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Novel Bulgarian *Lactobacillus* strains ferment prebiotic carbohydrates

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ABSTRACT

Prebiotics are non-digestible food ingredients that stimulate the growth or activity of the beneficial to human health bacteria in the digestive system. Generally, the prebiotic should increase the number of potentially probiotic lactic acid bacteria if they could convert it. The aim of this study is to isolate strains belonging to genus *Lactobacillus* and to examine their capability to grow in media containing prebiotic carbohydrates as a sole carbon source.

Thus, thirty two *Lactobacillus* strains were checked for its ability to convert the following mono-, di-, and trisaccharides: xylose, galactose, mannose, arabinose, fructose, rhamnose, cellobiose, melibiose, and raffinose, and also the polysaccharides inulin, xylan, carboxymethyl cellulose and pullulan. Our results revealed that all strains ferment to lactic acid galactose and mannose, and most of them - arabinose, cellobiose or fructose. Eleven strains convert melibiose (gal-glu), twelve - raffinose (gal-glu-fru). Observing the strains' capacity to hydrolyze long-chain carbohydrates, 2 strains were found to be able to ferment carboxymethyl cellulose, 4 – inulin, and no one converted xylan and pullulan. The putative gene responsible for the inulin degradation by two *Lactobacillus* strains was identified by PCR with specific primer pair. These results are important for the future application of the tested *Lactobacillus* strains in food industry. They may be useful for the development of functional foods containing prebiotic carbohydrates as well.

Key words: *Lactobacillus*, prebiotics, inulin

Introduction

Probiotics and prebiotics are food components that benefit the human health by their interactions with the gastro-intestinal tract. Prebiotics are a category of nutritional compounds grouped together, not necessarily by structural similarities, but by ability to promote the growth of specific beneficial (probiotic) gut bacteria. Many dietary fibers, especially soluble fibers, exhibit some prebiotic activity; however, non-fiber compounds are not precluded from being classified as prebiotics presuming they meet the requisite functional criteria (Kelly, 2008).

Inulin enhances the growth and activities of selected beneficial bacteria or inhibits growth or activities of certain pathogenic bacteria, hence promoting colonic health. In vitro inulin was found to selectively stimulate the growth of *Bifidobacterium* and *Lactobacillus*, which are health beneficial bacteria. This phenomenon is probably due to the fact that inulin affected short-chain fatty acids concentrations in the lumen (Muller & Seyfarth, 1997).

The fructan fermenters are mainly strains of the species *Lactobacillus paracasei* ssp. *paracasei*. There is a number of fructan-degrading enzymes in higher plants and micro-organisms distinguished by the different end-products formed during fructan hydrolysis. These include fructose, inulobiose or levanbiose, di-β-fructose dianhydrides, oligofructans and fructose-only oligomers (Ettalibi & Baratti, 1987).

The aim of this study was to observe the capability of newly isolated and potentially probiotic *Lactobacillus* strains to degrade different types of fructans, xylo-oligosaccharides

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and other prebiotic carbohydrates. Here we present new evidences for inulinase activity and the identification of the responsible genes in two novel isolates of *L. casei* group. Since many of the enzymes hydrolyzing inulin degrade also raffinose, melibiose, and other sugars and possess significant differences in substrate specificity and affinity, several di- and trisaccharides were included in the study as substrates as well.

Materials and Methods

Bacterial strains, media and cultivation conditions

Thirty lactic acid bacteria (LAB) isolates were obtained from Bulgarian traditional cereal-based fermented foods (Blagoeva et al., 2013). As a reference, the strain *Lactobacillus paracasei* B41, isolated from Bulgarian traditional fermented drink boza, prepared from wheat (Haskovo, Bulgaria) and deposited in DSMZ (German Collection of Microorganisms and Cell Cultures) under registration DSM 23505 (Petrova & Petrov, 2012) was used.

Lactococcus lactis ssp. *lactis* B84 was isolated from rye sourdough (Petrov et al., 2008). All LAB strains were maintained at 4°C (subcultured twice a month), or frozen at -80°C with 15% (w/w) glycerol added.

The detections of cellulase and xylanase activities were done by Congo red staining of agar plates as described by Samanta et al. (2011) and Carder (1986).

