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Development of Antioxidant Activity during Milk Fermentation by Wild Isolates of *Lactobacillus helveticus*

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Abstract

Background and Objective: Oxidative stress, due to free radicals, brings injury to the body by attacking large molecules and cell organs, and is the main reason of many diseases. Fermentation of foods containing large amount of proteins such as milk by special species of lactic acid bacteria is a potential way in enhancement of the antioxidative activity of foods. This study aimed at evaluating non-common starter species isolates of *Lactobacillus helveticus* for their capability to produce fermented milk enriched in antioxidant peptides.

Materials and Methods: Reconstituted skim milk (11%) was inoculated with 7 wild isolates of *Lactobacillus helveticus*, and after 24 h fermentation at 37ºC, the samples were kept 4ºC and for 14 days. Viable cell number, acidification and proteolysis degree in the milk fermented by each isolate were assessed in 1, 7 and 14 days. Development of antioxidant activity was measured using DPPH and $ABTS^{\bullet+}$ radical scavenging activities during the storage period.

Results and Conclusion: Though some slight strain-dependent differences were observed in growth, acidification and proteolysis, all the samples showed considerably strong antioxidant activity (at least 62.32±3.66% and 57.64±1.42% measured using DPPH and $ABTS^{\bullet+}$ radicals, respectively) through the whole storage period. In vitro simulated gastrointestinal digestion indicated that DPPH radical-scavenging activity of the antioxidative peptidic supernatants was not affected significantly by consecutive pepsin-pancreatin hydrolysis in most of the samples. These evidences support *Lactobacillus helveticus* as a promising functional culture able to promote health benefits in dairy-based functional foods.

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1. Introduction

Oxidative metabolism is a natural event in living bodies. Reactive oxygen species (ROS) comprise some oxygen containing molecules such as hydrogen peroxide (H_2O_2), superoxide ($O_2^{\bullet-}$), singlet oxygen ($^1O_2$), and hydroxyl radical ($^{\bullet}OH$) [1]. However, ROS can cause destructive and lethal cellular effects by oxidizing membrane lipids, cellular proteins, DNA and enzymes, resulting to numerous disorders such as diabetes and cancers for imbalance between the generations of oxygen derived radicals and the organism's antioxidant potential [2,3]. Antioxidants may function by preventing the formation of radicals or by scavenging of radicals, hydrogen peroxide and other peroxides [4]. Because of the increasing concerns regarding to consumption of artificial antioxidants, discovering and development of natural antioxidant components have found great attention recently. Fermentation of food materials is an attractive way in production of high value added products. Milk, due to its special diverse proteins, could be a unique substrate for releasing of bioactive peptides through fermentation

proceeds by lactic acid bacteria (LAB). Bioactive compounds are released during milk fermentation by proteolytic cleavage of milk proteins have some roles beyond their nutritional importance, such as immune-modulation, anticancer, antibacterial, anti-hypertensive and antioxidant activities [5]. Several peptide sequences with antioxidant activity from milk proteins, mostly from β- and κ-casein, have been discovered in recent years [6,7]. Different strains of LAB (like *Leuconostoc mesenteroides* ssp. *cremoris* strains, *Lactobacillus* (*L.*) *jensenii* (ATCC 25258), *L. acidophilus* (ATCC 4356) [8], *L. helveticus* spp. [5] and *L. casei* PRA205 [9] have been applied in milk fermentation in order to release antioxidant peptides. Proteolytic enzymes in selected bacteria seem to be the most important factor in releasing antioxidant peptides; this ability is very strain dependent. *L. helveticus* is known to produce abundant intracellular proteolytic enzymes, including cell-envelope proteinases, endopeptidases, aminopeptidases, and X-prolyl dipeptidyl aminopeptidase (PepX). Taking advantage of the powerful proteolytic system of this bacterium opens up opportunities to search for novel food-derived compounds with potential health promoting properties [10]. One strain of this species is very well-known for its cooperation in production of potent antihypertensive peptides (Val-Pro-Pro and Ile-Pro-Pro) [11].

Elfahri et al. reported that milk fermented by *L. helveticus* strains can release bioactive compounds with important multifunctional properties, and the characteristics and activities of these compounds appear to be highly strain- and fermentation time-dependent [5]. This study assessed the ability of 7 wild isolates of *L. helveticus*, previously isolated from traditional dairy products in Iran, in releasing bioactive compounds capable of exerting anti-oxidative activity, during fermentation of reconstituted skim milk and the refrigeration time.

