CHAPTER 4

DETERMINATION OF PROBIOTIC POTENTIAL OF PROTEOLYTIC LACTIC ACID BACTERIA AND ANTIOXIDATIVE ACTIVITY IN WHEY BUFFALO MILK

4.1 Introduction

Growth of public awareness regarding human and animal diets related to health benefits with food and feed containing bacteria including LAB or the LAB still conserves their beneficial properties including bioactive peptides after consumption (Donkor et al., 2007). Selection of probiotic microorganisms beginning from culturing bacteria in the fermentation stage is crucial to produce effective, and reliable bioactive peptides (Jaimez-Ordaz et al., 2019). However, not all LABs with proteolytic activity are considered as probiotic bacteria (Donkor et al., 2007). Characterisation of probiotics should be included in the microorganism's identification including LAB as a selective starter culture.

The well-known LABs that have been identified as probiotic bacteria were *Lactobacillus acidophilus, L. casei*, *L. rhamnosus*, and *L. plantarum* (Harzallah & Belhadj, 2013). Probiotic LABs are characterised by their ability to colonise in gastrointestinal tract (GIT), able to stabilize GIT microbiota, possible to counter back the action of harmful microorganisms, and may to stimulate the immune response in GIT (Florou-Paneri et al., 2013). During consumption of probiotic products, the product should contain a sufficient number of probiotic microorganisms which capable to survive and maintain viable populations especially through acidic and bile surrounding inside stomach and the GIT (Donkor et al., 2007; Salem et al., 2013).

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The colonisation and potential health benefits of probiotic LAB can only be anticipated when these viable populations are able to survive through these natural barriers of GIT. Shi et al. (2016) mentioned that the number of viable cells of probiotics in any product should be at least 10^6 colony-forming units per millilitre (cfu/ml) at the expiry date for health and functional claims. These probiotic LAB may improve a healthy balancing microbiota in GIT microbial ecosystem against gastrointestinal disorder, enhance immune system, reduce serum cholesterol, might prevent cancer, improve periodontal health, reduce blood pressure, and improve lactose metabolism (Shi et al., 2016; Wedajo, 2015).

Antioxidant peptides are generated from many protein sources and milk is one of the main sources. Most studies preferred cow milk as their main substrate because of the abundance availability and easy supply. However, an interesting finding found that protein content in buffalo milk is higher than human, cow, camel, and goat milks (Ahmad et.al, 2013; Hashmi & Saleem, 2015; Mahmood & Usman, 2010; Rosati & Van Vleck, 2002; Soliman, 2005). Protein composition from different types of milk revealed that buffalo milk is the second largest protein content (4.01 to 4.78 %) compared to cow milk (2.98 to 3.87 %) (Mahmood & Usman 2010). Furthermore, concentrations of casein and whey in buffalo milk were also higher than in cow milk (Arora & Khetra, 2017; Khedkar et al., 2016).

Previously, hydrolysis of protein in buffalo milk via enzymes was studied to generate antioxidative peptides. Proteolysis happened when buffalo milk casein was digested with different combinations of pepsin, trypsin, and chymotrypsin to produce antioxidative peptides (Shanmugam et al., 2015) while buffalo cheese whey was hydrolysed with different combinations of trypsin, chymotrypsin, and carboxypeptidase-A to produce antioxidative peptides with 24 kDa in molecular weight

(Bassan et al., 2015). Furthermore, potential antioxidative peptides against tert-butylhydroperoxide-induced colon cancer from water-soluble milk peptides were extracted from both cow and buffalo cheddar cheese. Antioxidant assay of ABTS radical scavenging was conducted for the water-soluble peptides extracted from buffalo cheddar cheese showed the higher antioxidant activity (15.95 %) than cow cheddar cheese (15.88 %) at 150th day throughout the cheese ripening period (Huma et al., 2018).

In the present study, previously, the potential of LAB isolates to generate antioxidative peptides from whey skimmed milk fermented at 37 °C for 24 h fermentation was determined using scavenging of 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical activity, and ferrous ions chelating activity (FICA) assay. Selection of LAB with proteolytic activity, and able to generate antioxidative activity in the whey skimmed milk were further evaluated for their probiotic potential to enhance the safety of LAB and to support self-assurance from consumers. Thus, the objectives of this present study were to evaluate the probiotic characteristics of selected LAB isolates, and to determine the antioxidant activity of peptides hydrolysed from whey buffalo milk by the isolated LABs using both DPPH free radical scavenging activity and FICA assays fermented at 37 °C for 24 h.

4.2 Materials and Methods

4.2.1 Preparation and Growth Conditions of Lactic Acid Bacteria

The bacterial culture preparation of LAB isolates namely isolates Bd2, Pk2, WG2, and S1 of this section was described as in Section 3.2.1. These four isolates were selected for determining its probiotic characteristics due to these isolates being able to hydrolyse protein to antioxidant peptides with the four highest antioxidant activity amongst all isolates.

4.2.2 Growth Conditions of Pathogenic Bacteria

Five selected pathogenic bacteria namely *Bacillus cereus* ATCC 10876 (*B. cereus*), *Bacillus subtilis* ATCC 21332 (*B. subtilis*), *Staphylococcus aureus* ATCC 25923 (*S. aureus*), *Salmonella typhimurium* ATCC 13311 (*S. typhimurium*), and *Escherichia coli* ATCC 25922 (*E. coli*) were obtained from the culture collection of Microbiology Laboratory of Universiti Sains Islam Malaysia (USIM). These bacteria were grown at 37 °C for 24 h on nutrient agar (Merck) plates to get a single colony for each targeted bacterium and was kept in 2 °C (cold room storage). Prior to analysis, each bacterial strain was sub-cultured in nutrient broth at 37 °C for 24 h, and until the growth concentration was to 10^6 cfu/ ml.

