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Isolation and characterization of *Lactobacillus brevis* C23 with ability to secrete antimicrobial substance for the inhibition of a foodborne pathogen *Listeria monocytogenes* ATCC 7644

Dharni Kuhan Sreedharan[1], Sahar Abbasiliasi[2], Paramasivam Murugan[1], Zhang Jin Ng[1], Arbakariya Bin Ariff[3,4] and Joo Shun Tan[1]*

[1]School of Industrial Technology, Universiti Sains Malaysia, 11800 Gelugor, Pulau Pinang, Malaysia.
[2]Halal Products Research Institute, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia.
[3]Department of Bioprocess Technology, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia UPM, Serdang 43400, Selangor, Malaysia
[4]Bioprocessing and Biomanufacturing Research Centre, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia UPM, Serdang 43400, Selangor, Malaysia
Email: jooshun@usm.my

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ABSTRACT

Aims: This study aims to isolate lactic acid bacteria (LAB) from various food sources to obtain a potent strain against *Listeria monocytogenes*.
Methodology and results: A total of 68 LAB isolates were selected to evaluate their antimicrobial activity against *L. monocytogenes*, a foodborne pathogen and a causative agent of listeriosis. The selected isolate was identified and characterized. The isolate C23 from cabbage showed the highest antimicrobial activity against *L. monocytogenes* ATCC 7644 with inhibition ability of 73.94%. The isolate was closely related to *Lactobacillus brevis* by 16S rRNA sequencing and subsequently deposited in GenBank with an accession number of MN880215, named as *L. brevis* C23. The cell free supernatant (CFS) of *L. brevis* C23 had high tolerance in low pH and was able to withstand up to 60 °C. The proteinaceous nature of the antimicrobial agent was also confirmed through the enzymatic test. The CFS was stable on different detergents as well as bile salts. Under transmission electron microscopy (TEM), the inhibitory effect of CFS against *L. monocytogenes* was proven by causing cell lysis.
Conclusion, significance and impact of study: Bacteriocin-like inhibitory substances (BLIS) of *L. brevis* C23 showed very promising potential in food industrial application.

Keywords: Lactic acid bacteria, *Lactobacillus brevis*, foodborne pathogen, *Listeria monocytogenes*, antimicrobial

INTRODUCTION

Foodborne diseases which have a massive impact on the economy worldwide is still not under control and its outbreaks can cause health and economic losses. For the past 20 years, there had been numerous outbreaks of foodborne diseases involving bacterial species of *Salmonella* spp., *Listeria* spp., and *Escherichia coli* (Amézquita and Brashears, 2002).

Listeria monocytogenes, a foodborne pathogen is a facultative anaerobic Gram-positive which is motile, non-spore forming, catalase positive and oxidase negative bacterium. *Listeria monocytogenes* are able to survive at various unfavourable environment, such as low pH and refrigeration temperature as well as tolerate high sodium chloride concentration (Složilová *et al*., 2014). Due to the highly adaptability nature, *L. monocytogenes* are found in raw meats and vegetables especially in ready to eat processed foods (Shamloo *et al*., 2019).

Chemical additives and preservatives are widely used in processed food to slow down pathogen growth, and extending its shelf life (Choi and Chin, 2003). Since *L. monocytogenes* are able to survive at most conditions, natural preservation such as curing and drying is futile. However, prolonged usage of chemical preservatives is dangerous for health. For instance, benzoates are known to worsen asthma, trigger allergies and cause urticarial while salicylates might cause child hyperactivity and other neurological disturbances (Abdulmumeen *et al*., 2012).

Biopreservation are in high demand due to its risk free to human health and environmental friendly (Ünlü *et al*., 2016). Bacteriocins are ribosomal synthesised protein produced by Gram-positive and some Gram-negative lactic acid bacteria (LAB). However, bacteriocin produced

***Corresponding author**

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only by LAB are used in food industry since it is Generally Regarded As Safe (GRAS status) (Nettles and Barefoot, 1993).

Antibacterial peptides or bacteriocins produced by many strains of LAB have been used as food preservatives for many years without any known adverse effects. Bacteriocins, especially those with broad antibacterial spectrum, are bactericidal against food spoilage and many pathogenic bacteria. In the commercialization of bacteriocins as food preservatives, there is a need to determine their antibacterial spectrum, biochemical characteristics, effectiveness and regulatory implications in the food system.

Bacteriocin exhibits a large antimicrobial spectrum which capable to be used in food packaging or as natural preservatives. Bacteriocin exert bactericidal effect by interrupting cell wall synthesis or by pore formation on target's plasma membrane (Güllüce *et al.*, 2013). However, the use of the bacteriocin is very limited due to technological or legislation barriers, but intensive studies are carried out to diversify its use in the food industry. The aim of this study is to investigate the potential use of bacteriocin-like inhibitory substances (BLIS) from locally isolated LAB strains against a foodborne pathogen *L. monocytogenes*. The cell-free supernatant (CFS) with the highest antimicrobial activity against *L. monocytogenes* ATCC 7644 was selected and characterized.

MATERIALS AND METHODS

Isolation of BLIS producing strains

A total of seven different food sources such as yoghurt, tofu, cabbage and guava were obtained from a Penang market, Malaysia; oil palm trunk sap was collected from I.K Panel (M) Sdn. Bhd. located at Nibong Tebal, Malaysia; homemade fermented rice and human breast's milk was obtained from a healthy mother in Penang, Malaysia. The samples were suspended into 0.85% (w/v) NaCl (Fisher Scientific, New Hampshire, Unites States) and homogenized well. The sample was serially diluted to 10-fold using 0.85% (w/v) sodium chloride (NaCl) and spread platted on de Man Rogosa Sharpe (MRS) agar (Merck, Darmstadt, Germany) and M17 agar (Merck, Darmstadt, Germany). The agar plates were incubated at 37 °C for 48 h. Distinctive colonies were selected and streaked to obtain pure colonies. The pure isolates were cultured in MRS broth for 24 h at 37 °C and kept at –80 °C in 30% (v/v) glycerol for long term storage (St. Louis, Missouri, US).

