Malaysian Journal of Microbiology, Vol 14(6) Special Issue 2018, pp. 490-496
DOI: http://dx.doi.org/10.21161/mjm.1461807

Malaysian Journal of Microbiology
Published by Malaysian Society for Microbiology
(In SCOPUS since 2011)

Antibacterial potential of lactic acid bacteria isolated from local pickled *Eleiodoxa conferta* (*kelubi*) against selected foodborne pathogens

Nur Ilida Mohamad[1,2], Musaalbakri Abdul Manan[3] and Norrakiah Abdullah Sani[1*]

[1]Centre for Biotechology and Functional Food, Faculty of Science and Technology, Universiti Kebangsaan Malaysia, 43600 Bangi, Selangor, Malaysia.
[2]Food Science Techology Research Centre, Malaysian Agricultural Research and Development Institute (MARDI), 43400 Serdang, Selangor, Malaysia.
[3]Biotechnology and Nanotechnology Research Centre, Malaysian Agricultural Research and Development Institute (MARDI), 43400 Serdang, Selangor, Malaysia.
Email: norrasani@ukm.edu.my

ABSTRACT

Aims: Pickled fruits are a popular condiment not only in Malaysia but throughout the world. Lactic acid bacteria (LAB) are known to be able to produce several antibacterial compounds during the pickling process. Pickled *Eleiodoxa conferta* or *kelubi* is among the commonly consumed pickled fruits in Malaysia and may be a promising source of LAB. This study was carried out to isolate and identify LAB from local pickled *E. conferta* and to determine their antibacterial activity against foodborne pathogens.
Methodology and results: The isolation of LAB was conducted using standard methods and the isolated colonies were preliminarily identified based on their morphology on De Man, Rogosa and Sharpe (MRS) agar, Gram-positive staining characteristics and their catalase-negative reactions and subsequently chosen for identification with polymerase chain reaction (PCR) amplification of the 16S rRNA gene and sequencing. A total of four LAB isolates were successfully isolated and identified: *Lactobacillus brevis* (LABK1 and LABK2), *L. plantarum* (LABK3) and *Leuconostoc mesenteroides* (LABK4). The antibacterial activity of the identified LAB was determined against 10 commonly known foodborne bacterial pathogens using LAB cell cultures by agar spot test method. The antibacterial activity was also evaluated using the cell free supernatant (CFS) of the LAB through agar well diffusion method. All four LAB cultures were able to demonstrate antibacterial activity and inhibited five Gram-positive and five Gram-negative bacterial pathogens.
Conclusion, significance and impact of study: This study demonstrated that the isolated LAB from *E. conferta* has antibacterial effects against several foodborne pathogens. These LAB strains may have potential as a natural food preservative.

Keywords: Eleiodoxa conferta, antibacterial, lactic acid bacteria, foodborne

INTRODUCTION

Foodborne illnesses which include food poisoning is a significant issue throughout the world. Resistant species of pathogens that causes foodborne illness have emerged due to microbial adaptation towards the changing environment. These environmental changes include changes in food production, preservation and packaging that have altered the microorganisms and caused the emergence of new pathogenic strains that previously may not have caused harm to the food industry (Lü *et al.*, 2014). Due to the existence of resistant species, the food industry is facing difficulties in fighting foodborne pathogens and needs to find ways to eliminate and inhibit these pathogens. Consumers prefer foods that are not only delicious but also safe and have fewer synthetic or chemical additives (Da Silva Sabo *et al.*, 2014). Therefore, the application of natural preservatives may have huge potential in ensuring food safety and at the same time complying with the consumers' needs (Sharif *et al*., 2017).

In relation to this, the lactic acid bacteria (LAB) are known to produce several antibacterial compounds such as organic acids, hydrogen peroxide and bacteriocins that make them a good source of natural preservatives. Other than that, the potential of LAB application as an alternative preservative to the commonly used synthetic or chemical preservatives is strengthened by the fact that it is regarded as safe (GRAS) to consume (Lü *et al*., 2014). To date, research exploring the potential of LAB as an alternative natural preservative is ongoing. A review done by Sharif *et al*. (2017) has mentioned that natural food preservatives are usually derived from plant, animal or microbial origin and these include the LAB and their

***Corresponding author**

ISSN (print): 1823-8262, ISSN (online): 2231-7538

Malays. J. Microbiol. Vol 14(6) Special Issue 2018, pp. 490-496
DOI: http://dx.doi.org/10.21161/mjm.1461807

metabolites. However there still remain gaps in knowledge and application procedures that need to be explored. These natural preservatives can become an alternative to the synthetic, artificial or chemically derived preservatives that can cause harm to human health if consumed excessively (Sharif *et al*., 2017). Sharif *et al.* (2017) also suggests that there are still many natural preservatives that can be explored and studied including potential natural preservatives from new sources. Soltan *et al.* (2017) suggests that other than dairy products, plant-based fermented products such as pickled fruits and vegetables can also be a good source of LAB.

