

Nutrient Physiology, Metabolism, and Nutrient-Nutrient Interactions

Dietary Fructooligosaccharides Affect Intestinal Barrier Function in Healthy Men

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ABSTRACT In contrast to most expectations, we showed previously that dietary fructooligosaccharides (FOS) stimulate intestinal colonization and translocation of invasive *Salmonella enteritidis* in rats. Even before infection, FOS increased the cytotoxicity of fecal water, mucin excretion, and intestinal permeability. In the present study, we tested whether FOS has these effects in humans. A double-blind, placebo-controlled, crossover study of 2 × 2 wk, with a washout period of 2 wk, was performed with 34 healthy men. Each day, subjects consumed lemonade containing either 20 g FOS or placebo and the intestinal permeability marker chromium EDTA (CrEDTA). On the last 2 d of each supplement period, subjects scored their gastrointestinal complaints on a visual analog scale and collected feces and urine for 24 h. Fecal lactic acid was measured using a colorimetric enzymatic kit. The cytotoxicity of fecal water was determined with an in vitro bioassay, fecal mucins were quantified fluorimetrically, and intestinal permeability was determined by measuring urinary CrEDTA excretion. In agreement with our animal studies, FOS fermentation increased fecal wet weight, bifidobacteria, lactobacilli, and lactic acid. Consumption of FOS increased flatulence and intestinal bloating. In addition, FOS consumption doubled fecal mucin excretion, indicating mucosal irritation. However, FOS did not affect the cytotoxicity of fecal water and intestinal permeability. The FOS-induced increase in mucin excretion in our human study suggests mucosal irritation in humans, but the overall effects are more moderate than those in rats. *J. Nutr.* 136: 70–74, 2006.

KEY WORDS: • fructooligosaccharides • humans • microflora • mucin • prebiotics

Fructooligosaccharides (FOS)² are nondigestible carbohydrates, which are assumed to be beneficial for the host health because they stimulate the protective intestinal microflora (1). FOS are found in varying concentrations in many foods such as wheat, bananas, asparagus, and garlic (2) and they are supplemented to several products, such as dairy products and infant formulas.

FOS are composed of linear chains of fructose units, linked by $\beta(2\rightarrow1)$ bonds and often terminated by a glucose unit. The number of fructose units ranges from 2 to 7. FOS are not hydrolyzed by human small intestinal glycosidases and reach the colon intact (3). In the colon, FOS may specifically stimulate growth of endogenous bifidobacteria and lactobacilli (4,5). The protective microflora and their subsequent production of organic acids may increase host resistance against acid-sensitive pathogens. However, this assumption is based largely on in vitro studies (6–8). Host defense against invasive pathogens, such as

salmonella, also depends on the barrier function of the intestinal mucosa. High concentrations of organic acids may induce injury to the intestinal mucosa (9), thereby impairing the barrier function (10).

Indeed, in several strictly controlled rat infection experiments, we showed that dietary FOS increased intestinal translocation of the invasive pathogen *Salmonella enteritidis* to extraintestinal organs (11–14). FOS increased infection-induced growth impairment (12–14), diarrhea (13), gut inflammation (14), and permeability of the large intestine (11). Before infection with salmonella, FOS increased cytotoxicity of the intestinal contents, mucin excretion, and intestinal permeability in rats (11–14). We speculate that the impaired intestinal barrier in our animal studies may result from cytotoxic fermentation metabolites formed by rapid fermentation of FOS within the proximal large intestine.

In view of the adverse effects of dietary FOS on the resistance to salmonella in rats and the fact that FOS has been added to a variety of products, such as dairy products and infant formulas, our aim was to show proof-of-principle in humans. Therefore, we tested whether the adverse effects of FOS that occurred before infection in rats would occur in humans. In the present study, we determined the effects of FOS on the

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² Abbreviations used: CrEDTA, chromium EDTA; DP, degree of polymerization; FOS, fructooligosaccharides; ICP-AES, inductively coupled plasma-atomic emission spectrophotometry; SCFA, short-chain fatty acids.

cytotoxicity of fecal water, mucin excretion, and intestinal permeability in healthy men.

