Stellingen :

I :

Bij de berekening van de calorische waarde van fructooligosacchariden gaan Roberfroid *et al.* er ten onrechte van uit dat alleen bifidobacteriën deze oligosacchariden afbreken (Roberfroid, M.; Gibson, G.R. and Delzenne, N., Nut. Rev. 51(1993)137-146)

II :

De door Gibson *et al.* gevonden antimicrobiële werking van bifidobacteriën wordt waarschijnlijk niet veroorzaakt door bacteriocines. *(Gibson, G.R. and Wang, X., J. Appl. Bact. 77(1994)412-420)*

III :

Het bifidogene effect waargenomen door Bouhnik *et al.*, wordt eerder veroorzaakt door de gebruikte methodiek, dan door een reële toename van bifidobacteriën. *(Bouhnik, Y.et al.; Nutr. Cancer 26(1996)21-29 en Bouhnik, Y. et al, J. Nutr. 127(1997)444-448)*

IV :

De door Brigenthi *et al.* waargenomen gasvorming van 30 ml/mg inuline na het eten van 10 gram hiervan, moet hoogst oncomfortabel zijn geweest voor de proefpersonen. *(Brighenti et al., Ital. J. Gastroent. 27(1995)122-128)*

V :

Door de samenstelling van het huidige huisdiervoeder, zijn honden en katten nauwelijks meer te beschouwen als vleeseters.

VI :

Door de gebrekkige regelgeving op het gebied van probiotica, zijn er een groot aantal producten op de markt, waarvan geen enkele werking is vastgesteld, maar waarvan de consument de indruk heeft dat ze gezond zijn.

VII :

De mate van protest tegen een verkeersbelemmerende maatregel is een goede maat voor de effectiviteit van de betrokken maatregel.

VIII :

De lage dunk die de onderzoekers op de Dreijen hebben van de bestuurders in het hoofdgebouw, wordt in de hand gewerkt door de geografische positie van zowel de Dreijen als het hoofdgebouw.

IX :

Groenland is groener dan Nederland.

X :

Hoewel in IJsland geen Eskimo's wonen, een nog veel gehoorde stelling, wonen de IJslanders wel in iglo's.

Stellingen behorend bij het proefschrift :

Prebiotic effects of non-digestible oligosaccharides

Ralf Hartemink, Wageningen, 07 juni 1999

PREBIOTIC EFFECTS OF NON-DIGESTIBLE OLIGO- AND POLYSACCHARIDES.

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PREBIOTIC EFFECTS OF NON-DIGESTIBLE OLIGO- AND POLYSACCHARIDES.

Ralf Hartemink

Proefschrift

ter verkrijging van de graad van doctor
op gezag van de rector magnificus
van de Landbouwuniversiteit Wageningen,
dr. C.M. Karssen,
in het openbaar te verdedigen op
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ABSTRACT

Prebiotic effects of non-digestible oligo- and polysaccharides.

Ph.D. thesis by Ralf Hartemink, Food Microbiology Group, Wageningen Agricultural University, the Netherlands

Oligo- and polysaccharides occur naturally in many foods, mainly of vegetable origin. Many of these carbohydrates are not, or partially, digestible by the human upper gastro-intestinal tract. The non-digested carbohydrates enter the large intestine, where the majority is degraded and fermented by the intestinal microflora. This flora is a complex mixture of several hundred bacterial species. These bacteria are for a considerable part dependent on non-digested dietary components for their growth and energy requirements. Some non-digestible carbohydrates are considered prebiotics, since they may cause changes in the bacterial composition, or the metabolic activity of the flora, and thus may have a positive influence on the host health. Two studies described in this thesis deal with the fermentation of commercial oligosaccharides by the oral flora, and the risk of dental caries. It is concluded that there is a potential risk for dental caries with one oligosaccharide preparation (fructooligosaccharides), but the other preparation (transgalactosyl-oligosaccharides) does not pose a risk for caries. Two additional studies are described that deal with the fermentation of non-digestible polysaccharides by the intestinal microflora and their use as sources for prebiotic oligosaccharides. It was concluded that the two substrates tested were fermented only by a limited number of species. Fermentation of several non-digestible carbohydrates was studied using faecal inocula. It was concluded that there are considerable differences in the faecal metabolic capacity in samples from different volunteers, indicating that the metabolic capacity is partly determined by genetic factors. Finally three studies are described that deal with new isolation media for two bacterial groups, lactobacilli and bifidobacteria. The new media were compared with other media and it is concluded that both media perform better than the other media tested.

Abbreviations used throughout this thesis :

Bacterial genera		Carbohydrates	
Ac.	*Actinomyces*	α-GOS	α-Glucooligosaccharides
B.	*Bacteroides*	CEL	Cellobiose
Ba.	*Bacillus*	CYC	Cyclodextrin
Bi.	*Bifidobacterium*	FOS	Fructooligosaccharides
C.	*Coprococcus*	GeOS	Gentiooligosaccharides
Ca.	*Candida*	GLL	Galactosyl-lactose
Ci.	*Citrobacter*	IMA	Isomaltose
Cl.	*Clostridium*	IMT	Isomaltotriose
E.	*Escherichia*	LAS	Lactosucrose
Ec.	*Enterococcus*	LAT	Lactulose
Ent.	*Enterobacter*	LOL	Lactitol
Eu.	*Eubacterium*	LTOS	Lactitol-oligosaccharides
F.	*Fusobacterium*	MAT	Maltitol
H.	*Hafnia*	NEP	Neosugar P
Kl.	*Klebsiella*	PAL	Palatinose
L.	*Lactococcus*	PAN	Panose
Lb.	*Lactobacillus*	PCO	Palatinose Condensate
Me.	*Megasphaera*	RAF	Raffinose
Meg.	*Megamonas*	STC	Stachyose
Mi.	*Mitsuokella*	TOS	Transgalactosyl-oligosaccharides
P.	*Peptostreptococcus*		
Pr.	*Prevotella*		
Pro.	*Propionibacterium*		
Prot.	*Proteus*		
R.	*Ruminococcus*		
Ri.	*Rikenella*		
S.	*Staphylococcus*		
St.	*Streptococcus*		
V.	*Veillonella*		

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GENERAL INTRODUCTION

The (human) gastrointestinal tract harbors a large number of bacteria, that live in symbiosis with the host. Especially in the large intestine, these bacteria can reach very high numbers per gram intestinal contents. These bacteria, commonly referred to as the intestinal microflora, are of great importance to the host. The bacteria degrade and ferment many of the food components that can not be absorbed in the small intestine. The fermentation results in end-products that can be absorbed through the intestinal wall. This results in additional energy and, possibly, add to the nutritional value of the food consumed. The intestinal flora also acts as a barrier against food-borne pathogenic bacteria. On the other hand, the intestinal microflora may produce harmful substances, that may be involved in the formation of intestinal cancers

Within the intestinal microflora several hundred bacterial species may be present, some of these are considered beneficial for human health, others are considered as possible pathogens or producers of possible harmful substances. In a healthy large intestine an equilibrium exists between beneficial and possibly harmful bacteria. Many factors determine this equilibrium, one of these factors is the diet.

The past few decades there is increasing interest in influencing the intestinal flora through the diet, especially to shift the equilibrium towards more beneficial bacteria. Two methods are being used to influence the intestinal flora, one using living bacteria (probiotics), another using specific growth substrates (prebiotics).

In the concept of probiotics, live beneficial bacteria, mainly from the genera *Lactobacillus* or *Bifidobacterium* are used. Of several species of these genera it has been proven that ingestion of large quantities reduces lactose intolerance and reduces the effects of diarrhea. Several other beneficial effects have been claimed, but, at present not been proven sufficiently. Probiotics mainly act in the small intestine.

The second concept deals with specific food components that affect the composition or the metabolism of the intestinal flora. Components that have a beneficial effect are known as prebiotics. Most prebiotics are carbohydrates that are fermented by a large number of intestinal bacteria and do have some effect on either the composition or the metabolic activity of the flora (O'Sullivan, 1996). Prebiotics are mainly effective in the large intestine.

To have a beneficial effect prebiotics should not be digestible in the small intestine, they should be fermentable by the desired bacteria and have measurable effects. The studies described in this thesis deal with the latter two prerequisites.

AIM AND OUTLINE OF THIS THESIS

This thesis forms part of a larger research project on Non-Digestible Oligosaccharides (NDO-project), carried out at the Wageningen Agricultural University. The project was industry funded and carried out at four departments of the university : Food Chemistry, Food Microbiology, Human Nutrition and Animal Nutrition. The three main objectives of the project were :

- production and characterization of oligosaccharides
- fermentation of oligosaccharides by intestinal micro-organisms, and
- effects of (commercial) non-digestible oligosaccharides on humans and animals

This thesis describes the results of the studies carried out at the Food Microbiology group within the NDO-project. The results of the studies in Human Nutrition and Animal Nutrition, as well as Food Chemistry will be published in separate theses.

In **Chapter 1** of this thesis the current knowledge of fermentation of non-digestible oligosaccharides is reviewed as an introduction to the studies carried out within the Food Microbiology group. Within these studies the following topics are distinguished :

1. fermentation of commercial and newly produced oligosaccharides by the gastro-intestinal microflora
   a) fermentation by the oral flora
   b) fermentation by the large intestinal flora
2. fermentation of (food) polysaccharides by the large intestinal microflora
3. analysis of the intestinal and faecal flora of humans and piglets

Ad 1) Several studies have been carried out on the fermentation of different oligosaccharides by the oral and intestinal microflora. It was observed that commercial fructooligosaccharides (FOS) could be degraded and fermented by members of the *Enterobacteriaceae* (Hartemink *et al.*, 1997) as well as by the oral streptococci. The latter studies are presented in **Chapter 2.** Fermentation of the other commercial oligosaccharides, used at present in the Netherlands, transgalactosyl-oligosaccharides

(TOS), has also been studied. Fermentation by intestinal bacteria was observed, but fewer bacterial species were capable of fermenting TOS. These studies were carried out in close co-operation with the Food Chemistry group. Similarly, TOS were not degraded or fermented by the oral microflora, as is described in **Chapter 3**.

In cooperation with Food Chemistry several plant cell wall polysaccharides were tested as substrates for intestinal species. Within the same studies induction and enrichment experiments were carried out.

All previously mentioned studies were carried out with pure cultures of intestinal bacteria. **Chapter 6** describes a series of batch fermentations, using faecal inocula from human volunteers, receiving the same diet.

Ad 2) Two plant polysaccharides were tested as substrates for intestinal bacteria. The polysaccharides chosen were xyloglucan and guar gum (galactomannan).
The results of the polysaccharide fermentation, can be used to predict fermentability of oligosaccharides derived from these polysaccharides. Fermentation of xyloglucan is described in **Chapter 4**, fermentation of guar in **Chapter 5**.

Ad 3) Within the NDO-project, the Human and Animal Nutrition groups have carried out several studies with NDOs in the diet of humans and piglets. One of the main aims of these studies was to determine changes in the faecal or intestinal microflora. It was thus necessary to have reliable methods to determine changes in the bacterial and chemical composition of the intestinal flora. As these methods turned out to be unreliable, new microbiological media had to be developed.

The main emphasis within the human and animal trials was on lactic acid bacteria, as these may be an indication of intestinal health. Two new isolation media were developed for the detection of bifidobacteria and lactobacilli, the two main genera of lactic acid bacteria in the intestinal flora. The development of the medium for bifidobacteria is described in **Chapter 7**, that for lactobacilli in **Chapter 8**. Finally, in **Chapter 9**, these two media are compared with other media used elsewhere.

1

NON-DIGESTIBLE OLIGOSACCHARIDES AND THEIR FERMENTATION IN THE GI TRACT.

THE GASTROINTESTINAL TRACT

The human gastrointestinal tract (GI tract) basically is a large tube, running through the body and which includes the mouth, oral cavity, esophagus, stomach, small intestine and colon. The small intestine can be divided into duodenum, jejunum and ileum, the large bowel is composed of the caecum, ascending colon, transverse colon, descending colon, and sigmoid rectum. The proximal colon consists of the caecum and ascending colon whereas the distal bowel comprises the descending colon and sigmoid rectum. The caecum, to which the appendix is attached, is located in the right lower abdomen. The ascending colon continues to the right upper abdomen, where it turns at the hepatic flexure to form the transverse colon. Distally, the large bowel then extends across the splenic flexure in the left upper abdomen, which marks the beginning of the descending colon. In the left lower abdomen, an S-shape loop runs through the pelvis, and empties into the sigmoid rectum. The rectum is about 15 cm long and terminates at the anus. In adults, the entire length of the colon is about 150 cm (Cummings and Macfarlane, 1991; Macfarlane and Cummings, 1991).

The principal functions of the GI tract include degradation and absorption of food components and water. Degradation mainly takes place in the upper part of the GI tract, whereas the major site of absorption are the (lower) small intestine and the large intestine. Degradation and absorption are enhanced by the excretion of a large number of digestive enzymes, such as glycosidases, lipases, peptidases and proteinases (Cummings and Macfarlane, 1991).

All non-digested food compounds as well as a substantial part of the intestinal secretions will reach the large intestine. In the colon most products are further degraded by the resident intestinal microflora. Many products of bacterial fermentation are absorbed and thus contribute to the nutritional value of the ingested foods (Cummings and Macfarlane, 1991).

The colon receives digesta from the terminal ileum approximately 5h after food ingestion. Thereafter, speed of passage slows progressively from the caecum towards the distal colon. Concurrent with this is an increase in water absorption, thus gut contents in the proximal colon are more or less liquid in nature but have a faeces like appearance distally (Cummings and Macfarlane, 1991).

In Western populations, the average gut transit time is 60h, with a range of 23-168h. The colon itself has a volume of approximately 500ml with about 220g

contents. The average stool size in Western Europe is 106 g/day, with a range of 19-415g. In general, stool weight correlates inversely with transit time. Studies with healthy volunteers have indicated that speeding up colonic transit times from 67 to 25h results in an increase in stool weight from 148 to 285 g/day. Conversely, when transit times are increased, stool weight decreases from 182 to 119 g/day (Cummings and Macfarlane, 1991; Stephen *et al.* 1987). The differences are mainly due to changes in the water content of the faecal mass.

The composition of faeces is highly variable. Bacteria may constitute up to 55% of the total solids, whilst fiber and other non-digested, non fermented compounds represent less than 17% of the weight of which about 24% is soluble material. Fecal water content may be as high as 70% of the total weight (Stephen and Cummings, 1980). Stool size is influenced by both dietary and endogenous factors. Endogenous factors mainly act through hormones on the intestinal motility. Well known are a decreased peristaltic movement during exercise and menstrual cycle. Dietary factors, like non-digestible fibers and polyalcohols (sorbitol), may retain water and thus increase stool weight. High amounts of these factors may cause diarrhea, due to the increased osmotic pressure (Cummings and Macfarlane, 1991; Stephen and Cummings, 1980).

THE GASTROINTESTINAL FLORA

The gastrointestinal flora has been described as one of the most complex bacterial ecosystems known. The whole GI tract is colonized by a large number of bacteria. Each part of the GI tract harbors a specific flora, adapted to the local conditions. In total over 400 species may be present in the GI tract of healthy humans, either as residents or in a transient state (Drasar and Hill, 1974).

Total counts vary between the different regions within the GI tract and are mainly dependent on the oxygen tension, pH and flow of the digesta. Due to the low pH the stomach generally shows the lowest number of live bacteria. The pH in the stomach of pigs and rats is much higher than in humans, this is reflected in the total counts, which are considerably higher in the stomach of these animals (Table 1.1)

The composition of the bacterial population and the relation to carbohydrate metabolism will be discussed in the next chapters.

Table 1.1 : Approximate bacterial counts (logN/g) in different parts of the intestinal tract of different animal species.

	Human	Rat	Swine	Mouse	Rabbit
Oral cavity	7-8	7-8	7-8	7-8	7-8
Stomach	<3	7	6-7	7	4
Jejunum	<3	7	8	7	4
Ileum	5	8	10	8	8
Colon	11	11	11	11	11

(based on Hill, 1995)

The oral cavity

Ingested food material first comes into the oral cavity. The oral cavity is composed of different niches, each with its own microbial population. In the oral cavity bacteria are the main group of microorganisms, but viruses and yeasts can also be found.

The main ecological habitats of the mouth are : 1) the mucosa of lips, cheeks and palate; 2) the tongue; 3) the tooth surface; 4) the saliva and 5) the tonsillar area (Macfarlane and Samaranayaka, 1989). The population of microorganisms is mainly depending on the presence of oxygen and nutrients as well as the flow rate of the saliva. The highest bacterial numbers can be found deep in the periodontal crevasses, the lowest numbers are found in the saliva (Fig. 1.1)

The major species in the oral cavity are lactic acid bacteria of the genera *Streptococcus*, *Lactobacillus* and *Bifidobacterium.* In dental plaque and oral infections, many anaerobic species have been isolated, mainly *Prevotella* and *Porphyromonas* species. Other genera include *Eubacterium*, *Actinomyces* and *Veillonella* (Marsh and Martin, 1992).

The main source of nutrients and energy for oral bacteria is the food. Especially carbohydrates are the main source of energy for the oral microflora. Due to the predominant presence of lactic acid bacteria, carbohydrates are rapidly metabolized to lactic and acetic acids. This results in a rapid drop in the pH of the saliva after ingestion of carbohydrates. Surplus carbohydrates can be incorporated in a large number of bacterial exopolysaccharides, which are used as energy storage compounds, and, on the other hand act as attachment factors (Marsh and Keevil, 1986; Marsh and Martin, 1992).

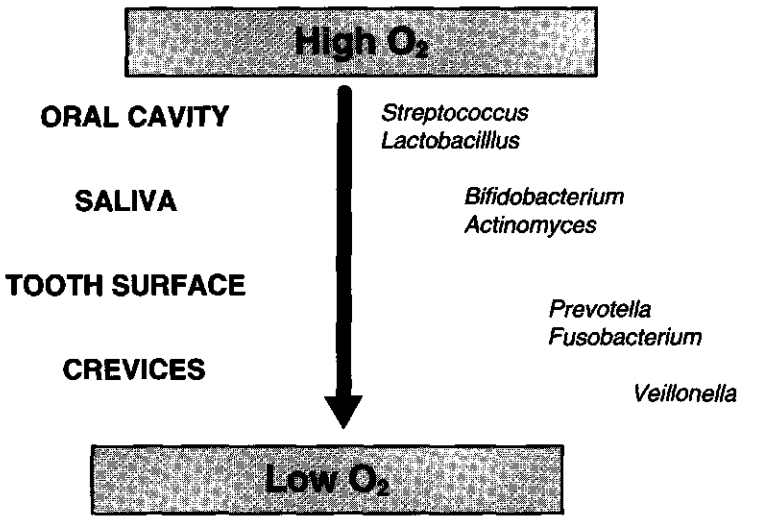


Figure 1.1. Relation between bacterial species, oxygen tension and habitat in the oral cavity.

The main effect of carbohydrate metabolism in the oral cavity related to human health is the process of dental caries (Fig. 1.2). Dental caries is the disease in which the upper protective layer of the teeth is degraded. Thereby small lesions appear, which may be the start of a larger cavity and, finally, dental infection. If not cured, the decayed tooth has to be removed. The process starts when the dental enamel (the upper layer of the tooth) starts to dissolve. The enamel starts to dissolve when the pH reaches a value of 5.5 or lower. Lactic acid is especially effective in dissolving the dental enamel. Formation of lactic and acetic acids from dietary carbohydrates thus is the main factor in the process of caries formation. The second main factor is the formation and presence of dental plaque. Dental plaque is the combination of bacteria (living and dead) and food components glued to the dental surface by exopolysaccharides produced by the different bacteria. Fermentation of carbohydrates in the dental plaque enhances caries formation as 1) the acid is produced directly on the dental surface, and 2) the neutralizing effect of the saliva is minimized, due to the protective layer of plaque (Marsh and Martin, 1992).

Several bacterial species in the oral cavity are considered cariogenic. Among these *Streptococcus mutans* is considered the most pathogenic species as it is capable of rapidly fermenting carbohydrates and is present in relatively high numbers. Other

lactic acid bacteria, such as lactobacilli, bifidobacteria and *Actinomyces* species, are also considered cariogenic (Marsh and Martin, 1992).

All fermentable carbohydrates have a potential cariogenic risk, the risk dependent mainly on the fermentation rate and the final pH reached after fermentation. Sucrose, as it is fermented very rapidly by a large number of species, is considered the main cariogenic carbohydrate. Glucose and fructose are considered slightly less cariogenic than sucrose, whereas lactose and galactose are much less cariogenic. Oligosaccharides, being carbohydrates, may thus also have cariogenic properties, especially oligosaccharides consisting of highly cariogenic monosaccharides, such as glucose and fructose.

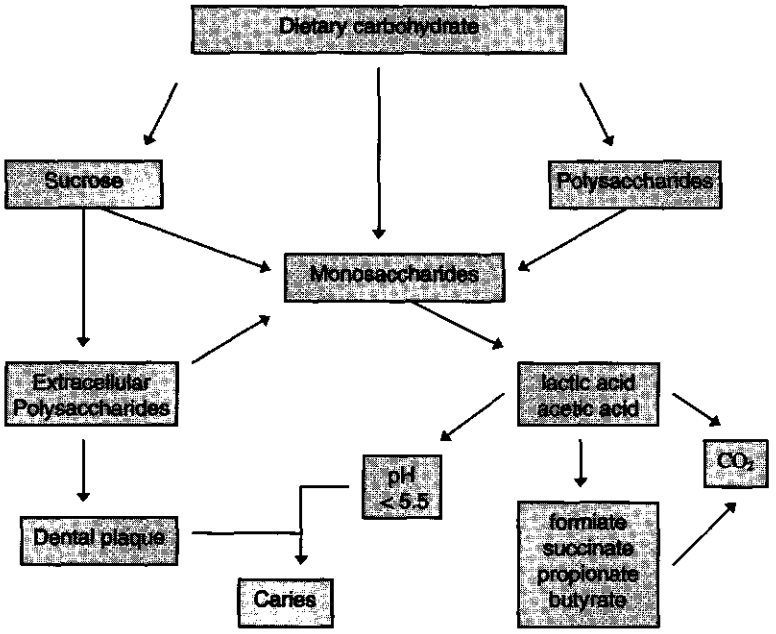


Figure 1.2. Carbohydrate metabolism by the oral flora and factors related to dental caries formation.

The stomach

The human stomach generally has a very low pH, which is the main factor preventing bacterial growth. The normal resting gastric juice pH is below 3.0, which prevents practically all bacterial growth, and which is bactericidal for most transient species. During and shortly after a meal the pH may increase to values around 6.0. This will

allow transient bacteria to survive and enter into the small intestine. The resident flora of the stomach lumen is highly acid tolerant and consists mainly of lactobacilli and streptococci. In the stomach mucosa the pH is much higher and bacterial populations may be higher. In addition to lactobacilli and streptococci, some other bacterial species and yeasts may be present (Hill, 1995a).

Fermentation of ingested carbohydrates in the stomach hardly occurs.

The small intestine

When the partly digested food enters the small intestine, it is mixed with intestinal secretions, such as bile, pancreatic enzymes and bicarbonate. Especially the bile has a strong bactericidal effect. Together with a strong fluid secretion by the intestinal mucosa, this prevents extensive colonization of the small intestine. Colonization mainly takes place in crypts and blind loops. In the lower parts of the small intestine the flow is somewhat reduced, the bile is diluted, the pH becomes more neutral and the oxygen tension drops rapidly. This favors the growth of different bacteria, initially mainly aerotolerant species, and in the ileum also strict anaerobes (Fig. 1.3) (Hill, 1995a; Lee, 1985).

Due to the flow rate and the low bacterial mass there is not much carbohydrate fermentation in the small intestine in healthy humans.

In animals the conditions in the small intestine differ widely. In pigs, where the stomach pH is much higher, the bile secretion is less and the flow rate is lower, there is extensive bacterial growth in the small intestine (Russell, 1979). This also results in extensive fermentation of ingested carbohydrates, including oligosaccharides.

The large intestine

In the large intestine the flow rate of the digesta decreases considerably. In addition to this, the bile is even more diluted and the pH is near to neutral. Together with an abundance of fermentable material, this results in extensive bacterial growth. Total logarithmic counts may reach up to 10^{11} bacteria/gram contents. Higher numbers have been reported, but it is physically impossible to reach numbers over 10^{12} bacteria/gram faecal dry weight, taking into account the average composition of faeces and the dimensions of an average bacterium (Stephen and Cummings, 1980).

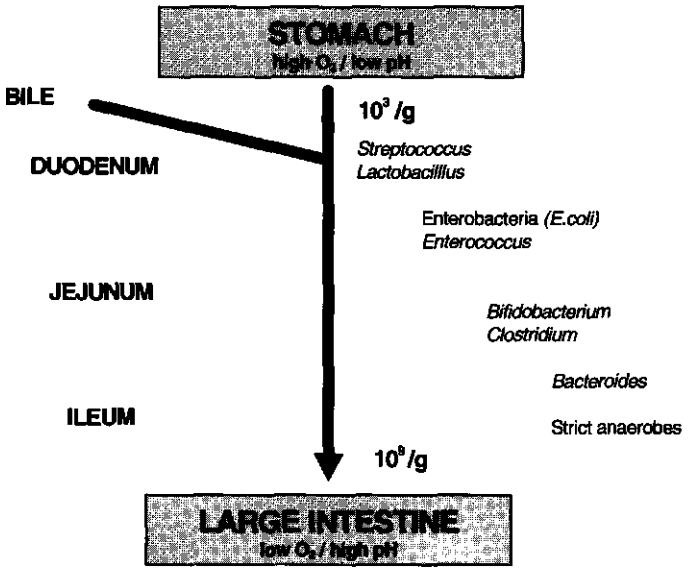


Figure 1.3 Appearance of bacterial species in the small intestine.

It is estimated that over 400 different bacterial species reside in the human large intestine. Of these about 200 have been validly described, but often non-identifiable strains are reported (Hill, 1995a; Holdeman and Moore, 1977; Benno *et al.* 1989). In addition to the resident bacteria, transient bacteria are often isolated (mainly aerobic species). The flora in the large intestine is fairly stable, especially the dominant flora (like *Bacteroides*, *Bifidobacterium*, anaerobic cocci). Large variations occur in the less dominant species, especially among the facultative or aerotolerant species like *E. coli* and lactobacilli (Benno *et al.*, 1989; Finegold *et al.* 1974, Holdeman *et al.*, 1976; Hentges, 1978). The numbers of the dominant species are also comparable in different populations (Fig. 1.4). Differences in counts are more often due to the methodology used, rather than actual differences. As in individuals, the counts of less dominant species differ widely between different populations.

Among the dominant bacterial groups are members of the genera *Bacteroides*, *Bifidobacterium, Coprococcus, Peptostreptococcus, Eubacterium* and *Ruminococcus.* Members of the following genera are often isolated and are present in lower numbers : *Fusobacterium, Streptococcus, Lactobacillus, Enterococcus, Veillonella, Megasphaera, Propionibacterium* and *Enterobacteriaceae.*

This complex microbial ecosystem has an enormous fermenting capacity. Practically all carbohydrates entering the colon can be fermented, either by a single species, or, for complex carbohydrates, by a combined action of more species. The main factors determining whether a specific carbohydrate is fermented and at what rate are the following : composition of the monomeric units, degree of polymerization (DP), degree of branching and solubility. In general, shorter carbohydrates are fermented faster than long chain carbohydrates. Similarly linear chains are fermented faster than branched chains and soluble carbohydrates are fermented faster than insoluble carbohydrates. In the next sections the fermentation of different oligo- and polysaccharides will be described in more detail.

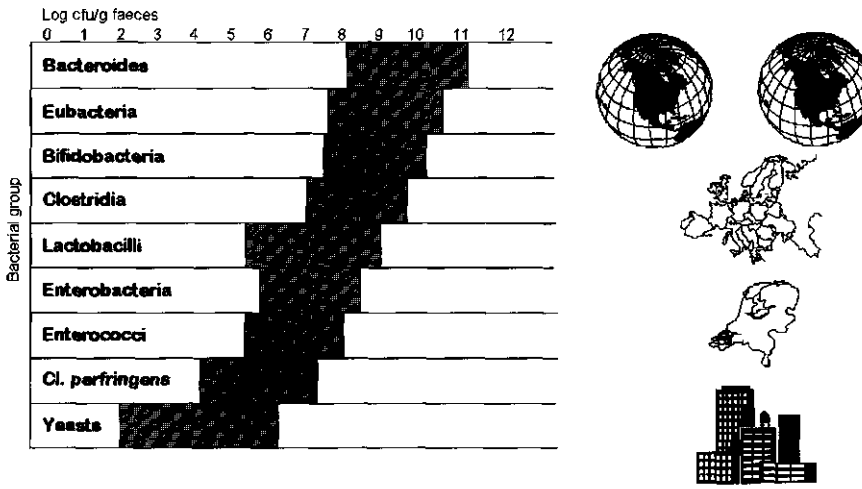


Figure 1.4 Faecal bacterial counts as compared to human population figures. Reported ranges of bacterial groups are shaded.

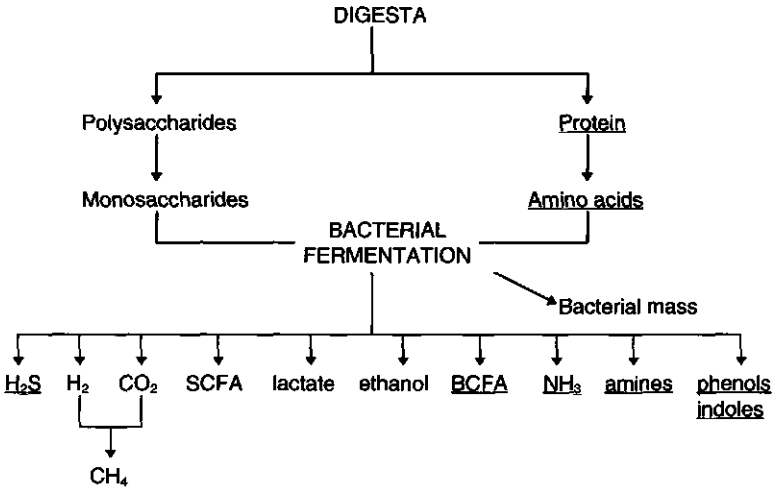
PREBIOTICS

Prebiotics are defined as : *non digestible food ingredients, that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improve host health* (Gibson and Roberfroid, 1995). Most

potential prebiotics are carbohydrates, but the definition does not exclude non carbohydrates to be used as a prebiotic. In theory, any antibiotic that would reduce the number of potentially harmful bacteria and favor health promoting bacteria or activities, can be considered a prebiotic.

This definition does not emphasize a specific bacterial group. Often, however, it is assumed that a prebiotic should increase the number and/or activity of bifidobacteria and lactic acid bacteria, as these groups of bacteria are claimed to have several beneficial effects on the host. A product that stimulates (or claims to stimulate) bifidobacteria is considered a bifidogenic factor. Some prebiotics may thus also act as a bifidogenic factor and vice versa, but the two concepts are not identical.

The definition also states that a change in metabolic activity may result in an improvement of the host health. This means that no specific group of bacteria has to be stimulated, but that the metabolic activity of the intestinal flora as a whole has to be modified. This generally means an increase in carbohydrate fermentation and a decrease in protein degradation and fermentation.



SCFA = Short Chain Fatty Acids, BCFA = Branched Chain Fatty Acids

Figure 1.5 Schematic fermentation of protein and carbohydrates by the intestinal microflora. Protein and fermentation products mainly derived from protein fermentation are underlined.

Carbohydrate fermentation generally results in harmless or even beneficial end products, whereas protein fermentation results in the production of potential harmful

products (Fig.1.5) (Hill, 1995). A change in metabolic activity does not have to correlate with an increase in the numbers of the possible beneficial bacterial groups. The concept of prebiotics thus is clear, but it is not certain that specific groups of micro-organisms, such as the bifidobacteria, are to be stimulated. If the aim is to stimulate bifidobacteria, a non-digestible carbohydrate has to be selectively fermented by bifidobacteria. If the aim is to modify the metabolic patterns, it is necessary to increase general fermentation, thus as many bacteria as possible need to be able to ferment the carbohydrate.

In both cases it is necessary to determine the fermentation by individual strains, in faecal slurries (batch or fermentor) and finally in human studies.

Fermentation by individual strains is often determined using three different methods:

1) Determination of change in pH. This method assumes that fermentation will always result in a drop in pH. However, not all strains produce enough acids to reduce the pH considerably. Secondly, many species metabolize protein at the same time, which results in the formation of alkaline products. These mask a possible drop in pH. This method thus may result in false negative results

2) Determination of change in optical density. This method is more reliable than the first method, but there is a risk for false positive results. Many species may grow on the carbohydrate-free medium, which results in an increase in optical density, without fermentation of the carbohydrate.

3) Determination of the actual carbohydrate content using HPLC. This method clearly shows fermentation, regardless of changes in pH or optical density. Fermentation and/or degradation of each individual compound in a mixture can be determined. No false positives or false negatives will occur, but the method is expensive and time consuming.

The differences in methods may result in different conclusions regarding the fermentability of a certain compound. This should be taken into consideration when interpreting the results in Table 1.3.

OLIGOSACCHARIDES

In the following paragraphs the different oligosaccharides are classified according to structure, not origin or production technique. Oligosaccharides in this classification are defined as carbohydrates or carbohydrate derivatives with a degree of polymerization (DP) of 2-10. Synthetic or natural oligosaccharides that are not generally present in

foods or feed and which have never been tested in studies aimed at fermentation by the intestinal flora (either *in vitro* or *in vivo*) are not included.

Digestible oligosaccharides, like sucrose, lactose, maltose, maltodextrins, gentiobiose and trehalose are not included.

Glucooligosaccharides

Cellobiose and cellodextrins

Cellobiose (CEL, 4-O-ß-D-glucopyranosyl-D-glucose) is a non-digestible disaccharide, which does not normally occur in nature. It can be formed, however, by degradation of cellulose, either industrially or by bacterial hydrolysis in the colon. It is known that the human intestine harbors cellulolytic bacteria. Some of these highly cellulolytic species were identified as *Bacteroides* sp. (Betian *et al.*, 1977). It is not known whether these bacteria actually produce cellobiose but, if so, it may be used as a carbon source by other bacteria. Cellobiose is often used in determining carbohydrate fermentation patterns in the identification of bacteria. As many intestinal species are capable of fermenting cellobiose (Table 1.3), it is not unlikely that cellobiose may have an effect on either the composition or the metabolic activity of the intestinal microflora. So far there have not been any studies to determine such an effect.

Cellodextrins ($(4\text{-O-ß-D-glucopyranosyl})_n$-D-glucose), like cellobiose, can be formed by hydrolysis of cellulose (Russell, 1985). The degree of polymerization ranges from n=3 (cellotriose) to n=6 (cellohexaose). No data are available on the fermentation of cellodextrins by intestinal microorganisms. It is, however, to be expected that cellulose degrading bacteria will also be able to degrade and ferment cellodextrins. Cellodextrins have as yet not been tested in human or animal studies as possible prebiotics.

Cyclodextrins

Cyclodextrins are cyclic molecules composed of α-1-4 linked D-glucose residues, α-cyclodextrin being a hexamer, ß-cyclodextrin a heptamer and γ-cyclodextrin an octamer (Suzuki and Sato, 1985).

So far cyclodextrins have not been proposed as non-digestible carbohydrates with functional food properties, but they are increasingly used in food and non-food products. Cyclodextrins readily form inclusion complexes with a number of chemicals. This complex formation has resulted in a large number of patents that propose the use

of cyclodextrins as nutritionally inert stabilizers in various food and pharmaceutical products (Antenucci and Palmer, 1984).

α- And ß-cyclodextrins are quite resistant against human α- and ß-amylases and will thus reach the colon unaltered. γ-Cyclodextrin, however, is readily degraded by α-amylase *in vitro* (Szejtli and Sebestyén, 1979; Suzuki and Sato, 1985; Antenucci and Palmer, 1984).

In a study with ß-cyclodextrin it was found that 91% of the ingested dose could be recovered from the effluent of ileostomy patients, but not from stools (Flourié *et al.*, 1993). Studies with labeled cyclodextrins have shown that ß-cyclodextrin is degraded rapidly by the intestinal flora (Szejtli *et al.*, 1980). α-Cyclodextrin on the other hand is degraded very slowly if at all (Suzuki and Sato, 1985).

Fermentation by individual strains has not been studied extensively. It has been shown that ß-cyclodextrin can be fermented by several human faecal bacteria, especially the *B. fragilis* group, *Lb. acidophilus* and three species of clostridia, among them *Cl. perfringens*. The 5 species of bifidobacteria tested were not able to ferment this oligosaccharide. Neither did 22 other species, among them several lactobacilli and enterobacteria (Table 1.3)(Mitsuoka *et al.*, 1987, Antenucci and Palmer, 1984).

Degradation of α- or γ-cyclodextrin by intestinal bacteria has not been studied in detail.

It can thus be concluded that α- and ß-cyclodextrins are non-digestible and, due to their fermentation, may have an effect on the composition or activity of the intestinal flora. No studies have been published yet on the influence of cyclodextrins on the intestinal flora of humans or animals.

Gentiooligosaccharides

Gentiooligosaccharides (GeOS) are a mixture of ß-1-6 linked D-glucose oligomers with a DP of 2-5. They are produced in Japan by transglucosylation and the commercial preparation is marketed as Gentose.

GeOS are not or partly hydrolyzed in the small intestine and will be fermented in the colon. The only available study shows fermentation by a large number of bacteria (Table 1.3), among these bifidobacteria and *Bacteroides spp.* (Nakakuki *et al.*, 1990).

It is also claimed that addition of GeOS will increase the number of bifidobacteria and decrease the protein fermentation, resulting in lower concentrations of putrefactive products (Nakakuki *et al.*, 1990; Playne and Crittenden, 1996).

As data are scarce it can not be concluded whether GeOS have a significant effect on the intestinal microflora or its metabolism.

Glucan hydrolysates

Glucan oligosaccharides are obtained after enzymatic hydrolysis of oat ß-glucan using a commercial enzyme mixture. Several lactic acid bacteria were screened for their hydrolyzing activity of the resulting oligosaccharide mixture. Only a single strain of *Lb. casei* was able to ferment the oligosaccharides. Three bifidobacteria and two other lactobacilli did not degrade the mixture (Jaskari *et al.*, 1994).

Glucooligosaccharides

Glucooligosaccharides (αGOS) are a mixture of α-D-glucose, with oligomers with a DP of 2-6. They are produced by transglucosidation using an α-glucosidase from *Leuconostoc mesenteroides*, and marketed in France by BioEurope (Djouzi *et al.*, 1995).

Glucooligosaccharides can partly be digested by the small intestinal enzymes in rats (Valette *et al.*, 1993). A substantial part of the mixture will thus reach the large intestine. Data on fermentation by individual strains are limited, but most *Bacteroides* species, as well as some bifidobacteria and clostridia are capable of fermenting these oligosaccharides. Most lactobacilli could not ferment αGOS (Djouzi *et al.*, 1995).

Some animal studies have shown that αGOS may influence both the metabolism and the composition of the intestinal microflora (Djouzi *et al.*, 1995; Gidenne, 1995; Monsan, 1997). As yet, data from studies with human volunteers are not available.

Isomaltooligosaccharides

Isomaltooligosaccharides consist mainly of linear α-1-6 linked glucose residues, some 1-4 linkages may also occur. Examples are isomaltose (IMA, 6-O-α-D-glucopyranosyl-D-glucose), isomaltotriose (IMT, 6-O-α-D-glucopyranosyl-(1-6)-α-D-glucopyranosyl-D-glucose) and panose (PAN, 6-O-α-D-glucopyranosyl-(1-6)-α-D-glucopyranosyl-(1-4)-D-glucose).

Isomaltooligosaccharides occur naturally in fermented foods as miso, soy sauce and sake as well as in honey (Kohmoto *et al.*, 1992). Commercial isomaltooligosaccharides are prepared from starch by the combined action of α-amylase, pullulanase and α-glucosidase. The resulting mixture is commercially available as Isomalto-900 in Japan (Kohmoto *et al.*, 1988).

Isomaltooligosaccharides can be degraded by oral bacteria, among these *St. mutans*. Fermentation rates, however, are fairly low, reducing the risk of caries formation. Studies using rats have shown that these oligosaccharides are low cariogenic *in vivo* (Tsunehiro *et al.*, 1997).

Some isomaltooligosaccharides are degraded to some extent by intestinal enzymes, especially isomaltase (Dahlquist *et al.*, 1963). Degradation was further confirmed in a study with ^{13}C labeled isomaltooligosaccharides, where some of the labeled carbon could be recovered from the breath of the volunteers. Similarly, a rise in blood glucose was observed, indicating absorption of free glucose and thus intestinal degradation (Kohmoto *et al.*, 1992).

The fraction of isomaltooligosacchrides reaching the colon, will be fermented by the intestinal flora. A large number of intestinal bacteria is capable of fermenting isomaltooligosaccharides, especially the *Bacteroides* and *Bifidobacterium* species (Table 1.3)(Kohmoto *et al.*, 1988).

Trials with human volunteers have shown that ingestion of isomaltooligosaccharides results in a change in the composition of the intestinal flora, with an increase in bifidobacteria in the faeces. Bifidobacterial numbers increased both in healthy adults and in hospitalized elderly people. (Table 1.2). No significant changes were observed in other bacterial groups (Kohmoto *et al.*, 1988).

Several other trials with human volunteers by the same research group have also shown selective increases of the number of bifidobacteria in faeces. Effects were already observed at a daily intake of 8.2 g/day for the commercial mixture (Kohmoto *et al.*, 1991), 10 g/day for the fraction with DP=2, and 5 g for the DP=3 fraction (Kaneko *et al.*, 1994), which may be partly due to digestion of the DP=2 fraction in the small intestine.

However, studies using Wistar rats did not show a significant increase in bifidobacteria for 12 months. In the same study only an significant increase in lactobacilli could be detected after 3 months. This increase was not detected after 6 or 12 months. No significant changes could be detected in any other bacterial group (Kaneko *et al.*, 1990).

It can thus be concluded that isomaltooligosaccharides are partly non-digestible and potentially have an effect on the intestinal flora. Data are, however, limited to some (poorly documented) Japanese studies. The effects were not observed in rats, but this may be due to the large differences in the faecal flora between man and rats, as well as the differences in experimental set-up (time, dose and diet were all markedly different).

Table 1.2: Effect of 13.5 g/day isomaltooligosaccharides on human intestinal flora.
A: healthy adults, B: hospitalized elderly patients

A: healthy adults

Bacterial group	day -2	day 0	day 7	day 10
Total anaerobes	10.4 (0.1)	10.4 (0.2)	**10.6 (0.2)**	**10.6 (0.1)**
bacteroides	10.1 (0.1)	10.2 (0.3)	10.3 (0.3)	10.2 (0.2)
bifidobacteria	9.5 (0.3)	9.4 (0.7)	**10.0 (0.1)**	**10.0 (0.3)**
enterobacteria	7.4 (0.8)	7.4 (1.1)	7.5 (0.8)	7.0 (0.9)

B: hospitalized elderly patients

Bacterial group	day -2	day 0	day 7	day 10
Total anaerobes	9.8 (0.3)	9.9 (0.3)	9.9 (0.3)	10.1 (0.3)
bacteroides	9.6 (0.3)	9.6 (0.4)	9.7 (0.3)	9.8 (0.4)
bifidobacteria	8.3 (0.6)	8.3 (0.6)	8.8 (0.5)	9.2 (0.5)
enterobacteria	7.4 (1.9)	7.1 (0.7)	7.3 (1.3)	7.0 (1.3)

Only those groups which all volunteers harbored are shown. Data in bold differ significantly ($P<0.05$) from day=0.

(data from Kohmoto *et al.*, 1988)

Leucrose

Leucrose (5-O-α-D-glucopyranosyl-D-fructopyranoside), is a sucrose isomer, which is prepared by enzymatic transglucosylation. It has been found to be a by-product of microbial dextran production by *Leuconostoc mesenteroides* (Stodola *et al.*, 1956) and may thus naturally be present in foods fermented by this bacterial species. Leucrose can be used as a sugar substitute, but at present there are no products available with added leucrose.

Leucrose was found to be non- or very low cariogenic *in vivo* and *in vitro* (Ziesenitz *et al.*, 1989a).

Leucrose is largely digested by the human small intestinal enzymes, although much slower than maltose (31%) or sucrose (63%). It is therefore likely that some leucrose will reach the colon (Ziesenitz *et al.*, 1989b). There are no data available on the

fermentation of leucrose by intestinal bacteria or its effect on the composition or activity of the intestinal flora.

Leucritol

Leucritol can be prepared from leucrose by catalytic hydrogenation. The resulting mixture consists of approx. 60% glucosyl-sorbitol and 40% of glucosyl-mannitol. Leucritol is not present in natural products and was developed as a sugar substitute. It is not used at present in any foods.

Leucritol is hydrolyzed to some extent by the human small intestinal enzymes, with relative hydrolysation rates of 3 and 7% as compared to maltose and sucrose (Ziesenitz *et al.*, 1989b). It is thus likely that a substantial amount of leucritol will reach the colon. There are no data available on the fermentation of leucritol by intestinal bacteria or its effect on the composition or activity of the intestinal flora.

Maltitol

Maltitol (MAT, 6-O-α-D-glucopyranosyl-D-sorbitol) is a disaccharide alcohol which is partially digestible in the small intestine. Maltitol is widely used as a non-cariogenic sweetener and sugar substitute. Maltitol is as yet not used as a possible prebiotic.

It has been estimated that about 50% of the ingested dose reaches the caecum (Nilsson and Jägerstad, 1987, Würsch *et al.*, 1989). Undigested maltitol is fermented rapidly by the intestinal flora, as could be concluded from hydrogen production (Würsch *et al.*, 1989) and absence of maltitol in faeces (Beaugerie *et al.*, 1990).

Fermentation of maltitol by individual strains of intestinal bacteria has not been studied extensively (Table 1.3). The available data show that maltitol can be fermented by species of several genera. As fermentation occurs, it is possible that maltitol has an effect on the composition or activity of the intestinal flora, but this has not yet been tested in trials using human volunteers.

Palatinose

Palatinose (PAL, 6-O-α-D-glucopyranosyl-D-fructose), is a sucrose isomer. It has also been referred to as isomaltulose (Takazoe, 1989) or isomalturose (Mitsuoka *et al.*, 1987).

Palatinose has been discovered in 1957 in the beet sugar manufacturing process. It has also been detected in honey and cane juice (Takazoe, 1989). In Japan palatinose is produced on a large scale since 1984 by the α-glucosidase reaction by *Protaminobacter rubrum* (Takazoe, 1989 Fuji and Komoto, 1991). At present palatinose is

used in Japan as a sweetener, although its sweetness is only 42% as compared to sucrose.

