

Phlotypes related to *Ruminococcus bromii* are abundant in the large bowel of humans and increase in response to a diet high in resistant starch

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Abstract

To further understand how diets containing high levels of fibre protect against colorectal cancer, we examined the effects of diets high in nonstarch polysaccharides (NSP) or high in NSP plus resistant starch (RS) on the composition of the faecal microbial community in 46 healthy adults in a randomized crossover intervention study. Changes in bacterial populations were examined using denaturing gradient gel electrophoresis (DGGE) of 16S rRNA gene fragments. Bacterial profiles demonstrated changes in response to the consumption of both RS and NSP diets [analysis of similarities (ANOSIM): $R = 0.341\text{--}0.507$, $P < 0.01$]. A number of different DGGE bands with increased intensity in response to dietary intervention were attributed to as-yet uncultivated bacteria closely related to *Ruminococcus bromii*. A real-time PCR assay specific to the *R. bromii* group was applied to faecal samples from the dietary study and this group was found to comprise a significant proportion of the total community when individuals consumed their normal diets ($4.4 \pm 2.6\%$ of total 16S rRNA gene abundance) and numbers increased significantly ($\pm 67\%$, $P < 0.05$) with the RS, but not the NSP, dietary intervention. This study indicates that *R. bromii*-related bacteria are abundant in humans and may be significant in the fermentation of complex carbohydrates in the large bowel.

Introduction

A recent large multinational study has demonstrated that there is a significant lowering of colorectal cancer risk with increased fibre consumption (Bingham *et al.*, 2003). This is in contrast to a recent metaanalysis of prospective studies, which failed to find such a relationship (Park *et al.*, 2005). Nevertheless, there is a substantial body of evidence which suggests that dietary complex carbohydrates are important for maintaining the health of the large bowel (Cassidy *et al.*, 1994; Topping & Clifton, 2001; Topping, 2007). Fibre can be regarded as plant components that are resistant to digestion in the small intestine and that undergo varying degrees of fermentation in the large intestine. This includes nonstarch polysaccharides (NSP) and other components such as resistant starch (RS). There is growing evidence that RS, which is abundant in foods such as grains and legumes, may contribute to a decreased risk of colorectal disease by

increasing faecal bulk and short-chain fatty acid (SCFA) levels, and decreasing faecal pH and transit time (Topping & Clifton, 2001; Topping, 2007). RS appears to counteract or prevent colonic DNA damage induced by high levels of dietary protein, and this is associated with the production of SCFA, especially butyrate (Toden *et al.*, 2007a, b). Butyrate appears to be most important in this regard because it is the preferred source of energy for colonocytes and helps maintain tissue integrity by promoting apoptosis in aberrant cells lining the colon (Hague *et al.*, 1993; Clausen & Mortensen, 1995; Ritzhaupt *et al.*, 1998; Topping & Clifton, 2001).

The effects of fibre, particularly RS, on the colonic microbiota are poorly understood but are likely to be central to understanding the activity of these organisms in the colon.

Studies of human gut microbial ecology have indicated the presence of a significant diversity of bacterial phlotypes, with current estimates for the human colon varying

between <150 and >500 individual species (Mai & Morris, 2004; Eckburg *et al.*, 2005). Although recent studies have concentrated on the composition of colonic flora through phylogenetic (Wilson & Blitchington, 1996; Suau *et al.*, 1999; Eckburg *et al.*, 2005; Abell & McOrist, 2007) and metagenomic (Gill *et al.*, 2006) analyses, studies of the dominant bacterial species involved in dietary metabolism are limited (Duncan *et al.*, 2007), with the majority of studies examining *in vitro* activity of faecal bacteria. Molecular studies of the effects of dietary fibre intervention on faecal microbiota in humans suggest that there is a change in the distribution of dominant bacteria among the major groups, with increases in the abundance of *Bifidobacteria* and lower numbers of some members of *Clostridiaceae* (Smith *et al.*, 2006). A recent study of the effect of prebiotic carbohydrates on mucosal flora demonstrated an increase in the number of culturable *Bifidobacteria* in humans after administration of oligofructose and inulin (Langlands *et al.*, 2004). Studies of the bacterial phylotypes colonizing insoluble faecal material by *in vitro* methods have demonstrated the predominance of phylotypes related to *Ruminococcus* and other groups colonizing starch particles (Leitch *et al.*, 2007). The *Bacteroides* and *Clostridial* groups, currently identified or classified by anaerobic culture or molecular approaches, dominate human colonic flora, but the degree to which species differ in distribution between individuals is largely unknown. The majority of cultivated butyrate-producing bacteria belong to clostridial clusters IV and XIVa (Barcenilla *et al.*, 2000), which include the *Eubacterium* and *Faecalibacterium* clusters. One study detected butyrate-producing members of the *Eubacterium/Roseburia* genera in all of their human volunteers (Hold *et al.*, 2003), and suggests that these species are among the most abundant butyrate-producing bacteria in human faeces.

In the study described here, healthy human volunteers supplemented their regular diets with foods high in NSP or high in NSP and RS to examine effects on the indices of large bowel health. As part of this effort, we have used molecular methods [PCR and denaturing gradient gel electrophoresis (DGGE)] to examine key bacterial population changes in faecal bacteria in response to these diets. These bacterial populations may significantly influence the production of colonic SCFA or other parameters associated with bowel health.

Materials and methods

Study design

The randomized crossover dietary intervention trial involved 46 healthy human volunteers. The ages of the subjects (16 men, 30 women) ranged from 25 to 66 years. Body mass index (BMI) ranged from 19.0 to 36.2 (average 26.40).

Approval for the study was obtained from the CSIRO Human Nutrition Human Ethics Committee and informed written consent was obtained from each volunteer. Participants completed a health questionnaire, and none were smokers, had a history of colorectal pathology or had used antibiotics in the preceding 3 months.

Volunteers consumed their normal diet with or without supplementation throughout the 14-week study period, but were told to avoid high-fibre food or foods known to affect bowel physiology (e.g. liquorice) during the supplementation periods. After 2 weeks of consuming their normal (regular) diets (N), faecal SCFA concentrations were measured and volunteers were randomly allocated to one of two cohorts normalized for age, gender and SCFA concentration. After a further 2 weeks on their regular diet (a total of 4 weeks), each cohort consumed one of two dietary supplements daily for 4 weeks, i.e. a supplement high in NSP consisting of 25 g total fibre and 1 g RS (NSP diet) or a supplement high in NSP and RS (RS diet) consisting of 25 g total fibre and 22 g RS (Table 1). This was followed by 2 weeks of their normal diet (NI, normal intervening) and then 4 weeks on the alternate supplement, i.e. individuals who consumed the NSP diet during the first supplementation period subsequently consumed the RS diet in the second supplementation period, and vice versa. Volunteers maintained a record of supplements consumed during the NSP or RS supplementation phases.