LAB is also an important group of industrial microorganisms that has a long-standing usage in food fermentation as natural colonisers or are intentionally added as starter cultures. In Malaysia, local fermented products such as pickles of various fruits and vegetables have long been in the market. *E. conferta* or *kelubi* in Malay is an under-utilised fruit that belongs to the Arecaceae family (Mokhtar and Abdul Aziz, 2015). It can be eaten fresh or processed into pickles. In Malaysia, the pickled *E. conferta* is commonly consumed together with other types of pickled fruits such as pickled mango (*Mangifera indica*), guava (*Psidium guajava*) and papaya (*Carica papaya*). In recent years, researchers have reported on the LAB species with antibacterial properties isolated from pickled vegetables including pickled radish, carrot, bitter bean, garlic and cabbage (Rahman *et al.*, 2017); and pickled fruits including jackfruit, plum, lemon, olive, apple and dates (Roy and Rai, 2017). *E. conferta* fruit extract however have shown antibacterial effect against several food pathogens at different fruit maturity (Mokhtar and Abdul Aziz, 2015). However, there are no reports on the LAB isolated from pickled *E. conferta* and their antibacterial property against foodborne pathogens. In view of these findings, this study was carried out to isolate and characterise LAB from local pickled *E. conferta* that is commonly consumed in Malaysia and also to identify if these LAB have antibacterial properties against commonly known Gram-positive and Gram-negative foodborne pathogens and therefore enabling it to be utlised as a natural food preservative.

MATERIALS AND METHODS

Sample collection

The pickled *E. conferta* samples were randomly collected from a variety of sources (stalls, wet markets and supermarkets) in the Hulu Langat (Selangor) area. Samples were kept chilled in an ice box with ice replaced during transportation and taken to the laboratory for analysis.

Isolation of lactic acid bacteria (LAB) from local pickled *E. conferta* samples

A 10 g sample was homogenised in a stomacher (Seward Medical, UK) for 30 sec in 90 mL of Ringers solution (Oxoid, UK). The 10 g of sample was obtained using sterile scissors and forceps and weighed using aseptic technique following the standard methodology for preparation of sample homogenate for microbiological analysis (FDA, 2003). The 90 mL Ringers solution was prepared according to manufacturer's instruction.

An amount of 1 mL of the diluent from the prepared homogenate was pipetted into 9 mL sterile Ringers solution for serial decimal dilution. The number of dilutions prepared depended on the microbial density of each sample. Then, 1 mL aliquot of each dilution was transferred to a sterile Petri dish using the pour plate method. The sterile De Man, Rogosa and Sharpe (MRS) agar (Difco, USA) of approximately 15 mL was poured into plates to form a layer, swirled and left to solidify. The MRS agar is a recommended medium for the cultivation of *Lactobacillus* and other lactic acid bacterial species. The solidified plates were inverted and placed in an anaerobic jar containing an anaerobic gas generator sachet (AnaeroGen, Oxoid, UK). The anaerobic gas generating sachet was used to create an anaerobic environment suitable for LAB growth before incubation at 37 ^{o}C ± 2 ^{o}C for 24 to 48 h in an incubator.

Each plate was examined and single colonies that were white or cream in colour and round were counted and recorded as the presumptive number of LAB and later expressed as colony forming unit/ gram of sample (CFU/g). The isolates of each sample were sub-cultured onto fresh MRS agar. The sub-culturing process was continued at least three times and the isolates that maintained their characteristics during each sub-culturing were subjected to Gram staining and catalase reaction test. The isolates were stored at - 20 ^{o}C ± 2 ^{o}C (Sanyo, Japan) in MRS broth (Difco, USA) supplemented with 50% glycerol (Sigma, Germany) until used.

Table 1: Lactic acid bacteria (LAB) isolates from local pickled *E. conferta* (*kelubi*).

Sample	Common name	Source and number of sample from eachsource	Total viable count on MRS agar (CFU/g)	Code of representative LAB isolates
Eleiodoxa conferta	*Kelubi*	Stall (2 samples)	2.0×10^2 and 2.0×10^4	LABK1, LABK2
Eleiodoxa conferta	*Kelubi*	Supermarket (2 samples)	1.0×10^3 and 1.0×10^4	LABK3
Eleiodoxa conferta	*Kelubi*	Wet market (3 samples)	2.0×10^2,1.0×10^3 and 1.0×10^4	LABK4

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

Malays. J. Microbiol. Vol 14(6) Special Issue 2018, pp. 490-496
DOI: http://dx.doi.org/10.21161/mjm.1461807

Morphological and biochemical identification of LAB isolates

The isolates that were viable following sub-culture and met the characteristics of LAB on agar (white colour, round shape) were subjected to Gram staining and catalase reaction test as done by Nurul Huda and Norrakiah (2015) and Nanasombat *et al*. (2012). LAB are Gram-positive bacteria and are catalase-negative.

