IDENTIFYING AND CHARACTERIZING NEW INGREDIENTS IN VITRO FOR PREBIOTIC AND SYNBIOTIC USE

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To Eemil

You’re the light of my life

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ABSTRACT

The endogenous microbiota, constituting the microbes that live inside and on humans, is estimated to outnumber human cells by a factor of ten. This commensal microbial population has an important role in many physiological functions, with the densest microbiota population found in the colon. The colonic microbiota is a highly complex and diverse bacterial ecosystem, and a delicate balance exists between the gut microbiota and its host. An imbalance in the microbial ecosystem may lead to severe symptoms in and also beyond the gastrointestinal tract.

Due to the important role of the gut microbiota in human health, means of its modification have been introduced in the dietary concepts of pro-, pre- and synbiotics. Prebiotics, which are usually carbohydrates, strive to selectively influence beneficial microbes resident in the colon with the aim of modifying the composition and functionality of the commensal microbial population towards a purportedly healthier one. The study of prebiotic effects on colonic micro-organisms is typically done by using human faecal material, though this provides relatively little information on bacterial populations and metabolic events in different parts of the colon. For this reason, several *in vitro* models have been developed to investigate the gut microbiota.

The aim of this doctoral thesis was to screen through some of the promising prebiotic candidates, characterize their effects on the microbiota through the use of two *in vitro* methods (pure microbial cultures and a colon simulator model) and to evaluate their potential as emerging prebiotics or synbiotics when combined with the probiotic *Bifidobacterium lactis*.

As a result of the screening work and subsequent colon simulation studies, several compounds with promising features were identified. Xylo-oligosaccharides (XOS), which have previously already shown promise as prebiotic compounds, were well fermented by several probiotic *Bifidobacterium lactis* strains in pure culture studies and in the following simulation studies utilizing the complex microbiota by endogenous *B. lactis*. Another promising compound was panose, a trisaccharide belonging to isomalto-oligosaccharides (IMO) that also was also able to modify the microbiota *in vitro* by increasing the number of beneficial microbes investigated. Panose has not been widely studied previously and therefore, this thesis work provided the first data on panose fermentation in mixed colonic microbiota. Galacto-oligosaccharide (GOS) is an established prebiotic, and it was studied

here in conjunction with another potential polygosaccharide polydextrose (PDX) and probiotic *B. lactis* Bi-07. In this final study, the synbiotics including GOS were more effective than the constituting pro- or prebiotics alone in modulating the microbiota composition, thus indicating a synergy resulting from the combination.

The results obtained in this *in vitro* work can be, and have already been, utilized in product development aimed at the nutritional modification of the human colonic microbiota. Some of the compounds have entered the human clinical intervention phase to investigate in more detail the prebiotic and synbiotic properties seen in these *in vitro* studies.

ABBREVIATIONS

BA = biogenic amines

BCFA = branched chain fatty acid

CD = Crohn's disease

CFU = colony forming unit

DGGE = denaturing gradient gel electrophoresis

DNA = deoxyribonucleic acid

EFSA = European Food Safety Authority

FISH = fluorescence in situ hybridization

FOS = fructo-oligosaccharides

GOS = galacto-oligosaccharides

GIT = gastrointestinal tract

GLP-1 = glucagon-like peptide -1

HPLC = high-performance liquid chromatography

IBS = irritable bowel syndrome

IBD = inflammatory bodel disease

IMO = isomalto-oligosaccharides

MRS = de Man, Rogosa, and Sharpe medium

OD = optical density

PDX = polydextrose

qPCR = quantitative polymerase chain reaction

SCFA = short chain fatty acid

SEM = standard error of mean

SHIME = simulator for human intestinal microbiological ecosystem

SOS = soy-oligosaccharides

TIM = TNO intestinal model

TSB = tryptic soy broth

V1 = vessel 1

V2 = vessel 2

V3 = vessel 3

V4 = vessel 4

XOS = xylo-oligosaccharides

LIST OF ORIGINAL PUBLICATIONS

I Mäkeläinen, H.S, Mäkivuokko, H.A., Salminen, S.J., Rautonen, N.E., and Ouwehand, A.C. (2007). The effects of polydextrose and xylitol on microbial community and activity in a 4-stage colon simulator. *Journal of Food Science* **72(5):** M153-159

II Mäkeläinen, H., Hasselwander, O., Rautonen, N., and Ouwehand, A.C. (2009). Panose, a new prebiotic candidate. *Letters in Applied Microbiology* **49(6):** 666-72

III Mäkeläinen, H., Forssten, S., Saarinen, M., Stowell, J., Rautonen, N., and Ouwehand, A.C. (2010). Xylo-oligosaccharides enhance the growth of bifidobacteria and *Bifidobacterium lactis* in a simulated colon model. *Beneficial Microbes* **1(1):** 81-91

IV Mäkeläinen, H., Saarinen, M., Stowell, J., Rautonen, N., and Ouwehand, A.C. (2010). Xylo-oligosaccharides and lactitol promote the growth of *Bifidobacterium lactis* and *Lactobacillus* species in pure cultures. *Beneficial Microbes* **1(2):** 139-148

V Mäkeläinen, H., Ottman, N., Forssten, S., Saarinen, M., Rautonen, N., and Ouwehand, A.C. (2010). Synbiotic effects of GOS, PDX and *Bifidobacterium lactis* Bi-07 *in vitro*. *International Journal of Probiotics and Prebiotics* **5(4):** 203-210

1. REVIEW OF THE LITERATURE

1.1. Introduction

The microbes that live inside and on humans constitute the resident microbiota, which is estimated to outnumber the human cells by a factor of ten (Turnbaugh *et al*., 2007). This huge commensal microbial population has an important role in many physiological functions and in the colon, where the most dense microbiota can be found, it takes part for example in the digestion of food, which is a complex co-operation of human and microbe driven functions (Macfarlane and Englyst, 1986). The colonic microbiota is a highly complex and diverse bacterial ecosystem, and a delicate balance exists between the gut microbiota and its host. An upset in the microbial ecosystem may lead to severe symptoms in and also beyond the gastrointestinal tract. The role of the microbiota in the health and disease of the host started to be unravelled by Metchnikoff and Tissier over 100 years ago. Metchnikoff ascribed the longevity of Bulgarians to the consumption of fermented sour milks, while Tissier discovered bifidobacteria in the faeces of breast-fed infants (Fuller and Gibson, 1997; Gibson and Fuller, 2000). More recently, the composition and activity of the endogenous microbiota of the gut have been intensively studied, which has led to the development of a vast array of new molecular techniques used to investigate the microbial ecology in health and the composition shifts in disease (Furrie, 2006; Tannock, 2008).

Due to the important role of the gut microbiota in human health, means of dietary modification have been introduced in the concepts of probiotics (Fuller, 1989) and prebiotics (Gibson and Roberfroid, 1995). These aim to modify the composition and/or activity of the microbiota towards a purportedly healthier one. Probiotics, being live microbial supplements, aim to do this through incorporating beneficial exogenous microbes into the endogenous microbiota, whereas prebiotics strive to selectively influence the beneficial microbes already resident in the colon. Synbiotics, combinations of pro-and prebiotics, try to benefit from both of its constituent components' features, and increase the probiotics function by providing specific nutrition to it through the prebiotic component (de Vrese and Schrezenmeir, 2008).

The study of prebiotic compounds has relied quite heavily on the investigation of human faecal samples as representative samples from other parts of the gastrointestinal tract (GIT) are rather difficult to obtain. *In vitro* models of the human GIT have proven to be useful tools

in studying prebiotic fermentation in more detail as they allow the monitoring of prebiotic fermentation in different artificial compartments of the GIT and provide information on the metabolites produced and the microbial groups affected by the different substrates. Furthermore, they allow the analysis of bacterial enzyme activities and toxin formations potentially associated with the beneficial and harmful effects and are good models for localizing the site of the prebiotic effect. The *in vitro* models have been successfully used in selecting the potential candidates showing prebiotic effects for further *in vivo* and human studies, which remain the obligatory steps in verifying the prebiotic effect of an ingredient (Roberfroid *et al.*, 2010).

1.2. The microbiota of the colon

1.2.1. Composition of the microbiota

The human gastrointestinal tract is colonized by microbes from the mouth to the end of the large intestine. The numbers of bacteria vary in different regions of the GIT in accordance with the prevailing conditions; the colon has the most favorable conditions for microbial growth, thus, it is the most heavily colonized part if the GIT (Vernazza *et al*., 2006b). In an adult, the colon is estimated to contain about 800 different bacterial species (Bäckhed *et al*., 2005), adding up to between 10^{13} and 10^{14} bacteria in total (Gill *et al*., 2006). The majority of these microbes are strict anaerobes, and facultative anaerobes and aerobes are present in lower numbers (Salminen *et al*., 1998). In addition to bacteria, the human colon also contains eukarya, archaea and viruses (Bäckhed *et al.*, 2005). During the past decade, the progress made in molecular techniques has enabled new ways to investigate human microbiota, the research of which had previously relied completely on the culturability of the microbes. At the turn of the century, it was estimated that between 60%and 80% of adult colonic microbes represented putative novel species, most of which have so far not been identified through cultivation (Suau *et al.*, 1999). Based on molecular analysis, the majority of colonic microbes belong to three major bacterial divisions: *Bacteroidetes, Firmicutes* (containing e.g. *Clostridium leptum, Cl. Coccoides-Eubacterium rectale* and *Lactobacillus* as subgroups) and *Actinobacteria* (e.g. *Bifidobacterium* and *Atopobium)*. The two dominant groups, *Cl. coccoides-Eubacterium rectale* (28%) and *Cl. leptum* (25%) make up more than half of the total microbiota and the most abundant microbial groups after them are *Bacteroides* (9%), *Bifidobacterium* (4%) and *Atopobium* (3%). The *Lactobacillus-Enterococcus* group represents approximately 2% of the total microbial population (Lay *et al*., 2005). Molecular

analyses have also revealed that the composition of the human intestinal microbial population is host-specific (Eckburg *et al.*, 2005; Ley *et al.*, 2006) and relatively stable over time (Rajilic-Stojanovic *et al.*, 2009; Seksik *et al.*, 2003). Furthermore, recent metagenome sequencing data suggest the existence of a common core human microbiome (Qin *et al*., 2010), although this core may exist more at the level of shared functional genes rather than shared common taxa (Turnbaugh *et al*, 2009); the high degree of diversity between individuals in the composition of the microbial community suggests a functional redundancy in the gut ecosystem. Many different bacterial species can probably carry out the same metabolic functions and thus there is an opportunity for different bacterial species to fill a specific ecological niche in the healthy, ”normal” ecosystem (Tannock, 2008). Perhaps, therefore, it has been rather difficult to define the composition of the ”normal” microbiota in humans, as large deviations within the ”normal range” can occur.

1.2.2. The role of microbiota composition in health and disease

The colonizing microbiota is essential to host development and health. Studies on germ-free animals have highlighted the importance of the microbial stimulus to the host, as the absence of microbiota causes adverse changes in intestinal morphology, absorptive function, motility, enteroendocrine and exocrine functions and development of immunity (Smith *et al*., 2007). The major functions attributed to the endogenous microbiota are the fermentation of undigested food components and the production of energy for the host (Bäckhed *et al*., 2005), the colonization barrier against invading pathogens (Gibson *et al*., 2005) and the stimulation of the immune system (Macpherson and Harris, 2004); the endogenous microbiota has been shown to influence the development of immune system in infants, and its correct establishment from birth onwards is considered to have long-lasting implications for the health and disease of the host (Kalliomäki *et al.*, 2001; Ouwehand *et al*., 2001; Gueimonde *et al*., 2007; Sjögren *et al*., 2009).

The composition of the microbiota in health and disease has been investigated intensively using molecular biological methods and the altered balance between the members of the microbiota has been identified in various gastrointestinal tract disorders, such as *Clostridium difficile* –associated diarrhea (Chang *et al*., 2008), irritable bowel syndrome (IBS; Salonen *et al*., 2010; Lee and Tack 2010; Cremon *et al*., 2010) and inflammatory bowel diseases (IBD; Manichanh *et al.* 2006; Qin *et al.*, 2010; Frank *et al*., 2007). Furthermore, even non-gut related disorders such as obesity (Ley *et al.*, 2010), atopic eczema (Björksten *et al*., 2001; Kalliomäki *et al*., 2001), rheumatoid arthritis (Eerola *et al*., 1994; Toivanen 2003; Vaahtovuo

et al., 2008) and autism (Parracho *et al.*, 2005) have been correlated with the altered composition and/or functionality of this microbial population.

According to recent metagenomic studies, the microbiota balance and functionality are also altered with advancing age, which might affect the homeostasis between the gut microbiota and the host's immune system and contribute to the progression of diseases and frailty in the elderly (Rajilic-Stojanovic *et al.*, 2009; Biagi *et al.*, 2010).

1.2.3. Metabolic acitivity of the microbiota

Through the fermentation of undigested food components, the colonic microbiota contributes greatly to the net energy gain of the host from its diet (Wostmann *et al.*, 1983), and the amount of energy that can be harvested from the food is affected by the microbiota composition (Bäckhed *et al.*, 2004; Turnbaugh *et al.*, 2006). In addition to energy production, the gut bacteria are also involved in vitamin synthesis, especially of vitamins B and K (Salminen *et al.*, 1998; Smith *et al.*, 2007), and have the capacity to produce and modify hundreds of metabolites that affect intestinal health and immunity, as recently highlighted in metabolomic studies (Jacobs *et al.*, 2009).

It has been estimated that, on a daily basis, 10-60g of carbohydrates and 6-18g of protein or amino acids reach the human colon and that these substrates are converted by bacteria into a range of metabolites (Cummings and Macfarlane, 1991), which are either used by the microbes, absorbed into the epithelia and systemic blood circulation, or excreted in feaces. The colonic microbiota contains bacteria that derive their energy solely from saccharolytic fermentation (e.g. *Bifidobacterium* and *Ruminococcus*) or proteolytic fermentation (e.g. *Peptococcus*) although many of the bacteria (e.g. *Clostridium*, *Bacteroides*, *Propionibacterium*, *Streptococcus Lactobacillus* and *Eubacterium*) are able toderive their energy through either type of fermentation, depending on the availability of the substrates (Salminen *et al.*, 1998; Hughes *et al.*, 2000). Furthermore, microbes are able to utilise, and may even depend on, metabolites produced by other microbes; for instance, lactate and acetate, produced by lactic acid bacteria, can be further converted to butyrate by other colonic microbes that possibly are not able to utilize the original substrate (Belenguer *et al.*, 2006; Duncan *et al.*, 2004). This cross-feeding reflects the ability of microbial species to adapt to the limited availability of nutrients, especially in the distal gut (Gibson and Wang, 1994c), as significant regional differences in the colon exist in terms of substrate availability. The caecum receives digesta from the small intestine and this proximal part of the colon is characterized by both high substrate availability and high bacterial activity in terms

saccharolytic fermentation; and a faster transit time and low pH, due to the high concentrations of fermentation metabolites. The distal part of the colon, on the other hand, becomes progressively more depleted in nutrients, the fermentation of proteins and amino acids is quantitatively more dominant in the left colon and the pH is close to neutral (Cummings and Macfarlane, 1991; Macfarlane *et al.*, 1992).

Fiber and oligosaccharides (e.g. lactose, raffinose, xylitol and prebiotics) that either resist digestion or are poorly digested in the small intestine are metabolized by the saccharolytic bacteria to produce short chain fatty acids (SCFA), lactate, hydrogen (H_2) and carbon dioxide (CO_2) (Wong *et al.*, 2006). SCFAs, namely acetate, propionate and butyrate, derive mainly from carbohydrate fermentation, although protein fermentation also contributes to their production, and the substrate availability, microbiota composition and intestinal transit time all affect the amount and types of SCFAs (and other metabolites) produced (Macfarlane and Macfarlane, 2003). SCFAs are very efficiently absorbed from the colon as only 5-10% of the produced SCFA is excreted in faeces (Cummings and Macfarlane, 1991), and their absorption is associated with enhanced water and sodium absorption and bicarbonate excretion from the colon. Amongst the SCFAs, butyrate is the most widely studied and has been implicated in the maintenance of colonic health. It is the preferred energy source of the colonic epithelial cells as between 70% and 90% of produced butyrate is metabolized by the colonocytes (Wong *et al.*, 2006), and some evidence exists that butyrate exerts various positive effects, such as the inhibition of colon carcinogenesis, decreased inflammation and oxidative stress, and improvement of the colonic barrier function (Hamer *et al.*, 2008; Scheppach and Weiler, 2004). Acetate is the principal SCFA in the colon and is mainly absorbed and utilised systemically in peripheral tissues (liver, brain, cardiac and skeletal muscle). It is used as a substrate for cholesterol and fatty acid synthesis in the liver, and also as a precursor for gluconeogenesis. Propionate is also absorbed from the colon and used by the liver cells in gluconeogenesis (Wong *et al.*, 2006). In animals, propionate inhibits the uptake of acetate into the cholesterol synthesis pathway, and reduces the levels of serum cholesterol. In humans, the same effect has not been as pronounced (Hara *et al.*, 1999; Salminen *et al.*, 1998).

Dietary protein enters the colon in small quantities, as do host-derived substances like mucin glycoproteins, digestive enzymes and exfoliated epithelial cells. Both dietary and host derived proteins contribute to proteolytic fermentation, which produces e.g. ammonia,

phenolic and indolic compounds, N-nitroso compounds, branched chain fatty acids, biogenic amines, hydrogen sulphide, CO_2 and H_2 (Cummings and Macfarlane, 1991). Although proteins are important sources of energy for microbes when carbohydrate sources are exhausted, some of the proteolytic metabolites (e.g. indoles, hydrogen sulphide and biogenic amines) are potentially harmful substances for the host and can exert toxic effects (Hughes *et al.*, 2000; Wong *et al.*, 2006). *In vitro, in vivo* and human studies have demonstrated that high colonic protein fermentation contributes to increased toxicity of the intestinal contents and an increased risk of colorectal cancer (Santarelli *et al.*, 2008; Hughes *et al.*, 2000; Macfarlane *et al.*, 1992), and implications between proteolytic fermentation and inflammatory bowel diseases also exist (van Nuenen *et al.*, 2004). Therefore, lowering the concentrations of proteolytic metabolites, especially in the distal colon where their concentration is highest, is considered to benefit the health of the host (Macfarlane *et al*., 1992). Fermentable carbohydrates have been shown to decrease ammonia *in vitro* and urinary phenol and p-cresol and faecal BCFA concentrations in humans, thus leading to the assumption that carbohydrates are the preferred energy source for the gut microbes at the expense of proteolytic metabolism (Blachier *et al.*, 2007; Hughes *et al.*, 2000).

1.3. Modulating the composition of microbiota

Due to the significance of the roles the microbiota play in both the health and disease of the host, attempts have been made to regulate the composition towards a purportedly healthier one. Probiotic and prebiotics, and their combination, synbiotics, have been introduced and also used successfully in balancing the microbiota.

