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RESEARCH ARTICLE

Identification and Antimicrobial Activity of Lactic Acid Bacteria Isolated from Tualang Bee Honey

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Abstract Malaysia multifloral Tualang honey usually collected from the combs of Asia rock bees (*Apis dorsata*), which build their hives high up in the Tualang tree (*Koompassia excelsa*). Tualang honey is used commonly as a medicinal product and as food in Malaysia. lactic acid bacteria (LAB) strains have been discovered to exhibit antimicrobial properties especially in Malaysia. However, the research on LAB found from honey are still scarce. The study was conducted to isolate and identify lactic acid bacteria (LAB) from Tualang honey bee from East Coast Peninsular Malaysia. The Tualang honey with LAB strains were further tested against several pathogenic bacteria by using well-diffusion method. The LAB were primarily identified by colony morphology, microscopy of Gram`s stain, biochemical tests and 16s rRNA sequencing method. The 10 LAB strains that has been identified as *Leuconostoc mesenteroides* (5 strains), *Lactobacillus kunkeei* (4 strains), and *Lactobacillus farraginis* (1 strains) were selected for the characterization of antimicrobial agents produced by LAB. The findings shows that 5 strains exhibited strong antimicrobial activity against *Staphylococcus epidermidis* and *Pseudomonas aeruginosa* results in excellent inhibition zones diameters larger than 15 mm. This study indicated that LAB isolated from Tualang honey has potential antimicrobial activity against pathogenic bacteria and can be further characterized for health benefits and potential use in food industry at Malaysia.

Keywords: Tualang Honey, LAB, pathogen, 16s rRNA sequencing, antimicrobial activity.

Introduction

The application of Lactic acid bacteria (LAB) in food fermentation and culture of food products has been practiced in the past 4000 years ago. According to Abdelbasset *et al*., (2014), LAB has been considered as the most important groups of microorganisms in food fermentation. Inhibition of spoilage bacteria through the production of growth-inhibiting substances and lactic acid by LAB has also contribute towards the texture, nutritional value and flavour of the fermented products. Combined with their long historical contribution and its widespread usage in food fermentation, LAB was accepted as GRAS (Generally Recognized as Safe) for human consumption (Parmjit, 2011).

Malaysian honey has been reported to contain several strains which showcases promising antibacterial properties. However, extensive exploration on the beneficial therapeutic effects of bee honey is still lacking. It has been known from many years before that honey possesses medicinal effect and has been used traditionally to fight against bacterial infections. Honey is a sweet liquid that are stored temporarily in the bee's stomach during flight and comprised of various amount of sucrose, glucose, and fructose (Babendreier *et al*., 2007). Tualang honey (TH) are known for having antimicrobial effects which are related to the osmotic effects of the sugar contents, pH and the peroxidase activity in the honey itself (Roslan *et al*., 2015).

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Several strains of bacteria were isolated from honey and were tested for its antimicrobial activity against both Gram positive and negative bacteria (Aween *et al*., 2010: Ibarguren *et al*., 2010: Lee *et al*., 2008). A study by Olofosson and Vasquez (2008) found out that Lactic acid bacteria of the genera *Lactobacillus* and *Bifidobacterium* were successfully isolated from the stomach of honeybees and honey samples. LAB has evolved through its stress response system and defences, allowing them to cope and survive in harsh condition such as selective pressure from environmental changes. The evolution also allowed them to exhibit antimicrobial action against pathogenic bacteria by producing metabolites such as lactic acid, formic acid, di-acetyl, and acetic acid, contributing to their host defence system (Vasquez *et al*., 2009: Forgsen *et al*., 2010). The aim this study is to isolate LAB from Tualang honey and to evaluate its antimicrobial activity in order to select those with promissory potential for future starter culture in dairy production.

Materials and Methods

Sample Collection and Preparation

Two (2) Tualang honey samples from Hulu Terengganu and Marang, Terengganu in the East Coast Peninsular of Malaysia region were collected and used for the study. All samples were freshly collected, placed in sterile containers and stored at room temperature until analysed. Unwanted material such as wax sticks, dead bees and particles of combs were removed by straining the samples through cheesecloth before analysis.

