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Investigation of probiotic properties of lactobacilli bacteria isolated from human gastrointestinal tract

Untersuchung von probiotischen Eigenschaften von Lactobacilli-Bakterien aus menschlichem Gastrointestinaltrakt isoliert

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Summary

In this study, the probiotic characteristics of *Lactobacillus* which were taken from the stool samples of 30 children, aged between 5 and 15 years, were studied. The stomach medium (low pH) and bile salt tolerance, bile salt hydrolysis activity, antagonistic activity and cholesterol assimilation quantity of *Lactobacillus* strains were determined. It was defined that on the whole *Lactobacillus* strains were resistant to high acidity (pH 2.0–2.5) and sensitive to high levels of bile salt (% 1.00 Oxgall), there was no bile salt hydrolysis activity, they were resistant to vancomycin, teicoplanin and bacitracin and had antagonistic effects on pathogenic bacteria. It was noted that only *L. curvatus* L26 strain, which produced bacteriocin, had a general antagonistic effect; was indulgent to gastric acid and bile salt; and in different bile salt medium assimilated cholesterol at a high degree (15.22–25.42 µg/ml). At the same time, the fact that *L. curvatus* L26 had ß-glucuronidase digesting prebiotics and ß-galactosidase digesting lactose is one of its most significant probiotic properties. In the light of this information, our opinion is that *L. curvatus* L26 can involve potential probiotic characteristics.

Keywords: Probiotic, *Lactobacillus*, Gastrointestinal tract

Zusammenfassung

In dieser Studie wurden die probiotischen Eigenschaften von *Lactobacillus*, die aus den Stuhlproben von 30 Kindern im Alter von 5 bis 15 Jahren gemacht wurden, wurden untersucht. Der Magen-Medium (niedriger pH-Wert) und der Gallensalztoleranz, Gallensalz-Hydrolyse-Aktivität, antagonistische Aktivität und Cholesterin Assimilation Menge von *Lactobacillus*-Stämme wurden bestimmt. Es wurde festgelegt, daß auf die gesamten *Lactobacillus*-Stämme waren resistent gegenüber hohen Säuregrad (pH-Wert 2,0–2,5) und empfindlich gegenüber hohen Niveaus von Gallensalz (1,00 % Ochsengalle), gab es keine Gallensäurensalz-Hydrolyse-Aktivität, sie waren resistent gegen Vancomycin, Teicoplanin und Bacitracin und hatte antagonistische Wirkungen auf pathogene Bakterien. Es wurde festgestellt, dass nur *L. curvatus* L26-Stamm, der Bakteriozin produziert, hatte eine allgemeine antagonistische Wirkung; war nachsichtig Magensäure und Gallensalz; und in verschiedenen Gallensalzmedium gleich Cholesterin auf einem hohen Grad (15,22 bis 25,42 g/ml). Gleichzeitig ist die Tatsache, dass *L. curvatus* L26 hatte ß-Glucuronidase und ß Verdauen Präbiotika-Galactosidase Verdauen Lactose einem seiner wichtigsten probiotischen Eigenschaften. Angesichts dieser Informationen ist unsere Meinung, dass *L. curvatus* L26 können potenzielle probiotischen Eigenschaften beinhalten.

Schlüsselwörter: Probiotische, *Lactobacillus*, Gastrointestinaltrakt

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Introduction

Probiotic microorganisms are those which are found in the natural human flora, tolerant of gastric acid and bile, can hold onto epithelial cells of the gastrointestinal (GI) system, are of anaerobic character, are non-pathogenic and have an antagonistic attribute (Kaur et al. 2002; Otles et al. 2003; Salminen et al. 2005; Shobha and Agrawal, 2007). For many years, lactic acid bacteria (LAB) have been used for the fermentation of various foods such as yoghurt, cheese, kefir, pickle, sausage, etc. (Şimşek et al. 2006). Probiotic LAB have been widely studied in recent years. Currently, *Lactobacillus casei* shirota (LCS), *L. rhamnosus* LGG, *L. acidophilus* LA7, *L. acidophilus* LA5, *L. acidophilus* DDS23, *L. casei* LC1, *Bifidobacterium longum* BB536, *B. lactis* BB12, *B. animalis* DN-173010 and *Saccharomyces boulardii* microorganisms are used as probiotics (Klein et al. 1998; Soccol1 et al. 2010).

Probiotics taken by oral route should preserve their intactness while passing through the gastric juice. Microorganisms taken from food stay in the stomach having a pH value between 2.0 and 3.0 and an enzymatic medium for 1 to 4 h (Kopp-Hoolihan, 2001). Most of the microorganisms are inhibited during this period while probiotic microorganisms are transferred unimpaired to the intestines.

Bile, which is formed in the liver and which is its major organic compound (Ceydilek and Beyler, 2005), is secreted into the small intestine at an amount of 600–1200 mL/day. Of this amount, 20–30 grams enter the large intestines and display an antimicrobial effect against microorganisms (Dunne et al. 1999). Previous studies showed that both conjugated and deconjugated bile salt acids inhibited the *in vitro* survival of bacteria such as *Escherichia coli* isolates, *Klebsiella* sp and *Enterococcus* sp (Lewis and Gorbach, 1972; Stewart et al. 1986). Klayraung and Okonogi (2009), stated that *L. fermentum* displays an antagonistic effect on *L. monocytogenes, S. aureus ssp aureus, S. typhi* and *Shigella sonnie* and this effect increases with bile salt addition.

Bacteriocin is a polypeptide produced by bacteria. Some strains of LAB produce bacteriocin that shows antagonistic effect on varied bacteria. Ghalfi et al. (2006) noted that bacteriocin of *Lactobacillus curvatus* CWBI-B28 strain displays antagonistic effect against *Listeria monocytogenes.*

Bile salts both enable digestion of fat and prevent the microorganisms from growing up in gut. It was reported that some LAB can reduce the emulsifying effect of the bile salts by hydrolyzing them with bile salt hydrolase enzymes (Erkkila and Petaja, 2000). In several studies, it is noted that some types and strains of LAB *(L. acidophilus, L. salivarius, L. plantarum, L. brevis L. fermentum)* hydrolyze bile salts (Dashkevicz and Feighner, 1989; Du Toit et al. 1998; Guijie et al. 2009).

There are studies demonstrating that lactobacilli are naturally resistant to vancomycin and teicoplanin (Klein et al. 1998; Klein, 2011) while lactobacilli of human origin are resistant to bacteriocin (Charteris et al. 1998; Charteris et al. 2001).

One of the desired properties in probiotic microorganisms is that prebiotics which cannot be absorbed or digested should be hydrolized through the ß-glucuronidase and ß-galactosidase enzymes intolerant people. *Lactobacillus* has these sorts of enzymes (Stromp and Laukova (2013). None of the strains showed any level of α-chymotrypsin, ß-glucuronidase, ß-glucosidase, N-acetyl-ß-glucosaminidase activities associated with intestinal diseases (Heavey and Rowland, 2004).

Another characteristic of probiotic microorganisms is that they possess an antagonistic attribute. Organic acids, carbon dioxide, diacetyl, acetaldehyde, biosurfactant substances, H_2O_2 and compounds with protein structure (bacteriocin and bacteriocin-like substances) produced by lactic acid bacteria have an antagonistic effect (Mishra and Lambert, 1996; Rolfe, 2000). Lactic acid bacteria were shown to make an inhibitor effect against pathogens like *Listeria monocytogenes* (Moroni et al. 2006) and *Helicobacter pylori* (Avonts and De-Vuyst, 2001).

In the world, death arising from high cholesterol is in the lead. High cholesterol causes embolism, heart attack and deaths. There are a lot of researches stating that probiotic bacteria can be used in order to lower cholesterol (Coeuret et al. 2004; Belviso et al. 2009; Zeng et al. 2010).

The objective of this study is to determine the probiotic characteristics of 30 isolated *Lactobacillus* bacteria of human gastrointestinal origin concerning gastric solution resistance, bile salt tolerance, antibiotic susceptibility, antagonistic activity, bile salt hydrolysis activity, enzymatic activity and cholesterol assimilation.

