

Characterization and Kinetics of Growth of Bacteriocin like Substance Produced by Lactic Acid Bacteria Isolated from Ewe Milk and Traditional Sour Buttermilk in Iran

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Abstract

Ethnic people of Iran consume variety of traditional fermented milk products including buttermilk made from ewe's milk. The purpose of this study was to isolate and characterize bacteriocin producing by Lactic acid bacteria from these products, and to exploit their potential as bio preservative. Ten strains of Lactic acid bacteria isolated from ewe's milk, traditional yoghurt and sour buttermilk from different areas in Azarbayjan-e-sharqi, Iran were screened for their ability to produce bacteriocin like inhibitory substances (BLIS). According to results, *Lactobacillus pentosus*, *Lactobacillus paracasei*, *Lactobacillus brevis*, *Pediococcus acidilactici* were shown to produce proteinaceous substances inhibitory against a number of Gram positive and negative bacteria including *Staphylococcus aureus*, *Listeria monocytogenes* and *Salmonella enteritidis*. The inhibitory activities of two Lactic acid bacteria (*Lactobacillus paracasei* and *Pediococcus acidilactici* isolated from ewe milk and buttermilk respectively) were unaffected by the action of pH neutralization and hydrogen peroxide while completely inhibited in the presence of proteolytic enzymes. The kinetic of bacteriocin like inhibitory substances against *Staphylococcus aureus* indicated a direct relationship between the growth rate and the amount of bacteriocin produced. The inhibitory activity of these lactic acid bacteria started in the early logarithmic phase and continued to the end of exponential phase. During ultrafiltration studies, bacteriocins produced by *Pediococcus acidilactici*, *Lactobacillus paracasei* were able to pass through the cellulose membranes with 10 and 30 KDa. Titre of bacteriocins produced by *Lactobacillus pentosus*, *Lactobacillus paracasei*, *Lactobacillus brevis* were estimated 1600 AU/mL while the titre for bacteriocin which produced by *Pediococcus acidilactici* was calculated as 3200 AU/mL.

Keywords: Antagonistic activity; Bacteriocin like inhibitory substances; Ewe milk; Lactic acid bacteria

Introduction

A great number of Gram (+) bacteria and Gram (-) bacteria produce substances of protein structure during their growth with antimicrobial activities, called bacteriocins. Among the Gram positive (+) bacteria, lactic acid bacteria (LAB) have gained particular attention nowadays, due to the production of bacteriocins [1]. Lactic acid bacteria (LAB) play an essential role in the majority of food fermentations, and a wide variety of strains are routinely employed as starter cultures in the manufacture of dairy, meat, vegetable, and bakery products. In addition, the growth of spoilage and pathogenic bacteria in food containing LAB is inhibited. This can be due to pH reduction by the organic acids produced or their ability to produce a variety of antimicrobial substances like ethanol, formic acid, acetoin, hydrogen peroxide, diacetyl and bacteriocins. The latest synthesized ribosomally, peptides or proteins which inhibit microorganisms that are usually closely related to the producer strain [2]. On the other hand, artificial chemical preservatives are employed to limit the number of microorganisms capable of growing within foods, but increasing consumer awareness of potential health risks associated with some of these substances has led researchers to examine the possibility of using bacteriocins produced by LAB as bio-preservatives [3,4]. In this study our aim was to investigate the properties of Lactic acid bacteria present in ewe's milk and traditional sour buttermilk. We analyzed the antimicrobial spectrum and physico-chemical properties of bacteriocin like substances (BLIS) produced by these locally isolated LAB strains.

Material and Methods

Sample preparation

Ewe milk (n:20), yoghurt (n:20) and buttermilk (n:20) were

collected from 30 sheep herds in Myaneh (15 herds) and Hashrood (15 herds) two cities in Azarbayjan-e-sharqi (north-west of Iran). All samples were collected according to EN ISO 707:2001 in sterile bottles of 250 mL and transported to the laboratory under refrigeration (4°C) within 36 h [5]. The sour buttermilk preparation operation is shown in Figure 1.

