Pertanika Journal of

TROPICAL AGRICULTURAL SCIENCE

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PERTANIKA JOURNALS

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Pertanika Journal of Tropical Agricultural Science
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About the Journal

Overview

Pertanika Journal of Tropical Agricultural Science is an official journal of Universiti Putra Malaysia. It is an open-access online scientific journal. It publishes the scientific outputs. It neither accepts nor commissions third party content.

Recognised 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.

Pertanika Journal of Tropical Agricultural Science 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 for submission by authors from all over the world.

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. Currently, as an interdisciplinary journal of agriculture, the revamped journal, *Pertanika* Journal of Tropical Agricultural Science now focuses on tropical agricultural research and its related fields.

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To publish journals of international repute.

Mission

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

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Pertanika Journal of Tropical Agricultural Science is now over 45 years old; this accumulated knowledge has resulted in *Pertanika* Journal of Tropical Agricultural Science being abstracted and indexed in SCOPUS (Elsevier), Clarivate Web of Science (ESCI), EBSCO, DOAJ, Agricola, ASEAN CITATION INDEX, ISC, Microsoft Academic, Google Scholar, National Agricultural Science (NAL), and MyCite.

Citing journal articles

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

Publication policy

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Authors who complete any submission 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 sent to reviewers. Authors are encouraged to suggest names of at least 3 potential reviewers at the time of submission of their manuscripts to *Pertanika,* but the editors will make the final selection and are not, however, bound by these suggestions.

Notification of the editorial decision is usually provided within 90 days 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.

The journal's peer review

In the peer review process, 2 or 3 referees independently evaluate the scientific quality of the submitted manuscripts. At least 2 referee reports are required to help make a decision.

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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Operating and review process

What happens to a manuscript once it is submitted to *Pertanika*? Typically, there are 7 steps to the editorial review process:

1. The journal's Chief Executive Editor and the Editor-in-Chief examine the paper to determine whether it is relevance to journal needs in terms of novelty, impact, design, procedure, language as well as presentation and allow it to proceed to the reviewing process. 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 2 or 3 reviewers. They are specialists in the subject matter of the article. The Chief Executive Editor requests that they complete the review within 3 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 research field.

3. The Editor-in-Chief examines the review reports and decides whether to accept or reject the manuscript, invite the authors to revise and resubmit the manuscript, or seek additional review reports. In rare instances, the manuscript is accepted with almost no revision. Almost without exception, reviewers' comments (to the authors) are forwarded to the authors. If a revision is indicated, the editor provides guidelines to the authors 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 authors may also submit a rebuttal if there is a need especially when the authors disagree with certain comments provided by reviewers.

5. The Chief Executive Editor sends the revised manuscript out for re-review. Typically, at least 1 of the original reviewers will be asked to examine the article.

6. When the reviewers have completed their work, the Editor-in-Chief examines their comments and decides whether the manuscript is ready to be published, needs another round of revisions, or should be rejected. If the decision is to accept, the Chief Executive Editor is notified.

7. The Chief Executive Editor reserves the final right to accept or reject any material for publication, if the processing of a particular manuscript is deemed not to be in compliance with the S.O.P. of *Pertanika.* An acceptance notification is sent to all the authors.

   The editorial office ensures that the manuscript 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 editorial office. 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 manuscript 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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Foreword

Welcome to the second issue of 2023 for the Pertanika Journal of Tropical Agricultural Science (PJTAS)!

PJTAS 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 20 articles; one review article, one short communication, and the rest are regular articles. The authors of these articles come from different countries namely Brazil, Canada, Hong Kong, Indonesia, Malaysia, Nigeria, United State of America, and Yemen.

A selected article entitled "Revisiting *In Vitro* Micropropagation Protocols of *Mimosa pudica* for Enhanced Seed Germination, Shoot Multiplication, and Root Initiation" outlined a procedure for seed germination, shoot multiplication, and root initiation of *in vitro* micropropagation of the undervalued medicinal plant, *M. pudica*. They found out that the developed micropropagation protocol of *M. pudica* could facilitate its large-scale cultivation, indicating its potential as a medicinal crop for the extraction of bioactive compounds. Full information of this study is presented on page 571.

A regular article entitled "*Parthenium hysterophorus* Weed Fecundity and Seed Survival at Different Soil pH and Burial Conditions" evaluated the effect of soil pH on *P. hyterophorus* weed growth and fecundity, as well as the effect of burial depths on *P. hyterophorus* seed survival and emergence. The findings suggested that *P. hyterosphorus* seed could survive a wide range of conditions with high germinability. The seed, however, showed a marked decline in viability if buried for more than 12 months without tillage. The further details of this study are found on page 593.

Azman Abd Samad and his team from Universiti Teknologi Malaysia extracted and purified protease from the crown of MD2 pineapple using anion exchange chromatography, gel filtration, and desalting. They also identified the protease using liquid chromatography-mass spectrometry (LC-MS) and determined the proteolytic activity using the well diffusion method and Casein Digestion Unit. The results showed that the purified enzyme that was extracted from pineapple crown was ananain-like protease, one type of cysteine protease like other papain family members. The detailed information of this article is available on page 607.

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.

We would like to express our gratitude to all the contributors, namely the authors, reviewers, and Editorial Board Members of PJTAS, who have made this issue possible.

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.

PJTAS 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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Short Communication

Pathogenicity of *Aeromonas hydrophila* in High-value Native Pangasius Catfish, *Pangasius nasutus* (Bleeker)

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ABSTRACT

Pangasius catfish, *Pangasius nasutus*, is a promising candidate for aquaculture due to its high market value. However, the presence of pathogenic bacteria in *Aeromonas hydrophila* is a major concern in *P. nasutus* farming in this country. This study determines the pathogenicity of *A. hydrophila* in *P. nasutus*. A total of 80 *P. nasutus* juveniles were intraperitoneally injected with 0, 10^3, 10^5, and 10^7 CFU mL^{-1} of *A. hydrophila* and monitored until 240 hr. The infected moribund fish's kidneys, livers, and spleens were collected for histopathological analysis. The $LD_{50\text{-}240hr}$ value was found at 0.8×10^4 CFU/ml of *A. hydrophila*. The percentage of mortality in 0, 10^3, 10^5, and 10^7 CFU/ml infected groups were found to be at 0, 40, 60, and 90%, respectively. The infected fish showed congestion at the base of the fin, ascites, enlarged gall bladder, and swollen spleen. It is the earliest report on *A. hydrophila*'s pathogenicity in high-value native fish, *P. nasutus*.

Keywords: *Aeromonas hydrophila*, $LD_{50,}$ pangasius catfish, *Pangasius nasutus*, pathogenicity

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INTRODUCTION

Pangasius catfish, *Pangasius* sp., is one of the most significant fish species, particularly in Asian countries such as Vietnam, Bangladesh, India, Indonesia, Malaysia, Myanmar, Philippines, and Thailand (Sirimanapong et al., 2014). *Pangasius nasutus*, locally recognized as "patin buah" in Malaysia, was regarded as among the highest-valued fish, with an estimated price of RM 70-300/kg (Jamaludin & Ting, 2021; Yusof & Nakajima, 2019), threefold higher compared to the common local black Pangasius catfish, *Pangasius micronemus* (Hashim et al., 2015). There is a significant market demand for this species due to its sweet, savory, and more appealing than the other commercial species. This market preference is evident in Malaysia and regions that could serve as export markets, such as Asia, Europe, and North America (Rafi et al., 2022).

However, like other *Pangasius* sp. in Malaysia, *P. nasutus* is prone to *Aeromonas hydrophila* infections (Mansor et al., 2020). *Aeromonas hydrophila* is a rod-shaped, Gram-negative bacterium that lives in aquatic habitats, infects different fish and significantly harms the aquaculture industry (Jiang et al., 2016; Mazumder et al., 2021). The pathogen was first recognized as the causal agent of hemorrhagic septicemia and has been considered the dominant cause of motile aeromonad septicemia (MAS) (Tartor et al., 2021; Zhang et al., 2019). *Aeromonas hydrophila* causes various pathogenic severities in fish, including hemorrhagic septicemia, abdominal edema, exophthalmia, ulcers, and respiratory infections (Laith & Najiah, 2014; Zhang et al., 2016). In the USA alone, the outbreaks of MAS have cost 60–70 million dollars in loss a year (Bøgwald & Dalmo, 2019). At the same time, significant mortalities due to *A. hydrophila* infection were recorded in the South and South-East Asia farmed fish (Laith & Najiah, 2014). In Malaysia, the *A. hydrophila* outbreak was first recorded in diseased catfishes from a local farm, exhibiting MAS's common clinical and histological symptoms (Anjur et al., 2021).

Aeromonas hydrophila was reported to be commonly infecting *Pangasius* spp. at all life stages (Sarker & Faruk, 2016). Previous studies have been conducted on the infection of *A. hydrophila* in *Pangasianodon hypophthalmus*, *Pangasius bocourti*, and *Pangasius pangasius* (Doan et al., 2013; Hayati & Prihanto, 2020; Le et al., 2018). Their studies found that *A. hydrophila* could infect *Pangasius* sp. severely, with an average mortality of more than 80%. It is crucial to determine the pathogenicity of *A. hydrophila* in *P. nasutus*, as this fish species is currently being promoted in aquaculture. Understanding the disease development will help in future preventive measures. It is the earliest report on the infectivity of *A. hydrophila* in high-value native fish of Malaysia, *P. nasutus*.

MATERIALS AND METHODS

Experimental Fish

One hundred (100) Pangasius catfish, *Pangasius nasutus* (8.95 ± 2.50 g), were purchased and transferred to the Aquatic

Animal Health Unit, Faculty of Veterinary Medicine, Universiti Putra Malaysia from a commercial fish farm in Rawang, Selangor. Fish were maintained in an aerated recirculating water system at a temperature of 26.23 ± 0.59°C, pH at 6.48 ± 0.24, dissolved oxygen at 6.38 ± 0.48 mg/L, and ammonia at 0.01 ± 0.00 mg/L. The fish were acclimatized under laboratory conditions for ten days before the experimental challenges. Twenty (20) fish were randomly selected to check for bacteria and parasitic infection. The fish was found healthy, and no clinical signs were ever observed.

Bacterial Culture and Confirmation

Aeromonas hydrophila strain Ah1sa5 was isolated and obtained from diseased tilapia (*Oreochromis* sp.) on a local farm in Tasik Kenyir Terengganu, Malaysia (Matusin, 2015). The isolate was sub-cultured on tryptic soy agar (Oxoid, United Kingdom) and incubated at 30°C for 16 hr. Pure colonies were inoculated in tryptone soy broth (TSB; Oxoid, United Kingdom) and incubated overnight at 30°C and 150 rpm.

Aeromonas hydrophila used in this study were subjected to polymerase chain reaction (PCR) identification following Azzam-Sayuti, Ina-Salwany, Zamri-saad, Yusof, et al. (2021). The isolate was cultured on tryptone soy agar (TSA, Oxoid, United Kingdom) on plates for 24 hr at 30°C. Afterward, the *A. hydrophila* isolates were grown into 200 ml of TSB broth and cultured for 24 hr with gentle shaking at 30°C.

Genetic DNA from each sample was extracted from a pure bacterial colony using the DNeasy Blood and Tissue Kit (Qiagen, Germany) following the manufacturer's protocol. The extracted DNA was subjected to PCR amplification using 16S rRNA (F: GGTTACCTTGTTACGACTT and R: AGAGTTTGATCCTGGCTCAG) and DNA gyrase B subunit (*gyr*B) (F: TCCGGCGGTCTGCACGCGGT and R: TTGTCCGGGTTGTACTCGTC) primers to detect the target region of the bacterial strain with a PCR product size of approximately 1,541 and 1,100 bp, respectively. The PCR reactions were made using REDiant 2× PCR Master Mix (FirstBase, Malaysia) in a final volume of 25 µl containing 2× PCR master mix, 1 µM of each primer, and 100 ng of template DNA. Each master mix of 50 µl contains 1× PCR buffer, 200 mmol/L of each dNTP, 2.0 mmol/L magnesium chloride ($MgCl_2$), 1 U *Taq* DNA polymerase, and 50 pmol of every primer. The thermal cycling was carried out on a Thermal cycler (Bio-Rad Laboratories, USA) using the amplification conditions as follows: 1 cycle of 95°C for 5 s (initial denaturation), 33 cycles of 95°C for 1 min, 59°C for 2 min 15 s and 72°C for 1 min 15 s, and then a final extension at 72°C for 10 s. The PCR products were sequenced at Firstbase (Malaysia), and the phylogenetic trees were constructed using the Molecular Evolutionary Genetics Analysis (MEGA) (version 10.1.8) bioinformatics software with 100 bootstrap trials and the neighbor-joining method.

Lethal Dose of *A. hydrophila* Against *P. nasutus*

Preparation of *A. hydrophila* Cultures. Briefly, 10 colonies of *A. hydrophila* were randomly selected and inoculated into 500 ml of tryptic soy broth (TSB, Oxoid, United Kingdom) and incubated at 30°C and 150 rpm for 16 hr. Ten-fold serial dilutions of the bacterial culture were conducted to determine the concentration of the bacterial stocks, and 100 µl of TSB from every dilution were plated on tryptic soy agar (TSA; Oxoid, United Kingdom) in duplicate. The culture was then incubated at 30°C overnight. Standard plate count was used to calculate the colony forming unit per milliliter (CFU/ml), according to Wohlsen et al. (2006). The bacteria were then harvested through centrifugation and washed thrice with phosphate buffer saline (PBS, pH 7.4). Finally, the bacterial pellets were suspended in PBS (pH 7.4) before being diluted to the desired concentrations.

Challenge Test. The median lethal dose (LD_{50}) of *A. hydrophila* was determined with 80 juvenile *P. nasutus*. The fish were equally disseminated in four tanks in duplicate (10 fish per tank) and not given any feed a day before the challenge trial. Fish were intraperitoneally injected (i.p.) with 0.1 ml of *A. hydrophila* suspension with the respective concentrations: 0 CFUml (control, PBS only), 10^3, 10^5, and 10^7 CFU/ml. The mortality pattern and gross lesion were monitored until 240 h. The $LD_{50–240hr}$ values were calculated as described by Reed and Munch (1938) and were performed using Statistical Product and Service Solutions (SPSS) Statistics software (ver. 26) (SPSS Inc., USA). Bacterial isolation from the challenged moribund fish and 16S rRNA PCR, and nucleotide Basic Local Alignment Search Tool (BLAST) analysis was done afterward to confirm the cause of their death from *A. hydrophila* infection and phylogenetic analyses using the neighbor-Joining method were conducted afterward in MEGA X (Kumar et al., 2018).

Histopathological Analysis

The infected moribund fish underwent histological examination of the kidneys, livers, and spleens to check for any gross lesions in the internal organs. The collected samples were fixed in 10% neutral-buffered formalin for at least 24 hr, dehydrated using a series of increasing concentrations of ethanol (50–100%), cleared in xylene, and embedded in paraffin. The paraffin sections (4 µm thick) were prepared using a microtome (Leica RM 2155, Germany) and stained with hematoxylin and eosin (H & E) dyes before being inspected microscopically (Rahman et al., 2022).

RESULTS

Bacterial Culture and Confirmation

As shown in Figure 1, the phylogenetic tree does not delineate the reference strains into separate groups. The bacteria isolated from the infected fish were distantly related to the *A. hydrophila* reference strain, MG984625.1 ATCC (Figure 1A) and AY987520.1 (Figure 1B). Amplification products for 16S rRNA

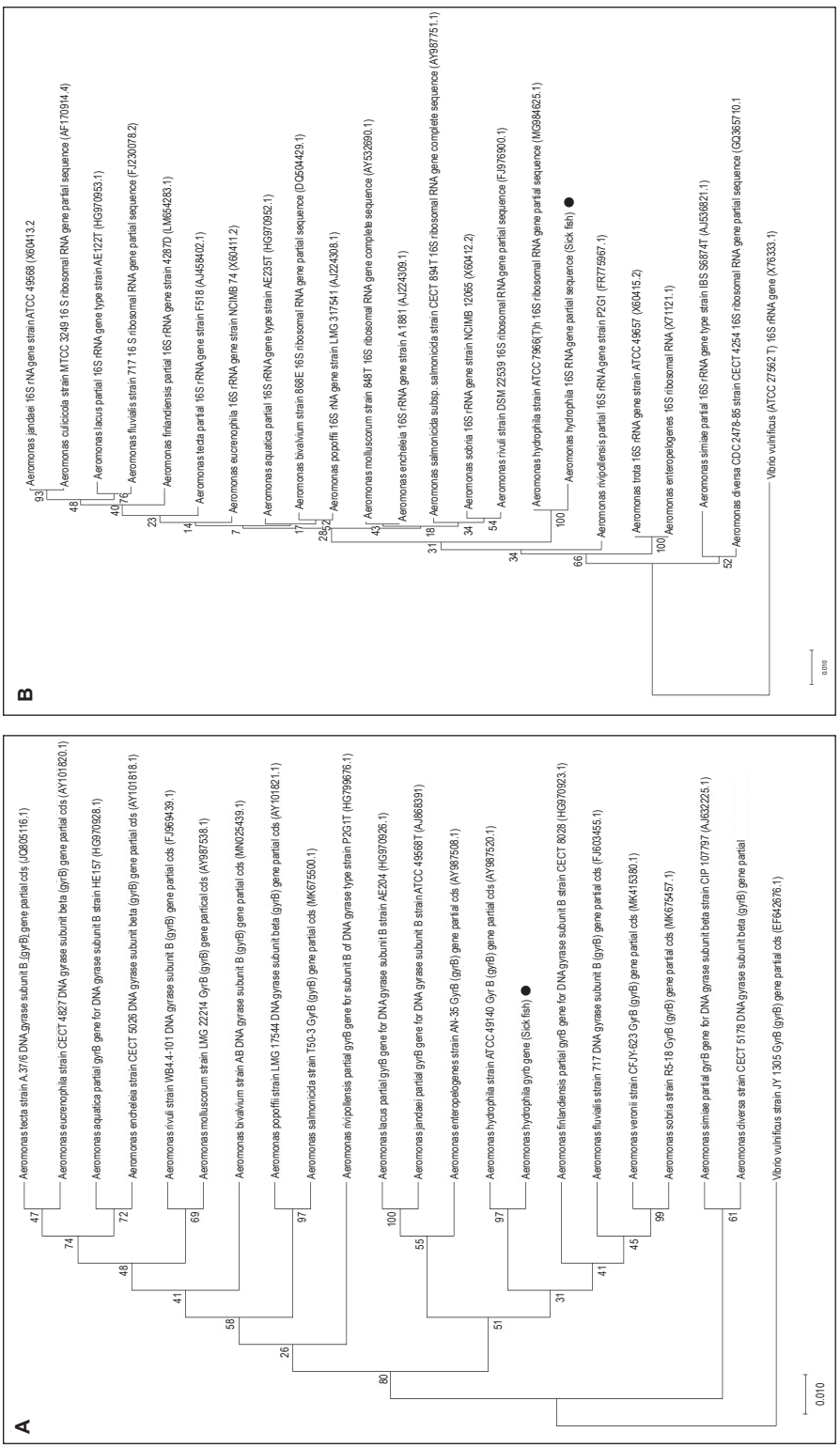

Figure 1. Phylogenetic analysis of *Aeromonas hydrophila* based on 16S rRNA (A) and *gyr*B (B) genes inferred using the neighbor-joining method with other *Aeromonas* sp., as a reference and one non-*Aeromonas* sp., *Vibrio vulnificus*, as the out-group. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown next to the branches. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. Evolutionary analyses were conducted in MEGA X (Kumar et al., 2018). The bacterial strain used in this study was marked with "●"

and *gyr*B genes were sequenced using the BLAST Nucleotide algorithm, and the similar sequences from the GenBank non-redundant (NR) data source were determined as *A. hydrophila* with 98% (Accession no.: JN711800.1) and 99% (Accession no.: MK937644.1) similarities, respectively.

Mortality Pattern and $LD_{50\text{-}240h}$ of *A. hydrophila* Against *P. nasutus*

After 24 hr post-challenge (hpc), mortality of *P. nasutus* was seen in concentration 10^7 CFU/ml and at 48 hpc for 10^3, 10^5, and 10^7 CFU/ml. After 24 hpc, 40% mortality was recorded in the 10^5 CFU/ml group, while at 72 hpc, the mortalities were the highest for concentrations of 10^3 and 10^5 CFU/ml at 20 and 30%, respectively. The mortalities lasted for 96 hpc for concentrations of 10^3, 10^5, and 10^7 CFU/ml, and 120 hpc for group 10^7 CFU/ml. By 240 hpc, the mortality rate was observed as 40, 60, and 90% for 10^3, 10^5, and 10^7 CFU/ml, respectively (Figure 2). No mortality was observed in the control samples throughout the experiment. Probit analysis revealed that the lethal dose ($LD_{50\text{-}240hr}$) of *A. hydrophila* for *P. nasutus* was observed at 0.8×10^4 CFU/ml. Nevertheless, the re-isolated bacteria from the experimentally infected *P. nasutus* was confirmed phenotypically and molecularly as *A. hydrophila*.

Pathological Symptoms of *P. nasutus* Post-infection with *A. hydrophila*

After intraperitoneal injection of *A. hydrophila*, all infected fish showed varying clinical signs and gross lesions typical of *Aeromonas* infection. They include reduced feed intake, isolation, irregular breathing, and swimming near the surface, which was not observed in the control group. Gross lesions include hemorrhagic foci around

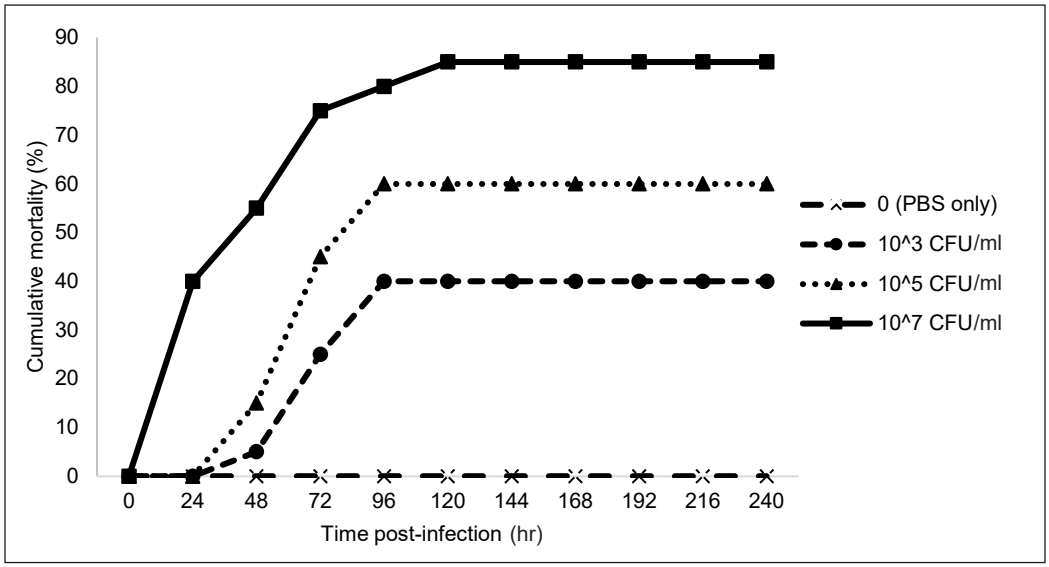

Figure 2. Cumulative mortality of *Pangasius nasutus* infected with different concentrations of *Aeromonas hydrophila*

the anal region and under the pectoral fin (Figure 3). The post-mortem examination showed swollen gall bladder, bloody-colored fluid in the abdominal cavity, and an enlarged spleen. These were not observed in control fish.

Histopathological Analysis

Histopathological lesions following *A. hydrophila* infection in the kidneys of *P. nasutus* showed varying degrees of necrosis and degeneration in the tubular epithelium and interstitial nephritis with inflammatory cell infiltration (Figure 4). Desquamated tubular epithelial cells with cell lysis and expansion in the glomerular cavity were also observed in infected kidneys. The infected livers showed multifocal congestion, dilatation of the sinusoids with vacuolar degeneration, and hepatocellular necrosis. The infected spleen showed subcapsular necrosis, multifocal necrosis, splenic hemorrhage with numerous red blood cells, and aggregation of marked melano-macrophage centers (MMC) with hemosiderin deposition. The spleens of the control fish showed normal architecture with the absence of MMCs. Indistinct pathological changes were observed after injection with PBS in the control group's kidney, liver, and spleen.

DISCUSSION

Pangasius nasutus was recently introduced as a new candidate for aquaculture (Tahapari et al., 2020). The fish is highly consumed in Malaysia, particularly in Pahang, where it is popularly cooked in a special local delicacy. The supply of *P. nasutus* primarily comes from the wild stock (Jaapar et al., 2021). The challenge experiment revealed typical *A. hydrophila* infection signs and gross lesions

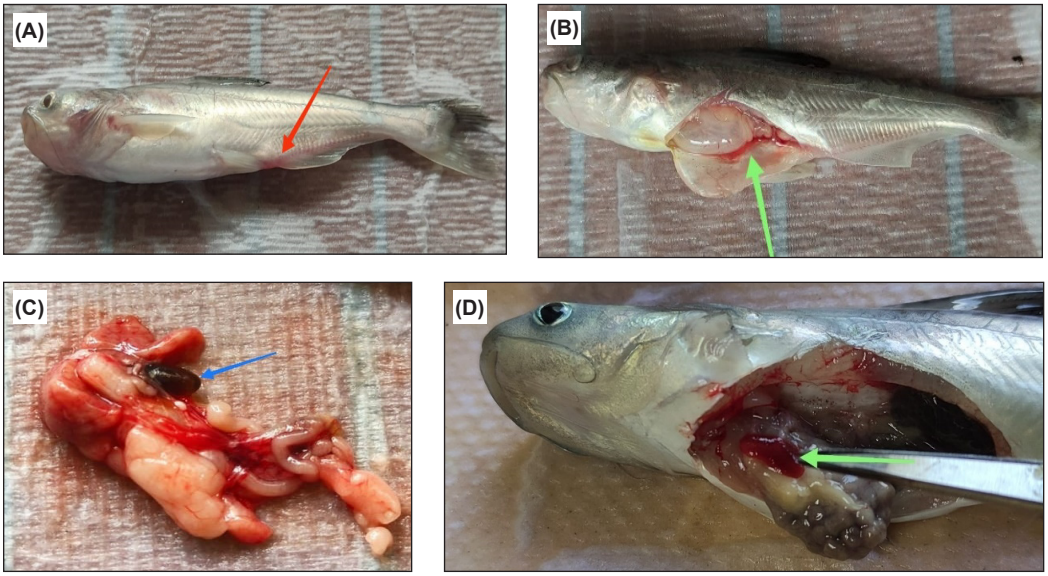

Figure 3. Gross lesions were found on the *Pangasius nasutus* infected with *Aeromonas hydrophila*. (A) Congestion at the base of the fin (orange arrow); (B) Fluid accumulation in the abdominal cavity (green arrow); (C) Enlarged gall bladder (blue arrow); (D) Swollen spleen (green arrow) in challenged *P. nasutus*

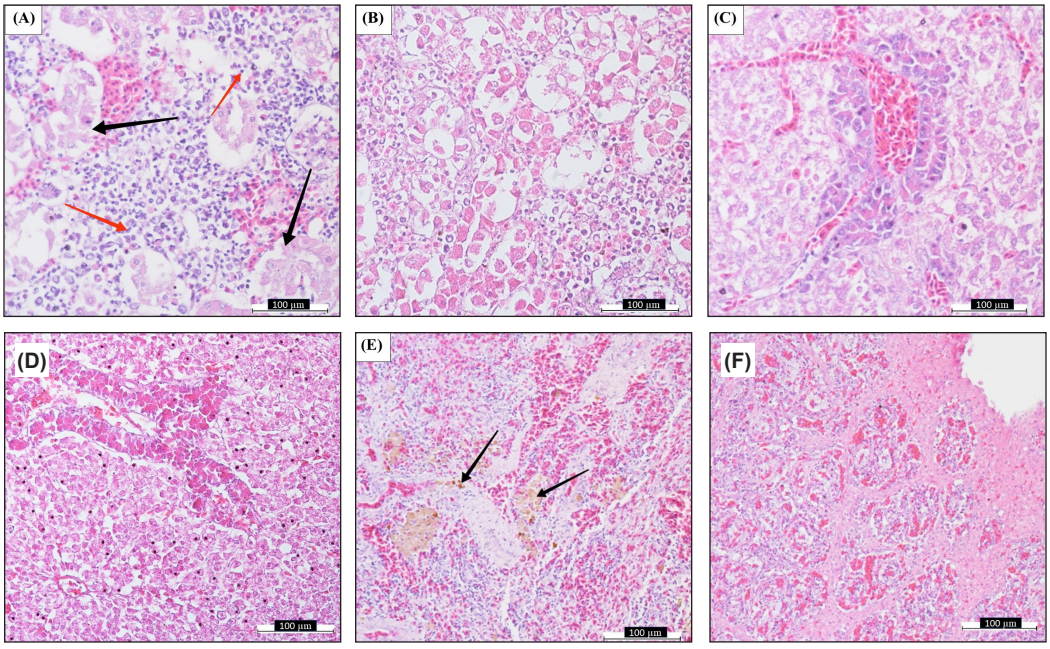

Figure 4. Histological detections of infected kidney, liver, and spleen and control *Pangasius nasutus* following *Aeromonas hydrophila* challenge. (A) Kidneys of infected fish showed necrosis with interstitial nephritis, especially with the tubular epithelial cells (black arrow) with inflammatory cell infiltration (red arrow) (400×); (B) Normal architecture with intact, healthy tubular epithelia in the kidneys of control fish (400×); (C) Infected liver showing dilatation of the sinusoids with vacuolar degeneration and necrosis (200×); (D) Liver of the control fish showed normal liver architecture and hepatocytes (200×); (E) Infected spleen showed subcapsular necrosis, splenic hemorrhage, and aggregation of hemosiderin (black arrows) (100×); (F) The spleen of the control fish showed normal splenic architecture (100×)

as reported in Asian catfish (*P. bocourti*) and snakehead (*Channa striata*) (Doan et al., 2013; Samayanpaulraj et al., 2019). With increasing bacterial concentration, the mortality rate increased due to more toxins being released, potentially damaging the fish's internal organs (Dong et al., 2017). Early mortality in the infected fish was observed at 24 hpc in 10^7 CFU/ml treatments (40%), while in 10^3 (5%) and 10^5 CFU/ml (15%) treatments, mortality started after 48 hpc, demonstrating the pathogenicity of the bacteria to cause acute-to-chronic illness in *P. nasutus*. While after 72 hpc, 25, 45, and 75% cumulative mortalities were recorded in 10^3, 10^5, and 10^7 CFU/ml treatments, respectively. The $LD_{50\text{-}240hr}$ of *A. hydrophila* strain Ah1sa5 for *P. nasutus* was found at 0.8×10^4 CFU/ml. In this study, the LD_{50} of *A. hydrophila* was lower than in Basa, *P. bocourti* (Doan et al., 2013), and in striped catfish, *P. hypophthalmus* (Sirimanapong et al., 2014), which both reported 10^8 and 10^5 CFU/ml, respectively. The differences in the LD_{50} value may cause by the *A. hydrophila* strains used in each study and the challenged host. *P. nasutus* was reported to have a higher sensitivity to environmental stressors and was difficult to be farmed (Zulkiflee et al., 2020).

The clinical manifestations seen in this study after the *A. hydrophila* infection in *P. nasutus* were mainly behavioral pattern changes and respiratory difficulties. The same clinical signs have been described in the intradermal or intraperitoneal infection of *A. hydrophila* and other fish species (Azzam-Sayuti, Ina-Salwany, Zamri-Saad, Annas et al., 2021; de Oliveira et al., 2011; Dias et al., 2016). It is believed that the effect of acetylcholinesterase secreted by *A. hydrophila* may lead to these signs due to its narcotic effects on the central nervous system (Dias et al., 2016). However, other reported macroscopic lesions, such as hemorrhages at the site of infection, scale loss, and dermal necrosis (Azzam-Sayuti, Ina-Salwany, Zamri-Saad, Annas et al., 2021; Dias et al., 2016), were not observed in *P. nasutus*. After *A. hydrophila* infection, the histopathological variations observed in the internal organs of *P. nasutus* were similar and common to the lesions found in other infected fish species (Abdelhamed et al., 2017; Chen et al., 2018; Saharia et al., 2018). However, polymorphonucleated cell infiltration and glomerular cell proliferation have also been reported in *A. hydrophila* infections in fish (Chen et al., 2018; Saharia et al., 2018).

CONCLUSION

It is the first report on the pathogenicity of *A. hydrophila* in high-value native fish of Malaysia, *P. nasutus*. This study indicates that *P. nasutus* is susceptible to *A. hydrophila* infection and could be a potential disease threat to cultured and wild *P. nasutus*. Further studies need to be conducted on the severity of *A. hydrophila* infection in *P. nasutus* vital organs either via histopathological changes scoring or at the molecular level. Besides, the possible transmission of *A. hydrophila* infection to the fish should be considered. This valuable information could result in significant disease preventive measures such as biosecurity and vaccine development programs.

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The Impact of Music on Milk Production and Behaviour of Dairy Cattle

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ABSTRACT

The influence of music on the milk performance of dairy cattle has been increasingly studied in recent years, although its efficacy is still being debated due to the inconsistent outcomes and a limited number of studies. This study aimed to investigate the impact of music on the milk production and behaviour of 24 lactating Jersey cattle, consisting of 10 primiparous and 14 multiparous cows. The experiment involved milk collection and behavioural observation before and after exposure to music at a dairy farm in Sitiawan, Perak. The results indicate that music and parity significantly affect the milk yield produced by a cow at $p < 0.05$. The cows produced significantly less milk after being exposed to music, while multiparous cows recorded significantly higher milk yields than primiparous cows. In terms of the observed behaviour, the cows displayed a significantly higher proportion of feeding and ruminating when no music was played. On the other hand, multiparous cows displayed a significantly lower proportion of feeding and ruminating, and a higher proportion of standing compared to primiparous cows. However, no significant effects of parity and music were observed in walking and lying behaviour. To conclude, music did not improve the milk yield of Jersey dairy cows in this study. Due to the inconsistencies in the outcomes of many studies related to the effect of music on milk yield, more long-term research should be conducted by considering factors that might influence milk yields, such as parity, breed, housing environment, and music selection.

Keywords: Behaviour, dairy cattle, milk yield, music, parity

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INTRODUCTION

Malaysia is one of the largest milk-importing countries in the world, after China, Mexico, Algeria, the Russian Federation, Indonesia, Saudi Arabia, and the Philippines (Faghiri

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et al., 2019). Malaysia's National Dairy Industry Development Program aims to achieve a 100% self-sufficiency level (SSL) for local fresh milk production by 2025, which is currently at 62% (Aman, 2021). Qatar's major dairy producer has aligned with two of Malaysia's largest dairy farms to enable the country to achieve higher milk SSL so it can be imported to Qatar as the country's demand for milk keeps increasing (Whitehead, 2019). Farmers have explored ways to enhance their cows' milk production in response to government requests. One of the easiest approaches is through music, but the effectiveness of this is still disputed. Music has been known to evoke reactions in humans and a wide range of animals. Music therapy has been proven successful in enhancing body immunity and psychology by reducing stress, anxiety, depression, and pain in humans (Dhungana et al., 2018).

Music is acknowledged as a non-invasive application, and its physical, psychological, and social impacts have been utilised throughout the years (Dağli & Çelik, 2022). Recent studies have shown that music can increase breast milk production in mothers with premature infants (Dağli & Çelik, 2022; Jayamala et al., 2015; Varişoğlu & Güngör Satilmiş, 2020). Listening to soothing music or sounds has been proven to help people relax, which is especially good for nursing mothers because the inhibition of stress hormones aids breast milk flow (Katili et al., 2021). Music therapy, which has a calming impact, lowers the risk of stress-related physical reactions in mothers of premature newborns who are hospitalised (Dağli & Çelik, 2022; Jayamala et al., 2015; Varişoğlu & Güngör Satilmiş, 2020). It is accomplished by helping the mothers feel positive and reducing their negative feelings about their babies (Dağli & Çelik, 2022).

Music has been proposed to improve animal well-being by eliminating unpleasant background noises, reducing anxiety and aggression, and creating auditory enrichment, depending on the animals (Alworth & Buerkle, 2013). Chickens exposed to country music have a significantly lower ratio of heterophilic lymphocytes, lower levels of physiological stress, and displayed grooming behaviours for twice as long (Rickard et al., 2005). Slow music can elicit a response from anxious buffalo cows, making them easier to handle (Dhungana et al., 2018). Reductions in aggressive behaviour and increases in inquisitiveness and sociability have also been observed in chimpanzees exposed to music (Alworth & Buerkle, 2013).

Such an effect can also be seen in dairy cattle, as music can ease them, thus increasing their welfare and milk production (Lemcke et al., 2021). Dairy cattle consider music exciting, which could result in their voluntary movement towards automatic milking systems (AMS) without much coercion (Lemcke et al., 2021). In a recent study, cattle demonstrated most physical signs of relaxation, such as chewing the cud and lowering the eyelids, indicating they were pleased with the slow music (Kemp, 2020). Most cows exhibited fewer stress behaviours, such as sleeping and relaxing, when playing lullaby music (Kemp, 2020).

Dairy cattle responded better to classical, country, and audiobook music, exhibiting fewer aberrant, such as tongue rolling, less vocalisation, and greater movement, which indicated reduced stress and increased welfare (Crouch et al., 2019). In addition, music has also been observed to affect milk production. Studies have shown the positive effects of music on dairy cattle's milk, although these remain sparse.

Dairy farms, particularly those with noisy mechanical milking parlours, can benefit from playing slow music to their cows to alleviate stress and increase milk production by up to 3% (Kochewad, 2022; Lemcke et al., 2021). Listening to classical music for a long time has increased milk production in dairy cattle (Dhungana et al., 2018; Ganesh, 2020; Liu et al., 2017; Ma & Wang, 2020). While some music genres have a soothing effect, which can reduce stress in dairy cattle and increase their milk supply, others have a numbing effect that could increase the cows' tension and reduce the milk supply (Donghai et al., 2018; Ganesh, 2020). Some studies found that music had no effect on milk production, although the cows demonstrated relaxed behaviours when some form of music was played (Kemp, 2020; Lemcke et al., 2021).

Due to the mixed results concerning the role of music on dairy cattle's milk production, more studies need to be conducted to investigate other factors that may have contributed to these inconsistent findings, such as breed, parity, nutrition, environment, stress, and song selection. In Malaysia, providing music therapy to dairy cattle is not common. Although Malaysia's milk production constantly increased between 2011 and 2017, this was still not enough to sustain milk consumption, which nearly quadrupled between those years (Suntharalingam, 2019). If music therapy can consistently increase milk production in dairy cattle, it would be highly beneficial to farmers as they could use this relatively cheap method of enhancing milk production on their farms.

Therefore, this study was conducted to investigate the effect of music on the milk performance and behaviour of primiparous and multiparous Jersey dairy cattle on a dairy farm in Perak, Malaysia.

METHODS

Sampling Site

This study was conducted at Cherry Alpha Sdn. Bhd. in Sitiawan, Perak, located at 4°14'58"N and 100°44'12"E. Cherry Alpha Dairy Sdn. Bhd. has been providing high-quality Jersey milk since its establishment in 2014 on a local family farm. The farm has 200 purebred Jersey cows: heifers, pregnant cows, and lactating cows.

Animals, Facilities, and Management

The farm's approximately 100 lactating Jersey cows were kept together in a 59 m × 40 m pen. Of these, 24 individuals which were healthy and not in heat were selected to undergo this study. The sample consisted of 10 primiparous and 14 multiparous individuals, with an average of 206 days after giving birth to calves. The dairy cattle

were milked twice daily at 7.30 a.m. and 4.00 p.m. using an automatic milking system (AMS). Using the AMS, a total of 48 Jersey dairy cattle could be milked at one time, and they would then be returned to their pen after the milking sessions. All the cattle on the farm were fed fresh straw *ad libitum*, with the addition of vitamin supplements and dairy cattle pellets in the morning. Drinking water with added minerals was also provided throughout the day. The cattle were also sanitised and cleaned before each milking session.

Data Collection

This study was conducted in March 2022 for a total of 13 days, and it involved milk collection and behavioural observation before and after exposure to music. The experiment started with data collection for five days without music, followed by a one-day habituation period when the cows were exposed to music without any milk collection and behavioural observation and continued with data collection for seven days with music (Figure 1).

During the final seven days, music was played during milking in the area (7.30 a.m. until 8.30 a.m.) and in the shed for three hours after milking (9 a.m. to 12 p.m.). Two wireless Bluetooth speakers (SonicGear Super P8000, China and Armaggeddon Puma III, Singapore) were used throughout the experiment. One Bluetooth speaker was set up in the milking area, approximately 0.96 m above the dairy cattle's heads. The sound pressure levels were kept below 85 dB, and frequencies were kept between 23 and 35 kHz; these were checked using Arduino Science Journal apps to ensure that the music reached the cattle's ears and did not disturb them (Alworth & Buerkle, 2013; Lemcke et al., 2021). A total of 23 songs were chosen and combined into one playlist, which consisted of French classical piano, flute music, Richard Clayderman's classical piano music, and Mozart's classical music since these types of music have been shown to increase milk production in the previous studies (Ganesh, 2020; Liu et al., 2017; Ma & Wang, 2020).

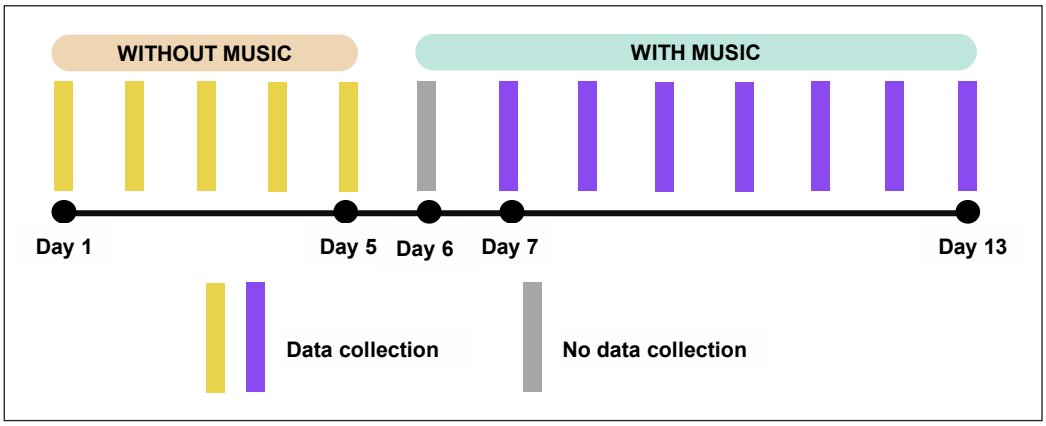

Figure 1. Experimental period with and without music

Milk Collection

The cows in this study were milked together based on their parity, and the starting order was alternated each day. The keeper took the cows to the milking parlour, and their teats were attached to the cups. The suckling process took around five minutes on average, depending on the cows (Figure 2). The milk was then drawn into the pipeline and the Mueller milk cooling tank (Mueller Model U Open Tank, U-2000, USA), as shown in Figure 3. The milk was then measured using a measuring dipstick. The volume of milk collected was recorded approximately two minutes after the suckling machine stopped.

Behaviour Observation

The cows' behaviour was recorded using instantaneous scan sampling 30 min after the morning milking session; three scans were performed, with a 10-min interval between each scan. One observer recorded all the cattle's behaviours to eliminate the effects of various interpretations. The observed behaviours are described in Table 1; some are shown in Figures 4, 5, 6, 7, and 8.

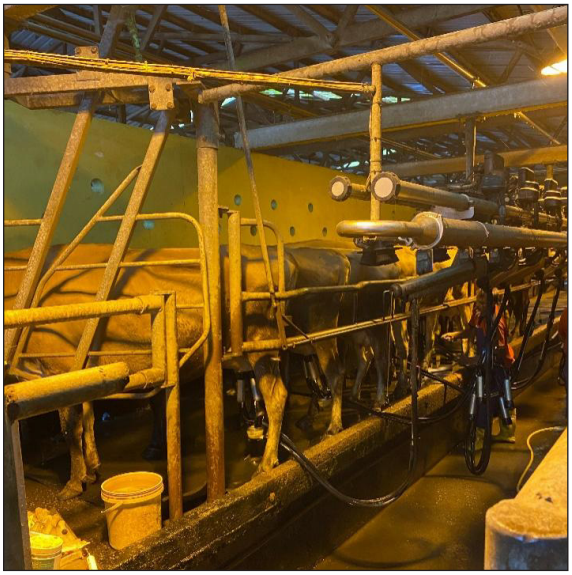

Figure 2. Suckling process

Figure 3. Mueller Model U Open Tank

Table 1
Description of dairy cattle behaviour

Behaviour	Description
Feeding	The cow lowers its head towards the feeding trough, puts the food (either maize, straw, or pellets) into its mouth, and chews before swallowing it
Rumination	The cow re-chews the cud completely before swallowing it with its head down, and its eyelids lowered while standing or lying
Drinking	The cow places its muzzle in the drinking trough and consumes water

Table 1 *(continue)*

Behaviour	Description
Standing	The cow stands with all four legs on the ground for at least five seconds
Walking	The cow moves forward more than three steps
Lying	The cow lowers its body, folds all its legs to the ground, and dozes off with its eyes open or closed and with its head down or up
Vocalisation	The cow makes different types of sounds, such as mooing, bellowing, snorting, and grunting
Aggression	The cow pushes or strikes another cow on the other side using its head or body
Tongue rolling	The cow flicks its tongue outside its mouth and then rolls it back inside
Grooming	The cow licks its own body or licks other cows on the head or neck
Licking minerals	The cow licks the mineral block to supply its body with minerals
Scratching	The cow rubs its body by moving back and forth against any object, such as a pole and fence

Figure 4. Feeding behaviours. The cows were eating pellets (A), fresh maize (B), and straw (C)

Figure 5. Standing behaviour

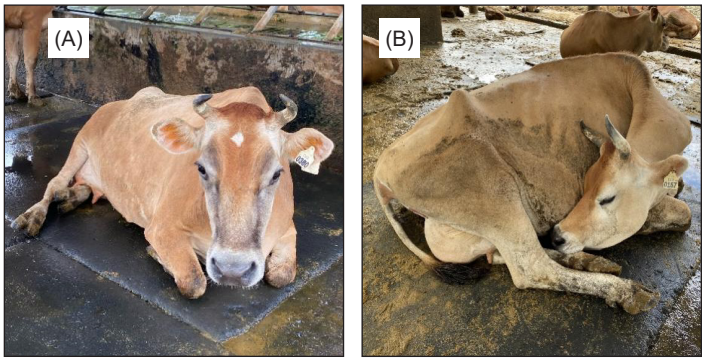

Figure 6. Lying behaviours. The cow was resting with its eyes open and all four legs on the ground (A), while the other cow was dozing with its head down (B)

Figure 7. Tongue rolling behaviour

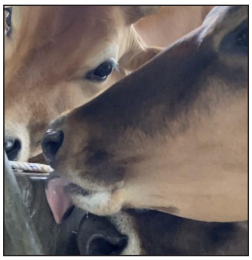

Figure 8. Licking behaviour

Statistical Analysis

The average milk yields were calculated by dividing the milk yield by the number of individuals, as the assessment referred to the milk collected from the whole group, not individuals. It was then analysed using the general linear model (GLM) univariate procedure, with music, parity, and their interaction fitted as fixed factors. For the general pen behaviours observed using instantaneous scan sampling, only feeding, rumination, standing, licking mineral, walking, and lying behaviours were analysed due to the infrequent display of the other behaviours. The behaviours were expressed as a proportion of the overall scan for each individual per day and analysed using Wald test in a generalised linear model (GsLM), which fitted a binomial distribution with a logit function. For all analyses, any significant difference was considered at $p < 0.05$. IBM® SPSS® Statistics (version 26) was used to analyse all the data.

RESULTS

The Average Milk Yield Before and After Music

The cows' milk production was found to be affected by music and parity (Table 2). However, the interaction between the two factors had no impact on the milk yield ($F = 0.008$, $df = 1$, $p = 0.928$).

Table 2
Average milk yield with Standard Error of Mean by female Jersey cattle based on music availability and parity

	Average milk yield (L)	*F*-test
Music availability		
No	3.454 (0.261)	$F_1 = 4.771$,
Yes	2.708 (0.220)	$p = 0.041$
Parity		
Primiparous	2.543 (0.241)	$F_1 = 9.943$,
Multiparous	3.619 (0.241)	$p < 0.05$

Behaviours Observation

Among the six behaviours analysed, feeding and rumination were found to be significantly affected by both music and parity (Table 3). When there was no music, the cows performed significantly higher proportions of feeding and rumination than when music was played (Table 3). Higher proportions of rumination and feeding were also observed in primiparous cows compared to multiparous cows. It contrasted with standing behaviour, whereby multiparous cows displayed a significantly higher proportion of standing than primiparous cows. However, no significant effect of music was found on the proportion of standing in the dairy cattle. There was a tendency for music and parity to affect licking mineral behaviour (Table 3). On the other hand, both music and parity did not significantly affect the proportion of walking or lying behaviour observed in the cows (Table 3).

Table 3
Mean proportion of observation with Standard Error of Mean, where specific behaviours were displayed by female Jersey cattle observed during scan sampling (feeding, rumination, standing, licking mineral, walking, and lying), based on music and parity

Behaviour	Music availability		Parity	
	No	Yes	Primiparous	Multiparous
Feeding	0.38 (0.029)	0.30 (0.020)	0.39 (0.027)	0.29 (0.022)
	Wald = 5.508, $df = 1$, $p = 0.019^{\#}$		Wald = 8.652, $df = 1$, $p < 0.05$*	
Rumination	0.38 (0.29)	0.31 (0.020)	0.40 (0.027)	0.29 (0.022)
	Wald = 4.788, $df = 1$, $p = 0.029^{\#}$		Wald = 9.219, $df = 1$, $p < 0.05$*	
Standing	0.15 (0.021)	0.16 (0.017)	0.09 (0.015)	0.26 (0.021)
	Wald = 0.169, $df = 1$, $p = 0.681$		Wald = 35.506, $df = 1$, $p < 0.05$*	
Licking mineral	0.02 (0.009)	0.04 (0.008)	0.02 (0.007)	0.04 (0.011)
	Wald = 1.959, $df = 1$, $p = 0.162$		Wald = 3.420, $df = 1$, $p = 0.064$	
Walking	0.04 (0.012)	0.02 (0.006)	0.03 (0.009)	0.04 (0.009)
	Wald = 2.100, $df = 1$, $p = 0.147$		Wald = 0.505, $df = 1$, $p = 0.477$	
Lying	0.20 (0.024)	0.22 (0.018)	0.22 (0.023)	0.20 (0.019)
	Wald = 0.302, $df = 1$, $p = 0.583$		Wald = 0.727, $df = 1$, $p = 0.394$	

Note. # = Significant differences in behaviours displayed between periods with and without music ($p < 0.05$); * = Significant differences in behaviours displayed between parity ($p < 0.05$)

DISCUSSION

In this study, the cows produced significantly less milk when exposed to music than when there was no music. It was similar to the findings obtained by Donghai et al. (2018) and Ganesh (2020), which showed a decrease in the milk yield produced by Holstein dairy cattle after being exposed to ragas, African percussion, and rock music (Donghai et al., 2018; Ganesh, 2020). However, the types of music used in this study were French classical piano, Richard Clayderman's classical piano, flute, and Mozart's classical music, which were similar to the genres used in previous studies that were found to generate a positive effect on milk production (Ganesh, 2020; Liu et al., 2017; Ma & Wang, 2020). One of the reasons for these contradictory results may be the ambient temperature in the cowshed. In the study conducted by Ganesh (2020), the cows recorded significantly lower milk yields when exposed to flute music in the summer compared to during the winter, which demonstrated the significant role of the interaction between music and ambient temperature in milk production. It reflects the finding in the current study as the weather during the experiment was constantly warm and sunny, which may have affected the cows' milk production to some extent, even though all the music played, including flute music, has been shown to have a positive impact on milk yields in previous studies. In addition, it was also possible that the selection of music used in this study may not have been adequate for calming the cows during milking, especially

when combined with the loud noise of the AMS engine, which may have stressed the cows. Uncomfortable environmental conditions can affect the secretion of adrenaline and pituitary gland hormones. Adrenaline hormones diffuse into the bloodstream, particularly into the umbilical cord, causing the udder muscles to tighten and resulting in decreased milk production (Mutiasari et al., 2018).

The decrease in milk yield after being exposed to music may be due to the cows' handling before the milking procedure, which might have distressed them. Man-animal interaction is one of the most common factors that may cause stress to livestock (Ciborowska et al., 2021). In this study, the selected cows had to be fetched, handled, and steered in the right direction for their milking sessions using the AMS, which was different from their normal days before the experiment was conducted. During the five days of the experiment, before the music was played, the cows were also fetched and steered, but the effects of the cows' stress perhaps began to show a few days later when the music started to be played. Further investigation may be needed to clarify this. However, some studies have identified a positive effect on milk production after cows were exposed to music (Ganesh, 2020; Liu et al., 2017; Ma & Wang, 2020).

Dairy cattle produce more milk when they hear music that mimics their normal pulse, which explains why light music increases lactation (Liu et al., 2017). An increase in the gamma-aminobutyric acid (GABA) content in the blood of cattle, thus promoting the secretion of growth hormones, could also be seen when light music was played (Ma & Wang, 2020). Other studies also found no association between milk yield and music (Kochewad et al., 2022; Lemcke et al., 2021). Even though the highest milk output occurred when no music was played, the cows demonstrated relaxed behaviours such as sleeping and relaxing when some music was played, indicating reduced stress and increased welfare (Kemp, 2020).

The milk yield for multiparous cows was significantly higher than that of primiparous cows. This result was supported by previous studies, which stated that multiparous Holstein cows produced more milk than primiparous cows (Meikle et al., 2004; Morales-Piñeyrúa et al., 2022; Pahl et al., 2015; Wathes et al., 2007). It was a biologically normal lactation curve for primiparous cows, which usually peak later, produce less milk, and are more persistent than multiparous cows (Siewert et al., 2019). Cows milked more frequently generate more milk, which is why multiparous cows produce a higher milk yield (Siewert et al., 2019).

Feeding is one of the major behaviours in ruminants, and they spend much time on it (Llonch et al., 2018). It was interesting to find that music impacted the feeding behaviour of the Jersey dairy cattle. In this study, the cows fed more without music than when music was played. There were mixed results regarding feeding behaviour and music availability. A study on other animals found that quails subjected to

random, metallic, reggae, or classical music consumed significantly less food than quails not exposed to any form of music (Cabaral et al., 2017). Some other studies have shown results that contradict this study, whereby music was found not to influence the feeding behaviour of piglets, while classical music was observed to enhance broilers' feed intake and time spent eating (Li et al., 2019; Zhao et al., 2020). According to Llonch et al. (2018), animal well-being is linked to feeding behaviour, while Kriengwatana et al. (2022) stated that music has been suggested to improve welfare in animals by increasing the complexity of the surroundings that animals might sense, perceive, and respond to, which triggers brain development and the expression of a wide range of behaviours.

Animals may respond differently to different sounds, frequencies, and intensities (Li et al., 2019). Dairy cattle can pick up frequencies that range from 23 to 35 kHz, which includes some of the ultrasonic range from 16–35 kHz and noises lower than 85 dB (Alworth & Buerkle, 2013; Lemcke et al., 2021). However, it has been reported that cattle exposed to sounds of 80 dB consumed less food, became agitated, and had faster heart rates (Ciborowska et al., 2021; Solan & Józwik, 2009). It could explain why the cows in this study fed less in periods with music, even though the maximum noise audible to cows was 85 dB, as other studies recommended, which was the level applied in this study (Arnold et al., 2007; Lemcke et al., 2021; Weeks et al., 2009). The music played in this study could have been misinterpreted by the cows as noise, causing them to eat less.

Parity also affected feeding behaviour, as primiparous cows were observed to feed significantly more than multiparous cows. Some studies have shown contradictory results, whereby multiparous cows consumed more food than primiparous cows due to their higher social dominance, which typically produces a greater quantity of milk (Bach et al., 2006; Maekawa et al., 2002). Primiparous cows ate significantly more during the 10-min scan sampling period than multiparous cows, which might be explained by the fact that they required more time to eat and had less competition at the manger to acquire their maximum feed intake (Maekawa et al., 2002). Furthermore, younger cows consume food more slowly and spend more time feeding than older cows (Beauchemin, 2018; Neave et al., 2017). The lower proportion of feeding by multiparous cows might also have been due to the longer time spent milking (Hart et al., 2013), although this parameter was not specifically recorded in this study.

Rumination was discovered to be voluntarily regulated by the animals, and if they were distracted in any way, such as when they were being milked or fed, they would discontinue ruminating (Beauchemin, 2018; Paudyal, 2021). The Jersey dairy cattle in this study ruminated more without music than when music was played. These results contrast with other findings that music can improve animal welfare for some animals like rats, chimpanzees, dogs, and even dairy cattle (Alworth & Buerkle, 2013; Dhungana et al., 2018; Kemp, 2020). However, as mentioned above, the cows in this study might have been disturbed by the

music played and perceived as noise due to its intensity (Dhungana et al., 2018). Some studies have stated that noises above 85 dB may potentially increase stress or anxiety (Alworth & Buerkle, 2013; Dhungana et al., 2018). The cows might still not have become used to having music because the data was collected only one day after they were habituated to the presence of music.

In this study, primiparous cows ruminated more than multiparous cows, which other studies have also found (Pollock et al., 2022; Stauder et al., 2020). These results could also be observed in other animals, such as early weaned primiparous beef cows, which ruminate more frequently than multiparous beef cows, and primiparous ewes, which ruminate significantly more frequently than multiparous ewes (Ungerfeld et al., 2011; Yusof, 2019). Cows with lower feeding rates due to feeding restrictions or diet composition tend to display higher rumination times to compensate for the longer particle sizes of the ingested feed (Beauchemin, 2018; Mikuła et al., 2022). It has also been observed that rumination peaks four hours after feeding (Beauchemin, 2018). It is consistent with the outcomes of this study, as the primiparous cows, which were lower on the social hierarchy than multiparous cows, ate later due to the presence of multiparous cows (Bach et al., 2006). Multiparous cows may be hungry and bothered by the cowherd's pellet barrels, thus reducing their rumination time (Paudyal, 2021; Schirmann et al., 2012).

It was not fully clear why multiparous cows stood more than primiparous cows in this study. Many multiparous cows might have been in the shed, which increased their competitiveness at the feed bunk (Proudfoot et al., 2009). Since multiparous cows almost always feed first during the provision of concentrate feed, this might have contributed to the greater competition between them. Additionally, it was observed that the cows started to stand approximately 15 min before their pellet feeding time or when they heard the pellet barrels, so that they may have been anticipating the provision of concentrate feed by the cowherd.

In this study, the cows tended to lick minerals in periods with music compared to when no music was played. Ruminants, particularly cows, would lick mineral blocks, which provide various minerals vital to their well-being; this may increase milk production with a high-fat content (Kikelomo, 2016). As this was the first study to investigate the relationship between the mineral-licking behaviour of cows with music available, the results cannot be explained in detail. Ruminants need to supplement their diet with mineral (salt) licking when forage or feed sources are mineral-deficient, which might also prevent other behaviours like licking each other, licking wood, and fence chewing (Kikelomo, 2016). It could be seen as a good sign, implying that the cows tended to add minerals into their body while the music was played because music has been suggested to improve their welfare. Multiparous cows tended to lick the mineral more than primiparous cows. Increased mineral intake improves milk production and growth rates,

according to McDowell (2002), which could be associated with higher milk production in multiparous compared to primiparous cows (Meikle et al., 2004; Morales-Piñeyrúa et al., 2022; Pahl et al., 2015; Wathes et al., 2007). Most of the studies on the use of music to milk production and behaviour have been conducted on Holstein dairy cows, which opens the possibility that breed is one of the factors that may have influenced the outcomes of this study.

In this study, no differences between parity and music could be observed in terms of walking and lying behaviours. Unfortunately, no study has yet been conducted to investigate the relationship between music and walking. However, previous findings in dogs showed a significant increase in lying behaviour when classical music was played (Bowman et al., 2017). The amount of time cows spent lying down might provide insights into their welfare and health (Lovarelli et al., 2020; Temple et al., 2016; Tucker et al., 2021). Therefore, the results suggest that the cows may already have been in good condition, or they were not bothered by the presence of music because they would still lie down or walk whether music was played or not.

CONCLUSION

Overall, the results showed that music negatively impacts milk production in primiparous and multiparous Jersey dairy cattle. It could be because the cows perceived the music as a cause of stress and disturbance. Interestingly, the music played during this study had an impact on the behaviour of the cows, whereby they were observed to display lower proportions of feeding and rumination when the music was played compared to when no music was played, possibly due to discomfort or anxiety. Despite the negative effect of music on milk production, this study may still provide useful information for dairy farmers pursuing music therapy for their dairy cows. As suggested by other researchers, music may have a long-term influence on milk production. Therefore, to better understand the effects of music on the milk yield and behaviour of dairy cattle, it may be beneficial to collect the data over a longer period and consider other factors such as parity, breed, housing environment, and music selection.

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Low Genetic Diversity of Vector-Borne Haemoparasites in Dogs and Their Ticks Revealed Local and Long-Range Transmission in Peninsular Malaysia

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ABSTRACT

Molecular methods coupled with phylogenetic analysis are sensitive tools for detecting and classifying parasites. This study used nuclear and mitochondrial gene markers to investigate the host-vector interaction of the vector-borne haemoparasites. The population genetic structures of important vector-borne haemoparasites in dogs, namely, *Anaplasma platys*, *Ehrlichia canis*, *Babesia vogeli*, and *Babesia gibsoni*, were determined from the nuclear gene of 16S or 18S rRNA gene, *gltA* and *groESL* and mitochondrial gene of *COX1* across dogs and vector ticks. A total of 220 blood samples and 140 ticks were collected from shelter dogs in Peninsular Malaysia. Out of the positive samples for the vector-borne haemoparasites, 28 positive blood isolates and six tick isolates were selected and characterised. There was a low diversity in tick sequences, while varying degree of variability was observed in dogs' sequences. Overlapped haplotypes were observed in sequences of dogs and ticks, revealing the possibility of the same infection origin. No regional separation was detected, but similar haplotypes from different regions were observed. These findings contribute to the epidemiology of vector-borne haemoparasites in dogs in Malaysia.

Keywords: Dogs, genetic structures, haemoparasites, ticks

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INTRODUCTION

Ticks can transmit a wide range of diseases, including viral, bacterial, and protozoan, to animal hosts (Otranto & Wall, 2008). *Rhipicephalus sanguineus* sensu lato (*R. sanguineus*), the brown dog tick, now known as *Rhipicephalus linnaei* (Šlapeta et al., 2021) is an important vector of canine vector-borne haemoparasites (VBHs) in Southeast Asia (SEA), and the primary tick infesting dogs in Malaysia (Dantas-Torres, 2010; Low et al., 2017; Nguyen et al., 2019; Prakash, Low, Vinnie-Siow, et al., 2018; Sipin et al., 2020). The higher prevalence of VBHs in dogs and low prevalence in ticks were evident in previous studies (Galay et al., 2018; Low et al., 2018; Prakash, Low, Tan et al., 2018; Prakash, Low, Vinnie-Siow, et al., 2018; Sipin et al., 2020). Nevertheless, stray dogs were more susceptible to VBHs infection compared to pet dogs due to the high exposure to arthropod vectors (Cao et al., 2015). The infection rate in dogs strongly correlates to exposure to ticks and VBHs (Jennett et al., 2013).

The development of molecular methods combined with phylogenetic analysis provides a highly sensitive diagnostic tool for detecting and classifying these VBHs into their closely related species according to genetic makeup. Molecular methods and phylogenetic trees have been used in phylogenetic studies of parasites in co-relation to their geographical distribution, climate change effects, and host-specificity (Clare, 2011; Martin et al., 2006; Martins et al., 2009; Morgan et al., 2012). Polymerase chain reaction (PCR)-based detection methods are commonly used for pathogen detection and species identification. The gene markers, namely 16S rRNA and 18S rRNA, are markers that are commonly used in canine VBHs detection using molecular techniques (Koh et al., 2016; Low et al., 2018; Mokhtar et al., 2013; Nazari et al., 2013; Prakash, Low, Vinnie-Siow, et al., 2018). However, these common nuclear gene markers were reported to be highly conserved (Harrus & Waner, 2011; Kamani, Baneth, et al., 2013; Vargas-Hernandez et al., 2012; Zhang et al., 2008). Higher resolution marker such as the mitochondrial DNA (mtDNA) gene (e.g., cytochrome c oxidase I (*COX1*)) is more variable than the nuclear gene. Thus, the present work attempts to use the *COX1* gene, *groESL* gene, and citrate synthase (*gltA*) gene for the haplotype and nucleotide diversities analyses of selected VBHs.

Several studies in Malaysia have started focusing on phylogenetic analysis of VBHs in dogs and their arthropod vector, tick (Low et al., 2018; Prakash, Low, Vinnie-Siow, et al., 2018; Sipin et al., 2020). Nonetheless, further studies are required on the correlation of the presence of VBHs in dogs with the arthropod vector to investigate the origin of the infection. Therefore, this study aims to characterise VBHs in dogs and their ticks using molecular techniques, which focus on investigating the genetic overlap of VBHs in dogs and ticks and the origin of infection using phylogenetic and haplotype network analyses.

MATERIALS AND METHODS

Ethical Approval

The Institutional Animal Care and Use Committee (IACUC) Universiti Putra Malaysia (AUP no: UPM/IACUC/AUP-R028/2018) approved the application for animal ethical clearance. Additionally, consent was obtained from the animal shelters before sampling activities were conducted. The owner of the shelters was briefed regarding the purpose of the study, and the handling of the animals during the sample collection was in accordance with animal ethics laws.

Parasites Isolation

Details of sample collection, tick identification, and DNA isolation were performed as described by Sipin et al. (2020). Briefly, a total of 220 dog blood samples and 140 tick samples were collected from 10 animal shelters in North (Pulau Pinang, Kedah, Perak), South (Johor), East (Pahang), and Central (Selangor) regions of Peninsular Malaysia for the detection of VBHs. Shelter dogs were restrained and subjected to blood and tick collection, where blood was sampled from the cephalic vein into ethylenediamine tetraacetic acid (EDTA) blood tubes, and ticks were removed using forceps and placed in small plastic tubes. The morphological inspection of ticks was performed under a stereomicroscope before DNA extraction.

A total of 28 positive blood isolates and six tick isolates screened from 220 shelter dogs were subjected to further analyses (Table 1). Out of the 28 positive blood isolates used in this study, 11 samples were from each North and Central region, five were from the East region, and only one was from the South region. In addition, four tick isolates used were from the North region and one isolate from each South and Central region.

Table 1
VBHs isolates subjected to molecular characterisation

Region	Sample	ID no.	VBH detected
North (N)	Blood	Nb15	*Anaplasma platys*
		Nb2	*Anaplasma platys*
		Nc13	*Anaplasma platys*
		Nd4	*Anaplasma platys*
		Na12	*Ehrlichia canis*
		Nb9	*Ehrlichia canis*
		Nc6	*Ehrlichia canis*
		Nc7	*Ehrlichia canis*
		Nd15	*Ehrlichia canis*
		Nb1	*Babesia gibsoni*
		Nd7	*Babesia vogeli*
	Tick	Nb17 (11)	*Ehrlichia canis*
		Nb17 (32)	*Ehrlichia canis*
		Nc15	*Ehrlichia canis*
		Nb4	*Babesia vogeli*

Table 1 *(continue)*

Region	Sample	ID no.	VBH detected
South(S)	Blood	Sa2	*Babesia gibsoni*
East(E)	Blood	Ea7	*Anaplasma platys*
		Ea8	*Anaplasma platys*
		Ea11	*Anaplasma platys*
		Ea6	*Ehrlichia canis*
		Ea14	*Ehrlichia canis*
	Tick	Ea8	*Anaplasma platys*
Central(W)	Blood	Wa19	*Anaplasma platys*
		Wd25	*Anaplasma platys*
		Wa19	*Ehrlichia canis*
		Wb20	*Ehrlichia canis*
		Wb23	*Ehrlichia canis*
		Wd1	*Ehrlichia canis*
		Wa1	*Babesia vogeli*
		Wb18	*Babesia vogeli*
		Wd2	*Babesia vogeli*
		Wb25	*Babesia gibsoni*
		Wd25	*Babesia gibsoni*
	Tick	Wd22	*Anaplasma platys*

Molecular Technique

DNA extraction of ticks and blood was conducted using commercially available extraction kits, DNEasy Blood and Tissue Kit (Qiagen, Germany). All DNA was eluted in 100 µl of elution buffer and stored at -20°C before PCR screening.

The PCR amplification for 16S rRNA and *groESL* gene for *A. platys*, 18S rRNA and *COX1* gene for *B. gibsoni*, 18S rRNA and *COX1* gene for *B. vogeli*, and 16S rRNA and *gltA* gene for *E. canis* from both blood and tick samples were performed with the cycling conditions indicated in Table 2.

PCR products were sequenced using the cycle sequencing technology (dideoxy chain termination/cycle sequencing) on ABI PRISM 3730xl Genetic Analyser (Applied Biosystems, USA). The sequences obtained were subjected to the Basic Local Alignment Search Tool (BLAST) identity search to compare with known sequences of *B. gibsoni*, *B. vogeli*, *A. platys*, and *E. canis* available in the NCBI GenBank.

Phylogenetic Analysis

The sequences obtained via sequencing were aligned using DAMBE5 (Xia, 2018). MEGA X was applied for phylogenetic relationship determination using a maximum likelihood (ML) algorithm (Kumar et al., 2018). At least 1,000 replicates were used to estimate each species' bootstrap values. Nucleotide sequences of *A. platys* (16S rRNA and

Table 2
Primers and annealing temperatures for PCR reaction used in this study

Haemoparasites	Primer sequence (5'-3')	Gene target (bp)	Annealing temperature (°C)	Reference
Babesia gibsoni	Gib599F (5'-CTCGGCTACTTGCCTTGTC-3') Gib1270R (5'-GCCGAAACTGAAATAACGGC-3')	18S rRNA (690)	62	Otranto et al. (2009)
	Bg-cox1-F (5'-CTTCAGCCAATAGCTTTCTGTTTG-3') BG-cox1-R (5'-CCTGAGGCAAGTAAACCAAATAT-3')	*COX1* (150)	62	Qurollo et al. (2017)
Babesia vogeli	C172F (5'-GTTTATTAGTTTGAAACCCGC-3') C626R (5'-GAACTCGAAAAAGCCAAACGA-3')	18S rRNA (450)	57.5	Otranto et al. (2009)
	CoxBV-F (5'-TGAGTGGCGCAAATTTTGTA-3') CoxBV-R (5'-TGTCTGTCAAGAAAAACCATTAGC-3')	*COX1* (166)	62	Primers were designed based on accession no. KX426022.1 in the National Center for Biotechnology Information (NCBI) GenBank
Anaplasma platys	PlatysF (5'-AAGTCGAACGGATTTTTGTC-3') PlatysR (5'-CTTTAACTTACCGAACC-3')	16S rRNA (500)	60	Beall et al. (2008)
	GroESL-F (5'-AAGGCGAAAGAAGCAGTCTTA-3') GroESL-R (5'-CATAGTCTGAAGTGGAGGAC-3')	*groESL* (724)	60	Inokuma et al. (2002)
Ehrlichia canis	ECA (5'-AACACATGCAAGTCGAACGGA-3') HE3 (5'-TATAGGTACCGTCATTATCTTCCCTAT-3')	16S rRNA (400)	60	Wen et al. (1997)
	Ecanis(gltA)-F (5'-GCTGATCATGAGCAAAATGC-3') Ecanis(gltA)-R (5'-TTGACCAAAACCCATTAGCC-3')	*gltA* (400)	62	Primers were designed based on accession no. LC428206.1 in NCBI GenBank

groESL), *B. vogeli* (18S rRNA and *COX1*), *B. gibsoni* (18SrRNA and *COX1*), and *E. canis* (16S rRNA and *gltA*) in blood and tick samples were used for analyses in the phylogenetic tree. Furthermore, selected sequences in GenBank were used as an outgroup reference sequence for comparison to sequences obtained from the molecular work.

The present study nucleotide sequences were submitted to the NCBI GenBank database under the following accession numbers: *B. gibsoni* 18S rRNA [Blood (MN068981-84)], *B. vogeli* 18S rRNA [Blood (MN075251-54), Tick (MN194598)], *A. platys* 16S rRNA [Blood (MN075275-83), Tick (MN159064-65)], *A. platys groESL* [Blood (OP104926-35), Tick (OP184817)], *E. canis* 18S rRNA [Blood (MN075258-68), Tick (MN159066-67)], and *E. canis gltA* [Blood (OP104926-35), Tick (OP104936-37)].

Pairwise differences (pi) indices and population genetic estimators were calculated in ARLEQUIN version 3.5.2.1 using individual and combined dogs or tick samples sequences data, haplotype diversity h, nucleotide diversity π (Excoffier & Lischer, 2010). Meanwhile, minimum spanning trees were computed using HapStar version 0.7 (Teacher & Griffiths, 2011). Population genetic differences were estimated using ARLEQUIN by analysis of molecular variance (AMOVA) using predefined groups based on the host, comprising dogs and ticks.

RESULTS

Sequence Alignment

The phylogenetic analysis of VBHs obtained in the study was inferred from sequences, as presented in Table 3. The size of each studied gene and the similarity between blood and tick sequences are shown in Table 4. The size of the studied sequence ranges from 116 to 681 bp. Most of the nucleotides in the blood sequence were monomorphic (95.7-100%), with a few variable sites for dogs ranging from 1 to 10 bp. All tick sequences for this study's studied gene of interest were monomorphic.

Table 3
Number of sequences inferred for phylogenetic analysis for VBH species in the study

VBH	Gene	No. of sequences
Anaplasma platys	16S rRNA	11
	groESL	9
Ehrlichia canis	16S rRNA	13
	gltA	12
Babesia vogeli	18S rRNA	5
	COX1	5
Babesia gibsoni	18S rRNA	4
	COX1	3

Phylogenetic Analysis

A phylogenetic tree based on the 16S rRNA gene of *A. platys* and *E. canis* revealed no significant clades and failed to reflect any correlation with the host and pattern of parasite transmission. Most of the sequences appeared similar and sequences from other regions of the world (Figure 1), with a few different sequences (highlighted in blue). A similar result was observed for the *groESL*

Table 4
Sequences alignment of VBH from the study against outgroup sequence in GeneBank

VBH	Gene	Samples	Monomorphic	Polymorphic
Anaplasma platys	16S rRNA	Blood	257 bp (97.70%)	6 bp (2.30%)
		Tick	263 bp (100%)	-
	groESL	Blood	681 bp (100%)	-
		Tick	Only one tick sequence is available	-
Ehrlichia canis	16S rRNA	Blood	374 bp (95.65%)	17 bp (4.35%)
		Tick	391 bp (100%)	-
	gltA	Blood	230 bp (99.57%)	1 bp (0.43%)
		Tick	231 bp (100%)	-
Babesia vogeli	18S rRNA	Blood	421 bp (99.76%)	1 bp (0.24%)
		Tick	Only one tick sequence is available	
	COX1	Blood	131 bp (100%)	-
		Tick	131 bp (100%)	-
Babesia gibsoni	18S rRNA	Blood	616 bp (98.40%)	10 bp (1.60%)
		Tick	None tick positive	
	COX1	Blood	114 bp (98.28%)	1 bp (1.72%)
		Tick	None tick positive	

gene of *A. platys* (Figure 2) and the *gltA* gene of *E. canis* (Figure 3). In addition, a similar result was observed in the phylogenetic tree based on the 18S rRNA gene (Figure 4) and *COX1* gene (Figure 5) of *B. gibsoni* and *B. vogeli*, revealing no significant clades and low diversity.

For the 16S rRNA gene of *A. platys* [Figure 6(a)] and *E. canis* [Figure 6(b)] haplotype network, four haplotypes were observed, with one common haplotype that was shared between both hosts. Only two haplotypes were observed for the *E. canis gltA* gene haplotype network, where one common haplotype was shared between both hosts [Figure 6(c)]. Additionally, four haplotypes were detected for the 18S rRNA gene of *B. vogeli*, comprising three diverse haplotypes in dogs and one separate haplotype for ticks [Figure 6(d)].

Haplotype and Nucleotide Diversity

The haplotype and nucleotide diversity for *A. platys* 16S rRNA, *E. canis* 16S rRNA, and *gltA* genes revealed no variation in the ticks and a low variation in dogs (Table 5). The haplotype and nucleotide diversity for *B. vogeli* 18S rRNA and *B. gibsoni* 18S rRNA and *COX1* genes depicted a low level of variation in dogs. Comparisons of haplotype and nucleotide diversity were generated for ticks as none of the tick samples were positive for *B. gibsoni*, while only one tick sample was positive for *B. vogeli* (Table 5).

DISCUSSION

The population genetic structure of *A. platys*, *E. canis*, *B. vogeli*, and *B. gibsoni* among dogs and the associated vector-

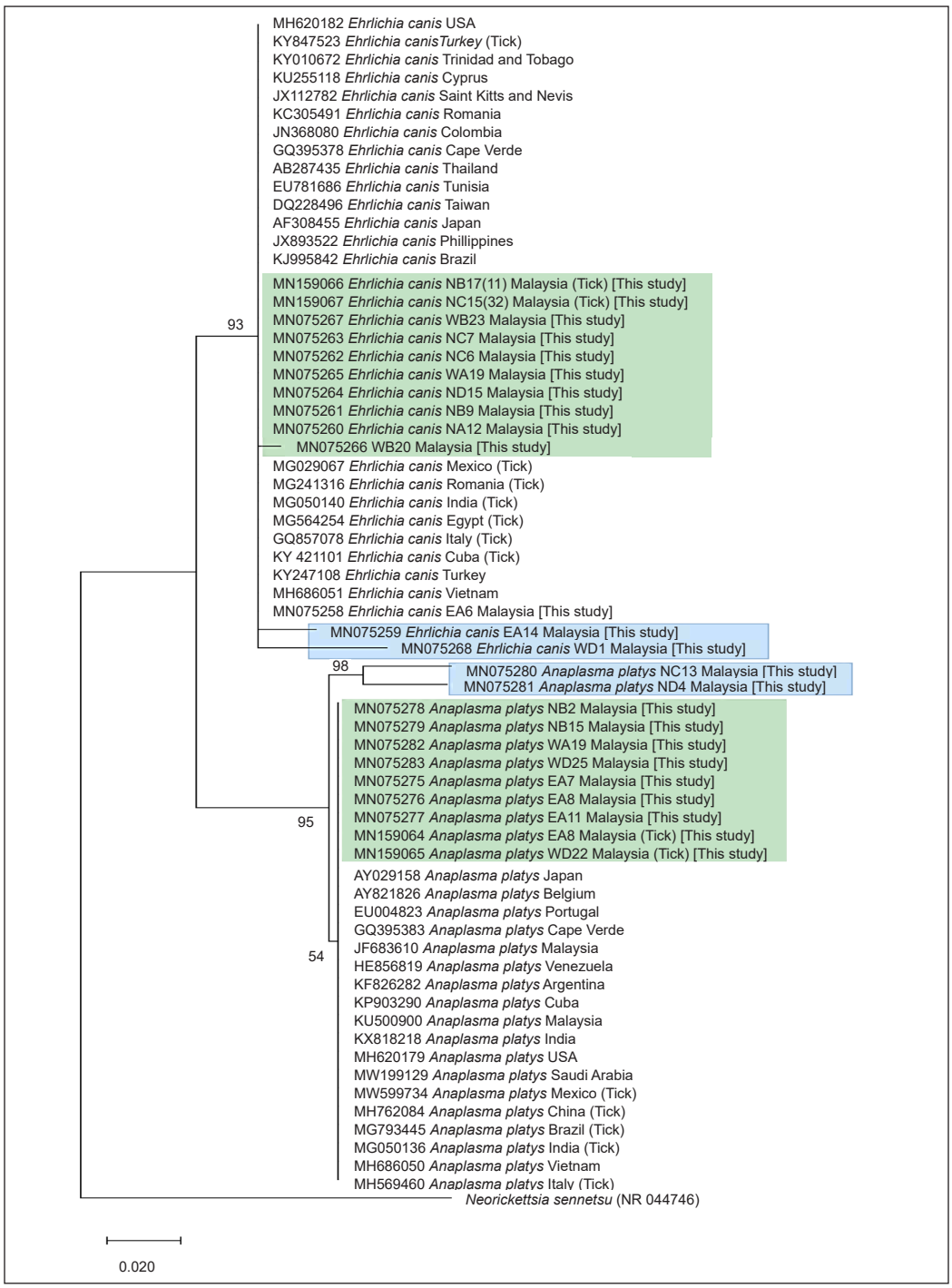

Figure 1. Phylogenetic analysis of *Anaplasma platys* and *Ehrlichia canis* based on the 16S rRNA gene obtained in this study and other countries using the Maximum Likelihood method (Kimura-two-parameter model). *Neorickettsia sennetsu* is provided as an outgroup species. MEGA X software with 1,000 bootstrap replications was used to create the phylogenetic tree

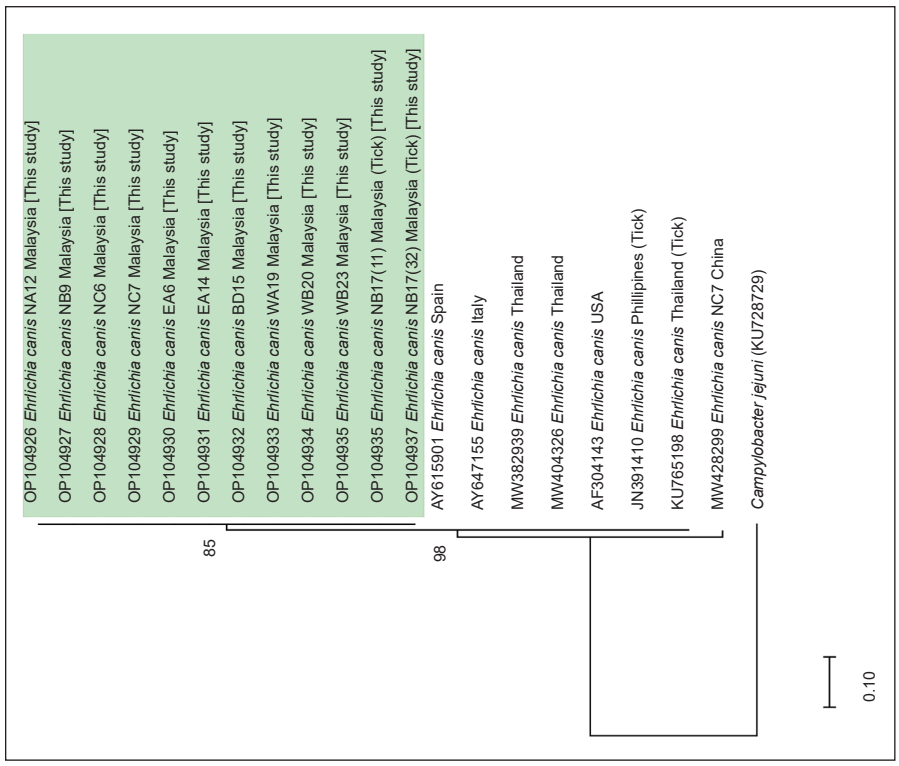

Figure 3. Phylogenetic analysis of *Ehrlichia canis* based on the *glnA* gene obtained in this study and other countries using the Maximum Likelihood method (Kimura-two-parameter model). *Campylobacter jejuni* is provided as an outgroup species. MEGA X software with 1,000 bootstrap replications was used to create the phylogenetic tree

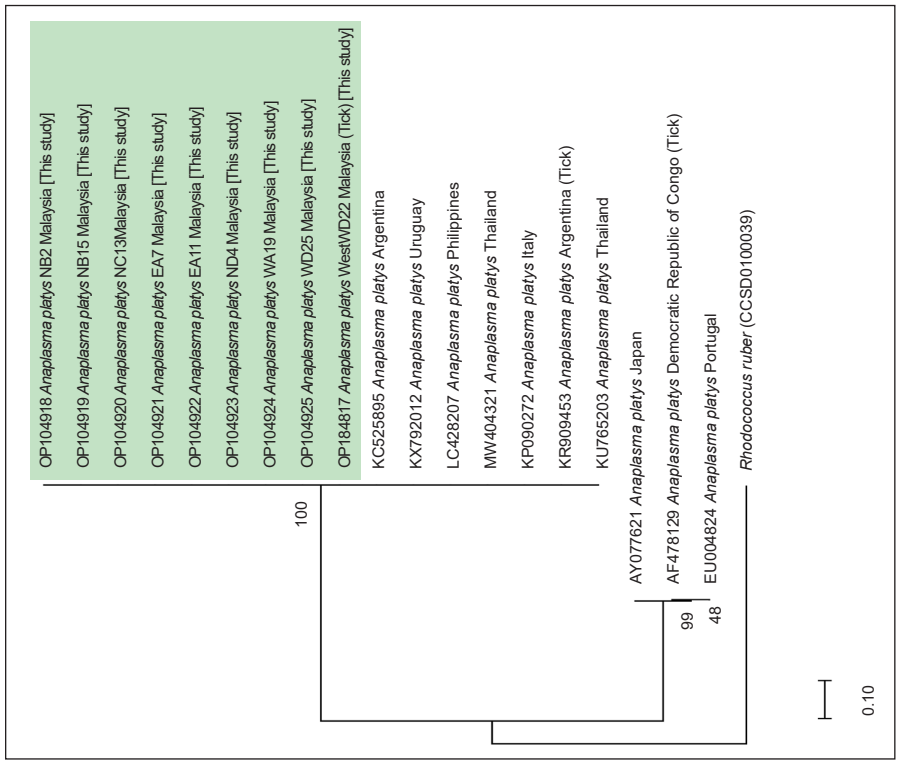

Figure 2. Phylogenetic analysis of *Anaplasma platys* based on the *groESL* gene obtained in this study and other countries using the Maximum Likelihood method (Kimura-two-parameter model). *Rhodococcus ruber* is provided as an outgroup species. MEGA X software with 1,000 bootstrap replications was used to create the phylogenetic tree

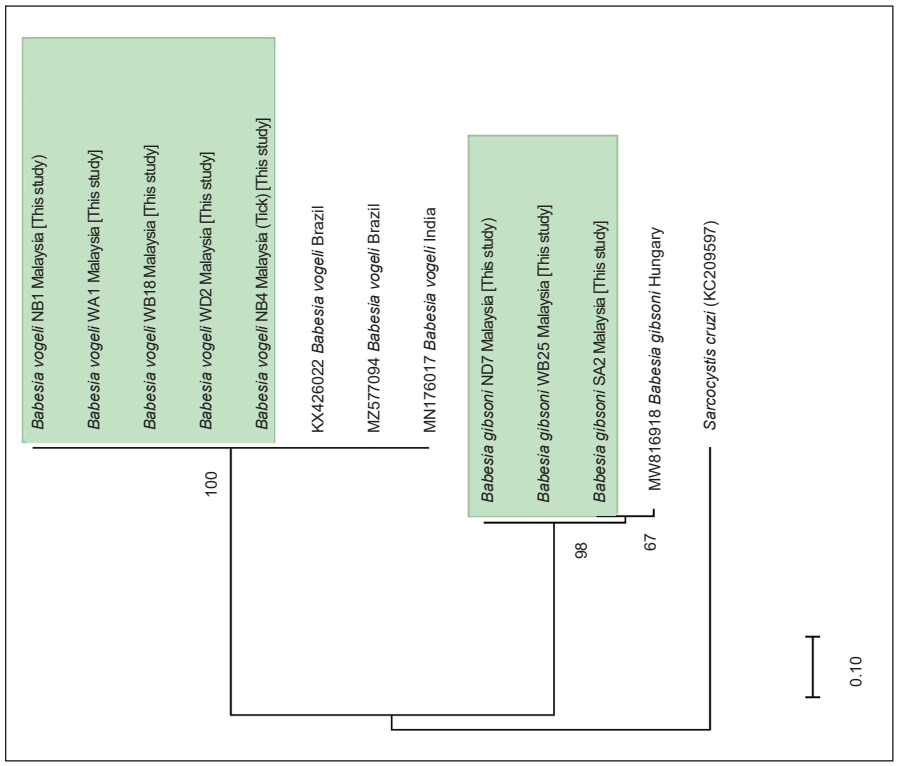

Figure 5. Phylogenetic analysis of *Babesia vogeli* and *Babesia gibsoni* based on the *COX1* gene obtained in this study and other countries using the Maximum Likelihood method (Kimura-two-parameter model). *Sarcocystis cruzi* is provided as an outgroup species. MEGA X software with 1,000 bootstrap replications was used to create the phylogenetic tree

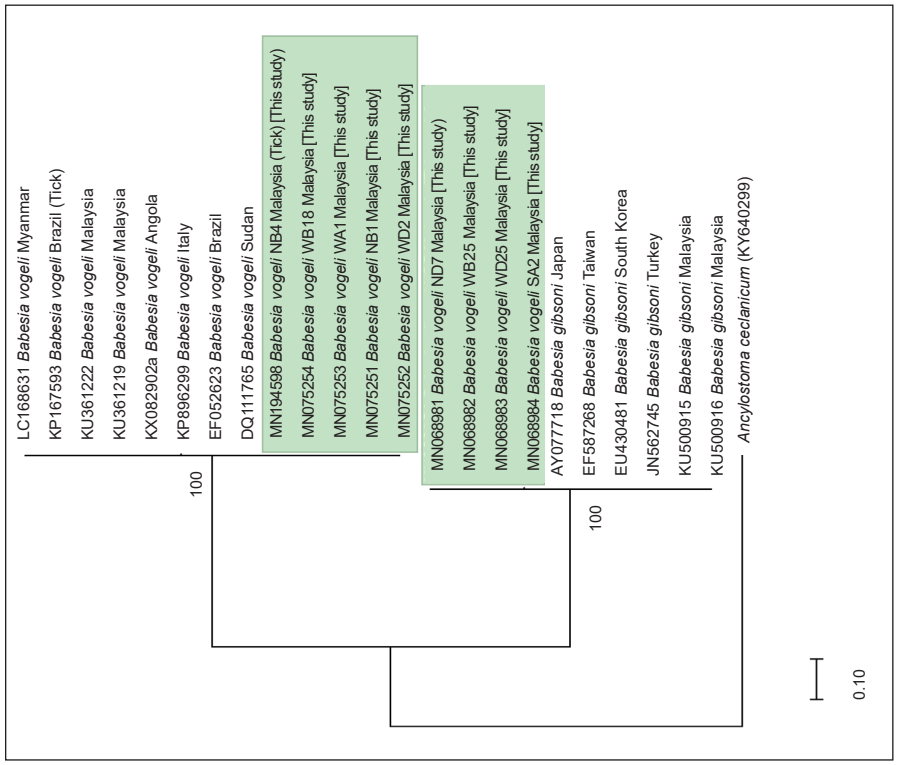

Figure 4. Phylogenetic analysis of *Babesia vogeli* and *Babesia gibsoni* based on the 18S rRNA gene obtained in this study and other countries using the Maximum Likelihood method (Kimura-two-parameter model). *Ancylostoma ceylanicum* is provided as an outgroup species. MEGA X software with 1,000 bootstrap replications was used to create the phylogenetic tree

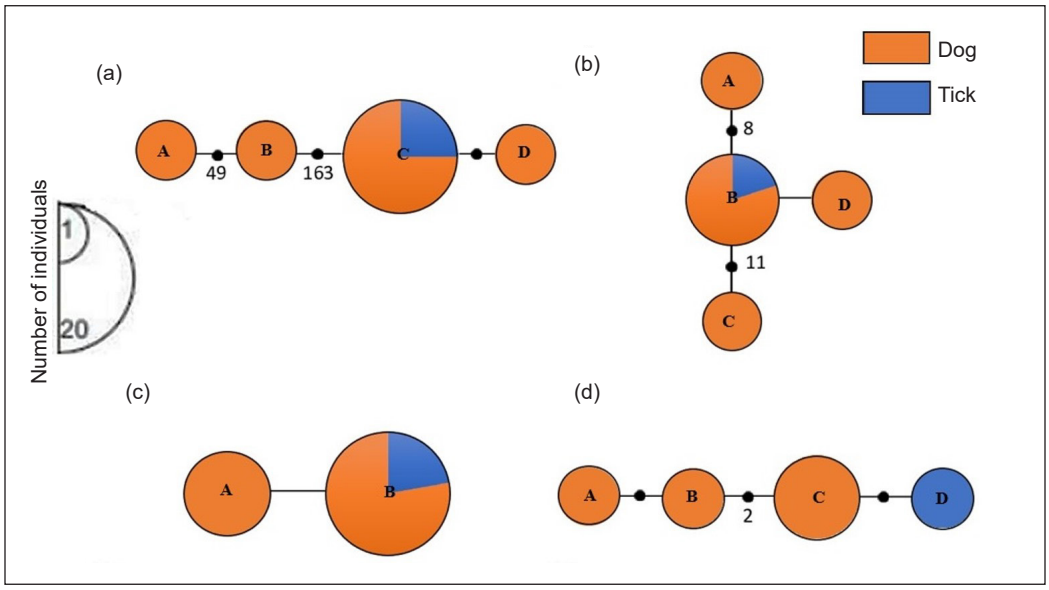

Figure 6. Haplotype network of *Anaplasma platys* 16S rRNA (a), *Ehrlichia canis* 16S rRNA (b), *Ehrlichia canis gltA* (c), and *Babesia vogeli* 18S rRNA (d) sequences. The size of the circles in the network is proportional to the frequency of the haplotype in the data set, and the coloured area identifies the sample collected

Table 5
Haplotype (h) and nucleotide diversities (π) based on the gene of interest of Anaplasma platys*,* Ehrlichia canis*,* Babesia vogeli*, and* Babesia gibsoni

Populations	Individuals	Haplotypes	Polymorphic sites	Haplotype diversity	Nucleotide diversity
Anaplasma platys 16S rRNA					
Blood	9	3	6	0.417	0.006
Tick	2	1	0	0.000	0.000
Ehrlichia canis 16S rRNA					
Blood	11	4	17	0.491	0.010
Tick	2	1	0	0.000	0.000
Ehrlichia canis gltA					
Blood	10	2	1	0.467	0.002
Tick	2	1	0	0.000	0.000
Babesia vogeli 18S rRNA					
Blood	4	2	1	0.667	0.002
Tick	-	-	-	-	-
Babesia gibsoni 18S rRNA					
Blood	4	2	10	0.500	0.008
Tick	-	-	-	-	-
Babesia gibsoni COX1					
Blood	3	2	2	0.667	0.013
Tick	-	-	-	-	-

borne haemoparasites in Malaysia were analysed in this study. This study revealed more variable genetic diversity in dogs as a host compared to the tick vector. Overlap was observed in ticks' and dogs' haplotypes except for the 18S rRNA gene of *B. vogeli*. However, the present study was unable to trace the origin of infection, most likely due to a low positive number of tick samples, overlapping of haplotypes between dogs and ticks, and the lack of clade separation according to regions in the phylogenetic tree analysis.

