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Dual Effects of *Lactobacilli* as a Cholesterol Assimilator and an Inhibitor of Gastrointestinal Pathogenic Bacteria

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Background: Probiotics are live microbial supplements which can improve the healthy intestinal microbial balance. *Lactobacilli* are a group of lactic acid producing bacteria (LAB) that are known as natural probiotics found in the dairy products.
Objectives: In this study, we aimed to detect the most potent Lactobacillus isolates of the Fars province local dairy products in cholesterol removal and investigate their antibacterial properties against some gastrointestinal pathogens.
Materials and Methods: Fifteen locally produced yogurt samples of the Fars province were collected and characterized with routine microbiology methods. Cholesterol removal ability of the *Lactobacilli* isolates were determined, and their growth inhibitory effect on some standard pathogenic strains pathogen was evaluated using the well-diffusion method.
Results: In this study, five common strains of *Lactobacilli* including *L. acidophilus, L. casei, L. fermentum, L. lactis,* and *L. bulgaricus* were identified in the samples obtained from the locally produced yogurt in the Fars province. *L. lactis* and *L. acidophilus* were determined as the two most active strains with the maximum rate of cholesterol assimilation (5.6 and 4.5 mg/mL, respectively) in the process of cholesterol removal. In the antibacterial activity assay, the two mentioned strains had significant inhibitory effect on all of the tested bacteria except for *B. subtilis*.
Conclusions: Cholesterol removal ability had a direct relation with bacterial growth, so it is suggested to use the probiotic bacteria in the growth phase to achieve better results.

Keywords: Lactobacillus; Enterobacteriaceae; Lower Gastrointestinal Tract

1. Background

There is a strong association between high levels of cholesterol in the blood and the incidence of cardiovascular diseases in human (1, 2). What has been less noticed is the fact that cholesterol has a crucial role as a component of cellular membranes and as a precursor of steroid hormones and bile acids. Cholesterol is an essential molecule in humans, but is not an essential component of the mammalian's diet (1). All cell types can synthesize cholesterol from simple precursors. The risk of coronary heart disease and death is approximately increased by 35% and 45% respectively, for each mmoL increase in the cholesterol level above the normal limit (1). A small reduction (~1%) in serum cholesterol level has been reported to reduce the risk of coronary heart disease (by apprixmately 2 to 3%) (3). It has been proposed that the dairy products containing probiotics can reduce the serum cholesterol level (2). Probiotics are regarded as "live microbial supplements which beneficially affect the host animal by improving its intestinal microbial balance" (2). For providing health benefits, probiotics have to overcome physical and chemical barriers such as bile and acids present in the gastrointestinal tract system. Fermented milk with lactic acid bacteria was first demonstrated to exhibit hypocholesterolemic effects in humans as early as 1963. Various studies have shown that some *Lactobacilli* could reduce the total cholesterol level and LDL (low-density lipoprotein) cholesterol. Exact mechanism of the effect of probiotics in serum cholesterol reduction is still unclear. However, there is some evidence that some strains of *Lactobacillus* spp. can secrete bile salt hydrolases (cholylglycine hydrolase). These enzymes catalyze the hydrolysis of taurine or glycine-conjugated bile salts into the free bile salts and amino acid residues. Free forms of bile salts are less soluble than the conjugated forms, resulting in a lower absorption in the intestinal lumen. Deconjugation process of bile acids reduces the level of serum cholesterol by increasing the formation of new bile acid needed to replace the bile acid pool of the enterohepatic circulation.

Liong et al. (2) proposed another mechanism for cholesterol reduction. They suggested that *L. acidophilus* might incorporate some of the removed cholesterol from the medium into the cell membranes during the growth phase. Cholesterol adhered to the cell wall or incorporated into the bacterial cells would be less available in the intestine to be absorbed into the blood (2, 4). It was postulated that

Implication for health policy/practice/research/medical education:
This investigation was performed to investigate the potential use of the local *Lactobacilli* strains in the food processing industry.

the cholesterol incorporated into the *L. acidophilus bacteria*, could alter the cellular membrane or cell wall of the organism. Gram staining of *L .acidophilus* was shown to be variable in the presence of cholesterol. All the bacteria grown in the absence of cholesterol are gram-positive (5, 6).

2. Objectives

Our aim was to detect the most potent *Lactobacillus* isolates of the local dairy products of Fars province (South of Iran) regarding their active cholesterol removal ability in the presence of bile salts; and to determine the antibacterial properties of the cholesterol removing *Lactobacilli* against some gastrointestinal pathogens (7, 8).

