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Full Length Research Paper

Antioxidant activity of lactic acid bacteria (LAB) fermented skim milk as determined by 1,1-diphenyl-2-picrylhydrazyl (DPPH) and ferrous chelating activity (FCA)

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Bioactive peptides can be generated from milk protein by fermentation with lactic acid bacteria. In this study, lactic acid bacteria (LAB) were isolated from different food samples. Isolates that showed clear zone on modified de Man, Rogosa and Sharpe (MRS) - $CaCO_3$ agar, catalase negative and Gram positive were considered as LAB and used for this study. Seven isolates that showed proteolytic activity on skim milk agar produced whey that have free radical scavenging activity ranging from 14.7 to 50.8% (v/v) after 24 to 72 h fermentation, respectively as determined by 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay. Ferrous chelating activity (FCA) of the whey showed similar values for EDTA after 24 h fermentation but decreased after 72 h for all LAB isolates with values between 41.8 to 97.6% (v/v) for 24 to 72 h, respectively. This study highlights that local LAB isolates have the potential to be used to generate peptides in whey with antioxidative activity from skim milk.

Key words: Antioxidant activity, fermented milk, lactic acid bacteria (LAB), 1,1-diphenyl-2-picrylhydrazyl (DPPH), ferrous chelating activity (FCA).

INTRODUCTION

Milk proteins are a rich source of biologically active peptides. It has received increasing attention as potential ingredients for health-promoting functional foods targeted at diet-related chronic diseases, such as cardiovascular disease, diabetes type II and obesity. These peptides are inactive within the sequence of the parent protein molecule and can be liberated by: (a) enzymatic hydrolysis by digestive enzymes, (b) fermentation of milk with proteolytic starter cultures and (c) proteolysis by enzymes derived from microorganisms or plants (Hannu, 2009). The fermentation of milk by lactic acid bacteria (LAB) releases a large number of peptides and amino acids with varying biological actions, such as angiotensin converting enzyme (ACE) inhibitory (Nielsen et al., 2009), immune modulatory (Coste et al., 1992), opioid (Meisel, 1986) and antioxidant activities (Pena-Rasmos et al., 2004).

Oxidative damage plays a significantly pathological role in human diseases. Cancer, emphysema, cirrhosis, atherosclerosis, and arthritis have all been correlated with oxidative damage (Halliwell and Gutteridge, 1984). Highly reactive free radicals especially oxygen derived radicals which are formed by exogenous chemicals or endogenous metabolic processes in the human body or in food systems are capable of oxidizing biomolecules resulting in cell death and tissue damage. Almost all organisms are well protected against free radical damage by enzymes such as, superoxide dismutase and catalase, or compounds such as ascorbic acid,

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tocopherol and glutathione (Niki et al., 1994). Synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and n-propyl gallate (PG) exhibit strong antioxidant activity against several oxidation systems. However, synthetic antioxidants pose potential risks *in vivo*; their use in food is restricted or prohibited in some countries. Antioxidants from natural sources are likely to be more desirable than those chemically produced because some synthetic antioxidants have been reported to be side effects (Osuntoki and Korie, 2010). Some proteins from certain foods have been reported to have the ability to scavenge active oxygen species and can function as natural antioxidant (Okada and Okada, 1998).

Antioxidants function in several ways including preventing the formation of radicals, scavenging free radicals, formation of hydrogen peroxide and other peroxides. Peptides generated from the digestion of milk proteins are reported to have antioxidative activities (Anne, 2006).

A variety of methods have been employed with the purpose of generating potentially functional peptides. For example, food-derived peptides with biological activities are primarily produced through enzymatic hydrolysis, fermentation, and chemical or enzymatic synthesis (Shahidi and Zhong, 2008). Amongst these processes, enzymatic hydrolysis is often the method of choice in which common digestive enzymes are exploited, such as trypsin and pepsin, to generate peptides with potential antioxidant properties (Kanda et al., 2007). During hydrolysis the protease specificity is essential because it dictates the amino acid sequence of the resultant peptides and their biofunctional properties (Murray and FitzGerald, 2007).

