Physiological effects of consumption of fructo-oligosaccharides and transgalacto-oligosaccharides

Martine S. Alles

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Martine S. Alles

Proefschrift

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Stellingen

1

Fructo-oligosacchariden en transgalacto-oligosacchariden worden volledig gefermenteerd in het colon van gezonde mensen *(dit proefschrift)*.

2

Fructo-oligosacchariden hebben geen gunstig effect op het glucose of lipidemetabolisme van niet-insuline afhankelijke diabeten *(o.a. dit proefschrift)*.

3

Studies naar het effect van niet-verteerbare oligosacchariden op de samenstelling van de darmflora zijn van weinig waarde als het effect niet wordt vergeleken met dat van een placebo behandeling *(o.a. dit proefschrift)*.

4

Bij onderzoek naar de fysiologische effecten van niet-verteerbare oligosacchariden zijn mogelijke negatieve effecten op lange termijn tot dusver onderbelicht.

5

Het is onduidelijk of bij gezonde mensen een verlaging van de concentratie homocysteïne in het plasma leidt tot een verlaging van het risico op hart- en vaatziekten *(o.a. Kuller LH, Evans RW. Homocysteine, Vitamins, and Cardiovascular Disease. Circulation 1998; 98:196-199)*.

6

Het effect van lijnen op het humeur ondermijnt het effect van lijnen op het lichaamsgewicht *(o.a. Wells AS. Alterations in mood after changing to a low-fat diet. British Journal of Nutrition 1998; 79:23-30)*.

7

De status van de zeehond als ambassadeur van de waddenzee is gebaat bij een zekere mate van schaarsheid van zeehonden *(Alles J. Selling Seals – Public Information and Education on the Ambassador of the Wadden Sea, Wadden Sea Newsletter 1996; 2:25-30)*.

8

Ver doorgevoerde bezuinigingen in de psychiatrie hebben geleid tot een toename van de ernstige gevallen van burenoverlast *(o.a. Riagg Oost-Veluwe. Evaluatieverslag project hulpverlening in het kader van de OGGZ 1997/1998).*

9

Opvoeders doen er goed aan het voorlezen van hun kinderen tot een ritueel te maken *(van de Wouw J. Rituelen, sfeerlezen en luisterspanning – Leesbevordering op de basisschool. Bibliotheek & Samenleving 1991; 19:310-313).*

10

Bij natuurbeschermers die zich verzetten tegen de plaatsing van windturbines speelt het NIMBY-effect ('Not In My Back Yard') een belangrijke rol.

11

In kringen van intellectuelen is het niet bevorderlijk voor je sociale status om te zeggen dat tv-kijken je hobby is.

12

Wie fecaliën onderzoekt moet gemakkelijk over poep kunnen praten.

Stellingen behorend bij het proefschrift
Physiological effects of consumption of fructo-oligosaccharides and transgalacto-oligosaccharides
Martine S. Alles. Wageningen, 18 december 1998.

Voor Jan en Annemiek

Abstract

Physiological effects of consumption of fructo-oligosaccharides and transgalacto-oligosaccharides

PhD thesis by Martine S. Alles, Division of Human Nutrition and Epidemiology, Wageningen Agricultural University, the Netherlands.

Oligosaccharides naturally occur in many raw materials that are used for the manufacture of human foods. Plant oligosacccharides are often not digestible in the upper part of the gastrointestinal tract, but may be fermented in the colon by the intestinal microflora. These non-digestible oligosaccharides are generally considered dietary fiber. We studied the effects of two types of oligosaccharides, fructo-oligosaccharides and transgalacto-oligosaccharides, in humans using placebo controlled interventions. Special emphasis was put on measuring fermentation and on the effects on the composition of the intestinal microflora, putative colon cancer risk markers and glucose and lipid metabolism. We showed that healthy volunteers ferment fructo-oligosaccharides and transgalacto-oligosaccharides completely, as measured by the recovery of the oligomers in feces. The apparent fermentability of fructo-oligosaccharides by patients with an ileal pouch-anal anastomosis was 83%. Fermentation of both fructo-oligosaccharides and transgalacto-oligosaccharides increased the excretion of breath hydrogen. When hydrogen is produced, most bacteria simultaneously produce short-chain fatty acids, but the fecal concentration of short-chain fatty acids after consumption of either fructo-oligosaccharides or transgalacto-oligosaccharides was not increased. Rapid absorption of the acids by the colonic mucosa probably occurred. We showed substrate specific changes of bacterial glycosidase activity in fecal water after dietary intervention with non-digestible oligosaccharides and conclude that the glycolytic activity of the intestinal bacteria might be a useful biomarker of the colonic metabolic activity. We found no effects of transgalacto-oligosaccharides on the composition of the intestinal microflora of healthy volunteers, when tested in a controlled feeding trial against a placebo treatment. There were no effects of fructo-oligosaccharides and transgalacto-oligosaccharides on stool weight or on the fecal concentration of butyrate, nor effects of transgalacto-oligosaccharides on fecal bile acid profiles or on the concentration of fecal toxic protein fermentation products. We showed that fructo-oligosaccharides do not favorably affect fasting glycaemia or serum lipids in non-insulin dependent diabetic patients that are under strict medical control for their diabetes. Although fructo-oligosaccharides and transgalacto-oligosaccharides are completely fermented, we were unable to find any evidence that they beneficially affect putative colon cancer risk markers, the composition of the intestinal microflora or glucose and lipid metabolism. It remains possible that they have effects in other study-populations, when tested against another background diet or when combined with other fermentable substrates.

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1

General introduction

Background

Oligosaccharides are naturally present carbohydrates in many raw materials that are used for the manufacture of human foods. The number of possible oligosaccharide structures is extremely large as each monosaccharide has several free hydroxyl groups that can form a link to another monosaccharide. Simple oligosaccharides, such as the α-galacto-oligosaccharides and ß-fructo-oligosaccharides that are present in crop plants, are energy stores in plant seeds and mobilized during germination. Plants also contain more complex oligosaccharides, which may be covalently linked to proteins in glycoproteins and to lipids as glycolipids. Their function is largely unknown (Alberts *et al*, 1994). Oligosaccharides can also be formed during food processes such as beer brewing (Lehtonen & Hurme, 1994), wine production (Castino, 1972), bread baking (Rodionova *et al*, 1995) and in the processing of fruits for fruit juices (Schols, 1995).

Many of the plant oligosaccharides are not digestible in the upper part of the gastrointestinal tract. These non-digestible oligosaccharides are considered dietary fiber by most professionals in the field (Lee & Prosky, 1995), however, with the official AOAC analysis of total dietary fiber, oligosaccharides are not measured and therefore often not mentioned in food tables (Deutsch, 1997). The mean intake of dietary fiber without oligosaccharides, based on the official methods of analysis, is 23 g per day in the Netherlands (Anonymous, 1996; Anonymous, 1993*b*). Little is known about the non-digestible oligosaccharide content of foods and the mean intake of non-digestible oligosaccharides. Van Loo *et al* (1995) estimated the average daily intake of plant ß(2-1)-fructans, inulin and fructo-oligosaccharides to be 1-4 g per day in the North American population. The main sources were wheat and onions. Obviously, oligosaccharides significantly contribute to the dietary fiber fraction in our diet.

Oligosaccharides can be made industrially by extraction from foods, transglycosylation reactions or by hydrolysis of polysaccharides. When extracted from foods, they are obtained in their natural form. Enzymatically produced oligosaccharides are not always naturally occurring food components.

Interest in the specific health effects of non-digestible oligosaccharides originates from Japan, where oligosaccharides are extracted on a large scale from soybean whey and used as additive in health foods (Fujii & Komoto, 1991). More than ten years ago, Japanese studies already pointed to possible favorable effects of non-digestible oligosaccharides on colonic health (Tanaka *et al*, 1983; Minami *et al*, 1985; Mitsuoka *et al*, 1987; Masai *et al*, 1987; Kohmoto *et al*, 1988). However, most of these Japanese studies had no strict study design and were restricted to elderly subjects. The recent commercialization of non-digestible oligosaccharides as functional food ingredients has triggered much research in Western countries. Non-digestible oligosaccharides

might alter both the composition and the activity of the colonic microflora, and may -via endproducts of fermentation- have favorable effects on colon cancer risk and on glucose- and lipidmetabolism.

Rationale of the thesis and project team

In our studies, several hypotheses concerning effects of fructo-oligosaccharides and transgalacto-oligosaccharides in humans with Western dietary patterns were tested using placebo controlled interventions. Fructo-oligosaccharides naturally occur in various edible plants, such as onions and bananas, and are defined as those oligosaccharides belonging to the fructan family (Van Loo *et al*, 1995). Transgalacto-oligosaccharides are ß-galacto-oligosaccharides that are produced from lactose by enzymatic transgalactosylation. Similar components and ß-galacto-oligosaccharides with a more complex structure are present in human breast milk. Special emphasis was put on measuring fermentation, on the composition of the intestinal microflora, on colon cancer risk markers and on glucose- and lipidmetabolism. Table 1 summarizes the treatment, design and key-variables in the studies that are described in ***Chapters 2-6***.

In ***Chapters 2, 4 and 5***, we studied effects of oligosaccharides in healthy volunteers. In ***Chapter 3***, a group of patients with an ileal pouch-anal anastomosis was studied. In these patients, the colon is removed and part of the distal ileum is constructed into an anal reservoir which takes over part of the function of the colon. In ***Chapter 6*** we studied the effects of fructo-oligosaccharides on glucose and lipidmetabolism in non-insulin dependent diabetic patients. Finally, in ***Chapter 7*** the main findings are presented, and methodological issues and possibilities for further research are discussed.

The thesis was part of a multi-disciplinary research program at the Wageningen Agricultural University on the role of non-digestible oligosaccharides in food and feed. Four departments were involved in the project: Human Nutrition and Epidemiology, Animal Nutrition, Food Microbiology and Food Chemistry.

In the following six paragraphs, the rational of this thesis will be explained in more detail.

Table 1.
Treatment, design and key-variables in the studies described in this thesis.

	Treatment	Design	Parameters
Chapter 2	Fructo-oligosaccharides Placebo	Healthy men Cross-over design	Absorption Fermentation products Colon cancer risk markers (fecal concentration of butyrate, stool weight)
Chapter 3	Fructo-oligosaccharides Resistant Starch Placebo	Pouch patients Cross-over design	Fermentation products Colon cancer risk markers (fecal concentration of butyrate, stool weight)
Chapter 4	Transgalacto-oligosaccharides Placebo	Healthy humans Parallel design	Fermentation products Composition of microflora Colon cancer risk markers (fecal concentration of butyrate and of protein fermentation products, stool weight, bile acid profiles)
Chapter 5	See chapter 2 and 4	See chapter 2 and 4	Fermentation Colon cancer risk markers (bacterial glycosidase activity)
Chapter 6	Fructo-oligosaccharides Placebo	Diabetic patients Cross-over design	Glucose- and lipidmetabolism

Structure of fructo-oligosaccharides and transgalacto-oligosaccharides

We studied the effects of two types of oligosaccharides; fructo-oligosaccharides (Raftilose P95®, ORAFTI, Tienen, Belgium) and transgalacto-oligosaccharides (Elix'or®, Borculo Whey Products, Borculo, Netherlands). These products were chosen for practical reasons as they had the legal status of food ingredients and were obtainable in large enough quantities for human trials.

The oligosaccharides from Raftilose® are made from inulin which is extracted from chicory roots. The degree of polymerization of the fructo-oligosaccharides present in Raftilose® ranges from 2 to 7. The oligomers consist of fructose-units with or without a terminal glucose-unit. Linkages between the monosaccharide units are ß-2,1. The general structure is given in figure 1.

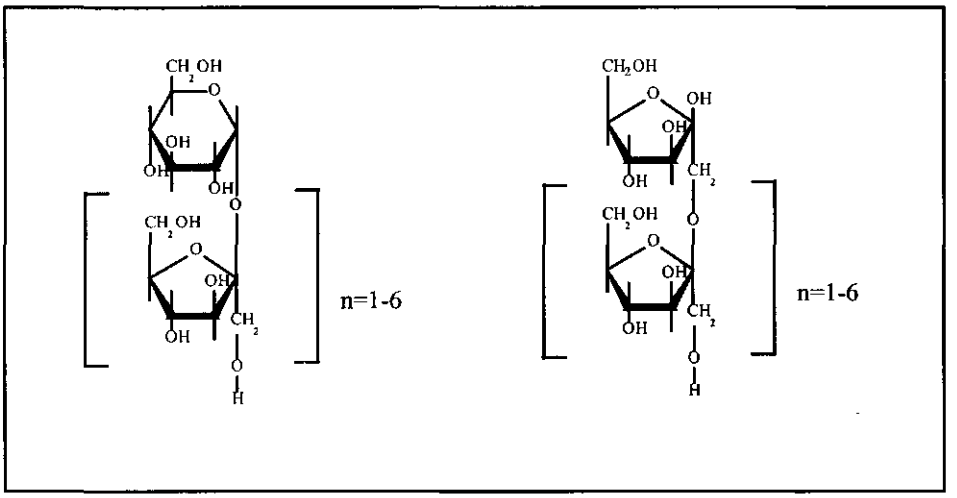
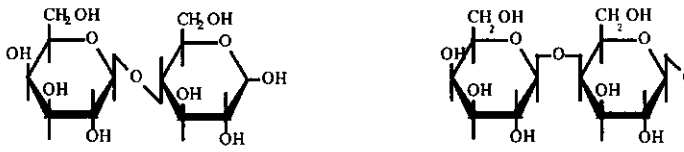


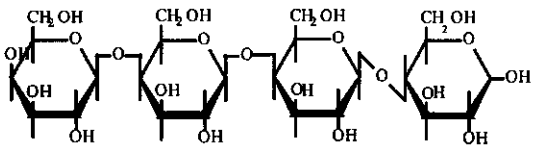
Figure 1. Fructo-oligosaccharides (Raftilose P95®, ORAFTI, Tienen, Belgium) with (left) or without (right) endstanding glucose molecule.

Elix'or® is produced from lactose by enzymatic transgalactosylation, using a β-galactosidase. It has a variety of oligomers consisting of glucose and galactose-units with mainly ß-1,4, but also ß-1,2, ß-1,3 and ß-1,6 linkages (Yanahira *et al*, 1995). The degree of polymerization of the transgalacto-oligosaccharides is 2 to 7. The structure is given in figure 2.



ß-D-Gal*p*-(1-4)-ß-D-Glc*p*

ß-D-Gal*p*-(1-4)-ß-D-Gal*p*-(1-4)-ß-D-Glc*p*



ß-D-Gal*p*-(1-4)-ß-D-Gal*p*-(1-4)-ß-D-Gal*p*-(1-4)-ß-D-Glc*p*

Figure 2. Examples of transgalacto-oligosaccharides made from lactose (upper left) (Elix'or®, Borculo Whey Products, Borculo, Netherlands).

Digestion and absorption

Human alimentary enzymes are not equipped with ß-fructosidase or ß-galactosidase activity. The enzyme lactase is an exception and only capable of degrading lactose. As a consequence fructo- and transgalacto-oligomers are able to survive partly the passage through the upper part of the gastro-intestinal tract (stomach and small bowel).

Digestion of fructo-oligosaccharides has been studied *in vitro* (Oku *et al*, 1984) and *in vivo* (Tokunaga *et al*, 1989; Bach Knudsen & Hessov, 1995; Molis *et al*, 1996). Three human trials were done to quantify the passage of fructo-oligosaccharides into the colon, two in ileostomy patients (Bach Knudsen & Hessov, 1995; Ellegard *et al*, 1997) and one in healthy volunteers, using an intubation technique (Molis *et al*, 1996). Both studies showed small losses (<13%) of the oligomers in the upper part of the gastrointestinal tract, probably due to hydrolysis in the stomach and microbial degradation in the small intestine. Hydrolysis by either pancreatic or intestinal enzymes did not occur.

Digestibility of transgalacto-oligosaccharides was studied *in vitro* showing that they are acid stable (unpublished results) and not hydrolyzed by lactase (Burvall *et al*, 1980). There are no studies on the small intestinal degradation of transgalacto-oligosaccharides *in vivo*.

A few studies investigated the possible absorption of the intact fructo-oligomers by the intestinal wall. Small traces of fructo-oligosaccharides were measured in some of the urine-samples of volunteers on fructo-oligosaccharide-rich diets (Rumessen *et al*, 1990; Molis *et al*, 1996). In ***Chapter 2*** we measured fructo-oligosaccharides in urine.

Fermentation

Substances that escape digestion by endogenous enzymes in the upper part of the gastrointestinal tract, may serve as substrates for the colonic microflora, which possesses a large enzymatic potential (Szylit & Andrieux, 1993). Colonic fermentation is the anaerobic process in which carbohydrates and proteins are metabolized by the intestinal microflora. Fermentation of carbohydrates will increase the production of gases, short-chain fatty acids such as acetate, butyrate and propionate and sometimes of lactate or ethanol. Protein fermentation increases the production of the branched chain fatty acids iso-butyrate and iso-valerate, and of phenolic compounds and ammonia. 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. It seems likely that the composition of the diet is of influence on the activity of the intestinal microflora (Cummings & MacFarlane, 1991).

The apparent fermentability of specific oligosaccharides can be measured in supplementation trials by determining the fecal excretion of the undigested and unfermented substance. In ***Chapters 2-4*** we studied the excretion of oligosaccharides in feces. Also, the endproducts of fermentation can be measured, such as hydrogen in breath or short-chain fatty acids in feces. To study the metabolic activity of the intestinal microflora, we measured different endproducts of fermentation after consumption of fructo-oligosaccharides (***Chapter 2***) and transgalacto-oligosaccharides (***Chapter 4***) in healthy humans and pouch patients ***(Chapter 3)***. In patients with ileal pouches ***(Chapter 3)***, we expected to find more profound effects on the fecal concentration of short-chain fatty acids. Fermentation metabolites are efficiently absorbed by the colon mucosa, but in pouch patients, the frequency of defecation is high and as a result, the time available for absorption is limited. We therefore hypothesized to find a lower absolute uptake of short-chain fatty acids by the pouch mucosal wall. The activity of bacterial glycolytic enzymes that are involved in the fermentation of oligosaccharides was studied in ***Chapter 5***.

Composition of the intestinal microflora

The microbial component of feces is estimated at 55% of the solids (Stephen & Cummings, 1980). Many different species of bacteria occur in the human large bowel. The majority is anaerobic, with the predominant genus being *Bacteroides*. Other important anaerobic species are bifidobacteria, lactobacilli and clostridia. The composition of the colonic microflora was always thought to be very stable over long periods of life (Simon & Gorbach, 1984), but some of the early Japanese studies showed that non-digestible oligosaccharides might induce beneficial changes (Tanaka *et al*, 1983; Minami *et al*, 1985; Mitsuoka *et al*, 1987; Masai *et al*, 1987; Kohmoto *et al*, 1988). Especially bifidobacteria were shown to be selectively stimulated by specific oligosaccharides. Bifidobacteria may comprise up to one quarter of the gut flora of normal healthy adults (Modler *et al*, 1990). They have a role in controlling the pH of the large intestine, through the production of lactic and acetic acid. A low pH might restrict the growth of many potential pathogens and putrefactive bacteria (Gibson & Wang, 1994; Modler *et al*, 1990) and might both depress the formation of secondary from primary bile acids and enhance the precipitation of bile acids (Hofmann & Mysels, 1992; Munster *et al*, 1994*b*; Nagengast *et al*, 1988*b*; Rafter *et al*, 1986). Cellular components of bifidobacteria may act as immunomodulators (Mitsuoka, 1990; Gibson & Roberfroid, 1995*b*).

Some recent studies confirmed the early reported effects of non-digestible oligosaccharides on the composition of the intestinal microflora (Ito *et al*, 1990; Buddington *et al*, 1996; Bouhnik *et al*, 1997; Bouhnik *et al*, 1996*b*; Gibson *et al*, 1995*a*). In most of these studies, a linear study design was used and possible time and placebo effects were thus neglected. In ***Chapter 4***, we compared the effects of transgalacto-oligosaccharides on the composition of the intestinal microflora with the effects of a placebo.

Colon cancer risk markers

Colorectal cancer is one of the most common malignancies in the Netherlands, as in most other Westernized countries (Netherlands Cancer Registry, 1990). Dietary patterns are considered to contribute to the etiology of sporadic (rather than inherited) colon cancer and especially dietary fiber might play a protective role here (Trock *et al*, 1990; Dwyer, 1993; Howe *et al*, 1992). Colon cancer risk may be modified by several fermentation related mechanisms (figure 3).

A low stool weight, as seen in many Westernized populations (80-120 g/d) is associated with increased colon cancer risk (Cummings *et al*, 1992). Fermentable carbohydrates are thought to increase fecal biomass (Cummings *et al*, 1992; Cummings & MacFarlane, 1991; Stephen &

Cummings, 1980; Gibson *et al*, 1995*a*). The effect of daily consumption of non-digestible oligosaccharides on fecal weight is reported in ***Chapter 2***, ***3*** and ***4***.

Colonic fermentation of non-digestible oligosaccharides may lead to an increase in the fecal concentration of butyrate (Younes *et al*, 1995; Roland *et al*, 1995; Campbell *et al*, 1997*b*). Butyrate is a putative protective factor towards colon cancer (Gamet *et al*, 1992; Scheppach *et al*, 1995; Csordas, 1996). It is a major fuel for the colonic mucosa and may inhibit cell proliferation and stimulate cell differentiation. The fecal concentration of short-chain fatty acids, including butyrate, was studied after consumption of fructo-oligosaccharides (***Chapter 2*** and ***3***) and transgalacto-oligosaccharides (***Chapter 4***).

Daily, about 5-10% of the bile acids that are used for the solubilization of lipids in the small intestine enter the colon. The primary bile acids (mainly cholate and chenodeoxycholate, conjugated with either glycine or taurine) are deconjugated and dehydroxylated by the colonic flora into the secondary bile acids (deoxycholate and lithocholate), which might have promoting effects on colonic carcinogenesis (Lapré & Meer, 1992; Bruce, 1987; Munster & Nagengast, 1993). Non-digestible oligosaccharides might lower the amount of secondary bile acids in fecal water (Rafter *et al*, 1986; Hofmann & Mysels, 1992; Reddy *et al*, 1992; Nagengast *et al*, 1988*b*; Munster *et al*, 1994*b*; Christl *et al*, 1997). In ***Chapter 4***, the effect of transgalacto-oligosaccharides on the profile of bile acids in fecal water is examined.

Endproducts of the bacterial degradation of proteins, such as ammonia, indoles and skatoles, might have adverse effects and are potentially associated with colonic cancer (Birkett *et al*, 1996; Clausen & Mortensen, 1992*a*; Clinton *et al*, 1988; Bone *et al*, 1976). Supplying the colon with fermentable carbohydrates might lower the concentration of the protein degradation products. In ***Chapter 3*** and ***4*** we examined the effects of fructo-oligosaccharides and transgalacto-oligosaccharides on the fecal concentrations of various protein fermentation products.

Some of the bacterial enzymes with glycolytic activity might have adverse effects in the colon. ß-glucuronidase is involved in the hydrolysis of glucuronide conjugates in the gut, which leads to the generation of toxic and carcinogenic metabolites (Rowland, 1988; Mallett & Rowland, 1987). The hydrolytic activity of ß-glucosidase is responsible for the generation of mutagenic aglycones (Mallett & Rowland, 1987). Several studies show that dietary fiber may lower these fecal bacterial enzymes (Reddy *et al*, 1992; Buddington *et al*, 1996; Mallett & Rowland, 1987). In ***Chapter 5***, we studied the bacterial glycosidase activity of fructo-oligosaccharides and transgalacto-oligosaccharides.

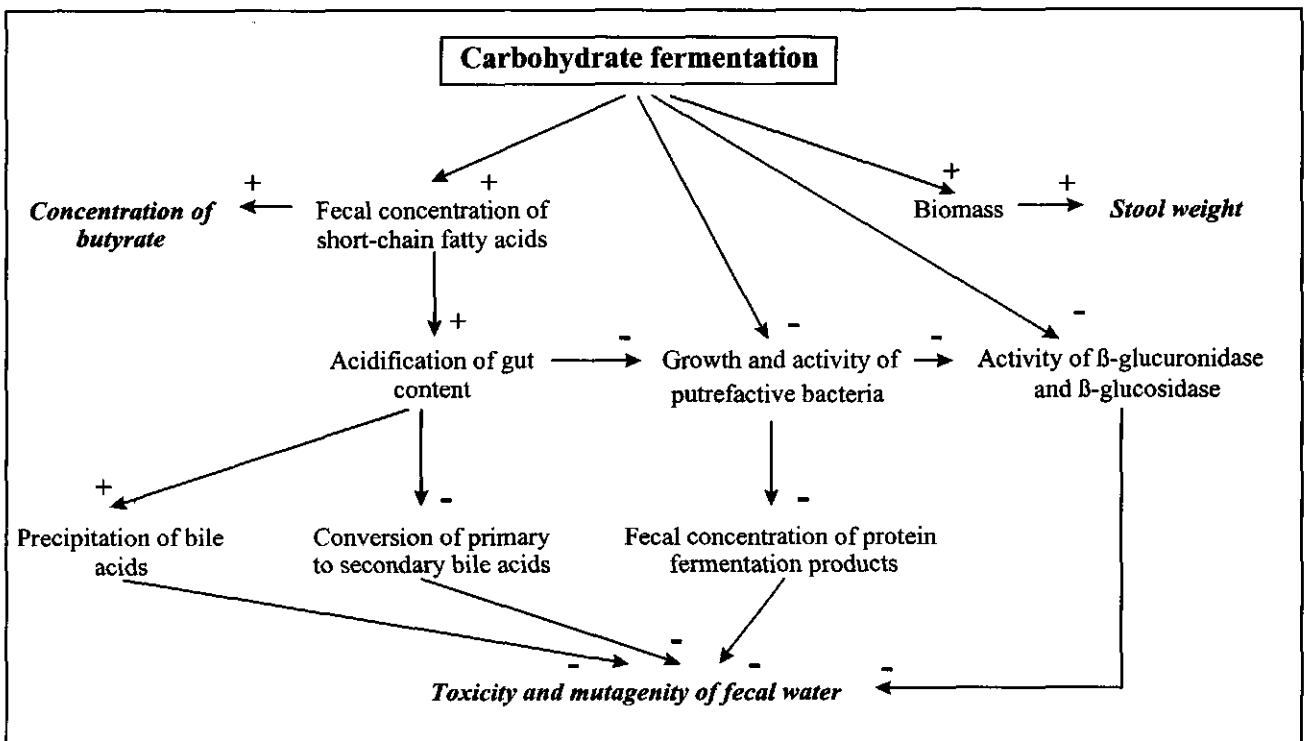


Figure 3. Hypotheses on the protective effects of colonic fementation of carbohydrates in relation to colon cancer risk markers.

Glucose- and lipid metabolism

The short-chain fatty acids that are produced during fermentation of non-digestible oligosaccharides are thought to be readily absorbed by the colonic mucosa (Pomare *et al*, 1985; Cummings *et al*, 1987*b*; Rombeau & Kripke, 1990). It is known that butyrate serves as a fuel for the mucosa, whereas acetate and propionate enter the portal blood and may influence systemic carbohydrate and lipid metabolism (Cummings *et al*, 1987*b*). One study with non-insulin dependent diabetic patients showed a decrease in both fasting blood glucose and serum lipids after consumption of fructo-oligosaccharides (Yamashita *et al*, 1984). Several rat experiments confirmed these results (Kok *et al*, 1996; Fiordaliso *et al*, 1995; Levrat *et al*, 1994; Delzenne *et al*, 1993), but two studies with healthy volunteers failed to show an effect of fructo-oligosaccharides on serum lipids or glucose metabolism (Luo *et al*, 1996; Pedersen *et al*, 1997). In ***Chapter 6***, we investigated the effects of fructo-oligosaccharides in non-insulin dependent diabetic patients on serum acetate concentrations, fasting glycaemia and serum lipids.

2

Fate of fructo-oligosaccharides in the human intestine

Martine S. Alles, Joseph G.A.J. Hautvast, Fokko M. Nagengast, Ralf Hartemink, Katrien M.J. Van Laere, Jan B.M.J..Jansen. *British Journal of Nutrition* 1996; 76:211-221.

Abstract

There is a need for studies on colonic fermentation in order to learn more about health and diseases of the colon. The aim of the present study was to evaluate the fate of two different doses of fructo-oligosaccharides (5 and 15 g/d) *v.* glucose in the intestine of healthy men. Twenty-four volunteers participated in a 5-week study. The study was a completely balanced multiple crossover trial using an orthogonal Latin-square design for three periods, with supplement periods of 7 d and two 7 d wash-out periods. Breath samples and fecal samples were collected. There was a clear gaseous response to the consumption of fructo-oligosaccharides. The highest dose significantly increased 24 h integrated excretion of breath hydrogen ($P < 0.05$). Breath hydrogen excretion after ingestion of 5 g fructo-oligosaccharides was higher than control, but did not reach significance. No effects on the total concentration of short-chain fatty acids in feces were observed, no modification of the molar proportions of the various short-chain fatty acids was observed. The fecal pH did not change. No changes in fecal weight were observed. No fructo-oligosaccharides were recovered in feces. We conclude that fructo-oligosaccharides added to the diet of young Western subjects are fully metabolized in the large intestine. The level of fermentation seems to be dose dependent.

Introduction

Studies on the effects of non-digestible oligosaccharides in elderly Japanese subjects suggest that dietary supplementation with fructo- or galacto-oligosaccharides is beneficial to the large intestine (Masai *et al*, 1987; Hidaka *et al*, 1986; Mitsuoka *et al*, 1987; Ito *et al*, 1990; Hayakawa *et al*, 1990). The studies show a higher activity of the colonic microflora in response to the consumption of oligosaccharides, as demonstrated by increased fermentation. During carbohydrate fermentation, gases and short-chain fatty acids are formed (Cummings *et al*, 1987*a*; Wang & Gibson, 1993). The production of short-chain fatty acids and the subsequent drop in colonic pH might protect against colonic cancer (Koo & Rao, 1991; Munster *et al*, 1994*b*). It is also suggested that oligosaccharides selectively stimulate bifidobacterial growth and thereby change the microfloral composition. Since bifidobacteria are considered to be beneficial genera, they may contribute to the establishment of a more healthy colonic environment (Mitsuoka, 1990; Modler *et al*, 1990; Gibson *et al*, 1995*a*).

However, because the Japanese studies often lacked a strict study design or focused primary on elderly people, we feel that they do not elucidate the metabolic fate of oligosaccharides in the large intestine. Also, observations made in Japan might not be applicable to populations with Western dietary patterns. Only a few studies have been performed within Western populations so far and they have not investigated to what degree the oligosaccharides are fermentable (Stone-Dorshow & Levitt, 1987; Rumessen *et al*, 1990; Gibson *et al*, 1995*a*). Thus, we need more information to evaluate the fermentation process of oligosaccharides.

The purpose of the present study was to examine the fate of fructo-oligosaccharides in the human intestine by studying several aspects of colonic fermentation in healthy young adults consuming a Western diet. The excretions of unfermented fructo-oligosaccharides and short-chain fatty acids in the feces, and hydrogen in the breath were measured.

Methods

Subjects and experimental design

Twenty-four healthy men aged 19-28 years volunteered to participate in the study. Women were excluded because of the possible influence of the menstrual cycle on fermentation (McBurney, 1991). All subjects had normal body weight (BMI 21.7 (SD 1.9) kg/m^2), had no history of diabetes or gastrointestinal disease, and had not recently been treated with antibiotics or laxatives. They were not under any medication and blood screening tests were normal. None of the volunteers smoked. Four volunteers were vegetarians, none of the volunteers used a special diet.