Isolation and identification of LAB

All collected samples were screened for the presence of LAB by morphological and biochemical tests. All isolated pure colonies were subjected to Gram staining and catalase test. To identify the species, the carbohydrate fermentation profiles using API 50 CHL medium (Bio-Merieux, France) according to the manufacturer's instruction was used.

Antimicrobial spectrum

The antimicrobial effects of selected LAB against Gram positive and negative pathogens were examined by agar well diffusion method described earlier [6]. Three pathogens namely *Staphylococcus aureus*

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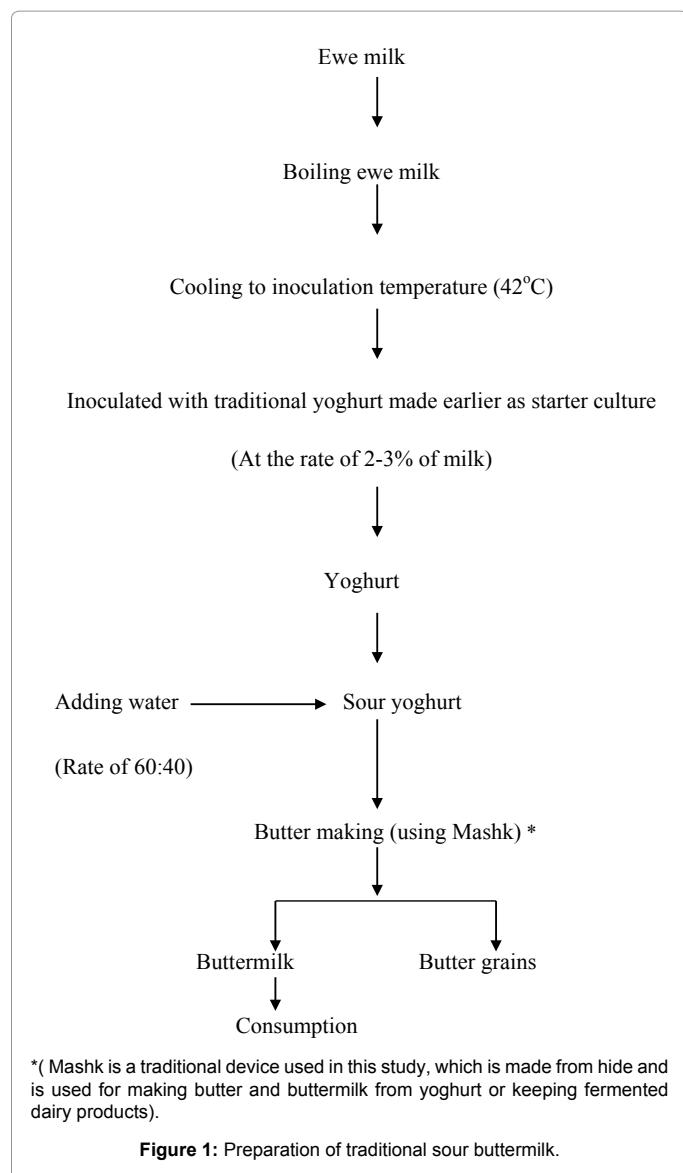


Figure 1: Preparation of traditional sour buttermilk.

(RTCC 1112), *Listeria monocytogenes* (RTCC 1298), and *Salmonella enteritidis* (local isolate) were used as indicator culture in the study. The antimicrobial activity was recorded as appearance of clear zone around the wells.

Sensitivity to enzymes

The cell free supernatants of the selected LAB strains was obtained by centrifuging, and adjusted to pH 6.5 with 1 N NaOH followed by filtration through a 0.22 µm pore size filters. The remaining activity was determined by agar well diffusion assay using *Staphylococcus aureus* as indicator strain. The supernatant fluid of the isolates which resisted pH neutralization were treated with catalase, lysozyme, lipase, pronase E, trypsin and proteinase K (Sigma) at a final concentrations of 1 mg/ml in phosphate buffer (pH 7.0). The remaining activity was determined as described earlier, after 2 h of incubation of the treated samples at 37°C [7].

