

The species composition of the human intestinal microbiota differs between particle-associated and liquid phase communities

Alan W. Walker,^{1†} Sylvia H. Duncan,¹
Hermie J. M. Harmsen,² Grietje Holtrop,³
Gjalt W. Welling² and Harry J. Flint^{1*}

¹Microbial Ecology Group and ³Biomathematics and Statistics Scotland, Rowett Research Institute, Greenburn Road, Bucksburn, Aberdeen, UK.

²Department of Medical Microbiology, University Medical Center Groningen, Groningen, The Netherlands.

Summary

Many of the substrates available as energy sources for microorganisms in the human colon, including dietary plant fibre and secreted mucin, are insoluble. It seems likely that such insoluble substrates support a specialized microbiota, and in order to test this hypothesis, faecal samples from four healthy subjects were fractionated into insoluble (washed particulate) and liquid fractions. Analysis of 1252 PCR-amplified 16S rRNA sequences revealed a significantly lower percentage of *Bacteroidetes* ($P=0.021$) and a significantly higher percentage of *Firmicutes* ($P=0.029$) among bacterial sequences amplified from particle-associated (mean 76.8% *Firmicutes*, 18.5% *Bacteroidetes*) compared with liquid phase (mean 65.8% *Firmicutes*, 28.5% *Bacteroidetes*). Within the *Firmicutes*, the most significant association with solid particles was found for relatives of *Ruminococcus*-related clostridial cluster IV species that include *Ruminococcus flavefaciens* and *R. bromii*, which together accounted for 12.2% of particle-associated, but only 3.3% of liquid phase, sequences. These findings were strongly supported by microscopy, using group-specific FISH probes able to detect these species. This work suggests that the primary colonizers of insoluble substrates found in the gut are restricted to certain specialized groups of bacteria. The abundance of such primary degraders may often be underestimated because of the

difficulty in recovering these bacteria and their nucleic acids from the insoluble substrate.

Introduction

The human large intestine harbours a dense bacterial community that plays an important role in mediating the effects of diet upon health (Eckburg *et al.*, 2005; Dethlefsen *et al.*, 2006; Flint *et al.*, 2007). The major energy sources that are available for microbial growth in the human large intestine are dietary residues that escape digestion in the small intestine, together with host secretions (Macfarlane and Gibson, 1997; Topping and Clifton, 2001). A significant fraction of this material exists in an insoluble form, for example dietary plant fibre that is rich in cell wall polymers such as cellulose and xylan, and particles of resistant starch. In addition, intestinal goblet cells produce abundant insoluble mucin that coats the gut wall and facilitates the passage of digesta through the gut.

It is well established that inert surfaces such as teeth and catheters allow the development of distinctive microbial communities, or biofilms (Kolenbrander *et al.*, 2005; Macfarlane *et al.*, 2005). The existence of specialized attached microbial communities on the more transient surfaces that exist within the healthy colon has been less clear, but recent evidence suggests that the metabolic activities of luminal bacteria and bacteria associated with insoluble digesta particles differ (Macfarlane and Macfarlane, 2006). The extended transit times for digesta passage through the large intestine (Cummings and Macfarlane, 1991) are therefore likely to be sufficient for the development of specialized communities on particulate material in the lumen of the gut. Tightly adherent bacteria are likely to play a key role in releasing energy from insoluble substrates in the gut.

In a recent study we used an *in vitro* continuous flow fermentor system to investigate colonization of three different insoluble substrates (wheat bran, insoluble starch and pig mucin) by human faecal bacteria (Leitch *et al.*, 2007). This work showed that particular species and phylotypes tended to become closely associated with a given substrate following a 24–48 h incubation period. *In vivo*, the insoluble component of faecal samples will consist of

Received 10 March, 2008; accepted 21 June, 2008. *For correspondence. E-mail hjf@rri.sari.ac.uk; Tel. (+44) 1224 712 751; Fax (+44) 1224 716 681. †Present address: Wellcome Trust Sanger Institute, Wellcome Trust Genome Campus, Hinxton, Cambridge, UK.

a complex mixture of incompletely degraded dietary residue, together with mucin and bacterial cells. The aim of the present study was to establish whether there is any detectable difference in the bacterial species composition of the particulate and liquid fractions of faecal samples that would support the existence of specialist substrate-attached bacterial populations *in vivo*. Analysis of 16S rRNA sequences and the use of targeted FISH probes provide evidence that the particle-associated and planktonic communities in human faecal material differ significantly, with a higher proportion of *Firmicutes*, especially clostridial cluster IV ruminococci, associated with particles, and a higher proportion of *Bacteroidetes* associated with the liquid fraction.