To avoid excess hydrogen being channeled into methane production, subjects chosen were non-methane producers. Three breath samples were collected on separate days, from each volunteer, before the study. Volunteers were classified as methane excreters, when in one or more of the samples the methane concentration exceeded 3 ppm above ambient air (Rumessen, 1992*a*; Nagengast *et al*, 1988*a*). Characteristics of the study population are given in Table 1.

Table 1.
Characteristics and dietary intake of the subjects

	Mean	SD
Age (y)	22.1	2.1
Weight (kg)	74.0	8.9
Height (m)	1.85	0.1
BMI (kg/m^2)	21.7	1.9
Energy intake (MJ)	13.7	3.6
Carbohydrate intake (%EI)	50.4	7.4
Fat intake (%EI)	34.2	6.2
Protein intake (%EI)	12.1	1.8
Dietary fiber (g)	39.7	11.9

Mean values for twenty-four male subjects and standard deviations between subjects (SD).
BMI, body mass index; EI, energy intake.

The study was a completely balanced multiple crossover trial using an orthogonal Latin-square design for three treatments, with supplement periods of 7 d and two 7 d wash-out periods between treatments. The volunteers consumed three different supplements in random order. At the end of each supplement period feces, breath and urine samples were collected.
All subjects gave their informed written consent before participating. The study protocol was approved by the Ethical Committee of the Department of Human Nutrition, Wageningen Agricultural University.

Supplements and food intake

Diet composition was calculated in every supplement period from 2 d estimated records, using the 1986-1987 release of The Netherlands nutrient data bank (Anonymous, 1987). Volunteers were asked to refrain from eating any fermented dairy products during the study.

The supplements consisted of fructo-oligosaccharides (Raftilose® P95; ORAFTI, Tienen, Belgium) and/or glucose (Cerestar Pur 01934; Cerestar Benelux BV, Sas van Gent, The Netherlands) in the following quantities: control, 0 g fructo-oligosaccharide+4.1 g glucose; SF5, 5 g fructo-oligosaccharide+2.7 g glucose; SF15, 15 g fructo-oligosaccharide+0 g glucose. They were dissolved in water and were of equal osmolarity.

Supplements were consumed in split dose together with breakfast and dinner. To estimate stool recovery, thirty barium impregnated rings were swallowed daily together with the supplements and were counted afterwards in the fecal samples. A small dose (240 μmol) of lithiumchloride (Merck, Darmstadt, Germany) was added to each supplement. To check for compliance, the recovery of lithium in 24 h urine was measured on the last 2 d of every supplement period (Sanchez-Castillo *et al*, 1987; Leclercq *et al*, 1990; Houwelingen *et al*, 1987).

Data collection

Body weight was measured on the first day of every supplement week, using electronic scales with a digital read-out to an accuracy of 0.05 kg (ED60-T; Berkel, Rotterdam, The Netherlands).

During the whole study period, volunteers kept a diary. They recorded time of supplement consumption, time of defecation, possible diseases or medication and deviations in eating, drinking or living behavior. Stool form was rated for all stools on a 5-point Likert scale which was based on the scale by Heaton *et al* (Heaton *et al*, 1992) (watery; soft, pudding-like; soft, snake-like; dry, cylindric; dry, hard pellets). Gastrointestinal complaints concerning flatulence, bloating, abdominal pains or cramps, eructations, nausea, vomiting and stomach pains or cramps were rated on a 4-point Likert scale (none; mild; moderate; severe).

Volunteers came to the Department of Human Nutrition twice at the end of each supplement period to defecate. Within 15 min after defecation the feces were weighed and immediately deep-frozen on dry ice to stop fermentation, then stored at -20 °C. On the last day of each supplement period, end-expiratory breath samples were taken at the Department of Human Nutrition at 4 h intervals from 08.00 hours (fasted) until 20.00 hours and again at 08.00 hours (fasted) the next morning. Urine (24 h) was collected on the last 2 d of every supplement period and 5 ml glacial acetic acid was added to all urine bottles before collection as a preserving agent.

Analytical procedures

The 24 h urine samples were pooled when both collections were complete. Lithium was measured as described previously (Leclercq *et al*, 1990) using an atomic absorption spectrophotometer (Type 2380, Perkin-Elmer, Norwalk, USA). Calibration was done with a standard concentration of 100 μmol/l and with water as a blank.

End-expiratory breath samples and ambient air samples were collected in plastic 60 mL syringes (Plastipak; Becton Dickinson, Dublin, Ireland). Methane was measured in end-expiratory breath samples, using gas chromatography (Chrompack 438 AS; Hewlett Packard 427, Middelburg, The Netherlands). Calibration was done (daily) with 5 ppm and 29 ppm methane-in-nitrogen gases (Intermar BV, Breda, The Netherlands). Within 2 h after collection the hydrogen concentration was measured using a standard electrochemical cell (Exhaled Hydrogen Monitor; Gas Measurement Instruments Ltd., Renfrew, Scotland). The cell was calibrated with a standard gas of 100 ppm hydrogen in air (Intermar BV)

All fecal samples were X-rayed before analysis to determine the number of barium rings (Philips Optimus M200, Eindhoven, The Netherlands). Dry weight of the feces was estimated by drying a portion for 4 d at 80 °C (Heraeus E45, Hanau, Germany). For the preparation of the aqueous fraction of stool the samples were thawed overnight at 4 °C. The two samples from each week were homogenized in a bowl and mixer. A portion was ultracentrifuged 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 analysis. The pH was measured in both fecal homogenate and fecal water with a digital pH meter (CD 620; WPA Ltd, Cambridge, United Kingdom).

Short-chain fatty acids were measured in fecal water as described by van Munster *et al* (1994*b*), using a Packard gas chromatograph (column: 10% SP1200 / 1% H_3PO_4 on 80/100 Chromosorb WAW, Hewlett Packard, Middelburg, The Netherlands). The fructo-oligosaccharides present in feces were determined in 0.25 g freeze-dried feces. This was suspended in 5 ml water, vortex-mixed and boiled for 10 min. The suspension was centrifuged at 4500 rev./min for 25 min. The supernatant fraction was analyzed by high-performance anion-exchange chromatography (Dionex BV, Breda, The Netherlands). The system consisted of a gradient pump and programmable pulsed electrochemical detector. Separations were performed using a Carbopack PA-100 analytical column (4*250 mm, Dionex BV, Breda, The Netherlands). The chromatographic mobile phase consisted of 0.1 M NaOH (A) and 1 M Na-acetate containing 0.1 M NaOH (B). A linear gradient was used from 100% A to 70% A plus 30% B in 25 min with a flow of 1 ml/min. Samples were compared with spiked samples, containing three oligomers from Raftilose P95® (GF2, GF3 and GF4). The same HPLC method was used in undiluted urine samples for the detection of fructo-oligosaccharides in urine.

Data analysis and statistics

The 24 h integrated breath-hydrogen excretion was estimated by calculating geometrically the area under the curve of concentration *v.* time (Wolever & Jenkins, 1986). Results are expressed as means and standard errors, unless stated otherwise. The distributions of the differences between the three

treatments were checked for normality by visual inspection of the normal probability plots. The significance of the differences between the three supplements in each subject was assessed by analysis of variance without interactions (General Linear Models procedure). Because of a small number of missing values in the data-set, leading to non-orthogonality of the model, the variable 'period' was added to the model. 'Period' did not contribute to the significance of the model, nor did it change any of the conclusions. Therefore the model used in the final analysis contained only 'subject' and 'treatment'. In cases of a significant difference ($P < 0.05$) between treatments, group means were tested with the Tukey or Scheffé tests.

An exception to the given method of analysis was made for the breath hydrogen data. Because of excessive numbers of missing values, we used the least-square means statement to estimate mean values for each supplement and to evaluate differences between treatments in a model including 'subject', 'period' and 'treatment'. This analysis accounts for unequal cell sizes; least-square means are estimators of the marginal means that would be expected had the design been balanced. The statistical analyses package SAS, version 6.09 (SAS Institute, Inc., Cary, NC) was used to perform the statistical analyses.

Results

Supplements and food intake

None of the volunteers experienced great difficulties with the consumption of the supplements. All volunteers completed the study successfully. The recovery of lithium in urine was significantly lower ($P < 0.05$) after consumption of SF15 (82%) than after consumption of control (90%). The results of the 2 d food records obtained during each supplement period revealed no changes in the average daily intakes of energy, protein, fat, carbohydrates or dietary fiber. Mean values of dietary intakes from 6 d food records are given in Table 1.

Diaries

The information from the diaries is presented in Table 2. Gastrointestinal complaints concerning flatulence were significantly higher ($P < 0.001$) during consumption of SF15 compared with the control supplement. Although flatulence did not rise significantly during SF5, there was a visible trend towards a higher level. All other symptom scores were low and equal for the three supplements.

Table 2.

Effect of dietary supplementation with 0 (control), 5 (SF5) or 15 (SF15) g fructo-oligosaccharides/d on gastrointestinal complaints, stool form and frequency of defecation.

	Flatulence[1]	Bloating[1]	Abdominal pains/cramps[1]	Stool form[2]	Frequency of defecation[3]
Supplement					
Control	0.5 ± 0.1	0.1 ± 0.1	0.0 ± 0.0	3.3 ± 0.1	1.2 ± 0.1
SF5	0.7 ± 0.1	0.1 ± 0.1	0.0 ± 0.0	3.2 ± 0.2	1.3 ± 0.1
SF15	1.0 ± 0.1	0.2 ± 0.1	0.1 ± 0.0	3.1 ± 0.1	1.2 ± 0.1
Differences					
SF5-control	0.2 ± 0.1	0.0 ± 0.0	-0.0 ± 0.0	-0.0 ± 0.1	0.1 ± 0.1
SF15-control	0.4[4] ± 0.1	0.1 ± 0.0	0.1 ± 0.0	-0.1 ± 0.1	0.1 ± 0.1

Mean values with their standard errors for 24 subjects.

[1] Subjects scored symptoms on a scale from 0 (none) to 3 (severe). Symptom scores for eructations, nausea, vomiting and stomach pains/cramps were zero for all supplements.

[2] Stool form was rated on a scale from 1 (watery) to 5 (dry, hard pellets).

[3] Mean frequency per day in each supplement week.

[4] $P < 0.001$.

There were no differences in recorded stool form between control and SF5. During SF15, stool form was rated somewhat lower and was more watery. This decrease, however, was not significant. No differences were found in frequency of defecation between the supplements.

Colonic fermentation

Because of a technical defect of the exhaled hydrogen monitor in the last week of the experiment, we only obtained sixteen observations of hydrogen excretion (instead of twenty-four) in each supplement group. Figure 1 shows the mean breath hydrogen curve for each supplementation. When comparing the separate hydrogen levels at different times we observed a significantly higher response ($P < 0.01$) at 12.00 hours for SF15.

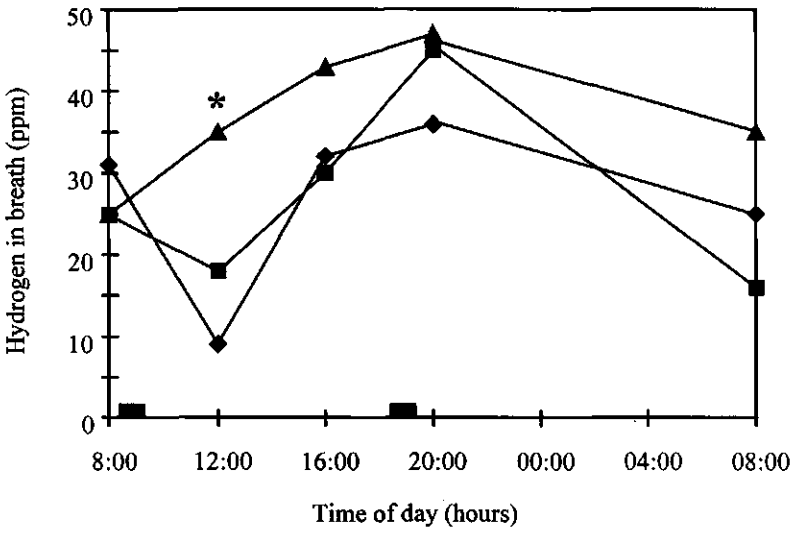


Figure 1. Least-square means (LS Means) of breath hydrogen concentration (ppm) at regular time intervals on the seventh day of supplementation with 0 (◆), 5 (■) or 15 g (▲) of fructo-oligosaccharides. LS Means are based on sixteen observations for each plotted point. (▬) time of supplement ingestion. * $P < 0.01$ in comparison with control.

Table 3.

Effect of dietary supplementation with 0 (control), 5 (SF5) or 15 (SF15) g fructo-oligosaccharides/d on breath hydrogen excretion, fecal weight, fecal pH and short-chain fatty acid concentrations.

	Breath hydrogen[1] (ppm.h)	Fecal wet wt[2] (g/d)	Fecal dry wt (g/kg wet wt)	Fecal pH[3]	Short-chain fatty acids[3] (mmol/L)
Supplement					
Control	682 ± 80	272 ± 26	245 ± 9	6.5 ± 0.1	151 ± 7
SF5	714 ± 77	279 ± 33	232 ± 12	6.4 ± 0.1	150 ± 7
SF15	986 ± 85	264 ± 22	237 ± 7	6.4 ± 0.1	153 ± 8
Differences					
SF5-control	32 ± 116	-32 ± 20	-13 ± 9	-0.1 ± 0.1	-4 ± 9
SF15-control	304[4] ± 120	-17 ± 25	-8 ± 8	0 ± 0.1	3 ± 7

Mean values with their standard errors for 24 subjects.

[1] Least-square means (LS Means) and standard errors of the LS Means; n 16 for each supplement group.

[2] Mean daily production as estimated from marker recovery. Four observations were excluded because markers were not swallowed.

[3] As measured in fecal water. Three observations were missing because there was too little fecal water extractable from feces.

[4] $P < 0.05$.

Table 3 summarizes the fermentation data. When expressing the hydrogen excretion as the area under the hydrogen-time curve (AUC), there was an increase in AUC from control (682 ppm.h) to SF5 (714 ppm.h; P=0.78, compared with control) to SF15 (986 ppm.h; P=0.02, compared with control).

No changes in fecal weight were observed. The observed difference in wet weight between control and SF15 (or SF5) did not vary in any systematic way over the range of measurements. There was no significant correlation between differences in wet weight in response to supplementation and average values of wet weight (for SF5: r -0.03; P = 0.89, for SF15: r -0.26; P = 0.26). Four observations were excluded from the analysis of wet weight because volunteers had forgotten to swallow all barium rings in that week, making it impossible to estimate daily productions.

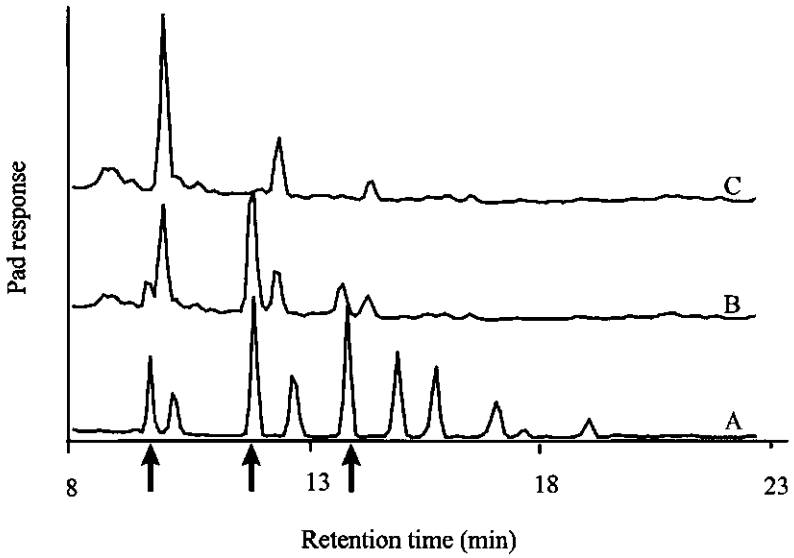


Figure 2. Typical HPLC patterns for one of the volunteers. Samples were (A) Raftilose P95®, (B) fecal sample after supplementation with fructo-oligosaccharides, spiked with three oligomers from Raftilose P95® (arrows), (C) the same fecal sample, unspiked.

Fecal dry weight, expressed as a percentage of wet weight, did not change in response to the supplementation. No traces of the fructo-oligosaccharides that were provided in the supplement were recovered in feces of any of the samples. Figure 2 gives an example of a typical HPLC elution

pattern for a fecal sample from one of the volunteers during SF15. Response A is Raftilose P95®, response B is the sample spiked with the Raftilose P95® oligomers GF2, GF3 and GF4, response C is the unspiked sample. In addition to fecal analyses, we also analyzed urine samples for fructo-oligosaccharides. In none of the samples were any fructo-oligosaccharides detected.

Concentrations of short-chain fatty acids were measured in fecal water. No accumulation of these fermentation products was observed. As shown in Figure 3, the relative contribution of different short-chain fatty acids did not change. The pH-values of fecal water and homogenate were not altered in response to the different supplements. Because there were three cases in which the amount of extractable fecal water was negligible, fecal short-chain fatty acids and pH of fecal water could not be measured for these observations. There was a significant negative correlation between the pH of fecal water and the total amount of short-chain fatty acids (r -0.57, $P < 0.0001$) as measured in fecal water.

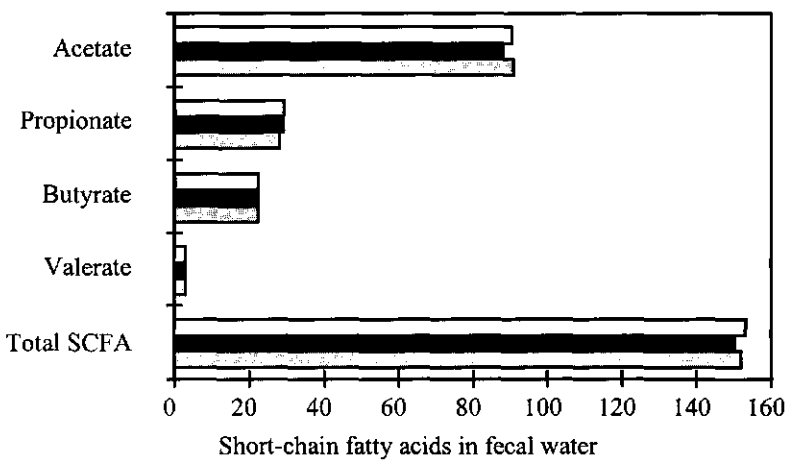


Figure 3. Mean concentration of short-chain fatty acids (SCFA) as measured in fecal water obtained from two pooled stools, after 5-7 d of dietary supplementation with 0 (white), five (black) or 15 g (grey) fructo-oligosaccharides.

Discussion

This study shows that fructo-oligosaccharides are completely fermented in the large intestine. Complete malabsorption in the small intestine was confirmed, since no fructo-oligosaccharides were

detected in any of the urine samples, and it has been shown that fructo-oligosaccharides are not metabolized by human digestive enzymes in the small intestine (Nilsson & Björck, 1988; Oku *et al*, 1984; Tokunaga *et al*, 1989) and thus reach the large intestine. We demonstrated fermentation positively through the observed increase of breath hydrogen excretion. This fermentation was complete, since no fructo-oligosaccharides were excreted in feces during the last two days of each supplementation period. The present study is the first to show the complete fermentation of fructo-oligosaccharides in an *in vivo* experiment.

The increase of hydrogen excretion during fermentation of fructo-oligosaccharides has also been observed by other investigators (Stone-Dorshow & Levitt, 1987; Rumessen *et al*, 1990; Gibson *et al*, 1995*a*). The level of breath hydrogen excretion seems to be dose dependent. During SF5 the excretion was higher than during the control period, although it did not reach significance. At the SF15 level, the excretion of hydrogen was significantly higher than during the control treatment. This dose effect was also observed by Rumessen *et al* (1990) using fructans, which are longer-chain oligomers. It is unlikely that the effect on hydrogen excretion during SF5 was missed because of a rapid fermentation and short-lived increases of breath hydrogen. The hydrogen response to fructo-oligosaccharides might be rapid, but the concentration is shown to be elevated for at least 6 h after supplementation (Stone-Dorshow & Levitt, 1987). A more likely explanation for the very small difference between control and SF5 is that the effect was masked by the meal-related hydrogen response. In the present study, it was impossible to test for linearity, since we only tested three doses of fructo-oligosaccharides. However, in a study by Rumessen *et al* (1990) the excretion of hydrogen in breath increased linearly with the consumption of up to 20 g lactulose.

We did not find any increase in short-chain fatty acid concentration in feces after supplementation with fructo-oligosaccharides and, consequently, no changes in the average pH-values of fecal water. Short-chain fatty acids are mainly produced in the proximal colon where the amount of available substrates is highest (MacFarlane *et al*, 1992). Rapid absorption by the colonic mucosa leads to a lower concentration in the feces (Höverstad *et al*, 1982; Cummings *et al*, 1987*a*). This is probably the reason that we did not find the expected changes in short-chain fatty acids or pH. Our results were in accordance with the recent findings of Gibson *et al* (1995*a*), who published a study investigating the effects of fructo-oligosaccharides on large-bowel microflora and colonic function in a controlled feeding trial (*n* 8). They did not observe any major differences in fecal short-chain fatty acid concentration between supplementation with 15 g fructo-oligosaccharides or sucrose. In an experiment by Mitsuoka *et al* (1987), the effects of fructo-oligosaccharides on intestinal microflora and fecal pH were evaluated. Elderly patients (*n* 23) participated in a 3-week experiment. They consumed 8 g of fructo-oligosaccharides/d for 2 weeks. Fecal collection took place before, during and after administration. A mean decrease of fecal pH (by 0.3 units) was

observed, but no information was given on variations or levels of significance. No control treatment was used.

In the present study, supplementation with fructo-oligosaccharides, even at the SF15 level, did not cause changes in stool form, wet or dry weight of feces or frequency of defecation. Our findings on fecal mass were surprising. We expected that increased fermentation would lead to an increase of total microbial mass. As this microbial mass is estimated to be about 55% of the total fecal solids, a fecal weight change was anticipated (Stephen & Cummings, 1980). Ito *et al* (1990) investigated the effects of administrating galacto-oligosaccharides on different variables. No changes in fecal weights were observed after testing different doses of galacto-oligosaccharides using a strict study design. Gibson *et al* (1995*a*) showed a trend towards higher fecal wet and dry weights. Since the excretion of nitrogen was significantly higher during supplementation with fructo-oligosaccharides, the authors concluded that the increase in fecal weight was probably due to an increase in biomass. Little effect was shown on total bacterial counts per gram feces.

The present study was performed under uncontrolled dietary conditions and therefore correction for dietary fiber intake was impossible. The bulking capacity of dietary fiber might have influenced the fecal weights in this study (Cummings *et al*, 1992) and possibly masked the effect of biomass on fecal weight. Moreover, it should be noted that fiber intakes were relatively high compared with the average intake by Dutch men of the same age category (40 g/d *v.* 27 g/d) (Anonymous, 1988). This is also reflected in the daily stool output (mean 270 g/d) which is considerably higher than reported values for healthy male adults in the UK (mean 122 g/d) (Cummings *et al*, 1992).

In general, the volunteers accepted the sweet taste of fructo-oligosaccharides well. The only negative remark concerned the increase in flatulence during SF15 supplements.

The fructo-oligosaccharide supplements were marked with lithium in order to check compliance of intakes. Lithium recovery was 82% during SF15, which was significantly lower than the 90% recovery during control. If this was due to a lower intake of SF15, then the intake of fructo-oligosaccharides at this level might not have been 15 g, but between 13.5 and 14 g/d. This would still have been high compared with the estimated daily consumption of fructo-oligosaccharides, which is only about 1 g/d (Spiegel *et al*, 1994).

We conclude that fructo-oligosaccharides added to the diet of young Western subjects are fully metabolized in the large intestine. The next step is to study in depth the specific effects of supplementation with fructo-oligosaccharides on microfloral growth and metabolism. Gibson *et al* (1995*a*) recently reported interesting results suggesting that supplementation with oligosaccharides might lead to the establishment of a more healthy colonic community, through the selective stimulation of bifidobacteria.

Acknowledgments

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3

Bacterial fermentation of fructo-oligosaccharides and resistant starch in patients with an ileal pouch-anal anastomosis

Martine S. Alles, Martijn B. Katan, Jan M.J.I. Salemans, Katrien M.J. Van Laere, Monique J.W. Gerichhausen, Marina J. Rozendaal, Fokko M. Nagengast. *The American Journal of Clinical Nutrition* 1997; 66:1286-1292.

Abstract

Patients with large bowel diseases may undergo ileal pouch-anal anastomosis, in which the colon is removed and part of the distal ileum is used to construct a pelvic reservoir. Competence of the ileal pouch to ferment carbohydrates is associated with the absence of pouchitis. However, the extent to which bacterial fermentation takes place and whether it is affected by diet are unclear. We investigated fermentation of two nondigestible carbohydrates, fructo-oligosaccharides and resistant starch, in 15 healthy patients with an ileal pouch by using a placebo-controlled crossover design (with glucose as a placebo). Apparent fermentability of fructo-oligosaccharides was 83%; that of resistant starch was 46%. Resistant starch increased fecal excretion of butyrate by 69% whereas fructo-oligosaccharides reduced excretion of the amino acid-derived isobutyrate by 94% and of isovalerate by 77%. Fructo-oligosaccharides also significantly increased fecal weight (651 compared with 541 g/d) and breath hydrogen excretion (286 compared with 85 ppm x h). Bacterial fermentation of nondigestible carbohydrates in pouches takes place to an appreciable extent and in a substrate-specific manner. The relation between such fermentation and inflammation of the pouch (pouchitis) deserves study.

Introduction

Excision of the colon followed by ileal pouch-anal anastomosis has become a frequently used surgical alternative to permanent ileostomy in patients with ulcerative colitis or familial adenomatous polyposis. In this operation part of the distal ileum is used to construct a reservoir or pouch that is connected to the anus and provides a measure of continence.

In the months or years after surgery, the pouch gradually acquires a bacterial content and mucosal morphologic characteristics that indicate that the pouch has taken over some colonic functions. Morphological adaptation of the ileal tissue of the pouch often results in transformation to colonic-type mucosa (de Silva *et al*, 1991; Tonelli *et al*, 1991; Campbell *et al*, 1994). Stasis of the luminal contents in the pouch probably promotes colonization by bacteria. Pouch effluent contains more anaerobic bacteria than does ileostomy effluent (nasmyth *et al*, 1989; Santavirta *et al*, 1991). Several studies showed considerable excretion of fecal short-chain fatty acids in pouch effluent (Mortensen *et al*, 1989; Nordgaard *et al*, 1993; Clausen *et al*, 1992*b*; Nasmyth *et al*, 1989; Ambroze *et al*, 1993) and, in one study, a breath-hydrogen response after consumption of the synthetic disaccharide lactulose was observed in half of the patients (Bruun *et al*, 1995). Short-chain fatty acids and hydrogen are both end products of fermentation and thus indicate the presence of an active bacterial mass in an ileal pouch.

In healthy humans fermentation depends to a high degree on the composition of the diet and availability of substrates to the intestinal microflora (Cummings & Englyst, 1987*a*). However, little or nothing is known about the fate of nondigestible dietary compounds in an ileal pouch and the extent to which bacterial fermentation takes place.

We therefore studied fermentation of two carbohydrates that are not digested or absorbed in the small intestine: fructo-oligosaccharides and resistant starch. We provided these carbohydrates as supplements to diets of patients with an ileal pouch-anal anastomosis. Fructo-oligosaccharides occur naturally in, for example, onions and leeks (Spiegel *et al*, 1994; Roberfroid *et al*, 1993; Van Loo *et al*, 1995). We used a native resistant starch that is enclosed in granules such as those in raw potatoes or unripe bananas (Englyst *et al*, 1992). In vitro studies showed that the end products of the fermentation of fructo-oligosaccharides are gases, such as hydrogen, and short-chain fatty acids, mainly acetate (Wang & Gibson, 1993). Resistant starch is a good substrate for production of butyrate (Wang & Gibson, 1993), the major fuel of colonic mucosa. In healthy humans, fructo-oligosaccharides are fermented rapidly whereas resistant starch has a slow, prolonged fermentation (Munster *et al*, 1994*a*; Rumessen *et al*, 1990; Stone-Dorshow & Levitt, 1987). Thus, these two carbohydrates span the range of fermentability in subjects with a normal gastrointestinal tract, which makes them suitable for examining the extent to which fermentation takes place in patients with an ileal pouch.

The following hypotheses were tested: 1) fructo-oligosaccharides are fermented more extensively than is resistant starch, as reflected in recovery of fructo-oligosaccharides and resistant starch in pouch effluent; 2) fructo-oligosaccharides increase breath hydrogen excretion and total fecal excretion of short-chain fatty acids, especially acetate, and decrease excretion of the amino acid-derived short-chain fatty acids; and 3) resistant starch increases breath hydrogen excretion and total fecal excretion of short-chain fatty acids, especially butyrate.

Subjects and methods

Subjects and experimental design

We recruited 16 patients (8 men and 8 women) who had undergone ileal pouch-anal anastomosis because of ulcerative colitis (n=15) or familial polyposis (n=1). Time since the operation was ≥ 12 months. The patients had never had clinical signs of pouchitis and were not treated with antibiotics for ≥ 3 months before the experiment. Subjects were asked to maintain their habitual medication regimen (strictly the same quantities) during the experiment. Five subjects took loperamide (4-16 mg/d) throughout the study; one subject also took prednisone (10 mg/d) and metformin (1500 mg/d). Two subjects smoked and one consumed a lactose-free diet. No subjects were vegetarian. The study protocol was approved by the Ethical Committee of the University Hospital St Radboud, Nijmegen, Netherlands. All subjects gave informed written consent before participating. After completing the study, patients received a small financial reward, the amount of which was based on the time they had spent on the experiment.

The study was a single-blinded, completely balanced, three period crossover trial with supplement periods of 7 d separated by 7-d wash-out periods. Each volunteer took each of three different supplements in random order. Fecal and breath samples were collected at the end of each supplement period. Subjects kept a diary in which they recorded stool frequency, possible diseases or discomfort, medications used, and deviations in eating, drinking or lifestyle behavior.

Supplements and food intake

Subjects were asked not to eat probiotic dairy products, which contain microorganisms able to survive passage through the upper gastrointestinal tract, or foods containing large amounts of fructo-oligosaccharides or resistant starch, such as beans, leeks, and onions. Subjects were given a list of these foods.

Participants recorded their habitual diet for two days in every supplement period. These food records were coded and nutrient and energy intakes were calculated with use of a modified version of the 1993 Netherlands Nutrient Data Bank (Anonymous, 1993*a*).

Composition of the supplements is shown in table 1. Subjects consumed half of each supplement at breakfast and the other half at dinner. Supplements were mixed with yoghurt, pudding or orange juice; no change in vehicle was allowed during the experiment. All supplements provided a total of 25.8 g carbohydrates/d. The fructo-oligosaccharide and resistant starch supplements each contained 14.3 g of nondigestible carbohydrates; the remainder was either digestible or readily absorbable.

Table 1.
Composition of the dietary supplements

	Treatment		
	Placebo	Fructo-oligosaccharides	Resistant starch
		g/d	
Fructo-oligosaccharides[1]	-	14.3	-
Resistant starch[2]	-	-	14.3
Digestible carbohydrates[3]	-	0.7	11.5
Added glucose[4]	25.8	10.8	-

[1] Raftilose P95®, Orafti, Tienen, Belgium; contains 95% oligosaccharides by weight as measured by the method described in this paper.