Isolation and Identification of Lactic Acid Bacteria (LAB) from Tualang Honey Samples

The preparation of media for LAB culture followed the methodology described by Aween *et al*. (2012). All plates will be incubated under anaerobic condition using Carbon dioxide incubator for 48 hrs. The presumptive of LAB colonies will be tested for Gram staining, catalase test and oxidase test. Cultures with Gram positive bacteria and negative for catalase will be maintained in MRS broth with 15% of glycerol and kept at - 20ºC for further study. Molecular approach was adopted by performing PCR followed by sequencing of the almost complete 16S rRNA gene amplified with universal primers pairs of 27F 5'-AGAGTTTGATYMTGGCTCAG-3' and 1492R 5'-TACCTTGTTACGACTT-3'. Genomic DNA was first extracted using Qiagen DNA Extraction Kit (QIAGEN, Germany). PCR reactions were performed as follows: an initial cycle of 95 °C for 5 min; 35 cycles of 95 °C for 30 seconds, 52 °C for 30 seconds, 72 °C for 1 min; and final extension at 72 °C for 10 min. The purified PCR products that were obtained from the PCR were sequenced by First BASE Laboratories Sdn. Bhd. (Shah Alam, Selangor, Malaysia). The 16S rRNA sequences were aligned and compared with other 16S rRNA genes in the GenBank by using the NCBI Database (Altschul *et al*., 1997)

Antimicrobial Activity Test by Agar Well Diffusion Method

The agar well diffusion method was used to determine the antimicrobial property of the LAB strains. A 24 hrs culture of the pathogens (Staphylococcus epidermidis, *Salmonella typhimurium*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Listeria monocytogenes*, and *Staphylococcus aureus*) grown in nutrient agar plates at 37°C will be prepared in Mueller-Hinton broth (Merck) and plated onto Mueller-Hinton agar (Merck) and swab in three directions by using sterile cotton swab. The plates were allowed to dry and a sterile stainless-steel borer of diameter (5 mm) was used to cut uniform wells in the agar. Each well was filled with 60µl of cell-free supernatant obtained from the LAB strains. The cell-free supernatant will be obtained from centrifugation of the isolated LAB at 12 000 rpm for 10 min at - 4˚C. The plates will be incubated at 30°C for 24 hrs. Then, the diameters of the inhibitory zone will be measured. Results were considered positive if the diameter (mm) of the zone of inhibition (ZOI) was greater than 1mm (Nigam *et al*., 2012). The experiment was carried out in triplicates and activity was reported as diameter of ZOI ± SD.

Results and Discussion

Isolation of LAB and Preliminary Identification of Bacterial Strains

The isolation of LAB from Tualang honey conducted using MRS agar, MRS agar with 0.8% $CaCO_3$, MRS agar with 10% glucose and M17 Agar. Colonies were purified with a streaking method. All LAB colony were further investigated by applying catalase test, oxidase test and Gram staining. The colonies selected for those tests were colony that have different size and shape. If the colonies having the same sizes and shape, only one colony was taken to undergo Gram staining, oxidase test and catalase test.

A total of 10 strains were selected from both the Hulu Terengganu and Marang Tualang honey samples. All the samples obtained were morphologically characterized. Table 1 shows the biochemical characteristics of the strains obtained along with their Gram reaction and microscopic examination. Only Gram positive, non-motile, catalase-negative, showing phenotypic characters similar to Lactobacillus species on MRS agar media were selected for further experiments.

Table 1. Preliminary identification of lab isolated from two Tualang honey samples.

Tualang honey Sample	Strains	Catalase Test	Gram Stain	Shape Morphology	Oxidase Test	Motility test
Hulu Terengganu	H001	-	+	cocci	-	-
	H002	-	+	cocci	-	-
	H003	-	+	cocci	-	-
	H004	-	+	cocci	-	-
	H005	-	+	cocci	-	-
Marang	M006	-	+	rod	-	-
	M007	-	+	cocci	-	-
	M008	-	+	rod	-	-
	M009	-	+	rod	-	-
	M010	-	+	rod	-	-

