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Antioxidant Activity of Skimmed Cow and Soy Milks Fermented by Lactic Isolates of Kefir Granules

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Abstract

Background and Objective: The proteolytic system of lactic acid bacteria hydrolyzes milk protein into several peptides, including those with antioxidative activities. The aim of this study was to assess antioxidant activities in cow and soy milks fermented by *Lacticaseibacillus rhamnosus* BD2, *Lentilactobacillus kefiri* YK4 and *Lentilactobacillus kefiri* JK17 previously isolated from kefir granules and their correlations with the peptide contents.

Material and Methods: Reconstitutes of skimmed cow and soy milks were fermented by the highlighted isolates at 37 ℃ for 0, 24, 48 and 72 h. Fermented products were analyzed for the isolates, pH, total titratable acidity, antioxidant activity (% radical 2,2-diphenyl-1-picrylhydrazil inhibition and antioxidant capacity expressed in µg AAE.ml^{-1} whey) and total peptides. Fermented skimmed cow and soy milks with the highest antioxidant activity were then partially fractionated using molecular filters of 10 and 3 kDa. Fractions with the highest activity were analyzed further for peptide identification. Statistical analysis was carried out using one-way analysis of variance and Duncan multiple range tests ($p \leq 0.05$) using SPSS software v.16.0.

Results and Conclusion: All isolates were able to grow in reconstituted skimmed cow and soy milks, while total count of reached to 9 log CFU.ml^{-1} with significant ($p \leq 0.05$) increases in titratable acidity and total peptides and decreased pH. Growth of the three isolates was mildly slower in soy milk than in skimmed cow milk. The maximum antioxidant activities were seen after 72-h fermentation of cow and soy milks. No differences were observed in antioxidant activity of cow milk fermented by the three isolates; however, *Lacticaseibacillus rhamnosus* BD2 produced the highest antioxidant activity in soy milk. In general, increases in antioxidant activities correlated with increases in the peptide contents. Fraction of less than 3 kDa of the two milks fermented by *Lacticaseibacillus rhamnosus* BD2 showed the highest antioxidant activity. Analyses of peptides present in these fractions using high resolution LC-MS/MS and *in silico* identification of peptides with antioxidant activity have been reported in this study. The present study suggests that the three isolates can be used as starter cultures in fermenting cow and soy milks to increase their antioxidant activities. Peptides with molecular weight of less than 3 kDa play key roles in antioxidant activity.

Conflict of interest: The authors declare no conflict of interest.

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1. Introduction

Normal cellular metabolism produces free radicals that are unstable and highly reactive due to their unpaired electrons. Free radicals, reactive oxygen species and reactive nitrogen species directly attack biological macromolecules such as DNA, RNA, proteins and other substances causing cell injury and induce oxidative stress that involve in various human diseases such as diabetes mellitus, neurodegenerative diseases, rheumatoid arthritis, cataracts, cardiovascular diseases, respiratory disorders and premature aging [1,2]. Because of their adverse effects on various physiological conditions leading to various diseases, additional dietary antioxidant intakes are critical to support defense mechanisms of the human body against free radicals as the natural antioxidants produced by the human body are not sufficient [3]. Foods containing antioxidants have received significant attentions to decrease activities of reactive

oxygen species and free radicals and hence prevent or decrease oxidative damages in the human body [2]. Fermented foods have been reported to contain antioxidant activities, including cow milk kefir [4], goat milk kefir [5,6], fermented cow and camel milks [7], fermented soy milk [8,9], fermented shrimp paste [10], fermented fish [11], fermented vegetables and kimchi [12] and tempeh [13].

Beneficial health effects of the fermented dairy products have been associated with bioactive peptides produced by lactic acid bacteria (LAB) [14], including those with antioxidative activities [3,14]. During milk fermentation, LAB such as Lactobacillus, Lactococcus and Streptococcus use milk proteins as their prime sources of essential amino acids to support their growth. Hydrolyses of protein during fermentation produce bioactive peptides [15] with health beneficial effects such as antimicrobial, metal-binding, antioxidant, immunomodulatory, cell-cycle and apoptosis modulating, antithrombotic, antihypertensive and cholesterol lowering effects [16]. The nature of protease enzymes produced by LAB, origin of proteins and conditions of hydrolyses affect sizes and sequences of the peptides released during fermentation and their bioactivities [17]. Each species of LAB produces various proteolytic activities leading to a large variety of protein hydrolysates [14]. Some LAB species have been known to produce antioxidants in fermented milks, including *Lactiplantibacillus plantarum* and *Leuconostoc mesenteroides* [18], *Streptococcus (S. thermophiles), Lactobacillus (L. delbrueckii) bulgaricus, L. lactis, L. acidophilus* and *L. helveticus* [2], *L. acidophilus* [19], *Pediococcus pentosaceus* [20], *L. rhamnosus* [21], *L. plantarum*, *L. paracasei* and *L. brevis* [22] and *L. lactis*, as well as *L. paraplantarum, L. kefiri, L. paracasei, L. gasseri, L. plantarum* [7].

Previous studies have shown that *Lentilactobacillus* (*L.*) *kefiri* YK4, *L. rhamnosus* BD2, *L. rhamnosus* YK12, *L. kefiri* BG8, and *L. kefiri* JK17 isolated from Indonesian kefir granules are capable of producing peptides with antioxidant activities in fermented cow milk [23]. The aim of the present study was to assess antioxidant activity in cow and soy milks fermented by *L. rhamnosus* BD2, *L. kefiri* YK4 and *L. kefiri* JK17 as well as their molecular fractions with high antioxidant activities. The other aim was to identify peptides with antioxidant activities in the fractions. Peptides analysis was carried out using high resolution liquid chromatography-electrospray ionization mass spectrometry (HR-LC-MS/MS) and identification of antioxidative peptides was carried out based on *in silico* studies, the approach used to predict biological functions of the bioactive compounds.

2. Materials and Methods

2.1 Preparation of the starters

In the present study, *L. kefiri* YK4, *L. kefiri* JK17 and *L. rhamnosus* BD2 previously isolated from kefir granules [23,24] were provided by the SEAFAST Center of IPB University, Indonesia. Each culture was revived in 10 ml of de Man, Rogosa and Sharpe (MRS) broth media (Oxoid, UK) and incubated at 37 ℃ for 24 h. Starter culture was prepared by inoculating 14% (w v^{-1}) reconstituted skimmed cow (NZMP, NZ) and soy (Metabolis, Indonesia) milks pasteurized at 95 ℃ for 10 min, with 2% (v v^{-1}) of the culture suspension to reach 10^6–10^7 CFU.ml^{-1} and incubated at 37 ℃ for 24 h [23,25].

2.2 Milk fermentation

Milk used for the fermentation included reconstituted skimmed cow milk (NZMP, NZ) and reconstituted soy milk (Metabolis, Indonesia) (14% w v^{-1}), which were pasteurized at 95 ℃ for 10 min [25]. Starter cultures of *L. kefiri* YK4, *L. rhamnosus* BD2 and *L. kefiri* JK17 were inoculated separately at 2% (w v^{-1}) to reach 10^6-10^7CFU.ml^{-1} into pasteurized skimmed cow or soy milk and then incubated (Medcenter Einrichtungen, Germany) at 37 ℃ for 0, 24, 48 and 72 h. Fermented milks with the highest antioxidant activity were further fractionated based on their molecular size by centrifugation at 3000 g for 40 min at 4 ºC using membrane filter tubes of 10 and 3 kDa (Amicon Ultra-4 ml centrifugal filters, Ireland) [23]. Fractions of > 10, 3-10 and < 3 kDa were collected and their volumes were adjusted to initial volume of whey (15 ml) using distilled water (DW). The fraction with the highest antioxidant activity was further analyzed for peptide identification.

2.3 Enumeration of lactic acid bacteria

The LAB was enumerated using pour plate method on MRS agar media (Oxoid, UK), then incubated at 37 ℃ for 48 h [24]. Total LAB was calculated based on the bacteriological analytical manual (BAM) method [26].

2.4 Assessment of pH and total titratable acidity

The pH value of fermented milk was assessed using pH meter (Eutech pH700; Thermo Scientific, USA) and total titratable acids (TTA) value was reported using titration method [27].