Materials and methods

Microorganisms

100 stool samples were collected from children, aged between 5 and 15 years, and one bacteria from each of them was isolated at the Microbiology Laboratory of Dr. Behçet Uz Pediatric Hospital. Of the stool, 1 g was homogenized using sterile physiological water, and the homogenization was used to prepare serial dilutions at a rate of 10^{-1}–10^{-7} (10^{-4}–10^{-7} dilutions were planted to De Man Rogosa Sharpe (MRS) Agar (Becton, Dickinson and Company, USA) and incubated at 37 °C for 18–24 h in an anaerobic jar). Whitish-gray colonies that grew on the plaques were collected, and gram positive and catalase negative bacilli were chosen for tests. After pilot experiments, 30 Lactobacilli strains were selected. API 50 CHL kit (Bio Mérieux La Bali Grottes, France) was used for the identification of possible Lactobacilli isolates. Lactobacilli isolates which were identified were kept in bead tubes (Cryobank, Mast Diagnostics, France) at –20 °C.

E. coli ATCC 25922, *P. aeruginosa* ATCC 27853, *S. aureus* ATCC 29213, *E. faecalis* ATCC 29212, vancomycin-resistant *S. aureus* (MRSA) and vancomycin-resistant *Enterococcus* spp (VRE) bacteria, used as indicator strains in the study, along with *C. albicans* and *C. parapsilosis* yeasts were acquired from the culture collection of the Microbiology Laboratory of Dr. Behçet Uz Pediatric Hospital and stored in bead tubes (Cryobank, Mast Diagnostics, France) at –20 °C.

Gastric juice tolerance

The medium was simulated by the use of pepzan tablet (Dr. F. Frik İlaç San. ve Tic. A.Ş.), which is employed in the treatment of digestive disorders. For that purpose, 2000 units pepsin +2000 mg glutamic acid hydrochloride (pepzan tablet) were dissolved in 1000 ml sterile water and filtered through a 0.45 µm pore filter (Millipore, Molsheim, France). This 25 ml solution was transferred to sterilized MRS broth and 75 ml culture medium and 1 N HCl (Merck) were used so as to adjust the pH to 2.0, 2.5 and 3.0 and pepsin to 0.5 U/ml pepsin (Park et al. 2002). *Lactobacillus* cultures are activated in anaerobic mediums at 37 °C during 18 h.

Using the activated lactobacilli cultures, 1 % (w/v) (≈log 8.50 cfu/ml) inoculations were made to culture media with low pH and pepsin and left to wait at 37 °C for 4 h. Then, all cultures were planted into MRS agar plaques and incubated at 37 °C for 24 h. The results were evaluated on the basis of the growth in the plaques.

Bile salt tolerance

Bile salts (Sigma) were added to MRS broth culture media (Becton, Dickinson and Company, USA) at rates of 0.25 %, 0.50 %, 0.75 % and 1.0 % and sterilized. From the cultures of active lactobacilli, 1 % (w/v) (≈log 8.50 cfu/ml) inoculations were made to MRS broth culture media and were left to incubate at 37 °C for 24 h. Following that, each sample was planted into MRS agar and again incubated at 37 °C for 24 h (Marshall, 1997). Bile salt tolerance was calculated according to the number of colonies obtained in the plaques after incubation.

Bile salt hydrolysis

Bile salt hydrolysis activity was done with disc agar method (NCCLS, 1997). After adding 0.5 % cholic acid and 0.37 g/L $CaCl_2$ and sterilizing for 15 minutes, MRS agar medium was poured to plates, lactobacilli cultures were activated at 37 °C in anaeobic medium for 18 h. Planting was done in plates with MRS agar and left to incubation at 37 °C in anaeobic medium for 72 h. The results were analyzed by means of a microscope. It was determined as (–) negative, (+) low hydrolysis, (++) medium hydrolysis and (+++) high hydrolysis according to the sedimentation and bile salt disintegration around colonies (Dashkevicz and Feighner, 1989; Du Toit et al. 1998).

Antibiotic sensitivity

The minimum inhibitory concentrations (MICs) of 14 antibiotics were determined using the PMIC/ID70 kit (Marylan, USA) following the manufacturer's recommendations in the Becton Dickinson (BD) PHOENİX 100™ system.

Enzymatic activity

For enzymatic activity, API-ZYM system (bioMérieux, Montalieu-Vercieu, France) was used with the manufacturer's recommendations. Each well was deposited with the inocula 65 µL of the McFarland standard 1 suspension. After 4 h of anaerobic incubation at 37 °C, enzymatic activity readings were recorded.

Antagonistic effect

Agar well diffusion method was used to determine the antagonistic effect of lactobacilli (Tona et al. 1991a). Lactobacilli cultures were incubated at 37 °C for 24 h in an anaerobic environment in the MRS broth culture medium. The cultures were centrifuged at 5.000 g for 15 min, after which the supernatant was drawn into an injector and passed through a filter (Millipore, Molsheim, France) with 0.45 µm pore diameter. Indicator microorganisms *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, *S. aureus* ATCC 29213, *E. faecalis* ATCC 29212, vancomycin-resistant *S. aureus* (VRSA) and vancomycin-resistant *Enterococcus* (VRE) bacteria were activated in sheep blood agar culture plate (Salubris, % 5 Sheep Blood Agar, Turkey). In order to test antagonistic activity, cultures with 0.5 McF of *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, *S. aureus* ATCC 29213 and VRSA were planted into Muller Hinton agar (Becton, Dickinson and Company, USA) and *E. faecalis* ATCC 29212 into VRE sheep blood agar plate (Salubris, % 5 Sheep Blood Agar, Turkey). *Candida* were activated in SDA (Becton, Dickinson and Company, USA) culture medium. For all culture plates, 100 µl non-neutralized and neutralized (pH 6.5) supernatant was added to 10 mm wells. The plaques were kept at room temperature for 2 h and left to incubate at 37 °C for 24 h. Diameters of zones that formed around wells were measured in mm (Harris et al. 1989).

It was observed that whether *L. curvatus* L26 strain, which displays inhibitive effect on neutralized supernatants of *S. aureus* (MRSA) tolerant to vancomycin and *S. aureus* ATCC 29213 produced bacteriocin or bacteriocin-like element or not. *L. curvatus* L26 was put separately in neutralized supernatant catalase 5 mg/ml (Sigma) and in proteinaz-K 10 mg/ml (Sigma) while being kept at 37 °C for 4 h. Previously prepared *S. aureus* ATCC and MRSA planted circles were opened and non neutralized supernatant to A circle, neutralized supernatant to B circle, supernatant with catalase to C circle, Proteinaz-K supernatant added 100 µl to D circle were put in plates with Muller Hinton Agar and left to incubation at 37 °C for 24 h. The formed zones were measured as mm.

Bile salt antagonistic activity

The effects of 0.25 %, 0.50 %, 0.75 % and 1.00 % bile salts (oxgall, cholic acid and taurocholic acid) against the indicator bacteria (*E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, *S. aureus* ATCC 29213 and VRSA) were tested using the diffusion method. The MRS broth of 0.25 % bile salts (oxgall, cholic acid and taurochilic acid) was prepared. Infusion was done at the rate of 1 % (w/v(≈log 8.50 cfu/ml) from the active cultures of *L. curvatus* L2, *L. curvatus* L26, *L. paracasei* ssp. *paracasei* L27 and *L. rhamnosus* L28 strains which are resistant to bile salt and gastric acid, and they were subject to incubation at 37 °C for 24 h in anaerobic medium. As in the antagonistic effect, bile salt was left to antagonistic activities through the agar diffusion method (Tona et al. 1991a).

