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Research Article

ANTIOXIDANT ACTIVITY OF MILK FERMENTED WITH LACTOBACILLUS PLANTARUM 1 AND LEUCONOSTOC MESENTEROIDES ISOLATED FROM NON-DAIRY SOURCES

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ABSTRACT

The aim of this study was to evaluate the antioxidant activity of skim milk hydrolysate fermented with lactobacilli isolated from non-dairy sources as determined by 1, 1-diphenyl-2-picrylhydrazyl (DPPH) assay, ferrous chelating activity (FCA) and reducing power (RP). The values of DPPH, FCA and RP increased with concentration of skim milk hydrolysate (SMH) and varied LAB isolates. The DPPH IC_{50} values of SMH fermented with Lb. plantarum1 was higher (2.92 mg/ml) than Ln. mesenteroides and Lb. plantarum ATCC8014. While, the IC_{50} values of SMH fermented with Lb. plantarum 1 and Ln. mesenteroides were 0.46 and 0.69 mg/ml, respectively greater than Lb. plantarum ATCC8014 (IC_{50} value 0.74 mg/ml) but lower than EDTA. All isolates showed poor reducing power compared to ascorbic acid. Among the LAB isolates Lb. plantarum ATCC8014 and Ln. mesenteroides seemed to generate peptides with similar reducing power activity. Lb. plantarum1 and Ln. mesenteroides isolated from non-dairy sources have probiotic properties and antioxidative properties which could benefit consumers.

Key words: *Antioxidant Activity, Lactobacillus Plantarum 1, Leuconostoc Mesenteroides, Fermented Milk,*

INTRODUCTION

The global interest in harnessing the beneficial properties of microbes and their metabolites for human health, coupled with the unique opportunity offered by fermented foods as vehicles for the delivery of bioactive agents produced by food-grade microbes make it important to explore potential uses of indigenous food grade lactobacilli in the development of functional foods and probiotics [1].

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Thus, increasing attention has been directed to the development of safe and effective functional foods and antioxidative agents from natural sources, especially peptides derived from hydrolyzed food proteins [2].

Milk proteins are currently the main source of a range of biologically active peptides. Many bioactivities are encrypted within the primary structure of milk proteins, requiring proteolysis for their generation from precursors. Bioactive peptides defines as specific protein fragment that insert a positive impact on body functions or conditions and may ultimately influence health are considered the most important source of bioactive peptides. Bioactive peptides with antioxidant activity are generated during milk ferment starter cultures or microbial enzymes from different sources, such as in yoghurt, sour milk and ripened cheese [3, 4]. The bioactive peptides have varying biological actions, such as angiotensin converting enzyme (ACE) inhibitory [5], immune modulatory [6], opioid [7] and antioxidant activities [8].

Lactic acid bacteria (LAB) play important role in traditional food fermentation producing desirable flavor and unique characteristic of fermented food. Some species of the genus *Lactobacillus (Lb.), Lactococcus (Lc.)* and *Leuconostoc (Ln.)* are widely used as starter cultures. In addition to their importance in food fermentation, *Lactobacillus* species are generally recognized as safe (GRAS) [9, 10]. In addition LAB has several beneficial effects, such as antimicrobial activity, ability to modulate immune response and anti-tumorigenic activity [11]. It has been shown that some lactobacilli have the ability to produce metabolites with antioxidant activity, and are able to reduce the risk of accumulation of reactive oxygen species (ROS) during ingestion of food [12].

Additionally, LAB have some probiotic functions, such as adjusting the balance of intestinal flora, reducing serum cholesterol, inhibiting and reducing the risk of tumors, and revitalizing the immune system among others [13, 14]. Probiotic fermented dairy products provide a healthy functional food for health properties. Many previous researches mentioned that, milk fermented by selected culture of lactic acid bacteria (LAB) has high biochemical activity and antioxidant activity [15, 16]. Among lactic acid bacteria, species of *Lactobacillus* have attracted a lot of attention for their potential probiotic effects in human health. *Lactobacillus* spp are important members of the healthy human microbiota [17]. Several dairy products and also fractions from them have been found to be antioxidative, e.g. milk, skim milk, whey, casein and lactoferrin [18]. Previous studies have shown that the *Lb. acidophilus*, *Lb. delbrueckii*, *Lb. fermentum, Lb. plantarum* 1 and *Le. mesenteroides* ssp. c*remoris* used to ferment milk for produced the whey have antioxidative activity, and were able to decrease the risk of accumulation of ROS [19].