[2] Hylon VII, Nat. Starch and Chemical Company, Bridgewater NJ, USA; contains 55.4% resistant starch by wt as measured in vitro according to the method of Englyst *et al* (1992).

[3] Other carbohydrates present in Raftilose P95® and Hylon VII, were mainly glucose, fructose, sucrose and digestible starch.

[4] Glucose Pur 01934, Cerestar Benelux BV, Sas van Gent, The Netherlands.

Breath samples

On the final day of each supplement period, subjects collected end expiratory breath samples at 2- or 3-h intervals from 08:00 (before eating) to 22:00 and again at 08:00 (before eating) the next morning. They used plastic 60-mL syringes (Sherwood, Ballymoney, Ireland) that were sealed and stored at 4 °C immediately after the sampling. Contents of the syringes were analyzed for hydrogen concentration by using a standard electrochemical cell (Lactoscreen, Hoekloos, Amsterdam, Netherlands) within 24 h after the first sample was obtained. The cell was calibrated with a standard containing 100 ppm hydrogen in air (AGA Gas BV, Amsterdam, Netherlands). The 24 h integrated breath-hydrogen excretion was estimated by calculating geometrically the area under the curve of concentration in relation to time (Wolever & Jenkins, 1986).

Stool Collection and Fecal Analyses

On the last day of each supplement period, subjects collected feces for 24 h and stored each stool immediately on dry ice. We collected stools at subjects' homes and stored them at -20°C. Feces were thawed overnight at 4 °C, weighed, pooled, and homogenized by using a bowl and mixer. The proportion of fecal dry material was estimated by freeze-drying aliquots in triplicate. Another aliquot was ultracentrifuged at 30000 x g for 120 min at 4°C for preparation of the aqueous fraction of stool. Fecal water was pipetted off and filtered through a 5 μm filter (Schleicher & Schuell, Dassel, Germany). The pH of the fecal water was determined and samples were stored at -20 °C.

Concentrations of short-chain fatty acids in fecal water were measured in duplicate as described by Tangerman *et al* (1996), by using gas chromatography (Chrompack Model CP 9001, 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). Fecal excretion of short-chain fatty acids was calculated as fecal weight X proportion of fecal wet material X concentration of short-chain fatty acids in fecal water. In this calculation, the proportion of fecal wet material was defined as 1 minus the proportion of fecal dry material.

Fructo-oligosaccharides in feces were measured in duplicate samples of 125 mg freeze dried feces resuspended in 2.4 mL distilled water. D-galacturonic acid (100 μL, 0.5% wt:vol) was added as an internal standard before extraction. The mixture was vortexed and heated for 15 min at 100°C and then centrifuged at 13000 x g for 30 min. The supernate was analyzed by high-performance anion-exchange chromatography (Dionex BV, Breda, Netherlands) by using a programmable pulse electrochemical detector, a Carbopack PA-100 analytical column (4*250 mm, Dionex) and a mobile phase consisting of 0.1 mol NaOH/L (A) and 1 mol Na-acetate/L containing 0.1 mol NaOH/L (B). A linear gradient was used from 100% A to 70% A plus 30% B in 25 min with a flow rate of 1 mL/min. A calibration curve was made with purified isokestose, which is one of the oligomers in the fructo-oligosaccharide supplement used in the study. Quantification of fructo-oligosaccharides in feces was based on this curve and a correction factor was obtained by comparing a known quantity of fructo-oligosaccharides with the calculated value.

Total starch in stools was determined by subtracting the amount of free glucose from the total amount of glucose obtained after incubation with a starch-degrading enzyme. This method was modified of that of Björk *et al* (1987). Fifty milligrams freeze-dried feces was suspended in 2 mL water and heated for 5 min at 100°C; free glucose was then measured with a test kit (catalog no. 716251, Boehringer Mannheim GmbH, Mannheim, Germany). For analysis of total glucose, defined as the sum of starch and free glucose, 50 mg of freeze-dried feces was suspended in 1 ml water and 1 ml of a solution containing 4 mol KOH/L and incubated for 30 min at room temperature. The suspension was neutralized with hydrochloric acid and diluted (1:1) with 0.4 mol

Na-acetate buffer/L (pH 4.75). The mixture was incubated with 20 µl amyloglucosidase (1.4×10^5 U/L, Boehringer Mannheim) for 30 min at 60 °C to hydrolyze the starch and centrifuged for 15 min at 1000 x g. Glucose in the supernate was determined by using the test kit. The amount of starch was calculated as the difference between total and free glucose. Mean recovery of starch if added to fecal samples before treatment of the samples was 102%. The CV between separately treated and measured duplicates was 8%.

Data Analysis

Results are expressed as means and standard errors, unless stated otherwise. The distribution of individual differences was checked for normality by visual inspection of the normal probability plots (univariate procedure, SAS Institute Inc. Cary, NC). The significance of the differences between treatments was assessed by analysis of variance without interactions using a model with *subject* and *supplement* (general linear models procedure). Adding *period* to this model did not contribute to the significance; thus there were no significant effects of time or sequence. In there was a significant difference between treatments ($P < 0.05$), group means were compared using the Tukey test if the model was orthogonal or the Scheffé test if there were missing values in the data set (general linear models procedure). Conventional Pearson's correlation coefficients (correlation procedure) were used to assess the relations between dependent variables.

Results

Characteristics of subjects who completed the study and dietary intakes during the study are given in table 2.

One subject withdrew at the end of the first washout period because of an endoscopically confirmed pouchitis; data from this subject were excluded from analyses. One subject did not complete the third supplement (fructo-oligosaccharides) period because of gastrointestinal complaints such as vomiting. For this subject we excluded only data from the fructo-oligosaccharide period from analyses. All other subjects completed the study without illness. Most subjects had flatulence during fructo-oligosaccharide supplementation; no other gastrointestinal problems were reported. There were no reports of problems with palatability of the supplements. The food records showed no changes in average daily intakes of energy, protein, fat, carbohydrates or dietary fiber between treatments.

Table 2.
Patient characteristics and background diets[1]

	Men (n=7)	Women (n=8)
Characteristics		
Age (y)	35.6 ± 6.5	36.5 ± 7.8
Height (m)	1.80 ± 0.1	1.66 ± 0.1
Weight (kg)	80.9 ± 10.4	66.4 ± 10.2
Body mass index (kg/m^2)	25.0 ± 3.0	24.1 ± 4.1
Diet[2]		
Energy (MJ/d)	12.1 ± 1.3	8.9 ± 0.6
Carbohydrate (% of energy)	45.1 ± 4.3	44.0 ± 6.0
Fat (% of energy)	37.1 ± 4.3	37.9 ± 4.7
Protein (% of energy)	15.0 ± 2.4	17.9 ± 3.6
Dietary fiber intake (g/d)	20.0 ± 6.6	13.5 ± 7.3

[1] Mean ± SD.

[2] Supplements not included; maximal contribution of supplements to energy intake was 0.45 MJ/d.

Bowel habit and stool composition

Data on bowel habits, fecal weight, fecal pH and fecal excretion of fructo-oligosaccharides and resistant starch are given in table 3. There was no fecal excretion of fructo-oligosaccharides in any subject taking placebo. During fructo-oligosaccharide supplementation, mean excretion was 2.4 g/d (95% CI, 1.1 to 3.7) or 17% of the supplemented dose. All constituents of the fructo-oligosaccharide supplement (table 1) were present in the feces; thus no selective breakdown of specific oligomers was observed. The fecal excretion of starch was 7.7 g higher per day while subjects were taking the resistant starch supplement than when they were taking placebo (95% CI, 5.1 to 10.4). Because 14.3 g resistant starch/d was added to the diet, 46% of dietary resistant starch was broken down in the alimentary tract.

Fructo-oligosaccharides increased mean stool frequency by 9% and fecal weight by 20% or 110 g/d. Percentage of dry matter was not affected by fructo-oligosaccharides but fecal excretion of dry matter increased by 28.8 g/d (95% CI, 9.5 to 48.1; $P = 0.0009$). In subjects taking resistant starch, values for mean stool frequency, fecal weight and fecal dry matter all tended to be slightly higher than placebo values, but the differences were not significant. Fecal water pH tended to be lower (although not significantly) in subjects taking fructo-oligosaccharides (95% CI, -0.4 to 0.1 pH-units) or resistant starch (95% CI, -0.4 to 0.1 pH-units) than in those taking placebo.

Table 3.
Bowel habits, fecal weight, fecal pH and fecal excretion of fructo-oligosaccharides and resistant starch after supplementation[1]

	Treatment		
	Placebo	Fructooligosaccharides	Resistant starch
Stools per day	6.4 ± 0.6	7.0 ± 0.5[2]	6.8 ± 0.5
Fecal dry matter (%)	12.5 ± 0.5	14.2 ± 0.7	13.4 ± 0.5
Fecal weight (g/d)	541 ± 60	651 ± 67[3]	587 ± 57
Fecal pH[4]	6.0 ± 0.2	5.8 ± 0.1	5.9 ± 0.1
Fecal excretion			
Starch (g/d)	4.8 ± 0.7	-	12.6 ± 1.3[5]
Fructo-oligosaccharides (g/d)	0 ± 0	2.4 ± 0.6[3]	-

[1] Mean ± SEM; $n = 15$, except for fructooligosaccharides for which $n = 14$.

[2] Significantly different from placebo ($P < 0.05$).

[3] Significantly different from placebo ($P < 0.01$).

[4] As measured in fecal water.

[5] Significantly different from placebo ($P < 0.0001$).

Short-chain fatty acids

Fecal excretion of various short-chain fatty acids during the three treatments is shown in table 4. Total amount of short-chain fatty acids excreted tended to be somewhat, although not significantly, higher during supplementation with fructo-oligosaccharides (95% CI, -3.4 to 7.6 mmol/d) or resistant starch (95% CI, -0.1 to 10.7 mmol/day) than with placebo. During supplementation with resistant starch, excretion of butyrate was elevated by 69% ($P = 0.0014$). Isobutyrate excretion was lowered by 94% ($P = 0.0006$) and isovalerate excretion by 77% ($P = 0.0023$) during fructo-oligosaccharide supplementation. We detected no caproate in any of the fecal samples. There was a negative correlation between number of stools per day and total excretion of amino acid-derived short-chain fatty acids ($r = -0.36$; $P < 0.05$).

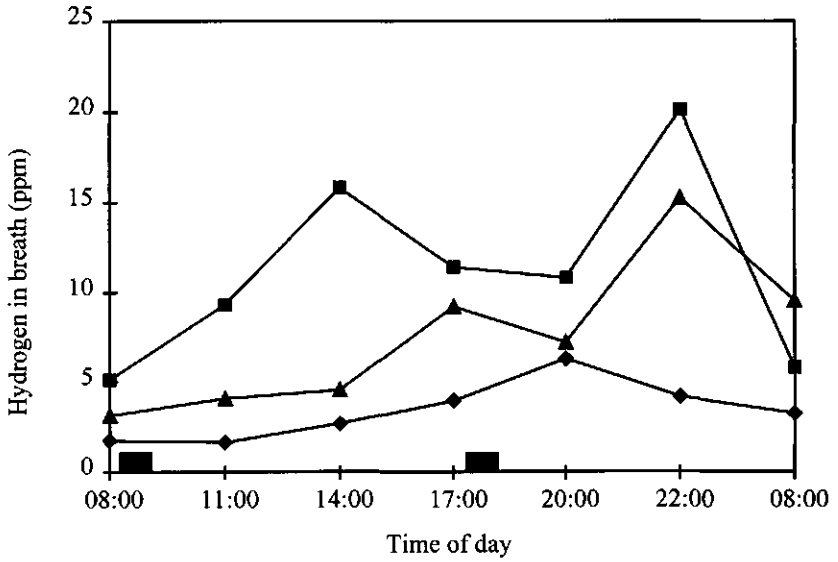

Figure 1. Mean breath-hydrogen concentration on the seventh day of supplementation with placebo (◆), fructo-oligosaccharides (■), or resistant starch (▲). Bars on the horizontal axis indicate time of ingestion of supplement.

Breath hydrogen

Breath-hydrogen curves over the last 24 hours of each supplement period are shown in figure 1. Integrated breath-hydrogen excretion expressed as area under the hydrogen-time curve, was higher

during fructo-oligosaccharide supplementation (286 ± 84 ppm x h) than during placebo consumption (85 ± 17 ppm x h, P = 0.004). Hydrogen excretion during resistant starch supplementation was elevated by 125 ppm x h (95% CI, -23.0 to 273.4) but the difference with placebo was not significant (P = 0.046) when the significance limit was corrected for multiple comparisons. There was no correlation between individual values for integrated breath-hydrogen excretion and fecal excretion of fructo-oligosaccharides (r = -0.14; P = 0.46) or resistant starch (r = -0.09; P = 0.64).

Discussion

We found that fructo-oligosaccharides and resistant starch, two nondigestible carbohydrates commonly found in foods, are well fermented in patients with an ileal pouch-anal anastomosis. To our knowledge this was the first quantitative study of carbohydrate fermentation in this patient group.

Apparent fermentability of fructo-oligosaccharides and resistant starch

The mean recovery of fructo-oligosaccharides in feces was 17% of the daily dose of supplement. Fructo-oligosaccharides are not metabolized by human digestive enzymes and most fructo-oligosaccharide molecules survive passage through the upper part of the gastrointestinal tract (Nilsson & Björck, 1988; Oku *et al*, 1984; Tokunaga *et al*, 1995; Bach Knudsen & Hessov, 1995; Molis *et al*, 1996). It is thus likely that most fructo-oligosaccharides in our study were fermented by bacteria in the ileum or pouch. No fructo-oligosaccharides were recovered from feces of any subject taking placebo, probably because of low amounts of fructo-oligosaccharides in the background diet (Spiegel *et al*, 1994; Roberfroid *et al*, 1993; Van Loo *et al*, 1995). In a previous study (Fleming *et al*, 1988) we found no fructo-oligosaccharides in feces of healthy men after supplementation with 15 g fructo-oligosaccharides (Alles *et al*, 1996). Thus colonic bacteria are evidently capable of fermenting 100% of fructo-oligosaccharide supplements in subjects with a complete colon. In our subjects with a pouch, the proportion was 83%.

Man fecal excretion of residual starch during placebo consumption was 5 g, which can be attributed to resistant starch present in the background diet. Average recovery of resistant starch from the supplemented dose was 54%. In healthy subjects, ≈ 20-30% of resistant starch supplement was recovered from feces (Philips *et al*, 1995). Patients with a pouch thus had more efficient fermentation of fructo-oligosaccharides than of resistant starch, but both nondigestible carbohydrates were fermented less extensively in such patients than in subjects with an intact colon.

Table 4.
Fecal excretion of short-chain fatty acids after supplementation[1]

	Treatment		
	Placebo	Fructooligosaccharides	Resistant starch
	mmol/d		
Total	28.8 ± 3.0	30.9 ± 3.5	34.1 ± 3.2
Acetate (2-carbon)	22.8 ± 2.3	25.2 ± 2.8	26.7 ± 9.2
Propionate (3-carbon)	3.6 ± 0.8	2.8 ± 0.9	3.4 ± 0.8
Buty-ate (4-carbon)	2.3 ± 0.4	2.9 ± 1.8	3.9 ± 0.6[2]
Iso-butyrate (4-carbon)	0.052 ± 0.02	0.003 ± 0.00[2]	0.022 ± 0.01
Valerate (5-carbon)	0.034 ± 0.02	0.008 ± 0.00	0.010 ± 0.01
Iso-valerate (5-carbon)	0.061 ± 0.02	0.014 ± 0.01[2]	0.030 ± 0.01

[1] Mean ± SEM. $n = 15$, except f or fructo-oligosaccharides, for which $n = 14$.

[2] Significantly different from placebo ($P < 0.01$).

Bowel habit and stool composition

An ileal pouch has a much smaller mucosal surface available for water resorption and a smaller volume than an intact colon. Thus, luminal contents that pass the pouch are relatively large as compared with those in an intact colon. The pouch thus fills up sooner, which results in a shorter stasis time of the luminal content, and faster transit (Öresland *et al*, 1990; de Silva *et al*, 1991). Low resorption of water from luminal contents causes a high stool weight and a lower proportion of dry matter than in subjects with an intact colon. During placebo supplementation, the mean fecal wet weight was 541 g/d with 12.5% dry matter. Fructo-oligosaccharides significantly increased 24 h fecal weight and fecal dry mass, although < 10% of the increase in fecal mass consisted of unfermented fructo-oligosaccharides. The proportion of fecal wet material was not affected by the fructo-oligosaccharides; therefore the increased fecal weight was probably not caused by an osmotic effect of fructo-oligosaccharide molecules. It is more likely due to an increase in fecal biomass, which is also found in healthy subjects (Gibson *et al*, 1995*a*).

Administration of resistant starch did not significantly alter fecal wet or dry weights. Unfermented resistant starch would be expected to have its greatest effect on fecal dry weight. Because of the lower apparent fermentability of resistant starch a smaller effect on biomass, reflected in fecal wet weight, might be expected. Intra-individual week-to-week variations in fiber intake possibly masked a small effect of resistant starch on dry weight and biomass.

The brief duration of stasis of the luminal contents in the pouch was also reflected in the frequency of defecation. During the control period, the patients reported a mean of 6.5 stools/d, which is similar to quantities found by other researchers (Nordgaard *et al*, 1993; Clausen *et al*, 1992*b*; Salemans *et al*, 1992). During the fructo-oligosaccharide supplementation, the frequency was even higher, probably as a result of the higher stool volumes. The mean time between pouch voidings was < 4 h. This interval was obviously long enough to allow bacteria to degrade most of the supplemented fructo-oligosaccharides. Because the fermentation of resistant starch is relatively slow, the interval between voidings may have been too short for microflora to ferment the resistant starch more extensively.

Breath hydrogen and fecal short-chain fatty acids

Bacterial degradation of fructo-oligosaccharides and resistant starch was reflected in the production of hydrogen and short-chain fatty acids. Bruun *et al* (Bruun *et al*, 1995) observed an increase in breath hydrogen in half of patients with a pouch who took the synthetic nondigestible disaccharide lactulose (10 g). Only 2 of the 15 subjects in that study had a hydrogen response to wheat starch. Those who did not respond to lactulose had poor functional pouch characteristics, such as incontinence. The authors concluded that fermentation of lactulose is associated with better pouch function.

In our study fructo-oligosaccharides increased hydrogen excretion by 236% relative to placebo values and resistant starch increased the hydrogen excretion by 147%. During supplementation with either fructo-oligosaccharides or resistant starch, half of the subjects did not respond, that is their hydrogen concentrations in expired air were never > 10 ppm. We did not find any significant correlations between hydrogen excretion and recovery of fructo-oligosaccharides or resistant starch in feces. This may indicate that the hydrogen response was determined by factors other than the mass of carbohydrates fermented. However, because of the small number of patients and the small range in fecal carbohydrate excretion, we may have missed a modest association.

Our data do not allow firm conclusions about the effect of either fructo-oligosaccharides or resistant starch on the total amount of short-chain fatty acids or on fecal pH. Short-chain fatty acids are produced simultaneously with hydrogen by most bacteria (Cummings & MacFarlane, 1991). MacFarlane *et al* (1992), 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 to rapid absorption of these acids by the colonic mucosa. A similar absorption by the pouch mucosa could explain why we did not find significant effects on total excretion of short-chain fatty acids or on pH in our study. We observed trends towards a greater excretion of total short-chain fatty acids, a greater excretion of acetate and a lower fecal pH, but our study probably lacked the power to detect small effects on these variables. Tonelli *et al* (Tonelli *et al*, 1995) found no increase in fecal short-chain fatty acids after administration of short-chain fatty acids into the pouch. They concluded that a rapid absorption by pouch mucosa was probably responsible.

Fructo-oligosaccharides significantly depressed excretion of isobutyrate and isovalerate. These short-chain fatty acids originate from the bacterial breakdown of amino acids (MacFarlane & MacFarlane, 1993; Rasmussen *et al*, 1988; Zarling & Ruchim, 1987). Similar effects were observed in healthy men given galacto-oligosaccharides (Ito *et al*, 1993*a*) or lactulose (Ito *et al*, 1993*b*; Mortensen *et al*, 1990). Protein fermentation was thus apparently depressed by the addition of a rapidly fermentable carbohydrate. Protein fermentation produces not only amino acid-derived short-chain fatty acids, but also indoles and ammonia. These products might have a toxic effect on mucosa (Prior *et al*, 1974; Lin & Visek, 1991; Clinton *et al*, 1988). Therefore, we suggest that adding fructo-oligosaccharides to the diet of patients with an ileal pouch-anal anastomosis might decrease the cytotoxicity of pouch contents.

Resistant starch increased the excretion of butyrate, an effect also found in studies in healthy subjects (Mcintyre *et al*, 1991; Philips *et al*, 1995). Butyrate is a major fuel for the colonic mucosa and may inhibit cell proliferation and stimulate cell differentiation. Moreover, it was suggested that ulcerative colitis is caused by diminished oxidation of butyrate (Roediger, 1980). Additionally, Nasmyth *et al* (1989) observed a negative correlation between concentration of butyrate in pouch

effluent and the severity of villous atrophy of pouch mucosa. Thus, it may be worthwhile to study the potentially beneficial effects of dietary resistant starch in patients with pouch inflammation.

Inflammation of the ileal pouch, pouchitis, is often regarded as a new manifestation of ulcerative colitis, because pouchitis is far more common in patients who were operated on because of ulcerative colitis than in those operated on because of familial adenomatous polyposis (Salemans *et al*, 1992; Shepherd, 1995). In this study we intended to study fermentation in a group of healthy patients with an ileal pouch-anal anastomosis. Patients had never had any clinical signs of pouchitis but we did not confirm the absence of pouchitis by endoscopic or histologic examinations of the pouch. Moreover, some of the patients were taking medications (loperamide and prednisone) that could have masked symptoms of pouchitis. As a result we might have included unintentionally some patients with pouchitis in our study.

Bacterial overgrowth was previously considered a cause of pouchitis (Santavirta *et al*, 1991; nasmyth *et al*, 1989; Sandborn *et al*, 1995). There is, however, no theoretical foundation for such an effect. Additionally, bacterial overgrowth of the pouch is present in virtually all patients with a pouch, independent of the presence or absence of pouchitis. Microbial analyses of pouch effluents did not show higher bacterial counts in patients with pouchitis compared to those without (O'Connell *et al*, 1986; Kmiot *et al*, 1993; Luukkonen *et al*, 1988). Thus bacterial growth does not contribute to pouchitis. In fact, there are indications that the pouch flora may help to maintain pouch health. A relative increase of aerobic bacteria and a decreased production of short-chain fatty acids, both possibly caused by a low supply of fermentable saccharides, were associated with pouchitis (Clausen *et al*, 1992*b*). Also, Bruun *et al* (Bruun *et al*, 1995) found that the ability to ferment lactulose, measured as a breath hydrogen response, was associated with a better pouch function.

Conclusion

Ileal pouch flora was competent to ferment nondigestible dietary carbohydrates. Fructo-oligosaccharides were largely fermented, and they depressed protein fermentation. Resistant starch caused an increase in excretion of butyrate. It appears justified to study the effects of such dietary carbohydrates on the development of pouchitis.

Acknowledgments

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4

Effect of transgalacto-oligosaccharides on composition and activity of the intestinal microflora

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Abstract

Non-digestible 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 content. We studied the effect of 2 doses of transgalacto-oligosaccharides on the composition and activity of the intestinal microflora in 18 women and 22 men as compared to a placebo. Strictly controlled experimental diets were supplied to three intervention groups (parallel design). The study was divided into two consecutive 3-week periods with participants consuming a run-in followed by an intervention diet which differed only in the amount of transgalacto-oligosaccharides: 0 g/d (placebo), 7.5 and 15 g/d. Breath and fecal samples were collected at the end of both run-in and intervention periods. Apparent fermentability of transgalacto-oligosaccharides was 100%. The highest dose of transgalacto-oligosaccharides significantly increased the concentration of breath hydrogen by 138% ($P < 0.01$) and the nitrogen density of the feces by 6.5% ($P < 0.05$). The number of bifidobacteria increased on both placebo and transgalacto-oligosaccharides, but the increases were not significantly different. Transgalacto-oligosaccharides did not 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, fecal pH or the composition of the intestinal microflora. We conclude that transgalacto-oligosaccharides 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.

Introduction

The recent commercialization of non-digestible oligosaccharides as food ingredient has triggered much research on their role in colonic health. Non-digestible oligosaccharides naturally occur in various edible food plants such as onions and leek (Van Loo *et al*, 1995). As they are not hydrolyzed by enzymes in the human small intestine, they reach the colon intact. Oligosaccharides are relatively small molecules. In a previous study we showed that fructo-oligosaccharides, added to the diet of young healthy subjects are fully fermented by the colonic microflora (Alles *et al*, 1996).

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 will mainly lead to the production of gases, the short-chain fatty acids acetate, butyrate and propionate, and sometimes of lactate or ethanol. Protein fermentation increases the branched chain fatty acids iso-butyrate and iso-valerate, phenolic compounds and ammonia (MacFarlane & MacFarlane, 1993; Cummings & MacFarlane, 1991). 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 (Cummings & MacFarlane, 1991). It seems likely that the composition of the diet is of influence on the activity of the intestinal microflora.

Transgalacto-oligosaccharides are produced from lactose by enzymatic transgalactosylation. The oligomers consist of lactose and several galactose molecules with mainly ß-1,4 linkages (Yanahira *et al*, 1995). Transgalacto-oligosaccharides are not hydrolyzed by human small-intestinal ß-galactosidase and will pass undigested into the colon (Burvall *et al*, 1980). Tanaka *et al* reported already in 1982 that administration of transgalacto-oligosaccharides leads to an increase in breath hydrogen excretion because of its non-digestibility. Moreover, they showed that transgalacto-oligosaccharides might stimulate the growth of resident bifidobacterial strains (Tanaka *et al*, 1983). Ten years later, these results were confirmed by studies of Ito *et al* (1990; 1993*a*) and Bouhnik *et al* (1997).

Bifidobacteria may comprise up to one quarter of the gut flora of normal healthy adults (Modler *et al*, 1990). They have a role in controlling the pH of the large intestine, through the production of lactic and acetic acid. A low pH might restrict the growth of many potential pathogens and putrefactive bacteria (Modler *et al*, 1990; Gibson & Wang, 1994) and might both depress the formation of secondary from primary bile acids and enhance the precipitation of bile acids (Munster *et al*, 1994*b*; Rafter *et al*, 1986; Nagengast *et al*, 1988*b*; Hofmann & Mysels, 1992). It thus seems important to identify food components that have a potential in increasing the number of indigenous bifidobacteria.

Most studies looking at the effects of transgalacto-oligosaccharides 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 (Cummings *et al*, 1992), bile acids (Reddy *et al*, 1992; Lapré & Meer, 1992) and putrefactive products (Clinton *et al*, 1988; Prior *et al*, 1974; Lin & Visek, 1991). We used a controlled feeding trial to study the effect of two doses of transgalacto-oligosaccharides as compared with a placebo on the composition of the intestinal microflora, the saccharolytic and proteolytic activity of the bacteria and on 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 comprising a health questionnaire, blood and urine analyses, a transgalacto-oligosaccharides tolerance test and a lactose breath test. Sixty-three volunteers started with the screening procedure. Five volunteers withdrew before the start of the study.

Biomedical inclusion criteria were as follows: age between 18 and 75, stable body weight, no (history of) gastrointestinal or gallbladder disorders, no use of antibiotics or laxatives in the 12 months prior to the experiment, not operated on in the 12 months prior to the experiment, no complaints of diarrhea, obstipation or abdominal pain, no use of medication known to affect gastrointestinal function, serum cholesterol concentration < 7.0 mmol/L, serum triacylglycerol concentration < 2.5 mmol/L, normal hemocytometric values, normal urinary values for protein, glucose and pH. Five volunteers were excluded because of biomedical reasons.

A lactose breath test was performed to exclude hydrogen responders to lactose. Subjects came to the department after an overnight fast and consumed a drink with 50 g of lactose. Breath samples were taken before and at 30 minutes intervals after the lactose-drink. An increase in the concentration of 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 of transgalacto-oligosaccharides to check for possible intolerance. None of the volunteers showed any signs of intolerance or discomfort. None of the volunteers was 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 fully explained to the

volunteers, who gave their informed written consent. The food was free during the experiment and after successfully completing the study, the volunteers received a small financial reward.

Design

The study was conducted using a six weeks parallel trial with three intervention groups. The study was divided into two consecutive 3-week periods during which each participant consumed a run-in diet in the first 3-week period and an intervention diet in the last 3-week period. The interventions consisted of either a diet high in transgalacto-oligosaccharides (aimed to be 15 g/d, high-TOS), or a diet with a low amount of transgalacto-oligosaccharides (aimed to be 7.5 g/d, low-TOS), or a diet with no transgalacto-oligosaccharides (placebo). At the end of the run-in and intervention period, fecal samples and breath samples were collected. The group was divided into 3 start-groups, each starting one week after the other, to be able to handle all fresh fecal samples at 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 (Feunekes *et al*, 1995; Feunekes *et al*, 1993). This allowed us to estimate the habitual energy intake of the subjects. The questionnaires were coded and diets were calculated by use of the Netherlands Food Composition Table (1996). The study diets were formulated at 21 levels of energy intake, ranging from 7.5 to 17.5 MJ per day, so that each subject received a diet that met his or her energy needs. Body weights were recorded three times weekly, and energy intake was adjusted when necessary as to maintain a stable weight.

The diets consisted of conventional foods, and 21 different menus were provided over the course of each 3-week period. The nutrient composition of each diet was similar, except for the oligosaccharides. The diets were rich in protein and low in fiber.

The transgalacto-oligosaccharides (Elix'or®) were provided by Borculo Whey Products, Borculo, Netherlands. Fruit juices 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 based on a transgalacto-oligosaccharides syrup which consisted of 75% dry matter of which 60% transgalacto-oligosaccharides with a degree of polymerization of 2-8. The remaining dry matter consisted of 20% disaccharides (mainly lactose) and 20% monosaccharides (mainly glucose). The low-TOS mixture was composed of the syrup with extra glucose and lactose, to correct for the "non-oligosaccharide" components in the syrup. The placebo mixture consisted only of glucose and lactose to equal amounts as present in the high-TOS and low-TOS mixtures.

The subjects were blinded to the nature of the intervention by using color codes for labeling

the supplied foods. All foodstuffs were weighed out for each subject. On week days at noon, hot meals were served and consumed at the department. All other food was supplied daily as a package. Food for the weekend and guidelines for its preparation were provided on Fridays. Approximately 90% of energy intake was from supplied foods, the remaining 10% was from products chosen by the subjects from a list of items with no fiber.

Subjects were urged not to change their selection of free-choice items throughout the study, and not to change their smoking habits. The participants kept diaries in which they recorded their frequency of defecation, any sign of illness, gastrointestinal complaints, medication used, phase of the menstrual cycle, the consumption of free-choice items, and any deviations from their diets and in their living 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 per week 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.

To estimate stool recovery, thirty Ba-impregnated grains were swallowed daily together with the meals in the last 10 days of run-in and intervention period and were counted back in the fecal samples.

Data collection and analytical procedures

Data collection

In the last week of run-in and intervention periods, two breath samples were taken at two consecutive days just before lunch.

In the last week of run-in and intervention periods, volunteers came to the department twice to defecate. A button in 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. Temperature of the feces was checked and within 5 minutes after defecation, a sample was weighed and transported into an anaerobic cabinet. The remaining feces was immediately deep-frozen on dry ice to stop fermentation, then stored at -80 °C. In the last weekend of run-in and intervention periods, subjects collected all stools and stored them immediately on dry ice.