Probiotics were first defined as "live microbial feed supplements, which beneficially affect the host by improving its intestinal microbial balance" (Fuller, 1989). The original definition has since been modified and a probiotic should therefore, in addition to merely modifying the intestinal microbiota, confer health benefits to the host when administered in adequate amounts (WHO/FAO, 2002). Probiotic microbes usually belong to the genera *Lactobacillus* or *Bifidobacterium*, which have a long and safe history of use in fermented foods (Mogensen *et al.*, 2002a; Mogensen *et al.*, 2002b) and are thought to provide protection towards inflammation in breast-fed babies (bifidobacteria), stimulate the immune system, and be partly responsible for the colonic barrier function (Macfarlane *et al.*, 2006; Meile *et al.*, 2008; Salminen *et al.*, 1998). In addition, the yeast, *Saccharomycces boulardii*, and some other bacteria such as *Enterococcus faecium* and *Lactococcus lactis*, have also been used as probiotics. In order for a probiotic microbe to confer health benefits to the host, it should

remain intact and viable in the GIT and interact with the host and/or the colonic microbiota (de Vrese and Schrezenmeir, 2008).

However, the efficacy and viability of probiotic microbes can be diminished by the protective barriers of the human body, such as a low pH and hydrogen chloride in the stomach, digestive enzymes in the small intestine and, in the colon, by the endogenous microbiota with which they are forced to compete for nutrients and an ecological niche (Tuohy *et al.*, 2005). Thus, an alternative prebiotic approach has been introduced (Gibson and Roberfroid, 1995). Prebiotics aim to target specific microbial groups already resident in the colon, thus, overcoming the main problem encountered with probiotic viability, and produce health promoting effects through the modification of the host microbiota composition and/or activity. However, the selective fermentation of a prebiotic only by those microbes with suggested beneficial activities has been difficult to establish (Saulnier, 2007); the main difficulty being that the large proportion of colonic bacteria that are still unidentified and their role as part of the healthy microbiota is as yet unknown. Also the inter-individual variation and the potential low numbers of beneficial microbes in the gut, e.g. due to diet, medication or age, could compromise the efficacy of a prebiotic (Macfarlane *et al*., 2006).

Synbiotics, which are combinations of pro- and prebiotics, could possibly overcome the limitations encountered with both of the individual components (Saulnier, 2007). Synbiotics were defined together with the introduction of prebiotics, and are mixtures of pro- and prebiotics that benefit the host by improving the survival and implantation of the selected, beneficial microbial dietary supplements. The prebiotic compounds should therefore be specific for the probiotic in question and enhance its efficacy in the colon, and should also modify the endogenous microbiota in a beneficial manner (Gibson and Roberfroid, 1995). However, although the synbiotic approach is an interesting one, only a few combinations of pro- and prebiotics can thus far be evaluated as being true synbiotics (Saulnier, 2007; Saulnier *et al.*, 2008). The amount of the prebiotic per serving is either too low to ascertain an effect, a bifidogenic prebiotic is combined with probiotic lactobacilli, or the synbiotic does not show any additional benefits when compared with its pre- and/or probiotic components (de Vrese and Schrezenmeir, 2008).

1.4. Prebiotics

Prebiotics were defined by Gibson and Roberfroid in 1995 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.” As

with probiotics, the original definition of prebiotics has been updated a few times to emphasize certain aspects of the definition (Gibson *et al.*, 2004; Pineiro *et al.*, 2008; Roberfroid, 2007) and, according to the latest definition, a prebiotic is a non-viable food component that confers a health benefit on the host associated with modulation of the microbiota (Pineiro *et al*., 2008). This defines prebiotics as food-grade components that deliver a measurable benefit to the host through the modulation of the microbiota.

The criteria that a prebiotic compound should fulfill are:

- The compound should be either completely indigestible or poorly digested and either not at all or poorly absorbed in the upper gastrointestinal tract, and should enter the colon intact.
- In the colon, the compound should be fermented by beneficial microbial groups amongst the endogenous microbiota and result in increased numbers of beneficial microbes and/or their metabolic products.
- The increased microbial mass and/or metabolites deriving from the prebiotic fermentation should lead to measurable health effects on the host.

Research into prebiotics began long before its definition, when bifidogenic factors in human milk were identified. Researchers concluded that some components of the milk improved the quality of the faeces in breast-fed infants (increased bifidobacteria) and in their health, in comparison to formula-fed ones. Thus, human milk oligosaccharides could be considered as the first and most natural prebiotic compounds (Coppa *et al*., 2004). Thereafter, several oligo- and polysaccharides have been addressed as being prebiotics over the past decades, but only a few are truly considered to be such (Gibson *et al*., 2004). Fructo-oligosaccharides (FOS) are the ”gold standard” of prebiotics and have been studied the most by far. Galacto-oligosaccharides (GOS) and lactulose also fulfill the current criteria of prebiotics. Nevertheless, as commercial interest towards functional food ingredients and gut health has grown, an increasing amount of compounds with promising prebiotic features have emerged. These include xylo-oligosaccharides (XOS), lactitol, polydextrose, isomalto-oligosaccharides (IMO) and soy-oligosaccharides (SOS) to name a few (Gibson *et al.*, 2004; Jie *et al.*, 2000; Tuohy *et al.*, 2005). Vey recently, cocoa-derives flavonols have also been reported to selectively modify the microbiota in humans (Tzounis *et al.*, 2011), thus expanding the field of prebiotic compounds from oligo- and polysaccharides to plant polyphenols.

Prebiotic compounds mediate their actions mainly through the microbiota (exogenous probiotics or resident gut microbes), inducing microbial growth and the resultant metabolic activity leads to desired health effects (Ouwehand *et al*., 2005). Through the endogenous microbes, prebiotics may influence the immune system (Moro *et al*., 2006; Arslanoglu *et al*., 2007, 2008), increase biomass in the colon (Gibson *et al*., 1995; Castiglia-Delavaud *et al*., 1998) and inhibit pathogenic growth by decreasing luminal pH and producing anti-microbial agents (Fooks and Gibson, 2002, 2003; Hopkins and Macfarlane, 2003). It has been also noted that prebiotics may increase the elimination of toxins (xenobiotics) from the colon by affecting the microbiota composition or activity (Reid *et al.*, 2003; Zampa *et al*., 2004; Kimura *et al*., 2004). Microbes produce SCFAs from prebiotics, and these acids can possibly influence several functions in the gut: relieve constipation, inhibit pathogenic growth, improve absorption of minerals and affects cholesterol synthesis in the liver (Topping and Clifton, 2001; Wong *et al*., 2006). Butyrate is especially important for the colonocytes as it provides nutrition for them and promotes correct cell differentiation (Hamer *et al.,* 2008). Prebiotics may also have direct effects on the colonocytes and immune cells in the gut epithelia (Roberfroid *et al.,* 2010).

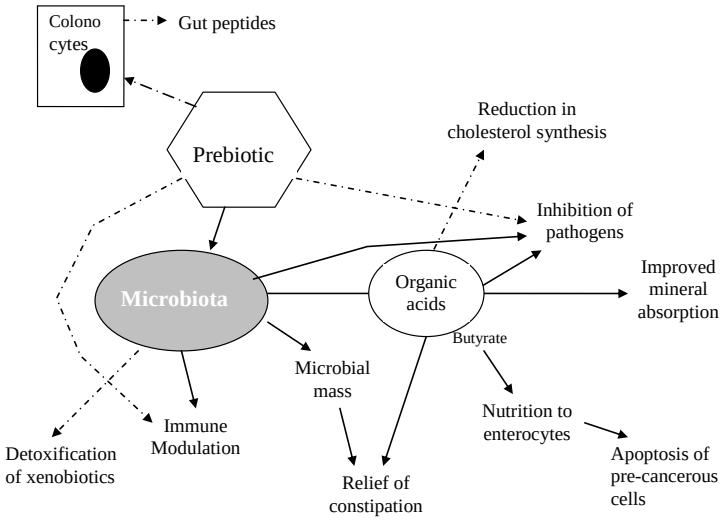

Figure 1. Possible mechanisms of prebiotic action in the colon. Solid lines indicate relatively established modes of action, whereas dotted lines indicate a less well-established mode of action (modified from Ouwehand *et al*., 2005).

The effects arising from the prebiotic actions in the colon have been intensively studied over the past two decades. The research has mainly focused on identifying the microbial groups among the gut microbiota that are affected by the prebiotic fermentation, and attempting to correlate these modifications with a health benefit. *Bifidobacteria* and *Lactobacillus* are the microbes that have traditionally been the most intensively studied because they benefit the host (Fuller and Gibson, 1997) and are easily quantified with classical cultivation techniques. A large number of human intervention studies have also linked the consumption of prebiotic ingredients to increased levels of bifidobacteria and/or lactobacilli while some studies have also reported reductions in the concentrations of commensal and potentially pathogenic bacteria, such as *Salmonella*, *Bacteroides* and clostridia, with prebiotic consumption (Roberfroid *et al*., 2010; Macfarlane *et al*., 2006). However, as knowledge on the previously unknown members of the microbiota is accumulating, the traditional division of microbiota into solely good (bifidobacteria and lactobacilli) and bad (clostridia and *Bacteroides*) components is perhaps too simplified, and it seems clear that other members of the gut microbiota are also affected by the prebiotics. For instance, the group *Bacteroides* contains species with not only potentially beneficial, but also detrimental properties (Wexler, 2007), and the beneficial prebiotic effects could be mediated through the increased numbers or activities of butyrate-producing microbes, e.g. the *Eubacterium rectale/Roseburia* species and *Feacalibacterium prausnitzii*, belonging to clostridial clusters IV and XIVa (Louis and Flint, 2009). The decreased levels of these microbes have been recently linked to IBD (Sokol *et al*., 2008; Takaishi *et al*., 2008), and their faecal levels strongly correlate with faecal butyrate concentrations. Furthermore, the levels of these microbes are modifiable with dietary carbohydrates and fibre (Benus *et al*., 2010; Duncan *et al*., 2007) and thus they may also respond to prebiotic supplementation. In addition to the various members of the gut microbiota, the role of their vast metabolic activity in improved function and/or the reduced risk of a disease associated with the prebiotic effect has been recently highlighted (Jacobs *et al*. 2009).

Although the definite causality of the prebiotic effect still remains to be answered, there is evidence that links the modification of the gut microbiota and its functionality to some gastrointestinal and systemic health effects. Table 1 summarizes the proposed targets for prebiotic effects, which are anticipated to be associated with the stimulation of growth and/or the activities of a limited number of gut microbes, and tentatively to a health effect and/or reduced risk of a disease (Roberfroid *et al*., 2010).

Table 1. Proposed targets for prebiotic effects (modified from Roberfroid *et al.*, 2010).

Modification of colonic microbiota composition
Improvement of gastrointestinal tract functions (faecal bulking, regularity, consistency)
Increase in mineral absorbtion and improved bone structure
Modulation of gastrointestinal peptide production related to satiety and energy intake
Initiation of proper immune function development and regulation after birth (atopic diseases, infections)
Improvement of intestinal barrier functions
Reducing the risk of gastrointestinal infections and inflammation
Reducing the risk of colon cancer
Modulation of cholesterol synthesis in liver
Reducing the risk of obesity, metabolic syndrome and adult-type diabetes

1.4.1. Established prebiotics; effects on the microbiota composition, functionality and health of the host

In Europe, inulin type fructans, galacto-oligosaccharides and lactulose have been shown to fulfil all the criteria of a prebiotic compound, based on results from *in vitro* studies and human interventions that provide sufficient evidence of their non-digestibility, ability to change the gut microbiota composition and confer health benefits to the consumer (Kolida and Gibson, 2007).

1.4.1.1. Fructo-oligosaccharides and inulin

Inulin and FOS are polymers of fructose, linked by β (2-1) bonds with a terminal glucose at the end of the molecule. FOS are oligosaccharides with a degree of polymerization between 2 and 10 and polysaccharides larger than that are referred to as inulin (Crittenden and Playne, 1996). Inulin and other fructans are present in relatively high concentrations in a number of natural foods, such as chicory, onion, Jerusalem artichokes and asparagus. Commercial FOS are produced by enzymatic hydrolysis from inulin, or synthesized from sucrose (Brown and Tuohy, 2006).

In one of the first articles published on FOS fermentation by colonic microbes, Gibson and Wang (1994a) demonstrated *in vitro,* using selective plating methods, the bifidogenic properties of FOS with concomitant suppression of *Bacteroides,* clostridia and coliforms in mixed faecal culture in a colon model. Later *in vitro* studies using culture-independent molecular techniques have also demonstrated the modulatory properties of FOS and inulin (Rycroft *et al.*, 2001a; Sghir *et al.*, 1998; van de Wiele *et al.*, 2007) and in several human

interventions, increased levels of *Bifidobacterium* and *Lactobacillus* and decreased levels of enterococci, fusobacteria and/or *Bacteroides* have been confirmed, utilising molecular techniques to assess the microbiota composition (Kolida *et al.*, 2007;Kruse et al., 1999; Bouhnik et al., 1999). Bifidogenicity is also evident when FOS and inulin are delivered in different food matrixes, such as bakery products (Tuohy *et al*., 2001; Kleessen et al., 2007) and fruit and vegetable drinks (Ramnani *et al*., 2010). The sufficient dose for the bifidogenic effect for inulin is between 5-8g/day (Kolida *et al*., 2007; Kolida and Gibson, 2007) and for FOS between 4-10g/day (Bouhnik *et al.*, 1999; Buddington *et al.*, 1996), although the magnitude of the increased proportion of bifidobacteria depends on the initial population levels (Tuohy *et al*., 2001; Kolida *et al*., 2007). The same holds true for other prebiotics, such as GOS and PDX (Tiihonen *et al*., 2008). The fermentation activity of the microbiota is also modified as a result of FOS and inulin consumption, which leads to increased SCFA production, especially of acetate and butyrate (Rycroft *et al.*, 2001a; Kleessen *et al*., 2001).

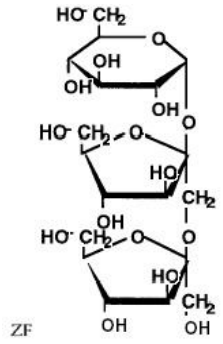

Figure 2. Molecular structure of FOS (dp=3)

The health effects associated with FOS and inulin have been investigated in relation to several conditions. For instance, in patients with IBD (Crohn's disease, CD), some evidence exists on the effectiveness of FOS and inulin to improve colonic inflammation and related symptoms (Hussey *et al*., 2003) with a concomitant increase in intestinal *Bifidobacterium* numbers (Lindsay *et al.*, 2006). Recent evidence also suggests that inulin-type fructans might be able to modulate the release of hormones associated with appetite control (plasma GLP-1 and peptide YY), thereby affecting the gut-brain axis of appetite regulation (Bosscher and Van Loo, 2008). Increased postbrandial satiety, lower hunger rates and a subsequently decreased total energy intake during meals following prebiotic consumption have been demonstrated (Cani *et al.*, 2006), and furthermore, satiety increasing effects were evident in both a short-term two-week intervention (Cani *et al.*, 2009) and in a long-term 12-month study on adolescent girls and boys (n=100) (Abrams *et al.*, 2007). Some indications of the role of FOS and inulin on improved mineral absorption (calcium and magnesium) from the colon and a consequently improved bone turnover rate exist (van den Heuvel *et al.*, 1999; Younes *et al*., 2001; Scholz-Ahrens *et al*., 2002; Holloway *et al*., 2007)

although the evidence is mainly experimental and human studies have shown contradictory results (Griffin *et al*., 2002; Tahiri *et al*., 2003; van den Heuvel *et al*., 2009). Nevertheless, consumption of high doses of FOS (20g/day) may also cause inverse gastrointestinal symptoms, such as flatulence and bloating, which could derive from the (too) rapid fermentation of FOS, leading to an accumulation of lactate that would otherwise normally be metabolized further into SCFAs, and inducing mucin secretion, reflecting irritation and impairment of the intestinal barrier (Ten Bruggencate *et al*., 2005, 2006). In rats, FOS and inulin have even been suggested in certain conditions to enhance the colonization and transloction of *Salmonella enteric* serovar Enteriditis (Ten Bruggencate *et al*., 2003, 2004).

1.4.1.2. Galacto-oligosaccharides

Galacto-oligosaccharides (GOS), sometimes referred to as trans-galacto-oligosaccharides (TOS), are naturally present in human milk but can also be manufactured from lactose by transglycosylation reactions. GOS consist of galactosyl derivatives of lactose with beta β (1-4) and β (1-6) –linkages and the degree of polymerization (dp) is usually between 2 to 5 (Crittenden and Playne, 1996). Alongside FOS, galacto-oligosaccharides (GOS) have an established prebiotic status and their health effects been extensively studied (Gibson *et al*., 2004; Roberfroid, 2007).

In pure cultures, GOS was fermented well by all bifidobacteria and moderately by a few *Bacteroides* and *Lactobacillus* among the 55 strains tested *in vitro* (Tanaka *et al*., 1983); and in faecal slurries, GOS most significantly increased bifidobacterial growth and decreased *Cl. histolyticum* group members in all the prebiotics tested (GOS, FOS, inulin, XOS, IMO, lactulose and SOS), as measured with fluorescence in situ hybridization (FISH; Rycroft *et al*., 2001a). Similarly in pigs, GOS (at 4% of the diet) consumption increased the numbers of bifidobacteria and lactobacilli in the proximal and distal colon, whereas other determined microbes (*Bacteroides* spp. and *Cl. histolyticum* group members) were unaffected; and GOS were more efficient in modulating the microbiota than inulin in the same experiment (Tzortzis *et al*., 2005). In human feeding trials, the bifidogenic properties of GOS have been demonstrated together with other modifications of the microbiota and microbial enzyme activities (Tuohy *et al*., 2005). With a daily dose ranging from 2.4g to 7g/day, GOS has been shown to significantly increase *Bifidobacterium* numbers (Depeint *et al*., 2008; Vulevic *et al*., 2008; Silk *et al*., 2009; Gopal *et al*., 2003) and to also affect other bacterial groups in the microbiota, as increased levels of *Lactobacillus-Enterococcus* spp. and *Cl. coccoides/Eub. rectale* group and decreased numbers of *Bacteroides-Prevotella* and *Cl. histolyticum* groups,

E. coli and *Desulfovibrio* spp. have been demonstrated with culture-independent techniques (Vulevic *et al*., 2008).

The fermentation of GOS in rats resulted in increased levels of SCFAs, a decreased colonic pH and a decrease in the production of the potentially toxic proteolytic metabolites p-cresol and indole (Tanaka *et al*., 1983). In humans, the faecal concentrations of acetate are increased after GOS consumption (Bouhnik *et al*., 1997) and the markers of proteolytic fermentation (BCFA, p-cresol and indole) are decreased (Ito *et al.*, 1993).

