CHAPTER 3

SCREENING OF LACTIC ACID BACTERIA FOR PROTEOLYTIC AND ANTIOXIDATIVE ACTIVITIES IN SKIMMED MILK

3.1 Introduction

Lactic acid bacteria (LAB) are Gram-positive and facultative anaerobe bacteria that convert lactose into lactic acid as the main product (Mokoena, 2017). The LABs were found about three billion years ago and some LAB species seem to be well adapted to both anaerobic and aerobic life conditions, tolerate with all necessary proteins for respiration, and several enzymes involved in fermentative pathways (Carr et al., 2002). In general, LAB lives in nature, these bacteria are distributed world-wide and have been isolated from plants, animals, and soils among many sources (Abubakr et al., 2012a; Henning et al., 2015; Husain et al., 2017). The recognition that LAB were Generally Recognised as Safe or GRAS resulted in them as starter cultures in most fermented food products especially in milk products (Widyastuti el al., 2014).

Each species of LAB is unique with its own specific characteristics. By choosing the suitable LAB species and manipulating their characteristics, the LAB become the most important group of microorganisms that have been used in food fermentation and pharmaceutical industries as well as in special dietary applications (Liu et al., 2014). One of the unique characteristics of LAB is proteolytic activity in which they are able to hydrolyse protein into peptides with bioactivity (Abubakr et al., 2012a). The proteolytic system of LAB consists of three major components which are the cell-wall bound proteinase, the peptidases, and the peptide transporters. These three proteolytic systems of LAB convert large proteins into small peptide fractions and/ or to amino acids, which is crucial for bacterial growth, provide flavour to fermented foods (such as in cheese making), and generate bioactive peptides with numerous physiological functionality (Broadbent & Steele, 2007; Korhonen & Pihlanto, 2006; Liu et al., 2010). There are several ways to determine LAB with proteolytic activity such as using spot cultivation, and well-diffusion tests with selective media (using skimmed milk media) (Moslehishad et al., 2017). The proteolytic activity of LAB can be determined by measuring the clear zone area around skimmed milk media after certain incubation conditions (Abubakr et al., 2012a; Moslehishad et al., 2017; Pailin et al., 2001; Phyu et al., 2015).

Antioxidants prevent or delay oxidative damage in several ways including preventing the formation of radicals and scavenging free radicals (Abubakr et al., 2012a). In fact, butylated hydroxylanisole (BHA), butylated hydroxytoluene (BHT), and tert-butyl hydroquinone (TBHQ) that are widely used in foods are commercial synthetic antioxidants and exhibit strong antioxidant activity could have negative effect towards human health (Race, 2009). These synthetic antioxidants may be effective at very low concentration, but when consumed at a high dosage may result in intoxication (Sarkar & Ghosh, 2016). Natural antioxidant peptides generated from various natural protein sources including meats, egg yolk, and beans have been reported by several researchers and the peptides usually are hydrolysed using pepsin, and pacreatin enzymes to generate antioxidative peptides (Carrasco-Castilla et al., 2012; Liu et al., 2016; Yousr & Howell, 2015).

Determination of antioxidant activity can be measured via different approaches and each antioxidant assay depends on the principle of the analysis, and type of samples. Methods such as scavenging of 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical activity, and ferrous ions chelating activity (FICA) assay are the two most commonly used assay methods. The DPPH radical scavenging assay is a stable free radical, and the method to evaluate the antioxidant property that scavenge free radicals is considered as easy, rapid, and sensitive (Nandhini et al., 2012). Furthermore, in FICA assay, the sample with antioxidant activity competes with ferrous ions might participate in hydroxyl-radical-generating Fenton reactions (Bamdad et al., 2017; Lim, 2013).

Milk and derived dairy products are considered as the most complete food for mammals. Milk protein either derived from whole milk, casein fractions, or whey proteins can be one of the sources of many bioactive components including biological active peptides (Davoodi et al., 2016; Hseih et al., 2014). The bioactive peptides in milk are latent and remain inactive in the original primary structure of native protein (Korhonen & Pihlanto 2006; Minervini et al., 2003; Park & Nam, 2015). These peptides can be released by proteolysis during gastrointestinal transit (digestive enzyme), during food processing either by fermentation of proteolytic starter cultures, or using enzymes derived from microorganisms, animals, and plants. Once the bioactive peptides are released, these peptides are able to function in gastrointestinal, cardiovascular, immune, endocrine, and nervous systems (Korhonen & Pihlanto, 2003). Fermentation of milk with LAB were reported to produce compounds that have antioxidative activities (Abubakr et al., 2012a; Osuntoki & Korie, 2010; Virtanen et al., 2007), however, were dependable to the LAB strains, and not directly connected to fermentation time (Virtanen et al., 2007).

