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ARTICLE

# Effect of Oligosaccharides on the Growth of *Lactobacillus delbrueckii* Subsp. *bulgaricus* Strains Isolated from Dairy Products

Tseteslava Ignatova,[†] Ilia Iliev,[‡] Nikolai Kirilov,[§] Tonka Vassileva,[‡] Michèle Dalgalarrondo,[∥] Thomas Haertlé,[∥] Jean-Marc Chobert,*[,∥] and Iskra Ivanova[§]

[†]Department of Functional Biology, Schoumen University, Schoumen, Bulgaria, [‡]Department of Biochemistry and Microbiology, Plovdiv University, Plovdiv, Bulgaria, [§]Department of General and Applied Microbiology, Sofia University, Bulgaria, and [∥]UR 1268, Biopolymères Interactions Assemblages, INRA, équipe FIPL, rue de la Géraudière, B. P. 71627, 44316 Nantes Cedex 3, France

Eighteen lactic acid bacteria (LAB) strains isolated from dairy products, all identified as *Lactobacillus delbrueckii* subsp. *bulgaricus*, were tested for their ability to grow on three different oligosaccharides: fructo-oligosaccharides (FOS), gluco-oligosaccharides (GOS) and galacto-oligosaccharides (GalOS). The growth of LAB on different oligosaccharides was very different. Study of the antimicrobial activities of these LAB indicated that the system of uptake of unusual sugars influenced in a specific way the production of antimicrobial substances (bacteriocins) specific against Gram-negative bacteria. The added oligosaccharides induced LAB to form end-products of a typical mixed acid fermentation. The utilization of different types of oligosaccharides may help to explain the ability of *Lactobacillus* strains to compete with other bacteria in the ecosystem of the human gastro-intestinal tract.

**KEYWORDS: Lactic acid bacteria; oligosaccharides; fermentation**

## INTRODUCTION

Galacto-oligosaccharides (GalOS), gluco-oligosaccharides (GOS), fructo-oligosaccharides (FOS), inulin, and other related carbohydrates have received considerable attention due to their potential health benefits. It is now well established that certain prebiotics stimulate specific fecal microbial populations both *in vitro* and *in vivo* (*1*). Gibson and Roberfroid (*2*) defined prebiotics as "non-digestible food ingredients that beneficially affect host health by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon and thus improve health". Various lactobacilli and bifidobacteria are among the resident intestinal bacteria that are stimulated by prebiotics (*3*, *4*). Modulation of colonic microbiota through prebiotics offers promise for reducing gut disorders that may be mediated through pathogens (*5*,*6*). According to Roberfroid (*7*), prebiotics are more than "mal absorbed", "non-digestible", or "resistant" carbohydrates because, when they reach the large bowel, they may be metabolized by a specific route, which can be afforded by advantageous rather than adverse bacteria.

Several studies have shown that the ability of lactobacilli and bifidobacteria to ferment prebiotic carbohydrates is both strain and substrate specific (*8*−*10*). In addition, it is not clear which prebiotics are the most suitable substrates for selective growth of specific strains. Recently, several quantitative approaches were conceived to determine the functional activity of prebiotics during *in vitro* fermentation conditions (*6*, *11*−*13*). Usually, these methods based on measurement of microbial populations, growth rate, substrate assimilation, and short-chain fatty acid (SCFA) production yield results reflecting the ability of a given prebiotic to produce specific effects. They showed that SCFA are involved in important physiological events. These include utilization of butyrate by rat colonic epithelial cells as the preferred energy substrate (*14*) and prevention of colon cancer in humans (*15*, *16*).

In recent years intensive studies have been performed with lactic acid bacteria (LAB) isolated from different ecological niches. In 1905 in Geneva the Bulgarian scientist Stamen Grigorov discovered the microbiota of the Bulgarian yogurt. This fact practically marked the beginning of the research on it, the name *Lb. bulgaricus* being connected with its discoverer's nationality. Special interest is the study of strains isolated from artisan milk products and traditional products from countries and regions that have saved the existing technologies and natural starter cultures. Bulgaria is the native land of yogurt.

Lactic acid production from LAB is of primary importance for the antimicrobial action, but bacteriocins and bacteriocin-like inhibitory substances may also play a role. In this context, the use of bacteriocinogenic probiotic strains for food fermentation looks attractive (*17*). Various carbohydrates were ranked for their potential to stimulate the growth of specific members of a mixed microbiota. However, because fermentation of prebiotics is dependent on the bacterial strains used, rather than based on species or genera, it is important to understand the extent to which metabolism of prebiotics occurs by specific strains of

*Corresponding author: Jean-Marc Chobert, INRA BIA-FIPL. Tel: + 332.40.67.52.30. Fax: +332.40.67.50.84. E-mail: chobert@nantes.inra.fr.

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bacteria, especially for those organisms whose intended use is as probiotics. The influence of these oligosaccharides on bacteriocin production remains unclear. Therefore, the aims of this work were (1) to determine the prebiotic activity score of various commercial prebiotics for selected strains of lactobacilli and (2) to study the effects of different prebiotics on antibacterial activity of the strains.

