UNIVERSITI PUTRA MALAYSIA

EVALUATION OF Bacillus spp. ISOLATED FROM STINGLESS BEE (Heterotrigona itama Cockerell) HONEY AS A POTENTIAL PROBIOTIC

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By

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Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Master of Science

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December 2020

Chairman : Norhasnida Zawawi, PhD
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In Malaysia, stingless bees are well known for producing special honey called "madu kelulut" that is high in antioxidant and antimicrobial activity. Due to its higher moisture content, it provides favourable condition for microbial growth. However, little is known about the types of microbes that resides in this honey. Probiotics are live microbes, which when administered in adequate amounts confer health benefits to the host. This study aimed to find new strains of spore-forming probiotic bacteria from stingless bee (*Heterotrigona itama* Cockerell) honey. Therefore, *Bacillus* spp. were isolated from honey produced by 5 different *H. itama* colonies which were then further identified via 16S rRNA sequencing. Out of 53 isolates, only 23 isolates that are Gram positive, catalase positive and can tolerate 7% NaCl were selected for further molecular identification. Gene sequence analysis revealed that the dominant *Bacillus* species were *Bacillus altitudinis* (34%) and *Bacillus pumilus* (33%) followed by *Bacillus megaterium* (13%), *Bacillus amyloliquefaciens* and *Bacillus aryabatthai* (8%), followed by *Bacillus subtilis* (4%). Antibacterial activity against pathogenic bacteria assessed using agar well diffusion method showed that *B. amyloliquefaciens* HTI-19 and *B. subtilis* HTI-23 have comparable inhibitory activity with commercial probiotic strain *Lactobacillus rhamnosus* GG. Based on their antibacterial activity, these two strains were evaluated for probiotic properties including acid and bile salt (0.3%) tolerance, hydrophobicity and autoaggregation ability. Both isolates exhibited high tolerance with more than 85% viability in simulated *in vitro* gastrointestinal (GIT) conditions. Adhesion activity assessed by hydrophobicity and autoaggregation test revealed that *B. amyloliquefaciens* HTI-19 has the highest autoaggregation ability (84.13%) but lowest hydrophobicity (53.64%) among tested isolates. However, it was not significantly different from *L. rhamnosus* GG. Both *Bacillus* spp. were susceptible to all antibiotics with different mode of action. *Bacillus amyloliquefaciens* HTI-19 was α-hemolytic while *B. subtilis* HTI-23 exhibited γ-hemolytic activity on blood agar plate. Acute and subacute oral toxicity tests were performed in Sprague-Dawley rats to assess the safety of newly isolated *B. amyloliquefaciens* HTI-19. The probiotic feeding in acute and sub-acute toxicity study showed no mortality or significant abnormalities in rats throughout the 14-day and 28-

day experimental period. The rats body weights were not affected by daily administration of probiotic *B. amyloliquefaciens* HTI-19 except for week 2 of acute study, when the body weight showed significant increase ($P<0.05$). By gross and microscopic examination of organs, no obvious and significant changes were observed in the morphology of organs from the treated rats. Serum biochemical tests and blood hematology test also revealed no treatment-related changes. Overall, these data indicated that oral administration of *B. amyloliquefaciens* HTI-19 culture up to 1 $x10^9$ CFU/ml for acute and subacute test were safe. The results of this study showed that *Bacillus* isolates from stingless bee honey, *B. amyloliquefaciens* HTI-19 and *B. subtilis* HTI-23, due to its spore forming ability, have remarkable probiotic properties.

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

PENILAIAN *Bacillus* spp. YANG DIASINGKAN DARI MADU LEBAH KELULUT (*Heterotrigona itama* Cockerell) YANG BERPOTENSI SEBAGAI PROBIOTIK