Previously, whey generated from fermented skimmed milk inoculated with *Lactobacillus brevis* (*L. brevis*) OK5 (isolated from wara), and *L. plantarum* (isolated from grape) obtained high DPPH radical scavenging activity after 24 h fermentation and 72 h fermentation, respectively (Abubakr et al., 2012a; Osuntoki & Korie, 2010) indicating have potential antioxidant peptides (Abubakr et al., 2012a). Furthermore, Lim (2013) reported that the FICA of yogurt and soya yogurt were ranged from 40.2 and 62.0 % (*L. casei* PC05 and *L. acidophilus* PC16 as starter culture) after eight days storage. These indicated that protein hydrolyse by proteolytic LAB have antioxidative activity.

Selection of LAB with proteolytic activity is crucial in order to apply for generating bioactive antioxidative peptides properties from milk because not all LAB are able to hydrolyse protein into peptides (Abubakr et al., 2012a; Phyu et al., 2015). Therefore, the purpose of this study was to isolate LAB from different food sources, and screen for its proteolytic activity. Isolates that obtained strong proteolytic activity were further evaluated for their antioxidative activity using DPPH free radical activity, and FICA assays in whey generated from fermented skimmed milk cultured with the isolated LAB.

3.2 Materials and Methods

3.2.1 Microorganisms and Culture Conditions

Twenty-five LABs were isolated from local fermented foods (seven isolates), fruits (eleven isolates), milk and milk products (six isolates), and soil (one isolate) were obtained from different places of Malaysia (Table 3.1). Prior to isolation, ten grammes of each sample was added to 90 ml 0.1 % peptone water in a stomacher bag. Next, the sample was homogenised using stomacher (Stomacher® 400 Circular Seward) and LAB from each sample was then isolated in serial dilutions. After that, an appropriate serial dilution of 0.1 ml was spread plated on de Man, Rogosa and Sharpe (MRS) agar (Oxoid CM0361) plates containing 0.8 % calcium carbonate ($CaCO_3$).

Table 3.1: Isolation of Lactic Acid Bacteria from Different Sample Sources

Source	Sample	LAB code
Fermented foods	Belacan	Bc
	Budu	Bd1, Bd2, Bd3
	Pekasam	Pk1, Pk2
	Tempoyak	Ty
Fruits	Banana	Bn1, Bn2, Bn3, Bn4
	Black grape	BG
	Dates	Dt
	Durian	Dr
	Pineapple	Pn
	Watery rose guava	RG
	White grape	WG1, WG2
Milk and Milk Products	Buffalo milk	Bf
	Cow milk	Cw
	Cultured drink	Vt1, Vt2
	Goat milk	Gt
	Homemade yogurt	Yg
Soil	Soil	Sl

All agar plates were incubated anaerobically in an aerobic jar at 37 °C for 48 h. Each of the isolates was tested for catalase by placing a drop of 15 % hydrogen peroxide (H_2O_2) solution on the cells. Immediate formation of bubbles indicated the presence of catalase in the cells. The isolates which were only catalase-negative were Gram-stained, and the morphology was observed using Nikon microscope (Nikon Eclipse 80i). All LAB isolates were purified and inoculated into MRS slant agar as a stock culture, and incubated at 37 °C for 24 h. Prior to beginning of further analysis, each bacterial strain was sub-cultured at least 3 times (1%, v/v) in MRS broth (Oxoid, CM0359) at 24 h intervals (Kheadr, 2006). Two commercial LABs of *L. plantarum* ATCC 8014, and *L. casei* ATCC 393 were used as positive control species.

3.2.2 Proteolytic Activity of Lactic Acid Bacteria on Skimmed Milk Agar

The skimmed milk agar (SMA) was prepared according to Pailin et al., (2001) with some modification. Twenty-five grammes of skimmed milk powder (Oxoid LP0031) was added with 250 ml of distilled water. Then, the mixture was stirred thoroughly and autoclaved at 110 °C for 10 min. Likewise, 500 ml of 2.5 % agar solution was prepared and autoclaved at 121 °C for 15 min. Both mixtures were held in a water bath at 50 °C. The skimmed milk solution was poured into the agar solution bottle and mixed thoroughly and, were then quickly poured the SMA into plates. Proteolytic activity of LAB was evaluated using SMA and incubated at 37 °C for 48 h in an anaerobic environment followed by cooling in a refrigerator at 4 °C for 3 d. Protein hydrolysis by LAB was observed through the production of clear zones surrounding a single isolate. Duplicate trials were conducted, and all results were averaged and reported as diameter in mm.

