World Applied Sciences Journal 14 (4): 552-560, 2011
ISSN 1818-4952

Assimilation of Cholestrol by *Lactobacillus* Species as Probiotics

[1]C.M. Raghavan, [2]Anima Nanda, [1]R. Yuvaraj, [3]D.J. Mukesh Kumar,
[4]A. Senthil Murugan and [5]R. Balaji Raja

[1]PG & Research Department of Microbiology, M.G.R. College, Dr. M.G.R. Nagar, Hosur, India
[2]Department of Biomedical Engineering, Sathyabama University, Jeppiar Nagar, Chennai, India
[3]Centre for Advanced Studies in Botany, University of Madras, Chennai, Tamil Nadu, India
[4]Department of Soil Sciences, Forest Research Centre, Mandar, Ranchi, Jharkhand, India
[5]Department of Biotechnology, National Institute of Technology, Raipur, Chhattisgarh, India

Abstract: Despite numerous therapeutic improvements, especially in the field of antibiotics, gastrointestinal infections and their consequences remain a major clinical problem. In addition, there has been a dramatic increase in the incidence of antibiotic-resistant microbial pathogens. There is a concern that industry will no longer be able to develop effective antibiotics at a rate sufficient to compete with the development of microbial resistance to old antibiotics. The study has been performed to reduce the cholesterol with *Lactobacillus* as probiotics to a significant level. From this study, we concluded that the high level of cholesterol could be removed by growing cell from media, which can be used as adjuvant to lower serum cholesterol *in-vitro*. The emergence of antibiotic-resistant bacteria and natural ways of suppressing the growth of pathogens has contributed to the concept of probiotics and these factors have renewed interest in the possibility of deliberately feeding beneficial microorganisms to humans as an alternative to antibiotic therapy in gastrointestinal disorders. Probiotics are also an attractive treatment alternative because antibiotics, which further delay recolonization by normal colonic flora, can be avoided.

Key words: Bacteriocin · Probiotics · Cholesterol · Gastrointestinal disorders · Lactic acid bacteria

INTRODUCTION

Increased serum cholesterol correlates highly with the incidence of coronary heart disease. Dietary adjustment is one way to decrease serum cholesterol, thereby reducing the risk of coronary heart disease. Consumption of dairy products containing *Lactobacillus* species can aid in the control of serum cholesterol in animals and humans. Assimilation of cholesterol in the intestines by *Lactobacillus* species may reduce the amount of dietary cholesterol absorbed into the body. Because the ability of *Lactobacillus* species to assimilate cholesterol during growth varies by strain, the *Lactobacillus* strains for use as a dietary adjunct to potentially reduce serum cholesterol should be carefully selected.

Some species of b acteria present in the intestinal tract, including *Lactobacillus acidophilus*, are able to deconjugate bile acids such as taurocholic or glycocholic acid. Deconjugation of bile acids may help to decrease serum cholesterol in humans because free bile acids are excreted from the intestinal tract more rapidly than are conjugated bile acids. As free bile salts are excreted from the body, the synthesis of new bile acids from cholesterol can reduce the total cholesterol concentration in the body [1].

Lactobacillus reuteri, a normal inhabitant of the gastrointestinal tract of humans and animals, can synthesize and secrete antimicrobial substances of proteic origin that have antagonistic actions against Gram-positive and Gram-negative bacteria, yeast, fungi, protozoa and viruses. The mechanisms involve the production of lactic and acetic acids, nutrient depletion, hydrogen peroxide production, change in oxidation/reduction potential and production of antibiotic-like compounds. Bacteriocin derived from lactic acid bacteria, usually small, heterogeneous, cationic proteins consisting of 30-60 amino acid residues, show marked variation in action spectrum, molecular weight and biochemical properties [2].

Corresponding Author: R. Balaji Raja, Department of Biotechnology, National Institute of Technology, Raipur, Chhattisgarh, India. Tel: +91-09942574378, E-mail: balajiraja_vlr@yahoo.com.

In the present work, probiotic *Lactobacillus* species was isolated, screened and selected for best alternate and their therapeutic value against enteropathogens from clinical samples. Also, its tolerance ability, cholesterol assimilation, antagonistic property was evaluated.

