Antimicrobial activity and antibiotic resistance of lactic acid bacteria isolated from Malaysian stingless bee's gut

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ABSTRACT

Aims: A study had been conducted to isolate lactic acid bacteria (LAB) from Malaysian stingless bee gut and to determine their population in 4 stingless bee species. The isolated LAB was subjected to antimicrobial activity against selected bacterial pathogens and antibiotic resistance towards several antibiotics.

Methodology and results: Lactic acid bacteria isolated from the gut of 4 Malaysian stingless bee species (*Heterotrigona itama*, *Geniotrigona thoracica*, *Tetragonula laeviceps* and *Tetrigona melanoleuca*) were subjected to antimicrobial activity test against selected pathogenic bacteria, antibiotic resistance assay and identification of potent LAB through molecular 16S rRNA gene sequencing. A total of 99 putative LAB isolated from 4 stingless bee species were found to exhibit LAB characteristics such as Gram positive, oxidase negative and catalase negative. Out of 99 isolates, only 7 LAB isolates viz. TIS 5, TIS 25, TID 18, TTD 6, TLH 13, TLH 16 and TMH 2 exhibited strong to intermediate inhibition against *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella typhimurium*, *Bacillus cereus*, and *Bacillus subtilis*. The antibiotic susceptibility test demonstrated that all 7 isolates were not susceptible to streptomycin (10 µg), gentamycin (10 µg), tetracycline (30 µg) and kanamycin (30 µg). Nevertheless, these 7 isolates exhibited intermediate susceptibility to antibiotic penicillin G (10 µg), carbenicillin (100 µg) and ampicillin (10 µg). These 7 potent LABS were identified genotypically as *Lactobacillus plantarum* (TIS 5 and TIS 25), *Fructobacillus tropaeoli* (TID 18 and TTD 6) and *Pediococcus pentosaceus* (TLH 13, TLH 16 and TMH 2).

Conclusion, significance and impact of study: Findings from this study demonstrated the stingless bee's gut as a reservoir for LAB with anticipating antimicrobial properties and tolerance to certain antibiotics.

Keywords: Lactic acid bacteria, insect gut microbiota, meliponine, human pathogen, *in vitro*

INTRODUCTION

Gastrointestinal microbiota plays a major role in the nutrient assimilation and immune function of the insect (Mahesh *et al.*, 2012). Yeasts, molds and bacteria are the main microorganisms in stingless bee colonies (Menezes *et al.*, 2013). The symbiotic microflora of the digestive tract of mature bees consists of Gram-negative, Gram-positive and Gram-variable bacteria, moulds, and yeasts (Raghavan *et al.*, 2013). Lactic acid bacteria (LAB) were discovered by researchers present in large amounts in bee gut (Olofsson *et al.*, 2014). Lactic acid bacteria (LAB) are found as commensals to host (Forsgren *et al.*, 2010).

Lactic acid bacteria are known to be good producers of antimicrobial substances such as organic acids, hydrogen peroxide and antimicrobial peptides (Forsgren *et al.*, 2010). Besides, the antimicrobial activity properties of LAB were also contributed by the production of bacteriocins (Raghavan *et al.*, 2013). Bacteriocins are proteins or protein complex which has inhibition activity

against Gram positive and negative bacteria (Aween *et al.*, 2012). Lactic acid bacteria demonstrated high antimicrobial activity against *Escherichia coli*, *Salmonella typhimurium* and *Enterobacter aerogenes* (Aween *et al.*, 2012). Findings of Olofsson *et al.* (2014) revealed the strong antimicrobial properties of LAB from bee's honey against severe wound pathogens viz. methicillin-resistant *Staphylococcus aureus* (MRSA), *Pseudomonas aeruginosa* and vancomycin-resistant *Enterococcus* (VRE).

Many scientific works reported the presence of LAB associated with honeybee gut. Olofsson and Vasquez (2008) reported that LAB associated with honeybee originates from honeybees' stomach and the lactic acid bacteria flora was composed of *Lactobacillus* and *Bifidobacterium*. The reports of Audisio *et al.* (2011) discovered that *Lactobacillus johnsonii*, isolated from *Apis mellifera* L. bee's gut, exhibited a beneficial effect on honeybee colonies. The findings of Mahesh *et al.* (2007) indicated that two microbial genera, *Lactobacillus*

predominantly present in significant numbers in the midgut of *Apis mellifera carnica*.

