Effect of transgalactooligosaccharides on the composition of the human intestinal microflora and on putative risk markers for colon cancer[1–3]

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ABSTRACT

Background: Nondigestible oligosaccharides have been claimed to benefit the health of the colon by selectively stimulating the growth of bifidobacteria and by decreasing the toxicity of the colon contents.

Objective: We compared the effect of 2 doses of transgalactooligosaccharides and a placebo on the composition and activity of the intestinal microflora in 18 women and 22 men.

Design: Strictly controlled experimental diets were supplied to 3 intervention groups in a parallel design. The study was divided into 2 consecutive 3-wk periods during which each participant consumed a run-in diet followed by an intervention diet that differed only in the amount of transgalactooligosaccharides: 0 (placebo), 7.5, and 15 g/d. Breath samples and fecal samples were collected at the end of both the run-in and intervention periods.

Results: Apparent fermentability of transgalactooligosaccharides was 100%. The highest dose of transgalactooligosaccharides significantly increased the concentration of breath hydrogen by 130% ($P < 0.01$) and the nitrogen density of the feces by 8.5% ($P < 0.05$). The number of bifidobacteria increased after both placebo and transgalactooligosaccharides ingestion, but the differences between these increases were not significantly different. Transgalactooligosaccharides did not significantly affect bowel habits; stool composition; the concentration of short-chain fatty acids or bile acids in fecal water; the concentration of ammonia, indoles, or skatoles in feces; fecal pH; or the composition of the intestinal microflora.

Conclusion: We conclude that transgalactooligosaccharides are completely fermented in the human colon, but do not beneficially change the composition of the intestinal microflora, the amount of protein fermentation products in feces, or the profile of bile acids in fecal water. Am J Clin Nutr 1999;69:980–91.

KEY WORDS Transgalactooligosaccharides, fermentation, stool composition, bifidobacteria, microflora, short-chain fatty acids, bile acids, ammonia, phenols, colon cancer, humans, Netherlands

INTRODUCTION

The recent commercialization of nondigestible oligosaccharides as food ingredients has triggered much research on their role in colonic health. Nondigestible oligosaccharides naturally occur in various edible food plants such as onions and leeks (1). Because they are not hydrolyzed by enzymes in the human small intestine, they reach the colon almost intact. Oligosaccharides are relatively small molecules. In a previous study we showed that fructooligosaccharides, added to the diet of young, healthy subjects, are fully fermented by the colonic microflora (2).

Colonic fermentation is the anaerobic process in which carbohydrates and proteins are metabolized by the intestinal microflora. End products of fermentation are substrate dependent. Fermentation of carbohydrates mainly leads to the production of gases; the short-chain fatty acids acetate, butyrate, and propionate; and sometimes lactate or ethanol. Protein fermentation increases the production of branched-chain fatty acids isobutyrate and isovalerate, phenolic compounds, and ammonia (3, 4). The colonic fermentative activity is regulated by the amount and type of substrate that enters the colon, such as endogenous compounds and undigested food components (4). It seems likely that the composition of the diet influences the activity of the intestinal microflora.

Transgalactooligosaccharides are produced from lactose by enzymatic transgalactosylation. The oligomers are linear and consist of lactose and several galactose molecules with β-1,6 or β-1,4 linkages (5). Transgalactooligosaccharides are not hydrolyzed by human small-intestinal β-galactosidase and will pass undigested into the colon (5). Tanaka et al (6) reported in 1983 that administration of transgalactooligosaccharides leads to an increase in breath-hydrogen excretion because of its nondigestibility.

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Moreover, they showed that transgalactooligosaccharides might stimulate the growth of resident bifidobacterial strains (6). Ten years later, these results were confirmed by the studies of Ito et al (7, 8) and Bouhnik et al (9).

Bifidobacteria may comprise up to one-quarter of the gut flora of healthy adults (10). They have a role in controlling the pH of the large intestine through the production of lactic and acetic acids. A low pH might restrict the growth of many potential pathogens and putrefactive bacteria (10, 11) and might both depress the formation of secondary from primary bile acids and enhance the precipitation of bile acids (12–15). It thus seems of great importance to identify food components that have a potential for increasing the number of indigenous bifidobacteria.

Most studies looking at the effects of transgalactooligosaccharides on bifidobacteria used a linear study design and did not exclude possible time and placebo effects. Also, the focus was always on the composition of the intestinal flora and not on possible colon cancer risk markers such as stool weight (16), bile acids (17, 18), and putrefactive products (19–21). We used a controlled feeding trial to study the effect of 2 doses of transgalactooligosaccharides compared with the effects of a placebo on the composition of the intestinal microflora, the saccharolytic and proteolytic activities of the bacteria, and the profile of bile acids in fecal water.

SUBJECTS AND METHODS

Subjects

Volunteers were recruited via advertisements in local newspapers and posters mounted in public buildings in Wageningen. The experiment was preceded by a screening procedure that included completion of a health questionnaire, blood and urine analyses, a transgalactooligosaccharides tolerance test, and a lactose breath test. Sixty-three volunteers started with the screening procedure. Five volunteers withdrew before the start of the study.

To be eligible for inclusion in the study, subjects had to be aged 18–75 y; have a stable body weight; have no history of gastrointestinal or gallbladder disorders; have not used antibiotics or laxatives in the 12 mo before the experiment; have had no surgery in the 12 mo before the experiment; have no complaints of diarrhea, obstipation, or abdominal pain; not be using any medication known to affect gastrointestinal function; have a serum cholesterol concentration <7.0 mmol/L; have a serum triacylglycerol concentration <2.5 mmol/L; have normal hemocytometric values; and have normal urinary values for protein, glucose, and pH. Five volunteers were excluded because they did not meet the biomedical inclusion criteria.

A breath-lactose test was performed to exclude hydrogen responders to lactose. Subjects came to the department after an overnight fast and consumed a drink containing 50 g lactose. Breath samples were taken before and at 30-min intervals after the lactose drink was ingested; a total of 7 samples were taken. An increase in breath hydrogen of 15 ppm above baseline was used as an exclusion criterion. Twelve volunteers showed hydrogen responses to lactose, probably because of lactase deficiency, and were excluded from the study. Subjects were challenged with a single dose of 10 g transgalactooligosaccharides to check for possible intolerance. None of the volunteers showed any signs of intolerance or discomfort and none were excluded because of intolerance. Forty-one volunteers (19 women and 22 men) entered the study.

The study protocol was approved by the Medical Ethics Committee of the Division of Human Nutrition and Epidemiology. The protocol and aims of the study were explained fully to the volunteers, who gave their informed, written consent. The food was provided at no cost during the experiment and the volunteers received a small financial reward after successfully completing the study.

