Iranian J Publ Health, Vol. 33, No. 2, pp.1 -7, 2004

Study of *Lactobacillus* as Probiotic Bacteria

J Nowroozi[1], M Mirzaii[1], M Norouzi[2]

[1]Dept. of Medical Microbiology, Medical School, Iran University of Medical Sciences, Tehran, Iran
[2] Dept. of Medical Virology, Medical School, Iran University of Medical Sciences, Tehran, Iran

Abstract
Because of inhibitory effect, selected probiotic lactobacilli may be used as biological preservative, so, the aim of this study was to present some data on *lactobacillus* as probiotic bacteria. Lactic acid bacteria were isolated from sausage. Each isolate of *lactobacillus* species was identified by biochemical tests and comparing their sugar fermentation pattern. Antibacterial activities were done by an agar spot, well diffusion and blank disk method. Enzyme sensitivity of supernatant fluid and concentrated cell free culture after treatment with α-amylase, lysozyme and trypsin was determined. The isolated bacteria were *Lacto. plantarum, Lacto delbruekii, Lacto. acidophilus, Lacto. brevis*. The isolated bacteria had strong activity against indicator strains. The antibacterial activity was stable at 100ºC for 10 min and at 56ºC for 30 min, but activity was lost after autoclaving. The maximum production of plantaricin was obtained at 25 - 30ºC at pH 6.5. Because, lactobacilli that used to process sausage fermentation are producing antimicrobial activity with heat stability bacteriocin, so, these bacteria may be considered to be a healthy probiotic diet. Lactobacilli originally isolated from meat products are the best condidates as probiotic bacteria to improve the microbiological safety of these foods.

Keywords: Lactobacillus*, Probiotic, Sausage, Antibacterial activity*, *Iran*

Introduction

Probiotic cultures have been associated historically with cultured of milks and dairy products, from which there is substantial evidence for positive effects on human health and general well-being (1, 2). Several in vitro and in vivo experiments on antagonism of different Lactobacillus strains against *Helicobacter pylori* and *Clostridium difficile*, *Campylobacter jejuni*, *E. coli* were performed. All tested human Lactobacillus strains were able to inhibit the growth of all strains of anaerobic human gastrointestinal pathogens (3, 4). In addition, bacteriocins have properties such as antitumour and anticholestrol activity. Chemical reactions associated with reduction of nitrate, improvements inimmu nological status and adsorption of vitamins B group (5). The transity lactic acid bacteria in the gastrointestinal tract are capable of delivering enzymes and other substances into the intestine which possibly help to control intestinal flora (6). Also, the antioxidative activity of lactic acid bacteria is reported (7). Because of inhibitory effect, selected probiotic lactobacilli may be used as biological preservative, so, the aim of this study was to present some data on isolation, growth, and antimicrobial activity, effect of pH, heat, and sensitivity to proteolytic enzymes of *lactobacillus* as probiotic bacteria.

Materials and Methods

Each of the following experiments was repeated thrice to get better results.

Isolation The isolation of lactic acid bacteria from sausages bought randomly from shops with different brand of factory was done by using MRS (pepton, meat extract, yeast extract, glucose, tween 80) medium. Briefly, 1g of sausage was mixed and vortexed into MRS broth medium, incubated at 37ºC for 24 h. Growth from MRS broth cultures was used to streak on MRS agar plate. *Lactobacillus* species of these

***Corresponding author:** P. O. BOX: 14155-6183, Fax: +98 21 8058719

isolates were identified by comparing their sugar fermentation patterns with the scheme described in Bergey's Manual of Systematic Bacteriology (8).

Growth of bacteria Lactobacilli were grown in MRS broth or MRS agar. One ml of an overnight culture of *lactobacillus* was used to inoculate 100 ml of MRS broth and incubation was continued at 20, 25, 30, 35, 37, 40, 45ºC for 24 h. Samples were removed at regular intervals (30 min) for the determination of turbidity (measured at 660 nm), culture pH and antibacterial activity. The experiment was repeated with broth in which the initial pH was adjusted to 2 to 12 with HCl or NaCl. Initial and final pH of all samples was also measured. Culture supernatant (200µl) was heated in a boiling water bath for 10 min and cooled rapidly on ice. Serial twofold dilutions of the heated supernatants were made in 0.2 N HCl, and 10µl of each dilution was spotted on to fresh, duplicate indicator lawns. Cultures were incubated for 24 h.