Screening of antimicrobial activity against *L. monocytogenes* ATCC 7644

A total of 59 isolates as well as 9 strains from our stock culture were screened for antimicrobial activity. The isolates were cultured in MRS and M17 broth for 24 h at 37 °C. The cultures were centrifuged at 8000 *g* for 20 min at 4 °C. Then, CFS was filter-sterilized using 0.22 µm filter membrane (Minisart®, Sartorius). *Listeria monocytogenes* ATCC 7644 was used as an indicator strain in which the CFS was tested for its antimicrobial activity. *Listeria monocytogenes* ATCC 7644 was cultured in Brain Heart Infusion (BHI) broth (Merck, Darmstadt, Germany) for 24 h at 37 °C and diluted to 0.02 ± 0.005 of optical density (OD) at 600 nm to give 10^6 CFU. After that, broth microdilution method was carried out in 96-well plate as described by Ng *et al.* (2020). In this assay, 100 µL of *L. monocytogenes* ATCC 7644 cell suspension was added with 100 µL of CFS into the 96-well plate. For the negative control, the CFS was replaced with MRS broth, while for positive control streptomycin (10 mg/mL) was used (Santa Cruz Biotechnology, Dallas, United State). The 96 well-plate was incubated at 37 °C for 24 h. Turbidity was measured at 596 nm and the percentage of growth inhibition of CFS against *L. monocytogenes* ATCC 7644 calculated using following equation:

$$Percentage\ of\ inhibition\ (I),\% = \frac{OD\ control - OD\ sample}{OD\ control} \times 100$$

Where, OD control represents the change in OD of growth control (*L. monocytogenes* ATCC 7644 mixed with MRS broth) and OD sample represents the change in the optical density of the sample (*L. monocytogenes* ATCC 7644 mixed with CFS).

Identification of BLIS producing isolate by 16S rRNA sequencing and phylogenetic tree analysis

The BLIS producing isolate which produced the highest percentages of inhibition against *L. monocytogenes* ATCC 7644 was analysed by 16S rRNA sequencing. The universal primers of 785F (5'-GGATTAGATACCCTGGTA-3') and 907R (5'-CCGTCAATTCMTTTRAGTTT-3') were used to purify and sequence the PCR products. The initial denaturation temperature of the PCR was at 94 °C for 3 min then continued by 35 cycles of denaturation for 30 sec at 94 °C, followed by annealing for 30 sec at 58 °C and extension for 30 sec at 72 °C (Ng *et al*., 2020). Multiple sequence alignment and the phylogenetic tree was conducted with MEGA X version 10.1.6 and neighbour joining method was used to construct phylogenetic tree (Figure 1).

Effect of physio-chemical factors on stability of CFS
Heat stability of CFS

Heat stability of CFS was tested by heating 10 mL of filtered sterilized CFS at 40 °C, 50 °C, 60 °C, 70 °C, 80 °C, 90 °C, 100 °C for 2 h. The CFS was then cooled down to room temperature (25 °C). The antimicrobial activity of the heat treated CFS were tested against the *L. monocytogenes* ATCC 7644 in a 96-well plate using broth microdilution method as mentioned above.

pH stability of CFS

The pH sensitivity of CFS was tested by adjusting the pH

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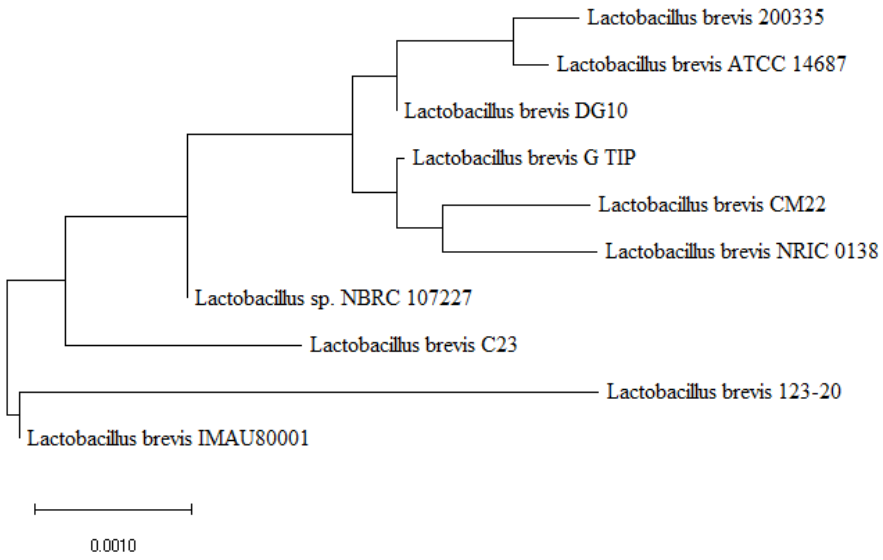

Figure 1: Neighbour-joining tree based on 16S rRNA sequencing showing the phylogenetic relationship between the isolated strain *Lactobacillus brevis* C23.

of CFS to 2, 3, 4, 5, 6, 7, 8, 9, and 10 using 1N hydrochloric acid (HCl) and 1N sodium hydroxide (NaOH). The pH-adjusted CFS was incubated at 37 °C for 2 h. The antimicrobial activity of the pH-adjusted CFS was tested against the *L. monocytogenes* ATCC 7644 in a 96-well plate using broth microdilution method as mentioned above.

Effect of enzymes on CFS

The effect of different enzymes including α-amylase (TCI, Tokyo, Japan), catalase (Nacalai Tesque, Kyoto, Japan), lipase (Himedia, Mumbai, India), papain, pepsin (Himedia, Mumbai, India), proteinase K (Merck, Darmstadt, Germany) and trypsin (Himedia, Mumbai, India) on CFS was tested at concentration of 0.1 mg/mL and 1 mg/mL. A total of 1 mL of CFS were incubated in the presence of enzymes at 1:1 ratio and incubated for 2 h at 37 °C. The mixture was heated at 85 °C for 10 min to inactivate the enzymes. The antimicrobial activity of the treated CFS was tested against *L. monocytogenes* ATCC 7644 using broth microdilution method as described above.

Effect of detergent on CFS

The effect of detergents on CFS activity was tested with urea (R&M Chemicals, Essex, United Kingdom), sodium dodecyl sulfate (Sigma, St. Louis, Missouri, United States), NaCl (Fisher Scientific, New Hampshire, Unites States), ethylenediaminetetraacetic acid (EDTA) (Amresco, Solon, Ohio, United States), Tween 20, Tween 80 and Triton X-100 (Sigma, St. Louis, Missouri, United States). The detergents were added into the CFS at a final concentration of 10 mg/mL (w/v). The CFS with the presence of detergent was incubated for 2 h at 37 °C. The antimicrobial activity of the treated CFS was tested against the *L. monocytogenes* ATCC 7644 using broth microdilution method.

Fermentation of carbohydrate

Sugar fermentation of selected isolate was analysed with API 50 CHL system kit, following the procedures provided by the manufacturer (BioMérieux, Lyon, France). The colour changes were recorded at 24 h and 48 h (Abbasiliasi *et al*., 2012).