The low diversity in tick sequences and the more variations in dog sequences could be due to the movement of dogs and ticks as the carriers of VBHs. In terms of the tick vector, the haplotype in ticks has a low diversity due to the restricted movement to allow the genetic mixing or admixture event to occur. On the other hand, dogs can move in a broader range of geographical areas; thus, dogs are more likely to spread genetic variation, resulting in better haplotype diversity. It might explain why more haplotypes were observed in dogs than in ticks, as shown in the haplotype networks. The movement of stray dogs before being brought to the shelter might contribute to spreading ticks and haemoparasites. The role of dogs as long-distance hosts and carriers of the infection, as well as the capability of the tick vectors to remain attached to their host during travelling, might lead to the introduction of the same infection in other regions of the country. These events may contribute to the presence of the same haplotype in different regions observed in this study. Moreover, some of the sampled shelters accepting dogs from other regions of the country could also contribute to the haplotype sharing between sequences obtained from different regions. Another theory that might play a role in the dispersal of pathogens from one region to another is the presence of a variety of hosts available for tick infestation. Although primarily infesting dogs, *R. sanguineus* sensu lato, now known as *R. linnaei* (Šlapeta et al., 2021), could also infest a wide range of hosts such as mammals, birds, and humans (Dantas-Torres, 2010). The mobility of these hosts throughout the country might be considered one of the factors influencing the genetic dispersal and transmission of the infection among dogs and ticks in different regions, which can also explain the different haplotypes observed in the same shelter.

The haplotype networks obtained for the 16S rRNA gene of *A. platys* (Figure 6a), 16S rRNA gene of *E. canis* (Figure 6b), and the *gltA* gene of *E. canis* (Figure 6c) revealed that the tick isolates were sharing the same haplotype with some of the blood isolates. The most likely explanation was that the infection in both dogs and ticks originated from the same source, which could be either the tick vector collected, different ticks feeding on the same infected dogs, or the movement mentioned earlier. On the other hand, the tick sampled for the 18S rRNA gene of *B. vogeli* (Figure 6d) showed a separate haplotype from the rest of the blood samples. It might indicate that the infection in ticks does not share the same origin with all the blood samples

collected. A haplotype network was not presented for the *groESL* gene of *A. platys* since the sequence alignment result was monomorphic. Likewise, the haplotype network was not presented for the *COX1* gene of *B. vogeli* since only one tick sample was found positive; thus, the haplotype and nucleotide diversity for tick samples could not be generated.

The 16S rRNA gene of *E. canis* reflected a slightly higher haplotype (0.491) and nucleotide (0.00954) diversities compared to the *gltA* gene of *E. canis*. This result was proportional to the haplotype network for both genes as the 16S rRNA gene of *E. canis* depicted the presence of four haplotypes, whereas only two haplotypes were observed for the *gltA* gene of *E. canis*. The *gltA* or citrate synthase gene encrypts an important enzyme in the tricarboxylic acid cycle, which is present in almost all living cells and acts as an important regulator for intracellular adenosine triphosphate (ATP) production (Wiegarg & Remington, 1986). These results indicated that the *gltA* gene was more conserved compared to the 16S rRNA gene in contrast to a previous study (Inokuma et al., 2001). The *gltA* gene was different among *Ehrlichia* spp. sequences compared to the 16S rRNA gene. Although the differences were small, the result suggests that the *gltA* could be a tool for advanced molecular work and suitable for comparing closely related species but not within species (Inokuma et al., 2001).

Although *E. canis* is widely distributed globally and has been identified for a long time, little is known about its genetic diversity and strains from geographically distant regions (Hsieh et al., 2010). Recent studies have focused on 16S rRNA genes for molecular work, while there is a dearth of information on other genes (Alhassan et al., 2021; Çelik et al., 2021; Low et al., 2018; Malik et al., 2018; Selim et al., 2021; Sipin et al., 2020). The 16S rRNA gene sequences of *E. canis* have high similarity despite originating from South America, North America, Asia, Europe, Africa, and the Middle East. The similar characteristic of the gene recommends significantly conserved and limited information on the gene diversity. Some other genes that were found to be conserved among isolates worldwide include the OMP-1 family, Dsb, TRP19, and TRP140 (Aguiar et al., 2008; Aguirre et al., 2004; C.-C. Huang et al., 2010; Kamani, Lee, et al., 2013; Yu et al., 2007; Zhang et al., 2008). Two main antibody epitopes: TRP36 and gp36, in the tandem repeat region, were considered to vary among *E. canis* isolates. The studies of the *trp36* gene in *E. canis* strains from the United States, Brazil (only a single strain), Cameroon, Nigeria, and Taiwan *E. canis* identified variations in the number of tandem repeats while a divergent *E. canis* genotype has been identified in Israel (Zhang et al., 2008). A phylogenetic tree of *E. canis* strains based on the gp36 amino acid sequences revealed that the Taiwanese isolates fell into a separate clade, indicating the presence of a novel strain that is yet to be characterised (Hsieh et al., 2010).

Anaplasma platys isolates in the current study presented four haplotypes using the 16S rRNA gene, and only one haplotype was observed in the *groESL* gene (haplotype

network not presented). These results were similar to previous studies reporting low variation in both genes in the sequences of *A. platys* strains (De La Fuente et al., 2006; H. Huang et al., 2005; Martin et al., 2005; Unver et al., 2003). The 16S rRNA and *groESL* sequences may be useful for phylogenetic studies of *Anaplasma* species (Inokuma et al., 2002); however, they are disadvantageous for within-species variation and strain differentiation (De La Fuente et al., 2006). The low genetic variation of *A. platys* compared to other *Anaplasma* species might be due to the limited movement of infected dogs compared to infected cattle in *Anaplasma marginale* and/or the narrow host range of *Anaplasma ovis* in comparison to *Anaplasma phagocytophilum* in humans (De La Fuente et al., 2005).

For the genes targeted for the detection of *B. gibsoni*, the *COX1* dataset displayed higher haplotype (0.667) and nucleotide diversities (0.01258) compared to the 18S rRNA gene of *B. gibsoni*. It is expected since the *COX1* gene was considered to vary significantly compared to the 18S rRNA gene (Bilgic et al., 2010; Haanshuus et al., 2013; Isozumi et al., 2015). Small canine piroplasms, originally known as *B. gibsoni*, were found to be divided into three genetically distinct species with similar morphology and clinical signs. *Babesia conradae*, described in dogs in the western United States, was previously thought of as *B. gibsoni* (Conrad et al., 1991; Kjemtrup et al., 2006; Ionita et al., 2012). However, only *B. gibsoni* and *B. vogeli* infections were observed in the present work.

In this study, the 18S rRNA gene for *B. vogeli* revealed the presence of three haplotypes in dogs and one in ticks, while *COX1* displayed 100% similarity in all sequences in dogs and ticks (haplotype network not presented). In 18S rRNA, the haplotype in a tick did not overlap with any of the sequences in dogs. It is most likely that the tick was newly introduced into the study area, given that the haplotype in a tick was new and yet to be established in the studied dogs. The present work was in contrast with previous work in Brazil, where only one haplotype was observed from the 18S rRNA gene of *B. vogeli* observed in dogs (Moraes et al., 2015). The researchers concluded that the genotypes observed were the most common in the South American continent (Moraes et al., 2015). In the present study, dogs and ticks shared no common haplotype for the 18S rRNA gene of *B. vogeli*, which corroborates the phylogenetic analysis by reflecting low variation in sequences and non-host-specific population structure.

Analysis of molecular variance (AMOVA) depicted a higher level of variation within populations than among populations (Table 6). It indicates that the infection in ticks used in this study has little to no variation from the infections in dogs, consistent with the haplotype networks. There were no significant differences in genetic variation within the population, although the percentage of variations was high. This result could indicate a low to no genetic differentiation of parasite isolates included in this study but a high gene flow of

Table 6
Analysis of molecular variance (AMOVA) results for the gene of interest of Anaplasma platys*,* Ehrlichia canis*, and* Babesia vogeli *for all samples as a group*

Gene/ Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation	FST
Anaplasma platys 16SrRNA					
Among population	1	14.182	- 5.750 Va	- 21.100	- 0.211
Within population	9	297.000	33.000 Vb	121.100	
Total	10	311.182	27.250		
Ehrlichia canis 16S rRNA					
Among population	1	0.336	- 0.460 Va	- 32.110	- 0.094
Within population	11	20.818	1.893 Vb	132.110	
Total	12	21.154	1.433		
Ehrlichia canis gltA					
Among population	1	0.150	- 0.018 Va	- 9.380	- 0.094
Within population	10	2.100	0.210 Vb	109.380	
Total	11	2.250	0.192		
Babesia vogeli 18S rRNA					
Among population	1	1.300	0.083 Va	6.670	0.067
Within population	3	3.500	1.167 Vb	93.330	
Total	4	4.800	1.250		

Note. d.f. = Degrees of freedom; FST = Fixation indices; Va = Among groups variance; Vb = Within groups variance

the vector-borne haemoparasites in the study area (Yin et al., 2013). This high gene flow could stem from the movements of the dog host in the study area, as mentioned earlier. However, the current result could also be due to the small number of parasite isolates used in this study since the genetic diversity could be affected by several factors, including the sample size (Araya-Anchetta et al., 2015). The haplotype networks showed that most parasite isolates were in the same haplotype, with only one parasite isolated in several other haplotypes. In addition, the pairwise differences (pi) demonstrated no significant results statistically (Table 7), indicating little genetic differentiation between the host, which agrees with the phylogenetic analysis.

All the phylogenetic trees generated from this study exhibited no specific pattern or separation between isolates of VBHs in dogs and ticks, as well as region-wise separation for all observed haemoparasites. Two main factors exist for the insufficient separation obtained in the present study. First, the insufficient sample size represents the population where 28 blood isolates and only four tick isolates were used for the characterisation and phylogenetic analyses. The low number of tick isolates was insufficient to represent the population, thus affecting the phylogenetic data analysis results. The low prevalence of VBHs in ticks was also observed in previous studies despite high detection in dogs

Table 7
Pairwise differences measures of haplotype frequencies (pi) based on the gene of interest of Anaplasma platys*,* Ehrlichia canis*,* Babesia vogeli*, and* Babesia gibsoni *sequences below diagonal and above diagonal,* p *values of significant (*=p < *0.05)*

Gene	Sample	Blood	Tick
Anaplasma platys 16SrRNA	Blood	-	0.991
	Tick	- 0.211	-
Ehrlichia canis 16S rRNA	Blood	-	0.991
	Tick	- 0.094	-
Ehrlichia canis gltA	Blood	-	0.991
	Tick	- 0.094	-
Babesia vogeli 18S rRNA	Blood	-	0.991
	Tick	0.067	-

(Chao et al., 2016; Foongladda et al., 2011; Galay et al., 2018; Inokuma et al., 2000; Livanova et al., 2018; Nguyen et al., 2019; Ybañez et al., 2012). Tick DNA isolation is difficult compared to blood due to the thick chitinous exoskeleton and the small amount of microbial nucleic acids usually present in ticks' DNA (Halos et al., 2004). Furthermore, inhibitors can interfere with PCR (Halos et al., 2004; Hill & Gutierrez, 2003; Hubbard et al., 1995). The other possible reason for the low prevalence of tick-borne haemopathogens (TBHs) in ticks might be due to the detachment of ticks infected with TBHs from the dog host before sample collection since most of the tick vectors rest in the environment (Dantas-Torres et al., 2008). The ticks sampled in this study were all attached to dogs during collection.

Second, the most likely reason is the conserved region of the genetic markers used in the present study. The use of *groESL* and *gltA* gene markers for *A. platys* and *E. canis* was due to the absence of previous works on the mtDNA marker for both pathogens. Low variation was observed despite using the *COX1* gene for *Babesia* spp. *COX1* is a mitochondrial gene that has been reported to be of more diversity than the nuclear gene. Mitochondrial genes have benefits in previous studies and have been used to identify variation within populations and cryptic species. Several mtDNA regions have served as relevant markers for genetic, epidemiological, and ecological studies (Hu & Gasser, 2006; Jex et al., 2010). mtDNA is acknowledged as a suitable marker in discovering the possibility of cryptic species when sequence data from small sample sizes were utilised (Blouin, 2002). However, *COX1* is still considered a conserved gene compared to other mtDNA. Other mtDNA genes have a more variable region and were suggested by researchers as useful in exploring variation in parasite populations due to the within-species variation, for example, NADH dehydrogenase subunit 4 (NAD4) and subunit 6 (NAD6) (Lv et al., 2012; Jabbar et al., 2013).

Most of the molecular studies have reported various isolates of the canine VBHs targeting the 18S rRNA or the 16S rRNA gene markers. Both gene markers were suitable and widely used for species identification of the VBHs worldwide (Abd-Rani et al., 2011; Adao et al., 2017; Andersson et al., 2017; Galay et al., 2018; Nazari et al., 2013; Pinyoowong et al., 2008; Yabsley et al., 2008). Nevertheless, the 18S rRNA and 16S rRNA genes are conserved and considered unreliable in distinguishing between strains or subspecies (Aktas et al., 2009; Criado-Fornelio et al., 2003; Tian et al., 2013). Most studies using these target genes reported amplifying fragments with a higher similarity percentage to other strains of that pathogen deposited in GeneBank.

CONCLUSION

A more variable gene marker region should be employed for detailed phylogenetic analysis (e.g., NAD4, NAD6, and mitochondrial DNA). The present study was unable to infer the actual transmission pattern in VBHs among dog and tick hosts, which may be due to the conserved region of the gene of interest and a low number of positive samples, especially from ticks. Future studies are recommended to identify and employ a more variable gene region, as well as a larger sample size, to allow a better resolution to elucidate the transmission patterns of VBHs among dogs and the infecting ticks.

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Preliminary *In Silico* Analysis of *CHS1* Gene in Commelinids Clade: Family Zingiberaceae, Costaceae, and Poaceae

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ABSTRACT

The chalcone synthase (*CHS*) gene families are known to be conserved in plants and have been well-studied in many plants, and they have an important role in the physiological and biological processes of plants. One of the studied *CHS* gene families is the *CHS1* gene. *CHS1* gene is known for its function in the flavonoid biosynthetic pathway. However, not many studies have been reported on the *CHS1* gene in the Commelinids clade, especially the evolution of this gene within three families: Zingiberaceae, Costaceae, and Poaceae. Thus, this study aimed to perform a preliminary *in silico* comparative analysis of the *CHS1* gene across these three families. Through this *in silico* comparative analysis, 20 partial sequences of the *CHS1* gene, which are restricted to 565 bp regions, were analysed. The partial sequences were extracted from the National Center for Biotechnology Information database comprised of 16 Zingiberaceae species, three Costaceae species, and one Poaceae species. From the analysis, these targeted regions showed a low polymorphic site (18.23%) with 103 positions of single nucleotide polymorphisms and three mutations (substitution, insertion, and deletion). Meanwhile, phylogenetic analysis showed no clear evolutionary pattern within the three studied families. In conclusion, the studied partial sequences of the *CHS1* gene in Zingiberaceae, Costaceae, and Poaceae showed that the gene is conserved within the Commelinids clade. Further studies to understand the consequences of low polymorphism and mutations as well as adaptive evolution in the *CHS1* gene,

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accompanied by biochemistry and gene expression studies, should be done in these 20 species of Zingiberaceae, Costaceae, and Poaceae.

Keywords: *CHS1* gene, commelinids, Costaceae, evolution, Poaceae, Zingiberaceae

INTRODUCTION

A group of genes can be grouped into a family according to their high sequence similarity, but adaptation or speciation can contribute to gene diversification (gene polymorphism) and evolution (Lynch & Conery, 2000; Nei & Rooney, 2005). The mutation is a factor that induces gene diversification and evolution. Gene diversification has largely arisen from duplication followed by functional divergence (Reams & Roth, 2015). Many duplicates are immediately lost during a gene duplication event because mutations accumulate in duplicated genes with redundant functions (Innan & Kondrashov, 2010). However, some mutations can lead to a higher degree of functional divergence of duplicates, which can be advantageous for adaptive evolution (Ezoe et al., 2021), consistent with the neutral theory of molecular evolution (Kimura, 1983). Gene divergence could be measured in multigene families through unequal crossing-over rate, mutation rate, gene conversion rate, and selection coefficient of the locus (Matsuo & Yamazaki, 1989).

The chalcone synthase (*CHS*) gene families are among the important genes continuously studied in many plants to understand their evolutionary patterns (Durbin et al., 2000; Glagoleva et al., 2019). The *CHS* gene families express enzymes that belong to type III polyketide synthases and are involved in flavonoid biosynthesis (Roslan, Huy, Kee et al., 2020; Roslan, Huy, Ming et al., 2020; Yuan et al., 2021). The *CHS* genes have been studied in many plants and showed up to 60% homologues sequences (Jiang & Cao, 2008). The *CHS* genes have been studied in *Arabidopsis thaliana* (Dao et al., 2011), *Juglans regia* (Cheniany et al., 2012), *Oncidium* Gower Ramsay (Liu et al., 2012), *Malus domestica* (Dare et al., 2013), *Garbera hybrida* (Deng et al., 2014), *Triticum aestivum* (Trojan et al., 2014), and some species of Zingiberaceae such as *Curcuma longa* (Ayer et al., 20108; Deepa et al., 2017; Resmi & Soniya, 2012), *Boesenbergia rotunda* (Chia et al., 2020; Roslan, Huy, Kee, et al., 2020; Roslan, Huy, Ming, et al., 2020), and *Alpinia oxyphylla* (Yuan et al., 2021). Comparative analysis of the *CHS* gene between and among families of plants to understand the evolutionary pattern of the *CHS* gene is still scarce, and no study has been done on the *CHS1* gene.

The *CHS1* gene involves in the flavonoid biosynthetic pathway. Flavonoid biosynthetic pathway genes (structural and regulatory genes) presented a model system to understand the variety of evolutionary processes, such as causes of evolutionary variation rate among genes, duplicated genes that presented an evolution of novel characters, and the relative importance of structural and regulatory genes that involved in important ecological characters (Rausher, 2006). Each of these processes

is important for plant adaptation, and it has been presumed that due to this reason, selection played a determining role in the evolution of the genes (Yang et al., 2004). However, *CHS* genes varied in plants across taxa leading to the evolution of those genes involved in the flavonoid pathway. Therefore, *in silico* comparative analysis of the *CHS1* gene between the Zingiberaceae, Costaceae, and Poaceae was done as a preliminary study to provide a fundamental idea of the evolutionary pattern in the *CHS1* gene within the Commelinids clade.

MATERIALS AND METHODS

A total of 22 *CHS1* sequences were downloaded from the National Center for Biotechnology Information (NCBI) database (Table 1; https://www.ncbi.nlm.nih.gov/genbank/). Those *CHS1* sequences belong to 16 species of Zingiberaceae (*Curcuma longa*, *Alpinia galanga*, *Alpinia luteocarpa*, *Alpinia vittata*, *Alpinia zerumbet*, *Curcuma amada*, *Curcuma aromatica*, *Curcuma caesia*, *Elettaria cardamomum*, *Globba marantina*, *Hedychium coronorium*, *Kaempferia elegans*, *Kaempferia galanga*, *Kaempferia rotunda*, *Etlingera elatior*, and *Zingiber officinale*), five species of Costaceae and only one Poaceae species. Of those *CHS1* sequences, two were complete *CHS1* sequences, and 20 were partial *CHS1* sequences, as shown in Table 1. All *CHS1* sequences belonging to Costaceae and Poaceae were subjected to dataset selection. The sequences were checked for their percentage of identity, query cover, and E-value with *CHS1* sequences of Zingiberaceae taxid using BLASTn (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Two *CHS1* sequences belonging to Costaceae (HM161806.1 and HM161808.1) were excluded from the final dataset because they showed no similarity with *CHS1* sequences belonging to the Zingiberaceae taxid (Table 2).

Thus, the final dataset consisted of 16 *CHS1* sequences belonging to Zingiberaceae, three *CHS1* sequences belonging to Costaceae, and one *CHS1* sequence belonging to Poaceae (Table 3). Then, multiple sequence alignment was performed using BioEdit 7.2 software (Hall, 1999), and all sequences were adjusted to well match each other by restricting the sequence size to 565 bp (Figure 2). Overall nucleotide dissimilarities among 20 *CHS1* sequences were manually determined to look for mutation and were recorded.

As shown in Figure 1, a similarity matrix was computed using MEGA X software (Kumar et al., 2018). After that, phylogenetic trees were constructed using unrooted neighbour-joining (NJ) trees with two iterations (100 and 1,000). Both iterations constructed similar trees. Hence, the phylogenetic tree constructed with 1,000 iterations was selected to show the *CHS1* evolutionary pattern within the Commelinids clade: Zingiberaceae, Costaceae, and Poaceae.

RESULTS

Twenty *CHS1* sequences belonging to Zingiberaceae, Costaceae, and Poaceae were aligned, and nucleotide dissimilarities

Table 1
List of CHS1 *gene sequences of Zingiberaceae, Costaceae, and Poaceae from the NCBI database*

Family	Species	Accession number	Sequence length (bp)	Sequence status
Zingiberaceae	*Curcuma longa*	AB573020.1	2,107	Complete
	Alpinia galanga	HM161832.1	568	Partial
	Alpinia luteocarpa	HM161830.1	568	
	Alpinia vittata	HM161834.1	568	
	Alpinia zerumbet	HM161836.1	568	
	Curcuma amada	HM161809.1	568	
	Curcuma aromatica	HM161810.1	568	
	Curcuma caesia	HM161811.1	568	
	Elettaria cardamomum	HM161813.1	568	
	Globba marantina	HM161814.1	568	
	Hedychium coronarium	HM161815.1	568	
	Kaempferia elegans	HM161819.1	565	
	Kaempferia galanga	HM161816.1	568	
	Kaempferia rotunda	HM161820.1	568	
	Etlingera elatior	HM161821.1	571	
	Zingiber officinale	DQ089697.2	578	
Costaceae	*Costus erythrophyllus*	HM161829.1	568	
	Costus malortieanus	HM161838.1	568	
	Costus pulverulentus	HM161806.1	568	
	Costus pictus	HM161826.1	568	
	Cheilocostus speciosus	HM161808.1	568	
Poaceae	*Sorghum bicolor*	AF152548.1	2,078	Complete

Table 2
The similarity index of CHS1 *sequences of Costaceae and Poaceae with Zingiberaceae taxid using BLASTn*

Query sequence	Match sequence	Query cover	E-value	Percentage of identity
HM161829.1	HM161832.1 (*Alpinia galanga* chalcone synthase (*CHS1*) gene, partial cds)	100%	6e-159	84.51%
HM161838.1	HM161830.1 (*Alpinia luteocarpa* chalcone synthase (*CHS1*) gene, partial cds)	97%	6e-134	82.29%
HM161806.1	-	-	-	-
HM161826.1	HM161819.1 (*Kaempferia elegans* chalcone synthase (*CHS1*) gene, partial cds)	100%	9e-123	80.84%
HM161808.1	-	-	-	-
AF152548.1	MT811929.1 (*Curcuma alismatifolia CHS1* mRNA, complete cds)	47%	0.0	79.90%

Note. Cds = Coding sequences

Table 3
The final dataset of CHS1 *gene sequences for in silico comparative* CHS1 *gene study*

Family	Species	Accession number
Zingiberaceae	*Curcuma longa*	AB573020.1
	Alpinia galanga	HM161832.1
	Alpinia luteocarpa	HM161830.1
	Alpinia vittata	HM161834.1
	Alpinia zerumbet	HM161836.1
	Curcuma amada	HM161809.1
	Curcuma aromatica	HM161810.1
	Curcuma caesia	HM161811.1
	Elettaria cardamomum	HM161813.1
	Globba marantina	HM161814.1
	Hedychium coronarium	HM161815.1
	Kaempferia elegans	HM161819.1
	Kaempferia galanga	HM161816.1
	Kaempferia rotunda	HM161820.1
	Etlingera elatior	HM161821.1
	Zingiber officinale	DQ089697.2
Costaceae	*Costus erythrophyllus*	HM161829.1
	Costus malortieanus	HM161838.1
	Costus pictus	HM161826.1
Poaceae	*Sorghum bicolor*	AF152548.1

	[Cl]	[Ag]	[Al]	[Av]	[Az]	[Ca]	[Cr]	[Cc]	[Ec]	[Gm]	[Hc]	[Ke]	[Kr]	[Ne]	[Zo]	[Ce]	[Cm]	[Cp]	[Sb]	[Kg]
[Cl]																				
[Ag]	0.4705																			
[Al]	0.5138	0.4525																		
[Av]	0.5155	0.4443	0.3651																	
[Az]	0.4913	0.0348	0.4865	0.4605																
[Ca]	0.4796	0.4412	0.3245	0.0519	0.4676															
[Cr]	0.4934	0.4444	0.3188	0.0462	0.4676	0.0145														
[Cc]	0.4861	0.4612	0.3216	0.0519	0.4849	0.0218	0.0181													
[Ec]	0.4775	0.0181	0.4593	0.4540	0.0348	0.4509	0.4509	0.4712												
[Gm]	0.4759	0.4580	0.3358	0.0813	0.4853	0.0558	0.0558	0.0675	0.4647											
[Hc]	0.4899	0.4476	0.3245	0.0635	0.4675	0.0386	0.0367	0.0442	0.4542	0.0558										
[Ke]	0.5031	0.4519	0.4855	0.5080	0.4720	0.4729	0.4794	0.4795	0.4584	0.4662	0.4691									
[Kr]	0.4791	0.4477	0.3358	0.0713	0.4710	0.0480	0.0461	0.0576	0.4543	0.0615	0.0385	0.4660								
[Ne]	0.5214	0.4355	0.4859	0.4624	0.4585	0.4427	0.4297	0.4394	0.4325	0.4493	0.4489	0.2356	0.4395							
[Zo]	0.5507	0.5137	0.5321	0.5481	0.5281	0.5110	0.5107	0.5145	0.5280	0.5187	0.5108	0.0836	0.5182	0.2737						
[Ce]	0.4841	0.1756	0.4696	0.4303	0.1848	0.4274	0.4240	0.4339	0.1779	0.4276	0.4208	0.4654	0.4115	0.4196	0.4852					
[Cm]	0.5178	0.4367	0.2171	0.3303	0.4629	0.3107	0.3107	0.3190	0.4399	0.3332	0.3219	0.4316	0.3218	0.4447	0.4780	0.4458				
[Cp]	0.4905	0.4149	0.4555	0.4509	0.4276	0.4310	0.4210	0.4307	0.4308	0.4212	0.4242	0.2239	0.4182	0.2075	0.2511	0.3837	0.4361			
[Sb]	0.5156	0.2969	0.4865	0.5023	0.2996	0.5207	0.5245	0.5356	0.3051	0.5096	0.5244	0.4974	0.5132	0.4489	0.5113	0.2600	0.4694	0.4436		
[Kg]	0.4830	0.4475	0.3301	0.0773	0.4710	0.0538	0.0519	0.0635	0.4542	0.0714	0.0442	0.4902	0.0386	0.4489	0.5405	0.4113	0.3274	0.4273	0.5131	

Figure 1. Similarity matrices of 20 *CHS1* sequences belong to 16 Zingiberaceae species, three Costaceae species, and one Poaceae species
Note. [Cl] *Curcuma longa*; [Ag] *Alpinia galanga*; [Al] *Alpinia luteocarpa*; [Av] *Alpinia vittate*; [Az] *Alpinia zerumbet*; [Ca] *Curcuma amada*; [Cr] *Curcuma aromatica*; [Cc] *Curcuma caesia*; [Ec] *Elettaria cardamomum*; [Gm] *Globba marantina*; [Hc] *Hedychium coronarium*; [Ke] *Kaempferia elegans*; [Kr] *Kaempferia rotunda*; [Ne] *Etlingera elatior*; [Zo] *Zingiber officinale*; [Ce] *Costus erythrophyllus*; [Cm] *Costus malortieanus*; [Cp] *Costus pictus*; [Sb] *Sorghum bicolor*; [Kg] *Kaempferia galanga*

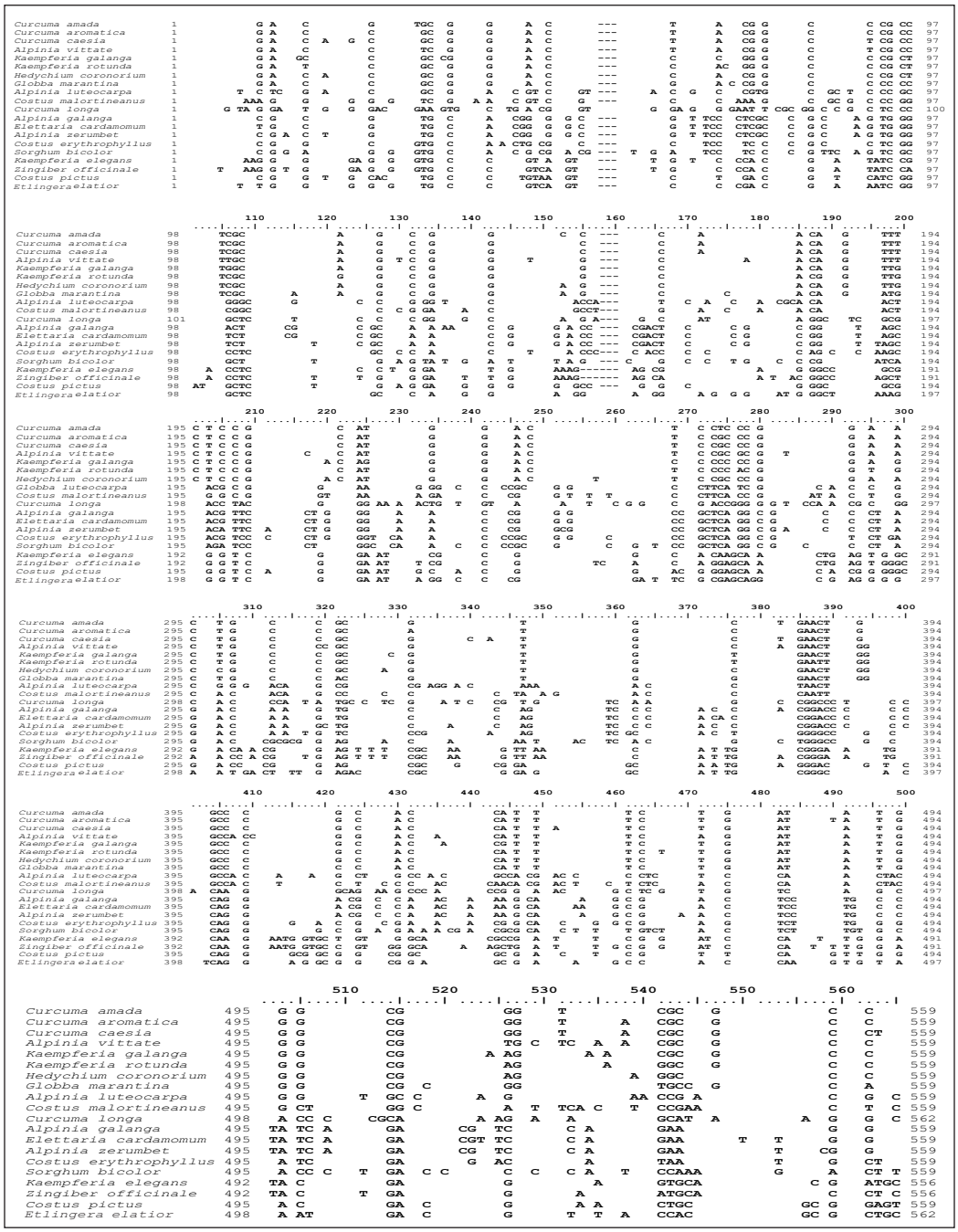

Figure 2. The 20 *CHS1* sequences aligned using BioEdit 7.0 software. The 16 Zingiberaceae species: *Curcuma longa*, *Alpinia galanga*, *Alpinia luteocarpa*, *Alpinia vittata*, *Alpinia zerumbet*, *Curcuma amada*, *Curcuma aromatica*, *Curcuma caesia*, *Elettaria cardamomum*, *Globba marantina*, *Hedychium coronorium*, *Kaempferia elegans*, *Kaempferia galanga*, *Kaempferia rotunda*, *Etlingera elatior*, and *Zingiber officinale*, three Costaceae species: *Costus erythrophyllus*, *Costus malortineanus*, and *Costus pictus*, and one Poaceae species: *Sorghum bicolor*

were observed among them (Figure 2). The observed nucleotide dissimilarities proposed three mutations, which were deletion, insertion, and substitution. Two minor deletions were observed at 155 to 157 in two species (*Kaempferia elegans* and *Zingiber officinale*). Insertion mutations of three nucleotides were observed in two sequences at two different positions in two species: (i) CTT were inserted at positions 57 to 59 in *Curcuma longa*, while (ii) GTC were inserted at positions 158 to 160 in *Etlingera elatior*. Substitution mutations were observed at 285 positions, equivalent to 50.44% of sequence variation. Moreover, 103 positions showed single nucleotide polymorphisms (SNPs). Hence, the results propose the studied 565 bp *CHS1* gene regions within the Commelinids clade; the Zingiberaceae, Costaceae, and Poaceae are conserved *CHS1* gene regions with low polymorphism (only 18.23% of SNPs).

Moreover, the evolutionary pattern of the *CHS1* gene within the Commelinids clade showed no clear evolutionary pattern (Figure 3), and this supports the previous results, which found *CHS1* gene is conserved within the Commelinids clade. The constructed phylogenetic tree showed two clades (Clade i and Clade ii). Clade i comprised 11 species, and Clade ii comprised two subclusters with nine species. In Clade i, 10 species belong to Zingiberaceae, while one belongs to Costaceae. In Clade ii, subcluster

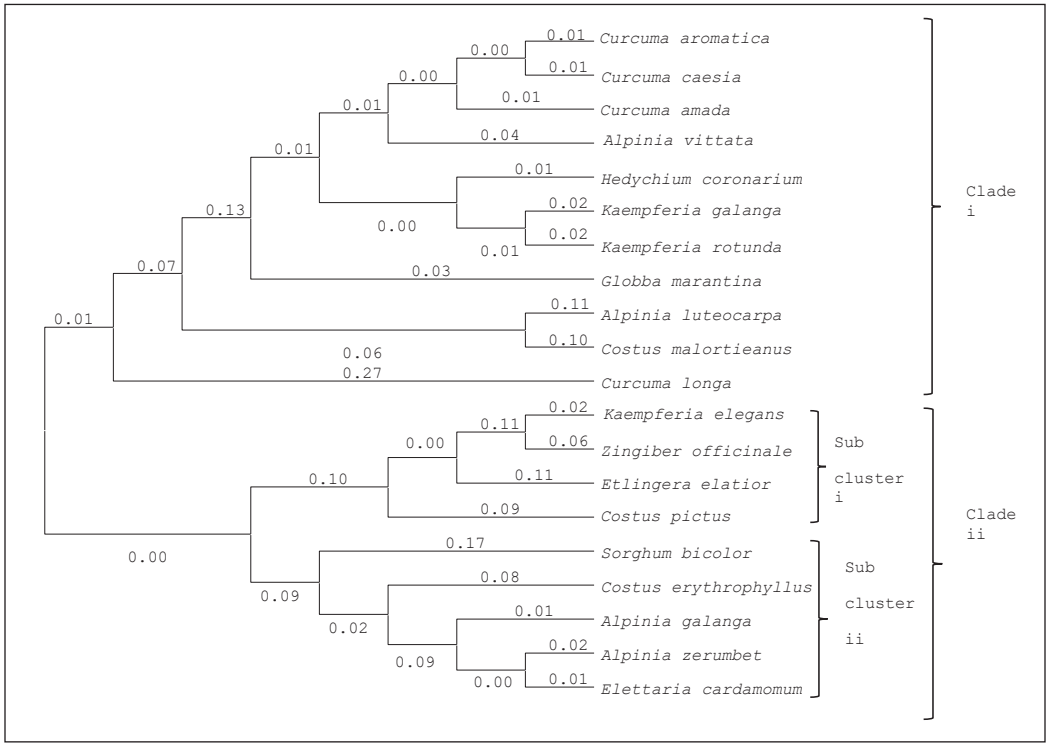

Figure 3. An unrooted neighbour joining (NJ) tree with 1,000 iterations showed an unclear evolutionary pattern of the *CHS1* gene within the 20 studied sequences of the Commelinids clade

i comprises three species that belong to Zingiberaceae, while only one species belongs to Costaceae. Whereas subcluster ii comprised three species belonging to Zingiberaceae, one species belonging to Costaceae, and one to Poaceae.

DISCUSSION

The present study investigated the evolutionary pattern in the *CHS1* gene of the Commelinids clade using 20 species belonging to Zingiberaceae, Poaceae, and Costaceae at a preliminary stage. Nucleotide dissimilarities were observed due to three mutations: substitution, insertion, and deletion. These mutations contribute to low polymorphism in the *CHS1* gene. In addition, the unrooted NJ tree showed no clear evolutionary pattern of the *CHS1* gene and supported the *CHS1* gene as a conserved gene with low polymorphism due to several mutations.

Two of the three mutations (i.e., substitution and deletion) observed in this study have been reported in previous *CHS* gene studies of different plant families. Deletion in the *CHS* gene has been reported at the promotor region of the *CHS4* gene in *Glycine max* (Tuteja et al., 2004). Short frameshift deletions in protein-coding regions of *CHS* genes (*Chs-A4T*, *Chs-A3*, and *Chs-A4T*) have been found in *Triticum aestivum* (Glagoleva et al., 2019). Truncated *CHS3-ICHS1* is presented in mutant soybean (*Glycine max*) due to deletion at 5' flanking or coding region of *ICHS1* in Ms-m mutant (Senda et al., 2002). Meanwhile, Jiang and Cao (2008) have observed nucleotide substitutions in *BcCHS-wf* at two positions (i.e., A to G at 37 and 970 bp, respectively) in both wild and mutant types of Chinese cabbage-Pak choi (*Brassica campestris* spp. *chinensis*). Another mutation reported in the *CHS* gene is duplication (Clegg et al., 1997; Lynch & Conery, 2000; Vision et al., 2000). It has been found in *Arabidopsis thaliana* (Lynch & Conery, 2000; Vision et al., 2000). However, duplication was not found in the present study. Duplication in a gene indicates polyploidisation, which was known to occur 100 million years ago (Durbin et al., 2000). Mutations at nucleotide sequences or amino acids are a primary step in gene evolution (Durbin et al., 2000; Vision et al., 2000). Mutations can lead to the divergence of gene families due to evolutionary forces, such as demographic history, mating system, and natural selection (Chiang et al., 2003; Chiang et al., 2004; Huang et al., 2004).

The *CHS* gene families are functional genes that control flavonoid production and are conserved genes that portray an adaptive evolution (De Meaux et al., 2006; Johnson & Dowd, 2004). For example, the *CHS* genes are structurally and functionally conserved in flowering plants, such as *Antirrhinum majus* (Sommer & Saedler, 1986). In functional and conserved genes, mutations usually occur in intergenic regions, including insertion, deletion, and a large amount of substitution (Mitchell-Olds, 2001). Previous studies also showed the *CHS* genes are conserved in many plant genera and families (Austin & Neol, 2003; Clegg et al., 1997; Durbin et al., 2000;

Huang et al., 2004; Koch et al., 2000; Koes et al., 1989; Yang et al., 2002) as a single gene (Feinbaum & Ausubel, 1988; Kreuzaler, 1983) or as multigene families (Anguraj et al., 2018; Christensen et al., 1998; Deng et al., 2014; Durbin et al., 2000; Glagoleva et al., 2019; Han et al., 2016, 2017; Koes et al., 1987, 1989; Ito et al., 1997; Radhakrishnan & Soniya, 2009; Schroder et al., 1998; Tuteja et al., 2004). For example, barley has seven copies of *CHS* genes (Christensen et al., 1998), *Pinus* has two copies of *CHS* genes (Schroder et al., 1998), and *Petunia hybrida* has eight complete and four partial *CHS* genes that are expressed in floral tissues and seedlings but not present in leaf, root, and stem (Koes et al., 1987).

CONCLUSION

The *in silico* comparative *CHS1* gene study within the Commelinids clade using 20 partial sequences (565 bp) belonging to 16 Zingiberaceae species, three Costaceae species, and one Poaceae species showed the *CHS1* gene is conserved with no clear evolutionary pattern. However, low polymorphism (18.23% of SNPs) and a few mutations, substitution, insertion, and deletion were observed. Hence, further studies are needed to explain the possible consequences of low polymorphism and mutations in the *CHS1* gene within the Commelinids clade. Understanding this might elucidate adaptive evolution in the *CHS1* gene. In addition, more species under the Commelinids clade should also be studied for their *CHS1* gene evolution.

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Specific Sound Frequency Improves Intrinsic Water Efficiency in Rice Leaf by Imparting Changes in Stomatal Dimensions

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ABSTRACT

Various attempts have been made to increase rice production, including breeding for high-yielding and stress-tolerant varieties, a good crop management system, and increased agricultural input in rice production. Soundwave stimulation has been demonstrated to affect plant growth; thus, this method can be employed in the current rice production methods to improve yield. The study aims to determine the effects of different sound wave qualities on the general growth, physiological, and morphological of rice seedlings. Rice seeds of the MR219 variety were grown under a glasshouse condition in a nested design with five replications and were stimulated with various sound wave frequencies. Various sound wave frequencies, 380, 359, 357, 353, and 350 Hz, were obtained by placing the pot at varying distances (80, 160, 240, 320, and 400 cm, respectively) from the sound source, except control treatment. There were significant effects in some of the parameters: plant height, leaf physiology, and stomatal pore and length when treated with varying sound wave qualities. Plants can be stimulated with 380, 357, and 350 Hz soundwaves frequencies for the best

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photosynthetic experience. In addition, 359 Hz of sound wave stimulation resulted in high water use efficiency, which is beneficial in improving crop performance in drought conditions. Thus, it was demonstrated that the sound wave stimulation method has the potential to enhance rice performance in addition to the regular agronomic practices of rice production in farmers' fields.

Keywords: Leaf physiology, *Oryza sativa* L., photosynthesis, rice growth, rice production, sound wave stimulation, stomatal morphology

INTRODUCTION

Rice (*Oryza sativa* L.) is largely regarded as a vital crop owing to its prominent roles in shaping histories, cultures, diets, and economics for half of humanity (Gomez, 2001). Rice feeds more than three billion people, in addition to one billion people depend on rice cultivation for their livelihood (Skinner, 2012). In 2020, the early report of the total rice production in Malaysia was around 2.3 million tonnes, and 78.4% came from the granary areas (Department of Agriculture [DOA], 2021). Although the government has made many efforts to improve the self-sufficiency level in Malaysia, rice production has only achieved a 63% self-sufficiency level, with the rest being imported to meet the needs of the 33 million people in this country (Department of Statistics Malaysia [DOSM], 2021). Although rice production is gradually increasing, consumption is also increasing, requiring more rice to be imported to meet local demand. Furthermore, the gap between rice production and consumption was projected to widen from 2018 to 2026 (Omar et al., 2019). Therefore, there is an urgent need to increase local rice production to ensure food security instead of relying on imports from neighboring countries to meet domestic consumption.

Various efforts are taken to address the issue of rice shortage, which includes breeding and the introduction of varieties resistant to pests and diseases. These include varieties such as Minghui63-Xa21 and Shanyou63-Xa21, which are resistant to bacterial blight, and Hanyou 2 and Hanyou 3, which have a tolerance to abiotic stresses such as flood responses and drought. (Luo, 2010; Zhai et al., 2001). Other researchers approached the issue from different angles (Stoop et al., 2002), such as the application of various good crop management systems, including a system of rice intensification (SRI) on infertile soils and reduced rate of irrigation while maintaining other agricultural inputs optimally (Kim et al., 2008). Traditional crop production practices involving applying more fertilizers and chemicals to improve performance are environmentally unfriendly. Since 2000, the rice harvested area in Malaysia has remained constant, but rice yield in Malaysia has increased due to the increasing use of nitrogen (N) fertilizers (Herman et al., 2015). Therefore, new eco-friendly approaches need to be developed to ensure sustainability in crop production and be environmentally friendly. Despite the increase of N fertilization, manipulating the photosynthesis of plants is considered a sustainable method to improve crop

production. For example, rice variety MR253 showed higher photosynthetic assimilation under the light-saturated condition when treated with 50% N concentration compared to 100% N concentration in a hydroponic system (Herman et al., 2015). This result proved that applying more fertilizer does not necessarily improve crop performance. Similarly, it is believed that sound wave manipulation can be used to enhance photosynthesis at the leaf level, which could potentially improve plant growth and yield rice production.

Interestingly, sound wave has been reported to affect many biological properties in plants (Hassanien et al., 2014). However, whether the effect of sound waves promotes, or limits growth depends on the frequency, intensity, and acting time of the sound wave is not well defined. Stimuli, such as sonic, supersonic, electromagnetic, microgravity, and mechanical vibration, have been previously shown to affect plants. For example, the selectively permeable cell membrane can be injured by environmental factors, such as temperature, salinity, and air pollution. The injury incurred can be thought of as 'micro-perforations' that improve the penetrability of the membrane, beneficial in regulating substance movement into or out of the cell. Sound waves with a frequency of 400 Hz can improve the float of the cell membrane and strengthen the mutual function between the membrane's lipid and protein region (Bochu et al., 2003).

Many studies have demonstrated that music will increase plant growth. Yiyao et al. (2002) reported that plant tissues could be enhanced at a certain range of intensity and frequency of sound waves, but the effect could become the opposite when the sound field is beyond a certain range. Some research also found that the mechanical acceleration wave is needed to promote seed germination (about 70 m/s^2), which is far more than the acceleration of gravity wave (9.8 m/s^2), although this purported value cannot be entirely confirmed due to vibrational acceleration changes that happen continuously in the testing system (Uchida & Yamamoto, 2002). Thus, it is hypothesized that there is a specific sound wave quality, with respect to the varying distances from the sound source, which will be beneficial to improve rice physiologically. In addition, varying sound wave treatments will have differing effects in altering the stomatal and epidermal properties of rice leaves that affect the genetics of rice plants, which could be beneficial in selection for breeding purposes. This study aims to determine the effects of different sound wave qualities on the general growth, physiological, and morphological of rice seedlings.

MATERIALS AND METHODS

Experimental Site

The experiment was conducted in 2018 in a greenhouse of the Institute of Tropical Agriculture and Food Security (ITAFoS), Universiti Putra Malaysia (UPM) (GIS location: 2.98414168 N, 101.7336908 E). Ten rice seeds of MR219 variety were sown in a pot (80 mm [D] × 80 mm [H])

containing paddy field soil (Serdang series), and all the pots were arranged in a nested design with five replications (five pots) per treatment. Treatments were imposed, and growth parameters were assessed within 30–35 days after sowing (DAS). Standard agronomic practices, such as thinning, fertilizing, weeding, and watering, were performed throughout the growing period to maintain good plant growth. Thinning of the seedlings was carried out after 10 days of sowing by leaving only one healthy plant within each pot. Rice plants were fertilized from 14 DAS once a week until 35 DAS with a proprietary blend of hydroponic nutrient solutions from a local garden store.

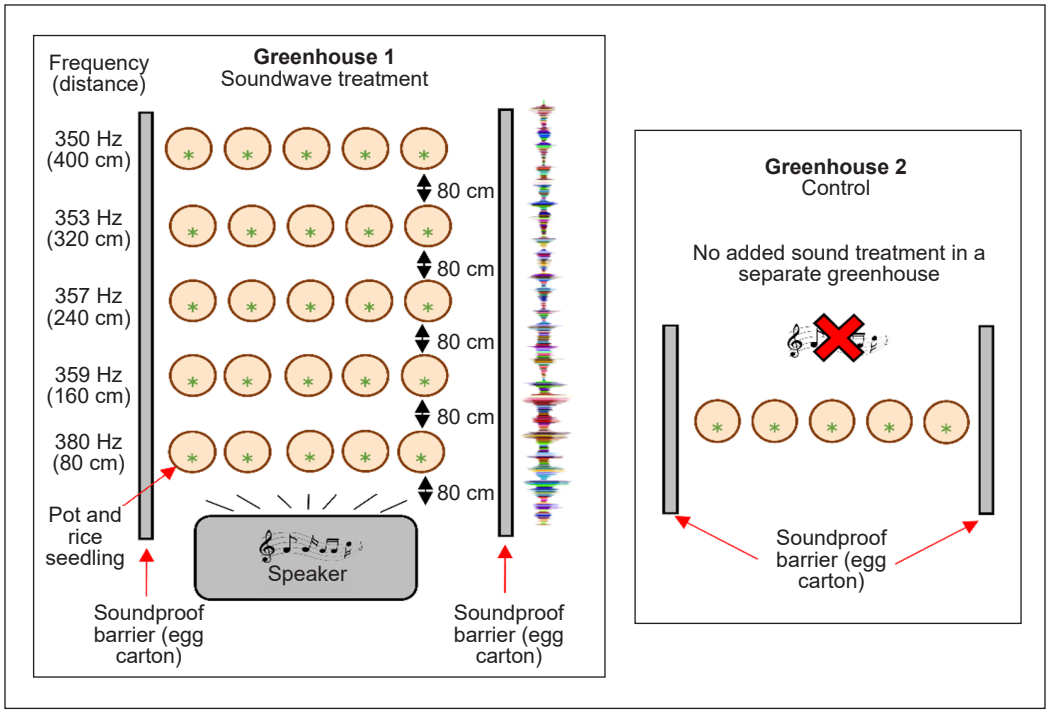
Rice plants were stimulated with different sound qualities in frequency (Hz). Six treatments were imposed on the seeds and seedlings, comprising five sound wave qualities and one control. Different sound qualities, which are 380, 359, 357, 353, and 350 Hz, were obtained by placing the pot at varying distances (80, 160, 240, 320, and 400 cm, respectively) from the sound source with a 600-watt loudspeaker (Aviano Precision, China) with the volume set to 5/10 (Figure 1). The decibel (dB) value, the scale of loudness (intensity of a wave) measured at these distances, is 78, 73, 69, 65, and 60 dB, respectively. The frequencies (Hz) and decibels (dB) were monitored using the FFTWave Android application on mobile phones for sound monitoring. The song used for the sound wave stimulation was a Mozart instrumental song by William McColl. The control treatment received no sound wave stimulation and was in a different greenhouse. The distance between control and treated plants is approximately 28 m (Figures 1a and 1b). The condition between the two greenhouses, especially the sunlight, is the same because the greenhouses are in the same area, and no other building close to this facility can create shade. Sound barrier and absorber using egg cartons were installed to the left and right of the plant rows to reduce the impact of ambient noise. The sound wave stimulation was performed every other day for a month, starting from day 1 of seed sowing for 4 hr in the morning. The duration of the song was 5 min and was repeated during a 4 hr of stimulation period. This whole experiment was conducted only once and was not repeated.

Data Collection

Growth and Appearance. Leaf number was counted manually, and the plant height was measured from the base to the tip of the highest shoot of rice plant using a measuring tape.

Leaf Physiology. Photosynthesis rate (assimilation, A_{400}) and stomatal conductance (g_{sw}) were performed using a portable photosynthesis system (LICOR-6400/XT, USA) to measure the physiological activity of leaves. Gas exchange was measured on the middle part of leaf 5. In the leaf chamber, light intensity, temperature, humidity, and carbon dioxide (CO_2) concentration were set at 1,600 $\mu mol \cdot m^{-2} \cdot s^{-1}$ PAR, 25°C, 60%, and 400 ppm, respectively. Intrinsic water use efficiency (iWUE) ($\mu mol \cdot CO_2 \cdot mol^{-1} \cdot H_2O$) was calculated as the ratio of assimilation

(a)



(b)

Figure 1. (a) A satellite image of two glasshouses used in the experiment and the distance between the experimental locations (about 28 m apart) of sound wave treatments and control treatment (no sound treatment); and (b) the experimental layout in the glasshouse. Rice seedlings treated with sound waves were placed in a different glasshouse (Greenhouse 1) than the non-treated rice seedlings (Greenhouse 2)

rate to stomatal conductance and calculated using the following formula:

$$\text{iWUE} = \frac{\text{Assimilation rate } (A_{400})}{\text{Stomatal conductance } (g_{sw})} \quad [1]$$

Leaf Stomatal Properties. A histology analysis was conducted to determine the properties of leaf stomata. The parameters evaluated were stomatal complex area, stomatal pore area, stomatal densities, percentage stomatal file, stomatal complex length, stomatal complex width, guard cell width, and cell file width. A section of about 1 cm of the fully expanded leaf 5 was cut and placed in Carnoy's fixative that was prepared according to Puchtler et al. (1968) without any modifications. All samples were placed in the Effendorf tubes and bleached with a 15% Clorox® (Malaysia) (sodium hypochlorite) solution for 24 hr until the samples became colorless (to remove the pigments). Then the bleached samples were cut in half, fixed on the slide with a chloralhydrate solution and Arabic gum, and covered with a cover slip.

Microscopy Analysis. Samples were observed using a LEICA DFC310 FX light microscope (United Kingdom) with a 400× magnification lens. The area of the stomatal complex, guard cell width, stomatal length, interveinal gap, and vein counting were performed using Image J software (version 1.48) (Schneider et al., 2012).

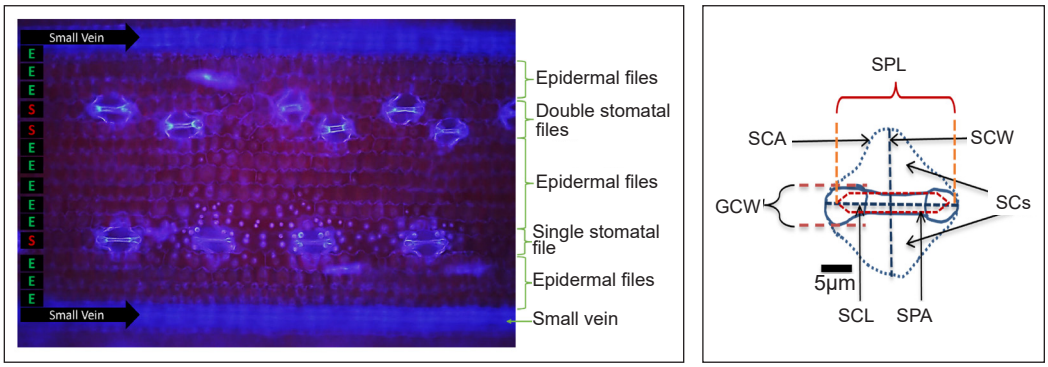
Measurement of Stomatal and Leaf Epidermal Characteristics. For the stomatal complex area, the width and length of the stomata were measured. The stomatal complex in rice consists of a pair of guard cells, and the area of the stomatal complex was calculated based on the measured values of the stomatal complex width and length using the following formula:

$$\frac{(2 \times \text{SPL} \times \text{GCW}) - \text{GCW}^2)}{2} \quad [2]$$

where, SPL = Stomatal pore length; GCW = Guard cell width (Yaapar, 2017)

The guard cell width was determined by drawing the lines from the top to the bottom of the guard cell, and the length of this line was measured as the guard cell width (Figure 2b). The stomatal density (per mm²) was calculated by counting the number of stomatal complexes between two interveinal gap areas. The percentage of stomata files was calculated by counting the number of files with at least one stoma over the total number of cell files. The stomata files were counted between the two parallel veins (Figure 2a).

Statistical Analysis. All data were analyzed in a one-way analysis of variance using SAS 9.4 software using PROC GLM. PROC MIXED was used to analyze data of parameters with missing data and non-constant variance (SAS Institute Inc., 2012). The data were subjected to a normality test to check residuals for normality and constant variance (SAS Institute Inc., 2012). When there were treatment differences, the means were compared and grouped into letter groupings using Fisher's protected least significant difference (LSD) ($P = 0.05$) with n = 5.



(a) (b)

Figure 2. Micrograph images of (a) typical epidermal features and stomatal patterning in a rice leaf (S stomatal files, E epidermal files); and (b) a stomatal complex outline in rice leaves indicating the stomatal complex area (SCA) (surrounded by blue dashed line), guard cell width (GCW), stomatal cell width (SCW), stomatal pore area (SPA) (surrounded by red dashed line), subsidiary cells (SCs), stomatal cell width (SCW), and stomatal pore length (SPL)

RESULTS

The effects of the sound wave in rice plants were evaluated on a few growth and physiological parameters, including plant growth, leaf physiology, and leaf stomatal properties. Each of these parameters corresponded to specific stages of rice growth, allowing a general correlation to be deduced about the vigor and potential yield of the plant. Plant growth parameters include the number of leaves and plant height. Physiological assessments were always performed on the mid-region of the leaf blade of fully expanded leaf 5, including assimilation and stomatal conductance rate. In addition, the assessment of stomata morphologies was performed using samples obtained from leaf 5.

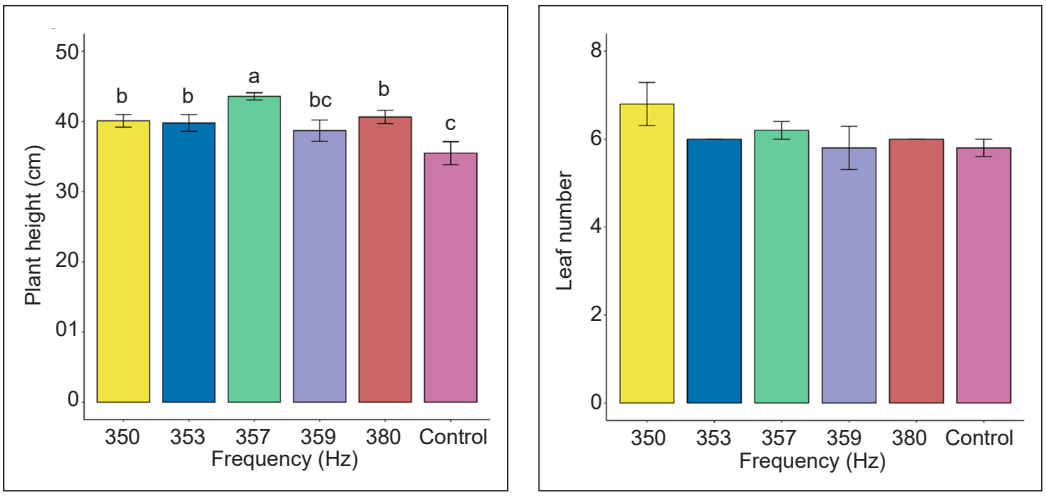
Growth and Appearance

Plant Height. The sound wave treatments significantly affected plant height. The frequency of 357 Hz resulted in the highest average plant height of 43.6 cm, approximately 21% higher than the control (Figure 3a). It was observed that the control and 359 Hz treatment had the lowest plant heights averaging 36 and 39 cm, respectively.

Leaf Number. There was no significant difference in leaf number between any sound wave treatments. However, there is a tendency for the plants that received a frequency of 350 Hz to produce higher leaf numbers compared to other treatments (Figure 3b).

Leaf Physiology. Assimilation rate (A_{400}), stomatal conductance (g_{sw}), and intrinsic water use efficiency (iWUE) were used to assess the performance of leaf 5 after being stimulated with a sound wave of different quality (Hz).

Photosynthesis (A_{400}). A_{400} measurements showed that stimulation with sound waves



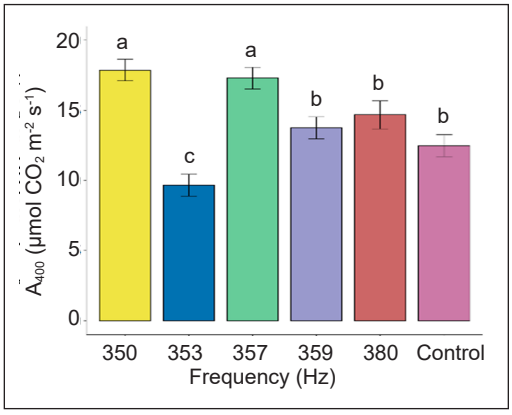
(a) (b)

Figure 3. Mean of (a) plant height and (b) leaf number of seedlings grown at different frequencies measured at 80 cm distance interval from the sound wave source. Means with the same letter are not significantly different at $P > 0.05$ using LSD (n = 5). Error bars indicate the standard error of the mean

of 357 and 350 Hz led to the highest rate of photosynthesis (17.3 and 17.9 µmol·CO_2·m^{-2}·s^{-1}, respectively) compared to the other treatments (Figure 4a). Compared to the control, these two treatments increase the assimilation rate by 39 to 43%. Intermediate assimilation rates were observed in plants receiving 380 and 359 Hz sound waves and control treatments (14.67, 17.86, and 12.48 µmol·CO_2·m^{-2}·s^{-1}, respectively). The lowest A_{400} value was observed in plants stimulated with 353 Hz soundwave (9.66 µmol CO_2 m^{-2} s^{-1}).

Stomatal Conductance (g_{sw}) and Intrinsic Water Use Efficiency (iWUE). Significantly high g_{sw} values were observed in treatments of 380 and 350 Hz (0.54 and 0.65 µmol·H_2O·m^{-2}·s^{-1}, respectively) compared to the control (0.37 µmol·H_2O·m^{-2}·s^{-1}) (Figure 4b). It is worth noting that the plants stimulated with sound waves of 359 to 353 Hz consistently produced significantly lower g_{sw} (and comparable to the control) compared to the plants stimulated at 380 and 350 Hz. When both A_{400} and g_{sw} were combined as a ratio to assess intrinsic water use efficiency (iWUE), the plants stimulated at 359, 357, and 353 Hz consistently produced significantly high iWUE (60.7, 48.3, and 48.5 µmol CO_2 mol H_2O^{-1}, respectively) compared to plants stimulated at 380 and 350 Hz (Figure 4c).

The iWUE for 359 Hz treatment was the highest among other treatments with an iWUE value of 60.6 µmol·CO_2·mol·H_2O^{-1}, indicating that plants at this distance have high water use efficiency, and it is expected that these plants can tolerate the drought condition well. However, this treatment was not significantly different from plants receiving 357 and 353 Hz with iWUE values of 48.2 and 48.5 µmol·CO_2·mol·H_2O^{-1}, respectively. The control, 380 and 350 Hz showed the lowest iWUE values, namely



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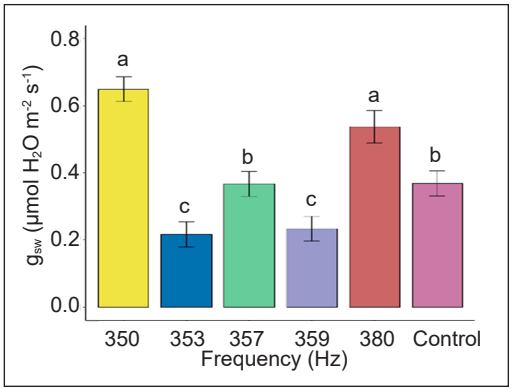

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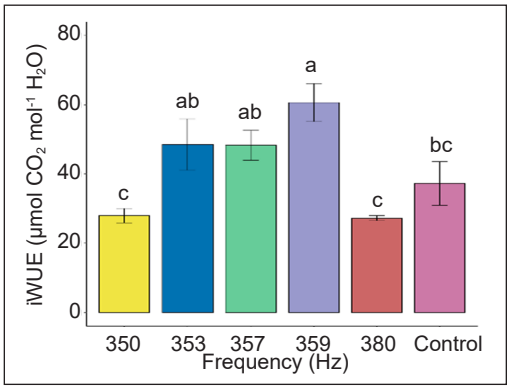


(c)

Figure 4. Mean of (a) assimilation rate (A_{400}); (b) stomatal conductance (g_{sw}); and (c) intrinsic water use efficiency (iWUE) of seedlings grown at different frequencies measured at 80 cm distance interval from the sound wave source. Means with the same letter are not significantly different at $P > 0.05$ using LSD (n = 5). Error bars indicate the standard error of the mean

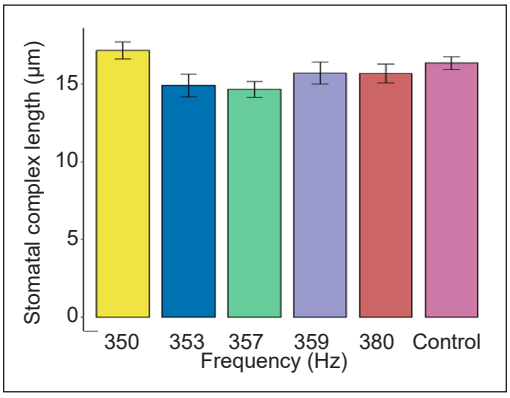
37.3, 25.1, and 28.0 µmol·CO_2·mol·H_2O^{-1}, respectively.

Leaf Stomatal Properties

Effects of Sound Wave on Stomatal and Epidermal Area. Stomata are small epidermal pores on leaves that regulate the movement of CO_2 and water in and out of the plants, respectively. Therefore, the attribute of stomata is crucial to ensure plants have a balanced gas exchange where they can maintain enough CO_2 for carbon fixation while minimizing water loss. The parameters quantified in this experiment included stomatal complex area (SCA), stomatal pore area (SPA), stomatal density (SD), percentage stomatal file (PSF), stomatal complex length (SCL), stomatal pore length (SPL), stomatal cell width (SCW), guard cell width (GCW), and cell file width (CFW) (Figure 2).

Stomatal Complex (Area, Length, and Width). Different frequencies of sound wave treatment have no significant effect on the SCL, SCW, and SCA (Figures 5a-5c). However, there is a tendency for the SCA to increase for plants stimulated with a 350 Hz sound wave (314.2 µm²) compared to the control treatment (293.6 µm²) (Figure 5c).

Stomatal Pore (Length and Area) and Guard Cell Width. It was observed that sound wave treatment had a significant impact in altering the length (SPL) and area of the stomatal pore (SPA) but not the width of the guard cell (GCW) (Figures 6a-6c). Significantly greater pore length was



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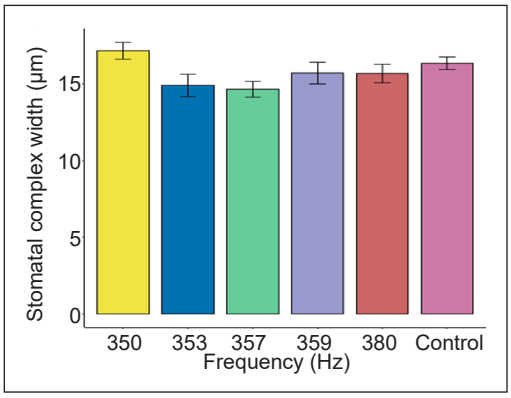

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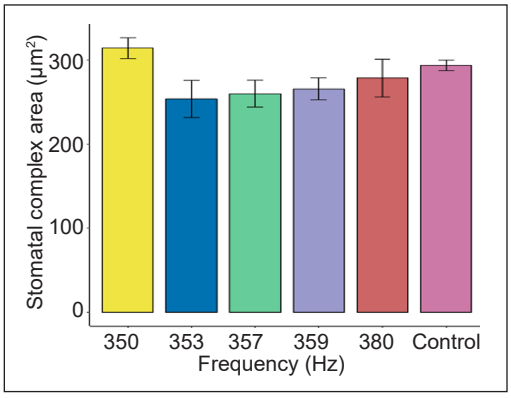
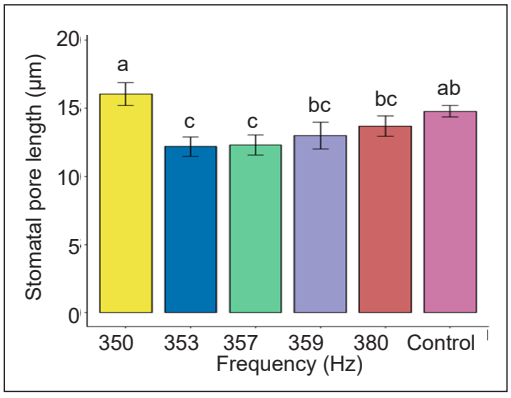
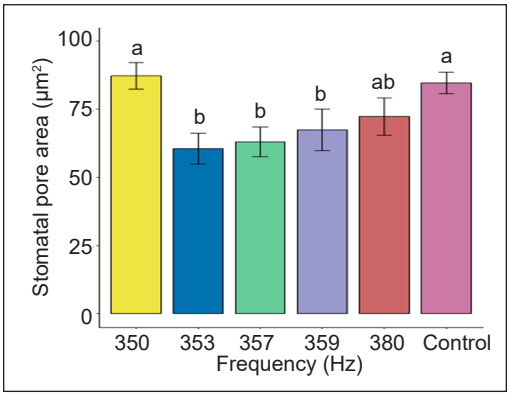


(c)

Figure 5. Mean of (a) stomatal complex length (SCL); (b) stomatal complex width (SCW); and (c) stomatal complex area (SCA) of seedlings grown at different frequencies measured at 80 cm distance interval from the sound wave source. Means with the same letter are not significantly different at $P > 0.05$ using LSD (n = 5). Error bars indicate the standard error of the mean



(a)



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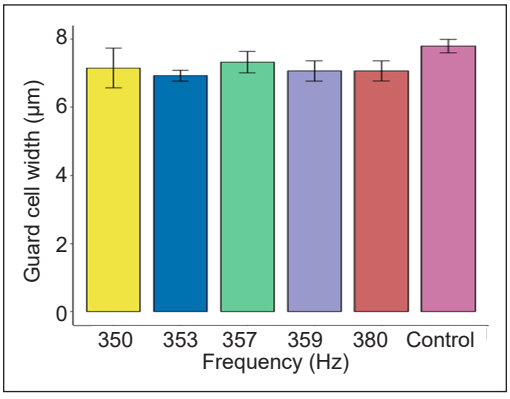


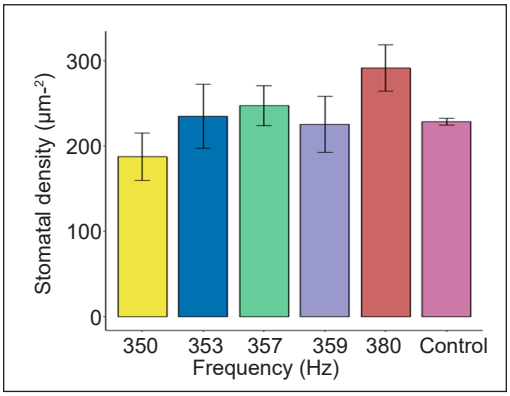
(c)

Figure 6. Mean of (a) stomata pore length (SPL); (b) stomatal pore area (SPA); and (c) guard cell width (GCW) of seedlings grown at different frequencies measured at 80 cm distance intervals from the sound wave source. Means with the same letter are not significantly different at $P > 0.05$ using LSD (n = 5). Error bars indicate the standard error of the mean

observed in 350 Hz stimulated plants and in the control treatment with mean values of 16.1 and 14.8 µm, respectively. It also showed that lower frequency has a significant effect in increasing the pore length. The shortest pore length was observed in plants receiving 353 Hz (12.19 µm) sound wave, but not significantly different from the 357, 359, and 380 Hz sound wave treatments with mean pore lengths of 12.3, 12.9, and 13.7 µm, respectively (Figure 6a). The 353 Hz sound wave stimulation produced the smallest pore length, and alikeuction was approximately 8 and 27% compared to control and 350 Hz treatments, respectively.

The largest SPA was observed in control, 380, and 350 Hz with a mean of 72.3, 87.23, and 84.63 mm², respectively. The smallest pore area was observed in plants that received 353 Hz, but this treatment is not significantly different from plants that received 380, 359, and 357 Hz. Sound wave treatments significantly reduced the pore area by 20–28% compared to the control. The GCW was not significantly affected by different sound wave treatments; however, the control treatment tends to have wider guard cells compared to other treatments.

Stomatal Density, Percentage Stomatal File, and Cell File Width. There was no significant difference in stomatal density (SD), percent stomatal file (PSF), and cell file width (CFW) of the rice plant when exposed to different sound wave treatments (Figures 7a-7c). The mean SD ranged from 187 to 291 per mm², with plants stimulated with 380 Hz tending to have a higher SD



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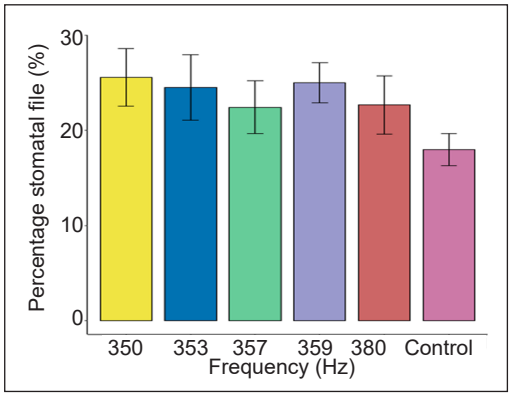

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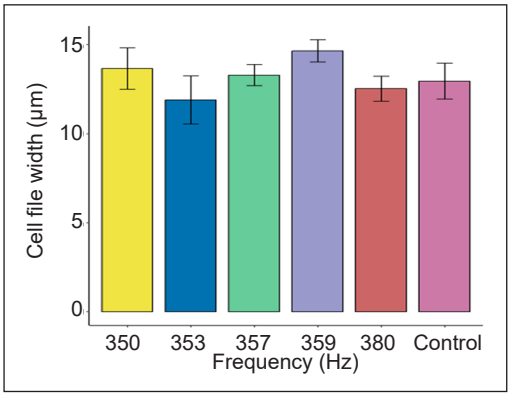

(c)

Figure 7. Mean of (a) stomatal density (SD), (b) percentage stomatal file (PSF), and (c) cell file width (CFW) of seedlings grown at different frequencies measured at 80 cm distance interval from the sound wave source. Means with the same letter are not significantly different at $P > 0.05$ using LSD (n = 5). Error bars indicate the standard error of the mean

and those stimulated with 350 Hz tending to have a lower SD. For PSF, the mean was between 18 and 26%, and there is a tendency for plants stimulated with 350 Hz to increase PSF, and the control treatment had a lower PSF as compared to other treatments. For CFW, the mean ranged from 11.9 to 14.65 μm, with plants exposed to 359 and 353 Hz sound waves tending to have higher and lower CFW, respectively.

Correlation Between Physiological and Morphological Attributes

The correlation analysis suggested that many of the stomatal morphological parameters have intermediate to strong correlations among each other, as compared to the correlation within growth/physiological parameters, as well as the correlation between growth/physiological and morphological parameters (Figure 8). The above results suggested that most stomatal characteristics are independent of leaf growth/physiological characteristics, except for the SPA and SPL, which had significant intermediate positive relationships with g_{sw}, a physiological parameter. Between the leaf physiological attributes, A_{400} shows a significant intermediate positive correlation with stomatal conductance. In addition, iWUE shows a significantly high positive correlation with stomatal conductance. On the other hand, within the leaf morphological attributes, intermediate positive correlations were observed between SCA and SCW, SCL and GCW, SCL and SCW, SPL and SCW, and SPA and GCW. Strong positive correlations were observed between SPL and SCA, SPA and SCA, SPA and SCW, SPA and SCA, and SPL.

DISCUSSION

This experiment studied the effect of sound wave qualities on three different components of crop performance in rice: plant growth, leaf physiology, and stomata morphology. In the first part of this study, the effects of sound waves on general plant growth and leaf physiological parameters were evaluated. The second part of the study evaluated the effects of sound waves on changing the morphological properties of the stomata and epidermis of rice leaves. The combined results from plants' physiological and morphological traits provide insight into how these components affect rice seedling growth.

Since ultrasound energy can increase the permeability and selectivity of cell membranes, thus promoting cell wall growth (Qi et al., 2010), it has been hypothesized that overall plant growth may benefit from enhanced permeability and selectivity of the cell membrane. However, sound wave qualities have a certain value that promotes or hinders plant growth. This study identified two soundwave frequencies, namely 357 and 350 Hz, which promote plant performance by significantly increasing several parameters, namely assimilation rate, stomatal conductance, and plant height. The above findings suggested that an increase or decrease in these parameters might correspond to the nature of the wave propagation, which has a peak and trough. The two peaks could correspond

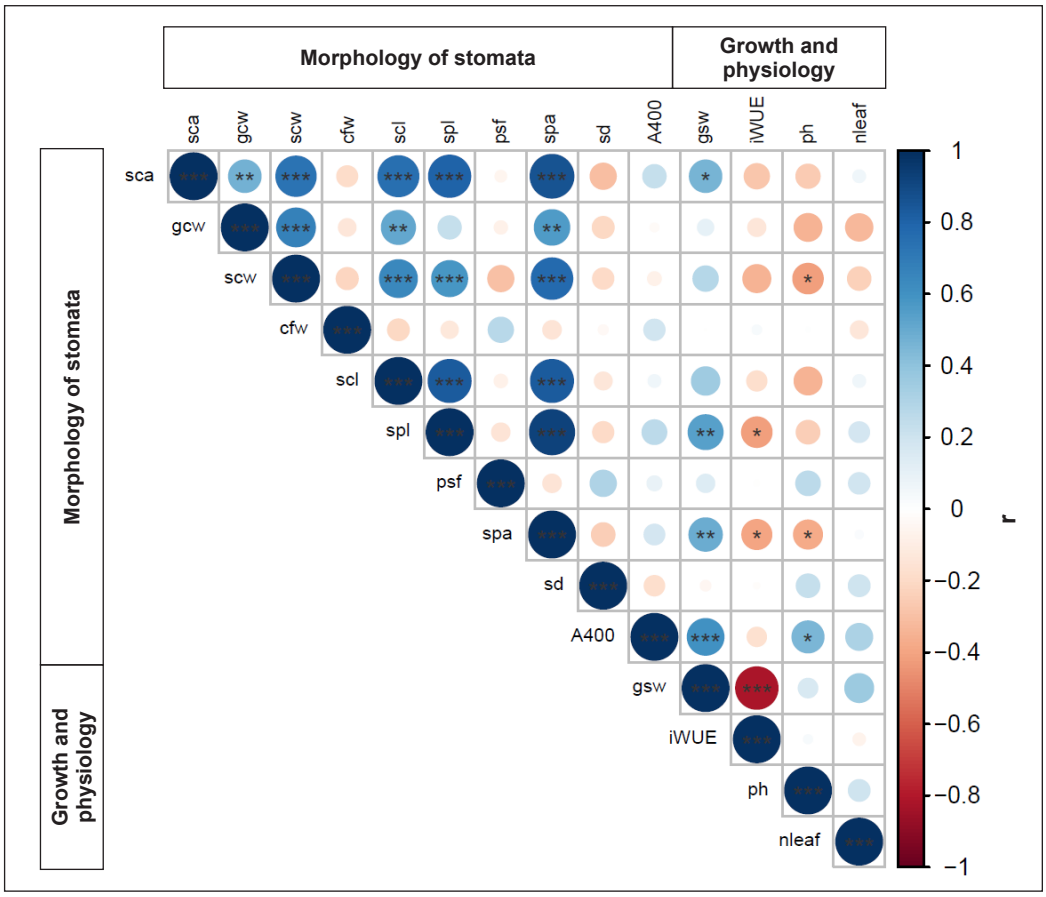

Figure 8. Pearson correlation coefficients between plant leaf characteristics, leaf physiology, and stomatal morphology of rice (MR219) grown at Ladang 15, UPM, in 2017

Note. *, **, *** Statistically significant at $P < 0.05$, 0.01, and 0.001, respectively; r = Correlation coefficient; sca = Stomata complex area; gcw = Guard cell width; scw = Stomata complex width; cfw = Cell file width; scl = Stomata complex width; spl = Stomata pore length; psf = Percentage of stomatal file; spa = Stomata pore area; sd = Stomatal density; A400 = Assimilation rate (CO_2 400 ppm); g_{sw}= Stomatal conductance; iWUE = Intrinsic water use efficiency; ph = Plant height at day 28; nleaf = Number of leaves at day 28

Non-star circle means not statistically significant at $P > 0.05$. Blue and red circles indicate positive and negative correlations, respectively. The size and color intensity of the circle indicates the strength of the correlation between parameters. Large circles mean a strong correlation, and small circle means a weak correlation

to the frequency of 357 and 350 Hz or 240 and 400 cm from the sound source (high values), while the trough could correspond to frequencies of 359 and 353 Hz or 160 and 320 cm from the sound source (low values). Although insignificant, other parameters also show a similar trend in sound wave propagation at slightly different distances. The above speculation needs to be proven, but a plausible explanation is that music can enhance the uptake of nutrients from the soil, resulting in better plant metabolism and increased growth and performance (Chowdhury et al., 2014). Sound waves

also can affect stomatal movement, so this may have an important impact on leaf gas exchange capacity (Cai et al., 2014).

Physiological data also revealed interesting findings which could be used to support the earlier results in general plant growth. It was previously reported that the frequencies of 357 Hz significantly improved plant height compared to plants that did not receive sound wave stimulation. The above finding is consistent with the photosynthesis measurements where the plants stimulated with similar sound wave quality also showed significantly higher assimilation rates compared to the control (Table 1).

The morphological study of stomatal and epidermal properties of rice leaf in this study included many dimensional categories of the stomata, including area, frequency, length, and width. The results showed that different sound wave qualities had minimal effects on stomatal and epidermal properties, suggesting that stomatal and epidermal properties are not very responsive to different sound wave qualities. It has been reported that stomata size is inversely related to stomata density (Büssis et al., 2006; Doheny-Adams et al., 2012), and the increase in stomata density is compensated by a decrease in stomata size (Büssis et al., 2006). It implies that plants with high stomata density will have small stomata and vice versa. It has been shown that mutants with low stomata density and large size have reduced transpiration rates when

Table 1
Summary of mean comparison when compared to control treatment only

When compared to the control			Frequency (Hz) [Decibel (dB)]				
			350 (60)	353 (65)	357 (69)	359 (73)	380 (78)
Growth and physiology	Growth	Leaf number (nleaf)					
		Plant height (ph)	↑	↑	↑		↑
	Leaf physiology	Assimilation rate (A_{400})	↑	↓	↑		
		Stomatal conductance (g_{sw})	↑	↓		↓	↑
		Water use efficiency (iWUE)				↑	
Morphology (stomata)	Area, width, and length	Stomatal complex length (SCL)				↓	↓
		Stomatal complex width (SCW)					
		Stomatal complex area (SCA)					
		Stomatal pore length (SPL)				↓	↓
		Stomatal pore area (SPA)		↓	↓	↓	

Note. ↑ Increase; ↓ Decrease
The green and red arrows indicate a significant increase or decrease, respectively. The mean comparison was performed using least significant difference (LSD) at a significant level of $P < 0.05$

grown in different CO_2 concentrations (200, 450, and 1,000 ppm) and water regimes (70 and 30%) (Doheny-Adams et al., 2012), suggesting that plants with these traits may be beneficial in drought conditions where water availability is limited.

In rice leaves, one of the most important properties in photosynthesis is the guard cell width, which controls the gas and water movement in and out of the leaf. In this study, most of the stomatal properties, namely stomatal complex length, stomatal complex width, stomatal complex area, guard cell width, stomatal density, percentage stomatal file, and cell file width, did not respond significantly to sound waves stimulation. However, significant responses were observed in stomatal pore length and area, indicating that stomatal cells have been stimulated by the sound wave, which could have resulted in the differential gene expression for these traits. It was reported that RNA and soluble protein content increased in chrysanthemum cell culture stimulated with sound waves suggesting an alteration in gene expression (Xiujuan et al., 2003). In addition, SPA (Figure 6b), which trend was also similar to the stomatal complex area, suggests a significant correlation between these two parameters (Table 1).

Sound vibration is a mechanical stimulus that can cause thigmomorphogenetic responses in plants (Telewski, 2006). Therefore, to a certain degree, soundwave treatments could alter the stomatal pore size. It was documented that stomatal features, including stomatal pore size, influenced stomatal conductance (Fanourakis et al., 2015). This study observed an intermediate positive correlation between conductance and stomatal pore length and area, suggesting the tendency of stomatal pore length and area to affect stomatal conductance (Table 1).

In addition, it was found that certain high frequencies tend to reduce the length and area of the stomata, while those of plants stimulated at 350 Hz (60 dB) were unaffected compared to the control treatment. The assimilation rate for plants stimulated with 350 Hz (60 dB) is the greatest compared to other treatments, including the control (Figure 4a). The current finding contradicts those of Hou et al. (2009), who reported that when using four speakers as sound wave treatment at a different planting distance in cotton, the minimum yield was obtained in plants grown at a relatively far distance (30 m) with a sound wave intensity range of 75–110 db. Evidence from another study showed that net photosynthesis measured weekly in strawberry plants treated with sound waves of 100 dB and a frequency of 40–2,000 Hz was not significant compared to the control (no sound wave), except during the fourth sound stimulation. Although insignificant, there were tendencies to improve net photosynthesis when treated with sound waves, potentially leading to higher yield. Similarly, the sound wave treatment tends to produce a higher fruit number compared to the control (Meng et al., 2012)—different ranges of sound intensity measured in decibels used for multiple frequencies in sound wave studies.

As previously mentioned, the intensity of a wave is the energy carried by the wave per unit of time per unit area at that point. On the other hand, frequency measures sound quality or the number of sound waves per second. At a given frequency, the intensity can vary depending on how much energy the sound wave carries. Therefore, when treating the plants with sound waves, the researcher can adjust the intensity of a specific frequency of interest.

Additionally, this study found that the stomata density of plants treated at higher frequencies (Figure 7a) tended to have smaller pore sizes (length and area) (Figures 6a and 5b). Although the effect between frequencies in stomata density was insignificant, the response pattern for stomata density and pore size (area and length) showed a similar trend to the results reported in a study that manipulated the genetics of stomata density in *Arabidopsis*. It was also implied that the plants with small pore sizes and high stomatal density showed significantly higher water use efficiency and were likely to adapt well to drought environments (Franks et al., 2015). In another study, rice plants with reduced stomatal density exhibited the ability to conserve water and tolerate drought while maintaining rice yield (Caine et al., 2019).

It was observed that a certain frequency of sound waves significantly changes the membrane protein's structure and affects the cell membrane's permeability and fluidity (Zhao et al., 2002). In addition, it was observed that the activity of plasmalemma H^+ ATPase (proton pumps), which regulates biochemical and physiological processes in plant growth, increases by 19.8% in plants treated with sound waves (Yi et al., 2003). It is believed that vibrations induced by sound waves can enhance the permeability of membranes and be useful in regulating the movement of substances in or out of the cell, thereby enhancing plant growth. Bochu et al. (2003) showed that sound waves with a frequency of 400 Hz could improve the buoyancy of the cell membrane and strengthen the mutual function between lipid and protein regions of the membrane.

It is worth mentioning that having a high photosynthetic rate alone, although sensible, means that resulting in relatively more vigorous plants will not always be helpful if the plant is experiencing water scarcity. There are two key findings in this study. First, if water is never an issue, rice plants can be stimulated with 380, 357, and 350 Hz soundwaves frequencies to achieve the best photosynthetic experience. Second, suppose water efficiency is the aim. In that case, stimulating the rice with a 359 Hz sound wave is the answer because the relatively high amount of carbon is assimilated for a one-unit amount of water lost. Nevertheless, it is worth noting that music comes with various sound qualities; thus, if the same physiological improvement is achieved, similar sound quality in terms of hertz and decibel should be applied regardless of the music type.

CONCLUSION

The current study aimed to determine the effects of sound wave quality on rice

plant characteristics, including height, stomatal properties, and physiological performances. These findings suggest that, in general, sound waves between the frequencies of 350 and 370 Hz could promote plant growth performance in terms of increased plant height. The proportionate carbon assimilation and stomatal conductance rates could be the reason for the growth increase, particularly at 350 Hz frequency. It was also shown that the pore dimensions of the stomata were alterable at frequencies higher than 350 Hz. The distinct significant effects of the sound wave in certain parameter classes suggest its useful potential as a stimulus like many other abiotic factors, such as light and temperature. The ability of sound wave stimulus to increase yield and achieve better water use efficiency indicates alternative options are always available in improving seedlings' establishment in rice, thus potentially leading to an enhanced overall rice yield and production. Also, since rice production is water intensive, reducing the water supply in the rice can adversely affect yield. Therefore, sound wave stimulation at a specific frequency, which can change the properties of stomata, resulting in the plant using water more efficiently, allows plants to produce a higher yield with limited water availability.

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Biodegradation of Expanded and Extruded Polystyrene with Different Diets by Using *Zophobas atratus* Larvae (Coleoptera: Tenebrionidae)

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ABSTRACT

Polystyrene waste pollutes the environment and poses a significant health risk to humans, animals, and marine ecology. This study aims to evaluate the effectiveness of degradation on expanded (EPS) and extruded (XPS) polystyrene with different diets using superworms (*Zophobas atratus* larvae) obtained in Malaysia. The growth and development of the larvae after consumption of EPS and XPS and the gut microbial community changes in response to high polystyrene consumption diets were also identified. The oatmeal, wheat bran, and cornmeal were used as supplement diets and showed significantly enhanced EPS and XPS consumption and degradation compared to sole diet treatment. Gel permeation chromatography was carried out using egested frass of *Z. atratus* larvae to characterize depolymerization of EPS and XPS, indicating a significant reduction in the average molecular weight and average molecular weight. The highest reduction occurred in the presence of oatmeal. Proton nuclear magnetic resonance and Fourier transform infrared spectroscopy analyses indicated functional group changes and chemical modification occurred with depolymerization and partial oxidation of EPS and XPS. The larvae length increased, while the number of instars and duration of larvae became shorter with the addition of supplement diets. Oatmeal is predominantly effective among other supplements in assisting *Z. atratus* larvae with EPS and XPS degradation. The results of this study support the ubiquity of polystyrene biodegradation in *Z. atratus* and the next-

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generation sequencing studies. *Kluyvera* sp., *Klebsiella* sp., and *Enterobacter* sp. were found to be strongly associated with degrading EPS and XPS polystyrene with oatmeal as a supplemental diet.

Keywords: Biodegradation, expanded polystyrene, extruded polystyrene, superworms, supplements, *Zophobas atratus*

INTRODUCTION

Plastic production worldwide is increasing yearly (Shanmugam et al., 2020). However, plastic waste takes the longest time to decompose naturally among different waste products. Improper plastic waste management, such as polystyrene, has caused accumulation in every corner of the world, adversely affecting wildlife and human health (Khoo et al., 2021). Besides, plastic trash accumulates in the sea creatures (Thushari & Senevirathna, 2020).

Insect larvae belonging to darkling beetles (Tenebrionidae), e.g., yellow mealworms (*Tenebrio molitor*) and superworms (*Zophobas atratus*), were found to degrade various plastic, especially polyethylene (PE), polystyrene (PS), and polypropylene (PP), among others, since 2015 (Brandon et al., 2018; Peng et al., 2019, 2022; Y. Yang et al., 2015, 2020; Yang, Brandon, et al., 2018; Yang, Wu, et al., 2018). The ubiquity of PS biodegradation of *T. molitor* was confirmed via tests of the larvae worldwide (Yang, Wu, et al., 2018). *Zophobas atratus* Fabricus 1775 (Coleoptera: Tenebrionidae) is a superworms commercially available animal feed worldwide (Rumbos & Athanassiou, 2021). Recently, *Z. atratus* strains from China and USA were reported to biodegrade PS (Peng et al., 2020, 2022) and low-density polyethylene (LDPE) via a gut microbial-dependent mechanism (L. Yang et al., 2021). However, the plastic degrading ability of *Z. atratus* in other areas has not yet been elucidated. Meanwhile, the effect of expanded polystyrene (EPS) and extruded polystyrene (XPS) consumption with different feeding diets on *Z. atratus* larvae remains unclear. Oatmeal, wheat bran, and cornmeal were used as a supplemental diet for *Z. atratus* larva to evaluate the consumption of EPS and XPS. Oatmeal, wheat bran, and cornmeal rich in essential nutrients are common feeding diets for *Z. atratus*. Several studies showed that oatmeal, wheat bran, and cornmeal manipulated the guts microbiota of *T. molitor* larvae and enhanced polystyrene consumption (D. Zhou et al., 2021; Gao et al., 2010; Matyja et al., 2020). Yang, Brandon, et al. (2018) first reported enhanced PS consumption by *T. molitor* larvae due to co-feeding bran and protein powders.

Insects asylum diverse microorganisms in the guts benefit their host, physiologically and ecologically (Singh et al., 2019). The major function of microorganisms harboring in the gut is to digest ingested food (Jang & Kikuchi., 2020). The capacity of *Z. atratus* and *T. molitor* larvae to degrade polystyrene (PS) within the gut concerning microorganism activity has been confirmed recently (S.-S. Yang & Wu, 2020; Y. Yang et al., 2015, 2020; Yang, Wu, et al., 2018). Thus, studying the *Z. atratus* larvae gut microbial community on the high

polystyrene (XPS and EPS) consumption diets is worth exploring.

This study investigated the supplementation of different co-diet to enhance *Z. atratus* larvae to degrade EPS and XPS. Besides, the growth and development of *Z. atratus* larvae after consuming expanded (EPS) and extruded (XPS) polystyrene was also evaluated. The gut microbial population alterations in response to high polystyrene consumption diets were also studied.

MATERIALS AND METHODS

Sources of *Zophobas atratus* Larval and Test Materials

Zophobas atratus beetles (120 individuals) with an average of 22.78 ± 2.36 mm length/individual and 500.30 ± 74.00 mg/individual were purchased from Ban Lee Agro (M) Sdn. Bhd. (Rawang), Malaysia then reared into a plastic container (18 cm length × 12 cm width × 7 cm height). After *Z. atratus* beetles undergo oviposition, the eggs are collected and allowed to hatch before the experimental study. A newly hatched *Z. atratus* larvae were immediately isolated into each plastic container (18 cm length × 12 cm width × 7 cm height) in a controlled environment (temperature: 28 ± 1°C and relative humidity [RH]: 50–70% RH). All larvae were healthy, without defects, and free from disease, antibiotics, additives, and hormones.