Identification of LAB Strains

Bacterial identification of the isolated lactic acid bacteria in this study was done by the sequencing of the 16S rRNA genes of the strains. All 10 strains had their 16S rRNA genes amplified as confirmed by agarose gel electrophoresis after polymerase chain reaction. When the sequences were compared with the GeneBank (NCBI) database, sequence similarity values of 90% or more were obtained. Table 2 shows that the strains were classified as *Leuconostoc mesenteroides* (five isolates from Hulu Terengganu Tualang honey), *Lactobacillus kunkeei* (four strains from Marang Tualang honey), and *Lactobacillus farraginis* (isolated also from Marang Tualang honey). Although phenotypic methods were the most commonly used method for the identification of bacterial strains (Corsetti *et al*., 2001), the genetic method such as 16S rRNA sequencing have been developed more recently to allow more consistent and accurate identification of individual strains (Tamang *et al*., 2008).

Table 2. Identification of the isolated lab by 16s rRNA gene sequencing showing percentage similarities.

Sample		**16S rRNA Gene Sequencing**	**Similarity**
		Most closely related type strain	
Hulu Terengganu	H001	*Leuconostoc mesenteroides* strain ATCC 8293	99%
	H002	*Leuconostoc mesenteroides* strain ATCC 8293	99%
	H003	*Leuconostoc mesenteroides* strain ATCC 8293	98%
	H004	*Leuconostoc mesenteroides* strain ATCC 8293	99%
	H005	*Leuconostoc mesenteroides* subsp. *suionicum* strain LMG 8159	99%
Marang	M006	*Lactobacillus kunkeei* strain YH-15	98%
	M007	*Lactobacillus farraginis* strain NRIC 0676	98%
	M008	*Lactobacillus kunkeei* strain YH-15	94%
	M009	*Lactobacillus kunkeei* strain YH-15	98%
	M010	*Lactobacillus kunkeei* strain YH-15	97%

Antimicrobial Activity against Selected Pathogens

The agar well diffusion method was used to assess the antimicrobial activity of the selected 10 LAB strains. The antimicrobial activity was tested against six food-borne pathogenic bacteria which consists of *Staphylococcus epidermidis*, *Salmonella typhimurium*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Listeria monocytogenes*, and *Staphylococcus aureus*. The result shows that the samples of LAB strains showed different spectrum range of inhibitory activities against the target bacteria by the well diffusion agar method. Table 3 shows the results for the antimicrobial activity of the strains in terms of diameter of the zone of inhibition (ZOI). According to Aween *et al*., (2012), clear zone length less than 10 mm is considered as weak resistance, while diameter ranges from 10 to 15 mm is considered moderate and diameter more than 15 mm is considered strong resistance to pathogens. LAB strain of H-001, H-002, H-003, H-004 and H-005 shows strong antimicrobial activity against almost all pathogens except *Listeria*

monocytogenes. Four pathogens compromise of *Salmonella typhimurium*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus* showed moderate resistance with more than 10mm diameter ZOI. However, a strong resistance with more than 21m ZOI when test against *Staphylococcus epidermidis*.

LAB strains of M-006, M-007, M-008, M-009, and M-010 only shows a moderate resistance with more than 10mm ZOI against *Staphylococcus epidermidis* and *Staphylococcus epidermidis* while the other four pathogenic bacteria shows no inhibition activity. According to Ammor *et al*., (2006) LAB produced a wide range of products from low molecular mass compounds, such as hydrogen peroxide, carbon dioxide and diacetyl, to high molecular mass compounds, such as bacteriocins. The use of LAB bacteriocins, separately or as bio preservative combinations, can be of significant use in enhancing food safety, particularly for traditional products (Mojgani and Amirnia, 2007). Organic acid produced by LAB can leads to a reduction in pH levels and increases the production of hydrogen peroxide (Ponce *et al*., 2008). These products exhibit antibacterial activity against various pathogenic microorganisms, including Gram-positive and Gram-negative bacteria (Maragkuodakis *et al*., 2009). The capacity to display antimicrobial activity against pathogenic bacteria is one of the significant WHO / FAO criteria for selecting organisms for probiotic purposes (Adeniyi *et al*., 2015). According to Batista *et al*., 2017, LAB strain with antimicrobial properties can be used in the production of fermented foods and this would assist in the inhibition of diseases in consumers.