DNA isolations and PCR amplifications

Total genomic DNA was isolated from 24 h-old cells, grown in MRS, using GeneJET Genomic DNA Purification Kit (Thermo Scientific), following manufacturer's recommendations. PCR amplification of 16S rRNA and *levH1* genes were prepared with *Pfu* DNA Polymerase (Thermo Scientific), in a total volume 50 µl and final concentrations of primers 5 pmol/µl (Macrogen Inc., Korea). QB-96 thermocycler (LKB) was used. The amplification of the 16S rRNA gene was performed with universal eubacterial primer pair: fD1: 5' AGAGTTTGATCCTGG CTCAG 3' and rD1: 5' AAGGAGGTGATCCAGCC 3'. The final volume of the template DNA was 2 ng/µl, the temperature profile was: 95°C for 5 min, 35 cycles consisting of 94°C for 1 min, 55°C for 1 min, 72°C for 2 min, followed by final elongation at 72°C for 5 min.

The amplification of the gene *levH1* was done using primer pair INF 5'ATGGATGAAAAGAAACATTAC AAGATG3' and INR 5'TTAGACTCGCTTCACCCG CCTC3', at the following temperature profile: 95°C for 3 min, 45 cycles consisting of 94°C for 1 min, 60°C for 45 sec, 68.5°C for 4 min and 30 sec, followed by final elongation at 72°C for 10 min. The corresponding PCR products were visualized in 1% agarose gel.

DNA sequencing and phylogenetic analysis

All obtained PCR amplification products were purified using GFX PCR DNA and gel band purification kit (Amersham Biosciences) and then sequenced by Macrogen Inc. (Korea). The primers, used for the sequencing were the described above fD1 and rD1 (for 16S rDNA). The sequence analysis was performed using programs Chromas and CAP3 (http://genome.cs.mtu.edu/cap). Sequence comparison with the GenBank data was done using BLAST and ClustalW programs.

Results

Carbohydrate utilization by the tested strains of lactic acid bacteria

The initial species identification of the isolates was done by morphological, biochemical and genetic criteria (16S rDNA sequencing). However, the microbial degradation of different carbohydrates is usually strain-specific feature. Having in mind the future biotechnological applications of newly isolated lactobacilli, it was important to elucidate their ability to utilize monosaccharides that compose the olygo- and poly-sugars, included in this study. At Table 1 are presented the strains, species affiliation and the monosaccharides that they are able to covert. All strains ferment glucose (not shown), galactose and mannose, and most of them – L(+) arabinose and fructose to lactic acid.

Observing the strains' potential to degrade di- and trisaccharides (Table 2), it was found that eleven strains convert melibiose (gal-glu), twelve - raffinose (gal-glu-fru). All isolates ferment cellobiose, except *L. pentosus* N3.

The tested LAB strains were able to hydrolyze long-chain carbohydrates too. Two isolates were found to ferment carboxymethyl cellulose (*L. fermentum* strain 1) and *L. sakei* strain 3/30). Four strains, initially identified as belonging to *L. casei*/*paracasei* group converted inulin. No one of the strains hydrolyzed xylan or pullulan (Table 3).

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Table 1. *Fermentation of monosaccharides. Designations: Xyl – D(+) xylose, Gal – D(+) galactose, Man – D(+) mannose, Ara – L(+) arabinose, Fru – D(+) fructose, Rham – L(+) rhamnose.*

Strain	Species	Xyl	Gal	Man	Ara	Fru	Rham
B41	*L. paracasei*	+	+	+	+	+	-
84	*Lactococcus lactis*	-	+	+	+	+	-
Ya2	*L. paracasei*	+	+	+	-	+	-
PD3	*L. casei/paracasei*	+	+	+	+	+	+
Da4	*L. paracasei*	+	+	+	+	+	+
Pr6	*L. paracasei*	+	+	+	+	+	-
M1	*Lactobacillus* sp.	+	+	+	+	+	-
S1	*Lactobacillus* sp.	-	+	+	+	+	-
H	*Enterococcus faecium*	+	+	+	+	+	-
1.2	*Enterococcus faecium*	+	+	+	+	+	-
BX3	*L. pentosus*	+	+	+	+	+	-
Lin2	*Pediococcus acidilactici*	+	+	+	+	+	+
A1	*L. casei*	-	+	+	-	+	-
Bom3	*L. plantarum*	-	+	+	+	+	-
LC1	*L. paracasei*	+	+	+	+	+	-
81	*L. plantarum*	-	+	+	+	+	-
D	*Streptococcus bovis*	+	+	+	+	+	-
65	*L. casei*	-	+	+	-	+	-
Bx1	*L. plantarum*	+	+	+	-	+	-
1	*L. fermentum*	-	+	+	-	+	-
2	*L. fermentum*	-	+	+	-	+	-
BB2	*L. plantarum*	+	+	+	-	+	-
3/30	*L. sakei*	-	+	+	-	+	-
N3	*L. pentosus*	-	+	+	-	+	-
7	*L. casei*	+	+	+	-	+	-
BX4	*L. pentosus*	-	+	+	-	+	-
95	*L. casei*	-	+	+	-	-	-
73	*L. casei*	-	+	+	-	+	-
BX2	*L. plantarum*	-	+	+	-	+	-
93	*E. faecium/durans*	-	+	+	+	+	-
91	*E. faecium/durans*	-	+	+	+	+	-
85	*L. casei*	-	+	+	+	+	-

