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Impact of polydextrose on the faecal microbiota: a double-blind, crossover, placebo-controlled feeding study in healthy human subjects

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Abstract

In this placebo-controlled, double-blind, crossover human feeding study, the effects of polydextrose (PDX; 8 g/d) on the colonic microbial composition, immune parameters, bowel habits and quality of life were investigated. PDX is a complex glucose oligomer used as a sugar replacer. The main goal of the present study was to identify the microbial groups affected by PDX fermentation in the colon. PDX was shown to significantly increase the known butyrate producer *Ruminococcus intestinalis* and bacteria of the *Clostridium* clusters I, II and IV. Of the other microbial groups investigated, decreases in the faecal *Lactobacillus–Enterococcus* group were demonstrated. Denaturing gel gradient electrophoresis analysis showed that bacterial profiles between PDX and placebo treatments were significantly different. PDX was shown to be slowly degraded in the colon, and the fermentation significantly reduced the genotoxicity of the faecal water. PDX also affected bowel habits of the subjects, as less abdominal discomfort was recorded and there was a trend for less hard and more formed stools during PDX consumption. Furthermore, reduced snacking was observed upon PDX consumption. This study demonstrated the impact of PDX on the colonic microbiota and showed some potential for reducing the risk factors that may be associated with colon cancer initiation.

Key words: Polydextrose: Human faecal microbiota: Genotoxicity: Biomarkers of colonic health

Prebiotics are growing in popularity amid evidence of a host of beneficial effects, including enhancing immune function[1] and improving mucosal structural[2] and cellular turnover[3]. Some studies even indicate anti-cancer potential[4] to be one among the many beneficial effects. A prebiotic is a selectively fermented ingredient that results in specific changes in the composition and/or activity of the gastrointestinal microbiota, thus conferring benefit(s) upon host health[5]. To date, much of the work on prebiotics has focused on fructo-oligosaccharides, inulin and galacto-oligosaccharides[6]. As the commercial interest in functional food ingredients and colonic health has grown worldwide, further prebiotics may eventually make their way into the market. However, the ultimate tests for prebiotic activity, the human volunteer trials, are scarce or lacking for the majority of these candidates[7]. Thus, more evidence on their efficacy is needed for a prebiotic status to be achieved.

A diet that contains sufficient fibre is likely to be advantageous for consumer health. Epidemiological studies have long suggested an inverse association between dietary fibre intake and a range of colonic and systemic human diseases including certain cancers and CVD[8]. The increased availability of carbohydrate within, especially, the distal part of the intestine means increased microbial saccharolytic activities and SCFA production. This leads to a potential reduction of proteolytic activities that are linked to carcinogen or genotoxin production[9]. In addition to decreased proteolytic activity, the independent role of increased SCFA concentrations, especially butyrate, has been speculated to be behind some of the beneficial effects associated with fibre[10]. Low- or non-fermentable fibres, such as cellulose, may act as bulking agents, improve intestinal transit time and, thereby, reduce the exposure of human mucosal tissue

Abbreviations: COX, cyclo-oxygenase; DGGE, denaturing gel gradient electrophoresis; FISH, fluorescence *in situ* hybridisation; PDX, polydextrose; qPCR, quantitative PCR.

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to genotoxic and carcinogenic compounds[11]. A prebiotic, on the other hand, has an additional property in that it selectively alters the intestinal microbiota, modulating the composition and/or activity to increase the relative population levels of potentially beneficial organisms, such as bifidobacteria[5].

Polydextrose (PDX) is a soluble fibre synthesised from glucose; it is frequently used to increase the fibre content of food. PDX has a highly branched structure with a spectrum of glycosidic linkages[12]. Previous *in vitro* research has shown that PDX is selectively fermented by the faecal microbiota, to stimulate bifidobacterial growth[13]. These findings are supported by a feeding trial where dose-dependent increases in bifidobacteria and lactobacilli and decreases of three species of bacteroides were observed[14]. However, it is worth noting that in the feeding trial of Jie *et al.*[15], enumeration is done using selective plating techniques, though such enumeration techniques are problematic[16]. *In vitro* studies suggest that PDX may have protective activities for markers of colorectal cancer; for example, cyclo-oxygenase (COX)-2 expression has been observed to be elevated in individuals with colon cancer[17], and fermentation products derived from PDX have been demonstrated to lower the expression of COX-2 *in vitro* by Caco-2 cells[18]. In addition, similar results were obtained in animal studies with reduced intestinal levels of branched chain fatty acids and biogenic amines[19,20]. Furthermore, in pigs, PDX tended to decrease the expression of mucosal COX-2, therefore possibly reducing the risk of developing colon cancer-promoting conditions in the distal intestine[20]. Furthermore, Peuranen *et al.*[20] reported an immune-stimulatory effect in the intestine by PDX with an associated change in the microbial metabolism, including butyrate production and reduction in putrefaction. Tools for the measurement of colon cancer risk factors are limited in short-term intervention studies. However, the use of single cell genotoxicity screening, or the comet assay, is gaining interest as a straightforward method for examining DNA damage and is, therefore, being considered as a biomarker for monitoring the effects of dietary intervention on cancer risk[21]. Furthermore, human intervention studies have shown that PDX may also provide benefits to colonic function[22].

The impact of PDX on the human colonic microbiota has been investigated to some extent, but would require further substantiation on the effects, in particular, on recognised beneficial bacteria, such as bifidobacteria and lactobacilli. To this end, a placebo-controlled, double-blind human feeding study was conducted on thirty-one healthy adult volunteers in a crossover manner. Changes in the colonic microbiota were determined by measuring bacterial population levels using fluorescence *in situ* hybridisation (FISH) and quantitative PCR (qPCR). In addition, a detailed qualitative study of the total microbiota diversity was performed on total faecal DNA extracts via denaturing gel gradient electrophoresis (DGGE). Faecal concentrations of SCFA were also determined; furthermore, gastrointestinal symptoms and stool characteristics were recorded (stool frequency, consistency, abdominal pain, intestinal bloating and flatulence) in order to assess tolerance. Additionally, markers of immune function and changes in the genotoxic nature of the faecal water and COX induction of the colon cells were assessed to determine whether the intervention would offer potential benefits against markers of colon cancer risk.

