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Isolation and characterisation of probiotic lactic acid bacteria from Malaysian fermented fish products *budu* and *bosou*

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Abstract

Budu (BUM) is mainly produced in Kelantan and Terengganu, while *bosou* (BO) is an ethnic fermented food originated from Sabah. These fermented foods are typically consumed as condiments for flavour enhancement of meals. In the present work, lactic acid bacteria (LAB) isolated from both fermented fish products were screened *in vitro* for their probiotic attributes, following the FAO/WHO guidelines. The acid and bile tolerance, haemolytic, and antimicrobial activities, as well as resistance against antibiotics were examined. A total of 42 isolates were characterised by a preliminary subtractive screening method (catalase-negative and Gram-positive cocci/bacilli). Of these, 14 isolates (four isolates from BO and ten isolates from BUM) could tolerate the high acidic conditions, thus were further tested for other probiotic characteristics, and molecularly identified by 16S rRNA sequencing. These isolates demonstrated survival rates above 90 and 50% when exposed to pH as low as 2.5 and 0.3% bile salts, respectively. These isolates also did not display β-haemolytic properties, and could retard the growth of all indicator pathogens to varying degrees. Based on 16S rRNA gene sequence analysis, the BO and BUM isolates were identified as *Lactobacillus plantarum* and *L. paracasei*, respectively, with 98% similarities to the nucleotide sequences existing in the GenBank database. These findings suggested that LAB isolated from both *budu* and *bosou* could be a promising probiotic potential, hence could be further developed as medicinal agents.

Introduction

The definition of probiotics is globally accepted as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" (FAO/WHO, 2002). Probiotics have been widely studied for various health potentials such as to lower serum cholesterol, alleviate lactose intolerance, prevent allergies, and produce bioactive metabolites such as vitamins (Bhat and Bajaj, 2019). Foods containing probiotics including fermented foods have been reported as health-promoting foods. This led to the research interest on various types of fermented foods, and the discovery of new probiotic strains. Research on new probiotic strains is also driven by the demand for non-dairy probiotics to overcome the increasing number of lactose intolerance cases around the world (Argyri *et al.,* 2013). The most investigated probiotic strains isolated from fermented foods are lactic acid bacteria (LAB) as they are the dominant microflora involved during fermentation (Abushelaibi *et al.,* 2017). In fact, LAB are Generally Recognized as Safe (GRAS) organisms, and dominant in the small intestine (Agaliya and Jeevaratnam, 2012). Numerous studies have also reported that LAB isolated from fermented foods possessed probiotic potentials (Ida Muryany *et al.,* 2017; Bindu and Lakshmidevi, 2021; Akman *et al.,* 2021).

Budu is a fish sauce widely produced in Kelantan and Terengganu, which are the states located on the east coast region of West Malaysia. To produce *budu*, fresh anchovies are mixed with salt before they are fermented for up to a year (Najafian and Babji, 2019). During fermentation, microorganisms release various metabolites including protease, which hydrolyse the proteinaceous raw materials. At the end of the fermentation period, some manufacturers cook the resulting sauce, while others do not.

Bosou (also known as *noonsom*) is an ethnic food of the Kadazan-Dusun tribe in Sabah, a state located on the northern region of East Malaysia.

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Bosou is almost similar to *pekasam*, which is widely consumed in West Malaysia. The differences are in terms of raw materials used to prepare *bosou*, and the way to consume it. It is usually made from small freshwater fish, and is consumed with salt, cold cooked rice, and pangi fruits (*Pangium edule* Reinw.) (Lajius, 2014). *Bosou* is served raw or cooked before consumption. Due to its strong odour, *bosou* is always kept in an airtight container.

Despite the extensive use of *budu* in Malaysian meals as traditional condiment, limited research has been done to characterise the probiotic potentials of its bacterial isolates. To date, few attempts have reported the characterisation of bacteria isolated from *budu*; Liasi *et al.* (2009) reported the antimicrobial activity and antibiotic sensitivity of LAB isolated from *budu*; Sim *et al.* (2012) reported the resistance profile of strains isolated from *budu* towards low pH and bile salts, as well as the inhibitory characteristics against foodborne pathogens, but lack of other probiotic features; and Khalil *et al.* (2018) focused on probiotic aspects of exopolysaccharide-producing *Lactobacillus* strains isolated from *budu*. For *bosou*, there has been no report on the probiotic potentials of its bacterial isolates. Therefore, the objective of the present work was to determine the probiotic characteristics of LAB isolated from *budu* and *bosou*.