Lactobacilli are commonly used to generate bioactive peptides via fermentation of milk proteins [20, 21]. Similarly, bioactive peptides have been found in the hydrolysates of meat and fish, and plant sources such as from barley hordein [22] and maize zein [23]. These bioactive proteins were reported to display antihypertensive, antioxidant, antimicrobial and antiproliferative effects [24, 25]. Some peptides derived from hydrolysed food proteins have been shown to have antioxidative activities against the peroxidation of lipids or fatty acids [26]. However, microbial fermentation of meat proteins has been less successful, presumably due to the poor proteolytic activity of the lactobacilli used in meat fermentations [27].

Osontoki and Korie reported that strains of *Lactobacillus* spp isolated from non-milk indigenous Nigerian fermented cereal-based food (ogi, ogi baba, kunnu and ugba) and wara (fermented milk) provide antioxidants in whey from fermented milk. Although their LAB from wara produced high antioxidant activity as determined by DPPH, their study demonstrated that lactobacilli from non-dairy food sources may serve as a delivery vehicle for probiotic lactobacilli and provide antioxidative activity from non-dairy source.

In vitro methods can be divided into two major groups: 1) Hydrogen atom transfer reactions like Oxygen Radical Absorbance Capacity (ORAC), Total radical trapping antioxidant potential (TRAP) and β carotene bleaching; 2) Electron transfer reactions like trolox equivalent antioxidant capacity (TEAC), reducing power (RP), radical scavenging assay (DPPH), Superoxide anion radical scavenging assay, Hydroxyl radical scavenging assay, Nitric oxide radical scavenging assay and total phenol assay [28]. These methods are popular due to their high speed and sensitivity. However, it is essential to use more than one method to evaluate antioxidant capacity of food materials because of the complex nature of food material and commonly used antioxidant assays along with various standards that can be used as positive control [29].

The 1,1-Diphenyl-2-picrylhydrazyl free radical (DPPH) is a rapid, simple, widely used and inexpensive method to evaluate antioxidant activity of foods. It has also been used to quantify antioxidants in complex biological systems in recent years [30].This assay measures the ability of a sample to donate hydrogen to DPPH radical [31]. In addition, ferrozine can quantitatively chelate with Fe^{+2} and form a complex with a red color. This reaction is limited in the presence of other chelating agents and results in a decrease of the red color of the ferrozine-Fe^{+2} complexes. Measurement of the color reduction estimates the chelating activity to compete with ferrozine for the ferrous ions [32]. Also reducing power is associated with antioxidant activity and may serve as a significant reflection of the antioxidant activity [33]. Compounds with reducing power indicate that they are electron donors and can reduce the oxidized intermediates of lipid peroxidation processes; so that they can act as primary and secondary antioxidants [34]. Therefore, the aim of this study was to evaluate the antioxidant activity of skim milk hydrolysates fermented with lactobacilli isolated from non-dairy sources using DPPH, FCA and RP.

MATERIAL AND METHODS

Microorganisms

Lb. plantarum 1 and *Ln. mesenteroides* ssp. isolates from grape and banana, respectively were used in this study (1). *Lb. plantarum* ATCC8014 was used as control.

Probiotic properties of LAB isolates

Bile tolerance

LAB isolates were inoculated into MRS broth and MRS broth containing 0.3% of bile (Sigma), incubated at 37°C (18). Growth of LAB was monitored hourly for 4 h by measuring absorbance at 560 nm using spectrophotometer (BioTek, USA) and spread plated on MRS agar incubated at 37°C for 24 h, anaerobically. Each test was carried out in triplicate.

Tolerance to acidic pH values

LAB isolates were grown in MRS broth at 37°C overnight, then sub-cultured into fresh MRS broth and incubated for another 24 h. The cultures were centrifuged at 5000 x *g* for 10 min at 4°C (Eppendorf, centrifuge 5804 R). The pellets were washed in sterile phosphate-buffer saline (PBS) pH 7.2 and re-suspended in PBS. PBS was modified to pH 2, 3, and 4 with 1 M HCl. Each LAB isolates were inoculated into the pH adjusted PBS at ratio 1:100 (µl). Growth of LAB was monitored hourly for 4 h by measuring absorbance at 560 nm using spectrophotometer (BioTek, USA) and spread plated on MRS agar incubated at 37°C for 24 h, anaerobically [35]. Each test was carried out in triplicate.