Results

Composition of insoluble particles from faecal samples

Faecal samples were obtained from four healthy volunteers consuming their normal diets, and were separated into liquid and washed, particulate fractions (see *Experimental procedures*). Analysis of the monosaccharides present in the insoluble material, following complete acid hydrolysis, revealed considerable similarity in composition between the four samples. Glucose accounted for between 39% and 48%, xylose 17.8–18.6%, arabinose 14–16.5%, galactose 9.6–13%, rhamnose 2.6–4.6% and fucose 2.4–3.9% of the neutral sugars present in the four samples. Only mannose showed any marked variation (11.9% in sample 4, 2–3.5% in the other three samples). Uronic acids were also lower in sample 4 (1.2% compared with 2.6–3.6% of dry weight for the other three samples). These data are consistent with plant cell wall material being the major source of the carbohydrate present in all four samples, with hemicellulose and pectin contributing xylose, arabinose, galactose, mannose, rhamnose, fucose and uronic acids. Cellulose is expected to be at

least as abundant in dietary residue as hemicellulose, and is assumed to be the main source of the glucose.

16S rRNA sequence analysis of bacteria associated with faecal particles

The liquid and washed, particulate fractions of these samples were used to construct 16S rRNA clone libraries. Extracted DNA was subjected to PCR amplification using broad eubacterial primers, and a total of 1252 partial 16S rRNA sequences (ranging between 139 and 177 clones for each fraction) were determined. The major bacterial phyla were unevenly distributed between the liquid and particulate fractions ($P < 0.001$, based on contingency table analysis). For all four samples the percentage of *Bacteroidetes* sequences was greater for the liquid than for the particulate fraction (means 28.5% and 18.5% respectively, $P = 0.021$) while the *Firmicutes* percentage was lower for the liquid than for the particulate fraction (means 65.9% and 76.8% respectively, $P = 0.029$) (Table 1). Detailed analysis of individual phylotypes revealed high diversity in both the particle-associated and liquid phase fractions. Diversity indices for all four samples were numerically slightly lower for particle-associated than for liquid fractions (Shannon index means 3.38 liquid, 3.21 fibre, $P = 0.116$; Simpson index means 0.949 liquid, 0.935 fibre, $P = 0.077$), but the differences were not statistically significant.

Table 2 summarizes the distribution between the particulate and liquid phases for the major phylogenetic groups, and for the 14 most abundant species groups that were detected across the four subjects. The species groups shown are those that occurred in at least three of the four individuals, and that comprised more than 2% of the total number of sequences in the study. It should be noted that these groups are based on the closest cultured species, but included between 1 and 15 different

Table 1. Distribution (% sequences) of bacterial phyla between liquid and washed particulate fractions of faecal samples from four healthy volunteers.

	Donor 1		Donor 2		Donor 3		Donor 4		Mean totals	
	Liquid	Particles	Liquid	Particles	Liquid	Particles	Liquid	Particles	Liquid	Particles
<i>Bacteroidetes</i>	31	19.2	29.1	20	21.4	9.5	33.3	26.6	28.5	18.5
<i>Firmicutes</i>	66.5	78.0	64.9	70.0	71.4	86.3	59.7	71.2	65.9	76.8
<i>Roseburia</i> + <i>E. rectale</i>	11.4	20.9	7.4	9.3	2.4	0.6	4.9	5.8	6.6	9.5
ruminococci (cl IV)	1.9	5.7	4.1	20	6.0	15.5	0.7	3.6	3.3	12.2
<i>Actinobacteria</i>	0	0	2	2	0	1.2	1.4	0	0.8	0.8
<i>Verrucomicrobia</i>	2.5	2.8	0.7	4.7	0.6	0	0	0	1	2
<i>Proteobacteria</i>	0	0	3.4	3.3	6	2.4	4.9	2.2	3.6	1.9
Others ^a	0	0	0	0	0.6	0.6	0.7	0	0.3	0.2
Total clones (no.)	158	177	148	150	168	168	144	139	618	634

a. Two *Cyanobacteria* and one *Lentisphaera* sequence.

