British Journal of Nutrition (2010), **104**, 701–708
doi:10.1017/S0007114510001078

A human volunteer study to assess the impact of confectionery sweeteners on the gut microbiota composition

Emma Beards, Kieran Tuohy* and Glenn Gibson

Food Microbial Sciences Unit, Department of Food Biosciences, The University of Reading, Reading RG6 6AP, UK

(Received 22 September 2009 – Revised 3 March 2010 – Accepted 5 March 2010 – First published online 7 April 2010)

Sweeteners are being sourced to lower the energetic value of confectionery including chocolates. Some, especially non-digestible carbohydrates, may possess other benefits for human health upon their fermentation by the colonic microbiota. The present study assessed non-digestible carbohydrate sweeteners, selected for use in low-energy chocolates, for their ability to beneficially modulate faecal bacterial profiles in human volunteers. Forty volunteers consumed a test chocolate (low-energy or experimental chocolate) containing 22·8 g of maltitol (MTL), MTL and polydextrose (PDX), or MTL and resistant starch for fourteen consecutive days. The dose of the test chocolates was doubled every 2 weeks over a 6-week period. Numbers of faecal bifidobacteria significantly increased with all the three test treatments. Chocolate containing the PDX blend also significantly increased faecal lactobacilli ($P=0{\cdot}00\,001$) after the 6 weeks. The PDX blend also showed significant increases in faecal propionate and butyrate ($P=0{\cdot}002$ and 0·006, respectively). All the test chocolates were well tolerated with no significant change in bowel habit or intestinal symptoms even at a daily dose of 45·6 g of non-digestible carbohydrate sweetener. This is of importance not only for giving manufacturers a sugar replacement that can reduce energetic content, but also for providing a well-tolerated means of delivering high levels of non-digestible carbohydrates into the colon, bringing about improvements in the biomarkers of gut health.

Chocolate: Microbiota: Prebiotics: Sweeteners: Polydextrose

With obesity becoming a global epidemic, it is now more apparent than ever that we must make more informed decisions about the food we eat. Energy-dense and nutrient-poor foods which are high in salt, sugar and saturated fats, combined with a lack of exercise, have led to obesity levels rising more than threefold since 1980[1]. With obesity come major, life-altering health and financial consequences. People who are obese and overweight are at a higher risk of developing CHD, type 2 diabetes and cancer and are predisposed to other chronic diseases, and health care costs for those who are obese are increasing. Until recently, the confectionery-manufacturing industry has largely used sucrose, but this has been impacted by the growing concern over health and diet. Manufacturers of these products are now producing lower energy alternatives using non-sucrose sweeteners in place of sucrose. There have been many human and animal studies looking into the correlation between sugar intake and the metabolic syndrome[2,3]. The metabolic syndrome is a combination of factors, such as weight, age or genetics, that increases the risk of CVD and diabetes. A recent study[4] in rats not genetically susceptible to diabetes has found that high level of sucrose intake (300 g/l of sucrose water for 42 weeks) significantly increased body weight and glucose intolerance compared with control animals. A further study with rats looked at the effects that a high-fat/sucrose diet had on the risk factors leading to atherosclerosis. After 2 years on the high-fat/sucrose diet, the rats were obese, hypertensive, hyperinsulinaemic and hypertriglyceridaemic compared with their counterparts on a low-fat complex carbohydrate diet[5].

The sugar substitutes used most frequently by the confectionery industry are bulk sweeteners. This group of sweeteners contains primarily sugar alcohols, which are not broken down in the stomach or small intestine, and non-digestible carbohydrates, which can be used in foods at similar levels to that of sucrose[6]. The term non-digestible in this context refers to the food ingredients being undigested in the upper gut with a large portion remaining for fermentation by the indigenous microbiota of the large intestine. Maltitol (MTL) is an example of a bulk sweetener, while polydextrose (PDX) and resistant starch (RS) are bulking agents, which can substitute for the texture properties of sucrose in confectionery products. All have been found to be fermented by the indigenous bacteria of the colon[7–10]. The human gut microbiota, comprising over 1000 different bacterial species, impacts greatly on host health and well-being[11–14]. Certain bacteria, namely bifidobacteria and lactobacilli, are regarded as health promoting, interacting with the host immune system, cells of the intestinal mucosa and other members of the gut microbiota, and play a role in immune homoeostasis and ability to fight off infections, mucosal integrity, production of vitamins, beneficial fats and other metabolites used by the host, and together with other members of the

Abbreviations: MTL, maltitol; PDX, polydextrose; RS, resistant starch.

* **Corresponding author:** Kieran Tuohy, fax +44 118 931 0080, email k.m.tuohy@reading.ac.uk

