Biological activities of lactobacilli relevant to cardiovascular health in skim milk

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Abstract In this study, skim milk was fermented using 14 *Lactobacillus* strains for 16 h at 42 °C or for 48 h at 25 °C. On conclusion of fermentation, the proteolytic, angiotensin converting enzyme-inhibitory (ACE-I), and antioxidant activities as well as the inhibition of thrombin and cholesterol micellar solubility were determined. The results revealed that *Lb. paracasei* B-4564 exhibited the highest ACE-I activity (68.11%) under the 42 °C for 16 h condition, while *Lb. rhamnosus* B-1445 demonstrated the highest ACE-I activity (92.23%) under the 25 °C for 48 h condition. *Lb. paracasei* B-4564 exhibited the highest inhibition rate of thrombin (42.43 and 48.10%) and cholesterol (68.60 and 87.01%) under the 42 °C for 16 h and 25 °C for 48 h conditions, receptively. *Lb. rhamnosus* B-442 exhibited the highest DPPH radical scavenging activity of 95.63 and 62.89% under the 42 °C for 16 h and 25 °C for 48 h conditions, receptively. *Lb. rhamnosus* B-1445 demonstrated the highest Fe²⁺ chelating activity and reducing power under both the tested fermentation conditions.

Keywords Lactobacilli · Biological activities · Cardiovascular health

Introduction

Lactobacilli are industrially important for the production of fermented milk products with health benefits through the production of large amounts of peptides with biological

activities and nutritional functionalities [1, 2]. Bioactive peptides are protein fragments with a specific amino acid composition and sequences containing 2–20 amino acids having molecular masses of < 6000 Da [3]. Bioactive peptides have positive effects on one or more factors relevant to cardiovascular diseases (CVD) including high blood pressure, high blood lipids, oxidative stress, obesity, and insulin resistance. Milk proteins are a primary source of bioactive peptides, which can be liberated via proteolysis with digestive enzymes or during microbial fermentation [4].

Many factors affect the production of bioactive peptides during the fermentation process including strains [5], inoculum size [6], fermentation temperature [6], incubation period [7], pH [8], and storage time [9]. Highly proteolytic strains are one of the main factors that increase the bioactive peptide concentration in fermented milks [10, 11]. There is a positive relationship between proteolysis and bioactive peptides production [12]. The proteolytic activity of lactobacilli is strongly affected by temperature and pH, and de Giori et al. [13] found that lactobacilli had the highest proteolytic activity at 15 °C or pH 5.2–5.6. Fast acidifying strains exhibit low proteolytic activities, whereas low acidifying strains exhibit the highest proteolytic activities [14, 15]. Decreasing the fermentation temperature to a below-optimum level prolongs the fermentation time and limits acidity development, which may allow an increase in both proteolysis and bioactive peptides production.

Nowadays, there is an increasing interest in dairy products containing specific bacterial species with potential health benefits. Thus, the purpose of this study was to perform a detailed investigation on the biological activities related to cardiovascular health of various lactobacillus strains in skim milk. Additionally, these bioactivities were

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enhanced by decreasing the fermentation temperature and extending the fermentation time.

Materials and methods

Materials

Cow milk, free from antibiotics, was obtained from an open nucleus herd belonging to the Cattle Information System of Egypt (CISE) and Technology Center of Agricultural Production, Faculty of Agriculture, Cairo University, defatted, and sterilized at 121 °C for 15 min.

The chemicals used included *O*-phthaldialdehyde (OPA), sodium tetraborate, sodium dodecyl sulfate, 2-mercaptoethanol, hippuryl-L-histidyl-L-leucine (Hip-His-Leu), 3-(2-Pyridyl)-5,6-diphenyl-1,2,4-triazine-*p*, *p'*-disulfonic acid monosodium salt hydrate (ferrozine), 1,1-diphenyl-2-picrylhydrazyl (DPPH), sodium tetraborate buffer HPCE (pH 8.0), ferrous chloride (FeCl₂), ferric chloride (FeCl₃), angiotensin I-converting enzyme (ACE, 0.25 U), fibrinogen, thrombin, and cholesterol and were obtained from Sigma-Aldrich (Egyptian International Centre for Import, Cairo, Egypt).

Fourteen *Lactobacillus* strains were procured from the Agriculture Research Service (ARS) Culture Collection, Norwegian Radio Relay League (NRRL) including *Lactobacillus acidophilus* (B-4495 and B-23431), *Lactobacillus rhamnosus* (B-442 and B-1445), *Lactobacillus casei* subsp. *casei* (B-1922 and B-441), *Lactobacillus paracasei* subsp. *paracasei* B-4564, *Lactobacillus fermentum* B-1932, *Lactobacillus brevis* B-3065, *Lactobacillus plantarum* B-4496, *Lactobacillus johnsonii* B-2178, *Lactobacillus reuteri* B-14172, *Lactobacillus gasseri* B-4240, and *Lactobacillus helveticus* B-734.