SUBJECTS AND METHODS

Subjects. Healthy men ($n = 34$; aged 18–55 y) participated in the study. Men were recruited by advertisements in regional newspapers and posters mounted in public buildings. Women were excluded because of the possible influence of the menstrual cycle on intestinal fermentation (15). It is also more difficult for women to collect urine and feces separately.

Subjects had no history of gastrointestinal disease, surgical operations of the small and large intestine, or lactose intolerance. Subjects had not used immunosuppressive drugs, antibiotics, anti-diarrheal drugs, laxatives, and pre- or probiotics in the last 3 mo before the study.

The study was fully explained to the subjects and they gave their written informed consent before participating. The study protocol was approved by the Ethical Committee of Wageningen University, the Netherlands. After successfully completing the study, the subjects received a small financial reward to compensate for the inconvenience.

Study design, diet and supplements. A double-blind, placebo-controlled crossover study design with 2 supplement periods of 2 wk separated by 1 washout period of 2 wk was used. Participants were stratified by age and then randomly divided in 2 groups. Throughout the 2 supplement periods, subjects were instructed to maintain their usual pattern of physical activity and their habitual diet but to abstain from all dairy products and other foods with a high calcium content. A low-calcium background diet was necessary because the FOS-induced adverse effects in the animal studies were inhibited by high dietary calcium intake (12). Foods containing large amounts of fermentable nondigestible carbohydrates (e.g., beans, leeks, onions) or pro- or prebiotics were forbidden (2). Alcohol consumption was restricted to a maximum of 4×250 mL/d and a maximum of 20×250 mL/wk. Participants received a list of the forbidden foods and drinks.

Subjects consumed either lemonade with 20 g of FOS (purity 93%, Raftilose P95, Orafit) or 6 g of sucrose (placebo) per day. The placebo lemonade was supplemented with sucrose to ensure equal taste, viscosity, and color of the 2 lemonades. This dose was divided into 3 daily portions of lemonade, which had to be consumed in the morning, afternoon, and evening.

The lemonade also contained the intestinal permeability marker, chromium EDTA (CrEDTA). The CrEDTA solution was prepared as described elsewhere (16). We did not use $^{51}\text{CrEDTA}$ to exclude chronic exposure to γ -rays. CrEDTA is a sensitive marker for intestinal permeability in humans (17,18) and daily intake of CrEDTA was shown to be equally sensitive as a single oral dose of CrEDTA in a previous study (11). The complete formation and stability of the CrEDTA complex were confirmed by passing the final CrEDTA solution through a cation exchange resin column (Chelex 100 Resin; Bio-Rad). No uncomplexed Cr^{3+} ions were present in the lemonade or in feces and urine samples of the subjects during the supplement periods (data not shown). Based on analysis of the lemonade by inductively coupled plasma atomic emission spectrometry (ICP-AES; Varian), CrEDTA intake was $150 \mu\text{mol/d}$ during the FOS and placebo period.

Nutrition diary. On the last 2 d of both supplement periods, subjects quantitatively reported all foods and drinks consumed in a nutrition diary. Mean daily energy and nutrient intake in each period was calculated using a computerized food consumption table (Dutch food composition database 2001, NEVO Foundation). Subjects were asked to rate the frequency and severity of gastrointestinal complaints such as flatulence, bloating, and abdominal pains or cramps on a visual analog scale from 0 (none) to 5 (severe).

Microbiological analyses of feces. On the last day of both supplement periods, 24-h fecal samples were collected for microbiological and biochemical analyses. Feces were frozen on dry ice immediately after defecation and stored at -40°C (aerobically). Thereafter, the whole sample was weighed, freeze-dried, and subsequently ground to obtain homogeneous powdered samples. DNA was isolated from feces, using the QIAamp DNA stool mini-kit, according to the instructions of the manufacturer, with slight modifications. After addition of

the lysis buffer, the samples were shaken 3×1.5 min at $2000 \times g$ together with glass beads in a Bead Beater. Real-time quantitative PCR was used to specifically quantify bifidobacteria, lactobacilli, and *Escherichia coli* in feces, as described and validated earlier (11,13,19,20). The within-assay CV was $<10\%$.