## MATERIALS AND METHODS

**Bacterial Strains and Culture Conditions.** A total of 18 *Lactobacillus* strains isolated from three different types of Balkan homemade yogurts, made from cow (C), sheep (Sh) and buffalo (B) milk, were used. All the strains were identified as *Lactobacillus delbrueckii* (*18*, *19*). The strains were cultured overnight (16−18 h) on MRS (de Mann, Rogosa, Sharpe broth, Merck, Darmstadt, Germany) at 37 °C and in limitation of oxygen (BBL Gas Pak anaerobic system Envelopes, Becton Dickinson, Franklin Lakes, NJ).

**Carbohydrates Used in This Study.** Three types of commercially available carbohydrates presenting different degree of polymerization (DP) were studied. FOS (Raftilose P95 from Orafti, Tienen, Belgium) contained 5% glucose, fructose, and sucrose; and 12% DP2, 48% DP4 and 35% DP7. GalOS (TOS-P from Yakult, Tokyo, Japan) contained 2% DP2, 48% DP3, 38% DP4 and 12% DP5. GOS (BioEcolians from Solabia, Pantin, France) contained 6% glucose and leucrose, and 24% DP4, 56% DP5, 7% DP6 and 7% DP7. The concentration of each carbohydrate was set to 2% to an MRS broth. Glucose (purity 99%, Merck) and raffinose (purity 99%, Fluka, Sigma Aldrich, Switzerland) were used as controls. Each carbohydrate was sterilized on 0.2 $\mu$m sterile filter (Sartorius, Labsystems, Sofia, Bulgaria), and pH was not adjusted. All measurements were performed at least twice.

**Fermentation.** Lactobacilli were grown in MRS broth (Merck) (*20*). Overnight grown cells were washed twice in saline (0.85% NaCl solution), and 10% of the bacterial suspension ($10^7$ cfu mL$^{-1}$) was used to inoculate modified MRS broth and agar medium (pH 6.8) containing either 2% glucose, 2% GOS, 2% FOS or 2% GalOS. The anaerobic fermentations were performed in 100 mL glass bottles at 37 °C for 48 h (BBL Gas Pak anaerobic system envelopes).

An MRS agar medium with different oligosaccharides was prepared by adding 2% (w/v) FOS, GalOS or GOS to MRS agar containing 0.05% L-cystein, 1.5% agar and 30 mg L$^{-1}$ bromocresol purple. The MRS basal medium (i.e., MRS without carbohydrate) was autoclaved, and the oligosaccharide (OS) was filter sterilized and then added to the agar. Each strain of lactobacilli was initially grown in MRS broth and then diluted and spread onto MRS-OS agar plates to give approximately 25 to 50 colonies. The plates were incubated anaerobically at 37 °C for 24 h. Strains that fermented FOS, GalOS or GOS (and produced acid end-products) grew as yellow colonies surrounded by a yellow zone ( > 3 mm) against a purple background. Nonfermenting colonies produced smaller white colonies without yellow zone.

**Microbial Growth.** To evaluate the influence of the above-mentioned different carbon sources on the growth of the bacterial strains, anaerobic fermentations were carried out in triplicate. Bacterial growth was measured by a turbidimetric method at 600 nm and calibrated against cell dry weight using a spectrophotometer (UV/vis Shimadzu, Kyoto, Japan). For each experiment, data were analyzed using the Excel statistical package. The OD readings and standard deviations were calculated from duplicate samples from three separate experiments.

Growth of the studied strains was monitored in MRS broth medium supplemented with the test oligosaccharides, at a concentration of 2%, as a sole carbon source. A 4% prewashed inoculum of a given strain overnight culture ($10^9$ cfu mL$^{-1}$) was used to examine the effect of the predetermined oligosaccharides on the growth of the studied strains. Cultures (200 mL per cultivation) were incubated at 37 °C for a period of 48 h under non-pH-controlled conditions. Growth of each strain was monitored by measuring the OD of the cultures at 0, 3, 4, 5, 6, 9, 12, 17, 24, 30, and 48 h at 600 nm.

**Analysis of Metabolites.** D-Lactic acid was determined enzymatically with L-lactate dehydrogenase and D-lactate dehydrogenase (commercially available kit code 10 139 084 035, Boehringer, Mannheim, Germany). Acetic acid was determined, enzymatically with acetyl-CoA synthetase, citrate synthase, malate dehydrogenase (commercially available kit code 10 148 261 035, Boehringer). Ethanol was determined enzymatically with alcohol dehydrogenase and aldehyde dehydrogenase (commercially available kit code 10 176 290 035, Boehringer).

**Table 1.** Test Organisms

| strains | origin[a] |
|---|---|
| *Lactobacillus delbrueckii* subsp. *bulgaricus* 11842 | ATCC |
| *Lactobacillus delbrueckii* subsp. *bulgaricus* B1 | LBB |
| *Lactobacillus delbrueckii* subsp. *bulgaricus* B3 | LBB |
| *Lactobacillus delbrueckii* subsp. *bulgaricus* B11 | LBB |
| *Streptococcus thermophilus* T32 | LBB |
| *Streptococcus thermophilus* T34 | LBB |
| *Streptococcus thermophilus* T36 | LBB |
| *Streptococcus thermophilus* T38 | LBB |
| *Streptococcus thermophilus* T39 | LBB |
| *Streptococcus thermophilus* T41 | LBB |
| *Leuconostoc mesenteroides* 8293 | ATCC |
| *Leuconostoc mesenteroides* NRLL-B512 | ATCC |
| *Enterococcus avium* 1278 | NBIMCC |
| *Bacillus subtilis* BRB1 | 1MB |
| *Bacillus subtilis* 6633 | ATCC |
| *Bacillus cereus* 4464 | ATCC |
| *Staphylococcus aureus* 745 | NBIMCC |
| *Listeria innocua* F | ENITIAA |
| *Escherichia coli* 3398 | NBIMCC |
| *Escherichia coli* HB 101 | IMB |
| *Escherichia coli* C600 | IMB |
| *Enterobacter aerogenes* 3691 | NBIMCC |
| *Salmonella typhimurium* 3591 | NBIMCC |