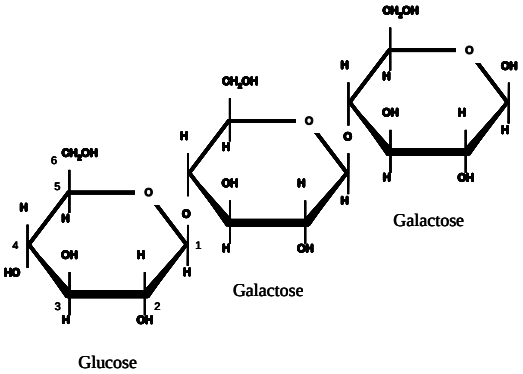

Figure 3. Molecular structure of GOS (dp=3)

The establishment of high levels of bifidobacteria in the infant gut has been attributed to the presence of galactose-containing oligosaccharides in human milk; thus, the inclusion of GOS to infant formulas has attracted considerable commercial interest (Crittenden and Playne, 1996) and these prebiotic supplemented formulas (GOS and FOS, 9:1) have been shown to modify the microbiota of the formula-fed babies in such a way that it resembles that of breast-fed babies (Rinne *et al*., 2005). Furthermore, the inclusion of prebiotics to infant formulas has been shown to reduce the cumulative incidence of intestinal and respiratory infections (Arslanoglu *et al.*, 2007; Bruzzese *et al*., 2009) and the cumulative incidence of atopic dermatitis in high risk populations during a six-month intervention (Moro *et al*., 2006) and the protective effects prevailed until at least the end of a two-year follow-up period (Arslanoglu *et al*., 2008). However, these studies have been criticized on several grounds for containing several weaknesses (EFSA, 2010) and thus the evidence of GOS (and FOS) on enhancing the immunity of infants remains inconclusive. GOS (in amounts between 3.5g and 7.0g per day) has been shown to help alleviate the symptoms of IBS by reducing flatulence and bloating while improving stool consistency in IBS patients during a 12-week study period, thus having significant therapeutic value for the IBS patients (n=44) (Silk *et al.*, 2009). Some evidence also exists of the preventive effects of GOS on the incidence and duration of traveller's diarrhea (Drakoularakou *et al*., 2010) and on the constipation of elderly people (Teuri and Korpela, 1998). A positive correlation between the GOS-induced bifidogenicity and the improved activity of the immune system (phagocytosis, natural killer cell activity and

production of anti-inflammatory and inflammatory cytokines) has also been demonstrated in healthy elderly volunteers (Vulevic *et al.*, 2008). Whether these changes in the immune system relate to actual improvements in immunity, i.e. reducing the risk and/or duration of an illness remains to be elucidated.

1.4.1.3. Lactulose

Lactulose is a synthetic disaccharide (D-galactopyranosyl-D-fructose) derived from the isomerization of lactose (Crittenden and Playne, 1996) and is traditionally used as a laxative and in the treatment of hepatic encephalopathy (Schumann, 2002). *In vitro*, lactulose has been shown to be fermented by the *Lactobacillus*, *Enterococcus* and *Streptococcus* strains of faecal or colonic biopsy origin (Kontula *et al.*, 1999) and to be fermented rapidly in mixed faecal cultures, resulting in the high production of SCFAs (Khan and Edwards, 2002). In pigs, lactulose feeding has been shown to decrease faecal proportions of culturable aerobic bacteria and *Cl. perfringens* (Krueger *et al*., 2002). In humans, lactulose ingestion (3-10 g/day) has been shown to increase faecal bifidobacteria, employing classical culture-based methods (Terada *et al*., 1992; Ballongue *et al*., 1997; Bouhnik *et al*., 2004) and culture-independent methods (FISH) (Tuohy *et al*., 2002); decreased clostridia were also demonstrated in the latter study. Increaed levels of faecal SCFAs with a concomitant decrease in potentially pro-carcinogenic faecal enzymes have been demonstrated (Terada *et al*., 1992). In addition to the constipation relieving properties of lactulose, some preliminary evidence exists on its beneficial effects on calcium absorption (van den Heuvel *et al*., 1999a), but this proposed health effect requires substantial further investigation.

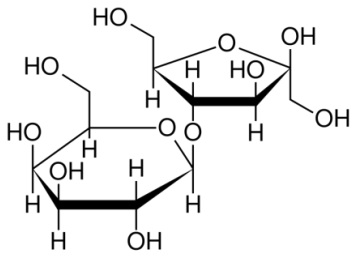

Figure 4: Molecular structure of lactulose

1.4.2. Emerging prebiotics; effects on the microbiota composition, functionality and health of the host

The market for prebiotics in foods is growing rapidly worldwide and in 2007 there were already over 400 prebiotic food products marketed (Pineiro *et al*., 2008). Most of the marketed prebiotic-containing products are inulin-based FOS or GOS components, though a variety of other potentially prebiotic compounds have been identified, such as isomalto-oligosaccharides, gentio-oligosaccharides, xylo-oligosaccharides, lactitol and polydextrose. However, the ultimate tests for the prebiotic activity, human volunteer trials, are either scarce or lacking for the majority of these candidates (Roberfroid *et al.*, 2010) and thus more evidence on their efficacy is needed for prebiotic status. Here, the prebiotic candidates used in this thesis project are presented in more detail.

1.4.2.1. Xylo-oligosaccharides

XOS are made up of xylose units and can be produced by enzymatic hydrolysis from xylan, the major component of plant hemicelluloses and therefore readily available in nature. XOS are relatively stable in acidic conditions, which may endow protection from decomposition when passing through the stomach (Imaizumi *et al*., 1991). The degradation of xylobiose (XOS dp=2) in the intestine has been studied *in vitro* with an artificial model of digestive enzymes (α-amylase, pancreatin, gastric juice and intestinal brush border enzymes) and no hydrolysation of xylobiose was observed (Koga and Fujikawa, 1993). This suggests that XOS may be non-digestible or low digestible carbohydrates and would reach the colon intact.

XOS have been reported to be preferentially fermented by bifidobacteria *in vitro*. Pure culture studies have indicated that XOS are metabolized by many bifidobacteria (*B. bifidum, B. longum, B. catenulatum, B. lactis* and *B. adolescentis*), whereas other enteric bacterial strains (lactobacilli, *Bacteroides*, clostridia) have not significantly metabolized XOS (Jaskari *et al*., 1998; Kontula *et al.*, 1998b; Yong *et al.*, 2001; Crittenden *et al.*, 2002; Palframan *et al*., 2003b). Faecal batch fermentations and semi-continuous culture fermentations have also shown that XOS stimulated the growth of bifidobacteria (Rycroft *et al.*, 2001b; Zampa *et al*., 2004). Trials with rats and mice have demonstrated that XOS stimulated the growth of caecal and faecal bifidobacteria at a higher level than FOS (Campbell *et al.*, 1997; Hsu *et al.*, 2004; Jaskari *et al*., 1998; Santos *et al.*, 2006). The growth of other intestinal microbes (clostridia, *Eubacterium*, enterococci, and *E. coli*) on XOS has been very limited with only some isolates

of *Bacteroides* and clostridia able to degrade XOS to some extent (Crittenden *et al.*, 2002; Jaskari *et al.*, 1998).

In human feeding trials, even low doses of between 2g and 5g of XOS per day have resulted in significant increases in culturable faecal bifidobacteria; culturable members of e.g. *Enterobacteriaceae*, *Lactobacillus*, *Bacteroidaceae*, *Eubacterium*, clostridia and *Bacillus* were not affected. Faecal SCFA concentrations were increased, and concomitantly pH and proteolytic metabolites and enzyme activites decreased, during the feeding trials (Nakakuki, 2003; Okazaki *et al.*, 1990). The limitations of these studies are the small number of subjects (five, nine and ten men), an uncontrolled study-design and the use of the plating method in enumeration of microbes in faeces. Some preliminary evidence exists on the ameliorating effects of XOS on constipation in women (Iino *et al.*, 1997; Tateyama *et al.*, 2005) although more controlled studies are needed to further assess the health benefits associated with XOS.

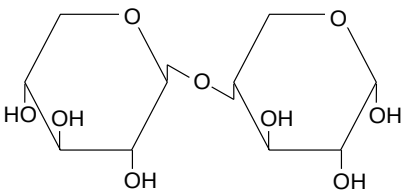

Figure 5. Molecular structure of xylobiose (dp=2)

1.4.2.2. Lactitol

Lactitol is a lactose-derived sugar alcohol constituting of galactose and sorbitol, which is only minimally hydrolyzed by the human upper gastrointestinal tract and reaches the colon virtually intact (Natah *et al.*, 1997; Nilsson and Jagerstad, 1987). *In vitro* studies have demonstrated that lactic acid bacteria are able to ferment lactitol in pure cultures (Klewicki and Klewicka, 2004; Kontula *et al.*, 1999; Saarela *et al.*, 2003) and in simulated colon fermentation studies with human faecal inoculum, lactitol was rapidly fermented in the proximal colon and increased *Bifidobacterium numbers*, decreased *Clostridium* cluster IV numbers, but did not affect *Lactobacillus* or *Bacteroidetes* numbers (Mäkivuokko *et al.*, 2010). Lactitol fermentation has been reported to increase the concentrations of SCFAs (especially butyric and acetic acids) in human faecal samples (Mäkivuokko *et al.*, 2010; Probert *et al.*, 2004), and also in rats (Peuranen *et al.*, 2004); and to lower pH, reduce the

concentrations of faecal aromatic compounds (e.g. phenol, cresol, indole and skatole) and pro-carcinogenic enzyme activities (e.g. azoreductase, glucuronidase and urease) in humans (Ballongue *et al*., 1997).

In healthy humans, lactitol consumption (2 x 10g per day) has been shown to increase culturable fecal bifidobacteria and lactobacilli while concomitantly decreasing the levels of culturable clostridia, *Bacteroides* and coliforms (Ballongue *et al*., 1997). The effective dose of lactitol was found to be 10g per day, which was sufficient to increase culturable *Bifidobacterium* numbers, decrease faecal pH and increase production of propionic and butyric acids (Finney *et al*., 2007). The *Bifidobacterium* increasing effects of lactitol have also been demonstrated in the healthy elderly, using culture-independent methods (Ouwehand *et al*., 2009), and it has been identified as an emerging prebiotic (Drakoularakou *et al*., 2007).

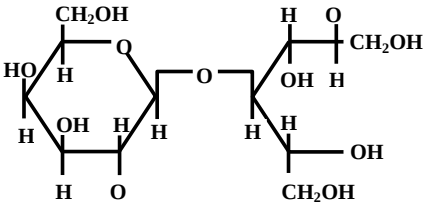

Figure 6. Molecular structure of lactitol

1.4.2.3. Polydextrose

Polydextrose (PDX) is a glucose polymer with a highly branched structure (average dp=12). It has been recognized as a soluble fiber, and human clinical, animal and *in vitro* studies have all demonstrated physiological effects associated with this feature (Craig *et al*., 2000). PDX reaches the large intestine intact, where it is gradually degraded by the colonic microbes (Lahtinen *et al.*, 2010) into butyric, acetic and propionic acids while concomitantly decreasing the levels of proteolytic metabolites (BCFA, biogenic amines, phenol, *p*-cresol and indole) (Mitsuoka, 1996; Jie *et al*., 2000; Mäkeläinen *et al.*, 2007). *In vitro*, PDX has been shown to increase bifidobacteria, assessed by molecular methods, in a mixed faecal population using a continuous-culture colon simulator (Probert *et al*., 2004), although in another similar apparatus, the bifidogenic effects were not reproduced, even if a gradual, slow fermentation of PDX by the microbiota was evident (Mäkeläinen *et al*., 2007; Mäkivuokko *et al.*, 2005). In a placebo-controlled, randomized, double-blind human intervention (n=120), significant increases in the numbers of culturable bifidobacteria and lactobacilli together with decreased *Bacteroides* numbers were demonstrated (Jie *et al*., 2000). Improved bowel function has been demonstrated with daily intake of 4–12g PDX with no adverse effects, such

as abdominal distension, cramps or diarrhea reported (Jie *et al*., 2000). Shortened oro-faecal time has also been demonstrated, implicating the role of PDX in alleviating constipation (Hengst *et al*., 2008). Therefore, some evidence on the prebiotic potential of PDX exists, although more research is needed to confirm the status.

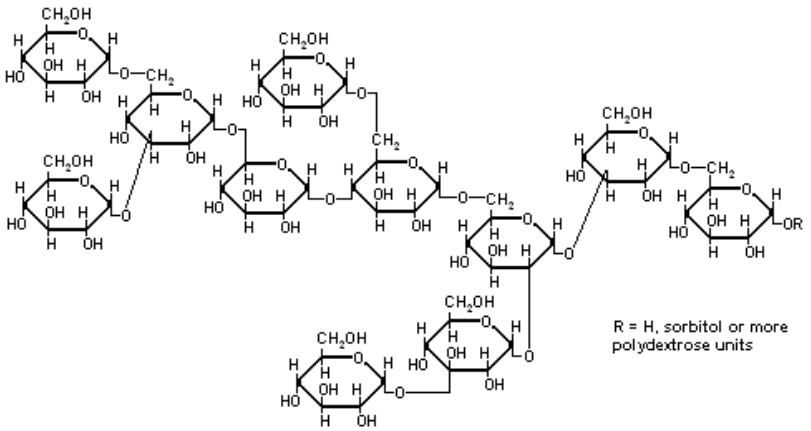

Figure 7. Molecular structure of polydextrose

1.6.2.4. Isomalto-oligosaccharides

Isomalto-oligosaccharides (IMO) are a mixture of oligosaccharides, consisting of glucose units (dp=2-5) which are bound with linear α (1-6) glycosidic linkages but which also contain branched molecules with α (1-4) linkages. IMOs can be produced from starch (Crittenden and Playne, 1996). The digestibility of IMO mixtures has been investigated in a study by Kohmoto and co-workers (1992), where the expiration rates of excess $^{13}CO_2$ and hydrogen were measured in six men after IMO ingestion. The results indicated that IMO would be partly digested and partly fermented by the endogenous microbiota in the colon. In another study, with rat jejunum loops, IMO was shown to be slowly digested and those components with higher degree of polymerization (isomaltotriose, -tetraose and –pentose) were less digestible than isomaltose (Kaneko *et al.* 1995).

IMOs have been shown to be very well fermented by bifidobacteria, *Bacteroides*, and *Enterococcus faecalis*, but not by *E. coli* or other tested bacterial populations in pure cultures (Kohmoto *et al*., 1988). In 24h static batch cultures, IMO increased *Bifidobacterium* numbers significantly and caused increased production of acetate, lactate and propionate (Rycroft *et al*., 2001a). In humans, IMO fermentation was shown to increase faecal *Bifidobacterium*

levels significantly in a dose-dependent manner (Kaneko *et al.*, 1994; Kohmoto *et al.*, 1988; Kohmoto *et al.*, 1991). Increased *Bacteroidaceae* levels were also measured in one of the human interventions (Kohmoto *et al*., 1988). The sufficient dose for bifidogenic effect seemed to be 5g per day, although higher doses (10-20g per day) resulted in more profound and faster increases in bifidobacteria. The evidence that supports the prebiotic status for IMO is therefore promising, but remains as yet insufficient (Gibson *et al*., 2004).

1.4.2.4. Panose

Panose is a trisaccharide (dp=3) with 1-4 and 1-6 linkages (alpha-D-Glc-(1→6)-alpha-D-Glc-(1→4)-D-Glc), and it is classified as an isomalto-oligosaccharide (IMO) (Kohmoto *et al*., 1988). Studies assessing the (in)digestibility of IMO mixtures containing panose in humans indicate that IMOs would be partly digested in the small intestine and partly fermented by the endogenous microbiota in the colon (Kohmoto *et al*., 1992). The fermentation of pure panose by colonic microbes has been studied only in pure cultures, where it was utilized by few *Bifidobacterium* and *Bacteroides* strains, *Enterococcus feacalis* and *Clostridium ramosum*. A range of other tested enteric bacteria (n=37, e.g. lactobacilli, *Eubacterium*, *E.coli*, clostridia, *Fusobacterium* stains) were not able to metabolize panose (Kohmoto *et al.*, 1988). In a few human studies, panose has been investigated only as part of an IMO mixture (Isomalto-900® containing 7% panose, or IMO3 containing 28% panose), which increased the culturable bifidobacteria in the faeces of healthy men and elderly persons without affecting a range of other bacterial groups and yeasts (Kaneko *et al.*, 1994; Kohmoto *et al.*, 1988; Kohmoto *et al.*, 1991). The fermentation of pure panose in human interventions has not yet been investigated.

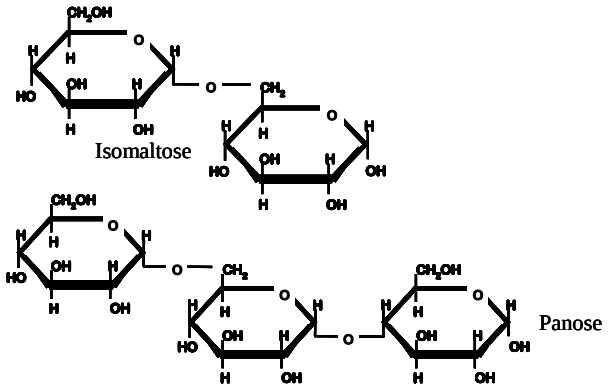

Figure 8. Molecular structures of IMO and panose

1.4.2.5. Pullulan

Pullulan is an extracellular polysaccharide excreted by the filamentous fungus *Aureobasidium pullulans*, consisting of maltotriose units (such as panose) linked through α (1→6) glycosidic bonds. Pullulan is partly digested and absorbed in the small intestine of humans, and partly fermented in the large intestine (Wolf *et al*., 2003). Fermentation of pullulan has been studied using human faecal bacteria and the increased production of SCFAs was measured (Spears *et al*., 2007). No studies on humans investigating the effects of pullulan on gut microbiota have been performed so far.

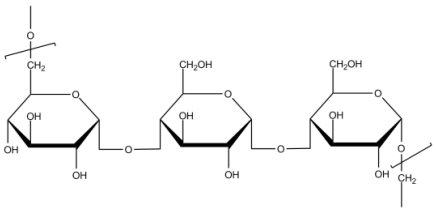

Figure 9. Molecular structure of pullulan

1.4.2.6. Sophorose

Sophorose is a disaccharide (dp=2) with 1-2 linkage between the glucose molecules (beta-D-Glc-(1→2)-D-Glc). No data on the indigestibility of sophorose in the small intestine was found. In a study by Sanz *et al.* (Sanz *et al*., 2005), sophorose was analyzed among tens of other carbohydrates with a small-scale *in vitro* batch fermentor with faecal inoculums. Of all the substrates, fermentation of sophorose resulted in the highest increases in bifidobacterial populations, even significantly higher than those resulting from the established prebiotic, FOS. When the changes in other measured microbial groups (*Bacteroides* and *Cl. histolyticum* groups) were also considered with molecular biology methods, sophorose showed the highest selectivity towards desirable colonic bacteria (Palframan *et al*., 2003a).