Experimental methods

Subjects

A total of thirty-three healthy volunteers (18–50 years of age) were enrolled from the Reading area; two volunteers dropped out due to personal reasons before the start of the intervention (n 31). Written informed consent was obtained from all participants and the study protocol was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving human subjects were approved by the Ethics and Research Committee of the University of Reading. Written informed consent was obtained from all subjects. Inclusion criteria for participation in the study were: a signed consent form, age 18–50 years inclusive, BMI 19–25 kg/m^2 inclusive and good general health. Volunteers with evidence of physical/mental disease, major surgery, history of drug abuse, severe allergy, a history of severe abnormal drug reaction and smokers were excluded. Volunteers were also excluded if pregnant, lactating or planning pregnancy. Intake of an experimental drug within 4 weeks before study, former participation in prebiotic, probiotic or laxative trials within 3 months, or use of antibiotics within 6 months before the study, chronic constipation, diarrhoea or other chronic gastrointestinal complaints (e.g. irritable bowel syndrome) were all exclusion criteria. Any intake of prebiotics, probiotics (live yoghurts, fermented milk drinks) and drugs active on gastrointestinal motility, antibiotic treatment or any class of laxative was not permitted. All medication taken throughout the duration of the study was recorded in diaries. Volunteers were instructed not to alter their usual diet or fluid intake during the trial.

Study design

The dietary intervention study was performed in a double-blind, randomised, placebo-controlled crossover manner. For a period of 14 d before the dietary intervention study, volunteers followed a restricted diet, refraining from consumption of yoghurts, prebiotic supplements and probiotics. A total of thirty-one subjects were randomly allocated into one of two groups. For volunteer randomisation, baseline bifidobacteria levels were enumerated and volunteers were allocated into the two groups in such a way that the two groups did not significantly differ for baseline bifidobacteria numbers.

The first group (n 16) consumed PDX powder (Litesse® Ultra, 8 g/d; Danisco UK, Redhill, UK) for 3 weeks, and then after a 3-week washout period, they consumed the equivalent placebo (maltodextrin powder, 8 g/d, Syral, Marckolsheim, France) for 3 weeks. The second group (n 15) received first the placebo for 3 weeks, and then after a 3-week washout period, PDX powder for another 3-week treatment period. During the 3-week washout period, no trial product was consumed.

All test products were packaged, labelled and randomised by Danisco before the study. The study was blinded to

investigators and volunteers. Volunteers were asked to keep diaries throughout the study to record stool frequency, consistency (constipation, hard, formed, or soft stool or diarrhoea), abdominal pain (none, mild, moderate or severe), intestinal bloating (none, mild, moderate or severe) and flatulence (none, mild, moderate or severe) on a daily basis. Any concomitant medication or adverse events were also recorded. Faecal samples were collected from each volunteer at five different time points, before and after each treatment: at baseline (before PDX intake, pre-PDX), following PDX intake, following washout 1 (post-PDX and before maltodextrin, pre-maltodextrin), following maltodextrin intake and following washout 2 (post-maltodextrin).

Quality of life questionnaires

Volunteers were issued quality of life questionnaires to complete at the end of the treatment periods 4 h following consumption of the test product. This questionnaire was to rate hunger, motivation, mood and mental alertness by marking along a line between 'not at all' and 'very'. The position along the line was quantified using a ruler to give rise to a value of between 1 and 10. These were used to assess general feeling of the volunteers following the treatments.

Collection and stool sample preparation

Freshly voided faecal samples were stored in an anaerobic cabinet (10 % H_2; 10 % CO_2; 80 % N_2; Don Whitley Scientific, Shipley, UK) for no longer than 2 h before processing.

For FISH and SCFA analysis, samples were diluted 1 in 10 (w/w) with PBS (0·1 M; pH 7·0) and mixed in a Stomacher 400 (Seward, Norfolk, UK) for 2 min at normal speed (460 paddle beats per min). Of the faecal slurries, 5 ml were transferred into 50 ml sterile plastic centrifuge tubes containing 2 g of glass beads (diameter = 5 mm) and vortexed for 30 s to further homogenise the samples.

Enumeration of faecal bacterial populations by *fluorescence* in situ *hybridisation*

FISH was performed as described previously[6]. All oligonucleotide probes were Cy3-labelled and synthesised by Sigma-Aldrich (Poole, UK). Table 1 gives the details of probes used in this study.

DNA extraction for denaturing gel gradient electrophoresis

For the isolation of bacterial DNA, 2 g of frozen faecal samples (−70°C in 7 ml sterile Sterlin tubes) were used. The samples were thawed at room temperature and extraction of bacterial DNA was carried out using QIAamp DNA Stool Mini Kit (QIAGEN Limited, Crawley, UK), essentially following the manufacturer's instructions. The protocol was modified by an initial disruption step using glass beads (diameter 0·1 mm) and a FastPrep beadbeater (Bio101 Savant FastPrep FP120, FastPrep® Instrument, Qbiogene, Cambridge, UK) set at speed 6 for two runs of 40 s. DNA quality was checked by running 5 μl aliquots of the samples on 0·8 % (w/v) ultraPURE agarose (Gibco-BRL, Paisley, UK) gels containing ethidium bromide (0·4 mg/ml), and visualising the bands using UV light. The quantity of DNA in each sample was determined using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Samples were stored at −20°C.

