CHAPTER 5

IDENTIFICATION OF LACTIC ACID BACTERIA AND DETERMINATION OF ANTIOXIDATIVE PEPTIDES OF WHEY BUFFALO MILK GENERATED BY AND *Enterococcus faecium* Bd2 AND *Lactobacillus plantarum* WG2

5.1 Introduction

Starter culture in the fermentation industry is one of the crucial substances in making fermented food and feed products. Selection of LAB as starter culture was preferred because this species had been generally recognised as safe or GRAS bacteria. Each LAB strains selected as starter culture mostly had specific characteristics as proteolysis and probiotic properties which provide a variety of beneficial health effects as one of the ingredients in the products (Jaimez-Ordaz et al., 2019; Mechai et al., 2014).

Exploring new LAB strains present in various sources may lead to the unique isolation species of these LAB with relevant technological future demand (Rodriguez et al., 2019). The step for the identification of LAB is important to determine specific LAB strains with special characteristics; and thus, the implementation of selected LAB could be specifically selected for industrial application. Generally, the identification of LAB was determined by phenotypic and genotypic approaches (Reginensi et al., 2013). A miniature kit named API 50 CHL is widely used because of rapid identification of LAB through biochemical tests based on assimilation and fermentation of 49 different compounds (Suhartik et al., 2014; Yuliana & Dizon, 2011). However, genotypic identification of LAB which is based on molecular approach is the most robust, superior, sensitive, and reliable method (Tilahun et al., 2018; Reginensi et al., 2013).

The genotypic LAB identification method widely used is 16S rRNA gene sequencing using polymerase chain reaction (PCR) amplification of LAB isolates (Tilahun et al., 2018; Caro et al., 2015; Park et al., 2010).

Several research has been developed to generate peptides from milk fermentation with health multifunctional properties. Over the decades, selected strains of LAB have been applied in different types of milk fermentation to generate peptides with numerous biological functional properties including antioxidant activity. Fermentation of goat milk by *Lactobacillus casei* (*L. casei*) L61 at 41 ℃ for 16 h also obtained antioxidative peptides and the percentage of scavenging of 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical activity rate reached up to 63.48 % from 41.97 % (Shu et al., 2018). In addition, the extraction of cow milk cheese made with rennet with different origins produced peptide fractions (<3 kDa) with antioxidant activity by scavenging of DPPH free radical activity, and ferrous ions chelating activity (FICA) assays. The scavenging of DPPH free radical activity and FICA for these fractions at 5 mg/ml ranged between 4.37 and 11.71 %, and between 28.90 and 79.04 %, respectively (Timón et al., 2019).

Indeed, limited studies reported LAB strains able to release bioactive peptides through buffalo milk fermentation process (Taha et al., 2017; Vankudre et al., 2015; Minervini et al., 2003). Previously, it was found that angiotensin I-converting-enzyme-inhibitory (ACE) and antibacterial peptides generated by *Lactobacillus helveticus* (*L. helveticus*) PR4 proteinase hydrolysed caseins for six types of milk including buffalo milk. The peptide concentration of buffalo milk sodium caseinate hydrolysates produced by the partially purified proteinase of *L. helveticus* PR4 was 1.238 mg/ml with ACE inhibitory activity (Minervini et al., 2003). However, there is a few data presented on the antioxidative peptides produced from buffalo milk fermented with selected LAB.

Therefore, the objectives of this study were to identify LAB species that had been used as cultures in buffalo milk fermentation and to determine a better antioxidative peptides of whey buffalo milk generated by this selective LAB.

5.2 Materials and Methods

5.2.1 Phenotypic Identification of Lactic Acid Bacteria by API 50 CHL Kit

The LAB isolates of WG2, Pk2, S1, and Bd2 were identified using API 50 CHL (API system, BioMérieux, France) assay. Overnight cultures of the isolates were grown on MRS plates (Oxoid) at 37 ℃ for 24 h anaerobically. Both LAB cultures were then washed and resuspended into API 50 CHL medium (API system, BioMérieux, France). Next, the turbidity of the suspension was determined using McFarland number 2 as following instructions provided by the manufacturer. Then, the suspension was transferred into 50 wells of the API 50 CH strips. To ensure the wells incubated in anaerobic condition, each well was layered with mineral oil and the strips were incubated at 37 ℃ for 24 and 48 h. The results were observed the changes in colour of the suspension and analysed with API WEB (BioMérieux).

5.2.2 Genotypic Identification of Lactic Acid Bacteria Using 16 S rDNA Sequencing

The 16S rRNA gene fragment was amplified using standard PCR protocol and the universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3') (Lawalata et al., 2011). The amplifications were performed with initial denaturation at 95 ℃ for 2 min, and 35 cycles of denaturation at 92 ℃ for 45 seconds, annealing at 54 ℃ for 1 min, extension at 72 ℃ for 1 min, and the final extension at 72 ℃ for 10 min (Personal Termocycler, Biometra).

The DNA was run on 1.5 % (w/v) agarose gel electrophoresis in 0.5 × Tris-acetate-EDTA (TAE) buffer at 110 V for 45 min, and was visualised and photographed with UV transilluminator (Bio-Rad Laboratories, Segrate, Italy). The partial 16S rDNA was determined by 1st Base, Malaysia and compared with databases (Gen- Bank).

5.2.3 Preparation of Cultures and Fermentations

5.2.3.1 Preparation of Lactic Acid Bacteria Isolates

The identified LAB Bd2, Pk2, WG2, and S1 were cultured by preculture, and direct culture methods of this section was described in section 4.2.7.1. For a comparison, fermentation of buffalo milk by LAB strains was carried out in triplicate at 37 °C for 24 h and 48 h, respectively.

5.2.3.2 Preparation of Whey Fraction from Fermented Buffalo Milk

The preparation of whey buffalo milk in this section was described same as in section 4.2.6.2. The supernatant filtrate was then freeze dried and stored at -20 °C for further analysis. The whey buffalo milk was used to analyse the antioxidant activity by scavenging of 1,1 diphenyl-2-picrylhydrazyl (DPPH) free radical activity, and Ferrous ions chelating activity (FICA) assays. The pasteurised buffalo milk with bacteria at 0 h was used as a control.

5.2.4 Determination of Antioxidant Activity of Whey Buffalo Milk

5.2.4.1 Scavenging of 1,1-Diphenyl-2-Picrylhydrazyl (DPPH) Free Radical Activity

The DPPH radical scavenging activity was evaluated using the Son and Lewis (2002) method with modification which was similar to the previous chapter as

mentioned in section 4.2.8.1.

5.2.4.2 Ferrous Ion Chelating Activity

The ferrous ion chelating activity or FICA of whey buffalo milk generated by LAB was evaluated using the Decker and Welch (1990) method with modification which was similar to the previous chapter as mentioned in section 4.2.8.2.