Faecal collection

Faecal specimens were collected from each volunteer at weeks 2 and 4 (initial normal dietary phase), 6 and 8 (first dietary intervention phase), 10 (normal diet between the two dietary phases, NI), and 12 and 14 (second dietary intervention phase). Each faecal specimen collection period was for 48 h. Upon passing, samples collected in sealable plastic bags were immediately transferred to freezers and stored at -20°C . Samples were defrosted at room temperature, and 48 h collections consisting of more than one sample were combined and termed the specimen. All specimens were weighed and homogenized gently by hand, and the pH and moisture content were measured. All processing occurred under anaerobic conditions (Bactron IV anaerobic chamber, Sheldon). Aliquots (0.1 g) of each specimen for DNA extraction and molecular analyses were then stored at -80°C until required.

DNA extraction

An aliquot (c. 0.1 g) of each specimen was defrosted at room temperature, resuspended in phosphate-buffered saline (137 mM NaCl, 10 mM phosphate, 2.7 mM KCl, pH 7.4), containing 2% w/v PVP K30, incubated for 5 min at 80°C and vortexed until homogeneous. DNA was extracted using

Table 1. Nutritional content of dietary supplements, quantity per 100 g of product

Component	High nonstarch polysaccharide (NSP) diet				High NSP plus Resistant Starch (RS) diet			
	BranPlus™	Carrots	Couscous	Unprocessed Bran	BarleyPlus™	Four bean mix	Greenwheat Freekah	HiMaize™
Energy (kJ)	1220	102	1448	1080	1521	497	1471	1259
Dietary fibre (g)	43	2.2	3.6	47.4	26.5	7.2	11	30.9
Resistant starch (g)	0.4	0.2	1.8	5	3.7	6	15.4	49
Protein (g)	13	0.8	11.3	14.6	15	7.7	12.6	0.5
Total fat (g)	3.9	0	1.3	2.7	7.2	0.7	4.3	0.5
Saturated fat (g)	0.6	0	0.3	1	0.4	0.1	1.6	0.1
Polyunsaturated fat (g)	0	0	0.2	0	1.4	0.2	1.3	0.2
Monounsaturated fat (g)	0	0	0.1	0	0.4	0	1.4	0
Carbohydrate (g)	29.2	5.2	71	23	61.3	16.2	74.9	63
Sugars (g)	14.4	5.2	1.3	2.4	7.8	2.2	3.5	0.1
Starch (g)	24.2	0.2	76.6	16.3	29.7	11.3	64.5	83.3
Water (g)	4.5	91.5	8.6	11.2	6.5	69.1	5.2	14
Thiamin (mg)	1.38	0	0.16	0.63	0.61	0.07	0.35	0.04
Riboflavin (mg)	1.08	0	0.08	0.26	0.9	0.02	0.22	0
Niacin (mg)	13	0.6	3.5	23.2	9.3	0.7	5.10	0.4
Niacin equivalents (mg)	15.17	0.73	5.38	28.2	11.8	1.98	7.2	0.48
Vitamin C (mg)	0	1	0	0	0	0	0	0
Total folate (µg)	191	22	20	252	222	40	42	1
Total vitamin A equivalents (µg)	250	620	0	1	0	0	0	0
B-Carotene equivalents (µg)	3	3710	0	5	3	1	0	0
Sodium (mg)	395	200	190	18	880	250	6	6.5
Potassium (mg)	952	48	166	1240	460	220	440	16
Magnesium (mg)	276	7	44	490	150	30	110	5
Calcium (mg)	500	27	24	87	34	43	53	8
Phosphorus (mg)	740	11	170	1001	340	89	319	42
Iron (mg)	10	0.5	1.1	11.9	6.7	2	4.5	0.8
Zinc (mg)	5	0.4	0.8	4.7	1.7	0.8	1.7	0.1

a modified method of Boom *et al.* (1990). Cell lysates were prepared by the addition of sodium dodecyl sulphate to a final concentration of 1%, followed by heating to 80 °C for 20 min, after which samples were cooled to room temperature and lysosyme was added to a final concentration of 20 mg mL⁻¹, incubated for 1 h at 37 °C and then treated with 0.5 mg mL⁻¹ proteinase K at 65 °C for 30 min. The lysate was extracted with an equal volume of a phenol:chloroform (1:1) mix and then with an equal volume of chloroform:isoamyl alcohol (24:1). To 100 µL of the resulting aqueous phase, five volumes of buffer 1 (6M sodium perchlorate, 50 mM Tris HCl, 10 mM disodium EDTA, pH 8.0) and 15 µL of sterile silica matrix (50% 5–10 µm particles in dH₂O v/v, pH 2.0) were added. Samples were vortex mixed and then incubated for 30 min at room temperature, followed by centrifugation at 10 000 g for 2 min, after which the supernatant was removed and a further 500 µL of buffer 1 was added. Samples were vortexed and spun again, then washed twice with 500 µL buffer 2 (20 mM Tris HCl, 2 mM disodium EDTA, 0.8 M NaCl, in 50% v/v absolute ethanol, pH 7.6) and dried, and the matrix resuspended in 100 µL of TE buffer [10 mM Tris HCl (pH 8.0); 1 mM EDTA]. DNA was eluted at 37 °C for 30 min, and the matrix was removed

by centrifugation at 10 000 g for 3 min. The DNA solution was removed to a fresh tube and stored at -20 °C. The purity and concentration of the nucleic acid were determined by spectrophotometry with purity assessed using the A_{260 nm}:A_{280 nm} ratio and the nucleic acid concentration quantified from the A_{260 nm} measurement.

PCR amplification of 16S rRNA genes

Universal bacteria 16S rRNA gene primer sets were used to amplify c. 500 bp of the 16S rRNA gene using the forward primer 907f (5'-AAA CTC AAA GGA ATT GAC GG-3') (Santegoeds *et al.*, 1998) and the GC-clamped reverse primer 1392rc (5'-CGC CCG CCG CGC CCC CGC CCG GCC CGC CGC CCC CGC CCC ACG GGC GGT GTG TRC-3') (Lane, 1991). The PCR comprised 1 U of BioTaq polymerase (Bioline), 10 mM deoxynucleotide triphosphates (dNTPs) and 12.5 pmol of each primer in a total volume of 25 µL. Approximately 100 ng genomic DNA from each faecal sample was added as template, but in some cases was varied to optimize the clarity of DGGE gels. PCR was performed using a touch-down protocol on a Hybaid PCR Express thermal cycler (Hybaid, UK): over 30 cycles of 1 min denaturation at 95 °C, 1 min annealing reduced

by 0.5° steps from 65 to 55 °C for the first 20 cycles, then 10 cycles at 55 °C and 2 min extension at 72 °C, followed by one cycle of a final 4 min 72 °C extension step.

