RESEARCH ARTICLE

In vitro fermentation of oat and barley derived β-glucans by human faecal microbiota

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Abstract

Fermentation of β-glucan fractions from barley [average molecular mass (MM), of 243, 172, and 137 kDa] and oats (average MM of 230 and 150 kDa) by the human faecal microbiota was investigated. Fractions were supplemented to pH-controlled anaerobic batch culture fermenters inoculated with human faecal samples from three donors, in triplicate, for each substrate. Microbiota changes were monitored by fluorescent *in situ* hybridization; groups enumerated were: *Bifidobacterium* genus, *Bacteroides* and *Prevotella* group, *Clostridium histolyticum* subgroup, *Ruminococcus–Eubacterium–Clostridium* (REC) cluster, *Lactobacillus–Enterococcus* group, *Atopobium* cluster, and clostridial cluster IX. Short-chain fatty acids and lactic acid were measured by HPLC. The *C. histolyticum* subgroup increased significantly in all vessels and clostridial cluster IX maintained high populations with all fractions. The *Bacteroides–Prevotella* group increased with all but the 243-kDa barley and 230-kDa oat substrates. In general β-glucans displayed no apparent prebiotic potential. The SCFA profile (51 : 32 : 17; acetate : propionate : butyrate) was considered propionate-rich. In a further study a β-glucan oligosaccharide fraction was produced with a degree of polymerization of 3–4. This fraction was supplemented to small-scale faecal batch cultures and gave significant increases in the *Lactobacillus–Enterococcus* group; however, the prebiotic potential of this fraction was marginal compared with that of inulin.

Introduction

β-Glucans are components of dietary cereal grains that are becoming increasingly recognized as functional ingredients in food and drink products (Angelov *et al.*, 2006; Naumann *et al.*, 2006). Cereal-derived β-glucans are unbranched hemicellulosic polymers containing β(1–4)-linked glucose residues, with β(1–3)-linked glucose residues every 2–3 units (Staudte *et al.*, 1983). A range of metabolic and physiological responses have been demonstrated from β-glucan consumption: lowered cholesterol levels in hypercholesterolaemic animals and humans (Davidson *et al.*, 1991, 1993; Braaten *et al.*, 1994; Behall *et al.*, 1997; Hecker *et al.*, 1998; Brown *et al.*, 1999; Kalra & Jood, 2000; Kerckhoffs *et al.*, 2002; Delaney *et al.*, 2003; Naumann *et al.*, 2006); blood insulin and glucose level responses (Bourdon *et al.*, 1999; Juntunen *et al.*, 2002; Frank *et al.*, 2004); and increased production of cholecystokinin (Anderson & Gustafson, 1988; Davidson *et al.*, 1991; Wood *et al.*, 1994; Bourdon *et al.*, 1999). In contrast, other studies refute an low-density lipoprotein (LDL) cholesterol-lowering effect (Leadbetter *et al.*, 1991; Lovegrove *et al.*, 2000).

A number of mechanisms have been proposed for the serum cholesterol lowering effect derived from β-glucan consumption; however, there is conflicting evidence for each (Malkki *et al.*, 1992; Horton *et al.*, 1994; Marlett *et al.*, 1994; Roy *et al.*, 2000; Sayar *et al.*, 2005). One such mechanism suggests that a hypocholesterolaemic effect occurs secondary to microbial fermentation of β-glucan in the large intestine; this results in short-chain fatty acid (SCFA) production, in particular propionate, which has a demonstrable hypocholesterolaemic effect (Chen *et al.*, 1984; Nishina & Freedland, 1990; Kishimoto *et al.*, 1995; Berggren *et al.*, 1996; Hara *et al.*, 1999; Han *et al.*, 2004). This involves a health-promoting role for β-glucans

in the lower gastrointestinal tract (GIT); however, some evidence challenges this (Beaulieu & McBurney, 1992).

Prebiotics are 'nondigestible (by the host) food ingredients that have a beneficial effect through their selective metabolism in the intestinal tract' (Gibson *et al.*, 2004). This effect is generally accepted to involve an increase in the populations and/or activity of *Bifidobacterium* spp., and *Lactobacillus* species. Hughes *et al.* (2007) demonstrated that a structure–function relationship existed between the molecular mass (MM) of wheat-derived arabinoxylan polysaccharide fractions and their fermentation properties *in vitro* by human faecal microbial communities; whereby the lowest MM AX fraction had the greatest selectivity for the *Bifidobacterium* genus and *Lactobacillus–Enterococcus* group, whereas the highest MM fraction was less selective for these groups. Other work has demonstrated that enzymatic pretreatment of polysaccharides to hydrolyse them into oligosaccharides before supplementation to the diet also increased their selectivity for bifidobacteria and lactobacilli (Mountzouris *et al.*, 1999; Olano-Martin *et al.*, 2000, 2002; van Laere *et al.*, 2000). β-Glucans and β-glucan oligosaccharides (β-GO) were previously shown to selectively stimulate the growth of lactobacilli populations in a rat model (Snart *et al.*, 2006), which suggested that prebiotic activity could occur in humans.

If SCFAs, and in particular propionate, are implicated in the hypocholesterolaemic effect of β-glucan it is necessary to determine the quantity and ratio of different SCFAs produced from the fermentation of relatively pure β-glucan fractions (unlike previous studies that used oats, or oat bran concentrate). Furthermore, it is important to correlate the specific groups of the microbial communities associated with β-glucan fermentation and the consequential SCFA profiles. This investigation will evaluate the fermentation properties of five fractions of commercially available β-glucans, either from barley or oats, in pH-controlled and stirred anaerobic batch culture fermenters. It is anticipated that the different MM fractions of β-glucan will influence the fermentation pattern adopted by the human faecal microbial communities. Furthermore, β-glucan was pretreated with endo-β-glucanase (lichenase) to produce β-glucan hydrolysates; their fermentation properties were tested in small-scale faecal batch cultures (Sanz *et al.*, 2005a).

Materials and methods

Materials

Five β-glucan fractions were provided by Megazyme Co. (Wicklow, Ireland), while inulin (Raftiline) was acquired from Orafti.

