Molecular Isolation, Probiotic Property, and Bacteriocin Production of *Enterococcus faecium* (TM81) and *Lactobacillus curvatus* (TM51) with Anti-Listerial Activity in Native Dairy Products of Iran

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ABSTRACT: *Lactic acid bacteria in dairy products are the important bacteriocin-producing group with an antibacterial activity effect. The aim of this study was the isolation and molecular identification of bacteriocin-producing enterococci and lactobacilli with a wide range of antibacterial activity. Bacteriocin-producing bacteria were isolated and purified from Iranian native dairy products. The effect of bacteriocins on the Gram-negative and Gram-positive indicator strains, as well as the impact of proteolytic enzymes, pH, and temperature changes on bacteriocin production, were investigated. A total of 431 bacteriocin-producing isolates were characterized from 100 traditional cheese and yoghurt samples. The resulting bacteriocin reduced the growth of these two strains. E. faecium TM81 had both bactericidal and bacteriostatic properties, whereas the L. curvatus TM51 only had bacteriostatic properties. The bacteriocin produced by both strains had the highest antibacterial effect among Gram-positive indicator strains such as Listeria monocytogenes and Bacillus cereus. Because produced bacteriocin (resistant to temperature and pH changes) has a wide range of inhibitory spectra on Gram-positive and Gram-negative bacteria, especially pathogenic bacteria, it is recommended to use it as a bio preservative in the food/pharmaceutical industry, and animal feed, as well as an alternative to chemical antibiotics.*

KEYWORDS: Bacteriocin, *Enterococcus faecium*, Lactobacillus, Bactericidal, Bacteriostatic.

INTRODUCTION

Consumers are looking for products without chemical preservatives and still maintain good shelf life and safety. In order to provide better food safety, chemical preservatives, antibiotics, or stricter physical

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processes are needed. All these methods change the physicochemical and nutritional properties of foods. There is an increasing demand for natural food preservatives. The use of microorganisms and/or metabolites to prevent spoilage of food products has gained the interest of food producers named bio-preservation technology. Biopreservation is a safe and well-extended shelf life method [1,2]. The bioactive metabolites that contribute to product preservation include simply organic acids, fatty acids, carbon dioxide, acetaldehyde, diacetyl, and hydrogen peroxide, or more specific components such as bacteriocins, which are produced by lactic acid bacteria [3,4].

The uses of bio-preservative organisms such as lactic acid bacteria (LAB) to control the spoilage organisms may constitute an economically viable approach towards reducing spoilage and thereby help to avoid associated wastage. One approach for long-lasting shelf life and storage with the most beneficial food properties is to introduce antimicrobial preferably the naturally occurring antimicrobials from LABs.

The use of LAB commonly associated with foods as protective cultures may demonstrate antagonism towards pathogenic and spoilage organisms in food preservation. The traditional use of LAB proves their lack of pathogenicity, and they are recognized as safe (GRAS). The inhibitory effect of LAB is the result of the impact of synergism among a number of mechanisms including competition for the production of mostly antimicrobial constituents such as bacteriocin-like substances [2].

LABs as the typical food fermentation bacteria have been assessed for their probiotic potential. *Enterococcus faecium* and *Lactobacillus curvatus* are widely used in the fermented food product industry. Due to distinctive probiotic properties and strong consumer demand for naturally bio preserved probiotic foods especially dairy products are an important collection of functional foods. Main criteria for the selection of probiotic bacteria include non-pathogenicity, survivability in the stomach, maintaining their viability and metabolic activity in the intestine, bile salts tolerance, competition with pathogenic bacteria, and resistance to antibiotics [5].

Today, most pathogenic bacteria are resistant to some antibiotics due to their excessive use in the treatment of infections which is one of the big challenges that threaten public health today. Bacteriocins are important as an alternative to antibiotics, especially for pathogenic bacteria, against which antibiotic resistance has been developed; but there are few studies in this regard [6].

The aim of this research was to isolate, screen, and identify bacteriocin-producing bacteria from native dairy products as a carrier for probiotic strains in Iran and to evaluate the antimicrobial and probiotic activity of the bacteriocin-producing strains as protective cultures to reduce the risk of food poisoning in food. It is important to determine the antimicrobial activity of native lactobacilli bacteriocin in traditional dairy products with a wide therapeutic potential. In this study, the antimicrobial, probiotic and molecular properties of LAB strains isolated from Iranian traditional dairy products were studied in order to be used in the field of medical treatment and food health. Probiotic properties of selected strains into the gastrointestinal tract, and to evaluate the viability of probiotic bacteria in dairy products during the storage period, the effect of gastric juice, bile salt, and antibiotics were examined.

EXPERIMENTAL SECTION

Materials and dairy samples

The diary samples used in the present research included 100 samples consisting of a total of 74 cheese samples (Kurdish, Lactic, White, Lighvan, Golpayegan, Motal, and Curd cheese) and 26 yoghurt samples from a different region of Iran. The samples were then transferred to the laboratory in ice. The samples were used to isolate bacteriocin-producing bacteria with different characteristics in terms of appearance and flavor.

In order to isolate, count, proliferate, and activate lactic acid bacteria, the specific media used in this study included De Man Rogosa and Sharpe agar (MRS), MRS Broth, M17, Brain heart infusion (BHI), and Mueller Hinton Agar (MHA), purchased from Oxoide Co. (England). Indicator strains, including *Lactobacillus brevis* F145 *and Lactobacillus bulgaricus* 340 were prepared from various domestic and international academic sources and the Centre of Industrial and Medical Fungi and Bacteria Collection affiliated to Iran's Scientific and Industrial Research Organization. The chemicals, Triton X-100, hydrogen peroxide 3% (Sigma-Aldrich, St. Louis, MO, USA), glycerol, sodium citrate, Tris, proteinase K, trypsin, α-chymotrypsin, catalase, α-amylase, lipase, Sodium Dodecyl Sulphate (SDS), and antibiotics including chloramphenicol, penicillin, tetracycline, gentamicin,

ampicillin, vancomycin, and ciprofloxacin were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA).

Identification of Bacteriocin-Like Inhibitory Substances (BLIS)-producing LAB isolates

Traditional dairy samples collected in sterile falcons were transferred to the laboratory in ice and stored in the refrigerator until the time of examination. The cheese and yoghurt samples were then homogenized. Then, serial dilutions (10^{-1}-10^{-10}) were made from each sample using sodium chloride 0.9% and cultured on MRS agar and M17 (1.5%w/v) medium. The culture medium was then incubated at 30°C and 37°C for 48 h under low aerobic conditions. The initial isolation was then carried out according to the modified antagonism test method proposed [8]. Plates containing 30-300 colonies were then used for each dilution on 0.8% MRS agar medium with indicator strains at 37°C for 24 h. The indicator strains included *L. bulgaricus* 340 and *L. brevis* F145. The colonies that were grown in a culture medium by creating a clear zone in the medium containing the strain indicator were randomLy cultured on an MRS Broth or M17 broth (Oxoid, UK) and then identified as strains producing antimicrobial agents. The isolates were purified through repeated culture in a suitable agar medium with microscopic observation. Purified isolates were then cultured on MRS or M17 medium and covered with 30% glycerol (w/v). The culture media were prepared twice in a suitable culture medium before the experiments. To isolate the bacteria, the modified antagonism test was performed according to the procedure proposed [7].

Testing the growth range of LABs

To stabilize the growth ability of isolated strains, they were cultured in MRS broth medium at 0, 10, and 45°C at 6% and 10% sodium chloride concentrations and pH values of 4, 6, and 9. After the incubation, the results of the experiment were reported to be positive in the case of having a turbid medium or sedimentation of substances. The incubation lasted for 3 days at 15-45°C and 7 days at 0-10°C [8].