4.2.3 Determination of Acid and Bile Tolerance of Lactic Acid Bacteria

4.2.3.1 Tolerance to Acid Condition

Ten ml of 24 h MRS each culture of LAB isolates (isolates Bd2, PK2, WG2, and S1) was harvested by centrifuging at 6,000 x g for 15 min (at 4 °C) (AllegraTM 25R centrifuge, Beckman CoulterTM), and re-suspended in an equal volume of MRS broth (Oxoid CM0359) with adjustment of pH to 2.0 using 1M HCl (Fisher Scientific). The suspended cells were incubated anaerobically at 37 °C for 90 min. Viable counts were determined before and after incubation by diluting samples in peptone water (0.1 %, w/v) (Liofilchem 610038) and spreading on the MRS agar plate appropriate dilutions onto MRS agar. Plates were incubated anaerobically at 37 °C for 48 h (Kheadr, 2006).

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4.2.3.2 Tolerance to Bile Condition

Ten ml of 24 h MRS each culture of LAB isolates (isolates Bd2, PK2, WG2, and S1) was harvested by centrifuging at 6,000 x g for 15 min (at 4 °C), re-suspended in an equal volume of MRS broth, and were adjusted the pH to 6.5 (containing 0.3 % (w/v) bile). The suspended cells were incubated anaerobically at 37 °C for 90 min. Viable counts were determined before and after incubation by diluting samples in peptone water (0.1 %, w/v) and plating appropriate dilutions onto MRS agar (Oxoid CM0361). Plates were incubated anaerobically at 37 °C for 48 h (Kheadr, 2006).

4.2.4 Preparation of Cell Free Supernatants of Lactic Acid Bacteria

The cell free supernatants (CFSs) of four LAB strains (isolates Bd2, PK2, WG2, and S1) with good proteolytic activity in the previous study were incubated in De Man Rogosa Sharpe (MRS) broth at 37 °C for 24 h. Bacterial cells were removed by centrifuging the culture at 8000 x g for 5 min (at 4 °C). The supernatants were kept sterilised, filtered through a filter with a pore size of 0.22 µm, and stored in a universal bottle at 4 °C until further analysis and for antimicrobial activity test (Vuyst et al., 2004).

4.2.5 Determination of Antibacterial Activity of Lactic Acid Bacteria Isolates

4.2.5.1 Dual Culture Overlay Method

The four LAB strains (isolates Bd2, PK2, WG2, and S1) were screened for their antibacterial activity using dual culture overlay method or agar spot test method. The dual culture overlay method was performed using MRS agar plates according to method of Schillinger and Lucke (1989) with modification. Initially, the overnight cultures of the LAB strains were inoculated onto the surface of MRS agar plates and incubated at 37 °C for 24 h in anaerobic condition to allow colonies to grow. The agar plate was

then overlaid with 2 ml of warm nutrient agar on which the LAB was grown. Each target bacteria with growth of 10^6 cells per ml was swabbed on hardened nutrient agar aseptically. After incubation at 37 °C for 24 h, all the plates were measured for inhibition zones. Triplicate tests were conducted for each LAB strain against five selected pathogenic bacteria. The diameter (mm) of inhibition zones were measured and were expressed as mean average in triplicate analysis (Klewicka & Libudzisz, 2004).

4.2.5.2 Agar Well Diffusion Method

The cell free supernatants (CFSs) of four LAB strains (isolates Bd2, PK2, WG2, and S1) were evaluated for its antibacterial activity using agar well diffusion against five targeted pathogenic bacteria. Each pathogenic bacteria was swabbed on the lawn nutrient agar plate antiseptically. Then, aliquots amount of 50 µL of CFS of each LAB strain was poured into 6 mm well in diameter on nutrient agar plate containing the pathogenic bacteria. The MRS broth was used as positive control and after incubation at 37 °C for 24 h, the clear zone of the inhibition zone surrounding the well was measured in diameter (mm). Triplicate tests were conducted for each CFSs of LAB strain against the five pathogenic bacteria. The diameter (mm) of inhibition zones were measured and were expressed as mean average in triplicate analysis (Batdory et al., 2006).

4.2.6 Collection of Buffalo Milk

Fresh raw whole buffalo milk (Murrah breed of *Bubalus bubalis*) was obtained from a local breeder at Bangi, Selangor, Malaysia. Buffalo milk was milked in the early morning starting at 5 a.m. with proper sanitation by a farm worker. The collected

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buffalo milk was weighed, wrapped tightly in a sterile plastic, and kept in a freezer temperature of -18 °C. Then, the buffalo milk sample was transferred from a freezer condition into a portable ice box container that containing ice in water to maintain the temperature of the surrounding buffalo milk sample inside the box ranged from 0 °C to 4 °C. The buffalo milk sample was delivered to analytical laboratory within 36 h after milk collection (Bernard & Glass, 1975).

4.2.7 Preparation of Bacterial Cultures and Fermentation Conditions

4.2.7.1 Preparation of Lactic Acid Bacteria Isolates

The four LAB isolates (isolates Bd2, PK2, WG2, and S1) were inoculated into 10 ml MRS broth and incubated at 37 °C for 24 h. The preculture of LAB was prepared similar to the preparation of preculture in Section 3.2.3.1. Initially, 1 % (v/v) of LAB culture was inoculated into sterilised skimmed milk (sterilised at 110 °C for 10 min) and incubated at 37 °C for 24 h for preculture preparation. After that, 2 % (v/v) of the precultured LAB strain was propagated into pasteurised buffalo milk (pasteurised at 62 °C for 30 min). The pasteurised buffalo milk without LAB culture was served as control experiment and was then incubated at 37 °C for 24 h. As comparison, a direct culture of 2 % (v/v) of LAB isolates was transferred directly from incubation at 37 °C for 24 h in MRS broth into buffalo milk. Fermentation of buffalo milk with LABs from both preculture and direct culture was carried out in trice replicated experiment for all the four selected LAB isolates.

