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Research Article
Bacteriocin-like Substances Produced by Specific Strains of Lactic Acid Bacteria Isolated from Milk Products

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Abstract

Background and Objective: The biopreservation of foods via bacteriocinogenic lactic acid bacteria (LAB) isolated directly from foods is an innovative approach. Bacteriocins derived from LAB isolates may show a promising antimicrobial activities and act as barriers against spoilage and/or pathogenic microorganisms in Egyptian dairy products. **Materials and Methods:** Eleven lactic acid bacteria (LAB) were isolated from raw buffalo milk, raw cow milk, yoghurt, raib, kareish cheese, domiati cheese and mish cheese. The isolated LAB were identified at the species level as *Pediococcus acidilactici*, *Streptococcus thermophilus*, *Lactococcus lactis* subsp *cremoris* (*Lact. lactis*), *Lact. plantarum*, *Lact. lactis* subsp. *lactis*, *Enterococcus italicus* (*Entero. italicus*), *Entero. camelliae*, *Lactobacillus delbrueckii* subsp *lactis* (*Lb. delbrueckii*), *Lb. helveticus*, *Lb. buchneri* and *Lb. fermentum*. The antimicrobial effects of these LAB species and three reference LAB strains were screened using an agar spot test and an agar well diffusion assay. **Results:** Crude bacteriocin supernatant fluid (CBSF) samples derived from these LAB species were active against the Gram-positive bacterium *Staphylococcus aureus* and the Gram-negative bacteria *Listeria monocytogenes* and *Escherichia coli*. The CBSFs derived from *Lact. plantarum*, *Lb. delbrueckii* subsp. *lactis* and *Lb. fermentum* were more effective against *Staph. aureus*. The CBSFs derived from species such as *S. thermophilus*, *Lact. Plantarum Lb. delbrueckii* subsp *lactis* and *Lb. fermentum* were effective against *Listeria monocytogenes*. In addition, CBSFs from *Lact. lactis* subsp. *cremoris*, *Lb. helveticus* and *Lb. fermentum* showed antimicrobial activity against *E. coli* and *Salmonella enterica*. The importance of major parameters (temperature, pH values and sensitivity to pepsin) for bacterial life and CBSF secretion was tested. The highest antimicrobial activity was observed at 30°C and a pH of 2.0-6.5 and pepsin inhibited the antimicrobial activity of the LAB by 90.9%. **Conclusion:** The antimicrobial activities of the bacteriocins produced by local LAB species acted as a barrier against spoilage and/or pathogenic microorganisms in dairy products.

Key words: Lactic acid bacteria, bacteriocin, antimicrobial activity, sensitivity of pepsin, crude bacteriocin supernatant fluid, dairy products

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INTRODUCTION

The growing need for naturally safe and healthy food has attracted increasing interest in the application of bacteriocin-producing lactic acid bacteria (LAB), which are currently used as protective cultures for the production of fermented foods and dairy products[1,2]. The LAB are used in food processing because they have the ability to improve the organoleptic characteristics of food.

In recent years, extensive study has been performed with bacteriocins and bacteriocin-producing LAB strains to investigate their potential use as bio-preservatives[3]. The LAB are generally recognized as safe (GRAS microorganisms) and play an important role in food and feed fermentation and preservation, either as natural microflora or as starter cultures that are added under controlled conditions. The preservative effects exerted by LAB are primarily attributable to the production of organic acids (such as lactic acid), which result in a lower pH[4].

Chemically, LAB bacteriocins are ribosomal proteins that exert bactericidal effects, have high molecular weights and are rapidly digested by the proteinases in the human digestive tract. LAB bacteriocins also have potential practical applications in food preservation or the prevention and treatment of Gram-positive and Gram-negative bacterial infections[5-7]. Study of Djadouni and Kihal[8] found that the LBbb 0141 strain of LAB produced an antimicrobial compound with a wide spectrum of action that inhibited the growth of ten Gram-positive and Gram-negative indicator strains. Certain LAB produce antibiotics that affect Gram-positive pathogenic bacteria and fungi[9]. The *in vitro* potential of nine bacteriocin-producing LAB strains isolated from vacuum-packaged, cold-smoked salmon (CSS) were tested for their possible use as bio-preservative cultures against *Listeria monocytogenes* by Tome *et al.*[10].

Recently, bacteriocin-producing by LAB is likely used due to their safe GRAS status use as safe additives especially in food industry as bio-preservatives[11]. Bacteriocins are active biologically complex protein against other bacteria, either within the same species or across genera[12,13]. Many bacteriocins are produced by food-grade LAB, a phenomenon that offers food scientists the potential to direct or prevent the development of specific bacterial species in food. This option is particularly useful for preservation or food safety applications, but it also has implications for the development of desirable flora in fermented food. Thus, bacteriocins are used to confer a rudimentary form of innate immunity in foodstuffs, allowing processors to extend their control over food flora long after manufacturing[13].

Nisin is the first studied bacteriocin that naturally produced by *Lactococcus lactis* for application in food technology. It has been applied as an additive to specific foods around the world[14]. Some food additives have been in use to realize the effectiveness of nisin vis various spoilage and pathogenic microorganisms such as *L. monocytogenes* and evaluate its potential application in several food products[15,16]. Although several bacteriocins from LAB such as pediocin have been characterized to date but they are not currently approved as antimicrobial food additives[17].

The antimicrobial activity of LAB may be attributable to the production of a number of antimicrobial substances such as lactic acid, hydrogen peroxide, diacetyl and bacteriocins[18]. Enterocin E-760 exerts broad antimicrobial activity against Gram-positive and Gram-negative bacteria[19]. The antimicrobial activity of Microgard™, either individually or in combination with nisin, was investigated against a *Listeria* inoculant in liquid cheese whey (LCW). Microgard™ did not reduce the initial count of the *Listeria* inoculant during storage at 7, 12, 20 and 25°C and it showed a response similar to that of untreated whey[15]. Another study by De Freitas *et al.*[16] evaluated the effectiveness of nisaplin, a commercial product in which nisin is the active component, for decreasing the staphylococcal population in refrigerated pizza dough. Two different nisaplin treatments were able to reduce the *Staphylococcus* sp. count (CFU g^{-1}) in refrigerated pizza dough and the authors suggested that nisin is a promising alternative to traditional antibiotics for controlling the survival of pathogenic microorganisms, particularly *Staphylococcus*, in foods such as refrigerated pizza dough.

Fresh fruits and vegetables harbour various microorganisms, some of which are psychotropic. *L. monocytogenes* is a pathogenic bacterium that is capable of growing at refrigerator temperatures. Moreover, it is also tolerant to acidic pH values and to salt concentrations up to 10%[20,21]. Therefore, it is important to seek bio-preservatives that control both spoilage and pathogenic microorganisms, including *L. monocytogenes*. Although several studies have indicated the presence of LAB species exerting antagonistic activities that improve the quality and safety of meat and dairy products[22,23], few reports have studied fresh produce[24]. From the mechanism point of view, the specific cell surface receptors can be recognized when the bacteriocin gain entry into the target and then kill the cell by forming ion-permeable channels in the cytoplasmic membrane by nonspecific degradation of cellular DNA, by inhibition of protein synthesis through the specific cleavage of 16S RNA or inducing cell lysis[25,26]. However, LAB contaminate milk obtained from

animals that have been treated with antibiotics and thus carry populations of resistant bacteria[27,28]. The isolation and screening of microorganisms from natural sources represents the most powerful method of obtaining useful and genetically stable strains for industrially important products[29].