Table 2. Distribution between particulate and liquid fractions of major phylogenetic groupings, and of the most abundant bacterial species groups, detected in 16S rRNA clone libraries from human faecal samples.

Bacterial group	No. of 16S rRNA sequences:			Occurrence ^a	% particle associated	P-value ^b
	Liquid	Particles	Total			
Total <i>Firmicutes</i>	407	487	894	4	54.5	0.029
Total clostridial cluster IV	129	157	286	4	54.9	0.161
<i>Ruminococcus</i> relatives (cl IV)	21	77	98	4	78.6	0.007
<i>R. flavefaciens</i> (12) ^c	9	46	55	4	83.6	0.039
<i>R. bromii</i> (4)	8	12	29	3	72.4	0.166
Other cl IV bacteria	108	80	188	4	42.6	0.103
<i>F. prausnitzii</i> (5)	36	31	67	4	46.3	0.642
<i>T. acetivus</i> (15)	19	9	28	4	32.1	0.148
Total, other <i>Firmicutes</i>	278	330	608	4	48.5	0.482
<i>R. obeum</i> (12)	39	35	73	4	47.9	0.614
<i>E. rectale</i> (2)	28	43	71	3	60.6	0.434
<i>C. clostridioforme</i> (9)	22	30	52	4	57.7	0.497
Unknown sp. SR1/5 (3)	15	27	42	4	64.3	0.323
<i>E. hallii</i> (9)	15	21	36	3	58.3	0.432
Unknown sp. SSC/2 (2)	11	16	27	3	59.3	0.503
Total <i>Bacteroidetes</i>	176	117	293	4	39.9	0.021
<i>B. vulgatus/dorei</i> (1)	37	26	63	4	41.3	0.236
<i>B. uniformis</i> (1)	27	23	50	4	46.0	0.647
<i>A. finegoldii</i> (7)	20	15	35	4	42.9	0.554
Other <i>Bacteroidetes</i>	92	53	145	4	36.6	0.044
Total, other phyla	35	30	65		46.2	
Total	618	634	1252			

a. Number of individuals (out of four) in which the group was detected.

b. Significance for the difference between particle-attached and liquid on binomial model with logistic link, allowing for effects of Donor and Fraction. Values given in bold were significant ($P < 0.05$).

c. Approximate number of phylotypes (in parenthesis) for which the named species was the closest cultured relative. Only those species groups that occurred in at least three individuals, and accounted for more than 2% of the total sequences, are listed.

phylotypes, some of which showed as little as 90% identity with the closest species type strain over the sequenced region. As observed in previous studies (Hold *et al.*, 2002; Eckburg *et al.*, 2005), there was substantial inter-individual variation at the phylotype level. On average, in each clone library, 58 phylotypes were detected of which 55% were represented by single sequences.

The group of cluster IV ruminococcus sequences related to *Ruminococcus flavefaciens*, *R. albus* and *R. bromii* showed a highly significant ($P = 0.007$) association with the particulate fraction, and largely accounted for the significant association seen for the *Firmicutes* as a whole (Table 2). Full-length sequences were obtained from eight representatives of these particle-associated phylotypes, and are compared with sequences from cultured clostridial cluster IV bacteria in Fig. 1. One phylotype, recovered from donor 2, was most closely related (94–95% identity) to known cellulolytic *Ruminococcus* isolates obtained from human faecal samples by Robert and Bernalier-Donadille (2003). A second, from donor 4, was 95% related to rumen strains of *R. albus*, but 98% related to a recent isolate (80/3) of human gut origin. Two further phylotypes, from donor 3, were related to *R. bromii* (98% and 96–97% respectively).

In contrast, the *Bacteroidetes* phylum, when considered in total, showed a significant association with the liquid phase (Table 2).

FISH analysis of bacteria colonizing faecal particles

In a second study, liquid and particulate fractions of faecal samples from five volunteers, including samples from donors 1 and 2 that had been analysed using 16S rRNA clone libraries, were also analysed by fluorescent *in situ* hybridization (FISH) with 16S rRNA-targeted probes. In agreement with the sequence analysis, *Bacteroides* relatives were readily detected with the probe Bac303 in the liquid phase, but not among cells attached to particles (data not shown).

