Role of Intestinal Microbiota in Ulcerative Colitis – Effects of Novel Carbohydrate Preparations

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Louise Kristine Vigsnæs
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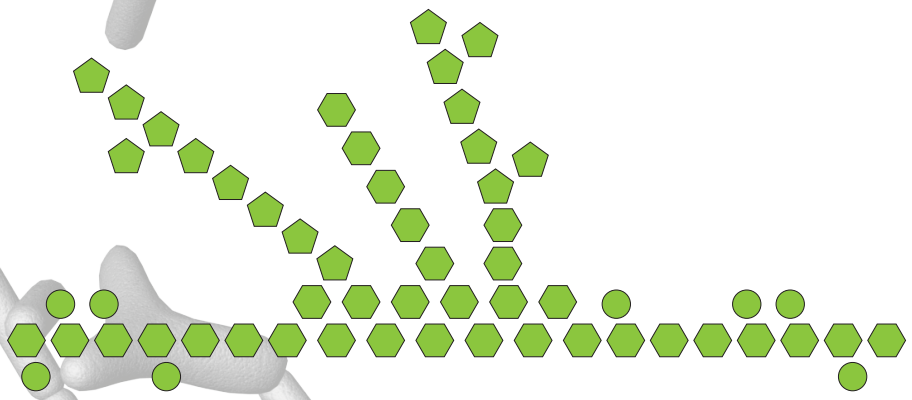

DTU Food
National Food Institute

Technical University
of Denmark

It is the microbes who will have the last word

- *Louis Pasteur*

Preface

This thesis presents the studies carried out at Bioscience and Technology, National Laboratory for Sustainable Energy, Technical University of Denmark (DTU) during the years 2007-2008 under the supervision of Associated Professor Peter Westermann and Professor Hanne Frøkiær and from the years 2008-2011 at the Division of Microbiology and Risk Assessment, National Food Institute, DTU under the supervision of Professor Tine Rask Licht.

The dynamic *in vitro* gut model experiments were carried out at Laboratory of Microbial Ecology and Technology (LabMET), Ghent University, Belgium.

The study was supported by the Danish Strategic Research Council's Committee on Food and Health, FøSu, Center for Biological Production of Dietary Fibers and Prebiotics, no. 2101-06-0067, and DTU.

The present thesis is submitted towards fulfilling the requirements for obtaining the degree of Ph.D at DTU.

Acknowledgment

This thesis would not have been possible without the support of many people.

First, I deeply thank my supervisor, Professor Tine Rask Licht, whose support, inspiration and enthusiastic guidance through my years of research was invaluable. Thank you for understanding me.

I would also like to thank Associated Professor Peter Westermann and Professor Hanne Frøkiær for their support and engagement during the initial year of my research.

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I am grateful to all my current and former colleagues at the "Gut Ecology" group for creating a supportive and pleasant working atmosphere and for the nice moments we have shared. A special thanks go to the technicians Kate Vibefeldt and Bodil Madsen for their help through the experimental part of the study.

Also a deep and sincerely thanks to the girls from "Office 007." Thank you for our scientific discussions, shared laughs and for listening to me through difficult times.

My warm thanks go to my parents and my fantastic sisters, Charlotte and Henriette. You have always believed in me, helped and supported me in every way possible. Thank you because you are there for me.

I also wish to thank my nearest friends for happy moments and being there when needed.

Last, but defiantly not least, I wish to express my deepest love and gratitude to my wonderful husband, Helge and my amazing son, Valder for their understanding, patience, support and endless love. Thank you Helge for loving me for who I am.

October 2011

Louise K. Vigsnæs

Summary

The microbiota of the human intestinal tract is complex with variable populations of bacteria who are either permanent gut residents (commensal bacteria) or transient inhabitants introduced from the environment. The commensal bacteria are believed to be important for human health due to actions such as protection against pathogens, induction of immune regulatory functions and nutrient processing. Hence, the composition of commensal bacteria is important to preserve colonic health.