Breath hydrogen

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

Preparation of stool samples

All fecal samples were X-rayed before analysis to determine the number of Ba-grains (Philips Optimus M200, Eindhoven, The Netherlands). The samples were then thawed overnight at 4 °C. All samples of a single person from each 3-week period were weighted and homogenized in a bowl and mixer. Dry weight of the feces was estimated by drying a portion at 80 °C in an oven (Heraeus E45, Hanau, Germany) to constant weight. One portion was used for the preparation of the aqueous fraction of stool and centrifuged at 26000 g for 90 min at 4 °C (MSE, Scientific Instruments, Crawley, Sussex, UK). Fecal water was carefully removed and stored at -20 °C until analysis of short-chain fatty acids and bile acids. The pH was measured in both fecal homogenate and fecal water with a digital pH meter (CD 620; WPA Ltd. Cambridge, Cambs, UK). One portion of the mixed feces was freeze-dried and ground and kept in a dry environment until analysis of nitrogen and transgalacto-oligosaccharides. The remaining feces was stored at -20 °C until analysis of ammonia, indoles and skatoles.

Transgalacto-oligosaccharides in feces and juices

Transgalacto-oligosaccharides were measured in duplicate samples of 125 mg freeze-dried feces re-suspended in 2.4 mL distilled water. D-galacturonic acid (100 µl, 1250 ppm) was added as an internal standard before extraction. The mixture was vortexed and heated for 15 min at 100 °C and then centrifuged at 10.000 g. The supernatant was analyzed by high-performance anion-exchange chromatography (Dionex BV, Breda, Netherlands) on a Spectra-Physics system (San Jose, CA, USA) equipped with a Carbopac PA-1 column (4x250 mm; Dionex, Sunnyvale, CA, USA). Elution was performed with a flowrate of 1 ml/min and a linear sodium acetate gradient of 0 to 0.2 M in 0.1 M NaOH for 30 min.

The fruit juices were centrifuged at 10.000 g and the supernatant was analyzed with high-performance anion-exchange chromatography, using similar conditions as mentioned above, to estimate the concentration of transgalacto-oligosaccharides. The elution profile and the areas under the peaks of transgalacto-oligosaccharides were compared with that of a water solution with known quantities of transgalacto-oligosaccharides.

Traces of transgalacto-oligosaccharides up to 10 µg/ml could be detected in fecal water and juices.

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 *et al* (Tangerman & Nagengast, 1996) 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 of one person were analyzed within one run.

For the analyses of bile acids, 150 µl of fecal water was freeze-dried and prepared according to Glatz *et al* (Glatz *et al*, 1985) 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 (Chrompack, Bergen op Zoom, The Netherlands), by using a Hewlett Packard gas chromatograph (model 5890 series II) and Flame Ionisation Detector. Initial pressure of the carrier gas (hydrogen) was 90 kPa. Splitless injection was performed using a liquid sampler (HP 7673). To cover the wide range of concentrations, injection volumes varied from 0.5 to 2.5 µl. In addition, the levels of calibration solutions were adapted according to the injection volume. The initial oven-temperature was 150 °C, then 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 275 °C. The amount of each bile acid was calculated from area response by using the internal standard method with a 7 points multilevel bracketing calibration with pure standards. Samples of one person were analyzed within one run. The coefficient of variation within runs was 10% for concentrations below 10 µmol/L and 5% for concentrations greater then 10 µmol/L.

Fecal nitrogen, ammonia, indoles and skatoles

Ammonia was extracted from 5 gram of homogenized feces with perchloric acid (20 ml/ 1M), then pH was set to 7 (± 0.1) with KOH (5 M). A commercial test kit (Ammonia UV-method, cat. no. 1112732; Boehringer Mannheim GmbH, 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).

Indoles and skatoles were extracted from 5 gram of homogenized feces with methanol (50 ml). The mixture was homogenized using an ultra-turrax (Janke en Kunkel, Tampson, Zoetermeer, The Netherlands), then filtered through a glass microfiber filter (Whatman GF/A) before analysis. Analyses were performed in duplicate according to the method described by Wilkens (Wilkins, 1990) using HPLC analysis with ultraviolet absorption detection.

Microbiological analyses

Microbiological analyses of each sample were done within 3h at the Department of Food Science, division of Food Microbiology. All analyses and preparations were done in an anaerobic chamber ($H_2/CO_2/N_2$; 10:10:80, 21 °C, Hoekloos SHK050H , Rotterdam, The Netherlands), unless stated otherwise. Feces was diluted (10^{-1}) in 70 ml of a solution of buffered peptone water, tween 80 (1

g/L) and cysteine (0.5 g/L), then homogenized using an ultraturrax. Aliquots of 1 ml were diluted in reduced physiological peptone water in decimal steps. Of each of the dilutions 0.03 mL was plated in duplicate onto selective media. All media were kept at least 24h in the anaerobic chamber before use.

Nutrient Agar (NA) was used to determine total aerobes (Oxoid CM 3, Basingstoke, United Kingdom) with dilutions of 10^{-2} - 10^{-5}. For *Escherichia coli* Eosine Methylene Blue Agar (EMB, Oxoid CM 69, Basingstoke United Kingdom) was used with dilutions of 10^{-2} - 10^{-5}. Total anaerobes were counted on Fecal Reinforced Clostridial Agar (FRCA), which consisted of Reinforced Clostridial Medium (Oxoid CM 149, Basingstoke, United Kingdom) 38 g/L, Hemine solution (Sigma H2250, 50 mg/100mL, Sigma, St Louis, USA) 1%, Vitamin K1 solution (0.5% in 95% ethanol, Bufa Pharmaceuticals, Uitgeest, The Netherlands) 0.02%, Agar (Oxoid L13, Basingstoke, United Kingdom) 18 g/L, and fecal extract 100 ml. Fecal extract was prepared by mixing equal volumes of swine feces and Buffered Peptone Water (Oxoid CM 509, Basingstoke, United Kingdom) to which cysteine.HCl (1 g/L) and Tween 80 (10 g/L, Sigma P1754) were added. The mixture was homogenized and sterilized. Dilutions of 10^{-6} - 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, United Kingdom) using dilutions of 10^{-3} - 10^{-7}. For lactobaccili, LAMVAB was used (Hartemink *et al*, 1997) with dilutions of 10^{-2} - 10^{-7}. Bifidobacteria were counted on Raffinose Bifidobacterium Agar (RB) in dilutions of 10^{-4} - 10^{-7} (Hartemink *et al*, 1996). All dilutions were plated in duplicate.

Plates of 6 cm diameter (Greiner, Kremsmünster, Austria) were used. All plates were incubated at 37 °C for 1-4 days. NA and EMB were incubated aerobically, all other media were incubated anaerobically. After incubation, colonies were counted according to colony morphology. Counts from duplicate plates were averaged. Media and incubation conditions were controlled using growth of selected test bacteria on each batch of medium. Anaerobic conditions were controlled using anaerobic indicator strips. All visible colony morphologies were tested microscopically to determine the selectivity of the media.

Statistical analyses

The average values for each person of both run-in and intervention periods were calculated and used for the determination of the differences between intervention and run-in diets. Differences were checked for normality by visual inspection of the normal probability plots (univariate procedure; SAS Institute Inc, Cary, NC). Bacterial counts were logarithmized 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 *start-group* to this model did not contribute to the 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 the Dunnett test. This method encompasses a downward adjustment of the significance limit for multiple testing.

Our strategy for controlling a type I error was based on our main outcome variable: bifidobacterial counts. A pilot experiment was performed prior to the study to assess the within-subject variation of bacterial counts, which was 5% for bifidobacteria (data not shown). The present study was designed to detect a 7% increase in bifidobacterial counts after correction for the placebo-treatment, which is about half the effect observed by Bouhnik *et al* (1997). We used data from literature and former studies at our department to estimate the variances for the other variables and used these to predict detectable responses to transgalacto-oligosaccharides for a given probability (Snedecor & Cochran, 1989).

The statistical analysis package SAS, version 6.09 (Statistical Analysis Systems Institute, Inc., Cary, NC, USA) was used to perform the statistical analyses.

Results

Characteristics of 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 completed the study successfully. Some volunteers in the low-TOS and high-TOS groups had flatulence. There were no reports of problems with the palatability of the diets or the juices.

Table 1.
Population characteristics[1]

	Placebo group (n=13)	Low-TOS group (n=13)	High-TOS group (n=14)
Sex (male/female)	7/6	7/6	8/6
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] Mean ± SD.

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.

Table 2.
Daily dietary intake during the study[1]

	Placebo group (n=13)	Low-TOS[2] group (n=13)	High-TOS[2] group (n=14)
Energy (MJ)	11.0 ± 0.4	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
Transgalacto-oligosaccharides (g)	0	8.5	14.4

[1] Mean ± SEM per day.

[2] TOS, transgalacto-oligosaccharides.

Energy supplied by the free-choice items did not differ between the dietary regimes and accounted for 11.2% of total energy, with a range of 7.7% to 14.4%. There was no significant difference between run-in and intervention or between treatments in the intake of protein, carbohydrates, fat and fiber. The low-TOS diet contained a mean of 8.5 g transgalacto-oligosaccharides per day, the high-TOS diet contained 14.4 g transgalacto-oligosaccharides per (table 2).

Breath hydrogen

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

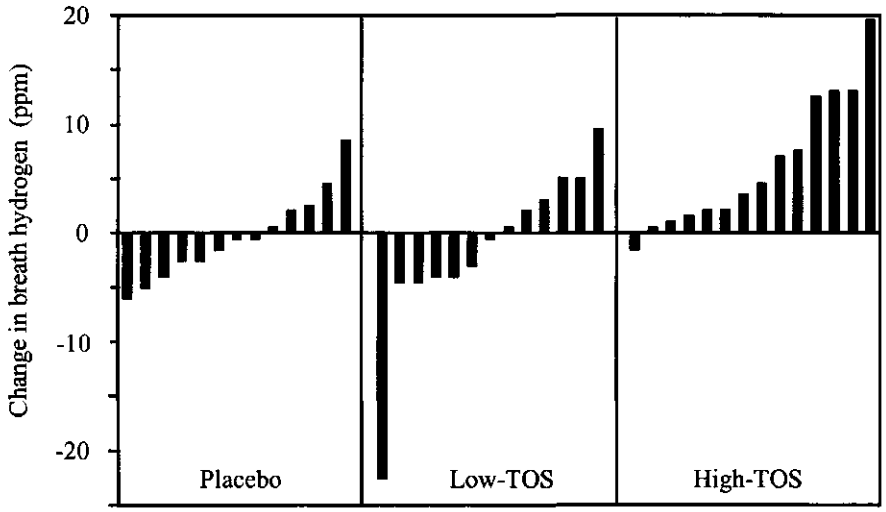

Figure 1. Individual changes in breath hydrogen concentration on a placebo diet, a diet with 8.5 g of transgalacto-oligosaccharides (low-TOS) or a diet with 14.4 g of transgalacto-oligosaccharides per day (high-TOS). Breath samples were taken twice at the end of the run-in period and at the end of the intervention period.

Bowel habit and stool composition

Data on bowel habit, fecal weight, fecal nitrogen density and fecal pH are given in table 3. There was no fecal excretion of transgalacto-oligosaccharides on any of the interventions. The transgalacto-oligosaccharides did not significantly affect reported stool frequency, percentage of fecal dry matter, fecal weight or fecal pH. There was a significant increase of the fecal nitrogen density in the high-TOS group after correcting for placebo (95% CI; 0.04, 0.62). On low-TOS the increase was not statistically significant after correcting for the change in the placebo group (95% CI; -0.14, 0.46).

Table 3.

Bowel habit, fecal weight, fecal nitrogen density and fecal pH[1]

	Placebo group (n=13)	Low-TOS[2] group (n=13)	High-TOS[2] group (n=14)	Differences (95% CI)[3]	
				Low-TOS compared with Placebo	High-TOS compared with Placebo
Number of stools per week					
Run-in	8.1 ± 0.7	6.2 ± 0.5	7.9 ± 0.7	-	-
Intervention	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	147 ± 11	113 ± 12	146 ± 22	-	-
Intervention	139 ± 14	127 ± 14	142 ± 18	22.3 (-21.2, 65.9)	3.52 (-39.3, 46.3)
Fecal dry matter (%)					
Run-in	23.3 ± 1.4	27.3 ± 1.6	25.7 ± 1.6	-	-
Intervention	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	5.2 ± 0.1	5.3 ± 0.1	5.1 ± 0.1	-	-
Intervention	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	6.8 ± 0.1	6.7 ± 0.1	6.6 ± 0.1	-	-
Intervention	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] Mean ± SEM; [2] TOS, transgalacto-oligosaccharides.

[3] Calculated as differences between changes (intervention minus run-in).

[4] Significantly different from placebo, $P < 0.05$.

Table 4.
Concentration of short chain fatty acids (mmol/L) and pH of fecal water[1]

	Placebo group (n=10)[5]	Low-TOS[2] group (n=12)[5]	High-TOS[2] group (n=12)[5]	Differences (95% CI)[3]	
				Low-TOS compared with Placebo	High-TOS compared with Placebo
Total SCFA[2] (mmol/L)					
Run-in	114.2 ± 9.6	108.6 ± 8.5	111.9 ± 8.8	-	-
Intervention	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 (C2, mmol/L)					
Run-in	71.8 ± 6.6	65.4 ± 5.4	69.2 ± 4.8	-	-
Intervention	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 (C3, mmol/L)					
Run-in	23.8 ± 1.8	20.3 ± 1.8	21.6 ± 2.3	-	-
Intervention	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 (C4, mmol/L)					
Run-in	11.4 ± 1.7	12.2 ± 1.6	11.9 ± 1.9	-	-
Intervention	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 iC4-C6[4] (mmol/L)					
Run-in	7.3 ± 1.0	10.6 ± 0.9	9.2 ± 1.2	-	-
Intervention	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	7.0 ± 0.1	7.0 ± 0.1	6.8 ± 0.1	-	-
Intervention	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] Mean ± SEM; [2] TOS, transgalacto-oligosaccharides; SCFA, short-chain fatty acids; [3] Calculated as differences between changes (intervention minus run-in).

[4] Sum of isobutyrate (4-carbon), valerate (5-carbon), isovalerate (5-carbon) and caproate (6-carbon). [5] Observations were missing because there was too little fecal water extractable from feces.

Short-chain fatty acids and bile acids in fecal water

Total short-chain fatty acid concentration in fecal water did not differ between the three interventions (table 4). The pH of fecal water and the concentrations of acetate, propionate, butyrate and the sum of iso-butyrate, valerate, iso-valerate and caproate were also not affected by the transgalacto-oligosaccharides. The molar ratio of acetate:propionate:butyrate was approximately 6:2:1 in all intervention groups. The total excretion of short-chain fatty acids per day was approximately 13 mmol/d. There were no differences in the excretion of short-chain fatty acids between the interventions.

Total concentrations of bile acids in fecal water decreased in all intervention groups, but there were no differences between groups (table 6). The ratio of hydrophobic (mono+dihydroxy) to hydrophilic (keto+trihydroxy) bile acids was not affected by the interventions. Secondary bile acids comprised about 85% of total bile acids in all intervention groups. (iso)Deoxycholic acid was the most abundant bile acid. The total excretion of bile acids was approximately 70 µmol/d, there were no differences in the excretion of bile acids between the interventions.

Ammonia, indoles and skatoles in feces

The fecal concentrations of ammonia, indoles and skatoles (table 5) were not affected by the transgalacto-oligosaccharides. The excretion of ammonia was approximately 85 mg/d, and did not differ between interventions. The excretion of indoles was 3 mg/d and of skatoles 1.3 mg/d, there were no differences between the interventions.

The intestinal microflora

Temperature of all samples for bacteriological analyses was checked. Mean temperature was 32.2 °C. The temperature of four samples was somewhat below 29.2 °C (= mean - 2 *SD). These were all very small samples that probably cooled down more quickly. None of the samples was excluded from analyses because of a low temperature. Counts of fecal bacteria were not affected by the interventions. There was an increase in the amount of bifidobacteria in the placebo group ($P<0.025$), the low-TOS group ($P<0.05$) and the high-TOS group ($P<0.05$). The differences between these changes were not statistically significant (table 7). Figure 2 shows the mean changes in fecal bacterial counts with their 95% confidence intervals on the three intervention diets. The ratio of bifidobacteria to the total amount of anaerobic bacteria was approximately 10 and was not affected by the interventions.

Table 5.

Fecal concentration of ammonia, indoles and scatoles (mg/100 g)[1]

	Placebo group (n=12)[4]	Low-TOS[2] group (n=13)	High-TOS group (n=14)	Differences (95% CI)[3]	
				Low-TOS compared with Placebo	High-TOS compared with Placebo
Ammonia (mg/100 g)					
Run-in	56.1 ± 4.4	76.3 ± 5.5	63.0 ± 5.1	-	-
Intervention	61.8 ± 5.1	75.9 ± 6.8	68.2 ± 6.1	-6.0 (-21.5, 9.5)	-0.4 (-15.7, 14.8)
Indoles (mg/100 g)					
Run-in	2.1 ± 0.4	2.3 ± 0.5	2.3 ± 0.4	-	-
Intervention	2.3 ± 0.4	1.9 ± 0.3	2.2 ± 0.4	-0.5 (-1.4, 0.4)	-0.3 (-1.1, 0.6)
Skatoles (mg/100 g)					
Run-in	0.8 ± 0.2	1.4 ± 0.4	1.5 ± 0.4	-	-
Intervention	0.9 ± 0.3	1.3 ± 0.4	1.4 ± 0.4	-0.3 (-1.0, 0.4)	-0.2 (-0.9, 0.4)

[1] Mean ± SEM.

[2] TOS, transgalacto-oligosaccharides.

[3] Calculated as differences between changes (intervention minus run-in).

[4] Observation was missing because there was urine in the feces.

Table 6.

Concentration of bile acids (μmol/L) in fecal water[1]

	Placebo group (n=11)[4]	Low-TOS[2] group (n=10)[4]	High-TOS[2] group (n=10)[4]	Differences (95% CI)[3]	
				Low-TOS compared with Placebo	High-TOS compared with Placebo
Total bile acids (μmol/L)					
Run-in	742.5 ± 136.1	680.7 ± 138.0	584.2 ± 132.8	-	-
Intervention	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[5]					
Run-in	141.2 ± 57.9	61.7 ± 30.2	85.3 ± 50.3	-	-
Intervention	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[5]					
Run-in	32.2 ± 11.3	23.8 ± 9.1	17.2 ± 5.9	-	-
Intervention	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[5]					
Run-in	321.0 ± 84.9	336.2 ± 60.1	282.0 ± 80.5	-	-
Intervention	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[5]					
Run-in	103.1 ± 28.3	155.0 ± 29.0	104.2 ± 41.0	-	-
Intervention	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[5]					
Run-in	62.8 ± 25.9	18.2 ± 5.1	22.4 ± 6.0	-	-
Intervention	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-Litocholic acid[5]					
Run-in	55.4 ± 5.4	62.9 ± 8.4	56.6 ± 6.9	-	-
Intervention	56.9 ± 4.7	53.5 ± 5.4	50.3 ± 6.9	-10.0 (-20.5, 0.4)	-5.1 (-15.6, 5.3)

Table 6 - continued.

	Placebo group (n=11)[4]	Low-TOS[2] group (n=10)[4]	High-TOS[2] group (n=10)[4]	Differences (95% CI)[3]	
				Low-TOS compared with Placebo	High-TOS compared with Placebo
7-keto-Deoxycholic acid[5]					
Run-in	26.7 ± 10.8	22.9 ± 6.4	16.5 ± 4.8	-	-
Intervention	26.5 ± 14.3	12.2 ± 2.3	13.7 ± 4.2	-9.8 (-23.4, 3.8)	-0.9 (-14.5, 12.7)
Ratio of hydrophobic: hydrophilic bile acids				-	-
Run-in	3.6 ± 1.2	4.5 ± 0.7	4.2 ± 1.4		
Intervention	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] Mean ± SEM; [2] TOS, transgalacto-oligosaccharides.

[3] Calculated as differences between changes (intervention minus run-in).

[4] Observations were missing because there was too little fecal water extractable from feces.

[5] Primary bile acids; cholic acid, chenodeoxycholic acid. Secundary bile acids; (iso)deoxycholic acid, (iso)lithocholic acid, ursodeoxycholic acid, 12-keto-litocholic acid, 7-keto-deoxycholic acid.

Table 7.
Fecal bacterial counts (log_{10} CFU/ g)[1]

	Placebo group (n=13)	Low-TOS[2] group (n=13)	High-TOS group (n=14)
Total anaerobes (log_{10} CFU/ g)			
Run-in	10.5 ± 0.1	10.4 ± 0.1	10.4 ± 0.1
Intervention	10.6 ± 0.1	10.6 ± 0.1	10.6 ± 0.1
Bifidobacteria (log_{10} CFU/ g)			
Run-in	9.4 ± 0.1	9.4 ± 0.1	9.2 ± 0.1
Intervention	9.8 ± 0.1	9.7 ± 0.1	9.6 ± 0.1
Lactobacilli (log_{10} CFU/ g)			
Run-in	5.0 ± 0.5	5.0 ± 0.5	5.9 ± 0.5
Intervention	5.0 ± 0.7	5.7 ± 0.7	6.5 ± 0.7
Clostridia (log_{10} CFU/ g)			
Run-in	7.9 ± 0.2	8.0 ± 0.1	8.0 ± 0.1
Intervention	8.1 ± 0.2	8.2 ± 0.1	7.9 ± 0.1
Total aerobes (log_{10} CFU/ g)			
Run-in	7.5 ± 0.2	7.5 ± 0.2	7.5 ± 0.2
Intervention	7.6 ± 0.2	7.3 ± 0.2	7.3 ± 0.1
E-coli (log_{10} CFU/ g)			
Run-in	7.1 ± 0.2	6.5 ± 0.6	6.2 ± 0.6
Intervention	7.1 ± 0.2	6.4 ± 0.4	6.7 ± 0.2

[1] Mean ± SEM; CFU, colony forming units.

[2] TOS, transgalacto-oligosaccharides.

Discussion

Our findings show that transgalacto-oligosaccharides, although fully fermented in the large intestine, do not beneficially change the composition or activity of the intestinal microflora. To our knowledge, this is the first placebo controlled feeding trial of transgalacto-oligosaccharide fermentation in humans.

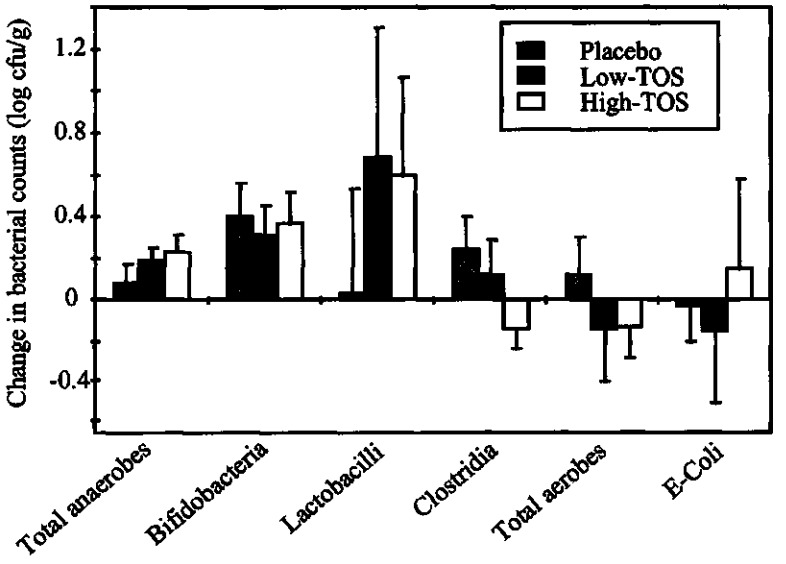

Figure 2. Changes in fecal bacterial counts (mean and 95% CI) on a placebo diet, a diet with 8.5 g of transgalacto-oligosaccharides (low-TOS) or a diet with 14.4 g of transgalacto-oligosaccharides per day (high-TOS).

Dietary intake, compliance to the treatment, fecal sampling and statistical power

We believe that the lack of effect in our study on the main outcome variables related to composition and activity of the intestinal microflora can not be explained by differences in dietary intake, non-compliance of the volunteers, inappropriate sampling and analysis of feces or by an insufficient statistical power of the experiment.

Our study was a completely controlled feeding trial in which all volunteers consumed a diet that was similar in nutrient composition. The energy intake was adjusted in order to maintain stable body weights which was warranted by body weight measurements three times weekly. The values for the analyzed duplicate portions were combined with the calculations based on the reported free-choice items. This allowed us to compute the energy and nutrient intakes of the volunteers during both run-in and intervention periods. There were no differences between the mean run-in and intervention diets for energy, macronutrients and dietary fiber, except for the amount of oligosaccharides in the diet. Total energy intake and the contribution of fat, protein and carbohydrates was similar for the intervention groups. We conclude that differences in dietary intake were negligible in our study and could not have affected the outcome.

The oligosaccharides in our study were dissolved in fruit juices. All diet items were color coded and the volunteers were blinded to their intervention group and to the vehicle of the oligosaccharides. There were no clear taste differences between the juices and none of the volunteers noticed anything unusual about them. The juices were divided over 3 portions of which one was taken together with the hot meal at our department. The other 2 portions were packed in sealed plastic bottles and consumed at home. Compliance as judged by the bottles that were emptied was near 100%. In an anonymous evaluation form we asked the volunteers about their compliance to the diet and it appeared to be extremely high for the juices. We therefore exclude the possibility that non-compliance of the volunteers affected the results. Moreover, consumption of the TOS supplements was confirmed by the increased reported flatulence in the high-TOS group.

When measuring the composition of the intestinal microflora, the handling and storing of fecal samples is of special importance (Bollongue, 1997; Haffejee, 1990). In a pilot study (Alles, 1997*a*) we found out that storing and freezing the fecal samples in a glycerol broth as described by Crowther *et al* (1971) decreased the bacterial counts of both bacteroides and *e.coli*, and increased the amount of clostridial counts. We therefore tried to minimize exposure to oxygen and avoid freezing and storing of the samples. Volunteers were informed about the importance of a quick handling of their fecal samples. Within 5 min after defecation, fecal samples were transported into an anaerobic cabinet. Freshness of the samples was checked by measuring the temperature. Control strains grew well on all media batches, indicating that the media were not inhibitory. No oxygen leaks were detected. Bifidobacterial counts were in accordance with other fecal flora studies (Buddington *et al*, 1996; Ito *et al*, 1993*a*; Ito *et al*, 1990; Guerin-Danan *et al*, 1998; Gibson *et al*, 1995*a*; Bouhnik *et al*, 1997). We are convinced that the fecal sampling in our study for the bacterial analyses was done carefully and could not have affected the results in any negative way.

The study protocol was designed to detect a 7% increase in bifidobacterial counts by consumption of transgalacto-oligosaccharides as compared to placebo. This a-priori power calculation was based on a 5% within-subject variation. The variance in the present study was somewhat lower (4.8%), probably because of the controlled diet. It provided an 80% chance of detecting any 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 transgalacto-oligosaccharides on bifidobacteria is probably smaller than 6.0%.

For most other end point variables, we were able to detect effects of 20% or smaller (range 2.8% to 18.8%) with 80% confidence. The within subject variation of fecal lactobacilli and bile acids was large (25 and 50%), as we had anticipated based on previous studies (Heijnen *et al*, 1998). We did not have data on the variation in indoles and skatoles prior to the experiment, it appeared to be high (30 and 40%). 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 exclude the possibility that an insufficient power influenced our main outcomes.

Apparent fermentability and gaseous response

No transgalacto-oligomers were recovered from any of the fecal samples. As the oligosaccharides are acid-stable and not hydrolyzed by human intestinal enzymes (*Burvall et al*, 1980), it is likely that all transgalacto-oligosaccharides in our study were fermented by bacteria in the colon. To our knowledge, this is the first study showing such complete fermentation of transgalacto-oligosaccharides through determination of the oligomers in the feces.

Bacterial degradation of TOS was reflected in the production of hydrogen. In the highest TOS-group, the hydrogen concentration was increased by 138 %, while there was no increase in the placebo group. In the low-TOS group, there was no difference in hydrogen concentration with placebo (95% CI: -7.5, 5.5 ppm). 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 non-hydrogen producers. Possible non-producers might have decreased the effect of TOS on mean hydrogen concentration.

A hydrogen response to TOS was found before in both rats (Djouzi & Andrieux, 1997; Meslin *et al*, 1993) and humans (Tanaka *et al*, 1983). Bouhnik *et al* (1997) compared hydrogen excretion in humans after 1 d of TOS consumption (10 g/d) to excretion after 7, 14 and 21 days. They observed a lower hydrogen response in the course of the study, possibly due to adaptation of the microflora to the substrate.

Bowel habits and stool composition

We did not observe any effects of TOS on 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 of the high TOS treatment.

We hypothesized that TOS would increase bacterial proliferation and thereby bacterial mass. As fecal bacteria contribute considerably to fecal weight (Stephen & Cummings, 1980; Hill, 1995), we expected to find an increase in total fecal weight, with a shift to a more watery constitution as bacteria are 80% water (Stephen & Cummings, 1980). We expected to find an increase in the frequency of defecation due to the laxative effects of an increased fecal mass. Although studies with other types of non-digestible oligosaccharides show an increase in stool weight (Alles *et al*, 1997*b*; Gibson *et al*, 1995*a*; Mortensen, 1992), or defecation frequency (Gibson *et al*, 1995*a*), our study and other TOS-studies (Ito *et al*, 1990; Bouhnik *et al*, 1997) did not show such effects. We did find a significant increase of 6.5% in the nitrogen density of the fecal dry matter on the high-TOS treatment, which increased the nitrogen excretion by 0.12 g/d after correction for placebo. This

increase is likely to be due to an increase in the bacterial mass (Gibson *et al*, 1995*a*; Stephen & Cummings, 1980; Levrat *et al*, 1993; Younes *et al*, 1995). The additional 0.12 g of N excreted is equivalent to approximately 1.9 g of bacterial solids (Stanier *et al*, 1979) and 9.5 g of wet stool (Stephen & Cummings, 1980). 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

Transgalacto-oligosaccharides 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 transgalacto-oligosaccharides on the production of short-chain fatty acids in fecal water or on the pH of the colon content. When hydrogen is produced, most bacteria simultaneously produce short-chain fatty acids (Cummings & MacFarlane, 1991). MacFarlane *et al* (MacFarlane *et al*, 1992), in an autopsy study of victims of sudden death, found high amounts of short-chain fatty acids in the proximal colon and lower amounts towards the end of the gastrointestinal tract. They attributed this to rapid absorption of these acids by the colonic mucosa. As oligosaccharides are rapidly fermented, possibly to a large extent in the proximal colon, fecal pH and the fecal concentration of short-chain fatty acids are not necessarily good indicators for saccharolytic activity. Other researchers also failed to show any effects of fermentable carbohydrates on short-chain fatty acids or fecal pH (Philips *et al*, 1995; Heijnen *et al*, 1998; Alles *et al*, 1996; Alles *et al*, 1997*b*; Munster *et al*, 1994*b*). 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 before (Munster *et al*, 1994*b*; Gibson *et al*, 1995*a*; Cummings, 1981; Heijnen *et al*, 1998), and did not differ among treatments.