3. Materials and Methods

In this study, the microbiota of domestic yogurt was analyzed. Fifteen yogurt samples were collected from three locations (Shiraz, Jahrom and Fasa) in Fars province. Ten grams of each yogurt sample was homogenized with 100 mL of Man Rogosa Sharpe (MRS) broth (Himedia Co.). The suspensions were plated on the surface of MRS agar (2, 9). After anaerobic incubation for 48 h at 37˚C, some colonies were selected randomly and catalase and oxidase tests were performed for the presumptive identification of the *Lactobacilli*. The isolated bacteria were specified as *Lactobacillus* with the following characteristics: rod shaped, gram positive, catalase negative and non-spore forming cells. Then sugar fermentation tests including mannitol, lactose, galactose, raffinose, sucrose, xylose, fructose, and TSI were applied to all of the isolates to characterize them to the species level (9). According to these properties, all isolates were categorized into five species of *Lactobacillus*, including *L. fermentum*, *L. acidophilus*, *L. casei*, *L. lactic,* and *L. bulgaricus*. In the next step, cholesterol assimilation of the isolates were assessed. The isolated species were sub-cultured three times in MRS broth containing 1% v/v inoculum and incubated at 37˚C for 20 hours (2). Then MRS broth was supplemented with 0.30% Oxgall (Merck. Germany. Co.) as a bile salt. Water-soluble cholesterol (polyoxyethylene cholesteryl sebacate, Merck Co.) was filter-sterilized and used at a final concentration of 70 to 100 μg/mL, for each strain (at 1% v/v), and incubated in anaerobic condition at 37˚C for 20 h. After the incubation period, bacterial cells were removed by centrifugation and the remaining broth medium was examined for cholesterol concentration by applying a modified colorimetric method, described previously by Rudel and Morris (10). Absorbance of the cholesterol-containing solution was read after 10 minutes of the treatment at 550 nm (2, 11, 12). To assay the absorbance of the cholesterol to the cell wall of the bacteria, prepared MRS broth containing Oxgall (0.30%) was inoculated with the studied *Lactobacilli* and incubated at 37˚C for 20 h. After the incubation period, cells were harvested by centrifuging at 10.000×g (Eppendorf) at 4˚C for 10 minutes. The cell pellets were washed twice with sterile double distilled water. For preparing heat-killed cells, the cell pellets were suspended in 10 mL of double sterile distilled water and autoclaved at 121˚C for 15 minutes. The heat-killed cells were suspended in freshly prepared MRS broth containing Oxgall (0.30%) and water-soluble cholesterol. For preparing resting cells, the cell pellets were suspended in 10 mL of sterile 0.05 M phosphate buffer (pH = 6.8) containing water-soluble cholesterol and Oxgall. All cultures were incubated at 37˚C for 20 h. The supernatant was evaluated for the cholesterol content as described above (2). Cholesterol assimilation by resting, growing and dead cells was calculated in dry weight to obtain uniformity across all the treatments (2, 13). The following equation was applied:

Cholesterol assimilation = (C1 − C2)/ (W2 − W1)

Where C1 and C2 were the amount of cholesterol present in the fermentation broths at time zero and 20h, respectively. W1 and W2 were the dry weight of each individual culture at time zero and 20 h, respectively. The results showed that two specific strains had the most activity in cholesterol assimilation. To evaluate the sensitivity of these two strains to common antibiotics, disk diffusion method was performed according to CLSI protocols with ampicillin (10 μg), vancomycin (30 μg), oxacillin (1 μg), cephalothin (30 μg), ceftazidime (30 μg), and tobramycin (10 μg) (MAST Co.). Antimicrobial susceptibility test was also performed on some reference strains: *E .coli* ATCC 35218, *S. aureus* ATCC 25923,*P. aeruginosa* ATCC 27853, *B. subtilis* ATCC 6633, and *K. pneumonia* ATCC 18833 (7, 8). To evaluate the antimicrobial activity, two solutions of the two *Lactobacillus* strains, *L. casei* and *L. acidophilus* were prepared as the following: first, the strains were incubated for 72 h at 37˚C in MRS broth containing 0.2% glucose, and then the broth was centrifuged; and the supernatant was filtered with microbiologic filters to remove any bacterial cells. To evaluate the activity of H_2O_2 and acidic compounds in these solutions, they were divided into two tubes; in one solution pH was adjusted to 6.5 with normal NaOH and the hydrogen peroxide was neutralized with catalase (5 mg/mL). The second solution was used without any changes in its composition. The prepared solutions were used as antimicrobial agents against the mentioned reference strains by the agar diffusion method (14, 15). Thereafter, the mentioned reference strains were incubated at 37˚C for 24 h in the nutrient broth, then a suspension of each strain with 0.5 McFarland turbidity (1.5×10^8 cfu/mL) was prepared and inoculated evenly on the nutrient agar plates. Two holes (0.5 mm diameter each) were punched in the agar media, in 30mm distance from each other. These wells were filled with the *Lactobacilli* supernatant solutions and incubated for 24 h at 37˚C. Finally, the diameters of inhibition zones were measured.