The aim of this study was first to determine the compatibility among selected species of isolated and identified LAB from raw buffalo milk, raw cow milk, yoghurt, raib, kareish cheese, domiati cheese and mish cheese and to identify their antagonistic activities against six pathogenic organisms in an agar spot test and an agar well diffusion assay. The second aim was to gauge the activity of bacteriocin in response to different temperatures, pH values and proteolytic enzymes.

MATERIALS AND METHODS

Materials: The dairy products (raw milk, yoghurt, raib, domiati, kareish and mish cheese) under investigation were collected from departmental stores, supermarkets and farms between 2015 and 2016.

Microorganisms: A selection of 11 species was identified according to previously identified criteria using morphological and biochemical methods[30,7]. Eleven species of LAB isolated from dairy products (raw milk, yoghurt, raib, domiati, kareish and mish cheese) were incubated on MRS agar[31] at 37°C for 24 h and on M17 agar (Biokar, Beau Vais, France)[32] at 30°C for 24 h[33,34]. Eleven LAB species consisted of six Gram-positive strains, *S. thermophilus*, *Lact. lactis* subsp *cremoris*, *Lact. plantarum*, *Lact. lactis* subsp *lactis*, *Entero. italicus* and *Entero. camelliae* and five Gram-positive rods, *Lb. delbrueckii* subsp *lactis*, *Lb. helveticus*, *Lb. buchneri* and *Lb. fermentum*.

Bacterial strains: Experiments were performed to detect the antagonistic activity of six indicator organisms, specifically *Staphylococcus aureus* ATCC 43300, which was kindly provided by the Department of Microbiology, Faculty of Medicine, Alexandria University and clinical species of *Candida albicans*, which were provided by the Department of Microbiology and Natural Products, National Research Center (NRC), Cairo, Egypt. *Listeria monocytogenes* NRRL1020, *E. coli* and *Klebsiella pneumoniae* were kindly provided by the Department of Microbiology, Medical Research Institute (MRI), Alexandria University, Egypt. *Salmonella enterica* PT4 was obtained from the Department of Food Science and Technology, Agriculture University of Athens, Greece.

Antibacterial activity: All LAB species were maintained at 4°C in MRS or M17 broth. All bacterial cultures were sub-cultured at 15 days intervals. Prior to their use in experiments, LAB back cultures were sub-cultured in MRS or M17 broth. To detect antimicrobial activity, an agar spot test and an agar well diffusion assay were performed.

Surface diffusion: Overnight cultures of 11 LAB species and the 3 reference LAB strains to be tested were grown in MRS or M17 broth at 30°C/16 h. Culture aliquots (5 µL) were spotted onto the surfaces of agar plates (MRS and M17 broth with 1.2% agar) and incubated at 37°C/24 h to allow colonies to develop. The spots on each plate were overlaid with approximately 7 mL of soft nutrient agar, soft Baird Parker agar, soft *Salmonella Shigella* (SS) agar, soft *Listeria*-selective agar or soft yeast mold (YM) agar (0.75% agar) and inoculated with 650 µL of a 10^{-1} dilution (10^5 CFU mL^{-1}) of an overnight culture of the relevant pathogenic organism.

The plates were incubated at 37°C/24 h[33,34]. Clear zones around the spots were measured and inhibition reactions were scored as positive if the width of a clear zone around a LAB colony was 5 mm or larger[1].

Mass diffusion: Suspensions of pathogenic cells stored at -20°C were used to inoculate nutrient agar, Baird-Parker-agar, SS-agar, *Listeria*-selective-agar or YM-agar growth medium at a final concentration of 10^5 CFU mL^{-1} at 45°C and poured into petri dishes, then cooled at 4°C for 2 h. After the plates solidified, four wells, each 6 mm in diameter, were cut into the agar and the base of each well was sealed with a drop of melted sterile water agar (20 g L^{-1} water).

The ability of a probiotic to inhibit a pathogen is considered its most important health-promoting property[35]. Eleven Gram-positive bacteriocin-producing LAB species were isolated from milk products. In addition, three reference LAB strains were screened and tested for their ability to produce inhibitory substances against six pathogenic organisms in a diffusion agar assay. The LAB species and reference strains were grown individually in MRS or M17 broth at 37°C/18 h. Screens were performed under anaerobic conditions using melted paraffin to avoid H_2O_2 formation[36]. Extracts of lactic cultures obtained from overnight cultures underwent one of the following treatments: (1) Centrifuged at 3000 rpm for 15 min, (2) Neutralized by adding 5 N NaOH to exclude the effects of organic acids and (3) Adjusted to pH 6.5 with 1 N NaOH and the possible inhibitory actions of H_2O_2 were eliminated by adding sterile catalase solution (1 mg mL^{-1}) at 25°C/30 min. Each solution was sterilized using a millipore membrane filter (0.45 µm pore diameter) before it was loaded into wells. These extracts were designated crude bacteriocin supernatant fluid (CBSF).

Fifty microliters aliquots of CBSF from each tested LAB species and 3 reference LAB strains were added to each well in seeded plates. The plates were then incubated at 37°C/24 h. After the incubation period, clear inhibition zones appearing around each well were measured in mm. An arbitrary unit (AU) of CBSF per µL was calculated by dividing the volume of the clear zone by the volume of the well[37].

Sensitivity of bacteriocins to heat treatment: The effects of temperature on bacteriocin activity for the selected microbes were determined as described previously[38]. Ten millilitres of CBSF from each test species (11) and the reference strains (three strains) were subjected to heat treatment in a water bath at 60°C/60, 100°C/20 and 121°C/15 min (autoclaving). CBSF antimicrobial activity against pathogenic microorganisms was then tested by using an agar well diffusion assay[39].

Activity of bacteriocins at different pH values: Bacteriocin stability was tested at different pH values by adjusting the pH of the CBSF of 11 LAB and three reference strains to pH 2.0, 6.5 and 9.0 with 1 N HCL and 10 N NaOH. An agar well diffusion assay was performed to evaluate residual activity[40,41].

Proteolytic enzyme inactivation: The proteolytic enzyme sensitivities of the inhibitory substances produced by 11 LAB species were tested individually by adding pepsin (Merck 7189) to the cells. Free culture supernatant at 37°C/60 min and residual supernatant activity were determined using an agar well diffusion assay as described previously by Bromberg *et al.*[42]. All measurements were performed in triplicate and average values are reported.

Statistical analysis: A one-way analysis of variance was performed to analyse the data using the General Linear Model procedure in SAS (Statistical Analysis System) 1998 (SAS Inst. Inc. Cary, NC)[43]. A previously described method by Duncan *et al.*[44] was used to compare the least significant difference (LSD) of the treatments at a p-value of $\leq$0.05. Mean data values were calculated in triplicate as the mean$\pm$standard error of the mean (SEM).

RESULTS AND DISCUSSION

Antibacterial activity of LAB: The inhibition diameter was calculated for each LAB species as the average of the inhibition diameter against every pathogenic organism, as shown in Table 1. Eleven LAB species were isolated from milk products, the three LAB used as reference strains showed antimicrobial activity against one or more test microorganisms according to the agar spot test. The Gram-positive bacterium *Staph. aureus* was quite sensitive to bacteriocins produced by different LAB strains compared with that produced by *C. albicans*, which exhibited no effect. The Gram-negative bacteria *L. monocytogenes*, *E. coli* and *S. enterica* showed considerable sensitivity. The obtained results were consistent with studies reporting on bacteriocin-producing LAB isolated from Munster cheese[45].