Molecular identification by amplification and sequencing of 16S rRNA gene of LAB

The Gram-positive and catalase-negative isolates were further identified by amplification and sequencing of 16S rRNA gene of LAB. The genomic DNA of the LAB isolates was extracted using QIAamp® DNA Mini Kit (QIAGEN, USA). The 16S rDNA gene was amplified by polymerase chain reaction (PCR) using primers 27-f : 5'- AGT TTG ATC CTG GCT CAG -3' and 1492-r : 5'- GTT TAC CTT GTT ACG ACT T -3' as mentioned by Tan *et al.* (2017). Both primers were synthesised at First Base Laboratories Sdn. Bhd. PCR was carried out in a 50 µL reaction mixture containing 25 µL *Taq* Mix Red, 2 µL of each primer, 1 µL of extracted DNA and PCR grade H_20. The PCR cycles were 1 cycle of 95 ºC for 15 sec; 40 cycles of 95 ºC for 15 sec, 55 ºC for 15 sec and 72 ºC for 30 sec; and 1 cycle of 72 ºC for 10 min. The PCR products were separated on 1% (w/v) agarose gel (GeneDirex, Taiwan) containing Gel Red (Biotium, USA) using electrophoresis with constant voltage of 80 V for 55 min in 1 × TBE buffer (UltraPure, USA). The sizes of the DNA fragments were estimated using 1000 base pair (bp) DNA ladder. Sequencing of the PCR products were done at First Base Laboratories Sdn. Bhd., Malaysia. Nucleotide sequences were then analysed using the BLAST program (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Table 2: Morphological and biochemical identification of lactic acid bacteria (LAB) isolates from *E. conferta*.

LAB code	Colony morphology	Cell shape	Gram staining	Catalase activity
LABK1	Circular. white colour	Rod	Gram +ve	Catalase-ve
LABK2	Circular, white colour	Rod	Gram +ve	Catalase-ve
LABK3	Circular, white colour	Rod	Gram +ve	Catalse-ve
LABK4	Circular, white colour	Rod	Gram +ve	Catalase-ve

LAB isolates and its culture conditions for antibacterial activity study

The antibacterial activity study was carried out from the stock cultures, where the strains were inoculated in 5 mL of MRS broth, tightly sealed in screw cap tubes and incubated at 37 ºC ± 2 ºC for 24 h. Later the strains were streaked onto MRS agar and placed in an anaerobic jar with AnaeroGen and incubated at 37 ºC ± 2 ºC for 48 h in an incubator. A single colony was cultured in 10 mL MRS broth and incubated for 18-24 h prior to antibacterial study.

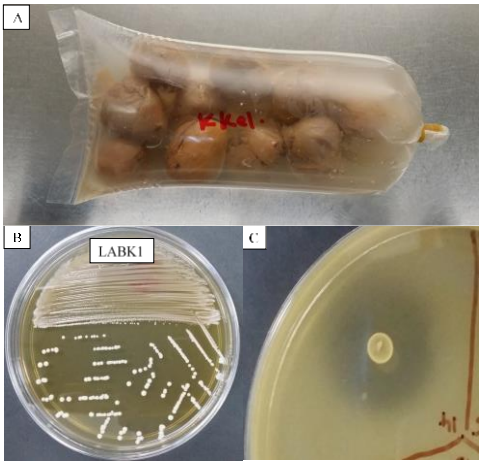

Figure 1: (A) Pickled *E. conferta* samples; (B) Morphology of LAB isolate of *E. conferta* (LABK1) on MRS agar with round shape and white colour colony; (C) Example of inhibition zone created by LABK4 isolated from pickled *E. conferta* using agar spot test method against *Salmonella enterica serovar* Typhimurium ATCC® 14028™

Preparation of pathogenic strains for antibacterial activity evaluation

Five each of Gram-positive and Gram-negative bacterial strains were used for the antibacterial study, consisted of 1) Gram-positive pathogens: *Staphylococcus aureus* ATCC® 25923™, *Enterococcus faecalis* ATCC® 19433™, *Listeria innocua* ATCC® 33090™, *L. monocytogenes* ATCC® 7644™, and *Bacillus cereus* ATCC® 10876™ and 2) Gram-negative pathogens: *Escherichia coli* ATCC® 48888™, *Salmonella enterica* serovar Poona NCTC 4840, *S. enterica* serovar Typhimurium ATCC® 14028™, *Vibrio parahaemolyticus* NCTC 10885 and *Cronobacter muytjensii* ATCC® 51329™.

