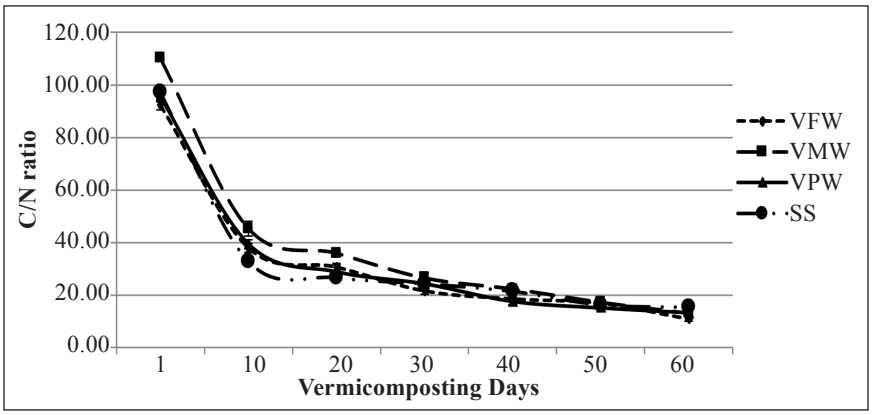

Figure 5. Change in C:N during vermicomposting

microorganisms by using phosphatases enzyme available in the gut, making it more available to plants (Karimi et al., 2017; Lim & Wu, 2016).

Table 3
Physico-chemical properties of final vermicompost in different treatments after 60 days of vermicomposting

Treatments	Inorganic phosphate concentration (mg/ml)	Chloride (mg/100g)	Calcium carbonate (%)	Sulphate (mg/100g)
VFW	0.59± 0.02	60.86± 0.04	0.11± 0.01	13.93± 0.05
VMW	0.54± 0.04	44.12± 0.05	0.09± 0.003	7.5± 0.02
VPW	0.79± 0.03	41.79± 0.02	0.14± 0.01	9.64± 0.01
SS	0.51± 0.002	38.57± 0.03	0.08± 0.004	6.96± 0.01

Values represent the mean of three replicates ±standard error of the mean

Potassium Content. The present findings recorded with the higher amount of K in VFW and VPW vermicompost (Figure 6). VMW and SS recorded with least K content in final vermicompost. Vermicomposting is one of the most efficient method for releasing maximum K content from organic material (Yadav et al., 2013). Releasing of K in vermicompost mainly happens due to enzymatic decomposition and grinding of organic substrate in earthworm gut, which may have caused its increase compared to the simple compost (Garg et al., 2006). The microorganisms present in the worm's gut probably converted insoluble K into the soluble form by producing microbial enzymes (Mahboub Khomami et al., 2016)

Chloride Content. Chloride play a critical role in plant development so its availability in soil matter allot for agriculture (Yaish et al., 2015). Chloride in soil is available in soluble form and predominantly dissolved in the soil water. However, it was demonstrated that higher Cl concentration in plant tissue could become toxic to plants, and stopped the agriculture practices in saline regions (Karnwal & Kumar, 2012). Our study

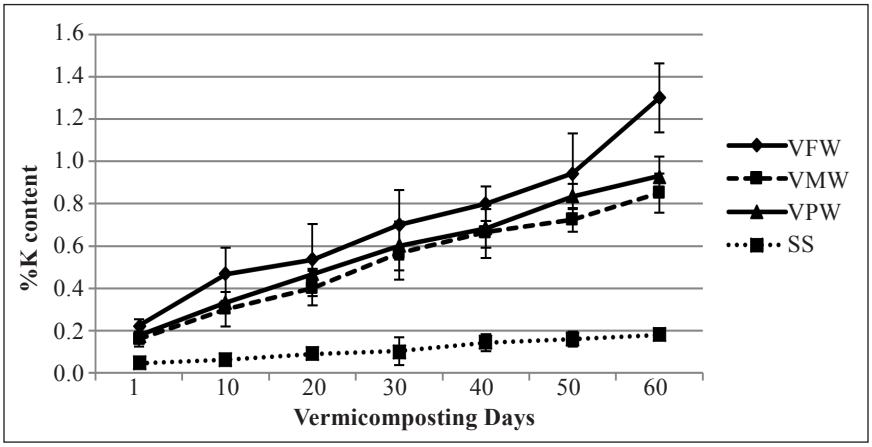

Figure 6. % Potassium content during vermicomposting process in different treatments

showed that in the various vermicompost samples (Table 3), maximum chloride content was recorded in VFW treatment (60.86 mg/g), followed by VPW (41.79 mg/g) and VMW (44.12 mg/g) whereas minimum chloride content was recorded in SS vermicompost (38.57 mg/g).

Calcium Carbonate Content. In present study, amount of calcium carbonate ranges from 0.08 to 0.14 % in final vermicompost (Table 3). Higher Ca content in VFW, VMW and VPW vermicompost relies on the enzymatic activity of carbonic anhydrase naturally available in calciferous glands of *Eudrilus eugeniae* that give rise calcium carbonate on the fixation of carbon dioxide (Yadav et al., 2013). Out of four treatments, minimum calcium carbonate was 0.08% in SS vermicompost whereas maximum Ca amount was recorded 0.14 % in VPW vermicompost followed by VFW and VMW treatments.

Sulphate Test. Sulphur is essential for optimal plant growth and development (Lim & Wu, 2016). It is one of the 17 essential plant nutrients. In natural system, plants are not able to consume sulphur in elemental form (Quilchano et al., 2002), They only consume sulphur from soil in sulphate form ($SO4^{2-}$). In present study, considerable increase in sulphate content appeared in final vermicompost (Table 3). Higher sulphate content was recorded in VFW followed by VPW vermicompost. Least level of sulphate was observed in SS treatment.

CONCLUSION

Bioconversion of solid waste by the use of earthworm into nutrition rich vermicompost can become an economical process for the farmers and local environment cleanup bodies, saving substantial amount of money and environmental security affected by deposition of solid waste in environment. It is concluded from present study that among the three type of solid waste (food waste, medical waste and paper waste), food waste provided best growth and nutritional value in produced vermicompost whereas paper and medical waste while exhibiting lower amount of nutrient value in vermicompost, they were still better than normal soil. The use of *Eudrilus eugeniae* is a suitable biological process for the bioconversion of food, medical and paper waste into organic nutrient rich compost that can decrease the burden of artificial fertilizers and enhance natural soil fertility by providing various plant nutrients in agriculture field.

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Short Communication

Histological Observations of Adventitious Root Derived from *in vitro* Plantlet and Shoot Bud of *Boesenbergia rotunda* (Zingiberaceae)

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ABSTRACT

Boesenbergia rotunda or locally known as 'Temu kunci' is renowned to possess some important bioactive compounds that are promising for pharmacological applications. It is essential to obtain structural information of the inner parts of the *in vitro* roots since growing these roots undertake various anatomical and morphological changes that influence their cells activities and nutrient uptake processes. Although root functions are thought to be significant for the growth of shoot, the morphological and anatomical knowledge of adventitious root for this plant are limited. This particular study aims to investigate and compare anatomical structures of *in vitro* adventitious root derived from *in vitro* plantlet with root derived from shoot bud of *B. rotunda*. Histological sections using resin were done to study the anatomical of adventitious root of *B. rotunda*. The root samples were fixed in a Glutaraldehyde-Paraformaldehyde-Caffeine (GPC) fixative, dehydrated, infiltrated, embedded, cut, stained and mounted with Surgipath mounting medium for observations under light microscope. From the histological observations, adventitious roots of *B. rotunda* had shown the presence of all the three main tissue systems and had the same internal structures containing epidermis, exodermis, suberized sclerenchyma cells, cortex and stele. Both adventitious roots derived from *in vitro* plantlet and shoot bud showed normal growth morphologically, had anatomically same normal cell structures and arrangements.

Keywords: Adventitious root, *Boesenbergia rotunda*, histological, *in vitro*, plantlet, shoot bud

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INTRODUCTION

Boesenbergia rotunda locally known as 'Temu kunci' in Malaysia and Indonesia is an herbaceous monocotyledon plant belonging to Zingiberaceae family. This medicinal herb is widely distributed throughout Southeast Asia regions mainly Southern China, Sri Lanka and India. Owing to its aromatic feature, this herb has been regularly used as a condiment in various Asian dishes to stimulate appetite. Traditionally, the rhizomes of *B. rotunda* have been used to treat ailments such as muscle pain, rheumatism, febrifuge, and gout. It is also capable to treat gastrointestinal diseases including stomach ache, flatulence, peptic ulcer, carminative and dyspepsia (Tan et al., 2012). *Boesenbergia rotunda* has been recognized by its valuable bioactive compounds derived from the flavonoids and essential oils. These compounds are known to be the most abundant secondary metabolites found in this plant.

There are three main groups of flavonoids particularly flavanones, flavones and chalcones that have been found in the rhizome of this plant (Chahyadi et al., 2014), which have been reported to have potentials as antimicrobial (Atun et al., 2018; Jitvaropas et al., 2012), anti-human immunodeficiency virus 1 (anti-HIV-1) (Cheenpracha et al., 2006) and showed significant antioxidant activity (Jitvaropas et al., 2012; Tanjung et al., 2013). Panduratin A and 4-hydroxypanduratin A in the rhizome of *B. rotunda* were found to display high inhibition towards dengue-2 virus protease (Eng et al., 2012; Kiat et al., 2006).

In recent years, plant biotechnology approaches including organ, tissue and cell cultures have been widely used to produce various secondary metabolites in medicinal plants that are worth for pharmaceuticals (Baque et al., 2012). Although plant cell culture has been acknowledged to be one of the efficient culture techniques to obtain secondary metabolites, the yields are often too little for commercialization level. Roots of plants are known to be the main location for synthesis and amassing of a wide range of highly potential secondary metabolites (Mahdieh et al., 2015). The adventitious roots induced from the manipulation of suitable plant growth regulators in culture medium under optimum and specific *in vitro* environments have revealed to be efficient in producing higher and stable root biomass and secondary metabolites. This organ culture is also sensitive towards the outer inducements which allow high secondary metabolites excretion into the culture medium. All these advantages have made adventitious roots highly potential and preferable method for future *in vitro* scale up (Ahmad et al., 2015).

As a monocotyledonous crop, ginger plant does not allow plentiful of explants range for micropropagation process. Rhizome and shoot bud were often used as source of responsive explants (Lincy & Sasikumar, 2010). Adventitious roots can be simply understood as roots that emerged from unusual plant parts (non-root tissues) other than the radicle including leaves, twigs, branches underground stems and aerial stems. It can also grow from old

roots or even as branches of secondary roots from the primary root itself (Steffens & Rasmussen, 2016). The development of a single root has always been illustrated through orthodox route involving division, elongation, and finally maturation phase (Alarcón et al., 2014). Consistently, the formation of the root initials is highly influenced by parenchyma cells. These cells are usually competence to revert themselves to meristematic activity that eventually will start to divide into real root as stated by Hassan and Dodd (1989) and Srikanth et al. (2016). This work has been done particularly to observe the morphological growth and compare the cell and tissue arrangements of *in vitro* adventitious root derived from *in vitro* plantlet and shoot bud of *B. rotunda.*

MATERIALS AND METHODS

Stock Plant

For the *in vitro* plantlet, micropropagation medium of *B. rotunda* was established by Yusuf et al. (2011), which was Murashige and Skoog (MS) medium supplemented with plant growth regulators; 2.0 mg/L 6-Benzylaminopurine (BAP) and 0.5 mg/L 1-Naphthaleneacetic acid (NAA). The medium had been used to culture and continuously maintained the *in vitro* plantlets of *B. rotunda* for subsequent uses. The cultures were placed in a controlled *in vitro* environment of culture room. This included constant lighting exposure from white fluorescent cylinder tube with the light intensity of 30 - 35 μE m^{-2} s^{-1}. Adventitious roots derived from shoot bud of *B. rotunda* were cultured onto the half strength of MS medium supplemented with combination of plant growth regulator and cytokinin; 0.5 mg/L 1-Naphthaleneacetic acid (NAA) and 0.1 mg/L Kinetin (KIN) (Azhar et al., 2018). The roots (three replicates of roots per category) used in this study were obtained from the *in vitro* plantlet and shoot bud of *B. rotunda* of four subculture cycles. The adventitious root cultures were maintained in total darkness at temperature of 25 ± 2ºC.

Microscopy

Both adventitious root samples were collected and cut into small pieces consist of longitudinal and transverse sections in 2 cm. The samples were fixed according to Jalil et al. (2008) in a Glutaraldehyde-Paraformaldehyde-Caffeine (GPC) (Sigma Chemical Co., USA) fixative (50 ml 0.2 M, pH 7.2 phosphate buffer; 20 ml 10% (v/v) paraformaldehyde; 4 ml 25% (v/v) glutaraldehyde; 1 g caffeine and distilled water to a total volume of 100 ml) for 24-48 hours at room temperature. The samples were then dehydrated at different concentrations of ethanol (EtOH); 30%, 30 min; 50%, 45 min; 70%, 45 min; 80%, 60 min; 90%, 60 min; 95%, 60 min and lastly twice for 60 min in pure EtOH. The tissues were infiltrated using Technovit 7100 resin for 24 hours at 4ºC. The specimens were embedded in a mould and left for 24 hours to solidify at room temperature before sectioning. Semi-thin sections of 3.5 μm were cut using a microtome. The slides were soaked in 100% EtOH. The sections were placed in distilled water in a glass

container with black paper underneath to facilitate picking up of the sections. The sections were arranged in clean dry slide and each section was stained with 0.5% (w/v) toluidine blue stain (Sigma, USA) to check tissues. Selected sections were double-stained with 1% (w/v) periodic acid for 5 min, then rinsed four times in distilled water (pH 4.5). Then they were soaked in Schiff's reaction in the dark for 20 min and were rinsed again in distilled water (pH 4.5) four times. Lastly, the slides were stained with napthol blue black (Sigma, USA) at 60ºC for 5 min and were rinsed well with distilled water. The slides were mounted with Surgipath mounting medium and were left dried for 24 hours. Photomicrographs were taken with a Leica camera on a Leica (LEITZ DMRB) light microscope (x20/0.5; x40/0.7; and x100/1.3). Observations of longitudinal and transverse sections with magnification in a range of 200× to 800× were made to determine the presence of three main components in tissue systems; epidermis, cortex, and the vascular bundle in the adventitious root of *B. rotunda.*

RESULTS AND DISCUSSION

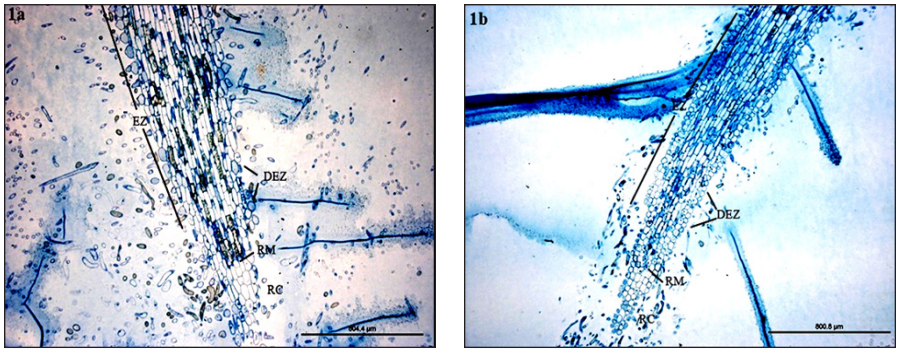
Based on morphological observations, we found that both adventitious roots of *B. rotunda* derived from *in vitro* plantlet and shoot bud mutually showed a similar growth pattern physically when cultured on their own specific medium formulation. Generally, the development of a single primary root can be studied by observing and defining the arrangement of its tissues through longitudinal and transverse sections which will be viewed under the light microscope. The arrangement of tissues in plant roots commonly made up of three main tissue systems which include the epidermis, cortex and vascular cylinder (Alarcón et al*.,* 2014). During staining procedure, it is very crucial to select the good quality of targeted tissues. Consequently, for any recalcitrant tissues, an extensive dehydration method could be carried out using 100% (v/v) butanol for three times with minimum 24 hours for each treatment to soften the tissue (Schwendiman et al., 1988). Periodic acid Schiff particularly stains polysaccharides (starch reserves and walls) while blue black naphtol particularly stains reserve protein blue-black or soluble (Fisher, 1968).

From the histological examinations, adventitious roots derived from *in vitro* plantlet and shoot bud of *B. rotunda* have showed the presence of all three main tissue systems which particularly similar in roots from monocot plants. The arrangement of tissues that form the body of the primary root can be studied through the longitudinal sections (Rost, 2011). Based on the histological examinations in Figure 1, both single segment of the adventitious roots (Figures 1a and 1b) showed physically cylindrical shaped roots. Both adventitious roots also displayed series of distinctive sequential root growth regions that consisted of root meristem, root cap, distal elongation zone and elongation zone. Root meristem and root cap could be seen clearly at the end of the distal elongation zone. This finding was supported by Jones and Dolan (2012), which had stated that a root cap was derived to protect the root apical meristem (RAM).

The RAM is important in continuously generating new cells as foundation to set up different targeted tissues in plant roots. RAM is made up of undifferentiated meristematic cells which are constantly and actively dividing to ensure continuance of root growth (Rost, 2011).

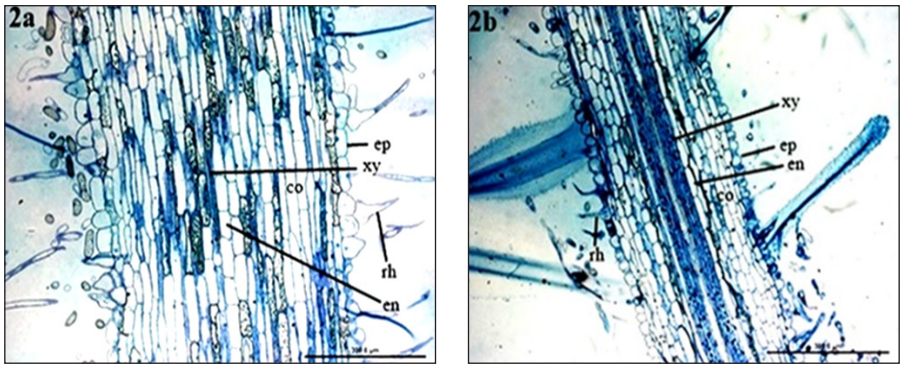
The root system of monocots which comprises of primary root is often temporary and only plays significant role at the initial stage of seedling growth while adventitious roots and seminal roots are considered more prominent. Both roots (adventitious and lateral) were capable in producing lateral roots which help in providing better support and sufficient nutrient uptakes for plants (Bell & Bryan, 2008). Based on Figure 2, there are no distinguishable differences in anatomical structures of adventitious roots derived from *in vitro* plantlet and shoot bud of *B. rotunda.* From the histological observations, there were presence of root hairs detected from both adventitious roots (Figures 2a and 2b) which indicated as good signs for root growth. Root hairs are normally grown as side extensions from the cells in the epidermis layer. Root hairs assist to increase the surface areas of both adventitious roots which allow the root system to boost up the water and nutrients uptake from the culture media (Leitner et al., 2010).

Like any other organs in plant, root also comprises three main specific tissue arrangements that include cortex, vascular bundle and epidermis. Both of the transverse sections of adventitious roots from *B. rotunda in vitro* plantlet and shoot bud (Figures 3a and 3b) showed the presence of important root anatomic structures including epidermis, endodermis, pericycle, phloem, cortex, late metaxylem and protoxylem pole. All these specifications are crucial for root growth in *in vitro* or *ex vitro* environments. The root cortex is a region which basically acts as storages for important organic products such as carbohydrates or other materials including essential oils and tannins, latex or resins. The cortical cells at the inmost layer of cortex will undergo cells



(a) (b)

Figure 1. Longitudinal cross section of adventitious roots segments of *B. rotunda* derived from **(1a)** *in vitro* plantlet. **(1b)** shoot bud. The root sections of both **(1a)** and **(1b)** displayed the cell structures and development that include **RM** root meristem, **EZ** elongation zone, **DEZ** distal elongation zone, and **RC** root cap, Magnification: ×800

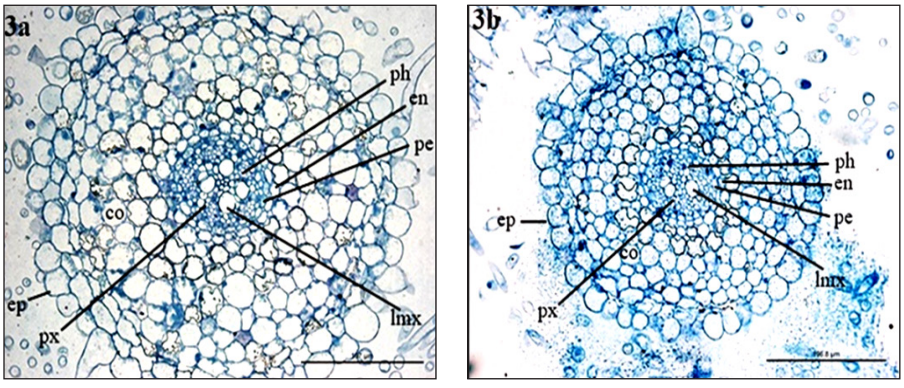


(a) (b)

Figure 2. Longitudinal cross section of column pericycle of *B. rotunda* adventitious roots derived from **(2a)** *in vitro* plantlet. **(2b)** shoot bud. Stained cross section showed **rh** root hair**, co** cortex, **ep** epidermis, **en** endodermis, **xy** xylem. Magnification: ×300

divisions to form endodermis. Endodermis is essential to ensure a one-way mode of water transport into the plant, whereas pericycle is usually where the new lateral roots initiated (Seago & Fernando, 2013).

Besides, the presences of vascular tissues were also highlighted in this histological inspection. Vascular tissues in roots specifically xylem and phloem are vital for conducting transportation of water, nourishments and yields from photosynthesis process to all over the parts of the plant. As an addition, these tissues help plants to adjust in unfamiliar and different environments together with the ability to act as aiding tool for mechanical support to build up the plant strength for continuous root growth (Bellini et al., 2014; Nanda & Melnyk, 2018). The cell and tissues arrangements from longitudinal and transverse sections of ten replicates each in this study were consistent.



(a) (b)

Figure 3. Transverse section of adventitious roots of *B. rotunda* segments for **(3a)** *in vitro* plantlet. **(3b)** shoot bud. Stained cross section showed, **co** cortex, **ep** epidermis, **en** endodermis, **ph** phloem**, lmx** late metaxylem**, pe** pericycle**, px** protoxylem pole. Magnification: ×200

CONCLUSIONS

This gives confirmation that the adventitious roots derived directly from shoot bud explants are comparable with the adventitious roots derived from *in vitro* plantlets in their morphological growth and they have same normal cell structures and tissue arrangements Although the growth and morphological characteristics of adventitious roots derived from shoot bud explants were seen as proficient as the roots from *in vitro* plantlets, histological analysis had to be done to further identify and clarify the root growth at cellular level. This study gives a validation that the adventitious roots of *B. rotunda* offer many interesting perspectives which can be useful for future studies.

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Microbial Contamination of Meat Contact Surfaces at the Selected Beef Processing Plants in Selangor and its Biofilm Formation Ability

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ABSTRACT

Cross-contamination of meat products with the surrounding contact surfaces does occur during slaughtering procedures. This study aimed to assess the microbial contamination level of meat contact surfaces at the selected local abattoirs. Swab samples of knives, splitting tools and air curtains were collected from two sites in Selangor. The presence of selected indicator and pathogenic microorganisms (total Aerobic Plate Count [APC], Enterobacteriaceae, *Escherichia coli* [*E. coli*] and *Salmonella* spp.) were determined using the plate count method. The isolates obtained were then tested in terms of the biofilm formation ability using the microtiter plate crystal violet assay. Overall results showed that the average total APC for all contact surfaces was 4.77±1.14 log CFU/cm^2 (mean±sd). Enterobacteriaceae was found on 75% of the samples at 3.31±1.14 log CFU/cm^2. *E. coli* was only detected on 11.36% of the total contact surfaces at 2.91±1.00 log CFU/cm^2 whereas 25% of the total samples were positive with *Salmonella* spp. Splitting tools were identified as the most contaminated meat contact surface. Variations in biofilm formation ability were observed between isolates although most of them formed weakly adherent biofilms, especially at

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4°C. The study findings help to enhance the systems used in the local abattoirs to ensure safe and top quality meat production.

Keywords: Abattoirs, beef, biofilm formation, meat contact surface, meat contamination

INTRODUCTION

Beef is one of the animal-based main diet for Malaysians and one of the important agro-based products in Malaysia (Arif et al., 2015). Even though 75% of the beef marketed in Malaysia is imported, the local beef production steadily increased from 48 835 metric tonnes in 2011 to 49 598 metric tonnes in 2017 (Department of Veterinary Services [DVS], 2018a). This is due to the increase of 1.7% p. a. in consumption per capita for this commodity over the 7 years' period (2011-2017) (DVS, 2018b). Malaysia is expected to have a total slaughter of more than 450,000 head (cattle) per year by 2020 (Arif et al., 2015). The upward trend of local beef production and demand increases the need to enhance the current ruminant industry performance in Malaysia.

Disease outbreak due to consumption of contaminated meat products with pathogenic microorganisms has been reported worldwide (Desmarchelier et al., 2007; Nørrung & Buncic, 2008). Pathogenic microorganisms are associated with meat contamination that include *E. coli*, *Listeria monocytogenes*, *Salmonella* spp. and *Campylobacter* spp. (Sofos, 2008; Sofos & Geornaras, 2010). Reports on microbial contamination of meat products in Malaysia involve pathogens such as *E. coli* O157:H7, *Listeria monocytogenes* and *Salmonella* spp. (Fauzi et al., 2016; Fazlina et al., 2012; Sahilah et al., 2010; Son et al., 1998; Sukhumungoon et al., 2011; Tan et al., 2019; Thung et al., 2017; Wong et al., 2012; Zulfakar et al., 2017). However, the studies focused on retail meat sold at wet markets and hypermarkets. The status of the microbial contamination level in the local abattoirs especially in terms of meat contact surfaces is still limited (Chong et al., 2017a, 2017b).

Abattoirs are the first place of beef production before being distributed to retail markets. Hence, abattoirs play a role in early prevention of microbial contamination of meat products. Contamination of meat products occurs during the slaughter and after slaughtering in the abattoir. Besides direct contact with the cattle faeces and viscera during the dehiding and evisceration steps, meat products are exposed to cross-contamination with the surrounding contact surfaces throughout the slaughter and post-slaughter procedures (Giaouris et al., 2014; Jackson et al., 2001). Hence, cleaning and disinfection of the meat processing areas as well as maintaining good hygienic practice among the meat handlers are pivotal in minimising the risk of microbial contamination.

Biofilm is the assemblages of microbial cells attached on a surface, forming a sessile community embedded in an extracellular polymeric substance (EPS) matrix (Giaouris et al., 2015). In fact, microorganisms exist in the form of biofilm on the surfaces exposed to the environment (Frank, 2001). The formation of biofilm

on contact surfaces has become a serious issue in the food processing industry and it is an ongoing threat in the meat industry (Wang 2019). Meat contact surfaces in the abattoir may harbour meat residues and nutritious detritus if the premise sanitation practice is compromised, thus providing a suitable environment for biofilm production and contribute to a continuous source of contamination (Sofos & Geornaras, 2010). Biofilm has high tolerance to the commonly used sanitisers and has high transferability potential between surfaces (Wang, 2019). These can jeopardise the abattoir hygienic state, thus affecting the safety and quality of the meat products.

This study aimed to assess the hygienic level of meat contact surfaces at two selected abattoirs in Selangor, Malaysia. The contamination level of selected indicator and pathogenic microorganisms (total APC, Enterobacteriaceae *E. coli* and *Salmonella* spp.) and the biofilm formation ability were determined.

MATERIALS AND METHODS

Sample Collection

Samples were collected from two abattoirs in Selangor, Malaysia. One of them is a large scale abattoir with a more systematic production line whereas another one has a smaller scale operation that only supplies for the local district markets. Sampling activities were conducted four times at each abattoir within nine months. Meat contact surfaces include knives (used to dehide the carcasses), splitting tools (used to eviscerate the carcasses) and air curtains (located in front of the chillers). The meat contact surfaces were chosen based on their potential to be in contact with beef carcasses thus increasing the risk of microbial contact and availability in both abattoirs. Sample collection from the meat contact surfaces was done prior to slaughtering as well as after slaughtering (that involved one batch of cattles) on the same day. Five swab samples were taken from each meat contact surface (knives and splitting tools). However, for air curtain samples, only one composite sample was taken at each sampling time. Sampling activities were conducted four times at each abattoir within nine months.

Pre-moistened sterile cotton swabs with buffered peptone water (BPW) (Merck, Germany) were used to collect samples from the meat contact surfaces (Midura & Bryant, 2001). The whole blade area of the knives and splitting tools were swabbed whereas a sterile 100 cm^2 template was used to sample the air curtains. Delineated areas of all samples were then swabbed for the second time with a dry cotton swab. Sterile swabs were then kept in 20 mL sterile BPW and maintained at 4°C before being transported to the laboratory for further analysis.

Sample Preparation

Samples were homogenised using vortex for 30 seconds. Ten mL of the homogenised sample were used to enumerate total aerobic plate count (APC), Enterobacteriaceae and *E. coli* while the remaining sample were used to detect *Salmonella* spp. (da Silva et al., 2013).

Detection and Enumeration of Total Aerobic Plate Count (APC), Enterobacteriaceae and *E. coli*. Plate count method was used to enumerate the presence of APC, Enterobacteriaceae and *E. coli* using plate count agar (PCA; Merck, Germany), violet red bile dextrose agar (VRBD; Merck, Germany) and eosin methylene blue agar (EMB; Merck, Germany) respectively. Series of 10-fold serial dilutions were performed on the homogenised samples before plating 0.1 mL aliquot from each dilution on the specified media. The plates were then incubated aerobically at 37°C for 24 h. The colonies were counted and recorded as log CFU/cm^2. Bacterial colonies from APC samples were further characterised based on the Bergey's Manual of Determinative Bacteriology (Williams, 2000) and Gram staining. Presumptive *E. coli* colonies detected on EMB agar were then subjected to standard biochemical tests (indole, methyl red, Voges-Proskauer and citrate tests) (da Silva et al., 2013).

Detection of *Salmonella* spp. Ten mL of the homogenised sample was incubated at 37°C for 20 h as pre-enrichment. An aliquot of 0.1 mL of the incubated samples was then transferred to a 10 mL Rappaport-Vassiliadis soy broth (RVS broth; Merck, Germany) and vortexed thoroughly before being incubated for 20 h at 40°C. A loopful of enriched suspension was then streaked onto xylose lysine deoxycholate agar (XLD; Merck, Germany) and incubated at 37°C for 24 h. *Salmonella* spp. was detected by the presence or absence of red colonies with black centres. Presumptive colonies were then subjected to standard biochemical tests (triple sugar iron agar, indole, methyl red, Voges-Proskauer and citrate tests) (da Silva et al., 2013).

Identification and Confirmation of Bacterial Isolates via PCR

DNA extraction was done according to Pui et al. (2011) with slight modifications. Bacterial culture was grown overnight on nutrient agar. One loopful of bacteria culture was suspended in 500 µL of sterile deionised water and centrifuged at 1000 × *g* for 5 min at 4°C. The supernatant was discarded and the pellet was resuspended with 200 µL phosphate buffered saline (PBS) (OXOID, Hampshire, England) and mixed thoroughly. The mixture was boiled at 95°C for 15 min and cooled at -20°C freezer for 15 min and recentrifuged at 1000 × *g* for 10 min at 4°C. The supernatant suspended with genomic DNA was transferred to a new tube and the each sample quality was confirmed using nanodrop spectrophotometer (Thermoscientific, Model 2000). The supernatant was used as the DNA template solution and kept at -20°C before PCR analysis.

PCR Amplification and Sequence Processing

The 16S rDNA amplification was conducted based on Suardana (2014) with some modifications. The PCR programme was carried out in 50 µL reaction solution containing 5 µL DNA template, 25 µL PCR

master mix (Promega, USA) and 5 µL of each primer. The primers used in this study were 27f (5'-AGA GTT TGA TCC TGG CTC AG-3') AND 1429r (5'-GGT TAC CTT GTT ACG ACT T-3'). The PCR cycling profile started with initial denaturation at 94°C for 2 min, followed by 35 denaturation cycles at 94°C for 1 min, annealing at 45°C for 1 min, followed by extension at 72°C for 2 min and final extension at 72°C for 10 min. About 5 µL PCR products was analysed by electrophoresis in 1% agarose gel with 4 µL sybr green as the DNA gel stain at 70 V for 40 min. Gel was visualised by UV trans-illumination.

The DNA sequencing of the PCR products was performed by First Base Sdn. Bhd., Malaysia. The sequences obtained were compared with NCBI database through basic local alignment search tool (BLAST) at www.ncbi.nih.nlm.gov/BLAST. For comparison, isolates with closely related sequences were searched. Identification of the isolates was done via sequence similarity.

Preparation of Bacterial Isolates for Biofilm Assay

All bacterial isolates obtained were stored as stock culture in tryptone soy broth (TSB; Oxoid, Hampshire, England) with 20% glycerol at -20°C. Working cultures were maintained on tryptone soy agar (TSA; Oxoid, Hampshire, England) and stored at 4°C no longer than a month. For experiment, a single colony was extracted from the working culture plate and grown overnight in TSB for 18-20h at 37°C without shaking. Cell suspensions were prepared by centrifuging the TSB culture at 1000 × *g* for 15 min. Cells were washed once in PBS at pH 7.3 (Oxoid, Hampshire, England) before being resuspended in fresh TSB to achieve bacterial concentration of approximately 10^7 CFU/mL. The optical density (OD) of 0.08 for the initial inoculum levels was standardised using a spectrophotometer (Shimadzu UV Mini 1240, Australia) at 625 nm. About 100 mL of diluted cell suspension was also plated on TSA in duplicate to confirm the inoculum level. Plates were incubated at 37°C for 24 h before counting the colonies.

Measurement of Biofilm Formation using Crystal Violet Staining

The bacterial isolate biofilm formation ability was measured according to Stepanović et al. (2004) with slight modifications. In short, 100 µL of bacterial suspension and 100 µL of fresh TSB (control wells) were added to 96-well flat bottom plate (Greiner Bio-One, Germany) in triplicate. The plates were then covered and incubated aerobically without shaking for 24 h at three temperatures (4, 25 and 37°C). After incubation, bacterial suspensions were aspirated by pipette and the wells were washed for three times with 200 µL PBS to remove loosely attached cells. Attached cells were heat-fixed at 55°C for 15 min and then stained with 100 µL of filtered 1% crystal violet for 15 min at 37°C. Wells were rinsed five times with sterile distilled water to remove excessive crystal violet stain and air-dried at 37°C. Next, 160 µL of 95% ethanol was added to test wells

to resolubilise the dye bound to the adhered cells. Biofilm formation level was quantified by measuring absorbance value using a 96-well microplate reader (Thermofisher, USA) at 650 nm. Experiments were repeated three times independently. The average OD reading for each sample and biofilm formation level for each bacterial isolate was classified according to Stepanović et al. (2004).

Statistical Analysis

The bacterial concentration results were presented as mean ± standard deviation (SD). One-way ANOVA followed by Games-Howell post hoc test were conducted to compare the bacterial contamination levels between meat contact surfaces. The statistical tests were performed using SPSS version 23.0. Results are deemed significant at $p<0.05$ unless otherwise stated.

RESULTS

The microbial loads of total aerobic plate count (APC), Enterobacteriaceae and *E. coli* found on meat contact surfaces from the selected abattoirs in Selangor were summarized in Table 1. Overall results showed that the average APC for all contact surfaces was 4.77±1.14 log CFU/cm^2 (mean±SD). Moreover, 75% of the samples were found to be positive with Enterobacteriaceae at 3.31±1.14 log CFU/cm^2 while only 11.36% of the contact surfaces were contaminated with *E. coli* (2.91±1.00 log CFU/cm^2).

Based on Table 1, knives sampled prior to slaughter had the lowest average count at 3.61±0.81 log CFU/cm^2. However, this is not significantly different ($p>0.05$) as compared to APC level for air curtain (4.28±0.65 log CFU/cm^2). Splitting tools sampled after slaughter were identified as

Table 1
Microbial load of Total Aerobic Plate Count, Enterobacteriaceae and E. coli *on meat contact surfaces from local abattoirs in Selangor*

Meat contact surface (n=20)[1]	Microorganisms detected on meat contact surfaces					
	Total Aerobic Plate Count		Enterobacteriaceae		*E. coli*	
	No. (%)[2]	Mean[3]±SD	No. (%)	Mean ± SD	No. (%)	Mean ± SD[4]
Air curtain	8 (100)	4.28±0.65^{cd*}	4 (50)	2.07±0.19^{b}	0	0
Knife (Before)	20 (100)	3.61±0.81^{d}	7 (35)	2.55±1.04^{ab}	1 (5)	2.20
Knife (After)	20 (100)	4.73±0.87^{bc}	19 (95)	3.54±1.08^{a}	7 (35)	2.78 ± 0.85
Splitting tool (Before)	20 (100)	5.27±1.06^{ab}	17 (85)	3.23±1.00^{a}	1 (5)	1.61
Splitting tool (After)	20 (100)	5.66±0.83^{a}	19 (95)	3.65±1.24^{a}	1 (5)	1.61
Total (N=88)	40 (100)	4.77±1.14	66 (75)	3.31±1.14	10 (11)	2.91±1.00

[1]No. of samples for each meat contact surfaces except for air curtains (n=8)
[2]No. of positive samples
[3]Mean bacterial counts expressed in Log CFU/cm^2
[4]Standard deviation data was only expressed for *E. coli* readings with more than one positive samples
*Different letters indicate significant difference between meat contact surfaces within the same column ($p>0.05$)

the most contaminated surface (5.66±0.83 log CFU/cm^2; p<0.05) as compared to other meat contact surfaces, save for splitting tools sampled prior to slaughter (p>0.05) with APC count of 5.27±1.06 log CFU/cm^2. Table 2 showed the bacterial isolates obtained from the APC counts, identified using the 16S rDNA method. Out of all isolates obtained, 63.6% are Gram-positive bacteria with the remaining 36.3% are Gram-negative (Table 2). From the APC counts of the isolates, 10 bacterial families were identified, with Staphylococcaceae has the highest frequency (27.3%), followed by Bacillaceae (22.7%), Enterobacteriaceae (13.63%) and Flavobacteriaceae (9.1%). Other families include Enterococcaceae, Moraxellaceae, Planobacteriaceae, Sphingobacteriaceae, Microbacteriaceae and Micrococcaceae (4.5% each), as depicted in Figure 1.

Save for one sample, both knives and splitting tools (95%) after slaughter were found to be positive with Enterobacteriaceae, recorded at 3.54±1.08 log CFU/cm^2 and 3.65±1.24 log CFU/cm^2 respectively (Table 1). The Enterobacteriaceae level on the meat contact surfaces showed an increase in the average bacterial count as compared to samples collected prior to slaughtering. The increase was also observed in APC

Table 2
Bacterial identification of isolates from Aerobic Plate Count (APC) plates via 16S rDNA PCR method

ID	Gram staining	Bacterial species
A3	Positive	*Staphylococcus fleuretti*
A4	Positive	*Bacillus cereus*
A7	Positive	*Staphylococcus saprophyticus*
A9	Positive	*Enterococcus hirae*
A10	Positive	*Bacillus thuringiensis*
A11	Negative	*Acinetobacter schindleri*
A12	Negative	*Wautersiella falsenii genomovar 2*
A14	Positive	*Kurthia populi*
A16	Negative	*Sphingobacterium daejeonense*
A31	Negative	*Macrococcus bovicus*
B1	Negative	*Enterobacter xianfangenesis*
B2	Negative	*Empedobacter falsenii*
B3	Negative	*Proteus mirabilis*
B4	Positive	*Microbacterium esteraromaticum*
G2	Positive	*Glutamicibacter creatinolyticus*
G14	Positive	*Bacillus cereus*
G18	Positive	*Staphylococcus sciuri subsp. sciuri*
G21	Positive	*Macrococcus caseolyticus*
G24	Positive	*Bacillus pumilus*
G29	Positive	*Staphylococcus sciuri*
G30	Positive	*Bacillus flexus*
G34	Negative	*Cronobacter malonaticus*

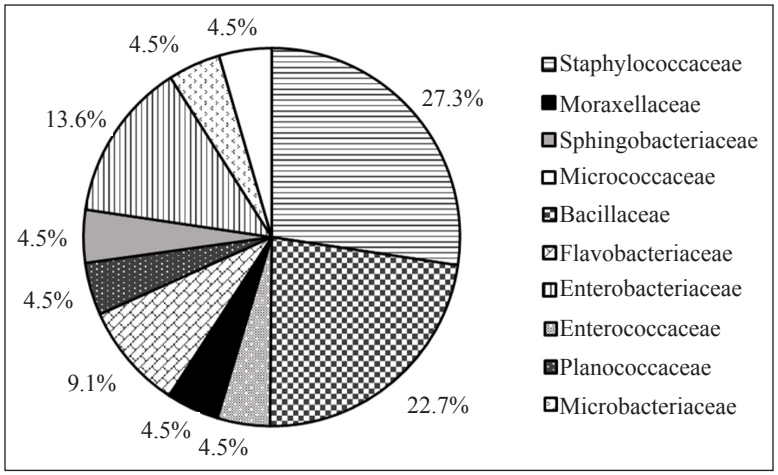

Figure 1. Diversity of bacterial isolates obtained from Aerobic Plate Count (APC) samples from meat contact surfaces at selected beef processing environment in Selangor

readings. However, there is no significant difference (p>0.05) in these readings save for the APC counts for knives. Air curtains had the lowest Enterobacteriaceae count at 2.07±0.19 log CFU/cm^2.

For *E. coli* detection, knives sampled after slaughter had the highest positive *E. coli* (58%) at 2.78 ± 0.85 log CFU/cm^2. No *E. coli* was found on the air curtains. Only one sample was found to be positive with *E. coli* for other contact surfaces. No statistical analysis was conducted to compare the average bacterial count between the surfaces due to insufficient samples that were tested positive. Analysis on the presence of *Salmonella* spp. showed that 25% of total samples were found to be positive. Out of them, splitting tools sampled after slaughter had the highest *Salmonella* spp. (41%) whereas knives sampled before slaughter had the lowest (Figure 2).