Methods

β-Glucan hydrolysate production

The high MM barley β-glucan fraction was hydrolysed using lichenase, which is a (1,3)-(1,4)-β-D-glucan-4-glucanohydrolase, derived from *Bacillus subtilis* (Megazyme Co.). It cleaved the 1,4-linkages of the 3-*O*-substituted glucose residues in β-glucan. Three β-glucan hydrolysate fractions were produced following lichenase hydrolysis, in a 60 °C waterbath; 1 U of activity corresponded to the formation of 1 nmol of reducing sugar (D-glucose) min^{-1}: hydrolysate 1 (H1) was treated with 1 U lichenase, for 20 min; hydrolysate 2 (H2) was treated with 1 U lichenase, for 60 min; and hydrolysate 3 (H3) was treated with 20 U lichenase, for 120 min, which ensured complete hydrolysis. In H3 84% of the degradation products were 3-*O*-β-cellobiosyl-D-glucose and 3-*O*-β-cellotriosyl-D-glucose, these were oligosaccharides with a degree of polymerization (DP) of 3 and 4, respectively (Wood *et al.*, 1991a). The remaining *c.* 16% of the fraction was higher DP oligosaccharide material that was derived from the longer sections of 1–4-linked glucose units in the parent β-glucan fraction, producing oligosaccharides of between DP 5 and 9 (Wood *et al.*, 1991a).

Complete hydrolysis of the oat and barley β-glucan fractions using the same β-glucanase as before, followed by analysis of the β-GO produced from each fraction, provided linkage information on the β-glucan fractions, i.e. the ratio of β(1–3) to β(1–4) linkages (Table 1).

Table 1. Physico-chemical properties of the β-glucan fractions

Average MW* (kDa)	Origin	% of fraction within stated MW range (kDa)						Calculated average MW (kDa)	Ratio of β(1-4): β(1–3) linkages	Viscosity* (cSt, 1%, 30 °C)
		> 359	< 359, > 245	< 245, > 183	< 183, > 123	< 123, > 40	< 40			
327	Barley	30.5	19.0	13.6	9.4	22.9	4.6	243	2.5 : 1	> 100
260	Barley	17.3	15.3	15.1	12.9	33.2	6.2	172	2.3 : 1	28
137	Barley	13.9	12.6	13.4	13.2	40.5	6.4	137	2.5 : 1	5.6
272	Oat	27.5	18.9	14.4	10.8	24.5	3.8	230	1.6 : 1	69
220	Oat	17.8	13.7	12.1	11.7	38.2	6.6	150	1.5 : 1	20–30

*Data provided by Megazyme, Co. Wicklow, Ireland.

Characterization of β-glucan and β-glucan hydrolysates

Solutions (1% w/v) of the β-glucan hydrolysates were prepared in HPLC grade water and analysed by SE-HPLC using TSK $G6000_{XL}$ and two TSK G4000 PW columns (Polymer Laboratories, Shropshire, UK). The eluent was 0.02% (w/v) sodium azide, with a 0.6 mL min^{-1} flow rate, at ambient temperature. Dextran standards, with MM between 1 and 670 kDa MM, were used to calibrate the column.

The specific binding of the calcofluor dye to β-glucan polymers was used as a method for quantifying the β-glucan fraction MM distributions; this binding caused an increase in calcofluor fluorescence which was related to the amount of β-glucan present (Wood *et al.*, 1991b; Rimsten *et al.*, 2003; Åman *et al.*, 2004). An online postsize exclusion column detection method was used and the MM distribution determined with a fluorescence detector that measured excitation at 410 nm and emission at 460 nm. The MM distribution of samples was calibrated using β-glucan standards (provided by Megazyme Co.) with average MM of 40–245 kDa. This method was not sensitive to β-glucan of < 10 kDa, therefore could not be used to analyse β-glucan hydrolysate samples (Jørgensen & Aastrup, 1988). The eluent was 0.02% (w/v) sodium azide, with a 0.5 mL min^{-1} flow rate, at ambient temperature. A 0.0005% calcofluor solution was added at 0.05 mL min^{-1} postcolumn, before entering a mixing coil.

HPLC analysis of β-glucan hydrolysate was carried out by producing solutions (1% w/v) of the β-glucan hydrolysates in HPLC grade water. This was subjected to HPLC analysis using the RSO oligosaccharide column (Phenomenex, Macclesfield, UK). The eluent was HPLC grade water with a 0.3 mL min^{-1} flow rate, at 75 °C temperature. A dextran homopolymer standard (Oxford Scientific, Oxford, UK), and malto-oligosaccharide standards (Sigma, Poole, UK) were used to calibrate the column.

In vitro fermentations

pH-controlled anaerobic batch cultures were used to study the growth of faecal bacteria during fermentation of the oat and barley derived β-glucans. It consisted of a water-jacket vessel, maintained at 37°C, that was filled with prereduced basal culture medium, and the pH maintained at 6.8 for the duration. This medium contained per litre: 2 g peptone water (Oxoid Ltd., Basingstoke, UK), 2 g yeast extract (Oxoid), 0.1 g NaCl, 0.04 g K_2HPO_4, 0.01 g $MgSO_4.7H_2O$, 0.01 g $CaCl_2.6H_2O$, 2 g $NaHCO_3$, 0.005 g haemin (Sigma), 0.5 g L-cysteine HCl (Sigma), 0.5 g bile salts (Oxoid), 2 mL Tween 80, 10 μL vitamin K (Sigma), and 4 mL of 0.025% (w/v) resazurin solution. 0.5 g (1% w/v) carbohydrate was partially dissolved for an hour in the medium before inoculation with a 10% (w/v) faecal slurry which was prepared by homogenizing fresh human faeces (10%, w/v) in phosphate-buffered saline (PBS 8 g L^{-1} NaCl, 0.2 g L^{-1} KCl, 1.15 g L^{-1} Na_2HPO_4 ,and 0.2 g L^{-1} KH_2HPO_4) pH 7.3 (Oxoid). The working volume of each culture was 50 mL. Cultures were conducted in triplicate, for each of three faecal donors, per carbohydrate ($n = 9$). The donors, one female and two male, were healthy, aged 24–29, had not received antibiotic treatment for at least 3 months before the study, had not knowingly consumed pre- or probiotic supplements, and had no history of bowel disorders. One sample was prepared as a control, without any carbohydrate addition, and another had inulin (Raftline; Orafti: Tienen, Belgium) supplemented as a positive prebiotic control (Gibson, 1999; Roberfroid, 1999; de Wiele *et al.*, 2004; Rossi *et al.*, 2005). Sample time points were 0, 5, 10, and 24 h (and 48 h for SCFA analysis).