Biochemical analyses of feces. Total fecal lactic acid was measured using a colorimetric enzymatic kit (Boehringer Mannheim), as described previously (21). A blank measurement for each individual sample was included to correct for the background signal in our lactate assay. Freeze-dried feces were reconstituted with double-distilled water to obtain fecal water with a dry weight percentage similar to the original sample (as determined by freeze-drying). Samples were mixed, incubated for 1 h at 37°C , and subsequently centrifuged for 1 h at $14,000 \times g$ (Hettich, Micro-rapid 1306). The pH of the fecal water was measured at 37°C , and the cytotoxicity of fecal water was determined using an erythrocyte assay as previously described and validated earlier with intestinal epithelial cells (22,23).

Mucins were extracted from freeze-dried feces and quantified fluorimetrically, as described earlier (21). Standard solutions of *N*-acetylgalactosamine (Sigma) were used to calculate the amount of oligosaccharide side-chains liberated from mucins. Fecal mucins are therefore expressed as μmol oligosaccharide equivalents. Control experiments showed that interfering oligosaccharides of dietary origin were completely removed by the molecular filtration step (data not shown).

To measure fecal CrEDTA and calcium, freeze-dried feces were treated with 50 g/L of trichloroacetic acid and centrifuged for 2 min at $14,000 \times g$. The supernatants were diluted with 0.5 g/L of CsCl, and chromium and calcium were analyzed by ICP-AES (Varian).

Biochemical analyses of urine. On the last 2 d of both supplement periods, complete 24-h urine samples were collected in plastic bottles containing 10 mL of 6 mol/L hydrochloric acid to prevent bacterial deterioration. To determine whether the 24-h urine collection was complete, urinary creatinine was measured by an enzymatic colorimetric method (CRE2U,0-512; Roche, Germany). Urinary CrEDTA and calcium were analyzed by ICP-AES, as described above.

Statistical analysis. The randomization code of the human intervention study was broken after finishing all laboratory analyses. All results are expressed as mean \pm SEM. A commercially available package (Statistica 6.1, StatSoft) was used for all statistics. Because we used a crossover design, a paired Student's *t* test (2-sided) was used to examine whether the placebo and FOS supplementation periods differed ($P < 0.05$). When data were not normally distributed, differences were tested with the Wilcoxon Matched Pairs Test.

RESULTS

Participant characteristics and diet. Participants were 27.7 ± 1.7 y and had a BMI of $23.2 \pm 0.5 \text{ kg/m}^2$. All participants completed the study successfully. However, 5 subjects did not collect feces in 1 supplement period and were excluded from all fecal analyses. The urinary CrEDTA results of 1 subject were excluded because of a very high urinary CrEDTA concentration in the FOS period. Daily intakes of energy and nutrients were determined by analysis of the nutrition diaries. As intended, there were no differences between the placebo and FOS period in energy and macronutrient intakes. The intakes of calcium (placebo, $298 \pm 11 \text{ mg/d}$; FOS, $315 \pm 14 \text{ mg/d}$) and fiber (placebo, $29.8 \pm 1.6 \text{ g/d}$; FOS, $30.6 \pm 1.6 \text{ g/d}$) did not differ between the 2 periods.

Compliance with the study protocol. Compliance of the subjects with the prescribed low-calcium diet was good, as indicated by the results of calcium excretion in urine and feces. The recommended daily allowance of calcium for adults in Western countries is 1000–1200 mg, depending on age (24). Calcium intake in the present study, as determined by the nutrition diaries, was $\sim 300 \text{ mg/d}$. The calcium intake determined by fecal and urinary calcium excretion was $\sim 400 \text{ mg/d}$. The daily urinary calcium excretion was $101 \pm 11 \text{ mg/d}$ in the

placebo period and 100 ± 10 mg/d in the FOS period. The daily fecal calcium excretion was 326 ± 30 mg/d in the placebo period and 288 ± 25 mg/d in the FOS period. Total CrEDTA intake in the present study was $150 \mu\text{mol/d}$. Based on the urinary and fecal excretions of CrEDTA, compliance with supplement intake was good (Table 1).

