International Journal of Agricultural Science and Research Volume 2, Number 1, Winter 2011(Serial #2)

A Study on the Antibacterial Activity of Lactic Acid Bacteria Isolated from Traditional Iranian Milk Samples

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Received: February, 19, 2011 *Accepted: June, 5, 2011*

ABSTRACT

198 Lactic acid bacteria (LAB) were isolated from Iranian milk samples. Almost all isolated LAB showed inhibitory activity towards *E. coli* and *S. aureus* as Gram negative and Gram positive indicators, respectively. No role was observed for hydrogen peroxide in the activity of all isolated strains while organic acids proved a prominent role in all detected inhibitory activities. Within isolated LAB, four strains represented the inhibitory activity against *S. aureus* based on the production of bacteriocin-like inhibitory substances (BLIS). Inactivation of the activity by proteolytic enzymes demonstrated the proteinaceous nature of the antimicrobial compounds. The selected producers were identified as *L. lactis, E. faecium* 1*, E. hirae* and *E. faecium* 2 based on biochemical tests according to Bergy's manual of systematic bacteriology. *L. lactis* had the highest Minimum Inhibitory Concentration (320 IU/ml) and showed bacteriostatic mode of activity towards the indicator bacteria.

Keywords: Lactic acid bacteria; Organic acids; Hydrogen peroxide; Bacteriocin; Minimum inhibitory concentration; Mode of action.

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INTRODUCTION

Lactic acid bacteria (LAB) have traditionally been used in food processing because of their potential ability to improve the organoleptic characteristics and healthiness of foodstuffs. Antimicrobial activities of LAB have been widely investigated in the past 20 years. Owing to an impending threat from foodborne pathogens and consumer demand for safe and fresh food products, LAB have come to the forefront as biopreservative agents (Cleveland *et al*., 2001; Gálvez *et al*., 2007; Zhu *et al*., 2000). It has been demonstrated that different antimicrobial agents produced by these bacteria can inhibit pathogenic and spoilage microrganisms, extending the shelf-life and enhancing the safety of food products. Lactic and acetic acid, hydrogen peroxide, carbon dioxide and antimicrobial peptides which are created during bacterial development are particular interest in this aspect (Gálvez *et al*., 2007; Zamfir *et al*., 1999; Zhu *et al*., 2000).

Variety traditional fermented milk products are available in Asia, Africa, the Middle East, Northern and Eastern Europe. The functional properties of traditional fermented products may be attributed to the metabolic products of the complex microbial content. The microbiological characteristics of fermented milk products have been studied in Indonesia (Yodoamijoyo *et al*., 1983), Zimbabwe (Feresu & Muzondo, 1990), Africa (Beukes *et al*., 2001), and Algeria (Guessas & Kihal, 2004). During the fermentation of dairy products, these cultures metabolize lactose to lactic acid. Acid production lowers the pH and creates an environment that is unfavorable to pathogens and spoilage organisms. Moreover, the low pH of fermented foods potentates the antimicrobial effects of organic acids (Aslim *et al*., 2005).

Although the acidic conditions created by these bacteria considered to be more responsible for their preservative effect in food (Zamfir *et al*., 1999), bacteriocins have extensively been examined for applications in microbial food safety and have been the subject of intensive investigation due to their potential use in the food industry as natural safe food preservatives (Cleveland *et al*., 2001; Ghrairi *et al*., 2008). The most promising antimicrobial peptides that particularly inhibit closely related species are known as bacteriocins that are characterized as a wide range of biologically active, usually ribosomally synthesized and extracellularly released, proteinaceous compounds produced by a variety of bacterial strains which exhibit a bactericidal or bacteriostatic mode of action mainly against closely related species (Cleveland *et al*., 2001; Deegan *et al*., 2006; De Vuyst & Leroy, 2007).

Hence, numerous bacteriocins from different groups of LAB have been isolated and studied in milk and fermented milks. Among tested samples, some bacteriocins have been shown to posses the ability to inhibit unrelated genera such as *Clostridia*, *Listeria* and gram-negative bacteria (Choi *et al*., 2000; Corsetti *et al*., 2004; Moreno *et al*., 2002; Simonova & Laukova, 2007). Finding these unique bacteriocin producers in traditional food products can be very useful from the industrial and clinical point of view.

The significant needs of this research is to explore new dimensions of the relevant area. It can be claimed that modification and recognition of new bacteriocins lead us both to new domains of scientific and industrial zones in the field of Food science and Technology.