MATERIALS AND METHODS

Characterization of *Lactobacillus* Species: In order to isolate better Probiotic agent, *Lactobacillus* strains were isolated from commercial yoghurt (KCA), using specific media Manne Rogosa Sharpe Agar (MRSA). The *Lactobacillus* culture was restreaked repeatedly in an MRSA medium and pure colonies were isolated. The culture was maintained in MRS broth in the refrigerator (4°C) and sub cultured at weekly intervals. Usually 48 h cultures grown at 37°C were used for the experiment. Identification of *Lactobacillus* species was performed by means of following tests: Gram staining, motility test and biochemical test such as indole test, methyl red test, Voges-Proskaeur test, citrate test, urease test, oxidase test, catalase test, nitrate reduction test. The bacterial strains identified were grown on Manne Rogosa Sharpe Agar media and pure cultures of strains were obtained.

Screening of enteropathogens from clinical samples: One ml of the sample was serially diluted from 10^{-1} to 10^{-5} using sterile peptone water. Each 0.1 ml of the diluted sample was inoculated onto nutrient agar plate by spread plate techniques. The plates were incubated for 24 h at 37°C. After incubation, the isolated colonies were stored in nutrient agar slant for future use. Identification of pathogenic microorganisms was done based on the same tests as performed earlier. The bacterial species identified were grown on respective selective media and in turn pure cultures of the species were obtained.

Experimental Analysis:

Bile Tolerance Method I: 0.01 ml of *Lactobacillus* culture was added to 100 ml of 10% pasteurized reconstituted skim milk containing 0-1.5% of oxgall and the mixture was incubated at 37°C for 5 h. The control was a culture without incubation. Viable counts were determined immediately after incubation on Reinforced Clostridial Agar (Oxoid) under anaerobic condition at 37°C for 72 h. All experiments were replicated twice.

The rate of bile tolerance was calculated as follows: Bile tolerance rate = (cfu/ml) bile/(cfu/ml) control.

Bile tolerance method II: The *Lactobacillus* culture was evaluated for rapidity of growth in a broth medium with and without bile acids. Over-night cultures of 1% (v/v) were inoculated into modified MRS broth containing 0.3% (w/v) oxgall and taurocholic acid, respectively and incubated anaerobically at 37°C for 7 h. The control comprised MRS broth without bile salt. Cultures were monitored every half an hour for growth spectrophotometrically at 620 nm. The absorbance values obtained were plotted against the incubation time and the bile tolerance of the culture was based on the time required for the absorbance value to increase by 0.3 units. pH values of all the fermentation broths at time = 0 were measured and another measurement was taken after the absorbance increased by 0.3 unit. All experiments were replicated twice.

Acid Tolerance: Overnight cultures of strains 10% (v/v) were inoculated into MRS broth (Oxoid) previously adjusted to pH 2.0. The mixtures were anaerobically incubated at 37°C for 2 h. 1 ml samples were taken at various times (0, 15, 30, 45, 60 and 120 min), serially 10-fold diluted in anaerobic diluents (half-strength peptone water plus 0.5 g of l-cysteine HCl liter^{-1}, pH 7.0) and plated in triplicate onto MRS agar (Oxoid). The plates were incubated at 37°C for 24 h under anaerobic conditions before enumeration. The experiments were repeated twice.

Cholesterol Removal Method: For cholesterol assimilation by growing cells 1% of *Lactobacillus* culture was inoculated into freshly prepared MRS broth, supplemented with 0.30% oxgall as a bile salt and filter sterilized, Water-soluble cholesterol at a final concentration of 100 μg/mL. For preparation of dead cells, freshly prepared MRS broth containing 0.30% oxgall was inoculated with *Lactobacillus* culture and anaerobically incubated at 37°C for 20 h. Cells were harvested after the incubation period by centrifuging at 10,000 x *g* at 4°C for 10 min. The cell pellet was washed twice with sterile distilled water.

For preparation of heat-killed cells, the cell pellet was suspended in 10 mL of sterile distilled water and autoclaved for 15 min at 121°C. The heat-killed cells were suspended in MRS broth containing 0.30% oxgall and water-soluble cholesterol. For preparation of resting cells, the cell pellet was suspended in 10 mL of sterile 0.05 M phosphate buffer (pH 6.8) containing oxgall and water-soluble cholesterol.

All three setup were anaerobically incubated at 37°C for 20 h. After the incubation period, cells were centrifuged and the remaining cholesterol concentration in the broth was determined using a modified colorimetric method [10].

Cholesterol assimilation by growing, resting and dead cells was expressed in dry weight to obtain uniformity in all treatments. The dry weight of culture was determined after drying the centrifuged cells to a constant weight in an 80°C oven. The following equation was used: Cholesterol assimilation = $(C_1-C_2)/(W_2-W_1)$, where C_1 and C_2 were the amount of cholesterol present in the fermentation broths at time = 0 and 20 h, respectively and W_1 and W_2 were the dry weight of the individual culture at time = 0 and 20 h, respectively, for all treatments studied. The experiments were repeated twice.