Preparation of pre-cultures and fermentations

Preparation of pre-culture

The isolates were cultured following the method described by Virtanen *et al.,* with modification. The isolate was inoculated into 10 mL MRS broth and incubated at 37°C for 24 h. The cultured broths was vortexed and used to inoculate sterilized skimmed milk (sterilized at 110 °C for 10 min) at a 1% (v/v) concentration, then incubated at 37°C for 24 h anaerobically to generate pre-cultures. These pre-cultures at 2% (v/v) were used to inoculate 10% skimmed milk (Oxoid LP0031) and pasteurized at 62 °C for 30 min. Fermentation was carried out in triplicate at 37°C for 48 h.

Preparation of skim milk hydrolysate (SMH)

The preparation of SMH essentially as described by Virtanen, Aliquots were collected from the fermented milk and the pH was adjusted to 4.6 with 1 M HCl followed by centrifuged at 10 000*g* for 20 min at 4°C. The supernatant was filtered using a 0.45µm filter (Millipore Corp, Billerica, MA, USA). Non-hydrolysed casein was discarded. The resulting supernatant was then freeze dried and stored at −20°C for further use.

Determination of IC_{50} antioxidant activity

IC_{50} scavenging of 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical activity

The DPPH radical scavenging activity was evaluated using the method of Son and Lewis [36]. DPPH radical solution (0.004%, w/v, Sigma Aldrich) in 95% ethanol was prepared. A volume of 2 ml of DPPH in ethanol was added to 2 ml of various concentrations of SMH (0.5 mg/ml to 3 mg/ml), well vortexed and incubated for 30 min in dark room at room temperature. Absorbance of each sample at 517 nm was measured using UV-Visible spectrophotometer (Varian Carry 50 Conc). Ethanol was used as a blank, while DPPH solution in ethanol served as control. BHT (Sigma, Germany), ascorbic acid (Sigma, Germany) and Trolox (Acros, USA) at a concentration of 0.02 mg/ml was used for comparison. The test was carried out in triplicate. The antioxidant activity was expressed as percentage of DPPH activity using the following formula:

DPPH activity (%) =

$$\frac{\text{Absorbance of blank - Absorbance of sample}}{\text{Absorbance of blank}} \times 100$$

The IC_{50} value for antioxidant activity was defined as the concentration of sample (mg/ mL) required to scavenging DPPH radicals by 50%.

IC_{50} ferrous chelating activity (FCA)

The ability of different peptides generated by LAB to chelate ferrous ions was assessed using the method of Decker and Welch [37]. One milliliter of various concentrations of SMH (0.5 mg/ml to 3 mg/ml) was first 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 shaken vigorously and left at room temperature for 10 min. Optical density of the reaction mixture was measured at 562 nm. A blank without 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:

Fe^{2+} chelating activity (%) =

$$\frac{\text{Absorbance of blank - Absorbance of sample}}{\text{Absorbance of blank}} \times 100$$

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

Reducing power activity assay (RP)

The reducing power was measured according to the method of Ahmadi *et al.,* [38]. 1 ml of various concentrations of SMH (0.5 mg/ml to 3 mg/ml) was mixed with 1 ml of phosphate buffer (pH 6.6) and 1 ml of 10 mg/ml potassium ferricyanide (Sigma, Germany). The mixture was incubated at 50 °C for 20 min. Then, 1 ml of 10% trichloroacetic acid (Sigma, Germany was added. After centrifugation at 1500 g for 10 min, 2 ml of the supernatant was collected and mixed with 2 ml of distilled water and 0.4 ml of 0.1% (w/v) ferric chloride (Sigma, Germany). After standing at room temperature for 10 min, the absorbance was measured at 700 nm. An equivalent volume of distilled water instead of the sample was used for the blank. Higher absorbance of the reaction mixture indicated higher reducing power. The test was carried out in triplicate.

Heat stability of SMH

The heat stability of SMH was evaluated. 10 ml SMH from bacterial fermentation samples were placed in bonjour bottles and immersed in water bath at 80 °C for 30 min then cooled on ice and stored at -20 °C until assay for DPPH, FCA and RP.