To compare the biofilm formation ability between the isolates, biofilm formation level was classified into four categories according to Stepanovic et al. (2004); non-adherent, weakly adherent, moderately adherent and strongly adherent. Table 3 compares the difference in biofilm formation

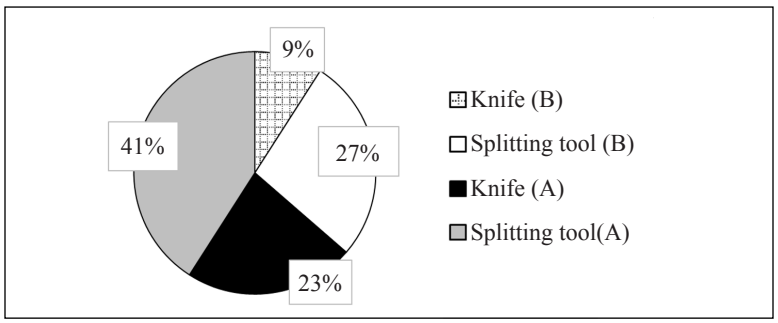

Figure 2. Distribution of *Salmonella* spp. positive samples on different types of meat contact surfaces (n=22)

Table 3
Classification of biofilm forming ability of isolates obtained from meat contact surfaces according to Stepanovic et al. (2004)[1]

ID	Temperature		
	4˚C	25˚C	37˚C
Isolates from APC plates			
A3	Non-adherent	Weak adherent	Weak adherent
A4	Non-adherent	Weak adherent	Weak adherent
A7	Moderate adherent	Moderate adherent	Weak adherent
A9	Non-adherent	Non-adherent	Weak adherent
A10	Weak adherent	Non-adherent	Weak adherent
A11	Non-adherent	Weak adherent	Weak adherent
A12	Non-adherent	Non-adherent	Weak adherent
A14	Non-adherent	Non-adherent	Non-adherent
A16	Weak adherent	Weak adherent	Weak adherent
A31	Weak adherent	Non-adherent	Moderate adherent
B1	Weak adherent	Weak adherent	Non-adherent
B2	Non-adherent	Weak adherent	Non-adherent
B3	Moderate adherent	Moderate adherent	Weak adherent
B4	Weak adherent	Weak adherent	Moderate adherent
G2	Non-adherent	Weak adherent	Weak adherent
G14	Non-adherent	Non-adherent	Weak adherent
G18	Weak adherent	Weak adherent	Weak adherent
G21	Weak adherent	Weak adherent	Weak adherent
G24	Non-adherent	Non-adherent	Non-adherent
G29	Weak adherent	Weak adherent	Weak adherent
G30	Weak adherent	Weak adherent	Moderate adherent
G34	Weak adherent	Weak adherent	Weak adherent
E.coli isolates			
A1	Moderate adherent	Strong adherent	Moderate adherent
EC3	Weak adherent	Weak adherent	Weak adherent
EC4	Weak adherent	Weak adherent	Weak adherent
EC7	Non-adherent	Weak adherent	Weak adherent
EC36	Weak adherent	Weak adherent	Weak adherent
EC37	Non-adherent	Weak adherent	Weak adherent
Salmonella isolates			
SL2	Non-adherent	Weak adherent	Weak adherent
SL4	Weak adherent	Weak adherent	Weak adherent
SL7	Weak adherent	Weak adherent	Weak adherent
SL8	Weak adherent	Weak adherent	Weak adherent
SL20	Weak adherent	Weak adherent	Weak adherent
SL21	Weak adherent	Weak adherent	Moderate adherent
SL25	Weak adherent	Weak adherent	Weak adherent
SL26	Non-adherent	Weak adherent	Weak adherent
SL27	Weak adherent	Weak adherent	Weak adherent

[1][$OD \leq OD_C$ = non-adherent, $OD_C < OD \leq 2 \times ODC$ = weak adherent; $2 \times OD_C < OD \leq 4 \times OD_C$ = moderate adherent and $4 \times OD_C < OD$ = strong adherent. OD= Optical density value for inoculated wells; OD_C = Optical density value for negative control wells]

ability between the isolates. Overall, results showed that there were two bacterial strains (A14 and G24) isolated from the APC plates with no ability to form biofilm at any temperatures whereas five isolates (A16, G18, G21, G29 and G34) showed weak biofilm adherence at all temperatures. Three isolates (A9, A12 and G14) only produced biofilm at 37°C while B2 produced biofilm at 25°C, although the biofilm adherence was weak. Other isolates that showed moderate biofilm adherence were A7 (25°C and 37°C), B3 (4°C and 25°C), A31, B4 and G30 (all at 37°C). Only one *E. coli* strain (A1) showed strong biofilm adherence but solely at 25°C. This particular isolate only formed moderate biofilm adherence at other temperatures. Other *E. coli* isolates formed weak biofilm adherence at all temperatures except for EC7 and EC37 that did not produce any biofilm at 4°C. Similar observation was seen with *Salmonella* isolates, where most strains showed weak biofilm adherence at all temperatures but both SL2 and SL26 did not form any biofilm at 4°C. However, one *Salmonella* isolate (SL21) had moderate biofilm adherence at 37°C. Based on Table 4, more than 50% of the isolates formed weak biofilm adherence at all temperatures. Meanwhile, the isolates had lower biofilm formation ability at 4°C in comparison to other temperatures.

DISCUSSION

Meat contact surfaces are identified as one of the contributing factors to microbial contamination of meat products in meat processing chain (Gounadaki et al., 2008). Regular inspection in terms of hygiene is essential to assure the quality and safety

Table 4
Distribution of biofilm production level of isolates obtained from meat contact surfaces across temperatures tested (4, 25 and 37°C)

Temperature	Biofilm Production Level	Total isolates	Percentage (%)
4˚C	Strong	0	0
	Moderate	3	8.1
	Weak	20	54.1
	Non-adherent	14	37.8
	Total	37	100
25˚C	Strong	1	2.7
	Moderate	2	5.4
	Weak	27	73.0
	Non-adherent	7	18.9
	Total	37	100
37˚C	Strong	0	0
	Moderate	5	13.5
	Weak	28	75.7
	Non-adherent	4	10.8
	Total	37	100

of the final meat products before being distributed to the public. The hygiene of the meat contact surfaces can be evaluated via indicator bacteria such as total aerobic mesophilic bacteria and Enterobacteriaceae counts (Tomasevic et al., 2016). In this study, the bacterial strains isolated are the bacteria commonly associated with microbial beef contamination (Doulgeraki et al., 2012). The average contaminations of the indicator microorganisms on the meat contact surfaces ranged from 3.61 to 5.66 log CFU/cm^2 and from 2.07 to 3.65 log CFU/cm^2 respectively. Currently, there is no specific regulation on the permissible range of microbial loads on meat contact surfaces in Malaysia. However, this study adopted the guidelines used by Australia and European countries, which suggest 10 CFU/cm^2 for total aerobic plate count (APC) and 1 CFU/cm^2 for Enterobacteriaceae (Gómez Ariño et al., 2012; New South Wales Government Food Authority [NSWFA], 2013). Based on the guidelines, the sanitation level of the meat contact surfaces in the selected abattoirs is deemed as poor.

It is a common practice for operators in the abattoirs worldwide to clean the hand tools such as knives by first rinsing them to remove any soil and have them sanitized in hot water at 82°C (Desmarchelier et al., 2007; Eustace et al., 2007; "EU Regulation 853/2004", 2004). This procedure is conducted for each carcass to prevent cross-contamination. However, based on the observation in the selected abattoirs involved in this study, this practice is not done rigorously, which may explain the high amount of APC and Enterobacteriaceae found on the meat contact surfaces. Another cause can be contamination due to the operators' hands. A study conducted in local abattoirs in Peninsular Malaysia (Shamsul et al., 2016) found that there was high prevalence of microbial contamination on the operators' hands during the meat processing procedures, which were due to inadequate practice of hand washing among the operators. However, this aspect is not covered in this study, whether the operators are aware and fully trained, or there is laxity among the operators in carrying out the procedures. Nevertheless, different meat processing environments apply different hygiene practices and the operators' knowledge, attitude and compliance with the abattoir laws vary between one another (Abdullahi et al., 2016; Ansari-Lari et al., 2010).

Although the number of samples positive with *E. coli* and *Salmonella* was low in this study, their presence on the meat contact surfaces poses a high risk of contamination, that will lead to foodborne illness if the meat products are consumed by the consumers. In fact, *E. coli* is one of the common microorganisms associated with contamination of beef products (Sofos 2008). Although *Salmonella* is more common in poultry products, there is a prevalence of this microorganism in Malaysian retail beef products (Tan et al., 2019). Hence there is a need to monitor the prevalence of the microorganisms in the abattoir environment to ensure the biosafety of beef products. The *E. coli* concentration found on meat contact surfaces indicates the level of pathogenic *E. coli* present in the abattoir environment

(Brown et al., 2000). The highest *E. coli* contamination level in this study was more than 2.5 log CFU/cm^2. This is dangerous as it has been established that the infectious dose for pathogenic *E. coli* such as O157:H7 is as low as 10-100 cells (Desmarchelier & Fegan, 2003). Furthermore, *Salmonella* spp. has the ability to colonise and persist on food contact surfaces and keep on being a contaminant to the final meat products (Joseph et al., 2001; Sallam et al., 2014).

It is surprising to find that the microbial contamination levels on knives and splitting tools prior to slaughter was already high. This raises the question on the effectiveness of cleaning and disinfection procedures in the abattoirs. However, detections of microorganisms on cleaned surfaces, which is up to 10^5 CFU/cm^2 were previously reported (Marouani-Gadri et al., 2009; Schlegelova et al., 2010). If the carcass gets contaminated with microorganisms due to the knives used during dehiding process, the contamination level will increase as the carcass moves along the production line and it can transfer the microorganisms to other meat contact surfaces. Although there is no significant difference, microbial loads on the meat contact surfaces showed an increase of microorganisms sampled after slaughtering procedures. A sufficient cleaning and disinfection regime must be at place to reduce microbial contamination in meat processing environment (Tomasevic et al., 2016).

Deficiency in hygiene practice during the cleaning and disinfection procedures will result in buildup of meat and fat residues on meat contact surfaces (Gill & McGinnis, 2004). This will cause the microbial concentration to accumulate on automated tools such as the splitting tools used for evisceration, which are much harder to clean. The complex structure of the tools causes meat residues to easily accumulate but hard to be accessed during cleaning. This in turn will support bacterial growth on the surfaces (Giaouris et al., 2014; Rivera-Betancourt et al., 2004). This is one of the factors that splitting tools were found to be the most contaminated contact surface in this study. The high microbial contamination subsequently increases the risk of contamination of the final meat products. In our previous study at the same meat processing environment (Chong et al., 2017a), beef carcasses were found to be contaminated with average APC of 4.00 log CFU/cm^2 with the presence of *Salmonella* spp. in some of the samples, that might be due to cross-contamination from the meat contact surfaces. Continuous accumulation and persistence of microorganisms on the meat contact surfaces can lead to biofilm formation on these surfaces (Sofos & Geornaras, 2010).

The high APC and Enterobacteriaceae levels found on the surfaces are sufficient to initiate biofilm formation. Biofilm formation involves many factors that include surface characteristics, properties of bacterial strains as well as environmental factors such as nutrient level, presence of antimicrobial agents, pH and temperature (Chmielewski & Frank, 2003; Giaouris et al., 2014). Although there is a limitation to reproduce the field conditions of the meat

contact surfaces in this study, microplate-based biofilm assays are extensively used to screen the biofilm formation capacity of bacterial isolates (Azredo et al., 2017). Although the surface materials used in the study are different than the actual meat contact surfaces available in the abattoir, the results can still be an indicator of the bacterial isolates' biofilm formation ability.

In this study, although more than 50% of the isolates obtained from the meat contact surfaces are regarded as weak biofilm producers at all temperatures, it still poses a threat to the meat processing environment, because there are reports that the standard sanitation procedures are not sufficient in removing biofilms (Giaouris & Simões, 2018; Joseph et al., 2001; Srey et al., 2013; Vogeleer et al., 2014). This can interfere with the cleaning procedures thus increase the risk of contamination of the meat products (Ayalew et al., 2015; Srey et al., 2013). Most *E. coli* and *Salmonella* isolates obtained in this study demonstrated weak biofilm production, which is in contrast with the previous studies (Speranza et al., 2011; Vogeleer et al., 2016; Wang et al., 2016). However, there are reports on variations between strains, even from the same species (Lianou & Koutsoumanis, 2012; Nesse et al., 2014; Pui et al., 2011; Wang et al., 2013, 2016). In this study, the variations of the isolates' biofilm formation ability at three temperatures are consistent with other reports (Di Bonaventura et al., 2008; Dourou et al., 2011; Rode et al., 2007).

Contamination of meat contact surfaces that leads to carcass contamination in the abattoir is inevitable during the slaughter and after slaughter (Niyonzima et al., 2015). The contamination resulting from the meat contact surfaces to the uncontaminated meat products must be strictly controlled. Even it is difficult to fully eliminate microbial contaminants from the meat processing environment, every meat producer is responsible to minimise the contamination level (Lowe et al., 2001). Good hygienic practice is essential to maintain the abattoir sanitation level, thus ensuring safe and top quality meat products. This can be achieved by the implementation of sanitation standards (HACCP, Good Hygiene Practices, Standard Sanitation Operating Procedures, etc.) and regular microbial quality assessment of the meat products and meat contact surfaces in meat processing establishments (Gómez et al. 2012; Tomasevic et al., 2016). The operators should be provided with training and regular supervision to ensure they understand the importance and roles of the programmes to make it a success (Kusumaningrum et al., 2003).

CONCLUSION

Meat contact surfaces were found to be contaminated even before slaughtering. The microbial contamination level of the meat contact surfaces was much higher than the permissible levels suggested by the international guidelines. Most of the isolates obtained from the surfaces were found to produce biofilms albeit their strength was low. This study has emphasized the need for better monitoring of local abattoir sanitation level by the relevant authorities.

Implementation of sanitation standards is suggested to facilitate better intervention strategies and policy in maintaining high hygiene level in the abattoirs, thus ensuring the safety and quality of the local beef supply.

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Short Communication

Trends on Isolation of *Staphylococcus aureus* and Distribution of the Virulence Genes in Hospital Universiti Sains Malaysia, Kelantan

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ABSTRACT

Staphylococcus aureus is one of the most common pathogens isolated from clinical specimens in many hospitals worldwide. The objectives of the present study was to determine the trends of the *S. aureus* isolated from clinical specimens and to detect the virulence genes of *S. aureus* namely *mec*A, *LukS*, *can*, *ica*A, *SdrE* and *hlg*. Data of *S. aureus* isolation in Hospital Universiti Sains Malaysia (HUSM) from 2002-2014 which is available in our WHONET system was analyzed. *Staphylococcus aureus* isolates were randomly collected from the archived culture and the virulence genes were detected by PCR. A total of 15176 *S. aureus* were isolated and reduction in the MRSA was observed during the study. This study showed that *S. aureus* infection is still an unresolved issue, where clinical specimen from swab contributes the highest number. The percentage of MRSA also is fluctuating and serves as a good indicator for a more vigilant infection control activity in the hospital setting.

Keywords: Malaysia, MRSA, trends, virulence genes

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INTRODUCTION

Staphylococcus aureus infection can be acquired through both hospital and community settings. The ability of *S. aureus* to cause disease depends on a wide range of virulence factors that contributes to colonization and disease in the host. In Malaysia, an increase in *S. aureus* isolation was observed from a total of 32,611 isolations in 2012, to 34,492 isolations in 2013, whereas the prevalence of methicillin-resistant *S. aureus* (MRSA) in Malaysian hospital had decreased from 26% in 2008 to 17.7% in 2013 (Akpaka et al., 2006). In the United States, an increase of 62% of *S. aureus*-related hospitalizations and an increase in the number of MRSA-related hospitalizations more than doubled (Klein et al., 2007).

There are limited studies on the prevalence of *S. aureus* over a longer period of time in Malaysia specifically in Kelantan. The study on trends of isolation of specific types of organisms helps the policy makers to make certain regulation especially related to infection prevention and control. Therefore, in this present study, the retrospective analysis of *S. aureus* isolation from 2002 till 2014 was done to determine the trends of *S. aureus* and MRSA isolation in Hospital Universiti Sains Malaysia (HUSM), Kelantan, Malaysia. Moreover, the prevalence of its virulence genes and its association with the specific clinical presentation of *S. aureus* infection were also determined. A comparison with *other* community in different geographical areas *may provide valuable information which may be important in the area of study*.

MATERIALS AND METHODS

Data of *S. aureus* infection of patients (location of wards or clinics and type of specimen) in HUSM, Kelantan from 2002 to 2014 was obtained retrospectively from WHONET database, a software used for the management and analysis of microbiology laboratory data. The types of wards include medical wards, intensive care units (ICUs), orthopedic wards, surgical wards, pediatric wards, and others. While the types of specimen collected in HUSM were swab, blood, pus, tracheal aspirates, wound, tissue, and others. The data was analyzed statistically by independent t-test and one-way ANOVA test using IBM SPSS Statistic software (version 21.0). Independent t-test was used to compare the prevalence of MRSA between years while one-way ANOVA test was used to compare the difference in frequency between each virulence genes. In this study, *S. aureus* ATCC 25923 and 80 clinical *S. aureus* isolates were randomly selected from the Medical Microbiology and Parasitology Laboratory, HUSM to study the virulence genes distribution (Table 1). For confirmation of *S. aureus*, all isolates were also evaluated for the presence of the species-specific gene *fem*A by PCR using forward primers CGCAAACTGTTGGCCACTAT and reverse primer CTCGCCATCATGATTCAAGT (Nik Zuraina, 2018).

DNA extraction was carried out by boiling method according to Queipo-Ortuño et al. (2008) with slight modification. Specific primers were used to amplify the virulence genes (Table 2). The PCR amplification was performed in 20 µl

of reaction mixture containing 20 ng of template DNA, 1 pmol/µl of primers (Integrated DNA Technologies, Singapore), 0.16 mM of dNTPs, 0.75 U Taq DNA polymerase, 2.5 mM $MgCl_2$ and 1X Taq polymerase buffer (Thermo Scientific, USA). The PCR was performed using a DNA thermal cycler MJ Research PTC-200 DNA Engine (MJ Research, Canada). All 80 isolates were amplified individually for all six genes using the specific primers, with 1 cycles of denaturation at 95°C for 1 min, 30 cycles of annealing at 56 °C for *LukS/F-PV* and *mec*A, 54°C for *ica*A, 48°C for *sdrE*, and 55.8°C for *hlg* and 46°C for *cna* for 1 min and elongation at 72 °C for 1.5 min; and followed by final extension at 72°C for 10 min. The same PCR cycling condition was used for amplification of *fem*A gene with an annealing temperature of 60°C for 1 min. Then, the PCR products were stained using DNA fluorosafe stain (1st Base Laboratories, Malaysia), and electrophoresed in 1.5% agarose gel at 90 V for 60 minutes. The PCR products were visualized using Alpha Innotech ChemiImager 5500 UV illuminator and image capturing unit (California, USA). The DNA band was analyzed and the size was determined by referring to the size of 100 bp DNA marker (Fermentas, USA).

Table 1
List of bacterial strains used in this study

Bacteria	Sources	No. of Strain
Staphylococcus aureus ATCC™ 25923	ATCC	1
S. aureus B1 – B20	Blood	20
S. aureus T1 – T20	Tissue	20
S. aureus SP1 – SP20	Sputum	20
S. aureus S1 – S20	Swab	20
Total		81

Table 2
Description of primers used for amplification of virulence genes

Gene	Putative function	Primer sequence (5'- 3')	Product size (bp)	References
LukS/ F-*PV*	Bicomponent leukocidin.	F: CAG GAGGTAATGGTTCATTT R: ATGTCCAGACATTTTACCTAA	151	(Al-Talib et al., 2009)
*mec*A	Methiclin resistance	F: ACGAGTAGATGCTCAATATAA R: CTTAGTTCTTTAGCGATTGC	293	(Al-Talib et al., 2009)
cna	Adhesin for collagen	F:AGTGGTTACTAATACTG R: CAGGATAGATTGGTTTA	744	(Peacock et al., 2002)
hlg	Bicomponent leukotoxins	F: GCCAATCCGTTATTAGAAAATGC R: CCATAGACGTAGCAACGGAT	937	(Peacock et al., 2002)
*ica*A	Polysaccharide intercellular adhesin	F: GATTATGTAATGTGCTTGGA R: ACTACTGCTGCGTTAATAAT	770	(Peacock et al., 2002)
sdrE	Putative adhesins	F: AGTAAAATGTGTCAAAAGA R: TTGACTACCAGGCTATATC	767	(Peacock et al., 2002)

RESULTS AND DISCUSSION

Over the period of 13 years (2002-2014), a total of 15,176 *S. aureus* was isolated in HUSM from 9584 patients. Whilst, a total of 4469 (29.4%) of *S. aureus* isolates were MRSA isolated from 2092 patients. This contributes to the MRSA prevalence of 21.8%. The average annual rate of MRSA infections was 344/year and the highest number of *S. aureus* infection was in 2013 (n=1790). The prevalence of *S. aureus* isolation in HUSM has increased by 1.8% from 2002 to 2014 and the prevalence of MRSA has declined from 41.6% in 2002 to 28% in 2014 (Figure 1). The differences between the years were significant (P<0.05). Similarly, a National Surveillance of Antibiotic Resistance Report by the Ministry of Health (MOH) (2013) reported that *S. aureus* isolation had increased from 32,611 *S. aureus* isolated in 2012 to 34,492 *S. aureus* isolated in 2013. Meanwhile, the MRSA prevalence has decreased from 26% in 2008 to 17.7% in 2013 (MOH, 2013).

In comparison, the MRSA prevalence in Hospital Kuala Lumpur (HKL) and Universiti Kebangsaan Malaysia Medical Centre (UKMMC) in 2007 to 2008 and 2009 were 44.1% and 26.6% respectively, were higher compared to the present study (Ghaznavi-Rad et al., 2010; Sapri et al., 2013). The MRSA rate among *S. aureus* isolates in this study was 29.4% which is within the range reported in Western Europe which was between 5% and 54% during 2000-2010 (Dulon et al., 2011). However, the rate cannot be compared directly with the previous study because of the different socio-demographic characteristics of the patients and hospitals. While in the USA, the MRSA infection rates declined 54% between 2005 and 2011, with 30,800 less severe MRSA infections (Dantes et al., 2013).

The MRSA isolation showed varying trends where a progressive decrease in MRSA infection rates was observed through 2002 to 2006, subsequently, the occurrence started to increase moderately from 2007 to 2010 and rapidly increased MRSA isolation through 2011 to 2013. Then, the MRSA isolation was slightly declined from 2013-2014 (Figure 1). The progressive decrease in MRSA infection rates during 2002 to 2006 in HUSM might be due to new prophylactic measures used, including strict isolation of patient whether suspected or proven to have MRSA infection; education improvements, hand hygiene, and use of hydro-alcoholic solutions; impact of surveillance; and better quality of care as a whole (Al-Talib et al., 2010).

The highest *S. aureus* isolated in HUSM were from medical wards (21.1%), followed by ICUs (18.0%), orthopedic wards (15.9%), surgical wards (14.2%), pediatric wards (3.9%) and others (26.9%). Other locations including Accidents and Emergency Department, Obstetrics and Gynecology Department, and Oncology and Ophthalmology Department. While in HKL, the highest *S. aureus* isolated from 2007 to 2008 were medical wards (20.1%), pediatrics (9.2%), surgical wards (11.2%), orthopedics (12.8%) and ICUs (5.1%). Findings in our current study

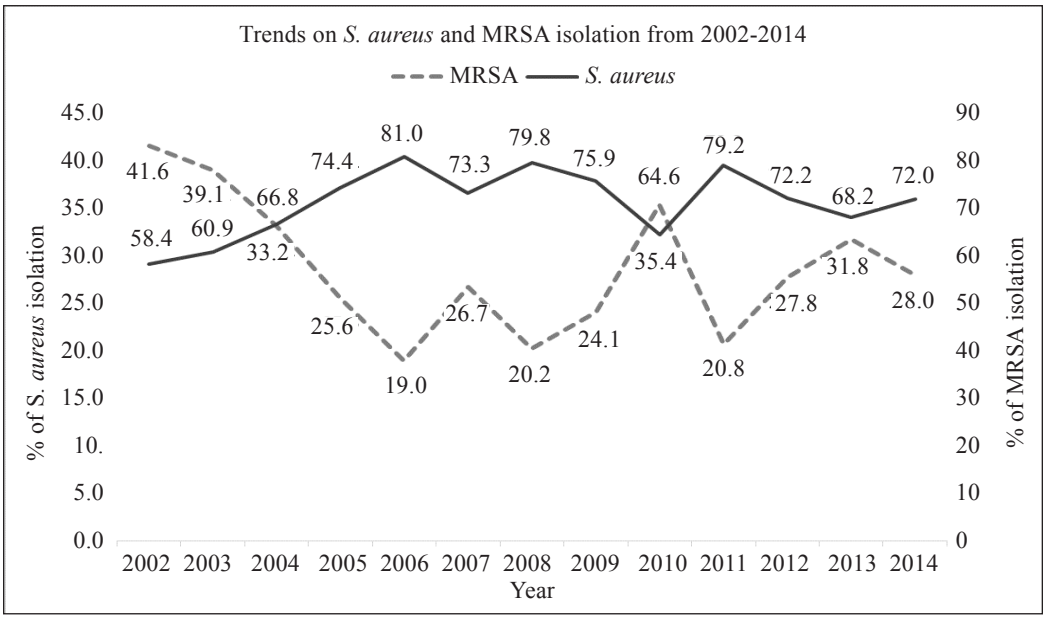

Figure 1. Trends of *Staphylococcus aureus* isolation (left) and MRSA isolation (right) from 2002 – 2014

are in agreement with the previous study conducted in HKL, Malaysia which showed that *S. aureus* was most frequently isolated from medical wards (Ghaznavi-Rad et al., 2010).

High-frequency *S. aureus* isolation from medical wards, orthopedic wards, and surgical wards reflects that these wards usually accommodate chronic patients that require prolonged hospitalization. Infected or colonized patients act as reservoirs, with transient hand carriage by healthcare workers and caretakers of these patients being the predominant mode of transmission from one person to another (McDonald, 1997). It could also be due to the traumatic and postoperative immunological suppression of the patients and environmental factors that were probably related to the higher rate of MRSA infection in these locations (Al-Talib et al., 2013).

In this study, swab specimen had the highest number of *S. aureus* isolation (22.61% of the total in HUSM) followed by blood (17.24%), pus (14.42%), tracheal aspirates (10.62%), wound (7.74%), tissue (7.53%) and others (19.84%). Other specimen includes urine, abdominal fluid, and cerebrospinal fluid. Additional PCR result showed the *fem*A gene (293 bp) was detected in all 80 isolates indicated the isolates were *S. aureus*. Then, PCR amplification of six virulence genes showed that only five genes (*mec*A, *LukS, cna, ica*A, *SdrE*) were present in some isolates except for *hlg* gene. The *mec*A gene was amplified in 13 isolates (16.25%), while 11 isolates (13.75%) were positive for carrying *LukS* gene, 4 isolates (5.0%) for *cna* gene, 20 isolates (25%) for *ica*A gene and 23 isolates (28.75%) for *SdrE* gene. However,no isolate was positive for carrying *hlg* gene.

The genes amplification did not occur consistently in all isolates where different genes were available in different isolates. However, some of the isolates were carrying two to three virulence genes. The highest coexistence of different genes was *mec*A + *SdrE* genes while the lowest frequency of gene coexistence were *mec*A + *cna*, *mec*A + *LukS* +*SdrE*, *mec*A + *cna* + *SdrE*, *LukS* + *ica*A and *LukS* + *SdrE* where each combination was detected in a single isolate. The frequency of virulence genes in this study was also lower compared with the previous study in the United Kingdom and India (Bhatty et al., 2013; Peacock et al., 2002).

The swab specimens were also recorded to have the highest number of virulence genes in 27 (33.8%) *S. aureus* isolates, followed by 19 (23.8%) tissue, 17 (21.4%) sputum, and 8 (10%) blood. The results showed that the separate frequency of each gene in *S. aureus* isolates indicated a significant difference, with $P<0.05$. There were also recorded 35 isolates were devoid of any gene. It is also suggested that *S. aureus* commonly causes non-complicated superficial skin infection in human. The largest number of virulence gene detected was putative adhesion (*SdrE*) suggested the gene play a role in virulence of *S. aureus* infection.

CONCLUSIONS

This study shows that *Staphylococcus aureus* infection is still an unresolved issue, where clinical specimen from swab contributes the highest number. The percentage of MRSA is fluctuating and serves as a good indicator for a more vigilant infection control activity in the hospital setting to curb the increasing trends of *S. aureus* and MRSA isolation. The present study has also demonstrated a variation in the occurrence of virulence genes populations of *S. aureus* as compared to other region. Studies related to the molecular diversity of *S. aureus* will be very helpful in understanding the molecular epidemiology of *S. aureus* in this hospital.

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Short Communication

Identification of Microfungi Isolated from Belian Fruits

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ABSTRACT

Belian tree (*Eusideroxylon zwageri* Teijsm. & Binn.) is one of the highly demanded commercial timber tree indigenous to the Southeast Asian region. The tree is threatened with over exploitation; habitat destruction and slow regeneration. While vascular plants are known as major reservoirs of fungi species, there have been no studies to identify the microfungi isolated from fruits of this endemic tree. By using internal transcribed spacers (ITS) sequence analysis, five genera were isolated and identified as *Annulohypoxylon*, *Daldinia*, *Hypoxylon*, *Lasiodiplodia* and *Trichoderma*. This result will be primarily used as baseline data for further investigations on microfungi diversity associated with Belian tree.

Keywords: *Annulohypoxylon,* Borneo ironwood, *Daldinia*, *Eusideroxylon zwageri*, *Hypoxylon*, Lauraceae, *Lasiodiplodia*, *Trichoderma*

INTRODUCTION

Fungi plays an important role in the optimal functioning of the ecosystem. In general, they involved in the breakdown of nutrients on the forest floor, which for ready uptake by the plants but in most cases the role of individual fungi in nature is unknown (Mueller & Bill, 2004). Apart from being an important contributors to the diversity of organisms in the forest, microfungi have been consistently utilized in the drug production, food processing, bio-control agents as well as in enzyme biotechnolgy (Kumar et al., 2013; Snaddon et al., 2012).

A study on microfungi associated with endemic plants in Mauritius reported more than 200 taxa of microfungi with approximately 90% of being new genus (Dulymamode et al., 2001) while study in Kenya reported four fungal taxa were new to science detected from their endemic and rare

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plants (Siboe et al., 2000). This is shown that, many unknown microfungi are likely to be rare and threatened to the same degree as their plant host.

However, very little is known and reported on the microfungi diversity isolated from the endemic and endangered trees in Malaysia. Belian tree, scientifically known as *Eusideroxylon zwageri* Teijsm. & Binn. is one of the most important commercial timber trees indigenous to Southeast Asian forests. It is native to Malaysia (Sarawak and Sabah), Brunei, Indonesia and The Philippines. The tree is taxonomically classified in the Lauraceae family and listed as a vulnerable species by the IUCN Global Red List of Threatened Species (version 2017.3) (International Union for Conservation of Nature [IUCN], 2017) resulting from the over exploitation and habitat destruction. To date, only one study of the microfungal diversity on the green and leaf litters of Belian tree from Kubah National Park, Sarawak has been reported (Lateef et al., 2015).

This study therefore, is aimed to identify microfungi community isolated from Belian fruits which will add value to the latest knowledge on Belian tree especially related to the diversity of microfungal. This information will aid further study on their numerous roles with respect to Belian tree.

MATERIALS AND METHODS

Materials

Ten available matured fruits were collected randomly under the adult Belian tree in May 2017 from Semenggoh Reserved Forest in Sarawak, Malaysia. The fruit samples were collected on the forest floor near to the mother tree and were kept in brown paper bags, labeled and transported to the laboratory for fungi isolation.

Isolation of Microfungi

The fruits were washed under tap water and were surface sterilized with 70% ethanol solution for 5 minutes, and then, the fruits were rinsed with sterilized distilled water for three times and blot-dried using sterilized filter paper (Kouame et al., 2010). The pericarp layer of the fruit samples was divided into three parts: tip, middle and bottom of the fruits to get an overall estimation of the fungi reside in the pericarp part of the fruits. Then it was aseptically cut into 1 cm^2 using sterile surgical blades and carefully transferred onto potato dextrose agar (PDA) plate supplemented with Streptomycin (20 mL/L of medium).

The plates were sealed and labeled by fruits sample part, initial culturing date and incubated at 28±2^oC. Observation and isolation of the growing microfungi on the surface of the media plates, colonies that differ in time of appearance, size, color, and elevation were recorded. A loop of each original pure fungi colony was picked up using plating technique, and re-plated onto a solid pure culture agar plates containing the same culture media but without antibiotics. The plates were sealed, labeled and incubated until the fungi grew to half of the plate.

The hyphae tip technique was performed to obtain pure culture isolate (Leslie & Summerell, 2006). Then the pure fungi isolates were transferred to PDA slants for short-term storage or in complete media xylose (CMX) 25% glycerol at -80°C for long-term preservation.

DNA Extraction and PCR Amplification

Total genomic DNA were extracted from ten successful pure cultured isolates using an Ultra Clean ® Microbial DNA isolation kit (MO-BIO, Carlsbad, CA, USA) according to manufacturer's instructions. Oligonucleotide primers ITS1 (5'-TCCGTAGGTGAACCTGCGG- 3') and ITS4 (5'-TCCTCCGCTTATTGATA TGC-3') (White et al., 1990) were used to amplify internal transcribed spacer (ITS) region of the nuclear ribosomal DNA. Each polymerase chain reaction (PCR) contained 5 uL of 5X PCR buffer, 4 uL of 25 mM $MgCl_2$, 0.5 uL of 10 mM dNTPs (Promega), 0.5 uL of each 10uM primer, 0.1uL of 5 units/uL GoTaq® DNA polymerase, 0.5 uL of 10X BSA and 2.0 uL of template DNA adjusted with nuclease free water to a final volume of 50uL. Amplification reactions were carried out in a thermal cycler (Eppendorf AG 22331, Hamburg). PCR cycling protocol was as follows: an initial pre-denaturation for 2 min at 95ºC, followed by 35 cycles of denaturation at 95ºC for 20 s, annealing at 50ºC for 30 s, and extension at 72ºC for 30 s; with a single cycle of final extension at 72ºC for 5 min. The PCR amplification products were separated by electrophoresis in 1.5% agarose gel in 1.0x Tris-boric acid-EDTA (TBE) buffer with Etb "Out" Staining Solution (YEASTERN BIOTECH Co. Ltd) and photographed under UV light. The images were captured with DOC PRINT system (Vilber Lourmat, USA). A good quality of amplified PCR products was sequenced in both directions using an Applied Biosystems 3730xl DNA Analyzer by MyTACG Bioscience Company, MY.

Data Analysis

ClustalW in Molecular Evolutionary Genetic Analysis (MEGA 7) was used to generate and aligned the consensus sequences. The consensus sequences were search against sequences in GenBank using Basic Local Alignment Search Tool (BLAST) (http://www.ncbi.nlm.gov) to determine the closest matched ITS sequence from the database. All the assembled DNA sequences were deposited in GenBank.

A Maximum Likelihood analysis was conducted using MEGA 7 with 1000 bootstrap replication value based on Tamura-Nei Model (Kumar et al., 2016). Branches corresponding to partitions reproduced in less than 50% bootstrap replicated were collapsed. The representative sequences of each species were obtained from GenBank and were included in the tree. The tree was rooted with *Puccinia graminis* (HM131358) as an out-group for this phylogenetic tree. The tree was constructed to comprehend the placement of each isolates and the reference sequences in the lineage.

RESULTS

The phylogentic tree was constructed to understand the placement among the isolates and the reference sequence (Figure 1) and colony features for each isolates were shown in Figure 2. All fungi isolates were identified belong to the phylum Ascomycota, which comprised nine taxa from five genera; *Annulohypoxylon* (2 species); *Daldinia* (1 species); *Hypoxylon* (1 species); *Lasiodiplodia* (1 species); and *Trichoderma* (4 species) (Table 1). The molecular fragment size of ITS region amplified was between 500 – 600 bp except for ITS fragment of *Annulohypoxylon* species which was > 800 bp. Based on BLAST search, the ITS sequences showed high percentages similarities (99% and 100%) with sequences in the GenBank database (http://www.ncbi.nlm.gov). All ITS sequences of the isolates were deposited into GenBank and the accession number are listed in Table 1.

The phylogenetic tree was divided into two main clades; I and II (Figure 1). Main clade I clustered all species under Class Sordariomycetes while main clade II grouped Class Dothideomycetes. Main clade I was further divided into subclades A and B which clustered the species into their specific order. Subclade A nested all species under Xylariales order and subclade B grouped Hypocreales species.

Subclade A contained three genera from *Xylariaceae* family; *Annulohypoxylon, Daldinia* and *Hypoxylon*. All the isolates are highly similar to their respective reference sequences from GenBank and well supported with 100% bootstrap value. Isolate Q2687 was identified as *Annulohypoxylon nitens* (KU684021) while isolate Q2688 was identified as *Annulohypoxylon viridistratum* (KX376325) both with 99% similarity. Colony of *A. nitens* was observed with filamentous white mycelium. The reverse side of the plate showed changes in

Table 1
Fungi species isolated from endocarp layer of Belian fruits which were characterized based on sequencing of the ITS region

Isolate No.	Sequence based identification	Deposited GenBank Accession No.	Percentage of similarities (%)	Accession No. of fungi closest match to GenBank database
Q2680	*Lasiodiplodia theobromae*	MG711820	100 %	KY473061
Q2681	*Trichoderma spirale*	MG711821	100 %	NR_077177
Q2682	*Trichoderma virens*	MG711822	100 %	KR296891
Q2683	*Trichoderma asperellum*	MG711823	100 %	HQ857121
Q2684	Trichoderma crassum	MG711824	100%	NR_134370
Q2685	Daldinia eschscholtzii	MG711825	99%	KU304335
Q2686	Hypoxylon investiens	MG711826	99 %	KC968925
Q2687	Annulohypoxylon nitens	MG711827	99 %	KU684021
Q2688	Annulohypoxylon viridistratum	MG711828	99 %	KX376325
Q2689	Daldinia eschscholtzii	MG711829	99 %	AB284189

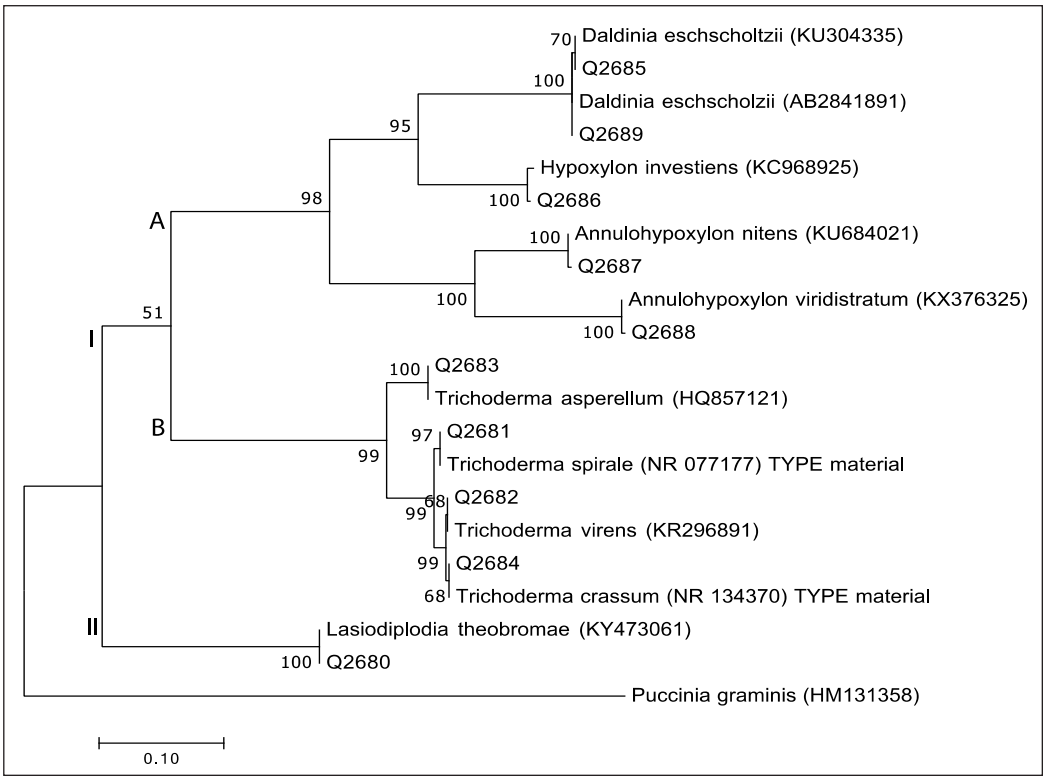

Figure 1. Maximum Likelihood (ML) tree, showing the relationship of fungi isolated from Belian fruits inferred from ITS region. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. *Puccinia graminis* was chosen as the out-group

pigmentation to orange brownish when aging. This differs from the colony feature of *A. viridistratum* which showed a reverse side of yellow pigmentation when aging (Figure 2).

Meanwhile, isolates Q2685 and Q2689 resulted with 99% similarity to two *Daldinia eschscholtzii* strains (KU304335 and AB2841891). Colony features of Q2685 were found to have a dusted white mycelium with yellow tint while colony of Q2689 only formed a dusted white mycelium. The reverse side of Q2689 was seen to form more blackish green pigmentation than Q2685 (Figure 2). Isolate Q2686 was observed with 99% similarity to *Hypoxylon investiens* (KC968925). The colony was found to form circular sparse and thin mycelium and brown ring pigmentation can be seen forming from the front and reverse side of the plate (Figure 2).

Subclade B, order Hypocreales strongly grouped four isolates (Q2681, Q2682, Q2683 and Q2684) to their respective reference sequences as *Trichoderma* species in *Hypocreaceae* family. Nucleotide BLAST for sequences of isolate Q2682 resulted with 99% similarity to *T. virens* (KR296891) while Q2681 resulted as *T. spirale* (NR_077177); Q2683 as *T. asperellum* (HQ857121)

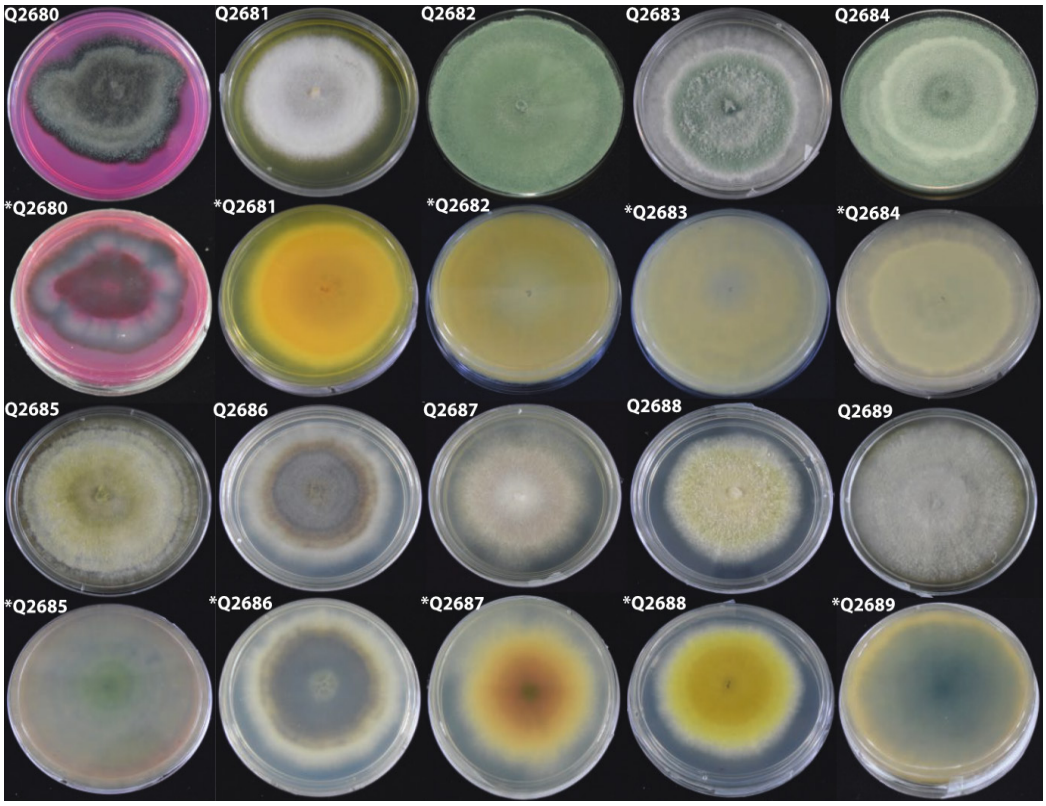

Figure 2. Colony features of the fungi isolated on Potato Dextrose Agar plate
* = pigmentation on reverse plate

and Q2684 as *T. crassum* (NR_134370) resulted with 100% similarity. Colony of *T. asperellum* were observed forming filamentous white floccose aerial mycelium while colony of *T. spirale* formed a thick circular cottony mycelium with green tint that changes the PDA media to yellowsih tan. While both colony of *T. virens* and *T. crassum* have similar colony characteristics of green dusted mycelium except that colony of *T. crassum* was also observed to form white dusted circular on the front features.