2. Materials and Methods

2.1. Cultures and materials

In our previous work [12], a number of predominant colonies (named as DY1, DY2, FK11, FMK1, BY24, HY21, VY22, FM11 and SY14) were isolated from different home-made fermented dairy products (yogurt and butter samples in Chahar-Mahale Bakhtiyari Province, Iran), all of which were identified as *L. helveticus*. All of these isolates (except DY2, which was supposed to be identical to DY1 and then eliminated from the current study) were applied in the current study in order to investigate their potential in development of antioxidant activity through milk fermentation. All the chemicals (O-phthaldialdehyde, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2, 2'-Azinobis 3-ethylbenzothiazoline-6-sulfonic acid (ABTS), pepsin (P7000), ox-gall (70168) and pancreatin (P3292) were purchased from Sigma (St Louis, USA). MRS broth and bacteriological agar were from obtained Biolife Laboratories (Milan, Italy). A commercial *L. helveticus* culture, LH-BO2 (Chr-Hansen, Hørsholm, Denmark), was included in experiments as control. In addition, a commercial yogurt sample (Falat Koohrang Dairy Co. Chahar-Mahalo Bakhtiyari, Iran) was included in the proteolysis and antioxidant activity assays.

2.2. Fermentation of milk

The *L. helveticus* strains were first activated in MRS broth at 37°C overnight, followed by a second passage (1% v v^{-1} inoculation) in sterilized reconstituted skim milk (RSM) at 37°C overnight. The RSM (11%) was prepared by suspending appropriate amount of skim milk powder (Rama Mehr Arya, Qom, Iran) in distilled water and sterilization at 110°C for 10 min. The cultures activated in RSM were used as starter cultures in the fermentation experiment. Each starter culture was inoculated (2% v v^{-1}) to sterilized RSM, and fermentation was completed by incubation of the inoculated samples for 24 h at 37°C. Then the fermented milk was transferred to the refrigerator (4°C) for a period of 14 days.

All fermentations were performed in triplicate. Aliquots of each fermented milk were taken immediately after 24 h of fermentation (day 1) and during refrigeration (in days 7 and 14) in order to determine the cells' viability, pH, proteolysis degree and antioxidant activity. Viable cell count was performed by pour-plating of appropriate dilutions in MRS agar and incubation at 42°C for 24-48 h under anaerobic conditions. For evaluation of proteolysis degree and antioxidant activity, it was necessary to remove non-hydrolyzed casein. For this propose, the aliquots were centrifuged (13000 ×g, 15 min at 5°C), and then the supernatant was filtered on a 0.45 μm filter [13]. The filtrates were then stored at -20°C until further use.

2.3. Determination of proteolysis degree

Proteolysis degree is defined as the percentage of peptide bonds that have been cleaved through fermentation. This analysis was done using O-phthaldialdehyde (OPA) reagent [14]. 20 μl of sample, blank (deionized water) and standard (peptone at concentration of 0.25-2 mg ml^{-1} in deionized water) was mixed with 1000 μl of OPA reagent, and after 2 min (room temperature), the absorbance was measured at 340 nm using spectrophotometer (PerkinElmer Lambda Bio UV/VIS Spectrometer; Perkin-Elmer, Norwalk, CT, USA). The samples were diluted with water, if necessary, to give a final absorbance between 0.3 and 1.2.

2.4. Determination of antioxidant activity

2.4.1. DPPH radical-scavenging activity assay

The scavenging activity of DPPH radical was measured according to the method of Wu and Chen (15) with certain modification. The samples were

diluted 1:4 with phosphate buffer (0.1 M), and 1 ml of the dilutions was added to 2.5 ml of 0.1 mM DPPH (in 60% methanol) and mixed vigorously. After standing in darkness at room temperature for 30 min, the absorbance was measured at 517 nm. The scavenging activity was calculated using the following equation:

$$\text{Inhibition (\%)} = [(A_{control}-A_{test}) / A_{control}] \times 100 \quad \text{Eq.1}$$

Where, $A_{control}$ is the absorbance of the control sample (1 ml phosphate buffer mixed with 2.5 ml DPPH solution), and A_{test} is the absorbance of the test sample.