***In vitro* Determination of Antimicrobial Activity:** The inhibitory activity against indicator microorganisms of isolates of Lactobacillus species was determined by the spot-on-the-lawn antagonism method and the well-diffusion simultaneous antagonism method.

Spot-On-The-Lawn Antagonism Method: The *Lactobacillus* isolates were seeded in point (20-μL) form into MRS agar plates and incubated at 37°C for 12 h under aerobic conditions. Culture medium containing the indicator microorganisms was transferred to tubes containing 5 mL of MRS broth for Lactobacillus and 5 mL of brain-heart infusion (BHI) broth (Oxoid) for the other bacteria *V. cholerae*, *Shigella*, *E. coli*, *Bacilllus*, *Klebsiella* and Salmonella and incubated at 37°C for 12 h under aerobic conditions. Then 200 μL of the suspension was transferred to 20 mL of MRS broth for *Lactobacillus* and 20 mL of BHI broth for the other bacteria. The mixture was supplemented with previously prepared 0.75% agar-agar and maintained in a water bath at 45°C. Each indicator culture was poured onto the plates cultured with Lactobacillus. After complete solidification of the upper layer, the plates were incubated for an additional 24 h at 37°C under aerobic conditions. After incubation the plates were observed for the formation of inhibition zones and were chosen for the well-diffusion simultaneous antagonism method.

Well-Diffusion Simultaneous Antagonism Method: Cell-free supernatants of the *Lactobacillus* isolate showing antimicrobial activity against the indicator microorganisms by the spot-on-the-lawn method were obtained by centrifugation at 7500 × g for 10 min in MRS broth supplemented with 0.05% glucose and incubated for 18 h at 37°C under aerobic conditions. Next, the pH was adjusted to 6 with 10 N NaOH and the samples were filtered through a Millex-GV micro filter membrane with a pore size of 0.22 μm and a diameter of 25 mm. The supernatant samples were stored at 8°C. Aliquots (20 μL) of the indicator cultures were transferred to 20 mL of Hektoen broth (Oxoid) supplemented with 0.75% agar-agar (*V. cholerae*, *Shigella*, *E. coli*, *Bacilllus*, *Klebsiella* and Salmonella), MRS broth supplemented with 0.75% agar-agar (Lactobacillus). The broths were then poured onto Petri dishes. After complete solidification, six wells with a size of 4-5 mm were punched and 10 μL, 20 μL, 30 μL, 40 μL and 100 μL of the cell-free supernatant was placed into wells. Sixth well was filled with unseeded broth to serve as control. The indicator cultures had an optical density of 0.102 at 600 nm, which corresponded to approximately 10^6 colony-forming units per milliliter. The plates were incubated at 37°C for 24 h under anaerobic conditions. The Lactobacillus isolate, again showed antimicrobial activity against the indicator microorganisms, observed as formation of an inhibition zone around the wells.

Detection of Bacteriophages: The sandwich method was used to detect Bacteriocin producing Lactobacillus strains. The isolate of Lactobacillus that showed antimicrobial activity against the indicator microorganisms by the well-diffusion method were seeded in point form onto Petri dishes containing MRS agar and incubated at 37°C for 12 h under aerobic conditions. A layer of nutrient broth supplemented with 1.5% agar-agar was added and left to solidify and then the BHI broth supplemented with 0.75% agar-agar containing indicator microorganisms was added. The plates were again incubated at 37°C for 24 h under anaerobic conditions. The action of antagonistic substances against the indicator microorganisms was demonstrated by the formation of inhibition zones around the wells. Lack of inhibition would indicate the action of bacteriophages against the indicator.

RESULTS AND DISCUSSION

Bile Tolerance of *Lactobacilli*: The effect of oxgall and taurocholic acid on the growth of *Lactobacillus* species was studied. Growth of *Lactobacillus* species in MRS broth without bile was used as a control. Taurocholic acid was used as the conjugated bile and oxgall contained both conjugated and deconjugate bile. Due to the addition of bile acids to the media, the effect of pH was monitored. Media containing oxgall acid had lower pH values compared with the other media containing taurocholic acid. The average reduction was highest from media supplemented with oxgall (1.23), whereas the lowest pH reduction was from media supplemented with taurocholic acid (0.81). These findings indicated that the initial pH of media with different bile sources had minimal influence toward subsequent growth and bile tolerance of the cultures and pH-related inhibitory actions of bile salts. The *Lactobacillus* species showed better growth in MRS broth without bile. The culture showed faster growth in

MRS broth-containing oxgall; slower growth was observed in the presence of taurocholic acid as shown in Table 3.