3.2.3 Preparation of Precultures and Fermentations of Skimmed Milk

3.2.3.1 Fermentation of Skimmed Milk with Selected Lactic Acid Bacteria

Each LABs isolate was inoculated into 10 ml MRS broth and incubated at 37 °C for 24 h. Initially, 1 % (v/v) of the culture was inoculated into sterilised skimmed milk (sterilised at 110 °C for 10 min) and incubated at 37 °C for 24 h to generate precultured LAB. After that, 2 % (v/v) of these precultured were propagated into pasteurised skimmed milk (pasteurised at 62 °C for 30 min), and pasteurised skimmed milk without the bacteria as a control experiment, before incubating at 37 °C for 24 h. The fermentation of skimmed milk by the LAB was carried out in a triplicate experiment (Abubakr et al., 2012a).

3.2.3.2 Preparation of Whey Skimmed Milk

The whey skimmed milk was prepared following the method described by Virtanen et al. (2007). Aliquots of fermented skimmed milk prepared as described in Section 3.2.3.1 were collected, and the pH of fermented skimmed milk was adjusted to 4.6 with 1M HCl. Next, the suspension was harvested by centrifugation at 10,000 x g for 20 min at 4 °C (Centrifuge Combi-514R, Korea). The non-hydrolysed casein was removed, and the supernatant was filtered using 0.45 µm filter (Millipore Corp, USA). The whey skimmed milk (filtrate) was further analysed for antioxidant activity using DPPH radical scavenging activity and FICA assays. The pasteurised skimmed milk without bacteria was used as a control experiment for both antioxidant assays.

3.2.4 Determination of Antioxidant Activity of Whey Skimmed Milk

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

Determination of 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity of whey skimmed milk was evaluated according to Son and Lewis (2002) method with modification. First, the DPPH solution (0.004 % w/v) in 95 % ethanol was prepared and two ml of the DPPH solution was added to 2 ml of whey skimmed milk prepared in Section 3.2.3.2. Next, the mixture was vortexed and incubated for 30 min in a dark place at room temperature (25 °C). The optical density (OD) of each mixture was measured at 517 nm using a visible spectrophotometer (Varian Cary 50 Conc). Ethanol was used as blank, while the DPPH solution in ethanol served as a control experiment. The ascorbic acid (Sigma, Germany) at a concentration 0.02 mg/ml was used as a positive control experiment and for standard curves assay. The antioxidant activity was expressed as a percentage of DPPH activity using the following equation.

$$\text{DPPH scavenging activity (100\%)} = \left(\frac{\text{Absorbance } (A_{517}) \text{ of blank - Absorbance } (A_{517}) \text{ of sample}}{\text{Absorbance}(A_{517}) \text{ of blank}} \right) \times 100$$

3.2.4.2 Ferrous Ion Chelating Activity

The ferrous ion chelating activity or FICA of whey skimmed milk generated by LAB was evaluated using the Decker and Welch (1990) method with modification. One ml of whey skimmed milk was mixed in 3.7 ml of distilled water. Prior to analysis, 0.1 ml of 2 mM ferrous chloride (Sigma Aldrich) was added to the mixture. Next, after 3 min the mixture was added with 0.2 ml of 5 mM ferrozine (Sigma Aldrich) and was vigorously shaken before leaving at room temperature (25 °C) for 10 min. The OD of the reaction mixture was measured at A_{562} nm using a visible spectrophotometer (Varian Cary 50 Conc). A blank without sample was prepared in same protocol and 0.1 mg/ml of ethylenediaminetetraacetic acid (EDTA) was used for comparison. The chelating capacity was calculated as a percentage using the following formula.

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

3.2.5 Statistical Analysis

The proteolytic activity of LAB on SMA and antioxidant activity of whey skimmed milk both DPPH and FICA assays were presented as mean ± standard deviations of average readings and were statistically analysed using one-way and two-way analysis of variance (ANOVA) using Minitab version 16 (Germany). Correlation analysis for antioxidant activity of whey skimmed milk generated by LAB was done using Pearson correlation between DPPH and FICA methods. The p-value (P<0.05) were considered statistically significant.

3.3 Results

3.3.1 Characteristics of Lactic Acid Bacteria Isolates

Twenty-five LABs isolated from different sources obtained clear zones on modified MRS-$CaCO_3$ agar, catalase negative, and Gram-positive were considered as LAB (Aween et al., 2012). Most of the LAB isolates were rod in shape except for the LAB isolates of Bc, Dr, and Gt which were in cocci shape that originated from goat milk, durian, and belacan, respectively (Table 3.2).