The strains were previously stored in glycerol (Sigma, Germany) at –20 ºC ± 2 ºC and were revived prior to use by transferring 20 µL of the glycerol stock into 5 mL Tryptic soy broth (TSB, Oxoid) and incubated at 37 ºC ± 2 ºC for 24 h. Later, 1 mL of these cultures were transferred into 10 mL TSB broth and incubated again at 37 ºC ± 2 ºC for 24 h. The bacterial suspension was then grown to a turbidity of 0.5 McFarland standard (~1 × 10^8 colony forming units CFU/ mL) by adding sterile distilled water into the suspension until the desired turbidity was achieved. The turbidity was determined by placing the suspension bottle into a McFarland nephelometer (Becton Dickinson, USA).

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

Malays. J. Microbiol. Vol 14(6) Special Issue 2018, pp. 490-496
DOI: http://dx.doi.org/10.21161/mjm.1461807

Antibacterial activity evaluation of LAB cell culture using agar spot test method

Initially, the antibacterial activity was tested using an agar spot test method. A volume of 5 µL of the overnight culture of all LAB isolates were inoculated as a spot in the middle of MRS agar plate and incubated at 37 ºC ± 2 ºC for 24 h anaerobically. Subsequently, a spot colony that grew in the middle of the plate, was overlaid with pathogenic strains (~1 × 10^8 CFU/ mL, prepared as mentioned in the above section) that had been mixed first with 4 mL of soft Tryptic soy agar (TSA, Oxoid). The overlaid plates were then incubated at 37 ºC ± 2 ºC for 24 h, aerobically. Zones of inhibition around the spots (diameter) were measured in mm and scored accordingly as done by Tejero-Sariñena *et al*. (2012). The LAB strains that were able to inhibit any of the pathogenic strains were observed as a clear zone, indicating the antibacterial potential of the LAB strains. These strains were further analysed using the agar well diffusion method.

Antibacterial activity evaluation of LAB cell free supernatant (CFS) using agar well diffusion method

A 10 mL of overnight cultures of each LAB was centrifuged using refrigerated centrifuge (Eppendorf, Germany) at 10 000 rpm for 10 min at 4 ºC. The pellet was discarded and the CFS was filter-sterilised through a sterile 0.45 µm-pore-size filter (Sartorius Stedim, France). The pH of CFS for each LAB was determined using a pH meter (Eutech, Singapore). The CFS was then divided into four portions and used for antibacterial activity determination of the LAB isolates using well diffusion method following the method described by Yang *et al*. (2012) with some modifications. The first portion was the original CFS which was identified as untreated CFS (pH 4.07 to pH 4.45) and used as a control for this study, while the other portions of the untreated CFS was treated as follows: (i) CFS was adjusted from their initial pH (pH 4.07 to pH 4.45) to pH 6.00 ± 0.20 using sterilised 1 N NaOH (Sigma, Germany) and filtered through sterile 0.45 µm-pore-size filters (Sartorius Stedim, France). This experiment was conducted to rule out antibacterial inhibition resulting from the production of organic acids and identified as neutralised CFS; (ii) CFS was treated with 1 mg/ mL of catalase (Sigma-Aldrich, USA) at 25 ºC ± 2 ºC for 30 min; and (iii) CFS was treated with 1 mg/ mL of proteolytic enzyme, trypsin (Sigma Aldrich, USA) at 37 ºC ± 2 ºC for 2 h This part was conducted to evaluate the possibility of antimicrobial inhibition by the LAB isolates due to hydrogen peroxide and bacteriocin, respectively.

Prior to applying the untreated CFS, neutralised CFS and both catalase and trypsin treated CFS for antibacterial activity using well diffusion method, the Luria Bertani (LB, Oxoid) agar plates surface were inoculated by spreading 1 mL of the pathogenic bacterial culture (~1 × 10^8 CFU/ mL, prepared as stated in the above section) over the entire agar surface using sterile cotton swab. A hole with a diameter of 5 to 6 mm was punched aseptically on each of the plates using the top edge of sterile tips. Then, a volume of 20 µL of the same agar was added to the well in order to seal the base and avoid leakage of the sample added to the well. An amount of 70 µL of the untreated CFS, neutralised CFS and enzyme-treated CFS (catalase and trypsin) was introduced into each well respectively and left to dry before the agar plates were placed in an anaerobic jar with AnaeroGen and incubated at 37 ºC ± 2 ºC for 48 h in an incubator. The inhibition zones (diameter) around the wells filled with each type of CFS were measured in mm and recorded accordingly.

Statistical analysis

Data were analysed using SAS 9.3 statistical software (SAS Institute Inc., USA). A one-way analysis of variance was performed to evaluate significant differences between sample means. The level of significance was set at α = 0.05. All experimental results were expressed as mean values obtained from three replicates (n = 3) unless stated otherwise.