The culture showed also a high tolerance to different concentration of oxgall. Multiplication of the culture in skim milk with or without bile was very slow, probably due to the restriction in nutrients. However, complete survival was observed after 5 h incubation in the presence of oxgall at 0.5%. More than half of the bacteria survived after 5 h incubation in 1.5% oxgall as indicated in Table 4.

Acid Tolerance of *Lactobacilli:* The effect of acid on viability of *Lactobacillus* species was studied. The culture showed tolerance to pH 2.0 for 2 h despite variations in the degree of growth. In general there was a greater reduction in total colony-forming unit of the culture for the first hour of incubation. However, the culture showed greater acid tolerance over the entire incubation period and the colony count decreased by 19.5-10.5 log cycles as shown in Table 5.

Cholesterol Removal by Growing, Dead and Resting Cells: The amounts of cholesterol removed by growing, resting and dead cells were studied. Our aim was also to find out whether non-growing cells could remove cholesterol. Heat-killed and resting cells showed a small degree of cholesterol removal, ranging from 10.21 to 13.07 μg/g of dry weight, compared with 21.61 μg/g of dry weight for growing cells. Although there was no significant difference between cholesterol removal by resting and dead cells, mostly the culture exhibited higher cholesterol removal when cells were resting suspended in phosphate buffer (pH 6.8), compared with heat-killed cells.

The presence of bile also affected the cholesterol removal from the media. The cholesterol removal was more in media containing oxgall as bile acid than taurocholic acid when compared to media without bile acids. 29.33% of total cholesterol was removed by growing cells from media containing oxgall where-as only 12.66-18.71% was removed by dead and resting cells, respectively. In media containing taurocholic acid, growing cells were able to remove only 16.88%, dead cells 3.76% and resting cells 9.07%, of the total cholesterol content, respectively. Cholesterol removal was much greater in media without bile acid when compared to media with taurocholic acid. Growing cells were able to remove 21.61%, dead cells 10.21 and resting cells 13.07% of the total cholesterol content, respectively (Table 6).

***In vitro* Determination of Antimicrobial Activity:** The isolates that demonstrated antimicrobial activity against Gram-positive and Gram-negative indicator microorganisms with the spot-on-the-lawn antagonism method were identified. The isolate inhibited indicator microorganisms of the genera *Salmonella*, *Vibrio*, *Escherichia*, *Shigella*, *Bacillus* and *Klebsiella* but showed no antagonistic activity against the *Lactobacillus* species by the well-diffusion simultaneous antagonism. The *Lactobacillus* isolate also showed antimicrobial activity against the same Gram-positive and Gram-negative indicator microorganisms with the well-diffusion antagonism method under anaerobic conditions The halo of inhibition ranged in diameter from 13 to 37 mm. Analysis of variance of the mean diameters demonstrated that the *Vibrio* (37 mm) were significantly more sensitive followed by *Shigella* (33 mm) *Salmonella* (28 mm), *Klebsiella* (25 mm) than the *Bacillus* (20 mm) and *Escherichia* (18 mm), for 0.1 ml of the antagonistic substances produced by *Lactobacillus* species (Table 7).

Table 1: The probiotic bacteria were identified and confirmed as *Lactobacillus* species by following biochemical tests.

Characters	Lactobacillus species
Gram staining	+ve
Motility	Non-motile
Indole test	-ve
Methyl red test	-ve
Voges-Proskaeur test	-ve
Citrate test	-ve
Urease test	-ve
Oxidase test	-ve
Catalase test	-ve
Nitrate reduction test	+ve

Table 2: The enteropathogens were identified and confirmed by the following biochemical tests.

Characters	Salmonella	Vibrio	Escherichia	Shigella	Bacillus	Klebsiella
Gram staining	-ve	-ve	-ve	-ve	+ve	-ve
Motility	Actively motile	Actively motile	Actively motile	Non motile	Actively motile	Non motile
Indole test	-ve	+ve	+ve	-ve	-ve	-ve
Methyl red test	+ve	+ve	+ve	+ve	-ve	-ve
Voges-Proskaeur test	-ve	+ve	-ve	-ve	-ve	+ve
Citrate test	-ve	+ve	-ve	-ve	-ve	+ve
Urease test	-ve	-ve	-ve	-ve	-ve	+ve
Oxidase test	-ve	+ve	-ve	-ve	+ve	-ve
Catalase test	+ve	+ve	+ve	+ve	+ve	+ve
Nitrate reduction test	+ve	+ve	+ve	+ve	+ve	+ve

Table 3: Bile tolerance of *Lactobacillus* species in different bile media.

