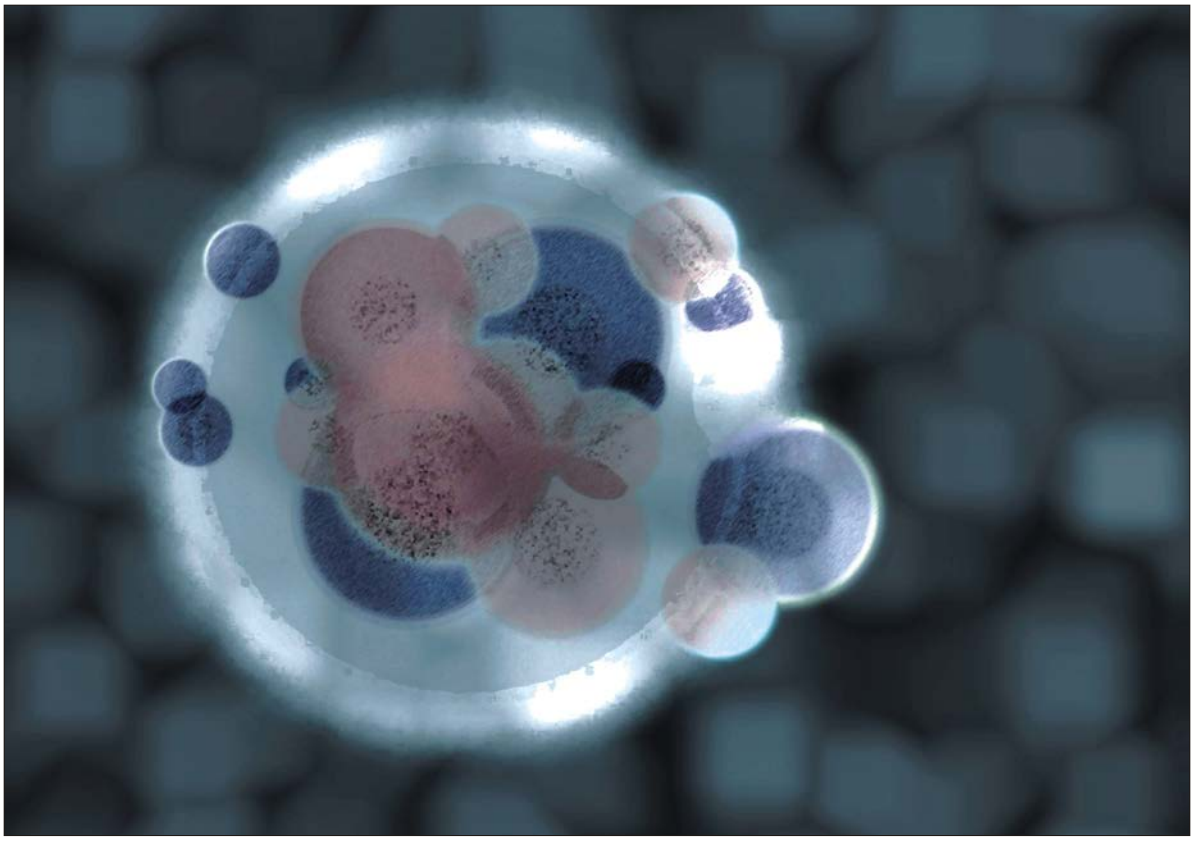

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Antimicrobial Effects of Bacteriocin Like Substance Produced by *L. acidophilus* from Traditional Yoghurt on *P. aeruginosa* and *S. aureus*

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Abstract: Lactic Acid Bacteria (LAB) commonly used in food as starter cultures are known to produce antimicrobial substances such as bacteriocins and have great potential as food bio-preservatives. Six *L. acidophilus* isolated from traditional yoghurt, were screened for the production of antimicrobial substances. *Lactobacillus acidophilus* isolated from bio-yoghurt showed the broadest spectrum of antimicrobial activity was selected for further characterization. The growth of isolates was investigated in MRS medium containing 1-2% glucose at pH 6.5-7. Purification of the active compound was achieved after gel filtration and ion exchange chromatography. As revealed by SDS-PAGE, active fractions were relatively homogeneous, showing a protein with molecular mass of 30 kDa. It was stable to heat (100°C for 15 min). The bacteriocin-like substance was active against the gram positive bacteria *Bacillus cereus* and *Staphylococcus* aureus and against *Pseudomonas aeroginosa* and *Klepsiella pneumona*. The antimicrobial activity against *P. aeroginosa* was stronger than *S. aureus*. These antibacterial activities of bacteriocins-like substance were determined under absence of organic acids and hydrogen peroxide in medium.