DGGE analysis

PCR-amplified 16S rRNA gene products were analysed using DGGE (INGENYphorU-2 gel, Ingeny International, NL) according to the manufacturer's method for perpendicular gels. Bacterial PCR products were separated on a 30–75% denaturing gradient (where 100% denaturant contains 7 mol L⁻¹ urea and 40% formamide), 6% acrylamide, TAE (40 mM Tris-acetate and 1 mM disodium EDTA, pH 8.0) gel. PCR product (15 µL) and of 6 × gel loading dye (3 µL) (40% w/v sucrose, 0.25% bromophenol blue and 0.25% xylene cyanol FF in dH₂O) were loaded per track and electrophoresed at 110 V for 16 h at 60 °C. Gels were stained for 30 min with 1 × SYBR-gold nucleic acid stain (Molecular Probes, Eugene, OR) in 100 mL of TAE. Gels were destained for 3 min in 100 mL of Milli-Q H₂O and photographed under UV light (DigiDoc System, Bio-Rad Laboratories). For comparison between gels, the positive control PCR reaction, containing a mixture of bands, was run in the outside lanes and used to standardize fragment migration. DGGE banding patterns were converted to a band-weight matrix using the GEL-QUANT software package (AMPL software).

DNA sequencing and phylogenetic analysis

DGGE bands of interest were extracted and reamplified according to the methods of Abell & Bowman (2005). Sequencing of DGGE bands was performed using BigDye (V3) chemistry and analysed on an ABI 3700 DNA sequencer (ABI), utilizing the 907F primer. The sequences were checked for chimeras (Ribosomal RNA Database Project, <http://www.rdp.cme.msu.edu>), and phylogenetic analysis was performed using the ARB software package (Ludwig *et al.*, 2004). Sequences from this study and closest reference sequences obtained using BLAST (<http://www.ncbi.nlm.nih.gov/blast>) were imported into the ARB database (release January 2005), automatically aligned and then corrected manually. Phylogenetic trees were calculated using the neighbour-joining method (Saitou & Nei, 1987), utilizing only sequences with > 1300 nucleotides. Shorter sequences were added to the tree using the parsimony method and applying the bacteria base frequency filter, without changing the overall tree topology. Sequences from this study were deposited into the GenBank database under accession numbers EF581095–EF581123 and EF591651–EF591667.

Design of PCR primers specific to the *Ruminococcus bromii* cluster

Primers specific to members of the *R. bromii* and closely related sequences from this study and the GenBank database

were designed using the probe design function in ARB. The primers were checked for specificity *in silico* with the probe check function in ARB, with BLAST against the NCBI database, and *in vitro* by PCR using DNA extractions of cultures from a number of divisions, including *Escherichia coli*, *Bacteroides distasonis*, *Faecalibacterium prausnitzii*, *Roseburia intestinalis*, *Lactobacillus lactis* and *Methanobrevibacter smithii*, to ensure no nonspecific amplification. Samples that were known to contain target DNA were also assayed to achieve optimal annealing and assay temperatures using melt curve analysis (OPTICON MONITOR Version 3.1, MJ Research). The specificity of the *R. bromii et rel* primer set was tested by cloning the PCR product, generated with the *R. bromii et rel* primer set and the thermal cycling conditions described below (with the exception that only 25 cycles were performed), from two samples using the p-GEM T-easy kit (Promega). Ten clones from each library were selected and sequenced as described previously (Abell & McOrist, 2007). All sequenced clones containing an insert grouped closely with *R. bromii* (97–100% homology, EF591651–EF591667).

Real-time PCR quantification of members of the *R. bromii* group

The relative abundance of *R. bromii et rel* was determined using real-time PCR. Real-time PCR reactions were prepared in hard-shell, thin-wall, 96-well microplates and carried out in a Chromo-4 thermocycler (MJ Research). Results were analysed with the OPTICON MONITOR 3 software (Version.3.1). Reactions (20 µL volumes) contained 1 U of BIOTAQ (Bioline), 0.6 µL MgCl₂ (50 mM), 0.4 µL 50 mM dUNTP mix (Bioline), 2 µL 10 × NH₄ reaction buffer, 0.2 µL of each of the primers R.brom-F and R.brom-R (5'-TAA ACT TCT TTT ATT AA -3' and 5'-ACT ACT GAC TTC GGG TAT T-3', respectively) (50 pmol µL⁻¹), 1 µL of 1 × SYBR Green I nucleic acid stain (Molecular Probes) and 50 ng of template DNA and sterile Milli-Q water. Assays were performed in triplicate using a thermocycling program consisting of an initial 5 min, 95 °C step followed by 35 cycles consisting of 95 °C for 30 s, 42 °C for 30 s and 72 °C for 30 s with fluorescent acquisition, and a further fluorescent acquisition step at 80 °C. A final melt curve analysis was performed after completion of all the amplification cycles with fluorescence acquired at 0.5 °C intervals between 55 and 100 °C. Fluorescence analysis was performed at a temperature at which all primer dimers had melted, but the specific product had not (80 °C for both assays). Amplified products from mixed template samples only contained a single peak, indicating that product length variability and G+C content did not have a significant effect on quantification. Positive control standards for the real-time PCR included a clone with an *R. bromii*-related sequence as well as known positive samples.

A dilution series of *R. bromii et rel* positive faecal DNA was also quantified using the assay and demonstrated a linear relationship between concentration and C_T value ($R^2 \geq 0.98$). A series of 10-fold dilutions of the control template was analysed in parallel with faecal DNA samples. The negative controls included samples lacking template DNA. All real-time PCR products were examined using agarose gel electrophoresis to ensure that products corresponded to the correct size and to ensure the absence of nonspecific products. Values were corrected for their initial sample weight and averaged to calculate the total 16S rRNA genes per gram wet weight of faecal material. The samples that tested negative for *R. bromii et rel* DNA were assigned an *R. bromii et rel* DNA concentration of 0 for the purpose of analysis. Total faecal 16S rRNA gene abundance was performed as described previously (Abell *et al.*, 2006), using the primer pair 519f (5'-CAG CMG CCG CGG TAA TAC-3') and 907r (5'-CCG TCA ATT CCT TTG AGT TT-3'). *Ruminococcus bromii et rel* abundance was expressed as a percentage of total 16S rRNA gene abundance.