Morphological, biochemical, and molecular identification of LABs

To identify a strain whose bacteriocin has the highest antibacterial activity, the primary tests included Gram stain, microscopic shape, colony shape and colour, motility, and catalase test. Gram-positive, negative catalase, bacilli, and cocci strains were identified as LABs.

Identification of strain genotypes using 16SrDNA fragment sequencing

Genomic DNA was extracted using 3 mL overnight culture kit inoculated with a single colony and kept at -20°C. The molecular identification of the isolated strains was carried out using Polymerase chain reaction and 16S rRNA sequencing was later performed. The PCR reaction was composed of 1µL template DNA, buffer, magnesium chloride, deoxyribonucleotide triphosphate (dNTP), primer, single polymerase enzyme, and total reaction volume of 50 µL. The PCR was then carried out in a Thermocycler (Techno, Barloworld scientific, Cambridge, UK). The template DNA model was denatured at 94°C for 5 minutes followed by DNA replication reaction in 35 cycles as follows: denaturation at 94°C for 60 seconds, primer annealing at 56°C for 1.15 minutes, and primer elongation at 72°C for 1.15 minutes. Final denaturation was carried out at 94°C for 1 minute and the final incubation was performed at 72°C for 5 minutes to ensure the completion and elongation of the primers. To detect PCR products, 1% agarose gel electrophoresis was carried out using a buffer (TBE 0.5 x (0.5 mM EDTA, pH 8.0, 44.5 mM Tris / Borate). The gel was stained by ethidium bromide (0.5 mg/mL) and then electrophoresed for 30 minutes at 100 v. UV-based identification of the PCR product was then investigated [9]. The PCR reaction was then carried out using a fully automatic fluorescence method. fD1 (5'-AGAGTTTGATCCTGGCTCAG-3 ') and rD1 (5' TAAGGAGGTGATCCAGGC3 ') primers were used for the amplification of the 16S rRNA gene. 16S rRNA sequencing was carried out by Pioneer Co. (South Korea). This sequence was analyzed by various software such as Finch TV, and Gene Runner. The results of the basic local alignment search tool (BLAST) of the intended sequence were examined at the NCBI sites

(http://blast.ncbi.nlm.nih.gov/blast)

and analyzed by MilleGen software [10].

Detection of the presence of bacteriocin and its antibacterial effect

To confirm that the antimicrobial effect is due to the production of bacteriocin rather than other substances

produced, such as organic acids, the effects of antimicrobial substances on organic acids was eliminated. To remove the inhibitory effect of the organic acids produced, the supernatant pH was adjusted to 6.5 to 7 using 1N NaOH or1 N HCl. Then the antimicrobial effect was studied according to the agar spot method [11].

The antibacterial effect of the cell-free supernatant was determined by the quantitative disk diffusion method [12]. Cell-free culture supernatants (16-18 h) were prepared by centrifugation (10,000 g, 15 min, 4°C) and adjusting pH to 6 using 1N NaOH. To prevent proteolytic degradation of bacteriocins, the Cell-Free Culture Supernatant (CFCS) was exposed to 80°C for 10 minutes. Agar medium 0.8% was prepared by adding the indicator strain. The wells were placed in the agar medium and 50µL of overnight culture supernatant was inoculated in wells. The plates were then incubated for 40 minutes at room temperature under sterile conditions prior to incubation at the appropriate temperature of the indicator microorganism *L. brevis* F145. A clear zone of inhibition with a minimum diameter of 2 mm was recorded as positive.

Antimicrobial activity

Antibacterial activity against pathogenic and non-pathogenic indicator strains

Various bacterial strains were used as indicator microorganisms to evaluate the inhibitory activity spectra. Before use, the strains were revived in a suitable broth solution overnight culture. Agar was prepared by adding 0.8% (w/v) of agar to the culture medium. Antimicrobial activity against different indicators was performed using the spot-on-lawn method [13]. CFCs (16-18 h) were prepared as described above. Agar culture was prepared in sterile Petri dishes after adding indicator strains to the growth medium during the initial growth phase in an enriched medium. After 30 minutes, 10µL of supernatants was stained on an agar layer containing a strain indicator. Before being incubated, plates remained at room temperature for 40 minutes under sterile conditions. A clear zone of inhibition with a minimum diameter of 2 mm was recorded as positive.

Antifungal activity

The antifungal activity test was performed using the modified method [14]. Strains were cultured in 13 mL MRS 0.8% in a concentrated agar as follows. pH was adjusted to 6.2 and sodium acetate was then removed. After incubation, fungal suspension spores (10^4 spores/mL) underwent spot culture on an agar plate for 48 h at 30°C, and the incubation continued for 72 h at 25°C in order to increase the fungal growth. The overall fungal growth was compared with the control group and described as follows: Non-inhibitory growth, unequal fungal growth; moderate or strong inhibition; inadequate and low fungal growth; very strong inhibition, no fungal growth.

Effect of heat, pH, enzymes and chemicals on bacteriocin function

To determine the effect of temperature on the bacteriocin activity, a CFCS with neutral pH was divided into several tubes and exposed to heat at 50°C for 30 minutes, at 100°C for 5, 15, 30 minutes, and at 121°C for 15 minutes [15]. Antibacterial activity of heated supernatants was studied against the indicator strains of *L. brevis* F145 using the disk-diffusion agar method. Untreated supernatant was used for the control group. To determine the resistance of the supernatant against pH changes, the supernatant of bacteriocin-producing strain was then isolated. The resulting supernatant was divided into several tubes and the supernatant pH of each tube was adjusted at 3 to 10 using NaOH 1N and HCl 1N. The tubes were then placed at 37^0C (room temperature). The pH was then adjusted to 6.5. The antibacterial activity of supernatant against indicator strains was investigated using the agar diffusion method. Untreated supernatant was used for the control group. Protein-degrading enzymes are used to prove that the antibacterial agent present in the supernatant has a protein nature. Trypsin, α-chymotrypsin, amylase, lipase, and pronase enzymes were prepared at a concentration of 1 mg/mL at pH 7. Then, 20 µL of the enzyme solution was added to 200 µL of the isolated supernatants. The enzyme-containing supernatant solution was incubated at 37 °C for 2 h. Then, to eliminate the enzyme effect, the solution was heated in a water bath at 100°C for 10 minutes. Finally, the effect of the enzyme-containing supernatant against the indicator strains was investigated using on agar diffusion method and this effect was compared with the effect of the enzyme-free supernatant [16].

Enzymes such as protease type X and α-chymotrypsin type II, protease type VIII, pronase E, lipase type VII (Sigma), catalase (Sigma), and amylase (Sigma) were used

in the current study. To determine the effect of various chemicals, the following substances were used: Sodium Dodecyl Sulfate (SDS) (1% (w/v)), TritonX-20, Triton X-80, Triton X-100, Triton100, β-mercaptoethanol (1% w/v), Ethylene Diamine Tetraacetic Acid Na- (EDTA) (1 mM), and sodium chloride NaCl (6.5%, w/v). The supernatant solution containing any chemical was incubated for two h at 37°C. Finally, the present study investigated the effect of the treated supernatant against the indicator strains of *L. brevis* F.145 using the disk-diffusion agar method and this effect was then compared with the effect of untreated supernatant.

