In vitro effects of selected synbiotics on the human faecal microbiota composition

Delphine M.A. Saulnier1,2, Glenn R. Gibson1 & Sofia Kolida1

1Food Microbial Sciences Unit, Department of Food Biosciences, The University of Reading, Reading, UK; and 2Department of Pathology, Baylor College of Medicine, Texas Children’s Hospital, Houston, TX, USA

Correspondence: Delphine Saulnier,
Department of Pathology, Baylor College of Medicine, Texas Children’s Hospital, 6621 Fannin MC 1-2261, Houston, TX 77030, USA.
Tel.: +1 832 824 3346; fax: +1 832 825 0164; e-mail: saulnier@bcm.edu

Received 28 January 2008; revised 9 June 2008; accepted 9 June 2008.
First published online 31 July 2008.
DOI:10.1111/j.1574-6941.2008.00561.x
Editor: Julian Marchesi

Abstract

Synbiotics are recognized means of modulating gut microbiota composition and activities. However, whether synbiotics are superior to prebiotics and probiotics alone in moderating the gut microbiota towards a purportedly healthy composition has not been determined. Eight selected synbiotics (short-chain fructooligosaccharides or fructooligosaccharides, each combined with one of four probiotics, Lactobacillus fermentum ME-3, Lactobacillus plantarum WCFS1, Lactobacillus paracasei 8700:2 or Bifidobacterium longum 46) were added to 24-h pH-controlled anaerobic faecal batch cultures. The prebiotic and probiotic components were also tested alone to determine their respective role within the synbiotic for modulation of the faecal microbiota. Effects upon major groups of the microbiota were evaluated using FISH. Rifampicin variant probiotic strains were used to assess probiotic levels. Synbiotic and prebiotics increased bifidobacteria and the Eubacterium rectale–Clostridium coccoides group. Lower levels of Escherichia coli were retrieved with these combinations after 5 and 10 h of fermentation. Probiotics alone had little effect upon the groups, however. Multivariate analysis revealed that the effect of synbiotics differed from the prebiotics as higher levels of Lactobacillus–Enterococcus were observed when the probiotic was stimulated by the prebiotic component. Here, the synbiotic approach was more effective than prebiotic or probiotic alone to modulate the gut microbiota.

Introduction

The protective nature of certain microorganisms, in particular lactic acid bacteria, has a long history as does the term probiotic, which is currently defined as ‘a live microorganism which, when administered in adequate amounts, confers a health benefit on the host’ (FAO/WHO, 2002). Several studies have confirmed the efficacy of the probiotic approach for conditions such as diarrhoea, certain allergies, irritable bowel syndrome and inflammatory bowel diseases. However, being ingested microorganisms, probiotics are not always able to survive host physicochemical protective barriers such as stomach acidity and bile acids. Once probiotics reach the lower gut, they then have to compete for nutrients and an ecological niche with the already established colonic microbiota. These factors can compromise probiotic establishment in the colon and therefore diminish efficacy.

For this reason, another approach, the use of prebiotics, has been introduced to target indigenous beneficial bacteria already established in the host. Prebiotics are ‘nondigestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one, or a limited number of bacteria in the colon that can improve the host health’ (Gibson & Roberfroid, 1995). Target genera for prebiotics are usually bifidobacteria and lactobacilli.

To capitalize upon the probiotic and prebiotic approaches, the two can be combined as a synbiotic. Potential advantages would be to increase the survival/activity of the probiotic in the host gastrointestinal tract (GIT), while stimulating the indigenous beneficial bacteria (Gibson & Roberfroid, 1995). The term synbiotic refers indirectly to a synergy, and that is why some authors have suggested that it should exclusively refer to products in which the prebiotic compound selectively favours the probiotics (Schrezenmeir & de Vrese, 2001).
Although the synbiotic approach has attracted interest, the development and evaluation of synbiotics is still at an early stage. Only a few combinations of pre/probiotics have been evaluated as synbiotics, with only a limited number determining effects on the human faecal microbiota using reliable molecular techniques (Saulnier, 2007). Moreover, the respective role of the probiotic and prebiotic to modulate the faecal microbiota in a synbiotic has not been fully investigated. Here, we describe the first stages in the development of a synbiotic, by first selecting prebiotics promoting the growth of probiotics of interest, then by evaluating their effect on the faecal microbiota in vitro, to assess whether the synbiotic can have a superior functionality as compared with its probiotic/prebiotic components.

Materials and methods

Probiotic and prebiotic selection in pure culture

Bacterial strains

Five probiotic lactobacilli [Lactobacillus plantarum 299v (DSM 9843), Lactobacillus paracasei 8700:2 (DSM 13434), Lactobacillus fermentum ME-3 (DSM 14241), Lactobacillus rhamnosus GG (ATCC 53103) and L. plantarum WCFS1 (NCIMB 8826)] and one probiotic Bifidobacterium [Bifidobacterium longum 46 (DSM 14583)] were investigated (Table 1). All test strains were maintained on Cryobank cryogenic beads (Prolab Diagnostics, UK) at −80 °C for long-term storage.

