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STIMULATION OF BIFIDOBACTERIA GROWTH BY OLIGOFRUCTOSE AND MUCIN

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Plant polysaccharides and mucins are probably the most important nutrition agents for bacterial growth and maintenance in the large intestine. The aim of the study was to determine the influence of mucin and oligofructose on bifidobacteria propagation *in vitro*. The growth and acidifying activity of the strains in the minimal nutrition medium containing 0.2 or 2.0% of oligofructose, mucin or a mixture of oligofructose and mucin at a ratio of 1:1 in comparison to lactose, as the control, was examined after 24-h incubation at a temp. of 37°C. Among ten strains tested the growth of six, belonging to *B. longum* and *B. animalis* subsp. *animalis,* and *B. animalis* subsp. *lactis*, in the presence of oligofructose was comparable or significantly higher in comparison to lactose. Mucin was utilized by *B. bifidum* strains and by one *B. breve* strain. The growth of five out of nine mucin- or oligofructose--utilizing strains in the presence of the higher dose of the substrate in the culture medium was significantly stimulated by the mixture of oligofructose and mucin ($p \leq 0.05$). The results observed indicate that intestinal mucins may influence the growth of *Bifidobacterium* and bacterial ecology in the intestine.

INTRODUCTION

Plant polysaccharides and mucins are probably the most important source of carbon and energy for resident saccharolytic bacterial groups in the large intestine, particularly in the distal bowel, where fermentable carbohydrates are limited. Fructooligosaccharides, inulin, galactooligosaccharides, and other indigestible carbohydrates belong to prebiotic carbohydrates defined as "nondigestible food ingredients that beneficially affect host health by selective stimulating the growth and/or activity of one or a limited number of bacteria in the colon [Gibson & Roberfroid, 1995]. Prebiotics alone or in combination with probiotic bacteria, as synbiotics, are recognized as having the ability to influence and improve the gastrointestinal health of humans [Tuohy *et al*., 2003]. According to current knowledge, food components that exert the best prebiotic effects are inulin-type fructans [Kleessen *et al*., 2001].

Although the principal substrates for gut bacterial growth are dietary carbohydrates, also amino acids, bacterial secretions and lysis products, exfoliated epithelial cells and mucin play an important role as well. Especially, mucin glycoproteins are a considerable source of carbohydrates for bacteria growing in the large intestine. The mucus is continuously produced and secreted into luminal contents by goblet cells in the colonic mucosa. Nevertheless, salivary, gastric, biliary, bronchial, and small intestinal mucins also enter the colon with the digesta from the upper gut. It is estimated that from 2 to 3 g of mucin enter human large bowel each day from the upper digestive tract [Stephen *et al.,* 1983]. Also, partly digested plant materials transferred in the gut are entrapped in this viscous and slippery mucosal gel, which must be broken down to facilitate the access of intestinal microorganisms to the food residues. On the other hand, too extensive mucin degradation nearby the mucosa might impair the protective mucin layer which lines the colon and, thus, increase the vulnerability of the mucosa to harmful substances in the colon contents and some types of colonic disease.

Lactobacilli and bifidobacteria are among known resident intestinal bacteria that are stimulated by prebiotics. So far, studies on the elaboration of efficient synbiotics for humans have shown that the ability of lactobacilli and bifidobacteria to metabolize prebiotic carbohydrates is both a strain- and substrate-specific feature [Schrezenmeir & de Vrese, 2001]. Although quality and quantity of the resident bacteria of the colon depend to a large extent on exogenous nutrients as substrates for energy and growth, it has been proved experimentally difficult to produce consistent changes by dietary manipulation [Venketeshwer, 2001; Santos *et al.,* 2006].

The main objective of this study was to examine mucin degradation activity *in vitro* of the *Bifidobacterium* strains as well as the influence of mucin on bifidobacteria growth in the presence of oligofructose. The ability of the *Bifidobacterium* strains to grow in the presence of mucins and plant polysaccharides may be significant in bifidobacteria ecology.

