In vitro Fermentability of Prebiotic Oligosaccharides by Lactobacilli

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Abstrakt

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Twelve strains of lactobacilli were tested for their growth and ability to utilise six prebiotics (pure substances and commercially available prebiotics) as a sole carbon source. All strains showed a considerable growth on all prebiotics tested. Inulin was the best carbohydrate source for lactobacilli, followed by lactulose and raffinose. A massive increase of viable cells on commercial prebiotic mixtures (Vivinal, Oligomate 55, and Orafti P95) was also observed. Lysozyme susceptibility was assayed in 13 strains of lactobacilli. Eight out of 13 strains were completely resistant to the lysozyme concentration of 400 μg/ml, in the rest of the strains a slight delay of the exponential phase of the growth curves was observed. Lactobacilli tolerated lysozyme well and were able to utilise all prebiotics.

Keywords: prebiotics utilisation; lactobacilli; fructooligosaccharides; galactooligosaccharides; lysozyme susceptibility

Prebiotics are oligosaccharides defined as "non-digestible food ingredients that, when consumed in sufficient amounts, selectively stimulate the growth and/or activity of one or a limited number of microbes in the colon resulting in documented health benefits" (Ouwehand *et al.* 2007). They positively affect the composition and metabolic activity of the intestinal microflora and a daily moderate supplement of these non-digestible oligosaccharides stimulates mineral (especially Ca and Mg) absorption (Van Loo *et al.* 1999). Prebiotic compounds can also have immunomodulatory properties, with and without the addition of probiotic bacteria (Reid 2008). In addition, the effects of prebiotics as stabilising agents in probiotic products during storage, freeze-drying, and spray-drying were reported by more authors (Desmond *et al.* 2005; Schwab *et al.* 2007). Other properties of oligosaccharides are, for example, a low calorific value, reduced sweetness, acting as anti-caries agents, posibility to modify the viscosity and freezing point of foods, etc. (Playne & Crittenden 1996).

Most of the studies on prebiotics concern fructooligosaccharides (FOS) and galactooligosaccharides (GOS). FOS can be found in plants (chicory roots, garlic, onion, etc.) while GOS are synthesised from lactose via enzymatic transgalactosylation using β-galactosidase (Boehm *et al.* 2004). Most companies produce several purity grades of their oligosaccharide mixtures, produced either as a powder or syrup (Playne & Crittenden 1996).

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According to Roberfroid (2007), the criteria for prebiotics classification are the following: resistance to gastric acidity, hydrolysis by mammalian enzymes and gastrointestinal absorption, fermentation by intestinal microflora and selective stimulation of the growth and/or activity of intestinal bacteria associated with health and well-being. According to the author, there are presently only two food ingredients that fulfill all these criteria – inulin and *trans*-galactooligosaccharides (TOS). As concerns candidates, the data are promising, but more studies are still required.

Lysozyme is an antimicrobial enzyme occurring naturally in tears, saliva, blood, breast milk and other body fluids. It catalyses the hydrolysis of polysaccharide chains consisting of *n*-acetyl-glucosamine units and the rest of the *n*-acetylmuramic acid, which are responsible for the strenght of bacterial cell walls (Vodrážka 1999). In the EU, lysozyme from hen egg white has a status of a food additive and hence it has an E-code, E 1105 (Directive 95/2/EC). As a food additive, lysozyme has been permitted for use in ripened cheeses for preventing the outgrowth of *Clostridium tyrobutyricum* spores (Meyer 2003). Lysozyme resistance is therefore desirable in lactic acid bacteria used in dairy industry.

The aim of this work was to investigate the fermentation properties of the lactobacilli strains (both industrial and of human origin) in *in vitro* conditions in media containing different prebiotics as a sole carbohydrate source. In addition, the susceptibility to antimicrobial enzyme lysozyme of 13 strains of lactobacilli was tested.

