



***BOTANICAL ORIGIN, NUTRITIONAL VALUES AND PROBIOTIC  
PROPERTIES OF BEE BREAD OF STINGLESS BEE (*Heterotrigona itama*  
Cockerell)***

**SALMA MALIHAN BINTI MOHAMMAD**

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*Cockerell*)**

By

**SALMA MALIAH BINTI MOHAMMAD**

**Thesis Submitted to the School of Graduate Studies, Universiti Putra  
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Science**

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*Dedication to those who has painted the dark universe with beautiful lights*

*And to those who seeks knowledge in between pages*

*“Handle a book as a bee does a flower, extract its sweetness but do not damage it.”*

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the degree of Master of Science

**BOTANICAL ORIGIN, NUTRITIONAL VALUES AND PROBIOTIC PROPERTIES OF BEE BREAD OF STINGLESS BEE (*Heterotrigona itama* Cockerell)**

By

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**May 2020**

**Chair : Norhasnida Binti Zawawi, PhD**  
**Faculty : Food Science and Technology**

Bee bread is bee-collected pollen, added with honey and bee salivary enzymes. It undergoes lactic acid fermentation by indigenous microbes as it is stored inside the closed pots in the beehive. In comparison to honey, bee bread is still an underutilized bee product with limited nutrient information. This study aims to identify the botanical origin and characterize the nutritional composition of stingless bee (*Heterotrigona itama*) bee bread and also isolate and further characterize the probiotic potential of bacteria from the bee bread. The melissopalynology analysis using a Scanning Electron Microscope (SEM) discovered *Biden pilosa* in bee bread samples from all geographical locations with *Asteraceae* as the predominant family plant. From the proximate analysis, *H. itama* bee bread was found to be high in carbohydrate ( $58.03 \pm 0.75\%$ ), protein ( $22.46 \pm 0.63\%$ ), lipid ( $5.29 \pm 0.53\%$ ) and ash ( $2.56 \pm 0.13\%$ ). The sugar profile analysis using High-Performance Liquid Chromatography- Evaporating Light Scattering Detector (HPLC-ELSD) found glucose as the most abundant followed by fructose. The amino acids were quantified with HPLC-Fluorescence Detector (FLD). Eight essential amino acids were present with phenylalanine as the most abundant with 2.317 g/100 g. The mineral content and heavy metals were analyzed using Inductively Coupled Plasma Mass Spectrometry (ICP-MS). The major mineral element was potassium (average 6705.9 mg/kg). Heavy metals such as lead, arsenic, cadmium and mercury were detected within the safe permitted levels. Ninety-seven (97) bacterial strains were isolated from *H. itama* bee bread. From these, 27 were presumed to be lactic acid bacteria (LAB) and *Bacillus* from Gram-staining and catalase test. The presumptive bacteria were identified using 16s rRNA gene sequencing. These bacteria were *Lactobacillus musae*, *Lb. crustorum*, *Lb. mindensis*, *Leuconostoc mesenteroides*, *Enterococcus faecalis*, *Fructobacillus fructosus*, *Bacillus safensis*, *B. amyloliquefaciens*, *B. megaterium*, *B. cereus* and *B. pumilus*. The bacteria were evaluated for its hemolytic ability on 5% blood sheep agar. All LAB

strains and *B. megaterium* MPS2 did not hemolyzed blood. Antagonistic activities against foodborne pathogens using agar well diffusion showed *L. musae* SGMT17 and *L. crustorum* SGMT22 with the highest inhibition zone, comparable to those of commercial strains *Lb. rhamnosus* GG. The bacteria with antibacterial properties were assessed for their viability in pH 3, 0.3% bile and digestive enzymes. All strains were able to tolerate the simulated conditions except for *F. fructosus* U45 and U47 as they recorded viability below 80% after treatment in pepsin and pancreatin simultaneously. The adhesion properties using autoaggregation and cell surface hydrophobicity (CSH) demonstrated *L. mindensis* SGMT22 with highest autoaggregation ability (41.16%) while *Lc. mesenteroides* U39 showed the highest CSH (80.52%). The antibiotic resistance patterns for the isolates against 11 antibiotics were assessed using the disc diffusion method and interpreted using standard of Clinical and Laboratory Standard Institute (CLSI). All strains were susceptible to chloramphenicol, ampicillin and tetracycline, but varied for eight antibiotics. From the result obtained, *Lb. musae* SGMT17 and *Lb. crustorum* SGMT20 showed the highest antibacterial activity and probiotic properties in the human *in vitro* digestive model. The outcomes of this study contribute towards the knowledge on the nutritional information of *H. itama* bee bread, its potential as commercialize food or food ingredients.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia  
sebagai memenuhi keperluan untuk ijazah Master Sains

