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**Survival and synergistic growth of mixed cultures of bifidobacteria and lactobacilli combined with prebiotic oligosaccharides in a gastrointestinal tract simulator.**

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ORIGINAL ARTICLE

# Survival and synergistic growth of mixed cultures of bifidobacteria and lactobacilli combined with prebiotic oligosaccharides in a gastrointestinal tract simulator

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**Background**: Probiotics, especially in combination with non-digestible oligosaccharides, may balance the gut microflora while multistrain preparations may express an improved functionality over single strain cultures. *In vitro* gastrointestinal models enable to test survival and growth dynamics of mixed strain probiotics in a controlled, replicable manner.

**Methods**: The robustness and compatibility of multistrain probiotics composed of bifidobacteria and lactobacilli combined with mixed prebiotics (galacto-, fructo- and xylo-oligosaccharides or galactooligosaccharides and soluble starch) were studied using a dynamic gastrointestinal tract simulator (GITS). The exposure to acid and bile of the upper gastrointestinal tract was followed by dilution with a continuous decrease of the dilution rate (de-celerostat) to simulate the descending nutrient availability of the large intestine. The bacterial numbers and metabolic products were analyzed and the growth parameters determined.

**Results**: The most acid- and bile-resistant strains were *Lactobacillus plantarum* F44 and *L. paracasei* F8. *Bifidobacterium breve* 46 had the highest specific growth rate and, although sensitive to bile exposure, recovered during the dilution phase in most experiments. *B. breve* 46, *L. plantarum* F44, and *L. paracasei* F8 were selected as the most promising strains for further studies.

**Conclusions**: De-celerostat cultivation can be applied to study the mixed bacterial cultures under defined conditions of decreasing nutrient availability to select a compatible set of strains.

Keywords: *mixed culture*; *prebiotic oligosaccharides*; *Bifidobacterium*; *Lactobacillus*; *gastrointestinal tract simulator*

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The human gastrointestinal microbiota is a complex ecosystem and its balance is crucial for human health through multiple functions, including nutrient digestion and absorption, vitamin synthesis, inhibition of pathogenic microorganisms, involvement in energy metabolism, and stimulation of the immune system (1–3).

Diet and specific nutritional factors have been shown to shape intestinal microflora and influence health (4–6). Compared to a Western diet, the ancient food was significantly less processed and refined, rich in dietary fibers and live microorganisms. Technological progress in the food industry (heat treatment, cooling, freezing, etc.) has resulted in a dramatic decrease of consumption of food-borne microbes and has coincided with an increased number of disorders including inflammatory bowel disease and atopic disorders such as asthma and food allergies (7). Foods and food additives containing probiotics (living beneficial microbes) may support the restoration of the healthy balance of the gut microflora (8) while, compared to single strain probiotics, multistrain preparations may express an improved functionality by combining benefits of different strains (9, 10). Currently, *Lactobacillus* and *Bifidobacterium* are the most studied genera regarding such health-promoting properties in prevention and treatment of various gastrointestinal

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disorders, and used in probiotic foods (11, 12). The species *Bifidobacterium breve*, *B. longum*, *B. animalis* subsp. *lactis*, and *B. pseudocatenulatum* have been reported as common inhabitants of the human gut mucosa both in adults and infants (13).

Dietary fibers, mostly oligo- and polysaccharides that are not degraded by human digestive enzymes, can promote the growth of indigenous microorganisms in the large bowel (2, 14). The fermentation products of these substrates, that is, short-chain fatty acids (SCFA) contribute positively to normal large bowel function as energetic substrates for epithelial cells and pH regulators to enhance scavenging of minerals from colon (15, 16). To overcome the fiber-deficiency of everyday food, prebiotic saccharides can be added to foods or consumed as dietary supplements. The best-studied prebiotics include galacto-oligosaccharides (GOS), inulin and fructo-oligosaccharides (FOS), and lactulose (17, 18).

A synbiotic concept to supply probiotics and prebiotics within the same product (19) has been reported to support viability and enhance metabolic activities of probiotic strains as well as to stimulate indigenous bifidobacteria and lactobacilli in the gut (20, 21). Thus, the ability to ferment prebiotics is an important criterion for the selection of probiotic strains (22) while selective fermentation is a prerequisite for a prebiotic substance (18).