We were unable to show any effects of transgalacto-oligosaccharides on the concentration of bile acids in fecal water. We hypothesized that acidification of the colon content, as a result of the fermentation of oligosaccharides, would lead to precipitation of the soluble deconjugated bile acids (Fini & Roda, 1987; Bruce, 1987; Munster & Nagengast, 1993) and to a suppression of the bacterial conversion of primary to secondary bile acids (Christl *et al*, 1997; Munster & Nagengast, 1993). 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 (Lapré *et al*, 1993). On a low-fiber diet, the production of acids by fermentation processes is probably low. Increasing our low-fiber diet with fermentable transgalacto-oligosaccharides might not have been effective in reaching the acidification necessary for the expected effects on bile acids. Due to 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, using different dietary treatments (Munster *et al*, 1994*b*; van Faassen *et al*, 1996; Nagengast *et al*, 1988*b*), we might have missed possible smaller effects in the present study.

Ammonia, indoles and skatoles in feces

Transgalacto-oligosaccharides did not lower fecal concentrations of the protein degradation products ammonia, indoles and skatoles.

We hypothesized that transgalacto-oligosaccharides would decrease protein fermentation products by two mechanisms. Firstly, we expected to find a depression of protein fermentation by providing the bacteria with an easy carbohydrate source. Both substrate competition and a low pH are thought to decrease the use of endogenous and dietary proteins as an energy source for bacteria (Mortensen *et al*, 1990; Alles *et al*, 1997*b*). Also, when transgalacto-oligosaccharides are used as an energy substrate by the bacteria, nitrogen sources are required for the subsequent increase in bacterial growth. Use of ammonia as a source of bacterial nitrogen, leads to decreased concentrations of fecal ammonia and an increase of bacterial nitrogen (Birkett *et al*, 1996; Mortensen *et al*, 1990; Levrat *et al*, 1993).

We did not observe a decrease in the fecal concentrations of the protein degradation products ammonia, indoles and skatoles. We also did not observe a decrease in the concentration of isobutyrate, valerate, isovalerate and caproate, which are short-chain fatty acids that originate from bacterial breakdown of amino acids (Rasmussen *et al*, 1988; MacFarlane & MacFarlane, 1993). Ito *et al* (1993*a*, 1993*b*) and Djouzi *et al* (Djouzi & Andrieux, 1997) showed a decrease in one or more of the above mentioned variables. Ito e.g. showed a 37.2% decrease in the concentration of indoles by supplementing the diet of Japanese men with 2.5 g of transgalacto-oligosaccharides per day for 3 weeks (Ito *et al*, 1993*a*). The power of detecting such an effect in our study was 80%.

We chose for a background diet with a relative high protein content (16%) in order to increase protein fermentation of dietary proteins (Gibson *et al*, 1976). We hereby expected to increase the chance of showing inhibiting effects of transgalacto-oligosaccharides on this fermentation. Levrat *et al* (Levrat *et al*, 1993) studied the effects of the fermentable carbohydrate inulin in rats fed a high versus a moderate protein diet. They showed that use of ammonia as a source of bacterial growth was increased on inulin, but more evident when the dietary protein level was moderate. In high protein diets, uremia and flux of urea from plasma to colon is high and an important determinant of fecal concentrations of ammonia (Levrat *et al*, 1993; Younes *et al*, 1995). We conclude that transgalacto-oligosaccharides have no significant effects on protein fermentation when supplemented to a high protein background diet.

Intestinal microflora

We did not observe any effects of transgalacto-oligosaccharides on the composition of the intestinal

microflora when tested in a controlled feeding trial against a placebo treatment.

It was striking that bifidobacterial counts increased by 4% in the course of the study, independently of the treatment. The differences between the increases on placebo and transgalacto-oligosaccharides were not statistically significant. Without the placebo group, the conclusion would have been that transgalacto-oligosaccharides selectively increase bifidobacteria in the gut. We can not explain the increase of bifidobacteria in all groups. There were no differences in the background diet or in the handling and analyzing of the fecal samples. There were no other bacteria that increased during the study. Moreover, test bacteria were analyzed every week and showed no tendency to rise during the study. We hypothesize that there might have been an adaptation of bifidobacteria to the background diet (high-protein, low-fiber). 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 (Koopman, 1984).

There was a non-significant rise in lactobacilli on both low-TOS (of 14%) and high-TOS (of 10%), which we were unable to detect because of the large variation.

Other researchers have shown that certain non-digestible oligosaccharides selectively stimulate the growth of bifidobacteria in the large intestine. Often linear study designs were used with measurements before and after the treatment (Ito *et al*, 1993*a*; Bouhnik *et al*, 1997; Gibson *et al*, 1995*a*; Buddington *et al*, 1996). Our results indicate the importance of a placebo treatment to exclude possible time, placebo and unknown factors.

Our volunteers were healthy volunteers, not especially selected for having low bifidobacterial counts. In this population, using a high-protein and low-fiber diet, we were unable to show any effects of transgalacto-oligosaccharides on the composition of the intestinal flora. It might be difficult to get diet-induced changes when there is a stable and healthy balance in the microfloral population.

Conclusions

We conclude that transgalacto-oligosaccharides, supplemented to a relative decadent diet, are completely fermented in the colon of healthy individuals, but do not beneficially affect the composition of the intestinal microflora nor the putative risk markers of colon cancer. We believe that the lack of effect in our study on the main outcome variables related to composition and activity of the intestinal microflora could not be explained by differences in dietary intake, non-compliance of the volunteers, inappropriate sampling of feces or by an insufficient statistical power of the experiment. It remains possible that transgalacto-oligosaccharides have effects in other study-populations such as people with low bifidobacterial counts, or when tested against another background diet.

Acknowledgments

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5

Fructo-oligosaccharides and transgalacto-oligosaccharides affect the bacterial glycosidase activity in human subjects

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Abstract

Non-digestible oligosaccharides have been claimed to benefit the health of the colon by affecting composition and activity of the intestinal microflora. We performed two human trials to test whether fructo-oligosaccharides (FOS-study) and transgalacto-oligosaccharides (TOS-study) would change the bacterial glycosidase activity in the colon. In the FOS-study we investigated fermentation of fructo-oligosaccharides in 24 healthy men by using a placebo-controlled crossover design (with glucose as the placebo). Fructo-oligosaccharides significantly decreased α-L-arabinofuranosidase activity by 35%. The TOS-study was a controlled feeding trial in which the effects of transgalacto-oligosaccharides were tested in 25 healthy volunteers receiving either transgalacto-oligosaccharides or placebo. Transgalacto-oligosaccharides significantly increased the activity of ß-galactosidase by 123%. Neither fructo-oligosaccharides nor transgalacto-oligosaccharides affected the activities of ß-glucosidase or ß-glucuronidase, which are enzymes involved in the generation of toxic compounds. We conclude that non-digestible oligosaccharides affect glycolytic activity of human intestinal bacteria in a substrate specific manner.

Introduction

Non-digestible oligosaccharides naturally occur in various edible food plants (Loo *et al*, 1995) and in human breast milk (Carlson, 1985; Coppa *et al*, 1993; Grönberg *et al*, 1989). As they are not hydrolyzed by the enzymes in the human small intestine, they reach the colon intact. Bacterial fermentation of oligosaccharides in the colon has been claimed to benefit its health by selectively stimulating the growth of bifidobacteria and by decreasing colon cancer risk markers (Ito *et al*, 1990; Bouhnik *et al*, 1997; Gibson *et al*, 1995*a*; Buddington *et al*, 1996; Bouhnik *et al*, 1996*b*).

Gut bacteria degrade non-digestible carbohydrates by using a wide range of depolymerizing enzymes. Polysaccharidase and glycosidase activities are found extracellularly as well as associated with bacterial cells (MacFarlane *et al*, 1991; Cummings & MacFarlane, 1991). By inducing these enzymes, dietary components may affect the metabolic activity of the microflora. Several studies were done to measure the end-products of bacterial breakdown of non-digestible oligosaccharides and often no effects were found on their fecal excretion (Alles *et al*, 1996; Alles *et al*, 1997*b*; Gibson *et al*, 1995*a*). This might be due to a rapid absorption and utilization of fermentation products by the colonic mucosa (Höverstad *et al*, 1982; Cummings *et al*, 1987). MacFarlane *et al* (1991) measured the glycosidase activities in different regions of the human large intestine and conclude that activities were found throughout the large intestine without significant regional differences. Therefore, the activity of glycolytic enzymes in feces might be a more relevant measure to study the metabolic activity of the intestinal microflora, then fecal end-products of bacterial fermentation. Little is known about the glycolytic responses to consumption of non-digestible oligosaccharides by humans, but in a rat experiment by Djouzi *et al* (1997) it was found that there are profound differences between oligosaccharides.

We studied bacterial glycosidase activity induced by consumption of non-digestible oligosaccharides compared with digestible carbohydrates in two human trials. The first experiment was a free-living study done with fructo-oligosaccharides, which are short-chain fructans. The second experiment was a controlled feeding trial, using transgalacto-oligosaccharides, which are produced from lactose by enzymatic transgalactosylation. This work is an extension of previous studies that describes the fate of fructo-oligosaccharides in the human intestine (Chapter 2) and the effects of transgalacto-oligosaccharides on microbial composition and colon cancer risk markers (Chapter 4).

Methods

Fructo-oligosaccharides study

Experimental methods are described in detail in Chapter 2. Briefly, twenty-four healthy men aged 19-28 years participated in a single-blind crossover trial with supplement periods of 7 d and a 7d wash-out period. The supplements where consumed in random order and consisted of either 15 g of fructo-oligosaccharides (Raftilose P95®, ORAFTI, Tienen, Belgium) or 4 g of glucose (Cerestar Pur 01934; Cerestar Benelux BV, Sas van Gent, The Netherlands). Fructo-oligosaccharides had a degree of polymerization of 2-7 monosaccharide units.

At the end of each supplement period volunteers came to the Department twice to defecate. Within 15 min after defecation the feces were weighed and immediately deep-frozen on dry ice to stop fermentation, then stored at -20°C.

Transgalacto-oligosaccharides study

This investigation formed part of a larger study, details of the experimental design and results on composition of the intestinal microflora and colon cancer risk markers are presented in Chapter 4. The experiment was preceded by a screening procedure, comprising a healthy questionnaire, blood and urine analyses, a transgalacto-oligosaccharides tolerance test and a lactose breath test. Forty-one volunteers (19 women and 22 men) entered a strictly controlled single-blind parallel experiment. The study was divided into two consecutive 3-week periods during which each participant consumed a run-in diet in the first 3 weeks followed by a 3-week intervention diet which differed only in the amount of transgalacto-oligosaccharides: 0 g/d (placebo) or 15 g/d (Elix'or®, Borculo Whey Products, Borculo, the Netherlands). Transgalacto-oligosaccharides were produced by transgalactosylation of lactose and had a degree of polymerization of 2-8 monosaccharide units. The diets consisted of conventional foods, and the nutrient composition of each diet was similar. Approximately 90% of energy intake was from supplied food, the remaining 10% was from products chosen by the subjects from a list of items. In the last week of both run-in and intervention period, volunteers came to the Department twice to defecate. Within 10 min after defecation the feces was deep-frozen on dry ice to stop fermentation, then stored at -20°C. In the last weekend of run-in and intervention periods, subjects collected all stools and stored them immediately on dry ice.

Both studies were approved by the Medical Ethics Committee of the Division of Human Nutrition and Epidemiology. The protocol and aims of the study were fully explained to the volunteers, who gave their informed written consent.

Preparation of stool samples

Samples were thawed overnight at 4 °C. Feces of a single person of one period were weighted and homogenized in a bowl and mixer. A portion was used for the preparation of the aqueous fraction of stool and centrifuged at 26000 g for 90 min at 4 °C (MSE, Scientific Instruments, Crawley, Sussex, UK). Fecal water was carefully removed and stored at -20°C until analysis of glycolytic enzymes.

Bacterial glycolytic activities

Glycosidases are enzymes which can cleave off single units of sugar residues from glycosylated compounds including the non-reducing site of oligomeric sugars. The p-nitrophenyl glycoside assay (pnp-assay) was used to measure the rate of release of p-nitrophenol from p-nitrophenylglycosides in fecal water. We tested the following bacterial glycosidase activities: α-L-arabinofuranosidase, α-glucosidase, β-glucosidase, α-galactosidase, β-galactosidase, β-glucuronidase, β-xylosidase, α-fucosidase. The reaction mixture contained 25 µl substrate solution (0.1% w/v, Sigma, St-Louis MI, USA) and 15 µl of the diluted fecal water (1:11) in 75 µl phosphate buffer (50 mM, pH 6.5). Incubation was at 37 °C and the release of p-nitrophenol from pnp-glycosides was measured spectrophotometrically at 405 nm after addition of 125 µl of glycine (0.5 M glycine NaOH buffer, pH 9.0 + 2 mM EDTA). The activity was calculated using a molar extinction coefficient of 13700 M^{-1} cm^{-1}. Incubation time changed between substrates. Enzyme activities were expressed as units, one unit corresponds to the release of 1 µmol glycoside min^{-1} under standard conditions. Absorbances exceeding 1 after correction for the blank, were diluted further and measured again.

Statistical methods

Differences were checked for normality by visual inspection of the normal probability plots. For the fructo-oligosaccharides study, paired t-tests were used to test the difference between placebo and fructo-oligosaccharides supplementation. For the transgalacto-oligosaccharides study, unpaired t-tests were used to test the differences between changes. Two-sided *P*-values less then 0.05 were considered significant. The statistical analysis package SAS, version 6.09 (Statistical Analysis Systems Institute, Inc.; Cary, NC, USA) was used to perform the statistical analyses.

Results

Fructo-oligosaccharides study

All volunteers completed the study successfully, were apparently healthy and had normal body weight (BMI 21.7 (SD 1.9) kg/m^2). In 4 fecal samples, there was too little fecal water extractable.

Compared with the placebo treatment, there was a significant decrease (of 34.8%) in the arabinofuranosidase activity on the fructo-oligosaccharides supplement. There was a non-significant increase in ß-galactosidase activity of 45.3% (P = 0.0605) and a non-significant decrease in ß-xylosidase of 18.8% (P = 0.075). There were no other changes in glycosidase activity (table 1).

Transgalacto-oligosaccharides study

One female subject withdrew in the first week of the experiment because of personal reasons; data from this subject were excluded from analyses. In four subjects, there was too little fecal water extractable from feces. All other subjects completed the study successfully. They were healthy and had normal body weight (BMI 22.8 (SD 2.3) kg/m^2).

Transgalacto-oligosaccharides significantly increased the activity of ß-galactosidase by 122.8%, after correction for placebo. There were no other changes in glycosidase activity (table 2).

Discussion

We found that fructo-oligosaccharides and transgalacto-oligosaccharides, two non-digestible oligosaccharides, affect the glycolytic activity of human intestinal bacteria in a substrate specific manner, without increasing those enzymes that are involved in the production of toxic and carcinogenic metabolites.

We measured the glycosidase activity aerobically in fecal water, which reflects the extracellular enzyme activity. As the feces was frozen before we prepared the fecal water, some intracellular enzymes might have been released due to lysis of bacteria. MacFarlane *et al* (MacFarlane *et al*, 1991) found that cell-bound activities are between 11 and 19 fold greater than those occurring extracellularly. The relative distribution of the enzymes however between extracellular and cell-bound enzymes did not differ much in their study.

Both the decrease of arabinofuranosidase on fructo-oligosaccharides and the increase of ß-galactosidase on transgalacto-oligosaccharides were not due to differences in the proportion of fecal wet weight. Correction for fecal wet weight did not change the results.

The availability of substrates to gut bacteria leads to the induction of substrate specific hydrolyzing enzymes. Fructo-oligosaccharides can be hydrolyzed by ß-fructosidase and transgalacto-oligosaccharides can be hydrolyzed by ß-galactosidase. We did not measure the activity of β-fructosidase, as no pnp-substrates are available for this enzyme. However, Bouhnik *et al* (1996*b*) described a method for measuring the β-fructosidase activity using High Performance Anion Exchange Chromatography. They showed an increase in ß-fructosidase activity after consumption

Table 1.

Bacterial glycolytic activities (IU/l fecal water) in 20 subjects receiving dietary supplements with 4 grams of glucose (placebo) or 15 grams of fructo-oligosaccharides in a cross-over design with supplement periods of 1 week*

	Placebo		Fructo-oligosaccharides		Difference (95% CI)†
	Mean	SEM	Mean	SEM	
α-L-Arabinofuranosidase	400	61	261	30	-139 (-256, -21)‡
α-Glucosidase	221	23	189	16	-32 (-81, 17)
β-Glucosidase	133	9	113	11	-21 (-49, 7)
α-Galactosidase	328	86	244	65	-84 (-292, 125)
β-Galactosidase	373	42	542	94	169 (-8, 347)
β-Glucuronidase	166	28	152	46	-14 (-98, 70)
β-Xylosidase	186	40	151	37	-35 (-75, 4)
α-Fucosidase	22	4	19	4	-4 (-11, 4)

α-L-Arabinofuranosidase, *EC* 3.2.1.55; α-glucosidase, *EC* 3.2.1.20; β-glucosidase, *EC* 3.2.1.21; α-galactosidase, *EC* 3.2.1.22; β-galactosidase, *EC* 3.2.1.23; β-glucuronidase, *EC* 3.2.1.31; β-xylosidase, 3.2.1.37; α-fucosidase, 3.2.1.51.

* Four observations were missing because there was too little fecal water extractable from feces.

† Fructo-oligosaccharides compared with placebo.

‡ Difference between fructo-oligosaccharides and placebo was significant, $P = 0.0229$.

of fructo-oligosaccharides. In the TOS-study, we showed an enhanced activity of ß-galactosidase by specific substrate induction by transgalacto-oligosaccharides, which was not due to differences in fecal weight. This was also found by Djouzi *et al* (1997) in rats.

Substrates can also affect the activity of non-specific glycolytic enzymes, due to either changes in microflora composition or shifts in the metabolic activity of individual species or strains (Djouzi & Andrieux, 1997; Buddington *et al*, 1996; Reddy *et al*, 1992). We observed a decreasing effect of fructo-oligosaccharides on the activity of α-L-arabinofuranosidase, which was also not due to differences in fecal weight. Arabinofuranosidase is the enzyme that degrades arabinosyl linkages from various hemicelluloses such as arabinans, (arabino)xylans, (arabino)galactans and arabinose-substituted xyloglycans (Kaji, 1984). Fructo-oligosaccharides also increased the activity of ß-galactosidase and decreased the activity of ß-xylosidase. Probably due to the small number of volunteers relative to the large variation in enzyme activities, these changes were not statistically significant.

Changing the activity of glycolytic enzymes may affect human health. Some of the enzymes help to generate energy for the colon mucosa in the form of butyrate. Increasing the activity of ß-galactosidase allows further degradation of lactose that escapes digestion and of arabinogalactans, which are important non-starch polysaccharides.

Other bacterial enzymes may have adverse effects in the colon. ß-Glucuronidase is involved in the hydrolysis of glucuronide conjugates in the gut, which lead to the generation of toxic and carcinogenic metabolites (Rowland, 1988; Mallett & Rowland, 1987). The hydrolytic activity of ß-glucosidase is responsible for the generation of mutagenic aglycones (Mallett & Rowland, 1987). We and others (Djouzi & Andrieux, 1997; Bouhnik *et al*, 1996*a*; Kleessen *et al*, 1997; Bouhnik *et al*, 1996*b*) did not show any effects of fructo-oligosaccharides or transgalacto-oligosaccharides on the activity of ß-glycosidase and ß-glucuronidase. Buddington *et al* (1996) showed a decrease of ß-glucuronidase on fructo-oligosaccharides but did not compare the effects with a placebo treatment and was thus unable to exclude possible time effects.

This study shows that the glycolytic activity of human intestinal bacteria in feces can be used as a biomarker of colonic metabolic activity, in dietary interventions with non-digestible oligosaccharides. Although our study design was not ideally suited to test the differences between fructo-oligosaccharides and transgalacto-oligosaccharides, we showed that non-digestible oligosaccharides affect the glycosidase activity in a substrate specific manner. Both substrates did not affect those enzymes that generate toxic and carcinogenic metabolites.

Table 2.

Bacterial glycolytic activities (IU/l fecal water) in 21 subjects receiving a run-in diet for 3 weeks, and a placebo diet (with no transgalacto-oligosaccharides) or a diet with 15 grams of transgalacto-oligosaccharides for the subsequent 3 weeks*

	Placebo (n=11)		Transgalacto-oligosaccharides (n=10)		Difference (95% CI)†
	Mean	SEM	Mean	SEM	
α-L-Arabinofuranosidase					
Run-in	398	49	254	61	-
Intervention	438	57	187	31	-107 (-244, 29)
α-Glucosidase					
Run-in	689	132	320	80	-
Intervention	657	140	486	264	198 (-244, 640)
β-Glucosidase					
Run-in	45	12	35	8	-
Intervention	51	8	31	7	-11 (-37, 16)
α-Galactosidase					
Run-in	513	134	205	43	-
Intervention	447	96	215	34	76 (-136, 288)
β-Galactosidase					
Run-in	787	121	474	53	-
Intervention	709	126	978	222	582 (141, 1022) ‡
β-Glucuronidase					
Run-in	87	20	57	12	-
Intervention	89	18	43	9	-16 (-55, 23)

Table 2 - continued.

	Placebo (n=11)		Transgalacto-oligosaccharides (n=10)		Difference (95% CI)[†]
	Mean	SEM	Mean	SEM	
β-Xylosidase					
Run-in	66	11	51	10	-
Intervention	69	9	47	9	-8 (-30, 15)
α-Fucosidase					
Run-in	29	8	19	4	-
Intervention	32	9	18	5	-4 (-20, 11)

Arabinofuranosidase, *EC* 3.2.1.55; α-glucosidase, *EC* 3.2.1.20; β-glucosidase, *EC* 3.2.1.21; α-galactosidase, *EC* 3.2.1.22; β-galactosidase, *EC* 3.2.1.23; β-glucuronidase, *EC* 3.2.1.31; β-xylosidase, 3.2.1.37; α-fucosidase, 3.2.1.51.

[*] Four observations were missing because there was too little fecal water extractable from feces.

[†] Transgalacto-oligosaccharides compared with placebo.

[‡] Difference between changes (intervention minus run-in) was significant, $P = 0.0124$.

6

Consumption of fructo-oligosaccharides does not favorably affect blood glucose and serum lipids in non-insulin dependent diabetic patients

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Abstract

Fructo-oligosaccharides have been claimed to lower fasting glycemia and serum total cholesterol concentrations, possibly via effects of short-chain fatty acids from fermentation. We studied the effects of fructo-oligosaccharides on blood glucose, serum lipids and serum acetate in 20 non-insulin dependent diabetic patients. In a randomized, single-blind crossover design, patients consumed either glucose as a placebo (4 g/d) or fructo-oligosaccharides (15 g/d) for 20 days each. Average daily intakes of energy, macronutrients and dietary fiber were similar on both treatments. Compliance, expressed as the proportion of supplements not returned, was near 100% during both treatments. Fructo-oligosaccharides did not significantly affect fasting concentrations of serum total cholesterol (95% CI; -0.07, 0.48 mmol/L), HDL cholesterol (95% CI; -0.04, 0.04 mmol/L), LDL cholesterol (95% CI; -0.06, 0.34 mmol/L), serum triacylglycerol (95% CI; -0.21, 0.44 mmol/L), serum free fatty acids (95% CI; -0.08, 0.04 mmol/L), serum acetate (95% CI; -0.01, 0.01 mmol/L) or blood glucose (95% CI; -0.37, 0.40 mmol/L). We conclude that 20 days of dietary supplementation with fructo-oligosaccharides has no major effect on blood glucose, serum lipids or serum acetate in non-insulin dependent diabetic patients. This lack of effect was not due to changes in dietary intake, insufficient statistical power or non-compliance of the patients.

Introduction

Fructo-oligosaccharides are non-digestible oligosaccharides that naturally occur in various edible food plants such as onions and leeks (Van Loo *et al*, 1995; Spiegel *et al*, 1994). The recent use of fructo-oligosaccharides as a food ingredient has triggered much research on their possible health effects. As fructo-oligosaccharides are not hydrolyzed by enzymes in the human small intestine, they reach the colon intact. In a previous study we showed that fructo-oligosaccharides, added to the diet of young healthy subjects, are fully metabolized by the colonic microflora (Alles *et al*, 1996). End products of carbohydrate fermentation are gases, lactate and short-chain fatty acids, such as acetate, propionate and butyrate. Fermentation of fructo-oligosaccharides probably takes place in the proximal colon leading to a rapid increase in breath hydrogen after consumption (Alles *et al*, 1996; Rumessen *et al*, 1990; Alles *et al*, 1997*b*). The short-chain fatty acids that are produced during this fermentation are thought to be readily absorbed by the colonic mucosa (Pomare *et al*, 1985; Cummings *et al*, 1987*b*; Rombeau & Kripke, 1990). It is known that butyrate serves as a fuel for the mucosa, whereas acetate and propionate enter the portal blood and may influence systemic carbohydrate and lipid metabolism (Cummings *et al*, 1987*b*). Because acetate and propionate have dissimilar effects on glycaemia (Akanji *et al*, 1989; Akanji & Hockaday, 1990; Thorburn *et al*, 1993; Wolever *et al*, 1991; Carman *et al*, 1993; Boillot *et al*, 1995) and serum lipids (Wolever *et al*, 1995; Wolever *et al*, 1991; Wolever *et al*, 1996; Kok *et al*, 1996), the pattern of fermentation of fructo-oligosaccharides might be of importance when predicting their metabolic effects.

Yamashita *et al* (1984) studied the systemic effects of adding 8 g of fructo-oligosaccharides to the daily diet of non-insulin dependent diabetics whose blood glucose and serum lipid levels were still uncontrolled. They found an 8% decrease in fasting blood glucose levels, a 6% decrease in total cholesterol and a 10% decrease in LDL cholesterol. Several rat experiments have been done since, all showing that fructo-oligosaccharides lower serum triacylglycerol, total-, LDL- and VLDL-cholesterol (Fiordaliso *et al*, 1995; Levrat *et al*, 1994; Kok *et al*, 1996; Delzenne *et al*, 1993). Two recent intervention studies were done in healthy humans. A study by Luo *et al* (1996) showed that chronic consumption of 20 g of fructo-oligosaccharides daily only increased basal hepatic glucose production but had no effect on insulin-stimulated glucose metabolism or serum lipids. Pedersen *et al* (1997) studied the effect of inulin on blood lipids and did not find any changes in either total cholesterol, HDL cholesterol, LDL cholesterol or triacylglycerol. Thus the effects of fructo-oligosaccharides on blood glucose and serum lipids are not yet clear.

In the present study we investigated the effects of fructo-oligosaccharides in twenty non-insulin dependent diabetic patients on serum acetate concentrations, fasting glycaemia, free fatty

acids, triacylglycerol, total, LDL and HDL cholesterol, in a 6-weeks placebo-controlled experiment using a cross-over design.

Materials and methods

Patients and experimental design

We recruited patients that were diagnosed with non-insulin dependent diabetes using WHO diagnostic criteria (WHO Study Group, 1994), from 5 general practices in the environment of Nijmegen. All practices were connected to the Nijmegen Monitoring Project, the registration network of the Department of General Practice of the University of Nijmegen. Twenty non-insulin dependent diabetic patients (9 men and 11 postmenopausal women) were selected. All patients had previous measures of fasting blood glucose levels exceeding 6.5 mmol/L and fasting serum cholesterol levels exceeding 6 mmol/L. Participants had no history of gastrointestinal disease, and had not been treated with antibiotics or laxatives in the 3 months prior to the experiment. Patients were treated for their diabetes by dietary advice and 17 patients used oral blood glucose lowering medication. Patients were also treated for diabetes related co-morbidity and used e.g. antihypertensive agents (9 subjects) and lipid-lowering drugs (1 subject).

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 fully explained to the patients, who gave their informed written consent. After successfully completing the study, the patients received a small financial reward.

A single-blinded placebo-controlled crossover design for 2x20 days was used; there was no wash-out period between the treatments. Each participant used each of two supplements in random order. Blood samples were collected at the end of each treatment period. Patients kept a diary in which they recorded time of supplement consumption, possible diseases or discomfort, medication used and deviations in eating, drinking or lifestyle behavior. Complaints of flatulence were rated on a 4-point Likert scale (none; mild; moderate; severe).

Supplements and food intake

Patients were instructed to maintain habitual eating, drinking and lifestyle behavior. They were asked not to eat probiotic dairy products which contain micro-organisms able to survive passage through the upper gastrointestinal tract, or to consume products containing large amounts of fructo-oligosaccharides, such as onions and leeks. Subjects were given a list of these foods.

The patients recorded their habitual diet for two days in each treatment period. These food records were coded and the nutrient and energy intakes were calculated with use of the modified version of the 1993 release of The Netherlands Nutrient Data Bank (Anonymous, 1993*a*).

On day 1 of the study, the patients were randomly assigned to daily treatment with 15 g of fructo-oligosaccharides (Raftilose P95®; ORAFTI, Tienen, Belgium) or 4 g of glucose as a placebo (Cerestar Pur 01934; Cerestar Benelux BV, Sas van Gent, The Netherlands). The supplements were aimed to be iso-energetic (≈ 70 kJ/d) (Roberfroid *et al*, 1993) and of equal sweetness (Franck-Frippiat, 1995). Ten patients started out on fructo-oligosaccharides and 10 on placebo. Patients consumed the supplements in split dose; half of the supplement at breakfast and the other half at dinner. The supplements were mixed with yogurt. To prevent any gastrointestinal complaints when starting with the fructo-oligosaccharides without adaptation, the dose was gradually increased during the first three days with steps of 5 grams per day.

Blood sampling and analyses

Blood was taken by the practice assistants on days 1, 18 and 21 (period 1) and days 39 and 42 (period 2) after an overnight fast (12 h) and in the sitting position.

For glucose measurements, capillary blood samples were taken from a finger. A drop of blood was placed on a test-strip and blood glucose was measured immediately with a blood glucose meter. The participating practices used different glucose meters, but standard procedures were used for each glucose meter and repeated measurements were done using the same equipment. All glucose meters were gauged before the experiment.

For the lipid analyses, blood was obtained by venipuncture using a standardized protocol. After the blood samples had been allowed to clot (15-30 min), the tubes were put on ice and transported to the laboratory where serum was obtained by low-speed centrifugation (10 min, 1500 g, 4°C; Sigma 4K10, Salm en Kipp BV, Breukelen, The Netherlands). Within 2 h after sampling serum was stored at -80°C until analyses.

Lipids were analyzed enzymatically for total cholesterol (Siedel *et al*, 1983), HDL cholesterol (Warnick *et al*, 1982) and triacylglycerol (Fossati & Prencipe, 1982) on a Spectrum Analyzer (Abbott Laboratories, Chicago, USA). The coefficient of variation within runs was 1.0% for total cholesterol, 1.4% for HDL cholesterol and 0.5% for triacylglycerol. The mean bias with regard to the target values from serum pools provided by the Centers for Disease Control and Prevention, Atlanta, was -1.4% for total cholesterol, 0.8% for HDL cholesterol, and 9.7% for triacylglycerol. LDL cholesterol was calculated by using the equation of Friedewald *et al* (Friedewald *et al*, 1972).

Free fatty acids in serum were measured enzymatically (Cat. no. 1383 175, Boehringer Mannheim GmbH, Mannheim, Germany). The mean recovery of the standard (palmitic acid) was 69% and a correction factor of 1.44 was used for all data.

Serum acetate was measured after deproteinizing the samples. Perchloric acid (0.5 ml, 1 M) was added to the serum samples (0.5 mL). After centrifugation (15 min, 1500 x g) potassiumhydroxide (45 ml, 4M) was added to the supernatant (0.6 mL) and after a second centrifugation step (10 min, 1500 g), acetate was measured enzymatically in the supernatant (Cat. no. 148 261, Boehringer Mannheim, Mannheim, Germany). Values were corrected for volumes, for the specific gravity and for the liquid fraction of serum. The mean recovery of the standard was 100%.