Molecular size estimation of bacteriocin-like substances

Approximate molecular weight of the antagonistic peptides was

determined by ultrafiltration. A 2 ml culture free supernatant fluid was ultrafiltered through cellulose membranes with 5, 10 and 30 KDa exclusion units (Centricon, Micro concentrations, USA). Bacteriocin activity in retained and eluted fractions was determined by well-diffusion agar [8].

Bacteriocin titration

Bacteriocin-like activities of selected LAB strains were examined by the dilution assay. Serial two-fold dilutions of bacteriocin were tested by the well-diffusion method. The antimicrobial activity of the bacteriocin was defined as the reciprocal of the highest dilution showing inhibition zone and was expressed in active units per milliliter (AU/ml) [9].

Kinetics of bacteriocin-like production

20 µl of overnight culture supernatants of LAB strains were inoculated into 50 ml of MRS broth, then the incubated at 37°C. At time intervals of one hour, the growth of cells was measured by the absorbance (OD 660 nm) and the antimicrobial activity [10].

Antibiotic resistance

The antimicrobial susceptibilities of the isolates and reference strains were preliminarily assessed by the disk diffusion method on MRS [11]. Ten antibiotics used in the treatment of the most common hospital infections were chosen, belonging to different categories: ampicillin, penicillin, tetracycline, vancomycin, erythromycin, gentamicin, clindamycin, Chloramphenicol, Kanamycin and Sterptomycin. The assay was performed as follows: swabs imbibed with bacterial cultures, adjusted to 0.5 McFarland, were plated, and paper disks with antibiotics were placed aseptically on the inoculated plates and incubated for 24 h at 37 1C in 5% CO₂. The diameters of the inhibition zones were measured (mm).

Result

Isolation and Identification of LAB

Seventy-seven bacterial isolates from the traditionally made dairy samples including yogurt and buttermilk were identified as LAB. All isolates were identified as catalase negative and Gram-positive bacilli or cocci in pairs or long chains, and/or coccobacilli. All isolates showed different level of activity against the tested pathogens. Ten isolates with higher activity against *Staphylococcus aureus*, *Listeria monocytogenes*, and *Salmonella enteritidis* were identified to species level as *Lactobacillus pentosus*, *Lactobacillus paracasei* (two strains), *Lactobacillus brevis*, *Pediococcus acidilactici* (four strains) and *Lactococcus lactis* (two strains) (Table 1).

Antimicrobial spectrum

Ten selected strains showing antibacterial activity against all pathogens. In addition, all isolates showed maximum antimicrobial zone against *Salmonella enteritidis* (Table 2).

Strains	Samples
<i>Lactobacillus pentosus</i>	Milk
<i>Lactobacillus brevis</i>	Milk
<i>Lactobacillus paracasei</i> (LP1,2)	Milk
<i>Lactococcus lactis</i> (LL1,2)	Milk
<i>Pediococcus acidilactici</i> (PA1,2)	Milk
<i>Pediococcus acidilactici</i> (PA 3,4)	Buttermilk

Table 1: Identification of the selected LAB isolates to species level using standard API 50CHL identification kit.

Sensitivity to enzymes

The antagonistic activity shown by *Lactococcus lactis* (LL1), *Lactobacillus paracasei* (LP1) and *Pediococcus acidilactici* (PA 1, 2, 3) appeared to be due to acid production as their activity was completely lost on pH neutralization of their supernatant fluids. The antibacterial activity of *Lactobacillus pentosus*, *Lactobacillus paracasei* (LP2), *Pediococcus acidilactici* (PA4) and *Lactobacillus brevis* are BLIS, as their antimicrobial activity were completely eliminated after treatment with lysozyme, pronase and proteinase (Table 3).