EPS (0.0375 g/cm^3 density) and XPS (0.1086 g/cm^3 density) polystyrene were used as a feedstock purchased from Yee Hup Foam and Packaging Industries Sdn. Bhd. (Shah Alam, Malaysia). Throughout gel permeation chromatography (GPC) analysis, the weight average molecular weight (Mw) and number average molecular weight (Mn) values of EPS were 399.90 ± 1.54 and 98.50 ± 0.96 kDa, respectively. Meanwhile, XPS polystyrene has Mw and Mn values of 360.60 ± 0.60 and 83.50 ± 0.67 kDa. Before the test, sterilized distilled water was used to clean the EPS and XPS polystyrene components, then dried for 48 hr at 30°C to ensure cleanliness and dirt removal.

Oatmeal (66.60% carbohydrates, 16.70% protein, 5.80% fat, 10.80% fiber, and others), wheat bran (64.20% carbohydrates, 15.80% protein, 4.20% fat, 12.90% fiber, and others), and cornmeal (76.70% carbohydrates, 8.30% protein, 3.30% fat, 7.50% fiber, and others) with 1.2 g, respectively, were used as supplements in the feedstock according to the treatment. All supplements were purchased from Ban Lee Agro (M) Sdn. Bhd. without adding pesticides and antibiotics. All supplement foods were sorted and appropriately filtered to eliminate impurities and contamination before the experiment.

Biodegradation of Expanded Polystyrene and Extruded Polystyrene

EPS and XPS polystyrene (1.2 g) used for each replicate according to treatment were washed with clean air to eliminate dirt particles. An isolated single *Z. atratus* larva was grown in each treatment container. During the cultivation period, *Z. atratus* larvae were raised in a controlled environment at 28 ±

1°C with a relative humidity of 50–70%, according to Yang, Brandon, et al. (2018), with slight modifications. Eight treatments were prepared according to the feeding conditions (Table 1): i) EPS only; ii) EPS + oatmeal; iii) EPS + wheat bran; iv) EPS + cornmeal; v) XPS only; vi) XPS + oatmeal; vii) XPS + wheat bran; and viii) XPS + cornmeal, respectively. Oatmeal, wheat bran, and cornmeal as supplements were supplied only once with 1.2 g, respectively, in the selected treatments. EPS and XPS consumption were identified based on the mass loss via weighing the unconsumed EPS and XPS within treatments. All treatments were carried out with sixty replicated.

Characterization of EPS and XPS Biodegradation with Frass Contents

Gel permeation chromatography with high temperature (HT-GPC) (Agilent 1260 Infinity II GPC/SEC system, USA) was used to identify the Mw and Mn of digested polystyrene in frass contents with minimal modifications based on Yang, Brandon, et al. (2018). Sample (frass) around 50 mg was deposited into a sterile glass vial (30 ml) that contained 10 ml tetrahydrofuran (THF) (Merck, Germany) to extract for 2 hr at 30°C. The extract was filtered twice using a polyvinyl difluoride (PVDF) sterile syringe filter with 0.22 µm (Bioflow, Malaysia). THF extract samples (100 µl) were inserted into a GPC running at 40°C with a THF eluent 1.0 ml/min (flow rate). The results of molecular weight (Mn and Mw) were recorded.

Proton nuclear magnetic resonance (^{1}H NMR) was used to examine the chemical changes between raw polystyrene and frass samples based on the Peng et al. (2020) protocol. Fresh frass samples (50 mg) were deposited in glass vials (10 ml), then 2,000 µl chloroform-D (Merck, Germany) was added and allowed thoroughly mix for 2 hr. The extracts were transferred to a clean glass vial (10 ml) after being filtered with PVDF sterile syringe filter with 0.22 µm (Bioflow

Table 1
The treatment used for the experimental study

Treatment	Information
Group 1 (Expanded polystyrene)	
T1	Expanded polystyrene only
T2	Expanded polystyrene + oatmeal (ratio 1:1)
T3	Expanded polystyrene + wheat bran (ratio 1:1)
T4	Expanded polystyrene +cornmeal (ratio 1:1)
Group 2 (Extruded polystyrene)	
T5	Extruded polystyrene only
T6	Extruded polystyrene + oatmeal (ratio 1:1)
T7	Extruded polystyrene + wheat bran (ratio 1:1)
T8	Extruded polystyrene + cornmeal (ratio 1:1)

Note. Expanded polystyrene = 1.2 g; Extruded polystyrene = 1.2 g; Supplements (oatmeal, wheat bran, cornmeal = 1.2 g. The experiment was recorded until pupation took place

Lifescience, Malaysia). Extracts (600–700 µl) were injected into the 1,000 µl NMR tubes. The ^{1}H spectra were measured using a Varian Inova 500 MHz NMR spectrometer (USA). The ^{1}H spectra were expressed in ppm, also known as parts per million, with a reference peak for residual chloroform-D (^{1}H–7.26 ppm).

The alterations in the major functional groups of polystyrene were identified using Fourier transform infrared spectroscopy (FTIR), known as Fourier Transform Infrared Spectroscopy (Brandon et al., 2018; Sekhar et al., 2016). FTIR spectroscopy (Perkin Elmer, Spectrum 100, USA) examined the frass samples. The absorbance was measured in the range of 650–4,000 per cm wavenumbers in the mid-IR region. A comparison among treatments was recorded when different peaks occurred, such as C=O carbonyl group, =C-H bend, C-O stretch, methylene (CH_2) deformation, C=C stretch (benzene ring), CH_2 bending (symmetrical), and CH_2 bending (asymmetrical).

Growth and Development of *Z. atratus* after Ingested Polystyrene

The growth and development of larvae after ingested polystyrene was investigated based on measuring body length (mm). The larvae length (mm) was measured using a digital vernier caliper (Senator DCS 200, Germany) after larval exuvium was observed (Kim et al., 2015). The larva length was measured from the tip of the head thorax until the end of the abdomen. During larva measurement, avoid direct contact because *Z. atratus* larvae are susceptible to external stimuli. It will shrink when touched, resulting in measuring errors.

The number of instars and total duration of larval instars (days) were recorded after the larval exuvium was observed. The exuvium of larvae was discarded after being observed to avoid duplicate counting. The number of instars and larval development duration can vary depending on food quality (Morales-Ramos et al., 2010). After pupation, the larvae-initiated curling and the number and duration of larval instar counting were stopped.

Microbial Community Analysis

The treatment focuses on potential supplement diets that effectively consume EPS or XPS. Three treatments were prepared according to the feeding conditions: (1) oatmeal only, (2) EPS + oatmeal, and (3) XPS + oatmeal, respectively. Twenty larvae with 10 to 12 instars from each treatment were randomly harvested to avoid individual variability between larvae with identical diets. The experiment was repeated three times. Larvae were collected to make a gut cell suspension used as a bacterial enrichment inoculum. The surfaces of the larvae were immersed in 70% absolute ethanol (Merck, Germany) for 60 s to eliminate surface germs. The larvae were washed with sterilized distilled water (Hu et al., 2018). The protocol by Brandon et al. (2018) and J. D. Zhou et al. (1996) was modified and used for bacterial genomic DNA extraction. Scalpel and clipper removed the guts walls from the

outer skin layer (cuticle). Guts walls were discarded, and DNA was extracted by mixing guts contents with 500 µl of 2% (w/v) sodium dodecyl sulfate (SDS, Merck, Germany) and lysis solution of 500 µl (ethylenediaminetetraacetic acid [EDTA, 0.5 M]) with pH 8.0, cetyltrimethylammonium bromide (CTAB, 0.1 M), Tris-hydrochloric acid (Tris-HCl, 10 Mm) with pH 7.5, sodium chloride (NaCl, 0.3 M), respectively in a microcentrifuge tube (2.0 ml) then vortexing for uniformly mixed.

The suspension formed was incubated at 37°C for 20 min in the water bath. The suspension was mixed with the same volume of phenol (Merck, Germany), chloroform (Merck, Germany), and isoamyl alcohol (Merck, Germany) with the ratio of 25:24:1 into a new 2.0 ml microcentrifuge tube and then centrifuged for 10 min with 12,298 x *g* (Eppendorf, Centrifuge 5415 R, Germany). The liquid formed at the aqueous phase was aliquoted into a new microcentrifuges tube (2.0 ml). The same amount of chloroform and isoamyl alcohol (24:1) was added and mixed well, then centrifuged for 10 min at 12,298 x *g*. The aqueous phase (top layer) containing genomic DNA was precipitated by adding 100% cold absolute ethanol (2.5 volumes) and mixed well. The mixture containing genomic DNA was incubated overnight at -4°C in the refrigerator. After overnight precipitation, the mixture was centrifuged for 10 min at 12,298 x *g*. The supernatant was discarded while the pellet consisting of genomic DNA was washed with 70% (v/v) cold absolute ethanol (700 µl) (Merck, Germany). The pellet containing 70% (v/v) absolute ethanol was centrifuged for 10 min at 12,298 x *g* and then allowed to air-dry in laminar airflow. The dried DNA pellet was then dissolved in 40 µl of TE buffer [Tris-HCI (10 mM) with pH 7.5 and ethylenediaminetetraacetic acid (1 mM with pH 8.0, Merck, Germany). The genomic DNA was then kept in a -20°C freezer.

The 16S rRNA gene in the V3-V4 region was sequenced using phasing amplicon sequencing (PCoA), as described by S.-S. Yang et al. (2021), with some modifications. Illumina MiSeq platform (next-generation sequencing [NGS]) was used to paired-end sequenced on purified amplicons. UCHIME (v4.2.4.0) was used to remove low-quality sequences. Ribosomal Database Project (RDP) used a 70% confidence level to evaluate the taxonomic of each 16S rRNA gene sequence against the SILVA 16S rRNA database. R packages (v3.4.3) and QIIME (v1.9.1) were used to run alpha diversity, principal coordinate analysis, and relative abundance analyses according to taxonomic tanks and operational taxonomic unit (OTU) sequences.

Statistical Analysis

All the research data was gathered and processed using statistical analysis (SAS) software and analysis of variance (ANOVA). The mean separation of treatment was done using Tukey's test to access total plastic consumption amount and changes in molecular weight, differences in body length, total number and duration of larval instars, and microbial diversity between diets. The study was conducted using a significance threshold of $p<0.05$.

RESULTS AND DISCUSSION

EPS and XPS Polystyrene Consumption by *Z. atratus* Larvae

The study of the EPS and XPS groups revealed that differing diets had a significant influence ($p<0.05$) on polystyrene consumption (Figure 1). Treatments utilizing oatmeal, wheat bran, and cornmeal as co-diet demonstrated a substantial polystyrene consumption by *Z. atratus* larvae in EPS and XPS groups. This phenomenon has proven that including nutrient-dense meals in one's diet may increase the consumption of EPS and XPS. Treatment EPS (oatmeal) as a co-diet produced higher total EPS consumption, 422.30 ± 21.44 mg, compared to treatment EPS only (57.40 ± 6.05 mg). In the XPS group, XPS (oatmeal) as a co-diet showed the highest total plastic consumption, 268.33 ± 11.08 mg, compared to treatment XPS only (43.73 ± 5.70 mg). The interaction of nutrients with the microbiome may impact the larva's behavioral decisions (Leitão-Gonçalves et al., 2017). Oatmeal has slightly higher essential nutrient compositions. Most importantly, it may help improve larva appetite and increase the specific variety of microbiota in the *Z. atratus* larva gut to consume EPS and XPS efficiently (Fu et al., 2020). Peña-Pascagaza (2020) and Yang, Brandon, et al. (2018) revealed that EPS and XPS polystyrene contain only hydrogen and carbon elements, thus unfavored polystyrene consumption by larvae. Treatment EPS (wheat bran) showed significantly higher EPS consumption (220.96 ± 10.17 mg) than EPS (cornmeal), with 91.17 ± 4.87 mg within the EPS group.

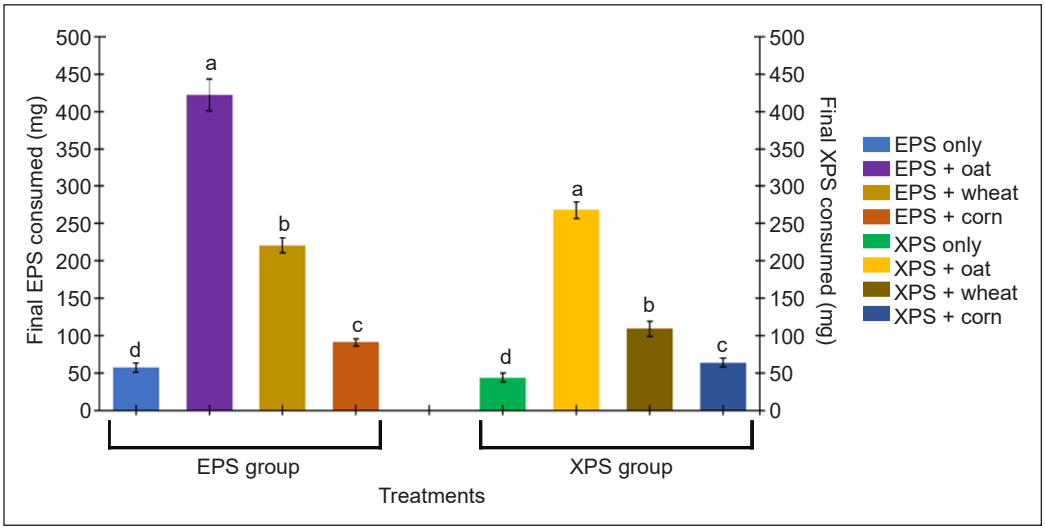

Figure 1. Final plastic consumption of larva in each treatment with EPS group and XPS group. Electronic balance was used to measure the plastic left by larval

Note. Initial EPS / XPS mass = 1.2 g; Supplement (oatmeal/wheat bran/cornmeal) mass = 1.2 g. The experiment was recorded until the pupation took place. All values were obtained from the mean of sixty replications with larvae reared individually and separately per replication. Mean followed by different letters are significantly different at $p<0.05$ level according to Tukey's one-way ANOVA. The same letter above the bar within the group represents no significant difference between treatments

In the XPS group, the treatment XPS (wheat bran) also showed significantly higher XPS consumption (109.12 ± 10.07 mg) than treatment XPS (cornmeal) with 63.97 ± 5.80 mg. Lou et al. (2020) discovered that wheat bran as a co-diet may modify the gut microbiota of *T. molitor* larvae and improve the capacity to break down polyethylene and polystyrene. Wheat bran has slightly lower essential nutrient compositions than oatmeal, reducing the favoring of microbiota production and larvae appetite on EPS and XPS consumption. Besides, cornmeal easily absorbs moisture from the surroundings, influencing shelf life and nutrient contents (McHargue, 1920). Thus, treatment with cornmeal as a co-diet with insufficient nutrients provided the growth of the gut microbiome, reducing EPS or XPS consumption.

Evidence of EPS and XPS Polystyrene Biodegradation Through Frass Contents Analysis

GPC was carried out using egested frass of *Z. atratus* larvae to characterize the depolymerization of EPS and XPS (Peng et al., 2020). The GPC method determines linear polymers' molecular weight (Mw and Mn), particularly polystyrene (Kissin, 1995). GPC results revealed significant changes (p<0.05) in Mw and Mn of the residual EPS and XPS polymers (Figure 2). Mn and Mw in EPS and XPS groups showed decreased values after EPS, and XPS degraded in the gut of *Z. atratus* larvae in all treatments. In the EPS group, GPC analysis on residual polymer showed that treatment EPS (oatmeal) produced the highest reduction with Mw (49.08 ± 2.08%) and Mn (28.24 ± 0.43%) compared among

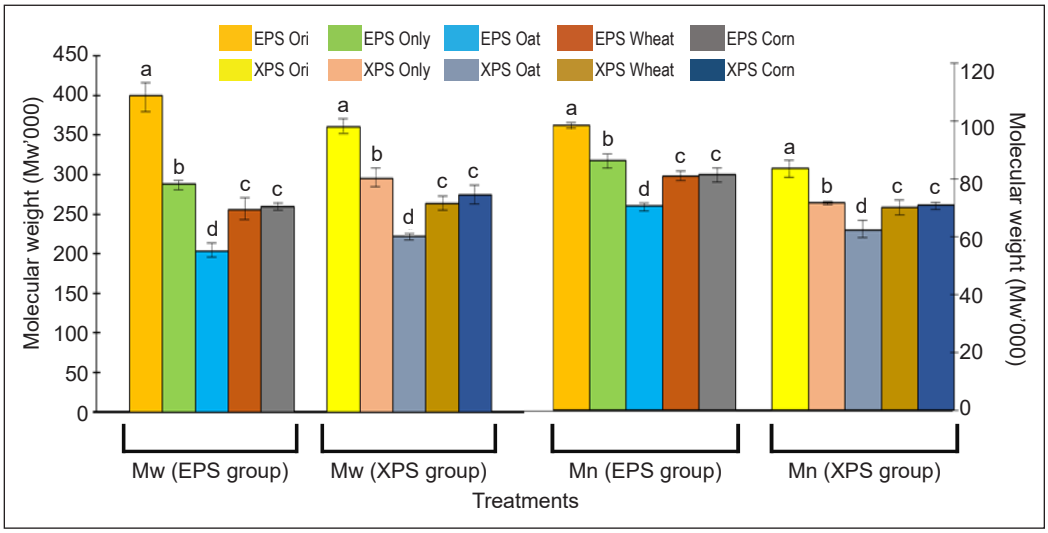

Figure 2. Weight average of molecular weight (Mw) and number average of molecular weight (Mn) of residual polymer from larval frass with treatment EPS group and XPS group

Note. GPC analytical technique was used to measure the molecular weight of degraded polymer after passing through the larval gut passage. Mean followed by different letters are significantly different at p<0.05 level according to Tukey's one-way ANOVA. The same letter above the bar within the group represents no significant difference between treatments

the EPS group. Meanwhile, the XPS group with treatment XPS (oatmeal) produced the highest reduction with Mw (38.37 ± 2.60%) and Mn (25.35 ± 0.33%) compared to the XPS group. Oatmeal with essential nutrients supports larvae growth and enhances EPS and XPS degradation. The higher Mw and Mn value reduction indicated degradation activity and depolymerization were highly effective than other treatments. The enhanced variety of gut microbial activity is responsible for the capacity to depolymerize polystyrene (Fu et al., 2020). Besides, treatment EPS (wheatmeal) and EPS (cornmeal) are insignificant, but both treatments showed significant residual polymer reduction in Mw and Mn when compared with the EPS original. The results obtained were similar to the XPS group as well. XPS (0.1086 g/cm^3) is generally denser than EPS (0.0375 g/cm^3). It could be why more PS mass was consumed and higher depolymerization extent of EPS (27.9% Mw, 12.4% Mn reduction) than XPS (18% Mw, 9.3% Mn reduction), although both foams had similar Mw and Mn. When ingested by larvae, wheat bran contains essential nutrients, triggering the gut microbiome to metabolize polystyrene (Brandon et al., 2018). Cornmeal also plays a similar role in helping *Z. atratus* larva metabolize polystyrene. However, wheat bran and cornmeal can enhance the reduction of Mw and Mn but are less efficient than oatmeal as supplements. The difference in PS consumption and depolymerization extent between oatmeal vs. wheat bran or cornmeal could be the difference in protein content of the co-diets. The results of GPC analyses indicated that both EPS and XPS degradation in *Z. atratus* larvae tested were performed via a broad depolymerization pattern or decrease in both Mw and Mn (L. Yang et al., 2021; Peng et al., 2022).

A comparison of FTIR spectra showed evidence of chemical changes and new functional group formation, indicating oxidation of residual polymer (Lin & Liu, 2021; Umamaheswari & Murali, 2013). Evidence of polystyrene degradation can be shown by monitoring the reduction in peak intensities and the new peak formation compared to the original polystyrene (J. Yang et al., 2014; Sekhar et al*.,* 2016). The spectrum of residual polymer from *Z. atratus* larvae fed with EPS and XPS treatment showed decreased peak intensities, and a new functional group occurred compared with the original EPS (EPS group) and XPS (XPS group) (Figures 3 and 4). In the EPS group, the peaks with (C=C stretch, 1,551 to 1,610 per cm) are known as benzene rings (Yang, Brandon, et al., 2018). It was found that treatment EPS only, EPS (cornmeal), EPS (wheat bran), and EPS (oatmeal) showed a decrease in intensity accordingly and dampened, indicating the evidence of ring cleavage. Besides, the -C-O stretch and -C=O carbonyl were responsible for the peak at 1,736 per cm and the lesser intensity at about 1,085 per cm. This peak was found in all EPS treatments, and EPS (oatmeal) treatment showed the most significant EPS degradation with new peaks and decreasing intensity compared to different treatments. This peak showed signs

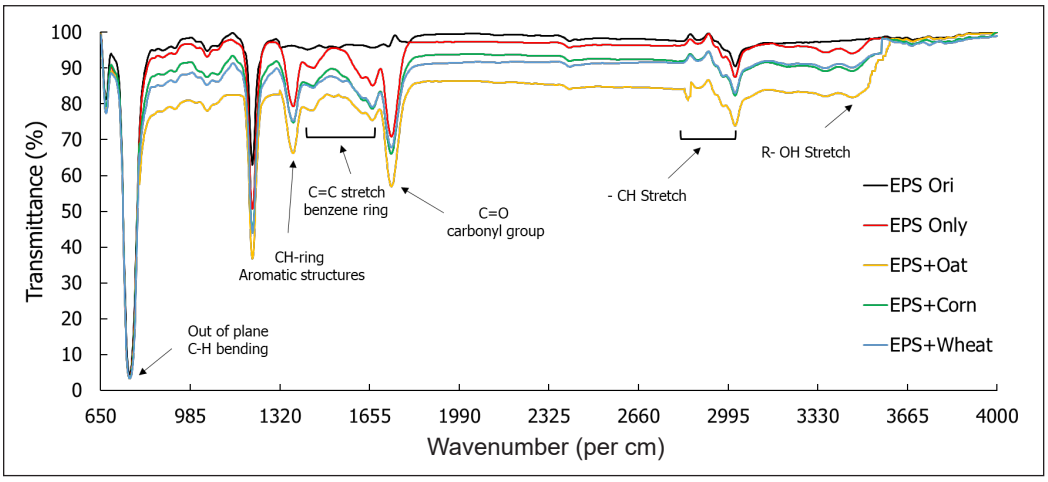

Figure 3. FTIR spectra of residual polymer (EPS group) from larva frass
Note. Fourier transform infrared spectroscopy analytical technique was used to characterize a major functional group of the degraded polymer after passing through the larva gut passage in the range of 650 - 4,000 cm^{-1}

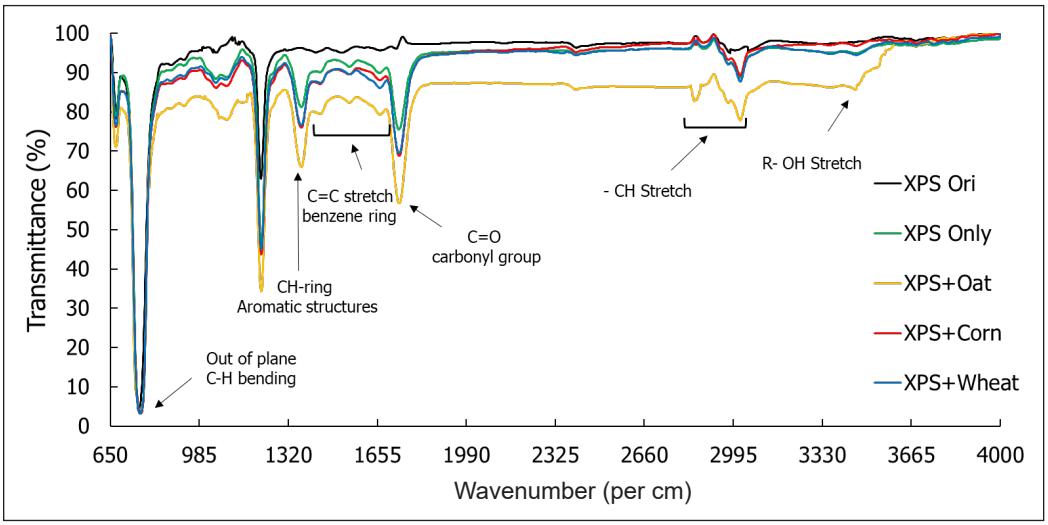

Figure 4. FTIR spectra of residual polymer (XPS group) from larval frass
Note. Fourier transform infrared spectroscopy analytical technique was used to characterize a major functional group of the degraded polymer after passing through the larva gut passage in the range of 650–4,000 per cm

of oxidation of ingested EPS (Brandon et al., 2018; Peng et al., 2020; Yang, Brandon, et al., 2018). The new peak appears at 1,369 per cm (CH- ring) in all EPS treatments assigned to the aromatic structures, as proved by Georgakopoulos (2003) and Yang, Brandon, et al. (2018). In the frass sample, a new absorption peak formed about 3,330–3,665 per cm. A new absorption peak ascribed to the hydroxyl group with OH stretch showed a change in surface characteristics from hydrophobic to hydrophilic (L. Yang et al., 2021). Overall, the

most effective EPS degradation by *Z. atratus* larva is treatment EPS oatmeal, followed by EPS (wheat bran), EPS (cornmeal), and weaker EPS alone based on the new peak appearance and level of intensities on peak decrease. The spectrum in the XPS group showed similar results as in the EPS group.

The aromatic structure and C=C stretch benzene ring were attributed to the peaks around per 1,360 cm (Yang, Brandon, et al., 2018). This peak appeared in all XPS treatments, while treatment XPS (oatmeal) showed a high peak indicating strong benzene ring cleavage. Besides, new peaks appeared around 1,160 and 1,710 per cm in all treatments, indicating the existence of C-O stretch and C=O carbonyl (J. Yang et al., 2014). The C-O stretch and C=O carbonyl on the polystyrene demonstrated that oxidation occurred, and deterioration was verified. The peak density showed that treatment XPS (oatmeal) is more distinct while treatment XPS (only) is the least. A peak was found at 2,839–3,000 per cm with distinct decreasing intensities. These peaks indicate that the C-H stretch was weaker, causing increasing vibration and instability, leading to the breakage of the C-H bond (Herman et al., 2015). Besides that, another new peak occurred around 3,330 per cm in all treatments attributed to the O-H stretch known as the hydroxyl band (S.-S. Yang et al., 2021). This peak indicates that the surface of XPS is changed to a hydrophilic character (Peng et al., 2019). EPS and XPS groups showed similar results, with the peak occurrence nearly identical, but each peak's intensity varies. The results showed that treatment with oatmeal as a co-diet demonstrated the most effective in helping larvae degrade EPS and XPS based on the occurrence of new peaks and intensity levels of the related peaks.

The proton nuclear magnetic resonance (^{1}H NMR) analysis was used to prove the chemical changes in the residual polymer (Kundungal et al., 2021). Brandon et al. (2018) characterized residual PS versus original PS foam using ^{1}H NMR. A comparison of ^{1}H NMR spectra among residual EPS and XPS treatment was examined by extracting the residual polymer with chloroform-D. The number of new peaks increased, indicating that the chemical structure of polystyrene has altered, resulting in the formation of new chemical bonds between functional groups (Sekhar et al., 2016). In the EPS group (Figure 5), new peaks occurred in regions of 2.3 ppm, denoting the formation of C=O (carbonyl group), 2.8 ppm, 3.8 ppm, 4.2 ppm denoting the formation of -OH (hydroxyl group), while 5.1 ppm and 5.5 ppm known as CH=CH- (alkenyl hydrogen) (Liu, 2021; Yang, Brandon, et al., 2018). The C=O (carbonyl group) and -OH (hydroxyl group) indicated that EPS oxidation occurs, and the surface is readily hydrophilic. The CH=CH- (alkenyl hydrogen) indicates a hydrogen shift to double-bond carbon, where the chemical shift is likely to occur (Liu, 2021). It was noticed that treatment EPS (oatmeal) appeared to have more peaks when compared to EPS origin, indicating the highest effectiveness on EPS degradation and depolymerization. The effectiveness

of EPS depolymerization starts from most effective to less with EPS (oatmeal), EPS (wheat bran), EPS (cornmeal), then EPS (only). In the XPS group (Figure 6), new peaks occurred among treatments with 2.3 ppm, 3.8 ppm, 4.6 ppm, and 5.1 ppm compared with XPS origin. The peaks in 2.3 ppm are C=O (carbonyl group), 3.8 ppm, and 4.6 ppm denoting the formation of -OH, while 5.1 ppm is CH=CH-. In the XPS group, XPS (oatmeal) treatment showed more peaks than XPS origin. It means that treatment XPS (oatmeal) is more effective in transforming and modifying XPS within the gut of the *Z. atratus* larva. Meanwhile, treatment XPS (wheat bran) and XPS (cornmeal) have more peaks than treatment XPS only when compared with XPS origin. The *Z. atratus* larva co-diet with wheat bran and cornmeal showed significant depolymerized XPS. EPS and XPS degradation are from most effective to less, such as oatmeal, wheat bran, cornmeal, and the sole diet.

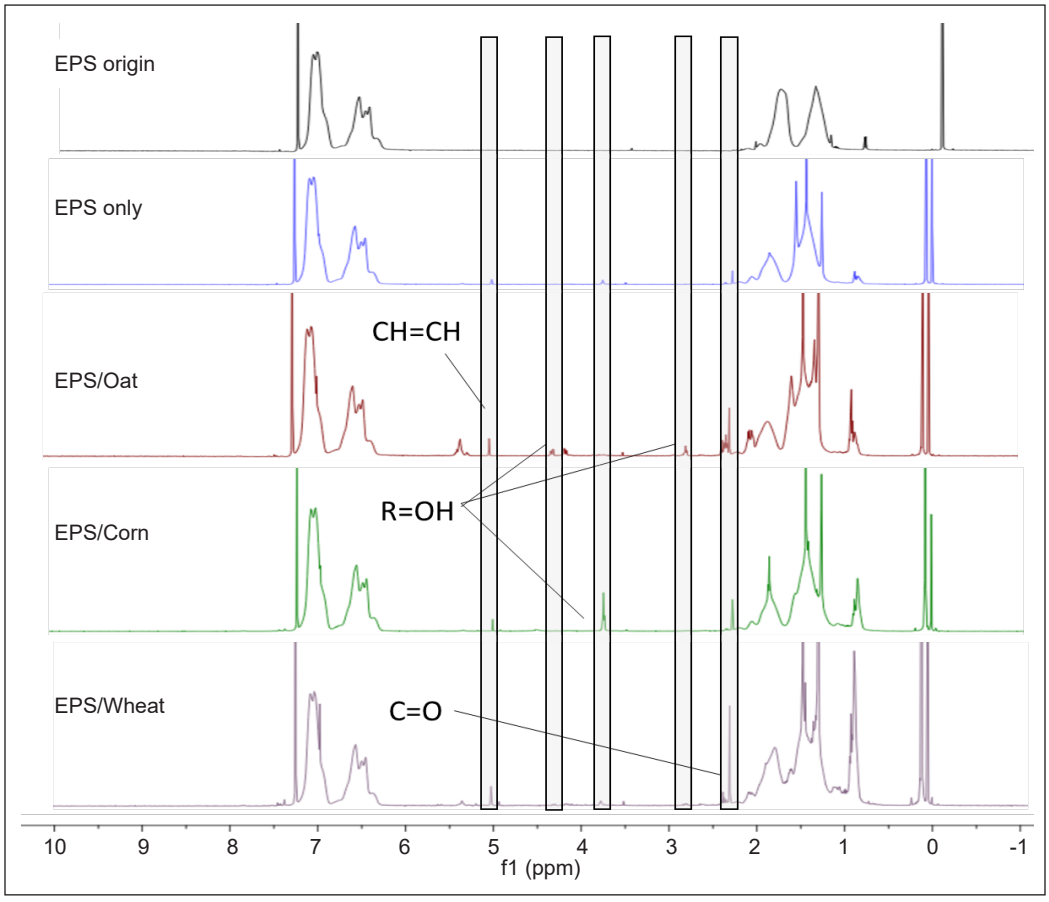

Figure 5. ^{1}H NMR spectra of residual polymer from larva frass (EPS group)
Note. ^{1}H nuclear magnetic resonance analytical technique was used to characterize changes in the end groups of the degraded polymer after passing through the larva gut passage

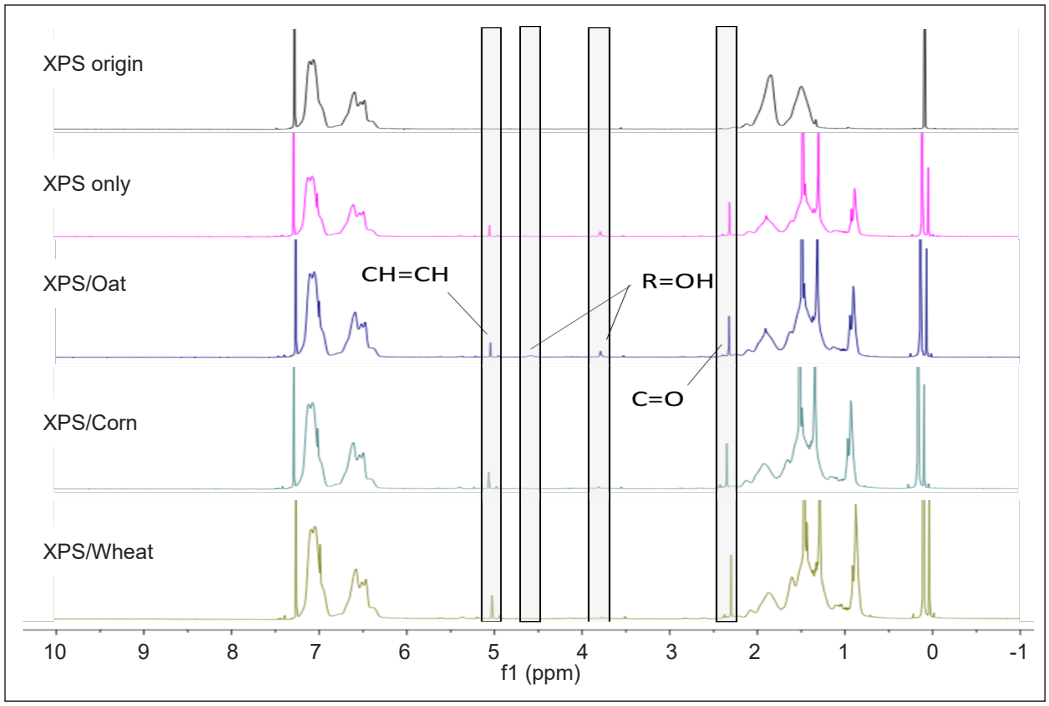

Figure 6. ^{1}H NMR spectra of residual polymer from larva frass (XPS group)
Note. ^{1}H nuclear magnetic resonance analytical technique was used to characterize changes in the end groups of the degraded polymer after passing through the larva gut passage

Growth and Development of *Z. atratus* Larvae After Ingested EPS and XPS Polystyrene

The growth and development of larvae were recorded throughout the study to compare the differences in *Z. atratus* larva growth after consuming EPS and XPS in different feeding diets. The *Z. atratus* larvae's growth and development depended on the types of diet and quantity of nutritional contents obtained. The final larvae length of *Z. atratus* larva among treatments revealed a significant difference (p<0.05) with different diets (Figure 7). The results showed that EPS (oatmeal), EPS (wheat bran), and EPS (cornmeal) produced longer larva lengths with 48.01 ± 3.32, 39.01 ± 2.29, 30.03 ± 1.36 mm, respectively, as compared to treatment EPS alone (26.10 ± 1.16 mm). In the XPS group, the larva's most extended final body length was attributed to treatment oatmeal as co-diet with 42.44 ± 2.50 mm, while the shortest was found in treatment XPS alone (24.87 ± 3.01 mm).

Oatmeal, wheat bran, and cornmeal provide essential nutrients for *Z. atratus* growth. These meals are protein-rich essentially for larvae growth (Emaleku et al., 2018; Onwulata et al., 2010; Rasane et al., 2015). Thus, all treatments in EPS and XPS groups containing supplements showed enhancement in final larva length compared to EPS or XPS alone. Larvae cuticles play an important role in larvae body elongation.

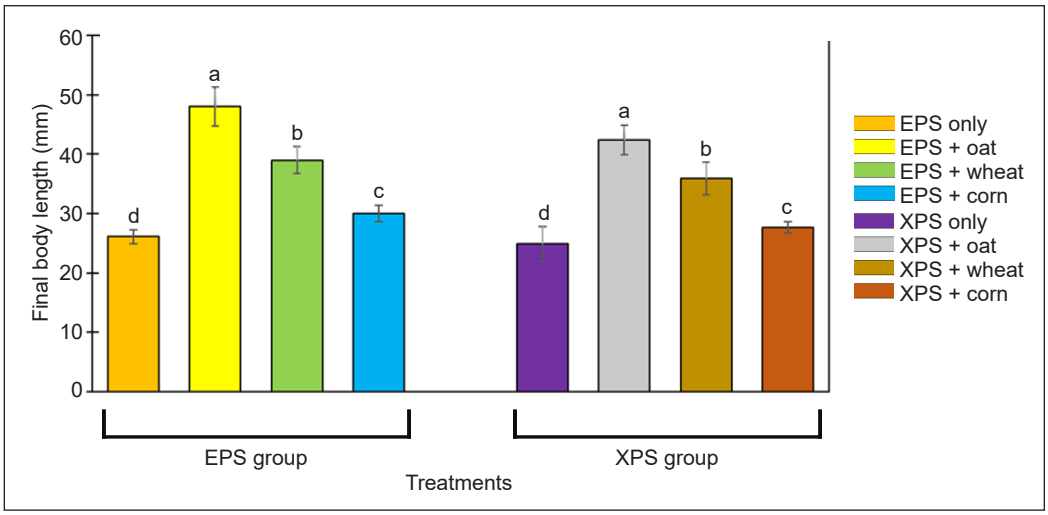

Figure 7. The final body length of the larva in each treatment with EPS and XPS groups. A digital vernier caliper was used to measure the larval body length

Note. All values were obtained from the mean of sixty replications with larvae reared separately per replication. Mean followed by different letters are significantly different at $p<0.05$ level according to Tukey's one-way ANOVA. The same letter above the bar within the group represents no significant difference between treatments

Protein is one of the components in larvae cuticle (Kaleka et al., 2019). Oatmeal and wheat bran-rich protein compared to cornmeal indicated larvae growth was more vigorous in a protein-rich meal. Besides, oatmeal could alter the gut microbiota to enhance digestion activity, improving nutrient uptake (Kristek et al., 2018). The larva length in oatmeal treatment as a co-diet showed a significant and highest value in the EPS and XPS groups. Meanwhile, wheat bran and cornmeal showed less efficiency in promoting *Z. atratus* larva growth than oatmeal. Treatment with EPS alone (EPS group) and XPS alone (XPS group) had shown the shortest larva length. Mlček et al. (2021) mentioned that the nutritional value of polystyrene-made materials is negligible but can convert into lipids after mineralization by providing a limited energy source for larvae growth. Thus, EPS and XPS made from polystyrene only provided limited nutrition to *Z. atratus* larvae growth.

The total number of larval instars was counted when the *Z. atratus* larva exuvium was observed, and larva instars counting stopped when the larva started to curl (Kim et al., 2015). In EPS and XPS groups, the analysis showed a significant difference in the total number of larval instars among treatments (Figure 8). In the EPS group, treatment with EPS only showed the highest number of instars (20 ± 2 instars), followed by EPS (cornmeal) with 18 ± 1 instars, then EPS (wheat bran) with 16 ± 1 instars, and the lowest was EPS (oatmeal) with 15 ± 2 instars. Meanwhile, treatment with XPS only (20 ± 1 instars) showed the highest number of larval instars among the XPS group, followed by XPS (cornmeal) with

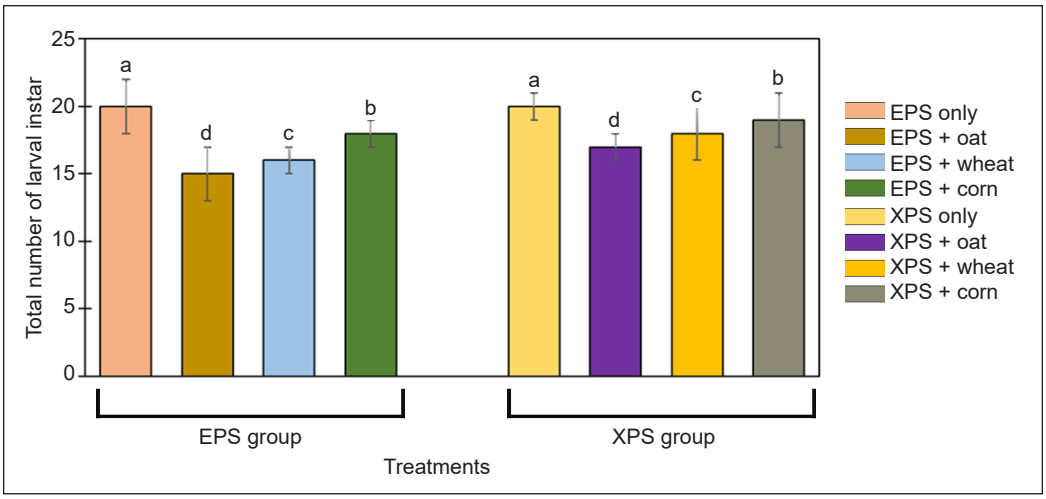

Figure 8. The total number of larval instars in each treatment with EPS and XPS groups. Each time of larvae molt (exuvium) was counted as a larval instar

Note. All values were obtained from the mean of sixty replications with larvae reared separately per replication. Mean followed by different letters are significantly different at $p<0.05$ level according to Tukey's one-way ANOVA. The same letter above the bar within the group represents no significant difference between treatments

19 ± 2 instars. XPS (wheat bran) with 18 ± 2 instars and XPS (oatmeal) with 17 ± 1 instars. Nutrient intake influences larva's instars (Ribeiro et al., 2018). The rising number of instars might be a lack of food or nutritional deficiencies (Esperk et al., 2007). Treatment with EPS only and XPS only showed the highest number of instars.

In contrast, treatment with supplements revealed a lower total number of instars in EPS and XPS groups. Oatmeal as a supplement showed the lowest number of instars in the EPS and XPS groups, followed by wheat bran and cornmeal. The total number of instars in the wheatmeal and cornmeal supplement treatments was shorter than in the EPS (only) and XPS (only). Protein is a critical component of all organisms' tissues and essential for growth and development (Ortiz et al., 2016). Protein content increases and the instars number will reduce (Morales-Ramos et al., 2010). Oatmeal and wheat bran contain many essential nutrients, especially protein. However, the structural characteristics and protein fraction distribution are different. Oat protein is soluble in salt or water, while wheat protein is insoluble in salt solutions (Rasane et al., 2015). Thus, oat protein is more readily digested and dissolves completely in the larvae's gut than wheat. Besides, Chojnacka et al. (2021) study revealed that cornmeal contains an insufficient amount of amino acids compared to wheat diets which play an important role in assisting protein digestibility. Therefore, the highest number of larval instars is treatment EPS or XPS as sole diets followed by cornmeal, wheat bran, and oatmeal.

In EPS and XPS groups, the analysis showed a significant difference among treatments in the total duration of larval (Figure 9). Treatment with EPS only showed the most prolonged larval duration with 223 ± 9 days in the EPS group. Meanwhile, treatment with XPS only (237 ± 5 days) also showed similar results to the EPS group. In contrast, co-diet meal treatment revealed a shorter larval instar duration in EPS and XPS groups. Ruschioni et al. (2020)'s study on *T. molitor* revealed that protein and carbohydrates are the primary nutrients that affect larval development time. Rho and Lee (2022) discovered that increased dietary nutrition, such as protein and carbohydrates, shortened larval development time.

The total duration of larva in the treatment-contained supplements was shown to be shorter than treatment EPS (only) and XPS (only). The shortened development time of larvae growth in treatment supplements is due to the sufficient nutrient (protein and carbohydrates) and other micronutrients provided. Oonincx et al. (2019) study proved that sufficient protein diets able to reduce development time in *T. molitor* and black soldier flies (*Hermetia illucens*). Oatmeal as a co-diet showed the shortest instar duration in the EPS group (157 ± 6 days) and XPS group (168 ± 4 days). Oatmeal is rich in macronutrients and micronutrients and helps to improve the diversity of larvae gut microbiota, indirectly improving

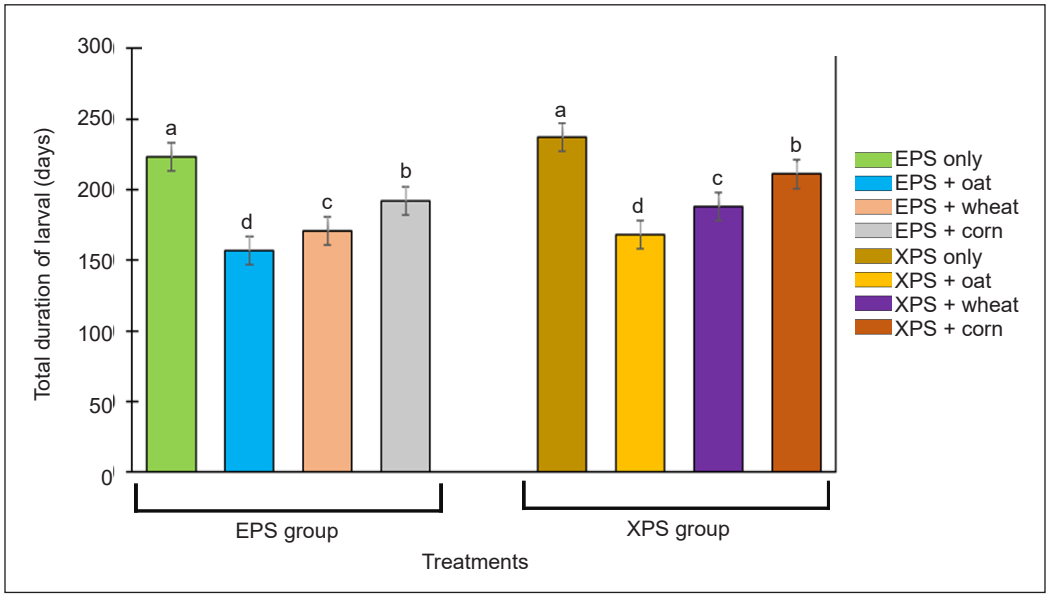

Figure 9. The total duration of larval in each treatment with EPS and XPS groups. Larvae curled were counted as the end of the larval stage

Note. All values were obtained from the mean of sixty replications with larvae reared separately per replication. Mean followed by different letters are significantly different at $p<0.05$ level according to Tukey's one-way ANOVA. The same letter above the bar within the group represents no significant difference between treatments

nutrient uptake. Urbanek et al. (2020) study revealed that *T. molitor* consuming oatmeal increases the variety of gut flora. It indicated that oatmeal, wheat bran, and cornmeal, with sufficient nutrients, supplied, reduced larval development time.

Gut Microbial Analysis

Microbial community analysis was done on oatmeal treatment due to high EPS and XPS polystyrene consumption. The alpha analysis resulted revealed that treatment OB (EPS) showed the highest value in Chao1 indices, Fisher alpha index, and Shannon and Simpson index as compared to other diets such as OB (XPS) and OB (only). Chao1 and fisher alpha indices indicated that the richness of bacterial species is slightly higher in the diet of OB (EPS) compared to other diets. Shannon diversity and Simpson index refer to the species diversity and also showed the highest values in OB (EPS) groups followed by OB (XPS), then OB only (Table 2). Jiang et al. (2021) revealed that the difference in diversity might be due to dietary differences and related to gut ecophysiology. Study by Peng et al. (2019) on *T. molitor* feeding with polystyrene also showed increasing microbial community diversity compared to normal diet feed. Therefore, the feeding meal that included EPS and XPS may lead the gut flora to grow, making it faster to digest. Similar phenomena were shown in four different diversity analyses where the treatment OB (EPS) and OB (XPS) showed higher value in terms of species richness and diversity compared to OB (only). Besides, density may affect the microbial population in the gut. High polymer density with high molar mass may cause microorganisms and oxidizing enzymes difficulty accessing polymer chains (Mohanan et al., 2020). Furthermore, XPS is denser than EPS, eventually reducing appetite and limiting the microbial population's growth. Thus, treatment OB (EPS) may have higher gut microbial diversity than OB (XPS).

The Bray-Curtis Distance dissimilarity measure was used to perform principal coordinate analysis (PCoA), which identified a cluster linked within treatment OB (only), OB (EPS), and OB (XPS). The microbiomes of *Z. atratus* larvae fed with treatment OB (EPS) and OB (XPS) were almost similar (Figure 10). However, the microbial community composition has differed from larvae fed between treatment OB (Only) and polystyrene (EPS and XPS) containing

Table 2
Bacterial diversity analyses based on illumine sequencing of the 16S rRNA gene amplicons for Zophobas atratus *larvae under different diet conditions (OB only, OB + EPS groups, OB + XPS)*

Samples	Chao1	Fisher	Shannon	Simpson
OB fed only	57.67	8.41	3.26	0.94
OB + EPS	66.00	9.89	3.62	0.96
OB + XPS	64.67	9.64	3.49	0.95

Note. OB = Oatmeal; EPS = Expanded polystyrene; XPS = Extruded polystyrene

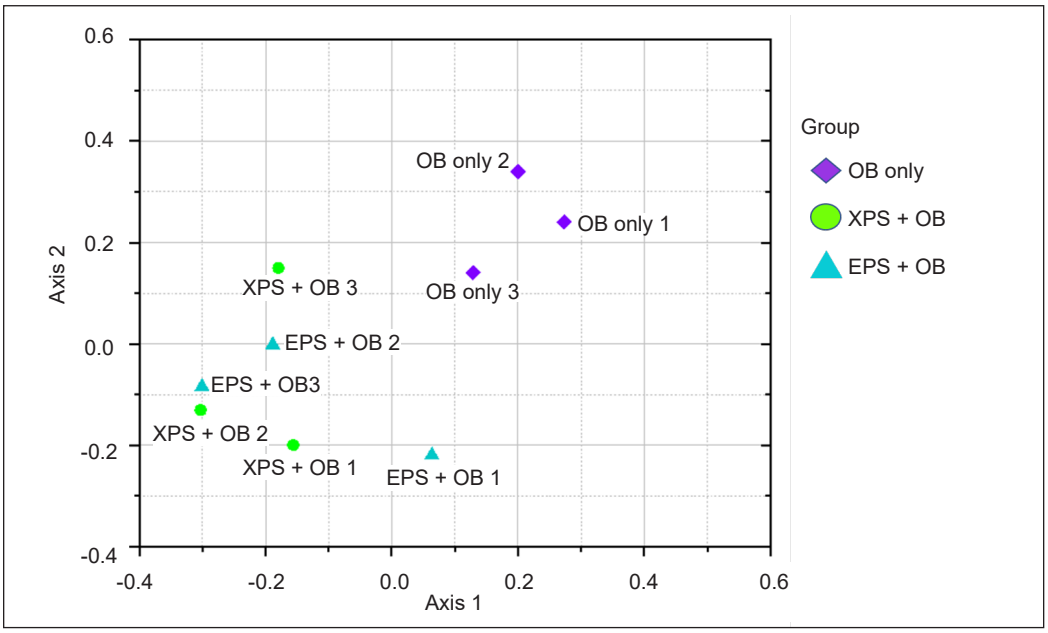

Figure 10. Analysis of gut microbiomes of *Zophobas atratus* larvae with PCoA
Note. Principal coordinate analysis (PCoA) on the population of gut microbiomes of *Z. atratus* larvae between different diets (OB only, XPS + OB, and EPS + OB fed) based on Bray Curtis Distance. OB = oatmeal; XPS = Extruded polystyrene; EPS = Expanded polystyrene

diets. EPS and XPS are made from styrene monomer, but the manufacturing processing method, cell structure, and density differ. Thus, the diversity of microbial species between treatment OB (EPS) and OB (XPS) may have high similarities. Oatmeal provides essential nutrients for the growth of *Z. atratus* larva and improves the appetite of larvae to ingest polystyrene (Fu et al., 2020). Therefore, adding EPS and XPS with oatmeal help improve larvae appetite and digestion, indirectly shifting the composition of specific microbial species to digest the EPS and XPS. Lou et al. (2021) study on yellow mealworms with different diets revealed that the gut microbiome showed distinct clustering between PS + bran co-diet and normal feeding diets. Therefore, the microbial community was distinct from treatment OB only compared to OB (EPS) and OB (XPS).

Relative abundance analysis was performed on the genus level. *Klebsiella* sp., *Kluyvera* sp., and *Enterobacter* sp. were discovered as OTUs with higher relative abundance in treatment OB (EPS) and OB (XPS) compared to OB (only) (Figure 11). These three OTUs were identified as facultative anaerobic bacteria at the genus level, meaning they can live in an environment with or without oxygen (Farmer III, 2015; Lopes et al., 2010; Maintinguer et al., 2017). *Klebsiella* sp. was a prominent genus in the gut microbiomes of *Z. atratus* larvae fed with treatment OB (XPS) and OB (EPS) with respective 8.49 ± 5.60% and 12.01 ± 1.39%, which increased by 6.31% and 9.83 % compared to treatment

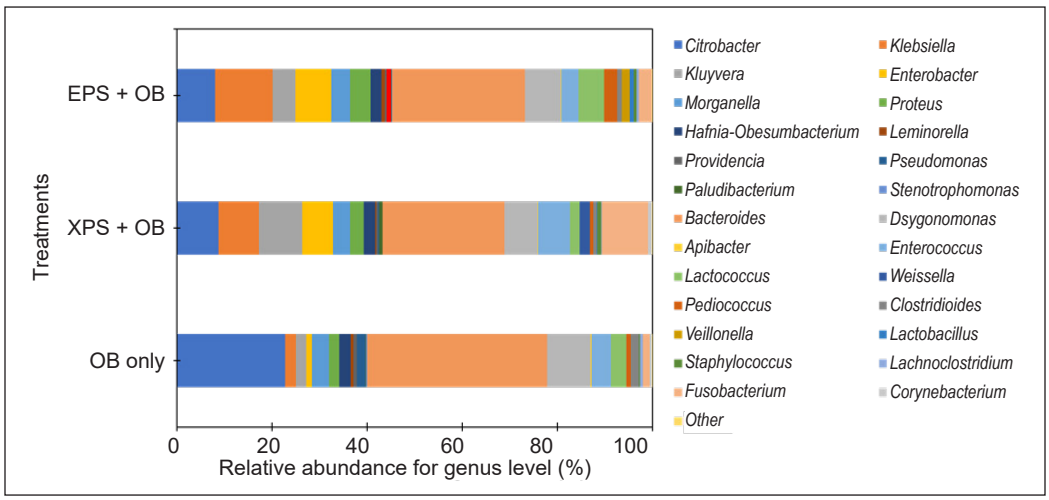

Figure 11. Analysis of gut microbiomes of *Zophobas atratus* larvae with relative abundance in genus level
Note. Relative abundance analysis on the population of gut microbiomes of *Z. atratus* larvae between different diets (OB only, XPS + OB, and EPS + OB fed) at the genus levels. OB = Oatmeal; XPS = Extruded polystyrene; EPS = Expanded polystyrene. All values represent mean ± standard deviation

OB (only) with the relative abundance of 2.18 ± 1.76%. *Klebsiella* sp. acts mutualistic relationship with insect hosts by digesting food in the gut and providing nutrients to the host (Sun et al., 2022). Besides, Machona et al. (2022) mentioned that *Klebsiella* sp. can degrade polystyrene in the gut system of *T. molitor*. Therefore, *Klebsiella* sp. showed higher abundance in OB (EPS), and OB (XPS) than OB (only) may be due to more *Klebsiella* sp. are require digesting oatmeal and EPS or XPS. Saygin and Baysal (2021) study revealed that *Klebsiella* sp. could degrade sub-microplastics through oxidation. The functional groups, such as the C=O carbonyl group, vinyl group, and oxygen-to-carbon ratio, were increased when the microplastics were treated by *Klebsiella* sp. Besides that, *Kluyvera* sp., as one of the OTUs members in the microbial community of treatment OB (XPS) and OB (EPS), showed a shift to slightly higher relative abundance (9.10 ± 1.23% and 4.89 ± 3.98%) respectively than treatment OB (only) with a relative abundance of 2.17 ± 0.95%. *Kluyvera* sp. can help *Z. morio* digest oatmeal diets and convert nutrients to the gut (Pivato et al., 2022).

Furthermore, XPS is a more closely packed structure than EPS, which takes time to digest (Turner, 2020). The relative abundance of *Kluyvera* sp. is higher in OB (XPS) than OB (EPS) may be due to more need for this species among the gut community to digest XPS. Thus, *Kluyvera* sp. might help degrade polystyrene in the gut of *Z. atratus* larval. *Enterobacter* sp. in treatment OB (XPS) and OB (EPS) with 6.46 ± 4.95% and 7.53 ± 4.10%, which increased by 5.28% and 6.35% as compared to treatment OB (only) with a relative abundance of 1.18 ± 0.22%. The symbiotic relationships between insects and *Enterobacter* species may be advantageous

to the host as it has varied capacities to catalyze nitrogen fixation, hydrolyze and ferment carbohydrates, and synthesize vitamins and pheromones for the host (Hendrichs et al., 2021). *Enterobacter* sp. needs a small amount to break down normal feeding diets (oatmeal) but adding EPS or XPS triggers rapid growth of *Enterobacter* sp., digested all the food sources. It also suggested that *Enterobacter* sp. can biodegrade food, including EPS and XPS. *Serratia marcescens* belonging to *Enterobacter* sp. showed overwhelming superiority in PS-fed gut microbiomes and was found in the PS-fed group (Lou et al., 2020). *Enterobacter* sp. should be investigated further since it is thought that various *Enterobacter* species may play a role in EPS and XPS polystyrene degradation. *Klebsiella* sp., *Kluyvera* sp., and *Enterobacter* sp. species in the genus were advised to be further validated by isolation and enzymatic testing to evaluate the capacity of plastic breakdown and the enzyme involved.

CONCLUSION

The results of this study demonstrated that superworms, *Zophobas atratus* larvae, from a Malaysian source could biodegrade EPS and XPS, supporting the ubiquity of PS degradation in this insect species. Furthermore, the PS consumption rate was enhanced significantly in the presence of co-diet with the sequence: oatmeal > wheat bran > cornmeal, positively depending on the protein content. FTIR and ^{1}H NMR analysis have further proven EPS and XPS polymer biodegradation. Incorporating co-diets such as oatmeal, wheat bran, and cornmeal in EPS and XPS groups showed an increase in *Z. atratus* larva length, total number of instar, and duration of instar. *Kluyvera* sp., *Klebsiella* sp., and *Enterobacter* sp. in the gut of *Z. atratus* larvae were discovered to be strongly associated with the XPS and EPS polystyrene degradation. Thus, further studies are needed to verify the role of gut microbes in depolymerization and biodegradation via antibiotic suppression tests. Besides, further study on PS degradation rates can be done by examining THF extracted fraction.

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Evaluation of Environmental Contamination with *Salmonella* spp. in a Large Animal Ward at a Veterinary Hospital in Malaysia

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ABSTRACT

Veterinary hospitals are important locations for various sick and immunocompromised animal patients. These centers may act as reservoirs for nosocomial diseases such as *Salmonella* infection, one of the most common causes of healthcare-associated infections in veterinary hospitals. The study was performed at the Large Animal Ward (LAW), University Veterinary Hospital, Universiti Putra Malaysia, to assess the environment's degree of *Salmonella* spp. contamination. Environmental samples were obtained from various floor and surface areas in the LAW using sterile, moistened gauze. *Salmonella* spp. was determined using conventional bacteriological culture on all samples. Positive *Salmonella* isolates were subject to antimicrobial sensitivity testing. A total of 6 out of 135 (4.4%) samples were found to be positive for *Salmonella* spp., with 5/116 (4.3%) samples obtained from the ward environment and 1/19 (5.3%) obtained from reusable equipment. Antimicrobial sensitivity testing revealed three resistance profiles: all isolates were resistant to penicillin and enrofloxacin, one isolate was resistant to streptomycin, and one was resistant to gentamicin. The results indicate that animal treatment areas within the LAW can become contaminated with *Salmonella* spp. This study highlights the importance of improving biosecurity programs to prevent nosocomial diseases in patients and the hospital environment.

Keywords: Environment, large animal, nosocomial, *Salmonella*, veterinary hospital

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INTRODUCTION

The most prevalent healthcare-associated infection in large animal veterinary clinics has been attributed to *Salmonella enterica,* and outbreaks of nosocomial salmonellosis have been documented (Hartmann et al., 1996; Schott et al., 2001; Steneroden et al., 2010). These outbreaks of salmonellosis have resulted in the closure of hospitals for extensive cleaning and disinfection, which has led to significant financial losses, including losses in client confidence, employee morale, and student educational opportunities (Aceto et al., 2007; Dallap Schaer et al., 2010; Ward et al., 2005). In addition to patients and resident animals being infected or acquiring the infection, they may also act as a reservoir for transmitting these microorganisms to other patients, hospital staff, students, and clients. *Salmonella* infection is a major concern for animal health; there is also a serious concern about its zoonotic potential, which significantly impacts public health (Bean & Griffin, 1990; Mead et al., 1999). Furthermore, there is a possibility that animals returning to their farms and stables after being discharged from the hospital may carry infectious agents to their herd mates, and therefore represent a further ethical obligation for veterinarians to prevent the spread of the disease outside of the hospital environment (Morley et al., 2013). The ethical and professional responsibility of veterinarians is to create a protective environment for hospitalized animals, students, staff, and clients to minimize infectious disease hazards in veterinary hospitals (Morley, 2013; Morley et al., 2013).

Stress is a factor that may instigate *Salmonella* fecal shedding by diseased animals (House et al., 1999; Wray et al., 1991). When animals infected with *Salmonella* are admitted to the hospital along with other immunocompromised animals, they may be subjected to environmental changes such as new locations, different feedings, and treatment regimens. These stressed animals pose a significant risk of nosocomial *Salmonella* infection to vulnerable animals (Pandya et al., 2009). Horses that have traveled an hour or more to the veterinary teaching hospital had antimicrobials before admission, abnormal nasogastric intubation findings, had an intravenous catheter placed, or rectal palpation performed, developed leukopenia, laminitis, or diarrhea during hospitalization, abdominal surgery performed (enterotomy or anastomosis) and were hospitalized for more than eight days had an increased likelihood of shedding *Salmonella* organisms in their feces (Burgess & Morley, 2019; Dallap Schaer et al., 2012; Ernst et al., 2004; Hird et al., 1984; Kim et al., 2001).

Hospitalized patients frequently have a compromised immune system because of the stress associated with transportation, management changes, and the underlying medical or surgical condition (Irwin, 1994; Radošević-Stasic et al., 1990; Sheridan et al., 1994; Tannock & Savage, 1974). Additionally, patients may have lowered resistance to gastrointestinal tract infections

because of decreased gastrointestinal tract motility and altered gastrointestinal tract flora because of anorexia, surgery, and antimicrobial therapy (Hird et al., 1984; Owen et al., 1983). Thus, eliminating nosocomial *Salmonella* infections requires two fundamental strategies: decreasing patient exposure to pathogenic organisms and limiting additional increases in patient vulnerability to pathogens.

In large animal facilities, the veterinary staff will likely perform duties in central services (e.g., pharmacy, storerooms, and laundry) and some specialties (e.g., radiology). Commonly, the individuals most likely to encounter patients are veterinary students, who is rotated throughout multiple sections and may be considered novices concerning patient management and infection control practices. The degree of integration and shared resources in the hospital can impact disease transmission even though infectious disease or environmental contamination in one area may likely affect patients managed in that region (Burgess & Morley, 2019; Steneroden et al., 2010). A study has demonstrated that the probability of *Salmonella* detection in the hospital environment increases as the caseload increases and the demand for personnel subsequently increases. Personnel may not comply with established infectious disease control protocols with increased demand of cleaning frequency and patient contacts, which creates more opportunities for infectious organisms' transmission between patients (Burgess & Morley, 2018; Dallap Schaer et al., 2010; Hartmann et al., 1996; Roberts & O'Boyle, 1981; Schott et al., 2001). These demonstrate that the ecological factors affecting the contamination of the hospital are complex and should be considered for the development of epidemics of nosocomial salmonellosis.

This study aims to ascertain the degree of *Salmonella* spp. contamination in the environment in a large animal veterinary hospital in Malaysia as one of the efforts to improve the biosecurity measures of the facility.

METHODOLOGY

Environmental samples were collected from 135 sites in the Large Animal Ward (LAW) at the University Veterinary Hospital (UVH), Faculty of Veterinary Medicine, Universiti Putra Malaysia. The selection of sample sites took into consideration the layout of the wards, the pattern of movement of the animals and staff in the ward, and the usage of space for treatment or procedures. A total of 17 pens/stable boxes were used to house resident animals and hospitalized patients in which the floors, walls, which are constructed from concrete and painted, feed, water buckets made of plastic, and the entrance gates were sampled. All wards are half-walled at the back, which faces outside or on the sides. Four stable boxes were used to ward horses, and four and nine pens were used to hospitalize large and small ruminant animals, respectively. Four walkways used to enter and exit the premises and five locations on treatment floor areas were also sampled. The other areas that were sampled were the cattle

crush and horse stock bars, and flooring, which is in the central area of the LAW and close to each other, and hand contact surfaces such as table surfaces and tap handles located at sinks for handwashing and near wards, the entrance gate handles, and table surfaces used for the treatment of patients and preparation of treatments kept in the central area, and cupboards handle at the drugs dispensary cabinet. Cleaning equipment (brooms, rakes, and scoops) and reusable equipment (nasogastric and orogastric tubes and stomach pumps) were also sampled. The frequency of specific sites sampled is demonstrated in Table 1.

Environmental samples such as taps, table surfaces, cupboard handles, equipment, and entrance handles were collected by swabbing the targeted surface for approximately 20 s with a sterile gauze saturated with sterile saline for 5 s. The gauze was then placed in a sealable and labeled plastic bag. All samples were collected aseptically using sterile gloves that were changed between sampling locations. One or three gauze swabs were swabbed along the entire surface for the sampling of table tops and restraint equipment. Three gauze swabs were swabbed to sample the floors and walls, covering an area of roughly 61 cm × 61 cm.

Standard aerobic bacterial culture of the environmental samples was performed. For enrichment, the gauze swab was placed in 90 ml of Buffered Peptone Water (BPW) (Thermo Scientific ™ Oxoid ™, USA), and the broth was incubated at 37°C for 24 hr in aerobic conditions. One ml of BPW mixture was transferred by micropipette into 9 ml of Rappaport-Vassiliadis (RV) broth (Thermo Scientific ™ Oxoid ™, USA) and incubated at 37°C for 24 hr in aerobic condition. A loopful of the RV broth was cultured onto brilliant green (BG) agar plates and xylose-lysine-deoxycholate (XLD) agar plates (Thermo Scientific ™ Oxoid ™, USA). The plates were incubated at 37°C for 24 hr in aerobic conditions. Three non-lactose fermenting, hydrogen sulfide-producing colonies would be selected and isolated. These colonies were then subjected to biochemical tests: urease agar, citrate utilization test, Sulfur Indole Motility (SIM) test, and triple sugars iron (TSI) agar slant (Thermo Scientific ™ Oxoid ™, USA), which were incubated at 37°C for 24 hr. TSI tubes were evaluated for changes that indicated alkaline (red) development in the slant and acid (yellow) and hydrogen sulfide (H_2S) precipitate in the butt, with or without gas presence. The absence of color in urease agar and color change from green to a blue indication of citrate utilization indicated positive *Salmonella* samples.

The antibiotic susceptibility of the positive isolates was tested using the standardized disc diffusion method (Bauer et al., 1966). Positive cultures were obtained from the nutrient agar collected using a wire loop, mixed into a test tube filled with 2–3 ml of sterile saline, and mixed using a vortex mixer until the density was visually equivalent to 0.5 MacFarland standard. The bacterial broth suspension was streaked evenly in 3 planes using sterile, non-toxic cotton swabs onto the surface of Mueller-

Hinton agar plates (Thermo Scientific ™ Oxoid ™, USA) and left to dry for 2 to 4 min. The antimicrobial sensitivity discs were then placed on the culture using a Disk Diffusion Dispenser (Oxoid™, USA). Antibiotic discs tested were penicillin 10 µg, streptomycin 300 µg, gentamicin 10 µg, and enrofloxacin 5 µg (Thermo Scientific ™ Oxoid ™ Antimicrobial Susceptibility Discs, USA). The plates were incubated at 37°C for 24 hr, and the size of the inhibition zone was interpreted as sensitive or resistant according to Clinical and Laboratory Standards Institute guidelines (Clinical and Laboratory Standards Institute [CLSI], 2020). According to Magiorakos' criteria, samples were classed as having Multiple Drug Resistance (MDR) when resistant to at least one drug in three or more antimicrobial classes (Magiorakos et al., 2012).

IBM SPSS Statistics for Windows (version 26.0) was utilized to generate descriptive statistics demonstrating *Salmonella* spp. contamination by different areas sampled and sampling locations.

RESULTS

Overall, 135 samples were collected from the walls (n = 49), floors (n = 10), hand-contact surfaces (n = 21), food and water buckets (n = 32), and equipment (cleaning, n = 10; reusable, n = 9; restraint, n = 4) at the large animal ward. Samples were evaluated by standard aerobic bacteriological culture and antimicrobial sensitivity testing. The results revealed that the large animal ward areas and equipment are contaminated with *Salmonella* spp. with an overall prevalence of 4.4% (6/135). *Salmonella* was successfully isolated from floors of walkways in the large animal section of the hospital ad flooring of a pen (3/26, 11.5%), walls of a stall box (1/16, 6.3%), and a water bucket (1/32, 3.1%). Additionally, only one (10%) sampled cleaning equipment recovered *Salmonella* spp., which was isolated from a rake used to scoop fecal materials from the wards. The overall prevalence of positive samples for *Salmonella* is demonstrated in Table 1. *Salmonella spp.* was not isolated from the treatment area, restraining equipment, or the commonly used veterinary equipment such

Table 1
The frequency of positively isolated Salmonella *spp. of environmental and equipment samples obtained from the LAW, UVH*

Type of sample	N	Positive	Negative
Walls	16	1 (6.3%)	15 (93.8%)
Floors	26	3 (11.5%)	23 (88.5%)
Hand-contact surfaces	38	0 (0%)	38 (100%)
Feed and water buckets	32	1 (3.1%)	31 (96.9%)
Equipment			
- Cleaning	10	1 (10%)	9 (90%)
- Reusable	9	0 (0%)	9 (90%)
- Restraint	4	0 (0%)	4 (100%)
Total	135	6 (4.4%)	129 (95.6%)

as nasogastric or orogastric tubes. There was no statistically significant difference between environment and equipment samples, χ (1) = 0.35, p = 0.852.

The results of the antimicrobial sensitivity testing revealed three antimicrobial-resistant profiles. Four isolates had an antimicrobial-resistance profile of penicillin-enrofloxacin, one isolate was penicillin-enrofloxacin-streptomycin, and one isolate was penicillin-enrofloxacin-gentamicin. None of the six *Salmonella* isolates were pan-susceptible: all isolates were resistant to penicillin and enrofloxacin. Five isolates were sensitive to streptomycin, while one was resistant. All isolates except one were sensitive to gentamicin (Table 2). Four of these isolates (the floor and water bucket of wards and floors of the two walkways) had similar antimicrobial patterns where isolates were sensitive to streptomycin and gentamicin and resistant to penicillin and enrofloxacin. Of these isolates, 2/6 (33.3%) were classified as having multiple drug resistance, as these were resistant to one agent in three or more antimicrobial classes.

DISCUSSION

Prevalence

The environmental contamination reported in this study is less prevalent than that reported at Ohio State University Veterinary Teaching Hospital (VTH) (5.9%) (Pandya et al., 2009), Colorado State University VTH (11.9%) (Burgess et al., 2004), and Equine Veterinary Hospital Santiago Chile (4.5%) (Soza-Ossandón et al., 2020), but higher than the prevalence reported by Michigan State University VTH (0.1%) (Ewart et al., 2001), and Purdue University VTH (2.1%) (Alinovi et al., 2003). However, it is challenging to directly compare the prevalence of *Salmonella* contamination in different hospital environments due to differences in microbiological culture techniques between institutions and their laboratories and various sampling techniques.

Various techniques and methods are used to recover or isolate *Salmonella* from environmental samples. The use of direct plating without enrichment, enrichment in a variety of broth media (including buffered peptone water, selenite, thioglycolate,

Table 2
The antimicrobial resistance profiles of the isolates of positively isolated Salmonella *spp. from environmental samples acquired from the LAW, UVH*

Salmonella spp. frequency	Antimicrobial resistance profiles	
4	Penicillin-enrofloxacin	
1	Penicillin-enrofloxacin-streptomycin	
1	Penicillin-enrofloxacin-gentamicin	
Antimicrobials	Sensitive	Resistance
Penicillin	0 (0%)	6 (100%)
Streptomycin	5 (83.3%)	1 (16.6%)
Gentamicin	5 (83.3%)	1 (16.6%)
Enrofloxacin	0 (0%)	6 (100%)

tetrathionate, and Rappaport-Vassiliadis broths), and culture on a variety of agar medium (including MacConkey, brilliant green, XLD, and xylose-lysine-tergitol-4 [XLT-4] agar) (Adzitey et al., 2012; Cummings et al., 2014; Rostagno et al., 2005; Ruple-Czerniak et al., 2014) are among the reported. Despite the numerous methods available to culture and isolate *Salmonella* spp., not all are equally effective at isolating the bacteria. A study compared two culture techniques during a *Salmonella* epidemic at a large animal hospital, one was the current method used in the diagnostic laboratory associated with the hospital, where samples were cultured in selenite broth (SEL) and plated on XLD (SEL-XLD), and the other method was reported for environmental surveillance in a veterinary teaching hospital where samples were incubated in tetrathionate broth with brilliant green (TBG), followed by Rappaport-Vassiliadis (RV) broth and plated on XLT-4 (TBG-RV-XLT) (Burgess et al., 2004). According to a study, the TBG-RV-XLT culture approach was significantly more sensitive than the SEL-XLD culture method for detecting *Salmonella* in environmental samples (Lyle et al., 2015).

A similar finding was also noted in another study which demonstrated that enrichment with tetrathionate and RV broth was three times more sensitive than a culture method that incubated in thioglycolate enrichment broth (Ruple-Czerniak et al., 2014). Failing to correctly detect *Salmonella* bacteria resulting from the use of poorly sensitive culture techniques that yield false negatives delays the application of strategies to curb nosocomial outbreaks and thereby facilitating the spread of *Salmonella* in hospital environments and between patients (Dallap Schaer et al., 2010; Hyatt & Weese, 2004). The more sensitive method could be attributed to increased testing that uses selective enrichment that encourages the growth of *Salmonella* spp.; however, more steps in the cultivation can cause a delay in the reporting of results. A rapid diagnosis is also vital to initiate proper treatment and infection control measures (Hyatt & Weese, 2004).

There is a general lack of sensitivity in all detection methods for *Salmonella* spp., which is why enrichment methods are typically recommended for use to aid in detection with culture (Hyatt & Weese, 2004). Recognizing that using enrichment broths in the culture process produces qualitative rather than quantitative results (positive vs. negative) is critical. It would preclude it from determining the degree of environmental contamination adequate to induce infection in animals or people (Burgess et al., 2004; Ruple-Czerniak et al., 2014). It is noted that there is a distinction between sampling strategies, such as the use of premoistened sponged, gauze sponges, gauze swabs, and electrostatic wipes. To date, there are no superior sampling or culture methods that have been determined; nonetheless, there have been two studies that have evaluated two distinct samplings and culture approaches. Both studies (Goeman et al., 2018; Ruple-Czerniak et al., 2014) agree that electrostatic wipes are more effective

at collecting bacteria and debris and using enrichment broths to aid in the detection of *Salmonella* bacteria. The sampling and recovery techniques applied in this study were deemed reliable and effective at isolating the bacteria, as the study used the lowest effective number of steps to isolate *Salmonella* spp. successfully. Bacterial growth may be inhibited by environmental factors such as disinfectants. Therefore, polymerase chain reaction (PCR) can be used to detect *Salmonella* bacteria in the environmental samples obtained. Studies have demonstrated that PCR was more sensitive in detecting positive *Salmonella* spp. in environmental samples than bacterial culture (Cohen et al., 1996; Ewart et al., 2001).

Impact

The study's findings imply that students and veterinarians working in the large animal hospital should be mindful of the dangers of nosocomial infections spreading to other patients and potential zoonotic infections spreading to themselves, clients, and visitors. Potential health problems that could be attributed to *Salmonella* infections in students, staff, and clients could not be identified as this was beyond the scope of the study. The two floors used as walkways are the most used entrances to the hospital by students and staff. Floor samples are the most frequently contaminated in veterinary hospital environments after floor drains (Alinovi et al., 2003; Pandya et al., 2009). It is a commendable finding that the treatment floor area, stock, crush, treatment tables, and the entrance floors and gates used by animals are not contaminated with *Salmonella* spp. A water bucket in one of the small ruminant wards was contaminated with *Salmonella* spp. It could be a potential source of nosocomial infection to the resident animals or patients, as *Salmonella* is transmitted by ingesting the bacteria. Nasogastric intubation, a routine procedure to treat colic in horses, has been established as a risk factor for *Salmonella* detection (Hird et al., 1984, 1986; Traub-Dargatz et al., 1990). In this study, it was found that these gastric tubes were not contaminated with *Salmonella* spp. However, one of the cleaning equipment, the rake, was found to be harboring *Salmonella* organisms. It could be a potential method to disseminate the bacteria throughout the rest of the hospital environment. *Salmonella* spp. in the hospital environment could be detrimental to hospitalized patients, resident animals, and personnel working due to increased nosocomial and zoonotic risks. It is essential to comprehend and manage the microbial contamination of components interacting with patients to prevent nosocomial infections.

Current Cleaning Protocols

There have been no outbreaks of nosocomial infections due to *Salmonella* in this veterinary hospital. The current cleaning and disinfection protocols used at the hospital where areas are cleaned using a high-pressure water hose while scrubbing with a brush broom and disinfected with a diluted bleach solution (1:32). There is a foot bath

that is cleaned and filled daily with a diluted bleach solution, along with a separate area to wash boots at one entrance to the hospital, with sinks and soap available for hand washing near the treatment area. The entrances and treatment areas are cleaned at least twice daily and immediately following each animal examination. When there was suspicion or confirmed cases of infectious disease, animals were hospitalized at the ward, and infection control programs and other biosecurity measures such as the use of disposable gloves and gowns and frequent hand washing, and sanitizing were enforced. Unfortunately, a large animal isolation facility for infectious diseases is not available at the current time. Based on the output of this study, an environmental surveillance report for *Salmonella* spp. has been issued, and a suggestion for routine disinfection, guidelines for best-practice prevention efforts, and environmental surveillance protocol have been brought forward to the management of the large animal ward. The suggestions have been accepted positively. Other measures to further help reduce the population of pathogenic organisms in the wards by adding disinfectant footbaths at all entrances into the large animal ward, and not just at the main entrance to the facility. Before entering the hospital premises, boots should be vigorously scrubbed and cleaned after each farm/stable visit. The boots should be immersed in a regularly changed footbath containing disinfectant such as 1:16 dilution of sodium hypochlorite (bleach) for the appropriate contact time, which has been shown to decrease microbial loads on the surface of the boots significantly (Hornig et al., 2016; Kirk et al., 2003; Morley et al., 2005; Stockton et al., 2006). Bleach is a very potent and inexpensive disinfectant used in veterinary settings that thoroughly inactivates numerous pathogens of concern. However, it is considerably deactivated by organic matter and has no detergent action (Steneroden, 2012). Disposable boot covers have significantly reduced nosocomial infections in hospital settings (Schott et al., 2001).

Infectious Disease Control Program

Infection prevention and control recommendations include wearing gloves when cleaning wards and treating animals, removing gloves, and washing hands immediately after work is complete. In places where patients are handled, hand-washing sinks or alcohol-based hand-sanitizing solutions should be readily available (Smith et al., 2004; Wright et al., 2005). All disinfectants require a clean and debris-free surface, and the disinfectant must be utilized at the manufacturer's recommended dilution and contact time. Stalls that housed discharged animals should be cleaned by removing all soiled bedding, followed by gently hosing down and scrubbing. Once the stall looks completely free of feed, bedding, feces, and blood, it should be cleaned extensively with a brush and detergents such as an iodophor detergent (Lindores*-30, Sunzen Biotech Berhad, Malaysia) to remove organic matter and biofilms. Follow up with rinsing

and application of the disinfectant, such as sodium hypochlorite, diluted at 1:32, allowing at least 10 min of contact time. In a study comparing the effectiveness of different types of disinfectants, bleach was the most effective in eliminating *Salmonella* organisms from all surfaces when detected using bacterial culture or PCR testing (Ewart et al., 2001). It is because of the mechanism of action of bleach, which causes the breakdown of the cellular lining but also destroys the DNA of the bacteria (Merritt et al., 2000).

It has been suggested that periodic environmental surveillance by dried environmental sampling should be collected and cultured for *Salmonella* spp. to determine the cleaning protocol's efficiency (Smith et al., 2004). While continuous monitoring is not required, it is preferable to undertake it on a set schedule, such as monthly or biannually (Burgess & Morley, 2015). It is also essential to ensure that any defect, chipped paint, loose flooring, floor mats, and damaged or rotted wood on surfaces of the ward and treatment areas are in good repair so that they can be easily cleaned and not become a nidus for pathogens to survive (Kramer & Ames, 1987). A study has shown that environmental sampling is indicated following the cleaning and disinfection protocol as a small proportion of stalls may remain contaminated with *Salmonella* spp.; therefore, a second round of cleaning and disinfection can be performed before the stall can be used for admissions (Alinovi et al., 2003). However, it has been noted that certain strains of *Salmonella* have been shown to survive in the environment despite rigorous cleaning and disinfection methods, functioning as a reservoir for hospital-acquired diseases and zoonoses. It was speculated that complete eradication might be difficult or unattainable in a large animal hospital environment (Pandya et al., 2009; Ruple-Czerniak et al., 2014; Steneroden et al., 2010).

Veterinary health care providers have an ethical and legal responsibility to minimize risks for infectious disease transmission in health care settings. To date, there have been few published discussions on the responsibilities of nosocomial infections in veterinary settings, and this aspect has to be emphasized and highlighted to the many veterinary care providers throughout the country. Several veterinary associations from various countries such as Australia, Britain, Canada, and the United States have released guidelines for infection control procedures (Australian Veterinary Association [AVA], 2017; Canadian Committee on Antibiotic Resistance [CCAR], 2008; Gerrad, 2021; Newbury et al., 2010; Stull et al., 2018). While Veterinary Associations set protocols, the employment and implementation of the procedures may vary from facility to facility. It was surmised from multiple studies that a significant number of veterinarians are either unaware of the risks of zoonoses or nosocomial infections or are complacent about the risks (Anderson & Weese, 2022; Lipton et al., 2008; McMillian et al., 2007; Tambuwal et al., 2009; Wright et al., 2008). An international group of experts recently developed a consensus on

opinions about infection control in equine populations (Morley et al., 2013). Therefore, at the current time, the best practice of infection control should be acknowledged and promoted in veterinary settings.

Antimicrobial Resistance

In this study, antimicrobial sensitivity testing tested the common antimicrobial drugs currently used in large animal practices in Malaysia. In this study, all the *Salmonella* isolates were resistant to penicillin (100%) and enrofloxacin (100%). Only two isolates had different antibiograms; one demonstrated resistance to gentamicin in addition to penicillin and enrofloxacin, and the other demonstrated resistance to streptomycin. It could be interpreted as three different species of *Salmonella* bacteria; however, further investigation with serotyping is warranted to assess this accurately. The emergence of resistance to antimicrobial drugs in salmonellae is a worldwide problem (Chaslus-Dancla et al., 2000; Dargatz & Traub-Dargatz, 2004). An amount of 28.6% of isolates in this study was resistant to one agent in three antimicrobial classes, which agrees with earlier reports in humans, horses, and other domestic animals (Mammina et al., 2002; van Duijkeren et al., 2002). Many of the documented nosocomial outbreaks associated with *Salmonella* in veterinary hospitals have been associated with MDR strains, even in isolates obtained from the environment (Amavisit et al., 2001; Castor et al., 1989; Dallap Schaer et al., 2010; Hartmann et al., 1996; Hartmann & West, 1995; Schott et al., 2001; Steneroden et al., 2010; Tillotson et al., 1997). Even routine environmental surveillance of veterinary hospitals has demonstrated isolates that possess MDR, where 6.25–40.1% of isolates demonstrated MDR (Pandya et al., 2009; Soza-Ossandón et al., 2020). These studies showed that isolates were resistant to streptomycin (Pandya et al., 2009) and gentamicin (Soza-Ossandón et al., 2020), similar to what was seen in this study; however, neither study reported resistance to enrofloxacin as seen in all isolates obtained from this study.

CONCLUSION

This study has established the presence and degree of *Salmonella* spp. contamination in the environment in this large animal veterinary hospital for the duration of sampling highlighted the importance of periodic environmental surveillance and monitoring. Implementing infectious disease control and biosecurity protocols is vital to controlling nosocomial infections, especially with *Salmonella* spp., and is considered an ethical and professional responsibility of any veterinary practice. It can be recommended that other veterinary facilities in this country consider evaluating the environmental contamination in their healthcare premises.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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Isolation and Characterization of *Avian Coronavirus* from Diagnostic Cases of Selected Bird Species in Malaysia

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ABSTRACT

Infectious bronchitis virus (IBV) in chicken (*Gallus gallus*) is the most common and well-studied *Avian coronavirus* (ACoV) in avian species. The study aims to molecularly characterize ACoV isolate of selected bird species other than chicken obtained from the archived samples of field diagnostic cases in the Northern Zone Veterinary Laboratory (MVZU), Malaysia. Twelve archived virus isolates from 2013 to 2019 were amplified using selected primers on the 3' UTR gene and S1 gene for oligonucleotide sequencing. These sequences were then molecularly characterized and compared with common IBV strains in chicken to determine the genetic diversity of the virus among selected avian species. Subsequent analyses of the nucleotides amplified on 3' UTR conserved region of 12 selected ACoVs isolates originating from peacocks (*Pavo cristatus*), turkey (*Meleagris*), jungle fowl (*Gallus gallus spadiceous*), guinea fowl (*Meleagris gallopavo domesticus*), goose (*Anser anser domesticus*), love bird (*Agapornis*), macaw (*Ara macao*), and bird (species unidentified) are classified as belonging to the *gammacoronavirus* (Gamma-CoV) genus and have a high degree of homology. The S1 complete gene sequence analyses of guinea fowl and jungle fowl showed that both ACoV isolates are Gamma-CoV and under genotype I and GI-13 lineages. Both are identified as having a high similarity of 98% and 99%, respectively, with IBV vaccine strain 4/91 (AF093793). Due attention should be given to ACoVs strains,

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especially the IBV vaccine strains detected in other bird species, because there is a high probability that other bird species could be the source of pathogenic ACoV infection in general and IBV infection in chickens, as reported in other countries.

Keywords: *Avian coronavirus*, diagnostic cases, *gammacoronavirus*, IBV vaccine strain, selected bird species

INTRODUCTION

The emergence of diseases caused by *Coronaviridae* that are zoonotic, highly contagious, and fatal in humans, such as Severe Acute Respiratory Syndrome Coronavirus (SARS-CoV) (2002), Middle East Respiratory Syndrome Coronavirus (MERS-CoV) (2012), and ongoing SARS-CoV2 (Covid-19) recently attracted the attention of researchers worldwide. It has opened the eyes of many parties, especially researchers. It has boosted research into the molecular and evolutionary mechanisms that control coronavirus cell tropism and interspecies transmission (Parkhe & Verma, 2021).

Most *Avian coronavirus* (ACoV) in birds are classified under Gamma-CoV, and only some numbers are in Delta-CoV (de Wit & Cook, 2020). The taxonomic designation comprises the extremely contagious infectious bronchitis viruses (IBVs) in chickens and ACoVs affecting other domestic birds like turkeys, guinea fowls, or quails (de Wit & Cook, 2020). Gamma-CoV has no zoonotic implications (World Organization for Animal Health [WOAH], 2018). IBV in chickens causes severe morbidity and impairs production, especially in poultry industries such as layer and broiler (Bande et al., 2016). IBV exhibits a wide variety of tissue tropism, including the renal and reproductive systems, despite mostly impacting the respiratory tract. Thus, the organ or tissue implicated and the pathotype or strain of the infecting virus may impact the disease's outcome (Bande et al., 2016). IBV could mutate and recombine genetically, resulting in antigenic drift and shift. As a result, control through vaccination alone is challenging (Cook et al., 2012), despite proper vaccination and biosecurity in farms (Guzmán & Hidalgo, 2020).

Vaccines are available in various strains and forms, depending on local conditions and legal dispositions. Live vaccinations, commonly attenuated via repeated passages in chicken embryos, offer higher local respiratory tract protection than inactivated vaccines (WOAH, 2018). The use of live vaccines carries the risk of back-passage in subsequent flocks via the excreted feces, thus, resulting in residual pathogenicity (WOAH, 2018). The vaccine 4/91 strain is a live attenuated type of vaccine and is most commonly and widely used to control infectious bronchitis (IB) together with biosecurity implementation in poultry farms (Guzmán & Hidalgo, 2020). As the live attenuated virus vaccine, the vaccine would undergo a normal infection process without causing disease; thus, effective mass vaccination results in the safe administration of live vaccines in most cases.

In Malaysia, many studies of ACoVs have been conducted, and most focus on the IBV in poultry species, which covers many aspects. Among the studies that have been conducted are the following; the sequence and phylogenetic analysis of selected genes of IBV by Zulperi et al. (2009), the molecular characterization of IBV strains in Malaysia based on partial genomic sequences by Khanh et al. (2017), the molecular characterization studies on the Malaysian IBV isolates by Leow et al. (2018), and the evaluation of the antigen relatedness and efficacy of a single vaccination with different infectious bronchitis virus strains by Ismail et al. (2020). There is a lack of studies involving the molecular characterization of ACoVs focusing on other bird species. Most ACoVs from other bird species studies in Malaysia and other countries are on the isolates obtained from the random sampling of the surveillance program. The virus isolates are collected from samples such as cloacal or tracheal swabs and feces from random, apparently healthy birds, with none of the dead birds testing positive for coronavirus (CoV) (Chamings et al., 2018).

In this study, isolates of ACoVs obtained from tissue samples of selected bird species with a history of swollen kidneys and sudden death from field diagnostic cases were molecularly characterized. Thus, this study provides a comprehensive overview of the ACoVs among selected bird species and their relationship with IBV in commercial chickens.

METHODOLOGY

Samples

Positive IBV and ACoV isolates were obtained from diagnostic cases of the Northern Region Veterinary Laboratory (MVZU), Department of Veterinary Service Malaysia (DVS), from 2013 to 2019. The viruses were obtained from post-mortem samples of organ tissues of the lung, kidney, brain, trachea, and gastrointestinal tract tissues of bird carcasses that had died and were referred to the laboratory. The tissues were further processed, and the viral suspension was inoculated into embryonated chicken eggs (ECE) and propagated for one or two passages. The allantoic fluids (AF) were harvested, and the IBV and ACoV were confirmed by detection with reverse transcriptase polymerase chain reaction (RT-PCR) assay targeting the untranslated region (UTR) (Adzhar et al., 1996). Allantoic fluids of the viral isolates were stored at -80°C. This study used virus isolates confirmed as ACoV from selected bird species other than commercial chickens, totaling 12 isolates.

PCR Extraction, Amplification, and Sequencing

Viral RNA was extracted from the allantoic fluid using TRIzol® LS reagent (Life-Technologies Inc., USA). The primer used for the amplification of the 3' UTR genomic region of IBV was selected based on several recommendations in the recent studies of ACoV by Adzhar et al. (1996), Hughes et al. (2009), and Ismail (2019). Amplifying the 3' UTR region of *Avian coronavirus* for initial RT-PCR uses both previously

published forward and reverse primers (Adzhar et al., 1996). For heminested-PCR, similar forward oligonucleotide primers were used as in initial RT-PCR but with another reverse primer (Hughes, 2009). Amplification of the full-length gene of the S1 region was conducted using primer pairs IBS_FQ1 and IBS-RQ1 (Ismail, 2018) to produce an amplicon at approximately 1,183 bp. The position of the full-length S1 gene is between the ORF1b and S2 genes, as listed in Table 1. Reverse transcription and amplification were using the OneStep RT-PCR Bioline kit (Bioline MyTaq One-Step RT-PCR kit, Meridian Bioscience®, United Kingdom) and performed in accordance with the manufacturer's protocols. The isolated DNA fragments were submitted for DNA purification and sequencing at Apical Scientific Sdn. Bhd. (Malaysia).

Analysis of S1 Gene Sequences

The complete S1 gene sequences from the isolates of the guinea fowl and jungle fowl were selected and further analyzed. The identity or similarity of sequences was analyzed using Molecular Evolutionary Genetic Analysis (MEGA) version X (Kumar et al., 2018) and the open-source Basic Local Alignment Search Tool (BLAST) program. The complete S1 sequenced isolates were aligned and compared with the available similar gene sequences in GenBank of the National Center for Biotechnology Information (NCBI). The query sequence in FASTA format is BLAST for the similarity with any available gene in the GenBank. Open reading frames (ORFs) were identified, and nucleotide sequences were translated into deduced amino acid sequences using Unipro UGENE version 37 (Okonechnikov et al., 2012) for codon-based tests. Homology analyses were conducted by pairwise alignment tests using the Multiple Sequence Alignment Distance Matrix for identity, similarity, gaps, and alignment scores of sequences. The similarity test was also conducted via open source pairwise alignment, the EMBOSS program using

Table 1
Type of primers used in the amplification of UTR region of Avian coronavirus *and amplification of the full-length SI gene of ACoV*

No.	Primer*	Sequences (5'-3')	Nucleotide location 5'-3'**	Product size (bp)	Reference
1.	UTR41+	5'-ATGTCTATCGCCAGGGAAATGTC-3'	27373-27395	214	Adzhar et al. (1996)
2.	UTR11-	5'-GCTCTAACTCTATACTAGCCTA-3'	27617-27638		
3.	UTR41+	5'-ATGTCTATCGCCAGGGAAATGTC-3'	27373-27395		Hughes et al. (2009)
4.	UTR-hemi	5'-CTTAAACTAAAATTTAGCTCTTCC-3'	27559-27582		
5.	IBS_FQ1	5'-AGTGGAAAAACACTGCACGC-3'	20140-20159	1,183	Ismail (2018)
6.	IBS_RQ1	5'-AGGGTGGTAGGACCCARACA-3'	21322-21307		

Note. F = Forward prime; R = Reverse primer; * = Oligonucleotide identification; ** = Location corresponds to that complete S1 sequence of GenBank Accession MN548289

optimal global alignment of two sequences using the Needleman-Wunsch algorithm.