In Table 2, some LAB species demonstrated clear inhibitory activity against the tested pathogenic organisms. *Lact. plantarum*, *Lb. delbrueckii* subsp. *lactis*, *Lb. fermentum*, *S. thermophilus*, *Lact. lactis* subsp. *lactis*, *Entero. camelliae* and *S. thermophilus* TH4 showed high activity against *Staph. aureus* (14.69, 14.69, 14.69, 13.44, 13.44, 13.44 and 13.44 AU, respectively). However, there was less inhibition demonstrated for *E. coli*, *S. thermophilus*, *Lact. plantarum*, *Lb. delbrueckii* subsp. *lactis*, *Lb. fermentum* and *S. thermophilus* (7.11, 6.25, 6.25, 4.69, 7.11, 5.44 and 4.69 AU, respectively). In addition, *S. thermophilus* TH4 demonstrated inhibition activity against *L. monocytogenes* (10.02, 10.02, 10.02, 10.02, 10.02 and 10.02 AU) as well as against *K. pneumoniae*, as shown in Table 2. Similar antimicrobial activity results were reported previously by Schillinger and Lucke[1], Carrasco *et al.*[46], Nespolo and Brandelli[47] and Kivanc *et al.*[48]. The results of this study showed that the antimicrobial activities of the bacteriocins derived from LAB species were generally greater against the tested Gram-positive bacteria than Gram-negative organisms according to the results obtained from the agar spot and agar well diffusion tests.

According to results from the agar spot test and agar well diffusion assay, all LAB species tested showed no effects against *C. albicans* and *K. pneumoniae*, while *K. pneumoniae* was sensitive to *Bifidobacteriun bifidum* (*B. bifidum*) (Bb12). Furthermore, there were no significant differences at $p<0.05$ between both methods in these presented results. In addition, the three reference strains, *S. thermophilus* TH4, *Lb. acidophilus* (CYX11-1) and *B. bifidum* (Bb12), gave the highest inhibition diameters and AU values compared with other tested species, as indicated in Table 2. By contrast, results reported previously by Fricourt *et al.*[49] indicated that *Lb. plantarum* showed inhibitory activity in an agar well diffusion test, while another study by Kivanc[50] reported that *Lb. plantarum* showed no inhibitory effects against *Staph. aureus*. Hence, there was no difference in performance between the two methods we adopted in this study.

Table 1: Lactic acid bacteria (LAB) species isolated from different milk products and reference strains that demonstrated positive inhibition against six pathogenic organisms (strains) in an agar spot test

		Pathogenic organism strains					
		Gram-positive		Gram- negative			
LAB-species	Source products	*Staphylococcus aureus*	*Candida albicans*	*Listeria monocytogenes*	*Escherichia coli*	*Salmonella enterica*	*Klebsiella pneumoniae*
LAB: Cocci							
Pediococcus acidilactici	Yoghurt+Raib	+	-	+	+	-	-
Staphylococcus thermophilus	Raw milk+Yoghurt+Ka+Domiati+Mish	+	-	+	+	-	-
Lactococcus lactis subsp. *cremoris*	Raw milk+Raib+Ka+Domiati+Mish	+	-	+	+	+	-
Lactococcus plantarum	Raib+Ka+Domiati+Mish	+	-	+	+	+	-
Lactococcus lactis subsp. *lactis*	Raw milk+Ka	+	-	+	+	+	-
Enterococcus italicus	Ka+Mish	+	-	+	+	-	-
Enterococcus camelliae	Ka+Mish	+	-	+	+	-	-
LAB: Bacilli							
Lactobacillus delbrueckii subsp. *lactis*	Raw milk+Yoghurt+Raib+Ka+Domiati+Mish	+	-	+	+	-	-
Lactobacillus helveticus	Raw milk+Yoghurt+Raib+Ka+Domiati+Mish	+	-	+	+	+	-
Lactobacillus buchneri	Raw milk+Ka+Mish	+	-	+	+	-	-
Lactobacillus fermentum	Raw milk+Yoghurt+Raib+Ka+Domiati+Mish	+	-	+	+	+	-
Reference LAB strains							
Staphylococcus thermophilus (TH_4)		+	-	+	+	+	-
Lactobacillus acidophilus (CYX_{11-1})		-	-	+	+	+	-
Bif. bifidum (Bb_{12})		-	-	+	+	+	+

+: Large inhibition zone (>1.0 mm), (+): Small inhibition zone (0.5-1.0 mm), Ka: Kareish cheese

Table 2: Antagonistic activities of the identified LAB isolated from different dairy products that showed inhibition zones against six pathogenic organisms in a well diffusion assay

	Pathogenic organisms												
	Gram-positive						Gram-negative						
	Staphylococcus aureus		*Candida albicans*		*Listeria monocytogenes*		*Escherichia coli*		*Salmonella enterica*		*Klebsiella pneumoniae*		
LAB-genera	Diameter of inhibition zone (mm)*	Inhibitory activity (AU)	Diameter of inhibition zone (mm)*	Inhibitory activity (AU)	Diameter of inhibition zone (mm)*	Inhibitory activity (AU)	Diameter of inhibition zone (mm)*	Inhibitory activity (AU)	Diameter of inhibition zone (mm)*	Inhibitory activity (AU)	Diameter of inhibition zone (mm)*	Inhibitory activity (AU)	Total No. of isolates
LAB: Cocci													
Ped. acidilactici	20±1.0^{a}	11.11±1.05^{a}	0	0	17±0.0^{b}	8.02±0.0^{b}	16±0.0^{b}	7.11±0.0^{a}	0	0	0	0	
S. thermophilus	22±0.0^{a}	13.44±0.0^{a}	0	0	19±0.0^{a}	10.02±0.0^{a}	13±0.0^{c}	4.69±0.0^{c}	0	0	0	0	
Lac. lactis subsp. *cremoris*	19±1.5^{a}	10.02±1.90^{a}	0	0	18±0.0^{a}	9.00±0.0^{a}	16±0.0^{b}	7.11±0.0^{b}	16±0.0^{b}	7.11±0.0^{b}	0	0	
Lac. plantarum	23±0.0^{a}	14.69±0.0^{a}	0	0	19±0.0^{a}	10.02±0.0^{a}	16±0.0^{b}	7.11±0.0^{b}	13±0.0^{c}	4.69 ±0.0^{c}	0	0	
Lac. lactis subsp. *lactis*	22±1.0^{a}	13.44±1.05^{a}	0	0	18±0.0^{a}	9.00±0.0^{a}	16±0.0^{b}	7.11±0.0^{b}	14±0.0^{c}	5.44±0.0^{c}	0	0	
Entero. italicus	20±0.0^{a}	11.11±0.0^{a}	0	0	17±0.0^{a}	8.02±0.0^{b}	15±0.0^{c}	6.25±0.0^{c}	0	0	0	0	
Entero. camelliae	22±0.0^{a}	13.44±2.44^{a}	0	0	17±0.0^{a}	8.02±0.0^{b}	14±0.0^{c}	5.44±0.0^{c}	0	0	0	0	
LAB: Bacilli													
Lb. delbrueckii subsp. *lactis*	23±0.0^{a}	14.69±0.0^{a}	0	0	19±0.0^{a}	10.02±0.0^{a}	15±0.0^{c}	6.25±0.0^{c}	0	0	0	0	
Lb. helveticus	19±1.5^{a}	10.02±1.5^{a}	0	0	18±0.0^{a}	9.00±0.0^{a}	14±0.0^{c}	5.44±0.0^{c}	16±0.0^{b}	7.11±0.0^{b}	0	0	
Lb. buchneri	18±2.0^{a}	9.00±2.10^{a}	0	0	17±0.0^{a}	8.02±0.0^{b}	16±0.0^{b}	7.11±0.0^{b}	0	0	0	0	
Lb. fermentum	23±0.0^{a}	14.69±0.0^{a}	0	0	19±0.0^{a}	10.02±0.0^{a}	15±0.0^{c}	6.25±0.0^{c}	17±0.0^{b}	8.02±0.0^{b}	0	0	
LAB reference strains													
S. thermophilus (TH4)	22±2.0^{a}	13.44±2.01^{a}	0	0	19±0.0^{a}	10.02±0.0^{a}	13±0.0^{c}	4.69±0.0^{c}	0	0	0	0	
Lb. acidophilus (CY-X_{11-1})	0	0	0	0	17±0.0^{a}	8.02±0.0^{b}	20±0.0^{a}	11.11±0.0^{a}	0	0	0	0	
Bifid. bifidum (Bb - 12)	0	0	0	0	15±0.0^{c}	6.25±0.0^{c}	20±0.0^{a}	11.11±0.0^{a}	0	0	14±0.0^{c}	5.44±0.0^{c}	