## ASAL USUL BOTANI, NILAI NUTRISI DAN CIRI-CIRI PROBIOTIK DALAM ROTI LEBAH KELULUT (*Heterotrigona itama Cockerell*)

Oleh

**SALMA MALIAH BINTI MOHAMMAD**

Mei 2020

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**Fakulti : Sains dan Teknologi Makanan**

Roti lebah adalah debunga yang dikumpul lebah, ditambah dengan madu dan air liur lebah sebelum disimpan di dalam sarang di mana ia menjalani proses penapaian oleh mikrob. Berbanding dengan madu, roti lebah merupakan produk kelulut yang kurang dimanfaatkan berikutan kekurangan maklumat mengenai nutrisinya. Kajian ini bertujuan untuk mengenalpasti asal usul pokok dan komposisi nutrisi roti lebah kelulut *Heterotrigona itama* dan selanjutnya mengisolasi dan mencirikan potensi probiotik bakteria yang diperoleh dari roti lebah. Analisis melissopalynogi menggunakan mikroskop imbasan electron (SEM) menunjukkan terdapat debunga dari pokok *Biden polisa* dalam sampel roti lebah dari setiap kawasan. Pokok dari keluarga *Astaracea* merupakan yang utama. Dari analisis proksimat, roti lebah *H. itama* mempunyai karbohidrat yang tinggi ( $58.03 \pm 0.75\%$ ), protein ( $22.46 \pm 0.63\%$ ), lemak ( $5.29 \pm 0.53\%$ ) dan abu ( $2.56 \pm 0.13\%$ ). Analisis gula menggunakan kromatografi cecair berprestasi tinggi yang dilengkapi dengan pengesan pengewapan serakan cahaya (HPLC-ELSD) mendapati glukosa sebagai gula yang paling banyak dan diikuti oleh fruktosa. Asid amino dikuantifikasi menggunakan kromatografi cecair berprestasi tinggi yang dilengkapi dengan pengesan pengewapan serakan cahaya (HPLC-FLD). Terdapat lapan asid amino penting, dimana phenylalanin merupakan tertinggi dengan nilai 2.317 g / 100 g. Kandungan mineral dan logam berat dianalisa menggunakan pasangan plasma induktif-mass spektroskopi (ICP-MS). Unsur mineral utama di dalam roti lebah ialah kalium (purata 6705.9 mg / kg). Logam berat seperti plumbum, arsenik, kadmium dan merkuri turut dikesan, tetapi dalam tahap yang selamat. Sembilan puluh tujuh (97) bakteria telah diasingkan dari roti lebah *H. itama*. Daripada jumlah ini, 27 diramalkan sebagai bakteria asid laktik (LAB) dan *Bacillus* setelah dilakukan pewarnaan Gram dan ujian katalase. Bacteria telah dikenal pasti menggunakan penjujukan gen rRNA 16s. Bacteria ini ialah *Lactobacillus musae*, *Lb. crustorum*, *Lb mindensis*, *Leuconostoc mesenteroides*, *Enterococcus faecalis*, *Fructobacillus fructosus*, *Bacillus safensis*, *B. amyloliquefaciens*, *B. megaterium*, *B. cereus* dan *B. pumilus*. Bacteria dinilai untuk keupayaan hemolitik pada agar yang

dicampur 5% darah kambing biri-biri. Semua strain LAB dan *B. megaterium* MPS2 tidak menghemolisis darah. Aktiviti antagonistik terhadap patogen bawaan makanan menggunakan penyebaran agar menunjukkan *L. musae* SGMT17 dan *L. crustorum* SGMT22 dengan kawasan inhibisi tertinggi, setanding dengan strain komersil *Lb. rhamnosus* GG. Bakteria dengan sifat antibakteria dinilai untuk keupayaan untuk dalam kondisi pH 3, 0.3% hempedu dan enzim secara asing. Semua strain mampu hidup dalam keadaan simulasi kecuali *F. fructosus* U45 dan *F. fructosus* U47 kerana mereka mencatatkan keupayaan hidup di bawah 80% selepas rawatan di dalam pepsin dan pancreatin secara serentak. Ciri-ciri adhesi menggunakan autoagregasi dan hydrofobisiti permukaan sel (CSH) menunjukkan *L. mindensis* SGMT22 dengan keupayaan autoagregasi tertinggi (41.16%) manakala *Lc. mesenteroides* U39 menunjukkan CSH tertinggi (80.52%). Corak rintangan antibiotik untuk isolat terhadap 11 antibiotik telah dinilai menggunakan kaedah penyebaran cakera dan ditafsirkan menggunakan piawaian dari institut standard klinikal dan makmal (CLSI). Semua strain sensitif kepada chloramphenicol, ampicillin dan tetracycline, tetapi berbeza bagi lapan antibiotik lain. Dari hasil yang diperolehi, *Lb. musae* SGMT17 dan *Lb. crustorum* SGMT20 menunjukkan aktiviti antibakteria tertinggi dan sifat probiotik dalam model pencernaan *in vitro* manusia. Hasil kajian ini menyumbang kepada pengetahuan mengenai maklumat nutrisi pemakanan roti lebah *H. itama*, berpotensi untuk dikembangkan sebagai produk komersil atau bahan makanan. Lebih banyak kajian perlu dilakukan pada bakteria yang dipilih untuk mengenalpasti nilai terapeutik lain dan keselamatannya menggunakan model *in vivo*