Kudoh et al. (2001) reported that a *κ*-casein-derived peptide with 2,2-diphenyl-1-picrylhidrazyl (DPPH) radical-scavenging activity was recently found in a milk fermented with *Lactobacillus delbrueckii* ssp *bulgaricus.* Osuntoki and Korie (2010) indicated the highest level of antioxidant activity using DPPH radical was observed in the whey from milk fermented with the strain of *Lactobacillus brevis* isolated from wara, a dairy product.

The purpose of this work is to evaluate lactic acid bacteria isolated from different food samples in Malaysia for their ability to produce peptides in whey with antioxidant activity through fermentation of skim milk. Two methods were used namely, DPPH and ferrous chelating activity (FCA) are essential to evaluate the antioxidant activity to indicate the possible mechanism of antioxidant activity.

MATERIALS AND METHODS

Isolation of lactic acid bacteria

Lactic acid bacteria were isolated from six fruit samples namely, dates, banana, black grapes, white grapes, pineapple, and apple obtained from markets in Bandar Baru Nilai, Negeri Sembilan, Malaysia. Ten gram of sample was added to 90 ml 0.1% peptone water and appropriate dilution was spread plated on de Man, Rogosa and Sharpe (MRS) agar (Oxoid CM0361) plates containing 0.8% calcium carbonate.

Plates were incubated anaerobically in anaerobic jar at 37 ± 1°C for 48 h. Each of the isolates was tested for catalase activity by placing a drop of 4% hydrogen peroxide solution on the cells. Immediate formation of bubbles indicated the presence of catalase in the cells. Only those isolates which was catalase-negative was Gram-stained and the morphology was observed using Nikon microscope (Nikon Eclipse 80i) and streaked on de Man, Rogosa and Sharpe (MRS) agar to obtain pure isolates. All bacterial strains used in this study were maintained in 15% glycerol stock and stored at -20°C. They were re-cultured in MRS broth (Oxoid CM0359) at 37 ± 1°C under anaerobic condition. Prior to beginning the experiments, each bacterial strain was sub-cultured at least three times (1%, v/v) at 24 h intervals (Kheadr, 2006). *Lactobacillus plantarum* ATCC8014 was included as positive control.

Detection of protein hydrolysis

Preparation of skim milk agar and cultures

Skim milk agar was made as follows: 25 g of skim milk was reconstituted with 250 ml of distilled water. The mixture was stirred thoroughly and autoclaved at 110°C for 10 min. Likewise, 500 ml of 2.5% agar solution was sterilized. For plating, skim milk and agar solutions were held in a water bath at 50°C and then the skim milk was poured into the agar bottle and mixed thoroughly. The skim milk agar was poured quickly into plates (Pailin et al., 2001).

Measurements of protein hydrolysis

To detect protein hydrolysis, the selected LAB were inoculated on skim milk agar plates and were incubated at 37 ± 1°C for 48 h in an anaerobic chamber followed by cooling in a refrigerator (4°C) for 3 days. Protein hydrolysis was observed by the production of clear halos surrounding isolated colonies. Duplicate trials were conducted and all results were averaged and reported as diameter in mm. LAB that show good proteolytic activity (> 6 mm) were used for future studies (Pailin et al., 2001).

Preparation of pre-cultures and fermentations

Selection of lactic acid bacteria isolates

The isolates were cultured following the method described by Virtanen et al. (2007) 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 ± 1°C for 24 h to generate precultures. These precultures were used to inoculate fresh skimmed milk (pasteurized at 62 °C for 30 min) at 2% (v/v) concentration and fresh pasteurized skimmed milk without bacteria served as control. Fermentation was carried out in triplicate at 37 ± 1°C for 24 to 72 h. The bacteria that have the highest antioxidant activity were identified and selected for further study.

Preparation of whey fraction from fermented skim milk

The whey fraction was prepared essentially as described by Virtanen et al. (2007). 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 0.45 µm filter (Millipore Corp, Billerica, MA, USA). Non-hydrolysed casein was discarded. Skim milk without bacteria was used as a control.