4.2.7.2 Preparation of Whey Fraction from Fermented Buffalo Milk

The whey fraction of fermented buffalo milk was prepared similar to the preparation of whey fraction from fermented skimmed milk as mentioned in Section

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3.2.3.2. However, in the buffalo milk fermentation, the pH of fermented buffalo milk was not adjusted to 4.6 as in fermented skimmed milk. The initial pH of fermented buffalo milk fermented from preculture of four isolates were pH 4.25, 4.19, 4.88, and 4.32 for isolates Bd2, Pk2, WG2, and S1, respectively. Indeed, the suspension was harvested by centrifuging at 10,000 x g for 20 min (at 4 °C) and the supernatant was filtered using 0.45 µm filter (Millipore Corp, USA). The whey buffalo milk was then analysed its antioxidant activity using DPPH radical scavenging activity and FICA assays. The pasteurised buffalo milk with bacteria at 0 h was used as a control experiment.

4.2.8 Determination of Antioxidant Activity of Whey Buffalo Milk

4.2.8.1 Scavenging of 1,1-Diphenyl-2-Picrylhydrazyl Free Radical Activity

Determination of 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity of whey buffalo milk was evaluated according to the method of Son and Lewis (2002) with modification as mentioned in Section 3.2.4.1.

4.2.8.2 Ferrous Ion Chelating Activity

The ferrous ion chelating activity or FICA of whey buffalo milk generated by four LAB isolates (isolates Bd2, PK2, WG2, and S1) was determined using the method of Decker and Welch (1990) with modification similar to as described in Section 3.2.4.2.

4.2.9 Statistical Analysis

All experiments regarding acid and bile tolerance of isolates, antibacterial activities of isolates, and antioxidant activities of whey buffalo milk were presented as

mean ± standard deviations. One-way and two-way analysis of variance (ANOVA) and Tukey tests were used to determine the significant differences between means ($P<0.05$). All data was analysed using Minitab software version 16 (Germany).

4.3 Results

4.3.1 Resistance to Acid and Bile

All four LAB isolates (isolates Bd2, PK2, WG2, and S1) survived in the acidic (pH 2.0) and bile (pH 6.5) environments with slightly reduction (maximally <1.05 log cycle) in viable cell counts. All LAB isolates reduced viability ranged from 0.06 to 1.05 log cfu/ml after exposuring 90 min at acidic condition (pH 2.0). The control probiotic *L. plantarum* ATCC 8014 obtained slight reduction in viable cell counts by 0.10 log cfu/ml while isolate WG2 obtained the lowest reduction ($P<0.05$) in cell viability by 0.06 log cfu/ml which demonstrated that this strain was able to survive in acidic stress compared to other isolates (Figure 4.1).

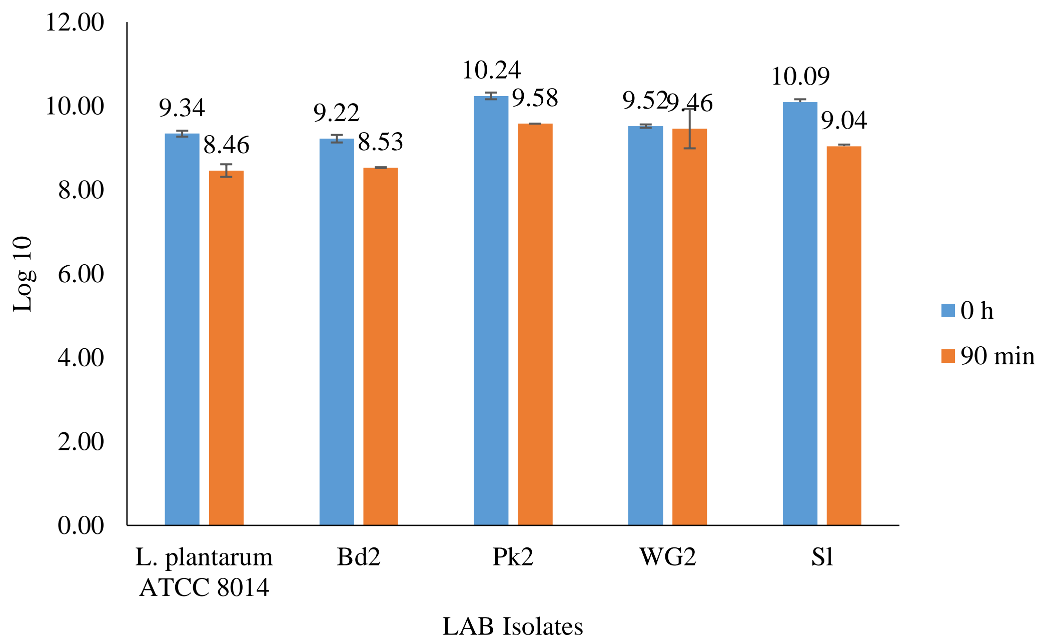

Figure 4.1: Viability of LAB Isolates at 0 and 90 min in pH 2

The ability of the bacterial isolates to support stomach aggressive conditions were tested by exposing to bile salt treatment. Results found that the LAB isolates could survive on bile stress (pH 6.5) with different stress ability. In general, exposure of the four LAB isolates in bile stress for 90 min resulted in slight increment (P<0.05) of viability except for isolates PK2 and S1. All four LAB isolates found significant viability (P<0.05) against bile salt environment. Control *L. plantarum* ATCC 8014 increased the highest viability with the log cycle 0.70 cfu/ml compared to isolate WG2 and Bd2. Isolate WG2 and Bd2 also displayed an increment in viable count with the log cycle of 0.03 cfu/ml and 0.37 cfu/ml viable cell counts, respectively (Figure 4.2).