MATERIALS AND METHODS

Bacterial strains and growth conditions

All strains used in the studies were isolated in our laboratory from the gastrointestinal tract of infants (*B. longum*

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KNA1 and KN4, *B. breve* KN3 and KN14), adults (*B. bifidum* KD6E and KD7E), rats (*B. animalis* subsp. *animalis* PS37 and KSP4), and from commercial bioyoghurts (*B. animalis* subsp. *lactis* BI11 and J38). The strains were identified to species using phenotypic and molecular methods [Wasilewska *et al*., 2003; Markiewicz *et al.,* 2009 (in press)].

Strains were maintained frozen at –70°C in reconstituted skim milk (5% dry wt.) supplemented with saccharose (10% w/v) at a ratio of 1:2. Before every experiment, strains from frozen stocks were subcultured twice in Garche's broth (G broth; [Teraguchi *et al.,* 1982], containing in grams per liter: bacto peptone – 20.0, yeast extract – 2.0, L-cysteine hydrochloride – 0.4, lactose – 10.0, CH_3COONa – 6.0, $MgSO_4$ x 7 H_2O – 0.12, Na_2HPO_4 x 12 H_2O – 2.5, KH_2PO_4 – 2.0, and agar – 1.0; pH after sterilization and cooling – 6.4. The broth was inoculated with 3% (v/v) of active bifidobacterial culture and incubated in anaerobic jars (AnaeroGen™, Oxoid) at a temp. of 37°C until stationary phase has been achieved (pH ~4.4).

Mucin and oligofructose utilization

Criteria for the evaluation of polysaccharides utilization were acidifying activity and bifidobacteria growth related to the growth in the control medium with lactose. Lactose was chosen a control substrate as being a well-metabolized source of carbon and energy for bifidobacteria and the strains tested were isolated and propagated in the lactose-containing medium. The growth of bifidobacteria in the presence of glucose was also checked for comparison. Ability of bifidobacteria to utilize mucin (Sigma; M2378) and oligofructose (BENEO P95, ORAFTI, Belgium) was examined in minimal Garche's broth (MG) without carbohydrate and with a lowered meat peptone content (1.0% w/v). MG broth was inoculated with 1% (v/v) of active bifidobacterial culture and poured quantitatively into tubes. To each tube 0.2 or 2.0% (w/v) of mucin, oligofructose, a mixture of mucin and oligofructose at a ratio of 1:1, as well as glucose or lactose (as the control) were added. Incubation was carried out anaerobically at a temp. of 37°C for 24 h. The number of bifidobacterial cells was determined as colony forming units (CFU) per millilitre directly after inoculation and after incubation. For CFU determination the cultures were serially diluted in 1% peptone water (containing: 0.5% (w/v) meat peptone – Peptobak, BTL, Poland; and 0.5% (w/v) pancreatic hydrolysate of casein – bio-Trypcase, bioMerieux, France; pH 7.0, agar 0.1% (w/v)), plated on the Garche's agar (agar 1.5% w/v) and incubated anaerobically at a temp. of 37°C for 48 h. The dilution rates of samples were taken into consideration during calculation of the results. Acidifying activity of the strains was determined as pH changes using HI 9025 microcomputer pH meter (Hanna Instruments). Each assay was performed in duplicate.

Statistical analysis

The results are presented as the average of at least two independent experiments with standard deviation. Each experiment was performed with two parallels to correct for an intra-assay variation. The results were analysed using Microsoft Office Excel 2003 statistical package and Student's t-test. Differences were considered significant at $p \leq 0.05$.