SCFA analysis

SCFA concentrations in faecal specimens were measured according to a modified method of Patten *et al.* (2002). Briefly, duplicate 1 g faecal specimens were prepared for SCFA distillation by the addition of 3 × volume for weight of 1.68 mM heptanoic acid, pH 7, mixed and centrifuged at 2500 g for 10 min at 5 °C. The supernatant (150 µL) was distilled under vacuum and the distillate was transferred to a glass vial. A volume (0.2 µL) of each distillate was loaded separately onto a Zebtron ZB-FFAP (Phenomenex) GC column (length 30 m, internal diameter 0.53 mm, film thickness 1 µm) within an Agilent 6890N Network GC system. The GC system used initial temperature 90 °C, hold 0.5 min, ramp 20 °C min⁻¹, final temperature 190 °C and total run time of 8.0 min. A gas flow of 7.7 mL min⁻¹ maintained a 3.26 psi column head pressure. Calibration standards consisted of the following amounts of acids: 26.22 mM acetic, 19.86 mM propionic, 3.24 mM isobutyric, 16.32 mM butyric, 5.40 mM isovaleric, 5.46 mM valeric, 4.74 mM caproic and 5.04 mM heptanoic. The standard mix (0.2 µL) was used to calculate retention times and create a standard plot, included in each GC run at five-sample intervals.

Statistical analysis

Statistical analysis of DGGE banding patterns was performed using the PRIMER 6 package (PRIMER-E Ltd, Plymouth, UK). Before statistical analysis, DGGE banding profiles were normalized by dividing individual band intensities by the sum of band intensities in the sample.

Subsequent statistical analysis was performed on square-root transformed data.

Similarity percentage analysis (SIMPER) was used to weight the contribution of DGGE bands to the similarity or dissimilarity within or between different dietary phases for each individual (Clarke, 1993). Bands that had > 10% contribution to differentiating either dietary phase were selected for extraction and sequencing. The relatedness of samples representing different dietary phases within each individual sample was calculated using the Bray–Curtis similarity calculation on square-root transformed data (Kenkel & Orloci, 1986; Minchin, 1987). Analyses of the mean change in similarity between different dietary phases across all individuals within the trial were divided into two groups according to diet order, and the mean similarity between samples was calculated for all individuals within that diet-order group. From this a similarity matrix was assembled and used to generate nonmetric multidimensional scaling (MDS) plots representing the mean similarity of dietary phases, across all volunteers in each diet-order group in two-dimensional space. The similarity between diets on the basis of DGGE banding patterns was calculated using the two-way crossed analysis of similarities (ANOSIM) routine (Clarke, 1993), where a value of 0 indicates no difference between two samples and a value of 1 indicates no similarity between samples. The ANOSIM differences were considered significant when $P < 0.05$. Analysis of bacterial diversity from DGGE profiles was performed using the Shannon–Weaver index (H') (Shannon & Weaver, 1949), based on relative band intensities (Abell & McOrist, 2007).

Results

Dietary compliance

All subjects consumed their normal diets, with or without supplement, for the duration of the study. On average, 93% and 91% of the amount of the fibre supplement distributed to each individual was consumed during the NSP and RS phases, respectively, and > 90% of the RS supplement was consumed during the RS phase.

Faecal SCFA

Data relating to faecal physical and biochemical analyses will be comprehensively described in a separate publication and are mentioned only briefly here (unpublished data). Faecal acetate, propionate, butyrate and total SCFA pools, as well as faecal weight and moisture, were all significantly higher ($P < 0.05$) relative to those collected during the consumption of regular diets when individuals consumed the NSP or RS diets. The total SCFA pools were increased from an average of 18.4 mmol for the regular diet (N) to 24.3 mmol by the NSP diet (a 32.1% increase) and to 25.1 mmol by the

RS diet (a 36.4% increase). However, when SCFA levels are calculated as concentrations instead of pools, the acetate, butyrate and total SCFA levels significantly increased relative to the regular diets by the RS diet only (from 82.3 to 90.6 mM, a 10.1% increase; $P < 0.001$). Butyrate concentrations were increased by 22.1% and acetate by 9.5% (both $P < 0.001$). The RS diet also significantly lowered faecal pH (-0.14 ; $P < 0.001$).

DNA extraction, PCR amplification and DGGE analysis

DNA was successfully extracted from 355 faecal specimens, and was shown to be of high molecular weight and free from protein and RNA contamination by agarose gel electrophoresis. PCR amplification of 16S rRNA genes and subsequent agarose gel electrophoresis of PCR product resulted in specific bands of the predicted size (*c.* 500 bp). PCR products were separated by DGGE and banding patterns were obtained from all DNA samples. Distinct bacterial profiles for each participant were observed (see Fig. 1 for profiles from three individuals; other profiles are not shown). In the regular dietary phase, individuals demonstrated between seven and 26 distinct DGGE bands (mean 15). For each individual, changes in the bacterial profiles involved an increase or decrease in the intensity of particular bands, with only minor variation in the number of bands from samples obtained during the course of the trial. Mean DGGE band numbers and H' demonstrated no significant difference between any of the diet groups, and there was no significant difference between diet orders.

Changes in DGGE banding patterns

An analysis of the similarity of DGGE banding patterns between diet groups, using the Bray–Curtis similarity measure, demonstrated a mean change in the composition of colonic flora with dietary intervention across both the

diet-order groups. Cluster analysis based on the distance matrix analysis of similarity between diet group samples demonstrated discrete clustering of each of the dietary phases (Fig. 2).

The separation of different dietary phases on MDS plots was supported by a two-way crossed ANOSIM analysis, which demonstrated a significant difference between all dietary phases in the first diet order; however, there was no significant difference between the NI and NSP dietary phases in the second dietary order (Table 2).

Analysis of selected DGGE bands

Analysis of the change in bacterial community associated with diet in each individual revealed a number of DGGE bands whose increase in intensity, in both samples from a dietary phase, was deemed to be associated with dietary intervention. Band excision and sequencing revealed 29 individual band sequences from 21 individuals. Phylogenetic analysis of the band sequences revealed phylotypes related to the *Bacteroides* (1), *Mollicutes* (6) and *Clostridiales* (22), with a significant number closely related to *R. bromii et rel* (Rbrom) group (45%) (Fig. 3).