Milk fermentation

The 14 *Lactobacillus* strains were activated in MRS broth (Lab M Ltd., Heywood, Lancashire, UK) at 37 °C overnight five times, followed by a sixth passage in sterilized skim milk at 37 °C overnight. Sterilized skim milk was fermented by activated strains individually at a ratio of 1% (w/v) and incubated at 42 °C for 16 h or 25 °C for 48 h. On conclusion of fermentation, the pH and the titratable acidity (TA) of the fermented milk were recorded. The proteolytic, antioxidant, ACE-I, and antithrombotic activities in addition to the inhibition of cholesterol micellar solubility were determined.

Methods

pH and titratable acidity

pH values were measured using a digital pH meter with a glass electrode (Jenway 3305, Jenway Limited, Essex, England). Titratable acidity was determined via titration with 0.1 N NaOH employing phenolphthalein as an indicator [16].

Preparation of water-soluble extract from fermented milk

The water-soluble nitrogen extract was prepared according to the method described by Shori et al. [17]. Fermented milk (10 g) was added to distilled water (40 mL) in screw-cap tubes. The mixture was homogenized, followed by holding at 40 °C for 1 h. The homogenate was then centrifuged at 10000×*g* for 30 min, and the obtained supernatant (water-soluble nitrogen extract, WSE) was harvested.

Proteolytic activity

The proteolytic activity of the WSE was determined by reacting free amino acids with OPA according to the method described by Luo et al. [18]. The OPA working reagent was prepared using 25 mL of 100 mM sodium tetraborate, 2.5 mL of 20% (w/w) sodium dodecyl sulfate, 40 mg of OPA, and 100 µL of β-mercaptoethanol. The volume of this solution was increased to 50 mL by adding distilled water. 50 µL of the WSE was mixed with 3 mL of the OPA working reagent, vortexed, and then incubated at room temperature (25 °C) for 2 min. The absorbance of the mixture was measured at 340 nm using a spectrophotometer (Jenway® Genova Life Science Spectrophotometer UV/Visible, Bibby Scientific Ltd., Stone, Staffordshire ST150SA, UK).

Angiotensin I-converting enzyme (ACE) inhibitory activity

The ACE inhibitory (ACE-I) activity was determined using the spectrophotometric method described by Luo et al. [18]. The hippuryl-L-histidyl-L-leucine (Hip-His-Leu) substrate solution was prepared by adding 8.3 mM Hip-His-Leu in 50 mM sodium borate buffer containing 0.5 M NaCl at pH 8.3. The WSE (50 µL) was mixed with an equal volume of an ACE solution (0.25 U). Following incubation at 37 °C for 10 min, 150 µL of the Hip-His-Leu substrate solution was added to the above mixture. The mixture was then incubated at 37 °C for 60 min, followed

by termination of the reaction by adding 250 μL of 1 N HCl. To extract hippuric acid, 1.4 mL of ethyl acetate was added to the mixture, which was vortexed for 5 s and centrifuged at $1500\times g$ for 10 min. Then, 1 mL of the upper organic phase was transferred into glass vials. The ethyl acetate in the glass vials was evaporated at room temperature for 1.5 h in a vacuum oven. After adding 2 mL of distilled water to dissolve the extracted hippuric acid, the absorbance of dissolved hippuric acid was measured at 228 nm using a spectrophotometer. The control sample was prepared by replacing the WSE with distilled water. The inhibition (%) was calculated using the following formula:

$$\text{Inhibition (\%)} = (A_{\text{control}} - A_{\text{sample}}) / A_{\text{control}} \times 100$$

where A_{control} and A_{sample} are the absorbance values of the control and WSE, respectively.

Thrombin inhibition activity

The inhibition of fibrinogen coagulation was evaluated according to the method discussed by Zhang [19]. A microplate reader was set at 37 °C. Thrombin and fibrinogen were independently dissolved in 0.05 M Tris–HCl buffer (pH 7.2) containing 0.12 mM NaCl. 0.1% fibrinogen solution (140 μL) and 40 μL WSE were first injected into the plate wells and mixed; the absorbance at 405 nm was then recorded (blank reading). Next, 10 μL of thrombin solution (12 IU/mL) was added into the wells to initiate the thrombin-catalyzed fibrinogen coagulation reaction. After 10 min of incubation, the absorbance of the WSE was recorded again. 40 μL Tris–HCl buffer (pH 7.2, 0.05 M) was instead of the WSE for measurement of the absorbance of the control blank and the control. The inhibitory ability was calculated as follows:

$$\text{Inhibition effect (\%)} = ([C - CB] - [S - SB]) / [C - CB] \times 100$$

where S, SB, C, and CB represent the absorbance of the WSE, WSE blank, control, and control blank, respectively.