Bacteriocin titer assay (kinetics of bacteriocin)

According to the strain tested, the time for bacteriocin production was set at 37º C and 30º C in MRS media. We inoculated the medium with overnight cultures (2% v/v). They were then incubated under a non-regulated pH environment. Antimicrobial activity (AU/mL) of the bacteriocin, and modifications in pH and optical density (OD_{600nm}) of the cultures were specified at regular intervals (1h) for 24 h. Using the spot-on-lawn method, the anti-microbial activity in AU/mL was scrutinized [13]. Cell-free supernatants were also obtained. The produced sample was serially diluted twofold with Na-phosphate (100mM, pH 6.5). Dilutions were spotted on the lawn of agar media which had a sensitive strain. The plates were incubated at 37°C overnight, and the titer was set as the reciprocal of the highest dilution (2^n) which led to inhibition of the indicator lawn. Thus, the AU of antimicrobial activity per milliliter was defined as $2^n \times 1000$ µL 10 µL. *L. brevis* F145 was used as a sensitive strain. AU of antimicrobial activity per milliliter was (AU /mL) = $2^n \times 1000$ µL 10 µL.

The bacteriocin titer is expressed as active units per milliliter and obtained based on Eq. (1) where n is the number of the last well in which the zone of inhibition is formed. In other words, the number of the last well, in which the indicator strain has no growth [17].

The effect of bacteriocin-containing CFCS on the growth of the indicator strains

Efforts were made to investigate the effect of supernatant containing bacteriocin produced by *E. faecium* TM81 and *L. curvatus* TM51 on the growth of *L. monocytogenes* 506 and *L. brevis* F145 indicator strains [18]. Primarily, bacteriocin-containing supernatant was isolated from the bacteriocin-producing strain. Indicator strains were cultured in the flask containing 50 mL of Mueller Hinton Broth (each strain was incubated in two flasks at 37°C). 2 h after culture, 5 mL of bacteriocin-containing supernatant, sterilized with a filter (0.20 µm, Millipore), was added to one of the flasks. The optical absorption was read by spectrophotometric apparatus every 1 h (Ultraspec 2000, Pharmacia Biotech, USA) at 600 nm for 24h and compared with the supernatant-free culture. Finally, the growth diagram was drawn according to time (24 h) and Optical Density (OD) [19].

Partial purification of bacteriocin

To isolate the CFS, the purified strains underwent 24-hour cultured in MRS broth and centrifuged at 10,000 g for 15 minutes. The supernatant was then filtered using a 0.22µm filter. This supernatant, which is in the form of an acidic extract and contains various bacterial metabolites, including bacteriocin, was used for bacteriocin purification purposes [20]. To reduce the inhibitory activity of the organic acids produced, the supernatant pH was adjusted to 6.5 to 7 using 5N NaOH and 5N HCl. To neutralize the effect of oxygenated water, the catalase enzyme (2 mg/mL) was used. Ammonium sulfate 70% (Merck, 31119) was used to sediment proteins and increases their concentration. Powdered ammonium sulfate was then added to the resulting supernatant and then kept refrigerated for 18 h, after which the samples were centrifuged at 15000 g for 30 minutes. The precipitates were collected and then dissolved in 0.06 M phosphate buffer at pH= 7. An equal volume of chloroform and methanol solution was then added to it at a ratio of 1:2 and kept at 4°C for 1 h. The centrifugation was carried out at 4 °C at 20000 g and the resulting the white precipitate was then extracted and dissolved in sterilized double-distilled water [18]. Then the antimicrobial property of the remaining precipitate and supernatant was tested [21].

Molecular identification of bacteriocin-producing genes

Genomic DNA was isolated using a purification kit from a 2 mL overnight culture inoculated with a single colony according to the instructions and stored at -20 °C until use. The presence of genes encoding different bacteriocin genes was tested by PCR, using primers and conditions described previously. PCR amplification

were performed in 100 μL reaction mixtures containing 40 ng of bacterial DNA, 1X reaction buffer, 1.5 mM $MgCl_2$, 1 U of Taq DNA polymerase (GoTaq, Promega, USA), 250 μM of dNTPs mix, and 300 nmol of each primer. A DNA thermal cycler model (Techno, Barloworld scientific, Cambridge, UK) was used to perform the amplification using a program with primary denaturation at 94°C for 4 minutes, followed by 35 cycles of denaturation at 94°C for 1 minute, annealing at an appropriate temperature which depended on mT of primers for 1 minute, elongation at 72°C for 1.0 minute and a final extension step of 7 minutes at 72°C. For each PCR, a negative control (sample without template DNA) was included [9]. Amplification products were separated on a 2.0% agarose gel in 0.5 x TAE buffer for 30 min at 100 V and made visible by UV trans-illumination after staining with ethidium bromide (0.5 mg/mL). The BLAST algorithm (http://blast.ncbi. nlm.nih.gov/Blast) was used to find the most related sequence relatives in the NCBI nucleotide sequence database.

Probiotic properties of LAB strains

Growth in bile salts in different acidity rates

The pH of the strains was adjusted to 0.3, 0.4, 0.5, 0.6, 0.7, 0.9, 0.11, and 0.13 with 1M HCl or 1M NaOH before autoclaving in MRS broth. The strains were grown in the MRS broth medium containing 0.2, 0.3, 0.5, 0.6, 0.1., 0.2.0, and 3.0% Oxbile (Sigma) to analyze their resistance to the bile salts. The cultures grown in bile salt-free MRS were considered as controls. All tests were carried out in 96-well microtiter plates (TPP; Zellkultur tetraplatte, Trasadingen, Switzerland). Each well was filled with 100μL of culture medium, and cultured with 5μL of overnight cultures. The Optical Density (OD) was read using a TPP and recorded at 600 nm for each 12 h.

Antibiotic resistance

The antibiotic resistance was tested using the disk diffusion method using 8 antibiotics of vancomycin, tetracycline, kanamycin, ampicillin, chloramphenicol, gentamicin, penicillin, and ciprofloxacin. The feasible concentration ranged from 0.2 to 512 μg/mL. The antibiotic- susceptibility levels included resistant, moderate, or susceptible according to the breakpoint recommended for enterococci according to the National Committee for Clinical Laboratory Standards (NCCLS) standards for antimicrobial disk susceptibility testing and for lactobacilli according to the Scientific Committee on Animal Nutrition [22].

Statistical analysis

Statistical analysis was carried out using SPSS software (SPSS, Windows version 20, SPSS Inc., Chicago, IL, USA) and the Tukey test was used to compare the mean of samples. In order to determine the samples with significant mean differences, one-way repeated measures ANOVA was applied.

RESULTS AND DISCUSSION

Isolation, purification, and identification of the bacteriocin-producing active strain

Among 100 native dairy samples collected from native dairy products, 430 various strains were isolated. Twelve strains with inhibitory effects against *L. innocua* CIP 80.11 and several reference indicators were selected (Table 1). Then, they showed the highest significant inhibitory effect against the indicator strain using the good diffusion method. Among all, only 2 strains having an antimicrobial effect against all the indicators, and the initial acid and thermal resistance were selected (Table 2). They were then examined using catalase test and Gram stain and bacilli, Gram-positive, catalase-negative cocci, LAB were identified. Their genus was then confirmed by uniform universal primers and the strain was also identified by 16S rRNA gene sequence analysis. The above primer pairs were used to amplify the gene. 16S rRNA gene sequence analysis showed that isolates belonged to *E. faecium* TM81 and *L. curvatus* TM51 isolated from Kermanshah and Ilam White cheese, respectively. *E. faecium* TM81 is a Gram-positive bacterium with white colony colour. It is composed of a group of non-moving negative-catalase and oxidase cocci morphologically and has alpha hemolysis. These bacteria are seen in different environments, but they are mainly present in human and animal digestive systems. The extensive distribution of *enterococci* in nature, as compared to other acidic bacteria, is due to their sustainability and their resistance to growth inhibitory factors such as heat, drought, acidity, salt, and chemicals. Some enterococcus species are considered hospital pathogens and have antibiotic resistance genes. However, there has not yet been a report on the hazardous nature of bacteriocin-producing enterococci used in various foods and these compounds

are known as harmless compounds. Bacteriocins produced by enterococci generally referred to as entertain have been studied in various studies and their antibacterial effect has also been proved. One of these bacteriocins is called AS-48, which is used as a bio preservative in various foods such as canned foods, fruit juices, dairy products, and sausages. Most of the bacteriocin-producing enterococcus strains belong to *E. faecium* and *E. faecalis* species.