Materials and methods

Sample collection

Bosou was purchased from Tamu Donggongon, Penampang, Sabah, while *budu* was purchased from Perusahaan Warisan Ketereh, Tumpat, Kelantan. Both products are fermented foods that do not experience any heat treatment during preparation as well as before packaging. The samples were immediately transported to Food Microbiology Laboratory, Universiti Sains Islam Malaysia, Negeri Sembilan, Malaysia. Samples were then stored in air-tight containers, and refrigerated at 4°C until further analysis.

Isolation and preliminary selection of LAB

The isolation of LAB from both fermented food samples was done on MRS agar (Oxoid, UK) according to Haitham *et al.* (2017). Briefly, the fermented food samples were homogenised in MRS broth (Oxoid, UK) using a stomacher at a sample-to-broth ratio of 1:9 (v/v). Next, a serial dilution using MRS broth up to 10^5 was prepared from the homogenate. Then, 100 µL of all dilutions was spread on MRS agar plates containing 0.8% calcium carbonate ($CaCO_3$), which were then incubated aerobically at 37°C for 48 h. Following incubation, white and creamy colonies with different morphologies and dissolved calcium circles surrounding the colonies were randomly chosen and purified on another MRS agar plate.

The pure cultures were then screened for their catalase reactions using the slide method. All catalase-negative isolates were further examined for Gram-reaction and cell morphology. Only catalase-negative and Gram-positive isolates were selected to be tested for their ability to grow in both aerobic and anaerobic conditions. The pure isolates were stored in MRS broth with 20% (v/v) glycerol at -80°C. Isolates were activated before each experiment by sub-culturing twice on MRS agar, and inoculated in MRS broth.

Determination of acidic pH tolerance

The tolerance the isolates to acidic pH was determined according to Zhang *et al.* (2016) with slight modification. Initially, MRS broth was inoculated with approximately 10^8 colony-forming units/mL (CFU/mL) of overnight-grown LAB culture and *Lactobacillus casei* strain Shirota from Yakult (Tokyo, Japan) as the reference strain. Next, 0.1 mL of each culture was aliquoted into 0.9 mL MRS broth, adjusted to pH 2.5, 3, and 6.2 (control) using hydrochloric acid (HCl), and incubated for 3 h at 37°C. Later, the numbers of viable bacteria ($\log_{10}$ values of CFU/mL) were determined. Further incubation for 48 h at 37°C was conducted after serial dilution and inoculation of the culture on MRS agar plate. The survival ability of isolates towards different pH levels was compared with the control. The experiment was performed in triplicates, and repeated twice. The percentage of resistance to acidic pH was calculated Using Eq. 1:

$$\text{Percentage resistance (\%)} = (\log_{10} T/\log_{10} C) \times 100 \quad \text{(Eq. 1)}$$

where, $\log_{10} T = \log_{10}$ CFU/mL at pH 2.5 or 3; $\log_{10} C = \log_{10}$ CFU/mL at pH 6.2.

Isolates with pH tolerance over 90% at pH 3 were considered acid tolerant, and investigated further for their probiotic potentials.

Determination of bile tolerance

Bile tolerance determination was evaluated following the methods of Kumar and Kumar (2015) with some modifications. Briefly, 25 µL of overnight-grown LAB culture in MRS broth and

L. casei strain Shirota as the reference strain (10^8 CFU/mL) were inoculated into 1 mL of MRS broth with 0.3% w/v bile salt (Oxoid, UK). MRS broth without bile salt served as control. These broths were incubated at 37°C, and the optical density (OD) was measured at wavelength 600 nm after 7 and 24 h incubation. The OD of each isolate grown in MRS broth with 0.3% bile salt was compared to the control. The experiment was performed in triplicates, and repeated twice. The percentage of resistance to bile was calculated using Eq. 2:

$$\text{Percentage resistance (\%)} = (OD_T/OD_C) \times 100 \quad \text{(Eq. 2)}$$

where, OD_T = absorbance of isolate in MRS broth with 0.3% bile salt at 7 or 24 h; OD_C = absorbance of isolate in MRS broth without bile salt at 7 or 24 h.

Isolates with bile tolerance over 50% at 0.3% bile salt after 7 h incubation period were considered bile tolerant, and selected for further investigation.