Main clade II segregated isolate Q2680 from the rest of the isolates. It was identified as *Lasiodiplodia theobromae* (KY473061) with 100% similarity, which belonged to *Botryosphaeriaceae* family in Dothideomycetes clade. The placement was well supported with 100% bootstrap value. The colony was observed forming irregular black velvety mycelium surface when cultured on PDA media (Figure 2). It formed white to maroon pigmentation when aging in the reverse side of the plate. It was observed that the colony also changed the PDA media to pink coloration when incubated at room temperature.

Puccinia graminis (HM131358) which was chosen as an out-group control for this tree belongs to Basidiomycota phylum, a sister phylum to Ascomycota phylum and distinctly separated from their main clade.

DISCUSSIONS

In this study, we used molecular application techniques to identify the isolated microfungi up to their species level. The use of molecular approaches in this study is more reliable, exhibited higher sensitivity and more time saving compared to morphological characterization techniques. This is due to their cryptic and often ephemeral character that make them difficult to identify with only using classical morphological identification especially when there is no basic information about the fungi diversity on the infected host of Belian fruits. We used the internal transcribed spacers, ITS 1 and 4 regions to amplify and identify the isolated microfungi. The ITS have a convenient target region for molecular identification of fungi due to the variability of length and nucleotide content among different species and amplify highly variable ITS1, ITS2 and the 5.8S-coding sequences (Martin & Rygiewicz, 2005).

It was observed that the size of ITS region for *Annulohypoxylon* species is more than 800 bp as compared to other genera. This was in agreement with a similar study conducted on the fungi isolation from Thailand, which revealed large differences in size from 479 to 936 bp in *Annulohypoxylon* species. A further sequencing analysis using alpha-actin and beta-tubulin was suggestive of the presence cryptic species (Suwannasai et al., 2013) This also supports the need to subject the species of *Annulohypoxylon* identified in this study for further identification processes using other markers. *A. viridistratum* identified in this study was recently reported as new tropical and subtropical species (Kuhnert et al., 2014). *Annulohypoxylon* species usually associated with dead dicotyledonous wood (Fournier & Lechat, 2016) and also frequently found as endophytes in seed plants (Ikeda et al., 2014).

Little is known on the diversity of fungi species associated with Belian tree especially related with their fruits, which will generally affect their seed germination and survival. Study on *Cecropia insignis* has shown that seed-associated fungi are highly diverse (U'ren et al., 2009). It is noted that the most common seed-associated fungi in tropical forest are host-generalists however; they still have profound effects on seed germination for a particular tree species as well as site-specific effects on seed survival. It is desirable to learn more about this understudied topic as fungi are one of the major causes of seed mortality in soil for a variety of tropical trees (U'ren et al., 2009) and it is of more concern when allied with one of the vulnerable endemic tree.

Furthermore, many scientist have acknowledged that various fungi species are associated with many endangered plant species (Buchanan at al., 2002; Fuchs & Haselwandter, 2008; Zubek et al., 2011). This fungi species has the potential for novel utilization in many scientific undertakings. Their association with endangered plant species put them at the risk of going into extinction with their host plants (Fuchs & Haselwandter, 2008). As a threatened species, this scenario is foreseen in Belian tree because of the diversity of their microfungi not yet well studied. It is hoped

that the findings reported in this study will serve as a baseline for further investigations that will enhance the conservation status of this economic tree and its associated microfungi.

CONCLUSION

Since this is a first attempt on studying the microfungi diversity of Belian fruits, data from this study will be used for future understanding of the biology of *E. zwageri* and can also be used to strengthen its conservation importance. It is hoped that the data presented here will lend an insight into further investigation of fungi diversity involved with rooted seed in Belian and as well serving as the starting point for potential novel fast seed germination processes derived from the seed-associated fungi. The outcomes is believed to be far reaching in the understanding, protection and conservation of Belian tree as well as in the conservation of the fungi species associated with endangered plant species which are proned to extinction in the nearest future.

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Review Article

The Potential of Biochar as an Acid Soil Amendment to Support Indonesian Food and Energy Security - A Review

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ABSTRACT

The future of Indonesian food and energy security is challenged by the limited availability of productive land due to the land conversion issue and, in particular, the leveling-off of rice soil productivity. Acid soils as major contributor to marginal soils occupy approximately 55% of the total terrestrial land in Indonesia. To support the Indonesian policy on food and energy security, acid dryland soil areas have targeted for agricultural land expansion. However, managing such soils for crop productivity with conventional amendments, such as lime was challenged by the availability of lime, its cost and the adverse effects of over-liming. Recent research findings indicate that biochar, with its liming capacity and other beneficial effects, could serve as an amendment to acid soils. A question then can be asked: can biochar be a potential solution for the multiple constraints of Indonesian acid soil? The objectives of this review are to explore the potential of biochar as an amendment to Indonesian acid dryland soils and to develop a research framework for future studies involving biochar so as to support the future of Indonesian food and energy security. Articles and conference papers were selected, studied and critically reviewed. Specific problems with Indonesian acid dryland soils and the utilization of biochar as a potential amendment to the suboptimal soil in Indonesia were investigated. Biochar is alkaline in nature and recent research findings strongly indicate that with its liming effect, water and nutrient retention capability, highly recalcitrant nature, and carbon sequestration capacity could be a potential solution for improving upland acid soil productivity. This could be supported by the huge and sustainable production of feedstock in Indonesia.

Keywords: Ameliorant, liming value, pyrogenic carbon, soil acidity, sustainable agriculture

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INTRODUCTION

The future of Indonesian food and energy security is challenged by the limited availability of productive land due to the high rate of agricultural land conversion (150,000-200,000 ha/year) and, in particular, the leveling-off of rice soil productivity (5.3 t/ha in average) (Badan Pusat Statistik [BPS], 2017). The Indonesian population is estimated to be 319 million in 2045 (BPS, 2017). Assuming that rice consumption per capita is 139 kg/year and wetland productivity is 5.341 t/ha, the total rice consumption should then be 42.381 million tons per year and it requires 9.935 million ha. However, Indonesian rice field area is projected to decrease to approximately 5.1 million ha in 2045 (based on the recent Google Earth's IKONOS, Quickbird, and Worldview with 8 to 12 year time differences) (Mulyani et al., 2016). To overcome the lack of required rice field area, suboptimal soils such as acid soil and climatic dryland soil has being targeted for the future agricultural land expansion (Syakir & Nursyamsi, 2015). The acid soil covers the largest dryland area in Indonesia. It occupies approximately 55% of the total land area (191.09 million ha) in Indonesia. About 107.36 million ha of all Indonesian acid soils is classified as dryland acid soils and the rest (14.93 million ha) as peat soil. Managing acid soils for agricultural land is challenged by the multiple constraints of soil acidity e.g. low pH and cation exchange capacity (CEC), low nutrient concentration and retention (high leaching), low beneficial microbe population, activity and diversity, toxicity of Al, Mn or Fe, high P fixation, and low Mo and other micronutrients.

Liming the soil or adding organic materials are considered the most conventional acid soils amendment strategies in Indonesia. However, there is the potential for over-liming the soil, which lead to adverse effects such as trace element deficiency and cation imbalances, the availability or cost of liming materials, and distribution of liming materials. Adding organic materials to acid soil can increase soil pH and decrease soil exchangeable Al. However, this beneficial effect is only short-lived. Therefore, an alternative solution is required for alleviating the multiple constraints on acid soils for the future agricultural development.

Biochar, the rich C byproduct of biomass pyrolysis under a limited supply of oxygen has being established as a soil amendment and agent of carbon sequestration. It can be produced from a single biomass source or a mixture of biomass materials at the high heat pyrolysis temperature of 300-700^{o}C. Biochar, based on recent research findings, has the liming potential with good pH buffering capacity, thus it could be potential for ameliorating soil acidity. Biochar has also being established as an adsorbent for heavy metal or pollutants removal. Moreover, it also has the capacity to retain water and nutrients, a characteristic that could potentially alleviate the leaching problem of highly weathered tropical acid soils, in addition to as nutrient sources for plant growth. In addition, biochar could also be a secure habitat for soil microbial

community due to its high porosity. Biochar has also being established as an agent of carbon sequestration, and greenhouse gas emission abatement, making it a climate change mitigation tool. These beneficial effects of biochar on acid soils have attracted the attention of researchers worldwide and research findings have being formulated to be government policies in some countries. There is a substantial opportunity to alleviate Indonesian soil acidity and climate change problems simultaneously by using biochar. However, there are very limited research findings on ameliorating highly weathered acid soils with biochar in Indonesia. Thus, the objectives of this overview are to summarize the benefits and the potential of using biochar to improve the productivity of Indonesian acid mineral soils and to provide a simplified framework for developing biochar as a sustainable amendment to Indonesian acid mineral soils.

Acid Soils in Indonesia

Acid soils in Indonesia are distributed amongst the big islands, such as Kalimantan (39.42%), Sumatera (28.81%), Papua (18.03%), Java (7.77%), and Sulawesi (6.95%) (Figure 1). Most of the acid soils in Indonesia are derived from old volcanic and sedimentary rocks under a humid tropics condition (annual rainfall > 2.000 mm or udic moisture regime, and temperature >22^{o}C), at the wide ranges of their development, and were dominated by order of Ultisols (41.92%), Inceptisols (40.89%), Oxisols (14.14%), Entisols (3.8%), and Spodosols (2.08%) (Mulyani & Sawarni, 2013).

In some sites, such as Jasinga (Bogor, West Java) and Guradog (Lebak, Banten), the exchangeable Al is extremely high (8.7-14.0 cmolc/kg) and the pH is also very low (3.9-4.0) (Berek & Hue, 2016). Consequently, the ameliorant should also

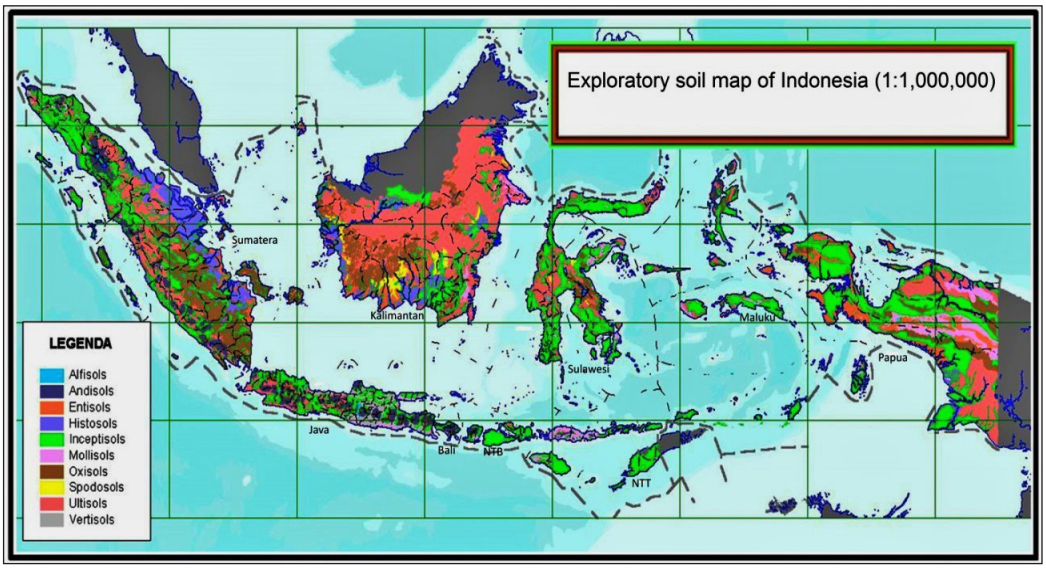

Figure 1. Exploratory soil map of Indonesia (Adapted from Pusat Penelitian Tanah dan Agroklimat Indonesia [Puslittanak], 2000)

be applied at a high rate for increasing the pH and alleviating the toxicity of Al. In addition to chemical constraints, water shortage is another problem of podzolic soils and podsols. The range of critical moisture content is narrow, thus improving water content of these soils could be considered for any amendment chosen (Notohadiprowiro, 1989). Furthermore, the soil acidity issue in Indonesia varies widely site to site. For example, acid soils samples collected from 31 sites (including Java, Sumatera, and Kalimantan) were shown to vary in soil type, dominant clay minerals, C and N content, pH and CEC (Martinsent et al., 2015).

The new challenging for Indonesian food and energy security in the future could be how to improve the productivity of the sub-optimal land, including upland acid soils. Thus, the most important question is: Can biochar help support the future of Indonesian food and energy security?

Biochar Alkalinity, Liming Effect, and pH Buffering Capacity

Biochar is alkaline in nature. The alkalinity of biochar is originated from inorganic and organic components of biochar (Fidel, 2012). More specifically, there are four partitions of biochar alkalinity determined by the quantification method: carbonates, other inorganics, low-pKa organic structural and other organics (Fidel et al., 2017). Carbonate content is responsible for biochar alkalinity (Hass et al., 2012; Mukome et al., 2013) particularly the high temperature pyrolysis biochar (Yuan et al., 2011). The close correlation (r^2 = 0.84) between alkalinity and the quantity of basic cations in the biochars has been shown by Fidel et al. (2017), and the carbonate content is closely correlated (r^2 = 90) with the quantity of basic cations (Figure 2) (Berek & Hue, 2016). During the pyrolysis, the major basic moieties in the feedstock ash become transformed to their carbonates or oxides, referred to as calcium carbonate equivalent (CCE). The liming value (expressed as the

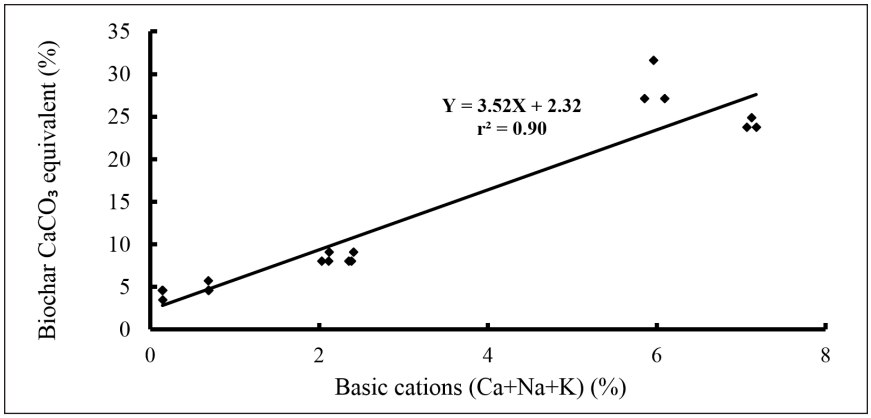

Figure 2. The relationship between biochars $CaCO_3$ equivalent and basic cations (Adapted from Berek and Hue, 2016)

cmol(OH-)/kg biochar) of measured $CaCO_3$ equivalent is then proportional to the liming value or alkalinity that was produced from the total quantity of basic cations in the biochars (Berek & Hue, 2016).

Oxygenic functional groups such as carboxylic acids and phenolics could also be responsible for increasing the alkalinity of low pyrolysis temperature biochar (Keiluweit et al., 2010; Wang et al., 2014; Yuan et al., 2011). The good buffering capacity of the biochars, the capacity of biochars to resist pH change, is originated mostly from their high cation exchange capacity. For example, the negative charge derived from the deprotonation of biochar's surface functional groups and the carbonates give biochars a high buffering capacity (Dai et al., 2014).

Beneficial Effects of Biochar on Acid Soils

A meta-analysis conducted by Biederman and Harpole (2013) revealed that biochar promoted plant productivity and yield allowing for positive short- and long-term effects. For example, biochar's ability to improve water holding capacity and introducing nutrients such as K and P were shown to be short-term effects, while liming effect and nutrient retention were shown to be long-term effects. Alkaline biochar was shown more effective (than acidic one) at increasing soil pH, reducing exchangeable Al and Fe, which in turn, would increase P availability in acid soils. Jeffery et al. (2017) performed a global-meta analysis and pointed out that enhancing crop yield through the use of biochar in the Tropics was more effective than via liming and fertilization of acid low nutrient soils received low fertilizer input. The effects and mechanisms by which biochar can improve acid soil productivity for Indonesian upland acid soils will be summarized and discussed in the following paragraphs.

As previously mentioned, increasing the pH, alleviating Al toxicity and to lesser extent Mn or Fe toxicity, and decreasing P fixation are considered the main expected beneficial effects of adding biochar to acid soils. Figure 3 shows the correlation biochar alkalinity and soil pH, while Figure 4 shows how soil exchangeable Al relates to biochar $CaCO_3$ equivalent. The main beneficial effect of biochar depends on its alkalinity, which is originated from the basic cations in the ash impurity, and the oxygenic surface functional groups attached on its surface (Chintala et al., 2013; Deenik et al., 2011; Joseph et al., 2010; Nguyen & Lehmann, 2009; Novak et al., 2009; Singh et al., 2010; Slavich et al., 2013; Smider & Singh, 2014; Streubel et al., 2011; Tryon 1948; Van Zwieten et al., 2010; Wang et al., 2014; Yamato et al., 2006; Yuan et al., 2011).

Basic cations within biochar are in the form of carbonates or oxides. These cations are the source of biochar alkalinity and will replace the function of lime in producing the ion OH^- to neutralize excess ion H^+ resulting in an increase soil pH (Berek & Hue, 2016). Alleviating Al toxicity may be attributed to the decreased activity of monomeric Al^{3+} and other species by precipitation due to the increased soil pH, the similar mechanism

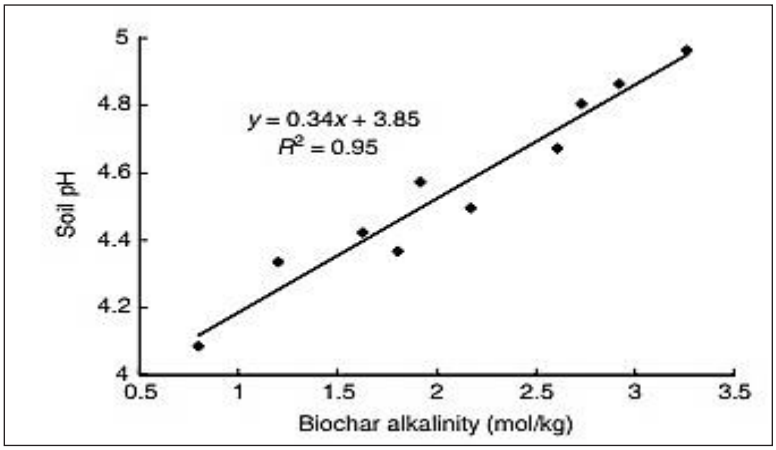

Figure 3. The correlation between biochar alkalinity and soil pH (Adapted from Yuan and Xu, 2011)

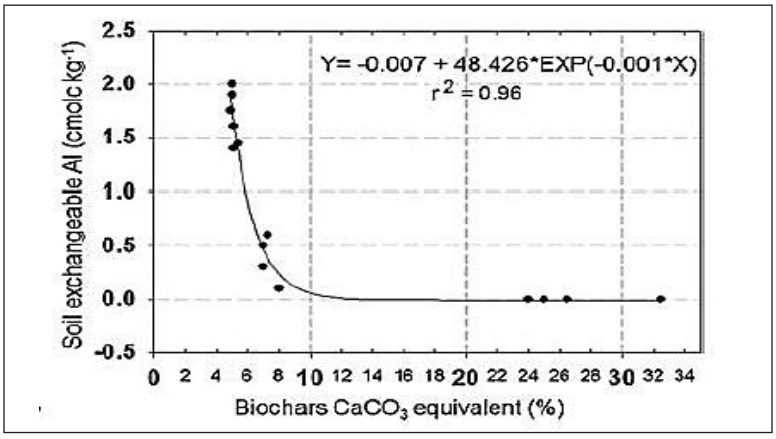

Figure 4. The relationship between soil exchangeable Al and biochars $CaCO_3$ equivalent (Adapted from Berek and Hue, 2016)

by which lime neutralize pH and detoxifies Al phytotoxicity. Increasing soil pH and precipitating Al and Fe will then release fixed P to available form to plant. Micronutrients (e.g., Zn and Mo) will become more readily available, and the activity of beneficial microbes (e.g., rhizobium, mycorrhizal fungi, and phosphate solubilized bacteria) will also become more pronounced after the decrease of soil acidity. Therefore, it seems likely that biochar could replace lime functions in correcting soil acidity problems.

Decarboxylation of organic anions and the negatively charged functional groups will consume protons and increase the soil pH (Wang et al., 2014). The oxygenic functional groups of biochar, such as phenolics and carboxylic acids can provide binding sites for Al adsorption. Carboxylic acids can also complex with Al in the soil solution and reduce its toxicity to plant growth (Figure 5) (Qian et al., 2013). These mechanisms are similar to the mechanisms by which organic amendments alleviating

Al toxicity in acid soils (Berek et al., 1993; Hue, 2011; Hue et al., 1986). Thus, biochar is expected to be more effective than conventional acid soil ameliorants.

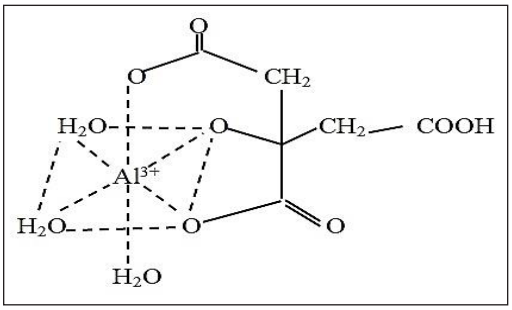

Figure 5. Al-citrate (Adapted from Motekaitis and Mortell, 1984)

Silicon-aluminum interaction (such as the formation of hydroxy-alumino-silicate) has also been shown its positive effect on reducing Al toxicity. For example, the presence of Si in nutrient solution significantly decreased monomeric Al concentration, thus supported corn root elongation (Barcelo et al., 1993). Therefore, another mechanism by which biochar may control highly toxic Al could be through precipitation reaction of Al with silicate or formation of a Al-Si compound in the epidermis root, especially in the Si-rich biochars, such as those from rice straw (Figure 6) (Qian et al., 2016).

Improving soil pH buffering capacity could be another benefit gained through the addition of biochar to low activity/ CEC acid soils (Table 1) (Xu et al., 2012). Highly weathered acid soils in the Tropics, such as Oxisols and Ultisols are low in pH buffering capacity due to low CEC and low organic matter, and dominated by kaolinitic and halloysitic minerals, making them prone to soil acidification. Incorporating biochars (with high CEC and pH) into acid soils could make those soils' be more resistant to pH change due to acidification processes (Shi et al., 2017).

Biochar has been shown to be capable of improving water and nutrient retention of soils, including acid soils, in the Tropics. Jeffery et al. (2011) highlighted that one

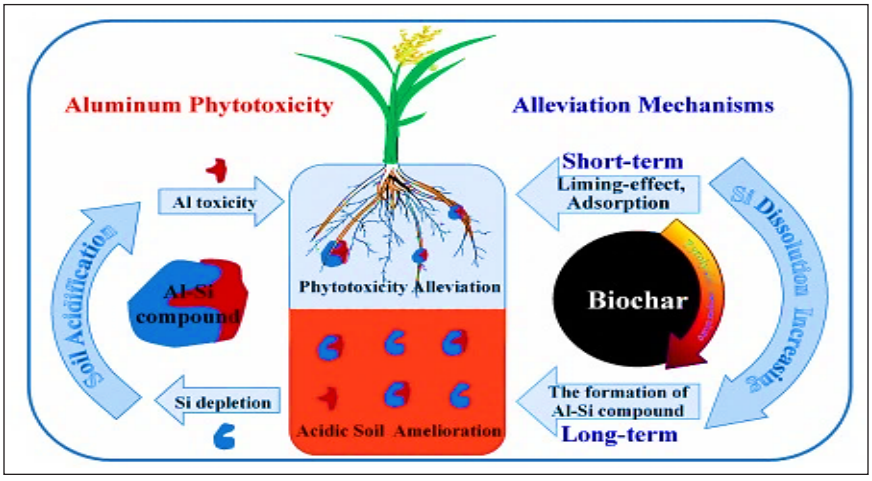

Figure 6. Alleviation mechanisms of Al phytotoxicity using biochar amendment with short-term effects and long-term effects (Adapted from Qian et al., 2016)

Table 1
Effect of two biochars incorporated on properties and pH buffering capacity of soils

Soil and location	Depth (cm)	Treatment	pH	Organic matter (g kg^{-1})	CEC (mmol kg^{-1})	pH buffering capacity (mmol kg^{-1} pH^{-1})
Ultisol from	60-120	Control	5.38	4.4	51.5	20.8
Lizhou,		3% CSBC	6.72	15.5	59.0	22.3
Guangxi		5% CSBC	7.46	23.0	61.7	27.3
		3% PSBC	6.83	27.7	82.6	30.5
		5% PSBC	7.35	41.2	92.8	36.1
Oxisol from	60-130	Control	5.05	8.4	59.7	20.1
Chengmai,		3% CSBC	6.68	19.1	61.2	23.0
Hainan		5% CSBC	7.29	26.3	71.4	27.0
		3% PSBC	6.85	31.0	80.1	29.4
		5% PSBC	7.29	44.4	90.3	38.6
Ultisol from	50-110	Control	5.00	10.9	53.0	15.5
Kunlun,		3% CSBC	6.70	21.4	65.3	18.4
Hainan		5% CSBC	7.47	26.8	70.4	23.6
		3% PSBC	7.04	32.9	78.0	25.7
		5% PSBC	7.45	46.2	96.9	34.6

Note: CSBC = canola straw biochar, PSBC = peanut straw biochar (Adapted from Xu, et al. 2012)

principle mechanism by which biochar enhanced crop yield was its ability to improve soil water holding capacity. High water retention of biochar is mainly attributed to its large surface area and high porosity (Brantley et al., 2015; Brown et al., 2006; Laird et al., 2010; Lua et al., 2004; Novak et al., 2012), which is affected by pyrolysis temperature and feedstock (Brantely et al., 2015; Karhu et al., 2011; Novak et al., 2009, 2012). The extent of which soil water retention is improved by biochar is determined by biochar feedstock, pyrolysis temperature, application rate, and soil properties. For example, adding greenwaste biochar (produced at 450^{o}C by a slow pyrolysis) to an Alfisol increased the field capacity water retained in the soil (Chan et al., 2007). Switch grass biochar, produced at 500^{o}C and applied at 40 Mg/ha, increased water retention of a loamy sand soil more than poultry litter, pecan shell and peanut hull biochars (Novak et al., 2009). Water retention of Hapludoll (from Iowa) was increased by adding a mixed wood biochar (Laird et al., 2010). Water retention of clay soil was improved by adding a mixed tree fruits biochar at 3% (Castellini et al., 2015). Water retention of a loamy sand soil was increased from 13.92% to 17-21.5% through the addition of 5% of biochar made from acacia wood, cashew wood or bamboo (Rattanakam et al., 2017).

Highly weathered soils in the Tropics such as Indonesian acid soils are often poor in nutrients because of leaching. The loss of nutrients not only increases the cost of plant production, but also causes environmental problems such as water pollution. Recent research revealed that adding biochar to

soils would decrease nutrient losses (Table 2) (Berek et al., 2018; Berek & Hue, 2016; Laird et al., 2010; Liu et al., 2014; Major et al., 2012; Singh et al., 2010; Ventura et al., 2012). The nutrient retention capacity of biochar could be attributed to its large surface area, high surface charge, high porosity, and other factors such as pH and ionic competition. Thus, adding biochar to acid soils could alleviate soil nutrient losses by electrostatic adsorption and physical entrapment of the nutrients inside the pores (Cheng et al., 2012; Jones et al., 2012; Kameyana et al., 2012; Laird, 2008; Lehmann et al., 2003; Prendergast-Miller et al., 2013).

In addition to improving acid soil productivity, biochar can also act as an agent of carbon sequestration and greenhouse gas (GHG) emission mitigation (Woolf et al., 2010). Biochar can store and lock atmospheric carbon in the soil system on a long-term basis depending on its recalcitrant nature. Biochar may also be reduce GHG emissions, such as N_2O and methane via several pathways (Fidel et al., 2019; Sánchez-García, et al., 2014).

It seems that biochar may not only replace lime or other conventional ameliorants functions in decreasing soil acidity, but also provides a new solution for mitigating climate change problems. Collectively, biochar may be necessary tool in alleviating the Indonesian food and energy security problems for the future.

Biochar Opportunity for Managing Indonesian Acid Soils

To support the Indonesian policy on food and energy security, acid upland soils a component of suboptimal soils has been targeted for agricultural land expansion.

Table 2
Nutrients leached over the 2005 and 2006 rainy seasons under a Colombian savanna Oxisol that received 0 or 20 t/ha biochar in 2002

Depth	Biochar	Total amount leached							
		Water	P	Sr	NH4-N	NO3-N	Ca	Mg	K
m	t ha^{-1}	mm	---------- kg ha^{-1} ----------						
0.15	0	2823	0.56	0.30b+	9.1b	168.9	61.2b	42.4b	206.9b
	20	2867	0.50	0.98a	69.1a	266.4	227.7a	130.3a	413.6a
0.3	0	2742	0.44a	0.57b	2.7	234.7b	125.4b	84.0	190.1a
	20	2750	0.38b	0.87b	2.2	330.9a	179.3a	92.0	185.9b
0.6	0	2593	0.40	0.41b	1.0b	196.0b	84.6b	55.5b	119.5b
	20	2588	0.36	1.00a	2.4a	399.8a	223.0a	116.7a	131.3a
1.2	0	2361	0.34	0.35a	1.0	110.2a	54.6a	33.5a	36.0a
	20	2346	0.32	0.30b	1.6	108.1b	47.2b	26.1b	24.7b
2.0	0	2329	0.35a	0.06b	0.6a	12.7	6.5b	3.1	14.9
	20	2296	0.26b	0.09a	0.5b	19.8	9.0a	4.3	13.6

Note: + Different letters represent significant differences ($P<0.00t$; $n = 3$) between treatents at a single depth. No letter indicates that differences are not significant (only flux dominated bu unsaturated flow) (Adapted from Major et al., 2012)

This opens up opportunities for biochar to be developed as a sustainable amendment to acid soils and an agent of climate change mitigation. This opportunity was also being supported by the ever-increasing research interest of Indonesian scholars and research agencies, such as the Indonesian Agency of Agricultural Research and Development (Balitbangtan) Ministry of Agriculture, the Indonesian Agency of Forestry Research and Development (Balitbanghut), and Indonesian Biochar Association (ABI) which was established several years ago.

A sustainable and available supply of feedstock is an essential requirement for biochar development. There are potential feedstocks for biochar that are available in abundance in Indonesia include palm oil empty fruits and kernels, sugar cane waste, sawdust, rice husks, sewage sludge, manures and urines mixtures, and municipal waste. Utilization of waste, municipal waste in particular, as a potential biochar feedstock could be a comprehensive solution from environmental, health, climate change mitigation, and agricultural perspectives.

Economic and socially viable and acceptance of biochar technology could be a noble chance for Indonesia small-scale farmers as a way out to face with the high cost and the decreased availability of fertilizer and other conventional amendments in rural areas. Most of Indonesia farmers experiencing the benefit of slash and burn traditional practices that provide short-term high yield resulted from the basic nutrient content in the ash. In many parts of this country, people produce charcoal for energy purposes. Therefore, this could also be the opportunity for biochar development in respect to the familiarity of the community to the production process.

Future Perspective

Initial biochar research reports, such as Berek and Hue (2016), Islami et al. (2011), Martinsen et al. (2015), Masulili et al. (2010), Sukartono et al. (2011) and Yamato et al. (2006) indicated that biochar could be potential as an amendment for Indonesian acid soils. Therefore, future research needs to be developed in Indonesia on improving soil productivity, increasing crop yield, and climate change abatement through the incorporation of biochar in acid soils. Thus, a research framework is required to be designed for researchers to go through. As previously mentioned, managing acid upland soils in Indonesia will be challenging due to the multiple constraints that vary from site-to-site. For example, acid upland soils and the availability of feedstock are widely distributed among the islands and even on the same island.

To overcome these high variability circumstances, a specific biochar system for Indonesian acid soils could be developed perhaps based on the engineered/designer biochar concept. The potential Indonesian biochar feedstock needs to be mapped, followed by establishing the production pathways. Next, the characterization of the products-biochars based on a general or local standard for acid soils that should be performed. Then, conducting pot/greenhouse and field trials are necessary to

test the biochar effects on soil properties, carbon sequestration, greenhouse gases emission, and plant growth and yield. The conceptual biochar research priority and framework for Indonesian acid soils is illustrated in Figure 7.

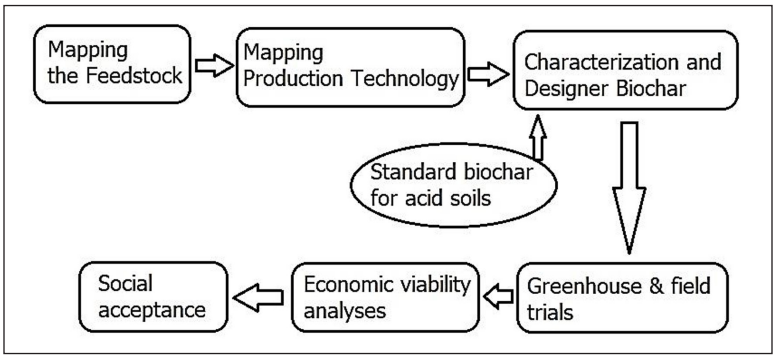

Figure 7. A simplified biochar research priority and a framework for Indonesian acid soils

CONCLUSION

Biochar has tremendous potential as an alternative solution for amending soil acidity, which in turn, will support the future of Indonesian food and energy security. The abundance availability of biochar feedstock, its wide distribution, cheaper production technology, high research interest, and familiarity within the community for product utilization, are all-significant opportunities for biochar development in Indonesia.

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Process and Characterization of Aggregate Stabilization in Degraded Inceptisols by Earthworms

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ABSTRACT

Earthworms are widely recognized as a means of enhancing soil quality. However, less is known about their role in aggregate formation, especially their interaction with organic materials and soil aggregate size, as well as the output of this process. The objectives of this research are to assess the process and characterization of aggregate formation on degraded inceptisols by earthworms. Experiments were conducted in a random block design with factorial pattern using a combination of soil aggregate size, presence and absence of earthworms, presence and absence of compost. Sixteen treatments and 6 replication were used. A total of 96 pots were prepared, and 32 pots were examined every month during three months. The changes of mmorphological and elemental composition were observed using a scanning electron microscopy coupled with an energy-dispersive X-ray spectrometer (SEM-EDS). Aggregate stability was measured using the water aggregate stability method. The results showed that micro- and macro-aggregates were formed in less than two months, the aggregate surface became coarser and more porous. The interaction of aggregate size, earthworms and compost could improve degraded inceptisol quality through aggregate stabilization. This process started after the first month and increased after the second month. During the third month, 76.61% stable aggregates (without compost), 80.56% (without earthworms) and 91.74% (with both earthworms and compost) were formed. This finding suggests that stable aggregates are influenced more by a combination of earthworm and compost, however aggregates formation is strongly influenced by earthworm which is faster than compost.

Keywords: Aggregate morphology, aggregate size, compost, interaction, stable aggregate

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INTRODUCTION

Soil degradation is a major constraint on agricultural production. Erosion is one of the major causes of soil degradation, which has affected about 500 million hectares of land in the tropics, including inceptisols (Lal, 2001). In tropical areas, the erosion rate of inceptisols by water is generally faster than the rate of soil formation. Erosion not only affects soil physically, but also influences the decline of chemical properties, such as loss of soil nutrients, loss of minerals charge positively. Eroded soil normally more acidic and less fertile. Degraded inceptisol by erosion affects the physical quality of soil by disintegration aggregate. Therefore, aggregate stability has become an important physical indicator of soil erosion, which reflects the measurement of the ability of the soil to retain its structure (Díaz-Zorita et al., 2002). Aggregate stability influences several aspects of soil's physical behavior, especially water infiltration and soil erosion. Splinters of soil particles get separated from aggregates by raindrop produced finer fragments that filled the soil pores (Nciizah & Wakindiki, 2014). This, in turn, decreases porosity, aeration, water infiltration (Díaz-Zorita et al., 2002), microbial community structure (Hattori, 1998), diffusion of oxygen (Hansel et al., 2008; Sexstone et al., 1985), and the regulation of water and cation exchange capacity (Barthès & Roose, 2002).

The biological properties of the soil deteriorate via loss of soil organic matter and loss of soil fauna (Lal, 2015). The restoration of soil quality through the aggregate formation process and stability is influenced by three main factors: soil fauna, soil organic matter (SOM), and soil minerals. This is known as the Biogenic Soil Structure concept. The interaction between these factors is essential for achieving healthy soil conditions (Wall et al., 2012).

Soil faunas, such as earthworms, have an apparent role in the formation of aggregate and aggregate stabilization. Through biological activity, earthworms convert leaf litters into fragments and destroy soil micro-structures by mixing mineral clays and organic materials in their gut system; consequently creating an organo-mineral structure with certain physical, chemical, and microbiological properties (Lavelle et al., 1997, 2006; Castellanos-Navarrete et al., 2012) as well as a biogenic structure in the form of casts, mounds, and fungus comb chambers (Bhadauria & Saxena, 2010). Earthworms have a higher stimulating effect on the formation of soil aggregates (macro-aggregates and micro-aggregates within macro-aggregates) when organic matters or residues are added (Fonte at al., 2009). Moreover, the addition of organic matters increases water, nutrients, and soil organic carbon (SOC) availability. Increasing SOC further triggers aggregate formation; which, in turn, increases porosity, aeration, and water infiltration simultaneously and adequately; thus improving soil structure stability (Food and Agriculture Organization [FAO], 2007, 2017).

Soil minerals also play a role in determining how aggregates are formed through cation exchange. Besides having a high oxide content, tropical soils, such

as inceptisols, are well known for having a large content of mineral particles with variable charges (oxide and 1:1 clay minerals, such as kaolinite). Contrary to soils in the sub-tropics, which are dominated by type 2:1, and where organic matter acts as the primary binding agent for soil aggregates (Six at al., 2002), organic materials in oxide-rich soil bind to oxides. This prevents the expression of an aggregate hierarchy (Oades & Water, 1994) since an aggregate hierarchy occurs in aggregate soils, and stabilization is formed by organic soil matter. Thus, in such types of soil, plentiful mineral particles provide an alternative way of producing macro-aggregates through physiochemical mineral bonds. The large capacity of inceptisols for producing macro-aggregates through this kind of process is supported by Six et al. (2002), who reported that the positive charge of inceptisols was high, as high as difference between maximum pH (8-10) with points of zero net charge for kaolinite of type 1 : 1 (3.5 to 4.6). This characteristic also plays a significant role in the accumulation of organo-minerals produced by earthworms, since large margin of positive charges provides a considerable capacity for adsorption.

Based on the research concept and the mechanism of aggregate formation, the process of microaggregate formation by earthworms is believed to improve soil quality through changes in aggregate surface, soil chemistry (element) composition, and aggregate stability. Therefore the objectives of this research are to assess the process and characterization of aggregate formation on degraded inceptisols by earthworms. The knowledge and information gained from this study will be valuable for the restoration of degraded inceptisols programs in the tropics.

MATERIALS AND METHODS

This research consisted of two phases. The first phase was the process of soil aggregate formation, which was studied by adding earthworms and/or compost to the soils of various aggregate sizes. The second phase was examination of morphological and aggregate stability under laboratory condition.

The soil in this experiment was collected from Gunung Geulis (=GG) area (832-943 m above sea level), with 45-60 % of slope. The geographical location of sample soil was between 06^{0}55.443' S - 06^{0}56.404' S and 107^{0}48.045' E - 107^{0}48.904' E at Sumedang Regency, West Java – Indonesia. In this area, three land-use systems are present: agrosystem, agroforestry, and forestry system. Based on land use, the agrosystem was classified into: banana garden, cereal crops, and mixed garden. Previous study had examined the soil profile and mineral type at agrosystems at GG. The results showed that the soil order was inceptisol (Supriyadi et al., 2014). Soil quality had been analyzed by scoring system, which integrated physical, chemical, and biological characters. The results revealed that the soil quality was degraded by erosion (Supriyadi et al., 2014).

The samples used in this experiment were taken from the agrosystem cereal crops, which were considered as degraded inceptisols. The experiment on soil

improvement process was conducted in the laboratory of School of Life Sciences and Technology, Institut Teknologi Bandung, Indonesia.

Soil Preparation

The soil samples of degraded inceptisols having aggregate size composition (27% macro-aggregate, 54% mesoagregate, and 19% micro-aggregate) were filtered and grouped into three different measures; (841 – 2000) µm, (250 – 841) µm, and (44 – 250) µm.

The compost used in the experiment was made from a mixture of 60% agriculture waste and forest litter (1:1 v/v), 20% bran, and 20% manure. A starter solution was added to facilitate the composting process. This solution consisted of 8% effective microorganisms (EM4), 8% molasses, and 83% water. The solution was kept at 40% humidity (Supriyadi, 2017). The preparation of the starter solution followed the method used by Wididana and Higa (1995). The precursor, EM4, was made the day before composting by dissolving EM4 (1 L) and brown sugar (500 g) into water to make a 50 L solution.

In order to obtain the desired organic soil carbon (SOC) for this experiment (2%--which represents a healthy soil condition (Bot & Benites, 2005), compost was added to plots assigned for compost treatment. The amount of compost added was calculated using a formula given below from Ellert and Bettany (1995).

$$Ms : 0.20\ (m) \times 10.000\ (m^2) \times BD\ (Mg/m^3) \quad [1]$$

$$To : (Ct - Ce) \times 1.724 \times Ms \quad [2]$$

Ct: The amount of soil organic carbon expected (%)
Ce: The amount of soil organic carbon existing (%)
BD: Bulk Density (Mg/m^3)
Ms: Sum of soil mass ($Mg\ ha^{-1}$)
To: Total compost added ($Mg\ ha^{-1}$)

For each treatment, 17 individual earthworms (about 20 g) were used. The earthworms were collected from a protected forest in the GG area. The earthworms were Anecic type and belonged to the species *Amynthas ilotus*, Megascolesidae family.

Experimental Design

The experiment on the process of soil aggregate formation was conducted using a combination of different sizes of soil aggregate (As) consist of four levels of soil aggregate size; as0 = the size of aggregate between (841 – 2000) µm, as1 (250 – 841) µm, as2 (44 – 250) µm, and as3 was the existing degraded soil (consist of different aggregate size: 27% 841 – 2000 µm, 54% of 250 – 841 µm, and 19% of 44 – 250 µm). Five kilograms of different sizes of soil aggregate and 1.98 kg of compost were mixed with 0.5 liter of water until the soil condition reached 70% of humidity and was placed in a pot (26 cm in diameter). To maintain the humidity, all the pots were

sprayed by water once every day. Each pot consisted of different size of degraded soil, the presence (ew1), and absence (ew0) of earthworms as well as presence (cp1) or absence (cp0) of compost.

These experiments were set in a random block design with a factorial pattern of three factors. A total of 16 treatments of different soil aggregate size, the presence and absence of earthworm and compost (Table 1) were prepared with six replications (in 96 pots). Every month, the samples were taken from each treatment (32 samples) to determine aggregate characteristics (morphology, stability) and element content of soil aggregate. The examination was repeated twice.