Quantitative-PCR (Q-PCR) of *R. bromii et rel*

Real-time PCR confirmed the presence of organisms closely related to *R. bromii* in all individuals participating in the study during at least one of the dietary phases. The mean abundance of this group in the normal dietary phase was $4.39 (\pm 2.6)\%$ of total 16S rRNA gene abundance, with between 0% and 20.5% detected in individuals during the normal diet phase. There was a significant increase in the abundance of the *R. bromii* group in the RS diets relative to the initial normal dietary period, irrespective of diet order (Table 3). There was no significant relationship between the abundance of this group and faecal SCFA concentrations or pH (data not shown).

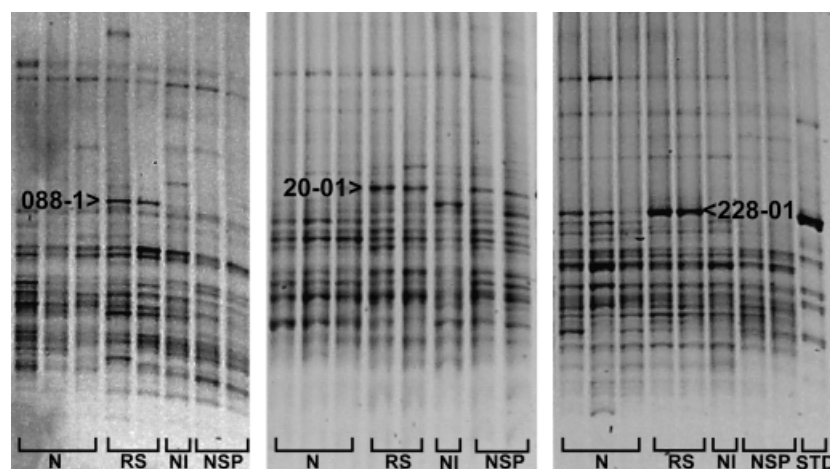


Fig. 1. DGGE profiles of three individuals demonstrating variation in banding profiles with diet. Bands indicated with arrows were deemed to be associated with diet and were subsequently extracted and sequenced (left to right DGGE bands 088-1, 20-01 and 228-1).

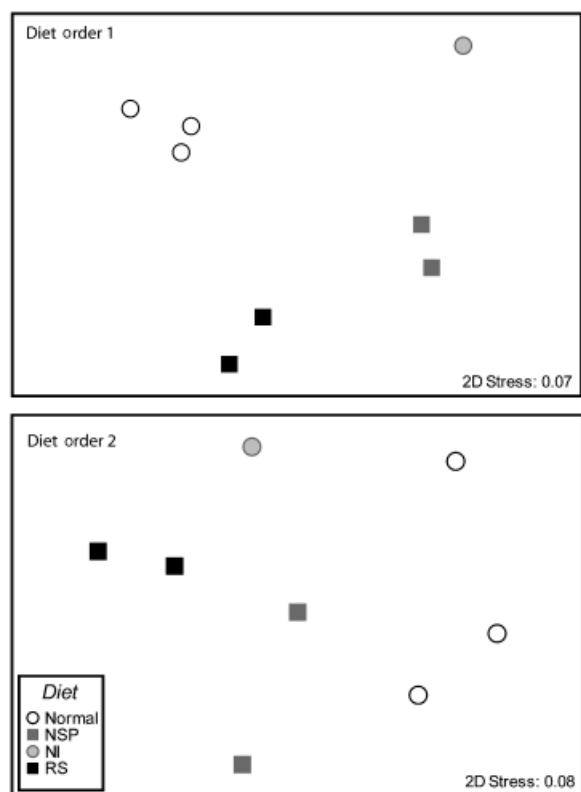


Fig. 2. MDS analysis of diets based on mean difference between diets within each individual across the whole trial. Diet order 1 (RS then NSP, top) and diet order 2 (NSP then RS, bottom). Stress levels for each analysis are shown at the bottom right of each plot, and indicate a good two-dimensional representation. Diet symbols are shown at the bottom left of the lower plot. Diets: ○, normal; ■, RS; ■, NSP; ●, NI.

Table 2. Two-way crossed analysis of similarity for DGGE band patterns between diet groups across all volunteer groups

Diet order 1 (RS–NSP)	Normal	RS	NI
RS	0.341**	–	–
NI	0.48***	0.6**	–
NSP	0.507***	0.537***	0.619***
Diet order 2 (NSP–RS)			
RS	0.479***	–	–
NI	0.359*	0.478**	–
NSP	0.434***	0.394**	0.3

Shaded cells are significant.

* $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$.

Numbers represent the degree by which two groups of samples differ (0, identical communities and 1, no similarity between communities).

NI, normal intervening; NSP, high NSP diet; RS, high NSP plus high RS diet.

Discussion

In this study we have shown significant shifts in the populations of bacteria in the large bowel of humans in response to diets high in NSP or NSP and RS. We found that

many of the bacteria that appeared to have increased in dominance in response to these diets, based on DGGE band intensity, formed a group within the clostridial cluster IV closely associated with *R. bromii*, suggesting that this group of organisms may play a significant role in the digestion of dietary fibre and carbohydrates in the human colon. A Q-PCR assay for phylotypes closely related to *R. bromii* was subsequently developed and demonstrated a surprising abundance of this specific group of bacteria, which increased significantly in response to the RS, but not the NSP, diets. Other bacteria related to *F. prausnitzii*, *Eubacterium rectale* and *Bacteroides thetaiotaomicron*, organisms known to contribute to SCFA production or starch degradation, appeared to increase in dominance in response to the RS diet in a number of subjects, and along with *R. bromii* related phylotypes may facilitate the health-promoting effects of RS in the large bowel.

Analysis of DGGE banding patterns from individuals demonstrated significant variation in the banding patterns between volunteers, consistent with a previous study of healthy adults (Abell & McOrist, 2007). Analysis of these patterns demonstrated that the differences between individuals were greater than the differences between dietary phases within each individual. For this reason, nested analysis was performed to determine whether a significant dietary effect was seen.

Ruminococci species are polyphyletic, forming two separate groups within the clostridial clusters IV and XIVa (Collins *et al.*, 1994). *Ruminococcus* are abundant in the large bowel, caecum or rumen of a large number of animals and humans and facilitate the fermentation of carbohydrates such as cellulose, pectin and starch (Herbeck & Bryant, 1974; Wang *et al.*, 1997; Leitch *et al.*, 2007). Numerous *Ruminococcus* species have been identified, many of which can be detected in human faeces. Wang *et al.* (1997) demonstrated the presence of *Ruminococcus albus*, *R. bromii*, *Ruminococcus obeum* and *Ruminococcus callidis* using a PCR assay specific for each of these species. Recent *in vitro* fermentations of human faeces by Leitch *et al.* (2007) have shown that *R. bromii*, which belongs to clostridial cluster IV (Collins *et al.*, 1994), is one of the most predominant bacteria to adhere to high-amylose starch *in vitro*.