Identification of antimicrobial property and growth amplification test of isolated strains

The present study investigated the antimicrobial spectrum of the studied strains against LABs, pathogens, and food spoilage agents. Both TM81 and TM51 strains showed antimicrobial activity against similar strains such as lactobacillus and enterococci and prevented the growth of *L. bulgaricus* 340 and 3 strains of *L. brevis* and *E. faecium*. Only *E. faecium* TM81 was able to inhibit the growth of LABs such as *L. paracasei* and *Lactococcus lactis*, and *Lactobacillus casei*. *L. curvatus* TM51 was not able to inhibit them. Both strains showed the inhibitory effect against *L. monocytogenes* and *Bacillus cereus* as food pathogens. Overall, the most anti listeria activity was observed among Gram-positive indicator strains. The enterococci bacteriocin showed antimicrobial effect mainly against Gram-positive bacteria that are considered as food spoilage agents, such as *L.monocytogenes* and *B. cereus*. These bacteriocins rarely affect Gram-negative bacteria; however, the strain isolated in the present research produced bacteriocins that had an antimicrobial effect on Gram-positive bacteria. No activity was reported against *E. coli*, *Salmonella*, and others. *Lactobacillus* TM51 showed antifungal activity only against *Cladosporium* and *Fusarium* and had no inhibitory effect against *Penicillium roqueforti*. *E. faecium* TM81 showed no antifungal activity. The anti-listeria activity showed by the studied strains, confirms their major role in biological protection against food pathogens and listeria disease. Antifungal activity of *L. curvatus* TM51 can also be an important milestone. There are few studies on their antifungal compounds and mechanisms [14]. The identification of the antifungal activity of the intended strain is also considered an important horizon for bio-preservation.

Effect of heat and pH of enzymes and chemicals on bacteriocin function

Bacteriocin-containing CFCS of both TM81 and TM51 strains were active at 100 °C for 30 minutes and at 121°C (autoclave temperature) for 15 minutes. The activity of both strains was maintained at different pH values of 2-10. The effect of amylase, lipase, catalase, and proteolytic enzymes also showed that bacteriocin activity disappeared after an hour, which shows their protein nature. The effect of various chemicals such as triton x 20 and 80 and 100, beta-mercaptoethanol, sodium EDTA, and sodium chloride on bacteriocin CFCS did not have an inhibitory effect on the indicator organism. The thermal stability of bacteriocin of strains studied is considered a very useful characteristic in food biopreservation because most of the food processing methods are carried out at high temperatures. pH stability is also a good tool in acidic food formulations.

Kinetics of bacteriocin production

The production of bacteriocin of the two above strains at 30°C and 37°C in MRS was investigated. The best bacteriocin production temperature for *E. faecium* TM81 and lactobacillus TM51 was 37 °C and 30 °C, respectively (Fig. 1). The TM51 strain began producing the bacteriocin three h later in the logarithmic phase (200 AU/ML). Maximum bacteriocin production was observed at the end of the exponential phase (3200 AU/ML), although it was decreased by the onset of an inactivity period (1600 AU/ML). A very low level of acid was observed after 24 h of incubation (200 AU/ML). Similar results were observed for the TM81 strain. This strain began to produce bacteriocin in the first 2 h of the exponential phase (200 AU/ML), and the maximum production of bacteriocin was reached within 5 h at the end of the exponential phase (3200 AU/ML). A decrease was observed in the inactivity phase. These results were consistent with the results of studies [29]. Many of the bacteriocins studied are produced during the period of exponential growth, which results in the maximum production of bacteriocin at the beginning or the end of the inactivity period.

Effect of bacteriocin-containing CFCS on the growth of the indicator strain

Adding the cell-free overnight culture supernatant on the *L. brevis* F145 and *L. monocytogenes* 506 indicator strains within the initial 3 h of the exponential growth phase prevented cell growth (Figs.1,2). *L. curvatus* TM51 strain supernatant showed bacteriostatic property against *L. brevis* F145 and *L. monocytogenes 506* strains.

Table 1: Antimicrobial activity of obtained LAB isolates.

Indicator strains	LAB isolates											
	TMN2	TMN2	TMN3	TMN4	TMN22	TM51	TM63	TM65	TM81	TMQ73	TMF11	TMF22
L. bulgaricus 340	+	+	+	+	+	+	+	+	+	+	+	+
L. brevis F145	+	+	+	+	+	+	+	+	+	+	+	+
L. brevis F1.114	-	-	-	-	-	+	-	-	+	-	-	-
L. brevis F1106	-	-	-	-	-	+	-	-	+	-	-	-
Listeria. innocua CIP 80.11	-	-	-	-	-	+	-	-	+	-	-	-

"+" - the presence of inhibition

"-" – the absence of inhibition

Table 2: Primers for detection of bacteriocin genes.

Target gene	Primer (5' – 3')	Product size (bp)	Ann. Temp (ºC)	Reference
CurvA	F: GTAAAAGAATTAAGTATGACA R: TTACATTCCAGCTAAACCACT	171	40	Remirger et al., 1996[23]
Ent A	F: GAG ATT TAT CTC CAT AAT CT R: GTA CCA CTC ATA GTG GAA	542	45	Aymerich et al., 1996[24]
Ent B	F: GAA AAT GAT CAC AGA ATG CCT A R: GTT GCA TTT AGA GTA TAC ATT TG	159	41	Toit et al., 2000[25]
Ent P	F: ATG AGA AAA AAA TTA TTT AGT TT R: TTA ATG TCC CAT ACC TGC CAA ACC	216	41	Gutiérrez et al., 2002[26]
Ent L50A	F: NNNNCCATGGGAGCAATCGCAAAA R: NNNNAAGCTTAATGTTTTTTAATCCACTCAAT	135	50	Hadji-Sfaxi et al., 2011 [27]
Ent P	F: GCTACGCGTTCATATGGTAATGGT R: ATGTCCCATACCTGCCAAACCAGAAGC	132	60	Moreno et al., 2006 [28]
Ent Q	F: GGAATAAGAGTAGTGGAATACTGATATGAGAC R: AAAGACTGCTCTTCCGAGCAGCC	653	60	Moreno et al., 2006 [28]
Ent AS48	F: GAGGAGTATCATGGTTAAAGA R: ATATTGTTAAATTACCAA	253	56	Moreno et al., 2006 [28]

No cellular degradation was observed after adding supernatant containing this strain to the culture of the indicator strains because the OD did not decrease, but the growth of the indicator bacteria stopped (Fig 1a and Fig. 2a). The effect of the CFCS of *E. faecium* TM81 was dependent on the strain type. The bacteriocin of this strain caused the cellular degradation of *L. brevis* F145 (Fig. 1b) because it resulted in a reduction in OD levels. This indicates the bactericidal activity of TM81 strain. Adding the supernatant of this strain to *L. monocytogenes* 506 had a bacteriostatic effect, similar to the bacteriocin produced by the TM51 strain (Fig. 2b). Also, the addition of CFCs of *E. faecium* AQ71 to the *L.monocytogenes* growth medium reduced the growth of this bacterium over a period of 20 h [30].