Key words: *Lactobacilus acidophillus*, traditional yoghurt, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, bacteriocin like substance

INTRODUCTION

Lactic Acid Bacteria (LAB) commonly used in food as starter cultures are known to produced antimicrobial substances such as bacteriocins and have great potential as food bio-preservatives (Jamuna and Jeevaratnuam, 2004; Avonts *et al*., 2004). During the fermentation of dairy products, the lactobacilli cultures metabolize lactose to lactic acid. Acid production lowers the pH and creates an acidic environment that is unfavorable for pathogens and spoilage organisms (Aslim *et al*., 2004).

In addition to acids, hydrogen peroxide, diacetyl and bacteriocins may also play inhibitory roles for pathogenic microorganism (Zhu *et al*., 2000). *L. acidophilus* has received more attention and has been the subject of much research due to its ability to produce antimicrobial agents against other bacteria. Bacteriocins are antimicrobial proteins or peptides, which are bactericidal to other, usually closely related bacteria (Due Toit *et al*., 2000). They have been the subject of extensive studies in recent years because of their potential use as natural food preservatives (Villiani *et al*., 2001).

Bacteriocin production may facilitate the establishment of a probiotic strain in the competitive environment of the gut (Klaenhammer and Kullen, 1999).

L. acidophilus is the main *Lactobacillus* species involved in the manufacture of mild and probiotic yoghurt (Mercenier *et al*., 2003). *L. acidophilus* 30SC produced a heat stable antimicrobial compound which inhibited a number of gram-positive bacteria including *Listeria ivanovii* (Oh *et al*., 1993). The ability of *L. acidophilus* to prevent pathogenic bacteria proliferation has been well documented. Bogovic-Matigasic *et al*. (1998) isolated two bacteriocin of *L. acidophilus* which had an antagonistic effect against *Bacillus cereus*, *Clostridium* sp. and *Listeria innocua*. Zamphir *et al*. (1999) showed antibacterial activity against *E. coli* and *Salmonella* by *L. acidophilus* IBB 801.

The aim of this study was to investigate both growth and bacteriocin production of *L. acidophilus* isolated from Iranian traditional yoghurt. The inhibitory effects of *L. acidophilus* extra-cellular products were also evaluated on indicator bacteria *B. cereus*, *S. aureus*, *P. aeroginosa* and *K. pneumonia*.

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MATERIALS AND METHODS

Bacterial strains, culture media and identification: *Lactobacillus acidophilus* was isolated from yoghurt by appropriate dilutions with ringer, plated on MRS agar and incubated anaerobically at 37°C for 48 h (Gaspack A, Merck, Germany). The well isolated colonies were picked up and transferred to MRS broth. Nutrient broth and agar were used for culturing of *P. aeruginosa* (8821 M), *S. aureus* (ATCC 29213), *B. cereus* and *K. pneumonia* (clinically isolates).

L. acidophillus strains were stored at -80°C in the MRS broth containing 20% glycerol as cryoprotectant. All *L. acidophilus* isolates were tested for their ability to produce bacteriocin.

Preparation of culture supernatant fluid: *Lactobacillus acidophilus* was grown for 48 h at 37°C at pH 6.5 in MRS (Merck, Germany) broth with 1-2% of glucose and without glucose. Culture broth was centrifuged at 5000x g for 10 min at 4°C. The supernatant then removed and the pH was adjusted to 6.5-7 with NaOH, treated with 5 mg mL^{-1} catalase to eliminate hydrogen peroxide and filter serilized using a 0.2 μm pore size filter (Schleicherand Schuell GMbH, Dassel, Germany).

Chemical reagents: All chemical reagents and enzymes (lysozyme, tripsin, pronase, proteinase K) were obtained from Sigma Ltd.