Determination of haemolytic activity

The selected isolates with tolerance towards acidic pH and bile were evaluated for their blood haemolysis ability. Briefly, the overnight-grown LAB cultures in MRS broth and the reference strain *L. casei* strain Shirota were streaked onto the surface of 5% sheep blood agar (Oxoid, UK). Inoculated agar plates were then incubated for 48 h at 37°C, following which the agar plates were observed for signs of β-haemolysis (clear zones around colonies), α-haemolysis (green-hued zones around colonies), or γ-haemolysis (no zones around colonies). The experiment was repeated thrice, and only isolates without β-haemolytic properties were evaluated for the next probiotic characteristics.

Determination of antagonistic properties

The inhibition of pathogenic bacteria was determined using agar spot antimicrobial assay method (Shokryazdan *et al.,* 2014) with two Gram-positive bacteria (*Bacillus cereus* ATCC® 11778™ and *Staphylococcus aureus* NCTC 6571) and two Gram-negative bacteria (*Escherichia coli* ATCC® 25922™ and unknown strain of *Salmonella enterica* serovar Typhimurium) as the indicator strains. Briefly, the overnight-grown LAB cultures in MRS broth (10^8 CFU/mL) were spotted on MRS agar plates. The plates were air-dried for 30 min at room temperature (25 to 27°C) before incubated for 24 h at 37°C. Then, 1% pathogenic bacteria (10^6 CFU/mL) were used to inoculate 10 mL of molten Mueller-Hinton agar (Merck Millipore, USA). The inoculated MH agar was overlaid on top of the spotted MRS plates. Following the solidification of MH agar, the plates were incubated for another 24 h at 37°C. Ampicillin disc (10 µg; Oxoid, UK) served as a positive control, MRS broth served as a negative control, while *L. casei* strain Shirota served as a reference strain. Growth inhibition zones of the indicator strains around the spotted LAB isolates were measured. A diameter of more than 1 mm around the spot was considered positive for antagonistic properties (Jacobsen *et al.,* 1999).

Determination of antibiotic resistance patterns

The antibiotic discs used for resistance determination were ampicillin (10 µg), bacitracin (10 µg), chloramphenicol (10 µg), nalidixic acid (30 µg), penicillin G (10 units), streptomycin (10 µg), tetracycline (30 µg), and vancomycin (30 µg) (Oxoid, UK), according to Angmo *et al.* (2016) with slight modifications. Prior to analysis, MRS agar plates were overlaid with 100 µL of LAB liquid culture (10^8 CFU/mL) using a sterile cotton swab and air-dried. Each antibiotic disc was placed on the inoculated plate under sterile conditions using forceps dipped and flamed with ethanol. The diameter of inhibitory zones was measured after 48 h of incubation at 37°C. Diameters of 20 mm and above were considered as susceptible, between 15 mm to 19 mm as intermediate, and 14 mm and below as resistant (Sharma *et al.,* 2016).

Genotypic identification of LAB

All isolates were molecularly identified based on 16S rRNA gene sequence analysis. Genomic DNA from each strain was extracted using the One-Tube Bacterial Genomic DNA Extraction Kit (Bio Basic, Canada) following the manufacturer's protocol with slight modification. Amplification of the 16S rRNA gene was carried out using universal prokaryotic primers, 27F (5' AGAGTTTGATCMTGGCTCAG 3') and 1492R (5' TACGGYTACCTTGTTACGACTT 3') (Lane, 1991). The following thermal cycle was used: 95°C for 1 min; 35 cycles of 95°C for 15 s (denaturation), 50°C for 15 s (annealing), and 72°C for 10 s (extension); and one cycle for final primer extension at 72°C for 5 min. The PCR product size was expected at approximately 1,500 bp, and sequenced by Macrogen Laboratory (Seoul, South Korea). All sequences were then further analysed and compared with other 16S rRNA genes deposited in the GenBank database using the nucleotide blast program provided by the online Basic Local

Alignment Search Tool (BLAST) from National Centre for Biotechnology Information (NCBI).

Statistical analysis

Data for each experiment was expressed as mean ± standard deviation (mean ± SD). Results were analysed using SPSS (Statistical Package for the Social Sciences) version 26.0. One-way ANOVA was carried out for normally distributed and equally variance data, while Kruskal-Wallis test was performed for data that did not follow these conditions. Significant difference was indicated by a probability value of less than 0.05 ($p < 0.05$).

