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ANTIBACTERIAL ACTIVITY AND ORGANIC ACIDS FORMATION BY *LACTOBACILLUS* SP. ORIGINATED FROM PICKLED GUAVA AND PAPAYA

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Abstract: Lactic acid bacteria (LAB) produce several antibacterial compounds, including organic acids that inhibit many types of pathogenic bacteria. The antibacterial activity of LAB with the ability to inhibit growth of pathogenic bacteria associated with foodborne illness is seen as a natural way to improve food safety. This study was carried out to isolate and identify LAB from local pickled guava (*Psidium guajava*) and papaya (*Carica papaya*) and to evaluate their antibacterial activity against selected foodborne pathogens. Standard method was used for the isolation of LAB, while identification was done based on their morphological characteristics, biochemical reaction and polymerase chain reaction (PCR) amplification of 16S rRNA gene and sequencing. This study evaluated the ability of cell free supernatant (CFS) of the identified LAB to inhibit the growth of selected Gram-positive and Gram-negative foodborne pathogens through microtiter plate method. Determination of the organic acids formation in the CFS that are responsible for the antibacterial activity of the LAB was also conducted using high-performance liquid chromatography (HPLC). The results showed that three LAB from the genus *Lactobacillus* have been successfully isolated and identified as *Lactobacillus plantarum* (LABP), *Lactobacillus reuteri* (LABR) and *Lactobacillus paracasei* (LABC). All three *Lactobacillus* sp. were able to demonstrate antibacterial activity against foodborne bacterial pathogens used in this study. The results also suggested that the antibacterial activity of CFS of all three *Lactobacillus* sp. was due to organic acids production.

Keywords: Lactic acid bacteria, cell free supernatant, antibacterial, pickled fruits, foodborne pathogen.

Introduction

Foodborne diseases cause by pathogenic bacteria are a major public health problem and remain a relevant issue throughout the world. Millions of people fall ill every year resulting from eating unsafe food contaminated with pathogenic bacteria, such as *Escherichia coli* and *Salmonella* sp. Application of antibacterial agent in the form of natural food preservatives is seen as having a huge potential to prevent or control the growth of pathogenic bacteria in the food industry (Thielmann, 2017). The application of antibacterial agents from natural sources will provide an alternative to synthetic chemical antibacterial agents widely used in industry that has been associated with long-term detrimental effects towards human health (Nuryana, 2019). LABs are generally recognised as safe (GRAS) by the United States Food and Drug Administration (USFDA), indicating it as safe for human consumption (George *et al*., 2018). Other than that, 50 of the recognised LAB members also have the qualified presumption of safety (QPS) status granted by the European Food Safety Agency where most of the LAB are from the *Lactobacillus* sp. (Ricci *et al*., 2017).

LAB are widely spread and can be found in many environments such as soil, plants, raw food, fermented food and the mucosal surfaces of human and animals. The various pressures and conditions in each environment are key factors for the LAB genomic diversity and variation (McAuliffe, 2018). Thus, isolation work of LAB from various and novel sources is an important

part in LAB research and must be done based on their intrinsic characteristics, intended use and future application. In recent years, researchers have reported on the LAB species with antibacterial property isolated from pickled vegetables and fruits. However, it was found that there was more LAB isolation work performed on pickled vegetables compared to pickled fruits, including among others, chili (Shahidah *et al*., 2016), cabbage, bitter bean, garlic and radish (Sukirah *et al*., 2017). LAB isolation studies on pickled fruits included jackfruit, plum, lemon, olive, apple and dates (Roy & Rai, 2017). Thus, this study was carried out to isolate and identify LAB from local pickled guava and papaya and also to evaluate their antibacterial activity using cell free supernatant (CFS) against commonly known foodborne pathogens.

Materials and Methods

Isolation of Lactic Acid Bacteria (LAB) from Local Pickled Guava and Papaya Samples

Pickle samples were collected randomly from a variety of sources, including supermarkets and wet markets in Hulu Langat, Selangor, Malaysia. Samples then were taken to laboratory for analysis. 10 grams of each sample were obtained using aseptic technique and was homogenised in a stomacher (Seward Medical, UK) for 30 seconds in 90 mL of Ringers solution (Oxoid, UK). Serial dilution was performed using 1 mL of the prepared homogenate into 9 mL sterile Ringers solution and three consecutive dilutions that could provide single colony growth were chosen. An amount of 1 mL aliquots of each dilution was transferred to a Petri dish using the pour plate method. The sterile warm (45℃ ± 2℃) molten deMan, Rogosa and Sharpe agar (MRS, Difco, USA) of approximately 15 mL was poured into plates to form a layer, swirled and left to solidify. The solidified plates were incubated anaerobically at 37℃ ± 2℃ for 24 to 48 hours.