Denaturing gradient gel electrophoresis

Fragments (230 bp) derived from the variable V3 region of the 16S rRNA gene were amplified using the universal primers P2 and P3[23]. Primers were synthesised by Sigma-Genosys (Haverhill, UK). DGGE was carried out as described previously[24]. The V20-HCDC DGGE system (BDH, Poole, UK) was used with gels comprising 35–60 % gradients and run at 100 V. Gels were silver-stained according to the previously published method[24].

The gel pictures were analysed by the Gel Compar II software (Applied Maths NV, Sint-Martens-Latem, Belgium). Following normalisation, bands were defined for each sample using the appropriate densitometric curves. Bands constituting less than 1 % of the total band area were omitted from further analysis. Similarity between DGGE profiles was determined by calculating the Pearson's correlation. Clustering of the sample profiles was done using the unweighted pair-group method using arithmetic average with a tolerance of 5 %.

Shannon-Weaver index, Simpson's index and Fisher's index were calculated using an implementation on Gel Compare II

Table 1. Probes used in this study for fluorescence *in situ* hybridisation analysis

Target*	Probe sequence (5′–3′)	Accession no.	Hybridisation – washing temperature (°C)
Erec482	GCT TCT TAG TCA RGT ACC G	pB-00963	50
Bac303	CCA ATG TGG GGG ACC TT	pB-00031	45
Fpra655	CGC CTA CCT CTG CAC TAC	pB-00734	58
Bif164	CAT CCG GCA TTA CCA CCC	pB-00037	50
Ato291	GGT CGG TCT CTC AAC CC	pB-00943	50
Lab158	GGT ATT AGC AYC TGT TTC CA	ND†	45
Chis150	TTA TGC GGT ATT AAT CTY CCT TT	pB-00962	50

ND, not deducted.

* Probe designation according to probeBase (http://www.microbial-ecology.net/probebase). All probes were used at the concentrations of 50 ng/μl[11].

† No information relating to these probes has been deposited in probeBase.

software (Applied Maths NV, Sint Martens-Latem, Belgium). They were used to compare changes in the diversity of microbial communities within treatments and baseline. Shannon-Weaver index (H'), Simpson's index (D) and Fisher's alpha index (α) were calculated as done previously[24].

DNA extraction for quantitative PCR

Microbial DNA was extracted from the faecal samples by an initial bead beating step of two 3 × 30 s cycles at 6800 rpm, and thereafter with the use of a modified Promega Wizard genomic DNA purification kit (Promega, Madison, WI, USA). DNA isolation was performed according to instructions of the manufacturer. DNA concentrations were measured by a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and samples were stored at −20°C until qPCR analyses.

Quantitative PCR

Extracted microbial DNA (1 ng) was analysed by qPCR, using primers specific for *Atopobium* spp., *Clostridium difficile*, *C. leptum* group (*Clostridium* cluster IV), *C. histolyticum* (clusters I and II), *C. perfringens*, *Desulfovibrio intestinalis*, *Roseburia intestinalis* and *Staphylococcus aureus* (Table 2[25–30]). Each sample was analysed in triplicate and all samples of one subject analysed in the same run. No template controls and DNA standards from bacterial strains were included on each plate to enable quantification of the bacterial content of the samples.

SCFA analysis by GC

SCFA concentrations were analysed by a method adapted from Zhao *et al.*[31], as described by Walton *et al.*[32]. Peaks were integrated using Atlas Lab managing software (Thermo Lab Systems, Mainz, Germany). Fatty acid concentrations were calculated by comparing their peak areas with those of the standards and were expressed as mmol/g faeces.

Immunological analyses

Concentrations of IgA and PGE_2 were measured from the soluble fraction of the faeces. The frozen samples were thawed and extracted with bovine serum albumin as described previously[20] and stored at −20°C before analysis. Concentrations of IgA and PGE_2 were determined using ELISA according to the respective manufacturer's instructions (E80-102; Bethyl Laboratories, Inc., Montgomery, TX, USA; Cayman Chemical Company Limited, Ann Arbor, MI, USA) and the results were expressed as mg or pg/g faeces (fresh weight), respectively.

Faecal water preparation

Faecal samples were diluted 1:1 (w/v) in 1 M-ice-cold PBS in a stomacher bag. Samples were homogenised in a stomacher for 2 min at high speed or until a uniform consistency was achieved. For each sample, 10 ml aliquots, in duplicate, were then transferred into ultracentrifuge tubes (Beckman Ultra-clear tubes; Beckman Limited, High Wycombe, UK) and the tubes stored at −70°C. Samples were thawed on ice before centrifugation, then ultracentrifuged at 64 000 ***g*** for 2 h at 4°C (Beckman Optima L90K Ultracentrifuge; Beckman Limited, High Wycombe, UK). Following centrifugation, the tubes were placed on ice and supernatants (faecal water) carefully removed and placed into sterile 7 ml Sterilin tubes. The faecal waters were then filtered through 0·2 μm syringe filter, dispensed into 0·5 ml aliquots and frozen at −70°C until analysis.

