Food Sci Biotechnol (2017) 26(5):1325–1332
DOI 10.1007/s10068-017-0160-9

Antioxidant and antibacterial activities of bioactive peptides in buffalo's yoghurt fermented with different starter cultures

Soad Taha[1] · Moniur El Abd[1] · Cristian De Gobba[2] · Mahmoud Abdel-Hamid[1] · Ensaf Khalil[3] · Doaa Hassan[3]

Received: 15 November 2016 / Revised: 6 May 2017 / Accepted: 23 May 2017 / Published online: 20 September 2017

Abstract The effect of *Lactobacillus acidophilus* 20552 ATCC (T2) or *Lactobacillus helveticus* CH 5 (T3) in combination with yoghurt starter (1:1) on the antioxidant and antibacterial activities of the bioactive peptides present in buffalo's yoghurt was studied. The SDS-PAGE results indicate that all caseins were completely hydrolyzed by both strains, whereas whey protein fractions were still present. All starter cultures have the ability to produce low-molecular-weight bioactive peptides, most of which were originated from β-casein and fewer from αs_1 casein. The antioxidant activity (%) of the water-soluble peptide extract from yoghurt samples increased in all samples during storage. Samples containing *Lb. helveticus* CH 5 showed the highest values. All yoghurt treatments displayed antibacterial activity against *Escherichia coli*. Control yoghurt and T3 showed higher antibacterial activity on *E. coli*, *Staphylococcus aureus,* and *Bacillus cereus* as compared to T2.

Keywords Buffalo yoghurt · Bioactive peptides · *Lactobacillus helveticus* CH 5 · *Lactobacillus acidophilus* 20552 ATCC · Antibacterial and antioxidant activities

✉ Soad Taha
soad_hassantaha@yahoo.com

Moniur El Abd
Moniur.Ibrahim@agr.cu.rdu.eg

Cristian De Gobba
gobba@food.ku.dk

Mahmoud Abdel-Hamid
mahmoudah3@yahoo.com

Ensaf Khalil
ensaf1966@yahoo.com

Doaa Hassan
doaafathy23@yahoo.com

[1] Dairy Science Department, Faculty of Agriculture, Cairo University, Giza 12613, Egypt

[2] Department of Food Science, Faculty of Science, University of Copenhagen, Rolighedsvej 30, 1958 Frederiksberg, Denmark

[3] Food Technology Research Institute, Special Foods Department, Ministry of Agriculture, Giza, Egypt

Introduction

Yoghurt is a widely enjoyed dairy product and has a number of health benefits such as increased bone mineralization [1] and gut-associated immune response and laxation [2]. It is an excellent vehicle for the production of functional foods, especially those containing probiotic bacteria [3].

The health effects of yoghurt can be enhanced by the release of bioactive peptides [4] as a result of proteolytic activities of the organisms triggering the fermentation [5].

These peptides are specific protein fragments that have a positive impact on body functions and influence on health [6]. They are released through one of the following ways: (1) hydrolysis by digestive enzymes, (2) enzymatic cleavage by proteases derived from microorganisms or plants, and (3) via food processing or manufacturing (e.g., by acids, alkali, heating).

Fermentation of milk by lactic acid bacteria releases a large number of peptides and amino acids with varying biological actions, including inhibition of angiotensin-converting enzymes (ACE) and antioxidant activities [7], immune modulation, and opioid effect [8].

The present work aims to study the effect of starter culture on the antioxidant and antibacterial activities of bioactive peptides during cold storage of buffalo yoghurt.

Materials and methods

Materials

Buffalo's milk and buffalo's skimmed milk were obtained from Animal Production Research Institute, Agricultural Research Center, Ministry of Agriculture, Dokki, Egypt. Yoghurt culture (*Streptococcus salivarius* subsp. *thermophilus* CH1 and *Lactobacillus delbrueckii* subsp. *bulgaricus* LB-12-DRI-VAC) and *Lactobacillus helveticus* CH 5 were obtained from Chr. Hansen laboratories, Hoersholm, Denmark, while *Lactobacillus acidophilus* 20552 ATCC was obtained from Cairo Microbiological Resources Centre (Cairo, MIRCEN), Faculty of Agriculture, Ain Shams University, Egypt. *Bacillus cereus* ATCC 6538, *Staphylococcus aureus* ATCC 25923, *Salmonella typhimurium* ATCC 9027 and *Escherichia coli* ATCC 2592 were obtained from Food Technology Research Institute, ARC, Ministry of Agriculture, Egypt, while *Pseudomonas aeruginosa* ATCC 9027 was obtained from Cairo University Research Park (CURP), Faculty of Agriculture, Giza, Egypt. Nutrient agar was obtained from Difico, Sparks, Maryland (USA). All chemicals were purchased from Sigma Chemical Company (Saint Louis, MO, USA).