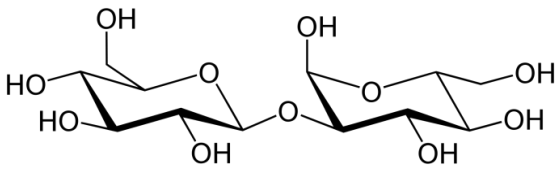

Figure 9. Molecular structure of sophorose

1.4.2.7. Gentiobiose

Gentiobiose is a disaccharide (dp=2) that is composed of glucose units with a β (1→6) linkage. No data on the indigestibility of gentiobiose in the small intestine has been reported,

and thus far, neither gentiobiose nor gentio-oligosaccharides have been studied in great depth. Sanz and co-workers (2005) studied the structure-function effects of a range of carbohydrates in small-scale *in vitro* fermentations with human faecal inoculum and in these studies gentiobiose did not enhance the growth of bifidobacteria or lactobacilli (*Bacteroides*, *Clostridium* histolyticum groups were also investigated, with FISH) but produced a high increase in lactic and acetic acids during the fermentation. Gentio-oligosaccharides with varying degree of polymerization (dp 1-5) have also been studied in a batch-fermentor, where gentio-oligosaccharides produced higher increases in bifidobacteria and lactobacilli populations than FOS (Rycroft *et al*., 2001b).

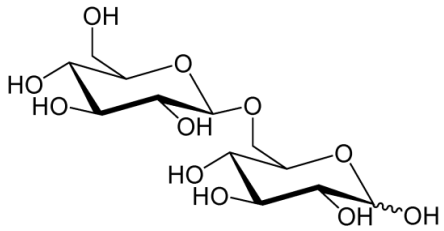

Figure 10. Molecular structure of gentiobiose

1.4.2.8. Xylitol

Xylitol is a five-carbon polyalcohol that is best known for its anticariogenic properties (Burt, 2006). It is widely used as a sugar substitute in chewing gums, dentistry products and confectionery (Burt, 2006; Touger-Decker and van Loveren, 2003); the European Food Safety authorities (EFSA) has positively evaluated the health claim (reducing the risk of tooth decay) in chewing gum and pastilles with 100% xylitol (EFSA, 2008). Ingestion of xylitol is associated with only minor changes in the plasma levels of glucose, insulin and glucose-independent insulinotrophic peptide (GIP), indicating that only a portion of xylitol is absorbed in the upper gastrointestinal tract and that it is mostly metabolized in the colon (Natah *et al.*, 1997; Salminen *et al.*, 1989). Estimates of the extent of xylitol fermentation in the colon range between 50% and 75% (Livesey, 2003). The microbial fermentation of xylitol in the colon is still largely unknown and has previously been studied only with selective plating methods (Salminen *et al.*, 1985).

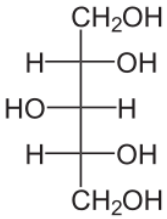

Figure 11. Molecular structure of xylitol

1.5. Characterizing prebiotic fermentation in the colon

Investigation of the prebiotic effects in humans is usually conducted by assessing the faecal microbiota composition and alterations occurring in it, as the invasive sampling of different parts of the human intestine presents technical difficulties and ethical issues. To this end, *in vitro* and *in vivo* animal models provide much-needed tools to characterize the prebiotic effects in more detail. The effects of prebiotic fermentation on gastrointestinal microbiota and its functionality (e.g. SCFA production) can be easily studied in *in vitro* culture systems, whereas animal models can be used to investigate e.g. the mechanisms by which the prebiotic affects the immune system and colonic cells in different parts of the gut *in vivo* (Rumney and Rowland, 1992). However, while the relevance of these models to the authentic human gastrointestinal tract can always be questioned, valuable information on the colonic breakdown of complex carbohydrates and their importance in maintaining gut health has been obtained with the aid of *in vitro* and *in vivo* models (Macfarlane and Macfarlane, 2007).

1.5.1. *In vitro* models

In vitro models that have been used to investigate prebiotic fermentation include pure cultures of bacteria, batch cultures, and different types of gastrointestinal simulators. All of the models use anaerobic conditions to support the growth of colonic microbes. Pure culture studies are the simplest way to study the ability of single microbial strains to ferment prebiotic compounds. Batch fermentors are usually small-scale bottle fermentors; whereas the different gastrointestinal simulator models are structurally more complex, containing sequentially connected anaerobic fermenting vessels representing different compartments of the human gastrointestinal tract. All batch and simulator models are kept at 37˚C, they use faecal material from single or several donors for inoculation and most employ artificial intestinal media originating from the media published by Gibson and co-workers (1988), mimicking the fluid of the human caecum. The simulators have similar pH set-points (ranging from 5.0 to 7.0) resembling those of the authentic human colon (Macfarlane *et al*., 1998) and pH levels and fluid transitions are computer-controlled. Samples collected from these models are analyzed for their microbial composition and metabolites produced through prebiotic fermentation (Mäkivuokko and Nurminen, 2006).

1.5.1.1. Batch-type models

Batch fermentors are the most common models with which to study prebiotic fermentation by colonic microbes. They are usually anaerobic, sealed bottles containing the pure cultures of a microbe, defined mixed microbial cultures or faecal slurry. They model a certain part of the gastrointestinal tract (depending on the pH) and the run-times range from 2 to 24 hours. Batch fermentations are useful tools in investigating the nature and amount of metabolites and gas produced as a result of the prebiotic fermentation but are perhaps not the best models with which to study microbiota composition as the accumulation of fermentation products (e.g. SCFAs) and the depletion of nutrients can alter the conditions and microbiota balance in the fermentor, thus affecting the *in vivo* relevance in longer simulations (Mäkivuokko and Nurminen, 2006; Rumney and Rowland, 1992).

1.5.1.2. Gastrointestinal simulators

The Reading simulator, introduced by Macfarlane and co-workers (1989), simulates the human large intestine in three connected vessels, each with a working volume of 300ml. Fresh feacal sample is suspended in buffer and used to inoculate each of the fermenting vessels, and left to reach equilibrium overnight. During the colonic transit simulation, medium is pumped into the system continuously and there is a continuous overflow of material through the three vessels. The Reading model is operated for 60 hours before the actual simulation with a test substrate in order to achieve microbiological steady-state conditions in the vessels. The test substrate is added to the model and it is run until a new steady-state is achieved, followed by a wash-out period. Samples are collected during and after the different stages and subjected to analysis. The model has been validated against samples obtained from GIT of sudden death victims and has been demonstrated, employing classical culturing techniques, to reproduce the bacterial community structure and metabolic activities of different parts of the colon (Macfarlane *et al*., 1998). By also using more modern molecular techniques (FISH), the composition of microbiota in the colon model has been shown to be very similar to that found in the original faecal inoculum (Child *et al*., 2006): all the major bacterial groups found in faeces (*Bacteroides*, *Bifidobacterium*, *Lactobacillus*, *Roseburia*, *Ruminococci*, *Cl. coccoides/Eub. rectale* and *Atopobium* groups) were also able to establish themselves as major groups in the colonic model, although some variation in the population dynamics between the three vessels was observed during the simulation run.

Molly and co-workers introduced a Simulator for Human Intestinal Microbiological Ecosystem (SHIME) (1993), which simulates the whole human gastrointestinal tract from stomach to colon. In this five-stage system, the first two glass vessels represent the small intestine and the subsequent three vessels simulate the colon and are inoculated with faecal suspension. Working volumes range from 300ml to as high as 1600ml (Alander *et al.*, 1999; Kontula *et al.*, 1998a). The microbiota in the SHIME model is sustained by feeding media into it every eight hours and the model is run for two weeks with media only before adding the test substance. Feeding of the substrate is continued for three weeks, followed by another two-week media-only period, during and after which samples are collected (Grootaert *et al.* 2009). The composition of microbiota in the model has been recently assessed by denaturing gradient gel electrophoresis (DGGE) and HITChip analysis (Van den Abbeele *et al.*, 2010) and found to, upon inoculation, display a reproducible microbial colonization and community development which was region specific; proximal parts of the model harboured more saccharolytic microbes (e.g. *Bacteroides* and *Eubacterium* spp.) whereas the population in the distal region was enriched in mucin-degrading microbes (e.g. *Akkermansia* spp.). The shift from the *in vivo* to *in vitro* situation was, however, characterized by a reduced diversity of the microbial population of the model, as the relative abundance of specific microbial groups were altered; members of the *Bacteroides* and *Clostridium* cluster IX were selectively stimulated whereas clostridial clusters IV and XIVa were reduced in the SHIME model.

The EnteroMix Colon simulator (Figure 12, materials and methods section), used in this thesis work, was introduced by Mäkivuokko and co-workers (2005). It has four parallel units, all of which have four connected glass vessels, thus allowing four simulations to be run simultaneously using the same faecal inoculum. This simulation system has significantly smaller working volumes than the Reading and SHIME models (from 6ml to 12ml), requiring only 50ml of faecal slurry. For inoculation, fresh faecal sample is diluted and incubated for 24 hours prior to the simulation. The colonic simulation takes two days to complete and during these 48 hours the transfer and feeding of the system occur semi-continuously in three-hour cycles. The residuals of fermented fluids and microbes are transferred between the vessels of the system (from V1 to V2, V3, V4, and eventually to the waste container), followed by the feeding of the proximal part (V1) of the model with fresh medium containing the test substrate. At the end of the simulation, all sample material is collected from the vessels and analyzed for microbiota composition and its metabolic activity (Mäkeläinen *et al.*, 2007; Mäkivuokko *et al.*, 2006). The microbiota composition and functionality of the

colon model has been measured against a pig model in which polydextrose fermentation was studied; similarities between the two models were seen in the gradual fermentation of PDX, the lack of bifidogenicity, and the production of SCFAs (Mäkivuokko, 2007). Further, the increased *Bifidobacterium* numbers resulting from fermentation of lactitol in the Enteromix model (Mäkivuokko *et al*., 2010) have been demonstrated in humans as well (Ouwehand *et al*. 2009), indicating that the model is able to reproduce *in vitro* the GIT conditions and prebiotic fermentation observed *in vivo* although an extensive analysis of the Enteromix microbiota composition and comparison to *in vivo* human colonic microbiota is still lacking.

A three-stage simulator of the infant gut has also been recently developed (Cinquin *et al*., 2004, 2006). In contrast to the other "free-cell culture" models described above, this model uses child faecal microbiota immobilized on gel polysaccharide beads, thus establishing two distinct bacterial populations on gel-beads and non-adherent in the liquid phase of the system. The free-cell suspension cultures may present stability problems, such as loss of less competitive bacteria during a long experiment, and this model is said to reproduce the high bacterial concentrations and diversity of the faeces to at least a general level, with a high stability in long-term experiments; and to describe more closely the microbial populations growing on food particles, biofilms and epithelia in the gut. One simulation experiment lasts for between 29 to 56 days, including colonization, stabilization, testing and re-stabilization periods, during which microbial and metabolite levels are analysed on several occasions. The model has been used in studying e.g. FOS, which was shown to differently modify the bead-bound fraction and the non-adherent fraction of the microbiota in the model. It has also been modified to represent the infected child gut (Zihler *et al*., 2010) and was used successfully to investigate the long-term effects of probiotic strains and prebiotics on *Salmonella* infection in infant GIT.

The TNO Intestinal Model (TIM) system is composed of two parts and was introduced by Minekus and co-workers (1995, 1999). The TIM-1 system simulates the stomach and small intestine with its eight sequentially connected glass vessels. The TIM-2 system consists of four glass modules in a loop representing the proximal colon. These dynamic models have peristaltic pumps to transfer the fluid from vessel to vessel, and there is a constant absorption of water and fermentation metabolites (e.g. SCFAs) through dialysis membranes. The volume of intestinal fluids in both models is 200ml and the simulation operation takes from one to three days. With the combination of TIM-1 and -2, the digestion and fermentation of

prebiotic compounds and other food ingredients can be modeled in the whole length of the human gastrointestinal tract. Also, of all the *in vitro* models, the continuous absorption of substrates perhaps enables TIM to simulate the authentic GIT the most accurately. Fresh, pooled feacal samples are used to prepare the standardized inoculum for the system, and this inoculum is either used straight away (Kovatcheva-Datchary *et al*., 2009) or after freeze-and-thawing (Rajilic-Stojanovic *et al*., 2010). The different inoculum preparation methods seem to have an impact on the composition of the establishing microbiota in TIM-2, as freeze-thawed microbiota profiles were different from those of the faecal samples of healthy adults (Rajilic-Stojanovic *et al*., 2010) whereas where the inoculum used in TIM-2 was fresh, microbiota resembling more closely the original faecal populations was produced (Kovatcheva-Datchary *et al*., 2009).

1.5.2. *In vivo* models

Animal models provide opportunities to study the metabolic and immunological activities of intestinal microbiota in more detail than in the *in vitro* models. Intestinal contents and also tissues and organs can be collected from dead animals at autopsy. Conventional, germ-free and human flora-associated rodent models have been used as models for the human gastrointestinal tract in health and disease although the gastrointestinal tract of pigs has more similarities with humans in terms of nutritional requirements, intestinal physiology, microbiota and metabolism (Fava *et al.*, 2007) than the rodent models (Tiihonen *et al*., 2008). The effects of non-digestible oligosaccharides and fiber on gut microecology and physiology have been studies in rats (Bielecka *et al.*, 2002; Dongowski *et al.*, 2002; Henningsson *et al.*, 2002; Peuranen *et al.*, 2004) and in pigs or piglets in several studies (Correa-Matos *et al*., 2003; Fava *et al.*, 2007; Krueger *et al.*, 2002; Martinez-Puig *et al.*, 2003). The animal models have clear advantages in studying the human gastrointestinal tract and microbiota over the *in vitro* models, because there are a wide range of similarities between the hosts. However, there are also many morphological and physiological differences between the species that may hinder the straight extrapolation of the results from animal models (especially with regard to rodent models) to humans (Henriksson, 2006).

The ultimate tests for prebiotic activity have to be performed in human volunteer trials (Roberfroid *et al.*, 2010). The colonic fermentation of complex carbohydrates can be studied with healthy volunteers using faeces or samples obtained from different parts of the GIT. The small intestinal fermentation can be studied, e.g., in ileostomy patients, using intubation tubes

positioned in the gastrointestinal tract. From samples obtained directly from the gastrointestinal tract, the fermentation of a substrate and the consequences on microbiota can be accurately measured (Englyst *et al.*, 1996; Englyst and Cummings, 1985; Silvester *et al.*, 1995; Stephen *et al.*, 1983). Also non-invasive methods with human subjects, such as breath hydrogen measurements (Stephen *et al.*, 1983) or measuring SCFAs in peripheral venous plasma or faeces can be used (Cummings *et al.*, 1996). However, since SCFAs are quickly absorbed from the colon and part is used by the colonocytes, these measurements cannot accurately gauge the total production of SCFAs resulting from prebiotic fermentation (Cummings and Englyst, 1991; Wong *et al.*, 2006). Human clinical interventions provide the best model for studying the microbiota and fermentation of prebiotics but they also suffer from many difficulties, such as the possible low compliance of the subjects, the practical and ethical limitations of sampling and the high costs of intervention trials (Macfarlane and Macfarlane, 2007).

On the whole, the study of prebiotic components is usually started with *in vitro* models: either simple pure culture or batch type fermentors can be utilized before proceeding to more complex *in vitro* simulator models and/or animal models to characterise the effects of prebiotics on microbiota composition and metabolic activity. Animal models can also be used in mechanistical studies, although the final proof of the health effects and benefits mediated by prebiotic consumption has to be demonstrated in controlled human intervention studies.

2. AIMS OF THE STUDY

The objectives of this series of *in vitro* studies were to screen new oligosaccharides for their prebiotic potential, compare these candidates in order to established reference prebiotics, and furthermore, to characterize their potential in modulating the colonic microbiota for subsequent product development. This was done by characterizing their effects on the colonic microbiota composition and functionality using *in vitro* models of differing complexicity.

The specific aims of the study were:

1. To screen a number of prebiotic candidates for their effects on the growth of selected probiotic, commensal and pathogenic microbes using a simple *in vitro* model (Studies II and IV).

2. To characterize the properties of the most promising candidates in modulating the composition and metabolic activity of the microbiota using an *in vitro* model of the human large intestine (Studies I, III and V).

3. To evaluate the potential of the candidates to modify the microbiota in relation to established reference prebiotics (Study III and V).

4. To analyze the potential of a novel synbiotic product formulation in modulating the colonic microbiota (Study V).

3. MATERIALS AND METHODS

3.1. Pure culture screening studies

3.1.1. Prebiotic compounds

The study compounds are listed in Table 2. Most of the oligosaccharides were chosen for the screening because they were either interesting new product candidates or emerging prebiotics that needed further testing. In addition, two commercially available prebiotics, FOS (Raftilose®, Beneo, Belgium) and XOS (Longlive 95P, Shandong Biotechnology co., China) were included into the growth assays, and glucose (α-D-glucose, Serva, Germany) was used as a nonselective control substrate (Vernazza *et al*., 2006a). Medium without any additional carbohydrates was used to determine the basal growth of the bacteria, and this basal growth was subtracted from the results.

A 10% suspension of each compound was prepared in sterile water and the suspension was sterilized with ultra-violet radiation or sterile-filtered (0.2 μm Minisart, Sartorius AG, Göttingen, Germany), depending on the degree of polymerization of the compound, and stored at 4°C until use.

Results regarding the first 13 compounds in the table (from FOS to xylan) are published in peer reviewed journal articles. The following six compounds were also tested but the results have not been published.

3.1.2. Bacterial strains and growth media

Bacterial strains were obtained from different culture suppliers and strain collections, and grown in appropriate growth mediums (MRS, TSB, or Bifidomedium; Table 3) under anaerobic conditions. Using mediums and test tubes prepared with the Hungate boiling system (Hungate, 1950), anaerobic growth conditions were generated. Microbial strains were activated from -70°C storage in appropriate, glucose-containing medium for 24 or 48 hours at +37°C. After the cultivation microbes were inoculated into a fresh growth medium containing glucose (MRS or TSB) and incubated for another 24 hours. Then a 1% cell suspension of single microbes was prepared (v/v) in the test medium, which did not include any carbohydrates other than the tested one, and used immediately for the growth assays.

Table 2. Oligosaccharides tested in the studies.