A small-scale *in vitro* fermentation method (validated against 150 mL pH-controlled batch cultures) (Sanz *et al.*, 2005a) was used to study the growth of faecal bacteria during the fermentation of β-GO. The fermentation of each treatment was conducted in triplicate on each of three faecal donors ($n = 9$). Two of the three donors were common to the β-glucan investigation above. Carbohydrate (10 mg) was mixed in autoclaved basal medium (same ingredients as described above) to give a final concentration of 1% (w/v). One sample was prepared as a control, without any carbohydrate addition, and inulin was used as a positive prebiotic control. Samples were inoculated with 1 mL faecal slurry (prepared as above). All additions, inoculations and incubations were carried out inside an anaerobic cabinet (10% H_2, 10% CO_2, and 80% N_2) at 37 °C, in Hungate tubes, that were shaken at 300 r.p.m. to ensure a homogenous distribution of carbohydrate. pH was not maintained. Samples were taken from the inoculum and at 12 h from the treated samples for bacterial counts.

Enumeration of bacteria

The fluorescent *in situ* hybridization (FISH) technique was used to quantify bacteria at four time points during the incubations. Samples (375 μL) were fixed overnight at 4 °C with 4% (w/v) filtered paraformaldehyde (pH 7.2) in a ratio of 1 : 3 (v/v), washed twice with filtered PBS, resuspended in 300 μL of a mixture of PBS/ethanol (1 : 1, v/v) and then stored at − 20 °C for up to 3 months. The hybridization was carried out as previously described (Rycroft *et al.*, 2001a, 2001b) using genus- and group-specific 16S rRNA gene-targeted oligonucleotide probes labelled with Cy3 (MWG Biotech, Ebersberg, Germany) or the nucleic acid stain 4′,6-diamidino-2-phenylindole for total cell counts. The bacterial groups were selected based on their high abundance within, and contribution to, the colonic microbiota. The probes (previously validated for the bacterial

groups) were: Bif164, specific for the *Bifidobacterium* genus (Langendijk *et al*., 1995); Bac303, specific for the *Bacteroides* and *Prevotella* group (Manz *et al*., 1996); His150, for the *Clostridium histolyticum* subgroup (Franks *et al*., 1998); Erec482 for the *Ruminococcus–Eubacterium–Clostridium* (REC) cluster (Franks *et al*., 1998); Lab158, for the *Lactobacillus–Enterococcus* group (Harmsen *et al*., 1999); Ato291 for the *Atopobium* cluster, including most *Coriobacteriaceae* species (Harmsen *et al*., 2000); and Prop853, for clostridia cluster IX (Walker *et al*., 2005).

SCFA analysis

Samples were centrifuged at 13,000 ***g*** for 20 min, and the supernatant was passed through a 0.2-μm filter before SCFA content analysis using a BioRad HPX-87H HPLC column (Watford, UK), at 50 °C, with a 0.005 mM H_2SO_4 eluant at a flow rate of 0.6 mL min^{-1} (Sanz *et al*., 2005a).

Results

β-Glucan characterization

Five β-glucan fractions, three derived from barley, and two from oats, were kindly provided by Megazyme Co. The SE-HPLC calcofluor detection method showed that each β-glucan fraction comprised a mixture of components with the MM distribution of the fractions overlapping (Table 1); the calculated mean MM for the three barley β-glucan fractions were 243, 172, and 137 kDa, and for the two oat β-glucan fractions were 230 and 150 kDa. The physico-chemical properties of the five β-glucan fractions are described in Table 1.

Microbial communities response

The response of the microbiota was determined using a set of FISH probes (Table 2). The probe set selected had coverage of 14–35% of the total at time 0 but this increased in all cases and accounted for 31–69% by 24 h.

There was a significant increase in the *C. histolyticum* group in response to all β-glucan fractions after 24 h (Table 2). A relationship was evident between increased *C. histolyticum* populations and decreasing MM, whereby *C. histolyticum* was greater on lower MM β-glucan. There was also a nonsignificant but continued increase in *C. histolyticum* in the no treatment control.

There were significant increases in the *Bacteroides–Prevotella* group by 24 h with both the 172-kDa barley and 150-kDa oat β-glucan fractions, and the 137-kDa barley β-glucan. However, there was no change in *Bacteroides–Prevotella* following fermentation of the higher MM fractions: 243-kDa barley and 230-kDa oat β-glucan.

Clostridia cluster IX was significantly lower with inulin and the no treatment control by 24 h. However, at 10 and/or 24 h for all β-glucan fractions (except 137-kDa barley β-glucan) clostridia cluster IX was significantly higher than the no treatment control. Visual inspection also suggested that the amount (i.e. the bio-volume) of bacteria in clostridia cluster IX was far greater following β-glucan treatment than with the controls. This change in bio-volume cannot be quantified by FISH, which simply enumerates group populations rather than bio-volume.

The only significant increase in the *Bifidobacterium* genus was at 24 h, resulting from inulin fermentation. β-glucan fermentation generally had little impact on the *Bifidobacterium* genus population. Lab158 positive cells (*Lactobacillus–Enterococcus* group) increased in all cultures at 5 h. However, their populations were not maintained past the 5/10 h peak as numbers decreased in all treatments at 24 h, and significantly so with inulin and both the oat β-glucan treatments.

The Erec482 probe detected faecal bacteria in the REC cluster (Franks *et al*., 1998). REC decreased in all treatments at 5 h. However, it increased significantly in all β-glucan treatments at 10 and/or 24 h; particularly for the 137-kDa barley β-glucan and the 150-kDa oat β-glucan fractions which caused increases of 73% and 53%, respectively, at 24 h compared with 5 h.