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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

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## LIST OF ABBREVIATIONS

°C	Degree Celsius
μL	Micro liter
μM	Micro molar
BLAST	Basic local alignment search tool
CLSI	Clinical and Laboratory Standard Institute
bp	Base pair
CO <sub>2</sub>	Carbon dioxide
CSH	Cell surface hydrophobicity
DNA	Deoxyribonucleic acid
DRI	Dietary reference intake
dNTP	2'-deoxy-nucleotide-5'-trphosphate
EDTA	Ethylene diamine tetra acetic acid
ELSD	Evaporating light scattering detector
FAO	Food and agriculture organization
FLD	Fluorescence detector
g	Gram
GIT	Gastrointestinal tract
H <sub>2</sub> O <sub>2</sub>	Hydrogen peroxide
HCL	Hydrochloric acid
HPLC	High-performance liquid chromatography
ICP-MS	Inductive coupled plasma mass spectroscopy
Kb	Kilo base pair
Kg	Kilogram
LAB	Lactic acid bacteria
M	Molar
MEGA 4	Molecular evolutionary genetics analysis version 4
MgCl <sub>2</sub>	Magnesium chloride
Min	Minute
ml	Milliliter
mM	Milimolar
MRS	De man, rogosa and sharpe
NaOH	Sodium hydroxide
NCBI	National center of biotechnology information
SEM	Scanning electron microscopy

## CHAPTER 1

### INTRODUCTION

#### 1.1 Research Background

Bee products are crucial for bee survivals and it is believed to be acquired and used by human since 8000 BC as medicine, offering, cosmetic and everyday uses (Mizrahi & Lensky, 2013). Gradually, it influences the emergence of beekeeping industry whereby beekeepers collect these products for commercial purposes or personal uses. Malaysia beekeeping industry plays an important role in the agricultural area; for socioeconomic development and biodiversity conservation. Its contribution towards Malaysia regional growth depends on efficient production and marketing of bee products and bee byproducts (Ismail, 2014). Although this industry is progressively expanding in Malaysia, research on Malaysia bee byproducts are disregarded (Ismail, 2016), especially on stingless bee (locally known as '*kelulut*') species *Heterotrigona itama*.

Bee bread is one the bee byproducts, made from pollen collected by bee, added with nectar and bee salivary enzymes before undergoes lactic acid fermentation in beehives (Kieliszek et al., 2018). It is an underutilized bee product, mostly overshadowed by honey well-known status as the money maker (Ismail, 2016). The local market price for raw unprocessed wet bee bread is ranged from RM150-RM400/kg. Bee bread has brought newfound interest among Malaysian consumers largely because of the many claims on its health benefits. Demand for bee bread is expected to rise due to its nutritional value and application in apitherapy (Kieliszek et al., 2018). The Malaysian beekeepers are seizing this opportunity for generating incomes (Lob et al., 2017).

Bee bread is high in carbohydrate (24-34%), protein (14-37%), lipids (6-13%) and contain other macronutrients such as minerals, vitamin (Kieliszek et al., 2018), phenolic compounds (Vit, Albore, et al., 2018) and essential amino acids which can't be synthesized by human (Bonvehí & Escola, 1997; da Silva et al., 2014; Human & Nicolson, 2006). However, the chemical composition of bee bread is varied depending on the botanical origin, geographical location, climatic condition, soil type, beekeepers activities or storage treatments in the commercial production (Ares et al., 2018; Pascoal et al., 2014). The current report on Malaysian bee bread nutritional value is limited. So far, (Ismail et al., 2018) only reported on the nutrient analysis for *H. itama* bee bread from Perak. More information is needed to confirm bee bread nutritional values across other Malaysia region. Because bee pollen is rich in nutrient, it becomes a source for the microbial growth in bee pollen. These microbes have a mutual relationship with bee. For the bee, microbes are essential to protect bee colonies against pathogens and to provide nutrient for bee growth (Engel et al., 2012). They also contribute towards bee pollen conversion into bee bread. Bee bread is