The results are presented as the mean value±SEM*: Inhibition zone around the well, ^{abc}Values expressed that were different in the treatment are significantly different at p<0.05, AU: Arbitrary unit, 0: Not active, <5 mm: No inhibition, 5-10 mm: Low inhibition, 11-20 mm: Moderate inhibition, 21-30 mm: High inhibition

Effects of different heat treatments and pH values on bacteriocin activity: As presented in Table 3, the CBSFs produced by *P. acidilactici*, *S. thermophilus*, *Lact. plantarum*, *Lact. lactis* subsp. *lactis*, *Lb. delbrueckii* subsp. *lactis*, *Lb. helveticus*, *L. buchneri*, *Lb. fermentum*, *S. thermophilus* TH4, *Lb. acidophilus* (CYX11-1) and *Bif. bifidum* (Bb12) demonstrated similar heat stability levels under different heat treatments (60°C/60, 100°C/20 and 120°C/15 min) against *L. monocytogenes* (8.02, 10.02, 10.02, 9.0, 10.02, 9.0, 8.02, 10.02, 10.02, 8.02 and 6.25 AU, respectively). After heating the samples at 60°C/60, 100°C/20 and 121°C/15min, a loss in heat stability was observed based on the activity against *Staph. aureus* presented in Table 3. The CBSFs produced by *P. acidilactici*, *S. thermophilus*, *Lact. plantarum*, *Lact. lactis* subsp *lactis*, *Lb. delbrueckii* subsp *lactis*, *Lb. buchneri*, *Lb. fermentum* and *S. thermophilus* TH4 showed stability at different heat treatments against *E. coli* (7.11, 4.69, 7.11, 7.11, 6.25, 5.44, 7.11, 6.25 and 4.69 AU, respectively) but were not heat stable under different heat treatments against *Staph. aureus*. As shown in Table 3, the antimicrobial substances produced by *Lact. lactis* subsp *lactis* and *Lb. buchneri* demonstrated similar heat stability levels at different heat treatments against *Staph. aureus* (13.44 and 9.0 AU, respectively), while *Lact. plantarum*, *Lact. lactis* subsp *lactis*, *L. helveticus* and *Lb. fermentum* were heat stable after treatment at 60°C/60, 100°C/20 and 121°C/15 min in terms of activity against *Salmonella enterica* (4.69, 5.44, 7.11 and 8.02 AU, respectively). The bacteriocin produced by *Lb. acidophilus* (CYX11-1) was stable at 60, 100 and 121°C against both *K. pneumoniae* (4 AU) and *L. monocytogenes* (8.0 AU). This bacteriocin demonstrated no heat stability against *E. coli*, but its antimicrobial activity was eliminated after being autoclaved at 121°C for 15 min. The heat stability of bacteriocins produced by different LAB at 121°C for 15 min was reported earlier for Lactocin RN 78 [51] and *L. brevis* OGI[52], these results were similar to this study finding showing the loss of activity of antibacterial substances produced by *Lactobacillus* sp. after heat treatment at 121°C for different amounts of time. Moreover, Fatima and Mebrouk[53] reported on the effects of heat and pH. The bacteriocins from two LAB strains were considered to be extremely heat stable because their antibacterial activity was not altered by heat treatment after 15 min at 121°C. With respect to temperature stability, the examined bacteriocins exhibited strong heat stability, placing them within the heat stable and low-molecular-weight group of bacteriocins. The antimicrobial activity of the extracted bacteriocin was destroyed by treatment with proteinase K because there was no inhibition zone compared with that produced by the untreated bacteriocin sample (control). Similar results were also reported by other studies Todorov *et al.*[40], Corsetti *et al.*[54], Hernandez *et al.*[55], Rattanachaikunsopon and Phumkhachorn[56]. Furthermore, the stability of bacteriocin in response to high temperatures was reported previously by Klaenhammer[57], the study identified the following four distinct classes o f LAB bacteriocins based on biochemical and genetic characterization: Lantibiotics (Class I), small, heat-stable, non-lanthionine peptides (Class II), large heat-labile proteins (Class III) and complex bacteriocins containing chemical moieties such as lipids and carbohydrates (Class IV).

Table 4 presents the pH stability of CBSFs studied over a ranged from 2.0-9.0. The results showed that the antimicrobial activity of the supernatants were highest at pH 2.0 against only *Staph. aureus*, *L. monocytogenes*, *E. coli* and *Salmonella enterica*. All examined CBSFs were completely inactive above pH 9.0. This finding was also previously reported by another study Todorov *et al.*[40], in which various physicochemical factors appeared to affect bacteriocin production as well as activity. The data presented in Table 4 show that inhibitory activity was decreased against *Staph. aureus*, *L. monocytogenes* and *E. coli* only at pH 6.5. By contrast, maximum activity was observed at pH 2.0 and 6.5 and at 30°C, as shown in Table 3 and 4. These results were consistent with other studies Thanh *et al.*[41], Parada *et al.*[58], Cheikhyoussef *et al.*[59,60]. The resulting bacteriocin was stable over a wide pH range, which is a common feature of many bacteriocins[51,53-57]. Bacteriocin characterization showed that these molecules are active over certain temperature and pH ranges[58]. Present results were also confirmed by a previous study Fatima and Mebrouk[53] that examined pH stability over a range from pH 2-12. These bacteriocins were active at pH values ranging from 2-6.0 but were reduced at higher pH values, as shown in Table 4. Thus, the observed wide range of pH tolerance indicates that these bacteriocins may be useful in acidic as well as non-acidic foods.

Sensitivity to a proteolytic enzyme: The pepsin sensitivities of the CBSFs produced by the eleven examined LAB species are presented in Table 5. In this study, most of the compounds were fully inactivated by the proteolytic enzyme, indicating their protein-based, aqueous nature. These species were completely inactivated by pepsin, particularly *E. italicus*, which is generally resistant to enzyme treatment. Similar results were reported previously by Bromberg *et al.*[42], the study found that pepsin inhibited the antagonistic activity of 90% of antibacterial substances produced by LAB strains isolated from meat and meat products. Table 5 shows that pepsin inhibited the antagonistic activity of 91.91% of LAB. Consistent with

Table 3: Antagonistic activities of CBSFs secreted by LAB under different heat treatments against tested microorganisms in a well diffusion assay