RESULTS

The tested strains of bifidobacteria differed in their ability to utilize all the compounds tested (Figures 1 and 2). None of the *B. animalis* subsp. *animalis* and *B. animalis* subsp. *lactis* strains tested grew in the presence of mucin, whereas, all of them well multiplied in the media containing lactose, glucose or oligofructose at both doses applied (Figure 1). After 24-h incubation at a temp. of 37°C, the population number of the strains ranged from 7.96 to 8.79 and from 8.43 to 9.27 log CFU/mL in the cultures containing 0.2 or 2.0% of the substrate tested, respectively. A clearly visible tendency for a better growth in the presence of 2.0% of the substrate in the culture medium was observed, however, only the population count of *B. animalis* subsp. *lactis* BI11 and *B. animalis* subsp. *animalis* PS37 strain was significantly ($p \leq 0.05$) higher in comparison to the growth in the presence of the lower dose of glucose and oligofructose, respectively (Figure 1). The presence of both oligofructose and mucin in the culture medium resulted in similar or enhanced growth in comparison to single compounds. Only the growth of *B. animalis* subsp. *lactis* J38 strain was, however, significantly ($p \leq 0.05$) higher in the culture containing 2.0% of the mixture of oligofructose and mucin in comparison to its growth in the culture containing 2.0% of oligofructose alone. An acidifying activity of the *B. animalis* strains was differentiated depending on the substrate and its concentration, and only to a smaller extent on the strain studied. The lowest pH values were in turn reported for lactose, glucose, oligofructose, the mixture of oligofructose and mucin, and mucin; and the values were higher in the cultures grown in the media containing the lower dose of the substrates tested (Table 1).

The human-originated strains differed in their ability to grow in the presence of all substrates tested (Figure 2). No significant growth of both *B. longum* strains tested was observed in the presence of mucin, independently of the applied dose of the substrate. The strains revealed also poor growth in the presence of 0.2% of lactose or glucose in the culture medium; however, they were observed to well utilize for their growth the applied low doses of oligofructose, and the mixture of oligofructose and mucin (Figure 2). In turn, both *B. longum* strains grew well in the presence of 2.0% of lactose, glucose or oligofructose in the culture medium. Their population number ranged from 8.25 to 9.19 log CFU/mL and in comparison to lactose was significantly ($p \leq 0.05$) higher in the presence of oligofructose. The mixture of oligofructose and mucin, as compared to single compounds, was also found to exert a significant ($p \leq 0.05$) synergistic effect on both *B. longum* strains. As regards the remaining human-originating strains, both *B. breve* strains were characterised by good ability to utilize both doses of lactose, but considerably poorer ability to utilize glucose, and by inability to utilize oligofructose (Figure 2). *B. breve* KN14 revealed growth only in the presence of 2.0% of mucin in the culture medium. The most differentiated growth in the presence of the compounds tested was observed for both *B. bifidum* strains. After 24-h incubation in the presence of lactose or glucose, the population count (independently from the amount of the substrate in the culture medium) ranged from 7.24 to 7.97 log CFU/mL (Figure 2), with the exception