Determination of antioxidant activity

Scavenging of 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical

The DPPH radical scavenging activity was evaluated using the method of Son and Lewis (2002). DPPH radical solution (0.004%, w/v) in 95% ethanol was prepared. A volume of 2 ml of DPPH in ethanol was added to 2 ml of whey fraction, 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 antioxidant activity was expressed as percentage of DPPH activity calculated as:

$$\text{DPPH activity (\%)} = \frac{\text{Absorbance of blank - absorbance of sample}}{\text{Absorbance of blank}} \times 100$$

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 (1990). One millilitre of peptide solution was first mixed with 3.7 ml of distilled water. A solution of 2 mM ferrous chloride (Sigma Aldrich) (0.1 ml) was added and after 3 min the reaction was inhibited by the addition of 5 mM ferrozine (Sigma Aldrich) (0.2 ml). 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 chelating capacity was calculated as a percentage using the following formula:

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

Identification of LAB isolates

The LAB isolates that show antioxidant activity in all the tests were identified by API 50 CH (API system, BioMérieux, France). The isolates were tested for catalase and Gram stain. Overnight cultures of selected LAB isolates were grown in MRS plates (Oxoid) at 37°C for 24 h. The pure colonies were suspended in API 50 CHL medium (API system, BioMérieux, France) (Conter et al., 2005). The suspension was transferred into each of the 50 wells of the API 50 CH strips. All wells were overlaid with paraffin oil to make it anaerobic. Strips were incubated at 30°C as recommended by the manufacturer. Changes in colour from wells were noticed after 24 and 48 h. The result was analyzed with API WEB (BioMerieux). Promising LAB isolates were further identified by 16 s rDNA using primer 16S forward: (5-AGAGTTTGATCCTGGCTC-3) and 16S reverse: (5-CGGGAACGTATTCAC-CG-3) (Magnusson et al., 2003). Primer was synthesized at 1st Base, Malaysia. The settings of PCR were as follows: initial at 95 °C for 2 min, denaturation at 92°C for 45 s, annealing at 54°C for 1 min and extension at 72°C for 1 min, with 35 cycles for each steps.

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) at using (Minitab, Inc.) version 15 (Germany). Values of $p \leq 0.05$ were considered statistically significant.

RESULTS

Isolation of LAB

12 from 35 LAB isolated from fruits showed clear zone on modified MRS-$CaCO_3$ agar, catalase negative and Gram positive and were considered as LAB (Table 1).

Protein hydrolysis of LAB isolates

Good proteolytic activity was observed from 7 out of 12 isolates when tested on skim milk agar. The clear halos surrounding the colonies were greater than 6 mm (Table 2 and Figure 1).

Antioxidant activity

Scavenging of 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical

Fermenting skim milk with different LAB strains resulted in increased in DPPH values compared to control. The DPPH values of whey from LAB fermented skim milk increased with the fermentation time ranging from (14.7 to 50.8% v/v) for 24 to 72 h, respectively. Milk fermented with Gr1 and Gr2 showed DPPH values of 50.8 and 50.7%, respectively and greater than *L. plantarum* ATCC8014. The DPPH values of BHT, natural ascorbic acid and Trolox® were 97.7, 98.5 and 96.7%, respectively. DPPH activity was significantly ($p \leq 0.05$) affected by the species of LAB used and fermentation time (Table 3).

Measurement of ferrous chelating activity (FCA)

Ethylenediaminetetraacetic acid (EDTA) was used as a positive control to compare the ferrous chelating activity (FCA) of the whey from LAB fermented skim milk. FCA values of whey were similar to EDTA after 24 h fermentation but decreased after 72 h fermentation for all LAB isolates. The FCA values were in the range of (97.6-41.8% v/v) for 24 to 72 h, respectively. Skim milk fermented with Gr1 and Gr4 produced whey with FCA values of 97.6 and 94.7% (v/v) similar to EDTA after 24 h fermentation. However, FCA values decrease significantly ($p \leq 0.05$) with longer fermentation time (Table 4).

Table 1. Phenotypic characteristics of LAB isolated.

S/N	Sample	Code	Catalase reaction	Gram reaction	Cell morphology
1	Dates	D	-	+	Cocci
2	Banana	Bn1	-	+	Cocci
		Bn2	-	+	Rod
		Bn3	-	+	Rod
		Bn4	-	+	Rod
3	Black grape	Gr1	-	+	Short rod
		Gr2	-	+	Short rod
4	White grape	Gr3	-	+	Short rod
		Gr4	-	+	Short rod
5	Apple	Wg1	-	+	Cocci
		Wg2	-	+	Short rod
6	Pineapple	Pi	-	+	Cocci

(+) positive, (-) negative reactions.