Molecular size estimation of bacteriocin-like substances

The fractions of the bacteriocins produced by *Lactobacillus pentosus* and *Lactobacillus brevis* showing antibacterial activity corresponded

to peptide molecules in the range of 5-10 KDa while *Lactobacillus paracasei* (LP2) and *Pediococcus acidilactici* (PA4) range of 10-30 KDa which was subsequently confirmed by subjecting the fractions to ultra-filtration using filtron membranes with 5, 10 and 30 KDa molecular weight cut off.

Bacteriocin titration

The highest titration of bacteriocin of *Lactobacillus pentosus*, *Lactobacillus paracasei* (LP2) and *Lactobacillus brevis* was 1600 AU/ml though in *Pediococcus acidilactici* (PA4) was 3200 AU/ml.

Kinetics of bacteriocin-like production

Figures 2 and 3 show the growth curves and the profiles of bacteriocin production of the *Lactobacillus paracasei* (LP2) and

strains	Pathogens	<i>Listeria monocytogenes</i>	<i>Staphylococcus aureus</i>	<i>Salmonella enteritidis</i>
<i>L.b pentosus</i>		+	+	+++
<i>L.b brevis</i>		++	+	+++
<i>L.c lactis 1</i>		+++	+++	+++
<i>L.c lactis 2</i>		+	+++	+++
<i>L.b paracasei 1</i>		+	+++	+++
<i>L.b paracasei 2</i>		+++	+++	+++
<i>Ped. acidilactici 1</i>		+++	+	+++
<i>Ped. acidilactici 2</i>		+	++	+++
<i>Ped. acidilactici 3</i>		+	+	++
<i>Ped. acidilactici 4</i>		+++	+	++

+: 3mm< zone
 ++: 3mm< zone <5mm
 +++: 5mm< zone <7mm

Table 2: Antagonistic activity cell free supernatant of selected strains against pathogens by agar well diffusion assay.

LAB isolates	pH	Catalase	Lysozyme	Pronase E	Proteinase K	Trypsin
<i>L.b pentosus</i>	+	+	-	-	-	+
<i>L.b brevis</i>	+	+	-	-	-	+
<i>L.c lactis 1</i>	-	Acid	Acid	Acid	Acid	Acid
<i>L.c lactis 2</i>	+	+	+	-	-	+
<i>L.b paracasei 1</i>	-	Acid	Acid	Acid	Acid	Acid
<i>L.b paracasei 2</i>	+	+	-	-	-	+
<i>Ped. acidilactici 1</i>	-	Acid	Acid	Acid	Acid	Acid
<i>Ped. acidilactici 2</i>	-	Acid	Acid	Acid	Acid	Acid
<i>Ped. acidilactici 3</i>	-	Acid	Acid	Acid	Acid	Acid
<i>Ped. acidilactici 4</i>	+	+	-	-	-	+

+: antimicrobial activity remaining
 -: no antibacterial activity

Table 3: Inhibitory activity of selected LAB isolates against *Staphylococcus aureus* after pH neutralization and enzyme treatments.