High % G+C content bacteria (*Actinobacteria*) are widely considered to be underestimated by clone library approaches, as counts obtained by FISH or by cultivation are generally higher than estimates from clone library analyses (Franks *et al.*, 1998; Eckburg *et al.*, 2005). Bifidobacteria were detected using the Bif164 probe in the liquid fraction of four of the five samples examined (not in donor 1) but were only detected on particles from the samples of donors 2 and 7. Specific probes were also used that target bacteria related to *Faecalibacterium prausnitzii*

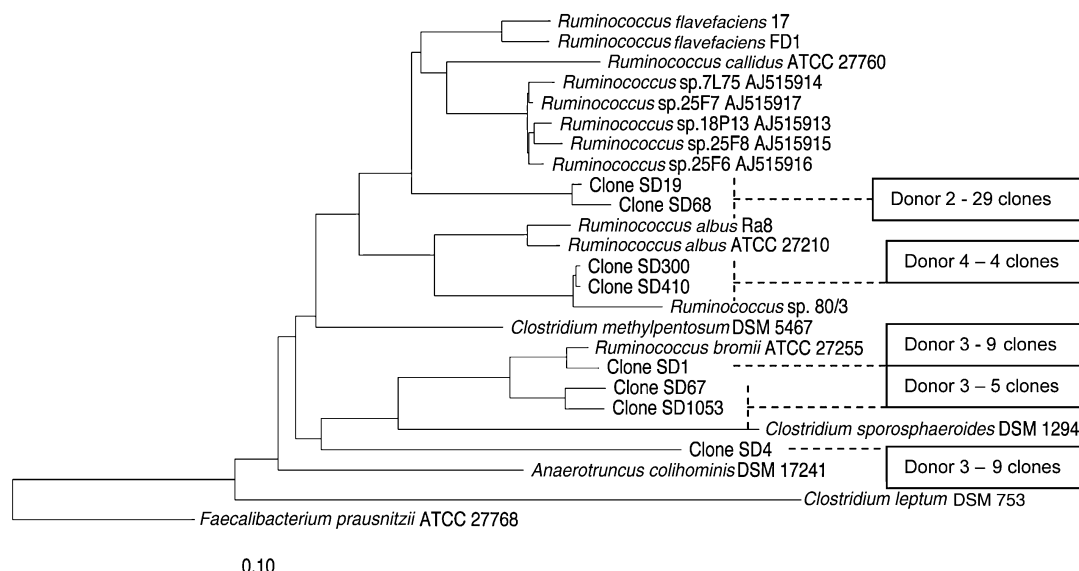


Fig. 1. Phylogenetic tree of 16S rRNA gene sequences (1450 base pairs) related to cluster IV ruminococci. Sequences labelled 'clone' are from the present study and are representative of certain phylotypes found to be associated with particles; the donor and number of closely related clones are indicated in each case. Other sequences represent cultured bacteria and are from the EMBL database. The five strains labelled *Ruminococcus* sp. (AJ515914–515918) were isolated by Robert and Bernalier Donadille (12). *Ruminococcus* sp. 80/3 (sequence Accession No. EU266551) is a recent human isolate from the Rowett Institute.

(Fprau645), *Atopobium* (Ato291), cluster IX clostridia (Prop853), cluster XVI clostridia (Ecyl387), *Lactobacillus* (Lab158) and *Enterobacteriaceae* (EnterobactD). All of these groups were detected in the luminal fraction in most samples, but only cluster XVI and bacteria detected by Fprau645 probe were abundant on the washed particulate fraction of some samples (results not shown).

Clostridial cluster IV *Ruminococcus* species detected by the Rfla729+Rbro730 probes were seen to be abundant on particles from all five faecal samples examined, as were clostridial cluster XIVa bacteria detected by the Erec482 probe. In order to examine the relative abundance of these two groups, the combined Rfla729+Rbro730 probes and the Erec482 probe were labelled with different fluorescent markers and used in parallel. Additional DAPI staining was used to reveal any bacteria that were not detected by either probe. Figure 2A shows that for insoluble material from donor 5, cluster IV ruminococci and cluster XIVa clostridia accounted for most of the particle-attached bacteria present. The proportion of DAPI-stained cells was higher for the donor 6 sample, however, perhaps reflecting significant colonization with bifidobacteria (Fig. 2B). Dual-probing with the Erec482 (cluster XIVa) and Rrec584 (*Roseburia* + *E. rectale* specific) probes (Fig. 2C) revealed that not all cluster XIVa bacteria adherent to the faecal particles were *Roseburia* spp. or *E. rectale*.