Oleh

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Di Malaysia, lebah kelulut dikenali kerana menghasilkan madu khas yang disebut "madu kelulut" yang tinggi antioksidan dan aktiviti antimikrobial. Kerana kandungan lembapannya yang lebih tinggi, ia memberikan keadaan yang baik untuk pertumbuhan bakteria. Probiotik adalah mikroorganisma hidup, yang apabila diberikan dalam jumlah yang mencukupi dapat memberikan manfaat kesihatan kepada tuan rumah. Kajian ini bertujuan untuk mencari jenis bakteria probiotik pembentuk spora baru dari madu lebah kelulut (*Heterotrigona itama* Cockerell). Oleh itu, *Bacillus* spp. diasingkan dari 5 koloni *H. itama* yang berbeza, yang kemudian dikenal pasti melalui penjujukan 16S rRNA. Daripada 53 isolat, hanya 23 isolat yang positif Gram, positif katalase dan boleh bertoleransi 7% NaCl dipilih untuk pengenalan molekul selanjutnya. Analisis urutan gen menunjukkan bahawa spesies *Bacillus* yang dominan adalah *Bacillus altitudinis* (34%) dan *Bacillus pumilus* (33%) diikuti oleh *Bacillus megaterium* (13%), *Bacillus amyloliquefaciens* dan *Bacillus aryabatthai* (8%), diikuti oleh *Bacillus subtilis* (4%). Aktiviti antibakteria terhadap bakteria patogen yang dinilai menggunakan kaedah penyebaran sumur agar menunjukkan bahawa *B. amyloliquefaciens* HTI-19 dan *B. subtilis* HTI-23 mempunyai aktiviti pencegahan yang setanding dengan strain probiotik komersial *Lactobacillus rhamnosus* GG. Berdasarkan aktiviti antibakteria mereka, kedua strain ini dinilai untuk sifat probiotik seperti toleransi asid dan garam hempedu (0.3%), hidrofobik dan kemampuan pengagregatan secara automatik. Kedua-dua isolat menunjukkan toleransi tinggi dengan daya maju lebih dari 85% dalam keadaan simulasi *in vitro* gastrointestinal (GIT). Aktiviti lekatan yang dinilai oleh ujian hidrofobisiti dan pengagregatan automatik menunjukkan bahawa *B. amyloliquefaciens* HTI-19 mempunyai kemampuan pengagregatan auto tertinggi (84.13%) tetapi hidrofobik terendah (53.64%) di antara isolat yang diuji. Walau bagaimanapun, ia tidak berbeza secara signifikan dari *L. rhamnosus GG*. Kedua-dua *Bacillus* spp. terdedah kepada semua antibiotik dengan cara tindakan yang berbeza. *Bacillus amyloliquefaciens* HTI-19 adalah α-hemolitik sementara *B. subtilis* HTI-23 menunjukkan aktiviti γ-hemolitik pada plat agar darah. Ujian ketoksikan oral akut dan subakut dilakukan pada tikus

Sprague-Dawley untuk menilai keselamatan *B. amyloliquefaciens* HTI-19 yang baru diasingkan. Probiotik yang diberikan dalam kajian ketoksikan akut dan sub-akut tidak menunjukkan kematian atau kelainan yang ketara pada tikus sepanjang tempoh percubaan 14 hari dan 28 hari. Berat badan tikus tidak dipengaruhi oleh probiotik *B. amyloliquefaciens* HTI-19 yang diberikan setiap hari, kecuali untuk minggu ke-2 kajian akut, berat badan tikus menunjukkan peningkatan yang ketara ($P < 0.05$). Dengan pemeriksaan organ secara kasar dan mikroskopik, tidak ada perubahan yang jelas dan ketara dalam morfologi organ dari tikus yang dirawat. Ujian biokimia serum dan ujian hematologi darah juga menunjukkan tidak ada perubahan yang berkaitan dengan rawatan. Secara keseluruhan, data ini menunjukkan bahawa pemberian oral kultur *B. amyloliquefaciens* HTI-19 hingga 1 $x10^9$ CFU / ml selama 28 hari adalah selamat. Hasil kajian ini menunjukkan bahawa *Bacillus* yang diasingkan dari madu lebah kelulut, *B. amyloliquefaciens* HTI-19 dan *B. subtilis* HTI-23, mempunyai sifat probiotik yang luar biasa kerana kemampuannya membentuk spora.