Determination of DNA damage by single cell gel electrophoresis (comet assay)

The comet assay (single cell gel electrophoresis) was used to microscopically detect DNA damage at the level of a single cell as described earlier[33]. The assay was conducted on the baseline faecal water sample for all volunteers. The nine volunteers whose baseline samples caused high–medium levels of DNA damage to the HT29 cells and two additional low-level faecal waters were investigated for the remaining time-points. Briefly, after 48 h of cultivation in Dulbecco's modified Eagle's medium supplemented with 10 % (w/v) fetal bovine serum and penicillin (50, 000 U/L) streptomycin (50 μg/ml) at 37°C in air with 5 % CO_2, sub-confluent cultures of HT29 were harvested by trypsination and adjusted to a concentration of 2×10^6 cells/ml in serum-free Dulbecco's modified Eagle's medium. Duplicate tubes containing 100 μl of cell suspension and 100 μl of the respective faecal waters or PBS (negative controls) were incubated at 37°C for 30 min with constant gentle shaking. After incubation, one series of

Table 2. Primers used in the quantitative PCR to enumerate the microbial groups of interest

Species	Primer name	Annealing temperature (°C)	References
Atopobium group	gAtop_F gAtop_R	58	Rinttilä *et al.*[25]
Clostridium difficile	cdif_F2 cdif_R2	60	Lahtinen *et al.*[26]
Clostridium leptum subgroup (cluster IV)	sg_Clep_F sg_Clep_R3	62	Matsuki *et al.*[27]
Clostridium perfringens	CPTAFW CPTARV	55	Tiihonen *et al.*[28]
Clostridium clusters I and II	g_Cperf_F g_Cperf_R	55	Rinttilä *et al.*[25]
Desulfovibrio intestinalis	apsA1F apsA1R	62	Tiihonen *et al.*[28]
Roseburia spp.	Roseint_F gRose_R	56	Mäkivuokko *et al.*[29]
Staphylococcus aureus	NUC1 NUC2	60	Brakstad *et al.*[30]

tubes was treated with 75 μM-H_2O_2 as a genotoxic model agent for 5 min on ice. Samples were centrifuged at 280 ***g*** for 5 min at 4°C and washed once with PBS. Following this, three 50 μl aliquots of each sample were centrifuged again and the resulting cell pellets re-suspended in warm, low melting point agarose and further processed as described[33]. Cell numbers and viability of HT29 were assessed before and after incubation using Trypan Blue exclusion assay.

Cyclo-oxygenase gene expression

Human colon-derived carcinoma cells, Caco-2 (ATCC HTB-37), were used to study the effects of faecal water samples on the COX gene expression of colonic epithelial cells. The Caco-2 cells were maintained at 37°C in a humidified air/5 % CO_2 atmosphere in basal culture of Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10 % fetal bovine serum (Invitrogen), 2 mmol/l stable glutamine (Invitrogen), 1 × non-essential amino acids (Invitrogen), 20 U/ml penicillin (Invitrogen), 20 μg/ml streptomycin (Invitrogen) and 0·5 μg/ml amphotericin (Invitrogen). The cells were seeded as $6{\cdot}4 \times 10^4$ cells/cm^2 on twelve-well cell culture plates (Greiner Bio-One GmbH). On the second day following seeding, the medium was replaced with the serum- and antibiotic-free culture medium. On the third day of culture, the Caco-2 cells were treated with 0·5 % (w/v) faecal water samples prepared in the serum- and antibiotic-free culture medium. In addition to the medium-only control, 5 mmol/l sodium butyrate (Sigma-Aldrich, Chemie GmbH, Steinheim, Germany) and 100 mmol/l NaCl (J.T. Baker, Deventer, The Netherlands) were used as positive and negative controls, respectively. The cells were incubated at 37°C in a humidified air/5 % CO_2 atmosphere for 20 h, after which the cells were lysed in 300 μl RA1 Lysis Buffer (Macherey-Nagel, Düren, Germany) containing 1 % β-mercaptoethanol (Sigma-Aldrich).

Total RNA was isolated from the lysed cell samples using a nucleic acid extraction robot (Corbett, Sydney, Australia), quantified with NanoDrop ND-1000 (NanoDrop Technologies; Thermo Fisher Scientific, Wilmington, DE, USA) and subjected to complementary DNA synthesis. Reverse transcription was conducted according to the manufacturer's instructions by using SuperScript III (Invitrogen) and random primers (Invitrogen). The concomitant COX-1 and COX-2 gene expression analyses were done with real-time qPCR using specific TaqMan® Gene Expression Assays (Applied Biosystems, Foster City, CA, USA). All assays were run with 7500 FAST Real-Time PCR System (Applied Biosystems). PCR results were analysed with the relative quantification method[34] describing the change in expression of the target gene in relation to a reference, which was the medium-only control. The amount of the target gene was also normalised to an internal control gene, a ribosomal large protein (RPLP0)[34].

Polydextrose measurements

The amounts of PDX excreted in the faeces were measured using the HPLC method as described previously[12].

Statistical analysis

The data from the crossover study were analysed with linear mixed-effects models in a repeated-measures manner assuming no carry-over effect, having a random effect for the subject accounting for repeated measures, and fixed effects for the order of treatments (first PDX, second placebo or vice versa) and for the treatment (placebo, PDX, washout or baseline), and finally having a baseline regression coefficient accounting for individual baseline differences between the subjects. Based on the residual analysis, some of the original variables were log-transformed. The linear model analyses were conducted with statistical programming language R (version 2.11.1; R-Core, Auckland, New Zealand) using the non-linear mixed-effects package (version 3.1-97; R-Core, Auckland, New Zealand).

Data for DGGE indices were analysed by using one-way ANOVA and paired Student's *t* test using GraphPad Prism 5·0 (GraphPad Software, La Jolla, CA, USA).

Results

Overall, thirty-three participants entered into this crossover study (sixteen female, seventeen male), two dropped out (both male) – due to moving from the local area. Therefore, a total of thirty-one volunteers (sixteen female, fifteen male) aged 22–52 years (average age 33 years) with an average BMI of 24·1 (SD 2·8) kg/m^2 completed the study.