5.2.4.3 Determination of IC_{50} by Ferrous Ion Chelating Activity

The ability of different peptides generated by LAB to chelate ferrous ions was assessed using the method of Decker and Welch (1990). One milliliter of various concentrations of whey buffalo milk (0.5 mg/ml to 3 mg/ml) was mixed with 3.7 ml of distilled water. A solution of 0.1 ml 2 mM ferrous chloride (Sigma Aldrich) was added and after 3 min the reaction was inhibited by the addition of 0.2 ml 5 mM ferrozine (Sigma Aldrich). The mixture was vigorously shaken and left at room temperature (25 °C) for 10 min. Optical density of the reaction mixture was measured at 562 nm. A blank without a sample was prepared in a similar manner. EDTA (0.1 mg/ml) was also run in the same way for comparison. The test was carried out in triplicate and the chelating capacity was calculated as a percentage using the following formula:

$$\text{Fe}^{2+} \text{ chelating activity (100\%)} = \text{Absorbance } (A_{562}) \text{ of blank} - \frac{\text{Absorbance } (A_{562}) \text{ of sample}}{\text{Absorbance } (A_{5627}) \text{ of blank}} \times 100$$

The IC_{50} value for antioxidant activity was defined as the concentration of sample (mg/ml) required to chelate ferrous ions by 50 %.

5.2.5 Determination of Molecular Weight of Peptides from Whey Buffalo Milk by Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS PAGE)

The molecular weight (M_w) of peptides from whey buffalo milk produced by precultured WG2 and Bd2 were estimated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) method as described by Laemmli (1970) (Garfin, 1990).

5.2.5.1 Preparation of Stock Solution

Preparation of sample buffer was as done as follows and the stock sample buffer (0.06 M Tris-C1, pH 6.8, 2 % SDS, 10 % glycerol, 0.025 % Bromophenol Blue) was prepared as shown in Table 5.1.

Table 5.1: Preparation of the Sample Buffer

Ingredients	Volume (ml)
H_2O	4.8
0.5 M Tris-HCl, pH 6.8	1.2
10 % (w/v) SDS	2.0
GIycerol	1.0
0.5 % Bromphenol Blue (w/v water)	0.5

Then, one liter of the running buffer (0.025 M Tris, 0.192 M glycine, 0.1 % (w/v) SDS, pH 8.3) was prepared as follows. Initially, 3 g Tris-base, 14 g glycine, 10 ml 10% SDS were mixed in 1000 ml of distilled water running buffer. No need to adjust the pH of the running buffer, but just mix the reagents together and confirm that the pH is near 8.3 ± 0.2.

5.2.5.2 Preparation of Casting Gels (Resolving and Stacking Gel)

Initially, a 12 % (v/v) of resolving gel was prepared by combining all the reagents from Table 5.2 except the ammonium persulfate (APS) and tetramethylethylenediamine (TEMED) in a disposable plastic beaker. The solution was deaerated under vacuum using dessicator. After that, APS and TEMED were mixed gently into the deaerated monomer solution. The monomer solution was added using a pipette between the gel plates up to the mark delimiting the resolving gel.

Next, immediately overlaid it with water saturated 2-butanol or *tert*-amyl alcohol to exclude air, which might inhibit polymerisation, from the surface of the monomer mixture. Then, the gel was allowed to polymerise for 45 min to 1 h. Polymerisation was evidenced by the appearance of a sharp interface beneath the overlay, which will start to become visible in about 15 min. Polymerisation was essentially completed in 90 min, but the stacking gel can be poured after an hour. The unused monomer was allowed to polymerise in the beaker and lastly the gel was discarded.

Table 5.2: Components of Resolving Gel Making

Component	**12 % Resolving gel**
Water	3.35 ml
1.5 M Tris-C1, pH 8.8	2.5 ml
10 % SDS	0.1 ml
Acrylamide/bis (30 % T, 2.7 % C)	4.0 ml
10 % ammonium persulfate	50 µL (0.05%)
TEMED	5 µL (0.05%)

For a stacking gel preparation, all reagents were mixed (Table 5.3) until volume reached 10 ml of stacking gel monomer solution. The solution was deaerated under vacuum by using a desiccator for at least 15 min. Finally, the top of the resolving gel was rinsed thoroughly with water and the area above it was dried with filter paper. A

well-forming comb was placed between the gel plates with a slight tilt at an angle to provide a way for bubbles to escape.

Table 5.3: Components of Stacking Gel Making

Component	Stacking gel
Water	6.1 ml
0.5 M Tris-C1, pH 6.8	2.5 ml
Acrylamide stock solution (30 % T)	1.3 ml
10 % SDS	0.1 ml

5.2.5.3 Preparation of Sample for SDS PAGE

The loading buffer was prepared by adding 50 µL of 2-mercaptoethanol to each 0.95 ml of stock sample buffer (to a final concentration of 5 % 2-mercaptoethanol). The freeze dried whey sample was diluted with at least 4 volumes of complete SDS-reducing buffer. Next, an aliquot amount of 20 µL sample solution was mixed with 20 µL of loading buffer followed by heating at 95 °C for 5 min by suspending the sample tubes into a water bath.

5.2.5.4 Electrophoresis

The electrophoresis tank was set up (Nyxtechnyx Electronyx Vertical Gel Tank (Global Medical Instrument, USA). The running buffer (0.025 M Tris, 0.192 M glycine, 0.1 % (w/v) SDS) with pH 8.3 was loaded and the comb from a stacking gel was removed. Then, 20 µL of each sample was loaded into the well of the stacking gel and electrophoresis was carried out at constant current 120 V for 120 min. After that, the gel was carefully removed from between the glass plates and was placed in the fixing solution consisting of 40 % (v/v) of methanol mixed with 10 % of acetic acid (v/v) for 30 min. This gel was stained for 3 h with Coomassie G-250 stain (BIO-RAD, Bio Rad

Laboratories, Inc). Then, after 3 h staining, the gel was destained using a destaining solution consisting ratio of (4: 5: 1) (v/v/v) methanol, water and acetic acid. Finally, the molecular weight (M_w) of the sample was determined using standard molecular weight markers 10 to 250 kDa (BIO-RAD, Bio Rad Laboratories, Inc).

5.2.6 Statistical Analysis

Antioxidant activity of whey buffalo milk by both DPPH radical scavenging activity and FICA assays were presented as mean ± standard deviations of average readings and were statistically analysed using two-way analysis of variance (ANOVA) using Minitab version 16 (Germany). The p-value ($P<0.05$) were considered statistically significant.