Experimental procedure

Buffalo's milk was standardized to 3% fat. The chemical composition of the milk was 11.70% total solids (TS), 3.5% total protein (TP), 0.78% ash, and 0.17% titratable acidity (TA). The milk was heated at 90 °C for 10 min, cooled to 38 ± 1 °C, and then divided into three portions. The first one (T1) was inoculated with yoghurt culture (2%) and served as control, while the second one (T2) was inoculated with 2% of a mixture of *Lb. acidophilus* 20552 ATCC + yoghurt culture (1:1).The third portion (T3) was inoculated with 2% of a mixture of yoghurt culture + *Lb. helveticus* CH 5 (1:1). All starter cultures were activated in MRS broth before use. The inoculated milk was packed in sterilized plastic cups and incubated at the optimum temperature for the growth of starter culture (38 °C for T2 and 42 °C for T1 and T3) until pH4.6 (0.6–0.7% titratable acidity). Yoghurt samples were cooled, maintained refrigerated at 4 °C, and analyzed at intervals.

Analysis methods

Chemical composition of milk and yoghurt

The milk fat, TS, protein content, and titratable acidity (TA%) of milk and yoghurt were determined according to the Association of Official Analytical Chemists (AOAC) [9].

Extraction of water-soluble peptides

Water-soluble peptide extract (WSPE) was prepared from yoghurt samples according to Bhardwaj and Singh [10]. The protein content of WSPE was determined according to Lowry et al. [11].

Determination of degree of hydrolysis

The degree of hydrolysis (DH) was determined according to Hoyle and Merritt [12].

$$\mathrm{DH}(\%) = [\text{Soluble protein(mg)}/\text{Total protein(mg)}] \times 100$$

Measurement of antioxidant activity

The antioxidant activity was evaluated using the following two different methods:

2,2′-Azino-bis-3-ethylbenzothiazoline-6-sulfonic (ABTS) assay

The antioxidant activity of WSPE of yoghurt samples, either fresh or during cold storage, was determined spectrophotometrically according to De Gobba et al. [13] at 405 nm. The addition of antioxidant compounds reduces the ABTS acid cations, thus causing a reagent decolorization, which is measurable spectrophotometrically, depending on the antioxidant type and concentration as well as the reaction time. The ABTS radical scavenging activity (%) was calculated using the following:

$$\text{Radical scavenging}\,(\%) = 100 - \left[100 \times \left(\mathrm{A_{sample}}\right)/\left(\mathrm{A_{ctrl}}\right)\right],$$

where A_{ctrl} is the absorbance of the control sample with water instead of hydrolysate and A_{sample} is the absorbance of the hydrolysate sample.

1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical assay

The DPPH radical scavenging activity of the WSPE of fresh and cold stored yoghurt samples was spectrophotometrically determined at 517 nm according to Ahmed et al. [14].

$$\text{Radical scavenging activity}(\%) = (\mathrm{A0} - \mathrm{A1}/\mathrm{A0}) \times 100,$$

where A0 is the absorbance of the control at 517 nm and A1 is the absorbance of the WSPE at 517 nm.

SDS-PAGE analysis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis of WSPE of either fresh or cold stored yoghurt samples was performed according to Dei Piu et al. [15] by using handcast 16% (v/v) polyacrylamide gels. Gel preparation and vertical electrophoresis were carried out using Bio-Rad (Mini-PROTEANW system) equipment. To evaluate the protein molecular weights, 5 µL of standard protein and 15 µL of each sample containing around 7 µg/mL of protein were loaded. Gels were stained in a water solution containing 0.05% (w/v) Coomassie Blue, 50% (v/v) methanol, and 10% (v/v) acetic acid at 50 °C for a few minutes. They were then destained by washing with water a few times at 50 °C.

Peptide profile by ultra-high performance liquid chromatography/tandem mass spectrometry (UHPLC/MS)

Peptides were characterized by LC–MS/MS analysis using an UHPLC + Ultimate 3000 (Thermo Fisher Scientific, 4000 Roskilde, Denmark) coupled with a Q Exactive Biotech mass spectrometer (Thermo Fisher Scientific, 4000 Roskilde, Denmark) as described by De Gobba et al. [16], with some modifications. Buffer A was 0.1% formic acid in water and buffer B was 0.1% formic acid in 80% acetonitrile. Ten microliters of the samples were injected. The flow rate was 0.25 mL/min and the gradient consisted of 100% buffer A for 5 min, followed by a linear increase from 0 to 60% buffer B in 70 min. UV spectra were recorded at 214 and 280 nm. On-line MS/MS spectra were recorded in the positive mode using first the Full MS method from 200 to 2000 *m/z* with a resolution of 7000. Mass spectra of peptides were exported and compared to the buffalo's proteome from the Swissprot database using Proteome Discoverer (v 1.4, Thermo Fisher Scientific, 4000 Roskilde, Denmark).

Antibacterial activity

The antibacterial activity of the WSPE of either fresh or cold stored yoghurt samples was examined according to Correa et al. [17] by using the disc diffusion assay. Indicator microorganisms were activated in the nutrient broth and the final concentration was 10^8 CFU mL^{-1}. Aliquots of 25 µL of sample (50 mg mL^{-1}) were spotted on the freshly prepared indicator strain, and plates were incubated at the optimal temperature for each test microorganism (37 °C for *E. coli, S. Aureus, and S. typhimurium*; 40 °C for *B. cereus* and 5–6 °C for *Ps. aeruginosa*). Subsequently, zone diameters of growth inhibition (represented by clear zone) were measured and presented as inhibition zones (mm). Streptomycin 500 mg was used as a positive control and sterilized water was used as a negative control.