Finally, urinary creatinine excretion was measured and indicated compliance with the collection of 24-h urine samples. Urinary creatinine excretions did not differ between the FOS and placebo periods and were within the normal range of $0.13\text{--}0.22$ mmol/kg body weight (data not shown).

Intestinal microflora. Dietary FOS consumption increased fecal bifidobacteria (Fig. 1A, $P < 0.0001$). Except for 1 subject, all subjects had higher numbers of fecal bifidobacteria during the FOS period. FOS also increased fecal lactobacilli (Fig. 1B, $P < 0.05$). Fecal *E. coli* numbers did not differ between the placebo ($6.7 \pm 0.2 \log_{10}/\text{g}$ wet feces) and FOS ($6.6 \pm 0.2 \log_{10}/\text{g}$ wet feces) periods.

Gastrointestinal complaints and fecal characteristics. Subjects more frequently reported flatulence ($P < 0.0001$) and bloating ($P < 0.005$) in the FOS period than in the placebo period (Fig. 2). Scores for abdominal pain and cramps tended to be higher during the FOS period ($P = 0.05$). FOS increased daily fecal wet weight ($P < 0.05$), but did not affect the percentage of fecal dry weight. This was confirmed by measuring fecal cation excretion (data not shown).

Fecal lactic acid and cytotoxicity. FOS consumption tended to decrease fecal pH (Table 1; $P = 0.06$) and increased total fecal lactic acid excretion (Fig. 3, $P < 0.05$). Fecal lactic acid concentrations were $3.3 \pm 0.7 \mu\text{mol/g}$ dry feces in the placebo period and $22.0 \pm 8.0 \mu\text{mol/g}$ dry feces in the FOS period ($P < 0.05$). Fecal calcium and inorganic phosphate concentrations were lower in the FOS period ($P < 0.0005$) due to the increased fecal dry weight. FOS consumption did not affect the cytotoxicity of fecal water (Table 1).

Fecal mucin excretion and intestinal permeability. FOS increased fecal mucin excretion (Fig. 3, $P < 0.05$). However, urinary CrEDTA excretion did not differ between the 2 periods (Table 1). Daily intake of CrEDTA was similar in the 2 supplement periods ($150 \mu\text{mol CrEDTA/d}$). Subjects excreted $2.72 \pm 0.28\%$ of the ingested CrEDTA in urine during the placebo period and $2.76 \pm 0.44\%$ during the FOS period, values within the normal range for healthy subjects (25,26). Fecal CrEDTA excretion did not differ between the dietary periods (Table 1).

TABLE 1

Effect of FOS on feces characteristics, the cytotoxicity of fecal water, and urinary CrEDTA in humans¹

	Placebo	FOS
Feces		
Wet weight, g/d	203.7 ± 28.3	$268.0 \pm 29.4^*$
Dry weight, g/d	44.2 ± 4.9	54.0 ± 5.1
Calcium, $\mu\text{mol/g}$	176.1 ± 9.5	$147.2 \pm 11.1^*$
Inorganic phosphate, $\mu\text{mol/g}$	270.3 ± 15.2	$208.5 \pm 17.7^*$
CrEDTA, $\mu\text{mol/d}$	146.7 ± 13.8	136.8 ± 11.1
Fecal water		
pH	6.1 ± 0.03	5.9 ± 0.06
Cytotoxicity, % of max lysis	43.4 ± 7.5	45.8 ± 7.3
Urine		
CrEDTA, $\mu\text{mol/d}$	3.3 ± 0.2	3.0 ± 0.3

¹ Values are means \pm SEM ($n = 29$ and 33 for measurements in feces and urine, respectively). *Different from placebo, $P < 0.05$.