Design

The 6-wk study had a parallel design. The subjects were divided into 3 treatment groups. All groups started with the same run-in diet for 3 wk. For the next 3 wk, they switched to an intervention diet that differed for each of the 3 groups: a diet high in transgalactooligosaccharides (aimed to be 15 g/d, high-TOS group), a diet low in transgalactooligosaccharides (aimed to be 7.5 g/d, low-TOS group), or a diet with no transgalactooligosaccharides (placebo group). At the end of the run-in and intervention periods, fecal samples and breath samples were collected. The volunteers were divided into 3 start groups. Each start group started the intervention 1 wk after the other so that the laboratory would be able to adequately process the fresh fecal samples sent to the laboratory. The interventions were equally distributed over the start groups.

Diets

Before the trial, trained dietitians used a questionnaire to ask the subjects about their habitual diets (22, 23). This allowed us to estimate the habitual energy intake of the subjects. The questionnaires were coded and the diets were formulated by using the Netherlands Food Composition Table (24). The study diets were formulated at 21 levels of energy intake, ranging from 7.5 to 17.5 MJ/d, so that each subject received a diet that met his or her energy needs. Body weights were recorded 3 times weekly and energy intake was adjusted when necessary to maintain a stable weight. The diets consisted of conventional foods, and 21different menus were provided over each 3-wk period. The nutrient composition of each diet was similar, except for the oligosaccharides. The diets were rich in protein, mainly animal protein ($\approx$76%), and low in fiber.

The transgalactooligosaccharides (Elix'or) were provided by Borculo Whey Products, Borculo, Netherlands. Fruit juices, which were also part of the run-in diets, were used as a vehicle for the interventions and were divided over 3 portions of 150 g each and consumed with each meal. Three mixtures were made to add to the juices. The high-TOS mixture was composed of a transgalactooligosaccharide syrup that consisted of 75% dry matter, of which 62% was transgalactooligosaccharides with a degree of polymerization of 2 (32% nondigestible disaccharides), 3 (35%), 4 (23%), 5 (8%), or >5 (2%). The remaining dry matter consisted of 20% lactose and 18% monosaccharides (mainly glucose). For the low-TOS mixture, we used less transgalactooligosaccharide syrup; therefore, we added extra glucose and lactose to equalize the amount of nonoligosaccharide components in the syrup. The placebo mixture consisted of only glucose and lactose, in amounts equal to those in the high-TOS and low-TOS mixtures.

Approximately 90% of the subjects' energy intake was from supplied foods; the remaining 10% was from products chosen by the subjects from a prepared list of non-fiber-containing foods. All foods supplied to the subjects were color coded to keep them unaware of which intervention they were receiving. Foods were supplied at the Department of Human Nutrition and Epidemiol-

ogy, Wageningen Agricultural University, Netherlands. On weekdays at noon, hot meals were consumed at the department. For all other meals on weekdays, food was packaged and provided daily. Food for consumption on the weekend and guidelines for its preparation were provided on Fridays.

Subjects were urged not to change their selection of free-choice items throughout the study and to maintain their usual smoking habits. The participants kept diaries in which they recorded their frequency of defecation, any sign of illness, gastrointestinal complaints, medications used, menstrual cycle phase, the free-choice food items consumed, and any deviations from their usual diet and lifestyle behavior.

Duplicate portions of the diets and the juices were collected every day for an imaginary participant with a daily energy intake of 11 MJ, stored at −20°C, pooled weekly, and analyzed after the study. Records of the free-choice items were coded and their energy and nutrient content were combined with the analyzed values of the food supplied.

Thirty barium-impregnated grains (Radio-Opaque Pellets; TD Medical, Eindhoven, Netherlands) were swallowed daily together with the meals in the last 10 d of the run-in and intervention periods and were counted in the fecal samples. Stool recovery was estimated by using the following formula: (30 × weight of the pooled feces)/(the number of barium-impregnated-grains in the pooled feces).

Data collection and analytic procedures

Data collection

In the last week of the run-in and intervention periods, 2 breath samples were taken on 2 consecutive days just before lunch. In the last week of the run-in and intervention periods, volunteers came to the department twice to defecate. A button on the study toilet was pressed immediately after defecation and a light and buzzer warned the analyst that a fresh sample was ready to be handled. The temperature of the feces was recorded and within 5 min after defecation, a sample weighing ≈10 g was weighed and transported into an anaerobic cabinet. The remainder of the sample was immediately deep-frozen on dry ice to stop fermentation and then stored at −80°C. Complete stool collections were made during the last weekend (2.5 d) of the run-in and intervention periods; subjects collected all stools and stored them immediately on dry ice.

Breath-hydrogen concentrations

End-expiratory breath samples and ambient air samples were collected in plastic, 60-mL syringes (Plastipak; Becton Dickinson, Dublin). Within 2 h after collection, the hydrogen concentration was measured by using a standard electrochemical cell (exhaled hydrogen monitor; Gas Measurement Instruments Ltd, Renfreq, Scotland). The cell was calibrated with a standard gas (100 ppm H_2) in air. Volunteers refrained from smoking in the hour before sampling.

Preparation of stool samples

All fecal samples (stools collected at the department plus stools collected at home) from a single subject from each 3-wk period were pooled. All fecal samples were X-rayed (Optimus M200; Philips, Eindhoven, Netherlands) before analysis to determine the number of barium-impregnated grains. The samples were then thawed overnight at 4°C, weighed, and homogenized in a bowl and mixer. The dry weight of the feces was estimated by drying a portion at 80°C in an oven (model E45; Heraeus, Hanau, Germany) to constant weight. One portion was used to prepare the aqueous fraction of stool and centrifuged at 26 000 × g for 90 min at 4°C (MSE; Scientific Instruments, Crawley, United Kingdom). Fecal water was carefully removed and stored at −20°C until analyzed for short-chain fatty acids and bile acids. The pH was measured in both the fecal homogenate and fecal water with a digital pH meter (CD 620; WPA Ltd, Cambridge, United Kingdom). One portion of the mixed feces was freeze-dried, ground, and kept in a dry environment until analyzed for nitrogen and transgalactooligosaccharides. The remaining feces were stored at −20°C until analyzed for ammonia, indoles, and skatole.

Transgalactooligosaccharides in feces and juices

Transgalactooligosaccharides were measured in duplicate samples of 125 mg freeze-dried feces resuspended in 2.4 mL distilled water. D-Galacturonic acid (100 μL, 1.25 g/L) was added as an internal standard before extraction. The mixture was vortex mixed, heated for 15 min at 100°C, and then centrifuged at 10 000 × g and 20°C. The supernate was analyzed by high-performance anion-exchange chromatography (Dionex BV, Breda, Netherlands) on a Spectra-Physics system (San Jose, CA) equipped with a Carbopac PA-1 column (4 × 250 mm; Dionex, Sunnyvale, CA). The eluent was monitored with a Dionex PED detector in the pulsed amperometric detection mode. Elution was performed with a flow rate of 1 mL/min and a linear sodium acetate gradient of 0–0.2 mol/L in 0.1 mol NaOH/L for 30 min.