[a] NBIMCC, National Bank Industrial Microorganisms & Cell Cultures, Sofia, Bulgaria; LBB, Collection of LB Bulgaricum, Sofia Bulgaria; IMB, Collection of Institute of Microbiology, BAS, Sofia, Bulgaria; ENITIAA, Ecole Nationale d'Ingénieurs des Techniques des Industries Agricoles et Alimentaires, Nantes, France; ATCC, American Type, Culture Collection.

**Analysis of Carbohydrates and Proteins.** The oligosaccharides were analyzed by HPLC using a Symmetry $C_{18}$ column (4.6 × 150 mm) and a Waters 1525 Binary HPLC Pump (Waters, Milford, MA). Oligosaccharides were detected by using a Waters 2414 refractive index detector. The products were identified in the chromatograms as described by Remaud-Simeon et al. (*21*).

Sugars (residual glucose, fructose, galactose and oligosaccharides in fermentation broth after fermentation) were determined by HPLC, using a Zorbax carbohydrate column (4.6 × 150 mm; Agilent, Santa Clara, CA), analytical guard column Zorbax NH2 (4.6 × 12.5 mm), and a mobile phase of 75/25 (v/v) acetonitrile/water. Breeze Chromatography Manager Software (Waters) was used for data treatment.

Proteins were assayed by the method of Lowry et al. (*22*).

Tricine−SDS−PAGE was performed according to Schagger and von Jagow (*23*) at room temperature on a 16.5% acrylamide running gel and 4% acrylamide stacking gel. The silver staining of the gel was done according to Nesterenko et al. (*24*).

**Antimicrobials.** Antimicrobial assay was performed as previously described (*25*) by the well diffusion method. After adjusting the pH to 6.5 by NaOH, the activity of the collected samples (60 $\mu$L) was checked against test organisms belonging to different species as listed in **Table 1**. For cultivation of all *Lactobacillus* strains, MRS and Elliker broth and agar were used. The test organisms were propagated in appropriate media as follows: *Escherichia coli* grown on LB (Luria−Bertani) agar medium (Sigma, St. Louis, MO), *Listeria innocua* F grown on BH (Brain Heart)-agar medium (Biokar Diagnostics, Beauvais, France), *Salmonella typhimurium* grown on Elliker, and *Bacillus* sp. grown on nutrition broth and agar. The plates were incubated overnight at 30 and 37 °C.

Effects of heat and hydrolytic enzymes (trypsin from Sigma, No. T-8253; proteinase K from Sigma, No. P-0390) on bacteriocin activity were determined according to Pantev et al. (*26*). Samples (100 $\mu$L) were treated for 2 h with 0.1 mg mL$^{-1}$ and 1 mg mL$^{-1}$ final concentration of trypsin or proteinase K, respectively. Antimicrobial activity of treated samples was tested against *E. coli* and *L. innocua*.

