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Growth characteristics of lactobacillii strains cultivated on media with fructooligosaccharides and their antimicrobial activity

ABSTRACT

Following classical microbiological techniques four strains including lactic acid bacteria were isolated from yogurt and cheeses samples from the different regions of Bulgaria. The isolated bacteria were studied for their ability to utilize carbohydrates as glucose and oligosaccharides - fructooligosaccharides (FOS). The kinetics of their growth and their antimicrobial activity were studied. Some of the strains showed activity against test cultures - *Staphilococcus aureus* ATCC39592, *Esherichia .coli* HB101 and *Bacillus subtilis*.

Key words: lactic acid bacteria, antimicrobial activity, oligosaccharides, fructooligosaccharides, prebiotics

Introduction

Lactic acid bacteria (LAB) are gram-positive, non-sporulating, catalase-negative, aero-tolerant, acid tolerant, nutritionally fastidious, strictly fermentative microorganisms that lack cytochromes and produce lactic acid as the major end product of carbohydrate metabolism. The LAB group consists mainly of strains from *Lactobacillus, Weissella, Carnobacterium, Streptococcus, Enterococcus, Lactococcus, Vagococcus, Leuconostoc, Oenococcus, Pediococcus*, and *Tetragenococcus* genera (Azcarate-Peril & Klaenhammer, 2010). Lactobacilli can be found in a variety of natural ecological niches, such as plants or plant-derived materials, animals and raw milk. Historically, lactobacilli have been deliberately introduced in fermented food products as a means of food preservation. Nowadays, they are considered as food additives for their putative probiotic properties. The concept of probiotics was first popularized at the turn of the century by the Russian Nobel laureate, Elie Metchnicoff. He proposed that a normal, healthy, gastrointestinal microbiota in humans and animals provided resistance to "putrefactive" intestinal pathogens (Bibel, 1988). His theorized that the intestinal flora influences the incidence and severity of enteric infections and either enhances or slows atrophy and aging processes. Metchnicoff proceeded to isolate a *Lactobacillus* culture from fermented milk consumed by Bulgarian peasants who were renowned for living long and healthy lives.

Several *Lactobacillus* and *Bifidobacteria* strains have been considered probiotics due to their beneficial effect on the host by improving the intestinal microflora, helping in the immune system maturation, and presenting inhibitory activity toward the growth and adhesion to epithelial cellsw or intestinal mucus of pathogenic microorganisms (Roberfroid, 2002; Ljungh & Wadstrom, 2007; Petrova et al., 2009). In our previous experiments indicated how prebiotics fructo-, galactooligosaccharides and soyo-oligosaccharides (rafinose) evaluated the fermentation of probiotics LAB strains and have considered the effect of oligosaccharides on antimicrobial activity (Ignatova et al., 2009). In this study, native *Lactobacillus* strains were isolated from Bulgarian yogurt and chesses, and the effect of oligosaccharides supplementation on *Lactobacillus* growth and antimicrobial activity was evaluated.

Materials and Methods

Strain

A total four *Lactobacillus sp.* was isolated from two types sheep and cow Balkan homemade yogurts and cheeses. The strains with prefics "K" and "O" correspond to strain isolated from cow and sheep yogurts and cheeses respectively. The

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strains were originated from the northeast part of Bulgaria, in a village near to Shumen. The strains were cultured overnight (16-18h) on Mann Rogosa Sharpe (MRS) broth at 37°C and in limitation of oxygen.

Media

The strain cultivated in media of MRS (de Mann Rogosa Sharpe) in composition, per liter: glucose – 20; Tween 80 – 1; pepton from casein – 10.0; meat extract – 8.0; yeast extract – 4.0; K_2HPO_4 – 2.0; sodium acetate – 5.0; ammonium citrate – 2.0; $MgSO_4.7H_2O$ – 0.2 and $MnSO_4$ – 0.05 g/l and media M17 (Terzaght & Sandine, 1975) (MERCK 1.15108.0500) in composition (g/l): peptone from soya– 5,0; peptone from meat – 2.5; peptone from casein – 2.5; yeast extract – 2.5; meat extract – 5.0; lactose – 5.0; ascorbic acid– 0.5; sodium – β-glicerophosphate – 19.0; $MgSO_4.7H_2O$ – 0.25; dhd – 12.75.