Effect of different sugar and NaCl concentration on production of bacteriocin Isolated *lactobacillus* was grown in MRS broth without beef extract, supplemented with different concentration of glucose, xylose, sucrose, furoctose, galactose, maltose and NaCl. Then, remaining activity against indicator strains was assayed.

Preparation of culture supernatant The bacteriocin producing strain was grown in MRS broth for 24 h at 25ºC. A cell free solution was obtained by centrifuge the culture, followed by filtration of the supernatant through a 0.2 µl pore size filter. The supernatant was adjusted to pH 6.5 or dialyzed for 24 h against MRS broth at 4ºC.

Mode of action one ml of cell free culture supernatant of isolated lactobacilli was added to 10 ml of a fresh culture logarithmic phase of indicator bacteria. Culture optical density were determined (at 660 nm) at appropriate intervals.

Antimicrobial activity For detection of antagonistic activities, an agar spot procedure, well diffusion assay and blank disk method were used. For the agar spot test, supernatant of overnight cultures of *lactobacillus* strains were spotted (1mm) onto the surface of BHI agar plates of indicator strains and incubated for 24 h at 37ºC to allow colony develop. For the agar-well diffusion assay, an overnight culture of the indicator strain was used to inoculate agar growth media at 37ºC. Wells of 5mm diameter were cut into agar plates and 50 µl of culture supernatant fluid containing antibacterial activity were added to each well. Supernatant fluid was obtained by growing the inhibitory producer strain overnight in MRS broth at 30ºC. Cells were then removed by centrifugation and the supernatant fluid placed in the wells and allowed to diffuse into the agar for 24 h at 4ºC. The plates were then incubated at optimum growth temperature of the indicator strains and examined after 24 h for inhibition zone. Five sterile paper blank disks were placed on the agar plate which was inoculated by indicator strains and 20 µl of the filtered supernatant of lactobacilli was applied. Plates were incubated and observed for zones inhibition.

Indicator strains used as indicator organisms for bacteriocin screening were *Staphylococcus aureus, Salmonella typhi, Yersinia enterocolitica, Bacillus subtilis, Listeria monocytogenes* and other lactobacilli isolated from sausage without antibacterial activity. The plates were incubated at 30ºC for 24-48 h or until growth of the test organism could be easily observed with naked eye.

Sensitivity to pH and heat To test sensitivity to pH, the supernatant was adjusted to pHs between 2 to 12 with HCl or NaOH and incubated. To test heat stability, the supernatant fluid was heated in boiling water for 10 min, at 56ºC for 15 min, or autoclaved at 121ºC for 15 min. In all cases, the activity remaining after treatment was measured by spotting procedure. This experiment repeated and the solutions were kept at 4 and -20ºC for 4 weeks, then antibacterial activity was measured.

Sensitivity proteolytic enzymes To test for

enzyme sensitivity, cell free culture supernatant fluid was treated for 1h at 30ºC with trypsin, α-amylase, lysozyme at final concentration of 0.5mg/ml, 220 IU/mg/ml and 22IU/mg/ml, and incubated at 37ºC for 1 h.Concentrated cell free culture supernatants were heated at 100ºC for 20 min and the remaining activity was determined by spotted procedure.

Bacteriocin concentration one liter lactobacilli culture was grown in MRS broth at 30ºC until the late logarithmic phase. The cell removed by centrifugation for 12 min. at 4ºC, and ammonium sulphate was gradually added to achieve 40% saturation. The sample was kept at 4ºC with stirring for 30 min. After centrifugation for 30 min, the resulting pellet was mixed and solubilized in 120 ml of 10 mM sodium phosphate buffer, pH 5.8. Then antimicrobial activity was measured against indicator bacteria (9).

