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Lactic Acid Bacterium with Antimicrobial Properties from Selected Malay Traditional Fermented Foods

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ABSTRACT

Traditional or local fermented foods have been the favourite sources for Lactic acid bacteria (LAB) used for starter cultures. Traditional food such as fermented cassava '*tapai ubi'*, fermented glutinous rice '*tapai pulut'* and fermented shrimp paste '*belacan'* have been local heritage consumed as Malay delicacies. In this work, 33 LAB samples were isolated from *tapai ubi, tapai pulut and belacan* and out of these only 5 LAB isolates (PG, PH, BG, UG and UL) showed inhibitory properties against selected indicator organisms (*Bacillus subtilis, Escherichia coli, Salmonella typhimurium,* and *Staphylococcus aureus*). Morphologically, isolate PG, PH, BG are cocci, UL is rod and UG is coccobacillus. Biochemically, isolates (PG, PH, BG UL and UG) are found to be Gram positive, non motile, lactose fermenter and catalase negative. The 16s Ribosomal RNA gene sequencing was carried out and each was identified with an accession number (PB: MT645488, PH: MT645489; BG: MT645490 UG: MT645491 and UL MT645492). Isolates PG and PH from *tapai pulut* belonged to *Pediococcus pentosaceus* (at 99% and 98%, respectively). Meanwhile, isolate from *Belacan* BG belonged to *Enterococcus faecium* (99%), and those from fermented shrimp, UG and UL belonged to *Weissella confusa* (99%), *and Lactobacillus fermentum* (98%), respectively. Majority of the isolates demonstrated broad spectrum inhibition against both Gram positive and negative indicator strains. Compared to the rest of isolates, PH exhibited the highest antibacterial activity against *Bacillus subtilis.* These results suggested that isolate PH are the most potent isolates which is producing antimicrobial agent with potential as food preservatives.

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Introduction

Recent consumer awareness and perception have renewed interest in the consumption of healthy food; food with no additives or preservatives; or so called 'natural' or 'traditional' foods. The consumption of fermented food can offer many benefits that it was suggested to be included in food consumption guides [1]. Fermented foods exist in many cultures and lactic acid bacteria (LAB), or yeast are the common starter cultures used in food fermentation aimed at enhancing the texture and flavour of the desired products [2].

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Fermentation of carbohydrates produces product such as lactic acid which reduces the pH thereby creating condition unfavourable for the growth of harmful or food spoilage organism.

As a result of urbanisation and commercial demand, the production of traditional food need to up-scaled to ensure its benefit or relevance. However, the large scale production of traditional food is still a challenge. According to Anal et al [3], fermented food which is usually prepared in a small scale set up is subjected to safety risk, especially due to microbial contamination following bad agricultural practises. Therefore, the use of selected microflora or a more define starter cultures is highly recommended in minimizing this risk. For years, works have been carried out to isolate useful LAB in an attempt to screen for the best strain to be used as starter culture. Lactobacillus, Lactococcus, Streptococcus, Pediococus and Leuconostac are among the commonest group of LAB which have been used as starter cultures [4]. Despite LAB can be found widely in other non-food sources such as gastrointestinal tract of human or animals, fermented food was still considered the favourite target of many works on isolation for LAB. LAB produces ribosomally synthesized antimicrobial peptides known as bacteriocins which were considered as potential alternative to chemical food preservatives and antimicrobial drugs [5]. The diversity of LAB, and their target spectrum of their antimicrobial activities have made them interesting target for bacteriocin isolation.

There have been several studies on the isolation LAB from several types of Malay traditional foods. These include *tapai* (fermented tapioca), *tempoyak* (fermented durian flesh), chilli puree and fresh goat's milk [6]; fermented fish; Chili bo (chili puree) [7]; bambangan [8]; *pekasam* (fermented fish), *jeruk maman* (fermented vegetable), *tapai* (fermented glutinous rice) and *tempoyak* (fermented durian)[9]; and a local vinegar [10]. In one of our previous study, *Staphylococcus piscifermentans* was isolated from Cincaluk (Malaccan fermented shrimp) [11]. These studies indicated that different or diverse groups of LAB are continually being isolated even from similar food sources. Moreover, the same type of food may not be similarly prepared or following a standard ingredients. In this study, Malay traditional fermented food such “tapai pulut”, “tapai ubi” and “belacan” were selected as the sources for lactic acid bacterium (LAB). This LAB strains were tested for their probiotic properties and their ability to produce antimicrobial

activities against selected pathogenic strains. The properties of this isolates could make them suitable candidate to be used as starter culture in food fermentation.