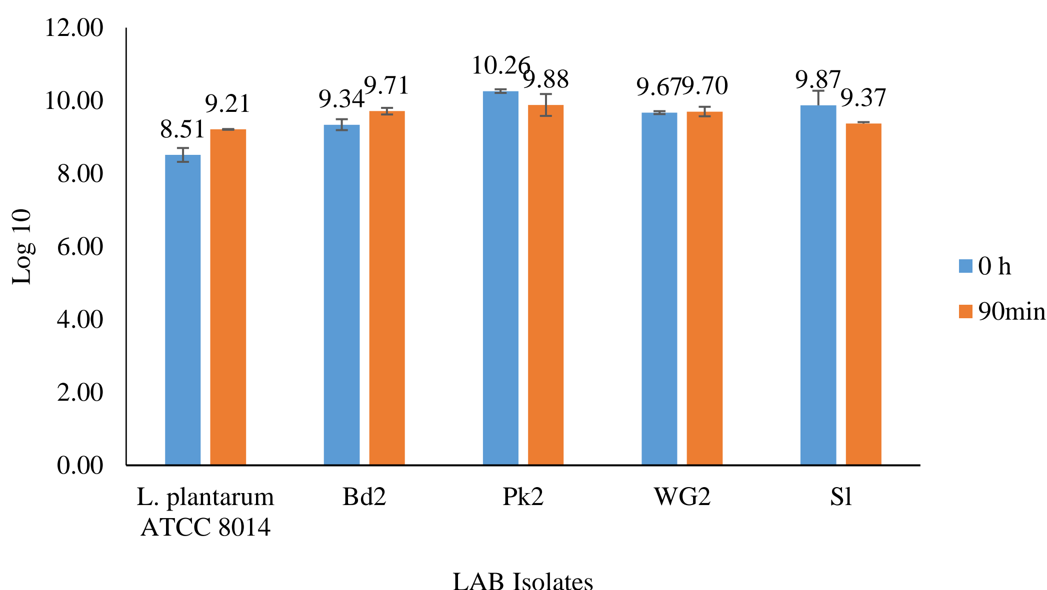

Figure 4.2: Viability of LAB Isolates at 0 and 90 min in pH 6.5

4.3.2 Antibacterial Activity of Lactic Acid Bacteria Isolates

The four isolates (isolates Bd2, PK2, WG2, and S1) were also tested for its antibacterial properties against common food pathogenic bacteria namely *B. cereus*

ATCC 10876, *B. subtilis* ATCC 21332, *S. aureus* ATCC 25923, *S. typhimurium* ATCC 13311, and *E. coli* ATCC 25922. The antibacterial activities of LAB isolates against these pathogenic bacteria were presented in diameter of inhibition zones (mm) using both dual culture overlay method (Table 4.1) and using agar well diffusion methods (Table 4.2). From the Table, it was found that all the four LAB isolates obtained inhibitory activities against the pathogenic bacteria using both assays.

In the dual culture overlay method, all the four LAB isolates indicated strong inhibitory activity against pathogenic bacteria *S. typhimurium* ATCC 1331, and *E. coli* ATCC 25922 (Table 4.1).

Table 4.1: Inhibition Zones (mm) of LAB Isolates Against Pathogenic Bacteria Using Dual Culture Overlay Method

LAB isolates	**Pathogenic Bacteria**				
	B. cereus ATCC 10876	*B. subtilis* ATCC 21332	*S. aureus* ATCC 25923	*S. typhimurium* ATCC 13311	*E. coli* ATCC 25922
Bd2	26.00 ± 6.93	24.00 ± 9.54	23.33 ± 10.41	85.00 ± 00.00	85.00 ± 00.00
Pk2	32.67 ± 6.81	29.33 ± 5.03	27.00 ± 2.65	85.00 ± 00.00	85.00 ± 00.00
WG2	18.33 ± 8.14	30.33 ± 15.70	24.67 ± 4.62	85.00 ± 00.00	85.00 ± 00.00
Sl	28.33 ± 7.64	25.00 ± 15.62	24.33 ± 6.03	85.00 ± 00.00	85.00 ± 00.00

Note: Results are presented in mean ± standard deviation of diameter (mm) of growth inhibitory zone of pathogenic bacteria which measured at 37 °C after 24 h incubation

It was observed that isolates Bd2, Pk2, WG2, and S1 obtained clear zone inhibition almost every area of petri dish plate containing nutrient agar against these pathogenic bacteria of *S. typhimurium* and *E. coli* ATCC 25922 with diameter of clear inhibitory zone of 85.00 mm. However, all these four LAB isolates inhibited pathogenic bacteria of the *B. cereus* ATCC 10876, *B. subtilis* ATCC 21332, and *S. aureus* ATCC 25923 moderately with inhibitory zone ranged between 18.33 and 32.67 mm (Table 4.1). The inhibition zone of all these LAB isolates to inhibit *B. cereus* ATCC 10876,

B. subtilis ATCC 21332, and *S. aureus* ATCC 25923 were found insignificant (*P*>0.05) between the LAB isolates.

Antibacterial activity of LAB isolates using agar well diffusion is shown in Table 4.2. From the Table 4.2, it was found that the inhibitory activity of the four LAB isolates was moderately inhibit all the pathogenic bacteria of *B. cereus* ATCC 10876, *B. subtilis* ATCC 21332, *S. aureus* ATCC 25923, *S. typhimurium* ATCC 13311, and *E. coli* ATCC 25922. Results found that all LAB isolates inhibited (*P*>0.05) *E. coli* ATCC 25922 with the maximum diameter of inhibition zone of 13.83 mm. However, the minimum inhibition zone (13.33 mm) against *E. coli* ATCC 25922 was shown by isolate WG2. Interestingly, isolate Pk2 exhibited the highest inhibition zone for pathogenic bacteria of *B. cereus* ATCC 10876, *B. subtilis* ATCC 21332, and *E. coli* ATCC 25922 with the inhibition zone of 10.67 mm, 11.50 mm, and 13.83 mm, respectively. On the contrary, isolate S1 found the lowest inhibition zone for pathogenic bacteria of *B. cereus* ATCC 10876, *S. aureus* ATCC 25923, and *S. typhimurium* ATCC 13311 with the inhibition zone of 9.33 mm, 8.00 mm, and 10.83 mm, respectively (Table 4.2).