	Time								
				pH					
	H			T_1			T_2		
Growth medium	Exp1	Exp2	Mean	Exp1	Exp2	Mean	Exp1	Exp2	Mean
MRS broth	2.1	2.3	2.2	6.2	5.62	5.91	5.93	5.61	5.77
MRS broth + 0.3% oxgall	2.2	2.4	2.3	6.3	5.92	6.11	5.06	4.7	4.88
MRS broth + 0.3% taurocholic acid.	4.4	4.2	4.3	6.24	5.86	6.05	5.88	4.6	5.24

H, time required to increase by 0.3 absorbance units at 620 nm in each medium; T_1, pH at time zero; T_2, pH at time after absorbance increased by 0.3 units.

Table 4: Effect of bile acids on the growth rate of *Lactobacillus* species in modified MRS broth.

	Rate of bile tolerance		
Concentration of oxgall (%)	Experiment I	Experiment II	Mean
0	0.8	0.84	0.82
0.5	0.6	0.63	0.62
0.75	0.3	0.24	0.27
1	0.17	0.19	0.18
1.25	0.14	0.12	0.13
1.5	0.05	0.048	0.049

Table 5: Effect of pH 2 on viability of *Lactobacillus* species

	Viable count (cfu/mL)		
Incubation period (min)	Experiment 1	Experiment 2	Mean
0	42	37	39.5
30	29	34	31.5
60	17	22	19.5
90	11	16	13.5
120	8	13	10.5

Table 6: Cholesterol assimilation (in µg/mL) by *lactobacillus* species in different bile media

	Growing cells			Dead cells			Resting cells		
Growth medium	Exp1	Exp2	Mean	Exp1	Exp2	Mean	Exp1	Exp2	Mean
MRS broth	22.12	21.1	21.61	10.13	10.29	10.21	13.85	12.29	13.07
MRS broth + 0.3% oxgall	30.35	28.31	29.33	12.36	12.96	12.66	19.32	18.10	18.71
MRS broth + 0.3% taurocholic acid	16.21	17.55	16.88	4.21	3.31	3.76	9.11	9.03	9.07

Table 7: Antimicrobial activity of *Lactobacillus* against various enteropathogens.

Concentration of cel free extract (µL)	Zone of inhibition (mm)						
	Salmonella	Vibrio	Escherichia	Shigella	Bacillus	Klebsiella	Lactobacillus
10	17	24	-	18	-	19	-
20	19	26	-	19	-	20	-
30	20	29	13	20	15	21	-
40	23	30	15	24	17	23	-
100	28	37	18	33	20	25	-
Control	-	-	-	-	-	-	-

The sandwich technique demonstrated the lack of bacteriophages, which would have prevented the antagonistic action against the indicator microorganisms.

Probiotics are the living microbial food supplements, which benefit the health of consumers by maintaining or improving their intestinal microbial balance. There are many studies, which have proven in the benefit of probiotics in the human being. Fuller [5] studied that the maintenance of healthy gut microflora may provide protection against gastrointestinal disorders. The similar findings of Salminen [8] stated that the use of probiotic bacterial culture stimulates the growth of preferred microorganisms crowds out potentially harmful bacteria and reinforces the body's natural defense mechanisms.

High level of serum cholesterol has been associated with risks of coronary heart disease. The use of probiotic bacteria in reducing serum cholesterol levels has attracted much attention. Probiotic bacteria are mostly delivered in a food system and must be acid and bile tolerant to survive in the human gastrointestinal tract. The time from entrance to release from the stomach has been estimated to be approximately 90 min, with further digestive processes requiring longer residence time. Stresses to organisms begin in the stomach, with pH between 1.5 and 3.0 and in the upper intestine that contains bile. Survival at pH 3.0 for 2 h and at a bile concentration of 1000 mg/L is considered optimal acid and bile tolerance for probiotic strains. The *Lactobacilli* species used in this study showed varying levels of viability at pH 2.0 after 2-h incubation. Growth observed in the presence of different bile sources suggested that conjugated bile (taurocholic acid) was more inhibitory toward *Lactobacilli* species compared with oxgall.

The findings of Conway and Goldin [3] stated that survival of lactic acid bacteria within the human stomach is closely related to pH, which can vary largely among individuals [3]. Some lactic acid bacteria survive much longer in human gastric juice over the pH range 1-5 and it is encouraging that the strain showing best survival in gastric juice, *L. acidophilus* ADH, also adhered best to human and pig ileal cells.