Materials and Methods

Media and reagents preparation

Isolation and culturing of lactic acid bacteria (LAB) was carried out using De Man Rogosa and Sharpe (MRS) media. Pathogenic strains used as indicator bacterium *Bacillus subtilis, Escherichia coli, Salmonella typhimurium* and *Staphylococcus aureus* were grown in nutrient agar (NA). Nutrient agar (NA) media was also used for the antimicrobial activities. Pathogenic strains *Bacillus subtilis, Escherichia coli, Staphylococcus aureus* and *Salmonella typhimurium* were obtained from the Kuliyyah of Sciences collection at International Islamic University Malaysia, Kuantan Campus.

Sample sources

Three types of Malay traditional food, *tapai pulut* (fermented glutinous rice), *tapai ubi* (fermented cassava) and *belacan* (fermented shrimp paste) were purchased from local fresh market in Kuantan. About 10 g of each food sample was added with 90 ml of peptone water. Samples were homogenised and serially diluted up to 1 x 10^{-3} with 0.1% peptone water. The diluted samples were vortexed and spread on (MRS) agar plates. The plates were incubated anaerobically in gas pack for 3 days (72 hours), at 37°C. New formed colonies were sub-cultured on new MRS agar and incubated anaerobically for 24 hours at 37°C, and the procedure was repeated until single colony was obtained for each isolate. The new LAB isolates were maintained in MRS broth with 40 % glycerol and stored at -80 °C

Identification of lactic acid bacteria (LAB)

Preliminary identification and characterization of selected isolates were based on lactose utilization test, Gram staining and catalase test. Carbohydrate fermentation was tested using bromocresol lactose agar which contained bromocresol purple (purple indicator) (0.025 g), peptone (5.0 g), beef extract (3.0 g), lactose (10.0 g), and nutrient agar (15.0 g) per 1 liter water. The gram staining were carried out on the isolates using standard protocols and examined under light microscope (Nikon) at 100X magnification (immersion oil).

Screening of antimicrobial activities of LAB

In this work, preliminary screening for inhibitory activities from LAB were detected using disk diffusion methods according to Kirby method [12] and *Bacillus subtilis, Eschericia coli, Staphylococcus aureus and Salmonella typhimurium* were used as indicator bacterium. The strains were grown in Mueller-Hinton broth until turbidity reached at 0.5 McFarland standard and spread evenly using sterile cotton swab on NA agar plates to make bacterial lawn. The test LAB isolates were incubated anaerobically in MRS broth medium at for 48 hours at 37°C. Filter paper disks (Whatman, 9.0 mm diameter) were loaded with 20 µl of fresh cultures and allowed to dry. The filter discs were then laid on NA plates pre-streaked with indicator strains with gentle press. Then, all of the plates were incubated at 37°C for 24 hours. Tetracycline was used as positive control while MRS broth was used as negative control. The antimicrobial activities of the selected strains were determined by inhibitory zones around the wells and diameter of zone inhibition was measured (in mm).

Cell free extraction (CFE) and agar well diffusion assay

Following disk diffusion method, inhibitory activities from cell free extract (CFE) were detected using agar well diffusion assay [13]. Each LAB was propagated in MRS broth and incubated at 37°C for 48 hours. The broths were centrifuged (12 000x g) for 5 minutes at 4°C. Collected supernatant was filtered (Millipore, 0.22 µm pore size) to form LABs' CFE. About 35 ml of molten NA was poured into 50 ml falcon tube and mixed with 30 µl of indicator strains. This mixture was then poured on sterile plate and left to solidify, in which holes were later punched on solidified agar using sterile glass dropper. Each well was filled with 70 µl of prepared CFE and incubated at 37°C for 24 hours. MRS broth was used as a negative control. Following incubation, the zone on inhibitions around the well were observed and measured (in mm).