MATERIAL AND METHODS

Tested strains. The list of isolates is shown in Tables 1 and 2. Industrial isolates (originating from different sources of dairy products) and human isolates of lactobacilli were included in this work. The industrial isolates were obtained from the Culture Collection of Dairy Microorganisms Laktoflora® – CCDM (Prague, Czech Republic) and the human isolates originated from biopsy samples or faeces of pediatric patiens (aged 2 to 18 years) with a wide range of gastroenterological diagnoses (non-specific inflammatory bowel disease, chronic diarrhoea, polyps, etc.) obtained from the Faculty Hospital in Prague-Motol (Czech Republic).

Susceptibility of strains to lysozyme. The susceptibility to lysozyme was tested according to Rada *et al.* (2010). Bacterial growth was determined using Densitometer DEN-1 (Dynex, Prague, Czech Republic) based on the OD_{540} values. The

Table 1. Tolerance of tested strains to lysozyme (400 μg/ml)

Strain	Origin	Resistant*	Partially sensitive**
Lbc. brevis 202	human faeces	+	–
Lbc. paracasei subsp. *paracasei* 212	human faeces	+	–
Lbc. fermentum RL25	human faeces	+	–
Lbc. rhamnosus 150	curd, CZ	+	–
Lbc. delbrueckii subsp. *bulgaricus* 66	yogurt, Turkey	+	–
Lbc. acidophilus 151	pill Biolacta	+	–
Lbc. casei subsp. *paracasei* DM1TA6–P	biopsy sample (colon)	+	–
Lbc. casei subsp. *paracasei* PE1TB-P	biopsy sample (colon)	+	–
Lbc. gasseri PHM-7E1	biopsy sample (colon)	–	+
Lbc. delbrueckii subsp. *bulgaricus* 767	yogurt, Switzerland	–	+
Lbc. casei subsp. *casei* 198	Eidam cheese, CZ	–	+
Lbc. animalis 382	raw goat milk	–	+
Lbc. helveticus 62	human faeces	–	+

*Lactobacilli in the group "resistant" tolerated lysozyme in the concentration 400 μg/ml without affecting their growth curves compared with control (without lysozyme addition)

**growth curves of strains in the group "partially sensitive" were influenced by the addition of lysozyme in smaller extent (exponential phases of the growth curves were slightly delayed)

No isolates were sensitive to lysozyme at the concentration 400 μg/m

results were evaluated in MS Excel 2007 (Microsoft, Redmond, USA).

Bacterial growth in the presence of prebiotics. Twelve isolates of lactobacilli (Table 2) were tested for their growth in the presence of the following prebiotics: galactooligosaccharides Vivinal® (Humana GmbH, Wiesbaden, Germany) and Oligomate 55NP (Yakult Pharmaceutical Ind. Co, Ltd., Tokyo, Japan), inulin and lactulose (both Sigma Aldrich, Prague, Czech Republic), raftilose – Orafti P95 (Beneo-Orafti, Tienen, Belgium), and D-raffinose (LaChema, Brno, Czech Republic). The bacterial growth in the presence of prebiotics was tested according to Rada *et al.* (2008) with some modifications. Briefly, 0.3 ml of the respective bacterial cultures (in the exponential growth phase) grown in Wilkins Chalgren broth (Oxoid, Basingstoke, UK) were injected into 10 ml of complex medium (tryptone 10 g, peptone 10 g, yeast extract 5 g, Tween 80 1 ml, L-cysteine hydrochloride 0.5 g, distilled water 1 l, pH = 7). The medium was supplemented with different prebiotic substrates: raffinose, lactulose, inulin, raftilose or galactooligosaccharides (2 g) each as a sole carbon source. The purity of the prebiotic Orafti P95 is 95%, the rest (5%) is made up of fructose and glucose. The purity of GOS – Vivinal and Oligomate is about 55–60%. The rest (40–45%) consists of unreacted lactose, further of glucose and galactose. Mono- and disaccharides do not have any prebiotic properties. For this reason, they were removed from the media using precultivation with *Lactobacillus helveticus* CCDM 40 at 37°C for 16 hours. This strain utilises only glucose, galactose, fructose, and lactose (tested by API 50 CHL, BioMerieux, Marcy l'Etoile, France). Monosaccharide contents were measured reflectometrically before and after cultivation using Reflectoquant RQ flex device (Merck, Darmstadt, Germany). When the mono- and disaccharide contents decreased below the detection threshold of the test (16 h), *Lactobacillus helveticus* was removed from the media by centrifugation (6000 g/7 min) and subsequently the supernatant was colected, pH was adjusted to 7, and the media were sterilised (114°C/20 min). The prebiotic media obtained (without mono- and disaccharides) were thusy ready for the inoculation with the tested strains. The growth ability was tested using 96-well microtitre plates (Böttger, Hamburg, Germany). Each well contained 100 µl of medium containing prebiotic and was inoculated with 10 µl of bacterial suspension which was in the exponential growth phase. All strains were grown in triplicate on each carbohydrate source. The microtitre plates were incubated in an anaerobic jar (Oxoid, Hampshire, UK) at 37°C for 24 hours. The counts of lactobacilli on hour 0 and 24 were determined using MRS agar (pH 5.7; Merck, Darmstadt, Germany). Lactic acid contents in the media containing inulin and lactulose were measured using Reflektoquant® Equipment (Merck, Darmstadt Germany).