Palatinose is partially digestible by isomaltase at a rate of about 25-30% of that of isomaltose (Kaga and Mizutani, 1985, Nilsson and Jägerstad, 1987, Tewari and Goldberg, 1991). Some palatinose may therefore reach the colon, where it can be fermented by a large number of intestinal bacteria (Table 1.3). Details on fermentation rate, short chain acid formation and gas production are not available (Mizutani, 1989).

In a single experiment with 6 healthy human volunteers, no statistical differences were observed in the faecal flora composition after ingestion of palatinose for 10 days (Mizutani, 1989). This single study is too limited to conclude that palatinose does not have an effect on the composition or activity of the intestinal microflora.

Palatinose condensate

Palatinose condensate (PCO), which is formed by heating palatinose under suitable conditions, consists of a mixture of oligosaccharides (tri- to octamers) of glucose and fructose. The exact composition of this melt is not known (Mizutani, 1989).

It is not known whether all components in the condensate are non-digestible, but studies with human volunteers have shown effects on bacterial composition. It is thus likely that a substantial part of the mixture consists of non-digestible compounds.

Fermentation of palatinose condensate has been tested with a limited number of bacterial species. It was observed that bifidobacteria and *Bacteroides* species were capable of fermenting the condensate to a large extent (Mizutani, 1989).

Several studies have shown that palatinose condensate intake has an effect on the intestinal flora in healthy Japanese adults. An increase in bifidobacteria was observed, whereas all other groups remained unaffected during the test period (Kashimura *et al.*, 1989; Mizutani 1989; Mizutani, 1989b). No significant changes in faecal pH and water content were observed during the test period, although the pH dropped from 6.3 to 6.0 with a dose of 24 g/day (Kashimura *et al.*, 1989).

A dose-response relation was observed between ingestion of palatinose condensate and percentage of bifidobacteria in the faeces. Without palatinose the percentage of bifidobacteria was around 6 %, with 2.4 g palatinose condensate per day this percentage increased to 10%, 4.9 g/day showed 12% bifidobacteria, whereas 10.8 g/day increased this percentage to 22% (Mizutani, 1989b).

It can be concluded that PCO may have a potential effect on the composition and the activity of the intestinal microflora. More studies are needed to prove this effect.

Palatinit

Palatinit is an equimolar mixture of 6-O-α-D-glucopyranoside-D-mannitol and 6-O-α-D-glucopyranoside-D-sorbitol (maltitol). It is prepared from palatinose by reduction of palatinose (Nilsson and Jägerstad, 1987).

Palatinit is degraded slowly by human small intestinal enzymes. This indicates that a substantial part of the ingested palatinit will reach the colon unaltered (Nilsson and Jägerstad, 1987).

Palatinit is fermented rapidly in the caecum, as can be judged from hydrogen production. Hydrogen production was measurable 1.5 h after digestion of 19 g palatinit. This value is similar to that for other polyols and lactulose (Würsch *et al.*, 1989).

Stool weight and stool frequency did not change significantly after ingestion of palatinit (Fritz *et al.*, 1985). Data on fermentation by individual strains and effects on composition or activity of the intestinal flora, however, are not available.

Theanderose

Theanderose (O-α-D-glucopyranosyl-(1-6)-O-α-D-glucopyranosyl-(1-2)-ß-D-fructofuranoside) is a naturally occurring sugar, present in honey and commercially produced using the fungus *Mucor javanicus* (Shimokawa *et al.*, 1995).

It is likely that theanderose is at least partly indigestible, as in a test using human volunteers, it was observed that the number of bifidobacteria increased and the concentrations of putrefactive products (protein degradation products) was decreased (Shimokawa *et al.*, 1995).

Galactooligosaccharides

Galactooligosaccharides are defined as oligosaccharides whose main component is galactose.

Digestible galactooligosaccharides, like lactose and melibiose are not included.

Lactitol

Lactitol (4-O-ß-D-galactopyranosyl-D-sorbitol) is a disaccharide alcohol, produced by hydrogenation of lactose (Booy, 1986). Lactitol is not present in nature (Harju, 1988). Lactitol is marketed as a non-caloric, non-cariogenic sweetener, and as a relieve agent against intestinal disorders, such as hepatic encephalopathy (Harju, 1988).

Lactitol is not, or very slowly fermented by oral bacteria and does not promote plaque formation (Booy, 1986, chapter 3). It thus does not form a risk for caries formation.

Neither is lactitol is hydrolyzed by human intestinal enzymes and thus reaches the caecum unaltered (Nilsson and Jägerstad, 1987, Patil *et al.*, 1987). Lactitol, like other sugar alcohols may have a laxating effect. The laxative threshold, however, is around 75 g/day with no symptoms occurring below 40 g/day (Patil *et al.*, 1987).

In the colon, lactitol is rapidly fermented. Fermentation of lactitol results in the formation of relatively large amounts of hydrogen, higher than that of other polyols or lactulose. Hydrogen production from lactitol was also more rapid as compared to other sugaralcohols, about 1.2 h after digestion (Würsch *et al.*, 1990, Es, van 1986, Piva *et al.*, 1996). Fermentation of lactitol by individual bacterial strains has been studied by several research groups. It can be concluded that lactitol can be fermented by a number of bacterial species, from different genera, among these gas producing genera, such as clostridia and enterobacteria (Kitler *et al.*, 1992).

Addition of lactitol to the diet of human volunteers with low initial bifidobacterial counts resulted in an increase in the number of bifidobacteria as well as a reduction of the putrefactive products (Ballongue *et al.*, 1997).

The results indicate that lactitol is fermented rapidly and may thus have an effect on intestinal metabolism. As there are no studies with healthy volunteers available, it can not be concluded that lactitol has an influence on the composition of the intestinal microflora.

Lactosucrose

Lactosucrose (O-ß-D-galactopyranosyl-(1-4)-O-a-D-glucopyranosyl-(1-2)-ß-D-fructofuranoside) or 4^G-ß-D-galactosylsucrose, a raffinose isomer, is produced in Japan by action of a fructofuranosidase from *Arthrobacter sp.* on a mixture of lactose and sucrose (Terada *et al.*, 1992b). It has, as yet, not been detected in any natural food product.

Lactosucrose is not digested by oral or intestinal enzymes to a large extent. Only 5% hydrolysis was observed after incubation with rat intestinal acetone powder (Fujita *et al.*, 1991). Non-digestibility and fermentability of lactosucrose was confirmed by the rapid formation of hydrogen; increases in breath hydrogen could be observed 1-3 h after ingestion. Hydrogen production was similar as for maltitol or FOS (Fujita *et al.*, 1991).

Lactosucrose can be fermented by a number of intestinal bacteria, especially bifidobacteria, bacteroides and clostridia (Fujita *et al.*, 1991) (Table 1.3).

Several Japanese studies have shown that lactosucrose intake in human volunteers may increase the number of bifidobacteria in the stools. Fujita *et al.* (1991)

used a preparation with only 55% lactosucrose and showed an increase of bifidobacteria in both subjects tested.

Yoneyama *et al.* (1992) used a more purified preparation in 6 volunteers. In all three doses tested (2.0; 5.0 and 10.0 g/day) a significant increase in bifidobacteria was observed. No significant changes were observed in other bacterial groups, faecal pH, SCFA, faecal dry weight or faecal wet weight.

Ogata *et al.* (1993) used a similar study design with 8 volunteers and doses of 1.0; 2.0 and 3.0 g/day. They observed an increase in bifidobacteria with all three concentrations used, as well as a decrease in *Cl. perfringens* and faecal ammonia. No effects could be observed on other bacterial groups, faecal wet or dry weight. Fecal pH had a tendency to decrease, but only at the higher dose. Fecal sulfide concentrations, however, increased.

Similar results have been obtained by Ohkusa *et al.* (1995) in healthy volunteers, in several patient groups (Teramoto *et al.*, 1996) and with animals, like cats (Terada *et al.*, 1993), dogs (Terada *et al.*, 1992b) and chickens (Terada *et al.*, 1994).

Even though the studies were carried out on a limited number of volunteers, the data suggest that lactosucrose may have an effect on either the composition or the metabolism of the intestinal microflora.

Table 1.3 : Fermentation of non-digestible oligosaccharides by intestinal bacteria.

Species	CEL *	MAT	β-CYC	PAL	PCO	GeOS	IMA	IMT	PAN	LOL	LAT	RAF	STC	TOS	LAS	LTOS	GLL	FOS	NEP
Ac. naeslundii	+**											+							
B. distasonis	+	+	+	+	+	+	+	+	+	+	+	v	-	+	+	+	+	+	v
B. eggerthii	-											-							
B. fragilis	-	-	+	-	-	+	+	+	+	v	+	+	-	+	+	+	+	+	v
B. ovatus	+	+	+			+	+	+	+	-	+	v		+			-	+	v
B. thetaiotaomicron	+	+	+	+	-	+	+	+	+	v	+	+		+	+		+	+	+
B. uniformis	+					+				-	+	v	-	+	+		v	v	v
B. vulgatus	-	-	+	-	-	+	+	+	+	-	+	+	-	+	+	+	+	v	v
Bi. adolescentis	+	v	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Bi. bifidum	-	-	-	-	-	+	-	-	-	-	+	-	-	+	v	+	+	-	-
Bi. breve	+	+	-	+	+	+	+	+	+	v	+	+	+	+	+	+	+	+	v
Bi. catenulatum	+											+						+	
Bi. dentium	+											+						+	
Bi. infantis	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Bi. longum	+	-	-	+	+	+	+	+	+	v	+	-	-	+	+	+	+	+	+
Bi. pseudocatenulatum											+	+			+			+	+
Cl. beijerinckii	+											+							
Cl. bifermentans	-			-	-		-	-	-	-	v	v		-	v	-	-	-	
Cl. butyricum	+	+	+	+	-	+	-	-	-	v	+	+	+	-	+	+	-	+	+
Cl. cadaveris	-					-					v	-	-						-
Cl. clostridioforme	+	-	-		-	-	-	-	-	v	v	v	-	+		+	-	v	v
Cl. coccoides	+			+	-		+	-	-	-	+	+			+		-		
Cl. difficile	-	-	-	-	-	-	-	-	-	-	-	-	-	+			-	-	-
Cl. glycolicum	-									-	+	-							
Cl. innocuum	+			+	-	-				-	v	-	-	+	-		v	+	-
Cl. novyi	-					-						-						-	
Cl. paraputrificium	+	-	+	-	-	+	-	-	-	v	+	-		-	-	-		v	-
Cl. perfringens	+/-	v	+	-	-	-	+	+	+	v	+	v	-	v	+	+	v	v	-
Cl. pseudotetanicum	+											+							
Cl. ramosum	+	+	-	-	+	+	+	+	+	+	+	+	-		+	+	-	+	+
Cl. sartagoforme	+											+							
Cl. septicum	+					-						-						-	-
Cl. sordelli	-					-						-	-	-				-	-

Table 1.3 (cont.) : Fermentation of non-digestible oligosaccharides by intestinal bacteria.

Species	CEL *	MAT	β-CYC	PAL	PCO	GeOS	IMA	IMT	PAN	LOL	LAT	RAF	STC	TOS	LAS	LTOS	GLL	FOS	NEP
Cl. sporogenes	-					-					+	-	-	-				-	v
Cl. tertium	+					+						-						-	
Ec. faecalis	+	-	+	-	-	+	+	+	+	-	+	v	-	v	v	-	-	v	-
Ec. faecium	+	-	+			+				+	+	v	-	v			v	+	-
Ec. hirae	+											-							
Ec. mundtii	+											-							
E. coli	-	-	-	-	-	-	-	-	-	-	v	v	-	+	-	-	-	-	-
Eu. aerofaciens	+	-	-	-	-	-	+	-	-	-	+	-	-	v	v		-	+	v
Eu. limosum	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-		-	-	-
Eu. nitrinogenes	+			-	-	-					-	-	-		-			-	-
Eu. tortuosum	+			+	-						+	+							
F. mortiferum	+					-						v						-	
Kl. pneumoniae	+	+	-			+	+	+	-	+	+	+	+	+			+	v	v
Lb. acidophilus	+	-	+	+	-	+	+	-	-	v	+	-	-	v	+	-	+	+	v
Lb. casei ssp casei	+	+	-	+	-	+	-	-	-	+	+	-		+	v	-	-	+	-
Lb. fermentum	-	-	-			-				-	+	+		+				-	-
Lb. gasseri	+					+				+	+	-	-	+	-		-	+	v
Lb. reuteri	-											+		+			+		
Lb. salivarius ssp salivarius	-	-	-	-	-	-	-	-	-	+	+	+	+	+	v		-		+
Megamonas hypermegas	+	+	-	+	-	+					v	+	+		-		+	+	v
Megasphaera elsdenii	-	-	-	-	-						-	-	-	-	-		-	-	-
Mitsuokella multiacidus	+	-	-	+	-	+	+	+	+	+	+	+	+	+	-		-	+	v
P. anaerobius	-			+	-		-	-	-		+	-	-				-		
P. parvulus	-	-	-	-	-		-	-	-		v	-					-		
Pro. acnes	-	-	-	-	-	-	-	-	-	-	v	-		-			-	+	-
Pro. granulosum	-					-						v	-					-	
Pro. jensenii	+											+							
Rikenella microfusus	-						-	-	-		-	-			+		-		+
R. albus	+											-							
R. productus	+			-	-	+	-	-	-	+	+	+	-	+			v		-
St. mutans												+						+	

Legend to Table 1.3.

* Cel = cellobiose, MAT = maltitol, ß-CYC = ß-cyclodextrin, PAL = palatinose, PCO = palatinose condensate, IMA = isomaltose, IMT = isomaltotriose, PAN = panose, GeOS = gentiooligosaccharides, LOL = lactitol, LAT = lactulose, RAF = raffinose, STC = stachyose, TOS = transgalactosyl oligosaccharides, LAS = lactosucrose, LTOS = lactitol oligosaccharides, GLL = galactosyllactose, FOS = fructooligosaccharides, NEP = Neosugar P (a commercial FOS preparation).

** Data are compiled from a number of studies. Bacteria known to be unable to ferment any carbohydrate, but which have been tested, are excluded from the table. This includes the following species : *Cl. indolis*, *Eu. lentum*, *Eu. moniliforme*, *F. necrophorum*, *F. nucleatum*, *F. varium*, *P. magnus*, *P. micros*, *P. prevotii* and *Veilonella* sp.

+=positive fermentation in one or more studies, v=variable results in a single study, or different results between studies, -= no fermentation observed in any of the studies.

Results from studies using different methodologies are compared. Studies used for the table (method of detection between brackets, pH = pH changes, OD = optical density at 630 or 660 nm) :

CEL : Holdeman and Moore, 1977 (GLC), **CYC** : Mitsuoka *et al.*, 1987 (OD_{660}); Antenucci and Palmer, 1984 (pH/gas), **PAL/PCO** : Mizutani, 1989 (pH), **IMA/IMT/PAN** : Kohmoto *et al.*, 1988 (OD_{660}), **GeOS** : Nakakuki *et al.*, 1990 (pH), **LOL** : Kitler *et al.*, 1992 (pH); Yanahira *et al.*, 1995 (OD_{650}), Watanabe *et al.*, 1995 (pH), **LAT** : Kitler *et al.*, 1992 (pH); Ohtsuka *et al.*, 1989 (OD_{660}); Mizutani, 1989 (pH); Sahota *et al.*, 1982 (TLC analysis) ; Kawaguchi *et al.*, 1993 (gas formation), Hara *et al.*, 1994 (pH); Watanabe *et al.*, 1995 (pH), **RAF** Masai *et al.*, 1987 (OD_{630}), Mizutani, 1989 (pH); Ohtsuka *et al.*, 1989 (OD_{660}), Holdeman and Moore, 1977 (GLC); Hara *et al.*, 1994 (pH), : Masai *et al.*, 1987 (OD_{630}), **GLL :** Ohtsuka *et al.*, 1989 (OD_{660}); Ishikawa *et al.*, 1995 (pH), **TOS** : Tanaka *et al.*; 1983 (pH); Ishikawa *et al.*, 1995 (pH), **LAS** Fujita *et al.*, 1991 (OD_{660}); Hara *et al.*, 1994 (pH), **LTOS** : Yanahira *et al.*, 1995 (OD_{660}), **FOS** : Asano *et al.*, 1994 (pH), Hayakawa *et al.*, 1991 (OD_{660}), Wada (pH); Asano *et al.*, 1994 (OD_{660}), **NEP** : Mitsuoka *et al.*, 1987 (OD_{660}); Hara *et al.*, 1994 (pH); Kawaguchi *et al.*, 1993 (gas formation); Nakakuki *et al.*, 1990 (pH)

Lactulose

Lactulose (4-O-ß-D-galactopyranosyl-D-fructose) is prepared by enzymatic or chemical isomerisation of lactose. Unlike lactose, lactulose is not hydrolyzed by human intestinal ß-galactosidase and reaches the colon unaltered (Dahlquist and Gryboski, 1965).

Lactulose is mainly marketed as a laxative and, recently, in the Netherlands in a product to promote intestinal health. Furthermore, lactulose is being used as a treatment in patients with hepatic encephalopathy and related intestinal disorders (Tamura *et al.*, 1993; Terada *et al.*, 1992a; Uribe-Esquivel, 1997).

In the caecum lactulose is fermented rapidly, as can be concluded from hydrogen production. Lactulose is often used a positive marker in carbohydrate intolerance studies, as ingestion results in large amounts of hydrogen (Peled and Gilat, 1979; Brighenti *et al.*, 1995; Kono and Nakae, 1996; Florent *et al.*, 1985; Würsch *et al.*, 1989; Kitler *et al.*, 1992; Sahota *et al.*, 1982).

It can be concluded from a number of studies (Kitler *et al.*, 1992; Ohtsuka *et al.*, 1989; Mizutani, 1989; Sahota *et al.*, 1982; Kawaguchi *et al.*, 1993; Hara *et al.*, 1994; Watanabe *et al.*, 1995) that lactulose is easily fermented by the intestinal flora. It is fermented by all bifidobacteria, bacteroides and lactobacilli tested (Table 1.3), as well as many other bacteria.

Gas production is relatively large on lactulose. The gas production is mainly due to fermentation by clostridia (*Cl. butyricum, Cl. paraputrificium, Cl. perfringens, Cl. sporogenes* and *Cl. clostridioforme*), as well as *Kl. pneumoniae* (Kawaguchi *et al.*, 1993).

Fermentation of lactulose in *in vitro* cultures by different species results in different end-products, mainly acetate and lactate. It was observed that a relatively large concentration of butyrate was produced by the faecal inoculum (Sahota *et al.*, 1982). The combination of the acids indicates a fermentation in which different bacterial groups are involved.

The effect of lactulose on the composition of the intestinal flora was determined using 8 healthy volunteers and 3 g/d of lactulose. Bacterial counts revealed an increase of bifidobacteria, whereas the number of bacteroides and clostridia decreased. All other groups tested remained constant. Similarly indole, skatole, phenols, faecal pH, ß-glucuronidase, nitroreductase and azoreductase activities decreased. Ammonia remained constant and water content increased (Terada *et al.*, 1992a). In another study addition of lactulose to the diet of human volunteers with low initial bifidobacterial counts resulted in an increase in the number of bifidobacteria as well as a reduction of the putrefactive products (Ballongue *et al.*, 1997).

Even though the studies are very limited, the results are an indication that lactulose may have an effect on both the bacterial composition and the metabolic activity of the intestinal flora.

Raffinose, stachyose and other soybean oligosaccharides

The raffinose series of oligosaccharides form a class of mixed oligosaccharides. Raffinose (RAF, O-α-D-galactopyranosyl-(1-6)-α-D-glucopyranosyl-ß-D-fructofuranoside), the main oligosaccharide in this group, is a trimer of fructose, galactose and glucose. Higher oligosaccharides of this group have additional galactose residues; stachyose (STC) has one and verbascose has two.

As this group of oligosaccharides occur in high concentrations in nature, especially in legumes, they have been studied already for a long time. They play a major role in the flatulence problem which occurs after eating too much legumes (Christofaro *et al.*; 1974).

Raffinose and its analogs are not degraded by the human digestive enzymes, and reach the colon unaltered. However, a small percentage of the oligosaccharides may be absorbed through the intestinal wall and are excreted with the urine (Christofaro *et al.*; 1974, Kato *et al.*, 1991).

In the colon these oligosaccharides are fermented rapidly by the intestinal flora, as can be judged by the formation of large amounts of hydrogen and increased flatulence, especially by *Cl. perfringens* and some *Bacteroides* species (Myhara *et al.*, 1988; Hayakawa, 1990; Kono and Nakae, 1996).

Among the species that are able to ferment raffinose and stachyose are most *Bacteroides* and *Bifidobacterium* species. Clostridia and other species ferment raffinose or stachyose, not or relatively slowly (Table 1.3) (Masai *et al.*, 1987; Mizutani, 1989; Ohtsuka *et al.*, 1989; Holdeman and Moore, 1977; Hara *et al.*, 1994). However, data on gas formation and fermentation are not very consistent, which is probably due to differences in experimental design and variations between the strains used. Regarding *B. distasonis*, both Ohtsuka (1989) and Mizutani (1989) find positive fermentation of raffinose, whereas Hayakawa *et al.* (1990) do not find any or very limited fermentation. Both Hayakawa *et al.* (1990) as Ohtsuka (1989) use similar media and methods. Similarly, in identification schemes (Holdeman and Moore, 1977) several species are listed as positive in over 95% of the strains tested, whereas in some studies using OD or pH measurements, the same species is listed as negative (no fermentation) (Masai *et al.*, 1987; Hara *et al.*,1994).

Studies with healthy Japanese volunteers have shown that ingestion of raffinose does increase the number of bifidobacteria in faeces, both in low (3-5 g/day) as high (15 g/day) doses (Benno *et al.*, 1987; Fujisaki *et al.*, 1994). Clostridial and bacteroides counts decreased significantly with the higher dose. All other bacterial groups tested remained unchanged. Fecal pH was somewhat reduced with the higher dose, although not significantly. No changes were observed in putrefactive products (Benno *et al.*, 1987; Fujisaki *et al.*, 1994). Some of the volunteers, however, initially developed diarrhea with the higher dose of raffinose used. The effects diminished during the study, indicating an adaptation of the flora (Benno *et al.*, 1987).

In a similar study soybean oligosaccharides were tested on 6 healthy male volunteers. Soybean oligosaccharides contained 23% stachyose, 7% raffinose, 44% sucrose and 23% other oligosaccharides. Each person ingested 10 g oligosaccharides per day, which corresponds with 3g raffinose and stachyose. Differences observed were an increase in bifidobacterial counts and a decrease in the numbers of *Cl.*

perfringens. Other bacterial groups tested remained unchanged. Similarly ß-glucuronidase and azoreductase activities decreased. No changes were observed in faecal moisture content and 2 of 6 volunteers showed decreased amounts of indole in their faeces (Masai *et al.*, 1987, Hayakawa *et al.*, 1990).

Furthermore, in an *in vivo* study using human faecal inocula in a continuous flow model addition of soybean oligosaccharides resulted in an increase in bifidobacteria. Of the enzymatic activities tested only azoreductase was lowered significantly. Nitroreductase, nitrate reductase, ammonia, ß-glucuronidase and ß-glucosidase were not changed (Saito *et al.*, 1992).

It can thus be concluded that ingestion of raffinose and/or raffinose containing soybean oligosaccharides, may have a significant effect on the composition and metabolic activity of the intestinal flora.

TOS

TOS, or transgalactosyl-oligosaccharides are produced by the action of ß-galactosidases on lactose. The mixture consists of several di-, tri-, tetra-, penta- and hexasaccharides, which consist of galactose residues linked to a terminal glucose. The linkage may be ß1-4, ß1-3 or ß(1-6) (Rowland and Tanaka, 1993). Although TOS are produced chemically it is likely that some structures will occur in nature. TOS is being prepared commercially both in Japan (Oligomate, Yakult Ltd.) and in the Netherlands (Borculo Whey Products). The actual composition of the two preparations is very similar, but the relative amounts of the components differ (Van Laere, pers. comm).

Although some minor degradation by human ß-galactosidase may occur, the main fraction of the TOS mixture is non-digestible. The main fraction of TOS thus reaches the caecum, where rapid fermentation takes place, as can be judged from breath hydrogen increases (Tanaka *et al.*, 1983; Ishikawa *et al.*, 1995; Kikuchi *et al.*, 1996; Kondo and Nakae, 1996; Bouhnik, 1997).

TOS can be fermented to some extent by a number of intestinal bacteria, especially bifidobacteria, bacteroides, enterobacteria and lactobacilli (Table 1.3) (Tanaka *et al.*, 1983; Ishikawa *et al.*, 1995).

Trials with Japanese volunteers have shown that ingestion of TOS, or TOS enriched in disaccharides increases the number of bifidobacteria in the faeces (Tanaka *et al.*, 1983; Ito *et al.*, 1990; Ito *et al.*, 1993; Ishikawa *et al.*, 1995).

The increase in bifidobacteria showed a linear relation to the dose of TOS used (Ito *et al.*, 1990). Fecal ammonia, stool weight and stool frequency were not affected. No

signs of diarrhea were observed in doses of up to 10 g/day (Tanaka *et al.*, 1983; Ito *et al.*, 1990). Simultaneous ingestion of TOS and *Bi. breve* did not affect the number of bifidobacteria, but reduced the number of bacteroides and enterobacteria. However, in this case some reduction in faecal ammonia was observed (Tanaka *et al.*, 1983).

Two other studies also showed an increase in bifidobacteria after administration of TOS, but in both cases the initial bifidobacterial numbers were rather low (Ito *et al.*, 1993; Bouhnik *et al.*, 1997).

Finally a well controlled study using a large number of volunteers did not show any effect of TOS on the bacterial composition (Alles *et al.*, in press).

Effects on the intestinal metabolism were studied using specific enzymes as markers. Rats associated with a human faecal flora were fed a diet with 5% TOS. Caecal pH was reduced, as were the activities of ß-glucuronidase and nitrate reductase. Both enzymes are associated with toxic effects on the host. Similarly the conversion of the dietary carcinogen IQ to its genotoxic 7-hydroxy derivative was reduced. On the other hand ß-glucosidase activity was increased (Rowland and Tanaka, 1993).

Lactitol-oligosaccharides

Lactitol-oligosaccharides (LTOS) are prepared by transgalactosylation using *Aspergillus oryzae* ß-galactosidase and lactitol as starting carbohydrate. The resulting mixture consists of several different components, all with a terminal sorbitol residue.

LTOS are non digestible in rats, but have not yet been tested in human studies. LTOS can be utilized by a number of intestinal bacteria (Table 1.3) among these bacteria from several genera (Yanahira *et al.*, 1995).

Increased numbers of bifidobacteria were observed in rat faeces after feeding 1-3 weeks of LTOS. Similarly peptococci increased, but no changes in other bacterial groups were observed. Concentrations of indole and cresol decreased significantly (Yanahira *et al.*, 1995).

These results indicate that LTOS may have an effect on the composition and the metabolic activity of the intestinal flora of rats, but data on humans are not available.

4´-Galactosyllactose

Galactosyllactose (GLL, O-D-galactopyranosyl-(1-4)-O-ß-D-galactopyranosyl-(1-4)-D-glucopyranose) is produced in Japan by the action of *Cryptococcus laurentii* on lactose. The product is

marketed as Cup-oligo powder or syrup. This preparation also contains traces of the tetramer $(gal)_3$-glu (Playne and Crittenden, 1996).

Galactosyllactose appears to be unaffected by human intestinal enzymes as feeding trials with human volunteers have shown an increase in bifidobacteria in the faeces.

Galactosyllactose can be fermented by a number of intestinal species (Table 1.3), especially bifidobacteria and bacteroides. None of the clostridia, lactobacilli, enterococci or *E. coli* were able to ferment galactosyllactose (Ohtsuka *et al.*, 1989; Ishikawa *et al.*, 1995).

Studies with 10 healthy human volunteers who received 8 g/day of GLL for 10 days showed a significant increase in the number of bifidobacteria. No changes in other bacterial groups were observed. Fecal pH decreased in most subjects during the trial period (Ohtsuka *et al.*, 1989).

Although the data are rather limited, it is not unlikely that GLL may have an effect on either the composition or the metabolic activity of the human intestinal microflora.

Synthetic galactooligosaccharides

Synthetic galactooligosaccharides were prepared chemically by means of the Koenigs-Knorr reaction (Minami *et al.*, 1985). Several products were prepared this way, namely neogalactobiose (NGB), isogalactobiose (IGB), galsucrose (GAS), three lactose isomers, Isolactose I (IL1) (ß-D-galactopyranosyl-ß-D-glucopyranoside), Isolactose II (IL2) (ß-D-galactopyranosyl-α-D-glucopyranoside) and Isolactose III (IL3) (α-D-galactopyranosyl-ß-D-glucopyranoside) as well as a trimer, GFF (α-D-galactopyranosyl-ß-D-fructofuranosyl-(2-6)-ß-D-fructofuranoside) (Minami *et al.*, 1983; Minami *et al.*, 1985).

It is not known whether these compounds are digested in the upper gastrointestinal tract. These products have not been tested for their influence on the intestinal flora in human or animal trials.

Fermentation by individual intestinal bacteria has shown that all substrates could be fermented by the 5 bifidobacteria and the *Klebsiella* species tested. *Lactobacillus acidophilus* and enterococci were not able to ferment any of the compounds, all *E. coli* strains could utilize only NGB, but some strains also utilized IGB, IL1, IL3I and GAS (Minami *et al.*, 1983; Minami *et al.*, 1985).

It was claimed that the use of these oligosaccharides may have some bifidogenic effect, even though other species grew well on these oligosaccharides. Also, these oligosaccharides have not yet been tested on other anaerobic species like *Bacteroides*

sp. or clostridia. As these substrates have as yet not been tested on humans or animals, their actual bifidogenic effect is still unknown.

Fructooligosaccharides

Fructooligosaccharides (FOS) are oligosaccharides, with fructose as the major monomeric residue.

Two different classes of FOS mixtures are produced commercially, based on inulin degradation or transfructosylation processes. As the chemical structure of the mixtures does not differ significantly, the two types will be discussed in one paragraph.

FOS can be produced by degradation of inulin, or polyfructose, a polymer of D-fructose residues linked by ß(2-1) bonds with a terminal α(1-2) linked D-glucose. The degree of polymerization of inulin ranges from 10-60. Inulin can be degraded enzymatically or chemically to a mixture of oligosaccharides with the general structure Glu-$(Fru)_n$ (GF_n) and Fru_m, (F_m), with n,m ranging from 1 to 7 (Playne and Crittenden, 1996). This process also occurs to some extent in nature, and these oligosaccharides can be found in a large number of plants, especially in Jerusalem artichoke and chicory (Roberfroid *et al.*, 1993; Campbell *et al.*, 1997). This type of FOS is prepared commercially by Orafti Ltd., Tienen Belgium, which markets the product as Raftilose. It has also been referred to as oligofructose, a term which also includes pure inulin. The main components are kestose (GF_2), nystose (GF_3), fructosylnystose (GF_4), bifurcose (GF_3), inulobiose (F_2), inulotriose (F_3) and inulotetraose (F_4).

The second class of FOS is prepared by the transfructosylation action of a ß-fructosidase of *Aspergillus niger* on sucrose. The resulting mixture has the general formula of GF_n with n ranging from 1 to 5. Contrary to the inulin derived FOS, the binding is not only ß(1-2), but other linkages do occur, though in limited numbers. This group is produced in Japan by Meiji Seika Kaisha LTD and marketed as Meioligo, Neosugar, Profeed, Actilight or Nutraflora (Hidaka *et al.*, 1986, Thiriet, 1989; Spiegel *et al.*, 1994).

Some reviews have been published previously on FOS describing the production, functionality and nutritional effects in more detail (Fishbein *et al.*, 1988; Hidaka *et al.*, 1986, Roberfroid, 1991; Hidaka *et al.*, 1990; Hidaka *et al.*, 1991; Hidaka and Hirayama, 1991).

FOS can be fermented by human oral bacteria, and can thus be considered cariogenic (Chapter 2).

Human intestinal enzymes do not, or very slowly, degrade FOS. This was determined using rat tissue homogenates (Oku, 1984), ileostomy patients (Alles *et al.*, 1997)

and human breath hydrogen measurements (Alles *et al.*, 1996; Alles *et al.*, 1997, Fujita *et al.*, 1991).

FOS are degraded and fermented rapidly by the intestinal flora. Many different species of intestinal bacteria are capable of fermenting FOS, among these bifidobacteria, lactobacilli, clostridia, enterobacteria, bacteroides and several others (Tables 1.3 and 1.4) (Asano *et al.*, 1994; Hayakawa *et al.*, 1991; Wada 1990.; Asano *et al.*, 1994; Mitsuoka *et al.*, 1987; Hara *et al.*, 1994; Kawaguchi *et al.*, 1993; Nakakuki *et al.*, 1990; Hartemink *et al.*, 1997).

Different studies often find contradictory results regarding the fermentation of the different FOS-components by strains of the same species. This is most often due to the strains and methodology used. Bailey *et al.* (1991) observed no growth on FOS by different enterobacteria, using OD-measurements and a medium poor in nutrients, whereas other groups observed growth (Wang and Gibson, 1993) or fermentation (Hartemink *et al.*, 1997). Similarly, several reports show no growth on FOS by Cl. perfringens (Mitsuoka *et al.*, 1987), whereas other groups observe good growth and gas formation by different strains of this species (Wang and Gibson, 1993; Hartemink and Rombouts, 1997). Methodology and growth conditions thus are important in determining the possible fermentation of FOS by a selected strain.

Gas formation has been observed by different groups (Wang and Gibson, 1993; Kawaguchi *et al.*, 1993; Hartemink and Rombouts, 1997), as well as an increase in flatulence and gastrointestinal complaints (Stone-Dorshow and Levitt, 1987). Gas formation was mainly due to *Cl. sporogenes*, *Cl. clostridioforme* and *Kl. pneumoniae* (Kawaguchi *et al.*, 1993) and *Cl. perfringens* (Hartemink and Rombouts, 1997; Kolsteren, 1997).

Studies using faecal slurries have observed production of short-chain fatty acids and a relatively high production of butyrate. Faecal pH was decreased in some animal studies. Changes in enzyme patterns have also been observed by different groups.
Ingestion of FOS was also shown to have an effect on the microbial composition in humans, pigs, rats and other animals, often an increase in bifidobacteria was observed (Mitsuoka *et al.*, 1987; Howard *et al.*, 1995; Bouhnik *et al.*, 1996; Gibson and Wang, 1993), whereas the other bacterial groups tested were unaffected.

Table 1.4 Specific growth rates of selected intestinal bacteria on FOS and glucose

species	FOS* (ΔOD/h)	glucose* (ΔOD/h)
Bi. adolescentis	0.160	0.124
Bi. angulatum	0.261	0.218
Bi. bifidum	0.183	0.180
Bi. breve	0.204	0.132
Bi. catenulatum	0.270	0.201
Bi. infantis	0.297	0.173
Bi. longum	0.208	0.215
Bi. pseudolongum	0.303	0.243
Cl. perfringens	0.216	0.259
E. coli	0.212	0.242

* Data adapted from Wang and Gibson, 1993 measured as OD_{650} in a stirred chemostat kept at pH 7.0 in PY broth + 0.5% carbohydrate. Measurements in triplicate on a single strain

Even though many of the human studies have been carried out with special populations, like older patients (Mitsuoka *et al.*, 1987, Mitsuoka *et al.*, 1986), people with very low initial bifidobacteria counts (Bouhnik *et al.*, 1996) or diabetics (Sanno, 1986), the data indicate that addition of FOS affect the bacterial composition and the metabolic pattern of the intestinal microflora.

The increase of bifidobacteria, however, is not consistent with the increase in gas formation (Wang and Gibson, 1993; Kawaguchi *et al.*, 1993; Hartemink and Rombouts, 1997) or butyrate production, as bifidobacteria produce neither gas nor butyrate (Sgorbati *et al.*, 1995). FOS thus are not selectively fermented by bifidobacteria as has been stated previously (Roberfroid *et al.*, 1993). The increase in bifidobacteria may be a combined effect of growth by these bacteria as well as a reduced transit time with subsequent better recovery of bifidobacteria in faeces.

Synthetic oligosaccharides

These oligosaccharides are synthesized completely by chemical, non-enzymatic, methods. Often they do not resemble natural oligosaccharides and are synthesized as possible non-digestible non-caloric bulking agents. As yet none of these oligosaccharides have been tested on individual bacterial strains. A number of these

compounds have been tested on faecal slurries and fermentation was measured by acid and gas production. It was found that sorbitol derivatives (like 2,3,4,6-tetra-O-ß-D-glucopyranosyl-D-sorbitol) and other highly branched oligosaccharides could be fermented slowly with the production of some acid and gases. Also, all the hexoses with a 5-C-hydroxymethyl group were found to be completely non-fermentable, suggesting a critical role for this group in metabolism (Mazur *et al.*, 1993).

As fermentation of these groups was found to be very slow, no significant effects on the intestinal flora will be expected.

Xylooligosaccharides

Xylooligosaccharides are prepared by enzymatic degradation of xylan by a xylanase from *Trichoderma* sp.. The resulting mixture consists of xylobiose, xylotriose, xylotetraose and xylopentaose (Imaizumi *et al.*, 1991; Cotta, 1993). Xylobiose is naturally present in bamboo shoots, which are consumed in Japan and China (Imaizumi *et al.*, 1991).

Xylooligosaccharides are not fermented by the human intestinal enzymes and reach the colon intact. In the colon, xylooligosaccharides are fermented by a number of species, among them most bifidobacteria, several clostridia, *Bacteroides* species, *E. coli* and some lactobacilli (Okazaki *et al.*, 1990; Van Laere *et al.*, 1997).

In a study using 5 male volunteers, it was observed that addition of xylooligosaccharides increased the number of bifidobacteria in the faeces. No changes were observed in other bacterial groups (Okazaki *et al.*, 1990).

Changes in the metabolic pattern of the intestinal microflora have been observed in rats, but as yet not in human feeding trials (Hoshi *et al.*, 1994; Imaizumi *et al.*, 1991; Campbell *et al.*, 1997). The results indicate that addition of xylooligosaccharides in the diet may have an effect on either the composition and/or the metabolic activity of the intestinal microflora.

Alginate oligosaccharides

This group consists of oligosaccharides prepared by enzymatic degradation of alginate and are rich in mannuronic and guluronic acid residues. The oligosaccharides tested could be distinguished in high mannuronate-oligosaccharides and high guluronate-oligosaccharides. Only seven intestinal bacteria were tested for growth on these two groups and growth was compared with glucose. Growth was poor for *Bi. infantis*, *Bi. breve*, *Bi. longum*, *Lb. acidophilus* and *Ec. faecalis*, whereas *B. thetaiotaomicron* and

Cl. perfringens were not able to use these oligosaccharides for growth (Kitamikado *et al.*, 1993).

Even though these oligosaccharides are poorly fermented by bifidobacteria, a bifidogenic effect has been observed by Terada *et al.* (1995) in healthy human volunteers.

Due to the limited data available, it is difficult to estimate whether alginate oligosaccharides have an effect on either the metabolism or the composition of the intestinal microflora.

Mucin-derived oligosaccharides

These oligosaccharides are produced in the upper intestine from the gastrointestinal mucosal glycoproteins. Mucin-derived oligosaccharides differ widely in structure, but are characterized by a complex composition, with many different monosaccharide building blocks, and different charged side chains.

In the human intestine only a few *Ruminococcus* and *Bifidobacterium* strains were found to be the most active in degradation of human ABH blood group antigens (a group of mucin-derived oligosaccharides). The 16 other bacteria tested, among them *Bacteroides* species, *E. coli, Ec. faecalis* and several other bifidobacteria did not degrade these products (Hoskins *et al.*, 1985; Hoskins *et al.*, 1992). These oligosaccharides have not yet been used in human or animal trials.

Mannooligosaccharides

Mannooligosaccharides are oligosaccharides, with mannose as the main component sugar. Two types of mannooligosaccharides can be distinguished; yeast derived mannooligosaccharides and guar gum hydrolysates (galactomannan hydrolysates). The latter will be dealt with in Chapter 5.

Commercial mannooligosaccharides are marketed by Alltech as a crude powder, with less than 50% oligosaccharides. These oligosaccharides have been successfully used in the animal feed industry as a growth promoter (Kumprecht *et al.*, 1997; Jacques *et al.*, 1997). There are no data available on the fermentation of these oligosaccharides either by individual bacterial strains, or from human feeding trials.

2

DEGRADATION AND FERMENTATION OF FRUCTOOLIGOSACCHARIDES BY ORAL STREPTOCOCCI.

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SUMMARY

Fructooligosaccharides (FOS) are claimed to have a positive effect on the intestinal flora. They are being used in functional foods in Japan and Europe. The degradation of two commercial FOS preparations by oral streptococci in order to predict the cariogenicity of these products was studied. Both preparations could be fermented to some extent by the species of oral streptococci tested.

The enzymes necessary for the degradation of FOS were inducible. Each strain showed a specific degradation pattern. All strains, particularly *St. mutans* rapidly produced acid, mainly lactic acid. *St. mitis* also produced high concentrations of acetic acid. Plaque formation by *St. mutans* was similar to the sucrose control. It is concluded that FOS are cariogenic to a similar extent as sucrose.

INTRODUCTION

Fructooligosaccharides are a group of oligosaccharides, consisting of several ß(1-2) or ß(1-6) linked fructose residues (Fn-series), which may be linked to a glucose residue (GFn-series). Two FOS mixtures are available commercially, 'Profeed' and 'Raftilose'. Profeed is produced enzymatically from sucrose using a fungal fructosyltransferase. The final product consists of a mixture of linear and branched oligosaccharides of the GFn-series. The product has been marketed as 'Neosugar', 'Meioligo', 'Actilight' or 'Nutraflora' (Hidaka *et al.*,1990).

Raftilose is produced enzymatically from inulin using an endo-inulinase. The final product consists of a mixture of linear oligosaccharides from both the GFn-series and the Fn-series. This product has also been referred to as 'Oligofructose' (Roberfroid *et al.*, 1993).

FOS are not degraded by the human digestive enzymes and thus reach the colon unaltered (Hidaka *et al.*, 1990). It has been shown for both commercial mixtures that a daily intake of FOS results in an increase of faecal bifidobacteria (Mitsuoka *et al.*, 1987; Wang and Gibson, 1993). An increase in faecal bifidobacteria is claimed to have a beneficial effect on health. FOS are therefore used as a health promoting agent in 'functional foods' (Spiegel *et al.*, 1994).

As FOS are widespread in nature, they are considered in food legislation as ingredients, rather than additives. In Japan this has resulted in the addition of FOS to a large range of products (Spiegel *et al.*, 1994).

As FOS are used in many food products it is likely that some product remains in the oral cavity after consumption. It is therefore possible that FOS are degraded by the oral microflora and that, consequently, they may be cariogenic. It is well known that oral

streptococci produce several fructanases, which can degrade inulin and other fructans (Hamada and Slade, 1980; Walker *et al.*, 1983; Takahasi *et al.*, 1985; Burne *et al.*, 1987).

Nystose (GF3), one of the components in both Raftilose as Profeed, has been shown to be non-cariogenic (Hirasawa *et al.*, 1984; Ikeda *et al.*, 1990), however, Ziesenitz and Siebert (1987) came to the opposite conclusion. Neosugar (Profeed) has been shown to be non-cariogenic, although some acid is produced by oral streptococci (Hirasawa *et al.*, 1984).

Among the genera present in the oral cavity, the streptococci are considered to be the main cariogenic group of bacteria. We have therefore tested the degradation of FOS by members of this group, mainly *St. mutans* to predict the cariogenicity of this group of oligosaccharides.

MATERIALS AND METHODS

Bacteria

The following strains of oral streptococci were obtained from Dr. F.H.M. Mikx, Dept. of Oral Microbiology, Catholic University of Nijmegen, The Netherlands: *St. mutans* 44 (Nijm. 591), *St. oralis* 45 (Nijm. 592), *St. mitis* biovar I 46 (Nijm. 593), *St. sanguis* biovar I 43 (Nijm. 594) and *St. gordonii* biovar II 42 (Nijm. 595). In addition *St. mutans* 36 (DSM 20523) was used. For the induction studies a *Streptococcus* strain was isolated from saliva of a human volunteer. It was identified as *St. salivarius*, using the Rapid ID 32 Strep and the APILAB identification program (Bio-Merieux, Marcy-l'Etoile, France).

Oligosaccharides

Raftilose P95 was obtained from Orafti (Tienen, Belgium). Profeed P95 was obtained from Nutreco (Boxmeer, The Netherlands). Both preparations consisted of at least 95% (P95) oligosaccharides according to the manufacturers. The remaining part consists of glucose, fructose and sucrose.

Screening

FOS agar was used to screen for growth and acid formation of oral isolates. FOS agar consisted of Buffered Peptone Water (Oxoid) to which were added (gl^{-1}): Raftilose P95 15, agar technical (Oxoid) 15, yeast extract (Oxoid) 5 and chlorophenol red (Merck) 0.05. Only Raftilose P95 was used, as most components of Profeed P95 are present in Raftilose P95 as well. Appropriate dilutions of the strains, pre-grown in Peptone-Yeast

extract broth supplemented with glucose (PY-G) were applied to FOS agar plates. PY-G was prepared according to Holdeman and Moore (1977). Growth was considered positive when the colony size was over one mm diameter. Acid production was considered positive when a yellow zone was formed around the colony. Raftilose P95 contains some fermentable mono- and disaccharides. Acid formation due to fermentation of these compounds was neutralized by the buffer in the medium. Any yellow discoloration is therefore due to fermentation of oligosaccharides. The plates were incubated at 37EC anaerobically, using an atmosphere of 80% nitrogen, 10% hydrogen and 10% carbon dioxide (HoekLoos, Rotterdam, The Netherlands).

Induction

Induction was studied using a *St. salivarius* strain which showed slow growth and slow acid production on FOS agar (i.e. no yellow zone within 24 h). The strain was cultured in 25 ml PY-G. After 24 h 100 µl was inoculated into 25 ml PY-broth with 0.5% Raftilose P95 (PY-Raf). This strain was subsequently subcultured daily in PY-Raf for 7 days.

Growth curves were determined by adding 25 µl of a 24 h PY-G culture to 250 µl PY-G and PY-Raf, respectively, in a microtiter plate. Similarly 25 µl of a 24 h old culture of *St. salivarius* subcultured 7 times on PY-Raf was added to PY-Raf. Growth curves were determined by measuring the absorbance at 620 nm every 15 minutes for 11 hours in a microtiterplate reader (EAR 400, SLT Instruments, Groedig, Austria).

Acid formation

Acid formation was studied by culturing the following strains in HPLC-broth : *St. mutans* 36, *St. mutans* 44, *St. oralis* 45, *St. mitis* 46, *St. sanguis* 43 and *St. gordonii* 42. HPLC broth is a medium which minimizes interference on the HPLC, it consists of (gl^{-1}): neutralized bacterial peptone (Difco) 5, yeast nitrogen base (Difco) 6, dipotassium hydrogen phosphate (anhydrous, Merck) 13, Raftilose P95 or Profeed P95 5. The pH was adjusted to 7.0 using 3 $moll^{-1}$ sodium hydroxide before sterilization for 15 min at 121°C. The strains were pre-cultured on PY-G for 16 h. All strains were grown anaerobically for 60 h at 37°C. Samples were taken at 12 h intervals for *St. mutans* 36 and after 60 h for the other strains.