Morphology of Aggregates

The morphological characteristics of soil were observed using Scanning Electron Microscope (SEM). The element composition was analysed by Energy Dispersive X-ray Spectroscopy (EDS). The type of SEM used was EVO MA10, Carl-Ziess SMT. Prior to the analysis, the sample was gold spurted with the aid of a sputter coater (model Polaron SC) to produce 515 $\pm$ 20 nm sample thickness. The mounting of samples used double coated conductive carbon tape. The surface of samples was kept clean from abrasive or cracks after being last polished, especially for EDS analysis. Subsequently, magnetic electron lens was focused on the sample. SEM and

Table 1
The treatments of soil aggregate formation experiments

No.	Treatments			Code
	Aggregate Size (As)	Earthworms (Ew)	Compost (Cp)	
1	(841 – 2000) µm	-	-	as0ew0cp0
2	(841 – 2000) µm	V	-	as0ew1cp0
3	(841 – 2000) µm	-	V	as0ew0cp1
4	(841 – 2000) µm	V	V	as0ew1cp1
5	(250 – 841) µm	-	-	as1ew0cp0
6	(250 – 841) µm	V	-	as1ew1cp0
7	(250 – 841) µm	-	V	as1ew0cp1
8	(250 – 841) µm	V	V	as1ew1cp1
9	(44 – 250) µm	-	-	as2ew0cp0
10	(44 – 250) µm	V	-	as2ew1cp0
11	(44 – 250) µm	-	V	as2ew0cp1
12	(44 – 250) µm	V	V	as2ew1cp1
13	Existing degraded soil	-	-	as3ew0cp0
14	Existing degraded soil	V	-	as3ew1cp0
15	Existing degraded soil	-	V	as3ew0cp1
16	Existing degraded soil	V	V	as3ew1cp1

v : treatment application

EDS analyses were applied only for the samples of the existing degraded soil added with earthworms (Table 1; no. 14).

Stability of Aggregates

Aggregate stability was measured using the water stability aggregate (WSA) method. This method is used to isolate the stable micro-aggregates from inside macro-aggregates (McCarthy et al., 2008; Six et al., 2002; Tisdall & Oades, 1982), and the SOM dynamic (Six et al., 2000, 2002, 2004). The stable aggregates were subsequently measured with a wet-sieving method, which can distinguish three aggregate fractions; stable aggregates, rather-stable aggregates, and unstable aggregates (Six et al., 2002, 2004; Sohi et al., 2001; Sui et al., 2011). Eight grams of dry soil samples were placed on a sieve (1000 µm) and immersed into the deionized water until the soil was completely submerged. The sieve was then moved up and down for three minutes at 20 cycle's min^{-1}. The sieved particles collected inside a metal container were then dried and classified as unstable aggregates. Afterwards, the collecting vessel was changed with a vessel filled with 5%. NaOH Unsieved particles were again sieved for eight minutes. The collecting vessel was taken and the sieved particles inside were dried and classified as somewhat-stable aggregates. The remaining unsieved particles were classified as stable aggregates. The weight of stable aggregates (unsieved particles) was determined after drying at 40^{o}C. The stable aggregate distribution was calculated following Sui et al. (2011), based on the total percentage of the mass of the stable aggregate in each aggregate fraction as follows:

$$\text{Stable Aggregate} = \frac{\text{Stable Aggregate Weight (g)}}{\text{Total Weighted Soil (g)}} \times 100 \quad [3]$$

Statistical Analysis

The effect of compost and earthworms on soil aggregate stability on different aggregate sizes was tested using ANOVA with $p = 0.05$ for the interaction between three factors; aggregate size, earthworms, and compost. The Duncan Multiple Range Test (DMRT) was applied to determine the effect of interaction between the three factors and aggregate stability. This statistical analysis was followed (Gomez & Gomez, 1984; Oehlert, 2010; Steel et al., 1997).

RESULTS

Experiment on the process of soil aggregate formation and its characteristic on degraded inceptisols were explained through the changes of aggregate morphology, element composition, aggregate stability, and the interaction between aggregate size, earthworms and compost.

Morphology of Aggregates

Earthworm activity in degraded inceptisols resulted in the changes in soil aggregate structure, shown by the micrograph analysis from SEM (Figure 1 a-d). Before earthworm treatment, the morphological surface of the

degraded soil was smooth with no clumps or pores (Figure 1a). After one month of earthworm treatment, the aggregate surface became rough, clumps and the pores had formed (Figure 1b). After two months, the clumps segregated and formed macroaggregates and microaggregates (Figure 1c). After three months, inside of macro-aggregates showed a crack which indicate the formation of micro-aggregates within the macro-aggregates (Figure 1d).

The change in the aggregate surface from smooth surface became rough and the formation of clumps indicated the improvement of soil structure due to micro-aggregates and macro-aggregates formation. Compared to smooth surfaces, the rough surface of macro-aggregates facilitates better storage of organic molecules through the process of flocculation and adsorption. This phenomenon was supported by Chenu and Plante (2006) that the rough surfaces could store organic carbon several times better than the smoother surface of degraded soil. According to Bronick and Lal (2004), the soil clumps in a macro-aggregate formation block the decomposition process of organic matters (Bossuyt et al., 2005), which may lead to the enrichment of stable C due to the combination of micro-porous space and cracks in coarse soil particles (Mayer, 1999). According to Trevisan

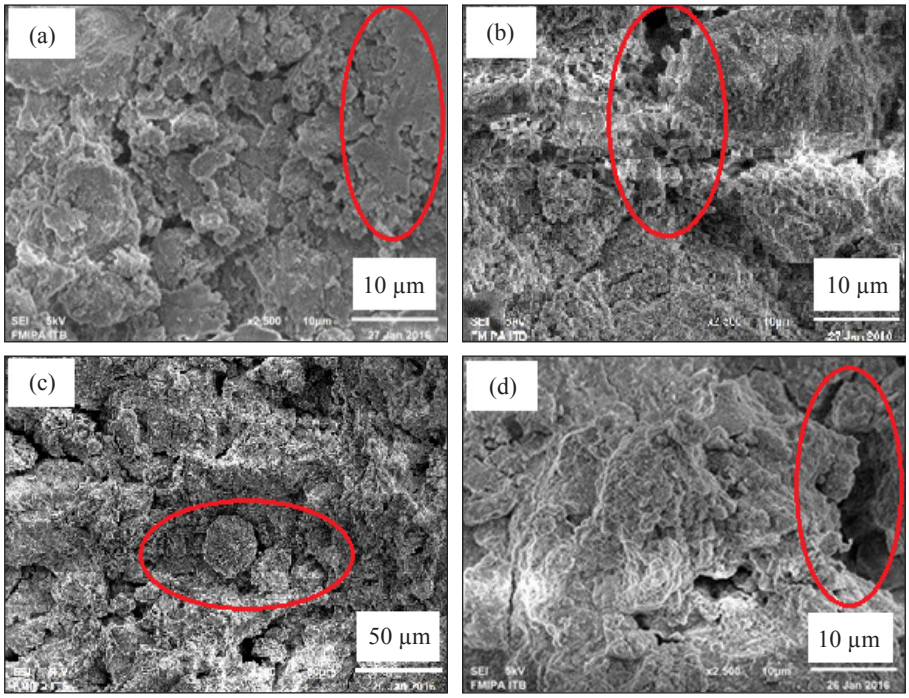

Figure 1. Morphological structure of aggregate formation by earthworm in existing degraded Inceptisols. (a) before earthworm treatment aggregate structure show smooth and flat surface; (b) after one month, the soil structure more rough with small clumps formation; (c) after two months, micro-aggregate formation appeared; (d) after three months, the clumps started to segregated signaling more microaggregate formation within macroaggregate

(2009), the porous soil particles and the cracked structure are more responsive to many soil characteristics. These results showed that earthworm activity triggering the aggregate formation in less than two months.

Element Compositions on Soil Aggregates

The elements C, N, Al, Si, K, Ca and Fe were consistently present throughout the treatment period with only slight changes in their relative abundance (Table 2). The presence of N and K in the early period (first month) suggests that the aggregation process was still unstable. The presence of Na tends to reduce aggregate stability as water can be easily absorbed by Na, which subsequently triggers soil dispersion. During the process of aggregate stabilization, Na can be considered as a labile element, or agent of dispersion. The decrease in Na and its disappearance after two months along with the higher abundance of cations with high valence (C_4^+, N, Al_3^+, Si_4^+ and Fe_3^+) throughout the treatment period indicate the process of aggregate stabilization. A strong aggregate stabilization process was supported by the increase in Fe_3^+, which has high capability of binding organic matter in aggregate stabilization (Bronick & Lal, 2004; Six et al., 2004). Meanwhile, the consistent high proportion of C and N during the treatment period indicates that earthworm activity could stabilize organic soil carbon (SOC) (Bertrand et al., 2015).

Aggregates Stability

In all aggregate size, earthworm treatment without compost (as0cp0ew1, as1cp0ew1, as2cp0ew2, and as3cp0ew3) resulted on the increased of aggregate stable (Figure 2a). After one month of treatment, the highest amount of stable aggregates formed (66.65%) was found in as0, followed by (48.06%) in as1, and (35.39%) in as2. This pattern was persistent in the periods two and three month of treatments.

Similar situation was also found in all aggregate sizes that were given compost treatment without earthworms (as0cp1ew0,

Table 2
Elements composition in soil aggregate of degraded inceptisols during three months period

Element	0 month	1 month	2 months	3 months
	Mass (%)	Mass (%)	Mass (%)	Mass (%)
C	24.26	24.22	29.64	27.41
N	28.46	28.42	32.69	29.10
Na	0.09	0.04	0.09	-
Al	20.45	19.45	14.45	16.97
Si	23.96	22.96	17.96	20.46
K	0.44	0.49	0.44	0.07
Ca	0.76	0.67	0.31	0.21
Fe	1.58	3.75	4.42	5.78
Total	100.00	100.00	100.00	100.00

as1cp1ew0, as2cp1ew0, and as3cp1ew) (Figure 2b). After one month of compost treatment, the highest amount of stable aggregates was formed 84.68% (in as0) followed by 83.67% (in as1). Meanwhile, aggregate stable in as2 was 7.86%. This value was lower than the amount of stable aggregate formed in as3 (degraded soil aggregate), which was 67.8%.

On larger aggregate size, treatment with compost or earthworm resulted in the higher aggregate stable. However, for all aggregate sizes, the levels of aggregate stable in soils with compost treatment were higher than those with earthworm treatment. This pattern was found during the second and third month of the experiments (Figure 2a and 2b). The influence of a single factor by adding earthworms (ew1) or compost (cp1) on all aggregate sizes [macroaggregate (as0), messoaggregate (as1), microaggregate (as2), and degraded soil (as3)] resulted in the highest stable aggregate on macroaggregate (a0), on mesoaggregate (as1) and microaggregate (as2). These results indicate the influence of aggregate size factor, as shown by the percentage of stable aggregates of non-treatment soil.

On the combined treatment of earthworm and compost (as0ew1cp1, as1ew1cp1, as2ew1cp1, and as3ew1cp1) for three month periods, the aggregate stability also increased, except in microaggregates where it decreased after two months. Unlike the two previous treatments (earthworm or compost only), the as0 consistently had higher aggregate stability than as1 and as2 during the three months treatment. In the combined treatment, however, the aggregate stability of as1 in the second and third months (91.94% and 94.12%) was higher than that of as0 (87.40% and 93.67%) (Table 3 and Figure 3). These results showed also that there was no correlation between aggregate stability and aggregate size (R_{xy} = 0.28).

At single treatment (compost or earthworm), aggregate size influences aggregate stability in that the larger aggregate

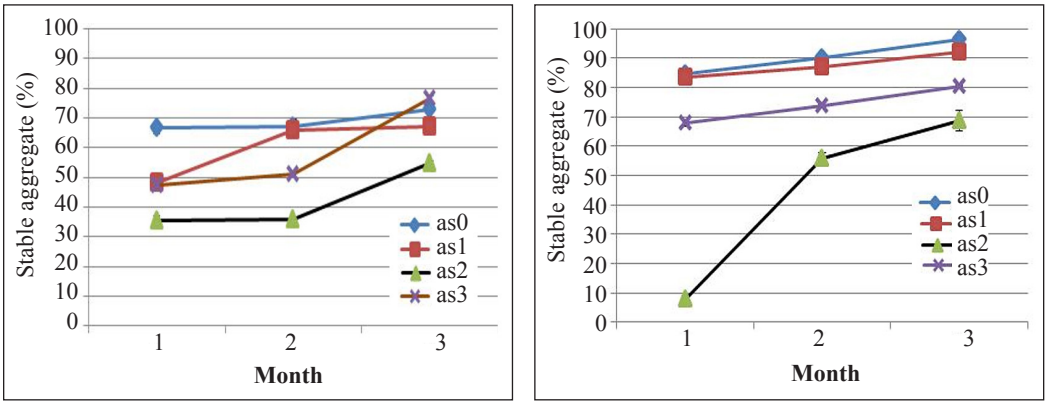

Figure 2. Stable aggregate formation on soils with different size of aggregates during three months of treatments by adding (a) earthworm and (b) compost separately

Table 3
Interactions among aggregate size, earthworm and compost on stable aggregate formation based on simple effect analysis

As	Cp	Ew (Earthworm)											
		After one month				After two months				After three months			
		Ew0		Ew1		Ew0		Ew1		Ew0		Ew1	
		---------- Stable Aggregate (%) ----------											
as0	cp0	52.81	d	66.65	d	50.58	d	67.04	c	45.90	c	73.02	c
		A		B		C		D		E		F	
	cp1	84.68	f	85.50	e	90.15	h	87.40	e	92.47	f	93.67	e
		A		A		D		C		E		E	
as1	cp0	19.87	c	48.06	b	19.99	b	65.90	c	21.75	b	67.14	b
		A		B		C		D		E		F	
	cp1	83.67	f	82.52	e	87.07	g	91.94	f	91.97	f	94.12	e
		A		A		C		D		E		F	
as2	cp0	2.26	a	35.39	a	2.96	a	36.02	a	6.05	a	54.80	a
		A		B		C		D		E		F	
	cp1	7.86	b	60.18	c	55.89	e	78.19	d	68.71	d	87.31	d
		A		B		C		D		E		F	
as3	cp0	23.02	c	47.57	b	23.65	c	51.01	b	25.03	b	76.61	c
		A		B		C		D		E		F	
	cp1	67.80	e	63.98	d	73.85	f	78.04	d	80.56	e	91.74	de
		B		A		C		D		E		F	

As = Agregates size, Ew = Earthworms, Cp = Compost
Note: The pair of numbers followed by same lowercase letters (on the vertical direction) and same capital letters (the horizontal direction) indicate no significant difference according to the Duncan's Multiple Range Test P=0.05

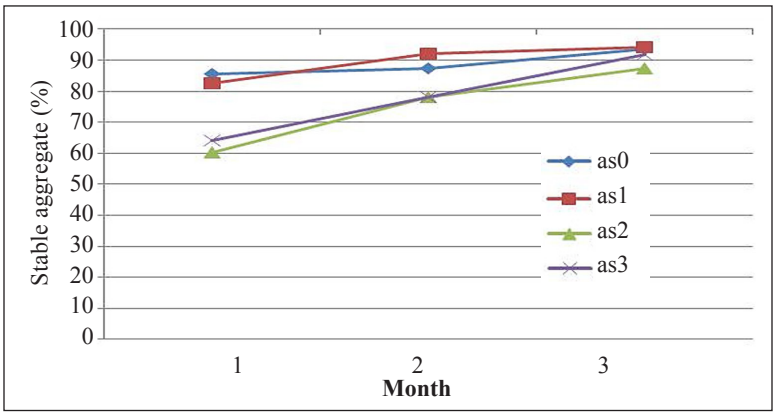

Figure 3. Formation of stable aggregate by earthworm and compost on different size of aggregates during three months period of treatments

size resulted in higher aggregate stability. However, on combined treatment (compost and earthworm), the effect of aggregate size was less. It seems that the role of organic materials (earthworm and compost) are more significant in increasing aggregate stability. Analysis on the effect of treatment on the degraded soil samples (Figure 2a, 2b and 3) showed that the earthworm treatment resulted in a faster increase of stable aggregates than other treatments. During the three months treatment, aggregate stability increased by 29.04%, 12.76%, and 27.76% in earthworm, compost and combined treatment respectively.

Interaction on Aggregate Size, Earthworms and Compost

Experiment on formation of stable aggregates showed by the results of WSA in the period of sample analysis during three month experiments (Table 3). After one month, as0 treatment with compost and earthworms (as0cp1ew1) resulted on 85.5% stable aggregate. Treatment without earthworms (as0cp1ew0) found 84.68% stable aggregate. Based on simple effect analysis on the stable aggregate interactions between (as0) and (as1), the addition of compost with and without earthworms resulted in zero interaction (Table 3). However, the simple effect between (as0cp1ew0) and (as0cp1ew1) showed no significant difference (A-A). Experiment as1, with compost and earthworms (as1cp1ew1) found 82.52% stable aggregate and treatment of as1 with compost without earthworm (as1cp1ew0) resulted in 83.67% of stable aggregate. The difference between treatments (simple effect) between the treatments of (as1cp1ew1) and (as1cp1ew0) showed no significant difference (A-A).

Experiment on as0, with compost and absence of earthworms (as0cp1ew0) resulted in 84.68% stable aggregate and experiment with as1, with compost and absence of earthworms (as1cp1ew0) resulted on 83.67% stable aggregate. Base on ANOVA and DMRT the simple effect (as0cp1ew0) with (as1cp1ew0) showed no significant difference (f-f). Experiment on as0 with compost and earthworms (as0cp1ew1) resulted in 85.5% of stable aggregate and experiment on as1 with compost and earthworms (as1cp1ew1) resulted on 82.52% stable aggregate. The difference between the two treatments showed no significant difference (e-e) or the simple effect between two treatments showed no significant different (e-e).

Experiment on as2 with compost and absence of earthworms (as2cp1ew0) resulted on 7.86% stable aggregate however at experiment as3 with compost and absence of earthworms (as3cp1ew0) resulted on 67.80%. The simple effect showed significant difference (b-e). In these experiments, the initial aggregate size (as2 and as3), the addition of compost or without compost and the presence or absence of earthworms showed the difference in stable aggregate. Addition of compost on different size of aggregate and the presence or absence of earthworms was significantly

differ than treatment without compost. If we compare the presence of earthworm at all aggregate size and the absence of earthworms showed significant difference in stable aggregate formation except in as0cp1 and as1cp1.

Based on simple effect analysis on the stable aggregate interactions between (as0) and (as1), the addition of compost and the presence or absence of earthworms resulted in zero interaction (Table 3). It can be concluded that the simple effect analysis on data during the first month showed that not all pairs resulted in interaction. There are pairs that did not show any interaction during the first month, that were: 1) [(as0cp1ew0-as1cp1ew0) and (as0cp1ew1 - as1cp1ew1)], 2) [(as1cp0ew0-as1cp0ew1) and (as3cp0ew0-as3cp0ew1)], 3) [as0cp0ew0-as0cp0ew1) and (as2cp0ew0-as2cp0ew1)].

Interactions of three-factor occurred during the second month period. The highest interaction found in (as1) with compost and the presence of earthworms (as1cp1ew1) resulted in (91.94% of stable aggregate. Based on a simple effect analysis for every aggregate size, the addition of compost provided significantly different results as compared to soil without compost. The addition of earthworms also furnished significantly different results as compared to soil without earthworms.

At the third month period, the formation of stable aggregate influenced by the presence of compost and earthworms at all aggregate size. Experiment of as1 with compost and earthworms (as1cp1ew1) resulted the highest stable aggregate (94.12%). The results of the simple effect analysis of the third month period were similar to those of the first month. Not all simple effect pairs displayed interaction. For all aggregate sizes, the effect of interaction on the formation of stable aggregate found at almost all of the pair of simple effect. Exceptions were shown by pairs of (as0cp0ew0 and as1cp0ew0) and also pairs of (as0cp0ew1 and as1cp0ew1). In this case, experiment with compost and the presence or absence of earthworms was not significantly different.

DISCUSSION

Erosion in degraded Inceptisols is one of the factors causing low soil quality, including physical, chemical, and biological quality of the soil (Lal, 2001). Aggregate stability and changes of the soil structure are important indicators in eroded soils (Bronick & Lal, 2004). This research reveals that the structure of degraded inceptisol due to erosion has smooth surface and low aggregate stability. Addition of compost, earthworms, or both (compost and earthworms) resulted on the change of soil structure and increase stable aggregate. This was occurred during the first, second and third months after treatments (Figure 1 and Table 3). Before treatment, the structure was visually fine (Figure 1a.). The presence of earthworms resulted in coarser soil structures (Figures 1b, 1c and 1d). The change in soil surface from smooth to rough was an indication of increasing number of pores and organic carbon content. This was

supported by (Bronick & Lal, 2004) that the rough surface of soil is an indication of soil aggregate formation. Rough surface can store organic carbon several times higher than a smooth surface (Chenu & Plante, 2006). The increased on organic carbon content inside the soil affects aggregate stability through the binding of cation and organic carbon. The binding agent produced by earthworms induces the formation of macroaggregates which strengthen the bond between soil particles (inter-particles binding) to form stable macroaggregates (Six et al., 2000, 2004; Tisdall & Oades, 1982). The stability of macroaggregates are due to presence of more labile organic carbon and cations that prevent dispersion (Kong et al., 2005; Pulleman et al., 2005). This research suggests that the size of aggregate is an important factor in aggregate stabilization (Table 3) as shown by the highest percentage of stable aggregates in treatment as0 (the size of aggregate between 841 – 2000 μm) without compost and earthworms (as0cp0ew0) compare with as1cp0ew0 and as2cp0ew0 in first month period. This is because larger size of soil aggregate has more soil organic carbon (SOC) content than the smaller one (Bronick & Lal, 2004).

Addition of both earthworms and compost facilitate the stable aggregate formation (Table 3). However, earthworms can facilitate faster stable aggregate formation than compost. The activity of earthworms in digging, burrowing soil and feeding on soil particles containing carbon and other organic materials produce cast containing organic carbon in the form of carbonic acid, fulvic acid and humic acid (Fonte et al., 2007; Knoepp et al., 2000; Pulleman et al., 2005; Zhang, et al., 2013). The increase of carbonic acid could trigger an increase in the negative charge on the surface of soil particles and then strengthen the binding of soil particle (Six et al., 2004; Tisdall & Oades, 1982).

Even though addition of compost facilitates the process of stable aggregate formation, the process was slower than in the treatment by earthworms (Table 3). The compost released cations and organic materials at a slower rate, because it had to undergo the decomposition and mineralization process first. In general, the release of cations and organic carbon from compost are slow (Maheswari et al., 2014). The difference in the release of binding agents by compost and earthworms resulted in a difference in the stable aggregate-forming process. Although stable aggregate-forming process induced by compost was slower than that of earthworms, but the aggregate stability might persist longer.

The presence of stable macroaggregates can protect soil organic carbon (SOC) from decomposition by microfauna or microorganisms. This facilitates the accumulation of stable organic carbon in the soil. (Six et al., 2004). Therefore, soil organic carbon and what aggregates mutually protect each other. SOC efficiently influences the soil aggregation (Six et al., 1998). On the other hand, SOC is physically protected and stabilized between and within micro-aggregates (Six et al., 1998, 2000, 2002,

2004). The effect of interaction among the size of aggregates, earthworms, and compost increases aggregate stability. Subsequently, aggregate stability increases formation of micro-aggregates within macro-aggregates. This interaction (aggregate stability and organic content) resulted in stable soil aggregate structure that has higher organic carbon contents as a consequence of a higher degree of physical protection.

CONCLUSIONS

The process of aggregate formation was indicated by the changes in the morphological structure of aggregate, i.e. the surface became rough and the aggregate has more pores. The size of the aggregate is an important factor in determining aggregate formation, however in the three-month period, the formation of stable aggregates was more influenced by the presence of earthworms and compost. Aggregate stabilization is initiated by the formation of macro-aggregates. Subsequently, aggregate stability increases with the formation of micro-aggregates within macro-aggregates. The interaction effects among the size of aggregates, earthworms, and compost become stronger and was related to the changes in cation composition that function as binding agents to stabilize the soil aggregates. In general, this research showed that the process of aggregate stabilization was visible after the first month of treatment and increased until the third month.

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Effect of Liming and Fertilizer Application on Mineralization of Nitrogen in Hemic and Sapric of Tropical Peat Material

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ABSTRACT

This study was conducted to investigate the effect of liming and fertilizer application on mineralization of nitrogen in hemic and sapric peat material of Tropical Histosols. The peat materials were left to decompose aerobically for 8 weeks under laboratory incubation and samples were taken for extractable ammonium at every 1 week interval. The trends in nitrogen mineralization were found to be similar between hemic and sapric peat materials for all treatments; however the rate and amount of ammonium release differed. The application of lime increased the pH of the peat material from around 3.7 to a pH of about 6.0 but no significant differences were found in the amount of NH_4^+ at 8 weeks of incubation between treatments with and without liming material in hemic and sapric peat material respectively. Fertilizer application significantly increased NH_4^+ in both peat materials, where NH_4^+ in hemic material was significantly higher (23.85 g/kg) compared to sapric (16.48 g/kg) peat material. This study showed that the practice of liming to increase soil pH did not necessarily improve the mineralization of nitrogen in Hemic and Sapric peat therefore was not prerequisite unless the crops to be planted are intolerant to acidity.

Keywords: Ammonium, hemic, incubation, nitrogen mineralization, peat, sapric

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INTRODUCTION

Tropical peat comprises soils that are formed from incompletely decomposed organic materials developed in low-lying depressional areas which are waterlogged for most parts of the year. These organic materials contain large amounts of nutrients that are essential for plants, since they are

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of vegetative origin (Andriesse, 1988; Bot & Benites, 2005; Lickacz & Penny, 2001).

Upon decomposition, these nutrients will eventually be released in plant available form (Lickacz & Penny, 2001).One of the most important nutrients generally found in Histosols chiefly peat soils is nitrogen whereby its total content varies from 0.3-4.0% (Lucas, 1982). Values between 1.3-1.5% are common in tropical Histosols (Gurmit et al., 1987). Although such values are considered high in soils, however, most of the nitrogen are unavailable for plants due to its high C:N ratio. The waterlogging condition associated with peat swamps as well as having high acidity due to organic acids further impedes decomposition and thus the release of nutrient (Brady & Weil, 2002).

It has been a standard practice in agricultural activities to lime soils that are high in acidity. This is especially true for mineral soils, however the effectiveness of liming in organic soils to facilitate in mineralization of nitrogen is still unclear. While reports have shown that the application of lime increase the mineralized N in acid mineral soil (Nyborg & Hoyt, 1978) such result may be otherwise in organic soils. Studies by Chapin et al. (2003) discovered that the addition of lime in Northern Minnesota decreased potentially mineralizable N as well as cumulative N mineralization in the fen. Whereas according to Rangeley and Knowles (1988), liming may result to an increase in the activity of aerobic organisms in peat, but this has little effect in anaerobic condition. Such findings demonstrated that the effect of liming cause variation in mineralizable N due to the difference in the type and nature of peats. Moreover, according to reports by Andriesse (1988), microelement contents such as copper, iron, cobalt, manganese, zinc and molybdenum varies at different depths of the peat deposit. It was found that the contents of most if not all of these microelements decrease as depth of the deposit increases. The same pattern also applies for the contents of macro and secondary elements such as nitrogen, phosphorus and magnesium where the surface layer of the sapric material commonly showed higher values compared to the subsurface layers of hemic and fibric peat materials. This is partly due to the difference in the degree of decomposition where the availability of the nutrients differs between peat materials. The parent material for sapric, hemic and fibric might originate from the same material, but the ash content in these three materials resulting from different degrees of decomposition might portray contrasting values.

Apart from liming, it is a prerequisite in agricultural practices to apply fertilizers where nutrients are suspected to be low. Peat soils are unique as they exhibit diverse physical and chemical properties which not every peat is of the same productivity as the other (Lim et al., 2012). The use of fertilizers tends to facilitate in accelerating the rate of decomposition, thus promoting more N to be released from the organic materials of the peat (Andriesse, 1988). However, McGreevy and Farrell (1984) found that

although fertilizers provide more available nutrients into the soil, however the liberation is often short-lived in ombrotrophic peats after initial stimulation.

There is limited information on studies of nitrogen in tropical peat emphasizing on the different types of peat material as a response to liming and fertilizer application. Given such situation it is of great magnitude to assess according to the types of peat since different kinds of peat response differently upon mineralization due to their varied nutrient contents and other abiotic condition. Such information may provide significant importance in understanding the nutrient dynamics of the peat material brought upon by anthropogenic activities. Therefore, this study aimed to investigate the effect of liming and fertilizer application on mineralization of nitrogen through the amount of NH_4^+ extracted in hemic and sapric material of tropical peat.

MATERIALS AND METHODS

Experimental Materials

The peat utilized in this study was of ombrogeneous (rain-fed) oligotrophic (nutrient-poor) tropical peat (Histosols) comprising two different depths of organic materials based on the degree of decomposition; hemic (less decomposed) and sapric (well decomposed) respectively. These samples were taken from a peat secondary forest in Kampung Kundang, Kuala Langat, Selangor, Malaysia (02°42'839"N 101°33'269"E). Surface (0-30 cm) and subsurface samples (40-60 cm) were collected by stratified random sampling method using a MaCaulay peat sampler (peat auger). Sapric material was located on the surface layer containing well decomposed peat as it was exposed to air for decomposition to occur while that of hemic material was found in the subsurface layer comprising the semi-decomposed peat material which was often below groundwater table and therefore waterlogged for most period of time.

Analysis of Physical and Chemical Properties

The types of peat were classified based on their degree of decomposition by using the von Post pressing method as quoted by Andriesse (1988). This test was done directly on the field where the fresh peat samples were first pressed on the palm. The colour of the extruded liquor and the proportions of the extruded matter were observed in detail to classify the peat materials as to match on the Von Post scale while the percentage of unrubbed fibre content was determined following the method by Andriesse (1988). Bulk density was measured using a core sampler which dried at 105 ºC to a constant weight. Moisture content was also determined using gravimetric method (American Society for Testing and Materials [ASTM], 1988). The pH of the samples was determined potentiometrically in soil suspensions of 1:10 volumetric ratio of air dried samples to water. The soil organic carbon (SOC)

was quantified using LECO Total Organic Carbon analyser whereas total nitrogen from the soil samples was determined following semi micro-Kjeldhal method.

Treatments

The treatments applied to each type of peat soil, hemic and sapric material respectively were no application of lime and fertilizer (T1), application of 20 g of liming material ($CaCO_3$) (T2) and application of 20 g of liming material ($CaCO_3$) with 5g of compound fertilizer 12:12:17:2 (T3). The lime and fertilizer treatments were based on a recommendation for oil palm seedling stage of up to 12 months (Gillbanks, 2003). The mineralization of nitrogen in hemic and sapric peat material following application of treatments were assessed using plastic containers of 15 cm (diameter) x 10.5 cm (depth) with laboratory temperature of 30°C. The initial weight of the peat with the container was recorded prior to application of treatments. Moisture was controlled by maintaining at 70% field capacity. As much as 800 g of fresh peat soil were placed into the container. The peat materials were left to decompose aerobically for 8 weeks and samples were taken for the determination of extractable ammonium (NH_4^+) at every 1 week interval.

Analysis on Nitrogen Mineralization

As much as 4.0 g of the peat samples were collected from the container in 4 replicates in accordance to the type of peat material and treatment applied. The determination of extractable N was based on mineral N (NH_4-N) where organic forms of nitrogen such as that of proteins were converted into simpler compounds of amino acids and the nitrogen was released as ammonium ion (NH_4^+) as it decomposed. Therefore by analysing the extractable NH_4^+ in the peat soil, nitrogen mineralization can be assessed. This was done by extracting the soil samples with 2 M KCl solution at 1: 5 ratio of soil to extractant (Maynard et al., 2008).

Statistical Analysis

Simple linear and non-linear regression was used to analyse the trend in extractable NH_4^+ for each of the treatments tested in both peat materials using SigmaPlot version 12.0. Analysis of variance (ANOVA) was conducted to test the effects of treatments applied onto the peat materials while meant comparison was done using Tukey's Test at 5% level using Statistical Analysis System (SAS) version 9.2.

RESULTS AND DISCUSSION

Physico-chemical Properties

Two types of peat material were used in this study where both types were determined based on their degree in decomposition measured by fibres these peat materials contained. The percentage of unrubbed fibre content was higher in hemic compared to sapric material as described in Table 1. This result corroborates with the findings in Soil Survey Staff (1975) where moderately decomposed materials such as hemic had more fibre content as compared to the well

Table 1
Selected physical and chemical properties of hemic and sapric peat material prior to incubation

Properties	Type of peat material		
	Hemic	Sapric	Range
von Post scale	H6	H8	
% unrubbed fibre	40± 2.719	26.67±2.723	
Munsell notation	10YR 7/4	10YR 3/4	
pH	3.74	3.67	3-4.5[a]
Total carbon (%)	63.95±1.26	62.2±1.14	12-60[a]
Total Nitrogen (%)	0.82±2.11	1.35±2.57	1-4[b]
Bulk density (g/cm)	0.15±2.83	0.18±2.851	0.1-0.2[c]
Moisture content (%)	72.11±0.62	68.57±0.85	

Source: [a]Andriesse (1988); [b]Lucas (1982); [c] Soil Survey Staff (1975)

decomposed sapric material due to the advanced mineralization leaving the more resistant materials remaining in the soil forming humic material. Thus, it is for this reason that the colour in sapric material as indicated by the Munsell notation has a higher value of 3/4 and is therefore much darker than that of hemic material which indicated a value of 7/4. This result also supports with the characteristics determined by Von Post Scale of humification where sapric material had a higher scale of humification of H8. It indicates a very highly decomposed peat where most of the materials are amorphous and exhibit very indistinct plant structure after squeezing between fingers. Hemic having a degree of humification of H6 implies moderately high decomposed peat where plant structures are more distinct after the material has been squeezed.

Bulk density value for hemic and sapric material was consistent with the range of values documented in Soil Survey Staff (1975). Bulk density has a direct relationship with fibre content. The more fibre of a soil material contains per unit volume, the lower its weight over its volume therefore the lesser of its bulk density. Hence material such as hemic which contains more fibre has a lower bulk density as compared to sapric material. Hemic material was saturated with water at the time of sampling, therefore it contains more moisture than the surface layer of sapric material. Thus, this explains the lower value of moisture content in sapric material (68.57%) as shown in Table 1 compared to the hemic material (72.11%). The pH for hemic was slightly acidic than that of sapric. Such difference is attributed to the distinct degree of decomposition. Nonetheless, the pH for both types of peat

material was typical for an ombrogenous oligotrophic tropical peat soil with a pH range in water of 3.0 to 4.5 (Andriesse, 1988). The higher degree of decomposition in sapric resulted in lower carbon content due to conversion to CO_2. Hence, lower carbon content may also imply progressive decomposition process. Consequently, element such as nitrogen (N) is higher in sapric material than the sub-surface hemic material.

Nitrogen Mineralization

The rate of nitrogen mineralization by determining the amount of extractable NH_4^+ for T1 increased throughout 8 weeks of incubation period regardless of the types of peat material (Figure 1). The trend in NH_4^+ release can be described by the quadratic equation where both types of peat material exhibit significant quadratic response at 1% level. Based on the response shown in Figure 1, it can be postulated that mineralization of nitrogen through the release of NH_4^+ will continue to increase after 8 weeks of incubation.

By using the quadratic equation of $Y=59.19 + 47x - 2.47x^2$ for sapric material, it can be estimated that the maximum release of NH_4^+ in sapric can be achieved at 9.5 week with as much as 282.8 mg/kg of extractable NH_4^+ . As for hemic, by applying the equation of $Y= 80.5167 + 39.398x – 1.98x^2$, the proposed maximum amount of extractable NH_4^+ will occur at week 10 with an estimated amount of 276.5 mg/kg of NH_4^+. Thus it can be understood that the two types of peat produced similar response on the amount of NH_4^+, however, their differences was shown where hemic released lower amounts of NH_4^+ which might stretch for an extended period of time compared to sapric.

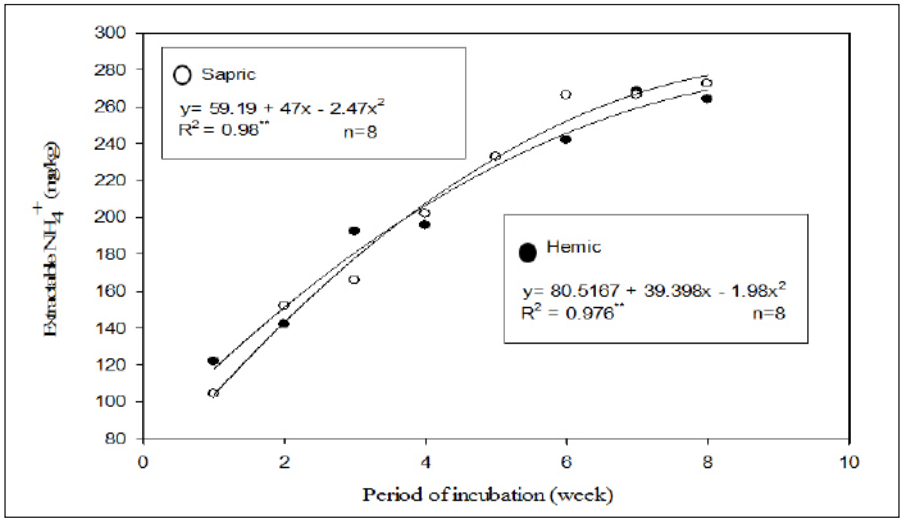

Figure 1. Extractable ammonium (NH_4^+) extracted throughout 8 weeks of incubation for hemic and sapric peat material under control condition (T1).

The response of NH_4^+ release for both hemic and sapric peat material under limed treatment (T2) was found to be linear at 1% significant level respectively (Figure 2). Although the trends of ammonium release throughout the incubation period were similar between the two types of peat where they increase exponentially, yet the amount of extractable NH_4^+ was lower in hemic material compared to sapric. Such observation was also found in T1 where hemic material responded in lower amounts of extractable NH_4^+. This can be explained based on the nature of the peat material itself where sapric material consists of well decomposed organic material containing higher amounts of nutrients such as nitrogen, capable of releasing higher amounts of NH_4^+ at a faster rate compared to hemic.

It should be emphasized that since the response was linear under limed treatment (T2), therefore the release of ammonium might continue to increase for a much longer period of time in contrast with T1 where the response was quadratic where the release of ammonium might follow a declining pattern afterwards soon after achieving their maximum amount at week 10. This demonstrates that liming peat materials resulted in more NH_4^+ being released from its organic pool due to the increase in pH of the soil that might have triggered a suitable condition for more microorganisms to perform decomposition resulting in rapid mineralization of nitrogen (Neale et al., 1997).

The response to lime and fertilizer treatment (T3) can be described by the quadratic equation and both peat materials showed significant quadratic relationship at 1% level (Figure 3). Hemic material attained higher amounts of extractable NH_4^+ compared to sapric; a condition

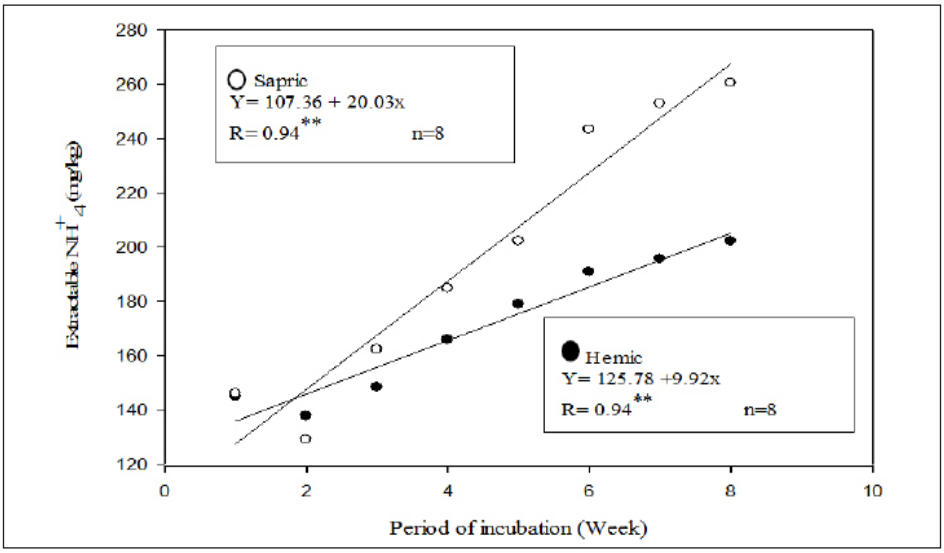

Figure 2. Extractable ammonium (NH_4^+) extracted throughout 8 weeks of incubation for hemic and sapric peat material under limed condition (T2)

which was otherwise comparing to the aforementioned treatments (T1 and T2) where the mineralization rate was higher in sapric than in hemic.

The maximum value of NH_4^+ extracted was found at week 5 for sapric with as much as 2.10% of extractable NH_4^+ while hemic material achieved 2.49% of extractable NH_4^+ also at 5th week. These values were achieved when the pH of the peat material was the highest. Sapric achieved its highest pH of 6.9 while hemic achieved its highest pH of 7.05. Such observation demonstrated that different types of peat material might exhibit similar response in nutrient release within similar soil condition (in this case, soil pH) however; the amount of the nutrient release may vary since different peat types have different chemical properties.

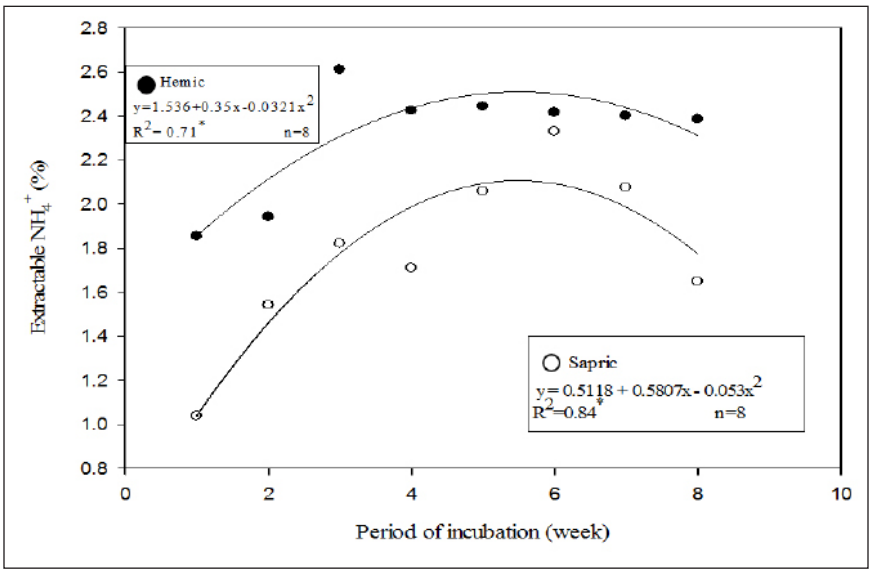

Figure 3. Extractable ammonium (NH_4^+) extracted throughout 8 weeks of incubation for hemic and sapric peat material under limed and fertilized condition (T3)

Comparison on Extractable NH_4^+ at 8 Weeks Incubation between Treatments

The extractable NH_4^+ in T1 and T2 for Hemic and Sapric peat material at 8 weeks of incubation as shown in Table 2 was significantly higher than in the initial condition (fresh peat material). However, no significant differences on the amount of extractable NH_4^+ in T1 and T2 regardless of the peat material.