Faecal microbiota

A primary objective of the study was to assess the impact of PDX on the intestinal microbiota. 16S rRNA-based FISH probes were used to target predominantly significant groups of human faecal bacteria (Table 1). Bacterial numbers at the end of each treatment period – either 8 g/d PDX or 8 g/d placebo – were compared to samples obtained before the respective treatment, and the results between the two treatment groups were also compared. Average faecal bacterial numbers for the volunteers, as determined by FISH, and expressed as $\log_{10}$ cells/g faeces ± standard deviations (*n* 31) are shown in Table 3. The FISH results for PDX supplementation showed significantly decreased levels of *C. histolyticum* group from baseline levels ($P<0{\cdot}05$). PDX treatment was, furthermore, associated with a trend of reduced levels of *C. histolyticum* group ($P=0{\cdot}0525$) as compared to the placebo treatment. Both PDX treatment and placebo were found to reduce the levels of lactobacilli/enterococci in relation to their respective baseline values (both $P<0{\cdot}001$). In addition, the placebo increased the bacterial numbers within the *Eubacterium rectale–C. coccoides* group ($P<0{\cdot}01$, in comparison to baseline); a trend of increased levels for this group was also observed after PDX treatment ($P=0{\cdot}0593$). No other significant changes in bacterial population number determined by FISH were observed following the supplementations.

qPCR analyses were conducted to assess the effects of PDX on selected pathogenic and commensal bacterial groups and species (Table 4). Bacterial cell numbers of *C. histolyticum*

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Table 3. Bacteriology of faecal samples determined by fluorescence *in situ* hybridisation in the placebo-controlled, double-blind, crossover, human feeding study investigating the effects of polydextrose (PDX; 8 g/d), as compared to the placebo (8 g/d), on the human faecal microbiota of a healthy adult population‡

(Mean cell numbers (log_{10}) and standard deviations, *n* 31 volunteers)

	PDX				Placebo			
	Baseline		Treatment		Baseline		Treatment	
	Mean cell number (log_{10})	SD	Mean cell number (log_{10})	SD	Mean cell number (log_{10})	SD	Mean cell number (log_{10})	SD
Total bacteria	10·91	0·42	10·89	0·30	10·91	0·27	10·90	0·27
Atopobium group	9·74	0·39	9·78	0·42	9·75	0·25	9·75	0·37
Bacteroides spp.	9·89	0·42	9·84	0·36	9·92	0·33	9·98	0·38
Bifidobacterium spp.	9·03	0·79	9·13	0·72	9·18	0·81	8·99	0·88
Eubacterium rectale/Clostridium coccoides group	10·11	0·31	10·16	0·25	10·15	0·25	10·19††	0·26
Clostridium histolyticum group	9·34	0·36	9·12†	0·36	9·4	0·28	9·25	0·28
Lactobacillus/Enterococcus spp.	9·18	0·39	9·16 ***	0·42	9·25	0·42	9·23†††	0·53
Faecalibacterium prausnitzii	9·77	0·28	9·7	0·25	9·81	0·28	9·74	0·27

***Mean cell numbers were significantly different between PDX and placebo treatments ($P<0·001$).
Mean cell numbers were significantly different between (PDX baseline *v.* PDX treatment or placebo baseline *v.* placebo treatment): †$P<0·05$, ††$P<0·01$, †††$P<0·001$.
‡ The aforementioned treatments were consumed in either order, unknown to the investigator and the volunteer.

(clusters I and II) group increased after PDX treatment in comparison to baseline ($P<0·01$) and to placebo ($P<0·01$), which was opposite to the effect measured with FISH. However, the species-specific assay for *C. perfringens* led to no detection within the samples. Faecal counts of *R. intestinalis* were significantly higher after PDX intervention than after the placebo treatment ($P<0·05$), and similarly, numbers of the *C. leptum* (cluster IV) were shown to increase with PDX treatment in comparison to baseline values ($P<0·001$), as well as compared to placebo treatment ($P<0·001$). *Atopobium* numbers remained unchanged following both the PDX and placebo treatments, as measured by both qPCR and FISH.

D. intestinalis was detected in the faecal samples of twenty-one subjects, of which eleven subjects had *D. intestinalis* in all their samples. PDX treatment did not seem to affect *D. intestinalis* numbers.

S. aureus was not detected in faecal samples collected from fourteen volunteers, while it was detected in twelve subjects at only one time point, and in one subject at all time points. *S. aureus* was detected in five samples at the PDX baseline, in five samples at PDX treatment, and also in five at PDX washout, two subjects had positive samples for all three of their PDX samples (baseline, treatment and washout); thus, no effects were seen due to PDX supplementation. In the placebo group, there were six positive samples at baseline, four after treatment and seven after the washout period.

The *C. difficile* assay showed only one positive sample, which was in the first washout. Hence, no analyses could be performed on these data.

PCR-denaturing gradient gel electrophoresis and cluster analysis

In order to ascertain if PDX and/or placebo had an influence on the diversity of faecal microbiota, PCR-DGGE cluster analysis

Table 4. Quantitative PCR results expressed as mean cell numbers (log_{10}) and standard deviation per g faeces

	Polydextrose				Placebo			
	Baseline		Treatment		Baseline		Treatment	
	Mean cell number (log_{10})	SD	Mean cell number (log_{10})	SD	Mean cell number (log_{10})	SD	Mean cell number (log_{10})	SD
Atopobium spp.	8·6	0·6	9·5	0·6	8·7	0·6	9·1	0·6
Clostridium difficile‡	–	–	–	–	4·6	–	–	–
Clostridium leptum gp	8·5	0·8	9·2***†††	0·7	8·4	0·9	8·5	0·7
Clostridium clusters I and II (*histolyticum*)	5·1	0·7	5·6**††	1	5·3	0·9	5·1	0·7
Clostridium perfringens	–	–	–	–	–	–	–	–
Desulfovibrio intestinalis	7·2	0·9	7·8	0·8	7	0·9	7·4	0·8
Ruminococcus intestinalis	7·8	0·8	8·2*	0·7	7·8	0·9	7·9	0·9
Staphylococcus aureus§	5·7	0·2	6·1	0·4	5·6	0·5	5·5	0·6

Mean cell numbers were significantly different between PDX and placebo treatments: *$P<0·05$, **$P<0·01$, ***$P<0·001$.
Mean cell numbers were significantly different between treatment and respective baseline values (PDX baseline *v.* PDX treatment or placebo baseline *v.* placebo treatment: ††$P<0·01$, †††$P<0·001$.
‡ Data from one subject only.
§ Data from positive samples only.