Cholesterol assimilation

Cholesterol assimilation was studied by a modified Searchy and Bergquist method (1960). MRS broth and MRS broth containing 0.25 % oxgall, cholic acid and taurochilic acid were prepared. Human plasma serum cholesterol, including high cholesterol at the rate of 15–20 % (500–600 mg/dl), was added to these broths. Infusion was done at the rate of 1 % (w/v(≈log 8.50 cfu/ml) from the active cultures of *L. curvatus* L2, *L. curvatus* L26, *L. paracasei* ssp. *paracasei* L27 and *L. rhamnosus* L28 strains which are resistant to bile salt and stomach acid and they were subject to incubation at 37 °C for 24 h in anaerobic medium. Tubes were centrifuged with 5000 g for 10 min. It was detected by taking 2 ml from the liquid part that rose to the top using the enzymatic method, SYNCHRON® Systems (Beckman Caulter, USA) kit and Unicel DxC800 model auroanalyzer (Beckman Caulter, USA) gadget. The results were changed from mg/dl to µg/ml (Belviso et al. 2009).

Statistical Analysis

Results of three experiments with duplicate or triplicate determinations were expressed as the mean and standard error mean. Statistical analysis was performed on the data by SPSS 19.0 Bivariate Correlation Analysis (SPSS Inc., Chicago, Ill., U.S.A.) with statistical significance determined at p

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< 0.01/0.05. The Pearson rank order correlation test was used for comparisons between EPS production and aggregation ($p < 0.01$), and cholesterol removal ($p < 0.05$). Concentration-viable counts (log cfu/mL) relationships were designated from the correlation and regression coefficients for the tolerance to pH and bile salts (Tulumoglu et al. 2013).

Results

Microorganisms

30 lactobacilli strains isolated from 100 stool samples were used in this study. The lactobacilli bacteria isolated from children stool were identified as *L. curvatus, L. rhamnosus,* and *L. paracasei* ssp paracasei.

Gastric juice tolerance

Results pertaining to the tolerance of lactobacilli to incubation in MRS culture medium with pH values of 2.0, 2.5 and 3.0 as well as 0.5 U/ml pepsin at 37 °C for 4 h are presented in Table 1. In the study, *L. curvatus* L25, *L. curvatus* L26 and *L. curvatus* L29 isolates were found tolerant of all (gastric juice) media with low pH and pepsin.

Bile salt tolerance

Table 2 shows the survivability of *Lactobacillus* species in MRS broth containing 0.25 %, 0.50 %, 0.75 % and 1.0 % bile salt. Bacteria in general were viable in 0.25 % bile salt, while fewer bacteria survived in 0.75 % bile salt. At 1.0 % bile salts, only two isolates survived. *L. curvatus* L8 isolates survived at a rate of log 5.56 cfu/ml in 0.25 % bile salt, log 4.10 cfu/ml in 0.50 % bile salt, log 4.10 cfu/ml in 0.75 % bile salt and log 3.05 cfu/ml in 1.00 % bile salt. *L. paracasei* ssp. *paracasei* L13 isolates were found to survive at a rate of log 7.08 cfu/ml in 0.25 % bile salt, log 6.31 cfu/ml in 0.50 % bile salt, log 3.95 cfu/ml in 0.75 % bile salt and log 3.90 cfu/ml in 1.00 % bile salt.

TABLE 1: *Viability rates of lactobacilli strains in media containing 0.5 U/ml pepsin and having different pH values.*

Isolates	Initial number of microorganisms Log (cfu/ml)	Different pH Values pH 2.0+0.5U/ml pepsin Number of microorganisms after 4 h incubation Log (cfu/ml)	pH 2.5+0.5U/ml pepsin Number of microorganisms after 4 h incubation Log (cfu/ml)	pH 3.0+0.5 U/ml pepsin Number of microorganisms after 4 h incubation Log (cfu/ml)
L. curvatus L1	8.62±0.03*)	4.23±0.02	6.06±0.08	8.43±0.09
L. curvatus L2	8.67±0.03	8.14±0.01	8.37±0.17	8.16±0.19
L. curvatus L3	8.57±0.10	8.46±0.04	8.32±0.31	8.45±0.15
L. paracasei ssp. *paracasei* L4	8.45±0.02	5.50±0.00	6.41±0.26	7.70±0.28
L. curvatus L5	8.66±0.01	5.27±0.03	5.35±0.20	7.38±0.31
L. rhamnosus L6	8.48±0.02	8.22±0.02	8.16±0.22	8.47±0.09
L. rhamnosus L7	8.56±0.12	5.65±0.07	7.34±0.31	7.72±0.10
L. curvatus L8	8.57±0.14	0	0	3.13±0.16
L. rhamnosus L9	8.57±0.10	7.31±0.01	7.25±0.35	8.72±1.15
L. rhamnosus L10	8.63±0.04	6.17±0.04	6.30±0.28	7.34±0.07
L. curvatus L11	8.48±0.02	7.32±0.03	7.26±0.04	8.27±0.38
L. paracasei ssp. *paracasei* L12	8.52±0.02	4.06±0.08	5.45±0.30	6.43±0.18
L. paracasei ssp. *paracasei* L13	8.52±0.03	8.10±0.14	8.37±0.45	8.23±0.16
L. rhamnosus L14	8.62±0.03	8.37±0.04	8.37±0.07	8.30±0.25
L. rahmnosus L15	8.69±0.00	7.78±0.02	8.03±0.02	8.40±0.14
L. paracasei ssp. *paracasei* L16	8.48±0.02	4.45±0.07	4.98±0.02	5.51±0.26
L. paracasei ssp. *paracasei* L17	8.56±0.12	3.26±0.04	4.31±0.16	6.52±0.25
L. paracasei ssp. *paracasei* L18	8.18±0.02	3.29±0.05	4.15±0.14	5.62±0.24
L. curvatus L19	8.63±0.04	–	–	4.37±0.18
L. curtavus L20	8.70±0.07	4.25±0.07	5.41±0.12	6.13±0.16
L. curvatus L21	8.42±0.04	3.51±0.26	4.32±0.15	5.48±0.12
L. rhamnosus L22	8.59±0.07	3.19±0.01	4.43±0.12	5.56±0.19
L. rhamnosus L23	8.62±0.03	4.23±0.02	6.06±0.08	8.43±0.09
L. curvatus L24	8.67±0.03	8.14±0.01	8.37±0.17	8.16±0.19
L. curvatus L25	8.57±0.10	8.46±0.04	8.32±0.31	8.45±0.15
L. curvatus L26	8.45±0.02	5.50±0.00	6.41±0.26	7.70±0.28
L. paracasei ssp. *paracasei* L27	8.66±0.01	5.27±0.03	5.35±0.20	7.38±0.31
L. rhamnosus L28	8.48±0.02	8.22±0.02	8.16±0.22	8.47±0.09
L. curvatus L29	8.56±0.12	5.65±0.07	7.34±0.31	7.72±0.10
L. rhamnosus L30	8.57±0.14	–	–	3.13±0.16

*): Arithmetic mean and Standard deviation

Antibiotic susceptibility

One of the required properties for probiotic strains is their safety for human consumption without harboring acquired and transferable antibiotic resistance. In this study, all lactobacilli strains were sensitive to 12 of 14 used antibiotics. However, all strains were resistant to vancomycin where only L3, L4, L11, L18, L23, L25 and L29 were susceptible to clindamycin (Table 3) according to the microbiological breakpoints defined by the Scientific Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) (Leuschner et al. 2010).

Enzymatic activities

All *Lactobacillus* strains showed (Table 4) esterase (C4), esterase-lipase (C8), leucine arylamidase, acid phosphatase, ß-galactosidase activities at high level. Additionally, weak to moderate level activities of valin arylamidase, cysteine arylamidase, naphthol-AS-BI-phosphohydrolase, α-galactosidase were recorded for several strains. None of the strains showed any level of α-chymotrypsin, ß-glucuronidase, ß-glucosidase, N-acetyl-ß-glucosaminidase activities which have been associated with intestinal diseases (Heavey and Rowland. 2004). In contrast, high ß-galactosidase activity of cells would help in lactose digestion and ameliorate the disorders associated with lactose intolerance. These results indicated that the isolated *Lactobacillus* strains are safe for probiotic use.