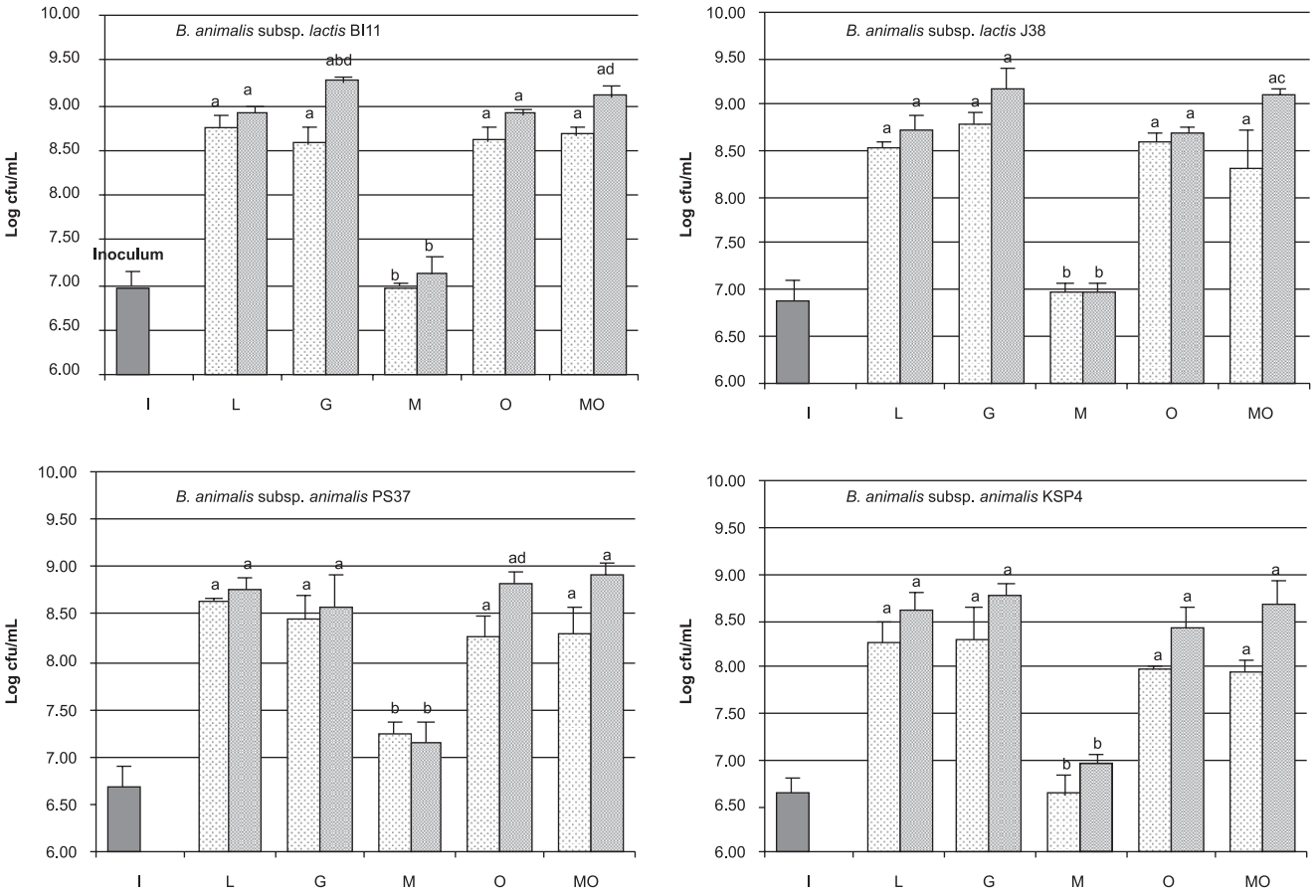

FIGURE 1. Growth of animal-originated *Bifidobacterium* strains cultured in minimal Garche's medium containing 0.2 ⬚ or 2.0% ▨ (w/v) of lactose – L, glucose – G, mucin – M, oligofructose – O and a mixture of mucin and oligofructose (at a ratio of 1:1) – MO. The cultures were incubated anaerobically at 37°C for 24 h. The letters above the columns means counts of bacteria different at the significance level $p \leq 0.05$, respectively from: [a] – the initial level (inoculum), [b] – the control growth in the presence of the same concentration of lactose, [c] – the well observed growth in the presence of the same concentration of mucin or oligofructose (regards only counts of bacteria cultured in the medium containing the mixture of oligofructose and mucin), [d] – the growth in the medium containing 0.2% of the same substrate.

of KD6E which did not grow at 0.2% of glucose. This strain did not utilize oligofructose either, whereas KD7E achieved the population number of 7.5 log CFU/mL at both concentrations tested. Both strains, however, utilized mucin achieving the population number of 7.95/8.14 and 8.65/8.64 log CFU/mL in the presence of 0.2 and 2.0% of mucin, respectively. The numbers were significantly higher ($p \leq 0.05$) in comparison to lactose, except for KD7E strain which achieved comparable to lactose growth in the medium containing 0.2% mucin. The synergistic effect of the mixture of oligofructose and mucin on the growth of both *B. bifidum* strains (in comparison to single compounds) was stated ($p \leq 0.05$) for the higher dose of oligofructose and mucin. An acidifying activity of human-originating strains cultured in the presence of the compounds tested revealed a similar tendency as described for animal- and yoghurt-originating strains, yet greater differences were observed between the strains (Table 1).