Table 3.2: Characteristics of Lactic Acid Bacteria Isolated from Different Sources

Source	Sample	LAB code	Catalase reaction	Gram reaction	Cell morphology
Fermented foods	Belacan	Bc	-	+	Cocci
	Budu	Bd1	-	+	Short rod in pairs
		Bd2	-	+	Rod in pairs
		Bd3	-	+	Rod in pairs
	Pekasam	Pk1	-	+	Short rod
		Pk2	-	+	Short rod in pairs
	Tempoyak	Ty	-	+	Short rod
Fruits	Banana	Bn1	-	+	Rod
		Bn2	-	+	Short rod
		Bn3	-	+	Short rod
		Bn4	-	+	Rod
	Black grape	BG	-	+	Rod in cluster
	Dates	Dt	-	+	Rod
	Durian	Dr	-	+	Cocci
	Pineapple	Pn	-	+	Short rod
	Watery rose guava	RG	-	+	Short rod in cluster
	White grape	WG1	-	+	Rod
		WG2	-	+	Short rod
Milk and Milk Products	Buffalo milk	Bf	-	+	Short rod
	Cow milk	Cw	-	+	Rod
	Cultured drink	Vt1	-	+	Rod in cluster
		Vt2	-	+	Short rod in pairs
	Goat milk	Gt	-	+	Cocci
	Homemade yogurt	Yg	-	+	Rod
Soil	Soil	Sl	-	+	Short rod
Commercial	Control	*L. plantarum* ATCC 8014	-	+	Rod
		L. casei ATCC 393	-	+	Rod

Notes: (+) positive; and (-) negative reactions

3.3.2 Proteolytic Activity of Lactic Acid Bacteria Isolates

The example of LAB isolates with proteolytic activity obtained clear halos on SMA plates (Figure 3.1). Results found that ten LAB isolates including two commercial LABs were found to be proteolytic LAB. Good proteolytic activity was observed from 6 out of 10 isolates where the clear halos surrounding the colonies were greater than 6 mm in diameter on SMA (Abubakr et al., 2012a).

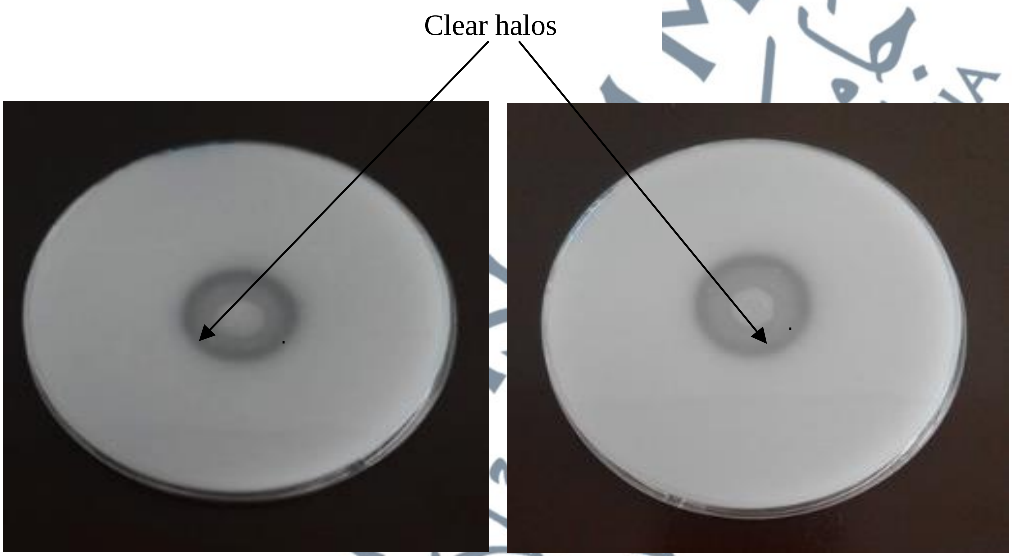

Figure 3.1: Clear Zones Around Colony on SMA Plate Indicating Proteolytic Activity

Table 3.3: Proteolytic Activity of LAB Isolates on Skimmed Milk Agar

Lactic acid bacteria	**Diameter of clear zone (mm)**
L. plantarum ATCC 8014	6.3 ± 0.35^{b}
L. casei ATCC 393	6.0 ± 0.00^{bc}
Bd2	14.5 ± 0.71^{a}
Pk2	7.3 ± 1.06^{b}
Bn2	3.5 ± 0.71^{d}
BG	4.0 ± 0.00^{cd}
Dt	4.0 ± 0.00^{cd}
WG2	12.5 ± 0.71^{a}
Yg	4.0 ± 0.00^{cd}
Sl	8.0 ± 0.00^{b}

