

Effect of isomalt consumption on faecal microflora and colonic metabolism in healthy volunteers

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Due to its low digestibility in the small intestine, a major fraction of the polyol isomalt reaches the colon. However, little is known about effects on the intestinal microflora. During two 4-week periods in a double-blind, placebo-controlled, cross-over design, nineteen healthy volunteers consumed a controlled basal diet enriched with either 30 g isomalt or 30 g sucrose daily. Stools were collected at the end of each test phase and various microbiological and luminal markers were analysed. Fermentation characteristics of isomalt were also investigated *in vitro*. Microbiological analyses of faecal samples indicated a shift of the gut flora towards an increase of bifidobacteria following consumption of the isomalt diet compared with the sucrose diet ($P < 0.05$). During the isomalt phase, the activity of bacterial β -glucosidase decreased ($P < 0.05$) whereas β -glucuronidase, sulfatase, nitroreductase and urease remained unchanged. Faecal polyamines were not different between test periods with the exception of cadaverine, which showed a trend towards a lower concentration following isomalt ($P = 0.055$). Faecal SCFA, lactate, bile acids, neutral sterols, N, NH₃, phenol and *p*-cresol were not affected by isomalt consumption. *In vitro*, isomalt was metabolized in several bifidobacteria strains and yielded high butyrate concentrations. Isomalt, which is used widely as a low-glycaemic and low-energy sweetener, has to be considered a prebiotic carbohydrate that might contribute to a healthy luminal environment of the colonic mucosa.

Isomalt: Bifidobacteria: Microbial metabolism: Healthy volunteers: Fluorescence *in situ* hybridization

Isomalt (Palatinit[®]) is used worldwide as a sugar replacer with technological properties comparable to those of sucrose. Chemically, isomalt is a mixture of the polyols 1-*O*- α -D-glucopyranosyl-D-mannitol and 6-*O*- α -D-glucopyranosyl-D-sorbitol. It is derived from sucrose in two major processing steps: (i) the enzymatic rearrangement of sucrose (2-*O*- α -D-glucopyranosyl-D-fructofuranose) into isomaltulose; (ii) the catalytic hydrogenation of isomaltulose into isomalt. Physiologically, isomalt is characterized as low in energy, non-cariogenic (Gehring & Karle, 1981) and low-glycaemic (Petzoldt *et al.* 1982; Gee *et al.* 1991; Hütter *et al.* 1993). As shown in ileostomy patients, isomalt is slowly and only partly digested and absorbed in the upper gastrointestinal tract (Langkilde *et al.* 1994). Undigested and/or unabsorbed portions reach the colon and are fermented by the gut microflora (Bär, 1990). On the basis of various studies in animals and human subjects, Livesey (2003) suggested that the undigested and fermented fraction of ingested isomalt is approximately 90%.

Non- or low-digestible saccharides such as fructo-oligosaccharides, inulin and sugar alcohols are potential prebiotics

(Cummings *et al.* 2001). This property is defined as follows: 'A prebiotic effect is a food-induced increase in numbers and/or activity predominantly of bifidobacteria and lactic acid bacteria in the human intestine' (Van Loo *et al.* 1999). Bifidobacteria and lactic acid bacteria are considered biomarkers of a well-balanced intestinal flora. In breast-fed infants the intestinal flora is dominated by bifidobacteria and lactobacilli, whereas the flora of classic formula-fed infants contains more bacteroides, clostridia and enterobacteriaceae (Balmer & Wharton, 1989). Human milk contains numerous bifidogenic factors like non-absorbable oligosaccharides, which are probably responsible for the difference in the flora of infants fed human milk and formula (Edwards & Prrett, 2002). In some gastrointestinal diseases such as irritable bowel syndrome (Pistoli *et al.* 2003) and Crohn's disease (Giaffer *et al.* 1991; Linskens *et al.* 2001) the counts of bifidobacteria and lactobacilli in faeces are reduced in comparison to healthy controls. Several preventive and therapeutic properties of bifidobacteria and lactic acid bacteria on antibiotic-associated intestinal disorders, gastroenteritis, lactose intolerance, intestinal infections with *Clostridium difficile*

Abbreviations: FISH, fluorescence *in situ* hybridization.

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and *Helicobacter pylori* or traveller's diarrhoea, inflammatory bowel diseases, irritable bowel syndrome and colon cancer have been discussed (Gibson & Roberfroid, 1995; Marteau *et al.* 2002). The mechanisms of these beneficial effects are not yet fully understood. Besides biotope competition, immunomodulation and production of antibiotic agents, the barrier function against pathogens, inflammatory diseases and neoplasms could be explained by the metabolic characteristics of bifidobacteria and lactic acid bacteria. Bifidobacteria form a major group of saccharolytic bacteria in the colon. By fermenting carbohydrates they produce lactate and SCFA, which results in lower pH values in the colon lumen that are not tolerated by the majority of pathogens. A more acidic milieu also inhibits protein degradation and microbial enzymes that liberate potentially harmful substances such as NH_3 , phenol derivatives, metabolites of bile acids and neutral sterols. Although lactobacilli and bifidobacteria do not produce considerable amounts of butyrate, an increase in bifidobacteria is associated with a high butyrate production, probably in symbiosis with other colon bacteria (Cummins *et al.* 2001). This SCFA is the main energy source for colonocytes (Roediger, 1982) and has regulatory effects on the cell cycle (Velazquez *et al.* 1996). Numerous attempts have been made to modulate the bacterial microflora by administering probiotic bacterial strains or prebiotic carbohydrates; the latter serve as substrates for the endogenous gut flora as well as external probiotics (Bezkorovainy, 2001). Today, inulin and fructo-oligosaccharides are the best studied prebiotics with bifidogenic effects (Gibson *et al.*, 1995; Tuohy *et al.* 2001b). The objective of the present study was to investigate if the widely used sweetener isomalt may also have prebiotic properties.