Cholesterol micellar solubility inhibition

The inhibition of cholesterol micellar solubility was measured using the method discussed by Zhong et al. [20]. In this method, a mixture of WSE (1.0 mL) and a micellar solution (7.0 mL) containing 10.0 mM sodium taurocholate, 2.0 mM cholesterol, 5.0 mM oleic acid, 132.0 mM NaCl, and 15.0 mM sodium phosphate (pH 7.4) was prepared by sonication and was incubated at 37 °C for 24 h; then, the mixture was ultracentrifuged at $100000\times g$ for 60 min at 37 °C. The supernatant was collected to

determine the total cholesterol content using a BT 1500 Fully Automated Biochemistry Analyzer (Biotechnica Instruments SpA, Rome, Italy).

Antioxidant activity properties

1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity

DPPH radical scavenging activity was determined according to the method described by Li et al. [21]. WSE (1 mL) was mixed with 0.1 mmol/L DPPH (1 mL) dissolved in 95% ethanol. The mixture was vortexed and left for 30 min at room temperature. The resulting solution's absorbance was measured at 517 nm. Distilled water was used as a blank instead of the sample. Ascorbic acid (0.2 mmol/L) was used as a positive control. The scavenging activity was calculated using the following equation:

$$\text{DPPH radical scavenging activity (\%)} = (A_0 - A_s) / A_0 \times 100$$

where A_0 is the absorbance of the blank at 517 nm and A_s is the absorbance of the WSE at 517 nm.

Metal ions chelating activity

Metal ions chelating activity was estimated using the ferrozine method [18]. WSE (1 mL) was mixed with distilled water (1 mL) and 2.0 mM FeCl_2 (50 μL), followed by resting at room temperature for 30 s. After adding 0.1 mL of 5 mM ferrozine to the above mixture, vortexing, and incubation at room temperature for 10 min, the absorbance was measured at 562 nm. The absorbance of control was determined similarly by replacing the supernatant with distilled water. EDTA (0.1 mg/mL) was used as a positive control. The metal ions chelating activity was calculated as follows:

$$\text{Chelating activity (\%)} = [1 - (A_{\text{sample}} / A_{\text{control}})] \times 100$$

where A_{sample} and A_{control} are the absorbance of WSE and control, respectively.

Reducing power

Reducing power was tested according to the method described by Luo et al. [18]. A 1-mL aliquot of the WSE was mixed with 1 mL of 0.2 M phosphate buffer saline (pH 6.6) and 1 mL of 1% (w/v) potassium ferricyanide. The mixture was incubated at 50 °C for 20 min, followed by cooling to room temperature. After adding 2.0 mL of

10% (w/v) trichloroacetic acid, the mixture was mixed with 0.3 mL of a 0.1% (w/v) ferric chloride solution. Following incubation for 10 min, the absorbance of the resulting solution was measured at 700 nm. Ascorbic acid (0.2 mmol/L) was used as a positive control. A high absorbance was indicative of strong reducing power.

Statistical analysis

Data are expressed as mean $\pm$ standard deviation (SD) values. A randomized complete block design and analysis of variance of factorial methods were performed using Mstat-C. All data were analyzed in three replications for each parameter. The least significant differences (LSD) test was used to compare the significant differences between the mean values for different treatments. Results were considered statistically significant at $P \leq 0.05$.

Results and discussion

pH and titratable acidity

The pH and TA values of milk fermented using 14 *Lactobacillus* strains after 16 h at 42 °C or after 48 h at 25 °C are listed in Table 1. All the used *Lactobacillus* strains varied in their acidity production under the fermentation conditions. The variation in acidity production may depend on the strain used; there were low and high acidifying strains. On conclusion of fermentation for 16 h at 42 °C, the pH and TA values ranged from 4.40 and 0.69% for *Lb.*

helveticus B-734 to 4.03 and 0.80% for *Lb. brevis* B-3065, respectively. After fermentation for 48 h at 25 °C, the pH and TA values ranged from 4.30 and 0.72% for *Lb. helveticus* B-734 to 3.98 and 0.81% for *Lb. gasseri* B-4240, respectively. These results indicate that the acidification ability varied among strains. By decreasing the fermentation temperature from 42 to 25 °C and prolonging the fermentation time from 16 to 48 h, 10 out of the 14 tested strains recorded a significant decrease in pH and a significant increase in TA values. The remaining strains exhibited non-significant variation in pH and TA values. The *Lb. helveticus* B-734 strain exhibited the highest pH (significant) and the lowest acidity (significant). For *Lb. plantarum* B-4496, this was vice versa. This finding indicates that the growth rate of these strains was affected by the change in the fermentation conditions, where acid production depends on the ability of *Lactobacillus* strains to ferment lactose in milk during their growth [22]. In this regard, Chen et al. [6] found a decrease in the pH of fermented milk due to 59 *Lb. helveticus* strains after 24 h of fermentation at 37 °C.