Notes: Means within column with different lowercase letters indicate significant differences (P<0.05) between LAB isolates

There was a significant difference (*P*<0.05) on the proteolytic activity of LAB between the isolates and both two commercial LABs of *L. plantarum* ATCC 8014 and *L. casei* ATCC 393. Isolates of Bd2, Pk2, WG2, and Sl, found high clear halos which were 14.5 mm, 7.3 mm, 12.5 mm, and 8.0 mm, respectively. However, both control species of *L. plantarum* ATCC 8014 and *L. casei* ATCC 393 found clear halos with 6.3 mm and 6.0 mm, respectively (Table 3.3). All the ten LAB isolates were further screened for their ability to hydrolyse milk into peptides with antioxidative activity in 24 h fermentation of skimmed milk.

3.3.3 Antioxidant Activity of Whey Skimmed Milk

3.3.3.1 Scavenging of Free Radical Capacity of Whey Skimmed Milk

The four highest DPPH free radical scavenging activity of whey skimmed milk produced by LAB isolates in descending order were isolates S1 (19.04 %), Bd2 (18.70 %), WG2 (12.82 %), and Pk2 (12.59 %) (Figure 3.2). The LAB isolates were from soil, budu, white grape, and pekasam, respectively. From the figure, it was obtained that whey skimmed milk fermented by *L. casei* ATCC 393 (LAB control) displayed the significantly (*P*<0.05) lowest capacity (4.25 %) to scavenge DPPH free radical. The values of scavenging activity obtained from whey skimmed milk were significantly (*P*<0.05) lower than ascorbic acid (98.91 %) (Data not shown in Figure 3.2). Other DPPH free radical scavenging activity of whey skimmed milk by LAB isolates in the descending order were isolate Dt (8.58 %), control *L. plantarum* ATCC 8014 (6.13 %), isolate Bn (5.26%), isolate Yg (5.15 %), and isolate WG2 (4.40 %).

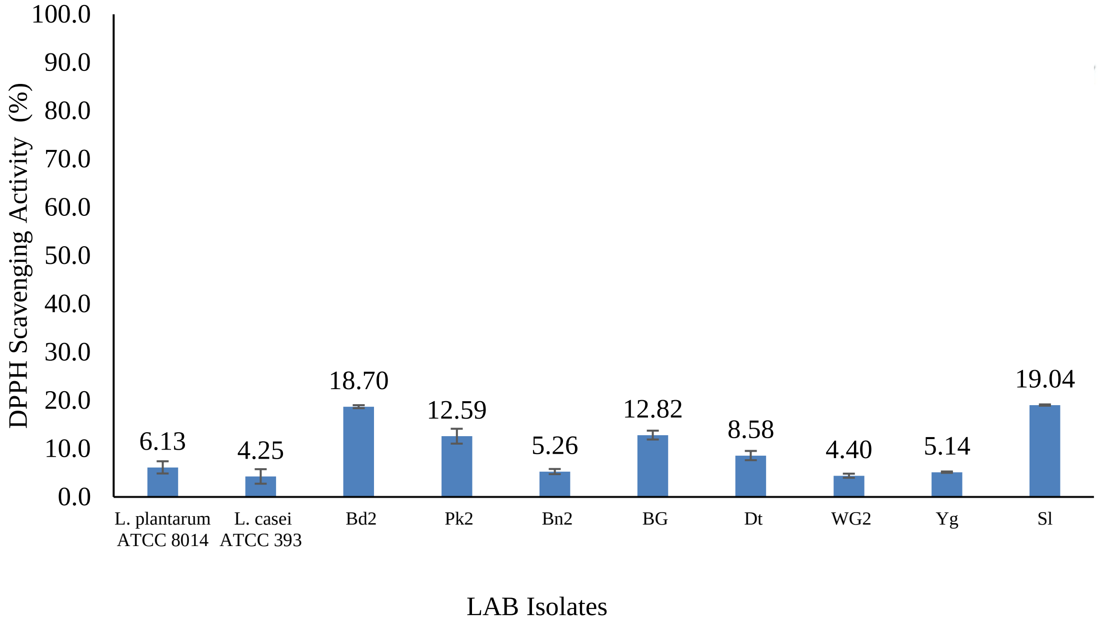

Figure 3.2: Antioxidant Activity of Whey Skimmed Milk Determined by DPPH Free Radical Scavenging Activity Assay

