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In vitro fermentation of chitosan derivatives by mixed cultures of human faecal bacteria

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Abstract

Stirred, pH controlled batch cultures were carried out with faecal inocula and various chitosans to investigate the fermentation of chitosan derivatives by the human gut flora. Changes in bacterial levels and short chain fatty acids were measured over time. Low, medium and high molecular weight chitosan caused a decrease in bacteroides, bifidobacteria, clostridia and lactobacilli. A similar pattern was seen with chitosan oligosaccharide (COS). Butyrate levels also decreased. A three-stage fermentation model of the human colon was used for investigation of the metabolism of COS. In a region representing the proximal colon, clostridia decreased while lactobacilli increased. In the region representing the transverse colon, bacteroides and clostridia increased. Distally a small increase in bacteroides occurred. Butyrate levels increased. Under the highly competitive conditions of the human colon, many members of the microflora are unable to compete for chitosans of low, medium or high molecular weight. COS were more easily utilised and when added to an in vitro colonic model led to increased production of butyrate, but some populations of potentially detrimental bacteria also increased.
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1. Introduction

Chitin is a natural polymer found in the exoskeletons of crustacea and insects and in the cell walls of certain fungi (Roller, 2003). It is the second most abundant polymer in nature, following cellulose (Sandford, 1988) and is therefore plentiful in supply, largely as a by-product of seafood processing. The chitin molecule consists of β 1–4 linked *N*-acetyl glucosamine residues and is insoluble in most solvents, restricting its biological activities. Full or partial deacetylation of chitin results in chitosan, a molecule with several biological activities some of which are described below.

The use of chitosan and corresponding oligosaccharides as antimicrobial agents has been studied and is mainly related to food safety applications. Organisms reportedly inhibited by chitosan and chitosan oligosaccharides include *Candida* sp. and *Rhodotorula* sp. (Rhoades & Roller, 2000), *Listeria monocytogenes*, *Salmonella typhimurium*, *Escherichia coli* O157 and *Staphylococcus aureus* (Tsai, Wu, & Su, 2000), *E. coli*, *S. typhi*, *Pseudomonas aerginosa*, *Streptococus mutans*, *Micrococcus luteus*, *Staphylococcus aureus*, *Staph. epidermidis, Bacillus subtilis*, *Lactobacillus* spp. and *Streptococcus faecalis* (Jeon, Park, & Kim, 2001), *Bacillus sp*. and *Enterobacter sakazakii* (No, Park, Lee, Hwang, & Meyers, 2002). The mechanisms of such inhibition are unknown but interaction between positively charged chitosans and negatively charged residues at the microbial cell surface have been suggested (Hadwiger, Chiang, Victory, & Horowitz, 1988). Hadwiger et al. (1988) also hypothesised that host immune responses may be directly triggered by the interaction of chitosans with the minor groove of the DNA double helix. Despite these antimicrobial properties, Lee, Park, Jung, and Shin (2002) proposed, through pure culture studies, that chitosan oligosaccharides were stimulatory to *Bifidobacterium bifidum* and *Lactobacillus* sp and found that in rich media (MRS), low concentrations of chitosan oligosaccharides can

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lead to increased cell numbers for some species. This ability to stimulate purportedly beneficial gut species opens up the possibility of their acting as prebiotics (Gibson & Roberfroid, 1995). However, *Streptococcus thermophilus* and *Lactobacillus brevis* were unable to grow at a chitosan oligosaccharide level of just 0.63% (w/v). No data are available on the fermentation properties of chitosans in mixed culture systems and so a prebiotic claim cannot be substantiated at the present time.

In vitro studies with chitosan have shown that it can bind 4–5 times its weight in micellar lipids (Nauss, Thompson, & Nagyvary, 1983) leading to claims on some brands of slimming pills for blocking fat absorption, although this has been shown not to have an effect on body weight or faecal fat excretion (Gades & Stern, 2002).