Bile salt tolerance test

Lactobacillus brevis C23 culture was cultured in MRS broth for 24 h at 37 °C. Bile salts (Oxoid, United Kingdom) were prepared at concentrations of 0.3%, 0.5% and 1% (w/v). One mL of overnight *L. brevis* C23 culture was inoculated into 10 mL of MRS broth in the presence of different concentrations of bile salts (0.3%, 0.5% and 1% (w/v)) and incubated at 37 °C for 5 h. The growth of *L. brevis* C23 were measured spectrophotometrically at OD600 nm and defined in terms of the cell dry weight (DCW). The relationship between dry cell weight and OD600 was observed as 0.779 g DCW/L/OD600.

Adsorption study

The adsorption of BLIS on the producer cells was carried out according to the methods described by Yang *et al.* (1992). *Lactobacillus brevis* C23 culture was grown at 37 °C for 24 h. The initial culture pH (pH 5.76) was adjusted to pH 6.5 and centrifuged at 8000 *g* for 20 min at 4 °C. The supernatant was discarded, and the pellet was washed with sterile 0.1 M phosphate buffer (pH 6.5). The

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cells were resuspended in 10 mL of sterile 100 mM NaCl (pH 2) (Fisher Scientific, New Hampshire, Unites States) and stirred for 1 h at 4 °C. The suspended cells with NaCl were centrifuged at 8000 *g* for 20 min at 4 °C and filtered using 0.22 µm filter membrane. The antimicrobial activity was tested against the *L. monocytogenes* ATCC 7644 in a 96-well plate using broth microdilution method.

Effect of CFS on *L. monocytogenes* ATCC 7644 using transmission electron microscope (TEM)

Listeria monocytogenes ATCC 7644 was cultured for 24 h at 37 °C. CFS was added into the culture and incubated for 24 h at 37 °C for the test and MRS broth was replaced with CFS and used as negative control. After 24 h, both cultures were centrifuged at 8000 × *g* for 20 min. The supernatants were discarded and the pellets from both cultures kept for TEM analysis. The pellets were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer for 4 to 6 h. The samples were centrifuged, and the supernatants pipetted to remove the fixative. A few drops of horse serum were added to each of the pellets. The coagulated pellets were then diced into 1 mm^3 pieces. Following three washings with sodium cacodylate buffer, the samples were post-fixed in 1% aqueous osmium tetroxide and dehydrated in ascending grades of acetone dilutions (30%, 50%, 75%, 80%, 95% and 100%). Samples were then infiltrated overnight with a 50:50 mixture of resin and acetone. The following morning, samples were infiltrated with 100% resin and dropped into resin-filled, pre-labeled beam capsules and polymerized at 60 °C for 16 h. Ultrathin sections on copper grids were stained with uranyl acetate and lead citrate and examined by TEM (Abbasiliasi *et al*., 2017).

Statistical analysis

All of the data obtained were analysed using SPSS version 22.0 (IBM, New York, USA). One-way analysis of variance was carried out to study the significance difference between means. All the data obtained from duplicates were expressed as means value. The significance level was set at α = 0.05 and the data were analysed by Tukey's test.

RESULTS

Isolation and screening of BLIS-producing LAB

A total of 59 unidentified isolates were selected based on the morphologies of the colonies such as size, colour and shape (Table 1). Most of the colonies were small in size and circular in shape. The notable colours of the colonies were white while some were creamy. However, 3 of the colonies obtained from the oil palm trunk sap were yellowish in colour with a web-like pattern on the colony surface.

The 59 unknown isolates and 9 identified strains were tested for the antimicrobial activity by broth microdilution method. Among the 59 different distinctive isolates, 12 isolates showed positive antimicrobial activity against of *L. monocytogenes* ATCC 7644 with four of it showed more than 50% inhibition (Figure 2). The highest growth of inhibition was at 74% from cabbage isolate (SCW 2) and the lowest growth of inhibition was at 0.90% from yoghurt (SCW 1). For identified strains, *L. bulgaricus* FTCC 0411 had the highest inhibition percentage of 70.94%, while *L. bulgaricus* FTDC 1211 showed the lowest inhibition percentage of 50.45%. The isolate from cabbage SCW2, which had the highest antimicrobial activity was selected for 16S rRNA sequencing identification.

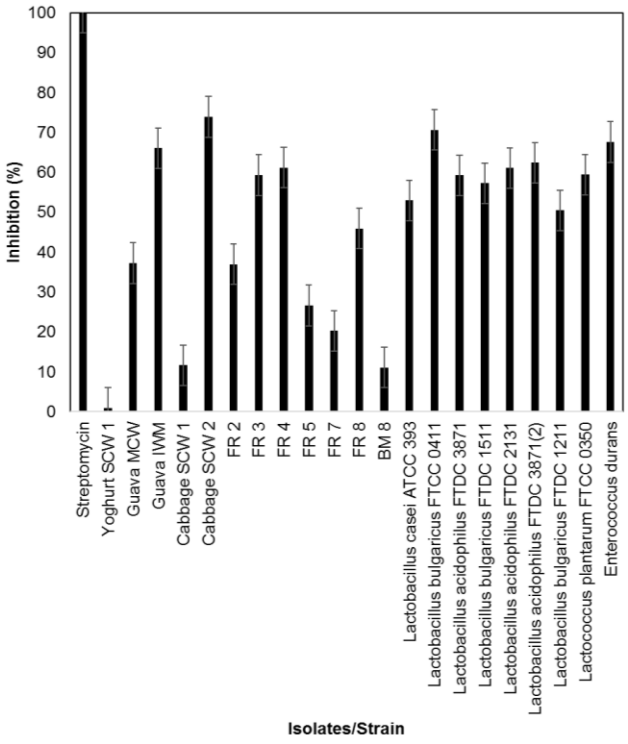

Figure 2: Percentage of inhibition (%) on *L. monocytogenes* ATCC 7644 by CFS from different LAB showing inhibitory effect. FR represents fermented oil palm sap; Streptomycin (10 mg/mL) acted as positive control. Results were expressed as mean and standard deviation, where tests were performed in triplicate.

Identification of selected isolates by 16S rRNA sequencing and phylogenetic analysis

The results from 16S rRNA sequencing revealed that SCW 2 with the nucleotide sequences (1521 bp) showed 99.8% of pairwise similarities to *L. brevis* G TIP (MK530232.1). It was showed the isolate SCW 2 was closely related to *L. brevis* and named as *L. brevis* C23. The 16S rRNA nucleotide sequence of *L. brevis* C23 was deposited in GenBank with the accession number of MN880215.

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Table 1: Isolation of LAB strains from different food sources on selective medium of MRS and M17 agar and their colony's morphologies.

