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The effects of inulin and fructo-oligosaccharide on the probiotic properties of *Lactobacillus* spp. isolated from human milk

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Abstract: This study aims to determine the effects of inulin and fructo-oligosaccharide (FOS) on the probiotic properties of five *Lactobacillus* spp. isolated from human milk. *Lactobacillus* spp. were isolated and identified, and the growth characteristics, acid and bile salt tolerance, antagonistic effects, and cholesterol assimilation of *Lactobacillus* strains were investigated in the presence of inulin and FOS. *Lactobacillus casei* L1 was able to utilize inulin and FOS as carbon source as well as glucose even other strains were able to use, including *Lactobacillus rhamnosus* GG. This strain also showed high tolerance to acid and bile salt, even at pH 2.5 and 0.5% bile salt levels, respectively. Inulin and FOS promoted the antimicrobial activity of *L. casei* L1 against pathogenic bacteria. Cholesterol assimilation was higher than in the other and control probiotic strains in the presence inulin and FOS, which were measured as 14 and 25 mg/dL, respectively. In conclusion, *L. casei* L1 can use both inulin and FOS to maintain its viability both at digestive conditions and also the relevant prebiotics, and show broad antagonistic activity and cholesterol assimilation.

Keywords: fructo-oligosaccharides (FOS); human milk; inulin; *Lactobacillus casei*; *Lactobacillus* spp.

1 Introduction

Consumer demand has shown a tendency toward convenience foods as a result of the modern lifestyle, with the main expectations of health and protective benefits. Fermented foods, including probiotic microorganisms, offer tremendous health benefits. Thus, sustaining the viability and the stability of probiotics in foods or the host is one of the targets of keeping them functional. Prebiotics are oligosaccharides that stimulate the development of probiotics, such as *Lactobacillus*, *Bifidobacterium*, and *Pediococcus*, which cannot be digested by either human or animal intestinal systems [1]. There are two types of prebiotics, i.e. prebiotics that occur naturally in plants such as bananas, asparagus, beans, and cereals, and prebiotics that are synthesized from the enzymatic digestion of polysaccharides, such as starch. Galacto-oligosaccharide, fructo-oligosaccharide (FOS), inulin, and soya-oligosaccharide are all natural prebiotics, whereas lacto-sucrose, lactulose, isomalto-oligosaccharide, gluco-oligosaccharide and xylo-oligosaccharide are synthesized examples [2].

Prebiotics play a role in increasing the stability of probiotics and also in contributing to the health of the host. A study reported that *Bifidobacterium* and *Lactobacillus* are increased by 31–59% in the digestive system of infants who are nourished with FOS and galacto-oligosaccharide supplements during their first 6 months [3]. In vivo studies have shown that the administration of probiotics and/or prebiotics can be effective in improving the lipid profiles of blood by reducing the total serum/plasma cholesterol, LDL-cholesterol, and triglycerides, or increasing HDL-cholesterol [4]. Oligosaccharides have been reported to increase immunity, thereby protecting infants against various diseases [5]. When FOS and galacto-oligosaccharide are fermented by *Lactobacillus crispotus*, *Lactobacillus jensenii*, and *Lactobacillus vaginalis*, the pathogens *Candida albicans*, *Escherichia coli*, and *Gardnerella vaginalis* are prevented in the intestinal system [6]. Similarly, Ignatova et al. [7] found that FOS and galacto-oligosaccharides inhibited *Escherichia coli* during the prebiotic fermentation of strains of *Lactobacillus delbrueckii* subsp. *Bulgaricus* B5 and B8.

In recent years, the biotherapeutic uses of probiotics on human metabolic diseases have increased substantially. There have been many studies that showed that some members of the LAB are capable of lowering the cholesterol level in the host, thereby preventing hypocholesterolemia and lowering the risk of heart attacks

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[4]. It has been shown further that cholesterol can be assimilated mostly during bacterial growth, being bound without transformations on the cellular surface and incorporated within the phospholipid bilayer membrane [8]. In addition, it has been demonstrated that the uptake may be accompanied by co-precipitation with bile salts and/or transformation to coprostanol, similarly resulting in cholesterol removal [9]. However, to our knowledge, the effects of prebiotics on the lowering of cholesterol by probiotic strains have not been previously reported. This study aims to investigate the effects of prebiotics on the probiotic properties of *Lactobacillus* spp. isolated from the human milk. In this respect, the cholesterol-lowering levels of *Lactobacillus* spp. were determined particularly when prebiotics were fermented. In this study, high inulin- and FOS-fermenting *Lactobacillus casei* L1 was identified that showed outstanding probiotic and as well as cholesterol lowering effects with both inulin and FOS.