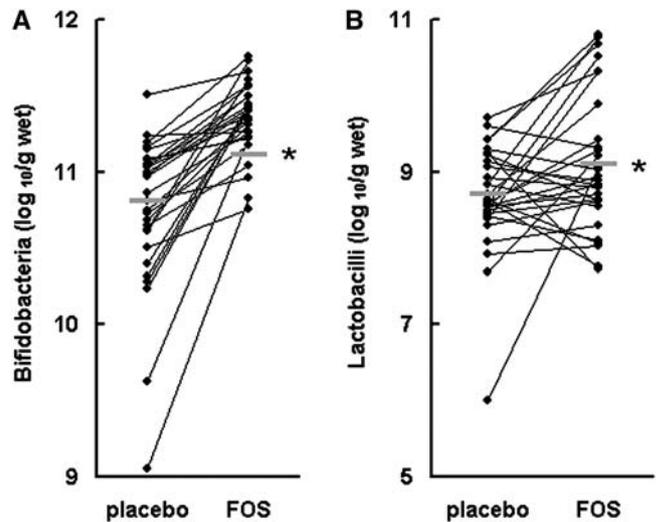


FIGURE 1 Effects of consuming 20 g/d FOS for 2 wk on the number of fecal bifidobacteria (A) and lactobacilli (B) in men. Individual (●) and mean values (—) are indicated ($n = 29$). *Different from placebo, $P < 0.05$.

DISCUSSION

In the present study, intestinal fermentation of FOS increased the numbers of fecal bifidobacteria and lactobacilli. Human studies from other research groups (4,27,28) and our previous animal studies (11–14) also showed that highly fermentable nondigestible carbohydrates such as FOS stimulate the growth of these bacteria.

As in our rat studies (11–14), intestinal fermentation of FOS in humans resulted in increased concentrations of fecal lactate. Lactate is a bacterial fermentation intermediate, which is normally metabolized to short-chain fatty acids (SCFA) by a range of intestinal bacteria (29,30). Therefore, lactate is either absent or present in low concentrations in feces from healthy subjects (30–32). However, lactate does accumulate during rapid carbohydrate breakdown because, unlike SCFA, it is poorly absorbed by the epithelial cells of the large intestine (29,33).

We speculate that the rapid production of organic acids in the proximal colon induces the mucin excretion in the present study (34,35). The organic acid-induced mucin secretion likely reflects irritation and impairment of the intestinal barrier in our earlier animal studies (11–14). In those studies, FOS supplementation resulted in 2-fold higher mucin secretion but

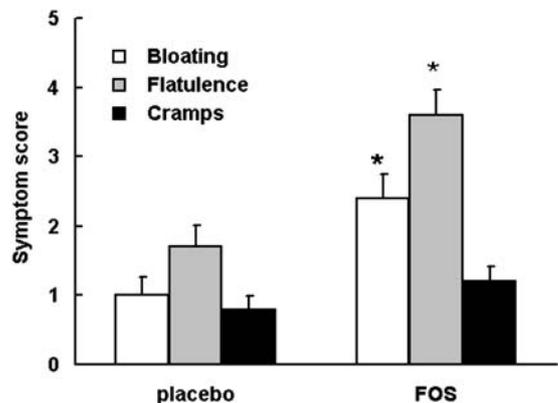


FIGURE 2 Effects of consuming 20 g/d FOS for 2 wk on flatulence, intestinal bloating and cramps in men. Results are expressed as means \pm SEM, $n = 34$. *Different from placebo, $P < 0.05$.