Subjects and methods

Subjects and study design

Twenty healthy volunteers were recruited for the trial. One volunteer dropped out because of tonsillitis, which required the administration of antibiotics. Nineteen volunteers (twelve women and seven men) aged 34.7 (SE 2.4) years (median 30.5 years, range 21–53 years) completed the study. The BMI of the women was 23.5 (SE 0.7) kg/m^2 (median 23.6 kg/m^2 , range 20.8–28.6 kg/m^2) and of the men 25.8 (SE 1.1) kg/m^2 (median 24.0 kg/m^2 , range 23.3–30.2 kg/m^2). Volunteers did not take any antibiotics for at least 6 weeks prior to the start of the study, nor did they take laxatives, motility-affecting medications or lipid-altering medications during the study. Exclusion criteria were a history of severe chronic disease, gastrointestinal diseases, severe abdominal discomfort, severe constipation, abnormal dietary habits, known or suspected lack of compliance with the study protocol and pathologic laboratory values except elevated blood lipids. All participants signed consent forms after written and oral information about the aim, course and potential hazards of the trial. The study was approved by the Ethics Committee of the Faculty of Medicine, University of Würzburg, Germany.

The study was conducted in a double-blind, placebo-controlled, cross-over design. During two 4-week study periods,

volunteers consumed a controlled diet with a washout phase of 4 weeks between periods. The diets were almost iso-energetic (difference in energy intake approximately 200 kJ/d) and composed as a typical Western diet. They provided 46% energy as carbohydrate, 40% energy as fat, and 14% energy as protein. Dietary fibre intake in the diets was limited to 1.4 g/MJ. Mean daily energy intake was 8.8 MJ (2100 kcal) for women and 11.3 MJ (2700 kcal) for men. A 7 d rotating menu including ready-to-eat foods (Eismann Tiefkühl-Heimservice, Mettmann, Germany) was used. Meals were weighed and packed in the university hospital's dietetic kitchen. All meals (breakfast, morning snack, lunch, afternoon snack and dinner) were given to the subjects to be eaten at home. Additional food was not allowed, and the volunteers were asked to consume the delivered portions completely. The diets were identical except that isomalt was added to one diet and sucrose to the other. Isomalt and sucrose were given in sweet foods (jam, biscuits, pudding, chocolate and candies) during the test periods in a randomized cross-over fashion. The dose was increased from 5 g/d to 30 g/d in the first week of each test phase. The test products were prepared and supplied by Suedzucker AG (Mannheim/Ochsenfurt, Germany). When significant weight loss (2 kg) in the course of the study was noted, the volunteers received a fibre-free formula diet (Biosorb; Pfrimmer Nutricia, Erlangen, Germany) to meet energy requirements. Water, tea, coffee were allowed *ad libitum*. Alcohol consumption was restricted to 500 ml beer or 250 ml wine per week. Sugar-free soft drinks were restricted to 500 ml daily. The macronutrient composition of the diet was calculated with the software package Prodi expert 4.502 based on the NutriBase database (Wissenschaftliche Verlagsgesellschaft GmbH, Stuttgart, Germany) and information from the label of the ready-to-eat foods. The diet has been described in detail previously (Gostner *et al.* 2005).

Urine collections (24 h) were obtained at the start and at weekly intervals in both phases. From day 24 to day 28 of each study period, stools were collected for quantitative assessment. Each stool was collected separately in a plastic box and frozen in the laboratory freezer as soon as possible (within 2 h as described previously; Hylla *et al.* 1998) and stored at -25°C . After each study period, 5 d stool collections were pooled without additives by using a grinder and frozen at -25°C until determination of lactate, SCFA, N, Ca, phosphate, phenol and *p*-cresol. For determination of bile acids and neutral sterols, aliquots of pooled faeces were lyophilized (Gamma IA apparatus; Christ, Osterode, Germany). For bacterial spectrum, stool spot samples were collected on 2 d in separate thin tubes without media. Volunteers were asked to bring the stool samples as soon as possible after defecation and to fill up the tube to avoid a head space. An aliquot was fixed in paraformaldehyde (see later) for analysis by fluorescence *in situ* hybridization (FISH) or in 0.9% NaCl for conventional microbiological culture techniques within 1 h. For determination of bacterial enzyme activities, stool specimens were collected in tubes with anaerobic media (PBS with 0.5 g cysteine/l and 10% glycerol, pH 7.4) and stored at -80°C until analysis. For polyamine analysis, faecal samples were frozen in liquid N_2 , lyophilized and stored at -80°C until analysis.

Faecal bacterial spectrum using conventional microbiological culture techniques and fluorescence in situ hybridization with 16S/23S rRNA

For enumeration of faecal bacteria with conventional microbiological culture techniques, 200 mg fresh stool specimen was weighed into a bottle containing 0.9% NaCl and frozen at -80°C . Analyses of faecal bacteria after serial dilution of the faecal slurry and plating on selective and non-selective media and enumeration were done by a commercial laboratory.

For whole-cell FISH with species- and group-specific oligonucleotide probes targeted to 16S/23S rRNA, 500 mg fresh faecal sample was fixed in paraformaldehyde and absolute ethanol respectively and stored at -25°C until analysis. Hybridization and enumeration were performed according to the method of Schwartz *et al.* (2000) using a charge-coupled device video camera (SensiCam; PCO, Kelheim, Germany) and the software package KS400 (Carl Zeiss GmbH, Jena, Germany).

Total bacteria were enumerated using a mixture of bacteria-directed oligonucleotide probes for hybridization (Eub338, Eub297, Eub785, Eub1055, Eub1088; Kleessen *et al.* 2001). The following group-specific oligonucleotides were chosen: HGC69a, Gram-positive bacteria with a high DNA G + C content (Roller *et al.* 1994); LGC354 group, Gram-positive bacteria with a low DNA G + C content (Meier *et al.* 1999); Fprau645, *Faecalibacterium prausnitzii* group (Suau *et al.* 2001); Erec482, *Clostridium coccooides*-*Eubacterium rectale*-*Ruminococcus* group (Franks *et al.* 1998); Ecy1387, *Eubacterium cylindroides* group (Harmsen *et al.* 2002a); Bac303, *Bacteroides*-*Prevotella* cluster (Manz *et al.* 1996); Bif164, *Bifidobacterium* spp. (Langendijk *et al.* 1995); Ato291, *Atopobium*, *Coriobacterium* and *Eggerthella lenta* (Harmsen *et al.* 2000); Lab158, *Lactobacillus/Enterococcus* (Harmsen *et al.* 1999); Gam42a, γ subclass of proteobacteria (Manz *et al.* 1992); Ec1531, *Escherichia coli* (Poulsen *et al.* 1995); Chis150, *Clostridium histolyticum* group (Franks *et al.* 1998); Clit135, *Clostridium lituseburense* group (Franks *et al.* 1998); Rint1102, *Roseburia intestinalis* group (Hold *et al.* 2003).