2 Experimental

2.1 Human milk and indicator bacterial strains

Human milk samples (n = 5) were collected from healthy first-time mothers who were in the early lactation period (within 80 days of delivery) and who visited the Dr. Behçet Uz Child Diseases and Surgery Training and Research Hospital, İzmir, Turkey. The milk samples were collected in sterile test tubes with minimal skin contact.

Escherichia coli (ATCC 259225), *Staphylococcus aureus* (ATCC 29213), methicillin-resistant *S. aureus* (MRSA) 316, *Pseudomonas aeruginosa* (ATCC 278853), *P. aeruginosa* 344, and vancomycin-resistant *Enterococcus faecium* (VRE 461) were obtained from the Dr. Behçet Uz Child Diseases and Surgery Training and Research Hospital, Clinical Microbiology Laboratory, Culture Collection. All LAB isolates and indicator strains were stored at –20 °C with the addition of 25% glycerol (Merck, Germany).

2.2 Isolation and identification of *Lactobacillus* spp.

The human milk samples (1 mL) were transferred to 9 mL of sterile saline (0.85% sodium chloride) and serially diluted. Appropriate dilutions of the samples were plated on a *Lactobacillus* de Man, Ragosa Sharpe (MRS) agar, with a total of 20 isolates collected and analyzed for colony morphology, catalase reaction, Gram staining, and carbohydrate fermentation (glucose, fructose, lactose, mannose, maltose, arabinose, sucrose, galactose, mannitol, sorbitol, and xylose).

The ultimate identification of the LAB isolates was carried out through 16S rRNA gene sequencing, and two primer sets (529F 5′-GTGCCAGCMGCCGCGG-3′, 1491R 5′ ACGGCTACCTTGTTACGACTT-3′ and 27F 5′-AGAGTTTGATCCTGGCTCAG-3′ – 780R 5′-TACCAGGGTATCTAATCCTGTT-3′) were used for the amplification of 1464 bp of the 16S rRNA gene. Polymerase chain reaction (PCR) was carried out according to the following program: 95 °C for 3 min, 30 cycles of 95 °C for 30 s, 57 °C for 30 s, 72 °C for 1 min, and 72 °C for 5 min final extension. The PCR products were run on a gel to check the amplification, and the amplicons were sent to Macrogen for sequencing. The obtained sequences were interrogated with the NCBI database using the BLAST algorithm, with a similarity criterion of 97–100%.

2.3 Determination of inulin and FOS fermentation capability of LABs

A modified MRS agar including 2% inulin and FOS was used to determine the fermenting capability of the LAB strains. Thus, an MRS agar was supplemented with 0.05% L-cysteine and 30 mg bromocresol purple after sterilization. Filter (0.22 µm)-sterilized inulin and FOS were added to the modified MRS agar. Each 10^3 CFU/mL *Lactobacillus* spp. was spread on the agar plates and incubated under anaerobic conditions at 37 °C for 24 h. The yellow color around the colonies was identified as inulin- and FOS-fermenting LAB strains. MRS agar with glucose was used as control [10].

2.4 Determination of the fermentation characteristics of *L. casei* L1

Lactobacillus casei L1 was cultivated at 37 °C for 24 h in a 1-L flask including MRS prepared with 2% inulin and FOS, with or without glucose, respectively. Each flask was inoculated with 10^7 CFU/mL, and the density of cells was measured with DENSIMAT (Biomérieux, France) at 0, 4, 8, 12, 16, 20, and 24 h.