The survival and metabolic activities towards prebiotic substrates of the candidate strains of probiotic bacteria prior to complex animal and human trials can be evaluated *in vitro*. A single-vessel dynamic simulator was developed by Sumeri et al. (23) to study the viability of probiotic strains under physiological conditions of the stomach and small intestine. The reliability of a similar simulator has been confirmed also in comparative *in vivo* experiments by Ritter et al. (24). In the current study, a novel algorithm was introduced to mimic, after the upper gastrointestinal tract passage, the conditions of the large intestine by diluting the fermenter content with gradually decreasing rate. The strains *L. plantarum* F44,

*L. paracasei* F8, *B. breve* 46, *B. longum* 6:18 and *B. lactis* 8:8, were previously characterized by high tolerance to acid and bile exposure, strong antimicrobial activity (AMA) against clinical *Clostridium difficile* strains including the strain NAP1/027, high cell surface hydrophobicity and prebiotic degradation capabilities (25, 26). *B. pseudocatenulatum* 1200[T] was used as a reference strain and *B. longum* 6:18 as a bile sensitive control. The robustness and compatibility of the multispecies probiotics combined with multiple prebiotic substrates were evaluated to select the most promising combinations for further studies.

## Materials and methods

### Strains and culture conditions
Strains of bifidobacteria and lactobacilli (Table 1) for testing as synbiotic combinations in the gastrointestinal tract simulator (GITS) were selected on the basis of their 1) acid and bile tolerance, 2) fermentation ability of specific prebiotic oligosaccharides, and 3) AMA against pathogenic strains of *C. difficile* (25). In two-strain cocultures in MRS broth no inhibition between the strains was observed (data not shown).

For biomass preparation, cultures of lactobacilli were grown in 25–50 mL of De Mann–Rogosa–Sharp (MRS) broth (LABM, UK) at 37°C. Cultures of bifidobacteria were grown in 50–100 mL (depending on the cell concentration) MRS broth with 0.05% Cysteine-HCl at 37°C in an anaerobic environment (AnaeroGen™, Gas Pack System, Oxoid, Inc., Basingstoke, UK). All cultures for the GITS experiments were prepared by harvesting the biomass from overnight cultures by centrifugation and resuspending the biomass in 10 mL mixture (1:1 vol/vol) of glycerol and growth medium (MRS with Cysteine-HCl or MRS broth for bifidobacteria and lactobacilli, respectively). The cultures were frozen and kept at −80°C until use.

*Table 1.* Probiotic strains used in gastrointestinal tract simulator experiments

| Strain | Source | Experiment no | | | | | |
| | | 1a | 1b | 1c | 2a | 2b | 2c |
| --- | --- | --- | --- | --- | --- | --- | --- |
| *B. breve* 46 | LMG P-26117 | + | + | + | | + | + |
| *B. longum* 6:18 | LMG P-26115 | + | + | + | | | |
| *B. animalis* subsp. *lactis* 8:8 | LMG P-26116 | | + | + | | + | + |
| *B. pseudocatenulatum* 1200[T] | JCM 1200 | | | | + | + | |
| *L. plantarum* F44 | LMG P-26120 | + | + | + | + | + | + |
| *L. paracasei* F8 | LMG P-26118 | | | | + | + | + |

Carbon sources used in experiments 1a–1c: galactooligosaccharides (GOS), fructooligosaccharides (FOS) and xylooligosaccharides (XOS), and in experiments 2a–2c: GOS & soluble starch (SS).
*B.* – *Bifidobacterium*; *L.* – *Lactobacillus*; LMG – culture collection of Lund University and Bacteria Collection of BCCM/LMG; JCM – Japanese Collection of Microorganisms.

Before experiments, the cultures were thawed, centrifuged (10,000 rpm/15 min, at $+4°C$) to remove the glycerol mixture, and the bacterial biomass was resuspended in a total volume of 200 mL culture medium with oligosaccharides as shown in Table 2.

### Prebiotic substrates

Galacto-, fructo-, and xylooligosaccharides (GOS, FOS and XOS, respectively) were used as substrates in this study. Information about the content of mono- and disaccharides and suppliers of the preparates is presented in Table 2. While fructo- and galactooligosaccharides are best-characterized prebiotics, XOS was chosen because of its high prebiotic potential. The GOS, FOS, and XOS were dissolved in deionized water and filter-sterilized with syringe filters (0.2 μm, FP 30/0.2 CA-S, Whatman) or Express[TM]PLUS, SteriTop (0.22 μm, Millipore). Soluble potato starch was mixed with water, heated to $100°C$, and sterilized at $121°C$ for 15 min. The prebiotic substrates GOS, FOS, and XOS were used in the experiments no. 1a–1c while mixture of GOS and SS was used in the experiments no. 2a–2c. Considering the average composition of the substrates (Table 2), about 5%, that is, 3.2 mM of the total carbohydrates in GOS, FOS, and XOS were in form of mono- and disaccharides.