Increases also occurred in the *Atopobium* cluster in response to the β-glucan fractions; these increases were significant at 10 and/or 24 h. There was no significant change in the total cell count in any culture, which fluctuated between 9.6 and 10.2 (log_{10}, cells mL^{-1} culture fluid) in all vessels.

SCFA and lactate production

Acetate was the most prevalent SCFA in all treatments; accounting for $>$ 40% of the SCFA produced with the β-glucan treatments; 67.3% in the no treatment control, and 63.3% with inulin, at 48 h (Table 3). However, fermentation of β-glucan resulted in higher total amounts and proportions of propionate; ranging from 30% with 150-kDa oat β-glucan treatment, to 37.1% with 172-kDa barley β-glucan, at 48 h. In fact, all the vessels containing β-glucan had $>$ 30% propionate throughout the experimental time period. The mean SCFA ratio, for all β-glucan treatments, at 24 h, was 51 : 32 : 17, acetate : propionate : butyrate (Table 4). Butyrate production from β-glucan fermentation was also higher, both in terms of total amounts and proportions of total SCFA produced, in comparison with inulin. Lactate production peaked in all cultures after 10 or 24 h of fermentation before a decline occurred. Lactate is rapidly converted into SCFAs by the cross-feeding of other bacteria (Duncan, 2002; Bourriaud, 2005), which is the likely cause of its decline in concentration by 48 h.

Table 2. Bacterial populations (log10 cells mL^{-1} batch culture fluid) in pH controlled and stirred batch cultures at 0, 5, 10 and 24 h using inulin and five fractions of barley and oat-derived fractions of β-glucan as substrates, compared with a no treatment control

Treatment	Time	Total cell population	*Atopobium* cluster and most *Coriobacteriaceae* spp.	*Bacteroides–Prevotella* group	*Bifidobacterium* genus	*Ruminococcus–Eubacterium–Clostridium* cluster	*Clostridium histolyticum* subgroup	*Lactobacillus–Enterococcus* group	Clostridium Cluster IX	% Probe Coverage[†]
No treatment	0	9.89 (0.07)	7.59 (0.04)	8.08 (0.06)	7.69 (0.05)	8.24 (0.05)	6.60 (0.07)	6.67 (0.03)	7.36 (0.06)	14.1
	5	9.61 (0.06)	7.61 (0.05)	8.46 (0.05)	7.55 (0.04)	8.08 (0.05)	6.69 (0.08)	6.99 (0.04)*	7.03 (0.05)	38.6
	10	9.65 (0.07)	7.59 (0.05)	8.61 (0.06)**	7.65 (0.05)	8.02 (0.04)	6.73 (0.09)	6.75 (0.03)	7.13 (0.06)	53.3
	24	9.68 (0.06)	7.53 (0.05)	8.33 (0.07)	7.72 (0.05)	8.08 (0.05)	6.87 (0.09)	6.73 (0.05)	6.89** (0.06)	52.6
Inulin	0	9.97 (0.06)	7.63 (0.05)	8.41 (0.06)	7.71 (0.04)	8.25 (0.05)	6.54 (0.08)	6.76 (0.04)	7.48 (0.07)	16.4
	5	9.67 (0.06)	7.49 (0.05)	8.65 (0.06)	7.62 (0.04)	8.03 (0.05)	6.60 (0.08)	6.98 (0.04)**	7.21 (0.07)	35.8
	10	9.70 (0.06)	7.92 (0.06)	8.58 (0.07)	7.86 (0.05)	8.13 (0.04)	6.62 (0.10)	6.93 (0.04)	7.21 (0.07)	64.7
	24	9.62 (0.06)	7.84 (0.05)	8.00 (0.06)**	8.07 (0.06)**	8.07 (0.05)	6.68 (0.09)	6.50 (0.04)*	7.06* (0.07)	33.6
Barley 243-kDa β-glucan	0	9.75 (0.06)	7.60 (0.05)	8.31 (0.06)	7.69 (0.04)	8.39 (0.06)	6.59 (0.08)	6.80 (0.04)	7.54 (0.07)	24.7
	5	9.62 (0.06)	7.85 (0.06)	8.54 (0.07)	7.72 (0.04)	8.02 (0.04)*	6.80 (0.08)	6.99 (0.04)	7.30 (0.07)	49.9
	10	9.88 (0.07)	8.35 (0.05)**	8.53 (0.06)	7.96 (0.05)	8.23 (0.07)	6.97 (0.09)	6.67 (0.03)	7.46 (0.09)	35.6
	24	9.79 (0.07)	8.12 (0.06)**	8.42 (0.05)	7.71 (0.05)	8.25 (0.06)	7.12 (0.09)**	6.64 (0.04)	7.37 (0.07)	30.5
Barley 172-kDa β-glucan	0	9.79 (0.06)	7.59 (0.05)	8.45 (0.08)	7.82 (0.05)	8.44 (0.07)	6.62 (0.08)	6.70 (0.04)	7.40 (0.07)	34.7
	5	10.02 (0.06)	7.75 (0.06)	8.44 (0.06)	7.69 (0.05)	8.22 (0.05)	7.07 (0.09)	6.93 (0.05)**	7.39 (0.07)	18.3
	10	10.07 (0.07)	8.09 (0.06)**	8.58 (0.06)	7.86 (0.05)	8.49 (0.07)	7.05 (0.10)	6.94 (0.03)**	7.53 (0.08)	34.8
	24	10.05 (0.09)	8.24 (0.05)**	9.03 (0.08)**	7.56 (0.05)	8.44 (0.07)	7.36 (0.10)**	6.81 (0.06)	7.57 (0.05)	69.3
Barley 130-kDa β-glucan	0	9.90 (0.07)	7.72 (0.05)	8.53 (0.06)	7.72 (0.05)	8.16 (0.06)	6.54 (0.06)	6.80 (0.05)	7.45 (0.07)	24.0
	5	9.73 (0.07)	7.92 (0.05)	8.38 (0.06)	7.75 (0.05)	7.89 (0.05)	7.14 (0.09)**	6.93 (0.05)	7.32 (0.07)	26.0
	10	9.94 (0.07)	7.95 (0.06)	8.37 (0.07)	7.88 (0.06)	7.97 (0.05)	7.17 (0.09)**	7.06 (0.04)	7.30 (0.08)	25.5
	24	10.12 (0.07)	8.03 (0.05)**	9.05 (0.06)**	7.75 (0.04)	8.45 (0.07)	7.33 (0.11)**	6.79 (0.05)	7.26 (0.06)	43.7
Oats 230-kDa β-glucan	0	9.89 (0.06)	7.83 (0.05)	8.42 (0.07)	7.70 (0.05)	8.19 (0.06)	6.56 (0.06)	6.84 (0.04)	7.49 (0.07)	22.6
	5	9.93 (0.06)	7.68 (0.05)	8.53 (0.07)	7.60 (0.05)	7.91 (0.05)	6.99 (0.08)	7.04 (0.04)	7.34 (0.07)	19.1
	10	10.05 (0.06)	8.18 (0.05)**	8.13 (0.07)	7.77 (0.06)	8.08 (0.05)	7.14 (0.09)**	6.98 (0.04)	7.43 (0.07)	13.7
	24	9.65 (0.08)	7.78 (0.06)	8.30 (0.08)	7.80 (0.06)	8.20 (0.06)	7.07 (0.09)**	6.47 (0.03)**	7.36 (0.05)	67.3
Oats 150-kDa β-glucan	0	9.93 (0.05)	7.60 (0.05)	8.21 (0.07)	7.68 (0.05)	8.33 (0.06)	6.61 (0.07)	6.96 (0.04)	7.49 (0.07)	25.3
	5	9.86 (0.06)	7.83 (0.06)	8.46 (0.07)	7.48 (0.04)	8.08 (0.04)	6.97 (0.09)	7.00 (0.05)	7.27 (0.07)	19.9
	10	10.04 (0.07)	8.00 (0.06)**	8.30 (0.07)	7.93 (0.05)	8.11 (0.06)	7.29 (0.10)**	6.91 (0.03)	7.27 (0.08)	22.9
	24	9.82 (0.07)	7.79 (0.05)	8.57* (0.06)	7.69 (0.05)	8.40 (0.07)	7.23 (0.08)**	6.55 (0.04)**	7.47 (0.05)	46.9