The pH of media MRS was adjusted to 6.5 with 1N NaOH and media M17 was adjusted to 7.2 with 1N NaOH. The basic medias was sterilized by autoclaving at 121°C for 20 min, and carbohydrates supplemented were sterilized using 0.22 µm filters (Manisart ®). An mMRS agar medium with different OS was prepared by adding 2% (w/v) FOS (Orafti, Belgium) and GOS (Bioecolia; Solabia, pantin Cedex, France) to MRS agar.

API 50CHL System Assay

Initial identification of all the strains was performed by API 59 CHL system (BioMerieux, Craponne, France), according to the manufacturer`s instruction. The fermentation profiles were read after incubation at 37°C in anaerobic condition, for 3 days.

Antimicrobial activity

Antimicrobial assay was performed as previously described (Beal et al., 1999) by the well diffusion method by using soft 0,8% agar. After adjusting the pH at 6.5 by NaOH, the activity of the collected samples (60 µL) was checked against *Escherichia coli* HB 101on Luria-Bertain agar media, *Bacillus subtilis* on meat extract agar media and *Staphilococcus aureus* ATCC 39592 on meat extract agar media. The plates were incubated overnight at 37°C. Antimicrobial activity of 24 h hydrolyzed samples was checked on the strains cultivated on media containing 2% glucose. The neutralized supernatants (pH 6.5) obtained after 24 h preculture in mMRS-FOS and 24h culture was checked for the activity against *E. coli*, *B. cereus* and *St. aureus*. All experiments were performed in triplicate.

Microbial growth

Bacterial growth was measured by a turbidimetric metod at 600 nm and calibrated against cell dry weight using a spectrofotometer (UV/vis JENWAY 6315). For each experiment, data were analyzed using the Excel statistical package. The OD reading and standard deviations were calculated from duplicate samples from three separate experiments. Growth of each strain was monitored by measuring the OD of the cultures at 0, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 30 and 48 h at 600 nm.

Results and Discussion

Following the classical microbiological methods were isolated strains from two different artesian cow and sheep cheese and cow and sheep yogurt. The microscopic photos are shown in Figure 1. All isolated strains were Gram-positive rods, non-spore forming, non-motile bacteria, and negative for catalase, indol, and oxidase tests. In addition, the strains presented an optimal growth at 35 or 40 C, while at 15 and 45°C all strains presented a moderate growth. These biochemical characteristics are in agreement with previous reports of *Lactobacillus* characterization. Using API tests the strains isolated from cow and sheep cheeses were identified as *Lactobacillus helveticus* and *Lactobacillus casei.* From cow and sheep yogurt were identified strains as *Lactobacillus plantarum.*

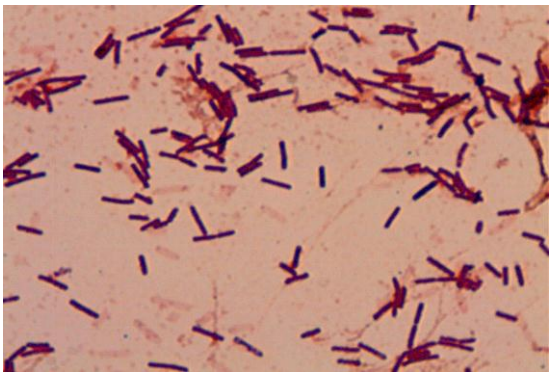

Figure 1. *Microscopic photo of Lactobacillus spp.*

The specific growth rate of 4 bacterial *Lactobacillus* strains in modified mMRS broth supplemented with fructooligosaccharide is shown in Table 1.