Results and discussion

Isolation and preliminary selection of LAB

In the present work, 42 LAB were isolated from both *bosou* and *budu*. The presumptions were made based on their ability to hydrolyse $CaCO_3$ on the MRS agar along with their cultural characteristics such as round colonies of white or creamy colour, and raised or convex elevation. Besides, the isolates were also catalase-negative and Gram-positive with rod shape. The isolates were labelled as BO1 to BO17 (isolates from *bosou*) and BUM1 to BUM25 (isolates from *budu*). All the isolates were facultative anaerobe, which was proven by their ability to grow aerobically and anaerobically, with no significant difference between both conditions ($p > 0.05$).

Determination of acidic pH tolerance

All isolates were examined for their resistance after 3 h incubation in MRS broth adjusted to pH 3 and 2.5, reflecting the gastric residence time (Maurer *et al.,* 2015). Of the 42 LAB isolates, only 14 isolates showed excellent resistance with values above 90% at pH 3, whereas only 12 isolates survived with the same percentage at pH 2.5 (Figure 1). The percentage of viable cells that tolerated pH 3 and 2.5 ranged from 90.5 to 99.4% and 87.4 to 97.5%, respectively. Meanwhile, the survival percentage of the commercial probiotic *L. casei* strain Shirota was 95.7% at pH 3 and 94.9% at pH 2.5. Results showed that the survival rates of BO16 (93.8% at pH 3; 87.4% at pH 2.5) and BUM18 (93.2% at pH 3; 90% at pH 2.5) isolates significantly ($p < 0.05$) decreased when exposed to pH 2.5, while no significant ($p > 0.05$) difference was observed for the remaining isolates and *L. casei* strain Shirota when pH decreased from pH 3 to 2.5.

The transit through the acidic surroundings of the stomach represents a primary defence mechanism that all ingested microorganisms must deal with, including pathogens and beneficial probiotics. According to FAO/WHO (2002), the potential probiotic microorganism must be alive to confer a health benefit on the host. Generally, the acid resistance of isolates are strain- and species-dependent with general decrement below pH 3 (Corcoran *et al.,* 2005). This theory validates our preliminary study of acid tolerance, in which the isolates could not withstand pH 2.5. Contrarily, Sim *et al.* (2012) reported the ability of their LAB strains to survive in pH as low as 1.5. Sagdic *et al.* (2014) reported that *L. plantarum* strains were able to grow at pH 2.5, but other strains included *L. casei, L. brevis*, and *L. buchneri* strains failed to survive. The high survival cut-off percentage of 90% was critically chosen based on the potential of LAB to withstand the first barrier, referring to the harsh

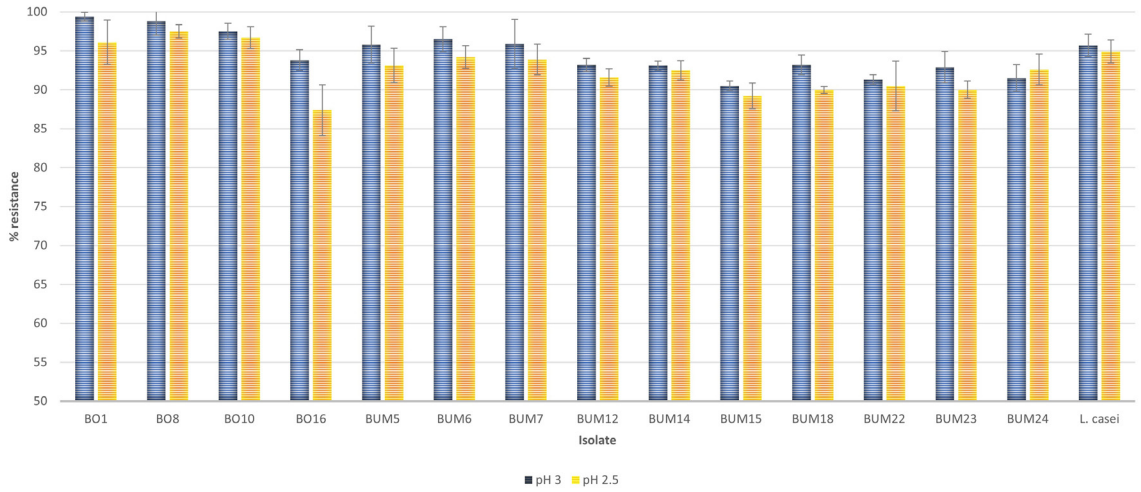

Figure 1. Effect of acidic pH on survival of BO isolates, BUM isolates, and *L. casei* strain Shirota following 3-hour incubation at 37°C.

acidic environment without losing its viability (Castorena-Alba *et al.,* 2018). The LAB must withstand the robust acidic environment, which varied between pH 1.5 to 3, before they could reach, colonise, and exert their benefits in the intestinal tract (Corcoran *et al.,* 2005; Liong and Shah, 2005). In general, LAB can induce an acid tolerance response under acidic stress, and result in pH homeostasis and repair processes, which eventually develop their resistance at low pH (Aarti *et al.,* 2017).