Genotypic characterisation

To all five LAB strains, the genomic DNA were extracted (GF-1, Vivantis) according to manufacturer's protocols. The gene for 16S rRNA were amplified using a pair of universal primer (forward: 5' – AGA GTT TGA TCC TGG CTC AG – 3' and reverse 5' – CCG TCA ATT CCT TTG AGT TT- 3') [14]. PCR reaction mixture contained 25 µl of master mix, 3 µl of DNA template, 5 µl of each primer and made up to 50 µl reaction volume with distilled H_20. The PCR runs were as follows: Initial denaturation at 94°C (2

minutes), denaturation at 94°C (1 minute), annealing at 55°C (1 minute), and extension at 72°C (1 minute). The amplification was repeated in 25 cycles followed by a final extension at 72°C (7 minutes). In this work, the DNA concentrations were checked using Nanodrop Spectrophotometer (NanoDropTM 2000, USA). The gel was visualised using 1% agarose gel electrophoresis and viewed using UV transiluminator (AlphaImager). The PCR products were electrophoresed and purified (Clean-Up kit,Vivantis) and sent to a sequencing agency (Apical scientific Sdn. Bhd.). The generated sequences were analysed using BLASTn which available at NCBI website (*http://blast.ncbi.nlm.nih.gov*). Selected sequences were aligned using ClustalX2, and MEGA 6 software were used to build up a phylogenetic tree. The 16S rRNA gene sequences of representative LAB strains were analysed using Neighbour-joining methods with bootstrap values based on 500 replications.

Results and Discussion

Morphological and biochemical characterisations

From 33 colonies, only five isolates (PG, PH, BG, UG and UL) were able to show lactose utilisation on MRS agar plate and these isolates were further subjected to biochemical, morphological and genotypic characterizations. As shown in **Fig 1**, the results of Gram staining, all isolates are gram positives in which three of the isolates (PG, PH and BG) are cocci. Meanwhile isolate UL appears rod-shaped and UG is coccobacillus (see to **Table 1**). The lactose fermentation was indicated by the changes in the colour of bromocresol dye from purple to yellow. Fermentation of lactose produces lactic acid as the main metabolite which in turn reduces the pH of media, a common feature exhibited by LAB [15]. LAB ferment sugar for carbon and energy sources while producing a variety of compounds such as organic acids, aromatic compounds and other substances beneficial to health. As shown in **Table 1**, all of these isolates were found to be catalase negative. Since LAB are adapted to anaerobic environment, they are lacking in hydrogen peroxide scavenging enzyme such as catalase [16]. Therefore, in catalase test there would be no bubble due to evolution of oxygen gas. Overall results from biochemical tests were consistent with other characterizations featured for LAB which are Gram positive, catalase negative, coccus, non-spore former, non-motile and anaerobic organisms.

Table 1 Morphological and biochemical tests on LAB

Characteristics	Lactic acid bacteria (LAB)				
	PG	PH	BG	UG	UL
Lactose test	+	+	+	+	+
Gram staining	+	+	-	+	-
Cell morphology	coccus	coccus	coccus	coccobacillus	bacillus
Catalase test	-	-	-	-	-

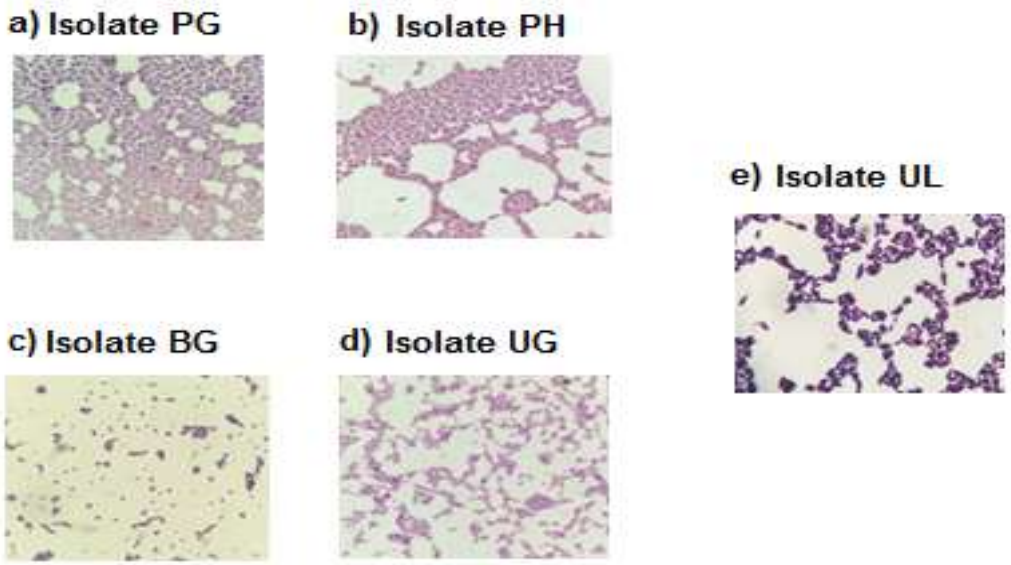

Fig 1 Morphologies of LAB isolates from Malay traditional food after Gram staining method, and viewed under light microscopic (Nikon, 100X magnification with oil immersion)