5.3 Results

5.3.1 Phenotypic and Genotypic Identification of Lactic Acid Bacteria

Thus, through phenotypic identification using API 50 CHL test kits and API web, these four LAB strains were identified as *L. plantarum* for isolate Bd2, WG2, and S1, respectively while *L. lactis cremaris* for isolate Pk2 (Table 5.4). Characterisation of LAB strains can be differentiated through carbohydrate fermentation from API 50 CHL test kits by observing 49 different carbon sources react (Yuliana & Dizon, 2011). However, using gene-based approach known as genotype identification of DNA that used universal primer obtained clear bands of LAB (Figure 5.1) with approximate molecular weight (M_w) 1400 base pair (bp) and the similarity (99%) for all LAB were determined as *E. faecium* for isolate Bd2, *L. paracasei* for isolate Pk2, *L. plantarum* for isolate S1 and WG2.

Table 5.4: Phenotypic and Genotypic Identification of LAB Isolates

Code of LAB	Source	Phenotype identification		Genotype identification	
		Identification	Similarity %	Identification	Similarity %
Bd2	Budu	*L. plantarum*	94.9	*E. faecium*	99.0
Pk2	Pekasam	*L. lactis cremaris*	93.7	*L. paracasei*	99.0
Sl	Soil	*L. plantarum*	92.5	*L. plantarum*	99.0
WG2	Grape	*L. plantarum*	99.9	*L. plantarum*	99.0

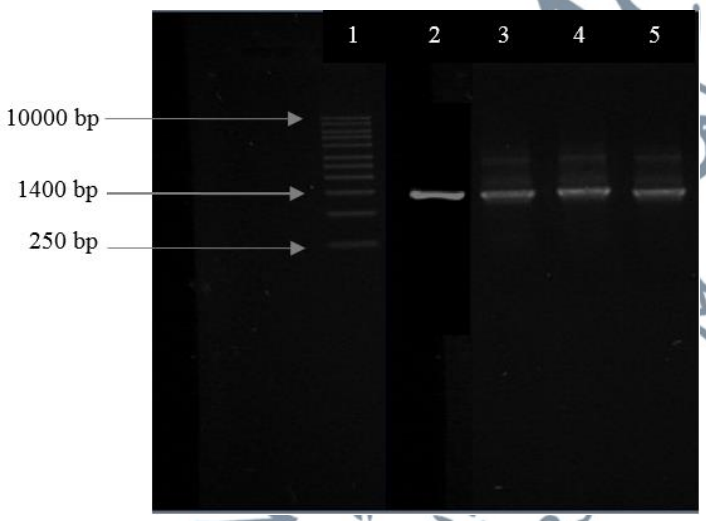


Figure 5.1: DNA Bands of LAB on 1.0 % Agarose Gel

Notes: Lane 1 = DNA ladder; Lane 2 = *E. faecium* Bd2; Lane 3 = *L. paracasei* Pk2; Lane 4 = *L. plantarum* S1, and Lane 5 = *L. plantarum* WG2

5.3.2 Antioxidant Activity of Whey Buffalo Milk Generated by Precultured LAB

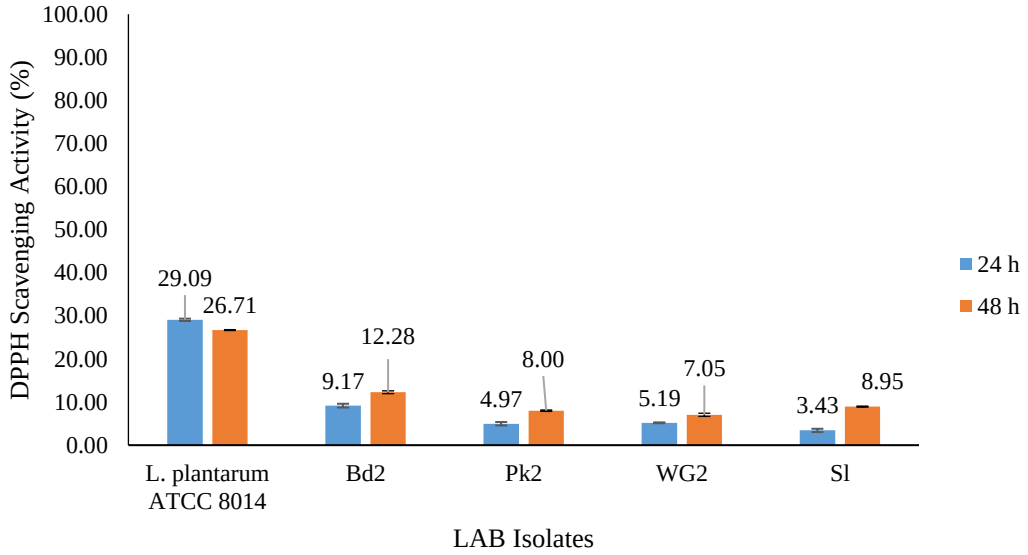
Antioxidant activity of whey buffalo milk produced by precultured LAB was not significantly (P>0.05) affected by fermentation time for both scavenging DPPH free radical activity and FICA assays. However, when increasing fermentation time from 24 h to 48 h for all four LABs resulted in increasing the percentage of antioxidant by both scavenging DPPH free radical activity and FICA assays except for FICA of whey buffalo milk generated by *L. paracasei* Pk2 and *E. faecium* Bd2 (Figure 5.2). The FICA of whey buffalo milk generated by *L. paracasei* Pk2 decreased from 69.94 % (24

h) to 56.58 % (48 h) while the FICA of whey buffalo milk generated by *E. faecium* Bd2 decreased from 78.22 % (24 h) to 73.44 % (48 h).