Despite major findings of LAB from honeybee, there is a big gap on discovery of LAB isolated from stingless bee gut. The information of LAB associated with stingless bee is limited. Furthermore, findings by Leonhardt and Kaltenpoth (2014) only reported LAB from 3 species of Australian stingless bee. Currently, there is a growing interest on microorganism associated with stingless bees in Malaysia, but no study has been conducted on gut microflora especially LAB. Therefore, this study was conducted to isolate LAB from Malaysian stingless bee's gut and investigate their antimicrobial potential against selected pathogenic bacteria and antibiotic resistance towards several antibiotics.

MATERIALS AND METHODS

Adult workers of four stingless bee species *Heterotrigona itama*, *Geniotrigona thoracica*, *Tetragonula laeviceps* and *Tetrigona melanoleuca*, were collected from three stingless bee farms viz. herbal garden (Laman Herba), vegetable garden (Laman Sayur) and durian orchard in Malaysian Agricultural Research and Development Institute (MARDI). Ten adult workers of each species were captured with sterile soft forceps, placed in sterile Falcon tubes, and chilled in ice box along transportation. After collection, the specimens were transferred into -20°C freezer for storage until processing (Keerthi *et al.*, 2013). Before dissection, the specimens were washed in 95% ethanol and the abdomen were separated from their thorax prior to dissection. The abdomen was then cut open with a sterile scalpel to obtain the crop and mid gut (Figure 1). The gut was then macerated with sterile micro pestle in 1.5 mL Eppendorf tube containing 0.85% NaCl solution (Mahesh *et al.*, 2012).

Figure 1: Stingless bee gut dissected to obtain crop (Cr) and midgut (Mg) part where LAB was isolated.

Isolation of LAB

The gut sample of an amount of 1 mL were transferred into inoculation tube added with 9 mL de Man Ragosa and Sharpe (MRS) broth (Oxoid, United Kingdom) prior to isolation. The samples were incubated for 48 to 72 h on rotary shaker until the broth appeared to be slightly turbid. After 48 h, the samples were serially diluted with 0.85% saline solution. An amount of 200 μL aliquots was spread

plated on de Man Ragosa and Sharpe (MRS) agar (Oxoid, United Kingdom) and incubated at 37°C for 24 to 48 h under anaerobic condition and extended to 72 h until visible colonies appeared on the plate (Keerthi *et al.*, 2013).

Biochemical test

The presumptive putative of LAB colonies were determined by the Gram staining, catalase test and oxidase test. Gram staining method was performed by mounting a single colony of LAB from MRS plate on slide and fixed with one drop of distilled water. After drying, the slides were stained by flooding in crystal violet for 1 min. The slides were washed with distilled water and flooded again by iodine for 1 min followed by washing with distilled water. Slides were decolorized by flooding with 95% alcohol for 10 sec and washed off with tap water and drain. Then, slide was flooded with safranin solution and counterstained for 30 sec. Slide was washed off with tap water and drained. Finally, slide was examined using light microscope under the oil immersion lens. Positive bacteria exhibited purple color of colony under microscope observation.

Catalase test was used to determine potential of microorganism to degrade hydrogen peroxide (H_2O_2) by producing the enzyme catalase. A single colony was touched with a loop and smeared onto a clean slide. 2 drops of H_2O_2 were added and smeared with the colony. The presence or absence of free oxygen gas bubbles formed was checked. Negative catalase reaction was determined when there is no indicative bubble formed. (Chakraborty and Bhowal, 2015). The oxidase test detected the presence of a cytochrome oxidase system that catalyzed the transport of electrons between electron donors in the bacteria and a redox dye-tetramethyl-p-phenylene-diamine. The dye was reduced to deep purple color. Lactic acid bacteria were identified as oxidase negative bacteria (Chakraborty and Bhowal, 2015). The Bactident® Oxidase strips (MERCK, United State of America) was used to determine the oxidase test on lactic acid bacteria.