The fruit juices were centrifuged at 10 000 × g and 20°C and the supernate was analyzed with high-performance anion-exchange chromatography, under conditions similar to those mentioned above, to estimate the concentration of transgalactooligosaccharides. The elution profile and the areas under the peaks of transgalactooligosaccharides were compared with those of a water solution with known quantities of transgalactooligosaccharides (Elix'or). The detection limit for the transgalactooligosaccharides in fecal water and juice was 10 mg/L.

Short-chain fatty acids and bile acids in fecal water

Concentrations of short-chain fatty acids in fecal water were measured in duplicate as described by Tangerman and Nagengast (25) by using a gas chromatograph (model CP 9001; Chrompack, Middelburg, Netherlands) and a column packed with 10% SP1200 silicone stationary phase and 1% H_3PO_4 on an 80–100 Chromosorb W acid-washed instrument (Chrompack). An internal standard was added to all samples before analysis (15 mmol 2-ethylbutyric acid/L in 100% formic acid). Samples from each subject were analyzed within one run.

For the analyses of bile acids, 150 μL fecal water was freeze-dried and prepared according to Glatz et al (26), with a few minor modifications. Samples were analyzed on a capillary fused silica column (length: 30 m; internal diameter: 0.25 mm) coated with CP-Sil-19 CB with a film thickness of 0.25 mm (Chrompack, Bergen op Zoom, Netherlands) by using a Hewlett-Packard (Palo Alto, CA) gas chromatograph (model 5890, series II) and flame ionization detector. The initial pressure of the carrier gas (hydrogen) was 90 kPa. Splitless injection was performed by using a liquid sampler (HP 7673; Hewlett-Packard). To cover the wide range of concentrations, injection volumes varied from 0.5 to 2.5 μL. In addition, the concentrations of the calibration solutions were adapted according to

the injection volume. The initial oven temperature was 150°C, which was raised to 225°C immediately after injection and increased gradually to 245°C at a rate of 1°C/min. The final temperature (275°C) was maintained for 40 min. The temperature of the injection port was 300°C and of the detector was 275°C. Each bile acid sample was calibrated by using 7 standard solutions with increasing concentrations. The calibrations were bracketed, ie, 2 calibration sets were measured (one before and one after sampling) to eliminate system drift. The average result was applied to the sample calculations. Calculations were performed by using the internal standard method: the area response of the peak of interest was compared with the area response of the internal standard peak (nor-DCA), yielding the area response ratio. The area response ratio, plotted against the amount ratio, resulted in a linear calibration curve, enabling calculations of the amount of each bile acid. Samples from one subject were analyzed within one run. The CV within runs was 10% for concentrations <10 μmol/L and 5% for concentrations ≥ 10 μmol/L.

Fecal nitrogen, ammonia, indoles, and skatoles

Ammonia was extracted from 5 g homogenized feces with 20 mL perchloric acid (1 mol/L) and then the pH was set to 7.0 ± 0.1 with 5 mol KOH/L. A commercial test kit (Ammonia UV-method, catalog no. 1112732; Boehringer Mannheim GmbH, Mannheim, Germany) was used to determine the concentration of ammonia. Total nitrogen was measured in freeze-dried feces by the Kjeldahl method (Kjeltec Autosampler System, 1035 Analyzer; Tecator Ltd, Bristol, United Kingdom). Indoles and skatoles were extracted from 5 g homogenized feces with 50 mL methanol. The mixture was homogenized with an ultra-turrax (Janke en Kunkel, Zoetermeer, Netherlands) and then filtered through a glass microfiber filter (E Merck BV, Amsterdam) before being analyzed. Analyses were performed in duplicate by using HPLC with ultraviolet absorption detection according to the method described by Wilkens (27).

Microbiological analyses

Microbiological analyses of each sample were done within 3 h of defecation at the Department of Food Science, Division of Food Microbiology, Wageningen Agricultural University. All analyses and preparations were done in an anaerobic chamber (H_2:CO_2:N_2, 10:10:80; 21°C; SHK050H; Hoekloos, Rotterdam, Netherlands) unless stated otherwise. Feces were diluted (10^{-1}) in 70 mL of a solution of buffered peptone water, Tween 80 (1 g/L; Sigma Chemical Co, St Louis), and cysteine (0.5 g/L), and then homogenized by using an ultra-turrax. Aliquots of 1 mL were diluted in reduced physiologic peptone water in decimal steps. From each of the dilutions, 0.03 mL was plated in duplicate onto selective media. All media were kept ≥ 24 h in the anaerobic chamber before being used.

Nutrient agar was used to determine total aerobes (Oxoid CM 3; Basingstoke, United Kingdom) with dilutions of 10^{-2} to 10^{-5}. For Escherichia coli, eosine methylene blue agar (Oxoid CM 69; Basingstoke) was used with dilutions of 10^{-2} to 10^{-5}. Total anaerobes were counted on Fecal Reinforced Clostridial Agar, which consisted of 38 g Reinforced Clostridial Medium/L (Oxoid CM 149; Basingstoke), 1% Hemine solution (H2250, 0.5 g/L; Sigma Chemical Co), 0.02% phylloquinone solution (0.5% in 95% ethanol; Bufa Pharmaceuticals, Uitgeest, Netherlands), 18 g Oxoid L13 agar/L (Basingstoke), and 100 mL fecal extract. Fecal extract was prepared by mixing equal volumes of swine feces and buffered peptone water (Oxoid CM 509; Basingstoke) to which cysteine·HCl (1 g/L) and Tween 80 (P1754, 10 g/L) were added. The mixture was homogenized and sterilized. Dilutions of 10^{-6} to 10^{-8} were used. Sulfite-reducing clostridia (mainly Clostridium perfringens) were counted on perfringens agar base and supplement (CSA, Oxoid CM 587, SR 47, and SR 88; Basingstoke) by using dilutions of 10^{-3} to 10^{-7}. For lactobacilli, LAMVAB medium was used (28) with dilutions of 10^{-2} to 10^{-7}. Bifidobacteria were counted on raffinose bifidobacterium agar by using dilutions of 10^{-4} to 10^{-7} (29). All dilutions were plated in duplicate.

Plates with a 6-cm diameter (Greiner, Kremsmünster, Austria) were used. All plates were incubated at 37°C for 1–4 d. Nutrient agar and eosine methylene blue agar were incubated aerobically; all other media were incubated anaerobically. After incubation, colonies were counted according to colony morphology. Counts from duplicate plates were averaged. The quality of the media and the incubation technique for each batch was checked by plating in duplicate selected test bacteria on the plates. Anaerobic conditions were controlled by using anaerobic indicator strips. All visible colony morphologies were tested microscopically to determine the selectivity of the media.