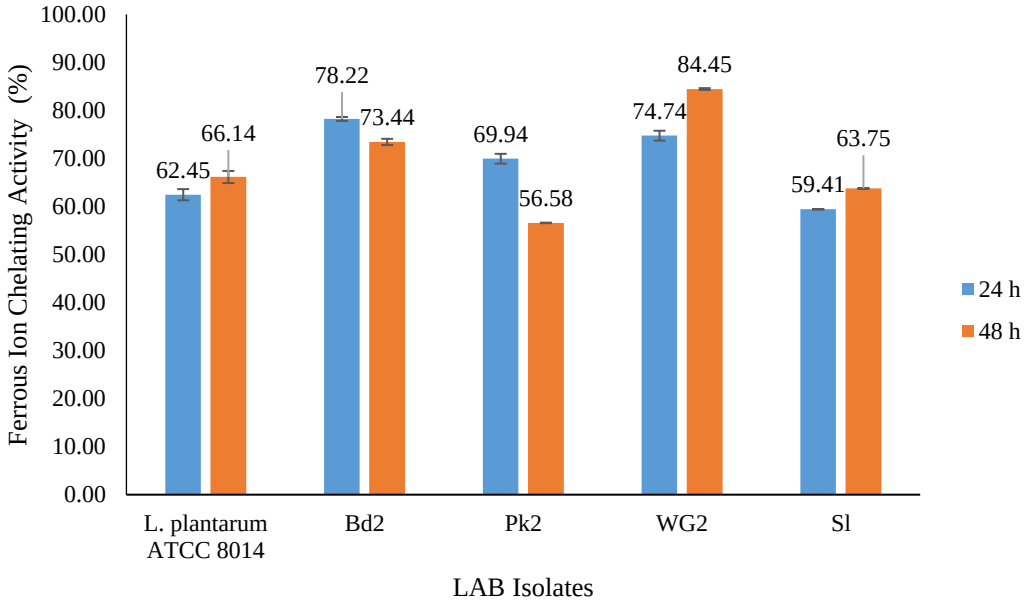
Each LAB strain significantly (P<0.05) showed different antioxidant values of whey buffalo milk for both scavenging DPPH free radical activity and FICA assays. The LAB strain identified as *E. faecium* Bd2 inoculated in buffalo milk fermentation by preculturing approach produced whey with the highest antioxidant activity by DPPH radical scavenging activity assay (12.28 %) but still significantly (P<0.05) lower than whey generated by the control *L. plantarum* ATCC 8014 (26.71 %) at 48 h fermentation time.

Meanwhile, the *L. plantarum* WG2 inoculated in buffalo milk fermentation by preculturing approach produced whey with the highest antioxidant activity (84.45 %) by FICA assay at 48 h fermentation time followed by whey generated by *E. faecium* Bd2 at 24 h fermentation time (78.22 %). Interestingly, these two isolates that produced high FICA were significantly (P<0.05) higher than whey buffalo milk generated by the control *L. plantarum* ATCC 8014 (66.14 %) at 48 h fermentation time.

Indeed, the antioxidant activity of whey buffalo milk generated by all four LAB isolates by preculturing approach significantly (P<0.05) resulted in high FICA when compared to DPPH radical scavenging activity values. The FICA values ranged between 56.58 and 84.45 % while the DPPH radical scavenging activity values ranged between 3.43 and 12.28 %, respectively.



(a)



(b)

Figure 5.2: (a) Scavenging DPPH Free Radical Activity; and (b) FICA of Whey Buffalo Milk Generated by Precultured LAB (%)

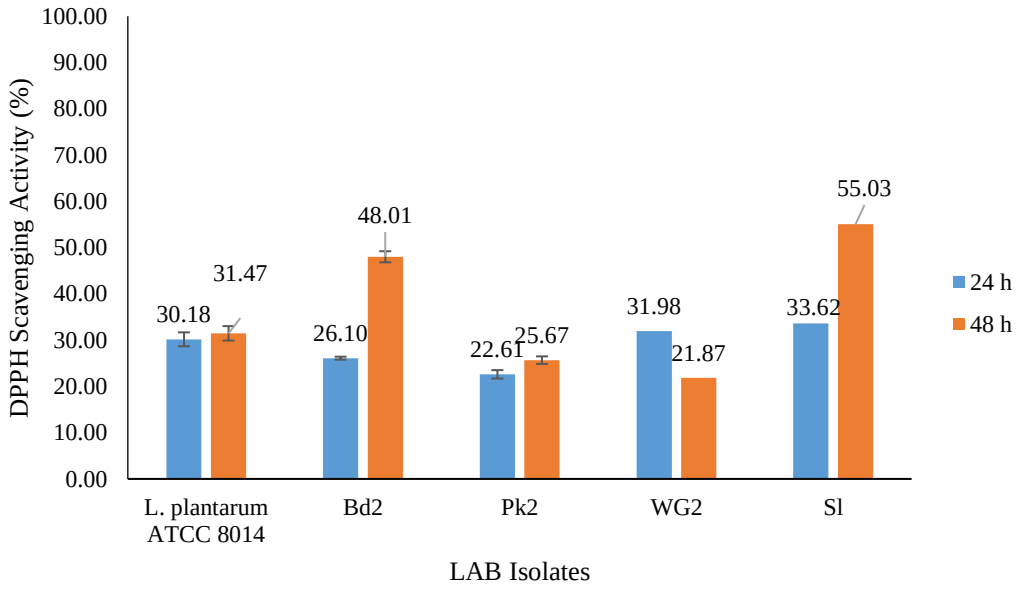
Notes: Bd2 = *E. faecium* Bd2; Pk2 = *L. paracasei* Pk2; WG2 = *L. plantarum* WG2; and Sl = *L. plantarum* S1

5.3.3 Antioxidant Activity of Whey Buffalo Milk Generated by Direct Cultured LAB

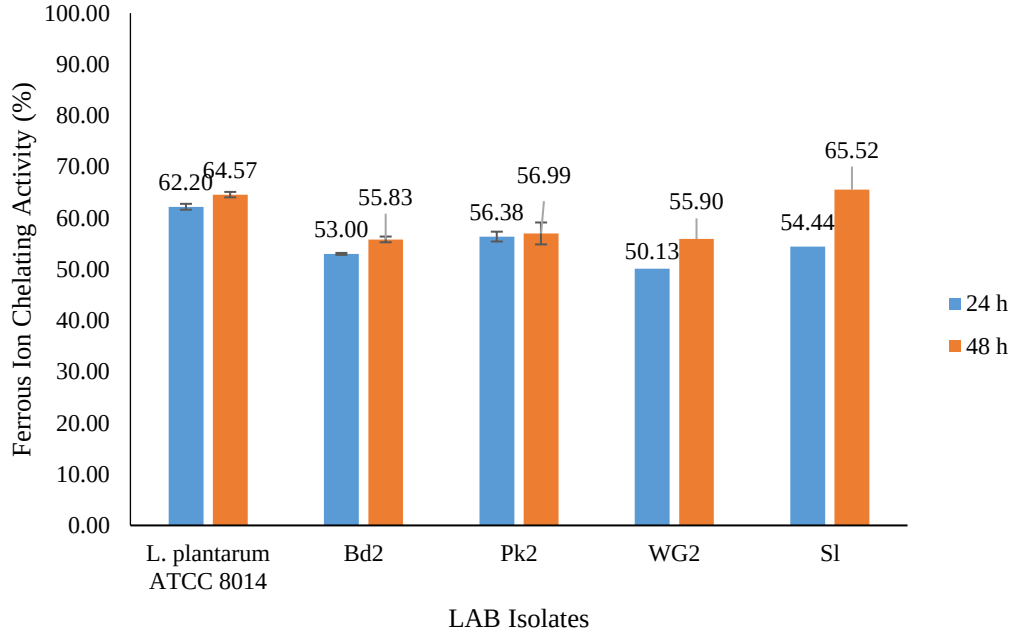
Different fermentation times in direct culture approach significantly (P<0.05) affected antioxidant activity of whey buffalo milk generated by LAB isolates. The antioxidant activity of whey buffalo milk generated by LABs at 48 h fermentation time determined by both scavenging DPPH free radical activity and FICA assays showed higher values than whey buffalo milk generated by the same LABs at 24 h fermentation time (Figure 5.3).