2.5 Determination of the acid and bile salt tolerance of LABs

To determine the acid and bile salt tolerance of the LAB strains in the presence of inulin and FOS, cells were harvested after overnight cultivation, and 10^{10} CFU/mL cells were subjected to the following stress conditions:

The harvested cells were resuspended in MRS, including 1% inulin and FOS, with the pH adjusted to 2.0, 2.5, and 3.0 with 1 N HCI (Merck, Germany). Afterward, the LAB strains were incubated for 1 h and spread on the MRS agar for the enumeration of viable cell numbers [12]. Similarly, to determine the bile salt tolerance, 10^{10} CFU/mL cells were resuspended in inulin and FOS including MRS prepared with bile salt (Oxgall, Sigma) at the ratios 0.3, 0.5, and 1.0%. After 1 h of incubation, the viable cell numbers were counted on the MRS agar [12].

2.6 Determination of the antagonistic effects of LABs

The antagonistic effects of the LAB strains against *E. coli* (ATCC 259225), *S. aureus* (ATCC 29213), MRSA 316, *P. aeruginosa* (ATCC 278853), *P. aeruginosa* 344, and VRE 461 were tested with an agar diffusion method [13]. The LAB strains were cultivated in MRS, including 2% inulin and FOS rather than glucose at 37 °C for 24 h, and the cell debris was removed by centrifugation. Finally, the supernatant was filtered and placed in wells opened on agar plates prepared after each indicator bacterium was inoculated with the Brain Heart Infusion agar (0.7%) and poured on the Mueller Hinton Agar (Merck, Germany) where the wells were formed in 5 mm diameter. All plates were then incubated at 37 °C for 24 h and screened for the presence of antimicrobial zones.

2.7 Determination of the cholesterol assimilation of LAB

To determine the cholesterol assimilation of LAB in the presence of inulin and FOS, human-derived cholesterol was used in MRS of approximately 300–400 mg/dL, together with inulin or FOS at 2% and bile salts at 0.3%. The overnight-cultivated *Lactobacillus* spp. strains were inoculated at 1% to the prepared modified MRS medium and incubated for 24 h at 37 °C. Afterwards, the cells were removed by centrifugation at 5000 *g* for 10 min, and the cholesterol content of the supernatant was determined using an enzymatic [SYNCHRON® Systems (Beckman Coulter, USA)] kit and a Unicell DxC800 model auto-analyzer (Beckman Coulter, USA).

2.8 Statistical analysis

Statistical analysis was carried using the SPSS software: version 15.0. A one-way analysis of variance (ANOVA) was applied, followed by a Tukey's range test for the analysis of the inulin and FOS fermentation of lactic acid bacteria, acid and bile salt tolerance, antagonistic effect, and cholesterol assimilation, with the level of significance set at $p < 0.05$.

3 Results

3.1 Inulin and FOS utilization

Figure 1 shows the growth characteristics of the *Lactobacillus* spp. strains in MRS, with and without glucose, inulin, or FOS. *L. casei* L1 was able to grow in the presence of 2% inulin and FOS in the MRS medium, as well as in MRS with glucose. However, the amount of *Lactobacillus* spp. strains was less than 5 log CFU/mL. When the growth level of *L. casei* L1 was compared in the presence of inulin and FOS, it was found that the lower molecular weight FOS promoted cell growth more than inulin. On the other hand, *L. casei* L1 started to form rapidly in both inulin and FOS, as well as in glucose, in the initial fermentation, and no significant differences were identified in the growth characteristics of *L. casei* L1 between the MRS media, including glucose or inulin and FOS. In all carbon sources, the growth of *L. casei* L1 reached the highest amount of >10 log CFU/mL (Figure 2), which is a clear indication that the *L. casei* L1 strain was very adept at utilizing both inulin and FOS as carbon source.

3.2 Acid and bile salt tolerance

Gastric acid tolerance (pH 2–3) is one of the barriers that probiotic bacteria must overcome to survive.