The resistance of the *Lactobacilli* toward deconjugate bile may be because conjugated bile salts have greater solubility and detergent activity and may, therefore be more toxic than their deconjugate counterpart. This was supported by the fact that the oxgall added to the fermentation broths was far less soluble than taurocholic acid, based on the solubility index.

Taurocholic acid was not accumulated by *Lactobacillus* due to its hydrophilicity Such preference was supported by previous reports [7], which showed that *Lactobacillus* species actively accumulated cholic acid in an ATP-dependent manner, or when they were energized by glucose. Our results suggest that under high concentration of conjugated bile, *Lactobacillus* species are likely to survive best, whereas in presence of deconjugate bile they would survive best. Growth in the presence of oxgall showed that *Lactobacillus* species survived best.

Cholesterol assimilation in the presence of different bile sources showed a good relationship with bile tolerance of the culture studied (Fig. 4). *Lactobacillus* species showing greater tolerance toward deconjugated bile exhibited higher overall cholesterol assimilation in the presence of oxgall, whereas which showed greater inhibition by taurocholic acid produced lower cholesterol assimilation. Also, the *Lactobacillus* isolate exhibited better growth in the presence of cholesterol, indicating that cholesterol stimulated their growth. Regression analyses suggested that cholesterol removal was closely associated with the bacterial growth. Cholesterol assimilation by growing cells was significantly higher than resting and dead counterparts; however, there was no significant difference in the level of cholesterol removal by resting and dead cells.

The capability of strains in dead and resting stages to remove cholesterol indicated that cholesterol might be removed via binding to cells. Higher cholesterol removal by growing cells indicated that the degree of bound cholesterol might be dependent on the growth of cells. Liong and Shah [7] in their studies reported that the

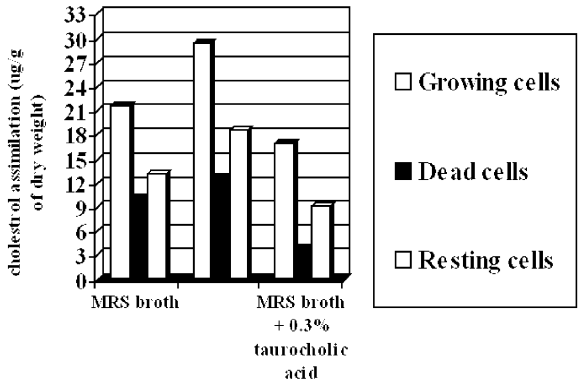

Fig. 1: Cholesterol removal by growing,heat killed,and resting cells of Lactobacillus

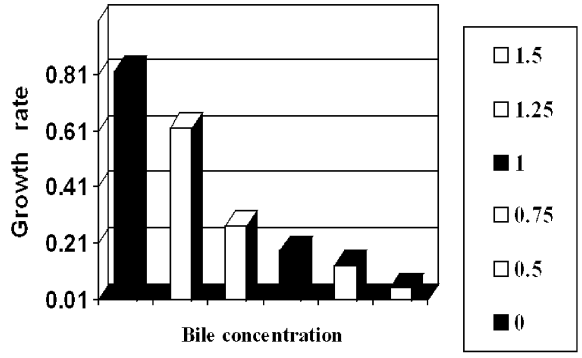

Fig. 2: Effect of bile acids on the growth rate of Lactobacillus species in mMRS brot

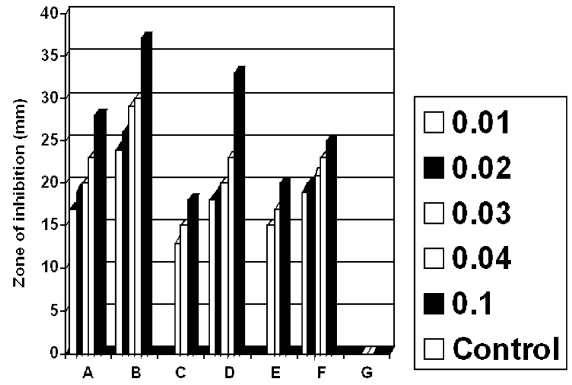

Fig. 3: Antimicrobial activity of Lacobacillus against various enteropathogens.

A - *Samonella*
B - *Vibrio*
C - *Escherichia*
D - *Shigella*
E - *Bacillus*
F - *Klebsiella*
G - *Lactobacillus* (control organism)

physiological pH in the intestinal tract of humans is usually neutral to alkaline. Although cholesterol assimilation occurred mainly with growing cells, results on cholesterol removal from media at pH 6.8 by heat-killed cells indicated the potential of nonviable cells to reduce cholesterol concentration in the gastrointestinal system.