2.4.2. $ABTS^{\bullet+}$ radical scavenging activity assay

$ABTS^{\bullet+}$ radical scavenging activity was assayed by a decolorization method as described by Ao and Li (16) with slight modification. In this assay, ABTS is oxidized to form the colored $ABTS^{\bullet+}$ radical, which is again reduced to a colorless compound if the sample of interest contains hydrogen donating antioxidants. Equal quantities of $ABTS^{\bullet+}$ stock solution (14 mM) and potassium persulphate (4.88 mM) were mixed, and then were allowed to react for 12-16 h at ambient temperature in the dark. This solution was diluted with 0.1 M phosphate buffer (pH 7.4) to reach the final absorbance of 0.70±0.05 at 734 nm as working solution. Ten microliter of sample or blank (phosphate buffer) was added to 200 µl of working $ABTS^{\bullet+}$ radical solution. Then it was shaken for 10 s at 25°C, and after 5 min, the absorbance was measured at 734 nm. The scavenging activity was calculated using the following equation:

$$\text{Inhibition (\%)} = [(A_{ABTS\ solution}-A_{test}) / A_{ABTS\ solution}] \times 100 \quad \text{Eq.2}$$

Where, $A_{ABTS\ solution}$ is the final absorbance of the $ABTS^{\bullet+}$ radical solution (0.70±0.05), and A_{test} is the absorbance of test sample.

2.5. In vitro simulated gastrointestinal digestion

To characterize the resistance of antioxidant materials in the fermented milks to simulated gastrointestinal digestion, the samples were digested sequentially with pepsin-pancreatin solutions [17]. The pH of fermented milks' supernatant was adjusted to 2.5 with 1 M HCl. One milliliter of the pH-adjusted sample was mixed with 1 ml of pepsin solution (0.3 g l^{-1}, in a solution of NaCl 125 mM, KCl 7 mM and $NaHCO_3$ 45 mM, pH 2.5). Then the mixture was incubated in water bath (37°C) for 1 h. Immediately, the pH of the solution was adjusted to 5.3 with $NaHCO_3$ solution (0.9 Mml) and further to pH 7.5 with 1 M NaOH. Sampling (1 ml) was carried out to measure antioxidant activity after acidic-pepsin digestion. In the next step, 1 ml of 0.4 g l^{-1} pancreatin (in 45 mM NaCl, pH 8.0) solution was added to 1 ml of acidic-pepsin digested solution, and further incubated in water bath (37°C) for 3 h. In order to inactivate pancreatin, the solutions were submerged in boiling water for 10 min. The digests were cooled down to room temperature and centrifuged at 11000 ×g for 15 min. The enzyme-digested samples were then assayed using DPPH radicals for antioxidant activity as described above.

2.6. Statistical analysis

GraphPad Prism, version 6.0 (GraphPad Software, La Jolla, Canada) was used for data analysis. The results were expressed as the mean and standard error. The data were subjected to analysis of variance (ANOVA) and the differences between means were evaluated by Tukey's test. Data were considered significantly different when the p-values were less than 0.05. All fermentation and analytical experiments were performed in triplicate.

3. Results and Discussion

Among the LAB, the species *L. helveticus* has the highest extracellular proteinase activity and thus the highest ability to release bioactive peptides to the fermented milk medium [5,10]. The principal aim of our study was to assess 7 wild isolates of *L. helveticus* to enrich milk with antioxidant peptides through fermentation.

3.1 Viability and acidification

The rates of growth and pH reduction are parameters indicative of the fermentative performance of a microbial culture and of the effectiveness of its proteolytic and glycolytic systems to sustain the microbial growth [18]. Many strains of *L. helveticus* have been extensively used in dairy studies due to their rapid growth in milk, ability to grow under acid stress, and high proteolytic activity [5].

Table 1 shows bacterial plate counts (log CFU ml^{-1}) and acidification after 24 h of fermentation at 37ºC (day 1) and after 7 and 14 days of storage at 4ºC. The count number of all isolates (except isolate FK11) and control culture LH-BO2 reached the higher than 8.00 log CFU ml^{-1}, and then decreased in a different way. The FMK1, FK11, DY1 and SY41 isolates showed about 2 log CFU ml^{-1} reduction during the refrigeration; however, in the milk fermented by SY41, bacterial reduction was significantly higher during 14 days of storage (Table 1). The reduction of viable cells in other fermented milks was less (ca. 0.8, 1 and 1.5 log CFU ml^{-1} for HY21, FM11 and VY22, respectively). The control culture (LH-BO2) presented no significant reduction in viable cell number during the refrigeration ($p \geq 0.05$). After 14 days of fermentation (storage at 4ºC), SY14 showed the lowest viable cell number followed by DY1, FK11, FMK1, VY22 and HY12, which were significantly lower than the commercial