Table 1. Probiotics evaluated in pure culture experiments

<table>
<thead>
<tr>
<th>Bacillus species</th>
<th>Strain</th>
<th>Reference</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>L. plantarum</td>
<td>299v</td>
<td>DSM 9843</td>
<td>Gastrointestinal mucosa of healthy adult</td>
</tr>
<tr>
<td></td>
<td>WCFS1</td>
<td>NCIMB 8826</td>
<td>Healthy adult saliva</td>
</tr>
<tr>
<td>L. fermentum</td>
<td>ME-3</td>
<td>DSM 14241</td>
<td>Infant faeces</td>
</tr>
<tr>
<td>L. rhamnosus</td>
<td>GG</td>
<td>ATCC 53013</td>
<td>Adult faeces</td>
</tr>
<tr>
<td>L. paracasei</td>
<td>8700:2</td>
<td>DSM 13434</td>
<td>Gastrointestinal mucosa of healthy adult</td>
</tr>
<tr>
<td>B. longum</td>
<td>46</td>
<td>DSM 14583</td>
<td>Gastrointestinal mucosa of healthy senior</td>
</tr>
</tbody>
</table>

ATCC, American Type Culture Collection; DSM, Deutsche Sammlung von Mikroorganismen; NCIMB, National Collection of Industrial and Marine Bacteria.

Carbohydrates

Nine commercially available carbohydrates with purported prebiotic properties were investigated along with two non-selective controls, digestible in the upper GIT, glucose (BDH, UK) and a polysaccharide (maltodextrin, Avebe, The Netherlands) as follows: fructooligosaccharides [FOS, Beneo™ P95, Orafti, Belgium, 5% glucose, fructose and sucrose, degree of polymerization (DP) = 2–10], short-chain fructooligosaccharides (scFOS, Actilight 950P, Beigins-Meijji, France, 5% glucose, fructose and sucrose, DP = 2–5), isomaltooligosaccharide (IMO, Isomalto 900P, Showa Sango, Japan, 3.9% glucose, 6.7% maltose, 35.2% isomaltose, 3.2% other disaccharides, DP = 3–7), low-molecular-weight inulin (Beneo™ ST, 8% glucose, fructose and sucrose, average DP = 11), high-molecular weight inulin, Beneo™ HP, 100% inulin, average DP = 23), FOS/inulin mixture (Beneo™ Synergy 1, 8% glucose, fructose and sucrose), lactitol (Danisco, UK, 100% lactitol), polydextrose (Litesse, Danisco, 90% polydextrose), and xylooligosaccharide (XOS, Suntory Limited, Japan, 95% XOS, DP = 3–6).

Determination of growth parameters with different carbohydrates

Bacterial growth on each test carbohydrate was measured with a spectrophotometer (Sherwood, UK) at OD660 nm. Probiotics were grown in 10 mL glucose-free MRS medium (De Man et al., 1960) containing one of the carbohydrate substrate (1% w/w) and inoculated with 100 mL of bacterial suspension to obtain an initial OD660 nm of 0.05. A negative control (NC) without any carbohydrate was also included. Probiotics were incubated for 24 h at 37 °C under anaerobic conditions (hydrogen 10%; carbon dioxide 10%; nitrogen 80%). Maximum OD660 nm (OD660 nm max) during the 24 h of fermentation was recorded. Plate counts on MRS agar were used to determine live bacterial counts at the start, in the exponential phase after 10 h and at the end of the growth curve experiments after 24 h. At the end of fermentation, culture pH was recorded as further indication of bacterial fermentation. All the experiments were carried out in triplicate.

Probiotic, prebiotic and their synbiotic combinations evaluation in 24-h faecal batch cultures

Probiotic strains and rifampicin variant selection

To track the growth of L. fermentum ME-3, L. plantarum WCFS1, L. paracasei 8700:2 and B. longum 46 in mixed culture, antibiotic (rifampicin)-resistant variants were selected by successive overnight anaerobic incubation in increasing rifampicin concentrations (from 0.0001 to 100 µg mL−1) in MRS broth (Sigma, Poole, UK). The variants were tested for fermentation of scFOS and FOS at 1% (w/v) by means of pure culture in glucose-free MRS broth.
Volunteer screening for rifampicin-resistant faecal lactobacilli and bifidobacteria

Volunteers were screened for rifampicin resistance in their faecal lactobacilli and bifidobacterial populations, in order to avoid false-positive growth from indigenous species. Faecal samples from three healthy donors were collected on site, in the Department of Food Biosciences, University of Reading, kept in an anaerobic cabinet (10% H₂, 10% CO₂, 80% N₂) and used within a maximum of 10 min following collection. A 1/10 (w/v) dilution in phosphate-buffered saline (PBS) solution (0.1 M phosphate buffer solution, pH 7.4) was prepared and the samples homogenized in a stomacher (Seward, UK) for 2 min at normal speed. Twenty microlitres of this preparation were plated onto media for lactobacilli and bifidobacteria, Rogosa (Rogosa et al., 1951) and Beermann agar (Berrens, 1990), respectively, containing 100 μg mL⁻¹ rifampicin (Sigma). Plates were incubated in the anaerobic cabinet at 37 °C for 48 h. Volunteers were excluded in cases where growth of lactobacilli or bifidobacteria occurred. Donors were free of any known metabolic and gastrointestinal diseases (e.g. diabetes, ulcerative colitis, Crohn’s disease, irritable bowel syndrome, peptic ulcers and cancer), were not taking probiotic or prebiotic supplements and had not taken antibiotics 6 months before faecal sample donation.