2.5 Assessment of antioxidant activity and capacity

Antioxidant activity expressed as % radical 2,2-diphenyl-1-picrylhydrazil (DPPH) inhibition was assessed using spectrophotometric DPPH method [2]. Whey was collected by centrifugation of the fermented skimmed cow milk and fermented soy milk at 3904.05 g for 15 min at 4 ℃ (Hermle Z 383 K, Germany). Supernatant was filtered using filter papers (Whatman, USA) and pH was adjusted using 0.1 N NaOH until reached pH 7. Then, it was recentrifuged and filtered using 0.22-µm sterile filters (Himedia, India). Supernatant or whey was analyzed for its antioxidant activity. Analysis of antioxidant activity was carried out by mixing 2 ml of sample with 2 ml of 0.2-mM DPPH (Sigma-Aldrich, Germany) solution in 95% (v v^{-1}) methanol (Merck,

Germany) and then incubated for 30 min at dark. Blank was prepared using DW. Furthermore, DPPH free radical inhibition activity was reported by measuring the absorbance at 517 nm using UV-Vis spectrophotometer (UV mini-1240; Shimadzu, Japan). The percentage of DPPH free radical inhibitory activity was calculated based on difference between the absorbance of blank and sample solution using the following equation (Eq. 1):

$$\text{DPPH radical scavenging activity (\% inhibition)} = \frac{\text{Abs blank} - \text{Abs sample}}{\text{Abs blank}} \times 10 \quad \text{Eq. 1}$$

The total antioxidant capacity was expressed as ascorbic acid equivalent (AAE) calculated based on the inhibition activity of DPPH free radicals [28]. The calculation used the equation generated from the standard curve of inhibitory of ascorbic acid at concentrations of 10, 20, 60 and 80 ppm or µg.ml^{-1} toward DPPH free radicals. The antioxidant capacity was expressed as µg AAE.ml^{-1} whey.

2.6 Assessment of total peptides

Analysis of the total peptides was carried out for similar sample solutions (neutralized supernatants) as for antioxidant activity analysis. This analysis referred to Lowry et al. [29]. Absorbance was measured at 750 nm using UV-Vis spectrophotometer (UV mini-1240; Shimadzu, Japan). Bovine serum albumin (BSA) was used as standard at concentrations of 0.5, 1.0, 1.5, 2.0, 2.5, 3.0 mg.ml^{-1} prepared by diluting BSA stock solution (10 mg.ml^{-1}).

2.7 Peptide identification

Fractions with the highest antioxidant activity were filtered through 0.22-µm PTFE filter membranes. Briefly, 10 µl of each fraction were injected into HR-LC-MS/MS instrument. The high-resolution instrument could analyze and identify peptides due to its capability to report accurate masses of peptide fragment ions up to 5 decimals, based on the mass analyzers using quadrupole mass filter and orbitrap mass analyzer. In this study, the ion mass analyzers were set at an ion mass scan range of 200-2000 m.z^{-1} and a scan speed of up to 12 Hz. The analysis was carried out using Ultimate 3000 RSLC Tandem Q Exactive HR-LC-MS/MS (Thermo Scientific, USA) with an electrospray ionizer at positive ionization mode. Peptide separation was carried out on PepMap RSLC C18 nanocolumn (0.075 × 150 mm, 3 µm particle size, 100Å pore size) using 0.1% (v v^{-1}) formic acid in water as mobile phase A and 0.1% (v v^{-1}) formic acid in acetonitrile as mobile phase B. Running of the mobile phases in gradient was programmed as follows: at 2-35% B for 30 min, 35-90% B for 15 min, 90% B for 15 min and 5% B for 15 min with a flow rate of 0.3 µl.min^{-1}. Then, mass spectral data (MS1 and MS2) achieved from HR-LC-MS/MS were analyzed using Proteome Discoverer 2.2 and Xcalibur software (Thermo Fisher Scientific, USA) to discover six or further amino acid (AA) sequences of the peptides based on *in silico* protein cleavage database (with trypsin which cleavages C-terminal to Lys and Arg; mass tolerance 10 ppm) from cow and soy milks. Peak retention time and mass spectrum of each identified peptide were read and analyzed from the chromatogram and mass spectrum files of HR-LC-MS/MS for assessing peptide sequences and peak area using the software. The relative area for each peak calculated as percentage of the peak area divided by total peak area was assessed to evaluate abundance of the peptides. Prediction of the antioxidant activity of the identified peptides was carried out *in silico* using Biopep-UMW database (www.uwm.edu.pl/biochemia/index.php/pl/biopep) under sequence matching). The BIOPEP-UWM database of bioactive peptides is a basic tool for the research on bioactive peptides derived from foods with the activity to prevent development of chronic diseases [30].

2.8 Statistical analysis

All analyses were carried out in triplicate and expressed as mean ±SD (standard deviation). data for total LAB, pH, TTA, total peptides and antioxidant activity (% radical DPPH inhibition and antioxidant capacity µg AAE.ml^{-1} whey) were analyzed using one-way ANOVA. Differences between the means were analyzed using Duncan multiple range test (DMRT) with $p \leq 0.05$. Statistical analysis was carried out using SPSS software v.16.0 (IBM, USA).

3. Results and Discussion

3.1 Total LAB, pH and total titratable acidity

Total LAB in the fermented skimmed cow milk showed increases during 72-h fermentation and reached 9.31 log CFU.ml^{-1} for *L. kefiri* YK4 Figure 1A), 9.34 log CFU ml^{-1} for *L. rhamnosus* BD2 (Figure 1B) and 9.39 log CFU.ml^{-1} for *L. kefiri* JK17 (Figure 1C). Statistical analysis showed significant ($p \leq 0.05$) increases in the number of total LAB after 24 h. Number of LAB in milk fermented with *L. kefiri* YK4 significantly increased up to 72 h of fermentation. Increases in *L. rhamnosus* BD2 and *L. kefiri* JK17 were not significant ($p \leq 0.05$). This indicated that *L. kefiri* YK4 grew slower in milk than that *L. rhamnosus* BD2 and *L. kefiri* JK17 did. Increases in the total LAB of the three isolates in fermented skimmed cow milk were similar with the increases in TTA and decreases in pH during 72 h of fermentation ($p \leq 0.05$). The longer the fermentation time, the lower the pH value of the fermented skimmed cow milk from an initial pH of 6.88 to 4.85 for *L. kefiri* YK4, 6.82 to 4.61 for *L. rhamnosus* BD2, 6.82 to 4.37 for *L. kefiri* JK17 after 72 h of fermentation. For yoghurt fermentation, the end point of fermentation is usually when the pH reaches 4.6 [31].