3.3.3.2 Ferrous Chelating Activity of Whey Skimmed Milk

The four highest reducing capacity of whey skimmed milk produced by LAB isolates were also displayed by isolates S1 (84.33 %), Bd2 (84.81 %), WG2 (78.61 %), and PK2 (82.71%) (Figure 3.3) similar to the DPPH free radical scavenging activity. The ferrous chelating capacity of whey skimmed milk fermented by *L. plantarum* ATCC 8014 (control LAB species) found significantly (P<0.05) the highest (86.95 %). However, all ferrous chelating capacity of whey skimmed milk produced by LAB were significantly (P<0.05) lower than commercial antioxidant, EDTA (97.77 %) (Data not shown in Figure 3.3). Indeed, the ferrous chelating activity of whey skimmed milk in this studied ranged between 56.02 and 86.95 % (Figure 3.3).

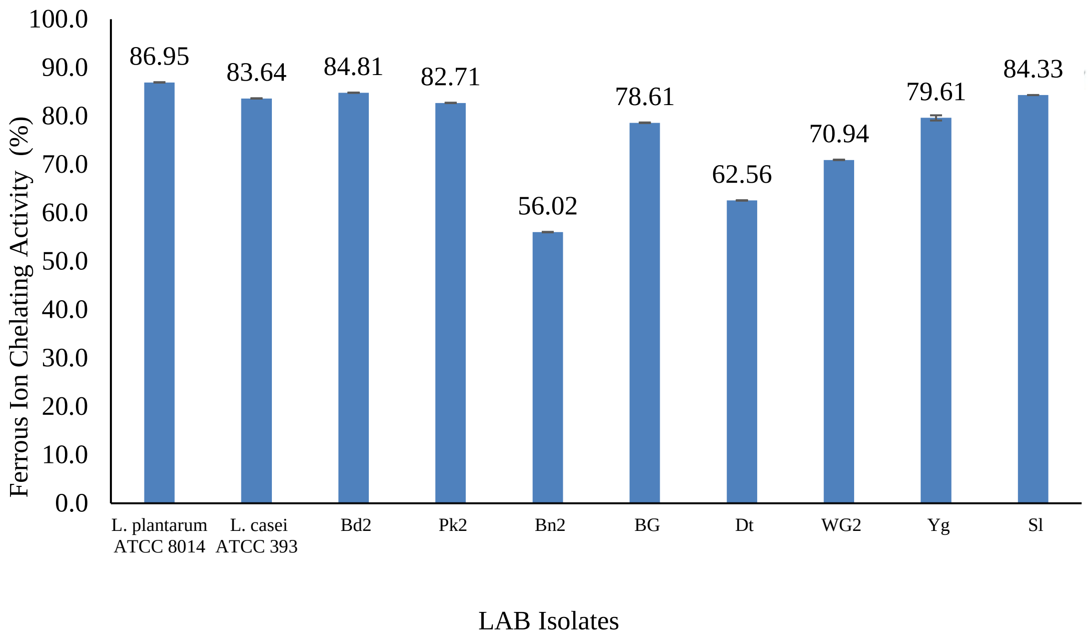

Figure 3.3: Antioxidant Activity of Whey Skimmed Milk Determined by FICA Assay

3.4 Discussion

Lactic acid bacteria (LAB) are widespread in nature and have been used in food fermentation especially in production of dairy products (Gemechu, 2015). One of the LABs characteristics is the ability to hydrolyse protein into peptides which have numerous biological activities including antioxidative peptides. The ability of LABs to hydrolyse milk protein into peptides can be determined by spotting the LAB culture onto the SMA and measuring the clear zone (Pailin et al., 2001).

In this study, eight LAB isolates with proteolytic activities were found in different sources which were from local fermented foods (Pk2, Bd2), fruits (Bn2, WG2, BG, Dt), yogurt (Yg), and soil (Sl). The highest clear zone on SMA was found in isolate Bd2 with the clear zone of 14.5 mm in diameter followed by isolate WG2 with the clear zone of 12.5 mm in diameter. Previously, a study reported that the isolated LABs from fruits were able to hydrolyse milk protein with the clear zone ranged between 2.0 and 10.0 mm (Abubakr et al., 2012a; Maryam & Wedad, 2017) in diameter.