Table 4.2: Inhibition Zones (mm) of LAB Isolates Against Pathogenic Bacteria Using Agar Well Diffusion Method

LAB isolates	**Pathogenic Bacteria**				
	B. cereus ATCC 10876	*B. subtilis* ATCC 21332	*S. aureus* ATCC 25923	*S. typhimurium* ATCC 13311	*E. coli* ATCC 25922
Bd2	9.67 ± 0.76	9.00 ± 0.00	11.67 ± 4.51	11.17 ± 3.18	13.83 ± 4.65
Pk2	10.67 ± 2.93	11.50 ± 1.00	11.33 ± 2.93	11.50 ± 3.12	13.83 ± 5.11
WG2	10.00 ± 2.00	9.17 ± 1.44	11.50 ± 4.27	12.00 ± 5.29	13.33 ± 6.03
S1	9.33 ± 3.82	10.00 ± 2.65	8.00 ± 7.00	10.83 ± 2.93	13.83 ± 5.92

Note: Results are presented in mean ± standard deviation of diameter (mm) of growth inhibitory zone of pathogenic bacteria which measured at 37 °C after 24 h incubation

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The example of the inhibition zone of LAB isolate (isolate W2) against pathogenic bacteria (*B. cereus* ATCC 10876) using dual culture overlay method is shown in Figure 4.3(a), while Figure 4.3(b) shows the example of inhibition zone of LAB isolates (isolates WG2, Pk2 and Bd2) against *E. coli* ATCC 25922 using agar well diffusion method.

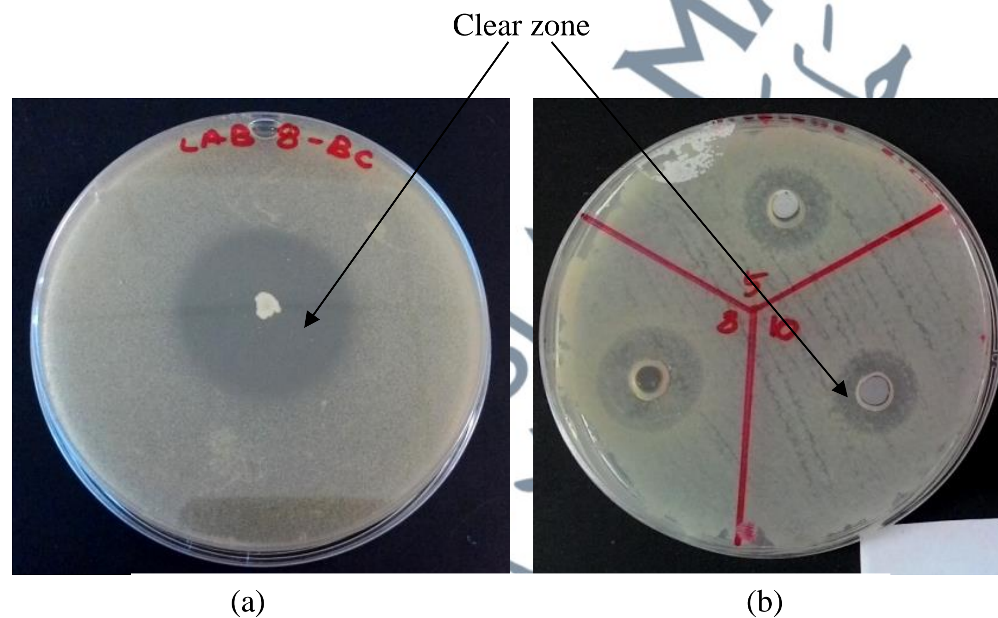

Figure 4.3: (a) Inhibition Zone of isolate WG2 Using Dual Culture Overlay Method against *B. cereus* ATCC 10876, and (b) Inhibition Zone of Isolates WG2, Pk2, and Bd2 Using Agar Well Diffusion Method Against *E. coli* ATCC 25922

4.3.3 Antioxidant Activity of Whey Buffalo Milk

Figure 4.4 shows DPPH free radical scavenging activity of whey buffalo milk produced by four LAB isolates (isolates Bd2, Pk2, WG2, and S1) by both preculture and direct culture of LAB isolates. Results clearly found that the DPPH free radical scavenging activity of whey buffalo milk fermented by direct culture inoculation of LAB isolates were significantly (P<0.05) higher than whey buffalo milk fermented by preculture of LAB isolates as starter cultures. The scavenging activity obtained from

whey buffalo milk fermented by direct culture inoculation of LAB isolates ranged from 22.61 to 33.62 % while the scavenging activity obtained by whey buffalo milk fermented by preculture culture of LAB isolates as starter culture cultranged from 3.43 to 9.17 %. However, the scavenging activity obtained from whey buffalo milk fermented by control *L. plantarum* ATCC 8014 either by direct inoculation or by preculture as starter culture found similar ($P>0.05$) with the amount of 30.18 and 29.09 %, respectively.