Molecular identification of the bacteriocin gene

In order to screen the bacteriocin genes in the genome of the strains, several primers specific for bacteriocins of LAB were employed (Table 2). For *L. curvatus* TM51 PCR amplicon of the expected size (171 bp) was obtained only with the pair of primers that targets the structural gene of curvacin A (*curA*) (Fig. 3a). For *E. faecium* TM81 PCR amplification resulted in detection of 3 entropion genes: *entA* (542 bp) (Fig. 3b), *entP* (Figure 3c) (216 bp) and *entL50A* (135 bp) (Fig. 3d)

The results of the analysis of nucleotide sequence fragment suggested 99% of identity with the *curA* gene of *L. curvatus* (GenBank: AB292465.1; *L. curvatus curA, cuiA* genes for pre-peptide of curvacin A, immunity protein for curvacin A, complete cds, gene – *curA* position 145.339) (Fig. 4).

Obtained amplicons were analyzed by sequence alignment and amino acid sequence alignment (https://blast.ncbi.nlm.nih.gov/Blast.cgi) based on reconstruction via genetic level, as it was shown before for *curA* gene amplification product of *L. curvatus* TM51 strain.

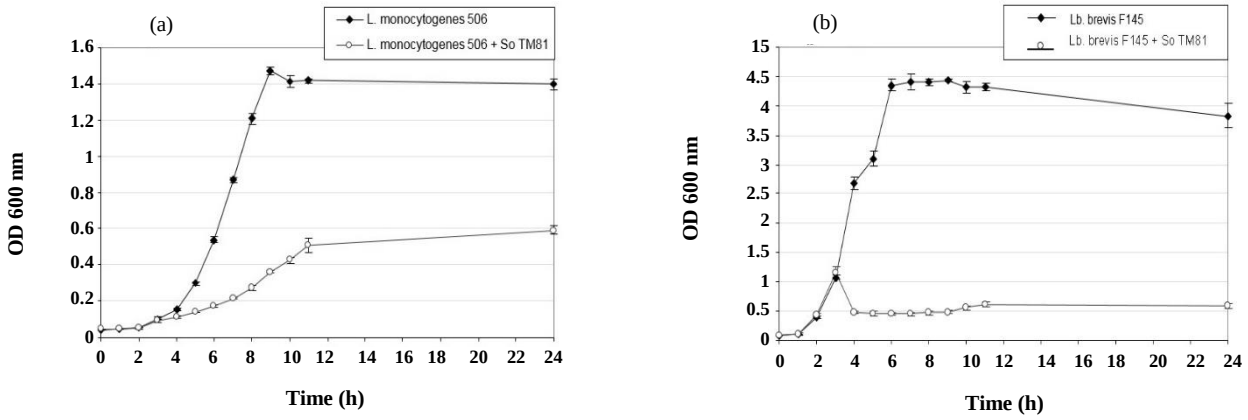

Fig. 1: Effects of bacteriocin-containing cell-free supernatants of strains Lb. curvatus TM51 (a) and E.faecium TM81 (b) on the growth of Lb. brevis F145.

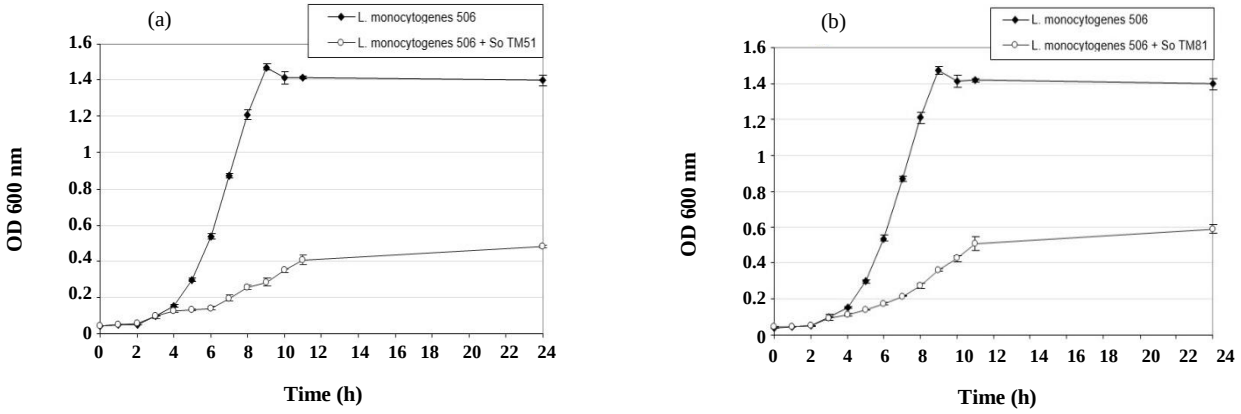

Fig. 2: Effects of bacteriocin-containing cell-free supernatants of strains Lb. curvatus TM51 (a) and E. faecium TM81 (b) on the growth of L. monocytogenes 506.

Nucleotide sequence analysis of amplified PCR products showed 99% of the identity of amplicons to corresponding accession numbers *entP* (AF005726), *entA* (AM746970.1), and *entL50A* genes (AJ223633.1). primers were designed to amplify a partial segment of the bacteriocin genes.

Probiotic property of isolated strains

The ability of isolated proteolytic and bacteriocinogenic strains to grow at different pH values and in the presence of different concentrations of bile salts was tested. Good growth of all tested *Enterococci* strains was recorded in MRS broth with initial pH values of 6.0, 7.0, 8.0, 9.0, and 11.0 (Fig. 5). At pH 13.0 growth level was lower. None of the *Enterococci* strains grows in MRS with an initial pH of 3.0 and 4.0. Obtained results are in accordance with the literature data about the optimal pH range for *Enterococci* [31].

For *Lactobacilli* strains obtained results were different. Strain *L. curvatus* TM51 was able to grow only in MRS with initial pH 6.0, 7.0, and 9.0. Further, decrease or increase of pH inhibited the growth of this strain. *L. helveticus* TM 705 grew well in MRS with an initial pH of 6.0 and 7.0. When the initial pH of the media was increased to 9.0 or decreased to 5.0 we also observe the growth, however, there was retard in entering the exponential growth phase. At pH 3.0, 4.0, and higher than 9.0, the strain didn't grow. *L. paracasei* TM581

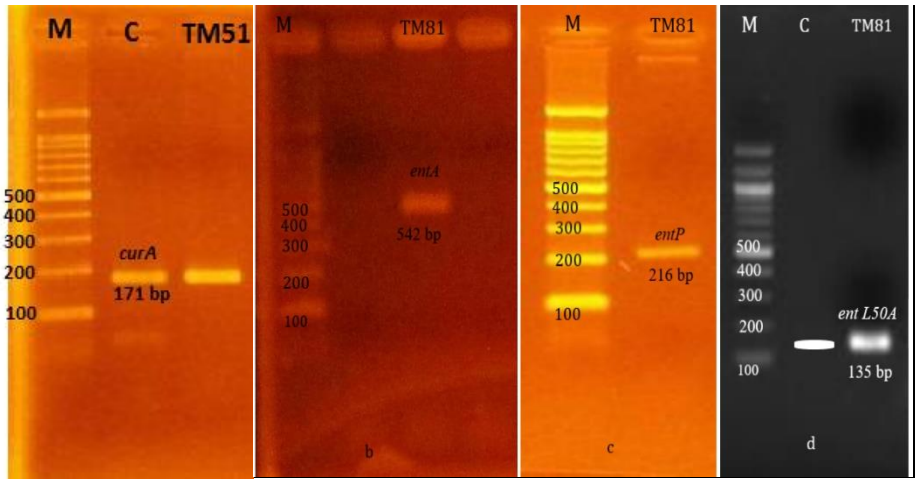

Fig. 3: (a) Electrophoresis gel of L.curvatus genus and species PCR products. The band at 171 bp is the product of the L. curvatus genus primers. TC, (L. curvatus AB292465.1); T1, template 1. L, 100 bp ladder. (b) Electrophoresis gel of E. faecium TM81 genus and species PCR products. The band at 542 bp is the product of the E. faecium TM81 ent A.T1, template 1. L, 100 bp ladder. (c) Electrophoresis gel of E. faecium TM81 genus and species PCR products. The band at 216 bp is the product of the E. faecium TM81 ent P.T1, template 1. L, 100 bp ladder. (d) Electrophoresis gel of E. faecium TM81 genus and species PCR products. The band at 135 bp is the product of the E. faecium TM81 ent L50A.T1, template 1. L, 100 bp ladder. TC, (E. faecium AJ223633.1).