Faecal bacterial enzymes

Thawed probes were mixed for 3 min using a vortex and glass beads (3 mm). The stool suspension was centrifuged (200 g for 10 min at 4°C) and the supernatant was used for enzyme assays as follows.

Faecal β -glucuronidase activity was assayed in duplicate using a colorimetric kit (kit no. 325-A; Sigma Chemical Co., St. Louis, MO, USA).

For β -glucosidase the enzyme reaction was run at 37°C in a total volume of 2 ml composed of 0.1 ml faecal suspension, 0.4 ml *p*-nitrophenyl glucoside (0.01 mol/l; Sigma Chemical Co.) and 1.5 ml acetate buffer (0.1 mol/l, pH 7.4). After 0, 20, 40 and 60 min of incubation, the reaction was stopped by adding 1 ml of a 1:1 mixture of glycine buffer (0.1 mol/l, pH 12) and distilled water to 0.2 ml of the reaction mixture. The *p*-nitrophenol liberated was measured photometrically at 405 nm.

Faecal sulfatase activity was assayed under similar conditions by using 0.4 ml *p*-nitrophenyl sulfate (0.1 mol/l; Sigma Chemical Co.), 0.4 ml faecal suspension and 1.2 ml

acetate buffer. Readings of liberated *p*-nitrophenol sampled after 0, 30 and 60 min were taken at 405 nm.

Faecal nitroreductase activity was measured according to the method of Wise *et al.* (1982) with minor modifications.

All the enzyme activities were calculated for the linear reaction range by using standard curves for each indicator (phenolphthalein, *p*-nitrophenol), and expressed as μmol liberated indicator/h per g fresh faeces and as total daily enzyme activity ($\mu\text{mol}\cdot\text{h/d}$).

Faecal urease activity was assayed in duplicate using a colorimetric kit (Ammonia-Kit 171; Sigma Chemical Co.) with minor modifications. Enzyme activity was expressed as μmol liberated NH_3/h per g fresh faeces and as total daily enzyme activity ($\mu\text{mol}\cdot\text{h/d}$) after subtraction of endogenous NH_3 .

Faecal pH, SCFA and lactate

Faecal pH was measured with a microprocessor pH meter (WTW, Weilheim, Germany) in triplicate in the pooled faecal samples.

SCFA were determined after conversion into their methyl esters using headspace GC (HP 6890 gas chromatograph; Agilent and PerkinElmer HS 40 XL headspace sampler; Agilent, Waldbronn, Germany) and a capillary fused silica column (PerkinElmer Elite 1701, 50 m \times 0.23 mm \times 1.0 μm ; Perkin Elmer, Rodgau, Germany) with flame ionization detection. The carrier gas was He. The temperature programme was 50°C for 0.3 min, $30^{\circ}\text{C}/\text{min}$ to 260°C , 260°C for 6 min. The concentrations of SCFA were calculated based on peak area ratios according to the internal standard method using chloroacetic acid as standard solution.

Faecal lactate (D- and L-lactate) was determined using a lactate dehydrogenase enzyme test (kit no. 1112821; Roche, Mannheim, Germany). Faecal samples (5 g) were clarified with Carrez reagents (5 ml $\text{K}_4(\text{Fe}(\text{CN})_6)\cdot 3\text{H}_2\text{O}$ (3.6 g/100 ml), 5 ml $\text{ZnSO}_4\cdot 7\text{H}_2\text{O}$ (7.2 g/100 ml), NaOH (2 mol/l) to pH 8) and filtered. The photometric measurement was performed at a wavelength of 365 nm.

Stool fat, stool nitrogen and ammonia

Aliquots of pooled faecal samples were stored at -25°C until analysis. Stool fat was analysed according to the method of van de Kamer *et al.* (1949).

N in faecal samples was analysed according to the Kjeldahl method (EN 25 663). NH_3 was assayed in duplicate in 50 μl faecal suspension (for preparation, see this page) using a colorimetric kit (Ammonia-Kit 171; Sigma Chemical Co.) after deactivation of endogenous urease activity at 80°C for 20 min. NH_3 concentration was expressed as $\mu\text{mol/g}$ wet weight and as total NH_3 excretion/d.

Faecal polyamines

The polyamines putrescine, spermine, spermidine, cadaverine, histamine, N8-acetyl-spermidine and N1-acetyl-spermine were analysed in lyophilized stool samples using HPLC according to the method of Noack *et al.* (1998).

Faecal bile acids and sterols

Faecal bile acids and neutral sterols were analysed in dried faeces by GC-MS after derivatization of faecal bile acids to their *n*-butyl esters, directly in stools, followed by trimethylsilylation of the sterols and bile acids, according to the method of Batta *et al.* (1999). The determination was performed using the HP 6890 gas chromatograph (Agilent), an HP 5973 mass-selective detector and an HP-5 column (30 m, 0.25 mm \times 0.25 μ m; Batta *et al.* 1999).

Faecal and urinary phenol and *p*-cresol

Phenol and *p*-cresol in urine and faeces were determined by GC after hydrolysis of the corresponding sulfate and glucuronic esters. The determination was performed by using the HP 6890 gas chromatograph with a Zebron ZB-5 column (60 m, 0.25 mm inner diameter, film thickness 0.25 μ m; Phenomenex, Aschaffenburg, Germany) and with He as carrier gas. Split ratio was 10:1 at a constant flow rate of 1.4 ml/min. Initial injector temperature was 40°C for 1 min, 10°C/min to 230°C. Temperature programme (column oven) was 50°C for 0.01 min, 680°C/min to 250°C (flame ionization detector, 350°C). The concentrations of the phenols were calculated based on peak area ratios according to the internal standard method with *o*-cresol as standard.