pH-controlled anaerobic faecal batch cultures

Probiotic *L. plantarum* WCFS1, prebiotics (FOS or scFOS) and symbiotic combinations (FOS or scFOS with *L. plantarum* WCFS1) were evaluated in anaerobic 24-h pH-controlled batch cultures with the faecal microbiota. An NC containing neither the probiotic nor the prebiotic was also included. Six batch fermenters were thus run in parallel and were inoculated with 15 mL of 10% (w/v) faecal slurry, prepared as described above. The experiment was performed in triplicate, using one faecal sample given by a different donor for each run of six batch fermenters. The total set-up of the experiment (three runs of six batch fermenters with the combinations – NC, probiotic, FOS, scFOS, probiotic together with FOS, probiotic together with scFOS) was then performed with *L. paracasei* 87002, *L. fermentum* ME-3 or *B. longum* 46 as probiotic. The same three donors provided the samples as described above. One hundred and thirty-five millilitres of sterile basal medium were aseptically added to each fermenter (300 mL working volume) [peptone water (Oxoid, UK) 2 g L⁻¹, yeast extract (Oxoid) 2 g L⁻¹, NaCl (BDH, UK) 0.1 g L⁻¹, K₂HPO₄ (BDH) 0.04 g L⁻¹, KH₂PO₄ (BDH) 0.04 g L⁻¹, MgSO₄ · 7H₂O 0.01 g L⁻¹, CaCl₂ · 2H₂O (BDH) 0.01 g L⁻¹, NaHCO₃ 2 g L⁻¹, cysteine · HCl 2 g L⁻¹, bile salts (Oxoid) 0.5 g L⁻¹, vitamin K 10 μL L⁻¹, Tween 80 (BDH) 2 mL L⁻¹, hemin 0.0005 g L⁻¹]. Resazurin (0.001 g L⁻¹) was added as an anaerobic indicator. Unless otherwise stated, all chemicals were supplied by Sigma. The fermenters were sparged overnight with O₂-free N₂ at a rate of 15 mL min⁻¹. Each vessel was then inoculated with 15 mL faecal slurry (as prepared above). Culture pH was regulated between 6.7 and 6.9 by means of automated pH controllers (Electrolab Ltd, UK) and temperature was maintained at 37 °C by means of a circulating water bath.

The test prebiotics were added to the basal medium just before the addition of the faecal slurry to a final concentration of 1% (w/v) and were the sole carbon source available for the 24-h fermentation period. In vessels inoculated with the test probiotics, 1 mL of a 10⁶ cells mL⁻¹ solution of rifampicin-variant strains was added. To prepare this solution, all strains were anaerobically grown overnight, in MRS broth containing 100 μg mL⁻¹ of rifampicin. Before addition, the probiotic culture was centrifuged for 5 min at 3000 g. The cells were then resuspended in PBS and centrifuged for a further 5 min at 3000 g. One millilitre culture fluid was serially diluted and plated on Beermann or Rogosa agar with 100 μg mL⁻¹ rifampicin for bifidobacteria and lactobacilli, respectively, to determine the initial concentration of each strain.

Sampling and enumeration of probiotic rifampicin variants

Samples were obtained at 0, 5, 10 and 24 h of batch culture fermentation. Five millilitres of culture fluid were removed from each vessel and prepared for FISH analysis. Samples were also serially diluted and plated out onto Rogosa or Beermann agar (for *Lactobacillus* strains) or Beermann agar (for *B. longum* 46) with 100 μg mL⁻¹ rifampicin, in triplicate, to follow probiotic growth throughout the 24-h batch culture fermentations. Plates were incubated anaerobically at 37 °C for 48 h.

FISH

The FISH technique was used to enumerate selected groups of bacterial components of the gut microbiota as described by Harmsen et al. (2006b). Briefly, 375 μL of culture fluid was diluted in 1125 μL of 4% (w/v) paraformaldehyde and fixed overnight at 4 °C. Bacterial cells were pelleted by centrifugation (10 000 g, 5 min) and washed twice in filter-sterilized PBS. Additional treatment with lysozyme and lipase was performed for the enumeration with the Lab 158 probe as described by Harmsen et al. (1999). Cells were then resuspended in 150 μL PBS and 150 μL ethanol. Samples were stored at −20 °C for a minimum of 3 h before further processing.