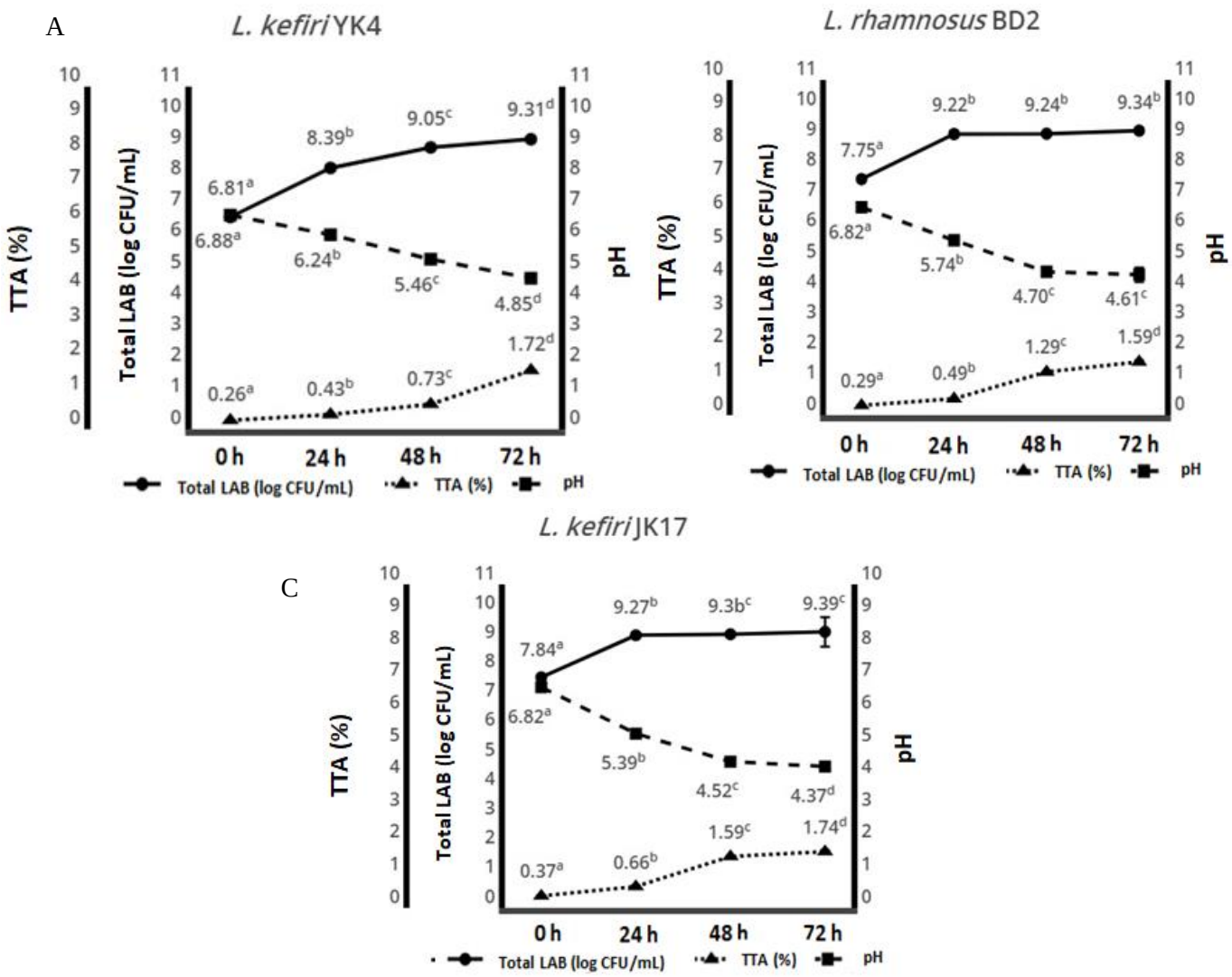

Figure 1. Effects of fermentation time of 0, 24, 48 and 72 h of skimmed cow milk fermented by LAB (A= *L. kefiri* YK4, B = *L. rhamnosus* BD2 and C = *L. kefiri* JK17) on total LAB (-●-), pH (-■-) and TTA (-▲-)

In addition, increases were seen in TTA from 0.26 to 1.72% for *L. kefiri* YK4, 0.29 to 1.59% for *L. rhamnosus* BD2 and 0.37 to 1.74% for *L. kefiri* JK17. Marya et al. [32] explained that pH of fermented milks depended on the organic acids produced by LAB. During fermentation process, LAB broke down lactose into lactic acid. Time to reach pH 4 varied between the LAB, showing differences in LAB growth rates [25].

In contrast to the microbial growth in skimmed cow milk, the total number of the three LAB isolates increased significantly ($p \leq 0.05$) up to 72 h of fermentation of soy milk. After 72 h of fermentation, LAB in fermented soy milk of *L. kefiri* YK4 reached 9.33 log CFU.ml^{-1} (Figure 2A), 9.27 log CFU.ml^{-1} of *L. rhamnosus* BD2 (Figure 2B) and 9.11 log CFU.ml^{-1} of *L. kefiri* JK17 (Figure 2C). Increases in LAB was accompanied with decreases in pH and significant increases in TTA up to 72 h of fermentation ($p \leq 0.05$). Moreover, LAB growth was affected by the media, environmental conditions and isolate types [33].

After 72 h of fermentation, TTA of the soy milk increased from 0.42 to 1.11% for *L. kefiri* YK4, 0.39 to 1.12% for *L. rhamnosus* BD2 and 0.37 to 0.99% for *L. kefiri* JK17. Moreover, pH of the fermented soy milk decreased from 6.77 to 4.32 at the end of fermentation for *L. kefiri* YK4, 6.72 to 4.24 for *L. rhamnosus* BD2 and 6.82 to 4.61 for *L. kefiri* JK17. The present results were similar with the results of a study by Yusmarini et al. [34], showing increases in TTA from 0.11 to 0.31-0.34% and decreases in pH from 6.7 to 4.6–4.8 at the end of soy milk fermentation by *L. plantarum*1 R.1.3.2, *L. plantarum* 1 R.11.1.2 and *L. acidophilus* FNCC 0051. Growth of *L. kefiri* YK4, *L. rhamnosus* BD2 and *L. kefiri* JK17 in skimmed cow milk was slower than that in soy milk.

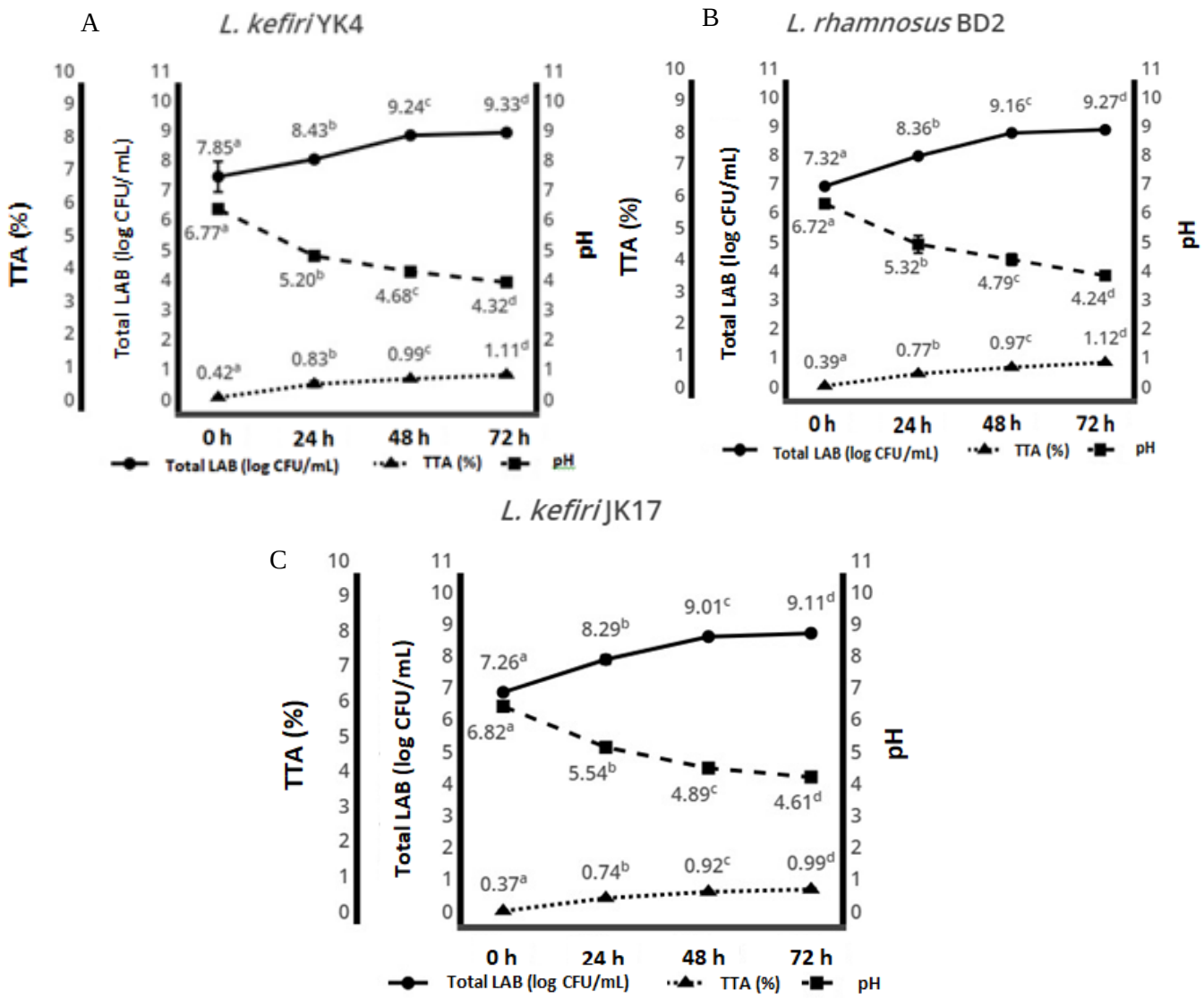

Figure 2. Effects of fermentation time of 0, 24, 48 and 72 h of soy milk fermented by LAB (A = *L. kefiri* YK4, B = *L. rhamnosus* BD2 and C = *L. kefiri* JK17) on total LAB (-●-), pH (-■-) and TTA (···▲···)

This was due to the differences in sugar types as carbon and energy sources available in cow and soy milks. Naturally, the major sugar in cow milk is lactose while soy milk contains sucrose, raffinose and stachyose [35-37]. The LAB isolates were originated from the kefir granules adapted to cow milk; hence, they are readily to grow in cow milk while their growth in soy milk needed adaptation.