For all blood variables, samples from one particular patient were analyzed in one run.

Table 1.
Patient characteristics and baseline blood values[1]

	Males (n = 9)	Females (n = 11)
Characteristics		
Age (y)	56 ± 5.2	62 ± 4.1
Height (m)	1.76 ± 0.07	1.65 ± 0.07
Weight (kg)	91.1 ± 14.4	74.3 ± 9.05
Body mass index (kg/m^2)	29.4 ± 4.22	27.4 ± 2.72
Baseline blood values		
Total serum cholesterol (mmol/L)	5.61 ± 1.34	6.26 ± 0.89
Serum triacylglycerol (mmol/L)	2.66 ± 1.46	2.95 ± 1.53
Blood glucose (mmol/L)	8.83 ± 2.30	8.06 ± 1.75

[1] Values are expressed as mean ± SD.

Statistical analyses

For statistical analyses, the values of two blood samples per person, taken at the end of each period, were averaged. Complaints of flatulence were averaged over the last two weeks of each supplement period.

The differences between the two treatments were normally distributed as judged by visual inspection of the normal probability plots (SAS, univariate procedure). We used an ANOVA model including *patient* and *treatment*. Adding *period* to this model did not contribute to the significance, indicating that there were no significant effects of time or sequence of the treatments (SAS, general

linear models procedure). We then used paired *t*-tests to test for differences between the placebo and fructo-oligosaccharide treatment. *P*-values of less than 0.05 were considered significant. The variation (sd) in the effect was calculated and used to estimate the detectable effect of the treatment with a given probability (Snedecor & Cochran, 1989).

The statistical analysis package SAS, version 6.09 (Statistical Analysis Systems Institute, Inc., Cary, NC, USA) was used to perform the statistical analyses.

Results

Characteristics of the study population are given in table 1. Cholesterol concentrations were lower than we had anticipated based on the patient records; half of the baseline values were below 6 mmol/L. Two participants had blood glucose levels lower than 6.5 mmol/l at baseline.

All participants completed the study successfully. Complaints of flatulence were significantly higher (P=0.008) during the supplementation with fructo-oligosaccharides than during the placebo treatment. No other gastro-intestinal complaints were reported.

Compliance, expressed as the proportion of supplements not returned, was 100% during both treatments. The diaries did not reveal relevant differences between the treatments in physical activity patterns, medication used and eating, drinking or lifestyle behavior. Body weight decreased significantly over time and was 0.6 kg lower at the end of the study compared to baseline (p=0.008). However, there were no differences in weight difference between the treatments. Mean body weight was 81.7 (SD 14.0) kg on placebo and 81.9 (SD 14.4) kg on fructo-oligosaccharides.

There were no differences in the average daily intakes of energy, protein, fat, carbohydrates or dietary fiber (table 2).

Blood parameters

Fasting concentrations of blood glucose, serum acetate, serum total cholesterol, HDL and LDL cholesterol and triacylglycerol did not differ between treatments. Table 3 gives the average values during both treatments. Figure 1 shows the mean differences with their 95% confidence intervals.

Table 2.
Reported dietary intake of 20 diabetic patients after supplementation with glucose (placebo) or fructo-oligosaccharides[1]

	Treatment	
	Glucose (placebo)	Fructo-oligosaccharides
Energy intake (MJ)	6.6 ± 3.2	6.1 ± 2.1
Carbohydrate intake (% EI)	38.5 ± 7.5	39.4 ± 7.2
Fat intake (% EI)	39.9 ± 9.0	36.7 ± 9.6
Protein intake (% EI)	21.5 ± 6.2	23.2 ± 6.1
Dietary fibre (g/MJ)	2.1 ± 0.9	2.1 ± 0.8

[1] Values are expressed as mean ± SD, supplements are not included.

Table 3.
Fasting serum lipids, serum acetate and blood glucose in 20 diabetic patients after 20 days of daily supplementation with glucose (placebo) or fructo-oligosaccharides[1]

	Treatment	
	Glucose (placebo)	Fructo-oligosaccharides
	mmol/L	
Total cholesterol	6.01 ± 1.18	6.21 ± 1.29
HDL cholesterol	1.09 ± 0.25	1.12 ± 0.23
LDL cholesterol	3.80 ± 1.04	3.99 ± 0.91
Triacylglycerol	2.44 ± 0.79	2.56 ± 1.22
Free fatty acids	0.83 ± 0.28	0.75 ± 0.26
Acetate	0.11 ± 0.02	0.11 ± 0.02
Glucose	8.59 ± 2.66	8.61 ± 2.61

[1] Values are expressed as mean ± SD.

Eight patients showed higher total cholesterol values on the placebo treatment than on the fructo-oligosaccharide treatment, one showed no difference, and 11 had lower values on placebo. For 11 patients, the concentration of blood glucose was higher on placebo than on the fructo-oligosaccharides, one patient showed no difference, and eight patients had lower values on placebo.

Data analyses for men and women separately did not reveal differences of the treatments between the sexes (data not shown). The sample size was too small to perform analyses in smaller subgroups such as patients using similar medication.

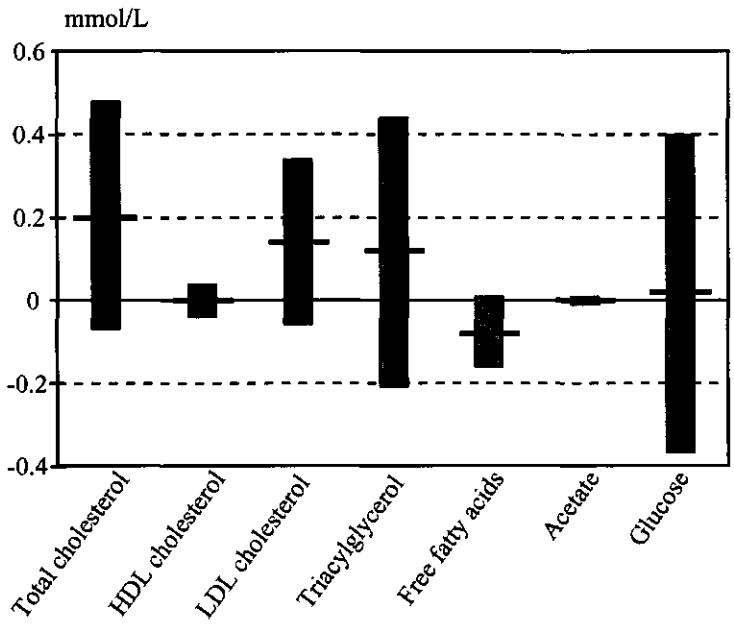

Figure 1. Net effect (mean and 95% confidence interval) of fructo-oligosaccharides on fasting concentrations of serum lipids, serum acetate and blood glucose in 20 diabetic patients, computed as the effect of a 20 days period of supplementation with fructo-oligosaccharides minus the effect of a placebo (glucose).

Discussion

Our findings show that consumption of 15 grams of fructo-oligosaccharides does not significantly affect blood glucose and serum lipids in non-insulin dependent diabetic patients.

Compliance of the participants, statistical power of the experiment and dietary intakes during the study

We believe that the lack of effect in our study can not be explained by insufficient compliance of the participants, a low statistical power, changes in intake of other nutrients, or by the fact that we studied both men and women.

Both reported compliance and compliance judged by the supplements that were not returned was near 100% and did not differ between treatments. Furthermore, consumption of the fructo-oligosaccharide supplements was confirmed by the anticipated increase in flatulence during this treatment.

Based on studies with soluble dietary fibers, such as pectin and guar gum, we hypothesized that 15 grams of fructo-oligosaccharides would decrease serum cholesterol by 10%. In our study, we were able to detect a decrease of 6.1% with 80% confidence and of 7.2% with 90% confidence. We were able to detect changes smaller then 10% in 3 out of 6 of the other parameters. As the variation in response was high for the variables acetate, free fatty acids and triacylglycerol, the detectable effects were also higher (15-21%, with 90% confidence). We conclude that the study design was powerful enough to detect biologically relevant changes in our main outcome variables. Contrary to our expectations, we observed small, non-significant increases in all of our variables, except for free fatty acids. The 9.2% decrease in free fatty acids might have been a true change, but was not detectable with the used study design.

We checked the dietary intakes of the patients by means of 2-days food records in both treatment periods. Reported energy intakes were very low in both men (8 MJ/d) and women (5 MJ/d) as compared to mean intake of Dutch men (10.5 MJ/d) and women (7.9 MJ/d) (Anonymous, 1993*b*), probably because of underreporting. The results however revealed no differences in the average intakes of energy, macronutrients or dietary fiber between treatments. There were also no differences between treatments in body weight. It is thus unlikely that the participants adjusted their dietary habits in response to the treatment.

Both men and women were included in our study population. It is often believed that women are less suitable patients for studying dietary effects on colonic fermentation or serum lipids because of confounding effects of the menstrual cycle (Kim & Kalkhoff, 1979; McBurney, 1991) or of the use of oral contraceptives (Demacker *et al*, 1982). However, all women were postmenopausal and none used oral contraceptives.

Dose and duration of the treatment

The study tested the effects of daily supplementation with 15 grams of fructo-oligosaccharides for 20 days. This choice of dose and duration of the treatment might have influenced the results.

To our experience, a dose of 15 grams gives rise to some gastrointestinal complaints, in particular flatulence, but is still acceptable to healthy humans (Alles *et al*, 1996). By supplementing the diet with 15 grams of fructo-oligosaccharides, we at least doubled the average daily intake of inulin plus fructo-oligosaccharides, which is estimated to be 1-10 g/d dependent on population and method of food analysis (Van Loo *et al*, 1995). It is thus unlikely that effects of the supplemented fructo-oligosaccharides were masked by effects of naturally occurring fructo-oligosaccharides.

Our results do not agree with those of several studies in rats (Levrat *et al*, 1994; Fiordaliso *et al*, 1995; Kok *et al*, 1996; Delzenne *et al*, 1993). However, this discrepancy can be explained by species differences or incomparable doses. In all rat-studies, animals were fed a 10% fructo-oligosaccharides diet, with a mean food intake of 23 g/d and an average body weight at the end of study of 300 g. For rats, the intake of fructo-oligosaccharides per kilogram of metabolic weight (body weight $^{0.75}$) was thus 5.7 g/kg. For our diabetic patients, with a mean body weight of 81 kg, the average intake was 0.6 g/kg metabolic weight. Rats thus consumed a dose of fructo-oligosaccharides which is comparable with a human consumption of 150 g per day, which is clearly not in the feasible range of intakes in humans.

In the human study of Yamashita *et al* (1984) 8 g of fructo-oligosaccharides per day lowered fasting glycaemia and serum lipids. The control group in their study consumed 5 g/d of sucrose which has a somewhat higher energy content than 8 g of fructo-oligosaccharides, but this difference is too small to explain their results. Participants in the study of Yamashita were patients with uncontrolled diabetes, who had a mean blood glucose level of 11 mmol/L at baseline. Their mean cholesterol level was 6.3 mmol/L at baseline. The largest treatment effects were found in the most hypercholesterolemic patients. The participants in our study were under strict medical control for their diabetes. Mean baseline concentration of blood glucose was 8.4 mmol/L and that of total serum cholesterol was 5.95 mmol/L. We used patients' blood values of their most recent medical check-up as a criterion for inclusion in the study. Baseline values in the experiment however were mostly lower, possibly due to differences in the analytical methods between the Human Nutrition laboratory and the Nijmegen Hospital Laboratory or due to the fact that our patients fasted overnight before blood was taken. The apparent effectiveness of medication in our patients might have masked a possible effect of the fructo-oligosaccharides.

It is possible that 20 days of supplementation might have been too short to induce differences in glucose and lipid metabolism. However, we think this is unlikely as serum lipid concentrations stabilize within 2 weeks after a dietary change (Stasse-Wolthuis *et al*, 1980; Keys *et al*, 1965; Wolever *et al*, 1994) in healthy humans and hypercholesterolemic patients. Dietary effects on fasting glycaemia in non-insulin dependent diabetic patients can be established within 2 weeks (Crapo *et al*, 1986).

Effects of short-chain fatty acids on glucose and lipid metabolism

The potential impact of fructo-oligosaccharides on glucose and lipid levels is based on the hypothetical effects of short-chain fatty acids from fermentation on glucose and lipid metabolism.

Several researchers found increased serum levels of acetate after treatment with other types of non-digestible carbohydrates (Muir *et al*, 1995; Pomare *et al*, 1985; Jenkins *et al*, 1991; Rumessen *et al*, 1992*b*). We did not observe this but we might have missed the effect because we measured fasting and not post-prandial serum concentrations of acetate. Fructo-oligosaccharides are rapidly fermented and a peak concentration of serum acetate would be expected after a few hours, as is seen with lactulose (Rumessen *et al*, 1992*b*; Pomare *et al*, 1985).

Acetate and propionate have dissimilar effects on glucose and lipid metabolism. Acetate is thought to facilitate the cellular uptake of glucose by suppressing lipolysis and thereby lowering the amount of free fatty acids in serum. Free fatty acids compete with glucose to enter the cell (Akanji *et al*, 1989; Akanji & Hockaday, 1990; Thorburn *et al*, 1993). The *in vivo* effect of propionate on fasting glycemia seems to depend on the route of administration. Rectal infusion of propionate stimulates gluconeogenesis (Wolever *et al*, 1991; Carman *et al*, 1993; Boillot *et al*, 1995), whereas orally administered propionate tends to reduce glycemia (Wolever *et al*, 1995; Wolever *et al*, 1991). Acetate may act as a precursor for cholesterol synthesis (Wolever *et al*, 1995; Wolever *et al*, 1991), whereas propionate might decrease the use of acetate as a precursor of cholesterol syntheses (Wolever *et al*, 1991; Wolever *et al*, 1995; Wolever *et al*, 1996; Kok *et al*, 1996). Thus, acetate is thought to decrease glycaemia and increase cholesterol levels, whereas fermentation-derived propionate probably increases glucose production and decreases cholesterol levels.

The *in vitro* fermentation of both lactulose and fructo-oligosaccharides was tested by Luo *et al* (Luo *et al*, 1996). The authors conclude that the ratio of acetate:propionate is higher after lactulose fermentation (11.4) than after fructo-oligosaccharide fermentation (5.4), which was confirmed by Wang and Gibson (1993). *In vivo*, acetate seems to dominate the net effects in the case of lactulose. Jenkins *et al* (1991) showed increased levels of total cholesterol after lactulose supplementation. With fructo-oligosaccharides the net effect on glucose and lipid levels seems to be zero.

Conclusions

We conclude that fructo-oligosaccharides do not have important effects on blood glucose and serum lipids in non-insulin dependent diabetic patients who are under strict medical control. The lack of effect in our study could not be explained by changes in dietary intake, insufficient power or non-compliance of the patients. Thus, our findings do not suggest that fructo-oligosaccharides are an effective means to favorably affect serum lipids or glucose. However, it remains possible that other

type non-digestible oligosaccharides, with different fermentation patterns do have effects on serum lipids or glycaemia.

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7

General discussion

Introduction

This thesis embodies four human trials in which we studied the physiological effects of dietary fructo-oligosaccharides and transgalacto-oligosaccharides. The studies were aimed at investigating fermentation of the oligosaccharides, effects on the composition of the intestinal microflora, on colon cancer risk markers and on glucose- and lipid metabolism. First, the main findings are summarized and related to other studies. Then, the methodology used in the studies is discussed. Finally, recommendations for further research are given at the end of this chapter.

Main findings in relation to other studies

Fermentation

Because of the non-digestibility of the oligosaccharides, most of the molecules will survive passage through the upper part of the gastrointestinal tract and reach the colon unchanged, were they may serve as a substrate for the colonic microflora. Oligosaccharides will be degraded by microbial glycosidases into monomeric or dimeric material, which can be transported into the bacterial cells and metabolized into energy and end products such as short-chain fatty acids and hydrogen.

Fecal excretion of oligosaccharides

Apparent fermentability can be determined by analyzing the fecal excretion of the specific oligosaccharides added to the diet. We used high performance anion exchange chromatography to quantify the amount of oligosaccharides in feces. In ***Chapter 2***, we showed that feces of healthy volunteers adapted to a diet with 5 or 15 g of extra fructo-oligosaccharides per day, contained no oligomers derived from the supplement. These results were confirmed by Molis *et al* (1996). In ***Chapter 3***, the fate of fructo-oligosaccharides was determined in patients with an ileal pouch-anal anastomosis. In these patients the colon is removed and part of the distal ileum is used to construct an ileal reservoir. Although the fermentation capacity of the ileal pouch was considered to be much smaller than that of the colon, bacterial fermentation obviously took place to an appreciable extent, as only 17% of fructo-oligosaccharides were recovered from feces.

In ***Chapter 4***, the fecal excretion of transgalacto-oligosaccharides was measured in healthy volunteers using different doses of transgalacto-oligosaccharides in the diet (0, 7.5 and 15 g/d). No transgalacto-oligomers were recovered in feces of any of the subjects.

Gas production

The gases hydrogen and methane that are produced by colonic fermentation are partly absorbed into the blood and can be measured in breath. In ***Chapter 2*** and ***3***, we studied the hydrogen response to fructo-oligosaccharides and observed significant increases of the integrated breath hydrogen excretion over 24 hours, at a dose of 15 g/d. Several other studies showed similar gaseous responses to consumption of fructo-oligosaccharides (Gibson *et al*, 1995*a*; Stone-Dorshow & Levitt, 1987; Rumessen *et al*, 1990). Stone-Dorshow and Levit (1987) showed a breath hydrogen response after consumption of fructo-oligosaccharides that was comparable to that after lactulose. Prolonged intake (12 d) of fructo-oligosaccharides resulted in higher breath hydrogen excretion. Rumessen *et al* (1990) also showed that the hydrogen production increased with increasing doses of fructans. On the other hand, in a study by Gibson *et al* (1995*a*), breath methane was not affected by fructo-oligosaccharides.

In ***Chapter 4***, a significant increase in the concentration of breath hydrogen was found after consumption of 15 g of transgalacto-oligosaccharides per day, but not after consumption of 7.5 g per day. Tanaka *et al* (1983) also reported on the excretion of hydrogen after supplementation with transgalacto-oligosaccharides. They observed an increase in hydrogen after administration of 0.5 g/kg body weight. This was confirmed by a study of Bouhnik *et al* (1997) who observed a high excretion of hydrogen after supplementation with 10 g/d. In their study, prolonged ingestion of transgalacto-oligosacccharides led to a significant drop in the expired hydrogen. Transgalacto-oligosaccharides did not significantly affect the excretion of methane in this study.

At doses of fructo- and transgalacto-oligosaccharides below 10 g/d, the effects on the excretion of hydrogen are less profound and probably masked by effects of other dietary components.

Fermentation related events, such as excessive gas production in the colon, may cause some gastro-intestinal discomfort. In ***Chapter 2***, we quantified gastro-intestinal discomfort by using a scoring system in which subjects scored the severity of symptoms on a four point scale. We observed an increased flatulence with increasing amounts of fructo-oligosaccharides (0, 5 and 15 g/d). All other scores were low and equal for the three treatments. Similar results were found in a study by Stone-Dorshow and Levit (1987). Briet *et al* (1995) evaluated the tolerance to a sweetener containing fructo-oligosaccharides. Hereto, fructo-oligosaccharides were ingested throughout the day either occasionally (one a week) or regularly (every day). Daily doses were increased until diarrhea and/or a symptom (such as cramps, bloating, flatus) rated as "severe" occurred. Excessive flatus was observed at doses higher than 30 g/d. Borborygmus and bloating appeared at higher levels (>40 g/d) and cramps at doses >50 g/d. The authors also concluded that chronic consumption of fructo-oligosaccharides does not improve tolerance.

We did not quantify the gastro-intestinal complaints after consumption of transgalacto-oligosaccharides. Ito *et al* (1990) studied the effect of transgalacto-oligosaccharides on feelings of abdominal discomfort in humans given different doses (0, 2.5, 5 and 10 g/d; for 7 d). Although the frequency of fullness, flatulence and abdominal pain increased with increasing doses of transgalacto-oligosaccharides, the increase was only significant for fullness.

Fecal short-chain fatty acids and pH

Several rat studies show an increase of cecal short-chain fatty acids pools and a decrease in cecal pH after supplementation with either fructo-oligosaccharides (Djouzi & Andrieux, 1997; Campbell *et al*, 1997*b*; Younes *et al*, 1995; Levrat *et al*, 1991) or transgalacto-oligosaccharides (Rowland & Tanaka, 1993; Djouzi & Andrieux, 1997; Levrat *et al*, 1991). Human studies so far did not detect any effect of non-digestible oligosaccharides (Munster *et al*, 1994*b*; Gibson *et al*, 1995*a*; Bouhnik *et al*, 1996*b*) on fecal pH or concentrations of short-chain fatty acids. In ***Chapter 2, 3*** and ***4*** we also failed to show an increase in the concentration of short-chain fatty acids or a decrease in pH in response to consumption of fructo-oligosaccharides or transgalacto-oligosaccharides.

Bacterial glycosidase activity

Gut bacteria degrade non-digestible carbohydrates by using a wide range of depolymerizing enzymes. Dietary components may affect the metabolic activity of the microflora by inducing these enzymes. Bouhnik *et al* (1996*b*) showed an increase in ß-fructosidase activity after consumption of fructo-oligosaccharides. We showed an enhanced activity of ß-galactosidase by specific substrate induction by transgalacto-oligosaccharides. This was also found by Djouzi *et al* (1997) in rats. Also non-specific enzymes may be altered in response to consumption of non-digestible oligosaccharides. We observed a decreasing effect of fructo-oligosaccharides on the activity of α-L-arabinofuranosidase, which is the enzyme that degrades arabinosyl linkages from various hemicelluloses (Kaji, 1984). These non-specific effects may be due to either changes in microflora composition or shifts in the metabolic activity of individual species of bacteria.

Healthy volunteers thus ferment fructo-oligosaccharides and transgalacto-oligosaccharides completely. Consumption of non-digestible oligosaccharides leads to a clear gaseous response, reflected in an elevated excretion of breath hydrogen and often to more complaints of flatulence. Short-chain fatty acids are produced simultaneously with hydrogen by most bacteria (Cummings & MacFarlane, 1991). MacFarlane *et al* (MacFarlane *et al*, 1992), in an autopsy study of victims of sudden death, found high amounts of short-chain fatty acids in the proximal colon and lower

amounts towards the end of the gastrointestinal tract. They attributed this to rapid absorption of the acids by the colonic mucosa. In ***Chapter 3***, a similar rapid absorption by the pouch mucosa could explain why we did not find significant effects on the fecal excretion of short-chain fatty acids or pH in this study. The fraction of the short-chain fatty acids found in feces does obviously not correspond to their intra-colonic production. An alternative biomarker for the colonic metabolic activity might be the glycolytic activity of intestinal bacteria in feces. In ***Chapter 5*** we showed that substrate specific changes of bacterial glycosidase activity can be measured in feces.

The intestinal microflora

Oligosaccharides from human breast milk are known to have a so-called bifidogenic effect and are responsible for the predominance of bifidobacteria in the feces of breast-fed infants. The responsible compounds are ß-galacto-oligosaccharides with various complex structures, consisting of glucose, galactose, N-acetylglucosamine, fucose and sialic acid (Coppa *et al*, 1993; Carlson, 1985; Grönberg *et al*, 1989). Growth of bifidobacteria in adults might possibly be manipulated by adding other types of oligosaccharides to the diet. Several human studies investigated the effects of fructo-oligosaccharides (Gibson *et al*, 1995*a*; Bouhnik *et al*, 1996*a*; Bouhnik *et al*, 1996*b*; Buddington *et al*, 1996) or transgalacto-oligosaccharides (Ito *et al*, 1990; Tanaka *et al*, 1983; Bouhnik *et al*, 1997; Ito *et al*, 1993*a*; Hayakawa *et al*, 1990) on the composition of the intestinal microflora. They all found a significant increase in the fecal bifidobacterial counts. In ***Chapter 4***, we found no effects of transgalacto-oligosaccharides on the composition of the fecal microflora of healthy volunteers, when tested in a controlled feeding trial against a placebo treatment.

Our volunteers were healthy and not especially selected for having a small number of bifidobacteria. In healthy volunteers with a stable and healthy balance in their microfloral population, it might be difficult to get diet-induced changes. Also, our findings do not exclude the possibility that changes in the composition of the flora occurred in the proximal part of the colon.

Colon cancer risk markers

As indicated in figure 3 of ***Chapter 1***, there are several mechanisms by which carbohydrate fermentation might affect colon cancer risk. In this thesis we measured the following colon cancer risk markers: stool weight, fecal concentrations of butyrate, bile acid profiles, fecal concentrations of protein degradation products and the activity of several glycolytic enzymes.

Stool weight

We hypothesized that non-digestible oligosaccharides would increase bacterial proliferation and thereby bacterial mass. As fecal bacteria contribute considerably to fecal weight (Stephen &

Cummings, 1980; Hill, 1995), we expected to find an increase in total fecal weight, with a shift to a more watery constitution as bacteria are 80% water (Stephen & Cummings, 1980). Due to the laxative effects of the larger fecal mass, we expected to find a higher frequency of defecation.

In healthy volunteers, we did not observe any significant changes in total stool weight or frequency of defecation in response to consumption of either fructo-oligosaccharides (***Chapter 2***) or transgalacto-oligosaccharides (***Chapter 4***). We did find a 6.3% higher nitrogen density of the fecal dry matter on transgalacto-oligosaccharides, which points to an increase in the bacterial mass (Stephen & Cummings, 1980; Levrat *et al*, 1993; Younes *et al*, 1995). The effect of this increase on total stool weight, which is theoretically 9.5 g per day, was probably too small to detect. Gibson *et al* (1995*a*) showed higher stool weights and an enhanced nitrogen excretion on fructo-oligosaccharides. In pouch patients ***(Chapter 3)***, fructo-oligosaccharides significantly increased 24 h fecal weight by 20.3%. The unfermented oligosaccharides and probably also the extra biomass were responsible for the increase in fecal weight.

Fecal butyrate

We did not show any increases in the fecal concentration of butyrate after consumption of either fructo-oligosaccharides or transgalacto-oligosaccharides (see under "fermentation").

Fecal bile acids

We were unable to show any effects of transgalacto-oligosaccharides on the concentration of bile acids in fecal water (***Chapter 4***). We hypothesized that acidification of the colon content, as a result of the fermentation of oligosaccharides, would lead to precipitation of the soluble deconjugated bile acids (Fini & Roda, 1987; Bruce, 1987; Munster & Nagengast, 1993) and to a suppression of the bacterial conversion of primary to secondary bile acids (Christl *et al*, 1997; Munster & Nagengast, 1993). We therefore expected to find a lower concentration of bile acids in fecal water and a smaller ratio of hydrophobic to hydrophilic bile acids. Although we have no information on the acidification in the colon, we did not find a decrease in the fecal pH. Due to the high within-subject variation, only large effects (of 50-60%) on the concentration of bile acids were detectable in our study. Although such effects have been shown by others, using different dietary treatments (Munster *et al*, 1994*b*; van Faassen *et al*, 1996; Nagengast *et al*, 1988*b*), we might have missed possible smaller effects.

Protein fermentation products

In ***Chapter 3*** we found indications that fructo-oligosaccharides would decrease protein fermentation. In patients with an ileal pouch anal anastomosis, we found a significant lower fecal

excretion of isobutyrate and isovalerate, which are short-chain fatty acids that originate from the bacterial breakdown of amino acids (Geypens *et al*, 1997; MacFarlane & MacFarlane, 1993; Rasmussen *et al*, 1988; Zarling & Ruchim, 1987). Similar effects were observed in other studies, using galacto-oligosaccharides (Ito *et al*, 1993*a*) or lactulose (Mortensen *et al*, 1990). Protein fermentation produces not only amino acid-derived short-chain fatty acids, but also indoles and ammonia. These products might have a toxic effect on the mucosa (Birkett *et al*, 1996; Clausen & Mortensen, 1992*a*; Clinton *et al*, 1988; Bone *et al*, 1976). We hypothesized that transgalacto-oligosaccharides would decrease protein fermentation products by two mechanisms. Firstly, we expected to find a depression of protein fermentation, as seen in pouch patients after consumption of fructo-oligosaccharides. Also, when transgalacto-oligosaccharides are used as an energy substrate by the bacteria, nitrogen sources such as amino acids and ammonia are used for the subsequent increase in bacterial growth (Birkett *et al*, 1996; Mortensen *et al*, 1990; Levrat *et al*, 1993). In healthy volunteers, we did not show any decreasing effect of transgalacto-oligosaccharides on the fecal concentration of protein degradation products ammonia, indoles and skatoles, an effect shown earlier by Ito *et al* (1993*a*) and Djouzi *et al* (1997). We also did not observe a decrease in the concentration of the short-chain fatty acids that originate from bacterial breakdown of amino acids.

Bacterial glycosidase activity

By affecting the composition and activity of the intestinal microflora, the activity of bacterial enzymes might be altered. Several studies showed that dietary fiber may lower some of the fecal bacterial enzymes that have adverse effects in the colon (Reddy *et al*, 1992; Mallett & Rowland, 1987), such as ß-glucuronidase and ß-glucosidase. We (***Chapter 5***) and others (Djouzi & Andrieux, 1997; Bouhnik *et al*, 1996*a*; Kleessen *et al*, 1997; Bouhnik *et al*, 1996*b*) did not show any effects of fructo-oligosaccharides or transgalacto-oligosaccharides on the activity of these enzymes.

Although there are many hypotheses linking fermentation of non-digestible oligosaccharides to lower putative risk factors for colon cancer, our results do not give sufficient evidence to support any of them. It should be noted that there is no conclusive evidence that the biomarkers that were used are indeed causally related to the development of colon cancer. We did not measure the cytotoxicity of fecal water or the colonic mucosal proliferation in rectal biopsies after consumption of the oligosaccharides.

Glucose- and lipid metabolism

Short-chain fatty acids that are produced during fermentation can be absorbed by the colonic mucosa (Rombeau & Kripke, 1990; Pomare *et al*, 1985; Cummings *et al*, 1987*a*) and used as fuel for the mucosa (butyrate) or entered into the portal blood (acetate and propionate). Acetate and

propionate may both affect systemic glucose (Akanji *et al*, 1989; Akanji & Hockaday, 1990; Thorburn *et al*, 1993; Wolever *et al*, 1991; Carman *et al*, 1993; Boillot *et al*, 1995) and lipid metabolism (Wolever *et al*, 1995; Wolever *et al*, 1991; Wolever *et al*, 1996; Kok *et al*, 1996). Via these fermentation products, non-digestible oligosaccharides may affect glucose and lipid metabolism. In ***Chapter 6***, we showed that fructo-oligosaccharides do not favorably affect fasting glycaemia or serum lipids in non-insulin dependent diabetic patients. Other studies with healthy volunteers also failed to show any effect on fasting blood glucose or lipids (Luo *et al*, 1996; Pedersen *et al*, 1997). These results do not agree with those of several studies in rats (Kok *et al*, 1996; Fiordaliso *et al*, 1995; Levrat *et al*, 1994; Delzenne *et al*, 1993). However, this discrepancy can partly be explained by incomparable doses of fructo-oligosaccharides. In all rat-studies, animals were fed diets containing approximately 5.7 g fructo-oligosaccharides per kg of metabolic weight. For our patients, the average intake was 0.6 g per kg of metabolic weight. In a human study by Yamashita *et al* (Yamashita *et al*, 1984), fructo-oligosaccharides significantly reduced fasting glycaemia and serum lipids in patients with uncontrolled diabetes. The participants in our study were under strict medical control for their diabetes.