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Table 5. Diversity index values calculated from denaturing gel gradient electrophoresis profiles during placebo-controlled, double-blind, crossover, human feeding study investigating the effects of polydextrose (PDX; 8 g/d), as compared to the placebo (8 g/d), on the human faecal microbiota of a healthy adult population*

(Mean values and standard deviations, *n* 31 volunteers)

	Simpson's		Shannon Weaver (log_{10})		Fisher's α	
	Mean	SD	Mean	SD	Mean	SD
Baseline	0·059†	0·03	1·349†	0·21	8·066†	3·72
PDX	0·054†	0·02	1·372†	0·2	8·191†	4·11
Placebo	0·039‡§	0·02	1·493‡§	0·18	10·091‡§	4·28

* The aforementioned treatments were consumed in either order, unknown to the investigator and the volunteer.
† Mean values were significantly different from those of placebo ($P<0·05$).
‡ Mean values were significantly different from those of baseline ($P<0·05$).
§ Mean values were significantly different from those of PDX ($P<0·05$).

was conducted. Dendrograms were constructed from DGGE profiles using Dice and unweighted pair-group method using arithmetic average. The values of diversity indices calculated from DGGE profiles are presented in Table 5. The DGGE data indices show that there were significant differences in the indices of the baseline profiles as compared to the placebo treatment ($P=0·008$ − Simpson's index) and the placebo treatment *v.* the PDX treatment ($P=0·014$ − Simpson's index).

SCFA concentrations

Table 6 shows the concentrations of faecal SCFA and branched chain fatty acids as determined by GC. No significant changes in faecal concentrations were observed after PDX or placebo treatment.

Analysis of bowel habits and gastrointestinal symptoms

From volunteer diaries, both the PDX and the placebo products were well tolerated (Table 7). However, less abdominal discomfort (on a scale none, mild, moderate to severe) and pain overall were recorded by the subjects while PDX was consumed (abdominal discomfort, none $P<0·05$). There was also a trend for less hard ($P=0·0719$) and more formed stools ($P<0·01$) during PDX consumption compared to placebo. Additionally, there was a trend for reduced snacking during the PDX period ($P=0·072$; Table 8).

Faecal IgA and PGE_2 levels

Following PDX treatment, the level of total faecal IgA was found to be lower (174 (SD 46) μg/g wet weight faeces) than after the placebo treatment (256 (SD 50) μg/g wet weight faeces; $P<0·05$). PGE_2 was not found to be different after PDX treatment (844 (SD 208) pg/g wet weight faeces) compared to placebo treatment (1116 (SD 315) pg/g wet weight faeces).

Determination of DNA damage by single cell gel electrophoresis (comet assay)

DNA-damaging potential towards the colonic epithelium exerted by the faecal water was analysed to assess the effect of the PDX and placebo treatment on exposure to genotoxins using the comet assay.

Genotoxicity data showed that the faecal water following PDX treatment led to significantly less genotoxic damage to HT29 DNA as compared to following the placebo treatment ($P<0·01$). No differences were observed compared to baseline values (Fig. 1).

Cyclo-oxygenase gene expression

Exposure of Caco-2 cells to faecal water collected after PDX treatment induced a 1·80 (SD 0·97)-fold increase in the relative mRNA levels in COX-1 and a 1·32 (SD 52)-fold increase in COX-2 expression in comparison to the reference medium (1·0). Following exposure to faecal water after placebo treatment, COX-1 expression increased 2·22 (SD 1·86)-fold and COX-2 expression increased 1·43 (SD 0·50)-fold. No statistically significant differences were seen between the treatments. Also, the COX-1:COX-2 ratio was not significantly altered between the treatments; 2·54 (SD 5·92) and 2·31 (SD 4·39) after PDX and placebo, respectively.

Table 6. Volatile fatty acid profiles (mmol/g faeces) determined by GC from placebo-controlled, double-blind, crossover, human feeding study investigating the effects of polydextrose (PDX), as compared to the placebo (8 g/d), on the human faecal microbiota of a healthy adult population*

(Mean values and standard deviations, *n* 31 volunteers)

	PDX				Placebo			
	Baseline		Treatment		Baseline		Treatment	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Acetic acid	29·5	14·6	23·9	15·3	25·6	14·4	24·7	14·7
Propionic acid	10·1	4·4	8·9	5·2	9·4	4·8	9·8	5·3
Iso-butyric acid	1·2	0·8	0·9	0·4	1·0	0·5	1·1	0·4
n-Butyric acid	9·3	7·1	6·5	5·2	6·5	4·7	6·5	5·8
Iso-valeric acid	1·7	1·4	1·3	0·6	1·4	0·7	1·5	0·7
n-Caproid acid	1·0	0·9	0·9	1·0	0·9	1·0	0·5	0·4

* The aforementioned treatments were consumed in either order, unknown to the investigator and the volunteer.