The samples were centrifuged for 15 min at 10.000 rpm to precipitate the cells. Lactic and acetic acids were determined using HPLC with an Aminex HPX-87H column and 0.01 mol l^{-1} sulfuric acid as the mobile phase (Voragen *et al.*, 1986).

Rapid acidification

For the rapid acidification experiments *St. mutans* 36 was precultured for 16 h in PY-G. Cells were harvested by centrifuging 100 ml culture and washed twice with reduced peptone physiological salt solution (RPFS). RPFS consisted of (gl^{-1}): neutralized bacterial peptone (Difco) 1.0, sodium chloride (Merck) 8.5 and cysteine.HCl (Sigma) 0.5. The pH was adjusted before sterilisation to 6.7 with 3 mol l^{-1} potassium hydroxide. After the second washing the cells were resuspended in 5 ml RPFS. For acidification tests 500 µl carbohydrate or carbohydrate/RPFS mixture was added to 5 ml RPFS. The mixture was placed in an incubator and the pH was measured continuously. When the pH was stabilized 500 µl cell suspension was added and the pH was measured continuously for at least 30 min.

Fermentation patterns

Fermentation patterns were determined by growing all strains anaerobically at 37°C in HPLC-broth supplemented with 20 gl^{-1} of Raftilose P95 for 60 h. Profeed P95 was not used in this experiment, as most components of Profeed P95 are present in Raftilose P95. After 60 h a sample (one ml) was taken and centrifuged at 10.000 rpm to precipitate the cells. Residual Raftilose P95 was analysed using High Performance Anion Exchange Chromatography (HPAEC). HPAEC was performed using a BIO-LC system (Sunnyvale, California, USA), equipped with a Dionex Carbopac PA-100 (4x250 mm) column and a Dionex pulsed electrochemical detector in the pulsed amperometric detection method (Voragen *et al.*, 1986). The oligosaccharides were analyzed using a gradient of 0 to 1.0 mol l^{-1} sodium acetate in 100 mmol l^{-1} sodium hydroxide.

Artificial plaque formation

Plaque formation was studied using *St. mutans* 36. The strain was pre-cultured anaerobically at 37°C for 24 h in PY-G broth. Plaque formation was determined using the procedure described by Hirasawa *et al.* (1984). PY supplemented with 10% Profeed, Raftilose or sucrose was used. In a second experiment 0.1 mol l^{-1} potassium dihydrogen phosphate was added and the pH adjusted to 7.0 using 3 mol l^{-1} sodium hydroxide.

RESULTS

Screening

All oral streptococci were able to grow on FOS agar. Good growth was observed within 24 h for all strains except *St. sanguis* 43, which showed good growth only after 48 h (Table 2.1). Acid production started within 24 h for *St. mutans* 36 and *St. mitis*. All strains showed acid production after 3 days, except for *St. oralis* which showed only weak acid production.

Similarly, FOS agar plates inoculated with samples of saliva from 40 healthy volunteers and 4 healthy piglets, all showed acid production and good growth within 48 h.

Induction

The induction experiments with a slow-growing *St. salivarius* strain showed that the production of FOS-degrading enzymes is inducible. Fig 2.1 shows that the main effect is a reduced lag-phase, indicating the induction of the necessary enzymes. The growth rate was unaffected by induction.

Table 2.1 : Growth of oral streptococci on FOS agar, containing 1.5% Raftilose P95.

Strain	growth and acid production after					
	day 1		day 2		day 3	
	growth	acid	growth	acid	growth	acid
St. gordonii 42	++*	+	++	++	++	++
St. mitis 46	++	+	++	++	++	++
St. mutans 36	++	+	++	++	++	++
St. mutans 44	++	+	++	+	++	++
St. oralis 45	+-	--	++	--	++	+
St. sanguis 43	-	--	++	+	++	+

*: growth ++ colony size over 1 mm diameter, + colonies small but clearly visible, +- colonies barely visible, -- no visible colonies

acid ++ yellow zone around colonies, + orange to yellow zone, -- no zone around colonies

Acid formation

All strains showed production of both lactic and acetic acids (Table 2.2). Lactic acid was generally present in higher concentrations than acetic acid. Most acid was produced by *St. gordonii*, *St. mutans* 36 and 44 and *St. mitis*. These strains also showed the fastest production of acid in the screening experiment. *St. mitis* also produced a relatively high concentration of acetic acid, indicating heterofermentative fermentation.

St. mutans 36 showed slower growth on Profeed, which resulted in lower concentrations of acid after 60 h. *St. mutans* 36 produced similar amounts of acid on Raftilose as on sucrose, indicating a similar and rapid fermentation.

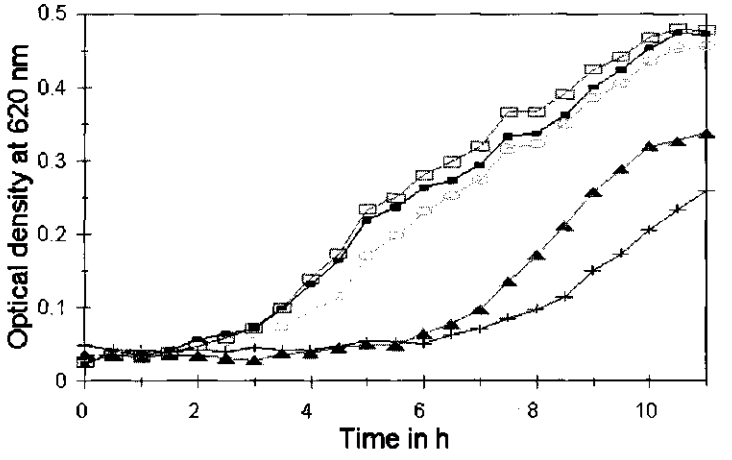

Fig. 2.1 : Induction of growth of *St. salivarius* on Raftilose P95.

St. salivarius was grown on PY-G. After 24 h the strain was subcultured in PY-Raf. This procedure was repeated every 24 h. Growth of the following cultures in PY-Raf was measured in a microtiter reader with absorbance at 620 nm : pre-grown on PY-G only (open rectangles), subcultured once on PY-Raf (crosses), subcultured twice on PY-Raf (triangle), subcultured three times on PY-Raf (line only) and four times of PY-Raf (closed rectangles)

Rapid acidification

Acid formation was very rapid as could be concluded from this experiment. A critical pH for dental caries of 5.5 was reached within 15 minutes for glucose as well as both FOS preparations (Fig 2.2). With a lower concentration of Raftilose (2.3 mmol l^{-1}) the critical pH

was reached within 30 minutes. No acid was produced in the absence of a fermentable carbohydrate.

Table 2.2 : Acid formation on 0.5 % Raftilose P95 and Profeed P95 by oral streptococci in HPLC broth.

Strain	time of incubation (h)	lactic acid (mmol l^{-1})	acetic acid (mmol l^{-1})
St. gordonii 42	60	107.0	6.9
St. mitis 46	60	49.6	38.4
St. mutans 36	12	1.1	9.7
	24	58.4	13.6
	36	105.3	12.9
	48	115.4	12.4
	60	123.7	10.3
St. mutans 44	60	124	8.9
St. oralis 45	60	38.2	10.3
St. sanguis 43	60	41.2	11.8

Acid production was determined using HPLC. See Materials and Methods section for details.

Fermentation patterns

The fermentation patterns of the different strains are shown in Figure 2.3. It is clear that the strains degrade the FOS mixture differently. The only common factor is a complete fermentation of sucrose (GF, at 11.5 minutes). *St. mutans* 44, *St. gordonii* and *St. mitis* showed the most extensive degradation. This is in accordance with both the screening and the acid formation experiments.

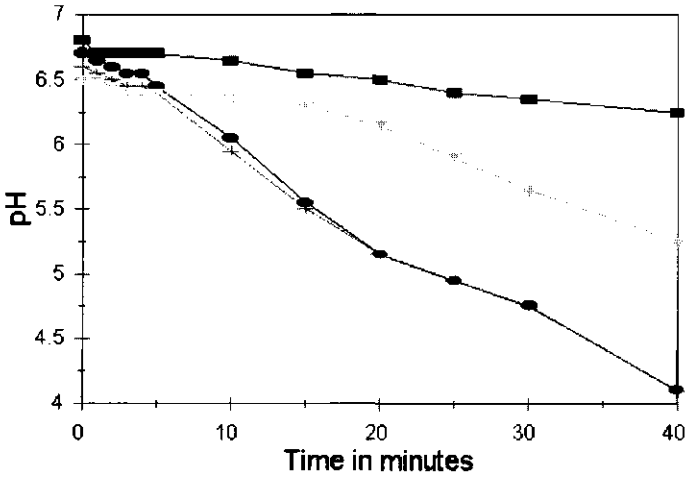

Figure 2.2 : Rapid acidification by *St. mutans* 36.

Cells of *St. mutans* 36 pregrown in PY-G for 16 h were washed twice with RPFS and added to 500 µl RPFS containing 23 mol l^{-1} carbon sources : (rectangles) no carbon source added, (crosses) glucose, (circles) Raftilose and (triangles) 2.3 mol l^{-1} Raftilose.

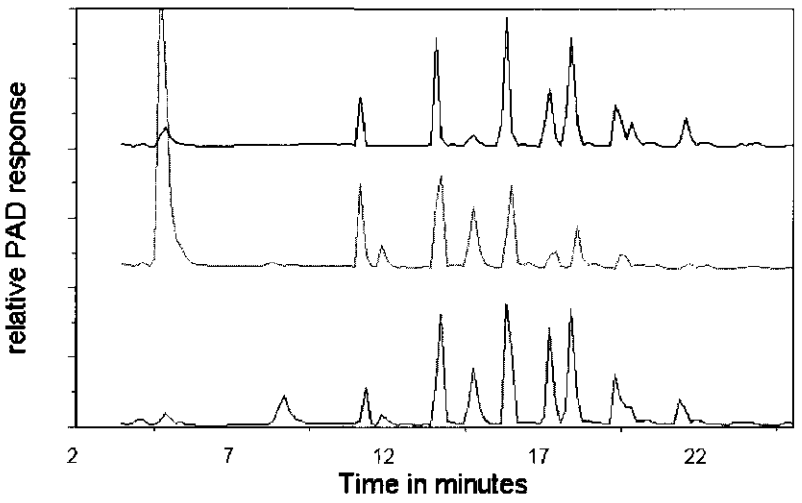

Figure 2.3: Change in composition of Raftilose upon fermentation by oral streptococci.

Composition patterns were determined by HPLC. For experimental conditions see Materials and Methods section. Lower line: Raftilose, Middle line: Raftilose after fermentation by *St. gordonii* 42, Upper line: Raftilose after fermentation by *St. mitis* 46.

St. gordonii degraded the higher oligosaccharides, both Fn and GFn series (Table 2.3), thereby producing fructose, F2 and GF2. The intermediate structures F3 and GF3 seemed to be unaffected, or their degradation and formation rates are in equilibrium. Similarly, glucose levels seemed to be unaffected. This may also be due to a preference for fructose by *St. gordonii*.

St. sanguis showed only limited degradation of Raftilose. Only GF5 and F4 were degraded completely. In contrast to *St. gordonii*, no fructose accumulation was observed. On the other hand an accumulation of F3 was observed, probably due to hydrolysis of a fructose residue from F4.

St. mutans 44 showed no production of GF2, which may be due to a better, more extensive degradation. Similarly no glucose could be detected. Large amounts of fructose and F2 were detected.

The degradation with time by *St. mutans* 36 differed from the pattern of strain 44, in that GF3 was degraded as rapidly as the other components. Similarly no increase in F3 was observed. From the data (Table 2.4) it can be seen that all components were degraded rapidly, the Fn series somewhat faster. Increases in fructose and glucose were only observed after 36 hours. *St. oralis* showed very limited degradation. Only glucose, sucrose and GF2 were degraded. Surprisingly no changes in fructose were observed.

St. mitis showed again extensive degradation. In contrast to *St. gordonii* and *St. mutans* 44 no degradation of GF3 or higher was observed. All linear fructose oligomers (F to F4) were degraded completely, as were sucrose and GF2. No accumulation of fructose could be detected. Glucose concentrations remained unaffected, similarly as in *St. gordonii*.

Artificial plaque formation

Plaque formation was determined using *St. mutans* 36. The results (Table 2.5) show that Raftilose induces the formation of plaque as rapidly as does sucrose. Growth and formation of plaque on Profeed was considerably slower. However, after three days of incubation, the difference was negligible. Use of a phosphate buffer, which was added to imitate the conditions in the mouth more accurately, clearly influenced the formation of plaque. The amount of plaque formed with Raftilose as a substrate even surpassed the amount formed with sucrose.

Table 2.3 : Changes in carbohydrate-pattern after incubation of 2% Raftilose P95 by oral streptococci.

Strain	Compound*										
	G	GF	GF2	GF3	GF4	GF5	F	F2	F3	F4	Fn
St. gordonii 42	/**	---	+	/	-	--	+++	++	/	--	--
St. sanguis 43	/	---	/	/	/	-	-	/	+	-	/
St. mutans 44	-	-	--	/	-	--	+++	++	/	--	-
St. oralis 45	-	---	-	/	/	/	/	/	/	/	/
St. mitis 46	---	---	/	-	/	/	+	---	--	-	/

* G = glucose unit, Fx = number (x) of fructose units, n = more than 4 fructose units

**/ : no visible changes, - some degradation (<20%), -- degradation (20-75%), --- : complete degradation, + slight increase (<20%), ++ clear increase (20-100%), +++ considerable increase (>100%)

Increases are due to degradation of larger oligosaccharides and incomplete fermentation.

Table 2.4: Changes in carbohydrate pattern after incubation of 2% Raftilose P95 by *St. mutans* 36.

	time (h)			
component	0	24	36	42
G* (glucose)	100	0	133	100
F (fructose)	100	0	1409	200
GF (sucrose)	100	0	6	0
GF2 (kestose)	100	19	29	0
F2	100	0	0	0
GF3	100	23	4	0
F3	100	0	0	0
GF4	100	24	1	0
F4	100	0	0	0
GF5	100	27	0	0

* G = glucose units, Fx = number (x) of fructose units

Values were determined using HPLC. For experimental details see Materials and Methods section. Values are relative percentages as compared to T=0.

Increases are due to degradation of larger oligosaccharides and incomplete fermentation.

Table 2.5 Artificial plaque formation by *St. mutans* 36. Values are average of duplicate measurements.

Carbon source (10%)	day 1 (mg plaque)	day 2 (mg)	day 3 (mg)
sucrose	11	14	16
sucrose + buffer*	15	16	25
Raftilose P95	12	10	18
Raftilose P95 + buffer	18	22	34
Profeed P95	8	5	13

*: buffer was 0.1 mol l^{-1} KH_2PO_4, pH adjusted to 7.0.

Plaque formation was determined by growing *St. mutans* 36 on the carbon source in pre-weighed plastic tubes. After growth the tubes were emptied, dried and weighed again.

DISCUSSION

Fermentation of fructooligosaccharides is very common among oral streptococci as can be concluded from our experiments. The presence of FOS fermenting streptococci is widespread among the population, as saliva of 40 volunteers and 4 piglets contained strains which were able to degrade FOS (data not shown). None of the volunteers or the piglets had ever consumed commercial FOS, as at the moment of the trial no products with added FOS were on the Dutch market. It has been shown previously (Walker *et al.*,1983) that oral streptococci possess fructanases. These fructanases are able to degrade levan- as well as inulin-type fructans. As the main fraction of FOS are oligomers of the inulin-type, it is not surprising that all the strains tested were able to degrade FOS.

The FOS-degrading enzymes may be inducible, as was shown for a strain of *St. salivarius*. This has also been demonstrated previously for *St. mutans* using D-fructans as a substrate (Burne *et al.*, 1987).

Various streptococci degraded FOS in a different way. Especially *St. mutans* and *St. gordonii* showed extensive degradation of all oligomers. *St. mitis*, however, showed mainly degradation of the Fn series, and hardly any degradation of the GFn series. This was surprising, as in a previous study no strains of *St. mitis* seemed to produce a fructanase, when pre-grown on glucose (Walker *et al.*, 1983). However, the latter studied

only extracellular fructanases using fructan as a substrate. It is possible that fructanases are not able to degrade oligosaccharides. It is possible that the FOS-degrading enzymes are not necessarily extracellular. Finally, it is also possible that the enzymes are inducible, and may be suppressed by pre-culturing on glucose.

The GF3 compound, nystose, seems to be relatively stable towards degradation by most strains. Only *St. mutans* 36 rapidly degraded nystose. *St. mutans* 44 degraded nearly all substrates except nystose. This would also explain the results obtained by other researchers. Ikeda *et al.* (1990) showed that nystose was slowly degraded or fermented by 5 strains of *St. mutans*, among them *St. mutans* NCTC 10449, which is the same as our strain 36. On the other hand Ziesenitz and Siebert (1987) showed that nystose was degraded rapidly by their strain of *St. mutans* NCTC 10449 and also with human mixed dental plaque bacteria. Walker *et al.* (1983) reported that fructanase activities ranged from 0 to 944 units per liter. In the same study they showed that the production of fructanase was dependent on pre-cultivation as well as medium conditions. Finally, fructanases produced by different strains showed different preferences towards fructans (Walker *et al.*, 1983). These factors may have contributed to the observed differences towards degradation of nystose.

All species produced acid from FOS, both from Raftilose and from Profeed. Acid production on Raftilose was similar to that on sucrose, about 0.11 mol l^{-1}. Profeed apparently is a less suitable substrate for *St. mutans* 36, as could be determined from the lower acid production and slower growth rate.

Rapid acidification studies with *St. mutans* 36 showed that acidification is as rapid on glucose as on FOS. The critical pH for dental caries (5.5-5.7) was reached within 15 minutes with 23 mmol l^{-1} FOS as substrate. This is somewhat longer than found with mixed dental plaque (Ziesenitz and Siebert, 1987), which is probably due to the lower cell concentration in our experiments. Even with a reduced concentration of Raftilose (2.3 mmol l^{-1}) the critical pH was reached within half an hour.

Artificial plaque formation was similar on Raftilose and on sucrose, around 17 mg was formed after 3 days. Plaque formation on Profeed was somewhat less, 13 mg after 3 days. This data is in contrast with the results of Hirasawa *et al.* (1984), who could not detect any plaque after 3 days incubation with Neosugar (Profeed). Even if only the sucrose unit of the GFn series would be utilized in plaque formation, it is to be expected that Neosugar, which consists entirely of the GFn series, would be a good substrate for

plaque formation. The slower production of plaque on Profeed may again be due to the slower growth rate of *St. mutans* 36 on Profeed.

Addition of buffer increased the plaque formation considerably to around 30 mg. Surprisingly plaque formation on Raftilose surpassed plaque formation on sucrose. As sucrose is the preferred substrate for *St. mutans* glycosyltransferases (Hamada and Slade, 1980) it is unlikely that Raftilose would be a better substrate. However, it has been described that oligosaccharides may act as a primer for *St. mutans* glycosyltransferase (Hamada and Slade, 1980) and this may be the explanation for the increased plaque formation. As growth and acid production on Profeed was slower than on Raftilose, it is likely that the Fn series are degraded more rapidly by the streptococci. This was confirmed with the degradation experiments. *St. mutans* 36 degraded the Fn series faster than the GFn series, and after 60 h considerably more degradation of the Fn series could be detected with the other strains.

As all strains grow and produce acid rapidly from Fn as GFn oligosaccharides, it can be concluded that both substrates are cariogenic, unless proven otherwise in animal studies on cariogenesis. Furthermore, artificial plaque formation occurs and this will enhance cariogenicity.

Comparison of both fructooligosaccharide preparations would lead to the conclusion that Raftilose is somewhat more cariogenic than Profeed, due to the slower growth rate and acid formation by the most cariogenic species, *St. mutans*, on Profeed.

3

IN VITRO CARIOGENICITY OF TRANSGALACTOSYL-OLIGOSACCHARIDES.

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SUMMARY

Transgalactosyl-oligosaccharides (TOS) are a class of oligosaccharides produced by transgalactosylation of lactose. TOS are used as bifidogenic factors in human and animal nutrition. TOS can be present in the oral cavity and form a risk for caries.

All oral bacteria tested were able to degrade and ferment both TOS and galactosyllactose (GLL), one of its components. Growth was improved as compared to carbohydrate-free media and acid was produced after 24 h incubation of the bacteria with TOS and GLL. Degradation patterns, using HPAEC, showed degradation of most components. Galactosyllactose was degraded only partially. Rapid acidification was only observed for *Streptococcus mutans*, resulting in a pH of 5.4 within 30 minutes. All other strains fermented TOS and GLL only slowly. Plaque formation could not be detected on both substrates.

It can be concluded that TOS and GLL possess only a small risk for caries formation, unless proven otherwise in animal studies.

INTRODUCTION

Transgalactosyl oligosaccharides (TOS) are a class of oligosaccharides produced from lactose using ß-galactosidase (EC 3.2.1.23). Several ß-galactosidases are known to possess transgalactosylation properties. These enzymes may be of fungal or bacterial origin (Smart, 1993, Zárate and López-Leiva, 1990; Toba *et al.*, 1981; Toba *et al.*, 1985; Asp *et al.*, 1980; Dumortier *et al.*, 1990). Commercial TOS is produced using ß-galactosidases from *St. thermophilus* (Greenberg and Mahoney, 1983), *Cryptococcus laurentii* or *Aspergillus oryzae* (Fujii and Komoto, 1991).

The composition of the resulting mixture is dependent on the enzyme and process conditions, like pH, temperature, salt and substrate concentration (Zárate and López-Leiva, 1990).

Several commercial TOS mixtures are available, both in Japan and in Europe. The general structure of the oligosaccharides in these mixtures is (gal)n-glu, with n ranging from 1 to 4. The glucosidic linkage between the residues may be ß-1,3, ß-1,4 or ß-1,6. This results in a large number of oligosaccharides in some of these mixtures (Tanaka *et al.* 1983; Smart, 1993).

TOS are marketed in Japan as bifidogenic factors. Several studies have shown that ingestion of TOS may result in an increase in bifidobacterial counts in the faeces in humans (Tanaka *et al.*, 1983; Ohtsuka *et al.*, 1989; Ito *et al.*, 1990; Fujita *et al.*, 1991; Yoneyama *et al.*,

1992; Ito *et al.*, 1993a,b; Ogata *et al.*, 1993) and animals (Terada *et al.*, 1992; Rowland and Tanaka, 1993; Terada *et al.*, 1993; Terada *et al.*, 1994).

Practically none of the oligosaccharides in the TOS mixtures can be digested by human intestinal lactase (ß-galactosidase). They are, however, rapidly degraded by the intestinal flora (Tanaka *et al.*, 1983; Fujita *et al.*, 1991). This implicates that many bacterial species have ß-galactosidases that are able to degrade this class of oligosaccharides. As these compounds are applied in food products, it may be possible that some part remains in the oral cavity. Many of the oral streptococci and lactobacilli are able to ferment lactose and thus possess ß-galactosidases (Zárate and López-Leiva, 1990). These enzymes may also degrade ß-galactooligosaccharides and fermentation of the resulting monomers may contribute to caries formation.

As little is known about the fermentation of these compounds by oral bacteria we determined their possibility to contribute to caries.

MATERIALS AND METHODS

Oligosaccharides

Galactosyllactose (GLL, Cup Oligo syrup) was a gift from Nissin Sugar Co., (Tokyo, Japan). This syrup also contains about 25% mono- and dimeric sugars (glucose, galactose and lactose) as well as traces of larger components as stated by the manufacturer.

TOS was obtained from Borculo Whey Products, Borculo Holland. This mixture contained less than 5% lactose and no monomeric sugars as stated by the manufacturer.

Strains

Oral bacteria used were the same as described previously (Hartemink *et al.*, 1995). These strains were : *St. mutans* 44 and *St. mutans* 36 (DSM 20523), *St. oralis* 45, *St. mitis* biovar I 46, *St. sanguis* biovar I 43 and *St. gordonii* biovar II 42. In addition a strain of *Lb. rhamnosus*, isolated from a human volunteer, was used.

Growth characteristics

Growth characteristics were determined by growing the strains in Peptone-yeast extract (PY) broth supplemented with 100 mmol l^{-1} phosphate buffer, pH 6.8 +/- 0.1, and 1% (w/v) of the test sugar. PY-broth was prepared according to Holdeman and Moore

(1977). Carbohydrates were sterilized separately at 121°C for 15 minutes and added before use.

Strains were cultured in 5 ml sugar-free PY or PY supplemented with lactose (PY-lac), galactosyl-lactose (PY-gll) or TOS (PY-tos) for 24 h at 37°C. After incubation 50 µl was added to 5 ml of the same medium. In addition 50 µl of the PY, PY-lac and PY-tos cultures were added to 5 ml of PY, PY-lac, PY-gll and PY-tos. This was done to determine induction by pre-culturing on lactose or TOS. From these tubes 250 µl was pipetted in triplicate in a microtiter plate. Growth was determined by measuring the optical density every 15 minutes for 20 h in a microtiter reader (SLT-240 ATCC, SLT Instruments, Groedig, Austria). From the resulting curves V_{max} and DOD were determined using the EasyKin software supplied with the reader.

Acidification studies

Acidification was determined by measuring the pH after 24 h incubation at 37°C in the media as described above.

Rapid acidification was determined by pre-growing *St. mutans* 36, *St. gordonii*, *St. oralis* and *Lb. rhamnosus* in 400 ml PY supplemented with 1% (w/v) sucrose for 24 h at 37°C. Cells were removed by centrifugation and washed twice with 400 ml reduced physiological saline solution (RPS). RPS consisted of (g l^{-1}) sodium chloride 8.5, cysteine.HCl 0.5 and bacto-peptone 1.0, pH was adjusted to 6.8 +/- 0.1 using 3 mol l^{-1} NaOH.

After the washing steps the pellet was dissolved in 7 ml RPS and stored at 7°C until use. To 5 ml pre-heated RPS or RPS supplemented with 1% (w/v) carbohydrate, 1 ml of the cell suspension was added. The tube was immediately placed in an incubator at 37°C and a pH electrode was added. The pH was measured continuously for 60 minutes. In another experiment the cell suspension was replaced by saliva. The saliva was a mixture of saliva of three healthy volunteers.

Carbohydrate analysis

After incubation as described previously, samples were taken for carbohydrate analysis and stored at -80°C. Carbohydrate analysis was performed to determine which compounds were degraded by the bacteria.

Carbohydrates were determined using a HPAEC (High Performance Anion Exchange Chromatography) system. HPAEC was performed using a BIO-LC system (Sunnyvale, California, USA), equipped with a Dionex Carbopac PA-100 (4x250 mm)

column and a Dionex pulsed electrochemical detector in the pulsed amperometric detection method as described previously (Hartemink *et al.*, 1995).

Acid analysis

After incubation as described above, samples were taken from the acidified tubes for acid analysis. Samples were stored at -80°C before analysis. Lactic and acetic acids were determined using HPLC with an Aminex HPX-87H column and 0.01 mol l^{-1} sulfuric acid as the mobile phase as described previously (Hartemink *et al.*, 1995).

Plaque formation

Plaque formation on TOS and GLL was determined as described previously (Hartemink *et al.*, 1995), using *St. mutans* 36 as the test strain.

RESULTS

Growth characteristics

All strains showed higher final optical density and increased growth rates with PY-lac, PY-gll and PY-tos as compared with the carbohydrate free PY, indicating fermentation of the carbohydrates.

Acidification studies

A final pH more than 0.5 units below the carbohydrate-free PY was considered positive for fermentation. All strains showed a clear decrease in pH in PY-lac, PY-gll and PY-tos, indicating fermentation of all substrates (Table 3.1). It should, however, be taken in consideration that significant concentrations of lactose are present in PY-gll.

Rapid acidification studies showed no rapid pH decrease for saliva, *Lb. rhamnosus*, *St. gordonii* and *St. oralis*. *St. mutans* fermented TOS rapidly, resulting in a final pH of 5.4 after 30 minutes.

Acid analysis

Acid analysis showed production of lactic and acetic acids from all substrates, indicating extensive fermentation (Table 3.1). Lactic acid was the major acid produced after fermentation of both GLL and TOS by all strains. Acetic acid production was higher for all strains on TOS as a substrate, as compared to GLL as a substrate. Total acid production on GLL ranged from 33.1 to 87.6 mmol l^{-1} and from 58.9 to 80.7 with TOS as the substrate. *Ac. naeslundii*, *Lb. rhamnosus* and *St. sanguis* produced similar

amounts of acid on both substrates. *St. gordonii* and *St. mutans* 44 produced more acid on GLL, whereas the other strains produced more acid on TOS as a substrate. Especially *St. mitis* produced only limited amounts of acid on GLL, but 80.7 mmol l-1 on TOS.

Table 3.1: Acid formation by oral bacteria after 24h incubation at 37°C on buffered PY medium supplemented with 1% (w/v) of the carbohydrate tested. The strains were precultured for 24h at 37°C in the same medium.

Species	Strain	PY			PY-lac			PY-gll			PY-tos		
		pH*	lactic** acid	acetic acid	pH	lactic acid	acetic acid	pH	lactic acid	acetic acid	pH	lactic acid	acetic acid
Ac. naeslundii	41	-	n.t.	n.t.	+	n.t.	n.t.	+	57.6	8.8	+	55.2	12.7
Lb. rhamnosus		-	n.t.	n.t.	++	n.t.	n.t.	+	63.7	7.8	+	61.3	9.3
St. gordonii	42	-	n.t.	n.t.	+	n.t.	n.t.	+	67.6	21.0	+	64.1	13.6
St. mitis	46	-	n.t.	n.t.	++	n.t.	n.t.	+	21.5	11.6	+	56.8	23.9
St. mutans	36	-	5.7	6.8	++	56.2	5.1	++	43.2	7.5	++	48.7	10.2
St. mutans	44	-	n.t.	n.t.	+	n.t.	n.t.	+	57.9	12.6	+	57.8	8.9
St. oralis	45	-	n.t.	n.t.	++	n.t.	n.t.	++	50.5	7.7	++	51.9	19.9
St. sanguis	43	-	n.t.	n.t.	+	n.t.	n.t.	+	57.0	8.5	+	56.3	8.6

* pH: - no decrease in pH (pH between 6.3 and 6.7), + pH between 5.0 and 6.0, ++ pH below 5.0
** acid in mmol l^{-1} n.t. : not tested

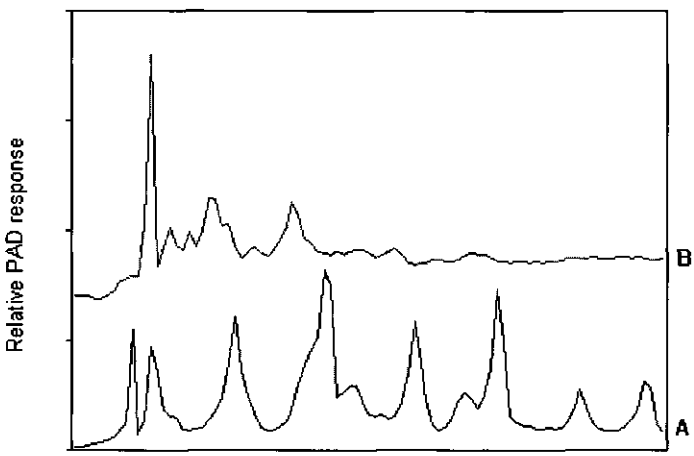

Figure 3.1: Carbohydrate degradation pattern of PY-tos after incubation with *St. oralis* 45 for 24h at 37°C; A : HPAEC pattern of PY-tos before incubation with *St. oralis* 45; B : HPAEC pattern of PY-tos after 24 h incubation at 37°C with *St. oralis* 45

Carbohydrate analysis

HPAEC analysis showed degradation of all major components of the TOS mixtures by all strains. Degradation was nearly complete for *St. oralis* (Fig 3.1) and *St. mitis*. The other species degraded mainly the higher components. Galactosyllactose in the TOS mixture was degraded only partially by most strains. Pure galactosyllactose was degraded slowly by all strains (Fig 3.2). No complete degradation was observed.

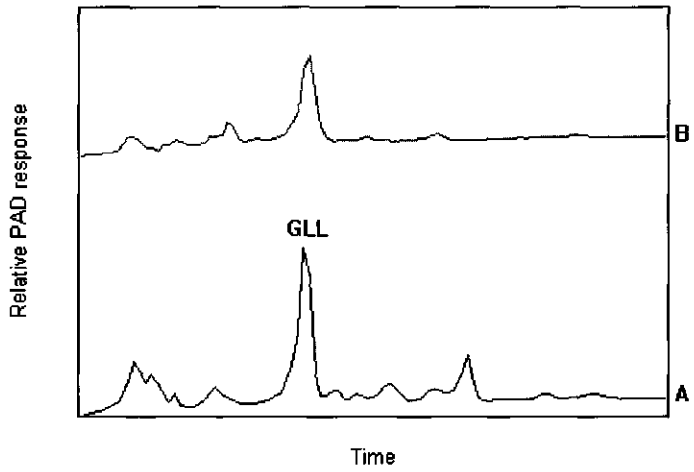

Figure 3.2: Carbohydrate degradation pattern of PY-gll after incubation with *St. gordonii* 42 for 24h at 37°C.; A : HPAEC pattern of PY-gll before incubation with *St. gordonii* 42; B : HPAEC pattern of PY-gll after 24 h incubation at 37°C with *St. gordonii* 42

Plaque formation

No plaque could be detected on any of the substrates tested after 3 days of incubation.

DISCUSSION

It has been shown that all oral bacteria tested were able to grow on both TOS and GLL. Growth was enhanced and final cell yield, as judged by optical density, increased.

All strains were able to produce acid from TOS and GLL. As all strains are able to grow on lactose, they possess intra- or extracellular ß-galactosidases. It is therefore not surprising that several components from the TOS mixture are degraded. Most strains produced lactic as well as acetic acid, indicating heterofermentative fermentation. Total acid production was somewhat higher on TOS than on GLL. This is in according with the degradation patterns obtained using HPAEC. Galactosyllactose was fermented

slowly by all the strains. Similarly GLL present in the TOS mixtures was not or not completely degraded. The reason for this is not known.

Rapid acidification showed that only *St. mutans* 36 was able to acidify the medium within 30 minutes to pH 5.4. The other strains degraded and fermented TOS and GLL too slow to show any significant pH drop within 90 minutes. Preculturing on TOS or GLL did not increase the acidification rate (data not shown). As *St. mutans* is the main cariogenic species, the rapid acidification may be a risk factor for caries. The other bacteria do not form a risk for caries formation, due to the slow acidification rate. Saliva has a buffering capacity that will neutralize the acid produced. This was confirmed by the test with saliva, where no rapid acidification could be detected.

Plaque formation was absent for *St. mutans*. Together with rapid acidification, plaque formation is a risk factor for caries formation. Plaque consists mainly of bacterial polysaccharides. These polysaccharides are both glucans and levans. As TOS and GLL are mainly composed of galactose, transglycosylation to plaque polysaccharides is unlikely (Schemmel *et al.*, 1982). These results are in accordance with Balekjian *et al.* (1977), who did not observe plaque formation on lactose. However, lactose was found to be cariogenic in rats (Schemmel *et al.*, 1982) and humans (Koulourides *et al.*, 1976).

It can therefore be concluded that oral bacteria are able to degrade and ferment TOS and GLL. Acid is produced, but, with the exception of *St. mutans*, the fermentation process is relatively slow. In addition plaque is not formed, which decreases the cariogenicity of these substances. The risk of caries formation from TOS and GLL can therefore be considered rather low. As acid is produced, the risk is not absent. Studies using animal models should be undertaken to determine the actual risk *in vivo*.

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4

FERMENTATION OF XYLOGLUCAN BY INTESTINAL BACTERIA

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SUMMARY

Xyloglucan, a cell wall polysaccharide and part of the dietary fiber fraction of the diet, can be degraded by the intestinal microflora. Degradation is not common among intestinal bacterial species. Of 138 strains (representing 75 species) only nine strains were able to degrade xyloglucan polymer. These strains, seven clostridia, one bifidobacterium and one bacteroides, all produced apparent extracellular enzymes, which degraded the glucan backbone. Gas production was detected with faecal slurries and the individual clostridia. The other two strains did not produce any gas. Selective elimination of bacterial groups from faecal slurries indicated that clostridia are the main group of intestinal bacteria responsible for degradation of xyloglucan polymer. In addition to the polymer-degrading strains, some other strains were able to degrade and ferment oligosaccharides produced from tamarind seed xyloglucan with a fungal endo-ß-glucanase. HPAEC analysis showed that the fungal oligosaccharides were identical to the oligosaccharides produced by the polymer-degrading species suggesting the presence of an endo-ß-glucanase in these bacteria. It is proposed that xyloglucan is degraded *in vivo* by endo-ß-glucanases produced mainly by clostridia, followed by fermentation of the oligosaccharides by a larger group of bacteria.

INTRODUCTION

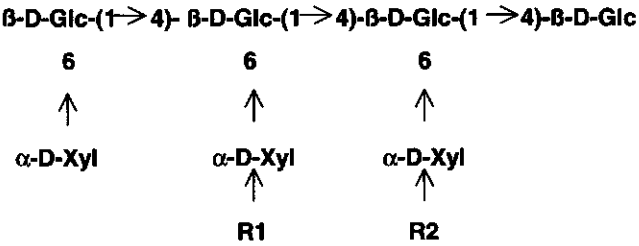
Non-digested polysaccharides from the diet can be separated in three different groups; resistant starch, hemicelluloses and cellulose. All three groups are fermented to some extent by the intestinal microflora to short chain fatty acids, hydrogen and carbon dioxide (Hill, 1995).

The hemicelluloses are a diverse group of plant cell wall polysaccharides, including galactomannans, arabinoxylans, pectic substances and many more. One of the lesser studied polysaccharides from cell wall material is xyloglucan. Xyloglucan consists of a ß-1-4 glucosidic backbone, with side chains made up of xylose, galactose and, occasionally, fucose (Belitz and Grosch, 1987; Maclachlan, 1987; York *et al.*, 1993). Xyloglucans are present in cell walls of many plants, like rice (Shibuya and Misaki, 1978), cotton (Hayashia and Delmer, 1988), mung beans (Kato and Matsuda, 1980a), peas, potatoes and wheat (Maclachlan, 1987). Xyloglucans are generally present in relatively small quantities. In tamarind (*Tamarindus indica*) seeds, however, xyloglucan is the predominant polysaccharide. This xyloglucan can be obtained rather easily, and is commercially available in large quantities (Belitz and Grosch, 1987). It lacks the fucose side

chains and consists of a glucan backbone with xylose-galactose side-chains attached at regular intervals (Fig 4.1) (York *et al.*, 1993).

Xyloglucan from tamarind seed is used as pectin substitute in the production of jellies and marmalades. Xyloglucan can also be used as a thickening agent and stabilizer in ice cream and mayonnaise (Belitz and Grosch, 1987).

It can thus reasonably be assumed that xyloglucans form a part of the daily intake of dietary fiber. Xyloglucans cannot be digested by the human small intestinal enzymes (Sone *et al.*, 1992). Xyloglucans thus reach the colon unaltered. It is to be expected that xyloglucans, like other hemicelluloses, can be degraded and fermented by the intestinal bacteria. Whereas no published data are available on fermentation of xyloglucan by bacteria, degradation and fermentation by other micro-organisms, such as fungi, have been studied in detail (Kato and Matsuda, 1980a,b).



1. **R1=R2=ß-D-Gal**
2. **R1=H, R2=ß-D-Gal**
3. **R1=ß-D-Gal, R2=H**
4. **R1=R2=H**

Fig 4.1 : Structure of xyloglucan-oligomers (York *et al.*, 1993).

Several mechanisms of xyloglucan degradation can be postulated. In the first mechanism the side-chains are degraded and fermented before the backbone is degraded. The second mechanism assumes degradation of the backbone into smaller oligosaccharides with the side chains still attached. In the third mechanism both processes occur simultaneously.

It is well known that intestinal bacteria possess a large number of cell-associated or extracellular enzymes, which are able to degrade polysaccharides. Polysaccharide degradation by intestinal bacteria has been reported for *Bacteroides* spp., *Bifidobacterium* spp. and *Clostridium* spp.(Salyers *et al.*, 1977; Crociani *et al.*, 1994; Englyst *et*

al., 1987). As several of these enzymes might be of commercial interest, we determined the ability of intestinal bacteria to degrade xyloglucan from tamarind seed.

MATERIALS AND METHODS

Xyloglucan

Xyloglucan polymer (XYG-P) from tamarind seed was obtained from Dainippon Pharmaceutical, Osaka, Japan. It was essentially free of oligosaccharides and monosaccharides as was judged by high-performance anion exchange chromatography (HPAEC).

Xyloglucan oligomers (XYG-O) were prepared from the polymer by enzymatic hydrolysis, using an endo-ß-glucanase obtained from *Trichoderma viride* (Beldman *et al.*, 1985). The resulting mixture consisted of four oligosaccharides and no monomeric carbohydrates could be detected using HPAEC (Vincken *et al.*, 1995). These oligosaccharides were used as standards for the products obtained after liquefaction.

Strains

All strains were from the culture collection from our Department. Most strains were of human origin, some strains originated from porcine faeces. A total of 135 strains was tested for XYG-P liquefaction: 10 strains of the *Bacteroides fragilis* group (5 species), 21 bifidobacteria (6 species), 37 clostridia (21 species), *Eubacterium limosum*, 7 lactobacilli (3 species), 12 enterococci (3 species), 28 enterobacteria (19 species or serotypes), 5 fusobacteria (3 species), 4 *Actinomyces* sp. (3 species), 2 *Prevotella* species, 6 streptococci (5 species) and two species of peptostreptococci.

Previously unidentified strains were characterized using the API 50CH system (BioMerieux, France) for carbohydrate fermentation combined with Gram staining, gas formation and spore formation (plating after heating for 15 min at 80°C) (Holdeman and Moore, 1977).

Liquefaction

Screening for xyloglucan degradation was done by liquefaction of a XYG-P gel. A 5% (w/v) XYG-P gel was prepared in PY broth. PY broth was prepared as described previously (Holdeman and Moore, 1977). This mixture was boiled to dissolve all XYG-P and, while still hot, poured in screw-capped test tubes. These were sterilized and kept closed at room temperature until use. Strains or faecal slurries were pre-grown in carbohydrate

free thioglycollate broth, or PY broth supplemented with 1% glucose (PYG) anaerobically at 37°C for 24-48 hour. XYG-P tubes were inoculated using sterile tooth picks, each pure culture was inoculated in triplicate, faecal slurries in duplicate. The inoculated tubes were incubated anaerobically at 37°C for 24-96 hours. Liquefaction indicated degradation of the polymeric xyloglucan. Syringes were attached to the tubes to determine gas production.

Table 4.1 : Treatment of faecal slurries for elimination of specific bacterial groups.

Treatment	Medium	Conditions/additions	Number of subcultures	Selected group	Reference
A	RCM	none	-	*all* faecal bacteria	
B	RCM	10 min 80°C	1	sporeformers	
C	RCM	10 min 80°C, aerobic	1	bacilli	
D	RCM	aerobic	2	aerobes	
E	RCM	vancomycin 2 mg/ml[1]	2	Gram-negative bacteria	Chow and Cheng, 1988
F	RCM	cefpirome 200 mg/l[2]	2	resistant anaerobes	Kato *et al.*, 1993 Scriver *et al.*, 1992
G	RCM	D, E and F combined	2	resistant aerobes	
H	RCM	1 ml acetic acid 96%, pH 5.2	2	lactic acid bacteria	
I	RB	pH 5.9	2	bifidobacteria	Hartemink *et al.*, 1996

1 : Vancomycin was obtained from Eli-Lilly, 2 : Cefpirome sulfate was obtained from Roussel-Uclaf.

Faecal studies

To determine whether faecal slurries were able to degrade XYG-P and XYG-O, liquefaction tests were performed. Faecal slurries were prepared by mixing fresh human faeces from several healthy volunteers with anaerobic storage medium (Crowther, 1971) in an anaerobic cabinet. Slurries were kept at -80°C before use. To determine which groups of micro-organisms were able to degrade XYG-P, faecal slurries were prepared in which certain groups were eliminated as follows: Three faecal slurries (3 ml each) were diluted in 80 ml Reinforced Clostridial Medium (RCM, Oxoid, Basingstoke, UK). From these mixtures 1 ml samples were transferred into 80 ml RCM supplemented with antibiotics (vancomycin, obtained from Eli Lilly, and cefpirome sulfate, obtained from Roussel-Uclaf) or in RB-medium (Hartemink *et al.*, 1996), according to Table 4.1. For some of the options this procedure was repeated after 24h incubation. After another 24h

incubation one ml was used to inoculate a 20 ml XYG-P gel. Liquefaction was determined as described above.

Oligosaccharide fermentation

Eighteen strains, both negative and positive in the liquefaction test, were further screened for XYG-O degradation and fermentation. Strains pre-grown in PYG or thioglycollate broth were subcultured in triplicate in PY broth supplemented with 1% XYG-O and 0.01% bromocresol purple as pH indicator. After anaerobic incubation for 24, 48 and 72 hours at 37 °C, pH was measured and a final pH of 1.0 pH unit below a control (non-supplemented PY) tube was considered positive. Strains were checked for morphology before and after the incubation.

HPAEC

Oligosaccharides produced from XYG-P by fungal endo-ß-glucanase and bacterial incubation were separated using HPAEC. The HPAEC system consisted of a CarboPac PA-100 column, with a 1 M acetic acid (A) in 100 mM NaOH (B) gradient and PAD detection. The gradient conditions were as follows: T=0 min : A 100 %, T=5 min A 97%, B 3%, T=45 min A 92%, B 8%, T=55 min A 80% B 20%, T=60 min B 100%, T=65 min B 100%, T=66 min A 100% (Vincken *et al.*, 1995).

Enzyme location

Strains positive for XYG-P liquefaction were used to determine whether the liquefying enzymes were extracellular. Strains were cultured in PYG broth at 37°C for 24-48 h. The culture was centrifuged 15 min at 10.000 rpm to precipitate the cells. The supernatant was added to a sterile test tube containing a 5% gel of XYG-P in water, prepared as described above. Liquefaction was monitored after incubation at 37°C for 24, 48, 72 and 96 h. Degradation was confirmed using HPAEC as described above.

RESULTS

Faecal studies

The results of incubation of XYG-P gels with faecal slurries are summarized in Table 4.2. All three untreated faecal slurries (A) liquefied XYG-P gels completely. Gas could be detected. Phase contrast microscopic analysis showed the presence of different types of cocci and rods. Heat treatment, which selectively isolates spores, resulted in complete liquefaction with gas production for the anaerobic samples (B).

Only one of the three heat-treated aerobic samples (C-3) showed complete liquefaction with gas formation. One other sample (C-1) showed some liquefaction without gas. Microscopy showed only short rods in samples C-1 and C-2, but also some streptococci in sample C-3.