Hence, the current research was conducted and targeted in order to isolate LAB strains with antibacterial activity against Gram negative and Gram positive indicators from Iranian dairy products. Evaluation of the antimicrobial agents responsible for their activity was performed afterwards and the BLIS producing isolates were identified and their activities were characterized.

MATERIAL AND METHODS

Bacterial strains and culture media

Bacteria tested for bacteriocin production were isolated from different milk and milk product samples. *Staphylococcus aureus* ATCC6538 and *E. coli* ATCC8739 were used as the indicator strains. Organisms used in this study were maintained as frozen stocks at –70 °C in 25% (v/v) glycerol. Before experimental use working cultures were propagated twice in appropriate broth

media from 18 to 24h. Agar and soft agar media were obtained by adding 1.5 and 1.0% agar (MERCK, Germany) respectively, to the broth media. All chemical reagents and enzymes (catalase, trypsin, and pepsin) were obtained from Sigma-Aldrich (St. Louis, MO, USA) and all culture media were supplied by MERCK in Germany.

Isolation of lactic acid bacteria

Fifty-four dairy samples were aseptically collected from rural households of different central region of Iran. The samples were brought to the laboratory in icebox flasks and maintained at 4–5 °C. Thereafter, each sample was homogenized and serially diluted tenfold with saline solution (0.9%l NaCl). Aliquots (1 ml) were spread onto MRS (Mann Rogosa & Sharpe) and CASO (Tryptic Soy) agar. After incubation aerobically and microaerobically (MART system, 5% CO_2, 5.9% O_2, 7.2% H_2, 79% N_2) at 37 °C for 24 and 48 h, the colonies were picked off and stabbed onto the fresh agar plates. Pure cultures were obtained by repeated streaking onto the media and initially identified by their colony morphology, Gram-staining, cell morphology, and the catalase test.

Detection of antimicrobial activity within isolated LAB

For antimicrobial activity detection, an agar spot test (Corsetti, *et al.,* 2004; Schillinger & Lucke 1989) was performed against E. coli and S. aureus; each isolated strain was cultured in MRS broth at 37 °C for 24 h. Aliquots (10 μl) of the culture were then spotted onto agar surface plates and the plates were incubated for approximately 18 h to allow colonies to develop. They were then overlaid with 6 ml CASO soft agar seeded with the cell suspension of the indicator bacteria at a final concentration of 10^6 CFU/ml for each of indicators. The plates were incubated for an additional 18 h, and then checked for the appearance of inhibitory zones around spots of the putative producers.

Estimation of the antimicrobial agents

Isolated strains showing inhibitory activities based on agar spot test, were tested furthermore for detecting the antimicrobial agents responsible for the activity. For this purpose, the inhibitory activity of cell-free supernatants of 15 h-old cultures against *S. aureus* was tested by using the agar well-diffusion assay (AWDA) (Batdorj *et al*., 2006; Schillinger & Lu¨cke 1989). Cell-free culture supernatant of each producer strain was obtained by centrifugation of the cultures at 5,000 × g for 15 min.

To rule out the possibility that the inhibition might have been caused by acidification of the media induced by LAB metabolism, supernatants were adjusted with NaOH (5 mol/l) to pH 6.5 and in order to elucidate whether the antimicrobial activity might derive from the production of hydrogen peroxide by LAB, 1 mg/ml, Bovine catalase was used to the supernatant extracts and the mixture was incubated at 37 °C for 2 h. Both treatments were applied for investigating the role of antimicrobial peptides for the inhibitory activity.

Resulted supernatant fluids were filter-sterilized with a 0.22 μm (Millipore Co., Bedford, MA, USA) filter membrane. Thereafter, 100 μl of the resulted samples were transferred into wells (6 mm in diameter) drilled into soft CASO agar plates seeded with *S. aureus* suspension using a sterile cotton swab. The strain suspension were prepared by suspending overnight colonies from appropriate agar media in 0.9% saline and adjusted photometrically at 600nm to a cell density equivalent to 1.5×10^8 CFU/ml of the indicator strain. For the pre-diffusion of the compounds contained in the supernatant, plates were kept at 4°C for 2 h, and consequently incubated for 16 h at 37 °C. Inhibition was detected by a clear zone around a test well.