Antimicrobial activity of LAB against selected pathogenic bacteria

The antimicrobial activity of LAB was determined by using agar well diffusion assay (Venkadesan and Sumathi, 2015). The tested pathogenic bacteria *S. aureus*, *E. coli*, *P. aeruginosa*, *S. typhimurium*, *B. cereus* and *B. subtilis* was subcultured in nutrient agar. All the bacteria were diluted with 0.85% NaCl to 0.5 McFarland. A bacterial suspension of pathogenic bacteria was swab on Mueller-Hinton agar (MHA) using sterile cotton bud. A volume of 100 μL cell free supernatant of LAB was filled in each agar well. Streptomycin was used as positive control. The culture was incubated at 37°C for 24 h. The diameter of the inhibition zone (DIZ) was measured with calipers in mm.

Antibiotic susceptibility test

The antibiotic susceptibility of isolated LAB was determined by using Kirby Bauer disc method (Jannah *et al.*, 2014). Isolated lactic acid bacteria used for antimicrobial activity were incubated in MRS broth for 48 h at 37 °C. Mueller Hinton sterile agar plates were swabbed with 100 µL of LAB suspension (approximately 2×10^8 CFU/mL). Subsequently, antibiotic discs (50 susceptibility disc Oxoid, United Kingdom) containing antibiotics of ampicillin (10 µg), gentamicin (10 µg), kanamycin (30 µg), Penicillin G (10 µg), streptomycin (10 µg), tetracycline (30 µg) and carbenicilline (100 µg) were placed on Mueller Hinton agar plates. The plates were incubated at 37 °C for 24 h. The diameter (mm) of inhibition zones was measured to determine antibiotic sensitivity of the isolates.

Molecular identification of LAB isolates through 16S rRNA gene sequencing

Total genomic DNA isolated LAB was subjected to DNA extraction and followed by 16S rRNA PCR amplification. The cells from a LAB culture (1 mL) were harvested by centrifugation (10 min, 13 000 ×g). The pellet was then suspended in 1 mL sterile saline solution (0.9% NaCl, w/w) to remove the residual media compounds from the cells and centrifuged twice more for a further 10 min at 13 000 ×g. For the extraction of total DNA, the GF-1 bacterial DNA extraction kit (Vivantis, United State of America) was used according to Manufacturer's instructions for Gram-positive bacteria. The cells were incubated with lysozyme (20 µL) at 37 °C for 2 h. The treatment with proteinase K was performed at 72 °C for 30 min. Finally, the DNA was dissolved in 100 µL elution buffer (GF-1 bacterial DNA extraction kit, Vivantis, United State of America) and used immediately or stored at -20 °C. PCR amplification of the 16S rRNA gene from each LAB DNA sample was performed using a thermal cycler (MJ Research PTC-200 Peltier Thermal Cycler, Bio-Rad). Each reaction mixture (final volume, 25 µL) contained: 2 µL of the template DNA (50 ng), 1 µL each primer at a concentration of 10 µM, 0.5 µL deoxynucleotide triphosphate (dNTP) at a concentration of 10 µM, 2.5 µL 10× PCR buffer for Taq DNA polymerase, and 0.5 µL of Taq DNA Polymerase (Vivantis, United State of America).

The universal oligonucleotide primers used for bacterial 16S rRNA were F8 (5'-AGAGTTTGATCMTGGCTC-3') and rP2 (5'-ACGGCTACCTTGTTACGA CTT 3') (Park *et al.*, 2010). The PCR conditions were as follows: initial denaturation of DNA for 5 min at 94 °C, then 30 cycles of denaturation of DNA for 1 min at 95 °C, annealing for 1 min at 55 °C, extension for 1 min at 72 °C, and final incubation for 7 min at 72 °C. The PCR products were detected by electrophoresis (MS Gel Electrophoresis System, Major Science, United State of America) on 1% agarose gel running in TAE (Tris-Acetate-EDTA) buffer at 100 V, and stained with SYBR® Safe DNA Gel Stain (Invitrogen,

Thermo Fisher Scientific, United State of America). The size of DNA fragments was estimated using VC 1 kb-Ex DNA ladder (Vivantis, United State of America). The purified PCR products were then sequenced by a sequencing company (1st BASE Sdn. Bhd., Kuala Lumpur, Malaysia). The DNA sequencing results were compared with available sequences in GenBank using BLASTN tools through the National Centre for Biotechnology Information (NCBI). Sequence homology of more than 97% was regarded as belonging to the same species (Jannah *et al.*, 2014).