After incubation period, each plate was examined and those containing single, white or cream round shape colonies were selected for sub-culturing onto fresh MRS agar following the method done by Kam *et al*. (2011). The sub-culturing process was done three times and the isolates that maintained their characteristics during each sub-culture were grown in MRS broth (Difco, USA) and supplemented with 20% glycerol (Sigma, USA) before being stored at -20℃ ± 2℃ until being used for further analysis and study.

Identification of LAB Isolates Through Biochemical and Molecular Methods

Identification of the isolated LAB was preliminary done using biochemical identification involving Gram staining and catalase reaction tests. The Gram staining was conducted following the standard protocol for Gram staining while catalase reaction was performed on sterile microscope glass slide using 3% hydrogen peroxide (Sigma, USA) according to Astuti (2016). The Gram staining result was recorded as to whether the isolates were of Gram-positive or Gram-negative bacteria, while the catalase reaction was recorded as either positive or negative of catalase activity.

The isolates having Gram-positive and catalase negative reaction were further identified using molecular method through amplification and sequencing of 16S rRNA gene following the method used by Nur ilida *et al*. (2018). The set of primers used were 27-f : 5'- AGT TTG ATC CTG GCT CAG -3' and 1492-r : 5'- GTT TAC CTT GTT ACG ACT T-3'. The QIAamp® DNA Mini Kit (QIAGEN, USA) was used to extract genomic DNA of each isolate and the 16S rRNA gene was amplified by polymerase chain reaction (PCR) using a thermal cycler in a total volume of 50 µL under the following conditions: 1 cycle of 95℃ for 15 seconds; 40 cycles of 95℃ for 15 seconds, 55℃ for 15 seconds and 72℃ for 30 seconds; and 1 cycle of 72℃ for 10 minutes. The PCR products were then separated on 1% (w/v) agarose gel (GeneDirex, Taiwan) containing GelRed (Biotium, USA) using electrophoresis with constant voltage of 80 V for 55 minutes in 1 x TBE buffer (UltraPure, USA). The sizes of DNA fragments were estimated using 1000 base pairs (bp) DNA ladder. The PCR products were submitted to First Base Laboratories Sdn Bhd

for sequencing and the nucleotide sequences obtained were later analysed using the BLAST program available online at *https://blast.ncbi.nlm.nih.gov/Blast.cgi*.

Preparation of LAB Isolates and Pathogenic Strains for Antibacterial Activity Study

The identified LAB isolates then were subjected to antibacterial activity study and the preparation of the LAB culture was done according to Arena *et al*. (2016). All of the LAB isolates which were previously maintained at -20℃ ± 2℃ in MRS broth supplemented with 20% glycerol a stock were taken. An amount of 0.1 mL of the LAB glycerol stock were inoculated in 5 mL MRS broth, tightly screwed cap and incubated at 37℃ ± 2℃ for 24 hours. Later, another 1 mL was taken from the overnight culture and transferred into 10 mL MRS broth and incubated for 24 hours prior to antibacterial study.

The pathogenic strains were also previously stored in glycerol at -20℃ ± 2℃ and were revived prior to use by transferring 20 µL of the glycerol stock into 5 mL Tryptic soy broth (TSB, Oxoid, UK) and incubated at 37℃ ± 2℃ for 24 hours. Later, 1 mL of these cultures were transferred into 10 mL TSB broth and again incubated at 37℃ ± 2℃ for 24 hours. The bacterial suspension was then prepared to a turbidity of 0.5 McFarland standard (~1 x10^8 colony forming units CFU mL^{-1}) by adding the sterile distilled water into the suspension until the desired turbidity was achieved. The desired turbidity was determined by using McFarland nephelometer (Becton Dickinson, USA). The pathogenic bacteria used were Gram-positive *Listeria monocytogenes* ATCC® 7644™ and two Gram-negative organisms, *E. coli* ATCC® 48888™ and *Salmonella enterica* serovar Typhimurium ATCC® 14028™.