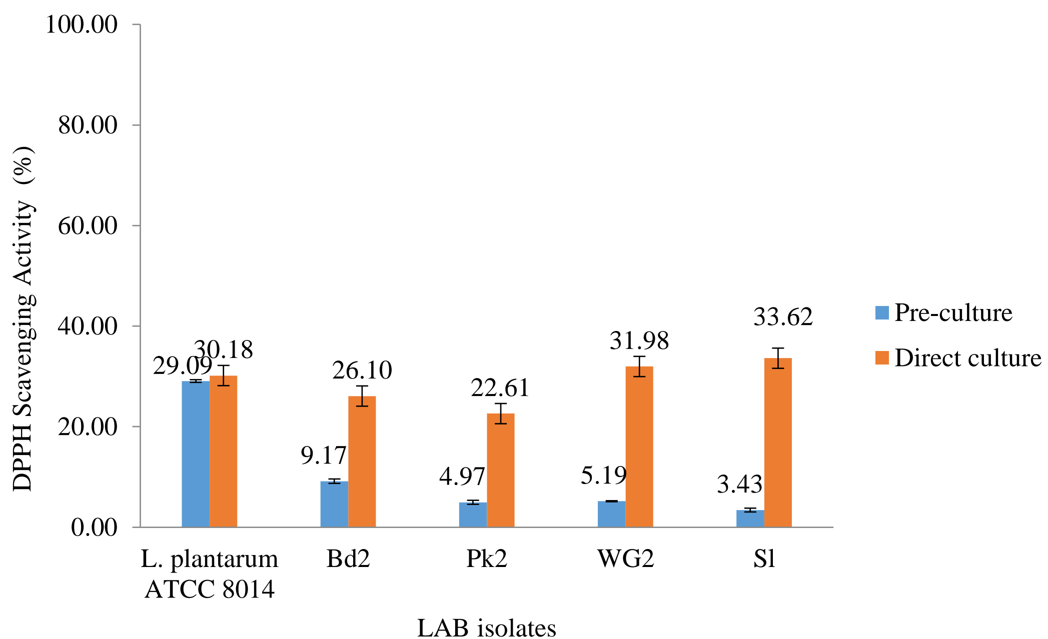

Figure 4.4: Antioxidant Activity of Whey Buffalo Milk by Scavenging of 1,1-Diphenyl-2-Picrylhydrazyl (DPPH) Free Radical Assay

The ferrous chelating capacity of whey buffalo milk fermented by *L. plantarum* ATCC 8014 (control LAB species) compared to the four LAB isolates (isolates Bd2, Pk2, WG2, and S1) is shown in Figure 4.5. On contrary, it was found that the DPPH free radical scavenging activity of whey buffalo milk fermented by preculture of LAB isolates as starter culture were significantly ($P<0.05$) higher than whey buffalo milk

fermented by direct culture inoculation of LAB isolates. However, the ferrous ion chelating activity (FICA) of whey buffalo milk was significantly ($P<0.05$) higher than the DPPH free radical scavenging activity for both fermented by preculture of LAB isolates as starter culture or by direct culture inoculation. The percentage of FICA of the whey buffalo milk for both preculture and direct culture as starter culture in the fermentation ranged between 50.13 and 78.22 % (Figure 4.5) and higher than the percentage of DPPH free radical scavenging activity of the whey buffalo milk for both preculture and direct culture as starter culture in the fermentation.

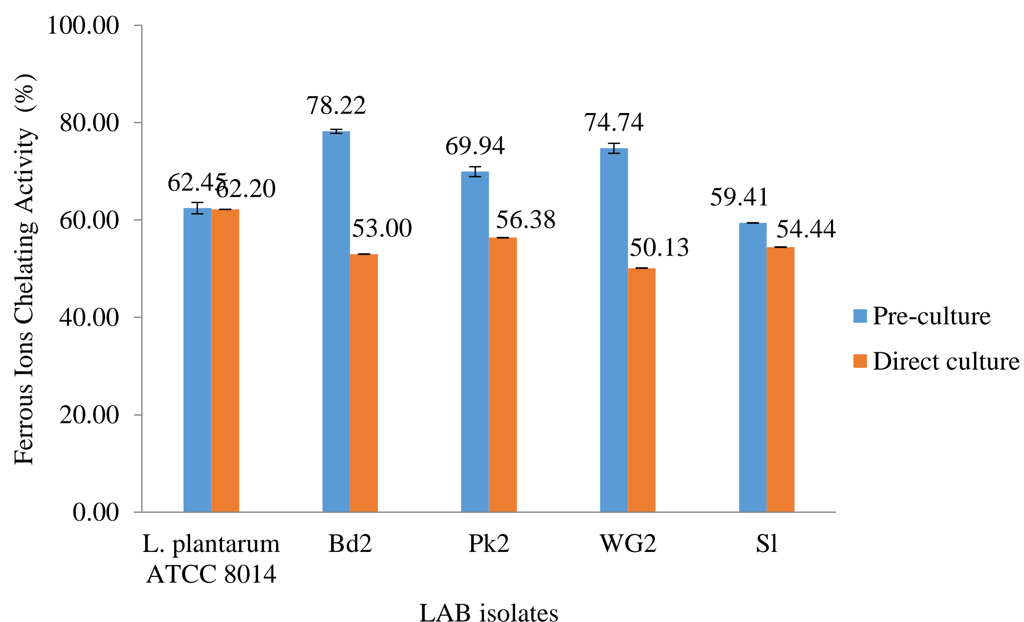

Figure 4.5: Antioxidant Activity of Whey Buffalo Milk by Ferrous Ions Chelating Activity (FICA) Assay

4.4 Discussion

Lactic acid bacteria (LAB) with proteolytic activity exhibited extra vital values for attributes in selection of probiotic organisms (Kim et al., 2019). The ability of proteolytic LAB with other probiotic characteristics was inevitable because a variety of

bioactive peptides may be released from this microbial action of LAB (Donkor et al., 2007). In the end, by confirming probiotic viability and multifunctional bioactive peptides benefited human and animal when ingested/consumed food or fed with LAB strains that have both proteolytic and probiotic abilities (Jaimez-Ordaz et al., 2019). Therefore, four LAB isolates with high in proteolytic activity from previous study were further evaluated as potential probiotic to support additional value of choosing this LAB by challenging LAB in acidic, bile stresses and determining its antimicrobial activity. Then, these proteolytic LAB with probiotic potential were further evaluated in buffalo milk fermentation for 24 h fermentation incubated at 37 ^{o}C as starter culture to generate whey with antioxidative activity by examining them using both DPPH free radical scavenging activity and FICA assays.