Many of the applications lead to the presence of chitosans in the human gut, which is home to a complex microbial ecosystem that plays a major role in host health, especially in the colon (Gibson & Roberfroid, 1995). It is not currently clear if there are any effects, on the composition of gut bacteria, which is known to contain positive and negative entities.

2. Materials and methods

2.1. Batch culture fermenters

Batch culture experiments were used to investigate the ability of the gut microflora to utilise low molecular weight chitosan (50–190 kDa, Aldrich, Dorset, UK), medium molecular weight chitosan (190–30 kDa, Aldrich), high molecular weight chitosan (310–375 kDa, Aldrich) and chitosan oligosaccharide lactate (COS, Mr < 5000, Aldrich). A range of molecular weights was chosen to determine possible molecular weight-associated effects as seen in previous experiments on food safety (Rhoades & Roller, 2000). Controls included glucose, a well fermented substrate which supports the growth of most bacteria and fructooligosaccharides (FOS, Raftilose P95, Orafti, Tienen, Belgium), as a positive control for prebiotic (bifidogenic) effects. Fermentation vessels (280 ml) were maintained anaerobically by constant sparging with O_2–free N_2. The vessels were maintained at 37 °C and pH controlled at pH 6.7 through a pH controller which added 1 mol l^{-1} NaOH or HCl as appropriate. Culture medium (200 ml) (peptone water (Oxoid) 2 g l^{-1}, yeast extract (Oxoid) 2 g l^{-1}, NaCl (BDH. Poole, Dorset, UK) 0.1 g l^{-1}, K_2HPO4 (BDH) 0.04 g l^{-1}, KH_2 PO_4 (BDH) 0.04, $MgSO_4 \cdot 7H_2O$ (BDH) 0.01 g l^{-1}, $CaCl_2 \cdot 2H_2O$ (BDH) 0.01 g l^{-1}, $NaHCO_3$ (BDH) 2 g l^{-1}, Tween 80 (BDH) 2 ml l^{-1}, Hemin (Sigma, Dorset, UK) 5 m g l^{-1}, Vitamin K1 (Sigma) 10 µl l^{-1}, Cysteine–HCl (Sigma) 0.5 g l^{-1}, Bile Salts (Oxoid) 0.5 g l^{-1}, Resazurin (Sigma) 1 mg l^{-1}) containing 10 g l^{-1} test carbohydrate was inoculated with 22 ml of a slurry of faeces, from one of three healthy human volunteers, in phosphate buffered saline (PBS, 0.16 mol l^{-1} NaCl, 0.003 mol l^{-1} KCl, 0.008 mol l^{-1} Na_2HPO_4, 0.001 mol l^{-1} NaH_2PO_4, pH 7.3, Oxoid). Samples were taken after 0, 5, 10 and 24 h for analysis and processed as described below.