DISCUSSION

Polysaccharide fermentation is one of the most important bacterial activities in the colon [McFarlane *et al.,* 1995]. However, the undigested chyme that reached the colon is not an optimum medium for bacterial growth [Ballongue, 1997]. Limited sources of carbon and energy cause increased competition for nutrients. Consequently, organisms that are predisposed to ferment prebiotic sugars are enriched, presumably at the expense of those that are not. The application of two doses of the substrate as a source of carbon and energy was aimed at a better differentiation of abilities of the tested bacterial strains to utilise the compounds examined. The experimental results revealed that the animal-originated strains of bifidobacteria grew well in the presence of both the higher and limited dose of the substrates tested with the exception of mucin. However, the growth of the human-originated strains, especially in the presence of the lower amount of substrate in the medium, was differentiated, both in the presence of lactose as control and other examined sugars. *B. longum* strains revealed poor ability to grow in the medium containing the low dose of lactose or glucose, whereas, both *B. bifidum* strains and one *B. breve* strain (KN14) revealed none or very poor growth at the low dose of glucose during 24-h incubation. As regards the compounds not digested in the upper gastrointestinal tract, both *B. bifidum* strains and one *B. breve* (KN14) strain, growing well in the presence of both or the higher dose of mucin, respectively, seem to be predisposed

TABLE 1. Acidifying activity of the *Bifidobacterium* strains cultured in minimal Garche's medium containing 0.2% or 2.0% (w/v) of lactose, glucose, mucin, oligofructose or a mixture of mucin and oligofructose (at a ratio of 1:1)[1].

Strain	Inoculum[2]	Addition of substrate – 0.02%					Addition of substrate – 2.0%				
		lactose[3]	glucose[3]	mucin[4]	oligofructose[3]	mucin and oligofructose[5]	lactose[3]	glucose[3]	mucin[4]	oligofructose[3]	mucin and oligofructose[5]
B. animalis subsp. *animalis* PS37	6.00 ± 0.01[6]	4.83 ± 0.04	5.13 ± 0.04	5.64 ± 0.06	5.15 ±0.06	5.42 ± 0.04	4.19 ± 0.04	4.48 ± 0.03	5.29 ± 0.06	4.44 ± 0.06	4.39 ± 0.05
B. animalis subsp. *animalis* KSP4	6.01 ± 0.02	4.87 ± 0.06	4.98 ± 0.03	5.79 ± 0.03	5.25 ±0.08	5.39 ± 0.04	4.13 ± 0.07	4.46 ± 0.06	5.35 ± 0.07	4.47 ± 0.06	4.39 ± 0.07
B. animalis subsp. *lactis* BI30	6.03 ± 0.02	4.93 ± 0.04	5.35 ± 0.05	5.84 ± 0.04	5.30 ± 0.06	5.51 ± 0.07	4.13 ± 0.04	4.60 ± 0.06	5.37 ± 0.01	4.82 ± 0.08	4.74 ± 0.07
B. animalis subsp. *lactis* J38	6.02 ± 0.01	4.87 ± 0.03	5.01 ± 0.01	5.83 ± 0.04	5.32 ± 0.06	5.58 ± 0.05	4.05 ± 0.04	4.38 ± 0.04	5.36 ± 0.02	4.80 ± 0.09	4.70 ± 0.04
B. longum KNA1	6.03 ± 0.04	5.17 ± 0.05	5.18 ± 0.04	5.87 ± 0.02	5.17 ± 0.02	5.36 ± 0.08	4.13 ± 0.24	4.05 ± 0.30	5.41 ± 0.05	4.52 ± 0.08	4.61 ± 0.15
B. longum KN4	6.08 ± 0.04	5.10 ± 0.15	5.19 ± 0.16	5.80 ± 0.07	5.23 ± 0.04	5.39 ± 0.06	4.12 ± 0.22	4.06 ± 0.32	5.32 ± 0.06	4.44 ± 0.04	4.48 ± 0.05
B. breve KN3	5.96 ± 0.01	4.98 ± 0.04	5.82 ± 0.03	5.89 ± 0.02	5.94 ± 0.03	5.87 ± 0.03	4.36 ± 0.08	4.87 ± 0.01	5.34 ± 0.15	5.67 ± 0.23	5.47 ± 0.17
B. breve KN14	5.98 ± 0.04	4.91 ± 0.13	5.29 ± 0.05	5.89 ± 0.01	5.85 ± 0.07	5.73 ± 0.04	4.07 ± 0.11	4.37 ± 0.08	5.15 ± 0.10	5.17 ± 0.01	5.26 ± 0.06
B. bifidum KD6E	6.13 ± 0.04	5.58 ± 0.11	6.08 ± 0.11	5.68 ± 0.11	6.07 ± 0.10	5.61 ± 0.08	5.34 ± 0.05	5.56 ± 0.08	4.93 ± 0.04	5.45 ± 0.33	4.57 ± 0.10
B. bifidum KD7E	6.08 ± 0.03	5.23 ± 0.04	5.23 ± 0.32	5.56 ± 0.08	5.44 ± 0.09	5.41 ± 0.08	4.85 ± 0.07	5.06 ± 0.06	4.65 ± 0.06	4.85 ± 0.08	4.45 ± 0.08