Determination of bile tolerance

The 14 isolates with high tolerance to acidic pH were further evaluated for their resistance at 0.3% bile salts concentration for 7 h, which simulated the duration in the intestines (Maurer *et al.,* 2015), and at 24 h. Generally, each isolate survived with a percentage above 50% after 7 h incubation period in MRS broth containing 0.3% bile salt (Figure 2). The survival percentages of all BUM isolates significantly ($p < 0.05$) decreased when exposed to 0.3% bile salts in a longer duration (24 h). Despite not reaching 50% survival in comparison to the control, BUM isolates were also growing, as evidenced by the increase in absorbance as compared to 7 h. BO1 (71.5% at 7 h; 79.4% at 24 h), BO8 (65.1% at 7 h; 80.3% at 24 h), and BO16 (51.1% at 7 h; 78.1% at 24 h) resistance patterns appeared contradictory with significant ($p < 0.05$) increments at 24 h. The commercial probiotic *L. casei* strain Shirota persisted in the 0.3% bile salt environment almost entirely with resistance values of 94.7% after 7 h, and 82.3% after 24 h.

Defeating the inhospitable acidic environment is worthless if the microorganism to be applied as probiotic is unable to survive in the intestine. As bile salts are naturally destructive towards bacteria, their presence becomes the next hurdle for the isolates to endure before they could remain active, grow, and colonise the intestinal tract (Song *et al.,* 2015). Although the commonly proposed bile salt concentrations to evaluate the probiotic characteristics are in between 0.15 and 0.5%, isolates that show above 50% survival rates at 0.3% bile salts are adequate to be considered bile resistant (Kumar and Kumar, 2015). In addition, Ruiz *et al.* (2013) also suggested that strains with resistance to other stress conditions such as acidic pH will also likely to be resistant towards bile. In the present work, most isolates obtained lower survival percentages at 24 h as compared to 7 h, while *vice versa* for BO1, BO8, and BO16, thus suggesting that the bile resistance properties might be strain-specific. The high growth of these isolates at an extended incubation period might be due to their capacity to accommodate the stress condition over a long period of incubation as compared to the other tested isolates

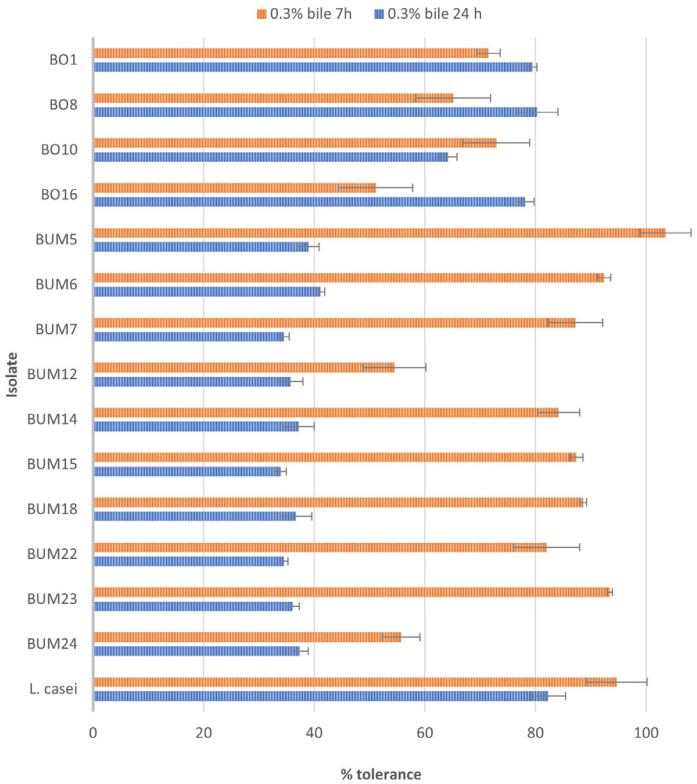

Figure 2. Effect of 0.3% bile salt on survival of BO isolates, BUM isolates, and *L. casei* strain Shirota following 7- and 24-hour incubation at 37°C.