Antibacterial Activity Study of LAB Cell Free Supernatant (CFS) Using Microtiter Plate Method

The antibacterial activity of the LAB CFS was conducted following the method used by Hor & Liong (2014). A 10 mL of overnight culture of each LAB was centrifuged at 10,000 rpm for 10 minutes at 4℃. The pellet was discarded and the CFS was filter-sterilized through a sterile 0.45 µm pore size filter (Sartorius Stedim, France). The pH of CFS for each of the LAB was evaluated using a pH meter (Eutech, Singapore). The CFS was then divided into four portions prior to antibacterial activity determination. The first portion (i) was the original CFS identified as untreated CFS (pH 4.07 to pH 4.45). The second and following portions of the CFS were treated as follows: (ii) CFS was adjusted from their initial pH (pH 4.07 to pH 4.45) to pH 6.00 ± 0.20 using sterilized 1 N NaOH and filtered through sterile 0.45 µm pore size filter (Sartorius Stedim, France); (iii) CFS was treated with 1 mg mL^{-1} of catalase (Sigma-Aldrich, USA) at 25℃ ± 2℃ for 30 minutes and (iv) 1 mg mL^{-1} of proteolytic enzyme, trypsin (Sigma-Aldrich, USA) at 37℃ ± 2℃ for 2 hours. An amount of 100 µl of the untreated CFS, neutralised CFS and enzymes treated CFS (catalase and trypsin) was pipetted into each well of the microtiter plate. Later, 100 µl of prepared pathogen suspension was pipetted and mixed with each of the different CFS in the well. Another well for control was used, where 100 µl of the pathogen suspension was mixed with 100 µl of MRS broth to replace untreated CFS and treated CFS. This experiment was performed using bacterial strains as mentioned in the above section. All microtiter plates were incubated at 37℃ ± 2℃ for 48 hours in an incubator. Bacterial growth of the pathogen after incubation was recorded as optical density (OD) reading and the reading was taken using VersaMax™ ELISA Microplate Reader (Molecular Devices, USA). The percentage of growth inhibition was calculated using the below equation:

$$\text{OD value (control) - OD value (sample)/OD value (control) x 100}$$

The value of OD for control was taken from the well containing pathogen with MRS broth while the OD for sample was taken from the well with pathogen in untreated CFS or treated CFS.

Statistical Analysis

Data were analyzed 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 $\alpha = 0.05$. All experimental results were expressed as mean values obtained from three replicates ($n = 3$) unless stated otherwise.

Results and Discussion

Biochemical and Molecular Identification of Isolated LAB

The colony morphology on MRS agar for all isolates (coded as LABP, LABR and LABC) were round in shape and white in color and met the LAB characteristic on MRS agar as described by Astuti (2016). The isolates were all Gram-positive bacteria in rod form, as observed during Gram staining, and they did not produce bubbles in the catalase reaction test, thus identified as catalase negative. Both tests (the Gram staining and catalase reaction) are the most common test used for preliminary identification of LAB based on biochemical reactions. The Gram staining is to identify bacteria based on cell wall characteristics and the LAB are known as Gram-positive bacteria that have a peptidoglycan-rich cell wall. The LAB are also generally known as catalase negative where they produce small amount of catalase enzyme thus are not able to break hydrogen peroxide into water and oxygen (Astuti, 2016).

The isolates then were identified as *Lactobacillus plantarum* (LABP), *Lactobacillus reuteri* (LABR) and *Lactobacillus paracasei* (LABC) through PCR amplification of 16S rRNA gene and sequencing. The sequencing results were compared for similarity with reference species of bacteria contained in genomic database bank using BLAST algorithm and the identification results are as shown in Table 1.

Lactobacillus sp. was a common LAB species successfully isolated from pickled fruits and vegetables as also discovered by other researchers. Kumari *et al*. (2018) isolated seven isolates of LAB from pickled wild Himalayan fig. Isolation of LAB from fermented vegetables done by Kazemipoor *et al*. (2012) have identified four types of *Lactobacillus* sp. from their samples which were *L. animalis*, *L. rhamnosus*, *L. fermentum* and *L. reuteri*. The species *L. reuteri* was also isolated in this study from the pickled papaya. Other than *Lactobacillus* sp., other researchers have also isolated other types of LAB genus from their pickled vegetables and fruits samples such as *Pediococcus* sp. (Roy & Rai, 2017) and *Leuconostoc* sp. (Nur ilida *et al*., 2018).