Statistical analysis

All experiments were carried out in triplicates and the mean values were tabulated. Differences between samples with respect to bioactivity were tested using one-way analysis of variance (GLM procedure), and means of samples were compared using Duncan's Multiple Range test (SPSS 23 static analysis, IBM).

Results and discussion

Chemical composition

Table 1 shows the chemical composition of yoghurt samples under cold storage. TS content ranged 11.71–11.75% in fresh samples and significantly increased ($p < 0.05$) during storage, up to 12.64–2.94%. The same trend was observed for both protein and fat contents. The TA increased significantly ($p < 0.05$) in all treatments during storage.

DH represents the percentage of peptide bonds cleaved. The degrees of protein hydrolysis during cold storage under the different treatments, as shown in Fig. 1, were 56.88, 70.14, and 79.45% for T1, T2, and T3, respectively; they increased in all treatments at the end of cold storage to reach 71.71, 80.62, and 81.62%, with significant difference ($p < 0.05$) between control samples (T1) and both T2 and T3. The highest values were recorded in T3, in which *Lb. helveticus* CH 5 was used owing to its higher proteolytic activity, followed by T2 with non-significant differences, and lastly T1. *Lb. helveticus* has higher proteolytic activity than most other lactobacilli and hydrolyses more casein in culture media than other species [18].

SDS-PAGE patterns of the WSPE of the yoghurt samples are shown in Fig. 2. The results indicate that all caseins were completely hydrolyzed by all strains, whereas the whey protein fractions were present even after 15 days of cold storage, suggesting the limited capacity to hydrolyze whey proteins. These results are in agreement with Gonzalez-Olivares et al. [19], who found that Lactic acid bacteria (LAB) degrade casein more than whey proteins. The intensity of β-LG band in T3, which contained *Lb. helveticus*, is light compared with that in T1 and T2, reflecting the ability of *Lb. helveticus* to hydrolyse β-LG. A similar result was reported by Bu et al. [20], who observed high hydrolysis of β-LG and α-LA with combined strains of *Lb. helveticus* and *S. thermophilus*. During cold storage, low-molecular-weight peptides appeared and their intensity increased with the storage period.

Peptide profiles of the WSPE of the fresh or cold stored yoghurt samples shown in Table 2 indicate that the molecular weight of the bioactive peptides in fresh control

Table 1 Chemical composition (%) of yoghurt during storage at 4 °C

Treatments*	Storage period (days)		
	Fresh	7	15
Total solids (TS)			
T1	$11.71^{cC} \pm 0.01$	$12.04^{bC} \pm 0.13$	$12.78^{aB} \pm 0.16$
T2	$11.78^{cA} \pm 4.43$	$12.66^{bA} \pm 0.05$	$12.94^{aA} \pm 4.78$
T3	$11.75^{cB} \pm 0.02$	$12.48^{bB} \pm 0.02$	$12.64^{cC} \pm 0.03$
Protein			
T1	$3.34^{bB} \pm 0.03$	$3.36^{bB} \pm 0.20$	$3.52^{aC} \pm 0.02$
T2	$3.52^{cA} \pm 0.02$	$4.0^{bA} \pm 0.10$	$4.17^{aB} \pm 0.02$
T3	$3.52^{cA} \pm 0.02$	$4.17^{bA} \pm 0.02$	$4.36^{Aa} \pm 0.02$
Fat			
T1	$3.03^{aA} \pm 0.03$	$3.03^{aB} \pm 0.057$	$3.03^{aB} \pm 0.05$
T2	$3.0^{bA} \pm 0.00$	$3.06^{aA} \pm 0.50$	$3.1^{aA} \pm 0.05$
T3	$3.0^{bA} \pm 0.00$	$3.06^{aA} \pm 0.057$	$3.1^{aA} \pm 0.05$
Titratable acidity (TA)			
T1	$0.65^{cB} \pm 0.005$	$0.90^{bB} \pm 0.01$	$1.02^{aC} \pm 0.02$
T2	$0.71^{cA} \pm 0.028$	$0.84^{bB} \pm 0.04$	$1.21^{aB} \pm 0.01$
T3	$0.70^{cA} \pm 0.005$	$1.02^{bA} \pm 0.01$	$1.31^{aA} \pm 0.028$

* *T1* yoghurt made with yoghurt starter, *T2* yoghurt made with a mixture of *Lb. acidophilus* 20552 ATCC + *yoghurt culture* (1:1), *T3* yoghurt made with a mixture of yoghurt culture + *Lb. helveticus* CH5 (1:1)

Superscripts (A, B, C…) at the same column indicate significant difference between treatments

Superscripts (a, b, c…) at the same row indicate significant difference between storage