[1] The cultures were incubated anaerobically at temp. 37°C for 24 h. [2] pH of the culture directly after inoculation – before incubation and substrate addition; [3] pH of the culture directly after inoculation and substrate addition amounted about 6.0; [4] pH of the culture directly after inoculation and substrate addition amounted about 5.9 and 5.4 for 0.2 and 2% addition of the substrate, respectively; [5] pH of the culture directly after inoculation and substrate addition amounted about 5.95 and 5.65 for 0.2 and 2% addition of the substrate, respectively; [6] Mean ± standard deviation.

to utilize mucin in the gut. Regarding dietary plant polysaccharides, oligofructose significantly stimulated the growth of both *B. longum* strains, whereas the abilities of *B. bifidum* and *B. breve* strains to utilize oligofructose during 24-h incubation were rather poor or none, respectively.

The abilities of *B. bifidum* strains to utilize mucin have already been described, nevertheless, the scope of mucin degradation by *B. bifidum* remains unknown [Crociani *et al.,* 1994]. Mucus covering the intestinal epithelium provides a protective barrier against the detrimental influence of lumen contents and pathogens that can penetrate the mucosa and reach tissue's interior. On the other hand, carbohydrate moiety of glycoproteins and glycolipids in the mucosal layer acts as a site for binding bacteria and large biomolecules (*e.g.* microbial toxins, surface proteins, hormones, antibodies), and also as a nutrient for commensal intestinal bacteria [Walker, 2000]. Generally, the enhancement of the activity of mucin-degrading enzymes indicates an increased number of enteropathogens, such as *Vibrio cholerae*, *Bacteroides fragilis, Shigella* spp., *Helicobacter pylori* and *Yersinia enterocolitica* [Colina *et al.,* 1996]. However, the ability of bifidobacteria to compete for such nutrients may be one of the defense mechanisms against gastrointestinal infections or overgrowth of opportunistic and other enteric bacteria, *e.g. Bacteroides*. The *B. bifidum* species is a natural inhabitant of human gut belonging to generally recognized as safe (GRAS) intestinal microflora, and the strains tested in this study were isolated from a healthy person, however, a great ability of *B. bifidum* strains to utilise mucin must be taken into account in experiments designed for probiotic or synbiotic elaboration. Our previous study revealed that both tested *B. bifidum* strains were also characterised by a high ability to bind to epithelium-like Caco-2 cells *in vitro* [Bielecka & Biedrzycka*,* 2001]. For a healthy organism well-balanced microflora is important for digestion and maintenance of the intestinal ecosystem, thus, despite of the natural origin of these strains, separate studies should be performed to confirm the non-invasive character of mucin-utilizing bifidobacteria as potential candidates for probiotic or synbiotics. So far, MacFarlane *et al.* [1989] have stated a significant increase of bifidobacteria number in the studies on the influence of mucin on enzymatic activities (glycosidase, protease and arylamidase) and growth of faecal microflora in simulated gastrointestinal conditions *in vitro*. Katayama *et al.* [2005] were first to clone and describe 1,2-α-L-fucosidase and endo-α-N-acetylgalactosaminidase genes – novel specific enzymes acting on oligosaccharides that exist mainly in mucin glycoproteins, produced by *B. bifidum* and *B. longum,* respectively. It seems to confirm preferential ability of some bifidobacteria to utilize host-produced oligosaccharides present in the intestinal ecosystem, which is likely to affect their capacity for colonization of different intestinal niches.