Fw

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amLicon  5  CGGCG-TGCTAGATCATATGGCAACGGTGTTTACTGTAATAATAAAAAATGTTGGGTAAA 63
            ||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||||
curA gene 207 CGGCGGTGCTAGATCATATGGCAACGGTGTTTACTGTAATAATAAAAAATGTTGGGTAAA 266

amLicon  64  TCGGGGTGAAGCAACACAAAGTATTATTGGTGGTATGATTAGCGGCTGGGCTAGTGGTTT 123
             |||||||||||||| ||||||||||||||||||||||||||||||||||||||||||||
curA gene 267 TCGGGGTGAAGCAACGCAAAGTATTATTGGTGGTATGATTAGCGGCTGGGCTAGTGGTTT 326

amLicon  124 AGCTGGAATGTAAA 137
             ||||||||||||||
curA gene 327 AGCTGGAATGTAAA 340
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Rev

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amLicon  6  ATA-CACC-ATAATACTTTGTGTTGCTTCACCCCGATTTACCCAACATTTTTTATTATTA 63
            ||| |||| |||||||||| ||||||||||||||||||||||||||||||||||||||||
curA gene 302 ATACCACCAATAATACTTTGCGTTGCTTCACCCCGATTTACCCAACATTTTTTATTATTA 243

amLicon  64  CAGTAAACACCGTTGCCATATGATCTAGCACCGCCGGTAATTGTTTGTAATTCTGTCATA 123
             |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
curA gene 242 CAGTAAACACCGTTGCCATATGATCTAGCACCGCCGGTAATTGTTTGTAATTCTGTCATA 183

amLicon  124 CTTAATTCTTTTACA 138
             ||||||||||||||
curA gene 182 CTTAATTCTTTTACA 168
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Fig. 4: Results of sequences alignment of obtained curA gene amplification product

was able to grow in MRS with initial pH ranging from 4.0 till 11.

All *Enterococci* strains grew well in the absence and in the presence of oxbile concentrations ranging from 0.2% to 3.0% (Fig. 6). For *Lactobacilli* growth was observed only at concentrations of oxbile 0.2% and 0.3%. Probiotic strains are considered to have the ability to grow in the presence of 0.3% bile salts.

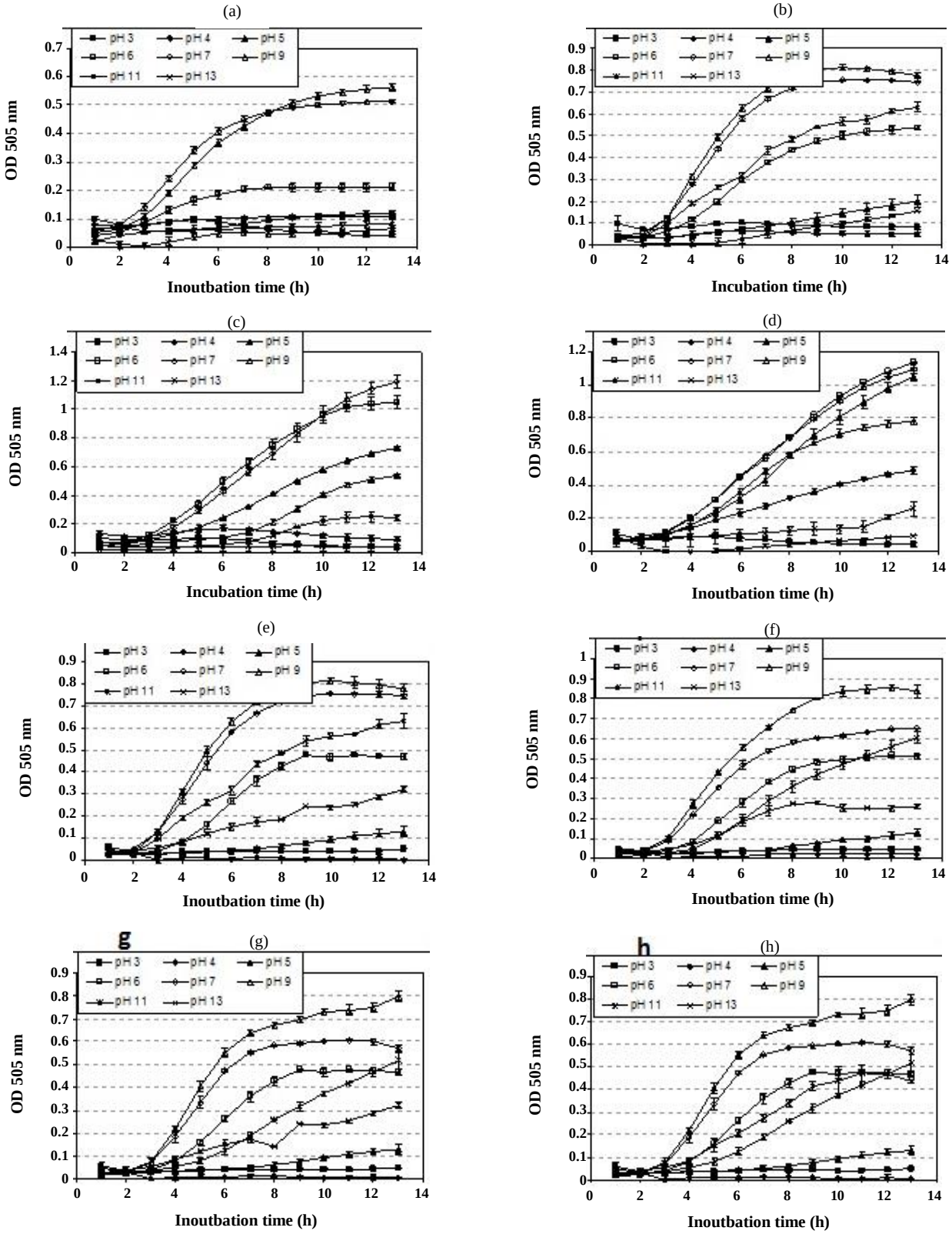

Fig. 5: Comparison of growth of L. curvatus TM51 (a), E.faecium TM81 (b), L. helveticus TM705 (c), L. paracasei TM581 (d), E. faecalis TMN10 (e), TM701 (f), TM121 (g), TM124 (h) in MRS broth adjusted to different pH values