commensal microbiota, in health, mount an effective barrier to invading pathogenic micro-organisms. Through saccharolytic fermentation, the microbiota is able to salvage energy for the host from foods that remain largely undigested in the upper gut, mainly producing the SCFA acetate, propionate and butyrate. Up to 95 % of the SCFA produced can be taken up and utilised by the host[15]. Once absorbed, they can be metabolised by the cells of the colonic epithelium and other extra-intestinal tissues[16,17]. Acetate and propionate provide energy for the heart, brain and muscle, while 50 % of the daily energy requirements of the gastrointestinal mucosa comes from butyrate[18,19]. SCFA are also involved in blood cholesterol and lipid regulation[20,21]. Studies have also shown that these SCFA may have a positive effect on human gastrointestinal health and diseases, for example, colon cancer, inflammatory bowel disease and gastrointestinal infections[22–25]. However, imbalances in the composition of the gut microbiota have been observed in association with a number of the same chronic human diseases, and more recently, in metabolic diseases such as diabetes and obesity. The European Union-supported Concerted Action PASS-CLAIM (QLK1-2000-00 086) defined a healthy or balanced intestinal microbiota as one that 'is predominantly saccharolytic and comprises significant numbers of bifidobacteria and lactobacilli'. While most non-digestible carbohydrates which reach the colon to some extent contribute to saccharolytic fermentation by the resident microbiota, fermentation of certain non-digestible carbohydrates results in elevated numbers of bifidobacteria and/or lactobacilli. Dietary prebiotics, recently defined as 'selectively fermented ingredient(s) that result(s) in specific changes in the composition and/or activity of the gastrointestinal microbiota, thus conferring benefit(s) upon host health'[26], have been repeatedly shown to bring about elevated numbers of faecal bifidobacteria in human feeding studies[27–29]. Many of the sugar replacements used in the confectionery industry are non-digestible in the upper gut, and therefore have the potential to be prebiotics. What may become problematic for consumers is that overconsumption of certain prebiotics has been reported to result in unwanted intestinal side effects such as increased flatulence or intestinal bloating or pain. Doses of about 15 g/d of fructo-oligosaccharides and inulin have been shown to induce laxation effects, increased stool frequency and gas production[30].

In the present study, we have measured the microbiota modulatory potential and intestinal tolerance of chocolate containing blends of sugar replacers (in place of sucrose) likely to be used in manufacturing low-energy confectionery, and compared that to traditional sucrose chocolate. To date, there are no data on the effects these sugar replacers may exert on the gut microbiota *in vivo*. The aim of the present study was therefore to assess the potential prebiotic supplementation of chocolate to selectively increase numbers of beneficial faecal bacteria, and to measure the tolerability of high level (45·6 g sugar replacer/d) consumption of this low-energy, high-non-digestible carbohydrate chocolate. For this, a placebo-controlled, double-blinded, dose–response human feeding study was conducted using forty healthy human volunteers in a parallel manner. Volunteer diaries were employed to monitor changes in bowel habit and gastrointestinal discomfort.

Materials and methods

Experimental design

Treatments. Placebo chocolate was a normal milk chocolate bar containing sucrose. Each 50 g of treatment chocolate was supplemented with 22·8 g of MTL, MTL and PDX, or MTL and RS in place of sucrose. Chocolate was supplied by Cadburys (Bournville, Birmingham, UK) and the number was coded so as to be blind to investigator and volunteers. Volunteers were split randomly into four groups, and they consumed either the placebo or one of the treatment chocolates in a parallel manner. The treatment was delivered as 50 g of chocolate consumed daily for 14 d, followed by 75 g for a further 14 d and 100 g for the final 14 d. This corresponded to a dose of non-sucrose sweetener of 22·8, 34·2 and 45·6 g for the three 2-week periods, respectively. Volunteers were free to consume the chocolate at any time of the day. The power of the study as calculated by MINITAB (University of Reading) was 0·71.

Outline of study protocol. Forty healthy volunteers (twenty-seven females and thirteen males) with an average age of 33 and average BMI of 22·7 kg/m^2 participated in the study. Written consent was obtained from each individual, and the study was approved by the ethics committee of the University of Reading. Test chocolates were administered to volunteers at the beginning of each 14 d period. Volunteers were asked to keep diaries while consuming the chocolate to record stool frequency, consistency, abdominal pain, intestinal bloating and gas on a daily basis. Any concomitant medication, adverse events or volunteer comments were also recorded.

Pre-trial assessment. Volunteers were assessed for good health, and were selected on the basis of adherence to inclusion and exclusion criteria. A pre-treatment stool sample was taken on day 0.

Dose period 1 (days 1–14). Volunteers consumed 50 g of their randomly assigned treatment chocolate, and kept daily diaries during the period. A stool sample was collected on day 15. There was no significant difference in age, BMI or sex between the three treatment groups.

Dose period 2 (days 15–29). Volunteers consumed 75 g of their treatment chocolate, and kept daily diaries throughout the period. A stool sample was collected on day 30.

Dose period 3 (days 30–44). Volunteers consumed 100 g of their treatment chocolate, and kept daily diaries during the period. A stool sample was taken on day 45.

Inclusion and exclusion criteria

Inclusion criteria. The volunteer inclusion criteria were a signed consent form, age 20–40 years inclusive, BMI 18·5–24·9 kg/m^2 inclusive and good general health as determined by a medical questionnaire.

Exclusion criteria. The volunteer exclusion criteria were any requirement to take long-term medication, especially those active on the gastrointestinal tract or for CVD, use of antibiotics within the previous 6 months, a history of alcohol or drug abuse, pregnancy or lactation or planning pregnancy, involvement in a study involving an experimental drug/medication within 4 weeks before entry into the study, history of chronic constipation or diarrhoea, or other chronic

British Journal of Nutrition

gastrointestinal complaints (e.g. irritable bowel syndrome), high cholesterol (as determined by medical questionnaire and use of cholesterol-lowering drugs/functional foods) and intake of other specific prebiotics or probiotics, drugs active on gastrointestinal motility or a laxative of any class within the 4 weeks before the start of the study. Those suffering with diabetes and those having any allergies for nuts, etc. were also not included in the study.