starter culture (LH-BO2). Among the isolates, FM11 had the highest viability, which was not significantly different from LH-BO2 ($p \geq 0.05$) (Table 1). In all samples, pH reached below 4.0 after 24 h of fermentation at 37°C (day 1), except HY21, which had the highest pH (4.32±0.18). All samples showed a slight reduction until the end of storage time at 4°C. Totally, all isolates were able to reduce the pH of milk efficiently and retain viability in high amount through fermentation; however, slight differences were recognizable. Chen et al. analyzed 59 strains of *L. helveticus* through milk fermentation, and recognized a range of pH 3.35-5.22 after 24 h of fermentation, which is accordance with our results [13].

3.2. Degree of proteolysis

Evaluation of proteolysis degree is a key parameter for monitoring the peptides releasing through fermentation. In fermentation, milk proteins are hydrolyzed by extracellular proteinases of lactobacilli, and therefore, resulting in an increase in the amount of NH_3 groups, which can be quantified by the OPA reagent [19]. Table 2 shows the peptide production (mg ml^{-1} peptone Eq) in the milk fermented using the isolates and the commercial culture of LH-BO2 after fermentation (day 1) and during the refrigeration (days 7 and 14). In order to have better comparison, commercial yogurt sample was included in the measurements. After fermentation, the lowest amount of proteolysis degree was for the milk fermented by FM11 (0.93±0.05 mg ml^{-1} peptone Eq), while in other samples, proteolysis degree reached to more than 1 mg ml^{-1}. According to the results given in Table 2 and considering proteolysis behavior, it is possible to divide the isolates into two groups. Group 1 (isolates FMK1, VY22 and DY1) increased proteolysis during storage time continuously; however, it was significant ($p \leq 0.05$) just for isolate VY22. In group 2 (remaining isolates and commercial starter culture LH-BO2), the samples reached the maximum of proteolysis at day 7, and then they showed reduction. The maximum proteolysis degree was detected for isolates HY12 and FK11 at day 7 with the values 2.13±0.24 and 2.11±0.07 mg ml^{-1} peptone Eq, respectively. Among all the fermented samples, commercial yogurt showed significantly the lowest proteolysis during the storage time ($p \leq 0.05$).

It is well known that strains of *L. helveticus* possess one of the most complex proteolytic systems among LAB [10]. Li et al. reported the highest amount of peptide releasing activity for *L. helveticus* (1.6 mg ml^{-1} peptone Eq after 48 h of milk fermentation) followed by *L. delbrueckii* spp. *bulgaricus* and *L. lactis* [20]. These results are similar to those reported by Leclerc et al. In addition, these authors reported the strain-dependent proteolytic activity within these species [19].

Proteolysis activity depends, firstly, on the proteinases of bacteria. In almost all strains of Lactic acid bacteria, there is a cell-envelope proteinase (CEP), located in the wall of the cell, and is responsible for breaking down the proteins out of the cell and releasing of peptides. However, some studies have reported the presence of more than one CEP in most of the studied *L. helveticus* strains [21], which make this species a powerful proteolytic starter among LAB. Broadbent et al. reported a high intraspecific diversity of genes encoding CEP among 51 strains of *L. helveticus*. In our previous study [12], the presence of 2 CEP genes, prtH and prtH2, among 8 isolates of *L. helveticus* (studied in the current research) has been assessed. Accordingly, all isolates possess gene encoding PrtH2 proteinase, but the presence of gene encoding PrtH proteinase was variable; it was just detected for isolates DY1, HY21, SY14 and VY22. It can explain somehow the different proteolysis degree observed here. In the current study, HY21 and FM11 showed the highest and lowest proteolysis degrees, respectively, which is probably explainable by the presence of higher number of cell wall proteinases. In addition, isolate HY21 showed one of the highest cell viabilities (7.16±0.18 log CFU ml^{-1}) among all the isolates, which along with possessing two CEPs it can possibly explain the high proteolysis degree of this isolate. However, the exact explanation of differences in proteolytic activity is not easily possible as *L. helveticus* relies on a complex proteolytic system to obtain all necessary free amino acids.