Product	Description	Composition	Supplier
FOS	Fructo-oligosaccharide powder, dp 2-7, prebiotic reference	95,5% FOS dp2-6; 4,5% other dp2 †	Beneo, Belgium
Gentiobiose	β-Gentiobiose powder	≥ 85 % gentiobiose (remainder primarily α anomer)	Sigma (G3000)
Glucose	Alpha-D-glucose powder	100% glucose	Serva, Germany
GOS	Galacto-oligosaccharide powder, dp 3-6	Purified from most mono- and disaccharides, 0.7% galactose, 1.1% glucose and 2.9% lactose	Danisco Cultures, France
Lactitol	Lactitol powder	99 % lactitol	Danisco Sweeteners, UK
Panose	D-Panose powder	≥ 98 % panose	Sigma (P2407)
PDX	Polydextrose, Litesse® *Ultra*™ powder, average dp 12	99 % polydextrose	Danisco Sweeteners, UK
Pullulan	Pullulan powder	Cosmetic grade	Hayashibara, UK
Sophorose	Sophorose powder	≥ 98 % sophorose	Sigma (S1404)
XOS dp2*	Xylo-oligosaccharide syrup, dp 2	100% XOS (7% dp1, 82% dp2, 10% dp3, 1% dp4) †	Lenzing AG, Austria
XOS dp2-10	Xylo-oligosaccharide powder, dp 2-10	99% XOS (13% dp2, 19% dp3, 11% dp4, 60% dp≥5) †	Lenzing AG, Austria
XOS Longlive 95P	Xylo-oligosaccharide powder, dp 2-5	84% XOS (43% dp2, 30% dp3, 10% dp4, 17% dp≥5); 13,5% other dp1 †	Shandong Biotechnology, China
Xylan	Liquid xylan hemisuspension, dp 35-40	97% XOS dp≥5 †	Lenzing AG, Austria
TOS (Bi^{2}muno)	Trans-galacto-oligosaccharide	16% dp=2 and 11% dp≥3 GOS, 49% lactose, 13% galactose and 11% glucose	Clasado, UK
HT XOS	Hydrothermolytically manufactured xylo-oligosaccharide powder, dp 2-10	93% XOS (50% dp2, 23% dp3, 10% dp4, 17% dp≥5) †	Lenzing AG, Austria
IMO-500	Isomalto-oligosaccharide powder	≥35% IMO, no knowledge on dp distribution	Genencor, USA
Meyprodor 5	Guar Gum powder	No knowledge on the specific structure of the product	Genencor, USA
Meyprodor 30	Guar Gum powder	No knowledge on the specific structure of the product	Genencor, USA
Sun Fiber	Partial hydrolysate of Guar Gum, powder	No knowledge on the specific structure of the product	Sun Fiber, Japan

*; dp = degree of polymerisation

†; composition analysed with chromatographic methods

Table 3. Bacterial strains used in the study and the media used for the growth of the strains.

Bacterial strains	Origin of microbes	Preculturing Media	Test Media (without glucose)
Bacteroides fragilis	ATCC [a] 25285	Meat medium [1]	MRS-[5]
Bacteroides vulgatus	DSM [b] 1447	Meat medium	MRS-
Bifidobacterium adolescentis	DSM 20083	Medium 58 [2]	MRS-
Bifidobacterium breve	Bb-03 [c]	Medium 58	MRS-
Bifidobacterium infantis	DSM 20088	Medium 58	MRS-
Bifidobacterium lactis	Bi-07 [c]	Medium 58	MRS-
Bifidobacterium lactis	Bl-04 [c]	Medium 58	MRS-
Bifidobacterium lactis	420 [c]	Medium 58	MRS-
Bifidobacterium lactis	DN-173 010 [d]	Medium 58	MRS-
Bifidobacterium lactis	HN019 [c]	Medium 58	MRS-
Bifidobacterium lactis	Bb-12 [e]	Medium 58	MRS-
Bifidobacterium longum	913, Wisby [c]	Medium 58	MRS-
Bifidobacterium longum	KC-1 [c]	Medium 58	MRS-
Bifidobacterium longum	DSM 20219	Medium 58	MRS-
*Clostridium difficile**	ATCC 9689	Meat medium	MRS-
Clostridium perfringens	ATCC 13124	TSB+ [3]	TSB- [6]
*Eubacterium biforme**	DSM 3989	MRS+ [4]	MRS-
Eubacterium limosum	ATCC 8486	Meat medium	MRS-
Escherichia coli	EHEC 0157:H7 [f]	TSB+	TSB-
Lactobacillus acidophilus	NCFM [c]	MRS+	MRS-
Lactobacillus bulgaricus	1260 [c]	MRS+	MRS-
Lactobacillus paracasei	Lpc-37 [c]	MRS+	MRS-
Lactobacillus rhamnosus	HN001 [c]	MRS+	MRS-
Salmonella typhimurium	EELA 4185/96 [g]	TSB+	TSB-
Staphylococcus aureus sp. a*ureus**	ATCC 10990	TSB+	TSB-
Staphylococcus epidermis	CCUG 37527 [h]	TSB+	TSB-
Streptococcus thermophilus	715 [c]	MRS+	MRS-

* Results of these strains are not presented as the growth on glucose was negligible

a) American Type Culture Collection, USA; b) Deutsche Sammlung von Mikroorganismen und Zellkulturen

c) Danisco; d) Danone e) Christian Hansen; f) EHEC 0157:H7 The German Resourse Centre for Biological material

g) Veterinary and Food Research Agency, Finland; h) Culture Collection, University of Göteborg, Sweden

1) DifcoTM Cooked meat medium; Beckton, Dickinson and Company, USA; 2) Bifidomedium, DSMZ

3) Tryptic soy broth (with glucose); LAB M, UK; 4) de Man, Rogosa and Sharpe medium with glucose (recipe: MRS broth, LAB M, UK); 5) de Man, Rogosa and Sharpe medium without glucose

6) BactoTM Tryptic soy broth without dextrose; Beckton, Dickinson and Company, USA

3.1.3. Measurement of the bacterial growth intensity

The growth of the bacterial strains on different carbohydrate substrates was measured with an automatic Bioscreen® C system (Labsystems, Helsinki, Finland) connected with a PC equipped with a Biolink® software package (Labsystems). The system recorded kinetic changes in the absorbance (600nm) of liquid samples in a 100-well plate and the growth rates of different oligo- and polysaccharide and microbe combinations were determined from this data.

The Bioscreen system was kept in an anaerobic hood (80% N_2, 10% CO_2 and 10% H_2) and all bacterial strains were cultured in anaerobic conditions and media. Test substrate solutions (10%) or glucose (10%) were added to each well of the plate (20µl), and the wells were filled with the diluted cell suspension (180µl) of a single bacterial strain (OD 0.2-0.6). Thus, the final concentration of the carbohydrate substrate in each well was 1% (w/v). For control wells with medium without added carbohydrates, 20µl of sterile water and 180µl of microbial suspension were added. Anaerobic conditions and techniques were used during the growth assay. Bacterial strains were incubated at 37ºC for 24 hours and the optical density of the samples was measured at 600nm once every 30 minutes. Plates were mixed for 10 seconds prior to measurement. At least two sets of experiments were conducted on each strain-and-carbohydrate combination with five replications.

The bacterial growth was determined as the area under the growth curve (OD_{600} x min) obtained from the Bioscreen® data. As previously described, the growth in the blank control medium (MRS or TSB without added carbohydrates) was subtracted from results as baseline growth (Jaskari *et al*., 1998). The relative growth rate of a bacterium was obtained by comparing the area under the growth curve of each strain and prebiotic combination to the growth of the same bacterial strain in glucose, the positive control, and converted to relative growth rates (Crittenden *et al*., 2002).

3.2. Colonic fermentation studies

3.2.1. Colon simulator

The semi-continuous Enteromix® human colon simulator has been used in studying potential prebiotic compounds and the structure and function of the system has been previously described in detail (Mäkivuokko *et al.*, 2005; Mäkivuokko *et al.*, 2006). The simulator consisted of four parallel units, each of which was comprised of four sequentially connected glass vessels, V1 to V4. The conditions in the vessels were adjusted to represent the different

compartments of the human colon: V1 represented the ascending colon, V2 the transverse colon, V3 the descending colon, V4 the end of descending colon and sigmoid/rectal area. The set pH levels in the simulator vessels (5.5, 6.0, 6.5 and 7.0) were similar to those measured in different parts of the human colon (Cummings and Macfarlane, 1991; Macfarlane *et al.*, 1992) and were adjusted with gaseous ammonia when the pH dropped below the target level. The volumes of microbial slurry in the vessels increased from the ascending to the distal end (6, 8, 9, and 12ml), mimicking the reduced flow. The simulator was kept anaerobic at 37°C in a thermostatic room. All run parameters, including pH control and gas and liquid transitions, were computer regulated by synchronized control of pumps and magnetic valves using customized software.

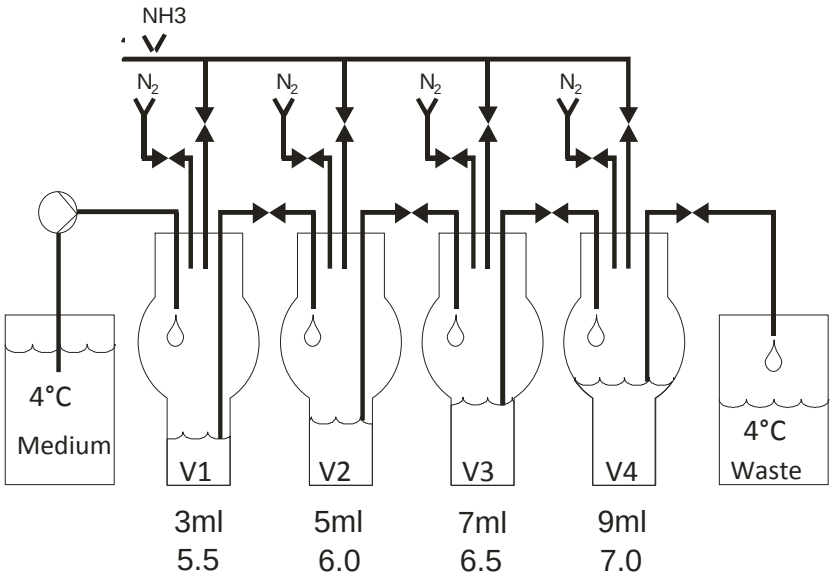

Figure 12. A single unit of the EnteroMix™ human colon simulator, consisting of four connected glass vessels (modified picture from Mäkivuokko *et al*., 2006). The volumes and pH of the different vessels are shown below the figure, V1 = 1st vessel, V2 = 2nd vessel, V3 = 3rd vessel, and V4 = 4th vessel, N_2 = nitrogen gas, NH_3 = ammonium gas.

3.2.2. Simulator medium and test substrates

Synthetic ileal fluid, originally described by Gibson et al. (1988), was used as the basic simulator medium in the colon model. The medium contained the following constituents (g/l) in distilled water: starch 5.0; peptone 0.05; tryptone 5.0; yeast extract 5.0; NaCl 4.5; KCl 4.5; mucine (porcine gastric type III, Sigma Chemicals Co., St.Louis, USA) 4.0; casein 3.0; pectin (citrus) 2.0; xylan (oatspelt) 2.0; arabinogalactan (larch wood) 2.0; $NaHCO_3$ 1.5; $MgSO_4$ 1.25; guar gum 1.0; inulin 1.0; cysteine 0.8; KH_2PO_4 0.5; K_2HPO_4 0.5; bile salts no.3 0.4; $CaCl_2$ x 6 H_2O 0.15; $FeSO_4$ x 7 H_2O 0.005; hemin 0.05; and Tween 80 1.0. Tested prebiotic compounds were suspended into this medium (2% concentration, w/v), and no other carbohydrates, in addition to the ones in the basic medium, were added to the baseline simulations.

3.2.3. Faecal inocula

Faecal samples were obtained from healthy human volunteers (aged between 4 and 85 years old; Study I n=4; Study II n=1; Study III n=4; and Study V n=7) and the faeces of a single donor were used to run a set of four parallel simulations. Fresh faecal material was diluted with the simulator media and filtered through a 0.3 mm metal mesh, after which it was incubated anaerobically for 24 hours at 37°C (Mäkivuokko *et al*., 2005). In the experiments with XOS (Study III), it was noticed in the first three simulation runs that the levels of *Bifidobacterium lactis* varied significantly between the donors and thus, in the last four simulation sets, the inoculum was spiked with *B. lactis* Bi-07 (Danisco Cultures, Niebüll, Germany) to ensure a basal level of this species. The amount of *B. lactis* added to the inoculum was, on average, 2.0×10^7 CFU/ml ($\pm$ 6.4×10^6), which accounted for approximately 20% of the average *B. lactis* numbers measured in the faecal matter used in Study III ($9.6 \times 10^7 \pm 4.4 \times 10^7$ CFU/ml). All of the test substrates were simulated once with non-spiked inoculum and twice with spiked inoculum and so the effect of additional *B. lactis* on the results was equal to all of the tested compounds. In other simulation experiments with other candidate prebiotics (Studies I, II and V), no spiking was required. In the synbiotic simulation experiments (Study V), probiotics were added to the medium in some of the assays (probiotic and synbiotic) but not in all (control and prebiotic).

3.2.4. Operating the simulator

Prior to inoculation, all of the tubing and vessels of the simulator were flushed with nitrogen gas for one hour and filled to pre-set levels (3, 5, 7, and 9ml respectively) with anaerobic NaCl-solution. The preconditioned faecal sample from a single donor was injected anaerobically into the V1 vessel of all four simulation units, mixed well, and part of the diluted inoculum was transferred to vessel V2. The transfer procedure was repeated between vessels V2 and V3, V3 and V4 and eventually between V4 and the waste container. The final volumes in vessels V1, V2, V3 and V4 were again 3, 5, 7, and 9ml respectively. Three hours after the initiation of the simulation experiments, fresh medium was pumped into the V1 vessel. The medium was fermented in the first vessel for three hours and then 3ml of the fermented fluid was transferred to the V2 vessel by gas pressure. The transitions of the medium and the feeding of fresh media occurred in the same manner at three-hour intervals and the fermented medium residuals moved from vessel to vessel and eventually into the waste container. Simulation was continued for 48 hours, after which the samples were collected. At this point the volumes in vessels V1, V2, V3 and V4 were 6, 8, 10, and 12ml respectively.

3.2.5. Analysis of microbial numbers

Total bacterial numbers from the simulator samples were determined by flow cytometry as previously described (Apajalahti *et al.*, 2002). Briefly, the bacterial samples for flow cytometry analysis were fixed with 4% formaldehyde immediately after the simulation and stored at 4°C until analysis. The microbes were stained with a fluorescent, nucleic acid binding dye, SYTO 24 (Molecular probes, Leiden, The Netherlands), prior to counting them with the FACSCalibur system (BD Biosciences, San Jose, CA, USA).

Quantitative PCR (qPCR) was utilized to determine *Bifidobacterium, Lactobacillus, Bacteroides-Prevotella-Porphyromonas* and *Clostridium histolyticum* (clusters I and II) group densities (Study I: only *Bifidobacterium*). Also *Bifidobacterium longum* (studies I and III) and *Bifidobacterium lactis* (Studies II, III and V) species densities were enumerated from simulator samples. Samples for the qPCR analyses were harvested using centrifugation at 48000 x g for 15 minutes; the pellets were stored at –70°C until DNA extraction. Microbes were first lysed with lysozyme and then with bead beating, DNA was extracted with chloroform-isoamylalcohol treatment as described previously (Apajalahti *et al.*, 1998) and DNA was purified using a commercial kit (Genomic DNA extraction kit, Qiagen, Hilden,

Germany). Oligonucleotide primers (Thermo Biosciences, Ulm, Germany or MedProbe, Lund, Sweden) and a probe for *Bifidobacterium* species detection (Applied Biosystems, Warrington, UK) were used. The assays were performed with ABI Prism® 7000, 7300 or 7500 FAST Sequence Detection System (Applied Biosystems, Foster City, CA), using optimized concentrations of mastermix ingredients and PCR cycle conditions (Table 4).

The amplification mixture (25µl) contained SYBR Green® or TaqMan® Mastermixes (Applied Biosystems, Warrington, UK), primers in optimized concentrations, H_2O and 1ng DNA template isolated from the microbial samples. The temperature profile for the annealing and amplification cycle consisted usually of ten minutes at 95˚C, followed by 30-40 cycles of 15 seconds at 95˚C, 30 seconds at 55-65˚C, and 30 seconds at 72˚C, with the exception of the PDX-xylitol study where 40 cycles of 15 seconds at 95˚C and one minute at 60-64˚C were used (Table 4).

3.2.6. Analysis of metabolites

Chromatographic analysis of volatile fatty acids and lactic acid was performed as previously described with pivalic acid as an internal standard (Ouwehand *et al.*, 2009). Briefly, samples collected from the simulator were mixed with pivalic acid, water and saturated oxalic acid then centrifuged and incubated at 4°C. The supernatant was analyzed by gas chromatography using a flame ionization detector and helium as the carrier gas. Concentrations of acetic, propionic, butyric, isobutyric, valeric, isovaleric, 2-methylbutyric and lactic acids were determined.

Biogenic amines were analyzed as dansyl derivates by reversed phase high-performance liquid chromatography (HPLC), using a method by Saarinen (2002). Heptylamine was used as an internal standard and the biogenic amines were extracted with perchloric acid. Biogenic amines were derivatised with dansyl chloride, separated on a reverse phase HPLC column and detected with a fluorescent detector. The concentrations of methylamine, ethylamine, tryptamine, 2-methylbutylamine, putrescine, cadaverine, histamine, tyramine, spermidine, and spermine were determined.

Xylo-oligosaccharides in the fermentation fluids were determined by high pH ion exchange chromatography with pulsed electrochemical detection, using a Carbo Pak-PA1 separation column and a mixture of water and 0.2 M NaOH as eluent. Fructo-oligosaccharides were analyzed as total fructose after acid hydrolysis. The acid hydrolysis was conducted by incubating the sample in sulphuric acid and thereafter fructose was determined by high pH ion exchange chromatography as described (Bach Knudsen, 1997). Polydextrose

concentrations were measured using a HPLC method described previously (Craig *et al*., 2000); and xylitol concentrations were determined by a commercial enzymatic colorimetric method (Boehringer Mannheim/R-Biopharm, Darmstadt, Germany). GOS or panose concentrations were not analysed from the samples as methods for their determination were not available at the time.

3.2.7. Statistical analysis

Data are reported as the mean values of the results from all the vessels of a simulator unit (from V1 to V4) and replicates (n = 3 or 4 for the test compounds and n = 4-8 for baseline simulations) and the standard error of the mean (SEM). The statistical analysis was performed using SPSS software (SPSS, Inc., Chicago, Ill., USA, 14.0 and16.0 versions). Data were assessed using ANOVA and t-tests or Tukey's multiple comparison tests to compare results from baseline simulations with the test substrates, and also to compare results between the commercial prebiotics and other substrates. The differences among the means were considered significant when $P<0.05$. Spearman correlation coefficiencies were also calculated from the whole data to search for correlations between microbial groups and various metabolites.