Ulcerative colitis (UC) is an inflammatory bowel disease and dysbiosis in the composition of commensals has been reported, which could affect colonic health. In the experimental part of this thesis, the fecal microbiota derived from UC patients in either remission or with active disease and healthy subjects was quantified using quantitative Real-Time PCR (qPCR) to examine the microbiota composition. The results demonstrated that the microbiota composition was different in UC patients in relapse compared to healthy subjects and the difference could be ascribed Gram-negative bacteria, hence indicating that an altered microbiota composition is associated with colonic inflammation. Additionally, results revealed that the microbiota composition in remission either resembled the composition in healthy or in relapse, demonstrating that the microbiota in remission is unstable.

The mucus layer lining the epithelium of the intestinal tract is important for the protection of the epithelium in humans. The commensal bacteria that colonize the colonic mucus are suggested to play an important role in stimulating regulatory immune responses compared to luminal bacteria, since they reside closer to the intestinal epithelial cells. The ability of fecal microbiota derived from healthy subjects and UC patients to colonize mucus was examined in a study of this thesis to elucidate, if the adhesion capacity is different depending on disease state. For this purpose, an *in vitro* dynamic gut model was used. Several bacterial taxa from both lumen and mucus were quantified using qPCR. The results revealed that the bacterial community of the mucus differed from that of the lumen and that lactobacilli and bifidobacteria derived from UC patients had a significant decreased capacity to colonize mucus than observed for similar bacterial groups originating from healthy subjects. This suggests that the inflammatory state in UC may influence the adhesion capacity of commensal bacteria such as beneficial Gram-positive bacteria lactobacilli and bifidobacteria.

Maintenance of a benign gut microbial composition may help decrease risk of mucosal inflammation in UC. This could be achieved by the consumption of prebiotics (include non-digestible carbohydrates), which are defined as "*selectively fermented ingredients that cause specific changes in composition and/or activity in the gastointestinal microbiota, which confer benefits upon host well-being and health*."

In the experimental part of this thesis, *in vitro* fermentation studies were performed using fecal samples from human volunteers either healthy and/or UC patients to investigate prebiotic properties of novel carbohydrate preparations. The results revealed that incubation with arabino-oligosaccharides (DP2-10) from sugar beet pulp could selectively stimulate fecal lactobacilli and bifidobacteria from UC patients. This may suggest protective properties of arabino-oligosaccharides against UC. In addition, *in vitro* fermentation studies demonstrated that high molecular weight fractions of galactose or arabinose chains either from potato or sugar beet pulp, respectively, selectively stimulated the growth of fecal bifidobacteria derived from healthy subjects. The prebiotic properties demonstrated by the high molecular weight carbohydrate preparations *in vitro* could be of importance *in vivo*, since long-chain carbohydrates may be fermented slowly by beneficial colonic bacteria, thus penetrating prebiotic effect all the way throughout the colon.

Dansk sammendrag

Menneskets tarmmikrobiota er komplekst og består af mange forskellige bakterie populationer, som enten er en del af den faste tarmbestand (kommensale bakterier) eller flygtige mikrober tilført fra kosten. De kommensale bakterier menes at have stor betydning for menneskets sundhed. Dette på grund af deres evne til at forhindre kolonisering af patogene bakterier, til at stimulere immunregulerende funktioner og til at generere energi fra ufordøjelige kostfibre. Sammensætningen af kommensale bakterier er derfor vigtig for at opretholde en sund tarm. Colitis ulcerosa (UC) er en inflammatorisk tarmsygdom, og det er tidligere blevet beskrevet, at UC patienter har en anderledes bakteriesammensætning i tarmen. Denne ændrede sammensætning menes at kunne have konsekvenser for tarmens sundhed. I den eksperimentelle del af denne afhandling er den fækale mikrobiota fra UC patienter og raske personer kvantificeret vha. kvantitative Real-Time PCR (qPCR) for at undersøge sammensætningen af forskellige bakteriegrupper fra UC patienter og raske personer. Resultaterne viste, at bakteriesammensætningen i UC patienter med aktiv colitis var forskellig fra raske personer og forskellen kunne tilskrives Gram-negative bakterier. Dette kunne tyde på, at en ændret bakteriesammensætning kan linkes til inflammation i tarmen. Forsøget viste ligeledes, at bakteriesammensætningen i UC patienter med inaktiv colitis enten var sammenlignelig med raske eller UC patienter med aktiv colitis. Dette kunne indikere, at UC patienter med inaktiv colitis har en ustabil tarmmikrobiota.