Sample hybridization was carried out as described previously (Sanz et al., 2006) using appropriate genus-specific 16S rRNA gene-targeted oligonucleotide probes labelled with the fluorescent dye Cy3 (MWG Biotech, Germany) for
selected bacterial groups, or the nucleic acid stain 4',6-diamidino-2-phenylindole (DAPI) for total cell counts. The probes used were as follows: Bif 164, specific for *Bifidobacterium* (Langendijk *et al.*, 1995), Bac 303, specific for the *Bacteroides–Prevotella* group (Manz *et al.*, 1996), His 150 for the *Clostridium histolyticum* subgroup (Franks *et al.*, 1998), Erec 482 for the *Clostridium cocoides–Eubacterium rectale* group (Franks *et al.*, 1998), Lab 158 for *Lactobacillus–Enterococcus* (Harmsen *et al.*, 1999), Ato 291 for the Atopobium cluster (Harmsen *et al.*, 2000a), Fpra 655 for the *Faecalibacterium prausnitzii* group (Hold *et al.*, 2003) and EC 1531 for *Escherichia coli* (Poulsen *et al.*, 1994). Probe sequences and hybridization conditions are listed in Table 2. The samples were then filtered using 0.2-μm pore size filters (Millipore Corporation, UK) and cells were counted using a Nikon Eclipse E400 fluorescence microscope (Nikon, UK). A minimum of 15 random fields of view was counted in each slide.

## Statistical analysis

### Prebiotic ranking in pure culture

For each probiotic, prebiotics were first ranked by decreasing OD$_{660 \text{ nm}}$ max obtained during the 24-h fermentation in order to visualize the best performing substrates. OD$_{660 \text{ nm}}$ max was chosen as a discriminating factor to classify the carbohydrates as this generally reflects the biomass formed through the time of fermentation. One-way ANOVA was performed to investigate whether there were significant differences of OD$_{660 \text{ nm}}$ max between the substrates, with 95% confidence. When this was the case, Fisher’s test with 95% confidence intervals was carried out for a pairwise comparison among the substrates and thus clustered the prebiotics. Prebiotics were ranked according to decreasing average OD$_{660 \text{ nm}}$ max calculated for the six probiotics, in order to visualize the prebiotics which were generally better fermented. One-way ANOVA and Fisher’s test were performed as previously in order to determine whether there was any significant difference between the prebiotics. The statistical analyses were performed using the MINITAB RELEASE 14.1 STATISTICAL SOFTWARE (Minitab Inc., UK).

### pH-controlled faecal batch cultures

Plate count data for the rifampicin-variant probiotics and FISH data are presented as mean ± SD. Growth of the rifampicin variants was evaluated by a paired $T$-test after 5, 10 and 24 h of fermentation compared with the initial counts at the start of the experiment. Growth was considered significantly different from 0 h of fermentation when $P < 0.05$. For each time point and for each strain, differences between the substrates were evaluated by one-way ANOVA with the substrates as factor, in randomized block, with the runs as blocks. Substrates were considered to be significantly different when $P < 0.05$. In this case, Fisher’s Least Significant Difference was performed to identify which substrate(s) was (were) different from the others with 95% confidence.

For the FISH data, growth of each group targeted by each probe was evaluated by a paired $T$-test after 5, 10 and 24 h of fermentation compared with the initial counts at the beginning of the experiment. Growth was considered significantly different from 0 h when $P < 0.05$. For each time point and for each probe, differences between the substrates were evaluated by one-way ANOVA. The analyses were performed using GENSTAT, 8th edition (VSN International Ltd, UK).

FISH results, for the single strain *L. plantarum* WCFS1 or *L. fermentum* ME-3 series of experiments, were combined and examined by principal component analysis (PCA) using MINITAB (Minitab Ltd, UK) in order to assess whether there were any different patterns of fermentation permitting to distinguish the different combinations.

## Results

### Prebiotic utilization is strain dependent in the pure culture experiment

Probiotic growth varied greatly between the different test substrates in the pure culture experiments (Table 3).

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**Table 2.** FISH oligonucleotide probes used in this study

<table>
<thead>
<tr>
<th>Probe name</th>
<th>Target bacterial group/species</th>
<th>Sequence from 5’ to 3’ end</th>
<th>$T_{h}^{*}$ (°C)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Erec 482</td>
<td><em>Eubacterium rectale–Clostridium cocoides</em> group</td>
<td>CGGTACCTGACTAAGAAGC</td>
<td>50</td>
<td>Franks <em>et al.</em> (1998)</td>
</tr>
<tr>
<td>Bac 303</td>
<td><em>Bacteroides–Prevotella</em></td>
<td>CCAATGTTGGGAGCCCTT</td>
<td>45</td>
<td>Manz <em>et al.</em> (1996)</td>
</tr>
<tr>
<td>Ato 291</td>
<td><em>Atopobium</em> group</td>
<td>GGTCGGCTCTCAACCC</td>
<td>50</td>
<td>Harmsen <em>et al.</em> (2000a)</td>
</tr>
<tr>
<td>Fpra 655</td>
<td><em>Faecalibacterium prausnitzii</em> group</td>
<td>CGGTACCTGACTAAGAAGC</td>
<td>58</td>
<td>Hold <em>et al.</em> (2003)</td>
</tr>
<tr>
<td>Bif 164</td>
<td><em>Bifidobacterium</em> spp.</td>
<td>CATCCGGCATATTACCCC</td>
<td>50</td>
<td>Langendijk <em>et al.</em> (1995)</td>
</tr>
<tr>
<td>His 150</td>
<td><em>Clostridium histolyticum</em> group</td>
<td>AAAGGAAGATAATACCGCATA</td>
<td>50</td>
<td>Franks <em>et al.</em> (1998)</td>
</tr>
<tr>
<td>Lab 158</td>
<td><em>Lactobacillus–Enterococcus</em> spp.</td>
<td>GGTATGACTGTGGTCCA</td>
<td>50</td>
<td>Harmsen <em>et al.</em> (1999)</td>
</tr>
<tr>
<td>EC 1531</td>
<td><em>E. coli</em> spp.</td>
<td>CACCGTATGGCCTCCTGATCA (23S rRNA)</td>
<td>37</td>
<td>Poulsen <em>et al.</em> (1994)</td>
</tr>
</tbody>
</table>