The DPPH radical scavenging activity values of whey buffalo milk at 48 h fermentation time ranged between 21.87 and 55.03 % which was higher than recorded for 24 h fermentation time except for whey buffalo milk generated by *L. plantarum* WG2 where the DPPH radical scavenging activity reduced from 31.98 % (24 h fermentation time) to 21.87 % (48 h fermentation time). The highest DPPH radical scavenging activity of whey was observed in buffalo milk inoculated with *L. plantarum* S1 (55.03 %) followed by *E. faecium* Bd2 (48.01 %) by direct culturing at 48 h fermentation time which were significantly (P<0.05) higher than the control *L. plantarum* ATCC 8014 (31.47 %) at 48 h fermentation time.

Meanwhile, buffalo milk fermented with LABs at 48 h produced whey with FICA values ranged between 55.83 and 65.52 % which was slightly higher than that recorded for 24 h fermentation time (50.13 to 62.20 %). The highest FICA was observed in whey buffalo milk inoculated with *L. plantarum* S1 (65.52 %) by direct culturing approach at 48 h fermentation time which was slightly higher than the control *L. plantarum* ATCC 8014 (64.57 %) at 48 h fermentation time.



(a)



(b)

Figure 5.3: (a) Scavenging DPPH Free Radical Activity, and (b) FICA of Whey Buffalo Milk Generated by Direct Cultured LAB (%)

Notes: Bd2 = *E. faecium* Bd2; Pk2 = *L. paracasei* Pk2; WG2 = *L. plantarum* WG2; and Sl = *L. plantarum* S1

Similar to preculture approach, different LAB strains used as starter culture by direct culturing approach in the fermentation of buffalo milk resulted in different antioxidant activity of whey for both scavenging DPPH free radical activity and FICA assays where all these four LABs can produce antioxidative whey from buffalo milk fermentation. The antioxidant activity of whey buffalo milk generated by all four LAB isolates by direct culturing approach significantly (P<0.05) resulted in high FICA values when compared to DPPH radical scavenging activity values. Whey produced from fermented buffalo milk inoculated with LABs by direct culturing approach produced FICA ranged between 50.13 and 65.52 % while the DPPH radical scavenging activity ranged between 21.87 and 55.03 % for both 24 h and 48 h fermentation time, respectively.

Generally, both scavenging DPPH free radical activity and FICA assays either by preculturing or direct culturing approaches at 24 h and 48 h buffalo milk fermentation successfully generated whey with antioxidant activity. The highest antioxidant activity of whey was determined by preculturing approach in fermenting buffalo milk for both 24 and 48 h fermentation time by FICA assay. In this study, the highest FICA was observed in whey buffalo milk generated by precultured *L. plantarum* WG2 at 48 h fermentation time followed by whey buffalo milk generated by precultured *E. faecium* Bd2 at 24 h fermentation time. Whey buffalo milk generated by both precultured *E. faecium* Bd2 and *L. plantarum* WG2 did not appear significant difference (P>0.05) when prolonging the fermentation time from 24 h to 48 h. Thus, these both strains *E. faecium* Bd2 and *L. plantarum* WG2 were continually used as starter culture to further examine their antioxidant activity by half concentration of inhibition (IC_{50}) using FICA assay to determine the concentration of whey buffalo milk required to chelate 50 % metal ions responsible for oxidation.

5.3.4 Determination of IC_{50} Ferrous Ion Chelating Activity (FICA)

The inhibition concentration of whey produced from fermented buffalo milk produced by precultured of *E. faecium* Bd2 and *L. plantarum* WG2 required to chelate 50 % ferrous ions were further calculated (Figure 5.4, Table 5.5). The percentage of FICA for standard EDTA and whey buffalo milk increased when the concentration increased (Figure 5.4). It was observed that there were no significant (P>0.05) changes of FICA of whey buffalo milk at concentration between 0.5 to 3.0 mg/ml for both whey buffalo milk precultured with *E. faecium* Bd2 and *L. plantarum* WG2.

However, when comparing with standard EDTA, there was a significant difference (P<0.05) between the FICA of whey buffalo milk fermented with both strains of *E. faecium* Bd2 and *L. plantarum* WG2. The IC_{50} values for whey buffalo milk fermented by both precultured *E. faecium* Bd2 and *L. plantarum* WG2 at 24 h fermentation time were similar (0.39 mg/ml). The IC_{50} values for whey buffalo milk fermented by both precultured *E. faecium* Bd2 and *L. plantarum* WG2 at 48 h fermentation were different. Precultured *E. faecium* Bd2 produced whey buffalo milk with IC_{50} value of 0.41 mg/ml but precultured *L. plantarum* WG2 produced whey buffalo milk with IC_{50} value of 0.37 mg/ml at 48 h fermentation time. The IC_{50} values for both *E. faecium* Bd2 and *L. plantarum* WG2 at 24 h and 48 h were significantly (P<0.05) lower than the IC_{50} value of standard EDTA (0.29 mg/ml).