It is known that fermentation of starch in the large bowel by bacteria results in the production of volatile SCFAs, including branched-chain acids (Topping & Clifton, 2001). Given the association of *R. bromii* with NSP and RS, it is likely that it plays an important role in the degradation and possibly fermentation of these substrates. Our study has given further support for an important role of *R. bromii* in the fermentation of starch in humans. Giving individuals a diet that contained high levels of RS along with a high NSP background (RS diet) resulted in an increased intensity of many *R. bromii*-related DGGE bands when bacterial 16S

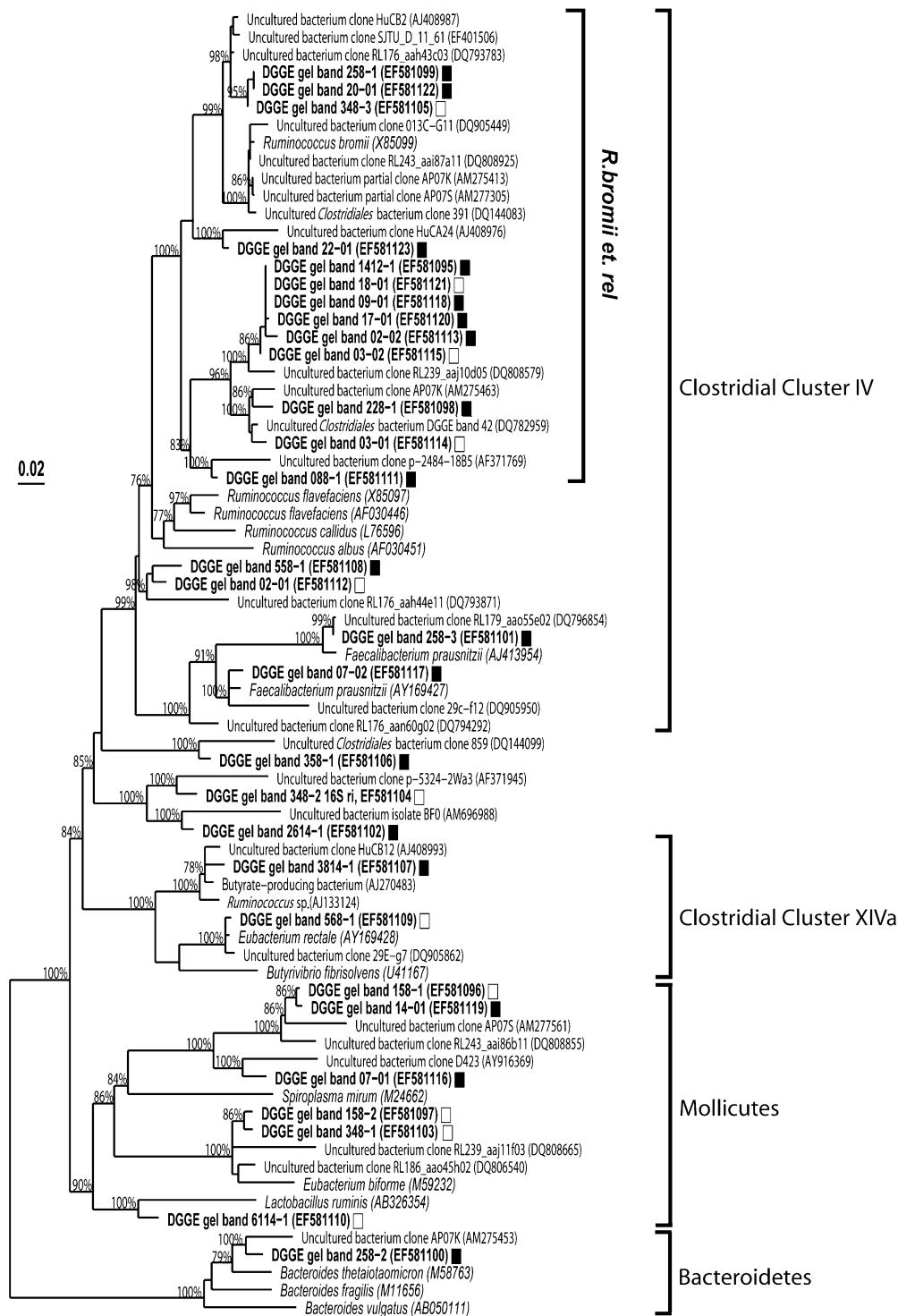


Fig. 3. 16S rRNA gene phylogenetic tree of DGGE band sequences that were associated with either the NSP or RS diets. Diets in which the bands were prominent are indicated. ■, RS; □, NSP.

rRNA gene PCR products of faecal DNA were separated. However, some of the *R. bromii*-related bands also increased in response to the NSP diet.

To obtain a more accurate measure of changes in the populations of organisms closely related to *R. bromii*, we

developed a Q-PCR assay. Although a PCR method for detecting *R. bromii* in human and animal faeces has previously been described (Wang *et al.*, 1997), we developed primers, suitable for real-time PCR, based on the more recent sequence data available. Our assay demonstrated that

Table 3. Q-PCR analysis of members of the *Ruminococcus bromii* cluster group

Diet order	Normal diet phase	Supplementation phase 1	NI diet phase	Supplementation phase 2
1 (NSP–RS)	3.9% (\pm 1.1)	4.6% (\pm 0.9)	5.9% (\pm 1.4)	6.6% (\pm 1.7)
2 (RS–NSP)	4.9% (\pm 1.1)	7.9% (\pm 2.1)	5.6% (\pm 1.4)	5.4% (\pm 1.5)

Values are expressed as a percentage of total bacterial 16S rRNA gene abundance, values in brackets represent SE. Shaded cells are significantly different from the normal diet ($P < 0.05$).

NI, normal intervening; NSP, high-NSP diet; RS, high-NSP plus high-RS diet.

R. bromii and closely related phylotypes increased with both the NSP and RS diets; however, this was only statistically significant in the RS, but not the NSP, diet.