RESULTS

Following the amplification of the conserved 3' UTR region, all the 12 selected ACoVs isolates were sequence analyzed. Analyses of their nucleotides showed that all ACoVs isolates are classified as Gamma-CoV genus and show high homologous to each other (Figure 1). There were represented by 5 peacocks (DVS/MVZU1007/14, DVS/MVZU1730/14, DVS/MVZU2114/14, DVS/MVZU4022/15, and DVS/MVZU102/180), and one each in turkey (DVS/MVZU1664/19), jungle fowl (DVS/MVZU1365/190, guinea fowl (DVS/MVZU3574/19), goose (DVS/MVZU1389/19), love bird (DVS/MVZU1649/13), bird (DVS/MVZU1691/19), and macaw (DVS/MVZU232/17).

Two selected ACoVs isolates of jungle fowl (DVS/MVZU2166/15) and guinea fowl (DVS/MVZU3574/16) were amplified in their S1 region. Their hypervariable region of the S1 complete gene was compared with 42 other reference strains. The phylogenetic tree showed both sequences classified under the Gamma-CoV genus. The reference strains consisted of 3 common IBV isolates, 32 prototype lineages reference isolates of

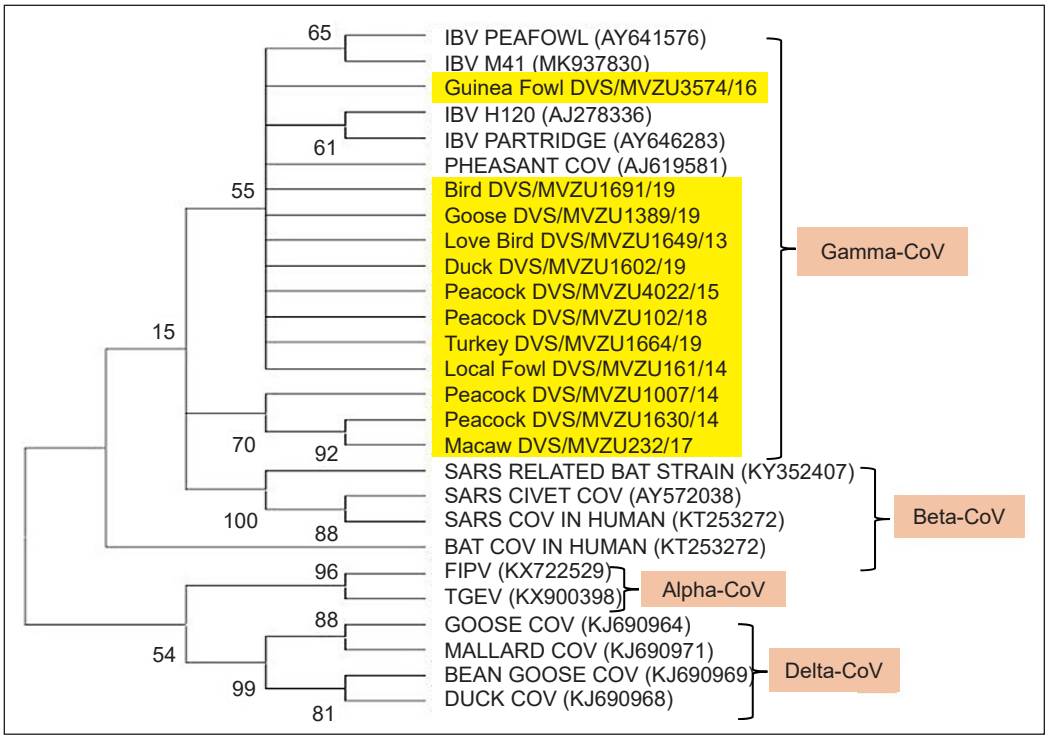

Figure 1. Phylogenetic consensus tree of 3' UTR gene analysis of coronavirus (CoV) for genus classification. The phylogenetic tree was constructed by the maximum likelihood method, and bootstrap was applied for 1,000 replications. The 12 local *Avian coronavirus* (ACoV) isolates were classified within the Gamma-CoV genus and highlighted in yellow.

the Gamma-CoV genus (Valastro, 2016), and 7 gene sequences to represent Alpha-CoV, Beta-CoV, and Delta-CoV. These gene sequences were used as the outgroup gene references, and the sequence details are listed in Table 2.

Further phylogenetic analyses of jungle fowl (DVS/MVZU2166/15) and guinea fowl (DVS/MVZU3574/16) for genotype and lineages were compared with 19 other reference strains based on the S1 gene full length of ACoV (Table 3).

Table 2
List of 44 avian coronavirus gene sequences used for phylogenetic analysis of complete S1 gene for genus classification

No.	Sequence name	GenBank accession reference	Amplicon (bp)
1.	Bulbul CoV[c]	NC_011547	26,487
2.	Camel CoV[d]	MF593473	27,389
3.	GI-1: 1937–2013; Beaudette USA 1937[b]	M95169	27,608
4.	GI-2: 1954–2006; Holte USA 1954[b]	GU393336	1,168
5.	GI-3: 1960–2006; Gray USA 1960[b]	L14069	1,738
6.	GI-4: 1962–1998; Holte USA 1962[b]	L18988	1,636
7.	GI-5: 1962–2012; N1/62 Australia 1962[b]	U29522	1,709
8.	GI-6: 1962–2010; VicS Australia 1962[b]	U29519	1,703
9.	GI-7: 1964–2012; TP/64 Taiwan 1964[b]	AY606320	1,617
10.	GI-8: 1965–1967; L165 USA 1965[b]	JQ964061	1,632
11.	GI-9: 1973–2011; ARK99 USA 1973[b]	M99482	1,974
12.	GI-10: 1970s–2000s; New Zealand 1970s[b]	AF151954	1,635
13.	GI-11: 1975–2009; UFMG/G Brazil 1975[b]	JX182775	1,610
14.	GI-12: 1978–2006; D3896 The Netherlands 1978[b]	X52084	1,776
15.	GI-13: 1983–2013; Moroccan-G/83 Morocco 1983[b]	EU914938	1,764
16.	GI-14: 1984–2006; B1648 Belgium 1984[b]	X87238	2,882
17.	GI-15: 1986–2008; B4 Korea 1986[b]	FJ807932	1,632
18.	GI-16: 1986–2011; IZO 28/86 Italy 1986[b]	KJ941019	1,780
19.	GI-17: 1988–1999; CA/Machado/88 USA 1988[b]	AF419315	1,632
20.	GI-18: 1993–1999; JP8127 Japan 1993[b]	AY296744	1,635
21.	GI-19: 1993–2012; 58HeN-93II China 1993[b]	KC577395	1,620
22.	GI-20: 1996–1999; Qu_mv Canada 1996[b]	AF349621	1,629
23.	GI-21: 1997–2005; Spain/97/314 Spain 1997[b]	DQ064806	1,620
24.	GI-22: 1997–2011; 40GDGZ-97I China 1997[b]	KC577382	1,635
25.	GI-23: 1998–2012; Variant 2 Israel 1998[b]	AF093796	1,614
26.	GI-24: 1998–2013; V13 India 1998[b]	KF757447	1,523
27.	GI-25: 2004–2013; CA/1737/04 USA 2004[b]	EU925393	1,626
28.	GI-26: 2006–2007; NGA/B401/2006 Nigeria 2006[b]	FN182243	1,611
29.	GI-27: 2008–2013; GA08 USA 2008[b]	GU301925	1,630
30.	GII-1: 1979–1984; D1466 The Netherlands 1979[b]	M21971	1,605
31.	GIII-1: 1988–2008; N1/88 Australia 1988[b]	U29450	1,712
32.	GIV-1: 1992–2003; DE/072/92 USA 1992[b]	U77298	1,654

Table 2 *(continue)*

No.	Sequence name	GenBank accession reference	Amplicon (bp)
33.	GV-1: 2002–2008; N4/02 Australia 2002[b]	DQ059618	1,614
34.	GVI-1: 2007–2012; TC07-2 China 2007[b]	GQ265948	1,638
35.	Guinea fowl DVS/MVZU3574/16	NA	1,129
36.	IBV Malaysian Variant Strain[a]	MK828778	3,502
37.	IBV QX-Like[a]	KU949746	6,789
38.	IBV H120[a]	MK937831	27,642
39.	Jungle fowl DVS/MVZU2166/15	NA	1,129
40.	SARS-related bat strain[c]	KY352407	29,274
41.	SARS civet CoV[e]	AY572038	29,683
42.	SARS CoV2[c]	NC_045512	29,903
43.	Thrush CoV[c]	FJ376621	26,396
44.	TGEV[d]	KX900398	28,521

Note. a = Reference group common IBV strain; b = Prototype lineages reference group; c = Outgroup *deltacoronavirus*; d = Outgroup *alphacoronavirus*; e = Outgroup *betacoronavirus*; NA = Not applicable

Table 3
List of 21 avian coronavirus gene sequences used for phylogenetic analysis of complete S1 gene for genotype and lineages identification

No.	Sequence name	GenBank accession reference	Amplicon (bp)
1.	Guinea fowl DVS/MVZU3574/16	NA	1,129
3.	IBV Malaysian Variant Strain[a]	MK828778	3,502
4.	IBV Malaysian QX-like strain[a]	KU949743	6,789
5.	IBV Malaysian Variant Strain IBS037A/2014[a]	KU949737	6,789
6.	IBV Malaysian Variant V9/04[a]	FJ518779	1,593
7.	IBV Malaysian Variant MH5365/95[a]	EU086600	1,720
8.	IBV Indonesian QX-like strain[b]	MH671338	383
9.	IBV China QX strain[b]	AF193423	1,657
10.	IBV China LX4 strain[b]	AY189157	3,495
11.	IBV CR88 vaccine strain[c]	JN542567	1,617
12.	IBV 793B vaccine strain[c]	GQ844991	1,617
13.	IBV 4/91 vaccine strain[c]	AF093793	1,617
14.	IBV 4/91 vaccine strain[c]	JN192154	3,492
15.	IBV D274 vaccine strain[c]	X15832	3,570
16.	Jungle fowl DVS/MVZU2166/15	NA	1,129
17.	Lineages GI-1; USA[d]	M95169	27,608
18.	Lineages GI-12; The Netherlands[d]	X52084	1,776
19.	Lineages GI-13; Morocco[d]	EU914938	1,764
20.	Lineages GI-19; China[d]	KC577395	1,620
21.	Lineages GI-24; India[d]	KF757447	1,523

Note. a = Common local Malaysian strain; b = Other common strain; c = Vaccine strain; d = Prototype lineages reference; NA = Not applicable

Based on the S1, complete gene sequence analyses showed that both ACoV isolates are Gamma-CoV and under genotype I and GI-13 lineages (Figure 2). Both are identified as having a very high similarity with IBV vaccine strain 4/91 (AF093793).

Reference sequences of Malaysian QX-like strains are in lineage G-19, and reference strains of QX-like Indonesia and QX/LX4 China strains. However, the reference sequences of Malaysian variant strains are forming unique clusters which

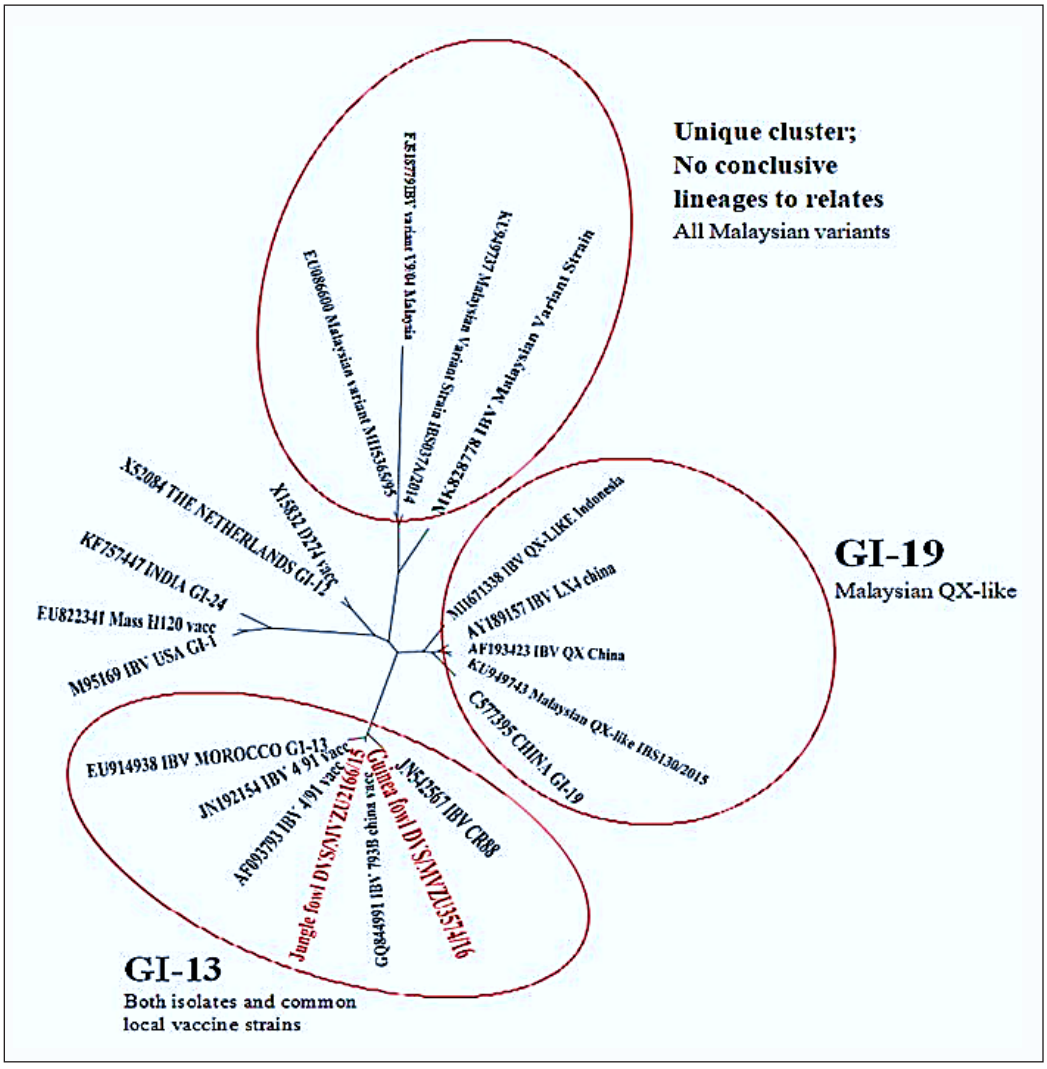

Figure 2. Phylogenetic radiation tree analyses of isolates sequences for identifying *Avian coronavirus* (IBV) genotypes and their lineages based on the S1 nucleotide complete gene. Sequences were analyzed by MAFFT v.7.475 and aligned using the MAFFT-FFT-NS-i method. The phylogenetic tree was constructed using the neighborhood joining with the Jukes-Cantor method and bootstrap applied for 1,000 replicates. Both jungle and guinea fowl isolates are closely distanced with lineage GI-13 and sequence references of common vaccine strain 4/91, 793B, and CR88. The vaccine sequence reference strains of H120 are in GI-1

significantly genetically distance against any existing lineage.

Deduced amino acids of S1 gene sequences were compared for similarity by NCBI using the open source of BLASTP for a protein similar in NCBI GenBank, indicating that both are highly similar to the S1 spike glycoprotein of IBV. The high similarity of the isolates with other ACoV S1 protein reference strains of GI-13 lineages by Multiple Sequence Alignment Distance Matrix, UGENE (Okonechnikov, 2012) showed 99% and 98% similarity of 4/91 vaccine strain reference with ACoV sequences of jungle fowl and guinea fowl, respectively. Other similarity analyses were conducted by Pairwise Sequence Alignment of EMBOSS Needle method, which also showed 100% and 98.4% similarity between 4/91 vaccine strain reference with jungle fowl and guinea fowl.

Pairwise sequence alignment by EMBOSS Needle method was performed between jungle fowl and guinea fowl ACoV isolates and showed 98.4% identification and 98.4% similarity.

DISCUSSION

Avian coronavirus has many hosts, from chickens and turkeys to wild birds (Suryaman et al., 2019). Coronaviruses have been identified in several avian hosts and mammals, including camels, bats, masked palm civets, mice, dogs, and cats (Lu et al., 2020). Infectious bronchitis is the only well-studied disease among ACoVs, especially in commercial chickens. This virus remains one of the most impacting and causes massive economic losses in the poultry industry (Legnardi et al., 2020). ACoVs have also been isolated from guinea fowl, domesticated and wild peafowls, partridge, pigeon, jungle fowl, pheasants, turkey, and non-Galliformes birds like teal. Although these ACoVs are mainly based on IBVs or IBV-like, molecular and sequence analyses showed they were genetically distant from IBVs (Promkuntod, 2016). Commercial IBV H120 vaccine strain has been detected in peafowl CoVs isolate (Liu et al., 2005).

Coronaviruses isolated from turkeys, pheasants, and guinea fowl are genetically similar to IBV by the highly conserved region of 3' UTR that shows 90% nucleotide identity (Cavanagh et al., 2002). Several recent studies have found that IBV has been isolated from other bird species, where IBV Massachusetts strains in wild peafowls (Sun et al., 2007), IBV of chicken strains detected in turkey (Cavanagh et al., 2001), IBV H120 vaccine strains isolated from peafowl is pathogenic to chicken (Felippe et al., 2010) as well as detection of Gamma-CoV in wild birds (Muradrasoli et al., 2010). Hence, the real potential role of the other bird species in the transmission of ACoVs and IBV in chickens has not been well determined.

Various aspects such as geographical conditions, living norms, social economy, and habitat and physiology of birds generally explain the role of other bird species in the transmission and replication of IBV among chickens and other bird species. Other studies of ACoVs showed that the IBV could replicate in other galliform and some non-galliform bird species with or

without clinical signs and lesions. It has been proposed that the other bird species act as a natural reservoir of the carrier of IBV (Promkuntod, 2016).

There are nine species of birds from the 12 selected samples of birds used in this study. Peacock is the most common species with 4 isolates, one isolate of each for guinea fowl (*Numida meleagris*), goose (*Anser anser domesticus*), turkey (*Meleagris gallopavo domesticus*), macaw (*Ara macao*), jungle fowl (*Gallus gallus spadiceous*), duck (*Anas platyrhynchos domesticus*), lovebird (*Agapornis*), and unclassified bird, respectively. Phylogenetic analysis of the 3' UTR gene of all these birds has shown that the ACoVs are Gamma-CoV, the same genus as IBV in chickens. It is consistent with the commonly known fact of CoVs genus classification stating that all ACoVs are classified mostly under Gamma-CoV and some under Delta-CoV (Zhou et al., 2021).

Hypervariable regions of the S1 gene of coronavirus are recommended genomic regions for genotyping and lineage identification and provide more bioinformatic information and are more accurate as compared with conventional strain identification by serotyping methods (Valastro, 2016). The information on lineages obtained from the phylogenetic analysis could give us an idea of the origin or closer lineages of the ACoVs with other reference strains. This study compared two isolates of jungle fowl and guinea fowl ACoVs with local Malaysian common IBV strains and vaccine strains. Both sample isolates are classified in genotype I and grouped in the GI-13 lineages with three other common local vaccine strains of CR88, 4/91, and 793B. The samples are distinctly separated from both the Malaysian IBV QX-like strain (in GI-19) and the Malaysian variant strain (unique unidentified lineages). Both isolates were highly similar to the IBV vaccine strain of live attenuated 4/91 vaccine, a broadly and commonly used vaccine strain in most commercial chicken farms worldwide, including Malaysia, for years (Guzmán & Hidalgo, 2020).

The phylogenetic analysis of the S1 hypervariable region and similarity analyses shows that the ACoVs from both guinea fowl and jungle fowl are grouped under GI-13 lineages and are highly similar at 98% and 99%, respectively to the live attenuated IBV 4/91 vaccine strain. This finding showed that IBV strain 4/91 is present in other than commercial chicken species: the jungle fowl and guinea fowl. The administration of the IBV vaccine to these two species has never been practiced in Malaysia; furthermore, both species are known as disease-resistant species in general (Syahar et al., 2014).

Jungle fowl or *Gallus gallus spadiceus* (local name known as 'ayam hutan merah') is the species available in Southeast Asia, including Malaysia. It is an endangered species, and hunting is only allowed during certain seasons by those with a hunting license from the Department of Wildlife (PERHILITAN) Malaysia. This jungle fowl is most popular and highly demanded on the illegal market for its meat and colorful feathers. The habitat of the jungle fowl is

in the bushes at the fringe of the jungle. In Malaysia, jungle fowls have frequently been seen on palm oil plantation farms because they sleep on trees, eat in the open areas in the morning and evening, and eat under a tree. It is an opportunistic feeder and eats various insects, animals, and plant components, including oil palm fruit (Syahar et al., 2014).

Guinea fowl or *Numida meleagris* (local name known as 'ayam piru') originated from an African tropical forest. They were once wild birds, and according to the Encyclopedia Britannica (n.d.), the modern birds are the domesticated form of the helmeted guinea fowl. It is a hardy and disease-resistant bird. It has become one of the choices for meat protein other than chicken or duck meat due to its tenderness and nutritious meat. It lives in flocks and walks about on the ground, feeding on seeds, tubers, and some insects.

Based on the background information of these two avian species, it can be postulated that the common IBV 4/91 strain isolated from these two species is most likely to be obtained through the environment that has been contaminated with feces from commercial chickens that received live attenuated strain vaccine 4/91. Since IBV 4/91 vaccine is a live attenuated virus vaccine, the virus vaccine could have been transmitted via contaminated feces of the commercial poultries. The shaded vaccine virus would have spread to the jungle and guinea fowls. It is a diagnostic case with a history of sudden death that was sent to the MVZU. The results of the postmortem findings only showed gross lesions of swollen kidneys. The case background information is unclear enough to state whether these two species of birds show clinical signs. Chicken farming is normally within the palm oil farms near the jungle fowl habitat (Syahar et al., 2014), and the jungle fowl occasionally search for food nearby and would have consumed the contaminated feed. DVS Malaysia livestock data shows that most backyard farms practice integrated farming with multi-avian species, including chickens, ducks, geese, guinea fowl, turkeys, and other avian species. This multispecies farming practiced by certain farmers has increased the likelihood of guinea fowl contracting the infection from soil contaminated by feces containing the virus.

Viral vaccine reversion to virulence may hinder the control of IB in the country as the source of the virus will now be in the wild, by the jungle fowl and guinea fowl, which will spread or be transmitted to other wild birds. Disease control procedures and policies on using the IBV vaccine, especially the live attenuated form, need to be improved to ensure this risk can be controlled. The farm biosecurity also needs to be improved. The question of whether the virus isolated from these two birds is pathogenic and causing the death of the birds needs to be determined. Moreover, there could also be other risk factors that aggravate the condition of the already infected birds. Further studies on the pathogenicity of the newly isolated virus and its protection studies need to be conducted to get more convincing answers. Surveillance

studies on how the IBV vaccine strains circulate among other bird species will be very useful. This information will provide significant input for developing effective vaccines using circulating local viruses.

CONCLUSION

Based on the UTR region, all the local ACoVs isolates of nine bird species were classified under Gamma-CoV. The molecular characterization demonstrated that ACoV isolates from diagnostic cases of jungle fowl (isolate 2015) and guinea fowl (isolate 2016) strains are homologous to common IBV vaccine 4/91 strains. Due attention should be given to ACoVs among other bird species because there is a high probability that other bird species could cause transmission of pathogenic ACoV infection (IBV) in chickens, as reported in other countries.

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Locule Position and Thawing Duration Affect Postharvest Quality of Freshly Cryo-Frozen Musang King Durian Fruit

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ABSTRACT

This study examined the effect of locule position and thawing duration on the physicochemical and nutritional characteristics of intact cryo-frozen Musang King durian fruit. Cryo-frozen durian that had 5 locules was thawed for 2 and 18 hr, and the fruitlets of each locule were analysed for colour (L*, a*, b*, C*, and h°), firmness, soluble solids concentration (SSC), titratable acidity (TA), pH, ascorbic acid (AA), total phenolic content, total flavonoid content, 1,1-diphenyl-2-picrylhydrazyl, 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid and ferric reducing antioxidant power assay (FRAP). Results show that L* and a* of pulp colour, firmness, SSC, pH, TA, AA, and FRAP of cryo-frozen durian fruit were affected by a significant interaction between locule position and thawing duration. It implies the postharvest quality of intact cryo-frozen durian fruitlet distinct from each other due to their locule position and thawing duration.

Keywords: Colour, cryogenic, eating quality, fruitlet, nutritional quality

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INTRODUCTION

Durian (*Durio zibethinus*) fruit, especially clones like Musang King and Black Thorn, is popular among durian connoisseurs due to their unique mouthfeel and pulp colour. As a result, the planting acreage of durian has increased to 72,391 ha with a total production of 210,874 MT/year (Md Nor, Ding, Sakimin, et al., 2022). Currently, Musang King durian has been exported

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to countries such as China, Singapore, Taiwan, Australia, and European countries (Razali et al., 2020). Musang King durian fruit is very perishable, with easily dehisce husk within 12 hr of harvest under harsh and poor handling environments. The fruit must be frozen using a cryogenic technique where liquid nitrogen of -110 to -90°C is used to freeze the intact fruit for at least one hour before storage and shipping under -20 to -18°C (Ding, 2018). As a result, the postharvest life of intact cryo-frozen Musang King durian fruit is longer than fresh fruit; thus, the fruit can be exported to long-distance markets using sea referral.

Since the husk of intact cryo-frozen durian is strong and cannot be dehisced, the fruit must be thawed before serving. Thawing can be as simple as leaving the intact cryo-frozen durian at room temperature or using electromagnetic radiation to generate heat within the fruit (Wu et al., 2021). During thawing, the ice crystals of water molecules in the frozen fruit will change from solid to liquid, an important deterioration phenomenon to the fruit quality as cell structure collapses and nutrients are lost. Thus, thawing duration may affect cell structure and nutrients. The information on the thawing duration of durian fruit quality is very scarce. Although some studies on cryo-frozen durian have been reported, their focus was not on the effect of thawing duration on fruit quality. Razali et al. (2022) thawed intact cryo-frozen Musang King durian fruit for 0, 12, 24, and 36 hr to compare the effectiveness of conventional and cryo-freezing on the fruit and concluded that cryo-freezing was better in preserving durian fruit. The Japanese group of researchers revealed that thawing conventional-frozen (keeping fruit in a -20°C freezer) Thai durian neither using iced water (~0°C) nor hot water (~90°C) affects fruit eating quality (Tagubase et al., 2016). There are no standard operating procedures to thaw the cryo-frozen durian fruit: some vendors advise thawing the fruit overnight, but most do thaw for 2 hr. Nevertheless, the effect of thawing duration on fruitlets eating quality of Musang King durian fruit is not known.

Apart from thawing, it is believed that fruit locule may affect the eating quality of durian fruitlets. Generally, a durian has three to seven locules, where fruitlets are formed from the flower ovary after successful pollination (Ketsa et al., 2020). Fruit such as pineapple has been evident that the parts (basal, medium, top) of a fruit affected flesh firmness, with the basal part showing lower firmness than the medial and top parts (Joomwong & Sornsrivichai, 2005). The central part of white-fleshed dragon fruit (*Hylocereus undatus*) contained higher soluble solids than other parts of the fruit; thus, the fruit core tastes sweeter than the pulp near the peel (Nomura et al., 2015). The pulp dry matter at the stem end of Hass avocado was higher than above the seed, and the dry matter gradient decreased from the outside (peel) to the core (seed) of the fruit, indicating the distribution of moisture content varied in different parts of a fruit (Phetsomphou, 2000). For durian, the information on the locule effect on fruit-

eating quality is almost nil. The information may enhance the knowledge of fruit quality from different locules of durian fruit.

Thus, the present study was conducted to study the effect of locule position and thawing duration on the postharvest quality of cryo-frozen Musang King durian.

MATERIALS AND METHODS

Fruit Samples

The fresh Musang King durian of export grade (having 5 locules and a weight of approximately 2 kg per fruit) was cryo-frozen using liquid nitrogen for 60 min at -110°C within 5 hr of harvesting. The freshly cryo-frozen fruits were packed in a polystyrene box with an ice gel pad and stored at -20°C overnight while waiting for transport to send to the laboratory. The frozen fruit arrived at the laboratory within 3 hr from Top Fruits Sdn. Bhd., Batu Pahat, Malaysia. Upon arrival, the fruits were removed from the polystyrene box and allowed to thaw at room temperature (25 ± 2°C) for 2 and 18 hr. After the thawing duration, the fruits were opened with the help of a chopping knife. Randomly, one of the dehisced locules was assigned as locule number 1 (L1), the following locules from the right of the L1 were assigned as locule number 2 (L2), locule number 3 (L3), locule number 4 (L4), and locule number 5 (L5), respectively. The L5 was to the left of L1. Each locule of durian fruit consisted of five to seven fruitlets. Analyses were immediately conducted once the fruitlets were removed from each locule.

Physico-Chemical Analysis

The durian pulp physio-chemical properties of each fruitlet, such as colour (L*, a*, b*, C*, and h°), firmness, soluble solids concentration (SSC), pH, titratable acidity (TA), and ascorbic acid contents (AA) were analysed (Md Nor et al., 2021). The colour and firmness were measured when the pulp was attached to the seed. The Durian pulp colour of each fruitlet was taken using a colourimeter (CR-400, Minolta Corp., Japan) that was calibrated with a standard white tile before measuring. Three random points of each fruitlet were taken and presented in lightness/darkness (L*), redness/greenness (a*), yellowness/blueness (b*), chroma (C*), and hue angle (h°) according to International Commission on Illumination CIELAB. The firmness was measured using Instron Universal Testing Machine (5543P5995, Instron Corp., USA) fitted with a 6-mm diameter cylindrical probe and a 5-kg load cell. Each durian fruitlet was penetrated with a probe to a depth of 3 mm at a crosshead speed of 20 mm/min. The reading was recorded in Newton (N) using the Instron Merlin Software version M12-13664-EN. For SSC determination, 10 g durian pulp was homogenised with 20 ml distilled water prior to cotton filtration. Two to three drops of the filtrate were placed on a digital hand-held pocket refractometer (ATAGO™ RX-5000α, Japan), and the reading was calculated and presented as %SSC. The remaining filtrate prepared for SSC analysis was used for pH and TA analysis. pH was measured using an electrode of a pH

meter, and TA was measured by acid-base titration (Ding & Raja, 2021). Titration was performed using 10 ml filtrate added with 2 drops of phenolphthalein against 0.1 N sodium hydroxide (NaOH) until a light pink solution appeared for at least 15 s, and results were expressed as a percentage of malic acid. The dye method was applied according to Zainal et al. (2019) with modifications for AA determination. One gram of pulp was first homogenised with 25 ml 2% cold metaphosphoric acid (HPO_3), followed by a second filtering step using cotton. The filtrate was made up of 25 ml solution, and 10 ml filtrate was then titrated with standardised dye solution until a permanent pink colour appeared. The result was expressed as mg/100 g of fresh weight (FW).

Antioxidant Analysis

Prior to antioxidant analysis, 0.50 g of durian pulp was extracted with 1.50 ml (75% methanol) (Merck, Germany) using a Wise Clean ultrasonic water bath (Wise Laboratory Instrument, Germany) at 35°C for 30 min. Then, the sample was centrifuged at 16,100 × *g* for 10 min. The supernatant was collected and further analysed for total phenolic content (TPC), total flavonoid content (TFC), 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay, and 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging assay analysis.

TPC was determined using 0.2 M Folin-Ciocalteau phenol reagent (SIGMA Chemical Co., USA) according to Ding and Choon (2021) with slight modification. A diluted extract of 200 µl was mixed with 25 µl Folin-Ciocalteu reagent for 4 min. Consequently, 20 µl 6% (w/v) sodium bicarbonate solution was added to the mixture and incubated in the dark for 30 min. Finally, the absorbance of the reaction mixture was measured at 750 nm using a spectrophotometer (Thermo Scientific, Multiskan™ Go Microplate Spectrophotometer, Finland). The result was extrapolated using a gallic acid standard curve, and the value was expressed as milligrams of gallic acid equivalents (GAE) in 100 g extract (mg GAE/100 g extract).

TFC was quantified using the aluminium trichloride method modified by Rebaya et al. (2015). A volume of 125 µl extract was added to 75 µl 5% sodium nitrite ($NaNO_2$) (SIGMA Chemical Co., USA). The mixture was allowed to mix for 6 min before adding 150 µl aluminium trichloride (10% $AlCl_3$) (Merck, Germany). Later, the mixture was incubated for 2 hr at room temperature with continuous shaking. Then, 750 µl 1 M NaOH (Merck, Germany) and 1,475 µl distilled water were added into the mixture and incubated for 15 min until a pink-peach colour solution formed. The absorbance was measured at 510 nm. Quercetin was standard, and the result was expressed as quercetin equivalents (QE) in mg/100 g FW.

The 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) radical scavenging assay was modified according to the procedure described by Ding and Choon (2021). The solution of 0.2 mM DPPH (Merck, Germany) was prepared in methanol. A total

volume of 20 µl fruit extract was added to 180 µl DPPH and incubated in the dark for 30 min at 25°C. Discolouration of DPPH was measured against a blank at 517 nm. Trolox solution was used as the reference standard, and DPPH free radical scavenging activity results were expressed as µmol Trolox equivalent (TE)/100 g FW.

The 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay was adapted from the method of Ding and Choon (2021) with some modifications. Firstly, 0.7 mM ABTS salt (Merck, Germany) and 0.24 mM potassium persulfate ($K_2S_2O_8$) (Merck, Germany). were mixed and incubated in the dark for overnight to form a stock solution. Then, 5 ml stock solution was mixed with 100% methanol (MeOH) (Merck, Germany) to form a 25 ml working solution. Fruit extract with volume 10 µl was added to 150 µl ABTS working solution and let to react in the dark within 6 min at room temperature. The $ABTS^{\bullet+}$ radical discolouration was measured at 734 nm. Trolox solution was used as a reference, and results were expressed as µmol Trolox equivalent (TE)/100 g FW.

The FRAP assay was prepared and modified according to the procedure applied by Ding and Choon (2021) using 2,4,6-tripyridyl-S-triazine (TPTZ) (Merck, Germany). The essay was prepared by mixing 25 ml acetate buffer (pH 3.6), 2.5 ml TPTZ solution (10 mM), and 2.5 ml ferric chloride hexahydrate ($FeCl_3 \cdot 6H_2O$) (20 mM) solution (Merck, Germany), warmed in the dark at 37°C just before conducting the analysis. Then, 25 µl of the sample solution was mixed with 175 µl FRAP essay and incubated for 10 min in the dark at 25°C. Absorbance was measured at 562 nm. Trolox was standard, and results were expressed in µmol Trolox equivalent (TE)/100 g FW.

Statistical Analysis

The experiments were conducted using a completely randomised design with a factorial arrangement of treatments (2 thawing duration × 5 locule) with 3 biological replications and 3 technical replicates. Statistical analysis was performed by SAS software (SAS Institute, USA). Data were analysed using analysis of variance (ANOVA), and means separation was determined by Tukey's post hoc tests at $p < 0.05$.

RESULTS AND DISCUSSION

Effects of Locule Position and Thawing Duration on Eating Quality of Cryo-Frozen Musang King Durian

The main effects of locule position and thawing duration and their interaction on the colour of durian pulp are presented in Table 1. The colour of L* and a* of durian pulp was affected significantly by the interaction between the locule position × thawing duration, while another colour parameter was not affected by the interaction. From Table 1, the L* (lightness) and a* (red/green coordinate) values of durian pulp varied among locules and changed with thawing duration, with fruit thawing for 18 hr having darker colour (lower L* and

higher a* values) than those thawing for 2 hr. The b* (yellow/blue coordinate), C* (colour chromaticity), and h^{o} (hue angle) of durian pulp colour was not affected by the interaction but affected by locules position (Table 1). During the thawing of conventional-frozen (using -20°C) Thai durian fruit using hot water, it was found that the fruit pulp was darker in colour than iced-water thawing (Tagubase et al., 2016). The temperature could affect thawed durian fruit's L* and a* values. The temperature of Musang King durian fruit thawed for 18 hr has elevated and higher than those thawed for 2 hr, thus causing darker pulp colour in fruit pulp thawed for 18 hr.

Table 1
The main and interaction effects of locule position and thawing duration on pulp colour (L, a*, b*, C*, and h^{o}) of intact cryo-frozen Musang King durian fruit*

Factors	L*	a*	b*	C*	h^{o}
The main effect of locule position					
L1	76.55^{a}	0.13^{c}	42.58^{b}	42.58^{b}	88.28
L2	71.24^{b}	1.05^{a}	48.07^{ab}	48.07^{ab}	88.59
L3	72.82^{b}	1.02^{ab}	47.62^{ab}	47.62^{ab}	88.95
L4	72.53^{b}	1.12^{a}	49.83^{a}	52.10^{a}	88.68
L5	72.04^{b}	0.49^{bc}	49.13^{ab}	49.83^{ab}	89.04
Levels of significance	**	**	*	*	ns
The main effect of thawing duration (hr)					
2	74.70^{a}	0.64^{b}	47.63	47.69	89.02
18	70.97^{b}	0.89^{a}	47.26	47.26	88.40
Levels of significance	**	*	ns	ns	ns
Interaction effect of locule position and thawing duration					
2 hr of thawing					
L1	76.31^{a}	0.10^{c}	41.45	41.41	86.72
L2	75.20^{ab}	0.70^{ab}	48.19	48.11	88.80
L3	74.21^{ab}	1.15^{a}	48.09	48.02	88.61
L4	74.80^{ab}	0.79^{ab}	52.10	52.11	89.11
L5	72.82^{b}	0.46^{bc}	48.30	48.30	88.62
18 hr of thawing					
L1	74.72^{a}	0.16^{c}	43.70	43.70	89.81
L2	67.22^{c}	1.40^{a}	47.95	47.95	88.33
L3	71.44^{b}	0.89^{ab}	47.14	47.14	89.23
L4	70.26^{b}	1.45^{a}	47.57	47.56	88.25
L5	71.21^{b}	0.52^{bc}	49.95	49.95	89.41
Levels of significance	*	*	ns	ns	ns

Note. Means (n = 3) followed by the same letter in the same column within factors are not significantly different at $p < 0.05$ according to Tukey's post hoc tests
ns, *, ** = Non-significant or significant at $p \leq 0.05$ or $p \leq 0.01$, respectively

Table 2 shows pulp firmness, SSC, pH, TA, and AA of cryo-frozen Musang King durian was affected by a significant interaction between locule position × thawing duration. Regardless of thawing duration, the firmness, SSC, pH, TA, and AA of durian fruitlets differed among locules (Table 2). For the eating quality of durian fruitlets, firmness, pH, TA, and AA of 18 hr-thawed fruitlets was lower than 2 hr-thawed while SSC of cryo-frozen durian fruit increased with thawing duration. Thawing conventional-frozen Thai durian using 90°C hot water at a high temperature but shorter

Table 2
The main and interaction effects of locule position and thawing duration on pulp firmness, soluble solids concentration, pH, titratable acidity, and ascorbic acid of intact cryo-frozen Musang King durian fruit

Factors	Firmness (N)	Soluble solids concentration (%SSC)	pH	Titratable acidity (%malic acid)	Ascorbic acid (mg/100 g)
The main effect of locule position					
L1	1.51^{b}	29.95^{a}	6.79^{c}	0.07^{a}	7.52^{b}
L2	1.53^{b}	27.22^{b}	7.22^{ab}	0.04^{b}	6.41^{b}
L3	1.96^{ab}	26.40^{b}	7.10^{b}	0.04^{b}	6.15^{b}
L4	1.97^{ab}	27.00^{b}	7.28^{a}	0.03^{c}	7.09^{b}
L5	2.51^{a}	21.95^{c}	7.32^{a}	0.04^{b}	10.34^{a}
Levels of significance	*	**	**	**	**
The main effect of thawing duration (hr)					
2	2.53^{a}	25.12^{b}	7.55^{a}	0.05^{a}	8.65^{a}
18	1.26^{b}	27.89^{a}	6.73^{b}	0.03^{b}	6.36^{b}
Levels of significance	**	**	**	**	**
Interaction effect of locule position and thawing duration					
2 hr of thawing					
L1	1.35^{c}	28.20^{a}	6.18^{e}	0.10^{a}	6.84^{b}
L2	2.13^{bc}	25.90^{b}	6.87^{c}	0.05^{b}	6.50^{b}
L3	2.92^{ab}	25.20^{b}	6.60^{d}	0.04^{c}	4.62^{c}
L4	2.62^{ab}	25.90^{b}	6.93^{b}	0.03^{d}	4.62^{c}
L5	3.64^{a}	20.40^{c}	7.08^{a}	0.05^{b}	9.23^{a}
18 hr of thawing					
L 1	1.68^{a}	31.70^{a}	7.40^{b}	0.04^{a}	8.21^{bc}
L 2	0.94^{b}	28.53^{b}	7.57^{ab}	0.02^{c}	6.33^{c}
L 3	0.99^{ab}	27.60^{b}	7.59^{ab}	0.03^{b}	7.69^{bc}
L 4	1.32^{ab}	28.10^{b}	7.63^{a}	0.02^{c}	9.57^{ab}
L 5	1.34^{ab}	23.50^{c}	7.57^{ab}	0.02^{c}	11.45^{a}
Levels of significance	*	*	**	**	*

Note. Means (n = 3) followed by the same letter in the same column within factors are not significantly different at $p < 0.05$ according to Tukey's post hoc tests
*, ** = Significant at $p \leq 0.05$ or $p \leq 0.01$, respectively

thawing duration caused higher moisture content to fruitlets compared to ice-water thawing with a longer period (Tagubase et al., 2016). At the same time, thawing methods did not affect other eating qualities of durian, such as texture profile (hardness, cohesiveness, and adhesiveness), SSC, pH, organic acids, and sugar contents.

In terms of durian fruitlet firmness, fruit thawing for 2 hr has a firmer texture than those thawing for 18 hr (Table 2). Firmer fruit is more acceptable than softer fruit since it gives a fresh perception to consumers (Md Nor & Ding, 2020). The softening of durian pulp in prolonged thawing duration may be due to the melting of crystalised water that breakdown the cell's structure. The greater SSC value in fruit thawed for 18 hr compared to 2 hr (Table 2) may indicate that starch has been actively converted to simple sugar as enzyme activity increases with temperature as the thawing process progresses. Additionally, the lower pH values in fruit thawed for 18 hr shows that durian pulp is acidic and exhibits a greater hydrogen dissociation rate than 2 hr-thawed fruit. TA reflects titratable organic acids content, and the lower TA in 18 hr-thawed fruit may be due to the utilisation of organic acids during metabolic respiration as a respiratory substrate or carbon skeleton (Md Nor, Ding, Abas, et al., 2022) during the thawing of durian fruit at room temperature. Lower AA in durian fruit thawed for 18 hr, signifying more degradation of AA has occurred during longer thawing duration compared to 2 hr thawing (Table 2). AA is water soluble and a powerful antioxidant that prevents or reduces the damage caused by reactive oxygen species in fruit (Khaliq et al., 2015). Oxidative damage could have taken place during longer thawing duration and utilise the natural AA in the fruit as a detoxification compound to fight against reactive oxygen species (Table 2).

Effects of Locule Position and Thawing Duration on Nutritional Quality of Cryo-Frozen Musang King Durian

Bioactive compounds such as phenolic and flavonoid, as part of the nutritional quality of fruit, were quantified as TPC and TFC in this study. At the same time, the oxidant systems of cryo-frozen durian were evaluated based on radical scavenging activity (DPPH and ABTS) and one assay based on the reducing potential of antioxidants (FRAP) (Table 3). The presence of bioactive compounds with antioxidant activity in a fruit indicates it is packed with nutrients that can combat oxidative stress-induced degenerative illnesses (Ding & Choon, 2021). This study's findings revealed no significant interaction between locule position × thawing time on the nutritional quality of cryo-frozen durian fruit except FRAP (Table 3). It indicates that regardless of thawing duration, every locule fruitlet in a cryo-frozen durian fruit has the same amount of nutritional quality except FRAP.

The pulp colour (L* and a*) of durian fruitlet that underwent longer thawing duration was darker and redder than those thawing for a shorter period (Table 1). The pulp darkening could be due to an enzymatic

browning reaction and degradation of natural pigment. This study has quantified that cryo-frozen durian contains polyphenol compounds as valued by TPC (Table 3). During thawing, cell decompartmentalisation may occur, permitting a reaction between enzymes and polyphenol compounds, consequently leading to browning (Wei

Table 3
The main and interaction effects of locule position and thawing duration on total phenolic content, total flavonoid content, DPPH, and ABTS a FRAP of intact cryo-frozen Musang King durian fruit

Factors	Total phenolic content (mg GAE/100 g)	Total flavonoid content (mg QE/100 g)	DPPH (μmol TE/100 g)	ABTS (μmol TE/100 g)	FRAP (μmol TE/100 g)
The main effect of locule position					
L1	16.54	0.50^{ab}	22.79	8.69	62.11
L2	12.76	0.54^{ab}	31.48	7.90	63.21
L3	15.75	0.77^{a}	28.07	7.15	68.63
L4	14.30	0.29^{b}	26.33	8.89	59.43
L5	15.86	0.42^{ab}	28.07	8.09	62.70
Levels of significance	ns	*	ns	ns	ns
The main effect of thawing duration (hr)					
2	17.09	0.48	30.45	8.38	73.55^{a}
18	12.99	0.53	26.61	7.91	52.88^{b}
Levels of significance	ns	ns	ns	ns	**
Interaction effect of locule position and thawing duration					
2 hr of thawing					
L1	15.97	0.56	19.54	9.66	58.22^{a}
L2	14.00	0.41	39.47	8.83	55.96^{a}
L3	21.37	0.70	30.52	7.59	59.29^{a}
L4	15.58	0.28	28.55	8.42	42.53^{b}
L5	18.54	0.46	34.14	7.39	48.40^{ab}
18 hr of thawing					
L1	17.11	0.45	26.05	7.71	65.99^{b}
L2	11.51	0.67	23.49	6.97	70.47^{ab}
L3	10.12	0.84	37.41	6.71	77.98^{a}
L4	13.02	0.30	24.10	9.36	76.32^{a}
L5	13.17	0.38	34.14	8.79	77.01^{a}
Levels of significance	ns	ns	ns	ns	*

Note. Means (n = 3) followed by the same letter in the same column within factors are not significantly different at $p < 0.05$ according to Tukey's post hoc tests
GAE = Gallic acid equivalent; QE = Quercetin equivalent; TE = Trolox equivalent; DPPH = 1,1-diphenyl-2-picryl-hydrazyl; ABTS = 2-2'-azino-bis(3-ethylbenzothiozoline-6-sulfonic acid); FRAP = Ferric reducing antioxidant power
ns, *, ** = Non-significant or significant at $p \leq 0.05$ or $p \leq 0.01$, respectively

et al., 2022). Furthermore, a carotenoid responsible for the intense yellowish colour of durian pulp (Tan et al., 2020) is heat labile and susceptible to degradation (Gheonea et al., 2020). Temperature equilibration between frozen fruit (-20°C) and room (25°C) could have degraded the carotenoid and thus manifested in a darker colour, especially when the thawing duration was prolonged.

CONCLUSION

Results from this study indicated that the locule position and thawing duration significantly interact with the postharvest quality of cryo-frozen Musang King durian fruit. Unfortunately, the locule position of durian fruit was assigned randomly. Thus, it could not corroborate the cause of variation in each locule towards the postharvest quality of durian fruit. However, the finding of this study indicates that the sampling methods employed in collecting data on an intact durian should consider the locule effect. Since the thawing duration of cryo-frozen durian affects the colour of durian pulp, thus vendors must provide proper advice or instruction to consumers in enjoying the intact cryo-frozen durian fruit.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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Subcutaneous Implantation of Unidirectional Self-Inflating Anisotropic Tissue Expander Has No Effect on the Physiological Parameters and Behaviours of Horses

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ABSTRACT

The tissue expansion technique is one of the most important innovations in skin reconstructive surgery in human and veterinary medicine. This study investigated horses' physiological and behavioural responses to subcutaneous implantation of a unidirectional self-inflating anisotropic tissue expander. The tissue expanders were subcutaneously implanted on six horses at three different locations: the frontal region of the head, the lateral side of the right shoulder, and the dorsomedial part of the cannon region of the right forelimb. Prior to the tissue expander implantation, each horse was clinically examined, and the observed vital parameters and behaviour were recorded as baseline data. For six days post implantations, the horses were clinically examined, implantation sites monitored, and the horse's behaviour was recorded both during the day by visual observation

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and at night with a video camera. The results showed that all horses tolerated the subcutaneous gradual skin expansion by implantation of the tissue expanders, and all surgical sites healed without any complications. The implantation of the tissue expanders does not affect the vital parameters or overall horse behaviour either during the day or at night. In conclusion, the subcutaneous implantation of unidirectional anisotropic tissue expanders in horses resulted in successful skin growth with no physiological and psychological discomfort; hence skin expansion is a good option to be considered when pursuing equine skin reconstructive surgery.

Keywords: Behaviour, horse, self-inflating tissue expander, subcutaneous expansion

INTRODUCTION

In equine practice, a large wound is commonly managed as an open wound with scar formation resulting in deformation of the injured area (Theoret & Schumacher, 2017; Zöllner et al., 2012). To ensure anatomical restoration of the injured or defective site, reconstructive surgery is usually required (De Lorenzi et al., 2018; Keller et al., 1994; Whittaker et al., 2020).

The tissue expansion technique is a surgical technique to generate excess skin adjacent to a defect mechanically (Fang et al., 2013). The significance of this technique is that it allows the replacement of lost tissue with analogous tissue with matching texture, colour, and other important local features (Agrawal & Agrawal, 2012; Tepole et al., 2012). In humans, the skin expansion technique is used to manage soft tissue defects because of neoplastic resection, burns, congenital conditions, and adjacent or distant skin expansion for use as a local or free flap, respectively. In the equine clinical setting, surgical coverage of large wounds, particularly at the distal part of the horse's legs and face, cannot be easily performed due to inadequate skin for closure (Zöllner et al., 2012). Thus, healing is characterised by excessive scar formation resulting in deformation of the injured area. Although skin grafting has been used to fill the skin defect, however, tissue expansion technique has been proposed as an alternative technique to skin grafting because it is more favourable in terms of higher success rates and cosmetic results since the defect is replaced with analogous tissue with matching texture, colour and other important local features (Agrawal & Agrawal, 2012; Al-Majhali et al., 2021; Whittaker et al., 2020).

Considering the recent awareness and the potential use of the tissue expansion technique in equine reconstructive surgery, the knowledge of possible behavioural responses to gradual subcutaneous expansion to unidirectional self-inflating tissue expander implantation became necessary. Currently, no research has evaluated the behaviour of horses to tissue expander implantation. Behaviour in animal species, including horses, is one of the most readily observed measures of welfare (Budras et al., 2012; Chung et al., 2018; Mason & Mench, 1997),

and it is mostly studied by assessing the animal response to its environment or procedures. To substantiate the significance of the behavioural evidence, including physiological assessment in the behavioural study is always considered an important step (Yarnell et al., 2013).

Therefore, in this study, we investigate the effect of subcutaneous implantation of unidirectional anisotropic tissue expanders on the physiological parameters and behaviour of horses. It was hypothesised that there would be no physiological parameters and behavioural changes to horses' subcutaneous implantation of tissue expanders.

MATERIALS AND METHODS

Ethical Consideration

The study was approved by the Institutional Animal Care and Use Committee of Universiti Putra Malaysia (UPM/IACUC/AUP-R066/2018).

Animals

A total of six horses comprising four geldings and two mares were used in this study. The horses were aged 17–22 and weighed 450 to 500 kg. They were housed separately in a clean stall box (3.5 m × 4.5 m × 4 m) with good bedding and fed on hay, pelleted feed, and clean water provided ad libitum. The horses had up-to-date vaccination against equine influenza, Japanese Encephalitis, and tetanus. All the horses were from Ceremonial Mounted Squadron, Malaysian Armed Forces.

Subcutaneous Implantation of Unidirectional Self-Inflating Anisotropic Tissue Expanders

The horses were implanted with the tissue expanders (Expaniderm, Oxtex®, United Kingdom) subcutaneously at three locations: the rostral part of the frontal region of the head, the lateral side of the right shoulder, and the dorsomedial part of the cannon region of the right forelimb. All procedures were performed under the aseptic condition with the horses on standing sedation with xylazine (Chanazine® 10%, Chanelle Pharma, Ireland) with normal saline infusion (100 µg/ml, at a constant infusion rate of 14 drops/min). The 2% lignocaine hydrochloride (DSN Pharma Sdn. Bhd, Malaysia) was infiltrated at the sites of the surgical implantation. After which, a horizontal incision was made at the implantation site, and the skin was then undermined with blunt dissection to create a subcutaneous pocket. A piece of anisotropic tissue expander was then gently inserted into the subcutaneous pocket and secured in position by placing a few tacking sutures to prevent migration of the tissue expander subcutaneously. The incision site was then sutured with 3-0 Ethilon® (USA) using a cross-mattress suture pattern. During the post-implantation, the antibiotic of procaine penicillin and dihydrostreptomycin sulphate (Norbrook, United Kingdom) (22,000 IU/kg, intramuscularly), and anti-inflammatory of flunixin meglumine (Norbrook, United Kingdom) (1.1 mg/kg, intravenously) was given once a day for three days. A period

of 14 days was allowed for the cutaneous expansion process as recommended by the manufacturer. The experiment lasted for 18 days.

Behavioural and Physiological Vitals Assessment

Three days prior to the tissue expander implantation, each horse was observed for normal behaviour in the stall box (Table 1). Physiological parameters were determined, including rectal temperature, heart rate, and respiratory rate. The physiological parameters were assessed 30 min before the behavioural observational section. The horse behaviour was observed and recorded at an interval of 10 min for four hours during the daytime and another four hours at night. All observation sessions were performed around 10:00 a.m. to 2:00 p.m. and between 9:00 p.m. to 1:00 a.m. The frequency and duration based on the ethogram before the implantation of tissue expanders were recorded and considered as baseline data (D_o). Two camera traps (Ltl Acorn, Ltl-5210A, United Kingdom) were installed in the stable where the horses are kept and adjusted to record the horses' behaviour and responses according to a schedule throughout the day/night.

Table 1
Description of horse behaviours examined before and after implantation of tissue expanders

Behaviour	Description
	Measured in Frequency (number of occurrences/hour)
Head shaking	Shaking the head from side to side
Head-turning	Turning the head together with the neck to a particular side
Nose scratching	Rubbing a nose area on the wall or any hard object
Circling	Radial locomotion in the stable
Eating	Mastication and swallowing
Drinking	Ingest water by dipping lips at or slightly below the surface of the water and drawing the water with a sucking action, and swallowing
	Measured in Duration (hour)
Lying	Lying down on the sternum with legs folded under the body
	Lying down on the side with legs stretched out
Scratching of the implant site	Rubbing of the forehead
	Rubbing of the shoulder area
	Nibbling, biting, licking, or rubbing the lower forelimb
Standing rest	Weight-bearing on all four legs
	Weight-bearing on three legs with right hindleg slightly flexed
	Weight-bearing on three legs with left hindleg slightly flexed
Walking	Forward and backward movement of more than 1 limb resulting in a new position within the stable
Exploring	Foraging without eating or smelling the local environment while standing or walking

Note. The ethogram description of horse behaviours was based on previous studies (Ashley et al., 2005; Price et al., 2003; Torcivia & Mcdonnell, 2020)

Post-surgical implantation of tissue expanders, the horses were observed for any change in behavioural patterns and comfort level for up to 6 days (D_1, D_2, D_3, D_4, D_5, and D_6). On each day prior to behavioural observation, the rectal temperature, heart rate and respiratory rate were taken 30 min before recording the horse's behaviour. The frequency and time duration of the specific behaviour were analysed based on the visual observation and recorded video of the horse in the stable.

Statistical Analysis

The data were expressed as mean and standard deviation. The mean frequency or percentage difference of the behavioural parameters between pre- and post-implantation was analysed using one-way analysis of variance (ANOVA) followed by Tukey's post hoc for multiple comparisons. The p-value < 0.05 was considered significant. All statistical analysis was performed using GraphPad Prism version 8.0.2 (GraphPad Software, USA).

RESULTS

Physiological Assessments

All horses tolerated the surgical implantation procedure, and the incision sites healed without complications. There was no evidence of suture breakage, implant rupture, hematoma, or discharge. The physiological parameters of the horses pre- and post-implantation of tissue expander subcutaneously in horses are shown in Figure 1. There was no significant effect ($p > 0.05$) of tissue expander implantation on the rectal temperature (Figure 1A) and respiratory rate over the six days observational period (Figure 1C). However, a significant difference ($p = 0.0245$) in heart rate within the observational days was observed among the horses (Figure 1B).

Behavioural Assessments

The mean proportion of time duration for each behaviour during the day and night of the pre-and post-implantation observational period are presented in Tables 2 and 3,

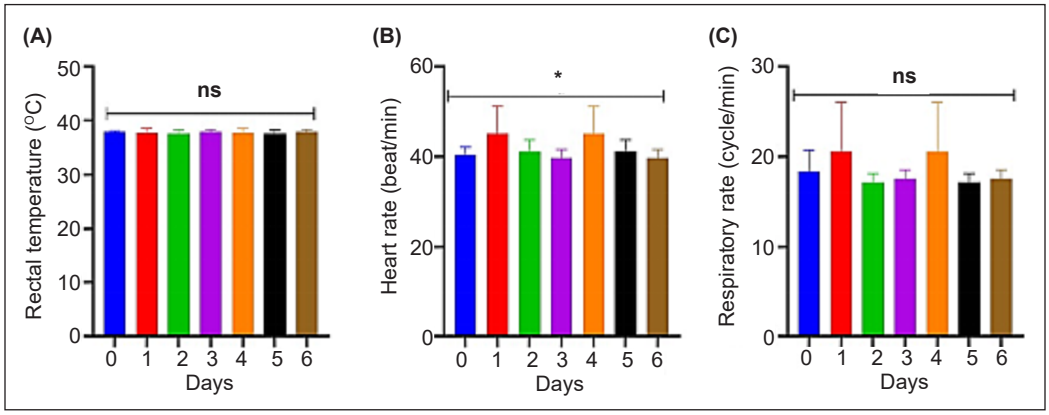

Figure 1. Rectal temperature (A), heart rate (B), and respiratory rate (C) determined pre and six days post-implantation of tissue expander subcutaneously in horses
Note. * $p < 0.05$ significance different over the time duration; ns = No significant difference between the time duration

Table 2
The proportion of time spent on each behavioural parameter observed during the daytime pre- and post-implantation period

Behaviour	Percentage of observation time/hour						
	Pre-implantation	Post-implantation					
	D_o	D_1	D_2	D_3	D_4	D_5	D_6
Standing rest	40.49±4.88	42.53±7.76	41.74±5.35	37.78±4.13	44.79±3.61	39.68±3.75	41.71±4.78
Walking	7.46±1.41	7.11±0.50	7.29±0.83	6.51±2.11	7.66±0.31	7.57±1.36	7.52±1.41
Exploring	11.25±2.86	10.65±4.03	12.35±3.09	9.03±5.20	12.22±1.86	12.36±2.67	11.81±2.07
Eating	37.23±5.70	38.61±4.47	36.32±6.47	39.54±7.34	33.24±4.81	37.87±1.15	37.34±4.46
Lying down	3.18±5.79	1.11±2.72	2.315±5.67	6.898±7.78	2.083±3.49	2.362±5.79	1.297±3.18
Forehead scratching	0.01±0.03[b]	0	0	3.36±5.15[a]	0	0	0.05±0.07[b]
Shoulder scratching	0.14±0.16	0	0.12±0.14	0.23±0.33	0.05±0.11	0.09±0.11	0.26±0.31
Lower limb scratching	0.21±0.25	0	0.17±0.19	0.26±0.39	0.05±0.11	0.17±0.14	0.33±0.39

Note. All values expressed in mean and standard deviation; [a,b] Means across the row with different superscripts are significantly different at $p < 0.05$

Table 3
The proportion of time spent on each behavioural parameter observed during the night pre- and post-implantation period

Behaviour	Percentage of observation time/hour						
	Pre-implantation	Post-implantation					
	D_o	D_1	D_2	D_3	D_4	D_5	D_6
Standing rest	62.08±10.39	58.59±5.68	62.18±7.82	61.71±16.65	56.94±9.67	67.32±7.53	68.84±6.36
Walking	4.08±0.85	4.19±0.86	4.44±1.39	4.77±1.21	4.35±1.68	4.49±1.60	5.16±0.87
Exploring	6.57±1.46	6.80±3.60	5.47±1.63	5.63±2.64	5.78±4.08	6.32±3.11	8.45±1.09
Eating	23.87±8.72	27.18±8.73	21.16±11.02	23.10±16.17	22.52±11.56	20.00±2.04	14.86±4.05
Lying down	3.12±3.01	3.24±3.56	6.67±8.17	4.58±7.34	10.28±19.39	5.28±12.93	2.45±3.81
Forehead scratching	0	0	0	0	0.05±0.11	0.05±0.11	0.02±0.06
Shoulder scratching	0.14±0.22	0	0.05±0.07	0.19±0.27	0.05±0.07	0.07±0.08	0.19±0.25
Lower limb scratching	0.04±0.06	0	0.05±0.07	0.02±0.06	0.02±0.06	0.05±0.07	0.02±0.06

Note. All values expressed in mean and standard deviation

respectively. During the day observation, the horses spent most of their time either standing rest (40.5%), exploring (11.3%), or eating (37.2%). There was no significant ($p > 0.05$) change in the time spent in all the behavioural parameters observed both during the pre-and post-implantation, except for forehead scratching, which was significantly ($p = 0.04$) higher in the daytime. Similarly, the time spent at night on each behaviour did not change significantly ($p > 0.05$) between the pre- and post-implantation periods. Compared to the daytime observations, the horses spent more time standing at rest, eating and recumbent rest (lying down), and less time exploring the stall box. Overall, the horses spent most of the time standing square on their 4 legs or with slightly flexed right or left hindlimb feet. There was no difference in the time spent in these different standing postures between the pre-and post-implantation day and night periods of observations (Figures 2A and 2B).

The frequency of other miscellaneous behaviour, such as headshaking, head-turning, circling, and scratching of the nose and/or abdomen, did not significantly change when compared between the pre- and post-implantation for day and night observation periods (Tables 4 and 5).

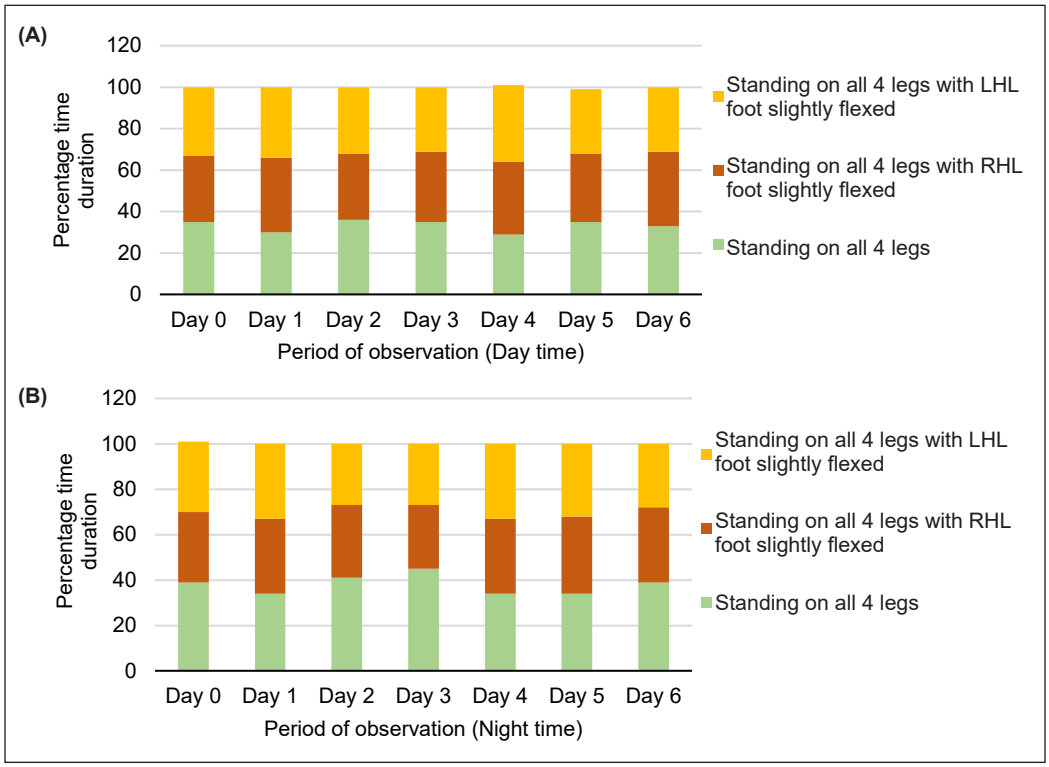

Figure 2. Average percentages of time spent standing without other activity during the day (A) and night (B) period of the observations
Note. LHL = Left hind limb; RHL = Right hind limb

Table 4
Mean frequency of other miscellaneous behaviours observed during the day

Behaviour	Mean frequency of observation time						
	Pre-implantation	Post-implantation					
	D_o	D_1	D_2	D_3	D_4	D_5	D_6
Head shaking	13.33±9.46	13.33±6.09	8.00±5.55	8.67±5.85	8.67±5.39	10.00±7.82	14.17±13.41
Head-turning	4.00±1.67	3.33±2.07	2.50±1.76	2.83±1.47	3.50±1.38	1.83±0.98	3.00±2.10
Circling	1.33±0.82	1.17±0.98	0.67±0.82	1.00±0.89	1.00±1.55	1.17±1.33	0.5±0.84
Drinking	2.83±4.62	1.50±3.67	3.17±6.31	5.00±6.87	1.83±2.14	0.83±0.75	2.167±3.92
Nose scratching	0.67±1.03	0.33±0.82	0.17±0.41	0.33±0.82	1.17±2.86	0.67±1.63	0.17±0.41
Abdomen scratching	0.50±0.84	0.17±0.41	0.33±0.82	0.50±0.84	0	0.17±0.41	0

Note. All values expressed in mean and standard deviation

Table 5
Mean frequency of other miscellaneous behaviours observed during the night

Behaviour	Mean frequency of observation time						
	Pre-implantation	Post-implantation					
	D_o	D_1	D_2	D_3	D_4	D_5	D_6
Head shaking	8.0±8.67	8.5±10.01	10.0±10.49	7.50±7.18	6.17±4.96	7.67±7.99	9.67±10.75
Head-turning	2.17±1.72	1.5±1.38	1.83±1.17	2.67±1.63	2.167±2.32	1.167±1.17	1.67±0.82
Circling	0.83±0.75	0.33±0.52	3.33±6.25	1.83±2.14	0.67±1.21	1.5±2.35	0.67±0.82
Nose scratching	0.67±0.82	0.33±0.82	0.17±.41	0	1.0±2.45	0.5±1.23	0.17±0.41
Abdomen scratching	0.5±0.84	0.17±0.41	0.5±1.23	0.83±0.98	0.5±0.84	0.33±0.52	0

Note. All values expressed in mean and standard deviation

DISCUSSION

It is the first study to describe that horse does not show any significant change in behaviour following subcutaneous tissue expander implantations with the hydrogel unidirectional self-inflating anisotropic tissue expanders. When assessed day and night, all horses showed no physiological and behavioural changes associated with the tissue expander implantation. All horses tolerated the implantation procedure, and the surgical sites healed without complication. There was no evidence of suture breakage, implant rupture, hematoma, or discharge. These findings are consistent with earlier observations (Al-Majhali et al., 2018; Chandawarkar et al., 2003; Whittaker et al., 2020). The elimination of pain after surgical implantation of the tissue expander by parenteral analgesia was to ensure the observed physiological and behaviour were not due to pain. The rectal temperature, respiratory, and heart rate of each horse over the six-days observation period, when compared with baseline data (pre-implantation), revealed normal vital parameters. Similarly, the rectal temperature and respiratory rate showed no significant change when considering all horses together. Although the heart rate revealed significant change over the observed period, the mean heart rate for each observed day is within the normal reference value. The slight changes in heart rate could be attributed possibly to the individual horse variation effect. This finding is similar to a previous behavioural study on pain where no correlation between the vital parameters and pain surgery, particularly following post-operative analgesia, was observed (Price et al., 2003).

In this study, the behavioural indices observed during the day and night pre-implantation of the tissue expanders considered normal for horses include standing, walking, exploring, lying down (recumbent rest), eating, and drinking. Based on the proportion of time, the horse spent most of the day standing, eating, and exploring the stall box. While at night, they spent the highest of the observed periods standing, resting and eating. Post-implantation, there was no significant change in the proportion of time spent on each of the behavioural parameters observed about the baseline (pre-implantation) during day and night over the six-day observational period. Scratching at the implantation areas was considered insignificant as it may reflect itching due to the inflammatory response to healing processes. Moreover, this behaviour was occasionally observed during the pre-implantation period suggesting a response to an external irritant (Velnar et al., 2009).

The findings of standing rest to be the predominating behaviour are not surprising, as it is typical for horses to spend most of the hours of the day and night in standing rest and lesser time in recumbent rest (Chung et al., 2018; Evans, 2000; Ransom & Cade, 2009). Horses can stand for long periods and even drowse and sleep while standing due to their unique passive stay apparatus of the equine forelimbs and hindlimbs. Thus, the horse could stand on their feet with minimum muscular effort (Dyce et al., 2009). Furthermore, horses may shift

weight between the hindlimb with slight flexing of the foot (Budras et al., 2012; Fuchs et al., 2016). This study's less time spent on recumbent rest seems lower than the average recumbent resting duration reported previously. In those reports, the type of bedding has been identified as an important factor that affects the duration of recumbent rest: sternal and/or lateral recumbency (Pedersen et al., 2004; Werhahn et al., 2010). Besides the bedding type, the depth and nature of bedding materials, such as cleanliness, texture, softness, and odour, could also influence the recumbent rest duration. However, the present observation of shorter recumbent rest duration is similar to what was documented for horses housed in shaving bedding, the same bedding material used in this study (Greening et al., 2013).

The duration of hay and/or concentrated eating during day and night, either pre- or post-implantation, were consistently normal for horses. Similar to the earlier report on the normal digestive behaviour of horses, the horses spent about 1/3 of the daytime and 1/4 of the night observation period engaged in eating (Greening et al., 2013).

When compared between the pre-and post-implantation period, the non-significant change in spent time on the behaviour of horses, such as eating, drinking, circling, head shaking or turning, and exploring during day and night horses tolerated the skin expansion technique without any discomfort or unwanted responses. Among the unwanted responses is reflected restlessness behaviour, which may be seen as either decreased eating, drinking, and exploring time or increased circling, head shaking, and/or turning (Ashley et al., 2005). More importantly, with a strong correlation between the longer time spent on standing rest and eating, standing rest behaviour has been described as an appropriate indicator of good welfare in horses (Ninomiya et al., 2007).

CONCLUSION

The subcutaneous implantation of unidirectional anisotropic tissue expanders in horses does not affect the physiological and behaviour of the horses. Hence tissue expansion technique is a good option to be considered when pursuing equine skin reconstructive surgery for equine skin reconstructive surgery.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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Review Article

Review on the Epidemiology, Milk Composition Changes, and Antimicrobial Susceptibility of Causative Agents of Bubaline Mastitis in Asia

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ABSTRACT

Mastitis is one of the diseases that cause economic losses worldwide due to the reduction in milk yield and the high treatment costs in dairy buffaloes. Although antibiotics are the mainstay treatment for this disease, the overuse of antibiotics has resulted in the emergence of antimicrobial resistance in animals and humans. Hence, this study aims to review and assess the available literature on bubaline mastitis in Asia. The prevalence of subclinical mastitis was higher in dairy buffaloes than in clinical mastitis, especially in Pakistan. Bubaline mastitis was commonly detected using the California mastitis test, surf field mastitis test, somatic cell count, and bacterial culture. In Asia, farm management and host factors were the primary causes of bubaline mastitis risk factors. Mastitis in buffaloes caused alterations in milk composition, such as increasing lactose levels, somatic cell count, and the presence of bacteria in the milk. However, protein, fat, and solid non-fat level variations were also affected by other factors such as the stage of lactation, breed, and age. The most prevalent isolated bacteria in bubaline mastitis milk samples were coagulase-negative staphylococci, *Staphylococcus aureus*, *Streptococcus* spp., and *Escherichia coli*. Most showed high resistance toward penicillin, amoxicillin, ampicillin, and streptomycin. The

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antimicrobial susceptibility of causative agents in Asia varies depending on the usage of common antibiotics to treat bubaline mastitis in each country. This review will help to understand bubaline mastitis better, although studies are limited in many Asian countries.

Keywords: Antimicrobial resistance, Asia, bubaline mastitis, buffalo, epidemiology, milk composition

INTRODUCTION

Mastitis is the inflammation and infection of the udders area, a global issue in the dairy buffaloes farming industry. Mastitis is classified as either subclinical mastitis (SCM) or clinical mastitis (CM). The risk factors of bubaline mastitis include milking practices, farm and milk management, age, number of parity, stage of lactation, udder and leg hygiene, and teat end morphology of dairy buffaloes (Salvador et al., 2012). Common mastitis treatments in dairy animals are streptomycin, ampicillin, cloxacillin, penicillin, and tetracycline (Bhosale et al., 2014). The consequences of mastitis in dairy animals included a reduction of milk yield, an increase in the spread of mastitis within the herd, high treatment costs, and high economic losses (Fareed et al., 2017; Fetrow, 2000; Jingar et al., 2017).

Although mastitis can be treated with antibiotics, the misuse and overuse of antibiotics can lead to another serious issue: antimicrobial resistance (AMR). The common causative agents of AMR in bubaline mastitis are *Staphylococcus* spp. (36.04%), *Enterococcus* spp. (19.52%), *Escherichia coli* (9.26%), and *Streptococcus* spp. (4.39%) (Patel et al., 2019). The consequences of AMR in mastitis are harmful to animals and humans due to the causative pathogens resistant to antibiotic treatment, increased mortality, high cost of treatment, and high economic losses.

The buffaloes are considered to be less susceptible to mastitis than cows (Fagiolo & Lai, 2007). However, once the buffaloes are infected, the causative pathogens can multiply rapidly due to the high nutrient content in buffalo milk. Buffaloes have tighter teat sphincters, which is one of the reasons the occurrence of mastitis in buffaloes is lower than in bovine mastitis, as well as contributes to a better barrier to pathogens invasion (Krishnaswamy et al., 1965), and the mucin-1 (*MUC1*) gene that mainly to protect the cell surface from the environment pathogens (da Rosa et al., 2020). Since the occurrence of mastitis in buffaloes is lower than in bovine mastitis, the information about the risk factors, milk composition changes, occurrence, and antimicrobial susceptibility of mastitis in dairy buffaloes, especially in Asian countries, is very limited.

For this review, the main objective was to review and assess available literature on the epidemiology, milk composition changes, and antimicrobial susceptibility of causative agents of bubaline mastitis in Asia.

Dairy Industry

The dairy industry consists of dairy farming and the processing industry. Dairy farming

is an agricultural class of animal husbandry that involves long-term milk production, mainly from cows and followed by buffaloes, goats, sheep, and camels. Cows, buffaloes, goats, sheep, and camels are the important dairy animals that contributed 81.05, 15.14, 2.25, 1.20, and 0.35%, respectively, to the world milk production in 2019 (Food and Agriculture Organization of the United Nations [FAO], n.d.).

Due to the high consumption of dairy products, especially cheese, there is still insufficient supply to satisfy the world's demand for dairy products, despite increases in milk production, dairy product manufacture, and the number of dairy animals. India and Pakistan are expected to contribute to milk production in the world over the next ten years, which includes milk production from cows and buffaloes (Organisation for Economic Co-operation and Development/Food and Agriculture Organization of the United Nations [OECD/FAO], 2020).

India and Pakistan have produced more milk from buffaloes than cows (FAO, n.d.). Buffaloes are the second-highest milk producer in the world, producing approximately 133,752,296 tonnes of milk, and have the fourth-highest dairy animal population in the world, with about 69,924,520 heads. A total of 98.21% of buffalo milk is produced in Asia countries, especially in India (70.03%) and Pakistan (26.16%). The world and Asia countries' dairy buffalo population and milk production in 2019 are shown in Table 1 (FAO, n.d.).

Table 1
World dairy buffaloes population and milk production in 2019

Countries	Population of dairy buffaloes (heads)	Production of milk (tonnes)
Total (World)	69,924,520	133,752,296
Total (Asia)	68,328,457	131,363,080
Bangladesh	90,934	35,790
Bhutan	261	261
Brunei	178	183
China (Mainland)	5,818,607	2,928,369
Georgia	10,177	6,180
India	45,000,000	92,000,000
Indonesia	117,441	85,474
Iran	45,000	128,000
Iraq	59,738	35,981
Malaysia	5,555	7,691
Myanmar	458,523	204,750
Nepal	1,560,584	1,372,905
Pakistan	14,959,000	34,371,000
Sri Lanka	91,790	73,566
Syria	3,940	6,378
Turkey	79,333	79,341
Vietnam	27,396	27,211

Buffaloes

Buffaloes can be categorized into river and swamp buffaloes. Both are used as human food sources such as meat and milk, by-products such as hide, fertilizer, and draught power (Deb et al., 2016), and river buffaloes produce more milk than swamp buffaloes, which each produce 1,800 to 2,000 kg per lactation and 500 to 800 kg per lactation, respectively (Borghese, 2011; Nanda & Nakao, 2003). The breeds of Asian swamp buffalo (Moioli & Borghese, 2005; Mokhber et al., 2018; Mukesh et al., 2009; Sethi, 2003) are listed in Table 2. Based on Table 2, the common breeds of river buffalo are

Table 2
Buffalo breed in Asia

Breed	Countries	Milk yield (kg/lactation)	Milk fat (%)
Sambalpuri	India	2,200–2,400	-
Kundi	Pakistan	2,000	7.0
Jafarabadi	India	1,800–2,700	8.5
Meshana	India	1,800–2,700	6.6–8.1
Murrah	India (common in Asian countries)	1,800–2,000	7.2
Nili Ravi	Pakistan, India (common in Asian countries)	1,800–2,000	6.5
Pandharpuri	India	1,502	-
Surti	India	1,300–2,090	6.6–8.1
Kuzestani	Iran, Iraq	1,300–1,865	6.6
Azeri	Iran, Azerbaijan	1,200–1,300	6.6
Paralakhemudi	India	1,200	-
Anatolian	Turkey	700–1,000	4.2–4.6
Bhadawari	India	780–1,150	7.2
Lime	Nepal	875	7.0
Parkote	Nepal	875	7.0

Murrah and Nili Ravi due to their high milk yields and high-fat content in milk (Siddiky & Faruque, 2017; Zhou et al., 2018).

Buffaloes can be readily acclimatized to varied climates and reared in Southeast Asian countries with hot and humid climates (Ayalew & Taye, 2004). They are known to be less prone to disease than cows and have better productivity with a high milk yield (Siddiky & Faruque, 2017). Buffalo's milk contains low water content and cholesterol levels as well as high total solids, lactose, proteins, and fat content, which are suitable for producing fat-based and solid non-fat (SNF) based dairy products such as cheese, butter, and ghee (Fundora et al., 2001).

Mastitis and the Effect of Bubaline Mastitis on the Dairy Industry

Dairy animal diseases can affect milk production in the dairy industry. Mastitis is one of the major economic diseases in dairy animals worldwide. The repercussions of mastitis in the dairy industry can be decreased milk quality and yield, high treatment costs and veterinary services, discarded milk, and milk withholding times, leading to economic losses in the dairy industry. One previous study on economic losses due to mastitis in Pakistan (2015) stated that the total daily cost of milk losses and mastitis treatment for dairy cows is $8.02 and $26.31, respectively (Fareed et al., 2017). India (2011) and the United States (US) (2009) reported that the annual economic losses on dairy cows were $47.14 and $2 billion, respectively (Jingar et al., 2017; Viguier et al., 2009). Due to a lower incidence of mastitis among buffaloes in India, the total loss in buffaloes ($18.77) was lower than in cows ($21.23) (Jingar et al., 2017).

Mastitis is a mammary gland or udder inflammation caused by several diverse pathogens, including bacteria, fungi, viruses, and others (Dalanezi et al., 2020). Pathogen invasion on the udder is followed by pathogen multiplication and the production of harmful substances, resulting in inflammation, decreased milk production, and altered milk quality (Oliver & Calvinho, 1995). Based on the clinical examination of dairy cattle for mastitis symptoms such as redness and swelling of the udders, altered milk consistency, and appearance; reduced milk production; lack of appetite, and fever, clinical mastitis can be characterized (Harmon, 1994). It is readily visible and easy to detect. In comparison, subclinical mastitis is difficult to distinguish from CM because there are no visible symptoms on the udder or milk. However, the number of somatic cells increases, and milk production diminishes, usually diagnosed based on somatic cell counts and inflammatory markers in milk (Schukken et al., 2003; Varshney & Mukherjee, 2002).

Prevalence and Detection of Bubaline Mastitis

Several methods that can be used to detect bubaline mastitis include the California mastitis test (CMT), surf field mastitis test (SFMT), white side test (WST), somatic cell count (SCC), electrical conductivity (EC), bromothymol blue (BTB), N-acetyl glucosaminidase (NAGase), and bacteria culture (BC). Combining CMT and SCC to detect mastitis in dairy animals is the best method than SCC, CMT, WST, and SFMT alone because the result is more precise and accurate (Hoque et al., 2014). SFMT was suggested as a cheaper and more user-friendly method for poor countries to use as SCM diagnostic test (Akhtar et al., 2012). BC is a gold standard in the detection method for determining the prevalence of causative pathogens in bubaline mastitis (Anirban et al., 2012). However, BTB and NAGase are rarely used to detect mastitis in dairy animals. These methods can be used for early diagnosis and prevention of SCM in dairy animals, which can prevent mastitis infection, boost milk quality and quantity, and prevent economic losses in the future (Panchal et al., 2016).

The prevalence of bubaline mastitis in Asia (2005 to 2021) is listed in Table 3. In Asia, the prevalence range of animal-based SCM and CM varies between 36.38 and 77.98% and between 10.20 and 24.60%, respectively. Meanwhile, the prevalence ranges for quarter-based SCM and CM are 9.77 to 64% and 2.81 to 9.33%, respectively. The prevalence of SCM was higher in dairy buffaloes than in CM, especially in Pakistan (Ashfaq & Muhammad, 2008). However, the prevalence of bubaline mastitis in Asia varies due to risk factors such as animal breed, lactation stage, immunological response, season, temperature, detection methods, and management practices, including poor environmental hygiene, improper hygiene, improper milking handling, and milking method (Aliul et al., 2020; Eberhart, 1986; Jingar et al., 2014; Kavitha et al., 2009; M. Z. Khan & Khan, 2006; R. Hussain et al., 2013; Salvador et al., 2012; Tapdasan et al., 2018).

Table 3
Prevalence and detection of bubaline mastitis in Asia (2005 to 2021)

Countries	Prevalence	Methodology	Sample size	Reference
Bangladesh	Animal-wise SCM: 56.66% (17/30 buffaloes) Quarter-wise SCM: CMT: 32.50% (39/120 milk samples) WST: 29.16% (35/120 milk samples) SFMT: 26.66% (32/120 milk samples)	CMT, WST, SFMT	30 buffaloes, 120 milk samples	Kisku and Samad (2013)
	SCM: 70% (21/30)	CMT	30 buffaloes	Talukder et al. (2013)
	SCM: 20% (14/70 buffaloes)	CMT	70 buffaloes	Biswas et al. (2020)
India	SCM: 32.90% (1878/5707 milk samples) CM: 18.74% (1070/5707 milk samples)	BC	2,057 buffaloes, 5,707 milk samples	Sharma and Sindhu (2007)
	Animal-wise SCM: CMT: 14.03% (8/57 buffaloes) EC: 10.53% (6/57 buffaloes) SCC: 12.28% (7/57 buffaloes) BC: 15.78% (9/57 buffaloes)	CMT, EC, SCC, BC	57 buffaloes, 228 milk samples	Neelesh et al. (2009)
	SCM: 13.60% (17/125 buffaloes) CM: 3.10% (31/1000 milk samples)	SCC, CMT	125 buffaloes, 1,000 milk samples	Kavitha et al. (2009)
	78.10% (1883/2411 milk samples)	CMT	2,411 milk samples	Bhanot et al. (2012)
	Animal-wise SCM: BC: 28.78% (19/66) SCC: 13.63% (9/66) Quarter-wise SCM: BC: 18.30% (48/262) SCC: 7.25% (19/262)	BC, SCC	66 buffaloes, 262 milk samples	Charaya et al. (2013)
	CM: 47.83% (22/46 milk samples)	CMT	46 milk samples	Sagi (2014)
	SCM: SCC: 48.40% EC: 40% CMT: 45.80% BTB: 61.10% NAGase: 61.60%	SCC, EC, CMT, BTB, NAGase	57 buffaloes, 190 milk samples	Preethirani et al. (2015)
	20% (48/240)	CMT, BC	240 milk samples	Maurya and Joshi (2021)
Iran	SCM: CMT: 34.82% (70/201 milk samples) SCC: 45.77% (92/201 milk samples)	CMT, SCC	51 buffaloes, 201 milk samples	Beheshti et al. (2011)

Table 3 *(continue)*

Countries	Prevalence	Methodology	Sample size	Reference
	Animal-wise SCM: 23.66% (71/300 buffaloes) Quarter-wise SCM: 13.87% (166/1200)	CMT	300 buffaloes, 1,200 milk samples	Vajdi et al. (2011)
Iraq	CM: 36.25% (29/80 buffaloes)	CMT, SCC	80 lactating buffaloes	Al-saadi and Alshakh (2015)
	CM: 12% (12/100 milk samples) SCM: 24% (24/100 milk samples)	CMT	25 buffaloes, 100 milk samples	Wahid et al. (2017)
Turkey	SCC: SCM: 9.77% (160/1637 milk samples) CM: 2.81% (46/1637 milk samples) CMT (CM and SCM): 12.58% (206/1637 milk samples)	SCC, CMT	1,637 milk samples	Özenç et al. (2008)
Nepal	SCM: 6.48% (23/355 buffaloes) CM: 93.52% (332/355 buffaloes)	EC	355 buffaloes	Dhakal et al. (2007)
	55.15% (75/136 buffaloes)	CMT	136 buffaloes	Ng et al. (2010)
Philippines	24.22% (93/384 buffaloes)	CMT	384 buffaloes	Badua et al. (2020)
Pakistan	SCM: 27% (54/200 milk samples) CM: 4% (8/200 milk samples)	SFMT	50 buffaloes, 200 milk samples	Z. Khan and Muhammad (2005)
	Animal-wise SCM: 77.98% (234/300 buffaloes) Quarter-wise SCM: 58.75% (705/1200 milk samples)	SFMT	300 buffaloes, 1200 milk samples	Bachaya et al. (2005)
	Quarter-wise CM: 9.33% (7/75 milk samples) SCM: 64% (48/75 milk samples)	SFMT	75 milk samples	Ashfaq and Muhammad (2008)
	SCM: 44% (264/600 buffaloes)	WST	600 buffaloes	M. A. Ali et al. (2011)
	Animal-wise: SCM: 36.38% (139/382 buffaloes) CM: 24.60% (94/382 buffaloes) Quarter-wise: SCM: 16.04% (222/1528 milk samples) CM: 8.04% (123/1528 milk samples)	SFMT	382 buffaloes, 1,528 milk samples	Hameed et al. (2012)

Table 3 *(continue)*

Countries	Prevalence	Methodology	Sample size	Reference
	SCM: 18.46% (240/1300 milk samples) CM: 4.62% (60/1300 milk samples)	SFMT	1,300 milk samples	A. Hussain et al. (2013)
	SCM: 41.80% (163/390 buffaloes) CM: 13.60% (53/390 buffaloes)	CMT	390 buffaloes	T. Ali et al. (2014)
	SCM: 38.80% (402/1036 buffaloes) CM: 10.20% (106/1036 buffaloes)	CMT	1,036 buffaloes	A. Hussain et al. (2018)

Note. SCM = Subclinical mastitis; CM = Clinical mastitis; CMT = California mastitis test; SFMT = Surf field mastitis test; WST = White side test; SCC = Somatic cell count; EC = Electrical conductivity; BTB = Bromothymol blue; NAGase = N-acetyl glucosaminidase; BC = Bacteria culture

One previous study about the prevalence of bovine and bubaline mastitis stated that the prevalence of bubaline mastitis is lower than bovine mastitis because the buffaloes have a tighter teat sphincter that provides a better barrier to the invasion of causative pathogens (Krishnaswamy et al., 1965), a longer teat and teat canal than cows so that pathogens must travel further through the teat to establish the infection within the mammary gland (Thomas, 2004), and the *MUC1* gene that mainly to protect the cell surface from the environmental pathogens (da Rosa et al., 2020).

Causative Agents of Bubaline Mastitis

There are several methods, such as BC, polymerase chain reaction (PCR), and metagenomics pyrosequencing, are utilized to detect the causative pathogens of bubaline mastitis (SCM and CM) (Patel et al., 2019; Preethirani et al., 2015). Using BC, bacteria are detected based on the morphology and biochemical tests, consequently allowing antibiotic susceptibility testing. At the same time, PCR utilizes primers to detect the conserved DNA sequences of bacterial genes that encode ribosomal RNA (Järvinen et al., 2009). PCR can also detect all genotypic resistance and underlying genetic mechanisms. The advantage of PCR is simple, less time-consuming (it can take less than 24 hr to complete compared to BC), and a less expensive method for processing milk samples (Abd El-Razik et al., 2010).

Metagenomics pyrosequencing of 16rDNA was performed to characterize the bacterial communities associated with milk samples from healthy, CM, and SCM in buffaloes. Quantitative Insights into Microbial Ecology (QIIME) analyzed the obtained sequencing data (Caporaso et al., 2010), whereas Paleontological Statistics (PAST) was used for statistical analysis (Hammer et al., 2001). Pyrosequencing produced 47.3 million base pairs reads (Patel et al., 2019). Phylogenetic profiles using the ribosomal database resulted in the

different genus-level prevalence of bacterial communities in healthy, CM, and SCM samples. Healthy samples are prevalent with *Lactobacillus*, *Paenibacillus*, and *Pseudomonas*; CM samples are prevalent with *Staphylococcus*, *Enterococcus*, *Escherichia*, and *Streptococcus*; and SCM samples are prevalent with *Staphylococcus*, *Ralstonia*, *Enterococcus*, and *Bacillus* (Patel et al., 2019). Genomic pyrosequencing can detect highly diverse and complex bacterial communities in three milk samples simultaneously. However, the bacterial communities included causative bacteria and non-causative bacteria. In the future, it can contribute to an increase in general insights into the pathogenesis of bubaline mastitis, which can lead to improved diagnostics (Patel et al., 2019).

The prevalence of causative pathogens of bubaline mastitis in Asia is listed in Table 4. The causative pathogens of bubaline mastitis can be divided into two types: contagious pathogens such as *Staphylococcus aureus* and *Streptococcus agalactiae*, and environmental pathogens such as *Streptococcus uberis*, *Streptococcus dysgalactiae*, *Escherichia coli*, *Klebsiella pneumonia*, and *Pseudomonas aeruginosa*. Environmental pathogens are present in feces, bedding surfaces, milking equipment, and milkers' hands (Fagiolo & Lai, 2007). However, *Streptococcus agalactiae* can be either contagious or environmental pathogens. The common causative pathogens in bubaline mastitis are *Staphylococcus* spp. including *S. aureus* and coagulase-negative staphylococci (CNS), including (*Staphylococcus albus*, *Staphylococcus epidermidis*, *Staphylococcus simulans*, *Staphylococcus hyicus*, *Staphylococcus lentus*, *Staphylococcus xylosus*, *Staphylococcus hominis*, and *Staphylococcus intermedius*); *Streptococcus* spp. including *S. agalactiea*, *S. dysgalactiae*, *S. uberis*, and *Streptococcus mitis*; *Escherichia* spp. such as *E. coli*; and *Entrococcus* spp.