suggested to be preserved by lactic acid bacteria (LAB) which have been previously discovered in bee bread and bee pollen of honeybee *Apis mellifera* (Anderson et al., 2014; Vásquez & Olofsson, 2015). Apart from LAB, *Bacillus* species also attain its niche in bee bread of *Apis mellifera* and stingless bee *H. itama* products (Gilliam, 1979; Ngaliimat, Raja Abd. Rahman, Yusof, Syahir, & Sabri, 2019). However, little is known on the probiotic potential of bacteria that facilitated the fermentation of bee pollen in stingless bee especially in the *H. itama* species.

Probiotic is “live microorganism”, when administered in adequate amounts, gives health benefits to host (Araya et al., 2002). Lactic acid bacteria (LAB) such as *Lactobacillus*, *Streptococcus*, *Leuconostoc*, *Lactococcus* etc are the most prominent bacteria in the probiotic industry. However, other bacterial genera such as *Bacillus* have also shown promising outcomes as probiotic candidates (Elshagabee et al., 2017). The general requirement for probiotic is to survive the gastrointestinal tract and safe for human consumption. Probiotic has been associated with functional foods because of the positive effect on gut microbiota (Ziemer & Gibson, 1998). Probiotic effect on host health is strain specific. Thus, the discovery of novel strains is desirable to improve the bio-therapeutic actions of probiotic bacteria incorporated in food and pharmaceutical products (Manap, 2008). Since certain bacteria species preferred different niche, exploring new source to isolate probiotic is to look-for. For this reason, this study also interested to investigate on the probiotic potential of bacteria isolated from bee bread.

## 1.2 Problem Statement

Although stingless bee *H. itama* is the dominant stingless bee species in Malaysia, the information on its by-products especially bee bread are still scarce and underexplored (Ismail, 2016). Current literature profoundly emphasized on honey from honeybee species (*Apis* spp.). This lack of information for Malaysia bee bread has led to a setback for its commercialization. Therefore, beekeepers loss the opportunities to generate additional profits from bee bread without heavily relying on honey as a source of incomes.

Reports have been disseminated on bee bread nutritional values, but these data largely depend on various factors such as botanical origin, geographical location, bee species, beekeepers activities and downstream processing (Ares et al., 2018; Pascoal et al., 2014). The beekeeping industry in Malaysia is flourishing. Unfortunately, beekeepers have not well equipped with scientific evidence to prove Malaysia bee bread nutritional value of its own. The nutritional claims exerted on local's bee bread mostly based upon studies from other countries which could mislead the consumers. With these lacks of data for Malaysia bee bread, the exact nutritional values are questionable leading to a lack of standardization for bee products. In addition, abundant literature has been reported on bacterial communities in bee bread but their probiotic potential remains unknown. The common public misconception upon probiotic relies upon the ideology that all probiotics work the same. Probiotic work in a



strain-dependent manner, therefore current studies which have documented on probiotic properties and health benefits cannot be extrapolated to other strains. Novel probiotic strains required extensive studies, on the basic level, is to characterize its viability in digestive tract and safety using *in vitro* studies. If not, the probiotic strains could not exert its benefits and could endanger the host well-being.

### **1.3 Objectives**

This study was carried out to fulfil the following objectives;

1. To determine the botanical origin and characterize the nutritional composition of bee bread of stingless bee (*Heterotrigona itama*).
2. To isolate and identify lactic acid bacteria and *Bacillus* spp. from bee bread of stingless bee (*H. itama*).
3. To determine the probiotic properties of bacteria isolated from bee bread of stingless bee (*H. itama*) using *in vitro* tests.

### **1.4 Significance of study**

This study will contribute toward the existing knowledge of bee bread of stingless bee (*Heterotrigona itama*) nutritional values in Malaysia. These data will provide valuable information to the Department of Standards Malaysia to establish the quality standard for bee bread. In line with the Ministry of Agriculture Malaysia's plan to promote the beekeeping industry, the outcome of this study will benefit the beekeepers to make profits from bee bread. Beekeepers will be more encouraged to develop bee bread into a commercialized product. The obtained information could also be used to promote the incorporation of bee bread as an ingredient for functional food development in local food. With the promising outcomes on bee bread nutritional values – yet limited, this could encourage other researchers to study bee bread from other Malaysia regions, especially in Borneo. The findings also provide insights towards bee bread as a potential source to isolate novel probiotics.

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