Sucrose replacers

The sugar alcohol MTL (Maltisorb P200; Roquette, Northampton, UK) was used singularly and as the base for each blend. It is commonly used in confectionery because of its high sweetness (approximately 90 % as sweet as sucrose). In ileostomy volunteers, 70 % of MTL reaches the colon intact[31]. MTL was blended with PDX (Litesse ultra; Danisco, Redhill, UK) and RS (Nutriose FB06; Roquette). PDX is a soluble fibre commonly used as a sugar replacer due to its low energy content. From a study by Figdor & Bianchine[32], it was found that PDX can survive almost intact into the colon, where 50 % is fermented by the microbiota. RS is able to escape digestion, with 85 % reaching the colon[33]. The chocolate containing the sucrose replacers, and the placeto sucrose-containing chocolate, were thoroughly microbiologically tested for health and safety, and were cleared for commercial use. The RS is found naturally in foods, and has been used in the food industry for over 10 years. Both MTL and PDX are used commercially as sugar replacements. The laxation threshold for MTL is 60–90 g/d. One study compared its tolerance with that of sucrose in chocolate, and found that neither 30 nor 40 g of MTL caused a significantly greater laxation than sucrose[34]. According to the European Commission Scientific Committee for Food EC/SCF, PDX has a laxation threshold of 90 g/d[35]. For the final 2 weeks of the trial, the maximum amount of these additions being consumed was 45·6 g.

Bacterial enumeration

Freshly voided faecal samples were diluted 1 in 10 (w/v) with anaerobic PBS, and mixed in a stomacher for 2 min. Changes in faecal bacterial populations were assessed through the use of fluorescent *in situ* hybridisation with molecular probes targeting 16S rRNA. Genotypic probes targeting the predominant components of the gut microbiota were tagged with fluorescent markers such that quantifiable changes may be determined. The probes used were Bif164[36], Bac303[37], cHis150[38], Lab158[39], Ato291[40], Erec482[38], Fprau0645[41], Rbro730 and Rfla729[42] specific for bifidobacteria, bacteroides, clostridia (*Clostridium perfringens/histolyticum* subgroup), lactobacilli/enterococci, *Atopobium* spp., *Eubacterium rectale, Faecalibacterium prausnitzii*, *Ruminococcus bromii* and *Ruminococcus flavefaciens* group, respectively. The probes were commercially synthesised and 5′ labelled with the fluorescent dye Cy3. The nucleic acid stain 4′-6-diamidino-2-phenylindole was used for total bacterial counts. Samples were diluted and fixed overnight in 4 % (w/v) paraformaldehyde. The cells were then washed with PBS, resuspended and stored. The cell suspension was then added to a hybridisation mixture. Hybridisation was carried out at appropriate temperatures for the probes. Subsequently, probes were vacuum filtered, and the filter was mounted onto a microscope slide and examined using a fluorescent microscope.

SCFA analysis

Freshly voided faecal samples were diluted 1 in 10 (w/v) with anaerobic PBS, and mixed in a stomacher to blend for 2 min. Changes in SCFA were assessed using GC. Ethyl butyrate served as the internal standard. Acetic acid, propionic acid, butyric acid, isobutyric acid, isovaleric acid, *n*-valeric acid, *n*-caproic acid and lactic acid were run as external standards and calibrated. One millilitre of the diluted faecal sample was centrifuged at 18 000 ***g*** for 10 min. Supernatant was filtered through 0·2 μm filter into a new eppendorf tube. To a GC vial containing 400 μl of ethyl butyrate, 100 μl of filtered supernatant were added, and the cap was applied. This was stored at 4 °C until use. Samples of 1 μl were injected into a GC system (HP 5890 A; Hewlett Packard GmbH, Böblingen, Germany) equipped with a flame-ionisation detector and a capillary column (25 m × 0·23 mm) impregnated with 20 m Carbowax (Hewlett Packard GmbH). The carrier gas was He used at a column flow rate of 12 ml/min with a split ratio of 1:12. The column temperature was 125°C[43].

Statistics

Data were analysed using ANOVA (Minitab 15). Differences were considered significant at $P<0{\cdot}05$. Volunteer diaries were analysed using a *t* test and the Mann–Whitney *U* test for non-parametric analysis, and differences were considered significant at $P<0{\cdot}05$.

Results

Bacterial enumeration using fluorescent in situ *hybridisation*

The bacterial populations as enumerated using fluorescent *in situ* hybridisation are given in Table 1. Significant increases in bifidobacteria were observed with all the test treatments. An increase of between 0·4 and 0·5 $\log_{10}$ cells/g was observed with the MTL, PDX and RS blends ($P=0{\cdot}0006$, 0·0009 and 0·001, respectively), while the 0·2 $\log_{10}$ cells/g increase by the placebo was not statistically significant. Similarly, lactobacilli were increased significantly by the test treatments, but not by the placebo. The PDX blend had the largest increase of 1·1 $\log_{10}$ cells/g ($P=0{\cdot}00\,001$), while MTL and the RS blends had increases of 0·8 and 0·6 $\log_{10}$ cells/g, respectively ($P=0{\cdot}001$ and 0·004). Enumeration of the *Clostridium histolyticum/perfringens populations* showed significant increases in all groups. The changes were between 0·8 and 0·4 $\log_{10}$ cells/g, with the RS blend having the largest increase after 4 weeks ($P=0{\cdot}002$) and the PDX blend having the smallest increase ($P=0{\cdot}007$). Bacteroide numbers were left unchanged by the placebo, but they were significantly increased by the test groups. The MTL blend showed the greatest increase of 0·7 $\log_{10}$ cells/g ($P=0{\cdot}001$), while the PDX and RS blends showed increases of 0·3 and 0·5 $\log_{10}$ cells/g, respectively ($P=0{\cdot}003$ and 0·002). *F. prausnitzii*, a commensal bacterium found in the gut, is a major member of the Firmicutes. Recent research has shown *F. prausnitzii* populations

British Journal of Nutrition

Table 1. Changes in bacterial populations during the 6-week human volunteer trial*