Results

A total of 28 lactic acid bacteria isolated from sausage were tested for antimicrobial activity. Only 4 strains (14.3%) of lactobacilli (*Lacto. plantarum, Lacto. delbruekii, Lacto. acidophilus, Lacto. brevis*) were shown to produce a bacteriocin-like substance. Their sensitivity varied greatly. *Lacto. plantarum* produced a more heat stable bacteriocin than the other isolated strains, which exhibited a broad spectrum of inhibitory activity. The antibacterial activity of plantaricin was more potent than the other isolated strains when sensitive strains were in the logarithmic growth phase, including cell lysis, as observed by decreased in optical density. No bacteriocin activity was found in cultures grown at 4 or 8ºC. However, bacteriocin production was observed at 20, 25, 30, 37, 40 and 45ºC. At all of these temperatures, the maximum antimicrobial activity in the growth medium was obtained in the late logarithmic phase growth and early of stationary phase.

The amounts of bacteriocin produced at 25 and 30ºC were similar. The bacteriocin activity in the supernatant was stable and no decrease in activity was detected after 5 days at 25ºC. The antibacterial activity was stable at 100ºC for 10 min and at 56ºC for 30 min, but all activity was lost after autoclaving. The antibacterial activity was not lost by freezing and thawing, and long term storage at 4 and -20ºC. When the supernatants of the cultures containing *Lacto. plantarum* were checked, a small zone of inhibition was first observed on plates after 6 h at 25ºC and larger zones of inhibition were detected after 24 h.

Factors affecting bacteriocin activity The antibacterial activity of bacteriocin was destroyed by trypsin treatment, but was unaffected by α-amylase and lysozyme.

The inhibitory activity remained stable over the pH range 2 to10, but was lost after incubation at pH 12, indicating its sensitivity to alkali treatment. All activity was lost after autoclaving. The antimicrobial properties of the Lacto-*lactobacillus* strains tested were very variable. Many of the strains showed weak or no inhibition of the pathogenic strains.

Only 4 strains (14.3%) inhibited the growth of pathogenic bacteria broadly. The maximum production of the bacteriocin was obtained at 25ºC at pH 6.5.

Bacteriocin production Maximum production of bacteriocin was obtained in MRS broth containing at least 1-2% glucose or xylose. Also, MRS medium with 1% NaCl found that, the antibacterial activity increased. The inhibitory activity was maximal at the beginning of the stationary phase and remained stable long after growth had ceased, even in the presence of the producer cells.

Zone inhibition of *Staph.aureus* against supernatant of lactobacilli by agar spot method, blank disk, and agar well diffusion assay are shown in Figs 1, 2, 3.