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This thesis was submitted to the Senate of the 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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α	Alpha
β	Beta
°C	Degree Celsius
%	Percentage
A_{600nm}	Optical density at wavelength 600 nanometer
µL	Microliter
µm	Micrometer
µmoles	Micromoles
bp	Base pair
DNA	Deoxyribonucleic acid
g	Gram
kb	Kilobase
L	Litre
M	Molar
CFU	Colony-forming unit
DNA	Deoxyribonucleic acid
FAO	Food and Agriculture Organization
WHO	World Health Organization
PA	Protective antigen
LF	Lethal factor
EF	Edema factor
BSH	Bile salt hydrolase
LA	Linolenic acid

GLP	Glucagon-like peptide-1
GI	Gastrointestinal
EU	European Union
GALT	Gut-associated lymphoid tissue
ETEC	Enterotoxigenic *Escherichia coli*
GIT	Gastrointestinal tract
EFSA	European Food Safety Authority
QPS	Qualified presumption of safety
GRAS	Generally Regarded As Safe
FDA	Food and Drug Administration
MIC	Minimum inhibitory concentrations
PCR	Polymerase chain reaction
NCBI	National Center of Biotechnology Information
CFS	Cell-free supernatant
PBS	Phosphate-buffered saline
SR	Survival rate
OD	Optical density
CLSI	Clinical and Laboratory Standard Institute
UHT	Ultra-high temperature processing
OECD	Organisation for Economic Co-operation and Development
LD	Lactate dehydrogenase
PCR	Polymerase chain reaction
MD	Medium dose
AST	Aspartate transaminase

ALT	Alkaline phosphatase
ALB	Albumin
ALP	Alkaline phosphatase
RBC	Red blood cells
MCV	Mean corpuscular volume
MCHC	Mean corpuscular haemoglobin concentration

CHAPTER 1

INTRODUCTION

1.1 Research background

Stingless bee species are mostly found in the tropics and subtropics of the world like Australia, Africa, Southeast Asia, and parts of Mexico and Brazil. They have significant role as pollinators in different crops and orchards for both wild and cultivated flowering plants (Seunghwan Lee, Duwal, & Lee, 2016). The bees produced honey by gathering the nectar or blossoms and combine them with specific substances to let it ripen and mature (Codex, 2001). Best known for its sour taste, it is also rich in nutritional and therapeutic value (Rao, Krishnan, Salleh, & Gan, 2016).

The main composition of honey are sugars and water and generally, honey is acidic in nature (pH 4) (Begum et al., 2015; Kahraman et al., 2010). Stingless bee honey has substantially higher moisture content, water activity, ash content and free acidity than honeybee honey, though pH and total soluble solid content are marginally lower (Lage et al., 2012). Fermentation of stingless bee honey may be attributed to the low reducing sugar and high moisture content which are favourable for microbial growth (Cheng et al., 2019; Nascimento et al., 2015). Besides that, the fungi (filamentous and yeast) and bacteria are also responsible for stingless bee honey fermentation, making stingless bee honey a abundant source of microorganisms (Lee et al., 2015; Ngalimat et al., 2019), with some of them showing probiotic qualities (Begum et al., 2015; Esawy et al., 2015).

Probiotics are defined as “live microorganisms in which, when administered in adequate amounts, will confer a health benefit to the host” (FAO/WHO, 2002). Probiotics already have a long history of safe use but have just been perceived to bring economic value during the 20th century. The worldwide probiotics market is expected to achieve a turnover estimation of US$46.55 billion by 2020 (Toole et al., 2017) and will be dominated by food enterprises, dietary supplement companies and committed probiotic manufacturing companies (Isolauri, Salminen, & Ouwehand, 2004). While in Malaysia The Malaysian Probiotics market was valued at US$218.750 million in 2019 and is expected to grow at a compound annual growth rate of 6.03% over the forecast period. (Malaysia Probiotics Market - Forecasts from 2020 to 2025, 2020). Bacteria that are generally classified as probiotics usually comes from the genera of *Lactobacillus*, *Lactococcus*, *Bifidobacterium*, *Leuconostoc*, *Pediococcus* and *Bacillus*. *Bacillus* species, for example, *B. subtilis*, *B. licheniformis*, *B. pumilus*, and *B. amyloliquefaciens* were among the potential probiotic bacteria isolated from the honey of stingless bee honey (Ngalimat et al., 2019; Silva et al., 2016).