Figure 3 shows images, at two different planes of focus, of particles that had been dual-probed with

Rfla729+Rbro730 and Erec482. These images demonstrate a spatial separation between the two groups of bacteria, with bacteria detected by the Erec482 probe apparently being more superficial, and the cluster IV ruminococci apparently more intimately associated with the particles.

Discussion

This work establishes that the species composition of the microbial community that is closely attached to insoluble faecal particles differs significantly from that in the liquid phase of the same sample. Faecal particles are expected to consist of incompletely degraded dietary fibre, colonizing bacteria and mucin (Macfarlane and Macfarlane, 2006; Leitch *et al.*, 2007). It seems likely that the relatively long transit time in the large intestine (Cummings and Macfarlane, 1991) allows time for the primary bacterial colonizers of insoluble substrates to develop a close association with the substrate, resembling a biofilm. Many other bacteria may exist in a looser association, probably benefiting from cross-feeding interactions (Belenguer *et al.*, 2006; Flint *et al.*, 2007).

Relatives of the clostridial cluster IV species *R. flavefaciens* and *R. bromii* accounted for 12.2% of particle-attached sequences, but only 3.3% of liquid phase sequences, and were the species most clearly associated with particles in the four faecal samples analysed here. Libraries of amplified 16S rRNA genes cannot

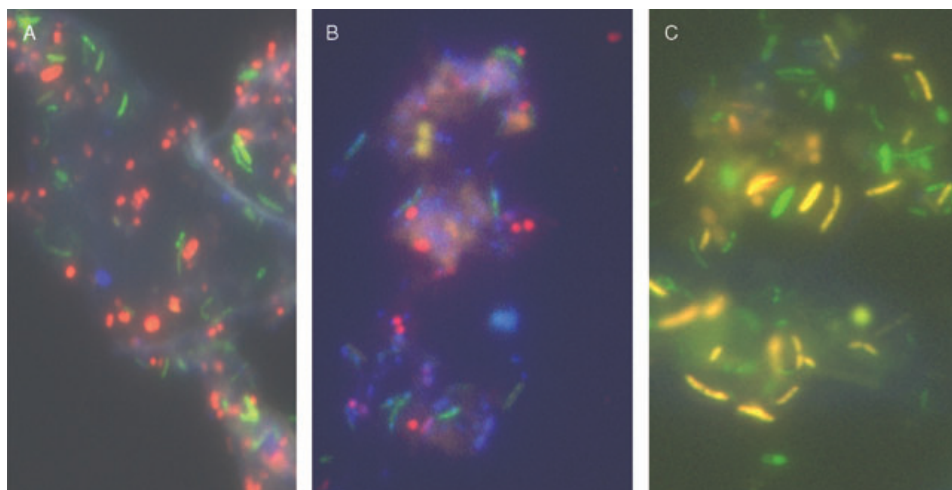


Fig. 2. Simultaneous FISH probing of faecal particles with different probes. In (A) and (B) (samples from donors 5 and 6 respectively) red cells represent *Ruminococcus* species detected with Rfla729+Rbro730, green cells represent cluster XIVa species detected with Erec482, and blue cells represent bacteria that have been stained by DAPI but not by the other two probes. In (C) (sample from donor 5) faecal particles were dual-probed with Erec482 and Rrec584 to show the presence of *Roseburia/E. rectale* cells (detected by both probes – yellow) and of other bacteria belonging to clostridial cluster XIVa (detected only with Erec482 – green).

be guaranteed to reflect the relative abundance of all bacterial groups present because of the possibility of differential DNA recovery and amplification. Close association with the substrate, together with a bacterial cell wall that is relatively resistant to lysis, may contribute to incomplete recovery of DNA from cluster IV ruminococci, and the particle-associated population may therefore be underestimated by this approach. It was therefore important to complement this information with FISH-probing studies. These strongly supported the view that cluster IV

ruminococci are abundant colonizers of faecal particles that show an intimate association with the substrate. *Ruminococcus flavefaciens* is one of the three main cellulolytic bacterial species identified from the rumen (Rincon *et al.*, 2005; Flint *et al.*, 2008) and related cellulolytic bacteria have been isolated from human faeces (Robert and Bernalier-Donadille, 2003). This group can therefore be expected to play a key role in the degradation of cellulose- and hemicellulose-rich plant material. Attachment and hydrolysis of cellulosic substrates by rumen