Previously, Phyu et al. (2015) reported a high clear inhibition zone by LAB (between 11 and 26 mm) of at incubation time of 24, 48, and 72 h. The widest clear inhibition zone was obtained by isolate L9 (26 mm) for 72 h incubation time followed by isolate L1 (25 mm) at 48 h incubation (both were incubated at 37 °C). Cultivation of LAB in MRS broth at 37 °C at different hours may also influent better proteolytic activity of LAB compared to this study (at 48 h). It was found that the LAB culture broth of both 48 and 72 h incubation times were more effective than 24 h. Nevertheless, all eight proteolytic LABs obtained in this study, were further tested at 24 h fermentation of skimmed milk to produce whey skimmed milk with antioxidative activity.

The percentage of DPPH free radical scavenging activity of whey skimmed milk by isolates LAB obtained were ranged from 4.25 and 19.74 % (Figure 3.2) and lower than previous studies (Abubakr et al., 2012a; Osuntoki & Korie, 2010; Uugantsetseg & Batjargal., 2014; Virtanen et al., 2007). In fact, the scavenging activity of whey skimmed milk to DPPH varied with LAB species used in the fermentation. Previously, Uugantsetseg and Batjargal (2014) reported the highest antioxidant activity of whey skimmed milk fermented was fermented with *Leuconostoc mesenteroides* (*Leu. mesenteroides*) ssp. *cremoris* strains followed by *L. acidophilus* ATCC 4356. The scavenging activity of whey skimmed milk by LAB isolated from a traditional Mongolian fermented milk called an airag ranged between 3.98 and 27.79 %. However, the scavenging activity value of whey fermented milk with lactobacilli isolated from Nigerian fermented foods ranged from 2.8 % to 31.5 % for 24 h fermentation (Osuntoki & Korie, 2010).

A similar study was done by Abubakr et al. (2012a) regarding DPPH free radical scavenging activity of skimmed milk fermented (24 h) by isolates LAB from fruits. The findings obtained that DPPH free radical scavenging activity were higher and ranged between 14.7 and 48.9 %. The scavenging activity of milk protein hydrolysed by LAB of fermented skimmed milk increased with increament of fermentation time and was in line with a previous study (Virtanen et al., 2007). Even though Virtanen et al. (2007) evaluated radical scavenging activity using ABTS assay, the radical scavenging concept in both assays is the slightly similar. DPPH assay is used to determine radical scavenging activity in hydrophobic antioxidant systems, however, ABTS assay indicate better reflection radical scavenging activity in hydrophilic antioxidant systems. The wavelength used to monitor coloured radical depletion in spectrophotometric measurements of DPPH and ABTS assays were 517 nm and 745 nm, respectively (Olszowy & Dawidowicz, 2018).

Several factors may influence DPPH free radical scavenging activity of whey skimmed milk. These include microorganism strains used to boost the fermentation process, fermentation time, fermentation temperature, and pH of the environment fermentation. In yogurt making with high antioxidative activity, it was found that a mixture culture of *L. casei*, *L. acidophilus*, and *Bifidobacterium bufidus* (*B. bufidus*) need to reach until 12 h fermentation, or until the pH reach up to 4.6. Meanwhile, yogurt making with a mixture of *Streptococcus thermophilus* (*S. thermophilus*), and *L. delbrueckii* ssp. *bulgaricus* (4 h fermentation) drastically increased DPPH radical scavenging activity up to 52.44 % compared to fermentation with a single species. Unfortunately, after three days of storage at 4 °C, the DPPH radical scavenging activity decreased to 39.43 % (Gjorgievski et al., 2014). Although the scavenging activity of whey skimmed milk by LAB in this present study was lower than previously but is still considered to have a potential act as antioxidant.

Several methods are usually required to evaluate antioxidant activities of a sample to determine the antioxidant capacity in parallel relationship. Based on Figure 3.3, it was shown that the FICA of whey skimmed milk were more than 50.00 % (56.02 and 86.95 %) after 24 h fermentation by isolates LAB. The highest and lowest (P<0.05) FICA were obtained from whey skimmed milk fermented by *L. plantarum* ATCC 8014 (86.95 %) and isolate Bn2 (isolated from banana) (56.02 %). Previously, Liu et al (2005a) reported that the percentage of FICA of milk and soya milk fermented by kefir grain were 25.89 % and 12.90 % (at the concentration 10 mg/ml), respectively, during 24 h fermentation which similar to the fermentation condition in this present study. The FICA percentage of 4.0 mg/ml of steriled cow and goat milk, as well as cow and goat milk propagated with kefir were approximately between 50.00 and 57.00 % after fermentation at 20°C for 20 h (Liu et al., 2005b). However, these studies did not determine the specific bacterial species in the kefir that possess proteolytic activity produce bioactive peptide, and certainly the kefir contain several cultures which cause the FICA lower than this present study.