Proteinaceous nature of the antimicrobial compounds

The cell free supernatants of isolated strains suspected of bacteriocin producing were adjusted to proteolytic enzyme treatments. Each supernatant was tested with different proteases:

proteinase K (EC 3.4.21.64), trypsin (EC 3.4.21.4) and pepsin (EC 3.4.23.1). Proteinase K and trypsin were dissolved in sterile phosphate buffer (0.1 mol/l, pH 7.5) and pepsin was dissolved in HCl solution (0.02 mol/l, pH 2.0) and in separate treatments each one was added to the cell free supernatants at a final concentration of 1 mg/ml. Incubation was performed at 37 °C in a water-bath for 2 h. Samples were then heated at 100 °C for 3 min to stop the reactions and the inhibitory activity was assayed. Negative controls for elucidating the possible role of other elements in the inhibitory activity including the buffer or HCl solution, buffer or HCl solution containing only the enzymes, and the cell free culture supernatants in buffer or HCl solution without enzymes were exposed to the same conditions.

Identification of bacteriocin-producing isolates

Unidentified Gram-positive and catalase-negative bacterial isolates showing BLIS inhibitory activity towards the indicator were grown on differential media and tested according to Bergey's Manual of Systematic Bacteriology. Thus the identification was carried out by the following phenotypic tests: growth at 40 and 50°C; growth at pH 9.6; growth in the presence of 4 and 6.5% NaCl and in the presence of 40% bile, and acid production from lactose, sucrose, raffinose, melibiose, mannitol, melezitose and glycerol.

Minimum inhibitory concentration

The minimum inhibitory concentration (MIC) of BLIS producing strains was tested on cell-free supernatant of a 15h old culture by well diffusion method. Cell-free supernatant was obtained as described previously. The resulting sample was serially diluted twofold with sterilized phosphate buffer (0.1 mol/l, pH 6.5) and filled in 6mm wells drilled into soft CASO agar. After incubation at 37°C for 16h, the antimicrobial activity was expressed as AU/ml. One AU was defined as the reciprocal of the highest dilution showing a clear zone of growth inhibition according to the method of Batdorj *et al*., (2006). The experiment was repeated three times and the average was calculated.

Mode of action

To study the inhibitory action of the BLIS produced by each isolated strain, ten ml of cell-free supernatant (pH 6.5) was filter-sterilized and added to 50 ml of CASO broth containing 10^5 CFU/ml of *S. aureus* and incubated at 37°C for 14 h. In control sample, ten ml of MRS broth was used instead of cell free supernatant. Changes in viable cell counts (CFU/ml) were determined by pour plate method on CASO agar at 2-h intervals.

RESULTS AND DISCUSSION

LAB isolates with antimicrobial activity

198 isolated strains within 54 collected dairy products were detected as catalase negative, Gram positive cocci, bacilli and coccobacilli bacteria and categorized as LAB while 56 catalase-positive isolates were known as non- LAB strains.

In the initial screening, based on the agar spot test, almost all LAB isolates showed a significant inhibition activity against one or both indicators (data not shown). Subsequently, the antimicrobial agents responsible for the activity were detected by testing the cell free supernatant samples of all LAB isolates by the agar well diffusion method. The activity of all tested isolates remained stable after the catalase treatment (data not shown). In contrast, the results showed that although the antimicrobial activity of all LAB isolates was decayed after neutralizing the supernatants, the inhibitory activity was remained in all tested isolates (data not shown). Meanwhile, the neutralized and catalase-treated supernatant of only four isolates, showed a measurable clear zone that confirmed the production of other antimicrobial compounds by these strains (Table 1). As shown in Table 1, the inhibitory activity was not affected by catalase treatment and was preserved in neutralized supernatant fluid. This suggests

that hydrogen peroxide did not involve in the inhibition and apart from organic acid, the activity could be related to the production of other inhibitory substances.

Table1: Effect of neutralization and enzyme treatment on the activity of the LAB isolate cell free supernatants, determined by agar well diffusion against *S. aureus*.

	L. lactis	E. faecium1	E. hirae	E. faecium2
Treatment	Diameter of inhabitation zone (mm) *			
Control **	25.5	28	24	27
Nutralized	15	14.5	12	13
Catalase + Neutralized	15.5	14.5	11.5	13
Catalase†	25	27.5	24	27.5
Proteinas K†	—	—	—	—
Trypsin†	10.5	—	—	—
Pepsin†	—	—	—	—

* Wells (6 mm) were filled 100 µl treated cell free supernatant
** Controls were defined as untreated supernatant samples.
—; No inhibitory zone
† The final enzyme concentrations were 1mg/ml.