The increase in pH due to the addition of liming material in T2 did not cause any significant increase in the amount of NH_4^+ during the period of incubation. Alternatively, the application of fertilizer as in T3 showed significant increase in the amount of NH_4^+ for both peat types compared to T1 and T2. This was attributed to the the introduction of fertilizers from the treatment that added in nitrogen which

could be easily dissolved in the soil solution. However, it was observed that hemic material in T3 accumulated significantly higher amount of NH_4^+ compared to sapric of the same treatment. Such result reveals that hemic and sapric materials behave differently upon the application of fertilizers albeit the same amount of treatment was added into the soil. The higher microbial population in sapric material as reported by Ivarson (1977) may cause most of the NH_4^+ to be incorporated into the biomass of microorganisms rendering it unavailable for extraction using potassium salt (KCl) which may explain the significantly lower values of NH_4^+ in sapric.

Table 2

Comparison on the amount of extractable NH_4^+ (mg/kg) between at initial condition and at 8 weeks of incubation for hemic and sapric peat material

Treatment	Extractable NH_4^+ (mg/kg)	
	Hemic	Sapric
Initial condition	102.88cA	86.84cB
T1 (no additives)	264.07bB	272.45bA
T2 (lime only)	202.13bcB	260.35bA
T3 (lime and fertilizer)	23,849.6aA	16,476.5aB

Note. Small letters display mean separation in a column by Tukey at 5% level. Capital letters display mean separation in a row using Tukey at 5% level

Changes in Total Carbon Content

Hemic material contained significantly higher amounts of carbon compared to sapric material initially and in T1 respectively (Figure 4). However, such condition was otherwise in T2 and T3 where sapric material accumulated significantly higher amounts of carbon in contrast to hemic material. The application of lime and fertilizer in T3 resulted in the lowest amount of total carbon. This shows that carbon was lost from the peat material when treatments were applied whereby the addition of lime and fertilizer stimulates more decomposition of the organic material in the peat soil. This result is corroborated with other findings (Zhang et al., 2012) that the incorporation of inorganic fertilizer enhances the mineralization of organic matter such that when N fertilizers are applied to the soil, it triggers the mineralization of recalcitrant organic N pool from the organic material. This implies that the agricultural practices of liming and adding fertilizers into peat soil will cause more carbon to be lost despite more nutrients are being released from the soil system, thus depleting the carbon

storage in peat soil that is well known for its capability to act as a carbon sink.

Many of the findings by other workers (Dong et al., 2012) observed the application of fertilizers resulted in build-up of carbon but these literatures were mainly referring to mineral soils. In order for mineralization to occur in organic soils, it requires the participation of microbes to breakdown the organic form of an element into inorganic. Hence, microbes will use the carbon source from the organic matter as energy to facilitate in the mineralization process leading to release of carbon dioxide, water and available nutrients as end products thus justifying the reduction of carbon in T3.

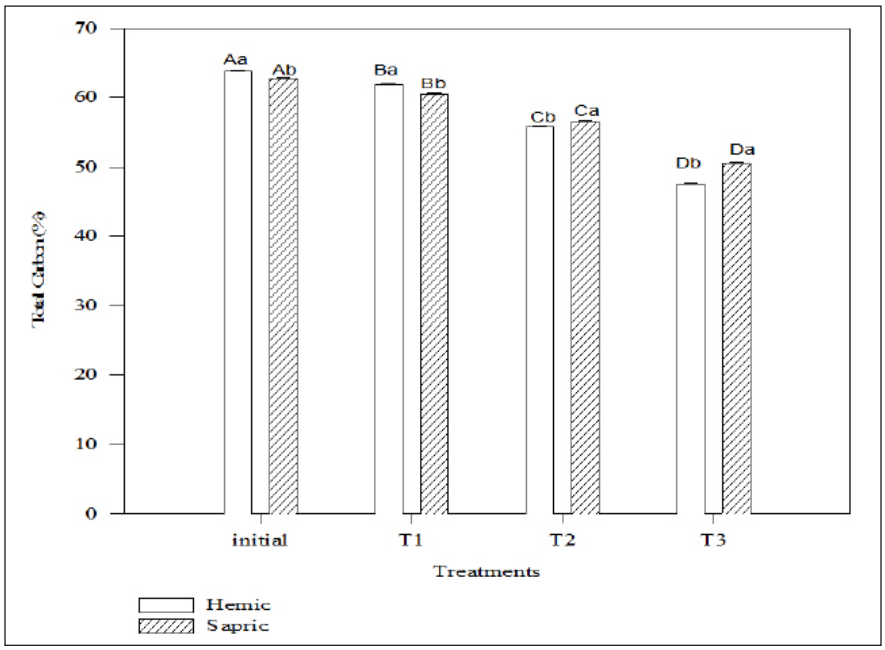

Figure 4. Differences in total carbon (%) in hemic and sapric peat prior to incubation (initial condition) and at 8 weeks incubation period (T1, T2 and T3).
Note. Capital letters display mean separation between treatments within a single type of peat material by Tukey at 5% level while small letters referring to mean separation between types peat material in a particular treatment using Tukey at 5% level

CONCLUSIONS

The crux was no significant differences in the amount of NH_4^+ accumulated at the end of 8 weeks incubation period in T1 and T2 for the two types of peat material, however, the pattern of nitrogen mineralization differed as it would likely to continue for T2 even after 8 weeks of incubation and thus releasing ammonium for a longer period of time compared to T1. Conversely, while the amount of NH_4^+ accumulated was highest in T3 for both peat materials comparing to T1 and T2, however, the mineralization

of NH_4^+-N was found to be temporal as it declined after 6 weeks of incubation.

This study shows that the application of lime (only) may not necessarily improve the nutrient availability in these two types of peat material although results showed that these peat materials that were limed underwent progressive decomposition process. Instead, it resulted in higher carbon loss due to rapid decomposition process which might lead to subsidence and this might affect the sustainability of the peat materials in years to come. This study may provide better understanding on the behaviour of hemic and sapric peat material upon the addition of lime and fertilizers in chemical aspects. Such information is useful in predicting changes in the nutrient dynamics of peat soils that are brought upon by anthropogenic inputs as to facilitate in better planning and application of lime and fertilizers.

RECOMMENDATION

Cropping on peat land is very different from that in mineral soils as one should always bear in mind that peat soils will disappear with time as carbon dioxide when inputs are much lesser than the outputs. Based on the results from this study, although it is highly recommended not to cultivate on peatlands, however, if the need is crucial, then this can be done by choosing acid tolerant crops, so that farmers will be able to maintain the acidity of the organic soils so decomposition process can be controlled and also not to be burdened by the high amounts of lime to be added. Apart from that, it is also suggested by choosing perennial crop than annual crops so as to not disturb the soil too much upon harvesting and replanting which may also accelerate the decomposition. Also, it is recommended to plant crops that have shallow and fibrous roots so that the water table can be maintained as high as possible so as not to expose much of the peat soils to oxidation.

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The Effect of Physical and Biological Pre-treatments of Oil Palm Fronds on *in vitro* Ruminal Degradability

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ABSTRACT

Physical pre-treatment of the oil palm frond (OPF) is known to loosen the lignocellulose while the biological pre-treatment is capable in degrading the lignin, making the substrates more accessible for rumen microbes. This study aimed at assessing the efficacy of physical, biological and combination of both pre-treatments of OPF on the *in vitro* ruminal degradability. Five different samples of OPF pre-treatments were used in this study; OPF was subjected to the physical pre-treatment (POPF), OPF to the biological pre-treatments using an enzyme extract of each *Ganoderma lucidum* (BGL) and *Lentinula edodes* (BLE), respectively. Another two samples were subjected to a combination of physical and biological pre-treatments of *G. lucidum* (CGL) and *L. edodes* (CLE) respectively. The control was non-treated OPF. Two fistulated Katjang goats consuming 440 g/kg OPF and 897 g/kg commercial pellet daily on dry matter basis were used as rumen fluid donors. *In vitro* incubation was carried out at 39°C for 24 hours. Proportions of volatile fatty acid were measured at the end of incubation by gas chromatography. Results showed that concentrations of lignin following all pre-treatment methods were significantly

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lower ($p<0.05$) at 150(POPF), 90(BGL), 119(BLE), 100(CGL) and 120(CLE) g/kg DM as compared to the FOPF (190 g/kg). After 24 hours of incubation, the cumulative gas of all treatment groups differed significantly from FOPF. Both BGL and CGL showed significantly higher propionate and butyrate concentrations as well as apparent rumen degradable carbohydrate with 6.57 mg and 6.54 mg, respectively as compared to the FOPF. It appeared that BGL and CGL resulted in higher lignin degradation that increased the *in vitro* rumen degradability. In conclusion, biological pre-treatment with enzyme extract of WRF, either alone or in combination are promising to improve the quality of OPF.

Keywords: Biological pre-treatment, *in vitro* ruminal degradability, lignin, oil palm frond, physical pre-treatment, white rot fungi

INTRODUCTION

Oil palm industry generates abundant amounts of by-products including oil palm fronds (OPF). The industry produces approximately 83 million tons of OPF annually. Oil palm frond has been widely utilized as ruminant feed as an alternative to grasses or roughages when feed is in short supply. Whole OPF consists of petiole and leaflets, which are fed to ruminants following chopping, and usually in combination with other feedstuff as total mixed rations. Indeed, adding the OPF in the diet of beef and dairy cattle could support live weight gain between 0.6 and 0.8 kg per day and milk yield of about 22 litre per day (Wan Zahari et al., 2003). Despite the previous reports of OPF as a promising source of roughage for ruminants, the use of OPF for ruminant feeding is still limited. In addition, OPF cannot be fed solely as animal feed due to its poor nutritive values with low metabolisable energy of 4.9 to 6.5 MJ/kg dry matter (DM) (Dahlan, 2000). The lignin content of OPF is also high at an average of 205 g/kg DM (Abdul Khalil et al., 2006) and can impair OPF intake and digestibility. These problems have encouraged lots of studies on investigating different technologies to improve the feeding value of OPF.

To date, some physical pre-treatments have been developed to upgrade the OPF, which include pressing using conventional sugarcane machine (Zahari et al., 2012), pelleting, grinding, chopping and steaming. Wan Zahari et al. (2003) reported that pelleting, grinding and steaming processes increased the intake and digestibility of OPF hence, improved the feed intake and growth performance of cattle. Furthermore, physical pre-treatment on OPF is considered practical, cost effective, easily operated, apart from low risk of health and safe.

The OPF could also be improved by chemical reactions using alkaline solutions such as sodium hydroxide (NaOH), ammonia (NH_3) and urea. This chemical pre-treatment requires maintenance of low temperature and pressure as compared to other pre-treatment strategies (Mosier et al., 2005). In addition, treatment with NaOH is also detrimental to the palatability of OPF in cattle, thus, urea pre-treated rice and wheat straws are considered safer, more palatable and cost-effective than NaOH

pre-treatment (Walli, 2010). However, there are some factors that should be considered for successful urea pre-treatment which are urea level, moisture content and duration of pre-treatment.

Studies have shown that biological pre-treatment with white rot fungi (WRF) can effectively degrade lignin and enhance digestibility of various lignocellulosic biomass (Hassim et al., 2012; Metri et al., 2018). In addition, the biological pre-treatment improves nutrient digestibility of poor quality roughages including wheat straw (Tuyen et al., 2012), rice straw (Sharma & Arora, 2010), sugarcane bagasse (Tuyen et al., 2013) and OPF (Rahman et al., 2011). In fact, *G. lucidum* and *L. edodes* could lessen and degrade approximately 40% of lignin content in the OPF (Rahman et al., 2011). However, the consequence of biological pre-treatment using WRF is time consuming which leads to dry matter losses (Rahman et al., 2011) due to the fungal metabolism. Nevertheless, study has shown that this limitation can be overcome if the WRF pre-treatment is done by using enzyme extract of that WRF as compared to the WRF mass. Indeed, the dry matter loss in wheat straw has been reported to reduce after using the enzyme extracts isolated from *Trametes versicolor*, *Bjerkandera adusta* and *Fomes fomentarius* (Rodrigues et al., 2008). This finding indicated that pre-treatment of wheat straw with enzyme extract isolated from WRF degraded the lignocellulose without the unnecessary polysaccharide consumption.

Although a number of studies has been conducted on physical and biological pre-treatments of OPF, combination of physical and biological pre-treatments using an enzyme extract of each *G. lucidum* and *L. edodes* have not yet been applied to OPF. Therefore, this study was carried out to assess the efficacy of physical, biological and the combination of both pre-treatments of OPF on *in vitro* ruminal degradability. The physical pre-treatment was done by pressing the OPF using conventional sugarcane machine, whereas the biological pre-treatment was done by pre-treating OPF with enzyme extracts from two types of WRF (*G. lucidum* and *L. edodes*).

MATERIALS AND METHODS

Oil Palm Fronds

Fresh OPF from 7-year old palm trees were used which were obtained from a palm plantation located in Felda Kemahang, Kelantan, Malaysia. The petiole of the OPF with leaflet was chopped off at approximately 2 metres length, which was 1/3 of the whole OPF length. The petioles were stored under shade at ambient temperature of 28−30^{o}C and relative humidity of 75–95% prior to pre-treatment. Sample weight and moisture content were recorded every 24 hours in a storage condition.

Physical Pre-treatment of OPF

Fresh whole OPF were taken with leaflets and chopped between 50 and 60 cm length. Then, they were pressed using a conventional sugarcane pressing machine

with three heavy duty steel rollers with specification of power 1500 watt and speed at 1400 RPM (Prasad et al., 2012; Zahari et al., 2012). The chopped OPF were inserted into the chamber and the spinning rollers pressed the petioles and leaflets to obtain the sample. The sample of whole OPF pressed fibre was dried at 60°C overnight prior to the chemical and *in vitro* analyses.

Biological Pre-treatment of OPF with Enzyme Extracts from WRF

Fungal Strains. Two white rot fungi, *G. lucidum* strain ATCC 64251 and *L. edodes* strain ATCC 52998 were cultivated in potato dextrose agar (PDA) plate at 30°C for 7 days. The fungi were then transferred into fresh PDA plate and incubated for another 7 days at 30°C. Seven-mm-diameter plugs were cut from each of fungal colony grown on purified culture, placed into a new PDA agar plate and cultured for 5 days to observe mycelia growth.

Enzyme Extraction

The enzyme extraction was performed in quadruplicate, as described by a published study (Dias et al., 2010). Enzymatic extracts were obtained from solid culture media containing 15 g of ground OPF with 22.5 mg of glucose in 250 mL Erlenmeyer flasks (Dinis et al., 2009). After that, 45 ml of deionised water was also added into the flask.

All flasks were autoclaved at 121°C for 20 min prior to treating OPF with WRF. After cooling, three plugs (10 mm in diameter) were taken from the isolated fungus and added to the sterile flask containing the ground OPF. The flask was then incubated at room temperature (37°C) for 45 days (Azmi et al., 2016). The content of culture flask was suspended in 150 ml of deionised water and the flask was placed on a rotary shaker (100 rpm) for 3 hours. Enzyme extracts were filtered (Whatman GF/A) and 0.06 g polyvinyl polypyrrolidone (PVPP) was added before centrifugation at 12000 × *g* for 10 minutes. The aliquots were used for determination of enzyme activity according to Azmi et al. (2016).

Oil Palm Frond Pre-treatment with Enzyme Extracts from WRF

Pre-treatments of OPF with each of the enzyme extracts were performed in triplicate. About 8 ml of the culture media of enzyme extracts were collected into 250 ml Erlenmeyer flask containing 70 ml citrate buffer (50 mM; per liter of distilled water: 10.5 g $C_6H_8O_7.H_2O$ and 14.7 g $C_6H_5Na_3O_7.2H_2O$; pH5.0) and 11 g of OPF. The flasks were autoclaved at 121°C for 20 minutes. After cooling, 20 ml enzymatic extract, 6.7 ml MnSO4 and 1 ml H_2O_2 were added into the flasks. The flasks were then put in a forced air oven at 40°C for 5 days (Hassim, 2012). Oil palm frond residues were obtained after filtration through paper filter. Samples were immediately dried in a forced air oven at 60°C, ground to pass a 1 mm screen (Retsch, Cutting mill, model SM1, Haan, Germany) and stored in airtight flasks at room temperature for later chemical analysis and *in vitro* incubation with rumen fluid.

Experimental Design

The experimental design was completely randomized with three replicates per treatment. The non-treated OPF (FOPF) was considered as control group in this study. The treatments included physical pre-treatment of OPF (POPF), biological pre-treatment of OPF with enzyme extracts from two WRF which were *G. lucidum* (BGL) and *L. edodes* (BLE) and combination of physical and biological pre-treatments of *G. lucidum* (CGL) and *L. edodes* (CLE) respectively.

Chemical Analyses

Proximate analysis. All samples used in the current study namely the control sample (FOPF), POPF, BGL, BLE, CGL, CLE and goat concentrates were analysed for chemical compositions (g/100g DM). Dry matter content of samples was determined by oven-drying at 105°C for 24 hours based on standard analytical method (Association of Analytical Chemistry [AOAC], 1990). Ash content was determined by combustion at 550°C for 4 hours in a muffle furnace. Determination of crude protein (CP) involved three different stages. First stage was the digestion process of the sample with concentrated sulfuric acid (H_2SO_4), followed by distillation process with sodium hydroxide (NaOH) by Kjeldahl system and finally titration against acid. The amount of nitrogen (N) found were converted to crude protein (CP= N*6.25. Crude fibre (CF) was determined by washing and boiling the sample in H_2SO_4 and NaOH using fibre bag technique. Ether extract was determined by extraction with petroleum ether. All experiments were done in triplicate.

Lignocellulose Content. The samples of FOPF, POPF, BGL, BLE, CGL and CLE were weighed 1 g per fibre bag. Insoluble fibres in the samples included cellulose, hemicellulose and lignin (Van Soest, 1963) were measured as neutral detergent fibre (NDF), acid detergent fibre (ADF) and acid detergent lignin (ADL) based on the Gerhardt application fibrebag-system protocol. All experiments were done in triplicate.

Neutral Detergent Fibre Analysis. Fibre bags were dried for 1 hour at 105°C and were allowed to cool in desiccator for 30 min. Approximately 1 g of sample was weighed in fibre bags. Neutral detergent fibre solution was prepared using EDTA ehtylenediamine tetra acetic acid-disodium salt and disodium tetra borate-decahydrate, dodecylsulphate-sodium salt, 2-ethoxyethanol, sodium dihydrogenphosphate and heat-stable α-amylase. The NDF (%) were determined as follow using the blank value as shown in equation below:

$$\text{NDF (\%)} = \frac{(m_3 - m_1) - (m_4 - m_5)}{m_2} \times 100$$

where m_1 – weight of fibre bag (g), m_2 – initial sample weight (g), m_3 – weight of crucible with dried fibre bag and sample residue after digestion, m_4- weight of crucible with ash (g), m_5 – blank value of empty fibre bag (g)

Acid Detergent Fibre (ADF) Analysis. Acid detergent fibre solution was prepared by diluting N-cetyl-N, N, N-trimethyl-ammoniumbromide in sulphuric acid. The protocol of ADF analysis was similar to the NDF analysis.

Acid Detergent Lignin (ADL) Analysis. For the ADL procedure, the ADF procedure was used as a preparatory step. However, the components of cellulose and lignin were not eluted from the feed by the acid detergent solution. The cellulose was therefore dissolved with 72% sulphuric acid in order to receive the crude lignin (ADL). Hemicellulose was calculated as NDF – ADF and cellulose as ADF – ADL (Van Soest, 1963).

***In vitro* Incubation with Rumen Fluid and Analysis.** The *in vitro* incubation with ruminal fluid was performed in syringes (100 ml volumes) according to the published method (Rahman et al., 2011). Rumen fluid was collected from two fistulated male Katjang goats before morning feeding at 0800h. Both goats were fed with OPF and a commercial pellet. The goats were cared for in accordance to the animal ethics guidelines of the Universiti Putra Malaysia (UPM/IACUC/AUP-R039/2016). The rumen fluid was mixed in a mixture (Waring Products Division, New Hartford, USA) for 30 seconds and filtered through four layers of cheesecloths. The rumen fluid (5 mL) was mixed with 20 ml of bicarbonate and phosphate buffer in 100 mL sterile gas-tight syringes containing 0.25 g of each sample (FOPF, POPF, BGL, BLE, CGL and CLE) following the modified method by Menke (1988). Air was removed from the syringes before the tip was closed. All treatments were done in triplicate and incubation was done at 39°C for 24 h in the oven. Syringes were shaken carefully to ensure complete mixing of the incubated contents. Gas production was measured and recorded by reading the scale on the syringe at 0, 2, 4, 6, 8, 10, 12 and 24 h of incubation. Following 24 h incubation, the samples were acidified with 25% metaphosphoric acid in water, centrifuged (10 min, 4°C at 15,000 × *g*) and filtered before the filtrate was used to determine the VFA.

Determination of Volatile Fatty Acid and Apparent Rumen Degradable Carbohydrate

At 0 and 24 h of incubation, 10 ml of rumen fluid was collected for VFA analysis and acidified with 0.2 ml phosphoric/formic acid (10/1, v/v). Samples were centrifuged for 10 min at 15,000 x *g* and the supernatant was recovered for VFA analysis by gas chromatography (Shimadzu GC-14A, Shimadzu Corporation, Hertogenbosch, The Netherland). Apparent rumen degradable carbohydrate (ARDC) was calculated based on the equation below (Rahman et al., 2011):

$$\text{ARDC (mg)} = \frac{\text{Ac}}{2} + \frac{\text{Pr}}{2} + \text{But} * \frac{162}{1000}$$

with 162 the assumed molecular weight of 1 mol fermented carbohydrates (Demeyer, 1991) and Ac, Pr and But expressed as net micro-molar production.

Statistical Analysis

Statistical analyses were performed using Statistical Package for Social Science 20.0 (SPSS software for Windows, release 20.0 SPSS, Inc., Chicago, IL, USA). All parameters were statistically evaluated separately using a one-way analysis of variance (ANOVA) at a significance level of 5% between the controls, physical and biological pre-treatments of OPF. Thus, the null hypothesis was rejected when $P<0.05$.

RESULTS AND DISCUSSION

Chemical Composition of Pre-treated OPF

Chemical compositions of FOPF, POPF, BGL, BLE, CGL and CLE are shown in Figure 1. The content of CF showed significant ($p<0.05$) difference in all pre-treated OPF as compared with FOPF (control), except following physical pre-treatment. However, the physical, biological and combined pre-treatments did not change the cellulose content of the OPF ($p>0.05$). The hemicellulose contents of all pre-treatments decreased significantly ($p<0.05$) compare to control, but no significant ($p<0.05$) differences were observed among pre-treatment. Similarly, the lignin contents of all pre-treated OPFs showed significant ($p<0.05$) decrease as compared with the non-treated OPF ($p<0.05$) and treatments with BGL and CGL showed significant ($p<0.05$) decrease as compared to other pre-treatments. The content of CP was highest in both BGL and CGL (80 g/kg DM), followed by CLE (78.2 g/kg DM), BLE (75 g/kg DM), FOPF (50 g/kg DM) and POPF (47.07 g/kg DM).

In the current study, fresh OPF consisted of 21% hemicellulose, 49% cellulose and 19% of lignin (Figure 1), nearly similar value with the 19.2% hemicellulose (Hong et al., 2012) and 20% lignin reported earlier (Dahlan, 2000), except for the cellulose (31.5%). Previous study also reported higher content of hemicellulose at 30.34% (Hermiati et al., 2013) in OPF obtained from Banten, Indonesia. This may be due to factors such as geographic location, age and climate. In the current study, fresh OPF from 7-year old palm trees were used. Furthermore, handling and different portion of OPF used may also contribute to the difference of chemical composition. In the current study, 1/3 of the whole OPF length was used.

In this study, the lignocellulose content decreased following physical and biological pre-treatments with *G. lucidum* and *L. edodes*. However, only lignin and hemicellulose contents showed significant decrease following physical pre-treatment, which were consistent with the results of a previous study that demonstrated the 11% decrease of lignin and 13% of hemicellulose following physical pre-treatment with hot compressed water at 200°C (Goh et al., 2012). The report also showed that the cellulose content remained similar as the current study.

Biological pre-treatments either with enzyme extracts from *G. lucidum* and *L. edodes* or in combination with physical pre-treatment did not change the cellulose content but reduced the hemicellulose and lignin contents. By partially removing

the lignin, the accessibility of enzymes to cellulose was markedly improved as lignin acts as physical barrier (Pan et al., 2005). It is expected that the reduction of hemicellulose as well as lignin gives a positive effect on OPF digestibility. *Ganoderma lucidum* and *L. edodes* have been shown to produce ligninolytic enzymes, including the

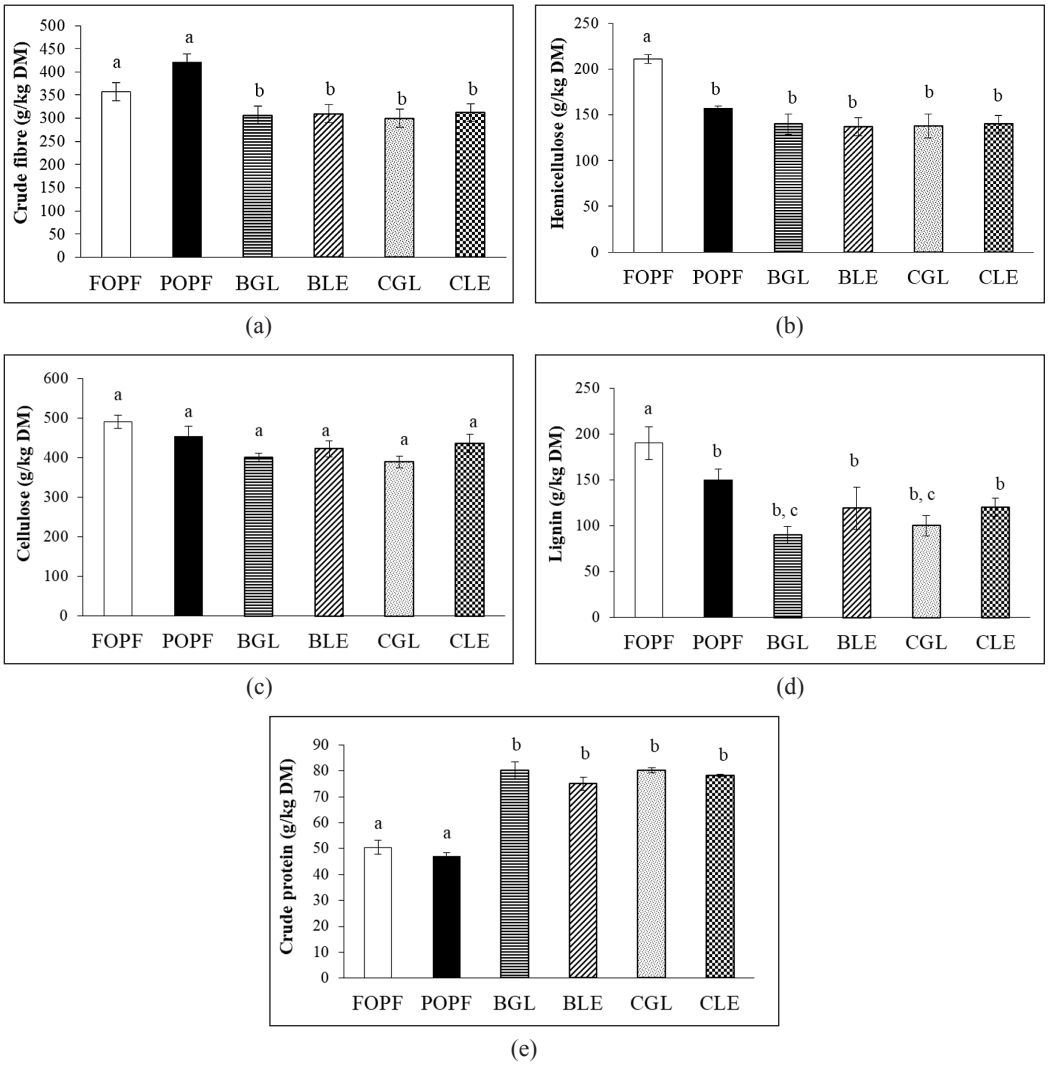

Figure 1. Comparison of chemical compositions of (a) crude fibre, (b) hemicellulose, (c) cellulose (d) lignin and (e) crude protein in non-treated OPF as control, physical pre-treated OPF, biological pre-treated OPF with enzyme extract of *G. lucidum* and *L. edodes* as well as combination of pre-treated OPF with enzyme extract of *G. lucidum* and *L. edodes*

Notes: Bars indicate standard error of mean (n=4). [abc] means in the same row with different superscript are significantly different ($p<0.05$). FOPF: non-treated OPF; POPF: physical pre-treated OPF; BGL: biological pre-treated OPF with enzyme extract of *G. lucidum;* BLE: biological pre-treated OPF with enzyme extract of *L. edodes*; CGL: combination of pre-treated OPF with enzyme extract of *G. lucidum*; CLE: combination of pre-treated OPF with enzyme extract of *L. edodes*

laccase, manganese peroxidase (MnP) and lignin peroxidase (LiP) that decompose hemicellulose, cellulose and lignin of OPF by oxidising the phenolic and non-phenolic lignin polymer and mineralise the insoluble lignin (Datta et al., 2017).

Total Gas Production Following 24 hours *in vitro* Incubation

There was a steady increase in the volume of gas produced by all substrates with time of incubation (Figure 2). Total gas productions for all pre-treatment groups (POPF, BGL, BLE, CGL and CLE) were significantly ($p<0.05$) higher than the FOPF. The highest cumulative gas production was in BGL (48 ml) whereas FOPF was the lowest (27.5 ml) following 24 hours incubation.

The *in vitro* gas production has a good correlation with *in vivo* digestibility of ruminant feed (Bhatta et al., 2007; Liu et al., 2011). Therefore, gas production technique is often used to evaluate the ruminal degradability of feed mixtures. The current study reported an increase in gas production with increasing time of incubation as reported by Rahman et al. (2011). However, the amount of gas production in biological pre-treatment was lower than the previous finding (Rahman et al., 2011), probably due to the use of different fungal strain and different mechanism of action. Indeed, biological pre-treated OPF using enzyme extracts from *G. lucidum* resulted in higher gas production in the current study as compared to the non-treated and other treatments.

The differences between the gas volume in controls and treatment samples may be attributed to the high content of soluble carbohydrate in dietary treatments especially in biological pre-treated OPF using enzyme extracts from *G. lucidum*. To date, there is no report on the response of OPF *in vitro* gas production and rumen fermentation following pressing method using conventional sugarcane machine as well as combined pre-treatment. Physical pre-treatment using sugarcane machine was considered practical, cost effective and easily operated as it didn't require high energy and it was suitable to be operated in small-scale farms. Physical pre-treatment also increased the gas production as compared to the non-treated OPF, indicating increased ferment ability. This was observed in the previous study that other physical pre-treatment, such as steam explosion was proven as a promising biomass pre-treatment which improved the nutritional contents of the fibrous feeds (Goh et al., 2012) and increased the fibrous feed digestion (Viola et al., 2008). Benefits of pre-treatment to ruminants feed include a shift of lignocellulosic biomass to fermentable sugars, allowing the access of cellulose to the microbial enzymes and convert the carbohydrate polymers into fermentable sugars (Saritha et al., 2012). Biological pre-treated OPF using enzyme extracts from *G. lucidum* resulted in higher gas productions as compared to non-treated OPF. The result suggested that WRF used were able to decrease the lignin content in the OPF as well as increase carbohydrate availability for ruminal degradation.

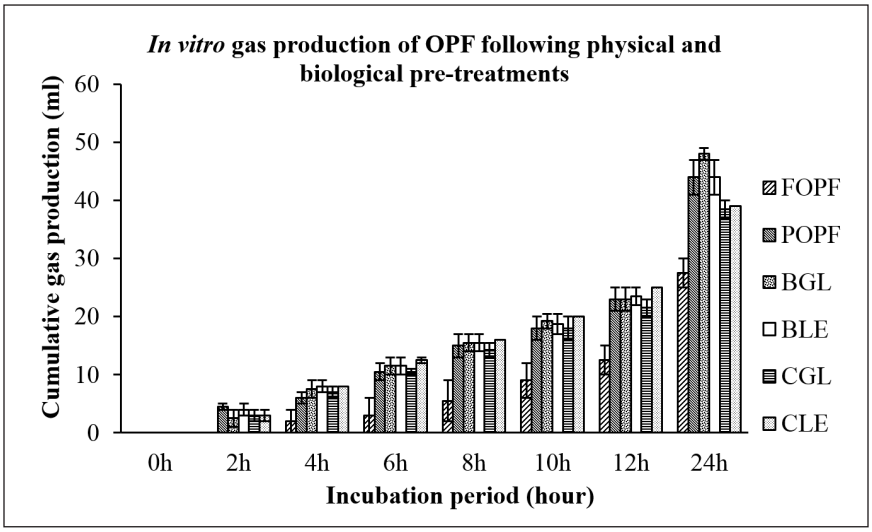

Figure 2. *In vitro* gas measurement (ml) of OPF following physical pre-treatment by pressing using conventional sugarcane machine and biological pre-treatment with enzyme extract of WRF; *G. lucidum* and *L. edodes*

Notes: Data shown are average of triplicates with standard error bars. FOPF: non-treated OPF (control); POPF: physical pre-treated OPF; BGL: biological pre-treated OPF with enzyme extract of *G. lucidum*; BLE: biological pre-treated OPF with enzyme extract of *L. edodes*; CGL: combination of pre-treated OPF with enzyme extract of *G. lucidum*; CLE: combination of pre-treated OPF with enzyme extract of *L. edodes*

Effect of the Pre-treatment on *in vitro* Rumen Fermentation Parameters

The VFA production is shown in Table 1. The levels of acetic, propionic and butyric acids were consistent in all pre-treatment groups and significantly higher ($p<0.05$) than the rumen fluid only (no substrate). In general, the acetic acid showed the highest level followed by the propionic and the butyric acids in all groups (Table 1). Both BGL and CGL were significantly higher ($p<0.05$) in propionic and butyric acids as compared to the FOPF.

There was also an increase in the ARDC of all treatments and the highest ARDC was in BGL with 6.57 mg. The treatments that showed significantly higher ($p<0.05$) ARDC than FOPF were the BGL (6.57$\pm$0.02 mg) and the CGL (6.54$\pm$0.05 mg). Physical, biological and/or combination pre-treatments with enzyme extracts from *L. edodes* failed to change the level of ARDC.

Volatile fatty acids or short chain fatty acids are produced by the anaerobic microbial fermentation in the rumen and supplied an estimated 70-80% of the energy in ruminant (Aluwong et al., 2010). Based on Figure 1, almost 21% of lignin in OPF was lost following pressing method and about 48% of lignin was degraded in OPF pre-treating with enzyme extract of *G. lucidum* and *L. edodes*. The results suggested that the ligninolytic enzymes namely laccase, LiP and MnP decreased the lignin content and in turn improved rumen degradability based on higher VFA production (Table 1). However,

one previous study reported that the lignin loss was not always correlated with VFA production or *in vitro* gas production (Rahman et al., 2011). The same study also demonstrated few WRF including *G. lucidum* that was promising in improving ruminal degradability and the observation agreed with the current study. In the present study, the BGL and CGL produced a significant effect on VFA production and ARDC compared to the FOPF and other pre-treated OPF. The biological pre-treatment of OPF using enzyme extract from *G. lucidum* was observed to be more effective as compared to enzyme extract of *L. edodes* since *G. lucidum* had the optimal enzyme activity for pre-treating the OPF (Azmi et al., 2016). The lignolytic potential needs to be at the highest in order to degrade the lignin content and allow the accessibility of rumen microbes to the cellulose and hemicellulose (Azizi-Shotorkhoft et al., 2016). Meanwhile, it is recognised that low level of cellulose and hemicellulose enzyme activity can maintain the structures of both cellulose and hemicellulose. Hence, this allows the rumen microbes to digest the cellulose and hemicellulose for VFA production.

A tremendous amount of works has been done on the techniques and pre-treatment strategies in order to improve the value of OPF. The physical pre-treatment has been sometimes abandoned due to its poor quality in increasing the nutritive value of roughage. However, in the present study, the physical treatment applied was practical because it only required conventional sugarcane machine which was economically feasible and environmental-friendly. Using pressing method combined with enzyme extract of WRF may be an alternative way to improve the quality of OPF and increase VFA as well as ARDC. Meanwhile, biological pre-treatment of OPF with enzyme extract of *G. lucidum*, either alone or in combination gives better results in terms of lignocellulosic content, gas production as well as the VFA. Previous studies also reported that *G. lucidum* showed substantial ability to degrade lignin (58%) and hemicellulose (74.8%), but not cellulose in wheat straw (Ćilerdžić et al., 2017). This indicates that *G. lucidum* is the best fungus to be used in pre-treatment in various fibrous by-products which contain high lignin. Some factors which are likely to contribute to the good finding in *G. lucidum* compared to other WRF include the production of a significant amount of ligninolytic enzyme by *G. lucidum*, the enzyme extract from *G. lucidum* matched easily with OPF and enzyme extract from *G. lucium* are not easily denatured to the temperature or any condition during pre-treatment.

Although the combination of pressing method with biological pre-treatment has not been documented yet, different combination strategy for OPF was clearly reported (Metri et al., 2018). It has been shown that combination of few WRF inoculation, namely *Ceriporiopsis subvermispora*, *G. lucidum*, *L. edodes*, *Phlebia brevispora* and *Pleurotus eryngii* for 3 or 9 weeks with enzyme supplementation was not successful in increasing the rate of gas production and

ARDC in OPF except for the high dose of commercial enzyme product, Hemicell to *Pleurotus eryngii* inoculated OPF (Metri et al., 2018).

Table 1
Volatile fatty acid (VFA, mmol/l) production and apparent rumen degradable carbohydrate (ARDC, mg) after 24 hour in vitro ruminal fermentation of pre-treatment and non-treated groups (mean±SE)

Parameters	FOPF	POPF	BGL	BLE	CGL	CLE
Total VFA (mmol/ incubation)	89.03±1.04	92.02±6.64	95.45±0.74	85.17±3.45	95.24±0.40	87.59±1.21
Acetate (mmol)	44.39±0.21^{a}	44.12±2.50^{a}	46.15±0.30^{b}	42.30±0.20^{a}	45.39±0.68^{a}	42.78±1.52^{a}
Propionate (mmol)	13.67±0.17^{a}	14.69±1.18^{a}	15.03±0.08^{b}	13.83±0.16^{a}	15.15±0.08^{b}	13.81±0.29^{a}
Butyrate (mmol)	8.87±0.16^{a}	9.50±0.02^{a}	9.93±0.01^{b}	6.08±2.07^{a}	10.08±0.06^{b}	8.69±0.02^{a}
ARDC (mg)	6.18±0.14^{a}	6.09±0.04^{a}	6.57±0.02^{b}	5.95±0^{a}	6.54±0.05^{b}	5.99±0.15^{a}

Notes: Data shown are average of triplicates. FOPF: non-treated OPF; POPF: physical pre-treated OPF; BGL: biological pre-treated OPF with enzyme extract of *G. lucidum;* BLE: biological pre-treated OPF with enzyme extract of *L. edodes*; CGL: combination of pre-treated OPF with enzyme extract of *G. lucidum*; CLE: combination of pre-treated OPF with enzyme extract of with *L. edodes.* ^{abc} means in the same row with different superscript are significantly different ($p<0.05$)

CONCLUSIONS

In this work, biological pre-treatment with enzyme extract of *G. lucidum* and *L. edodes* showed a similar pattern in degradation of the structural carbohydrates in OPF as well as in increasing the CP content. However, comparing these two WRF, *G. lucidum* seemed more promising for improving the *in vitro* rumen degradability. Biological pre-treatment with enzyme extract of *G. lucidum* combined with physical pre-treatment improved the nutritional values of OPF by decreasing the lignin contents, consequently improving the ruminal digestibility along with high total gas production, high VFA and high ARDC.

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Short Communication

Preliminary Foliar Anatomical Assessment of Four *Vanilla* Species (Orchidaceae) from Perak, Malaysia

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ABSTRACT

Assessment of the leaf anatomy among *Vanilla griffithii*, *Vanilla kinabaluensis*, *Vanilla sanguineovenosa* and *Vanilla* sp. 1 from Perak showed generic characterization of the epidermal layer and leaf lamina, suggesting the genus to possess plesiomorphic characters from its progenitor except for the leaf margin outlines. The species were found to differ interspecifically and acknowledged to possess taxonomic value. Leaf margin among the species showed similarity in tapering outline but distinguished in the overall shape and apical curve.

Keywords: Leaf anatomy, marginal outlines, taxonomy, *Vanilla*

INTRODUCTION

The genus of *Vanilla* Plum. ex Mill. characters are diagnosed as hemiepiphytic vinous monopodial growth habit, with roots produced at each internode and having fleshy fruits and wingless seeds with a hard seed coat (Soto-Arenas & Cribb, 2010). Furthermore, the plant structures can be classified by having membranous to coriaceous leaves, axillary racemose to paniculate inflorescence, spreading sepals and petals, labellum usually fused to the column, column complex consisting of pollen in monads, stigmata and rostellum, articulated ovary to the perianth with dehiscent capsule (Pridgeon et al., 1999).

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Recently, Soto-Arenas and Cribb (2010) had updated the classification of the genus into two subgenera *Vanilla* and *Xanata* based on the taxanomic reviews that congruent with the phylogenetic analysis. The subgenera *Xanata* which represents the majority and distributed pantropically is further classified into two sections namely *Xanata* and *Tethya* with the introduction of grouping system within the system.

In Peninsular Malaysia, *Vanilla* is considered as understudied in comparison to the other genera of Orchidaceae. Previous checklists stated that there were five species native to this region namely *Vanilla aphylla*, *Vanilla borneensis*, *Vanilla griffithii*, *Vanilla kinabaluensis* and *Vanilla montana* (Govaerts et al., 2017) and two new taxa to science, *Vanilla sanguineovenosa* R. Go et A. Raffi (Raffi et al., 2017a) and *Vanilla norashikiniana* R. Go et A. Raffi (Raffi et al., 2017b). However, the genus is considered taxonomically difficult due to the high degree in vegetative resemblance and flowers that are scarce and ephemeral (Soto-Arenas & Dressler, 2009). With more species expected to be described from this highly species-rich region, it is important to evaluate different techniques that can aid in the identification process. The investigations to distinguish the species had been conducted via several approaches of which among them anatomical description was rarely used. Anatomical assessment is an important approach to get a better understanding of the cell characters which are valued as secondary data in taxonomic studies. Among the plant parts, leaves are noted to be widely used in taxonomic descriptions based on their highly variables in shapes, sizes, textures and colours and most importantly, they are most prominent part across terrestrials plant. The leaves anatomy can be defined by the abaxial epidermis, adaxial epidermis, lamina, midrib and margin (Cutler et al., 2008).

The comparative leaf anatomy of *Vanilla* was previously assessed by Stern and Judd (1999) who also compiled results from previous works on various species. Among the species examined, only two taxa also distributed in Peninsular Malaysia namely *Vanilla pilifera* (synonym to *V. borneensis*) and *V. aphylla* were included. Therefore, this study was conducted to investigate the characters in four leaf parts of four species obtained from different localities in Perak which are *V. griffithii* collected from coastal hill forest (Lumut), *V. kinabaluensis* from lower montane forest with the highest annual precipitation in Malaysia (Bukit Larut), *V. sanguineovenosa* from lower montane forest (Tapah), and the unidentified *Vanilla* sp. 1 from the 130 million years old dipterocarp forest (Sg. Enam). The significance of the cell characters in the systematics of the local genus was evaluated and served as the supplementary data to the previous description.