Aerobic incubation of faecal slurries (D) resulted in good growth after two days of pre-incubation. Many different bacterial groups were present as could be judged by microscopy. However, no liquefaction was observed. Only in sample D-2 some limited liquefaction occurred, the sample was somewhat fluid but far from liquefied. No gas was detectable.

Treatment with antibiotics (E-G) resulted in good growth in all samples after the second subculturing step, but did not result in any liquefaction. No gas was detected in any sample. In the vancomycin-treated samples (E) mainly long rods were visible with some streptococci. In sample E-3 the streptococci were more abundant than the rods. In the cefpirome-treated samples (F) predominantly streptococci were visible. Combination of the antibiotics with aerobiosis did not result in any growth after 96h of incubation. Growth of lactic acid bacteria was favored by acidification of the medium (H). In all three samples long and short rods were visible, with some bifidobacteria in sample H-3. Some liquefaction was detectable in all three samples. Gas production was absent. Pre-incubation in RB medium (I) resulted in predominantly bifidobacteria, with some straight rods in samples I-2 and I-3. Only samples I-1 and I-2 showed some liquefaction, without gas formation.

Liquefaction

Only 9 strains of 135 showed liquefaction of the XYG-P gel after 96 h of incubation. These strains are shown in Table 3. Gas was produced by all clostridia, especially *Cl. beijerinckii* Cl-7, *Cl. novyi* Cl-8, *Cl. butyricum* Cl-26 and *Cl. sartagoforme* Cl-27. The two other strains, *Bi. infantis* Bi-223 and *B. ovatus* B-1 did not produce significant amounts of gas.

Table 4.2 : Liquefaction of XYG-P with faecal slurries treated to eliminate specific groups.

Treatment	sample	selected group	pH 24h	pH 48h	growth 24h	growth 48h	gas 24h	gas 48h	morphology after pre-incubations*	lique-faction	morphology in liquefied sample	pH	gas
A*	1	all faecal	n.t.#	n.t.	n.t.	n.t.	n.t.	n.t.	long + short rods, cocci, streptococci	++	rods, cocci	5.1	++
	2	bacteria	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	long + short rods, cocci, streptococci	++	rods, cocci	4.9	++
	3		n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	long + short rods, cocci, streptococci	++	rods, cocci	5.2	++
B	1	spore-	5.4	n.t.	++	n.t.	++	n.t.	rods	++	rods	5.8	++
	2	formers	5.4	n.t.	++	n.t.	++	n.t.	rods	++	rods	5.3	++
	3		5.3	n.t.	++	n.t.	++	n.t.	rods + duplococci	++	rods + duplococci	4.9	++
C	1	bacilli	5.4	n.t.	++	n.t.	++	n.t.	rods	+	rods	n.t.	-
	2		n.t.		-	n.t.	-	n.t.					
	3		n.t.		++	n.t.	++	n.t.	rods + streptococci	++	rods + streptococci	5.0	++
			5.5	n.t.									
D	1	aerobes	4.7	4.8	++	++	-	-	long + short rods, cocci, streptococci	-			
	2		4.7	4.7	++	++	-	-	long + short rods, cocci, streptococci	+		n.t.	-
	3		4.8	4.7	++	++	-	-	long + short rods, cocci, streptococci	-			
E	1	Gram	5.8	4.5	+	++	-	-	long rods	-			
	2	negative	5.4	4.5	+	++	-	-	long rods, streptococci	-			
	3	bacteria	6.4	4.7	+	++	-	-	streptococci, long rods	-			
F	1	resistant	7.0	5.3	+	+	-	-	streptococci	-			
	2	anaerobes	6.6	4.7	+	++	-	-	streptococci, rods	-			
	3		6.8	4.7	+	++	-	-	streptococci	-			
G	1	resistant	n.t.	n.t.	-	-	n.t.	n.t.					
	2	aerobes	n.t.	n.t.	-	n.t.	n.t.	n.t.					
	3		n.t.	n.t.	-	n.t.	n.t.	n.t.					
H	1	lactic acid	5.1	5.1	++	++	-	-	long and short rods	+		n.t.	-
	2	bacteria	5.1	5.2	++	++	-	-	long and short rods	+		n.t.	-
	3		5.7	5.2	+	++	-	-	long and short rods, bifidobacteria	+		n.t.	-
I	1	bifido-	5.5	5.2	++	++	-	-	bifidobacteria	+		n.t.	-
	2	bacteria	5.4	5.2	++	++	-	-	bifidobacteria, short rods	+		n.t.	-
	3		5.9	5.8	+	+	-	-	bifidobacteria, long rods	-			

* For treatments, see Table 1. # n.t. : not tested, * cocci = single coccoid cells, duplococci = paired coccoid cells, streptococci = long or short strains of coccoid cells, bifidobacteria = branched or pleomorphic rods

Table 4.3 : Strains positive in the liquefaction of XYG-P, with the final pH reached after liquefaction.

Species	strain	origin	final pH
Bacteroides ovatus	B-1	ATCC 8483 (type)	5.3
Bifidobacterium infantis	Bi-223	DSM 20223	4.4
Clostridium beijerinckii	Cl-7	human faeces	4.7
Cl. beijerinckii	Cl-30	human faeces	5.9
Cl. butyricum	Cl-26	human faeces	5.0
Cl. clostridioforme	Cl-3	human faeces	4.7
Cl. novyi	Cl-8	human faeces	4.8
Cl. sartagoforme	Cl-27	human faeces	5.3
Cl. sordelli	Cl-9	ATCC 9714	5.3

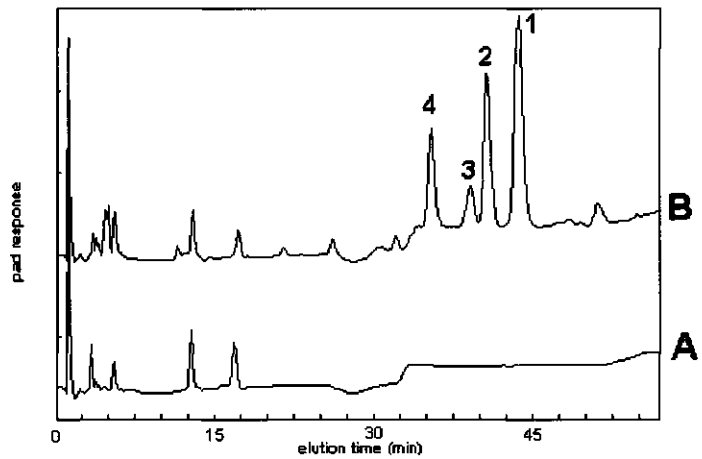


Figure 4.2 : Degradation of xyloglucan polymer and formation of oligomers after incubation of a xyloglucan polymer gel with and *Cl. sartagoforme* Cl-27.

A: Blank sample of tamarind seed xyloglucan polymer, B: Sample after incubation with *Cl. sartagoforme* Cl-27 The numbers refer to the structures as shown in Fig 4.1.

HPAEC revealed production of oligosaccharides from the XYG-P gel. The degradation patterns of *Cl. butyricum* Cl-26 and *Cl. sartagoforme* Cl-27 are shown in Fig 4.2. The 4 major peaks co-eluted with the four peaks that were present after incubation of XYG-P with fungal endo-ß-glucanase. The structures of these oligosaccharides are shown in Fig 4.1 (York *et al.*, 1993).

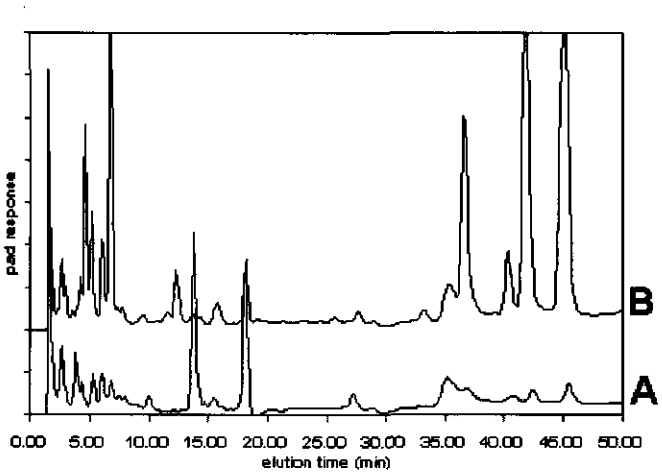


Fig 4.3 : Degradation of xyloglucan oligomers after incubation with *Lb. acidophilus* 128

A: Sample of xyloglucan oligomers as obtained after degradation of xyloglucan polymer from Tamarind seed by endoglucanase from *Trichoderma viride* after incubation with *Lb. acidophilus* 128. B: Blank sample.

Table 4.4 Ability of selected faecal bacteria to degrade* and ferment XYG-O.

Species	origin	final pH	degra-dation	Species	origin	final pH	degra-dation
- (blank)		7.2	-	*Cl. perfringens*	human faeces	6.8	-
B. fragilis	ATCC 25285	6.9	-	*Cl. ramosum*	human faeces	7.9	-
B. ovatus	ATCC 8483	6.3	-	*Cl. sartagoforme*	human faeces	7.6	+
B. thetaiotaomicron	ATCC 29741	**5.8**	-	*Cl. sporogenes*	human faeces	7.8	-
B. vulgatus	ATCC 8482	**6.0**	-	*E. coli*		7.6	-
Bi. adolescentis	DSM 20083	**5.7**	-	*E. coli*	human faeces	6.6	+
Bi. breve	DSM 15700	6.9	-	*Kl. pneumoniae*	human faeces	6.9	++
Bi. infantis	DSM 20088	6.4	-	*Lb. acidophilus*	swine faeces	7.0	+
Bi. longum	DSM 20219	**5.1**	-	*Lb. casei*	Yakult	**5.7**	-
Cl. beijerinckii	human faeces	7.5	++	*Lb. fermentum*	swine faeces	**6.1**	-
Cl. clostridioforme	human faeces	**6.0**	++	*R. productus*	ATCC 35244	**5.5**	-

pH values shown in bold are considered positive for fermentation.

* - : no clear degradation or fermentation, + : some degradation, ++ : complete degradation

Oligosaccharide fermentation

The results of the degradation of XYG-O are summarized in Table 4.4. Of the 18 strains tested only 8 were able to ferment XYG-O as was judged by a drop in pH. However, HPAEC analysis revealed that not all these strains were able to degrade the oligosaccharides. Complete fermentation could only be observed with three strains: *Cl. butyricum* Cl-26, *Cl. clostridioforme* Cl-3 and *Kl. pneumoniae* 119. Partial degradation was observed with *E. coli* 103 and *Lb. acidophilus* 128 (Fig 4.3).

Enzyme location

All strains that were positive in the liquefaction test (Table 3) produced extracellular enzymes as was judged by liquefaction of an aqueous XYG-P gel. HPAEC analysis of the resulting fluid revealed the presence of the same oligosaccharides as could be produced with the fungal endo-ß-glucanase. In addition some small unidentified oligomeric structures and larger oligosaccharides could be detected. No monomeric sugars could be detected.

DISCUSSION

Xyloglucan can be fermented rapidly by the human intestinal microflora as was shown with faecal inocula. Selective elimination of specific groups from faecal slurries indicated a major role for clostridia. Samples were heated at 80°C to eliminate all vegetative cells. To distinguish between clostridia and bacilli, a series of heat-treated samples was incubated aerobically. All three anaerobic samples showed complete liquefaction and production of gas. Microscopic analysis revealed the presence of streptococci in two samples. This was also reflected in a lower final pH (Table 4.2). Further purification of these strains and subsequent liquefaction showed that the streptococci were not able to degrade XYG-P, whereas the rods liquefied the gel and produced gas (data not shown). The liquefaction in all samples was therefore due to the rod shaped bacteria. As aerobic incubation showed much less or no liquefaction, it can be concluded that in the anaerobic samples liquefaction was due to anaerobic spore-forming gas producing Gram-positive rods. This strongly indicates *Clostridium* species. The presence of streptococci in two samples indicates that the heating procedure was not sufficiently long for these samples. As some liquefaction occurred under aerobic conditions it can be concluded that aerotolerant *Clostridium* species or *Bacillus* species may be responsible for liquefaction. It is known that some *Clostridium*

species are aerotolerant (*Cl. perfringens*) or able to grow under aerobic conditions (*Cl. tertium*) (Cato *et al.*, 1986).

The use of vancomycin against Gram-positives (Chow and Cheng., 1988; Holliman and Bone, 1988; Green *et al.*, 1991) did not result in liquefaction. It can be concluded that the strains responsible for liquefaction are probably Gram-positive.

Literature data on cefpirome are limited, but indicate resistance to the concentrations used for *Bacteroides* sp., *Fusobacterium* sp. and *Cl. difficile*. Aerobes are susceptible (Kato *et al.*, 1993; Scriver *et al.*, 1992). It can be concluded that the strains responsible for liquefaction are susceptible to cefpirome, which excludes most *Bacteroides* sp., *Fusobacterium* sp. and *Cl. difficile*.

Combination of antibiotics and aerobic treatment resulted in complete absence of growth. This indicated that the resistant organisms were anaerobic.

Selective isolation of lactic acid bacteria (*Lactobacillus*, *Bifidobacterium*, *Enterococcus* and *Streptococcus* species) was carried out using acetic acid and a low pH. After two subculturing steps only rods were observed. In the third sample some bifidobacteria were present. As most other rods in faeces (clostridia, bacteroides, fusobacteria, enterobacteria) fail to grow in the presence of acetic acid at these low pH values (Cato *et al.*, 1993) the rods present were considered *Lactobacillus* species. No complete liquefaction was observed, although the gel was somewhat fluid. Gas was not produced. It is concluded that lactobacilli are not responsible for the liquefaction in faecal slurries.

Bifidobacteria can selectively be isolated using RB medium (Hartemink *et al.*, 1996). After two subculturing steps only bifidobacteria were present in the samples. In samples 2 and 3 some rods were present, most likely unbranched bifidobacteria. Liquefaction did not occur, gas was not produced. It is concluded that bifidobacteria are not responsible for the liquefaction in faecal slurries.

The results mentioned above indicate that the strains responsible for liquefaction are anaerobic and Gram-positive. Bifidobacteria, enterococci, streptococci and lactobacilli could be excluded. Heat treatment did not affect liquefaction. These results strongly suggest that clostridia are the main group of bacteria responsible for the liquefaction of xyloglucan.

To test whether pure strains of intestinal bacteria were able to degrade XYG-P 135 strains were tested, representing 75 species. Of these only 9 were able to degrade the polymer and liquefy the sample. Seven of these strains were clostridia and all

produced gas. Both other strains, *Bi. infantis* Bi-223 and *B. ovatus* B-1, did not produce any gas. Liquefaction with the latter two strains was also slower than with the clostridia. These data are consistent with the data observed with the faecal slurries, indicating strongly the role of clostridia in the degradation of xyloglucan.

Degradation of XYG-P gels resulted in formation of oligosaccharides. These oligosaccharides were similar to oligosaccharides produced with a fungal endo-ß-glucanase. This result suggests the presence of an endo-ß-glucanase in the positive strains. It was also very likely that the endo-ß-glucanase was excreted, although it can not be excluded that the enzyme originates from dead cells. No other degradation products could be observed in large amounts using culture supernatants. This result indicates that other enzymes necessary for degradation are cell-bound or intracellular. It also implies that degradation of xyloglucan with endo-ß-glucanase results in oligosaccharides that may be available for all other bacteria. This conclusion confirms the results with faecal slurry studies, where many other bacteria were present in liquefied samples that were not precultured. Similarly, in heat-treated samples where streptococci were present, the final pH was lowered, possibly indicating growth on degradation products of xyloglucan.

We therefore tested the degradation of XYG-O with a selection of intestinal bacteria. XYG-O was produced as described previously, which resulted in a mixture that is similar to the oligosaccharide mixture produced by the different clostridia. It was shown that some other intestinal bacteria, which were not able to liquefy the polysaccharide, were able to ferment the oligosaccharides completely. Not all species that were able to liquefy the gel were able to degrade and ferment the oligosaccharides. This may be due to two factors, either the strains possess an endo-ß-glucosidase, but not the enzymes for further degradation, or the growth medium alone supported growth and the necessary enzymes for degradation were not induced. As liquefaction of the gel results in a low final pH and gas production, it is unlikely that the strains do not possess the other enzymes. It can thus be concluded that the oligosaccharides are a 'difficult' substrate for many species and complete degradation will only occur under substrate-limiting conditions.

It was also noted (Table 4.4) that a decrease in pH did not always correspond with degradation of the oligomers and vice-versa. This may be due to the presence of other fermentable material in the XYG-O preparation. On the other hand, some species (*Cl. beijerinckii*) may ferment the oligosaccharides slowly. Simultaneous protein

degradation, which results in an increase in pH, thus masks the oligosaccharide fermentation. It is therefore necessary for substrates that are fermented slowly to determine degradation using HPLC.

In conclusion we propose the following model for xyloglucan degradation by the intestinal microflora. Xyloglucan is degraded by endo-ß-glucanases, mainly produced by *Clostridium* species, to oligosaccharides. These oligosaccharides are further degraded both by some of the same species, as well as some other bacteria. The complete mechanism of this latter degradation remains to be elucidated. Contrary to many other plant cell wall polysaccharides (Salyers *et al.*, 1977; Salyers *et al.*, 1978; Degnan and Macfarlane, 1995), xyloglucan is degraded mainly by clostridia, not by bacteroides or bifidobacteria. This may result in a relatively large production of gas after ingestion of this polysaccharide.

Acknowledgements

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5

DEGRADATION OF GUAR GUM BY INTESTINAL BACTERIA.

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Bioscience and Microflora, in press

SUMMARY

Guar gum is widely used in the food industry as a thickening agent. Guar and other galactomannans are ingested as a normal part of the human diet. Guar is completely degraded in the large intestine. Often large amounts of gas are produced. It was observed that only a limited number of species is able to degrade and ferment guar. Guar degrading species are present in faeces in numbers over 10^8/g and in saliva in numbers over 10^5/g. Guar degrading strains could be isolated from faecal samples of all volunteers and in 90% of the saliva of volunteers.

The main species isolated from humans were *Bi. dentium* and *Cl. butyricum*. From several samples of animal faeces *St. bovis* could be isolated. In addition some strains of *Bacteroides ovatus* were able to degrade guar to a limited extent.

Fermentation resulted in the production of short-chain fatty acids and, when *Cl. butyricum* was present, in a large gas production. Competition experiments showed that *Cl. butyricum* degrades guar faster than both other species under simulated physiological conditions.

INTRODUCTION

Guar gum consists of a (1→4)-ß-linked D-mannan backbone which carries single unit (1→6)-α-linked D-galactose-residues with a mannose-galactose ratio of 1:2 (Figure 5.1). It is obtained from the seeds of the guar plant or cluster bean (*Cyamopsis tetragonoloba*), a leguminous tree. Guar gum is widely used in many food applications (Table 5.1) (Reid and Edwards, 1991). The annual world production of guar exceeds 100.000 tons, mainly produced in India, Pakistan and the USA (Gierschner, 1986). It can thus be stated that guar is often ingested as part of a Western-style diet.

Guar gum can not be degraded by the human small intestinal enzymes and thus reaches the colon unaltered. Although it has been widely observed that guar is degraded completely by the intestinal microflora (Nyman and Asp, 1982; Tomlin *et al.*, 1986; Nyman *et al.*, 1986; Bourquin *et al.*, 1996), very little is known which species are involved in degradation and fermentation of guar and related galactomannans.

Fermentation of guar has been described for *B. ovatus* (about 25% of the strains tested), *Bi. adolescentis* (1 strain), *Bi. dentium* (all strains), an unidentified *Bacteroides* species and *R. albus* (all strains) (Okubo *et al.*, 1994; Salyers *et al.*, 1977; Balascio *et al.*, 1981; Crociani *et al.*, 1994). Only for *B. ovatus* the fermentation has been characterized in more

detail. In addition to this, fermentation of partially hydrolyzed guar gum (PHGG) has been tested by Japanese researchers (Okubo *et al.*, 1994). They observed a bifidogenic effect of the PHGG in humans, but none of the 16 bifidobacterial strains tested, was able to ferment PHGG. They did not, however, test *Bi. dentium*, which is mainly an oral species, although some strains have been isolated from intestinal contents. In addition, they tested 67 other intestinal bacteria. Of these only *Cl. butyricum*, *Cl. coccoides*, *B. ovatus* and *R. productus* were able to ferment the PHGG to some extent (Okubo *et al.*, 1994).

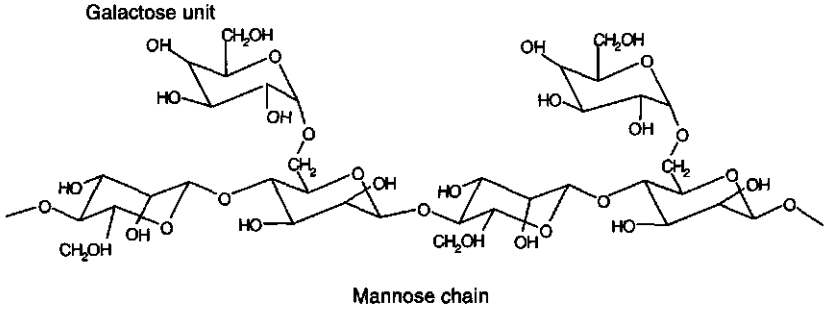

Figure 5.1: structure of guar.

Table 5.1 : Use of guar and other galactomannans in food and consumer products.

Product group	Function and application	Food products
Bakery products	thickener	icings, bread
Beverages	thickener	chocolate milk
Dairy	thickener, stabilizer, fat substitute	ice cream, milk desserts, cheese
Desserts	thickener	jellies
Diabetic products	thickener	coffee whiteners, infant formula
Instant products	thickener	desserts, hot milk puddings
Pharmaceuticals	thickener, stabilizer	tooth paste
Seasonings	thickener	sauces

Adapted from Reid and Edwards (1991) and Gierschner (1986)

Fermentation of guar results, as is the case with most undigestible carbohydrates, in the production of short chain fatty acids and gases. It has been noted in several studies

that the fermentation of guar results in relatively large amounts of butyrate. However, none of the species known to ferment guar produces butyrate as a major fermentation end product. *B. ovatus* produces mainly acetate and propionate, bifidobacteria produce only acetate and lactate. Only *R. albus* produces small amounts of butyrate, but mainly acetate (Holdeman and Moore, 1977). It is not likely that butyrate is formed from either lactate, propionate or acetate. Of the PHGG fermenting strains, only *Cl. butyricum* produces butyrate, *Cl. coccoides* and *R. productus* produce succinate and acetate as main end products, respectively (Holdeman and Moore, 1977; Kaneuchi *et al.*, 1976).

Fermentation of guar often, but not always, results in gas production. Trials using human volunteers have shown increased intestinal pressure and flatulence, as well as hydrogen and methane excretion in the breath (Robb, *et al.*, 1991; Wolever and Robb, 1992; Cummings *et al.*, 1978; Todd *et al.*, 1990). As with the formation of butyrate, no hydrogen is produced in large amounts by the strains known to ferment guar. Some *B. ovatus* strains produce large amounts of carbon dioxide, only the three PHGG fermenting species may produce hydrogen (Holdeman and Moore, 1977).

To answer some of the discrepancies mentioned above, we decided to study the fermentation of guar by the intestinal flora in more detail.

MATERIALS AND METHODS

Galactomannans

The guar used was a commercial product, intended for use in foods (Vellinga Food Ingredients, Oudewater, The Netherlands). Other galactomannans, with a galactose-mannose ratio of 1:1, 1:3 and 1:4 were obtained from Diamalt (Diamalt, Munich, Germany). Pure mannan from yeasts was obtained from Sigma (M 7504, Sigma, Saint Louis, USA).

Strains

The strains used for the screening experiment were either type strains or isolates from human or swine faeces. Strains used for the initial screening included *Ac.* species (3 strains), *B. fragilis* group (49), bifidobacteria (17), clostridia (27), *Eu. limosum*, *F. necrophorum* (2), lactobacilli (29), *Me. elsdenii* (5), anaerobic cocci (3), propionibacteria (3), enterobacteria (6), streptococci (3) and 22 unidentified faecal isolates.

Liquefaction

Degradation of guar was determined by liquefaction. A 1.5% guar solution was prepared in carbohydrate free thioglycollate broth. This broth was prepared from the ingredients of thioglycollate broth (Oxoid CM 391). In addition 0.1% cysteine.HCl (Sigma, C 7880) was added. The pH was adjusted to 6.7 +/- 0.1, unless otherwise stated. The guar was dissolved by mixing the guar slowly into the broth, while stirring rapidly. This highly viscous solution was rapidly poured into test tubes (10-15 ml/tube) and sterilized 15 min at 121°C. After sterilisation the tubes were placed in an anaerobic chamber for at least 16h before use. The anaerobic atmosphere consisted of : nitrogen 80%, carbon dioxide 10% and hydrogen 10% (SHK050H, HoekLoos, Rotterdam, The Netherlands). Tubes were inoculated with either culture medium (0.1-1 ml) or test material using sterile loops. The tubes were incubated up to 5 days anaerobically at 37°C. Guar degradation was considered positive when the tubes were completely liquefied.

HPGPC

In some samples checking for liquefaction was combined with degradation measurements using High-Performance Gel Permeation Chromatography (HPGPC). The HPGPC system consisted of three combined (linear) Biogel TSK columns, 40XL, 30XL and 20XL, each column being 300 x 7.5 mm. The columns were eluted using 0.4 M sodium acetate buffer at pH 3.0 and a flow rate of 0.8 ml/min. Detection was by refraction index measurements, using a Shodex SE-61 detector. The column was kept at 30 °C and the injection volume was 20 µl.

Isolation of guar degrading strains

Guar degrading strains were isolated from faeces or saliva of healthy human volunteers or faecal samples obtained from different animals. Guar tubes were inoculated with different serial dilutions of the samples. After liquefaction 100 µl of the liquid was transferred into new guar tubes. This procedure was repeated twice. After the third liquefaction 5 µl of the liquid was plated onto agar plates with Reinforced Clostridial Agar (RCA, Oxoid CM 151). These plates were incubated anaerobically and colonies were purified, using the same medium. Purified colonies were tested for guar liquefaction. Strains positive in the liquefaction test were characterized using morphology, spore formation, aerobic growth, catalase and oxidase tests, as well as a

API 50CH test (BioMerieux, France). For identification the procedure described by Holdeman and Moore was used (Holdeman and Moore, 1977).

As only a limited number of species could be isolated, the procedure was changed to improve isolation of other species. To improve isolation of lactic acid bacteria the pH of the guar tubes was decreased to 5.0. To isolate lactobacilli tubes with pH 5.0 and vancomycin (20 mg/l) were used (Hartemink *et al.*, 1997). To isolate Gram-negative bacteria vancomycin was added. To isolate aerobes the whole procedure was carried out aerobically and cysteine was omitted from the guar tubes.

In addition it was tried to isolate guar degrading strains using solid media. The media used were MRS, Raffinose Bifidobacterium Agar (RB) (Hartemink *et al.*, 1996), Kanamycin Azide Agar (KAA, Oxoid CM 591), Bacteroides Bile Esculin Agar (BBE) (Summanen *et al.*, 1993), which were prepared from the ingredients, replacing glucose by 0.25% guar. Samples were plated on these media and colonies were purified and tested for guar degradation using the liquefaction procedure.

Gas measurements

Gas produced by fermentation of guar was determined with a pressure meter. Strains or faecal samples were grown in 50 ml screw-capped glass bottles, with a butyl-rubber gas-tight septum. A maximum of 30 ml culture medium was added to the bottles. Gas was measured using a needle attached to a three-way valve, which was connected to the pressure meter and a syringe. The needle was pierced through the septum and the pressure was read. The pressure was reduced to zero by pulling the syringe. The volume of gas produced was read from the syringe.

Faecal inocula

Fermentation of guar was determined using faecal inocula. Faecal inocula were prepared from fresh faeces in buffered peptone water with cysteine.HCl (0.5 g/l) in approximately 10-fold dilution. In an anaerobic chamber a 1 ml sample of this dilution was transferred into a screw-capped bottle with 25 ml of the following medium (Medium 1): yeast extract 5 g/l (Oxoid L21), hemin solution 5 ml/l (Sigma H 2250), salts solution 40 ml/l, LabLemco powder 5 g/l (Oxoid L29), potassium phosphate buffer 25 mmol/l (Merck 1.05104), sodium thioglycollate 0.5 g/l (Sigma T 0632), guar 5 g/l and cysteine.HCl 0.5 g/l. The pH was adjusted to 6.7 +/- 0.1 using a 6 N NaOH solution. The bottles were sterilized by autoclaving 15 min at 121°C. After inoculation the bottles were incubated at 37°C for 36 h. The gas production was measured as described

above at three different time intervals. Final pH was measured after 36h. Some samples were frozen at -80 °C and stored for later analysis of short-chain fatty acid profile (acetate, propionate, butyrate) and lactate concentrations using HPLC (Lee, 1990).

Faecal inocula were prepared from 10 volunteers. Use was made of faecal samples obtained in a large nutrition trial. During this trial, which was not related to our experiments, the volunteers received a completely controlled diet. The diet was essentially free of guar and other galactomannans. All volunteers received the same diet. Faeces was sampled twice in the third week as well as twice in the sixth week of the trial, resulting in 40 faecal inocula. All volunteers were of good health. The bacterial counts of all the samples were within a 7% range for the following bacterial groups: total anaerobes, total aerobes, bifidobacteria, *E. coli*, clostridia and lactobacilli (unpublished data).

Competition experiments

Strains used for the competition experiments were *Cl. butyricum* G-13 (isolated from human faeces), *Bi. dentium* DSM 20484^T and *St. bovis* G-2 (obtained from ID-DLO, Lelystad, The Netherlands). *Clostridium butyricum* G-13 was chosen as it produced the highest amount of gas, compared to the other *Clostridium* isolates (data not shown). The other two strains were chosen as these also liquefied guar rapidly, and were well characterized strains.

Competition was determined using gas measurements as described above. The strains were pre-cultured overnight in carbohydrate free RCM broth (made from the ingredients according to the RCM broth (Oxoid CM 149)), with 0.25% glucose or guar added. In addition the pH of the RCM medium was adjusted to pH 5.5, 6.0, 6.5 or 7.0 using 1 M HCl and 1 M NaOH.

After incubation 1 ml of the overnight culture, or appropriate dilution in reduced physiological salt solution, was added to 25 ml of the same medium in a screw-capped glass bottle. Either single cultures or combinations of the test strains were added.

All dilutions and inoculations were performed in an anaerobic chamber to maintain anaerobic conditions.

After inoculation the bottles were incubated at 37°C for 120h. Gas was measured at regular time intervals and after 120h the final pH was determined.

RESULTS

Screening and isolation.

None of the identified strains tested in the initial screening experiment was able to liquefy guar, with the exception of the *Bi. dentium* type strain and one strain of *B. ovatus* (WAU B-203). Samples of human or animal faeces as well as saliva liquefied guar within 24 hours, most often even within 12 hours.

From human faecal samples only two types of guar-degrading bacteria could be isolated. These were identified as strains of *Cl. butyricum* and *Bi. dentium/adolescentis*. The latter were identified as *Bi. dentium* on their ability to degrade guar (Crociani *et al.*, 1994), carbohydrate fermentation pattern (Holdeman and Moore, 1977) and presence of ß-glucuronidase (Roy and Ward, 1992). Identification was further confirmed, as known strains of these species from culture collections were also capable of degrading guar.

Fermentation of guar by strains of *Bi. dentium* resulted in a final pH of 4.2-4.6, whereas the final pH of the *Cl. butyricum* strains was between 5.0 and 5.6. All *Cl. butyricum* strains fermented guar with the production of large amounts of gas, none of the bifidobacteria produced any gas.

In addition to the human strains some guar-degrading strains were isolated from animal faeces. Strains were isolated from the following species : pig (*Sus scrofa*), pekari (*Tayassu tajacu*), kudu (*Tragelaphus strepsiceros*), wallaby (*Wallabia sp*), watussi (*Bos taurus*), giraffe (*Giraffa camelopardia*), cheetah (*Acinonyx jubatus*), camel (*Camelus bactrianus*), water antelope (*Kobus ellipsiprymus*), yak (*Bos grunniens*), elephant (*Elephant maximus*), oryx (*Taurotagus oryx*), lama (*Llama glama*) and bison (*Bison bison*). With the exception of three strains, all strains were streptococci. The strains were identified as *St. bovis* using the API-strep system (BioMerieux, France). The identity was again further confirmed by testing known strains of *Strep bovis* (type strain and strains obtained from pigs and cows at the ID-DLO institute, Lelystad, The Netherlands) for guar degradation. No gas was produced by any of the strains, the final pH was between 4.5 and 5.0. The three non-streptococci, isolated from pigs and cheetah, were identified as *Cl. butyricum*. These strains produced large amounts of gas.

Selective isolation from faecal samples using specific media, selective agents, aeration or antibiotics did not result in any additional species, neither from human nor from animal samples.

In addition to the faecal isolates we could isolate *Bi. dentium* from the saliva of 37 out of 40 volunteers as well as from 10 out of 10 samples of dental plaque (obtained using dental floss). No other guar degrading strains could be isolated from the oral cavity.

Faecal inocula

Fermentation of guar by 40 faecal inocula resulted in the production of gas and short-chain fatty acids. Gas production was between 7 and 50 ml, with an average of 26 ml. The final pH was between 4.4 and 6.6 with an average of 5.25 (Figure 5.2). Only 8 samples had a pH below 5.0. Incubation of the same faecal inocula in the same medium without carbohydrate resulted in a gas formation of less than 5 ml and a pH of 6.5 +/- 0.1.

Short chain fatty acids and lactate were determined in 12 samples. The total acid concentration was between 24.5 and 69.8 mmol/l, with an average of 54.9 mmol/l (Table 5.2). The major acid produced was acetate, followed by butyrate, propionate and lactate. The average percentage of butyrate, expressed as percentage of all acids, was 28%.

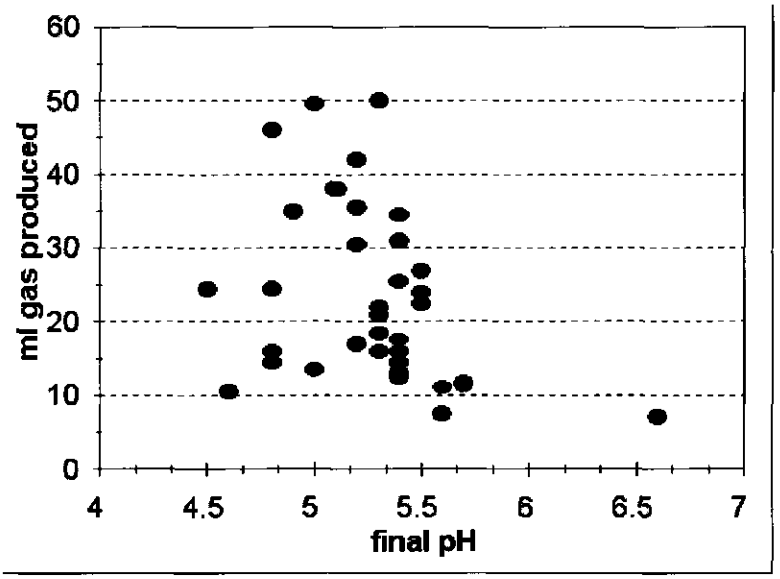

Figure 5.2 : Gas produced and final pH after fermentation of guar by faecal inocula.

Table 5.2 : Final pH, gas, lactate and short-chain fatty acids after batch fermentation of guar with faecal inocula from 12 healthy human volunteers.

sample	ml gas	final pH	concentration (mmol/l)*				total (mmol/ l)	relative percentage			
			L	A	P	B		L	A	P	B
1	35	4.9	12.55	29.55	9.37	18.32	69.79	17.99	42.34	13.43	26.25
2	42	5.2	4.73	18.56	3.27	17.71	44.26	10.69	41.93	7.38	40.00
3	31	5.4	2.58	20.94	10.74	17.00	51.26	5.03	40.85	20.96	33.16
4	49.5	5.0	11.40	21.07	8.06	25.95	66.48	17.15	31.69	12.13	39.03
5	34.5	5.4	4.06	18.15	6.93	21.34	50.48	8.05	35.95	13.73	42.27
6	7	6.6	10.26	7.94	2.72	3.53	24.45	41.97	32.46	11.14	14.43
7	22	5.3	1.59	24.05	17.74	17.19	60.57	2.62	39.71	29.29	28.38
8	35.5	5.2	5.29	20.04	8.88	23.99	58.20	9.09	34.43	15.26	41.22
9	7.5	5.6	5.45	22.78	12.36	9.78	50.37	10.82	45.22	24.53	19.43
10	11	5.6	2.14	27.75	12.28	14.23	56.40	3.80	49.20	21.77	25.23
11	11.5	5.7	2.66	29.76	25.07	6.62	64.11	4.14	46.42	39.11	10.33
12	17	5.2	4.02	33.83	14.63	9.95	62.43	6.44	54.19	23.43	15.93
average	25.29	5.43	5.56	22.87	11.00	15.47	54.90	11.48	41.20	19.35	27.97
SD	13.86	0.42	3.60	6.56	5.92	6.59	11.68	10.33	6.59	8.60	10.79

L= lactate, A = acetate, B= butyrate, P= propionate

HPGPC

The degradation of guar was determined by HPGPC for seven strains, one *B. ovatus* (WAU B-203), three strains of *St. bovis*, one strain of *Cl. butyricum* and two strains of *Bi. dentium*. After 24h incubation all guar gels were completely liquefied. Gas was produced by the *Cl. butyricum* strain. The HPGPC patterns showed that the degradation was very limited for the *B. ovatus* strain (Figure 5.3). Both the *St bovis* and *Bi. dentium* strains degraded the guar to smaller compounds and monomers. It was impossible to obtain a degradation pattern from the *Cl. butyricum* strain, the reason for this is unknown.

All strains tested also liquefied gels made with galactomannans with other mannose-galactose ratios. Final pH values were similar as obtained with guar. Final pH on pure mannan generally was higher, indicating less fermentation (data not shown).

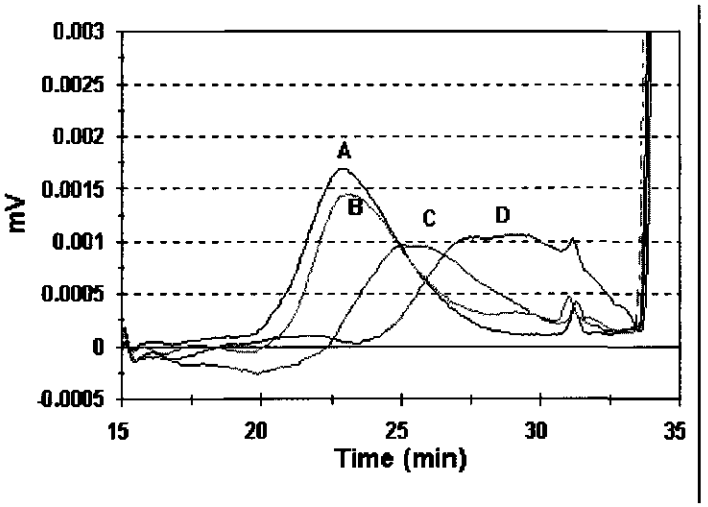

Figure 5.3. HPGPC Elution pattern of guar incubated 24 h with the following strains: A Blank guar, B *B. ovatus* WAU B-203, C *Bi. dentium* G-6, D *St. bovis* G 41. *B. ovatus* WAU B-203 was obtained from a local hospital, *Bi. dentium* G-6 was obtained from saliva of a human volunteer, *St. bovis* G 41 was isolated from pig faeces.

Competition experiments

The competition experiments between *Cl. butyricum* G-13 and the *Bi. dentium* (type) showed that guar is fermented faster by *Bi. dentium* than by the *Clostridium* strain tested (Table 5.3). Gas production was around 27 ml for the *Cl. butyricum* strain alone. Addition of *Bi. dentium* reduced the gas production, indicating fermentation by *Bi. dentium*. In a ratio 1:100 (*Bi dentium* vs. *Cl .butyricum*) *Bi. dentium* reduced the overall gas production by 20-40%, the strongest at the lower pH values. When equal amounts of both strains were added, gas production was reduced by half for pH 6.5 and 7.0 and by 80% for the two lower pH values. In a ratio of 10:1 (*Bi. dentium* vs. *Cl. butyricum*) gas production was practically zero at the lower pH values and reduced by 70-80% at pH 6.5 and 7.0.

Table 5.3. Competition experiments between *Cl. butyricum* G-13 (C) and *Bi. dentium* type (B)). Total gas production and final pH after 120h incubation at 37°C at four different initial pH values. Guar concentration 0.25%. Initial cell counts were $3*10^8$/ml +/- $2*10^8$/ml. Data are mean of three different bottles.

Inoculation level		Initial pH of media				Initial pH of media			
		5.5	6.0	6.5	7.0	5.5	6.0	6.5	7.0
ml C	ml B	Total gas produced				Final pH			
1	0	26.7	27.3	27.3	27.3	5.9	5.8	6.0	5.9
1	0.01	15.3	14.3	23.7	20.7	5.3	5.6	5.9	5.8
1	1	4.3	5.7	17.3	12.3	5.1	5.1	5.4	5.6
0.1	1	0.7	1.7	5.7	8.0	4.9	4.9	5.1	5.1
0	1	0.7	n.d.	0.7	n.d.	4.9	4.8	5.0	4.9

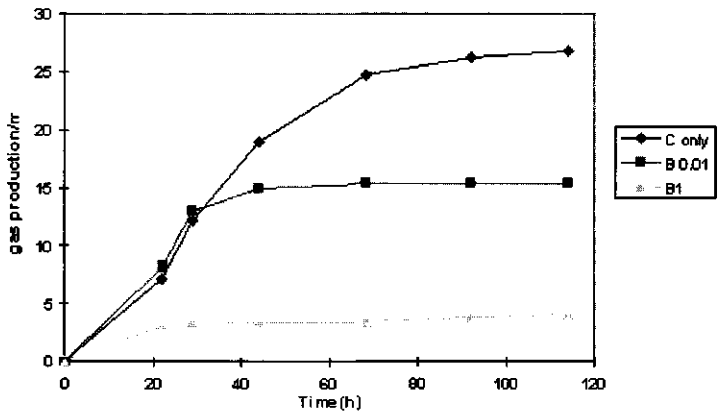

Figure 5.4 : Effect of initial inoculum of *Bi. dentium* on the gas production resulting from the fermentation of guar in the presence of a fixed inoculum of 10^8/25 ml *Cl. butyricum* at pH 5.5. C only is no *Bi. dentium* added, B 0.01 is 0.01 ml *Bi. dentium* added, B1 is 1 ml *Bi. dentium* added. Concentration of *Bi. dentium* was 3.10^8/ml.

Final pH values also showed a similar trend, indicating fermentation of guar by *Bi. dentium*.

Gas production was relatively slow, probably due to the low concentration of guar (Figure 5.4). The maximum amount of gas produced was not reached within 3 days of

incubation. The influence of *Bi. dentium* became noticeable directly when equal amounts of both strains were added, but only after 40h when added in the ratio 100:1 (*Bi. dentium* vs. *Cl. butyricum*). At higher pH values growth and gas production was faster, reaching a maximum already after 50 h at pH 6.5.

The competition between Cl. *butyricum* G-13 and *St. bovis* G-2 showed a different pattern (Figure 5.5). No effect of *St. bovis* on the gas formation could be observed at pH 6.5 or 7.0, not even at the initial ratio of 100:1 (*St. bovis* vs. *Cl. butyricum*). At pH 6.0 the gas production was reduced by 20% at a ratio of 1:1 or 10:1 and by 75% at a ratio 100:1. At pH 5.5 gas production was clearly reduced, even at a 1:1 ratio, final gas production was reduced by 75% under these conditions.

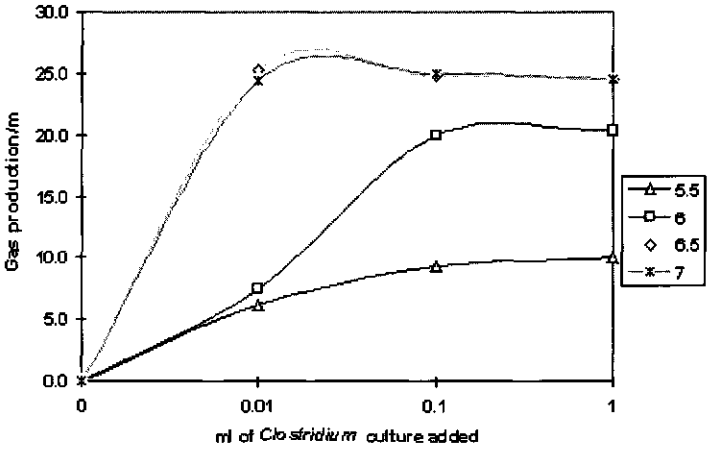

Figure 5.5 : Effect of initial inoculum of *Cl. butyricum* on the gas production resulting from the fermentation of guar in the presence of a fixed inoculum (1 ml) of *St. bovis* at different pH values: 5.5; 6.0; 6.5 and 7.0. Concentration of *Cl. butyricum* was $2.5*10^8$/ml, that of *St. bovis* was $7*10^8$/ml.

DISCUSSION

The results show that guar can only be degraded by a limited number of individual bacterial species from the intestinal contents. We have only been able to isolate strains from three species, *Bi. dentium*, *St. bovis* and *Cl. butyricum* that are able to degrade and ferment guar. In addition, some strains of *B. ovatus* were able to liquefy guar, but probably did not completely ferment the mannose chain. Besides *B. ovatus* and *Bi. dentium*, only *R. albus* has been isolated previously. In addition to this, *Cl. coccoides*

and *R. productus* have been found to be able to degrade PHGG. None of the latter two strains, however has been isolated previously as a guar fermenting species from human or animal faeces. Even though we have found only a limited number of guar degrading strains, it is not unlikely that a combination of strains from different species are able to degrade and ferment guar and galactomannans *in vivo*.

The strain most frequently isolated from human faecal samples was *Cl. butyricum*. The numbers *of Cl. butyricum* were found to be around or over 10^9/g faeces in most samples. Although this species has been isolated previously from human faeces in similar numbers, we have isolated this species from most humans. Previous results did only show a low frequency of isolation, probably due to the presence of other clostridial species. *Cl. butyricum* has not been isolated previously as a guar degrading strain, but it was found to be able to degrade PHGG (Okubo *et al.*, 1994) and glucomannan (Nakajima and Matsuura, 1997).

The presence of *Bi. dentium* in faeces has been described previously (Crociani *et al.*, 1994). Literature results indicate that *Bi. dentium* is present in relatively small numbers compared to other bifidobacterial species. The isolation of *Bi. dentium* from faeces therefore requires selective techniques. Also, *Bi. dentium* is difficult to differentiate from *Bi. adolescentis* and *Bi. catenulatum*, which are both more common in faeces (Holdeman and Moore, 1977). It is thus likely that some *Bi. dentium* isolates have previously misidentified as *Bi. adolescentis* or *Bi. catenulatum*. The two selective tests to differentiate between the species are the presence of ß-glucuronidase and guar fermentation, both of which are positive for *Bi. dentium* (Crociani *et al.*, 1994; Roy and Ward, 1992).