Table 4
Prevalence of causative pathogens of bubaline mastitis in Asia

Countries	Prevalence	Methodology	Total of isolated bacteria	References
Bangladesh	Single infection: *Staphylococcus* spp. (30.77%) *Streptococcus* spp. (20.51%) *Bacillus* spp. (15.39%) *Escherichia coli* (12.82%) Mixed infection (12.82%) Unclassified bacterial growth (7.69%)	BC	39 isolated bacteria	Kisku and Samad (2013)
	Staphylococcus spp. (50%) *Escherichia coli* (28.57%) *Enterobacter* (14.29%) *Bacillus* spp. (4.76%) *Proteus* spp. (2.38%)	BC	42 isolated bacteria	Talukder et al. (2013)

Table 4 *(continue)*

Countries	Prevalence	Methodology	Total of isolated bacteria	References
	Bacteria culture: *Staphylococcus aureus* (44.83%) *Escherichia coli* (31.03%) *Bacillus* spp. (13.79%) *Streptococcus* spp. (10.34%) PCR: *Staphylococcus aureus* *Escherichia coli*	BC, PCR	29 isolated bacteria	Biswas et al. (2020)
Turkey	*Candida* spp. (41.91%) CNS (20.59%) *Staphylococcus aureus* (16.91%) *Penicillium* spp. (2.21%) *Bacillus* spp. (1.47%) Mixed infections (16.91%)	BC	136 isolated bacteria	Özenç et al. (2008)
India	*Staphylococcus* spp. (coagulase positive) (30%) *Escherichia coli* (18.75%) Coagulase-negative staphylococci (17.50%) *Streptococcus agalactiae* (7.50%) *Corynebacterium pyogenes* (6.25%) *Klebsiella pneumonie* (6.25%) *Corynebacterium bovis* (5%) *Bacillus cereus* (5%) *Pseudomonas aeruginosa* (3.75%)	BC	80 isolated bacteria	Das and Joseph (2005)
	Staphylococcus spp. (38.81%) *Streptococcus* spp. (32.40%) *Escherichia coli* (11.80%) *Corynebacterium* spp. (5.20%) *Bacillus* spp. (1.36%) *Klebsiella* spp. (2.03%) *Pseudomonas aeruginosa* (0.78%) *Proteus* (0.14%) Mixed infections (7.33%)	BC	3,447 isolated bacteria	Sharma and Sindhu (2007)
	Staphylococcus spp. (20.11%) *Staphylococcus aureus* (11.73%) *Escherichia coli* (16.76%) *Streptococcus* spp. (11.17%) *Streptococcus dysgalactiae* (5.03%) *Streptococcus uberis* (3.91%) *Streptococcus agalactiae* (2.23%) Mixed infections (29.05%)	PCR, BC	179 isolated bacteria	Kumar (2009)
	Staphylococcus spp. (43.60%) *Streptococcus* spp. (21.80%) *Escherichia coli* (16.3%) *Klebsiella* spp. (5.40%)	BC	-	Bhanot et al. (2012)

Table 4 *(continue)*

Countries	Prevalence	Methodology	Total of isolated bacteria	References
	Corynebacterium pyogenes (5.40%) *Pseudomonas aeruginosa* (3.60%) *Bacillus* spp. (3.60%)			
	Staphylococcus spp. (64%) *Streptococcus* spp. (36%)	BC	50 isolated bacteria	Charaya et al. (2013)
	Coagulase-negative staphylococci (64.80%) *Streptococcus* spp. (18.10%) *Escherichia coli* (9.80%) *Staphylococcus aureus* (7.30%)	BC, Monoplex PCR, multiplex PCR	195 isolated bacteria	Preethirani et al. (2015)
	Staphylococcus spp. (51.16%) *Streptococcus* spp. (37.94%) *Escherichia coli* (8.41%) *Corynebacterium pyogenes* (1.62%) *Pseudomonas aeruginosa* (0.46%) *Klebsiella* (0.19%) Mixed infections (10.38%)	BC	2,580 isolated bacteria	Sharma et al. (2018)
	CM causative pathogens: *Staphylococcus* (25.95%) *Enterococcus* (10.80%) *Escherichia* (8.88%) *Streptococcus* (3.97%) SCM causative pathogens: *Lactococcus* (23.96%) *Staphylococcus* (10.09%) *Ralstonia* (12.72%) *Enterococcus* (8.72%) *Bacillus* (4.29%)	Metagenomic pyrosequencing	-	Patel et al. (2019)
	Escherichia coli (29.17%) *Staphylococcus aureus* (54.17%)	BC	48 isolated bacteria	Maurya and Joshi (2021)
Iran	*Staphylococcus* spp. (48.55%) CNS (36.18%) *Staphylococcus aureus* (14%) *Lactobacillus* (14%) *Corynebacterium bovis* (8%) *Bacillus subtilis* (7%)	BC	173 isolated bacteria	Beheshti et al. (2011)
	CNS (38.24%) *Corynebacterium bovis* (11.75%) *Bacillus subtilis* (11.75%) *Streptococcus agalactiae* (5.87%) *Staphylococcus aureus* (2.95%) Mixed infections (29.41%)	BC	34 isolated bacteria	Vajdi et al. (2011)
	CNS (66.08%) *Staphylococcus aureus* (33.91%)	BC, PCR	171 isolated bacteria	Ahmadi et al. (2020)

Table 4 *(continue)*

Countries	Prevalence	Methodology	Total of isolated bacteria	References
Iraq	*Escherichia coli* (27.78%)	BC	36 isolated bacteria	Wahid et al. (2017)
Nepal	CNS (36.96%) *Micrococcus* (21.74%) *Staphylococcus aureus* (20.65%) *Clostridium* spp. (8.69%) *Bacillus subtillis* (7.61%) *Escherichia coli* (5.43%) *Klebsiella pneumonia* (2.17%) *Providencia* spp. (2.17%) *Pseudomonas* spp. (2.17%) *Streptococcus mitis* (1.09%) *Corynebacterium diphtheria* (1.09%) Mixed infections (4.35%)	BC	92 isolated bacteria	Dhakal et al. (2007)
	CNS (35.70%) *Micrococcus* (23.20%) *Clostridium* spp. (10.70%) *Bacillus subtillis* (8.90%) *Escherichia coli* (7.10%) *Klebsiella pneumonia* (3.60%) *Providencia* spp. (3.60%) *Pseudomonas* spp. (3.60%) Mixed infections (3.60%)	BC	56 isolated bacteria	Dhakal and Nagahata (2018)
Pakistan	*Staphylococcus aureus* (45.16%) *Streptococcus agalactiae* (22.58%) *Escherichia coli* (17.74%) *Bacillus* spp. (14.52%)	BC	62 isolated bacteria	A. Z. Khan and Muhammad (2005)
	Staphylococcus aureus (44%) *Streptococcus agalactiae* (22%) *Escherichia coli* (16%) *Bacillus* spp. (4%) Mixed growth (14%)	BC	50 isolated bacteria	Kisku and Samad (2013)
	Staphylococcus aureus (48.08%) *Streptococcus agalactiae* (21.15%) CNS (13.45%) *Corynebacterial* spp. (3.9%) Undifferentiable (nontypable) Coagulase-negative staphylococci (3.8%) *Bacillus* spp. (3.8%) *Staphylococcus hominis* (1.92%) *Escherichia coli* (1.9%)	BC	52 isolated bacteria	Ashfaq and Muhammad (2008)
	Staphylococci (28.32%) *Escherichia coli* (16.18%) *Pseudomonas* (13.29%)	BC	364 isolated bacteria	M. A. Ali et al. (2011)

Table 4 *(continue)*

Countries	Prevalence	Methodology	Total of isolated bacteria	References
	Bacillus (12.42%) Streptococci (7.51%) *Salmonellae* (7.22%) *Corynebacterium* (6.64%) *Klebsiella* (5.20%) Enterococci (3.17%)			
	CNS (61.67%) *Staphylococcus aureus* (38.33%)	BC	180 isolated bacteria	A. Hussain et al. (2013)
Philippines	*Staphylococcus aureus* (41.94%)	BC	93 isolated bacteria	Badua et al. (2020)

Risk Factors of Bubaline Mastitis

In Asia, the risk factors of bubaline mastitis can be divided into 3 main categories, which are climatic and seasonal, host, and management. In countries with hot and humid climates, such as Southeast Asia, the prevalence of bubaline mastitis is higher than in countries with cold and humid and hot and dry climates, especially in the rainy season. In temperate countries, the prevalence of bubaline mastitis is higher in winter than in other seasons due to the low temperature-humidity index (THI)-associated with cold stress during the night as the buffaloes are in loose housing systems (Aliul et al., 2020; Jingar et al., 2014).

The host factor consists of animal age, breed, number of parities, stage of lactation, the shape of udders, teat end morphology, teat and udder lesion, leakage from the teats, and position of quarters. The prevalence of bubaline mastitis is higher in buffalo aged 7 to 18 than in those aged 3 to 6 due to the structural changes in the udder and teats and the gradual suppression of the buffalo immune system (Tapdasan et al., 2018). The prevalence of bubaline mastitis is higher in crossbred buffalo than in indigenous breed buffaloes, as the crossbred buffaloes can produce more milk (Tapdasan et al., 2018). Buffaloes with more than 4 parities had a higher chance of getting infected with mastitis than buffaloes with less than 3 parities (Kavitha et al., 2009; T. Ali et al., 2014). The prevalence of bubaline mastitis in the early lactation stage (14 to 100 days) is higher than in the late lactation stage (more than 200 days) and the mid-lactation stage (100 to 200 days) due to a gradual increase in milk production (Kavitha et al., 2009; T. Ali et al., 2014; Tapdasan et al., 2018).

It will lead to physiological stress in newly calved buffalo, with various contaminations of causative pathogens of mastitis during parturition. The prevalence of bubaline mastitis is higher in buffaloes with bowl- or round-shaped udders than cup shape udders and higher in cylindrical and round teat ends than pointed teat ends (R. Hussain et al., 2013). The presence of teat and udder lesions leads to a high prevalence

of bubaline mastitis because the causative pathogens enter easily through the teat canal. The prevalence of bubaline mastitis is also high in the presence of leakage from the teats due to incomplete milk removal in the udders after milking. It is important to completely remove the milk from the udder to decrease the bubaline mastitis prevalence (R. Hussain et al., 2013). The hind-quarters are more easily infected with bubaline mastitis than the fore-quarters because the hind-quarters have greater chances of getting soiled with tail and urine (Kavitha et al., 2009; R. Hussain et al., 2013; Salvador et al., 2012; T. Ali et al., 2014).

Management factors for bubaline mastitis include herd size, type of bedding, cleanliness of the farm, milking method, milking hygiene practices, history of the postparturient disease, as well as dry cow management and antibiotic therapy (Biswas et al., 2020; Kaur et al., 2015; T. Ali et al., 2014). Due to the differences in the farm management systems, bubaline mastitis is higher in big herds than in medium or small herds (Aliul et al., 2020). The prevalence of bubaline mastitis in free-range feeding areas is higher than in stall-feeding areas (Aliul et al., 2020), and there is a higher prevalence for buffaloes raised on the floor without bedding compared to concrete and sand flooring (Hameed et al., 2012; Kavitha et al., 2009). It is due to the close contact of the buffaloes with pathogens from the environment. The poor condition and cleanliness of the farm also lead to contaminated environments with increased exposure to causative pathogens of bubaline mastitis (Biswas et al., 2020; T. Ali et al., 2014). The hand milking method, including full hand, knuckling, and stripping, contributed to a high prevalence of bubaline mastitis rather than machine milking (Kaur et al., 2015; Kavitha et al., 2009; T. Ali et al., 2014). The milking method is closely related to milking hygiene practices themselves. Improper milking handling and cleaning of milking equipment, as well as poor milker hygiene habits, also cause a higher prevalence of bubaline mastitis (Biswas et al., 2020; Sharif & Ahmad, 2007) due to the transmission of causative pathogens from equipment and humans to buffaloes and from buffaloes to humans through the milk. Post antiseptic teat dipping, cleaning the milking equipment and milker hand with hot water and detergent, and drying it before milking can help to prevent and reduce bubaline mastitis (Aliul et al., 2020; M. Z. Khan & Khan, 2006; Rathva et al., 2019; Sah et al., 2020). However, the knowledge of good milking practices, such as post-antiseptic teat dipping in Asia countries, is very poor and limited. The presence of dry cow management and antibiotic therapy can reduce bubaline mastitis prevalence by eliminating 70% of environmental causative pathogens such as *Streptococcus* spp. (Eberhart, 1986; M. Z. Khan & Khan, 2006).

Milk Composition Changes in Bubaline Mastitis

Bubaline mastitis causes a reduction in milk quantity and alteration in milk composition, such as increased lactose levels, somatic cell count, and bacteria in the milk. Variations

in protein level, fat level, and solid non-fat level were also affected by other factors such as stage of lactation, breed, and age. Some studies found that mastitis in buffaloes changes milk composition, especially in protein, lactose, fat, and SNF (M. Singh et al.,2017; R. Hussain et al., 2012; Swami et al., 2017; Uallah et al., 2005). Low lactose concentration is due to the lactose efflux from milk to blood, and increased tissue permeability between the udder milk duct and blood results in blood components leakage into the udder and milk composition changes (Sharif et al., 2007). One previous study done on the difference in mineral and traces element profiles in milk between healthy buffaloes and buffaloes infected with SCM stated that there is an increase in sodium (Na) and chloride (Cl) content and a decrease in protein, fat, and zinc (Zn), iron (Fe), potassium (K), calcium (Ca), and selenium (Se) content. The value of protein, fat, Zn, Fe, Na, K, Ca, Cl, and Se in healthy buffaloes and buffaloes infected with SCM are listed in Table 5 (M. Singh et al., 2017). Fat percentage decrease in bubaline mastitis due to an increase in lipolysis rate. Na, Cl, and K decrease due to intramammary infection (IMI) response, causing the breakdown of ductal cells and secretory epithelium. Ca decreases due to the unbalanced permeability of calcium transport from milk to blood and the disruption of a junctional complex of mammary epithelium by udder pathogens (Aslam & Tucker, 1998). The IMI causes an increase in somatic cells, which leads to changes in milk's mineral and trace element profile, as well as oxidative stress due to free radical production and reactive oxygen that leads to mammary gland damage (K. V. Singh et al., 2017).

Treatment and Prevention for Bubaline Mastitis

The common treatment for bubaline mastitis is β-lactam antibiotics such as penicillin, tetracycline, streptomycin, ampicillin, and cloxacillin (Bhosale et al., 2014; Preethirani et al., 2015), and dry antibiotic therapy are enrofloxacin and oxytetracycline (Kashif et al., 2013). Both have a high cure rate

Table 5
Nutritional value between healthy buffaloes and buffaloes infected with SCM

Nutritional value	Healthy buffaloes	Buffaloes infected with SCM
Protein	4.44±0.10%	3.65±0.08%
Fat	7.08±0.14%	6.38±0.24%
Zn	5.96±1.44 mg/L	4.22±0.69 mg/L
Fe	6.99±1.37 mg/L	4.97±0.76 mg/L
Na	450±52 mg/L	606±152 mg/L
K	1063±90 mg/L	856±156mg/L
Ca	566±90 mg/L	475±77 mg/L
Cl	616±47 mg/L	828±113 mg/L
Se	0.22±0.04 mg/L	0.14±0.06 mg/L

of 91.67 and 70%, respectively (Kashif et al., 2013), administered intramammary to eliminate the existing IMI and prevent the new IMI. In return, dry therapy helps to control mastitis (Kashif et al., 2013). Another study was done on non-antibiotic and non-proprietary formulations containing trisodium citrate, vitamin C, zinc sulfate, and copper sulfate, which were equivalently effective in treating CM compared to antibiotic treatment with cure rates were 70.21 and 72.09%, respectively (Manzoor et al., 2020).

The prevention of bubaline mastitis is by introducing the latest technologies for mastitis diagnosis and implementing good milking hygiene and practice guidelines provided by National Mastitis Council (NMC) to the farmers. The mastitis-field test kit, such as CMT, SFMT, and WST, can be introduced to the farmers, which can help in the early detection of bubaline mastitis. One example of milking hygiene and practice guidelines are washing their hands with water and soap and drying them properly, washing the teats and udder of dairy animals with sanitizing solution and drying it with paper towels or individual towels, using the chlorhexidine gluconate to clean the udders, dipping the teats using germicidal teat dip solution to provide a protective germicidal barrier film on teats skin before and after milking, drying and wiping off the excess germicidal teat dip solution on the teat with paper towels or individual towels, wipe the end teat with a soaked cotton swab of 70% alcohol to kill the pathogens, and always start to cleaning the farthest teat to the nearest teat from the milkers to prevent contamination at the teat ends (A. Singh & Ramachandran, 2020; NMC, n.d.). The importance of knowledge of good milking hygiene and practices guidelines can benefit the farmers, such as reducing mastitis occurrence on farms and increasing milk yield and quality (Sah et al., 2020).

Antimicrobial Resistance (AMR) and the Impact of AMR on the Buffalo Dairy Industry

To control mastitis in dairy animals, antibiotics treatment is commonly used. It has raised another global concern on AMR due to the overuse and misuse of antibiotics, inadequate infection prevention and control measures, and lack of surveillance and monitoring. The development of AMR reduces the effectiveness of existing treatments, with the need to develop new antibiotics. The development of AMR in dairy animals leads to high potential resistant infections to humans, high pressure in the antimicrobial selection, high mortality in dairy animals, and high economic losses (Loo et al., 2019).

The World Health Assembly of the World Health Organization (WHO) endorsed a global action plan to tackle AMR in May 2015. The global action plan on AMR has five strategic objectives, including improving awareness and understanding of antimicrobial resistance through effective communication, education, and training; strengthening the knowledge and evidence base through surveillance and research;

reducing the incidence of infection through effective sanitation, hygiene, and infection prevention measures; to optimize the use of antimicrobial medicines in human and animal health; and to develop the economic case for sustainable investment that takes account of the needs of all countries and increase investment in new medicines, diagnostic tools, vaccines, and other interventions. All countries are expected to develop national action plans on AMR (WHO, 2015).

Antimicrobial Susceptibility of Causative Agents of Bubaline Mastitis

There are several methods for determining the antimicrobial susceptibility of causative pathogens of bubaline mastitis, such as the disk diffusion method, microdilution assay, and PCR (Clinical Laboratory Standards Institute [CLSI], 2019; Hoque et al., 2022). Disk diffusion method is commonly used to determine the antimicrobial susceptibility causative pathogens of bubaline mastitis in Asia countries because it is simple, reliable, and can detect the isolated resistant colonies (Mayrhofer et al., 2008; Milici et al., 2007). PCR detected the virulence genes in isolated bacteria (Hoque et al., 2022).

Kirby-Bauer disk diffusion assay was performed to determine the antimicrobial susceptibility of the isolated pathogen colonies of mastitis in buffaloes in which the commercially prepared antimicrobial disks containing common antibiotics used to treat bubaline mastitis were put on each inoculated plate (Maurya & Joshi, 2021). The inhibition zones are then measured using a precision caliper and interpreted as susceptible or sensitive, intermediate, or resistant following the suggested breakpoints diameter according to the Clinical Laboratory Standards Institute standard (CLSI, 2019).

The antimicrobial susceptibility of causative pathogens of bubaline mastitis in Asia is listed in Table 6. Most showed high resistance towards penicillin, amoxicillin, ampicillin, and streptomycin, except for cefoxitin (Gram-positive bacteria), ceftriaxone, and cefotaxime (Gram-negative bacteria). In Asia, the antimicrobial susceptibility of causative agents of bubaline mastitis countries varies based on the common antibiotics used to treat mastitis. The isolated bacteria are resistant to the common antibiotics used to treat mastitis and susceptible to antibiotics not commonly used to treat bubaline mastitis. The antibiotics susceptible to isolated bacteria can potentially become the new antibiotics to treat bubaline mastitis (Biswas et al., 2020).

CONCLUSION

The prevalence of SCM was higher in dairy buffaloes than in CM, especially in Pakistan. SFMT and CMT are usually used to detect subclinical mastitis in dairy buffaloes since both methods are cheaper, reliable, and can be used in the field. In Asia, the main risk factors of bubaline mastitis are caused by farm management, including improper milking handling and host factors such as lactation stage, age, and quarters position. Farmers can be educated on proper milking

Table 6
Antimicrobial susceptibility of causative pathogens of bubaline mastitis in Asia

Countries	Isolated bacteria	Antimicrobial susceptibility	Number of antibiotics used	Methodology	References
India	*Streptococcus agalactiae*	Resistant: Penicillin	8 antibiotics	Disk diffusion method	Das and Joseph (2005)
	Klebsiella pneumonie, *Pseudomonas aeruginosa*	Resistant: Penicillin and erythromycin			
	Staphylococcus spp. (CPS and CNS), *Corynebacterium bovis*, *Corynebacterium pyogenes*, *E. coli*, *Bacillus cereus*	Susceptible: Ciprofloaxacin, penicillin, chloramphenicol, gentamycin, erythromycin, kanamycin, ampicillin, and streptomycin			
	Staphylococcus spp.	Susceptible: Ampicillin, cloxacillin, amoxycillin, ceftriaxone, cefoperazone, penicillin, chloramphenicol, tylosin, and enrofloxacin	14 antibiotics	Disk diffusion method	Charaya et al. (2013)
	Streptococcus spp.	Susceptibility: Chloramphenicol, ampicillin, enrofloxacin, amoxycillin, ceftriaxone, and cefoperazone			
	Staphylococcus aureus	Disk diffusion method: Resistant: Penicillin, methicillin, amoxycillin, ceftriaxone, amoxycillin + clavulanic acid, and ceftriaxone + tazobactum	6 antibiotics (disk diffusion method), 3 antibiotics (microdilution assay)	Disk diffusion method, microdilution assay	Sagi (2014)
		Microdilution assay: Resistant: Amoxycillin and amoxycillin + clavulanic acid microdilution assay Susceptible: Amoxycillin + clavulanic acid			

Table 6 *(continue)*

Countries	Isolated bacteria	Antimicrobial susceptibility	Number of antibiotics used	Methodology	References
	Staphylococcus aureus	Resistant: Penicillin, vancomycin, and nalidixic acid Intermediate: Cefixime, methicillin, novobiocin, amoxiclav, colistin, pipemidic acid, ofloxacin, streptomycin, sulphamethizole, ampicillin/sulbacta, cefalexin, cefazolin, cefoperazone, enrofloxacin, floxidin, and meropenem Susceptible: Cefuroxim, ciprofloxacin, clindamycin, gentamicin, levofloxacin, norfloxacin, and tetracycline	38 antibiotics	Disk diffusion method	Nigam (2015)
	CNS	Resistant: Methicillin, amoxycillin/sulbactam, and penicillin G Intermediate: Ceftriaxone/sulbactam, cefoxitin, and cefotaxime Susceptible: Co-trimoxazole, chloramphenicol, and gentamicin	15 antibiotics	Disk diffusion method	Preethirani et al. (2015)
	Staphylococcus aureus	Resistant: Cefoxitin, penicillin G, and ceftriaxone/sulbactam Intermediate: Enrofloxacin Susceptible: Co-trimoxazole and oxacillin			

Table 6 *(continue)*

Countries	Isolated bacteria	Antimicrobial susceptibility	Number of antibiotics used	Methodology	References
	Streptococcus spp.	Resistant: Methicillin, streptomycin, cefoxitin, and penicillin G Intermediate: Gentamicin and ceftriaxone Susceptible: Chloramphenicol and Oxacillin			
	Escherichia coli	Resistant: Amikacin, amoxycillin/sulbactam, ampicillin, cefotaxime, cefoxitin, ceftriaxone/sulbacta, methicillin, penicillin G, and streptomycin Intermediate: Enrofloxacin Susceptible: Cotrimazole and chloramphenicol			
	Staphylococcus aureus	Susceptible: Gentamicin and streptopenicillin	12 antibiotics	Disk diffusion method	Maurya and Joshi (2021)
	Escherichia coli	Susceptible: Tetracycline, gentamicin, enrofloxacin, streptopenicillin, ceftriaxone + sulbactum, streptomycin, ceftriaxone, and methicillin			
Bangladesh	*Staphylococcus* spp., *Streptococcus* spp., *Bacillus* spp., *E. coli*	Resistant: Ampicillin, Amoxycillin and streptomycin High and intermediate susceptible: Gentamicin, ciprofloxacin, Endrofloxacin and chloramphenicol	7 antibiotics	Disk diffusion method	Kisku and Samad (2013)

Table 6 *(continue)*

Countries	Isolated bacteria	Antimicrobial susceptibility	Number of antibiotics used	Methodology	References
	All isolated bacteria	Resistant: Amoxicillin Susceptible: Chloramphenicol and ciprofloxac	10 antibiotics	Disk diffusion method	Talukder et al. (2013)
	Staphylococcus spp.	Resistant: Amoxicillin, erythromycin, and azithromycin Susceptible: Ceftriaxone, trimethoprim, chloramphenicol, gentamycin, levofloxacin, and amoxicillin			
	Escherichia coli	Resistant: Ceftriaxone, trimethoprim, Azithromycin and nitrofurantoine Susceptible: Ciprofloxacin, azithromycin, trimethoprim, chloramphenicol, and levofloxacin			
	Staphylococcus aureus, *E. coli*, *Streptococcus* spp., *Bacillus* spp.	Resistant: Penicillin, amoxicillin, cefoxitin, and tetracycline Intermediate: Ceftriaxone, enrofloxacin, vancomycin, and erythromycin Susceptible: Gentamicin and ciprofloxacin	12 antibiotics	Disk diffusion method	Biswas et al. (2020)
Iraq	*Escherichia coli*	Resistant: Cefotaxime and ampicillin Susceptible: Tetracycline, amikacin, trimethoprim/sulfamethaxol, gentamicin, and ciprofloxacin	7 antibiotics	Disk diffusion method	Wahid et al. (2017)

Table 6 *(continue)*

Countries	Isolated bacteria	Antimicrobial susceptibility	Number of antibiotics used	Methodology	References
Nepal	*Escherichia coli*	Resistant: Ceftriaxone and ciprofloxacin	-	Disk diffusion method	Bhandari et al. (2021)
Pakistan	All isolated bacteria	Resistant: Ampicillin, streptomycine, chloramphenicol, penicillin, and amoxicillin Susceptible: Ciprofloxacin Sensitive: Gentamicin and norfloxacine	8 antibiotics	Disk diffusion method	Mustafa et al. (2013)
	Staphylococcus aureus	Susceptible: Co-trimaxazole, oxytetracycline, ciprofloxacin, chloramphenicol, amoxicillin, gentamycin, ampicillin, novobiocin, and enrofloxacin	9 antibiotics	Disk diffusion method	A. Hussain et al. (2013)
	Staphylococcus aureus	Susceptible: Trimethoprim, erythromycin, ciprofloxacin, doxycycline, streptomycin, gentamycin, tylosin, kanamycin, amoxicillin, and chloramphenicol	10 antibiotics	Disk diffusion method	A. Hussain et al. (2020)
Philippines	*Staphylococcus aureus*	Resistant: Penicillin Intermediate: Erythromycin Susceptible: Chloramphenicol, clindamycin, trimethoprim-sulfamethoxazole, tetracycline, rifampicin, ciprofloxacin, and gentamycin	9 antibiotics	Disk diffusion method	Badua et al. (2020)

practices and farm management to help reduce the prevalence of bubaline mastitis. Mastitis in buffaloes causes changes in milk composition, such as increased lactose levels, SCC, and bacteria in the milk. However, protein, fat, and solid non-fat level variations were also affected by other factors such as the stage of lactation, breed, and age. The most prevalent bacteria in bubaline mastitis milk samples were CNS, *S. aureus*, and *Streptococcus* spp., including *S. agalactiae* and *S. uberis*, and *E. coli* were detected using bacterial isolation and identification and PCR. Penicillin, amoxicillin, ampicillin, and streptomycin are highly resistant to the isolated bacteria, according to previous studies, but not cefoxitin for Gram-positive bacteria or ceftriaxone for Gram-negative bacteria. However, the antimicrobial susceptibility of causative agents in Asia varies depending on the usage of common antibiotics to treat bubaline mastitis in each country. This review will help better understand bubaline mastitis, although studies are limited in Asia except for Pakistan and India, which both have the most buffalo and produce the highest amount of buffalo milk. However, Southeast Asian countries, except Thailand, have conducted no studies on bubaline mastitis. More studies on the epidemiology, diagnostic tests, milk composition changes, and antimicrobial susceptibility of causative agents of bubaline mastitis in Asian countries are needed to fill the knowledge gap in the future.

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Revisiting *In Vitro* Micropropagation Protocols of *Mimosa pudica* for Enhanced Seed Germination, Shoot Multiplication, and Root Initiation

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ABSTRACT

Mimosa pudica is a medicinal plant worthy of therapeutic properties. It is often overlooked as one of the weed species, and the potential was underappreciated despite its abundance in nature, particularly in tropical climate countries. Considering the aptitude of this species, the micropropagation protocol of *M. pudica* was revisited and enhanced. The seed surface sterilization and germination were assessed, followed by shoot multiplication rate and root initiation efficiency. Seeds of *M. pudica* were best surface sterilized with 35% of Clorox and recorded the highest germination rate at 65.55% in media of three-quarter strength Murashige and Skoog (MS) combined with 30 g of sucrose. The explants were harvested and subcultured into multiplication media fortified with 6-benzylaminopurine, kinetin, and coconut water. The highest number of new shoots and leaves were obtained at 3 shoots and 3.27 cm in media of MS combined with 17.6 µm kinetin. Half-strength MS media devoid of hormone was recommended for root induction, resulting in the 5 healthy roots at 2.06 cm after 4 weeks of culture. Overall, the developed

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micropropagation protocol of *M. pudica* could facilitate its large-scale cultivation, indicating its potential as a medicinal crop for the extraction of bioactive compounds.

Keywords: Medicinal weed, micropropagation, *Mimosa pudica*

INTRODUCTION

Mimosa pudica is a medicinal plant known for its sensitive trait in which its leaves fold inward and droop as a form of defensive response. In previous reports, *M. pudica* has been demonstrated to possess promising antioxidant and antidiabetic properties (Baharuddin et al., 2021). The significant amount of phenols and flavonoids in *M. pudica* extract, especially when extracted using optimal solvent extraction techniques, has positioned it as the next valuable medicinal plant species to be regenerated *in vitro*.

Among many advantages of *in vitro*-cultured plants is the ability to synthesize and accumulate many of the same important bioactive compounds as the parent plant in nature (Baskaran et al., 2014). It also enables biosynthetic pathways to be manipulated to boost the synthesis and accumulation of compounds of interest (Dias et al., 2016). The most significant aspect is the capacity to produce a continuous, sustainable, affordable, and viable synthesis of natural substances within a controlled microenvironment (Anand, 2010). However, studies on the protocols of *in vitro* micropropagation of *M. pudica* are limited (Hassan et al., 2010; Ramesh et al., 2013). In general, the medium composition is one of the many factors that influence the success of an *in vitro* micropropagation protocol. The result from the most recent micropropagation study of *M. pudica* by Bianchetti et al. (2017) in the germination part was not reproducible in this present study. To investigate this issue, other germination media components not addressed in the previous study, such as the type and strength of the basal medium and sucrose concentration, were assessed to establish a reproducible *in vitro* germination media for *M. pudica* seeds. Also, an organic additive was tested in the shoot multiplication test, and 2,4-dichlorophenoxyacetic acid (2,4-D) was used in the rooting part of this experiment to fill in the gap in the previous studies.

Therefore, the present study aimed to develop reliable micropropagation protocols for the *in vitro* seed germination, shoot multiplication, and root initiation of *M. pudica*. Through a series of experiments, the effects of media composition were determined: (1) effects of basal medium type, basal medium strengths, and sucrose concentration in response to seed germination, (2) effects of different types of cytokinins and organic additive in response to the shoot multiplication, and (3) effects of Murashige and Skoog (MS) basal medium strength and 2,4-D in response to root initiation of *M. pudica* plantlets.

MATERIALS AND METHODS

Surface Sterilization, Seed Germination, and Culture Initiation

Seeds of *M. pudica* were obtained from a single source from Beijing, China, to

ensure uniformity of origin. The seeds were put in a conical flask and washed under running tap water, followed by a Decon 90 (Decon, United Kingdom) wash. The seeds were treated with fungicide (Ancom Thiram 80, Malaysia) containing several drops of Tween-20 (Merck Schuchardt OHG, Germany) for 30 min on a rotary shaker before being rinsed with distilled water. Next, the seeds were transferred to a laminar airflow chamber (BIOBASE, BBS-V800, China) and soaked in 70% ethanol (Systerm Chemicals, Malaysia) for 1 min. Subsequently, the seeds were treated with three different treatments of 35, 70, and 100% Clorox© (contains 5% sodium hypochlorite, NaOCl (Systerm Chemicals, Malaysia) as an active ingredient) for 15 min (Table 1). In each treatment, 100 seeds were used with three replicates. The surface-sterilized seeds were rinsed with sterile distilled water five times before culturing them into media containing MS basal medium. The rate of contamination and germination was recorded after 14 days of culture using equations 1 and 2 (Ahmadi et al., 2012). The appropriate concentration of Clorox© was selected for the subsequent seed germination experiment.

Table 1

Mimosa pudica *seed sterilization treatment steps*

Treatment no.	Sterilizing agents with exposure time			
	Step 1 (30 min)	Step 2 (30 min)	Step 3 (1 min)	Step 4 (15 min)
1	Decon 90 + Teepol	Thiram + Tween 20	70% ethanol	35% Clorox
2				70% Clorox
3				100% Clorox

Equation 1:
Contamination percentage (%) = (Number of seeds with microbial growth/Total number of seeds) × 100%

Equation 2:
Germination percentage (%) = (Number of grown seeds/Total number of seeds) × 100%

To assess the effects of media composition on seed germination of *M. pudica*, the surface-sterilized seeds were cultured into two different basal mediums (Murashige and Skoog, MS and Gamborg, B5) at different basal medium strengths (half, three-quarter, and full) with different sucrose (MSM Holdings Berhad, Malaysia) concentrations (15, 30, 45 g/L). The media used were adjusted to a pH of 5.7 using sodium hydroxide (NaOH) (Systerm Chemicals, Malaysia) or hydrochloric acid (HCl) (Systerm Chemicals, Malaysia) and solidified with 3% agar (Gelrite, Sigma Aldrich, USA) before autoclaving at 121°C and 103 kPa for 20 min. The flasks were then sealed with parafilm M® (Bemis Company, USA), and the cultures were incubated in a culture room at 25 ± 2°C, illuminated by white fluorescent light at an intensity of 3,000 lux (OSRAM Licht AG, Germany),

with a photoperiod of 16 hr light and 8 hr darkness. The same condition of the culture room was used for subsequent *in vitro* experiments. Cultures were checked routinely for any contamination. The seed germination percentage, number of shoots, and shoot length were recorded on the 30th day of culture.

Shoot Multiplication of *M. pudica*

To determine the effects of different types of cytokinin, i.e., 6-benzylaminopurine (BAP) and kinetin (KIN), and coconut water (CW) to shoot multiplication of *M. pudica*, sterile nodal segments of 2.0 – 3.0 cm obtained from culture initiation with decapitated leaves were vertically cultured on MS containing 3% sucrose and 3 g/L of agar with varying concentration of BAP and KIN (2.2, 4.4, 8.8, 17.6, and 35.2 µM) and CW (10, 20, 30, 40, and 50% v/v). CW was acquired from fresh coconuts sold by a local vendor. MS was the control group without adding plant growth hormone or organic additives. The number of new shoots, shoot length, and morphology were visually assessed and recorded after 30 days of culture.

Root Initiation of *M. pudica*

To evaluate the effects of MS basal medium strength and auxin concentration, which was 2,4-D to root initiation of *M. pudica*, sterile plantlet clusters of about 4 cm having at least 4 shoots were excised and cultured into rooting media treatment with different MS strength (half and full) and varying 2,4-D concentrations (5.33 and 10.66 µM). Days to root emergence, number of roots, root length, and morphology were recorded after 30 days of culture.

Statistical Analysis

All the experimental data were given as mean ± standard error of the mean and submitted to analysis of variance (ANOVA) to detect significant differences between means. Means differing significantly were compared using the Tukey post hoc test (p<0.05) using the statistical software program SPSS (version 23).

RESULTS AND DISCUSSION

Effect of Different Sterilization Treatments on Contamination and Seed Germination Rate

Surface sterilization of seeds is a crucial step in plant tissue culture since it is necessary not only to disinfect seeds from seed-borne microorganisms but also to ensure they can grow into healthy plantlets. Failure to optimize this step will result in microorganisms competing for nutrients with the growing explants (Tiwari et al., 2012). Furthermore, this procedure is required to eliminate or destroy contaminants while preserving the biological activity of the explants (Felek et al., 2015).

Clorox is a commercial bleach commonly used in sterilization treatments with 5% NaOCl as its active ingredient in an undiluted solution. In the control group (0% Clorox), contamination recorded was 100%, observed in each culture, and the germination rate was 9.67% (Figures 1A and 1B). Furthermore, the 35% Clorox treatment indicated 13.66 and 41.33% contamination and germination rates,

respectively. Contrariwise, the 70% Clorox treatment resulted in a marginally lower contamination rate of 12.33% with a significantly reduced seed germination rate of 27.33% (p<0.05). In 100% Clorox, zero contamination was observed, resulting in extremely few germinated seeds. The morphology and survival of explants are known to be affected by sterilizing agent dosages and exposure time. Explants must be disinfected for a specific amount of time and at a specific concentration, as overdosing might result in plant tissue death (Oyebanji et al., 2009). Even though there was no contamination, 100% Clorox was shown to be ineffective for seed surface sterilization of *M. pudina* since it dried out the seeds (Figure 1C) and impaired their propensity to germinate.

The same pattern was observed in the surface sterilization of *Zea mays* seeds, where the combination of 70% ethanol and 10% NaOCl was agreed to be the most effective treatment in eliminating seeds-associated microbes. It resulted in the highest number of germinated seeds with the lowest contamination rate, as compared to the treatments with 10% hydrogen peroxide (H_2O_2) or 10% sodium hypochloride (NaOCl) (Davoudpour et al., 2020). Similarly, using 4% NaOCl to surface-sterilize the seeds of *Althaea officinalis* showed the lowest contamination rate compared to using mercuric chloride. The germination percentage recorded was also comparable to the treatment that resulted in the highest germination rate (Younesikelaki et al., 2016).

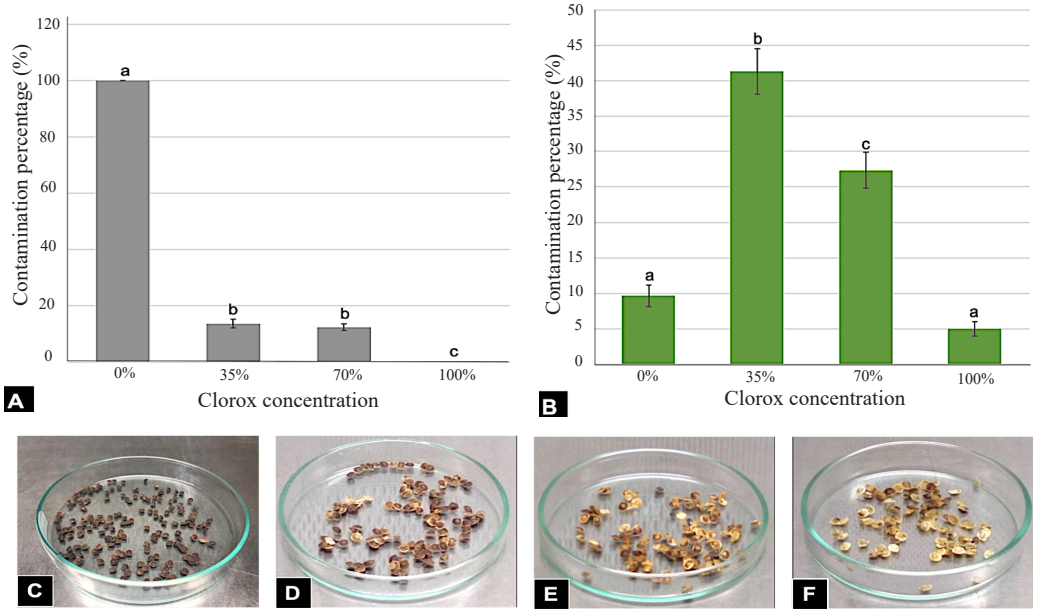

Figure 1. A) Contamination rate of *Mimosa pudica* after different sterilization treatments; B) Germination rate of *M. pudica* after different sterilization treatments. Values are expressed as mean ± standard error (n = 3). Different letters indicate statistically significant differences between factors (one-way ANOVA + Tukey post-hoc test at p<0.05); C-F) Seeds of *M. pudica* after surface sterilization with different concentrations of Clorox ranging from 0, 35, 70, and 100%, respectively

Seed Germination of *M. pudica* Seeds and Plantlets Development in Response to Different Medium Composition

Seed germination was recorded starting from day five after inoculation in germination media as the hypocotyls emerged (Figure 2A). Following that, the dicotyledons appeared (Figure 2B), the first true leaves were formed (Figure 2C), and the shoot was established (Figure 2D). The most recent micropropagation study of *M. pudica* by Bianchetti et al. (2017) incorporated a full-strength MS basal medium and recorded 87% germination. In this study, other germination media parameters, such as type and strength of the basal medium and sucrose concentration, need to be assessed to establish a reproducible *in vitro* germination media for *M. pudica* seeds, which were not addressed in the previous study.

Therefore, different media compositions were incorporated in the basal medium, MS and B5, with varied concentrations (i.e., ½, ¾, and full), including sucrose with concentrations ranging from 15, 30, to 45 g/L. The overall results (Table 2) demonstrated that the ½ MS + 15 g/L sucrose recorded the highest percentage of germinated seeds (68.85%), the ¾ MS + 30 g/L sucrose recorded the highest number of new shoots (3.22 cm), and full MS + 15 g/L sucrose recorded the longest shoot length (3.78 cm). In contrast, the full B5 + 45 g/L sucrose recorded the lowest values for all three responses. Therefore, based on the results, the ¾ MS + 30 g/L sucrose was selected as germination media for plantlet production for the subsequent study shoot multiplication study.

All three factors demonstrated statistically significant differences (p<0.05) in seed germination percentage. However, only the basal medium type was found significant to the number of new shoots

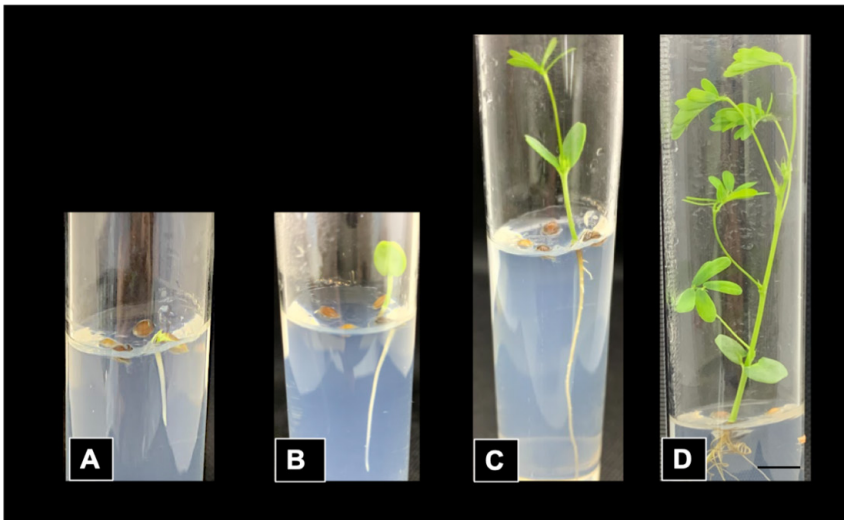

Figure 2. Culture initiation of *Mimosa pudica* seed. A) Emergence of hypocotyls; B) Emergence of dicotyledons; C) Formation of first true leaves; D) Formation of shoots

Note. Bar = 1.0 cm

formed and the shoot length. In terms of interactions between the factors, overall interactions were significant to germinated seeds percentage. Nevertheless, the factors' interactions had no notable effect on the number of shoots. The same applies to the length of the shoot, except that basal medium strength and sucrose were the only significant interaction factors (Table 3).

Table 2

The effects of the type and concentration of the basal medium and the sucrose concentration in the germination media on the percentage of seed germination, number of shoots, and shoot length of M. pudica

Basal medium	Basal medium strength	Sucrose concentration (g/L)	Germinated seeds (%)	Number of shoots	Shoot length (cm)
-	Control	-	$50.00 \pm 5.6^{b,c}$	2.78 ± 0^{a}	1.18 ± 0.2^{b}
MS	1/2	15	68.85 ± 4.5^{a}	2.22 ± 1.4^{a}	3.09 ± 1.3^{a}
		30	$52.20 \pm 1.1^{a,b,c}$	2.11 ± 0.7^{a}	$2.86 \pm 1.3^{a,b}$
		45	$36.70 \pm 1.1^{c,d}$	2.11 ± 0.7^{a}	$3.36 \pm 0.7^{a,b}$
	3/4	15	47.75 ± 5.6^{c}	2.61 ± 0.7^{a}	$2.88 \pm 0.8^{a,b}$
		30	$65.55 \pm 3.3^{a,b}$	3.22 ± 1.4^{a}	3.38 ± 0.6^{a}
		45	47.80 ± 1.1^{c}	2.33 ± 0.7^{a}	$3.68 \pm 1.1^{a,b}$
	Full (1)	15	$44.45 \pm 2.3^{c,d}$	3.11 ± 1.4^{a}	$3.78 \pm 0.2^{a,b}$
		30	$40.00 \pm 2.2^{c,d}$	2.67 ± 0.7^{a}	$3.16 \pm 0.4^{a,b}$
		45	$38.9 \pm 3.3^{c,d}$	2.11 ± 0.7^{a}	3.04 ± 0.9^{a}
B5	1/2	15	$44.4 \pm 2.2^{b,c}$	2.44 ± 0.8^{a}	$2.90 \pm 0.6^{a,b}$
		30	$36.67 \pm 5.6^{c,d}$	2.11 ± 0.7^{a}	$3.19 \pm 0.4^{a,b}$
		45	42.22 ± 0^{d}	1.78 ± 0.8^{a}	$1.92 \pm 0.7^{a,b}$
	3/4	15	28.89 ± 2.2^{d}	1.67 ± 0.6^{a}	$2.10 \pm 0.8^{a,b}$
		30	$27.78 \pm 1.1^{c,d}$	1.44 ± 0.8^{a}	$2.58 \pm 0.9^{a,b}$
		45	$43.33 \pm 1.1^{c,d}$	2.22 ± 1.3^{a}	3.38 ± 1.1^{a}
	Full (1)	15	$51.11 \pm 2.2^{c,d}$	2.67 ± 1.2^{a}	$3.04 \pm 0.6^{a,b}$
		30	$42.22 \pm 2.2^{c,d}$	2.11 ± 1.1^{a}	$2.59 \pm 1.1^{a,b}$
		45	$27.78 \pm 1.1^{c,d}$	1.44 ± 1.3^{a}	$2.08 \pm 1.4^{a,b}$

Note. Data were collected and expressed as the mean ± standard error after 30 days of culture of three replicates, in which each replicate contains three explants. The data were tested for statistical differences by three-way ANOVA followed by the Tukey post hoc test. The criterion for significance was set at $p<0.05$. Means that do not share a letter are significantly different

MS = Murashige and Skoog
B5 = Gamborg B5

Table 3

The p *value of interactions between factors affecting seed germination of* M. pudica

Factor's interaction	Response	*p* value
Basal medium type * Basal medium strength	Germinated seeds	0.000*
Basal medium type * Sucrose		0.003*
Basal medium strength * Sucrose		0.000*
Basal medium type * Basal medium strength * Sucrose		0.009*
Basal medium type * Basal medium strength	Number of shoots	0.219
Basal medium type * Sucrose		0.697
Basal medium strength * Sucrose		0.458
Basal medium type * Basal medium strength * Sucrose		0.557
Basal medium type * Basal medium strength	Length of shoots	0.787
Basal medium type * Sucrose		0.495
Basal medium strength * Sucrose		0.021*
Basal medium type * Basal medium strength * Sucrose		0.368

Note. Value with * is significant ($p<0.05$)

MS and B5 have their properties and potential effects on *in vitro* plantlets. Generally, the most notable difference between MS and B5 media is the decreased ammonium/nitrate (NH_4^+ / NO_3^-) ratio in the B5 medium (Russowski et al., 2006). The saturated nutritional content of full-strength MS might be inappropriate for the small, immature seeds of *M. pudica*, which hinders germination and healthy growth of the plantlets. On the other hand, the low inorganic nutrients of B5 (e.g., calcium and magnesium) might cause nutrient deficiency, thus compromising the germination and growth of seedlings (Li & Zhang, 2018). MS contains high inorganic salts (e.g., nitrate, potassium, and ammonium), facilitating plant nutrient uptake and development (Koné et al., 2015). This study supports evidence from previous observations on *Dendrobium hookerianum* seeds (Paul et al., 2012), where the highest percentage of germination and plantlets growth was recorded in those of MS medium. Similar to the case of embryo germination of *Juglans regia*, the MS medium demonstrated outstanding proliferation from the embryonic axis compared to the other media (Sánchez-Zamora et al., 2006). According to these data, it can be generally inferred that MS basal medium is more suitable than B5 for micropropagation of *M. pudica*.

Regarding the MS basal medium strength, the results confirmed that high MS strength was unnecessary to improve overall seed germination. This observation might be attributed to the increased quantity of a certain nutritional salt, which inhibits the plant's ability to absorb other mineral nutrients. The ½ MS resulted in the highest

germination percentage, whereas full MS resulted in a 24% lower germination rate. The finding is inconsistent with those observed on *Vanilla planifolia* by Jing (2016), in which seed germination performed better in a full-strength MS. Nevertheless, fewer shoots were recorded in the same media at 2.22 shoots per explant and a reduced shoot length at 3.09 cm. This result indicated that a low macro- and micronutrient concentration in the culture media might promote germination but not plantlet development. No significant difference was detected between the different strengths of MS in response to the number of new shoots of each plantlet and the shoot length, which exhibited a similar trend to a study on *Vigna subterranea* by Koné et al. (2015). Thus, the ¾ strength or 3.3 g/L of MS is sufficient for germinating *M. pudica* seeds.

Plantlets grown in culture vessels are called semi-autotrophic plants since their leaves may not acquire photosynthetic competency due to a lack of carbon dioxide (Hazarika, 2003). Therefore, a carbohydrate source like sucrose is commonly added to provide a sufficient supply of carbon for *in vitro* multiplication and growth of plant cells, tissues, organs, and entire plantlets. Carbon sources are also required as osmotic agents in the culture medium, which can alter cell physiology, proliferation, and differentiation (Gibson, 2000). Different carbohydrate sources can affect the development of plantlets in different ways. Improper type and concentration selection could cause morphogenesis to be delayed and physiological diseases like vitrification to develop. Sucrose has been widely used as a carbon source in most studies involving *in vitro* shoot induction and development, root induction, callogenesis, embryogenesis, and regeneration (Yaseen et al., 2012). Sucrose is a significant carbon and energy source in plant tissue culture since it is the most frequent carbohydrate in phloem sap and regulates many development stages (Zahara et al., 2016). The high solubility in water, electrical neutrality, and lack of inhibitory effect on most metabolic processes are all linked to sucrose's favorable effects on explant growth *in vitro* (Liu et al., 2006). In this study, sucrose concentration was found significant to the percentage of germinated seeds of *M. pudica*, although it did not significantly affect the plantlets' development in terms of the number of new shoots and length. The highest seed germination percentage was observed from the ½ MS + 15 g/L of sucrose, while higher sucrose concentrations up to 45 g/L resulted in a lower germination rate. At high sucrose levels, seed germination and plantlet development were stunted, implying that the seeds were either subjected to osmotic stress at greater molarities or that sucrose hydrolysis products produced during autoclaving hampered their growth and development (Johnson et al., 2011).

Koné et al. (2015) reported that the plant height and biomass of *Bambara* groundnuts were increased when the sucrose content was increased from 1 to 3%. However, there was no significant variation in the number of leaves and root length among the different

sucrose concentrations examined. Studies on *Ruscus hypoglossum* and *Cornus alba* also found similar results (Ilczuk & Jacygrad, 2016). A high sucrose concentration also hindered orchid plantlets' growth, particularly the shoot and root numbers (Wotavová-Novotná et al., 2007). In *Drosera intermedia*, maximum shoot length and shoot number were reported on liquid medium containing 10 and 20 g/L sucrose, respectively (Rejthar et al., 2014). On the contrary, *Abelmoschus esculentus* did not thrive in a medium containing 10 and 15 g/L sucrose, where the explants displayed shoot tip necrosis and could not produce leaves. This finding indicated that lowering sucrose content influenced cellular division directly owing to a lack of carbon supply and energy, resulting in poor shoot regeneration and growth (Woldeyes et al., 2021). The contrasting difference is due to the variation in genotype, growth regulators, and culture conditions. In the case of *M. pudica* seeds, 3% sucrose concentration in the basal medium, equivalent to 30 g/L, is sufficient for normal plant growth.

Shoot Multiplication of *M. pudica* in Response to Different Plant Growth Hormone and Organic Additives at Different Concentrations

Nodal segments of *M. pudica* obtained from germinated seeds cultured in germination media were inoculated in media of MS basal medium supplemented with different concentrations of exogenous cytokinin hormones (i.e., BAP and KIN) and organic additive CW. The results revealed that plant growth hormone or additive types and the interaction with the concentration had statistically significant differences ($p<0.05$) to shoot multiplication, determined by shoot number and shoot length. However, the difference in plant growth hormone or additive concentration alone was significant only to shoot number but not to the shoot length. Based on Table 4, media supplemented with 35.2 µM KIN exhibited the highest shoot number formed (4.87), which was two-fold higher as compared to MSO (control). MSO is Murashige and Skoog only with no plant growth regulator added. All concentrations of CW treatments and 17.6 µM and 35.2 µM BAP treatments recorded the lowest shoot number of below 2. Treatments with 2.2 µM BAP, 35.2 µM BAP and 35.2 µM KIN produced more than four shoots per explant. While all CW treatments yielded the longest shoot length of over 3 cm, they produced a relatively small number of shoots. The shortest shoot of 0.32 cm was exhibited by 35.2 µM BAP.

Leaves production and basal callus formation were visually assessed and scored as – (absent), + (low), ++ (moderate), and +++ (high). MSO (control) and 17.6 µM BAP produced moderate leaves (++). A low concentration of BAP resulted in shoots with leaves, but leaves were not produced at a concentration of 17.6 µM and above. As for KIN, at 17.6 µM, leaves produced were twice as produced in BAP and coconut water, but at other concentrations, leaves were minimal (+) or absent (-). CW, at all concentrations, consistently produced

Table 4

Effects of types of plant growth regulators (PGR), additive, and their concentrations on shoot multiplication of M. pudica

Plant growth regulator	Concentration	Number of shoots	Shoot length (cm)	Leaves	Initiated callus
MSO (Control)	-	$2.33 \pm 1.4^{a,b,c,d,e}$	$2.89 \pm 2.0^{a,b,c}$	++	-
BAP (µM)	2.2	$4.07 \pm 0.96^{a,b}$	$3.06 \pm 0.4^{a,b,c}$	+	++
	4.4	$2.87 \pm 1.6^{b,c,d}$	$2.17 \pm 1.2^{b,c}$	+	++
	8.8	$3.8 \pm 2.3^{a,b}$	$3.2 \pm 1.4^{a,b,c}$	+	-
	17.6	$1.67 \pm 1.0^{d,e}$	2.07 ± 0.9^{c}	-	-
	35.2	0.07 ± 0.3^{e}	0.32 ± 1.2^{d}	-	-
KIN (µM)	2.2	$4.0 \pm 1.1^{a,b}$	$3.18 \pm 0.9^{a,b,c}$	+	+
	4.4	$3.53 \pm 1.3^{a,b,c}$	$3.09 \pm 1.0^{a,b,c}$	-	++
	8.8	$3.93 \pm 2.1^{a,b}$	$2.79 \pm 1.0^{a,b,c}$	+	-
	17.6	$3.6 \pm 2.3^{a,b,c}$	$3.27 \pm 1.0^{a,b,c}$	++	+
	35.2	4.87 ± 1.2^{a}	$3.65 \pm 0.9^{a,b}$	-	+
CW (%)	10%	$1.27 \pm 0.7^{d,e}$	$3.43 \pm 1.1^{a,b,c}$	+	-
	20%	$1.27 \pm 1.0^{d,e}$	3.8 ± 1.8^{a}	+	-
	30%	$1.73 \pm 0.8^{d,e}$	3.98 ± 1.5^{a}	+	-
	40%	$1.93 \pm 0.7^{c,d}$	$3.1 \pm 1.6^{a,b,c}$	+	-
	50%	$1.67 \pm 0.6^{d,e}$	4.2 ± 0.8^{a}	+	-

Note. Data were collected and expressed as the mean ± standard error after 30 days of culture. The data were tested for statistical differences by two-way ANOVA followed by the Tukey post hoc test. The criterion for significance was set at $p<0.05$. Means that do not share a letter are significantly different. Leaves and basal callus rating: – (absent), + (low), ++ (moderate), and +++ (high)

MSO = Murashige and Skoog without plant growth regulator
BAP = 6-benzylaminopurine
KIN = kinetin
CW = coconut water

shoots with a small number of leaves. Basal callus formation was observed in plantlets treated with 2.2 - 4.4 µM BAP (Figure 3C) and all concentrations of KIN except for one at 8.8 µM while MSO (control) and CW exhibited zero basal callus. The basal callus is a common issue in most plants during shoot induction; however, the severity varies by species. It was most likely caused by a disruption in endogenous hormones in the tissue, as a common physiological stress reaction. In several cases, callus is formed due to the accumulation of endogenous auxin at the wounded parts of the plants (Fehér, 2019). Above all, MS basal medium fortified with 17.6 µM KIN (Figure 3A) was selected as the best media for shoot multiplication of *M. pudica* due to the satisfactory shoot number and shoot length, a moderate amount of leaves with low basal callus formation.

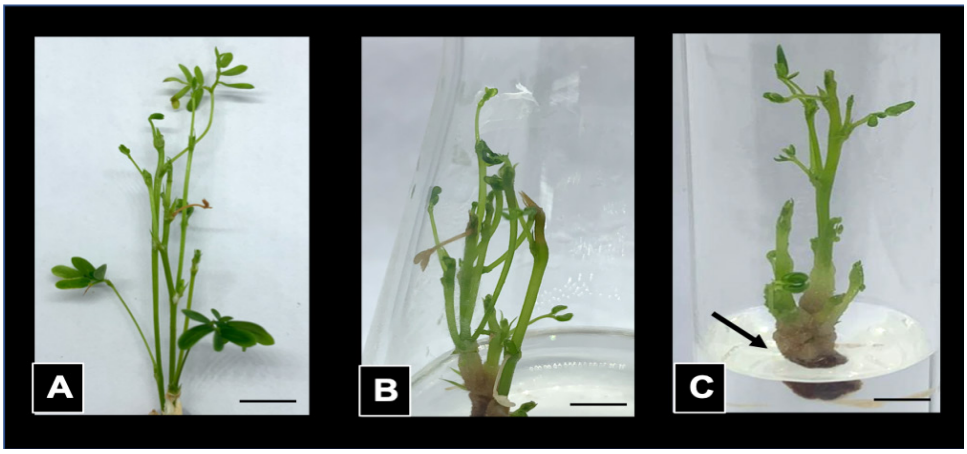

Figure 3. Shoot multiplication of *Mimosa pudica*. A) Shoot multiplication in media fortified with 17.6 µM KIN; B) Shoot multiplication in media fortified with 35.2 µM KIN; C) Shoot multiplication in media fortified with 4.4 µM BAP, the arrow pointing to formed basal callus

Note. Bar = 1.0 cm
KIN = kinetin
BAP = 6-benzylaminopurine

Consistent with the previous observation by Bianchetti et al. (2017), the highest number of shoots recorded in 5 µM BAP was 5 shoots/explant, which was just marginally higher than those in the current study (4.87 shoots/explant in 35.2 µM KIN). It was also reported that increasing BAP concentration hindered shoot number and elongation, with plantlets reaching an average height of 2.5 cm, which was lower than the shoot length achieved in this study, which was over 3 cm. On the contrary, they found that supplementing *M. pudica* shoots with KIN did not significantly increase their proliferation with no significant differences compared to the control. In addition, supplementing with 1-naphthaleneacetic acid (NAA) and BAP did not affect shoot elongation, demonstrating a negative relationship between multiplication rate and shoot length.

The cytokinin signaling system involves coordinating three types of proteins: histidine kinase receptors for signal perception, histidine phosphotransfer proteins for the signal relay, and response regulators for signal output (Keshishian & Rashotte, 2015). When the signal is relayed to the nucleus, it will be conveyed to two types of gene expression regulators that govern the plant's developmental stages. The signal receptor perceives different types of cytokinin differently, which explains the differences in plant response even though it comes from the same group of PGR (Schepetilnikov & Ryabova, 2017). Plant growth hormone supplementation in cultures causes the transcription of specific genes that modulate each stage of plant growth. When the groups of genes responsible for a particular plant growth process are identified, the media culture can

be better optimized, promoting the defined hormones with their effective concentration to influence gene expression throughout the development process (Jiang & Asami, 2018).

Cytokinin is commonly used in the induction and proliferation stages of explants. Aside from PGR, organic additives like CW, apple juice, tomato juice, and peptone may help plant tissues grow and develop (Gupta, 2016; Kaur & Bhutani, 2016; Prando et al., 2014). CW contains sugars, amino acids, vitamins, enzymes, and organic acids, as well as endogenous cytokinin that promotes explant development and regeneration by stimulating cell division (Ge et al., 2005). When plant portions are excised into smaller sizes, the production of cytokinin in plants is restricted owing to the damage (Gallavotti, 2013). Therefore, in the present study, cytokinin was added to the basal media to stimulate shoot induction, cell division, and cell differentiation. In the case of *M. pudica* plantlets, this study revealed that KIN was the most suitable cytokinin option for its shoot multiplication and proliferation.

On the contrary, previous research on *Justicia gendarussa* found that supplementing BAP in MS basal medium resulted in the highest percentage of shoot induction, the number of shoots, and shoot length compared to KIN and thidiazuron (TDZ) (Janarthanam et al., 2011). The same finding was observed in *Clinacanthus nutans*, in which 12 µM BAP was more effective than the other cytokinins in the early stages of shoot induction (Haida et al., 2020). Moreover, comparable research on *Clitoria ternatea* and *Pisum sativum*, which belong to the same family as *M. pudica*, reported that the proportion of shoot regeneration and the number of shoots increased when BAP was incorporated into the culture media (Das et al., 2014; Mukhtar et al., 2010).

According to this study, CW supplementation in *M. pudica* culture media did not have a significant effect on the number of shoots, but it did have a significant effect on shoot length. It accords with a previous report (Buah & Agu-Asare, 2014), which revealed that CW treatment only exhibited 25 shoots on the Cavendish banana plant compared to 37 shoots by BAP. Nevertheless, the banana plantlet's height was 3 cm longer in CW treatment compared to BAP. Conversely, other species like *Pomacentrus amboinensis* thrived in a medium fortified with CW in terms of root elongation, leaf size, and plantlet elongation (Utami & Hariyanto, 2019). The same trend was observed in plantlets of *Renanthera imschootiana*, in which 10% (v/v) of CW supplementation in ¼ MS enhanced the growth of the protocorms (Wu et al., 2014). The same pattern was seen in *in vitro* cultivated olive embryos, where CW supplementation enhanced the number of leaves but did not affect shoot length or other features (de Souza et al., 2013). The discrepancies in plantlet responses could be related to the CW treatment's reaction being relatively species-specific (Villa et al., 2010).

Leaves are the most critical organ to develop to initiate rooting. A classical study

of *in vitro* rooting on avocado cuttings revealed that the number of leaves preserved on the cuttings was linked with the number of root plantlets. The role of leaves in ensuring the continuation of photosynthesis has resulted in a build-up of carbohydrates at the cuttings' base (Reuveni & Raviv, 1980). In *Plectranthus scutellarioides*, longer roots were produced at stem cuttings with apical leaves, as compared to leafless stem cuttings. The correlation between the presence of leaves and rooting performance was due to the leaves providing reserves, hormones, and co-factors that stems alone lack sufficient quantity (Belniaki et al., 2018). The same behavior was observed in bitter melon cuttings, where leaf-on cuttings had a major effect on roots, with rooting remaining extremely modest even when the incubation time was increased to 10 days. While bitter melon failed to root on leafless cuttings even with auxin hormone treatment, sweet potato cuttings can root easily without leaves (Malik et al., 2012). A moderate 17.6 µM KIN supplementation was deemed the most suitable shoot multiplication media for *M. pudica*. After that, it was used for the next *in vitro* rooting study.

Rooting Initiation of *M. pudica* in Response to Different MS Strength and Auxin Hormone, 2,4-D Concentrations

The effect of MS basal medium strength and supplementation of exogenous auxin hormone 2,4-D on *in vitro* rooting of *M. pudica* was evaluated. Based on Table 5, only two treatments, ½ and full MS were shown to respond with rooting, both of which were devoid of 2,4-D addition. Other treatments supplemented with 2,4-D resulted in no rooting response, and gall formation was observed at the basal part (Figure 4B). The emergence of roots in plantlets in ½ MS media was recorded after 5.93 days; for full MS, they took 7.27 days to root. In addition, the number of roots produced in ½ MS was higher (5.13) as compared to full MS (1.47). However, both treatments exhibited the same root length of 2.06 cm.

Table 5

Effects of Murashige and Skoog (MS) strength and 2,4-dichlorophenoxyacetic acid (*2,4-D) concentration on* in vitro *rooting of* M. pudica

MS strength	2,4-D (µM)	Days to root	Number of roots	Length of root (cm)	Morphology of root
½	-	5.93 ± 3.28[a]	5.13 ± 2.61[a]	2.06 ± 1.41[a]	Thin, long
½	5.33	-	0	0	Gall formed
½	10.66	-	0	0	Gall formed
Full	-	7.27 ± 2.87[a]	1.47 ± 0.83[b]	2.06 ± 1.41[a]	Thick, rotten
Full	5.33	-	0	0	Gall formed
Full	10.66	-	0	0	Gall formed

Note. Data were expressed as the mean ± standard error of fifteen replicates. The data were tested for statistical differences by two-way ANOVA followed by the Tukey post hoc test. The criterion for significance was set at $p<0.05$. Means that do not share a letter are significantly different

The roots of *M. pudica* in ½ MS treatment appeared healthy, thin, and long (Figure 4A), while in full MS, the single root that emerged were thick, and some were observed rotten (Figure 4 C). It may be inferred that adding 2,4-D did not help the rooting initiation of *M. pudica*; hence PGR incorporation was unnecessary. Additionally, ½ MS is sufficient for rooting since full MS resulted in low rooting efficacy, with only 1–2 thick and rotting roots emerging, compared to a higher number of healthy roots in ½ MS.

These results reflect those of Bianchetti et al. (2017), who found that no significant difference was observed between control and NAA-fortified treatments of *M. pudica*, indicating that this PGR did not promote rooting regardless of the concentration. Since there has been little research on 2,4-D as a source of auxin in *M. pudica* rooting, it was examined in this study.

Even when auxins are present in culture media, excessive salt concentrations in basal medium might hamper root development. Therefore, it is necessary to reduce macro- and micronutrient concentrations to half their normal levels during the rooting phase of most plant species (Woldeyes et al., 2021). Similar observations were recorded in *Ruscus hypoglossum* (Dahab et al., 2005), *Drosera intermedia* (Rejthar et al., 2014), and *Abelmoschus esculentus* (Rizwan et al., 2018) in which ½ strength MS media produced the highest root number and length. However, at a much lower concentration of MS (¼ MS strength), the least weak root development and proliferation were recorded in *Abelmoschus esculentus* (0.70 root number and 0.54 cm root length).

Figure 4. Root initiation of *Mimosa pudica*. A) Plantlet in rooting media fortified with ½ MS void of 2,4-D; B) Plantlet in rooting media fortified with ½ MS + 1.0 mg/L 2,4-D, an arrow pointing to rotten gall formed; C) Plantlet in rooting media fortified with full MS void of 2,4-D

Note. Bar = 1.0 cm
MS = Murashige and Skoog
2,4-D = 2,4-dichlorophenoxyacetic acid

When 2,4-D is administered at effective concentrations to dicotyledonous plants, it will be absorbed by the roots, stems, and leaves and translocated to the plant's meristems (Munro et al., 1992). As a result, the roots thicken and become stunted, stem phloem and xylem tissue disintegrate, and leaf development ceases, followed by stem curling, leaf withering, and eventually plant death (Grossmann, 2009; Song, 2013). The molecular mode of action of auxin is administered by the auxin receptor residing at the plasma membrane, which involves an amino acid permease-like protein that mediates auxin inflow (Swarup et al., 2008), efflux carriers that mediate auxin outflow and ATP-binding cassette, which serve as auxin transporters (Petrásek et al., 2006). The structure or size of their auxin binding pockets on the receptor, which is regulated by its aromatic ring size, may explain the difference in the effectiveness of various auxin types on the plants (Calderon-Villalobos et al., 2010).

According to observations in vine rootstock cuttings, 2,4-D did not stimulate the formation of adventitious roots or sprouting. However, it did reduce mortality, attributed to the stimulation of ethylene biosynthesis, which promoted senescence in various tissues (Tofanelli et al., 2014). The rooting of *Lippia* species cuttings treated for 24 hr with IBA, NAA, or 2,4-D showed that 2,4-D did not promote the formation of adventitious roots and that 100% of the treated cuttings died (Pimenta et al., 2007), which recorded similar response to this study. Despite this, 2,4-D is a key synthetic plant regulator, acting similarly to auxins in promoting lateral roots and preventing root stretching (Simon & Petrášek, 2011). On the one hand, 2,4-D might produce harmful metabolic and biochemical alterations in nucleic acids, cell wall flexibility, and the function of the RNA-polymerase enzyme causing excessive cell proliferation in tissues, in stem epinasty and phloem disruption (Tofanelli et al., 2014). It can be deduced that *M. pudica* can root without supplementing exogenous auxins such as IBA, NAA, and 2,4-D.

CONCLUSION

This study outlined a procedure for seed germination, shoot multiplication, and root initiation of *in vitro* micropropagation of the undervalued medicinal plant, *M. pudica*. The best basal medium for seed germination and culture initiation was ¾ MS supplemented with 30 g/L of sucrose. MS fortified with 35.2 µM of kinetin was the most satisfactory treatment for shoot multiplication. Roots of *in vitro M. pudica* were effectively initiated in ½ strength of MS devoid of any plant hormone. The impact of other main media components not explored in this study could improve the culture medium. Further research is warranted to gauge the adaptability of the *in vitro* plantlets to the environment. Overall, *in vitro* culture is a sustainable alternative to harvesting wild plants for their medicinal properties and an appealing approach that may provide sufficient preliminary materials for the large-scale cultivation of medicinal plants.

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Parthenium hysterophorus Weed Fecundity and Seed Survival at Different Soil pH and Burial Conditions

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ABSTRACT

Parthenium hysterophorus L. is regarded as one of the most invasive weed species. This study evaluated the effect of soil pH on *P. hyterophorus* weed growth and fecundity, as well as the effect of burial depths on *P. hyterophorus* seed survival and emergence. The first study evaluated five soil pH levels (acidic, sub-acidic, neutral, sub-basic, and basic) in a randomised complete block design. Seed germinability and subsequent seedling growth (height, leaf area, biomass, and seed number/plant) were evaluated. This study showed that *P. hysterophorus* seeds have a similar germination capacity under varying soil pH conditions. However, acidic soil indicated accelerated growth (25% higher biomass with 15.2% more leaf area) and fecundity (13.4% faster to enter the flowering stage at 74 days after sowing). In the second study, two factors were tested; burial depths (0.5, 5, 10, and 20 cm) being nested into seven burial durations (0, 2, 4, 6, 8, 10, and 12 months). Seed viability (final germination percentage and germination rate index), electrical conductance, and emergence percentage were assessed with the burial conditions. The results showed that only seed buried at 0.5 cm depth indicated emergence (81.3% of cumulative emergence). Forty per cent of the seeds remained viable after exhumation at 0.5–5 cm depth, while only 19–27% survived as the burial depth increased beyond 10 cm depth within 12 months. The seed remained viable for more than 12 months as a seed bank, despite faster deterioration at increasing soil depth.

Keywords: *Parthenium hysterophorus*, seed, survival

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INTRODUCTION

Parthenium hysterophorus L. is one of the most invasive weeds from the Asteraceae family, which has spread and distributed throughout almost the entire globe (Kaur et al., 2014; Tanveer et al., 2015). It was originally native to North and South America (Bajwa et al., 2019; Kaur et al., 2014). In Malaysia, the invasion of *P. hyterophorus* was officially reported in 2013, which had then spread to several states, mainly Kedah, Perak, Negeri Sembilan, and a few other regions within Peninsular Malaysia (Karim et al., 2018). *Parthenium hysterophorus* infestations were mainly associated with human and animal health issues such as dermatitis, asthma, bronchitis, agricultural losses, and a significant threat to biodiversity (Allan et al., 2018; Kaur et al., 2014). Significant loss of grains and forage yields by 40 to 90% was reported, as it exhibited a strong allelopathic influence, which increased its competitive advantage for soil available moisture and nutrients. These allelochemicals, such as parthenin and hysterin, were proven to inhibit agronomic crops and vegetables germination and growth (Bajwa et al., 2019; Tanveer et al., 2015). Currently, an integrative management approach is utilised to control *P. hysterophorus* infestations, including the usage of chemical herbicides (mainly clomazone, metribuzin, atrazine, glyphosate, metsulfuron methyl, butachlor, bentazone, dicamba, and metsulfuron-methyl), extracts of allelopathic plants (herbs, grasses, and trees that showed inhibitory effect on seed germination), intercropping with competitive plant species, and usage of biological control agents such as Mexican beetle, seed-feeding weevils, leaf-mining moth, and sap-feeding plant hopper (Tanveer et al., 2015).

Parthenium hysterophorus is regarded as a highly prolific seed producer as a single plant could produce up to 28,000 seeds in a life cycle (Kaur et al., 2014; Tanveer et al., 2015), which in turn contributed to a large and persistent seed bank in the soil (Navie et al., 2004; Nguyen et al., 2017). The small sized seed (2 mm in length) are easily dispersed by water and wind, but most of the spread across the regions were mainly through farm vehicles and machinery and as contaminants in the exported animals' feed as well as other crop seed lots (Kaur et al., 2014). The rapid spread of *P. hysterophorus* can be attributed to its seed production's prolific nature and its high capacity to adapt to a wide range of environmental conditions (Annapurna & Singh, 2003; Bajwa et al., 2020). Nevertheless, some variation may persist from the plant-environment interactions, ultimately influencing weed invasiveness or infestations (Vilà et al., 2021). The soil conditions, including pH, moisture levels, and temperatures, affect seed germination, seedling growth, and the buried seedbanks' longevity (Merino-Martín et al., 2017; Schwartz-Lazaro & Copes, 2019).

From a seed germination perspective, studies have shown that the pH range can promote or suppress weed seed germination. Pierce et al. (1999) suggested that *Digitaria sanguinalis* seed showed

higher germination and growth under acidic and sub-acidic conditions (pH 5-6). In contrast, *Cyperus esculentus* (L.) showed that acidic pH resulted in 33% less germination in comparison to neutral-alkaline conditions. Similarly, *Sida spinosa* (L.) seed germination was highest in alkaline (more than pH 8) soil (Singh & Singh, 2009). Interestingly, for *Eleucine indica*, a pH ranging from 5 to 10 did not influence seed germination (Chauhan & Johnson, 2008). From the seedling growth aspect, the plant characteristics such as heights, leaf area or lateral spread, dry biomass, number of flowers, and pollen quality are known to be influenced by soil pH conditions (Jiang et al., 2017). Gentili et al. (2018) reported that common ragweed (*Ambrosia artemisiifolia* L.) showed a higher growth rate with earlier flowering time, as well as more inflorescences and pollen production under acidic soil conditions. Based on these studies, soil pH may greatly affect weed fecundity and spread.

As mentioned previously, one of the main characteristics contributing to a weed's prolific nature is its persistence in the soil as a seed bank. However, it is important to note that the seed's survival upon burial will be influenced by the soil's specific conditions (such as moisture and temperature). In arid, semi-arid, or temperate regions, most of the seed could exhibit long seed bank persistence due to inconsistent rainfall, which reduces germination. A tropical region such as Malaysia, where the monthly rainfall ranges from 150–350 mm (Wong et al., 2009), could be an ideal condition for germination commencement, thus accelerating the seed-to-seed cycles. Nevertheless, for the same reason, the buried seed also may experience a higher level of deterioration due to the higher moisture level, reducing the seed's long-term survival. Thus, this study aims to determine the effect of soil pH on *P. hyterophorus* weed growth and seed production and the effect of burial depths and duration on *P. hyterophorus* seed survival and emergence.

MATERIALS AND METHODS

Seed Collection

The seed samples were collected from the Department of Veterinary Services Ladang Infoternak, Sungai Siput Perak (4.7844° N, 101.1089° E). Mature seeds of *P. hysterophorus* were collected and stored in labelled paper bags. The samples were immediately transported to Seed Technology Laboratory, Universiti Putra Malaysia (UPM), and kept inside desiccators prior to further analysis.

Study Location

The study was conducted at the Field 15, Universiti Putra Malaysia (UPM).

Seedling Growth and Seed Production as Influenced by Soil PH

Site Preparation. This study was conducted inside a 2 m × 10 m double-layered shelter unit at Field 15 UPM to prevent weed infestation. An organic soil mix was used, and the desired pH range was obtained by adjusting the soil's calcium magnesium

carbonate (dolomite) amount. Two kilograms (kg) of soil mix was then transferred into a 36 cm × 30 cm plastic pot for each experimental unit. Triangular spacing of 0.5 m was used to minimise light competition along with ease of access for data collection and pest control application. Each of the pots was watered with approximately 500 ml of water daily.

Experimental Design. Five levels of soil pH were evaluated (5-acidic, 6-sub-acidic, 7-neutral, 8-sub-basic, and 9-basic) in a randomised complete block design (RCBD), with four replications; a total of 20 experimental units. The pots were blocked according to the sunlight direction in the field.

Seed Germination. Four replications of 25 seeds were directly sown into the pot and monitored for 14 days. Germination was counted upon radicle emergence from the seed. The germination parameters were assessed as described by Ranal et al. (2009), including:

Final germination percentage, FGP (%) = The total seeds germinated at the end of the trial/Number of initial seeds used × 100 %

Germination index, GI = $(20 \times n_1) + (19 \times n_2) + ... + (1 \times n_{14})$; where n_1, n_2 ...n_{14} is the number of germinated seeds on the first, second, and subsequent days until the 14th day and the multipliers are weights given to the days of the germination

Mean germination time, MGT (day) = ΣFx/ΣF, where F is the number of seeds germinated on day x

Germination rate index, GRI (% / day) = G1/1 + G2/2 + ... + Gi/I, where Gn is the germination percentage on day n

Time spread of germination, TSG (day) = Differences between final dan first day of germination (days)

Seedling Vigour Index, SVI = Seedling length (cm) × Germination (%)

Seedling Evaluation. Seedling growth data were collected 4, 8, 12, and 16 weeks after sowing. Four replications of three seedlings were used in all data collection. Seedling height (cm) was measured using a measuring tape. The height was taken from the base of the stem until the base of the highest fully opened leaves. Stem diameter was also recorded by using callipers. Total leaf area (cm^2) was determined using a benchtop Li-COR Area Meter (model LI-3100, USA) at Plant Physiology Laboratory, UPM. Both shoot and root were dried separately using an oven at 70°C for at least 76 hr until no change in mass was observed. The dry biomass was measured using the Mettler Toledo balance (B303-S, USA). The numbers of developing inflorescences and seeds per plant were also recorded.

Seed Survival and Seedling Emergence at Different Burial Conditions

Site Preparation and Experimental Design. A nested design was used; the first

factor was burial depths (0.5, 5, 10, and 20 cm), nested into seven burial durations (0, 2, 4, 6, 8, 10, and 12 months), with three replications. The 15 cm × 15 cm burial plots were made for 20 cm depth, with 30 cm spacing between plots. A nylon mesh bag (6 cm × 8 cm) containing 100 mg seeds was laid flat onto the bottom of the plot. The hole was covered with soil until 10 cm depth before placing another bag containing seeds onto it. The process was repeated until 0.5 cm depth before the surface was lightly topped with soil and labelled accordingly. This part will provide information on the ability of the seeds to be viable under buried conditions. It was followed by a seedling emergence study using polybags to prevent the possibility of spreading the species. Three replications of 25 seeds were placed inside 20 cm x 30 cm polybags at respective burial depths, with 0.5 m spacing between rows. The soil pH was taken using Takemura Soil pH Meter DM-15 (Japan) at each data collection point. The soil moisture was determined gravimetrically by drying 100 g of soil samples at 105°C for 24 hr, as a percentage of dry weight basis. The emergence test will reveal the ability of the seeds to emerge from different depths without tillage.

Seed Germination. Three replications of 25 seeds were exhumed and germinated using the top paper method (International Seed Testing Association [ISTA], 2016). The final germination percentage and rate index were calculated as described previously (Ranal et al., 2009).

Seedling Emergence. Seedling emergence was monitored at 2 monthly intervals for 12 months. Cumulative emergence is the total number of seedlings from the soil/25 × 100 (%). At each count, emerged seedlings were removed due to limited space in the polybag.

Electrical Conductivity. Measured on three replications of 100 seed samples (approximately 50 mg) per lot weighed on an analytical balance (0.0001 g) and soaked in a container containing 50 ml deionised water for 24 hr at 20°C (Dutra & Vieira, 2006). Reading was made using a digitised conductivity meter. Results were expressed as $\mu S \cdot cm^{-1} \cdot g^{-1}$.

Statistical Analysis

Analysis of variance (ANOVA) was performed by Microsoft Excel and Statistical Analysis Software, SAS 9.4 (SAS Institute, USA). Significance levels of $p \leq 0.05$ were used to calculate the least significant differences (LSD) throughout this study. Pearson correlation analysis was performed among studied parameters by CORR procedure using SAS 9.4

RESULTS

Germination, Seedling Growth, and Seed Production as Influenced by Soil PH

Parthenium hysterophorus seeds showed similar final germination (FGP) values for all the soil pH, ranging from 78 to 88% (Table 1).

No mean germination time (MGT) differences were recorded, as most seeds germinated between days 6 and 7

Table 1

Final germination (FG), germination index (GI), mean germination time (MGT), germination rate index (GRI) and time spread of germination (TSG), and seedling vigour index (SVI) for seeds germinated under acidic and basic soil condition

pH	FGP (%)	GI	MGT (day)	GRI (%/day)	TSG (day)	SVI
5 (Acidic)	79.00 ± 4.43^{a}	171.25 ± 9.36^{abc}	6.33 ± 0.10^{a}	14.56 ± 0.83^{abc}	8.25 ± 0.48^{a}	407.00 ± 18.62^{bc}
6 (Sub-acidic)	87.00 ± 4.12^{a}	184.75 ± 12.60^{ab}	6.53 ± 0.24^{a}	15.76 ± 1.08^{ab}	6.25 ± 0.58^{b}	510.90 ± 22.64^{a}
7 (Neutral)	88.00 ± 2.83^{a}	192.25 ± 10.08^{a}	6.26 ± 0.33^{a}	16.45 ± 0.92^{a}	7.00 ± 0.91^{ab}	508.87 ± 15.96^{a}
8 (Sub-basic)	80.00 ± 5.16^{a}	162.75 ± 10.61^{bc}	6.82 ± 0.47^{a}	13.28 ± 1.01^{bc}	6.00 ± 0.87^{b}	469.10 ± 32.40^{ab}
9 (Basic)	78.00 ± 2.58^{a}	155.50 ± 6.65^{c}	7.02 ± 0.30^{a}	13.25 ± 0.57^{c}	8.25 ± 0.63^{a}	393.07 ± 18.62^{c}

Note. Mean with different letters indicate significant differences between treatments

after sowing. In terms of time spread of germination (TSG), no specific pattern was observed as the seeds germinated within 6 to 9 days after sowing. However, based on the germination index (GI) and germination rate index (GRI) values, seeds sown at pH 7 and below showed slightly higher yet significant GI (more than 171.25) and GRI (more than 14% per day). Interestingly, the seedling vigour index (SVI) was higher for soil pH ranging from 6 to 7 (more than 469 SVI), as compared to acidic (pH 5) and basic (pH 9) conditions. Within the first 8 weeks, generally, no differences were observed for all treatments, except for plant height, where acidic soil recorded a significantly higher value of 17.17 cm compared to less than 15.2 cm, as shown by another soil pH (Table 2).

Table 2

Plant height, stem diameter, florets, and seed number of Parthenium hysterophorus *seedlings under different soil pH conditions (Note: no florets and seeds were recorded in week 4 and 8)*

Time (weeks)	pH	Plant height (cm)	Stem diameter (mm)	Florets number/plant	Seed number/plant
4	5 (Acidic)	5.87 ± 0.03^{a}	2.92 ± 0.19^{a}	0	0
	6 (Sub-acidic)	5.14 ± 0.02^{b}	2.75 ± 0.12^{a}	0	0
	7 (Neutral)	5.78 ± 0.05^{a}	3.08 ± 0.19^{a}	0	0

Table 2 *(Continue)*

Time (weeks)	pH	Plant height (cm)	Stem diameter (mm)	Florets number/plant	Seed number/plant
8	8 (Sub-basic)	5.85 ± 0.04[a]	2.91 ± 0.23[a]	0	0
	9 (Basic)	5.06 ± 0.20[b]	2.83 ± 0.17[a]	0	0
	5 (Acidic)	17.17 ± 0.61[a]	5.75 ± 0.18[a]	0	0
	6 (Sub-acidic)	13.92 ± 0.51[bc]	5.03 ± 0.23[ab]	0	0
	7 (Neutral)	13.42 ± 0.57[c]	4.42 ± 0.23[b]	0	0
	8 (Sub-basic)	15.17 ± 0.55[b]	4.59 ± 0.19[b]	0	0
	9 (Basic)	13.42 ± 0.51[c]	5.08 ± 0.26[ab]	0	0

Note. Mean with different letters indicate significant differences between treatments

However, at 12 weeks after sowing, a marked increase in height and stem diameter was recorded by acidic soil, with 20 and 13% higher than the average treatment values, respectively. At 16 weeks, this trend continued where acidic soil showed 14 and 10% higher values of plant height and stem diameter, respectively—the average days to flowering commenced as early as 74 days after sowing for acidic soil, followed by sub-acidic and sub-basic (83 days), basic (86 days), and neutral pH soil (90 days). By the end of the 16th week, acidic soil recorded an average of 2,850 total seeds/plant, followed by neutral, sub-acidic, and sub-basic (ranged from 647–1,258 seeds/plant), and basic soil conditions (740 seeds/plant). All soil pH conditions showed similar shoot and root growth values and the total leaf areas within the first 4 weeks (Table 3). At 8 weeks after sowing, acidic soil (pH 5) indicated a significantly higher leaf area of 304.58 cm^2, whereas other treatments recorded less than 268.00 cm^2. By the 12th week, acidic soil generally showed 10% higher total biomass and 14% more leaf area values than other treatments. This pattern remained until 16 weeks after sowing, when the acidic soil showed a 25% higher overall biomass with 15.2% more leaf area values.

Table 3

Total dry biomass and leaf area of Parthenium hysterophorus *seedlings under different soil pH conditions*

Time (weeks)	pH	Biomass (g)		
		Shoot	Root	Leaf area (cm^2)
4	5 (Acidic)	0.112 ± 0.004^{a}	0.045 ± 0.003^{b}	42 ± 1.73^{ab}
	6 (Sub-acidic)	0.116 ± 0.006^{a}	0.047 ± 0.006^{b}	37.083 ± 1.83^{b}
	7 (Neutral)	0.113 ± 0.006^{a}	0.044 ± 0.002^{b}	43.58 ± 2.18^{a}
	8 (Sub-basic)	0.109 ± 0.004^{a}	0.038 ± 0.001^{b}	42.08 ± 2.06^{ab}
	9 (Basic)	0.11 ± 0.005^{a}	0.066 ± 0.007^{a}	41.583 ± 2.39^{ab}
8	5 (Acidic)	1.971 ± 0.0450^{a}	0.211 ± 0.007^{a}	304.58 ± 11.06^{a}
	6 (Sub-acidic)	1.545 ± 0.077^{c}	0.183 ± 0.015^{ab}	246.58 ± 12.42^{b}
	7 (Neutral)	1.774 ± 0.077^{ab}	0.16 ± 0.008^{b}	245.33 ± 10.59^{b}
	8 (Sub-basic)	1.745 ± 0.079^{bc}	0.182 ± 0.011^{ab}	267.5 ± 10.25^{b}
	9 (Basic)	1.792 ± 0.088^{ab}	0.199 ± 0.014^{a}	258.08 ± 11.04^{b}
12	5 (Acidic)	9.135 ± 0.189^{a}	4.407 ± 0.196^{a}	491.67 ± 10.11^{a}
	6 (Sub-acidic)	7.867 ± 0.341^{b}	3.536 ± 0.164^{b}	410.33 ± 18.07^{b}
	7 (Neutral)	8.425 ± 0.22^{ab}	3.726 ± 0.09^{b}	428.58 ± 13.90^{b}
	8 (Sub-basic)	8.327 ± 0.313^{ab}	3.765 ± 0.111^{b}	425.83 ± 13.54^{b}
	9 (Basic	7.911 ± 0.396^{b}	3.609 ± 0.126^{b}	393.5 ± 15.16^{b}
16	5 (Acidic)	16.975 ± 0.376^{a}	7.195 ± 0.153^{a}	792.5 ± 20.23^{a}
	6 (Sub-acidic)	12.467 ± 0.20^{ab}	5.392 ± 0.183^{ab}	670.58 ± 18.03^{bc}
	7 (Neutral)	13.859 ± 0.883^{a}	6.116 ± 0.212^{ab}	619.83 ± 19.97^{c}
	8 (Sub-basic)	11.601 ± 0.430^{b}	4.921 ± 0.153^{b}	684.25 ± 17.36^{b}
	9 (Basic)	12.551 ± 0.58^{ab}	5.299 ± 0.269^{ab}	672.5 ± 25.14^{bc}

Note. Mean with different letters indicate significant differences between treatments

Seed Survival and Seedling Emergence at Different Burial Conditions

In terms of seedling emergence, only seeds buried at 0.5 cm depth indicated emergence throughout the 12 months evaluation period. Cumulative emergence of 81.3% was recorded at 2 months after burial, with no additional emergence recorded afterwards (Figure 1).

At the beginning of this study, the FGP and GRI ranged from 81–88% and 11.4–16.4, respectively (Figure 2). As the

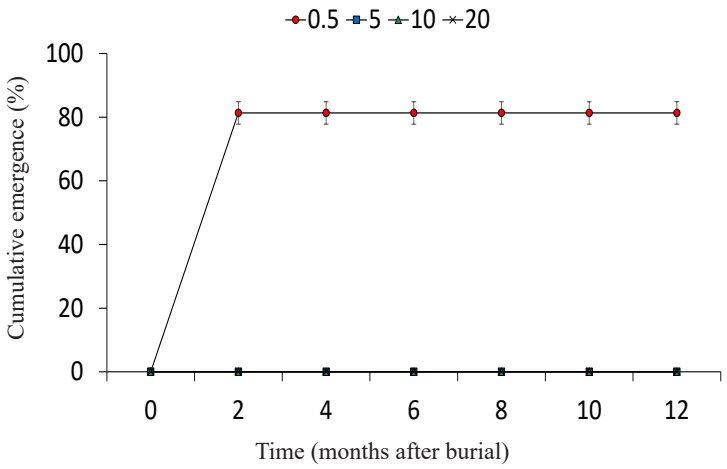

Figure 1. Percentage of seedling emergence for *Parthenium hysterophor*us at 0.5, 5, 10, and 20 cm depths upon 12 months of burial

burial duration increased, it was evident that *P. hysterophorus* viability decreased by an average of 25.5% after 6 months and 59.2% towards the end of 12 months burial period. Higher seed viability loss was found at a deeper burial depth of 10 and 20 cm. At 10 months of burial, the differences in FGP between 0.5 and 20 cm burial was more than 25%. The seed electrical conductance values were positively correlated with burial time and depth. Initially, the seeds recorded 8–11 $\mu S \cdot cm^{-1} \cdot g^{-1}$ of EC, and the values increased up to 60 $\mu S \cdot cm^{-1} \cdot g^{-1}$ within 4 months, with no significant differences among depths. However, at 6 months onwards, higher EC values were recorded for 10 and 20 cm depths compared to less than 5 cm burial.

By 12 months after burial, 10 and 20 cm depth indicated higher than 200 $\mu S \cdot cm^{-1} \cdot g^{-1}$ of EC. The soil moisture content was positively correlated with depth, and the values remained consistent throughout the study period. Both 0.5 and 5 cm depths generally showed 3–13% soil moisture, whereas, for 10 and 20 cm depths, the values were between 7–25%. Based on the Pearson correlations values, there was a strong positive correlation between FP with GRI (R^2 = 0.914) and moderate negative correlations between FGP and GRI with EC (R^2 = 0.627 and 0.683, respectively) values. The seed EC value was found to have a moderate positive correlation (R^2 = 0.684) with the soil moisture levels.

DISCUSSION

This study showed that *P. hysterophorus* seeds had a similar capacity to germinate for most germination parameters under varying soil pH conditions, except for GI and GRI, which were found to be slightly higher when the pH was 7 or lower. Pérez-Fernández et al. (2006) suggested that pH generally had no significant effect on total

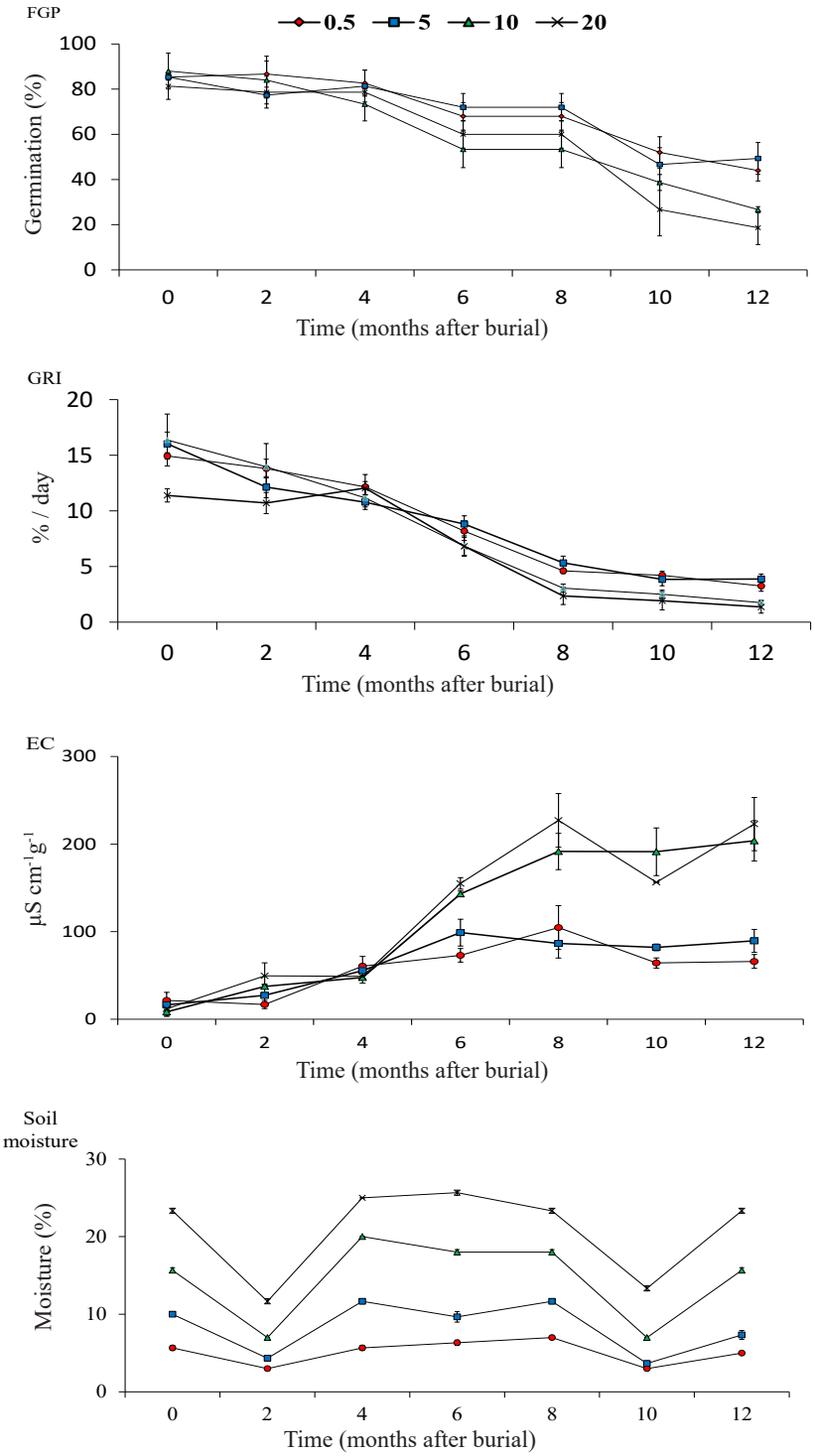

Figure 2. Final germination percentage (FGP), germination rate index (GRI), electrical conductivity (EC), and soil moisture content of *Parthenium hysterophorus* at 0.5, 5, 10, and 20 cm depths upon 12 months field burial

germination percentage, but the reduction in germination speed was found particularly in high pH environments. It simply means that *P. hysterophorus* can germinate under a wide range of soil pH, giving it a competitive advantage compared to other seeds, hence facilitating its spread. On the other hand, Laghmouchi et al. (2017) reported that *Origanum compactum* seed favours a more neutral pH as maximum germination of 71% was obtained at pH 7. The influence of soil pH was more prominent as the germinated seed entered the early seedling stage. It is widely known that soil pH affects plant growth based on its influence on nutrient availability. Generally, most micronutrients, including iron, manganese, boron, and copper, are more available to plants under acidic and sub-acidic conditions than neutral-basic ones, thus favouring growth (Lončarić et al., 2008). Findings from the subsequent seedling analysis indicated that *P. hysterophorus* seedlings showed accelerated growth and fecundity in acidic soil conditions. As a result, the seedling enters the flowering stage at 74 days after sowing, resulting in a 13.4% quicker rate compared to pH 6 and above (with 85 days average).