	Bif	Bac	Clos	Lac	Erec	Ato	Rfla	Fprau	Rbro	Total
Control base	8·9	9·2	8·2	8·1	9·2	8·8	8·5	8·6	8·6	9·9
Control 2 weeks	9	9·2	8·7	8·2	9·3	8·9	9·2	9·2	9·2	10·2
Control 4 weeks	9·1	9·1	8·8	8·4	9·3	8·8	9·1	9·2	9·2	10·4
Control 6 weeks	9·1	9·2	8·8†	8·2	9·2	8·9	9·4†	9·3†	9·3†	10·5†
MTL base	8·9	8·7	8·2	8·3	8·9	8·5	8·8	8·4	8·5	9·9
MTL 2 weeks	9·1	9·1	8·7	8·8	9·1	8·7	9·1	9·1	9·1	10·2
MTL 4 weeks	9·2	9·3	8·6	9	9·4	8·8	9·2	9·3	9·3	10·4
MTL 6 weeks	9·4†	9·4†	8·7†	9·1†	9·5†	8·9†	9·3†	9·4†	9·3†	10·6†
PDX blend base	8·9	9	8·3	8·1	9	8·7	8·9	8·9	9	10
PDX blend 2 weeks	9·1	9·1	8·6	8·7	9·1	8·9	9	9	9	10·2
PDX blend 4 weeks	9·2	9·2	8·7	9·1	9·2	8·9	9·2	9·3	9·3	10·4
PDX blend 6 weeks	9·3†	9·3†	8·5†	9·2†	9·4†	8·9	9·3†	9·4†	9·3	10·5†
RS blend base	8·8	9·1	8·2	8·2	9·2	8·8	8·9	9·1	9	9·9
RS blend 2 weeks	9	9·3	8·7	8·6	9·3	8·9	9·2	9·2	9·1	10·3
RS blend 4 weeks	9·1	9·4	9	8·8	9·5	9	9·3	9·3	9·2	10·4
RS blend 6 weeks	9·2†	9·6†	8·9†	8·8†	9·6†	9	9·3†	9·4	9·2	10·5†
SEM	0·04	0·04	0·06	0·09	0·04	0·04	0·06	0·07	0·06	0·06

Bif, bifidobacteria, Bac, *Bacteroides*; Clos, *Clostridium*; Lac, lactobacilli; Erec, eubacteria; Ato, *Atopobium*; Fprau, *Fusobacterium prausnitzii*; Rfla, *Ruminococcus flavefaciens*; Rbro, *Ruminococcus bromii*; MLT, maltitol; PDX, polydextrose; RS, resistant starch.

* At 2 weeks, volunteers consumed either 49 g of control chocolate containing sucrose or 49 g of chocolate containing 22·8 g of supplemented substrates. At 4 weeks, supplemented substrate was increased to 34·2 g in 75 g chocolate, and at 6 weeks, it was increased to 45·6 g in 100 g chocolate. Data of bacterial enumeration using fluorescent *in situ* hybridisation are given. Results are mean $\log_{10}$ cells/g. Probes for Bif, Bac, Clos, Lac, Erec, Ato, Fprau, Rfla, Rbro and the total bacteria count are given below.

† A significant increase over the time period according to an ANOVA.

to be reduced in patients suffering from Crohn's disease[44]. Significant increases in *F. prausnitzii* populations were observed in all groups except in the RS blend group. The largest increase of 1 $\log_{10}$ cells/g was seen in the group that consumed MTL ($P=0{\cdot}0001$). *E. rectale*, *R. flavefaciens* and total bacteria also showed significant increases by all the test treatments, while *Atopobium* was increased significantly by MTL, and *R. bromii* populations showed significant increases with MTL and the placebo.

SCFA analysis

The SCFA measurements in faeces are given in Table 2. Acetate showed the largest increase of 17·6 mM ($P=0{\cdot}003$) in groups that consumed the RS blend. Significant increases were also seen with MTL and PDX blends, 10·3 and 13·6 mM, respectively ($P=0{\cdot}007$ and 0·005), but not with the placebo, which only increased acetate levels by 2·8 mM. The PDX blend showed the most significant increases in butyrate and propionate with 16·5 and 11·8 mM, respectively ($P=0{\cdot}002$ and 0·006), over the 6-week period. Significant increases of 9 mM in propionate were also observed over the trial in the MTL and RS blend groups ($P=0{\cdot}009$). With butyrate, all groups including the placebo group had significant increases as follows: 4·6 mM (placebo), 10·4 mM (RS blend) and 11 mM (MTL) ($P=0{\cdot}04$, 0·008 and 0·007).

Volunteer diaries

Table 3 summarises data on stool frequency, stool consistency and digestive tolerance (abdominal pain, bloating and gas) recorded by volunteers during the trial. Small increases in the amount of stools produced per person per 2-week period were seen for all groups, but these changes were NS. Stool consistency was graded by volunteers as hard, formed or soft. The placebo and MTL groups showed a change from soft to hard stools during the 6-week period, which was a change of approximately 11 %. The group that consumed the PDX blend showed a switch of 6 % from formed to soft stool, but the reports of hard stools were unchanged. Conversely, the group that consumed the RS blend showed the opposite effect of a 7 % change from soft to formed stool. The occurrence of abdominal pain, bloating and gas was also recorded daily by volunteers under the headings none, mild, moderate and severe. The largest changes for the group that consumed the placebo were a 10 % change in bloating from none to mild/moderate; however, decreases in the reports of moderate and severe gas over the trial led to increases in mild, although there was a 7 % decrease in volunteers recording no gas. The group that consumed MTL

Table 2. Changes in SCFA produced during 6-week human volunteer trial*

	Acetate	Propionate	Butyrate
Control base	14·7	2·4	2·3
Control 2 weeks	16·6	4·4	4·4
Control 4 weeks	17·8	5·1	6·6
Control 6 weeks	17·5	5·8	6·9†
MTL base	13	2·4	2·5
MTL 2 weeks	16·9	4·9	5·3
MTL 4 weeks	20·5	7·6	8·4
MTL 6 weeks	23·3†	11·3†	13·5†
PDX blend base	14·8	2·3	2·2
PDX blend 2 weeks	17·9	5·2	5·6
PDX blend 4 weeks	23·7	10·6	11·3
PDX blend 6 weeks	28·4†	14·1†	18·7†
RS blend base	14·7	2·5	2·4
RS blend 2 weeks	18·8	5·8	6·1
RS blend 4 weeks	22·1	9·1	9·4
RS blend 6 weeks	26·3†	11·5†	12·8†
SEM	1·11	0·93	1·19

MLT, maltitol; PDX, polydextrose; RS, resistant starch.