**Table 2.** Oligosaccharide Utilization by *Lactobacillus* Strains

| strains[a] | growth on MRS[b] | OD glucose[c] | growth on MRS-FOS[b] | OD FOS[c] | growth on MRS-GOS[b] | OD GOS[c] | growth on MRS-GalOS[b] | OD GalOS[c] |
|---|---|---|---|---|---|---|---|---|
| C2 | + | 4.9 ± 0.15 | − | 0.1 ± 0.02 | + | 0.6 ± 0.03 | + | 0.6 ± 0.04 |
| C3 | + | 4.3 ± 0.12 | − | 0.1 ± 0.02 | + | 1.8 ± 0.05 | + | 1.5 ± 0.1 |
| C5 | + | 4.2 ± 0.15 | − | 0.3 ± 0.03 | − | 0.2 ± 0.02 | − | 0.1 ± 0.02 |
| C6 | + | 3.1 ± 0.08 | − | 0.1 ± 0.02 | − | 0.2 ± 0.02 | + | 1.7 ± 0.1 |
| C7 | + | 3.6 ± 0.1 | − | 0.1 ± 0.02 | − | 0.1 ± 0.02 | + | 0.7 ± 0.05 |
| C14 | + | 4.8 ± 0.15 | − | 0.1 ± 0.02 | − | 0.2 ± 0.02 | − | 0.5 ± 0.03 |
| C15 | + | 4.1 ± 0.12 | − | 0.4 ± 0.03 | − | 0.3 ± 0.02 | − | 0.2 ± 0.02 |
| C17 | + | 5.2 ± 0.12 | − | 0.1 ± 0.02 | − | 0.3 ± 0.02 | − | 0.1 ± 0.02 |
| C20 | + | 4.0 ± 0.12 | − | 0.1 ± 0.02 | − | 0.1 ± 0.02 | + | 1.8 ± 0.1 |
| C21 | + | 5.1 ± 0.15 | − | 0.1 ± 0.02 | − | 0.3 ± 0.02 | + | 0.7 ± 0.04 |
| C22 | + | 4.0 ± 0.12 | − | 0.2 ± 0.02 | + | 0.4 ± 0.03 | + | 1.9 ± 0.1 |
| C26 | + | 3.9 ± 0.1 | − | 0.1 ± 0.02 | − | 0.2 ± 0.02 | − | 0.4 ± 0.03 |
| C27 | + | 4.1 ± 0.12 | − | 0.1 ± 0.02 | − | 0.3 ± 0.03 | − | 0.5 ± 0.04 |
| Sh42 | + | 4.2 ± 0.15 | − | 0.2 ± 0.02 | + | 0.5 ± 0.03 | + | 0.7 ± 0.04 |
| Sh43 | + | 3.9 ± 0.1 | − | 0.1 ± 0.02 | + | 0.8 ± 0.05 | − | 0.4 ± 0.04 |
| B5 | + | 4.4 ± 0.1 | + | 0.5 ± 0.03 | + | 0.5 ± 0.03 | + | 2.0 ± 0.02 |
| B7 | + | 3.9 ± 0.1 | − | 0.2 ± 0.02 | − | 0.1 ± 0.02 | − | 0.4 ± 0.02 |
| B8 | + | 4.2 ± 0.12 | + | 0.7 ± 0.04 | − | 0.3 ± 0.03 | + | 1.5 ± 0.1 |

[a] C: cow. Sh: sheep. B: buffalo. [b] Growth on MRS, MRS-FOS, MRS-GOS and MRS-GalOS agar was positive (+) if colonies were surrounded by a yellow zone and negative (−) if no yellow zone was visible. [c] Optical density (OD) at 600 nm after 24 h in MRS, MRS-FOS, MRS-GOS and MRS-GalOS broths. For each experiment, data were analyzed using the Excel statistical package. The OD readings and standard deviations were calculated from duplicate samples from three separate experiments.

## RESULTS

**Selection of *Lactobacillus delbrueckii* Subsp. *bulgaricus* from Dairy Products To Utilize Different Types of Oligosaccharides.** Growth of 18 bacterial *Lactobacillus* strains in modified MRS broth supplemented with different carbohydrate sources is shown in **Table 2** and **Figure 1**. Growth was evaluated in terms of maximum optical density at 600 nm and specific growth rate achieved during 24 h fermentation. Growth kinetics on glucose were used as control. All studied *Lactobacillus* strains fermented FOS, GOS and GalOS in different manner. Only 2 strains (B5 and B8) were able to grow on a medium containing FOS. GOS was fermented only by 6 strains (C2, C3, C22, Sh42, Sh43, and B5). The third oligosaccharide used in this study, GalOS, was metabolized by more strains (10) than the others. Five strains reached the optical density of 1.5 after 24 h of incubation.

**Residual Oligosaccharide Analysis.** The extent of carbohydrate utilization by strains B5 and B8 was analyzed by HPLC. *Lactobacillus delbrueckii* subsp. *bulgaricus* B5 and *Lb. delbrueckii* subsp. *bulgaricus* B8 showed different preferences for the types and degree of polymerization (DP) of studied oligosaccharides (**Figure 2**).

GOS rich in DP5 and DP4 was better consumed by strain B8. Hydrolysis of DP4 and DP5 was accompanied by the appearance of DP2 and DP3. Strain B5 was unable to use DP6 and DP7 from GOS while 50% of these oligosaccharides were hydrolyzed by strain B8.

GalOS was hydrolyzed to the same extent by strains B5 and B8, strain B5 hydrolyzing DP4 more than strain B8, and strain B8 hydrolyzing DP3 more than strain B5.

When using FOS, rich in DP7 and DP4, DP7 was completely hydrolyzed by strain B8 with concomitant appearance of DP5. Strain B5 hydrolyzed also, to a lesser extent, DP7, but the products of hydrolysis were different as less DP5 appeared. Most likely a significant portion of FOS was metabolized to fructose. DP4 was also well hydrolyzed particularly by strain B5 as compared with strain B8 as already observed in the case of GalOS. Some DP3 appeared after hydrolysis by the two strains as observed in the case of GOS hydrolysis (**Figure 2**).

**Production of Lactic Acid, Acetic Acid and Ethanol.** The fermentation pattern depends on the physiological conditions of the growing cells. Homofermentative LAB can ferment hexoses via glycolysis with 90% of the glucose being metabolized to lactic acid. When cultivated on different oligosaccharides, the studied strains B5 and B8 produced different amounts of acetic acid, lactic acid and ethanol (**Table 3**). When the fermentation end-products obtained using oligosaccharides as growth substrates were compared to those observed on glucose, the main effect was that the production of lactic acid was lower while the production of acetic acid and ethanol increased. Both studied strains showed the same behavior.