### Culture media and additives

The dilution medium contained (g $L^{-1}$): tryptone – 5, yeast extract – 2.5, Tween 80 – 0.5, $K_2HPO_4$ – 1, Cys-HCl – 0.5, $MgSO_4 \cdot 7H_2O$ – 0.2, $MnSO_4 \cdot 5H_2O$ – 0.04, pH $6.5 \pm 0.1$ before autoclaving. The mixture of prebiotic carbohydrates comprised (g $L^{-1}$ in the final medium): 1) FOS – 3.33, GOS – 3.33, XOS – 3.33, or 2) GOS – 5 and soluble starch – 5. Yeast extract and tryptone were purchased from LABM (UK), phosphate buffer from POCH Chemicals SA (Gliwice, Poland), Cys-HCl from Merck (Germany) and $MgSO_4 \cdot 7H_2O$, $MnSO_4 \cdot 5H_2O$ and Tween 80 from Sigma-Aldrich (US).

Native porcine bile was pooled from 10 slaughtered pigs and sterilized through a 0.45 μM filter (VWR International, Stockholm, Sweden) and stored at $-20°C$ until use

(26). Before use, the bile preparation was thawed and diluted to 30% (v/v) bile solution with deionized water.

### The gastrointestinal tract simulator

The GITS (23) consisted of a 1 L 'Biobundle' fermenter (Applikon Biotechnology, Schiedam, The Netherlands), a biocontroller ADI 1030 and balances (Sartorius, Germany) connected to the PC and controlled by the cultivation program 'BioXpert' (Applikon, The Netherlands). The temperature ($37°C$), pH, culture volume ($V = 300$ mL), agitation (200 rpm), deaeration (flushing with $N_2$) and speed rates of liquid pumps (HCl, $NaHCO_3$, bile salts, feeding medium) were controlled by this program (Fig. 1).

Two hundred milliliters of suspension of the selected strains of lactobacilli and bifidobacteria was added into fermenter containing 100 mL of 0.01M HCl simulating an empty stomach (27). Secretion of gastric acid was simulated by adding 1M HCl at a rate of 20 mmol $h^{-1}$ (28) to pH = 3.0 (about 30 min). The bioreactor content was then neutralized to pH 6.0 by adding 1 M $NaHCO_3$ at a rate of 4.5 mL $min^{-1}$ (approximately 3–5 min), the 30% porcine bile solution was added at a rate of 4 mL $min^{-1}$ up to concentration of 3% bile in the medium and incubated under these conditions for 30 min. In the previous GITS experiments, we have used maximum 0.4% of pure bile salts (Sigma Aldrich, USA) (23, 29) which lethal effect was found to correlate with about 3% of porcine bile (data not shown).

The fermenter content was then diluted with the dilution medium containing prebiotic oligosaccharides, pH 6.0 with a decreasing rate from $D = 0.4$ $h^{-1}$ to 0.04 $h^{-1}$ up to the total experiment length of 24 h.

### Enumeration of bacteria

The colony forming units of bacteria were determined from four time-points: the beginning (immediately after inoculation), 30 min after exposure of maximum bile concentration, 6.5 and 21 h and from the end (24 h). The following agar media and incubation conditions were used: 1) MRS-agar with 0.05% Cysteine-HCl (MRS-C), anaerobic incubation at $37°C$, 72 h for total bacterial

Table 2. Composition and supplier of prebiotic oligosaccharides and soluble starch

| Carbohydrate source | Purity (%) according to specification | Other ingredients | Supplier |
|---|---|---|---|
| GOS90 PF, powder | 96 | 2.3% lactose, 1.2% glucose, 0.3% galactose | Friesland Campina (Amersfoort, Netherlands) |
| FOS, powder | 95 | 5%: glucose, fructose and sucrose | Orafti (Tienen, Belgium) |
| XOS, powder | 95 | <5% xylose, arabinose | Sweet Town Biotech (Taipei, Taiwan) |
| Soluble starch from potato | 100 | ND | Merck (Darmstadt, Germany) |