It, in turn, shortens the seed-to-seed cycle as each seedling enters maturity significantly faster compared to higher soil pH conditions, again providing means for quick infestation. Gentili et al. (2018) reported similar findings as *A. artemisiifolia* seedlings showed faster growth (larger size, more dry weight) at pH 5 and 6, along with a higher number of inflorescences and pollen production. This study showed that *P. hysterophorus* seeds buried at 5 cm depth and below could not emerge from the soil. However, the non-emerged seeds were generally still viable upon exhumation, at a varying level, depending on the burial conditions. More than 40% of the seeds survived when buried at 0.5–5 cm depth, while only 19–27% survived as the burial depth increased beyond 10 cm at 12 months. The lower germinability observed at increasing burial depth can be linked to a higher rate of deterioration from higher soil moisture levels. It is generally known that seed longevity is influenced by several factors, including relative humidity or moisture, temperature, and oxygen level (Rajjou & Debeaujon, 2008).

Low relative humidity could reduce the deterioration rate even with high storage temperature conditions, suggesting that moisture level might play a larger role in determining seed longevity. In general, seed EC value could indicate membrane integrity perseverance which protects the seed tissue from oxidative damage; and can be linked to the seed germinability. From the Pearson correlation analysis, both EC and soil moisture indicated a moderate positive correlation, which could explain the higher reduction in exhumed seed viability for 10 and 20-cm burial depths. Higher EC values recorded at both depths suggested that the electrolytes were leached out due to a higher degradation level of the outer membrane (Nakagawa et al., 2020; Wang et al., 2018). These findings suggest that *P. hysterophorus* weed seed bank emergence and survival would vary depending on the burial depth, regional rainfall, and soil

moisture level. It may exhibit secondary dormancy that relates mainly to water availability. Navie et al. (2004) suggested that *P. hysterophorus* does not confer the typical physical dormancy characteristics since the seed can emerge immediately within two weeks upon exposure to moisture or rainfall.

In the case of *Parthenium argentatum* seed, the onset of prolonged rainfall was reported to trigger germination immediately in desert conditions. It ensures that germination inhibitors are leached when there is sufficient water level to prevent untimely germination of seeds, particularly in arid environments. As a consequence, the emerged seedlings would be able to sustain the growth stages and reproduce. The results obtained in this study on the viability of seeds upon burial showed that only seeds at the 0.5 cm depth can germinate, while the rest will remain viable for slightly more than a year before total deterioration occurs. It, however, is the situation if no-tillage is done.

CONCLUSION

The overall findings from this study suggested that *P. hyterosphorus* seed could survive a wide range of conditions with high germinability. However, the environment affected the subsequent seedling growth and flowering time. Higher plant height, leaf areas, and overall seedling biomass were recorded in lower pH conditions, enabling the seedlings to enter the earlier flowering stage. It reduces the seed-to-seed cycle and increases overall weed fecundity. The seed, however, showed a marked decline in viability if buried for more than 12 months without tillage.

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Isolation of Proteolytic Enzyme from Pineapple Crown

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ABSTRACT

The pineapple waste from the pineapple industry has contributed to an increase in waste in Malaysia and worldwide every year. A major type of endopeptidase enzymes found in pineapple is fruit bromelain, stem bromelain, ananain, and comasain. This study aims to extract and purify protease from the crown of MD2 pineapple. Protease was extracted and purified using anion exchange chromatography, gel filtration, and desalting before being identified using liquid chromatography-mass spectrometry (LC-MS). Proteolytic activity was determined using the well diffusion method and Casein Digestion Unit. In the present study, the proteolytic assay showed that 1 kg crown of MD2 cultivar produced an activity of 126.0 ± 3.86 U/ml, a specific activity of 3937.50 U/mg. In the present study, the proteolytic assay showed that 1 kg crown of MD2 cultivar produced an activity of 126.0 ± 3.86 U/mL, a specific activity of 3937.50 U/mg and the total activity of 3.94×10^9 U. The molecular weight of the purified enzyme was in the range of 25 to 35 kDa under the optimum condition of pH 7 and 37°C. Purification of the extract yielded a band at the molecular weight of 20–25 kDa at the optimum pH of 3 and 9 at 60°C. From LC-MS analysis, the purified enzyme from the crown extract was similar to ananain under accession number A0A199VSS3 (according to Uniprot). It had five unique peptides and covered 97/356 amino acids (44.9% coverage). The ananain (EC 3.4.22.31) is classified in the subfamilies of cysteine protease C1A (clan CA, family C1), a peptidase family related to papain. In conclusion, protease was extracted and identified as an ananain-like protease from the crown.

Keywords: Ananain, bromelain, crown, MD2, proteolytic activity

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INTRODUCTION

Pineapple (*Ananas comosus*), locally known as 'nanas,' is a member of the Bromeliaceae family. It is the most common fruit cultivated worldwide, particularly in China, Indonesia, Malaysia, Thailand, Kenya, the Philippines, and India (Ramli et al., 2017). Today, world pineapple production continues to increase yearly, including in Malaysia. Three common cultivars that are commercially cultivated in Malaysia are Spanish (also known as Josapine, Maspine, and hybrid pineapple), Queen, and Smooth Cayenne (Sarawak pineapple) (Hidayat et al., 2012). The introduction of MD2 (hybrid variety) into the pineapple market in Malaysia has increased demand and a gradual shift from the export of Smooth Cayenne to MD2. Among pineapples, MD2 and N36 are cultivated for export due to their longer shelf life, whereas Moris, Sarawak, and Josapine are grown for the local market. Therefore, pineapple cultivars MD2 are a benchmark in developing future cultivars for fresh consumption. The MD2 pineapple weight is uniform, approximately 1.5 kg each, and has a shelf life of 30 days, like papaya (Falah et al., 2015; Thalip et al., 2015).

According to the Malaysia Pineapple Industry Board (MPIB), in 2017, Malaysia had a total cultivation area of 13,733 ha with a production of 432,812 metric tonnes and import of USD 24 million, while exports of USD 47 million for all varieties of pineapple (Lembaga Perindustrian Nanas Malaysia [LPNM], 2018). After durian and banana, the pineapple industry is one of Malaysia's most important fruit sectors. The production is expected to reach 1.7 million metric tonnes by 2025. For MD2, the production has increased and reached 3,600 metric tonnes by the end of the year 2017. The MD2 pineapple production at 3,800 metric tonnes by 2019 saw an increase of 200 metric tonnes. Malaysia currently targets USD 42 million to export pineapple products, with a total production of 253,227 metric tonnes by 2023. Three hundred sixty hectares of MD2 pineapple plants are being grown commercially in Ulu Tiram, Kluang, and most recently, in Tanah Abang, Mersing, Johor. The plan was to expand the crop to 1,000 ha by 2021. The aim is to produce 100 containers together with 1,800 tonnes of pineapple per month, including 50 containers or 900 tonnes of pineapple would be exported. Growing pineapple crops in Malaysia generates millions of tons of plant waste. During harvesting activities, a large amount of leftover from pineapple growing, known as agro-waste, is created in the agricultural process. According to Nor et al. (2015), the waste proportions are twice the proportion of fruit for the flesh, core, peel, and crown of the Smooth Cayenne pineapple. In addition, approximately one billion tons of agricultural waste are produced globally, with 1.2 million tonnes of agro-waste disposed of into landfills in Malaysia each year (Neh & Ali, 2020). In Malaysia, agricultural waste is projected to be 0.210 ($kg \cdot cap^{-1} \cdot day^{-1}$) in 2025 (Ngoc & Schnitzer, 2009). Generally, harvest leftovers from crops such as pineapple waste are usually just burnt or left to rot, releasing carbon dioxide and methane gas

into the atmosphere. Recovery of nutrients from fruit processing wastes could minimize environmental issues (Mirabella et al., 2014). It was revealed that some parts of the pineapple waste, such as the core, crown, and peel, were sources of protease enzyme (Ketnawa et al., 2012).

Pineapple waste has been discovered as a possible source of proteases like bromelain (Abreu & Figueiredo, 2019). Bromelain comprises proteases such as fruit bromelain (EC 3.4.22.33), stem bromelain (EC 3.4.22.32), ananain (EC 3.4.22.31), and comosain, whereas non-proteases components consist of carbohydrates, peroxidases, cellulases glucosidases, glycoproteins, and phosphatases (Sahoo & Das, 2017). Therefore, it suggested that protease enzymes extracted from pineapple can be utilized to fulfill the demand in industrial processes, including the foods, textile, and medical cosmeceutical industries. Protease enzyme has been shown to aid in the tenderization of meat (M. S. Arshad et al., 2017), the relaxation and suppression of shrinkage in baking industries (Sahoo & Das, 2017), the improvement of silk and wool quality (Z. I. M. Arshad et al., 2014), and the removal of tooth stains in tooth whitening products (Munchow et al., 2016).

In addition, the topical application of protease for the debridement of skin wounds and burns has been introduced (Muhammad & Ahmad, 2017). It acts as a fibrinolytic agent, which increases fibrinolysis by activating the conversion of plasminogen to plasmin (Kwatra, 2019), an anti-cancer agent by suppressing carcinogenesis during the cancer development (Rathnavelu et al., 2016), and an anti-inflammatory agent (Barrera-Núñez et al., 2014). As a result, there is an increasing demand to exploit the sustainable utilization of pineapple wastes for industrial applications that can be commercialized as an environment-friendly alternative for carbon source utilization. However, enzyme purity is necessary for industrial and commercial applications (Nor et al., 2015). Since a high degree of purity is required, entire downstream processing is necessary, including extraction, purification using ion-exchange chromatography, and identification of purified enzyme. Therefore, pineapple stems, fruits, and waste products such as crowns, leaves, peel, and core are extracted and purified with different homogenization processes and the addition of different extraction buffers to produce beneficial products (Wan et al., 2016; Youryon et al., 2018).

MATERIALS AND METHODS

Plant Materials

Six pineapple wastes (crown and stem) from different cultivars were brought from a local market at Taman Universiti, Skudai, Johor, Malaysia (Figure 1). The six pineapple cultivars included Madu Kaca, Josaphine, Morris, Yankee, N36, and MD2. All the pineapples were verified by Hj Musliman bin Tasim from Lembaga Pekan Nanas Malaysia (LPNM). The wastes (crown and stem) were directly taken to the laboratory (Plant Biotechnology Lab, T02 UTM) for enzyme assay. However, the wastes (MD2 crown) were taken from LPNM,

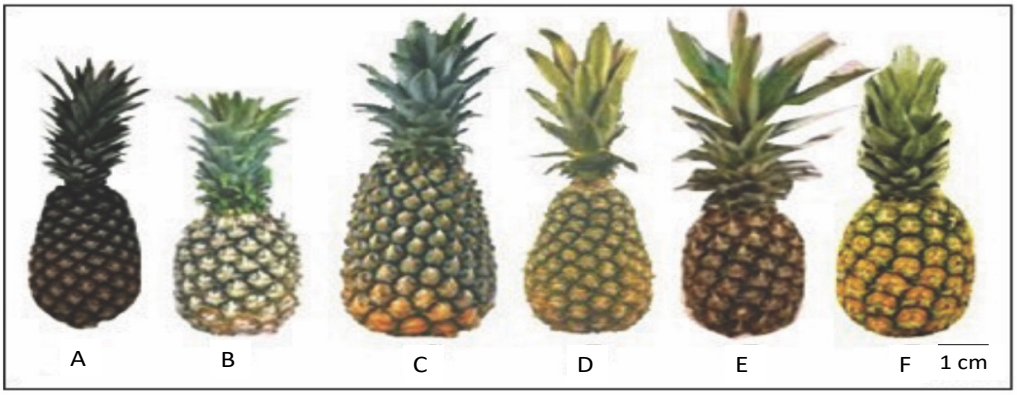

Figure 1. Six pineapple cultivars: (A) Madu Kaca; (B) Josaphine; (C) Morris; (D) Yankee; (E) N36; and (F) MD2

Pekan Nanas, to the laboratory (Plant Biotechnology Lab, T02 UTM) a day before the purification step. All the samples were kept at room temperature.

Preparation of Crude Protease Extract from the Pineapple Waste

A known quantity (1 kg) of each crown was ground into a fine piece using a blender (Panasonic MX-GM1011 G, Malaysia). Then 100 mM sodium acetate buffer solution (pH 7.0) (Bio-Rad, USA) was added and left at room temperature for 3 min. Next, the mixture was filtered through a filter (Minisart PES 28, Sartorius, Germany) and centrifuged at 10,108 x *g* for 20 min at 4°C. After that, the supernatant (crude enzyme extract) was collected, measured, and stored at -20°C before the experiment.

Determination of Proteolytic Activity Using Casein Digestion Unit (CDU)

The CDU method determined the enzyme activity (Murachi, 1976). This study chose casein as substrate, and L-tyrosine was used as standard. In this method, the proteolytic activity of hydrolyzed casein was measured. One unit of proteolytic activity was defined as 1 g of tyrosine released in 1 min/ml of a sample when casein was hydrolyzed under the standard conditions at 37°C and pH 7.0 for 10 min. The activity of the protease was estimated by the following Equation 1:

$$Proteolytic\ activity\ \left(\frac{Units}{ml}\right) = \frac{\mu moles\ of\ tyrosine\ \times\ Reaction\ vol}{Sample\ vol\ \times\ Reaction\ time\ \times\ Vol\ assayed} \quad (1)$$

Determination of Proteolytic Activity Using Well Diffusion Method

The enzyme activity is used well diffusion as described by Vijayaraghavan and Vincent (2013) with a minor modification. About 1% (w/v) casein skimmed milk (Sunlac, New Zealand) was added into the agar and autoclaved at 121°C for 15 min. Then, the agar was poured into disposable Petri dishes and solidified for 30 min. Approximately 8 mm diameter was punched into the solidified agar using tip collars of 1 ml pipette tips. A 70 µl crude supernatant volume was pipetted

into the holes and incubated overnight at 37°C. A 70 µl of acetate buffer (Bio-Rad, USA) was used as a negative control.

Protein Concentration

The concentration of the crude enzyme extract was determined as described by the Bradford method (Bradford, 1976) and bovine serum albumin (BSA) as a standard. Coomassie Brillant Blue G-250 (Bio-Rad, USA) was a dye bound to an unknown protein and formed a complex detected spectrophotometrically at 595 nm.

Specific Activity of Enzyme

The specific activity of the enzyme was determined by the following formula and expressed as U/mg protein.

$$Specific\ activity\left(\frac{Units}{mg}\right) = \frac{Proteolytic\ activity}{Protein\ concentration} \quad (2)$$

Purification of Protease Enzyme

The purification steps were constructed according to Costa et al. (2014). However, the buffers used in each stage of purification, on the other hand, are slightly different. Firstly, the resuspended extracts were transferred into centrifugal filter devices (Amicon Ultra-15 10K, Merck, Germany) for fast ultrafiltration and centrifuged at 1,792 x *g* for 20 min at 4°C. The concentrate was collected from the filter device sample reservoir using a pipette. The extract was concentrated using the Amicon Ultra-15 (Merck, Germany) before being loaded onto a 5 ml HiTrap Q HP (5 × 5 ml) (Cytiva, USA) column pre-equilibrated with buffer A (50 mM Tris hydrochloride [Tris-HCL] buffer, pH 8.0) (Bio-Rad, USA). The protein was eluted using buffer B (50 mM Tris-HCL, pH 8, and 1 M sodium chloride (NaCl) (Bio-Rad, USA). The eluate was collected in a 96-deep well plate container after elution at a flow rate of 2 ml/min. Aliquots of anion exchange chromatography were collected and subjected to gel filtration chromatography purification. The Sephadex 200 column (Merck, Germany) was prepared with pH 8 buffer (25 mM Tris + 150 mM NaCl + 0.5 mM ethylenediamine tetraacetic acid (EDTA) (Bio-Rad, USA). The sample was eluted at a flow rate of 0.5 ml/min. The purified enzyme was loaded onto a 5 ml HiTrap Q HP (5 × 5 ml) (Cytiva, USA) equilibrated with sodium acetate buffer (pH 7) (Bio-Rad, USA) and then eluted at a flow rate of 3 ml/min. All procedures were conducted at 4°C. The desalted enzyme was stored at 4°C.

Determination of Protein Molecular Weight by SDS-PAGE

The sodium dodecyl-sulfate polyacrylamide gel (SDS-PAGE) electrophoresis estimated the molecular weight of the purified protein with different gel concentrations of 12 and 15% (w/v). First, each 20 µl protein sample was mixed with 10 µl loading buffer (Bio-Rad, USA) comprising 0.2 M Tris-HCl of pH 6.8, 20% (v/v) glycerol, 10% (w/v) SDS, 10 mM beta-mercapto-ethanol, and 0.05% (w/v) bromophenol blue. Then, the protein sample was heated at 95°C for 15 min. Then,

the protein sample was electrophoresed at 150 V for 120 min and stained with 0.02% Comassive Brilliant Blue G-250 (Bio-Rad, USA) for 30 min. Finally, the gel was detained with a mixture of acetic acid and methanol solution for 1 hr till the bands were visible. The molecular weight markers used were Precision Plus Protein™ Dual Color Standards (Bio-Rad, USA).

pH and Temperature Characterizations

The pH profile of protease was determined by assaying its proteolytic activity at various pHs ranging from 3 to 10 according to the previous method (Ketnawa et al., 2012). The residual proteolytic activity was measured and expressed as the relative proteolytic activity. In addition, the effect of temperatures (30, 37, 40, 50, 60, 70, 80, and 90°C) on proteolytic activity was performed for 10 min. All assays were carried out using casein as the substrate. The caseinolytic activity was expressed as the relative proteolytic activity compared to the control.

LC-MS

Amino acid sequence analysis was recorded using LC-MS. The purified enzymes were digested with ammonium bicarbonate which consisted of 12.5 ng/µl mass spectrometry grade Trypsin Gold. The Liquid Chromatography analysis was carried out in Dionex Ultimate 3000 RSLCnano, and full scan spectra were collected using orbital MS (OTMS) following the parameters of scan range of 310–1800 m/z, resolving power of 120000, AGC target of 4.0 e5 (400 000), and maximum injection time of 50 ms. LC-MS data were investigated using Thermo Scientific™ Proteome Discoverer™ 4 Software Version 2.1.

Phylogenetic Analysis

Five peptide sequences obtained from LC-MS analysis were subjected to sequence analyses using National Center for Biotechnology Information (NCBI-Bethesda, Maryland, USA) BLASTP. Then, the full sequences of ananain of *Ananas comosus* under the accession number AOA199VSS3 were retrieved from the NCBI Genbank database. Ten sequences with 100% sequences identity (AOA199UL32, AOA199UUY2, AOA199W8N4, AOA199VSB5, AOA199W3D2, AOA199W9F2, AOA199W8R4, O24641, and P0074.8) to AOA199VSS3 were aligned using Clustal Omega. Ten aligned sequences were used to construct the phylogenetic tree using the MEGA-X version software. The domain was identified using InterProScan against multiple databases, including Pfam, PRINTS, SMART, PROSITE, ProDom, Panther, GO lookup, TMHMM, and SignalP_EUK with pathway.

Statistical Analysis

All data obtained from three or more independent variables were analyzed using One-way ANOVA of Statistical Package for Social Science (SPSS) version 22. The Bonferroni method evaluated differences between means of treatments by the post-hoc test. The test was used to compare the means at a significance level of $\alpha = 0.05$.

RESULTS AND DISCUSSION

Proteolytic Activity in Crown and Stem of Pineapple from Different Cultivars

Major endopeptidase enzymes found in bromelain extract are stem bromelain (EC 3.4.22.32), fruit bromelain (EC 3.4.22.32), ananain (EC 3.4.22.31), and comasain (Kahiro et al., 2017; Ketnawa et al., 2012). These enzymes were found in pineapple fruit and industrial waste, such as the crown, core, leaves, and peel (Z. I. M. Arshad et al., 2014). However, each extract's enzyme activity and protein content are influenced by different proteolytic enzymes (Ketnawa et al., 2012). This study revealed that the MD2 crown obtained higher proteolytic activity than the MD2 stem, and different protease enzymes were found in both the crown and stem extracts.

The extracts from the MD2 crown (A) recorded higher proteolytic activity than stem (B), with values of 126.27 ± 3.86 and 118.5 ± 1.19 U/ml in 100 g of pineapple wastes, respectively (Figure 2). This study recorded that the MD2 crown exhibited a higher yield than the MD2 stem (p<0.05). The MD2 crown also showed a larger diameter of the halo zone in casein and skim milk plates, where it was 9.3 mm and 11.0 mm, respectively (Figures 3A and 3C). The halo zone diameters of the stem in the casein and skimmed milk plates were 6.0 mm and 9.7 mm, respectively (Figures 3B and 3D). The resulting halo zone may be related to the amount of protease presented in the crude extract. Similar findings also showed that the crown of *Ananas comosus* L. contained higher proteolytic activity compared to other parts (Hebbar et al., 2008, 2011, 2012; Kahiro et al., 2017; Ketnawa et al., 2012; Neta et al., 2012; Z. I. M. Arshad et al., 2014). According to Neta et al. (2012), proteolytic activity depends on the part of the plant from which the sample was extracted.

Furthermore, some researchers also found that crown extracts from both cultivars (*Nang Lae* and *Phu Lae*) contained bromelain as the major protease enzyme (Ketnawa et al., 2012; Neta et al., 2012)—pineapple cv. *Vitória* has high proteolytic activity than other cultivars (Costa et al., 2014). *Ananas comosus* also showed the highest proteolytic activity than other varieties of pineapples (Chaurasiya & Hebbar, 2013; Martins et al., 2014). In addition, the expression of the proteolytic enzyme was influenced by environmental conditions such as salt concentration, temperature, soil moisture, and agroecological zone (Kahiro et al., 2017). Thus, it shows that the proteolytic activity varies depending on the cultivar and the part of the examined and extracted plant. The MD2 cultivar showed the highest proteolytic activity in this study, with the crown being the best part. The MD2 crown was purified for further characterization. In conclusion, cultivars and different parts of plant extracts gave differences in enzyme activity and protein content.

Purification of Protease Enzyme

Protease purification using anion exchange chromatography yielded 147.09 U/ml of proteolytic activity, 171.03 U/mg of specific activity, and a purification

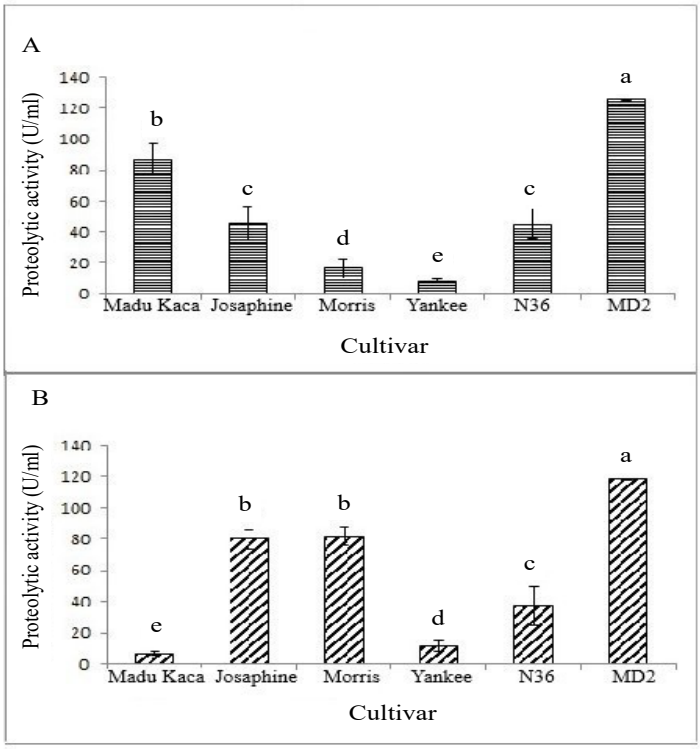

Figure 2. Comparison of proteolytic activity (U/ml) in (A) crown and (B) stem of different pineapple cultivars

Note. Different alphabetical letters indicate the significant differences (p>0.05)

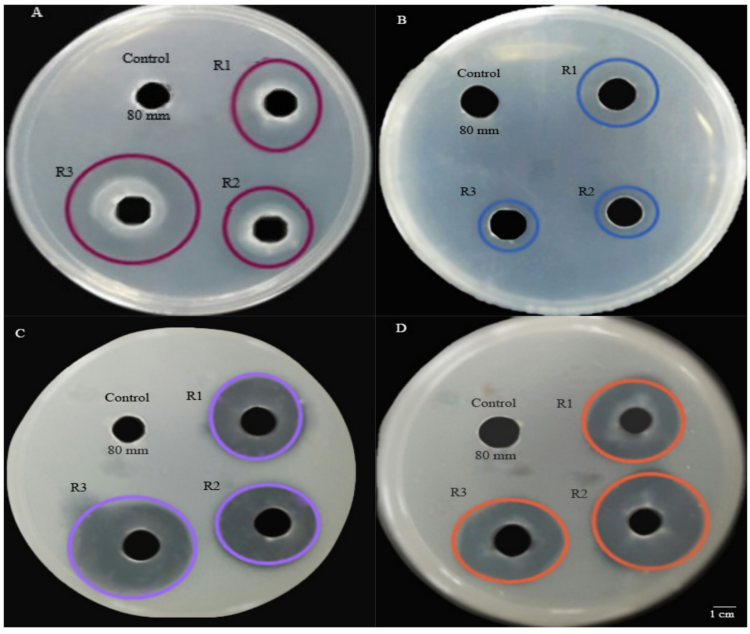

Figure 3. Halo zone formation on casein plate by extracts from the crown (A) and stem (B) and halo zone formation on skim milk plates by extracts from the crown (C) and stem (D)

fold of 4.33 (Table 1). The proteolytic and specific activities were higher than previously reported bromelain purification by Costa and co-workers (2012). They reported the purification of bromelain from carboxymethyl cellulose (CMC) that exhibited specific enzyme activity with a purification fold of 69.50 U/mg and 3.01, respectively. It indicated that the purified protease in this study showed higher proteolytic activity than other reported pineapple proteases using anion exchange chromatography. The high proteolytic activity obtained in this study could be attributed to using 50 mM Tris-HCl buffer (pH 8) instead of 1 M acetate buffer (pH 4.5) at the ion exchange chromatography step. Novaes et al. (2016) reported that bromelain exhibited an enhanced capacity to bind to acid 8-aniline-1-sulfonic acid without tertiary contact at pH 8. Optimization of the extraction conditions is necessary to extract the protease enzyme from the main contaminants, thus greatly enhancing the purity of the enzyme extracted (Vicente et al., 2016).

Table 1

Purification steps of crown extract

Steps	Vol (ml)	Total protein (mg)	Total activity (Units)	Protein concentration (mg/ml)	Proteolytic activity (U/ml)	Specific activity (U/mg)	Fold purification	% yield
Step 1 (After crude extract)	200	640	25200	3.20	126.27	39.46	1.00	100
Step 2 (After anion exchange chromatography)	150	129	22060.50	0.86	147.09	171.03	4.33	0.87
Step 3 (After gel filtration chromatography)	0.50	0.24	75.47	0.48	150.90	314.38	1.84	0.03
Step 4 (After desalt)	0.50	0.42	72.01	0.84	144.02	171.45	0.55	0.95

After ion exchange chromatography, fractions pooled I (fractions D12-E6), II (fractions 2 A10-2B9), and III (fractions 2 C12-2 D9) (Figure 4) were used for the gel filtration chromatography step. The gel filtration chromatogram showed that the C10/C11 peak was eluted at 17 ml (Figure 5). After the gel filtration chromatography step, the concentration, proteolytic activity, and specific activity of the purified enzyme were 0.48 mg/ml, 150.90 U/ml, and 314.38 U/mg, respectively (Table 1). Based on these results, high solution concentrations make the enzyme more stable, resulting in decreased proteolytic activity, whereas low concentrations make the enzyme less stable, resulting in increased proteolytic activity. According to Hale (2005), low bromelain concentrations are more susceptible to spontaneous inactivation of proteolytic activity than concentrated bromelain solution. In addition, stem bromelain

was more stable than fruit bromelain and ananain. Plus, the higher number of steps involved in purification could remove more natural protease enzyme inhibitors, causing activity to rise (Bala et al., 2012).

Initially, during the purification process, it was observed that purified protease did not show a clear and distinct band on SDS-PAGE gel even though proteolytic activity was detected when assayed. It indicates that the purified proteases were actively being degraded and most likely were self-degrading. During gel filtration chromatography, ethylene diamine tetra acetic acid (EDTA) was added to the buffer to chelate metal ions by forming a stable complex with divalent ions, such as calcium ions (Ca^{2+}). EDTA is a cation-chelating agent that eliminates essential cations required for proteases. That way, EDTA inactivates protease activity so that the purification process will proceed with low or negligible proteolytic degradation, and the purified enzyme can be detected on an SDS-PAGE gel. However, EDTA must be removed before catalytic activity characterization so the protease can be activated again. Removal of EDTA from the purified sample was achieved by using desalting chromatography.

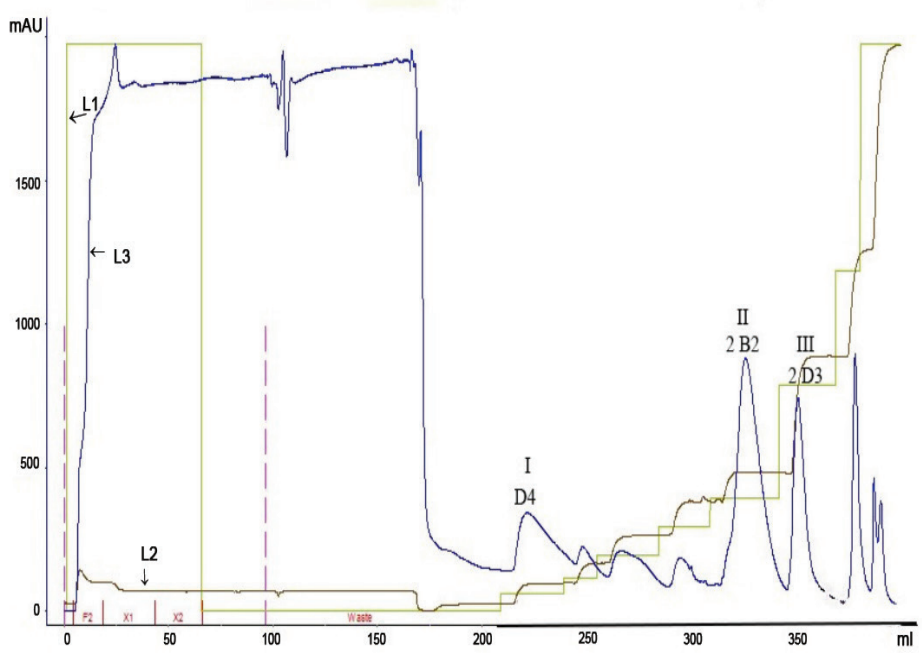

Figure 4. Anion exchange chromatography using step gradient for the crown extract. Green (L1), brown (L2), and blue (L3) lines represent the percent concentration of buffer B (%), the conductivity of protein (mS/cm), and protein sample flow. Y-axis represents the intensity of absorbance (mAU), and the x-axis represents the volume of the sample (ml)

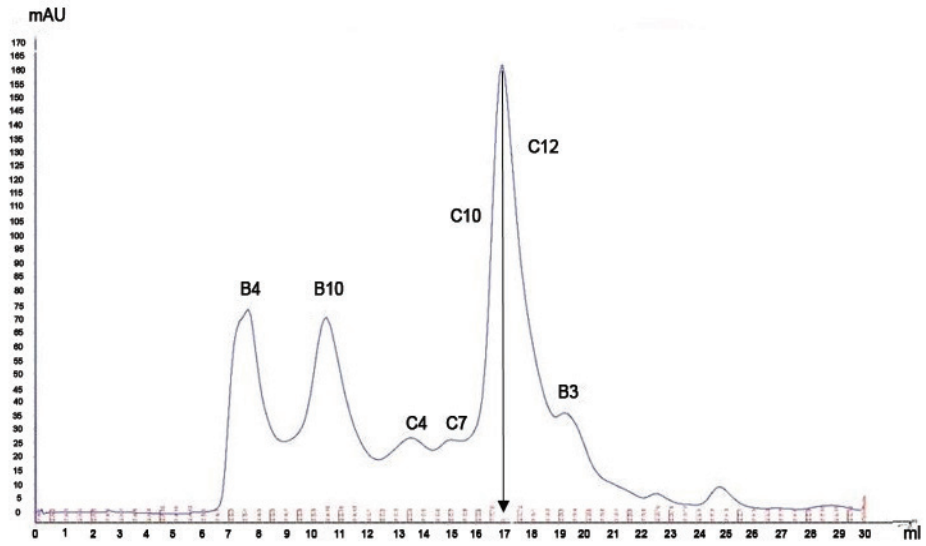

Figure 5. Gel filtration chromatography of crown extract. Y-axis represents the intensity of absorbance (mAU), and the x-axis represents the sample volume (ml). The arrow indicated the elution of purified enzyme at 17 ml

After desalting chromatography purification, the purified enzyme's proteolytic activity and specific activity decreased to 144.02 U/ml and 171.45 U/mg, respectively. The reduced activity could be due to the loss of enzyme during the desalting chromatography step and the inhibitory effect conferred by residual EDTA that may still be in the sample. Figure 6A depicts SDS-PAGE with 15% acrylamide for improved separation. Lane C was the crude sample, D4, D3, and B2 were from anion exchange chromatography peaks, and C10/11 was from gel filtration chromatography. The C10/C11 fraction has high protein band intensities with 25–32 kDa MW. The purified enzyme was then desalted, and a single band on SDS-PAGE in Lane I showed an MW of 25–32 kDa (Figure 6B). It demonstrates that it is a single polypeptide, and the protease was purified to apparent homogeneity. According to certain studies, protease enzyme isolated from the crown has a molecular weight of 25–27 kDa and a more intense band of 28–30 kDa (Bresolin et al., 2013; Ketnawa et al., 2012; Krishnan & Gokulakrishnan, 2015).

pH and Thermal Profiles

Based on a comparison among cultivars, crowns from the MD2 cultivar showed the highest proteolytic activity. Thus, the purified protease enzyme from the MD2 crown was used for further characterization. The effect of pH on proteolytic activity from the crown was measured and expressed as a relative protease activity against control. Purified protein from the MD2 crown exhibited the highest proteolytic activity at pH 7. Lower proteolytic activity was detected in acidic conditions (pH 4–5) and alkaline conditions (pH 8) (Figure 7). The

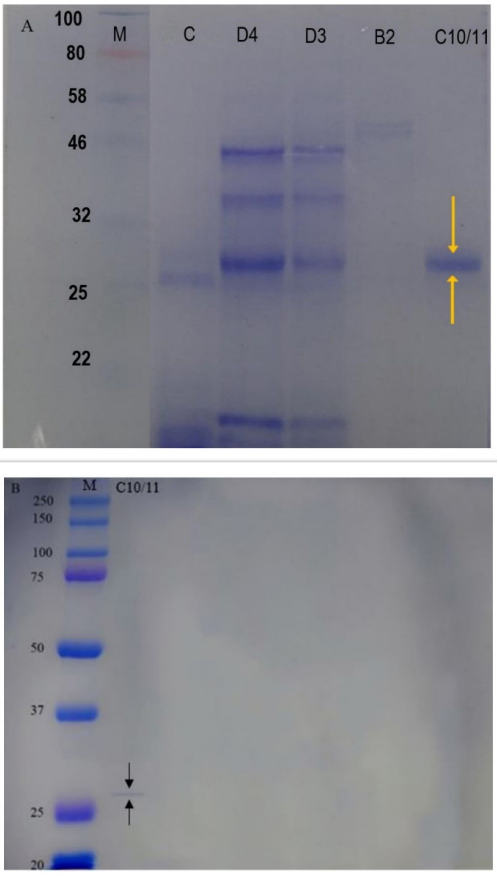

Figure 6. (A) 15% SDS-PAGE; C is the crude extract, D4, D3, and B2 are from anion exchange chromatography and C10/11 from gel filtration. (B) Desalting step of purified protein in the lane I. The arrow shows the band of the purified enzyme

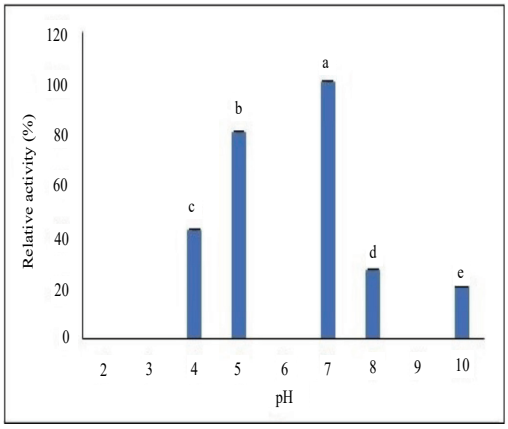

Figure 7. Effect of pH on the proteolytic activity of purified protein extracted from crown MD2 cultivar

Note. Different alphabetical letters indicate the significant differences (p>0.05)

major components of protease enzyme in pineapple were stem bromelain (80%), fruit bromelain (10%), and ananain (5%) (Kahiro et al., 2017). The types of protease enzymes depend on the extracted part of the fruit. Some researchers found that the activation of protease enzyme was at the pH range of 3–7 for all parts of pineapple and showed high proteolytic activity and specific activity at pH 7 and gradually declining after attaining the optimum pH (Ketnawa et al., 2012; Krishnan & Gokulakrishnan, 2015; Novaes et al., 2016; Omotoyinbo & Sanni, 2017). However, the proteolytic activity of fruit bromelain was found to be optimum at pH 4.5 with 4.429 U/ml (Mohan & Sivakumar, 2016), whereas stem bromelain recorded the optimum pH in the range of 7.5–10 (Han et al., 2018). In addition, peel and pulp extracts exhibited optimum pH at 7.5 and slightly decreased when pH changed to alkali or acidic (Indrajeet et al., 2017). Pineapple leaf and pulp also recorded the maximum activity at pH 6 and 8, respectively (Ramalingam et al., 2012). It revealed that different extracted parts of pineapple possessed different optimum pH due to the surface charge on the adsorbent materials (Omotoyinbo & Sanni, 2017).

However, no activity was recorded at pH 3, 6, and 9. Different pHs affect the enzyme's structure and enzyme activity by changing the ionization state of basic or acidic amino acids. Basic amino acids

have amine-containing functional groups, whereas acidic amino acids have carboxyl functional groups on their side chains. The ionic bonds hold three dimensional of a protein; therefore, if the ionized state of the amino acids in the protein changes, the protein structure will also alter. It may result in the inactivation of enzymes or alterations in protein function. Thus, extremes of acidity (pH 2 to 3) or alkalinity (pH 9) can cause the total loss of enzyme activity. Aside from that, no activity was shown at pH 6. However, adjusting the pH of the reaction mixture to the optimal pH restored the highest activity observed at pH 7. The pH of the reaction mixture may promote substrate dissociation and hence impact activity by its action on the substrate (Piper & Fenton, 1965).

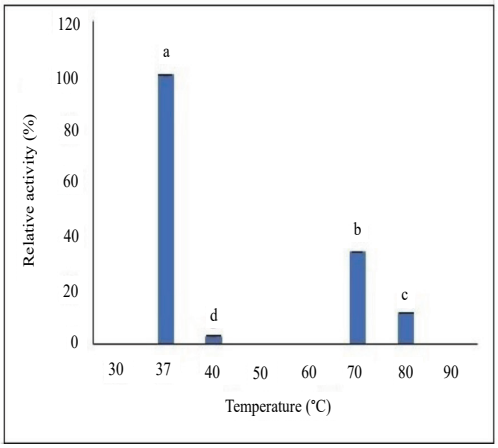

Figure 8. Effect of temperature on the proteolytic activity of purified protein extracted from crown MD2 cultivar

Note. Different alphabetical letters indicate the significant differences (p>0.05)

The temperature condition for activity was between 30–40°C (Figure 8). As the temperature rises, more molecules have adequate kinetic energy to react up to the optimal point, when the peptide bonds begin to degrade, resulting in protein misfolding and structural alteration (Han et al., 2018; Ketnawa et al., 2012). As a result of the irreversible inactivation of the enzyme, the proteolytic enzyme, and its function decreased (Ataide et al., 2018; Soares et al., 2012). Hence, bromelain has optimum activity ranging from 30 and 40°C. As a result, the purified enzyme showed an optimum pH of 7 and temperature between 30–40°C with a molecular weight of 25 to 32 kDa. However, the fruit bromelain showed an optimum pH of 7 and a temperature between 30 and 40°C with a molecular weight of 24.5 kDa (Ferreira et al., 2011). Even though the purified protein seemed to resemble fruit bromelain, the activity was drastically reduced at temperatures ranging from 40 to 60°C. It showed that the purified protein was not a glycosylated enzyme (Mamo & Assefa, 2019). Generally, stem and fruit bromelains consist of 1–2 sites of glycosylation (Ramli et al., 2018). Thus, the purified protein was most likely to be ananain as it was a non-glycosylated protein, while comosain differed from ananain but had similar carbohydrate composition closely resembling stem bromelain (Napper et al., 1994). Unlike stem bromelain and fruit bromelain, the purified protein from the crown more resembled ananain. The purified protein was then identified via LC-MS.

LC-MS

The use of LC-MS is to provide protein identification information and quantitative measurements in proteomic research. From this analysis, five unique sequences (Query ID: 55577, 46136, 15596, 35225, and 36722) were subjected to sequence analyses using NCBI BLASTP, which revealed that 100% of the amino acids sequences were identical with the sequences of ananain of *Ananas comosus* from the family Bromeliaceae under accession number OAY80104.1 (according to NCBI) or AOA199VSS3 (according to Uniprot) (Table 2). The five unique peptide sequences (in colors) show that 100% of sequences were identified with the full sequences of ananain of *Ananas comosus* from the family Bromeliaceae under accession number AOA199VSS3 (Figure 9). It covered 97/356 amino acids with 44.9% coverage. In addition, this study revealed that the ananain from *Ananas comosus* (AOA199VSS3) had the closest relationship with ananain (accession number AOA199UL32, AOA199UUY2, and AOA199W8N4), fruit bromelain (AOA199VSB5, AOA199W3D2, AOA199W9F2, and AOA199W8R4), bromelain (O24641), and papain (P0074.8).

Thus, the phylogenetic relationships of 10 proteases were constructed (Figure 10). Based on the MEROPS database (the peptidase database), the evolutionary tree indicated that family C1 was categorized into two subfamilies: C1A and C1B, which are closely related to the papain and bleomycin hydrolase subfamily, respectively. Ananain (EC 3.4.22.31) was classified in the subfamilies of C1A, a peptidase family related to papain (Figure 10). The molecular weight of ananain is in the range of 20 to 35 kDa and, thus, grouped in the clan CA (superfamily cysteine protease). In conclusion, a comparative analysis of this sample revealed that the purified enzyme had highly matched with the sequences of ananain from *A. comosus* under accession number A0A199VSS3 (according to Uniprot). It has five unique peptides and covers 97/356 amino acids (44.9% coverage). Thus, the purified enzyme extracted from pineapple crown was ananain-like protease, one type of cysteine protease like other papain family members.

Table 2

Identification of five unique peptides sequences from the purified enzyme

No.	Query ID	Sequences	Read length	Total score	% identity
1.	55577	KGILEPLSEQQVLDCAK	17	56.6	100 % OAY80104.1 (Ananain)
2.	46136	GVASGAIYPYK	11	37.1	100 % OAY80104.1 (Ananain)
3.	15596	QPITVAVDANANFQYYK	17	58.7	100 % OAY80104.1 (Ananain)

Table 2 *(Continue)*

No.	Query ID	Sequences	Read length	Total score	% identity
4.	35225	SGVFNGPCGTSLNHAVTAIGYGQDSNGKK	29	91.8	100 % OAY80104.1 (Ananain)
5.	36722	DVSSSSGICGIAIDSLYPTLESR	23	73.6	100 % OAY80104.1 (Ananain)

MAWKVQLVFLFLFLCVMWASPSAASADEPSDPMMKRFEEWMVEYGRVYKDNDEKMRRFQIFKNNV
NHIETFNSRNENSYTLGINQFTDMTNNEFIAQYTGGISRPLNIEREPVVSFDDVDISAVPQSIDWRDYGA
VTSVKNQNPCGACWAFAAIATVESIYKIKKGILEPLSEQQVLDCAKGYGCKGGWEFRAFEFIISNKGVAS
GAIYPYKAAKGTCKTNGVPNSAYITGYARVPRNNESSMMYAVSKQPITVAVDANANFQYYKSGVFNGP
CGTSLNHAVTAIGYGQDSNGKKYWIVKNSWGARWGEAGYIRMARDVSSSSGICGIAIDSLYPTLESRAN
VEAIKMVSESRSSV

Figure 9. The full sequences of ananain of *Ananas comosus* under the accession number AOA199VSS3. The colors indicated the five unique peptides sequences (Query ID: 55577, 46136, 15596, 35225, and 36722)

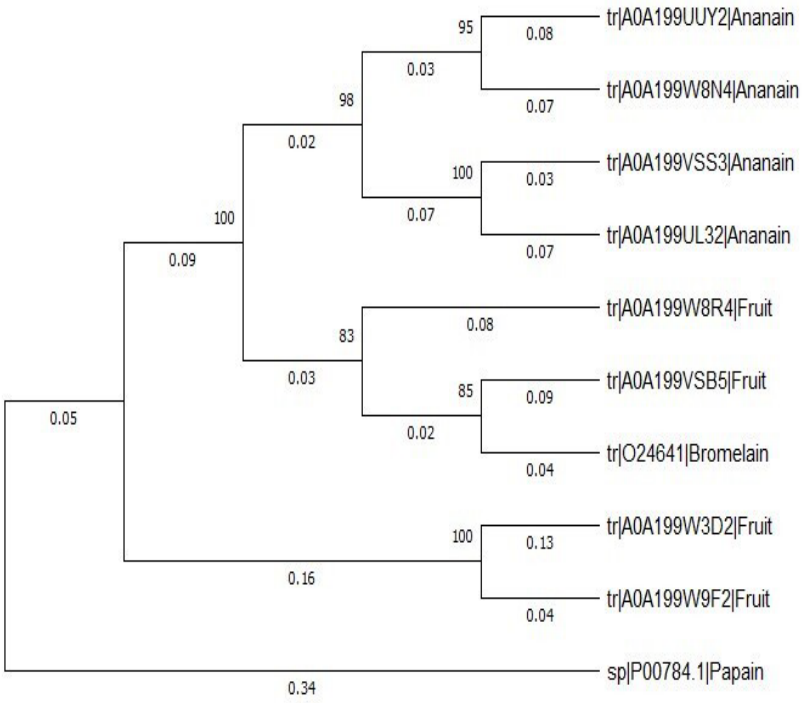

Figure 10. The phylogenetic relationship of ananain from *Ananas comosus*

CONCLUSION

The proteolytic activity of the MD2 crown was 126.0 ± 3.86 U/ml, the specific activity was 3937.50 U/mg, and the specific activity in 1 kg of waste was 3.94×10^9 U. Therefore, in 50 tonnes of a crown, it is possible to obtain 1.97×10^{14} U of bromelain. MD2 crown recorded the highest proteolytic activity, as shown by larger halo zone formation on both casein and skim milk plates. As a result, the MD2 cultivar was the best cultivar with the most proteolytic activity. The purified enzyme (ananain) extracted from the pineapple's crown contained high proteolytic activity and specific activity of 144.02 U/ml and 171.43 U/mg, respectively. Purification of the extract yielded a band at a molecular weight of 25–32 kDa MW. The optimum conditions for the proteolytic activity of this purified enzyme were pH 7 at 37°C. The purified protein was characterized, and LC-MS confirmed that it resembled more ananain than comosain, stem bromelain, or fruit bromelain. The sample highly matched with ananain from *A. comosus*, under accession number A0A199VSS3 (according to Uniprot), and it has five unique peptides and covered 97/356 amino acids (44.9 percent coverage). Ananain (EC 3.4.22.31) was classified in the subfamilies of C1A, a peptidase family related to papain. The molecular weight of ananain is in the range of 20 to 35 kDa and, thus, grouped in the clan CA (superfamily cysteine protease). Thus, the purified enzyme extracted from pineapple crown was ananain-like protease, one type of cysteine protease like other papain family members.

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Effects of Spacing on Early Growth Rate and Yield of Hybrid *Eucalyptus* Stands

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ABSTRACT

Optimizing tree spacing in a forest plantation is one of the main management techniques to improve stand quality and productivity. Its influence on growth from an early age is an important matter for forest management. This study aims to evaluate the effect of tree spacing on early growth rate and yield over time in *Eucalyptus grandis* × *Eucalyptus camaldulensis* hybrids. The data were obtained from an experiment in Itamarandiba, Minas Gerais, Brazil. The plots were composed of five planting spacing (3.00 m × 0.50 m, 3.00 m × 1.00 m, 3.00 m × 1.50 m, 3.00 m × 2.00 m, and 3.00 m × 3.00 m) measured at the ages of 7, 12, 24, 36, 48, 61, 77, 85, and 102 months. Growth and yield were analyzed by fitting the Gompertz model and a baseline exponential model up to 36 months of age to evaluate the influence of early growth on the harvest age. A Pearson correlation matrix was also generated to find out the relationship between the mean annual increment in the respective treatments during the studied period. It was observed that a positive correlation in the average annual increase in the 3 × 2 and 3 × 3 spacings. It was verified that tree spacing influenced the yielded wood volume and the optimal harvest age. The early growth rate influences the optimal

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harvest, which may explain a possible loss of yield during the productive cycle of the forest stand.

Keywords: Growth and yield modeling, harvest planning, mean annual increment, optimal harvest age

INTRODUCTION

Brazil is one of the major suppliers of forest products and by-products (da Silva et al., 2017). The Brazilian forest-based industry has been highlighted for presenting the highest productivity with a short-rotation intensive culture (Moreira et al., 2017). *Eucalyptus* is an especially important species in Brazil due to its rapid growth, low-density canopy shape, and multiple uses for its wood (Souza et al., 2020). *Eucalyptus* timber is used for various purposes, such as producing timber plates, plywood, charcoal, wood foils, sawn timber, pulp, furniture, and building purposes (Gonçalves et al., 2008; Instituto Brasileiro de Geografia e Estatística [IBGE], 2021).

The growth rate of a forest stand is determined by factors such as the site`s productive capacity species, number, and spatial distribution of trees. It is also linked to soil conditions, as high salt concentrations can cause reductions in plant growth and productivity, such as for early growth eucalyptus clones (Lacerda, 2016). These characteristics need to be accounted for by the forest manager to select silvicultural treatments and harvest strategies (Clutter et al., 1983).

Mathematical models are often used to predict the growth and yield of a forest stand to aid in the decision-making process. Models are especially useful for reasoning, forecasting, and decision-making and aim to abstract and summarize the problem in real life (Buongiorno & Gilless, 2003). These models must be dependable to describe as precisely as possible the complex dynamics of the nonlinear system that is the growth of forest stands (Porté & Bartelink, 2002). The accuracy of growth estimates directly affects forest planning (Bettinger et al., 2017; Campos & Leite, 2017).

The spacing and spacing arrangement definition directly reflect the silvicultural activities in the stands. These activities influence the availability of resources for plant growth, forest management, wood quality, and production costs (Corrêa et al., 2020; de Oliveira Neto et al., 2003; Moulin et al., 2017).

Some factors must be taken into account to select the best number of trees per unit area: the population density, the local productive capacity, intended wood product, technological aspects (e.g., the equipment to be used for harvesting), silvicultural, ecological, and economic aspects, represented by climate, soil, relief, condition with higher or lower plantation density and management (Campos & Leite, 2017; Salles et al., 2012). At high-density spacings, the growth resources become limited more rapidly, resulting in suppressed individuals and a low population growth rate at relatively young ages (Soares et al., 2004).

In addition, the growth in diameter might also be influenced by the tree spacing, where the larger the spacing, the less competition among the trees and, thus, the greater development in diameter of the trees (Berger

et al., 2002; Cockerham, 2004; dos Santos Leles et al., 2001; Pinkard & Neilsen, 2003).

Denser spacings generally provide trees with a smaller diameter and lower survival percentage but higher basal area per hectare and higher total volume per hectare (Stape & Binkley, 2010). However, denser plantations result in smaller trees, shorter rotation, and higher costs of mechanized harvesting, as tree spacing is one factor that defines the trees' growth rate. Meanwhile, the effects of tree spacing on early growth rates and final yields of hybrid *Eucalyptus* stands have not yet been decided. Based on the above, the study aimed to test if the spacing between eucalyptus trees affects the final yield evaluated from the early growth rate (less than 2 years). This study aims to evaluate the effects of spacing on early growth rate and yield over time in *Eucalyptus grandis* × *Eucalyptus camaldulensis* hybrid stands.

MATERIAL AND METHODS

Study Site and Experiment Design

The dataset for the development of the study was from a tree spacing experiment installed in a hybrid *E. grandis x E. camaldulensis* stand in Itamarandiba, in the Jequitinhonha Valley, Minas Gerais, Brazil (17.86° S latitude and 42.86° W longitude). The average annual temperature is 21.2°C, and the climate is tropical altitude with two well-defined seasons. The region has an altitude ranging from 645 to 1,648 m, with an average rainfall of 1,130 mm per year (Alvares et al., 2013).

The experiment started in 2002 and used a randomized block design with five treatments (spacing) and three blocks. Each experimental plot had six planting rows with 28 trees (168 trees) with a distance between rows equal to 3 m. The distance within rows varied according to the treatment and was defined as 0.5, 1.0, 1.5, 2.0, and 3.0 m. A double border was used with 48 measurable trees in each plot (Table 1). The detailed experiment design and dendrometry data by age and treatment in hybrid stands of *E. grandis* × *E. camaldulensis* are shown in Table S1.

Data Collection, Height, and Volume Estimates

The plots were measured at ages 7, 12, 18, 24, 36, 48, 61, 77, 85, and 102 months. In these ages, the circumference at the breast

Table 1
Experiment design and treatments to study the effects of spacing on the early growth rate and yield over time in hybrid Eucalyptus *stands*

Treatment	Spacing (m × m)	Area per tree (m²)	N° of tree in the block	N° of the tree on the border	N° of measurable tree per plot	Plot area (m²)
1	3.00 × 0.50	1.5	168	120	48	72
2	3.00 × 1.00	3.0	168	120	48	144
3	3.00 × 1.50	4.5	168	120	48	216
4	3.00 × 2.00	6.0	168	120	48	288
5	3.00 × 3.00	9.0	168	120	48	432

height of all trees in each experimental plot was measured using a measuring tape. From the age of 48 months onward, the heights of the first 12 normal trees in each plot were measured (Paulino, 2012). A Haglöf hypsometer (Sweden) was used to measure these heights. A hypsometric equation (Equation 1) was fitted to estimate the tree heights that were not measured.

A total of 245 sample trees were scaled by measuring the diameter at every 1-meter section of the stem and calculating their total stem volumes using Smalian's formula. The data from these trees fit the Schumacher and dos Santos Hall (1933) model to estimate the volume for all trees in the plots (Equation 2).

$$LnHt = \beta_0 + \beta_1 Dbh^{-1} + \varepsilon \quad (1)$$

$$LnV = \beta_0 + \beta_1 LnDbh + \beta_2 LnHt + \varepsilon \quad (2)$$

where, V = Volume (m^3); Dbh = Diameter at breast height (cm); Ht = Total height (m); Ln = Natural logarithm; $\beta_0, \beta_1, \beta_2$ = Regression coefficient; and ε = Random error

Growth and Yield Modeling

The growth and yield model were developed using the measurements from 7 to 102 months old stands. The Gompertz model (Gompertz, 1833) (Equation 3) was fitted for each treatment in the experiment. The Gompertz model is the most suitable for analyzing growth as a function of age for eucalypt stands (Reis et al., 2022):

$$Y = \beta_0 e^{-e\beta_1 - \beta_2 A} \quad (3)$$

where, Y = Estimated volume outside bark; $\beta_0, \beta_1, \beta_2$ = Regression coefficients ; and A = Age (months)

The equations to generate the current monthly increment (CMI) and acceleration curves were obtained through the first (Equation 4) and second (Equation 5) derivatives of the Gompertz model, respectively. The curves were created by treatment.

1st derivative of the Gompertz model:

$$Y' = \beta_0 \beta_2 e^{\left(-e^{(\beta_1 - \beta_2 X)} - \beta_2 A + \beta_1\right)} \quad (4)$$

2nd derivative of the Gompertz model:

$$Y'' = \beta_0 \beta_2 (\beta_2 e^{(\beta_1 - \beta_2 A)} - \beta_2) e^{\left(-e^{(\beta_1 - \beta_2 A)} - \beta_2 A + \beta_1\right)} \quad (5)$$

The model was evaluated using the correlation $ry\hat{y}$ between observed and estimated volume (Equation 6), percent root mean square error (RMSE%) (Equation 7), and bias (Equation 8). The scatterplots between observed and estimated values were also analyzed.

$$ry\hat{y} = \frac{n^{-1}\sum_{i=1}^{n}(\hat{Y}_i - \hat{Y}_m)(Y_i - \overline{Y})}{\sqrt{n^{-1}\sum_{i=1}^{n}(\hat{Y}_i - \hat{Y}_m)^2 n^{-1}\sum_{i=1}^{n}(Y_i - \overline{Y})^2}} \quad (6)$$

$$RMSE\% = 100\overline{Y_i}^{-1}\sqrt{\frac{\sum_{i=1}^{n}(\hat{Y}_i - Y_i)^2}{n}} \quad (7)$$

$$bias = \frac{1}{n}\sum_{i=1}^{n}\left(\hat{Y}_i - Y_i\right) \quad (8)$$

where, Y_i and $\hat{Y}_i$ = Observed and estimated volumes, respectively; $\overline{Y}$ and $\hat{Y}_m$ = Mean

of the observed and estimated values, respectively; n = Number of cases

Assessment of Early Growth Rate and Yield

The exponential model was fitted for each plot and treatment for the early ages of 7 to 36 months to evaluate the influence of the early growth rate on the mean annual increment (MAI) 6 (MAI 6) and 7 years (MAI 7). The parameter estimates were then related to yield at 6 and 7 years.

$$Y = \beta_0 A^{\beta_1} \quad (9)$$

where, Y = Yield (m^3 ha^{-1} $year^{-1}$); β_0, β_1 = Parameters of the equation; A = Age in months, up to the age of 3 years (36 months)

An exponential reference model, up to 36 months of age, was fitted with a higher early growth rate. Finally, the influence of early growth on the cutting age was evaluated by comparing parameter β_1s with MAI 6 and MAI 7.

A Pearson correlation matrix was generated in R studio (R Core Team, 2020) using the corrplot package (Wei et al., 2021), and the Shapiro-Wilk normality test was performed using the nortest package (Gross & Ligges, 2015) for each treatment to figure out the relationship between MAI in the respective treatments over the studied period. In this way, it was possible to infer in each treatment if the early yield has any positive relationship with the final yield.

RESULTS

It was observed that all treatments slightly underestimated the total volume. Treatment 4 had the highest bias, and Treatment 2 had the highest RMSE%. The lowest RMSE% was obtained in Treatment 5. All treatments showed a Pearson correlation between observed and estimated volume higher than 98%. The model results and parameters for each treatment can be seen in Table 2, and its distribution graph is in Figure 1.

Figure 2 illustrates the growth curves obtained for the outside bark wood volume as a function of the age in months, the current monthly increment (CMI), mean

Table 2
Parameter estimates and performance statistics for the Gompertz model for each treatment in hybrid eucalypt stands

Treatments	Spacing (m × m)	Parameters					
		β_0	β_1	β_2	Bias	RMSE%	Ryy′
1	3.00 x 0.50	299.3482	1.3215	0.0578	-1.2246	15.2907	0.9896
2	3.00 x 1.00	293.994	1.4198	0.0476	-1.1218	16.2654	0.9878
3	3.00 x 1.50	296.6093	1.467	0.0462	-0.8735	14.9302	0.9898
4	3.00 x 2.00	278.3436	1.4564	0.0479	-1.2320	15.9876	0.9872
5	3.00 x 3.00	252.1766	1.5925	0.0461	-0.6413	7.8878	0.9962

Note. $\beta_0, \beta_1, \beta_2$ = Parameters of the equation $Y = \beta_0 e^{-e^{\beta_1 - \beta_2 A}}$, where Y = volume outside bark (m^3/ha) and A = age (months); RMSE% = Percentage of root mean square error; Ryy′ = Correlation between the observed and estimated volume

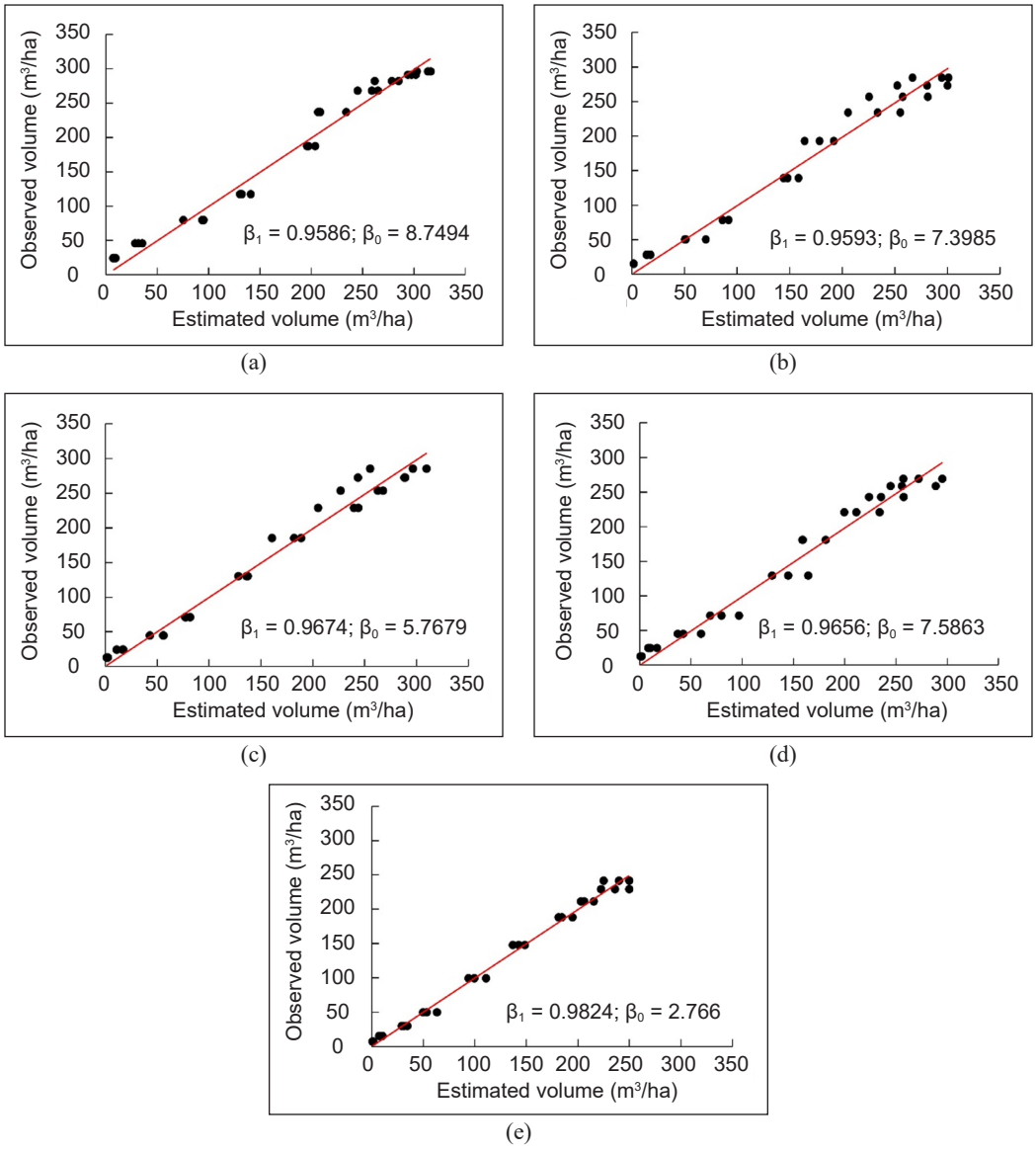

Figure 1. Distribution of observed volumes (m³/ha) on the y-axis and estimated volumes (m³/ha) on the x-axis by treatment for the ages of 7 to 102 months in hybrid eucalypt stands: (a) Treatment 1; (b) Treatment 2; (c) Treatment 3; (d) Treatment 4; and (e) Treatment 5

monthly increment (MMI), and Acceleration curves. The growth acceleration curve is null when the maximum current monthly increment (MCMI) is reached, and they must occur at the same age.

The null hypothesis of normality was not rejected in 33 of 35 cases, with $0.1041 \leq p\text{-value} \leq 0.9869$. In the other three cases, the *p*-values were 0.0289, 0.0308, and 0.0372 (Table S3). The highest positive

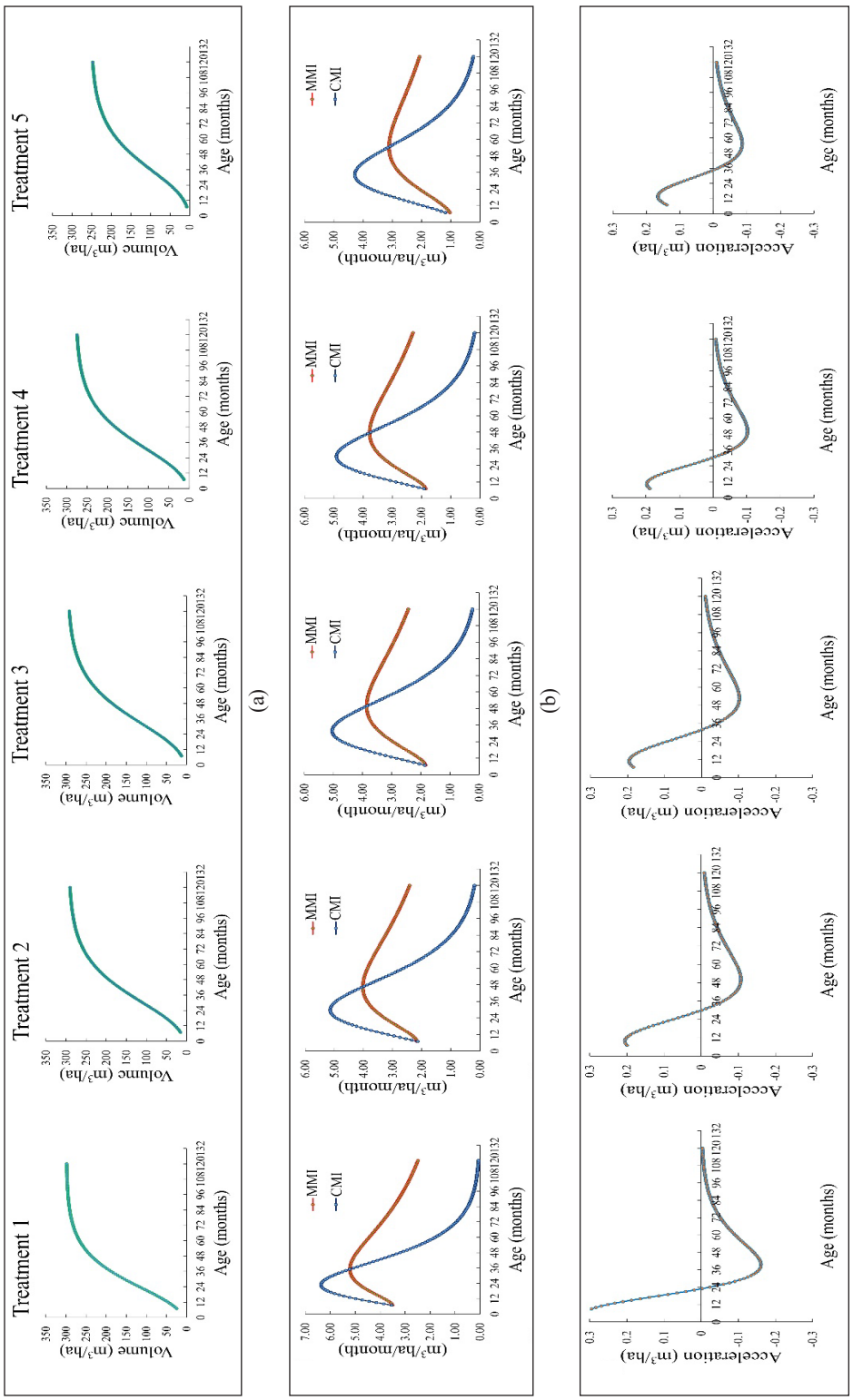

Figure 2. Relationship between growth and yield for each treatment studied in hybrid eucalypt stands: (a) Production; (b) Current monthly increment (CMI), Mean monthly increment (MMI); and (c) Acceleration curves

correlations were observed for Treatments 4 and 5, representing 3 × 2 and 3 × 3-meter spacings (Figure 3).

Table 3 shows the parameter estimates for IMA6 and IMA7. The parameter β_1 of the exponential model indicates the growth rate. Treatment 1 had the lowest parameter value and the highest yield and Treatment 5 had the highest and lowest yield. As shown in Figure 4, the trend was for a lower yield (i.e., lower MAI6 and MAI7) when the parameter β_1 value was higher (i.e., higher early growth rates). This behavior was both for increases at ages 6 and 7.

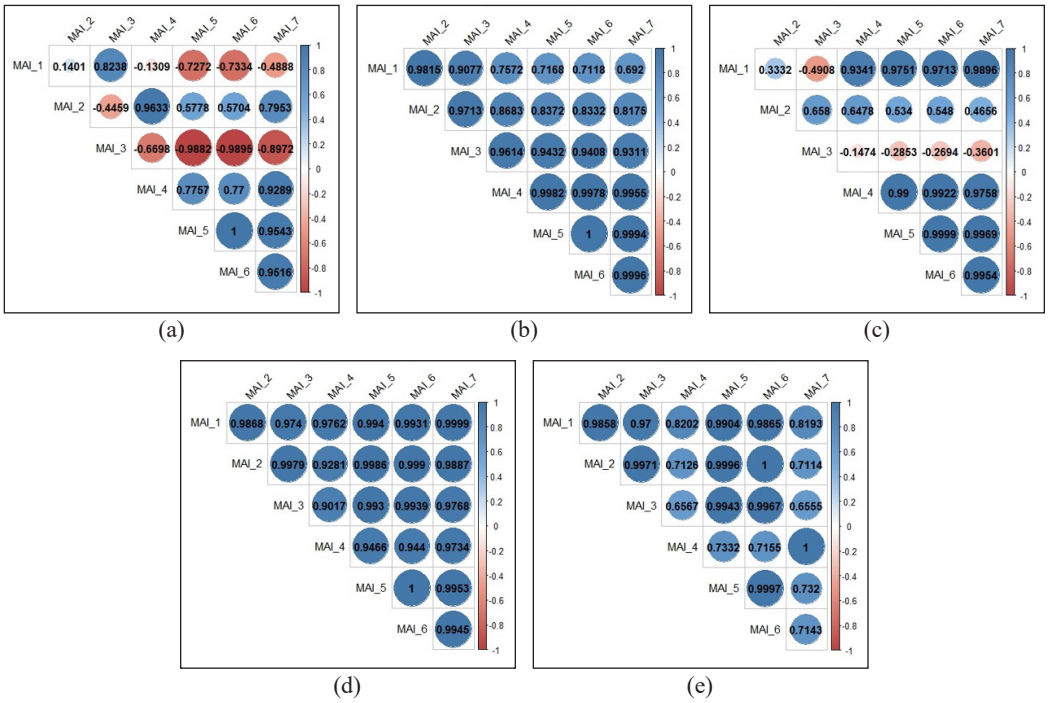

Figure 3. Pearson correlation matrices for mean productivities in the ages of 1 to 7 years in hybrid eucalypt stands: (a) Treatment 1; (b) Treatment 2; (c) Treatment 3; (d) Treatment 4; and (e) Treatment 5

Table 3
Estimation of parameter β_1 of the exponential model and mean annual increment at 6 and 7 years in hybrid eucalypt stands

Treatment	Spacing (m × m)	Plot	β_1	MAI 6	MAI 7
1	3.00 × 0.50	1	0.53	47.5	42.6
1	3.00 × 0.50	6	0.49	43.7	41.5
1	3.00 × 0.50	11	0.54	46.4	42
2	3.00 × 1.00	2	0.7	46.9	42.4
2	3.00 × 1.00	7	0.74	42.9	39.6
2	3.00 × 1.00	12	0.69	37.6	35.6
3	3.00 × 1.50	3	0.76	44.6	40.8

Table 3 *(continue)*

Treatment	Spacing (m × m)	Plot	β_1	MAI 6	MAI 7
3	3.00 × 1.50	8	0.83	43.8	40.7
3	3.00 × 1.50	13	0.7	37.8	34.4
4	3.00 × 2.00	4	0.68	42.9	40.7
4	3.00 × 2.00	9	0.73	39.2	36.1
4	3.00 × 2.00	14	0.75	37.3	34.5
5	3.00 × 3.00	5	0.82	35.9	35.3
5	3.00 × 3.00	10	0.81	34.4	31.4
5	3.00 × 3.00	15	0.89	33.8	33.3

Note. β_1 = Parameter of the equation $MAI_i = \beta_0 A^{\beta_1}$, where *MAI* = Mean annual increment (m³/ha/year) at 6 and 7 years and A = Age in months, up to the age of 3 years (36 months)

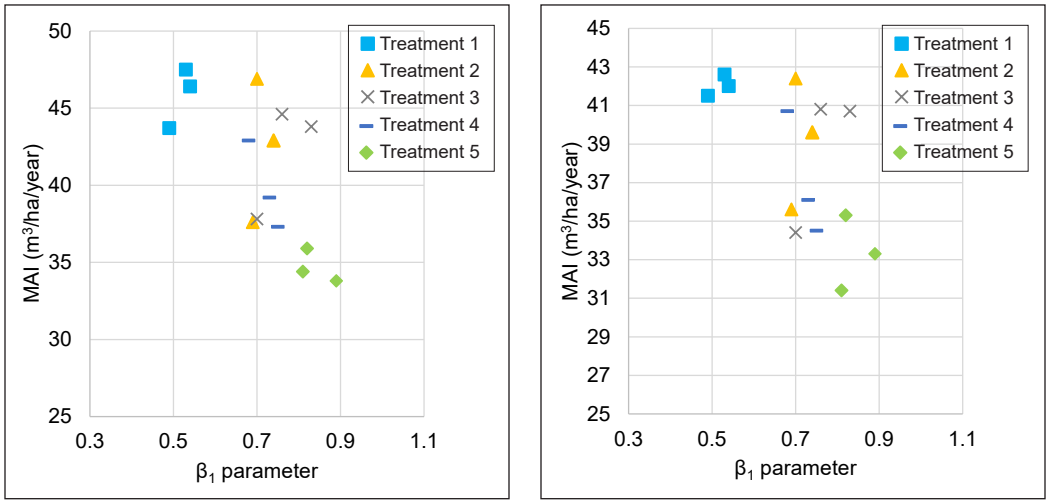

Figure 4. Correlation analysis of parameter (β_1) of the exponential model with mean annual increment (MAI) in hybrid eucalypt stands at (a) 6 and (b) 7 years, respectively

DISCUSSION

This study evaluated the effect of tree spacing on early growth rate and final yield. It was observed that distance within trees in a row influences turnover and yield. Using inventory data in ages lower than two years can lead to inefficiencies in modeling. It, associated with economic issues, reinforces the proposal to carry out a continuous forest inventory of *Eucalyptus* stands from two years of age.

Growth and yield were evaluated by fitting the Gompertz model and found that it was unbiased and allowed the evaluation of the characteristics of each treatment, such as optimal harvest age, zero acceleration age, and MCMI, and to analyze the effect of spacing. Leite et al. (2006), studying

Pinus taeda stands, concluded that spacing influences the volume growth trend per hectare as a function of fertilization and tree spacing; they also observed higher volume yield per unit area at smaller spacings in *E. camaldulensis* stands (de Oliveira Neto et al., 2003). However, Schneider and Schneider (2008) affirmed that the total volume per hectare increases with the number of trees until a certain planting density is reached (critical density), after which the increase in the number of trees causes the reduction of the total volume per hectare due to mortality for competition.

With the increase in tree spacing, it is expected a delay in the optimal harvest age (MMI = CMI). It was observed in Treatments 1, 2, 3, and 5, where the optimal harvest age is about 34 months for Treatment 1, 42 months for Treatment 2, 49 months for Treatment 3, and about 59 months of age for Treatment 5.

Treatment 4 had an unexpected behavior because an ideal harvest age between 49 and 59 months was expected. According to the intersection point of the MMI = CMI curves, the optimal harvest age is 48 months for Treatment 4. On the other hand, Treatment 1 presents the intersection point at 34 months. It may be explained due to the denser spacing in Treatment 1, so the age for harvest tends to be early. More details can be found in Table S2.

Treatments 1, 3, and 5 had an expected behavior where the null acceleration must coincide with the same period of the MCMI. Treatment 1 was at 31 months, Treatment 3 at 32 months, and Treatment 5 at 43 months of age. On the other hand, Treatments 2 and 4 did not have similar behavior. They did not present the same period of null acceleration with the MCMI. Treatment 2 had the maximum increment at 31 months and the null acceleration at 29 months. Whereas, for Treatment 4, the maximum increment was observed at about 29 months and null acceleration at 30 months. Furthermore, relatively low correlations were observed between early yield (1 and 3 years) and yield at 6 and 7 years.

Positive correlations were seen only in Treatments 4 and 5, characterized by spacings 3 × 2 and 3 × 3 meters within rows. It is possible to infer that an increase in the yield at early ages results in an increase in the yield at final ages and, therefore, a reduction in the rotation period. It shows the risk when selecting genotypes based on clonal tests or other types of experiments based on the results of measurements made at early ages, mainly before 3 years.

This trend, however, may not occur in a high-quality site. In this case, the environment supports high growth rates for longer. On the other hand, if the environment is not able to support the early growth rate, there will be a possible reduction in yield at the harvest age (7 years).

Figure 4 shows the estimates for the parameter β_1 in the function of the yield at 7 years, where the higher the value of the parameter (i.e., higher early growth rate), the lower the MAI 7 in relation to the maximum mean annual increment (MMAI) (about 3 years). However, this trend may not occur if the site's quality is extremely high.

Genetic materials with high early growth rates present a greater difference between MMAI and MAI 7 regardless of the tree spacing, with MAI 7 < MMAI. Plots with denser tree spacing tended to have a higher total yield which is also observed in the works of Corrêa et al. (2020) as well as Watzlawick and Carla Benin (2020), with a shorter cutting cycle. Nonetheless, it is important to consider wood use, where a greater total yield does not imply a higher economic return. For instance, a higher total yield may imply higher economic return for energy purposes since there is no effect on wood density and calorific value. Thus, choosing genetic materials with lower early growth rates based on site quality may be an alternative for forest-based companies to reduce yield losses at the end of the cutting cycle.

The growth of a forest stand depends on the genetic material, age, productive capacity, the degree of utilization of the productive potential of the site, silvicultural treatments, and the most relevant environmental factors are the availability of water for the plants throughout the years, temperature, and solar radiation (Campos & Leite, 2017; de Alcantra et al., 2018). The clones developed in Minas Gerais showed a good adaptation to the environmental conditions of the site, with high production (dos Santos et al., 2017).

CONCLUSION

Tree spacing directly affects eucalypt stands growth rates. This study found that distance between trees affects the early growth rate of *Eucalyptus* trees for a fixed distance between rows. It is especially highlighted in early ages (< 2 years) and was noted for the most common tree spacing designs used in eucalyptus plantations. The results suggest that growth and yield models can be affected using datasets collected early in the growth stages. Therefore, forest yield prognosis using only data from early age inventory can have relatively low accuracy. It highlights the importance of continuously collecting inventory data, especially using information from stands older than 2 years to infer the growth and yield of eucalyptus forest plantations.

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APPENDICES

Supplementary Table 1
Dendrometric data by age and treatment in hybrid eucalypt stands

Treatment	Spacing (m × m)	Age (months)	QMD (cm)	BA (m^2/ha)	Volume (m^3/ha)
1	3.00 × 0.50	7	2.77	4.02	7.85
1	3.00 × 0.50	12	4.00	8.38	31.63
1	3.00 × 0.50	18	5.52	16.00	87.97
1	3.00 × 0.50	24	6.36	21.15	134.51
1	3.00 × 0.50	36	7.08	24.69	199.00
1	3.00 × 0.50	48	7.38	26.92	216.42
1	3.00 × 0.50	61	7.84	29.72	256.37
1	3.00 × 0.50	72	8.04	31.02	275.18
1	3.00 × 0.50	85	8.27	32.77	297.89
1	3.00 × 0.50	102	8.57	33.64	310.96
2	3.00 × 1.00	7	3.04	2.30	1.82
2	3.00 × 1.00	12	4.65	5.68	15.60
2	3.00 × 1.00	18	6.78	12.08	57.47
2	3.00 × 1.00	24	7.65	15.32	88.24
2	3.00 × 1.00	36	8.78	18.91	150.33
2	3.00 × 1.00	48	9.22	21.00	178.31
2	3.00 × 1.00	61	9.82	23.29	231.40
2	3.00 × 1.00	72	10.04	24.56	254.77
2	3.00 × 1.00	85	10.42	25.86	277.71
2	3.00 × 1.00	102	10.83	26.64	287.46
3	3.00 × 1.50	7	3.07	1.66	1.94
3	3.00 × 1.50	12	5.00	4.38	15.04
3	3.00 × 1.50	18	7.33	9.40	51.70
3	3.00 × 1.50	24	8.32	12.08	78.75
3	3.00 × 1.50	36	9.87	16.38	133.90
3	3.00 × 1.50	48	10.60	19.07	177.12
3	3.00 × 1.50	61	11.25	21.05	229.49
3	3.00 × 1.50	72	11.44	21.86	252.46
3	3.00 × 1.50	85	11.92	23.62	273.62
3	3.00 × 1.50	102	12.37	24.32	287.16
4	3.00 × 2.00	7	2.96	1.16	1.15
4	3.00 × 2.00	12	5.16	3.52	11.89
4	3.00 × 2.00	18	7.83	8.08	46.38
4	3.00 × 2.00	24	9.40	11.59	81.63
4	3.00 × 2.00	36	10.91	14.77	146.04
4	3.00 × 2.00	48	11.77	17.23	166.42
4	3.00 × 2.00	61	12.59	19.28	214.90
4	3.00 × 2.00	72	12.85	20.28	238.77

Supplementary Table 1 *(continue)*

Treatment	Spacing (m × m)	Age (months)	QMD (cm)	BA (m²/ha)	Volume (m³/ha)
4	3.00 × 2.00	85	13.32	21.59	262.99
4	3.00 × 2.00	102	13.75	21.98	274.70
5	3.00 × 3.00	7	3.31	0.96	0.84
5	3.00 × 3.00	12	5.64	2.79	8.35
5	3.00 × 3.00	18	8.48	6.28	31.55
5	3.00 × 3.00	24	10.07	8.86	55.49
5	3.00 × 3.00	36	12.10	12.31	101.41
5	3.00 × 3.00	48	13.06	14.67	142.68
5	3.00 × 3.00	61	14.07	16.68	186.78
5	3.00 × 3.00	72	14.27	17.00	208.30
5	3.00 × 3.00	85	15.11	18.84	236.13
5	3.00 × 3.00	102	15.52	18.85	238.26

Note. QMD = Quadratic mean diameter; BA = Basal area

Table S2
Table of production for each of the treatments in hybrid eucalypt stands

Age (months)	Treatment 1				Treatment 2				Treatment 3				Treatment 4				Treatment 5			
	Volume (m^3/ha)	MMI (m^3/ha/month)	CMI (m^3/ha)	Acceleration (m^3/ha)	Volume (m^3/ha)	MMI (m^3/ha/month)	CMI (m^3/ha)	Acceleration (m^3/ha)	Volume (m^3/ha)	MMI (m^3/ha/month)	CMI (m^3/ha)	Acceleration (m^3/ha)	Volume (m^3/ha)	MMI (m^3/ha/month)	CMI (m^3/ha)	Acceleration (m^3/ha)	Volume (m^3/ha)	MMI (m^3/ha/month)	CMI (m^3/ha)	Acceleration (m^3/ha)
7	24.54	3.51	3.55	0.31	15.16	2.17	2.14	0.20	12.88	1.84	1.87	0.18	12.94	1.85	1.90	0.19	7.17	1.02	1.18	0.14
8	28.24	3.53	3.85	0.30	17.40	2.18	2.34	0.20	14.84	1.85	2.05	0.19	14.94	1.87	2.09	0.19	8.42	1.05	1.32	0.15
9	32.25	3.58	4.15	0.29	19.84	2.20	2.54	0.21	16.99	1.89	2.25	0.19	17.13	1.90	2.29	0.20	9.81	1.09	1.47	0.15
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31	160.31	5.17	5.79	-0.13	114.09	3.68	5.14	-0.01	105.45	3.40	5.04	0.01	105.30	3.40	4.90	-0.01	77.68	2.51	4.22	0.03
32	166.03	5.19	5.66	-0.13	119.22	3.73	5.12	-0.02	110.50	3.45	5.04	0.00	110.20	3.44	4.89	-0.02	81.92	2.56	4.25	0.02
33	171.62	5.20	5.52	-0.14	124.32	3.77	5.09	-0.03	115.54	3.50	5.04	-0.01	115.08	3.49	4.87	-0.03	86.17	2.61	4.27	0.01
34	*177.07*	*5.21*	*5.38*	*-0.15*	129.39	3.81	5.05	-0.04	120.57	3.55	5.02	-0.02	119.93	3.53	4.84	-0.04	90.44	2.66	4.28	0.01
35	182.37	5.21	5.23	-0.15	134.42	3.84	5.00	-0.05	125.57	3.59	4.99	-0.03	124.75	3.56	4.80	-0.05	94.72	2.71	4.28	0.00
36	187.52	5.21	5.07	-0.16	139.40	3.87	4.95	-0.06	130.55	3.63	4.95	-0.04	129.52	3.60	4.75	-0.05	98.99	2.75	4.27	-0.01
37	192.51	5.20	4.91	-0.16	144.32	3.90	4.88	-0.07	135.48	3.66	4.91	-0.05	134.23	3.63	4.69	-0.06	103.25	2.79	4.25	-0.02
38	197.35	5.19	4.75	-0.16	149.17	3.93	4.81	-0.07	140.36	3.69	4.86	-0.06	138.89	3.66	4.62	-0.07	107.49	2.83	4.23	-0.03
39	202.02	5.18	4.59	-0.16	153.94	3.95	4.74	-0.08	145.19	3.72	4.80	-0.06	143.48	3.68	4.55	-0.07	111.70	2.86	4.19	-0.04
40	206.53	5.16	4.43	-0.16	158.64	3.97	4.66	-0.08	149.95	3.75	4.73	-0.07	148.00	3.70	4.48	-0.08	115.88	2.90	4.15	-0.04
41	210.88	5.14	4.27	-0.16	163.25	3.98	4.57	-0.09	154.64	3.77	4.66	-0.08	152.44	3.72	4.40	-0.08	120.01	2.93	4.11	-0.05
42	215.07	5.12	4.11	-0.16	*167.78*	*3.99*	*4.48*	*-0.09*	159.26	3.79	4.58	-0.08	156.79	3.73	4.31	-0.09	124.09	2.95	4.06	-0.05
43	219.11	5.10	3.95	-0.16	172.21	4.00	4.38	-0.10	163.80	3.81	4.50	-0.08	161.05	3.75	4.22	-0.09	128.12	2.98	4.00	-0.06
44	222.98	5.07	3.80	-0.15	176.54	4.01	4.28	-0.10	168.26	3.82	4.41	-0.09	165.23	3.76	4.13	-0.09	132.09	3.00	3.94	-0.06
45	226.70	5.04	3.64	-0.15	180.77	4.02	4.18	-0.10	172.62	3.84	4.32	-0.09	169.31	3.76	4.03	-0.10	135.99	3.02	3.87	-0.07
46	230.27	5.01	3.49	-0.15	184.90	4.02	4.08	-0.10	176.90	3.85	4.23	-0.09	173.29	3.77	3.93	-0.10	139.83	3.04	3.80	-0.07

Supplementary Table 2 *(continue)*

	Treatment 1				Treatment 2				Treatment 3				Treatment 4				Treatment 5			
Age (months)	Volume (m^3/ha)	MMI (m^3/ha/month)	CMI (m^3/ha)	Acceleration (m^3/ha)	Volume (m^3/ha)	MMI (m^3/ha/month)	CMI (m^3/ha)	Acceleration (m^3/ha)	Volume (m^3/ha)	MMI (m^3/ha/month)	CMI (m^3/ha)	Acceleration (m^3/ha)	Volume (m^3/ha)	MMI (m^3/ha/month)	CMI (m^3/ha)	Acceleration (m^3/ha)	Volume (m^3/ha)	MMI (m^3/ha/month)	CMI (m^3/ha)	Acceleration (m^3/ha)
47	233.69	4.97	3.35	-0.15	188.93	4.02	3.97	-0.11	181.08	3.85	4.13	-0.10	177.17	3.77	3.83	-0.10	143.60	3.06	3.73	-0.08
48	236.96	4.94	3.20	-0.14	192.85	4.02	3.87	-0.11	185.16	3.86	4.03	-0.10	*180.96*	*3.77*	*3.73*	*-0.10*	147.29	3.07	3.65	-0.08
49	240.09	4.90	3.06	-0.14	196.66	4.01	3.76	-0.11	*189.14*	*3.86*	*3.93*	*-0.10*	184.64	3.77	3.63	-0.10	150.90	3.08	3.57	-0.08
50	243.09	4.86	2.93	-0.13	200.37	4.01	3.65	-0.11	193.03	3.86	3.83	-0.10	188.22	3.76	3.53	-0.10	154.43	3.09	3.49	-0.08
51	245.95	4.82	2.79	-0.13	203.97	4.00	3.55	-0.11	196.81	3.86	3.73	-0.10	191.69	3.76	3.42	-0.10	157.88	3.10	3.41	-0.08
52	248.68	4.78	2.67	-0.13	207.47	3.99	3.44	-0.11	200.49	3.86	3.63	-0.10	195.06	3.75	3.32	-0.10	161.25	3.10	3.32	-0.08
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56	258.38	4.61	2.20	-0.11	220.38	3.94	3.02	-0.10	214.20	3.83	3.22	-0.10	207.54	3.71	2.92	-0.10	173.86	3.10	2.98	-0.09
57	260.53	4.57	2.09	-0.10	223.35	3.92	2.92	-0.10	217.37	3.81	3.12	-0.10	210.41	3.69	2.82	-0.10	176.80	3.10	2.89	-0.09
58	262.57	4.53	1.99	-0.10	226.22	3.90	2.82	-0.10	220.45	3.80	3.02	-0.10	213.18	3.68	2.72	-0.10	179.65	3.10	2.81	-0.09
59	264.51	4.48	1.89	-0.10	228.99	3.88	2.72	-0.10	223.42	3.79	2.93	-0.10	215.85	3.66	2.63	-0.09	*182.41*	*3.09*	*2.72*	*-0.08*
60	266.35	4.44	1.80	-0.09	231.67	3.86	2.63	-0.10	226.30	3.77	2.83	-0.10	218.44	3.64	2.54	-0.09	185.09	3.08	2.64	-0.08
61	268.11	4.40	1.71	-0.09	234.25	3.84	2.53	-0.09	229.09	3.76	2.74	-0.09	220.93	3.62	2.44	-0.09	187.69	3.08	2.56	-0.08
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118	298.13	2.53	0.07	0.00	289.59	2.45	0.21	-0.01	291.17	2.47	0.25	-0.01	274.18	2.32	0.20	-0.01	246.85	2.09	0.24	-0.01
119	298.20	2.51	0.07	0.00	289.79	2.44	0.20	-0.01	291.41	2.45	0.24	-0.01	274.37	2.31	0.19	-0.01	247.09	2.08	0.23	-0.01
120	298.26	2.49	0.06	0.00	289.99	2.42	0.19	-0.01	291.64	2.43	0.23	-0.01	274.56	2.29	0.18	-0.01	247.32	2.06	0.22	-0.01

Note: MMI = Mean month increment; CMI = Current month increment; Underlined values = The production at the optimal harvest age

Table S3
Shapiro-Wilk normality test for mean productivity in the ages of 1 to 7 years in hybrid eucalypt stands

Treatment	Period	W	*p*-value
1	MAI_1	1.00	0.89
1	MAI_2	0.90	0.38
1	MAI_3	0.86	0.26
1	MAI_4	0.81	0.14
1	MAI_5	0.95	0.55
1	MAI_6	0.94	0.54
1	MAI_7	1.00	0.87
2	MAI_1	0.88	0.33
2	MAI_2	0.77	0.04
2	MAI_3	0.93	0.50
2	MAI_4	1.00	0.97
2	MAI_5	0.99	0.86
2	MAI_6	0.99	0.84
2	MAI_7	0.99	0.79
3	MAI_1	0.85	0.25
3	MAI_2	0.80	0.10
3	MAI_3	0.86	0.27
3	MAI_4	0.92	0.45
3	MAI_5	0.83	0.18
3	MAI_6	0.84	0.21
3	MAI_7	0.76	0.03
4	MAI_1	0.92	0.45
4	MAI_2	0.98	0.76
4	MAI_3	1.00	0.88
4	MAI_4	0.76	0.03
4	MAI_5	0.97	0.66
4	MAI_6	0.97	0.67
4	MAI_7	0.93	0.47
5	MAI_1	0.83	0.18
5	MAI_2	0.93	0.50
5	MAI_3	0.97	0.65
5	MAI_4	1.00	0.98
5	MAI_5	0.92	0.44
5	MAI_6	0.93	0.49
5	MAI_7	1.00	0.99

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Improvement of Growth and Development of Sweet Basil (*Ocimum basilicum* L.) Through the Application of Chitosan at Different Plant Maturity Stages

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ABSTRACT

Sweet basil is one of the most popular culinary, medicinal, and fragrance herbs in Mediterranean, Asian, and Western countries. This study aims to increase the growth performance of sweet basil via different concentrations of chitosan, which is applied at three growth stages. The study was arranged in a factorial randomized complete block design with four replications. The plants were divided into three growth stages, which were the vegetative stage (S1), the reproductive stage (S2), and both the vegetative and reproductive stages (S1 + S2). Those plants were then treated with four different concentrations of chitosan (0, 2, 4, and 6 ml/L) either on S1, S2, or S1 + S2. The results indicated that plants treated with chitosan at S1 showed greater performance. Chitosan concentration of 4 ml/L produced greater plant height (55.09 ± 1.75 cm/plant), stem diameter (11.08 ± 0.89 mm/plant), and a number of leaves (296.57 ± 11.61 leaves/plant). It is also interesting to observe that the lowest chitosan concentration was non-significantly different, with 4 ml/L at S1 in some parameters. Plants in those treatments showed the highest average length of internode, number of branches, total root length, average root diameter, total root volume, and total root surface area. Besides, correlation analysis proved that all the parameters significantly correlated positively. As the concentration of 4 ml/L showed a superior effect, especially on the number of yields, thus it is recommended for growers to apply chitosan at 4 ml/L during S1.