3.2 Antioxidant activity

Antioxidant activity was assessed based on the ability of the sample to inhibit DPPH free radicals by donating hydrogen atoms [38]. Fermentation of the skimmed cow milk by the LAB strains of *L. kefiri* YK4, *L. rhamnosus* BD2 and *L. kefiri* JK17 significantly increased its DPPH free radical inhibition activity from 0 h to 72 h of fermentation (Table 1). The highest inhibition activity produced by the three LAB at 72 h of fermentation with inhibition activity of 76.53% ±0.59 for *L. kefiri* YK4, 78.03% ±4.07 for *L. rhamnosus* BD2 and 73.13% ±3.40 of *L. kefiri* JK17. The *L. rhamnosus* BD2 produced the highest antioxidant activity than that the *L. kefiri* YK4 and *L. kefiri* JK17 did. Within 24 h of fermentation, antioxidant activity of *L. kefiri* JK17 was significantly different from that of *L. kefiri* YK4 and *L. rhamnosus* BD2 ($p \leq 0.05$). At 48 h and 72 h, no significant differences were demonstrated between the isolates ($p > 0.05$). This finding was similar to a finding by Li et al. [2], who showed differences between the species in the ability to produce antioxidant activities. Yusuf et al. [23] reported that cow milk fermented by *L. rhamnosus* BD2 and *L. kefiri* YK4 for 24 h included antioxidant activities of 74.53% ±2.06 and 80.92% ±3.03, respectively. Using similar isolates in this study, antioxidant activities of the fermented skimmed cow milk were slightly lower that might be due to the various compositions of milk. The highest increase in antioxidant activity was achieved at 24 h of fermentation. The antioxidant activity of LAB (Table 1) correlated with their growth in skimmed cow and soy milks (Figures 1 and 2). Production of substances with antioxidant activity

declined at the end of logarithmic phase or at the initial stationary phase [39].

The DPPH free radical inhibition activities of the fermented skimmed cow milk by *L. kefiri* YK4, *L. rhamnosus* BD2 and *L. kefiri* JK17 were different from that of the fermented soy milk. Results showed significant increases after 72 h of fermentation of the soy milk ($p \leq 0.05$) using the three isolates of *L. kefiri* YK4, *L. rhamnosus* BD2 and *L. kefiri* JK17 (Table 1). Similar to the fermented skimmed cow milk, the highest antioxidant activity was reported in soy milk that fermented for 72 h by *L. rhamnosus* BD2 (64.97% ±0.75), followed by *L. kefiri* YK4 (62.83% ±0.55) and *L. kefiri* JK17 (61.75% ±0.46). However, the highest increase in the antioxidant activity in fermented soy milk occurred after 48 h of fermentation. Increases in antioxidant activity between 48 to 72 h of fermentation were less than those of 24 to 48 h with significant increases ($p \leq 0.05$). Furthermore, *L. rhamnosus* BD2 was able to produce the highest antioxidant activity and showed significant differences, compared to other two isolates ($p \leq 0.05$). Increases in antioxidants were seen being align with the growth of the three isolates in fermented soy milk.

During fermentation, proteins were hydrolyzed by extracellular proteinases that produced by *Lactobacillus*, increasing number of the peptides and free AAs [16]. This was demonstrated by *L. kefiri* YK4, *L. rhamnosus* BD2 and *L. kefiri* JK17 during fermentation of skimmed cow and soy milks. Increases in antioxidant activity of the fermented cow and soy milks were addressed as results of production of bioactive peptides. The present study was similar to the previous studies on the antioxidant activity of LAB [2,40-42]. Peptides that produced in the fermented cow and soy milks were different, where the antioxidant peptides of the fermented cow milk were derived from their parent protein casein of α_{S1}-casein, α_{S2}-casein, α-lactalbumin and β-lactoglobulin [23,40]. Additionally, antioxidant peptides of the fermented soy milk derived from β-conglycinin and glycinin [43,44]. Glycinin derived peptides were more effective than β-conglycinin peptides [43,44]. Moreover, proteolytic activity of the LAB produced bioactive compounds in soymilk and increases in antioxidant activities of the fermented soy milk were due to the deliberation of other compounds during fermentation such as isoflavone aglycones that acted as further effective hydrogen donors to chelate DPPH free radical [8]. The β-glucosides were hydrolyzed into daidzein and genistein by β-glucosidase enzyme that has presently been reported in *L. rhamnosus* CRL 981, producing antioxidant activity of 71.2% ±4.0 of DPPH inhibition after 24 h of fermentation [8].

At 0 h of fermentation, considerable proportions of DPPH free radical inhibition activity presented in skimmed cow (36.67-47.93% inhibition) and soy (40.52-41.94%) milks. This was caused by the presence of other compounds in skimmed cow milk and fermented soy milk that acted as antioxidants. Lipophilic antioxidant compounds (e.g., carotenoids, α-tocopherol, vitamins A and D3, phospholipids and coenzyme Q) and hydrophilic antioxidants (e.g., proteins, caseins, peptides, vitamins, minerals and low molecular weight thiols) play major roles in maintaining pro-oxidant and antioxidant homeostatic in oxidation systems [45]. Antioxidant capacity of the casein subunits (α-casein, β-casein and κ-casein) can inhibit thiobarbituric reactive substances (TBARS) and lipid peroxides. Furthermore, milk contains enzymes that act as antioxidants, including superoxide dismutase (SOD), glutathione peroxidase and catalase as well as several phenolic compounds such as phenol, cresol, thymol and carvacrol [45].

The calculated antioxidant capacities (μg AAE.ml^{-1} whey) of skimmed cow and soy milks fermented by *L. kefiri* YK4, *L. rhamnosus* BD2, *L. kefiri* JK17 are showed in Table 2. Vitamin C equivalent antioxidant capacity to show the total antioxidant capacity of a food calculated on the weight basis is more desirable than other methods as this method is reported as simple and reliable [28]. Similar to the DPPH free radical inhibition activity, antioxidant capacity showed increases during 72-h fermentation of skimmed cow and soy milks. After 48 and 72 h of fermentation, no significant differences were shown between the antioxidant capacities of the isolates ($p>0.05$). The highest antioxidant capacity (58.89 ±4.20 μg AAE.ml^{-1} whey) in fermented skimmed cow milk was achieved after 72 h of fermentation by *L. rhamnosus* BD2, followed by *L. kefiri* YK4 (57.34 ±0.61 μg AAE.ml^{-1} whey) and *L. kefiri* JK17 (53.83 ±0.84 μg AAE.ml^{-1} whey). Results of this study were weaker than that reported by Ramos et al. [46], showing that the antioxidant capacity of yoghurt was 310 μg AAE.g^{-1} yoghurt and Park et al. [47], demonstrating an antioxidant capacity of 100 μg AAE.ml^{-1} with an inhibition of DPPH free radical activity of 79.21%. Significant increases of antioxidant capacity of the fermented milk were reported after 24-h fermentation of the skimmed cow and soy milks and further increases were seen until 72 h of fermentation (Table 2). After fermentation for 72 h, *L. rhamnosus* BD2 produced the highest antioxidant capacity of the skimmed cow and soy milks, which was significantly different from that of *L. kefiri* JK 17 for the fermented skimmed cow milk and *L. kefiri* YK4 and *L. kefiri* JK17 for the fermented soy milk (Table 2). In general, antioxidant capacity of the fermented skimmed cow milk was higher than that of the fermented soy milk, correlating with the growth of LAB in cow and soy milks.