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Table 7. Volunteer response diary data; changes in volunteer responses during the feeding study
(Mean values and standard deviations)

		Percentage of occurrence														
		Consistency			Abdominal discomfort				Bloating				Flatulence			
	Stools/d number	Hard	Formed	Soft	None	Mild	Moderate	Severe	None	Mild	Moderate	Severe	None	Mild	Moderate	Severe
Placebo																
Mean	1·5	12·7	57·2	30·1	70·4	21·4	6·6	1·7	68·8	23·6	6·5	1·2	45	36·4	14·7	3·8
SD	0·4	19·5	21·5	22·3	30·8	23·0	14·9	6·2	34·5	26·7	13·0	5·0	33·9	26·9	19·4	8·0
PDX																
Mean	1·3	7·8	69·7**	22·5	83·5*	13·4	2·5	0·5	76·4	17·3	4·9	1·3	40·9	43·7	11·7	3·8
SD	0·4	12·4	23·0	19·3	23·9	17·8	8·7	2·8	23·6	18·2	10·3	4·2	32·0	26·0	14·9	7·6

PDX, polydextrose.
Mean values were significantly different between PDX and placebo treatments: *$P<0·05$, **$P<0·01$

Faecal polydextrose

Faecal PDX excretion was found to be significantly higher after the PDX treatment compared to the placebo treatment and baseline values (8·05 (SD 4·47) *v.* 0·25 (SD 0·96) *v.* 0·05 (SD0·23) g/kg faeces, respectively; $P<0·0001$).

Discussion

To date, few studies have been conducted investigating the prebiotic effects of PDX in human intervention studies. The present crossover study aimed to assess the effects of such supplementation in a European-based population.

Increased faecal PDX concentrations were detected after PDX intervention, thus demonstrating that PDX was not completely fermented by the colonic microbes and some PDX reached the distal part of the large intestine. Assuming a faecal output of approximately 100 g[22], 0·8 g (or 10 %) of the consumed PDX (8 g) was excreted in the faeces. A slow but constant fermentation of PDX has been demonstrated previously[35,36], and this feature could provide benefits for the health of the distal colon, as a substrate for the saccharolytic type fermentation is constantly available. Increased proteolytic fermentation in the distal part of the colon can contribute towards an increased risk of genotoxicity[35]; thus, increasing saccharolytic fermentation at the expense of proteolytic microbial metabolism should therefore benefit the health of the colon[37].

Decreased genotoxic effects of the faecal water on colonocytes after PDX consumption were demonstrated in this trial. The induction of DNA damage and mutations, brought about by a range of environmental (dietary) genotoxins, are key factors in the early stages of carcinogenesis initiation. Thus, this study provides evidence that PDX may aid in the reduction of such genotoxic events. The cytotoxic effects of faecal water are considered to reflect the ability of these genotoxins to damage the colonic epithelium, resulting in a hyperproliferative response characteristic of early stages of tumour promotion in the colon[38]. The *COX-1* gene is expressed constitutively in the gastrointestinal tract, whereas the *COX-2* gene is over-expressed in conditions such as gastrointestinal inflammation and cancerous diseases[39]. No effects of the PDX faecal water on *COX* gene expression in Caco-2 colon cancer cells were demonstrated.

Analysis of selected members of the intestinal microbiota during this double-blind, placebo-controlled, randomised crossover study demonstrated that PDX fermentation leads to modulations of the colonic microbiota. Decreased numbers of faecal *C. histolyticum* (clusters I and II) and lactobacilli/enterococci were measured with FISH, but the levels of *Bifidobacterium, Bacteroides* and *Atopobium* genera remained unchanged. According to qPCR analysis, however, PDX increased numbers of the *Clostridium* clusters I, II and IV and *R. intestinalis*. *C. leptum* cluster IV and *E. rectale–C. coccoides* group which includes important butyrate-producing microbes, such as *Faecalibacterium prausnitzii* and *R. intestinalis,* and faecal levels of these microbes have been correlated to faecal butyrate concentrations[38,40]. However, in the present study, no concomitant change in faecal butyrate levels, or in any other SCFA, was observed. The major part of research on prebiotic effects has, to date, mostly focused on the bifidogenic (and lactobacilli-increasing) properties of the candidate products, whereas in the future it may be worth investigating the impact of dietary intervention

Table 8. Volunteer response diary data; changes in volunteer responses during the feeding study*
(Mean values and standard deviations)

	Hungry		Full		Desire to eat		Snack appetite		Contented		Irritability		Sleepy		Alert		Ability to concentrate	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Placebo	4·75	2·98	4·67	2·50	4·71	3·18	5·17	3·40	6·58	1·71	3·13	2·72	4·17	2·76	6·29	2·39	6·54	2·40
Polydextrose	4·09	2·57	5·17	2·72	3·71	2·58	3·57	2·59	6·73	2·12	2·43	2·02	3·39	2·78	5·65	2·81	6·43	2·39

*On a scale from 0 to 10; 0 being not at all; 10 being very.

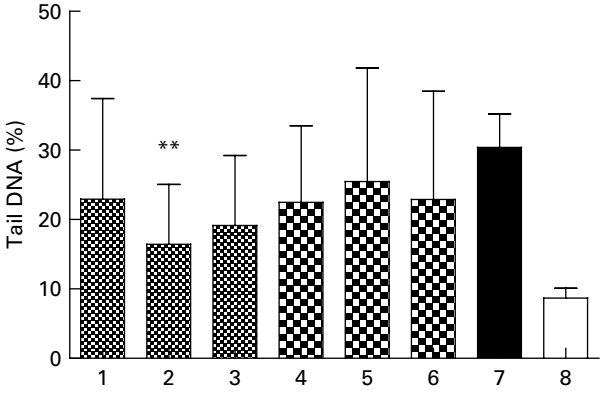

Fig. 1. Changes in faecal water genotoxicity, as determined by DNA tail length following the comet assay. Values are means and standard deviations represented by vertical bars, *n* 11 volunteers. (1) Pre-polydextrose (PDX) treatment; (2) PDX treatment; (3) post-PDX washout; (4) pre-placebo treatment; (5) placebo treatment; (6) post-placebo washout; (7) positive control – H_2O_2; (8) negative control – PBS. ** Mean values were significantly different to placebo treatment ($P<0{\cdot}01$).

on other genera, such as *Roseburia, Feacalibacterium* and *Eubacterium*[7].