Table 4. Oligonucleotide primers and probe sequences, qPCR mastermixes and annealing temperatures used in the assays to identify bacterial strains and species of interest

Target species	Primer	Sequence 5´-3´	Master Mix	Annealing temp. (°C)	Reference
Bacteroidetes gp.	gBacter_F gBacter_R	GGTGTCGGCTTAAGTGCCAT CGGAYGTAAGGGCCGTGC	SYBR Green® Core Reagents	64	Rinttilä *et al*. 2004
Bifidobacterium spp. (Studies II, III and V)	BF BGR Bprobe	CCTGGTAGTCCACGCCGTAA CAGGCGGGATGCTTAACG FAM-ATCCAGCATCCACCG-MGB	TagMan® Universal	60	Mäkivuokko *et al.* 2005
Bifidobacterium genus (Study I)	Bifidogenus 5' Bifidogenus 3'	GAT TCT GGC TCA GGA TGA ACG C CTG ATA GGA CGC GAC CCC AT	Power SYBR Green® Universal	60	Guiemonde *et al.* 2004
Bifidobacterium lactis spp.	Blact_1 Bflact5	GCATGTTGCCAGCGGGT CACACCACACAATCCAATAC	SYBR Green® Core Reagents	65	Mäkeläinen *et al.* 2009 Ventura *et al.* 2001
Bifidobacterium longum spp.	Blongum_F Blongum_R	TTC CAG TTG ATC GCA TGG TCT TCT GGC TAC CCG TCG AAG CCA CG	Power SYBR Green® Universal	60/64	Rinne *et al*. 2005
Clostridium histolyticum gp. (clusters I and II)	gCperf_F gCperf_R	ATGCAAGTCGAGCGAKG TATGCGGTATTAATCTYCCTTT	SYBR Green® Core Reagents	55	Rinttilä *et al.* 2004
Lactobacillus spp.	Lac1 Lab-0677r	AGCAGTAGGGAATCTTCCA CACCGCTACACATGGAG	SYBR Green® Core Reagents	56	Walter *et al.* 2001 Heilig *et al.* 2002

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4. RESULTS

4.1. Pure culture screening studies

The growth of tested bacterial strains in pure cultures on different test substrates is presented in Table 5. In Study II, panose and sophorose were tested in pure cultures with ten microbial strains due to the limited availability of the products. In Study IV, a range of probiotic, commensal and pathogenic microbial strains (n=23) were used.

4.2. Colon simulator studies

The characterization of interesting prebiotic candidates was continued with the *in vitro* colon simulator model with the aim of mimicking prebiotic fermentation in the human large intestine by complex microbiota. In these studies (I, II, III and V), fermentation of XOS compounds (dp2, dp2-10 and Longlive), xylan, FOS, panose, GOS and PDX (all 2% w/v) were investigated. Furthermore, in Study I, PDX and xylitol were a tested combination (PDX 1% + xylitol 1%, w/v); and in Study V, PDX and GOS were analysed together with probiotic *B. lactis* (in synbiotic GOS+PDX+*B. lactis*; PDX 1% + GOS 1%, w/v). The results from all four vessels of the simulator model were combined and thus one simulator unit was treated as one entity in order to make results more comprehensible. Moreover, results regarding the metabolic activity of the microbiota were also separately analysed from vessels.

Table 5. Results of the pure culture screening studies. The growth rate for each bacteria on the various prebiotic compound was calculated relative to its growth rate on glucose, which was used as a positive control for microbial growth: '++++'=100% or more of the growth rate on glucose, '+++'=70 - 99% of the growth rate on glucose, ++'=40 - 69% of the growth rate on glucose, '+'=10-39% of the growth rate on glucose, '0'=1-10% of the growth obtained with glucose, '-'=0% or less of the growth rate on glucose. 'nt'=not tested.

Relative growth rates	XOS dp2	XOS dp2-10	XOS Longlive	Xylan	FOS	GOS	PDX	Lactitol	Gentio-biose	Pullulan	Panose	Sophorose	Meyprodor 30	Meyprodor 5	Sun Fiber	GOS Bimuno	IMO-500	XOS HT
B.breve Bb-03	0	+	0	0	++	++++	0	-	++	-	nt	nt	0	+	+	+++	+	0
B.longum KC-1	0	-	0	0	++	++++	0	0	-	-	0	-	0	0	0	++	++	0
B.longum 913	+	0	0	-	+	+++	0	-	0	-	nt	nt	0	0	-	+	+	0
B.longum DSM20219	0	-	0	0	++	+++	0	-	-	-	nt	nt	0	0	0	nt	nt	nt
B.infantis	+	+	+	+	++++	++++	+	+++	+	-	nt	nt	0	0	+	nt	nt	nt
B.adolescentis	++	++	+++	0	++++	++++	0	0	+	-	nt	nt	+	0	0	nt	nt	nt
B.lactis HN019	+++	+++	+++	0	+++	++	0	-	0	-	++++	-	-	0	+	+++	+++	0
B.lactis Bb-12	+++	++	++++	0	+++	++++	0	0	+	+	nt	nt	+	+	+	+++	++++	0
B.lactis Bi-07	++	++	+++	0	+++	+	+	+	+++	+	+++	+++	-	+	+	+++	++	0
B.lactis 420	++	++	+++	-	+++	++	+	0	++	+	nt	nt	+	+	+	+++	+++	0
B.lactis Bl-04	++	+++	+++	-	+++	++	0	-	0	0	nt	nt	+	+	+	++	+++	0
B.lactis DN-173010	++	++	++	-	++	+	0	-	+	-	nt	nt	0	0	0	++	+++	0
L.acidophilus NCFM	+	-	+	0	+++	+++	+	+++	+++	-	+	+++	-	+	+	++++	+++	0
L.bulgaricus	-	+	-	-	-	+	-	-	-	-	++	-	0	0	+	++	+	0
L.paracasei Lpc-37	-	-	-	-	+++	0	+	++	++	-	-	+	-	-	+	+++	+	0
L.rhamnosus HN001	-	-	0	-	0	0	+	++	++	-	0	+++	-	-	+	+++	++	0
Strept.thermophilus	0	0	0	-	0	++	0	0	0	0	0	0	+	0	0	++	0	0
E.coli EHEC	+	-	0	-	+	+++	+	0	-	0	nt	nt	-	-	++	+++	++	0
Salm.typhimurium	0	-	0	0	0	0	0	0	0	-	0	0	-	-	0	+++	+++	0
Cl. perfringens	-	-	-	-	+	+++	+	-	-	++	0	-	-	-	+	++++	+++	0
Staph.epidermis	0	-	-	-	++	0	0	-	-	-	nt	nt	-	-	0	+++	++	0
Bact.fragilis	+	+	0	0	+	+	0	0	++	+	nt	nt	0	+	+	+++	++	0
Bact.vulgatus	+	+	+	0	++++	++++	0	-	+++	+	nt	nt	+	+	+	nt	nt	nt
Eu.limosum	0	+	0	0	+++	++++	0	-	++	0	nt	nt	-	-	+	0	0	0

4.2.1. Microbiota composition

Results regarding the investigated bacterial groups from all studies (I, II, III and V) are presented in Table 6 as mean values (log10 ± SEM) per ml of fermentation fluids from all four vessels (V1 to V4). *Bifidobacteria* was the group most commonly affected by various prebiotics but other measured bacterial groups, namely *Lactobacillus, Bacteroides-Prevotella-Porphyromonas* and *Clostridium histolyticum* (clusters I and II), were also modified by different substrates. The species densities of either *B. lactis* and/or *B. longum* were also investigated in all studies; in Study III, XOS enhanced the growth of *B. lactis* significantly more than FOS ($P<0.05$ for XOS Longlive and $P<0.01$ for XOS dp2-10), whereas FOS increased the growth of *B. longum* more than XOS compounds ($P<0.05$ for XOS dp2-10 and xylan, and $P<0.01$ for XOS Longlive).

In Study V the different synbiotics were further compared to the constituting pre- and probiotic components. The PDX+*B. lactis* combination resulted in higher numbers of total microbes ($P<0.01$) and greater production of acetate ($P<0.01$) and propionate ($P<0.01$) in comparison to the probiotic *B. lactis*, whereas, in relation to the prebiotic component, increased levels of *B. lactis* ($P<0.01$) and *Bifidobacterium* species ($P<0.05$), as well as and acetate and total biogenic amines, were measured. Synbiotic GOS+*B. Lactis,* in comparison to the probiotic, had significantly more effects on the levels of total *Bifidobacterium* and *Bacteroides-Prevotella-Porphyromonas* species and several metabolites; acetate ($P<0.001$) and butyrate ($P<0.001$) concentrations were increased while the total levels of amines ($P<0.001$) and isovaleric acid ($P<0.01$) were decreased. In comparison to prebiotic GOS, the synbiotic increased the numbers of *B. lactis* ($P<0.05$) while concomitantly decreasing *Bacteroides-Prevotella-Porphyromonas* ($P<0.05$) group members. An increased trend of *Bifidobacterium* ($P=0.057$) and decreased *Lactobacillus* ($P=0.06$) groups were also measured. When PDX was added to the synbiotic GOS+*B. lactis*, further modifications on the microbial metabolism were measured, as propionic acid production was increased ($P<0.05$) and that of acetate decreased ($P<0.001$).

Table 6. The effects of prebiotic fermentation on selected members of the colonic microbiota, expressed as mean cell numbers (log10±SD) per ml of the simulator fluids.

Study I [a]	Baseline	PDX (2%)	Xylitol (2%)	PDX+Xylitol (1%+1%)
Total microbes	10.0 ± 9.1	10.1 ± 9.3	10.0 ± 9.1	10.0 ± 9.2
Bifidobacterium gp.	7.9 ± 7.2	7.9 ± 7.2	7.6 ± 7.0	7.7 ± 7.0
B.longum spp.	7.8 ± 7.0	7.7 ± 7.0	7.4 ± 6.9	7.5 ± 6.8

Study II [b]	Baseline	Panose
Total microbes	9.6 ± 8.7	9.6 ± 8.6
Bifidobacterium gp.	7.9 ± 7.1	**8.9 ± 8.1 *** **
B.lactis spp.	7.7 ± 7.2	**8.6 ± 8.1 ***
Lactobacillus gp.	6.8 ± 6.4	6.8 ± 6.4
Bacteroides-Prevotella-Porphyromonas gp.	8.7 ± 7.6	**8.2 ± 7.5 *** **
Clostridium clusters I and II	6.6 ± 6.4	5.8 ± 5.4

Study III	Baseline	XOS dp2	XOS dp2-10	Xylan	XOS Longlive	FOS
Total microbes	9.8 ± 8.7	10.0 ± 9.2	10.2 ± 9.5	10.1 ± 9.4	9.9 ± 9.2	10.0 ± 9.1
Bifidobacterium gp.	8.5 ± 7.8	**8.9 ± 8.2 ** **	**9.0 ± 8.4 ***	**8.8 ± 8.3 ***	**9.4 ± 9.0 ***	**9.6 ± 9.2 ***
B.lactis spp.	7.8 ± 7.3	8.2 ± 7.8	**8.4 ± 7.8 ***	7.8 ± 7.6	**9.0 ± 8.6 ***	7.5 ± 7.2
B.longum spp.	8.3 ± 7.7	8.7 ± 8.3	8.5 ± 8.1	8.5 ± 7.7	8.3 ± 7.7	**9.0 ± 8.2 ** **
Lactobacillus gp.	7.9 ± 7.4	8.6 ± 8.2	8.8 ± 8.6	**7.3 ± 6.5 ***	8.8 ± 8.5	**7.4 ± 6.9 ***
Bacteroides-Prevotella-Porphyromonas gp.	8.0 ± 7.5	7.8 ± 7.0	7.7 ± 7.2	8.5 ± 8.1	**7.3 ± 6.8 ***	7.6 ± 7.1
Clostridium clusters I and II	6.7 ± 6.6	5.5 ± 5.4	4.5 ± 3.9	4.8 ± 4.4	4.4 ± 3.9	5.6 ± 5.5

Study V	Baseline	PDX	GOS	B.lactis Bi-07	PDX+B.lactis	GOS+B.lactis	PDX+GOS+B.lactis
Total microbes	9.7 ± 8.6	**9.9 ± 9.1 ***	9.8 ± 9.0	**9.8 ± 8.7 ***	**10.1 ± 9.1 ****	**9.9 ± 8.7 ****	**10.0 ± 8.9 ****
Bifidobacterium gp.	8.0 ± 7.1	8.1 ± 7.5	**9.2 ± 8.3 ****	**8.8 ± 8.0 ****	**8.7 ± 8.1 ***	**9.5 ± 8.7 ****	**9.3 ± 8.5 ****
B.lactis spp.	7.3 ± 6.6	**6.8 ± 6.2 ** **	**7.9 ± 7.4 ***	**8.8 ± 8.2 ** **	**8.7 ± 8.1 ** **	**8.5 ± 8.0 ** **	**9.0 ± 8.2 ****
Lactobacillus gp.	6.0 ± 5.5	5.8 ± 5.3	5.5 ± 4.9	6.0 ± 5.7	6.9 ± 7.1	**5.1 ± 4.5 ** **	**5.5 ± 4.9 ***
Bacteroides-Prevotella-Porphyromonas gp.	8.5 ± 7.4	8.6 ± 8.1	8.0 ± 7.3	8.3 ± 7.6	8.6 ± 7.9	**7.9 ± 7.4 ** **	8.4 ± 7.8
Clostridium clusters I and II	5.7 ± 4.8	**6.1 ± 5.4 ***	5.4 ± 4.7	**5.9 ± 5.0 ***	**6.1 ± 5.5 ***	**5.2 ± 4.4 ****	**5.3 ± 4.5 ** **

[a] Values are mean cell numbers (log10) ± SD

[b] Stars mark the significant difference in microbial numbers in comparison to respective baseline levels; * P<0.05; ** P<0.01; *** P<0.001

4.2.2. Metabolic activity of the microbiota

The metabolic functionality of the microbes in the whole length of the colon model was modified very similarly by most of the prebiotic substrates; increased production of different SCFAs was measured with a concomitant decrease in the BCFA (Table 7). The total concentrations of measured biogenic amines remained mainly unaffected, as only GOS (and GOS+*B. lactis*) significantly decreased the production of these metabolites and xylitol increased it. Individual biogenic amine concentrations were affected by a few of the compounds; some compounds increased the production of amines while others decreased it, e.g. tyramine production was decreased by panose and XOS dp2 fermentation, but increased in XOS Longlive and FOS simulations.

Significant correlations between some metabolite concentrations and bacterial numbers were observed in the combined data set from all simulations. SCFA acetate and butyrate and lactic acid levels were positively correlated with *Bifidobacterium* group numbers, whereas an inverse correlation between bifidobacteria and propionate was found. On the contrary, propionate concentrations were positively correlated with *Bacteroides-Prevotella-Porphyromonas* and *Cl. histolyticum* group numbers. Total biogenic amines and all BCFAs were also inversely correlated with bifidobacteria, whereas a positive correlation was found between total amines and *Lactobacillus. Bacteroides-Prevotella-Porphyromonas* group numbers were also positively correlated with total amines and all BCFAs while *Cl. histolyticum* group levels correlated negatively with acetate and butyrate.

Table 7. (Continued on next page) The concentration of metabolites after the fermentation of prebiotic compounds in the colon model (mean ± SEM). The concentrations of biogenic amines are given for total amount of measured amines and individually for the most abundant and significantly altered amines. The statistical difference from the respective baseline simulations is marked with stars: * P < 0.05; ** P < 0.01; *** P < 0.001.

	Study I	**Baseline**		**PDX**			**Xylitol**			**PDX+Xylitol**		
Volatile	Acetate	24.4 ± 1.7		62.5 ± 4.7	***		13.1 ± 2.6	**		25.6 ± 3.3		
	Propionate	7.8 ± 0.5		18.5 ± 2.1	***		8.8 ± 0.8			14.2 ± 1.5	***	
	Butyrate	8.7 ± 0.6		21.7 ± 1.4	***		56.2 ± 5.6	***		46.0 ± 5.6	***	
fatty acids	Lactic acid	0 ± 0		0.176 ± 0.1704			0.76875 ± 0.4151			0.284 ± 0.196		
	Isobutyric acid	0.9 ± 0.1		0.6 ± 0.1	**		0.6 ± 0.2			0.5 ± 0.1	***	
	2-methylbutyric acid	0.5 ± 0.1		0.2 ± 0.1	***		0.2 ± 0.1	***		0.2 ± 0.0	***	
	Isovaleric acid	0.9 ± 0.1		0.4 ± 0.1	***		0.2 ± 0.1	***		0.3 ± 0.1	***	
(mM)	Valeric acid	1.4 ± 0.2		1.3 ± 0.2			1.5 ± 0.3			1.3 ± 0.3		
Biogenic	Methylamine	28.8 ± 7.9		30.2 ± 4.1			29.2 ± 3.5			22.5 ± 4.6		
	Putrescine	27.6 ± 10.2		19.4 ± 6.8			44.5 ± 10.9			80.6 ± 17.9	*	
	Piperidine	9.2 ± 4.8		3.2 ± 1.4			3.0 ± 1.3			4.8 ± 1.6		
amines	Cadaverine	205.5 ± 29.9		178.2 ± 23.7			264.4 ± 25.1	*		258.0 ± 33.6		
	Histamine	8.3 ± 1.8		3.9 ± 1.4	**		5.7 ± 1.6	*		6.2 ± 1.7	*	
	Tyramine	17.9 ± 2.3		15.5 ± 2.1	**		17.1 ± 2.2			16.8 ± 2.1		
(µM)	Total amines	313.4 ± 35.0		266.1 ± 29.4			382.4 ± 32.4	*		403.9 ± 51.4		

	Study II	Baseline	Panose	
Volatile	Acetate	23.2 ± 1.5	**122.5 ± 13.3**	***
	Propionate	7.6 ± 0.8	9.5 ± 2.7	
	Butyrate	10.1 ± 1.3	**49.1 ± 7.5**	***
fatty acids	Lactic acid	0.0 ± 0.0	5.8 ± 5.8	
	Isobutyric acid	1.2 ± 0.1	**0.6 ± 0.3**	***
	2-methylbutyric acid	0.8 ± 0.1	**0.3 ± 0.2**	***
	Isovaleric acid	1.3 ± 0.1	**0.4 ± 0.2**	***
(mM)	Valeric acid	1.1 ± 0.5	0.5 ± 0.4	
Biogenic	Methylamine	72.9 ± 5.1	**55.8 ± 4.9**	*
	Putrescine	202.7 ± 52.5	112.5 ± 20.2	
amines	Piperidine	6.9 ± 1.5	**11.3 ± 0.9**	*
	Cadaverine	29.9 ± 12.7	10.9 ± 2.1	
	Tyramine	105.4 ± 17.8	**52.7 ± 7.3**	*
(µM)	Total amines	512.9 ± 86.1	328.3 ± 32.6	