Univariate ANOVA and Tukey's multivariate comparison tests were used to determine a significant increase/decrease of bacterial populations compared with 0 h, within each treatment.

*$P < 0.05$, and

**$P < 0.01$. Standard error is shown in parentheses.

[†]Percent of the microbial communities diversity enumerated by the probe set compared with DAPI.

Table 3. SCFA and lactic acid concentration (mM) in pH-controlled and stirred batch cultures at 0, 5, 10, 24 and 48 h using inulin and five fractions of barley and oat-derived β-glucan substrates, compared with a no treatment control

Concentration (mM)	Time	Total SCFA	SE	Lactate	SE	Acetate	SE	Propionate	SE	Butyrate	SE
No treatment	0	1.67	(0.48)	0.04	(0.03)	0.00	(0.00)	1.43	(1.27)	0.20	(0.18)
	5	3.96	(0.32)	0.42	(0.22)	2.25	(0.54)	1.03	(0.26)	0.26	(0.15)
	10	11.43	(0.98)	0.13	(0.10)	8.13	(2.29)	2.55	(0.50)	0.62	(0.29)
	24	11.43	(2.31)	1.76	(0.38)	18.27*#	(5.31)	3.00	(0.87)	2.56	(1.44)
	48	20.69	(1.57)	0.00	(0.00)	13.92	(3.04)	4.15	(0.95)	2.62	(0.73)
Inulin	0	1.16	(0.23)	0.14	(0.11)	1.02	(0.58)	0.00	(0.00)	0.00	(0.00)
	5	11.51	(1.31)	2.05	(0.58)	6.93	(2.68)	1.99	(0.59)	0.54	(0.30)
	10	30.73*	(2.51)	2.87	(1.29)	21.00*	(5.72)	4.88	(1.33)	1.98	(0.80)
	24	38.90*#	(2.77)	0.92	(0.43)	22.83*#	(5.02)	9.93*	(2.61)	5.22	(1.73)
	48	37.81*	(2.81)	0.00	(0.00)	22.52*	(5.65)	9.60	(2.34)	3.42	(1.28)
Barley 243-kDa β-glucan	0	1.80	(0.45)	0.09	(0.08)	1.03	(0.67)	0.68	(0.60)	0.00	(0.00)
	5	13.66	(1.31)	2.43	(1.24)	7.21	(1.50)	3.35	(1.24)	0.67	(0.27)
	10	21.42	(1.61)	3.66	(1.66)	10.35	(2.07)	5.85	(1.42)	1.56	(0.36)
	24	38.82*	(2.56)	3.07	(1.74)	18.51*	(2.65)	11.82*	(2.83)	5.42	(1.13)
	48	45.19*#	(2.96)	0.34	(0.30)	22.81*	(3.16)	13.86*#	(3.03)	8.18*#†	(1.68)
Barley 172-kDa β-glucan	0	0.67	(0.17)	0.09	(0.08)	0.58	(0.42)	0.00	(0.00)	0.00	(0.00)
	5	38.90	(1.24)	3.59	(1.41)	5.18	(0.76)	5.55	(1.90)	0.89	(0.33)
	10	37.81*	(1.69)	4.09	(2.01)	11.57	(1.91)	7.27	(1.43)	2.28	(0.75)
	24	47.46*#	(2.14)	1.09	(0.59)	21.30*#	(3.82)	15.77*#	(1.89)	9.30*#†	(1.83)
	48	46.98*	(3.17)	0.61	(0.46)	19.25	(3.97)	17.20*#	(3.14)	9.92*#†	(2.12)
Barley 130-kDa β-glucan	0	0.99	(0.19)	0.00	(0.00)	0.96	(0.58)	0.03	(0.02)	0.00	(0.00)
	5	11.25	(0.90)	2.60	(1.15)	5.29	(0.96)	2.86	(0.65)	0.49	(0.14)
	10	23.59	(1.78)	4.86	(2.92)	10.55	(1.57)	6.20	(0.99)	1.97	(0.51)
	24	36.71*	(2.46)	2.62	(1.78)	18.02*	(3.16)	9.55*	(1.76)	6.52*#	(1.71)
	48	41.38*	(1.87)	0.00	(0.00)	11.42	(0.91)	9.66*	(2.25)	9.12*#†	(2.75)
Oats 230-kDa β-glucan	0	0.30	(0.08)	0.00	(0.00)	0.19	(0.11)	0.09	(0.09)	0.03	(0.04)
	5	8.53	(0.68)	1.27	(0.58)	4.47	(1.01)	2.18	(0.45)	0.62	(0.31)
	10	11.43	(1.70)	3.42	(1.79)	10.43	(2.12)	5.79	(1.50)	1.22	(0.52)
	24	32.78*	(2.63)	0.24	(0.21)	16.74	(3.66)	9.91*	(2.37)	5.90*	(1.60)
	48	45.62*	(3.36)	0.00	(0.00)	22.39*#	(5.03)	14.15*#	(3.09)	9.08*#†	(2.05)
Oats 150-kDa β-glucan	0	1.58	(0.25)	0.18	(0.16)	0.88	(0.31)	0.47	(0.31)	0.05	(0.08)
	5	10.88	(1.02)	2.50	(1.51)	4.64	(0.72)	3.09	(0.98)	0.65	(0.20)
	10	25.74	(1.76)	5.45	(2.95)	11.63	(1.98)	6.75	(1.30)	1.91	(0.51)
	24	43.57*	(2.72)	6.11	(2.96)	19.61	(2.08)	12.96*	(1.44)	4.89	(0.70)
	48	45.54*#	(2.44)	0.09	(0.07)	24.64*#	(4.25)	13.65*	(2.11)	7.17*	(1.40)