4. Results

Five common strains of *Lactobacilli L. acidophilus*, *L. casei*, *L. fermentum*, *L. lactis*, and *L. bulgaricus* were identi-

fied considering the biochemical tests and fermentation properties. Table 1 shows the range of cholesterol assimilation by different isolated strains of *Lactobacilli* after 24 h based on the presence or absence of bile salts. As the main bile salt in the peptic system is the cholic acid, all the evaluations were performed in the presence of this salt. The amounts of cholesterol assimilated into the cells and membranes were expressed as micrograms per milliliter of protein ranging from 7.82 μg/mL for the *L. lactis* in the presence of cholic acid, to 34.96 μg/mL for the *L. casei* in the presence of Oxgall. The amounts of cholesterol assimilation were maximum for *L.acidophilus* and *L. casei*. Generally, all mentioned strains of *Lactobacilli* had accentuated growth in the presence of cholesterol. Table 2 presents cell wall cholesterol assimilation capability of *Lactobacilli* according to the equation proposed by Liong and Shah. *L. acidophilus* and *L. lactis* had the maximum rate of cholesterol assimilation (5.6, and 4.5 mg/mL, respectively). Table 3 summarizes antibiotic susceptibility patterns of the two isolated strains of *Lactobacilli*; *L.casei* and *L. acidophilus*. *L. casei* was resistant to all of the tested antibiotics. *L. acidophilus* was resistant to all of the antibiotics except for vancomycin and Tobramycin. Antimicrobial activity of *L. acidophilus* and *L. casei* against *S. aureus*, *P. aeruginosa*, *B. subtilis*, *K. pneumonia* and *E. coli* are shown in Table 4.

Table 1. Cholesterol Assimilation of *Lactobacillus* Strains in the Presence and Absence of Bile Salts [a], [b]

Strain	MRS Broth	MRS Broth +, 0.3% Cholic Acid	MRS Broth +, 0.3% Oxgall	MRS Broth +, 0.3% Taurocholic Acid
L. acidophilus	16.67 ± 0.17	29.33 ± 0.16	28.80 ± 0.22	8.68 ± 0.27
L. casei	20.68 ± 0.21	32.25 ± 0.42	34.69 ± 0.45	20.80 ± 0.25
L. bulgaricus	18.75 ± 0.46	12.66 ± 0.37	23.2 ± 0.64	12.14 ± 0.17
L. lactis	10.00 ± 0.44	20.38 ± 0.31	10.94 ± 0.32	7.82 ± 0.35
L. fermentum	21.61 ± 0.19	22.09 ± 0.21	27.6 ± 0.25	11.93 ± 0.85

[a] Abbreviations: MRS, Man Rogosa Sharpe broth.
[b] All data are presented as mean ± SD

Table 2. Amount of Cholesterol Assimilation According to the Dry Weight of *Lactobacilli*[a]

Strain	C1, μg/mL	C2, μg/mL	W1, g	W2, g	Result, mg/mL
L. acidophilus	100	94.50	6.25	7.15	5.6
L. casei	100	96.82	8.75	10.15	2.27
L. bulgaricus	100	98.30	3.95	4.45	3.4
L. lactis	100	95.50	6.85	7.75	4.5
L. fermentum	100	95.35	6.55	7.95	3.32

[a] C1 and C2, the amount of cholesterol present in the fermentation broths at time = 0 and 20 h, respectively; W1 and W2, the dry weight of the individual cultures at time = 0 and 20 h, respectively.

Table 3. Antibiotic Susceptibility Pattern of *L. casei* and *L.acidophilus*[a]

Strains	Tobramycin, 10 μg	Cefodizime, 30 μg	Cephalothin, 30 μg	Oxacillin, 1 μg	Vancomycin, 30 μg	Ampicillin, 10 μg
L. casei	R	R	R	R	R	R
L. acidophilus	S	R	R	R	S	R

[a] Abbreviations: R, resistant; S, sensitive.