The stimulating effect of prebiotic carbohydrates of different polymerization degree on bifidobacteria growth has already been described [Kaplan & Hutkins, 2000; Schrezenmeir & de Vrese, 2001; Bielecka *et al*., 2002; Janer *et al.,* 2004]. In the present study, we compared the growth of bifidobacteria in the presence of low (0.2%) and high (2.0%) amount of oligofructose in the medium. The applied lower dose of oligofructose in the medium was efficient for the growth of all

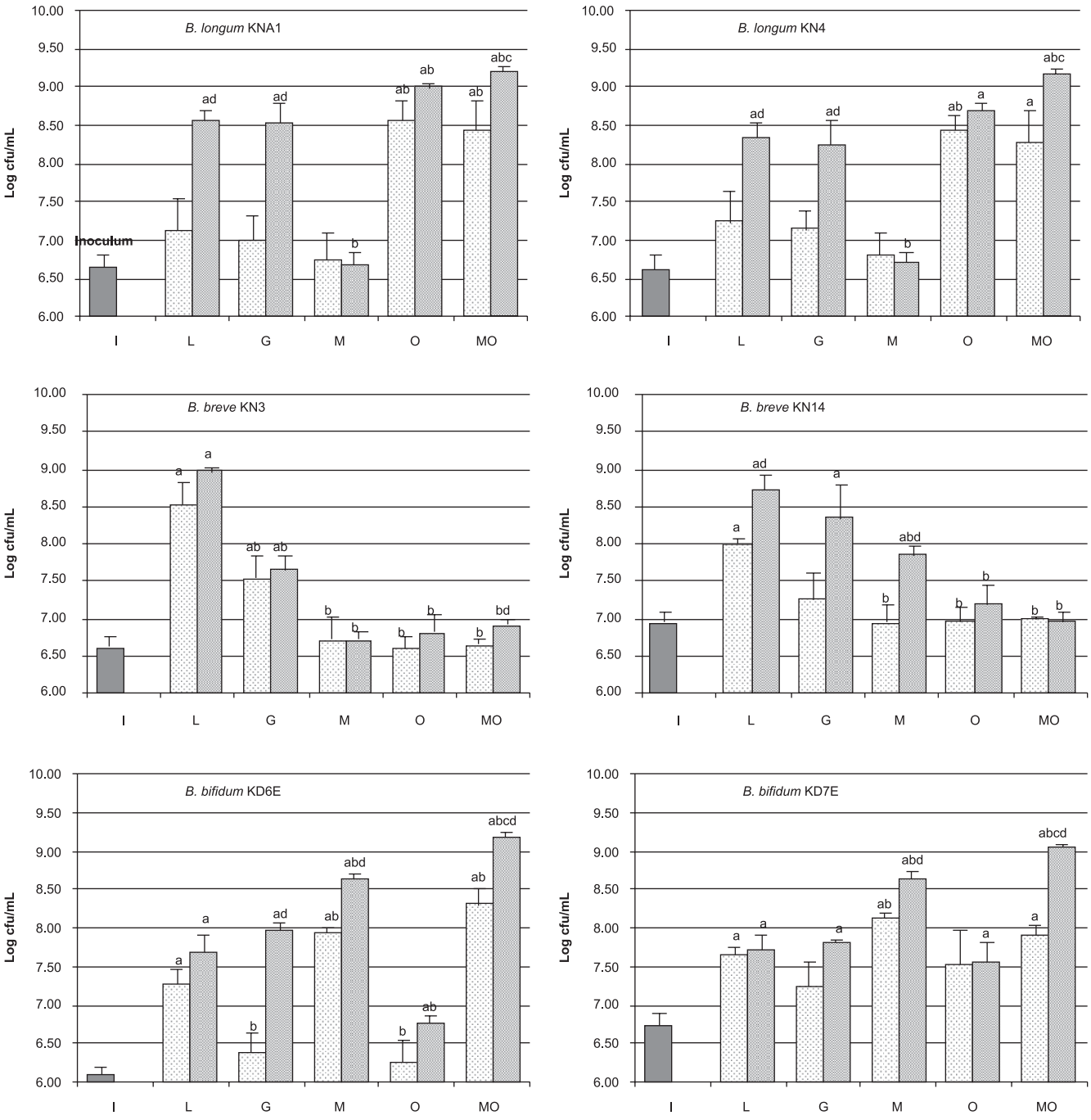

FIGURE 2. Growth of human-originated *Bifidobacterium* strains cultured in minimal Garche's medium containing 0.2 ☐ or 2.0% ▨ (w/v) of lactose – L, glucose – G, mucin – M, oligofructose – O and a mixture of mucin and oligofructose (at a ratio of 1:1) – MO. The cultures were incubated anaerobically at temp. 37°C for 24 h. The letters above the columns mean counts of bacteria different at the significance level $p \leq 0.05$, respectively from: [a] the initial level (inoculum), [b] the control growth in the presence of the same concentration of lactose, [c] the well observed growth in the presence of the same concentration of mucin or oligofructose (regards only counts of bacteria cultured in the medium containing the mixture of oligofructose and mucin), [d] the growth in the medium containing 0.2% of the same substrate.

examined strains of *B. animalis* and *B. longum* to an extent comparable or, in the case of the latter species, even higher than the control growth in the presence of lactose. It confirms a great potential of oligofructose for preferential stimulation of some bifidobacteria in the gut, where dietary carbohydrates are limited. As we have previously described, the ability of bifidobacteria to metabolize fructan-type oligosaccharides seems to be a more species-dependent feature, and only to a small extent a strain-dependent one [Bielecka *et al*., 2002]. Both the previous and present study indicate, however, that *B. longum* and *B. animalis* subsp. *lactis* or *B. animalis* subsp. *animalis* strains utilise oligofructose the best, whereas *B. bifidum* and *B. breve* strains probably possess poor or none ability to utilise oligofructose. These results are in accordance with findings of other researchers [Kaplan & Hutkins, 2000; Schrezenmeir & de Vrese, 2001; Janer *et al.,* 2004].

To the best of our knowledge, there are no reports on the influence of mucin on the growth of *Bifidobacterium*