Univariate ANOVA and Tukey's multivariate comparison tests were used to determine a significant increase in each SCFA concentrations compared with 0 h, within each treatment.

*Significant from initial value $P < 0.05$.

#Significant difference from 5 h value, $P < 0.05$.

†Significant difference from 10 h value, $P < 0.05$. SE is shown in parentheses.

Less SCFA was produced after 10 h from the fermentation of β-glucan than from inulin. However, unlike with inulin, fermentation of β-glucan (except for the 172-kDa barley β-glucan fraction) was more prolonged because the total concentrations continued to rise between 24 and 48 h, whereas with inulin SCFA production did not rise after a 10 h peak (Table 3).

β-Glucan hydrolysates and their fermentation

Three β-glucan hydrolysate fractions were analysed by size-exclusion-HPLC (Fig. 1) and HPLC that separated oligosaccharide material with DP 1–9. H1 and H2 contained a large range of material according to the size-exclusion-HPLC analysis, with a MM below 150 kDa, with most material below 5 kDa (relative to the dextran standard equivalents). Nearly all of H3 was below 5 kDa according to size-exclusion-HPLC analysis (Fig. 1), and of the material detected by HPLC analysis 84% was composed of β-GO with DP 3–4, in a ratio of *c.* 2.5 : 1. The remaining 16% of H3 detected by HPLC had oligosaccharides with DP 5–9.

The three hydrolysates were fermented by human faecal microbiota, and compared with the parent barley derived 243-kDa β-glucan, inulin, and a no treatment control. H1

Table 4. SCFA ratio following 48 h fermentation of different treatments, in stirred, pH-controlled batch cultures, at 37 °C; % refers to the proportion of each SCFA produced with respect to total SCFA produced (i.e. acetate + propionate + butyrate)

	SCFA ratio (%)		
Treatment	Acetate	Propionate	Butyrate
No treatment	67.3	20.0	12.6
Inulin	63.3	25.4	11.3
Barley 243-kDa β-glucan	50.9	30.9	18.2
Barley 172-kDa β-glucan	41.5	37.1	21.4
Barley 130-kDa β-glucan	43.1	33.0	23.8
Oats 230-kDa β-glucan	49.1	31.0	19.9
Oats 150-kDa β-glucan	54.2	30.0	15.8

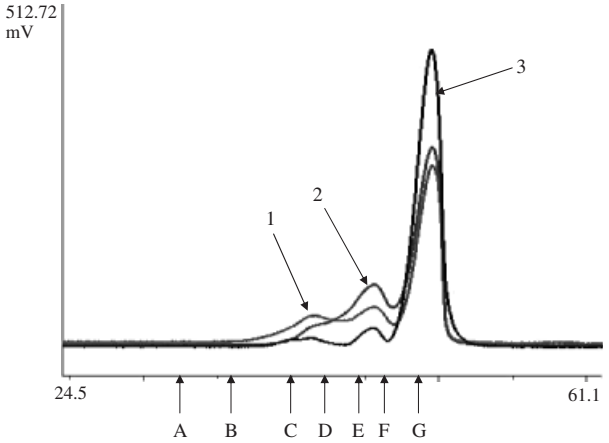

Fig. 1. SE-HPLC chromatogram of the three β-glucan hydrolysates fractions, 1 (1), 2 (2) and 3 (3), on a TSK G6000$_{XL}$ and two TSK G4000 PW columns. Dextran standard elution peaks: A, 670 kDa; B, 150 kDa; C, 77 kDa; D, 25 kDa; E, 11.6 kDa; F, 5 kDa; and G, 1 kDa.

and H2, when fermented, had no unique selectivity for any group of the microbial communities, as shown in Table 5. Total cell numbers decreased in all treatments, except for the β-glucan treatment where there was an increase. H3 also had no significant impact on the microbial communities, apart from a selective increase in the *Lactobacillus–Enterococcus* group; yet this was still lower than the increase in the *Lactobacillus–Enterococcus* group resulting from all the other carbohydrates tested. All hydrolysates gave significant decreases in clostridia cluster IX. Hydrolysate 2 gave a significantly greater increase in populations of the *Atopobium* cluster, the *Bifidobacterium* genus and *C. histolyticum* groups than hydrolysates 1 and 3. Inulin gave the greatest increases in *Bifidobacterium*, with a 0.22 log increase in the population.