Sulphide, produced by sulphate-reducing bacteria, is toxic to colonic epithelial cells, and may counteract the beneficial effects of butyrate[41]. *D. intestinalis*, a sulphate-reducing bacterium, was not affected by PDX consumption; neither were the levels of potentially pathogenic organisms such as *C. difficile, C. perfringens* and *S. aureus*. These microbes were mostly not detected or detected only in part of the samples, indicating that they are either not present or present at such low concentrations that they are below the detection limits of the qPCR assays.

Decreasing numbers of the *C. histolyticum* (clusters I and II) upon PDX treatment were detected by FISH; however, the same clusters were significantly increased according to qPCR analysis. This contradiction in results obtained with different methods has also been reported in previous studies[42,43]. The difference could be due to variability in rRNA operon copy numbers in species covered by the probe; hence, amplification in PCR will be influenced by this variation. Changes in bacterial levels within the group may affect the number outcome differently; for example, it is known that *C. histolyticum* possesses 10 rRNA copies[44], whereas *C. beijerinckii* possesses thirteen copies – both species are targeted by primers for the *Clostridium* I and II qPCR assay. Thus, with the present probe, the FISH analysis may be more accurate. *C. perfringens* was not detected in the samples (analysed by species-specific qPCR), indicating that PDX treatment does not effectively increase this potentially pathogenic microbe to detectable numbers but instead increases other members of the *Clostridium* clusters I and II, therefore demonstrating an encouraging outcome of PDX treatment.

Comparison of the microbial changes observed in this study to the previously published study of Jie *et al.*[15] has shown some differences in the effects; they observed that PDX led to dose-dependent increases in bifidobacteria and lactobacilli and decreased three *Bacteroides* species. However, it should be considered that the research of Jie *et al.* was performed in a parallel study design and used the less quantifiably accurate plating techniques. In the present study, the numbers of *Bifidobacterium* and *Bacteroides* remained unchanged, while those of *Lactobacillus–Enterococcus* decreased. This reduction was modest, but statistically significant. The biological relevance, however, would need to be determined. These differences could derive from the variation of responses between the two different study populations (Chinese *v.* European), and the different methods used in enumeration of the microbes. An inverse correlation between the initial levels of bifidobacteria and the magnitude of prebiotic bifidogenicity has been demonstrated for some prebiotic substrates, such as fructo-oligosaccharides[45] and galacto-oligosaccharides[28], where high initial levels of bifidobacteria tended to remain unchanged after prebiotic intervention. In this study, the baseline levels of bifidobacteria, as measured using FISH-technique, were above $\log_{10}$ 9·0 cells/g and no effects on bifidobacteria were seen.

Previously, fermentation of PDX by colonic bacteria has been shown to increase the production of SCFA in *in vitro* models[13,36], in rats[20], pigs[19] and in human subjects[15]. In particular, the levels of butyrate and acetate have been highly elevated in the previous studies due to PDX fermentation. Such changes were, however, not observed in the present study.

Faecal immune markers IgA and PGE_2[46] were also measured and decreased levels of IgA were demonstrated after PDX consumption. In previous studies, increases in the intestinal immune parameters such as IgA and PGE_2 levels have been measured after prebiotic and probiotic consumption[46], but also unchanged[26,47] and decreased levels have been reported[26]. It has been reported that many factors including stress and exercise can impact IgA levels; therefore discrepancies in changes may be a result of this.

In the present study, the placebo, maltodextrin, was seen to have an effect on the diversity of the faecal microbiota, leading to increased diversity – the same effect was not observed for PDX. The increase in diversity was relatively small. Little information is available on increased microbial diversity; however, the research of Scanlan *et al.*[44] associated increased diversity with colonic polyps and cancer formation. However, the absence of a change in genotoxicity in this incidence was not the case. Further knowledge of the bacterial groups involved in this increased diversity would help to clarify these findings.

The effects of PDX consumption on the quality of life and bowel habits of the subjects were also assessed during the feeding trial. PDX did not influence the measured aspects of quality of life (hunger, motivation, mood and mental alertness), although a trend towards reduced snacking was observed. This agrees with previous findings in another human feeding study, where PDX was shown to increase post-prandial satiety[48]. However, the effects on bowel habits were more profound, as less abdominal discomfort (none on a scale none, mild, moderate, severe) was recorded by the subjects while the PDX was consumed. There was also a trend towards less hard and more formed stools during PDX consumption.

Previously, an *in vitro* study showed that PDX fermentation led to less gas production compared to the prebiotics tested (fructo-oligosaccharides, galacto-oligosaccharides and inulin)[49]. Furthermore, the members of *Clostridium* cluster IV are *Ruminococcus*, *Eubacterium* and *Faecalibacterium* that are butyrate-producing commensals of which, in particular the latter has been suggested to be beneficial. Minimal gas production may explain the sensation of less abdominal pain by subjects during the feeding trial. Improved stool consistency (together with shortened oro-faecal transit time) has also been previously demonstrated in constipated subjects[22], and indicates the fibre-like properties of PDX.

PDX led to a significant decline in faecal water genotoxicity of volunteers following consumption. This shows the potential of PDX for reducing the risk factors that may be associated with colorectal cancer. This change coincides with increases in the numbers of a bacterial population that includes known butyrate producers. However, further investigation would be required to determine the activities of genes involved in the butyrate formation[50].

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