Antimicrobial activities of LAB

Based on disc diffusion assay, five isolates showed antimicrobial activities against Gram positive and Gram negative indicator bacterium (see **Table 2** and **Fig 2**). Both isolate PG and PH were able to show inhibitions against all indicator strains tested. Isolate PH showed strong inhibitions against almost all strains except a moderate inhibition against *S. thyphimurium.* Isolate BG was only active against gram negative strains (*E .coli* and *S. thyphimurium*). Meanwhile, Isolate UG and UL were lacking of inhibitory properties except against gram negative *E. coli.*

Table 2 Antimicrobial activity of the isolates against selected pathogenic bacteria using the disc diffusion assay

Pathogens	Inhibitory activities				
	PG	PH	BG	UG	UL
Bacillus subtilis	++	++	-	-	-
Eschericia coli	+++	+++	+++	++	+++
Salmonella typhimurium	+++	++	++	-	-
Staphylococcus aureus	++	+++	+	-	-

Sign denotes the degree of inhibition: '+' indicates low inhibition zone (0.9±05 mm); '++' moderate inhibition zone (1.0-1.2±05 mm); '+++' strong inhibition zone (1.3-1.6±05 mm); and '-' no inhibition zone.

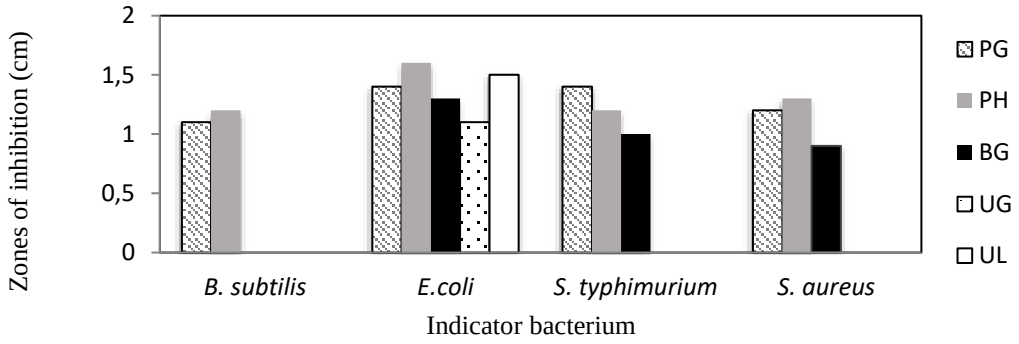

Fig 2 Zones of inhibition produced by LAB against selected pathogenic strains using agar disk diffusion methods

Table 3 Inhibitory activities using agar well diffusion assays

Pathogens	Inhibition activities				
	PG	PH	BG	UG	UL
Bacillus subtilis	+++	+++	+++	+++	+++
Eschericia coli	+++	+++	++	++	+++
Salmonella typhimurium	++	+++	+++	+++	++
Staphylococcus aureus	+++	++	++	-	-

Sign denotes the degree of inhibition: '+' indicates low inhibition zone (0.9±05 mm); '++' moderate inhibition zone (1.0-1.2±05 mm); '+++' strong inhibition zone (1.3-1.6±05 mm); and '-'no inhibition zone.

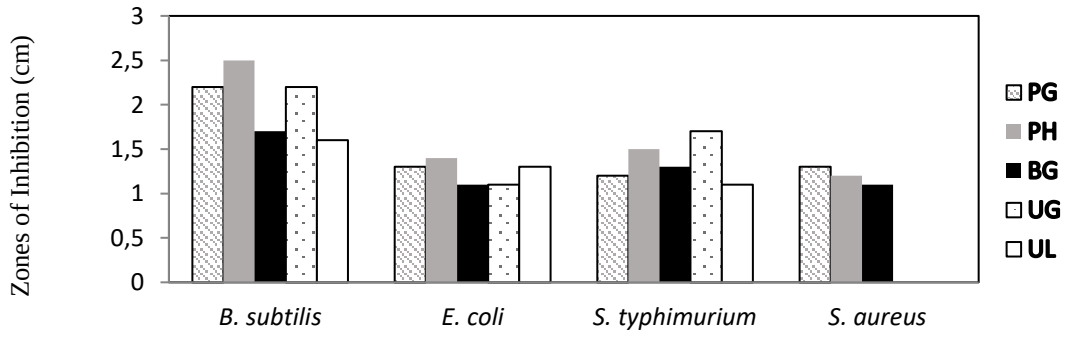

Fig 3 Zones of inhibition produced by LAB against selected pathogenic strains using agar well diffusion methods