Keywords: Chitosan, drenching, physiology, reproductive stage, vegetative stage

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INTRODUCTION

The Lamiaceae family is one of the most important and largest pharmaceutical and aromatic plants, where sweet basil (*Ocimum basilicum* L.) is the main valuable member (Mosadegh et al., 2021). Sweet basil is an annual, warm-season, and sun-loving herb (Zulfiqar et al., 2021). Sweet basil poses a valuable source of nutraceuticals such as proteins, carbohydrates, minerals, and vitamin C (Corrado et al., 2020). Due to widespread culinary, medicinal, and aromatic consumption, sweet basil is a popular herb in Mediterranean, Asian, and Western countries (Bufalo et al., 2015; Delbeke et al., 2015; Harnafi et al., 2013; Incrocci et al., 2019). Sweet basil is a naturally eatable antioxidant (Patriani et al., 2021). The antioxidant compounds present in sweet basil provide treatment for human cell cancers (Hanachi et al., 2021). Traditionally, it is used to treat inflammation and helminthic (Osei-Akoto et al., 2020).

Regarding dietary, sweet basil is used as a fresh vegetable alone or with many foods and salads (Mirzajani et al., 2015). Commonly, sweet basil is produced for the fresh market (Corrado et al., 2020; Delbeke et al., 2015; Fattahi et al., 2019; Klintham et al., 2018). Since as a medicinal herb, the whole plant is used in traditional pharmacies (Ghasemzadeh et al., 2016). The total fresh biomass is considered as yield (Scagel et al., 2019). Hence, the yield is assumed to be associated with plant growth and development. In recent years the demand for sweet basil has increased (Acharya et al., 2020; Ciriello et al., 2021; Pandey et al., 2019).

Sweet basil is the most consumed herb and is a highly demanded fresh market, but still, the production is insufficient to meet the demand. In sweet basil, the yield is associated with growth parameters. Thus, the growth and yield of sweet basil could be affected by environmental factors (Elhindi et al., 2017). One of those factors is a chitin-based biopolymer plant growth promoter named chitosan (Govindaraju & Arulselvi, 2018). Since chitosan is obtained from waste materials of seafood industries, the use of chitosan could be actively attributed to reducing pollution (El-Amerany et al., 2020). Chitosan is biodegradable, biocompatible, and ecologically friendly with low economical cost (Jiao et al., 2018). Chitosan has been tested in crops other than sweet basil and showed greater growth performance (Monfared et al., 2020; Turk, 2019). For instance, Mondal et al. (2016) suggested that *Solanum lycopersicum* be treated with 75 mg/L to obtain maximum plant growth and yield components. In addition, a concentration of 5 mg/L chitosan gave a higher number of branches, length of branches, and a number of leaves compared to other common growth regulators such as cytokinins, kinetin, and auxins in *Ipomoea purpurea* L. (Acemi et al., 2018). Similarly, Acemi (2020) suggested that chitosan could be a good alternative to other growth regulators, such as 6-benzylaminopurine and jasmonic acid, in improving plant growth and development. Also, Chamnanmanoontham et al. (2015) reported that 40 mg/L chitosan resulted in greater growth performance in terms of shoot and root in *Oryza sativa* L. plants.

Since herbal yield is associated with growth parameters as well as the number of leaves per plant, chitosan was reported to increase herbal yield in several plants such as *Thymus vulgaris* L., *Mentha* × *Piperita* L., *Nigella sativa* L., and *Lavandula officinalis* Chaix. (Fahmy & Nosir, 2021; Goudarzian et al., 2020; Ibrahim, 2020; Waly et al., 2020).

Although chitosan is considered a growth-promoting substance, its effectiveness depends on the crop species, concentration, and plant's growth stage to be treated (Heidari et al., 2020). Hence, it would be a great contribution for the producers to determine the effective concentration of chitosan and the suitable time of application for sweet basil. Therefore, in the current study, four concentrations of chitosan were tested at three different growth stages on sweet basil.

MATERIALS AND METHODS

Experiment

To evaluate four different levels of chitosan, including 0, 2, 4, and 6 ml/L at three different stages of growth, including the growing stage (S1: 45 days after sowing [DAS]), reproductive stage (S2: 65 DAS), and both (S1 + S2) on sweet basil cv. 213, the experiment was arranged in a randomized complete block design (RCBD) with four replications. The blocking was oriented against sunlight movement. Five plants were included in each replication, and the total number of observed plants was 240 (12 × 4 × 5). Furthermore, all 240 observations contributed to the data belonging to plant height, stem diameter, length of internode, number of branches, number of leaves, total root length, average root diameter, total root volume, total root surface area, and total fresh biomass.

Field Conditions and Activities

The experiment was performed in Field 15 of the research farm in the Faculty of Agriculture, Universiti Putra Malaysia. Seeds were purchased from Green World Genetics Sdn. Bhd., Malaysia. Seeds germinated in peat moss, and seedlings were produced under 50% shade and transplanted at 25 days old prior to 4 leaf stages. Plants grew in 14" polybags with a commercial soil mixture (Bio-soil, Melayu Impra™, Malaysia). Plants were oriented at 3,500 cm^2 planting density in open field conditions and treated with concentrations of 0, 2, 4, and 6 ml/L chitosan (KitosanPlus+, Malaysia) at the growing stage (S1, 45 DAS), reproductive stage (S2, 65 DAS), and both stages (S1 + S2). Finally, plants were harvested at 85 DAS.

Data Collection and Analysis

The physiological parameters such as plant height, stem diameter, and leaf number were collected prior to harvesting at 84-85 DAS in the field. Briefly, plant height was measured from the base form of the stem to the shoot tip using a ruler (100 cm stainless steel), and the average was taken (Hassnain et al., 2020). Stem diameter was measured by using an electronic digital calliper (Insize, Resolution: 0.01mm/0.0005", Accuracy: ± 0.2mm/0.1", Germany), and the average was taken. The internode length was measured using a ruler (30 cm), and the average was taken. Branches from the base

of the stem to the end were counted, and the average was taken for data on the number of branches. Leaves of the whole plant were counted, and the average was recorded as data on the number of leaves per plant. After this, plants were harvested and delivered to the laboratory for further investigation. Both shoot and root were weighed using a digital balance (Model B303-S, Mettler Toledo, Switzerland) and recorded as data of total fresh biomass. The root systems were detached from the stems and subjected to a root scanner (WinRHIZO Pro 2019a, Canada) for picture analysis. Data of root parameters such as total root length, average root diameter, total root volume, and total root surface area were taken from the output.

The data were analyzed using Statistical Analysis Software version 9.4 (SAS 9.4). Analysis of variance was performed using least significant differences (LSD) at 95% significant difference, and means were separated. Besides, Pearson correlation analysis was performed to evaluate the relationships between 10 parameters—the results of correlation analysis are presented in Table 1.

RESULTS

Plant Height

The results showed that plant height was significantly affected by the interaction of chitosan concentrations and plant maturity stages at ($p \leq 0.05$). Concentrations of 2 and 4 ml/L of chitosan applied at the vegetative stage had significantly increased plant height from 45.35 ± 2.59 to 55.08 ± 1.75 cm and decreased to 42.20 ± 2.23 cm at 6 ml/L (Figure 1). The non-treated chitosan plants produced shorter plant heights, where all the plants were less than 40 cm tall. However, applying chitosan at all concentrations in the reproductive stage was not significantly

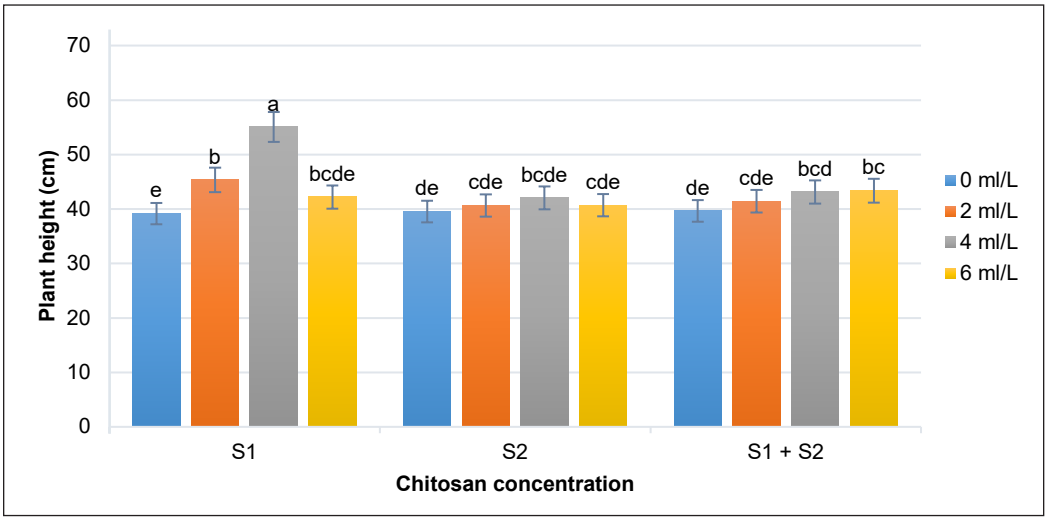

Figure 1. Effect of application of different chitosan concentrations in plant height of sweet basil. Means with the same letters are not significantly different at p ≤ 0.05, and the error bar shows the standard error
Note. S1 = 45 days after sowing; S2 = 65 days after sowing

different compared to the control. When chitosan was applied two times (vegetative and reproductive stage), only plants treated with 6 ml/L showed significantly taller compared to the control, which was 43.36 ± 1.12 cm. The differences in plant height can be seen in Figure 2.

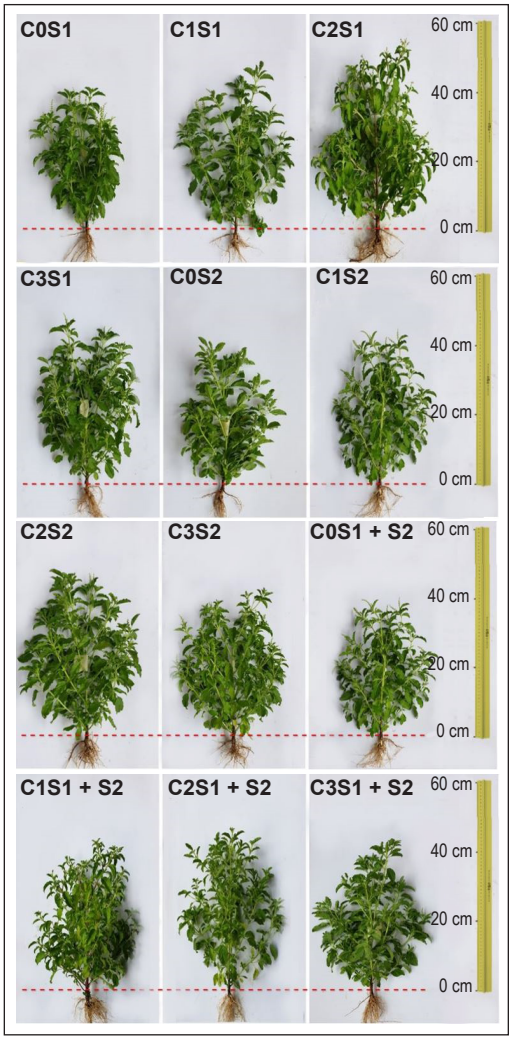

Figure 2. Representative sweet basil plants treated with different concentrations of chitosan. Where C0, C1, C2, and C3 are 0, 2, 4, and 6 ml/L chitosan, respectively
Note. S1 = 45 days after sowing; S2 = 65 days after sowing

Stem Diameter

The result showed that stem diameter was significantly affected by different concentrations of chitosan and the plant's maturity stage at $p \leq 0.5$. Plants treated with chitosan showed bigger diameters compared to non-treated plants (Figure 3). Single application of chitosan at vegetative stage increased the stem diameter from 10.04 ± 0.71 (2 ml/L of chitosan) to 11.08 ± 0.89 mm (4 ml/L of chitosan). A further increase to 6 ml/L of chitosan applied at the vegetative stage reduced the stem diameter to 9.02 ± 0.26 mm, which is still bigger than the control (8.06 ± 0.80 mm). Those plants were not significantly different from others that received 6 ml/L at both the reproductive and vegetative stages.

Applying chitosan at the reproductive stage showed all plants having less than 9 mm of stem diameter, including the control. However, those applied two times (at vegetative and reproductive stages) showed a stem diameter of more than 9 mm, with 4 ml/L recorded at 10.04 ± 0.70 mm of stem diameter, which was significantly bigger compared to other treatments at this time of application.

Length of Internode

The results showed that the internode length was significantly affected by the interaction of different concentrations of chitosan and plant maturity stages at $p \leq 0.05$. Applying chitosan at 4 ml/L at the vegetative stage significantly increased the internode length from 39.30 ± 4.30 (control) to 50.43 ± 4.30 mm per internode. It was the highest

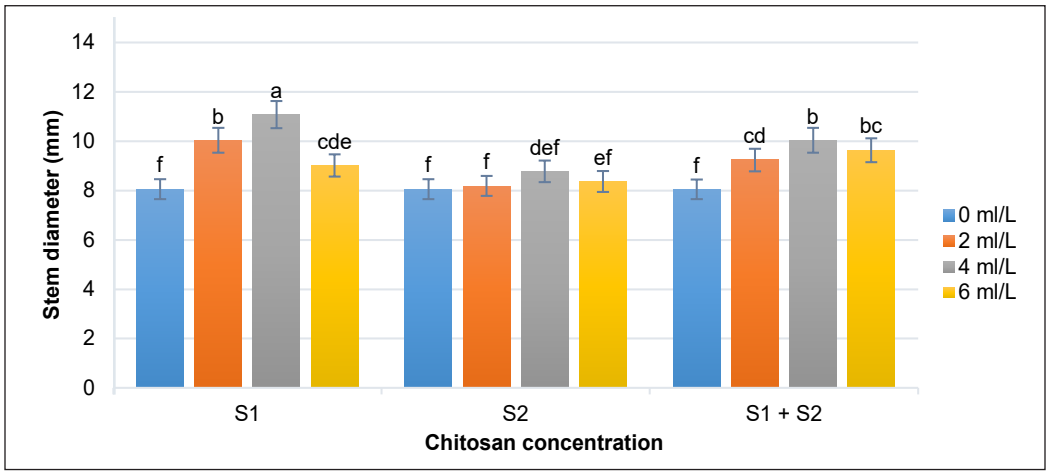

Figure 3. Effect of application of different chitosan concentrations in stem diameter of sweet basil
Note. Means with the same letters are not significantly different at $p \leq 0.05$, and the error bar shows the standard error. S1 = 45 days after sowing; S2 = 65 days after sowing

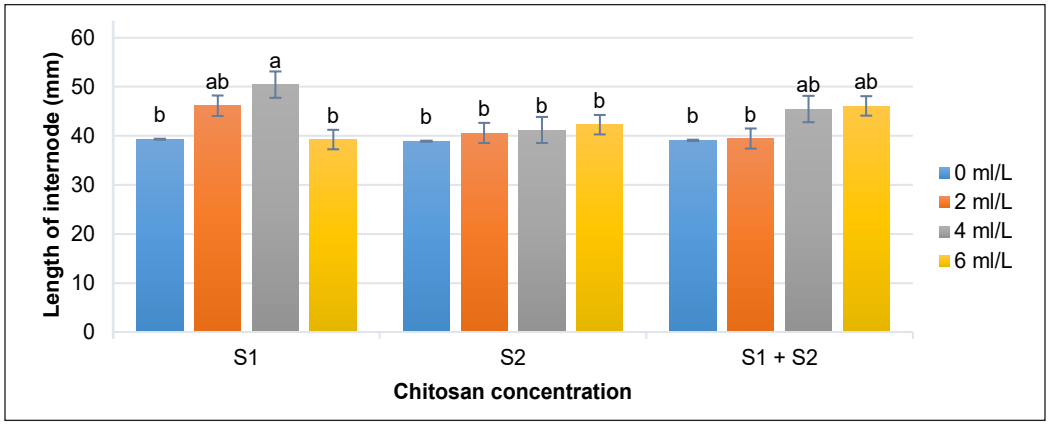

Figure 4. Effect of application of different chitosan concentrations in the length of internode of sweet basil
Note. Means with the same letters are not significantly different at $p \leq 0.05$, and the error bar shows the standard error. S1 = 45 days after sowing; S2 = 65 days after sowing

value recorded in this study's internode length. Twice application of chitosan does not effectively enhance the length of the internode (Figure 4).

Number of Branches

A number of branches were significantly affected by the interaction of chitosan concentrations and plant maturity stages at $p \leq 0.05$. Based on the results, plants treated with concentrations of 2, 4, and 6 ml/L chitosan in the vegetative stage produced 16.04 ± 0.77, 16.70 ± 0.21, and 15.16 ± 0.33 branches per plant, which are significantly higher compared to none treated plants (13.83 ± 0.96 branches). Concentrations of 2 and 4 ml/L chitosan applied on the reproductive stage showed similar results to the control,

while 6 ml/L chitosan resulted from a significantly higher number of branches (15.24 ± 0.95 branches per plant) compared to the control. Twice the application of chitosan, which was at vegetative and reproductive stages at 2 ml/L, showed similar results to control plants, where plants treated with concentrations of 4 and 6 ml/L chitosan showed a significantly higher number of branches by 15.36 ± 0.49 and 15.57 ± 0.83 branches per plant, respectively (Figure 5).

Number of Leaves

Results showed that number of leaves was significantly affected by the interaction of chitosan concentrations and plant maturity stages at $p \leq 0.05$. Treated plants with chitosan concentrations significantly increased the number of leaves compared to the control (Figure 6). From the result, the application of chitosan at the vegetative stage positively influenced the number of leaves per plant, followed by the frequent

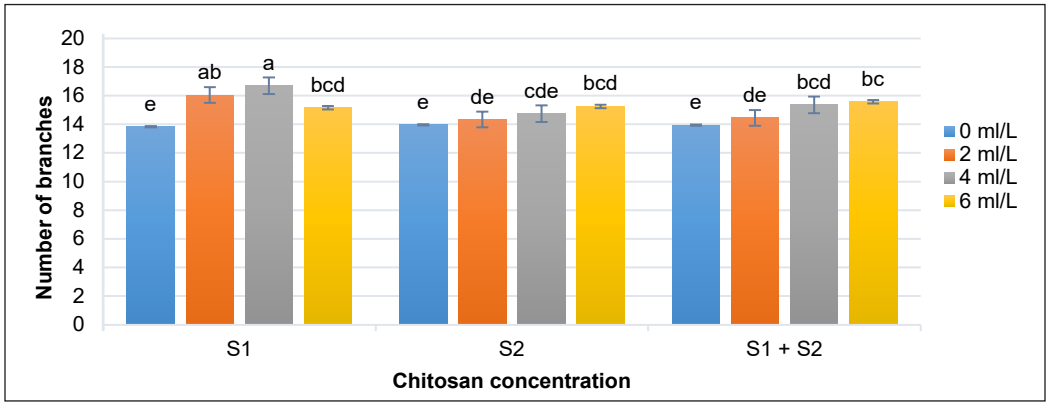

Figure 5. Effect of application of different chitosan concentrations in the number of branches of sweet basil
Note. Means with the same letters are not significantly different at $p \leq 0.05$, and the error bar shows the standard error. S1 = 45 days after sowing; S2 = 65 days after sowing

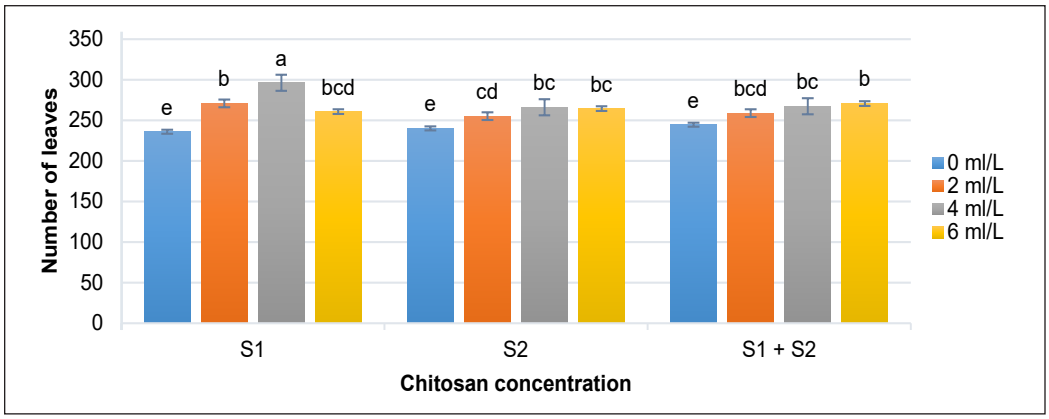

Figure 6. Effect of application of different chitosan concentrations in the number of leaves of sweet basil
Note. Means with the same letters are not significantly different at $p \leq 0.05$, and the error bar shows the standard error. S1 = 45 days after sowing; S2 = 65 days after sowing

application at vegetative and reproductive stages compared to at reproductive stage. Increasing the chitosan concentration from 2 to 4 ml/L increased the number of leaves from 270.90 ± 3.01 to 296.57 ± 11.61 per plant. Further increased to 6 ml/L showed a reduction in the number of leaves where the plants only produced 260.71 ± 8.85 leaves per plant. However, those in control showed fewer leaves produced, only 235.91 ± 10.17 per plant.

Applying chitosan at the reproductive stage showed no significant difference among different concentrations of chitosan. However, those plants were still producing a higher number of leaves compared to the control. The same pattern was also found when chitosan was applied at both vegetative and reproductive stages, where all chitosan-treated plants showed non-significant differences in the number of leaves but were still higher than the control.

Total Root Length

The underground part of sweet basil plants was also investigated. Results indicated that the interaction of different concentrations of chitosan with plant maturity stages significantly affected total root length at $p \leq 0.05$. Plants treated with chitosan with a single application at the vegetative stage and twice application at the vegetative and reproductive stages showed significantly higher root lengths compared to non-treated plants (Figure 7). Total root length was increased with the increase in chitosan concentration until it reached 4 ml/L (454.90 ± 16.35 cm) and reduced at 6 ml/L (405.26 ± 33.87 cm) for plants treated at the vegetative stage. However, control plants have only 339.02 ± 27.02 cm of total root length. It showed that chitosan effectively enhanced root growth and development in sweet basil. However, a different pattern was found when chitosan was applied at the reproductive stage of plant growth. All

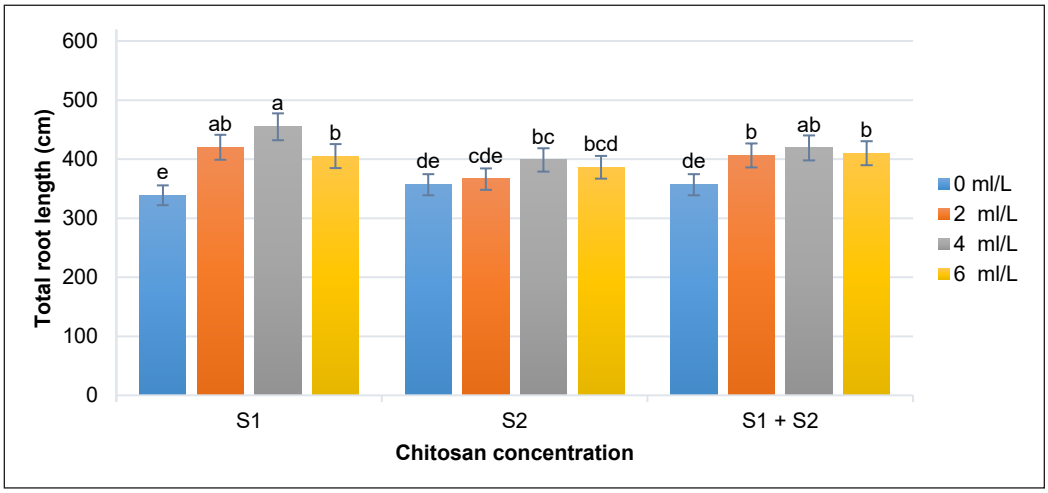

Figure 7. Effect of application of different chitosan concentrations in total root length of sweet basil
Note. Means with the same letters are not significantly different at $p \leq 0.05$, and the error bar shows the standard error. S1 = 45 days after sowing; S2 = 65 days after sowing

plants treated with a single application of chitosan at the reproductive stage showed no effectiveness in enhancing root growth in sweet basil, whereas all plants under this treatment showed a total root length of less than 400 cm. Thus, it is confirmed that chitosan can only help root development when applied at the vegetative stage and not at the reproductive stage. Furthermore, when chitosan was applied twice during the vegetative and reproductive stages, the total root length for all plants was more than 400 cm. Despite different concentrations being applied in this treatment, the root length was not significantly different from each other. The differences in root appearance as well as root length can be seen in (Figure 8).

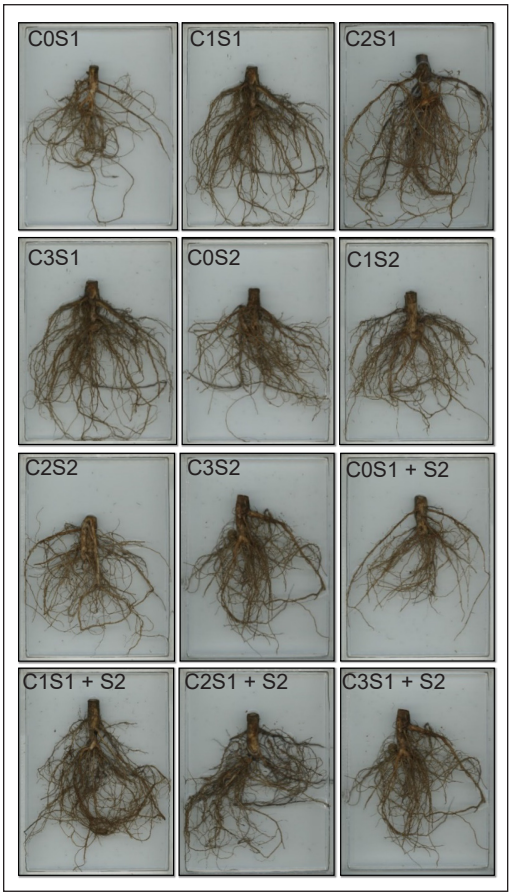

Figure 8. Roots of representative sweet basil plants treated with different concentrations of chitosan at different plant maturity stages in 15 cm × 20 cm containers. Where C0, C1, C2, and C3 are 0, 2, 4, and 6 ml/L chitosan, respectively

Note. S1 = 45 days after sowing; S2 = 65 days after sowing

Root Average Diameter

Root average diameter was investigated to qualify the root system of sweet basil plants. Results indicated that the interaction of chitosan concentrations and plant maturity stages at $p \leq 0.05$ significantly affected average root diameter. Chitosan-treated plants showed significantly higher average root diameters than non-treated plants, except those treated once at the reproductive stage.

Application of chitosan when plants are at the vegetative stage and concentrations of 2, 4, and 6 ml/L increased the average root diameter by 1.16 ± 0.01, 1.20 ± 0.01, and 1.15 ± 0.08 mm significantly, compared to control (1.05 ± 0.05 mm). Surprisingly, all plants treated with chitosan at the reproductive stage showed a similar average of root diameter to control. This study's finding proves that the time of application of chitosan is important to sweet basil, as it only influences root development, particularly the enlargement of the root when applied at the vegetative stage. The result from plants treated at vegetative and reproductive stages (two times) again proved this: the average root diameter increased by 1.16 ± 0.03, 1.13 ± 0.01, and

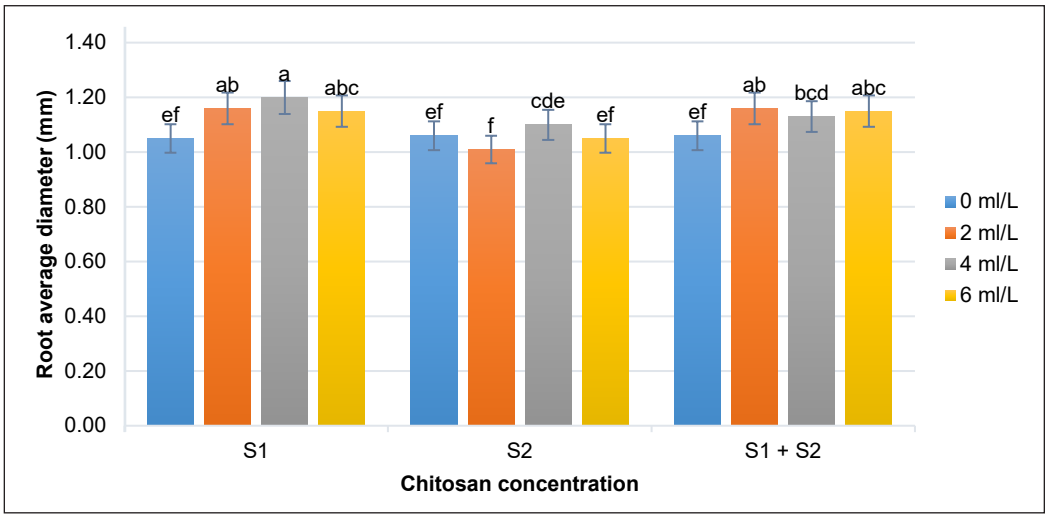

Figure 9. Effect of application of different chitosan concentrations in average root diameter of sweet basil
Note. Means with the same letters are not significantly different at $p \leq 0.05$, and the error bar shows the standard error. S1 = 45 days after sowing; S2 = 65 days after sowing

1.15 ± 0.02 mm, respectively, increase in concentrations compared to control (Figure 9). It is then assumed that the increase in diameter for those plants was caused by the application at the vegetative stage and not at the reproductive stage. Thus, a single application at the vegetative stage only is needed to enlarge the diameter of the sweet basil root.

Total Root Volume

In sweet basil plants, root volume was significantly and positively affected when plants were treated with different concentrations of chitosan at different plant maturity stages ($p \leq 0.05$). Treated plants showed greater root volume compared to non-treated plants, except those treated with 2 and 4 ml/L chitosan in the reproductive stage (Figure 10). Based on single-factor analysis, the difference between concentrations of chitosan was insignificant, where application at the vegetative stage showed a greater value of root volume compared to the application at the reproductive stage and both vegetative and reproductive stages. The interaction of both factors showed that the highest root volume (4.76 ± 0.34 and 5.09 ± 0.38 cm^3 per plant) were observed at concentrations of 2 ml/L and 4 ml/L, respectively, where higher concentration (6 ml/L) resulted in 4.02 ± 0.27 cm^3 per plant, which is still higher compared to control (3.13 ± 0.20 cm^3 per plant). Applying 6 ml/L chitosan during the reproductive stage significantly increased root volume from 3.16 ± 0.25 to 4.57 ± 0.54 cm^3, where there was no significant difference between 2 and 4 ml/L with control. When plants were treated two times on vegetative and reproductive stages with concentrations of 2, 4, and 6 ml/L chitosan, the root volume significantly increased by 4.17 ± 0.58, 4.66 ± 0.26, and 4.00 ± 0.14

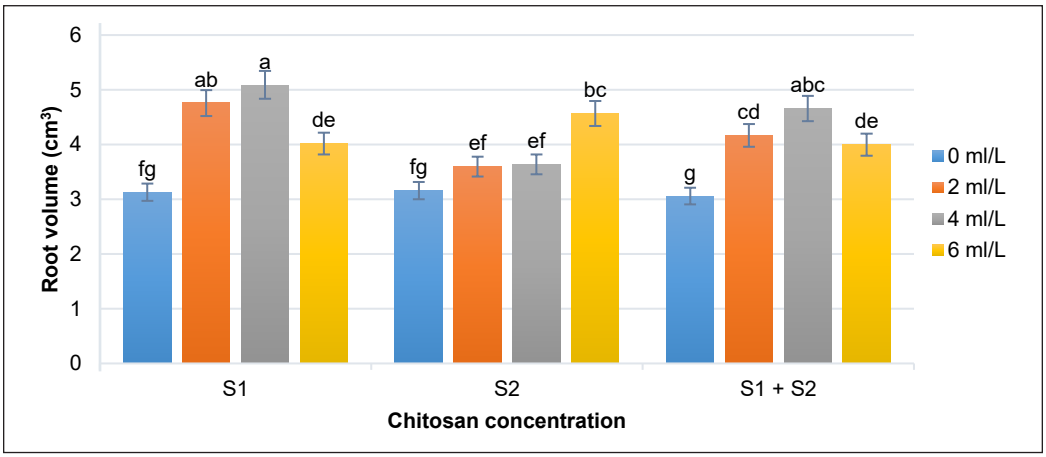

Figure 10. Effect of application of different chitosan concentrations in total root volume of sweet basil
Note. Means with the same letters are not significantly different at $p \leq 0.05$, and the error bar shows the standard error. S1 = 45 days after sowing; S2 = 65 days after sowing

cm^3, respectively compared to control. This result shows that the highest root volume is 62.62% greater than the control.

Total Root Surface Area

Analysis of variances for root surface area showed a significant difference in the interaction of chitosan concentrations and plant maturity stages at $p \leq 0.05$. Plants treated with chitosan showed significantly higher root surface area compared to non-treated plants, except for those treated with 2 ml/L at the reproductive stage (Figure 11). A concentration of 2 ml/L applied at the

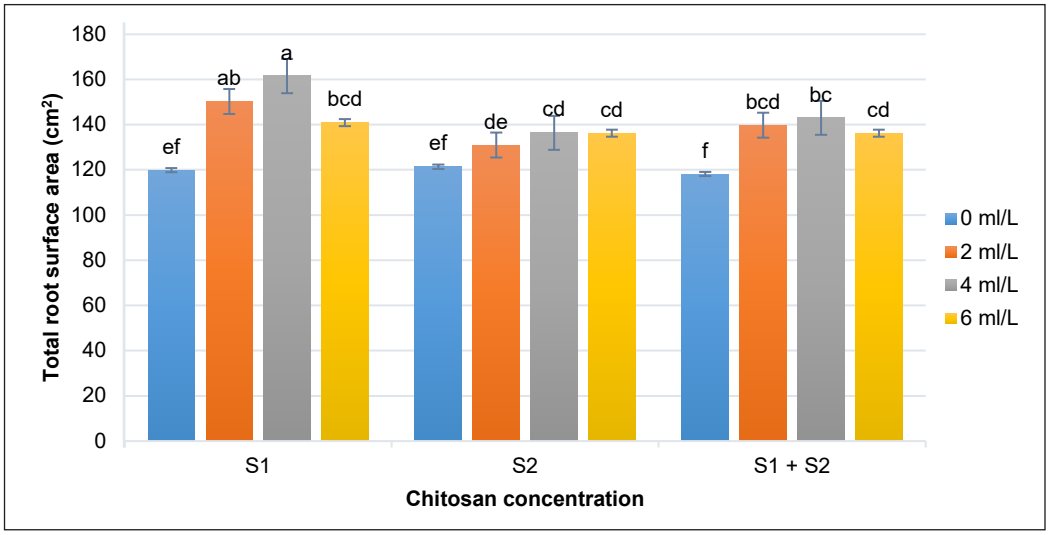

Figure 11. Effect of application of different chitosan concentrations in total root surface area of sweet basil
Note. Means with the same letters are not significantly different at $p \leq 0.05$, and the error bar shows the standard error. S1 = 45 days after sowing; S2 = 65 days after sowing

reproductive stage resulted in 130.97 ± 8.33 cm^2 of total root surface area, which was non-significant with control (121.32 ± 4.39 cm^2). At the same time, 4 and 6 ml/L chitosan concentrations showed significantly higher root surface area by 136.34 ± 13.54 and 136.19 ± 9.56 cm^2, respectively. Applying chitosan at both vegetative and reproductive stages seems ineffective, as all concentrations showed non-significantly differences with chitosan applied at the reproductive stage. The peak value of root surface area was found in those treated at the vegetative stage, where a concentration of 4 ml/L was 34.79% higher compared to the control. It showed that a specific chitosan concentration enhances the root surface area when applied at the correct plant's age.

Total Fresh Biomass

The data on total fresh biomass showed a significant difference at $p \leq 0.05$ between a combination of chitosan concentrations and plant maturity stages. All concentrations of chitosan applied at the vegetative stage showed greater value compared to the control (Figure 12). The highest total fresh biomass (109.49 ± 2.01 g per plant) was obtained when plants were treated with 4 ml/L at the vegetative stage, followed by the second highest (103.42 ± 2.59 g per plant) at 2 ml/L. A higher concentration of chitosan applied at the vegetative and reproductive stages or both vegetative and reproductive stages showed total fresh biomass ranging from 93.65 ± 5.31 to 94.85 ± 2.93 g per plant, still higher compared to the control. Those plants treated with low concentrations (2 or 4 ml/L) at the reproductive stage or both vegetative and reproductive stages showed total fresh biomass ranging from 89.43 ± 1.9 to 94.85 ± 2.9 g per plant, which are similar to the control.

Correlation Between Growth and Yield Components

The Pearson correlation analysis showed a significant positive correlation between

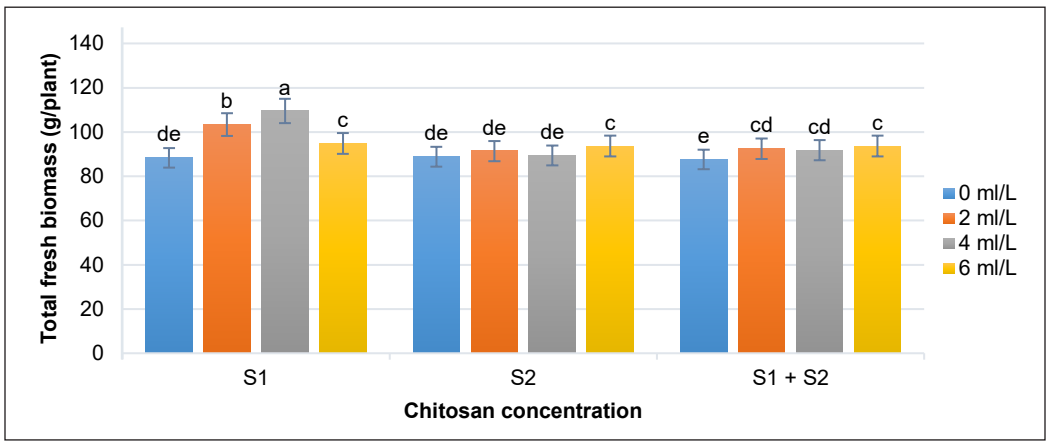

Figure 12. Effect of application of different chitosan concentrations in total fresh biomass of sweet basil
Note. Means with the same letters are not significantly different at $p \leq 0.05$, and the error bar shows the standard error. S1 = 45 days after sowing; S2 = 65 days after sowing

all growth parameters and biomass yield (Table 1). Based on the results, plant height has a strong positive and intermediate correlation with stem diameter and length of internode at r = 0.79 and r = 0.71, respectively. Similar to this, a positive intermediate correlation was found between plant height and the number of branches (r = 0.47). Thus, a positive intermediate correlation was found between the number of leaves with plant height and the number of branches at r = 0.54 and r = 0.58, respectively. In addition, total root length showed a positive intermediate correlation with plant height, stem diameter, length of internode, number of branches, and number of leaves at r = 0.63, 0.60, 0.58, 0.42, and 0.59, respectively. The total fresh biomass positively correlated with aerial parts such as plant height, stem diameter, length of internode, and the number of branches and leaves at r = 0.61, 0.77, 0.54, 0.62, and 0.68, respectively. Similarly, a significant and positive correlation was found between total fresh weight and the root parameters such as total root length, average root diameter, total root volume, and total root surface area at r = 0.66, 0.72, 0.70, and 0.57, respectively.

DISCUSSION

The results show that specific chitosan concentration acts differently according to the plant's growth stage in enhancing plant height. Besides, without a doubt, applying chitosan led to enhancing plant height. It agrees with Mukta et al. (2017), who found that a concentration of 250 mg/L chitosan was applied on *Fragaria* × *ananassa* Duch, produced the tallest plant height (19 cm) compared to the control, which was only 17.30 cm. Similarly, Rahman et al. (2018) reported that foliar application of chitosan at a concentration of 500 mg/L on *Fragaria*

Table 1
Correlation matrix between yield components of sweet basil

Variable	1	2	3	4	5	6	7	8	9	10
PH	1									
SD	0.79***	1								
LOI	0.71**	0.72**	1							
NOB	0.47**	0.55**	0.71**	1						
NOL	0.54**	0.65**	0.67**	0.58**	1					
TRL	0.63**	0.60**	0.58**	0.42*	0.59**	1				
RAD	0.71**	0.70**	0.59**	0.48*	0.69**	0.60**	1			
TRV	0.73**	0.74**	0.72**	0.61**	0.59**	0.76**	0.68**	1		
TRSA	0.61**	0.61**	0.71**	0.55**	0.59**	0.85***	0.47*	0.80***	1	
TFB	0.61**	0.77***	0.54**	0.62**	0.68**	0.66**	0.72**	0.70**	0.57**	1

Note. PH = Plant height; SD = Stem diameter; LOI = Length of internode; NOB = Number of branches; NOL = Number of leaves; TRL = Total root length; RAD = Root average diameter; TRV = Total root volume; TRSA = Total root surface area; TFB = Total fresh biomass
** and *** = Significant intermediate correlation and significant strong correlation, respectively

× *ananassa* Duch. plants resulted in higher plant height (25.10 cm) compared to the control (19.50 cm). The mechanism behind chitosan's effectiveness in increasing plant height is regulated by gibberellic acid inside the plant tissue (Lopez-Moya et al., 2019). Gibberellic acid can be found in all plants and is responsible for incrementing the number and length of cells in the plant's tissues (Guttridge & Thompson, 1959).

Similarly, the increment of stem diameter under the application of chitosan agrees with the previous reports of Choudhary et al. (2017), Dwyer et al. (1995), and Ullah et al. (2020), who confirmed the ability of chitosan to increase stem diameter. A bigger stem diameter can lead to a strong and stable stem, which can help the plants support a greater number of leaves and protect the plants from strong wind situations. Besides, stems with large diameters transport materials essential for plant growth and development as well as plant leaf biomass and contribute to biomass yield (Sun et al., 2019). Therefore, a bigger stem diameter in sweet basil is assumed to contribute to yield, which is leaf biomass.

Since chitosan regulates the accumulation of gibberellic acid inside the plants (Lopez-Moya et al., 2019), gibberellic acid was proven responsible for incrementing internode length (Mahmoody & Noori, 2014). This result agrees with Avestan et al. (2017), who reported the increment of internode length in chitosan-treated apple cuttings. This increment first appears in the length of internodes and then contributes to the increment of plant height (Atait & Qureshi, 2020; Brian, 1958; Wang et al., 2017).

Plants treated with 4 ml/L chitosan in the vegetative stage showed the best performance in relation to the number of branches. The increment of lateral branches under the application of chitosan agreed with Salehi et al. (2017), who reported the enhancement of a number of branches under the application of chitosan in *Satureja hortensis* plants. Similarly, Mondal et al. (2016) also stated that chitosan at a concentration of 100 mg/L can increase the number of branches in *Solanum lycopersicum* L. plants by 38.81%. In the case of sweet basil, a higher number of branches will bring benefits whereby it gives more surface for the leaves to develop and thus increasing its yield.

Also, chitosan enhanced the number of leaves in sweet basil plants. Similar to the results of the current study, early reports confirmed the increment of the number of leaves under the application of chitosan in *Curcuma longa* L., *Oryza sativa*, *Amaranthus hybridus*, *Hordeum vulgare*, and *Stevia rebaudiana* (Anusuya & Sathiyabama, 2016; Berliana et al., 2020; Divya et al., 2019; Hafez et al., 2020). For instance, chitosan at the rate of 75 ml/L applied as seed soaking increased the number of leaves from 14 to 16 per plant in *Capsicum frutescens* Linn. (Sari et al., 2020). Chitosan was claimed to be related to stimulating plant nutrition, where it has been reported that chitosan provides essential nutrients for plants (Sharif et al., 2018) to enhance plant growth and

development and produce leaves. Besides, soil application of chitosan was said to increase the absorption of essential nutrients for plants by increasing the number of beneficial soil microorganisms (Boonlertnirun et al., 2008). Water is assumed to transfer nutrients from the soil to different organs. Interestingly, chitosan increases water absorption in plants by increasing the growth and development of the root system (Hidangmayum et al., 2019). Thus, applying chitosan at the vegetative stage resulted in more leaves than in the reproductive stage. It is supported by Oosterhuis (1990), which stated that the early growing stage (after the seedling stage) is the time for *Gossypium hirsutum* plant to develop a leaf canopy. It is also assumed that plants are more active at an early growth stage and could easily respond to chitosan concentrations. At the late growing stage, plants may not absorb enough chitosan or cannot fully respond. However, more investigation is needed to clarify the mechanism of the physiological response of sweet basil plants to chitosan at different growth stages.

Since nitrogen is a mobile element in the soil and can be easily leached deeper, plants should provide long roots to search for this essential element. Therefore, reports say plants often produce long roots when facing nitrogen deficit only (Gruber et al., 2013). The results do not agree with this since the literature says that chitosan provides essential nutrition for plants as well as nitrogen elements (Xu & Mou, 2018). Thus, the results showed increased total root length because of chitosan treatment. It is supported by Sathiyabama and Parthasarathy (2016), who reported an increment of total root length to 31.25% in *Cicer arietinum* seedlings under the application of 0.10% chitosan. It is near the best treatment, resulting in a 34.18 % higher total root length in sweet basil. Similarly, Khan et al. (2011) observed an increment of total root length by 35% in *Arabidopsis thaliana* plants under the application of chitosan at a concentration of 10 µM. Furthermore, Iglesias et al. (2019) mentioned that applying chitosan could increase auxin accumulation in the plant's root system. At the same time, auxin is well-known as a root growth hormone (Tanimoto, 2005; Went, 1935). Therefore, the enhancement of root length in this study could be related to the potential of chitosan in stimulating the biosynthesis of auxin, which functioned in the root system. Longer root growth and development can help plants to reach more minerals to support their growth, besides increasing the anchoring of the plants to the soil, which in return is more stable.

From the results, sweet basil's growing stage was able to influence the effects of chitosan on the root system as well as total root length. The early growth stage was more efficient than the late growing stage for sweet basil plants to respond to treatment applications. Although, there was no significant difference between the vegetative and at both vegetative and reproductive stages of growing to apply chitosan on sweet basil plants based on single factor analysis.

The application of chitosan at reproductive phase has no effect on the root development, particularly on the root length. Furthermore, the effectiveness of chitosan at frequent application could be mostly related to the effectiveness at the vegetative stage. It is well supported by Ljung et al. (2001), which declared that auxin as root growth hormone could be synthesized at a maximum rate when the plant is at the early growth stage. To interoperate this, at the early growing stage of sweet basil plants, chitosan can improve the accumulation of auxin, where this hormone optimizes its function and increases root growth as well as prolonging of roots.

Root diameter is another important parameter of the root system (Bouma et al., 2000). Plants with thicker root diameters could survive longer and easily (Baddeley & Watson, 2005). It could be helpful for plants in finding water and nutrient elements from the soil. In addition, roots with higher diameters can absorb and transport a large amount of water and nutrient solution over the plants (Hutchings & John, 2003). Therefore, the thick root diameter in the root system could contribute to the overall growth of the plant canopy. Root diameter was improved due to applying chitosan at the vegetative stage. The result from this study agrees with Khan et al. (2011), who reported an increment of root diameter under the application of chitosan at a concentration of 100 µM chitosan in *A. thaliana* plants. The increment in average root diameter could be related to the potential of chitosan in manipulating auxin in the root system (Iglesias et al., 2019).

According to Davis and Jacobs (2005), root volume is an important parameter in the quality assessment of the root system. The higher root volume is said to provide larger contact with soil and a chance for more nutrient uptake by the plant (Haase & Rose, 1994). The root length, root diameter, and density of the roots contribute data for the root volume. Based on the results of this study, it was proven that the drench application of chitosan at a suitable stage could increase root volume in sweet basil plants. The increment of root volume is directly linked to the enhancement of nutrient uptake by the plants (Marschener, 1998). At the same time, Farouk et al. (2011) reported a positive correlation between root volume and nutrient uptake in *Raphanus sativus* L. var. *sativus* plants. Hence, an increment in root volume may contribute to growth and development.

Chitosan could also increase root surface area, which agreed with Guo et al. (2020), who reported the increment of root surface area in *Eleutherococcus senticosus*. According to Tagliavini et al. (1993), the increment of root surface area was influenced by the increment of root length and average diameter. Moreover, the effectiveness of water and nutrient uptake from the soil was attributed to the large root surface area (Marschener, 1998; Yang et al., 2009). For instance, Genc et al. (2007) reported that the increment of root surface area had positively influenced zinc uptake by *Hordeum vulgare* plants. In trees such as *Larix gmelinii*, the increment of leaf biomass was related to the increment in root

surface area (Meng et al., 2018). Therefore, as the application of chitosan increased the root surface area in this study, it has been proven beneficial to sweet basil.

The optimum total fresh biomass yield per plant (109.49 ± 2.01 g) obtained in this study is 23.93% higher than control plants, equal to 21.14 g per plant. Normally, the conventional cultivation density of sweet basil is 140,000 plants/ha (Guerrero-Lagunes et al., 2020). Considering this, treating sweet basil plants with 4 ml/L chitosan will increase the biomass yield by 2959.60 kg/ha. It could be a great contribution to the producers. In sweet basil plants, it is assumed that the yield is associated with growth and development, and several parameters may contribute to the increment of total fresh biomass. However, the increment in yield of sweet basil, as well as total fresh biomass due to chitosan, which was applied at the vegetative stage, agrees with the previous report by Mondal et al. (2011), who confirmed the increment of fresh biomass in *Basella alba* L. plants under 75 mg/L at the early stage of growth. It is 21.56% higher when compared with the control.

From the results of correlation analysis, plant height has a strong positive correlation and a positive intermediate correlation with stem diameter and length of the internode. It is assumed that a big stem could provide sufficient water and organic materials transportation to allow the plant to grow higher (Sun et al., 2019). Furthermore, the stem cell enlargement led to an increase in the internode length, which directly influences plant height. A positive intermediate correlation exists between plant height and the number of branches. In sweet basil, branches are produced from the nodes, which proves that in addition to the length of the internode, the number of nodes also contributes to the height of sweet basil plants. A positive intermediate correlation of the number of leaves with plant height and the number of branches revealed that the increment of plant height and the number of branches provided a larger surface for the development of leaves through the canopy of sweet basil plants. In addition, there is notably a relationship between sweet basil plants' underground and aerial parts. In plants, to be facilitated growth and development, it is assumed that the root system provides mechanical support, water, and essential nutrients for the shoot to produce organic materials through photosynthesis and distribute overall the plant. Thus, the previous report confirmed a positive correlation between shoots and roots in *A. thaliana* plants (Bouteillé et al., 2012). Considering this, all the aerial and underground parameters strongly contributed to yield performance. The current study's finding proves it, whereas the total fresh biomass had a significantly positive correlation with both aerial and underground parts in sweet basil plants.

CONCLUSION

A field experiment was carried out to evaluate the performance of sweet basil under the application of chitosan at different plant maturity stages. Results showed

that application of chitosan improved growth parameters, contributing to yield performance. Based on the findings, sweet basil plants should be treated with chitosan at one time at the vegetative stage compared to the reproductive stage or both. Therefore, it is recommended for sweet basil plants be treated with 4 ml/L chitosan at the vegetative stage. The use of commercial growing media could be a limitation of this study, and minor differences may occur if growers use field soil. Future study is needed to investigate phytochemicals as well as their postharvest quality.

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Evaluation of Avian Papillomavirus Occurrences and Effective Sampling Materials for Screening Purpose in Bird Species Through Systematic Review and Meta-Analysis

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ABSTRACT

Papillomaviruses (PVs), double-stranded circular DNA viruses, typically cause regressing papillomas (warts) on mucosal or keratinized epithelia of a wide spectrum of species. The viruses largely infect mammals, whereby PV infections in humans, bovines, and rabbits are extensively reported. However, studies on non-mammalian PVs, particularly avian ones, are relatively lacking and worthy of investigation. This study performed a meta-analysis post-systematic review based on the Preferred Reporting Items for Systematic Reviews and Meta-Analyses statement guidelines to evaluate the occurrences of avian papillomaviruses (APVs) in bird species and effective materials used for virus detection. The electronic databases Science

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Direct, Medline via PubMed, and Google Scholar were used to search for the journal articles. Upon article eligibility check, the QUADAS-2 was employed to assess the data. Of 1139 records, 31 were eligible for full-text review, but only 9 were significant for the final review. The results showed that APVs are highly prevalent among the Fringillidae family, with a proportion of 81%, followed by Laridae (30%) and Anatidae (13%). The pooled prevalence of APV in tissue samples was 38%, while in swab samples was 13%. Only one study reported positive APV from fecal materials (0.4%); hence, the reliability comparison between these three samples was not performed. This study concluded that APVs are most prevalent in the Fringillidae bird family, while tissues are the most suitable biological samples for APV screening and should be considered as a single sample material. From epidemiology, knowledge of APV incidences and distribution may assist in controlling papillomatosis in bird species.

Keywords: Avian, bird, meta-analysis, papillomavirus, virus

INTRODUCTION

Papillomaviruses (PVs) are relatively small, non-enveloped, icosahedral viruses belonging to the family *Papillomaviridae*. PVs contain circular double-stranded DNA with a complete genome size ranging from 6.9 to 8.6 kb. The genome is divided into three codon regions (early, E; late, L; and long codon region, LCR) that encode the replication proteins E1, E2, E4, the oncoproteins E5, E6, and E7, and the capsid proteins L1 and L2 (Araldi et al., 2017). More than 130 species in more than 50 genera of PV were identified (Canuti et al., 2019). Most PVs are detected in mammals; however, the number of PVs detected in birds is increasing. To date, 11 complete genomes and 9 partial sequences of avian papillomaviruses (APVs) have been reported. The APV with complete genome sequences were discovered in chaffinch (FcPV1) (Terai et al., 2002), Northern fulmar (FgPV1) (Gaynor et al., 2015), yellow-necked francolin (FlPV1) (Van Doorslaer et al., 2009), Adélie penguin (PaPV1 and PaPV2) (Varsani et al., 2014), African gray parrot (PePV1) (Tachezy et al., 2002), and Yorkshire canary (ScPV1) (Truchado, Moens, et al., 2018). The complete genome sequence of APV was identified in Atlantic puffin (PuPV-1), American herring gull (GuPV-1), mallard and American black duck (DuPV-3), and black-legged kittiwake (KiPV-2), while APV with partial sequence were identified in mallard (Duck PV), gull (GuPV-2 and GuPV-3), and black-legged kittiwake (KiPV-1, KiPV-3, KiPV-4, KiPV-5, KiPV-6, and KiPV-7) (Canuti et al., 2019).

PVs primarily infect and replicate in the mucosal and keratinized epithelia, which may induce the development of benign and malignant neoplastic lesions. The lesions or neoplasms are discovered in various body parts among different bird species. Chaffinch papillomatosis is cauliflower-shaped neoplasms on the tarsi and digits (Lina et al., 1973). Small featherless wartlike growths were found in the unfeathered areas around the canary's beak (Dom et al., 1993). Cutaneous papilloma-like lesions

in African gray parrots are discovered on the beak's head, eyelids, and commissure (Latimer et al., 1997). However, APV was also discovered in the healthy skin of a yellow-throated francolin (Van Doorslaer et al., 2009) and non-obvious lesions in the oral mucosa and tongue of a captive Yorkshire canary (Truchado, Moens, et al., 2018). Papilloma-like lesions also appeared in the legs of some bird species with no APV detected (Katoh et al., 2010).

Knowledge regarding APV prevalence is still very limited; thus, efforts need to be made to increase the sampling and screening of APV. Several diagnosis methods were implemented to identify APV. The first identification of APV was demonstrated using electron microscopy in the 1970s (Lina et al., 1973). The virus was examined in the nuclei of cells isolated from proliferative lesions on the legs of chaffinches (*Fringilla coelebs*). Electron microscopy also revealed leg papillomatosis in six chaffinches in the Czech Republic and one in Germany (Literák et al., 2003). Molecular technique such as PCR is developed to detect the presence of the virus in griffon vultures (Di Francesco et al., 2019), ducks (Williams et al., 2018), and other wild birds (Canuti et al., 2019; Padzil et al., 2022). The viruses were detected from various sample types, including skin, internal epithelium, fecal material, and oropharyngeal and cloacal swabs (Truchado, Williams, et al., 2018). PVs are highly host-specific DNA viruses; thus, the immunity is species-specific (King et al., 2011). Although data on APV prevalence are still very limited, a significant number of non-human PVs were identified in different species, especially birds. A proper diagnosis method and appropriate sample materials are needed to detect APV. Therefore, the objectives of this meta-analysis are to observe the distribution of APV in bird species and to determine the prevalence of APV in different sample materials. Thus, the most favorable biological sample type for APV screening can be determined.

MATERIALS AND METHODS

Protocol and Search Strategy

The protocol for the systematic review was performed according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) statement guidelines to specify the search strategy, eligibility criteria, objectives, and methods (Moher et al., 2009). The electronic databases Science Direct, Medline via PubMed, and gray literature (Google Scholar) were searched for papers published in any year. The following terms were used: "papillomavirus", "avian papillomavirus", "avian papillomavirus prevalence", or "avian papillomavirus detection". Reference lists cited in all article searches were also checked. An updated search was performed on November 13, 2020. Relevant citations from each database were extracted, and duplicate files were removed.

Eligibility Criteria

Titles and abstracts retrieved from journal articles were screened for eligibility. All the relevant journal articles were reviewed in full text, and those fulfilling inclusion criteria

were extracted for their data. All abstracts were screened by one author (NN). The journal articles were included if they met all the following eligibility criteria: (1) targeted PV in all avian species at any ages, not in human or other non-mammalian species, (2) screened using any laboratory-confirmed methods, (3) used any sample materials, and (4) published in English in any year. Studies that did not clearly state the presence of PVs in the avian species were excluded.

Data Extraction and Bias Assessment

Data extracted from the included studies were compiled in a developed data extraction sheet. Table 1 shows the information extracted from the selected studies. The risk of bias in each study was assessed using the Diagnostic Precision Study Quality Assessment Tool (QUADAS-2) recommended by the Cochrane Collaboration.

Data Analysis

The random effects model meta-analysis method was used to analyze the pooled prevalence of APV in different avian species and sample materials. The heterogeneity among the studies was analyzed using the Higgins test (I^2), which shows the percentage of variation among studies (Higgins et al., 2003). These analyses were compiled using the Review Manager Software (version 5.4) (Moher et al., 2009). The odds ratio (OR) test, with a 95% confidence interval (95% CI), was calculated to measure the probability of APV infection for symptomatic compared to asymptomatic birds.

RESULTS

Search results returned a total of 1,170 articles after duplicate removal. Of these, 1,139 studies were excluded due to irrelevant titles and abstracts during screening, while 31 were eligible for full-text review. After full-text articles were extracted, only nine studies indicated the presence of PVs in the avian species. Therefore, only these studies were considered completely relevant and thus included in the final review (Canuti et

Table 1
Datasheet extracted from articles and records included in this systematic review

Data	Range
Year	Any year
Sample taken	Avian species only
Sample size	1–500
Symptoms	General symptoms Appearance of papilloma Appearance of lesion Respiratory problem Dead Other Asymptomatic
Stages of samples	Juvenile Adult Other
Epidemiological unit	Wild bird species Wild habitat Zoo Natural park
Method of testing	Histopathology Molecular Combination (Histopathology and molecular)
Sample type	Biopsy Swab Feces Mixed
Avian papillomavirus prevalence	0–100%

al., 2019; Dom et al., 1993; Latimer et al., 1997; Pérez-Tris et al., 2011; Prosperi et al., 2016; Sironi & Gallazzi, 1992; Truchado, Williams, et al., 2018; Van Doorslaer et al., 2017; Williams et al., 2018). The result of the search strategy is shown in a PRISMA flow chart (Figure 1).

The included studies were published from 1992 to 2019. Despite the 27 years of time scale, the limited relevant studies affirm the insufficient knowledge of PVs in avian species. Out of nine, two studies were conducted in the United States and Italy, whereas Spain, Georgia, Sweden, Canada, and Belgium contributed with one study each. Six studies mentioned that symptoms appeared on the collected birds, while three did not report whether the birds were symptomatic or asymptomatic. The information on the stages and sex of the birds was insufficiently provided in all studies, so the criteria were not reported here. Meta-analysis of diagnostic accuracy of the APV detection methods was also not performed in this study due to inadequate data, such as sensitivity value, specificity value, true/false-positive value, and true/false-negative value.

Nine studies screened for APV from 711 bird samples of 47 different specified species and 3 unspecified species. The APV was detected in 17 species that are categorized under 6 families. Among the family Alcidae, *Fratercula arctica* was detected with a prevalence of 9.8%. Two studies detected APV in the family Anatidae, with four species (American black duck × mallard hybrid, *Anas platyrhynchos*, *Anas platyrhynchos domesticus*, and *Anas rubripes*) that were positive for APV infection. Four species among the family Fringillidae that showed positive APV

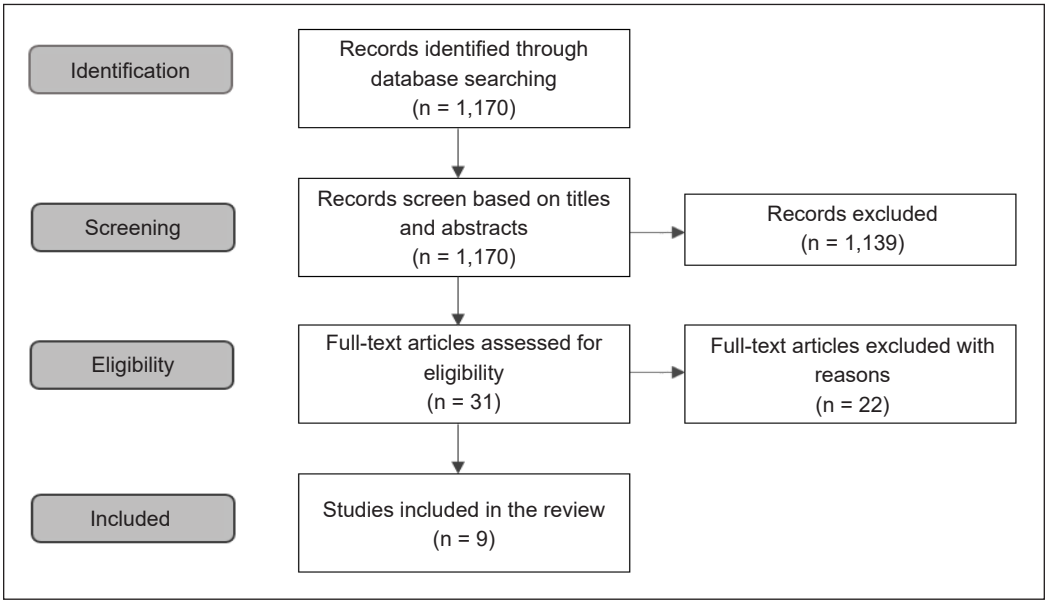


Figure 1. The process of article selection is based on PRISMA. Out of 31 eligible articles, only 9 reported on APV infection and thus were included in the final review

infection were *Carduelis chloris*, *Fringilla coelebs*, *Fringilla montifringilla*, and *Serinus canaria*, whereas the viruses were detected in three species among the family Laridae, namely, *Larus marinus*, *Larus smithsonianus*, and *Rissa tridactyla*. The families of Psittacidae, Spheniscidae, and Sylviidae had one species detected with PV infections: *Psittacus erithacus*, *Pygoscelis adeliae*, and *Sylvia atricapilla*. Table 2 presents the distribution of APV in the collected birds according to their species.

Among the included studies, five performed APV screening on the wild birds, whereas four performed screening at the aviary, with one performed screening on both wild and captive birds. One study performed the screening using archived tissue samples. The pooled prevalence of APV can be analyzed among the families Anatidae (duck species), i.e., 13% (95% CI = −0.03–0.29, I^2 = 90%), Fringillidae, i.e., 81% (95% CI = 0.56–1.05, I^2 = 0%), and Laridae, i.e., 30% (95% CI = 0.04–0.56, I^2 = 96%) (Figure 2).

Five studies included the descriptions of APV in biopsy samples from symptomatic birds. Seven types of biopsy samples involved a total of 43 samples, which were skin (n = 10), larynx/trachea (n = 2),

Table 2
Distribution of avian papillomavirus in the collected birds according to their species. Out of 798 birds of various species reported by nine studies, 95 were positive for APV

Family	Bird species	Number of bird sample	Number of birds with positive APV	Reference
Alcidae	*Alca torda*	30	0	Canuti et al. (2019)
	Fratercula arctica	51	5	
	Uria aalge	41	0	
	Uria lomvia	2	0	
Anatidae	American black duck × mallard hybrid	4	1	Canuti et al. (2019)
	Anas crecca	35	0	Williams et al. (2018)
	Anas penelope	1	0	Williams et al. (2018)
	Anas platyrhynchos	246	6	Williams et al. (2018)
		10	1	Canuti et al. (2019)
	Anas platyrhynchos domesticus	17	2	Williams et al. (2018)
	Anas rubripes	102	30	Canuti et al. (2019)
Burhinidae	*Burhinus oedicnemus*	1	0	Pérez-Tris et al. (2011)
Cacatuidae	*Cacatua moluccensis*	2	0	Latimer et al. (1997)
Columbidae	*Ducula oceanica*	1	0	Pérez-Tris et al. (2011)
	Leptotila rufaxilla	1	0	
	Streptopelia orientalis	1	0	
	Cyanocorax yncas	1	0	Pérez-Tris et al. (2011)
Emberizidae	*Emberiza leucocephalos*	1	0	Pérez-Tris et al. (2011)

Table 2 *(continue)*

Family	Bird species	Number of bird sample	Number of birds with positive APV	Reference
Fringillidae	*Carduelis chloris*	1	0	Pérez-Tris et al. (2011)
		2	2	Sironi and Gallazi (1992)
	Euphonia musica	1	0	Pérez-Tris et al. (2011)
	Fringilla coelebs	6	5	Pérez-Tris et al. (2011)
		5	5	Prosperi et al. (2016)
	Fringilla montifringilla	1	1	Prosperi et al. (2016)
	Loxia curvirostra	1	0	Pérez-Tris et al. (2011)
	Pyrrhula pyrrhula	1	0	Pérez-Tris et al. (2011)
	Pyrrhula pyrrhula griseiventris	1	0	Pérez-Tris et al. (2011)
	Serinus canaria	4	3	Truchado, Williams, et al. (2018)
		2	2	Dom et al. (1993)
Laridae	*Larus delawarensis*	9	0	Canuti et al. (2019)
	Larus glaucoides	4	0	
	Larus marinus	38	1	
	Larus smithsonianus	94	16	
	Rissa tridactyla	16	13	
Paridae	*Cyanistes caeruleus*	1	0	Pérez-Tris et al. (2011)
	Parus afer	1	0	Pérez-Tris et al. (2011)
	Periparus ater	1	0	Pérez-Tris et al. (2011)
Passeridae	*Passer domesticus*	7	0	Pérez-Tris et al. (2011)
	Passer griseus	1	0	Pérez-Tris et al. (2011)
Psittacidae	*Amazona aestiva*	1	0	Latimer et al. (1997)
	Amazona amazonica	1	0	Latimer et al. (1997)
	Amazona autumnalis	1	0	Latimer et al. (1997)
	Amazona farinosa	1	0	Latimer et al. (1997)
	Amazona ochrocephala	1	0	Pérez-Tris et al. (2011)
		1	0	Latimer et al. (1997)
	Amazona ochrocephala auropalliata	2	0	Latimer et al. (1997)
	Amazona sp. - unspecified	4	0	Latimer et al. (1997)
	Ara ararauna	6	0	Latimer et al. (1997)
	Ara chloroptera	1	0	Latimer et al. (1997)
	Ara sp. - unspecified	2	0	Latimer et al. (1997)
	Aratinga erythrogenys	1	0	Latimer et al. (1997)
	Aratinga sp. - unspecified	2	0	Latimer et al. (1997)
	Psittacus erithacus	2	1	Latimer et al. (1997)
Spheniscidae	*Pygoscelis adeliae*	25	1	Van Doorslaer et al. (2017)
Sylviidae	*Sylvia atricapilla*	3	0	Pérez-Tris et al. (2011)

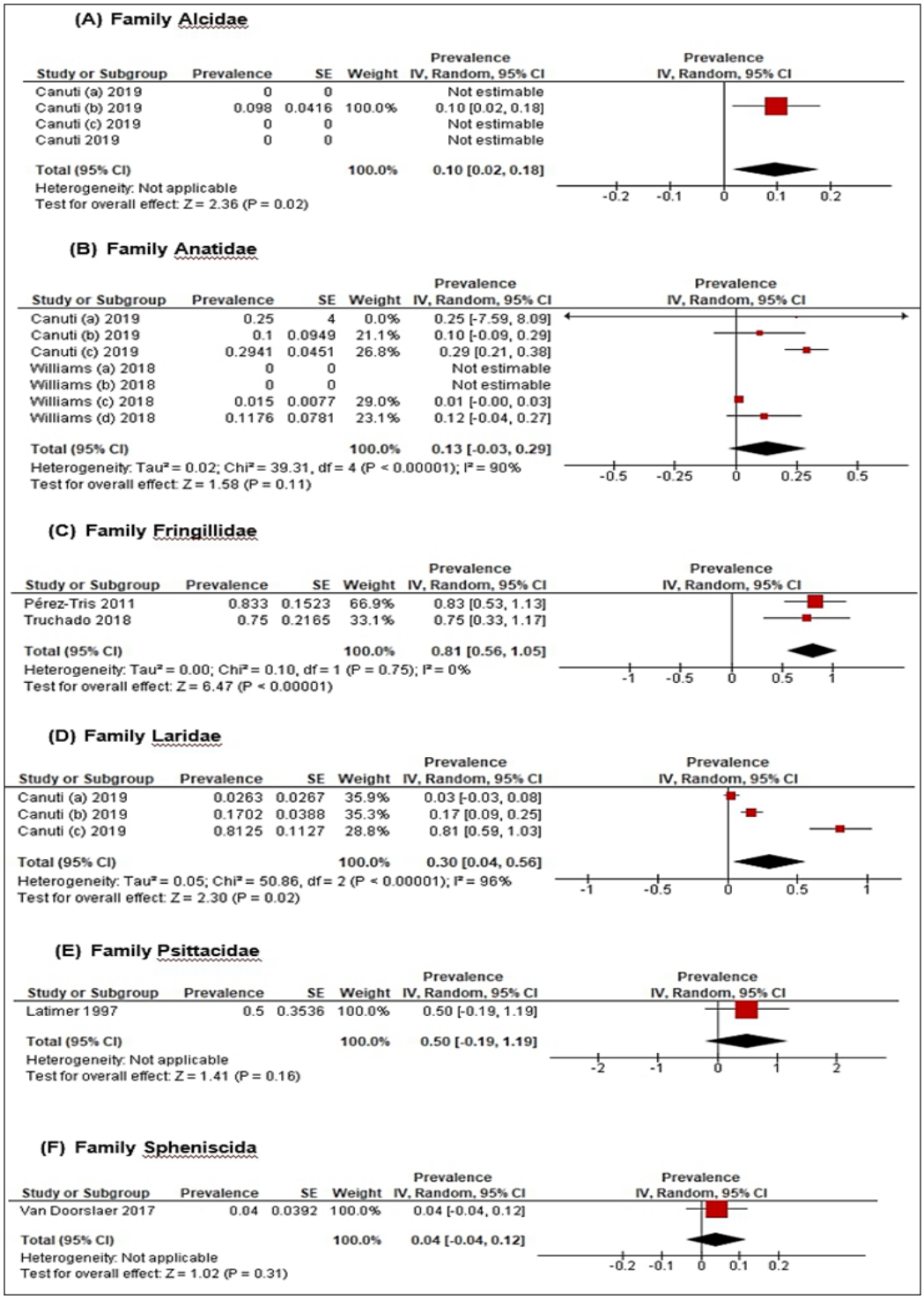


Figure 2. Forest plot of random-effect meta-analysis of avian papillomavirus in different bird families. Data shows that APVs are highly prevalent among the Fringillidae family, followed by Laridae and Anatidae

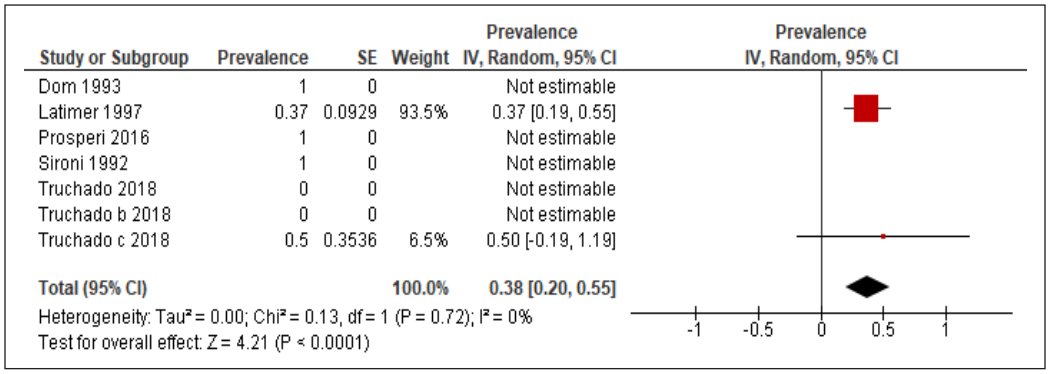
digestive tract (n = 2), tongue (n = 2), and cloacal/oral (n = 27). Of these, 12 samples were positive for APV infection, with 10 from the skin, 1 from the tongue, and 1 from cloacal/oral papilloma. Skin biopsies were performed in three studies, whereas other types of biopsy samples were reported by one individual study separately. Only one study screened APV in three biopsy samples: larynx/trachea, digestive tract, and tongue. The high prevalence of APV on bird skins corresponds to the known type of human cutaneous PV, represented by the beta and gamma genera, which reside widely on the skin surface. Therefore, a similar skin commensalism/mutualism between APV with their avian hosts and APV occurrences would best be diagnosed by symptom manifestations on the skin is proposed.

Four studies included descriptions of APV in swab samples. Three types of swab samples involved a total of 641 samples, which were oral swabs (n = 164), cloacal swabs (n = 25), and paired oropharyngeal and cloacal swabs (n = 452). Of these, 96 samples were positive for APV, with 20 from oral swab samples, 4 from cloacal swab samples, and 72 from paired oropharyngeal and cloacal swab samples. Two studies screened APV in oral swab samples, whereas cloacal swab and paired oropharyngeal and cloacal swab samples were separated in one study. One study involving 33 tissue biopsies and skin swab samples was excluded because it did not mention the virus in which sample types accordingly (Pérez-Tris et al., 2018). The pooled prevalence of APV in tissue samples was 38% (95% CI = 20–55, I^2 = 0%), and the pooled prevalence of APV in swab samples was 13% (95% CI = 5–21, I^2 = 83%), as shown in Figures 3(a) and 3(b), respectively. Only one study screened APV in fecal material, where 4 out of 968 (0.4%) samples were positive for APV.

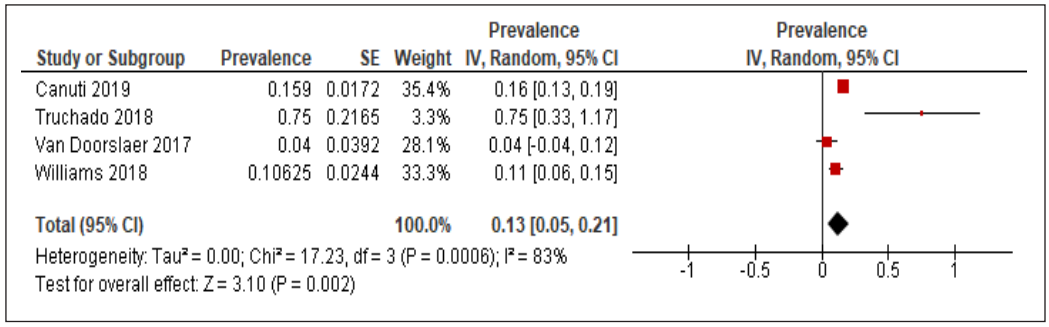
Out of the 711 samples, 68 birds were symptomatic, and 6 birds were asymptomatic, while clinical symptoms were not mentioned in the remaining 637 birds. The reported symptoms include the appearance of papillomas, pododermatitis, breathing problems, and lesions at the eyelid, leg, beak, head, and toe. Two birds with positive APV infection were reported dead. Only two studies screened for APV in both symptomatic and asymptomatic birds. The meta-analysis showed no significant occurrence of APV cases in asymptomatic birds and symptomatic birds (pooled OR = 2.89, 95% CI = 0.27–31.32, p = 0.71) (Figure 3(c)).

DISCUSSION

This study systematically collated published literature on the detection of APV in non-mammalian species, particularly in bird species, to observe the distribution of APV. This meta-analysis also aimed to determine the most favorable sample materials for APV screening. The information regarding accuracy parameters, such as sensitivity, specificity, true/false-positive, and true/false-negative values, was insufficiently provided in the studies included in the analysis. As such, a meta-analysis on the



(a)



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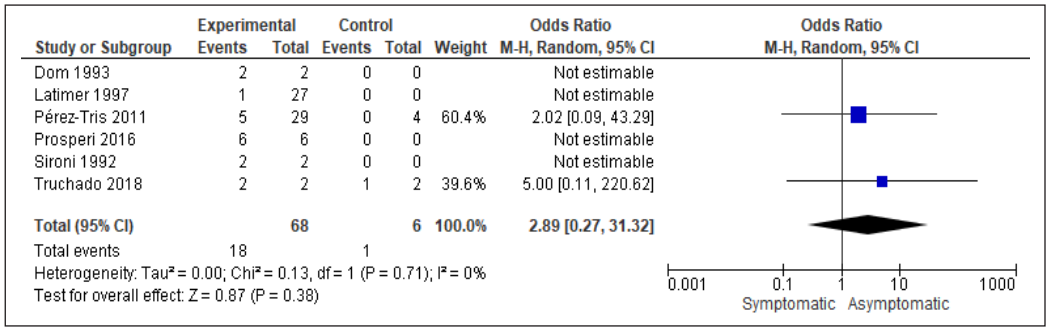

(c)

Figure 3. Forest plot of random-effect meta-analysis of avian papillomavirus (AVP) screened in (a) tissue biopsy samples, (b) swab samples, and (c) symptomatic vs. asymptomatic birds. Data shows the pooled prevalence of APV in tissue samples higher than in swabs, and no significant occurrence of APV cases in symptomatic birds compared to asymptomatic birds

diagnostic accuracy of APV screening was not performed.

Based on our findings, APV is highly distributed among the families Fringillidae, followed by Laridae and Anatidae. The high prevalence of APV in the American black duck population and a lower circulation rate in mallards were related to seasonality in infections (Canuti et al., 2019). There is also an assumption that the sexual route is

a possible viral transmission mode because APV was significantly more prevalent in adult ducks during the pre-breeding season (Canuti et al., 2019; Williams et al., 2018).

Five studies performed APV screening on the wild birds, whereas four performed screening on birds at the aviary, with one of the studies screenings on both wild and aviary birds. The incidence of APV infection among wild bird populations is found to be low (Prosperi et al., 2016). Nonetheless, the species-specific nature of the virus allows local transmission even with no direct cutaneous or mucosal contact, which may jeopardize the health status of either captive or wild bird populations. The prevalence of APV from tissue and swab samples was 38 and 13%, respectively, with only one study screened from fecal material (0.4%). As such, this study did not compare the reliability between these three samples. APV commonly infects the cloaca; hence, feces are preferable to other sample materials (Varsani et al., 2015). Besides, fecal sampling is considered a fast and noninvasive screening technique since it requires no physical contact and does not cause stress to exotic birds (Zanon et al., 2018). However, a study, which conducted virus screening in only fecal or swab samples, reported a lower incidence rate (Williams et al., 2018) compared to screening in paired oropharyngeal and cloacal swabs samples (Canuti et al., 2009). There were cases of APV detection in oropharyngeal swab samples but not in cloacal swabs (Canuti et al., 2009). It affirms the importance of selecting the correct sample materials to avoid false-negative results.

A specific, sensitive, and rapid method is important to improve our knowledge of the agents causing avian cutaneous lesions. Initially, the primary investigation method for avian papillomatosis relied on histologic examination of the lesions. This method involved tissue fixation, staining, and optical microscope observation (Di Francesco et al., 2019). There was no identity confirmation of the causative agents following histologic examination. As such, the unknown causative agent can be any papilloma-caused virus or bacteria. Electron microscopy provides evidence of PV in symptomatic samples based on the virus particle. PVs can be differentiated from other viruses based on their non-enveloped icosahedral structure with a diameter of 50–60 nm (Doorbar et al., 2015). Using negative contrast electron microscopy, Sironi and Gallazi (1992) demonstrated that PVs in green finches were 52.6 nm in diameter. However, the intranuclear rounded PV-like particles in canaries were shown to be smaller, i.e., approximately 45 nm in diameter (Dom et al., 1993). The same study reported that the smaller size might be due to the fixation artifacts in the ultrathin sections compared to negative staining electron microscopy (Sironi & Gallazi, 1992). A molecular diagnostic technique, polymerase chain reaction (PCR), was developed to detect the presence of PVs in avian species. Most PCR techniques target the *L1* gene due to its highly conserved region (Padzil et al., 2021; Van Doorslaer et al., 2016).

A multiplex PCR was developed to screen more than one papilloma-causative agent, PV, and poxvirus concurrently (Pérez-Tris et al., 2011; Truchado, Williams, et al., 2018). Thus, molecular diagnostic provides reliable results as they can differentiate the absence or presence of the target organisms.

APV infection is usually characterized by papilloma lesions at the base of the tongue or on the glottis among psittacines (Truchado, Williams et al., 2018). The infection of certain APVs, such as FcPV1, FgPV1, and PePV1, is associated with cutaneous papillomas (Jones et al., 2020). However, the occurrence of APV infection is not always presented in the clinical symptoms of the disease. The meta-analysis showed no significant APV cases in asymptomatic and symptomatic birds. Besides, there are cases where PV is not detected in birds, despite showing clinical signs (Di Francesco et al., 2018; Johne et al., 2002; Jones et al., 2020). It is due to the other viruses or bacteria that can cause similar clinical signs with PV infections. For example, viruses like poxvirus and herpesvirus may cause the development of nodules or papillomas, while bacterial abscesses or neoplastic diseases may cause epithelial tumors and soft tissue sarcomas (Di Francesco et al., 2018; Johne et al., 2002; Pérez-Tris et al., 2011). To further validate and reduce the gap of knowledge of APV occurrences in asymptomatic birds and symptomatic birds, increasing the number of APV screenings is recommended.

This study encountered several limitations, i.e., (1) the sample number varied from as low as 2 to 452 bird samples, thus did not provide a standardized data comparison, (2) few studies did not specifically mention the pre-deposited clinical signs of birds, either they were symptomatic or asymptomatic, (3) the diagnostic techniques were varied among those included studies, which might cause an argument in the meta-analysis study, and (4) a high heterogeneity in the results was also observed despite the small number of reviews being included. It was believed to be due to the random effects model, which was selected as the meta-analysis model. A further subgroup analysis or meta-regression model, which includes study settings, sample size, publication year, and phenotype search terms (Sun & Feng, 2019), can be explored in future work. Additionally, a reliability comparison can be conducted by reproducing the experiments to confirm the findings.

CONCLUSION

APV is most prevalent among three families, i.e., the Fringillidae, Laridae, and Anatidae. The distribution of APV among wild and captive birds could not be predicted efficiently due to insufficient rapid diagnostic kits targeting APV of birds compared to other species such as bovines and humans. Using paired samples in the virus screening is important because inconsistent results can occur in different sample materials collected from the same bird. However, the prevalence of APV in tissue samples is high, which can be used as a single sample material. The occurrence of APV cases among

symptomatic and asymptomatic birds is not significant. It was assumed that a high virus load is needed to develop the clinical signs among infected birds. Thus, this meta-analysis study helps determine the most suitable sampling methods for retrieving the PV in avian species. The information gathered from this study can significantly increase the chances of isolating the APV from tissue samples and studying them on molecular and structural levels.

CONFLICT OF INTERESTS

The authors have no conflicts of interest to declare.

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Comparative Study on Leaf Anatomy in Selected *Garcinia* Species in Peninsular Malaysia

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ABSTRACT

A comparative study of leaf anatomy was attempted on *Garcinia* species in Peninsular Malaysia to identify anatomical features useful in species identification and classification. The species are *Garcinia mangostana* var. *mangostana*, *Garcinia mangostana* var. *malaccensis*, and *Garcinia celebica*. Leaves were collected from two different regions: Kuantan, Pahang and Kepong, Kuala Lumpur. The leaf anatomical study was done using the methods of leaf peeling, leaf venation, leaf cross-section, and scanning electron microscopy. The assessment of the leaf anatomy found that these three *Garcinia* species showed similarities in anatomical features, including the presence of paracytic stomata on the abaxial surface, a straight to wavy anticlinal wall of both adaxial and abaxial surfaces, a thick cuticle wax layer, the presence of druses, mucilage canal, petiole vascular bundle, the presence of collenchyma cells in the midrib, and also the presence of sclerenchyma cells in midrib and petiole. Meanwhile, the notable anatomical variation observed in this study included three types of midrib vascular bundles: the outline of the leaf margin, the presence of tanniferous idioblast, leaf marginal, and laminal venation as six types of epicuticular waxes present on epidermal surface. Overall, this study highlighted the anatomical features that are taxonomically valuable, which could be used to identify selected *Garcinia* species in Malaysia.

Keywords: *Garcinia*, *Garcinia celebica*, *Garcinia mangostana* var. *malaccensis*, *Garcinia mangostana* var. *mangostana*, leaf anatomy, taxonomy

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INTRODUCTION

Plant systematics is established in the fundamental elements of evolution theory, and it purposely reveals and reconstructs phylogeny among species, genera, and families (Simpson, 2020). Plant anatomy serves as a tool that aids in plant systematics in terms of identification and classification based on specific characteristics. However, more than anatomical features are needed to implement the established classification, as it exclusively provides extra details to external morphological features (Metcalfe & Chalk, 1950). It only can be used as secondary evidence to the initial classification based on morphological features. Given the significance of anatomy as a tool for systematics and taxonomy, acquiring anatomical data is essential to overcome classification and phylogeny challenges. Due to inadequate morphological details, plant anatomy sometimes delivers primary evidence for classification at the species or subspecies level (Maiti et al., 2012). Both morphological and anatomical data can obtain more accuracy in plant classification.

The characteristics of the leaf epidermis provide important information for identifying plant species within various plant families (Adedeji et al., 2007), such as trichomes, stomata types, stomata pore, stomata index, and subsidiary cells (Ullah et al., 2018). These micromorphological features assist species delimitation (Stefano et al., 2008). Several *Garcinia* species reported the absence of trichomes (Gupta et al., 2018; Priya et al., 2018). Some features in the petiole cross sections, including vascular pattern, the appearance of sclerenchyma cells, and the presence of medullary vascular bundles and trichomes types, are helpful in species identification, especially in Dipterocarpaceae (Noraini et al., 2016). The structure, composition, and tissue arrangement in the leaf midrib provide excellent additional sources for plant delimitation (da Silva et al., 2015; Dalvi et al., 2014; Mantovani et al., 2009). Besides, the utilisation of plant anatomy is widespread for plant physiology and ecology (Sokoloff et al., 2021). Previous studies have incorporated the correlation of anatomies and environmental conditions, such as water stress on *Garcinia brasiliensis* (de Souza et al., 2013) and *Garcinia madruno* (Abreu et al., 2017), as well as *in vitro* gamma irradiation on *Garcinia mangostana* var. *mangostana* calli in combating photosynthesis deficiency (Qosim et al., 2011).

Garcinia is a notably large genus from Clusiaceae and subfamily Clusioideae Burnett (Stevens, 2007). Approximately 240 *Garcinia* species were discovered across Asia and Africa, predominantly in Southeast Asia, and 49 *Garcinia* species were distributed in Peninsular Malaysia (Nazre et al., 2007). The species range from small to large trees or shrubs in tropical forests (Nazre et al., 2018). Moreover, *Garcinia* can be distinguished through gummy latex and hard timber. It is also mentioned that *G. mangostana* var. *mangostana* (mangosteen), labelled the "Queen of Fruit", is a well-known cultivated species in Southeast

Asia. Some of *Garcinia* species such as *G. mangostana* var. *mangostana* and *Garcinia mangostana* var. *malaccensis* can adapt better to tropical climates and organic rich soils (Lim, 2012a, 2012b). Meanwhile, the coastal nature of *Garcinia celebica* tolerates salty conditions well, especially in sandy, rocky, and acid clay soils or areas with heavy rainfall and drought environments (Lim, 2012c).

For *Garcinia* species, the classification and phylogeny have been well constructed based on the morphological and genetic features from previous studies over the decades. The morphological features are widely documented by including the most important parts: inflorescences, stamens, and fruits in *Garcinia*. The recognition of *Garcinia* species is also possible through vegetative features such as the leaf, particularly in *G. mangostana* var. *mangostana* (Nazre et al., 2018). However, Nazre et al. (2018) also stated that leaf features must be carefully treated for species isolation due to the convergence of vegetative features, as they inaccurately showed upper-level relationships in all instances. Besides, the anatomical studies of *Garcinia* still need to be widely utilised, and only a few pieces of data were documented recently. Additional material, such as leaf anatomy, can provide more evidence and justification for species identification in *Garcinia*. Thus, this study aims to identify the leaf anatomical features of the selected *Garcinia* species in Malaysia, which are *G. mangostana* var. *mangostana*, *G. mangostana* var. *malaccensis*, and *G. celebica*.

MATERIALS AND METHODS

Plant Collection and Preparation

The study was conducted on three selected *Garcinia* species, namely *G. mangostana* var. *mangostana*, *G. mangostana* var. *malaccensis*, and *G. celebica*. The leaves samples were collected from Glasshouse Nursery Complex at International Islamic University Malaysia (IIUM), Kuantan Campus, Pahang and Forest Research Institute Malaysia (FRIM), Kepong, Kuala Lumpur. The leaves samples were then compressed and dried in the oven for approximately two weeks. The voucher specimens were preserved in the IIUM Herbarium for future reference and analysis.

Leaves Peeling

The fresh leaves samples at the epidermal surface of the abaxial and adaxial were scraped off using a sharp blade until the desired epidermis could be reached. The epidermal peels were placed on the glass slide and stained with safranin solution (Systerm, Malaysia) for 5 min. The features of the epidermal surface were viewed under a light microscope (Leica ICC50 HD, Germany).

Leaves Venation

The fresh leaves samples in a petri dish were submerged in a basic fuchsin solution (Bendosen, Malaysia) as a clearing agent. The petri dish was placed in the oven at 60^{o}C until the leaves samples were fully cleared. The cleared leaf sample was placed on a

glass slide, and the features of the veins were viewed under a light microscope (Leica ICC50 HD, Germany).

Leaves Cross Section

The fresh leaves parts (margin, petiole, midrib, and lamina) were sliced in a range of thickness (35-40 µm) using a sliding microtome. The sliced leaves samples were stained in safranin (Systerm, Malaysia) and Alcian blue (Sigma, USA) for approximately 5 min for each staining. The stained leaves samples were dehydrated using alcohol (ethanol) solutions at different concentrations (50, 70, 95, and 100%). The method, including slicing, staining, and dehydration, were modified following Johansen's (1940) and Sass's (1958) recommendations. The samples were placed on a slide and mounted in Euparal. Anatomical images were captured using the LAS EZ software (version 3.0.0) attached to a microscope (Leica ICC50 HD, Germany). The anatomical features were described properly.