Treatments	Pathogenic organisms											
	Gram-positive						Gram-negative					
	Staphylococcus aureus		*Candida albicans*		*Listeria monocytogenes*		*Escherichia coli*		*Salmonella enterica*		*Klebsiella pneumoniae*	
	Diameter of inhibition zone (mm)*	Inhibitory activity (AU)	Diameter of inhibition zone (mm)*	Inhibitory activity (AU)	Diameter of inhibition zone (mm)*	Inhibitory activity (AU)	Diameter of inhibition zone (mm)*	Inhibitory activity (AU)	Diameter of inhibition zone (mm)*	Inhibitory activity (AU)	Diameter of inhibition zone (mm)*	Inhibitory activity (AU)
Pediococcus acidilactici												
Neutralized	20±1.0^{a}	11.11±1.05^{a}	0	0	17±0.0^{b}	8.02^{b}±0.0^{b}	16^{b}±0.0^{b}	7.11±0.0^{b}	0	0	0	0
Heat												
60°C for 60 min	20±0.0^{a}	11.11±0.0^{a}	0	0	17±0.0^{b}	8.02±0.0^{b}	16±0.0^{b}	7.11±0.0^{b}	0	0	0	0
100°C for 20 min	16±0.0^{c}	7.11±0.0^{c}	0	0	17±0.0^{b}	8.02b±0.0^{b}	16±0.0	7.11±0.0^{b}	0	0	0	0
121°C for 15 min	16±0.0	7.11±0.0^{c}	0	0	17±0.0^{b}	8.02±0.0	16±0.0	7.11±0.0^{b}	0	0	0	0
Streptococcus thermophilus												
Neutralized	22±0.0^{a}	13.44±0.0^{a}	0	0	19±0.0^{a}	10.02±0.0^{a}	13±0.0^{c}	4.69±0.0^{c}	0	0	0	0
Heat												
60°C for 60 min	20±0.0^{a}	11.11±0.0^{a}	0	0	19±0.0^{a}	10.02±0.0^{a}	13±0.0^{c}	4.69±0.0^{c}	0	0	0	0
100°C for 20 min	18±0.0^{a}	9.00±0.0^{a}	0	0	19±0.0^{a}	10.02±0.0^{a}	13±0.0^{c}	4.69±0.0^{c}	0	0	0	0
121°C for 15 min	15±0.0^{c}	6.25±0.0^{c}	0	0	19±0.0^{a}	10.02±0.0^{a}	13±0.0^{c}	4.69±0.0^{c}	0	0	0	0
Lactococcus lactis* subsp. *cremoris												
Neutralized	19±1.50^{a}	10.02±1.90^{a}	0	0	19±0.0^{a}	10.02±0.0^{a}	16±0.0^{b}	7.11±0.0^{b}	16±0.0^{b}	7.11±0.0^{b}	0	0
Heat												
60°C for 60 min	15±0.0^{c}	6.25±0.0^{c}	0	0	16±0.0^{b}	7.11±0.0^{b}	13±0.0^{c}	4.69±0.0^{c}	15±0^{c}	6.25±0.0^{c}	0	0
100°C for 20 min	13±0.0^{c}	4.69±0.0^{c}	0	0	14±0.0^{c}	5.44±0.0^{c}	13±0.0^{c}	4.69±0.0^{c}	0	0	0	0
121°C for 15 min	13±0.0^{c}	4.69±0.0^{c}	0	0	13±0.0^{c}	4.69±0.0^{c}	13±0.0^{c}	4.69±0.0^{c}	0	0	0	0
Lactococcus plantarum												
Neutralized	23±0.0^{a}	14.69±0.0^{a}	0	0	19±0.0^{a}	10.02±0.0^{a}	16±0.0^{b}	7.11±0.0^{b}	13±0.0^{c}	4.69±0.0^{c}	0	0
Heat												
60°C for 60 min	23±0.0^{a}	14.69±0.0^{a}	0	0	19±0.0^{a}	10.02±0.0^{a}	16±0.0^{b}	7.11±0.0^{b}	13±0.0^{c}	4.69±0.0^{c}	0	0
100°C for 20 min	22±0.0^{a}	13.44±0.0^{a}	0	0	19±0.0^{a}	10.02±0.0^{a}	16±0.0^{b}	7.11±0.0^{b}	13±0.0^{c}	4.69±0.0^{c}	0	0
121°C for 15 min	22±0.0^{a}	13.44±0.0^{a}	0	0	19±0.0^{a}	10.02±0.0^{a}	16±0.0^{b}	7.11±0.0^{b}	13±0.0^{c}	4.69±0.0^{c}	0	0
Lactococcus lactis* subsp. *lactis												
Neutralized	22±1.0^{a}	11.11±0.0^{a}	0	0	18±0.0^{a}	9.00±0.0^{a}	16±0.0^{b}	7.11±0.0^{b}	14±0.0^{c}	5.44±0.0^{c}	0	0
Heat												
60°C for 60 min	22±0.0^{a}	13.44±0.0^{a}	0	0	18±0.0^{a}	9.00±0.0^{a}	16±0.0^{b}	7.11±0.0^{b}	14±0.0^{c}	5.44±0.0^{c}	0	0
100°C for20 min	22±0.0^{a}	13.44±0.0^{a}	0	0	18±0.0^{a}	9.00±0.0^{a}	16±0.0^{b}	7.11±0.0^{b}	14±0.0^{c}	5.44±0.0^{c}	0	0
121°C for 15 min	22±0.0^{a}	13.44±0.0^{a}	0	0	18±0.0^{a}	9.00±0.0^{a}	16±0.0^{b}	7.11±0.0^{b}	14±0.0^{c}	5.44±0.0^{c}	0	0
Enterococcus italicus												
Neutralized	20±0.0^{a}	11.11±0.0^{a}	0	0	17±0.0^{b}	8.02±0.0^{b}	15±0.0^{c}	6.25±0.0^{c}	0	0	0	0
Heat												
60°C for 60 min	15±0.0^{c}	6.25±0.0^{c}	0	0	15±0.0^{c}	6.25±0.0^{c}	13±0.0^{c}	4.69±0.0^{c}	0	0	0	0
100°C for 20 min	13±0.0^{c}	4.69±0.0^{c}	0	0	12±0.0^{c}	4.00±0.0^{c}	12±0.0^{c}	4.00±0.0^{c}	0	0	0	0
121°C for 15 min	13±0.0^{c}	4.69±0.0	0	0	12±0.0^{c}	4.00±0.0^{c}	12±0.0^{c}	4.00±0.0^{c}	0	0	0	0
Enterococcus camelliae												
Neutralized	22±0.0^{a}	13.44±0.0^{a}	0	0	17±0.0^{b}	8.02±0.0^{b}	14±0.0^{c}	5.44±0.0^{c}	0	0	0	0
Heat												
60°C for 60 min	18±0.0^{a}	9.00±0.0^{a}	0	0	15±0.0^{c}	6.25±0.0^{c}	12±0.0^{c}	4.00±0.0^{c}	0	0	0	0
100°C for 20 min	13±0.0^{c}	4.69±0.0^{c}	0	0	14±0.0^{c}	5.44±0.0^{c}	12±0.0^{c}	4.00±0.0^{c}	0	0	0	0
121°C for 15 min	13±0.0^{c}	4.69±0.0^{c}	0	0	12±0.0^{c}	4.00±0.0^{c}	12±0.0^{c}	4.00±0.0^{c}	0	0	0	0