**Antimicrobial Activity.** In our previous study (*19*) it was shown that some of the studied strains i.e. C2, C26, C27, and Sh42 were active against *E. coli* and *L. innocua* F when cultivated in MRS-glucose. The supernatants obtained after fermentation of studied strains on MRS-glucose, MRS-GalOS, MRS-GOS and MRS-FOS were tested for their antimicrobial activity after pH adjustment to 6.5. For the strain B5, the zones of inhibition of growth of *E. coli* were significantly bigger when the strain was cultivated in the presence of GalOS or FOS. Strain B8 showed an antimicrobial activity against *E. coli* when cultivated on GalOS or FOS. All the strains were active against *L. innocua* F. It is clear that the different sources of energy induced the production of antimicrobials. For this reason, supernatants were analyzed for the possible synthesis of bacteriocins. Determination of antimicrobial activity focused on strains B5 and B8, because of their inhibitory spectrum (**Table 4**). The strains B5 and B8 were found to possess antibacterial activity against one or more strains of the *Lactobacillus* species tested, as well as against other LAB species (*Streptococcus thermophilus* and *Leuconostoc mesenteroides*). A spectrum of activity was detected against the test strains representing species of food-borne pathogens. Three strains of *E. coli* were sensitive to the active substance. The pathogenic strain of *Enterobacter aerogenes* and the nonpathogenic strain, *L. innocua* F, were also inhibited as well as *Bacillus* species, *Staphylococcus aureus* and *Salmonella typhimurium*.

In order to measure the stability of produced antimicrobial proteins (peptides), the neutralized supernatants from strains B5 and B8 grown on oligosaccharides (FOS, GOS and GalOS) were boiled for 10 min, or hydrolyzed by trypsin and proteinase K. A complete inactivation was observed after treatment of the cell free supernatants with proteinase K and trypsin, which indicated the proteinaceous nature of the antimicrobial agent. The antimicrobial substance in neutralized active culture supernatant was relatively heat labile as it was inactivated completely after 10 min at 100 °C.

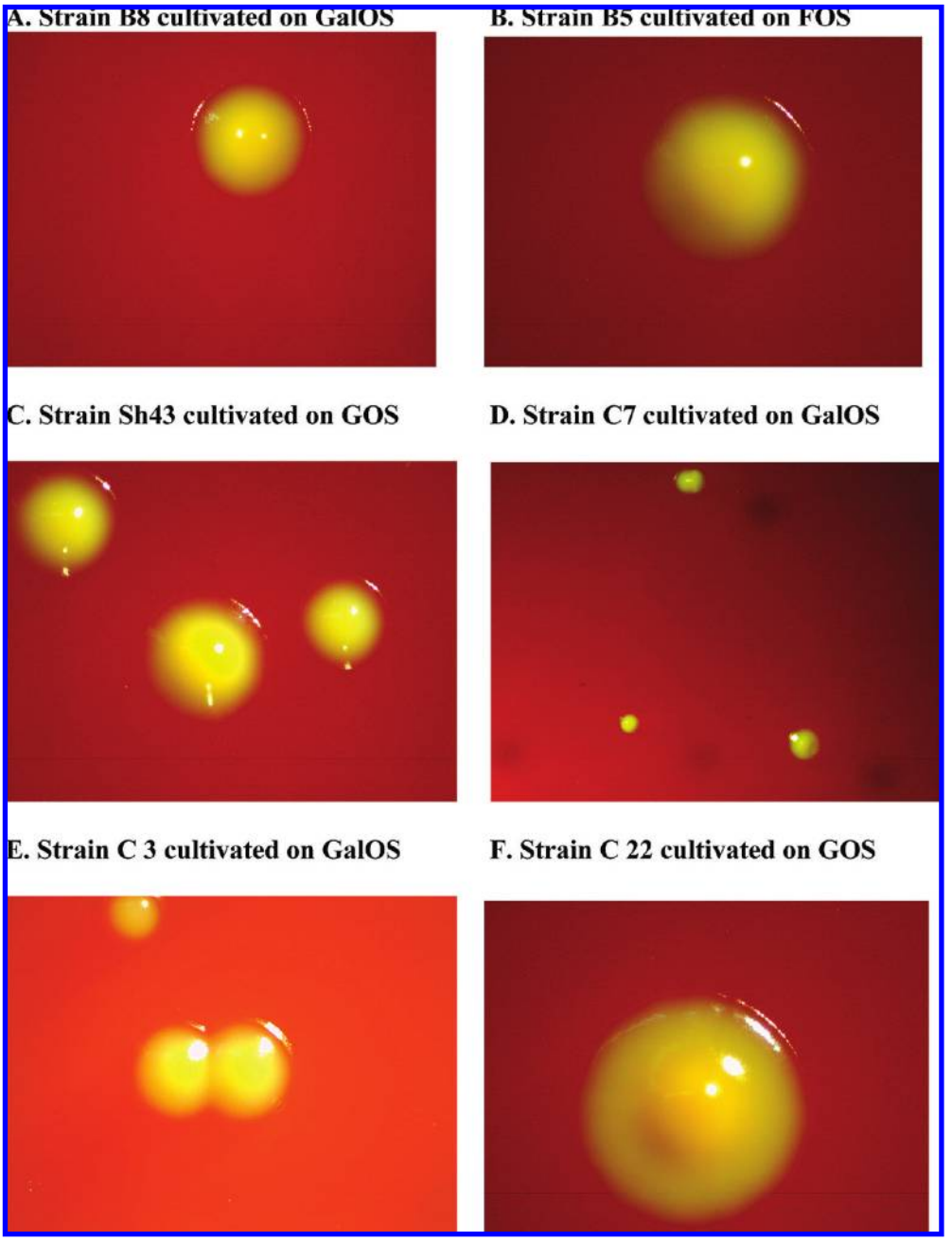


**Figure 1.** Microscopic analysis of *Lactobacillus* strains. Pictures are made with a Zoom-stereo microscope (OLYMPUS, model SZ61TR): basic configuration with wide field eyepiece with magnification 10× and field number 22.