*$T_{h}$ hybridization temperature
Lactobacillus rhamnosus GG (LGG) was not able to ferment any of the test prebiotics whereas L. paracasei 8700:2 fermented a range of carbohydrates. Apart from glucose, which was the nonselective control, scFOS and IMO were in general, the best fermented substrates. All the strains tested, except LGG, were able to ferment scFOS. FOS was not, or fermented to a lesser extent than scFOS by certain probiotics (LGG, L. plantarum ME-3, L. fermentum WCFS1, L. plantarum 299v, L. paracasei WCFS1).

The substrates that were less well used in 24 h by the majority of the test probiotics were fructans with a high DP such as Beneo™ HP, but also XOS, polydextrose and lactitol. Only L. paracasei 8700:2 was able to degrade all the test fructans. The two L. plantarum strains tested (299v and WCFS1) had similar profile of fermentation, with the exception of maltodextrin. Substrates that gave a high OD600 nm max had a low pH, indicative of production of acids as end products of fermentation, and high viable counts after 24 h fermentation (data not shown).

**Probiotic stimulation by prebiotics in batch cultures is strain dependent**

In the batch cultures, the growth of rifampicin-variant probiotics on the test prebiotics was variable, depending on the strain (Fig. 1). Lactobacillus plantarum WCFS1 and B. longum 46 increased with both prebiotics after 5 and 10 h of fermentation compared with the control. At 24 h, B. longum 46 returned to initial levels while that of L. plantarum WCFS1 remained at similar levels as 10 h. Lactobacillus fermentum ME-3 and L. paracasei 8700:2 were not stimulated by the presence of the prebiotics. In both the presence and absence of FOS and scFOS, L. fermentum ME-3 decreased slightly at 5, 10 and 24 h compared with 0 h. Concentrations of L. paracasei 8700:2 at 5, 10 and 24 h were similar to 0 h. No spontaneous rifampicin variants appeared during batch culture fermentation where no probiotic had been added (data not shown).

**FISH analysis in the runs of batch culture fermented with L. plantarum WCFS1**

FISH analysis was used to monitor how prebiotics, probiotics and their symbiotic combinations could modulate major groups of the faecal microbiota. In fermentations with NC most bacterial groups remained at similar levels after 5 and 10 h compared with the time of the inoculation (Fig. 2) and tended to stay at the same level or decrease after 24 h. The exception was E. coli, which increased after 5, 10 and 24 h compared with 0 h.

The only difference when comparing the fermentation with NC and the probiotics added alone was a higher count of Lactobacillus—Enterococcus. In contrast, prebiotic addition (FOS or scFOS) led to more noticeable changes in the faecal microbiota. The main effect was a significant increase in bifidobacterial levels after 5, 10 and 24 h of fermentation compared with 0 h. An increase in the Atopobium cluster was reported, especially in the faecal inoculum from one volunteer whereby the increase was to the same extent for bifidobacteria after 5, 10 and 24 h (data not shown). Because of high variations between the volunteers at 5, 10 and 24 h, the increase in the Atopobium cluster was not significant compared with 0 h. Compared with the vessels without carbohydrate (NC and L. plantarum WCFS1 alone), the E. rectale—C. cocoides group did not decrease from 10 to

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**Table 3.** Maximum OD600 nm (OD600 nm max) obtained during 24 h of pure culture fermentation in glucose-free MRS broth with 1% (w/v) test carbohydrates of six probiotics