* Data of SCFA measurements using GC. Results are in mean mmol.

† Significant increase following fermentation using an ANOVA.

British Journal of Nutrition

British Journal of Nutrition

Table 3. Summary of data recorded from volunteer diaries during 6-week human volunteer trial*

	Frequency	Consistency			Pain			Bloating				Gas			
	Stools	Hard	Formed	Soft	None	Mild	Moderate	Severe	None	Mild	Moderate	Severe	None	Mild	Moderate	Severe
Control 2 weeks	12·6	17	49	34	63	32	5	0	71	24	5	0	47	33	15	5
Control 4 weeks	13·4	18	52	30	64	32	4	0	66	30	4	0	45	47	8	0
Control 6 weeks	13	30	48	22	62	29	8	1	61	32	7	0	40	47	11	2
MTL 2 weeks	12·8	9	47	44	68	20	10	2	43	33	15	9	25	43	19	13
MTL 4 weeks	13·5	21	44	35	64	18	13	5	54	29	6	11	27	37	18	18
MTL 6 weeks	13·4	18	49	33	63	19	13	5	55	24	14	7	33	36	21	10
PDX blend 2 weeks	12·7	4	63	33	75	21	4	0	56	36	8	0	28	51	20	1
PDX blend 4 weeks	13·2	2	52	46	74	22	3	1	73	19	7	1	19	48	27	6
PDX blend 6 weeks	13·5	4	57	39	65	33	1	1	67	29	4	0	20	39	31	10
RS blend 2 weeks	14·4	18	55	27	73	25	1	1	81	17	1	1	42	52	5	1
RS blend 4 weeks	14·5	18	54	28	86	13	1	0	76	22	2	0	36	50	12	2
RS blend 6 weeks	14·6	15	65	20	71	19	10	0	51	35	13	1	41	49	9	1

MLT, maltitol; PDX, polydextrose; RS, resistant starch.
* Stool frequency is given as total stools per person per 2-week period. Consistency, pain, bloating and gas are given as percentage of responses per group. According to *t* test and Mann–Whitney *U* test, changes were NS.

showed decreases of 9 % from mild, 1 % from moderate and 2 % from severe relating to a 12 % increase in no reporting of bloating. This was related to gas in those initially reporting mild decreasing 6 % to none and simultaneously in those initially reporting severe changing to moderate over the trial. Similar to the other groups, those who consumed the PDX blend also reported increases in pain from none to mild of 10 %, although one volunteer reported severe after 4 and 6 weeks of the treatment. There were decreases in bloating, but increases of gas of 11 % for moderate and 9 % for severe after the 6 weeks. The RS blend followed a similar pattern with 9 % increases in pain from none/mild to moderate. The largest change throughout the trial was seen in reports of bloating. A 30 % decrease in people reporting none led to an 18 % increase in mild and a 12 % increase in moderate. This was not mirrored by the gas results as they remained fairly consistent with 3–4 % change from mild to moderate. Although changes in bowel symptoms were reported throughout the trial, these were not statistically significant and were not of major concern to the volunteers as none withdrew from the trial for this reason.

Discussion

The choices that we make about food influence our health and well-being. In the growing light of the obesity epidemic, people are looking for healthier alternatives of the food they enjoy. Manufacturers, aware of consumer concern, are sourcing substitutes for their unhealthier ingredients, such as sucrose. Some of the sugar substitutes already available to the confectionery industry may have other health effects aside from reducing energetic value of the product. Some of these sugar replacements have been seen to reach the colon intact and be fermented by the gut microbiota. Although research has shown that these substrates individually can be fermented by the gut microbiota[7,45,46], there have been no studies looking at the effects these blends of sweeteners may induce. In addition, little is known about the tolerability of consumers to elevated levels of these sucrose replacers in terms of gas production, bloating or abdominal pain. Reports of increased flatulence and other intestinal symptoms with more than 20 g/d of rapidly fermented compounds such as lactulose or fructo-oligosaccharides have limited wider application of these low-energy products in foods.

The present study tested the microbiota modulatory ability and intestinal tolerability of three different test chocolate products, each containing a different blend of sucrose replacers, in forty healthy volunteers compared to those of a traditional sucrose-containing chocolate. Bacterial populations in stool samples collected before and after each treatment period were enumerated using fluorescent *in situ* hybridisation, and faecal SCFA were measured using GC. Volunteer diaries recorded any other effects such as abdominal pain, bloating and gas. All three of the low-energy chocolates containing the sucrose replacers significantly increased the numbers of faecal bifidobacteria as determined by fluorescent *in situ* hybridisation ($P=0{\cdot}0006$, 0·0009 and 0·001). These results concur with the previous studies, whereby RS increased bifidobacteria significantly ($P<0{\cdot}01$) in human flora-associated rats compared with sucrose[45]. A similar result was observed *in vitro* with 1 and 2 % PDX[7]; however,