2.2. Fluorescent in situ hybridisation (FISH)

Culture fluid (375 µl) was added to 40 g l^{-1} paraformaldehyde (1125 µl) and left to fix overnight at 4 °C. Bacterial cells were pelleted by centrifugation for 5 min at 13400 × *g* and washed twice in PBS (Oxoid) before being resuspended in 150 µl PBS and 150 µl ethanol. Samples were stored at this stage at −20 °C for no longer than 6 months. Probes were used to allow the direct enumeration of bacteroides (Bac303 5′CCAATGTGGGGGACCTT3′, 45 °C, Manz, Amann, Ludwig, Vancanneyt, & Schleifer, 1996), bifidobacteria (Bif164 5′CATCCGGCATTACCACCC3′, 50 °C, Langendijk et al., 1995), clostridia (histolyticum/perfringens group (Chis150 5′TTATGCGGTTATTAATCT(C/T)CCTTT3′, 45 °C, Franks et al., 1998) and lactobacilli/enterococci (5′GGTATTAGCA(T/C)CTGTTTCGA3′, 45 °C, Harmsen, Elfferich, Schut, & Welling, 1999). This panel of probes has been used to characterise changes in the major groups of human faecal bacteria in volunteer trials (Tuohy, Kolida, Lustenberger, & Gibson, 2001). Probes were fluorescently labelled with Cy5 at the 5′ end and obtained from MWG Ebersberg, Germany. The fixed cell suspension (16 µl) was added to 264 µl pre-warmed hybridisation buffer (30 mmol l^{-1} Tris–HCl, 1.36 mol l^{-1} NaCl, 52 mmol l^{-1} sodium dodecyl sulphate, pH 7.2.) This mixture (135 µl) was added to 15 µl of a 25 nmol l^{-1} solution of the appropriate probe and incubated overnight. The cells were then washed in buffer containing 20 mmol l^{-1} Tris–HCl, 0.9 mol l^{-1} NaCl, pH 7.2. 4′-6-Diaminido-2-phenylindole (DAPI) was added to allow the enumeration of total bacteria and the tubes were incubated for 30 min to remove excess probe. The mixture was then filtered under vacuum onto a 0.2 µm pore size membrane filter (Millipore, Watford, UK). The filter was then mounted onto a glass slide with a drop of Slowfade® (Molecular Probes, Lieden, Netherlands). Cells were counted using a Nikon Eclipse E400 fluorescence microscope (Nikon, Kingston-Upon-Thames, UK) fitted with a 355 nm excitation filter (DAPI) and a 550 nm excitation filter (Cy5). Statistical analysis was performed by ANOVA using the Dunnett's test for comparisons of populations at various time points with those of time 0. Student's *t*-tests were used to compare between substrates.

2.3. Polymerase chain reaction/denaturing gradient gel electrophoresis (PCR/DGGE)

Culture fluid (750 µl) was added to the same volume of 30% glycerol Samples were stored at this stage at −20 °C. The thawed samples were centrifuged for 5 min at 13400 × *g* to produce a pellet. The pellet was then subjected to DNA

extraction using the QIAmp DNA Stool Mini Kit (QIAgen Ltd, Crawley, UK) as per the kit instructions with the initial lysis step being carried out at 95 °C. Extracted DNA was then amplified by PCR. Primers directed to the V3 region of the 16S rRNA and were as follows, Primer 1: 5′ATTACCGCGGCTGCTGG 3′; Primer 2: 5′CGCCC-GCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGG-GGCCTACGGGAGGCAGCAG 3′ (Muyzer, De Waal, & Uitterlinden, 1993). 50 μl reactions each contained 2 μl extracted DNA suspension, 100 μmol each dNTP, 25 pmol each primer, 5 μl of 100 mmol l^{-1} Tris–HCl, 15 mmol l^{-1} $MgCl_2$, 500 mmol l^{-1} KCl, pH 8.3, (10× PCR reaction buffer, Roche, Basel, Swizerland). The volume was made up to 49.5 μl with water. Samples were then incubated at 5 min at 94 °C to minimise non-specific primer binding and cooled to 4 °C before the addition of 2.5 U *Taq* polymerase (Roche). A touchdown PCR program was run using an MJ DNA engine thermocycler (MJ Research, Reno, USA) as follows: 94 °C for 1 min, 65 °C for 1 min, 72 °C for 1.5 min (7×), 94 °C for 1 min, 60 °C for 1 min, 72 °C for 1.5 min (7×), 94 °C for 1 min, 55 °C for 1 min, 72 for 1.5 min (7×). DGGE was performed using the Ingeny PhorU system (Ingeny, Goes, Netherlands). PCR product (15 μl) was loaded onto an 80 g l^{-1} polyacrylamide gel in 20 mmol l^{-1} Tris acetate buffer containing 0.5 mmol l^{-1} ethylenediamine tetraacetic acid (EDTA), pH 8. A denaturant gradient of 0–80% was achieved through mixture of solutions 0 and 100% denaturant containing 7 mol l^{-1} urea and 40% (v/v) formamide. Electrophoresis was performed at 100 V and 60 °C. The gel was then stained with ethidium bromide (0.5 mg l^{-1}) for 1 h and destained in Purite water for 1 h before being visualisation using UV. Bands of interest were excised and the DNA removed by soaking in Purite water overnight. Re-amplification was performed using the same PCR conditions. Sequencing was performed using a capillary sequencer. Sequences were aligned using the BLAST algorithm (www.ncbi.nlm.nih.gov/BLAST, Altshul, Gish, Miller, Muyers, & Lipman, 1990).