Table 2. Proteolysis activity of LAB isolated on skim milk agar.

Code of sample	Diameter of clear zone (mm)
D	3
Bn1	4
Bn2	7
Bn3	7
Bn4	3
Gr1	7
Gr2	10
Gr3	7
Gr4	6
Wg1	4
Wg2	7
Pi	3

Identification of LAB isolates

Phenotypic identification of four LAB isolates that generated antioxidative peptides in the whey namely, Gr1, Gr2, Gr3, Gr4 and Wg2 were identified as *L. plantarum* 1, while isolates Bn2 and Bn3 were identified as *L. pentosus* (Table 5). Identity of Gr and Bn by 16 s DNA confirmed they were *L. plantarum* 1 and *Leoconostoc mesenteroides*, respectively (Figure 2).

DISCUSSION

Lactic acid bacteria are known to have proteolytic activity that hydrolyses protein to produce peptides with bioactivity. Several studies have reported antioxidant activities in lactobacilli using milk casein as substrate. A number of methods can be used to evaluate the antioxidant activity of the protein hydrolysate for example, DPPH, FCA, reducing power, ABTS radical scavenging activity, linoleic acid and superoxide anion radical-scavenging activity assay. Although several studies have reported antioxidant activities in lactobacilli, a direct comparison of results is difficult because of the variety of assay methods used, the numerous ways in which the results are expressed, the use of nonstandardized inoculum size and various other discrepancies (Osuntoki and Korie, 2010). In this study, the antioxidant activity of whey generated by fermentation of skim milk using local LAB isolates was reported.

DPPH is a stable free radical with a maximum absorbance at 517 nm in ethanol. When DPPH encounters a proton-donating substance such as an antioxidant, the radical is scavenged and the absorbance is reduced (Yang et al*.,* 2008). Our finding indicated that whey skim milk fermented with the different strains of LAB isolates with good proteolytic activity produced higher antioxidant activity compared to control skim milk as determined by DPPH. Similarly, Liu et al. (2005) reported that *Leoconostoc helveticus* isolated from kefir grains that possessed highly proteolytic activities produced fermented milk and soy milk with good antioxidant activity. The measured DPPH activities varied with strain and free radical scavenging activity ranging from 14.7 to 50.8% v/v for 24 to 72 h fermentation. Milk fermented with *L. plantarum* 1 (Gr1 and Gr2) showed

Table 3. Scavenging activity (%) of whey from milk fermented with LAB isolates at various fermentation time[a].

Samples	Fermentation time (h)		
	24	48	72
BHT	97.7 ± 0.2	98.7 ± 0.2	98.5 ± 0.2
Ascorbic acid	99.0 ± 0.5	99 ± 0.5	99 ± 0.5
Trolox	99.0 ± 0.1	99 ± 0.1	99 ± 0.1
Control milk[b]	10 ± 0.7	10 ± 0.4	10 ± 0.2
ATCC 8014	35.8 ± 2.4	40.5 ± 1.7	41.6 ± 1.8
Gr1	38.6 ± 5.8	35.2 ± 5.7	50.8 ± 4.5
Gr2	48.9 ± 7.5	31.4 ± 4.6	50.7 ± 16.3
Gr3	21.7 ± 3.1	22.5 ± 1.1	29.7 ± 5.4
Gr4	14.7 ± 1.8	24.8 ± 3.9	31.6 ± 1.7
Bn2	28 ± 3.3	36.9 ± 8.2	42.7 ± 10.7
Bn3	29.3 ± 7.7	41.6 ± 1.8	45.4 ± 2.5
Wg2	29 ± 2.3	38.6 ± 1.5	37.5 ± 7.4

[a]Results are mean values of triplicate determinations ± s.d. [b] Control milk (without bacteria).

Table 4. Ferrous chelating activity of whey from milk fermented with LAB isolates at various fermentation time[a].