Table 4. Antimicrobial Activity of *L. casei* and *L.acidophilus* Against Standard Strains of Enteropathogens (Diameter Of Inhibition Zone In mm)

	S. aureus **(ATCC25923)**	*P. aeruginosa* **(ATCC27853)**	*B. subtilis* **(ATCC6633)**	*K. Pneumoniae* **(ATCC 18833)**	*E. coli* **(ATCC 35218)**
L. casei	9	8	-	7	8
L. acidophilus	8	8	-	7	8

5. Discussion

Hypercholesterolemia is a known predisposing factor for atherosclerosis and coronary artery diseases (3). Use of probiotics as a new attractive means to decrease serum cholesterol level has been proposed recently (16). Probiotics are defined as microbial supplements beneficially affecting the host immunity by improving the intestinal microbial balance (2). Probiotics mostly enter the human body via foods, and must be acid- and bile- resistant to survive in the gastrointestinal tract. The interval time from food entering and leaving the stomach is estimated as approximately 90 minutes, and efficient probiotic bacteria are those able to actively survive in the digestive tract for at least a few hours (17). Organisms undergo stress beginning in the stomach, with a pH between 1.5 and 3.0, which continues in the upper intestine due to the presence of bile. According to the results of a previous study, *Lactobacilli* can tolerate pH = 3.0 for 2 hours and also bile concentration of 1000 mg/L (18). Conjugated bile compounds (taurocholic acid) have more inhibitory effect on the growth of lactic acid bacteria strains compared to the cholic acid as a de-conjugated bile form and Oxgall. Conjugated bile salts have greater detergent activity and solubility, and so may be more toxic than their deconjugated counterparts. This was supported by the fact that the cholic acid added to the fermentation broths was less soluble than taurocholic acid, based on the solubility index. Lactic acid bacteria are commonly used as starter in the production of fermented dairy products (19).

We identified five strains of *Lactobacillus* spp. in our local dairy products with good cholesterol assimilation ability, which was comparable with the results of other studies (2, 11). Cholic acid is the most prevalent bile salt in the human intestine, and its presence in the environment was well tolerated by our native strains of *Lactobacilli*. Cholesterol binding to the cell wall of *Lactobacilli* is a probable mechanism for cholesterol assimilation as proposed by Kimoto et al. (13), but in our study most of the cholesterol molecules were decomposed to their intermediate metabolites and just a small amount of cholesterol was assimilated into the cell wall. We propose the analysis of cellular lipids and their composition with gas chromatography to understand the actual mechanism of cholesterol assimilation by *Lactobacilli*. We also evaluated the inhibitory effect of *Lactobacilli* on common enteropathogens. It was found that *L. casei* and *L. acidophilus* isolates had a weak antibacterial effect (7-9 mm zone of inhibition) against the reference strains studied. The main antibacterial effects of lactic acid bacteria are due to pH reduction through the production of acetic acid, lactic acid, and also diacetyl compounds, fatty acids, hydrogen peroxide, aldehydes and other compounds. Antibacterial effects of *L. sake* and *L. plantarum* strains isolated from the meat and meat products against several bacteria has been reported (20). In addition, *L. acidophilus* and *L. paracasei* sub sp. *paracasei* strains isolated from feces of infants had weak antibacterial activity against *Yersinia enterocolitica* and *Escherichia coli*. A total of 192 strains of lactic acid bacteria were isolated from Artisanal minas cheese. No correlation was found between bactericidal activity and lactic acid and hydrogen peroxide production. They found that *Lactobacillus* strains produced H_2O_2, but did not demonstrate any inhibitory effect. *Lactococcus lactis* subsp. *cremoris* Z20S strain produced maximum lactic acid but not H_2O_2. Both of these strains had an inhibitory effect against *S. aureus,* but not against *E. coli* and *P. aeruginosa*. Lactic acid bacilli involved in the fermentation industry had an antimicrobial effect against various food-borne pathogens, and the inhibitory products were extracellular and soluble. Inhibitory properties of lactic acid bacilli is influenced by the medium they grew in. Based on the results of this study, *L. casei* and *L.acidophilus* were resistant to routinely used antibiotics. This resistance is often intrinsic and non-transmissible. Interestingly, inherently antibiotic-resistant probiotic strains may benefit patients whose normal intestinal microbiota is unbalanced or greatly reduced in numbers due to administration of various antimicrobial agents. In the case of resistance to antimicrobial agents, vancomycin resistance is of major concern, because vancomycin is the last choice against some clinically important multi drug resistant pathogens. *L. casei* was resistant to all of the antibiotics used in this study, but *L. acidophilus* isolate was resistant to all of the antibiotics except for tobramycin and vancomycin. In conclusion, the presence of *Lactobacilli* species with a good cholesterol assimilation ability and remarkable resistance against commonly used antibiotics in our local dairy products can make them a good choice for use as fermentation starters used in the production of dairy products which are currently imported from other countries.

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Authors' Contribution

Both authors participated in all parts of the manuscript.

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