in the presence of oligofructose, and inversely. The performed studies revealed a significant increase in population counts of the strains cultured in the presence of the mixture of oligofructose and mucin in comparison to their growth in the medium containing single compounds. Such a synergistic effect was clearly visible, however, only in the presence of the higher amounts of the substrate in the medium and mainly for the human-originated strains. Individual bacteria in the colon exist in a multiplicity of different microhabitats and metabolic niches, containing certainly different amounts of mucin or oligosacharides. The observed synergistic effect of oligofructose and mucin on bifidobacteria growth indicates that host-produced glycoproteins may support the growth and maintenance of bifidobacteria in the intestine. Nevertheless, additional studies should be performed to understand the mechanism of such stimulation, also for better selection of potential probiotic strains. Van der Meulen *et al.* [2004] studied fermentation kinetics of fructans and some mono- and disaccharides by *B. animalis* DN-173 010. On the basis of changes in metabolite composition observed during the fermentation, the authors suggested that some enzymes breaking up di- and oligosaccharides can be induced in the presence of an adequate substrate. It is also likely that for most bacteria in the gut, polymer degradation is a cooperative activity with enzymes produced by many different bacteria taking part in the process [Macrfarlane *et al*., 1998]. Adaptation of the strains to use the intestinal nutrients could probably enhance the observed synergistic effect in the gut. Recent genome sequence analysis of *B. longum* NCC2705 has revealed that more than 8.5% of the total predicted proteins were involved in the degradation of oligo- and polysaccharides, perhaps reflecting the superior ability of this organism to adapt to its environment [Schell *et al*., 2002]. Investigations of abilities of the strains to adapt to gastrointestinal conditions may be crucial to future studies with probiotics and selection of the strains able to persist in the gut.

CONCLUSIONS

The performed studies confirmed diverse ability of bifidobacteria to utilize oligofructose and mucin. *B. animalis* subsp. *animalis, B. animalis* subsp. *lactis* and *B. longum* strains appeared to be the best predisposed to utilize oligofructose *in vitro*, whereas *B. bifidum* strains to utilize mucin. Furthermore, the research demonstrated the stimulation of the growth of mucin-degrading bacteria in the presence of oligofructose, and inversely, oligofructose-degrading bacteria in the presence of mucin. It suggests that intestinal mucin may play a role in controlling the composition and activities of bacteria in the large intestine, which indicates the need for further studies on the potential mechanism of such a synergistic effect.

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