GOS – galactooligosaccharides; FOS – fructooligosaccharides; XOS – xylooligosaccharides.
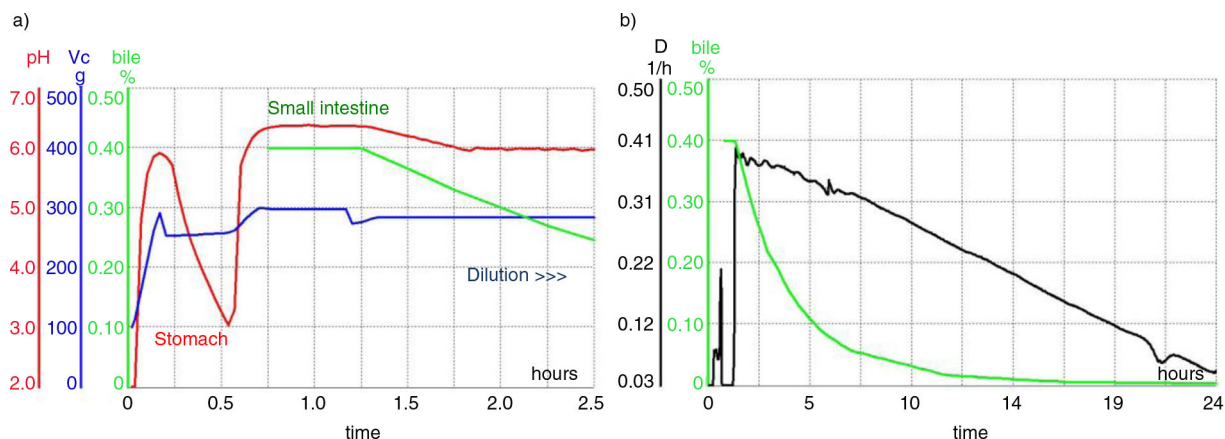
*Fig. 1.* Graphic presentation of the changes of acidity (pH), bile concentration (bile) and culture volume (Vc, mL) during 2.5 h of experiment (a); Decrease of the dilution rate (D) and bile concentration up to 24 h in the simulation experiments (b).

counts (bifidobacteria plus lactobacilli); 2) MRS agar, aerobic incubation at 37°C for 48 h for lactobacilli. *L. plantarum* F44 and *L. paracasei* F8 could be enumerated selectively on the basis of different colony size on MRS agar.

### Repetitive-PCR (Rep-PCR) analysis
Bacterial DNA was stored by applying 30 µL of the liquid culture of a single colony onto FTA Card (Whatman, Maidstone, UK). For the analyses, the DNA was extracted from FTA Cards according to the manufacturer's instructions. To specify the strain distribution in the population, Rep-PCR fingerprinting using (GTG)$_5$ primer (30) as described previously in Sumeri et al. (29) was performed on average for 30 selected colonies from the beginning and 30 colonies from the end of each experiment. The typing (identification) data were extrapolated onto the whole population (total numbers on MRS-C agar) to evaluate the proportion of each strain in the culture.

### Determination of organic acids and ethanol
The concentrations of lactate, acetate, formate, and ethanol in the culture media were determined by liquid chromatography (Alliance 2795 system, Waters Corp., Milford, US), using a BioRad HPX-87H column (Hercules, CA) with an isocratic elution of 0.005 M $H_2SO_4$ at a flow rate of 0.6 mL min$^{-1}$ and at 35°C. Refractive index (RI, 410 nm) detector (model 2414; Waters Corp., US) was used for quantification of organic acids and ethanol. Samples for HPLC were centrifuged (11,000 g, 5 min), and 0.25 mL of 10% sulfosalicylic acid was added to 1 mL sample centrifuged and the supernatants decanted and stored at −20°C until analysis. Before injection, the samples were thawed and centrifuged. Analytical grade standard substances were used for calibration. Detection limit of the analytical method was 0.1 mM.

### Calculation of growth characteristics
The average specific growth rate of a strain after acid and bile exposure, that is, during the dilution phase (between 6.5 and 24 h) was calculated as $\mu(\text{strain}) = D + d(N)/(dt*N)$, where $N$ is cell count in the fermenter, cfu mL$^{-1}$, $D$ is the dilution rate (1 h$^{-1}$), and $dt$ – the time interval between measurements. Total consumption of carbohydrates in hexose equivalents (Cons(hexose), mmol gdw$^{-1}$) was calculated based on the assumption that 2 moles of acetate, lactate or ethanol are produced per one mole of hexose (sum of lactate, acetate and ethanol divided by two). The biomass yield $Y_{XS}$ was expressed as dry biomass (cfu mL$^{-1}$ multiplied by the average dry mass of one cell, i.e., 0.25 pg) produced from total hexose equivalents.

## Results

### Dynamics of bifidobacteria and lactobacilli in the mixed cultures
The robustness and growth dynamics of synbiotic formulations containing lactobacilli, bifidobacteria and mixed prebiotic substrates was studied using a single vessel GITS. Three simulation experiments with two different prebiotic compositions were carried out as shown in Table 1. The lowest numbers of total colony-forming units were observed in the samples taken 5 h after exposure to maximum bile concentration 3% (6.5 h from the beginning of experiment). For the end of experiments the total bacterial numbers recovered to or exceeded the initial numbers, however, with different strain proportions. Two strains, *L. plantarum* F44 and *B. breve* 46, that were tested with both prebiotic combinations, form the basis of the comparisons, whilst *L. paracasei* F8 was included only in the second prebiotic set. The impact of *B. animalis* subsp. *lactis* 8:8 (experiments 1b, 1c, 2b, 2c), *B. longum* 6:18 (1a, 1b, 1c) and *B. pseudocatenulatum* JCM 1200 (in experiments 2a, 2b)

to overall metabolic patterns was very small due to significantly lower numbers, thus assumption was made that three experiments with each prebiotic set can be analyzed as parallels.