(Sahadeva *et al.,* 2011). The protective mechanism in LAB towards bile could be due to several reasons. For example, the production of bile salt hydrolase enzyme (BSH) could hydrolyse the bile, and lower the lethal effect (Kumar and Kumar, 2015). Besides, the isolates could also modify their composition of the cell membrane, and perform active efflux of bile to neutralise the deadly effect of bile (Ruiz *et al.,* 2013).

Determination of haemolytic activity

FAO/WHO (2002) recommended the haemolysis test to ensure the safeness of potential probiotics based on the absence of haemolytic ability. Results showed that all isolates and the commercial probiotic *L. casei* strain Shirota did not demonstrate undesirable β-haemolytic or complete haemolytic activities. Some arguments were reported in considering the types of haemolytic activity that were deemed safe to develop as probiotics. For instance, Abushelaibi *et al.* (2017) and Chopade *et al.* (2019) did not consider their isolates that exhibited partial haemolysis or α-haemolytic properties to be further investigated for probiotic potentials. However, other studies recommended that only strain with β-haemolysis was deemed to be destructive (Argyri *et al.,* 2013; Touret *et al.,* 2018), as α-haemolytic bacterial strain might harbour very minimal virulence ability, and commonly found among lactobacilli from foods and dairy products (Halder *et al.,* 2017). This is because α-haemolysin is sensitive to trypsin, which is the enzyme found in the small intestine that breakdowns proteins, and active in the presence of calcium ion (Aghemwenhio *et al.,* 2017).

Determination of antagonistic properties

Table 1 shows the antagonistic properties of BO and BUM isolates assessed against four pathogens. All isolates inhibited the growth of all pathogens in varying degrees, and were strain-dependent, similar to the findings by Kumar and Kumar (2015) and Chopade *et al.* (2019). Isolate BO8 exhibited the highest inhibitory activity against *E. coli* (14.17 mm), while BUM23 exhibited the lowest against *B. cereus* (5.50 mm). Although all

Table 1. Mean inhibition zones of BO isolates, BUM isolates, and *L. casei* strain Shirota against pathogenic bacteria.

Isolate	Inhibition zone (from outward edge of isolates to outward edge of clear region) (mm)			
	B. cereus	***S. aureus***	***E. coli***	***S.* Typhimurium**
BO1	8.67 ± 0.52	9.33 ± 0.82	13.17 ± 0.75	10.00 ± 1.41
BO8	8.67 ± 0.52	9.17 ± 0.98	13.67 ± 2.66	12.17 ± 1.17
BO10	10.67 ± 1.63	9.50 ± 0.55	10.83 ± 1.94	12.17 ± 1.60
B016	8.40 ± 0.55	9.67 ± 1.03	12.67 ± 1.03	11.83 ± 0.75
BUM5	6.67 ± 1.21	7.33 ± 1.03	11.50 ± 0.55	10.00 ± 0.89
BUM6	5.67 ± 1.03	7.80 ± 0.45	9.17 ± 0.98	10.80 ± 0.84
BUM7	6.00 ± 0.89	7.80 ± 1.64	8.00 ± 2.00	7.83 ± 0.75
BUM12	6.33 ± 0.52	7.67 ± 1.21	9.33 ± 0.52	11.83 ± 3.12
BUM14	6.67 ± 0.82	8.33 ± 0.82	10.83 ± 1.47	11.33 ± 2.50
BUM15	7.33 ± 1.21	10.00 ± 2.76	9.50 ± 2.88	12.33 ± 2.25
BUM18	6.33 ± 1.37	8.67 ± 2.66	9.00 ± 1.26	10.67 ± 2.42
BUM22	6.33 ± 0.82	7.67 ± 1.21	9.17 ± 0.98	9.00 ± 0.71
BUM23	5.50 ± 0.55	8.17 ± 2.14	9.33 ± 1.03	10.50 ± 2.26
BUM24	6.33 ± 1.21	8.00 ± 1.55	9.67 ± 0.81	11.00 ± 2.68
L. casei	6.17 ± 0.75	8.20 ± 1.48	9.67 ± 2.34	10.17 ± 1.17
Ampicillin (10 µg)	1.50 ± 0.55	11.00 ± 0.71	6.83 ± 0.75	11.50 ± 0.84

Values are means ± SD of two independent experiments, each in three replicates.