Methodological considerations

Storing and freezing feces before microbiological analyses

Collection of stools in dietary intervention trials is often difficult to control due to variations in defecation patterns. Often, volunteers are asked to freeze their stools at home or at the laboratory, immediately after defecation. The method of collection is of importance in the quality of the subsequent fecal analyses. Methods for quantifying bacteria in feces are based on viable counts on selective media. Because of the high vulnerability of most bacteria, special conditions must be created to ensure survival during sampling, processing and analysis. The effect of preservation of the stool sample on the bacterial counts in feces was studied by Crowther *et al* (1971). They compared different methods of storing at different freezing temperatures. After freezing at -80 °C, all species tested were recovered from suspensions of feces frozen in broth, with glycerol as a protective agent. No significant changes were observed. In a more recent study (Bollongue, 1997), Ballongue tested the survival of bifidobacteria during storage and confirmed the results of Crowther. Both studies based their conclusions on the analysis of one single fecal sample. It is possible that the sample size was too small and that errors in the measurement masked a possible effect of storing and freezing. We replicated the validation of Crowther with a larger sample size of different stools (n=23). The methods for the bacterial enumeration are similar as those described in

Chapter 4. The results (Figure 1), indicate a significant reduction in total aerobes, *E.coli* and bacteroides and an increase in clostridia, caused by storing the feces in the described way.

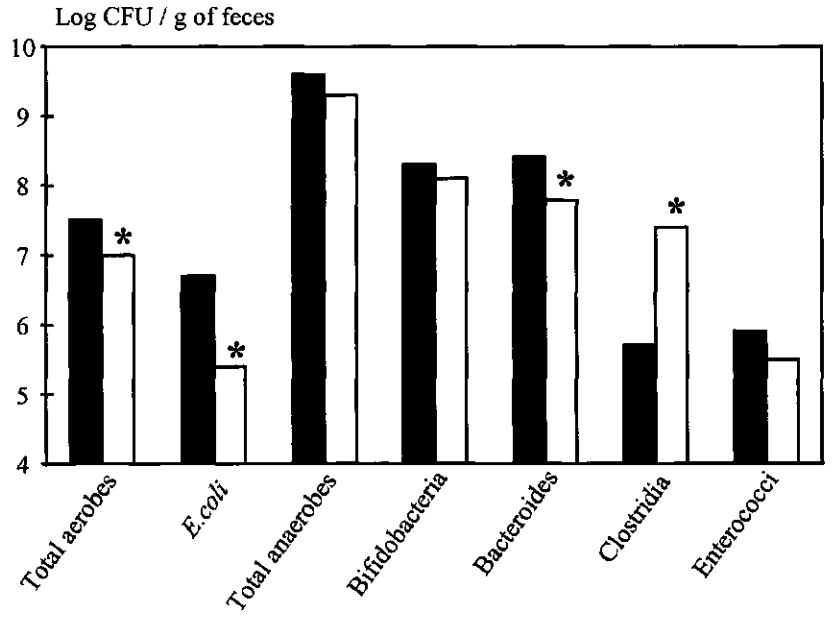

Figure 5. Effect of storing and freezing fecal samples (n=23), according to the method of Crowther *et al* (1971), on the composition of the intestinal microflora. Values are logarithms of the mean counts of colony forming units (CFU) per gram of feces in fresh samples (black bars) or after freezing (white bars). * Mean values are significantly different from those in the fresh samples ($P<0.05$).

We conclude that freezing the feces before using selective media in determining fecal bacteria, significantly affects the bacterial counts, with different effects for different bacterial groups.

Design of studies on diet and colonic microflora

To determine the required sample size for the study described in ***Chapter 4***, we measured the intra-individual variation in bacterial counts over a period of 2 weeks. The within-subject variation was only 5% for bifidobacteria and below 11% for all other bacteria that were tested (Table 1).

Table 1.

Intra-individual variation in fecal bacterial counts as measured in 3 different stools over a period of 2 weeks[1].

Bacteria	Mean	CV%
Total aerobes	6.4	8.3
E. coli	6.4	8.4
Total anaerobes	9.7	3.6
Bifidobacteria	8.6	4.9
Bacteroides fragilis-group	8.6	6.4
Lactobacilli	6.8	10.9

[1] Values are logarithms of the mean counts of colony forming units per gram of feces. CV%, day-to-day variation in bacterial counts.

In the study described in ***Chapter 4***, bifidobacterial counts increased by 4% independently of the treatment, but the differences between the increases on placebo and transgalacto-oligosaccharides were not statistically significant. With the exception of our study and 2 others (Ito *et al*, 1990; Bouhnik *et al*, 1996*b*), all studies on the effects of non-digestible oligosaccharides on the composition of the intestinal microflora were done using a linear study design, with measurements before and after the treatment. Without the placebo group, the conclusion from our study would have been that transgalacto-oligosaccharides selectively increase bifidobacteria in the gut.

We conclude that it is necessary to compare the effects of non-digestible oligosaccharides with a control treatment, to exclude possible time effects.

Background diet

The background diet of our volunteers was not controlled in ***Chapters 2, 3, 5*** and ***6***. Although we did not find any significant deviations in eating and drinking behavior in the dietary records, it remains possible that dietary components masked the effects of our intervention. The volunteers in ***Chapter 2*** e.g. consumed an habitual diet with 2.9 gram of dietary fiber per MJ. In ***Chapter 4***, we used a controlled feeding trial to test the effects of transgalacto-oligosaccharides. We chose for a background diet with a relative low fiber content (1.5 g/MJ) to overcome the above mentioned masking problems. The chosen diet was also high in protein (16%) in order to increase fermentation of dietary proteins (Gibson *et al*, 1976; Geypens *et al*, 1997). We hereby hoped to increase the chance of showing inhibiting effects of the oligosaccharides on this protein fermentation. Levrat *et al* (1993) studied the effects of the fermentable carbohydrate inulin in rats fed a high versus a moderate protein diet. They showed that use of ammonia as a source of bacterial growth was

increased on inulin, but more evident when the dietary protein level was moderate. In high protein diets, uremia and flux of urea from plasma to colon is high and an important determinant of fecal concentrations of ammonia (Levrat *et al*, 1993; Younes *et al*, 1995). This mechanism might explain the negative results in our study with regard to protein fermentation products.

Although the day-to-day variation in the composition of the intestinal microflora seems to be small, it is known from animal studies that sudden extreme changes in diet can have profound effects on this composition (Koopman, 1984). The low-fiber, high-protein diet that we provided in our study with transgalacto-oligosaccharides might have been too extreme for some of our volunteers. We hypothesize that at first, the diet upset the balance of microflora of the volunteers and that the flora then gradually adapted to the changes in substrate supply. This is our only possible explanation for the increase in bifidobacteria that was found independently of the treatment.

We conclude that the background diet is of great importance in studies on the effects of diet on the composition and activity of the intestinal microflora.

Recommendations

1. More information on the exact content of non-digestible oligosaccharides in foods is necessary, to be able to assess the possible effects of these compounds on human health in epidemiological studies.
2. There is a need for high quality and practical methods for measuring the composition of the intestinal microflora. The recent development of molecular techniques for this purpose is promising.
3. Effects of fructo-oligosaccharides and transgalacto-oligosaccharides on the composition of the intestinal microflora need to be confirmed in placebo controlled intervention trials.
4. Studies on non-digestible oligosaccharides should not only focus on expected positive effects. There is little information on the role of non-digestible oligosaccharides in the potentially harmful effects of: extreme pH decreases in the proximal colon, increases of proteolysis in the distal colon, *in vivo* growth and survival of specific pathogens or bacterial overgrowth in the small intestine.
5. Fructo-oligosaccharides and transgalacto-oligosaccharides are probably fermented in the proximal colon. In order to provide the whole colon with fermentable substrate, it might be useful to test combination effects of non-digestible oligosaccharides with a more slowly fermentable carbohydrate.

In conclusion

Fructo-oligosaccharides and transgalacto-oligosaccharides are completely fermented in the colon of healthy individuals, which leads to a clear gaseous response. We did not find any evidence that they beneficially affect putative colon cancer risk markers, the composition of the intestinal microflora or glucose and lipid metabolism. It remains possible that they have effects in other study-populations, when tested against another background diet or when combined with other fermentable substrates.

References

Anonymous. *NEVO Table 1986-1987, Nederlands voedingsstoffenbestand (Dutch Nutrient Database).* The Hague: The Netherlands Bureau for food and Nutrition Education, 1987.

Anonymous. *NEVO Table 1993, Nederlands voedingsstoffenbestand (Dutch Nutrient Database).* The Hague: The Netherlands Bureau for food and Nutrition Education, 1993*a*.

Anonymous. *NEVO Table 1996, Nederlands voedingsstoffenbestand (Dutch Nutrient Database).* The Hague: The Netherlands Bureau for food and Nutrition Education, 1996.

Anonymous. *Wat eet Nederland: resultaten van de Voedselconsumptiepeiling 1987-1988 (results of the Dutch food consumption survey 1987-1988).* Rijswijk: Ministerie van Welzijn, Volksgezondheid en Cultuur en Ministerie van Landbouw en Visserij, 1988.

Anonymous. *Zo eet Nederland 1992: resultaten van de Voedselconsumptiepeiling 1992 (results of the Dutch food consumption survey 1992).* Den Haag: Voorlichtingsbureau voor de voeding, 1993*b*.

Akanji AO, Hockaday DR. Acetate tolerance and the kinetics of acetate utilization in diabetic and nondiabetic subjects. *Am.J.Clin.Nutr.* 1990;51:112-118.

Akanji AO, Humphreys S, Thursfield V, Hockaday TDR. The relationship of plasma acetate with glucose and other blood intermediary metabolites in non-diabetic and diabetic subjects. *Clin.Chim.Act.* 1989;185:25-34.

Alberts B, Bray D, Lewis J, Raff M, Roberts K, Watson JD. *Molecular biology of the cell.* New York: Garland Publishing Inc., 1994.

Alles MS. The effect of non-digestible oligosaccharides on composition and activity of the intestinal microflora. In: Hartemink R, ed. *Proceedings of the International Symposium "Non-digestible oligosaccharides: healthy food for the colon?".* Wageningen: Gratuate Schools VLAG and WIAS, 1997*a*;79-89.

Alles MS, Hautvast JGAJ, Nagengast FM, Hartemink R, Van Laere MKJ, Jansen JBMJ. Fate of fructo-oligosaccharides in the human intestine. *Brit.J.Nutr.* 1996;76:211-221.

Alles MS, Katan MB, Salemans JMJI, et al. Bacterial fermentation of fructo-oligosaccharides and of resistant starch in patients with an ileal pouch-anal anastomosis. *Am.J.Clin.Nutr.* 1997*b*;66:1286-1292.

Ambroze WL, Pemberton JH, Phillips SF, Bell AM, Haddad AC. Fecal short-chain fatty acid concentrations and effect on ileal pouch function. *Dis.Colon.Rectum.* 1993;36:235-239.

Bach Knudsen KEB, Hessov I. Recovery of inulin from Jerusalem artichoke (Helianthus tuberosus L.) in the small intestine of man. *Br.J.Nutr.* 1995;74:101-113.

Birkett A, Muir J, Phillips J, Jones G, O'Dea K. Resistant starch lowers fecal concentrations of ammonia and phenols in humans. *Am.J.Clin.Nutr.* 1996;63:766-772.

Björck I, Nyman M, Pedersen B, Siljeström M, Asp N, Eggum BO. Formation of enzyme resistant starch during autoclaving of wheat starch: studies in vitro and in vivo. *J.Cereal Sci.* 1987;6:159-172.

Boillot J, Alamowitch C, Berger A, et al. Effects of dietary propionate on hepatic glucose production, whole-body glucose utilization, carbohydrate and lipid metabolism in normal rats. *Br.J.Nutr.* 1995;73:241-251.

Bollongue J. Technical problems related to in vitro study of colon flora. *Scand.J.Gastroenterol.* 1997;32:S14-S16.

Bone E, Tamm A, Hill M. The production of urinary phenols by gut bacteria and their possible role in the causation of large bowel cancer. *Am.J.Clin.Nutr.* 1976;29:1448-1454.

Bouhnik Y, Flourié B, Andrieux C, Bisetti N, Briet F, Rambaud J-C. Effects of Bifidobacterium sp fermented milk ingested with or without inulin on colonic bifidobacteria and enzymatic activities in healthy humans. *Eur.J.Clin.Nutr.* 1996*a*;50:269-273.

Bouhnik Y, Flourié B, D'Agay-Abensour L, et al. Administration of transgalacto-oligosaccharides increases fecal bifidobacteria and modifies colonic fermentation metabolism in healthy humans. *J.Nutr.* 1997;127:444-448.

Bouhnik Y, Flourié B, Riottot M, et al. Effects of fructo-oligosaccharides ingestion on fecal bifidobacteria and selected metabolic indexes of colon carcinogenesis in healthy humans. *Nutr.Cancer* 1996*b*;26:21-29.

Briet F, Achour L, Flourié B, et al. Symptomatic response to varying levels of fructo-oligosaccharides consumed occasionally or regularly. *Eur.J.Clin.Nutr.* 1995;49:501-507.

Bruce WR. Recent hypotheses for the origin of colon cancer. *Cancer Res.* 1987;47:4237-4242.

Bruun E, Meyer JN, Rumessen JJ, Gudmand-Hoyer E. Breath hydrogen analysis in patients with ileoanal pouch anastomosis. *Gut* 1995;37:256-259.

Buddington RK, Williams CH, Chen S-C, Witherly SA. Dietary supplement of neosugar alters the fecal flora and decreases activities of some reductive enzymes in human subjects. *Am.J.Clin.Nutr.* 1996;63:709-716.

Burvall A, Asp N, Dahlqvist A. Oligosaccharide formation during hydrolysis of lactose with "saccharomyces lactis" lactase - part 3: digestibility by human intestinal enzymes in vitro. *Food Chemistry* 1980;5:189-194.

Campbell AP, Merrett MN, Kettlewell M, Mortensen NJ, Jewell DP. Expression of colonic antigens by goblet and columnar epithelial cells in ileal pouch mucosa: their association with inflammatory change and faecal stasis. *J.Clin.Pathol.* 1994;47:834-838.

Campbell JM, Bauer LL, Fahey GC, Hogarth AJCL, Wolf BW, Hunter DE. Selected fructooligosaccharide (1-kestose, nystose, and 1f-b-fructofuransylnystose) composition of foods and feeds. *J.Agric.Food Chem.* 1997*a*;45:3076-3082.

Campbell JM, Fahey GC, Wolf BW. Selected indigestible oligosaccharides affect large bowel mass, cecal and fecal short-chain fatty acids, pH and microflora in rats. *J.Nutr.* 1997*b*;127:130-136.

Carlson SE. N-acetylneuraminic acid concentrations in human milk oligosaccharides and glycoproteins during lactation. *Am.J.Clin.Nutr.* 1985;41:720-726.

Carman RJ, Tassell RLV, Wilkins TD. The normal intestinal microflora: ecology, variability and stability. *Vet.Human.Toxicol.* 1993;35:11-14.

Castino M. Residual sugars in wines. *Vini-d'Italia* 1972;14:69-80.

Christl SU, Bartram HP, Paul A, Kelber E, Scheppach W, Kasper H. Bile acid metabolism by colonic bacteria in continuous culture: effects of starch and pH. *Ann.Nutr.Metab.* 1997;41:45-51.

Clausen MR, Mortensen PB. Fecal ammonia in patients with adenomatous polyps and cancer of the colon. *Nutr.Cancer* 1992*a*;18:175-180.

Clausen MR, Tvede M, Mortensen PB. Short-Chain fatty acids in pouch contents from patients with and without pouchitis after ileal pouch-anal anastomosis. *Gastroenterology* 1992*b*;103:1144-1153.

Clinton SK, Bostwick DG, Olson LM, Mangian HJ, Visek WJ. Effects of ammonium acetate and solium cholate on N-methyl-N'-nitro-N-nitrosoguanidine-induced colon carcinogenesis of rats. *Cancer Res.* 1988;48:3035-3039.

Coppa GV, Gabrielli O, Pierani P, Catassi C, Carlucci A, Giorgi PL. Changes in carbohydrate composition in human milk over 4 months of lactation. *Pediatrics* 1993;91:637-641.

Crapo PA, Kolterman AOG, Henry RR. Metabolic consequence of two-week fructose feeding in diabetic subjects. *Diabetes care* 1986;9:111-119.

Crowther JS. Transport and storage of faeces for bacteriological examination. *J.Appl.Bacteriol.* 1971;34:477-483.

Csordas A. Butyrate, aspirin and colorectal cancer. *Eur.J.Canc.Prev.* 1996;5:221-231.

Cummings JH. Short chain fatty acids in the human colon. *Gut* 1981;22:763-779.

Cummings JH, Bingham SA, Heaton KW, Eastwood MA. Fecal weight, colon cancer risk, and dietary intake of nonstarch polysaccharides (dietary fibre). *Gastroenterology* 1992;103:1783-1789.

Cummings JH, Englyst HN. Fermentation in the human large intestine and the available substrates. *Am.J.Clin.Nutr.* 1987*a*;45:1243-1255.

Cummings JH, MacFarlane GT. The control and consequences of bacterial fermentation in the human colon. *J.Appl.Bacteriol.* 1991;70:443-459.

Cummings JH, Pomare EW, Branch WJ, Naylor CPE, MacFarlane GT. Short chain fatty acids in human large intestine, portal, hepatic and venous blood. *Gut* 1987*b*;28:1221-1227.

de Silva HJ, Millard PR, Soper N, Kettlewell M, Mortensen N, Jewell DP. Effects of the faecal stream and stasis on the ileal pouch mucosa. *Gut* 1991;32:1166-1169.

Delzenne NM, Kok N, Fiordaliso M, Deboyser DM, Goethals FM, Roberfroid MB. Dietary fructooligosaccharides modify lipid metabolism in rats. *Am.J.Clin.Nutr.* 1993;57:S820.

Demacker PNM, Schade RWB, Stalenhoef AFH, Stuyt PMJ, van 't Laar A. Influence of contraceptive pill and menstual cycle on serum lipids and high-density lipoprotein cholesterol concentrations. *Br.Med.J.* 1982;284:1213-1215.

Deutsch MJ. Vitamins and other nutrients. In: Cunniff P, ed. *Official methods of analysis of AOAC international, volume II.* Gaithersburg: AOAC International, 1997;70-71.

Djouzi Z, Andrieux C. Compared effects of three oligosaccharides on metabolism of intestinal microflora in rats inoculated with a human faecal flora. *Br.J.Nutr.* 1997;78:313-324.

Dwyer J. Dietary fiber and colorectal cancer risk. *Nutr.Rev.* 1993;51:147-148.

Ellegard L, Andersson H, Bosaeus I. Inulin and oligofructose do not influence the absorption of cholesterol, or the excretion of cholesterol, Ca, Mg, Zn, Fe, or bile acids, but increase energy excretion in ileostomy subjects. *Eur.J.Clin.Nutr.* 1997;51:1-5.

Englyst HN, Kingman SM, Cummings JH. Classification of resistant starch in foods and food products. *Eur.J.Clin.Nutr.* 1992;46:S33-S50.

Faassen A van, van den Bogaard AE, Hazen MJ, Geerlings P, Hermus RJ, Janknegt RA. Effects of calcisorb on fecal bile acids and fatty acids in human volunteers. *Dig.Dis.Sci.* 1996;41:2319-2325.

Feunekes GIJ, van Staveren WA, de Vries JHM, Burema J, Hautvast JGAJ. Relative and biomarker-based validity of a food frequency questionnaire estimating the intake of fats and cholesterol. *Am.J.Clin.Nutr.* 1993;58:489-496.

Feunekes GIJ, van Staveren WA, Graveland F, de Vos J, Burema J. Reproducibility of a semiquantitative food frequency questionnaire to assess the intake of fats and chelesterol in the Netherlands. *Int.J.Food Sci.Nutr.* 1995;46:117-123.

Fini A, Roda A. Chemical properties of bile acids. IV. Acidity constants of glycine-conjugated bile acids. *J.Lipid Res.* 1987;28:755-759.

Fiordaliso M, Kok N, Desager J, et al. Dietary oligofructose lowers triglycerides, phospolipids and cholesterol in serum and very low density lipoproteins of rats. *Lipids* 1995;30:163-167.

Fleming SE, Fitch MD, Stanley DW. Influence of processing on physical form of beans and on intestinal fermentation. *J.Food.Sci.* 1988;53:777-782.

Fossati P, Prencipe L. Serum triglycerides determined colorimetrically with an enzyme that produces hydrogen peroxide. *Clin.Chem.* 1982;28:2077-2080.

Franck-Frippiat A. Innovative food products with inulin and oligofructose. In: Anonymous, eds. *Proceedings of the first ORAFTI research conference.* 1995;182-185.

Friedewald WT, Levy RI, Fredrickson DS. Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. *Clin.Chem.* 1972;18:499-502.

Fujii S, Komoto M. Novel carbohydrate sweeteners in Japan. *Zuckerind.* 1991;116 (3):197-200.

Gamet L, Daviaud D, Denis-Pouxviel C, Rémésy C, Murat JC. Effects of short-chain fatty acids on growth and differentiation of the human colon-cancer cell line HT29. *Int.J.Cancer* 1992;52:286-289.

Geypens B, Claus D, Evenepoel P, et al. Influence of dietary protein supplements on the formation of bacterial metabolites in the colon. *Gut* 1997;41:70-76.

Gibson GR, Beatty ER, Wang X, Cummings JH. Selective stimulation of bifidobacteria in the human colon by oligofructose and inulin. *Gastroenterology* 1995*a*;108:975-982.

Gibson GR, Roberfroid MB. Dietary modulation of the human colonic microbiota: introducing the concept of prebiotics. *J.Nutr.* 1995*a*;1251:1401-1412.

Gibson GR, Wang X. Regulatory effects of bifidobacteria on the growth of other colonic bacteria. *J.Appl.Bacteriol.* 1994;77:412-420.

Gibson JA, Sladen GE, Dawson AM. Protein absorption and ammonia production: the effects of dietary protein and removal of the colon. *Br.J.Nutr.* 1976;35:61-65.

Glatz JFC, Schouten FJM, den Engelsman G, Katan MB. Quantitative determination of neutral steroids and bile acids in human feces by capillary gas-liquid chromatographic analysis of total fecal bile acids. In: Beynen AC, Geelen MJH, Katan MB, Schouten JA, eds. *Cholesterol metabolism in health and disease: studies in the Netherlands.* Wageningen: Ponsen & Looijen, 1985;103-112.

Grönberg G, Lipniunas P, Lundgren T, Erlansson K, Lindh G, Nillson B. Isolation of monosialylated oligosaccharides from human milk and structural analysis of three new compounds. *Carbohydr.Res.* 1989;291:261-278.

Guerin-Danan C, Chabanet C, Pedone C, et al. Milk fermented with yogurt cultures and Lactobacillus casei compared with yogurt and gelled milk: influence on intestinal microflora in healthy infants. *Am.J.Clin.Nutr.* 1998;67:111-117.

Haffejee IE. Cow's milk-based formula, human milk, and soya feeds in acute infantile diarrhea: a therapeutic trial. *J.Pediatr.Gastroenterol.Nutr.* 1990;10:193-198.

Hartemink R, Domenech VR, Rombouts FM. LAMVAB - A new selective medium for the isolation of lactobacilli from faeces. *J.Microbiol.Meth.* 1997;29:77-84.

Hartemink R, Kok BJ, Weenk GH, Rombouts FM. Raffinose-Bifidobacterium (RB) agar, a new selective medium for bifidobacteria. *J.Microbiol.Meth.* 1996;27:33-43.

Hayakawa K, Mizutani J, Wada K, Masai T, Yoshihara I, Mitsuoka T. Effects of soybean oligosaccharides on human faecal flora. *Microb.ecol.health dis.* 1990;3:293-303.

Heaton KW, Radvan J, Cripps H, Mountford RA, Braddon FEM, Hughes AO. Defecation frequency and timing, and stool form in the general population: a prospective study. *Gut* 1992;33:818-824.

Heijnen M-LA, Amelsvoort JMM van, Deurenberg P, Beynen AC. Limited effect of consumption of uncooked (RS2) or retrograded (RS3) resistant starch on putative risk factors for colon cancer in healthy men. *Am.J.Clin.Nutr.* 1998;67:322-331.

Hidaka H, Eida T, Takizawa T, Tokunaga T, Tashiro Y. Effects of fructooligosaccharides on intestinal flora and human health. *Bifidobacteria Microflora* 1986;5:37-50.

Hill MJ. Bacterial fermentation of complex carbohydrate in the human colon. *Eur.J.Canc.Prev.* 1995;4:353-358.

Hofmann AF, Mysels KJ. Bile acid solubility and precipitation in vitro and in vivo: the role of conjugation, pH, and Ca2+ ions. *J.Lipid Res.* 1992;33:617-626.

Houwelingen R van, Nordoy A, Beek E vd, Houtsmuller U, Metz M de, Hornstra G. Effect of a moderate fish intake on blood pressure, bleeding time, hematology, and clinical chemistry in healthy males. *Am.J.Clin.Nutr.* 1987;46:424-436.

Howe GR, Benito E, Castelleto R, et al. Dietary intake of fiber and decreased risk of cancers of the colon and rectum: evidence from the combined analysis of 13 case-control studies. *J.Natl.Cancer Inst.* 1992;84:1887-1896.

Höverstad T, Böhmer T, Fausa O. Absorption of short chain fatty acids from the human colon measured by the 14CO2 breath test. *Scand.J.Gastroenterol.* 1982;17:373-378.

Ito M, Deguchi Y, Matsumoto K, Kimura M, Onodera N, Yajima T. Influence of galactooligosaccharides on the human fecal microflora. *J.Nutr.Sci.Vitaminol.* 1993*a*;39:635-640.

Ito M, Deguchi Y, Miyamori A, et al. Effects of administration of galactooligosaccharides on the human faecal microflora, stool weight and abdominal sensation. *Microb.ecol.health dis.* 1990;3:285-292.

Ito M, Kimura M, Deguchi Y, Miyamori-Watabe A, Tajima T, Kan T. Effects of transgalactosylated disaccharides on the human intestinal microflora and their metabolism. *J.Nutr.Sci.Vitaminol.* 1993*b*; 39:279-288.

Jenkins DJA, Wolever TMS, Jenkins AL, et al. Specific types of colonic fermentation may raise low-density-lipoprotein-cholesterol concentrations. *Am.J.Clin.Nutr.* 1991;54:141-147.

Kaji A. L-Arabinosidases. *Adv.Carbohydr.Chem.Biochem.* 1984;42:383-394.

Keys A, Anderson JT, Grande F. Serum cholesterol response to changes in the diet. IV. Particular saturated fatty acids in the diet. *Metabolism* 1965;14:776-787.

Kim H-J, Kalkhoff RK. Changes in lipoprotein composition during the menstrual cycle. *Metabolism* 1979;28:663-668.

Kleessen B, Sykura B, Zunft H-J, Blaut M. Effects of inulin and lactose on fecal microflora, microbial activity, and bowel habit in elderly constipated persons. *Am.J.Clin.Nutr.* 1997;65:1397-1402.

Kmiot WA, Youngs D, Tudor R, Thompson H, Keighley RB. Mucosal morphology, cell proliferation and faecal bacteriology in acute pouchitis. *Br.J.Surg.* 1993;80:1445-1449.

Kohmoto T, Fukui F, Takaku H, Machida Y, Arai M, Mitsuoka T. Effect of isomalto-oligosaccharides on human fecal flora. *Bifidobacteria Microflora* 1988;7:61-69.

Kok N, Roberfroid M, Robert A, Delzenne N. Involvement of lipogenesis in the lower VLDL secretion induced by oligofructose in rats. *Br.J.Nutr.* 1996;76:881-890.

Koo M, Rao AV. Long-term effect of Bifidobacteria and Neosugar on precursor lesions of colonic cancer in CF1 mice. *Nutr.Cancer* 1991;16:249-257.

Koopman J. Kolonisatie resistentie van het maagdarmkanaal (in Dutch). *Tijdschr.Diergeneeskd.* 1984;109:1017-1026.

Lapré JA, Meer Rvd. Diet-induced increase of colonic bile acids stimulates lytic activity of fecal water and proliferation of colonic cells. *Carcinogenesis* 1992;13:41-44.

Lapré JA, Vries HTd, Termont DSML, Kleibeuker JH, Vries EGEd, Meer Rvd. Mechanism of the protective effect of supplemental dietary calcium on cytolytic activity of fecal water. *Cancer Res.* 1993;53:248-253.

Leclercq C, Avalle V, Ranaldi L, Toti E, Ferro-Luzzi A. Simplifying the lithium-marker technique used to assess the dietary intake of discretionary sodium in population studies. *Clin.Sci.* 1990; 79:227-231.

Lee SC, Prosky L. International survey on dietary fiber: definition, analysis, and reference materials. *J.AOAC.Int.* 1995;78:22-36.

Lehtonen P, Hurme R. Liquid chromatographic determination of sugars in beer by evaporative light scattering detection. *J.Institute Brewing* 1994;100:343-346.

Levrat M, Favier M, Moundras C, Rémésy C, Demigné C, Morand C. Role of dietary propionic acid and bile acid excretion in the hypocholesterolemic effects of oligosaccharides in rats. *J.Nutr.* 1994;124:531-538.

Levrat M, Rémésy C, Demigné C. High propionic acid fermentations and mineral accumulation in the cecum of rats adapted to different levels of inulin. *J.Nutr.* 1991;121:1730-1737.

Levrat M, Rémésy C, Demigné C. Influence of inulin on urea and ammonia nitrogen fluxes in the rat cecum: consequences on nitrogen excretion. *J.Nutr.Biochem.* 1993;4:351-356.

Lin H, Visek WJ. Large intestinal pH and ammonia in rats: dietary fat and protein interactions. *J.Nutr.* 1991;121:832-843.

Loo JV, Coussement P, De Leenheer L, Hoebregs H, Smits G. On the presence of inulin and oligofructose as natural ingredients in the western diet. *Crit.Rev.Food.Sci.Nutr.* 1995;35:525-552.

Luo J, Rizkalla SW, Alamowitch C, et al. Chronic consumption of short-chain fructooligosaccharides by healthy subjects decreased basal hepatic glucose production but had no effect on insulin-stimulated glucose metabolism. *Am.J.Clin.Nutr.* 1996;63:939-945.

Luukkonen P, Valtonen V, Sivonen A, Sipponen P, Järvinen H. Fecal bacteriology and reservoir ileitis in patients operated on for ulcerative colitis. *Dis.Colon.Rectum.* 1988;31:864-867.

MacFarlane GT, Gibson GR, Cummings JH. Extracellular and cell-associated glycosidase activities in different regions of the human large intestine. *Lett.Appl.Microb.* 1991;12:3-7.

MacFarlane GT, Gibson GR, Cummings JH. Comparison of fermentation reactions in different regions of the human colon. *J.Appl.Bacteriol.* 1992;72:57-64.

MacFarlane GT, MacFarlane S. Factors affecting fermentation reactions in the large bowel. *Proc.Nutr.Soc.* 1993;52:367-373.

Mallett AK, Rowland IR. Dietary modification of intestinal bacterial enzyme activities - potential formation of toxic agents in the gut. *Scand.J.Gastroenterol.* 1987;129:S251-S257.

Masai T, Wada K, Hayakawa K, Yoshihara I, Mitsuoka T. Effects of soybean oligosaccharides on human intestinal flora and metabolic activities. *JPN.J.Bacteriol.* 1987;42:313-325.