Effect of enzymes on antioxidant activity of SMH

The SMH was treated with pepsin (Sigma Aldrich) and Proteinase K (Sigma Aldrich) separately. 1µl of each enzyme was inoculated to 1 ml of SMH and left for 1 h at room temperature. After that, SMH was tested for antioxidant activity as described above.

Statistical analysis

The results of antioxidant activity were presented as mean ± standard deviations of triplicate determinations and were statistically analyzed by two-way analysis of variance (ANOVA) using (Minitab, Inc.) version 15 (Germany), $p \leq 0.05$ were considered statistically significant. Linear regression analysis was used to calculate the IC_{50} values.

RESULTS AND DISCUSSION

Probiotic properties of LAB isolates

The probiotic potential of the LAB isolates was determined by growing the bacteria in MRS broth with 0.3% bile and at pH 2.0 - 4.0. All isolates grew in 0.3% bile after 4 h incubation at 37°C; similar growth pattern was also observed in the absence of bile (Table 1). Significant increase ($p \leq 0.5$) *Lb. plantarum* 1 and *Ln. mesenteroides* ssp. was observed in MRS with 3% bile better than growth without bile (Table 1). The pH-stressed *Lb. plantarum* 1 and *Ln. mesenteroides* ssp. cells were able to tolerate pH 2.0 - 4.0 but grow better between pH 3 and 4 (Table 2 and 3). It was observed that *Lb. plantarum* 1 able to tolerate low pH stressed than *Ln. mesenteroides* ssp. Probiotic LABs namely, *Lactobacillus acidophilus*, *Bifidobacterium longum*, *Lactobacillus fermentum* and *Lactobacillus sake* [39, 40, 41] had been shown possessing antioxidative activity, and were able to decrease the risk of accumulation of ROS.

Zhang et al. reported that intact cells and cell-free extract of *Lactobacillus casei* subsp. *casei* and *Lactobacillus delbrueckii* subsp. *Bulgaricus* isolated from Chinese traditional yogurt have antioxidant activity as determined DPPH free radicals activity and ferrous ion chelating capacity. However, this study observed that the species of lactobacillus form non-dairy sources have the probiotic properties were also have a high potential for inhibition of oxidative stress. This observation further supported the results reported by Osuntoki and Korie that lactobacilli from non-dairy food sources may serve as a delivery vehicle for probiotic lactobacilli and provide antioxidative activity from non-dairy source. Additionally, the probiotic properties of the LAB isolates would be useful in the dairy manufacturing industry. They could beneficially affect the consumer by providing effective and security dietary source of antioxidants or by providing probiotic bacteria with the potential of producing antioxidants during their growth in the intestinal tract.

Table 1: Growth of LAB strains in MRS broth with 0.3% of bile incubated at 37^{o}C^{a}

LAB isolates	***Media***	***Time (h)***			
		1	**2**	**3**	**4**
Lb. plantarum 1	With bile	0.1195	0.1225	0.1229	0.1231
	Without bile	0.1194	0.1225	0.1228	0.1230
Ln. mesenteroides	With bile	0.1420	0.1425	0.1431	0.1434
	Without bile	0.1418	0.1420	0.1431	0.1433

Table 2: Survival of pH-stressed Lb. plantarum 1 in MRS incubated at 37 ^{o}C^{abc}

pH-stressed time (h)	***pH***		
	2.0	**3.0**	**4.0**
1	0.3251	0.5238	0.9382
2	0.3290	0.5231	0.9330
3	0.3320	0.5205	0.9278
4	0.3328	0.5202	0.9253

Table 3: Survival of pH-stressed Ln. mesenteroides in MRS incubated at 37 °C[abc]

pH-stressed time (h)	*pH*		
	2.0	*3.0*	*4.0*
1	0.2200	0.3240	0.4320
2	0.2250	0.3210	0.4290
3	0.2290	0.3150	0.4251
4	0.2298	0.3120	0.4224

Antioxidant activity

In vitro antioxidant activities of skim milk hydrolysate were studied using scavenging activity of 1,1-diphenyl-2-picrylhydrazyl free radical (DPPH), ferrous chelating activity (FCA) and reducing power assay (RP).