isolates yielded lower inhibition zones against *B. cereus* as compared to other pathogens, most of the isolates' activities were significantly higher ($p < 0.05$) as compared to the positive control. Meanwhile, all BUM isolates except BUM15 inhibited the growth of *S. aureus* significantly ($p < 0.05$) as compared to the positive control. In terms of growth inhibition of *E. coli*, all BO isolates, as well as four BUM isolates (BUM5, BUM14, BUM15, and BUM24) exhibited significantly ($p < 0.05$) higher antagonistic activities than ampicillin (10 µg). Results also showed that only BUM7 and BUM22 showed significantly lower ($p < 0.05$) inhibition against *S.* Typhimurium as compared to the positive control, while the others were not significantly ($p > 0.05$) different. When compared to the reference strain, BO1, BO8, and BO16 produced significantly ($p < 0.05$) greater inhibition zone against *E. coli.*

This finding is in line with Shokryazdan *et al.* (2014), in which all *Lactobacillus* isolates demonstrated antagonistic properties against the tested pathogens. In addition, Khalil *et al.* (2018) reported that *L. fermentum* strains of BU11 and BU14 isolated from *budu* were able to inhibit the growth of indicator pathogens such as *S.* Typhimurium, *E. coli*, *Pseudomonas aeruginosa*, methicillin-resistant *S. aureus, S. aureus,* and *Listeria monocytogenes* with different levels of inhibitory activities. In contrast, Chopade *et al.* (2019) reported that some of their cultures could not inhibit the growth *S. aureus, E. coli,* and *S.* Typhimurium, while one of the isolates was unable to inhibit any of the pathogens tested. The ability to produce antimicrobial compounds is crucial for probiotics to compete with, inhibit growth of, or kill pathogens (Sakandar *et al.,* 2019). Bacteriocins are the example of protein produced by bacteria, which hinder the growth of similar or closely related strains in the same species or across genera (Yang *et al.,* 2018). The unique characteristics of bacteriocins include non-toxic properties to humans as well as stability at low pH and under heat. The organic acids produced by metabolisms in LAB, for example, lactic acid, acetic acid, and formic acid inhibit the growth or kill the pathogens by decreasing the pH of the environment. The organic acids also diffuse through the pathogens' cell membrane, dissociate, and release hydrogen ions, thus causing the cytoplasm's acidification which eventually leads to cell death (Tharmaraj and Shah, 2009). Another common antimicrobial substance of LAB is hydrogen peroxide which causes a powerful oxidising impact on the bacterial cell. The impact leads to the denaturation of cell proteins, and the peroxidation of membrane lipids, which increase membrane permeability (Lindgren and Dobrogosz, 1990). The present work, however, did not inspect further the possible substance produced, which caused the growth inhibition of all the tested pathogens.

Determination of antibiotic resistance patterns

The isolates were further tested for their antibiotic resistance patterns against eight different antibiotics using disc diffusion assay. All isolates were susceptible to ampicillin, chloramphenicol, penicillin G, and tetracycline, while showing resistance to nalidixic acid, streptomycin, and vancomycin (Table 2). BO1, BO10, and BO16 showed intermediate antibiotic resistance against bacitracin, while BO8 was resistant to the same antibiotic. Oppositely, all BUM isolates and the reference strain *L. casei* strain Shirota were susceptible to bacitracin. These findings concur with those reported by Goswami *et al.* (2017) in which their LAB strains isolated from *kahudi*; a traditional fermented food, were susceptible to similar antibiotics. Ampicillin and penicillin G are β-lactams that inhibit the synthesis of bacterial cell walls, while chloramphenicol and tetracycline inhibit the protein synthesis of bacteria (Shaikh *et al.,* 2015). These results are in agreement with Khalil *et al.* (2018) who found out that all their strains isolated from different Malaysian fermented foods showed resistance to vancomycin, nalidixic acid, and ciprofloxacin, but were either susceptible or resistant towards bacitracin. Much earlier, Liasi *et al.* (2009) reported the resistant profile of LAB isolated from *budu* against several antibiotics, including streptomycin, bacitracin, and nalidixic acid. Streptomycin is aminoglycosides that inhibit protein synthesis, while bacitracin is polypeptide that inhibits the synthesis of the bacterial cell wall. Nalidixic acid is quinolone that interferes with the synthesis of nucleic acid in Gram-negative bacteria, which explains its resistance against Gram-positive LAB (Shaikh *et al.,* 2015).