McBurney MI. Starch malabsorption and stool excretion are influenced by the menstrual cycle in women consuming low-fibre western diets. *Scand.J.Gastroenterol.* 1991;26:880-886.

Mcintyre A, Young GP, Taranto T, Gibson PR, Ward PB. Different fibers have different regional effects on luminal contents of rat colon. *Gastroenterology* 1991;101:1274-1281.

Meslin JC, Andrieux C, Sakata T, et al. Effects of galactooligosaccharides and bacterial status on mucin distribution in mucosa and on large intestine fermentation in rats. *Br.J.Nutr.* 1993;69:903-912.

Minami Y, Yazawa K, Nakamura K, Tamura Z. Selectivity and efficiency of utilization of galactosyl-oligosaccharides by bifidobacteria. *Chem.Pharm.Bull.* 1985;33:710-714.

Mitsuoka T. Bifidobacteria and their role in human health. *J.Ind.Microb.* 1990;6:263-268.

Mitsuoka T, Hidaka H, Eida T. Effect of fructo-oligosaccharides on intestinal microflora. *Nahrung* 1987;31:427-436.

Modler HW, McKellar RC, Yaguchi M. Bifidobacteria and bifidogenic factors. *Can.Inst.Food Sci.Technol.J.* 1990;23:29-41.

Molis C, Flourié B, Ouarne F, et al. Digestion, excretion, and energy value of fructooligosaccharides in healthy humans. *Am.J.Clin.Nutr.* 1996;64:324-328.

Mortensen PB. The effect of oral-administered lactulose on colonic nitrogen metabolism and excretion. *Hepatology.* 1992;16:1350-1356.

Mortensen PB, Hegnhoj J, Rannem T, Rasmussen HS, Holtug K. Short-chain fatty acids in bowel contents after intestinal surgery. *Gastroenterology* 1989;97:1090-1096.

Mortensen PB, Holtug K, Bonnén H, Clausen MR. The degradation of amino acids, proteins, and blood to short-chain fatty acids in colon is prevented by lactulose. *Gastroenterology* 1990;98:353-360.

Muir JG, Zhong XL, Young GP, Cameron-Smith D, Collier GR, O'Dea K. Resistant starch in the diet increases breath hydrogen and serum acetate in human subjects. *Am.J.Clin.Nutr.* 1995;61 :792-799.

Munster IP van, Boer HM de, Jansen MC, et al. Effect of resistant starch on breath-hydrogen and methane excretion in healthy volunteers. *Am.J.Clin.Nutr.* 1994*a*;59:626-630.

Munster IP van, Nagengast FM. The role of carbohydrate fermentation in colon cancer prevention. *Scand.J.Gastroenterol.* 1993;200:S80-S86.

Munster IP van, Tangerman A, Nagengast FM. Effect of resistant starch on colonic fermentation, bile acid metabolism, and mucosal proliferation. *Dig.Dis.Sci.* 1994*b*;39:834-842.

Nagengast FM, Erp J van, Koopman J, Tongeren J van. The relationship between methane (CH4) production in vitro and excretion in breath. *Gastroenterology* 1988*a*;94:a319

Nagengast FM, Hectors MPC, Buys WAM, Tongeren JHM van. Inhibition of secondary bile acid formation in the large intestine by lactulose in healthy subjects of two different age groups. *Eur.J.Clin.Inv.* 1988*b*;18:56-61.

Nasmyth DG, Godwin PGR, Dixon MF, Williams NS, Johnston D. Ileal ecology after pouch-anal anastomosis or ileostomy. *Gastroenterology* 1989;96:817-824.

Netherlands Cancer Registry. *Incidence of cancer in the Netherlands. Second report of the Netherlands Cancer Registry.* 1990.

Nilsson U, Björck I. Availability of cereal fructans and inulin in the rat intestinal tract. *J.Nutr.* 1988;118:1482-1486.

Nordgaard I, Clausen MR, Mortensen PB. Short-chain fatty acids, lactate and ammonia in ileorectal and ileal pouch contents: a model of cecal fermentation. *J.Par.Ent.Nutr.* 1993;34:324-331.

O'Connell PR, Rankin DR, Weiland LH, Kelly KA. Enteric bacteriology, absorption, morphology and emptying after ileal pouch-anal anastomosis. *Br.J.Surg.* 1986;73:909-914.

Oku T, Tokunaga T, Hosoya N. Nondigestibility of a new sweetener, "Neosugar", in the rat. *J.Nutr.* 1984;114:1574-1581.

Öresland T, Fasth S, Nordgren S, Akervall S, Hultén L. Pouch size: the important functional determinant after restorative proctocolectomy. *Br.J.Surg.* 1990;77:265-269.

Pedersen A, Sandström B, Amelsvoort JMMv. The effect of ingestion of inulin on blood lipids and gastrointestinal symptoms in healthy females. *Br.J.Nutr.* 1997;78:215-222.

Philips J, Muir JG, Birkett A, et al. Effect of resistant starch on fecal bulk and fermentation-dependent events in humans. *Am.J.Clin.Nutr.* 1995;62:121-130.

Pomare EW, Branch WJ, Cummings JH. Carbohydrate fermentation in the human colon and its relation to acetate concentrations in venous blood. *J.Clin.Inv.* 1985;75:1448-1454.

Prior RL, Topping DC, Visek WJ. Metabolism of isolated chick small intestinal cell. Effects of ammonia and various salts. *Biochemistry* 1974;13:178-183.

Rafter JJ, Eng VWS, Furrer R, Medline A, Bruce WR. Effects of calcium and pH on the mucosal damage produced by deoxycholic acid in the rat colon. *Gut* 1986;27:1320-1329.

Rasmussen HS, Holtug K, Mortensen PB. Degradation of amino acids to short-chain fatty acids in humans. *Scand.J.Gastroenterol.* 1988;23:178-182.

Reddy BS, Engle A, Simi B, Goldman M. Effect of dietary fiber on colonic bacterial enzymes and bile acids in relation to colon cancer. *Gastroenterology* 1992;102:1475-1482.

Roberfroid M, Gibson GR, Delzenne N. The biochemistry of oligofructose, a nondigestible fiber: an approach to calculate its caloric value. *Nutr.Rev.* 1993;51 (5):137-146.

Rodionova NA, Kilimnik AYU, Kaprel'yants LV, et al. Enzyme preparation from mycelial fungi that cleave cereal grain polymers. *Appl.Biochem.Microbiol.* 1995;31:370-376.

Roediger WEW. The colonic epithelium in ulcerative colitis: an energy deficiency disease? *Lancet* 1980;2:712-715.

Roland N, Nugon Baudon L, Andrieux C, Szylit O. Comparative study of the fermentative characteristics of inulin and different types of fibre in rats inoculated with a human whole faecal flora. *Br.J.Nutr.* 1995;74:239-249.

Rombeau JL, Kripke SA. Metabolic and intestinal effects of short chain fatty acids. *J.Par.Ent.Nutr.* 1990;14:181S-185S.

Rowland IR. Factors affecting metabolic activity of the intestinal microflora. *Drug Metab.Rev.* 1988;19 :243-261.

Rowland IR, Tanaka R. The effects of transgalactosylated oligosaccharides on gut flora metabolism in rats associated with a human faecal microflora. *J.Appl.Bacteriol.* 1993;74:667-674.

Rumessen JJ. Hydrogen and methane breath tests for evaluation of resistant carbohydrates. *Eur.J.Clin.Nutr.* 1992*a*;46:S77-S90.

Rumessen JJ, Bodé S, Hamberg O, Gudmand-Hoyer E. Fructans of Jerusalem artichokes: intestinal transport, absorption, fermentation, and influence on blood glucose, insulin, and C-peptide responses in healthy subjects. *Am.J.Clin.Nutr.* 1990;52:675-681.

Rumessen JJ, Franck YS, Gudmand-Hoyer E. Acetate in venous blood for determination of carbohydrate malabsorption. *Eur.J.Clin.Nutr.* 1992*b*;46:S135-S136

Salemans JMJI, Nagengast FM, Lubbers E-JC, Kuijpers HJH. Postoperative and long-term results of ileal pouch-anal anastomosis for ulcerative colitis and familial adenomatous polyposis. *Dig.Dis.Sci.* 1992;37:1882-1889.

Sanchez-Castillo CP, Seidell J, James WPT. The potential use of lithium as a marker for the assessment of the sources of dietary salt: cooking studies and physiological experiments in men. *Clin.Sci.* 1987;72:81-86.

Sandborn WJ, Tremaine WJ, Batts KP, et al. Fecal bile acids, short-chain fatty acids, and bacteria after ileal pouch-anal anastomosis do not differ in patients with pouchitis. *Dig.Dis.Sci.* 1995;40:1474-1483.

Santavirta J, Mattila J, Kokki M, Matikainen M. Mucosal morphology and faecal bacteriology after ileoanal anastomosis. *Int.J.Colorect.Dis.* 1991;6:38-41.

Scheppach W, Bartram HP, Richter F. Role of short-chain fatty acids in the prevention of colorectal cancer. *Eur.J.Cancer* 1995;31A:1077-1080.

Schols H. *Structural characterization of pectic hairy regions isolated from apple cell walls. Thesis.* Wageningen Agricultural University, the Netherlands. 1995.

Shepherd NA. Pouchitis and neoplasia in the pelvic ileal reservoir. *Gastroenterology* 1995;109:1381-1396.

Siedel J, Hägele EO, Ziegenhorn J, Wahlefeld AW. Reagent for the enzymatic determination of serum total cholesterol with improved lipolytic efficiency. *Clin.Chem.* 1983;29:1075-1080.

Simon GL, Gorbach SL. Intestinal flora in health and disease. *Gastroenterology* 1984;86:174-193.

Snedecor GW, Cochran WG. *Statistical methods*. Ames: Iowa State University Press, 1989.

Spiegel JE, Rose R, Karabell P, Frankos VH, Schmitt DF. Safety and benefits of fructooligosaccharides as food ingredients. *Food.Techn.* 1994;1:85-89.

Stanier RY, Adelber EA, Ingraham JL. *General microbiology*. London: MacMillan, 1979.

Stasse-Wolthuis M, Albers HFF, van Jeveren JGC, et al. Influence of dietary fiber from vegetables and fruits, bran or citrus pectin on serum lipids, fecal lipids, and colonic function. *Am.J.Clin.Nutr.* 1980;33:1745-1765.

Stephen AM, Cummings JH. The microbial contribution to human faecal mass. *J.Med.Microbiol.* 1980;1345:45-56.

Stone-Dorshow T, Levitt MD. Gaseous response to ingestion of a poorly absorbed fructooligosaccharide sweetener. *Am.J.Clin.Nutr.* 1987;46:61-65.

Szylit O, Andrieux C. Physiological and pathophysiological effects of carbohydrate fermentation. In: Simopoulos AP, Corring T, Rérat A, eds. *Intestinal flora, immunity, nutrition and health*. Basel: Karger, 1993;88-122.

Tanaka R, Takayama H, Mortomi M, et al. Effects of administration of TOS and bifidobacterium breve 4006 on the human fecal flora. *Bifidobacteria Microflora* 1983;2:17-24.

Tangerman A, Nagengast FM. A gas chromatographic analysis of fecal short-chain fatty acids, using the direct injection method. *Anal.Biochem.* 1996;236:1-8.

Thorburn A, Muir J, Proietto J. Carbohydrate fermentation decreases hepatic glucose output in healthy subjects. *Metabolism* 1993;42 (6):780-785.

Tokunaga T, Oku T, Hosoya N. Utilization and excretion of a new sweetener, fructooligosaccharide (neosugar), in rats. *J.Nutr. 1989;119:553-559.*

Tokunaga T, Oku T, Hosoya N. Influence of chronic intake of new sweetener fructooligosaccharide (neosugar) on growth and gastrointestinal function of the rat. *J.Nutr.Sci.Vitaminol.* 1995.

Tonelli F, Bianchini F, Lodovici M, Valanzano R, Caderni G, Dolara P. Mucosal cell proliferation of the rectal stump in ulcerative colitis patients after ileorectal anastomosis. *Dis.Colon.Rectum.* 1991;34:385-390.

Tonelli F, Dolara P, Batignani G, et al. Effects of short chain fatty acids on mucosal proliferation and inflammation of ileal pouches in patients with ulcerative colitis and familial polyposis. *Dis.Colon.Rectum.* 1995;38:974-978.

Trock B, Lanza E, Greenwald P. Dietary fiber, vegetables, and colon cancer: critical review and meta-analyses of the epidemiologic evidence. *J.Natl.Cancer Inst.* 1990;82:650-661.

Wang X, Gibson GR. Effects of the in vitro fermentation of oligofructose and inulin by bacteria growing in the human large intestine. *J.Appl.Bacteriol.* 1993;75:373-380.

Warnick GR, Benderson J, Albers JJ. Dextran sulfate-Mg2+ precipitation procedure for quantitation of high-density-lipoprotein cholesterol. *Clin.Chem.* 1982;28:1379-1388.

WHO Study Group. *Prevention of diabetes mellitus*. Geneva: World Health Organization, 1994;11-18

Wilkins CK. Analysis of indole and skatole in porcine gut contents. *Int.J.Food Sci.Technol.* 1990;25:313-317.

Wolever TMS, Fernandes J, Venketeshwer Rao A. Serum acetate:propionate ratio is related to serum cholesterol in men but not women. *J.Nutr.* 1996;126:2790-2797.

Wolever TMS, Jenkins DJA. The use of the glycemic index in predicting the blood glucose response to mixed meals. *Am.J.Clin.Nutr.* 1986;43:167-172.

Wolever TMS, Jenkins DJA, Mueller S, et al. Method of administration influences the serum cholesterol-lowering effect of psyllium. *Am.J.Clin.Nutr.* 1994;59:1055-1059.

Wolever TMS, Spadafora P, Eshuis H. Interaction between colonic acetate and propionate in humans. *Am.J.Clin.Nutr.* 1991;53:681-687.

Wolever TMS, Spadafora PJ, Cunnane SC, Pencharz PB. Propionate inhibits incorporation of colonic [1,2-13]acetate into plasma lipids in humans. *Am.J.Clin.Nutr.* 1995;61:1241-1247.

Yamashita K, Kawai K, Itakura M. Effects of fructo-oligosaccharides on blood glucose and serum lipids in diabetic subjects. *Nutr.Res.* 1984;4:961-966.

Yanahira S, Kobayashi T, Suguri T, et al. Formation of oligosaccharides from lactose by Bacillus circulans beta-galactosidase. *Biosci.Biotech.Biochem.* 1995;59:1021-1026.

Younes H, Garleb K, Behr S, Rémésy C, Demigné C. Fermentable fibers or oligosaccharides reduce urinary nitrogen excretion by increasing urea disposal in the rat cecum. *J.Nutr.* 1995;125:1010-1016.

Zarling EJ, Ruchim MA. Protein origin of the volatile fatty acids isobutyrate and isovalerate in human stool. *J.Lab.Clin.Med.* 1987;109:566-570.

Samenvatting

Oligosacchariden komen van nature voor in veel gewassen die worden gebruikt als grondstof bij de productie van voedingsmiddelen. De oligosacchariden die voorkomen in planten zijn vaak niet verteerbaar in het bovenste gedeelte van het menselijke spijsverteringskanaal en worden meestal beschouwd als voedingsvezel. Met de officiële AOAC analyse voor totaal voedingsvezel zijn ze echter niet in voedingsmiddelen te meten.

De studies in dit proefschrift beschrijven de fysiologische effecten van twee typen niet-verteerbare oligosacchariden: fructo-oligosacchariden (Raftilose P95®, ORAFTI, Tienen, België) en transgalacto-oligosacchariden (Elix'or®, Borculo Whey Products, Borculo, Nederland). De oligosacchariden in Raftilose® worden gemaakt van inuline dat wordt geëxtraheerd uit de wortel van cichorei. De oligomeren zijn opgebouwd uit fructose-eenheden met of zonder glucose molecuul. Elix'or® wordt gemaakt door middel van enzymatische transgalactosylering van lactose. Het bestaat uit verschillende oligomeren die zijn opgebouwd uit galactose-eenheden met of zonder glucose.

In vier placebo gecontroleerde interventie studies werd gekeken naar het lot van de oligosacchariden in het maagdarm kanaal en naar eindproducten van de fermentatie, en naar het effect van de oligosacchariden op de samenstelling van de darmflora, mogelijke risicofactoren voor dikke darm kanker en het glucose en lipiden metabolisme bij diabetes patiënten.

In het eerste experiment (***Hoofdstuk 2***) vergeleken we het effect van twee doses fructo-oligosacchariden (5 en 15 g/d) met een placebo behandeling (glucose) op fermentatie in de dikke darm van gezonde mannen. Er werden geen fructo-oligosacchariden gevonden in de urine en ontlasting. De hoogste dosis fructo-oligosacchariden zorgde voor een significante toename van de 24-uurs excretie van waterstof in de uitademingslucht ($P < 0.05$). De excretie van waterstof was ook hoger na consumptie van 5 g fructo-oligosacchariden, maar deze toename was niet significant. Er waren geen effecten op de fecale pH of op de absolute of relatieve hoeveelheid van korte keten vetzuren in de ontlasting. We concluderen uit deze studie dat fructo-oligosacchariden volledig worden gefermenteerd in de dikke darm van gezonde vrijwilligers.

Vervolgens onderzochten we in ***Hoofdstuk 3*** de fermentatie van twee typen niet-verteerbare koolhydraten, fructo-oligosacchariden en niet-verteerbaar zetmeel, in 15 gezonde patiënten met een ileale pouch-anale anastomose. Bij deze patiënten is de dikke darm verwijderd en is een deel van het distale ileum gebruikt om een reservoir – ofwel pouch - te maken, welke wordt verbonden met de anus. De schijnbare fermentatie van fructo-oligosacchariden was 83% en van niet-verteerbaar zetmeel 46%. Niet-verteerbaar zetmeel verhoogde de fecale excretie van butyraat met 69%, terwijl fructo-oligosacchariden de excretie verlaagde van het, uit de eiwit fermentatie afkomstige, iso-butrate (met 94%) en iso-valeraat (met 77%). Fructo-oligosacchariden verhoogden het fecaal

gewicht (met 110 g/d) en de excretie van waterstof in uitademingslucht (met 201 ppm.h). De studie suggereert dat bacteriële fermentatie van niet-verteerbare koolhydraten in de pouch in grote mate plaatsvindt. Fructo-oligosacchariden worden voor een groot gedeelte afgebroken in de pouch en zijn kennelijk een gemakkelijk fermenteerbaar substraat voor de darmflora.

In een gecontroleerde voedingsproef, beschreven in ***Hoofdstuk 4***, onderzochten we het effect van twee doses transgalacto-oligosacchariden (7.5 en 15 g/d) in vergelijking met een placebo (een mengsel van glucose en lactose) op de samenstelling en activiteit van bacteriën in de dikke darm. Er werden geen transgalacto-oligosacchariden gevonden in de ontlasting. De hoogste dosis zorgde voor een significante stijging van de waterstofconcentratie in adem met 138% ($P < 0.01$) en van het stikstofgehalte van de ontlasting met 6.5% ($P < 0.05$). Transgalacto-oligosacchariden hadden geen significant effect op de frequentie van defecatie, het gewicht van de ontlasting, de samenstelling van de ontlasting, de concentratie korte keten vetzuren of galzuren in fecal water, de fecale concentratie van ammonia, indolen of skatolen of op de fecale pH. Het aantal bifidobacteriën in de ontlasting nam significant toe gedurende de studie maar er waren geen verschillen tussen de stijging tijdens de placebo-behandeling en die tijdens interventie met transgalacto-oligosacchariden. We concluderen uit deze studie dat transgalacto-oligosacchariden volledig worden gefermenteerd in de dikke darm. We konden geen positieve effecten aantonen op de fecale concentratie van toxische producten uit de eiwit fermentatie, op het galzuurprofiel in fecaal water, of op de samenstelling van de darmflora.

In ***Hoofdstuk 5*** van dit proefschrift, combineren we gegevens uit de twee studies met gezonde vrijwilligers (***Hoofdstuk 2 en 4***) om het effect van fructo-oligosacchariden en transgalacto-oligosacchariden te onderzoeken op de bacteriële glycosidase activiteit in fecaal water. Transgalacto-oligosacchariden verhoogden de activiteit van β-galactosidase met 123%. Fructo-oligosacchariden verlaagden de activiteit van arabinofuranosidase met 35%. Beide oligosacchariden hadden geen effect op de activiteit van glucosidase of β-glucuronidase; deze enzymen zijn betrokken bij de vorming van giftige stoffen. We concluderen dat oligosacchariden een substraat specifiek effect hebben op de glycolytische activiteit van de bacteriën in de darm. Het meten van de activiteit van deze enzymen is een biomarker voor de metabole activiteit van bacteriën in de dikke darm.

In ***Hoofdstuk 6*** onderzochten we het effect van fructo-oligosacchariden (15 g/d) in vergelijking met een placebo (4 g/d) op bloed glucose, serum lipiden en serum acetaat, bij niet-insuline afhankelijke diabetes patiënten. De gemiddelde inname van energie, macronutriënten en voedingsvezel veranderde niet tijdens de studie. De compliance was bijna 100% gedurende beide interventies. Fructo-oligosacchariden hadden geen significant effect op nuchtere concentraties van serum totaal cholesterol (95% CI van het verschil; -0.07, 0.48 mmol/L), HDL cholesterol (95% CI van het verschil; -0.04; 0.04 mmol/L), LDL cholesterol (95% CI van het verschil; -0.06, 0.34

mmol/L), serum triacylglycerol (95% CI van het verschil; -0.21, 0.44 mmol/L), serum vrije vetzuren (95% CI van het verschil; -0.08, 0.04), serum acetaat (95% CI van het verschil; -0.01, 0.01 mmol/L) of bloed glucose (95% CI van het verschil; -0.37, 0.40 mmol/L). We concluderen dat het gedurende 20 dagen consumeren van een voedingssupplement met fructo-oligosacchariden geen duidelijke effecten heeft op bloed glucose, serum lipiden of serum acetaat in niet-insuline afhankelijke diabetes patiënten. Het ontbreken van een effect was niet te wijten aan veranderingen in het voedingspatroon, onvoldoende statistische power of een slechte compliance van de proefpersonen.

In ***Hoofdstuk 7*** beschrijven we een aantal methodologische aspecten van het onderzoek zoals beschreven in dit proefschrift. In een pilotstudie hebben we onderzocht wat het effect is van het bewaren en invriezen van ontlasting monsters alvorens de verschillende bacteriën te kwantificeren. Hieruit blijkt dat het invriezen van de ontlasting significante effecten heeft op de bacterie tellingen en dat het effect sterk afhankelijk is van het type bacterie. In een andere pilotstudie vonden we dat de binnen-persoons variatie in de hoeveelheid darmbacteriën zeer klein is (< 11%). Het is opvallend dat veel studies waarin gekeken wordt naar het effect van niet-verteerbare oligosacchariden op de samenstelling van de darmflora, gebruik maken van een lineaire opzet, zonder placebo behandeling. Zonder placebo groep zou de conclusie van ***Hoofdstuk 4*** zijn geweest dat transgalacto-oligosacchariden de groei van bifidobacteriën stimuleren. We concluderen dat het belangrijk is om de effecten van niet-verteerbare oligosacchariden te vergelijken met een placebo, om een mogelijk tijdseffect uit te kunnen sluiten.

Het is duidelijk dat fructo-oligosacchariden en transgalacto-oligosacchariden volledig worden gefermenteerd in de dikke darm van gezonde vrijwilligers. Deze fermentatie leidt tot gasvorming. We vonden geen bewijs voor positieve effecten van de oligosacchariden op kenmerken van de ontlasting die samenhangen met het risico op dikke darmkanker. We zagen geen positieve effecten op de samenstelling van de darmflora of op het glucose en lipiden metabolisme. Het is mogelijk dat fructo-oligosacchariden of transgalacto-oligosacchariden wel een effect hebben in andere studie populaties of in combinatie met een ander achtergronds dieet of andere fermenteerbare voedingsvezels.

Nawoord

Het onderzoek dat in dit boekje wordt beschreven is de afgelopen vijf jaar uitgevoerd op de Vakgroep Humane Voeding en Epidemiologie. Het was een leuke tijd en ik vind het fijn om als afsluiting iedereen te bedanken die heeft bijgedragen aan de totstandkoming van het proefschrift. Allereerst wil ik mijn promotor en copromotor bedanken. Professor Jo Hautvast, met u heb ik een bijzondere band gekregen. U heeft altijd een enorm vertrouwen in mij gehad, ook toen ik na 3 jaar AIO-zijn besloot om mijn promotie onderzoek verder te combineren met een baan in de industrie. Ik heb van u geleerd om strategische beslissingen te nemen en om op de juiste momenten een vuist te maken. Bedankt!

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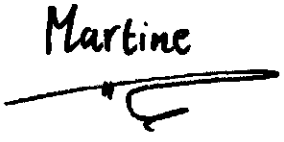

Curriculum Vitae

Martine Alles was born on April 04, 1969 in Haarlem, the Netherlands. In 1987 she graduated from secondary school (Athenaeum) at the Rölingcollege in Groningen. She started her studies at the polytechnic in Groningen were she obtained her propaedeutical degree in nursing in 1988. In September 1988 she moved to Maastricht for a study Biomedical Sciences at Maastricht University with a main subject in Human Nutrition. After finishing the theoretical part of her studies in Maastricht, she moved to Wageningen and did some subsidiary subjects in Human Nutrition, Education and Management of Organisations. She passed her exams in 1992. As part of the training in Human Nutrition, she then spent 6 months at the Dunn Clinical Nutrition Centre in Cambridge, United Kingdom. In August 1993 she obtained her MSc degree in Biomedical Sciences. From October 1993 till July 1998 she was appointed as PhD-fellow at the Division of Human Nutrition and Epidemiology of Wageningen Agricultural University. During this period, she performed the research described in this thesis. She participated in a multidisciplinary project on the role of non-digestible oligosaccharides in food and feed. She was a member of the PhD-excursion committee that organised a study-tour to the United States of America in 1995. She was a member of the editorial board of the PhD-newsletter "Newtrition" and she was an appointed member of the Permanent Committee for Research of the Wageningen Agricultural University. She participated in international courses on "Ecophysiology of the gastrointestinal tract" and "Regulation of food intake". She was one of the organisers of a workshop on dietary fibre for members of the Dutch Society for Nutrition and Food Technology in 1996. She was elected to participate in the fourth European Nutrition Leadership Programme in Luxembourg in 1997. Since December 1996 she has been working, at first part-time, as a research scientist nutrition at the Research Centre Deventer of Friesland Coberco Dairy Foods. She combined the writing up of her thesis with her work in industry.

Publications

Full papers

1. Geerling BJ, **Alles MS**, Murgatroyd PR, Goldberg GR, Harding M, Prentice AM. Fatness in relation to substrate oxidation during exercise. *International Journal of Obesity* 1994;18: 453-459.
2. **Alles MS**, Hautvast JGAJ, Nagengast FM, Hartemink R, Van Laere KMJ, Jansen JBJM. Fate of fructo-oligosaccharides in the human intestine. *British Journal of Nutrition* 1996;76:211-221.
3. **Alles MS**, Katan MB, Salemans JMJI, Van Laere KMJ, Gerichhausen MJW, Rozendaal MJ, Nagengast FM. Bacterial fermentation of fructo-oligosaccharides and resistant starch in patients with an ileal pouch-anal anastomosis. *The American Journal of Clinical Nutrition* 1997; 66:1286-1292.
4. **Alles MS**, de Roos NM, Bakx JC, van de Lisdonk E, Zock PL, Hautvast JGAJ. Consumption of fructooligosaccharides does not favourably affect blood glucose and serum lipids in non-insulin dependent diabetic patients. *The American Journal of Clinical Nutrition* (In press).
5. **Alles MS**, Hartemink R, Meyboom S, Harryvan JL, Van Laere KMJ, Nagengast FM, Hautvast JGAJ. Effect of transgalacto-oligosaccharides on composition and activity of the intestinal microflora. (Submitted for publication).
6. **Alles MS**, Van Laere KMJ, Voragen AGJ, Hautvast JGAJ. Fructo-oligosaccharides and transgalacto-oligosaccharides affect the bacterial glycosidase activity in human subjects. (Submitted for publication).

Other publications

1. **Alles MS**, Hautvast JGAJ. Bacterial fermentation of fructo-oligosaccharides in humans. *Proceedings of the fifth seminar on inulin* 1995: 93-97.
2. **Alles MS**, Hautvast JGAJ, Jansen JBMJ, Nagengast FM. The effect of fructo-oligosaccharides on colonic fermentation. *Proceedings of the first ORAFTI research conference* 1995:236.
3. **Alles MS**, Nagengast FM, Hautvast JGAJ. Bacteriële fermentatie van fructo-oligosacchariden. *Voeding* 1996;57 (6): 29 (In Dutch).

4. **Alles MS**, Katan MB, Salemans JMJI, Van Laere KMJ, Gerichhausen MJW, Rozendaal MJ, Nagengast FM. Bacterial fermentation of oligofructose and resistant starch in patients with ileal pouch-anal anastomosis. *Gastroenterology* 1996;110(sup):A789 (Abstract).
5. Tangerman A, **Alles MS**, Katan MB, Nagengast FM. Methanogenesis and sulphate reduction in patients with an ileal pouch-anal anastomosis. *Gastroenterology* 1996;110(sup):A1025 (Abstract).
6. Katan MB, **Alles MS**, Heijnen MLA, Zock PL. Yoghurts in de storm. *Voeding* 1996;57 (5): 12 (In Dutch, comment).
7. **Alles MS**, Hartemink R, Meyboom S, Nagengast FM, Hautvast JGAJ. Het effect van transgalacto-oligosacchariden op de bacteriële activiteit in het colon. *Samenvattingen van lezingen en posters van het congres "Trends in Voedingswetenschappen"* 1997: (In Dutch, abstract).
8. **Alles MS**, de Roos NM, Bakx, JC, van de Lisdonk E, Hautvast JGAJ. Dietary fructo-oligosaccharides do not modify blood glucose and serum lipids in non-insulin dependent diabetic subjects. *Research Abstracts in the Report of the Fourth European Nutrition Leadership Programme* 1997; 61 (Abstract).
9. **Alles MS**. The effects of non-digestible oligosaccharides on composition and activity of the intestinal microflora. *Proceedings of the International symposium "Non-digestible oligosaccharides healthy food for the colon?"* 1997;79-89.
10. **Alles MS**, de Roos NM, Bakx JC, van de Lisdonk E, Zock PL, Hautvast JGAJ. Consumption of fructo-oligosaccharides does not affect blood glucose and serum lipids in non-insulin dependent diabetic patients. *Proceedings of the International symposium "Non-digestible oligosaccharides healthy food for the colon?"* 1997;166 (Abstract).
11. **Alles MS**, Klerk M, Wijsman MR. Gezondheidsaspecten van sinaasappelsap, een functional food van moeder natuur. *Voeding* 1997;58 (11): 21-23 (In Dutch).
12. **Alles MS**, de Roos NM, Hautvast JGAJ. Niet-insuline afhankelijke diabetes: effect van fructo-oligosacchariden op glucose- en lipidemetabolisme. *Voeding* 1998;59 (3): 28 (In Dutch).
13. Siebelink E, Roosemalen K, de Vries JHM, **Alles MS**, Brouwer IA, Castenmiller JJM, van Staveren WA. Self-reported energy intake by a food frequency questionnaire compared with energy need to maintain body weight in 170 non-obese adults. European Journal of Clinical Nutrition 1998;52:S21 (Abstract).