TABLE 2: *Effect of different bile salt concentrations on growth of lactobacilli strains.*

Isolates	Initial number of microorganisms Log (cfu/ml)	Different bile salt concentrations 0.25 % Number of micro-organisms after 4 h incubation Log (cfu/ml)	0.50 % Number of micro-organisms after 4 h incubation Log (cfu/ml)	0,75 % Number of micro-organisms after 4 h incubation Log (cfu/ml)	1.00 % Number of micro-organisms after 4 h incubation Log (cfu/ml)
L. curvatus L1	8.62±0.03	–	–	–	–
L. curvatus L2	8.67±0.03	8.25±0.07	4.85±0.07	2.05±0.07	–
L. curvatus L3	8.57±0.10	–	–	–	–
L. paracasei ssp. *paracasei* L4	8.45±0.02	7.70±0.28	–	–	
L. curvatus L5	8.66±0.01	6.56±0.08	–		–
L. rhamnosus L6	8.48±0.02	7.61±0.26	–	–	–
L. rhamnosus L7	8.56±0.12	7.04±0.06	–	–	–
L. curvatus L8	8.57±0.14	5.56±0.19	4.10±0.14	4.10±0.14	3.05±0.14
L. rhamnosus L9	8.57±0.10	4.67±0.03	3.56±0.19	–	–
L. rhamnosus L10	8.63±0.04	6.41±0.41	2.35±0.21	–	–
L. curvatus L11	8.48±0.02	3.45±0.34	3.89±0.00		
L. paracasei ssp. *paracasei* L12	8.52±0.02	7.38±0.40	2.24±0.27	–	–
L. paracasei ssp. *paracasei* L13	8.52±0.03	7.08±0.12	6.31±0.26	3.95±0.07	3.90±0.16
L. rhamnosus L14	8.62±0.03	7.95±0.07	–	–	–
L. rahmnosus L15	8.69±0.00	7.88±0.02	–	–	–
L. paracasei ssp. *paracasei* L16	8.48±0.02	7.35±0.21			
L. paracasei ssp. *paracasei* L17	8.56±0.12	5.10±0.14	–	–	–
L. paracasei ssp. *paracasei* L18	8.18±0.02	6.10±0.13	–	–	–
L. curvatus L19	8.63±0.04	3.41±0.12	–	–	–
L. curtavus L20	8.70±0.07	6.40±0.42	3.85±0.07	2.31±0.26	–
L. curvatus L21	8.42±0.04	5.62±0.11	2.46±0.04	–	–
L. rhamnosus L22	8.59±0.07	5.21±0.27	2.31±0.26	–	–
L. rhamnosus L23	8.62±0.03	6.30±0.28	0		
L. curvatus L24	8.67±0.03	8.25±0.07	0	–	–
L. curvatus L25	8.57±0.10	8.37±0.38	4.85±0.07	–	–
L. curvatus L26	8.45±0.02	7.70±0.28	2.30±0.27	2.05±0.07	–
L. paracasei ssp. *paracasei* L27	8.66±0.01	6.56±0.08	6.60±0.14	–	–
L. rhamnosus L28	8.48±0.02	7.61±0.26	–	–	
L. curvatus L29	8.56±0.12	7.04±0.06	–		–
L. rhamnosus L30	8.57±0.14	5.56±0.19		–	–

*): Arithmetic mean and Standard deviation

Antagonistic effect

Lactobacilli isolates were tested for their effect against indicator bacteria and yeasts, and the results are presented in Table 5. Non-neutralized supernatant and neutralized supernatant of the lactobacilli isolates were established to have an inhibitor effect on at least two of the bacteria from among *E. coli* ATCC 25922. *P. aeruginosa* ATCC 27853. *S. aureus* ATCC 29213. *E. faecalis* ATCC 29212 and VRE, all of which were used as indicator bacteria. However, none of the isolates was found to have an antagonistic activity against *C. albicans* and *C. parapsilosis.*

In Table 6, the effect of the bacteriocin or bacteriocin-like element of *L. curvatus* L26 strain on *S. aureus* ATCC 29213 is seen. It is in A: non-neutralized supernatant effect (20.50 mm), B: neutralized (pH 6.5±0.1) supernatant effect (18.25 mm), C: supernatant with catalase enzyme (17.10 mm) and D: supernatant effect including proteinaz-K (0 mm). The zone is the biggest in A because of common effect of lactic acid and bacteriocin (or bacteriocin-like). In B, the zone is smaller than A because it was neutralized supernatant and its acidity was removed. In spite of catalase addition, zone formation continues in C. This situation shows that inhibition effect does not arise from peroxide. No inhibition zone was formed in D. The reason is that it removed the inhibition effect by segmenting bacteriocin or bacteriocin-like polypeptide element in the protein medium because of proteinaz-K addition. As it is well known, some species of lactobacilli and their strains produce some bacteriocin or bacteriocin-like elements. In other words, it shows that *L. curvatus* L26 produces bacteriocin or bacteriocin-like elements.

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Bile salt hydrolysis activity

Lactobacillus strains did not hydrolyze oxgall, cholic and taurocholic acid bile salts yet.

Bile salt antagonistic activity

It was tested whether selected *L. curvatus* L2, *L. curvatus* L26, *L. paracasei* ssp. *paracasei* L27 and *L. rhamnosus* L28 bile salts' lactic acid bacteria increased antagonistic activity against indicator bacteria or not. As seen in Table 7, by selecting *L. curvatus* L2, *L. curvatus* L26, *L. paracasei* ssp. *paracasei* L27 and *L. rhamnosus* L28 strains, there is no occasion which increases the antagonistic effect on indicator bacteria with 0.25 % bile salts (oxgall, cholic acid, taurocholic acid) in MRS broth medium.

Cholesterol assimilation

In Table 8, the strains which can be potential probiotic (*L. curvatus* L2, *L. curvatus* L26, *L. paracasei* ssp. *paracasei* L27 and *L. rhamnosus* L28) and the cholesterol amount assimilated in bile salts and MRS broth medium by bacteria are seen. *Lactobacillus* strains did MRS broth 12.10-21.10 µg/ml, MRS broth 0.25 % oxgall 15.30–25.42 µg/ml, MRS broth 0.25 % cholic acid 13.25–20.70 µg/ml and MRS broth 0.25 % taurocholic acid 7.67–15.22 µg/ml cholesterol assimilation. In all feed-lots, the highest cholesterol assimilation was done by *L. curvatus* L26 strain with MRS broth 21.10 µg/ml, MRS broth 0.25 % oxgall 25.42 µg/ml, MRS broth 0.25 % cholic acid 20.70 µg /ml and MRS broth 0.25 % taurocholic acid 15,22 µg/ml cholesterol assimilation.

Discussion

30 lactobacilli, isolated from children stool, were identified as *L. curvatus, L. rhamnosus,* and *L. paracasei* ssp *paracasei.* Mikelsaar et al. (2002) isolated *L. acidophilus, L. delbrueckii, L. crispatus, L. salivarius, L. paracasei, L. plantarum, L. curvatus, L. brevis* and *L. fermentum, L. buchneri* and *L. coprophilus* from stool samples of Estonian and Sweedish children. Arici et al. (2004) isolated 21 lactobacilli from the stool of newborns. The study indicated that there were *L. rhamnosus, L. paracasei, L. fermentum, L. buchneri, L. brevis, L. curvatus* and that stool samples were rich in terms of lactobacilli.