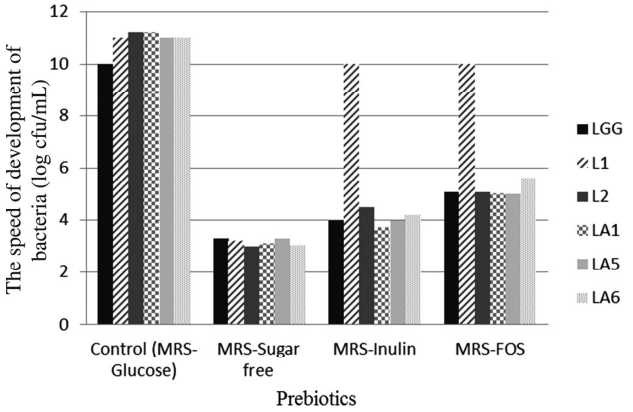

Figure 1: The growth of the *Lactobacillus* spp. in MRS without carbon source or with glucose, inulin and FOS under 37 °C for 24 h anaerobically.

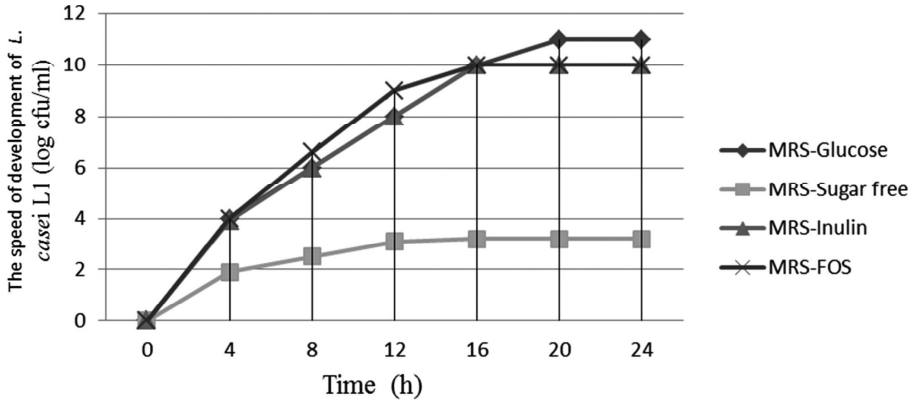

Figure 2: The growth of the *L. casei* L1 in MRS without carbon source or with glucose, inulin and FOS under 37 °C for 24 h anaerobically.

All *Lactobacillus* spp., apart from *L. casei* L1, survived between 3.07 and 4.29 log CFU/mL in pH 3. However, under the same condition, *L. casei* L1 showed a high level of survival (9.68 log CFU/mL) when inulin was used as carbon source (Table 1). When the pH of MRS was reduced to 2.5, only *L. casei* L1 and *Lactobacillus rhamnosus* GG were able to retain their viability, although the viable amount of *L. casei* was higher than that of the common probiotic strain *L. rhamnosus* GG, which means that this strain showed strong probiotic behavior in the presence of inulin. At pH 2.0, no cell growth was seen in the *Lactobacillus* spp. strains, including the control strain *L. rhamnosus* GG.

All strains were resistant to 0.3% oxgall at an intermediate level, although, among the *Lactobacillus* strains, *L. casei* L1 was able to tolerate a high oxgall level. As shown in Table 2, the viable cell amount of *L. casei* L1 was 9.84 log CFU/mL, while that of *L. rhamnosus* GG was 3 log CFU/mL lower. When the oxgall concentration was increased to 0.5%, only *L. casei* L1 could remain viable at 4.04 log CFU/mL. These results show that *L. casei* L1 can tolerate a high bile salt level, indicating its ability to survive in the harsh gastrointestinal environment.

3.3 Antagonistic effects

Among the *Lactobacillus* spp. strains, including the control *L. rhamnosus* GG, only *L. casei* L1 showed

Table 1: Low pH tolerance of *Lactobacillus* spp. strains isolated and identified from human milk in MRS including 2% inulin.

LAB Strains	Initial bacterial counts (log cfu/mL)	pH 2.0	pH 2.5	pH 3.0
L. rhamnosus GG	10.00±0.10	–	2.01±0.13	4.29±0.23
L. casei L1	10.00±0.12	–	3.68±0.24	9.68±0.24
L. casei L2	10.00±0.24	–	–	4.07±0.32
L. plantarum LA1	10.00±0.24	–	–	3.07±0.32
L. pentosus LA5	10.00±0.35	–	–	3.30±0.20
L. plantarum LA6	10.00±0.40	–	–	3.87±0.32

Table 2: Bile salt tolerance of *Lactobacillus* spp. strains isolated and identified from human milk in MRS including 2% inulin.