Table 1: LAB isolates identification by sequencing of 16S rRNA gene

Sample	Code of LAB isolates	Best match in BLAST analysis	Accession number of the best match in BLAST analysis	Identity score (% similarity)
Pickled guava	LABP	*Lactobacillus plantarum*	NR_104573.1	95%
Pickled papaya	LABR	*L. reuteri*	NR_113820.1	97%
Pickled papaya	LABC	*L. paracasei*	NR_117987.1	97%

Antibacterial Activity of Identified LAB Cell Free Supernatant (CFS)

The antibacterial study has shown that the untreated CFS of LABP, LABR and LABC having the ability to inhibit the growth of pathogenic species used in this study which were *L. monocytogenes* ATCC® 7644™, *E. coli* ATCC® 48888™ and *S. enterica* serovar Typhimurium ATCC® 14028™ (Table 2). The results also showed that the inhibition was at different range of percentage depending on both CFS LAB isolates (LABP, LABR or LABC) and pathogen strains used.

The LAB produced various inhibitory compounds that are responsible for its antibacterial activity usually the organic acids, hydrogen peroxide, bacteriocin and these compounds were produced during their growth and excreted extracellularly (Ozcelik *et al*., 2016). Thus, the antibacterial activity evaluation in this study was designed to also able to determine the inhibitory compound that might contribute most to the antibacterial activity of the LAB other than basic screening of their CFS antibacterial performance against pathogens used. For that purpose, the antibacterial activity of LABP, LABR and LABC was evaluated using a few different portions of CFS, namely untreated CFS (original prepared CFS without any treatment and maintain their original condition), neutralized CFS, catalase treated CFS and trypsin treated CFS. It was found that the neutralization of the CFS of all LAB isolates showed very low growth inhibition if compared to the growth percentage caused by the untreated CFS and also catalase and trypsin treated CFS (Figure 1, Figure 2 and Figure 3).

The results indicate that untreated CFS with acidic condition due to the presence of organic acid might have contributed to the antibacterial

Table 2: Antibacterial ability of untreated CFS of LABP, LABR and LABC against selected pathogens

Code of LAB Isolates	**Pathogen Growth Inhibition (%)**		
	***Listeria monocytogenes* ATCC® 7644™**	***Escherichia coli* ATCC® 48888™**	***Salmonella enterica* Serovar Typhimurium ATCC® 14028™**
LABP	75.63±0.44	89.68±2.32	89.43±1.75
LABR	67.32± 2.54	86.67±0.19	80.46±0.24
LABC	76.30± 2.71	85.31±0.65	85.08±1.26

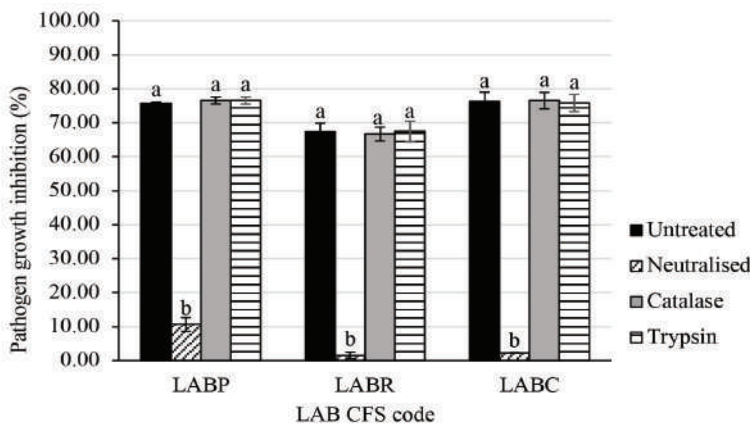

Figure 1: Antibacterial effect of untreated CFS, neutralized CFS, catalase and trypsin treated CFS on the pathogen growth inhibition of *Listeria monocytogenes* ATCC® 7644™. Error bars represent standard error of means (n=3). [a-b]Different lowercase letters indicate that the percentage within the same LAB CFS code are significantly different ($p<0.05$)