3.3. Development of antioxidant activity during fermentation

This article describes the scavenging of stable radical 1,1-diphenyl-2-picryl-hydrazyl free radical (DPPH) and 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) cation radical ($ABTS^{\bullet+}$) to evaluate the radical scavenging properties of fermented milk samples. DPPH radical is commercially available, and $ABTS^{\bullet+}$ radical can be generated freshly before the assay by oxidizing the neutral molecules with potassium persulphate [22]. DPPH free radical scavenging assay is widely common to investigate the antioxidant capacity of natural compounds [22]. Antioxidant substances can act as a quencher of DPPH radicals by providing hydrogen atoms or by electron donation and reducing the stable radical DPPH to the yellow-coloured non-radical diphenyl-picrylhydrazine (DPPH-H) [23]. $ABTS^{\bullet+}$ radicals are more reactive than DPPH radicals, and the reactions with $ABTS^{\bullet+}$ radicals involve a single-electron transfer process [22]. Depending to several factors, such as food matrix composition, overall peptidic profile of food, and amino acid composition of individual peptides, it would be possible to gain different results from the DPPH and ABTS assays.

Table 1. Growth and acidification of milk samples fermented by 7 isolates of *L. helveticus* and a commercial culture of *L. helveticus* LH-BO2 after 24 h of incubation at 37ºC (day 1) and 14 days storage at 4ºC

	Viable cell number (log CFU ml^{-1})			pH		
Isolate	1	7	14	1	7	14
FMK1	8.64±0.29 ^{aA}	7.88±0.16 ^{aBC}	6.60±0.43 ^{bAB}	3.75±0.12 ^{aA}	3.55±0.06 ^{bAB}	3.37±0.11 ^{cA}
FM11	8.16±0.19 ^{aA}	7.87±0.07 ^{abBC}	7.23±0.16 ^{bBC}	3.75±0.12 ^{aA}	3.47±0.15 ^{bAB}	3.37±0.04 ^{bA}
VY22	8.22±0.29 ^{aA}	7.96±0.14 ^{aC}	6.77±0.28 ^{bAB}	3.62±0.09 ^{aA}	3.47±0.06 ^{abAB}	3.42±0.04 ^{bA}
HY12	8.00±0.18 ^{aA}	7.55±0.06 ^{abABC}	7.16±0.18 ^{bAB}	4.32±0.18 ^{aB}	3.95±0.14 ^{bC}	3.90±0.12 ^{bC}
FK11	8.00±0.18 ^{aA}	6.82±0.43 ^{bA}	6.51±.163 ^{bAB}	3.72±0.09 ^{aA}	3.52±0.06 ^{bAB}	3.45±0.06 ^{bAB}
DY1	8.20±0.09 ^{aA}	7.61±0.25 ^{aABC}	6.25±0.45 ^{bAB}	3.62±0.09 ^{aA}	3.42±0.04 ^{bA}	3.40±0.05 ^{bA}
SY41	8.14±0.40 ^{aA}	6.90±0.36 ^{bAB}	6.01±0.34 ^{cA}	3.50±0.09 ^{aA}	3.40±0.05 ^{bA}	3.45±0.06 ^{bAB}
LH-BO2	8.13±0.06 ^{aA}	8.05±0.08 ^{aC}	7.84±0.08 ^{aC}	3.67±0.05 ^{aA}	3.62±0.05 ^{aB}	3.62±0.04 ^{aB}

* Different small letters in the same raw within the same isolate indicate significant differences ($p \leq 0.05$).
** Different capital letters in the same column within the same time indicate significant differences ($p \leq 0.05$).
*** Mean±SE (n=3)

Table 2. Degree of proteolysis (mg ml^{-1} peptone Eq) of milk samples fermented by 7 isolates of *L. helveticus* and a commercial culture of *L. helveticus* LH-BO2 after 24 h of incubation at 37ºC (day 1) and 14 days storage at 4ºC. Commercially produced yogurt sample was used as control

	Peptide concentration (mg ml^{-1} peptone Eq) during the storage time (days)		
Isolates	1	7	14
Commercial yogurt	0.31±0.01 ^{aA}	0.40±0.05 ^{aA}	0.45±0.06 ^{aA}
FMK1	1.25±0.14 ^{aB}	1.39±0.15 ^{aB}	1.66±0.13 ^{aB}
FM11	0.93±0.05 ^{aB}	1.29±0.20 ^{aB}	0.84±0.12 ^{aA}
VY22	1.22±0.12 ^{aB}	1.50±0.24 ^{abBC}	1.95±0.05 ^{bB}
HY12.	1.51±0.24 ^{aB}	2.13±0.24 ^{bC}	1.45±0.05 ^{aB}
FK11	1.35±0.22 ^{aB}	2.11±0.07 ^{bC}	1.59±0.16 ^{abB}
DY1	1.23±0.14 ^{aB}	1.31±0.08 ^{aB}	1.61±0.08 ^{aB}
SY41	1.25±0.22 ^{aB}	1.73±0.35 ^{aBC}	1.53±0.24 ^{aB}
LH-BO2	1.26±0.12 ^{aB}	1.55±0.07 ^{aBC}	1.41±0.14 ^{aB}