after supplementation of 1 % PDX, the researchers found that 55 % of the glucose in the PDX was resistant to attack by gut bacteria. This agrees with the findings of Figdor & Bianchine[32] who observed that although PDX can survive almost intact into the colon, only 50 % is fermented by the indigenous microbiota. For this human trial, MTL and PDX were mixed in a 50:50 ratio where it is possible for only 50 % to be fermented, while MTL, although only 70 % reaches the colon[31], is fully fermented by the microbiota[47]. It could therefore be said that it is the MTL that is leading to the changes in bifidobacterial populations, as this can be seen from its individual results from the trial. Lactobacilli were seen to be significantly increased by all the test treatments, but most effectively by the PDX blend. MTL may also be playing a significant role in increases in the lactobacilli seen with the PDX blend. Increases in these two bacterial populations are important markers for assessing prebiotic activity. These bacteria are of importance for health benefits they bring as probiotics. Recent studies have found links between these two bacteria and the positive effects that they can have against obesity and its related diseases[48,49]. It could be said that all the test treatments show potential as prebiotics by increasing the numbers of both bacteria significantly. Populations of the *C. histolyticum/perfringens* group were also seen to increase significantly in all the test groups, but most prominently in the RS blend group. *C. perfringens* is a known gas producer, and this may explain the increase in incidents of bloating and gas reported by the volunteers in this treatment group. Increases in the population of bacteroides were also seen to increase. This may appear as a result of harmful effects due to their prevalence in patients suffering from ulcerative colitis[50]; however, recent studies have highlighted the role bacteroides may have against obesity. The researchers found that in obese human subjects and animals, the numbers of *Bacteroidetes*, which includes the genus bacteroides, are reduced in favour of *Firmicutes* [51–53].

Acetate, propionate and butyrate are the SCFA end products of bacterial carbohydrate fermentation. The SCFA were increased significantly by all the test treatments, with the RS blend being most effective at increasing acetate and the PDX blend being most effective at increasing propionate and butyrate. PDX generated the largest increases in bifidobacteria and lactobacilli, which are acetate and lactate producers. *F. prausnitzii* is a known butyrate producer, and studies have shown that it is able to do this by acetate utilisation[54]. In the present study, *F. prausnitzii* and *Roseburia* spp. were grown in the presence of acetate and glucose only, which resulted in 85–90 % butyrate production. In mixed culture studies, this would be seen as cross-feeding, whereby the end products from one bacterial species' fermentation provide substrates that can be fermented by another species, thus producing a different end product. The PDX blend was seen to increase the populations of *F. prausnitzii* significantly.

Ingestion of some prebiotics in large doses may induce unwanted side effects such as increased gas production, bloating and a laxative effect[55]. H_2 and CO_2 are the major gases produced in the colon by bacteria such as enterobacteria and clostridia. When dealing with ingredients that may have applications in the food industry, monitoring of any negative side effects is of importance. Volunteers were able to record their levels of abdominal pain, bloating and gas, which were graded as either none, mild, moderate or severe. The placebo group showed little change other than a 10 % increase in bloating from none to mild, which may be due to the amount of chocolate they were asked to consume in the final 14 d period. The MTL group showed positive effects with decreases in bloating and gas. Those groups that consumed the PDX and RS blends, however, did appear to encounter some negative effects. Volunteers who consumed the PDX blend reported increases in pain and gas by week 6, whereas those who consumed the RS blend reported increases in pain and a 30 % change from none to mild/moderate for bloating but no change in gas/flatulence after the 6 weeks, which may be indicative of its change in the *Clostridium* population. However, when looking at reports from the lower dosages after 2 and 4 weeks, it can be seen that the RS blend only sustained a 5 % change in bloating from none to mild, and at 4 weeks, the number of people reporting no abdominal pain increased by 13 %. This is also consistent with the PDX blend which showed that reports of no abdominal pain remain the same at the end of weeks 2 and 4, and that the recording of no bloating increased to 73 % at 4 weeks. From this, it can be said that an optimal dose of 34·2 g of the sweeteners in 75 g of chocolate kept gastrointestinal discomfort low. After 4 weeks, the PDX blend increased bifidobacterial populations by 0·3 $\log_{10}$ cells/g and lactobacilli by 1 $\log_{10}$ cells/g while also increasing the levels of acetate, propionate and butyrate by about 9 mM each.

With bifidobacteria, lactobacilli and the SCFA being the most prominent markers of prebiotic activity, the results from this trial indicate that the sugar substitute of PDX–MTL blended together, at an optimal dose of 34·2 g, may have a beneficial effect on the gut microbiota as well as may keep abdominal discomfort low and reduce the energetic value of confectionery products. These findings are of importance not only for the health effects for the consumers, but also for the financial implications for the manufacturers. Consumers are now being given a wider and more varied choice of products available to them to aid them in a healthier lifestyle. Manufacturers need to keep up with the demand through sourcing alternative ingredients that combine a range of benefits for the consumers. In the present study, it can be seen that at the optimal dose, the PDX blend could not only lower the energetic value of chocolate, but also provide prebiotic effects for the consumers.

Acknowledgements

The present work was supported by Cadburys, Birmingham, UK. The authors have declared no conflict of interest. All the work was carried out solely by E. B., and is part of a PhD project funded by Cadburys. The work was supervised by K. T. and G. G. who are employed directly by the University of Reading, and receive their research funding from government (research councils, European Union), charities and many food industries. The authors have received no additional funding from Cadbury's outside this PhD scholarship. In conclusion, none of the contents of the paper was affected.