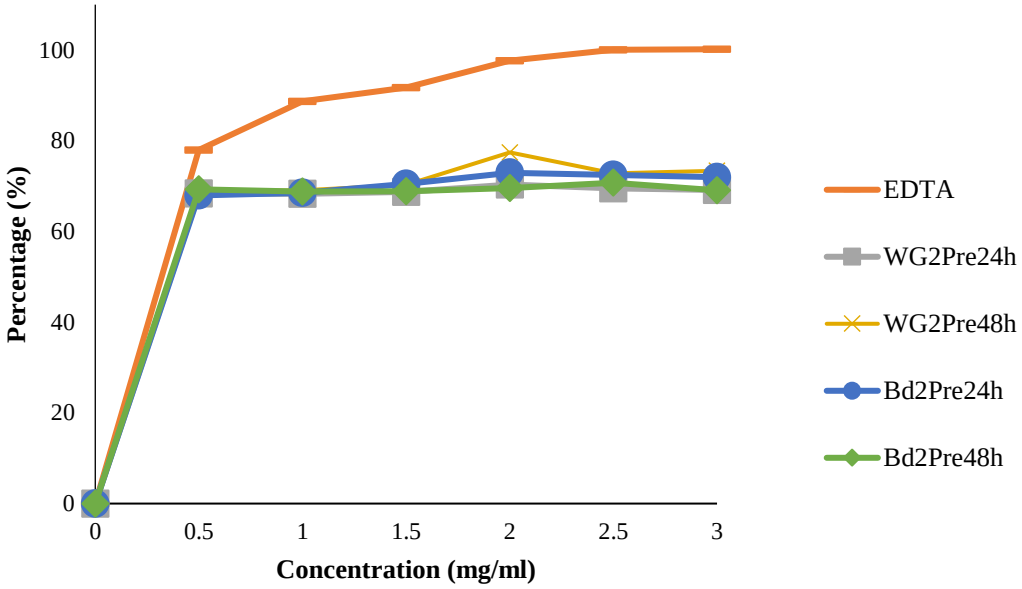


Figure 5.4: IC_{50} Value of FICA for EDTA and Selected Whey Buffalo Milk

Notes:
IC_{50}= Concentration of inhibitor required to inhibit 50 % of the FICA
EDTA: standard antioxidant EDTA
Bd2Pre24h: Whey produced by 24 h fermented buffalo milk with precultured *E. faecium* Bd2
WG2Pre24h: Whey produced by 24 h fermented buffalo milk with precultured *L. plantarum* WG2
Bd2Pre48h: Whey produced by 48 h fermented buffalo milk with precultured *E. faecium* Bd2
WG2Pre48h: Whey produced by 48 h fermented buffalo milk with precultured *L. plantarum* WG2

Table 5.5: IC_{50} values of EDTA and whey buffalo milk (mg/ml)

Code	Sample	IC_{50}[a] (mg/ml)
EDTA	EDTA	0.29
Bd2Pre24h	*E. faecium*	0.39
WG2Pre24h	*L. plantarum*	0.39
Bd2Pre48h	*E. faecium*	0.41
WG2Pre48h	*L. plantarum*	0.37

Notes:
IC_{50}= Concentration of inhibitor required to inhibit 50 % of the FICA
EDTA: standard antioxidant EDTA
Bd2Pre24h: Whey produced by 24 h fermented buffalo milk with precultured *E. faecium* Bd2
WG2Pre24h: Whey produced by 24 h fermented buffalo milk with precultured *L. plantarum* WG2
Bd2Pre48h: Whey produced by 48 h fermented buffalo milk with precultured *E. faecium* Bd2
WG2Pre48h: Whey produced by 48 h fermented buffalo milk with precultured *L. plantarum* WG2

5.3.5 Protein Separation of Whey Buffalo Milk by Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis

The result of Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis or SDS PAGE pattern for the whey buffalo milk generated by precultured *L. plantarum* WG2 and *E. faecium* Bd2 for 24 h and 48 h of fermentation time is shown in Figure 5.5. The bands indicate the M_w of whey buffalo milk obtained the same pattern. It was observed that the M_w of the whey buffalo milk ranged between 150 and 50 kDa, and between 20 and 10 kDa for all whey buffalo tested milk. The big M_w displayed to produce sharp bands while the small M_w displayed to have broader bands.

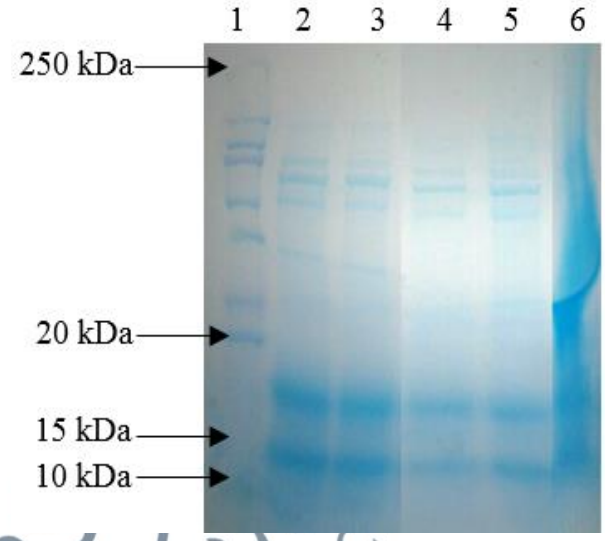


Figure 5.5: Protein Separation of Whey Buffalo Milk by SDS PAGE

Notes:
Lane 1: Marker
Lane 2: Peptides generated by 24 h of precultured *E. faecium* Bd2,
Lane 3: Peptides generated by 24 h of precultured *L. plantarum* WG2
Lane 4: Peptides generated by 48 h of precultured *E. faecium* Bd2
Lane 5: Peptides generated by 48 h of precultured *L. plantarum* WG2
Lane 6: Pasteurised buffalo milk (control)

5.4 Discussion

Lactic acid bacteria (LAB) are universal, economic, and safe microorganisms applied by the manufacturers in producing varieties of fermented foods including dairy products (Reginensi et al., 2013). Usually, these LABs were selected and used as starter cultures, probiotic supplements, and as microbial cell factories in the food/feed system (Rodriguez et al., 2019).

The isolation and identification of LAB are important to determine the most suitable strains to be applied in the products with specific characteristics. For examples, in a dairy industry of making fermented milk, selection of suitable strains of LAB as starter culture are crucial because the selected species of LAB should considered some importance technological properties such as ability to degrade proteins, probiotic properties for survivability in acid and bile environment, antimicrobial activity against pathogens as well as antibiotics resistance (Mechai et al., 2014; Uugantsetseg & Batjargal., 2014; Salem et al., 2013; Erkkilä, & Petäjä, 2000).

There are a great number of studies in the identification of LAB (Rodriguez et al., 2019; Tilahun et al., 2018; Caro et al., 2015; Suhartik et al., 2014; Baradaran et al., 2012; Yuliana & Dizon, 2011). Phenotypic and genotypic identification of LAB was determined using API 50 CHL kit and 16 S rDNA sequencing using PCR, respectively. There was a difference in LAB identification for Bd2 and Pk2 isolates using both methods. For Bd2 isolate, phenotypically about 94.9 % was similar to *L. plantarum* but when identified by genotypic approach, Bd2 isolate was identified as *E. faecium* with 99 % similarity. Phenotypically, 93.7 % of Pk2 isolate was similar to *L. lactis cremaris* but using genotypic approach, Pk2 isolate was identified as *L. paracasei* with 99 % similarity. Basically, phenotypic identification of LAB depends mainly on morphological, physiological, and biochemical criteria (Reginensi et al., 2013).