RESULTS AND DISCUSSION

Isolation of LAB from stingless bee gut

A total number of 226 isolates were isolated from the gut of 4 species of stingless bee (*H. itama*, *G. thoracica*, *T. laeviceps* and *T. melanoleuca*). The gut flora of worker honeybee was chosen as the source of sample since it possesses an abundant, diverse and ancient LAB microbiota with beneficial effects (Raghavan *et al.*, 2013). As shown in Table 1, bacterial isolates were found abundantly in species *H. itama* with number of 30 isolates from location A (herbal garden in MARDI, Serdang, Selangor), 28 from location B (vegetable garden in MARDI, Serdang, Selangor) and 39 from location C (Durian orchard in MARDI, Serdang, Selangor) followed by *T. laeviceps* (25 from location B and C, 20 from location A) and *T. melanoleuca* (22 isolates from location A). Vast number of presumptive LAB that was isolated from *H. itama* guts demonstrated that this species harbors a vast number of beneficial microflorae dominating 3 other species of stingless bee. To the best of our knowledge, this is the first study on the gut microbiota of the local stingless bee species. Hence, no evidence to support which species of local stingless bee harbor high number of LAB. Detail information on microbiota of all social and solitary bees that had been study so far includes widespread LAB that are not host specific (Leonhardt and Kaltenpoth, 2014). Most LAB such as fructophilic lactobacilli which is flower inhabiting microbes were found abundantly in the gut of flower's foraging bees. The host specificity of LAB is rare in Hymenoptera and can be preserved in social bees by disseminating symbionts among nestmates and transmitting them through workers during colony fissioning from one generation to the next (McFrederick *et al.*, 2012).

Biochemical test

Lactic acid bacteria were classified as Gram positive, catalase negative and oxidase negative (Olofsson *et al.*, 2014). In this study, screening process was narrowed down on all 226 isolates to biochemical test results including Gram reaction, catalase and oxidase test to obtain the desired LAB. Summary of all screening LAB on biochemical test were showed in Table 2.

Table 1: Isolation source and number of bacterial isolated from stingless bee.

Stingless bee species	Location	Name of isolates	Number of isolates
<i>H. itama</i>	A	TIH	30
	B	TIS	28
	C	TID	39
<i>G. thoracica</i>	A	TTS	20
	C	TTD	17
<i>T. laeviceps</i>	A	TLH	20
	B	TLS	25
	C	TLD	25
<i>T. melanoleuca</i>	A	TMH	22
Total isolates			226

Note: A, Herbal garden in MARDI, Serdang, Selangor; B, Vegetable garden in MARDI, Serdang, Selangor; C, Durian orchard in MARDI, Serdang, Selangor.

Table 2: Number of bacterial isolates from the stingless bees characterized based on biochemical reaction (Gram staining, catalase and oxidase reaction).

Biochemical reaction	Number of isolates
Gram positive, catalase negative, oxidase negative	99
Gram positive, catalase positive, oxidase positive	26
Gram positive, catalase positive, oxidase negative	21
Gram positive, catalase negative, oxidase positive	28
Gram negative, catalase negative, oxidase negative	8
Gram negative, catalase positive, oxidase negative	16
Gram negative, catalase positive, oxidase positive	20
Gram negative, catalase negative, oxidase positive	8
Total isolates	226

A total number 99 isolates out of 226 numbers of isolates were characterized as Gram positive, catalase negative and oxidase negative while, 26 isolates were characterized as Gram positive, catalase positive and oxidase positive. Furthermore, out of 226 isolates, 21 of them are Gram positive, catalase positive and oxidase negative followed by 28 isolates as Gram positive, catalase negative and oxidase positive. All 99 isolates identified as Gram positive, catalase negative and oxidase negative potent LAB were subjected to antimicrobial activity test.

Antimicrobial activity of lactic acid bacteria against pathogenic microorganisms

All 99 potent LAB isolates were tested for antimicrobial activity against common pathogenic microorganisms. The activities were determined based on diameter of the inhibition zone (DIZ) and classified into classes: weak (6-9 mm), intermediated (10-13 mm), strong (14-16 mm), no growth (0) (Venkadesan and Sumathi, 2015). The results are shown in Table 3.