Table 3: Continue

Treatments	Pathogenic organisms: Gram-positive: *Staphylococcus aureus* Diameter of inhibition zone (mm)*	*Staphylococcus aureus* Inhibitory activity (AU)	*Candida albicans* Diameter of inhibition zone (mm)*	*Candida albicans* Inhibitory activity (AU)	*Listeria monocytogenes* Diameter of inhibition zone (mm)*	*Listeria monocytogenes* Inhibitory activity (AU)	Gram-negative: *Escherichia coli* Diameter of inhibition zone (mm)*	*Escherichia coli* Inhibitory activity (AU)	*Salmonella enterica* Diameter of inhibition zone (mm)*	*Salmonella enterica* Inhibitory activity (AU)	*Klebsiella pneumoniae* Diameter of inhibition zone (mm)*	*Klebsiella pneumoniae* Inhibitory activity (AU)
Lactobacillus delbrueckii* subsp. *lactis												
Neutralized	23±0.0^{a}	14.69±0.0^{a}	0	0	19±0.0^{a}	10.02±0.0^{a}	15±0.0^{c}	6.25±0.0^{c}	0	0	0	0
Heat												
60°C for 60 min	23±0.0^{a}	14.69±0.0^{a}	0	0	19±0.0^{a}	10.02±0.0^{a}	15±0.0^{c}	6.25±0.0^{c}	0	0	0	0
100°C for 20 min	16±0.0^{b}	7.11±0.0^{b}	0	0	19±0.0^{a}	10.02±0.0^{a}	15±0.0^{c}	6.25±0.0^{c}	0	0	0	0
121°C for 15 min	16±0.0^{b}	7.11±0.0^{b}	0	0	19±0.0^{a}	10.02±0.0^{a}	15±0.0^{c}	6.25±0.0^{c}	0	0	0	0
Lactobacillus helveticus												
Neutralized	19±1.5^{a}	10.02±1.5^{a}	0	0	18±0.0^{a}	9.00±0.0^{a}	14±0.0^{c}	5.44±0.0^{c}	16±0.0^{b}	7.11±0.0^{b}	0	0
Heat												
60°C for 60 min	18±0.0^{a}	9.00±0.0^{a}	0	0	18±0.0^{a}	9.00±0.0^{a}	14±0.0^{c}	5.44±0.0^{c}	16±0.0^{b}	7.11±0.0^{b}	0	0
100°C for 20 min	15±0.0^{c}	6.25±0.0^{c}	0	0	18±0.0^{a}	9.00±0.0^{a}	14±0.0^{c}	5.44±0.0^{c}	16±0.0^{b}	7.11±0.0^{b}	0	0
121°C for 15 min	13±0.0^{c}	4.69±0.0^{c}	0	0	18±0.0^{a}	9.00±0.0^{a}	14±0.0^{c}	5.44±0.0^{c}	16±0.0^{b}	7.11±0.0^{b}	0	0
Lactobacillus buchneri												
Neutralized	18±2.0^{a}	9.00±2.10^{a}	0	0	17±0.0^{b}	8.02±0.0^{b}	16±0.0^{b}	7.11±0.0^{b}	0	0	0	0
Heat												
60°C for 60 min	18±0.0^{a}	9.00±0.0^{a}	0	0	17±0.0^{b}	8.02±0.0^{b}	16±0.0^{b}	7.11±0.0^{b}	0	0	0	0
100°C for 20 min	18±0.0^{a}	9.00±0.0^{a}	0	0	17±0.0^{b}	8.02±0.0^{b}	16±0.0^{b}	7.11±0.0^{b}	0	0	0	0
121°C for 15 min	18±0.0^{a}	9.00±0.0^{a}	0	0	17±0.0^{b}	8.02±0.0^{b}	16±0.0^{b}	7.11±0.0^{b}	0	0	0	0
Lactobacillus fermentum												
Neutralized	23±0.0^{a}	14.69±0.0^{a}	0	0	19±0.0^{a}	10.02±0.0^{a}	15±0.0^{c}	6.25±0.0^{c}	17±0.0^{b}	8.02±0.0^{b}	0	0
Heat												
60°C for 60 min	22±0.0^{a}	13.44+±0.0	0	0	19±0.0^{a}	10.02±0.0	15±0.0^{c}	6.25±0.0^{c}	17±0.0^{b}	8.02±0.0^{b}	0	0
100°C for 20 min	22±0.0^{a}	13.44±0.0^{a}	0	0	19±0.0^{a}	10.02±0.0	15±0.0^{c}	6.25±0.0^{c}	17±0.0^{b}	8.02±0.0^{b}	0	0
121°C for 15 min	20±0.0^{a}	11.11±0.0^{a}	0	0	19±0.0^{a}	10.02±0.0	15±0.0^{c}	6.25±0.0^{c}	17±0.0^{b}	8.02±0.0^{b}	0	0
***Streptococcus thermophilus* (TH4)**												
Neutralized	22±2.0^{a}	13.44±0.0^{a}	0	0	19±0.0^{a}	10.02±0.0^{a}	13±0.0^{c}	4.69±0.0^{c}	0	0	0	0
Heat												
60°C for 60 min	20±0.0^{a}	11.11±0.0^{a}	0	0	19±0.0^{a}	10.02±0.0^{a}	13±0.0^{c}	4.69±0.0^{c}	0	0	0	0
100°C for 20 min	18±0.0^{a}	9.00±0.0^{a}	0	0	19±0.0^{a}	10.02±0.0^{a}	13±0.0^{c}	4.69±0.0^{c}	0	0	0	0
121°C for 15 min	15±0.0^{c}	6.25±0.0^{c}	0	0	19±0.0^{a}	10.02±0.0^{a}	13±0.0^{c}	4.69±0.0^{c}	0	0	0	0
***Lactobacillus acidophilus* (CY-X_{11-1})**												
Neutralized	0	0	0	0	17±0.0^{b}	8.02±0.0^{b}	20±0.0^{a}	11.11±0.0^{a}	0	0	12±0.0^{c}	4.0±0.0^{c}
Heat												
60°C for 60 min	0	0	0	0	17±0.0^{b}	8.02±0.0^{b}	19±0.0^{a}	10.02±0.0^{a}	0	0	12±0.0^{c}	4.0±0.0^{c}
100°C for 20 min	0	0	0	0	17±0.0^{b}	8.02±0.0^{b}	18±0.0^{b}	7.11±0.0^{b}	0	0	12±0.0^{c}	4.0±0.0^{c}
121°C for 15 min	0	0	0	0	17±0.0^{b}	8.02±0.0^{b}	16±0.0^{b}	7.11±0.0^{b}	0	0	12±0.0^{c}	4.0±0.0^{c}
Bifidobacterium bifidum												
Neutralized	0	0	0	0	15±0.0^{c}	6.25±0.0^{c}	20±0.0^{a}	11.11±0.0^{a}	0	0	16±0.0^{c}	7.11±0.0^{c}
Heat												
60°C for 60 min	0	0	0	0	15±0.0^{c}	6.25±0.0^{c}	19±0.0^{a}	10.02±0.0^{a}	0	0	16±0.0^{c}	7.11±0.0^{c}
100°C for 20 min	0	0	0	0	15±0.0^{c}	6.25±0.0^{c}	19±0.0^{a}	10.02±0.0^{a}	0	0	13±0.0^{c}	4.69±0.0^{c}
121°C for 15 min	0	0	0	0	15±0.0^{c}	6.25±0.0^{c}	19±0.0^{a}	10.02±0.0^{a}	0	0	13±0.0^{c}	4.69^{c}±0.0^{c}

The results are presented as the mean value±SE. ^{Abc}Values expressed with different treatments are significantly different at $p<0.05$, *: Inhibition zone around the well, AU: Arbitrary unit, 0: Not active, 5 mm: No inhibition, 5-10 mm: Low inhibition, 11- 20 mm: Moderate inhibition

Table 4: Antimicrobial activities (clear zones) of CBSFs derived from LAB grown at different pH values against the tested microorganisms in a well diffusion assay