Results were obtained ± SEM from three seperate trails determined within the time interval from 0 to 24 h.

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Table 1. *Oligosaccharide utilization by Lactobacillus strain.*

Growth rate (μ^{-1})/strains	*L. plantarum* K1	*L. plantarum* O1	*L. helveticus* K	*L. casei* O
μ on MRS-glucose	0,29±0,008	0,35±0,014	0,23±0,012	0,26±0,017
μ on MRS-FOS	0,12±0,017	0,30±0,008	0,10±0,005	0,27±0,005

Growth was evaluated in terms of maximum optical density of 600 nm and specific growth rate archived during a fermentation period of 24 hours. For control was used the growth kinetics on glucose. All studied *Lactobacillus* strains fermented FOS in different manner. FOS was fermented by *L. plantarum-O1* with growth rate as those when grown on glucose. The same is the pattern for *L. casei*. *Lactobacillus helveticus* and the strain *L. plantarum* isolated from cow cheese have low growth rate. These results show the differences in the ability to metabolize FOS by the isolated strains, which could be owed to different production levels of β-fructofuranosidase to break up the β(2→1) bonds presented in FOS. Noteworthy, most of the strains were not able to consume the FOS with the highest degree of polymerization (DP=5). Several authors have reported the capability for metabolizing FOS by different strains that belong to the *Lactobacillus* and *Bifidobacterium* genera (Sanz et al., 2009; Mandagjieva et al., 2011). Recently it was shown by us that *Lactobacillus delbrueckii subsp. bulgaricus* B5 a preference for FOS with a DP=7, metabolizing 80% of the initial amount (Ignatova et al., 2009).

For FOS hydrolysis can involve three different enzymes: a sucrose phosphoenolpyruvate-dependent phosphotransferase (PTS), a fructofuranosidase, and a fructokinase; from which the sucrose PTS II that is involved in the sucrose uptake across the cytoplasmic membrane and its phosphorylation seems to be no specific and acts on FOS. For *L. plantarun* and *Bifidobacterium breve* have been predicted the intracellular production of β-fructofuranosidase showing that the ability to degrade FOS is related to their ability to transport them into the cell. In this sense, a deficit or decrease in the sucrose transporter function would be reflected in a low FOS degradation. In this sense, the results suggest that the isolated strains could follow an intracellular transport of FOS before being hydrolyzed, as has been previously reported.

Antimicrobial activity determined by the agar well diffusion method in resulted protein extracts obtained after culturing the isolated strains in MRS and MRS-FOS media for 24 and 48 h. Data are presented as the media of six independent measures ($\phi_{final} - \phi_{well}$) ± SD (Figure 2).