IC $_{50}$ scavenging activity of 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical:

Proton-radical scavenging is recognized as being an important mechanism for antioxidation. DPPH is a compound that possesses a proton free radical and this feature of DPPH was used to determine its proton-radical scavenging action. DPPH exhibits a characteristic absorption at 517 nm and its purple color fades when it encounters proton radical scavengers [42]. In the present work, DPPH values of BHT, ascorbic acid, Trolox and SMH fermented with LAB isolates increased significantly ($p \leq 0.05$) with skim milk concentration between 0 - 0.5 mg/ml, but increased slightly with increasing concentration (Fig.1). SMH fermented with *Lb. plantarum*1 generated peptides with the highest radical scavenging activity (44.28 % at 3.0 mg/ml) with the IC_{50} 2.92 mg/ml than the other isolates but lower than BHT, ascorbic acid and Trolox (Table 4). The results also demonstrated that the radical scavenging activity of SMH fermented with *Ln. mesenteroides* was 18.85 % at 3.0 mg/ml with IC_{50} 8.81 mg/ml.

Hydrolysis of protein food by microbial enzymes generates peptides with free radical scavenging activity and are affected by concentration of protein used. Hydrolyzing bovine whey with different enzymes resulted in variable IC_{50}; IC_{50} of the bovine whey hydrolysed by fungal protease was 9.5 mg/ml, the IC_{50} of the bovine whey hydrolysed by papain was 9.2 mg/ml while the IC_{50} of the bovine whey hydrolysed by double enzyme (fungal protease and papain) was 8.8 mg/ml [43]. Shu-Hua and Chi-Yue, [44] observed that the IC_{50} DPPH of pH 4.6 adjusted bovine colostrums casein and the resultant whey proteins were 14.50 mg/ml and 16 mg/ml, respectively. Additionally, the methanolic extract of *Cassia fistula* exhibited a mild DPPH activity with 50% of inhibition (IC_{50}) at 11.07 mg/mL of extract [45]. The lower IC_{50} indicates higher free radical scavenging ability. It can be concluded that the present study indicates that fermenting skim milk with the LAB isolates could generate peptides with better DPPH activity with lower IC_{50} compared to other food protein hydrolysates and plant extract.

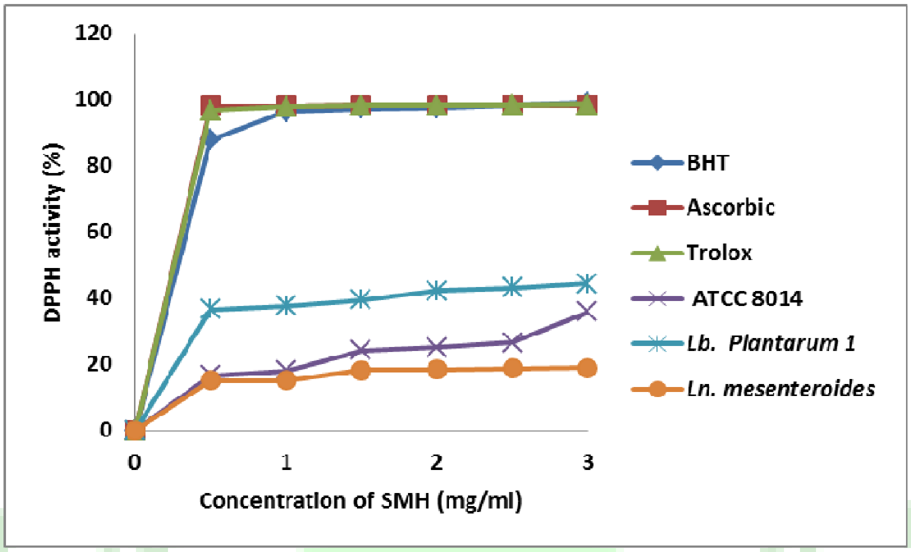

Fig.1: 1,1-diphenyl-2-picrylhydrazyl radical scavenging activity of standard and SMH fermented with different LAB isolates

Table 4: IC_{50} values of standard and SMH fermented with different LAB isolates

Samples	***$IC_{50}{}^{a}$ (mg/ml)***
Standards	
BHT	0.08
Ascorbic acid	0.11
Trolox	0.09
LAB isolates	
Lb. plantarum ATCC8014	4.53
*Lb. plantarum*1	2.92
Ln. mesenteroides	8.81

IC_{50} ferrous chelating activity (FCA)