Epitelet i mave-tarm kanalen er dækket af et slimlag (mucus), hvilket er vigtigt for at beskytte tarmcellerne mod infektion. Mucus er beboet af kommensale bakterier, som anses for at spille en vigtig rolle mht. stimulering af immunrespons i forhold til luminale bakterier, da de er lokaliseret tættere på tarmceller.

I et studie af denne afhandling blev det undersøgt, om der er en forskel på fækale bakteriers evne til at kolonisere mucus, alt efter om de kommer fra UC patienter eller raske personer. En dynamisk *in vitro* tarmmodel blev anvendt for at belyse dette. Forskellige bakteriegrupper fra både lumen og mucus blev kvantificeret vha. qPCR og resultaterne viste, at de bakterier, som kunne kolonisere mucus adskilte sig fra dem fra lumen. Ligeledes viste resultaterne, at lactobacillus- og bifidobakterier fra UC patienter med aktiv colitis havde en signifikant lavere evne til at kolonisere mucus end lignende bakterier fra raske personer. Dette kunne tyde på, at bakteriers evne til at

adhedere er påvirket af den inflammatoriske tilstand i UC, hvilket især har indflydelse på gavnlige Gram-positive bakterier som lactobacillus- og bifidobakterier.

Opretholdelse af en gavnlig bakteriesammensætning i tarmen menes at kunne være med til at reducere risikoen for tilbagefald hos UC patienter. Indtagelse af præbiotika har vist at medføre en sundhedsfremmende ændring af tarmens bakterier. Præbiotika er "*en selektivt fermenteret fødevarekomponent, som giver specifikke ændringer i sammensætningen og/eller i aktiviteten af mavetarmkanalens mikrobiota, og herved medfører gavnlig effekt på værtens sundhed.*" *In vitro* fermenteringsforsøg blev udført i den eksperimentelle del af afhandlingen, hvor potentielle kulhydratpræparater blev undersøgt for præbiotiske egenskaber. Fækale prøver fra raske personer og/eller UC patienter blev anvendt som bakteriel inokula. Resultaterne fra forsøgene viste, at arabino-oligosakkarider (DP2-10) fra sukkerroe pulp kan stimulere fækale lactobacillus- og bifidobakterier fra UC patienter. Dette kunne tyde på, at arabino-oligosakkarider (DP2-10) kan fremme en gavnlig bakteriesammensætning i UC patienter. Fermenteringsforsøgene viste ligeledes, at galaktose eller arabinose kæder med høj molekyle vægt fra henholdsvis kartoffel eller sukkerroe pulp kunne stimulere væksten af fækale bifidobakterier fra raske personer. Kulhydrater af lange kæder kan have en gavnlig effekt *in vivo,* da disse fermenteres langsomt og derved ville være i stand til at stimulere væksten af sundhedsfremmende bakterier - ikke kun i den proximale del af tyktarmen, men også den distale.

Introduction and objectives

Since the beginning of the last century from the early work of Elie Metchnikoff, it is believed that a synergistic interaction exists between intestinal microbes and their host.

Metchnikoff observed that:

"*… different susceptibilities of people to the harmful action of microbes and their products. Some can swallow without any evil result a quantity of microbes which in the case of other individuals would produce a fatal attack of cholera. Everything depends upon the resistance offered to the microbes by the invaded organism*" (Metchnikoff, 1908).

Hence, an unfavorable composition of the gut microbiota could play a role in intestinal infections and diseases. Consumption of indigestible carbohydrates or dietary fibers is suggested to improve gut health by modulating the gut environment through selective stimulation of bacteria who are able to promote beneficial events in the host by inhibition of pathogenic bacteria, and induction of immune regulatory functions (Vernazza *et al.*, 2006).