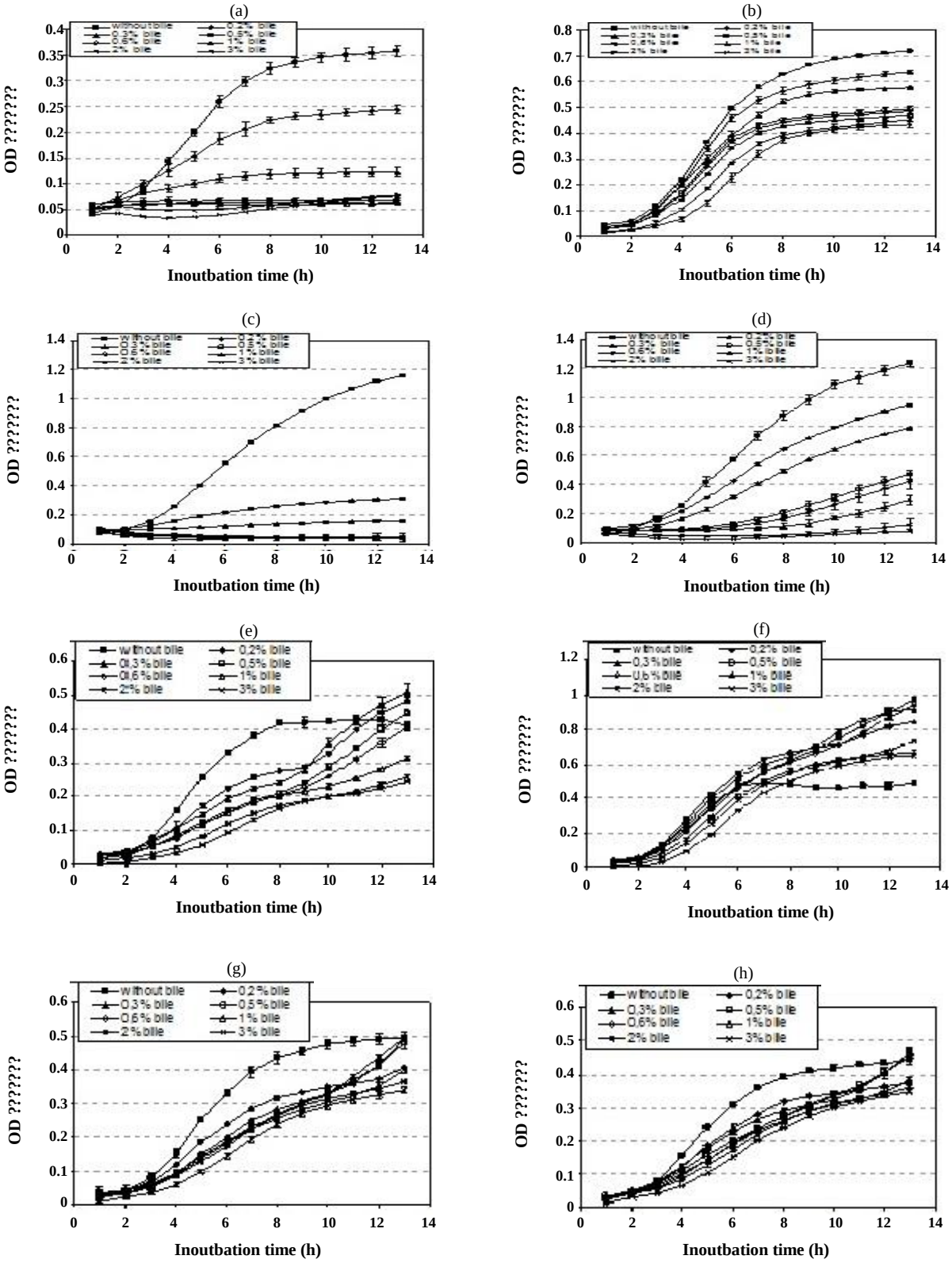

Fig. 6: Comparison of growth of L. curvatus TM51 (a), E.faecium TM81 (b), L. helveticus TM705 (c), L. paracasei TM581 (d), E. faecalis TMN10 (e), TM701 (f), TM121 (g), TM124 (h) in MRS broth supplemented with different concentrations of oxbile.

Table 3. Antibiotic susceptibility of LAB isolates.

	MIC (μg/mL)[1]							
LAB isolates	AMP	CHL	CIP	VAN	GEN	KAN	PEN	TET
E. faecalis TMN10	< 8	= 8	< 4	< 4	< 500	> 32	< 4	< 4
E. faecalis TM121	< 8	= 8	< 4	< 4	< 500	> 32	< 4	< 4
E. faecalis TM124	< 8	= 8	< 4	< 4	< 500	> 32	< 4	< 4
E. faecalis TM1221	< 8	= 8	< 4	< 4	< 500	> 32	< 4	< 4
E. faecalis TM1232	< 8	= 8	8	< 4	< 500	> 32	< 4	< 4
E. faecalis TM701	< 8	= 8	< 4	16	< 500	> 32	< 4	< 4
E. faecium TM81	< 8	< 8	< 4	< 4	< 500	ND	< 4	< 4
L. curvatus TM51	< 8	< 8	< 64	< 4	< 500	ND	< 4	< 4
L. helveticus TM705	< 8	< 16	< 64	< 8	-	ND	< 4	< 2
L. paracasei TM581	< 8	< 16	< 32	< 16	-	ND	< 4	< 2
E. faecium ATCC BAA-2317	32 <	-	8 <	32 <	-		64 <	32 <

AMP=Ampicillin, CHL=Chloramphenicol, CIP=Ciprofloxacin, VAN=Vancomycin, GEN=Gentamycin, KAN=Kanamycin, PEN=Penicillin, TET=Tetracycline

Antibiotic resistance

In addition to investigating the technological characteristics of strains prior to their use in the food industry, it is also necessary to evaluate their antibiotic resistance. Therefore, the bacterial resistance genes are transferred horizontally between strains. In this study, the antibiotic susceptibility of all LAB strains was examined with 8 antibiotic disks are listed in Table 3. All of the enterococci strains were susceptible to ampicillin (MIC<8 μg/mL), gentamicin (MIC<500μg/mL), and tetracycline (MIC<4 μg/mL). Chloramphenicol susceptibility was induced at 8 μg/mL breakpoint. Strains were evaluated as susceptible to ciprofloxacin (MIC<4 μg/mL). Vancomycin resistance was detected only for TM701 (MIC = 16 μg/mL). High resistance to kanamycin (without inhibition at all concentrations used) was observed for all strains. The β-lactam antibiotics susceptibility of food-dependent enterococci is also proved by other authors [32]. Vancomycin-resistant *enterococci* have been seen as a frequent cause of hospital infections in the past decade. Only one of the studied isolates was resistant to vancomycin. Food isolates showed little resistance to this antibiotic [33]. The antibiotic susceptibility was induced for lactobacilli strains according to the following breakpoint: ampicillin (2 μg/mL), chloramphenicol (16 μg/mL), penicillin (4 μg/mL), tetracycline (16 μg/mL); ciprofloxacin (4 μg/mL); vancomycin (64 μg/mL). Therefore, all ampicillin-resistant lactobacillus strains (MIC <8 μg/mL) and gentamicin (no susceptibility up to 500 μg/mL) were resistant. The MIC level was lower than the breakpoints for other antibiotics, so the suspension was considered susceptible According to references, lactobacilli are usually susceptible to ampicillin and chloramphenicol [34].