Out of 99 LAB strains isolated from four species of stingless bee only 53 isolates show inhibitory activity against broad spectrum of pathogenic human bacteria such as *S. aureus*, *E. coli*, *P. aeruginosa*, *S. typhimurium*, *B. cereus* and *B. subtilis*. Out of the 53 LAB that have wide spectrum of inhibition against pathogenic bacteria, 7 LAB isolates exhibited strong to intermediate inhibition against *S. aureus*, *E. coli*, *P. aeruginosa* and *S. typhimurium*. The isolates are TIS 5, TIS 25, TID 18, TTD 6, TLH 13, TLH 16 and TMH 2. Among these seven, isolate TID 18 has the strongest inhibition against four pathogenic bacteria.

The antibacterial properties of LAB might be contributed by the production of acetic and lactic acids that lowered the pH of the medium or competition for nutrients, or due to production of bacteriocin or antibacterial compounds (Tambekar *et al.*, 2009). According to Darsanaki *et al.* (2012) bacteria with an average inhibition of 7.91 mm indicate good capacity of inhibiting pathogenic bacteria.

Antibiotic susceptibility

Safety of any strains of LAB intended to be used as probiotic must be evaluated for antibiotic susceptibility profile and verification of antibiotic resistance genes (Sadrani *et al.*, 2014). Antibiotic resistance of probiotic strains does not constitute a safety concern in itself, when mutations or intrinsic resistance mechanisms are responsible for the resistance phenotype. In fact, some

probiotic strains with intrinsic antibiotic resistance could be useful for restoring the gut microbiota after antibiotic treatment.

However, specific antibiotic resistance determinants carried on mobile genetic elements, such as tetracycline resistance genes, are often detected in the typical probiotic genera, and constitute a reservoir of resistance for potential food or gut pathogens, thus representing a serious safety issue to the probiotic strain itself (Gueimonde *et al.*, 2013).

In this study, susceptibility of LAB isolates to 8 antibiotics was determined. Streptomycin did not inhibit the growth of all the isolates tested. Isolates TIS 5 and TIS 25 were less susceptible to Ampicillin (10 µg) with diameter of inhibition at 13.00±0.00 mm and 13.33±0.33 mm respectively. All isolates were susceptible to the antibiotic's penicillin G (10 µg) and carbenicillin (100 µg) indicated by the size of inhibition zone (Table 4). All isolates not susceptible to gentamycin (10 µg), tetracycline (30 µg) and kanamycin (30 µg). According to study done by Yadav *et al.* (2016), *L. plantarum* was found to be sensitive to most tested antibiotics including ampicillin (10 µg), gentamicin (10 µg), tetracycline (30 µg), streptomycin (10 µg), and erythromycin (15 µg). This species is found to be resistant to kanamycin (30 µg), nalidixic acid (30 µg), vancomycin (30 µg) and ciprofloxacin (5 µg).

Resistance of the probiotic strains to some antibiotics can be used for both preventive and therapeutic purposes in controlling intestinal infections. In addition, their resistance to antibiotics was describing their potential in minimizing the negative effects of antibiotic therapy on the host bacterial ecosystem (Sadrani *et al.*, 2014). A study done by Gueimonde *et al.* (2013) revealed that some antibiotics such as penicillin G, chloramphenicol, oleandomycin, erythromycin and clindamycin intake can significantly drop *Lactobacillus* spp. from intestinal microflora. On the other hand, four antibiotics for instance kanamycin, norfloxacin, vancomycin and tobramycin will not influence the growth of lactobacilli population. Studies have reported that lactobacilli are typically sensitive to ampicillin (Gueimonde *et al.*, 2013).

The antibiotic susceptibility of strain is crucial from the safety point of view for their use as potential probiotics because probiotic bacteria may act as potential reservoir of antimicrobial resistance genes and which can be transferred to gastrointestinal tract (Hoque *et al.*, 2010).

Microscopic and macroscopic morphological observation and molecular identification of LAB isolates

Lactic acid bacteria are generally Gram positive, usually non-motile, non-spore forming rods and cocci (Khalisanni, 2011). Morphological properties of lactic acid bacteria were characterized as cocci, rod or bacilli or tetrad

formation (Figure 2). Based on microscopic and macroscopic examination, Table 5 showed summary of the colony morphology of isolates TIS 5, TIS 25, TID 18 TTD 6, TLH 13, TLH 16 and TMH 2.