Isolates/strain	Sources	Agar plates	Colony's morphology
SCW 1	Yoghurt	MRS	Small, whitish, circular
MCW 1		MRS	Medium size, whitish, circular
SCW 2		M17	Small, whitish, circular
MCW 2		M17	Medium size, whitish, circular
SCW 1	Tofu	M17	Small, whitish, circular
VSCW 1		M17	Very small size, whitish, circular
SCW 2		MRS	Small, whitish, circular
VSCW 2		MRS	Very small size, whitish, circular
MCW	Guava	MRS	Medium size, whitish, circular
LCW		MRS	Large, whitish, circular
SCW		M17	Small, whitish, circular
IWM		MRS	Medium size, whitish, irregular
SCW 1	Cabbage	MRS	Small, whitish, circular
SCW 2		M17	Small, whitish, circular
VSCW 1		MRS	Very small size, whitish, circular
FR 1	Fermented rice	MRS	Large, pale whitish, irregular
FR 2		MRS	Medium size, creamy, circular
FR 3		MRS	Small, creamy, oval
FR 4		MRS	Small, whitish, circular
FR 5		MRS	Small, whitish, oval
FR 6		MRS	Medium size, creamy, circular
FR 7		MRS	Small, whitish, circular
FR 8		MRS	Small, whitish, oval
FR 9		MRS	Medium size, whitish, circular
FR 10		MRS	Small, whitish, star shape
FR 11		MRS	Small, whitish, oval
FR 12		MRS	Medium size, whitish, irregular shape
FR 13		M17	Very large, creamy, irregular shape
FR 14		M17	Medium size, creamy with white border, irregular shape
FR 15		M17	Very large, creamy, irregular shape
FR 16		M17	Medium shape, creamy, circular
FR 17		M17	Large, creamy, irregular shape
FR 18		M17	Very large, whitish, irregular shape
MM 1	Mother's milk	MRS	Small, whitish, circular
MM 2		MRS	Small, whitish, circular
MM 3		MRS	Medium shape, milky, irregular shape
MM 4		MRS	Small, whitish, circular
MM 5		MRS	Medium shape, creamy, circular
MM 6		MRS	Medium shape, whitish, circular
MM 7		MRS	Small, creamy, oval
MM 8		MRS	Medium, creamy, irregular shape
MM 9		MRS	Small, whitish, oval
MM 10		MRS	Small, creamy, circular
MM 11		MRS	Very small, creamy, oval
MM 12		M17	Very small, creamy, irregular shape
MM 13		M17	Small, whitish, circular
MM 14		M17	Small, creamy, oval
MM 15		M17	Small, creamy, oval
MM 16		M17	Small, whitish, circular
MM 17		M17	Very small, creamy, oval
MM 18		M17	Very small, creamy, circular
MM 19		M17	Very small, creamy, oval
MM 20		M17	Medium shape, creamy, circular
FOP 1	Fermented oil palm sap	M17	Very large, whitish, irregular
FOP 2		M17	Medium shape, yellowish, circular

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FOP 3	M17	Very small, creamy, oval
FOP 4	M17	Medium shape, yellowish, circular
FOP 5	M17	Very large, creamy, irregular shape
FOP 6	M17	Medium shape, yellowish, circular

Characterization of CFS from *L. brevis* C23

Heat stability of CFS

Heat stability of CFS from *L. brevis* C23 was tested at 40 °C, 50 °C, 60 °C, 70 °C, 80 °C, 90 °C and 100 °C and results are as in Table 2. The CFS of *L. brevis* C23 was stable up to 60 °C. The growth inhibition of *L. monocytogenes* showed there is no significant difference ($p < 0.05$) between them at 40 °C, 50 °C and 60 °C. The CFS tested at temperature 40 °C recorded the highest growth inhibition against *L. monocytogenes* ATCC 7644 at 85.37%, followed by 50 °C (85.15%). When the temperature increased to 70 °C, the growth of *L. monocytogenes* exhibited very low inhibition (6.83%) which is lower as compared to 60 °C (82.67%). There was no growth inhibition of *L. monocytogenes* when temperatures increased to 80 °C, 90 °C and 100 °C.

pH stability of CFS

The pH stability test of CFS from *L. brevis* C23 from acidic to alkaline environment was conducted. From Table 2, the CFS favoured acidic condition as the highest growth inhibition of 87.02%, 81.77% and 86.13%, against *L. monocytogenes* ATCC 7644 was obtained at pH 2, 3 and 4, respectively. The results of growth inhibition of CFS against *L. monocytogenes* ATCC 7644 from pH 2 to pH 4 were markedly higher than pH 5. The growth inhibition recorded at pH 5 was 53.86%, which is 33.15% lower as compared to pH 2 to 4. There was no growth inhibition when the pH increased from 6 to 10.

Effect of different enzymes on CFS stability

Results from effect of different enzymes (α-amylase, catalase, lipase, papain, pepsin, proteinase K and trypsin) on CFS are as in Table 2. The growth inhibition of CFS against *L. monocytogenes* ATCC 7644 was relatively stable when treated with different enzymes at 0.1 mg/mL. There is no significant difference for the growth inhibition by CFS treated with the enzymes at concentration of 0.1 mg/mL, but it was significantly higher as compared to the enzymes at 1 mg/mL ($p < 0.05$). As for the CFS treated with enzymes at concentration of 1 mg/mL, only the CFS treated with catalase, α-amylase and pepsin showed positive inhibition against *L. monocytogenes* ATCC 7644. The highest growth inhibition against *L. monocytogenes* ATCC 7644 was 45.86% from catalase, followed by α-amylase (36.19%) and pepsin (0.84%). There was no growth of inhibition when CFS treated with enzyme proteinase K, trypsin, lipase and papain at 1 mg/mL.

Table 2: The stability test of CFS at different temperature, pH, enzymes and detergents against *L. monocytogenes* ATCC 7644.