Statistical analyses. Statistical analyses were done using STATISTICA Cz 9.1 (StatSoft, Prague, Czech Republic) and Statgraphics® Centurion XV (StatPoint, Inc., Warrenton, USA). The significance of differences between the counts of lactobacilli were evaluated by the multiple range comparison with multiple range tests – LSD test by Statgraphics and then Tukey's HSD test by STATISTICA Cz version 9.1.

RESULTS AND DISCUSSION

Lysozyme susceptibility testing

The influence of the addition lysozyme on the growth of the tested strains is summarised in Table 1. In resistant strains (8 out of 13), the concentration of lysozyme (400 µg/ml) had no effect on the growth of the tested strains where growth curves were not affected as compared with controls without lysozyme. They were completely lysozyme-resistant. In 5 out of 13 lactobacilli, the growth curves were partially influenced and after lysozyme addition (in the first hour of incubation), a slight delay of the exponential phase of the growth curves was observed (Figure 1). However, the final counts of lactobacilli after 24-h of incubation were not affected in comparison to the control. None of the tested strains was classified as susceptible to the above mentioned concentration of lysozyme and the origin of the strain had no influence on its lysozyme susceptibility. It seems that lactobacilli, in general, tolerate lysozyme well, regardless of their origins. Guglielmotti *et al.* (2007) tested the strains of *Lactobacillus delbrueckii* and their phage mutants resistant to lysozyme. Heterogenous behaviour in the ability to grow in the presence of lysozyme at concentrations of 25, 50, and 100 ppm was observed, while the phage resistant mutants seemed to be more resistant to lysozyme. In the study of Neujahr *et al.* (1973), the sensitivity of the strain *L. fermentum* to lysozyme varied with

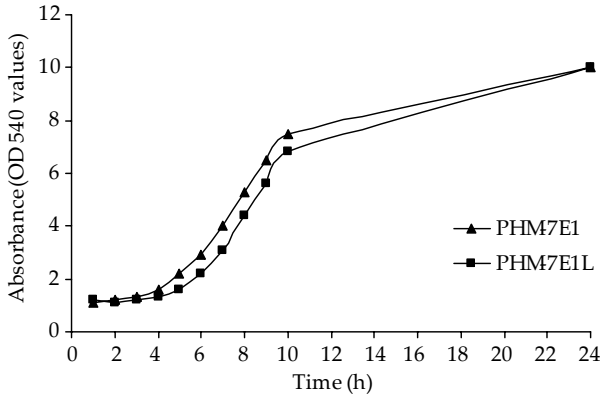

Figure 1. Growth of *Lactobacillus gasseri* PHM-7E1 without addition of lysozyme (▲) and in lysozyme (400 μg/ml) presence (■)

the growth phase, the peak having been observed between the mid-exponential and early stationary phases of the growth, while the cells from the stationary growth phase were resistant to lysozyme.