* Different small letters in the same raw within the same isolate indicate significant differences ($p \leq 0.05$).
** Different capital letters in the same column within the same time indicate significant differences ($p \leq 0.05$).
*** Mean±SE (n=3)

DPPH assay is more efficient when the peptidic system is more hydrophobic, however, ABTS assay is a better choice for assessing the antioxidant activity of highly pigmented or phenol-rich foodstuffs (e.g. fruits and vegetables) [24]. DPPH and $ABTS^{\bullet+}$ radical scavenging activities of the milk supernatants fermented by 7 isolates of *L. helveticus*, control culture LH-BO2 and a commercial yogurt sample are shown in Table 3. As LAB are known to have antioxidant activity, the fermented milk was centrifuged, and the suspension was filtered to eliminate the effect of bacterial cells on the results.

As shown in Table 3, the percentages of antioxidant activity measured using both free radicals of DPPH and ABTS were greater than 57.64% for all the fermented milks during the whole storage period. The milk sample fermented with all isolates showed significantly higher ($p \leq 0.05$) activity than the commercial yogurt sample (maximum activity 29.03±2.47%). Although some differences were observed in scavenging activity among the samples fermented using different isolates and commercial starter culture LH-BO2, the differences were almost not significant ($p \geq 0.05$). The only exception was isolate SY41 that could significantly ($p \leq 0.05$) improve antioxidant activity during the storage period, and showed significantly higher ($p \leq 0.05$) antioxidant activity than other isolates and culture LH-BO2 measured by DPPH (Table 3). In the antioxidant activity measured by ABTS method, however, no significant differences ($p \geq 0.05$) were observed. Tang et al. in their study on peptides isolated from zein hydrolysate showed that peptides with great hydrophobicity exhibited strong $DPPH^{\bullet}$ and $O_2^{\bullet-}$ scavenging ability, while those with intermediate hydrophobicity displayed the maximum $ABTS^{\bullet+}$ scavenging activity [25]. Accordingly, we can conclude that most of the peptides have released during the fermentation of milk by our *L. helveticus* isolates have hydrophobic properties, as in almost all cases, the activity measured by DPPH assay was higher than that of ABTS assay.so DPPH results are discussed more. Overall, no statistical correlation was found between the concentration of peptides and antioxidant activities that is in accordance with some other previous studies [26].

Virtanen et al. indicated that although development of radical scavengers is connected to the development of proteolysis, high radical scavenging activity was not directly connected to the high degree of proteolysis [8].

Table 3. Antioxidant activity (%) of milk samples fermented by 7 isolates of *L. helveticus* and a commercial culture of *L. helveticus* LH-BO2 after 24 h of incubation at 37ºC (day 1) and 14 days storage at 4ºC. Commercially produced yogurt sample was used as control