References

1. World Health Organization (2008) Information sheet on obesity and overweight. http://www.who.int/dietphysicalactivity/media/en/gsfs_obesity.pdf
2. Stanhope KL & Havel PJ (2008) Endocrine and metabolic effects of consuming beverages sweetened with fructose, glucose, sucrose or high fructose corn syrup. *Am J Clin Nutr* **88**, 1733S–1737S.
3. Fried SK & Rao SP (2003) Sugars, hypertriglyceridemia and cardiovascular disease. *Am J Clin Nutr* **78**, 873S–880S.
4. Kawasaki T, Kashiwabara A, Sakai T, *et al.* (2005) Long term sucrose-drinking causes increased body weight and glucose intolerance in normal male rats. *Br J Nutr* **93**, 613–618.
5. Barnard RJ, Faria DJ, Menges JE, *et al.* (1993) Effects of a high-fat, sucrose diet on serum insulin and related atherosclerotic risk factors in rats. *Atherosclerosis* **100**, 229–236.
6. Mela DJ (1997) Impact of macronutrient-substituted foods on food choice and dietary intake. *Ann N Y Acad Sci* **819**, 96–107.
7. Probert HM, Apajalahti JHA, Rautonen N, *et al.* (2004) Polydextrose, lactitol and fructo-oligosaccharide fermentation by colonic bacteria in a three stage continuous culture system. *Appl Environ Microbiol* **70**, 4505–4511.
8. Ghoddusi HB, Grandison MA, Grandison AS, *et al.* (2007) *In vitro* study on gas generation and prebiotic effects of some carbohydrates and their mixtures. *Anaerobe* **13**, 193–199.
9. Arrigoni E, Brouns F & Amadò R (2005) Human gut microbiota does not ferment erythritol. *Br J Nutr* **94**, 643–646.
10. Tsukamura M, Goto H, Arisawa T, *et al.* (1998) Dietary maltitol decreases the incidence of 1,2-dimethylhydrazine induced cecum and proximal colon tumors in rats. *J Nutr* **128**, 536–540.
11. DiBaise JK, Zhang H, Crowell MD, *et al.* (2008) Gut microbiota and its possible relationship with obesity. *Mayo Clin Proc* **83**, 460–469.
12. Steer T, Carpenter H, Tuohy KM, *et al.* (2000) Perspectives on the role of the human gut microbiota in health and methods of study. *Nutr Res Rev* **13**, 229–254.
13. Egert M, de Graaf AA, Smidt H, *et al.* (2005) Beyond diversity: functional microbiomics of the human colon. *Trends Microbiol* **14**, 86–91.
14. Blaut M & Clavel T (2007) Metabolic diversity of the intestinal microbiota: implications for health and disease. *J Nutr* **137**, 751–755.
15. Cummings JH, Pomare EW, Branch WJ, *et al.* (1987) Short chain fatty acids in human large intestine, portal, hepatic and venous blood. *Gut* **28**, 1221–1227.
16. Cook SI & Sellin JH (1998) Review Article: short chain fatty acids in health and disease. *Aliment Pharmacol Ther* **12**, 499–507.
17. Hijova E & Chmelarova A (2007) Short chain fatty acids and colonic health. *Bratisl Lek Listy* **108**, 354–358.
18. Macfarlane GT & Gibson GR (1997) Carbohydrate fermentation, energy transduction and gas metabolism in the human large intestine. In *Gastrointestinal Microbiology*, pp. 269–318 [RI Mackie and BH White, editors]. London: Chapman & Hall.
19. Roediger WEW (1989) The utilisation of nutrients by isolated epithelial cells of the rat colon. *Gastroenterology* **83**, 424–429.
20. Demigné C, Morand C, Levrat MA, *et al.* (1995) Effect of propionate on fatty acid and cholesterol synthesis and on acetate metabolism in isolated rat hepatocytes. *Br J Nutr* **74**, 209–219.
21. Wolever T, Spadafora P & Eshuis H (1991) Interaction between colonic acetate and propionate in humans. *Am J Clin Nutr* **53**, 681–687.
22. Emenaker NJ, Calaf GM, Cox D, *et al.* (2001) Short chain fatty acids inhibit invasive human colon cancer by modulating Upa, TIMP-1, TIMP-2, mutant p53, Bcl-2, Bax, p21 and pcna protein expression in an *in vitro* cell culture model. *J Nutr* **131**, 3041–3046.
23. Hong FU, Ying Qiang SHO & Shan Jin MO (2002) Effect of short chain fatty acids on the human colonic adenocarcinoma cell line Caco-2. *Chin J Dig Dis* **5**, 115–117.
24. Mortensen PB & Clausen MR (1996) Short chain fatty acids in the human colon: relation to gastrointestinal health and disease. *Scand J Gastroenterol* **31**, 132–148.
25. Galvez J, Rodriquez-Cabezas ME & Zarzuelo A (2005) Effects of dietary fibre on inflammatory bowel disease. *Mol Nutr Food Res* **49**, 601–608.
26. Gibson GR, Probert HM, Van Loo JAE, *et al.* (2004) Dietary modulation of the human colonic microbiota: updating the concept of prebiotics. *Nutr Res Rev* **17**, 257–259.
27. Kolida S & Gibson GR (2007) Prebiotic capacity of inulin-type fructans. *J Nutr* **137**, S2503–S2506.
28. Kolida S, Tuohy KM & Gibson GR (2002) Prebiotic effects of inulin and oligofructose. *Br J Nutr* **87**, S193–S197.
29. Gibson GR (1999) Dietary modulation of the human gut microflora using prebiotics oligofructose and inulin. *J Nutr* **129**, 1438S–1441S.
30. Cummings JH, Macfarlane GT & Englyst HN (2001) Prebiotic digestion and fermentation. *Am J Clin Nutr* **73**, S415–S420.
31. Langkilde AM, Anderson H, Schweizer TF, *et al.* (1994) Digestion and absorption of sorbitol, maltitol and isomalt from the small bowel. A study in ileostomy subjects. *Eur J Clin Nutr* **48**, 768–775.
32. Figdor SK & Bianchine JR (1983) Caloric utilization of [^{14}C]polydextrose in man. *J Agric Food Chem* **31**, 389–393.
33. Silvester KR, Englyst HN & Cummings JH (1995) Ileal recovery of starch from whole diets containing resistant starch measured *in vitro* and fermentation of ileal effluent. *Am J Clin Nutr* **62**, 403–411.
34. Storey DM, Koutsou GA, Lee A, *et al.* (1998) Tolerance and breath hydrogen excretion following maltitol incorporated at two levels into milk chocolate consumed by healthy young adults with and without fasting. *J Nutr* **128**, 587–592.
35. Flood MT, Auerbach MH & Craig SAS (2004) A review of the clinical tolerance studies of polydextrose in food. *Food Chem Toxicol* **42**, 1531–1542.
36. Langendijk PS, Schut F, Jansen GJ, *et al.* (1995) Quantitative fluorescent *in situ* hybridisation of *Bifidobacterium* spp. with genus-specific rRNA-targeted probes and its application in faecal samples. *Appl Environ Microbiol* **61**, 3069–3075.
37. Manz W, Amann R, Ludwig W, *et al.* (1996) Application of a suite of 16S rRNA-specific oligonucleotide probes designed to investigate bacteria of the phylum cytophaga–flavobacter–bacteroides in the natural environment. *Microbiology* **142**, 1097–1106.
38. Franks AH, Harmsen HJ, Raangs GC, *et al.* (1998) Variations of bacterial populations in human faeces measured by fluorescent *in situ* hybridisation with group specific 16S rRNA-targeted oligonucleotide probes. *Appl Environ Microbiol* **64**, 3336–3345.
39. Harmsen HJM, Elfferich P, Schut F, *et al.* (1999) A 16S rRNA-targeted probe for detection of lactobacilli and enterococci in faecal samples by fluorescent *in situ* hybridization. *Microb Ecol Health Dis* **11**, 3–12.
40. Harmsen HJM, Wilderboer-Veloo ACM, Grijpstra J, *et al.* (2000) Development of 16S rRNA-based probes for the *Coriobacterium* group and the *Atopobium* cluster and their application for enumeration of *Coriobacteriaceae* in human feces from volunteers of different age groups. *Appl Environ Microbiol* **66**, 4523–4527.
41. Suau A, Rochet V, Sghir A, *et al.* (2001) *Fusobacterium prausnitzii* and related species represent a dominant group within the human faecal flora. *Syst Appl Microbiol* **24**, 139–145.
42. Harmsen HJM, Raangs GC, He T, *et al.* (2002) Extensive set of 16S rRNA-based probes for detection of bacteria from human faeces. *Appl Environ Microbiol* **68**, 2982–2990.