RESULTS AND DISCUSSION

Isolation, morphological and biochemical identification

A total number of seven pickled *E. conferta* samples were collected for LAB isolation analysis as listed in Table 1. All samples were acidic and initially were packed in a pickling medium by the sellers/ suppliers. One of the samples is shown in Figure 1 (A). The viable LAB count found in the samples ranged from 2.0 × 10^2 to 2.0 × 10^4 CFU/ g as shown in Table 1. LAB isolated from traditional pickled fruits and vegetables procured randomly from Kolkata, India was discovered to have low amounts of viable LAB (2.0 × 10^1 CFU/ g) (Roy and Rai, 2017). Even though fermented products were expected to have a high count of LAB, Roy and Rai (2017) suggested that a low count may be due to intentional chemical acidification during early pickling process and not due to fermentation. On the other hand, Rahman *et al*. (2017) acknowledged that pickles contain high sugar and salt and are very acidic but a low number of LAB might be able to withstand the conditions.

Table 3: Lactic acid bacteria (LAB) identification by sequencing of 16S rRNA gene.

LAB Code	Best match in BLAST analysis	Accession number of the best match in BLAST analysis	Identity score (% similarity)
LABK1	*L. brevis*	NR_116238.1	97%
LABK2	*L. brevis*	NR_044704.2	98%
LABK3	*L. plantarum*	NR_042394.1	95%
LABK4	*Leuconostoc mesenteroides*	NR_074957.1	99%

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

Malays. J. Microbiol. Vol 14(6) Special Issue 2018, pp. 490-496
DOI: http://dx.doi.org/10.21161/mjm.1461807

Table 4: Antibacterial activity of isolated lactic acid bacteria (LAB) cells against Gram-positive bacterial pathogens by agar spot test.

LAB code	Diameter of inhibition zone (mm)				
	SA	EF	LI	LM	BC
LABK1	+++	++	+++	+++	+
LABK2	+++	++	+++	+++	+
LABK3	+++	++	+++	+++	++
LABK4	+++	++	+	+++	+

The different scores reflect the different degree of inhibition zone expressed in mm as the mean of three replicates, as below:
(-) no inhibition
(+) zone of inhibition between 5 and 10 mm
(++) zone of inhibition between 11 and 17 mm
(+++) zone of inhibition >17 mm
Gram-positive bacterial pathogens: SA (*Staphylococcus aureus* ATCC® 25923™), EF (*Enterococcus faecalis* ATCC® 19433™), LI (*Listeria innocua* ATCC® 33090™), LM (*Listeria monocytogenes* ATCC® 7644™), BC (*Bacillus cereus* ATCC® 10876™)

The four LAB isolates (LAB1 to LAB4) were selected after they survived the sub-culturing process and met the characteristics of LAB on MRS agar which were round shaped and white in colour (Table 2 and Figure 1B) as described by Astuti (2016) on shape and colour of LAB colonies on MRS agar. The isolates were Gram-positive bacteria in rod/ bacilli form as observed during Gram staining. All isolates were catalase negative. The isolates were then subjected to phenotypic identification using molecular methods and were identified as *Lactobacillus brevis* (LABK1 and LABK2), *L. plantarum* (LABK3) and *Leuconostoc mesenteroides* (LABK4) from BLAST results as shown in Table 3.

Table 5: Antibacterial activity of isolated lactic acid bacteria (LAB) cells against Gram-negative bacterial pathogens by agar spot test.

LAB code	Diameter of inhibition zone (mm)				
	EC	SP	ST	VP	CM
LABK1	++	+++	+++	+++	++
LABK2	++	+++	+++	+++	++
LABK3	+++	+++	+++	+++	++
LABK4	+++	+++	+++	+++	++

The different scores reflect the different degree of inhibition zone expressed in mm as the mean of three replicates (±SD), as below:
(-) no inhibition
(+) zone of inhibition between 5 and 10 mm
(++) zone of inhibition between 11 and 17 mm
(+++) zone of inhibition >17 mm
Gram-negative bacterial pathogens: EC (*Escherichia coli* ATCC® 48888™), SP (*Salmonella enterica serovar* Poona NCTC 4840), ST (*Salmonella enterica serovar* Typhimurium ATCC® 14028™), VP (*Vibrio parahaemolyticus* NCTC 10885), CM (*Cronobacter muytjensii* ATCC® 51329™)

Table 6: Antibacterial activity of cell free supernatant (CFS), neutralised CFS and enzyme (catalase and trypsin) treated CFS of lactic acid bacteria (LAB) isolates against Gram-positive bacteria.