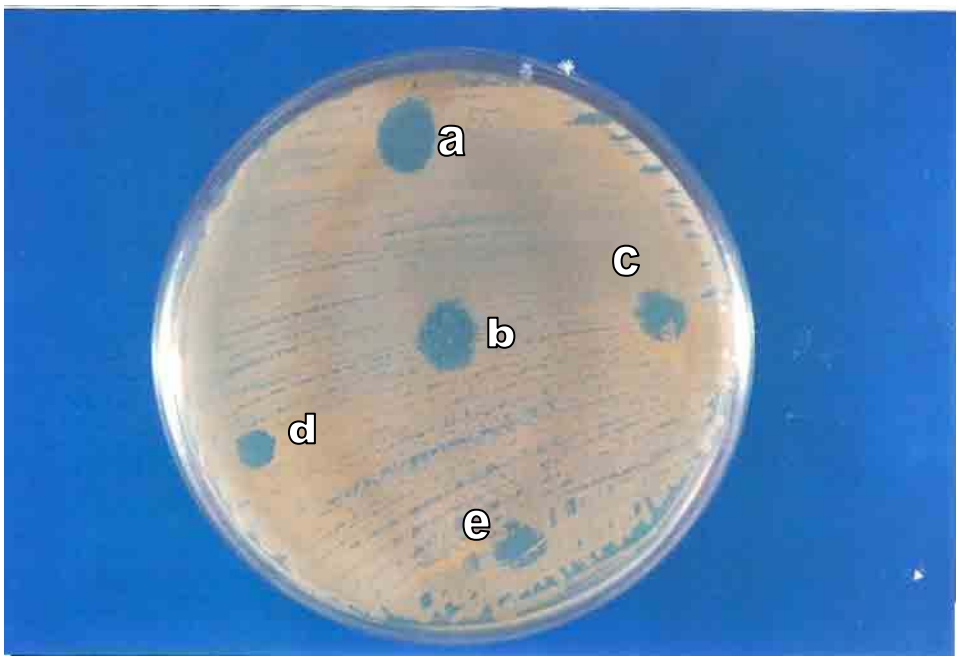

Fig. 1 : Zone inhibition of *Staph. aureus* against supernatant of *Lacto. plantarum* by agar spot method.
a: *Lacto. Plantarum*, b: *Lacto. delbruekii,* c: *Lacto. acidophilus,* d: *Lacto. brevis,* e: *Lacto. casei*

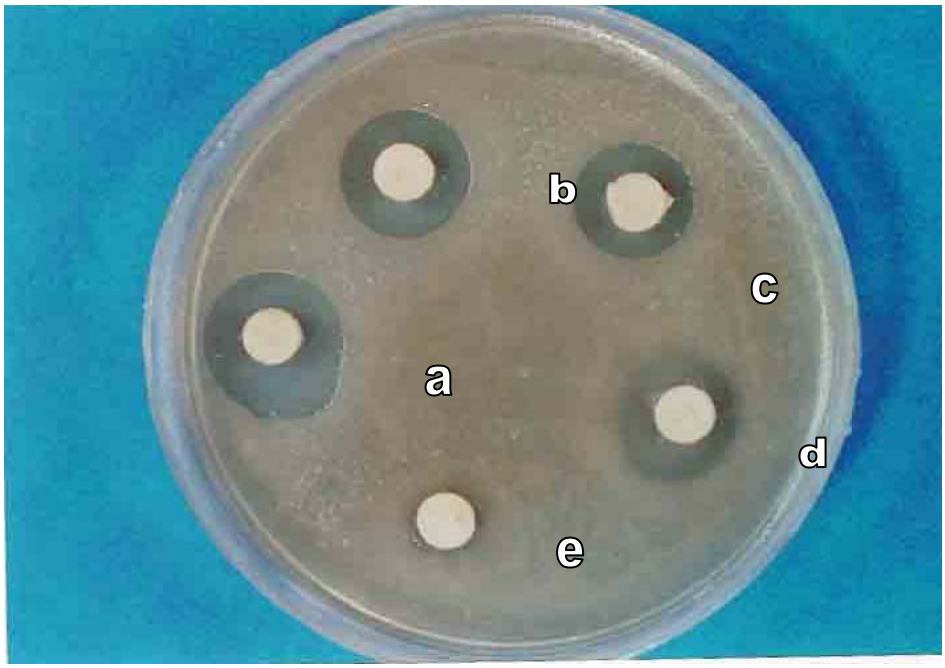

Fig. 2: Zone inhibition of *Staph. aureus* against supernatant of *Lacto. plantarum* by blank disk method.
a: *Lacto. plantarum*, b: *Lacto. delbruekii,* c: *Lacto. acidophilus,* d: *Lacto. brevis,* e: *Lacto. casei*