LAB Strains	Initial bacterial counts (log cfu/mL)	Oxgall 0.3%	Oxgall 0.5%	Oxgall 1.0%
L. rhamnosus GG	10.00±0.10	6.34±0.12	–	–
L. casei L1	10.00±0.20	9.84±0.10	4.04±0.03	–
L. casei L2	10.00±0.30	4.04±0.03	–	–
L. plantarum LA1	10.00±0.24	5.84±0.03	–	–
L. pentosus LA5	10.00±0.35	4.76±0.02	–	–
L. plantarum LA6	10.00±0.40	5.80±0.03	–	–

Table 3: Antagonistic effect of cell-free extracts of *Lactobacillus* spp. strains cultivated in MRS including 2% inulin or FOS at 37 °C for 24 h.

Cell-free extract	*E. coli* ATCC59225	*S. aureus* ATCC29213	MRSA 316	*P. aeruginosa* ATCC 278853	*P. aeruginosa* 344	VRE 461
L. rhamnosus GG						
MRS-inulin	–	–	–	–	–	–
MRS-FOS	–	–	–	–	–	–
L. casei L1						
MRS-inulin	14	14	12	14	15	–
MRS-FOS	15	14	13	16	16	–
L. casei L2						
MRS-inulin	–	–	–	–	–	–
MRS-FOS	–	–	–	–	–	–
L. plantarum LA1						
MRS-inulin	–	–	–	–	–	–
MRS-FOS	–	–	–	–	–	–
L. pentosus LA5						
MRS-inulin	–	–	–	–	–	–
MRS-FOS	–	–	–	–	–	–
L. plantarum LA6						
MRS-inulin	–	–	–	–	–	–
MRS-FOS	–	–	–	–	–	–

MRSA, methicillin-resistant *S. aureus* 316; VRE, vancomycin-resistant *E. faecium* 461.

antagonistic activity at its neutralized supernatant when cultivated in MRS and when inulin and FOS were used as a carbon source, which indicated that this strain produces an antimicrobial metabolite, possibly a bacteriocin. *Lactobacillus casei* L1 showed more antimicrobial activity against pathogenic bacteria when cultivated with FOS than inulin, while antimicrobial activity levels were the same against all the used indicator strains, which showed no activity against vancomycin-resistant *Enterococcus* (Table 3).

3.4 Cholesterol assimilation

All *Lactobacillus* spp. strains reduced cholesterol amounts by between 25 and 59 mg/dL in MRS with glucose, with the highest and lowest cholesterol removal determined in the cultures of *Lactobacillus plantarum* LA1 and *L. casei* L2, respectively. The cholesterol assimilation levels of *Lactobacillus* spp. decreased significantly when cultivated in MRS, including inulin and FOS, or together with 0.3% bile salts (oxgall, colic acid, and teutonic acid). The highest cholesterol assimilation among the *Lactobacillus* spp. strains in MRS, including inulin and FOS, was observed with *L. casei* L1 (Table 4). This strain reduced the cholesterol content by twofold with inulin and threefold with FOS. Also, cholesterol was assimilated to a higher extent with FOS than inulin when used as a carbon source for *L. casei* L1. However, the control strain *L. rhamnosus* GG was found to be unsatisfactory in cholesterol assimilation with inulin or with FOS together with bile salts (Table 4), which indicated that the high inulin and FOS utilization ability along with the bile salt tolerance of *L. casei* L1 provides an opportunity to assimilate cholesterol from the medium.

4 Discussion

In this study, five *Lactobacillus* spp. strains isolated from human milk were tested for their inulin and FOS utilization ability in an MRS background and for their probiotic behaviors in the presence of inulin or FOS. Multidimensional approaches were used to identify a potential probiotic LAB strain.