In vitro experiments

In vitro fermentation experiments with mixed human gut bacteria. Freshly voided mixed faeces of three volunteers were used to prepare a 10% (w/v) faecal slurry in anaerobic phosphate buffer (50 mM, pH 7.0, 0.5 g cysteine-HCl/l). Batch culture *in vitro* fermentations was carried out under anaerobic conditions for 28 h at 37°C under slight stirring with 1% faecal slurry in the following basic medium: tryptone 1.5 g, yeast extract 1.0 g, KH₂PO₄ 0.24 g, Na₂HPO₄ 0.24 g, (NH₄)₂SO₄ 1.24 g, NaCl 0.48 g, MgSO₄·7H₂O 0.10 g, CaCl₂·2H₂O 0.06 g, FeSO₄·7H₂O 2 mg, resazurin 1 mg, cysteine-HCl 0.5 g, vitamin solution 0.5 ml, trace element solution 9.0 ml, NaHCO₃ 2.0 g ad 1000 ml H₂O, pH 7.0, with or without 0.5% (w/v) isomalt. The fermentation experiments were performed five times in duplicate. At different time intervals, samples of fermentation broth were centrifuged for 10 min at 12 000 *g* at 4°C and subsequently sterile-filtered. Cell-free supernatants were kept at -20°C. The analysis of SCFA was performed by headspace GC as described earlier. Residual isomalt was analysed by high-pH anion-exchange chromatography with pulsed amperometric detection on a CarboPac-PA1 column (Dionex, Idstein, Germany) (Na-acetate gradient 1-27%, 30 min, room temperature, flow rate of 1 ml/min).

Fermentation experiments with human gut bifidobacteria. *Bifidobacterium adolescentis* (DSM 20 083, DSM 20 086, DSM 20 087), *Bifidobacterium angulatum* (DSM 20 098, DSM 20 225), *Bifidobacterium breve* (DSM 20 213), *Bifidobacterium catenulatum* (DSM 20 103, DSM 20 224), *Bifidobacterium infantis* (DSM 20 223) and *Bifidobacterium pseudocatenulatum* (DSM 20 438) were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany). Bifidobacteria were grown

anaerobically in 10-ml Hungate tubes on the following medium: peptone 10 g, meat extract 5 g, yeast extract 5 g, Na₂HPO₄ 1.44 g, NaH₂PO₄ 0.24 g, KH₂PO₄ 6 g, Tween-80 1 g, resazurin 1 mg, cysteine-HCl 0.5 g, vitamin solution 0.5 ml, trace element solution 9.0 ml, NaHCO₃ 2.0 g, carbohydrate (isomalt or glucose) 10 g ad 1000 ml H₂O, pH 7.0. The medium was inoculated with an exponential culture (1 ml) and incubated for 48 h at 37°C. The fermentation experiments were performed in duplicate. Preparation and analysis of the culture supernatants for residual carbohydrate, acetate and lactate were performed as described earlier. Growth was monitored at 578 nm using a Hitachi 2000 spectrophotometer (Hitachi, Tokyo, Japan).

Statistical analyses

Values are given as means with their standard errors. The non-parametric Wilcoxon rank-sum test for paired data was used for comparisons. Correlations were obtained by Pearson's test. Analyses were performed using SigmaStat for Windows, version 2.03 (SPSS Inc., Chicago, IL, USA). Differences were considered significant at $P < 0.05$.

Results

Subjects

Nineteen volunteers completed the study. Consumption of 30 g isomalt daily in food was well tolerated. Compliance of the volunteers was confirmed by urinary mannitol excretion (Gostner *et al.* 2005).

Faecal microflora

Total bacterial counts, per g faeces wet weight and per d, analysed by the FISH technique, did not differ between the two test periods. However, there were differences with regard to counts of certain bacterial groups (Tables 1 and 2).

Using conventional microbiological culture technique on selective media, counts of bifidobacteria were significantly higher ($P < 0.01$) with isomalt consumption compared with sucrose (Table 1). As shown in Fig. 1, growth stimulation of bifidobacteria was confirmed using the FISH method. Compared with sucrose, the proportion of bifidobacteria increased by 65% ($P < 0.05$) and total bifidobacteria cell counts by 47% ($P < 0.05$; Fig. 1). The mean faecal counts of bifidobacteria were 29% higher after isomalt than after sucrose consumption, but this figure did not reach statistical significance (Table 2).

Cell counts of lactobacilli, bacteroides, *E. coli* and enterococci reported by conventional culture techniques were not different between the test periods (Table 1). FISH analysis also showed a significant proportional increase of the atopobium group ($P < 0.05$) and of actinobacteria (Gram-positive bacteria with high G + C DNA content; $P < 0.01$), together with higher cell counts of actinobacteria ($P < 0.05$). Fewer cell counts of *Roseburia intestinalis* (counts/g faeces wet weight and counts/d: $P < 0.01$ and $P < 0.05$, respectively) and of Gram-positive bacteria with low G + C content (counts/d: $P < 0.05$) as well as of bacteroides species (counts/g faeces wet weight: $P < 0.05$) were seen (Table 2).

Table 1. Mean bacterial counts (cfu $\times 10^8$ /g faeces wet weight) and counts/d (cfu $\times 10^9$ /d) determined by conventional culture methods in stool samples of nineteen healthy volunteers after 4 weeks' consumption of either 30 g isomalt or 30 g sucrose daily (Mean values with their standard errors)

Genus	Isomalt (counts/g wet weight)		Sucrose (counts/g wet weight)		Isomalt (counts/d)		Sucrose (counts/d)	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Bifidobacteria	50.2**	13.6	21.2	4.0	505.8*	130.7	186.6	34.3
Bacteroides	68.2	20.8	42.7	16.5	752.9	261.2	320.3	98.3
<i>Escherichia coli</i>	0.2	0.1	0.1	0.1	2.2	0.9	0.9	0.3
Lactobacilli	0.002	0.001	0.002	0.002	0.03	0.02	0.02	0.01
Enterococci†	0.002	0.001	0.006	0.01	0.02	0.01	0.1	0.1

cfu, colony-forming units.

Mean values were significantly different from those of the sucrose group (Wilcoxon test for paired data): * $P < 0.05$, ** $P < 0.01$.

† $n = 9$.

For details of subjects and procedures, see p. 41.