Anderson and Gilliland [6] in their studies reported that daily intake of fermented milk (FM) products containing selected strains of *L. acidophilus* has the potential to significantly reduce serum cholesterol concentrations. Reductions of serum cholesterol concentrations of 3-4% are clinically meaningful since every 1% reduction in serum cholesterol concentrations leads to a 2-3%reduction in estimated risk for coronary heart disease. Thus, regular intake of FM containing an active cholesterol-reducing *L. acidophilus* could decrease estimated risk for coronary heart disease by 6-10%.

Probiotics are the living microbial food supplements, which benefit the health of consumers by maintaining or improving their intestinal microbial balance. There are many studies, which have proven in the benefit of probiotics in the human being. Fuller [5] also studied that the maintenance of healthy gut microflora may provide protection against gastrointestinal disorders. The similar findings of Salminen *et al.* [8] stated that the use of probiotic bacterial culture stimulates the growth of preferred microorganisms crowds out potentially harmful bacteria and reinforces the body's natural defense mechanisms.

Selected *Lactobacillus* strains are used as probiotics with supposed health promoting activites. Yuksekdag *et al.* [13] insisted that *Lactobacillus* species are frequently said to have promoting effects in the human and animal intestinal tract. The probiotic effects are conferred by the production of antimicrobial substances or bacteriocins that inhibits undesirable pathogens causing disease in the human intestine [18]. The probiotics exerted by the production of lactic acid and wide variety of antimicrobial substances including proteinacous and non proteinacous substances. Savage [17] also stated that the mode of action of potential probiotic in animal feeds he insisted that the endogenous *Lactobacillus* species are known to associate with epithelial surface in the alimentary canal. Sudirman *et al.* [16] states that a number of antimicrobial substances produced, such as lactic acid, hydrogen peroxide, diacetyl and bacteriocin having a strong antimicrobial properties. Kim and Worobo [15] have reported that a thermostable bacteriocin and low molecular weight substance are major substances, which exhibits strong inhibitory effect against various pathogens.

In our study it was found that the *Lactobacillus* dose had significant antagonistic effect against various enteropathogens. The efficacy and spectrum of action of lactic acid bacteria against pathogenic microorganisms are based on the action of bacteriocins and a combination of antimicrobial substances such as hydrogen peroxide, organic acids and bacteriophages. The antagonistic methods used in the present study, spot-on-the-lawn and well diffusion, were effective in demonstrating the sensitivity of the indicator strains; however, the size of the bacterial inhibition zone varied according to the antagonistic method used. The activity of bacteriocin can be estimated by the size of the inhibition zones produced in a diffusion test. In the present study, from the diameter of the inhibition zone we determined that the *Vibrio*, *Salmonella*, *Shigella* and *Klebsiella* were more sensitive than the *Bacillus* and *E. coli* to the antagonistic substances produced by *Lactobacillus* species.

By use of the well-diffusion simultaneous antagonism method with anaerobic incubation, the action of hydrogen peroxide was precluded, demonstrating that the inhibition zones formed by the supernatants against the enteropathogens were due to proteic action specifically. The action of organic acids could also be precluded since modified MRS culture medium supplemented with only 0.05% glucose and adjusted to pH 6 was used, thus reducing fermentation and, consequently, the production of organic acids. The use of the sandwich technique prevented the diffusion of bacteriophages.

There were many findings that illustrated the antimicrobial activity of *Lactobacillus* against various gastrointestinal pathogens. The same was added by Yuksekdag *et al.* [13], reported that the antimicrobial activity of *Lactobacillus* against *E. coli* and *Staphylococcus aureus* was significant. They have reported that the Lactic acid and acetic acid have the ability to inhibit the bacterial growth and act synergistically. The above findings were also supported by Sissons [14]. They have shown that antimicrobial proteins from selected lactic acid bacteria are capable of inhibiting *Listeria monocytogenes*.

The similar findings were reinvestigated by Yuksekdag *et al.* [13]. They have found that *Lactobacilli* inhibit the growth of *E. coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*. Similar work carried out by Ronka *et al.* [12] reported that *Lactobacillus brevis* strongly inhibited *Bacillus cereus* and to some extends *Staphylococcus aureus* and other harmful microorganisms. Lene Coconnier *et al.* [11] in their studies reported that the human *L. acidophilus* strain LB produces an antibacterial activity effective *in vitro* against Gram-negative and Gram-positive pathogens, *in vitro* against an enteroinvasive pathogen which adheres to and enters cultured human enterocytic cells and *in vivo* in the *S. typhimurium*-infected-mouse model. Considering from the results they concluded the component(s) secreted by *L. acidophilus* LB that supports the antimicrobial activity could contain an unusual acidic amino acid present in a novel peptidic agent.