<table>
<thead>
<tr>
<th>Carbohydrate</th>
<th>OD600 nm max*</th>
<th>OD600 nm max*</th>
<th>OD600 nm max*</th>
<th>OD600 nm max*</th>
<th>OD600 nm max*</th>
<th>OD600 nm max*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Average six</td>
<td>L. fermentum</td>
<td>L. fermentum</td>
<td>L. plantarum</td>
<td>L. plantarum</td>
<td>L. plantarum</td>
</tr>
<tr>
<td></td>
<td>probiotics</td>
<td>ME-3</td>
<td>GG</td>
<td>299v</td>
<td>WCFS1</td>
<td>8700:2</td>
</tr>
<tr>
<td>Glucose</td>
<td>2.36 ± 0.70 a</td>
<td>2.10 ± 0.26 a</td>
<td>3.40 ± 0.30 a</td>
<td>2.57 ± 0.10 a</td>
<td>2.87 ± 0.40 a</td>
<td>2.13 ± 0.58 a</td>
</tr>
<tr>
<td>scFOS</td>
<td>1.26 ± 0.66 b</td>
<td>0.70 ± 0.01 b</td>
<td>0.40 ± 0.01 d</td>
<td>1.85 ± 0.15 b</td>
<td>1.87 ± 0.15 b</td>
<td>1.97 ± 0.64 ab</td>
</tr>
<tr>
<td>IMO</td>
<td>1.05 ± 0.44 bc</td>
<td>0.46 ± 0.01 c</td>
<td>0.48 ± 0.02 cd</td>
<td>1.13 ± 0.06 c</td>
<td>1.53 ± 0.06 c</td>
<td>0.97 ± 0.11 efg</td>
</tr>
<tr>
<td>Beneo™ Synergy 1</td>
<td>0.77 ± 0.39 cd</td>
<td>0.46 ± 0.05 c</td>
<td>0.46 ± 0.05 c</td>
<td>0.52 ± 0.02 ef</td>
<td>0.59 ± 0.02 e</td>
<td>2.00 ± 0.61 ab</td>
</tr>
<tr>
<td>FOS</td>
<td>0.76 ± 0.54 cde</td>
<td>0.43 ± 0.01 cd</td>
<td>0.44 ± 0.03 d</td>
<td>0.53 ± 0.02 def</td>
<td>0.59 ± 0.02 e</td>
<td>1.43 ± 1.2 cd</td>
</tr>
<tr>
<td>Beneo™ ST</td>
<td>0.71 ± 0.39 cde</td>
<td>0.44 ± 0.03 cd</td>
<td>0.73 ± 0.02 b</td>
<td>0.60 ± 0.01 de</td>
<td>0.62 ± 0.02 e</td>
<td>1.55 ± 0.35 bcd</td>
</tr>
<tr>
<td>Maltodextrin</td>
<td>0.66 ± 0.50 cde</td>
<td>0.29 ± 0.04 d</td>
<td>0.43 ± 0.05 d</td>
<td>0.35 ± 0.04 g</td>
<td>1.63 ± 0.06 c</td>
<td>0.40 ± 0.02 gh</td>
</tr>
<tr>
<td>Polydextrose</td>
<td>0.53 ± 0.24 cde</td>
<td>0.30 ± 0.03 d</td>
<td>0.60 ± 0.05 bc</td>
<td>0.67 ± 0.02 d</td>
<td>0.87 ± 0.06 d</td>
<td>0.67 ± 0.04 fgh</td>
</tr>
<tr>
<td>XOS</td>
<td>0.48 ± 0.17 cde</td>
<td>0.23 ± 0.03 de</td>
<td>0.66 ± 0.02 b</td>
<td>0.41 ± 0.03 g</td>
<td>0.62 ± 0.05 e</td>
<td>0.62 ± 0.02 fgh</td>
</tr>
<tr>
<td>Lactitol</td>
<td>0.39 ± 0.51 de</td>
<td>0.18 ± 0.01 de</td>
<td>0.36 ± 0.02 d</td>
<td>0.22 ± 0.02 h</td>
<td>0.24 ± 0.03 f</td>
<td>1.53 ± 0.25 bcd</td>
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<tr>
<td>Blank</td>
<td>0.48 ± 0.36 de</td>
<td>0.46 ± 0.05 c</td>
<td>0.44 ± 0.01 d</td>
<td>0.20 ± 0.02 h</td>
<td>0.29 ± 0.02 f</td>
<td>1.03 ± 0.61 def</td>
</tr>
</tbody>
</table>

*OD600 nm max with different letter within the same column are significantly different with the Fisher’s test (P < 0.05).

Carbohydrates are ranked by decreasing OD600 nm max for the average of six probiotics.
24 h, and consequently a higher count occurred when the prebiotic was present at this time.

No major differences were seen when comparing the effects of scFOS and FOS for modulating the faecal microbiota. When comparing the effects of synbiotics and prebiotics, the major difference was an increase in *Lactobacillus–Enterococcus* with the synbiotics.

**FISH analysis in the series of batch culture fermented with *L. paracasei* 8700:2, *L. fermentum* ME-3 and *B. longum* 46 (supplementary data 1–3)**

In this series of experiments, the growth of each probiotic was not enhanced up to 24 h of fermentation. Combinations, NC and probiotic alone had similar effect upon the faecal microbiota (data not shown). Prebiotics and synbiotics led to more noticeable changes in the faecal microbiota compared with the probiotics or NC as it has been described above. However, here, prebiotics and synbiotics had similar effects on the faecal microbiota.

**PCA analysis with FISH data for the runs of batch culture fermented with *L. plantarum* WCFS1 and *L. fermentum* ME-3**

An advanced statistical technique (PCA) was further used to reveal the variance–covariance structure of the time of fermentation for the species/groups enumerated by FISH in order to highlight possible differences between the combinations. This analysis was performed with the FISH results obtained at the four sampling times among all the samples, in the three runs of six fermentations performed with *L. plantarum* WCFS1 or *L. fermentum* ME-3 as a probiotic.