43. Zhao G, Nyman M & Jönsson JA (2006) Rapid determination of short chain fatty acids in colonic contents and faeces of humans and rats by acidified water-extraction and direct injection gas chromatography. *Biomed Chrom* **20**, 674–682.
44. Sokol H, Pigneur B, Watterlot L, *et al.* (2008) *Faecalibacterium prausnitzii* is an anti-inflammatory commensal bacterium identified by gut microbiota analysis of crohns disease patients. *Proc Natl Acad Sci U S A* **105**, 16731–16736.
45. Silvi S, Rumney A, Cresci A, *et al.* (1999) Resistant starch modifies gut microflora and microbial metabolism in human flora associated rats inoculated with faeces from Italian and UK donors. *J Appl Microbiol* **86**, 521–530.
46. Lesmes U, Beards E, Gibson GR, *et al.* (2008) Effects of resistant starch type III polymorphs on human colon microbiota and short chain fatty acids in human gut models. *J Agric Food Chem* **56**, 5415–5421.
47. Oku T, Akiba M, Lee MH, *et al.* (1991) Metabolic fate of ingested [^{14}C]-maltitol in man. *J Nutr Sci Vitaminol* **37**, 529–544.
48. Cani PD, Neyrinck AM, Fava F, *et al.* (2007) Selective increases of bifidobacteria and gut microflora improve high-fat-diet-induced diabetes in mice through a mechanism associated with endotoxemia. *Diabetologia* **50**, 2374–2383.
49. Lee HY, Park JH, Seok HJ, *et al.* (2006) Human originated bacteria, *Lactobacillus rhamnosus* PL60, produce conjugated linoleic acid and show anti-obesity effects in diet-induced obese mice. *Biochem Biophys Acta* **1761**, 736–744.
50. Lucke K, Miehlke S, Jacobs E, *et al.* (2006) Prevalence of *Bacteroides* and *Prevotella* spp. in ulcerative colitis. *J Med Microbiol* **55**, 617–624.
51. Ley RE, Bäckhed F, Turnbaugh PJ, *et al.* (2007) Obesity alters gut microbial ecology. *Proc Natl Acad Sci U S A* **102**, 11070–11075.
52. Ley RE, Turnbaugh PJ, Klein S, *et al.* (2006) Microbial ecology: human gut microbes associated with obesity. *Nature* **444**, 1022–1023.
53. Turnbaugh PJ, Ley RE, Mahowald MA, *et al.* (2006) An obesity-associated gut microbiome with increased capacity for energy harvest. *Nature* **444**, 1027–1031.
54. Duncan SH, Holtrop G, Lobley GE, *et al.* (2004) Contribution of acetate to butyrate formation by human faecal bacteria. *Br J Nutr* **91**, 915–923.
55. Cummings JH & Macfarlane GT (2002) Gastrointestinal effects of prebiotics. *Br J Nutr* **87**, 5145–5515.

British Journal of Nutrition