LAB code	Type of CFS	Inhibition zone (mm)				
		SA	EF	LI	LM	BC
LABK1	Untreated	11.33±0.58^{a}	9.00±0.00^{a}	9.00±0.00^{a}	10.33±0.58^{a}	9.00±0.00^{a}
	Neutralised	0.00±0.00^{b}	0.00±0.00^{b}	0.00±0.00^{b}	0.00±0.00^{b}	0.00±0.00^{b}
	Catalase	11.67±1.53^{a}	9.67±0.00^{a}	10.33±0.58^{a}	10.67±0.58^{a}	10.00±0.00^{a}
	Trypsin	12.00±1.73^{a}	9.33±0.58^{a}	9.00±0.00^{a}	9.67±0.00^{a}	8.67±0.58^{a}
LABK2	Untreated	9.67±0.58^{a}	9.33±0.58^{a}	9.00±0.00^{a}	10.67±0.58^{a}	9.33±0.58^{a}
	Neutralised	0.00±0.00^{b}	0.00±0.00^{b}	0.00±0.00^{b}	0.00±0.00^{b}	0.00±0.00^{b}
	Catalase	9.00±0.00^{a}	9.00±0.00^{a}	9.00±0.00^{a}	9.00±0.00^{a}	9.00±0.00^{a}
	Trypsin	9.33±0.58^{a}	10.00±0.00^{a}	10.33±0.58^{a}	9.67±0.00^{a}	10.00±0.00^{a}
LABK3	Untreated	15.33±0.58^{a}	13.67±1.15^{a}	17.00±1.00^{a}	15.33±0.58^{a}	11.33±1.15^{a}
	Neutralised	0.00±0.00^{b}	0.00±0.00^{b}	0.00±0.00^{b}	0.00±0.00^{b}	0.00±0.00^{b}
	Catalase	14.67±0.58^{a}	12.00±0.00^{a}	16.67±1.15^{a}	15.00±0.00^{a}	12.00±1.7^{a}
	Trypsin	15.00±0.00^{a}	13.00±1.00^{a}	17.00±0.00^{a}	15.00±0.00^{a}	12.67±1.15^{a}
LABK4	Untreated	11.00±0.00^{a}	9.00±0.00^{a}	9.00±0.00^{b}	10.00±0.00^{a}	9.00±0.00^{a}
	Neutralised	0.00±0.00^{b}	0.00±0.00^{b}	0.00±0.00^{b}	0.00±0.00^{b}	0.00±0.00^{b}
	Catalase	10.00±0.00^{a}	9.00±0.00^{a}	9.00±0.00^{a}	10.00±0.00^{a}	9.00±0.00^{a}
	Trypsin	10.33±0.58^{a}	9.00±0.00^{a}	8.67±0.58^{a}	10.33±0.58^{a}	10.00±0.00^{a}

The results are the inhibition zones expressed in mm as the mean of three replicates, ± standard deviations
$^{a-b}$Different superscripted lowercase letters indicate that the zones in the same column, within the same LAB code are significantly different
Gram-positive bacterial pathogens: SA (*Staphylococcus aureus* ATCC® 25923™), EF (*Enterococcus faecalis* ATCC® 19433™), LI (*Listeria innocua* ATCC® 33090™), LM (*Listeria monocytogenes* ATCC® 7644™), BC (*Bacillus cereus* ATCC® 10876™)

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

Malays. J. Microbiol. Vol 14(6) Special Issue 2018, pp. 490-496
DOI: http://dx.doi.org/10.21161/mjm.1461807

Table 7: Antibacterial activity of cell free supernatant (CFS), neutralised CFS and enzyme (catalase and trypsin) treated CFS of lactic acid bacteria (LAB) isolates against Gram-negative bacteria.

LAB code	Type of CFS	Inhibition zone (mm)				
		SA	EF	LI	LM	BC
LABK1	Untreated	11.33±1.15[a]	9.67±0.58[a]	11.67±2.89[a]	10.67±1.15[a]	10.33±0.58[a]
	Neutralised	0.00±0.00[b]	0.00±0.00[b]	0.00±0.00[b]	0.00±0.00[b]	0.00±0.00[b]
	Catalase	11.00±1.00[a]	9.00±0.00[a]	11.00±1.00[a]	10.00±0.00[a]	10.00±0.00[a]
	Trypsin	11.00±0.00[a]	9.00±0.00[a]	10.00±1.00[a]	11.00±0.00[a]	10.00±0.00[a]
LABK2	Untreated	12.33±0.58[a]	10.00±1.00[a]	10.33±0.58[a]	10.33±0.58[a]	10.00±0.00[a]
	Neutralised	0.00±0.00[b]	0.00±0.00[b]	0.00±0.00[b]	0.00±0.00[b]	0.00±0.00[b]
	Catalase	13.00±1.00[a]	10.00±0.00[a]	9.67±0.58[a]	10.00±0.00[a]	9.67±0.58[a]
	Trypsin	12.00±0.00[a]	10.00±0.00[a]	10.00±0.00[a]	11.00±0.00[a]	9.00±0.00[a]
LABK3	Untreated	18.00±1.00[a]	14.67±0.58[a]	15.67±1.15[a]	11.67±0.58[a]	10.33±0.58[a]
	Neutralised	0.00±0.00[b]	0.00±0.00[b]	0.00±0.00[b]	0.00±0.00[b]	0.00±0.00[b]
	Catalase	17.00±1.00[a]	14.00±0.00[a]	15.00±0.00[a]	11.33±1.15[a]	9.67±0.58[a]
	Trypsin	16.67±1.15[a]	15.00±0.00[a]	15.00±0.00[a]	12.00±0.00[a]	11.00±0.00[a]
LABK4	Untreated	15.00±0.00[a]	11.67±0.58[a]	13.00±1.73[a]	10.00±0.00[a]	10.33±0.58[a]
	Neutralised	0.00±0.00[b]	0.00±0.00[b]	0.00±0.00[b]	0.00±0.00[b]	0.00±0.00[b]
	Catalase	14.33±0.58[a]	11.00±0.00[a]	13.67±0.58[a]	9.67±0.58[a]	10.00±0.00[a]
	Trypsin	15.00±0.00[a]	9.00±0.00[a]	13.00±0.00[a]	10.00±0.00[a]	10.00±0.00[a]