PCR amplification of the gene levH1, encoding putative inulinase

As the inulin belongs to a class of fructans - dietary fibers with wide application in food and medicine, it was important to identify the putative enzyme, responsible for its hydrolysis. Primer pair, targeting putative inulinase gene *levH1* in *L. casei*/*paracasei* was designed and used for PCR amplification (Figure 1). PCR amplicon, corresponding to *levH1* gene (3891 bp) was detected in two of four inulin-degrading strains: *L. paracasei* B41 and *L. casei/paracasei* LC1. Unspecific PCR product with different molecular size (≈800 bp) was received when DNA of *L. casei/paracasei* PD3 was used as a template, and no PCR amplification was obtained for *Lactobacillus* sp. strain 7.

Discussion

Probiotics and prebiotics improve human health through direct or indirect effects on the colonizing microbiota. Prebiotics, in part due to their function as a special type of soluble fiber, can contribute to the health of the general population. Two particular prebiotics then fully met this definition: trans-galactooligosaccharide and inulin (Roberfroid, 2007). Acacia gums (Gum Arabic) are considered the richest natural source. Other traditional dietary sources of prebiotics include beans, inulin sources (such as Jerusalem artichoke, jicama, and chicory root), raw oats, unrefined wheat, unrefined barley, and yacon. Some of the oligosaccharides that naturally occur in breast milk are

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believed to play an important role in the development of a healthy immune system in infants (Moshfegh et al., 1999).

Inulin-type prebiotics contain fructans of the inulin-type. Fructans are a category of nutritional compounds that encompasses naturally occurring plant oligo- and polysaccharides in which one or more fructosyl-fructose linkages comprise the majority of glycosidic bonds. To be "inulin-type" a fructan must have beta (2−1) fructosyl-fructose glycosidic bonds, which gives inulin its unique structural and physiological properties, allowing it to resist enzymatic hydrolysis by human salivary and small intestinal digestive enzymes.

Microbial enzymes attacking prebiotic carbohydrates were classified according to their affinity for the type of linkage of fructan and for the site of cleavage inside the fructose chain. They belong to GH32 family (about 1400 enzymes) that includes invertases, inulinases, levanases, sucrose-6-phosphate hydrolases, fructanotransferases, and fructosyltransferases. Most of the fructan hydrolases are classified into this family and have been separated into groups. The first group, the unspecific β-D-fructofuranosidases (β-D-fructan-fructohydrolase, EC 3.2.1.80 and β-D-fructofuranosidefructohydrolase, EC 3.2.1.26) hydrolyse the terminal unsubstituted fructose residue from the fructan chain.

Table 2. *Fermentation of di- and trisaccharides.*

Strain	Species	Cellobiose	Melibiose	Raffinose
B41	*L. paracasei*	+	+	+
84	*Lactococcus lactis*	+	-	-
Ya2	*L. paracasei*	+	+	+
PD3	*L. casei/paracasei*	+	-	+
Da4	*L. paracasei*	+	+	+
Pr6	*L. paracasei*	+	+	+
M1	*Lactobacillus* sp.	+	-	-
S1	*Lactobacillus* sp.	+	-	-
H	*Enterococcus faecium*	+	-	-
1.2	*Enterococcus faecium*	+	-	-
BX3	*L. pentosus*	+	-	-
Lin2	*Pediococcus acidilactici*	+	-	-
A1	*L. casei*	+	-	-
Bom3	*L. plantarum*	+	-	-
LC1	*L. paracasei*	+	+	+
81	*L. plantarum*	+	-	-
D	*Streptococcus bovis*	+	-	-
65	*L. casei*	+	-	-
Bx1	*L. plantarum*	+	-	-
1	*L. fermentum*	+	+	+
2	*L. fermentum*	+	+	+
BB2	*L. plantarum*	+	-	-
3/30	*L. sakei*	+	+	+
N3	*L. pentosus*	-	+	+
7	*L. casei*	+	+	+
BX4	*L. pentosus*	+	-	-
95	*L. casei*	+	-	-
73	*L. casei*	+	-	-
BX2	*L. plantarum*	+	-	-
93	*E. faecium/durans*	+	+	+
91	*E. faecium/durans*	+	-	-
85	*L. casei*	+	-	-