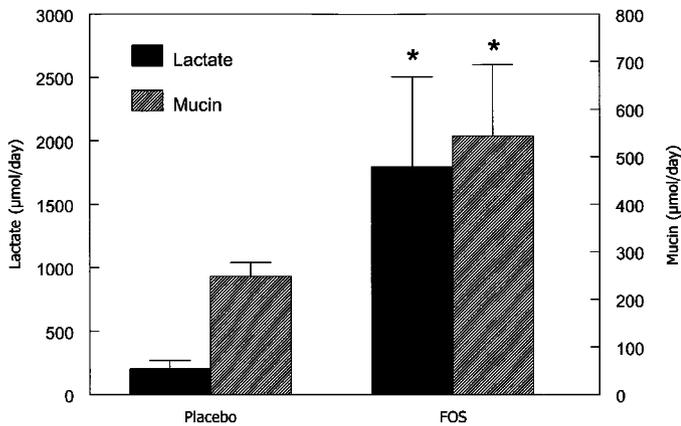


FIGURE 3 Effects of consuming 20 g/d FOS for 2 wk on daily fecal lactic acid and fecal mucin excretion in humans. Results are expressed as means \pm SEM, $n = 29$. *Different from placebo, $P < 0.05$.

concomitantly adversely affected intestinal barrier function as indicated by increased translocation of salmonella to extra-intestinal sites (11–14). We do not dispute that mucin secretion itself is an important and beneficial host defense response. However, the mucosa produces mucin in response to diverse insults (36) and irritating components such as bacterial pathogens (37–39), endotoxins (40), bile acids (41,42), or organic acids (34,35). Thus, in our opinion, the stimulated mucin secretion indicates the necessity of the epithelial layer to protect itself against harmful substances.

In our earlier rat studies (11–14) as well as in the present human study, FOS increased fecal lactic acid bacteria, fecal wet weight, fecal lactate, and intestinal mucin secretion. Thus, based on these physiological variables, rats seem to be an appropriate model for humans. Moreover, assuming a daily dry food intake of 500 g in humans, the total concentration of dietary FOS in the present study (20 g/500 g or 4%) was comparable to that of our rat (3–6%) studies (11–14).

However, FOS did not increase the cytotoxicity of fecal water and large intestinal permeability in humans. Considering the important role of calcium phosphate in intestinal resistance (12,14), we speculate that the discrepancy in cytotoxicity and large intestinal permeability between our human and rat studies can be explained largely by differences in intestinal calcium (and hence phosphate) concentration. The dietary calcium concentrations in our human and rat studies were comparable, 30 $\mu\text{mol Ca/g}$ diet in rats and 20–25 $\mu\text{mol Ca/g}$ diet in humans (12,13). Nevertheless, the intestinal calcium concentration was lower in rats than in the men of the present study (Table 1). This is likely due to the higher calcium absorption in growing rats than in adult humans. Fecal calcium concentration in rats was only 100 $\mu\text{mol Ca/g}$ feces in control and 55 $\mu\text{mol Ca/g}$ feces in FOS-fed rats (12).

In addition to the differences in luminal calcium concentrations between the studies in rats and humans, the lack of effect of FOS on the cytotoxicity of fecal water and large intestinal permeability in humans could be explained by a number of study limitations. First, the variable composition of the background diet of the subjects might have resulted in a high variation in luminal cytotoxic components. Second, the background diet of the participants contained relatively large amounts of fiber. This could have resulted in a more gradual fermentation of FOS and hence have decreased the adverse effects of rapid fermentation (43).

Are the results of the present human intervention relevant to the population at large? Daily intake of fermentable non-

digestible carbohydrates (derived mainly from wheat and onions) in Europe and the United States has been estimated at up to 10 g (44). This estimation does not take into account consumption of specific meals and products supplemented with inulin or FOS, typically 3–10 g/portion (44). In view of this, 20 g FOS/d (as in the present study) is realistic for human consumption. The recommended daily intake of calcium for adults in Western countries is \sim 1000 mg (24). The low calcium intake (400 mg/d) in the present study is comparable to the daily calcium intake of several large population groups, e.g., the elderly (45), lactose-intolerant subjects (46), and African-Americans (47) in industrialized societies, and people living in developing countries (48).

We conclude that the adverse effects of FOS on the resistance to intestinal infections in our animal studies (11–14) and the present human intervention study do not support the concept that stimulating the endogenous microflora and intestinal organic acid production by rapid fermentation of non-digestible carbohydrates is beneficial for the intestinal barrier in humans.

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