MATERIALS AND METHODS

Fresh materials were fixed in 70% of formalin acetic acid (FAA). Matured leaf samples of *V. griffithii*, *Vanilla* sp. 1, *V. kinabaluensis* and *V. sanguinevenosa* (Figure 1) were cut into 30 – 50 µm thick

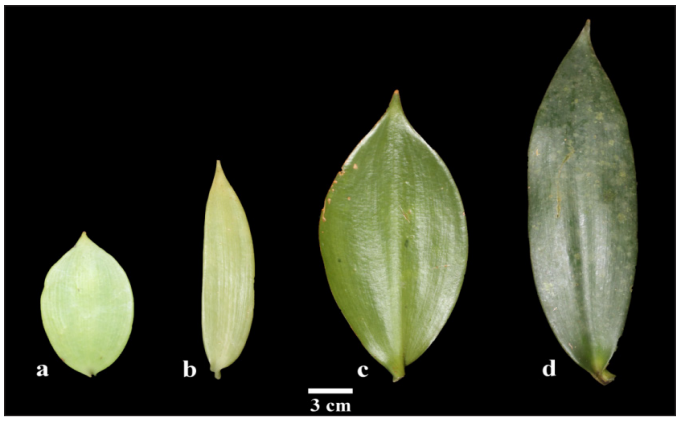

Figure 1. Leaves variation of *Vanilla* species from Perak. a) Elliptic shape: *Vanilla griffithii*; b) Oblong shape: *Vanilla* sp. 1; c) Large leaf, elliptic: *Vanilla sanguineovenosa*; d) Large leaf, narrowly elliptic: *Vanilla kinabaluensis*

slices using a sliding microtome into three main transverse sections; lamina, margin and midrib. They were subjected to bleaching process using 5% sodium hypochlorite (Chlorox®) solution for five minutes. The bleached samples were stained using Safranin and Alcian Blue for five minutes. The samples were then dehydrated twice by series of soaking in 50%, 70%, 95% and 100% alcohol and subsequently mounted onto specimen slides using Canada Balsam. The slides were oven dried at 60°C for one week. Four slides were prepared for each accession. The micrographs of the specimens were captured with an Olympus BX41 compound microscope and Olympus software.

Foliar anatomical characters were described from epidermal peels of adaxial and abaxial surfaces which were stained using Safranin and the three transverse sections. Stomatal density (mm^{-2}) and stomatal index (s / (s + e) x 100; where s = number of stomata per unit area and e = number of epidermal cells in the same unit area) were calculated in each accession. Stomatal density enumeration was obtained from nine epidermal layers while seven epidermal layers were observed in stomatal index calculation for each accession. Data generated were subjected to statistical analyses using IBM SPSS (Version 22.0, IBM Corp., Chicago, IL, USA). Basic descriptive statistic reported as mean ± standard deviation was performed. The data were also tested for fitness to a normal distribution by the Shapiro-Wilk test. One way ANOVA and a post-hoc Tukey pair wise comparison were used to test the significant difference among species. Statistical significant difference was set at confidence level of 95% (alpha = 0.05).

RESULTS

Description of Epidermal Layers

Epidermal cells anticlinal walls: straight to curve in adaxial and abaxial epidermis. **Epidermal cells shape:** basically isodiametric, polygonal and some elongated

in adaxial and abaxial epidermis (Figure 2). **Stomatal complexes:** hypostomatous, scattered among epidermal cells basically tetracytic with the presence of anisocytic and anomocytic types in all species, guard cells ovate (Figure 3). Data comprising stomatal size, density and index are summarized in Table 1.

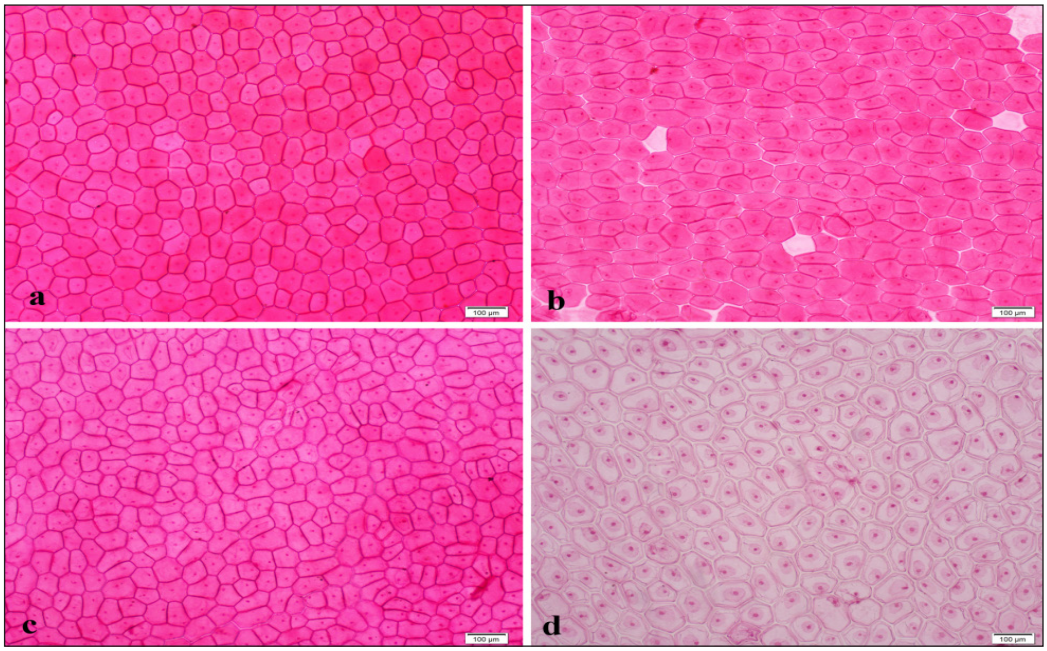

Figure 2. Cells architecture on leaf epidermal layers. a) *Vanilla griffithii*; b) *Vanilla* sp. 1; c) *Vanilla kinabaluensis*; d) *Vanilla sanguineovenosa*

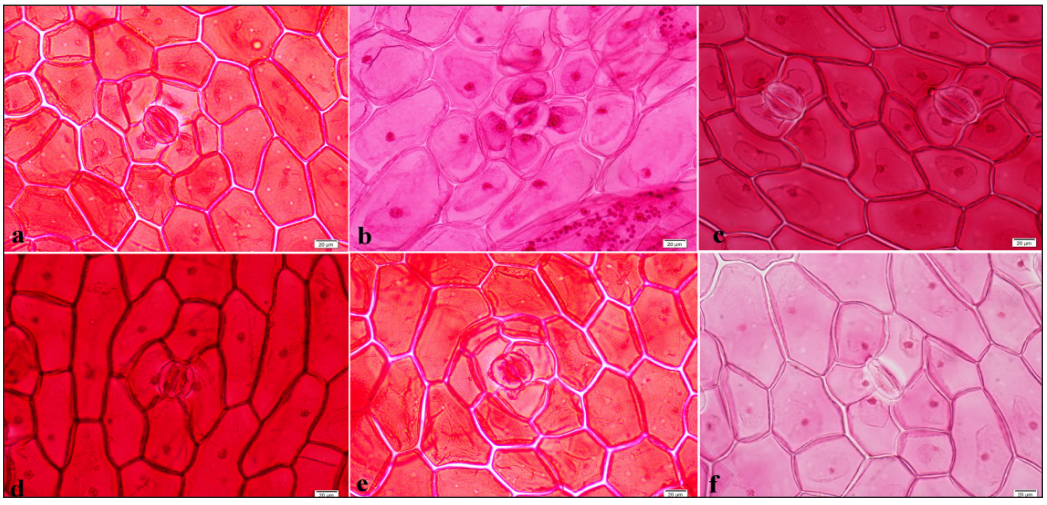

Figure 3. Stomatal types of *Vanilla* species from Perak. a-d) Tetracytic type of stomatal apparatus of (a) *Vanilla griffithii*, (b) *Vanilla kinabaluensis*, (c) *Vanilla sanguineovenosa* and (d) *Vanilla* sp. 1; e) abaxial of *Vanilla griffithii* showing anisotypic stomatal apparatus; f) anomocytic stomata in *Vanilla sanguineovenosa*

Table 1
Summary of the stomatal size, density and index of Vanilla species from Perak. Means with the same letters within the same column were not significantly different at $\alpha \leq 0.05$

Species	Stomatal size (Length × width, µm)	Stomatal density (mm^{-2})	Stomatal index (%)
Vanilla griffithii	40.00 – 46.67 x 29.33 – 40.00	14.33 ± 0.726 **ab**	4.371 ± 0.522 **a**
Vanilla sp. 1	38.67 – 45.33 x 29.33 – 38.67	12.11 ± 0.512 **a**	4.15 ± 0.171 **a**
Vanilla kinabaluensis	42.67 – 64.00 x 32.00 – 58.67	19.44 ± 1.192 **d**	5.35 ± 0.284 **ab**
Vanilla sanguineovenosa	41.33 – 50.67 x 32.00 – 40.00	16.22 ± 0.572 **c**	6.74 ± 0.919 **c**

Description of Transverse Sections of Leaf Lamina and Margin

Cuticles: present adaxially and abaxially. **Adaxial epidermal layers:** single epidermis layer of 1:2 ratio (length: width) except 1:3 ratio in *V. kinabaluensis*. **Abaxial epidermal layers:** single epidermis layer of 1:2 ratio except 1:1 ratio in *V. kinabaluensis*. **Hypodermis:** uniseriate at adaxial and abaxial epidermal layer. **Mesophyll:** homogenous, isodiametric in shape, section width. ***Vanilla griffithii*** = 16 – 18 cells, ***Vanilla* sp. 1** = 14 – 17 cells, ***Vanilla kinabaluensis*** = 10 - 13 cells and ***Vanilla sanguineovenosa*** = 15 – 16 cells, cells smaller and compact at both polar region but largest and loose towards the centre (Figure 4). **Cell inclusions:** mucilaginous idioblast present and scattered. **Vascular bundles:** collateral, arranged in one row, alternately between small and large bundle, centrally in the mesophylls of ***Vanilla griffithii*** and ***Vanilla sanguineovenosa*** and close the adaxial layer in ***Vanilla* sp. 1** and ***Vanilla kinabaluensis***, sclerenchyma cells ensheathing the vascular bundles, more on the phloic side. **Bundle sheaths:** basically isodiametric, some polygonal. **Outline of leaf midrib:** obscure. **Outline of leaf margin:** ***Vanilla griffithii***, rounded with

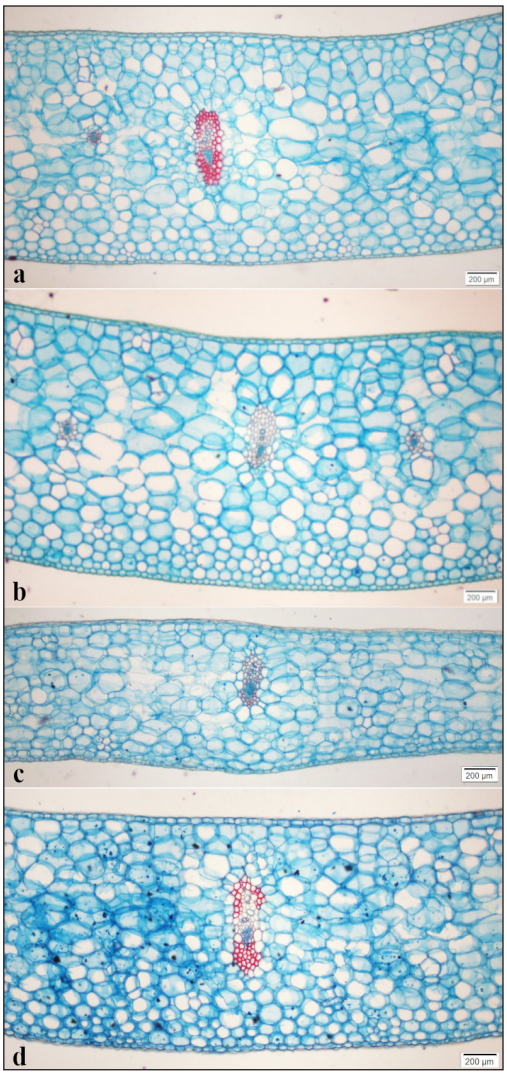

Figure 4. Lamina transverse sections of *Vanilla* species from Perak. a) *Vanilla griffithii*; b) *Vanilla* sp. 1; c) *Vanilla kinabaluensis*; d) *Vanilla sanguineovenosa*

convex-shaped outline, apex acute, recurved 10º towards the abaxial layer, no dent present; ***Vanilla* sp. 1**, rounded with convex-shaped outline, apex acute, dent present; ***Vanilla kinabaluensis***, pointed with blunt outline, apex obtuse, recurved 20º towards the abaxial layer, no dent present; ***Vanilla sanguineovenosa***, pointed with needle-shaped outline, apex acute, recurved 45° towards the abaxial layer, no dent present (Figure 5).

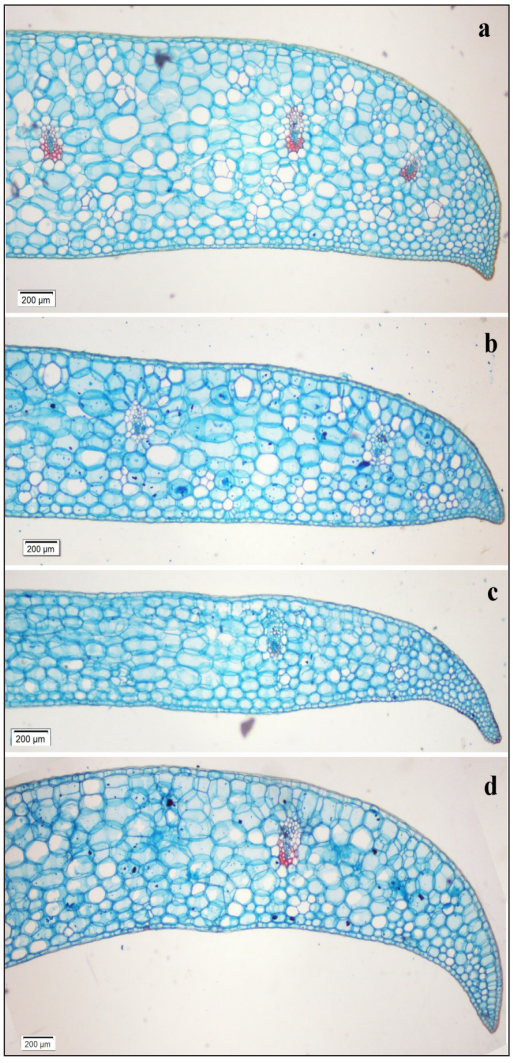

Figure 5. Margin transverse section of *Vanilla* species from Perak. a) *Vanilla griffithii*; b) *Vanilla* sp. 1; c) *Vanilla kinabaluensis*; d) *Vanilla sanguineovenosa*

DISCUSSION

Variations in Leaf Anatomy of Four Species from Perak

Information on the architecture of the cell has a significant role in plant systematics (Silva et al., 2014) since these cytological components displayed traits that are genetically controlled (Carlsbecker & Augstein, 2018). The inclusion of anatomical evaluation with the data in morphology is vital in plant conservation as it allows reliable identification of the conservation units (Heywood & Iriondo, 2003). As for the *Vanilla* species from Perak, their characterization of the cells composition in both epidermal surfaces and lamina transverse sections were generically congruent with the descriptions by Stern and Judd (1999) on the American and African species suggesting the genus to share plesiomorphic characters from its progenitor. However, crystalliferous idioblast documented in that study were absence among the assessed species but in the form of mucilage suspected to result from genetic and environment differentiation caused by biogeographical divergence (Konyar et al., 2014). Stomatal characteristics enumeration in the epidermal layer showed that the length, density and frequency were in accordance with the respective leaf sizes; lesser in smaller leaves and larger in broader ones. Since the stomatal size of plant species were reported to show significant positive

correlation to its genomic size (Hodgson et al., 2010) while the density and index were found to aid in the identification of *Ficus* species (Ogunkule & Oladele, 2008), it implies that these data could serve as discriminating factors. Furthermore, the stomatal size in angiosperms was noted as key to plant survival as species with smaller stomata would be able to adapt faster to the environmental shifts (Drake et al., 2013). This was in agreement with the stomatal characteristics depicted by the widely distributed *V. griffithii* as the species used in this study was collected in the uncharacteristic locality of xeric condition. Another eminent variation was noted in the mesophyll layer thickness of the leaf lamina. Each species displayed different range in the number of layers which *V. griffithii* appeared to have the thickest. This characteristic should be treated as a promising identification tool as it was demonstrated to be one of the delimitating characters in *Cussonia* (De Villiers et al., 2010**)**. The species examined also depicted two types of vascular bundle arrangements which were centrally and adaxially and grouped them into two respective groups sharing similarity in overall leaf shape; a) elliptic (*V. griffithii* and *V. sanguineovenosa*) and b) oblong (*Vanilla* sp. 1 and *V. kinabaluensis*). The importance of vascular bundles orientation and arrangement in providing species clustering were shown in the taxonomic study on *Passerina* species by Bredenkamp and Van-Wyk (2001). However, the assessment of these characteristics is only useful in the lamina part as its arrangement in all species is more adaxially positioned when moving towards the margin. Besides that, the studied species showed no prominent midrib outline confirming the foliar architecture of *Vanilla* orchids by Cameron and Dickison (1998). However, margin outlines were found to differ interspecifically and acknowledged to possess taxonomic value.

Taxonomic Significance of Transverse Section of Leaf Marginal Outlines

In general, the leaf margin outline among the species can be described as tapering, apex consisting of mesophyll cells flanked by hypodermal layers of which cells division at the adaxial layer determined the part orientation, vascular bundles present towards the marginal edge and presence of scattered mucilaginous inclusion. This study proposed the marginal outline to serve as a delimiting character in the leaf parts of the examined *Vanilla* (Figure 6). This is concordance with the anatomical assessment of different plant habits, from shrubs to woody, which pinpointed the incorporation of leaf marginal outline to be beneficial in plant identification (Hussin et al., 2000; Kantachot et al., 2007; Srinual & Thammathaworn, 2008; Talip et al., 2003, 2012). However, comparative anatomy investigations on the marginal outline were not aware to be reported in *Vanilla* or other genera in Orchidaceae except for the characterization of its cells composition in certain species (Stern, 2014). The character of marginal outline is usually neglected compare to petiole and midrib anatomical

characteristics (Talip et al., 2003). Hence, this study should serve as a preliminary data on the utilization of the distinguishing character. A proposed dichotomous key depicting the classification of each accession using leaf marginal outlines are provided as follows:

Tentative Key to the Species from Perak, Malaysia

1 Marginal outline rounded with convex-shaped ……..... 2
Marginal outline pointed with various shapes ……..……..…… 3

2 Apex straight, dented on abaxial ……...……...…….....*Vanilla* sp. 1
Apex recurved, smooth (no dent present) on abaxial .....*Vanilla griffithii*

3 Apex acute, recurved 45º towards abaxial.....*Vanilla sanguineovenosa*
Apex obtuse, recurved 20º towards abaxial ……...*Vanilla kinabaluensis*

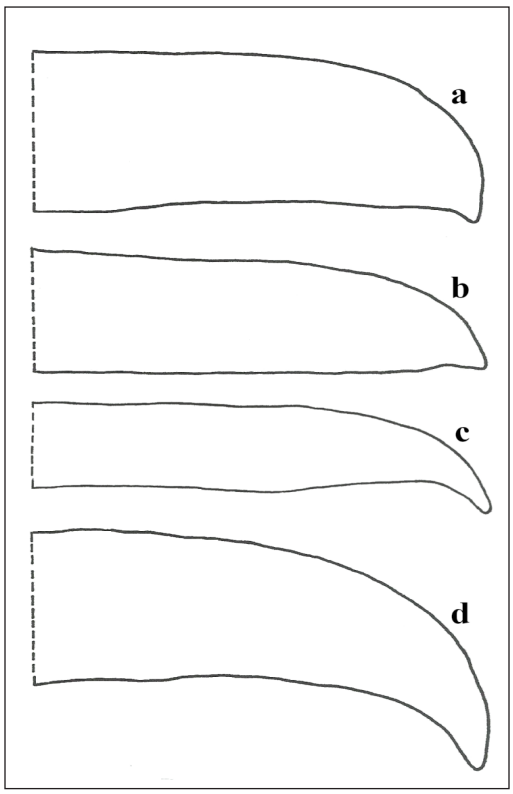

Figure 6. Schematic diagram of the marginal outlines of *Vanilla* species from Perak. a) *Vanilla griffithii*; b) *Vanilla* sp. 1; c) *Vanilla kinabaluensis*; d) *Vanilla sanguineovenosa*

CONCLUSION

Leaf marginal outlines were acknowledged to be one of the distinguishing anatomical characters among *Vanilla* species from Perak.

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Morphotaxonomic Study of Algal Epiphytes from *Ipomoea aquatica* Forssk. (Convolvulaceae) found in Laguna de Bay (Philippines)

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ABSTRACT

Epiphytic algae existing on submerged leaves, stems and roots of *Ipomoea aquatica* Forssk. (Convolvulaceae) found in Laguna de Bay was studied. Examination of the prepared specimen showed a total of 15 infrageneric taxa belonging to 10 orders, 13 families, 15 genera and 15 species were identified during the study period. Of these taxa, the occurrence of a rare photosynthetic euglenoid, *Cryptoglena skujae* Marin and Melkonian is reported as new record for the Philippines. Two species are also presented here based on current accepted taxonomic names and these are *Limnococcus limneticus* (Lemmermann) Komárková, Jezberová, O.Komárek & Zapomelová, and *Anabaenopsis circularis* (G. S. West) Woloszynska & V. Miller in V. Miller. The algal taxonomic records reported in this survey expand the knowledge regarding diversity and distribution of epiphytic algae from aquatic macrophytes found in Philippine freshwater environment.

Keywords: Epiphytic algae, Laguna de bay, macrophyte, morphotaxonomy, water spinach

INTRODUCTION

Ipomoea aquatica Forsk. (water spinach) is an edible aquatic macrophyte characterized by having a long, hollow stem (allowing a large number of air passages) with rooting at the nodes growing floating or prostrate in surface water or marshy ground (Baysa et al., 2006; Nagendra et al., 2008). This aquatic macrophyte is capable of forming tightly-packed masses of entangled vegetation, consequently forming thick layer above the water surface causing restriction in light distribution into the body of water. It is widely distributed geographically in tropical countries and is being planted and raised

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abundantly in Myanmar, Philippines, India, China, Bangladesh, Thailand, Indonesia and Vietnam, (Mandal et al., 2008; Naskar, 1990). Macrophytes (such as *Ipomoea aquatica*) play a crucial role in preserving and enhancing the water quality of a lake. These aquatic plants have a huge capability of assimilating nutrients and other harmful substances from a water system and thus lowering the content of pollutants (Dhote, 2007). Effective withdrawal of nitrogen, carbon, biochemical oxygen demand (BOD), chemical oxygen demand (COD), suspended solids, organic, phosphorus, heavy metals and the like from lake water were observed from aquatic macrophytes showing its potential for restoration and bioremediation of a polluted aquatic ecosystem (Dhote, 2007; Gupta, 1980).

Algal epiphytes are microalgae and cyanobacteria attached directly on aquatic macrophytes by means of secretion of jelly-like substances colonizing the leaf, roots and stem surfaces of the macrophyte and sediment surfaces (Adam et al., 2017). In addition, these microorganisms can colonize the aquatic plant and are responsible in the accumulation of large amounts of carbonate on submerged stem, leaves and roots of the macrophyte (Adam et al., 2017; Gaiser et al., 2011). Epiphytic algae play a crucial role as primary producers in lake and shallow freshwater ecosystems by acting as natural food to many grazers, zooplankton, and fish. Colonizing epiphytic microalgae and cyanobacteria are beneficial to macrophytes by reducing water movement in the aquatic ecosystem and by provision of alternative source of organic nutrients important for growth and proliferation of the aquatic plant (Adam et al., 2017). The diversity and density of algal epiphytes are influenced by several ecological factors such as water level, water temperature, location, light, seasonal change and morphology of macrophyte host as well as abundance and growth phases of host plant (Adam et al., 2017; Hassan et al., 2007).

In the Philippines, limited information is available on the distribution and diversity of epiphytic algae in submerged aquatic macrophytes (Rañola et al., 1990). Therefore, this study was conducted to do a preliminary survey and taxonomic account of some noteworthy epiphytic algae attached to submerged parts *Ipomoea aquatica* found abundantly in Laguna de Bay.

MATERIALS AND METHODS

Epiphytic Algae Sampling from Water Spinach

Samples of water spinach (*Ipomoea aquatica* Forsk.) where evident growth of epiphytic algae were observed were collected from the littoral to sublittoral zone of Laguna de Bay (14^{o} 10'- 14^{o} 35' N, 121^{o}-121^{o}30' E). Thereafter, the samples were placed in polyethylene plastic bags filled with lake water for laboratory analysis and examination. Twelve *Ipomoea aquatica* samples were analyzed during the conduct of the study. The epiphytic algae attached on the water spinach were separated following the procedure done by Zimba and Hopson (1997). Epiphytic algae observed in submerged stem, leaves and roots of water

spinach were separated by manual scraping and shaking for 30 minutes. The shaking procedure was repeated several times to make sure that the bulk of the attached organisms were separated. The samples were then filtered using 100 µm mesh sieves to separate the host aquatic plant and other large particles (Arguelles, 2019). The concentrated microalgae were then preserved using 4% neutralized formalin. The collected scraped epiphyte samples were thoroughly mixed and a 50 mL portion was kept for analysis and identification of diatoms. Scraped samples for analysis of diatom flora were digested following the protocol of Tunca et al. (2014) and Utermöhl (1958). An aliquot of treated diatom was air dried and mounted onto coverslips. The remaining water sample was transferred into a sterile graduated cylinder and allowed to settle for 24 hours. Thereafter, 45 mL of water was removed and the remaining 5 mL of water was placed into a sterile drum vial for microscopic observation (Tunca et al., 2014; Utermöhl, 1958).

Micrometry, Photomicrography and Identification

Morphotaxonomic description and identification of the epiphytic algae were done using an Olympus CX31 binocular research microscope with a built in Infinity X digital camera. The identification of microalgal species documented in this study was identified and described using the monographs and standard works of Desikachary (1959), John and Tsarenko (2011), McGregor et al. (2007), Presscott (1962), Velasquez (1962), Whitton (2011) and Wolowski (2011). Identification of the recovered epiphytic algae was done up to the species level using each of the available information. Current accepted taxonomic names of each of the alga are presented in the paper which was based on Guiry and Guiry (2018).

RESULTS AND DISCUSSION

Morphotaxonomic notes and illustrations of the algal taxa observed and identified in the samples collected in March, 2018 are presented below. A total of 15 microalgal and cyanobacterial species were identified from the studied water macrophyte, of which five species belong to Cyanophyceae, four species to Euglenophyceae, three species for Bacillariophyceae, two species for Trebouxiophyceae and one species for Chlorophyceae. References used in the identification of the various algal taxa are listed directly below the name of the relevant taxon. Also, short description of the habitat where the algae were collected is presented in the paper.

Morphotaxonomic Description of the Isolates

Chlorophyta
Class: Trebouxiophyceae
Order: Chlorellales
Family: Chlorellaceae
Genus: *Chlorella* Beyerinck [Beijerinck]

***Chlorella vulgaris* Beyerinck [Beijerinck] (Figure 1a)**

BASIONYM: *Chlorella pyrenoidosa var. duplex* (Kützing)

REFERENCES: Arguelles and Monsalud (2017); John and Tsarenko (2011); Ortega-Calvo et al. (1993); Zafaralla (1998).

DESCRIPTION: Cells are greenish, spherical and solitary with smooth and delicate cell wall; single parietal chloroplast that is cup-shaped with a single pyrenoid; diameter of the vegetative cell is 2.5 - 3.5 µm; cell proliferation through production of autospores (2 or 4) released from the mother cell.

SPECIMEN: LUZON, Laguna, Los Baños (Tadlak), E.DLR. Arguelles *s.n*. Photograph prepared from the mounted specimen. Observed existing as a greenish covering on leaves and stems of water spinach mixed with other planktonic algae and aquatic molds.

Class: Chlorophyceae
Order: Chlamydomonadales
Family: Chlorococcaceae
Genus: *Chlorococcum* Meneghini

***Chlorococcum infusionum* (Schrank) Meneghini (Figure 1b)**

SYNONYM: *Chlorococcum humicola* (Nägeli) Rabenhorst 1868
BASIONYM: *Lepra infusionum* Schrank

REFERENCES: Arguelles and Monsalud (2017); Kumar et al. (2012); Vijayan and Ray (2015).

DESCRIPTION: Cells are greenish in color, spherical, solitary or sometimes in groups of several cells crowded together forming a stratum; occurring either as uni- or multinucleate; chloroplast is parietal with one or more pyrenoids; cells 8.0-15.0 µm in diameter.

SPECIMEN: LUZON, Laguna, Victoria (San Benito), E.DLR. Arguelles *s.n.* Photograph prepared from the mounted specimen. Observed existing as a brownish to greenish crust on stem and leaves of water spinach mixed with other diatoms and cyanobacteria.

Class: Chlorophyceae
Order: Sphaeropleales
Family: Scenedesmaceae
Genus: *Tetradesmus* G.M. Smith

***Tetradesmus obliquus* (Turpin) M. J. Wynne (Figure 1c)**

SYNONYM: *Scenedesmus obliquus* Turpin (Kützing)

BASIONYM: *Achnanthes obliqua* Turpin

REFERENCES: Bose et al., (2016); Kim (2015); Hegewald and Silva (1988).

DESCRIPTION: Colony comprised 2-4 celled, linearly arranged or in alternating cells in 1 or 2 rows, rarely to be observed in solitary cells, joined side by side with almost three-quarters of the algal cell length. Cells are usually fusiform in shape with pointed end (12.0-28.5 µm long and 6.0-9.0 µm

wide). The marginal cells are shaped like a bow (arcuate) while the inner cells are straight. Cell walls are smooth and without teeth or spines. Cells have a parietal chloroplast with a single pyrenoid.

SPECIMEN: LUZON, Laguna, Calamba (Pansol), E.DLR. Arguelles *s.n*. Photograph prepared from the mounted specimen. Observed existing as a brownish to greenish crust on submerged stem and leaves of water spinach mixed with other filamentous cyanobacteria.

Bacillariophyta
Class: Bacillariophyceae
Order: Cymbellales
Family: Gomphonemataceae
Genus: *Gomphonema* Ehrenberg

***Gomphonema gracile* Ehrenberg (Figure 1d)**

REFERENCES: Bartozek et al. (2013); de Souza Santos et al. (2012).

DESCRIPTION: Valves are lanceolate in shape gradually attenuating at the end portion with round apices; valves are longer than wide, length 36.9-79.5 µm, width 7.5-12.5 µm; central area is characterized by having a shortened median stria; a stigma is located at the central nodule at the end of the central stria; striae uniseriate, parallel to slightly radiate towards the ends; striae density 10.0-12.0 in 10.0 µm.

SPECIMEN: LUZON, Laguna, Bay (Sto. Domingo), E.DLR. Arguelles *s.n*. Photograph prepared from the mounted specimen. Observed existing as a brownish crust on submerged stem and leaves of water spinach mixed with other microalgae and cyanobacteria.

Class: Coscinodiscophyceae
Order: Aulacoseirales
Family: Aulacoseiraceae
Genus: *Aulacoseira* Thwaites

***Aulacoseira granulata* var. *angustissima* (Otto Müller) Simonsen (Figure 1e)**

BASIONYM: *Melosira granulata* var. *angustissima* Otto Müller

REFERENCES: Cavalcante et al. (2013); Joh (2017).

DESCRIPTION: Frustules are linear, cylindrical forming colonies. Valves are longer than wide, 3.5–4.5 µm in diameter with a mantle height of 7.0-18.0 µm. The mantle height to valve diameter ratio is more than 3 (high mantle). The areolae are characterized to be square to round, but frequently elongate. Spines are situated at the end of each pervalvar mantle costa.

SPECIMEN: LUZON, Laguna, Los Baños (Tadlak), E.DLR. Arguelles *s.n*. Photograph prepared from the mounted specimen. Observed existing as a brownish crust on stem and leaves of water spinach mixed with other filamentous cyanobacteria.

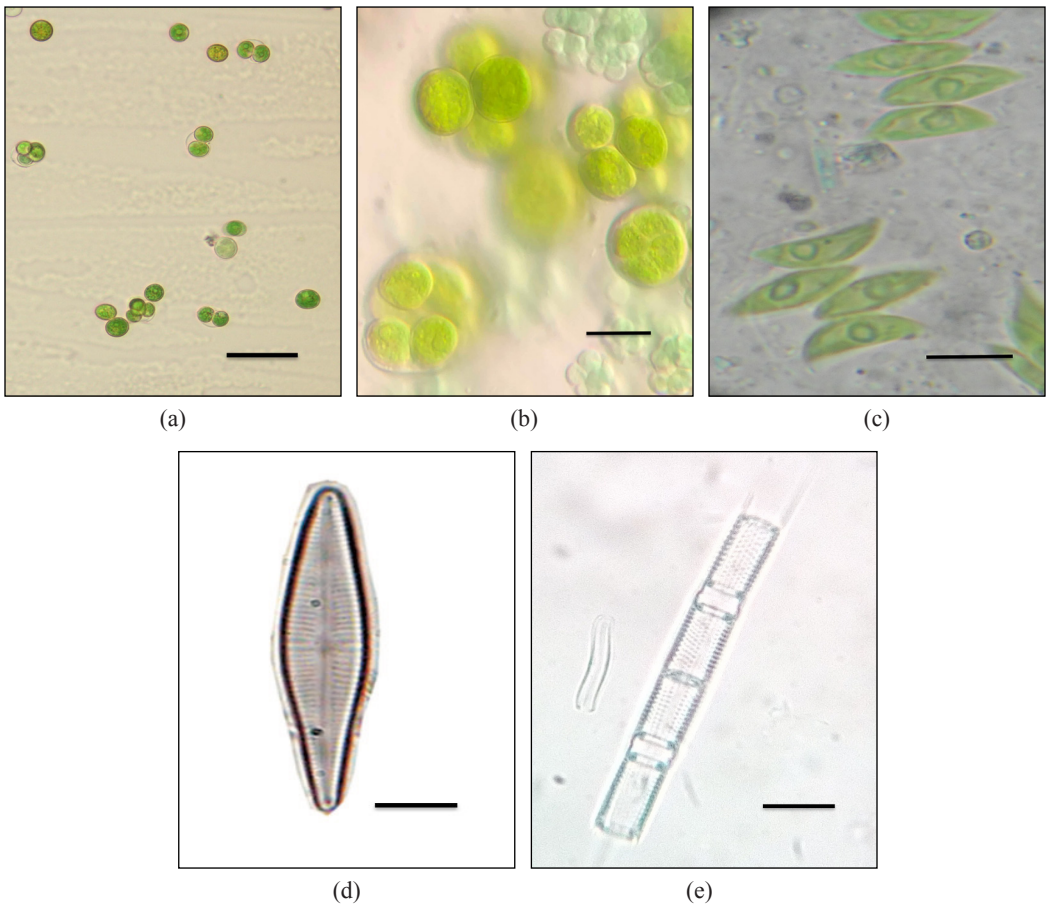


Figure 1. Photomicrographs of (a) *Chlorella vulgaris* Beyerinck [Beijerinck], (b) *Chlorococcum infusionum* (Schrank) Meneghini, (c) *Tetradesmus obliquus* (Turpin) M. J. Wynne, (d) *Gomphonema gracile* Ehrenberg, (e) *Aulacoseira granulata* var. *angustissima* (Otto Müller) Simonsen. All scale bars = 10 µm

Class: Mediophyceae
Order: Stephanodiscales
Family: Stephanodiscaceae
Genus: *Cyclotella* (Kützing) Brébisson

***Cyclotella meneghiniana* Kützing (Figure 2a)**

REFERENCES: Akbulut (2003); Costa et al. (2017); Leira et al. (2017); Marra et al. (2016).

DESCRIPTION: Cells rounded with central and marginal areas. Valves are small and disc-shaped with a narrow mantle; cells 7.0-15.0 µm in diameter and are characterized by having marginal chambered striae with flat and smooth central area covering 1/3 of the valve surface.

SPECIMEN: LUZON, Laguna, LUZON, Laguna, Victoria (San Benito), E.DLR. Arguelles *s.n.* Photograph prepared from the mounted specimen. Observed existing as a brownish crust

on submerged roots of water spinach mixed with other filamentous fungi and green microalgae.

Euglenophyta
Class: Euglenophyceae
Order: Euglenales
Family: Euglenaceae
Genus: *Trachelomonas* Ehrenberg

***Trachelomonas volvocina* (Ehrenberg) Ehrenberg (Figure 2b)**

BASIONYM: *Microglena volvocina* Ehrenberg

REFERENCES: Kouassi et al. (2013); Wolowski et al. (2017).

DESCRIPTION: Lorica are globular (13.5–21.0 µm in diameter) with smooth wall; reddish- brown in color; flagellum at the anterior part of the cell without a collar and is three times longer than lorica; presence of two lateral chloroplasts with double sheathed pyrenoids.

SPECIMEN: LUZON, Laguna, Los Baños (Mayondon) E.DLR. Arguelles *s.n*. Photograph prepared from the mounted specimen. Observed existing as a greenish film on submerged leaves of water spinach mixed with other green microalgae.

Class: Euglenophyceae
Order: Euglenales
Family: Phacaceae
Genus: *Lepocinclis* Perty

***Lepocinclis steinii* Lemmerman (Figure 2c)**

REFERENCES: Arguelles et al. (2014); Wolowski (2011); Wolowski et al. (2013).

DESCRIPTION: Cells are fusiform to elliptical (30.5–31.0 µm in length and 9.50– 11.0 µm in diameter) with short, pointed cauda at the posterior end with visible paramylon bodies occurring as large rings; numerous disc-shaped chloroplasts are present and pellicle is longitudinally striated.

SPECIMEN: LUZON, Laguna, Los Baños (Mayondon) E.DLR. Arguelles *s.n*. Photograph prepared from the mounted specimen. Observed existing as a greenish film on submerged leaves and stems of water spinach mixed with other filamentous cyanobacteria with other planktonic algae.

Class: Euglenophyceae
Order: Euglenales
Family: Euglenaceae
Genus: *Cryptoglena* Ehrenberg

***Cryptoglena skujae* Marin & Melkonian (Figure 2d)**

SYNONYM: *Phacus agilis* Skuja

REFERENCES: Alves-Da Silva and Bicudo (2009); Araujo et al. (2012); Roy and Pal (2016); Wolowski (2011).

DESCRIPTION: Cells are small and elliptical, 13.5-21.0 µm in length, 9.0-13.0 µm in width; anterior end slightly indented in the central portion; posterior pole rounded and without caudal process; exhibit longitudinal furrow extending along the length of

the ventral surface of the cell; a single red eyespot (stigma) is observed near the anterior end of the cell; pellicle is smooth and rigid (no metaboly); presence of two chloroplasts that are lateral discs in shape; presence of two large lobed structures (paramylon bodies) are present.

A New Record for the Philippines. SPECIMEN: LUZON, Laguna, Bay (Sto. Domingo), E.DLR. Arguelles *s.n*. Photograph prepared from the mounted specimen. Observed existing as a greenish film on submerged leaves of water spinach mixed with other filamentous algae.

Class: Euglenophyceae
Order: Euglenales
Family: Euglenaceae
Genus: *Monomorphina* Mereschkowsky

***Monomorphina pyrum* (Ehrenberg) Mereschkowsky (Figure 2e)**

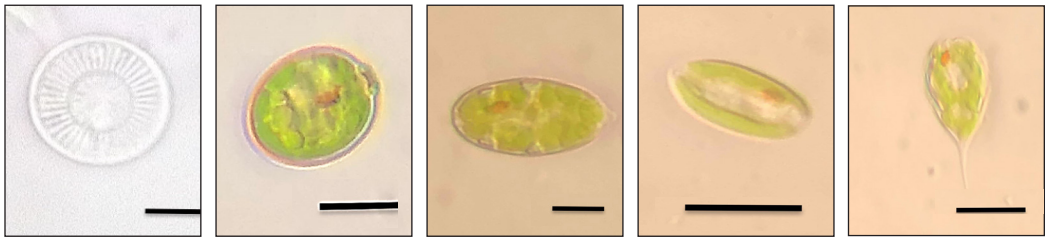
BASIONYM: *Euglena pyrum* Ehrenberg

REFERENCES: Alves-Da Silva and Bicudo (2009); Arguelles et al. (2014); Boonmee et al. (2011); Satpati and Pal (2017); Wolowski et al. (2013).

DESCRIPTION: Cells are oval-shaped (24.0–26.0 µm long and 10.5–12.0 µm wide); cells are gradually tapered at the posterior end forming a short, pointed caudus; anterior end cell is broadly rounded; pellicle is spirally striated (arranged in an S-shaped pattern) overlaying the entire cell; chloroplasts are small and numerous with two lateral paramylon bodies.

SPECIMEN: LUZON, Laguna, Bay (Sto. Domingo), E.DLR. Arguelles *s.n*. Photograph prepared from the mounted specimen. Observed existing as a greenish film on submerged leaves of water spinach mixed with other filamentous algae.

Cyanobacteria
Class Cyanophyceae
Order: Synechococcales
Family: Merismopediaceae



(a) (b) (c) (d) (e)

Figure 2. Photomicrographs of (a) *Cyclotella meneghiniana* Kützing, (b) *Trachelomonas volvocina* (Ehrenberg) Ehrenberg, (c) *Lepocinclis steinii* Lemmerman, (d) *Cryptoglena skujae* Marin & Melkonian, (e) *Monomorphina pyrum* (Ehrenberg) Mereschkowsky. All scale bars = 10 µm

Genus: *Limnococcus* (Komárek & Anagnostidis) Komárková, Jezberová, O. Komárek & Zapomelová

***Limnococcus limneticus* (Lemmermann) Komárková, Jezberová, O.Komárek & Zapomelová (Figure 3a)**

BASIONYM: *Chroococcus limneticus* Lemmermann

REFERENCES: Desikachary (1959); Komárková et al. (2010); Martinez (1984); McGregor (2013); McGregor et al. (2007); Prescott (1962); Whitton (2011); Zafaralla (1998).

DESCRIPTION: Colonies microscopic, free-floating, with mucilaginous slime, composed of sphaerical, subsphaerical to hemisphaerical cells. Cells are irregularly arranged, bright blue-green or sometimes appearing as greyish blue-green, with protoplast that is finely granulated, without aerotopes, 7.0-11.5 (-21.0) μm in diameter. Cell division is by three perpendicular planes in successive generations. Colonial mucilage is colorless, delicate, homogeneous, clearly delimited or diffluent at the margin, outer margin of colony usually distinct, sometimes scarcely visible, not lamellate, distant from the cells.

SPECIMEN: LUZON, Laguna, Calamba (Pansol), E.DLR. Arguelles *s.n*. Photograph prepared from the mounted specimen**.** Observed existing as a bluish-green layer attached on leaves and stems of water spinach mixed with other filamentous cyanobacteria and plaktonic green algae.

Class Cyanophyceae
Order: Chroococcales
Family: Chroococcaceae
Genus: *Chroococcus* Nägeli

***Chroococcus major* Komarék & Komákova-Legnerová (Figure 3b)**

REFERENCES: Comas-Gonzales et al. (2017); Komárek and Komákova-Legnerová (2007); McGregor (2013).

DESCRIPTION: Colonies are small, solitary, spherical or sometimes ellipsoidal, usually occurring with 2-4 cells characterized by having a well-defined colorless, homogeneous or sometimes slightly lamellated colonial envelopes. Cells blue-green in color, spherical to hemispherical in shape, 15.0-21.5 μm in diameter. Cell reproduction is by binary fission in 2-3 planes that are perpendicular to one another and is propagated by release of cells and/or group of cells.