Both agar disk diffusion and well diffusion methods were commonly used in studying antimicrobial activities. Due to its simplicity and cheap, disk diffusion method is commonly employed in antimicrobial screening work [17]. The trend of inhibition observed using disk diffusion method was however not necessarily replicated when tested in agar well diffusion method. In agar well diffusion assay (**Table 3** and **Fig 3**), all pathogenic strains showed inhibitions except for *Staphylococcus aureus* which showed resistance against isolates UG and UL. However, some of the zero inhibitions observed in disk diffusion method were able to shown inhibition in agar well method. Agar well diffusion methods were shown to be more sensitive than disk methods [18]. Disk diffusion method sometime produces smaller inhibition zones compared to agar well methods [19], and even the type of agar media used may affect its sensitivity [20]. The discordant we observed here were due to the phyco-chemical environment of these two methods and differences in the nature of inhibitory compounds produced by microorganism. For instance, inhibition due to low pH may be ineffective or not favourable when using disk diffusion method, a condition which is more effective for small organic or bacteriocin. Nevertheless, ability to show antagonisms is one of the desirable properties of LAB. Based on inhibitory strength and number of antagonised strains, the degree of antagonistic property among the isolates was in the order of PH > PG > BG > UG > UL. In term of spectrum of inhibition, Isolates PG, PH and BG are notable based on their ability to inhibit both Gram positive (*Bacillus subtilis, Staphylococcus aureus*) and Gram negative (*Escherichia coli and Salmonella typhimurium*) bacterium. Some of these inhibitions were due to the ability of LAB strains to produce bacteriocin or antimicrobial peptides.

Genotypic characterisations

The PCR amplification results for all isolates generated 1.5 kb fragment of 16s RNA (see **Fig 4**). Each of these sequence was assigned with an accession number (MT645488 for PB; MT645489 for PH; MT645490 for BG; MT645491 for UG; and MT645492 for UL) following gene submission at NCBI registery. The 16s ribosomal sequencing for the 5 LAB isolates indicated the following similarities: PG and PH with *Pediococcus pentosaceus* (at 99% and 98%, respectively); BG with *Enterococcus faecium* (99%), and UG with *Weisella confusa* (99%) and UL with *Lactobacillus fermentum* (98%), respectively. A phylogram was constructed as shown in **Fig 5,** incorporating the 16S

rRNA sequences of LAB strains from diverse genera such as Pediococcus, Lactobacillus, Enterococcus and Weisella.

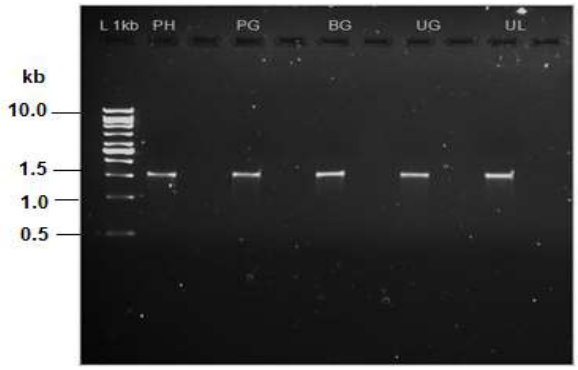

Fig 4 Ethidium bromide stained 1 % agarose gel displaying the bands of purified PCR amplification fragments product of 16s rRNA genes from LAB.