Table 2. Mean bacterial counts (counts $\times 10^9$ /g faeces wet weight), counts/d (counts $\times 10^{11}$ /d) and proportion (%) of total bacteria determined by fluorescence *in situ* hybridization in stool samples of nineteen healthy volunteers after 4 weeks' consumption of either 30 g isomalt or 30 g sucrose daily (Mean values with their standard errors)

Genus	Isomalt (counts/g wet weight)		Sucrose (counts/g wet weight)		Isomalt (counts/d)		Sucrose (counts/d)		Isomalt proportion (%)		Sucrose proportion (%)	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Total microflora	126	13	161	15	146	21	156	21	100	—	100	—
<i>Atopobium</i> group	2.8	0.4	1.7	0.2	3.1	0.6	1.8	0.3	2.7*	0.4	1.3	0.2
<i>Bacteroides</i> , <i>Prevotella</i>	18.3*	1.8	26.8	2.5	21.0	3.5	26.4	3.7	16.2	1.8	18.2	1.7
Bifidobacteria†	12.0	2.1	9.3	1.2	12.2*	2.3	8.3	1.0	10.6*	2.0	6.4	0.9
<i>Clostridium histolyticum</i> group‡	0.003	0.001	0.005	0.001	0.004	0.001	0.005	0.001	0.003	0.001	0.004	0.001
<i>Clostridium lituseburensense</i> group§	0.004	0.001	0.003	0.001	0.005	0.001	0.003	0.001	0.004	0.001	0.003	0.001
<i>Escherichia coli</i>	0.3	0.04	0.4	0.1	0.4	0.1	0.4	0.1	0.3	0.03	0.3	0.04
<i>Eubacterium cylindroides</i> cluster	1.5	0.6	1.3	0.4	2.1	0.9	1.6	0.5	1.1	0.3	1.1	0.5
<i>Eubacterium rectale</i> cluster	10.3	1.3	9.8	1.5	11.7	1.9	9.4	1.5	8.6	1.2	8.0	1.5
<i>Faecalibacterium prausnitzii</i>	0.4	0.1	0.4	0.1	0.4	0.1	0.4	0.1	0.4	0.2	0.3	0.1
Proteobacteria γ -group†	3.4	0.5	2.7	0.5	3.7	0.5	2.7	0.6	2.4	0.4	2.4	0.6
<i>Lactobacillus/Enterococcus</i> group	1.0	0.4	1.9	0.5	1.4	0.7	2.1	0.7	0.8	0.2	1.6	0.6
Gram ⁺ bacteria LGC¶	0.04	0.01	0.14	0.1	0.1*	0.01	0.2	0.1	0.04	0.01	0.1	0.1
Actinobacteria (Gram ⁺ bacteria HGC)	12.0*	2.3	8.0	1.6	12.1*	2.4	6.7	1.0	11.0**	2.4	5.1	1.0
<i>Roseburia intestinalis</i>	0.3**	0.1	0.6	0.1	0.4*	0.19	0.6	0.1	0.3	0.1	0.5	0.1

LCG, low G + C content (of DNA), HGC, high G + C content (of DNA).

Mean values were significantly different from those of the sucrose group (Wilcoxon test for paired data): * $P < 0.05$, ** $P < 0.01$.

Number of determinations/samples: † $n = 18$; ‡ $n = 15$; § $n = 12$; || $n = 14$; ¶ $n = 10$.

For details of subjects and procedures, see p. 41.

Faecal bacterial enzyme activity

Total faecal β -glucosidase activity decreased significantly by 40% after isomalt consumption compared with sucrose ($P < 0.05$). Activities of β -glucuronidase, sulfatase, nitroreductase and urease were not significantly modified by isomalt (Fig. 2).

Faecal pH, SCFA and lactate

Stool pH was similar in the isomalt (6.7 (SE 0.1)) and sucrose test phases (6.6 (SE 0.1); NS). Faecal concentration (isomalt: 7.7 (SE 0.5) v. sucrose: 8.0 (SE 0.5) mg/g faeces wet weight) and daily excretion (isomalt: 867.2 (SE 105.6) v. sucrose:

834.3 (SE 108.4) mg/d) of total SCFA showed no significant differences between test phases. The same also applied to faecal concentrations and daily excretions of the individual SCFA, i.e. acetic, propionic, n-butyric, iso-butyric, iso-valeric and n-valeric acids (data not shown). In both test phases acetate, propionate and butyrate were the main SCFA in stool samples, with a molar ratio of acetate:propionate:butyrate of 62:19:19 after the isomalt test phase. Faecal concentrations of D-lactate (isomalt: 0.28 (SE 0.11) v. sucrose: 0.28 (SE 0.14) mg/g faeces wet weight) and L-lactate (isomalt: 0.22 (SE 0.07) v. sucrose: 0.21 (SE 0.09) mg/g faeces wet weight) were similar in both test periods.

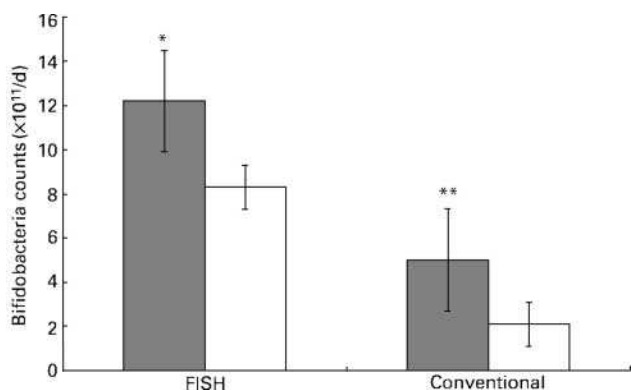


Fig. 1. Daily cell counts of bifidobacteria with isomalt consumption (■) and sucrose consumption (□) determined by fluorescence *in situ* hybridization (FISH) and conventional microbiological culture techniques. Values are means with their standard errors shown by vertical bars. Mean values were significantly different compared with the sucrose test phase (Wilcoxon test for paired data): * $P < 0.05$, ** $P < 0.01$.

Stool fat, nitrogen and ammonia

Stool fat excretion was not affected by isomalt consumption compared with sucrose (percentage of stool fat: 2.3 (SE 0.2) for isomalt *v.* 2.5 (SE 0.3) for sucrose, NS; daily stool fat excretion: 2.1 (SE 0.2) g/d for isomalt *v.* 2.3 (SE 0.2) g/d for sucrose, NS).