SPECIMEN: LUZON, Laguna, Bay (Sto. Domingo), E.DLR. Arguelles *s.n.* Photograph prepared from the mounted specimen**.** Observed existing as a slimy, bluish-green covering on submerged leaves and stems of water spinach mixed with other filamentous cyanobacteria, diatom and green microalgae.

Class Cyanophyceae
Order: Oscillatoriales
Family: Oscillatoriaceae
Genus: *Oscillatoria* Vaucher ex Gomont

***Oscillatoria limosa* C. Agardh *ex* Gomont (Figure 3c)**

REFERENCES: Desikachary (1959); Martinez (1984); Pantastico (1977); Prescott (1962); Velasquez (1962).

DESCRIPTION: Trichomes blue green in color, filamentous, straight and slightly constricted to crosswalls showing typical oscillatory movement; anterior end cells are rounded or flattened, not attenuated and without calyptra; specialized cells (heterocytes and akinetes) are absent; cells 9.0-11.0 µm long and 2.5-4.5 µm wide, protoplasm is slightly granulated; crosswalls often granulated.

SPECIMEN: LUZON, Laguna, Calamba (Pansol), E.DLR. Arguelles *s.n*. Photograph prepared from the mounted specimen. Observed existing as a bluish-green layer on submerged leaves roots and stems of water spinach mixed with other filamentous fungi and diatoms.

Class Cyanophyceae
Order: Oscillatoriales
Family: Microcoleaceae
Genus: *Arthrospira* Sitzenberger ex Gomont

***Arthrospira platensis* Gomont (Figure 3d)**

REFERENCES: Barman et al. (2015); Desikachary (1959).

DESCRIPTION: Trichomes, solitary, blue green, 5.0-8.0 µm wide, trichome ends not attenuated, regularly spirally (screw-like) coiled. Spirals 25.0-36.0 µm broad, distance between the spirals 42.0-54.0 µm; cells nearly as long as broad, or shorter than broad; cells nearly as long as broad, or shorter than broad, 2.0-6.0 µ long, cross walls granulated; end cells rounded.

SPECIMEN: LUZON, Laguna, Los Baños (Tadlak), E.DLR. Arguelles *s.n.* Photograph prepared from the mounted specimen. Observed existing as a bluish-green crust on submerged roots and stems of water spinach mixed with other green microalgae and cyanobacteria.

Class Cyanophyceae
Order: Nostocales
Family: Aphanizomenonaceae
Genus: *Anabaenopsis* (V.V. Miller)

***Anabaenopsis circularis* (G.S. West) Woloszynska & V. Miller (Figure 3e)**

BASIONYM: *Anabaena flosaquae* var. *circularis* (G.S. West)

REFERENCES: Aguilera et al. (2016); Komárek (2005); Martinez (1984).

DESCRIPTION: Trichomes free floating, are circular and spirally-coiled (1-3 coils), contricted at crosswalls without mucilaginous envelopes. Cells cylindrical to barrel-shaped, slightly curved, 6.0–12.0 µm long and 2.5–5.5

μm wide, with aerotopes. Heterocytes are spherical or ovoid, 2.5–4.5 μm in diameter. Akinetes are elliptical to oval and solitary, 8.8–14.5 × 4.5–6.5 μm.

SPECIMEN: LUZON, Laguna, Victoria (San Benito), E.DLR. Arguelles *s.n.* Photograph prepared from the mounted specimen. Observed existing as a bluish-green film on submerged roots and stems of water spinach mixed with other filamentous cyanobacteria, molds and diatoms.

Epiphytic microalgae and cyanobacteria from aquatic macrophytes are ubiquitous in freshwater bodies but have not been investigated properly. In this survey, a preliminary enumeration of algal epiphytes associated with water spinach (collected from selected sites in the vicinity of Laguna de Bay) was taxonomically studied. A total of 15 infrageneric taxa belonging to 10 orders, 13 families, 15 genera and 15 species were identified over the study period. Of these taxa, the occurrence of a rare photosynthetic euglenoid, *Cryptoglena skujae* Marin & Melkonian represents a new record for the Philippines. Dominant algal epiphytes such as diatoms, cyanobacteria, and photosynthetic euglenophytes were observed in the sampling sites. A number

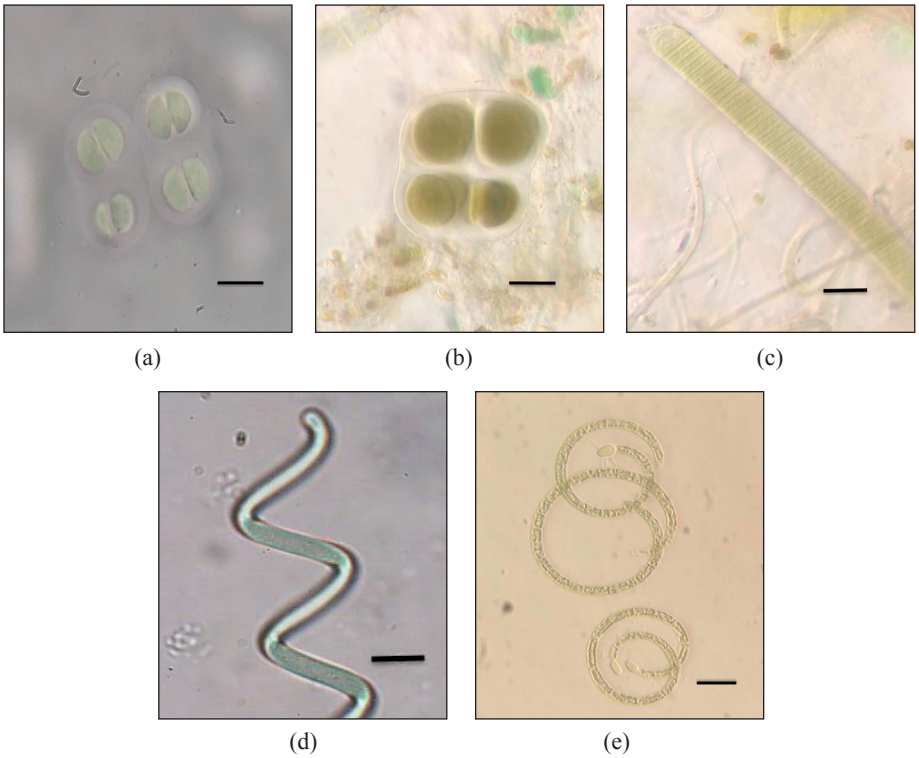


Figure 3. Photomicrographs of (a) *Limnococcus limneticus* (Lemmermann) Komárková, Jezberová, O.Komárek & Zapomelová, (b) *Chroococcus major* Komarék & Komákova-Legnerová, (c) *Oscillatoria limosa* C. Agardh ex Gomont, (d) *Arthrospira platensis* Gomont, (e) *Anabaenopsis circularis* (G.S.West) Woloszynska & V. Miller. All scale bars = 10 μm

of filamentous forms of cyanobacteria composed of the genera *Arthrospira, Oscillatoria,* and *Anabaenopsis,* together with unicellular forms such as *Chroococcus* and *Limnococcus* were observed from submerged stem, leaves and roots of the macrophyte. On the other hand, certain species of diatoms and green microalga that are considered ubiquitous and cosmopolitan genera such as *Gomphonema, Cyclotella, Chlorella* and *Tetradesmus* were present in all of the sampling sites. Strains of photosysnthetic euglenoids were represented by four taxa but their distribution was limited in two sampling sites only. The study shows that there is a variance observed in the distribution of the reported algal epiphyte species in the host macrophyte. The number and distribution of algal epiphytes on the host plant is dependant on several factors such as grazing pressure (fish and macro and micro-invertebrate grazing), changes in light intensity, growth form of macrophyte, texture of the plant parts, leaf arrangement and surface area of the host plant **(**Albay & Aykulu, 2002; Dunn et al., 2008; Salman et al., 2014). These factors can change the number of species, biomass and distribution of algal epiphytes on aquatic macrophytes. On the other hand, the growth and proliferation of aquatic macrophytes in bodies of water are also affected by epiphytic algae under elevated levels of nutrients in the water body. An increase in the level of water nutrients such as phosphorus and nitrogen promotes the growth and proliferation of both submerged aquatic macrophytes and the attached epiphytic algae, however, the proliferation of epiphytic algae interfere aquatic plants' growth by lowering the chlorophyll content and stimulating peroxidation of lipids in cell membrane of plants (Song et al., 2017).

CONCLUSION

From this preliminary survey it appears that the diversity of algal epiphytes are much more than it is expected. The algal taxonomic records reported in this survey expand the knowledge regarding diversity and geographic distribution of algal epiphytes in the Philippines. This highlights the significance of taxonomic studies of epiphytic algae in other aquatic macrophytes found in freshwater environments in the country. Further studies are necessary in order to increase knowledge of the taxonomy, distribution and ecology of these alga in plants and seaweeds found in the marine environment.

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Physicochemical and Sensory Properties from Indonesian White Shrimp (*Penaeus merguiensis*) Jerky

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ABSTRACT

Dendeng or jerky is the Indonesian traditional dried meat. It is commonly produced by using some spices and addition of starch at various levels. In this research, jerky was made by using fresh white shrimp flesh. White shrimp jerky is a versatile food product for consumption as a snack or meal. This research used three formulations with tapioca flour additions at 0% (A0, control), 5% (A1), 10% (A2), and 15% (A3) based on shrimp flesh weight. Shrimp does not have a wide surface like beef meat, so the processing of shrimp jerky involved only two steps, namely grinding and pressing. The protein, ash and water contents decreased with an increase in the percentage of tapioca flour. The sensory analysis of quantitative data revealed that no parameters were significantly different, except for textural properties. Overall, the addition of tapioca flour not exceeding 10% produced better physicochemical and sensory properties, similar to those of A0, the control. Shrimp jerky produced with or without flour could be a potential food product for consumption as a snack or meal.

Keywords: Jerky, physicochemical, sensory, tapioca flour, white shrimp

INTRODUCTION

Dendeng or jerky is the Indonesian traditional dried meat. It is commonly produced by using some spices and addition of starch at various levels. Therefore, its flavor is spicy, tasteful, and stable for several weeks at room temperature. Jerky is considered one of the oldest types of meat products. An et al. (2010) stated that jerky was commonly preserved through salting and drying to reduce moisture content and water activity.

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Jerky is convenient to eat, lightweight, and nutritious, as it is high in protein and low in fat (Choi et al., 2006).

Jerky is most often made from beef rather than other meats. Jerky can be produced using several types of meats, such as pork (Han et al., 2007), and poultry (Pegg et al., 2006). Shrimp jerky is a versatile product that could help The Healthy and Agriculture Department Programs. In this research, jerky was made by using fresh white shrimp (*Penaeus merguiensis*) flesh. The processing of shrimp jerky includes only two steps, namely, grinding and pressing. The characteristics of jerky are strongly influenced by curing, temperature and drying time, in terms of water content, Aw (water activity), chemical composition, etc. All these factors may ultimately affect the tenderness and acceptability of the final product. Konieczny et al. (2007) noted that the technological processing step, meat type, spices, extender or filler, and additives affected the characteristics of jerky. Many studies have used various processing procedures to investigate the effect of such factors on jerky quality, such as the marination method, drying time and temperature, and type of flour used as filler (Choi *et al*., 2006; Han *et al*., 2008; Konieczny et al., 2007). As known, consumers have commonly associated high quality jerky with good texture, color, flavor, and nutritional value (Konieczny et al., 2007). To improve the quality of jerky, our research introduced shrimp jerky as a new product with the addition of carbohydrates, such as a tapioca flour, as filler. The tapioca flour in jerky as the filler or binder, should have a strong binding, but weak emulsification properties. Therefore, binders, such as flour from wheat, tapioca, and sago, usually have a high carbohydrate content and low protein content.

Tapioca flour is commonly used to make a snack food in Indonesia. Jerky was expected to be a food product with high nutritional value that would add variety existing food. In any case, white shrimp and tapioca flour are used in many applications, especially for food diversity. The ratio of white shrimp and flour influences the physicochemical and sensory attributes of shrimp jerky. This research aimed to evaluate physicochemical properties such as proximate analysis, color, texture, structure morphology, and water holding capacity (WHC), and then conduct sensory evaluation by using a hedonic scale for white shrimp jerky.

MATERIALS AND METHODS

Preparation of Shrimp Jerky

The white shrimp (*Penaeus merguiensis*) jerky was prepared based on the method of (Garnida et al., 2015) with minor modifications. Fresh shrimp flesh was bought from a commercial slaughterhouse in Sungsang, Banyuasin, South Sumatera, Indonesia. The fresh shrimp flesh was ground, added to anchovy and brown sugar, and mixed with spices (garlic, onion, tamarind, coriander, galangal). The tapioca flour was added and mixed until homogeneous. The shrimp dough was molded using a roller on a pan with a

thickness of ± 3 mm, and dried in an oven at 60°C for 6 h. The addition of tapioca flour was based on the weight of fresh shrimp meat on a wet weight basis. The formulations of shrimp jerky were 5% addition of tapioca flour (A1), 10% addition of tapioca flour (A2), and 15% addition of tapioca flour (A3). The formulations of shrimp jerky are shown in Table 1.

Table 1
Formulation of the shrimp jerky

Components	Formulation A0 (g)	Formulation A1 (g)	Formulation A2 (g)	Formulation A3 (g)
Fresh shrimp flesh	500	475	450	425
Tapioca flour	0	25	50	75
Anchovy	20	20	20	20
Brown sugar	15	15	15	15
Onions	5	5	5	5
Garlic	1.5	1.5	1.5	1.5
Coriander	2	2	2	2
Galangal	2	2	2	2
Tamarind	3	3	3	3
Salt	2	2	2	2

The Proximate Analysis

Samples of shrimp jerky were analyzed for nutrient substances, such as moisture, ash, protein, fat, and carbohydrate contents. These were determined based on AOAC methods (Association of Official Analytical Chemist [AOAC], 2006). Amino acid composition was analyzed by using High Performance Liquid Chromatography determined by AOAC (2006) methods, wherein Alpha Amino Butyrate Acid (AABA) was used as a standard solution.

Color Measurement

The color of shrimp jerky sample surfaces was determined by using a Spectrophotometer CM-3500d, Konica Minolta Sensing, Inc., Osaka, Japan, and Hunter color values, L* (lightness), a* (redness), and b* (yellowness). The instrument was calibrated to standard black and white plates before analysis. The Hunter values were monitored by a computerized system using spectra magic software (Konica Minolta Sensing, Inc., Japan) and the measurements were performed in triplicate.

Hardness Analysis

An Instron Universal Testing Machine (Model 4400, Instron Co., USA) type TA18 (12.7MM DIA) probe was used for analysis. The samples were prepared as a uniform shape (1.0×2.0×0.3 cm) were measured by a cylindrical probe (12.7 mm diameter) to obtain the hardness data. A texture analyzer was prepared, and then a new test parameter was arranged. The test run was pressed and continued, then the probe above the sample

dropped and cut the sample. Then, the biting force data (Network / sec)) obtained were stored.

Structural Morphology

The morphology of shrimp jerky was examined by using an SEM (Scanning Electron Microscope) JEOL-2200 series. The control (shrimp jerky without addition tapioca flour) was also analyzed as a standard. All of the treatments were determined with three replicates.

Water Holding Capacity (WHC)

The WHC of shrimp jerky was determined by the method of Zhuang et al. (2007) with slight modifications. A sample of shrimp jerky (1 g) was added to 25 mL distilled water in centrifuge tube, stirred then incubated at 10^{o}C for 1 hour. After incubation, the sample was centrifuged at 3000 rpm for 20 min. The supernatant was separated and measured. The ability of shrimp jerky to bind water was determined with the supernatant. The supernatant refers to the water holding capacity, and is then calculated using the following formula:

$$WHC = \frac{\text{Water holding (mL)}}{\text{Sample (g)}}$$

Sensory Evaluation

The sensory analysis was conducted by 30 panelists who were trained to analyze sensory like or dislike of jerky. Furthermore, all of the sample treatments were assessed for their color, flavor, texture, and palatability with a 5 – point hedonic scale, where 5 implies extreme like and 1 implies extreme dislike. The panelists evaluated all the qualities. The samples were placed on a transparent plastic dish, and labeled randomly by assignment of a 3-digit numerical code. Each panelist was given mineral water for rinsing their oral cavity after every sample. All procedures were conducted in triplicates.

Statistical Analysis

The collected data were analyzed using one-way analysis of variance (ANOVA) using SAS 9.1.3. programme, and Duncan's multiple range test to detect if there was a significant difference between treatments.

RESULTS

Proximate and Amino Acid Analyses

In this study, we determined not only the components of protein, fat, carbohydrate (crude fiber), ash, and moisture but also performed amino acid analysis. The addition of tapioca flour to shrimp jerky had a statistically significant effect on the components of jerky substances. The control (A0) contained 46.71 ± 0.16% water content, which was higher than that of the other treatments. A decrease in ash and protein content was observed, otherwise carbohydrate and fat content gradually increased with the percentage of tapioca powder addition, compared to the control (Table 2).

The protein content of shrimp jerky supplemented with 15% tapioca powder (A3) was lower than that of the A1 and A2 treatments. Nevertheless, the A3 protein

Table 2
The proximate analysis of white shrimp (Penaeus merguiensis) *jerky (% g/100 g, dry basis)*

Treatment	Water (wet basis)	Ash	Fat	Protein	Carbohydrate
Control	46.71 ± 0.16a	8.06 ± 0.14a	0.32 ± 0.03c	77.61 ± 0.57a	13.37 ± 0.16d
A1	37.70 ± 0.35c	6.88 ± 0.16b	0.45 ± 0.04d	61.14 ± 0.41b	31.34 ± 0.34c
A2	45.27 ± 0.05b	6.13 ± 0.28c	0.72 ± 0.12b	55.79 ± 0.27c	36.84 ± 0.07b
A3	38.17 ± 0.07c	5.39 ± 0.02d	0.95 ± 0.07a	47.53 ± 0.17d	45.80 ± 0.24a

Note: Different letters in the same column show significantly different values (P <0.05)
Control : no addition of tapioca flour
A1 : 5% addition tapioca flour
A2 : 10% addition tapioca flour
A3 : 15% addition tapioca flour

content was still considered high. The protein content as the main substance in jerky product, our study stated that A1 was the best treatment compared to the other treatments, as its protein content decreased only 20% in relation to the control. The amino acid composition of shrimp jerky changed after the addition of tapioca powder. Glutamic and lysine acids were the highest amino acid components in the shrimp jerky. However, the levels of glutamic and lysine acids decreased from 8.72 ± 0.02% to 7.05 ± 0.01% and 5.70 ± 0.01% to 4.50 ± 0.01%, respectively, after the addition of tapioca flour (Table 3). The decrease in amino acids was greater for tryptophan and cystine, which decreased more than 50% compared to the control.

Table 3
The amino acid profiles of white shrimp (Penaeus merguiensis) *jerky (% w/w protein, dry basis)*

Amino acids	No addition of tapioca (control)	With addition of tapioca (the best treatment, A1)
Tryptophan	0.83 ± 0.02	0.32 ± 0.01
Cystine	0.54 ± 0.30	0.09 ± 0.01
Methionine	1.64 ± 0.02	1.39 ± 0.02
Serine	2.38 ± 0.02	2.03 ± 0.02
Glutamic acid	8.72 ± 0.02	7.05 ± 0.01
Phenylalanine	2.41 ± 0.03	2.26 ± 0.01
Isoleucine	2.47 ± 0.02	2.14 ± 0.03
Valine	2.33 ± 0.02	2.03 ± 0.01
Alanine	3.14 ± 0.01	2.60 ± 0.04
Arginine	4.20 ± 0.02	3.72 ± 0.01
Glycine	2.78 ± 0.04	2.40 ± 0.02
Lysine	5.70 ± 0.01	4.50 ± 0.01
Aspartic acid	4.83 ± 0.01	3.88 ± 0.01
Leucine	4.53 ± 0.01	3.98 ± 0.01
Tyrosine	1.57 ± 0.02	1.22 ± 0.02
Proline	1.51 ± 0.01	1.33 ± 0.03
Threonine	2.32 ±0.02	2.09 ±0.01
Histidine	1.19 ± 0.02	1.07 ± 0.02

The Morphological Characteristics

For this research, the characteristics of shrimp jerky including color, hardness, and WHC, are shown in Table 4. The addition tapioca powder as a filler in shrimp jerky affected the physical properties of the jerky. The textural properties of A1, A2 and A3 treatments were neither tender nor easily broken. A0, the control, could hold a large amount of water, so it had a texture that was tenderer and easily broken than that of jerky added tapioca powder (A1, A2 and A3) (Table 4). There was a correlation

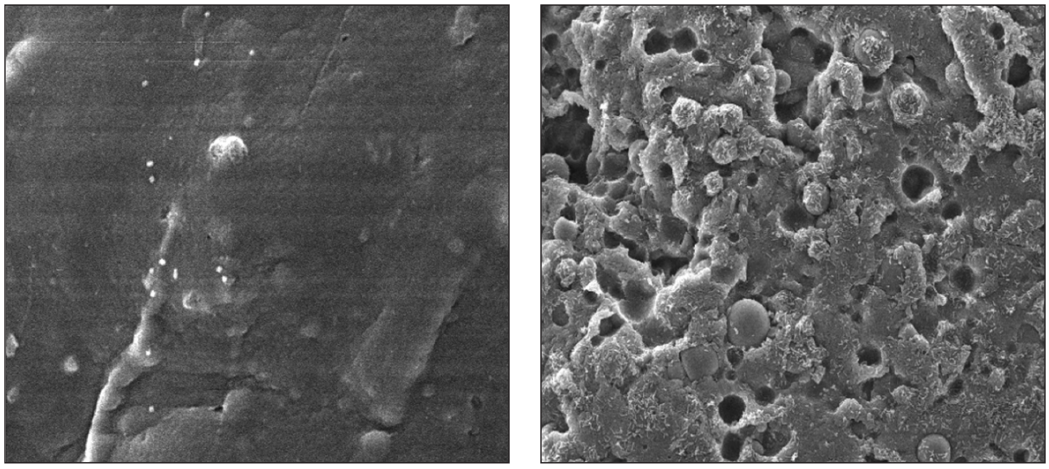
between hardness and WHC, indicating that shrimp jerky that could hold more water had a better texture than of the others jerkies, based on the WHC and hardness from the A0 treatment, conversely, A3 had 103.46 ± 10.63% hardness, and 1.33 ± 1.06% WHC. The colors of A2 and A3 were lighter, increasing the a* and b* values, compared to those of the A0 treatment. This means that the addition of tapioca powder influenced color.

Table 4
Hardness, color, water holding capacity (WHC) of white shrimp (Penaeus merguiensis) *jerky*

Treatment	Hardness (N/s)	WHC (ml/g)	Color		
			L*	a*	b*
A0	51.26±6.31b	7.94±1.02a	43.50 ± 5.02b	9.53 ± 1.25b	8.56 ± 4.65b
A1	52.33±10.65b	2.69±1.62b	50.06 ± 0.20b	10.53 ± 0.46ba	10.90 ± 0.26b
A2	77.93±10.95b	2.16±0.86b	55.70 ± 1.24a	11.83 ± 0.32a	15.60 ± 0.91a
A3	103.46±10.63a	1.33±1.06b	55.20 ± 0.43a	11.20 ± 0.60a	14.96 ± 0.15a

Note: Different letters in the same column show significantly different values (P <0.05)
Control : no addition of tapioca flour
A1 : 5% addition tapioca flour
A2 : 10% addition tapioca flour
A3 : 15% addition tapioca flour

The surface structure of shrimp jerky visualized with SEM (scanning electron microscopy) showed the existence of gaps or cavities on shrimp jerky added tapioca (500× magnification), shown in the following Figure 1.



(a) (b)

Figure 1. Microscopic analysis of shrimp jerky without adding tapioca starch as a control (a); and shrimp jerky with the addition of tapioca starch (b) at 500× magnification

The Sensory Evaluation

The sensory evaluation of the products prepared with different levels of tapioca flour, is shown in Table 5. There were no differences between the control and other treatments in color, taste, or flavor attributes, except for the texture (hardness) of the jerky. In these cases, A3 had a score of 2.96 ± 0.88 for textural properties. Otherwise, overall, the jerky palatability did not significantly differ among treatments or the control, and ranged from 3.16 ± 0.80 to 3.68 ± 0.52. An et al. (2010) reported similar results for sensory evaluations was under the same treatment (addition of kimchi powder) and the control (no addition kimchi powder) for semidried pork jerky.

Table 5
The sensory parameter of white shrimp (Penaeus merguiensis) *jerky*

Treatment	Sensory				
	Color	Taste	Flavor	Texture	Palatability
A0	3.20±0.76a	3.24±0.92a	3.28±0.79a	3.16±1.14a	3.16±0.80a
A1	3.48±0.96a	3.52±0.71a	3.44±0.82a	3.08±0.99a	3.24±1.09a
A2	3.84±0.68a	3.88±0.72a	3.88±0.66a	3.52±1.04a	3.68±0.52a
A3	3.72±0.67a	3.48±0.82a	3.60±1.00a	2.96±0.88b	3.44±0.86a

Note: Different letters in the same column show significantly different values (P <0.05)
Control : no addition of tapioca flour
A1 : 5% addition tapioca flour
A2 : 10% addition tapioca flour
A3 : 15% addition tapioca flour

DISCUSSION

Food processing can increase or decrease the nutritional value of foods. In this study, the contents of ash and protein decreased, and the amino acid content also decreased with addition of tapioca flour. It was inversely related to the carbohydrate and fat nutrients, which increased due to processing. The nutritional components and amino acids from white shrimp jerky changed due to food processing, cooking time and temperature, the addition of spices (ginger, tamarind and galangal), in the presence or absence of filler. William and Phillips (2000) stated that the chemical composition of meat depended on animal species, animal condition, carcass meat type, preservation process, storage and packing method. The chemical composition of meat is strongly influenced by its fat content. The content of fat in meat increased such that the protein and water contents decreased (William & Phillips, 2000). Jerky product is a high moisture food product, although these products in this study were heated for 6 h to prevent spoilage. Some studies of jerky have not only used seasoning but also increased drying time. This still resulted in an increase in the moisture of the meat product. In this study, the water content of shrimp jerky was decreased not only by water binding to other compounds but also by water release during the 6 h heating process.

The protein content decreased when the tapioca flour percentage increased (Table 2). This research agreed with studies by King (2002), Mohamed et al. (1989) and Yu (1991). However, their studies with varying percentages of addition differed in flour and spices. In Table 2, the fat content was less than 1% for all treatments. The fat content of tapioca flour is only 0.1% (Yu, 1991). However, protein could be maintained as the main component of shrimp jerky. The method to produce a more nutritional food product was to adjust carbohydrates and protein, either lower or higher.

The physicochemical characteristics of white shrimp jerk also changed after adding tapioca powder as a filler during processing (Table 3 and 4). The color and hardness of raw shrimp jerky increased, but sensory panelist judgments on the attributes of color, taste, aroma, and appearance of raw shrimp jerky did not differ significantly between treatments (Table 5). The hardness properties of shrimp jerky were different. The texture of raw shrimp jerky (A0 as control) was softer because the hardness level was low. For sensory properties, regarding the texture of the shrimp jerky the panelist indicated that a preference for shrimp jerky with added tapioca flour not exceeding 10% over A0 as the control (Table 5). The addition of tapioca as a binder made the texture of shrimp jerky more elastic than the control. Beef jerky is generally chewier than shrimp jerky (Ashlan et al., 2010; Kim et al., 2014).

The hardness values can be associated with the sensory evaluation of the shrimp jerky texture. A study on the effect of using flour as a binder or filler on the sensory properties of shrimp jerky showed that the texture of the shrimp jerky was acceptable by panelists (Chu-Ja & Cha-Sung, 2007). The higher quantity of tapioca flour used beyond 10%, the less panelists preferred the texture of shrimp jerky produced. This may be caused by the difficulty in cutting the jerky and the hardness when eaten.

Type and quantity of flour could influence texture, for example hardness, crispiness, and chewiness (Han *et al*., 2008). Texture is influenced by water content, fat content and type of filler, plus the morphological properties of the product (Carr et al., 1997). The function of filler (flour or starch) is to bind water. Subsequent binding of water can reduce the hardness of shrimp jerky, similar to the control, A0 (Table 3). These results agree with those of Yang et al. (2009) who reported that the moisture content affected the shear force or hardness.

Color and appearance are the most important attributes of food quality for users to enable them to visually detect the rate of quality damage and deterioration (Mardiah et al., 2010). The color of a food product is an index of quality in relation to aspects of damage and deterioration of fresh or cured product nutritious food, those with good texture and other appropriate features will not be eaten unless they have suitable and attractive colors (Pegg et al., 2006). The surface color values of shrimp jerky can change according to the drying time at the same temperature, i.e., the Hunter L-values and a* and b* values increased

with increasing of drying time up to 6 h. However, in the present study, because the same drying conditions, such as temperature (60^{o}C) and time (6 h), were applied to the treatments, the change in jerky color was also caused by tapioca powder. Peralta et al. (2008) noted that the brown color intensity of shrimp jerky increased with increasing of the tapioca percentage. In the present study, tapioca flour caused a steep increase in the surface color values, especially a* (redness), and b* (yellowness). The shrimp flesh replaced with 5% (A1), 10% (A2), and 15% (A3) tapioca flour experienced increased a* and b* values compared with the control. The variation in protein content was responsible for the degree of increase in the L* value. The increase in the L*value was higher in samples with decrease in the ratio of shrimp flesh.

The use of tapioca as a filler could significantly change the value of L*,a* and b*, increasing the texture value during the later stage of drying. There is a similar trend that is seen with unfried samples: increasing the tapioca flour but decreasing the shrimp flesh amount will contribute lighter color to the product, as shown by the higher L*value. Shrimp flesh has some pigments that contribute more to the color of the shrimp jerky, than does the color of tapioca flour. There are browning reaction between proteins and carbohydrates, called the Maillard reaction. In this study, the colored compounds formed due to H_2O released from amino acids (King, 2002). The difference in the myoglobin of meat could make the color of the product different. The light red color of myoglobin was kermesinus, oxymyoglobin, but taupe brown was from metmyoglobin. Actually, the drying process could also oxidize myoglobin. The research of Xiaolei et al. (2015) showed that hot air drying sometimes could not improve jerky color.

There are several factors that can cause variation in the water binding capacity of meat such as: pH, maturation treatment, cooking or heating, and biological factors such as muscle type, animal type, gender and livestock age. Similarly feeding factors, transport, temperature, humidity, storage and preservation, animal health, precutting and intramuscular fat treatment also affected WHC. Water binding capacity by a meat protein also called Water Holding Capacity (WHC), is defined as the ability of the meat to retain water and the influence of that on strength, such as effects on meat cutting, heating, grinding, and pressure. Meat also has the ability to absorb water spontaneously from water-containing environments (water absorption).

There are three forms of water bonds in muscle. Water that is chemically bonded by a muscle protein of 4 - 5% is as the first monomolecular layer. The two water bonds are rather weak as the second layer of water molecules to the hydrophilic group, amounting to approximately 4% this second layer is bound by the protein when the vapor pressure increases. The third is a layer of free water molecules between protein molecules, with a magnitude of about 100%. Denaturation of proteins will not affect the molecular changes in bound water (first and

second layer), while the free water between the molecules will decrease when the meat protein is denatured (Bourne, 1978; Carr et al., 1997).

The muscle proteins with extremely high water holding abilities are firm, with a tight structure, and a dry or sticky texture. Conversely, the network with low water binding ability has a soft, open structure (loose) and the texture is wet or seeded. The differences between intracellular water equilibrium in the first case and extracellular water in the latter case were related to water-binding ability. This is because tapioca flour contains carbohydrates, especially starch and cellulose in large amounts. The high carbohydrate content in food will cause free bonded water to be easily released because the water is physically bonded, followed by a decrease in the WHC of shrimp jerky (Table 4). The addition of more starch or flour should increase water absorption because of the hydroxyl group (OH) that can bind water (hydrophilic). Nevertheless, amylose in the material could bind water and will be more easily released during drying, as shown in the A3 treatment.

Zhuang et al. (2007) stated that the number of hydroxyl groups containing starch was large enough to have a great ability to absorb water. This hydrogen bond has not only bound water molecules to one another, but also water and other compounds with elements of O or N, such as carbohydrates with OH (hydroxyl) groups (Rabah & Abdalla, 2012; Suryati et al., 2014). Although, the amount of carbohydrate in the tapioca flour has the ability to bind water, it cannot emulsify fat (Rabah & Abdalla, 2012; Singh et al., 2001; Zhuang et al., 2007).

SEM data showed that, compared with the control, tapioca as a binder could improve the myofiber shape in jerky because the shrinkage of myofiber and sarcomere was reduced. The water on the surface of and inside was spread evenly, which could improves jerky texture. Cheow et al. (2004) studied the microstructure of shrimp jerky. In their research, they found that poorly-expanded shrimp jerky contained large aggregates of added flour. This prevented the starch from expanding in hot cooking oil, and made the filaments denser and thicker. Therefore, the final result was an increase in the hardness value of the jerky (Table 4). Water in starch grains could not move freely because the molecules were bound by the hydroxyl group in the starch molecules. Thus, cavities in the shrimp jerky were observed for jerky with added tapioca flour, compared to the control treatment (Figure 1). Furthermore, starch granules can swell excessively and irreversibly. The higher amounts of tapioca flour in the shrimp jerky formed more gaps or cavities in jerky product.

For the sensory data, this study did not show any significant improvement in color, taste, flavor, texture and palatability with the treatments used. The panelist reacted the same to the shrimp jerky, with and without added tapioca flour. This means that tapioca flour not exceeding 10% could be used to make shrimp jerky. If the addition is higher than that, the shrimp jerky will

very hard, observed in the A3 treatment. The sensory analysis could also determine the hardness, commonly by using the terms for crispiness. A low crispiness score will show high hardness. All the sensory data showed that the panelists could still accept the shrimp jerky product with the addition of flour not exceeding 10%. Mohamed et al. (1989) reported that fish crackers with lower hardness values along with rising linear expansion would decrease the hardness score of fish crackers. Similar to the shrimp jerky without tapioca powder in this research. To improve the physical characteristics of white shrimp jerky, this study can offer a better theoretical foundation for the application of tapioca as a filler on shrimp jerky to improve the physical and sensory characteristics, compared to shrimp jerky without tapioca flour.

CONCLUSION

The addition of tapioca flour as a filler at a rate less than 15% could improve the color, texture, and sensory properties of white shrimp jerky, although the characteristics did not differ significantly between treatments. For the nutrient component, the addition of tapioca powder decreased the ash, protein, and amino acid contents. However, the shrimp jerky could still maintain shrimp as the main source of protein. There was a negative correlation between WHC and hardness properties after the addition of tapioca flour. Overall, 5% tapioca flour had been added to white shrimp jerky, showing not only low hardness properties but also high protein, similar to shrimp jerky without flour (control treatment). This research can offer better options for using tapioca flour as a binder or filler in shrimp jerky. Thus, this study could be utilized by producers and fishermen for making jerky from white shrimp with or without tapioca as a versatile food product for consumption.

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Effects of Gibberellic and Abscisic Acids on Germination and Seedling Growth of Okra (*Abelmoschus esculentus* L.) under Salt Stress

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ABSTRACT

This study aimed to evaluate the effect of gibberellic acid (GA_3) and abscisic acid (ABA) on the germination of okra seeds (*Abelmoschus esculentus* L.) exposed under salinity constraint. In the first part, seeds were germinated in hormonal solutions of GA_3 (5 µM and 10 µM) and ABA (5 µM and 10 µM), in the presence of NaCl (100 mM). Results showed that NaCl reduced significantly the precocity of seeds germination without influencing its final rate; contrarily, it had a negative effect on hypocotylar growth, fresh weight and seedlings water content. The application of GA_3 treatment attenuated the depressive effect of NaCl on germination by stimulating it from the first day of sowing (after 24 h) with 20% and 26.66% under the effect of 5 µM and 10 µM of GA_3 respectively. The findings showed that, this phytohormone seemed positively influencing the hypocotylar length, fresh and dry weight as well as the water content of the okra seedlings. On the contrary, ABA was not effective in inducing tolerance to salinity.

Keywords: ABA, GA_3, germination, NaCl, okra, salinity

INTRODUCTION

In their natural environment, plants are subjected to a large number of environmental constraints of biotic and abiotic stresses that will influence their growth and development (Chen et al., 2015). By 2025, global food production will need to increase by about 38% and 57% by 2050 (Wild, 2003). Most of the appropriate land has been cultivated and expansion into new areas to increase

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food production is practically possible or desirable. Additional efforts are needed to improve productivity as more and more land is degraded (Rengasamy, 2006). It is estimated that about 15% of the world's total land area has been degraded by soil erosion and physical and chemical degradation, including soil salinization (Wild, 2003).

Soil salinity is one of the major abiotic factors limiting agricultural production in arid and semi-arid regions. Nearly 400 million hectares of land is affected by salinization, 80% of which is of natural origin and 20% of anthropogenic origin (Food and Agricultural Organization [FAO], 2015) with a resultant monetary loss of 12 billion US$ in agricultural production (Shabala, 2013). In Algeria, 3.2 million hectares of land is affected by salinity (Belkhodja & Bidai, 2004). This salinity is due mainly to the presence of high amounts of minerals such as Na^+ and Cl^- in soils and water (Tavakkoli et al., 2010) which affects crop growth and development through many diverse pathways, including water stress, nutritional disorders, ion toxicity, oxidative stress, alterations to metabolic processes, cell membrane disorganization, and reduced cell expansion and division (Hanin et al., 2016; Munns, 2002; Teige et al., 2004; Zhu, 2002, 2016).

Okra (*Abelmoschus esculentus* L. Moench.) is a popular vegetable crop belonging to Malvaceae family which is widespread in the tropical, subtropical and Mediterranean regions (Abid et al., 2002). This crop has high nutritive value, and it is used as a vegetable very rich in vitamin C and calcium. The productivity of this crop in Algeria is limited because of the lack of knowledge of its pedoclimatic requirements and its adaptation especially to salty soils. Since this vegetable is often grown under irrigation, especially where dry summers are more common, principally in the Mediterranean countries, it can be subjected to salt stress (Habib et al., 2016). Salinity retarded the growth, yield and physiological growth parameters of okra (Achour, 2016; Ayub et al., 2018; Ifediora et al., 2014).

Germination is considered as a critical step in plants development cycle; indeed, it prepares the seedling's installation, its adaptation to the environment and its subsequent productivity (Zhu, 2016). In saline environments, high salt levels impair seed germination and emergence in both glycophytes such as okra (*Abelmoschus esculentus* L.) (Achour, 2016; Dkhil et al., 2014), wheat (*Triticum durum* (Desf.)) (Otu et al., 2018), broad bean (*Vicia faba* L.) (Anaya et al., 2015), rice (*Oryza sativa* L.) (Liu et al., 2018), cowpea (*Vigna unguiculata* L. Walp.) (El-Shaieny, 2015) and halophytes (Gul et al., 2013, Haraguchi & Matsuda, 2018) and perturbs the hormonal balance in plants (Atia et al., 2009). For that, the treatments that can reduce the effect of salinity during this phase will be essential.

In the last two decades, physiological treatments such as hormone inputs have been intensively studied, assuming that the depressive effect of salinity on germination could be related to either a decrease or an increase in endogenous hormone levels

(Bahrani, 2015). Reduction in amounts of phytohormons especially of cytokinins and gibberellic acid and increase in the abscissic acid (Wang et al., 2001), in several species of plants, under salt and drought stresses, have been reported (Cao et al., 2014; Mizrahi et al. 1971; Zhang et al. 2006). Under saline stress, ABA can improve water permeability (Glinka & Reinhold, 1971) and induces late-embryogenesis abundant proteins (LEA proteins), and the osmolyte biosynthesis (Xiong et al., 2001).

Gibberellic acid plays an important role at different stages of plant development from germination to flowering; it participates in the lifting of seed dormancy, growth of stems and leaves, and flower development (Gupta & Chakrabarty, 2013; Yamaguchi, 2008).

Baskin and Baskin (2014) reported that gibberellins were often used to overcome seed dormancy, and could significantly improve seed germination in many species, mainly through the activation of embryo growth, mobilization of reserves, and weakening of the endosperm layer.

The GA_3 is the most active to attenuate the inhibitory effect of salinity on germination, by increasing nutrient uptake, dry weight, seedlings growth under saline conditions (Bahrani & Pourreza, 2012; Tsegay & Andargie, 2018; Waleed et al., 2019). It has also been proven that the growth of wheat, rice, cotton and some halophytes has been significantly improved in the presence of GA_3 under salt stress (Colebrook et al., 2014; Javid et al. 2011; Iqbal & Ashraf, 2013). Such improvement can be attributed to increased level of some endogenous GA_3 with a concurrent decrease in level of ABA and improvement of ions uptake under salinity-stressed conditions.

For this purpose the exogenous application of GA_3 on germination and seedling growth in saline conditions offers an attractive approach to alleviate the harmful effects of salt stress.

Therefore, the objectives of this experiment were to study the effects of gibberellic acid (GA_3) and abscisic acid (ABA) on the germination and seedlings growth of okra seeds under salinity constraint.

MATERIALS AND METHODS

Vegetal Material

Seeds were harvested in July 2012 from the fruits of okra (*Abelmoschus esculentus* L.) that was grown on a parcel of agricultural land.

Seed Preparation for Germination Tests

Seeds were disinfected with 2% of sodium hypochlorite during 3 minutes, rinsed thoroughly with distilled water and then dried on filter paper and placed in Petri dishes lined with two layers of sterile filter paper. Each test was carried out on 30 seeds which made 3 repetitions of 10 seeds per Petri dish. Control seeds received 10 ml of distilled water and the same volume of the different saline and hormonal solutions: NaCl (100 mM), ABA (5 and 10 µM), GA_3 (5 and 10 µM), ABA / NaCl, GA_3 / NaCl was added to the treated seeds.

The Petri dishes were put in an incubator that was set at 28°C. Daily, a count of germinated seeds was carried out for a week. The germination was marked by the exit of the radicle out of the seed's coat.

During this test the following parameters were studied:

The Precocity of Germination. Represented by the rate of the first germinated seeds, so be after 24 hours of sowing (Belkhodja & Soltani, 1992).

Final Rate of Germination (Tg). This parameter is the best way to identify the germination ability of the seeds under the different treatments used. It was calculated after 7 days from sowing date and expressed as percentage according to the equation as described by (Kandil et al., 2012). It is expressed as the ratio of the percentage of germinating seeds (Ni) to the total number of seeds recorded at the end of the test (Nt).

$$Tg = Ni \times 100 / Nt$$ (Tg: germination rate)

Hypocotyl Length. This parameter is a good step to evaluate the salt stress effect and the hormonal treatments effect on the hypocotyl's growth, this length is estimated using a caliper at the end of the germination test (after 7 days of sowing) (International Seed Testing Association [ISTA], 1996).

Water Content (WC %). The water content of the seedlings is determined by the calculation of seedlings fresh weight (FW) of the before drying them in the oven at 80°C during 48 hours. The dry weight is then determined (DW) and the water content is calculated by the following equation: WC (%) = (FW-DW/FW) * 100 (Chen et al., 2009).

Each treatment was replicated three times and results were statistically analyzed by calculating variance and the standard error (Sx) of the mean. Statistical analysis was performed based on SPSS (version 17.0) program. In order to detect the significance of differences ($p<0.05$) of variables, a multiple comparison (LSD) test was performed.