Molecular technique is significant for genotypic bacterial identification method and provide accurate information for LAB than the conventional phenotypic method (Shehata *et al.*, 2016). In this study, the 16S rRNA of the total genomic DNA from 7 selected LAB isolates was amplified and sequenced for identification. The sequences of the selected isolates were aligned with the 16S rRNA gene sequences from the GenBank database (website) to identify the selected LAB. From the 16S rRNA gene sequencing analysis data as shown in Table 6, isolates TIS 5 and TIS 25 showed high percentage of similarity (100%) to *L. plantarum*. Furthermore, LAB isolates TID 18 and TTD 6 showed high percentage of similarity (99%) to *Fructobacillus tropaeoli*. As for LAB isolates TLH 13, TLH 16 and TMH 2, the results showed high percentage of similarity (99-100%) to *Pediococcus pentosaceus*.

Lactic acid bacteria genera that were found associated with bees identified based on genotypic and phenotypic traits are *Lactococcus*, *Lactobacillus*, *Pediococcus*, *Enterococcus*, *Leuconostoc* and *Fructobacillus* (Vasquez and Olofsson, 2009; Belhadj *et al.*, 2010). The genus *Lactobacillus*, an important group from the LAB, is usually found as commensal bacteria and is commonly used as probiotics in humans and animals (Ouwehand *et al.*, 2002).

Fructobacillus tropaeoli was grouped into family *Leuconostocaceae* (Endo *et al.*, 2011). This species was found in fructose-rich environments such as flowers, fermented fruits or bee guts, and are characterized as fructophilic lactic acid bacteria (FLAB) (Endo *et al.*, 2015). This statement supported the presence of *F. tropaeoli* in the gut since stingless bee foraging for flower's nectar for the source of food.

Interestingly, research done by Endo and Salimen (2013) found that FLAB had probiotic potential for bee where FLAB exhibited high antimicrobial activity against *Melissococcus plutonius*, a causative pathogen of European foulbrood, and several pathogenic bacteria and yeast. Through our findings in the antimicrobial assay, strong antimicrobial performance showed by LAB isolates TID 18 and TTD 6 supported by findings of Endo and Salimen (2013).

Genus *Pediococcus* was found to be associated with most bees (Belhadj *et al.*, 2014). *Pediococcus* sp. has been also reported to be the producer of bacteriocin which may be of use as preservative in food industry. In study conducted by Sukumar and Ghosh (2010), the strain showed the ability to inhibit the growth of *S. aureus*, *P. aeruginosa* and *E. coli*.

Table 3: Summary of antimicrobial activities of 7 LAB isolates against selected bacterial pathogens.

LAB isolates	Antimicrobial activity against selected bacterial pathogen expressed in cm (inhibition zone)					
	<i>E. coli</i>	<i>S. typhimurium</i>	<i>P. aeruginosa</i>	<i>S. aureus</i>	<i>B. cereus</i>	<i>B. subtilis</i>
TIS 5	12.17±0.17	11.33±0.67	14.43±0.30	10.50±0.30	13.00±0.29	11.97±0.03
TIS 25	12.50±0.29	15.17±0.17	15.83±0.17	11.00±0.00	13.00±0.00	11.33±0.33
TID 18	14.53±0.26	15.07±0.23	14.00±0.00	14.27±0.15	14.07±0.07	14.90±0.10
TTD 6	13.00±0.00	13.17±0.17	14.17±0.17	10.50±0.29	14.50±0.29	14.50±0.26
TLH 13	10.83±0.44	15.50±0.29	10.67±0.33	12.50±0.29	14.43±0.29	13.27±0.14
TLH 16	10.67±0.33	15.50±0.29	10.83±0.44	13.17±0.17	14.00±0.00	13.77±0.19
TMH 2	10.17±0.17	15.20±0.20	11.00±0.58	13.17±0.17	14.53±0.29	13.40±0.058
Streptomycin (control)	11.93±0.58	11.83±0.73	11.17±0.60	14.53±0.26	23.00±0.00	22.33±0.33

Note: TIS, *H. itama* from herbal garden; TIS, *H. itama* from vegetable garden; TID, *H. itama* from durian orchard; TIS, *G. thoracica* from vegetable garden; TTD, *G. thoracica* from durian orchard; TLH, *T. laeviceps* from herbal garden; TLH, *T. laeviceps* from vegetable garden; TLD, *T. laeviceps* from durian orchard; TMH, *T. melanoleuca* from herbal garden.