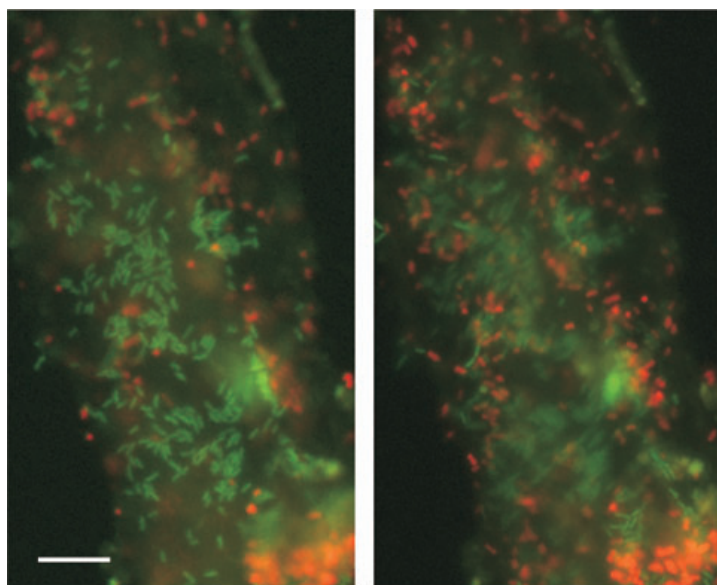


Fig. 3. Combined images of hybridizations performed with Rfla728+Rbro730 (shown in orange) and with Erec482 (shown in green) probes, at two planes of focus. Bar = 10 μ m.

strains of *R. flavefaciens* is known to involve a large cel-
lulosomal enzyme complex and specific binding proteins
(Ding *et al.*, 2001; Rincon *et al.*, 2005; 2007), while pili
may be involved in substrate binding in related species
(Rakotoarivonina *et al.*, 2005). *Ruminococcus bromii* is a
starch-utilizing species that was recently shown to asso-
ciate closely with starch particles in an *in vitro* fermentor
system inoculated with human faecal bacteria (Leitch
et al., 2007). Cluster IV ruminococci were also found to be
enriched among particle-associated bacterial sequences
from the rumen (Larue *et al.*, 2005). Interestingly, a
previous study (Robert and Bernalier-Donadille, 2003)
reported the isolation of cellulolytic ruminococci only from
methane-excreting human subjects. In the present work
donors 1 and 2 were methane excretors, while donors 3
and 4 were not. We detected fibre-associated *R. albus*-
related sequences in the non-methanogenic donor 4, and
R. flavefaciens-related sequences were detected in all
four donors (Table 2).

Clostridial cluster XIVa species, including members of
the *Roseburia/E. rectale* group, were also shown to be
abundant colonizers of particulate material in faeces by
FISH, although sequence analysis did not demonstrate
a statistically significant preferential association. FISH
microscopy suggested that, by comparison with the
cluster IV ruminococci, these bacteria have a more super-
ficial association with particles. It is likely that these, and
many other groups of gut bacteria, are capable of existing
readily in either the insoluble substrate-attached or plan-
ctonic state.

Despite the fact that *Bacteroides* species show a wide
range of polysaccharide-degrading activities (Salyers
et al., 1977a,b; Robert *et al.*, 2007) and very large
numbers of polysaccharidase genes (Xu *et al.*, 2003),
this group was less prominent among particle-attached
sequences than among sequences from the liquid phase.
Similarly, the proportion of *Bacteroidetes* (CFB phylum)
in the rumen community was reported to be lower on solid
fractions than in the liquid phase (Tajima *et al.*, 1999). This
suggests that the *Bacteroides* group may depend to a
large extent on utilization of solubilized polysaccharides
that are released by the activities of other bacteria (Dehor-
ity, 1991). The enzymatic and transport machinery for
starch in *B. thetaiotaomicron* (Shipman *et al.*, 2000) and
xylan in *Prevotella bryantii* (Miyazaki *et al.*, 1997) appears
designed for the efficient sequestration, uptake and utili-
zation of soluble polysaccharides (Flint *et al.*, 2008). The
possibility should be considered that there might have
been some differential loss of *Bacteroidetes*, e.g. due to
greater osmotic fragility, during the washing procedures.
The same washing procedures were used in a previous
study, however, in which certain *Bacteroides* spp. from
faecal samples were found to associate with insoluble
wheat bran (mainly *B. eggerthii*) or pig mucin (mainly

B. vulgatus) (Leitch *et al.*, 2007). There is no inconsis-
tency between this finding, that some *Bacteroides* are
able to bind specific insoluble substrates, and the present
work which suggests that a higher proportion of total
Bacteroides in faecal samples are liquid- rather than
particle-associated. The same strains may be able to exist
in the planctonic and particle-associated state.