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Table 3. *Fermentation of polysaccharides.*

Strain	Species	Inulin	Xylan	Carboxymethyl cellulose	Pullulan
B41	*L. paracasei*	+	-	-	-
84	*Lactococcus lactis*	-	-	-	-
Ya2	*L. paracasei*	-	-	-	-
PD3	*L. casei/paracasei*	+	-	-	-
Da4	*L. paracasei*	-	-	-	-
Pr6	*L. paracasei*	-	-	-	-
M1	*Lactobacillus* sp.	-	-	-	-
S1	*Lactobacillus* sp.	-	-	-	-
H	*Enterococcus faecium*	-	-	-	-
1.2	*Enterococcus faecium*	-	-	-	-
BX3	*L. pentosus*	-	-	-	-
Lin2	*Pediococcus acidilactici*	-	-	-	-
A1	*L. casei*	-	-	-	-
Bom3	*L. plantarum*	-	-	-	-
LC1	*L. paracasei*	+	-	-	-
81	*L. plantarum*	-	-	-	-
D	*Streptococcus bovis*	-	-	-	-
65	*L. casei*	-	-	-	-
Bx1	*L. plantarum*	-	-	-	-
1	*L. fermentum*	-	-	+	-
2	*L. fermentum*	-	-	-	-
BB2	*L. plantarum*	-	-	-	-
3/30	*L. sakei*	-	-	+	-
N3	*L. pentosus*	-	-	-	-
7	*L. casei*	+	-	-	-
BX4	*L. pentosus*	-	-	-	-
95	*L. casei*	-	-	-	-
73	*L. casei*	-	-	-	-
BX2	*L. plantarum*	-	-	-	-
93	*E. faecium/durans*	-	-	-	-
91	*E. faecium/durans*	-	-	-	-
85	*L. casei*	-	-	-	-

Raffinose (melitose, melitriose) is a trisaccharide composed of galactose, glucose, and fructose. It can be hydrolyzed to D-galactose and sucrose by the enzyme α-galactosidase (α-GAL), an enzyme not found in the human digestive tract. Melibiose is a reducing disaccharide formed by an alpha-1,6 linkage between galactose and glucose (D-Gal-α(1→6)-D-Glc). Our results revealed that the majority of the strains that convert raffinose, digest melibiose too, suggesting that these isolates display α-galactosidase activity. One exception is the strain *L. casei* PD3, which did not degrade melibiose, but hydrolyzed raffinose and inulin, most probably due to the action of β-D-fructofuranosidases.

The *levH1* gene encoded a protein LevH1 (Kuzuwa et al., 2012), which calculated molecular mass and pI were 138.8 kDa and 4.66, respectively. LevH1 (1296 amino-acids long) was predicted to have a four-domain structure, containing (i) an N-terminal secretion signal of 40 amino-acids, (ii) variable domain of about 140 residues whose function is unclear, (iii) a catalytic domain of about 630 residues with glycoside-hydrolase activity consisting of two modules (Figure 2). The presence of *levH1* gene, encoding exo-type inulinase (fructan β-fructosidase) from GH32 family in two of the newly isolated strains explains their capability to degrade inulin.

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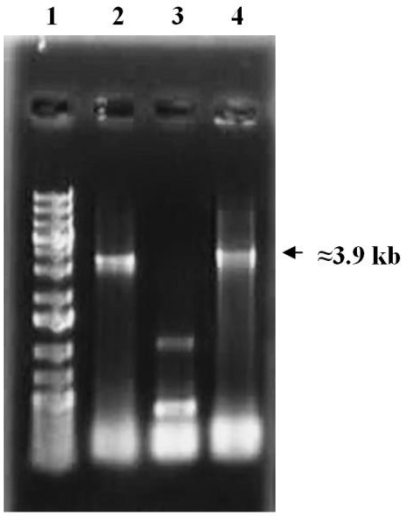

Figure 1. *PCR amplification with primer pair, targeting levH1 gene in L. casei/paracasei. Lanes and samples: 1) 1 kb plus ladder (Thermo Scientific), 2) L. paracasei B41, 3) L. casei PD3, 4) L. paracasei LC1.*

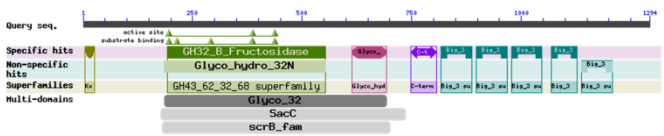

Figure 2. *Domain structure of LevH1, a putative inulinase of L. casei/paracasei (http://blast.ncbi.nlm.nih.gov/Blast.cgi).*

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