(±) SD

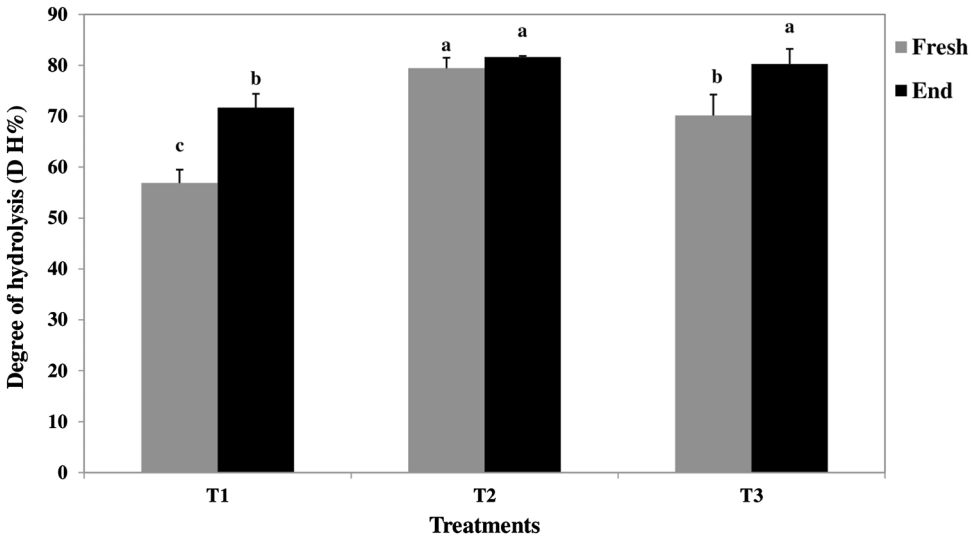

Fig. 1 Degree of hydrolysis (DH) of yoghurt samples during cold storage at 4 °C. *T1* yoghurt with yoghurt starter, *T2* yoghurt made with a mixture of *Lb. acidophilus* 20552 ATCC + yoghurt culture (1:1), *T3* yoghurt made with a mixture of yoghurt culture + *Lb. helveticus* CH5 (1:1). Different superscripts (a, b, c…) indicate significant difference between treatments

yoghurt (T1) ranged 0.68–1.99 kDa. During cold storage, lower-molecular-weight peptides were released and ranged 0.68–1.08 kDa at the end of storage. The molecular weight of the bioactive peptides in fresh acidophilus yoghurt (T2) ranged 0.78–2.33 kDa. As the storage period increased, new peptides were released with molecular weights 0.78–2.22 kDa. In fresh helveticus yoghurt (T3), smaller peptides were released compared to those in control and acidophilus yoghurt, with molecular weight ranging 0.68–1.09 kDa. At the end of storage, the molecular weight of the obtained peptides ranged 0.77–1.15 kDa. These results suggest that *Lb. helveticus* followed by *Lb. acidophilus* and yoghurt starter have the ability to produce low-molecular-weight bioactive peptides during fermentation. Most of the bioactive peptides originated from β-casein and fewer came from K-and α_{s1} casein. However, no

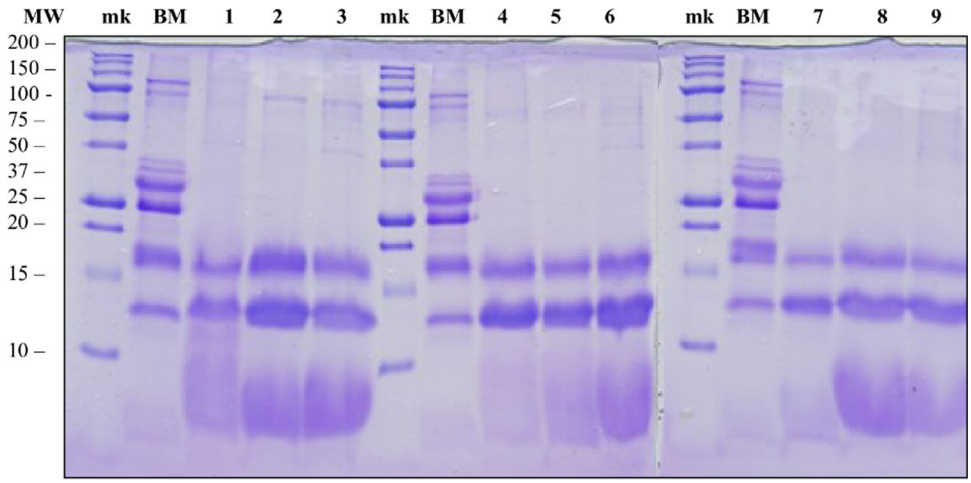

Fig. 2 SDS-PAGE patterns of the WSPE of yoghurt samples during cold storage at 4 °C. *MW* molecular weight (KDa), *Mk* internal marker, *BM* buffalo milk protein marker, *1* T1 control yoghurt (fresh), *2* (7 days), *3* (15 days), *4* T2 acidophilus yoghurt (fresh), *5* (7 days), *6* (15 days), *7* T3 helveticus yoghurt (fresh), *8* (7 days), *9* (15 days)