The purpose here is to determine the extent to which probiotics are tolerant of low pH and enzymatic medium through the use of gastric juice formed by simulating the human stomach medium. One of the probiotic characteristics of bacteria is its strength to gastric juice. In gastric juice medium, a taken nutrient passes to guts in 3–4 h. Results pertaining to the tolerance of lactobacilli to incubation in MRS culture medium with pH values of 2.0, 2.5 and 3.0 as well as 0.5 U/ml pepsin at 37 °C for 4 h are presented in Table 1. In the study, *L. curvatus* L25, *L. curvatus* L26 and *L. curvatus* L29 isolates were found tolerant of all (gastric juice) media with low pH and pepsin. Dunne et al. (2004) reported in their study that *L. casei* 161, *L. acidophilus* 1748, *L. casei* F19, *L. fermentum* KDL, *L. paracasei* 2123, *L. acidophilus* 242, *L. salvarius* UCC 118 bacteria remained viable for 60 min at 2.5 pH, while *Bifidobacterium* sp. 35658 was unviable. In a study (Marshall, 1997). including 17 Kanjika isolates, it was noted that 11 grew at 37 °C in an anaerobic medium at low pH (2.0 and 2.5) for 4 h and that *L. plantarum* were tolerant of low pH at a rate of 75 to 80 %. Denkove et al. (2007) found that *L. helveticus* H,

TABLE 3: *Minimal inhibitory concentrations (MIC) (in µg mL^{-1}) of lactobacilli strains to antibiotics.*

Antibiotics	L1	L2	L3	L4	L5	L6	L7	L8	L9	L10	L11	L12	L13	L14	L15	L16	L17	L18	L19	L20	L21	L22	L23	L24	L25	L26	L27	L28	L29	L30
Ampicillin	≤2	≤2	≤2	≤2	≤2	≤2	≤2	≤2	≤2	≤2	≤2	≤2	1.5	≤2	≤2	≤2	≤2	≤2	≤2	≤2	≤2	≤3	≤2	≤2	≤2	≤2	≤2	≤2	1.5	0.5
Cefazolin	≤2	≤2	≤2	≤2	1	≤2	4	1	≤2	4	≤2	≤2	≤2	≤2	1	≤2	1	1.5	1	≤2	≤2	≤2	≤2	1	≤2	1	1	1	≤2	4
Clidamycin	>8	>8	4	4	>8	>8	>8	>8	>8	>8	4	>8	>8	>8	>8	>8	>8	4	>8	>8	>8	4	4	4	4	>8	>8	>8	4	>8
Diaptomycin	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25
Erythromycin	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5
Gentamicin	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25
Levofloxacin	>2	>2	>2	1	>2	>2	≤2	>2	>2	≤2	1	1	1	1	>2	>2	≤2	>2	>2	≤2	1	1	>2	>2	≤2	1	>2	>2	≤2	1
Linezolid	≤1	2	≤1	≤1	2	≤1	>2	≤1	>2	≤1	>2	≤1	2	≤1	≤1	2	≤1	≤1	≤1	≤1	2	≤1	≤1	2	≤1	≤1	≤1	2	2	≤1
Moxifloxacin	1	0.5	1.5	2	1	1	2	1	2	0.5	1	0.5	1.5	2	1	1	2	1	2	0.5	1	0.5	1.5	2	1	1	2	1	1	2
Oxacillin	0.5	2	0.5	0.5	2	1	0.5	0.5	2	2	0.5	2	0.5	0.5	2	1	0.5		0.5	2	0.5	0.5	2	1	0.5	0.5	2	1	2	1
Penicillin G	≤0.0625	≤0.125	≤0.0625	≤0.0625	≤0.125	≤0.0625	1	1	≤0.0625	≤0.125	≤0.0625	≤0.0625	≤0.125	≤0.0625	1	1	1	≤0.0625	≤0.125	≤0.0625	≤0.0625	≤0.125	≤0.0625	1	≤0.0625	≤0.125	≤0.0625	1	≤0.125	≤0.0625
Tetracycline	1	1	1	2	1	2	1	2	2	2	1	2	1	2	1	2	1	1	2	2	1	2	0.5	1	2	1	1	2	1	1
Trimethoprim-Sulfamethoxazole	≤1/19	≤1/19	≤1/19	≤1/19	≤1/19	≤1/19	≤1/19	≤1/19	≤1/19	≤1/19	≤1/19	≤1/19	≤1/19	≤1/19	≤1/19	≤1/19	≤1/19	≤1/19	≤1/19	≤1/19	≤1/19	≤1/19	≤1/19	≤1/19	≤1/19	≤1/19	≤1/19	≤1/19	≤1/19	≤1/19	≤1/19

TABLE 4: *Enzymatic activities of L. fermentum strains assayed by the API-ZYM system (BioMérieux).*

	Alkaline phosphatase	Esterase (C4)	Esterase Lipaz (C8)	Lipase (C14)	Leucine arylamidase	Valin aryl-amidase	Cystine arylamidase	Trypsin	α-chymo-trypsin	Acid phosphatase	Naphthol-AS-BI-phosphohidrolase	α-galac-tosidase	β-galac-tosidase	β-glucu-ronidase	α-gluco-sidase	β-gluco-sidase	N-acetyl-β-glucosamindase	α-manno-sidase	α-fuco-sidase
L. curvatus L1	1*)	5	4	1	5	4	1	0	0	4	0	4	5	0	0	0	0	0	0
L. curvatus L2	0	5	5	0	5	3	3	0	0	5	1	0	5	0	0	0	0	0	0
L. curvatus L3	1	5	4	1	5	4	1	0	0	4	0	4	5	0	0	0	0	0	0
L. paracasei ssp. *paracasei* L4	0	5	5	0	5	3	2	0	0	5	1	0	5	0	0	0	0	0	0
L. curvatus L5	1	5	4	0	5	5	3	0	0	5	1	0	5	0	5	3	0	0	1
L. rhamnosus L6	1	5	4	1	5	4	1	0	0	4	1	4	5	0	0	0	0	0	0
L. rhamnosus L7	0	5	5	0	5	3	2	0	0	3	0	5	5	0	0	0	0	0	0
L. curvatus L8	1	5	4	0	5	5	3	0	0	5	0	0	5	0	5	3	0	0	0
L. rhamnosus L9	2	5	4	0	5	5	3	0	1	5	0	1	4	0	1	5	0	0	5
L. rhamnosus L10	1	4	3	1	5	5	1	0	0	4	0	0	0	0	5	3	0	0	1
L. curvatus L11	2	5	2	0	4	4	1	0	0	5	0	0	5	0	4	3	0	0	0
L. paracasei ssp. *paracasei* L12	1	5	4	1	5	4	1	0	0	4	0	4	5	0	0	0	0	0	0
L. paracasei ssp. *paracasei* L13	0	4	3	0	5	5	3	0	0	1	0	2	5	0	2	5	0	0	0
L. rhamnosus L14	0	5	3	0	5	5	3	0	0	2	0	2	5	0	1	5	0	0	0
L. rahmnosus L15	2	5	2	0	4	4	1	0	0	5	0	0	5	0	4	3	0	0	0
L. paracasei ssp. *paracasei* L16	1	5	4	1	5	4	1	0	0	4	1	4	5	0	0	0	0	0	0
L. paracasei ssp. *paracasei* L17	0	5	5	0	5	3	2	0	0	5	1	0	5	0	0	0	0	0	0
L. paracasei ssp. *paracasei* L18	1	5	4	1	5	4	1	0	0	4	0	4	5	0	0	0	0	0	0
L. curvatus L19	2	5	4	0	5	5	3	0	1	5	1	1	4	0	1	0	0	0	0
L. curtavus L20	1	4	3	1	5	5	1	0	0	4	0	0	4	0	5	0	0	0	0
L. curvatus L21	2	3	2	0	4	4	1	0	0	5	0	0	5	0	4	0	0	0	0
L. rhamnosus L22	1	5	4	1	5	4	1	0	0	4	0	4	5	0	0	0	0	0	0
L. rhamnosus L23	0	4	3	0	5	5	3	0	0	1	0	2	5	0	2	0	0	0	0
L. curvatus L24	0	4	3	0	5	5	3	0	0	2	0	2	5	0	1	0	0	0	0
L. curvatus L25	1	5	4	1	5	4	1	0	0	4	0	4	5	0	0		0	0	0
L. curvatus L26	1	5	4	1	5	4	1	0	0	4	1	4	5	0	0		0	0	0
L. paracasei ssp. *paracasei* L27	1	5	4	1	5	4	1	0	0	4	1	4	5	0	0	0	0	0	0
L. rhamnosus L28	0	5	5	0	5	3	2	0	0	5	1	0	5	0	0	0	0	0	0
L. curvatus L29	1	5	4	1	5	4	1	0	0	4	0	4	5	0	0	0	0	0	0
L. rhamnosus L30	1	4	3	1	5	5	1	0	0	4	1	0	3	0	5	3	0	0	0

*): 0, 1 and 2 : no enyzimatic activity, 3, 4 and 5 : enyzimatic activity.