Samples	Fermentation time (h)		
	24	48	72
EDTA	99.8 ± 0.3	99.5 ± 0.3	97.7 ± 0.3
Control milk[b]	20 ± 0.7	20 ± 0.6	20 ± 0.9
ATCC 8014	76 ± 5.4	52 ± 1.9	54 ± 0.8
Gr1	94.7 ± 3.0	79.6 ± 2.4	41.8 ± 3.6
Gr2	89.7 ± 2.1	79.7 ± 3.8	48.2 ± 4.4
Gr3	95.6 ± 2.0	59.4 ± 0.5	57.7 ± 2.7
Gr4	97.6 ± 0.3	63.5 ± 7.0	59.3 ± 0.6
Bn2	94 ± 0.6	55 ± 1.7	56 ± 1.9
Bn3	89.9 ± 3.9	60.5 ± 13.1	53.5 ± 1.9
Wg2	85 ± 5.4	52 ± 1.2	55 ± 2.6

[a]Results are mean values of triplicate determinations ± s.d. [b]Control milk is no bacteria included.

Table 5. Identification of the isolated strains using API50 CH kit.

Code	Source	Isolated strains	ID (%)	T-index
Gr1	Black grape	*Lactobacillus plantarum* 1	99.9	0.93
Gr2	Black grape	*Lactobacillus plantarum* 1	99.9	0.95
Gr3	Red grape	*Lactobacillus plantarum* 1	99.3	0.93
Gr4	Red grape	*Lactobacillus plantarum* 1	99.7	0.72
Bn2	Banana	*Lactobacillus pentosus*	82.0	0.66
Bn3	Banana	*Lactobacillus pentosus*	96.9	0.86
Wg2	Apple	*Lactobacillus plantarum* 1	99.8	0.71

DPPH values of 50.8 and 50.7%, respectively greater than *L. plantarum* ATCC8014 ($p \leq 0.05$) and that reported by Osuntoki and Korie (2010). The LAB isolated from African fermented foods scavenged between 2.8

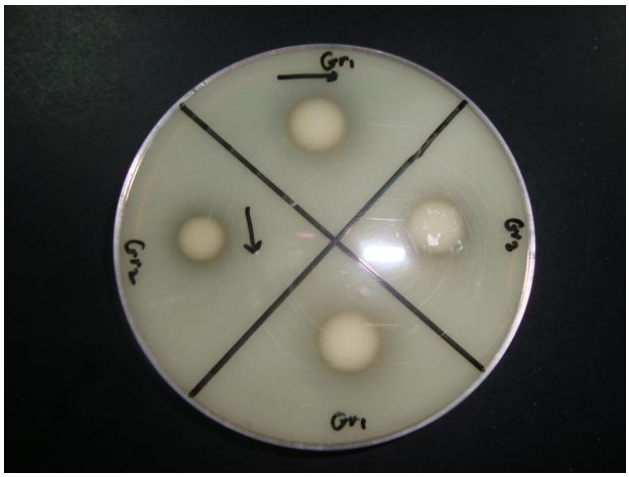

Figure 1. Clear zone surrounding the colonies indicate proteolysis activity.

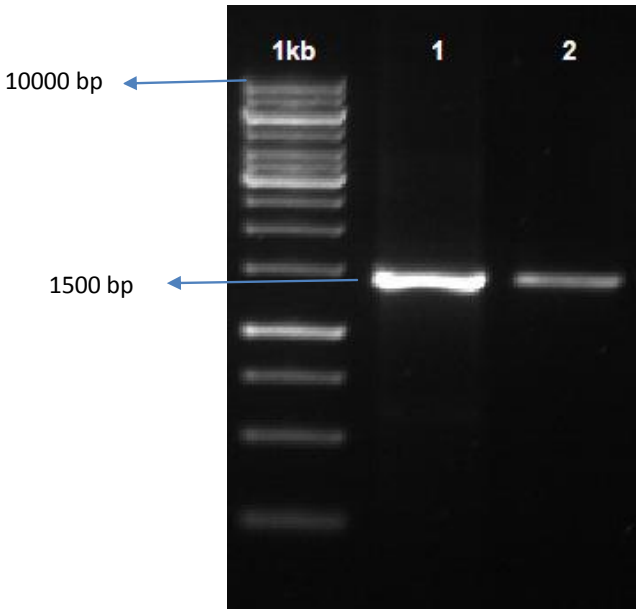

Figure 2. The DNA bands of LABs on the 1.5 % agarose gel using primers 16S.S: (5-AGAGTTTGATCCTGGCTC-3) and 16S.R: (5-CGGGAACGTATTCACCG-3), lane1kb, DNA ladder; Lane 1. Gr, 2. Bn.