However, together with genomic DNA extraction of LAB isolates for further analysed in PCR reactions to amplify the 16S rDNA gene sequencing of these LAB isolates produced a reliable LAB identification. Based on phenotypic identification by API CHL 50 kit, the LAB isolate of K1, K3 and K5 obtained 97 %, 85 % and 99.9 % homology to the *Pediococcus pentosaceus* (*P. pentosaceus*), *L. lactis* and *L. curvatus*, respectively. But, by analysing genotypic identification by 16S rDNA gene sequencing, the same LAB isolates were 99 %, 97 % and 99 % confirmed to be *L. lactis*, *P. pentosaceus* and *L. curvatus*, respectively (Baradaran et al., 2012). Similarly, LAB strain T29 isolated from Indonesian fermented foods was identified as *L. plantarum* using phenotypic identification but when analysed using genotypic identification, the same LAB confirmed to be *P. acidilactici* (Suhartik et al., 2014).

Oxidative stress is a situation caused by an imbalance between oxidants and antioxidants (Esfandi et al., 2019). It is a condition where the production and accumulation of reactive species such as superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), and hydroxyl ($HO^{\bullet}$) exceed normal production which caused toxicity to cells and tissues (Pizzino et al., 2017). When this phenomenon happens, the reactive species could overtake the antioxidant defense of cells and could cause oxidative damage in lipids, proteins, and/or nucleic acids which leads to various pathological conditions. (Bandyopadhyay et al., 1999).

Consumption of antioxidant compounds in a daily diet could prevent or treat various human diseases caused by oxidative stress. In this study, whey with antioxidative peptides produced from buffalo milk fermented with different LAB strains identified as *E. faecium* Bd2, *L. paracasei* Pk2, *L. plantarum* WG2, and *L. plantarum* S1. Previous studies reported fermentation for varieties of milk by LAB strains including bovine milk (skimmed milk), goat milk, and camel milk produced

antioxidative peptides (Shu et al., 2018; Soleymanzadeh et al., 2016; Abubakr et al., 2012a). Skimmed milk fermented with *L. plantarum* 1 and *Leoconostoc mesenteroides* (*Leu. mesenteroides*) produced whey with antioxidant activity (Abubakr et al., 2012a). In addition, goat milk fermented with *L. casei* L61 also produced antioxidant peptides (Shu et al., 2018). Furthermore, fermented skimmed milk and camel milk with different LAB strains such as *L. plantarum, L. paraplantarum, L. kefiri, L. gasseri, L. paracasei, Leu. lactis, Weissella cibaria* (*W. cibaria*), and *E. faecium* also generate antioxidant peptides when analysing using scavenging DPPH free radical activity and 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging activity assays (Soleymanzadeh et al., 2016).

To date, a few studies was done on buffalo milk fermentation with LAB strains could also produce antioxidative peptides using both in-vitro and in-vivo approaches (Padghan et al., 2018; Taha et al., 2017; Vankudre et al., 2015). The LAB strains of *L. lactis* and *L. delbeurkii* were used in both skimmed milk and buffalo milk to generate antioxidant whey analysed using scavenging DPPH free radical activity assay (Vankudre et al., 2015). The antioxidant peptides were also present in yogurt produced from buffalo milk with a mixture of yogurt culture of *L. acidophilus* and *L. helveticus* (Taha et al., 2017).

The consumption of Lassi containing a mixture of Dahi culture and *L. acidophilus* as routine daily diet in mice for 15 to 30 days was evaluated using in vivo study and was found that the level of antioxidant activity increased by observing the antioxidant enzymes superoxide dismutase (SOD), catalase, and glutathione peroxidase (GSHPx) with Lassi made with standard Dahi culture and Lassi made with *L. acidophilus* as compared to that of fed with oxidised oil (Padghan et al., 2018). Using other types of milk rather than buffalo milk, the selection of LAB strains to generate

antioxidative peptides via buffalo milk fermentation was similar to other different types of milk where the LAB strains should be able to hydrolyse proteins into peptides and/or amino acids and could also potential as probiotic LAB (Jaimez-Ordaz et al., 2019; Mechai et al., 2014).

It was also found that different LAB strains generated whey buffalo milk with different scavenging DPPH free radical activity and FICA. Both antioxidant assays had their own concept in determining antioxidant compounds including antioxidants from proteins. Antioxidant assay by the scavenging DPPH free radical activity can be categorised in primary or chain-breaking antioxidants while antioxidant assay by FICA is another approach of assay that can be categorised as secondary or preventive antioxidants (Apak et al., 2016). In scavenging DPPH free radical activity assay, the antioxidant samples have ability to act as free radical scavengers or hydrogen donours which prevent formation of free radicals formation (Shekar & Anju, 2014). While in FICA assay, the antioxidant samples have ability to chelate transition of metal Fe^{2+} ions by inhibiting Fenton reaction formation (Genaro-Mattos et al., 2015). In this study, whey generated from fermented buffalo milk with selected LAB strains able to act as antioxidant compounds (peptides form) by both actions as chain-breaking antioxidants which involved in the termination of free radical reactions and metal ferrous ion-chelating ability, respectively.

Fermentation time by 24 h and 48 h did not significantly (P>0.05) affect antioxidant activity of whey buffalo milk by both scavenging DPPH free radical activity and FICA assays. In contrast, this finding was different to most previous studies that found fermentation time did affect the antioxidant activity of whey or peptides (Niu et al., 2013; Abubakr et al., 2012a; Chang et al., 2009). The scavenging DPPH free radical activity value of whey from fermented skim milk with *L. plantarum* 1 increased when

fermentation time increased from 24 h (48.9 %) to 72 h (50.7 %) (Abubakr et al., 2012a). In the study of peptides from defatted wheat germ produced by *B. subtilis* B1, it was found that optimisation of fermentation conditions including fermentation time could affect production of peptides generated by selected microorganisms (Niu et al., 2013). However, several reports that production of peptides by fermentation of microorganisms including LAB was not influenced by time. The development of antioxidative activity was generally increased during fermentation but was not directly connected to fermentation time. This might be happened due to each LAB strains applied in the fermentation as starter culture have their own optimisation temperature to work at best to produce antioxidative whey/peptides or extracts (Chang et al., 2009; Virtanen et al., 2007).

This study also found that different culturing approaches of LAB into buffalo milk for fermentation produced whey with high DPPH radical scavenging activity by direct cultured LAB and high FICA by precultured LAB. The highest antioxidative values by scavenging DPPH free radical activity assay obtained from whey produced from fermented buffalo milk inoculated with direct cultured *L. plantarum* S1 at 48 h (65.52 %) and *L. paracasei* Pk2 at 24 h (56.99 %). Meanwhile the highest antioxidative values by FICA assay obtained from whey produced from fermented buffalo milk inoculated with precultured *E. faecium* Bd2 (78.22 %) and *L. plantarum* WG2 at 48 h (84.45 %), respectively. There were a few conditions need to be concerned to produce antioxidative whey buffalo milk generated by LAB through fermentation process such as ability to lysis protein, have probiotic properties, and each of them need an optimal environment conditions like temperature, time and inoculation size at a specific amount (Chen et al., 2019). From this study, the finding of culturing LAB using either preculture or direct culture approach has been proven can optimise functionality of LAB

to generate an optimum antioxidative whey buffalo milk with high value.