A total of 216 values were included for each analysis. In the series of runs with *L. plantarum* WCFS1, spots representing the vessels NC and *L. plantarum* WCFS1 were separated from those representing FOS alone or with *L. plantarum* WCFS1 on the first principal component axis (horizontal axis) (Fig. 3). The second components separated the vessels based on the presence of the probiotic (vertical axis). Thus, it could be seen that the two prebiotics clustered together, and were clearly separate from the two synbiotics.
The relationships between the 36 variables on the figures are represented on the same figure. Variables that correlated were grouped together using PCA. Moreover, the further away a variable lies from the plot origin, the stronger effect the variable has on the model.

On the horizontal axis, combinations containing the prebiotics and synbiotics were separated because they contained, in general, a higher number of the different groups targeted by FISH, *E. coli* being the exception, at times 5, 10 and 24 h. On the vertical axis, it was mostly *Lactobacillus – Enterococcus*, at all time points, which were positively correlated with the presence of *L. plantarum WCFS1*. *Atopobium* and *C. histolyticum* were on the contrary negatively correlated with the presence of the probiotic.

With the series of runs of batch culture with *L. fermentum ME-3*, as with *L. plantarum WCFS1*, combinations containing the prebiotics and synbiotics were separated from NC and the probiotic alone on the horizontal axis because they contained, in general, a higher number of the different groups (Fig. 4). However, in contrast to results obtained...
with the series with *L. plantarum* WCFS1, prebiotic and synbiotic combinations were not clearly separated. Moreover, no specific group at different time point could be clearly distinguished to explain the PCA results.

**Discussion**

In this study, we have described the first stages in the development of a synbiotic, by first selecting the prebiotics promoting the growth of probiotics of interest, then evaluating their effect upon the faecal microbiota *in vitro*. We
have shown that certain synbiotics, but not all, had a superior efficacy to their probiotic component alone to modulate the faecal microbiota towards a purportedly healthy composition.

Prebiotic screening for selected probiotics during 24 h growth experiments showed that fermentation was strain-specific. scFOS was the best fermented substrate for most of the probiotics tested here. However, another fructooligosaccharide (FOS) did not give the same increase for several of the test Lactobacillus strains. In previous pure culture studies whereby scFOS has been evaluated, 12 out of 16 Lactobacillus strains tested were able to grow on this substrate (Kaplan & Hutkins, 2003) which seems to confirm that scFOS is generally a well-used substrate by lactobacilli. Degradation of scFOS has been studied at the gene expression level and has shown that a specific transport system was involved exclusively in the intake of the shortest FOS fractions in L. plantarum WCF51 (Saunier et al., 2007). Other transport systems have been described for FOS utilization in other Lactobacillus strains such as Lactobacillus acidophilus NCFM (Barrangou et al., 2006) and L. paracasei 1195 (Kaplan & Hutkins, 2003; Goh et al., 2006). It is interesting to note that in the present study, the only strain that was able to degrade FOS was also an L. paracasei strain.

The second best performing prebiotic after scFOS in pure culture was IMO. Increase in lactobacilli with IMO has not been previously reported, but this has been shown with bifidobacteria (Schell et al., 2002). Results obtained with IMO in these pure cultures should be, however, interpreted with caution as the product tested (Isomalto 900P) contained more than 40% (w/w) of mono and disaccharides such as glucose, maltose and isomaltose, which are not considered to be prebiotics because of their absorption in the upper GIT. Moreover, even though it has been demonstrated in rats that IMO was digested slowly in the jejunum and that higher DP IMO were less digestible in the upper GIT (Kaneko et al., 1995), it cannot be presently concluded with certainty that this is also the case in humans, as no data are available.

From this pure culture study, scFOS was retained to be further evaluated in 24-h pH-controlled batch cultures inoculated with the faecal microbiota. FOS was also retained to assess whether differences observed in pure culture would persist in a mixed bacterial environment. For the probiotics, four strains (L. fermentum ME-3, L. plantarum WCF51, L. paracasei 8700:2 and B. longum 46) out of six were selected. LGG was excluded, as it could not ferment any of the test prebiotics. Lactobacillus plantarum 299v was excluded because fermentation of the prebiotics was similar to that of L. plantarum WCF51.

In the batch culture experiments, growth of probiotic strains among the faecal microbiota was monitored to see whether the addition of a prebiotic stimulates also their growth in this more complex environment. The selection of rifampicin variants from the probiotic wild types and plating onto selective agar was a reliable method to track growth of the probiotics during the 24-h fermentation period. This method has previously been applied in several studies with mice and piglets (Murphy et al., 1999; Simpson et al., 2000) that demonstrate the power of antibiotic selectivity in tracking exogenous or probiotic organisms introduced into the intestinal tract. An interesting finding was that enhancement of probiotic growth by the prebiotic in mixed culture was strain specific. Some of the probiotics did not increase in mixed culture studies possibly because they did not compete well with the rest of the gut microbiota. In contrast to results obtained in the pure culture study with B. longum 46 and L. plantarum WCF51, no preferential growth was observed with scFOS compared with FOS. This observation may be due to a partial degradation of FOS by other members of the gut microbiota whose products could be used by the probiotics.

