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An *in vitro* study of the effect of probiotics, prebiotics and synbiotics on the elderly faecal microbiota

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ABSTRACT

The use of dietary intervention in the elderly in order to beneficially modulate their gut microbiota has not been extensively studied. The influence of two probiotics (*Bifidobacterium longum* and *Lactobacillus fermentum*) and two prebiotics [isomaltooligosaccharides (IMO) and short-chain fructooligosaccharides (FOS)], individually and in synbiotic combinations (*B. longum* with IMO, *L. fermentum* with FOS) on the gut microbiota of elderly individuals was investigated using faecal batch cultures and three-stage continuous culture systems. Population changes of major bacterial groups were enumerated using fluorescent *in situ* hybridisation (FISH). *B. longum* and IMO alone significantly increased the *Bifidobacterium* count after 5 and 10 h of fermentation and their synbiotic combination significantly decreased the *Bacteroides* count after 5 h of fermentation. *L. fermentum* and FOS alone significantly increased the *Bifidobacterium* count after 10 h and 5, 10 and 24 h of fermentation respectively. *B. longum* with IMO as well as *B. longum* and IMO alone significantly increased acetic acid concentration during the fermentation in batch cultures. In the three-stage continuous culture systems, both synbiotic combinations increased the *Bifidobacterium* and *Lactobacillus* count in the third vessel representing the distal colon. In addition, the synbiotic combination of *L. fermentum* with scFOS resulted in a significant increase in the concentration of acetic acid. The results show that the elderly gut microbiota can be modulated *in vitro* with the appropriate pro-, pre- and synbiotics.

1. Introduction

The human colon harbours a highly complex bacterial community which changes with age both in relative numbers of different populations and in species diversity [1,2]. These changes can have negative consequences for the health of the individual as the gut microbial community plays an important role in the prevention of certain inflammatory and metabolic diseases and in resistance to infection [3]. Claesson et al. [4], in a study comparing people aged 65 and over with younger adults, concluded that the faecal microbiota of the elderly harboured a greater proportion of *Bacteroides* spp. and distinct abundance patterns of *Clostridium* groups. Mueller et al. [5], found that enterobacteria population was higher in the elderly compared to the adult group and bifidobacteria populations were higher in the Italian elderly population compared to all the other groups studied. In contrast, it has also been shown [6] that institutionalised elderly had significantly higher numbers of *Bacteroides* and lower numbers of bifidobacteria and *Clostridium* cluster IV in their faeces compared to the younger volunteers. Low fibre intake by the elderly and increased use of antibiotics could cause a reduction of colonic short-chain fatty acids (SCFA) [7]. Thus nutritional intervention in the elderly could be beneficial since it is known that pro-, pre- and synbiotics help to increase the production of SCFA in the colon. In order to select appropriate pro-, pre- and synbiotics, it is rational to investigate their influence on mixed faecal cultures. Different methodologies have been used to determine the properties of a test substrate. A rapid and simple way of evaluating various properties, ranging from the survivability of probiotics to the end products of fermentation such as SCFA, is the use of batch cultures inoculated with faecal slurry [8]. A multi-stage continuous culture system, with vessels representing the proximal, transverse and distal areas

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of the human colon, has also been used as a "gut model" [9]. This model has been validated through comparisons to the bacteriology and enzymology of gut contents from human sudden death victims and has been widely used since [10–12].

The aims of the present study were to investigate *in vitro* the effect of *Lactobacillus fermentum*, *Bifidobacterium longum*, scFOS, IMO and synbiotic combinations of *L. fermentum* with scFOS and *B. longum* with IMO upon intestinal bacterial populations and SCFA production associated with elderly people.

2. Materials and methods

2.1. Strains

L. fermentum 907 and *B. longum* 1011 (laboratory designated numbers), isolated from faeces of healthy elderly individuals were used based on their probiotic properties and antimicrobial activity as described by Likotrafiti et al. [13,14].

2.2. Chemicals

All media constituents were purchased from Oxoid Ltd., Basingstoke, UK and all chemicals were purchased from either Sigma–Aldrich, Poole, UK or BDH Ltd, Poole, UK unless otherwise stated. Media used consisted of Beerens' agar, for the enumeration of *B. longum* (prepared as described by Beerens [15]), Rogosa agar, for the enumeration of *L. fermentum*, peptone water, phosphate-buffered saline (PBS pH 7.3, 8 g/L NaCl) and de Man Rogosa Sharpe (MRS) broth were also used. Media were autoclaved according to the manufacturer's instructions. Peptone-yeast extract broth (PY, pH 6.9) was prepared as described by Likotrafiti et al. [13]. Rifampicin stock solution was prepared by diluting 1 g of rifampicin in 20 ml of dimethylsulphoxide (DMSO). Media used for the three-stage culture models (adapted by Macfarlane et al. [9]) was prepared as described by Probert et al. [11], with the addition of 4 g mucin (porcine gastric type III). 0.5 N and 1 N NaOH and HCl were used to adjust and maintain the pH of the batch cultures and the three-stage continuous culture models respectively. The carbohydrates used were as follows: short-chain fructooligosaccharides [scFOS, Actilight 950P (95% oligosaccharides, 5% glucose, fructose and sucrose, Cyril-Beghin Meiji, France, DP = 2–5)] and isomaltooligosaccharides [IMO Isomalto 900P, Showa Sangyo, Japan (88.6% oligosaccharides, 2.5% glucose, 2.4% maltose, 5% moisture)]; glucose was used as a non-selective control in the batch cultures. Both prebiotics were received in powder form, diluted as appropriate in distilled water, filter sterilised and stored at 4 °C for up to five days.