Faecal N excretion did not differ between isomalt and sucrose test periods (N concentration: 13.6 (SE 1.0) mg/g faeces wet weight for isomalt *v.* 14.1 (SE 0.7) mg/g faeces wet weight for sucrose, NS; daily N excretion: 1.4 (SE 0.1) g/d for isomalt *v.* 1.4 (SE 0.1) g/d for sucrose, NS).

NH₃ concentration and daily excretion were 11 and 20% lower, respectively, with isomalt, although differences between isomalt and sucrose were not significant (NH₃ concentration: 27.7 (SE 2.5) $\mu\text{mol/g}$ faeces wet weight for isomalt *v.* 31.1 (SE 3.0) $\mu\text{mol/g}$ faeces wet weight for sucrose, NS; NH₃ excretion: 3071.7 (SE 417.3) $\mu\text{mol/d}$ for isomalt *v.* 3859.8 (SE 430.8) $\mu\text{mol/d}$ for sucrose, NS).

Faecal polyamines

Faecal concentration and daily excretion of the polyamines putrescine, spermidine and spermine were similar following consumption of either isomalt or sucrose (data not shown). There was a tendency towards lower cadaverine concentrations in the isomalt test period (isomalt: 0.3 (SE 0.1) *v.* sucrose: 0.5 (SE 0.1) $\mu\text{mol/g}$ faeces wet weight, $P = 0.055$). Putrescine and spermidine were detectable in all stool samples, whereas cadaverine was detectable in thirteen and spermine in three stool samples from both periods. The biogenic amines histamine, N8-acetyl-spermidine and N1-acetyl-spermine were found only sporadically.

Faecal bile acids and neutral sterols

Faecal concentration (isomalt: 11.1 (SE 0.9) *v.* sucrose: 11.5 (SE 1.0) mg/g faeces dry weight, NS) and daily excretion of total bile acids (isomalt: 298.8 (SE 21.4) *v.* sucrose: 282.0 (SE 35.5) mg/d, NS) were similar in both test periods, as was faecal concentration (isomalt: 5.7 (SE 0.8) *v.* sucrose: 5.7 (SE 0.5) mg/g faeces dry weight) and daily excretion (isomalt: 163.8 (SE 22.3) *v.* sucrose: 143.1 (SE 20.5) mg/d) of total neutral sterols. Comparable concentration and daily excretion in both periods was also observed for individual bile acids (primary or secondary) and neutral sterols (data not shown).

Faecal and urinary phenol and *p*-cresol

Urinary concentration/excretion of *p*-cresol was comparable in the isomalt and sucrose phases. With regard to stool specimens, the concentration and excretion of phenol and *p*-cresol were similar in both experimental periods. Urinary phenol excretion decreased significantly during the sucrose diet whereas it remained unchanged during the test phase with isomalt. Consequently, urinary phenol excretion was significantly lower in week 4 of the sucrose test phase than in week 4 of the isomalt phase (Table 3).

A positive correlation was found between faecal and urinary phenol excretion with isomalt ($r = 0.69$, $P < 0.05$) but not with

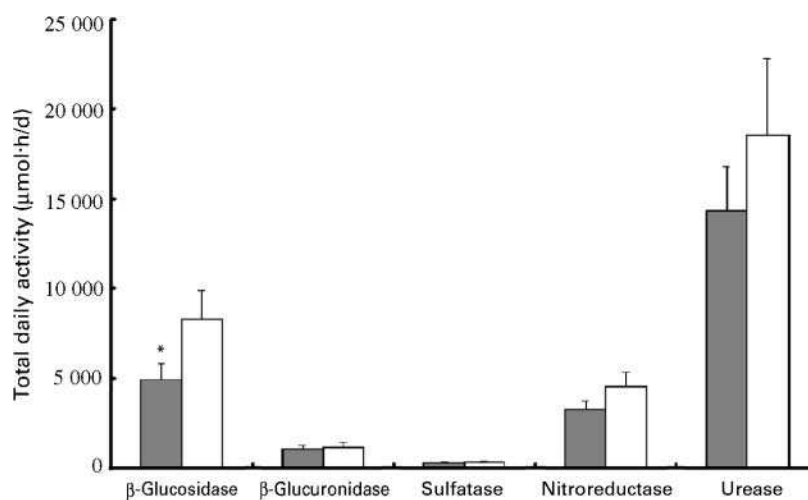


Fig. 2. Total daily faecal enzyme activities during isomalt consumption (■) and sucrose consumption (□). Values are means with their standard errors shown by vertical bars. Mean values were significantly different compared with the sucrose test phase (Wilcoxon test for paired data): * $P < 0.05$.

Table 3. Faecal (mg/kg) and urinary (mg/l) concentration and faecal and urinary excretion (mg/d) of phenol and *p*-cresol in nineteen healthy volunteers consuming either 30 g isomalt or 30 g sucrose daily (Mean values with their standard errors)

	Week	Concentration				Excretion			
		Isomalt (mg/l; /kg)		Sucrose (mg/l; /kg)		Isomalt (mg/d)		Sucrose (mg/d)	
		Mean	SE	Mean	SE	Mean	SE	Mean	SE
Urinary phenol	0	9.5	3.2	6.7	2.0	10.8	3.0	9.5	1.8
	4	8.9	3.2	3.7	0.8	11.0	3.5	5.4*†	1.1
Urinary <i>p</i> -cresol	0	19.4	3.9	13.8	3.7	24.1	3.3	23.6	6.3
	4	13.9	2.0	14.9	2.6	19.8	2.9	17.9	2.1
Faecal phenol	4	5.5	1.4	6.7	1.5	0.58	0.13	0.63	0.14
Faecal <i>p</i> -cresol	4	38.8	4.5	39.3	6.2	4.1	0.47	3.4	0.42

Mean value was significantly different from that at baseline (Wilcoxon test for paired data): * $P < 0.05$.

Mean value was significantly different from that of the isomalt phase (Wilcoxon test for paired data): † $P < 0.05$.

For details of subjects and procedures, see p. 41.

sucrose ($r = 0.13$), and not with *p*-cresol in both test phases (isomalt: $r = 0.4$; sucrose: $r = 0.3$). Faecal phenol excretion was negatively correlated with the mean transit time (as reported elsewhere, Gostner *et al.* 2005; $r = -0.54$, $P < 0.05$). No correlations were found between mean transit time and *p*-cresol excretion with isomalt ($r = 0.07$) or between mean transit time and phenol and *p*-cresol in the sucrose test phase ($r = -0.44$ and $r = 0.43$, respectively).