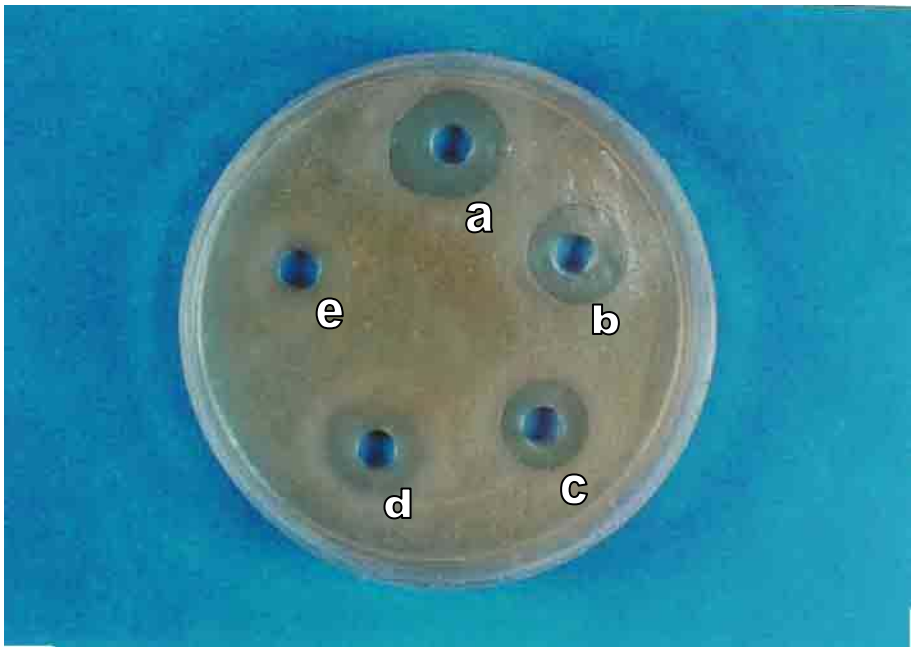

Fig. 3 : Zone inhibition of *Staph. aureus* against supernatant of *Lacto. plantarum* by agar well diffusion method.
a: *Lacto. plantarum*, b: *Lacto. delbruekii,* c: *Lacto. acidophilus,* d: *Lacto. brevis,* e: *Lacto. casei*

Discussion

Of 28 lactobacilli isolated from fermented sausage, 4 (14.3%) lactobacilli had antibacterial activity against indicator strains (*L. monocytogenes, Y. enterocolitica, Staph. aureus, B. subtilis, S. typhi* and other isolated lactobacilli with no antibacterial activity), which were further characterized. Their antimicrobial substances inactivated by trypsin and designed as bacteriocin (plantaricin). *Lacto. plantarum* (plantaricin) showed the broadest range of inhibitory action. This is agreeing by the results of Kelley et al (9) and not agrees elsewhere (10). The latter reported *Lacto. casei* with a potent antimicrobial activity.

Since antibacterial activity decreased after treatment with trypsin, but not affected with lysozyme and α-amylase, so, the bacteriocins have probably a pertinacious nature. This was in accordance with Gonzalez et al (11). Also, the protein nature of plantaricin K was confirmed by its sensitivity to trypsin (12).

Bacteriocin of *Lacto. plantarum* on the basis of its stability in the medium, its broad spectrum of activity on some pathogenic and spoilage food bacteria and its high potency of plantaricin production is recommended as food preservative. Our results showed that bactericidal action of the bacteriocin against indicator strains were on logarithmic phase and early stationary phase and cell lyses in actively growing cells, thereby causing a decrease in culture optical density. This was also confirmed by Gao et al (13).

In this study, production of plantaricin was best in MRS broth, or in a medium containing peptone, yeast extract, beef extract, glucose, sodium acetate and Tween 80. Glucose could be replaced by xylose without a decrease in the amount of plantaricin, but other carbohydrates resulted in less bacteriocin being produced.

Maximum production was coincided with onset of logariyhmic phase and early of stationary phase, and these conditions of low pH and high cell number have also been found to be necessary for the production of high levels of bacteriocins. Maximum production of plantaricin KW30 (9) and bacteriocin of *Lacto. delbrueckii* (14) were in MRS broth. Their results are similar to our results.

Our results showed that bacteriocin activity was very stable under a series of different conditions such as storage at room temperature for 5 days, 4ºC and -20ºC, and heating (100ºC for 10 min or 56ºC for 30 min). This is confirmed by Rekhif et al (15).

In general, bacteriocin are from lactobacilli specially *Lacto. plantarum* relatively heat stable with promising inhibitory spectra of antimicrobial activities.

Their general heat stability is an advantage, temperature stability being a very important parameter if a bacteriocin is to be used as a food preservative because many proc- essing procedures involve a heating step.

However, the bacteriocins from *Lacto. plantarum* described in this paper appear quite promising as potential biopreservatives. Our results are confirmed by some researchers (9, 11, 15).

Lactic acid bacteria originally isolated from meat, meat products and dairy products are probably the best candidates as probiotic bacteria to improve the microbiological safety of these foods. Since, they are well adapted to the conditions in meats and dairy products and should therefore be more competitive than from other sources.