3.3 Total peptides

Total peptides of the fermented skimmed cow and soy milks showed significant increases since 24 h of fermentation (Table 3).

Table 1. The 2,2-diphenyl-1-picrylhydrazil free radical inhibition activity in fermented skimmed cow milk and fermented soy milk

Fermentation time (h)	DPPH free radical inhibition (%)*		
	L. kefiri YK4	*L. rhamnosus* BD2	*L. kefiri* JK17
Skimmed cow milk			
0	41.49 ± 1.66^{aAB}	47.93 ± 0.81^{aB}	36.67 ± 6.03^{aA}
24	65.38 ± 3.34^{bB}	67.10 ± 2.22^{bB}	57.84 ± 1.96^{bA}
48	70.18 ± 3.53^{bA}	72.57 ± 3.73^{bcA}	69.42 ± 1.65^{cA}
72	76.53 ± 0.59^{cA}	78.03 ± 4.07^{cA}	73.13 ± 3.40^{cA}
Soy milk			
0	40.52 ± 0.42^{aA}	41.94 ± 0.82^{aB}	40.86 ± 0.42^{aAB}
24	46.94 ± 0.68^{bA}	49.74 ± 0.86^{bB}	45.81 ± 1.47^{bA}
48	60.88 ± 0.09^{cB}	62.24 ± 0.12^{cC}	59.21 ± 0.89^{cA}
72	62.83 ± 0.55^{dA}	64.97 ± 0.75^{dB}	61.75 ± 0.46^{dA}

*The results were achieved from three repetitions ± standard deviation. Different superscripts in one column (a-d) and different capitalized superscripts in one row (A-C) showed significant differences ($p \leq 0.05$) using Duncan's test. L.=Lactobacilus

Table 2. Antioxidant capacities of the fermented skimmed cow milk and fermented soy milk

Fermentation time (h)	Antioxidant capacities (µg AAE.ml^{-1} whey)*		
	L. kefiri YK4	*L. rhamnosus* BD2	*L. kefiri* JK17
Skimmed cow milk			
0	21.22 ± 1.72^{aA}	27.85 ± 0.84^{aAB}	16.25 ± 6.21^{aB}
24	45.85 ± 3.44^{bB}	47.62 ± 2.29^{bB}	38.07 ± 2.02^{bA}
48	50.79 ± 3.64^{bA}	53.26 ± 3.85^{bcA}	50.01 ± 1.70^{cA}
72	57.34 ± 0.61^{cC}	58.89 ± 4.20^{cC}	53.83 ± 3.51^{cA}
Soy milk			
0	18.19 ± 0.48^{aA}	19.79 ± 0.93^{aB}	18.57 ± 0.47^{aAB}
24	25.45± 0.77^{bA}	28.63 ± 0.98^{bB}	24.17 ± 1.66^{bA}
48	41.24 ± 0.09^{cA}	42.78 ± 0.14^{cB}	39.35 ± 1.02^{cC}
72	43.45 ± 0.62^{dA}	45.87 ± 0.84^{dB}	42.22 ± 0.52^{dA}

*The results were obtained from three repetitions ± standard deviation. Different superscripts in one column (a-d) and different capitalized superscripts in one row (A-C) showed significant differences ($p \leq 0.05$) using Duncan's test.

Table 3. Total peptides in the fermented skimmed cow milk and fermented soy milk

Fermentation time (h)	Total peptides (mg.ml^{-1})*		
	L. kefiri YK4	*L. rhamnosus* BD2	*L. kefiri* JK17
Skimmed cow milk			
0	1.24 ± 0.12^{aA}	1.37 ± 0.19^{aA}	1,21 ± 0,11^{aA}
24	1.56 ± 0.06^{bA}	1.74 ± 0.15^{bA}	1,43 ± 0,24^{abA}
48	1.78 ± 0.11^{cA}	2.31 ± 0.14^{cB}	1,72 ± 0,16^{bcA}
72	1.95 ± 0.14^{cA}	2.52 ± 0.16^{cB}	1,92 ± 0,06^{cA}
Soy milk			
0	1.12 ± 0.02^{aB}	1.25 ± 0.04^{aC}	1.04 ± 0.04^{aA}
24	1.21 ± 0.04^{bB}	1.40 ± 0.02^{bC}	1.13 ± 0.03^{bA}
48	1.79 ± 0.02^{cB}	2.02 ± 0.06^{cC}	1.68 ± 0.01^{cA}
72	1.93 ± 0.06^{dA}	2.22 ± 0.03^{dB}	1.89 ± 0.02^{dA}

*The results were obtained from three repetitions ± standard deviation. Different superscripts in one column (a-d) and different capitalized superscripts in one row (A-C) showed significant differences ($p \leq 0.05$) using the Duncan's test.

This was in accordance with increases in antioxidant activity. After 72 h of fermentation, total peptides produced by the *L. kefiri* YK4 and *L. rhamnosus* BD2 in fermented skimmed cow milk were not significantly different from those in their respective fermentation time of 48 h. Breaking down of proteins into peptides and AAs by proteolytic enzymes plays important roles in supplying cells with the nitrogen compounds necessary for their growth (15,48). When the growth ceased as shown by no significant increases in the cell number after 48 h (Figure 2 and 3), cells did not need more AAs and peptides; hence, no increases in the total peptides were detected in the media. Total peptides at 24 h of fermentation were not significantly different between the isolates ($p \leq 0.05$). After 48 h, the *L. rhamnosus* BD2 produced total peptides (2.31 ±0.14 mg.ml^{-1}) that were significantly different with that the *L. kefiri* YK4 and *L. kefiri* JK17 did. The highest total peptides of the milk fermented by the three LAB isolates were detected at 72 h with no significances with that of 48 h. The present results suggested that *L. rhamnosus* BD2 exhibited the highest proteolytic activity.