Table 2 Peptide profiles of the WSPE of yoghurt samples during cold storage

Sequence	Precursor protein	MW (kDa)	Activity	Storage period (days)									Ref.
				T1*			T2*			T3*			
				1	7	15	1	7	15	1	7	15	
LQDKIHP	β-Casein	0.8504	Antioxidant	+	+	+	−	−	−	+	+	+	[14]
VLPVPQK	β-Casein	0.7804	Antioxidant	−	+	+	+	+	+	+	+	+	[32]
KIHPFAQTQ	β-Casein	1.069	Antioxidant	−	−	−	+	−	−	−	−	−	[14]
VYPFPGPIPK	β-Casein	1.1146	Antioxidant	+	+	−	+	−	+	−	−	−	[13]
FVAPFPE	αS1-Casein	1.0895	ACE and Antioxidant	−	−	+	−	−	−	−	−	−	[32]
YPFPGPIPK	β-Casein	1.015	Antimicrobial	−	+	+	−	+	+	−	+	+	[31]
LVYPFPGPIPK		1.2271	−	−	−	−	−	−	−	−	−	−	–
LVYPFPGPIPKSLPQN		1.7668	−	+	−	−	+	−	−	+	−	−	–
IKHQGLPQ	αS1-Casein	0.9775	Antimicrobial	−	+	+	−	−	−	−	−	−	[33]
YQEPVLGPVRGPFPIIV	β-Casein	1.8815	Antimicrobial	−	−	−	−	+	+	−	−	−	[34]
LYQEPVLGPVRGPFPIIV		1.9944	ACE, Antioxidant	+	−	−	+	−	−	−	−	−	[33]

* *T1* yoghurt made with yoghurt starter, *T2* yoghurt made with a mixture of yoghurt culture + *Lb. acidophilus* 20552 ATCC (1:1), *T3* yoghurt made with a mixture of yoghurt culture + *Lb. helveticus* CH5 (1:1)

peptides originated from whey proteins; this result agrees with the previous reports on other types of fermented milk [21, 22].

Antioxidant peptides present in food play a vital role in the maintenance of antioxidant defense systems by preventing the formation of free radicals or by scavenging free radicals and active oxygen species, which induce oxidative damage to biomolecules and possibly cause many diseases [23]. Antioxidants from natural sources are more desirable than synthetic ones due to their carcinogenic effects [24]. Fermented milk products are an excellent source of bioactive peptides because of the proteolytic activities of the organisms involved in the fermentation [25, 26]. These peptides have an antioxidative effect [5, 27]. Figure 3(A, B) reveal a significant increment in the antioxidant activity of the WSPE of the fresh or cold stored yoghurt samples. In addition, significant differences were recorded between all treatments by the two methods. The rate of increment of the antioxidant activity (%) as determined by the ABTS assay was the highest in T1 (control 13.7%), followed by T2 (13.2%) and lastly T3 (10.5%), in which *Lb. helveticus* was used in combination with yoghurt starter.

The DPPH method is based on the ability of a sample to scavenge free stable DPPH radicals by hydrogen donation [28]. The DPPH scavenging activity of the WSPE of the yoghurt samples was 56.88–71.71% in T1, 70.14–80.62% in T2, and 79.45–81.62% in T3, in which *Lb. helveticus* was used. *Lb. helveticus* has been shown to exhibit strong proteolytic activity. The DPPH radical scavenging activity of crude yoghurt (0.2 mg/mL) was 94.47% of different