The objectives of this thesis were to investigate, if the gut microbiota and its ability to colonize mucus are different in patients suffering from the intestinal bowel disease, ulcerative colitis (UC) than in healthy subjects, hence playing a role in colonic inflammation. Additionally, investigate the ability of novel indigestible carbohydrates or fibers from sugar beet and potato pulp to induce beneficial changes in fecal microbiota communities *in vitro* obtained from human volunteers both healthy and UC patients.

The first part of this thesis gives a theoretical introduction to the phylogenetic composition of the human intestinal microbiota, the location of the microbiota within the colon and their interactions with the host. Additionally, UC and the colonic microbiota composition observed in UC patients will be described. Finally, modulation of the intestinal microbiota by prebiotics will be addressed together with a description of the production of prebiotics and novel carbohydrates. The second part contains a description of the methodologies used in the studies of this thesis and six papers that demonstrate the experimental work. The last part is discussion and conclusion based on the theoretical and experimental work of this thesis.

List of manuscripts

Paper 1

Vigsnæs, L.K., Brynskov, J., Wilcks, A., and Licht, T.R. Gram-negative bacteria account for differences in fecal microbiota between patients with ulcerative colitis and healthy controls. (2011) Submitted to BMC Microbiology, under review.

Paper 2

Vigsnaes, L.K., Van den Abbeele, P., Van de Wiele, T. and Licht, T.R. Fecal lactobacilli and bifidobacteria from ulcerative colitis patients display reduced ability to colonize mucus in the M-SHIME. (2011) Paper in preparation.

Paper 3

Vigsnæs, L.K., Holck, J., Meyer, A.S, and Licht, T.R. (2011) *In vitro* Fermentation of Sugar Beet Arabino-Oligosaccharides by Fecal Microbiota Obtained from Patients with Ulcerative Colitis Selectively Stimulates the Growth of *Bifidobacterium* spp. and *Lactobacillus* spp. *Applied and Environmental Microbiology*. 77:8336-8344

Paper 4

Holck, J., Lorentzen, A., **Vigsnæs, L.K.**, Licht, T.R, Mikkelsen, J.D. and Meyer, A.S. (2011) Feruloylated and non-feruloylated arabino-oligosaccharides from sugar beet pectin selectively stimulates the growth of *Bifidobacterium* spp. in human fecal *in vitro* fermentation. *Journal of Agricultural and Food Chemistry*. 59:6511-6519.

Paper 5

Thomassen, L.V., **Vigsnæs, L.K.**, Licht, T.R., Mikkelsen, J.D., and Meyer, A.S. (2011) Maximal release of highly bifidogenic soluble dietary fibers from industrial potato pulp by minimal enzymatic treatment. *Applied Microbiology and Biotechnology*. 90:873-884.

Paper 6

Holck, J., Hjernø, K., Lorentzen, A., **Vigsnæs, L.K.**, Hemmingsen.L., Licht, T.R., Mikkelsen, J.D., Meyer, A.S. (2011) Tailored enzymatic production of oligosaccharides from sugar beet pectin and evidence of differential effects of a single DP chain length difference human fecal microbiota composition after *in vitro* fermentation. *Process Biochemistry*. 46 :1039-1049.

Not included in this thesis

Bergström, A., Licht, T.R., Wilcks, A., Grønlund, H., Andersen, J.B., **Vigsnæs, L.K.**, and Bahl, M. The Gut Low Density Array 'GULDA' – A validated approach to rapid quantification of the key bacterial taxa present in intestinal samples. (2012) Paper in preparation.

Vigsnæs, L.K., Nakai, H., Hemmingsen, L., Lahtinen, S., Rasmussen, L.E., Hachem, M.A., Meyer, A.S, Licht' T.R., Svensson, B. Selective *in vitro* growth of individual human gut bacteria on potential prebiotic oligosaccharides produced by chemoenzymatic synthesis. (2012) Paper in preparation.