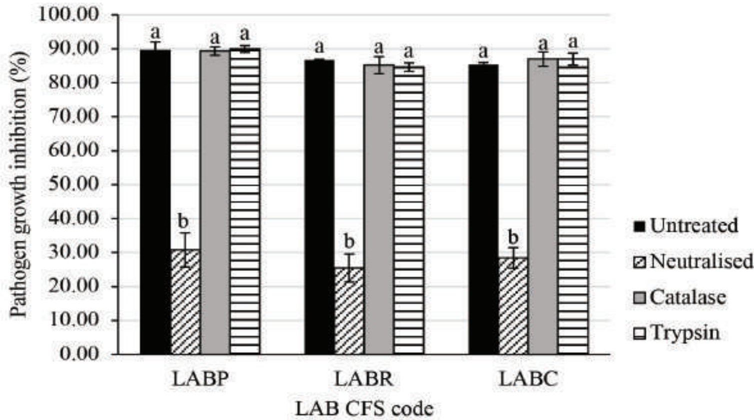

Figure 2: Antibacterial effect of untreated CFS, neutralized CFS, catalase and trypsin treated CFS on the pathogen growth inhibition of *Escherichia coli* ATCC® 48888™. Error bars represent standard error of means (n=3). [a-b]Different lowercase letters indicate that the percentage within the same LAB CFS code are significantly different ($p<0.05$)

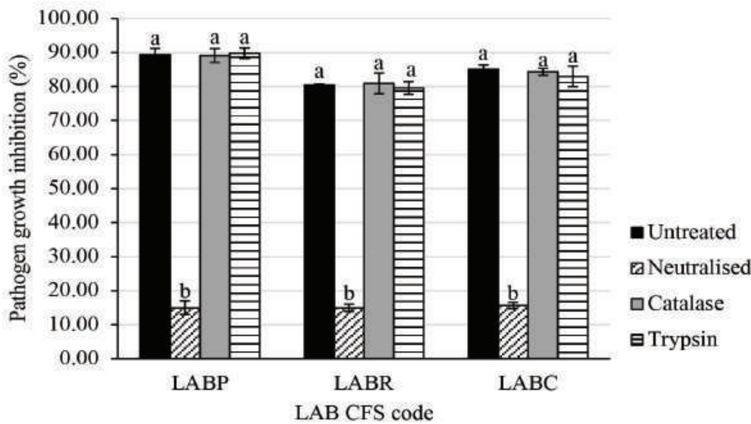

Figure 3: Antibacterial effect of untreated CFS, neutralized CFS, catalase and trypsin treated CFS on the pathogen growth inhibition of *Salmonella enterica* serovar Typhimurium ATCC® 14028™. Error bars represent standard error of means (n=3). [a-b]Different lowercase letters indicate that the percentage within the same LAB CFS code are significantly different ($p<0.05$)

activity of the isolates as also mentioned by Hor & Liong (2014). The LAB produced organic acids naturally as a major fermentation product of their metabolite pathways (Nuryana, 2019). Enzyme treatment on CFS using catalase and trypsin have no significant effect to the antibacterial activity of all LAB isolates against all pathogens if compared to untreated CFS. This was witnessed by the growth inhibition percentage of all pathogens were almost similar when in contact with untreated CFS and also with catalase and trypsin treated CFS. The catalase treatment was performed to rule out the antibacterial activity of CFS was due to hydrogen peroxide. The catalase enzyme will break hydrogen peroxide compound into water and oxygen and reduce the hydrogen peroxide amount in the CFS (Ozcelik *et al*., 2016). The ability of catalase treated CFS with reduced amount of hydrogen peroxide to maintain their inhibitory effect against pathogens confirms that hydrogen peroxide might not contribute to the

antibacterial activity of the strains. It has been recorded that LAB also were able to produce hydrogen peroxide that can inhibit pathogenic bacteria through superoxide anion chain reaction that will produce toxic oxidation (Vieco-Saiz *et al*., 2019). Inhibition action by hydrogen peroxide however, depends a lot on environmental factors, such as pH and temperature. *Lactobacillus* sp. namely *L. johnsonii* and commensal vaginal LAB, such as *L. crispatus*, *L. jensenii* and *L. gasseri* are among commonly known hydrogen peroxide producing species (Hertzberger *et al*., 2014). *Enterococcus faecium* and *Lactococcus lactis* were also among LAB strains reported as able to produce hydrogen peroxide with strong antibacterial effects against pathogens (Surendran *et al*., 2017).

Other than organic acids and hydrogen peroxide, proteinaceous compounds, such as bacteriocin, might be present, or at least in very low amount, in the CFS as an antibacterial compound. Proteolytic enzymes, including trypsin, will inactivate the proteinaceous compound when added to the CFS (Vieco-Saiz *et al*., 2019). The ability of the trypsin treated CFS in this study to maintain their inhibitory effect after inactivation of proteinaceous compound when compared to untreated CFS shows that the proteinaceous compound might not be responsible for the antibacterial activity of the CFS. In a study conducted by Burgenstock *et al*. (2020), they found that 10 out of 21 LAB isolated from traditionally processed sausages have lost their antibacterial activity against selected pathogens after neutralising of the CFS. Another 11 LAB isolates that were not affected by neutralisation however, lost their antibacterial activity after CFS enzymatic treatment with trypsin. They have concluded that the antibacterial activity of the 11 LAB isolates was due to bacteriocins.