The first interesting finding of this study was that *L. casei* L1 was the efficient in utilizing both inulin and FOS, which resulted in this strain being able to reach 10 log CFU/mL after 24 h of incubation. Previous studies had also identified LAB strains that could utilize inulin or FOS in a culture medium and reach higher viable cell counts. For instance, Manderson et al. [14] reported that the cell amount of *Bifidobacterium* strains was 8.63 log CFU/mL in MRS, including FOS after 24 h. In another study, *Lactobacillus acidophilus* NIT200 and *L. plantarum* NIT202 were shown to utilize FOS [15]. There have also been studies reporting that *L. casei* AP and *L. casei* AG strains could be grown in MRS containing 4% inulin [16]. To our knowledge, however, there have been no studies to date that identified a single strain that could utilize more than one prebiotic as a carbon source. The present study has shown

Table 4: Cholesterol assimilation (mg/dL) of *Lactobacillus* spp. in MRS including 2% glucose, inulin, and FOS with or without bile salts 0.3%.

Strains	Glucose	Inulin	Inulin+oxgall	Inulin+cholic acid	Inulin+teutonic acid	FOS	FOS+oxgall	FOS+cholic acid	FOS+teutonic acid
L. rhamnosus GG	25±0.5	1±0.1	1±0.2	0±0.0	2±0.2	9±0.8	10±0.0	0±0.0	5±0.4
L. casei L1	30±2.2	11±0.2	14±0.7	5±0.5	7±0.3	25±0.9	25±0.8	4±0.5	20±0.1
L. casei L2	24±1.3	6±0.4	5±0.4	1±0.0	2±0.1	6±0.4	6±0.9	1±0.0	4±0.5
L. plantarum LA1	59±5.0	5±0.6	5±0.5	0±0.0	1±0.0	9±5.5	8±2.5	2±1.2	12±3.2
L. pentosus LA5	37±5.5	6±0.7	5±0.8	0±0.0	1±0.4	6±5.5	12±2.5	5±1.5	10±2.4
L. plantarum LA6	41±5.0	5±0.2	5±0.5	0±0.0	1±0.2	10±4.6	7±1.0	8±1.7	12±2.0

that *L. casei* L1 isolated from human milk and identified with a whole 16S rDNA gene sequence is capable of utilizing prebiotics, which means that it may contain interesting genes related to prebiotic fermentation.

The high prebiotic fermentation ability of *L. casei* L1 provided acid and bile salt tolerance, as well as antagonistic activity against serious pathogens. These features will make this strain even more competitive against the harsh gastrointestinal environment. Probiotic strains are able to tolerate severe acidic and bile salt stresses through their special bile salt hydrolase activity or intracellular proton pumps [17]. However, *L. casei* L1 has been shown to be able to overcome the obstacles using inulin and FOS.

The neutralized supernatant (pH 6.5) of *L. casei* L1 produced with inulin and FOS showed antimicrobial activity against both gram-negative and gram-positive pathogens. To our knowledge, this is the first time that a LAB strain has been reported to produce antimicrobial metabolites using prebiotics. Although the nature of this antimicrobial metabolite should be investigated in more detail, this finding is significant for this strain, which shows its potential in eliminating pathogens in the digestive system in the case of their intake. In fact, the bacteriocins produced from the probiotics in particular are suggested for the treatment of colitis [18].

The other expectation from probiotics is their ability to enhance the metabolic health of the body, as they have been shown to successfully reduce the cholesterol level of plasma through assimilation [19]. In this study, *L. casei* L1 was found to be very successful in the assimilation of cholesterol in the culture media even when inulin or FOS was used together with several bile salts. This result demonstrates the ability of this strain to assimilate cholesterol in the intestinal environment, based on its extraordinary tolerance of bile salt and its utilization of inulin and FOS.

5 Conclusion

This study introduced a new probiotic strain, *L. casei* L1, that was isolated and identified from human milk, and that was able to utilize both inulin and FOS. The strain showed considerable probiotic effects when inulin or FOS was used in a culture medium. In particular, the acidic and bile salt tolerance was enhanced, an antagonistic effect against pathogens was observed, and a high amount of cholesterol was assimilated with the relevant used prebiotics. Based on these findings, this study proposes that probiotic's benefit may be improved if prebiotic fermenting strains such as *L. casei* L1 are consumed with prebiotics, such as inulin and FOS.

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