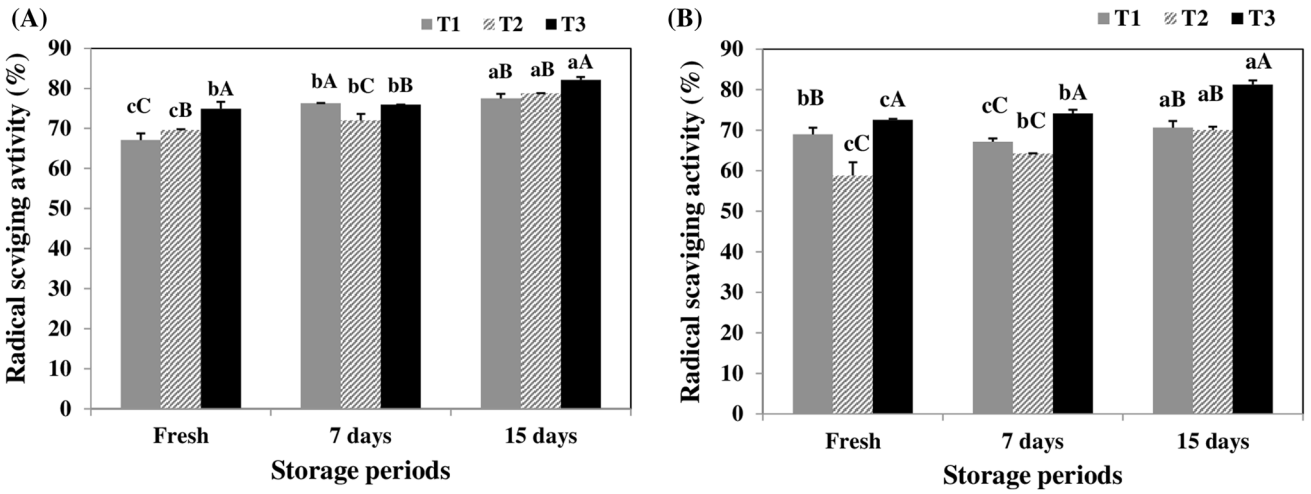

Fig. 3 Antioxidant activity (%) of the WSPE of yoghurt samples during cold storage at 4 °C. (**A**) ABTS assay, (**B**) DPPH assay. *T1* yoghurt made with yoghurt starter, *T2* yoghurt made with a mixture of *Lb. acidophilus* 20552 ATCC + yoghurt culture (1:1), *T3* yoghurt made with a mixture of yoghurt culture + *Lb. helveticus* CH5 (1:1). The superscripts a, b, c… indicate significant difference between treatments. The superscripts A, B, C… indicate significant difference between storage periods

fractions of yoghurt [29]. The authors [29] suggested that the oxidative stability of yoghurt depends on the antioxidant peptides released during the fermentation of milk by lactic acid bacteria. These peptides act as electron donors and can react with free radicals to convert them to more stable products. Milk fermented with *Lb. acidophilus* recorded the highest antioxidant activity, 63.99%, after the completion of fermentation. The lowest antioxidant activity of the same culture, which was recorded on the 10th day of storage at 4 °C, was 48.60%. The lowest antioxidant activity was seen in the milk fermented with the symbiotic cultures *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus,* which was 39.43%, as measured on the third day of storage [30].

Table 2 lists several peptides identified in the different samples. Among them, only LQDKIHP, LVYPFPGPIPKSLPQN, VYPFPGPIPK, LYQEPVLGPVRGP, LVYPFPGPIPK, VYPFPGPIPK, VLPVPQK, VAPFPE, KVLPVPQ, YQEPVLGPVRGPFPII, and VLPVPQ are already known for their antioxidant activity [13, 14, 31–34]. The lower-molecular-weight protein fractions were found to possess greater antioxidative capacity than higher-molecular-weight protein fractions. These peptides therefore can be considered as majorly responsible for the increased antioxidant activity of the samples. It is indeed known that peptides rich in Y, P, and F can contribute to the antioxidant activity [13].

The antibacterial activities of the WSPE of the fresh and cold stored yoghurt samples are shown in Table 3. Clearly, all the treatments displayed antibacterial activity against *E. coli* and this effect significantly increased at the end of cold storage, which is in accordance with Sah et al. [35]. In addition, T3 containing *Lb. helveticus* had the highest inhibition activity, followed by T1 and T2.

Concerning the antibacterial activity against *S. aureus* and *B. cereus*, both T1 and T3 had positive effect, which effect increased toward the end of the storage period, while T2 had an opposite trend.

The antibacterial activity of the WSPE of the yoghurt samples against *S. typhimurium* of all treatments decreased significantly as the storage period increased. The highest effect was observed for T1 (control yoghurt), followed by T3 and then T2.

The antibacterial activity of the control yoghurt against *P. aeruginosa* had a positive effect, which significantly increased with the storage period, while the antibacterial activity of both T2 and T3 significantly decreased toward the end of storage.

As shown in Table 2, only the peptides IKHQGLPQ, YQEPVLGPVRGPFPIIV, YPFPGPIPK, LVYPFPGPIPK, and LVYPFPGPIPKSLPQN were already known for their antibacterial activity [31, 33, 34]. The different behaviors of the samples against different strains allow us to speculate that the peptides can act against only some of the bacteria and that they can also have some synergistic effect, since there is no clear pattern for the samples with the presence of a particular peptide and antimicrobial activity. Most antimicrobial bioactive peptides can act either by penetrating and disrupting microbial membrane integrity or by translocating across the membrane and acting on internal targets [35]. *Lb. helveticus* has higher proteolytic activity (because of the secretion of proteolytic enzymes) than most other lactobacilli, and it hydrolyses more casein in culture media than other species [18].

Table 3 Antibacterial activity of the WSPE of yoghurt during cold storage (inhibition zone in mm) at a concentration of 50 mg/mL