Proteolytic activity

The proteolytic activities of *Lactobacillus* strains in skim milk were determined by measuring the release of free NH_3 groups following the OPA method and are presented in Table 2. All the used *Lactobacillus* strains exhibited proteolytic activity of significantly different degrees, where *Lb. paracasei* B-4564 exhibited the highest (0.450) value (significant) and *Lb. plantarum* B-4496 exhibited the

Table 1 pH and titratable acidity of skim milk fermented using different *Lactobacillus* strains

Strains	pH		Titratable acidity (TA, %)	
	42 °C/16 h	25 °C/48 h	42 °C/16 h	25 °C/48 h
<i>Lb. acidophilus</i> B-4495	4.19 $\pm$ 0.10 ^{ef}	4.19 $\pm$ 0.11 ^{ef}	0.77 $\pm$ 0.01 ^{de}	0.78 $\pm$ 0.01 ^{cd}
<i>Lb. acidophilus</i> B-23431	4.22 $\pm$ 0.11 ^{cde}	4.21 $\pm$ 0.02 ^{de}	0.78 $\pm$ 0.01 ^{cd}	0.79 $\pm$ 0.01 ^{bc}
<i>Lb. helveticus</i> B-734	4.40 $\pm$ 0.10 ^a	4.30 $\pm$ 0.10 ^{bcd}	0.69 $\pm$ 0.03 ⁱ	0.72 $\pm$ 0.04 ^{gh}
<i>Lb. casei</i> B-441	4.30 $\pm$ 0.10 ^{bcd}	4.09 $\pm$ 0.019 ^{gh}	0.72 $\pm$ 0.02 ^{gh}	0.75 $\pm$ 0.01 ^f
<i>Lb. casei</i> B-1922	4.30 $\pm$ 0.11 ^{bcd}	4.19 $\pm$ 0.02 ^{ef}	0.72 $\pm$ 0.03 ^{gh}	0.76 $\pm$ 0.01 ^{ef}
<i>Lb. rhamnosus</i> B-442	4.30 $\pm$ 0.09 ^{bcd}	4.19 $\pm$ 0.013 ^{ef}	0.71 $\pm$ 0.03 ^h	0.76 $\pm$ 0.01 ^{ef}
<i>Lb. rhamnosus</i> B-1445	4.10 $\pm$ 0.10 ^{fg}	4.03 $\pm$ 0.11 ^{ghi}	0.73 $\pm$ 0.01 ^g	0.79 $\pm$ 0.01 ^{bc}
<i>Lb. brevis</i> B-3065	4.03 $\pm$ 0.14 ^{ghi}	4.00 $\pm$ 0.15 ^{hi}	0.80 $\pm$ 0.01 ^{ab}	0.81 $\pm$ 0.01 ^a
<i>Lb. fermentum</i> B-1932	4.22 $\pm$ 0.12 ^{cde}	4.00 $\pm$ 0.16 ^{hi}	0.77 $\pm$ 0.01 ^{de}	0.81 $\pm$ 0.02 ^a
<i>Lb. gasseri</i> B-4240	4.10 $\pm$ 0.11 ^{fg}	3.98 $\pm$ 0.02 ⁱ	0.75 $\pm$ 0.01 ^f	0.81 $\pm$ 0.01 ^a
<i>Lb. plantarum</i> B-4496	4.35 $\pm$ 0.19 ^{ab}	3.99 $\pm$ 0.11 ⁱ	0.72 $\pm$ 0.04 ^{gh}	0.80 $\pm$ 0.03 ^{ab}
<i>Lb. paracasei</i> B-4564	4.33 $\pm$ 0.18 ^{ab}	4.10 $\pm$ 0.10 ^{fg}	0.71 $\pm$ 0.02 ^h	0.75 $\pm$ 0.03 ^f
<i>Lb. reuteri</i> B-14172	4.30 $\pm$ 0.19 ^{bcd}	3.99 $\pm$ 0.021 ⁱ	0.72 $\pm$ 0.02 ^{gh}	0.81 $\pm$ 0.03 ^a
<i>Lb. johnsonii</i> B-2178	4.31 $\pm$ 0.022 ^{abc}	4.30 $\pm$ 0.17 ^{bcd}	0.72 $\pm$ 0.03 ^{gh}	0.71 $\pm$ 0.02 ^{gh}
LSD	0.0912		0.0169	

Means in the same column or row for each parameter with different superscript letters differ significantly

Table 2 Proteolytic, ACE-I, anti-thrombotic, and anti-cholesterol activities for different *Lactobacillus* strains in skim milk