2.4. Short chain fatty acid (SCFA) analysis

Culture fluid (1 ml) was centrifuged at 13400×*g* for 5 min to remove bacteria and particulate matter The supernatant (30 μl) was then subjected to high performance liquid chromatography (HPLC) using an Aminex HPX-87H column (BioRad, Hemel Hempstead, UK). The eluent was degassed 0.005 mol l^{-1} H_2SO_4 at a flow rate of 0.6 ml h^{-1} and the column was maintained at 35 °C. Acetic, lactic, butyric and propionic acids were detected by UV absorbance at 210 nm.

Statistical analysis was performed on triplicate values using paired *T*-tests.

2.5. Gut model investigation

An in vitro model of the human large intestine (Macfarlane, Macfarlane, & Gibson, 1998) was used to further investigate the effects of chitosan oligosaccharide lactate (COS) on the gut microflora COS was chosen for its butyrate-producing properties in batch cultures and its suggested prebiotic properties (Lee, Park, Jung, and Shin (2002)). The system consisted of three fed vessels with effluent from the first flowing into the second and from the second into the third to simulate the proximal, transverse and distal colonic regions. The first vessel was slightly smaller (80 ml) than the following vessels (100 ml) to achieve faster transit times observed in the proximal colon. Culture pH in the separate vessels was controlled at 5.5, 6.2 and 6.9, reflecting physiological conditions. The entire system was maintained anaerobically under O_2–free N_2 at a rate of approximately 15 ml min^{-1} and held at 37 °C using a circulating water jacket. Each vessel was half filled with fermentation medium containing (g l^{-1}) Potato Starch (Sigma) 5, Peptone water (Oxoid) 5, Tryptone (Oxoid) 5, Yeast extract (Oxoid) 4.5, NaCl (Fisher) 4.5 KCl (Fisher) 4.5, Pectin (Sigma) 2, Casein (Sigma) 2, Xylan (Sigma) 2, Larchwood Arabinogalactan (Fluka) 2, $NaHCO_3$ (Fisher) 1.5, $MgSO_4$ (BDH) 1.25, Gum guar (Sigma) 1, Inulin (Orafti) 1, Cysteine-HCl, (Sigma) 0.8, KH_2PO_4 (BDH) 0.5, K_2HPO_4 (BDH) 0.5, Bile Salts (Oxoid) 0.4, $CaCl_2$ (Sigma) 0.15, Haemin (Sigma) 0.05, Vitamin K (Sigma) 0.01, $FeSO_4$ (Sigma) 0.005, and Tween 80 (BDH) 1 ml l^{-1}. The other half of the vessels' volume was made up of a faecal inoculum from a healthy human volunteer. The inoculum was prepared by addition of warm PBS to faeces at a weight volumetric ratio of 4:1. After reaching steady state, all three vessels were sampled and 10 g l^{-1} COS added (equivalent to a dose of ca. 6 g d^{-1}) to the feed medium. Sampling was carried out again at the second steady state.

3. Results and discussion

3.1. Levels of bacteria in batch culture fermentations

The data in Table 1 show that glucose, FOS, COS, low, medium and high molecular weight chitosans could all be used as a sole carbon source for the growth of some members of the human gut microflora. Different populations were able to grow to different degrees dependent on the molecular weight of the substrate tested. Bacteroides tended to decrease compared to non-selective glucose and the established prebiotic, FOS. This pattern was also reflected in bifidobacterial numbers. The numbers of bacteroides and bifidobacteria showed an inverse relationship with the molecular weight of the chitosans provided, possibly due to an inability of these genera to degrade the longer molecules. A molecular weight based effect in the antimicrobial activities of chitosan oligomers where higher molecular weights had a greater effect against foodborne pathogens and spoilage organisms has been reported (Jeon et al., 2001; Rhoades & Roller, 2000) and could also be the cause of the decrease in numbers of these populations seen here.