		Antioxidant activity (%) during the storage time (days)		
Activity tested	Isolates	1	7	14
DPPH				
	Commercial yogurt	14.01±2.59 ^{aA}	14.20±2.37 ^{aA}	26.81±7.18 ^{aA}
	FMK1	75.21±5.50 ^{aBC}	81.97±6.95 ^{aBC}	77.43±8.15 ^{aBC}
	FM11	72.73±2.70 ^{aBC}	75.28±3.47 ^{aBC}	75.26±6.63 ^{aBC}
	VY22	62.32±3.66 ^{aB}	73.71±8.49 ^{aBC}	67.12±7.18 ^{aB}
	HY12	74.39±8.59 ^{aBC}	68.75±9.38 ^{aBC}	73.03±6.72 ^{aBC}
	FK11	74.79±6.51 ^{aBC}	73.93±8.56 ^{aBC}	73.91±7.55 ^{aBC}
	DY1	70.35±9.36 ^{aBC}	74.92±9.76 ^{aBC}	78.46±5.04 ^{aBC}
	SY41	84.39±3.31 ^{abC}	82.89±6.19 ^{abC}	89.02±4.87 ^{bC}
	LH-BO2	75.53±3.67 ^{aBC}	65.82±1.33 ^{aB}	71.34±6.03 ^{aB}
ABTS				
	Commercial yogurt	26.46±2.97 ^{aA}	29.03±2.47 ^{aA}	22.10±4.12 ^{aA}
	FMK1	63.39±1.04 ^{aB}	64.67±4.00 ^{aB}	64.96±3.16 ^{aB}
	FM11	63.39±3.23 ^{aB}	65.64±2.18 ^{aB}	69.35±2.70 ^{aB}
	VY22	64.64±2.84 ^{aB}	64.17±3.68 ^{aB}	66.25±1.50 ^{aB}
	HY12	57.64±1.42 ^{aB}	59.03±2.20 ^{aB}	62.17±2.06 ^{aB}
	FK11	58.85±3.24 ^{aB}	69.53±2.20 ^{bB}	68.53±3.46 ^{bB}
	DY1	61.28±3.21 ^{aB}	67.17±2.99 ^{aB}	65.21±2.29 ^{aB}
	SY41	68.39±3.90 ^{aB}	65.28±2.83 ^{aB}	65.10±1.80 ^{aB}
	LH-BO2	68.17±3.33 ^{aB}	67.60±1.90 ^{aB}	64.96±1.28 ^{aB}

*Different small letters in the same raw within the same isolate indicate significant differences ($p \leq 0.05$).
**Different capital letters in the same column within the same time indicate significant differences ($p \leq 0.05$).
***Mean±SE (n=3)

Several researchers suggest that some other parameters like amino acid sequence of new peptides release from proteins (currently available oligopeptides) during fermentation are more important factors in the antioxidant properties of product [27, 28].

3.4. Resistance

It is possible that the antioxidant activity of peptides may be higher or lower after proteolytic digestion. Thus, to evaluate the impact of gastrointestinal proteases on the antioxidant activity, an in vitro simulating GI digestion using pepsin-pancreatin was used to illustrate its effect on the antioxidant activity of peptidic extract, and compared with the untreated samples (Figures 1A and 1B, related to supernatant of samples after 7 and 14 days of fermentation, respectively). According to the results, acidic-pepsin digestion for 1 h reduced DPPH radical-scavenging activity in all samples (except HY12 that showed increase) after 7 days of fermentation though it was not significant ($p>0.05$) in all samples except in SY41 that showed significantly lower antioxidant activity after acidic-pepsin treatment. Similar trend were observed after treatment with pancreatin juice. About the samples of 14 days of fermentation, in most of cases, acidic-pepsin digestion resulted to higher antioxidant activity; however, they were not significant. Again among the samples of 14 days of fermentation, it was the milk fermented by SY14 that showed significantly lower antioxidant activity after acidic-pepsin digestion. Overall, the antioxidant activity after consecutive digestion with pepsin-pancreatin was not less than 60% in all of the samples. Change in the bioactive activity of peptidic solutions after exposure to gastrointestinal proteolytic enzyme highly depends to their amino acid composition and sequence [29]. Gastrointestinal enzymes cleave the peptides in a very specific way. For example, it is known for pepsin that it is an aspartic protease with broad specificity, and preferentially cleaves the bonds containing Phe, Tyr, Trp and Leu in the positions that should be broken [30]. The enzyme does not cleave at Val, Ala, or Gly. You et al. reported that GI digests had increased the reducing power and the hydroxyl radical-scavenging activities of loach peptides (previously prepared by papain digestion) by 77% and 12%, respectively [31]. Therefore, the antioxidant peptides are most likely stable in real digestion system after ingestion in both the stomach and the intestines.

Ao and Li observed two antioxidant activity profiles after GI digestion [16]. Negatively charged fraction (NCFshowed decreasing in antioxidant activity after gastric digestion and then returned to the initial level of undigested one after intestinal digestion; however, the antioxidant activity of positively charge fraction (PCF) decreased significantly after GI digestion.