	Study III	Baseline	XOS dp2		XOS dp2-10		Xylan		XOS Longlive		FOS	
Volatile	Acetate	21.0 ± 0.7	**85.4 ± 11.1**	**	**81.4 ± 10.3**	***	**37.3 ± 5.4**	**	**88.4 ± 9.8**	***	**90.6 ± 8.7**	***
	Propionate	6.8 ± 0.5	15.8 ± 3.0		13.7 ± 4.1		**10.6 ± 2.2**	**	6.7 ± 1.5		10.9 ± 3.4	
	Butyrate	9.1 ± 0.3	**39.9 ± 5.6**	***	**29.8 ± 4.7**	**	**15.4 ± 2.0**	*	**41.1 ± 6.5**	***	**37.8 ± 7.0**	**
fatty acids	Lactic acid	0.0 ± 0.0	0.5 ± 0.4		2.6 ± 2.3		0.0 ± 0.0		6.3 ± 5.2		14.4 ± 6.9	
	Iso-butyric acid	1.0 ± 0.1	**0.6 ± 0.1**	***	**0.5 ± 0.2**	*	1.0 ± 0.1		**0.5 ± 0.2**	*	**0.4 ± 0.1**	***
	2-methyl-bytyric acid	0.6 ± 0.0	**0.2 ± 0.0**	***	**0.2 ± 0.1**	***	0.5 ± 0.1		**0.2 ± 0.1**	***	**0.2 ± 0.1**	***
	Iso-valeric acid	1.0 ± 0.1	**0.4 ± 0.1**	***	**0.4 ± 0.1**	***	0.9 ± 0.1		**0.5 ± 0.1**	***	**0.5 ± 0.2**	**
(mM)	Valeric acid	1.7 ± 0.4	0.4 ± 0.1		0.9 ± 0.2		2.1 ± 0.5		0.4 ± 0.2		1.0 ± 0.6	
Biogenic	Putrescine	270.5 ± 72.7	48.2 ± 16.2		211.7 ± 90.3		306.3 ± 81.1		140.5 ± 46.7		177.8 ± 54.0	
amines	Cadaverine	328.8 ± 60.1	223.9 ± 68.7		538.3 ± 108.8		235.6 ± 60.5		**47.0 ± 17.8**	***	172.0 ± 55.9	
	Tyramine	27.3 ± 2.2	**10.3 ± 1.0**	***	60.0 ± 16.4		29.7 ± 2.7		**72.3 ± 15.3**	*	**132.6 ± 47.9**	*
(µM)	Total amines	659.1 ± 112.1	320.5 ± 73.6		850.6 ± 178.7		602.3 ± 114.4		323.4 ± 50.4		508.3 ± 104.1	

	Study V	Baseline	PDX		GOS		*B. lactis* Bi-07		PDX+*B. lactis* Bi-07		GOS+*B. lactis* Bi-07		PDX+GOS+*B. lactis* Bi-07	
Volatile	Acetate	20.2 ± 0.8	**51.7 ± 4.0**	***	**92.9 ± 5.4**	***	**30.7 ± 2.9**	*	**69.7 ± 6.8**	***	**106.2 ± 6.4**	***	**74.2 ± 3.1**	***
	Propionate	6.1 ± 0.3	**19.4 ± 2.8**	**	3.8 ± 0.7		6.2 ± 0.8		**22.4 ± 3.6**	**	4.0 ± 0.9		**15.0 ± 1.8**	**
	Butyrate	10.7 ± 0.4	**26.1 ± 2.1**	***	**40.8 ± 4.3**	***	10.9 ± 1.6		**19.9 ± 2.8**	*	**41.0 ± 5.0**	***	**46.3 ± 4.4**	***
fatty acids	Lactic acid	0.1 ± 0.1	1.2 ± 0.9		4.4 ± 3.5		1.3 ± 0.9		1.4 ± 1.4		5.9 ± 4.5		1.0 ± 1.0	
	Iso-butyric acid	0.9 ± 0.1	**0.5 ± 0.1**	*	**0.4 ± 0.1**	**	1.0 ± 0.2		0.7 ± 0.1		**0.5 ± 0.1**	*	**0.4 ± 0.1**	**
	2-methyl-bytyric acid	0.5 ± 0.0	**0.2 ± 0.1**	**	**0.4 ± 0.1**	**	0.6 ± 0.1		**0.1 ± 0.1**	**	0.4 ± 0.0		**0.2 ± 0.1**	***
	Iso-valeric acid	0.9 ± 0.1	**0.2 ± 0.1**	***	**0.4 ± 0.1**	***	1.0 ± 0.2		**0.2 ± 0.1**	***	**0.4 ± 0.1**	**	**0.3 ± 0.1**	***
(mM)	Valeric acid	1.2 ± 0.2	2.0 ± 0.6		0.8 ± 0.2		1.2 ± 0.4		2.8 ± 0.8		1.1 ± 0.3		1.9 ± 0.6	
Biogenic	Methylamine	168.8 ± 23.5	211.8 ± 32.7		0.0 ± 0.0		208.0 ± 45.3		224.6 ± 52.5		0.0 ± 0.0		155.9 ± 34.7	
	Putrescine	199.3 ± 43.0	103.5 ± 35.9		**22.5 ± 4.8**	***	114.5 ± 55.2		107.2 ± 47.1		**18.8 ± 3.9**	***	153.1 ± 31.5	
amines	Cadaverine	55.0 ± 23.5	72.6 ± 34.9		0.0 ± 0.0		**184.4 ± 54.7**	**	**355.8 ± 98.3**	**	1.5 ± 1.5		124.2 ± 22.1	
	Tyramine	41.7 ± 5.0	25.5 ± 1.3		26.5 ± 1.5		54.8 ± 9.1		54.8 ± 9.6		27.3 ± 1.7		32.0 ± 1.7	
	Spermine	1.4 ± 0.9	**26.3 ± 8.6**	*	1.5 ± 0.7		0.0 ± 0.0		11.9 ± 5.9		1.3 ± 1.0		0.5 ± 0.5	
(µM)	Total amines	467.1 ± 63.8	439.8 ± 56.1		**54.8 ± 6.8**	***	561.7 ± 62.2		**754.3 ± 119.1**	*	**56.2 ± 7.7**	***	465.8 ± 60.9	

When individual vessels of the colon model were investigated separately, differences in the production rate of SCFA and BCFA were observed in different parts of the colon model (Table 8). GOS, panose, FOS, xylitol and PDX all initiated the production of SCFA as early as in the proximal vessel V1 of the colonic model, where significantly increased levels of acetate and/or butyrate/propionate were measured. Fermentation of XOS compounds led to significantly increased SCFA levels in the middle and distal parts of the colon model, as increased concentrations of butyrate and/or acetate were measured from vessel V2 onwards. The production of proteolytic metabolites (BCFA and/or biogenic amines) were already depressed by GOS, panose and xylitol as early as in the proximal (V1) part, whereas PDX-containing compounds and XOS dp2 decreased BCFA production also distally (V4), in the colon model. Probiotic *B. lactis* did not affect the metabolism of microbiota in individual vessels although the synbiotic PDX+*B. lactis* and GOS+*B. lactis* had similar effects on metabolite production as the prebiotic components alone had. Furthermore, PDX(1%)+GOS(1%)+*B. lactis* showed the combined effects of the two synbiotic products from vessel V1 onwards.

Table 8. Significantly (P<0.05 or more) altered levels of most abundant metabolites in different vessels of the colon simulator after 48-hr fermentation with prebiotic, probiotic and synbiotic substrates. Bold capital letters indicate increased production, whereas small letters in italics indicate the decreased production of that substrate in the specific vessel.

	Simulator vessel			
	V1	V2	V3	V4
PDX	**A,B***	**A,P,B**,*bcfa*	**A,P,B**,*bcfa*	**A,P,B**,*bcfa*
Xylitol	**A,B**,*bcfa*	**B**,*bcfa*	**B**	**B**
PDX+xylitol		**P,B,***bcfa*	**P,B,***bcfa*	**P,B,***bcfa*
Panose	**A**,*bcfa*	**A,B**,*bcfa*	**A,B**,*bcfa*	**A,B**
XOS dp2		**B**, *bcfa*	**A,B,**bcfa	**A,B**,*bcfa*
XOS dp2-10		**B**,*bcfa*	**B**	**B**
xylan			**BA**	
XOS Longlive		**A,B**,*bcfa*	**A,B**	**A,B,P**
FOS	**P**	**A,** *bcfa*	**A**, *bcfa*	**A**
GOS	**A,** *bcfa,ba*	**A,B**,*bcfa*	**A,B**,*bcfa,ba*	**A,B**
B.lactis Bi-07				
PDX+*B.lactis*	**A**	**A, P, B**	**A, P, B**,*bcfa*	**A, P**,bcfa
GOS+*B.lactis*	**A,P,**ba	**A,B**,*bcfa*	**A,B**,*bcfa*	**A,B**
PDX+GOS+*B.lactis*	**A**	**A,P,B**,*bcfa*	**A,P,B**,*bcfa*	**A,P,B**,*bcfa*

* A=acetate; P=propionate, B=butyrate; BA=biogenic amines;
BCFA=branchad chain fatty acids

4.2.3. Degradation of tested compounds in the colonic model

Slow degradation of PDX and a faster degradation of xylitol were observed (Study I). Part of the added PDX was still left in the distal colon, whereas xylitol was degraded by the microbes in the proximal and middle parts of the model. The degradation of both PDX and xylitol was also similar in the combination simulations, where these carbohydrates were present in lower concentrations. Oligosaccharides XOS and FOS were more rapidly consumed by the microbes in the simulated colon (Study III) as the concentrations of residual oligosaccharides were very low in the proximal part of the model and non-measurable in the distal part (Figure13).

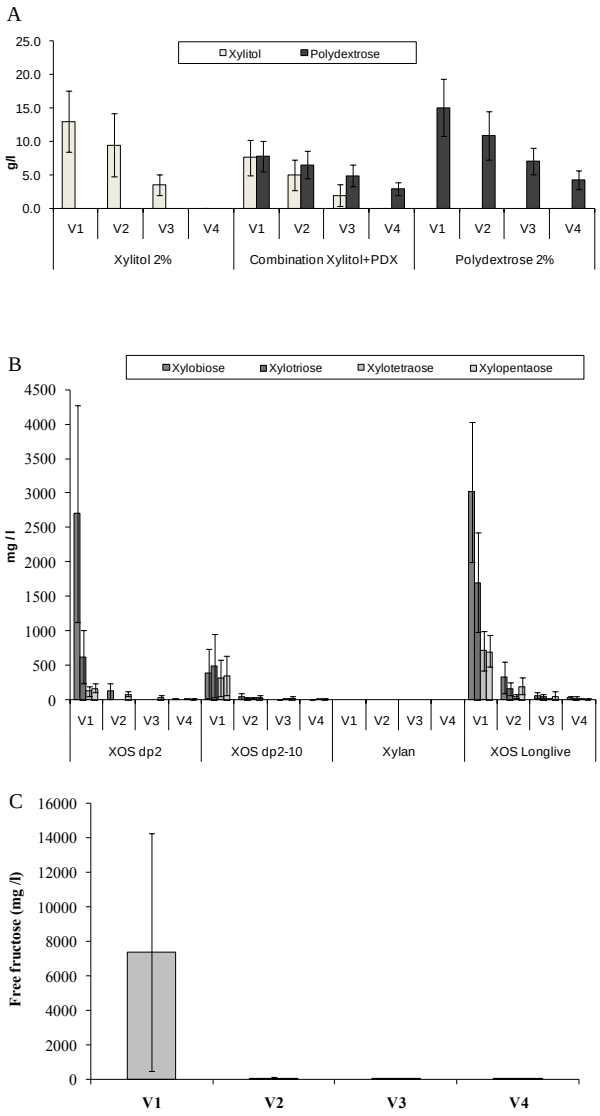

Figure 13. The concentrations of A) PDX and xylitol, B) XOS and C) FOS (as free fructose) in simulator vessels after 48-hour fermentation by the faecal microbiota.

5. DISCUSSION

The study and invasive sampling of different parts of the human GIT presents numerous methodological and ethical problems and therefore several *in vitro* models of the human colon have been introduced (Mäkivuokko and Nurminen, 2006; Cinquin *et al.*, 2004, 2006). *In vitro* models provide the means to investigate the microbiota in different parts of the colon and analyse bacterial metabolites in more detail as they are absorbed and utilized by the host *in vivo*. These models do suffer from a few limitations, such as the absence of host epithelial and immune cells and also the inoculation with faecal material, which represents the microbiota of the distal part of the human colon and which differs significantly from the mucosa- and biofilm-associated bacterial communities (Zoetendal *et al*., 2002; Cinquin *et al*., 2004, 2006). However, by varying the parameters that influence the fermentation processes (pH, dilution factor, and nutrient availability), the prevailing conditions in the simulator can be made to resemble the different parts of the colon (Macfarlane *et al*., 1998; Egert *et al*., 2006). In addition, the microbes in faeces are mostly of luminal origin and it is thus reasonable to assume that most of these microbes in closest proximity to the food bolus will carry out the fermentation of fiber and prebiotics in the colon (Rumney and Rowland, 1992). Therefore, i*n vitro* methods have been videly used in studying the processes that occur in the lumen of the colon during the fermentation of a prebiotic (Egert *et al.*, 2006; Rumney and Rowland, 1992); the Enteromix colon simulator has also been used previously in modelling human colonic microbiota and its functionality (Mäkivuokko *et al*., 2005, 2006, 2007) and was also utilised in this thesis work in the characterization of prebiotic candidate compounds, their fermentation properties and their potential in synbiotic combinations.

Prior to the colon simulator, a simple *in vitro* screening tool (Bioscreen®) was used to scan through a group of candidate compounds. Fermentation of prebiotics was tested with a range of probiotic, commensal and pathogenic bacterial strains in pure cultures and, according to these results, a few interesting compounds were selected for further characterization with the colon model. It was demonstrated that XOS compounds with varying degree of polymerization were well fermented by *Bifidobacterium lactis* strains; *B. adolescentis* was the only other species to metabolize XOS to a great degree. In line with previously reported studies (Jaskari *et al.*, 1998; Kontula *et al.*, 1998b), lactobacilli and most commensal and pathogenic strains were not able to metabolize XOS efficiently, with the exception of *Bacteroides* strains that exhibited some growth (Crittenden *et al.*, 2002). In previous studies, *Bacteroides* strains have, however, been shown to mainly utilize the

monosaccharide (xylose) fraction in the products (Jaskari et al., 1998). Similarly to XOS, panose, sophorose and lactitol were only fermented by a few probiotic strains in pure cultures; lactitol by probiotic lactobacilli and *B. infantis*, panose by two *B. lactis* and *L. bulgaricus* strains and sophorose by one probiotic *B. lactis* and two probiotic *Lactobacillus* strains. Previously, the fermentation of panose by *Bifidobacterium* spp., *Bacteroides* fragilis, *Ent. faecalis* and *Cl. ramosum*, but not with a range of other bacteria in pure cultures (n=37), has been reported (Kohmoto et al., 1988), as well as the utilization of lactitol by *Lactobacillus* strains (Kontula *et al.*, 1999). On the other hand, the established prebiotics, FOS and GOS, were fermented by a wider range of tested microbes: *Bifidobacterium, Lactobacillus*, and even some potentially pathogenic microbes (e.g. *E. coli*, *Cl. perfringens* and *Staph. epidermis*), were able to utilize them. Previous studies have also reported the non-selectivity of FOS in pure culture studies, where FOS enhanced not only the members of genera generally considered beneficial, but also other intestinal microbes such as *Bacteroides* spp, *Clostridium* spp, Enterobacteria and *Eubacterium* spp. (Hartemink *et al.*, 1997; Jaskari *et al.*, 1998). The prebiotic candidates, gentiobiose and IMO-500, were also fermented by a range of the tested microbial strains, whereas polydextrose, xylan and pullulan were only limitedly fermented in pure cultures, which could be due to the long-chained and/or highly branched structure of these compounds making it difficult for a single microbial strain to hydrolyse them (Crittenden *et al*., 2002). A few of the products were omitted from published results as the composition of the product made the interpretation of the results difficult: the poor growth of bacterial strains with guar gum samples was probably due to the high viscosity of the compounds, and the commercial GOS (Bi^2muno) contained a large amount of galactose, glucose and lactose (more than 70% in the commercial compound) which were difficult to separate from the product and adversely affected the growth rates. Hydrothermolytically produced HT XOS also inhibited the growth of all tested microbes, although it had similar structure to XOS dp2-10, and so the compound contained some unknown substrates, deriving from the production process, which had anti-microbial properties.

Pure culture studies can assist in understanding the fermentative capacity of individual strains and the mechanisms of polysaccharide break-down within the microbiota (Crittenden *et al.*, 2002). However, as fermentation in the colon is a complex process involving various bacterial enzymes (endo- and exosaccharidases) and cross-feeding between micro-organisms (Jaskari *et al.*, 1998), it is thus evident that studies done with single microbes in pure cultures do not wholly describe prebiotic fermentation in a complex microbial ecosystem *in vivo* in the large intestine (Roberfroid, 2007). FOS and GOS have been demonstrated to quite selectively enhance bifidobacteria in the presence of

complex gut microbiota in several studies (Macfarlane *et al.*, 2008; Rycroft *et al.*, 2001c; Wang and Gibson, 1993) even though in pure culture experiments they were fermented by a variety of tested microbial strains (Hartemink *et al.*, 1997; Jaskari *et al.*, 1998; Kneifel *et al.*, 2000). This probably relates to the fact that although several bacterial strains may be able to utilize the substrate in pure cultures, some of the microbes (such as bifidobacteria) have the high-affinity oligosaccharide transporters and multiple glycolytic enzymes specific for oligosaccharides which enable them to efficiently utilise non-digestible carbohydrates in the competitive environment of the gut (Schell *et al.*, 2002; van den Broek *et al.*, 2008; Vernazza *et al.*, 2006a). It has been demonstrated that when bifidobacteria (*B. bifidum*) and lactobacilli (*L. plantarum*) are co-cultured with pathogens, e.g. *Escerichia coli* and *Campylobacter jejuni*, which are potentially able to utilize the prebiotic in pure culture, the probiotic microbe inhibits the growth of the pathogenic microbes and lowers the pH as a result of the prebiotic fermentation, thus making conditions harder for the potential pathogen to succeed in (Fooks and Gibson, 2002, 2003; Gibson and Wang, 1994b). Consequently, the critical issue in the fermentation of oligosaccharides in the gut is how they are fermented in the presence of complex microbiota. Therefore, the most interesting candidates were chosen after the preliminary screening assays, namely XOS compounds, xylan, panose, FOS, PDX and GOS, for further characterization in the simulated human colon inoculated with human faecal microbiota. Athough sophorose performed well in the screening study, it was not analysed in the colon model as such data on sophorose fermentation already exists (Sanz et al., 2005). The potential of lactitol was also recognised in the pure culture experiments, after which the efficacy of lactitol and *L. acidophilus* NCFM has been investigated using the Enteromix *in vitro* colon model (Mäkivuokko *et al*., 2010) and in a human clinical trial (Ouwehand *et al*., 2009). The results indicate that this synbiotic combination could confer beneficial effects on the composition of the microbiota, its metabolic activity (production of butyrate) and immunity, especially with elderly subjects.