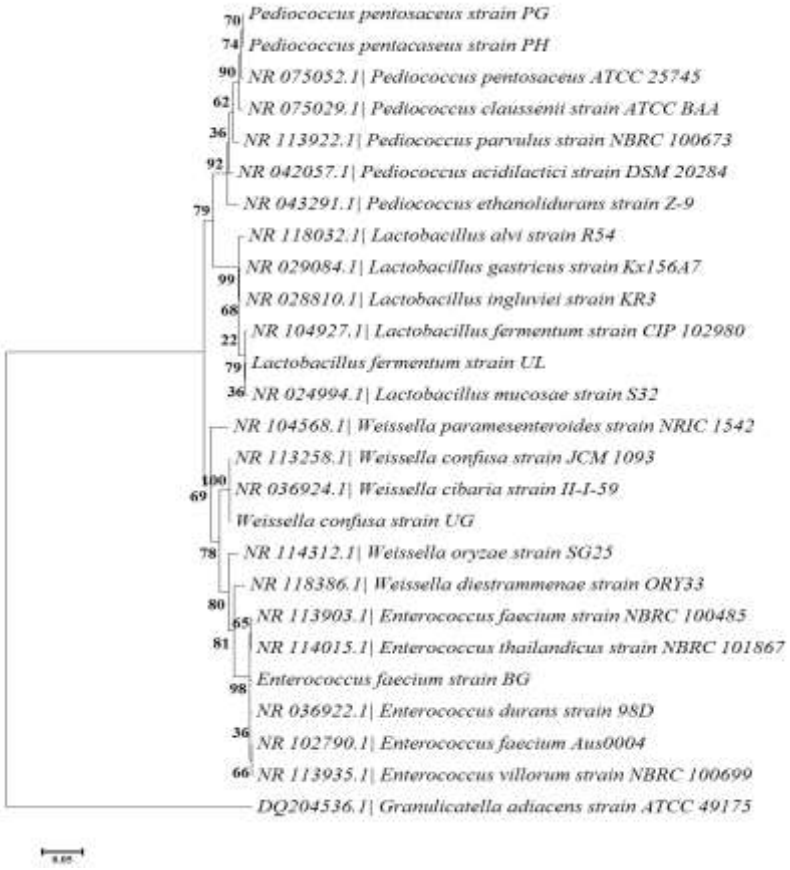

Fig 5 Phylogenetic tree was constructed using Neighbour-joining method (MEGA 6 software). The tree was constructed incorporating five 16S RNA sequences for five of the LAB isolates from Malay traditional fermented food (PG, PH, UL, UG and BG) and other 16s RNA sequences for LAB from other genera (Pediococcus, Lactobacillus, Enterococcus and Weissella). *Granulicatella adiacens* was selected as an out group in this tree construct.

All of the 5 isolates belonged common LAB isolated from various fermented foods throughout the world. For instance, *Pediococcus pentosaceus* were isolated from Kimchi (Korean fermented vegetable) [21], Korean sea food [22] and several Indonesian [23] and Tanzanian [24] fermented food. The isolation of *Lactobacillus fermentum* were common from fermented food product in many countries such as China [25, 26]; Turkey [27]; Tanzania [24] and Iran [28]. Diversity of LAB is notable as some of the strains could belong to opportunistic pathogen of which these were still being debated for being considered as probiotic. These include *Weissella confusa* [29] and nosocomial pathogen *Enterococcus faecium* [30]. *Weisella confusa* can be isolated from Indian fermented food [31]; Tanzanian [24] and Kimchi (Korea) [32]. Meanwhile, *Enterococcus faecium* are ubiquitous organism which can be isolated from cheese [33]; and other food sources [34].

Conclusion

In this study, five lactic acid bacteria strains designated as isolates PG, PH, BG, UG and UL have been successfully isolated from three types of traditional Malay food i.e., "tapai pulut", "tapai ubi" and "belacan". The phenotypic identifications indicated that these isolates showed typical properties of LAB with the abilities to antagonize selected pathogens. Antimicrobial activities studies showed varying degree of inhibition against pathogenic strains and most of these isolates showed broad spectrum inhibition. Based on 16S rRNA sequencing analysis, with more than 98% similarity; isolate PG was identified as *Pediococcus pentasaceus*, isolate UG as *Enterococcus faecium*, isolate UL *as Weissella confusa* and isolate BG as *Lactobacillus fermentum*. These isolates can be potential starter culture for traditional food preparation. Inhibitory studies may indicate that these isolates can be a potential sources for antibacterial agent such as bacteriocins which has potential to be used as natural bio-preservatives and in preventing the growth of spoilage pathogens in various food products.

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Conflict of Inerest
The authors declare no conflict of interest.

Abbreviations
LAB: lactic acid bacteria, CFE: cell free extract, rRNA: ribosomal ribonucleic acid

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