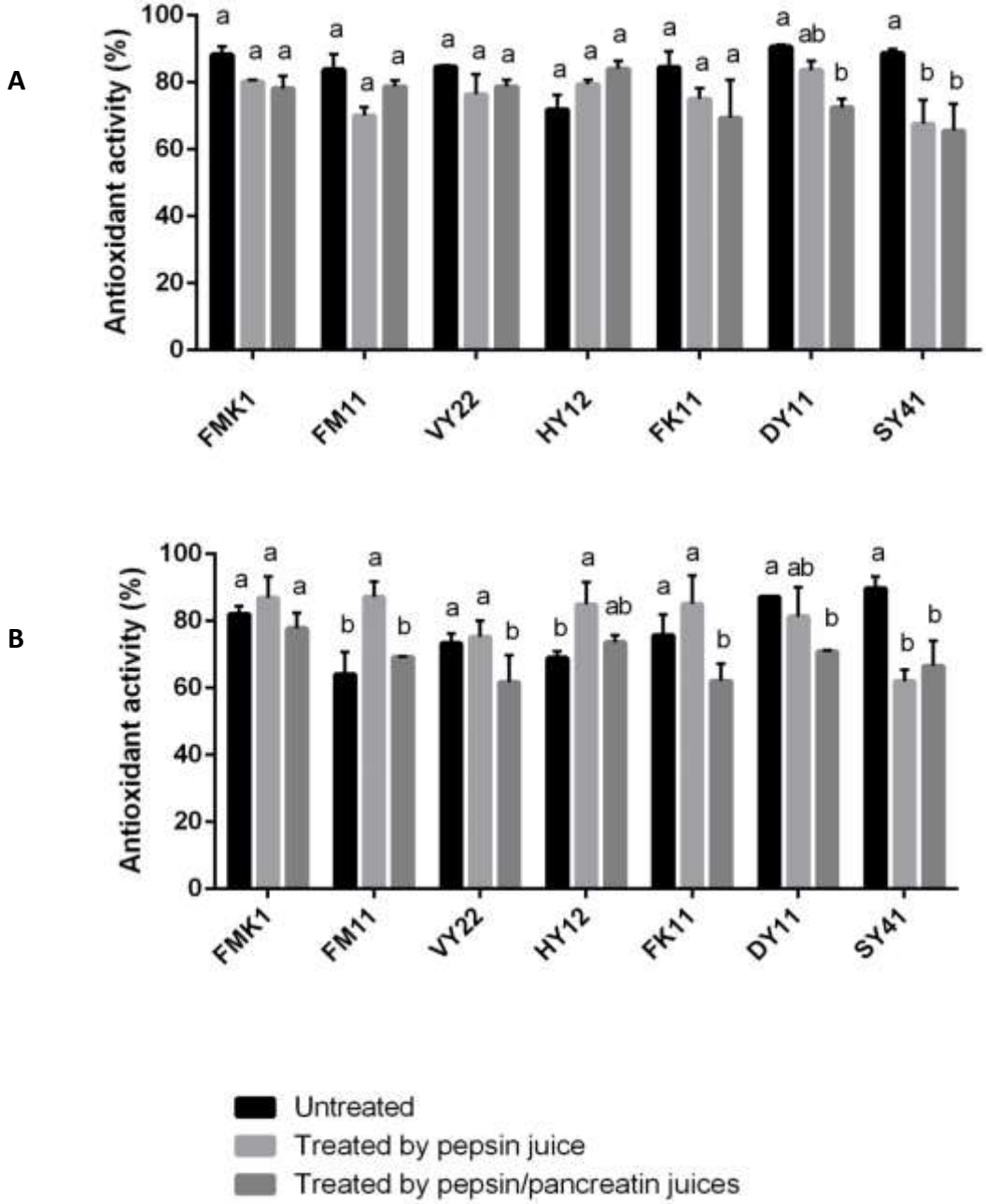

Figure 1. Antioxidant activity (%) of fermented milks after exposure to simulated gastrointestinal conditions. Supernatants of the fermented milk after 7 (A) and 14 (B) days of refrigeration have been analyzed. Different letters (a–c) show statistical differences between the strains in each treatment ($p \leq 0.05$). Error bars indicate SE.

4. Conclusion

This study reported the remarkable ability of wild *L. helveticus* isolates in realizing antioxidant peptides through milk fermentation. According to the results, fermentation with all isolates increased the anti-oxidant activity of milk during the fermentation, which retained in the whole storage period, and also after simulated gastrointestinal digestion. All isolates showed considerably high viability (higher than 6.0 log CFU ml^{-1} in 14 days of storage) in the fermented milk. As a result, in case that these isolates can demonstrate high resistance to gastrointestinal tract and present probiotic characterizations through further studies, they can be promising cultures as probiotic culture, and can successfully be applied in production of functional milk with high antioxidant activity and high content in health-promoting dairy-based functional products.

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6. Conflict of interest

The authors declare that there is no conflict of interest.

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