Among the many metal ions involved that catalyze oxidative reactions, iron ions are highly reactive, an important catalysts for ROS formation resulting in the cell membrane damage. Therefore, the ability of the tested strains to generated peptides from fermented skim milk that have antioxidatative property by chelating iron ions was investigated by FCA method. The IC_{50} values of SMH fermented with *Lb. plantarum* 1 and *Ln. mesenteroides* were 0.46 and 0.69 mg/ml, respectively greater than *Lb. plantarum* ATCC8014 which showed IC_{50} value 0.74 mg/ml but lower than standard (Table 5). The FCA activity was significantly ($p \leq 0.05$) affected by the different strains of LAB used.

In This study the FCA values of EDTA and SMH increased with concentration of skim milk used. However, at concentration between 0.5 - 3.0 mg/ml no significant change in FCA values was observed in all samples. There was no significant difference between the FCA values of SMH fermented with *Ln. mesenteroides* and *Lb. plantarum* 1, but a lower FCA values than EDTA (Fig. 2). The results showed a wide range of Fe^{+2} chelating ability of SMH fermented with *Lb. plantarum*1 and *Ln. mesenteroides*. SMH fermented with *Lb. plantarum*1 was higher than

Ln. mesenteroides and ATCC8014 generated peptides with FCA with 80.84, 78.75 and 72.64 % at 3 mg/ml skim milk, respectively. The FCA values obtained in this study was higher than the study of bovine colostrums casein adjusted to pH 4.6 and the resultant whey proteins which were 39 and 30 % at a concentration 2.0 mg/ml. Similarly, fermenting milk casein with selected LAB isolates resulted in FCA higher than that reported for Holy basil [46]. IC_{50} of FCA determines the iron ion chelating ability of an antioxidant, the smaller the values the greater is the antioxidant activity. It was observed that FCA IC_{50} values of SMH fermented with *Lb. plantarum*1 and SMH fermented with *Ln. mesenteroides* were 0.46 and 0.69 mg/ml, respectively greater than SMH fermented with *Lb. plantarum* ATCC8014 (IC_{50} value 0.74 mg/ml) but lower than standard (Table 5), thus further supports the high antioxidant activity of the peptides generated by selected LAB strains. Additionally, the IC_{50} obtained in this study was lower than that reported for plant extracts. Lee et al. [47] reported that the *Echinacea purpurea* L. extracts have chelating ability of IC_{50} 3.5 mg/ml while Dhan et al. [48] indicated that the IC_{50} values for seed kernel of *Mangifera indica* was 2.44 mg/ml. High IC_{50} was reported for pH 4.6 adjusted bovine colostrums casein and the resultant whey proteins, 8.0 mg/ml and 3.0 mg/ml, respectively. LABs produce enzymes that can break proteins into small peptides fragments. Peptide cleavages led to an enhanced Fe^{+2} binding due to an increased concentration of carboxylic groups (COO^{-}) and amino groups in branches of the acidic and basic amino acids, thus removing the pro-oxidative free metal ion from the hydroxyl radical system. The direct relationship between soluble protein/peptide concentration and the increase in the chelation capability supported this premise. It is possible that the LAB produced enzymes that break the milk protein into small fragments which may contribute to higher FCA than unfermented skim milk.

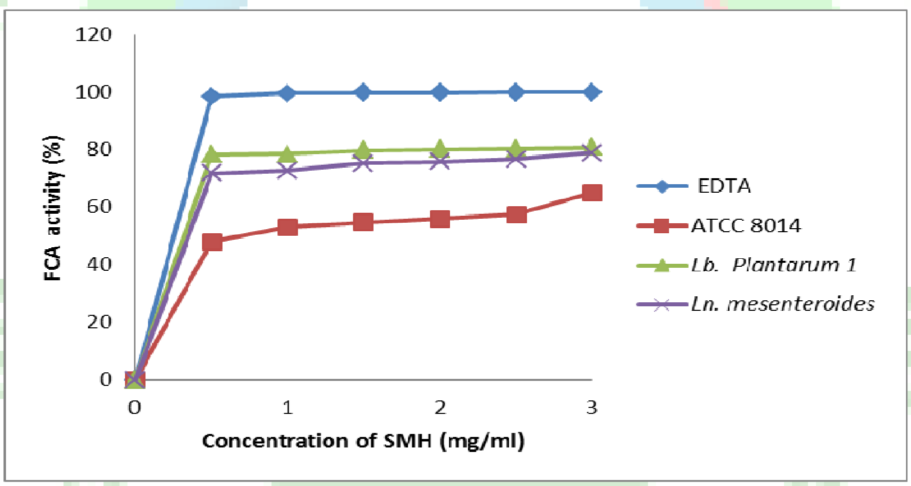

Figure 2: Ferrous chelating activity of EDTA and SMH fermented with different LAB isolates