Utilization of isomalt by human gut bacteria *in vitro*

To study the fermentation properties of isomalt escaping digestion in the small intestine and its metabolism by human gut bacteria, an *in vitro* fermentation assay was developed and extensively validated. Fig. 3 shows the data of *in vitro* fermentation assays with gut bacteria from mixed faecal samples of three human volunteers. Isomalt was degraded completely by human gut bacteria over a 14 h fermentation period, and the main SCFA formed were acetate, propionate and butyrate. Total SCFA concentration at the end of the fermentation was 35.6 (SE 3.3) mmol/l, while in the fermentation control (medium without supplemented carbohydrate) only 18.8 mmol/l was measured. A comparatively high concentration of butyrate was formed (14.2 (SE 1.8) mmol/l), which was more than that obtained with known butyrogenic substrates such as resistant starches (11.4 (SE 2.6) mmol/l, data

not shown). An acidification of the medium to pH 6.2 (SE 0.3) was observed at the end of the fermentation compared with an almost unchanged pH in the control (pH 7.2, data not shown).

Fermentation experiments with human gut bifidobacteria

As shown in Table 4, human bifidobacteria strains utilized isomalt and grew on a medium with isomalt as sole carbohydrate source. Some strains, e.g. *B. adolescentis* (DSM 20086), *B. catenulatum* (DSM 20103, 20224) and *B. infantis* (DSM 20223), metabolized isomalt almost completely. At the end of the fermentation experiment the isomalt content in the growth medium was reduced to less than 10% of the initial value and the concentration of SCFA in the medium increased to 60 mmol acetate/l and 32 mmol lactate/l. The optical density of these bacterial cultures at the end of the fermentation experiment was comparable to cell densities obtained with glucose as sole carbohydrate source (data not shown).

Discussion

The human colon harbours approximately 10^{11} – 10^{12} bacterial cells per gram of colon content with more than 400 different species of bacteria. The composition of the intestinal microflora varies among individuals and has traditionally been

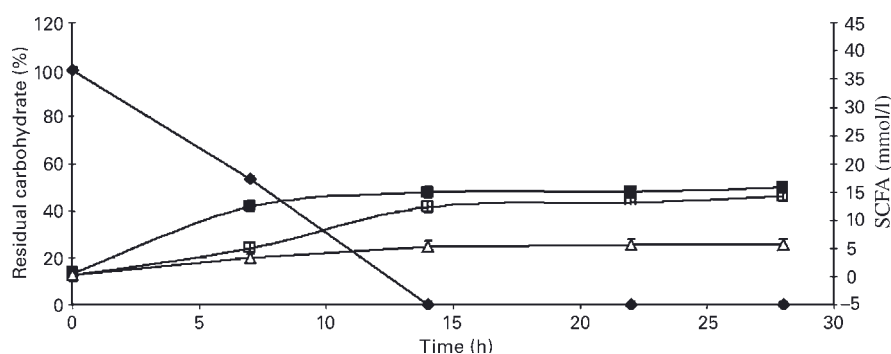


Fig. 3. *In vitro* fermentation of isomalt (◆) into SCFA (■, acetate; △, propionate; □, butyrate) by human gut bacteria. Values are means with their standard errors shown by vertical bars.

Table 4. Fermentation experiments with human gut bifidobacteria strains

Species	DSM no.	Acetate (mmol/l)	Lactate (mmol/l)	Residual isomalt (%)	OD ₅₇₈
<i>Bifidobacterium adolescentis</i>	20083	47.9	23.7	19.7	1.9
	20086	35.7	27.6	5.5	1.8
	20087	45.9	20.5	23.5	2.5
<i>Bifidobacterium angulatum</i>	20098	46.5	9.9	29.0	2.5
	20225	35.4	4.4	49.7	2.4
<i>Bifidobacterium breve</i>	20213	26.1	2.6	64.3	2.0
<i>Bifidobacterium catenulatum</i>	20103	43.1	23.1	8.3	3.7
	20224	57.9	31.9	3.1	4.2
<i>Bifidobacterium infantis</i>	20223	59.8	21.1	8.5	3.6
<i>Bifidobacterium pseudocatenulatum</i>	20438	42.4	17.6	21.8	1.5

OD₅₇₈, optical density measured at 578 nm; DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen.

thought to be very stable. However, it is now well recognized that the microflora of the intestine can be modulated by dietary intervention. A focus of many studies has been specific stimulation of the growth of resident bifidobacteria and lactobacillus species by feeding prebiotic low-digestible carbohydrates (Gibson & Roberfroid, 1995).

The major novel finding of the present trial has been the prebiotic effect of isomalt, which is used widely as a low-glycaemic and low-calorie food sweetener. Using two independent methods (conventional microbiological culture techniques and FISH with 16S/23S rRNA) we observed a significant increase in the faecal number and proportion of bifidobacteria by 47 and 65%. To our knowledge this is the first study that demonstrates that isomalt has also bifidogenic effects. This finding was further confirmed by *in vitro* experiments demonstrating the utilization of isomalt by several isolated human bifidobacteria strains. In addition to changes in bifidogenesis, an increase in the proportion and counts of bacteria belonging to the atopobium cluster was observed in faecal samples. This cluster includes several bacterial species, e.g. lactic acid-producing coriobacteria. The higher proportion and cell counts of actinobacteria (Gram-positive bacteria with high G + C DNA content) confirmed the increased counts of bifidobacteria and the atopobium cluster, as these are included therein. Proportions of other bacteria or bacteria groups were comparable in both periods and not affected by isomalt. Counts of the *R. intestinalis* group and of bacteroides (mean counts) were lower in the isomalt period. *R. intestinalis* represents a small group of the bacterial spectrum and also includes butyrate-producing bacteria species. *In vitro* fermentation with gut bacteria demonstrated that high butyrate concentrations are obtained from isomalt. Thus, lower counts obtained for the *R. intestinalis* group may indicate that bacteria of this group do not contribute considerably to the butyrate generation from isomalt.