Proteinaceous nature of the antimicrobial compounds

The proteinaceous nature of the antibacterial compounds produced by four isolated strains, were initially assessed by enzyme treatment (Table 1). Treatment of all cell-free supernatant samples with proteinase K and pepsin induced complete inactivation of the antimicrobial activity while, the inhibitory activity was eliminated by trypsin in all samples, except the cell free supernatant of *L. lactis* which partially lost its activity upon treatment with this enzyme. No antimicrobial activity was reported in control samples. Thus, the proteinaceous nature of the antimicrobial compounds was partially demonstrated by the preliminary tests.

Identification of BLIS-producing isolates

The isolates strains with BLIS activity were primarily identified as Gram-positive, catalase-negative cocci that was nonmotile, and nonsporulating with no gas production from glucose. The identity of the strains was conducted by phenotypic identification. Two strains were able to grow in the presence of 6.5% NaCl and 40% bile, and at 45 and 50 °C. These strains fermented lactose, glycerol and mannitol but did not show acid production from sucrose, melibiose, rafinose and melezitose. Consequently, these isolates were confirmed as *Enterococcus faecium*. One strain was approved as *E. hirae* based on growth at 45 °C and in the presence of 6.5 % NaCl, growth inhibition at 50 °C and its fermentation pattern. On the basis of fermentation patterns and positive results due to growth at 40 °C and in the presence of 4% NaCl the fourth isolate was tentatively identified as *Lactococcus lactis* subsp *lactis*.

Table 2: Characterization of BLIS producing isolates

Strain	Source	Location
Lactococcus lactis subsp lactis	Goat milk	Garmuk, Esfahan, Iran
Enterococcus faecium 1	Cow milk	Chadegan, Esfahan, Iran
Enterococcus hirae	Goat milk	Garmuk, Esfahan, Iran
Enterococcus faecium 2	Yoghurt	Emamzade Abdollah, Kohgiluyeh-Boyer Ahmad, Iran

Minimum inhibitory concentration

Figure 1, shows the activity intensity of each cell free supernatant. *L. lactis* represented the highest activity by 320 AU/ml that was followed by *E. faecium* 1 and 2. The lowest activity was detected in the case of *E. hirae* (40 AU/ml).

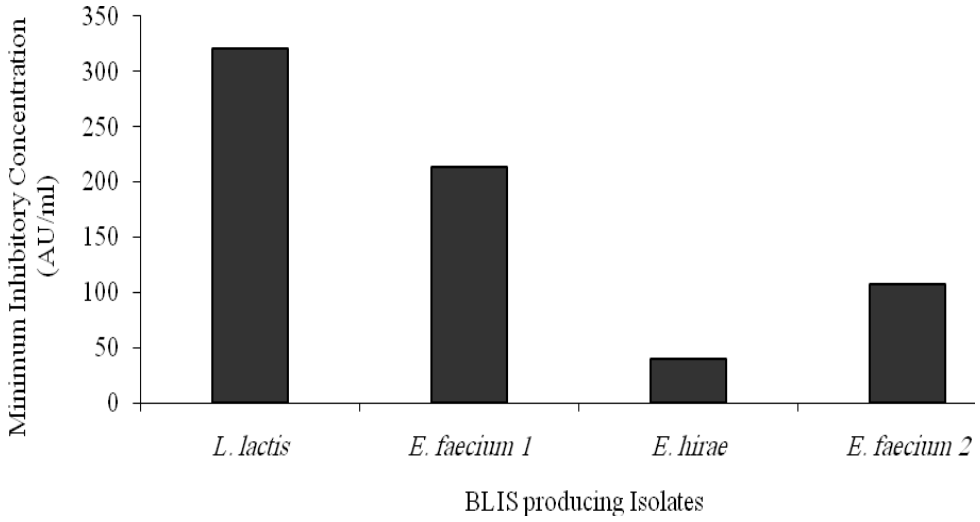

Fig.1: Minimum inhibitory concentration (MIC) of BLIS activity by LAB strains isolated from Iranian milk samples towards *S. aureus*

Mode of action

The activity of BLIS produced by the isolates LAB strains, against the growth of *S. aureus* is shown in Figure 2. Addition of active supernatant *L. lactis* to an initial cell density of about 5 log CFU/ml, inhibited the growth of indicator and just 0.5 log cycle per milliliter growth was detected while, bacterial growth in the control sample (not treated with BLIS) was measured as more than 2.8 log cycle, over the same period. Addition of cell free supernatant of *E. faecium* 1 and 2 resulted in a restricted (2 log cycles) growth of *S. aureus* after 16 h. On the other hand cell growth was not significantly affected by *E. hirae*.

Furthermore, no recovery was reported for bacterial growth during 16 hours so that, retention of BLIS activity was indicated during this period.