Interest in lactic acid bacteria is growing. Also, bacteriocins produced by lactic acid bacteria are great interest to the food fermentation industry because they may inhibit the growth of many food spoilage and pathogenic bacteria. Therefore, an investigation of bacteriocins in lactic acid bacteria may offer potential applicability in food preservation.

Acknowledgements

We would like to thank to laboratory staff in Dept. of Microbiology, Iran University of Medical Sciences, Iran this study was a part of Mr Mehdi Mirzaii thesis.

References

1. Kaenhammer TR (2000). Probiotic bacteria: today and tomorrow. *J Nut*, 130(2S suppl): 415S-16S.
2. Reuter G (2001). Probiotics-Possibilities and limitations of their application in food, animal feed, and in pharmaceutical preparations for men and animals. *Berl Munch Tierarztl Wochenschr*, 114(11-12): 410-9.
3. Strus M, Pakosz K, Gosciniak H, Przondo-Mordarska A, et al (2001). Anatgonistic activity of *Lactobacillus* bacteria strains against anaerobic gastrointestinal tract pathogens (*Helicobacter pylori, Compylobacter coli, Campylobacter jejuni, Clostridium difficile*). *Med Doew Mikrobiol*, 53(2): 133-42
4. Roberfroid MB (2002). Prebiotics and probiotics: are they functional foods? *Am J Clin Nutr*, 71(6 Suppl): 1692S-7S; discussion 1688S-90S.
5. Elmafa I, Heinzle C, Majchrzak Foissy H (2001). Influence of a probiotic yoghurt on the status of vitamin B (1), B (6) in the healthy adult human. *Am Nutr Metab*, 45(1): 13-8.
6. Collins M, Glenn D, Gibson R (1999). Probiotics, prebiotics and symbiotics: Approches for modulating the microbial ecology of the gut. *American J Clin Nutri,* 69(5): 1052s-57s.
7. Terahara M, Kurama S, Takemoto N (2001). Prevention by lactic acid bacteria of the oxidation of human LDL. *Biosci Biotechnol Biochem*, 65(8): 1864-68.
8. Kanlder O, Weiss N (1986). Regular, non-sporing Gram positive rods In: *Bergey's*

Manual of Systematic Bacteriology. Volume 2. Section 14, 1208-60.

9. Kelly WJ, Asmundson RV, Huang CM (1996). Characterization of plantaricin KW30, a bacteriocin produced by *Lactobacillus* plantarum. *J Appl Bacteriology*, 81: 657-62.
10. Vignolo GM, de Kairuz MN, de Ruiz Holgado AAP, Oliver G (1995). Influence of growth conditions on the production of lactocin 705, a bacteriocin produced by Lactobacillus casei CRL 705. *J Appl Bacteriol*, 78, 5-10.
11. Gonzalez B, Arca P, Mayo B, Suarez JE (1994).Detection and partial charaterization of plantaricin C, a bacteriocin produced by a Lacto. plantarum strain of dairy origin. *Appl Environ Microbiology* 60, 2158-63.
12. Olukoya DK, Tichazek PS, Butsch A, Vogel RF, Hammes WP (1993). Charaterization of the bacteriocins produced by Lactococcus pentosus DK7 isolated from ogi and Lactococcus plantarum DK9 from fufu. *Chem Microbiol Technol Lebensm.*15. 65-68.
13. Gao FH, Abee T, Konings WM (1991). Mechanism of action of the peptide antibiotic nisin in liposomes and cytochrome c oxidase containing proteoliposomes. *Appl Enviro Microbiol*, 2164-2170.
14. Boris S, Jimenez – Diaz R, Caso JL, Barbes C (2001). Partial characterization of a bacteriocin produced by *Lactobacillus* delbrueckii subsp. Lactis U0004, an intestinal isolate with probiotic potential. *J Appl Microbiol*, 91(2): 328-33.
15. Rekhif N, Atrih A, Lefebvre G (1995). Activity of plantaricin SA6, a bacteriocin produced by *Lactobacillus* plantarum SA6 isolated from fermented sausage. *J Appl bacteriology*, 78. 349-58.