Statistical analyses

The average values of each endpoint variable in the feces and breath from both the run-in and intervention periods for each subject were calculated and used to determine the differences between intervention and run-in diets. Differences were checked for normality by visually inspecting the normal probability plots (univariate procedure). Bacterial counts were log transformed to fit a normal distribution. The significance of the differences between the interventions was assessed by analysis of variance without interactions by using a model with subject (general linear models procedure). Adding the start group to this model did not contribute to significance; thus, there were no significant effects of time.

If there was a significant difference between treatments ($P < 0.05$), group means were compared by using Dunnett's test. This method encompasses a downward adjustment of the significance limit for multiple testing. Our strategy for controlling a type II error was based on our main outcome variable: bifidobacterial counts. A pilot experiment was performed before the study to assess the within-subject variation of bacterial counts (after they were log transformed), which was 5% for bifidobacteria (data not shown).

The present study was designed to detect a 7% increase in bifidobacterial counts with 80% confidence after correction for the placebo treatment, which is about half the effect observed by Bouhnik et al (9). We used data from the literature and from former studies at our department to estimate the variances for the other variables and used these to predict detectable responses to transgalactooligosaccharides. The variation in effect of the treatments was calculated and used to estimate the detectable effect of the treatment for a given probability (30). The statistical analysis package SAS (version 6.09; Statistical Analysis Systems Institute, Inc, Cary, NC) was used to perform the statistical analyses.

RESULTS

The characteristics of the subjects who completed the study are given in **Table 1**. One female subject withdrew in the first week of the experiment because of personal reasons; data from this subject were excluded from analyses. All other subjects

TABLE 1
Subject characteristics[1]

	Placebo group (n = 7 M, 6 F)	Low-TOS group (n = 7 M, 6 F)	High-TOS group (n = 8 M, 6 F)
Age (y)	37.8 ± 17.6	36.5 ± 17.6	42.9 ± 14.8
Weight (kg)	72.9 ± 10.5	71.0 ± 7.5	71.3 ± 10.6
Length (m)	1.79 ± 0.09	1.75 ± 0.09	1.76 ± 0.12
Body mass index (kg/m^2)	22.7 ± 2.6	23.4 ± 3.1	23.0 ± 2.1

[1] $\bar{x}$ ± SD. TOS, transgalactooligosaccharides. There were no significant differences between groups.

completed the study. Some volunteers in the low-TOS and high-TOS groups reported flatulence, but there were no reports of problems with the palatability of the diets or the juices.

Initial energy intakes were adjusted in 21 of the volunteers to maintain stable body weights. The mean daily intakes of energy and the composition of the diets, as determined by chemical analyses of duplicate diets and juices plus calculated contribution of free-choice items, are given in **Table 2**. Energy supplied by the free-choice items did not differ significantly between the dietary regimens and accounted for 11.2% of total energy (range: 7.7–14.4%). There were no significant differences between the run-in and intervention periods or between treatment groups in intakes of energy, protein, carbohydrate, fat, and fiber. The low-TOS diet provided a mean of 8.5 g transgalactooligosaccharides/d and the high-TOS diet 14.4 g transgalactooligosaccharides/d.

Breath-hydrogen concentrations

Individual changes in breath-hydrogen concentration between the intervention and run-in periods are given in **Figure 1**. The mean change (intervention minus run-in) was significantly higher ($P < 0.01$) in the high-TOS group than in the placebo group (95% CI: 1.8, 11.2 ppm). There were no significant differences between the low-TOS and placebo groups.

Bowel habits and stool composition

Data on bowel habits, fecal weight, fecal nitrogen density, and fecal pH are given in **Table 3**. There was no fecal excretion of transgalactooligosaccharides with any of the interventions. Transgalactooligosaccharide consumption did not significantly affect reported stool frequency, percentage of fecal dry matter, fecal weight, or fecal pH. There was a significant increase in fecal nitrogen density in the high-TOS group after correction for placebo, but not in the low-TOS group.

Short-chain fatty acids and bile acids in fecal water

Total short-chain fatty acid concentrations in fecal water did not differ significantly between the 3 groups (**Table 4**). The pH of fecal water and concentrations of acetate, propionate, butyrate, and the sum of isobutyrate, valerate, isovalerate, and caproate were also not significantly different between groups. The molar ratio of acetate to propionate to butyrate was ≈62:19:10 in all 3 groups. The total daily excretion of short-chain fatty acids was ≈13 mmol/d. There were no significant differences in the excretion of short-chain fatty acids between the 3 groups.

Total concentrations of bile acids in fecal water decreased after intervention in all 3 groups, but there were no significant differences between groups (**Table 5**). The ratio of hydrophobic (mono + dihydroxy) to hydrophilic (keto + trihydroxy) bile acids was not affected significantly by the interventions. Secondary bile acids made up ≈85% of total bile acids in all 3 groups; (iso)deoxycholic acid was the most abundant bile acid. The total excretion of bile acids was ≈70 μmol/d. There were no significant differences in the excretion of bile acids between the 3 groups.

Ammonia, indoles, and skatoles in feces

Mean fecal concentrations of ammonia, indoles, and skatoles did not change significantly after transgalactooligosaccharide consumption (**Table 6**). The excretion of ammonia was ≈85 mg/d and was not significantly different between the 3 groups. The excretion of indoles was 3 mg/d and of skatoles 1.3 mg/d; there were no significant differences between the groups.

Intestinal microflora

The mean temperature of all samples for bacteriological analyses was 32.2°C. The temperature of 4 samples was <29.2°C (mean temperature minus 2 SD).These samples were

TABLE 2
Daily dietary intake during the study[1]

	Placebo group (n = 13)	Low-TOS group (n = 13)	High-TOS group (n = 14)
Energy (MJ)	11.0 ± 0.4[2]	10.5 ± 0.4	11.0 ± 0.5
Protein (% of energy)	16.2 ± 0.1	16.1 ± 0.1	15.9 ± 0.1
Fat (% of energy)	37.5 ± 0.3	37.3 ± 0.1	36.9 ± 0.3
Carbohydrates (% of energy)	44.3 ± 0.6	45.3 ± 0.5	45.7 ± 0.4
Alcohol (% of energy)	2.1 ± 0.7	1.3 ± 0.5	1.5 ± 0.5
Dietary fiber (g/MJ)	1.77 ± 0.02	1.74 ± 0.02	1.76 ± 0.01
Transgalactooligosaccharides (g)	0	8.5	14.4[3]

[1] TOS, transgalactooligosaccharides.
[2] $\bar{x}$ ± SEM.
[3] Significantly different from the placebo and low-TOS groups, $P < 0.0001$.