An interesting question is how the bifidogenic effect of isomalt observed in the present trial compares with known prebiotics such as fructo-oligosaccharides and other low-digestible carbohydrates. The increase in bifidobacteria of 0.3 log cells/g faeces observed in the present study falls within the range reported by others (Gibson *et al.* 1995; Buddington *et al.* 1996; Kleessen *et al.* 1997; Menne *et al.* 1997; Alles *et al.* 1999; Bouhnik *et al.* 1999; Brighenti *et al.* 1999; Kruse *et al.* 1999; Zhong *et al.* 2000; Rao, 2001; Tuohy *et al.* 2001a,b; Harmsen *et al.* 2002b). In these studies, daily ingestion of 2.5 to 40 g low-digestible carbohydrates led to

an increase in bifidobacteria of 0.1–1.3 log cells/g faeces. Several authors found that the relative increase of faecal bifidobacteria in response to a prebiotic carbohydrate was highly dependent on the initial bacterial count (more pronounced prebiotic effect with low starting counts; Rao, 2001; Tuohy *et al.* 2001b). The magnitude of effect observed in the present study is equivalent to the 0.2–0.3 log increase found in other trials with healthy volunteers and high initial counts of faecal bifidobacteria (Brighenti *et al.* 1999, Tuohy *et al.* 2001b; Harmsen *et al.* 2002b).

Some hydrolytic and reductive bacterial enzymes catalyse reactions that release (co)carcinogens or promoters of colon carcinogenesis. For example, β -glucuronidase and sulfatase activate potentially toxic substances such as bile acids by deconjugation of the non-toxic conjugated substrates. The enzyme β -glucosidase hydrolyses plant glycosides and releases toxic aglycons (Mallett *et al.* 1987). The formation of aromatic amines by nitroreductase causes the production of potentially genotoxic intermediates such as reactive *N*-hydroxy compounds (Wheeler *et al.* 1975). Cytotoxic NH₃, which at high concentrations plays a role in hepatic encephalopathy, is produced by urease (Blei, 2000). There is evidence that a high-fibre diet could modulate microbial enzymatic activities (Johanson *et al.* 1990; Grasten *et al.* 2000). In the present study, total β -glucosidase activity was 40% lower at the end of the isomalt period compared with sucrose. Several mechanisms are possible for the observed reduction in faecal enzyme activity. The effect may be mediated by dilution effects, alterations in the composition of the intestinal microbial flora, modulation of the microbial environment such as pH value and substrate availability, or by direct or indirect substrate inhibition. In the present study a simple dilution effect is not likely, since specific activity and stool weight were not significantly altered by isomalt. As *Bacteroides ovatus* is known as a main producer of β -glucosidase (McBain & Macfarlane, 1998), it is imaginable that the significant decrease in faecal bacteroid concentrations of about 32% was one cause of the reduction in β -glucosidase activity. However, some strains of bifidobacteria are also known to synthesize β -glucosidase (McBain & Macfarlane, 1998, 2001).

There is a wide variety of bacterial metabolic processes in the human colon that may be influenced by nutritional factors, especially the intake of low-digestible carbohydrates. A more saccharolytic profile of the intestinal microflora with fermentation of carbohydrates in the colon is associated with

production of SCFA and lactate. SCFA, especially butyrate, are essential for the maintenance of physiological colonocyte morphology and function (Scheppach, 1994; Scheppach *et al.* 2001). In the present study, however, the faecal concentrations or daily excretions of the following metabolites/end products seemed to be not affected by isomalt consumption.

SCFA, lactate and pH value in faeces were similar in both test periods. Nevertheless, *in vitro* fermentation experiments showed that isomalt is completely fermented by human intestinal bacteria and that this fermentation pattern results in an increase in SCFA, especially butyrate, and a decrease in pH. The divergence between *in vivo* and *in vitro* is plausible because of the high absorption rates of SCFA in the colon. Thus, differences in SCFA production and pH in the colon between isomalt and sucrose may not be detectable in faeces.

In the present study faecal N was similar in both study periods. This observation differs from results of a study in pigs where the feeding of isomalt led to an increase of faecal N (van Weerden & Huisman, 1993). It has been proposed that the fermentation of carbohydrates stimulates bacterial proliferation and, thus, the fixation of N in bacterial matter. The finding for faecal N seems to be plausible, as there was no increase in the total bacteria counts in our study.

According to Weber (1997), the incorporation of N in bacteria would also lower luminal NH₃ concentrations. High concentrations of NH₃ are cytotoxic and may promote colonic carcinogenesis (Lin & Visek, 1991). In the present study faecal concentrations and daily output of NH₃ were 11 and 20% less with isomalt, although this difference did not reach significance.

Considerable amounts of polyamines have been detected in the human colon (Benamouzig *et al.* 1997). While putrescine, spermidine and spermine can be synthesized by mucosal cells or luminal bacteria, cadaverine is exclusively of bacterial origin. In this study, a trend towards lower faecal cadaverine concentration was observed following ingestion of isomalt whereas the faecal excretion of other polyamines was comparable between the two test periods.

Phenol and *p*-cresol are metabolites of the bacterial fermentation of aromatic amino acids (phenylalanine, tyrosine) and may play a role in the initiation of colon (Bone *et al.* 1976) and bladder cancer (Bryan, 1971). They are partially absorbed and excreted in the urine. In the present study no differences in the faecal concentrations of phenol and *p*-cresol were shown. Daily urinary phenol excretion did not change during the isomalt test period whereas it was reduced during the test period with sucrose. This finding is difficult to explain because there is no evidence in the literature that sucrose may affect protein fermentation.

Faecal concentration and daily excretion of bile acids and neutral sterols were not affected by isomalt consumption. Despite research in this field it is still unclear whether neutral sterols and bile acids are involved in large bowel carcinogenesis. In previous human intervention studies with acarbose (Bartram *et al.* 1991) and resistant starch (Hylon VII; Hylla *et al.* 1998) our group has shown a reduction of faecal concentrations of secondary bile acids and neutral sterols. In accordance with the present study, Alles *et al.* (1999) found no effect of low-digestible carbohydrates.

In conclusion, the present study showed for the first time that the widely used sweetener isomalt is bifidogenic in

healthy volunteers and is thus a candidate prebiotic carbohydrate that might contribute to a healthy gut flora and function. The extent of the bifidogenic effect obtained with isomalt was in good accordance with other studies examining prebiotic carbohydrates in healthy individuals with high initial bifidobacteria counts.

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