Table 1
Mean bacterial populations in triplicate batch culture fermentations from 3 volunteers (S.D.) after 0, 5, 10 and 24 h

Group	Glucose				FOS			COS			Low			Med			High		
	0	5	10	24	5	10	24	5	10	24	5	10	24	5	10	24	5	10	24
Total Count	9.57	9.63	10.01	10.24	9.78	9.62	9.93	9.37	9.66	9.41	9.52	8.90	9.06	9.25	9.07	9.12	9.52	8.94[a]	8.80
	(0.19)	(0.03)	(0.17)	(0.52)	(0.11)	(0.23)	(0.15)	(0.19)	(0.05)	(0.35)	(0.21)	(0.80)	(0.28)	(0.59)	(0.60)	(0.67)	(0.23)	(0.93)	(0.48)
Bacteroides	8.70	8.57	8.94	8.76	8.60	8.69	8.62	8.51	8.25	8.76	8.34	8.59	7.98	8.58	8.02	6.60[a]	8.58	8.94	7.55
	(0.42)	(0.89)	(0.51)	(0.16)	(0.46)	(0.43)	(0.47)	(0.61)	(0.76)	(0.24)	(0.59)	(0.61)	(0.76)	(0.11)	(1.33)	(0.48)	(0.68)	(0.00)	(0.97)
Bifidobacteria	7.77	8.63	8.25	9.57	8.73	7.87	8.89	7.73	7.80	8.32	7.89	8.24	8.11	7.48	7.84	7.23	7.18	6.77	7.27
	(0.78)	(1.10)	(1.38)	(0.15)	(1.18)	(0.97)	(1.23)	(0.20)	(0.90)	(0.15)	(0.45)	(0.10)	(0.38)	(1.01)	(0.26)	(0.45)	(1.02)	(1.00)	(0.46)
Clostridia	7.72	7.44	7.99	8.05	7.30	7.67	8.17	7.45	7.88	8.68	7.23	8.42	7.69	6.19	7.58	7.08	7.20	6.71	7.38
	(0.81)	(0.22)	(0.85)	(0.88)	(0.45)	(1.13)	(0.81)	(0.20)	(0.96)	(1.09)	(0.52)	(0.79)	(0.58)	(0.00)	(1.00)	(0.28)	(0.09)	(0.90)	(0.80)
Lactobacilli	7.76	7.44	7.17	6.34	7.23	8.07	6.73	7.34	6.97	7.33	7.16[a]	7.62[a]	7.06[a]	7.36	7.13	7.27	7.48	7.77	6.89
	(0.70)	(0.22)	(0.72)	(0.21)	(0.47)	(0.92)	(0.76)	(0.75)	(0.59)	(0.34)	(0.76)	(0.00)	(0.38)	(0.08)	(0.90)	(0.54)	(0.54)	(0.00)	(0.99)

[a] Significant reductions compared to $T=0$, $p<0.05$.

Lactobacilli were also unable to utilise chitosans in competition with the rest of the faecal flora, as demonstrated by their decreasing numbers during the 24 h fermentation period. With low molecular weight chitosan in particular populations decreased significantly ($p<0.05$) compared to their starting levels. As this was not seen with COS and medium molecular weight chitosan, it would seem that this particular molecular weight may even be directly toxic. Numbers of clostridia on low, medium and high molecular weight chitosan declined throughout the 24 h fermentation. This decline lead to a significant decrease of clostridia with COS when compared to glucose ($p<0.01$) after 10 h, but this was reversed after 24 h when clostridia increased. The decrease may be due to a lack of enzymes able to attack the larger chitosan molecules. In complex microbial ecosystems such as the human gut bacteria may work in concert to degrade molecules with certain groups able to metabolise polymers and others lower molecular weight carbohydrates. Competition in densely populated areas like the human gut could also deprive some bacteria of nutrients, thus causing them to decline whilst other groups thrive. This may have occurred in the fermentations described here, as the total number of bacteria remained relatively stable during all the experiments.