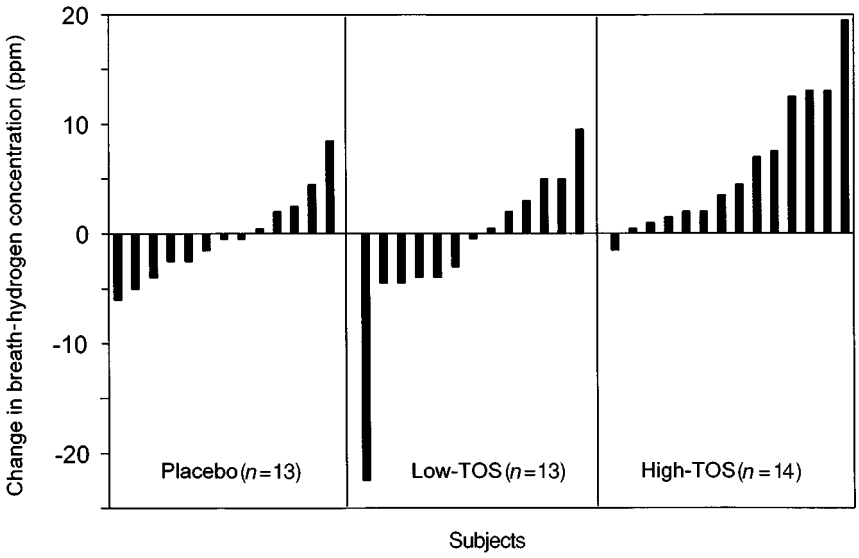

FIGURE 1. Individual changes in breath-hydrogen concentrations in the placebo (high-protein, low-fiber diet), low-TOS (high-protein, low-fiber diet supplemented with 8.5 g transgalactooligosaccharides/d), and high-TOS (high-protein, low-fiber diet supplemented with 14.4 g transgalactooligosaccharides/d) groups. Breath samples were taken twice at the end of the run-in period and twice at the end of the intervention period.

small and probably cooled down more quickly than the others. No sample was excluded from analyses because of a low temperature. Fecal bacteria counts were not affected significantly by the interventions. Control strains grew well on all media batches, indicating that the media did not inhibit growth. No oxygen leaks were detected.

There was a significant increase in the amount of bifidobacteria in the placebo ($P < 0.025$), low-TOS ($P < 0.05$), and high-TOS ($P < 0.05$) groups. The differences between these changes were not significant (**Table 7**). Mean changes (and 95% CIs) in fecal bacterial counts in the 3 groups are shown in **Figure 2**. The ratio of bifidobacteria to the total amount of anaerobic bacteria was ≈10 and was not affected significantly by the interventions.

DISCUSSION

Our findings showed that transgalactooligosaccharides, although fully fermented in the large intestine, did not beneficially affect putative risk markers of colon cancer or change significantly the composition of the intestinal microflora. To our knowledge, this is the first placebo-controlled feeding trial of transgalactooligosaccharide fermentation in humans.

Dietary intake, compliance with treatment, fecal sampling,

TABLE 3
Bowel habits and fecal weight, nitrogen density, and pH[1]

	Placebo group (n = 13)	Low-TOS group (n = 13)	High-TOS group (n = 14)	Difference (95% CI)[2] Low-TOS compared with placebo	Difference (95% CI)[2] High-TOS compared with placebo
Number of stools per week					
Run-in period	8.1 ± 0.7[3]	6.2 ± 0.5	7.9 ± 0.7	—	—
Intervention period	7.8 ± 0.6	6.5 ± 0.3	8.0 ± 1.0	0.69 (−1.0, 2.38)	0.38 (−1.27, 2.04)
Fecal weight (g/d)					
Run-in period	147 ± 11	113 ± 12	146 ± 22	—	—
Intervention period	139 ± 14	127 ± 14	142 ± 18	22.3 (−21.2, 65.9)	3.52 (−39.3, 46.3)
Fecal dry matter (%)					
Run-in period	23.3 ± 1.4	27.3 ± 1.6	25.7 ± 1.6	—	—
Intervention period	24.6 ± 1.3	26.0 ± 1.3	25.7 ± 1.5	−2.61 (−5.55, 0.33)	−1.36 (−4.25, 1.52)
Nitrogen density (% dry matter)					
Run-in period	5.2 ± 0.1	5.3 ± 0.1	5.1 ± 0.1	—	—
Intervention period	5.3 ± 0.2	5.5 ± 0.1	5.6 ± 0.1	0.16 (−0.14, 0.46)	0.33 (0.04, 0.62)[4]
Fecal pH					
Run-in period	6.8 ± 0.1	6.7 ± 0.1	6.6 ± 0.1	—	—
Intervention period	6.7 ± 0.1	6.7 ± 0.1	6.7 ± 0.1	0.03 (−0.24, 0.30)	0.18 (−0.08, 0.45)

[1] TOS, transgalactooligosaccharides.
[2] Calculated as differences between changes (intervention minus run-in).
[3] $\bar{x}$ ± SEM.
[4] Significantly different from placebo group, $P < 0.05$.

TABLE 4
Concentration of short-chain fatty acids and pH of fecal water[1]

	Placebo group (n = 10)	Low-TOS group (n = 12)	High-TOS group (n = 12)	Difference (95% CI)[2]	
				Low-TOS compared with placebo	High-TOS compared with placebo
Total short-chain fatty acids (mmol/L)					
Run-in period	114.2 ± 9.6[3]	108.6 ± 8.5	111.9 ± 8.8	—	—
Intervention period	110.5 ± 9.6	112.5 ± 7.9	116.5 ± 8.7	4.2 (−13.3, 21.8)	5.9 (−11.3, 23.5)
Acetate (mmol/L)					
Run-in period	71.8 ± 6.6	65.4 ± 5.4	69.2 ± 4.8	—	—
Intervention period	70.4 ± 6.2	69.9 ± 5.4	71.6 ± 4.8	4.5 (−6.7, 15.7)	2.5 (−8.7, 13.7)
Propionate (mmol/L)					
Run-in period	23.8 ± 1.8	20.3 ± 1.8	21.6 ± 2.3	—	—
Intervention period	22.6 ± 2.3	20.6 ± 1.6	23.0 ± 3.0	0.5 (−5.1, 6.0)	2.2 (−3.3, 7.8)
Butyrate (mmol/L)					
Run-in period	11.4 ± 1.7	12.2 ± 1.6	11.9 ± 1.9	—	—
Intervention period	10.5 ± 1.5	12.1 ± 1.2	11.9 ± 1.5	0.2 (−2.3, 2.7)	0.7 (−1.8, 3.1)
Sum of iso-C_4-C_6 fatty acids (mmol/L)[4]					
Run-in period	7.3 ± 1.0	10.6 ± 0.9	9.2 ± 1.2	—	—
Intervention period	7.0 ± 0.8	9.9 ± 0.5	9.9 ± 1.0	−0.9 (−2.8, 1.0)	0.5 (−1.4, 2.4)
pH of fecal water					
Run-in period	7.0 ± 0.1	7.0 ± 0.1	6.8 ± 0.1	—	—
Intervention period	7.0 ± 0.1	7.0 ± 0.2	7.1 ± 0.1	−0.1 (−0.4, 0.3)	0.2 (−0.2, 0.5)