^{abcd} Means in the same column followed by different superscript letters are significantly different ($P < 0.05$). Results are expressed as mean±SE, and each value is the average of three replications.

Table 4: Antibiotic susceptibility of LAB isolates against several antibiotics.

Antibiotics (µg)	Name of LAB isolates						
	TIS 5	TIS 25	TID 18	TTD 6	TLH 13	TLH 16	TMH 2
Streptomycin (10)	NS	NS	NS	NS	NS	NS	NS
Penicillin G (10)	11.00±0.57	10.17±0.17	10.67±0.88	7.93±0.81	9.27±0.82	8.17±0.44	9.67±0.88
Gentamycin (10)	NS	NS	NS	NS	NS	NS	NS
Tetracycline (30)	NS	NS	NS	NS	NS	NS	NS
Kanamycin (30)	NS	NS	NS	NS	NS	NS	NS
Carbenicillin (100)	13.00±0.00	13.33±0.33	13.33±0.88	9.67±0.33	10.33±0.33	11.33±0.88	11.33±0.88
Ampicillin (10)	13.00±0.00	13.33±0.33	NS	NS	NS	NS	NS
Negative control (sterile water)	NS	NS	NS	NS	NS	NS	NS

Note: ^{abcd} Means in the same column followed by different superscript letters are significantly different ($P < 0.05$).

*Results are expressed as mean ± SE, and each value is the average of three replications.

Diameter of the inhibition zone (mm): NS (not susceptible).

Table 5: Microscopic and macroscopic features of the 7 LAB isolates.

LAB isolates	Gram reaction (microscopic features)	Colony morphology (macroscopic features)
TIS 5	Gram positive short bacilli	Small size, round, opaque white, convex and entire colony
TIS 25	Gram positive short bacilli	Medium size, round, opaque cream, raised and entire colony
TID 18	Gram positive long bacilli	Small size, round, translucent white, flat and entire colony
TTD 6	Gram positive long bacilli	Medium size, round, translucent white, flat and entire colony
TLH 13	Gram positive coccus. The colonies group in pairs or tetrads	Medium size, round, opaque cream, umbonate and entire colony
TLH 16	Gram positive coccus. The colonies group in pairs or tetrads	Small size, round, opaque cream, convex and entire colony
TMH 2	Gram positive coccus. The colonies group in pairs or tetrads	Medium size, round, opaque cream, convex and entire colony

Figure 2: Macroscopic and microscopic examination of LAB isolates. A-G, Colony morphology of TIS 5, TIS 25, TID 18 TTD 6, TLH 13, TLH 16 and TMH 2 grow on MRS agar plate after 24 to 48 h. H-N, Microscopic observation of LAB isolates observed under compound microscope (1000 × magnification).

Table 6: Molecular identification of LAB isolates based on 16S rRNA gene sequencing.

LAB isolates	Most closely related type strain	Similarity %	Accession numbers
TIS 5	<i>L. plantarum</i> strain IPhp-GM10	100%	KX943030.1
TIS 25	<i>L. plantarum</i> strain IPhp-GM10	100%	KX943030.1
TID 18	<i>F. tropaeoli</i> strain R-46388	99%	HE590766.1
TTD 6	<i>F. tropaeoli</i> strain R-46388	99%	HE590766.1
TLH 13	<i>P. pentosaceus</i> strain JN18	99%	KT719234.1
TLH 16	<i>P. pentosaceus</i> strain JN18	99%	KT719234.1
TMH 2	<i>P. pentosaceus</i> strain JN18	100%	KT719234.1

CONCLUSION

The present study exhibited that LAB associated with 4 stingless bee species (*H. itama*, *G. thoracica*, *T. laeviceps* and *T. melanoleuca*) were dominated with the strain of *L. plantarum*, *F. tropaeoli* and *P. pentosaceus* with potential of antimicrobial properties and tolerance to selected antibiotics. Further study should explore the potential probiotics of the identified strains for the application in food or apiculture industry.

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