RESULTS

Precocity of Germination

In Different Hormones Concentration. The LSD test indicated significant effects of GA_3 on the germination precocity ($p<0.05$); the first germinations occurred from the first day of sowing for the control seeds and those treated with GA_3. It should be noted that the germination rate increased considerably for the seeds receiving the hormonal solution at 5 and 10 µM of GA_3 with respective levels 76.66% and 83.33%. On the other hand, significant effect of ABA on germination was noted ($p<0.05$), Indeed, any germination was observed on seeds under the ABA treatment during the first 48 hours; beyond that, the seeds germination appeared in a slow way compared to those treated with GA_3 (Figure 1).

In Different Hormone and NaCl Combination. Analysis of variance results indicate significant effect of NaCl and NaCl/hormone on okra seeds germination ($p<0.05$) compared to control seeds. Under 100 mM, the seeds germinate only from the second day of sowing. When the seeds receive the GA_3 at 5 and 10 µM associated with the saline solution, the germination starts the first day of sowing with respective rates of 20% and 26.66% (Figure 1). On the other hand, a very pronounced germination delay is recorded in the seeds treated with ABA.

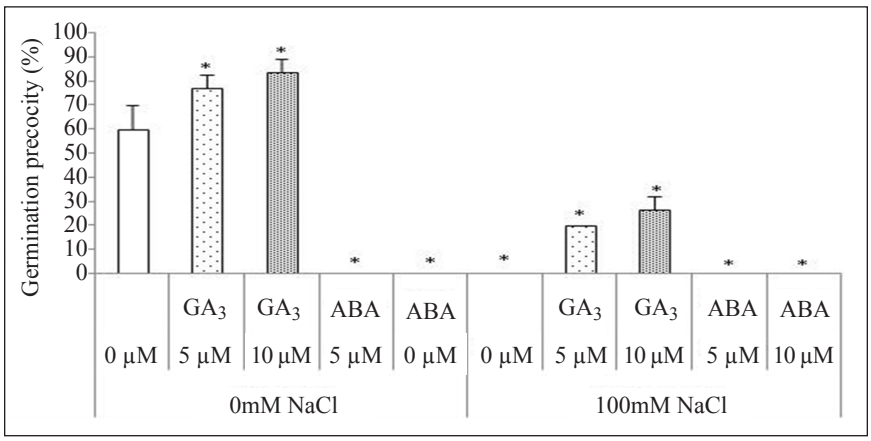

Figure 1. Effect of hormone/salinity interaction on germination precocity (%)

Table 1
Values are mean and standard errors of measurement made on three replicates. Asterisks within the means of each column (*) indicate significant difference among the means (LSD test, p <0.05) compared to control

Treatments	Germination precocity (%)	Final rate of germination (%)	Hypocotyl length (cm)	Seedlings fresh weight (g)	Seedlings dry weight (g)	Seedlings water content (%)
Control	60±10	100	8±1.11	0.46±0.04	0.03±0.003	93.26
5µM GA_3	76.66±5.77*	$100^{(ns)}$	$8.1±0.95^{(ns)}$	$0.48±0.03^{(ns)}$	$0.032±0.002^{(ns)}$	$93.33^{(ns)}$
10µM GA_3	83.33±5.77*	$100^{(ns)}$	12±0.98*	0.58±0.06*	$0.035±0.002^{(ns)}$	$94.03^{(ns)}$
5µM ABA	0*	$93.33±5.77^{(ns)}$	0.28±0.03*	0.07±0.005*	$0.03±0.004^{(ns)}$	55*
10µM ABA	0*	76.66±5.77*	0*	0.07±0.01*	$0.03±0.003^{(ns)}$	55.68*
100 mM NaCl	0*	$96.66±5.77^{(ns)}$	1.25±0.5*	0.14±0.04*	$0.03±0.006^{(ns)}$	77.93*
5µM GA_3/ NaCl	20*	$100^{(ns)}$	$8.47±0.73^{(ns)}$	$0.45±0.06^{(ns)}$	$0.035±0.005^{(ns)}$	$92.22^{(ns)}$
10µMGA_3/ NaCl	26.6±5.77*	$100^{(ns)}$	10.4±1.39*	$0.58±0.06^{(ns)}$	$0.036±0.003^{(ns)}$	$92.96^{(ns)}$
5µM ABA/ NaCl	0*	23.33±11.54*	0 *	0.06±0.01*	$0.03±0.002^{(ns)}$	52.34 *
10µMABA/ NaCl	0*	6.66±11.54*	0*	0.06±0.005*	$0.03±0.002^{(ns)}$	44.92*

Note: $^{(*)}$ signifiant at 0.05 level; $^{(ns)}$ non-signifiant

Final Rate of Germination

In Different Hormones Concentration. Any significant effect of GA_3 treatment was noted on final rate of germination (Table 1). Indeed, a final rate of 100% was recorded in the control seeds and those irrigated with GA_3. This rate decreased to 93.33% in the presence of ABA at 5 µM while the lowest rate was recorded in seeds receiving 10 µM ABA with 76.66% sprouted seeds.

In Different Hormone and NaCl Combination. The final rate of germinated seeds reached 93.33% then it increased until all of the seeds were germinated under the combination of GA_3 with NaCl solution (Figure 2), on the other hand, the combined ABA/ NaCl treatments (5 and 10 µM of ABA) decreased significantly final rate of germination with respective rates of 23.33% and 6.66%.

Hypocotyl Length

In Different Hormones Concentration. The hypocotylar length was positively influenced by the presence of GA_3 in the imbibing medium; in fact GA3 increased significantly ($p<0.05$). The highest length was recorded in seeds receiving 10 µM of GA_3 (12 cm) followed by control seeds and those receiving 5 µM of GA_3. In addition, it should be noted that treatment with ABA acted by slowing the hypocotylar growth (Figure 3).

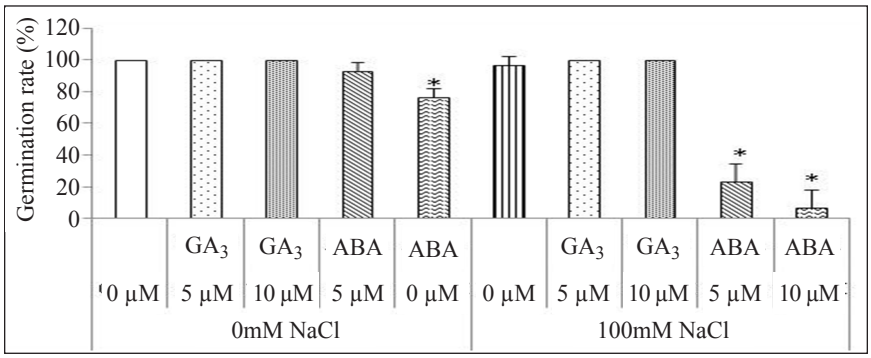

Figure 2. Effect of hormone/salinity interaction on the final rate of germination (%)

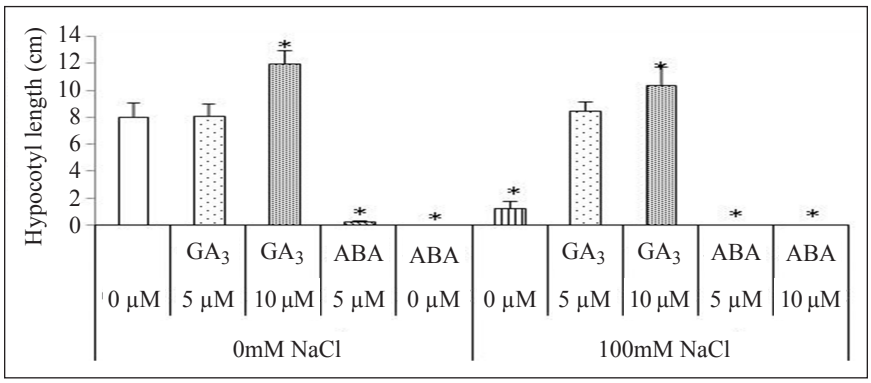

Figure 3. Effect of hormone/salinity interaction on the hypocotyl length (cm)

In Different Hormone and NaCl Combination. The hypocotyl's growth was slowed down, whereas the treatment with GA_3 (10 and 5 µM) associated with NaCl had positively affected this parameter so that the respective lengths varied between 10.4 cm and 8.47 cm. On the other hand, it should be noted that the growth of the hypocotyl was stopped in the seeds subjected to the treatment ABA/NaCl which was confirmed by the statistical study showing a significant effect of combined treatment ABA/salt on the hypocotyl growth of okra seedlings ($p<0.05$).

Seedling Fresh and Dry Weight

In Different Hormones Concentration. The FW seems positively influenced by the GA_3 effect. Thus, the highest FW was noted in treated seeds at 10 µM GA_3 (0.58 g). In contrast, the seeds exposed to ABA record a relatively low FW (0.07 g).

On the other hand, it should be noted that the DW did not seem to be influenced by the different treatments. Indeed, statistical analysis using the LSD test showed no significant effect of the different treatments compared to the control on the dry weight of seedlings ($p>0.05$) except for GA_3 at 10 µM. The DW of seedlings oscillated between 0.03 g and 0.035 g (Figure 4).

In Different Hormone and NaCl Combination. The fresh weight of the seedlings decreased significantly (0.14 g). On the other hand, it amounted to 0.45 g in seeds receiving GA_3 at 5 µM combined with NaCl and reached up to 0.58 g in the presence of 10 µM. The treatment with ABA at 5 µM and 10 µM combined with NaCl induced a significant reduction (($p<0.05$) of FW (0.064 g and 0.061 g) (Figure 5).

It should be noted that the DW did not seem to be influenced by the different treatments. In fact, the dry weight of seedlings varied between 0.03 g and 0.036 g.

Seedlings Water Content

In Different Hormones Concentration. The highest water contents were recorded in control seedlings and those treated with gibberellic acid with a maximum value of

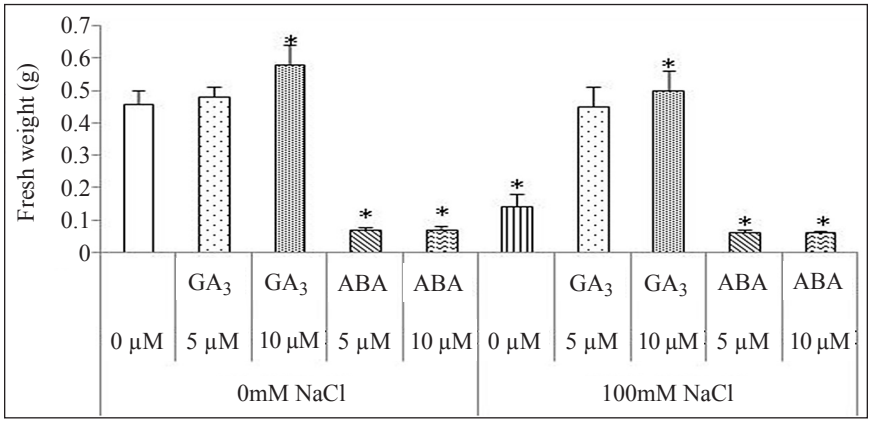

Figure 4. Effect of hormone/salinity interaction on seedlings dry weight (g)

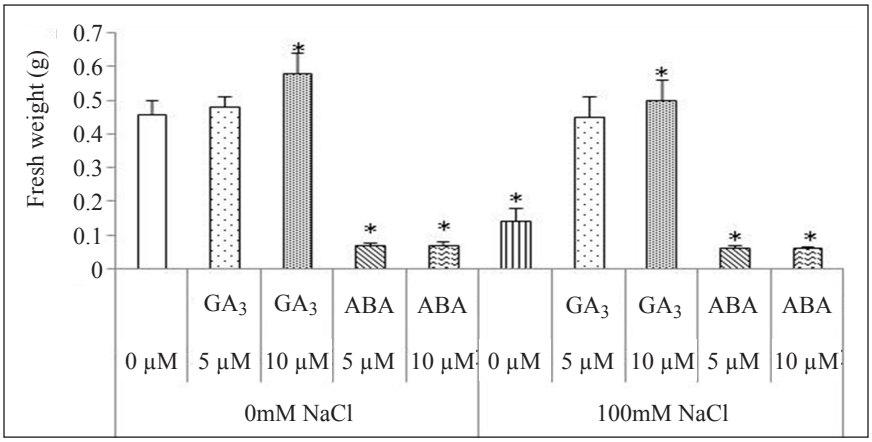

Figure 5. Effect of hormone/salinity interaction on seedlings fresh weight (g)

94.03% in the presence 10µM GA_3. The application of the ABA at 5 µM and 10 µM decreased significantly water content at 55% and 55.68% respectively.

In Different Hormone and NaCl Combination. The water content of seedlings reached only 77.93%, and increased to 92.22% and 92.96% in the presence of GA_3 in saline solution. However, the application of NaCl in combination with ABA greatly reduced the seedlings' water content (Figure 6), which was confirmed by the LSD test which showed significant effect of combined treatment ABA / salt on the water content of okra seedlings ($p<0.05$).

DISCUSSION

The results of this study showed that the application of salt stress at 100 mM significantly reduced the precocity of okra seeds germination without affecting its final rate. This delay of germination caused by this level of salinity is due to a difficulty of seeds' hydration because of a high osmotic potential and can be

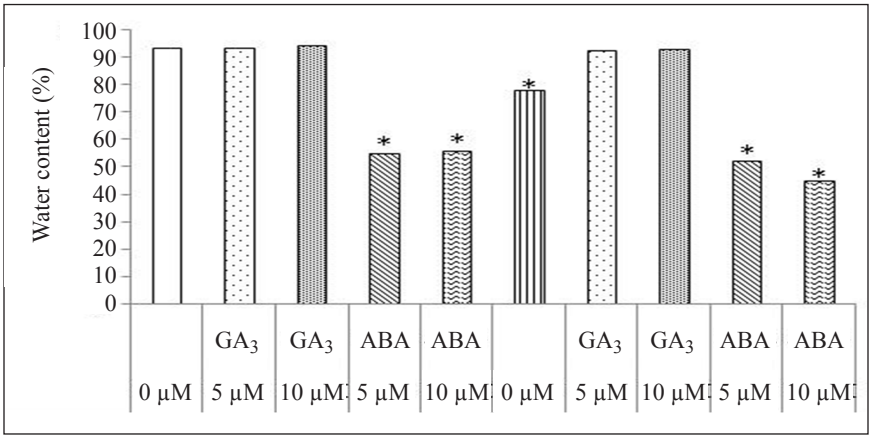

Figure 6. Effect of hormone/salinity interaction on seedlings water content (%)

explained by the time necessary for the seeds to trigger the mechanisms allowing them to adjust their osmotic pressure (Diallo et al., 2013; Ouis et al., 2015), which was also reported by Zemani (2009). Results showed that the NaCl acted negatively on the hypocotylar length and seedlings water content (Table 1), indicating that NaCl affected not only germination rate but also the seedlings' growth. Similar results have been highlighted by Bahrani and Pourreza (2012) on wheat. According to Werner and Finkelstein (1995), high salinity can inhibit the lengthening of roots and hypocotyls by slowing water absorption by the plant.

During seeds germination and seedling development stages, salinity negatively regulates GA biosynthesis (Horvath et al., 2015; Zhang et al., 2016). Shu et al. (2017) reported that NaCl delayed soybean seed germination by negatively regulating gibberellin (GA) while positively mediating abscisic acid (ABA) biogenesis, inducing a decrease in the GA/ABA ratio.

This study showed that the application of GA_3 at 5 µM and 10 µM in the 100 mM saline solution modified the responses of the seeds in particular the precocity and the final rate of germination which corroborates with the work of Chouhim (2011) on the same species and Samad and Karmoker (2012) on triticale seeds. Similarly, this phytohormone has a positive effect on hypocotylar growth, fresh and dry weights and water content of seedlings. The same results were reported by Turkyilmaz (2012) on soft wheat, Abdel-Hamid and Mohamed (2014) on barley, Achour (2016) on okra and Waleed et al. (2019) on wheat.

Under salt stress, gibberellins induce the lifting of physiological seeds' dormancy, increase water absorption and stimulate the synthesis and the activation of hydrolytic enzymes mainly α-amylase, thus releasing the reducing sugars and amino acids that are essential for embryo development (Ajmal Khan et al., 2004). Liu et al. (2018) demonstrated that NaCl treatment significantly reduced the activity of α-amylase and the germination rate of rice seeds. These effects can be mitigated by exogenous GA_3 during germination of rice. In addition, they found a positive relationship between bioactive GA content and α-amylase activity and between α-amylase activity and germination rate of rice seeds. Il the other side, gibberellins improve seeds germination by inhibiting ABA activity by either activating the enzymes involved in its catabolism or by blocking the pathway of its biosynthesis (Miransari & Smith, 2014).

In parallel, the application of ABA associated with NaCl on okra seeds does not seem to attenuate the depressive effect of salinity on the studied parameters. This observation expresses the inhibitory effect of ABA on germination was already confirmed by the work of Thakur and Sharma (2005). However, this ABA acts by limiting water absorption and inhibiting the synthesis of germination-specific enzymes such as α-amylase by counteracting the stimulatory effect of GA_3 on them (Kondhare et al., 2014). The decrease in germination rate observed under ABA treatment can be attributed to the induction of secondary dormancy and the

inhibition of seeds germination by limiting the availability of energy and metabolism (Leymarie et al., 2008). Besides GA, ABA also plays an important role in regulating seed germination. It was recognized that GA and ABA antagonistically regulate seed germination (Li et al., 2016; Shu et al., 2016), and NaCl inhibited soybean seed germination by decreasing the ratio of GA/ABA via decreased bioactive GA and increased ABA contents (Shu et al., 2017).

CONCLUSION

During seeds germination stage, the presence of NaCl at 100 mM negatively affected the precocity, but did not appear to influence the final rate of sprouted seeds. However, at the seedlings growth phase, this saline concentration significantly reduced seedlings growth and their development. The hormonal supply of GA_3 increased the seeds germinal capacity in the presence and in the absence of NaCl, moreover this phytohormone positively affected the fresh weight, the hypocotylar length and the water content of okra seedlings under the two tested concentrations. On the other hand, the presence of ABA in the imbibing medium did not improve the response of the seeds under salt stress, in particular under the concentration of 10 µM of ABA.

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Nest Characteristics of Stingless Bee *Heterotrigona itama* (Hymenoptera: Apidae) upon Colony Transfer and Splitting

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ABSTRACT

Stingless bees are in a diverse group of highly eusocial bees (meliponines) which are reared for their products and pollination potentials. The main source of stingless bee colonies is depending on feral colony hunting that potentially affects the ecosystem. Therefore, establishment of a good practise in colony transfer and splitting may extend the survival of a colony in a new location. This study describes a colony transfer strategy from log to hive and nest characteristics of stingless bee *Heterotrigoma itama* upon colony splitting from the hive. All 15 colonies from the hollow trunks of trees could survive after one month of transfer to hives. Pre-colony splitting data of 10 daughter colonies showed that the height of brood cells was 14.5 ± 1.20 cm, the number of brood layer was 8.9 ± 2.13, while the number of queen cell was 5.2 ± 1.32. After 12 weeks, the height of brood cells became 16.42 ± 1.05 cm, while the numbers of brood layer and virgin queen eggs were increased to 12.1 ± 1.85 and 6.4 ± 1.65, respectively. However, the correlation between the number of brood layer and virgin queen egg was significantly low (r = 0.421). These results show the ideal strategy for colony transfer from log and colony splitting, and the number of brood layer does not correlate to the presence of virgin queen egg which is crucial for survival of the colony.

Keywords: Colony splitting, *Heterotrigona itama*, stingless bee

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INTRODUCTION

Stingless bee (Meliponini) are eusocial insects which live in a colony consist of a single queen which controls thousands of workers. Similar to honeybee (*Apis*), stingless bee exhibits complex social, navigational and communication behaviours (Kärcher et al., 2013). In social insect colonies, the duration for the colony development process will influence the community expansion together with the substitution of the workers. These will affect the colony's ability to assemble and supply nutrient assets, which will then influence the colony's condition, dominance and duration to survive (Halcroft et al., 2013). The duration of life for an insect is mostly based on food uptake (Haydak, 1970), thus proper nursing in the early stage of life will increase the lifespan of juvenile stingless bee (Carey, 2001). Unlike Apini, the slower digestion of food by Meliponini larvae may affect its colony growth rate (Moo-Valle et al., 2004).

Meliponini can easily be found in tropical regions globally (Heard, 1999). In Malaysia, about 45 species of stingless bee were reported until 2014 (Jaapar et al., 2016). The *Heterotrigona itama* and *Genotrigona thoracica* are among the main species being reared individually or commercially (Kelly et al., 2014; Mustafa et al., 2018). As the valuable physicochemical, antioxidant and antimicrobial properties of the Malaysian stingless bee honey have gradually been recognized recently (Bakar et al., 2017; Ranneh et al., 2018; Tuksitha et al., 2018), the demand has also increased. The current market price of stingless bee honey in Malaysia reaches RM85 per 150 g.

The name stingless bee portrays the underdeveloped stinger (Shackleton et al., 2015), which makes this species less harmful than the stinger bees. Due to this factor, meliponiculture or stingless bee beekeeping activity has become a trend in many tropical countries (Cortopassi-Laurino et al., 2006). Traditionally, the tree trunks which contain the wild colonies are cut and relocated to a bee farm. Besides promoting deforestation and destruction of forest due to illegal hunting for stingless bee logs (Sommeijer, 1999), keeping the colonies in their own tree trunks restricts the observation of the internal structure and condition. To achieve a successful domestication, a good practice in transferring the colonies from logs into artificial house (hive) and performing colony splitting or multiplication may help to sustain the ecosystem (Singh, 2013). Colony splitting requires proper skills and wide knowledge on the insects' behaviour as it involves appropriate time, season and ability of the personnel to identify the perfect condition and location of the colony to be split (Cortopassi-Laurino et al., 2006). The new colony that is obtained from a splitting technique is relatively weak and vulnerable towards its natural threat such as ants, as the nursery process upon splitting mainly involves ensuring sufficient food to the new colony, rather than protection (Jaffé et al., 2015).

A few bee hive constructs has been patented and utilised successfully (Quezada-Euan et al., 2001). However, scientific documentation on colony transfer and

splitting techniques and nest structure of economically-important stingless bee species of Malaysia, such as *H. itama,* is relatively scarce (Saufi & Thevan, 2015). Therefore, this study explores those limitations and analyses the nest characteristics of the colonies upon splitting.

MATERIALS AND METHODS

Selection of Hive and Colony

All procedures were carried out at a bee farm located at Tumpat, Kelantan, Malaysia (6° 8' 26.5''N; 102° 13' 15.9'' E). A total of 15 colonies of *H. itama* was used for colony transfer from logs to hives. The MUSTAFA-Hive used in this study was designed by Dr. Mohd Zulkifli Mustafa (Jalil & Roubik, 2017).

Colony Transfer Technique from Log to Hive

Data on nest entrance and height of tree trunk with wild colony by Kelly et al. (2014) were referred. The hollow tree trunks with wild colonies (n=15) were removed with care, accordingly (Kelly et al., 2014). The

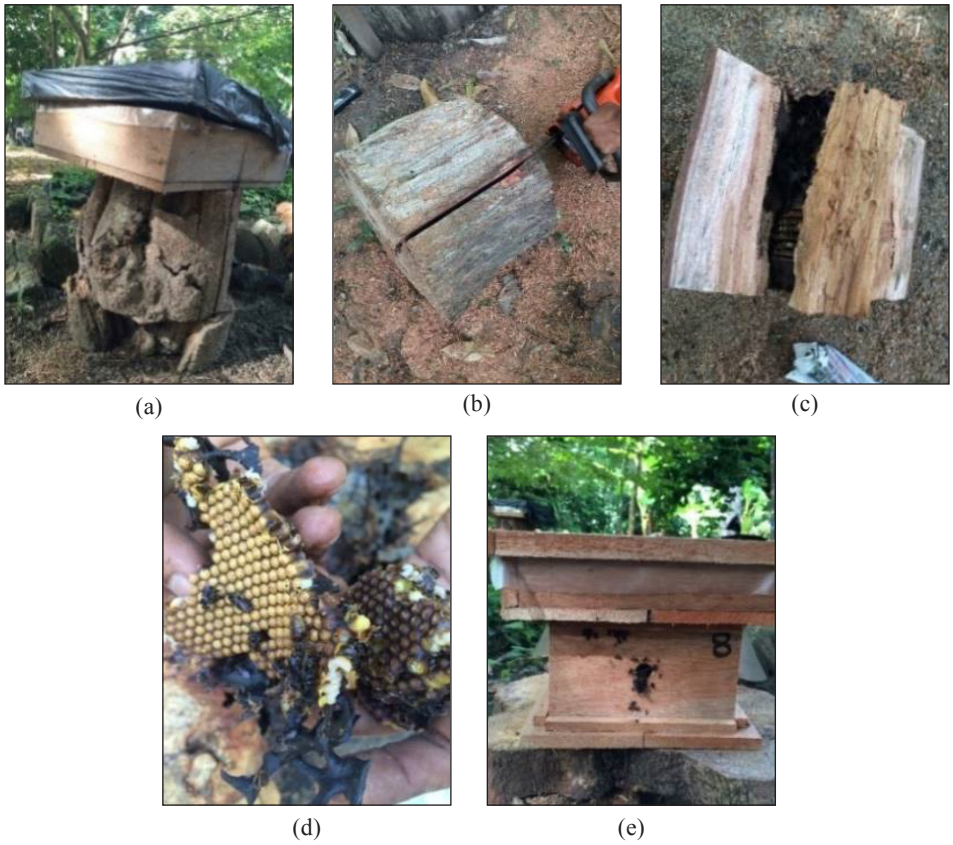

Figure 1. Colony transfer technique from logs to hives. (a) Log was cut and placed at designated farm. (b) Log was lined with a chainsaw. (c) After two weeks, log was twisted to expose the intact brood layers. The layers were transferred gently to a new hive. (d) Presence of egg-laying queen will ensure survivability of the colony. (e) The hive was placed at the previous location of the log

funnel entrance was sealed with clay at night and removed once the log had reached the designated farm. The log was left for at least two weeks to allow adaptation and repairing process by the bees. Next, the log was moved from the original location and lined using a chainsaw. The original location was replaced with a dummy box to avoid the foragers from being enraged of their missing nest. At the new location, the lined trunks were gently twisted to expose the intact brood layers which were then easily transferred to a new hive. This process was done meticulously to avoid destroying the queen or eggs. Presence of egg-laying queen will ensure survivability of the colony. Recovered honey and bee bread were removed to circumvent natural predators from attacking the fragile colony (Jaapar et al., 2016). The top space inside the hive was sealed with propolis to assist in colony repairing and survivability of the bees. Once done, the hive was placed at the previous location of the log (Figure 1). All colonies survived after one month of transfer to hives.

Colony Splitting Technique

Hives (n=10) were relocated from original location. The chamber was opened to expose the internal nest structure. Brood layers were removed and divided equally, i.e. the

Figure 2. Colony splitting strategy from one hive into two hives. (a) Hive was relocated from original location. (b) The hive chamber was opened. (c) Brood layers were removed and (d) divided equally. (e) Presence of egg-laying queen or virgin queen cells will ensure survivability of the colony. (f) Both hives were returned to the original location and stacked, where the old hive containing mother colony was placed at the top of the new, daughter colony (new hive)

old hive contained half of the old and new broods with the presence of egg-laying queen (mother colony), while the new hive contained half of the new and old broods with the presence of a virgin queen cell (daughter colony). The division of brood layer, either horizontally or vertically, depending entirely on the location of the new and old broods. Then, both hives were returned to the original location and stacked, where the old hive containing mother colony was placed at the top of the new, daughter colony (Figure 2). All colonies could survive upon 12 weeks of observation. Parameters in height of brood cells, number of brood layer and number of queen cell were recorded immediately (week 0) and biweekly for 12 after colony splitting.

Statistical Analysis

Statistical analysis was performed using one-way ANOVA, where each variable (height of brood cells, number of queen cell and number of brood layer) was analysed against one particular week. Correlation between the numbers of brood layer and queen cells was also performed. The data was transferred to graph pad Prism 7 for tabulation and analysis.

RESULTS AND DISCUSSION

Colony Transfer and Splitting Techniques

There are many ways to transfer or relocate a stingless bee colony, i.e. by colony expansion, drumming strategy or by using a dummy hive. In this study, the stingless bee colonies were transferred from logs to MUSTAFA-Hive. The bees, queen, broods, honey, bee bread and propolis were transferred immediately from the log after the inner structure of the log was exposed. The hive was closed and placed at the original location for colony repairing process by the bees. To facilitate the process, stingless bee honey was added in the hive regularly as extra food supplement for the bees. This technique requires less time for colony recovery compared to colony expansion or drumming strategies. A colony transfer is considered successful upon observation of new broods.

Colony splitting allows amplification of bee colonies which are reared in hives (Cortopassi-Laurino et al., 2006). The first well-documented colony splitting technique using stingless bee *Trigona carbonaria* reported an evenly split colonies, where the structure and the positioning of the brood chamber were well distributed (Heard, 1988). Amano (2004) showed colony splitting technique using stingless bees *T. carbonaria* and *Steatoda bipunctata*. The layer of the brood cells was divided equally and several large cells containing developing queen cells were scattered throughout the brood in the new colony. Compared to Amano (2004), our study recorded the number of queen cells distribution specifically and observation of these queen cell was made regularly. In addition, the position of the new and old hives was changed every week (top and bottom) to allow an equally active foraging activity of stingless bee worker and forager in both hives.

There were several parameters used to determine the readiness of colonies before splitting (data not shown); i) the minimum number of brood layers was more than ten, ii) there was a presence of egg-laying queen inside the colony, iii) the colony had actively performed the provisioning and oviposition process (POP), indicated by the full stocks of food and egg laying process by the queen, iv) the presence of a queen cell in the colony, and v) the broods inside the hive were full until they entered the honey cassette area. By placing the right amount of fully formed virgin queen cell inside the new hive, the chances of colony survivability will be high.

Nest Characteristics and Correlation between Numbers of Brood Layer and Queen Cell

Biweekly analysis of height of brood cells, number of brood layer and number of queen cells upon colony splitting were significantly different ($P<0.05$) except for height of brood on weeks 0 and 2 ($P>0.05$) (Table 1). The high value of standard deviation for numbers of brood layer and queen cell was due to the nature of the colony where the size and growth rate of each colony differs from one another. The height of brood cells increased over time upon colony splitting. For the first four weeks, the increment was slow as the workers were focusing in recovering the nest and ensuring enough food storage for the colony. This resulted in reduced number of brood layer during this stage. However, after four weeks, the colony has grown in its strength hence the gradual increase of height of brood cells, proportional to the number of brood layer, indicating a successful colony reconstruction.

Table 1
Biweekly analysis of height of brood cells, number of brood layer and number of queen cells upon colony splitting

Week	Parameters (mean ± SD)		
	Height of brood cells (cm)	Number of brood layer	Number of queen cell
0	14.5±1.20*	8.9± 2.13	5.2±1.32
2	14.5±1.22*	7.8±2.15	3.8±1.25
4	14.8± 1.19	6.7± 2.26	2.4±1.35
6	15.1± 1.13	8.5± 2.27	2.8±1.40
8	15.6±1.07	9.9± 2.23	4.0±1.76
10	15.9± 1.06	11.1±2.13	5.4±1.65
12	16.2± 1.05	12.1±1.85	6.4±1.65

Note. Analysis were performed to daughter colonies (n=10) and data were presented as mean ± standard deviation (SD). Significant differences among daughter colonies on a particular week (*), were determined by one-way ANOVA ($P<0.05$)

The number of queen cell in each colony which was reduced gradually within six weeks after colony splitting might reflect on the competency of workers. In honey bee *Apis mellifera*, it was found that workers regulate the queen rearing process by differentially constructing the cells (Hatch et al., 1999). After building different numbers of queen cells, over half (53%) of the constructed cells were destroyed by the workers themselves, in a non-random manner, prior to their emergence (Hatch et al., 1999). Therefore, a gradual increase of number of queen cells after six weeks, as shown by our study, indicating a positive gain of the workers aptitude towards colony survival and strength. The success of mother colonies were indicated by the presence of

new queen cells, addition of brood layers and the formation of new broods by the queen. However, the data was not recorded in detail.

Despite a proportionally inclined (α) pattern, it was a weak correlation between numbers of brood layer and queen cells where the correlation coefficient (r=0.421), was very close to 0 (Figure 3). These data suggest that a high number of brood layer does not represent an increase in number of queen cells. Hence, the survival of a colony could not be determined based on number of brood layer.

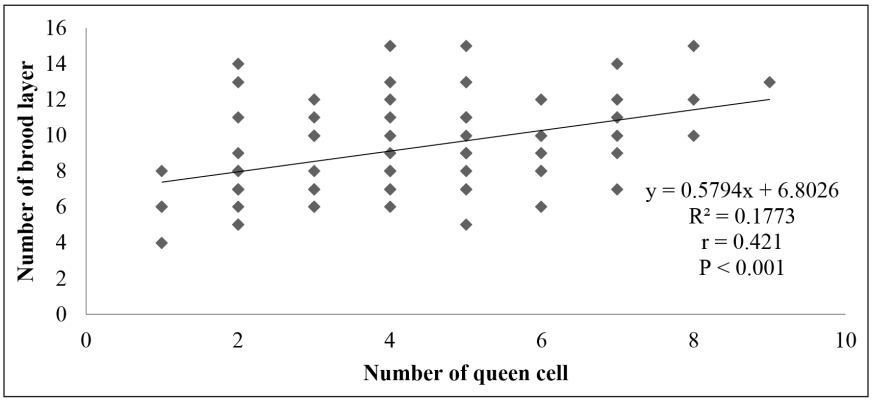

Figure 3. Correlation analysis between numbers of brood layer and queen cells

CONCLUSIONS

This study successfully described the colony transfer strategy from log to hive and the nest characteristics of height of brood cells, number of brood layer and number of queen cell for stingless bee *Heterotrigona itama* upon colony splitting. The colony transfer and splitting strategies proves to maintain survival of the daughter colony, which was indicated by the gradual increase of height of brood layer and number of queen cell. Judging from the weak correlation between numbers of brood layer and queen cell, further studies should consider the influence of workers in increasing the numbers of brood layer and queen cells for colony survival.

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CONFLICT OF INTEREST

None of the authors of this paper have any financial or personal relationships with other people or organizations that could inappropriately influence or bias the content of this paper.

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UTHM	*Universiti Tun Hussein Onn Malaysia*

While every effort has been made to include a complete list of referees for the period stated above, however if any name(s) have been omitted unintentionally or spelt incorrectly, please notify the Chief Executive Editor, *Pertanika* Journals at executive_editor.pertanika@upm.my

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Our goal is to bring high quality research to the widest possible audience

INSTRUCTIONS TO AUTHORS

(Manuscript Preparation & Submission Guide)

Revised: May 2019

Please read the Pertanika guidelines and follow these instructions carefully. Manuscripts not adhering to the instructions will be returned for revision without review. The Chief Executive Editor reserves the right to return manuscripts that are not prepared in accordance with these guidelines.

MANUSCRIPT PREPARATION

Manuscript Types

Pertanika accepts submission of mainly **four** types of manuscripts for peer-review.

1. **Regular article**

Regular articles are full-length original empirical investigations, consisting of introduction, materials and methods, results and discussion, conclusions. Original work must provide references and an explanation on research findings that contain new and significant findings.

Size: Generally, these are expected to be between 6 and 12 journal pages or not exceeding 6000 words (excluding the abstract, references, tables and/or figures), a maximum of 80 references, and an abstract of 100–200 words.

2. **Review article**

These report critical evaluation of materials about current research that has already been published by organizing, integrating, and evaluating previously published materials. It summarizes the status of knowledge and outline future directions of research within the journal scope. Review articles should aim to provide systemic overviews, evaluations and interpretations of research in a given field. Re-analyses as meta-analysis and systemic reviews are encouraged. The manuscript title must start with "Review Article:".

Size: Generally, these are expected to be between 8 and 12 journal pages or not exceeding 6000 words (excluding the abstract, references, tables and/or figures), a maximum of 80 references, and an abstract of 100–200 words.

3. **Short communications**

They are timely, peer-reviewed and brief. These are suitable for the publication of significant technical advances and may be used to:

(a) report new developments, significant advances and novel aspects of experimental and theoretical methods and techniques which are relevant for scientific investigations within the journal scope;

(b) report/discuss on significant matters of policy and perspective related to the science of the journal, including 'personal' commentary;

(c) disseminate information and data on topical events of significant scientific and/or social interest within the scope of the journal.

Size: These are usually between 2 and 4 journal pages and have a maximum of three figures and/or tables, from 8 to 20 references, and an abstract length not exceeding 100 words. Information must be in short but complete form and it is not intended to publish preliminary results or to be a reduced version of Regular or Rapid Papers.

4. **Others**

Brief reports, case studies, comments, concept papers, Letters to the Editor, and replies on previously published articles may be considered.

Language Accuracy

Pertanika **emphasizes** on the linguistic accuracy of every manuscript published. Articles must be in **English** and they must be competently written and argued in clear and concise grammatical English. Contributors are strongly advised to have the manuscript checked by a colleague with ample experience in writing English manuscripts or a competent English language editor.

Author(s) **may be required to provide a certificate** confirming that their manuscripts have been adequately edited. **All editing costs must be borne by the author(s)**. This step, taken by authors before submission, will greatly facilitate reviewing, and thus publication if the content is acceptable.

Linguistically hopeless manuscripts will be rejected straightaway (e.g., when the language is so poor that one cannot be sure of what the authors really mean). This process, taken by authors before submission, will greatly facilitate reviewing, and thus publication if the content is acceptable.

MANUSCRIPT FORMAT

The paper should be submitted in one column format with at least 4cm margins and 1.5 line spacing throughout. Authors are advised to use Times New Roman 12-point font and *MS Word* format.

1. Manuscript Structure

Manuscripts in general should be organised in the following order:

Page 1: *Running title*

This page should **only** contain the running title of your paper. The running title is an abbreviated title used as the running head on every page of the manuscript. The running title should not exceed 60 characters, counting letters and spaces.

Page 2: *Author(s) and Corresponding author information.*

This page should contain the **full title** of your paper not exceeding 25 words, with name(s) of all the authors, institutions and corresponding author's name, institution and full address (Street address, telephone number (including extension), hand phone number, and e-mail address) for editorial correspondence. First and corresponding authors must be clearly indicated.

The names of the authors may be abbreviated following the international naming convention.
e.g. Salleh, A. B.[1], Tan, S. G.[2]*, and Sapuan, S. M.[3]

Authors' addresses. Multiple authors with different addresses must indicate their respective addresses separately by superscript numbers:

Abu Bakar Salleh[1] and Mohd Zamri Saad[2]

[1]Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia
[2]Faculty of Veterinary, Universiti Putra Malaysia, 43400 Serdang, Selangor Malaysia

A **list** of number of **black and white / colour figures and tables** should also be indicated on this page**.** Figures submitted in color will be printed (upon request) in colour. See "*5. Figures & Photographs"* for details.

Page 3: *Abstract*

This page should **repeat** the **full title** of your paper with only the **Abstract** (the abstract should be less than 250 words for a Regular Paper and up to 100 words for a Short Communication), and **Keywords**.

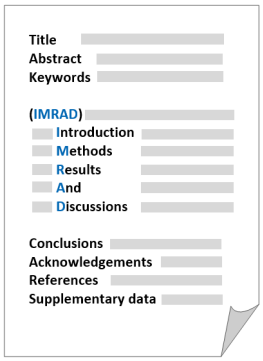
Keywords: Not more than eight keywords in alphabetical order must be provided to describe the contents of the manuscript.

Page 4: *Introduction*

This page should begin with the **Introduction** of your article and followed by the rest of your paper.

2. *Text*

Regular Papers should be prepared with the headings *Introduction, Materials and Methods, Results and Discussion*, *Conclusions, Acknowledgements, References, and Supplementary data* (if avavailble) in this order.



MAKE YOUR ARTICLES AS CONCISE AS POSSIBLE

Most scientific papers are prepared according to a format called IMRAD. The term represents the first letters of the words Introduction, Materials and Methods, Results, And, Discussion. It indicates a pattern or format rather than a complete list of headings or components of research papers; the missing parts of a paper are: Title, Authors, Keywords, Abstract, Conclusions, and References. Additionally, some papers include Acknowledgments and Appendices.

The Introduction explains the scope and objective of the study in the light of current knowledge on the subject; the Materials and Methods describes how the study was conducted; the Results section reports what was found in the study; and the Discussion section explains meaning and significance of the results and provides suggestions for future directions of research. The manuscript must be prepared according to the Journal's instructions to authors.

3. *Equations and Formulae*

These must be set up clearly and should be typed double spaced. Numbers identifying equations should be in square brackets and placed on the right margin of the text.

4. *Tables*

All tables should be prepared in a form consistent with recent issues of Pertanika and should be numbered consecutively with Roman numerals. Explanatory material should be given in the table legends and footnotes. Each table should be prepared on a new page, embedded in the manuscript.

When a manuscript is submitted for publication, tables must also be submitted separately as data - .doc, .rtf, Excel or PowerPoint files- because tables submitted as image data cannot be edited for publication and are usually in low-resolution.

5. *Figures & Photographs*

Submit an **original** figure or photograph. Line drawings must be clear, with high black and white contrast. Each figure or photograph should be prepared on a new page, embedded in the manuscript for reviewing to keep the file of the manuscript under 5 MB. These should be numbered consecutively with Roman numerals.

Figures or photographs must also be submitted separately as TIFF, JPEG, or Excel files- because figures or photographs submitted in low-resolution embedded in the manuscript cannot be accepted for publication. For electronic figures, create your figures using applications that are capable of preparing high resolution TIFF files.

6. *References*

References begin on their own page and are listed in alphabetical order by the first author's last name. Only references cited within the text should be included. All references should be in 12-point font and double-spaced.

NOTE: When formatting your references, please follow the **APA reference style** (6th Edition). Ensure that the references are strictly in the journal's prescribed style, failing which your article will **not be accepted for peer-review**. You may refer to the *Publication Manual of the American Psychological Association* for further details (http://www.apastyle.org/).

7. *General Guidelines*

Abbreviations: Define alphabetically, other than abbreviations that can be used without definition. Words or phrases that are abbreviated in the introduction and following text should be written out in full the first time that they appear in the text, with each abbreviated form in parenthesis. Include the common name or scientific name, or both, of animal and plant materials.

Acknowledgements: Any ndividuals and entities who have contributed should be acknowledged appropriately.

Authors' Affiliation: The primary affiliation for each author should be the institution where the majority of their work was done. If an author has subsequently moved to another institution, the current address may also be stated in the footer.

Co-Authors: The commonly accepted guideline for authorship is that one must have substantially contributed to the development of the paper and share accountability for the results. Researchers should decide who will be an author and what order they will be listed depending upon their order of importance to the study. Other contributions should be cited in the manuscript's Acknowledgements.

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Footnotes: Current addresses of authors if different from heading may be inserted here.

Page Numbering: Every page of the manuscript, including the title page, references, tables, etc. should be numbered.

Spelling: The journal uses American or British spelling and authors may follow the latest edition of the Oxford Advanced Learner's Dictionary for British spellings.

SUBMISSION OF MANUSCRIPTS

All submissions must be made electronically using the **online submission system ScholarOne™**, a web-based portal by Thomson Reuters. For more information, go to our web page and click "**Online Submission**".

Submission Checklist

1. **MANUSCRIPT**: Ensure your MS has followed the Pertanika style particularly the first four pages as explained earlier. The article should be written in a good academic style and provide an accurate and succinct description of the contents ensuring that grammar and spelling errors have been corrected before submission. It should also not exceed the suggested length.

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Any queries may be directed to the **Chief Executive Editor's** office via email to executive_editor.pertanika@upm.my.

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