Bacterial growth in the presence of prebiotics

The counts of the tested strains on the defined prebiotic substrates after 24-h incubation are shown in Table 2. The average count of lactobacilli before cultivation in media with prebiotics was approximately 4.29 log CFU/ml. Based on the counts of viable cells, it was found that the most fermented prebiotic was inulin, followed by lactulose and raffinose with which the counts of viable cells increased by 3 to 4 orders of magnitude. Ccommercially available prebiotic mixtures, Vivinal, Oligomate, and Orafti P95, were utilised to a somewhat smaller extent. On these substrates, the counts of lactobacilli increased by 2 to 3 orders of magnitude. It can be stated that the growth of all lactobacilli assayed in this study was stimulated by all prebiotics tested. However, a statistically significant difference was observed between the bacterial growth on particular prebiotics (Table 2) with the best growth on inulin. Between the pairs lactulose – rafinose and Vivinal – Oligomate no significant difference was recorded. The prebiotic fermented to the smaller extent was Orafti P95. The bacterial growth was accompanied by an increase of lactic acid content in media. The average production of lactic acid in 12 strains tested was 671 mg/l in the medium with inulin and 527 mg/l in the medium with lactulose.

Table 2. Utilisation of different prebiotic substrates after 24-h incubation

Strain	Vivinal	Oligomate	Inulin	Lactulose	Orafti P95	Raffinose
CCDM 198†	6.72 ± 0.11	6.86 ± 0.05	7.95 ± 0.05	7.72 ± 0.03	6.62 ± 0.15	7.88 ± 0.10
CCDM 150†	7.19 ± 0.10	6.90 ± 0.04	8.24 ± 0.15	7.72 ± 0.06	6.60 ± 0.03	8.05 ± 0.13
CCDM 151†	6.82 ± 0.14	6.83 ± 0.05	8.08 ± 0.07	7.96 ± 0.05	6.71 ± 0.11	7.94 ± 0.07
CCDM 767†	6.82 ± 0.10	6.83 ± 0.09	7.92 ± 0.04	7.90 ± 0.10	6.55 ± 0.05	7.96 ± 0.06
CCDM 66†	6.80 ± 0.09	6.88 ± 0.10	7.25 ± 0.14	7.98 ± 0.07	6.56 ± 0.15	7.82 ± 0.12
RL 25‡	6.92 ± 0.03	7.03 ± 0.13	7.90 ± 0.06	7.61 ± 0.02	6.45 ± 0.05	7.69 ± 0.20
DM1TA6-P‡	6.88 ± 0.18	6.86 ± 0.06	8.48 ± 0.05	8.08 ± 0.08	6.53 ± 0.06	8.08 ± 0.17
PE1TB-P‡	6.76 ± 0.15	6.91 ± 0.09	8.54 ± 0.16	8.18 ± 0.11	6.63 ± 0.05	7.97 ± 0.20
PHM-7E1‡	6.73 ± 0.04	6.64 ± 0.08	8.41 ± 0.10	8.00 ± 0.15	6.63 ± 0.08	7.97 ± 0.14
CCDM 62‡	6.42 ± 0.12	6.48 ± 0.17	8.89 ± 0.10	8.20 ± 0.08	6.20 ± 0.04	7.51 ± 0.30
CCDM 212‡	6.64 ± 0.05	6.51 ± 0.10	7.86 ± 0.12	7.35 ± 0.05	6.47 ± 0.07	7.56 ± 0.15
CCDM 202‡	6.32 ± 0.10	6.43 ± 0.16	8.87 ± 0.07	8.06 ± 0.05	6.10 ± 0.10	8.26 ± 0.18
Average	6.75 ± 0.23[b]	6.76 ± 0.20[b]	8.20 ± 0.47[d]	7.90 ± 0.25[c]	6.50 ± 0.18[a]	7.89 ± 0.22[c]