2.3. Selection of rifampicin-resistant variants

In order to detect and enumerate the probiotic strains throughout the batch and three-stage continuous cultures, rifampicin resistance variants were generated as described by Saulnier et al. [16]. Growth rates of the rifampicin variants were measured by optical density at 660 in PY broth containing 1% (w/v) scFOS for *L. fermentum* and 1% (w/v) IMO for *B. longum*. Growth rates were calculated and compared with those from the wild-type strains in triplicate.

Before adding the probiotics in the batch and three-stage continuous cultures, MRS broths were supplemented with 100 μg/ml of rifampicin and the strains grown under anaerobic conditions at 37 °C for 24 h. Overnight grown cultures were then centrifuged (13,000 ×*g* for 5 min) washed and resuspended in 1 ml (~10^8 CFU/ml) of sterile anaerobic PBS before being added to the fermenters. Similarly, all agar constituents used for enumeration of the resistant variants were supplemented with 100 μg/ml of rifampicin.

2.4. FISH

The FISH technique was used to enumerate selected groups of bacteria. Sample fixing and bacterial enumeration was performed as described by Saulnier et al. [16]. Sample hybridisation was carried out as described by Rycroft et al. [17], using genus-specific 16S rRNA 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* [18], Bac 303, specific for the *Bacteroides–Prevotella* group [19], His 150 for the *Clostridium histolyticum* subgroup [20] and Lab 158 for the *Lactobacillus–Enterococcus* [21]. The sequences of the probes and the hybridisation temperature are described by Saulnier et al. [16].

2.5. Stirred, pH-controlled, anaerobic faecal batch cultures

Faecal samples were donated by a 69-year-old woman (Donor A) and a 70-year-old man (Donor B). Both volunteers were healthy and did not take any antibiotics for six months prior to collection of the faecal samples. Before each experiment the faecal sample was screened for rifampicin resistant *Lactobacillus* and *Bifidobacterium* strains through plating onto appropriate agar containing 100 μg/ml of rifampicin.

Seven batch fermenters were run in parallel, filled with sterile pre-reduced PY broth and inoculated with 15 ml of 10% (w/v) faecal slurry up to a total volume of 150 ml. Slurries were prepared by homogenising faeces in anoxic PBS (pH 7.3). The fermenters were kept anaerobic by continuous sparging with O_2-free N_2 (15 ml/min) throughout the experiment, culture pH was automatically controlled at pH 6.9 using 0.5 N HCl and 0.5 N NaOH solutions, vessels were continuously stirred and the temperature held at 37 °C using a circulating water-bath. The set-up of the batch cultures was as follows: vessel 1 (V1) contained *B. longum* + 1% (w/v) IMO, vessel 2 (V2) *B. longum* + 1% (w/v) glucose, vessel 3 (V3) 1% (w/v) IMO, vessel 4 (V4) contained 1% (w/v) glucose, vessel 5 (V5) *L. fermentum* + 1% (w/v) scFOS, vessel 6 (V6) *L. fermentum* + 1% (w/v) glucose and vessel 7 (V7) contained 1% (w/v) scFOS. V1 and V5 were run in order to evaluate the synbiotics, V2 and V6 were run in order to evaluate the probiotic and V3 and V7 were run in order to evaluate the prebiotic. V4 was run with glucose and was used as non-selective control. Samples were obtained at 0, 5, 10 and 24 h of batch culture fermentation, for enumeration of bacterial counts by plating onto the appropriate agars and by FISH. Three independent runs were undertaken for each substrate using one faecal sample from one donor for each run (two samples from the Donor A and one from the Donor B).

2.6. Three-stage continuous culture system

All faecal samples used in the three-stage continuous culture models were donated by Donor A. Before each experiment, the faecal sample was screened for rifampicin resistant *Bifidobacterium* and *Lactobacillus* strains through plating onto the appropriate agars containing 100 μg/ml of rifampicin.

The model used consisted of three vessels: vessel 1 (V1), vessel 2 (V2) and vessel 3 (V3) representing the proximal, transverse, and distal region of the colon. The method was as described by Probert et al. [11]. Overall retention time of the model was evaluated at ~43 h with retention time in V1 of 13.6 h (dilution rate of 0.074/h) and 14.5 h (dilution rate of 0.069/h) in V2 and V3 respectively.