Partial purification of bacteriocins: Cell free supernatant from previous stages were concentrated by ammonium sulfate (40% saturation) and the precipitate, pelleted by centrifugation (5000 g, 30 min), dissolved in a small amount of sodium phosphate buffer (pH 6.5) and then dialyzed against the same buffer for three times. After filter sterilization, an aliquot of the supernatant fluid was loaded onto an ion exchange CM-Sepharose (Amersham Pharmacia Biotech, Appsala, Sweden) column. A linear gradient from 0-0.5 mol L^{-1} NaCl was used to elute the purified bacteriocin, at a flow rate 1 mL min^{-1}. OD of fractions was measured at 280 nm and fractions with maximum OD were pooled and concentrated with freeze drying.

SDS-PAGE: Partially purified supernatant of *L. acidophilus* concentrated with freeze- drying. A 10 μL aliquot of the sample and 5 μL aliquot of the molecular weight standards were loaded to the gel. Electrophoresis was performed in a vertical slab gel apparatus at a constant voltage (60-80V) for approximately 4 h. After electrophoresis, the gels were stained with Coomassie Brilliant Blue (Merck) and photographed with Polaroid camera.

Effect of proteolytic enzymes and heat treatment: The sensitivity to proteolytic enzymes was tested by treating the purified bacteriocins with 1 mg mL^{-1} of proteinase K, trypsin, lysozyme and pronase in phosphate buffer 10 mmol L^{-1}, pH 7, for 2 h at 37°C. Residual activity was determined by the well diffusion agar method. The effect of heat was assessed at temperatures of 80, 90 and 100°C for 10, 20, 30, 40 and 60 min in a thermostatic water bath and at 121°C (autoclaved) for 15 min. The effect of pH was tested by adjusting the pH in the range of 3-9 and examining the activity after incubation at 2, 4 and 24 h.

Effect of cultivation condition on growth and optimization of bacteriocins production: To study the glucose effect on growth and production of bacteriocins, a series of 250 mL of MRS broth with 0, 0.5, 1, 1.5 and 2% of glucose inoculated with 10^7 L. acidophilus and an-aerobically incubated at 37°C for 48 h. At the end of incubation period, cultures were examined for viable lactobacilli counts and antibacterial activity.

Inhibitory effect by the agar-well diffusion method: The inhibitory effects of *L. acidophilus* on indicator bacteria were carried out by well diffusion assay. Petri dishes with 10 mL of nutrient agar that were previously inoculated with 0.1 mL of a 24 h old nutrient broth culture of individual test bacteria, once solidified, dishes were stored for 2 h at 4°C. Four wells were made and the wells were filled with 60 μL culture filtrate, treated supernatant, partially purified bacteriocin and MRS broth as a control. The inoculated plates were kept at 4°C for 2 h and then incubated at 37°C for 24 h. The diameter of the inhibition zone was measured according to the methods outlined by Aslim *et al.* (2004).

RESULTS AND DISCUSSION

A total of 6 *L. acidophilus* isolated from Iranian traditional yoghurt was tested for antibacterial activity against *P. aeruginosa*, *K. pneumonea* and *S. aureus* and *B. cereus*. All of *L. acidophilus* isolated from yoghurt exhibited antibacterial activity, but *L. acidophilus* isolated from bio-yoghurt showed stronger antibacterial activity. Bacteriocin production of all *L. acidophilus* isolates showed a peak activity at the end of the exponential growth phase. The antibacterial activities were persisted

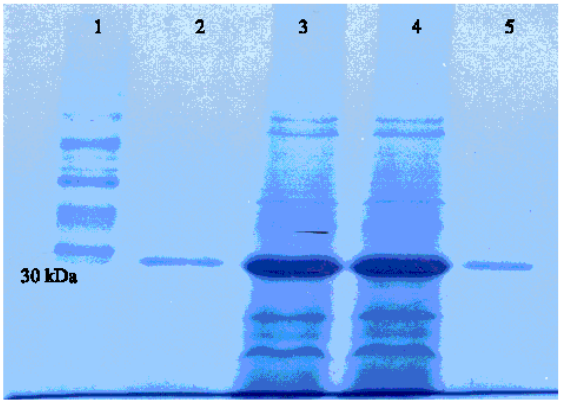

Fig. 1: SDS-PAGE of bacteriocins like substance from *L. acidophilus* isolated from bioyoghurt. Lane 1. High range standard marker; Lane 2, 5: bacteriocins like substances after chromatography. Lane 3, 5: bacteriocin before chromatography