Temperature (°C)	Concentration (mg/mL)	Inhibitory percentage (%)
Control		81.63 ± 3.861^{A}
40		85.37 ± 1.030^{A}
50		85.15 ± 0.178^{A}
60		82.67 ± 1.201^{A}
70		6.83 ± 14.709^{B}
80		0
90		0
100		0
pH		
Control		81.63 ± 3.86^{A}
2		87.01 ± 1.755^{A}
3		81.77 ± 3.665^{A}
4		86.13 ± 0.980^{A}
5		53.86 ± 10.984^{B}
6		0
7		0
8		0
9		0
10		0
Enzymes		
Control	-	76.79 ± 0.572^{A}
α-amylase	0.1	78.51 ± 3.807^{A}
	1	36.19 ± 7.633^{AB}
Catalase	0.1	73.50 ± 6.203^{A}
	1	45.86 ± 1.435^{AB}
Lipase	0.1	64.23 ± 9.071^{A}
	1	0
Papain	0.1	82.45 ± 2.174^{A}
	1	0
Pepsin	0.1	83.62 ± 8.080^{A}
	1	0.84 ± 35.615^{B}
Proteinase-K	0.1	78.18 ± 5.453^{A}
	1	0
Trypsin	0.1	63.56 ± 6.530^{A}
	1	0
Detergent		
Control	-	83.43 ± 5.322^{A}
Urea	10	92.42 ± 17.257^{A}
SDS	10	81.45 ± 5.682^{A}
NaCl	10	86.15 ± 2.914^{A}
EDTA	10	81.67 ± 6.081^{A}
Tween 20	10	86.91 ± 1.740^{A}
Tween 80	10	80.08 ± 14.785^{A}
Triton X-100	10	72.05 ± 1.990^{A}

^{A,B}Different superscript indicates quantity are significantly different ($p < 0.05$).
Control indicate CFS of *L. brevis* C23 grown in MRS media with no pH (pH 6.5) adjustment and incubated at 37 °C.

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Effect of detergent on CFS stability

Different types of detergents with concentration of 10 mg/mL were tested for the stability of CFS (Table 2). The growth inhibition of CFS against *L. monocytogenes* ATCC 7644 was relatively stable when treated with all types of detergent. There was no significant difference ($p < 0.05$) of the CFS when treated with the detergents. The highest growth inhibition of *L. monocytogenes* by the CFS was 92.42% which treated with urea. Tween 80, Tween 20, NaCl, EDTA and SDS showed 80.08%, 86.91%, 86.15%, 81.67% and 81.45% of growth inhibition of *L. monocytogenes*, respectively. The growth inhibition of CFS tested with Triton X-100 was 72.05%, which was the lowest among the other detergents.

Fermentation of carbohydrate by *L. brevis* C23

The carbohydrate metabolism reaction of isolate was analysed with API 50 CHL system kit (BioMérieux, Lyon, France). The results for carbohydrate metabolism profile after 24 h and 48 h were as observed in Table 3. *Lactobacillus brevis* C23 able to metabolize 4 different carbohydrates including D-ribose, D-xylose, D-fructose, D-maltose and partially metabolize potassium gluconate and potassium 5-ketogluconate at 24 h. At 48 h, *L. brevis* hydrolysed additional 4 carbohydrate and partially metabolized 1 carbohydrate, L-arabinose, D-glucose, methyl-αD-glucopyranoside, D-melibiose and N-acetylglucosamine.

Bile salt tolerance of *L. brevis* C23

Tolerance of the isolates to bile salts was conducted with the culture of *L. brevis* C23 suspended in different concentrations of bile salts (0%, 0.3%, 0.5%, and 1.0%). Based on Figure 3, the *L. brevis* C23 culture without bile salts showed the highest cell dry weight (0.716 mg), while culture with 0.5% bile salts had the lowest cell dry weight (0.630 mg) after 5 h of incubation. On the other hand, the culture with 0.3% and 1% of bile salts gave a cell dry weight of 0.647 mg and 0.641 mg, respectively. The difference of cell dry weight of *L. brevis* C23 culture with 1% of bile salts in comparison to the highest cell dry weight at 5th hour was 0.075 mg.

Adsorption study

In Table 4, the CFS of *L. brevis* C23 was used as control and the growth inhibition of the CFS against *L. monocytogenes* ATCC 7644 was 94.36%. No antimicrobial activity was detected after treatment of *L. brevis* C23 with 100 mM NaCl solution at pH2, in which the percentage of inhibition on *L. monocytogenes* ATCC 7644 by the *L. brevis* C23 cells (without pH adjusted) was 0.25% and pH adjusted *L. brevis* C23 cells (pH 6.5) was 0%.

Table 3: Biochemical analysis of carbohydrate fermentation of *L. brevis* C23 using API 50CH system.

Carbohydrate	24 h	48 h	Carbohydrate	24 h	48 h
control	-	-	esculin/ ferric citrate	-	-
glycerol	-	-	salicin	-	-
erythritol	-	-	D-cellobiose	-	-
D-arabinose	-	-	D-maltose	++	++
L-arabinose	-	++	D-lactose(bovine)	-	-
D-ribose	++	++	D-melibiose	-	++
D-xylose	++	++	D-saccharose(sucrose)	-	-
L-xylose	-	-	trehalose	-	-
D-adonitol	-	-	inulin	-	-
methyl-ßD-xylopyranoside	-	-	melezitose	-	-
D-galactose	-	-	raffinose	-	-
D-glucose	-	++	amidon	-	-
D-fructose	++	++	glycogen	-	-
D-mannose	-	-	xylitol	-	-
L-sorbose	-	-	gentiobiose	-	-
L-rhamnose	-	-	D-turanose	-	-
dulcitol	-	-	D-lyxose	-	-
inositol	-	-	D-tagatose	-	-
D-mannitol	-	-	D-fucose	-	-
D-sorbitol	-	-	L-fucose	-	-
methyl-αD-mannopyranoside	-	-	D-arabitol	-	-
methyl-αD-glucopyranoside	-	++	L-arabitol	-	-
N-acetylglucosamine	-	+	potassium gluconate	+	+
amygladin	-	-	potassium 2-ketogluconate	-	-
arbutin	-	-	potassium 5-ketogluconate	+	+

Interpretation of *L. brevis* sugar utilisation in API 50CH system at 24 hours and 48 hours; ++ indicates utilisation of sugar (yellow); + indicates partial utilisation of sugar (green); - indicates no utilisation of sugar.

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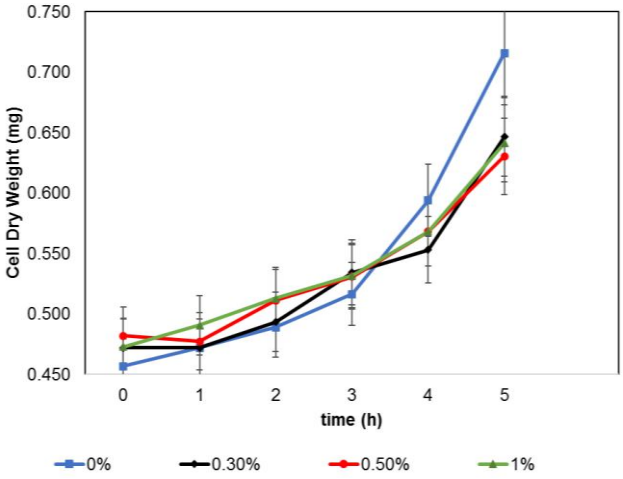

Figure 3: Tolerance of *L. brevis* C23 against different concentrations of bile salts. 0% represents the cell dry weight of *L. brevis* without bile salts; 0.3% represents the cell dry weight of *L. brevis* C23 with addition of 0.3% of bile salts; 0.5% represents the cell dry weight of *L. brevis* with addition of 0.5% of bile salts; 1% represents the cell dry weight of *L. brevis* with addition of 1% of bile salts.