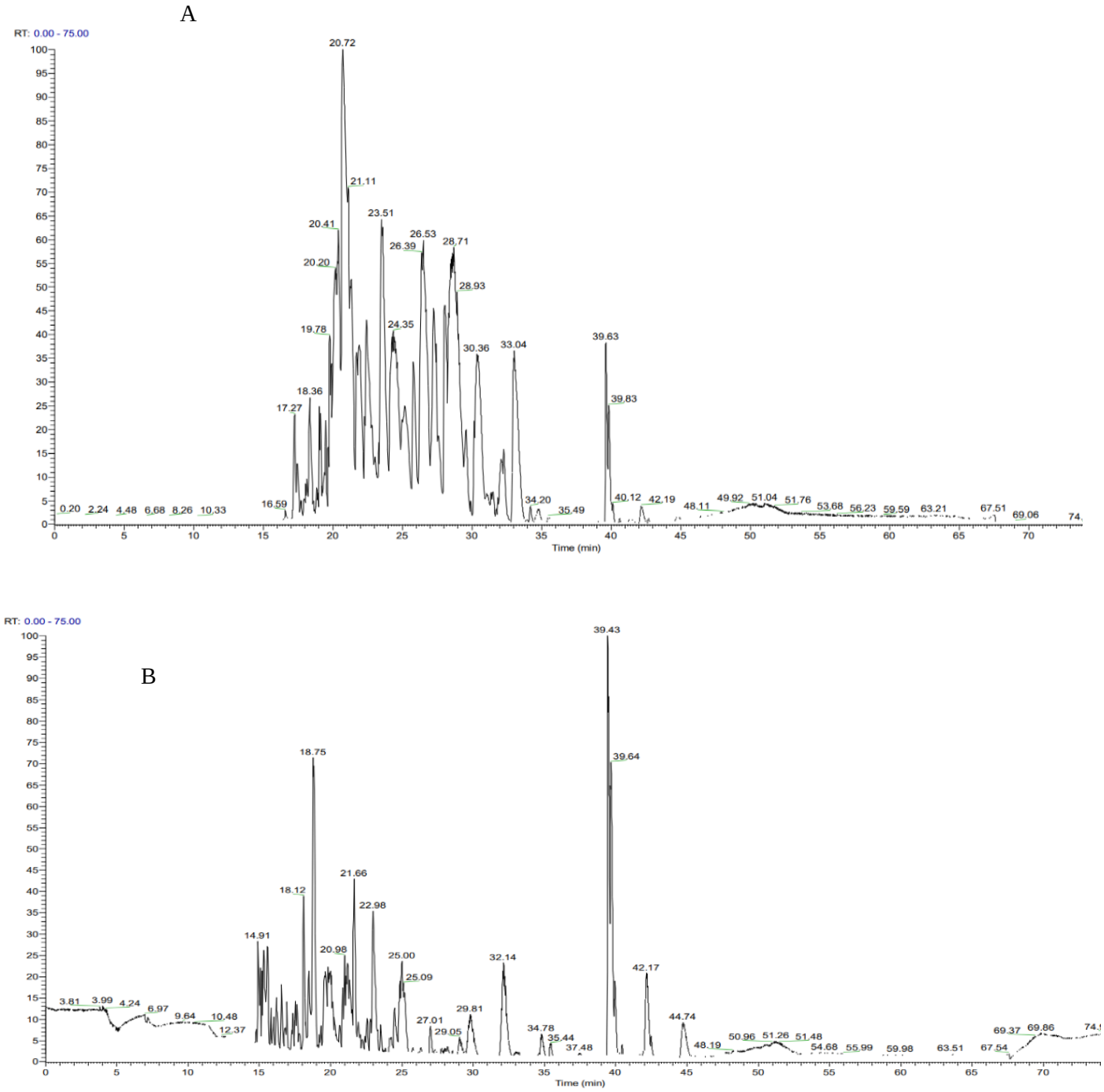

Figure 3. Chromatograms of the < 3 kDa fractions with relatively high antioxidant activities of 48 h fermented skimmed cow milk (A) and fermented soy milk (B). Fermentation used *L. rhamnosus* BD2 as selected LAB culture for the intention to produce functional foods

Previous studies reported that skimmed cow milk fermented by *L. kefiri* JK17 and *L. kefiri* YK4 for 24 h produced total peptides of 3.98 ±0.17 mg ml^{-1} and 3.54 ±0.07 ml^{-1} with no significant differences ($p \leq 0.05$) [25]. Peptide content of the cow milk fermented by *L. rhamnosus* BD2 was not significantly different from that by *L. kefiri* YK4 [23].

Similar to the patterns of the LAB growth in skimmed cow milk, the highest total peptides in fermented soy milk were reported after 72 h of fermentation, significantly different from that of 48 h ($p \leq 0.05$). In soy milk fermentation of 72 h, *L. rhamnosus* BD2 produced total peptides of 2.22 ±0.03 mg/ml, followed by *L. kefiri* YK4 of 1.93 ±0.06 mg.ml^{-1} and *L. kefiri* JK17 of 1.89 ±0.02 mg.ml^{-1}. Significant increases of the total peptides in soy milk fermentation from 48 h to 72 h were correlated with significant increases of the total LAB (Figure 2). As previously stated, LAB hydrolyze proteins to meet their needs of the AAs to support their growth [15,48]. Sanjukta and Rai [16] reported that soy protein was

hydrolyzed by the extracellular proteinase of *Lactobacillus* during lactic fermentation, resulting a number of bioactive peptides that acted as antioxidants. Results of the present study showed that increases in total peptides were in accordance with increases in antioxidant activities (Tables 1,2 and 3). Other researchers showed increases in proteolytic activity as well [41] total peptides [16,37], positively correlated with increases in antioxidant activities. Their studies revealed that the higher peptide content, the higher antioxidant activity.

3.4 Partial Fractionation and Antioxidant Capacity

Milk fermented by *L. rhamnosus* BD2 for 48 h was reported as a sufficient potential source for antioxidants as only slight increases in antioxidant activity were seen when the fermentation was extended to 72 h, especially in fermentation skimmed cow milk. At 48 h of fermentation, its antioxidant activity was higher than that of the other two isolates (Tables 1 and 2). To predict molecules responsible for the relatively high antioxidant activity, fractionation was carried out based on molecular sizes of <3, 3-10 and >10 kDa and then further analyzed for DPPH inhibition activity and total peptides. The highest DPPH inhibition activity and total peptides were observed in the fraction with a molecular weight of<3 kDa (Table 4). The<3 kDa fraction showed that the DPPH inhibition activity of the fermented soy milk (81.13% ±1.04) was higher than that of the fermented skimmed cow milk (73.68% ±2.29). The total peptides of<3 kDa fraction in the fermented soy milk (2.14 ±0.11 $mg.ml^{-1}$) were lower than those in the fermented skimmed cow milk (3.02 ±0.02 $mg.ml^{-1}$). Free radical inhibition capacity of the fermented soy milk was due to the presence of bioactive compounds such as isoflavones released during fermentation as well as bioactive peptides. This caused a higher DPPH inhibition activity at the lower peptide contents (Table 4). Other studies showed that fractionated yoghurt contained peptides with molecular sizes of >10 and 3–10 kDa with AA residues of threonine and serine from the parent proteins of α-lactalbumin and β-casein. Additionally, molecular sizes <3 kDa included AA residues of tyrosine, glutamine, tryptophan, histidine and leucine from the parent protein of casein [40], often found in peptides with antioxidant activities. Vasconcellos et al. [43] reported that the antioxidant activity of soy milk peptides depended on the parent protein of *β*-conglycinin and glycinin and glycinin derived peptides were more effective than conglycinin derived peptides. The <3 kDa fraction of the fermented skimmed cow milk and fermented soy milk were subjected further for peptide identification with antioxidant activity.

3.5 Peptide identification by HR-LC-MS/MS and in silico activity prediction

In general, HR-LC-MS/MS chromatogram profiles of the <3 kDa fractions of the fermented skimmed cow milk and fermented soy milk are present in Figure 3. The two profiles are recognized as peptide profiles, which are apparently different even they are achieved from the fermentation by similar LAB cultures (*L. rhamnosus* BD2) and fermentation lengths (48 h). This was due to the various parent proteins present in the two types of milk (animal and vegetable milks) as discussed previously and present in Tables 5 and 6. The profile in Figure 3 from the animal source is highlighted by the presence of more peaks, indicating that more peptides were present in the fraction of the fermented skimmed cow milk. This could explain distinct total peptide contents between the fermented skimmed cow and soy milks as presented in Table 4. From a total of 1550 spectra data achieved from HR-LC-MS/MS for the fraction of <3 kDa of the fermented skimmed cow milk and 2579 spectra data from that of fermented soy milk, 40 peptides were identified in the fraction of fermented skimmed cow milk (Table 5) and 25 peptides in the fraction of fermented soy milk (Table 6).

The peptide lengths in the fraction of fermented skimmed cow milk included 7-19 AA residues (averaged length of 12 residues). In the fraction of soy milk, longer peptide lengths of 9–24 AA residues (averaged 14 residues) were detected. Therefore, the average molecular weight (1554.1 Da) of peptides in the fermented soy milk fraction was higher than that (1331.5 Da) of the fermented skimmed cow milk fraction.

All identified peptides of the <3 kDa fraction of the fermented skimmed cow and soy milks included antioxidant activities (Tables 5 and 6).

Table 4. Antioxidant activities and total peptides of the fermented milk (by *L. rhamnosus* BD2 for 48 h) after fractionation based on the molecular sizes

Substrates*	DPPH inhibition activity (%)			Antioxidant capacitiy (μg AAE.ml^{-1})			Total peptides ($mg.ml^{-1}$)		
	>10 kDa	3-10 kDa	<3 kDa	>10 kDa	3-10 kDa	<3 kDa	>10 kDa	3-10 kDa	<3 kDa
Skimmed cow milk	43.06± 2.99^{C}	61.29± 2.03^{B}	73.68± 2.29^{A}	21.23± 3.37^{C}	41.71± 2.28^{B}	55.63± 2.57^{A}	2.20± 0.08^{B}	2.46± 0.63^{B}	3.02± 0.03^{A}
Soy milk	50.94± 0.89^{C}	69.35± 0.74^{B}	81.1± 1.04^{A}	30.08± 1.01^{C}	50.77± 0.83^{B}	64.00± 1.17^{A}	1.58± 0.21^{B}	1.65± 0.08^{B}	2.14± 0.11^{A}

*The results were obtained from three repetitions ± standard deviation. Different superscripts in one row (A-C) showed significant differences ($p \leq 0.05$) using the Duncan's test.