[1] TOS, transgalactooligosaccharides. Observations were missing for all 3 groups because there was too little fecal water extractable from feces. There were no significant differences between treatment groups.
[2] Calculated as differences between changes (intervention minus run-in).
[3] $\bar{x}$ ± SEM.
[4] Sum of isobutyrate (iso-C_4), valerate (C_5), isovalerate (iso-C_5), and caproate (C_6).

and statistical power

We believe that the lack of effect on the main outcome variables cannot be explained by differences in dietary intake, noncompliance of the volunteers, inappropriate sampling and analysis of feces, or insufficient statistical power. We carefully controlled the energy contents and nutrient compositions of the diets during the study: there were no significant differences between the run-in and intervention periods or between the diets of the 3 groups. We conclude that differences in dietary intakes were negligible throughout the study and could not have affected the outcome.

The oligosaccharides used were dissolved in fruit juices. One of the 3 daily servings was consumed with a hot meal at our department. The other 2 servings were consumed outside the department; however, compliance was near 100% based on inspection of the juice bottles. Therefore, we excluded the possibility that noncompliance of the volunteers affected the results.

We carefully handled and stored the fecal samples that were used for measuring the composition of the intestinal microflora to minimize exposure to oxygen (31–34). All fecal samples were transported into an anaerobic cabinet within 5 min of defecation and bifidobacterial counts agreed with those in other fecal flora studies (7–9, 35–37). We conclude that the fecal sampling in our study was done carefully and could not have affected the results in any negative way.

The variance in bifidobacteria in the present study was somewhat lower (4.8%) than we had anticipated (ie, 5%), probably because of the controlled diet. The protocol provided an 80% chance of detecting a 6.0% increase in the amount of bifidobacteria after correction for placebo. We conclude from this a posteriori power analysis that the actual effect of transgalactooligosaccharides on bifidobacteria is probably <6.0%.

For most other outcome variables we were able to detect effects of ≤20% (range: 2.8–18.8%) with 80% confidence. The within-subject variation of fecal lactobacilli and bile acids was large (25% and 50%, respectively), as we had anticipated on the basis of previous studies (38). We had no data on the variation of indoles and skatoles seen in other studies; however, it appeared to be high (30% and 40%, respectively). For lactobacilli, bile acids, indoles, and skatoles, a treatment-induced effect of 30–60% was detectable with the present study design. The detectable effects in our study were largely as we had anticipated and we therefore excluded the possibility that an insufficient power influenced our main outcomes.

Apparent fermentability and gaseous response

No transgalactooligomers were recovered from any of the fecal samples. Although it cannot be excluded that transgalactooligosaccharides are degraded to some extent in the stomach or small intestine, we know from in vitro experiments that oligosaccharides are acid-stable and are not hydrolyzed by human intestinal enzymes (N Asp, unpublished observations, 1994). It is thus likely that all the transgalactooligosaccharides in our study were fermented by bacteria in the colon. To our knowledge, this is the first study showing such complete fermentation of transgalactooligosaccharides on the basis of the content of oligomers in the feces.

Bacterial degradation of transgalactooligosaccharides was reflected by the production of breath hydrogen. In the high-TOS group, breath-hydrogen concentrations increased by 130%, whereas there was no increase in the placebo group. There was no significant difference in breath-hydrogen concentrations between the low-TOS and placebo (95% CI: −7.5, 5.5 ppm) groups. Other dietary fibers might have masked the effect in the low-TOS group. We excluded hydrogen responders to lactose from our study, but we did not check for nonhydrogen producers. Possible nonhydrogen producers might have decreased the effect of trans-

TABLE 5
Concentration of primary and secondary bile acids in fecal water[1]

	Placebo group (n = 11)	Low-TOS group (n = 10)	High-TOS group (n = 10)	Difference (95% CI)[2]	
				Low-TOS compared with placebo	High-TOS compared with placebo
Total bile acids (μmol/L)					
Run-in period	742.5 ± 136.1[3]	680.7 ± 138.0	584.2 ± 132.8	—	—
Intervention period	681.1 ± 110.0	520.8 ± 82.1	479.3 ± 83.9	−80.8 (−527.7, 366.0)	4.7 (−442.2, 451.5)
Cholic acid					
Run-in period	141.2 ± 57.9	61.7 ± 30.2	85.3 ± 50.3	—	—
Intervention period	94.8 ± 44.5	29.5 ± 7.3	61.4 ± 35.2	12.0 (−60.6, 84.6)	29.4 (−43.2, 102.0)
Chenodeoxycholic acid					
Run-in period	32.2 ± 11.3	23.8 ± 9.1	17.2 ± 5.9	—	—
Intervention period	25.8 ± 9.3	13.1 ± 2.0	17.7 ± 4.7	−4.3 (−24.7, 16.0)	6.0 (−14.4, 26.3)
(iso)Deoxycholic acid					
Run-in period	321.0 ± 84.9	336.2 ± 60.1	282.0 ± 80.5	—	—
Intervention period	298.2 ± 48.7	277.5 ± 53.8	225.9 ± 42.9	−27.0 (−275.3, 221.3)	−6.0 (−254.3, 242.3)
(iso)Lithocholic acid					
Run-in period	103.1 ± 28.3	155.0 ± 29.0	104.2 ± 41.0	—	—
Intervention period	105.4 ± 20.7	120.9 ± 26.4	98.1 ± 33.3	−27.1 (−167.8, 113.7)	0.6 (−140.1, 141.4)
Ursodeoxycholic acid					
Run-in period	62.8 ± 25.9	18.2 ± 5.1	22.4 ± 6.0	—	—
Intervention period	73.5 ± 39.7	14.2 ± 2.9	12.3 ± 2.1	−14.5 (−53.2, 24.1)	−19.2 (−57.9, 19.4)
12-keto-Lithocholic acid					
Run-in period	55.4 ± 5.4	62.9 ± 8.4	56.6 ± 6.9	—	—
Intervention period	56.9 ± 4.7	53.5 ± 5.4	50.3 ± 6.9	−10.0 (−20.5, 0.4)	−5.1 (−15.6, 5.3)
7-keto-Deoxycholic acid					
Run-in period	26.7 ± 10.8	22.9 ± 6.4	16.5 ± 4.8	—	—
Intervention period	26.5 ± 14.3	12.2 ± 2.3	13.7 ± 4.2	−9.8 (−23.4, 3.8)	−0.9 (−14.5, 12.7)
Hydrophobic:hydrophilic bile acids					
Run-in period	3.6 ± 1.2	4.5 ± 0.7	4.2 ± 1.4	—	—
Intervention period	4.4 ± 0.9	4.7 ± 0.9	3.8 ± 0.9	−0.4 (−3.7, 2.8)	−0.9 (−4.2, 2.3)