Bacteria from the genus *Bacillus* are mostly safe and not pathogenic to mammalians, except for *B. cereus and B. anthracis* (Cutting, 2011a). In fact, a broad range of functional secondary metabolites or also called antimicrobials were produced by *Bacillus* spp. This metabolites includes bioinsecticides, enzymes, and lipopeptides like iturin, surfactin, fengycins, bacteriocins, and bacteriocin-like inhibitors (BLIS) (Stein, 2005). *Bacillus* spp., due to their heat-resistant spores have advantages over non-spore formers like lactic acid bacteria (LAB) (Cutting, 2011b). These biologically and commercially relevant features make them a promising candidate for utilization as probiotic bacteria (Desai & Banat, 1997; Sabaté et al., 2009). For example, different strains of *B. subtilis* have been incorporated in fermented foods such as natto, soybean, or any other probiotic supplement as a dietary supplement in both human and animal diets (Jeon, Lee, Yang, Kim, & Paik, 2017; Quigley, 2010; S. Zhou et al., 2019).

Bacillus spp. has been utilized to produce food-grade enzymes (Ghani et al., 2013; Ouattara et al., 2017) vitamins and carotenoids for the synthesis of many health supplements for human use (Mohammed et al., 2014; Takano, 2016; Tanaka, Takanaka, & Yoshida, 2014). Nevertheless, despite the above advantages, because of their association with few human pathogens, these bacteria have not gained much importance and interest in the current functional food industry. Some *Bacillus* spp. particularly, *B. cereus, B. weihenstephanensis, B. anthracis,* and *B. thuringiensis* species were reported to generate different toxins such as ematic or enterotoxin (Cereulide), Bipartite exotoxins: protective antigen- lethal factor (PA-LF) and PA-edema factor (PA-EF), Cry and Cyt. *B. cereus* and *B. weihenstephanensis* are able to produce Cereulide, a major cause of food borne intoxications; while *B. anthracis* secretes PA-LF and PA-EF toxins, which are responsible for deadly diseases among humans and animals (reviewed in Elshaghabee et al., 2017).

1.2 Problem statements

Most studies of the microorganisms associated with stingless bees have been carried out with the objective of describing the bacterial and fungal communities only. There is still not enough scientific information on stingless bee honey in order for the bee farmers in Malaysia to commercialize and make profit out of it. Eventhough there is growing market demand for probiotics in Malaysia, the study on probiotic potential of microorganisms from the honey of *Heterotrigona itama* is still limited. As a spore-former, *Bacillus* spp. have advantage over non-spore former as they have better tolerance to acidic conditions in GIT as compared to LAB. Furthermore, the effect of probiotics is strain dependent. Therefore, the results on currently available probiotics cannot be used to justify the probiotic potential of a new strain. The ability to survive in GIT and the safety of a newly isolated strains need to be studied. This study aimed to find new probiotic candidates from stingless bee (*Heterotrigona itama*) honey.

1.3 Significance of study

Identification of *Bacillus* isolates with probiotic characteristics might add commercial value to the honey of stingless bee, which will eventually boost the socioeconomic standards of Malaysian's bee farmers. Probiotic bacteria isolated from stingless bee honey can be further exploited as functional food for food industries. Moreover, probiotic bacteria are strain specific, therefore the discovery of new probiotic strainswould contribute a lot to the biotherapeutic industry.

Due to their substantial production ability of extracellular enzymes, scientific findings supported by proof of safe use and long record of consumption would support the candidacy of spore formers such as *Bacillus* spp. as potential probiotics and as nutritional supplements. To our knowledge, information of *Bacillus* strains and its bioactivity in Malaysian raw stingless bee honey are still inadequate, and therefore this study would offer some valuable data on the probiotic properties and safety of the nonpathogenic *Bacillus* strains isolated from the *H. itama* honey.

1.4 Objectives

This work aims to find new probiotic candidates from the honey of *H. itama* from different meliponiculture places in Malaysia. The specific objectives of this research are:

1) To isolate and identify the potential probiotic bacteria from stingless bee (*H. itama*) honey.
2) To evaluate the probiotic properties such as antimicrobial activity, tolerance to GIT conditions and adhesion ability of selected bacteria.
3) To evaluate the safety of the potential probiotic bacteria *in vitro* and *in vivo*.

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LIST OF PUBLICATIONS

Journals

Amin, F. A. Z., Sabri, S., Ismail, M., Chan, K. W., Ismail, N., Mohd Esa, N., ... & Zawawi, N. (2020). Probiotic Properties of *Bacillus* Strains Isolated from Stingless Bee (*Heterotrigona itama*) Honey Collected across Malaysia. *International journal of environmental research and public health*, *17*(1), 278. (Published)

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