Table 5: IC_{50} values of EDTA and SMH fermented with different LAB isolates

Samples	*IC_{50}^{a} (mg/ml)*
EDTA	0.13
Lb. plantarum ATCC8014	0.74
Lb. plantarum1	0.46
Ln. mesenteroides	0.69

Reducing power activity assay (RP)

Reducing power measures the potential antioxidant activities of bioactive compounds in different products, including peptides [49]. In this assay, the presence of antioxidants caused the reduction of the Fe^{+3}/ ferricyanide complex to the ferrous form, and the yellow color of the test solution changed to various shades of green and blue depending on the reducing power of each compound. Changes in Fe^{+2} was then monitored by measuring the formation of Perl's Prussian blue at 700 nm [50, 51]. In this study it was observed that the reducing power activity increased with increased concentration of SMH (Table 6). However, all isolates the results showed poor reducing power compared to ascorbic acid. Among the LAB isolates *Lb. plantarum* ATCC8014 and *Ln. mesenteroides* seemed to generate peptides with similar reducing power activity; may be the peptides generated were not unable to reduce ferric ion (Fe^{+3}) to ferrous ion (Fe^{+2}). The reducing power of SMH fermented with LAB isolates increased with increasing concentrations. A similar observation has been reported by Huda-Faujan et al. [51] and Mohamed et al. [52].

Table 6: Reducing power of ascorbic acid and SMH fermented with different LAB isolates

Concentration of SMH mg/ml	*Ascorbic acid*	*Lb. plantarum ATCC8014*	*Lb. plantarum1*	*Ln. mesenteroides*
0.5	0.0042±0.0001	0.0035±0.0003	0.0013±0.0001	0.0038±0.0006
1.0	0.0138±0.0001	0.0054±0.0001	0.0016±0.0003	0.0042±0.0001
1.5	0.0177±0.0001	0.0057±0.0001	0.0019±0.0002	0.0058±0.0002
2.0	0.0351±0.0001	0.0064±0.0003	0.0024±0.0003	0.0083±0.0001
2.5	0.0490±0.0003	0.0079±0.0006	0.0028±0.0004	0.0085±0.0003
3.0	0.0522±0.0002	0.0090±0.0001	0.0033±0.0002	0.0096±0.0001

Heat stability

Heating at 80 °C for 30 min did not significantly change ($p \geq 0.05$) the antioxidant activity of all the SMH produced by all the LAB isolates as assayed by DPPH, FCA and RP (Table 7, 8 and 9). The effects of heat treatment on the antioxidant properties of milk, whey and their fractions have been investigated by several authors [53]. It was observed that no significant change in the antioxidant activity as measured by DPPH, FCA and RP after heating 80 ^{0}C for 30 min SMH fermented with the selected LAB strains, indicating that the heat stability of the peptide generated. The results are consistent to that reported by Korpela et al. [54] and Taylor and Richardson [55].

Table 7: Antioxidant activity of SMH fermented with Lb. plantarum ATCC8014 before and after heated at 80 ^{0}C for 30 min.

Concentration of SMH (mg/ml)	*DPPH (%)*		*FCA (%)*		*RP*	
	Before	*After*	*Before*	*After*	*Before*	*After*
0.5	16.44	15.04	47.94	46.12	0.0035	0.0032
1.0	18.39	16.73	53.11	51.43	0.0054	0.0049
1.5	24.1	22.91	54.79	53.55	0.0057	0.0051
2.0	25.05	24.75	55.96	54.96	0.0064	0.0063
2.5	26.58	25.01	57.39	55.31	0.0079	0.0074
3.0	35.76	33.71	64.98	63.23	0.009	0.0087

Table 8: Antioxidant activity of SMH fermented with Lb. plantarum1 before and after heated at 80 ^{0}C for 30 min.