Table 4: Adsorption of the BLIS in CFS of *L. brevis* C23 towards the surface of the producer cells by inhibition determination against *L. monocytogenes* ATCC 7644.

CFS	Inhibition percentage (%)*
Control (CFS) (pH 5.76)	94.36 ± 2.42^{A}
Cells I	0.25 ± 16.17^{B}
Cells II	0

Control represents cell free supernatant (CFS).
Cells I represents CFS without pH adjustment before suspending in NaCl (pH 2).
Cells II represents CFS with pH adjusted to pH 6.5 before suspending in NaCl (pH 2).
*Results are expressed as mean ± standard deviation (n = 2). Different superscript indicates quantity are significantly different ($p < 0.05$).

Evaluation of the effect of CFS on *L. monocytogenes* ATCC 7644 using TEM

Listeria monocytogenes ATCC 7644 with and without treatment of CFS from *L. brevis* C23 was viewed under TEM to elucidate effect of CFS on bacterial cells. In Figure 4B, *L. monocytogenes* ATCC 7644 when treated with CFS was severely damaged. The cell wall and cytoplasm membrane breached, releasing the cytoplasmic content out of the cell which resulted in cell death. On the contrary, *L. monocytogenes* ATCC 7644 without treatment with CFS showed the cell retained in its rod shape (Figure 4A). A smooth surface of cell wall and cytoplasm membrane was observed by enveloping the cells. Cytoplasm content also remained intact within the cytoplasm membrane.

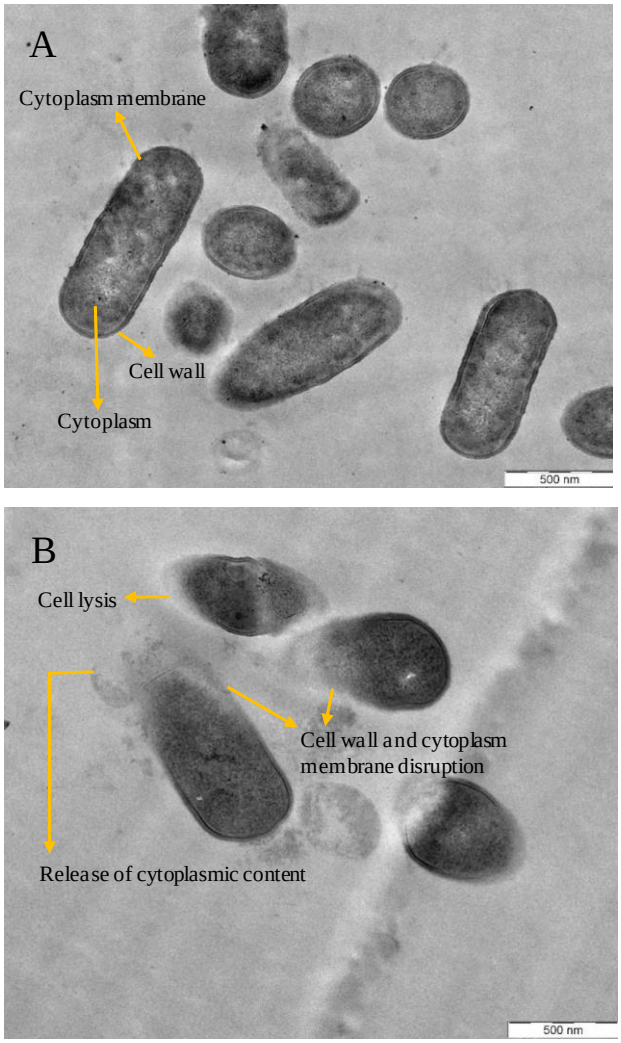

Figure 4: Morphology of *L. monocytogenes* ATCC 7644 under transmission electron microscope. *L. monocytogenes* without treatment (A) and with treatment (B) of CFS from *L. brevis* C23.

DISCUSSION

In this study, the BLIS from *L. brevis* C23 showed high antimicrobial activity against *L. monocytogenes* ATCC 7644, is similar to the previous report in which the *L. brevis* (MF179529) isolated from cow faecal sample also exhibited significant antilisterial activity (Riaz *et al*., 2019). The heat stability test showed that CFS of *L. brevis* C23 was sensitive to temperature beyond 60 °C. This is in contrast to a previous study on heat stability of bacteriocin from *L. brevis* OG1 which exhibited maximum bacteriocin activity at 121 °C for 60 min (Ogunbanwo *et al*., 2003). It was reported bacteriocin produced by *Lactobacillus* species able to resist in high temperature (Yi *et al*., 2016). However, some bacteriocins produced by lactic acid

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bacteria are heat labile peptide. For instances, the bacteriocin from *Streptococcus thermophilus* 81 was stable until 50 °C (Ivanova *et al*., 1998). Similar result was also observed in helveticin J produced by *L. helveticus* 1829 with heat stability until 50 °C (Vaughan *et al*., 1992). The pH sensitivity test of the CFS of *L. brevis* C23 showed high antimicrobial activity and there was no significance difference ($p > 0.05$) among the pH from 2 to 4. The antimicrobial activity was stable even at pH 2, in which this BLIS of *L. brevis* C23 can be widely applied in food preservation especially acidic food. However, the antimicrobial activity decreased from pH 5 and there were no activities beyond pH 6. A similar study on lactocin XN8-A produced by *L. coryniformis* XN8 displayed the highest antimicrobial activity from pH 4 to pH 6 and the activity decreased thereafter. This is due to protein denaturation at high pH level by strong intramolecular electrostatic interactions when the amino and carboxyl splits (Yi *et al*., 2016).

The enzyme sensitivity test of the CFS of *L. brevis* C23 showed that CFS was sensitive to proteolytic enzymes such as trypsin, pepsin, proteinase K and papain at 1 mg/mL. There was no antimicrobial activity recorded when tested with these enzymes confirming the proteinaceous nature of BLIS from *L. brevis* C23. It is in agreement with the results of Avaiyarasi *et al*. (2016) who reported that antimicrobial activity of bacteriocin produced by *Lactobacillus sakei* GM3 was lost after the treatment of pepsin, trypsin and proteinase K. As for treatment with catalase at 0.1 and 1 mg/mL, the antimicrobial activity of CFS was not fully inhibited, confirming that the antimicrobial activity of CFS was not due to the presence of hydrogen peroxidase (Ogunbanwo *et al*., 2003). The antimicrobial activity of CFS was not fully inhibited when treated with α-amylase at 0.1 and 1 mg/mL indicating that the bacteriocin is not glycosylated. A similar result was reported on antimicrobial activity of bacteriocin produced from *Pediococcus acidilacti* HA-6111-2 and *P. acidilacti* HA-5692-*3* after treating with catalase and α-amylase. When the CFS was treated with lipase, there was no antimicrobial activity at 1 mg/mL suggesting that the bacteriocin is lipid moiety (Albano *et al*., 2007).