The numbers of adherent bacteria belonging to different
phylotypes varied widely between the four samples
studied here. Such variation was also observed in a pre-
vious study that used a fermentor model system to study
adherent bacteria (Leitch *et al.*, 2007). It is not possible
to conclude from this study whether this variation is
mainly due to temporal and dietary differences, or to
inter-individual variation in gut microbiota composition,
although the latter is likely to be a factor (Zoetendal *et al.*,
2001; Lay *et al.*, 2005).

Two recent studies have reported effects of weight loss
diets with reduced total carbohydrate (including dietary
fibre) intake upon faecal microbiota in obese human
volunteers. In one study the % *Bacteroidetes* was
reported to increase gradually during weight loss over
12 months (Ley *et al.*, 2006), while in the other there was
no detectable change in % *Bacteroides*, but a reduction in
the *Roseburia + E. rectale* group of *Firmicutes*, over 4 or
8 weeks (Duncan *et al.*, 2007). The present work sug-
gests that variation in the insoluble fibre content of diets
will affect the species composition of the gut community,
but detailed human studies that specifically address this
question have yet to be reported.

Far from being a homogenous community, microscale
variation is likely to prove a very important feature of the
gut microbiota. The nature of communities that may be
associated with mucus and with the gut mucosa remains
to be established, but this work establishes the existence
of a specialized community associated with insoluble par-
ticles that pass through the human large intestine.

Experimental procedures

Separation of faecal samples into luminal and particulate fractions

Faecal samples were donated by seven volunteers. The
donors were healthy adults who had no history of colonic
disease and had consumed no drugs known to influence the
microbiota for at least 6 months prior to sampling date. All
were omnivorous, except for donor 1, who was a vegetarian.
Among the four donors whose samples used for 16S rRNA
clone library analysis, donors 1 and 2 were methanogenic
and donors 3 and 4 non-methanogenic. The methanogenic
status of the remaining three donors, whose samples were
examined only by FISH microscopy, is unknown.

Freshly voided faecal samples were mixed, and a 2 g
sample placed in a sterile 15 ml tube for separation into
particulate and luminal fractions. 8 ml of filter sterilized

phosphate buffered saline (PBS) and five 3 mm diameter glass beads were added followed by vigorous vortex mixing for 5 min and centrifugation at 700 *g* for 1 min to settle large particulate matter. Aliquots were removed from the supernatant fraction (luminal samples). To remove non-attached and loosely attached bacteria the insoluble faecal particles were re-suspended in 10 ml PBS, vortex-mixed vigorously for 1 min and then pelleted at 700 *g* for 5 min. This process was repeated 5 times in PBS and twice in PBS containing 0.1% Tween 80. To remove any excess Tween 80 the samples were washed and vortex-mixed in PBS one final time and the supernatant removed.

Samples from donors 1, 2, 3 and 4 were analysed by constructing libraries of amplified 16S rRNA genes. Samples from donors 1, 2, 5, 6 and 7 were analysed by FISH.

Sugar analysis

Samples of washed faecal particles were treated with α -amylase (termamyl), pullulanase (promozyme) and pancreatin, and ethanol-precipitable material (non-starch polysaccharides), then subjected to hydrolysis in 60% H₂SO₄. Neutral sugars were analysed by GC (Hoebler *et al.*, 1989) and uronic acids estimated colorimetrically (Scott, 1979) in the ethanol-insoluble material. Relatively little sugar was detected in the ethanol-soluble material.