3.2. Short chain fatty acid generation in batch culture fermentations

Short chain fatty acid levels from batch culture investigations are shown in Table 2 Butyrate levels were decreased with low, medium and high molecular weight chitosans compared to the glucose control after 24 h ($p<0.02$). COS did lead to a generation of butyrate but this was less than seen with glucose or FOS. High molecular weight chitosan gave rise to significantly less butyrate than did the oligosaccharide ($p<0.05$). Propionate levels were not affected in a significant manner. COS and low molecular weight chitosan gave less acetate than glucose ($p<0.05$). Lactate levels remained low in all the experiments, with very small amounts being generated from low, medium and high molecular weight chitosans. Some short chain fatty acids produced by bacteria are an important source of energy for the human gut and some are transported to other sites around the body for use. For example, acetate is primarily used in muscle tissue (Topping & Clifton, 2001) and propionate mainly by the liver (Wolever, Spadafora, & Eshuis, 1991). Butyrate is the preferred energy source for colonocytes which should be constantly renewed to maintain health (Cummings, 1984). In this study, as well as supporting bacterial growth, it can be seen that COS fermentation lead to the generation of butyrate, though not at such high levels as through the fermentation of glucose or FOS. This suggests that COS of the length used in these experiments may be beneficial for the gut ecosystem as, when metabolised by the gut flora, they may provide an energy source preferred by colonocytes although the in vivo

Table 2
Short chain fatty acid levels (mM) (SD) in batch culture fermentations from 3 volunteers after 0 and 24h

	Glucose		FOS		COS		Low		Med		High	
	0	24	0	24	0	24	0	24	0	24	0	24
Lactate	0.00 (0.00)	0.00 (0.00)	0.09 (0.16)	0.07 (0.12)	9.41 (4.04)	5.22 (9.04)	0.05 (0.09)	0.09 (0.16)	0.10 (0.17)	0.09 (0.15)	0.00 (0.00)	0.26 (0.45)
Acetate	0.14 (0.24)	35.04 (7.24)	0.15 (0.26)	31.75 (15.36)	0.00 (0.00)	14.78 (2.15)	0.11 (0.19)	11.45 (1.08)	0.11 (0.06)	8.85 (7.71)	0.11 (0.19)	8.66 (7.90)
Propionate	0.35 (0.43)	13.03 (6.97)	1.26 (1.15)	12.19 (3.16)	2.32 (2.32)	2.99 (0.46)	1.92 (1.24)	3.42 (1.09)	1.87 (0.52)	1.70 (1.48)	2.24 (0.25)	1.02 (1.28)
Butyrate	0.00 (0.00)	5.53 (0.99)	0.00 (0.00)	5.84 (0.85)	0.00 (0.00)	2.87 (1.47)	0.00 (0.00)	1.10 (0.44)	0.00 (0.00)	1.16 (1.02)	0.00 (0.00)	0.20 (0.35)

situation is undoubtedly more complex than this. Low, medium and high molecular weight chitosans produced only low butyrate levels. This presents further evidence that they are not well metabolised. Lactate levels were also very low with all substrates tested and at all time points. Lactate in the gut is relatively short-lived as it is a preferred electron sink product in anerobic metabolism may be quickly used up by bacteria such as the sulphate reducing bacteria (Gibson, 1990). The presence of lactate in vessel 1 when COS was added was due to the COS product tested being its lactate salt and therefore no conclusion may be drawn regarding an increased lactate production with COS.