From this study, the LAB strains of WG2, Pk2, S1 and Bd2 showed tolerance at low pH of 2.0 (acidic environment) (Figure 4.1). It has been demonstrated that acid tolerance is one of the important criteria for characterisation of probiotic organisms and tested acid stress analysis should be run with standard pH value of 2.0 to 3.0 (Ji et al., 2015). The selection of the standard pH value was supported from previous study where the acidic environment was adjusted to pH 2.0 with time simulation for GIT for food to pass through the human stomach was about 20 to 90 min (Kim et al., 2006). Similarly, 90 min of time interval for acid stress analysis mimicking the acidic environment of GIT with time for food to pass through the stomach had been observed by other study with similar pH level of approximately 2 to 3 (Kheadr, 2006).

The next most crucial criterion for LAB strains to be recognised as probiotic organisms is bile tolerance (Ji et al. 2015). As for bile resistant challenge, LAB strains of WG2, Pk2, S1, and Bd2 survived in bile salt environment at pH 6.5 (Figure 4.2). In fact, not all microorganisms can survive in a bile salt environment (Hassanzadazar et

al. 2012; Sahadeva et al. 2011). The exposure to bile salts disturbs cellular homeostasis which then causes dissociation of lipid bilayer and integral protein of bacterial cell membranes resulting in bacterial content leakage which finally causes cell death. After incubation either in acid at pH 2.0 or bile condition at pH 6.5, most LAB isolates showed reduction in viable counts, however, interestingly WG2 and Bd2 isolates resulted in increment of viable count after exposed in 0.3 % (w/v) bile concentration. It could be because of the stress adaptation mechanism of certain bacteria after exposure in a certain stress environment like in bile salts.

Indeed, all tested four LAB strains were tolerant to acid and bile stresses for 90 min. Previously, it was reported that survival of different strains of *Lactobacillu*s reduced about 0.3 to > 6.0 log cfu/ml after 90 min of acid exposure. Among the strains, the most acid tolerant strains were *L. plantarum* R1096, R1078, and R0202 with the viability ranged between 0.3 and 0.6 log cycle. In a bile environment, the survival of *Lactobacillus* slightly reduced from 0.2 to 0.7 log cfu/ml depending on strains tested (Kheadr, 2006).

Food spoilage caused by pathogenic bacteria such as *B. cereus*, *B. subtilis*, *S. aureus*, *S. typhimurium* and *E. coli* may result in food contamination which lead to foodborne diseases (Xiao et al., 2019; Mostafa et al., 2018). Indeed, foodborne outbreak by *B. cereus*, *B. subtilis*, *S. aureus*, *S. typhimurium*, and *E. coli* were reported in many countries (Yang et al., 2017; Cavallo et al., 2015; Tong et al., 2015; Pavic et al., 2005; Granum & Lund, 1997). Previous studies reported that LAB strains could inhibit the growth of some pathogenic bacteria that are responsible for food spoilage (Aween et al., 2012; Muhialdin et al., 2012). In this study, LAB strains of WG2, Pk2, S1, and Bd2 revealed successfully inhibit *B. cereus*, *B. subtilis*, *S. aureus*, *S. typhimurium*, and *E. coli*, respectively. Inhibition of these bacteria by LAB may benefit future industry. The

LAB with antibacterial activity could be used as an antimicrobial agent and can be implemented into food or feed as biopreservative. In fact, LAB with inhibitory activity against pathogenic bacteria could be add up into food to prevent the growth of spoilage and foodborne bacteria as well as to prevent the production of the different toxins in foodstuff by these bacteria (Muhialdin et al., 2012).

In this study, it was found that the lowest diameter of clear inhibitory zone for Bd2 isolate against *B. subtilis* using dual culture overlay method was 24.00 mm which was greater than *L. acidophilus* strains. The strains of *L. acidophilus* inhibit germination spores of *B. subtilis* with diameter of inhibitory activity ranging between 9.00 and 14.00 mm (Klewicka & Libudzisz, 2004). Selection of LAB strains with good antimicrobial activity are important to prevent any related food outbreak. There was a case of food poisoning that occurred in a kindergarten in Croatia caused by milk powder containing *B. subtilis* and *B. licheniformis*. This outbreak of foodborne intoxication happened because of poor food handling preparation of making milk and cocoa beverages to the children there and caused twelve children to have symptoms of nausea, headache, and vomiting with no fever (Pavic et al., 2005).

Using the agar well diffusion method, it was found that the diameter of clear inhibitory zone for LAB strains of WG2, Pk2, S1, and Bd2 against *E. coli* ranged between 13.83 and 13.33 mm which was greater than *L. plantarum* W21 (Table 4.2). Furthermore, LAB strain LBD02 identified as *E. faecium* exhibited inhibitory activity toward tested *E. coli* with diameter clear zone of 13.50 mm (Gaamouche et al., 2014). In addition, *Enterococcus* sp. including *E. faecium* are capable as antimicrobial agents because this species could produce bacteriocins which are responsible for the effectiveness of wide range against pathogenic and spoilage bacteria (Hanchi et al.,

2018). Similarly, LAB of *L. plantarum* W21 also inhibited *E. coli* O157: H7 with the inhibition of clear zone of 12.00 mm (Campana et al., 2017).