Strains	Proteolytic activity (A_{340nm})		ACE-I activity (%)		Anti-thrombotic activity (%)		Cholesterol inhibition (%)	
	42 °C/16 h	25 °C/48 h	42 °C/16 h	25 °C/48 h	42 °C/16 h	25 °C/48 h	42 °C/16 h	25 °C/48 h
<i>Lb. acidophilus</i> B-4495	0.277 ± 0.015 ^p	0.397 ± 0.017 ⁱ	42.91 ± 2.20 ^q	54.11 ± 2.11 ^k	10.01 ± 0.11 ^p	16.86 ± 0.25 ^m	23.88 ± 1.41 ^r	35.82 ± 1.39 ^p
<i>Lb. acidophilus</i> B-23431	0.295 ± 0.017 ^{lmn}	0.401 ± 0.02 ^{hi}	45.99 ± 2.11 ^o	57.36 ± 1.89 ^j	19.64 ± 0.13 ^l	29.53 ± 0.36 ^h	37.31 ± 1.23 ^o	49.25 ± 1.29 ^j
<i>Lb. helveticus</i> B-734	0.415 ± 0.01 ^g	0.480 ± 0.003 ^d	64.90 ± 1.90 ^g	72.13 ± 1.33 ^d	40.04 ± 0.90 ^e	40.94 ± 0.96 ^d	58.96 ± 1.15 ^g	62.88 ± 1.11 ^e
<i>Lb. casei</i> B-441	0.224 ± 0.011 ^r	0.401 ± 0.01 ^{hi}	36.81 ± 2.12 ^s	56.91 ± 1.91 ⁱ	5.89 ± 0.70 ^s	11.28 ± 0.21 ^o	13.43 ± 1.52 ^x	18.96 ± 1.76 ^u
<i>Lb. casei</i> B-1922	0.409 ± 0.011 ^{gh}	0.486 ± 0.012 ^d	63.91 ± 1.76 ^h	70.50 ± 1.56 ^e	24.46 ± 0.22 ⁱ	39.67 ± 0.89 ^e	53.73 ± 1.11 ⁱ	61.19 ± 1.11 ^f
<i>Lb. rhamnosus</i> B-442	0.273 ± 0.014 ^p	0.288 ± 0.013 ^{no}	41.11 ± 2.01 ^r	44.10 ± 1.98 ^p	9.25 ± 0.31 ^q	13.38 ± 0.19 ⁿ	17.90 ± 1.49 ^v	22.39 ± 1.51 ^s
<i>Lb. rhamnosus</i> B-1445	0.431 ± 0.003 ^f	0.819 ± 0.01 ^a	65.11 ± 1.33 ^g	92.33 ± 1.11 ^a	40.94 ± 0.81 ^d	46.52 ± 0.91 ^b	67.16 ± 1.01 ^d	80.60 ± 0.87 ^b
<i>Lb. brevis</i> B-3065	0.243 ± 0.013 ^q	0.505 ± 0.016 ^c	43.93 ± 2.03 ^p	73.12 ± 1.77 ^c	6.97 ± 0.76 ^f	11.54 ± 0.18 ^o	14.93 ± 1.61 ^w	20.90 ± 1.62 ^t
<i>Lb. fermentum</i> B1932	0.293 ± 0.012 ^{mno}	0.299 ± 0.013 ^{lm}	46.41 ± 1.99 ^{no}	52.98 ± 1.87 ^l	13.56 ± 0.21 ⁿ	21.16 ± 0.46 ^k	35.81 ± 1.71 ^p	47.76 ± 1.30 ^l
<i>Lb. gasseri</i> B-4240	0.286 ± 0.01 ^o	0.360 ± 0.017 ^j	46.10 ± 1.64 ^o	55.98 ± 1.66 ^j	10.52 ± 0.31 ^p	19.90 ± 0.51 ^l	28.36 ± 1.66 ^q	40.30 ± 1.41 ⁿ
<i>Lb. plantarum</i> B-4496	0.212 ± 0.012 ^s	0.303 ± 0.014 ^{kl}	33.61 ± 2.12 ^t	46.61 ± 2.22 ⁿ	3.39 ± 0.12 ^t	10.52 ± 0.29 ^p	10.99 ± 1.69 ^y	14.93 ± 1.71 ^w
<i>Lb. paracasei</i> B-4564	0.450 ± 0.03 ^e	0.729 ± 0.01 ^b	68.11 ± 1.66 ^f	83.20 ± 1.51 ^b	42.43 ± 0.56 ^c	48.10 ± 0.99 ^a	68.66 ± 1.51 ^c	87.01 ± 0.91 ^a
<i>Lb. reuteri</i> B-14172	0.311 ± 0.011 ^k	0.359 ± 0.012 ^j	51.01 ± 2.02 ^m	53.91 ± 1.81 ^k	22.18 ± 0.29 ^j	32.57 ± 0.78 ^g	43.28 ± 1.32 ^m	48.82 ± 1.31 ^k
<i>Lb. johnsonii</i> B-2178	0.414 ± 0.01 ^g	0.426 ± 0.01 ^f	63.98 ± 1.59 ^h	73.08 ± 1.42 ^c	24.94 ± 0.41 ⁱ	35.11 ± 0.69 ^f	56.72 ± 1.12 ^h	62.69 ± 1.09 ^e
LSD	0.0082		0.4774		0.5153		0.4207	