There is mounting evidence that dietary fermentable carbohydrate in the form of RS is important for maintaining bowel health. This is thought to be a result of the high fermentability of RS and the subsequent production of SCFA. Of the SCFA, butyrate is of particular importance because it acts to maintain the integrity of colonic tissues through induction of apoptosis in damaged cells (Topping & Clifton, 2001) and is a major energy source for mucosal cells lining the colon. The dietary intervention described in this study revealed that diets rich in RS and NSP, but not NSP alone, produced significant increases in concentrations of all of the major SCFA, with the exception of propionate. In particular, butyrate concentrations were increased by 22%. While we were unable to demonstrate a significant relationship between faecal SCFA concentration and *R. bromii* abundance, we did find an increase in the intensity of DGGE bands whose sequences are closely related to known SCFA-producing bacteria. These include one with a 16S rRNA gene sequence very closely related to that of *F. prausnitzii* and another with high 16S rRNA gene similarity to *Butyrivibrio fibrisolvens*. A band related to *E. rectale* was also increased in intensity by the RS diet. *Eubacterium rectale* belongs to clostridial cluster XIVa, which contains many butyrate producers (Hold *et al.*, 2003) and, like *R. bromii*, has been found attached to starch particles during *in vitro* fermentations of high-amylose maize starch in human faeces (Leitch *et al.*, 2007).

The lactic acid bacteria generally represent $< 1\%$ of the human faecal microbiota (Sghir *et al.*, 2000). In our study, the NSP diet resulted in an increased intensity of a DGGE band whose 16S rRNA gene sequence clusters with *Lactobacillus ruminus*, a predominant member of the community of lactic acid bacteria in the gut of a range of animals and humans (Reuter, 2001; Heilig *et al.*, 2002; Al Jassim, 2003). Given the low percentage of the *Lactobacillus* community within the faecal microbiota, it is not surprising that our DGGE method targeting the dominant microbial community has not detected more changes in this grouping. The role of this particular bacterium and whether it contributes to the health of the large bowel is unknown,

but our results indicate that its abundance may increase in concert with changes in the indicators of improved bowel health (increased levels of SCFA and decreasing digesta pH), suggesting that it may be worthy of further investigation.

In contrast to the lactic acid bacteria, *Bacteroides* spp. are one of the most numerically dominant groups of bacteria in human faeces (Wilson & Blitchington, 1996; Suau *et al.*, 1999; Hold *et al.*, 2002; Eckburg *et al.*, 2005) and are capable of degradation or utilization of starch and other less digestible polysaccharides (Salysers *et al.*, 1977; Xu *et al.*, 2003; Leitch *et al.*, 2007). However, based on our DGGE analysis of broad bacterial population changes in faeces, only one member of this group appeared to be associated with the RS or NSP diets. The bacterium was closely related to *B. thetaiotaomicron*. Genome sequencing of *B. thetaiotaomicron* has revealed an extensive range of enzymes suited to the breakdown of complex carbohydrates, including amylases, necessary for the breakdown of starch (Xu *et al.*, 2003). The bacteria have a starch utilization system that enables them to bind to and digest starches using periplasmic α -amylases (Cho & Salysers, 2001).

It is likely that the dietary intervention has influenced less numerically dominant members of the colonic microbiota; however, these may be present at levels below the detection limit of DGGE (1%, Casamayor *et al.*, 2000). It is also possible that the application of a real-time PCR assay encompassing a broader range of clostridia cluster IV organisms may have demonstrated a different relationship to dietary intervention and SCFA production.

This study supports data from *in vitro* models that have demonstrated *R. bromii*-related organisms to be an important player in starch colonization and digestion. The study also further confirmed the notion that colonic microbial populations are particularly stable and resilient to change in healthy adults (Zoetendal *et al.*, 1998; Abell & McOrist, 2007), suggesting that short-term dietary intervention may have a limited capacity for modifying colonic flora in the long term. Further study of the effect of longer-term dietary intervention on the colonic microbiota is needed if we are to understand the role of the gastrointestinal microbial population in human responses to dietary supplements and devise approaches to manipulate these for enhanced human health outcomes.