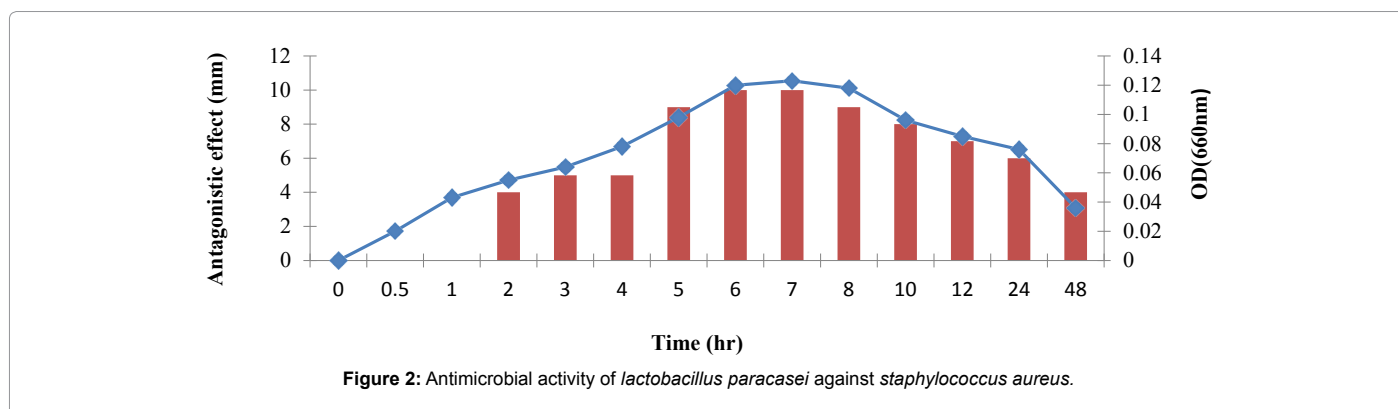


Figure 2: Antimicrobial activity of *lactobacillus paracasei* against *staphylococcus aureus*.

Pediococcus acidilactici (PA4) strains, respectively. In all these cases, bacteriocin production was initially detected in the logarithmic phase of growth, and the maximum levels of antimicrobial activity were found at different stages of the stationary phase of growth, depending on both the producer strain and the indicator microorganism. Both bacteriocins were decreased after 24 and 48 h.

Antibiotic resistance

Ten selected isolates were resistance to tetracycline and chloramphenicol. Furthermore all isolates were sensitive to kanamycin, streptomycin, vancomycin, clindamycin (Table 4).

Discussion

The traditional fermented dairy products can potentially be a good source of potential probiotic organisms. The microbial ecology and beneficial health effects of fermented dairy products such as buttermilk have not been reported earlier. These products are popular especially in the rural areas because of their good natural tastes and flavors. During our results, we isolated *Lactobacillus pentosus*, *Lactococcus lactis*, *Lactobacillus paracasei*, *Lactobacillus brevis* from ewe milk and *Pediococcus acidilactici* from buttermilk. Similar to our result, Tajabadi et al. [12] isolated LAB from different kinds of yogurt, cheese, fermented milk, dough and kashk. As a functional probiotic, anti-pathogen activity is one of important properties to be considered. The spectrum of activity of the antagonistic compound produced by these isolates

indicated their action against certain important food borne pathogens. *Staph.aureus* is considered a potential public health risk due to its production of enterotoxins that cause food poisoning [7]. In addition, food borne transmission of *L.monocytogenes* has been implicated of listeriosis in human involving the consumption of various foods [13]. Our study indicated that the selected LAB strains were more effective in inhibiting the growth of *Salmonella enteritidis* compared to other pathogens. A number of metabolites such as acid, Hydrogen peroxide and bacteriocin are believed to contribute to antimicrobial activity. The sensitivity of the inhibitory agent to lysozyme, pronase, trypsin and proteinase K indicates of their proteinaceous nature and thus might be considered as bacteriocin. Similar to this result, inhibition of pathogens like *Listeria monocytogenes* and *Staphylococcus aureus* by LAB was reported [14,15]. In this research, Bacteriocins were detected early in the logarithmic growth phase. Studies produced continuously during this phase supporting the fact that bacteriocins display primary metabolite kinetics. Bacteriocin activity showed a maximum level at the stationary growth phase. A decline in inhibitory activity during the late stationary growth phase was observed. This may be due to proteolytic degradation, adsorption to cells or bacteriocin aggregation [16]. In contrast to our result, production of bacteriocin by *Lactobacillus paracasei* occurred throughout logarithmic growth [17]. Also in contrast to our result, Ogunbanwo showed that bacteriocin of *Lactobacillus brevis* was unable to pass through 1,000 and 10,000 KDa molecular weight cut-off membranes. A tendency to aggregate with other proteins has been