Bi. dentium is normally associated with dental caries. We have shown, using guar degradation, that this species was present in saliva in higher incidence than observed previously (Scardovi and Crociani, 1974).

St. bovis has not previously been isolated as a guar degrading species. We isolated strains of *St. bovis* from most samples of animal faeces. *St. bovis* has been isolated previously from many animals, but also from human faeces (Hardie and Whiley, 1995). As all our isolates grew aerobically, they could not be misidentified with *R. productus* or *R. albus*, two anaerobic species that were associated with guar fermentation in previous studies. Also, the type strain of *St. bovis*, as well as strains that we obtained from other institutes, were capable of degrading and fermenting guar.

In contrast to previous research we failed to isolate guar degrading *B. ovatus* strains. The reason for this is unknown. It was shown in previous studies that about 25% of all strains of *B. ovatus* were capable of degrading and fermenting guar (Salyers *et al.*, 1977). The average log counts of *B. fragilis* group organisms in the tested faecal inocula was generally above 10^{10}/g, indicating that the conditions were not inhibiting to *B. ovatus*. Even with the addition of vancomycin to suppress the Gram-positive species, no liquefaction of guar gels was observed. In a similar technique as Bayliss and Houston (1984), using Bacteroides Bile Esculin Agar with guar as the only carbohydrate source, we isolated many Gram-negative rods, none of which liquefied guar in subsequent tests. *B. ovatus* is not present in faeces of all humans. Finegold *et al.* (1974) isolated *B. ovatus* only in 8 out of 18 persons on a Western style diet. Similarly *B. ovatus* was isolated only in 9 out of 70 samples of infant faeces (Benno *et al.*, 1984), 13 out of 30 samples of older persons (Benno *et al.*, 1989), 4 out of 13 samples of healthy vegetarian adults and 3 out of 14 non-vegetarian adults (Finegold *et al.*, 1977). It is unknown whether *B. ovatus* is common in healthy Dutch adults and/or our donors. *B. ovatus* is also rarely isolated from intestinal or faecal samples in general hospitals in the Netherlands [pers comm].

Guar fermentation by faecal inocula resulted in the production of gas and butyrate as a major short-chain fatty acid. Some lactate was produced as well. This is in agreement with other studies (Tomlin *et al.*, 1986; Bourquin *et al.*, 1996). The data presented in Table 5.2 include SCFA and lactate produced from the medium components. However, the total concentration of acids produced from the medium without carbohydrate source was less than 15 mmol/l, indicating that most acids were produced from the carbohydrate (unpublished data). Butyrate is formed by direct fermentation of carbohydrates, it is not formed from other acids, such as acetate or lactate by faecal bacteria. Of all guar fermenting species isolated in our or previous studies, only *Cl. butyricum* produces butyrate.

Gas production varied between the samples (Figure 5.2), but was always present. *In vivo* studies have also shown production of considerable amounts of gases, resulting in intestinal bloating, flatulence and hydrogen excretion (Robb *et al.*, 1991; Wolever and Robb, 1992; Cummings *et al.*,1978; Todd *et al.*, 1990). Of all species isolated in our or previous studies only *Cl. butyricum*, *Cl. coccoides* and *R. productus* produce hydrogen, some of the *B. ovatus* strains are reported to produce carbon dioxide (Holdeman and Moore, 1977). As we failed to isolate the latter three species, it can be concluded that *Cl. butyricum* is

the species responsible for the gas production. Gas may also be produced as a secondary metabolite from lactate by *Me. elsdenii* or *Veillonella* species (Holdeman and Moore, 1977). This would result in high concentrations of acetate and propionate. Similarly, the *B. ovatus* strains do not produce hydrogen, nor butyrate (Holdeman and Moore, 1977). The presence of large concentrations of butyrate, therefore, emphasizes the role of *Cl. butyricum*. Some secondary fermentation yielding butyrate may, however, not be excluded, neither *in vivo* nor *in vitro*.

The final pH of guar fermentation by faecal inocula was relatively high as compared with other substrates (unpublished data). With a few exceptions, the final pH was above 5.0. This further emphasizes the role of *Cl. butyricum* in many samples, as this strain does not acidify below 5.0, whereas the two other species can acidify below 5.0.

The *B. ovatus* strain tested did not completely degrade guar, but probably only removed the galactose units. The molecular mass was slightly less than that of the original guar, but the sample was completely liquefied. Removing of the galactose results in liquefaction of the gel, as mannan does not form gels. This *B. ovatus* strain thus was positive in the liquefaction test, but does not completely ferment guar. The two necessary enzymes for guar degradation (α-galactosidase and mannanase), have been described for *B. ovatus*. Two different α-galactosidases have been described, both of which are not active on intact guar gum. α-Galactosidase I is only active on partially degraded guar gum (Gherardini *et al.*, 1985). In another study it was shown that α-galactosidase activity is induced by guar gum and that these enzymes are acting towards guar (Macfarlane *et al.*, 1990). A mannanase has been described, but not purified (Gherardini *et al.*, 1985). Whether both enzymes are present in all strains is not known.

Repeated incubation and storage (over 1 year) in non-guar containing media (cooked meat medium) or at -80°C with subsequent inoculation of guar tubes, did not result in the loss of the guar degrading capacity for the isolated strains (data not shown). Similarly, the culture collection strains, which were most likely never tested on guar degradation, as it is normally not included in identification schemes, did liquefy guar rapidly. These data indicate that the enzymes are constitutive and not, or very rapidly, induced by guar.

The competition experiments showed that *Bi. dentium* was capable of influencing the gas production by *Cl. butyricum* at all pH values tested. The strongest inhibition was

observed at the lower initial pH values. This influence was also reflected in lower final pH values. As *Bi. dentium* grows faster than *Cl. butyricum* at physiological conditions (pH 6.5-6.8), isolation of *Bi. dentium* using guar degradation should occur, when both species are initially present in equal amounts, or even when *Cl. butyricum* outnumbers *Bi. dentium* by a factor 10-100. However, we could not detect *Bi. dentium* in liquefied guar gels using faecal samples. This indicates that *Bi. dentium* is present in much lower numbers than *Cl. butyricum.* It is therefore unlikely that *Bi. dentium* is responsible for the bifidogenic effect of PHGG as has been reported previously (Okubo *et al.*, 1994). The addition of PHGG to the diet of volunteers increased the numbers of bifidobacteria from (log counts) 9.9 to 10.3. As only *Bi. dentium* is capable of fermenting guar and *Bi. dentium* in our results is present only in (log) numbers below 6, the increase observed by this study would indicate an (unlikely) increase in numbers by 4 log units. It is not expected that the (Japanese) population tested has a much higher faecal count of *Bi. dentium* than our volunteers, as the presence of this species has not been reported previously by Japanese researchers in high numbers in faeces (Benno *et al.*, 1984; Benno *et al.*, 1989). Therefore the bifidogenic effect of PHGG as described by Okubo *et al.* (1994) can not readily be explained.

Competition experiments between *St. bovis* and *Cl. butyricum* indicated that *St. bovis* has no influence on the gas production by *Cl. butyricum* under physiological conditions, not even when *St. bovis* outnumbers *Cl. butyricum* with a factor 100. Only at pH 6.0 gas production was reduced. It can thus be concluded that in human faeces it is not likely to isolate *St. bovis* using guar gel liquefaction when *Cl. butyricum* is present. As we isolated all our strains from animals it can be concluded that, in these samples, *Cl. butyricum* was present in much lower numbers than *St. bovis.*

The fermentation of guar results in the formation of relatively large concentrations of butyrate (Tomlin *et al.*, 1986; Bourquin *et al.*,1996). Formation of butyrate is considered to have a beneficial effect in the intestine (Brøbech Mortensen and Rye Clausen, 1996). Guar does not directly stimulate the growth of beneficial bacteria such as bifidobacteria and lactobacilli, with the exception of *Bi. dentium.* On the contrary, guar seems to stimulate mainly *Cl. butyricum*, and the stimulation of clostridia is not considered beneficial. The effects of guar on intestinal health are thus difficult to determine.

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6

FERMENTATION OF SELECTED CARBOHYDRATES BY FAECAL INOCULA FROM VOLUNTEERS ON A CONTROLLED DIET LOW IN FIBRE.

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SUMMARY

The fermentation of 9 different carbohydrates (lactose, lactulose, lactitol, raffinose, guar gum, fructooligosaccharides (FOS), transgalactosyl-oligosaccharides (TOS), xyloglucan and inulin) was studied in a batch system using faecal samples from 10 volunteers. The volunteers all received the same controlled diet for six weeks, with an addition of TOS during the last three weeks. Two samples were taken both in the third and sixth week of the study. Carbohydrates were fermented for 48h and after incubation, final pH, gas production, lactate and short-chain fatty acids were determined. Gas production and pH differed widely between the substrates. Our batch fermentation results indicate extensive fermentation by clostridia of guar and xyloglucan. Lactose, raffinose, FOS and lactulose were probably fermented to a large extent by lactic acid bacteria, most likely bifidobacteria. Fermentation of TOS, lactitol and inulin could not be attributed to one or two specific bacterial groups.

Butyrate production in our system was highly correlated with gas production, indicating that gas measurements are a cheaper and faster method to screen for butyrate producing substrates.

The overall metabolic pattern was highly dependent of the volunteer donating the sample, as different samples from a single volunteer resulted in a similar profile. Results from different volunteers, consuming the same diet for 6 weeks, were markedly different, indicating that host factors are important for the metabolic capacity of the flora.

INTRODUCTION

Dietary carbohydrates can be divided in digestible and non-digestible carbohydrates. Components of the latter group will enter the colon unaltered and may act as a substrate for the colonic microflora. The non-digestible carbohydrates may be divided in the classical dietary fibres (mainly polysaccharides), resistant starch and non-digestible oligosaccharides.

Fermentation of carbohydrates by the colonic microflora results in the production of microbial mass, short-chain fatty acids (SCFA), lactate and the gases carbon dioxide and hydrogen. Subsequent metabolism of these compounds results in the formation of secondary products, such as methane (Hill, 1995).

Different carbohydrates are fermented by different subgroups of the bacterial population, due to structural changes of the carbohydrates and the (specific) enzymes necessary for degradation, which are not present in all bacterial species. Some carbohydrates are claimed to be selectively fermented by specific bacterial groups, such as fructooligosaccharides by bifidobacteria (Gibson and Wang, 1993; Bouhnik *et al.*,

1996). Other carbohydrates may be degraded and fermented by many different species from different genera (Table 1.3, Chapter 1). Complex carbohydrates, such as mucins, can only be degraded and fermented by different bacteria acting together (Hoskins *et al.*, 1992).

During the last decades many studies have been carried out on the fermentation of different carbohydrates, dietary fibres and crude substrates by the intestinal microflora. Studies have been performed using individual bacterial species (Chapter 1) as well as in batch and continuous fermentations using faecal inocula (McBurney and Thompson, 1987; Englyst and MacFarlane, 1987; Bourquin *et al.*, 1996). Due to the large differences in experimental setup the different studies are difficult to compare.

Fermentations are often carried out using a single faecal sample or a mixture of faecal inocula from different volunteers. As it is generally considered necessary to use a fresh faecal inoculum to avoid damage due to freezing or oxygen damage (Alles, 1998), repetition of the results may be difficult. Similarly, as the bacterial composition of the faeces may be dependent of the diet (Hentges, 1980; Edwards, 1993), faecal inocula, even when taken from the same donor, may be considerably different due to dietary changes.

To determine the variation in fermentative characteristics between faecal inocula of the same donor as well as the variation between inocula of different donors the present study was carried out.

Volunteers participating in a large nutritional trial that was aimed to determine the effects of transgalactosyl-oligosaccharides (TOS) on the intestinal microflora of healthy adults (Alles, *et al.*, 1999), provided the faecal samples. All volunteers received a controlled diet for the six weeks of study. During the last three weeks the volunteers received either the same diet (placebo) or the diet supplemented with two different doses of TOS.

The fermentation of nine different carbohydrates was investigated. With the exception of lactose, all carbohydrates tested are non-digestible. Lactose, lactulose and lactitol were used to compare chemical structure and fermentation products. Raffinose was used as it is supposed to be involved in the flatulence due to the digestion of legumes. Fructooligosaccharides and inulin were used to determine differences due to chain length. TOS was used to determine adaptation due to addition of TOS to the diet. Finally xyloglucan and guar gum were used as these are more complex polysaccharides, which have been found to be fermented mainly by clostridia (Chapters 4 and 5).

MATERIALS AND METHODS

Medium

The medium used for the batch fermentation consisted of : yeast extract 5.0 g/l, Lab Lemco powder (Oxoid) 5.0 g/l, sodium thioglycollate 0.5 g/l, cystein.HCl 0.5 g/l, hemin solution 5 ml/l (Holdeman and Moore, 1977), salts solution 40 ml/l (Holdeman and Moore, 1977) in 25 mM potassium phosphate buffer. The pH was adjusted to 6.6 +/- 0.1 using 1 M NaOH or 1 M HCl solutions. The pH was not affected after addition of the test carbohydrates or sterilisation. The medium was supplemented with the selected carbohydrates to a concentration of 5 g/l. The medium was then distributed in 25 ml screw-capped gas tight bottles, with a butyl rubber piercable septum. The bottles were sterilized 15 min at 121°C and kept at refrigeration temperatures until use. Prior to use, the bottles were kept overnight at 37°C in an anaerobic chamber to check sterility, to remove oxygen from the headspace and to avoid a temperature shock to the faecal inoculum.

The following carbohydrates were used : blank (no carbohydrate added), lactose (Merck, Darmstadt, Germany), lactulose (Pharmachemie, Haarlem, the Netherlands), lactitol (Purac, Gorinchem, the Netherlands), transgalactosyl-oligosaccharides (TOS, Borculo Whey Products (now Borculo Domo Ingredients), Borculo, the Netherlands), raffinose (Sigma, St. Louis, USA), fructooligosaccharides (FOS, Raftilose P95, Orafti, Tienen, Belgium), inulin (Raftiline LS, Orafti), guar gum (Vellinga Food Ingredients, Oudewater, the Netherlands) and xyloglucan (Dainippon Pharmaceutical, Osaka, Japan). Except for TOS, which contained up to 15% lactose and monomeric sugars, all carbohydrates contained less than 2% impurities.

Faecal inocula

Faecal inocula were obtained during a large experiment carried out in collaboration between the Human Nutrition and Food Microbiology groups at the Wageningen Agricultural University. This study has been described in detail by Alles *et al.* (1999, in press). In short, all volunteers received the same controlled diet, which was low in fibre and relatively high in protein content. During the first three weeks all volunteers received the same diet, the second three weeks of the study the diet was supplemented with a placebo, a low or a high dose of TOS. Faecal inocula were taken from 10

volunteers, selected at random from the subgroups, 5 of which received a high dose of TOS (15 g/d), 5 received a low dose of TOS (7.5 g/d). Faecal inocula were taken twice in the third week of the study (no addition of TOS to the diet), and twice in the sixth week (after 3 weeks of TOS supplementation). None of the volunteers had taken antibiotics or other medicine at least 6 months prior to the study date. None of the volunteers was lactose intolerant.

Volunteers defaecated in a special toilet near the laboratory. Faecal samples were transferred immediately into an anaerobic chamber. The whole procedure between defaecation and transfer into the chamber took less than 5 minutes. Within the anaerobic chamber (atmosphere 80% N_2, 10% CO_2 and 10%H_2, SHK 050H, HoekLoos, Rotterdam, the Netherlands), the samples were homogenized using an Ultra-Turrax mixer and diluted 100 fold with reduced physiological peptone solution (rps, peptone 1 g/l, NaCl 8.5 g/l, cysteine.HCl 0.5 g/l, pH 6.7 +/- 0.1). One milliliter of these dilutions was added to the medium containing the test carbohydrates. The bottles were then closed and transferred from the anaerobic chamber to be incubated for 48h at 37°C.

Gas measurements

Gas was measured using a needle attached to a three-way valve, which was connected to the pressure meter and a syringe. The needle was pierced through the septum and the pressure was read. The pressure was reduced to zero by pulling the syringe. The volume of gas produced was read from the syringe. Gas was measured after 24 and 48h of incubation at 37°C.

Analysis of acids

After 48h of incubation as described above, final pH was measured and samples were taken from the acidified tubes for acid analysis. To 5 ml sample 100 µl 50% sulfuric acid was added to kill all bacteria and stop the fermentation reactions. Acidified samples were immediately stored at -80°C before analysis. Prior to HPLC analysis 1 ml samples were centrifuged for 15 minutes in an Eppendorf centrifuge to remove all bacterial cells and insoluble material. Short chain fatty acids (SCFA, acetate, propionate and butyrate) and lactate were determined using HPLC with an Aminex HPX-87H column and 0.01 mol l^{-1} sulfuric acid as the mobile phase.

RESULTS

Gas production

Gas production differed widely between the substrates (Table 6.1, Fig. 6.1). The blank samples produced less than 10 ml of gas, with an average of 5.4 ml from faecal inocula taken from volunteers before and 6.6 ml from faecal inocula during the intervention of TOS. The highest average gas production was observed on guar gum (33.8 ml) in faecal inocula from the low-TOS group before the intervention.

The addition of TOS, in either dose, to the diet did not have any effect on the blank gas production, or on the gas production on inulin, lactose, lactulose, lactitol, raffinose, guar gum or xyloglucan. The gas production, however, was reduced in both the high-TOS and low-TOS intervention on FOS as a substrate.

Gas production differed widely between the faecal inocula of the same person as well as between persons (Fig 6.2). Large variation between individuals was observed for the polysaccharides guar gum and xyloglucan, lactitol and FOS. Variations were generally small for lactose, lactulose and TOS.

Table 6.1 : Gas produced on the different substrates by different faecal inocula derived from the two groups of volunteers before and during the intervention with high- or low TOS.

	before intervention with				after intervention with			
substrate	low TOS		high TOS		low TOS		high TOS	
	mean	SD	mean	SD	mean	SD	mean	SD
bla	6.3	1.6	4.8	1.6	6.8	1.2	6.4	0.6
fos	13.4	6.7	9.3	4.3	9.5	2.1	6.7	2.7
gua	33.8	12.7	21.5	12.5	22.1	5.1	21.1	11.2
inu	12.6	3.7	11.0	3.0	14.1	6.2	11.5	6.1
lac	6.2	2.7	5.9	2.3	6.2	1.3	5.0	1.3
lat	7.1	3.5	5.9	1.5	8.4	2.4	8.0	3.1
lol	14.6	4.7	13.7	7.2	13.9	4.4	13.3	3.9
raf	11.9	4.0	9.0	3.8	10.1	2.9	6.3	2.2
tos	7.7	2.9	7.2	2.2	9.4	3.2	7.0	3.2
xyg	24.8	12.0	17.1	9.8	17.2	5.3	16.0	7.5

bla = blank sample (no carbohydrates added), fos = fructooligosaccharides, gua = guar gum, inu = inulin, lac = lactose, lat = lactulose, lol = lactitol, raf = raffinose, tos = transgalactosyl-oligosaccharides, xyg = xyloglucan.

pH

The final pH differed widely between the substrates (Fig. 6.1C, 6.1D, Table 6.2). The final pH was practically unchanged for the blank samples, due to the presence of the buffer in the medium. A drop in pH was thus considered to be due to fermentation of the test carbohydrates. The final pH was on average below 5.0 for lactose, raffinose and FOS. Fermentation of all other substrates, except xyloglucan, resulted in an average final pH between 5.0 and 5.5.

Table 6.2 : Final pH after fermentation of a test carbohydrate with different faecal inocula the two groups of volunteers before and during the intervention with high- or low TOS.

	before intervention with				after intervention with			
substrate	low TOS		high TOS		low TOS		high TOS	
	mean	SD	mean	SD	mean	SD	mean	SD
bla	6.55	0.60	6.63	0.15	6.61	0.09	6.61	0.10
fos	4.85	0.28	4.66	0.16	4.76	0.19	4.64	0.22
gua	5.15	0.26	5.51	0.42	5.28	0.26	5.06	0.29
inu	4.93	0.28	5.08	0.13	5.11	0.27	4.96	0.37
lac	4.58	0.34	4.51	0.10	4.56	0.12	4.47	0.08
lat	5.56	0.37	5.50	0.27	5.80	0.33	5.77	0.32
lol	5.20	0.34	5.35	0.63	4.99	0.14	5.10	0.34
raf	4.78	0.25	4.72	0.15	4.86	0.18	4.69	0.22
tos	5.49	0.24	5.41	0.33	5.64	0.39	5.50	0.37
xyg	5.44	0.26	5.72	0.35	5.48	0.21	5.67	0.31

bla = blank sample (no carbohydrates added), fos = fructooligosaccharides, gua = guar gum, inu = inulin, lac = lactose, lat = lactulose, lol = lactitol, raf = raffinose, tos = transgalactosyl-oligosaccharides, xyg = xyloglucan.

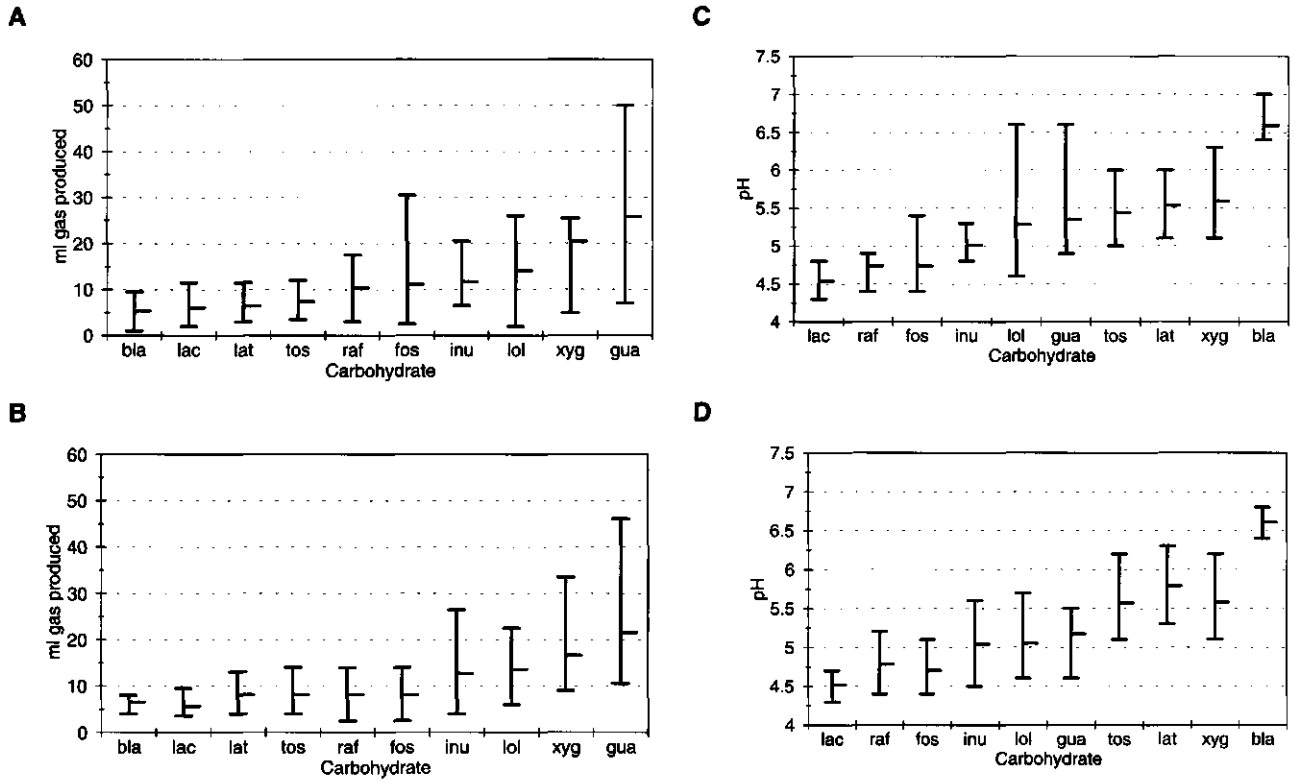

Figure 6.1 :
Overall gas production and final pH by faecal inocula. Lines show range and average of gas production (A,B) or pH (C,D) of all 20 faecal inocula derived from volunteers before (A,C) and after (B,D) the TOS supplementation to the diet.
bla = blank sample (no carbohydrate added), lac = lactose, lat = lactulose, tos = transgalactosyl-oligosaccharides, raf = raffinose, fos = fructooligosaccharides, inu = inulin, lol = lactitol, xyg = xyloglucan, gua = guar gum

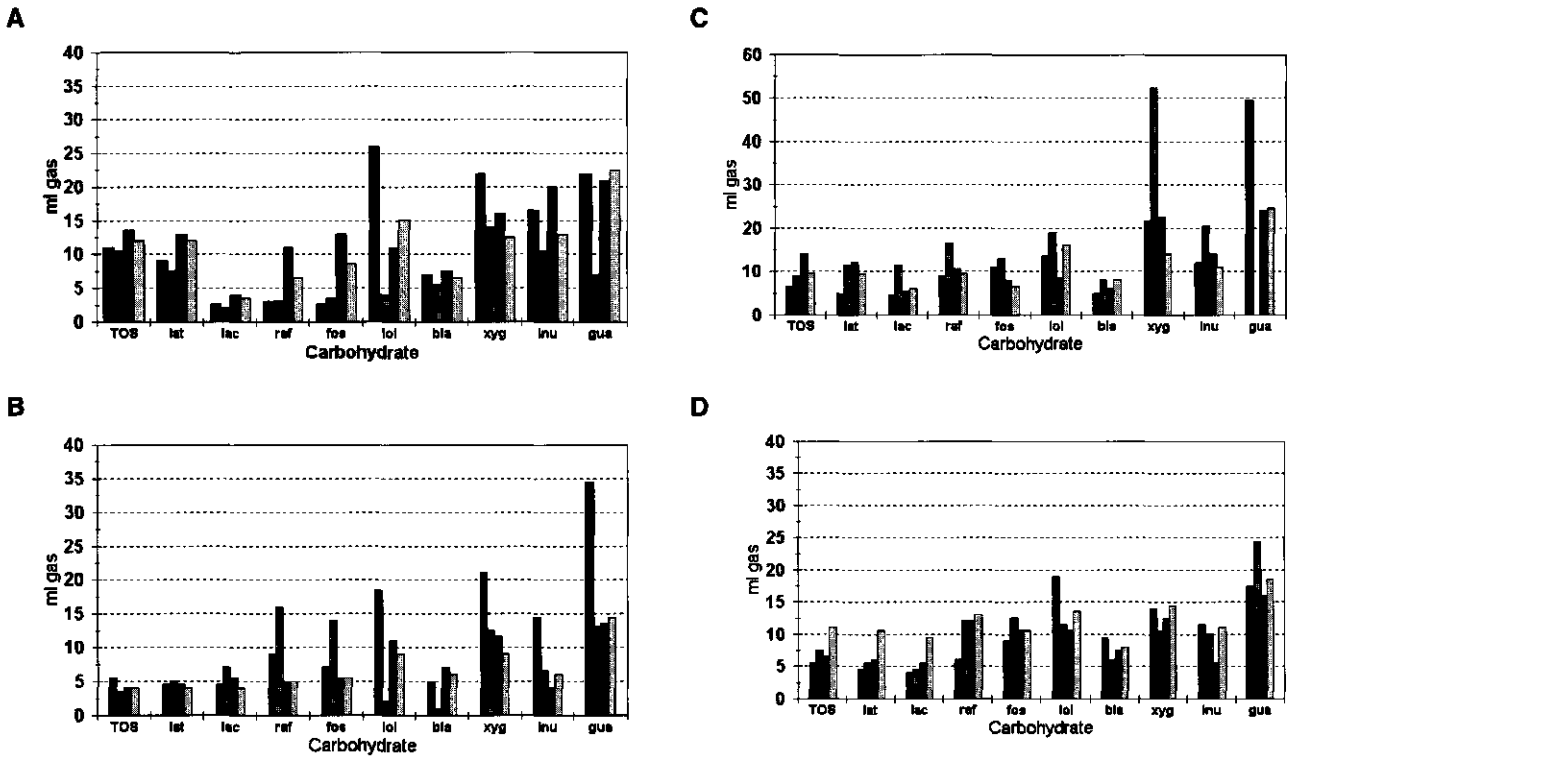

Figure 6.2 :
Examples of variation in gas production within single volunteers for all substrates. Faecal samples were taken within one week before (first two columns) and during (last two columns) the intervention with TOS in the diet. Graphs A and B : volunteers receiving 15 g/d TOS; graphs C and D : volunteers receiving 7.5 g/d TOS during the intervention period. bla = blank sample (no carbohydrate added), lac = lactose, lat = lactulose, tos = transgalactosyl-oligosaccharides, raf = raffinose, fos = fructooligosaccharides, inu = inulin, lol = lactitol, xyg = xyloglucan, gua = guar gum

Gas production versus pH

There was no relation between gas production and final pH when all data were plotted (Fig 6.3A). For some substrates, however, there was a clear relation between the amount of gas produced and the final pH. This correlation was high for TOS (r^2=0.86, Fig 6.3B) and lactulose (r^2=0.84), weak for FOS (r^2=0.55) and raffinose (r^2=0.45, Fig 6.3D). Other substrates did not show any correlation, an example is given in Fig 6.3C for lactitol (r^2=0.01).

SCFA and lactate

It was observed that the production of short-chain fatty acids and lactate varied widely between and within substrates (Table 6.3). Acetate was the main short-chain fatty acid produced, but for four substrates lactate was produced in higher concentrations.

Lactate concentrations were especially high after fermentation of lactose, raffinose and FOS. Fermentation of guar, xyloglucan and lactitol on the other hand, resulted in a very low concentration of lactate, but high percentages of butyrate and propionate.

Table 6.3 : Short-chain fatty acids and lactate produced after 48h of fermentation by different faecal inocula for all volunteers on the different substrates shown as relative percentages of total production (in mol%).

substrate	Lactate		Acetate		Propionate		Butyrate	
	%	SD	%	SD	%	SD	%	SD
fos	47.2	12.7	34.5	8.5	9.9	4.6	8.3	9.0
gua	11.5	10.3	41.2	6.6	19.4	8.6	28.0	10.8
inu	37.5	12.2	45.1	11.4	12.1	3.3	14.5	9.0
lac	55.3	9.8	35.1	9.4	6.6	1.9	3.1	2.3
lat	40.9	9.1	37.7	11.6	13.0	5.4	8.5	8.3
lol	15.1	9.3	49.8	9.2	17.2	8.6	17.9	12.2
raf	50.4	10.1	36.0	8.6	8.7	1.6	4.9	3.3
tos	26.5	12.8	48.6	7.9	10.6	4.8	14.3	15.2
xyg	9.5	7.8	44.4	4.8	21.8	9.7	24.3	10.9

fos = fructooligosaccharides, gua = guar gum, inu = inulin, lac = lactose, lat = lactulose, lol = lactitol, raf = raffinose, tos = transgalactosyl-oligosaccharides, xyg = xyloglucan.

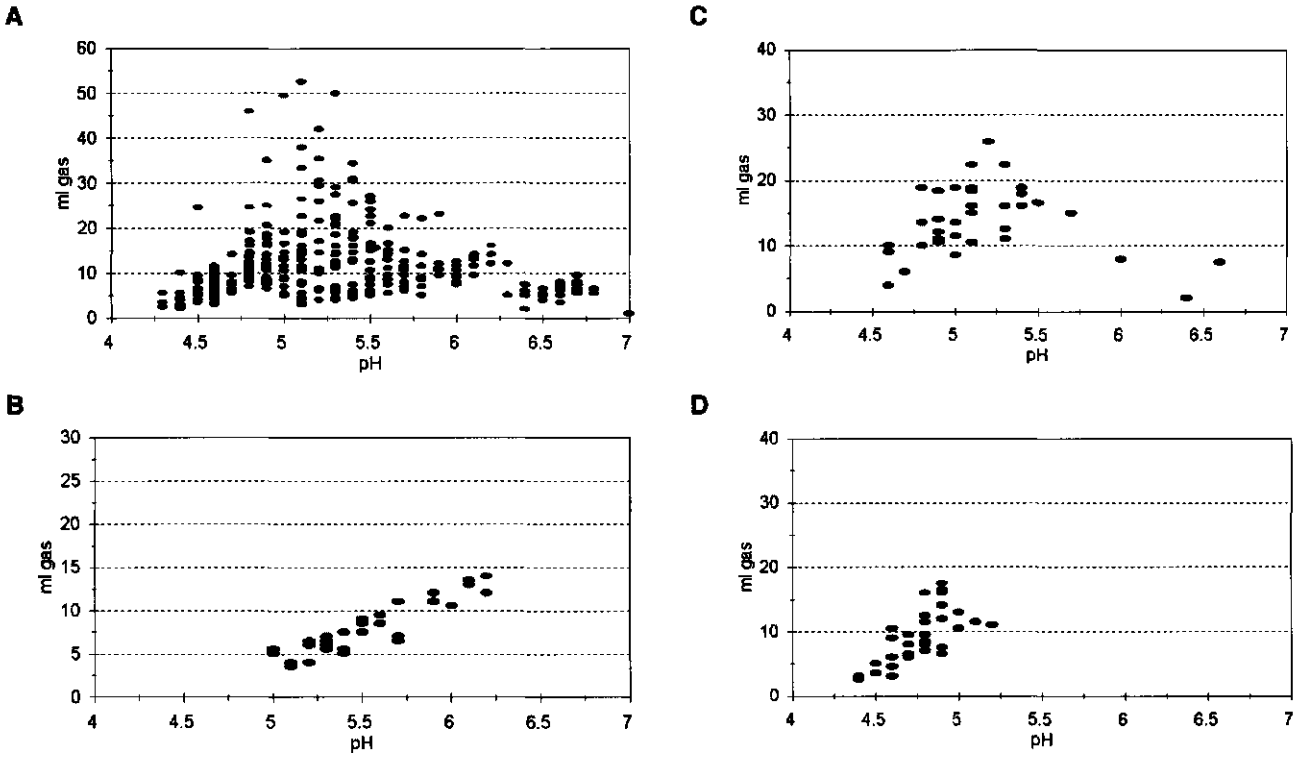

Figure 6.3 :
Gas production as a function of pH. A : All samples, all substrates; B : All samples with TOS only; C : All samples with LOL only; D : All samples with RAF only

Most volunteers showed similar relative proportions of SCFA and lactate in all samples (Fig 6.4, volunteers G,H,I), whereas other volunteers showed large variations in the relative proportions of acids between samples (Fig 6.4, volunteer F).

There was no correlation between the production of any of the acids with the final pH for any of the substrates tested, although samples with high lactate concentrations generally had a low pH, and samples with high butyrate generally showed a relatively high pH. There was no correlation between the production of acetate or propionate with the formation of gas. For butyrate, however, there was a relatively strong correlation (r^2=0.78, Fig 6.5A), the more gas produced, the larger the proportion of butyrate. An opposite relation was observed between lactate and gas formation. A relatively large concentration of lactate corresponds with a low gas production (Fig 6.5B).

DISCUSSION

All different substrates were fermented completely, as analysis of carbohydrates in a random series of fermentation bottles did not show residual carbohydrates (data not shown).

Fermentation resulted generally in a reduction of pH as well as production of acids and gases. Large differences were observed both between the different substrates as the different faecal inocula.

Gas production was highest for xyloglucan and guar gum. This was expected as both substrates are fermented mainly by clostridia (Chapter 4 and 5). Clostridia generally produce large amounts of gas. Production of hydrogen has also often been reported after the ingestion of guar by human volunteers (Robb, *et al.*, 1991; Wolever and Robb, 1992; Cummings *et al.*, 1978; Todd *et al.*, 1990). Similarly, fermentation of these two substrates also resulted in a relatively high final pH, and in the production of much propionate and butyrate and a small amount of lactate. These findings confirm the fermentation by clostridia. Guar is known to be fermented nearly exclusively by *Cl. butyricum*, which does produce large amounts of butyrate and very little lactate (chapter 5, Holdeman and Moore, 1977). The final pH of a *Cl. butyricum* fermentation hardly ever reaches values below 5.5.

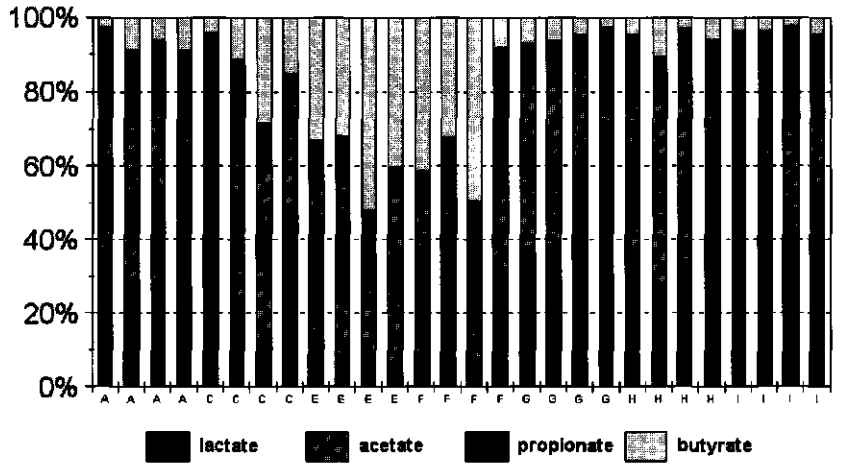

Fig 6.4 : Proportion of short-chain fatty acids and lactate produced with TOS as a substrate after fermentation by four faecal inocula from the same volunteer. Letters on X-axis indicate different volunteers. First two bars represent samples before intervention with TOS in the diet, last two bars during intervention.

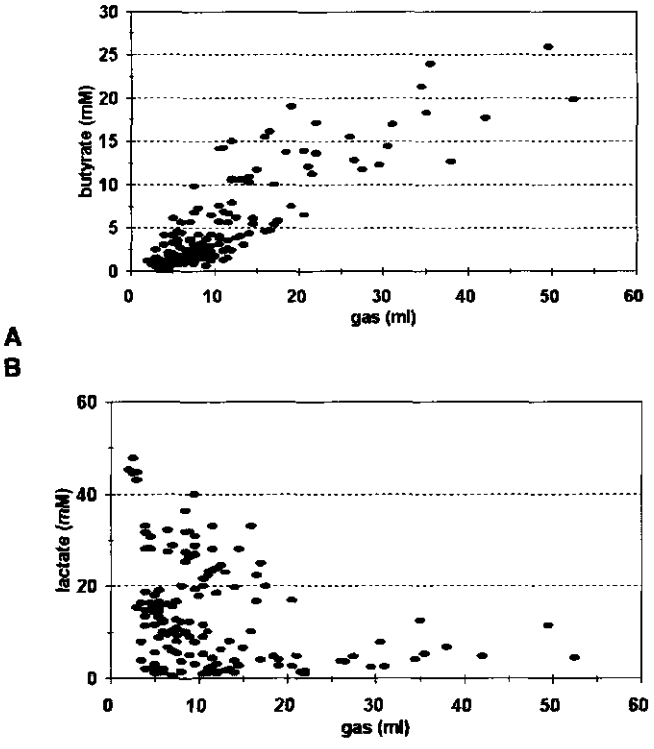

Fig. 6.5 :
Relation between the production of butyrate (A) and lactate (B) and gas by faecal inocula of all volunteers on all substrates.

Fermentation of raffinose, which has often been implicated in the flatulence observed after the ingestion of beans and related products (Christofaro *et al.*, 1974), did not result in the formation of much gas in the inoculum of any of the volunteers. This may either be due to differences between *in vivo* studies and our *in vitro* fermentations, or raffinose may not be the main flatulence factor in beans. The final pH after the fermentation with raffinose was low, with a high percentage of lactate among the acids produced, and small amounts of propionate and butyrate. Raffinose can be fermented by a large number of intestinal bacteria (Table 1.3, chapter 1). Raffinose has been proposed as a bifidogenic factor (Benno *et al.*, 1987; Fujisaki *et al.*, 1994) and as a selective agent in a medium for bifidobacteria (Chapter 7). Our *in vitro* batch fermentation results also indicate that bifidobacteria may play an important role in the fermentation, as bifidobacteria do not produce gas, butyrate or propionate, but they do produce acetate and lactate (Bezkorovainy and Miller-Catchpole, 1989).

Fermentation of FOS and inulin, which share a similar chemical composition, only differing in chain length, also resulted in relatively large gas production. On the other hand, the final pH after fermentation was relatively low for both substrates. Fermentation of FOS resulted in slightly more lactate and less propionate and butyrate than fermentation of inulin. Ingestion of FOS is known to result in an increase in breath hydrogen as well as increased flatulence (Wang and Gibson, 1993; Kawaguchi *et al.*, 1993; Hartemink and Rombouts, 1997; Stone-Dorshow and Levitt, 1987; Alles *et al.*, 1996; Alles *et al.*, 1997, Fujita *et al.*, 1991). Some gas formation was thus to be expected. The differences between the low gas production in our system and the reported increased flatulence *in vivo* can be explained by a rapid acidification due to fermentation by bifidobacteria. A low pH inhibits gas producing organisms, such as clostridia. *In vivo* the intestinal contents are highly buffered and the acids are rapidly absorbed (Hill, 1995). The pH will thus not drop as rapidly as in our system and some substrate will be fermented by gas producing organisms.

Both FOS and inulin can be fermented by a large number of intestinal bacterial species (Table 1.3), and have been proposed as bifidogenic factors (Mitsuoka *et al.*, 1987; Howard *et al.*, 1995; Bouhnik *et al.*, 1996; Gibson and Wang, 1993). As with raffinose, bifidobacteria seem to play an important role in the fermentation of FOS in our system, as can be seen from the large percentage of lactate. The effect of a larger chain length was reflected in the production of a little more butyrate and propionate. Inulin is fermented more slowly by bifidobacteria than FOS (Hopkins *et al.*, 1998), resulting in a

slower acidification rate. As discussed above, a slower acidification rate will result in increased fermentation of the substrate by other bacteria, including butyrate and propionate producing organisms. Our data from gas, butyrate and lactate production, indicate a more mixed fermentation for inulin than for FOS. This has also been shown previously in rats, where fermentation of inulin resulted in relatively large amounts of propionate and butyrate (Levrat *et al.*, 1991).

Lactitol fermentation resulted in the highest butyrate and propionate concentrations of the three galactosyl disaccharides, as well as relatively much gas. Very little lactate was formed from lactitol. The relatively high concentration of butyrate formed from lactitol, is probably due to the fermentation of the sorbitol residue of lactitol. Sorbitol fermentation was shown to result in the production of butyrate and propionate (Brøbech-Mortensen *et al.*, 1988). Lactitol has been proposed as a bifidogenic factor (Ballongue *et al.*, 1997), but our data do not indicate extensive fermentation by bifidobacteria, which would have resulted in large concentrations of lactate and little butyrate. In accordance with our results is the observation that most bifidobacteria cannot ferment sorbitol (Holdeman and Moore, 1977, Table 1.3). Which genera are responsible for the fermentation of lactitol cannot be deduced from the data or table 1.3.

Lactose and lactulose did, unlike lactitol, not give rise to much gas. Lactose fermentation resulted in the lowest average final pH and the largest percentage of lactate. Practically no butyrate and propionate were formed. Lactose (galactosylglucose) and lactulose (galactosylfructose) differ only slightly chemically, but lactulose fermentation resulted in a higher final pH. Lactose normally does not reach the large intestine, except in lactose-intolerant persons. In lactose-intolerant persons large amounts of gas are formed, causing flatulence and cramps. Considerable gas production on lactose was thus to be expected. The high lactate concentrations indicate fermentation by lactic acid bacteria, in faeces most likely bifidobacteria. Rapid fermentation and subsequent lowering of the pH, may have prevented gas production by other bacterial groups, such as clostridia.

Lactulose is known to be fermented rapidly with the formation of gas, as can be deducted from breath hydrogen studies (Peled and Gilat, 1979; Brighenti *et al.*, 1995; Kono and Nakae, 1996; Florent *et al.*, 1985; Würsch *et al.*, 1989; Kitler *et al.*, 1992; Sahota *et al.*, 1982). It has also been proposed as a bifidogenic factor (Terada *et al.*, 1992a). Many different bacterial species can ferment lactulose (Table 1.3). Our data indicate a mixed fermentation,

which is in accordance with the observed gas production *in vivo* as well as the bifidogenic effects. From our data the fermentation can not be attributed to any specific group of intestinal bacteria.

Fermentation of TOS resulted in a low gas production, and a similar final pH as lactulose. Slightly more propionate and butyrate were formed as compared to lactulose, and more as compared to lactose. On the other hand, little lactate was produced. No differences were observed in the two groups of volunteers. TOS is considered a bifidogenic factor, and it is supposedly fermented preferentially by bifidobacteria (Tanaka *et al.*, 1983; Ito *et al.*, 1990; Ito *et al.*, 1993; Ishikawa *et al.*, 1995 Bouhnik *et al.*, 1997). Fermentation by bifidobacteria would have resulted in a large percentage of lactate, as with lactose, FOS and raffinose. On the other hand not much gas was formed either, which should have indicated clostridial fermentation. It can thus not be concluded which bacterial groups are mainly involved in fermentation of TOS in our system.

Lactate was still present in most of the samples after 48h of fermentation. Presence of lactate was correlated with a low pH in our studies. Lactate may be further metabolized to butyrate, propionate, acetate and gas by different bacterial species. This further metabolism may be prevented by a low pH, which may (partly) explain the correlation between low pH and lactate. In a continuous, pH controlled system, as well as *in vivo*, lactate may be further metabolized. A low pH and high lactate concentration may thus indicate rapid fermentation, as lactate would have been further metabolized, before a critical low pH was reached. Similarly, when the pH does not drop rapidly, lactate may be formed, but not detected, due to subsequent fermentation.

It was observed that butyrate production correlates highly with the production of gases. The measurement of gases may thus be used as a simple and cheaper screening method for butyrate production. Butyrate can be produced directly from carbohydrates by many different intestinal species, especially clostridia. Similarly, some *Clostridium* species can further metabolize lactate to butyrate, carbon dioxide and water (Schlegel, 1993). To our knowledge, no other intestinal species is capable of this reaction. In conclusion it can be stated that a high concentration of butyrate indicates substantial fermentation by *Clostridium* species, either directly from the carbohydrates, or through lactate.

An interesting observation from this study is that samples from several volunteers showed a similar pattern of end products over a six-week period. Similarly, there were marked differences between the different volunteers, even though all volunteers had

received the same diet for six weeks. It can thus be concluded that factors other than diet (including TOS intervention) determine for a large part the metabolic capacity of a specific faecal flora in an *in vitro* batch system. This has been shown previously, but only for a single volunteer (McBurney and Thompson, 1987). Recent studies using molecular techniques have also shown that genetic factors determine the composition and metabolic activity of the intestinal flora *in vivo*, which is remarkably stable in time (McCartney *et al.*, 1996; Zoetendal et al, 1998).