In the detergent stability test, CFS was relatively stable in all the detergents at 10 mg/mL. The stability against surfactants and detergents favours a broad application of the bacteriocin because of its potential to retain its structure and function during different purification steps. Previous study showed that the plantaricin LPL-1 from *L. plantarum* LPL-1 had no difference in the antimicrobial activity when tested with EDTA, Tween 20, Tween 80, and urea, hence the BLIS is suitable to be used for emulsified food (Wang *et al*., 2018). Similar other study also reported that bacteriocin from *L. plantarum* bacST202Ch and *L. plantarum* bacST216Ch showed high resistance on the detergents (Todorov *et al*., 2010). There are many studies on the stability of bacteriocins on surfactants but mostly did not highlight the bactericidal effect of individual detergent. In the study of Millette *et al.* (2007) has reported that control was performed to verify the antimicrobial potential of detergents assayed such as Tween-80, Triton X-100, SDS and urea at 1% of concentration, and none demonstrated antimicrobial activity per se.

Lactobacillus brevis C23 was able to survive and grow at 0.3%, 0.5% and 1% of bile salts for 5 h. In the study by Gilliland *et al*. (1984), 0.3% of bile salts are considered as a threshold concentration for screening of bile salts resistant strains. The methods of determination of resistant strains (delay of growth, $d \leq 15$ min), tolerant strains ($15 \text{ min} < d \leq 40$ min), weakly tolerant strains ($40 \text{ min} < d < 60$ min) and sensitive strains ($d \leq 60$ min) were used. In this study, *L. brevis* C23 at 0.3%, 0.5% and 1% of bile salts was able to grow parallel to the control strain up to 4 h, indicating that the *L. brevis* C23 is highly tolerant to bile salts. Similar studies showed that *Pediococcus pentosaceus* CE65 able to survive at 0.3% bile salts (Manini *et al*., 2016). Another study with *L. mucosae* AN1 also showed resistance towards bile salts from 0.1 to 0.7% (Repally *et al*., 2018).

For the adsorption study, the antimicrobial activity of CFS (pH 2) after the cell washing had almost no activity indicating that all the antimicrobial peptides did not adhere to the surface of the producer cells (Banerjee *et al*., 2013). Similar studies were observed for pediocin ST18, brevicin FPTLB3, and plantaricin C19 where no antimicrobial activity was recoded at pH 2 suggesting the bacteriocin did not adhere on the producer cells (Atrih *et al*., 2001; Todorov and Dicks, 2005; Banerjee *et al.*, 2013).

Listeria monocytogenes ATCC 7644 when treated with CFS, the cell wall and cytoplasm membrane were disintegrated and spewing the cytoplasmic content out. This shows the CFS from *L. brevis* C23 facilitated the cell wall and cytoplasm permeability thus reducing the integrity of the membrane resulting the dispersion of the cytoplasmic content and cell death (Qiao *et al*., 2020). Similar results was reported on the *Bacillus cereus* when treated with bacteriocin from *Weissella confusa* A3 and *L. monocytogenes* CMCC 1595 when treated with enterocin TJUQ1 (Goh and Philip, 2015; Qiao *et al*., 2020).

CONCLUSION

Lactobacillus brevis C23 isolated from cabbage had shown a very high potential in food preservation or usage as coating for food packaging application. The CFS showed a significantly high antimicrobial activity against food pathogen, *L. monocytogenes* ATCC 7644. The BLIS of *L. brevis* C23 was proteinaceous nature. The CFS of *L. brevis* C23 did not favour alkaline environment but showed high antimicrobial activity at acidic environment. Hence, the CFS able to withstand at lower pH and is useful in packaging or preserving acidic and pickled foods. In addition, the CFS was not affected by detergents which play an important role in food industry. Moreover, the CFS able to withstand bile salts even at critical concentration. Combining with the survivability at low pH and critical bile salts concentration, the CFS able to withstand the digestive environment in our body. The antimicrobial activity of CFS was elucidated as the cell

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lysis was occurred to the *L. monocytogenes*. However, additional studies are required to verify *in vivo* the effectiveness of selected strains and analyse the components that are present in the CFS using SDS-PAGE method.

ACCESSION NUMBER

Lactobacillus brevis strain C23 16S ribosomal RNA gene, partial sequence, MN880215.

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REFERENCES

Abbasiliasi, S., Tan, J. S., Bashokouh, F., Ibrahim, T. A. T., Mustafa, S., Vakhshiteh, F., Sivasamboo, S. and Ariff, A. B. (2017). *In vitro* assessment of *Pediococcus acidilactici* Kp10 for its potential use in the food industry. *BMC Microbiology* **17(1), 1-11.**

Abbasiliasi, S., Tan, J. S., Ibrahim, T. A. T., Ramanan, R. N., Vakhshiteh, F., Mustafa, S., Ling, T. C., Rahim, R. A. and Ariff, A. B. (2012). Isolation of *Pediococcus acidilactici* Kp10 with ability to secrete bacteriocin-like inhibitory substance from milk products for applications in food industry. *BMC Microbiology* **12(1), 1-12.**

Abdulmumeen, H. A., Risikat, A. N. and Sururah, A. R. (2012). Food: Its preservatives, additives and applications. *International Journal of Chemical and Biochemical Sciences* **1, 36-47.**

Albano, H., Todorov, S. D., van Reenen, C. A., Hogg, T., Dicks, L. M. and Teixeira, P. (2007). Characterization of two bacteriocins produced by *Pediococcus acidilactici* isolated from "Alheira", a fermented sausage traditionally produced in Portugal. *International Journal of Food Microbiology* **116(2), 239-247.**

Amézquita, A. and Brashears, M. M. (2002). Competitive inhibition of *Listeria monocytogenes* in ready-to-eat meat products by lactic acid bacteria. *Journal of Food Protection* **65(2), 316-325.**

Atrih, A., Rekhif, N., Moir, A. J. G., Lebrihi, A. and Lefebvre, G. (2001). Mode of action, purification and amino acid sequence of plantaricin C19, an anti-Listeria bacteriocin produced by *Lactobacillus plantarum* C19. *International Journal of Food Microbiology* **68(1-2), 93-104.**