[1] TOS, transgalactooligosaccharides. Observations were missing for all 3 groups because there was too little fecal water extractable from feces. Primary bile acids: cholic acid and chenodeoxycholic acid; secondary bile acids: (iso)deoxycholic acid, (iso)lithocholic acid, ursodeoxycholic acid, 12-keto-lithocholic acid, and 7-keto-deoxycholic acid. There were no significant differences between groups.
[2] Calculated as differences between changes (intervention minus run-in).
[3] $\bar{x}$ ± SEM.

galactooligosaccharides on mean hydrogen concentrations.

Hydrogen production in response to transgalactooligosaccharides was found previously in both rats (39–41) and humans (6). Bouhnik et al (9) compared hydrogen excretion in humans after 1 d of transgalactooligosaccharide consumption (10 g/d) with excretion after 7, 14, and 21 d. They observed a lower hydrogen response with time, possibly because the microflora adapted to the substrate.

Bowel habits and stool composition

We did not observe any significant effects of transgalactooligosaccharides on the reported frequency of defecation, daily fecal weight, or fecal dry matter. We did show a significant effect on the nitrogen density of the fecal dry matter with the high-TOS intervention. We hypothesized that transgalactooligosaccharides would increase bacterial proliferation and thereby increase bacterial mass (42–44). We found a significant increase of 6.3% in the nitrogen density of the fecal dry matter after the high-TOS intervention, which increased nitrogen excretion by 0.12 g/d after correction for placebo. This increase was likely due to an increase in bacterial mass (37, 42, 45–47). The additional 0.12 g N excreted is equivalent to ≈1.9 g bacterial solids (48) and 9.5 g wet stool (42). The subsequent theoretical changes in fecal wet weight and fecal dry matter due to the increased bacterial mass were probably too small to detect.

Short-chain fatty acids and bile acids in fecal water

Transgalactooligosaccharides did not significantly affect fecal pH or the concentrations of short-chain fatty acids or bile acids in fecal water. Our data do not allow firm conclusions about the effect of transgalactooligosaccharides on the production of short-chain fatty acids in fecal water or on the pH of the colon contents. When hydrogen is produced, most bacteria simultaneously produce short-chain fatty acids (4). Macfarlane et al (49), in an autopsy study of victims of sudden death, found high amounts of short-chain fatty acids in the proximal colon and lower amounts toward the end of the gastrointestinal tract. They attributed this finding to rapid absorption of these acids by the colonic mucosa. As oligosaccharides are rapidly fermented, possibly to a large extent in the proximal colon, neither fecal pH nor the fecal concentration of short-chain fatty acids is a good indicator of saccharolytic activity. Other researchers also failed to show any effects of fermentable carbohydrates on short-chain fatty acids or fecal pH (2, 12, 38, 44, 50). Of the total amount of short-chain fatty acids, 62% was acetate, 19% was propionate, and 10% was butyrate. These proportions are in the same range as reported previously (12, 37, 38, 51) and did not differ significantly between the 3 groups.

We were unable to show any effects of transgalactooligosaccharides on the concentration of bile acids in fecal water. We

TABLE 6
Fecal concentration of ammonia, indoles, and skatoles[1]

	Placebo group (n = 12)[2]	Low-TOS group (n = 13)	High-TOS group (n = 14)	Difference (95% CI)[3]	
				Low-TOS compared with placebo	High-TOS compared with placebo
	μg/g	μg/g	μg/g	μg/g	
Ammonia					
Run-in period	561 ± 44[4]	763 ± 55	630 ± 51	—	—
Intervention period	618 ± 51	759 ± 68	682 ± 61	−60 (−215, 95)	−4 (−157, 148)
Indoles					
Run-in period	21 ± 4	23 ± 5	23 ± 4	—	—
Intervention period	23 ± 4	19 ± 3	22 ± 4	−5 (−14, 4)	−3 (−11, 6)
Skatoles					
Run-in period	8 ± 2	14 ± 4	15 ± 4	—	—
Intervention period	9 ± 3	13 ± 4	14 ± 4	−3 (−10, 4)	−2 (−9, 4)

[1] TOS, transgalactooligosaccharides. There were no significant differences between groups.
[2] One observation was missing because there was urine in the feces.
[3] Calculated as differences between changes (intervention minus run-in).
[4] $\bar{x}$ ± SEM.

hypothesized that acidification of the colon contents, as a result of oligosaccharide fermentation, would lead to precipitation of the soluble deconjugated bile acids (52–54) and to suppression of the bacterial conversion of primary to secondary bile acids (54, 55). This would lead to lower concentrations of bile acids and a smaller ratio of hydrophobic to hydrophilic bile acids in the aqueous phase of feces, resulting in a bile acid profile with smaller cytolytic capacity (56). With a low-fiber diet, the production of acids by fermentation processes is probably low. The amount of fermentable transgalactooligosaccharides added to the low-fiber diet used in the present study might not have been sufficient to achieve the acidification necessary for the expected effects on bile acids. Because of the high within-subject variation, only large effects on the concentration of bile acids were detectable with our study design, which allowed us to show effects of 60% with 90% confidence and of 50% with 80% confidence. Although such effects have been shown by others, who used different dietary treatments (12, 14, 57), we might have missed possible smaller effects.

Ammonia, indoles, and skatoles in feces

Transgalactooligosaccharides did not lower fecal concentrations of the protein degradation products ammonia, indoles, and skatoles. We hypothesized that transgalactooligosaccharides would decrease protein fermentation products by depressing protein fermentation (44, 58) and by stimulating the use of ammonia as a nitrogen source in bacterial growth (46, 58, 59). We did not observe a decrease in the fecal concentrations of the protein degradation products ammonia, indoles, and skatoles or in the concentration short-chain fatty acids that originate from bacterial breakdown of amino acids (3, 60). Ito et al (8, 61) and Djouzi and Andrieux (39) showed a decrease in one or more of the above-mentioned variables. Ito et al (8) showed a 37.2% decrease in the concentration of indoles after Japanese men consumed a diet supplemented with 2.5 g transgalactooligosaccharides/d for 3 wk. The power of detecting such an effect in our study was 80%.