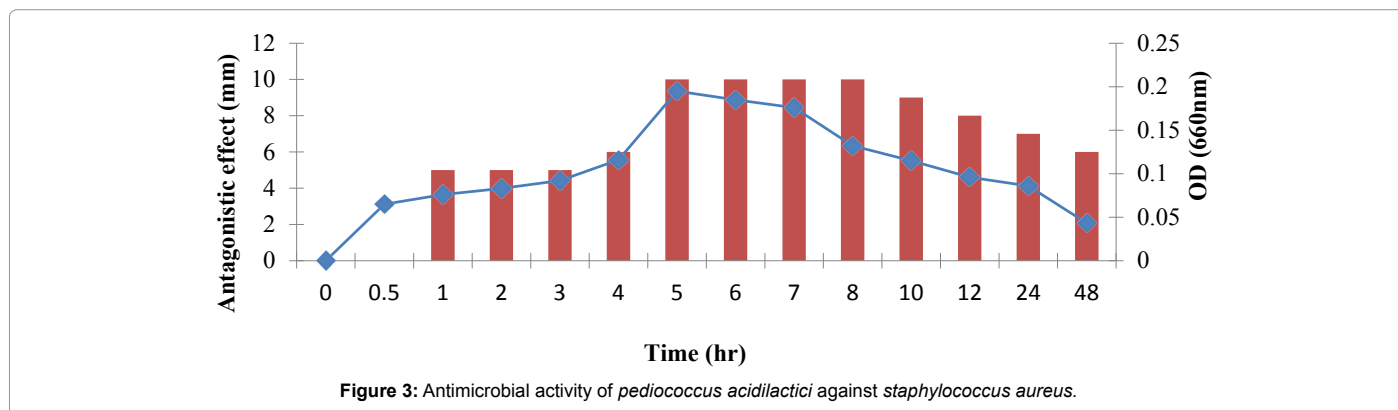


Figure 3: Antimicrobial activity of *pediococcus acidilactici* against *staphylococcus aureus*.

LAB isolates	Kanamycin	Gentamicin	Sterptomycin	Ampicillin	Vancomycin	Tetracycline	Chloramphenicol	Penicillin	Clindamycin	Erythromycin
<i>L.b pentosus</i>	-	-	-	5	-	8	10	5	-	9
<i>L.b brevis</i>	-	5	-	6	-	8	10	10	-	10
<i>L.c lactis 1</i>	-	-	-	-	-	7	5	-	-	-
<i>L.c lactis 2</i>	-	-	-	5	-	5	6	5	-	5
<i>L.b paracasei 1</i>	-	-	-	5	-	6	8	8	-	9
<i>L.b paracasei 2</i>	-	-	-	-	-	7	10	10	-	10
<i>Ped. acidilactici 1</i>	-	-	-	5	-	6	6	5	-	6
<i>Ped. acidilactici 2</i>	-	-	-	6	-	8	10	6	-	10
<i>Ped. acidilactici 3</i>	-	-	-	5	-	6	10	10	-	10
<i>Ped. acidilactici 4</i>	-	-	-	5	-	5	7	7	-	10

mm : zone of inhibition
 - : no antibiotic resistances

Table 4: Antibiotic resistances of selected LAB isolates by agar well diffusion assay.

reported in bacteriocins produced by other lactic acid bacteria, and may have been a contributing factor why the bacteriocins could not pass through the membrane with low molecular weight cut-off.

The EFSA considers antibiotic resistances, especially transferable resistances, a safety concern and a decision criterion for determining a strain's QPS status [18]. Antibiotic resistance genes carried by LAB can be transferred to human pathogenic bacteria either during food manufacture or during passage through the GIT [19]. In this research showed that *Lactococcus lactis* (LL2) was the most sensitive and *Lactobacillus brevis* and *Pediococcus cidilactici* (PA2) were the most resistance to different antibiotics. In compare to our result, Hummel et al., showed that lactic acid bacteria like *lactobacillus* and *pediococcus* were unaffected by gentamicin, streptomycin.

Conclusion

There is a wide variety of traditional dairy products especially in rural areas of Iran. These products mostly made from unpasteurized milk are appreciated by local people regarding its proven health benefits. This study showed the presence of viable probiotic LAB micro flora in these products. The antagonistic activity possessed by these isolates might be used for the control of unwanted pathogens mainly in dairy products, and could be exploited further for use in fermented dairy products.

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