Treatments	Pathogenic organisms											
	Gram-positive						Gram-negative					
	Staphylococcus aureus		*Candida albicans*		*Listeria monocytogenes*		*Escherichia coli*		*Salmonella enterica*		*Klebsiella pneumoniae*	
	Diameter of inhibition zone (mm)*	Inhibitory activity (AU)	Diameter of inhibition zone (mm)*	Inhibitory activity (AU)	Diameter of inhibition zone (mm)*	Inhibitory activity (AU)	Diameter of inhibition zone (mm)*	Inhibitory activity (AU)	Diameter of inhibition zone (mm)*	Inhibitory activity (AU)	Diameter of inhibition zone (mm)*	Inhibitory activity (AU)
Pediococcus acidilactici												
Neutralized	20±1.0^{a}	11.11±1.05^{a}	0	0	17±0.0^{b}	8.02±0.0^{b}	16±0.0^{b}	17.11±0.0^{b}	0	0	0	0
pH												
2.0	0	0	0	0	21±0.0^{a}	12.25±0.0^{a}	22±0.0^{a}	13.44±0.0^{a}	0	0	0	0
6.5	0	0	0	0	18±0.0	9.00±0.0^{a}	13±0.0	4.69±0.0^{c}	0	0	0	0
9.0	0	0	0	0	0	0	0	0	0	0	0	0
Streptococcus thermophilus												
Neutralized	22±0.0^{a}	13.44±0.0^{a}	0	0	19±0.0^{a}	10.02±0.0^{a}	13±0.0	4.69±0.0^{c}	0	0	0	0
pH												
2.0	28±0.0^{a}	21.77±0.0^{a}	0	0	23±0.0^{a}	14.69±0.0^{a}	22±0.0^{a}	13.44±0.0^{a}	0	0	0	0
6.5	0	0	0	0	20±0.0^{a}	11.11±0.0^{a}	22±0.0^{a}	13.44±0.0^{a}	0	0	0	0
9.0	0	0	0	0	20±0.0^{a}	11.11±0.0^{a}	20±0.0^{a}	11.11±0.0^{a}	0	0	0	0
Lactococcus lactis* subsp. *cremoris												
Neutralized	19±1.50^{a}	10.02±1.90^{a}	0	0	19±0.0^{a}	10.02±0.0^{a}	16±0.0^{b}	7.11±0.0^{b}	16±0.0^{b}	7.11±0.0^{b}	0	0
pH												
2.0	23±0.0^{a}	14.69±0.0^{a}	0	0	21±0.0^{a}	12.25±0.0^{a}	20±0.0^{a}	11.11±0.0^{a}	22±0.0^{a}	13.44±0.0^{a}	0	0
6.5	20±0.0^{a}	11.11±0.0^{a}	0	0	21±0.0^{a}	12.25±0.0^{a}	20±0.0^{a}	11.11±0.0^{a}	0	0	0	0
9.0	0	0	0	0	20±0.0^{a}	11.11±0.0^{a}	19±0.0^{a}	10.02±0.0^{a}	0	0	0	0
Lactococcus plantarum												
Neutralized	23±0.0^{a}	14.69±0.0^{a}	0	0	19±0.0^{a}	10.02±0.0^{a}	16±0.0^{b}	7.11±0.0^{b}	13±0.0^{c}	4.69±0.0^{c}	0	0
pH												
2.0	28±0.0^{a}	21.77±0.0^{a}	0	0	23±0.0^{a}	14.69±0.0^{a}	25±0.0^{a}	17.36±0.0^{a}	18±0.0^{a}	9.00±0.0^{a}	0	0
6.5	18±0.0^{a}	9.00±0.0^{a}	0	0	22±0.0^{a}	13.44±0.0^{a}	18±0.0^{a}	9.00±0.0^{a}	0	0	0	0
9.0	0	0	0	0	21±0.0^{a}	12.25±0.0^{a}	0	0	0	0	0	0
Lactococcus lactis* subsp. *lactis												
Neutralized	22±1.0^{a}	11.11±0.0^{a}	0	0	18±0.0^{a}	9.00±0.0^{a}	16±0.0^{b}	7.11±0.0^{b}	14±0.0^{c}	5.44±0.0^{c}	0	0
pH												
2.0	26±0.0^{a}	18.77±0.0^{a}	0	0	29±0.0^{a}	23.36±0.0^{a}	24±0.0^{a}	16.00±0.0^{a}	19±0.0^{a}	10.02±0.0^{a}	0	0
6.5	19±0.0^{a}	10.02±0.0^{a}	0	0	24±0.0^{a}	16.00±0.0^{a}	22±0.0^{a}	13.44±0.0^{a}	19±0.0^{a}	10.02±0.0^{a}	0	0
9.0	0	0	0	0	18±0.0^{a}	9.00±0.0^{a}	0	0	0	0	0	0
Enterococcus italicus												
Neutralized	20±0.0^{a}	11.11±0.0^{a}	0	0	17±0.0^{b}	8.02±0.0^{b}	15±0.0^{c}	6.25±0.0^{c}	0	0	0	0
pH												
2.0	25±0.0^{a}	17.36±0.0^{a}	0	0	27±0.0^{a}	20.25±0.0^{a}	24±0.0^{a}	16.00±0.0^{a}	0	0	0	0
6.5	19±0.0^{a}	10.02±0.0^{a}	0	0	25±0.0^{a}	17.36±0.0^{a}	17±0.0^{b}	8.02±0.0^{b}	0	0	0	0
9.0	0	0	0	0	0	0	16±0.0^{b}	7.11±0.0^{b}	0	0	0	0
Enterococcus camelliae												
Neutralized	22±0.0^{a}	13.44±2.44^{a}	0	0	17±0.0^{b}	8.02±0.0^{b}	14±0.0^{c}	5.44±0.0^{c}	0	0	0	0
pH												
2.0	26±0.0^{a}	18.77±0.0^{a}	0	0	27±0.0^{a}	20.25±0.0^{a}	22±0.0^{a}	13.44±0.0^{a}	0	0	0	0
6.5	22±0.0^{a}	13.44±0.0^{a}	0	0	22±0.0^{a}	13.44±0.0^{a}	18±0.0^{a}	9.00±0.0^{a}	0	0	0	0
9.0	0	0	0	0	0	0	15±0.0^{c}	6.25±0.0^{c}	0	0	0	0