The FICA of whey skimmed milk fermented by bacterial isolates were affected by fermentation time. For example, fermentatian at 37°C for 24 h, the FICA percentage of whey skimmed milk fermented by *L. plantarum* ATCC 8014 and isolated Gr4 (*L. plantarum* 1 isolated from red grape) were 76.00 % and 97.60 %, respectively (Abubakr et al., 2012a). However, when the fermentation process was extended up to 72 h, the FICA percentage of the whey skimmed milk decreased until 54.00 % and 59.30 %, respectively. This was associated to the limited subtrate in the batch fermentation process in the experiment.

Antioxidant activity of fermented soya bean by fungal species were reported by Lin et al. (2006) and Sitanggang et. al. (2020), respectively. Using FICA technique, antioxidant activity of koji (made from fermented soya bean) with fungi *Aspergillus sojae* (*A. sojae*) BCRC 30103, *Actinomucor taiwanensis* (*A. taiwanensis*), and *Rhizopus* sp. at the concentration of 10 mg/ml obtained up to 84.00 % after 3 days fermentation. Furthermore, Guan et al. (2016) used *Actinomucor elegans* DCY-1 to ferment okara and found a gradual increment in antioxidant activities at 24 h, 36 h, 48 h, and 60 h fermentation time as indicated by the enhanced FICA activity. During this solid-state fermentation model, isoflavones-derived compounds, small molecular weight peptides, and amino acids might be produced and could enhance the antioxidant activities of fermented okara.

During milk and soya bean fermentation, proteins were hydrolysed to several peptides by microorganisms through proteolytic activity. These peptides mostly possess bioactivity functions including antioxidant properties (Shu et al., 2018; Tagliazucchi et al., 2019; Taha et al., 2017). For examples, peptide antioxidants from milk whey protein include VGINYWLAHK are derived from α-lactoalbumin, and LAFNPTQLEGQCHV are derived from β-lactoglobulin (Correa et al., 2014; Mann et al., 2015). However, different microorganism species (Abubakr et al., 2012a; Embiriekah et al., 2016; Uugantsetseg & Batjargal, 2014) might hydrolyse protein with different mechanisms, different conditions, and fermentation time. Longer fermentation time decreased antioxidant activity of peptide antioxidants in batch fermentation model (Abubakr et al., 2012a; Virtanen et al., 2007) but the highest antioxidant activities of peptide antioxidant from fermented okara were achieved using solid-state fermentation model (Rashad et al., 2011; Sitanggang et. al. 2020). Thus, the peptide antioxidant activity was greatly associated with the type of fermentation model in the conducted experiment.

Pearson correlation coefficient (r) was applied to express the relationship and/or direction of the proteolytic activity of LAB and antioxidant activity. No direct relationship (r=0.067) between the diameter of milk hydrolysis of LAB isolates on SMA and the ability of LAB to produce high or low antioxidant percentage for DPPH scavenging activity and FICA. Furthermore, there was a strong positive correlation (r=0.973) for antioxidant activity of whey skimmed milk generated by LAB between DPPH and FICA methods. However, the antioxidant activity using FICA was higher than the DPPH scavenging activity and was in accordance with Abubakr et al. (2012a).

Indeed, the DPPH is a stable free radical that has been widely used for assessment of radical scavenging activity. When DPPH meets an antioxidant compound (test sample), or other proton-donating substance (mainly hydrogen ions), the absorbance is reduced and the appearance of deep violet colour fades because the DPPH radical is scavenged or neutralised by tested sample (Kedare & Singh, 2011). However, in FICA method, transition of free ferrous ions promotes reaction of free radicals to generate oxidation (mainly produce hydroxyl radicals). With addition of whey skimmed milk containing antioxidant properties, free ferrous ions are chelated, and decreased ferrous ion-ferrozine complex concentration to form colorless complex. (Gülçin, 2005). Thus, FICA determine the total ion ferrous-ferrozine complex binding percentage (by inhibiting production of hydroxyl radicals) compared to DPPH assay which measure the radical scavenge activity (by producing of non-radical DPPH-H) which cause FICA amount is higher than DPPH.

3.5 Conclusion

From this study, it can be concluded that LABs could hydrolyse skimmed milk into bioactive peptides which exhibited antioxidant activity within 24 h fermentation. Peptide antioxidants were also successfully hydrolysed from whey skimmed milk by isolates LABs. The peptides fermented by four isolates LAB namely isolates Bd2, Pk2, WG2, and S1 for 24 h fermentation obtained amongt the four highest antioxidant activity using both DPPH and FICA. Thus, these peptide antioxidants from fermented whey skimmed milk have a potent to be a good dietary supplement into food formulations or nutraceuticals in the future research.