To evaluate the effects of probiotics, prebiotics and synbiotics upon the faecal microbiota, different groups were monitored using FISH. The FISH probes used were chosen either because they allowed to enumerate numerically predominant groups of the faecal microbiota (E. rectale–C. coccoides, Bacteroides, F. prausnitzii and Atopobium), or groups considered beneficial for the host (Bifidobacterium or Lactobacillus–Enterococcus spp.) or potentially pathogenic (C. histolyticum group) or species against which the test probiotics have been reported to have an antagonistic effect in vitro such as E. coli (Hütt et al., 2006). Both FOS stimulated the growth of bifidobacteria. This is in agreement with numerous in vitro and in vivo studies, which have reported the bifidogenic effect of these substrates (Sghir et al., 1998; Kruse et al., 1999; Boulhnik et al., 2004). However, bifidobacteria was not the only group to be stimulated by the prebiotic. It was also the case at the later time of fermentation with the E. rectale–C. coccoides group. An increase in this group has been reported in vitro and in humans with FOS (Langlands et al., 2004). Barcenilla et al. (2000), who have looked for butyrate-producing bacteria in human faecal samples, found that most were related to this group. Butyrate has an important role in the metabolism and normal development of colonic epithelial cells and has been implicated in protection against colon cancer and ulcerative colitis (Hague et al., 1997). Certain species belonging to this group, such as Roseburia spp., were shown to be able to degrade fructans and increased in mixed faecal culture (Duncan et al., 2003). Another explanation for the increase in the E. rectale–C. coccoides group, could be cross feeding between bacterial groups. Some of the metabolic products produced during test prebiotic fermentation by one bacterial group may then provide substrates to support growth of other populations (Belenguer et al., 2006). The Atopobium cluster also increased with the addition of the
scFOS and FOS, especially in one of the three volunteers. Increases in the Atopobium cluster with scFOS and FOS have not been previously described, either in vitro or in vivo, when this FISH probe was used (Clavel et al., 2005). However, it has been reported that an increase of staining intensity in the RNA-denaturing gradient gel electrophoresis profiles corresponding to a member of Atopobium group, namely Collinsella aerofaciens was observed in humans consuming 2.5 g of FOS day⁻¹ (Tannock et al., 2004).

Increases in Lactobacillus–Enterococcus were only observed when the synbiotics contained Lactobacillus spp. From these results, it appeared that fermentation of the synbiotics was selective and affected mostly members of the microbiota considered as beneficial.

This series of mixed culture studies was performed also to determine whether effects of the synbiotic were different from the probiotic and prebiotic added separately to the faecal microbiota. PCA was a useful method to cluster the substrates and also to highlight the differences between them. Differences between the combinations, in particular between the prebiotics and synbiotics may have been small and difficult to detect. PCA is becoming popular in microbial ecology (Ramette, 2007). The three runs of fermentation with L. plantarum WCFS1 and L. fermentum ME-3 were chosen for applying PCA to emphasize different impacts on the faecal microbiota when the probiotic added was able to be stimulated by the prebiotic (L. plantarum WCFS1) or not (L. fermentum ME-3). With the series containing L. plantarum WCFS1, differences were detected between the synbiotics and prebiotics due to the Lactobacillus–Enterococcus group, which was higher with the synbiotic. Moreover, the synbiotics containing L. plantarum ME-3 were only observed with higher counts in one of the three volunteers. The effect of increasing these bacteria considered beneficial and reducing those considered detrimental in the GIT; however, this remains to be proven in vivo.

**Acknowledgements**

This work has been carried out with financial support from the Commission of European Communities, specific RTD programme ‘Quality of Life and Management of Living Resources’, QLRT-2001-00135 ‘Functional Assessment of Interactions Between the Human Gut Microbiota and the Host’. It does not necessarily reflect its views and in no way anticipates the Commission’s future policy in this area.

**References**


**Supporting Information**

Additional Supporting Information may be found in the online version of this article:

**Table S1.** Bacterial populations (log_{10} cells mL^{-1}) in pH controlled batch culture after 0, 5, 10 and 24 h of fermentation with negative control (NC), *L. fermentum* ME-3, fructooligosaccharides (FOS), short-chain fructooligosaccharides (scFOS), *L. fermentum* ME-3 with FOS, and *L. fermentum* ME-3 with scFOS.

**Table S2.** Bacterial populations (log_{10} cells mL^{-1}) in pH controlled batch culture after 0, 5, 10 and 24 h of
fermentation with negative control (NC), *L. paracasei* 8700:2, fructooligosaccharides (FOS), short-chain fructooligosaccharides (scFOS), *L. paracasei* 8700:2 with FOS, and *L. paracasei* 8700:2 with scFOS.

**Table S3.** Bacterial populations (log_{10} cells mL^{-1}) in pH controlled batch culture after 0, 5, 10 and 24 h of fermentation with negative control (NC), *B. longum* 46, fructooligosaccharides (FOS), short-chain fructooligosaccharides (scFOS), *B. longum* 46 with FOS, and *B. longum* 46 with scFOS.

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