The most robust and competitive strain among bifidobacteria was *B. breve* 46 (Table 3). Previously, this strain was shown to survive under physiological concentrations of acid and bile and to metabolize GOS, FOS and SS in pure culture (25). In the current study, the viable cell counts of *B. breve* 46 upon a sequential acid and bile exposure decreased to about 100 times from 7.2–8.1 to below 7 log(cfu) mL$^{-1}$ (Table 3, experiments no. 1a, 1b, 1c, 2b and 2c). *B. breve* 46 had the highest average specific growth rate on both substrate combinations ($\mu_{ave} =$

$0.43 \pm 0.09$ and $0.33 \pm 0.03$ h$^{-1}$, in GOS-FOS-XOS and GOS-SS media, respectively) and recovered during the dilution phase in all simulations (Table 3). Better average recovery of *B. breve* 46 (Change(B46)) was observed in the medium containing GOS, FOS and XOS (experiments 1a–1c) compared to the medium containing GOS and SS ($48 \pm 39$ vs. $11 \pm 6.4$ times, respectively) (Table 4). In two experiments (no. 1a and 1c), *B. breve* 46 was detected as the dominating strain in the end of dilution.

The *B. animalis* subsp. *lactis* strain 8:8 exhibited a substantial acid and bile tolerance in the screening studies (25). However, in multistrain cultures, after good survival during acid and bile exposure the numbers of strain B 8:8 were around $10^7$ cfu * mL$^{-1}$ at the end of most

*Table 3.* Bacterial numbers (expressed as log(cfu mL$^{-1}$)) determined by plate counts and corrected according to REP-PCR typing of the selected isolates

| Experiment no, sampling point | Time, h | Strains, log(cfu mL$^{-1}$) | | | | |
| --- | --- | --- | --- | --- | --- | --- |
| | | B46 | B88 | B1200 | F44 | F8 |
| **1a** | | | | | | |
| Start | 0 | 7.61 | – | – | 8.60 | – |
| Bile 30 min | 1.5 | 6.70 | – | – | 8.30 | – |
| Dilution 5 h | 6.5 | 6.70 | – | – | 8.08 | – |
| End 24 h | 24 | 8.54 | – | – | 8.11 | – |
| **1b** | | | | | | |
| Start | 0 | 7.77 | 7.53 | – | 8.72 | – |
| Bile 30 min | 1.5 | 6.99 | 6.99 | – | 8.55 | – |
| Dilution 5 h | 6.5 | 6.94 | 6.94 | – | 8.17 | – |
| End 24 h | 24 | 7.43 | 7.19 | – | 8.15 | – |
| **1c** | | | | | | |
| Start | 0 | 7.20 | 7.20 | – | 8.54 | – |
| Bile 30 min | 1.5 | 7.00 | ND | – | 8.19 | – |
| Dilution 5 h | 6.5 | 6.73 | ND | – | 7.98 | – |
| End 24 h | 24 | 8.59 | ND | – | 8.10 | – |
| **2a** | | | | | | |
| Start | 0 | – | – | 8.79 | 8.57 | 8.07 |
| Bile 30 min | 1.5 | – | – | 8.12 | 8.08 | 7.52 |
| Dilution 5 h | 6.5 | – | – | 7.59 | 7.95 | 6.62 |
| End 24 h | 24 | – | – | 7.70 | 8.28 | 8.28 |
| **2b** | | | | | | |
| Start | 0 | 7.92 | 8.10 | 8.22 | 8.22 | 8.34 |
| Bile 30 min | 1.5 | <7.00 | <7.00 | <7.00 | 8.45 | 8.32 |
| Dilution 5 h | 6.5 | 6.99 | 6.99 | 6.99 | 7.98 | 8.17 |
| End 24 h | 24 | 7.81 | 7.00 | 7.00 | 7.99 | 8.49 |
| **2c** | | | | | | |
| Start | 0 | 7.55 | 7.71 | – | 8.40 | 8.63 |
| Bile 30 min | 1.5 | 7.00 | <7.00 | – | 8.18 | 8.39 |
| Dilution 5 h | 4.5 | 6.75 | 7.75 | – | 7.34 | 7.94 |
| End 24 h | 24 | 7.95 | 7.00 | – | 8.50 | 8.87 |

Strain names: B46 – *B. breve* 46, B88 – *B. animalis* subsp *lactis* 88, B1200 – *B. pseudocatenulatum* 1200, F44 – *L. plantarum* F44, F8 – *L. paracasei* F8.
$<10^7$ – not detected ($<10^7$ cfu/mL); – strain not used in this experiment.