After sterile filtration of neutralized supernatants on 10 kDa cutoff Millipore membrane, antibacterial activity was found only in the concentrated sample. The molecular mass of antimicrobials was estimated at 12 kDa by tricine−SDS−PAGE (**Figure 3**).

## DISCUSSION

It is well-known that while most bifidobacteria strains can use oligosaccharides easily, only a few strains from other genera, including lactobacilli, possess this ability. It has been demonstrated in this study that, in the presence of different oligosaccharides such as FOS, GOS and GalOS, the studied strains showed very different preferences. The most important growth was obtained in the case of *Lb. delbrueckii* subsp. *bulgaricus* B5 and *Lb. delbrueckii* subsp. *bulgaricus* B8 isolated from yogurt made from buffalo milk. Our findings were to some extent similar to the results shown by Kaplan and Hutkins (*27*). We would like to note that *Lactobacillus* strains ordinarily used for yogurt manufacture are FOS nonfermenting. In this aspect our preliminary results on the utilization of FOS demonstrated *in vitro* the capacity of *Lb. delbrueckii* subsp. *bulgaricus* B5 and B8 to metabolize it. The preference toward shorter oligosaccharides in cultures with FOS mixture has been observed in *Lb. delbrueckii* subsp. *bulgaricus* B8. In contrast, the preference toward oligosaccharides with DP7 in cultures with FOS mixture has been observed in the case of *Lb. delbrueckii* subsp. *bulgaricus* B5. To the best of our knowledge, only some species of lactobacilli whose metabolism is associated with FOS have been characterized: *Lb. acidophilus, Lb. paracasei* and *Lb. plantarum*. The first two are able to assimilate it (*9*, *27*, *28*).

The antimicrobial activity of strains B5 and B8 determined against closely related LAB as well as against some food-borne pathogens after cultivation on oligosaccharides also indicated

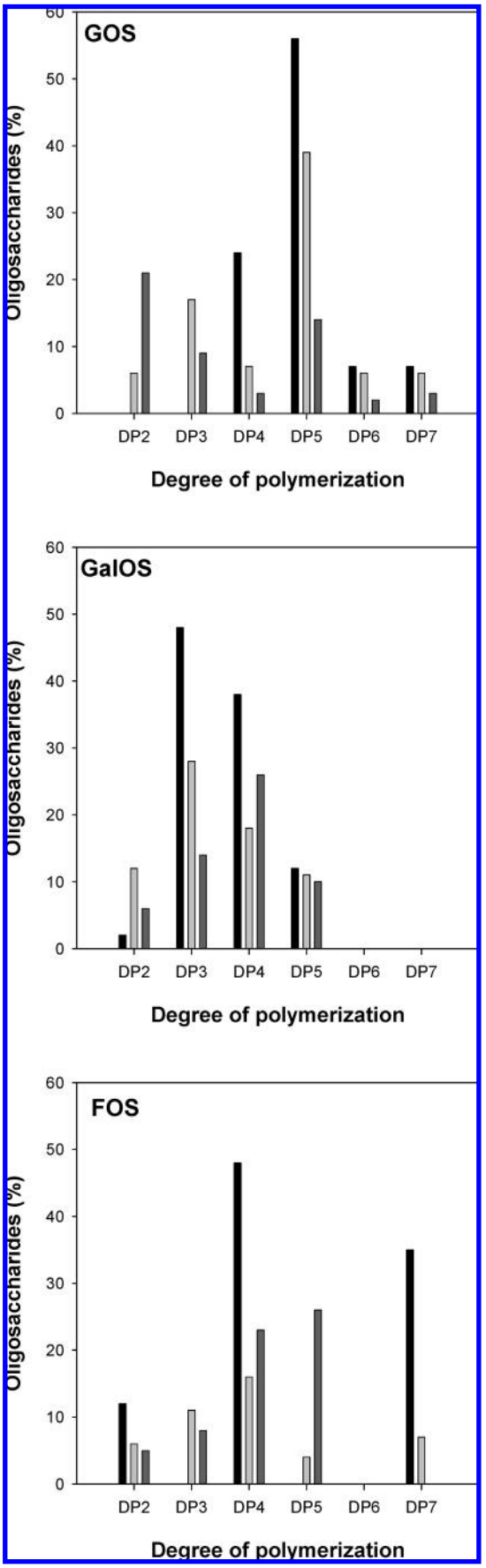


**Figure 2.** Oligosaccharides utilization by fermentation of *Lactobacillus delbrueckii* subsp. *bulgaricus* strains B5 and B8 on MRS with 2% oligosaccharides (composition at t0, black; B5, gray; B8, dark gray).