Vermeiren, J., Van den Abbeele, P., Laukens, D., **Vigsnæs, L.K.**, Vos, M.D., Boon, N., and Van de Wiele, T. (2011) Decreased colonization of *Clostridium* cluster XIVa in an *in vitro* dynamic gut model during incubation of the fecal microbiota from ulcerative colitis patients. *FEMS Microbiology Ecology*. DOI: 10.1111/j.1574-6941.2011.01252.x

Abbreviations

AG	Arabinogalactans
AOS	Arabino-oligosaccharides
CD	Crohn's disease
DBRPC	Double-blind randomized placebo controlled
DCs	Dendritic cells
DP	Degree of polymerization
DSS	Dextran sulfate sodium
FOS	Fructo-oligosaccharides
GalA	Galacturonic acid
GBF	Germinated barley foodstuff
GH	Glycoside-hydrolases
GI tract	Gastrointestinal tract
GMO	Oligosaccharides from goat milk
GOS	Galacto-oligosaccharides
HG	Homogalacturonan
IBD	Inflammatory bowel disease
IECs	Intestinal epithelial cells
IgA	Immunoglobulin A
IL	Interleukin
LPS	Lipopolysaccharides
LTA	Lipoteichoic acids
MAMPs	Microorganism-associated molecular patterns
NF-κB	Nuclear factor kappa B
OFI	Oligofructose-enriched inulin
PAMPs	Pathogen-associated molecular patterns
PG	Peptidoglycans
PRRs	Pattern-recognition receptors
PCA	Principal Component Analysis
qPCR	Quantitative Real-Time PCR
RGI	Rhamnogalacturonan I
RGII	Rhamnogalacturonan II
RPC	Randomized placebo controlled
SCFAs	Short-chain fatty acids
SCID	Severe combined immune deficiency
SHIME	Simulator of the human intestinal microbial ecosystem
SPF	Pathogen free conditions
TLRs	Toll-like receptors
TNBS	Trinitrobenzene sulfonic acid
TNF-α	Tumor Necrosis Factor-alfa
Treg cells	Regulatory T cells
UC	Ulcerative colitis
XOS	Xylo-oligosaccharides

List of contents

Theoretical part

1. The intestinal environment

The gastrointestinal (GI) tract of a fetal is sterile, but it is inoculated with both aerobic and anaerobic microorganisms shortly after birth from the mother's vagina and feces, and from the environment (Collins and Gibson, 1999). The microbial colonization of the GI tract of an infant the first year of life is influenced by milk-feeding and weaning. After weaning, children start to develop a more stable microbiota profile similar to adults (Vernazza *et al.*, 2006). The microbiota of the adult human GI tract is a complex and very dynamic microbial ecosystem, consisting of various bacterial populations, which are either permanent gut residents (including commensal bacteria) or transient inhabitants introduced from the diet or environment (Ventura *et al.*, 2009). In the following chapter, the phylogenetic composition of the intestinal microbiota in adult humans will be addressed together with the function and physiology of the large intestine.

1.1. The diversity of the intestinal microbiota

It has been estimated that the intestine harbors 10^{13} to 10^{14} microorganisms (Gill *et al.*, 2006), which means that the bacteria cells in the intestine are ten times more numerous than the total number of human body cells (Palmer *et al.*, 2007). Additionally, the collective genome of the microbiota, the microbiome, contains at least 100-fold more genes than the complete human genome (Gill *et al.*, 2006;Ventura *et al.*, 2009). This complex intestinal microbiota is considered to serve numerous important functions for its human host, including protection against pathogens (Tlaskalova-Hogenova *et al.*, 2004;Endt *et al.*, 2010;Jarchum and Pamer, 2011), induction of immune regulatory functions (Round *et al.*, 2011;Atarashi *et al.*, 2011), nutrient processing (Vander *et al.*, 1998;Vernazza *et al.*, 2006), and metabolic functions (Wikoff *et al.*, 2009). Despite the important role the intestinal microbiota plays on human health, little is still known with respect to the microbial composition, and species diversity. The main lack of knowledge could be due to the cultivation insensitivity, because only a small fraction (20-40%) of the intestinal species is accessible through culture-based techniques (Eckburg *et al.*, 2005;Qin *et al.*, 2010). However, recently developed culture-independent studies based on 16S rRNA gene sequences have helped to evaluate the composition of the human adult intestinal microbiota (Rajilic-Stojanovic *et al.*, 2007;Ventura *et al.*, 2009).