Organic Acids Detection in LAB Cell Free Supernatant (CFS)

The type of organic acids formed by each LAB in MRS broth in this study was also evaluated using HPLC and the results are summarised in Figure 4. Several types of organic acids were detected, which were lactic acid, acetic acid, citric acid, tartaric acid and succinic acid. The result in this study shows that all three LAB isolates produced lactic acid at sginificantlt higher concentrations if compared to acetic acid, citric acid, tartaric acid and succinic acid ($p<0.05$). LAB produced many types of organic acids as end products during growth and fermentation. These organic acids provide an acid environment to the medium and become unfavorable for the growth of other types of microorganisms, including pathogenic and spoilage microorganisms (Teusink & Molenaar, 2017).

Several types of organic acids usually produced by LAB and the type and amount secreted varies depending on several factors, including LAB strains and their growth substrate (Nuryana *et al*., 2019). Hor and Liong (2014), found that the LAB strains in their study produced higher concentration of lactic acid than acetic acid. Lactic acid usually produced by LAB as a result of carbohydrate catabolism through either homo or hetero-fermentative pathways (Mora-Villalobos *et al*., 2020) and many LAB strains were found to usually produce more than 250 mg/L in MRS broth (Ozcelik *et al*., 2016). The *L. plantarum* in MRS broth produced concentration of lactic acid of 509.40 mg/L while *L. acidophilus* produced 515.09 mg/L in a study done by Ozcelik *et al*. (2016). In another study, the *L. plantarum* produced lactic acid concentration of up to 6080 mg/L in MRS broth (Vodnar *et al*., 2010). Poppi *et al*. 2015 reported that several LAB species in their study produced almost same amount of lactic acid in MRS broth where *L. plantarum* produced up to 15 000 mg/L of lactic acid, while *L. reuteri* (16 100mg/L) and *L. casei* (8600 mg/L). Thu *et al*. (2013) and Oulkheir *et al*. (2015) also found the ability of LAB strains to produce several types of organic acids, while Bae and Lee (2015) said that the differences in concentration of each organic acid produced in the growth media depended on the LAB strain and each acid will also cause different antibacterial activity.

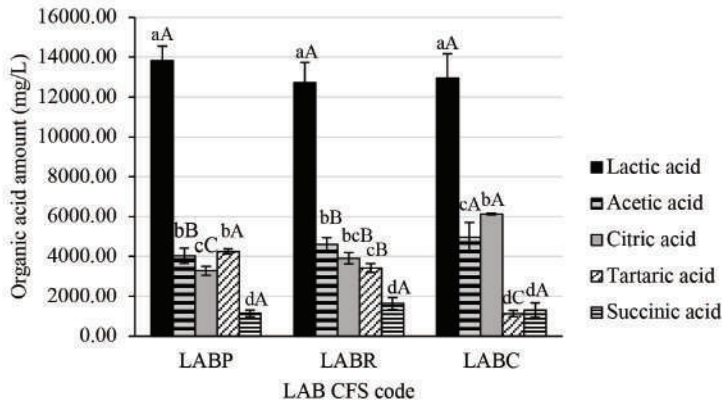

Figure 4: Organic acid formation (mg/L) by LAB in MRS broth. Error bars represent standard error of means (n=3). [a-d]Different lowercase letters indicate that the different organic acid type amount within the same LAB code are significantly different ($p<0.05$). [A-C]Different capital letters indicate that the same organic acid type amount between the different LAB CFS code are significantly different ($p<0.05$)

Conclusion

Three LAB isolates identified as *L. plantarum* (LABP), *L. reuteri* (LABR) and *L. paracasei* (LABC) were isolated from pickled guava and papaya samples. The antibacterial activity study shows that all isolates have inhibitory growth effect against *L. monocytogenes* ATCC® 7644™, *E. coli* ATCC® 48888™ and *S. enterica* serovar Typhimurium ATCC® 14028™ and the effect was due to organic acid. This finding suggested that these LAB isolates may have potential to be used as natural food preservatives. The study also provided a basis for further research regarding the antibacterial component of the isolated LAB and methods for their application in the food industry as an antibacterial agent.

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