**Table 3.** pH and Fermentation End-Products (Lactic Acid, Acetic Acid and Ethanol) Obtained after 24 h Growing of *Lactobacillus delbrueckii* Subsp. *bulgaricus* B5 and *Lactobacillus delbrueckii* Subsp. *bulgaricus* B8 on Glucose and Oligosaccharides

| sugar | strain | lactic acid ($\mu$mol mL$^{-1}$) | acetic acid ($\mu$mol mL$^{-1}$) | ethanol ($\mu$mol mL$^{-1}$) |
|---|---|---|---|---|
| glucose | | | | |
| 3 h | B5 | 9.0 ± 0.7 | 0 | 0 |
| 6 h | B5 | 16.0 ± 1.1 | 0 | 0 |
| 12 h | B5 | 34.0 ± 1.2 | 0 | 0 |
| 24 h | B5 | 48.0 ± 1.2 | 0 | 0 |
| 3 h | B8 | 7.5 ± 0.5 | 0 | 0 |
| 6 h | B8 | 11.5 ± 0.7 | 0 | 0 |
| 12 h | B8 | 27.0 ± 0.8 | 0 | 0 |
| 24 h | B8 | 37.5 ± 0.8 | 0 | 0 |
| GOS | | | | |
| 3 h | B5 | 4.2 ± 0.2 | 0.3 ± 0.02 | 0.4 ± 0.02 |
| 6 h | B5 | 7.4 ± 0.2 | 0.8 ± 0.02 | 0.6 ± 0.02 |
| 12 h | B5 | 15.2 ± 0.4 | 1.7 ± 0.03 | 2.1 ± 0.04 |
| 24 h | B5 | 21.0 ± 0.4 | 2.3 ± 0.02 | 3.4 ± 0.04 |
| 3 h | B8 | 2.3 ± 0.1 | 0.3 ± 0.01 | 0.7 ± 0.01 |
| 6 h | B8 | 4.1 ± 0.2 | 1.3 ± 0.01 | 1.1 ± 0.02 |
| 12 h | B8 | 7.9 ± 0.2 | 2.1 ± 0.01 | 3.8 ± 0.02 |
| 24 h | B8 | 11.0 ± 0.15 | 2.7 ± 0.01 | 5.7 ± 0.02 |
| GalOS | | | | |
| 3 h | B5 | 4.7 ± 0.1 | 0.5 ± 0.02 | 1.6 ± 0.06 |
| 6 h | B5 | 8.4 ± 0.3 | 1.1 ± 0.02 | 3.1 ± 0.06 |
| 12 h | B5 | 17.0 ± 0.8 | 1.9 ± 0.03 | 6.3 ± 0.10 |
| 24 h | B5 | 23.0 ± 1.1 | 2.4 ± 0.02 | 11.0 ± 0.10 |
| 3 h | B8 | 3.5 ± 0.2 | 0.3 ± 0.01 | 0.4 ± 0.02 |
| 6 h | B8 | 7.8 ± 0.5 | 1.1 ± 0.03 | 1.6 ± 0.03 |
| 12 h | B8 | 18.6 ± 0.7 | 1.9 ± 0.03 | 3.8 ± 0.04 |
| 24 h | B8 | 27.0 ± 0.7 | 2.4 ± 0.03 | 5.6 ± 0.04 |
| FOS | | | | |
| 3 h | B5 | 3.1 ± 0.05 | 0.2 ± 0.01 | 1.2 ± 0.02 |
| 6 h | B5 | 6.4 ± 0.1 | 0.6 ± 0.01 | 2.6 ± 0.05 |
| 12 h | B5 | 11.2 ± 0.2 | 1.0 ± 0.03 | 4.8 ± 0.10 |
| 24 h | B5 | 14.5 ± 0.2 | 1.3 ± 0.04 | 7.2 ± 0.10 |
| 3 h | B8 | 3.5 ± 0.1 | 0.3 ± 0.01 | 0.5 ± 0.01 |
| 6 h | B8 | 7.3 ± 0.1 | 0.6 ± 0.01 | 1.3 ± 0.01 |
| 12 h | B8 | 16.3 ± 0.4 | 1.6 ± 0.02 | 1.6 ± 0.02 |
| 24 h | B8 | 22.5 ± 0.6 | 2.7 ± 0.03 | 4.5 + 0.05 |

**Table 4.** Inhibitory Spectrum of *Lactobacillus delbrueckii* Subsp. *bulgaricus* B5 and *Lactobacillus delbrueckii* Subsp. *bulgaricus* B8

| | no. of strains inhibited/no. of strains tested | |
|---|---|---|
| indicator species | *Lb. delbrueckii* subsp. *bulgaricus* B5 | *Lb. delbrueckii* subsp. *bulgaricus* B8 |
| *Lactobacillus delbrueckii* subsp. *bulgaricus* | 2/4 | 1/4 |
| *Streptococcus thermophilus* | 3/6 | 2/6 |
| *Leuconostoc mesenteroides* | 1/2 | 1/2 |
| *Enterococcus avium* | 0/1 | 0/1 |
| *Bacillus subtilis* | 2/2 | 2/2 |
| *Bacillus cereus* | 1/1 | 1/1 |
| *Staphylococcus aureus* | 1/1 | 1/1 |
| *Listeria innocua F* | 1/1 | 1/1 |
| *Escherichia coli* | 3/3 | 3/3 |
| *Enterobacter aerogenes* | 1/1 | 0/1 |
| *Salmonella typhimurium* | 1/1 | 1/1 |

that the system of uptake of unusual sugars influenced in a specific way the production of antimicrobial substances. It is important to note that the activity against Gram-negative bacteria was shown for some bacteriocins isolated from *Lb. delbrueckii* and *Streptococcus thermophilus* (*29*, *30*). The mechanism of this stimulation remains unclear. Similar data were previously obtained (*31*).