and 31.5% DPPH radical for 24 h fermentation (Osuntoki and Korie, 2010). The highest development of DPPH scavenging activity was observed with *L. brevis* strain isolated from wara, followed by a strain of *L. fermentum* isolated from ugba. The least activity was shown by *L. delbrueckii* strain from ogi baba. Virtanen et al. (2007) also observed that development of antioxidant activity in whey during milk fermentation varies with strains used.

DPPH values of whey from skim milk increased with fermentation time and the strains of LAB used. Whey from skim milk fermented with *L. plantarum* 1 resulted in higher DPPH than whey from skim milk fermented with *L. mesenteroides*, after 72 h fermentation. The same phenomenon was also reported by other researchers that determine antioxidant activity of whey fraction fermented with different strains of LAB. *L. casei* (OK1), *L. brevis* (OK5), *L. plantarum* (OK6) and *L. fermentum* (OK8) isolated from different fermented foods in Nigeria showed increased in DPPH values over 24 hr fermentation (Osuntiki and Korie, 2010).

Recently, Monajjemi et al. (2012) and Virtanen et al. (2007) reported the DPPH values of milk protein hydrolysate from LAB fermented skim milk increased with the fermentation time. Other researchers also reported that longer fermentation time resulted in higher antioxidant activity as observed by Chang et al. (2009) that ferment soybean for 0, 1, 2, 5 and 10 days with *Rhizopus oligosporus* to produce tempeh. Fermentation of tempeh for 10 days exhibited the highest antioxidant activities than the others. Similarly, Mao-tofu fermented for 3, 5, 7 and 9 days by a strain of *Mucor* sp. showed that longer fermentation time of Mao-tofu gave the extracts a higher extraction yield, higher degree of hydrolysis of the protein fractions and higher antioxidant activity (Hang and Zhao, 2011). This may be attributed by the peptides released as a result of protein hydrolysis of substrates, extracellular metabolites and/or product of cell lysis (Osuntiki and Korie, 2010).

Free ferrous iron is quite sensitive to oxygen and gives rise to ferric iron and superoxide and generates hydrogen peroxide. Reaction of ferrous ion with hydrogen peroxide generates the hydroxyl radical, which oxidizes the surrounding bio-molecules. In this process, known as the Fenton reaction, hydroxyl radical production is directly related to the concentration of copper or iron (Sahu and Gray, 1997). Chelating power measures the effectiveness of compounds to compete with ferrozine for ferrous ion. A high chelating power extract reduces the free ferrous ion concentration by forming a stable iron (II) chelate and thus decreasing the extent of Fenton reaction which is implicated in many diseases (Halliwell and Gutteridge, 1992).

Our findings showed that the whey from fermented skim milk by the LAB isolates have a high potential for FCA similar to EDTA, but higher than control. Saiga et al. (2003) reported that protease treatment of porcine myofibrillar proteins produced peptides that showed metal chelating potencies. They suggested a direct relationship between soluble protein peptide concentration and the increase in the chelation capability. To the best of our knowledge this is the first report of high FCA obtained from fermentation of skim milk by LAB.

Fermentation time did not seem to affect the FCA suggesting that the high FCA values obtained in this study may be also contributed by proteins such as lactoferrin, serum albumin and casein (Tong et al, 2000) and peptides generated in whey. In general, milk fraction

contain a greater number of phosphoseryl serine groups that have greater affinity for iron, although the carboxyl group of the amino acids asparagine and glutamine can bind iron as well (Wong and Kitts, 2003).

Conclusion

The present study focused on antioxidant activity of LAB strains on fermentation of skim milk. *L. plantarum* 1 and *L. mesenteroides* isolated from local foods possess good antioxidative properties better than that previously reported. The whey of fermented skim milk has the potential to be a good dietary supplement into food formulations or cosmetics for prevention of oxidative stress related diseases such as atherosclerosis, coronary heart disease and cancer.

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