Probiotics represent a potentially significant therapeutic advance. In an effort to decrease reliance on antimicrobials, the time has clearly come to increase the exploration of the therapeutic applications of probiotics. There are too many reports describing the beneficial effects of probiotics to dismiss this concept for preventing and treating a variety of intestinal disorders. Probiotics offer dietary means to support the balance of the intestinal flora. They may be used to counteract local immunological dysfunction, to stabilize the intestinal mucosal barrier function, to prevent infectious succession of pathogenic microorganisms and to influence intestinal metabolism.

REFERENCES

1. Buck, M. and S.E. Gilliland, 1994. Comparisons of freshly isolated strains of *Lactobacillus acidophilus* of human intestinal origin for ability to assimilate cholesterol during growth. J. Dairy Sci., 77: 2925-2933.
2. Ogunshe, A.A.O., M.A. Omotoso and J. Adeleye Adeyeye, 2007. In vitro antimicrobial characteristics of bacteriocin producing *Lactobacillus* strains from Nigerian indigenous fermented foods. African J. Biotechnol., 6: 445-453.
3. Conway, F.L. and B.R. Goldin, 1987. Survival of lactic acid bacteria in the human stomach and adhesion to intestinal cells. J. Dairy Sci., 70: 1-12.
4. Belifore, C., P. Castellano and G. Vignolo, 2007. Reduction of *E. coli* population following treatment with bacteriocins from *Lactobacillus* and chelators. Food microbiol., 24: 223-229.
5. Fuller, R., 1973. Probiotics in man and animals. J. Applied Bacteriol., 66: 356-378.
6. Anderson, J. and S. Gilliland, 1999. Effect of fermented milk (yogurt) containing *Lactobacillus acidophilus* L1 on serum cholesterol in hypercholesterolemic humans. J. the American College of Nutrition, 18: 43-50.

7. Liong, M.T. and N.P. Shahj, 2005. Acid and bile tolerance and cholesterol removal ability of *Lactobacilli* strains. American Dairy Science Association, 88: 55-66.
8. Salminen, S., A.C. Quwehand and E. Isolauri, 1998. Clinical applications of probiotic bacteria. Indian J. Dairy Sci., 84: 563-572.
9. Rial D. Rolfe, 2000. The role of probiotics cultures in the control of gastrointestinal health. J. Nutrition, 130: 396-402.
10. Zurkowski, P., 1964. A rapid method for cholesterol determination with a single reagent. Clinica Chimica Acta, 10: 451-453.
11. Lene Coconnier, M.H., V. Lievin, M.F. Bernet-Camard, S. Hudault and A.L. Servin, 1997. Antibacterial effect of the adhering human *Lactobacillus acidophilus* strain LB. Antimicrobial Agents and Chemotherapy, 41: 046-1052.
12. Ronka, E., E. Malmen, M. Saarela, M. Rinta Koski, J. Aaarnikunnas and A. Pavia, 2003. Role of dietry *Lactobacilli* in gastrointestinal microecology. International J. Food Microbiol., 83: 63-68.
13. Yuksekdag, T., J. Stevenson and K. Kim, 2002. Inhibiting factors produced by lactic acid bacteria. Applied Environment Microbiol., 70: 337-349.
14. Sissons, J.W., 1989. Potential of probiotic organism to prevent diarrhoea and promote digestion in farm animals. J. food and Agricultural Sci., 49: 1-13.
15. Kim, S.H. and R.W. Worobo, 2000. Characterization and purification of a bacteriocin produced by a potential probiotic culture, *Lactobacillus acidophilus* 30sc. J. Dairy Sci., 83: 2747-2752.
16. Sudirman. I., F. Mathieu, M. Michel and G. Lefebvre, 1993. Detection and properties of curvaticin 13, a bacteriocin produced by *Lactobacillus curvatus* SB13. Current Microbiol., 27: 35-40.
17. Savage, D.C., 1997. Microbial ecology of the gastrointestinal tract. American J. Clininical Nutrition, 32: 113-117.
18. Lee, Y.J., W.K. Yu and T.R. Rev, 2003. Models for study of the specificity by which indigenous *Lactobacilli* adhere to murine gastric epithelia. International J. Antimicrobial Agents, 21: 340-344.