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7

RB AGAR, A NEW SELECTIVE MEDIUM FOR BIFIDOBACTERIA.

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SUMMARY

A new selective medium for the detection of bifidobacteria was developed. The medium owns its selectivity to the presence of propionate (15 g/l) and lithium chloride (3 g/l) as inhibitory agents, and raffinose (7.5 g/l) as a selective carbon source. In addition casein (5 g/l) is used as a protein source, which results in a zone of precipitation around the colonies of bifidobacteria. The medium, Raffinose-Bifidobacterium (RB) Agar is free of antibiotics and easy to prepare. Bifidobacteria growing on RB agar, show a yellow colony with yellow halo and precipitation zone around the colony. All human and dairy bifidobacteria grew well on RB-agar, except for some *Bi. bifidum* strains. Some uncommon or animal species, *Bi. gallicum*, *Bi. asteroides*, *Bi. animalis*, *Bi. pullorum*, and some *Bi. bifidum* did either not grow or did not show the characteristic reactions. No non bifidobacterial strains used in dairy products grow with the distinctive characteristics. Some faecal strains showed good growth and acid production, resulting in characteristic reactions. These strains were *Actinomyces* species, a *Cl. perfringens* strain and some lactobacilli from animal origin. Comparison with other media indicated that RB agar is more selective than other media used.

INTRODUCTION

Bifidobacteria are Gram-positive anaerobic branched or pleomorphic rods. Bifidobacteria can be isolated from a number of sources, mainly faeces, but also sewage and from the oral cavity. Their main habitat is the large intestine of humans and animals. Bifidobacteria are one of the major groups of intestinal bacteria. At present about 25 species of bifidobacteria are recognized. Bifidobacteria can be distinguished from other genera by the presence of fructose-6-phosphate phosphoketolase (F6PPK), a key enzyme in the 'bifidus pathway' (Scardovi, 1986; Bezkorovainy and Miller-Catchpole, 1989).

The main species present in humans are *Bi. adolescentis*, *Bi. bifidum*, *Bi. infantis*, *Bi. breve* and *Bi. longum* in the colon and *Bi. dentium* in the oral cavity. Five more species have been isolated from human sources, *Bi. catenulatum*, *Bi. pseudocatenulatum*, *Bi. angulatum* and *Bi. gallicum* (Scardovi, 1986; Bezkorovainy and Miller-Catchpole, 1989; Lauer, 1990).

It is claimed that a high number of bifidobacteria in the colon is positive for human health. A high number of bifidobacteria may prevent colonization of pathogens, and may have positive effects on intestinal peristalsis, the immunesystem, cancer prevention, cholesterol metabolism and carbohydrate metabolism in the colon (Mitsuoka, 1990; Ishibashi and Shimamura, 1993). For these reasons bifidobacteria are added as probiotics, especially in dairy products (Tamime *et al.*; 1995). To stimulate the growth of

bifidobacteria specific carbon sources, oligosaccharides, are used as bifidogenic factors (Modler, 1994). It is assumed that these carbohydrates are selectively fermented by bifidobacteria. Among oligosaccharides with bifidogenic activity are: lactulose, transgalactosyl-oligosaccharides (TOS), fructooligosaccharides (FOS), isomaltooligosaccharides, raffinose and soybean oligosaccharides (Terada *et al.*; 1992; Ito *et al.*, 1993; Mitsuoka *et al.*, 1987; Kohmoto *et al.*, 1988; Hayakawa *et al.*, 1990).

As there is growing interest in using bifidobacteria as probiotics as well as in the use of bifidogenic factors, it is necessary to have a rapid, reliable and relatively inexpensive method to determine bifidobacteria in both faeces and food products.

To detect bifidobacteria in faeces or dairy products several methods could be applied, like plate count methods, DNA-based methods (Ito *et al.*, 1992) or enzymatic methods (Scardovi, 1986). For quality control measurements in dairy products, or for a large number of samples in faecal flora studies, plate count methods are still preferable. It is therefore necessary to have a medium that selectively promotes the growth of bifidobacteria, whereas other bacteria are suppressed.

Many different media for bifidobacteria have been described (Table 7.1). As it is known that several of these media are not selective for bifidobacteria (Ishibashi and Shimamura, 1993) and as previous results showed a large number of other species growing on the media we used for the isolation of bifidobacteria, it was decided to develop a new medium for the selective detection of bifidobacteria.

The medium should enhance the growth of (most) bifidobacteria, inhibit the growth of (most) other faecal and dairy isolates, lactobacilli and streptococci. As there is very little known about the sensitivity and resistance to antibiotics in bifidobacteria, lactobacilli and other non-pathogenic intestinal bacteria (Sozzi *et al.*, 1990; Pacher and Kneifel, 1996; Lim *et al.*, 1993) as well as emerging resistance patterns, the medium should preferably be free of antibiotics. Furthermore, Pacher and Kneifel (1996) observed that the three media containing antibiotics tested in their survey (NPNL ,MPN and MSB) showed lower growth rates for bifidobacteria than media without antibiotics. As bifidobacteria need carbohydrates for growth (Bezkorovainy and Miller-Catchpole, 1989) and ferment many oligosaccharides (Terada *et al.*; 1992; Ito *et al.*, 1993; Mitsuoka *et al.*, 1987; Kohmoto *et al.*, 1988; Hayakawa *et al.*, 1990; Yazawa and Tamura, 1978; Yazawa *et al.*, 1978) it was decided to use an oligosaccharide as a carbon source. From the literature it was known that most bifidobacteria possess the enzyme α-galactosidase and are able to ferment raffinose (Scardovi, 1986; Bezkorovainy and Miller-Catchpole, 1989; Holdeman and Moore, 1977).

Table 7.1 : Selection of media used for the enumeration of bifidobacteria from faeces and dairy products.

Medium	Selectivity based on*	Used for	references
AL-agar	lactose, acetylglucosamine	faeces	Yazawa *et al.*, 1984
AMC-agar	nal, polymyxin B, kan, iac, TTC, LiCl, prop	*Bi. longum*	Arroyo *et al.*, 1995
BBM-agar	nal, rifampicine, raffinose	faeces	Cole and Fuller, 1989
BiBl-agar	aniline blue, blood	faeces, pharmaceutics	Rasiç, 1990
Bif-medium	human whey, nal, paro, aztreonam, netilmycin	dairy products	Pacher and Kneifel, 1996
BIM-25	kan, nal, iac, neo, polymyxin B	sewage	Muñoa and Pares, 1988
BS-agar	LiCl, neo, paro, prop	faeces	Rasiç, 1990
CB-agar	specific color of china blue	faeces	Mevissen-Verhage *et al.*, 1987
GL-agar	galactose, LiCl	dairy products	Iwana *et al.*, 1993
LCL-agar	lactose, liver infusion	faeces	Rasiç, 1990
LP-agar	LiCl, prop, lactose	dairy products	Lapierre *et al.*, 1992
MPN-agar	lactose, nal	faeces	Tanaka and Mutai, 1980
MRS-LP-agar	prop, LiCl	dairy products	Gomes *et al.*, 1995
NPNL-agar	LiCl, nal, neo, paro, prop	faeces, dairy products	Teraguchi *et al.*, 1978
propionate agar	propA, pH 5.0	faeces	Beerens, 1990
RCM (modified)	low pH	dairy products	Rasiç, 1990
RCM + stain	Loeffler's methylene blue stain	dairy products	Rasiç, 1990
Rogosa	low pH	dairy products, faeces	Rasiç, 1990
Rogosa (mod)	neo, paro, prop, LiCl	dairy products	Rasiç, 1990
Rogosa-N	low pH, nal	faeces	Rasiç, 1990
TCPY-agar (basic)	tomato juice	faeces	Rasiç, 1990
TOS-agar (basic)	TOS	faeces, dairy products	Sonoike, 1986
TOS-agar (mod)	TOS, nal, neo, paro	dairy products	Wijsman *et al.*, 1989
TPYd-agar	dicloxacillin	dairy products	Sozzi *et al.*, 1990
TTC-agar	TTC	faecal contamination	Gyllenberg and Niemela, 1957
VF Agar (mod)	LiCl, prop, neo, sodium lauryl sulfate	dairy products	Calicchia *et al.*, 1993
YN-6	lactose, nal, neo, bromocresol green	faeces, sewage	Resnick and Levin, 1981

* iac = iodoacetic acid, kan = kanamycin, LiCl = lithiumchloride, nal = nalidixic acid, neo = neomycin, paro = paromomycin, prop = propionate, propA = propionic acid, TOS = transgalactosyl oligosaccharides, TTC = 2,3,5-triphenyl-tetrazoliumchloride.

In addition raffinose is available in large quantities and high purity. Bromocresolpurple was used as a pH indicator to determine fermentation.

Bifidobacteria may be stimulated by short-chain fatty acids, like propionate and butyrate (Kaneko *et al.*, 1994). Several other media also use propionate either alone or in combination of lithium chloride (Table 7.1). Kaneko *et al.* (1994) still observed good growth of bifidobacteria at concentrations of propionate, that are higher than the concentrations used in these media. Therefore it was decided to add propionate in a high concentration in combination with lithium chloride.

Casein was chosen as a protein source. Bifidobacteria are able to utilize casein and may be stimulated by specific casein fractions (Bezkorovainy and Miller-Catchpole, 1989).

This report describes the selectivity of the newly developed Raffinose-Bifidobacterium (RB) medium as compared to other media.

MATERIALS AND METHODS

Raffinose Bifidobacterium (RB)-medium

The RB-medium has the following composition (g/l or ml/l):
Agar bacteriological No 1 (OXOID, Haarlem, The Netherlands) 18.0; D(+)-raffinose (Sigma, St. Louis, USA) 7.5; sodium caseinate (Sigma C0376) 5.0; yeast extract (OXOID) 5.0; lithiumchloride (Merck, Darmstadt, Germany) 3.0; sodium propionate (Verdugt, Tiel, The Netherlands) 15.0; L-cysteine.HCl (Sigma) 0.5; sodium thioglycollate (Sigma) 0.5; bromocresolpurple 1% solution (Merck) 15 ml and salts-solution 40 ml. The salts solution contained (g/l): $MgSO_4$ 0.2; $CaCl_2$ 0.2; K_2HPO_4 1.0; KH_2PO_4 1.0; $NaHCO_3$ 10.0 and NaCl 2.0. The final pH of the medium was adjusted to 6.7 +/- 0.1, using 4N NaOH or HCl.

Other media

The following media were prepared according to the instructions by the authors: AL (Yazawa *et al.*, 1984), Bifidus Blood Agar, BiBl (Rasiç, 1990), BIM-25 (Muñoa and Pares, 1988), China Blue Agar, CB (Mevissen-Verhage, 1987), Galactose Lactate Agar, GL (Iwana *et al.*, 1993), LCL (Rasiç, 1990), Lithium chloride - sodium Propionate agar, LP (Lapierre *et al.*, 1992), NPNL (Teraguchi *et al.*, 1978; Laroia and Martin, 1991), Propionate agar, prop (Beerens, 1990), TCPY (basic) (Rasiç, 1990), TOS (basic) (Wijsman *et al.*, 1989) and TTC (Gyllenberg and

Niemela, 1957). Agar was omitted from these media when used as broths. Thioglycollate medium, thio, (OXOID CM 173) with or without agar (12.0 g/l) was used as a non-selective control medium. These media were chosen on the basis that they were either free of antibiotics or used by several research groups. Media which were essentially similar to one of the media chosen were omitted, as were known non-selective media (f.e. Rogosa) or media requiring laborious determinations (BBM).

Strains

The strains used are listed in Table 7.2, as well as over 100 unidentified faecal isolates of lactic acid bacteria, bifidobacteria and streptococci. The strains were obtained from culture collections, dairy starters, our departmental strain collection as well as from hospitals in the Netherlands. All species tested may be present in faeces or dairy products.

All the unidentified faecal strains were isolated from human and swine faeces using media other than RB-agar and were characterized on the basis of cell morphology, aerobic and anaerobic growth, katalase and oxidase tests, Gram stain and acid tolerance (growth at pH 5.0).

All strains were kept as stock solutions at -80°C in thioglycollate medium with 30% (v/v) glycerol.

Growth on RB medium

All strains were pre-cultured from the stock cultures in thioglycollate broth for 24-48h anaerobically at 37°C. After incubation RB-agar plates were inoculated with these cultures using the surface streak method and incubated 72 h anaerobically at 37°C (80% N_2, 10% CO_2, 10% H_2, HoekLoos, Rotterdam, The Netherlands). Growth (colony size) and colony morphology were determined visually.

Comparison of RB with other media

Broth media were prepared according to the literature without the agar. It was decided to use broth cultures in the screening for practical purposes. Media (Table 7.1) used were AL, BiBl, CB, GL, LCL, LP, prop, TCPY, TOS, TTC and RB. The media were all free of antibiotics. A selection of strains (6 bifidobacteria and 26 non-bifidobacteria) were precultured in thioglycollate broth for 24-48 h anaerobically at 37°C. Screw capped tubes containing 7 ml medium were inoculated with 500 µl of these cultures.

The tubes were closed and incubated for 72 h (120 h for the prop medium) anaerobically at 37°C. Fresh thioglycollate tubes were inoculated with the same

Table 7.2 : Strains used in this study

Species	Strains	Origin*	Species	Strains	Origin*
Ac. israelii	Ac-1	human faeces	*Cl. difficile*	Cl-14	human faeces
Ac. naeslundii	Ac-2	human faeces	*Cl. fallax*	ATCC 19400	
Ac. naeslundii	041	oral isolate	*Cl. paraputrificium*	Cl-2	human faeces
Ac. viscosus	040	oral isolate	*Cl. perfringens*	NCTC 8239	
Ac. viscosus	Ac-3	human faeces	*Cl. perfringens*	Cl-35 and 657	human faeces
B. fragilis	ATCC 25285		*Cl. ramosum*	Cl-12	human faeces
B. ovatus	ATCC 8483		*Cl. septicum*	ATCC 6008	
B. thetaiotaomicron	ATCC 29714		*Cl. sordelli*	ATCC 9714	
B. vulgatus	ATCC 8482		*Cl. sporogenes*	ATCC 19404	
Bi. adolescentis	ATCC 15703, 15704, 15705		*Cl. tertium*	Cl-15	human faeces
Bi. angulatum	ATCC 27535		*Ec. faecalis*	ATCC 19433	
Bi. animalis	ATCC 25527		*Ec. faecium*	Ec-5 and Ec-6	human faeces
Bi. asteroides	ATCC 25910		*Ec. hirae*	ATCC 9790	
Bi. bifidum	ATCC 29521, 35917, DSM 20082, 20215, NCIMB 8810		*E. coli*	114, E-22 and E-23	human faeces
Bi. boum	ATCC 27917		*Eu. aerofaciens*	ATCC 25986	
Bi. breve	ATCC 15698, 15700		*F. mortiferum*	ATCC 9817	
Bi. catenulatum	ATCC 25739		*F. necrogenes*	ATCC 25556	
Bi. dentium	ATCC 27534		*F. necrophorum*	DSM 20698	
Bi. gallicum	ATCC 49850		*Kl. pneumoniae*	119	human faeces
Bi. infantis	ATCC 15697, 25962		*L. lactis* ssp. *lactis*	ATCC 19435, DSM 20661	
Bi. infantis	BBI	RhonePoulenc	*Lb. acidophilus*	126 and 128	swine faeces
Bi. longum	NCFB 2259, ATCC 15707		*Lb. acidophilus*		Rhone-Poulenc
Bi. longum	BBL	RhonePoulenc	*Lb. acidophilus*	L acid	NIZO
Bi. pseudocatenulatum	ATCC 27919		*Lb. acidophilus*	La-5	Christian Hanssen
Bi. pseudolongum ssp *pseudolongum*	ATCC 25526		*Lb. casei*	Shirota	Yakult
Bi. pullorum	ATCC 27685		*Lb. casei*	LC-10	RhonePoulenc
Bi. subtile	ATCC 27537		*Lb. delbrueckii ssp. bulgaricus*	DSM 20080	
Bi. suis	ATCC 27533		*Lb. fermentum*	123	swine faeces
Bi. suis	003	swine faeces	*Lb. helveticus*	NCIMB 8652, NCFB 860	
Bi. thermophilum	ATCC 25525		*Lb. plantarum*	ATCC 8014	
Bifidobacterium sp.	Bb 12	Christian Hanssen	*Me. elsdenii*	Meg-1	swine faeces
Bifidobacterium sp.	Bi-2	human faeces	mixed starter	NCFM	RhonePoulenc
Bifidobacterium sp.	Bd Bo	NIZO	*P. prevotii*	ATCC 9321	
Bifidobacterium sp.	Bf. species 420	Wiesby	*R. productus*	ATCC 35244	
Cl. beijerinckii	Cl-7	human faeces	*Salmonella infantis*	E-7	human faeces
Cl. bifermentans	ATCC 17836		*Shigella dysenteriae*	E-29	human faeces
Cl. butyricum	ATCC 8260		*St. thermophilus*	DSM 20259,NCFB 2075	
Cl. clostridioforme	Cl-3	human faeces	*Yersinia enterocolitica*	E-25	human faeces
Cl. difficile	ATCC 19397				

* The starter cultures were obtained through the following distributors : Christian Hanssen, Houten, The Netherlands ; NIZO, Netherlands Institute for Dairy Research, Ede, The Netherlands; Rhone Poulenc, Vellinga Food Ingredients, Oudewater, The Netherlands; Wiesby, Niebüll, Germany and Yakult BV, Almere, The Netherlands

cultures and used as controls. Growth was determined by optical density measurements at 620 nm (OD620, EAR 400, SLT Instruments, Groedig, Austria) and final pH was determined (pH PocketFet, Sentron, Roden, The Netherlands). For three media, BiBl, CB and RB only final pH was determined, for one medium, prop, only OD620 was determined. Growth was considered positive when either the OD620 was ≥ 0.2 units higher than the non-inoculated medium or the pH was ≥ 1.0 units lower than the non-inoculated control.

Solid media (AL, BiBl, BIM-25, CB, GL, LCL, LP, NPNL, prop and RB) were prepared according to the literature (Table 7.1). Thioglycollate agar was used as the control medium. A selection of 4 bifidobacteria and 11 non-bifidobacteria were pre-cultured in thioglycollate broth for 24-48 h anaerobically at 37°C. Plates were inoculated with these cultures and incubated 72 h anaerobically at 37°C. Growth and colony morphology were determined visually.

RB modifications

Several modifications of RB-medium were tested to increase the selectivity. The media were prepared as broths. Propionate concentrations were changed to 10 g/l (RB-9), 20 g/l (RB-11), 25 g/l (RB-12) or 30 g/l (RB-13). Propionate was replaced by butyrate (RB-17) or valerate (RB-18). Sodium caseinate was reduced to 2.5 g/l (RB-14) or replaced by acid casein hydrolysate (RB-8) and ammonium-iron sulfate (RB15), and raffinose was replaced by TOS (RB-5). TOS was free of glucose, lactose and galactose (Borculo Whey Products, Borculo, The Netherlands). The strains tested showed possible false positive results on RB agar (one actinomyces, one clostridium and four lactobacilli). In addition some bifidobacteria (two type strains, one isolate from swine faeces and one 'wild' strain from human faeces) were used. For RB-5, replacing raffinose with TOS, only 15 bifidobacteria were tested, among these all *Bi. bifidum* strains.

RESULTS

Growth on RB medium

Most bifidobacteria grew well on RB agar. Growth was defined as visible colonies of over 1 mm diameter. Visible colonies with smaller diameter were considered pin-point colonies. Of 27 strains, representing 18 species, six failed to show characteristic growth. Characteristic growth was defined as a yellow colony over 3 mm diameter with

a yellow opaque halo, due to acid formation and casein precipitation. Of these six strains only the type strain of *Bi. asteroides* failed to grow completely. The type strain of *Bi. pullorum* and four of the *Bi. bifidum* strains did grow, but failed to produce acid and thus showed no yellow halo or precipitation. Two other strains, *Bi. gallicum* and *Bi. animalis*, did show some acid formation, but no clear halo or precipitation zone. All other strains, including one *Bi. bifidum* (NCIMB 8810) showed the characteristic reactions.

Most non-bifidobacteria failed to grow on RB-medium. Only four out of 48 identified strains (representing 40 species) showed characteristic growth. These strains were *Cl. perfringens* 657 (a human isolate) and three lactobacilli, *Lb. fermentum* 123 and *Lb. acidophilus* 126 and 128. These last three strains were isolated from pigs. Three out of five actinomycetes, *Ac. naeslundii* Ac-2 and 041 and *Ac. viscosus* 040, showed growth, but with limited acid production. Casein precipitation was sometimes observed. The four *Bacteroides* species, 12 other clostridia, 6 enterobacteria, 3 enterococci, 4 lactobacilli, 2 streptococci, 2 lactococci, 3 fusobacteria, *Pe. prevotii*, *Eu. limosum* and *Me. elsdenii* showed either no growth or pin-point colonies. All *Lb. acidophilus* or *Lb. casei* containing starter cultures did either not grow at all, or showed pin-point colonies. Four out of six starters containing bifidobacteria showed characteristic colonies. Bf. species 420 and the *Bi. infantis* from the MSK B2 mixture failed to grow. The failure to recover bifidobacteria from the starter MSK B2 is probably due to the pre-culturing conditions. It is possible that the two other species (*Lb. acidophilus* and *St. thermophilus*) will outgrow bifidobacteria in thioglycollate broth. Subsequent plating on RB will therefore fail to show bifidobacteria. Preculturing in RB broth instead of thioglycollate and subsequent plating on RB agar did result in characteristic colonies, indicating that the *Bi. infantis* strain does grow on RB medium.

Of the unidentified faecal isolates all strains with a typical bifidobacterium morphology grew well on the medium and showed the characteristic reactions. None of the cocci showed characteristic reactions, neither did most of the lactobacilli. Only lactobacilli isolated from swine faeces showed acid production, but no casein precipitation.

Table 7.3 : Comparison of growth of bifidobacteria and faecal strains in broth cultures.

species	strain	Medium											
		thio	AL	BiBl	CB	GL	LCL	LP	prop	TCPY	TOS	TTC	RB
Bi. adolescentis	ATCC 15703	+*	+	+	+	+	-	+	-	+	+	+	+
Bi. bifidum	ATCC 29521	+	+/-	+	+	+	-	+	-	+	+	+	-
Bi. breve	ATCC 15700	+	+	+	+	+	+	+	+	+	+	+	+
Bi. infantis	ATCC 15697	+	+	+	+	+	+	+	+	+	+	+	+
Bi. longum	ATCC 15707	+	+	+	+	+	-	+	-	+	+	+	+
Bi. suis	ATCC 27533	+	+	+	+	+	+	+	-	+	+	+	+
B. fragilis	ATCC 25285	+	+	+	+	+	-	+	-	+	+	+	+/-
B. ovatus	ATCC 8483	+	-	+	+	+/-	-	+	-	+	-	+	+/-
B. thetaiotaomicron	ATCC 29741	+	+	+	+	+	+	+	-	+	+	+	+/-
B. vulgatus	ATCC 8482	+	-	+	+	+	-	n.d.	-	+	+	+	+/-
Cl. beijerinckii	Cl-7	+	+	+	+	+	+	+	+/-	+	+	+	-
Cl. butyricum	ATCC 8260	+	+	+	+	+	+	+	+	+	+	+	-
Cl. clostridioforme	Cl-3	+	+	+	+	+	+	+	-	+	+	+	-
Cl. paraputrificium	Cl-2	+	-	+	+	+/-	-	+	-	+	+	-	-
Cl. ramosum	Cl-12	+	+	+	+	+	+	-	-	+	+	+	+/-
E. coli	114	+	+	+	+	+	+	+	+	+	+	+	+/-
E. coli	E-22	+	+	+	+	+	+	+	+	+	+	+	+/-
E. coli	E-23	+	+	+	+	+	+	+	+	+	+	+	-
Ec. faecalis	ATCC 19433	+	+/-	+	+	+	+	+	-	+	+	+	-
Ec. faecium	Ec-6	+	+	+	+	+	+/-	+	+	+	+	+	-
Ec. faecium	Ec-7	+	+	+	+	+	+	+	+	+	-	+	-
Ec. hirae	ATCC 9790	+	+	+	+	+	+/-	+	+	+	+	+	-
F. mortiferum	ATCC 9817	+	-	+	+	+	+	+	-	+	+	+	-
F. necrogenes	ATCC 25556	+	+	+	+	+	+	+	-	+	+	+	-
Kl. pneumoniae	119	+	+	+	+	+	+	+	+	+	+	+	+/-
Lb. acidophilus	128	+	-	+	+	+	+	+	+/-	+	+	+	+
Lb. fermentum	123	+	+/-	+	+	+	+	n.d.	-	+	+	+	+
Me. elsdenii	Meg-1	+	-	+	+	+	+	+	-	+	+	+	-
R. productus	ATCC 35244	+	+/-	+	+	+	+	+	-	+	+	+	+/-
Salm. infantis	E-7	+	+	+	+	+	+	+	+	+	+	+	-
Sh. dysenteriae	E-29	+	+	+	+	+	+	+	-	+	+	+	-
Y. enterocolitica	E-25	+	+	+	+	+	+	+	+	+	+	+	-

* growth was considered positive (+) if either the final OD620 was 0.2 units higher or the final pH 1.0 unit lower as compared to the non-inoculated control. A final pH between 0.5 and 1.0 units lower than the control, or a final OD620 between 0.1 and 0.2 higher than the control were considered doubtful +/-. No differences with the non-inoculated control were considered negative (-). n.d. = not determined.

Comparison of growth in broths

The results of the comparison of the broth cultures are summarized in table 7.3. The six bifidobacteria were able to grow in all media except for *Bi. bifidum* ATCC 29521 (on LCL, prop and RB), *Bi. adolescentis* ATCC 15703 (on LCL and prop) and *Bi. longum* ATCC 15707 (on LCL and prop). Most non-bifidobacteria grew well in all media, except for the prop and RB media.

Comparison of growth on solid media

The results of the comparison of the solid media are summarized in table 7.4. Growth was defined as visible colonies of over 1 mm diameter. Visible colonies with smaller diameter were considered pin-point colonies. The four bifidobacteria were able to grow on all media, except for *Bi. pseudolongum ssp pseudolongum* ATCC 25526 on the prop medium. All other strains grew on thioglycollate medium (control), BiBI and BIM-25 medium. On the latter medium bifidobacteria and lactic acid bacteria showed a characteristic pink color, whereas other species did not.

Table 7.4 : Comparison of growth of bifidobacteria and other strains on agar plates.

species	strain	Medium										
		thio	AL	BiBI	BIM	CB	GL	LCL	LP	NPNL	prop	RB
Bi. adolescentis	ATCC 15703	+*	+	+	+#	+	+	+	+	+	+	+
Bi. breve	ATCC 15700	+	+	+	+	+	+	+	+	+	+	+
Bi. infantis	ATCC 25962	+	+	+	+#	+	+	+	+	+	+	+
Bi. pseudolongum ssp pseudolongum	ATCC 25526	+	+	+	+#	+	+	+	+	+	-	+
B. vulgatus	ATCC 8482	+	+	+	+	+	+	+	+	+	+	-
Cl. bifermentans	ATCC 17836	+	+	+	+	n.d.	n.d.	-	n.d.	-	-	-
Cl. difficile	ATCC 19397	+	+	+	+	n.d.	n.d.	-	n.d.	-	-	-
Cl. tertium	Cl-15	+	-	+	+	n.d.	n.d.	-	n.d.	-	-	-
Ec. faecalis	ATCC 19433	+	+	+	+	+	+	+		+	+	-
Ec. faecium	Ec-6	+	+	+	+	+	+	+	+	+	+	-
Ec. hirae	ATCC 9790	+	+	+	+	+	+	+	+	-	+	-
Lb. acidophilus	Lb. acid	+	+	+	+	+	+	+	+	-	+	-
Lb. casei	Shirota	+	+	+	+	+	+	+	+	+	+	-
Lb. delbrueckii ssp bulgaricus	DSM 20080	+	+	+	+#	+	+	+	+	-	+	-
St. thermophilus	NCFB 2075	+	+	+	+#	+	+	+	+	-	+	-

* + = good growth, colonies over 1 mm diameter, - = no growth or pin-point colonies (<1 mm diameter). n.d. = not determined # = red colonies

The CB, GL and LP agars also supported growth of all strains tested. Bifidobacteria showed the characteristic brown color on CB agar, whereas other species showed blue

colonies. *Cl. tertium* CI-15 failed to grow on AL agar and none of the clostridia grew on LCL or prop agar. Seven strains failed on NPNL, among them all dairy strains. None of the non-bifidobacterial strains grew on RB agar.

RB modifications

The results of RB with different propionate concentrations are given in table 7.5. The lower propionate concentrations (10 and 15 g/l) enhanced the growth of *Lb. acidophilus*. Increasing the concentration to 20 or 25 g/l inhibited only the *Lb. acidophilus* strains from dairy products. With 30 g/l propionate some bifidobacteria were inhibited, whereas other strains were not inhibited.

Addition of butyrate did not increase the selectivity. All 10 strains showed the same growth in the medium with propionate as in the medium with butyrate. Addition of valerate resulted in inhibition of two out of six bifidobacteria, whereas other bacteria showed the same growth as with propionate.

Replacing casein by casein hydrolysate did not affect the growth of any of the bacteria tested. The precipitation of casein, one of the main characteristics of RB medium, however, was not visible with casein hydrolysate. Addition of ammonium-iron sulfate (RB-15) resulted in decreased growth of bifidobacteria (lower final OD values) and no growth of some lactobacilli. The *Actinomyces* species were not inhibited.

Replacing raffinose by TOS resulted in increased growth of *Bi. bifidum* strains, none of the other bifidobacteria were inhibited.

Table 7.5: Effect of propionate concentration on the growth of selected bacteria.

species	strain	medium				
		RB-9*	RB-10	RB-11	RB-12	RB-13
Bifidobacterium sp.	Bi-2	+	+	+	+	-
Bi. infantis	ATCC15697	+	+	+	+	+
Bi. longum	ATCC15707	+	+	+	+	-
Bi. suis	003	+	+	+	+	+
Ac. naeslundii	Ac-2	+	+	+	+	-
Cl. bifermentans	ATCC17836	+	-	-	-	-
Lb. acidophilus	126	+	+	+	+	+
Lb. acidophilus	L. acid	+	-	-	-	-
Lb. fermentum	123	+	+	+	+	+
Lb. fermentum	124	+	+	+	+	+

* RB-9 10 g/l propionate, RB-10 15 g/l, RB-11 20 g/l, RB-12 25 g/l and RB-13 30 g/l

DISCUSSION

As most bifidobacteria possess the enzyme α-galactosidase we used raffinose as a selective carbon source. The same principle has been used previously, based on the detection of the enzyme α-galactosidase (Chevalier *et al.*, 1991). However, the chromogenic substrate used by these authors is expensive and difficult to obtain. Replacing raffinose by other carbohydrates as lactulose, fructooligosaccharides (FOS) and TOS did not result in increased selectivity. Many non-bifidobacteria strains did grow on the FOS and lactulose media (data not shown). TOS showed similar results as raffinose, but with increased growth of *Bi. bifidum*. However, TOS is as yet not available commercially in large quantities and high purity. Use of commercial TOS (with 10-15% lactose and galactose) showed good growth of a large number of other bacteria (data not shown), due to the mono-and disaccharides present. When TOS is available in high purity and large quantities, it has to be established whether replacing raffinose with TOS increases the selectivity.

Propionate was added in a high concentration. Bifidobacteria are stimulated by short-chain fatty acids, like propionate and butyrate (Kaneko *et al.*, 1994), whereas many other species are inhibited. Increasing the propionate concentration to 25 or 30 g/l did not improve the selectivity, the three lactobacilli showing false positive results were not inhibited. As propionate is cheaper, available as an (odorless) salt and as effective as butyrate, it was decided not to use butyrate.

Casein was added as bifidobacteria may be stimulated by casein fractions (Bezkorovainy and Miller-Catchpole, 1989). The fermentation of raffinose, and subsequent decrease in pH, resulted in casein precipitation. Although not intended, this precipitation was useful in characterizing bifidobacteria on RB agar. Replacing casein with casein hydrolysate or ammoniumsulfate resulted in loss of a precipitation zone. Increased selectivity was not observed by these replacements.

Finally bromocresolpurple was added as a pH indicator. Unexpectedly, some clostridia, which only showed pin-point colonies, did show a clear halo. However, this halo was neither yellow, nor was a precipitate present. Other experiments showed that this was due to degradation of the bromocresolpurple. Degradation of this indicator has not been demonstrated in the literature. This degradation will not yield in false-positive results, as the characteristic reactions are not present.

Growth of bifidobacteria on RB-medium was characteristic, due to acid formation from raffinose. This resulted in a yellow colony and halo and a precipitation of casein, due to the drop in pH. Twenty-one strains, out of 27 (75%) as well as four out of six (67%) starter cultures showed the characteristic reactions. Except for *Bi. bifidum*, these strains represent all major human species as well as the species used as probiotics. Raffinose fermentation by *Bi. bifidum* is strain specific (Scardovi, 1986; Bezkorovainy and Miller-Catchpole, 1989; Holdeman and Moore, 1977), which may result in a lower isolation of this species. From the bifidobacteria that did not show characteristic growth on RB-agar, *Bi. asteroides* and *Bi. pullorum* have so far only be isolated from honeybees and poultry respectively, *Bi. animalis* has been isolated from a number of animal species, whereas *Bi. gallicum* is a rare human species (only one strain has been described until 1996). The failure to recover bifidobacteria from the starter MSK B2 was due to the pre-culturing conditions as described above.

Growth of non-bifidobacterial strains on RB agar was restricted to actinomycetes, one *Cl. perfringens* and several lactobacilli. Actinomycetes are closely related to bifidobacteria and it is therefore not surprising that some growth will occur (Cross and Goodfellow, 1973). All lactobacilli that showed characteristic growth on RB agar were all isolated from pigs. As none of the lactobacilli from humans or dairy products showed any growth, the characteristic growth of these strains may be due to porcine origin. The reason for this difference remains unknown.

From the results it can be concluded that most antibiotic-free media tested do support the growth of bifidobacteria as well as many non-bifidobacteria. This was confirmed both in broth cultures as well on agar plates. Media with antibiotics (NPNL, BIM-25) supported the growth of bifidobacteria, but also of several other species. RB-broth and RB-agar support the growth of most bifidobacteria but only very few non-bifidobacteria.

RB-medium is therefore a more selective medium for the detection of bifidobacteria than the other media tested.

However, as with all media for bifidobacteria, all colonies showing doubtful characteristics should be confirmed as bifidobacteria by enzymatic or DNA-based methods (Scardovi, 1986; Bezkorovainy and Miller-Catchpole, 1989).

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8

LAMVAB - A NEW SELECTIVE MEDIUM FOR THE ISOLATION OF LACTOBACILLI FROM FAECES.

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SUMMARY

The development of a new selective medium, LAMVAB, for the isolation of *Lactobacillus* species from faeces is described. The medium is highly selective due to a low pH and the presence of vancomycin (20 mg L^{-1}). In addition the medium contains cysteine.HCl to enhance anaerobic conditions, and bromocresol green as a pH indicator.

Competing faecal flora is inhibited by the low pH (enterobacteria, bacteroides and other Gram-negative anaerobes, clostridia) and the vancomycin (enterococci, bifidobacteria, clostridia).

Characterization of the strains isolated on LAMVAB from human faeces included all common faecal lactobacilli and no other lactic acid bacteria. In one sample vancomycin-resistant cocci were present and in another sample yeasts were isolated.

It can be concluded that the new medium is highly selective and permits the growth of all common faecal lactobacilli.

The new medium can also be applied as a selective medium for lactobacilli in probiotics, which contain mixed populations of lactobacilli, bifidobacteria and enterococci.

INTRODUCTION

Lactobacilli are Gram-positive anaerobic or facultative aerobic rods, which can be isolated from a large number of sources. Lactobacilli can be isolated from the human and animal body (oral cavity, stomach, intestines, vagina), plants and material of plant origin, sewage and fermented products. Lactobacilli are strictly fermentative and acid-resistant. Growth is enhanced by acidic conditions. Final pH in broth cultures is generally below 4.5 (Hammes and Vogel, 1995).

Lactobacilli are considered to have beneficial effects on human and animal health. Therefore several species of lactobacilli are used as probiotics. Worldwide many products with probiotic lactobacilli are marketed (Fuller, 1992; Sanders, 1993). To have a beneficial effect on the health of the host, it is necessary that the probiotic strain is able to survive in the gastrointestinal tract (Fuller, 1992). It is therefore necessary to determine the number of lactobacilli in faeces and, if possible, to identify the different species directly. This can be done using specific selective media, DNA probes or immunological techniques.

Lactobacilli are generally isolated on rich, slightly acidic media, such as MRS, LBS and Rogosa agars (Mitsuoka *et al.*, 1973; Kleessen *et al.*, 1995; Minelli *et al.*, 1993). For most

(fermented) food products these media allow selective isolation of lactobacilli, as they are generally the most abundant group of bacteria present. In faeces, however, lactobacilli are present in relatively low numbers (Mitsuoka and Ohno, 1977; Finegold *et al.*, 1977). In faecal samples several lactobacilli have been isolated (Table 8.1), in humans the main lactobacilli are *Lb. acidophilus*, *Lb. casei* and *Lb. paracasei* (Hammes and Vogel, 1995). The counts of lactobacilli in faeces are highly variable between species and persons, even within a single person (Mitsuoka *et al.*, 1973; Mitsuoka and Ohno, 1977; Finegold *et al.*, 1977). The media mentioned above are not selective as growth of other acid-resistant bacteria is not restricted and in particular bifidobacteria, streptococci and enterococci are able to grow on these media (Cole and Fuller, 1989; Nelson and George, 1995).

Growth of other intestinal bacteria is restricted, but not negligible. It is therefore necessary to have a highly selective medium, which allows the isolation of low numbers of lactobacilli in the presence of high numbers of bifidobacteria, streptococci and/or enterococci.

In this report we describe the development of a new selective medium for the isolation of faecal lactobacilli.

Table 8.1 : Species of lactobacilli isolated from human or animal faeces (Hammes and Vogel, 1995)

Species	Isolated from	Species	Isolated from
Lb. acidophilus	human, animals	*Lb. hamsteri*	hamster
Lb. amylovorus	pig	*Lb. intestinalis*	mouse, rat
Lb. aviarius	chicken	*Lb. johnsonii*	chicken, calf, mouse, pig
Lb. brevis	human, cow, rat	*Lb. murinus*	mouse, rat
Lb. casei	human	*Lb. paracasei*	human
Lb. coryniformis	cow	*Lb. plantarum*	human
Lb. crispatus	human	*Lb. reuteri*	human, animals
Lb. curvatus	cow	*Lb. rhamnosus*	human
Lb. fermentum	human	*Lb. salivarius*	human, hamster, chicken
Lb. gallinarium	chicken	*Lb. vaccinostercus*	cow
Lb. gasseri	human		

Lactobacilli are highly acid resistant, growth is possible at an initial pH of 5.0 (Hammes and Vogel, 1995). Most other intestinal bacteria are not able to grow at this acidic pH. Bifidobacteria, enterococci and streptococci are not inhibited (Hardie *et al.*, 1995; Sgorbati *et al.*, 1995; Devriese and Pot, 1995). Contrary to other Gram-positive bacteria, most lactobacilli are resistant to the antibiotic vancomycin, while Gram-negative bacteria are

generally resistant (Swenson *et al.*, 1990; Holliman and Bone, 1988; Lentsner *et al.*, 1981; Chow and Cheng, 1988). Vancomycin has already been applied previously for the selective isolation of lactobacilli from brewery products (Simpson *et al.*, 1988). It is not useful as a selective agent in dairy products containing *Leuconostoc* or *Pediococcus* species, as these are vancomycin resistant (Swenson *et al.*, 1990; Bille *et al.*, 1992; Tsakri and Maniatis, 1991). Enterococci, streptococci and bifidobacteria are susceptible to vancomycin (Potgieter *et al.*, 1992; Lim *et al.*, 1993; Green *et al.*, 1991). Furthermore resistance to vancomycin is not inducible and only few vancomycin resistant enterococci have been isolated (Huygens, 1995). Vancomycin resistant bifidobacteria have not been reported.

Although lactobacilli are aerotolerant, anaerobic conditions favor their isolation and growth. It was decided to add cystein as a reducing agent to improve the isolation of lactobacilli.

The final medium consisted of MRS agar, with vancomycin, low pH (5.0) and cystein as selective and elective agents, and was named Lactobacillus Anaerobic MRS with Vancomycin and Bromocresol green, or LAMVAB.

MATERIALS AND METHODS

LAMVAB

The medium consists of three different components:
Solution A: MRS broth (Merck 1.10661) 104.4 g/l, with cystein.HCl 0.5 g/l and bromocresol green 0.05 g/l. The pH of this solution was adjusted to 5.0 +/- 0.1 using 4M HCl before autoclaving. Solution B: Agar 40 g/l. Solution C: Vancomycin hydrochloride (local pharmacy, >95% purity) 2 mg/ml in water. Solution C should be kept at 0-4 °C and was found to be stable at least three months.

Solutions A and B are sterilized at 121°C for 15 min. Solution C is sterilized by filtration using a 0.2 µm filter.

Preparation of the medium involves sterilization of equal volumes of solutions A and B. Solution B is cooled down to 50 °C in a water bath. Solution A is cooled down to room temperature. To 500 ml solution A 10 ml of solution C is added aseptically. Finally solution B is added to the MRS-vancomycin (A+C) mixture. Plates have to be poured immediately after mixing. This procedure results in a final vancomycin concentration of 20 mg/l.

Growth of different intestinal bacteria on LAMVAB

Selected intestinal bacteria from stock cultures were pre-cultured anaerobically in thioglycollate broth for 24-48h at 37°C. From these cultures a streak was made, using a sterile cotton swab, on LAMVAB and RCA (Reinforced Clostridial Agar, Oxoid) plates. RCA was used as a control medium.

The following strains were used: lactobacilli (150 strains, >10 species), bacteroides (10 strains, 5 species), clostridia (15 strains, 13 species), bifidobacteria (25 strains, >12 species), eubacteria (2 strains, 2 species), streptococci, lactococci and enterococci (50 strains, >10 species), anaerobic cocci (5 strains, 3 species), fusobacteria (5 strains, 3 species), enterobacteria (15 strains, 7 species), actinomyces (4 strains, 3 species) and two strains of *Megasphaera elsdenii*.

Inhibition of intestinal bacteria by LAMVAB

To determine which component of the LAMVAB medium inhibits different groups of intestinal bacteria, a selection of isolates (Table 8.2) was tested for growth on LAMVAB, LAMVAB with pH 6.5 (L65), and LAMVAB without vancomycin (L-). The inoculation was carried out as described above.

Comparison of LAMVAB with MRS

To compare the growth of intestinal lactobacilli and possible competing flora on MRS and LAMVAB, 71 strains of lactobacilli, bifidobacteria, *Actinomyces* species, streptococci and lactococci (Table 8.3) were grown in MRS broth. From these broths decimal dilutions were prepared as described above, and inoculated on MRS and LAMVAB agars. The plates were incubated anaerobically at 37°C for 72 h .

Isolation of lactobacilli from faeces

To determine the selectivity of LAMVAB as a medium for the isolation of lactobacilli, faecal samples (from humans and animals) were taken and dilutions were plated on MRS and LAMVAB agars. Fecal samples were taken as fresh as possible and an aliquot was transferred into a pre-weighed bottle with buffered medium. This medium consisted of Buffered Peptone Water (Oxoid) with cystein.HCl 0.5 g/l and Tween 80 1 ml/l. The pH was adjusted to 6.5 +/- 0.1 before autoclaving at 121°C for 15 min. After sampling the bottles were imported into an anaerobic chamber, with an atmosphere of 80% nitrogen, 10% hydrogen and 10% carbon dioxide (HoekLoos SHK 050H, HoekLoos, Rotterdam, The Netherlands). The samples were mixed thoroughly using an

Ultra Turrax mixer. A decimal dilution series was prepared of the homogenates using RPS. RPS consisted of Neutralized Bacterial Peptone (Oxoid), 0.5 g/l, NaCl (Merck) 8.0 g/l and cystein.HCl 0.5 g/l. The pH was adjusted to 6.7 +/- 0.1 before autoclaving at 121 °C for 15 min. The tubes were kept anaerobically at least 16h before use. Finally 30 µl of the 3rd, 5th and 7th dilutions was pipetted on LAMVAB or MRS plates. The plates were incubated anaerobically at 37 °C for 72 h.

RESULTS

Growth of different intestinal bacteria on LAMVAB

None of the intestinal strains, except for the lactobacilli and one strain of *St. gordonii* were able to grow on LAMVAB. All lactobacilli from all origins were able to grow on LAMVAB. Growth was determined as colonies over 1 mm diameter and a yellow discoloration of the medium, due to acid production. Most colonies were green or blue, due to the uptake of bromocresol green into the cell. As not all lactobacilli showed this color and remained white, the color of the colonies was not considered characteristic. Several isolates from fish failed to grow on LAMVAB. Further identification showed that these isolates were carnobacteria and not lactobacilli (M. Dapkevicius, Wageningen Agricultural University, personal communication). Carnobacteria fail to grow at pH 5.0.

Table 8.2 : Growth of selected bacteria on LAMVAB, LAMVAB at pH 6.5 (L65) or LAMVAB without vancomycin (L-).

Species	L65	L-	LAMVAB	Species	L65	L-	LAMVAB
Ac. naeslundii	No	Yes	No	*E. coli*	Yes	No	No
Aeromonas hydrophila	Yes	No	No	*Lactococcus lactis*	No	Yes	No
Bacillus cereus	Yes	No	No	*Listeria innocua*	Yes	No	No
Bacillus megatherium	Yes	No	No	*Proteus mirabilis*	Yes	No	No
B. fragilis	Yes	No	No	*Proteus vulgaris*	Yes	No	No
B. ovatus	Yes	No	No	*Pseudomonas fragi*	Yes	No	No
Bi. adolescentis	No	Yes	No	*Pseudomonas sp.*	Yes	No	No
Bi. dentium	No	Yes	No	*Salmonella infantis*	Yes	No	No
Bi. infantis	No	Yes	No	*Salmonella montevideo*	Yes	No	No
Bi. longum	No	Yes	No	*Staphylococcus aureus*	No	Yes	No
Enterobacter cloacae	Yes	No	No	*St. mutans*	No	Yes	No
Ec. faecalis	Yes	No	No	*St. oralis*	No	Yes	No
Ec. species	No	Yes	No	*Yersinia enterocolitica*	Yes	No	No

Growth was considered positive if colonies were visible. This included pinpoint colonies.

Table 8.3 : Counts of selected lactobacilli and possible competing flora on MRS (control medium) and LAMVAB. Counts are expressed as logarithmic numbers.