Scanning Electron Microscopy

The selected leaf samples were collected from the dried herbarium samples. The lamina part from the samples was excised in 1 cm^2 measurements, attached to aluminium stubs, and mounted on a mounting holder. The mounted samples were sputter-coated with a film layer of gold. The sample`s notable features, such as waxes and stomata structure, were observed under a scanning electron microscope Zeiss Model EVO 50 (Germany).

RESULTS AND DISCUSSION

The findings of this study showed that all three *Garcinia* species shared eight common anatomical features, which are the presence of paracytic stomata on the abaxial surface (Figure 1A), a straight to wavy anticlinal wall of both adaxial and abaxial surfaces (Figure 1B), a thick cuticle wax layer (Figure 1C), the presence of druses (Figure 1D), mucilage canal (Figure 1E), petiole vascular bundle (Figure 1F), the presence of collenchyma cells in midrib (Figure 1G), as well as the presence of sclerenchyma cells in midrib and petiole (Figure 1H).

This study also reported the notable variations that can be used to differentiate all three *Garcinia* species studied. First, three types of midrib vascular bundles were observed in the three *Garcinia* species in this study (Table 1). The midrib of the vascular bundle consists of a main vascular bundle (closed system with a non-continuous ring of the vascular bundle and O-shaped) and a medullary vascular bundle (opened system with a continuous ring of the vascular bundle and U-shaped), which can be found in both *G. mangostana* var. *mangostana* (Figure 2A) and *G. celebica* (Figure 2B). These two species only varied in the number of additional vascular bundles (opened system with continuous rings of vascular bundles). Meanwhile, the medullary vascular bundle was not observed in *G. mangostana* var. *malaccensis* (Figure 2C). Another variation is the outline of the leaf margin (Table 2). *G. mangostana* var. *mangostana* and *G. mangostana* var. *malaccensis* showed a 20° curved downward pattern toward the abaxial

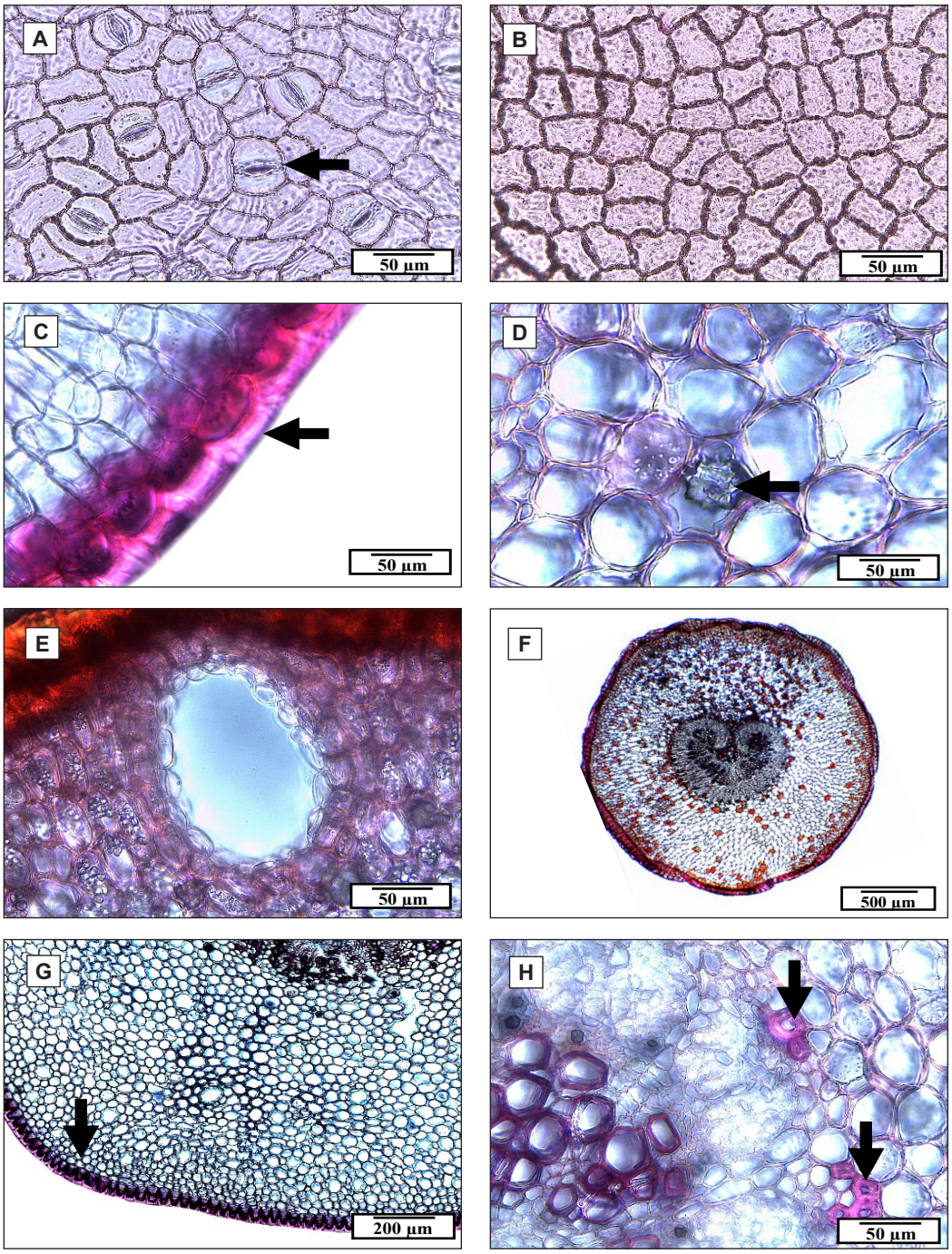


Figure 1. Common features of leaf anatomy and micromorphology in *Garcinia*. (A) Presence of paracytic stomata (black arrow), (B) Straight to the wavy anticlinal wall of adaxial and abaxial surfaces, (C) Presence of thick cuticular wax (black arrow), (D) Presence of druses (black arrow), (E) Presence of mucilage canal, (F) Petiole vascular bundle, (G) Presence of collenchyma cells in midrib (black arrow), and (H) Presence of sclerenchyma cells in midrib and petiole (black arrow)

Table 1
The identification and classification of midrib vascular bundles

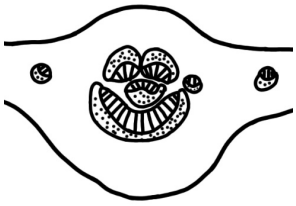
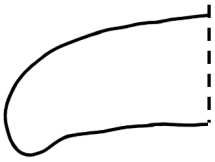
Type	Description	Species	Illustration
1	Closed system (non-continuous ring of the vascular bundle) with O-shaped, one medullary vascular bundle (opened system with a continuous ring of the vascular bundle) with U-shaped, five additional vascular bundles (opened system with continuous rings of vascular bundles)	*Garcinia mangostana* var. *mangostana*	
2	Closed system (non-continuous ring of the vascular bundle) with O-shaped, four additional vascular bundles (opened system with continuous rings of vascular bundles), no presence of medullary vascular bundle	*Garcinia mangostana* var. *malaccensis*	
3	Closed system (non-continuous ring of the vascular bundle) with O-shaped, one medullary vascular bundle (opened system with a continuous ring of the vascular bundle) with U-shaped, three additional vascular bundles (opened system with continuous rings of vascular bundles)	*Garcinia celebica*	

Table 2
The classification and description of the outline of the leaf margin

Type	Description	Species	Illustration
1	Tapered with a blunt end, 20° curved downward towards the abaxial epidermis. The size decreases gradually toward the edge of the margin	*Garcinia mangostana* var. *mangostana* *Garcinia mangostana* var. *malaccensis*	
2	Tapered with a blunt end, straight. The size decreases gradually toward the edge of the margin	*Garcinia celebica*	

epidermis (Figure 2D), while *G. celebica* showed a straight pattern (Figure 2E).

Next is the presence of tanniferous idioblast (Figure 2F), which can be found only in *G. celebica* and *G. mangostana* var. *malaccensis*. Furthermore, complete leaf marginal venation (Figure 2G) was observed in *G. mangostana* var. *mangostana,* while incomplete venation (Figure 2H) was found in *G. mangostana* var. *malaccensis* and *G. celebica*. For lamina venation, minority opened, and majority closed venation with

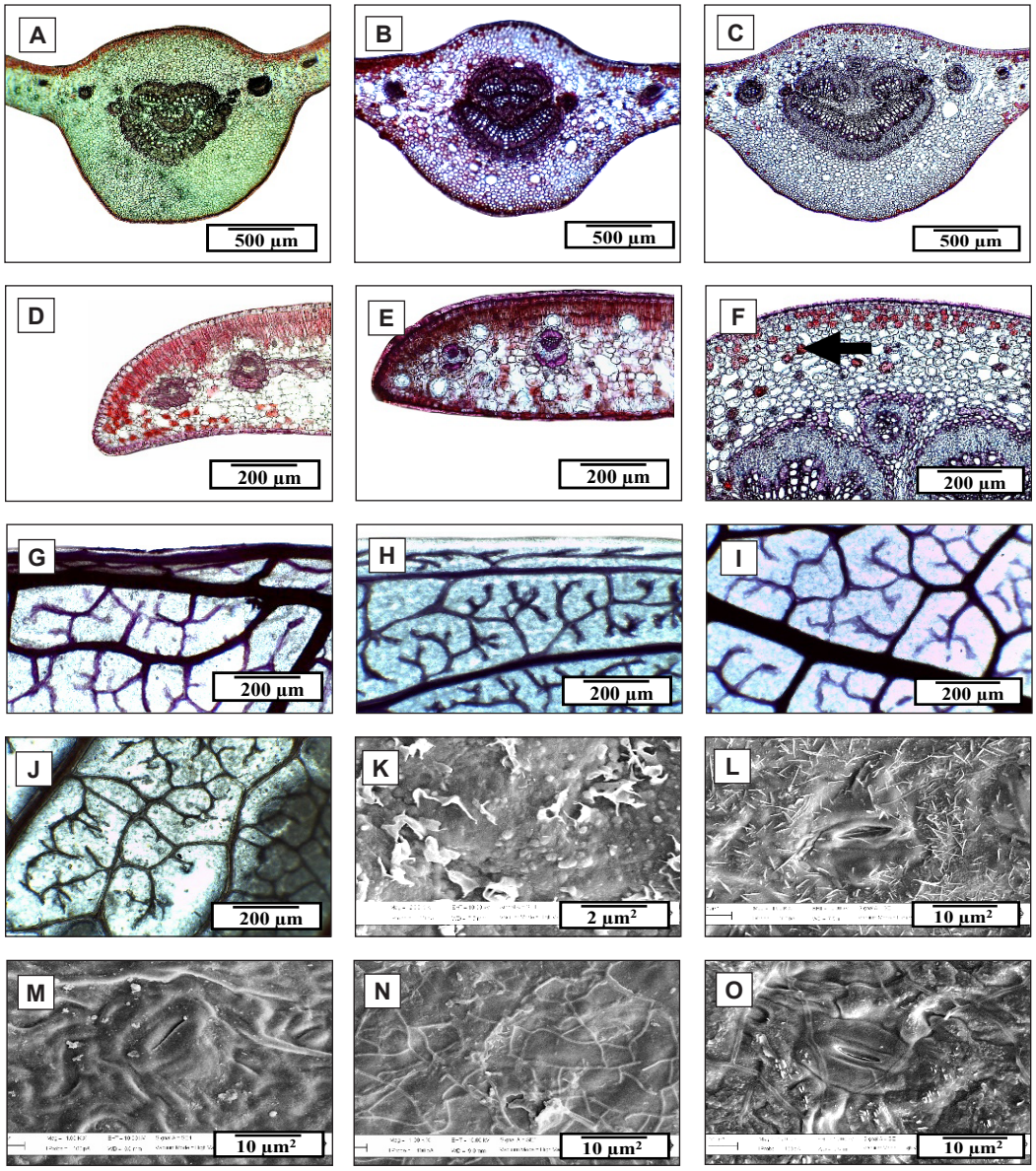


Figure 2. Variation characteristics of leaf anatomy and micromorphology in *Garcinia*. (A) Midrib of *Garcinia mangostana* var. *mangostana*, (B) Midrib of *Garcinia celebica*, (C) Midrib of *G. mangostana* var. *malaccensis*, (D) Marginal leaf outline of *G. mangostana* var. *mangostana* and *G. mangostana* var. malaccensis, (E) Marginal leaf outline of *G. celebica*, (F) Presence of tanniferous idioblast (black arrow) in *G. mangostana* var. *malaccensis* and *G. celebica*, (G) Complete marginal venation in *G. mangostana* var. *mangostana*, (H) Incomplete marginal venation in *G. mangostana* var. *malaccensis* and *G. celebica*, (I) Lamina venation with minority opened and majority closed with swollen tracheid in *G. mangostana* var. *mangostana* and *G. mangostana* var. *malaccensis*, (J) Lamina venation with minority closed and majority opened with swollen tracheid in *G. celebica*, (K) Irregular platelets cuticular wax on leaf abaxial of *G. mangostana* var. *mangostana*, (L) needle-like cuticular wax on abaxial part of *G. mangostana* var. *mangostana*, (M) Smooth layer cuticular wax on abaxial part of *G. mangostana* var. *malaccensis*, (N) Crust and granules cuticular wax on leaf adaxial of *G. mangostana* var. *malaccensis*, and (O) flakes cuticular wax on leaf abaxial of *G. celebica*

swollen tracheid (Figure 2I) was reported in *G. mangostana* var. *mangostana* and *G. mangostana* var. *malaccensis,* while minority closed and majority opened with swollen tracheid was seen in *G. celebica* (Figure 2J). Lastly, six types of epicuticular waxes presented on both epidermal surfaces were reported in all *Garcinia* species studied, which are irregular platelets (Figure 2K), needle-like wax (Figure 2L), smooth layer (Figure 2M), granules (Figure 2N), crust (Figure 2N), and flakes (Figure 2O). The variation in terms of epicuticular wax in *Garcinia* species in this study was summarised in Table 3.

Watson (1962) stated that the stomata arrangement and distribution are taxonomically essential above the species level. The stomatal distribution on the leaf surface and the type of stomata were similar among all *Garcinia* species in this study. All *Garcinia* species showed hypotomatous stomata, which means the stomata are distributed on the leaf`s lower surface (abaxial part). The distribution of stomata is affected by the surrounding environment, especially in mesophytic habitats. In adapted aerial habitats, plants with hypostomatous stomata usually reduce water loss (Stebbins & Khush, 1961). The type of stomata is significant in determining numerous ranks, establishing the linkage among distinct taxa, and clarifying taxonomic challenges (Baranova, 1992). Stomata are determined into various shapes through the guard's position and neighbouring cells' position. Van Cotthem (1970) listed several types of stomata, such as anomocytic, anisocytic, paracytic, diacytic, actinocytic, cyclocytic, and tetracytic. Paracytic stomata are identified when the stomata are enclosed by two subsidiaries, which are arranged side by side to the stomatal pore and the guard cells. This study found that all *Garcinia* species shared similar paracytic types of stomata. The paracytic pattern observed in *G. mangostana* var. *mangostana* complements the finding from the previous research done by Priya and Hari (2019). Hypostomatous and paracytic stomata were also reported on *Garcinia brasilliensis* (Cardoso et al., 2013), *Garcinia cambogia* (Sreelakshmi et al., 2014), *Garcinia zhangpuensis* (Wang et al., 2018), *Garcinia dulcis* (Begum, 2020), and *Garcinia atroviridis* (Susilowati et al., 2022). These findings supported the evidence that these two features of stomata are common in the *Garcinia* genus (Pathirana & Herat, 2004) and the Clusiaceae family (Metcalfe & Chalk, 1950).

Cutler et al. (1978) highlighted that plant species could be identified through

Table 3
The identification and classification of epicuticular wax

Plant species	Types of cuticular wax	
	Adaxial	Abaxial
Garcinia mangostana var. *mangostana*	Irregular platelets, granules	Smooth layer, needle-like wax
Garcinia mangostana var. *malaccensis*	Crust, granules	Smooth layer, granules
Garcinia celebica	Crust, granules	Crust, flakes, granules

leaf epidermis characterisation. Shah et al. (2018) mentioned that the structure and pattern of anticlinal walls are believed to be a form of environmental adaptation, given that xerophytes plant located in a dry area typically have straight walls, while mesophytic species located in average to hot areas typically have sinuous walls (Gifford & Foster, 1989). The findings of this study showed that all *Garcinia* species exhibited straight-to-wavy anticlinal walls on the abaxial and adaxial epidermis. According to Pathirana and Herat (2004), all *Garcinia* taxa for the adaxial and abaxial epidermis consist of rectangular to square cells. A similar pattern was also observed in *G. zhangpuensis* (Wang et al., 2018), *Garcinia indica*, and *Garcinia livingstonei* (Priya et al., 2018). The types of epidermal cells which are straight, undulated, and sinuate observed in the *Tomovita* species can be utilised in the phylogenetic study of morphological features and determination of linkages across species (Gahagen, 2015). In *Garcinia* cases, epidermal cells of *G. dulcis* (Begum, 2020) and *G. madruno* (Abreu, 2017) showed sinuosity, while *G. cambogia* exhibited undulating to irregular cells (Sreelakshmi et al., 2014). An epidermal anticlinal wall pattern can be a suitable anatomical feature for species delimitation in *Garcinia*.

Furthermore, all *Garcinia* species in this study exhibited a thick cuticular wax in the leaf midrib, petiole, lamina, and margin. Metcalfe and Chalk (1950) mentioned that cuticle thickness is essential to characterise *Garcinia* species. Data collected on the Sri Lanka *Garcinia* species included dorsiventral leaves and a thick cuticle (Pathirana & Herat, 2004). Other *Garcinia* species, such as *G. livingstonei* and *G. indica*, also showed thick cuticles on the leaf midrib, petiole, and lamina (Priya et al., 2018; Priya & Hari, 2019), except for *G. zhangpuensis*, which has a thin cuticle on the leaf abaxial part (Wang et al., 2018). This study proposed a thick cuticle wax to serve as a delimiting feature in the examined *Garcinia* species. The presence of cuticles attests to the interaction between plants and the environment. A previous study by Abreu et al. (2017) found thick cuticle wax on *G. madruno* under flooded conditions. They pointed out that this condition led to low water potential (Medri et al., 2011); thus, thick wax offered more protection to *G. madruno* (Abreu et al., 2017).

This study observed abundant mucilage canals or secretory cavities in all parts of the leaf cross-section. Mucilage canals were widely distributed surrounding the tissues of the vascular bundle, parenchyma, spongy mesophyll, and palisade mesophyll. This finding was justified by the report of Metcalfe and Chalk (1950). They also mentioned that the distribution of the secretory canal is important for characterising the *Garcinia* species. As Nnamani and Nwosu (2012) mentioned, the mucilage canal is used in closed and distinct related families since it possesses taxonomic value (Vieira et al., 2001). The taxonomic value of secretory cavities is demonstrated in numerous genera of the angiosperm families (Metcalfe & Chalk, 1957). The appearance of mucilage

canals was reported in *G. brasiliensis* (Cardoso et al., 2013) and *Garcinia kola* (Nnamani & Nwosu, 2012). Meanwhile, the presence of this feature was restricted to *G. dulcis* (Begum, 2020).

The presence of calcium oxalate crystal, commonly in a woody plant (Wu & Huang, 1997), justified the abundance of druses observed in all *Garcinia* species studied. This study found that the druses (Figure 1D) exhibited compact and spherical aggregates of angular crystal appearance. Beck (2010) explained that druses are commonly found in plants beside raphides, which appear as needle-like crystals. The presence of druses was observed in every tissue of the leaf cross-section. However, it was restricted to the leaf margin of *G. mangostana* var. *mangostana* and *G. mangostana* var. *malaccensis*. Druses could be used as a diagnostic character to identify *Garcinia* species, as observed in *Garcinia morella*, *Garcinia spicata* (Pathirana & Herat, 2004), *G. madruno* (Abreu et al., 2017), *G. indica* (Priya & Hari, 2019), *Garcinia dioica* (Wulansari et al., 2020), and *G. dulcis* (Begum, 2020). All of these *Garcinia* species exhibited a similar appearance of druses. Oxalate removal from the metabolic process, calcium storage, light regulation, and physical protection are all related to the occurrence of crystals (Franceschi & Nakata, 2005).

Moreover, a similar pattern of the vascular bundle in the petiole (Figure 1F) was observed in all *Garcinia* species in this study. Pathirana and Herat (2004) described the pattern of the petiole vascular bundle as consisting of a single arc-pattern collateral strand, open on the adaxial part with incurved distal ends that almost connect to form a complete tube. This pattern was also comparable according to the study reported by Priya and Hari (2019) on *G. mangostana* var. *mangostana*, where the vascular bundle consists of an incurved vascular strand with a narrow gap. A similar pattern of a vascular bundle in petiole was also found in other *Garcinia* species, which are *Garcinia talbotii* (Palkar et al., 2017) and *G. indica* (Priya et al., 2018). Some *Garcinia* species showed an incurved pattern but with a large gap on the adaxial part of the vascular bundle, such as *G. livingstonei* (Priya et al., 2018) and *G. spicata* (Palkar et al., 2017). The pattern of the vascular bundle is utilised to delimit the genus taxa in Clusiaceae (D'Arcy & Keating, 1979). Delimitation is crucial for allocating individuals to the same species or different species. In the Dipterocarpaceae family, the petiole vascular bundle is taxonomically essential to identify species and categorise different genera (Noraini et al., 2016).

The presence of collenchyma cells in the midrib is necessary for support against the strong wind (Amri et al., 2019) by facilitating bending to avoid vascular damage (Leroux, 2012). This statement could validate the presence of collenchyma cells in *G. celebica*, where the plants are commonly found in coastal areas (Nazre et al., 2018). In this study, about 2 to 3 layers of collenchyma cells were concentrated underneath the adaxial and abaxial epidermis in the midrib of all *Garcinia* species. This anatomical feature

could be used as a promising identification tool. Ibrahim et al. (2016) demonstrated the taxonomic significance of the number of collenchyma layers on two species, *Datura innoxia* and *Datura stramonium*. In the family Euphorbiaceae, the distribution of collenchyma is significantly important as the continuous rings of collenchyma were observed in the petiole of three *Acalypha s*pecies (Tadavi & Badhane, 2014). The morphology of collenchyma was considered for a taxonomical study on Cucurbitaceae (Abbas et al., 2022).

Sclerenchyma cells include numerous cell types with thick secondary walls that will lignify in maturity (Dickison, 2000). In this study, sclerenchyma cells were scattered as smaller patches encompassing vascular bundles in the petiole and midrib of all *Garcinia* species. However, these cells were absent in the midrib of *G. celebica* only. These patches of sclerenchyma cells were observed in *Garcinia pedunculata* (Gupta et al., 2018) and most Euphorbiaceae species studied (Tadavi & Bhadane, 2014). According to Noraini et al. (2016), the appearance of sclerenchyma cells was a notable feature in the petiole vascular bundle. Besides, the selected *Parashorea* species can be identified by the appearance of sclerenchyma cells surrounding the main and additional vascular bundles in the lamina (Noraini & Cutler, 2009). Sclerenchyma layers were included as a diagnostic feature of taxonomy value in Cucurbitaceae (Abbas et al., 2022). Noor-Syaheera et al. (2015) stated that both sclerenchyma and collenchyma cells act as structural support but vary in elasticity (Coyle, 2004). Sclerenchyma cells play a role in ecological adaptation under seasonal water shortage, soil nutrient deficits, leaf protection against herbivores, or leaf carbon uptake improvement, as highlighted by Edwards et al. (2000).

The previous study by Pathirana and Herat (2004) delivered six main groups of midrib vascular bundles that could be utilised to distinguish the *Garcinia* taxa in Sri Lanka. They also described the pattern of the midrib vascular bundle in *G. mangostana* var. *mangostana*, which consisted of two adaxial and abaxial vascular arcs opposing each other with a middle vascular plate, each individually encircled by a bundle sheath. A similar pattern was observed in *G. mangostana* var. *mangostana*, as seen in Table 1. Table 1 shows three different types of midrib vascular bundles that could be used to separate the *Garcinia* taxa individually. The presence of a medullary vascular bundle and the number of additional vascular bundles were two keys point used to distinguish these *Garcinia* species studied. Priya et al. (2018) also described three different shapes of the midrib's vascular zone, including the appearance of cowry, owl, and cordate in *G. mangostana* var. *mangostana*, *G. livingstonei*, and *G. indica*, respectively. Noor-Syaheera et al. (2015) reported multiple types of midrib vascular bundles (opened system) in selected taxa in Acanthaceae by including the shape of the vascular bundle (O-shaped, V-shaped, and U-shaped) and the number of separated vascular bundles. Numerous studies have

documented the systematic importance of the pattern of vascular bundles, including those on *Parashorea* (Noraini & Cutler, 2009) and the family Rhizophoraceae (Nurnida, 2012). Thus, it is justifiable that this feature is taxonomically significant.

Furthermore, this study suggested that the outline of the leaf margin could be used for species delimitation, as seen in Table 2. This study revealed two different patterns of a marginal outline: curved downward and straight. Similarly, these two patterns were also reported in certain species under the family Anacardiaceae (Norfaizal & Latiff, 2013) and selected *Mangifera* species (Tipmontiane et al., 2018). Noraini et al. (2012) revealed that two shapes (rounded and pointed) of the marginal outline act as diagnostic features in the selected *Johannesteijsmannia* species (Arecaceae). The comparative study based on the marginal leaf outline is not widely reported in *Garcinia*. A similar case happened in Orchidaceae because the marginal leaf outline is unrecognisable for anatomical comparison compared to midrib and petiole (Raffi et al., 2019). Raffi et al. (2019) also proposed that the marginal leaf outline should provide preliminary evidence for the characterisation and delimitation of *Vanilla* species.

The current study also showed that tanniferous idioblasts were scattered within the parenchyma tissue of the petiole and midrib except in *G. mangostana* var. *mangostana*. However, the absence of tanniferous idioblast in *G. mangostana* var. *mangostana* was contrary to evidence provided by Pathirana and Herat (2004), where these phenolic idioblasts were highly distributed in this respected species. Regardless, this feature could isolate *G. mangostana* var. *mangostana* from other species in this study. The abundance of tanned cells in the family Clusiaceae was considered by Metcalfe and Chalk (1957) as one of the diagnostic keys besides secretory canals. The distribution pattern of tanned cells in selected species, particularly *G. kola*, may be taxonomically useful by considering the intensity of the cells (Nnamani & Nwosu, 2012). The occurrence of tanniferous idioblast was one of the taxonomically valuable features in the petiole of the *Microcos* genus in the family Malvaceae (Nurul-Aini et al., 2013). This feature may be applicable for identifying and classifying species. Tanniferous idioblast is also linked with protection against stress (da Silva Lobato et al., 2020).

Leaf venation is linked with plant evolution and is taxonomically essential for plant systematics (Hickey, 1973). The architectural features of leaf venation are intensively studied due to their significance for systematic classification (Zetter, 1984), for instance, leaf venation patterns. This study identified two types of marginal venation, complete and incomplete, that can be potentially used to distinguish *Garcinia* species in this study. Complete marginal venation (Figure 2G) is visualised as higher vein orders attached to a vein that runs just beyond the margin, while incomplete venation (Figure 2H) is viewed as freely ending veinlets straight alongside

the margin (Badron et al., 2014). Similar types were also described in the *Ficus* genus (Badron et al., 2014) and the *Carallia* genus (Nurshahidah et al., 2011). Nurul-Aini et al. (2010) revealed that the two distinctive features: incomplete and complete in leaf venation, were significant for distinguishing the selected taxa, namely *Grewia* and *Microcos* (Grewioideae), respectively. This study also reported two different lamina venations with swollen tracheids in the *Garcinia* species studied; hence, these features can be important for classifying species. Leaf venation is considered an underutilised feature, but it has been used for species delimitation in Combretaceae by revealing intra- and intergeneric variation among all the species studied (Akinsulirea et al., 2020). Akinsulirea et al. (2020) also considered the areolar (lamina) venation and the number of veinlets endings useful to characterise the species. Incomplete areolar venation with swollen veinlet ending and closed areolar venation was reported for the *Carallia* species (Nurshahidah et al., 2011). Besides, the system of leaf venation mainly works on structural stability as well as water and solute transportation (Roth-Nebelsick et al., 2001).

Epicuticular waxes are also taxonomically significant for species identification and characterisation, as confirmed by Barthlott (1998). Several attempts have been made to utilise epicuticular wax as taxonomy characteristics to isolate the group of species within a genus or family (Maffei, 1996; Mimura et al., 1998). The irregular granules, flakes, smooth layer, crust, and needle-like epicuticular wax exhibited by each *Garcinia* species, as seen in Table 3, could distinguish these three species. The variation of epicuticular waxes in terms of type, density, and distribution found in the selected *Ficus* species could contribute as extra material for taxonomy (Araújo et al., 2014). The granules type of epicuticular waxes in the *Ficus* species (Araújo et al., 2014) was similar to the *Garcinia* species in this study. The interspecific variation among the selected *Hypericum* species can be seen through abundant waxes, listed as granules, flakes, crust, layer, road, and filament. These features can also aid *Hypericum*'s taxonomy and evolutionary study (Perrone et al., 2013). Perrone et al. (2013) also stated that epicuticular waxes prevent water loss by improving water binding, lessening solar ray interference, and limiting mechanical impairment caused by fungi and insects (Eglinton & Hamilton, 1967).

CONCLUSION

The leaf anatomical features in this study are essential for identifying and classifying the *Garcinia* genus. This study's description of anatomical features might be valuable for plant systematics by providing additional evidence for classification. The findings of this study provide some common features, which are hypostomatic and paracytic stomata, anticlinal walls pattern, thick cuticle wax layer, druses, mucilage canal, petiole vascular bundle, collenchyma cells, and sclerenchyma cells in all three *Garcinia* species studied. This study proposed that these common features could be used for

species delimitation among the *Garcinia* genus. On the other hand, a variation of features was also detected, such as three types of midrib vascular bundles, marginal leaf outline, tanniferous idioblast, leaf venation, and six types of epicuticular waxes on epidermal surfaces. These features possess taxonomical value to distinguish *Garcinia* species. Overall, the findings of this study significantly supported the establishment of species identification and classification among species and genera.

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The Effect of Edible Coatings (Spirulina and Chitosan) on the Quality and Shelf Life of Starfruit (*Averrhoa carambola* L. cv. B10) Throughout Storage

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ABSTRACT

The edible coating is one of the preservation methods widely applied by food industries as it is beneficial in suppressing respiration, minimising moisture loss, and reducing food wastage. This study investigates the effects of edible coating (*Spirulina platensis* and chitosan) on the quality and shelf life of B10 throughout storage at room temperature 27°C. The quality analysis of colour (L*, a*, b*, and hue), browning index, fresh weight and physical appearance were evaluated on days 0, 4, 8, 12, and 14. There was a significant difference for all quality analyses between storage days ($p < 0.05$). The physical appearance showed that at day 8, B10 coated with spirulina maintained the greenish colour while chitosan-coated and controlled turned the fruit bright yellowish, indicating ripening. Control samples were observed to have major browning at day 12, whereas samples coated with spirulina and chitosan only showed early signs of browning. Samples with spirulina coating have the least a* (greenness-redness) and b* (blueness-yellowness) values, which showed that spirulina was able to slow down the ripening process in comparison to chitosan and control ($p < 0.05$). For the hue value, samples with chitosan coating showed the least colour changes ($p < 0.05$), followed by spirulina and control with 9.04, 9.43, and 30.82°, respectively. It proved that coated samples provide the best results in slowing

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down the colour changes and browning of the carambola compared to the control. Besides, the regression analysis resulted in a good fitness (R^2 near 1) for browning and weight loss analysis for all coatings, which were agreed to be reliable and had a good predictive indicator power when the storage days were extended. Hence, these results would be potentially useful for the fresh produce industry to prolong the shelf-life of B10 during distribution, transportation, and commercialisation.

Keywords: Carambola, chitosan, colour, quality, regression analysis, shelf-life, *Spirulina platensis*, weight loss

INTRODUCTION

Averrhoa carambola L. cv. B10 is a member of oxalidaceae with star-shaped and greenish-waxy skin during unripe, which turns yellowish during ripening. The flesh and skin are crisp and juicy, while the amount of oxalic acid content mostly influences the taste of the fruit. Consumers like starfruit due to its sweet and sour taste; it also contains abundant antioxidants such as carotenoids, vitamin C, and vitamin E (Gol et al., 2015). Malaysia is the main producer and exporter of carambolas. This fruit is exported to Europe, such as the Netherlands, France, Switzerland, Canada, and other countries in Asia, such as Singapore and Hong Kong, with annual mean export values of RM 24 million (Ibrahim, 2019). It makes carambola one of Malaysia's leading exports that help increase economic growth.

However, due to the unstable internal environment and temperature along the supply chain, the fruits will be exposed to mechanical injuries, microbial attacks, and pathogen contamination. The quality of starfruit can be easily degraded due to the high moisture content level, which will lead to spoilage (Gol et al., 2015). Benkeblia (2018) reported that green and yellow starfruit are easily damaged and can easily bruise. It was also reported that, when starfruit was stored at 16 and 25°C, necrotic lesions, browning, and shrivelling of the ribs appeared at two and three weeks of the storage, respectively. The outbreak of foodborne illness caused by the microbial attack on the fruit can harm consumers as the fruit is often eaten raw (Warriner et al., 2009). Full attention is needed from the producer, storage operator, processor, and retailer when handling the postharvest fruit to maintain the quality and lengthen the shelf-life to reduce waste and food loss (Mahajan et al., 2014).

Various postharvest physical, chemical, and gaseous treatments can be applied to maintain the fresh-like quality without changing the nutritional value of fresh produce. The food industry widely uses physical treatment such as coating, and it has proven its capability of improving food quality and prolonging shelf-life (Lin & Zhao, 2007). The edible coating improves mechanical strength barrier properties and controls the food components' mass transfer (Khwaldia et al., 2004). It can control the gas exchange and oxidation of food products. Besides, specific properties of the edible coating are accountable for decelerating the organic vapour, such as solvent and/or aroma; water vapour; solutes, such as salts,

lipids, food additives and pigments; and gaseous such as carbon dioxide, oxygen, and nitrogen (Kumar & Neeraj, 2019).

There are a few edible coatings: lipid-based, polysaccharide-based, and protein-based. Chitosan coating is a polysaccharide-based coating made from the shell component of crustaceans. It has shown effectiveness in decreasing weight loss, delaying ripening and changes in colour, titratable acidity, and pH and improving the texture of frozen-thawed strawberries (Han et al., 2004). According to Vargas et al. (2006), chitosan can enhance antimicrobial activity and improve water vapour resistance, thus preserving the quality of strawberries better than the uncoated samples. For application on carambola, 0.3% of chitosan obtained significant results in maintaining the quality of carambola as it is proven to delay weight loss, decay percentage, titratable acidity, pH, total soluble solid, sugar accumulation, pigment degradation, and preserving higher concentration of total phenolics content besides excellent in inhibiting enzyme activity (Gol et al., 2015). Despite this, a higher concentration of chitosan in the coating solutions increased anaerobic respiration, followed by rises in fruit weight loss. In another study, chitosan coating was concluded to have good antibacterial and antifungal properties that could minimise bacteria and pathogen attacks and help slow down decay (Kerch, 2015).

Other than chitosan, *Spirulina platensis*, blue-green microalgae is developed to improve the productive performance of the cultivated plant and fresh produce, such development of structural biofilms for fruits coating during postharvest (Byantara & Dianursanti, 2021). Its protein-based coating is safe to consume as a nutritional supplement due to its chemical properties, including phytohormone, antibacterial, and antifungal compounds. De Oliveira et al. (2020) reported in the previous study that 1% *S. platensis* was able to slow down the colour changes and maintained sugar levels and ascorbic acid content while reducing the astringency after taste of carambola in comparison to that 0, 2, 3, and 4% of coating concentration. A study of guar-based edible coating with *S. platensis* and *Aloe vera* extract on mango by Ebrahimi and Rastegar (2020) showed that the coating with spirulina shows significant results in higher firmness and improving the bioactive compound (ascorbic acid, phenol, and flavonoids) throughout storage in comparison to control (uncoated) samples, thus help in maintaining the quality and further the shelf-life.

However, there is a lack of study on *S. platensis* and chitosan coatings on carambola B10. Thus, the effect of edible coatings (*S. platensis* and chitosan) on the quality and shelf-life of carambola B10 is determined in this study.

MATERIALS AND METHODS

Sample Preparation

Starfruit (*Averrhoa Carambola* L. cv. B10) index 2 was freshly plucked from the same tree early in the morning before being transported from Kuala Pilah, Negeri Sembilan, Malaysia to Universiti Putra

Malaysia (UPM), Serdang, Selangor by car in a three-hour journey. Starfruits, index 2, were harvested around 50 to 55 days after the fruit sets. Samples were carefully sorted to ensure uniformity in colour, size, and maturity with approximately no defect. No defect means the fruit must be free from pest attack and any bruise due to mechanical injury or limited to three or below spots ($n \leq 3$) for each fruit. The samples were washed with 0.05% sodium hypochlorite (NaOCl, Systerm, Malaysia) before coating and air dried on the drying racks. Three sets of samples (including control) were prepared, and each set contained three replications.

Preparation of Spirulina and Chitosan Coatings

Spirulina coating solution was prepared following the method by Cardoso et al. (2017). A total of 4% of starch (Systerm, Malaysia), 0.66% spirulina powder (JoyMix, Malaysia), and 0.66% gelatine (Halagel, Malaysia) were added into a solution containing 1% of glycerol (Systerm, Malaysia) and 93% of distilled water. The solution was heated on a heating plate and stirred for 15 min until a boiling point was reached. The coating was covered with the stretch film packaging wrap and was left at room temperature (26 ± 1°C) to reach homogeneity.

The chitosan coating solution was prepared following the method by Gol et al. (2015). The 0.3% (w/v) of chitosan coating solution was prepared by adding 0.3 g of chitosan powder (Chemiz, Malaysia) into 100 ml of distilled water and 0.5 ml (v/v) of glacial acetic acid (R&M Chemicals, Malaysia). The 0.75% of glycerol monostearate (EvaChem, Malaysia) was then mixed into the solution, which acts as a plasticiser to enhance the flexibility and strength of the chitosan coating. A magnetic stirrer was used to constantly stir the solution at room temperature (26 ± 1°C) for 24 hr to achieve complete dispersion. Then, 1 N of sodium hydroxide (ORC Chem Technologies Sdn. Bhd., Malaysia) was added until the pH reached 5.6 and lastly, 0.1 ml Tween 80 (EvaChem, Malaysia) was inserted to stabilise the mixtures.

The fruit samples were then directly dipped into the spirulina and chitosan coating for 2 min, and samples dipped into distilled water served as control. The samples were placed on the cooling racks and let air dried at 26 ± 1°C before being transferred into a box with tissue paper padding at the bottom. Each sample was wrapped with tissue paper to avoid physical contact between one fruit with another. Samples were left at room temperature (26 ± 1°C) until the end of storage days. The coated fruits were subjected to changes throughout storage, and the physical appearance, colour, and fresh weight were observed at days 0, 4, 8, 12, and 14.

Physical Appearance

An image of the samples was taken inside the black box. The LED light ring was placed on top of the box with the light setting of power 12 W, white colour, outer diameter 26 cm, and inner diameter 20 cm. Images were taken using a digital camera

with a fixed setting (Table 1). The images were taken on days 0, 4, 8, 12, and 14. The physical appearance of the samples was observed.

Table 1
Digital camera setting for a physical image of B10

Options	Settings
Megapixels	12 MP
Aperture size	F1.8
Focal length	26 mm
Optical zoom	1.7x
Shutter speed	½ s
Flash	Off
Focus pixel	Autofocus

Colour

The colour of the starfruits was measured at three points (a top, middle, and bottom) using a Minolta Chroma meter CR-400 (ECMinolta, Japan) and the average values were taken. The value of L*, a*, b*, hue, and browning index was calculated. A positive L* value indicates lightness, while a negative L* reading represents darkness. The a* value represents redness to greenness (redness is towards the positive value while greenness is towards the negative value). Besides, the b* value shows yellowness to blueness (yellowness is towards the positive value while blueness is towards the negative value). The hue value was calculated to represent the colour changes of the fruit where the result of 0° = red purple, 90° = yellow, 180° = blue-green, and 270° = blue. The hue value was calculated using Equation 1. The browning of the sample during storage was carried out by calculating the browning index (BI) shown in Equation 2.

$$\text{Hue angle, } \theta \text{ value} = \tan^{-1} |b^*/a^*| \quad \text{(Equation 1)}$$

$$\text{Browning index (BI)} = [100\ (x - 0.31)] / 0.17 \quad \text{(Equation 2)}$$

where, $x = (a^* + 1.75\ L^*) / (5.645\ L^* + a^* - 0.3012\ b^*)$ according to Ruangchakpet and Sajjaanantakul (2007).

Fresh Weight Loss

The fresh weight of the samples was taken using an analytical weighing balance (OHAUS Pioneer, USA). The percentage of fresh weight loss was calculated by Equation 3.

$$W_{loss} = ([W_{initial} - W_{final}]/W_{initial}) \times 100\% \quad \text{(Equation 3)}$$

W_{loss} is the weight loss in unit percentage, $W_{initial}$ is the initial sample weight in unit gram (g), and W_{final} is the final weight of the samples in unit gram (g).

Statistical Analysis

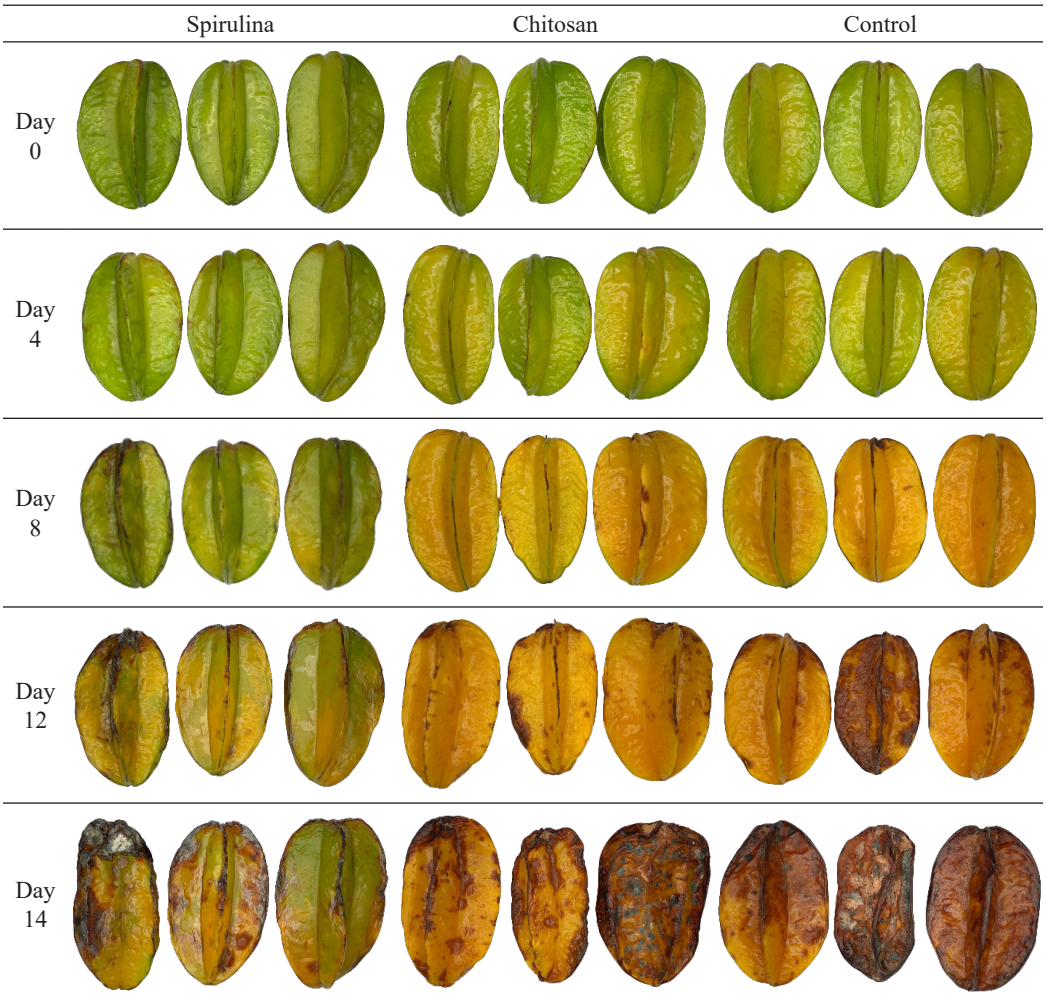
Analysis of variance (ANOVA) was performed using RStudio (version 4.1.2) statistical tools to compare the mean difference between groups. The post hoc test was performed using Tukey's honestly significant difference (HSD) to find the least significant difference ($p < 0.05$) between the compared groups. Linear regression used the first-order kinetic model to observe the relationship between experimental and theoretical data for all the quality tests.

RESULTS AND DISCUSSION

Physical Appearance

The physical appearance of the fruits is the most important quality attribute of fresh and minimally processed produce, where consumers' primary concerns are the size, colour, and defects on the skin finish (Aked, 2000). Spirulina coating maintained the greenish colour, while chitosan coating and control had turned bright yellowish on day 8 of storage (Table 2). The brownish spot on the fruit surface was believed to be an oxidative browning reaction polyphenol oxidase (PPO) oxidises phenolic content into melanin, a brown pigment (Ding & Yap, 2014). The control sample had developed major browning on the skin of the carambola at day 12, besides spirulina and chitosan coatings only showed

Table 2
The physical appearance of B10 coated with 0.66% of spirulina and 0.3% of chitosan coating throughout storage days 0, 4, 8, 12, and 14



early signs of browning development. Spirulina maintained the greenish colour and minimised browning until day 12 but had shown signs of decay compared to chitosan. It showed that spirulina could slow down ripening by minimising colour changes of the carambola but had a poor mechanical barrier, which was susceptible to microbial attack. The control showed major decay on day 12, and the vigorous result showed on day 14 to all samples. Microbial growth on the fruit was due to the decay relative to the gradual respiration and high production of ethylene gas resulting from ripening (Mahajan et al., 2014). According to Butler et al. (1996), polysaccharide properties in chitosan coating act as a good mechanical barrier that helps decrease decay and prevent pathogen infection.

Colour

There were significant differences for L* values in all treatments between the storage days of B10 ($p < 0.05$), and it showed a decrease in the trend as the storage days increased (Figure 1). The lightness (L*) ranged from 0 to 100, while lower values indicate darker colour (Ding & Yap, 2014). However, there was no significant difference ($p > 0.05$) in L* values between coated and uncoated samples on the final day of storage. On the other hand, a* value (redness-greenness) and b* value (yellowness-blueness) showed significant differences ($p < 0.05$) between coatings and storage days. The a* value increased (to redness) as the storage days increased because the ripening process took place. Spirulina coating had the least changes of a* value from the initial to the final storage days, followed by chitosan and control, which proved that spirulina could slow down the ripening process compared to chitosan and control. Suhaimi et al. (2021) agreed that starfruit coated with pectin (PE), maltodextrin (M), and 100 ppm sodium chloride (SC) mixtures had maintained the greenness (positive a*) value ($p < 0.05$) compared to uncoated starfruit. On the other hand, the b* value increased towards intense-yellowish colour, but spirulina had the least difference in b* value from day 0 to day 14, which is 6.36, 18.3, and 9.85 for spirulina, chitosan, and control, respectively (Zaki et al., 2012) supported that slowly increased in the b* values were obtained by the carambola coated with chitosan: stearin compared to uncoated carambola.

For the hue value, chitosan coating had the least colour changes ($p < 0.05$), followed by spirulina and control with the value of 9.04, 9.43, and 30.82°, respectively (Figure 2). The results proved that samples with coatings could minimise colour changes compared with control samples throughout storage days. Colour changes in fruits could be due to dehydration and moisture loss caused by the nonenzymatic browning, caramelisation, and denaturation of proteins in the fruit (Assis & Britto, 2014). A similar result was obtained from the previous study, where samples coated with pectin, maltodextrin, and 100, 200, and 300 ppm of sodium chloride recorded higher hue values compared to control samples due to the coating properties that were able to

reduce ripening process of fruits (Suhaimi et al., 2021). In line with the physical appearance result, there was a significant difference ($p < 0.05$) in the browning index between coated samples and the control. On day 14, spirulina and chitosan coating obtained a low browning index of 52.08 and 98.53, respectively, compared to the control, 107.20. There was also a significant difference ($p < 0.05$) between the browning index and storage days (Figure 3). In the study of strawberries coated with *Aloe*

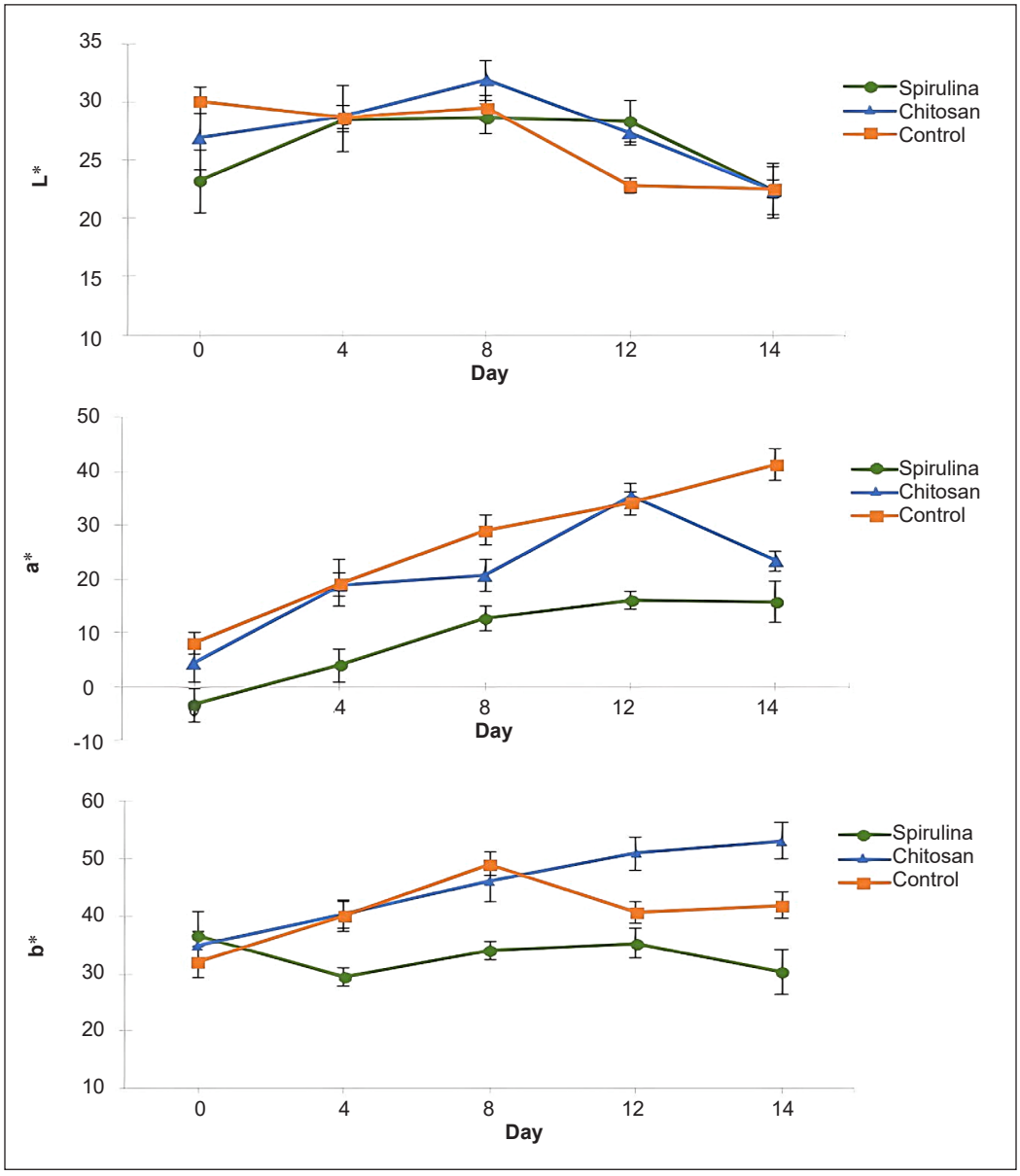


Figure 1. L* a* b* results for B10 coated with 0.66% spirulina and 0.3% chitosan at days 0, 4, 8, 12, and 14 of the storage days

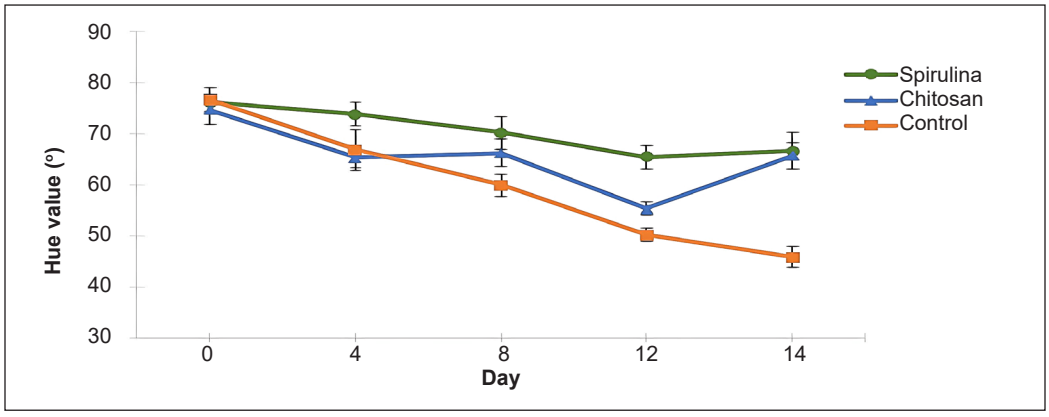


Figure 2. Hue values for B10 coated with 0.66% spirulina and 0.3% chitosan at days 0, 4, 8, 12, and 14 of the storage days

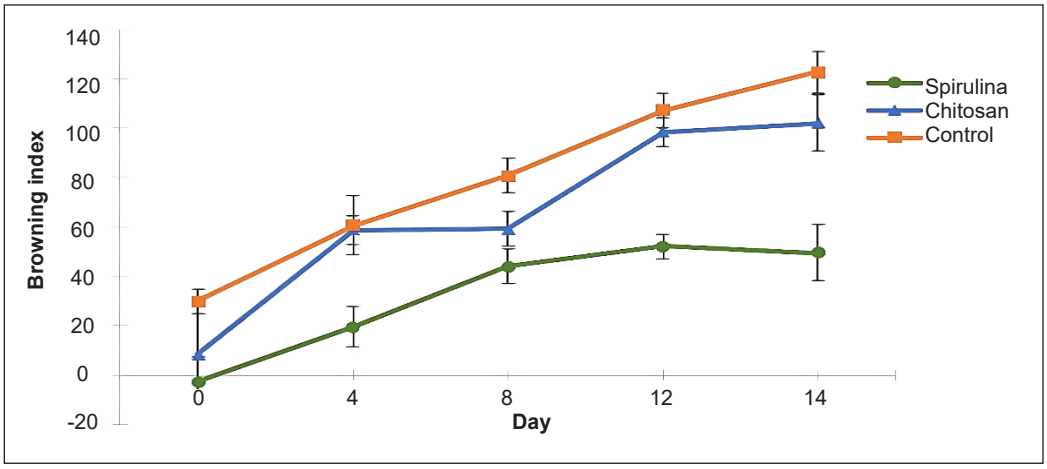


Figure 3. Browning index for B10 coated with 0.66% spirulina and 0.3% chitosan at days 0, 4, 8, 12, and 14 of the storage days

vera, uncoated ripe strawberries tend to become darker, less vivid, and browning compared to coated strawberries (Nasrin et al., 2017). It shows that coated fruits can delay oxidative browning, thus improving the quality.

Fresh Weight Loss

The percentage of fresh weight loss during storage is $p < 0.05$, while there was no significant difference in fresh weight loss of starfruit between different coatings (Figure 4). The mean weight loss showed an increase corresponding with storage days. It showed that spirulina coating had lower water vapour permeability properties than chitosan, resulting in reduced transpiration and weight loss (Hassan et al., 2018). It was agreed by Rastegar and Atrash (2021) that samples coated with spirulina with sodium alginate had resulted in low water loss compared to the uncoated sample of

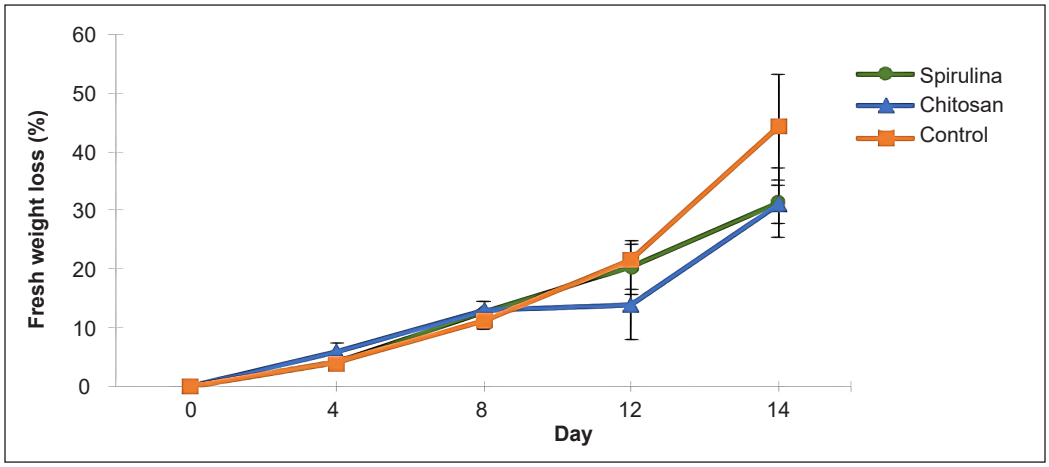


Figure 4. Fresh weight loss (%) for B10 coated with 0.66% spirulina and 0.3% chitosan at days 0, 4, 8, 12, and 14 of the storage days

mango fruit in cold storage. Cardoso et al. (2017) also supported that bell pepper incorporated with spirulina had lower mass loss throughout 14 days of storage. The study of carambola from Karamsad, a village of Anand District, Gujarat, India, proved that carambola coated with 0.3% chitosan had the least weight loss ($p < 0.05$) compared to gum arabica and alginate coatings due to its strong barrier properties in minimising the moisture loss (Gol et al., 2015).

Regression Analysis

The a* and b* were regressions of coefficients, while R^2 was the coefficient of determination obtained from linear regression analysis (Table 3). The range of R^2 values in all samples was from 0.16 to 0.99. Browning index and weight loss analysis obtained significant results ($p < 0.05$) for the determination coefficient at all coatings with $R^2 > 80\%$. It showed that the samples had a good correlation between models and experimental data. Thus, it could access the predictive power of the sampling trend when storage is extended (Ozili, 2022). Besides, low R^2 values such as L* for chitosan coating and b* for control samples indicate a poor relationship between the dependent variable (L* or b*) and the independent variable (storage days).

Furthermore, R^2 values for quality tests of a* and hue angle on chitosan coating were the lowest compared to spirulina and control samples. It might be due to the internal reaction, such as initiating the metabolic reaction in the chitosan-coated sample, which led to drastic changes in the colour throughout storage corresponding to the data obtained in the colour analysis. Rahman et al. (2013) reported in the previous study that papaya stored at 30°C encountered an initiation of anaerobic respiration rate, which caused a sudden increase in the respiratory quotients that were an indication of fermentation threshold, which affected the low R^2 value.

Table 3
The regression coefficient for analysis of L, a*, b*, hue angle, browning index, and weight loss for B10 coated with 0.66% spirulina and 0.3% chitosan*

Quality analysis	Coating	Regression coefficients		R^2
		a*	b*	
L*	Spirulina	24.878	0.386	0.68
	Chitosan	29.333	-0.241	0.16
	Control	31.143	-0.579	0.78
a*	Spirulina	-1.861	1.431	0.94*
	Chitosan	8.299	1.616	0.69
	Control	9.284	2.247	0.99*
b*	Spirulina	32.028	0.154	0.87
	Chitosan	35.112	1.315	0.99*
	Control	36.197	0.602	0.32
Hue angle	Spirulina	76.349	-0.775	0.95*
	Chitosan	72.022	-0.861	0.51
	Control	76.400	-2.175	0.99*
Browning index	Spirulina	2.716	3.921	0.91*
	Chitosan	17.034	6.368	0.92*
	Control	31.413	6.426	0.99*
Weight loss	Spirulina	-2.606	2.144	0.94*
	Chitosan	-1.371	1.864	0.83*
	Control	-5.114	2.804	0.82*

Note. The regression analysis was significant ($p < 0.05$), which indicates that the changes in predicted values were correlated with the changes in the experimental variable

CONCLUSION

By observing the samples' physical appearance, spirulina coating maintained the greenish colour, while chitosan-coated and control samples had turned yellowish on day 8. Despite this, spirulina and control started to decay on day 12, which showed that spirulina could reduce ripening but had a poor mechanical barrier compared to chitosan. Immense browning appeared on the control sample, while an early sign of browning had only shown on spirulina and chitosan coatings at day 12 with the value of 52.08, 98.53, and 107.20 BI, respectively, with symptoms of decay and necrotic lesion. The carambola's lightness (L*) decreased while a* and b* increased in values throughout storage. In addition, coated samples resulted in a lower intensity of hue angle, which were 9.04 and 9.43° for chitosan and spirulina coatings, respectively, compared to control with the value of 30.82°. Samples coated with spirulina showed the least change at a*, b*, and hue angle analysis compared to chitosan and control samples. Gradual weight loss

($p < 0.05$) was determined for all samples throughout storage, but the uncoated sample exhibited the highest weight loss on the final storage day compared to the coated samples. These proved that the coated samples could minimise physical and colour change, including weight loss relative to the storage days ($p < 0.05$). Browning and fresh weight loss showed better fitness with the independent variable (storage days) with the significant value ($p < 0.05$), $R^2 > 80\%$ in the regression analysis. Hence, these results would have the potential for commercialisation and exports between short-distance regions due to edible coatings' performance in maintaining quality and prolonging the shelf-life of the fruits at 27°C. Further studies on the storage of carambola under low temperatures would be further conducted to improve the export quality of carambola between distant freight.

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Phytase Enzyme Improves Growth Performance and Body Chemical Composition of Sangkuriang Catfish (*Clarias gariepinus* var. Sangkuriang) Juvenile

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ABSTRACT

Due to their nutritional value, alternative vegetable materials such as soybean are needed to promote sustainable aquaculture development. However, phytate in soybean could interfere with the protein digestion of the fish body. This study examines the effectiveness of phytate enzymes in enhancing protein digestibility, growth performance, mineral digestion, and body chemical composition of Sangkuriang catfish (*Clarias gariepinus* var. Sangkuriang) juveniles. The study was completely randomized design with 4 treatments and 3 repetitions. One hundred thirteen Sangkuriang catfish juveniles (7.65 ± 0.14 g) were used for each repetition. The fish were fed with an experimental diet supplemented with various doses of phytase enzyme: 0 (A), 500 (B), 1,000 (C), and 1,500 (D) FTU/kg of feed. The mineral digestibility, protein digestibility, protein efficiency ratio (PER), feed conversion ratio (FCR), the efficiency of feed utilization (EFU), apparent digestibility coefficients (ADCp), relative growth rate (RGR), and survival rate (SR) were observed. Data were analyzed using analysis of variance followed by Duncan's multiple range test. The results showed that adding 1,000 FTU/kg of phytase enzyme (C) exhibited the highest PER, FCR, EFU, ADCp, and RGR

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value of Sangkuriang catfish juveniles compared to other treatments. All treatment groups (B-C) had the same SR value (100%) and had the highest crude protein and ash content compared to the A group. All group treatments also exhibited a higher phosphorus, calcium, potassium, magnesium, iron, zinc, manganese, copper, and cobalt. Therefore, phytase enzyme supplementation could improve protein digestibility, mineral digestibility, growth performance, and body chemical composition of Sangkuriang catfish juveniles.

Keywords: Mineral, phytase enzyme, phytic acid, protein, Sangkuriang catfist

INTRODUCTION

The Sangkuriang catfish (*Clarias gariepinus* var. Sangkuriang) is a popular and frequently consumed freshwater fish in Indonesia. The Ministry of Maritime Affairs and Fisheries Indonesia noted that catfish production in Indonesia reached 1.06 million tonnes in 2021. The catfish production increased by 2.95% compared to the previous year, which amounted to 1.03 million tonnes (Widi, 2022). This fish is commonly consumed due to its relative ease of preparation, affordability, and high protein content. In addition, this fish is also cultivated due to its relatively rapid growth rate and increased customer demand, which drives catfish farmers to engage in intensive cultivation (Rachmawati et al., 2022). Artificial feed is fish culture feed made from various materials that have good nutritional content according to the needs of fish and is manufactured with careful consideration for the nature and size of the fish. Artificial feed has a significant role in determining the success of intensive Sangkuriang catfish cultivation. Fish meal represents up to 50% of the animal protein in artificial feed, making it the most expensive protein element (Tacon & Metian, 2008). Protein, essential amino acids, vital fatty acids, cholesterol, vitamins, and mineral attractants are abundant in fish feed, making it the primary dietary source (National Research Council [NRC], 2011). With the development of fish farming activities, the demand for fish feed increases, resulting in a restricted supply of fish feed and an increase in the rate of unsustainable overfishing in natural environments (Tacon & Metian, 2008).

Alternative vegetable materials are needed to promote the development of environmentally friendly and sustainable aquaculture (Shapawi et al., 2013). Soy flour is one of the vegetable ingredients used as a source of protein due to its availability, relative affordability, and balanced profile of key amino acids (Akiyama & Dominy, 1989; Tacon & Metian, 2008). However, the fish feed cannot contain soy flour due to anti-nutritional factors (ANF) in phytic acid. Phytic acid can generate phytate-protein and phytate-mineral-protein complexes, reducing protein digestion and utilization by inhibiting protein digestion (Cao et al., 2007). The phytate protein complex cannot be digested by proteolytic enzymes (Ravindran et al., 1995), and phytic acid inhibits the activity of certain proteolytic enzymes, including pepsin, amyloperoxidase, and amylase (Cao et al.,

2007). Additionally, phytic acid chelates minerals such as calcium, magnesium, zinc, iron, and copper to create insoluble complexes, limiting the absorption and bioavailability of macro and micro elements required by fish (Papatryphon et al., 1999).

Applying phytase enzyme in feed can hydrolyze phytate into penta-, tetra-, tri-, di-, and monophosphate myoinositol and neutralize the detrimental effect of phytic acid on protein and other nutrients in feed. Furthermore, the presence of phytase enzymes in feed can enhance the bioavailability of phosphorus and nitrogen bound to phytate, hence limiting the release of phosphorus (P) and nitrogen (N) into the aquatic environment (Cao et al., 2007). Phytase enzyme enrichment in feed has boosted growth, feed conversion ratio, and protein and mineral digestibility in some fish, such as *Pangasius pangasius* (Debnath et al., 2005), *Ephinephelus fuscoguttatus* (Shapawi et al., 2013), *Marsupenaeus japonicas* (Bulbul et al., 2015), *Psetta maxima* (von Danwitz et al., 2016), *Cirrhinus mrigala* (Hussain et al., 2020), *Sparus aurata* (Salem et al., 2022), *Oreochromis niloticus* (Shahzad et al., 2022), *Cyprinus carpio* (Shahzad et al., 2021), *Carassius auratus* (Nie at al., 2017), and *Labeo rohita* (Hussain et al., 2017).

There is little information regarding the phytase enzyme enrichment in Sangkuriang catfish juvenile feed; therefore, further research is required. This study aimed to examine the effectiveness of phytate enzymes in enhancing protein digestibility, growth performance, mineral digestion, and body chemical composition of Sangkuriang catfish (*Clarias gariepinus* var. Sangkuriang) juveniles.

MATERIALS AND METHODS

Animal Preparation and Research Design

This study was conducted in Balai Budidaya Ikan Air Tawar (BBIAT), Muntilan, Jawa Tengah, Indonesia. The current research used a completely randomized design (CRD), 4 treatments of phytase supplementation at different doses, and each treatment was repeated three times. The stocking density of fish was 113 fish/research container fiber for each repetition. The fish used was Sangkuriang catfish (*Clarias gariepinus* var. Sangkuriang) juveniles procured from BBIAT, weighing an average of 7.65 ± 0.14 g/head and rearing for 56 days. Juvenile Sangkuriang catfish were selected before a treatment based on their uniform size and weight, absence of deformity, active swimming, and good health (Rachmawati et al., 2017). During 1 week of acclimatization, fish were fed with commercial fish feed that did not contain phytase enzyme. Fish were not fed the day before receiving treatment to remove residual food from initial feedings.

Experimental Feed Preparation

The experimental fish feed was artificial pellets which contained 32% protein, isoenergy (301 kcal) (Rachmawati et al., 2022), 1% chromium (III) oxide (Cr_2O_3) as a protein digestibility indicator (NRC, 2011), phytase enzymes at different doses: 0 (A), 500 (B), 1,000 (C), and 1,500 (D) FTU/kg feed (Table 1). The phytase

enzyme (Nathupos®E 10,000 G, BASF SE, Germany) was made of light brown granules.

Feed preparation begins with weighing the raw materials according to the feed formulation, mixing the feed ingredients from tiny to large quantities, using a mixer machine, and adding fish oil (Ultra Omega 3-D™, NOW Foods, USA), corn oil (Mazola®, Saudi Arabia), and water to taste. Furthermore, the dough was placed within the extruder floating pellet molding machine. After stamping, the feed was air-dried at room temperature (around 26°C), then packaged and stored in airtight plastic.

Table 1

Feed formulation (1,000 g)

Material (g)	Treatment			
	A	B	C	D
Fish flour	345.00	345.00	345.00	345.00
Soybean	240.00	240.00	240.00	240.00
Corn flour	150.00	150.00	150.00	150.00
Bran	145.00	144.95	144.90	144.85
Tapioca flour	80.00	80.00	80.00	80.00
Fish oil	10.00	10.00	10.00	10.00
Corn oil	10.00	10.00	10.00	10.00
Mineral and vitamin mix[1]	10.00	10.00	10.00	10.00
Phytase enzyme (FTU)	0	0.05	0.1	0.15
Chromium (III) oxide (Cr_2O_3)	10.00	10.00	10.00	10.00
Total	1,000	1,000	1,000	1,000
	Proximate analysis results			
Protein (%)*	30.21	31.19	32.20	30.20
Fat (%)*	7.87	8.40	8.99	8.73
Nitrogen Free Extract/NFE (%)*	35.22	35.36	34.8	32.73
Energy (kcal)[2]	301.34	301.82	301.19	301.98
Ratio of energy/protein (E/P) (cal/g)[3]	9.98	9.97	9.97	9.90

Note.

[1]Mineral and vitamin mix/kg: Sodium (Na) 117 mg, selenium (Se) 150 mg, vitamin B1 52 mg, magnesium (Mg) 1,900 mg, vitamin B2 97 mg, vitamin B6 46 mg, potassium (K) 150 mg, calcium (Ca) 219 mg, copper (Cu) 9 mg, iron (Fe) 90 mg, vitamin C (coated) 68,800 mg activity, zinc (Zn) 90 mg, iodine (KI) 1.8 mg, cobalt (Co) 450 mg, vitamin B12 60 mg, vitamin A 36.000 I.U., vitamin D3 9.000 I.U., manganese (Mn) 105 mg, panthothenic acid 93 mg, inositol 225 mg, biotin 450 mg, vitamin E 187 mg, vitamin K3 19 mg, niacin 130 mg, and folic acid 10 mg

[2]Determined based on Digestible Energy (NRC, 2011), for 1 g of protein equal to 3.5 kcal/g, 1 g of fat equal to 1 kcal/g, and 1 g of carbohydrate equal to 2.5 kcal/g

[3]Based on NRC (2011), the E/P value for the optimum growth of fish ranges from 8–12 kcal/g

*Proximate analysis results from Laboratorium Ilmu Makanan Ternak, Fakultas Peternakan dan Pertanian, Universitas Diponegoro in the year 2022

Container Preparation

The study used a container fiber tub of 1.5 m x 1.5 m x 1 m with up to 12 units supplied with a recirculation system to maintain water quality within the optimal range. The maintenance media contained mountain water deposited in a reservoir. The study was initiated by placing 113 test fish with a known average initial weight into each of the 113 research containers. Determining fish density refers to Rachmawati et al. (2022), in which 50 fish were stocked per square meter. The feed was administered three times daily, at 06.00 a.m., 12.00 p.m., and 6.00 p.m., according to the *at-satiation* method. During the study, the weight gain of the test fish was monitored weekly using digital scales. Two hours after feeding, excrement, and feed residues were sucked up using a siphon to keep them clean and suitable for fish life.

Proximate Analysis

Based on Jayant et al. (2018), proximate analysis was conducted to assess the test feed and fish carcasses. A semi-automatic Kjeltec™ 2300 Analyzer Unit (FOSS Analytical, Denmark) was used to determine protein content. The fat content was evaluated using an ether extraction method based on the Soxhlet technique using Soxtec™ 2043 Fat Extraction System (FOSS Analytical, Denmark). Test feed samples and fish were burned in a furnace at 550°C for 24 hr to assess their ash content.

Protein Digestibility Analysis

According to Pérez-Jiménez et al. (2009), protein digestibility is determined using the indirect approach by adding 1% Cr_2O_3 to indicate protein digestibility to the test feed. During the 56-day study, fish feces were collected every morning, afternoon, and evening after the fish were fed. Feces are collected using a short plastic tube whose end is connected to a wooden stick to make it easier to maneuver while collecting feces, and the collected feces are then placed in a bucket. Feces were filtered using a plankton cloth net, and the filtrate is placed in small plastic bottles and refrigerated at 4°C. The feces were dried in a gravity oven (Fisherbrand™, Fisher Scientific, Belgium) at 6°C for 24 hr before analysis. The protein and Cr_2O_3 content in the feces were then measured using atomic absorption spectrophotometry (AAS) with a wavelength of 350 nm.

Mineral Content Analysis. At the end of the test, the mineral content of the fish was determined by randomly selecting twelve fish from each treatment (1 fish per replication). After drying each fish sample in an electric oven at 70–80°C until it attained a constant weight, approximately 2 g of each sample was weighed. Afterward, the sample was dissolved with strong nitric acid. The estimation of fish body minerals was performed with an atomic absorption spectrophotometer following the Association of Official Analytical Chemists (AOAC) (2019) guidelines.

Observed Parameters. The observed parameters consist of protein efficiency ratio (PER), feed conversion ratio (FCR), the efficiency of feed utilization (EFU), apparent digestibility coefficients (ADCp) (Pérez-Jiménez et al., 2009), relative growth rate (RGR), and survival rate (SR) (NRC, 2011); where each parameter was calculated using the following formulas:

$$ADCp\ (\%) = 100 - \left\{\frac{100 \times Cr_2O_3\ in\ the\ feed}{\%\ Cr_2O_3\ in\ the\ feces} \times \frac{\%\ protein\ in\ the\ feces}{\%\ protein\ in\ the\ feed}\right\} \quad (1)$$

$$EFU\ (\%) = \frac{Final\ weight - Initial\ weight}{Weight\ of\ diet\ consumed} \times 100 \quad (2)$$

$$RGR\ (\%) = 100 \times \frac{(Final\ weight - Initial\ weight)}{(Times\ of\ experiment \times Initial\ weight)} \quad (3)$$

$$FCR = \frac{Feed\ intake\ (g)}{Body\ weight\ gain\ (g)} \quad (4)$$

$$PER = 100 \times \frac{(Final\ weight - Initial\ weight)}{The\ amount\ of\ diet\ consumed \times Protein\ content\ of\ diet} \quad (5)$$

$$SR\ (\%) = 100 \times \left(\frac{Final\ count}{Initial\ count}\right) \quad (6)$$

Statistical Analysis. Data were analyzed using analysis of variance (ANOVA) using SPSS ver. 19.0 (USA). If the ANOVA results had a significant effect (p<0.05) or had a highly significant effect (p<0.01), Duncan's multiple range test was conducted to determine the difference in the mean values between treatments (Steel et al., 1997).

RESULTS

The addition of phytase enzyme to the feed significantly (p<0.05) increased PER, FCR, EFU, ADCp, and RGR, but not (p>0.05) in SR parameters (Table 2). The PER, FCR, EFU, ADCp, and RGR values of juvenile Sangkuriang catfish supplemented with phytase enzyme were greater than in the absence of supplementation. Based on the highest PER, FCR, EFU, and RGR values, adding 1,000 FTU/kg of feed phytase enzyme was the optimal dose for juvenile Sangkuriang catfish.

Table 3 shows the influence of phytase enzyme supplementation on the chemical composition of juvenile Sangkuriang catfish. Sangkuriang catfish juveniles fed with phytase enzyme had the highest crude protein content and ash content compared to those fed without phytase enzyme. There was no significant (p>0.05) difference between treatments regarding lipid and dry matter.

Table 2

Data of protein efficiency ratio (PER), feed conversion ratio (FCR), the efficiency of feed utilization (EFU), apparent digestibility coefficients (ADCp), relative growth rate (RGR), and survival rate (SR) of juvenile's Sangkuriang catfish

Parameter	Treatment			
	A	B	C	D
PER	1.63 ± 0.12^{d}	2.84 ± 0.18^{b}	3.49 ± 0.10^{a}	2.42 ± 0.13^{c}
FCR	1.82 ± 0.10^{d}	1.43 ± 0.14^{b}	1.02 ± 0.11^{a}	1.65 ± 0.17^{c}
EFU (%)	57.45 ± 0.22^{d}	69.28 ± 0.15^{b}	78.30 ± 0.18^{a}	65.29 ± 0.16^{c}
ADCp (%)	68.32 ± 0.25^{d}	75.46 ± 0.27^{b}	80.15 ± 0.23^{a}	72.52 ± 0.29^{c}
RGR (%/day)	2.10 ± 0.18^{d}	2.98 ± 0.12^{b}	3.27 ± 0.17^{a}	2.62 ± 0.19^{c}
SR (%)	100 ± 0.0^{a}	100 ± 0.0^{a}	100 ± 0.0^{a}	100 ± 0.0^{a}

Note. The alphabetical superscript indicates significant differences between treatments. The juvenile Sangkuriang catfish were fed with an experimental diet supplemented with various doses of phytase enzyme: (A) 0, (B) 500, (C) 1,000, and (D) 1,500 FTU/kg of feed

Table 3

The body chemical composition (%) of juvenile Sangkuriang catfish

Treatment	Dry matter	Protein	Lipid	Ash
A	24.38 ± 0.57^{a}	53.42 ± 0.13^{b}	24.60 ± 0.52^{a}	17.39 ± 0.25^{b}
B	23.67 ± 0.48^{a}	55.26 ± 0.54^{a}	23.65 ± 0.49^{a}	19.83 ± 0.62^{a}
C	23.54 ± 0.76^{a}	55.48 ± 0.29^{a}	23.57 ± 0.62^{a}	19.24 ± 0.28^{a}
D	24.73 ± 0.35^{a}	56.19 ± 0.32^{a}	24.22 ± 0.70^{a}	18.57 ± 0.59^{a}

Note. The alphabetical superscript indicates a significant difference between treatments. The juvenile Sangkuriang catfish were fed with an experimental diet supplemented with various doses of phytase enzyme: (A) 0, (B) 500, (C) 1,000, and (D) 1,500 FTU/kg of feed

The influence of feeding with phytase enzyme supplementation on the macro and micro mineral composition of juvenile Sangkuriang catfish is presented in Figures 1 (a–d) and 2 (a–e). The highest phosphorus value was obtained in juvenile Sangkuriang catfish, which was given treatment C (phytase enzyme 1,000 FTU/kg feed), followed by treatment D (phytase enzyme 1,500 FTU/kg feed), treatment B (phytase enzyme 500 FTU/kg feed), and the lowest phosphorus value was found in treatment A (phytase enzyme 0/kg feed) (Figure 1a). Juvenile Sangkuriang catfish fed with phytase enzyme supplementation had higher calcium and magnesium values than those without supplementation (Figures 1b and 1d). The potassium value showed no significant difference between juvenile Sangkuriang catfish fed with phytase enzyme supplementation and without supplementation (Figure 1c).

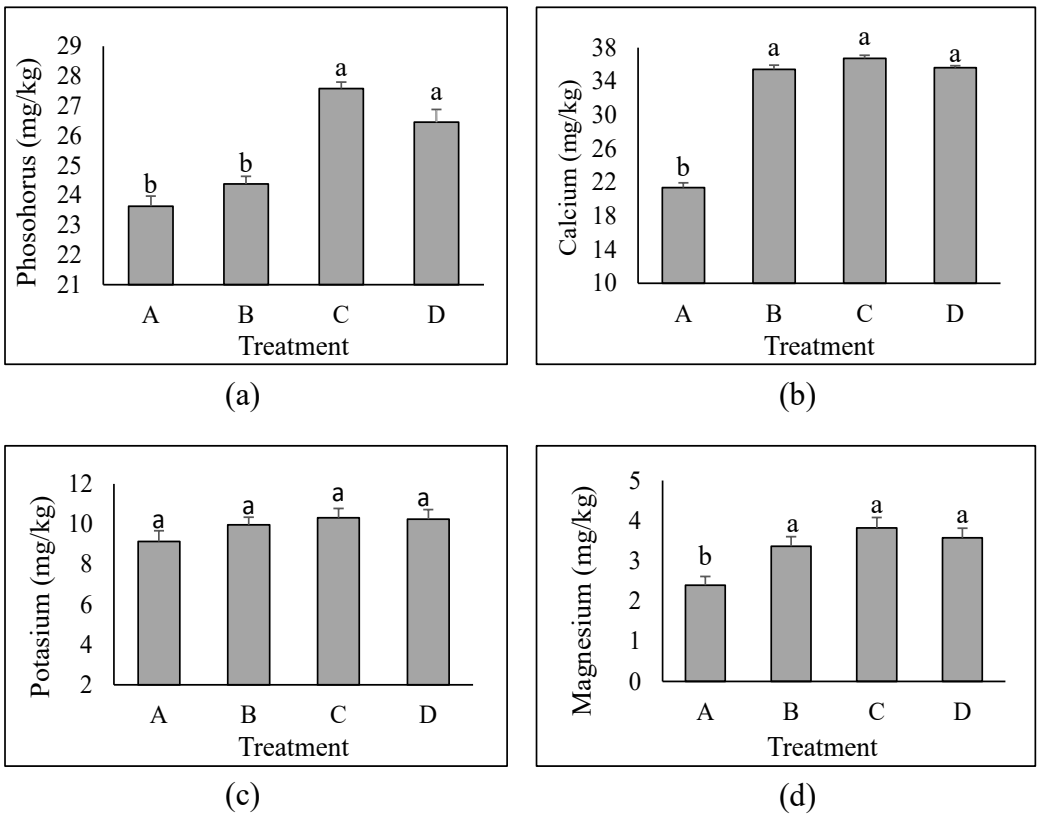

Figure 1. The macro-mineral composition including (a) phosphorus, (b) calcium, (c) potassium, and (d) magnesium of juvenile Sangkuriang catfish after fed with an experimental diet supplemented with various doses of phytase enzyme: (A) 0, (B) 500, (C) 1,000, and (D) 1,500 FTU/kg of feed

Note. The alphabetical superscript indicates a significant difference between treatments

The iron value of juvenile Sangkuriang catfish fed with phytase enzyme supplementation was higher than without phytase enzyme (Figure 2a). Juvenile Sangkuriang catfish fed with phytase enzyme supplementation also had higher zinc, manganese, and copper values than those without supplementation (Figures 2b, c, and d). The highest cobalt value gave the highest yield in juvenile Sangkuriang catfish that was given treatment C (phytase enzyme 1,000 FTU/kg feed), followed by treatment D (phytase enzyme 1,500 FTU/kg feed), treatment B (phytase enzyme 500 FTU/kg feed), and the lowest in treatment A (phytase enzyme 0 FTU/kg feed) (Figure 2e).

DISCUSSION

The phytase enzyme supplementation in the feed was substantially different (p<0.05) in the PER, FCR, EFU, ADCp, and RGR parameters but not in the SR parameter (p>0.05) (Table 2). It demonstrates that adding phytase enzyme to fish feed can hydrolyze protein phytate complex

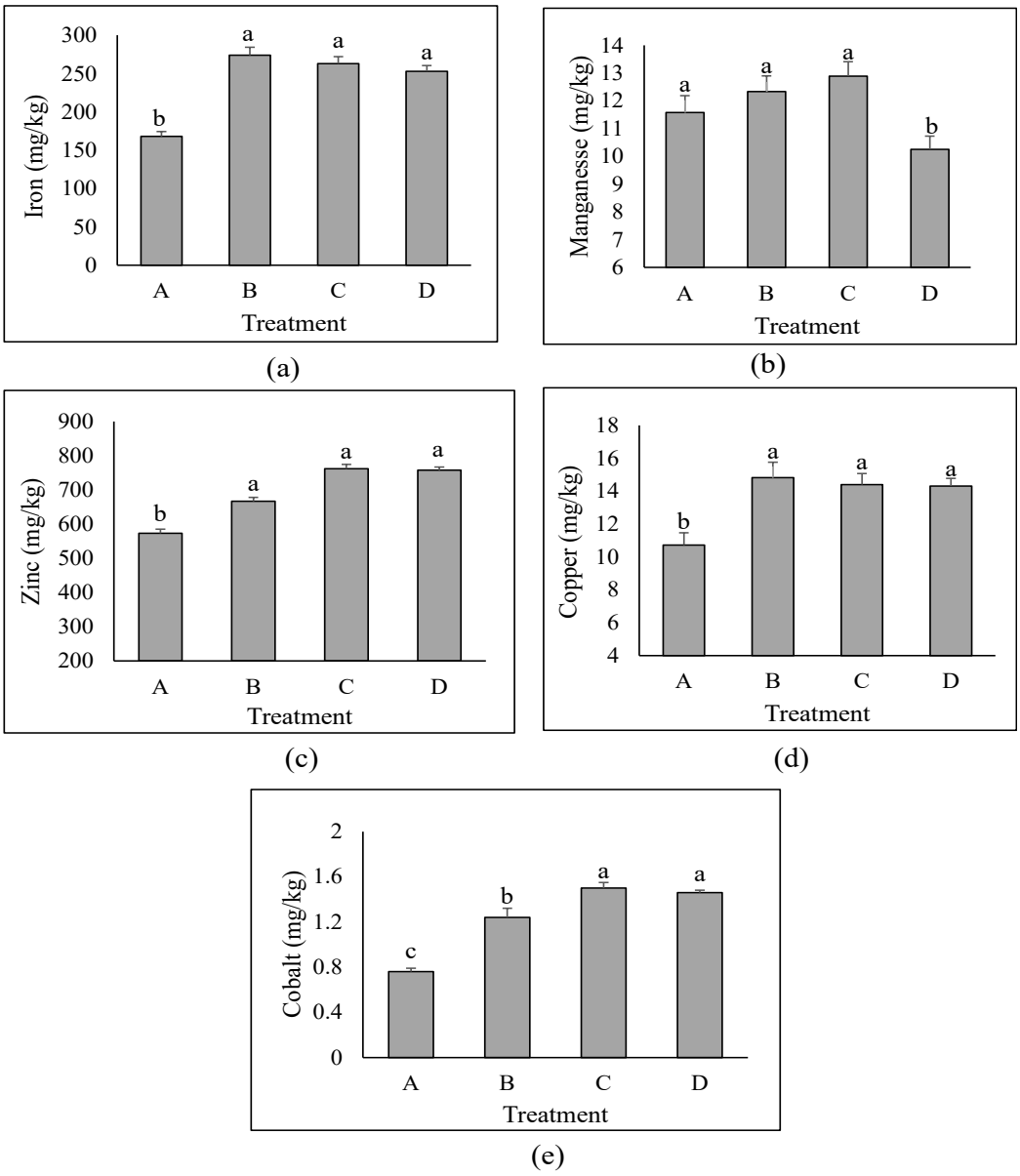

Figure 2. The micro-mineral composition including (a) iron, (b) manganese, (c) zinc, (d) copper, and (e) cobalt of juvenile Sangkuriang catfish after fed with an experimental diet supplemented with various doses of phytase enzyme: (A) 0, (B) 500, (C) 1,000, and (D) 1,500 FTU/kg of feed

Note. The alphabetical superscript indicates a significant difference between treatments

compounds into easily digestible amino acids for fish growth (Haghbayan & Mehrgan, 2015).

Spinelli et al. (1983) found that feed containing 0.5 % phytic acid had a negative effect on rainbow trout growth due to a decrease in protein availability in the feed. Phytic acid functions as a chelator to produce protein phytate complex molecules, influencing proteins and minerals'

bioavailability (NRC, 2011). The results of a similar study were reported by Debnath et al. (2005), Hussain et al. (2017), Salem et al. (2022), Shapawi et al. (2013), and von Danwitz et al. (2016), who reported that the addition of phytase enzymes to feed had a significant effect in the PER, FCR, EFU, ADCp, and RGR parameters but had no significant effect on SR.

The PER, FCR, EFU, ADCp, and RGR parameters of juvenile Sangkuriang catfish fed with phytase enzyme supplementation at 1,000 FTU/kg of feed were the highest compared to other treated feeds. The 1,000 FTU/kg feed of phytase enzyme is believed to be effective at hydrolyzing antinutrient factors (phytic acid) in feed (Cao et al., 2007). The highest PER, FCR, EFU, ADCp, and RGR values after 1,000 FTU/kg feed of phytase enzyme supplementation were also reported in striped bass (Papatryphon et al., 1999), rainbow trout (Vielma et al., 2000), Atlantic salmon (Sajjadi & Carter, 2004), *Sparus aurata* (Salem et al., 2022), and *Cirrhinus mrigala* (Hussain et al., 2017).

The results showed that the supplementation of phytase enzyme in the feed caused the FCR value to decrease. Phytase enzymes in the feed are believed to hydrolyze phytate, thereby breaking the complex bond between phytate and protein and minerals. It has a beneficial effect on converting trypsinogen to trypsin enzymes, which can break down protein into its constituent amino acids (Hussain et al., 2017). Thus, feed consumption efficiency is maximized, lowering the feed conversion ratio. Administration of phytase enzymes in feed can improve FCR, as it was reported in rainbow trout (Wang et al., 2009), *Labeo rohita* (Baruah et al., 2007), *Marsupenaeus japonicas* (Bulbul et al., 2015), *Cirrhinus mrigala* (Hussain et al., 2017), and *Oreochromis niloticus* (Shahzad et al., 2022).

All aquatic organisms require minerals for vital physiological and biochemical activities, as well as for the maintenance of normal life processes. Fish need both macro-minerals (phosphorus, calcium, magnesium, and potassium) and micro-minerals (cobalt, copper, iron, manganese, and zinc) (NRC, 2011). The results (Figures 1 and 2) demonstrated that supplementing Sangkuriang catfish juvenile feeding with phytase enzyme improved the macro- and micro-mineral content of the fish body. Adding phytase enzymes to the diet enhances the availability of phosphorus in freshwater fish, as was reported in *Cirrhinus mrigala* (Hussain et al., 2017), *Sparus aurata* (Salem et al., 2022), *Oreochromis niloticus* (Shahzad et al., 2022), *Carassius auratus* (Nie et al., 2017), and *Cyprinus carpio* (Shahzad et al., 2021).

In addition, Cian et al. (2019) reported that feeding Pacu fish (*Piaractus mesopotamicus*) with phytase enzyme supplementation increased the availability of minerals in the fish body (phosphorus [P], zinc [Zn], and iron [Fe]) due to a decrease in gastrointestinal phytic acid and an increase in mineral bioavailability compared to fish fed without supplementation. Supplementation of the phytase enzyme considerably enhanced the content of

minerals (phosphorus [P], calcium [Ca], magnesium [Mg], and zinc [Zn]) in the spine of juvenile red sea bream (*Pagrus major*) (Laining et al., 2012). Different amounts of phytase enzyme supplementation affected the consumption of phosphorus, minerals, and protein in rainbow trout (*Oncorhynchus mykiss*) (Sugiura et al., 2001). In addition, adding phytase enzymes to the feed can improve the fish's ability to digest minerals. Cheng and Hardy (2003) showed that adding phytase enzyme to the diet of rainbow trout increased the digestibility of Mg, Mn, Zn, and P, with P being more digestible than the other minerals.

As indicated in Table 3, adding phytase enzyme to the feed affects the protein content of juvenile Sangkuriang catfish. It may be noticed that the feed supplemented with phytase enzyme has a larger protein content than feed without the addition. It may be owing to the phytase enzyme's ability to enhance protein availability by completely hydrolyzing the phytate-protein complex in the fish intestine and negating the negative effect of phytate on protein (Liebert & Portz, 2005). This study's rise in PER supported the increase in juvenile Sangkuriang catfish protein content. According to Tables 2 and 3, adding phytase enzyme to the feed of juvenile Sangkuriang catfish enhanced the protein content of the fish body and the PER. In addition, the results revealed that juvenile Sangkuriang catfish fed with phytase enzyme supplementation at a dose of 1,000 FTU/kg feed (treatment C) had the highest increase in protein digestibility, which would result in the highest increase in feed utilization efficiency.

The addition of phytase enzymes in feed could increase protein digestibility and the efficiency of feed utilization in several fish (Biswas et al., 2019), including *Ephinephelus fuscoguttatus* (Shapawi et al., 2013), *Marsupenaeus japonicas* (Bulbul et al., 2015), *Sparus aurata* (Salem et al., 2022), *Carassius auratus* (Nie et al., 2017), and *Labeo rohita* (Hussain et al., 2017). The body ash content of juvenile Sangkuriang catfish that was given phytase enzyme supplementation was higher than that without supplementation. Bulbul et al. (2015) stated that adding phytase enzymes in feed can increase the concentration of bone ash and phosphorus in the body of *Marsupenaeus japonicas*. Liebert and Portz (2005) also reported that the ash content on the scales and spine of *Oreochromis niloticus* increased significantly after being fed a diet containing the phytase enzyme.

CONCLUSION

Adding 1,000 FTU/kg of phytase enzyme (C) exhibited the highest PER, FCR, EFU, ADCp, and RGR value of Sangkuriang catfish juvenile compared to other treatments. All treatment groups (B-C) had the same SR value (100%) and had the highest crude protein and ash content compared to the A group. All group treatments also exhibited a higher phosphorus, calcium, potassium, magnesium, iron, zinc, manganese, copper, and cobalt. Therefore, phytase enzyme supplementation could improve protein digestibility, mineral digestibility, growth performance, and body chemical composition of Sangkuriang catfish juveniles.

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