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TABLE 5: *Antagonistic effect of lactobacilli strains against indicator microorganisms..*

Isolates	Inhibition zones against indicator microorganisms (mm)*)							
	E. coli ATCC 25922	*P. aeruginosa* ATCC 27853	*S. aureus* ATCC 29213	*E. faecalis* ATCC 29212	Vancomycin-resistant *S. aureus* (MRSA)	Vancomycin-resistant *Enterococus* (VRE)	*C. albicans*	*C. parapsilosis*
L. curvatus L1	18±0.00	20±0.00	14±0.09	18±0.06	–	20±0.02	–	–
L. curvatus L2	16±0.32	20±0.18	12±0.12	14±0.10	–	18±0.30	–	–
L. curvatus L3	14±0.23	18±0.09	14±0.54	16±0.21	–	16±0.09	–	–
L. paracasei ssp. *paracasei* L4	18±0.40	20±0.03	16±0.00	16±0.32	–	16±0.21	–	–
L. curvatus L5	16±0.62	18±0.52	–	14±0.09	–	–	–	–
L. rhamnosus L6	16±0.33	18±0.03	14±0.32	16±0.10	–	18±0.07	–	–
L. rhamnosus L7	16±0.23	18±0.07	14±0.09	14±0.05	–	20±0.10	–	–
L. curvatus L8	16±0.30	20±0.10	14±0.21	–	–	18±0.00	–	–
L. rhamnosus L9	16±0.64	20±0.31	12±0.05	–	–	14±0.17	–	–
L. rhamnosus L10	14±0.90	20±0.29	16±0.01	16±0.03	–	16±0.38	–	–
L. curvatus L11	16±0.00	18±0.07	–	–	–	14±0.07	–	–
L. paracasei ssp. *paracasei* L12	14±0.33	16±0.10	–	16±0.00	–	18±0.03	–	–
L. paracasei ssp. *paracasei* L13	–	16±0.91	12±0.04	14±0.18	–	16±0.00	–	–
L. rhamnosus L14	–	20±0.12	–	16±0.29	–	14±0.27	–	–
L. rahmnosus L15	–	16±0.87	–	14±0.07	–	12±0.03	–	–
L. paracasei ssp. *paracasei* L16	–	16±0.07	–	–	–	12±0.32	–	–
L. paracasei ssp. *paracasei* L17	18±0.42	20±0.09	14±0.42	14±0.03	–	20±0.22	–	–
L. paracasei ssp. *paracasei* L18	18±0.53	20±0.33	14±0.09	20±0.45	–	18±004	–	–
L. curvatus L19	14±0.31	16±0.06	14±0.04	16±0.08	–	16±0.98	–	–
L. curtavus L20	16±0.09	14±0.04	14±0.12	16±0.01	–	12±0.05	–	–
L. curvatus L21	16±0.62	14±0.62	16±0.08	18±0.07	–	20±0.04	–	–
L. rhamnosus L22	–	20±0.45	14±0.09	18±0.65	–	16±0.08	–	–
L. rhamnosus L23	16±0.77	18±0.87	12±0.37	12±0.80	–	20±0.17	–	–
L. curvatus L24	14±0.39	18±0.03	14±0.06	14±0.00	–	18±0.43	–	–
L. curvatus L25	16±0.30	20±0.07	14±0.04	16±0.68	–	20±0.32	–	–
L. curvatus L26	18±0.35	16±0.32	20±0.00 **)18±0.12	14±0.01	20±0.12 **)18±0.02	14±0.39	–	–
L. paracasei ssp. *paracasei* L27	12±0.90	20±0.00	14±0.08	14±0.07	–	18±0.59	–	–
L. rhamnosus L28	16±0.08	16±0.24	12±0.31	14±0.04	–	16±0.57	–	–
L. curvatus L29	18±0.45	14±0.06	14±0.00	20±0.08	–	12±0.00	–	–
L. rhamnosus L30	16±0.52	14±0.11	–	16±0.14	–	20±0.02	–	–

*): <12 mm no effect, 12–14 mm : low effect, 14–16 mm: medium effect, 16–20 mm: high effect, **): Neutralized (pH 6.5) MRS supernatant inhibition effect, ***): Diameter of circle 8 mm.

L. acidophilus A, *L. casei* C and *L. plantarum* 221-4 bacteria survived after waiting for 4 h at 37 °C in an MRS culture medium at pH 2.0 and 0.7 U/ml pepsin. When results of this study are compared with literature data, it can be said that lactobacilli isolates are highly tolerant of the media with low pH and pepsin. Generally lactobacilli strain in low pH (pH 2.0–3.0) MRS broth medium containing pepsin continues growing. Mostly they are tolerant.

TABLE 6: *Bacteriocin or bacteriocin like effect of L. curvatus L26 strain on S. aureus ATCC 29213 bacteria (mm).*

L. curvatus L26	S. aureus ATCC 29213 (mm)*)
A: non-neutralized supernatant effect	20.50±0.70**)
B: neutralized (pH 6.5±0.1) supernatant effect	18.25±0.30
C: neutralized (pH:6.5±0.1) supernatant and catalase	17.10±0.10
D: neutralized (pH:6.5±0.1) supernatant and proteinase-K	–

N mm no effect. 12–14 mm : low effect. 14–16 mm: medium effect. 16–20 mm: high effect. **): Arithmetic mean and Standard deviation :Note: diameter of circle 8 mm.

One of the desired characteristics of probiotic bacteria is being tolerant to bile salts. Table 2 shows the survivability of *Lactobacillus* species in MRS broth containing 0.25 %, 0.50 %, 0.75 % and 1.0 % bile salt. Bacteria in general were viable in 0.25 % bile salt, while fewer bacteria survived in 0.75 % bile salt. At 1.0 % bile salts, only two isolates survived. *L. curvatus* L8 isolates survived at a rate of log 5.56 cfu/ml in 0.25 % bile salt, log 4.10 cfu/ml in 0.50 % bile salt, log 4.10 cfu/ml in 0.75 % bile salt and log 3.05 cfu/ml in 1.00 % bile salt. *L. paracasei* ssp. *paracasei* L13 isolates were found to survive at a rate of log 7.08 cfu/ml in 0.25 % bile salt, log 6.31 cfu/ml in 0.50 % bile salt, log 3.95 cfu/ml in 0.75 % bile salt and log 3.90 cfu/ml in

TABLE 7: *Antagonistic effects of potential probiotic bacteria on indicator microorganisms of 0.25 % bile salts (mm).*

Isolates	Inhibition zones against indicator microorganisms (mm)*)					
	E. coli ATCC 25922	*P. aeruginosa* ATCC 27853	*S. aureus* ATCC 29213	*E. faecalis* ATCC 29212	MRSA	VRE
***L. curvatus* L2**						
MRS broth	16±0.02	20±0.32	15±0.22	–	20±0.32	18±0.00
MRS broth + 0.25 % oxgall	12±0.32	–	–	–	–	–
MRS broth + 0.25 % cholic acid	–	12±0.02	–	–	–	–
MRS broth + 0.25 % taurochilic acid	16±0.71	18±0.21	–	14±0.00	–	16±0.08
***L. curvatus* L26**						
MRS broth	18±0.21	16±0.09	20±0.00	14±0.04	20±0.09	14±0.12
MRS broth + 0.25 % oxgall	–	14±0.00	–	–	–	–
MRS broth + 0.25 % cholic acid	–	–	–	–	15±0.01	–
MRS broth + 0.25 % taurochilic acid	18±0.01	16±0.09	12±0.03	14±0.02	18±0.04	14±0.23
***L. paracasei* ssp. *paracasei* L27**						
MRS broth	12±0.09	20±0.09	14±0.23	14±0.09	–	15±0.00
MRS broth + 0.25 % oxgall	–	–	–	–	–	–
MRS broth + 0.25 % cholic acid	–	–	14±0.07	12±0.56	–	14±0.09
MRS broth + 0.25 % taurochilic acid	12±0.04	18±0.05	14±0.00	14±0.29	–	18±0.54
***L. rhamnosus* L28**						
MRS broth	16±0.02	16±0.11	12±0.04	14±0.33	–	16±0.01
MRS broth + 0.25 % oxgall	–	–	–	–	–	–
MRS broth + 0.25 % cholic acid	–	–	–	–	–	–
MRS broth + 0.25 % taurochilic acid	16±0.01	16±0.00	–	14±0.00	14±0.16	16±0.10

MRSA:Vancomycin- resistant *S. aureus*, VRE:Vancomycin- resistant *Enterococus*, *): <12 mm no effect, 12–14 mm : low effect, 14–16 mm: medium effect, 16–20 mm: high effect, Note: diameter of circle 8 mm.