Concentration of SMH (mg/ml)	DPPH (%)		FCA (%)		RP	
	Before	After	Before	After	Before	After
0.5	36. 51	35.25	78.37	76.21	0.0013	0.0011
1.0	37.68	36.11	78.57	76.13	0.0016	0.0015
1.5	39.36	38.32	79.71	77.54	0.0019	0.0019
2.0	42.13	40.42	80.08	79.11	0.0024	0.0021
2.5	43.09	42.67	80.39	79.23	0.0028	0.0027
3.0	44.28	42.98	80.84	79.67	0.0033	0.0030

T*able 9: Antioxidant activity of SMH fermented with Ln. mesenteroides before and after heated at 80 ^{0}C for 30 min.*

Concentration of SMH (mg/ml)	DPPH (%)		FCA (%)		RP	
	Before	After	Before	After	Before	After
0.5	14.99	13.95	71.82	70.65	0.0038	0.0032
1.0	15.12	13.91	72.64	71.34	0.0042	0.0043
1.5	18.16	17.05	75.16	73.21	0.0058	0.0049
2.0	18.45	17.98	75.81	73.75	0.0083	0.0076
2.5	18.68	18.23	76.64	75.77	0.0085	0.0084
3.0	18.85	19.98	78.89	76.65	0.0096	0.0093

Effect of enzymes on antioxidant activity of SMH

Treating the SMH with pepsin and proteinase K destroyed the antioxidant activity for all the LAB isolates. Antioxidant activity of SMH was destroyed after enzyme treatments; this further supports the protein-like nature of the hydrolysate (Table 10, 11 and 12).

Table 10: Antioxidant activity of SMH fermented with Lb. plantarum ATCC8014 after treatment with pepsin and Proteinase K for 1h.

Concentration of SMH (mg/ml)	DPPH (%)		FCA (%)		RP	
	Pepsin	Proteinase K	Pepsin	Proteinase K	pepsin	Proteinase K
0.5	0.95	0.76	0.66	0.66	0.001	0.002
1.0	1.12	0.89	1.11	0.83	0.003	0.004
1.5	1.23	0.93	1.16	1.10	0.004	0.005
2.0	2.39	1.27	1.40	1.33	0.006	0.007
2.5	2.41	1.39	1.68	1.54	0.008	0.008
3.0	2.84	1.42	2.13	1.67	0.009	0.009

Table 11: Antioxidant activity of SMH fermented with Lb. plantarum1 after treatment with pepsin and Proteinase K for 1h.

Concentration of SMH (mg/ml)	DPPH (%)		FCA (%)		RP	
	Pepsin	Proteinase K	pepsin	Proteinase K	pepsin	Proteinase K
0.5	1.01	1.27	1.05	1.55	0.001	0.003
1.0	1.20	1.50	1.26	1.96	0.003	0.005
1.5	1.71	2.01	2.03	2.23	0.004	0.007
2.0	2.01	2.75	2.36	2.69	0.007	0.008
2.5	2.31	2.91	2.81	3.01	0.009	0.009
3.0	2.52	3.11	3.04	3.21	0.001	0.009

Table 12: Antioxidant activity of SMH fermented with Ln. mesenteroides after treatment with pepsin and Proteinase K for 1h.

Concentration of SMH (mg/ml)	DPPH (%)		FCA (%)		RP	
	Pepsin	Proteinase K	pepsin	Proteinase K	pepsin	Proteinase K
0.5	0.82	0.61	0.44	0.49	0.001	0.001
1.0	0.98	0.79	0.75	0.61	0.002	0.003
1.5	1.21	0.98	0.93	0.79	0.004	0.004
2.0	1.35	1.08	1.11	0.91	0.005	0.006
2.5	1.62	1.34	1.47	1.19	0.006	0.008
3.0	1.84	1.55	1.76	1.24	0.008	0.009

CONCLUSION

Lb. plantarum 1 and *Ln. mesenteroides* obtained from Malaysian fruits, non-dairy sources, generated protein-like compounds from SMH with antioxidant activity. The DPPH free radical scavenging activity, FCA and RP were affected by concentration of SMH and LAB strains. The protein-like compounds are heat stable and thus could be used as potential antioxidant in food system. Further works should be done to isolate and identify the specific peptides in SMH that are responsible for the overall antioxidative capability.

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