The results are the inhibition zones expressed in mm as the mean of three replicates, ± standard deviations
[a-b]Different superscripted lowercase letters indicate that the zones in the same column, within the same LAB code are significantly different
Gram-negative bacterial pathogens: EC (*Escherichia coli* ATCC® 48888™), SP (*Salmonella enterica serovar* Poona NCTC 4840), ST (*Salmonella enterica serovar* Typhimurium ATCC® 14028™), VP (*Vibrio parahaemolyticus* NCTC 10885), CM (*Cronobacter muytjensii* ATCC® 51329™)

Antibacterial activity of isolated and identified LAB cell culture using agar spot test method

In this study, all four LAB isolates were tested for their ability to inhibit 10 bacterial pathogens in MRS agar. Table 4 and Table 5 summarise the findings and showed that the four LAB isolates were able to inhibit all five Gram-positive bacterial pathogens which were *S. aureus*, *E. faecalis*, *L. innocua*, *L. monocytogenes* and *B. cereus* and also all five Gram-negative bacterial pathogens which were *E. coli*, *S.* Poona, *S.* Typhimurium, *V. parahaemolyticus* and *C. muytjensii.* However, the sizes of inhibition zones were varied depending on LAB and pathogen strains. Figure 1C shows an example of inhibition of *S.* Typhimurium by the LABK4 of *E. conferta*. All LAB isolates were able to inhibit the test strains of pathogenic organisms from the range of 5 mm to more than 17 mm indicating their potential as antibacterial agent (Quyen and Tu, 2016).

Antibacterial activity evaluation of CFS using agar well diffusion method

The LAB isolates that showed inhibitory activity based on preliminary evaluation using agar spot test were further tested for antibacterial activity using the agar well diffusion method. The antibacterial activity of LAB may be due to the production of antibacterial compounds produced during growth (Quyen *et al.*, 2016) and excreted extracellularly (Hor and Liong, 2014). The LAB secreted various inhibitory compounds that are responsible for its antibacterial activity such as organic acids, hydrogen peroxide and bacteriocin (Hor and Liong, 2014). All untreated CFS of four LAB isolates showed antibacterial effect against selected pathogens and the sizes of the inhibition zones varied as summarised in Table 6 and Table 7. However, the neutralised CFS of all four isolates showed no inhibition against all tested bacterial pathogens. This indicates that the acidic condition of the CFS due to the presence of organic acid might have contributed to the antibacterial activity of the isolates as mentioned by Hor and Liong (2014). Enzyme treatment using catalase and trypsin have no significant effect (p>0.05) on the antimicrobial activity of all LAB isolates against all pathogens if compared to the untreated CFS. The summary of the results are as expressed in Table 6 and Table 7. Catalase treatment was performed to rule out antibacterial activity by hydrogen peroxide from CFS. The catalase enzyme breaks down hydrogen peroxide into water and oxygen therefore reducing the hydrogen peroxide content in the CFS (Kormin *et al*., 2001). Therefore, the inhibitory effect of CFS against pathogen is not attributed to hydrogen peroxide. Other than organic acids and hydrogen peroxide, proteinaceous compounds such as bacteriocin might possibly be present in a very low amount in the CFS as antibacterial compounds. Proteolytic enzymes including trypsin will inactivate the proteinaceous compound when added to the CFS (Ghanbari *et al*., 2013). In this study, the ability of the trypsin-treated CFS to maintain their inhibitory effect when compared to untreated CFS shows that the proteinaceous compound might not be responsible for the antibacterial activity of the CFS.

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

Malays. J. Microbiol. Vol 14(6) Special Issue 2018, pp. 490-496
DOI: http://dx.doi.org/10.21161/mjm.1461807

CONCLUSION

Four lactic acid bacteria (LAB) identified as *Lactobacillus brevis* (LABK1 and LABK2), *L. plantarum* (LABK3) and *Leuconostoc mesenteroides* (LABK4) isolated from pickled. *E. conferta* (*kelubi*) possessed antibacterial activity against a total of 10 Gram-positive and Gram-negative foodborne pathogens. The antibacterial activity of the LAB was probably due to organic acid.