Avaiyarasi, N. D., Ravindran, A. D., Venkatesh, P. and Arul, V. (2016). *In vitro* selection, characterization and cytotoxic effect of bacteriocin of *Lactobacillus sakei* GM3 isolated from goat milk. *Food Control* **69, 124-133.**

Banerjee, S. P., Dora, K. C. and Chowdhury, S. (2013). Detection, partial purification and characterization of bacteriocin produced by *Lactobacillus brevis* FPTLB3 isolated from freshwater fish. *Journal of Food Science and Technology* **50(1), 17-25.**

Choi, S. H. and Chin, K. B. (2003). Evaluation of sodium lactate as a replacement for conventional chemical preservatives in comminuted sausages inoculated with *Listeria monocytogenes*. *Meat Science* **65(1), 531-537.**

Gilliland, S. E., Staley, T. E. and Bush, L. J. (1984). Importance of bile tolerance of *Lactobacillus acidophilus* used as a dietary adjunct. *Journal of Dairy Science* **67(12), 3045-3051.**

Goh, H. F. and Philip, K. (2015). Purification and characterization of bacteriocin produced by *Weissella confusa* A3 of dairy origin. *PLoS ONE* **10(10), e0140434.**

Güllüce, M., Karadayı, M., and Barış, Ö. (2013). Bacteriocins: Promising natural antimicrobials. *In:* Microbial Pathogens and Strategies for Combating Them: Science, Technology and Education. Méndez-Vilas, A. (ed.). FORMATEX, Madrid, Spain**. pp. 1016-1027.**

Ivanova, I., Miteva, V., Stefanova, T. S., Pantev, A., Budakov, I., Danova, S., Moncheva, P., Nikolova, I., Dousset, X. and Boyaval, P. (1998). Characterization of a bacteriocin produced by *Streptococcus thermophilus* 81. *International Journal of Food Microbiology* **42(3), 147-158.**

Manini, F., Casiraghi, M. C., Poutanen, K., Brasca, M., Erba, D. and Plumed-Ferrer, C. (2016). Characterization of lactic acid bacteria isolated from wheat bran sourdough. *LWT - Food Science and Technology* **66, 275-283.**

Millette, M., Dupont, C., Archambault, D. and Lacroix, M. (2007). Partial characterization of bacteriocins produced by human *Lactococcus lactis* and *Pediococccus acidilactici* isolates. *Journal of Applied Microbiology* **102(1), 274-282.**

Nettles, C. G. and Barefoot, S. F. (1993). Biochemical and genetic characteristics of bacteriocins of food-associated lactic acid bacteria. *Journal of Food Protection* **56(4), 338-356.**

Ng, Z. J., Zarin, M. A., Lee, C. K., Phapugrangkul, P. and Tan, J. S. (2020). Isolation and characterization of *Enterococcus faecium* DSM 20477 with ability to secrete antimicrobial substance for the inhibition of oral pathogen *Streptococcus mutans* UKMCC 1019. *Archives of Oral Biology* **110, 104617.**

Ogunbanwo, S. T., Sanni, A. I. and Onilude, A. A. (2003). Characterization of bacteriocin produced by *Lactobacillus plantarum* F1 and *Lactobacillus brevis* OG1. *African Journal of Biotechnology* **2(8), 219-227.**

Qiao, X., Du, R., Wang, Y., Han, Y. and Zhou, Z. (2020). Purification, characterization and mode of action of enterocin, a novel bacteriocin produced by *Enterococcus faecium* TJUQ1. *International Journal of Biological Macromolecules* **144, 151-159.**

Repally, A., Perumal, V., Dasari, A., Palanichamy, E. and Venkatesan, A. (2018). Isolation, identification of *Lactobacillus mucosae* AN1 and its antilisterial peptide

purification and characterization. *Probiotics and Antimicrobial Proteins* **10(4), 775-786.**

Riaz, A., Noureen, S., Liqat, I., Arshad, M. and Arshad, N. (2019). Antilisterial efficacy of *Lactobacillus brevis* MF179529 from cow: An *in vivo* evidence. *BMC Complementary and Alternative Medicine* **19(1), 1-9.**

Shamloo, E., Hosseini, H., Abdi Moghadam, Z., Halberg Larsen, M., Haslberger, A. and Alebouyeh, M. (2019). Importance of *Listeria monocytogenes* in food safety: A review of its prevalence, detection, and antibiotic resistance. *Iranian Journal of Veterinary Research* **20(4), 241.**

Složilová, I., Purkrtova, S., Kosova, M., MlhULOVÁ, M., Šviráková, E. and Demnerová, K. (2014). Antilisterial activity of lactic acid bacteria against *Listeria monocytogenes* strains originating from different sources. *Czech Journal of Food Sciences* **32(2), 145-151.**

Todorov, S. D. and Dicks, L. M. T. (2005). Pediocin ST18, an anti-listerial bacteriocin produced by *Pediococcus pentosaceus* ST18 isolated from boza, a traditional cereal beverage from Bulgaria. *Process Biochemistry* **40(1), 365-370.**

Todorov, S. D., Ho, P., Vaz-Velho, M. and Dicks, L. M. T. (2010). Characterization of bacteriocins produced by two strains of *Lactobacillus plantarum* isolated from Beloura and Chouriço, traditional pork products from Portugal. *Meat Science* **84(3), 334-343.**

Ünlü, G., Nielsen, B. and Ionita, C. (2016). Inhibition of *Listeria monocytogenes* in hot dogs by surface application of freeze-dried bacteriocin-containing powders from lactic acid bacteria. *Probiotics and Antimicrobial Proteins* **8(2), 102-110.**

Vaughan, E. E., Daly, C. and Fitzgerald, G. F. (1992). Identification and characterization of helveticin V‐1829, a bacteriocin produced by *Lactobacillus helveticus* 1829. *Journal of Applied Bacteriology* **73(4), 299-308.**

Wang, Y., Qin, Y., Xie, Q., Zhang, Y., Hu, J. and Li, P. (2018). Purification and characterization of plantaricin LPL-1, a novel class IIa bacteriocin produced by *Lactobacillus plantarum* LPL-1 isolated from fermented fish. *Frontiers in Microbiology* **9, 2276.**

Yang, R., Johnson, M. C. and Ray, B. (1992). Novel method to extract large amounts of bacteriocins from lactic acid bacteria. *Applied and Environmental Microbiology* **58(10), 3355-3359.**

Yi, L., Dang, J., Zhang, L., Wu, Y., Liu, B. and Lü, X. (2016). Purification, characterization and bactericidal mechanism of a broad spectrum bacteriocin with antimicrobial activity against multidrug-resistant strains produced by *Lactobacillus coryniformis* XN8. *Food Control* **67, 53-62.**

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