TABLE 8: *The cholesterol assimilation of potential probiotic strains in varied bile salts (µg/ml).*

Isolates	MRS broth + Cholesterol (µg/ml)	MRS broth + Cholesterol + 0.25 % Oxgall (µg/ml)	MRS broth + Cholesterol + 0.25 % Cholic Acid (µg/ml)	MRS broth + Cholesterol + 0.25 % Taurochilic (µg/ml)
L. curvatus L2	12.10±0.14*)	15.30±0.28	16.35±0.49	8.26±0.04
L. curvatus L26	21.10±0.14	25.42±0.03	20.70±0.42	15.22±0.31
L. paracasei ssp. *paracasei* L27	13.45±0.07	15.41±0.40	13.25±0.07	9.75±0.07
L. rhamnosus L28	18.45±0.07	21.10±0.16	13.40±0.28	7.67±0.18

*): Arithmetic mean and Standard deviation

1.00 % bile salt. Tsuda et al. (2007) demonstrated that *L. acidophilus* 140B2. *L. casei* 2082. *L. helveticus* 130. *L. homohiochii* L14-2. *L. paracasei* 931102 and *L. plantarum* 301102 bacteria survived (were tolerant) in 0.3 % oxgal concentration. In their research. Dunne et al. (1999) reported that *L. salivarus* tolerated 5.0 % bovine salt and *Bifidobacterium* sp. tolerated 1.5 % bovine salt. In another study, *L. helveticus* H, *L. acidophilus* A, *L. casei* C and *L. plantarum* 221-4 bacteria were shown to be viable at a high rate in 0.3 % and 1.0 % bile salts although there was a decrease in comparison to the bacteria numbers at the beginning (Denkove et al. 2007). Being highly tolerant to bile salts increases the living chance of lactobacilli strain. At the same time, it is one of the candidate characteristics to be potential probiotic.

Finding out the natural resistance of *Lactobacillus* to antibiotics shall be useful both in clinical terms and in terms of recognizing their probiotic characteristics. That is because the use of clinical antibiotic/probiotic combinations is regarded a critical measure in the prevention of diarrhea, preservation of the urogenital system and protection against pathogens.

One of the required properties for probiotic strains is their safety for human consumption without harboring acquired and transferable antibiotic resistance (Leuschner et al. 2010). In this study, all lactobacilli strains were sensitive to 12 of 14 used antibiotics. However, all strains were resistant to vancomycin where only L3, L4, L11, L18, L23, L25 and L29 were susceptible to clindamycin (Table 3) according to the microbiological breakpoints defined by the Scientific Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) (Leuschner et al. 2010)). These results showed that lactobacilli strains have similar antibiotic resistance patterns. The conclusion of Tulini et al. (2013), Yüksekdağ et al. (2004) and Delgado et al. (2007) for *Lactobacillus* strains that these strains have inherent resistance to glycopeptides, such as vancomycin, are true for this study. Furthermore, the sensitivity of lactobacilli strains to erythromycin, penicillin G, and tetracycline were similar to lactobacilli strains of infant feces, in studies conducted by these researchers.

All isolates are susceptible to erythromycin, sulbactam/cefoperazone, ampicillin and penicillin. Salminen et al. (2006) established that *L. gasseri* and *L. jensenii* isolated from 85 blood cultures were highly resistant to vancomycin. Similarly, *L. rhamnosus* and *L. paracasei* bacteria of human stool origin were shown to be resistant to vancomycin, colylic sulfate, gentamicin and oxolinic acid (Verdenelli et al. 2009). In Estonia, 10 lactobacilli isolated from the intestinal flora of children aged between 1 and 2 years were tested against ampicillin, cefuroxime, cefoxitin, gentamicin, ciprofloxacin, tetracycline, vancomycin, metronidazole and erythromycin, and only one was found to be resistant to ampicillin, gentamicin and erythromycin, and 73 % resistant to vancomycin (Mandar et al. 2001). It was established in the same study that lactobacilli species were susceptible to penicillin, ampicillin, sulbactam/cefoperazone and rifampin antibiotics. The 100 % resistance of bacteria to

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antibiotics shows that this bacteria has natural resistance. Unlike *Enterococcus* there is no resistance gene and plasmid of *Lactobacillus* strains against vancomycin and teicoplanin (glycopeptide). In this situation, it can be said that glycopeptide resistance cannot be translated to other bacteria (Tynkkynen et al. 1998; Klein et al. 2000). This point shows that *Lactobacillus* can be used securely as probiotic bacteria.

When considered in the context of use of clinical antibiotic/probiotic combinations to prevent diarrhea, to protect the urogenital system and to provide protection against pathogens, it can be argued that isolates obtained in the study have the necessary potential.

All *Lactobacillus* strains showed (Table 4) esterase (C4), esterase-lipase (C8), leucine arylamidase, acid phosphatase, ß-galactosidase activities at high level. Additionally, weak to moderate level activities of valin arylamidase, cysteine arylamidase, naphthol-AS-BI-phosphohydrolase, α-galactosidase were recorded for several strains. None of the strains showed any level of α-chymotrypsin, ß-glucuronidase, ß-glucosidase, N-acetyl-ß-glucosaminidase activities which have been associated with intestinal diseases (Heavey and Rowland. 2004). In contrast, high ß-galactosidase activity of cells would help in lactose digestion and ameliorate the disorders associated with lactose intolerance. These results indicated that the isolated *Lactobacillus* strains are safe for probiotic use.

All *Lactobacillus* strains showed esterase (C4), estarase-lipase (C8), leucine arylamidase, valin arylamidase, cid phosphatase and ß-galactosidase enyzimatic activities. Nevertheless, Alcaline phosphatase, Lipase (C14), Trypsin, α-chymotrypsin, Naphthol-AS-BI-phosphohdrolase ß-glucuronidase, α-glucosidase, ß-glucosidase, N-acetyl-ß-glucosamindase, α-mannosidase ve α-fucosidase did not show any enyzimatic activities. Moreover, it was observed that the activities of Cystine arylamidase and Cystine arylamidase were weak or absent and that the activity of α-galactosidase was seen in some strains while not in some others. In our study, α-glucosidase, ß-glucosidase and N-acetyl-ß-glucosamindase related with intestinal diseases did not show any enyzimatic activities. The microorganisms having these enzymes cannot be used as probiotics (Delgado et al. 2007). On the other hand, the hydrolisation of undigested prebiotics by means of ß-galactosidase enzyme in ß-glucuronidase and lactose intolerant people is regarded a major property while choosing probiotic organisms. In the study, the isolated *Lactobacillus* strains have both of the enyzmes mentioned. Our study shows similarity with the enyzimatic activities of *Lactobacillus* strains isolated from human gastrointestinal origin (Delgoda et al. 2007) and lactic acid bacteria isolated from dog and primate stools (Strompfová and Lauková 2013).