ACKNOWLEDGEMENTS

The authors would like to acknowledge that this work forms part of a research project funded by FRGS/1/2017/WAB01/UKM/02/4, FRGS/1/2014/STWN03/UKM/02/1 and DIP-2014-007 funds.

REFERENCES

FDA. (2003). Food sampling and preparation of sample homogenate. *In:* Bacteriological Analytical Manual, Edition 8, Revision A. Andrews, W. and Hammack, T. (eds.). AOAC International, Washington D. C., USA. **pp. 1-19.**

Astuti, I. (2016). Isolation, characterization and identification lactic acid bacteria from chicken waste faeces that potential as probiotics. *International Journal of Scientific and Research Publication* **6(5), 180-191.**

Ghanbari, M., Jami, M., Kneifel, W. and Domig, K. J. (2013). Antimicrobial activity and partial characterization of bacteriocins produced by lactobacilli isolated from Sturgeon fish. *Food Control* **32(2), 379-385.**

Hor, Y. Y. and Liong, M. T. (2014). Use of extracellular extracts of lactic acid bacteria and bifidobacteria for the inhibition of dermatological pathogen *Staphylococcus aureus. Dermatologica Sinica* **32(3), 141-147.**

Kormin, S., Rusul, G., Radu, S. and Ling, F. H. (2001). Bacteriocin-producing lactic acid bacteria isolated from traditional fermented food. *The Malaysian Journal of Medical Science* **8(1), 63-68.**

Lü, X., Yi, L., Dang, J., Dang, Y. and Liu, B. (2014). Purification of novel bacteriocin produced by *Lactobacillus coryniformis* MXJ 32 for inhibiting bacterial foodborne pathogens including antibiotic-resistant microorganisms. *Food Control* **46, 264-271.**

Mokhtar, S. I. and Abd Aziz, N. A. (2015). Organic acid content and antimicrobial properties of *Eleiodoxa conferta* extracts at different maturity stages. *Journal of Tropical Resource and Sustainable Science* **3(100), 72-76.**

Nanasombat, S., Phunpruch, S. and Jaichalad, T. (2012). Screening and identification of lactic acid bacteria from raw seafoods and Thai fermented seafood products for their potential use as starter cultures. *Songklanakarin Journal of Science and Technology* **34(3), 255-262.**

Nurul Huda, H. and Norrakiah, A. S. (2015). Isolation of lactic acid bacteria with potential protective culture characteristics from fruits. *AIP Conference Proceedings* **050042, 1-6.**

Quyen, T. and Tu, T. M. (2016). Selection of lactic acid bacteria producing bacteriocin. *Journal of Vietnamese Environment* **8(5), 271-276.**

Rahman, S. A., Kahar, A. A., Mansor, A., Murni, D. L., Hussin, A., Sharifudin, S. A., Geok, T., Yuhasliza, N., Rashid, A., Othaman, M. A. and Long, K. (2017). Potential indigenous microbe from local fermented vegetables with antimicrobial activity. *Galeri Warisan Sains (GWS)* **1(1), 1-3.**

Roy, A. and Rai, C. (2017). Isolation and characterization of lactic acid bacteria with probiotic potential from pickles. *Bioscience Discovery* **8(4), 866-875.**

Da Silva Sabo, S., Vitolo, M., González, J. M. D. and de Souza Oliveira, R. P. (2014). Overview of *Lactobacillus plantarum* as a promising bacteriocin producer among lactic acid bacteria. *Food Research International* **64, 527-536.**

Sharif, Z., Mustapha, F., Jai, J., Mohd Yusof, N. and Zaki, N. (2017). Review on methods for preservation and natural preservatives for extending the food longevity. *Chemical Engineering Research Bulletin* **19, 145-153.**

Soltan D. M. M., Zamaniahari, S., Davoodabadi, A., Hosseini, M. and Rajabi, Z. (2017). Identification and characterization of probiotic lactic acid bacteria isolated from traditional persian pickled vegetables. *GMS Hygiene and Infection Control* **12, 1-7.**

Tan, W. C., Lim, S. J. and Wan Mustapha, W. A. (2017). Pencirian bakteria asid laktik dan sebatian aroma ikan pekasam. *Sains Malaysiana* **46(3), 439-448.**

Tejero-Sariñena, S., Barlow, J., Costabile, A., Gibson, G. R. and Rowland, I. (2012). *In vitro* evaluation of the antimicrobial activity of a range of probiotics against pathogens: Evidence for the effects of organic acids. *Anaerobe* **18(5), 530-538.**

Yang, E., Fan, L., Jiang, Y., Doucette, C. and Fillmore, S. (2012). Antimicrobial activity of bacteriocin-producing lactic acid bacteria isolated from cheeses and yogurts. *Applied and Industrial Microbiology Express* **2(48), 1-12.**

 ISSN (print): 1823-8262, ISSN (online): 2231-7538