Strain	Our nr	Origin*	LogN counts on MRS	LAMVAB	Strain	Our nr	Origin*	LogN counts on MRS	LAMVAB
Lb. acidophilus	CH	dairy starter	8.40	8.62	*Lb. species*	622	human faeces	8.43	8.48
Lb. acidophilus	rp	dairy starter	8.67	8.74	*Lb. species*	613	human faeces	8.58	8.71
Lb. acidophilus	128	swine faeces	7.05	6.99	*Lb. species*	620	human faeces	8.12	8.26
Lb. acidophilus	126	swine faeces	6.88	6.90	*Lb. species*	604	human faeces	7.60	7.43
Lb. bulgaricus	Lb-1	IM	8.10	8.05	*Lb. species*	204	human faeces	7.13	7.09
Lb. casei	casCH	dairy starter	8.45	8.40	*Lb. species*	SST-7	oral cavity	6.88	6.88
Lb. casei	casY	dairy starter	8.09	7.99	*Ac. israelii*	Ac-1	MA	8.09	<2.0
Lb. fermentum	124	swine faeces	8.21	8.11	*Ac. naeslundii*	41	oral cavity, Nijm	7.95	<2.0
Lb. fermentum	123	swine faeces	7.64	7.73	*Ac. naeslundii*	Ac-2	MA	8.92	<2.0
Lb. helveticus	Lb-4	type	8.14	8.01	*Ac. viscosus*	40	oral cavity, Nijm	8.03	<2.0
Lb. plantarum	Lb-9	type	7.40	7.58	*Bi. adolescentis*	88	type	6.00	<2.0
Lb. reuteri	Lb-10	type	7.94	7.95	*Bi. animalis*	Bi-35	type	7.48	<2.0
Lb. sake	Lb. sake	ATO	8.20	8.17	*Bi. boum*	Bi-37	type	8.92	<2.0
Lb. species	626	human faeces	8.25	8.20	*Bi. choerinum*	Bi-42	type	8.71	<2.0
Lb. species	824	swine faeces	8.15	8.19	*Bi. dentium*	Bi-39	type	8.80	<2.0
Lb. species	199	human faeces	6.95	7.13	*Bi. globosum*	Bi-41	type	8.27	<2.0
Lb. species	168	human faeces	7.50	7.48	*Bi. infantis*	223	type	8.33	<2.0
Lb. species	856	swine faeces	7.30	7.43	*Bi. longum*	Bi-17	human faeces	8.50	<2.0
Lb. species	818	swine faeces	7.15	7.18	*Bi. longum*	219	type	8.78	<2.0
Lb. species	812	swine faeces	7.27	7.35	*Bi. pseudocatenulatum*	Bi-40	type	7.43	<2.0
Lb. species	711	human faeces	8.43	8.40	*Bi. pseudolongum*	Bi-33	type	8.12	<2.0
Lb. species	819	swine faeces	7.03	7.02	*Bi. pullorum*	Bi-38	type	7.33	<2.0
Lb. species	TR-3	oral cavity	7.45	7.18	*Bi. species*	383	human faeces	8.62	<2.0
Lb. species	SST-1	oral cavity	7.77	<2.0	*Bi. species*	628	human faeces	8.50	<2.0
Lb. species	SST-3	oral cavity	8.26	<2.0	*Bi. species*	665	human faeces	8.85	<2.0
Lb. species	SST-11	oral cavity	6.78	6.52	*Bi. suis*	211	type	8.67	<2.0
Lb. species	TST-1	oral cavity	7.21	7.17	*Ec. faecalis*	Ec-1	ATO	8.09	<2.0
Lb. species	TR-6	oral cavity	8.00	7.74	*Lactococcus. lactis*	Lc-4	NIZO	7.88	<2.0
Lb. species	624	human faeces	7.74	7.99	*L. lactis*	Lc-3	NIZO	7.92	<2.0
St. mutans	44	oral cavity, Nijm	8.21	<2.0	*L. lactis*	Lc-1	NIZO	7.86	<2.0
St. mutans	36	type	8.01	<2.0	*St. gordonii*	42	oral cavity, Nijm	8.32	7.07
St. oralis	45	oral cavity, Nijm	7.65	<2.0	*St. mitis*	46	oral cavity, Nijm	8.23	<2.0

Legend to Table 8.3

*These strains were obtained from : type : type strains, obtained from DSM, Braunsweig, Germany; MA = Maastricht University, The Netherlands; NIZO = Netherlands Institute for Dairy Research, Ede, The Netherlands; ATO = Agro Technological Institute, Wageningen, The Netherlands, IM = Dept. Industrial Microbiology, Wageningen Agricultural University, The Netherlands; Nijm = Dept. Oral Microbiology, Nijmegen University, The Netherlands

Inhibition of intestinal bacteria by LAMVAB

Comparing the growth on LAMVAB with LAMVAB at pH 6.5 (L65) and LAMVAB without vancomycin (L-) showed that Gram-negative bacteria were mainly inhibited by the low pH (Table 8.2) and Gram-positive bacteria by the vancomycin.

Comparison of LAMVAB with MRS

Comparing the new LAMVAB medium with MRS as a reference medium showed that only two out of 38 lactobacilli failed to grow on LAMVAB (Table 8.3). These two strains were both isolated from the oral cavity. All other lactobacilli showed no reduction in counts on LAMVAB. None of the bifidobacteria, *Actinomyces* species and cocci grew on LAMVAB, with the exception of *St. gordonii*, a strain isolated from the oral cavity.

Isolation of lactobacilli from faeces

Lactobacilli could be selectively isolated from samples of human faeces. Out of 20 samples of human faeces, lactobacilli could be detected in 15 samples. The counts ranged from (logN) 2.5 to 9.0. With the exception of enterococci in one sample and yeasts in another sample, no other bacteria were observed.

Fecal samples from 19 different animals were plated on MRS and LAMVAB (Table 8.4). Growth on MRS was observed in all samples. Bifidobacteria, cocci and spore-forming organisms were observed in 17 samples. In only two samples (from 3 and 34) only lactobacilli were detected on MRS. Growth on LAMVAB was observed in 13 samples. Lactobacilli were present in all 13 samples. Only in one sample some cocci were observed. Bifidobacteria, yeasts or spore-forming organisms were not observed. In only two samples presumptive lactobacilli were observed on MRS, but no growth was observed on LAMVAB.

Table 8.4: Morphology of bacteria isolated from animal faeces.

Animal	Medium	Colonies on plate[1]	Colonies tested[2]	possible lactobacilli[3]	Cocci[4]	Bifid[5]	Other[6]
Grevy's Zebra	MRS	9	8	7	1	0	0
(*Equus grevyi*)	LAMVAB	5	5	5	0	0	0
Capybara	MRS	8	8	4	4	0	0
(*Hydrochaeris hydrochaeris*)	LAMVAB	8	4	4	0	0	0
Giant Bat	MRS	8	8	0	8	0	0
(*Pteropus vampyrus*)	LAMVAB	n.g.					
Bongo	MRS	36	8	3	5	0	0
(*Boocercus euryceros*)	LAMVAB	12	6	6	0	0	0
White rhinoceros	MRS	52	8	8	0	0	0
(*Cerathoterium simum*)	LAMVAB	33	8	8	0	0	0
Gorilla	MRS	14	8	5	1	2	0
(*Gorilla gorilla*)	LAMVAB	3	3	3	0	0	0
Chimpanzee	MRS	9	8	6	1	1	0
(*Pan troglodytes*)	LAMVAB	6	6	6	0	0	0
Chimpanzee	MRS	17	8	3	0	5	0
	LAMVAB	4	4	4	0	0	0
Chimpanzee	MRS	15	8	5	0	3	0
	LAMVAB	50	8	8	0	0	0
Three-toed sloth	MRS	9	8	8	0	0	0
(*Bradipus tridactylus*)	LAMVAB	6	5	5	0	0	0
Celebes Hornbill	MRS	61	8	3	4	1	0
(*Aceros cassidix*)	LAMVAB	22	4	4	0	0	0
Giraffe	MRS	31	8	2	6	0	0
(*Giraffa camelopardalis*)	LAMVAB	n.g.					
Mouse deer	MRS	8	8	0	1	1	6 (spores)
(*Tragulus javanicus*)	LAMVAB	4	3	3	0	0	0
Llama	MRS	8	8	0	8	0	0
(*Lama glama*)	LAMVAB	n.g.					
Elephant	MRS	214	8	1	1	6	0
(*Loxodonta africana*)	LAMVAB	25	8	6	2	0	0
Yak	MRS	9	8	0	6	2	0
(*Bos grunniens*)	LAMVAB	n.g.					
Cheetah	MRS	12	8	0	0	0	8 (spores)
(*Acinonyx jubatus*)	LAMVAB	n.g.					
Watussi	MRS	19	8	1	6	0	1 (spores)
(*Bos taurus*)	LAMVAB	n.g.					
Bactrian Camel	MRS	37	8	3	3	2	0
(*Camelus bactrianus*)	LAMVAB	3	2	2	0	0	0

[1] Total number of colonies on the plate tested; [2] The number of colonies tested, preferably of the same dilution for both media, the colonies taken were mainly selected on differences in colony morphology, not incidence; [3] Possible lactobacilli were rod shaped straight or slightly curved, not branched bacteria and showed no spores; [4] Cocci were all coccal-shaped bacteria, mono-, duplo-, or streptococci; [5] Bifid were rod shaped bacteria with branches, included possible *Bifidobacterium*, *Actinomyces* and *Propionibacterium* species; [6] Others included spore-forming rods, yeasts and moulds. n.g. = no visible growth

DISCUSSION

The isolation of lactobacilli from faeces is generally difficult, due to the presence of competing micro-organisms, like bifidobacteria, streptococci and enterococci, which may be present in higher numbers than lactobacilli. Most media used for the isolation of lactobacilli, also support growth of these competing micro-organisms (Nelson and George, 1995; Cole and Fuller; 1989). The results presented above indicate that LAMVAB does not support the growth of these bacteria, whereas growth of lactobacilli is unaffected.

LAMVAB is easy to prepare, even though the three different components have to be sterilized separately. Due to the low pH of the medium, it is impossible to add the agar to the MRS broth directly. The medium does not require expensive chemicals or antibiotics. Vancomycin is widely used, relatively inexpensive and easy to obtain from pharmacies.

The medium is selective due to the low pH, which inhibits the most common Gram-negative bacteria in faeces, as well as the presence of vancomycin, which inhibits the competing Gram-positive flora (Table 8.2). This is in accordance with the literature.

Vancomycin inhibits Gram-positive bacteria, except lactobacilli. Several studies have shown that faecal lactobacilli are not inhibited by vancomycin (Swenson *et al.*, 1990; Holliman and Bone, 1988; Lentsner *et al.*, 1981; Chow and Cheng, 1988). Only one study indicated susceptibility of faecal lactobacilli towards vancomycin (Pacher and Kneifel, 1996). Susceptibility was reported for *Lb. acidophilus* and *Lb. bulgaricus* towards very low concentrations of vancomycin (1 and 4 mg/l). Our results show that most lactobacilli are not affected by the vancomycin, as there is no significant reduction in logarithmic counts between MRS and LAMVAB. Only two unidentified oral isolates failed to grow on LAMVAB. These results are in accordance with the literature (Swenson *et al.*, 1990; Holliman and Bone, 1988; Lentsner *et al.*, 1981; Chow and Cheng, 1988).

On only a few occasions other bacteria could be isolated on LAMVAB. These were cocci, probably enterococci or streptococci and it is known that a small percentage of enterococci are vancomycin resistant (Potgieter *et al.*, 1992; Lim *et al.*, 1993; Green *et al.*, 1991). Also, we observed growth of *St. gordonii*, an oral species, which may be present in small numbers in faeces.

Comparison of LAMVAB with MRS indicated a low selectivity of MRS, whereas LAMVAB was highly selective for lactobacilli. Even though we did not compare

LAMVAB with other common media, such as Rogosa and LBS agar, it is known that the growth of the bacteria tested on these media is similar to MRS (Nelson and George, 1995; Cole and Fuller; 1989).

LAMVAB was successfully applied to isolate lactobacilli from faeces from humans and a number of animals. Lactobacilli were present in most of the animals tested (Table 8.4), with the exception of the giant bat, giraffe, llama, yak, cheetah and watussi. In those samples other bacteria were isolated on MRS medium, indicating lower selectivity for this medium.

In only two samples (from the giraffe and watussi) presumptive lactobacilli were isolated on MRS, whereas no colonies were visible on LAMVAB. It is not known whether these isolates were true lactobacilli or other non-sporeforming non-branched rods.

LAMVAB may also be a suitable medium for the isolation of lactobacilli from probiotics. These products often contain lactobacilli and bifidobacteria. As bifidobacteria fail to grow on LAMVAB selective isolation of the lactobacilli is possible. We have successfully used LAMVAB to isolate different species of lactobacilli from dairy products in the presence of streptococci and bifidobacteria (data not shown).

It can be concluded that LAMVAB is highly selective for lactobacilli. It allows growth of all faecal lactobacilli tested and suppresses all other bacteria encountered in faecal material, with an occasional exception of cocci and yeasts. The medium is more selective than the media commonly used for the isolation of lactobacilli from faeces and probiotics.

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9

COMPARISON OF MEDIA FOR THE DETECTION OF BIFIDOBACTERIA, LACTOBACILLI AND TOTAL ANAEROBES FROM FAECAL SAMPLES.

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SUMMARY

The interest in functional foods, probiotics and prebiotics requires a proper method to determine specific bacterial groups in the intestinal flora, especially bifidobacteria and lactobacilli. Three media for lactobacilli (MRS, Rogosa, LAMVAB), three media for bifidobacteria (RB, NPNL, Beerens medium) and nine media for total anaerobes have been tested for selectivity and recovery. For total anaerobes Faecal Reinforced Clostridial Agar (FRCA) showed the highest cfu/g, followed by Columbia Blood Agar and BHI Blood agar. There were no significant differences between the media tested. Reduced physiological salt solution was found to be the best dilution medium. For bifidobacteria and lactobacilli samples of human faeces, cat faeces and pig ileal contents were used. Bifidobacteria could reliably be determined on all three media tested in human faeces, but not on pig ileal contents or cat faeces. Absolute counts were highest in human samples. No lactobacilli could be isolated on MRS in either sample, none of the colonies in the countable plates were lactobacilli. For Rogosa over 90% of the colony types observed in human samples were not lactobacilli. For cat faeces this was 58%, but no false positives were observed in the pig ileal samples. For LAMVAB the percentages of false positive colony types were 9, 14 and 0% for human, cat and pig samples.

It can be concluded that for bifidobacteria RB and Beerens medium show comparable results, and can be used to quantify bifidobacteria in human faeces, but none of the media tested is suitable for reliably counting bifidobacteria from pig and cat samples. For lactobacilli LAMVAB shows the highest sensitivity.

INTRODUCTION

In the last decade there has been an increasing interest in the effect of live bacteria (as probiotics) and non-digestible oligosaccharides (as prebiotics) on the intestinal microflora. It is claimed that both probiotics and prebiotics may have an effect on the composition and/or the metabolic activity of the intestinal flora, both in humans and in animals (Gibson and MacCartney, 1998). As probiotics mainly members of the genera *Lactobacillus* and *Bifidobacterium* are being used. Several types of non-digestible oligosaccharides, such as fructooligosaccharides and galactooligosaccharides, are commercially available as prebiotics (Gibson and MacCartney, 1998; Playne and Crittenden, 1996). In addition to these two categories much research is being carried out on the effect of antibiotics and polysaccharides (fibres) on the composition of the intestinal flora (Finegold *et al.*, 1983; Sugawara *et al.*, 1992; Bourquin *et al.*, 1996).

One major prerequisite for studying the effect of a certain compound on the flora, is the ability to quantify the different groups of intestinal bacteria accurately. For this

there are several methods, based on plate counts, molecular techniques, fluorescence and immunological techniques. Of these the first method is the most widely used. Molecular techniques are increasingly being used, but the other methods are so far used very rarely in applied studies. For all techniques it is necessary to know the accuracy, selectivity and possible problems and drawbacks (Charteris *et al.*, 1997).

The intestinal flora of humans and animals is a very complex microbial ecosystem, in which several hundred bacterial species have been isolated and identified. Detection of a single group of bacteria will always involve elimination of most of the other bacteria. It is thus obvious that contamination (plate counts) or cross-hybridization (DNA-techniques) is a serious problem, especially in the detection of groups or species that are not among the numerically dominant groups. Unfortunately data on selectivity are often not available or, for many media, only described for other purposes than faecal flora studies (Rasiç, 1990; Pacher and Kneifel, 1996).

The bacterial groups studied most extensively are total anaerobes, bifidobacteria, lactobacilli and *Escherichia coli*. For none of these groups there seems to be a standard medium available, as a large number of media have been used for the detection of these bacteria from faeces. Some of the media used for the detection of these groups are combined in Tables 9.1 (total anaerobes), 9.2 (bifidobacteria) and 9.3 (lactobacilli). For *E. coli* mainly MacConkey and Eosine Methylene Blue agars are used, both of which seem selective for faecal coliforms (Baron *et al.*, 1992; Adami and Cavazzoni, 1996).

Table 9.1 : Media used for the detection of total anaerobes from faeces

Medium	Class*	Reference
BB-Agar	1	Summanen *et al.*, 1993
Brain Heart Infusion Blood Agar	1	Roberts *et al.*, 1992
Columbia Blood Agar	1	Bartram *et al.*, 1994
Eggert Gagnon Agar	1	Mitsuoka *et al.*, 1973
RCA	2	Venketeshwer Rao *et al.*, 1994
Wensinck Agar	1	Wensinck *et al.*, 1981
Wilkins Chalgren Agar	1	Bearne *et al.*, 1990

* class : 1 = with blood, 2 = without additional ingredients

Total counts in faeces normally do not exceed 10^{11}/g wet faeces. Counts can reach as high as 11.3 (logN/g wet weight), but higher counts are physically impossible. Combining the results of 65 studies in healthy human adults the average count was determined as 10.7 (Fig 9.1.).

Media used for the detection of bifidobacteria can be classified in 5 different groups; known non selective media (MRS, Rogosa), media with elective carbohydrates, media with antibiotics, media with propionate and media with elective substance and/or low pH (Table 9.2).

Table 9.2 : Media used for the detection of bifidobacteria from faeces

Medium	Group*	Reference
Acetylglucosamine - Lactose Agar (AL-agar)	2	Yazawa *et al.*, 1984
Bifidobacterium selective agar (BS-Agar)	3, 5	Mitsuoka *et al.*, 1973
Bifidobacterium selective medium (BBM-agar)	2, 3	Cole and Fuller, 1989
Bifidus Blood Agar	5	Rasiç, 1984
Bifidobacterium Iodoacetate Medium (BIM-25 Agar)	3	Muñoa and Pares, 1988
China Blue Agar	5	Mevissen-Verhage *et al.*, 1987
Liver Cystine Lactose Agar (LCL-agar)	2	Rasiç, 1990
Modified Rogosa Agar	3, 5	Rasiç, 1990
MPN-agar	2, 3	Tanaka and Mutai, 1980
MRS	1	Venketeshwer Rao *et al.*, 1994
MRS Agar with LiCl and antibiotics (MRS-NN)	3, 5	Norin *et al.*, 1991
Neomycin Paromomycin Lithium Nalidixic acid Agar (NPNL)	3, 4	Teraguchi *et al.* ,1978
Propionate agar (Beerens-Agar)	4	Beerens, 1990
Raffinose - Bifidobacterium Agar	2, 4	Hartemink *et al.*, 1996
Reinforced Clostridial Agar with Cephalothin and Blood (RCB)	3	Yaeshima *et al.*, 1997
Rogosa agar	1	Rasiç, 1990
Tomato Casein Peptone Yeast Agar (TCPY)	5	Rasiç, 1990
Tomato Casein Peptone Yeast Agar (TCPY) with azide	5	Rasiç, 1990
Tomato Casein Peptone Yeast Agar (TCPY) with sorbic acid	5	Rasiç, 1990
Tomato Casein Peptone Yeast Agar (TCPY) with antibiotics	3	Roberts *et al.*, 1992
Transgalactosyloligosaccharide Agar (TOS-agar)	2	Sonoike *et al.*, 1986
TTC-agar	5	Gyllenberg and Niemela, 1958
x-Gal medium	5	Chevalier *et al.*, 1991
YN-6 Agar	2, 3, 5	Resnick and Levin, 1981

* group : 1 = non selective medium, 2 = medium without antibiotics but with elective carbohydrate, 3 = medium with antibiotics, 4 = medium with propionate, 5 = medium with elective substance and/or low pH

Combinations, media belonging to more than one group, are also used. From the large number of media used it can be concluded that there is no standard medium for the detection of bifidobacteria. Bifidobacteria are, in humans, normally present in numbers between (log) 9.0 and 10.5 per gram wet weight as can be seen in Figure 9.2., which has been derived from data of 65 studies.

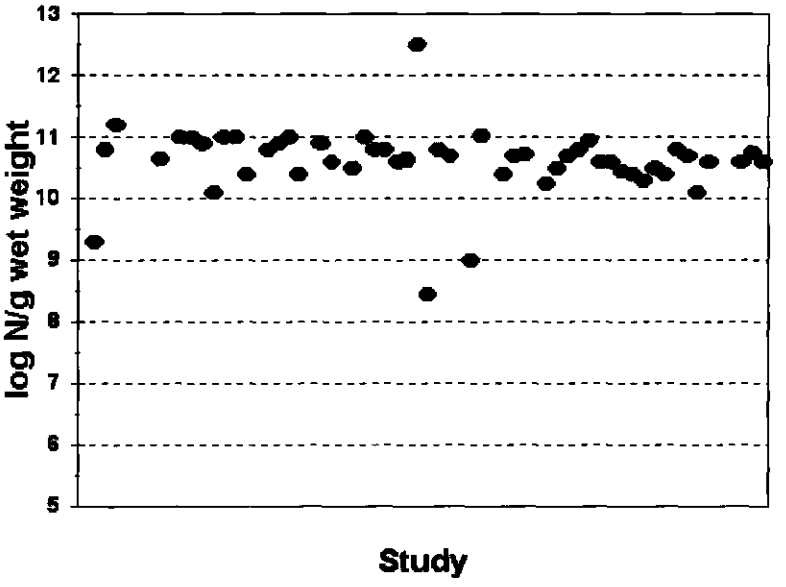


Fig. 9.1 : Total anaerobic counts as derived from 65 studies. All data were reported from fresh faeces obtained from healthy adult volunteers and expressed as $\log_{10}$ cfu/g wet weight.

The number of media used for the detection of lactobacilli is limited. Most media are not claimed to be selective for faecal lactobacilli, with the exception of the LAMVAB medium (Hartemink *et al.*, 1997). Lactobacilli are mainly inhabitants of the small intestine in humans and animals. Logarithmic counts in (human) faeces vary considerably between persons and even within a single person, as can be seen in Figure 9.2 (Mitsuoka and Ohno, 1977; Ogata *et al.*,1997).

In this study we examined the selectivity of different media for the quantification of total anaerobes, bifidobacteria and lactobacilli. Media were chosen on the basis of selectivity (from literature data) and usage in different studies. All media were tested with fresh human faeces, ileal samples of pigs (for small intestinal flora) and faecal samples of cats (a carnivorous animal), to obtain three types of samples, which may be markedly different in bacterial composition.

Table 9.3 : Media used for the detection of lactobacilli from faeces

Medium	Group*	Reference
LAB Agar	2, 4	Norin *et al.* , 1991
LAMVAB	3, 4	Hartemink *et al.*, 1997
LBS Agar	4	Mitsuoka *et al.*, 1973
LBS Agar with Tomato Juice	4	Summanen *et al.*, 1993
MRS	1	Mitsuoka *et al.*, 1973
Rogosa	4	Mitsuoka *et al.*, 1973

*group : 1 = non selective medium, 2 = medium without antibiotics, with elective carbohydrate, 3 = medium with antibiotics, 4 = medium with elective substance and/or low pH

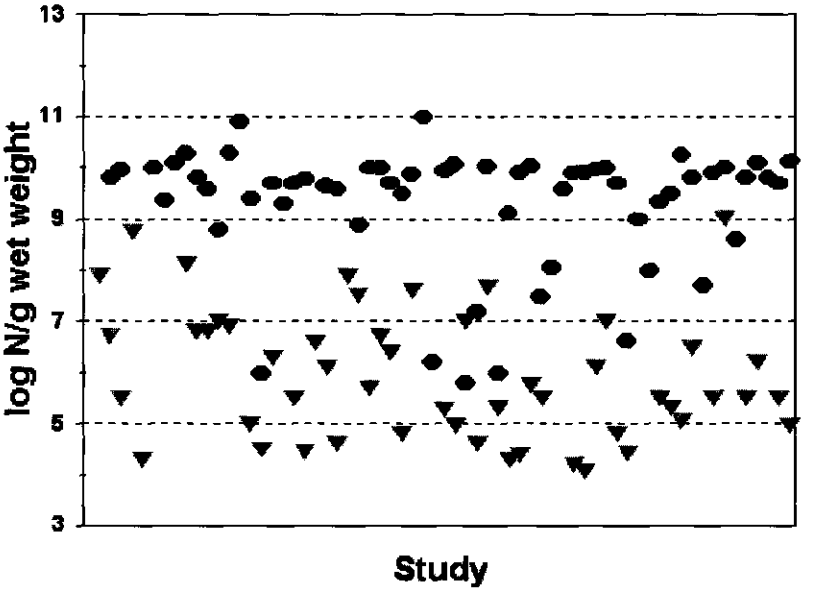


Fig. 9.2 : Counts of lactobacilli (triangles) and bifidobacteria (circles) as derived from 65 studies. All data were reported from fresh faeces obtained from healthy adult volunteers and expressed as $\log_{10}$ cfu/g wet weight.

MATERIAL AND METHODS

Faecal samples

Human faecal samples were collected from healthy human volunteers, who had not used antibiotics for the last 6 months prior to the sampling date, and had no recent

history of gastrointestinal complaints. Volunteers defaecated in a plastic bag and immediately the sample was homogenized by kneading the bag and a sub sample of approximately 10 grams was put in a pre-weighed glass container with 90 ml anaerobic buffered peptone water (Oxoid) with 0.5 g/l cysteine.HCl. The container was then closed, weighed to determine the actual sample size and immediately imported into an anaerobic cabinet (atmosphere : nitrogen 80%, carbon dioxide 10% and hydrogen 10%, HoekLoos SHK 050H, Rotterdam, the Netherlands). The whole procedure between defaecation and importing in the cabinet generally took less than 5 minutes. The samples were further homogenized using an Ultra Turrax blender inside the cabinet. Mixed samples were diluted further using reduced physiological salt solution (rps, peptone 1 g/l, cysteine.HCl 0.5 g/l and NaCl 8 g/l) or test media (see below). Finally the samples were plated on the appropriate media and incubated at 37°C. Unless otherwise stated, mixing, diluting, plating and incubation was carried out anaerobically.

Samples from pigs were obtained during a trial at the Department of Animal Nutrition. The trial conditions were not intended to affect the intestinal flora composition. Pigs were slaughtered and intestinal contents were sampled. For this study samples taken at the terminal ileum were used. Samples were collected and kept in a glass container with anaerobic buffered peptone water, flushed with nitrogen to remove oxygen from the headspace and imported into the anaerobic cabinet. Total time between sampling and importing took less than one hour. Samples were further treated as described above.

Samples from cats were taken at the local animal shelter. Samples were taken immediately after defaecation and placed in a glass container with anaerobic peptone water. Samples were imported into an anaerobic cabinet within 2 hours. Samples were further treated as described above.

Determination of total anaerobic counts

To determine the best medium for the determination of total anaerobic counts the following 9 media were compared : 1) Brucella Blood Agar (Summanen *et al.*, 1993), 2) CDC Anaerobe Blood Agar (Dowell *et al.*, 1981), 3) Reinforced Clostridial Agar (RCA, Oxoid, CM 151), 4) FRCA (see below), 5) RCA with 5 g/l bile salts (Oxoid L55), 6) Columbia Blood Agar (CAB, Oxoid CM 331), 7) Schaedler Blood Agar (Oxoid CM 437), 8) BHI Blood Agar (Difco 0037-17-8 with 1.6% agar and 50 ml sheep blood/l) and 9) Thioglycollate Agar (Oxoid CM 173). Faecal Reinforced Clostridial Agar (FRCA) was prepared from RCA with the following additions per liter medium : faecal extract (swine

faeces diluted 1:1 with buffered peptone water and sterilized 20 min 121°C) 50 ml, PY salts solution (Holdeman and Moore, 1977) 30 ml, hemin solution (Summanen *et al.*, 1993) 10 ml, vitamin K solution 0,2 ml. All media were poured inside an anaerobic cabinet to avoid exposure to oxygen. Media were kept in the anaerobic cabinet 24h before use. A total of 5 fresh human faecal samples were plated on all media. Plates were incubated anaerobically for 4 days at 37 °C.

Effect of dilution medium

The effect of dilution medium was tested with 9 different dilution media; 1) physiological salt solution (ps), 2) reduced physiological salt solution (ps with 0.5 g/l cysteine.HCl, pH 6.7), 3) dilution medium according to McCann *et al.* (McCann *et al.*, 1996), 4) thioglycollate broth (Oxoid CM 173), 5) Reinforced Clostridial Medium (RCM), 6) reduced transport medium (Atlas and Parks, 1993), 7) reduced salt solution medium (Atlas and Parks, 1993), 8) rps with 0.1% Tween 80 and 9) reduced salt solution medium at pH 6.7. All media were kept in an anaerobic cabinet for at least 24 h before use. Two different samples were diluted in all 9 media. Samples were plated on FRCA plates and incubated anaerobically for 4 days at 37°C.

The effect of diluting under aerobic and anaerobic conditions was tested for ps and rps. When diluting aerobically no vortex mixer was used, but samples were diluted by gently mixing with a pipette. Vortex mixing under aerobic conditions will result in rapid reduction of viable anaerobes (data not shown).

Media for bifidobacteria

The following media for bifidobacteria were tested : Beerens propionic acid medium (PROP (Beerens, 1990)), Raffinose-Bifidobacterium medium (RB, (Hartemink *et al.*, 1996)) and Neomycin-Paromomycin-Nalidixic acid-Lithium chloride agar (NPNL (Teraguchi *et al.*,1978)). PROP and NPNL were made according to the instructions by the authors, RB was kindly prepared by Merck (Darmstadt, Germany). In total 10 fresh human faecal samples, 7 samples of ileal contents of pigs and 9 faecal samples of cats were tested. Samples were treated and diluted in rps as described above. All diluting and plating procedures were carried out anaerobically. Plates were poured under aerobic conditions and stored in the anaerobic cabinet at least 20h prior to use. All plates were incubated anaerobically at 37 °C for 5 days. Total and typical colonies were counted. For RB medium, where bifidobacteria are supposed to form yellow-green colonies with a yellow halo, morphologies of typical and non-typical colonies were compared

(Hartemink *et al.*, 1996). No typical colonies have been described for PROP or NPNL. All colonies with different morphologies were examined microscopically. All bifid-shaped rods were tentatively considered bifidobacteria.

Media for lactobacilli

The following media for lactobacilli were tested : de Man, Rogosa and Sharpe Agar (MRS, Merck 1.10661), Rogosa Agar (Oxoid CM 627) and LAMVAB agar (Hartemink *et al.*, 1997). MRS and Rogosa were prepared according to the manufacturer's instructions, LAMVAB according to the instructions by the authors.

Samples and procedures for plating were the same as for the bifidobacteria. All plates were incubated anaerobically at 37 °C for 3 days. Total and typical colonies were counted. Typical colonies could not be defined for MRS and Rogosa, but all green to blue colonies on LAMVAB were considered typical (Hartemink *et al.*, 1997). Yellow or large white colonies were considered non-typical. All colonies with different morphologies were examined microscopically. All non-spore forming straight rods were tentatively considered lactobacilli.

RESULTS

Determination of total anaerobic counts

From Table 9.4 it can be concluded that total anaerobic counts on different media do not differ much from each other. Still each medium was assigned a rank number for each sample, based on absolute counts. Highest counts received a rank number of 1, lowest counts of 9. In general FRCA, BHI-blood agar and CAB show the highest counts, resulting in the lowest rank numbers. The average rank numbers for samples 2-5 are : FRCA 2.0; CAB and BHI 2.5; RCA 4.75; CDC 5.25; Schaedler 6.0; Thioglycollate 6.25; RCA/bile 7.25 and Brucella 7.5. Due to a heavily spreading species, no proper rank numbers could be assigned for sample 1. Total counts were in agreement with other studies (Fig 9.1), only sample 4 showed relatively low counts, which was mainly due to the high moisture content of the sample itself (relatively loose stools).

Colony morphologies were determined visually. On each medium different colony morphologies could be distinguished. On Schaedler, CDC, BHI and FRCA 5 different colony morphologies could be seen on the countable dilutions (10-100 colonies/plate), on CAB, Brucella and Thioglycollate 4, on RCA 3 and on RCA/bile only 2 different morphologies. Microscopic evaluation showed that in most cases the colonies consisted

of small pleomorphic rods or (duplo)cocci, only 3 colonies with bifid-shaped bacteria were observed (on FRCA, BHI and RCA).

Colony size was the largest on FRCA where 73% of the colonies on the countable plates had a diameter of 3 mm or more. Corresponding percentages for the other media were RCA 42, RCA/bile 33, BHI 29, CAB 21, CDC 9, Schaedler 6, Thioglycollate 5 and Brucella 1%.

Table 9.4 : Anaerobic counts in human faeces using 9 different media. Counts are expressed as $\log_{10}$ cfu/g wet weight and relative rank.

Medium	Sample 1*		Sample 2		Sample 3		Sample 4		Sample 5	
	logN/g	rank	logN/g	rank	logN/g	rank	logN/g	rank	logN/g	rank
1 Brucella	9.87	9	10.06	6	9.72	9	9.65	8	9.92	7
2 CDC	> 10.5	1	10.31	4	9.90	8	9.92	5	9.82	8
3 RCA	> 10.5	1	10.32	3	10.35	3	9.75	7	10.00	5
4 FRCA	> 10.5	1	10.33	2	10.71	2	10.15	1	10.12	3
5 RCA/bile	> 10.5	1	9.52	9	9.93	7	9.07	9	10.07	4
6 CAB	> 10.5	1	10.34	1	10.30	4	9.96	4	10.34	1
7 Schaedler	> 10.5	1	10.02	8	10.15	5	9.83	6	10.00	5
8 BHI	10.21	8	10.25	5	10.77	1	10.04	2	10.18	2
9 Thioglycollate	> 10.5	1	10.05	7	10.10	6	9.97	3	9.52	9

* counts could not accurately be determined, due to spreading of one of the species present

Effect of dilution medium

The effect of the dilution medium on the total plate counts is shown in Table 9.5. With the exception of the salt solution medium at pH 6.7 (medium 9) or 9.2 (medium 7) there were no differences between the different dilution media. Physiological salt and reduced physiological salt showed the highest results. These two media were further tested on the effect of anaerobic or aerobic conditions. No significant differences were observed between diluting and plating the samples aerobically or anaerobically. Nor were the results between ps and rps significantly different.

Table 9.5 : Effect of dilution medium. Counts are expressed as $\log_{10}$ cfu/g wet weight on FRCA plating medium.

Dilution medium	Sample 1	Sample 2
1 physiological salt	10.3	10.4
2 reduced physiological salt (rps)	10.2	10.3
3 salt solution acc. McCann	10.1	10.4
4 thioglycollate broth	10.1	9.9
5 RCM	10.0	10.0
6 reduced transport medium	9.8	10.3
7 reduced salt solution pH 9.2	9.9	9.7
8 rps with Tween 80	10.0	10.2
9 reduced salt solution pH 6.7	9.5	9.8

Media for bifidobacteria

The three media for bifidobacteria tested, showed a large variation in counts for the different samples (Fig. 9.3). Absolute counts were highest for the human faecal samples, followed by cat faeces and pig ileal contents. Variations were highest for the pig ileal samples, where, within the same sample, differences of two log units were observed between the three different media. NPNL always showed the highest counts, followed by RB in 8 of 9 samples. PROP showed the lowest counts. For cat faecal samples the variations were generally very small between NPNL and RB, but in three samples PROP showed lower counts, up to two log units. For human faecal samples the differences between the three media were small, generally less than one log unit.

Selectivity was determined by microscopic observations of all different colony morphologies on all countable (between 10 and 150 colonies/plate) plates (Table 9.6). Based on morphologies, selectivity was highest for human faeces with NPNL with 29% false positive colonies (growth, but no bifidobacterial morphology). PROP showed 39% false positives and RB 50% false positives. For cat faeces the results were much worse, with PROP and RB both showing 61% false positives and NPNL even 77%. For the pig ileum, PROP showed no less than 90% false positive colonies, both other media showed around 70%. False negatives (non typical colonies, but bifid morphology) could only be determined on RB, as this is the only medium for which typical colonies were described, but no false negatives were observed.

Most false positive colonies were different cocci (mono-, duplo- or streptococci), spore-forming rods and short rods. Only one yeast was observed on PROP in a pig ileal sample.

Based on actual counts, selectivity could only be determined for RB, as the colonies of bifidobacteria and non-bifidobacteria could not be determined for the other media and not all colonies were tested for morphology. Selectivity as a percentage of non-typical colonies ranged from around 5% for the human samples, to over 75% for some of the pig samples. Cat samples showed 10-50% non typical colonies per plate.

Table 9.6 : Selectivity of media for bifidobacteria and lactobacilli. Colonies of different shapes were tested microscopically. Bacterial morphology was determined and typical and non-typical morphologies were determined. Typical morphology for bifidobacteria was branched or bifid-shaped rods; for lactobacilli typical morphology was straight, non-sporeforming rods.

	human faeces			cat faeces			pig ileum		
Medium		morphology			morphology			morphology	
	n	typical	non-typ.*	n	typical	non-typ.	n	typical	non-typ.
RB pos**	24	12	12 (50)	23	9	14 (61)	15	4	9 (73)
RB neg	4	0	4 (0)	10	0	10 (0)	13	0	13 (0)
PROP	18	11	7 (39)	23	9	14 (61)	21	2	19 (90)
NPNL	28	20	8 (29)	31	7	24 (77)	33	11	22 (67)
LAMVAB pos	24	22	2 (9)	14	12	2 (14)	12	12	0 (0)
LAMVAB neg	0	0	0	4	0	4 (0)	10	0	10 (0)
MRS	33	5	28 (85)	29	13	16 (55)	22	1	21 (95)
Rogosa	27	1	26 (96)	26	11	15 (58)	6	6	0 (0)

* number in brackets is the percentage of false positive (typical colony, non typical morphology) or false negatives (non-typical colony, typical morphology) of the colonies tested.

**pos = colonies showing characteristics for bifidobacteria or lactobacilli, neg = colonies not showing characteristics for bifidobacteria or lactobacilli. For bifidobacteria characteristics were defined as yellow-green colonies with a yellow halo, for lactobacilli characteristics were defined as green to blue colonies and a yellow halo. These characteristics could only be determined on RB and LAMVAB, as no characteristics were defined for the other media.

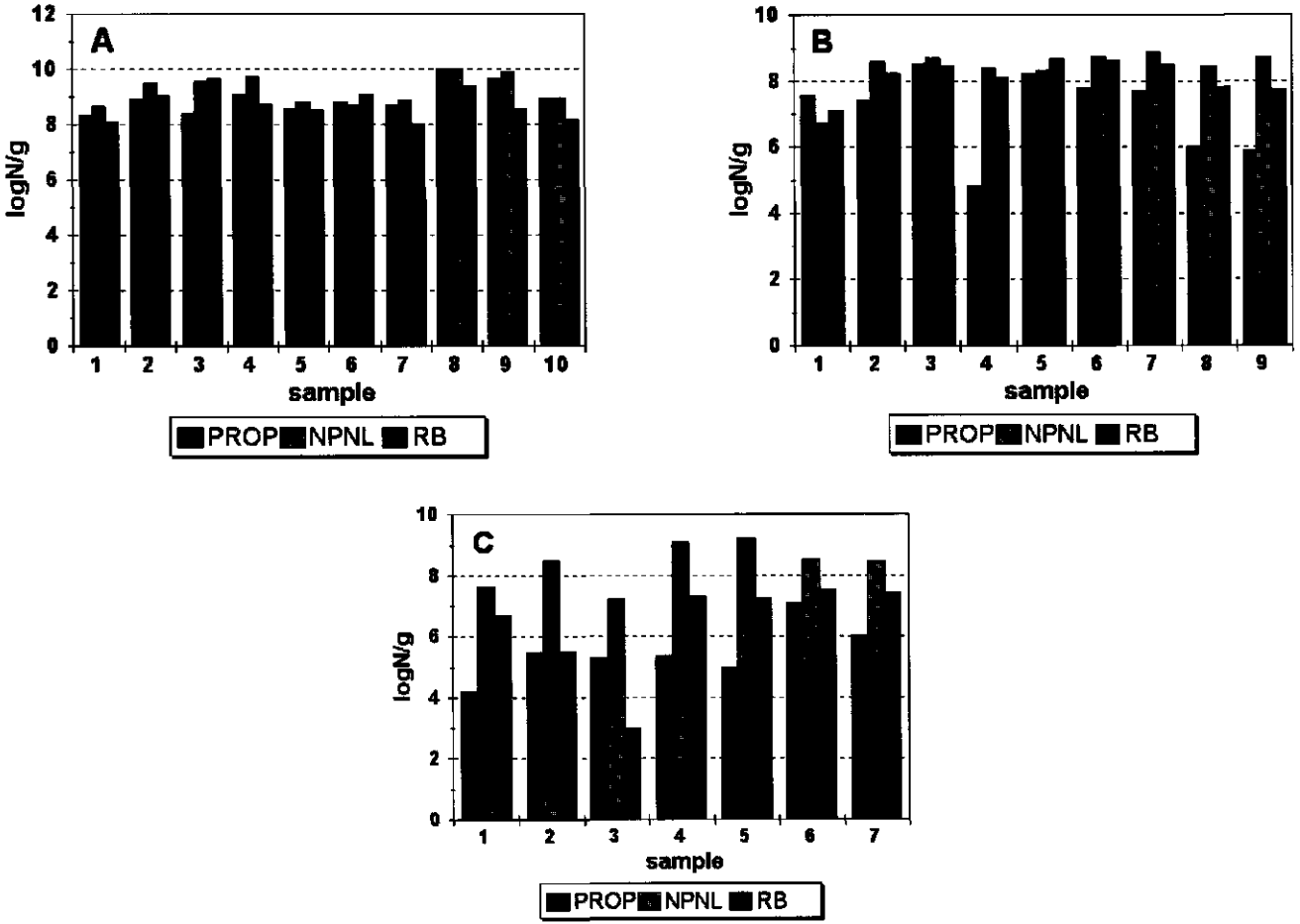


Fig 9.3. Counts (log_{10} cfu/g wet weight) on PROP, NPNL and RB media in human faeces (A), cat faeces (B) and pig ileal samples (C).

Media for lactobacilli

The three media for lactobacilli tested also showed a large variation in counts for the different samples (Fig 9.4). Absolute counts were highest for the human faecal samples, followed by cat faeces and pig ileal contents. Variations between the media were very high. In most samples LAMVAB showed the lowest counts, sometimes 5 log units lower than the counts on MRS. Rogosa showed similar or slightly lower counts than MRS, except for pig ileal samples, where the counts were comparable with LAMVAB.

Selectivity was determined by microscopic observations of all colonies with different morphologies on all countable plates. Selectivity on MRS was really poor, 85% of the different colony types for human faeces were not considered lactobacilli, for cat and pig samples these figures were 55 and 95% respectively. All straight, non spore-forming rods, were initially considered lactobacilli. Of the possible *Lactobacillus* colonies on MRS, 10 (3 from humans, 6 from cats and 1 from a pig sample) were tested for growth in MRS at pH 5.0. None of the strains grew at this pH, indicating that these strains were not lactobacilli (Hammes and Vogel, 1995). Other morphologies observed were bifid shaped bacteria, duplo- and streptococci, yeasts, spore forming anaerobes and *Megasphaera* species.

The figures for Rogosa were slightly better for the cat samples (58% false positives) as compared to MRS, but similar for human samples (96% false positive). For pig ileal samples, all isolates were possible lactobacilli. As with MRS, bifid shaped bacteria and different cocci were commonly isolated, no yeasts were observed. Only one spore-forming rod and several *Megasphaera* colonies were observed.

LAMVAB clearly was the most selective medium based on colony morphologies, no false positive colonies were observed for the pig samples, and only 9 and 14% in human and cat samples. No false negatives were observed on LAMVAB (non typical colonies, but possible lactobacilli). All non typical colonies (large white colonies, most often with a white halo) were found to be *Megasphaera* species. In addition to these, two isolates of duplococci were found.

Based on actual counts, selectivity could only be determined for LAMVAB, as the colonies of lactobacilli and non-lactobacilli could not be determined for the other media and not all colonies were tested for morphology. Selectivity as a percentage of non-typical colonies ranged from 0% for the pig samples, to around 5% for some of the cat samples.

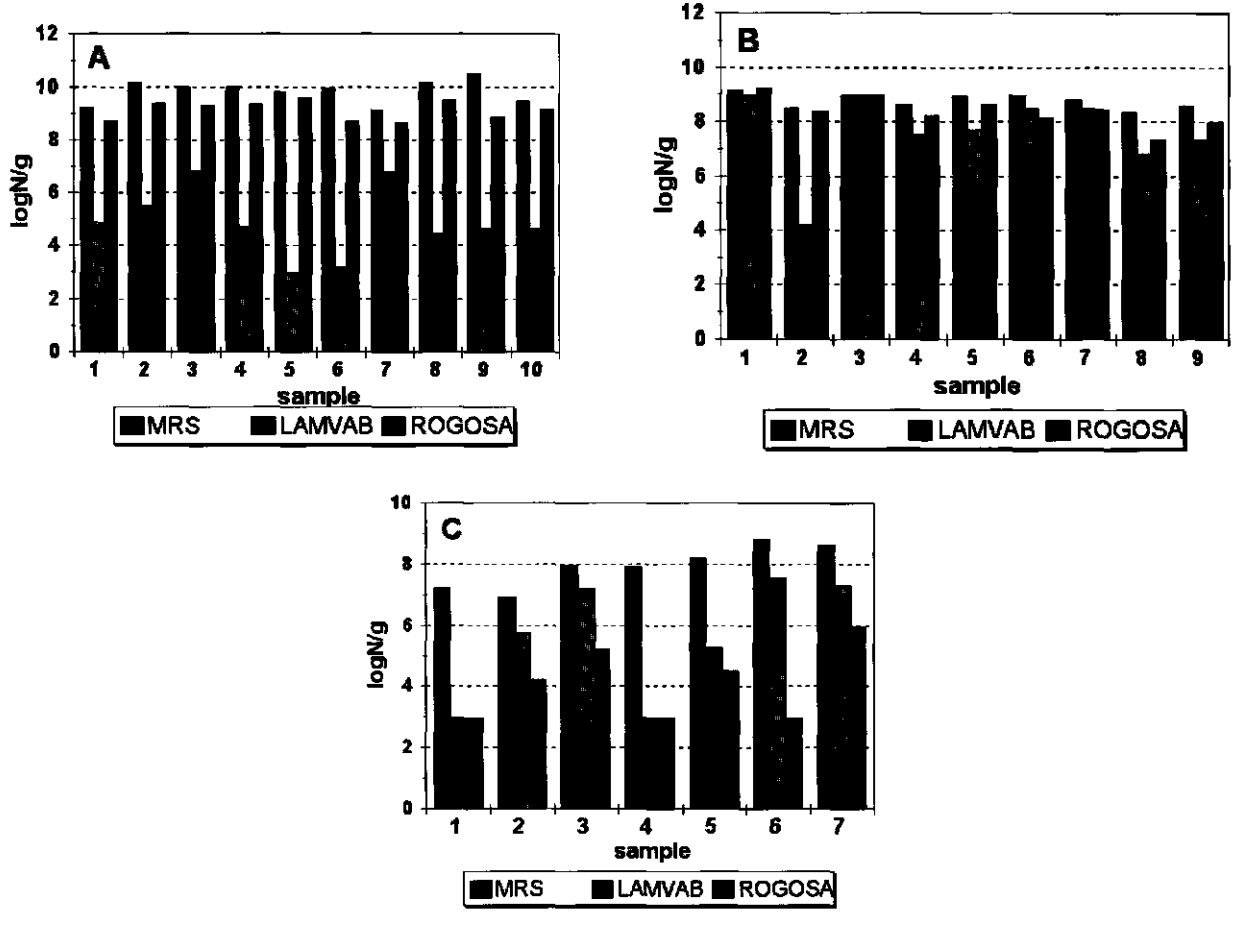


Fig 9.4. Counts (log_{10} cfu/g wet weight) on MRS, LAMVAB and ROGOSA media in human faeces (A), cat faeces (B) and pig ileal samples (C).