**Figure 3.** Tricine−SDS−PAGE of bacteriocin isolated from *Lactobacillus delbrueckii* subsp. *bulgaricus* B8 cultivated in MRS with glucose (lane 4) and oligosaccharides (lane 1, FOS; lane 2, GOS; lane 3, GalOS).

*In vitro* studies have shown that a number of prebiotics mimic eukaryotic cell surface receptors to which virulent bacteria adhere as part of the pathogenicity process. For example, GOS was shown to inhibit attachment of EPEC (enteropathogenic *E. coli*) to Hep-2 and Caco-2 cells, and to be more effective than either inulin or FOS (*32*). The activity found against some bacteria in our study could enable prebiotic oligosaccharides to be protective in colon. However, it is unclear whether sufficient amounts could be delivered to have any significant effect.

In the present study we have shown the production of heat-sensitive bacteriocin-like substances of 12 kDa. Recently, an effective enhancement of bacteriocin production was observed when FOS or trehalose was used by strains of *Lactococcus* and *Enterococcus* (*8*). However, studies relating to the antibacterial properties of *Lb. delbrueckii* have been limited (*29*, *33*). Most likely the diversity of the energy sources seemed to stimulate the production of some antimicrobials in the studied strains B5 and B8.

It is proposed that in the gut those LAB, which have specific enzyme activities for prebiotic substrate, may have potential to compete with other microbes. SCFA production is one of the most important physiological processes mediated by colonic microorganisms. The influence of certain prebiotics on production of SCFA by LAB is considered as a highly interesting parameter, and for this reason it has been another object of attention in different studies (*34*−*36*). The fermentation end-products obtained after cultivation in the presence of FOS, GOS and GalOS were compared to those observed on glucose. Production of lactic acid was decreased, while production of acetic acid and ethanol was increased. These results indicated that the used oligosaccharides induce LAB to form end-products of a typical mixed-acid fermentation. Similar observations were proposed by Kontula et al. (*35*) when $\beta$-gluco-oligosaccharides and xylo-oligosaccharides were used as fermentative substrates and by Sjöberg (*37*) who observed that in a homofermentative *Lactococcus lactis* strain metabolism was altered to mixed-acid fermentation by maltose. The mechanism inducing the metabolic shift to mixed-acid fermentation has not been explained in detail although the shift can be identified by gene expression (*38*). Starvation has been suggested as one of the factors changing the metabolism from homofermentative to mixed-acid production (*39*, *40*). Data obtained in the present study suggest that strains of LAB are able to change fermentation patterns, depending on the available substrates and metabolic pathways of the strains. In the colon, both monosaccharides and oligosaccharides are present, what means that both fermentation routes are possible.

It is known that a system constituted by a proton transport system (PTS) and a $\beta$-fructo furanosidase (invertase) is responsible for the assimilation of two types of $\beta$-(2−1) fructans (Fn and GFn). Despite significant commercial interest in using oligosaccharides as prebiotic substrates, little is known about how these oligosaccharides are metabolized by LAB and related bacteria. LAB accumulate sugars by secondary active transport (mainly by proton motive force, PMF), the PTS, or an ATP-mediated system. For the oral strain of *Streptococcus mutans*, protein dependent multiple sugar metabolism (MSM) transport system was described that transported the trisaccharides, raffinose and isomaltotriose, and disaccharide melibiose. The MSM genes coding for this system are organized in a cluster typical of ABC operons in that it contains genes coding for ATP- and oligosaccharide-binding proteins, and two membrane-spanning domains (*27*). From the other side most bacteriocins are exported across the cytoplasm membrane by a dedicated ABC-binding cassette/ABC transporter (*27*). Some speculation on the dual action of ABC system could be made in the case of FOS import and bacteriocin production in the studied strains *Lb. delbrueckii* subsp. *bulgaricus* B5 and *Lb. delbrueckii* subsp. *bulgaricus* B8. However, more studies should be performed in order to elucidate the pathways of utilization of oligosaccharides in these *Lactobacillus* strains.

In conclusion, 18 LAB strains belonging to species *Lb. delbrueckii* subsp. *bulgaricus* were shown to be able to utilize different oligosaccharides. To our knowledge, it is the first large-scale study on *Lactobacillus* strains and especially those isolated from fermented buffalo milk (strains B5 and B8). *Lb. delbrueckii* subsp. *bulgaricus* strains B5 and B8 possessed antimicrobial activity against nearly related LABs as well as against some food-borne pathogens and some Gram-negative bacteria. The induction of different end-products typical of mix-acid fermentation has been demonstrated.

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