Discussion

Traditionally, LABs identification is based on cellular morphology, analysis of fermentation products, the activity of similar enzymes, and the ability to use different carbohydrate substrates. The nucleic acids are universal in cellular biology and base nucleotide sequences of this molecule are not affected by the culture medium conditions. Genetic information (genome sequencing) is preferred over phenotypic information from two perspectives: greater ease, more reliability, and more precise interpretation of the results. Basically, genetic information contains more useful and more comprehensive information than phenotypic information. New molecular techniques have gradually come to the fore in the field of microbiology since 1965 and are considered by some researchers as a useful bacterial classification method. Characterization of genetic traits, DNA bases, nucleic acid

hybridization studies, cell wall analysis, and protein sequencing (though in a small amount) were gradually used for valid phylogenetic grouping of bacteria. Sequencing the 500bp region of the 16S rRNA gene (especially the variable regions of V1 and V2) was used to detect *L. acidophilus* strains. This technique successfully detected a wide range of strains. *Nithya et al.* (2011) identified the *L. fermentum* bacterium with high bacteriocin-producing ability in whey using the sequencing method [35]. Rajaram et al. (2010) showed that *L. lactis* bacterium, isolated from the aquatic environment, exhibited strong antimicrobial activity against food spoilage bacteria and can be used as a natural preservative [36]. This research also demonstrates the high ability of the 16S rRNA marker in detecting the different strains of *E. faecium* and *L. curvatus*. Another noteworthy point is the grouping of isolated natives isolated in a cloud, which could be due to the occurrence of mutations and the pressure caused by the natural selection applied to the native Iranian microbial population, which caused the determination of isolates from existing reference bacteria in GenBank, which in turn indicates high genetic diversity in native bacterial populations in Iran. LABs play an important role in the health, nutritional value, and physicochemical properties of milk. Carminati et al. (2001) observed that the number of *Streptococci* and *Enterococcus* bacteria increased compared to other bacteria by incubating milk at 37°C [37]. Research has shown that the presence of *E. faecalis* in a large number of foods is not always associated with fecal contamination. Enterococci are of little value in terms of contamination index in the industrial stages of food. The optimum temperature for the growth of enterococci bacteria and the best temperature for the production and activity of existing bacteriocins produced by *E. faecalis* was reported to be 37 °C. At this temperature, *E. faecalis* bacteriocin had an inhibitory effect on the growth of several species of enterococcus genus and several other genera. On the other hand, among the enterococci bacteria, *E. faecalis* is known for its high resistance to lysozyme [38]. This can justify the results of this study that almost all enterococcus species in the milk belonged to the fecal species. Today, *E. faecalis* is considered as one of the opportunistic antibiotic-resistant LAB. The ability of bacteria to synthesize one or more bacteriocins is considered an advantage because it can eliminate rival bacteria and provide an opportunity for their survival and amplification. In this study, out of a total of 100 samples and 431 isolates, 304 samples (about 70%) were reported to be lactic acid cocci. In another study on 270 samples of raw and pasteurized milk samples, Aleksieva (1980) showed that the prevalence of *E. faecium* and *E. faecalis* was reported to be 43.2 % and 24.6 %, respectively [39]. This research reported the inhibitory activity of enterocins against *L.monocytogenes* and bacillus cereus. Other researches also reported the inhibitory activity of enterocins against the *L.monocytogenes* and *Staphylococcus aureus* of other species [40]. Özdemir et al. reported that the frequency of enterocin genes of L50A / B and P, B, A, in enterococci isolates, isolated from different sources, was 62.9,72.2, 98.1, and 100, respectively [41]. However, the present study reported the presence of Enterocin A, P and L50A genes. Enterocins A, P were the most structural genes found in *Enterococcus* strains. Enterocin A was found more frequently in *E. faecium*, but the enterocin gene of B, P, L50B was found in both *E. faecium* and *E. faecalis*. Bacteriocin production is a regulated process. In other words, environmental conditions affect bacteriocin production. The results of this research and other researches show that the presence of a large number of enterotoxin genes in *E. faecalis* isolates isolated from milk products and confirm that enterocin production occurs extensively in milk and its products. Since enterocins have recently been considered, due to their potential use, as bio-preservatives in human and animal food, the exact identification of enterococcus species producing antimicrobial peptides is necessary to provide appropriate strains as probiotic agents and food bio-preservatives. The widespread distribution of the structural enterocin A gene is probably due to the remarkable ability of enterococci to release and receive the genetic materials between the strains and even genera (including *Staphylococci* and *Enterococci*). The enterocin prevalence varies according to its origin. In order to achieve valid results, it is necessary to investigate larger groups of the strains isolated from different geographical areas and compare independent studies. *Edalatian et al.* (2012) investigated the enterocin-coding genes in 15 enterocin-producing strains isolated from two Iranian traditional kinds of cheese. The results showed that the genes coding enterocin A, B, P, X, and 31 were identified in 10, 8, 5, and 2 strains, respectively. The results of this study are consistent with the results of the present study in terms of an order of frequency of the enterocins A,

P L50A genes [42]. Many studies have proved the effectiveness of enterococcal bacteriocins on *L.mono*cytogenes. Previous studies have proved the antibacterial activity of *E. faecium* bacteriocin (FAIR-E198) against *L. monocytogenes* [43]. Further studies also showed the effect of the *E. faecium* bacteriocin on *Bacillus cereus*, which is consistent with the findings of the present study [44]. There is a severe anti-listeria activity in both strains, but in contrast to the present study, these studies showed that the bacteriocin of *E. faecium* and *L. curvatus* strains have no effect on *S. aureus*. Most bacteriocins have anti-Gram-positive bacteria and are mainly ineffective on Gram-negative bacteria. One of these bacteriocins, which is widely used in the food industry, is nisin produced by *Lactococcus lactis*. Nisin has a bactericidal effect on Gram-positive bacteria but does not affect Gram-negative bacteria [6]. While bacteriocin o the *L. curvatus* bacteria isolated in present research have an inhibitory effect not only against Gram-positive bacteria but also *Fusarium* and *Cladosporium* fungi. The susceptibility of the produced bacteriocin was evaluated by the trypsin enzyme. This enzyme has been responsible for the degradation of antimicrobial activity of the bacteriocin. This finding confirms the protein nature of the resulting antimicrobial compound and indicates the bacteriocin nature of this metabolite. The *E. faecium* AQ71 bacteriocin is affected by the proteolytic enzymes of trypsin and alpha-chymotrypsin, and antimicrobial activity of bacteriocin was eliminated in the presence of these enzymes [30]. The bacteriocin produced by *E. faecalis* KT2W2G lost its activity in the presence of trypsin, pepsin, alpha-chymotrypsin, and Proteinase K . The bacteriocin produced in this research is resistant to high temperatures up to 121 °C. Thermal stability is an important property forbacteriocins since the use of these bacteriocins ensures anti-microbial protection for many foods and drugs that require high temperatures during the production process. *E. faecalis* KT2W2G bacteriocins also maintain their activity at temperatures up to 121 °C. The result of investigating the bacteriocin stability in isolated strains showed their high resistance to pH changes, which tolerated changes in pH from 2 to 10. The bacteriocin produced by *E. faecalis* KT2W2G and *E. faecium* AQ71 strains were active at different pH values (range 2 to 12) [45].

CONCLUSIONS

The results of the present research showed that bacteriocin supernatants produced by *E. faecium* and *L. curvatus* isolated from traditional cheese and yoghurt had antibacterial activity to both Gram-positive and Gram-negative bacteria and also reduces the growth of pathogenic strains. It also maintains its activity at pH changes (2 to 10) and is resistant to heat changes. Therefore, its purification and use as an alternative to chemical antibiotics can be useful in the treatment of antibiotic-resistant infections and as bio preservative in controlling foodborne and fermentative pathogens. The result also showed the presence of various probiotic and bacteriocin-producing LABs is an effective step in the food and pharmaceutical industries. Therefore, the molecular method-based identification of probiotic strains is increasing significantly. Due to consumers demand high quality, additive-free, safe, healthy, nutritious, vitamin-rich, minimally processed, fresh-tasting, lightly preserved, and functional foods with extended shelf life, identification and isolation of probiotic bacteria from such rich sources as well their preservation is necessary. They also should be used as natural preservatives in industrial applications in order to improve the quality and shelf-life of food products and be made available to the general public.

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Conflicts of Interest

The authors declare no conflicts of interest.

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