Means in the same column or row for each parameter with different superscript letters differ significantly

lowest (0.212) value (significant) for fermentation for 16 h at 42 °C. Further, *Lb. rhamnosus* B-1445 and *Lb. rhamnosus* B-442 exhibited the highest (0.819) and lowest (0.288) values (significant) for fermentation for 48 h at 25 °C. By comparing the proteolytic activities of each *Lactobacillus* strain at 42 and 25 °C, it was observed that decreasing the fermentation temperature resulted in a significantly increased proteolytic activity for all strains except *Lb. fermentum* B-1932, and the increment rate ranged from 2.82% for *Lb. johnsonii* B-2178 to 51.88% for *Lb. brevis* B-3065. de Giori et al. [13] noted the same trend, indicating that the temperature needed for developing highly enzymatic activity may be different from that needed for fast growth and that proteolytic activity varies with incubation temperature. Donkor et al. [23] reported that the extent of proteolysis varied among strains and appeared to be time dependent. They found that proteolytic activity increased slightly when the fermentation time increased from 0 to 12 h for some strains (*Lb. acidophilus* L10, *Lb. acidophilus* La 4962, *B. lactis* B94, *B. longum* B1 536, *Lb. casei* L26, and *Lb. casei* Lc 279), whereas it increased significantly for all strains when the fermentation time was increased from 12 to 24 h.

From the data in this study, there was no relationship noted between acidity and proteolytic activity of the used *Lactobacillus* strains, which is in agreement with the results obtained by Hassaïne et al. [14].

ACE inhibitory (ACE-I) activity

ACE is a key factor in elevating blood pressure and does so by catalyzing the production of vasoconstrictor angiotensin II and inactivating the vasodilator bradykinin. Therefore, inhibiting this enzyme can cause an antihypertensive effect. Synthetic ACE inhibitors are available as pharmacological drugs, although they have adverse side effects such as dry cough, skin rashes, and angioneurotic edema. Thus, developing safe and natural ACE-inhibitors is necessary for future treatment and prevention of hypertension. Milk products such as fermented milk are the main sources of ACE-I peptides that demonstrate potential to control hypertension [8]. Li et al. [24] reported that these peptides with ACE-I activity have branched aliphatic amino acids (Leu, Ile, or Val) at their N terminus and Trp, Phe, Tyr, or Pro at their C terminus.

The ACE-I activities of the tested *Lactobacillus* strains in skim milk are shown in Table 2. This activity varied significantly among all the used strains under both fermentation conditions. The *Lb. paracasei* B-4564 strain exhibited the highest (68.11%) ACE-I activity (significant), while *Lb. plantarum* B-4496 exhibited the lowest (33.61%) value (significant) for fermentation at 42 °C for 16 h. However, after 48 h of fermentation at 25 °C, the highest

and lowest ACE-I percentages (92.23 and 44.10%, respectively; significant) were noted for *Lb. rhamnosus* B-1445 and *Lb. rhamnosus* B-442, respectively. Fuglsang et al. [10] found that most lactic acid bacteria produced ACE inhibitors in varying amounts during milk fermentation, which depended on the strain used. The results listed in Table 2 show that ACE-I percentage increased significantly with decreasing fermentation temperature and increasing fermentation time for all strains. These results are in agreement with those by Gonzalez-Gonzalez et al. [8], who reported that ACE-I activity increases with increasing fermentation duration.

It was observed that there was a high positive correlation between the proteolytic and ACE-I activities of all the tested strains when performing fermentation for 16 h at 42 °C ($r = 0.98$) or for 48 h at 25 °C ($r = 0.94$). These results have the same trend as that reported by Pihlanto et al. [25], who noted a positive correlation between the ACE-I and proteolytic activities depending on the strain and fermentation time.

Anti-thrombotic activity

The platelet aggregation process is one of the major factors in CVD because of the interaction between the thrombin and fibrinogen-forming fibrin clots. Antiplatelet agents and drugs such as aspirin and ticlopidine have abilities to prevent arterial thrombi during chronic use [26]. Food-derived peptides that inhibit blood platelet aggregation can be useful as natural ingredients for preventing thrombosis [27]. Milk antithrombotic peptides inhibit platelet aggregation and combine with the receptor site, consequently preventing fibrinogen binding with blood platelets [28]. The anti-thrombotic activities of the used *Lactobacillus* strains in skim milk are listed in Table 2. All the tested strains exhibited different levels of anti-thrombotic properties. Under the fermentation conditions used herein (42 °C/16 h or 25 °C/48 h) and among all the tested strains, *Lb. paracasei* B-4564 and *Lb. plantarum* B-4496 exhibited the highest and lowest inhibition rates (significant) of thrombin, respectively. The inhibition rate of thrombin for each strain increased significantly (with different rates) according to the decrease in fermentation temperature and increase in fermentation time, between 2.19% for *Lb. helveticus* B-734 and 67.78% for *Lb. plantarum* B-4496. Furthermore, our results revealed that there was a positive correlation between the proteolytic and anti-thrombotic activities of all the strains tested at 42 °C for 16 h ($r = 0.93$) and at 25 °C for 48 h ($r = 0.72$). In a previous study, Rojas-Ronquillo et al. [29] found that bovine caseins fermented using *Lb. casei* Shirota exhibited a thrombin inhibitory activity of 80.7% after 24-h fermentation. Oh et al. [30] found that the hydrolysates from

fermented Maillard reaction products (MRP) exhibited an increase in the anti-thrombotic effect compared with non-fermented MRP. The anti-thrombotic effect may be due to the casoplatelin, liberated by trypsin and chymosin hydrolysis of bovine κ -CN, inhibited adenosine diphosphate (ADP)-induced platelet aggregation, and binding of the γ -chain of fibrinogen in a dose-dependent manner [31]. Rojas-Ronquillo et al. [29] showed that the C-terminal fragment of β -CN (f193-209), corresponding to the amino acid sequence Tyr-Gln-Glu-Pro-Val-Leu-Gly-Pro-Val-Arg-Gly-Pro-Phe-Pro-Ile-Ile-Val, inhibited the proteolytic activity of thrombin on fibrinogen.