Table 4: Continue

Treatments	Pathogenic organisms											
	Gram-positive						Gram-negative					
	Staphylococcus aureus		*Candida albicans*		*Listeria monocytogenes*		*Escherichia coli*		*Salmonella enterica*		*Klebsiella pneumoniae*	
	Diameter of inhibition zone (mm)*	Inhibitory activity (AU)	Diameter of inhibition zone (mm)*	Inhibitory activity (AU)	Diameter of inhibition zone (mm)*	Inhibitory activity (AU)	Diameter of inhibition zone (mm)*	Inhibitory activity (AU)	Diameter of inhibition zone (mm)*	Inhibitory activity (AU)	Diameter of inhibition zone (mm)*	Inhibitory activity (AU)
Lactobacillus delbrueckii* subsp. *lactis												
Neutralized	23±0.0^{a}	14.69±0.0^{a}	0	0	19±0.0^{a}	10.02±0.0^{a}	15±0.0^{c}	6.25±0.0^{c}	0	0	0	0
pH												
2.0	27±0.0^{a}	20.25±0.0^{a}	0	0	26±0.0^{a}	18.77±0.0^{a}	17±0.0^{b}	8.02±0.0^{b}	0	0	0	0
6.5	0	0	0	0	22±0.0^{a}	13.44±0.0	17±0.0^{b}	8.02±0.0^{b}	0	0	0	0
9.0	0	0	0	0	0	0	0	0	0	0	0	0
Lactobacillus helveticus												
Neutralized	19±1.5^{a}	10.02±1.5^{a}	0	0	18±0.0^{a}	9.00±0.0^{a}	14±0.0^{c}	5.44±0.0	16±0.0^{b}	7.11±0.0^{b}	0	0
pH												
2.0	28±0.0^{a}	21.77±0.0^{a}	0	0	27±0.0^{a}	20.25±0.0	22±0.0^{a}	13.44±0.0^{a}	25±0.0^{a}	17.36±0.0^{a}	0	0
6.5	22±0.0^{a}	13.44±0.0^{a}	0	0	23±0.0^{a}	14.69±0.0^{a}	22±0.0^{a}	13.44±0.0^{a}	23±0.0^{a}	14.69±0.0^{a}	0	0
9.0	0	0	0	0	0	0	20±0.0^{a}	11.11±0.0^{a}	21±0.0^{a}	12.25±0.0^{a}	0	0
Lactobacillus buchneri												
Neutralized	18±2.0^{a}	9.00±2.10^{a}	0	0	17±0.0	8.02±0.0^{b}	16±0.0^{b}	7.11±0.0	0	0	0	0
pH												
2.0	25±0.0^{a}	17.36±0.0^{a}	0	0	26±0.0^{a}	18.77±0.0^{a}	22±0.0^{a}	13.44±0.0^{a}	0	0	0	0
6.5	21±0.0^{a}	12.25±0.0^{a}	0	0	25±0.0^{a}	17.36±0.0^{a}	21±0.0^{a}	12.25±0.0^{a}	0	0	0	0
9.0	0	0	0	0	0	0	20±0.0^{a}	11.11±0.0^{a}	0	0	0	0
Lactobacillus fermentum												
Neutralized	23±0.0^{a}	14.69±0.0^{a}	0	0	19±0.0^{a}	10.02±0.0^{a}	15±0.0^{c}	6.25±0.0^{c}	17±0.0^{b}	8.02±0.0^{b}	0	0
pH												
2.0	28±0.0^{a}	21.77±0.0^{a}	0	0	24±0.0^{a}	16.0±0.0^{a}	22±0.0^{a}	13.44±0.0^{a}	24±0.0^{a}	16.0±0.0^{a}	0	0
6.5	23±0.0^{a}	14.69±0.0^{a}	0	0	25±0.0^{a}	17.36±0.0^{a}	22±0.0^{a}	13.44±0.0^{a}	22±0.0^{a}	13.44±0.0^{a}	0	0
9.0	0	0	0	0	0	0	0	0	20±0.0^{a}	11.11±0.0^{a}	0	0
***Streptococcus thermophilus* (TH4)**												
Neutralized	22±2.0^{a}	13.44±0.0^{a}	0	0	19±0.0^{a}	10.02±0.0^{a}	13±0.0^{c}	4.69±0.0^{c}	0	0	0	0
pH												
2.0	28±2.0^{a}	21.77±0.0^{a}	0	0	28±0.0^{a}	21.77±0.0^{a}	22±0.0^{a}	13.44±0.0^{a}	0	0	0	0
6.5	0	0	0	0	0	0	22±0.0^{a}	13.44±0.0^{a}	0	0	0	0
9.0	0	0	0	0	0	0	20±0.0^{a}	11.11±0.0^{a}	0	0	0	0
***Lactobacillus acidophilus* (CY-X_{11-1})**												
Neutralized	0	0	0	0	17±0.0	8.02±0.0^{b}	20±0.0^{a}	11.11±0.0	0	0	12±0.0^{c}	4.0±0.0^{c}
pH												
2.0	0	0	0	0	23±0.0^{a}	14.69±0.0^{a}	22±0.0^{a}	13.44±0.0^{a}	0	0	22±0.0^{a}	13.44±0.0^{a}
6.5	0	0	0	0	21±0.0^{a}	12.25±0.0^{a}	0	0	0	0	16±0.0^{b}	7.11±0.0^{b}
9.0	0	0	0	0	0	0	0	0	0	0	16±0.0^{b}	7.11±0.0^{b}
Bifidobacterium bifidum												
Neutralized	0	0	0	0	15±0.0^{c}	6.25±0.0	20±0.0^{a}	11.11±0.0^{a}	0	0	16±0.0^{c}	7.11±0.0^{c}
pH												
2.0	0	0	0	0	24±0.0^{a}	16.0±0.0^{a}	22±0.0^{a}	13.44±0.0^{a}	0	0	25±0.0^{a}	17.36±0.0^{a}
6.5	0	0	0	0	22±0.0^{a}	13.44±0.0^{a}	0	0	0	0	16±0.0^{c}	7.11±0.0^{c}
9.0	0	0	0	0	0	0	0	0	0	0	16±0.0^{c}	7.11±0.0^{c}

The results are presented as the mean value±SE. ^{Abc}Values expressed with different treatments are significantly different at $p<0.05$, *: Inhibition zone around the well, AU: Arbitrary unit, 0: Not active, 5 mm: No inhibition, 5-10 mm: Low inhibition, 11- 20 mm: Moderate inhibition

Table 5: Sensitivities of inhibitory substances produced by 11 LAB species and 3 reference strains to treatment with a proteolytic enzyme

	Sensitivity to enzyme	
Genus	No treatment	Pepsin treatment
Pediococcus acidilactici	+	-
Streptococcus thermophilus	+	-
Lactococcus lactis subsp. *cremoris*	+	-
Lactococcus plantarum	+	-
Lactococcus lactis subsp. *lactis*	+	-
Enterococcus italicus	+	+
Enterococcus camelliae	+	-
Lactobacillus delbrueckii subsp. *lactis*	+	-
Lactobacillus helveticus	+	-
Lactobacillus buchneri	+	-
Lactobacillus fermentum	+	-
LAB reference strains		
Strept. thermophilus TH4	+	-
Lb. acidophilus CY-X11-1	+	-
Bif. bifidum Bb12	+	-

these findings, pepsin sensitivity has been demonstrated for other bacteriocins, specifically plantaricin 35D and sakacin A[61] and enterocin 416 KI[1]. Present results strongly suggest that the inhibitory activities of CBSFs derived from LAB species are attributable to a protein-based aqueous compound. Insignificant results at $p<0.05$ were obtained when comparing the agar spot and agar well diffusion methods when bacteriocin was applied to *C. albicans*, as shown in Table 5. In contrast to Table 5, bacteriocins were more effective against pathogenic microorganisms, some bacteriocins produced by LAB exhibited higher antimicrobial activity than the antibacterial activities of certain antibiotics, as presented in Table 5. Typically, nisin and lacticin 3147 are effective agents for treating mastitis[14] and nisin is considered effective for the treatment of stomach ulcers caused by *Helicobacter pylori*[62]. Thus, present results suggest that the bacteriocins produced by certain local LAB demonstrate potential as food bio-preservatives and probiotics that are alternatives to the usually prescribed antibiotics.

CONCLUSION

In study, 11 LAB species were isolated from different sources, namely, raw buffalo milk, raw cow milk, yoghurt, raib, kareish cheese, domiati cheese and mish cheese. The only promising CBSFs identified were derived from *Lact. plantarum*, *Lb. delbrueckii* subsp *lactis* and *Lb. fermentum* and were effective against *Staph. aureus*. *Lact. lactis* subsp *cremoris*, *Lb. helveticus* and *Lb. fermentum* showed the highest antimicrobial activity at 30°C, pH 2.0-6.5 and pepsin inhibited the antimicrobial activity of LAB by 90.9%. Accordingly, the bacteriocins derived from LAB isolates showed promising antimicrobial activities and may act as barriers against spoilage and/or pathogenic microorganisms in dairy products. *In vitro* studies are needed to determine the mechanism(s) involved in mitigating the antagonistic activities and bacteriocins produced by promising isolates.

SIGNIFICANCE STATEMENT

This study discovers the antimicrobial activity of 11LAB isolates and *Lact. lactis* subsp *cremoris*, *Lb. helveticus* and *Lb. fermentum* showed the highest antimicrobial activity up to 90.9% in Egypt milk product. This study will help the researchers to uncover the antimicrobial activity of isolates that many researchers were not able to explore. Thus, the new theory on antioxidant activity of isolates in preservation of food milk and milk products may be arrived at.

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