In the colon simulator model, rapid fermentation of commercial XOS Longlive and hardwood-derived XOS dp2 and dp2-10 compounds led to the enhanced growth of bifidobacteria. In the same study (Study III), FOS was shown to significantly enhance *Bifidobacterium* growth, and in contrast to previously published results from *in vitro* colonic fermentation studies (Sghir *et al*., 1998; Zampa *et al*., 2004), decreased *Lactobacillus* growth was also measured. *Bacteroides-Prevotella-Porphyromonas* and *Cl. histolyticum* groups remained unchanged during XOS and FOS fermentation. Interestingly, XOS and FOS seemed to target different species among the bifidobacterial population, as XOS (two of the oligosaccharides with larger dp distribution) significantly enhanced the growth of *B. lactis*, whereas FOS enhanced the *B. longum* species, and

the numbers of these species were significantly different between the substrates. In pure cultures, FOS has been shown to be fermented by *B. longum* strains and XOS by *B. lactis* strains in both this and previous studies (Crittenden *et al*., 2002; Hopkins *et al*., 1998). These *in vitro* studies, however, demonstrated the possibility of prebiotics targeting different species of *Bifidobacterium* in the mixed faecal flora. These results warrant further investigations in the future on the synbiotic efficacy of these combinations.

Xylan, a larger polysaccharide composed of xylose units, also increased the total *Bifidobacterium* numbers but it did not, however, specifically enhance the growth of either the *B. lactis* or *B. longum* species. The other investigated microbial groups also remained unchanged.

Similarly to XOS, panose fermentation (Study II) significantly enhanced the growth of total bifidobacteria and B. *lactis* species but also influenced other investigated bacteria: the significantly reduced growth of *Bacteroides* and a decreased trend of *Clostridium histolyticum* group growth was observed. These studies provided the first data on pure panose fermentation in mixed human faecal microbiota, as only pure culture studies have been previously performed on this substrate (Kohmoto *et al*. 1988).

The fermentation of PDX was studied in two of the experiments; in Study I it was analysed together with xylitol and in Study V together with GOS and probiotic *B. lactis* Bi-07. In both of the studies, a sustained assimilation of PDX was seen as its concentration gradually decreased in the simulator vessels. The complex structure of the PDX molecule was not easily utilized and the molecule was degraded only by a consortium of microbes, since in pure cultures it was very poorly fermented by single bacterial strains. The sustained fermentation of PDX has been demonstrated in humans as well (Hengst *et al*., 2008) and might benefit the health of the colon by increasing saccharolytic-type fermentation distally. Previously, mixed results have been obtained on the bifidogenicity of PDX (Probert *et al*., 2004, Mäkivuokko *et al*., 2005; Tiihonen *et al*., 2008) and in this study PDX did not affect *Bifidobacterium*, *Lactobacillus* or *Bacteroides-Prevotella-Porphyromonas* group numbers in the colonic model, but increased the growth of *Clostridium histolyticum* group (Study V). This group contains potentially pathogenic clostridial strains (*Cl. difficile* and *Cl. perfringens*) and therefore the effects of PDX on the microbiota composition will have to be investigated in more detail in the future. The contadictory results on the bifidogenicity of PDX (as well as other prebiotic substrates) may derive from the fact that an increase in *Bifidobacterium* numbers as a result of successful probiotic or prebiotic intervention can be detected only if the initial levels of these microbes are low; high initial levels tend to remain stable (Kolida *et al.*, 2007; Ouwehand *et al.*, 2006; Rao, 1999; Tuohy *et al.*, 2005). In these simulator studies the levels of bifidobacteria were at moderate levels, as the numbers of total bifidobacteria ranged from $1x10^8$ to $5 x10^8$ copies / ml of

faecal slurry in the simulator vessels. Previously, as reported by Tiihonen and others (2008), low levels of bifidobacteria ($1x10^7$ CFU / g faeces) have been increased after PDX supplementation. Nevertheless, in the same simulator experiments, GOS fermentation increased *Bifidobacterium* levels by one log. GOS also significantly increased *B. lactis* strain densities but other measured microbial groups remained unchanged, even though GOS was generally well-fermented in the pure culture experiments by various microbes. This is in agreement with numerous studies where the bifidogenicity has been demonstrated *in vitro* (Palframan *et al.*, 2002; Rabiu *et al.*, 2001; Sharp *et al.*, 2001), in animal models (Djouzi and Andrieux, 1997; Holma *et al.*, 2002; Tzortzis *et al.*, 2005) and in humans (Depeint *et al.*, 2008; Vulevic *et al.*, 2008).
No conclusion on the effects of xylitol on the gut microbiota can be drawn, based on these *in vitro* studies, as only total numbers of *Bifidobacterium* species and *B. longum* were measured in Study I and those remained unchanged during xylitol fermentation, also when xylitol was combined with PDX.

Unlike prebiotics, synbiotics have not received much attention since their introduction in 1995. Synbiotic products can be combinations of any pro- and prebiotics but the effectiveness of any combination has been questioned recently (Saulnier *et al*., 2007, 2008; de Vrese and Schrezenmeir, 2008); the issue seems to be whether the prebiotic should be selective for the probiotic component and enable them to complement each other in such a way that additional benefits are mediated in comparison with the constituting pro- and prebiotic components. In the last study (Study V), the synbiotic combinations of a probiotic (*B. lactis* Bi-07) and prebiotics (GOS and/or PDX) were also investigated. The probiotic *B. lactis* was naturally shown to increase bifidobacteria and *B. lactis* in the colon model but it also increased *Clostridium histolyticum* group numbers. Synbiotic PDX+*B. lactis* did not change the microbial population any differently than the probiotic *B. lactis* or PDX alone, with the exception that PDX decreased *B. lactis* numbers. Therefore, no additional benefits on the microbiota composition were achieved with this combination. In contrast, synbiotic GOS+*B lactis* increased the levels of *Bifidobacterium* and decreased numbers of *Lactobacillus*, *Bacteroides-Prevotella-Porphyromonas* and *Clostridium histolyticum* groups in comparison to baseline measurements. Furthermore, the composition of the microbiota was altered significantly more with the synbiotic than with the constituting pre- and probiotic components, as increased levels of bifidobacteria and decreased levels of *Bacteroides-Prevotella-Porphyromonas* were measured in relation to *B. lactis* and GOS fermentations alone, respectively. Also the combination of PDX+GOS+*B.lactis* had similar beneficial effects on the microbiota, with the exception that *Bacteroides* levels remained unchanged. Thus, the synbiotic combinations with GOS showed

significant bifidogenic effects on the microbial populations as other microbial groups were depressed.

The metabolism of the microbiota in the colon model was significantly altered by the fermentation of all tested compounds. Most of the investigated prebiotics, in particular the short-chained oligosaccharides XOS, GOS, FOS and panose, significantly increased the levels of both acetate and butyrate during the simulated fermentation; propionate production was increased by polysaccharides, PDX and xylan. Increased butyrate concentration has often been hypothesized to be the mediator of beneficial effects associated with prebiotics and fiber (Hamer *et al*., 2008, 2009), and epidemiological studies show an inverse correlation between dietary fiber intake and the incidence of colorectal cancer (Bingham *et al*., 2003). Nevertheless, although several animal studies support the beneficial effects of butyrate on e.g. colon cancer, the evidence on humans is still inconsistent and inconclusive. Increased acetate and propionate production may also benefit the host, as these substrates are absorbed and utilised systemically in liver and muscle cells. There is some evidence indicating that increased dietary fiber and non-digestible carbohydrates are associated with increased levels of serum acetate, which could affect blood total and HDL cholesterol concentrations (Wolever *et al*., 2002). In previous *in vitro* studies the increased production of acetate and butyrate by FOS has also been demonstrated (Gibson and Wang, 1994b; Rycroft *et al.*, 2001a). In this study high levels of butyrate were measured after XOS, FOS, GOS and panose fermentations, but bifidobacteria, the numbers of which were mainly increased by these substrates, does not produce this fatty acid (Salminen *et al.*, 1998). Therefore, other microbial groups such as *Clostridial* clusters IV and XIV, containing butyrate-producers, which were not measured in this work, might have been affected by the fermentation of the prebiotics resulting in increased levels of butyrate. Alternatively, cross-feeding between different microbial groups could also have contributed to increased levels of butyrate as lactate utilizing microbes have been shown to produce butyrate (Belenguer *et al.*, 2006; Duncan *et al.*, 2004) and lactate levels in the colon simulator were very low or non-existent. The correlations that were found between bifidobacterial numbers and butyrate concentrations further indicate that increased levels of bifidobacteria were accompanied with increases in the activity of butyrate-producing microbes, either through enhanced cross-feeding or the increased numbers of these microbes.

The concentrations of all three BCFAs (isovaleric, 2-methylbutyric and isobutyric acid) were significantly decreased in the whole colon model by most of the prebiotics investigated; only xylan did not have an effect on the BCFA levels and xylitol decreased two of them. BCFAs originate

exclusively from the breakdown of proteins and thus, represent a good marker for protein fermentation in the colon. BCFAs represent some 5 to 10% of the total SCFA produced, and may serve as a substituting energy for the colonocytes if butyrate availability is decreased but they do not, however, seem to have the same effect on the proliferation and differentiation of colonocytes as butyrate (Blachier *et al*., 2007). BCFAs themselves are not toxic substrates but are still indicators of proteolytic fermentation, which can result in the production of potentially toxic metabolites (indolic and phenolic compounds) that have been linked with adverse affects in the colon (Hughes et al., 2000). The production of BCFA *in vitro* in the colon simulator reflected well the *in vivo* situation in the human gut, as increased levels of these metabolites were measured in distal parts of the colon model (Macfarlane *et al*., 1998). However, biogenic amines, those other markers of proteolytic fermentation, behaved differently. The highest concentrations were measured in the first vessels of all simulations and those concentrations decreased towards the distal vessels even though less carbohydrate was present in the simulator medium. This may reflect the decrease also in protein source originating from the inoculum and simulator medium. In the absence of a significant endogenous protein source, many of the tested substrates had only minor effects on biogenic amine production in the whole length of the colon model. GOS was the only substrate that was able to strongly shift the fermentation to the saccharolytic type and almost completely suppress the production of total biogenic amines. Biogenic amines are commonly present in living cells and in the colon. They are either of dietary origin, secreted from gastrointestinal cells or produced by the microbes; and are detoxified by monoamine and diamine oxidases in the gut mucosa and liver (Hughes *et al*., 2000). The exact function of biogenic amines in cells is still unclear, but they are required in large quantities in rapidly growing tissues as they are involved in DNA, RNA, protein and hormone synthesis (Bardócz, 1995). An increase in biogenic amines (e.g. spermine and spermidine) has been positively correlated with the consumption of probiotic bifidobacteria (*B. lactis* LMK512) and has been associated with reduced inflammation (Matsumoto and Benno, 2006), mutagenicity (Matsumoto and Benno, 2004) and improved epithelial growth in colon (Osborne and Seidel, 1989). However, they are also potential precursors for the formation of carcinogenic N-nitroso compounds (Cummings and Macfarlane, 1991) and have linked to neoplastic proliferation in the colon (Linsalata and Russo, 2008). Some biogenic amines (histamine, tyramine) have been related to food spoilage and outbreaks of food poisoning (Silla Santos, 1996) and high levels have even been linked to migraine and hypertension (Hughes *et al*., 2000). Thus, low levels of these amines are required for normal cellular function but too high a level can exert toxic effects (Jacobs *et al*., 2009). Therefore, it is difficult to draw any definite conclusions as to whether the changes in biogenic amines observed with different prebiotics in these simulated colon fermentations were

beneficial or detrimental. As with BCFAs, decreased levels of proteolytic fermentation markers are preferred, especially distally in the colon, which is the predominant site of several gastrointestinal disorders, e.g. ulcerative colitis and colon cancer. It has been hypothesised that low concentrations of SCFA accompanied with increased metabolites of protein fermentation could be involved in the pathogenesis of these diseases (Hamer *et al*., 2008).

The metabolic acitivity of the microbiota was also investigated in different parts of the colon model. Overall, oligosaccharides (GOS, panose, XOS, FOS) that were quickly fermented, mainly affected the acetate and propionate production in the whole length of the model (from vessels V1 or V2 to V4) and suppressed proteolytic fermentation in the proximal and middle part of the model (V1/V2 to V3). The polyalcohol xylitol increased saccharolytic and decreased proteolytic fermentation in the proximal colon (V1 and V2), whereas long-chained PDX, which was still present in the distal part of the colon model, increased the production of all SCFAs and decreased the production of BCFA throughout the colon model (from vessels V1/2 to V4). The other compound with a long-chained structure, xylan, also increased the concentrations of all three SCFAs in the whole model, but the levels of acetate and butyrate were significantly lower than in FOS simulations, and higher levels of BCFA and biogenic amines indicated a poorer fermentation of this substrate by the colonic microbes. GOS increased the production of acetate and propionate in all vessels and very efficiently suppressed the proteolytic fermentation in the proximal and middle parts of the model. The combination of PDX and GOS in the synbiotic product resulted in the high production of all SCFAs and decreased production of BCFA, also in the distal part of the model, even though the concentrations of both prebiotics was 50% lower than in the prebiotic fermentation simulations. The significant correlations between bifidobacteria and acetate and butyrate, and the inverse correlation with total biogenic amines and all three BCFA concentrations indicates that increased levels of *Bifidobacterium* species are specifically linked with increased saccharolytic-type fermentation. On the other hand, the other measured microbial groups, *Lactobacillus*, *Cl. histolyticum* and *Bacteroides-Prevotella-Porphyromonas*, have a role in the production of proteolytic metabolites, as significant correlations between the increased numbers of these microbes and BCFA and/or biogenic amines were demonstrated. The production of biogenic amines by a few *Lactobacillus* strains has been demonstrated previously (Bover-Sid and Holzapfel, 1999).

The purity and good quality of samples used in *in vitro* tests is essential to obtain reliable results. As seen with some of the samples tested, other saccharides and impurities (mono-and oligosaccharides in GOS Bi^{2}muno and unknown compounds in the HT XOS compound) may have affected the outcome of the study in such a manner that the results are not describing the fermentation of the

prebiotic candidate itself, but are instead due to impurities. In addition, the chemical properties of the product can make the testing of the sample impossible *in vitro;* for instance, the high viscosity of guar gum samples made reliable testing of these products impossible *in vitro*. It has to be noted as well that the commercial XOS Longlive also contained some monosaccharides (13.5% of it consisted of glucose, arabinose and galactose) which were difficult to separate from the product. Rarely are the commercial oligosaccharides pure (Rycroft *et al*., 2001a) and thus the commercial FOS also included 5% of other oligosaccharides. In human consumption, these monosaccharides are absorbed in the upper gastrointestinal tract and do not reach the colon. *In vitro*, however, these monosaccharides are present in the growth broth and are available as substrates for the tested microbes (Crittenden *et al*., 2002). It is therefore possible that part of the growth observed with Longlive XOS and FOS was due to the microbial utilization of the contaminating monosaccharides and not due to consumption of oligosaccharides.

According to the modified definition of prebiotics (Roberfroid, 2007), a health effect must be demonstrated in conjunction with the prebiotic consumption. Due to the incomplete understanding of the "normal" microbiota composition and activity on a population basis, it is difficult to deduce concrete preventive or curative effects from changes in bacterial cell counts or activities only, even if those (such as bifidogenicity or SCFA production) are considered beneficial. Therefore, conclusive proof of the health effects and improvements in clinical endpoints mediated by prebiotic consumption should be demonstrated in controlled human intervention studies. In the future, the potential prebiotics and synbiotic combinations that were identified in this thesis work will be assessed in more detail in humans. A few of the products, namely XOS (Longlive) and *B. lactis* (Bi-07); GOS and *B. lactis* (Bi-07); and PDX, have already entered the human intervention phase to investigate their prebiotic and synbiotic properties in feeding trials. It would also be interesting to study the novel prebiotic candidate, panose, in the human intervention phase to further assess the potential prebiotic properties seen *in vitro*. However, first the indigestibility or low digestibility of pure panose in the upper gastrointestinal tract should be confirmed, since only data on IMO mixtures exist to date. Furthermore, a novel cost-effective production method is needed for the production of panose as the current high price of the product will make it a commercially unprofitable emerging candidate.

With regard to the Enteromix colon model, an upgrade on the current microbiota analysis is needed in the future to meet the high level of microbiological research today. Accumulating evidence on the newly identified bacterial groups and dominant species in the gut highlights the need to expand the analysis of the microbiota to also include other microbial groups in addition to the traditional

bifidobacteria, lactobacillus, *Bacteroides* and clostridia. For instance, numbers of butyrate-producing genera, such as *Eubacterium, Roseburia* and *Faecalibacterium* and also other major components (e.g. dominating clostridial clusters) of the microbiota should be investigated in order to be able to draw conclusions on the selective nature of prebiotic fermentation. Furthermore, validation of the Enteromix colon simulator against *in vivo* human results is needed and can be undertaken when results from the ongoing human interventions are obtained.

6. CONCLUSIONS

The first goal of this thesis work was to screen a large group of potential and interesting prebiotic product candidates for their fermentation properties by colonic microbes. This was done with a simple *in vitro* screening tool which enabled the identification of several interesting candidates, such as xylo-oligosaccharides, lactitol and panose.

The second goal of the study was to further characterize the prebiotic potential of these compounds in a model mimicking the human large intestine, in which fermentation of these compounds by the colonic microbiota was assessed. It was shown *in vitro* that XOS compounds with varying degree of polymerization were fermented particularly well by probiotic and endogenous *B. lactis* in the colonic microbiota, without affecting the other investigated microbial groups. Furthermore, a novel prebiotic candidate, panose, was identified. The fermentation of pure panose was characterized for the first time by human faecal microbiota, and it was shown to have bifidogenic properties *in vitro* and to decrease the growth of *Bacteroides-Prevotella-Porphyromonas* group. Similarly to XOS, panose also increased the endogenous *B. lactis* growth in the colon model. The microbiota functionality was also affected by both compounds and the production of fermentation metabolites was altered in a manner considered beneficial; saccharolytic-type fermentation increased at the expense of proteolytic fermentation.

The third goal of the study was to compare the candidate compounds with established prebiotics. XOS and commercial FOS were analysed in the same colonic fermentation studies, and it was shown that these two oligosaccharides target different *Bifidobacterium* species; XOS was superior in terms of increasing the growth of *B. lactis* while FOS increased the levels of *B. longum*, which was unaffected by XOS. This finding differentiates these two oligosaccharides from each other and warrants further studies in the search of species-specific synbiotic products. Another comparison was made between the slowly-fermented PDX and the more rapidly metabolized GOS; the latter being superior in its modulatory capabilities on microbiota composition in these studies.

The final aim of the study was to assess the properties of potential synbiotic product combinations. The prebiotic GOS and probiotic *B. lactis* were combined and they were shown to together significantly influence the composition of the microbiota more than the constituting components alone. The inclusion of PDX into this synbiotic further modified the activity of the microbial population by also decreasing the production of proteolytic metabolites in the distal part of the colon model. As the fermentation of GOS increased also the endogenous *B. lactis* growth, the synbiotic combination could prove to be very effective.

In vitro models are useful tools in studying the prebiotic fermentation in the colon. However, the final proof of the health effects and benefits mediated by prebiotic consumption should be demonstrated in controlled human intervention studies. A few of the products identified in these *in vitro* studies, namely XOS (Longlive) and *B. lactis* (Bi-07); GOS and *B. lactis* (Bi-07); and PDX, have already entered the human intervention phase to investigate their prebiotic and synbiotic properties in humans in more detail.

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