3.3. Denaturing gradient gel electrophoresis of DNAs recovered from batch culture fermentations

DGGE analysis of batch culture samples showed that species level changes occurred (Fig. 1). Few species were present in the lanes representing cultures on low, medium and high molecular weight chitosan, whilst others, such as *Escherichia coli* and the bacterium homologous to *Shigella flexneri* (this is likely to be *E. coli* as the two species are very similar in 16SrRNA sequence) were present with all carbohydrate sources. COS generated two bands that were not visible with the other carbohydrates: *Bilophila wadsworthia* and *Clostridium disporicum* were selectively increased. Bacteroides species were also more prominent when COS was added to the gut model system. Bands representing bifidobacteria were not recovered from fermentations with COS. This would suggest that bifidobacteria are not strongly encouraged by chitosan or its derivatives.

Bifidobacterium longum was particularly stimulated by FOS. These data would support the findings of Bouhnik et al., (1999) who found that faecal bifidobacteria increased when human volunteers had their diets supplemented with FOS.

3.4. Gut model investigation of chitosan oligosaccharide

COS was investigated in a more complex gut model system in view of these batch culture results and previous publications indicating a prebiotic potential (Lee et al., 2002). FISH of culture fluid from the gut model showed that the addition of COS did have a major effect on the microflora, when substrates other than the test molecule were available. In vessel 1 (representing the proximal colon) a decrease was observed in numbers of clostridia. This was accompanied by a concomitant increase in lactobacilli but this may be due to the addition of chitosan oligosaccharide as its lactate salt to ensure solubility. This effect was seen to a small extent in the further two vessels and may also be due to the addition of lactate. Clostridia are considered to exert a negative effect upon human health (Gibson & Roberfroid, 1995) so their decrease may be considered beneficial. In vessel 2 (simulating the transverse colon), however, bacteroides and clostridia were

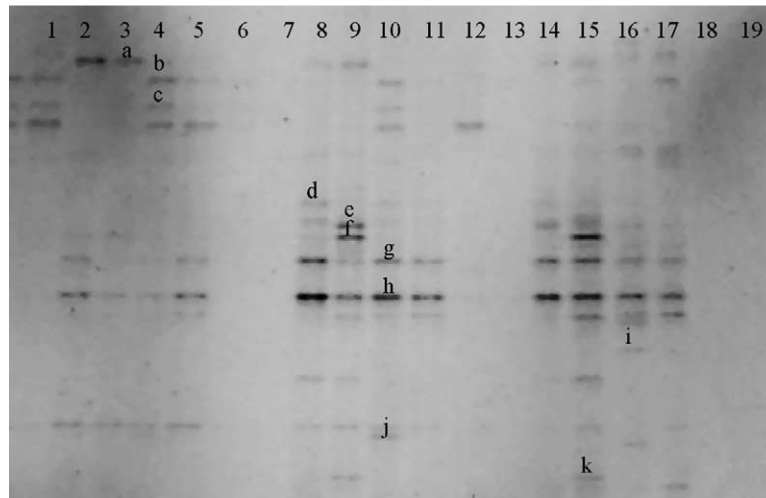

Fig. 1. DGGE showing bands amplified from batch culture experiments from one volunteer. Lane numbers refer to 1: Inoculum, 2–7: $T=5$ h with Glucose, FOS, COS, Low, Medium and High molecular weight chitosan, 8–13: $T=10$ h with the same substrates as 2–7, 14–19: $T=24$ h with the same substrates as 2–7. Lettered bands were sequenced and homologues (and identities) are as follows, a: uncultured Clostridium (100%); b: uncultured Bacteroides (98%); c: uncultured Bacteroides (97%); d: *Shigella flexneri* (99%); e: *Faecalibacterium prausnitzii* (98%); f: uncultured bacterium (98%); g: *Shigella flexneri* (98%); h: *Escherichia coli* (96%); i: *Bilophila wadsworthia* (97%); j: *Clostridium disporicum* (100%); k: *Bifidobacterium longum* (99%).