Treatments*	Storage period (days)		
	Fresh	7	15
E. coli			
T1	$6.3^{bAB} \pm 0.5$	$7.3^{bB} \pm 0.5$	$9.6^{aA} \pm 0.5$
T2	$5.6^{bB} \pm 2.1$	$7.3^{aB} \pm 2.5$	$7.6^{aB} \pm 3.2$
T3	$7.3^{bA} \pm 2.2$	$9.0^{bA} \pm 2.7$	$11.0^{aA} \pm 3.4$
S. Aureus			
T1	$1.3^{cC} \pm 0.5$	$4.3^{bB} \pm 0.5$	$10.6^{aA} \pm 1.0$
T2	$10.3^{aA} \pm 4.8$	$7.3^{bA} \pm 2.5$	$6.3^{bC} \pm 3.7$
T3	$7.0^{bB} \pm 0.5$	$8.3^{aA} \pm 0.0$	$8.3^{aB} \pm 0.5$
B. cereus			
T1	$3.6^{bB} \pm 1.5$	$8.0^{aA} \pm 1.0$	$8.0^{aB} \pm 1.0$
T2	$11.0^{aA} \pm 4.5$	$8.0^{bA} \pm 2.6$	$7.6^{bB} \pm 3.2$
T3	$4.3^{cB} \pm 0.5$	$6.0^{bB} \pm 0.5$	$11.3^{aA} \pm 0.5$
S. typhimurium			
T1	$8.3^{aA} \pm 0.5$	$7.0^{bA} \pm 0.0$	$6.0^{cA} \pm 0.0$
T2	$7.0^{aA} \pm 2.6$	$5.3^{abB} \pm 2.4$	$4.0^{bB} \pm 0.0$
T3	$8.0^{aA} \pm 0.0$	$5.6^{bB} \pm 2.1$	$2.3^{cC} \pm 2.1$
P. aeruginosa			
T1	$2.6^{cB} \pm 0.5$	$3.6^{bA} \pm 0.5$	$5.0^{aA} \pm 0.5$
T2	$5.6^{aA} \pm 2.0$	$3.0^{bB} \pm 1.1$	$1.3^{cB} \pm 2.3$
T3	$3.6^{aB} \pm 0.5$	$2.3^{bB} \pm 0.5$	$1.3^{bB} \pm 0.5$

* *T1* yoghurt made with yoghurt starter, *T2* yoghurt made with a mixture of *Lb. acidophilus* 20552 ATCC + yoghurt culture (1:1); *T3* yoghurt made with a mixture of yoghurt culture + *Lb. helveticus* CH5 (1:1)

Superscripts a, b, c… indicate significant difference between treatments

Superscripts A, B, C… indicate significant difference between storage periods

(±) SD

In conclusion, fermentation of the milk by lactic acid bacteria releases a large number of peptides and amino acids with varying biological actions. In this study, the yoghurt starters, *Lb. acidophilus 20552* ATCC and *Lb. helveticus* CH 5, had variable proteolytic activity. The WSPE of yoghurt fermented with *Lb. helveticus* CH 5 displayed the highest antioxidant and antibacterial activities as compared to both yoghurt culture and *Lb. acidophilus* 20552.

Acknowledgements The authors thank Dr. Hashem Mohamed Abdel-Latif, Lecturer, Department of Agronomy, Faculty of Agriculture, Cairo University, Egypt for his valuable help in the statistical analysis.

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

References

1. Bonjour JP, Benoit V, Payen F, Kraenzlin M. Consumption of yogurts fortified in vitamin D and calcium reduces serum parathyroid hormone and markers of bone resorption: a double-blind randomized controlled trial in institutionalized elderly women. J. Clin. Endocrinol. Metab. 98: 2915–2921 (2013)
2. Adolfsson O, Meydani SN, Russell RM. Yogurt and gut function. Am. J. Clin. Nutr. 80: 245–256 (2004)
3. Mckinley CM. The nutrition and health benefits of yoghurt. Int. J. Dairy Technol. 58: 1–11 (2005)
4. Shah N. Functional cultures and health benefits. Int. Dairy J. 17: 1262–1277 (2007)
5. Moller NP, Scholz-Ahrens KE, Roos N, Schrezenmeir J. Bioactive peptides and proteins from foods: Indication for health effects. Eur. J. Nutr. 47: 171–182 (2008)
6. Kitts DD, Weiler K. Bioactive proteins and peptides from food sources. Applications of bioprocesses used in isolation and recovery. Curr. Pharm. Des. 9: 1309–1323 (2003)
7. Solieri L, Rutella GS, Tagliazucchi DD. Impact of non-starter lactobacilli on release of peptides with angiotensin-converting enzyme inhibitory and antioxidant activities during bovine milk fermentation. Food Microbiol. 51: 108–116 (2015)
8. Mohanty DP, Mohapatra S, Misra S, Sahu PS.Milk derived bioactive peptides and their impact on human health—A review. Saudi J. Biol. Sci. 23: 577–583 (2016)
9. AOAC. Official Methods of Analysis of AOAC Intl. 17th ed. Method 947.05. Association of Official Analytical Chemists, Arlington, VA, USA (2000)
10. Bhardwaj G, Singh B. Anti-oxidative potential of bioactive peptides released during fermentation of bovine milk with Lactic Acid Bacteria. Int. J. Cell Sci. Biotechnol. 3: 1–3 (2014)
11. Lowry OH, Rosebrough NJ, Farr AL, Randall R. Protein measurement with the Folin Phenol reagent. J. Biol. Chem. 193: 265–275 (1951)
12. Hoyle NT, Merritt JH. Quality of fish protein hydrolysates from herring (*Clupea harengus*). J. Food Sci. 59: 76–79 (1994)
13. De Gobba C, Espejo-Carpio JF, Skibsted HL, Otte J. Antioxidant peptides from goat milk protein fractions hydrolysed by two commercial proteases. Int. Dairy J. 39: 28–40 (2014)
14. Ahmed SA, El-Bassiony T, Elmalt LM, Ibrahim HR. Identification of potent antioxidant bioactive peptides from goat milk proteins. Food Res. Int. 74: 80–8 (2015)
15. Dei Piu L, Tassoni A, Serrazanetti DI, Ferri M, Babini E, Tagliazucchi D, Gianot A. Exploitation of starch industry liquid by-product to produce bioactive peptides from rice hydrolyzed proteins. Food Chem. 155: 199–206 (2014)
16. De Gobba C, Olsen K, Skibsted LH. Components of wheat flour as activator of commercial enzymes for bread improvement. Eur. Food Res. Technol. 242: 1647–1654 (2016)
17. Correa FPA, Daroit JD., Coelho J, Meira SM, Lopes CF, Segali J, Risso HP, Brandelli A. Antioxidant, antihypertensive and antimicrobial properties of ovine milk caseinate hydrolyzed with a microbial protease. J. Sci. Food Agric. 91: 2247–2254 (2011)
18. Beganovi J, Kos B, Lebos AP, Uroic K, Petra Dzidara P, Suskovic J. Proteolytic activity of probiotic strain *Lactobacillus helveticus* M92. Anaerobe 20: 58–64 (2013)
19. Gonzalez-Olivares L, Anorve-Morga J, Neda-Ovando AC, Contreras-Lope E, Jaimez-Orda J. Peptide separation of commercial fermented milk during refrigerated storage. Food Sci. Technol. (Campinas) 34: 674–679 (2014)
20. Bu G, Luo Y, Zhangb Y, Chen F. Effects of fermentation by lactic acid bacteria on the antigenicity of bovine whey proteins. J. Sci. Food Agric. 90: 2015–2020 (2010)