Discussion

The β-glucan fractions varied appreciably in MM range and this variation correlated positively with viscosity. The linkage ratios of the fractions broadly corresponded with the ratios observed in β-glucans from barley and oats in a recent study where β1-4 linkages were more prevalent than β1–3 linkages, and the ratio of β(1–4):(1–3) linkages was *c.* 2.0–2.4 in oats, and 3.0 in barley (Papageorgiou *et al.*, 2005). This trend was confirmed in the present study (Table 1); although the dominance of β(1–4) linkages was slightly less, with ratios of 1.5–1.6 for oats and 2.3–2.5 for barley.

There was a considerable increase in propionate concentrations following β-glucan fermentation, which has been documented in previous studies (Monsma *et al.*, 2000); a number of bacterial groups could be implicated in this increase. Firstly, growth of the *C. histolyticum* group was stimulated by all the β-glucan fractions tested. The *C. histolyticum* subgroup is a minor group within the human microbial communities and has previously been associated with butyrate production (Salminen *et al.*, 1998; Zigova *et al.*, 1999). Yet, the significant increase in *C. histolyticum* combined with the fact that some species within the *C. histolyticum* group can produce propionate (such as *Clostridium homopropionicum*), indicates they could be implicated in the elevated propionate production resulting from β-glucan fermentation. Furthermore, within this group are *Clostridium cellulovorans*, *Clostridium longisporum* and *Clostridium acetobutylicum*, which all have genes encoding endo-β-glucanase enzymes (Zappe *et al.*, 1988; Foong *et al.*, 1991; Mittendorf & Thomson, 1993) that enable them to degrade β-glucan polysaccharides.

Secondly, *Bacteroides–Prevotella* are a dominant group of the gut microbial communities and known producers of propionate (Macy & Probst, 1979). A significant increase in the *Bacteroides–Prevotella* group from β-glucan fermentation may explain the increase in propionate production; however, this group did not increase significantly in response to all the β-glucan fractions tested, indicating that groups other than *Bacteroides–Prevotella* were involved in elevating propionate concentrations. Nonetheless, *Bacteroides fragilis*-type bacteria are a prevalent group in the human gut microbial community and produce substantial quantities of propionate from succinate and fumarate (Macy *et al.*, 1978). It has been proposed that *Bacteroides thetaiotaomicron*, *Bacteroides distasonis*, and *B. fragilis* are probably involved with most β-D-(1–3)-glucanase activity in the colon, and these species have been demonstrated to efficiently degrade a β1–3-linked β-D-glucose polysaccharide, called laminarin (Salyers *et al.*, 1977). Furthermore, the activity of β-D-(1-3)-glucanase was suggested as either an extracellular occurrence or it was loosely associated with the membrane. Other species in the *Bacteroides–Prevotella* group (e.g. *Bacteroides ovatus*) produce succinate, particularly when carbon is in excess (Macfarlane & Gibson, 1991), which was akin to the carbon concentration in the cultures reported here. Supposing succinate was produced other

Table 5. Bacterial populations (log_{10} cells g^{-1} faeces) in 10 mL batch cultures following 12 h fermentation, using inulin, β-glucan, and three β-glucan hydrolysate fractions, produced by lichenase hydrolysis, compared with a no treatment control, and the inoculum (i.e. 0 h)

Treatment	Total cell population	*Atopobium*	*Bacteroides-Prevotella*	*Bifidobacterium*	*Ruminococcus–Eubacterium–Clostridium*	*Clostridium histolyticum*	*Lactobacillus–Enterococcus*	Clostridia cluster IX
Inoculum	9.22 (0.06)	6.75 (0.02)	7.70 (0.05)	7.11 (0.03)	7.81 (0.05)	5.97 (0.01)	6.35 (0.03)	7.46 (0.04)
No treatment	9.14 (0.05)	6.79 (0.02)	7.72 (0.05)	7.33 (0.03)*	7.31 (0.03)	5.97 (0.01)	6.45 (0.03)	7.28 (0.04)
Inulin	9.06 (0.05)	7.23 (0.04)*	7.66 (0.05)	7.60 (0.04)*	7.97 (0.05)	5.98 (0.01)	6.90 (0.02)*	7.23 (0.04)*
Parent β-glucan[#]	9.43 (0.05)	7.23 (0.03)*	7.77 (0.04)	7.37 (0.03)*	8.23 (0.07)*	6.25 (0.02)*	6.64 (0.03)*	7.33 (0.04)
H1	9.20 (0.06)	7.02 (0.04)*	7.69 (0.06)	7.27 (0.03)*	8.01 (0.05)	5.99 (0.01)	6.56 (0.03)*	7.23 (0.05)*
H2	9.11 (0.05)	6.94 (0.04)*	7.98* (0.06)	7.44 (0.04)*	8.02 (0.05)	6.18 (0.03)*	6.67 (0.03)*	7.26 (0.04)*
H3	9.05 (0.05)	6.78 (0.03)	7.75 (0.05)	7.07 (0.04)	7.66 (0.05)	6.00 (0.01)	6.50 (0.03)*	7.16 (0.04)*

Univariate ANOVA and Tukey tests were used to determine significant differences for each SCFA concentration.
*Significant differences ($P < 0.05$) among the different carbohydrate sources for each bacterial genus. SE is given in parentheses.
[#]243-kDa barley β-glucan.

bacteria in the *Bacteroides–Prevotella* group were potentially converting it into propionate.

Alternatively, higher propionate production could be attributed to clostridia cluster IX, which maintained high populations and bio-volume following β-glucan fermentation. This cluster is known for its propionate production (Walker *et al.*, 2005) and species within it (e.g. *Succiniclasticum ruminis* and *Succinispira mobilis*) have been reported to quantitatively convert succinate into propionate (van Gylswyk, 1995; Janssen & O'Farrell, 1999). This may indicate a level of cross-feeding between succinate-producing *Bacteroides* spp. and succinate-to-propionate converting clostridia cluster IX bacteria.

The amount of butyrate produced with β-glucan fermentation was higher than that produced with inulin. The *C. histolyticum* group, which increased significantly, comprises a number of butyrate-producing bacteria (Salminen *et al.*, 1998; Zigova *et al.*, 1999) that may have been responsible for the butyrate produced from the β-glucans. There was also a steady increase in the REC cluster with β-glucan; REC includes numerous butyrate-producing bacteria (Barcenilla *et al.*, 2000) that could have been responsible for the increase in butyrate.