*Table 4.* Growth characteristics of probiotic strains in the GITS experiments, calculated according to data from the dilution phase (6.5–24 h), after acid and bile exposure

| Experiments Substrates (total 1%) | 1a, 1b, 1c GOS-FOS-XOS | | 2a, 2b, 2c GOS-SS | |
|---|---|---|---|---|
| | Average | Stdev | Average | Stdev |
| Change(B46) | 48.1 | 39.0 | 11.1 | 6.4 |
| Change(F44) | 1.1 | 0.2 | 6.0 | 7.6 |
| Change(F8) | NA | NA | 18.8 | 23.6 |
| Bif(%), 24 h | 52.7 | 26.1 | 12.4 | 4.4 |
| $\mu$, B46, $h^{-1}$ | 0.43 | 0.09 | 0.33 | 0.03 |
| $\mu$, F44, $h^{-1}$ | 0.26 | 0.04 | 0.27 | 0.07 |
| $\mu$, F8, $h^{-1}$ | NA | NA | 0.33 | 0.09 |
| Lact/(Ace + EtOH), mM/mM | 0.29 | 0.09 | 0.30 | 0.18 |
| Yxs, gdw/g(hexose) | 0.02 | 0.01 | 0.04 | 0.02 |
| Cons(hexose), mmol/gdw | 302.4 | 180.9 | 183.8 | 90.2 |
| Lact, mmol/gdw | 138.7 | 115.6 | 85.4 | 74.4 |
| Ace, mmol/gdw | 347.8 | 179.4 | 225.8 | 100.8 |
| EtOH, mmol/gdw | 91.7 | 51.1 | 41.3 | 13.7 |
| For, mmol/gdw | 117.6 | 52.3 | 113.6 | 45.7 |

Strain names as shown in Table 3.
Change(strain), cfu $mL^{-1}$ – change of bacterial counts during the dilution phase (from 6.5 to 24 h), as the final counts divided by counts in the beginning of dilution; Bif(%), 24 h – proportion of bifidobacteria from the total bacterial count in the end of experiment; $\mu$, strain, 1/h – average specific growth rate of a strain between 6.5 and 24 h (dilution phase); $Y_{XS}$, gdw/g(hexose) — biomass yield per hexose equivalents consumed; Cons(hexose), mmol/gdw – consumption of the carbohydrates (in hexose equivalents) per biomass formed; Lact, Ace, For, EtOH, mmol/gdw – formation of lactate, acetate, formate and ethanol per dry biomass; hexose – sum of carbohydrates in hexose equivalents; gdw – dry weight, expressed in grams of dry bacterial biomass.
NA – not analyzed.

experiments (Table 3), possibly due to outcompeting by other strains because of either lower average specific growth rate or lower affinity for the substrates compared to *B. breve* 46. A similar outcompeting effect was also observed for strain *B. pseudocatenulatum* JCM 1200. Therefore growth characteristics for *B. animalis* subsp. *lactis* 8:8 and *B. pseudocatenulatum* JCM 1200 could not be calculated. Experiments 1a–1c revealed the acid and bile sensitivity and poor survival in mixed cultures of the strain *B. longum* 6:18 (final counts below $10^7$ cfu/mL, Table 3). As this strain could not contribute to the overall metabolism and population dynamics, the data were not included in the calculations.

The lactobacilli *L. plantarum* F44 and *L. paracasei* F8 were previously characterized as robust strains, resistant to exposure of physiological concentrations of gastric acid and porcine bile (unpublished data). Our results showed a sharper decrease in total viable numbers of lactobacilli during the acid and bile exposure, but better recovery thereafter in a medium containing GOS and SS compared to a medium containing GOS, FOS and XOS (Table 3).

The average specific growth rate of *L. plantarum* F44 on both sets of prebiotic substrates used in the simulation experiments was high ($\mu_{ave} = 0.26 \pm 0.04$ and $0.27 \pm 0.07$ $h^{-1}$, for the first and second prebiotic combination, respectively) and this strain dominated at the end of most simulations (Tables 3 and 4). This could be explained by an efficient fermentation of GOS by *L. plantarum* F44 that is quite atypical trait among lactobacilli. *L. paracasei* F8 also survived well the sequential exposure to acid and bile and was able to grow during the dilution with prebiotic substrates (Tables 3 and 4).

### Metabolism of oligosaccharides

Production of organic acids and ethanol revealed utilization of the prebiotic oligosaccharides by the strains studied (Table 4). Considerable variation of proportions of lactate, acetate and ethanol was observed between two substrate combinations. Assuming that two moles of acid can be produced from one mole of hexose consumed, in average 13% of the total metabolic products (sum of lactate, acetate, ethanol) could be formed from mono-and disaccharides of the oligosaccharide preparations (about 5%, i.e. 3.2 mM of the total carbohydrates, Table 2).