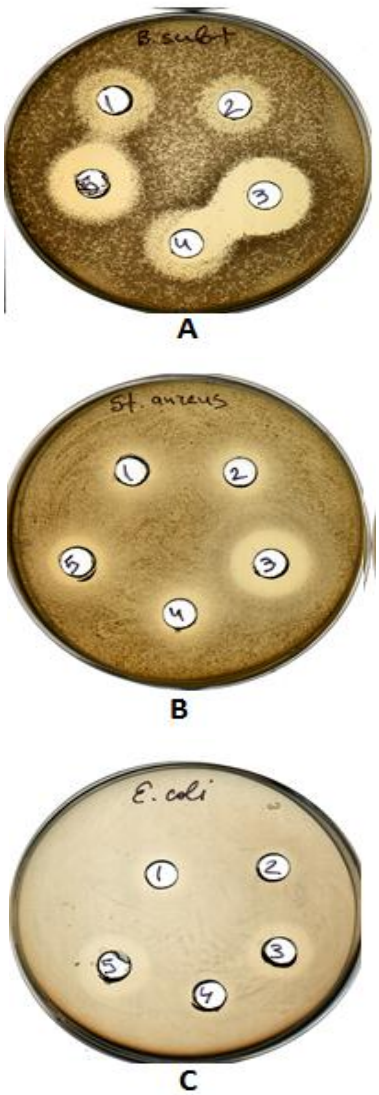

Figure 2. *Inhibitory activity of strains Lactobacillus cultivated in mMRS-FOS.* ***A*** *– B. subtilis;* ***B*** *– St. aureus;* ***C*** *– E. coli. 1-strain L. plantarum-K1 cultivated of FOS; 2 - strain L. plantarum-K1 cultivated of GOS; 3 - strain L. casei-O cultivated of FOS; 4 - strain L. casei-O cultivated of GOS; 5 - strain L. plantarum-O1 cultivated of FOS.*

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Evaluation of antimicrobial activity was conducted by the agar well diffusion method, using the neutralized and filtrated cell free supernatants. Antimicrobial activity was observed against *S. aureus, B. subtilis and E. coli* .None of the studied strains did not show any activity when cultivated on MRS with glucose. It can be noticed that *B. subtilis* was the most sensitive of the indicator strains since all showed antimicrobial activity against this strain after cultivation on mMRS-FOS. The zone of inhibition of *E. coli* was considerably bigger for strains *L.plantarum-O1* and *L. casei-O*. The supernatants from all tested strains showed zones of inhibition of *St. aureus* ,but the effect was more clear in the case again for L. casei and L. plantarum after cultivation in the presence of fructooligosaccharides. From these results it is clear that the different energy source induced the production of antimicrobials substances. Despite that it has been shown that the production of antimicrobials is related to the energy source presented in the culture medium, it is not clear the mechanism by which is realized.

The most interesting capacity showed strains *Lactobacillus plantarum-O* and *Lactobacillus casei-O* isolated from sheep yogurt and chesse. The antimicrobial activity determined after cultivation on oligosaccharides also indicate that the system of uptake of unusual sugars influence in a specific way the production of antimicrobial substances. However, more studies should be conducted to elucidate the pathways of utilization of oligosaccharides in these lactobacillus strains.

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References

Azcarate-Peril MA, Klaenhammer TR. 2010. Genomics of lactic acid bacteria: the post-genomics challenge – from sequence to function. – Wiley-Blackwell, Oxford, UK.

Beal C, Skokanova J, Latrille E, Martin N, Corrieu G. 1999. Combined effects of culture condition and storage time on acidification and viscosity of stirred yogurt. J. of dairy Science, 82: 673-781.

Bibel DJ. 1988. Elie Metchnikoff`s bacillus of long life. ASM News, 54: 661-665.

Ignatova T, Iliev I, Kirilov N, Vasileva T, Dalgalarrondo M, Haertle T, Chobert JM, Ivanova I. 2009. Effect of oligosaccharides on the growth of *Lactobacillus delbrueckii subsp. bulgaricus* strains isolated from dairy products. J. Agric. Food Chem., 57: 9496-9502.

Ljungh A, Wadstrom T. 2007. Lactic acid bacteria as probiotics. Curr. Issues Intestinal Microbiol., 7: 73-90.

Mandagjieva T, Ignatova-Ivanova T, Kambarev S, Iliev I, Ivanova I. 2011. Utilization of different prebiotics by *Lactobacillus spp.*, and *Lactococcus spp*. Biotechnol. & Biotechnol. EQ., 25(4) Suppl., 117-120.

Petrova M, Georgieva R, Dojchinovska L, Kirilov N, Iliev I, Antonova S, Hadjieva N, Ivanova I, Danova S. 2009. Lactic Acid Bacteria against pathogenic microbes. Trakia Journal of Sciences, 7(2): 33-39.

Roberfroid MB. 2002. Functional food concept and its application to prebiotics. Digestive and Liver Disease. 34: S105–S110.

Sanz ML, Polemis N, Morales V, Corzo N, Drakoularakou A, Gibson GR, Rastall RA. 2005. In vitro investigation into the potential prebiotic activity of honey oligosaccharides. Journal of Agricultural and Food Chemistry. 53: 2914–2921.