DISCUSSION

The differences between the media for the determination of total anaerobic counts were relatively small, and not statistically different. The experimental medium RCA/bile clearly reduced the counts compared to RCA alone, most likely due to sensitivity of some of the more common species to the concentration of bile used. Addition of faecal extract instead of blood did result in slightly, but not significant, higher counts. Faecal extract was chosen as a substitute for blood as it would mimic the environment in the intestine more than blood. Growth on FRCA clearly was improved as compared to blood-based media, as could be seen from the size of the colonies, which were larger than on most other media. This makes counting colonies easier, and may reduce the incubation period. As with the blood-based media, counting is difficult as the media are not or poorly translucent. The major disadvantages of this medium are the smell and the difficulty in obtaining faecal extract. Taking these aspects into account, CAB or BHI with blood would be the medium of choice to use in daily practice.

The effects of dilution media were relatively small as well. The dilution media commonly used are similar to rps or ps. These two simple solutions also gave the highest counts. Diluting aerobically or anaerobically did not affect the log counts significantly. Counts will decrease rapidly however, when the plates are kept aerobically for some time before incubation. In this study we only compared the effect of the dilution medium, therefore we did not use a vortex mixer and all plates were kept in the anaerobic cabinet prior to the plating procedure and immediately re-imported in the cabinet after plating.

The three categories of samples, human faeces, pig ileum contents and cat faeces, were chosen as we expected large differences in competing flora in the three types. Human faeces was chosen as the reference, as most studies have been performed on human faeces. Cats, as most carnivorous animals, do not regularly consume carbohydrates. Both lactobacilli and bifidobacteria require carbohydrates for growth. For cats we thus expected a high number of protein-fermenting species (clostridia, eubacteria) and relatively small numbers of lactobacilli and bifidobacteria. The pig ileum is a more aerobic environment, which will result in a different bacterial composition than present in faeces. Similarly, pigs generally consume large amounts of fibrous materials, which would also be beneficial for lactobacilli and bifidobacteria.

For the determination of bifidobacteria none of the three media tested was very selective. In our studies we mainly determined the occurrence of false positive or false

negative colonies. The lowest occurrence of possible false positive colonies was observed on NPNL, but in all three media the number of non-bifidobacteria capable of growing on the selective media was very high. When many different species are capable of growing on the medium, an increase of one of these species may result in serious mistakes in calculating bifidobacteria. NPNL and RB gave slightly higher counts than PROP. The incidence of false positives, based on morphologies, on RB was comparable with that on PROP and somewhat higher than that on NPNL. In human faeces the incidence of competitive flora was relatively low (less than 10% of the total colonies on the plates) and bifidobacteria could reliably be counted. This was to be expected, as bifidobacteria are one of the main groups of intestinal bacteria in humans (Fig. 9.2). In cat and pig samples, where the incidence of bifidobacteria was expected to be lower than in human faeces, the number of false positives increased, both in incidence as in percentage of colony morphologies. The latter was observed for all three media tested, but the incidence could only be determined for RB. In these samples *bifidobacteria could not be counted very reliably on either medium.* The competitive flora consisted mainly of different cocci (cat, pig, human), clostridia (cat, human) and lactobacilli (pig).

PROP medium has been described as the best medium for the determination of bifidobacteria by Silvi *et al.* (1996), but they also concluded that the total bifidobacterial counts were significantly lower on PROP than on the other media tested. Similarly Favier *et al.* (1997) concluded that PROP underestimated bifidobacteria in several of their samples. Both studies used human faeces as the test substrate. Several other studies in which PROP agar is used, also show considerably lower bifidobacterial counts than most other studies (Favier *et al.*, 1997; Bouhnik *et al.*, 1996). The total counts in human faeces as determined in our study were comparable to counts observed by other groups and with studies using molecular techniques (Fig 9.2., Welling *et al.*, 1997; McCartney *et al.*, 1996). This was also true for the PROP medium, which did not show significantly lower counts.

NPNL, which has been described as the medium of choice to determine bifidobacteria in dairy products (Teraguchi *et al.*, 1978), was found to be insufficient for pig faeces. This was in accordance to Silvi *et al.* (1996), who observed, like in our studies, many cocci on this medium. In the same study BIM-25 was tested and this medium was also found to be non specific.

In conclusion, all three media performed reasonably well for human faeces and bifidobacteria can reliably be counted. For cat faeces none of the three media performed well. For pig ileal samples, bifidobacterial colonies can be distinguished, but for reliable counts all different colonies should be identified. The results for lactobacilli indicated that both MRS and Rogosa, which are most often used, are highly non-selective. On MRS more than 95% of the colonies from human or pig faeces were no tentative lactobacilli. Of the possible lactobacilli, none grew at pH 5.0, indicating that probably no lactobacilli were present on the countable plates (10-100 colonies/plate). This low selectivity also been observed previously (Cole and Fuller, 1989; Nelson and George, 1995). The selectivity on Rogosa was clearly better than on MRS, but still considerable numbers of non-lactobacilli were determined.

The selectivity of LAMVAB was very high, both based on colony morphology as on actual numbers. The total counts were sometimes very low, compared to the other two media. It is known that counts of lactobacilli vary considerably between persons (Table 9.2) and within a single person (Mitsuoka and Ohno, 1977; Ogata *et al.*, 1997). Very often not all persons in a test population harbor detectable numbers of lactobacilli, generally this figure is around 75% (Ogata *et al.*, 1997). In our studies in 9 out of 10 samples lactobacilli could be determined in human faeces on LAMVAB. Similarly, there have been some reports that sensitivity towards vancomycin within the *Lb. acidophilus* group differs widely. This may result in underestimation of the *Lactobacillus* counts in some samples (Hamilton-Miller and Shah, 1998).

Surprisingly, all cat samples contained relatively high numbers of lactobacilli. For pig ileal samples, lactobacilli could only be observed in 5 out of 7 samples on LAMVAB.

Even though some lactobacilli do not grow on LAMVAB, the major advantage of LAMVAB is that very little false positive and no false negative colonies were observed. In most studies in which lactobacilli were determined accurately, laborious identification procedures were applied (either morphologically or biochemically). This is not necessary when LAMVAB is used. Still, there is no really better alternative to LAMVAB, as MRS and Rogosa are clearly unreliable media. Similarly, LBS, another medium, which has sometimes been used, was shown to be non-selective (Nelson and George, 1995).

10

GENERAL DISCUSSION

INTRODUCTION

This thesis describes studies related to the microbial effects of Non-Digestible Oligosaccharides (NDOs) on the intestinal microflora. The studies can be divided in three separate themes : effects on the oral microflora, fermentation of plant polysaccharides, as parent compounds for certain NDOs, and development of new isolation media for intestinal micro-organisms.

In this chapter the results of these studies are summarized and compared with other studies. Results obtained after the publication of some of the chapters by other groups will be discussed. Finally recommendations for further research are given at the end of this chapter.

ORAL MICROFLORA

Several NDOs are used commercially as prebiotics and/or bifidogenic factors in functional foods. Functional foods are defined as new foods, that are specially designed with additional health promoting ingredients. At present, most functional foods are regular foods with additional ingredients (fortified foods) or foods in which normal ingredients have been replaced by other, comparable, but health-promoting, ingredients (e.g. polyunsaturated fatty acids instead of saturated fatty acids). Foods, that are aimed to enhance the beneficial effects of the intestinal flora in general, or bifidobacteria in particular, are either fortified with NDOs or contain NDO-rich ingredients as replacement of regular ingredients.

As NDOs are added for their health promoting effects, they should not have any negative effect on the host health. This includes no negative effects in the oral cavity. NDOs, being carbohydrates, are a potential risk for caries and tooth decay. In **Chapters 2 and 3** the *in vitro* effects of NDOs on the oral flora are described.

The effects of FOS on the oral microflora are described in Chapter 2. It is concluded that FOS are fermented by the oral microflora, and that plaque formation is initiated *in vitro*. This has been confirmed by other studies (Ziesenitz and Sieber, 1987) for nystose, one of the components in FOS. Japanese studies on the other hand, did not observe any degradation of this compound by oral streptococci (Hirasawa *et al.*, 1984). No recent studies have been published, in which FOS is tested for its potential cariogenicity, neither *in vivo* nor *in vitro* (Moynihan, 1998).

The effects of TOS and lactulose are described in Chapter 3. It is concluded that TOS and lactulose can be degraded by the oral microflora, but the rate of acid and plaque formation is relatively slow. The risk of caries due to TOS is thus considered low. These studies have been confirmed for lactose and lactulose, both *in vivo* and *in vitro*. Moynihan *et al.* (1998) confirm the degradation and fermentation of lactulose by the oral microflora. Like in our studies they observed a relatively slow acidification rate.

In *in vivo* studies they also confirmed degradation and fermentation, but the oral pH did not reach values low enough to be a risk for dental caries (Moynihan et al, 1998). To our knowledge, no other studies on TOS and cariogenicity, either *in vitro* nor *in vivo* have been published prior to 1999.

Overall it can be concluded that FOS are cariogenic, but TOS are not. The actual risk of caries due to ingestion of FOS, however, is low as compared to sucrose, starch or other highly cariogenic carbohydrates. The total production of FOS in 1995 was limited to 12000 tonnes (Playne and Crittenden, 1996), which is just a fraction of the production of saccharose (over 100 million tonnes annually), glucose syrup (7 million tonnes) and starch (Belitz and Grosch, 1987). Daily intake of FOS from natural sources ranges from 2-10 g/day (Roberfroid *et al.*, 1993; Campbell *et al.*, 1997). FOS from functional foods will increase this amount, although it is difficult to estimate how much, as the number of foods with added FOS is still low and limited to a few food categories (product information Orafti, Tienen, Belgium).

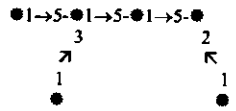
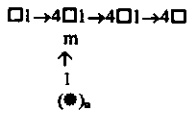
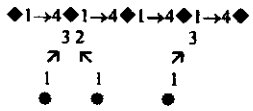
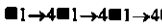
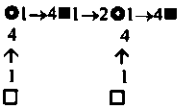
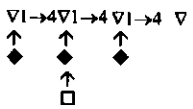
In **Chapter 5** the fermentation of guar gum is described (see below). Surprisingly, we observed that one of the few strains capable of degrading guar, was an oral species, *Bi. dentium*. This species has been isolated regularly from the oral cavity, but only from carious lesions. Using guar as a substrate, we have been able to demonstrate that *Bi. dentium* was present in the saliva of 19 out of 20 volunteers. Final pH of guar degradation was below pH 5.0, and guar can thus be considered cariogenic. The amount of guar consumed, as well as the low numbers of *Bi. dentium* reported, however, indicate that the risk of caries from guar can be considered to be low.

Guar can also be used in an easy and rapid procedure to isolate *Bi. dentium* from the oral flora. Quantitative counting of this species using guar-containing isolation media was tested, but this turned out to be non-reliable. Enrichment studies using guar tubes clearly showed the presence of *Bi. dentium*, but plating of the same samples did not result in the isolation of this species in around 50% of the samples (unpublished data).

DEGRADATION OF PLANT CELL WALL OLIGO- AND POLYSACCHARIDES

A large part of the human diet consists of vegetable material. Part of this vegetable material consists of non-digestible cell wall components, mainly polysaccharides. Therefore it can be expected that oligosaccharides, derived from plant (cell wall) polysaccharides are also (partly) non-digestible. Since plant cell wall polysaccharides are completely or partly degraded by the intestinal microflora these compounds may be a new source for NDOs. Oligosaccharides from plant cell walls can be produced enzymatically, using specific endo-glycanases (Van Laere, 1997). Oligosaccharides from

different plant cell walls were prepared (Fig. 10.1) and the fermentation of these oligosaccharides were tested.

polysaccharide	enzyme	oligosaccharide structure
arabinan	endo-arabinanase	
(arabino)-galactan	endo-galactanase	
(arabino)-xylan	endo-xylanase	
homogalacturonan	polygalacturonase	
rhamnogalacturonan	rhamnogalacturonase	
xyloglucan	endo-glucanase	

● : α -L-arabinofuranose, ▽ : ß-glucopyranose, □ : ß-galactopyranose, ◆ : ß-xylopyranose,
❍ : α-L-rhamnopyranose ■ : α-D-galacturonopyranose

Figure 10.1 : Production of oligosaccharides by enzymatic hydrolases of plant polysaccharides (Van Laere *et al.*, 1997).

Arabinoxylan oligosaccharides were prepared from arabinoxylan by incubation of arabinoxylan with endo-xylanase III from *Aspergillus awamori.* Of the 25 bacterial strains of intestinal origin tested, only 4 were able to degrade these oligosaccharides. These were *Bi. adolescentis, B. vulgatus, B. ovatus* and *Cl. sporogenes* (Van Laere *et al.*, 1995a).

Other oligosaccharides were prepared from pectins. Pectins are a complex class of plant cell wall polysaccharides. They comprise a family of acidic polymers like homogalacturonans and rhamnogalacturonans. These polysaccharides are often

associated with neutral polymers like arabans, galactans and arabinogalactans. Three types of oligosaccharides were produced by incubating polygalacturonan with a polygalacturonidase (galacturonooligosaccharides); methoxylated pectin with pectate lyase (pectic oligosaccharides) and rhamnogalacturonan with rhamnogalacturonidase (rhamnogalacturonooligosaccharides).

The galacturonooligosaccharides were fermented by *Cl. clostridioforme, Cl. ramosum* and *B. ovatus*, while the pectic oligosaccharides were fermented by *Bi. bifidum, Cl. clostridioforme, Cl. novyi, Cl. beijerinckii, B. thetaiotaomicron* and *B. ovatus*. The rhamnogalacturonooligosaccharides were only fermented by *B. fragilis, B. ovatus, B. thetaiotaomicron* and *B. vulgatus*. The oligosaccharides were completely fermented, which was determined using high performance anion exchange chromatography (Van Laere *et al.*, 1995b).

As preparation of oligosaccharides from polysaccharides is time-consuming, expensive and laboratory preparation results in only small quantities of pure oligosaccharides, it was decided to determine the fermentation of interesting polysaccharides. Two different polysaccharides were chosen, xyloglucan and galactomannan (guar gum). Xyloglucan is present in the cell wall of most plants, whereas galactomannan, which is present in most edible plants in small quantities only, is used regularly as a food additive (guar gum). The results of these studies is described in **Chapter 4 and 5**.

From the studies on oligo- and polysaccharides described above, it can be concluded that mainly *Clostridium* and *Bacteroides* species are capable of degrading and fermenting plant cell wall oligo-and polysaccharides. Some strains of bifidobacteria were also capable of degrading some components. Fermentation of polysaccharides by *Bacteroides* species has been described previously in many studies (Salyers *et al.*, 1977; Salyers et al, 1978). Fermentation by intestinal clostridia has been observed previously, but this has not been studied in detail. In our studies we observed that both xyloglucan and guar are fermented mainly by clostridial species *in vitro*. Fermentation of the plant cell wall poly- and oligosaccharides by bifidobacteria was limited. That is why the substrates tested may not be very effective to increase the number of intestinal bifidobacteria.

In **Chapter 6** fermentation of guar and xyloglucan was studied using faecal inocula. A third polysaccharide, inulin, was also included. Inulin, or polyfructose, is used as a bifidogenic factor (see Chapter 1). Inulin is a plant polysaccharide, not a cell wall polysaccharide, but rather an energy storage compound.

From the results described in Chapter 6, it can be concluded that xyloglucan and guar gum are degraded to a large extent by gas- and butyrate forming bacteria. Combining these results with those from chapters 4 and 5, it can be concluded that clostridia also play a major role in guar and xyloglucan degradation and fermentation in complex batch fermentations. This is further confirmed for guar in studies using human volunteers. In all cases an increase in breath hydrogen was observed, and sometimes an increased flatulence was reported.

No evidence was found that fermentation of inulin can be attributed mainly to a single group of bacteria. As with the other polysaccharides, butyrate was produced, but less than from the other polysaccharides. Also, more lactate and acetate and less gas was produced from inulin than from guar or xyloglucan. As it is known that bifidobacteria are capable of fermenting inulin, it is suggested that inulin is fermented by several bacterial groups, which is reflected in a mixture of fermentation end-products.

In conclusion it can be stated that the polysaccharides and plant cell wall oligosaccharides tested, are not primarily fermented by bifidobacteria. Degradation and fermentation of these substrates *in vitro* is mainly due to clostridia and bacteroides. *In vivo* other bacteria may benefit from the initial degradation of the polysaccharides into oligosaccharides. This has to be confirmed with studies using human volunteers. Only one such study has been performed with one of the substrates tested in our studies. Japanese researchers observed a bifidogenic effect of ingested hydrolyzed guar gum. They did not observe *in vitro* degradation by bifidobacteria, but an increased number of bifidobacteria in faeces (Okubo et al, 1994). It is thus likely that bifidobacteria may benefit from degradation products from the oligosaccharides tested, produced by other species, such as clostridia.

ANALYSIS OF FAECAL FLORA

Ingestion of NDOs has been reported to influence the composition of the intestinal microflora (Chapter 1). Most often an increase in bifidobacteria is reported. Changes in the number of bifidobacteria, and other bacteria, are thus to be determined accurately. The two main methods used for determining the number of (intestinal) bacterial groups are plate counts on selective media and molecular techniques. The plate count method is more often used, although the last few years more studies have made use of molecular techniques. In our studies on the faecal flora of humans and animals, we analysed many different bacterial groups (Alles *et al.*, 1999; Houdijk *et al.*, 1997; Houdijk *et al.*,

1999), using plate count techniques. Although molecular techniques are becoming increasingly popular, we used selective plating techniques, since availability of, and experience with molecular techniques was rather limited. We also wanted to use selective media to isolate and further characterize bacteria capable of fermenting oligosaccharides.

The main bacterial groups investigated were the bifidobacteria, lactobacilli, clostridia and *E. coli* as specific genera, as well as total aerobic and anaerobic bacteria. As the media generally used for the first three groups turned out to be unreliable, we developed new selective media for bifidobacteria and lactobacilli. The results of these studies are described in **Chapters 7, 8 and 9.**

The medium for bifidobacteria, RB, is suitable as a selective medium to isolate bifidobacteria from human faeces. Its selectivity was observed to be equal or better than other media used. As described in Chapter 9, it is also suitable for pig faeces, but not suitable for cat faeces. It is also not suitable for many other animal samples, such as samples from camels, elephants and seals. On the other hand it was a suitable medium for the isolation of bifidobacteria from gorillas and chimpanzees (Winsemius, 1996). For cats and pigs the medium, however, still performed better than the two other media tested.

RB medium has also successfully been tested in a multi-laboratory ring test using dairy bifidobacteria and lactobacilli. In the dairy industry viable bifidobacteria are to be determined in lyophilized starter cultures as well as in consumer products. In both products bifidobacteria are stressed, and thus are more susceptible for the acids or antibiotics used in most media (Table 7.1). Using RB, the recovery of bifidobacteria did not exceed 5% as compared with a non-selective medium. Still, this number was higher than obtained on Beerens medium (Beerens, 1990), with a high concentration of propionate at a relatively low pH. Using a double-layer technique and a four hour resuscitation period on non-selective medium, the recovery of bifidobacteria from starter cultures was increased to 90% or more. In the ring test most laboratories reported better results with RB than with the media used so far in their laboratory (unpublished results).

RB medium has also successfully been applied by the Swiss Office for Public Health (Grand and Baumgartner, 1997). The same authors report the effect of different agar bases on the recovery of bifidobacteria using the RB formula. Over 10 percent differences were observed in counts of the same product using different agar bases. As

most of the media used for the detection of intestinal micro-organisms are not available commercially, results from different groups may vary due to the use of different medium ingredients.

The development of a new selective medium for lactobacilli is described in **Chapter 8**. This medium was observed to be more selective than the other two media generally used (Chapter 9). We have used LAMVAB medium for the isolation of human and animal lactobacilli. We have isolated lactobacilli from over 50 species of animals, with none or very few non *Lactobacillus* isolates. LAMVAB has also successfully been used by other research groups (pers. comm, unpublished results).

The search for a new medium for clostridia was not successful. Combinations of different selective agents have been tried, but no selective medium could be developed. Clostridia are a very heterogeneous group, it is likely that in the near future, genetic information will make it possible to divide this group in several new genera. It may be possible to develop selective media, or specific probes, for these new genera.

The methodology used for the determination of the total aerobic and anaerobic counts has been evaluated in **Chapter 9**. It was observed that the procedures used by our group were reliable and the total (anaerobic) counts observed are of the same order of magnitude as obtained by other groups.

In conclusion it can be stated that the methodology used for the analysis of the (human) faecal flora, including the use of the two newly developed media, is reliable and the results obtained are comparable with other techniques. The main drawback of our methodology, however, is that the samples are to be treated fresh. Freezing the samples for later analysis was shown to cause unreliable and non-reproducible results (unpublished data). This drawback causes several methodological and logistic problems, when a large number of samples has to be processed. Similarly, due to oxygen stress, the samples have to be treated completely under anaerobic conditions. This may also cause problems in larger trials.

Molecular techniques may overcome part of these problems. For analysis with molecular techniques samples may be frozen and do not have to be kept anaerobically. On the other hand, when specific strains have to be isolated, molecular techniques cannot be used. A major drawback of molecular techniques, especially in frozen samples, is that it is not possible to distinguish between living and dead cells. At present, quantitative analysis of specific groups using molecular techniques is still very difficult as most probes have a high detection limit (over 10^7 cells/g) and there are no

probes for all groups available. Quantification of lactobacilli, which are often present in relatively small numbers, or total aerobic bacteria, using molecular techniques is not possible at present.

Molecular techniques may be used for specific purposes, but for each study it has to be determined on scientific and economic grounds which method is the most suitable for the purpose.

CHANGES IN THE INTESTINAL MICROFLORA

Prebiotics are defined as : *non digestible food ingredients, that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improve host health* (Gibson and Roberfroid, 1995). Most compounds that at present are used or marketed as prebiotics, are oligosaccharides.

Even though the definition does not mention any bacterial group specifically, most studies with prebiotics aim for an increase in the number of faecal bifidobacteria. Subsequently, many studies are performed to determine whether bifidobacteria are capable of degrading and fermenting new oligosaccharides. At present (Table 1.3) no carbohydrates are known that are selectively fermented by bifidobacteria. FOS, the best studied oligosaccharide mixture, for example, can be partly or completely fermented by all major groups of intestinal bacteria (Chapter 1).

An increase in bifidobacteria is used as an aim, as high numbers of bifidobacteria are considered positive for human health. This theory has its origins in Japan in the 1970s (Mitsuoka, 1990) are is based on the observations that infants receiving breastmilk had higher number of bifidobacteria than infants receiving artificial milk. A high number of bifidobacteria is thus considered the natural state. Many health aspects have been attributed to bifidobacteria (Mitsuoka, 1990), and as yet no negative effects have been attributed to bifidobacteria. On the other hand, there is no proof that people without bifidobacteria are less healthy or have intestinal disorders, as no such people have ever been reported (Fig. 9.2). Similarly, there is no proof, nor indication, that increased numbers of bifidobacteria make a person more healthy.

From the definition it can be concluded that any change in bacterial numbers or bacterial metabolism, which is beneficial for the host, can be used as a parameter for the effect of presumptive prebiotics. An increase of the number of faecal bifidobacteria may be considered as one of the possible parameters. Similarly, a decrease in possible

pathogenic bacteria or a decrease in putrefactive products, can be used as parameters.

As many characteristics of the intestinal microflora are host-specific (**Chapter 6**), and many different internal or external factors influence the intestinal flora or its metabolism (**Chapter 1**), large inter- and intrapersonal variations occur for each parameter. To determine the effect of prebiotics, these variations should be determined and taken into account. This problem may partly be overcome, by using a set of metabolic and microbial parameters combined in a health index.

Table 10.1 : Possible health parameters to be included in a health index.

a) microbiological parameters
- bifidobacteria
- total anaerobes
- total aerobes
- lactobacilli
- coliforms
- ratios between these groups to minimize intrapersonal variations

b) chemical parameters
- gases
- pH
- ammonia
- bile salts

c) enzyme activities
- glycosidases
- proteases
- tryptophanase

d) physiological parameters
- gastrointestinal complaints
- flatulence
- diarrhoea

A health index may consist of a defined set of reliable parameters. Average values and standard deviations of each of these parameters have to be determined in a specific population, similar to the test population. Changes due to a dietary intervention will be expressed as percentages above or below the average. From the changes in

individual parameters an overall average can be determined, the health index number. The deviation of this number from the health index number obtained from control subjects, determines the overall effect of the test compound. If the deviation correlates with an overall beneficial effect, the test compound can be considered a prebiotic.

To determine which parameters have to be included, it has to be established first what the importance of a specific parameter is for general health. Secondly, parameters should be quantifyable and with a high degree of reproducibility. Finally, inter- and intrapersonal variations should be determined and, when too high for practical purposes, a parameter should be excluded. Some possible parameters are described below. These parameters have been described in the literature, but other, as yet undetermined, parameters may be included. Neither the actual effect on the host health, nor the inter- and intrapersonal variations of these parameters are well established as yet.

A health index consisting of several of the parameters described above, or new parameters, may thus be more reliable to predict effects of presumptive prebiotics than the studies used so far.

RECOMMENDATIONS

Taking into account the considerations in the previous paragraphs, the following topics for future research can be recommended :

1. Determining the fermentation of other plant cell wall polysaccharides, as possible prebiotics.
2. Development of reliable parameters to determine gastrointestinal health.
3. Studies towards the changes of the intestinal flora within single individuals to determine inter- and intrapersonal variations.
4. Development of a health index.
5. Well controlled studies to the effect of presumptive prebiotics, using the health index concept.
6. Development of traditional or modern methods for the quantitative determination of intestinal bacteria.
7. More studies to the effect of genetic (host) factors on the composition and metabolism of the intestinal flora.

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SUMMARY

This thesis is the result of work carried out within a four-year multi-disciplined program, entitled ' Non-digestible oligosaccharides in foods and feed'. Within the project, four Ph.D. students were employed at the Food Chemistry, Food Microbiology, Human Nutrition and Animal Nutrition groups of the Wageningen Agricultural University. This thesis describes the studies carried out at the Food Microbiology group.

Oligosaccharides are carbohydrates, with an average chain length of 2-10 sugar residues. Most oligosaccharides cannot be digested by the enzymes in the upper gut, nor can these compounds be absorbed. These oligosaccharides are considered non-digestible, and reach the large intestine unaltered. Non-digestible oligosaccharides (NDOs) are mainly of vegetable origin and are a normal part of the human diet. Some of the natural NDOs are now produced commercially using enzymatic methods.

Most NDOs are completely or partially degraded and fermented by the bacterial populations in the large intestine. Some of the NDOs are considered to have a beneficial effect on the health of the host, due to the specific fermentation by two groups of intestinal bacteria, the bifidobacteria and lactobacilli. Commercial NDOs are marketed as a healthy ingredient, due to this selective fermentation, in several Western countries.

Chapter 1 describes the gastrointestinal tract and the bacterial composition in each part thereof. The same chapter gives an overview of the current knowledge of the fermentation of NDOs by intestinal bacteria and the effects on the host health, as far as known.

Chapters 2 and 3 describe the effect of the two types of NDOs, currently available on the Dutch market, on the etiology of dental caries. When consumed, residues of NDOs in foods may remain in the oral cavity. In the oral cavity many different bacteria are capable of degrading and fermenting carbohydrates, which results in the formation of acid and, possibly, dental lesions and caries. NDOs, being carbohydrates, may thus be fermented and are, in theory, a risk factor for dental caries.

In Chapter 2 the degradation and fermentation of fructooligosaccharides (FOS) by the oral microflora is described. It can be concluded that this class of NDOs can be fermented by the most common bacterial species. These NDOs can be considered

cariogenic, *in vitro*, but *in vivo* studies have to be carried out to determine the actual risk for dental caries.

In Chapter 3, the degradation and fermentation of transgalactosyl-oligosaccharides (TOS) by the oral microflora is described. It was concluded that this class of NDOs is not, or very slowly, degraded and fermented. These NDOs are not considered a risk factor for dental caries.

Within the framework of the project, the Food Chemistry group synthesized and purified a large number of oligosaccharide mixtures from plant cell walls. As these purifications are laborious and the total quantities of pure oligosaccharides are very small, it was decided to determine the fermentation of plant cell wall compounds by intestinal bacteria. These plant cell wall compounds are available in large quantities and thus could be used for screening studies.

Chapter 4 describes the degradation and fermentation of such a plant cell wall polysaccharide, xyloglucan. Xyloglucan is present in many edible plants, but it is commercially prepared from tamarind seeds. The more (chemically) complex the compound the more enzymes are necessary for degradation, and the less bacteria are capable of fermenting the compound. Xyloglucan has a relatively simple chemical structure, but, surprisingly, only very few intestinal bacteria were capable of degrading this compound. The second remarkable conclusion was that most of the bacteria capable of degrading xyloglucan, belonged to the genus *Clostridium.* Previously, this genus has not been considered of major importance for polysaccharide degradation in the intestine.

Chapter 5 describes the degradation of a second plant cell wall polysaccharide, guar gum, a galactomannan. Like xyloglucan, galactomannans are part of the cell wall of many plants. Guar is commercially produced from the seeds of the *Cyamopsis tetragonoloba* tree and used as a thickening agent in many foods. Guar also has a relatively simple structure. Nevertheless, only three different bacterial species, capable of degrading guar, could be isolated from human and animal faeces. One of these, *Bifidobacterium dentium* was considered to be mainly an oral species, but, using guar, could also be isolated from faeces. The same species could also be isolated from samples of saliva from 19 out of 20 volunteers. A second species, *Streptococcus bovis* could only be isolated from animal faeces, whereas the third species, *Clostridium butyricum* was present in human and animal faeces. The latter species produced large

amounts of gas, and can thus be considered responsible for the increased flatulence observed after the ingestion of guar.

Chapter 6 describes the differences in the fermentation of different oligosaccharides by human faecal inocula. In addition three polysaccharides were used in these studies. All donors had received the same diet and four samples were taken from each volunteer. The results show large differences between test compounds within the same volunteer, and large differences between volunteers on the same test compound. It can be concluded that the fermentation is largely dependent on host (genetic) factors, and not on dietary factors. It was also concluded that formation of gas is correlated with the formation of butyric acid. Butyric acid is considered to be important for the health of the intestinal wall. Gas production can thus be used as a simple screening method for butyrate production.

Within the project the variations in the bacterial composition of human and pig faeces have been studied. It was concluded in the early stages of the project that no methods existed for the reliable quantification of two major intestinal bacterial groups, *bifidobacteria and lactobacilli. Two new methods had to be developed for the* quantification of these bacterial groups.

Chapter 7 describes the development of a new medium for bifidobacteria, the RB medium. Selectivity is based on raffinose, propionate and lithiumchloride. The medium is not yet an ideal medium for the isolation and quantification for bifidobacteria but, compared with media currently used, it is more selective.

Chapter 8 describes the development of a new medium for lactobacilli, the LAMVAB medium. Selectivity is based on vancomycin and a low pH (5.0). The combination of vancomycin and low pH inhibits practically all other intestinal bacteria. LAMVAB has successfully been employed to isolate lactobacilli from faeces from a large number of animals.

The two newly developed media were compared with two other media, that are used regularly. The results of this comparison is described in **Chapter 9.** The media were used to isolate bifidobacteria and lactobacilli in human and cat faeces and pig small intestinal contents. The three media for bifidobacteria performed equally well for human faeces, but for the other two kinds of samples, the RB medium performed better. For lactobacilli, LAMVAB performed better for all three types of samples tested.

Chapter 10 discusses the results of this thesis and some recommendations for further research are given.

As conclusion it can be stated that very few NDOs are degraded and fermented selectively by bifidobacteria. This was confirmed in Chapters 2 and 6, in which degradation and fermentation of FOS by other bacterial groups is described.

Although FOS and TOS are found to be possibly cariogenic, it is not likely that either oligosaccharide will cause caries under normal conditions.

Xyloglucan and guar are degraded only by a limited number of bacteria. Unexpectedly, clostridia played a major role in the degradation of both substrates. Both substrates may be a good substrate for the production of new NDOs, but considering the results it is unlikely that these oligosaccharides are a good substrate for lactobacilli or bifidobacteria.

RB and LAMVAB are new media, which are suitable for the quantitative isolation of bifidobacteria and lactobacilli from human faeces. LAMVAB is also suitable for animal faeces. Both media are more selective than the media used at present.

SAMENVATTING

Dit proefschrift is tot stand gekomen binnen een vierjarig programma getiteld 'Niet verteerbare oligosacchariden in voeding en voeder'. Binnen dit project hebben vier AIO's gewerkt, gedetacheerd bij de vakgroepen Levensmiddelenchemie, Levensmiddelenmicrobiologie, Humane Voeding en Veevoeding. Dit proefschrift beschrijft de resultaten op het gebied van de microbiologie.

Oligosacchariden zijn koolhydraten met een gemiddelde ketenlengte van 2-10 suikerresiduen. De meeste oligosacchariden kunnen in de dunne darm van mens en dier niet opgenomen of verteerd worden. Deze oligosacchariden worden derhalve niet-verteerbare oligosacchariden genoemd. Deze komen van nature voor in de voeding van mens en dier, met name in plantaardig materiaal. Tevens zijn er tegenwoordig enzymatisch vervaardigde mengsels van oligosacchariden verkrijgbaar.

Deze niet-verteerbare oligosacchariden (in het vervolg NDOs genoemd, naar de Engelse afkorting Non-Digestible Oligosaccharides) worden door de bacteriën in het maagdarmkanaal gefermenteerd. Van een aantal van deze NDOs wordt gezegd dat ze de gezondheid van de gastheer zouden bevorderen. Dat zou gebeuren door selectieve fermentatie door twee groepen darmbacteriën, namelijk de bifidobacteriën en de lactobacillen. Om deze reden worden NDOs tegenwoordig geproduceerd in een aantal landen en als specifiek gezondheidsbevorderend ingrediënt aan levensmiddelen toegevoegd.

In **Hoofdstuk 1** wordt een overzicht gegeven van het maagdarmkanaal en de daarin voorkomende bacteriën. Ook worden hier de NDOs beschreven, waarvan op dit moment informatie beschikbaar is. Voor zover bekend is ook aangegeven door welke darmbacteriën deze NDOs gefermenteerd kunnen worden, en of een gezondheidseffect te verwachten is.

In de volgende twee Hoofdstukken (**Hoofdstuk 2 en 3**) worden de mogelijke effecten van de twee in Nederland verkrijgbare commerciële NDOs op de vorming van cariës ('gaatjes') beschreven. Als NDOs aan levensmiddelen worden toegevoegd, dan bestaat er het risico dat een deel in de mondholte achterblijft. In de mondholte komen vele bacteriën voor, en afbraak van de achtergebleven NDOs kan leiden tot zuur- en plaquevorming en daarna tot gaatjes. In Hoofdstuk 2 is beschreven welke bacteriën uit de mondholte fructooligosacchariden, FOS, kunnen afbreken en fermenteren. Geconcludeerd kan worden dat dit type oligosacchariden goed afgebroken en

gefermenteerd kan worden. Er bestaat dus een theoretisch risico dat deze oligosacchariden tot cariës kunnen leiden. Dit dient nog *in vivo* verder onderzocht te worden.

De in **Hoofdstuk 3** beschreven transgalactosyl-oligosacchariden, TOS, en aanverwante verbindingen, zijn veel moeilijker of zelfs niet af te breken door de bacteriën uit de mondholte. Er kan dan ook geconcludeerd worden dat deze NDOs geen risico vormen voor cariës.

Binnen het project zijn door de groep van Levensmiddelenchemie een groot aantal mengsels van oligosacchariden gemaakt uit plantencelwanden. Het maken en zuiveren van dergelijke mengsels is bijzonder arbeidsintensief en bovendien zijn de opbrengsten erg laag, honderden milligrammen zuivere stof. Dit is te weinig om uitgebreide studies met darmbacteriën mee uit te voeren. Daarom is besloten om eerst een aantal polysacchariden te bestuderen, waaruit, bij gunstige resultaten, later oligosacchariden bereid zouden kunnen worden. De polysacchariden, van plantaardige oorsprong, zijn wel in grote hoeveelheden voorhanden.

In **Hoofdstuk 4** wordt beschreven hoe xyloglucaan, een polysaccharide aanwezig in vele (eetbare) planten, maar commercieel gewonnen uit tamarindezaad, afgebroken wordt door darmbacteriën. Opvallend was dat maar een zeer beperkt aantal darmbacteriën in staat was om dit polysaccharide af te breken. Opvallend, omdat de chemische structuur vrij simpel is en het dus de verwachting was dat veel bacteriën in staat zouden zijn dit polysaccharide af te breken. Een tweede opvallende conclusie is dat van de bacteriën, die xyloglucaan konden afbreken, een groot deel behoorde tot het geslacht *Clostridium*. Hoewel bekend is dat dit geslacht in staat is plantencelwanden af te breken, worden ze meestal niet in verband gebracht met de afbraak van plantaardig materiaal in de darm.

In **Hoofdstuk 5** wordt de afbraak van een tweede polysaccharide, guar gum, een galactomanaan, beschreven. Guar wordt veel in levensmiddelen toegepast als verdikkingsmiddel, en vergelijkbare structuren komen voor in veel plantaardig materiaal. Ook dit polysaccharide heeft een vrij eenvoudige structuur. Desondanks blijkt guar maar door een drietal soorten darmbacteriën afgebroken te worden. Één daarvan, *Bifidobacterium dentium* was eigenlijk alleen bekend als een mondbacterie, maar blijkt ook in de darm voor te komen. Door de unieke eigenschap van guar afbraak is ook aangetoond dat deze bacterie in de mond van 19 van de 20 geteste personen voorkwam. Een tweede bacterie, *Streptococcus bovis*, werd alleen uit dierlijke

faecesmonsters geïsoleerd, terwijl de derde soort *Clostridium butyricum* zowel bij mensen als bij dieren het meeste geïsoleerd werd. Deze laatste bacterie is waarschijnlijk ook verantwoordelijk voor de gasvorming die bij veel personen wordt waargenomen na het eten van (te grote) hoeveelheden guar.

Hoofdstuk 6 beschrijft de verschillen in fermentatie van verschillende oligosacchariden en de hierboven beschreven polysacchariden. Er is gebruik gemaakt van faecale monsters van proefpersonen, die meededen aan een grote voedingsproef. Alle deelnemers kregen hetzelfde dieet, hierdoor werden mogelijke dieetinvloeden uitgesloten. De resultaten laten zien dat er grote verschillen bestaan tussen de monsters van verschillende proefpersonen, ook al gebruiken ze hetzelfde dieet. Geconcludeerd kon worden dat de fermentatieve capaciteit van de flora meer afhangt van gastheer-factoren dan van het dieet. Ook bleek er een goede correlatie te zijn tussen de hoeveelheid gevormd gas en boterzuur. Het meten van gas kan dus gebruikt worden als een makkelijke manier om te selecteren op boterzuurproductie. Boterzuur is een belangrijk eindproduct, omdat het mogelijk darmkanker kan tegengaan.

Binnen het project is ook veel onderzoek verricht naar de effecten van NDOs op de darmflora van mensen en biggen. Al vroeg bleek dat er geen betrouwbare methoden beschikbaar waren om de belangrijkste bacteriegroepen, de bifidobacteriën en de lactobacillen, betrouwbaar aan te tonen. Daarom werd het noodzakelijk om nieuwe methodieken te ontwikkelen voor het aantonen van deze twee bacteriegroepen.

In **Hoofdstuk 7** wordt de ontwikkeling van een nieuw medium voor bifidobacteriën beschreven. Dit medium is selectief door het gebruik van een NDO, raffinose, as koolstofbron en propionaat en lithiumchloride als remstoffen. Het medium is nog niet ideaal, maar het is een selectiever medium, dan de tot nu toe beschreven media. Het blijkt ook zeer bruikbaar te zijn voor het tellen en isoleren van bifidobacteriën uit humane faeces.

In **Hoofdstuk 8** wordt vervolgens een medium voor lactobacillen in faeces beschreven. De selectiviteit van dit medium is gebaseerd op een antibioticum, vancomycine, en een pH van 5.0. Door een combinatie van deze twee factoren worden de meeste andere in de darm voorkomende bacteriën geremd. Het blijkt ook zeer geschikt voor de isolatie van lactobacillen uit dierlijke faeces.

In **Hoofdstuk 9** worden de twee bovenstaande media vervolgens vergeleken met een aantal media, zoals die tot nu toe veelvuldig worden gebruikt. Uit de vergelijking blijkt dat de nieuwe media beter zijn dan de andere geteste media voor humane

monsters. Bij varkens zijn geen van de drie geteste media voor bifidobacteriën ideaal te noemen, maar het in Hoofdstuk 7 beschreven RB medium was wel het meest geschikt, omdat er goed onderscheid te maken was tussen bifidobacteriën en ongewenste bacteriën. Bij monsters van katten, die maar weinig bifidobacteriën in hun darm hebben, bleken de drie media niet geschikt. In varkens en katten bleken de twee oude media voor lactobacillen bijzonder ongeschikt, het in Hoofdstuk 8 beschreven LAMVAB werd echter zeer geschikt bevonden.

Tenslotte worden in **Hoofdstuk 10** de resultaten kritisch belicht en wordt ook een vergelijking gemaakt met de andere resultaten in het gehele NDO-project.

Concreet kan afgeleid worden uit Hoofdstuk 1 dat er maar weinig NDOs zijn, die selectief door bifidobacteriën of lactobacillen worden afgebroken. Dit werd ook bevestigd in Hoofdstuk 2, waarin FOS door de meeste mondbacteriën werd afgebroken, en Hoofdstuk 6, waarin faecale fermentaties nader zijn bestudeerd.

FOS en TOS blijken in de praktijk niet tot een risico op cariës te leiden, hoewel FOS, in tegenstelling tot TOS wel potentieel cariogeen zijn.

Xyloglucaan en guar blijken moeilijk afbreekbaar te zijn, ondanks de relatief eenvoudige structuur. Opvallend is de rol van clostridia in de afbraak. Beide polysacchariden zijn misschien een goede bron voor NDOs, maar door de moeizame fermentatie, is niet te verwachten dat bifidobacteriën en/of lactobacillen gestimuleerd zullen worden.

RB en LAMVAB zijn media die zeer goed te gebruiken zijn voor humaan faecaal onderzoek. RB is minder geschikt voor dierlijke monsters, terwijl LAMVAB geschikt is voor zeer diverse diersoorten. De media zijn beter geschikt dan de tot nu toe gebruikelijke media.

NAWOORD

Het in dit proefschrift beschreven onderzoek is hoofdzakelijk uitgevoerd bij de leerstoelgroep Levensmiddelenmicrobiologie van het Departement Levensmiddelentechnologie en Voedingswetenschappen, van de Landbouwuniversiteit Wageningen. Terminologie die anders was aan het begin van het onderzoek en over een jaar zullen zowel de universiteit als de leerstoelgroep wel weer anders heten. Een situatie vergelijkbaar met de wereld van de darmflora; de namen van de verschillende bacteriën veranderen ook, lijkt het, iedere paar jaar, wat het interpreteren van de resultaten soms sterk bemoeilijkt.

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CURRICULUM VITAE

Ralf Hartemink werd geboren op 22 januari 1966 in Hilversum. In 1984 behaalde hij het VWO diploma aan het Gemeentelijk Gymnasium aldaar. In hetzelfde jaar werd begonnen aan de studie Levensmiddelentechnologie aan de toenmalige Landbouwhogeschool Wageningen. Hij studeerde af in september 1989 met als gecombineerde afstudeerrichting Levensmiddelenchemie en Levensmiddelenmicrobiologie. Afstudeervakken en stages vonden plaats bij het RIKILT te Wageningen, de sectie Levensmiddelenmicrobiologie, Coberco Research in Deventer en Hollustuvernð Ríkisins in Reykjavík, IJsland. Tijdens zijn studie was hij actief in de studentenbadmintonvereniging De Lobbers, waarin hij vier jaar in het bestuur zat en de NSK Badminton 1989 organiseerde.

Na afstuderen vertrok hij opnieuw naar IJsland, waar hij twee jaar werkte bij het IJslands Visserijinstituut en anderhalf jaar bij de Universiteit van IJsland, als wetenschappelijk onderzoeker. In januari 1993 keerde hij terug naar Wageningen om te beginnen aan het in dit proefschrift beschreven promotieonderzoek, dat tot juni 1997 duurde. In 1998 was hij tevens de organisator van het op 4 en 5 december gehouden internationale symposium, ter gelegenheid het einde van het NDO-project. Sinds maart 1998 is hij aangesteld bij de sectie levensmiddelenmicrobiologie als tijdelijk wetenschappelijk medewerker, en bij het OWI Technologie en Voeding als een van de drie studiecoördinatoren Levensmiddelentechnologie, met als hoofdtaak de voorlichting en PR van de studierichting., alsmede als secretaris van de Richtings Onderwijs Commissie.

Sinds 1993 is hij tevens 's zomers reisleider van Nederlandse groepen in IJsland en Groenland en gedurende 1993-1996 is hij lid geweest van de AIO-raad van de onderzoeksschool VLAG, waarvan in 1995 als voorzitter.

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