In pure cultures, *L. plantarum* F44 metabolized effectively GOS, FOS and XOS within 24 h, while *L. paracasei* F8 showed only a very limited ability to ferment these substrates, especially GOS during 48 h incubation (unpublished data). Neither of these lactobacilli fermented soluble starch. In pure cultures of *L. plantarum* F44 and *L. paracasei* F8 (grown 24 h in MRS broth containing 20 g $L^{-1}$ glucose), the molar lactate to acetate ratio (lac/ace) was higher than two, and that of the bifidobacteria was about 0.35, while ethanol was produced only by bifidobacteria (data not shown). In the multistrain cultures grown on oligosaccharide media the average ratio of lactate to acetate was $0.36 \pm 0.1$ that resembles a typical metabolic pattern of bifidobacteria. The proportion of bifidobacteria (Bif(%)) remained lower in the GOS-SS medium compared to GOS-FOS-XOS medium ($12.4 \pm 4.4$ vs. $52 \pm 26.1\%$, respectively) that was accompanied by lower average ethanol production per biomass ($41.3 \pm 13.7$ vs. $91.7 \pm 51.1$ mmol $gdw^{-1}$, in GOS-SS vs. GOS-FOS, XOS, respectively, Table 4). As no significant differences were observed in acetate and lactate formation, between the two media containing either GOS-SS or GOS-FOS-XOS (for acetate $225.8 \pm 100.8$ vs. $347.8 \pm 179.4$ mmol/gdw$^{-1}$ and for lactate $85.4 \pm 74.4$ vs. $138.7 \pm 115.6$ mM gdw$^{-1}$, respectively, Table 4) the excess of

NADH may obviously be oxidized by alcohol dehydrogenase in the GOS-FOS-XOS medium.

An interesting finding was multiplication of *L. paracasei* F8 to high cell numbers in the medium containing GOS and SS, the substrates poorly or not metabolized by this strain in pure cultures. This indicates a possible cross-feeding phenomenon by other strains, capable of releasing monomers from these oligo- and polysaccharides.

## Discussion

Compared to conventional chemostat cultures the decelerostat technology enables better simulation of a continuous decrease of the bile concentration (from 3 to 0.001%), exhaustion of substrates and decrease of the movement rate of the luminal content in the gastrointestinal tract (from 0.4 to 0.04 $h^{-1}$), and thus, better mimics natural competition for substrates. Twenty four hours was chosen as an optimal time for the simulation experiments since a considerable inter-individual variability of the whole gut transit times with an average of 24 h for men and about 50 h for women has been reported (31).

Bacterial growth is mediated mostly by substrate availability and pH of the environment and *in vivo*, each segment of the gut has a different composition and acidity (32, 28). The pH of the gut lumen, varying from 5.5 to 7.5, is likely to be a key factor that determines the dynamics of the microbial community of the human colon (33, 34). For example, Cinquin et al. (35) showed that a more acidic pH accelerated the growth of *Bifidobacterium* spp. (7 vs. 9.8 log CFU $mL^{-1}$ at pH 6.8 and 5.7, respectively), whereas a more basic pH (6.9–7.5) decreased the numbers of *Lactobacillus* spp. (7.7–5.9 log CFU/mL) and increased the *Bacteroides* cell counts (6.9–8.2 log CFU/mL) in continuous cultures. Another study with a fecal microflora and FOS as the primary carbon and energy source revealed that lactobacilli can use FOS and outcompete bifidobacteria at pH 5.2–5.4 while in batch fermentations with pure cultures at pH = 6.5 with FOS as a single substrate, bifidobacteria grew faster than lactobacilli (36). Thus the constant pH = 6.0 kept in our model during the whole dilution phase, was supposed to give equal growth possibilities for both bifidobacteria and lactobacilli.

Microbial fermentation in the human colon is considered to be energy rather than nitrogen limited. In our experiments the dilution medium containing different oligosaccharides (GOS, XOS, FOS, or SS) with a total amount of 10 g $L^{-1}$ as carbon sources was used. Similar quantities of energetic substrates have been reported by others in batch culture studies (20) and continuous culture experiments with intestinal bacteria (33).

To elucidate the growth and competition processes occurring in complex microbial populations, characterization of simple mixed cultures is essential. Beside environmental parameters such as acidity variations and substrate limitation, the strain balance and activities of microorganisms are determined by an interplay between consortium members like antagonism, competition for substrates, or symbiosis by cross-feeding (37). As cross-feeding between bifidobacteria and butyrate producing bacteria in colon has been shown, the same phenomenon obviously exists between other phyla. Our co-culture experiments (unpublished data) as well as the current study reveal that growth of *L. paracasei* F8 is enhanced in presence of *B. breve* 46.