Different antibiotic resistance patterns of probiotics have both advantages and disadvantages. The former involves the bacterial survival in the gastrointestinal tract of patients receiving antibiotic treatment, while the latter involves the potential transfer of acquired resistance genes to pathogenic bacteria found in the intestine (Jose *et al.,* 2015). For the LAB to pass their antibiotic resistance genes to other bacteria, they must communicate with each other through the aid of plasmids and transposons. Plasmids are commonly present in enterococci, lactococci, leuconostocs, pediococci, lactobacilli,

Table 2. Antibiotic resistance patterns of BO isolates, BUM isolates, and *L. casei* strain Shirota against eight antibiotics.

	AMP	BCR	CHL	NAL	PEN	STR	TET	VAN
BO1	S	I	S	R	S	R	S	R
BO8	S	R	S	R	S	R	S	R
BO10	S	I	S	R	S	R	S	R
B016	S	I	S	R	S	R	S	R
BUM5	S	S	S	R	S	R	S	R
BUM6	S	S	S	R	S	R	S	R
BUM7	S	S	S	R	S	R	S	R
BUM12	S	S	S	R	S	R	S	R
BUM14	S	S	S	R	S	R	S	R
BUM15	S	S	S	R	S	R	S	R
BUM18	S	S	S	R	S	R	S	R
BUM22	S	S	S	R	S	R	S	R
BUM23	S	S	S	R	S	R	S	R
BUM24	S	S	S	R	S	R	S	R
L. casei	S	S	S	R	S	R	S	R

AMP: ampicillin, BCR: bacitracin, CHL: chloramphenicol, NAL: nalidixic acid, PEN: penicillin G, STR: streptomycin, TET: tetracycline, and VAN: vancomycin. Degree of inhibition = susceptible (S): inhibition zone ≥ 20 mm; intermediate (I): inhibition zone 15 - 19 mm; resistance (R): inhibition zone ≤ 14 mm (Sharma *et al.,* 2016).

and bifidobacteria, while transposons are found in enterococci, lactococci, and streptococci (Mathur and Singh, 2005). However, the extensive use of LAB in fermented foods has a good long record of safety, and has the GRAS status, as most of the strains contain intrinsic resistance genes that are non-transferable horizontally (Jose *et al.,* 2015). Therefore, the risk of infection derived from the ingestion of these bacteria is extremely limited. According to Abriouel *et al.* (2015), vancomycin-resistance and chloramphenicol-resistance genes in *Lactobacillus* species are highly conserved in the chromosome of each species, and probably non-transferable to other bacteria.

Genotypic identification of LAB

The presumptive LAB isolates based on phenotypic properties were genotypically identified, and confirmed as such by the molecular method. The 16S rRNA sequences of all BO isolates matched those of *L. plantarum*, while those of BUM isolates matched those of *L. paracasei* with a minimum of 98% similarities in the GenBank database. All sequences were consequently deposited into the GenBank database with accession numbers of MT163340 (BO1), MT163358 (BO8), MT163341 (BO10), MT163342 (BO16), MT163343 (BUM5), MT163344 (BUM6), MT163345 (BUM7), MT163346 (BUM12), MT163347 (BUM14), MT163359 (BUM15), MT163348 (BUM18), MT163360 (BUM22), MT163349 (BUM23), and MT163350 (BUM24). Previously, the LAB strains isolated from *budu* were identified as *L. casei, L. plantarum*, and *L. paracasei* (Liasi *et al.,* 2009); Sim *et al.* (2012) isolated *L. plantarum* from their *budu* samples; and Khalil *et al.* (2018) isolated *L. fermentum*, a strain that produced exopolysaccharides, from their *budu* samples.

Conclusion

The increasing demand for products

associated with health benefits has increased the research on underexplored foods and their potentials as probiotic sources. In the present work, *budu* and *bosou*, the fermented fish products of Malaysia, were investigated for the presence of lactic acid bacteria and their potential probiotic characteristics. Results revealed that 14 of 42 strains showed strong resistance towards the acidic physiological environment. These strains also exhibited promising probiotic characteristics such as bile salt tolerance, antagonism against pathogenic bacteria, and safe for consumption due to the absence of haemolytic activities. The strains isolated from *bosou* and *budu* were identified as *L. plantarum* and *L. paracasei*, respectively. More research is required to better understand the role of these isolates on human health. Further research could investigate the mechanisms involved for each probiotic characteristic, interactions with enterocytes, as well as their impact on the gene expressions of the enterocytes.

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