21. Chianese L, Caira S, Pizzolongo F, Melck D, Ferranti P, Merusi P. Production of a probiotic yogurt with increased levels of bioactive peptides. In Fermented milk (pp. 290–301). Brussels: Int. Dairy Fed. (IDF special issue. (2003)
22. Papadimitriou CG, Vafopoulou-Mastrojiannaki A, Silva SV, Gomes A, Malcata FX, Alichanidis E. Identification of peptides in traditional and probiotic sheep milk yoghurt with angiotensin I-converting enzyme (ACE)-inhibitory activity. Food Chem. 105: 647–656 (2007)
23. Gupta ABM, Kumar R, Sangwan RB. Antioxidant activity of Cheddar cheeses at different stages of ripening. Int. J. Dairy Technol. 62: 339–347 (2009)
24. Liu JR., Chen MJ, Lin CW. Antimutagenic and antioxidant properties of milk-kefir and soymilk-kefir. J. Agric. Food Chem. 53: 2467–2474 (2005)
25. Algaron FG, Le Bars MD, Monnet V. Milk fermentation by Lactococcus lactis with modified proteolytic systems to accumulate potentially bio-active peptides. Lait 84: 115–123 (2004)
26. Donkor ON, Henriksson A, Singh TK, Vasiljevic T, Shah NP. ACE-inhibitory activity of probiotic yoghurt. Int. Dairy J. 17: 1321–1331 (2007)
27. Pihlanto A. Antioxidative peptides derived from milk proteins. Int. Dairy J. 16: 1306–1314 (2006)
28. Karadag A, Ozcelik B, Saner S. Review of methods to determine antioxidant capacities. Food Anal. Methods 2: 41–60 (2009)
29. Farvin SKH, Baron CP, Nielsen NS, Otte J, Jacobsen C. Antioxidant activity of yoghurt peptides: Part 2—Characterisation of peptide fractions. Food Chem. 123: 1090–1097 (2010)
30. Gjorgievski N, Tomovska J, Dimitrovska G, Makarijoski B, Shariati MA. Determination of the antioxidant activity in yogurt. Journal of Hygienic Engineering and Design 8: 88–92 (2014)
31. Minervini F, Algaron F, Rizzello CG. Fox PF, Monnet V, Gobbetti V. Angiotensin I-Converting-Enzyme-Inhibitory and Antibacterial Peptides from *Lactobacillus helveticus* PR4 Proteinase-Hydrolyzed Caseins of Milk from Six Species. Appl. Environ. Microbiol. 69: 5297–5305 (2003)
32. Hernandez-Ledesm B, Miralles B, Amigo L, Ramos M, Recio I. Identification of antioxidant and ACE-inhibitory peptides in fermented milk. J. Sci. Food Agric. 85: 1041–1048 (2005)
33. Birkemov GA, O'Sullivan OO, Ross RP, Hill C. Antimicrobial activity of two peptides casecidin 15 and 17, found naturally in bovine colostrum. J. Appl. Microbiol. 106: 233–240 (2009)
34. Eisele T, Stressler T, Kranz B, Fischer L. Bioactive peptides generated in an enzyme membrane reactor using *Bacillus lentus* alkaline peptidase. Eur. Food Res. Technol. 236: 483–490 (2013)
35. Sah BNP, Vasiljevic T, McKechnie S, Donkor ON. Antibacterial and antiproliferative peptides in synbiotic yogurt— Release and stability during refrigerated storage. J. Dairy Sci. 99: 4233–4242 (2016)