Unlike the selective fermentation of inulin by species within the *Bifidobacterium* genus, β-glucan displayed no apparent selectivity for species within this genus. Curdlan, which is similar in structure to β-glucan (but comprises only β(1–3)-D-linked glucose), was previously shown to selectively increase the total bifidobacteria population, compared with cellulose and gellan gum, in the caecum of curdlan-fed rats (Shimizu *et al.*, 2001). It is possible that the mixed linkages [i.e. β(1–3) and β(1–4) linkages] present in the β-glucan fractions were responsible for this difference in selectivity for the *Bifidobacterium* genus.

Although there were initially increases in the *Lactobacillus–Enterococcus* group their populations collectively decreased by 24 h with β-glucan treatment. This conflicts with previous work that displayed a 'lactobacillogenic' effect of β-glucan on the gut microbial communities of rats (Snart *et al.*, 2006). This previous investigation demonstrated an increase in lactobacilli rRNA, as a proportion of the total microbial communities rRNA, when grown on high-viscosity β-glucan compared with cellulose treatment in mixed culture, and the population of *Lactobacillus acidophilus* was *c.* 40-fold higher in a pure culture study. These conflicting results may arise from differences in the experimental techniques used; the work by Snart *et al.* (2006) used *in vivo* rat caecal microbiota, and the present study was an *in vitro* investigation using human microbiota.

A previous *in vitro* study (Monsma *et al.*, 2000) using rat caecal inocula to ferment (predigested) oat bran resulted in a SCFA ratio of 69 : 19 : 12, acetate : propionate : butyrate; this was considered a high proportion of propionate in comparison with the SCFA profile generated by other substrates (NB acetate is the dominant SCFA in most fermentation studies of this nature). A further study demonstrated that supplementing oat bran to the diet of rats had no significant effect on the caecal SCFA profile compared with diets supplemented with various cereal brans, inulin, and a no-fibre control diet (Grasten *et al.*, 2002). The propionate-enriched ratio of 51 : 32 : 17, in the present study, agrees with the work by Monsma *et al.* (2000). This may have resulted from the high purity (> 96%) of the β-glucan being fermented, compared with the more crude forms of β-glucan (e.g. oat bran) previously tested. Another study (Hughes *et al.*, 2007) investigating the fermentation of relatively pure arabinoxylans (> 94%) demonstrated an average SCFA ratio of 63 : 12 : 25, which was deemed an example of a butyrate-enriched SCFA profile.

The elevated propionate production could relate to the hypocholesterolaemic effects associated with β-glucan consumption. A murine study (Chen *et al.*, 1984) demonstrated an association between propionate and hypocholesterolaemic effects; whereby the supplementation of propionate to

rats consuming a cholesterol-rich diet caused serum and cholesterol levels to significantly decrease in comparison with rats fed the cholesterol diet without propionate. However, whether the microbial communities can mediate serum cholesterol levels by increasing propionate production is debateable. A conflicting study demonstrated that the viscosity of different fractions of oat bran concentrates was the primary factor responsible for hypocholesterolaemic effects in rats, which implied an upper GIT mechanism for influencing serum cholesterol levels (Malkki *et al.*, 1992). It was suggested that this upper GIT effect probably occurred because the high viscosity reduced the diffusion rates of fat and cholesterol, which in turn reduced their absorption. However, viscosity is a physical property of β-glucan present in the upper GIT, which is lost on arrival in the large intestine when presented with the milieu of bacterial-derived carbohydrate-degrading enzymes. Therefore, Malkki *et al.* (1992) stated that the viscous property of β-glucan was unlikely to exert any effect in the microbial-rich lower GIT where viscosity becomes redundant, and that the benefits of β-glucan were probably restricted to the upper GIT.

Fermentation of H1 and H2 showed no apparent prebiotic activity with regards to selectively increasing groups of the microbial communities that are considered to be beneficial to health, for example *Bifidobacterium* spp., and *Lactobacillus* spp. This indicated that partial hydrolysis of β-glucan was not sufficient to generate any prebiotic activity. Prebiotic ingredients are often oligosaccharides, for example fructo-oligosaccharide and galacto-oligosaccharide (Hughes & Kolida, 2007), which is consistent with our observation that H3, containing *c.* 84% β-GO of DP 3–4, exerted a selective increase in the *Lactobacillus–Enterococcus* group, which has been observed before (Snart *et al.*, 2006). All the other group populations remained the same or decreased and the total cell number also decreased, accentuating this 'lactobacillogenic' effect of β-GO. Inulin, the positive prebiotic control, caused a greater increase in the *Lactobacillus–Enterococcus* group in addition to an increase in the *Bifidobacterium* genus and the REC cluster, which demonstrated a greater prebiotic effect of inulin than β-GO. Initial findings suggest that completely hydrolysing β-glucan into β-GO, using lichenase, was an effective approach to increasing the *Lactobacillus–Enterococcus* group population, and thus increase the prebiotic activity. This warrants further detailed investigation using more advanced *in vitro* culture techniques that more accurately mimic colonic conditions.

This study has revealed that the main group of human faecal microbial communities involved in β-glucan fermentation was the *C. histolyticum* group, and to a lesser extent clostridia cluster IX and the *Bacteroides–Prevotella* and *Atopobium* groups. A particularly high proportion of propionate production may indicate a lower GIT role for the hypocholesterolaemic effects of β-glucan following their fermentation by the microbiota, because propionate has previously been shown to reduce serum cholesterol levels (Chen *et al.*, 1984). There was little evidence to support a relationship between the structural and physical properties of β-glucan and its fermentation by the microbial communities. Although β-glucan was not 'prebiotic' in the classical sense of increasing bifidobacteria and lactobacilli, it significantly modulated the microbial communities and the resulting SCFA profile. If this modulation was proven to reduce cholesterol levels in a human trial, without any concurrent adverse affects arising form that modification, β-glucan would deservedly come under the heading 'prebiotic'. So further *in vivo* studies are required to establish whether hypocholesterolaemic effects result from increased serum propionate in humans and whether these are secondary to β-glucan fermentation by the human gut microbiota.

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