The study of FICA is important because antioxidant as protector against oxidative damage by chelation therapy mentioned that the main defense to avoid generation of ROS is associated with redox active metal catalysis involves chelating of the metal ions especially ferrous ions (Ebrahimzadeh et al., 2008). Thus, half-maximal inhibitory concentration (IC_{50}) value of antioxidant activity of whey buffalo milk generated by precultured *E. faecium* Bd2 and *L. plantarum* WG2 was evaluated to determine the stronger metal chelating ability. In this study, whey buffalo milk exhibited ferrous ions chelating activity although it was significantly ($P<0.05$) lower than that of EDTA. Indeed, the IC_{50} values for metal chelating ability of skimmed milk hydrolysates fermented with *L. plantarum* 1 and *Leu. mesenteroides* were lower than this finding which were 0.46 mg/ml and 0.69 mg/ml, respectively (Abubakr et al., 2014). However, the IC_{50} value of goat milk casein hydrolysate was higher than this finding which was 0.048 mg/ml but this casein hydrolysate was obtained by enzymatic hydrolysis of protease, alkaline protease, papain and trypsin, not by LAB or other microorganism fermentation (Li et al., 2013). The IC_{50} values that represent the chelating effect of whey buffalo milk to ferrous ions obtained from this study found antioxidant capability and, thus, the findings of antioxidative peptides of whey buffalo milk could protect cells against oxidative damage.

Buffalo milk fermentation treated with selected LAB caused protein to become hydrolysed and fractionised into smaller protein, peptides and/or amino acids. These fractions can be known by observing the total band formed at SDS PAGE and comparing it with the control and selected marker. The electrophoresis processing showed the total bands of whey buffalo milk fermented with precultured and *E. faecium* Bd2 and *L. plantarum* WG2 were observed more than bands of pasteurised buffalo milk

(control). The protein hydrolysis of buffalo milk fermentation was reached optimally by both precultured *E. faecium* Bd2 and *L. plantarum* WG2 at different fermentation times. Similar to other bioactive peptides, these antioxidant peptides can only become activated or functional when degradation of latent sequence of protein takes place (Korhonen and Pihlanto, 2003).

The M_w of band fractions for the whey buffalo milk responsible for antioxidant activity in this study had been obtained between 150 to 50 kDa and 20 to 10 kDa. There was band with M_w obtained similar to the M_w for beta-lactoglobulin (β-lactoglobulin) and lactoferrin which was 80 kDa and 18 kDa, respectively (Abdou & Elbarbary, 2016; Aich et al., 2015). It was supported by the findings of previous studies mentioned that by culturing LAB in milk fermentation may produce bioactive peptides such as β-lactoglobulin and lactoferrin (Mahdi et al., 2018). The β-lactoglobulin is a major protein in whey, approximately 10 to 15 % of total milk proteins. It is a globular protein that consists of 162 amino acids. The β-lactoglobulin possesses a mild antioxidant activity whose potency is less than that of vitamin E and probucol; a synthesised antioxidant that clinically had been used to reduce cholesterol level (Liu et al., 2007). While lactoferrin is an iron binding glycoprotein and can act as antioxidant compounds (Safaeian et al., 2015). The antioxidant lactoferrin plays a protective role in oxidative stress induced damage by inhibiting production of ROS in the cell membrane (Eipper et al., 2016).

Previous studies demonstrated that lower M_w peptide possessed high antioxidative activity (Timón et al., 2019; Liu et al., 2013). The peptide separation by Liquid Chromatography with tandem mass spectrometry (LC-MS/MS) analysis method was done for fermented goat milk yogurt resulting in three peaks of fractions responsible for antihypercholerolemia. Each peptide sequence of fermented goat milk

yogurt composed of 16 amino acids where the M_w less than 3 kDa was identified as LYQEPVLGPVRGPFPI, YQEPVLGPVRGPFPIL and VQSWMHQPPQPLSPT, respectively (Mahdi et al., 2018).

Hydrolysis using neutral and alkaline proteases generated goat milk casein hydrolysates with antioxidative activity and the M_w of five antioxidative fractions obtained was less than 1 kDa ranged between 931.47 and 524.28 Da. These five fractions were identified using nano-electrospray ionisation quadrupole-time-of-flight-tandem MS and each amino acid sequence was as followed: Val-Tyr-Pro-Phe, Phe-Gly-Gly-Met-Ala-His, Phe-Pro-Tyr-Cys-Ala-Pro, Tyr-Val-Pro-Glu-Pro-Phe, and Tyr- Pro-Pro-Tyr-Glu-Thr-Tyr, respectively (Li et al., 2013). The production of peptides from whey proteins were rather amphiphilic in nature, due to the presence of hydrophilic residues such as Lys, Asp, Glu, and Ser that scattered along the peptides with other hydrophobic residues (Zou et al., 2016). In addition, some of the possible reason for antioxidant peptides was because of hydrophobic amino acid residues including Phe, Pro, Gly, and Ala, positions of Pro, Val, and Phe residues in the N- or C-terminal, as well as His residues in the C-terminal position in the sequences which trigger antioxidant peptides to interact with fatty acids and improved the capturing ability for lipid free radicals (Li et al., 2013).

5.5 Conclusion

Through LAB identification, all four LAB were identified as *L. plantarum*, *L. paracasei*, *L. plantarum* and *E. faecium* for LAB strains of WG2, Pk2, S1 and Bd2, respectively. These four LABs successfully generated whey buffalo milk with antioxidative activity. The fermentation of buffalo milk with either or *E. faecium* Bd2 and *L. plantarum* WG2 by preculturing approach successfully generated whey with high

antioxidant activity by ferrous chelation as preventive antioxidants with the FICA of 84.45 % (v/v) at 48 h fermentation with IC_{50} value of 0.37 mg/ml and 78.22 % (v/v) at 24 h fermentation with IC_{50} value of 0.39 mg/ml, respectively.

Separation of peptides of whey buffalo milk revealed antioxidative peptide fractions in bands formation by size of M_w between 150 and 50 kDa, and between 20 and 10 kDa. Further study can be done to identify these antioxidative peptide fractions because instead of probability of β-lactoglobulin and lactoferrin as antioxidant, the possibility of other antioxidative peptides or novel finding could be determined so that the next new findings could provide other alternative to choose natural antioxidant to be implemented in food, feed, and pharmaceutical industries.