FISH analysis

Samples were prepared as described previously (Franks *et al.*, 1998). Briefly, after separating the fractions, 50 μ l of liquid samples or 50 μ g of washed faecal particles was added to 450 μ l of filter sterilized 4% paraformaldehyde and left at 4°C overnight to fix. These samples were then applied to gelatin coated slides and oligonucleotide probes were allowed to hybridize overnight in hybridization buffer. Vectashield (Vector Laboratories, Burlingame, CA) was added to each slide to prevent fading and fluorescing cells were visualized with a Leica DMRXA epifluorescence microscope. Probe sequences and specificities were reported as previously for Eub338, Rbro730, Rfla729, Ecyl387, Lab158, Ato291, Bac303, Erec482, Bif164 (Franks *et al.*, 1998; Harmsen *et al.*, 2002), Prop853 and Rrec584 (Walker *et al.*, 2005; Aminov *et al.*, 2006), EnterobactD (Ootsubo *et al.*, 2002) and Fprau645 (Suau *et al.*, 2001).

Multi-probe FISH analysis

Slides were hybridized as described above with different probes labelled with either Cy3 or FITC or with DAPI stain for total cell numbers. One hundred microlitres of a 400 μ l PBS + 1 μ l DAPI (500 ng μ l⁻¹) solution was dispensed onto the slides and incubated for 5 min at room temperature in a darkroom following overnight hybridization with fluorescently labelled probes. Separate images were taken of the same field of view using a different filter cube to detect each probe used. The images obtained were then overlaid on top of each other and edited using Photo-paint software (Corel, Maidenhead, UK).

DNA extraction and amplification of 16S rRNA gene

Frozen samples were thawed and the DNA extracted using the FastDNA® SPIN kit for soil and FastPrep® instrument (Qbiogene). The broad eubacterial primer pair 7f and 1510r, previously shown to amplify bifidobacteria (Leitch *et al.*, 2007), was used in this study, and these oligonucleotides were obtained from MWG Biotech. Amplification was performed using the following conditions: initial denaturation at 94°C for 5 min; then 20 cycles of denaturation (2 min at 94°C), annealing (30 s at 57°C), extension (2 min at 72°C); and a final extension at 72°C for 10 min. PCR products were run on a 1% agarose gel and bands of approximately 1500 bp were excised, cleaned using the Wizard PCR product purification kit (Promega, Southampton, UK) and concentrated by ethanol precipitation. Cleaned products were ligated into pGEM-T easy vector plasmids (Promega) and transformed into JM109 *E. coli* cells (Promega). Partial 16S rRNA sequences of random clones (minimum 350 bases) were obtained on a Beckman capillary sequencer. Full-length sequences were obtained for selected inserts using internal 16S rRNA primers.

Phylogenetic analyses

Sequences were aligned automatically using MUSCLE (Edgar, 2004), manually inspected and corrected using the sequence editor function in the ARB package (Ludwig *et al.*, 2004). The sequences (deposited in GenBank under accession numbers EU201195 to EU202445) were examined to give a broad classification at the phylum level using the Classifier tool at the Ribosomal Database Project website (Wang *et al.*, 2007). To obtain more detailed taxonomic information the sequences were then divided into phylotypes by generating distance matrices in ARB (with Olsen correction), which were then entered into the DOTUR program (Schloss and Handelsman, 2005) set to the furthest neighbour and 99% similarity setting. The resulting phylotypes were then assigned similarities to nearest neighbours using BLAST. Full-length sequences were tested for chimeras (Huber *et al.*, 2004) and any sequences that appeared to be chimeric were removed. Phylogenetic trees based on nearly full-length sequences (1450 bases) were constructed and bootstrap values calculated using PHYLIP (Felsenstein, 2005).

Statistical analysis – 16S rRNA sequence data

First, the association between the type of fraction (liquid, particulate) and distribution of clones among bacterial groups was tested with contingency table analysis using the chi-squared test (where data were combined over donors). Second, the probability that a clone belongs to a given bacterial strain or group was modelled as binomial with logistic link, with effects for donor and type of fraction. The binomial models allowed for overdispersion, but when its estimate was less than one the data were re-analysed with dispersion fixed to one (to avoid overly significant results).

Diversity indices were calculated for each fraction for each donor, using the Shannon (1948) and Simpson (1949) diversity indices. Differences between diversity indices for the

liquid and particulate fractions were tested using a paired *t*-test. All analyses were performed with Genstat 10th Edition Release 10.1 (VSN International, Hemel Hempstead, Hertfordshire, UK). *P*-values of 5% or less were regarded as significant.

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