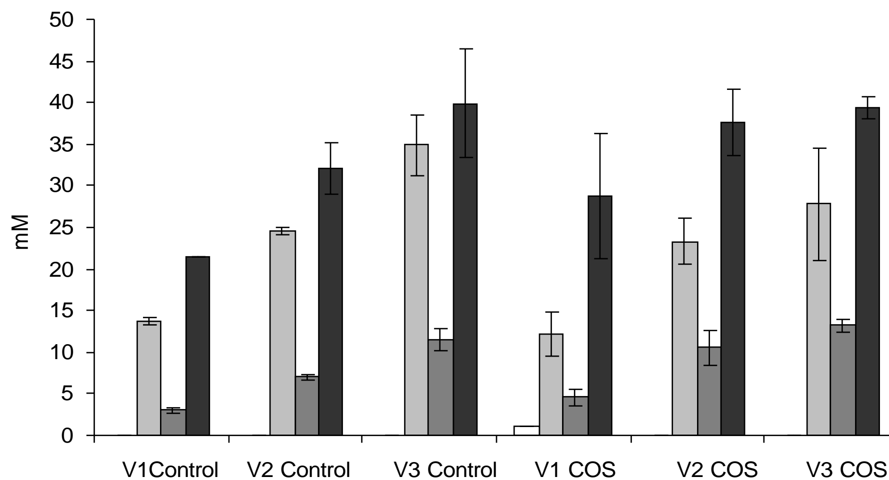

Fig. 2. Short chain fatty acid levels in gut model. Error bars are ±SE; □, Lactate; ▒, Acetate; ▒, Propionate; ■, Butyrate.

increased. Bacteroides are also considered to be a group with some negative effects, although saccharolytic species are likely not to be especially harmful. In vessel 3 (distal colon), where substrate availability was lowered due to degradation in the previous vessels, effects were diluted and bacteroides were only slightly increased compared to the control period. Bifidobacteria were unaffected by the presence of COS in all vessels.

Table 3
Bacterial populations in gut model (SE) after control media (control) and control media plus chitosan oligosaccharide lactate (COS) in vessel 1 (V1), vessel 2 (V2) and vessel 3 (V3)

	V1		V2		V3	
	Control	COS	Control	COS	Control	COS
Total count	10.26 (0.13)	10.19 (0.13)	10.36 (0.02)	9.97 (0.20)	9.90 (0.28)	9.99 (0.14)
Bacteroides	8.01 (1.22)	7.92 (0.28)	9.19 (0.22)	9.53 (0.30)	9.14 (0.14)	9.39 (0.35)
Bifidobacteria	10.27 (0.06)	10.21 (0.00)	10.13 (0.04)	10.01 (0.03)	9.85 (0.09)	9.88 (0.05)
Clostridia	7.67 (0.78)	7.03 (0.00)	6.49 (0.09)	8.00 (0.09)	6.58 (0.09)	6.86 (0.37)
Lactobacilli	8.00 (0.43)	8.66 (0.24)	7.98 (0.45)	8.14 (0.48)	7.79 (0.33)	8.10 (0.32)

Short chain fatty acid levels in the gut model are shown in Fig. 2. Analyses showed very low levels of lactate throughout (a small amount was detected in vessel 1 when COS was added as discussed above). Acetate generally decreased in all three vessels but propionate increased in all three vessels on the addition of COS and butyrate increased in vessels 1 and 2 (proximal and transverse colon). As mentioned, the generation of butyrate is seen as desirable for optimum gut health (Table 3).

4. Conclusions

This study has investigated the effects of chitosan and its oligosaccharides on the gut flora. Lee et al. (2002) showed that COS may be a prebiotic in that it promoted the growth of bifidobacteria and lactobacilli species in pure culture. The work presented here shows that despite the ability of some bifidobacteria and lactobacilli to grow on COS, bifidobacteria were not increased in a mixed culture model system. Groups of bacteria considered to be negative in the gut microflora increased in the gut model, principally clostridia. With respect to the short chain fatty acids produced in the gut model it can be concluded that the effects of COS on the gut flora are not wholly negative as butyrate levels were increased. Low, medium and high molecular weight chitosans were less able to support growth of bacteria, with a trend for the longer molecules to be less well metabolised. Further investigation of the full range of chitosan molecules reaching the gut should thus be undertaken to verify the safety of these molecules.

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