Data are expressed as log CFU/ml; values are means from triplicate determination ± standard deviation (SD)

†industrial isolates; ‡human isolates

[a–d]data in lines with different superscripts differs ($P < 0.05$)

CCDM 198 – *Lactobacillus casei* subsp. *casei*; CCDM 150 – *Lactobacillus rhamnosus*; CCDM 151 – *Lactobacillus acidophilus*; CCDM 767, 66 – *Lactobacillus delbrueckii* subsp. *bulgaricus*; RL 25 – *Lactobacillus fermentum*; DM1TA6-P, PE1TB-P – *Lactobacillus casei* subsp. *paracasei*; PHM-7E1 – *Lactobacillus gasseri*; CCDM 62 – *Lactobacillus helveticus*; CCDM 212 – *Lactobacillus paracasei* subsp. *paracasei*; CCDM 202 – *Lactobacillus brevis*

Monosaccharide contents before preincubation with *Lactobacillus helveticus* in the tested media were as follows: in the medium with Vivinal® 413 mg/l, in the medium with Oligomate 378 mg/l, and in the medium with Orafti P95 195 mg/l. After precultivation all media contained less than 65 mg/l (detection treshold of the Reflectoquant test) of monosaccharides.

According to the available literature, one of the best effect on the growth of lactobacilli seems to be exhibited by fructooligosaccharides (Rycroft *et al.* 2001; Barrangou *et al.* 2003). The growth of *Lactobacillus delbrueckii* strains and their phage resistant mutants on prebiotics was investigated by Guglielmotti *et al.* (2007). The most fermented prebiotic for all strains assayed was inulin which corresponds to our results. Lactulose was also fermented, while xylitol and raffinose were poorly fermented by all strains. Pan *et al.* (2009) tested the growth of two strains of lactobacilli (*L. plantarum, L. acidophilus*) in the presence of FOS, xylooligosaccharides (XOS), chitooligosaccharides (COS) and mannooligosaccharides (MOS). All oligosaccharides tested were fermented by lactobacilli, and XOS obviously improved their maximal growth. In addition, the stress resistance of lactobacilli was significantly improved. Similarly, Rycroft *et al.* (2001) also demonstrated that XOS and FOS were good carbohydrate sources and enhanced the increase of lactobacilli population.

The ability of microorganisms to utilise prebiotics is strain and substrate specific (Shah 2001; Pan *et al.* 2009). Based on our results, there was no significant difference between the growth of industrial isolates (from dairy products) and human isolates (from biopsy samples and faeces). More studies pointed out a possible relationship between the bacterial count and polymerisation degree (DP) of prebiotics. Al-Tamimi *et al.* (2006) observed an increase of bifidobacteria numbers as DP decreased. Similarly, a study using three strains of bifidobacteria showed a decreased rate of FOS utilisation and decreased lactate and acetate productions as the DP increased (Perrin *et al.* 2002). In our study, the growth on inulin obtained from chicory was excellent despite the fact that the DP was rather high (n = 36). We expected a better growth on the prebiotic Orafti P95 (with the DP about 4) but our expectations were not confirmed. We suppose that an important role may have been played by the precultivation with *Lactobacillus helveticus* CCDM 40 in the case of Orafti P95. Inulin from chicory was not precultivated because it is a pure substance and does not contain monosacharide units. One of posssible reasons for this finding may be the fact that the precultivation significantly reduced the amount of the growth substrate for bacteria.

In conclusion, we showed that lactobacilli are able to utilise the most commonly used prebiotic oligosaccharides while the best growth was observed on inulin, followed by lactulose and raffinose. In the presence of these prebiotics statistically significant differences in the growth of lactobacilli were observed. Especially inulin do we reccommend as a prebiotic additive to dairy products containing lactobacilli. In addition, the antimicrobial enzyme lysozyme was tolerated very well by all tested strains which is desirable in lactic acid bacteria used in dairy processing.

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