Inhibition of cholesterol micellar solubility

High plasma cholesterol levels have been associated with an increased risk of CVD. Suppression of micellar cholesterol solubility is associated with inhibition of cholesterol absorption in the jejunum in vivo and may be closely related to the lowering of serum cholesterol [20]. The inhibition of cholesterol micellar solubility for the tested *Lactobacillus* strains in skim milk is shown in Table 2. Our findings indicate that all the used strains exhibited cholesterol micellar solubility inhibition with various rates. Under both the fermentation conditions used herein (42 °C/16 h or 25 °C/48 h) and among all the tested strains, *Lb. paracasei* B-4564 and *Lb. plantarum* B-4496 exhibited the highest and lowest rates of cholesterol micellar solubility inhibition (significant), respectively. Our results are in agreement with those by Oh et al. [30], who found that 20 lactic acid bacteria strains, except *L. fermentum* H4, reduced micellar cholesterol solubility after fermentation of MRP for 48 h at 37 °C. By decreasing the fermentation temperature (42 to 25 °C) and increasing the fermentation time (16–48 h), the inhibition of cholesterol micellar solubility of each strain increased significantly with different levels ranging between 6.65% for *Lb. helveticus* B-734 and 50% for *Lb. acidophilus* B-4495. The variation in cholesterol micellar solubility inhibition may be attributed to the relation between both of the hydrophobic amino acids and peptide size as well as the decrease in cholesterol absorption and cholesterol micellar solubility [32]. Our results also revealed that there was a positive correlation between the proteolytic activity and the inhibition of cholesterol micellar solubility for all the strains tested for 16 h at 42 °C ($r = 0.97$) and for 48 h at 25 °C ($r = 0.73$).

Antioxidant activity

The antioxidant activities of the tested *Lactobacillus* strains in skim milk were determined using three methods—DPPH radical scavenging activity, metal ions (Fe^{2+}) chelating

activity, and reducing power- and the results are illustrated in Figs. 1, 2 and 3.

DPPH radical scavenging activity

DPPH is a stable free radical that has been used to evaluate the ability of antioxidant peptides as free radical scavengers or hydrogen donors. Among all the strains used in this study, when incubated for 16 h at 42 °C, *Lb. rhamnosus* B-442 and *Lb. fermentum* B-1932 exhibited the highest and lowest DPPH radical scavenging activities (95.63 and 39.12%, respectively; significant), respectively (Fig. 1). In this regard, Moslehishad et al. [33] reported that milk fermented using *Lb. rhamnosus* PTCC 1637 exhibits a higher antioxidant activity than milk fermented using *Leuconostoc mesenteroides* spp. *cremories* and *Lb. jensenii*. The DPPH radical scavenging activities of *Lb. rhamnosus* B-442 and *Lb. johnsonii* B-2178 in skim milk increased significantly compared with that of ascorbic acid (positive control). Under low fermentation temperature (25 °C) and long fermentation time (48 h) conditions, *Lb. rhamnosus* B-442 and *Lb. acidophilus* B-23431 exhibited the two highest (62.89 and 62.20%, respectively; significant) DPPH radical scavenging activities, whereas *Lb. fermentum* B-1932 and *Lb. plantarum* B-4496 exhibited the lowest activity (26.14%; significant). The antioxidant peptides released during fermentation may have the ability to neutralize free DPPH radicals either by donating hydrogen atom or by direct reduction through electron transfer [34]. Our results exhibit the same trend as those by Abubakr et al. [35], who indicated that the stress of radical scavenging for milk fermented using lactic acid bacteria depends on the strain used. The DPPH radical scavenging activities of all the *Lactobacillus* strains tested for 48 h at 25 °C were significantly less than that of the positive control (Fig. 1). By decreasing the fermentation temperature (42 to 25 °C) and increasing the fermentation duration (16 h to 48 h), the DPPH radical scavenging activities of all the used strains decreased significantly.