In the GOS-FOS-XOS supplemented medium (Experiments 1a–1c), bifidobacteria comprised about half of total population at the end of experiments (52 ± 26%) while in the GOS-SS containing medium (Experiments 2a–2c) proportion of bifidobacteria was significantly lower (12.4 ± 4.4%, Table 4). It can be explained by lower growth rate of *B. breve* 46 in GOS-SS medium (0.33 versus 0.43 $h^{-1}$ in GOS-FOS-XOS medium), whilst the growth rate of lactobacilli was comparable in both media (0.26 ± 0.04, and 0.27 ± 0.07 $h^{-1}$ for F44, and 0.33 ± 0.09 $h^{-1}$ for F8 in GOS-SS, Table 4). Another explanation of lower proportion of bifidobacteria (higher proportion of total lactobacilli) in medium containing GOS and SS could be inclusion of the strain *L. paracasei* F8 in experiments 2a–2c (GOS-SS), which was not used in experiments 1a–1c (GOS-FOS-XOS). The ability to metabolize various oligosaccharides in pure culture and high AMA against *C. difficile* in *in vitro* experiments suggest the strain *B. longum* 6:18 as a potential probiotic if protected, for example by encapsulation to survive passage through the upper gastrointestinal tract.

The metabolism of prebiotic oligosaccharides is species- and strain-specific depending on their composition, structure (linkage types, branching) and degree of polymerization (38–41). Metabolism of mono-, di- and trisaccharides is generally faster and energetically less costly for bifidobacteria and lactobacilli compared to substrates with DP > 3 (42, 43). Growth substrates may influence the metabolic features in proteome and metabolome as well as survival under gastrointestinal conditions (41). Van der Meulen et al. (44) studied the growth of bifidobacteria on different energy sources and showed that glucose was consumed with the highest rate and oligofructose with the lowest rate by strain *B. longum* BB536 (specific sugar consumption rate 3.5 vs. 1.3 mmol $CDM^{-1} h^{-1}$) while for the strain *B. animalis* subsp. *lactis* Bb-12 the fastest consumption of lactose and slowest for oligofructose were observed (3.9 vs. 0.5, respectively).

Under carbon-limited anaerobic growth conditions like the environment of the large intestine, lactate, acetate, formate and ethanol can be produced by both bifidobacteria and lactobacilli. In the simulated large intestine studies of Cinquin et al. (35), acetate production positively correlated with the concentration of bifidobacteria ($R = 0.76$, $P < 0.05$) suggesting acetate

production mainly by the 'bifidus' pathway, which theoretically yields two lactate and three acetate from two hexose molecules. Our study revealed correlation between higher proportion of bifidobacteria, acetate concentration as well as ethanol production (Table 4). That is in agreement with the pure culture data.

Based on the product and biomass formation and the contents – about 5% – of readily available free sugars (mono- and disaccharides) in oligosaccharide preparations (Table 2), on average 47–48% of total carbohydrates available (10 g L$^{-1}$) were metabolized during 24 h, suggesting that the differences in growth characteristics in two different media resulted clearly from differences of oligosaccharide metabolism by lactobacilli and bifidobacteria. The total biomass yield per carbohydrates consumed ($Y_{XS}$) was less than 4%, while about two times lower average $Y_{XS}$ which coincided with more intensive product formation (lactate, acetate, ethanol) was observed in GOS-FOS-XOS compared to GOS-SS medium (Table 4). However, similar ratios of lactate/(acetate + ethanol) in both media suggest similar ATP production and NAD$^+$ regeneration strategies of the cells in both media.

The largest limitation of the current system is the absence of the complex microbiota of the large intestine since the main objective of the current study was to select the most robust strains in regard to acid and bile resistance that are able to grow synergistically on the selected prebiotic substrates. Co-fermentations of the selected synbiotic combinations with the fecal microflora and other carbon sources will be considered in future studies.

We have shown that a single vessel de-celerostat based GITS can be used to study the survival, recovery and dynamics of mixed bacterial cultures under changing environmental conditions of gastrointestinal tract. As a result of this study, *B. breve* 46, *L. plantarum* F44, and *L. paracasei* F8 as the most resistant strains against acid and bile exposure able to grow synergistically in mixed culture were selected for further evaluation. For better understanding of the concomitant influence of pH, dilution rate and medium composition on metabolism of oligosaccharides in mixed cultures of bifidobacteria and lactobacilli, also steady state cultivations with the dynamic changes of dilution rate, pH or substrate ratios should be carried out.

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