Table 1: Antimicrobial activity of *L. acidophilus* isolated from different Iranian traditional yoghurt against indicator bacteria

L. Acidophilus Isolated from Yoghurt	CFF	CFBH	BLS
1	++	+	+
2	+	+	-
3	++	+	-
4	+	-	-
5	-	-	-
6	+++	++	++

Diameter of the inhibition zone: -, no inhibition zone; Weak (8-10 mm), ++, intermediate (13-20 mm), +++, strong (20-30 mm); CFF (culture supernatant); CFBH (neutralized culture supernatant); BLS (bacteriocin like substance)

during concentration of cell free culture supernatants by ammonium sulphate precipitation and chromatography procedures. The inhibitory activities of *L. acidophilus* bacteriocins like substances were stable at 100°C for 10 min. No decrease were observed in inhibitory activities after 7 days at 4°C. Maximum production of Bacteriocins-like Substance (BLS) were obtained in MRS broth containing 1-2% glucose. The electrophoresis mobility of BLS protein presented in the fractions of *L. acidophilus* revealed to be a molecular weight of 30 kDa (Fig. 1).

In the present study, culture supernatants (CFF) obtained from different *L. acidophilus* exhibited varying degrees of inhibitory activity against *S. aureus*, *B. cereus* and *P. aeruginosa* and *K. pneumonea*.

We concluded that the inhibitory effects of supernatants from bio-yoghurt were due to bacteriocin-like substances. The inhibitory activities exhibited by cultures of *L. acidophilus* were observed (Table 1). No inhibitory effects were detected when MRS medium were used as control.

Table 2: Antimicrobial activity of *L. acidophilus* isolated from Bio-yoghurt

Indicator bacteria	CFF	CFBH	BLS
S. aureus	++	+	+
P. aeroginosa	+++	++	++
K. pneumonia	++	++	++
B. cereus	++	+	+

Diameter of the inhibition zone: (-) no inhibition zone, (+) Weak (8-10 mm), (++) intermediate (13-20 mm), (+++) strong (20-30 mm) CFF (culture supernatant), CFBH (neutralized culture supernatant), BLS (bacteriocin like substance)

The bacteriocin preparation lost antimicrobial activity when treated with proteolytic enzymes but not with catalase.

It was observed that neutralized culture supernatant (CFBH) and catalase treated supernatant (CFB) of *L. acidophilus* exhibited weak inhibitory activity against the *S. aureus* and intermediate activity against the *P. aeruginosa* (Table 2). An earlier study with bacteriocin originated from *L. acidophilus* has been shown the intermediate activity against *S. aureus* (Aslim *et al*., 2004). The ability of *L. acidophilus* to prevent proliferation of pathogenic bacteria has been well documented. In addition to lactic and other acids, *L. acidophilus* has the capacity to produce numerous metabolites that kill pathogenic bacteria (Oh *et al*., 1993; Gupta *et al*., 1983; Gukasian *et al*., 2002). Bacteriocidal proteins with antagonistic activities, termed bacteriocin, are produced by some strains of *L. acidophilus*. Among lactobacilli, strains belonging to species of the *L. acidophilus* and *L. casei* are frequently used as probiotic agents (Kalenhammer and Kullen, 1999). Bacteriocin production and antibacterial activity has been shown for *L. acidophilus* isolated from dairy product and intestinal tract (Barefoot and Kleanhammer, 2003; Zamphir *et al*., 1999; Ioth *et al*., 1995).

In the similar studies, *L. acidophilus* IBB 801 isolated from dairy products have been nominated as the best bacteriocin producing strain and displayed antibacterial activity against *E. coli* and *Salmonella* (Zamphir *et al*., 1999; Avonts *et al*., 2004). According to the present results, it would be concluded that bacteriocins originated from *L. acidophilus* could have some antagonist activities against pathogens and food contaminants. The use of lactobacilli and their bacteriocins as bio-preservator in food and dairy products is now approved in several countries and could be a suitable and effective candidate for chemical preservatives.

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