Table 5. Antioxidant peptides present in < 3 kDa fraction of the fermented skimmed cow milk, analyzed using HR-LC-MS/MS and *in silico* antioxidant activity prediction (Biopep-UWM)

Retention time (min)	Accession no.	Parent protein	Identified Peptides*	Molecular weight (Da)	Relative area (%)
16.60	P80195	Glycosylation-dependent cell adhesion molecule	RSSRQPQSQNPKLP	1621.8607	0.10
17.21	P02666	*Beta*-casein	HQPHQPLPPT	1150.5860	0.10
17.24	P02663	*Alpha*-S2-casein	FLKKISQRYQK	1437.8418	1.76
17.51	A0A140T8A9	*Kappa*-casein	FMAIPPKKNQDK	1415.7534	1.76
17.52	P80195	Glycosylation-dependent cell adhesion molecule	SRQPQSQNPKLP	1378.7283	1.16
18.15	A0A140T8A9	*Kappa*-casein	FMAIPPKKNQDKTE	1645.8471	1.16
18.36	J9UHS4	*Beta*-casein	GPIHNSLPQN	1075.5383	0.51
19.02	P02453	Collagen *alpha*-1(I)	KTGPPGPAGQDGRPGPP	1584.8099	2.85
19.48	P80195	Glycosylation-dependent cell adhesion molecule	ILNKPEDETHL	1307.6707	0.60
19.77	P02666	*Beta*-casein	QQQTEDELQDKIHP	1707.8056	1.09
20.18	E1BE96	*Gamma*-aminobutyric acid	SKVLTRAPILQSTPVTP	1807.0505	2.24
20.19	P02666	*Beta*-casein	SKVLPVPQKAVP	1261.7740	2.16
20.40	A0A140T8A9	*Kappa*-casein	FMAIPPKKNQD	1287.6626	2.16
20.41	P02666	*Beta*-casein	SKVLPVPQKAVPYPQRD	1921.0786	3.70
20.72	P02666	*Beta*-casein	WMHQPHQPLPPT	1467.7067	3.70
21.12	P02663	*Alpha*-S2-casein	FYQKFPQ	956.4737	15.35
21.23	A0A140T8A9	*Kappa*-casein	MAIPPKKNQDKTEIPTINT	2138.1312	3.93
21.66	P02666	*Beta*-casein	SWMHQPHQPLPPT	1554.378	1.80
21.66	P02662	*Alpha*-S1-casein	YKVPQLE	875.4740	2.90
22.90	A5D7Q2	Uncharacterized protein	FTWNPTGGKTA	1178.5703	2.90
22.96	P02666	*Beta*-casein	GPIPNSLPQN	1035.5328	1.20
22.97	A0A140T8A9	*Kappa*-casein	IAKYIPIQ	944.5670	1.08
23.51	A0A140T8A9	*Kappa*-casein	WQVLSNTVPAKS	1328.7046	1.08
23.51	A0A140T8A9	*Kappa*-casein	WQVLSNTVPAK	1241.6740	5.02
24.34	P02666	*Beta*-casein	QNIPPLTQTP	1107.5908	5.02
25.78	P02662	*Alpha*-S1-casein	VAPFPEVFGKEKV	1445.7905	1.03
26.29	P02666	*Beta*-casein	MPFPKYPVEP	1203.5953	4.96
26.55	P02666	*Beta*-casein	VLGPVRGPFP	1037.6016	3.58
27.16	F1N0C3	Transmembrane protein 201	FAPGAPLPPTL	1079.6003	2.22
27.50	J9UHS4	*Beta*-casein	LVYPFPGPIHN	1252.6555	4.65
28.25	J9UHS4	*Beta*-casein	SLVYPFPGPIHN	1339.6915	1.92
28.68	J9UHS4	*Beta*-casein	LVYPFPGPIHNSLPQN	1791.9278	5.78
28.93	P02666	*Beta*-casein	FPGPIPNSLPQN	1279.6529	1.21
29.55	F1MJP2	Ring finger protein 165	PLPTLQF	814.4583	1.19
30.34	P02663	*Alpha*-S2-casein	GPIVLNPWDQ	1137.5803	0.96
31.46	P02663	*Alpha*-S2-casein	GPIVLNPW	894.4951	2.24
32.24	G3N1T8	Potassium voltage-gated channel subfamily A member 6	FRADGRGGSNGGGVSPGSR	1789.8715	0.6
33.05	P02666	*Beta*-casein	VVVPPFLQP	994.5830	0.12
34.24	P02666	*Beta*-casein	VVVPPFLQPEVMG	1410.7573	3.34
34.71	P02666	*Beta*-casein	VVVPPFLQPEVM	1353.7354	0.37

All identified peptides are predicted to have antioxidant activity by considering the peptide sequences matched with 2 to 10 residual amino acid sequences of antioxidant peptides in Biopep-UWM (www.uwm.edu.pl/biochemia/index.php/pl/biopep) for the HR-LC-MS/MS results from fermented skimmed cow milk fraction.

Predicted activity of the peptides was verified by considering the peptide sequences matched with AA sequences of the antioxidant peptides using Biopep-UWM. For HR-LC-MS/MS results from the fermented skimmed cow milk fraction, sequences of the identified peptides were matched with 2–10 AA sequences of the antioxidant peptides listed in Biopep-UWM (Table 5). Moreover, 2-5 AA sequences of the antioxidant peptides were matched for the results of the fermented soy milk fraction. Nearly 70% of the predicted antioxidant peptides from the skimmed cow milk source were formed from beta, alpha and cappa-caseins, whereas almost 80% of the predicted antioxidant peptides from the soy milk source were formed from uncharacterized proteins, dehydrin, oleosin and maturation proteins.

In this study, IPP and VPP, quantified as antihypertensive and antioxidative tripeptides in cow milk after fermentation by lactobacilli [49], were in the sequence of the identified peptides from the fraction of the fermented cow milk, including FMAIPPKKNQDK from kappa-casein and QNIPPLTQTP and VVVPPFLQP from beta-casein.

Table 6. Antioxidant peptides present in < 3 kDa fraction of the fermented soy milk, analyzed using HR-LC-MS/MS and *in silico* antioxidant activity prediction (Biopep-UWM)

Retention time (min)	Accession no.	Parent protein	Identified Peptides*	Molecular weight (Da)	Relative area (%)
14.90	Q9SB11	Glycinin	DEDEDEDEDKPRPSRPSQGK	2328.0052	0.00
15.28	I1N747	Oleosin	VQVHTTTHRYE	1369.6711	9.09
15.51	K7LEQ5	Uncharacterized protein	IDTDRQQHGTTGGYAGDTGRQHGN	2541.1288	4.17
15.80	Q70EM0	Dehydrin	IDTDRQQHGTTGGYAGDTGRQHG	2427.0980	4.35
16.21	B3TDK6	Lipoxygenase	NDLGDPDKGENHARP	1633.7429	0.00
16.55	Q70EM0	Dehydrin	IDTDRQQHGTTGGYAG	1675.7533	6.25
16.89	Q01527	Maturation protein	AMPGHGTGQPTGHVTEG	1632.7320	0.00
16.90	C6SVX2	Uncharacterized protein	VGGTTGDPMHKPAT	1367.6496	0.00
17.46	A0A0R0KKD6	Uncharacterized protein	VIKPPTDEQQQRPQ	1662.8674	0.00
17.47	A1KR24	Dehydrin	DIGRDHGTTG	1027.4676	0.00
18.10	P05046	Lectin	TFYAPDTKR	1097.5492	22.22
18.11	I1NH22	Uncharacterized protein	TTVMETSSGEAVAAH	1489.6721	0.00
18.12	A0A0R0KKD6	Uncharacterized protein	VIKPPTDEQQQRPQEE	1920.9500	0.00
18.55	Q9XET0	Seed maturation protein PM30	LGLGEHDQDNRRNY	1685.7844	7.14
18.56	K7KTR9	Oleosin	DQPRGSYSY	1071.4605	22.22
18.72	Q70EM0	Dehydrin	GNVEKQTDEYGNPVHA	1756.7985	6.25
18.73	F8WQS0	*Beta*-conglycinin *beta* subunit	FRSSNSFQT	1072.4917	22.22
18.75	K7LDT9	Uncharacterized protein	ATKTQYPLPGSHDQ	1541.7457	7.14
19.53	Q9SWB2	Seed maturation protein PM41	VHDPAGKGGPVFG	1236.6237	7.69
19.93	Q39898	Kunitz trypsin inhibitor	VSDDEFNNYK	1229.5179	20.00
19.94	Q9SWB2	Seed maturation protein PM41	VHDPAGKGGPVFGA	1307.6666	7.14
19.96	K7KTR9	Oleosin	ISTDQPRGSYSY	1372.6230	16.67
21.10	P0DO16	*Beta*-conglycinin *alpha*	DERQFPFPRPPHQKEE	2035.9859	12.50
21.68	D6PAW5	Lipoxygenase	LPEKGTPEYEEM	1421.6345	8.33
24.46	A0A0R0KVB4	Uncharacterized protein	TQQTGLGEL	945.4754	0.00