Table 3. Antimicrobial activity of lactic acid bacteria against pathogenic microorganisms

Pathogens	**Lactic Acid Bacteria (Hulu Terengganu Tualang Honey)**				
	H-001	H-002	H-003	H-004	H-005
Staphylococcus epidermidis	23±1.0	23±1.0	23±1.73	23.7±0.577	22±1
Salmonella typhimurium	12±1.0	13±1.0	13±1.73	12±0	12.7±1.53
Escherichia coli	13.5±0.707	15.5±0.707	13±1.41	13±1.41	13.5±0.707
Pseudomonas aerugenosa	14.3±0.577	14±1.73	13±1.0	14.67±0.577	13±0
Listeria monocytogenes	0	0	0	0	0
Staphylococcus aureus	11±0	11±0	11±0	11.3±0.577	11.7±0.577
Pathogens	**Lactic Acid Bacteria (Marang Tualang Honey)**				
	M-006	M-007	M-008	M-009	M-010
Staphylococcus epidermidis	11.3±0.577	11.3±0.577	12.7±0.577	10.7±0.577	10.7±0.577
Salmonella typhimurium	0	0	0	0	0
Escherichia coli	0	0	0	0	0
Pseudomonas aerugenosa	11.7±0577	12.3±0.577	12.3±0.577	12±1.0	12±1.0
Listeria monocytogenes	0	0	0	0	0
Staphylococcus aureus	0	0	0	0	0

*diameter of zoi (zone of inhibition) around the well (mm)

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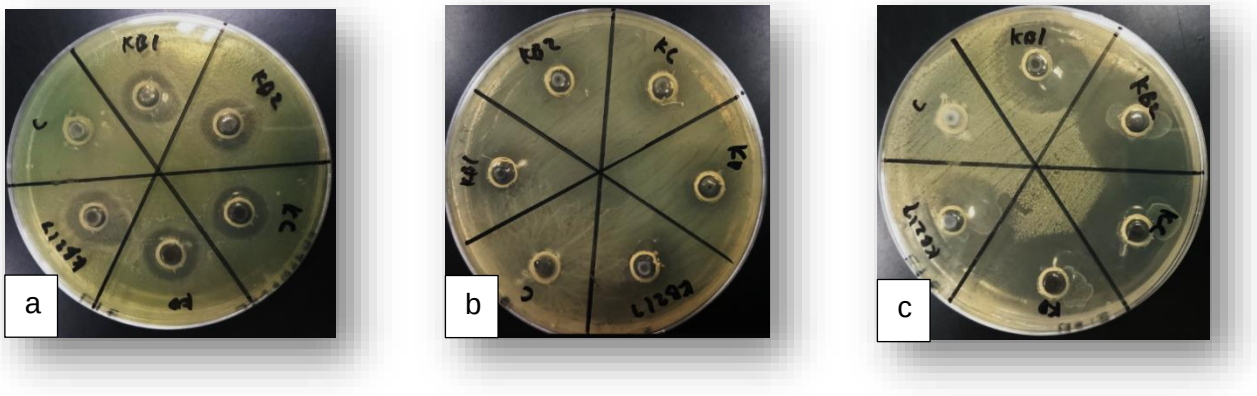

Figure 1. Inhibitory zone of lab on *Pseudomonas aeruginosa* (a), *Escherichia coli* (b), *Staphylococcus epidermidis* (c)

Conclusions

In this study, the LAB strains were successfully isolated from Tualang honey samples and were also identified using the 16s rRNA sequencing method. Three LAB species have been identified as *Leuconostoc mesenteroides*, *Lactobacillus kunkeei* and *Lactobacillus farraginis*. The LAB strains also exhibited antimicrobial attributes against several pathogenic bacteria. It has shown strong antimicrobial activity against *Staphylococcus epidermidis* and *Pseudomonas aeruginosa*. The antagonistic activity may occur due to the presence of organic acid, hydrogen peroxide and bacteriocin, which act as antimicrobial agent. These studies highlight the possibility that these bacteriocins and LAB will be notified as of great interest in terms of security especially in combination with other antimicrobial compounds in further research. However, further study is required regarding the LAB strains identified especially in terms of its suitability for its utilization as probiotic strains in food production.

Conflicts of Interest

The author(s) declare(s) that there is no conflict of interest regarding the publication of this paper.

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