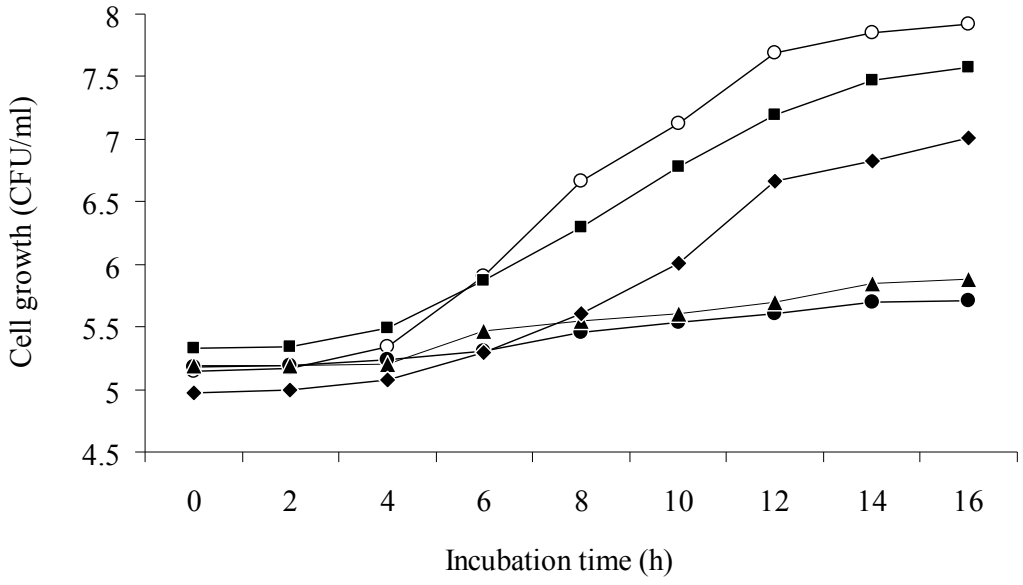

Fig.2: Viable counts (log CFU/ml) of *S. aureus* in CASO medium at 37 °C, in the presence of BLIS from *L. lactis* (—●—), *E. faecium* 1 (—▲—), *E. hirae* (—■—), *E. faecium* 2 (—✱—) or absence (—○—) of any BLIS compound.

CONCLUSION

It is clearly shown that almost all isolated LAB from dairy samples presented the inhibitory activity towards Gram positive (*S. aureus*) and Gram negative (*E. coli*) indicators. The activity was not affected by catalase treatment which suggests that hydrogen peroxide did not induce any inhibitory activity. On the other hand, the inhibitory effect was completely eliminated after neutralization, except four isolates. It can be concluded that in almost all LAB isolates, organic acids played a major role in the antibacterial activity.

Remaining the reduced activity after neutralization in the case of the LAB isolates, indicated that the inhibitory activities of this isolates were relevant to the production of other antimicrobial compounds, while organic acids were also involved in their activity. Proteolytic enzyme inactivation demonstrated the proteinaceous nature of the antimicrobial agents in these four isolates and induced these antimicrobial compounds in the category of bacteriocins. As the protein compounds were not characterized for amino acid sequences, it will be referred to BLIS.

The prevalence of four BLIS producers was mostly evidenced in goat milk and E. faecium was the most abundant producer. Although, the prevalence of Enterococcus (Alvarado *et al*., 2005; Chen *et al*., 2007; Moreno *et al*., 2002; Rehaiem *et al*., 2010) and Lactococcus species (Choi *et al*., 2000; Mitra *et al*., 2005; Ponce *et al*., 2008; Tukel *et al*., 2007) as BLIS producing bacteria, in foods has been reported in other surveys, this is the first report on the isolation and characterization of these isolates from Iranian dairy foods. The comparison between minimum inhibitory concentration of the cell free supernatant of isolated strains introduced BLIS from L. lactis as the most intensive antibacterial agent while, E. hirae showed the lowest activity. E. faecium strains, on the other hand, represented intermediate activities.

Therefore, according to the growth inhabitation pattern of indicator bacteria, the mode of activity of BLIS from L. lactis was suggested to be bacteriostatic, a characteristic which has been reported also for Lactobacillus plantarum TF711 (Herna´ndez *et al*., 2005), E. faecium isolates (Moreno *et al*., 2002) Lactobacillus plantarum ST8KF (Powell *et al*., 2007) and some other LAB (Corsetti *et al*., 2004).

ACKNOWLEDGMENTS

The authors would like to acknowledge the Science and Research Branch, Islamic Azad University for financial support.

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