One prominent probiotic feature of lactic acid bacteria is their antagonistic effect. This effect is attributable to organic acid, H_2O_2, diacetyl, CO_2 and bacteriocins. Thus, lactobacilli isolates were tested for their effect against indicator bacteria and yeasts, and the results are presented in Table 5. Non-neutralized supernatant and neutralized supernatant of the lactobacilli isolates were established to have an inhibitor effect on at least two of the bacteria among *E. coli* ATCC 25922. *P. aeruginosa* ATCC 27853. *S. aureus* ATCC 29213. *E. faecalis* ATCC 29212 and VRE. all of which were used as indicator bacteria. However, none of the isolates was found to have an antagonistic activity against *C. albicans* and *C. parapsilosis.* Verdenelli et al. (2009) established in their study that *L. rhamnosus* and *L. paracasei* which have probiotic characteristics inhibited the development of *E. coli* ATCC 11775, *S. aureus* ATCC 25923, *C. albicans* ATCC 10291 and *C. perfringens.* It was found in another study that *L. casei* subsp. *rhamnosus* LCR35 strain possessed an inhibitor effect on pathogenic bacteria like *E. coli* (ETEC), *E. coli* (EPEC), *Klebsiella pneumonia, Shigella flexneri, Salmonella typhimurium. Enterobacter cloacae, P. aeruginosa, E. faecalis* and *Clostridium difficile* (Forestier et al. 2001). Still in another study, lactobacilli isolated from the stool of newborns were shown to exert varying degrees of antagonistic effect against *B. cereus* FMC19, *E. coli* ATCC 25922, *S. aureus* ATCC 2392, *S. aureus* ATCC 2813 and *Y. enterocolitica* ATCC 1501 (Arici et al., 2004). These studies are parallel to ours.

L. curvatus L26 strain displays inhibition effect against neutralized supernatant *S. aureus* ATCC 29213 and VRSA bacteria. In order to understand this effect, a study was done with different enzymes.

In Table 6, the effect of bacteriocin or bacteriocin-like element of *L. curvatus* L26 strain on *S. aureus* ATCC 29213 is seen. It is in A: non-neutralized supernatant effect (20.50 mm), B: neutralized (pH 6.5±0.1) supernatant effect (18.25 mm), C: supernatant with catalase enzyme (17.10 mm) and D: supernatant effect including proteinase-K (0 mm). The zone is the biggest in A due to the common effect of lactic acid and bacteriocin (or bacteriocin like). In B, the zone is smaller than A because it was neutralized supernatant and its acidity was removed. In spite of catalase addition, zone formation continues in C. This situation shows that inhibition effect does not arise from peroxide. No inhibition zone formed in D. The reason is that it removed the inhibition effect by segmenting bacteriocin or bacteriocin-like polypeptide element in the protein medium because of proteinaz-K addition. As it is well known, some species of lactobacilli and their strains produce some bacteriocin or bacteriocin-like elements. In other words, *L. curvatus* L26 produces bacteriocin or bacteriocin-like elements. In different studies, the information reveals that there are antagonistic (bacteriocin) effects against *L. curvatus* bacteria (Mataragas et al. 2002; Chung and Yousef, 2005). Messens et al. (2003) stated that *L. curvatus* LTH 1174 strain produces bacteriocin (curvasin A) elements against *Listeria innocua* LMG 13568. In another study, Kawahara et al. (2010) noted that *L. curvatus* Y108 strain shows antibicrobial activity against *L. curvatus* JCM1090, *L. monocytogenes* JCM7671, *S. aureus* ssp. *aureus* JCM20624 and *S. marcescens* JCM20612. They determined that this activity depends on bacteriocin. It was pointed that *L. curvatus* L26 strains produce active bacteriocin against *S. aureus.* Probiotic bacteria producing bacteriocin increase antagonistic effect and helps protecting food, animals and people against pathogens.

It was tested whether selected *L. curvatus* L2, *L. curvatus* L26, *L. paracasei* ssp. *paracasei* L27 and *L. rhamnosus* L28 bile salts' lactic acid bacteria increase antagonistic activity against indicator bacteria or not. As seen in Table 7, by selecting *L. curvatus* L2, *L. curvatus* L26, *L. paracasei* ssp. *paracasei* L27 and *L. rhamnosus* L28 strains, there is no occasion which increases the antagonistic effect on indicator bacteria with 0.25 % bile salts (oxgall, cholic acid, taurocholic acid) in MRS broth medium. In different

studies, although it was stated that bile salts show different antagonistic effects on bacteria (Lewis and Gorbach, 1972; Stewart et al., 1986; Klayraung and Okonogi, 2009), our research showed that 0.25 % bile salt (oxgall, cholic acid and taurocholic acid) has little or no antagonistic effect against indicator bacteria. This case indicates that there should be more studies on antagonistic effects of bile salts.

In Table 8, the strains which can be potential probiotic (*L. curvatus* L2, *L. curvatus* L26, *L. paracasei* ssp. *paracasei* L27 and *L. rhamnosus* L28) and the cholesterol amount assimilated in bile salts and MRS broth medium by bacteria are seen. *Lactobacillus* strains did MRS broth 12.10–21.10 µg/ml, MRS broth 0.25 % oxgall 15.30–25.42 µg/ml, MRS broth 0.25 % cholic acid 13.25–20.70 µg/ml and MRS broth 0.25 % taurocholic acid 7.67–15.22 µg/ml cholesterol assimilation. In all feed-lots, the highest cholesterol assimilation was done by *L. curvatus* L26 strain with MRS broth 21.10 µg/ml, MRS broth 0.25 % oxgall 25.42 µg/ml, MRS broth 0.25 % cholic acid 20.70 µg /ml and MRS broth 0.25 % taurocholic acid 15,22 µg/ml cholesterol assimilation. Our study shows similarity with the studies of Loing and Shah (2005). In this research, they noted that *Lactobacillus* assimilates MRS broth 10.00–21.61 µg/ml and 0.30 % oxgall MRS broth 12.03–32.25 µg/ml cholesterol. Gilliland et al. (1985) stated that *L. acidophilus* strains carry out 18.8–63.0 µg/ml plasma serum assimilation in MRS broth and % 0.5 oxgall medium. In a study, Lys et al. (1994) pointed out that *L. acidophilus* strains did 20.5–83.3 µg/ml cholesterol assimilation, *L. casei* strains did 16.9–74.3 µg/ml cholesterol assimilation in MRS broth feed-lot (Brashears et al. 1998). Loing and Shah (2005) stated that *Lactobacillus* did MRS broth 10.00–21.61 µg/ml and 0.30 % oxgall MRS broth 12.03–32.25 µg/ml cholesterol assimilation. Lye et al. (2010) noted that *Lactobacillus* did MRS broth 14.22–27.89 µg/ml and 0.30 % oxgall MRS broth 11.17–62.42 µg/ml cholesterol assimilation. It is observed that cholesterol assimilation of *Lactobacillus* strains is different in type and strain level. Using strain, which does high cholesterol assimilation probiotically, reduces the level of blood serum and helps to decrease cardiovascular illnesses.

Conclusion

It was detected that generally lactobacilli strains are resistant to high acidity (pH 2.0–3.0) and sensitive to high bile salt (1.00 % Oxgall); are resistant to vancomycin, teicoplanin and bacitracin; show antagonistic effects against pathogen bacteria; do not hydrolysis bile salts. It was stated that with bile salt addition, *L. curvatus* L2, *L. curvatus* L26, *L. paracasei* ssp. *paracasei* L27 and *L. rhamnosus* L28 strains did not show antagonistic activity increasing effect. It was noted that only *L. curvatus* L26 strain produces bacteriocin against *S. aureus* (MRSA) which is resistant to *S. aureus* ATCC 29213 and vancomycin, and assimilates high cholesterol (15.22–25.42 µg/ml) in different bile salt mediums. The fact that *L. curvatus* L26 has ß-glucuronidase digesting the prebiotics and ß-galactosidase digesting lactose is a significant probiotic property.

At the same time, *L. curvatus* L26 strain can participate in the control of *S. aureus* which is the leading in common infections. In the light of these evaluations, we are of the opinion that *L. curvatus* L26 can have potential probiotic characteristics.

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