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References

- Abell GC & Bowman JP (2005) Ecological and biogeographic relationships of class *Flavobacteria* in the Southern Ocean. *FEMS Microbiol Ecol* **51**: 265–277.
- Abell GCJ & McOrist AL (2007) Assessment of the diversity and stability of faecal bacteria from healthy adults using molecular methods. *Microbiol Ecol Health Dis* **19**: 229–240.
- Abell GCJ, Conlon MA & McOrist AL (2006) Methanogenic archaea in adult human faecal samples are inversely related to butyrate concentration. *Microbiol Ecol Health Dis* **18**: 154–160.
- Al Jassim RAM (2003) *Lactobacillus ruminis* is a predominant lactic acid producing bacterium in the caecum and rectum of the pig. *Lett Appl Microbiol* **37**: 213–217.
- Barcenilla A, Pryde SE, Martin JC *et al.* (2000) Phylogenetic relationships of butyrate-producing bacteria from the human gut. *Appl Environ Microbiol* **66**: 1654–1661.
- Bingham SA, Day NE, Luben R *et al.* (2003) Dietary fibre in food and protection against colorectal cancer in the European Prospective Investigation into Cancer and Nutrition: an observational study. *Lancet* **361**: 1496–1501.
- Boom R, Sol CJ, Salimans MM, Jansen CL, Wertheim-van Dillen PM & van der Noordaa J (1990) Rapid and simple method for purification of nucleic acids. *J Clin Microbiol* **28**: 495–503.
- Casamayor EO, Schafer H, Baneras L, Pedros-Alio C & Muyzer G (2000) Identification of and spatio-temporal differences between microbial assemblages from two neighboring sulfurous lakes: comparison by microscopy and denaturing gradient gel electrophoresis. *Appl Environ Microbiol* **66**: 499–508.
- Cassidy A, Bingham SA & Cummings JH (1994) Starch intake and colorectal cancer risk: an international comparison. *Br J Cancer* **69**: 937–942.
- Cho KH & Salyers AA (2001) Biochemical analysis of interactions between outer membrane proteins that contribute to starch utilization by *Bacteroides thetaiotaomicron*. *J Bacteriol* **183**: 7224–7230.
- Clarke KR (1993) Non-parametric multivariate analyses of changes in community structure. *Aust J Ecol* **18**: 117–143.
- Clausen MR & Mortensen PB (1995) Kinetic studies on colonocyte metabolism of short chain fatty acids and glucose in ulcerative colitis. *Gut* **37**: 684–689.
- Collins MD, Lawson PA, Willems A *et al.* (1994) The phylogeny of the genus *Clostridium*: proposal of five new genera and eleven new species combinations. *Int J Syst Bacteriol* **44**: 812–826.
- Duncan SH, Belenguer A, Holtrop G, Johnstone AM, Flint HJ & Lobley GE (2007) Reduced dietary intake of carbohydrates by obese subjects results in decreased concentrations of butyrate and butyrate-producing bacteria in feces. *Appl Environ Microbiol* **73**: 1073–1078.
- Eckburg PB, Bik EM, Bernstein CN *et al.* (2005) Diversity of the human intestinal microbial flora. *Science* **308**: 1635–1638.
- Gill SR, Pop M, DeBoy RT *et al.* (2006) Metagenomic analysis of the human distal gut microbiome. *Science* **312**: 1355–1359.
- Hague A, Manning AM, Hanlon KA, Huschtscha LI, Hart D & Paraskeva C (1993) Sodium butyrate induces apoptosis in human colonic tumour cell lines in a p53-independent pathway: implications for the possible role of dietary fibre in the prevention of large-bowel cancer. *Int J Cancer* **55**: 498–505.
- Heilig HGJ, Zoetendal EG, Vaughan EE, Marteau P, Akkermans ADL & deVos WM (2002) Molecular diversity of *Lactobacillus* spp. and other lactic acid bacteria in the human intestine as determined by specific amplification of 16S ribosomal DNA. *Appl Environ Microbiol* **68**: 114–123.
- Herbeck JL & Bryant MP (1974) Nutritional features of the intestinal anaerobe *Ruminococcus bromii*. *Appl Microbiol* **28**: 1018–1022.
- Hold GL, Pryde SE, Russell VJ, Furrie E & Flint HJ (2002) Assessment of microbial diversity in human colonic samples by 16S rDNA sequence analysis. *FEMS Microbiol Ecol* **39**: 33–39.
- Hold GL, Schwiertz A, Aminov RI, Blaut M & Flint HJ (2003) Oligonucleotide probes that detect quantitatively significant groups of butyrate-producing bacteria in human feces. *Appl Environ Microbiol* **69**: 4320–4324.
- Kenkel NC & Orloci L (1986) Applying metric and nonmetric multidimensional scaling to some ecological studies: some new results. *Ecology* **67**: 919–928.
- Lane DJ (1991) 16S/23S rRNA sequencing. *Nucleic Acid Techniques in Bacterial Systematics* (Stackebrandt E & Goodfellow M, eds), pp. 115–175. John Wiley & Sons, New York.
- Langlands SJ, Hopkins MJ, Coleman N & Cummings JH (2004) Prebiotic carbohydrates modify the mucosa associated microflora of the human large bowel. *Gut* **53**: 1610–1616.
- Leitch EC, Walker AW, Duncan SH, Holtrop G & Flint HJ (2007) Selective colonization of insoluble substrates by human faecal bacteria. *Environ Microbiol* **9**: 667–679.
- Ludwig W, Strunk O, Westram R *et al.* (2004) ARB: a software environment for sequence data. *Nucleic Acids Res* **32**: 1363–1371.
- Mai V & Morris JG Jr (2004) Colonic bacterial flora: changing understandings in the molecular age. *J Nutr* **134**: 459–464.
- Minchin PR (1987) An evaluation of the relative robustness of techniques for ecological ordination. *Plant Ecol* **69**: 89–107.
- Park Y, Hunter DJ, Spiegelman D *et al.* (2005) Dietary fiber intake and risk of colorectal cancer: a pooled analysis of prospective cohort studies. *JAMA* **294**: 2849–2857.
- Patten GS, Abeywardena MY, McMurchie EJ & Jahangiri A (2002) Dietary fish oil increases acetylcholine- and eicosanoid-induced contractility of isolated rat ileum. *J Nutr* **132**: 2506–2513.

- Reuter G (2001) The *Lactobacillus* and *Bifidobacterium* microflora of the human intestine: composition and succession. *Curr Issues Intestinal Microbiol* **2**: 43–53.
- Ritzhaupt A, Ellis A, Hosie KB & Shirazi-Beechey SP (1998) The characterization of butyrate transport across pig and human colonic luminal membrane. *J Physiol* **507**: 819–830.
- Saitou N & Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**: 406–425.
- Salyers AA, Vercellotti JR, West SHE & Wilkins TD (1977) Fermentation of mucins and plant polysaccharides by strains of *Bacteroides* from the human colon. *Appl Environ Microbiol* **34**: 529–533.
- Santegoeds CM, Ferdelman TG, Muyzer G & de Beer D (1998) Structural and functional dynamics of sulfate-reducing populations in bacterial biofilms. *Appl Environ Microbiol* **64**: 3731–3739.
- Sghir A, Gramet G, Suau A, Rochet V, Pochart P & Dore J (2000) Quantification of bacterial groups within the human fecal flora by oligonucleotide probe hybridization. *Appl Environ Microbiol* **66**: 2263–2266.
- Shannon CE & Weaver W (1949) *The Mathematical Theory of Communication*. University Illinois Press, Urbana, IL.
- Smith S, Choy R, Johnson S, Hall R, Wildeboer-Veloo A & Welling G (2006) Lupin kernel fiber consumption modifies fecal microbiota in healthy men as determined by rRNA gene fluorescent *in situ* hybridization. *Eur J Nutr* **45**: 335–341.
- Suau A, Bonnet R, Sutren M *et al.* (1999) Direct analysis of genes encoding 16S rRNA from complex communities reveals many novel molecular species within the human gut. *Appl Environ Microbiol* **65**: 4799–4807.
- Toden S, Bird AR, Topping DL & Conlon MA (2007a) Dose-dependent reduction of dietary protein-induced colonocyte DNA damage by resistant starch in rats correlates more highly with caecal butyrate than with other short chain fatty acids. *Cancer Biol Ther* **6**: 253–258.
- Toden S, Bird AR, Topping DL & Conlon MA (2007b) High red meat diets induce greater numbers of colonic DNA double-strand breaks than white meat: attenuation by high amylose maize starch. *Carcinogenesis* **28**: 2355–2362.
- Topping DL (2007) Cereal complex carbohydrates and their contribution to human health. *J Cereal Sci* **46**: 230–229.
- Topping DL & Clifton PM (2001) Short-chain fatty acids and human colonic function: roles of resistant starch and nonstarch polysaccharides. *Physiol Rev* **81**: 1031–1064.
- Wang R-F, Cao W-W & Cerniglia CE (1997) PCR detection of *Ruminococcus* spp. in human and animal faecal samples. *Mol Cell Probes* **11**: 259–265.
- Wilson K & Blitchington R (1996) Human colonic biota studied by ribosomal DNA sequence analysis. *Appl Environ Microbiol* **62**: 2273–2278.
- Xu J, Bjursell MK, Himrod J *et al.* (2003) A genomic view of the human-*Bacteroides thetaiotaomicron* symbiosis. *Science* **299**: 2074–2076.
- Zoetendal EG, Akkermans ADL & de Vos WM (1998) Temperature gradient gel electrophoresis analysis of 16S rRNA from human fecal samples reveals stable and host-specific communities of active bacteria. *Appl Environ Microbiol* **64**: 3854–3859.