Metal ions chelating activity

Among metal ions, free ferrous ions are involved in catalyzing oxidation reactions, leading to the formation of reactive oxygen species (ROS) that are implicated in carcinogenesis and CVD. Ferrozine can quantitatively chelate with Fe^{2+} to form a red colored complex, and the intensity of red color decreases in the presence of other chelating agents [35]. Measurement of color reduction, therefore, allows the estimation of chelating activity of the coexisting chelator [35]. From the obtained results shown in Fig. 2, among all the tested strains and the EDTA (positive control), *Lb. rhamnosus* B-1445 exhibited the highest ability

Fig. 1 DPPH radical scavenging of *Lactobacillus* strains in skim milk. Ascorbic acid serves as a positive control

Fig. 2 Fe²⁺ chelating activity of *Lactobacillus* strains in skim milk. EDTA serves as a positive control

(significant) to chelate metal ions (Fe²⁺) in skim milk under either of the fermentation conditions used herein. *Lb. brevis* B-3065 and *Lb. paracasei* B-4564 (tested for 16 h at 42 °C) and *Lb. paracasei* B-4564 (tested for 48 h at 25 °C) had significantly lower Fe²⁺ chelating activity than the other strains and the positive control. By decreasing the fermentation temperature (42 to 25 °C) and increasing the fermentation time (16–48 h), the Fe²⁺ chelating activity of

all the tested strains declined significantly except *Lb. casei* B-1922 and *Lb. rhamnosus* B-442, which did not reflect a significant change. The chelating activity against pro-oxidative metal ions may be caused by the termination of certain radical chain reactions through specific side-chain groups of amino acid residues of antioxidant peptides [36].

Previous studies reported that *St. thermophilus* 821, *B. longum* 15708, *Lb. casei* KCTC 3260, and *Lb. plantarum*

Fig. 3 Reducing power of *Lactobacillus* strains in skim milk. Ascorbic acid serves as a positive control

C88, C10, and K25 exhibited strong hydroxyl radical scavenging activity, probably due to their capability of chelating metal ions such as ferrous ions [37].

Reducing power

A reducing power assay is used to evaluate the ability of antioxidant peptides to donate an electron or hydrogen ion. As shown in Fig. 3, *Lb. rhamnosus* B-1445, *Lb. brevis* B-3065, and *Lb. acidophilus* B-23431 strains showed the significant strongest reducing power, respectively, which were similar to that of ascorbic acid (positive control). However, *Lb. casei* B-441 and *Lb. rhamnosus* B-442 strains exhibited the significant lowest reducing power at 42 °C for 16 h, which was equivalent to 53.62–61.84% of the reducing power of the positive control. Under the fermentation for 48 h at 25 °C condition, *Lb. acidophilus* B-4495, *Lb. helveticus* B-734, *Lb. paracasei* B-4564, and *Lb. reuteri* B-14172 strains exhibited a significantly stronger reducing power than the other strains. All tested *Lactobacillus* strains had a significantly less reducing power than the positive control. Li et al. [21] found that the peptides from milk fermented using *Lb. delbrueckii* ssp. *bulgaricus* and *Lb. helveticus* showed high reducing power, equivalent to 60–67% of the reducing power of the positive control. They attributed the variations of reducing power among the strains to the reducing ability of the amino acid present in antioxidant peptides that react with free radicals, leading to blocking of the oxidation reaction. Due to the decrease in fermentation temperature and the increase in fermentation time, the reducing power decreased

significantly for *Lb. acidophilus* B-23431, *Lb. casei* B-1922, *Lb. rhamnosus* B-1445, *Lb. brevis* B-3065, *Lb. fermentum* B-1932, *Lb. gasseri* B-4240, *Lb. plantarum* B-4496, and *Lb. johnsonii* B-2178 and a non-significant reduction was recorded for the other strains. The reduction in antioxidant activities due to a decreased fermentation temperature and a prolonged fermentation time is attributed to further hydrolysis of some antioxidant peptides, leading to a change in the size and composition of peptides that are related to bioactivity [38].

All *Lactobacillus* strains showed a positive correlation ($r = 0.11$ – 0.36) between the proteolytic and antioxidant activities, which are assessed using three different methods at 42 °C for 16 h or 25 °C for 48 h. By decreasing the fermentation temperature and prolonging the fermentation time (25 °C/48 h), a negative correlation was found between the proteolytic activity and both DPPH radical scavenging activity ($r = -0.14$) and reducing power ($r = -0.10$), while the correlation was low positive between the proteolytic and Fe^{2+} chelating activities ($r = 0.11$). In this regard, Virtanen et al. [39] observed that the antioxidant activity is dependent on the strain used, and it generally increased during fermentation, which was not directly related to fermentation time. These results may be attributed to the type and sequence of amino acids in oligopeptides that are released during fermentation, as previously mentioned by Abubakr et al. [35].

In conclusion, all used *Lactobacillus* strains produced bioactive peptides related to CVD with different levels under the tested fermentation conditions. The *Lb. rhamnosus* (B-442 or B-1445) and *Lb. paracasei* B-4564 strains

exhibited the greatest activities for these peptides. An increase in fermentation time and decrease in fermentation temperature led to elevated proteolytic, ACE-I, anti-thrombotic, and anti-cholesterol activities for each strain, while the antioxidant activity had the opposite trend. A positive correlation was observed between the proteolytic activity and the ACE-I, anti-cholesterol, or antithrombotic activities for all used strains. Our findings have revealed that valuable strains and suitable fermentation conditions could be identified for producing functional fermented dairy products to protect from the risks of CVD.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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