Bioactive peptides produced from milk including buffalo milk had been one of interest of many researchers. Fermentation of buffalo milk by LAB exhibited bioactive peptides that benefit human health including antioxidative peptides (Taha et al., 2017; Vankudre et al., 2015). Similar to other milk fermentation, some molecular changes occurred during buffalo milk fermentation could yield to the production of different compounds such as peptides and free amino acids that are responsible for antioxidant activity (Tavakoli et al., 2019).

Antioxidant activity of whey buffalo milk cultured with selected LAB (proteolysis and probiotic potential) was investigated using DPPH scavenging radical activity assay (Figure 4.4). The results revealed that isolates WG2, Pk2, S1 and Bd2 could be an option as starter culture in buffalo milk fermentation to hydrolyse milk protein into peptides and/or amino acids with low to moderate antioxidant activity in inhibiting lipid peroxidation. The value for scavenging DPPH radical activity of whey buffalo milk fermented by direct cultured S1 isolate was 33.62 % followed by WG2 isolate was 31.98 % at 37 °C for 24 h and were still lower than previous studies (Tavakoli et al., 2019).

Antioxidant activity of yogurt made from skimmed milk was enhanced by the presence of commercial starter culture of *L. acidophilus* L10 with the highest value of scavenging DPPH radical activity was 55.39 % at day 14 storage time incubated at maximum temperature of 37 °C for 72 h maximum time under anaerobic conditions (Tavakoli et al., 2019). However, antioxidant activity of yogurt made from buffalo milk, the scavenging DPPH radical activity of water-soluble peptide extract of yogurt was from 79.45 to 81.62 % between day 0 to day 15 storage with a mixture of

Lactobacillus helveticus (*L. helveticus*) incubated at 38 °C until reached pH 4.6 (Taha et al., 2017). In fact, *L. helveticus* was recognised as proteolysis and probiotic bacteria which enhanced fermented milk with variety of health promoting properties (Griffiths & Tellez, 2013; Taverniti & Guglielmetti, 2012). It was suggested that the oxidative stability of yogurt might be due to antioxidant peptides released during the fermentation of milk by selection of LAB (Farvin et al., 2010). Instead of DPPH radical scavenger compounds in scavenging DPPH radical activity assay, the responsible antioxidative peptides act as electron donours and react with free radicals which convert them to more stable products (Taha et al., 2017).

The ability of isolates WG2, Pk2, S1, and Bd2 generated peptides from whey buffalo milk that have antioxidative properties by chelating ferrous ions was investigated by FICA assay (Figure 4.5). Results found that the same potential probiotic LAB strains were able to produce whey buffalo milk with antioxidant activity via FICA assay with moderate to high FICA percentage values. The highest FICA value of whey buffalo milk by precultured LAB was determined by Bd2 isolate (78.22 %) followed by precultured WG2 isolate (74.74 %) at 37 °C for 24 h. Antioxidant activity study of bioactive peptides in whey buffalo hydrolysates enhanced by protease obtained from *Bacillus* sp and commercial alcalase (Gregory et al., 2016). However, the antioxidant activity study of hydrolysed whey buffalo milk initiated by protease enzyme incubated at 45 °C for 4 h with FICA value of 72.90 % were lower than this finding (Gregory et al., 2016). The study applied enzymes and not choosing bacteria as protein hydrolysis to generate antioxidative peptides from buffalo milk. Thus, currently, there is still limited study regarding antioxidative peptides from whey buffalo milk generated by LAB using FICA assay.

Indeed, without antioxidant compounds with the ability in binding or chelating metal ions through determination of antioxidant activity by FICA assay, the reactive hydroxyl radical of ferrous ions stimulate lipid peroxidation through Fenton reaction by means of the decomposition of hydroperoxides to peroxyl radicals (Halliwell & Gutteridge, 1990). However, hydrolysed proteins or peptides are able in binding metal ions such as ferrous ions to prevent or terminate free radical-mediated oxidative reactions resulting in cell membrane damage (Walters et al., 2018).

Different culturing approaches by preculture and direct culture of LAB isolates significantly ($P < 0.05$) affected antioxidative values by both DPPH radical scavenging activity and FICA assays. The scavenging DPPH radical activity values by direct cultured LAB were observed higher than the scavenging DPPH radical activity values by precultured LAB. In contrast, the FICA values by direct cultured LAB were observed lower than the FICA values by precultured LAB. The effects of fermentation conditions including culture temperature, culture time, inoculation size, and reconstituted milk concentration on the angiotensin-converting enzyme (ACE) inhibitory peptide of fermented milk generated by *L. bulgaricus* LB6 (Chen et al., 2019). Under optimal fermentation conditions, peptides with specific functional properties may work best. Fermentation of defatted wheat germ by *B. subtilis* B1 found the optimal production of peptide at optimal fermentation conditions of 8 % in inoculums size at 31 °C fermentation incubation temperature for 48 h (Niu et al. 2013). Production of antioxidative peptides generated by fermented buffalo milk with LAB was also affected by selection of potential probiotic LAB strains and methods of culture either by preculture or direct culture approaches. Different LAB strains needed different fermentation conditions including culturing method. Therefore, optimisation of LAB

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as starter culture in milk fermentation to generate bioactive peptides should be further studied.

4.5 Conclusion

Isolate LAB strains of WG2, Pk2, S1, and Bd2 confirmed to have probiotic potentials used as starter culture to generate peptides from buffalo milk through fermentation and have proteolytic activity and the probiotic LAB successfully produces whey with antioxidative activity. Direct culture approaches with different strains of LAB provide antioxidative whey buffalo milk with moderate scavenging DPPH radical activity while preculture approaches with different strains of LAB provide antioxidative whey buffalo milk with high FICA. Further study on optimisation of fermentation conditions like selection of LAB with potential probiotics as starter culture and methods of culturing approach should be investigated because each LAB strain has their own standardisation to function at the best with specific conditions during fermentation.

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