* All identified peptides are predicted to have antioxidant activity by considering the peptide sequences matched with 2 to 5 residual amino acid sequences of antioxidant peptides in Biopep-UWM (www.uwm.edu.pl/biochemia/index.php/pl/biopep) for the HR-LC-MS/MS results from fermented soy milk fraction

However, the highest abundance peptide in the fraction of fermented cow milk (based on the percentage of the relative area) was WMHQPHQPLPPT. Longer peptides in the fraction of the fermented soy milk compared to those in the fermented cow milk was previously seen in the soy milk fermented by *L. plantarum* [37]. Different peptides from the fraction of the fermented soy milk were reported in the present study. The highest abundant peptides from the fraction of the fermented soy milk were GNVEKQTDEYGNPVHA, FRSSNSFQT and ATKT-QYPLPGSHDQ. In general, the whole peptide sequence in the low molecular fractions of the fermented foods reported in the present study are important for the advancement of functional foods that are processed on animal and vegetable milk proteins by the LAB fermentation.

4. Conclusion

Three LAB isolates from kefir granules of *L. kefiri* YK4, *L. rhamnosus* BD2 and *L. kefiri* JK17 could ferment skimmed cow and soy milks and increase the antioxidant activity. The three isolates produced similar antioxidant activities in fermented skimmed cow milk. However, *L. rhamnosus* BD2 produced significantly a higher antioxidant value in fermented soy milk than those the other two isolates did. The highest antioxidant activity of fermented milk (using *L. rhamnosus* BD2) was achieved for<3 kDa fractions. The antioxidant activity of < 3 kDa fraction was higher in fermented soy milk than fermented skimmed cow milk; however, the peptide content was lower. It can be concluded that low molecular peptides and other compounds (isoflavones in soy milk) play significant roles as antioxidants. Peptides with the predicted antioxidant activity were present in the two<3 kDa fractions. All identified peptides generated from proteins in skimmed cow and soy milks were predicted to include antioxidant activities. Number of the predicted antioxidant peptides in fermented skimmed cow milk fraction was higher than that in fermented soy milk fraction. Peptide identification and averaged length of the sequences were different between the fermented skimmed cow milk and fermented soy milk. Findings of this study can be further verified, needing studies on antioxidant bioactive peptides.

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6. Conflict of Interest

The authors report no conflicts of interest.

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فعالیت ضداکسایشی شیرهای بدون چربی گاو و سویای تخمیر شده توسط جدایه لاکتیک اسید دانه های کفیر

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- شیر تخمیر شده
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- *لنتی‌لاکتو باسیلوس کفیری*
- پپتیدها
- کشت‌های آغازگر

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چکیده

سابقه و هدف: سیستم پروتئولیتیک باکتری‌های لاکتیک اسید ، پروتئین شیر را به چندین پپتید، از جمله آنهایی که فعالیت ضداکسایشی دارند، هیدرولیز می‌کند. هدف از این مطالعه ارزیابی فعالیت‌های ضداکسایشی در شیر گاو و سویا تخمیر شده توسط *لاکتی‌کازیی باسیلوس رامنوسوس* BD2، *لنتی‌لاکتو باسیلوس کفیری* YK4 و *لنتی‌لاکتو باسیلوس کفیری* JK17 ، جداشده از دانه های کفیر و همبستگی آنها با محتوای پپتید بود.

مواد و روش ها: شیرهای گاو بدون چربی و سویای بازسازی شده توسط جدایه‌های مورد اشاره در دمای ۳۷ درجه سلسیوس به مدت ۰، ۲۴، ۴۸ و ۷۲ ساعت تخمیر شدند. در فرآورده‌های تخمیر، pH، اسیدیته قابل تیتراسیون کل، فعالیت ضداکسایشی (درصد مهار رادیکال ۲،۲-دی فنیل-۱-پیکریل هیدرازیل و ظرفیت ضداکسایشی براساس میکروگرم $AAE.ml^{-1}$ آب پنیر) و پپتیدهای کل اندازه‌گیری شد. سپس شیرهای تخمیر شده بدون چربی گاو و سویا دارای بالاترین فعالیت ضداکسایشی با استفاده از فیلترهای مولکولی ۱۰ و ۳ کیلو دالتون، تفکیک نسبی شدند. بخش‌های با بالاترین فعالیت، بعدا برای شناسایی پپتید مورد آنالیز قرار گرفتند. تجزیه و تحلیل آماری با استفاده از آزمون تحلیل واریانس یک طرفه و آزمون چند دامنه ای دانکن ($p\geq$۰/۰۵) با استفاده از نرم افزار SPSS v.16.0 انجام شد.

یافته‌ها و نتیجه‌گیری: همه جدایه‌ها قادر به رشد در شیرهای گاو بدون چربی و سویای بازسازی شده بودند، در حالی‌که تعداد کل میکروارگانیسم‌ها به ۹ $\log CFU.ml^{-1}$ افزایش معنی‌داری ($p \geq$ ۰/۰۵) داشت، افزایش اسیدیته قابل تیتراسیون و پپتید کل و کاهش pH مشاهده شد. رشد سه جدایه در شیر سویا نسبت به شیر گاو بدون چربی کمی آهسته‌تر بود. بیشینه فعالیت ضداکسایشی پس از ۷۲ ساعت تخمیر شیر گاو و سویا مشاهده شد. هیچ تفاوتی در فعالیت ضداکسایشی شیر گاو تخمیر شده توسط سه جدایه مشاهده نشد. با این حال، *لاکتی‌کازیی باسیلوس رامنوسوس* BD2 بالاترین ضداکسایشی را در شیر سویا موجب شد. به‌طورکلی، افزایش فعالیت‌های ضداکسایشی با افزایش محتوای پپتید ارتباط دارد. بخشی با وزن مولکولی کمتر از ۳ کیلو دالتون در دو شیر تخمیر شده توسط *لاکتی‌کازیی باسیلوس رامنوسوس* BD2، بیشترین فعالیت ضداکسایشی را نشان داد. آنالیز پپتیدهای موجود در این بخش‌ها با استفاده از LC-MS/MS با وضوح بالا و شناسایی پپتیدهای دارای فعالیت ضداکسایشی *درون تراشه‌ای*[۱] در این مطالعه گزارش شده است. مطالعه حاضر پیشنهاد می‌کند که این سه جدایه می‌توانند به عنوان کشت آغازین در تخمیر شیر گاو و سویا برای افزایش فعالیت ضداکسایشی آن‌ها استفاده شوند. پپتیدهای با وزن مولکولی کمتر از ۳ کیلو دالتون نقش کلیدی در فعالیت آنتی اکسیدانی دارند.

تعارض منافع: نویسندگان اعلام می‌کنند که هیچ نوع تعارض منافعی مرتبط با انتشار این مقاله ندارند.

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