We chose to use a background diet with a relatively high protein content (16%) to increase the protein fermentation of dietary proteins (62). We thus expected to increase the chance of showing inhibiting effects of transgalactooligosaccharides on protein fermentation. Levrat et al (46) compared the effects of the fermentable carbohydrate inulin on protein fermentation in rats fed high- or moderate-protein diets. They showed that inulin in the diets increased the use of ammonia as a source of bacterial growth. This effect was most prominent when the dietary protein content was moderate and not high. In high-protein diets, the fecal ammonia concentration is determined by uremia and a high flux of urea from plasma to the colon (46, 47). We conclude that transgalactooligosaccharides have no significant effects on protein fermentation when used as a supplement in high-protein diets.

Intestinal microflora

We did not observe any significant effects of transgalactooligosaccharides on the composition of the intestinal microflora in a placebo-controlled feeding trial. It was striking that bifidobacterial counts increased by 4% during the study, independently of the treatment. The differences in increases in bifidobacterial counts between the placebo and transgalactooligosaccharide-supplemented groups were not significant. If a placebo group had not been used, the conclusion would have been that transgalactooligosaccharides selectively increase bifidobacteria in the gut. We cannot explain the increase in bifidobacterial counts throughout the study. The background diets were the same and the fecal samples were collected and analyzed in the same way for all 3 groups. None of the other bacteria counted increased during the study. Moreover, test bacteria were analyzed every week and showed no tendency to rise during the study. We hypothesized that the bifidobacteria might have adapted to the high-protein, low-fiber background diet. It is known from animal studies that sudden extreme changes in diet can have profound effects on the composition of the intestinal microflora. The flora will adapt gradually to the changes in substrate supply (63). There was a nonsignificant rise in lactobacilli in both the low-TOS (by 14%) and high-TOS (by 10%) groups, which we were unable to detect because of the large variation. Other researchers have shown that certain nondigestible oligosaccharides selectively stimulate the growth of bifidobacteria in the large intestine. In many of these studies, linear study designs were used and measurements were made before and after

TABLE 7
Fecal bacterial counts[1]

	Placebo group (n = 13)	Low-TOS group (n = 13)	High-TOS group (n = 14)
		$\log_{10}$ CFU/g	
Total anaerobes			
Run-in period	10.5 ± 0.1	10.4 ± 0.1	10.4 ± 0.1
Intervention period	10.6 ± 0.1	10.6 ± 0.1	10.6 ± 0.1
Bifidobacteria			
Run-in period	9.4 ± 0.1	9.4 ± 0.1	9.2 ± 0.1
Intervention period	9.8 ± 0.1	9.7 ± 0.1	9.6 ± 0.1
Lactobacilli			
Run-in period	5.0 ± 0.5	5.0 ± 0.5	5.9 ± 0.5
Intervention period	5.0 ± 0.7	5.7 ± 0.7	6.5 ± 0.7
Clostridia			
Run-in period	7.9 ± 0.2	8.0 ± 0.1	8.0 ± 0.1
Intervention period	8.1 ± 0.2	8.2 ± 0.1	7.9 ± 0.1
Total aerobes			
Run-in period	7.5 ± 0.2	7.5 ± 0.2	7.5 ± 0.2
Intervention period	7.6 ± 0.2	7.3 ± 0.2	7.3 ± 0.1
Escherichia coli			
Run-in period	7.1 ± 0.2	6.5 ± 0.6	6.2 ± 0.6
Intervention period	7.1 ± 0.2	6.4 ± 0.4	6.7 ± 0.2

[1] $\bar{x}$ ± SEM. There were no significant differences between groups. TOS, transgalactooligosaccharides; CFU, colony forming units.

the treatment (8, 9, 35, 37). Our results indicate the importance of a placebo treatment to exclude the possible effects of time, placebo, and unknown factors.

Our volunteers were healthy and were not necessarily selected for having low bifidobacterial counts. Consumption of the transgalactooligosaccharide-supplemented high-protein, low-fiber diets did not significantly effect the composition of the intestinal flora. However, it may be that diet-induced changes on intestinal microflora are unlikely to occur when there is a stable and healthy balance of the microfloral population in the intestine.

Conclusions

We conclude that transgalactooligosaccharides, as a supplement to a Western diet, are completely fermented in the colon of healthy individuals, but do not beneficially affect the composition of the intestinal microflora or the putative risk markers of colon cancer. We believe that the lack of such an effect on the main outcome variables of our study (ie, composition and activity of the intestinal microflora) was not explained by differences in dietary intakes, noncompliance of the volunteers, inappropriate sampling of feces, or insufficient statistical power. It is possible that transgalactooligosaccharides might have such a beneficial effect in

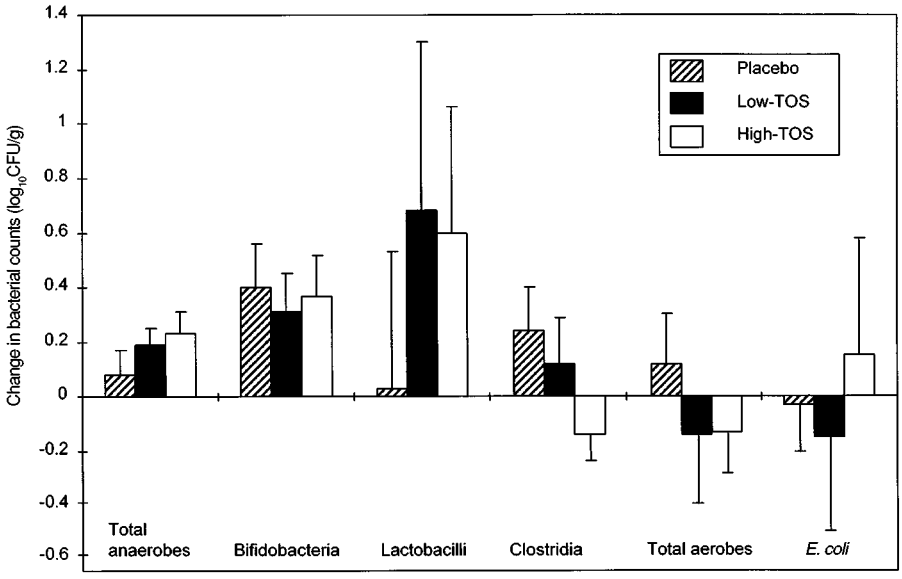

FIGURE 2. Mean changes (and 95% CIs) in fecal bacterial counts in the placebo (high-protein, low-fiber diet), low-TOS (high-protein, low-fiber diet supplemented with 8.5 g transgalactooligosaccharides/d), and high-TOS (high-protein, low-fiber diet supplemented with 14.4 g transgalactooligosaccharides/d) groups. There were no significant differences between groups. CFU, colony forming units.

other study populations (eg, persons with low bifidobacterial counts) or when used as a supplement to a background diet different from the one we used in this study.

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