Metagenomic studies have revealed that the majority of gut microbiota sequences belong to the Bacteria, reflecting their predominance in the human adult gut (Eckburg *et al.*, 2005;Qin *et al.*, 2010;Arumugam *et al.*, 2011). Despite the complexity of the human intestinal ecosystem, the majority of the bacteria are members of only a limited number of dominating bacterial phyla, such as *Bacteroidetes*, *Firmicutes*, *Actinobacteria, Proteobacteria*, *Verrucomicrobia*, and *Fusobacteria* with *Firmicutes,* and *Bacteroidetes* being the most abundant phyla (Figure 1)(Eckburg *et al.*, 2005;Tap *et al.*, 2009;Arumugam *et al.*, 2011). Within the *Firmicutes* phylum, 95% of the phylogenetic types are members of the *Clostridia* class, and of these a substantial number are related to butyrate-producing bacteria, all of which fall within the clostridial clusters IV, XIVa, and XVI (Eckburg *et al.*, 2005;Tap *et al.*, 2009). At genus level, *Bacteroides* has shown to be the most abundant genus in the human gut microbiota of adults, followed by the genera *Faecalibacterium*, *Bifidobacterium, Lachnospiraceae*, *Roseburia,* and *Alistipes* (Arumugam *et al.*, 2011). Another genus normally detected in the human gut is *Lactobacillus*, although only present in low levels depending on age and individuals (Mueller *et al.*, 2006;Frank *et al.*, 2007).

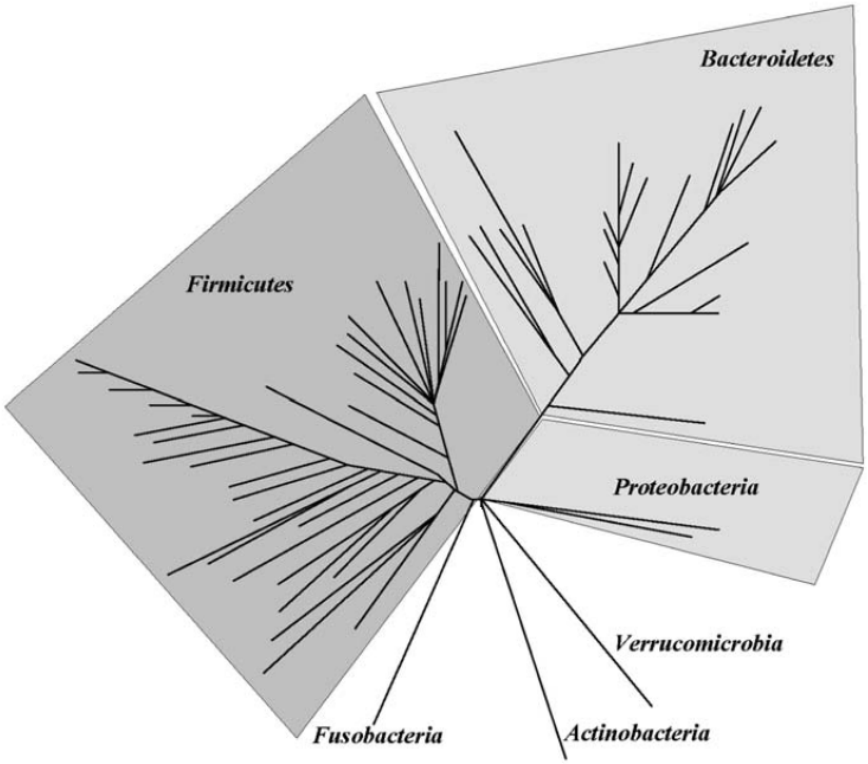

Figure 1: The microbial diversity of the main phylotypes in the human intestinal microbiota (Turroni *et al.*, 2009).

1.2. Function and physiology of the large intestine

The main functions of the large intestine are food storage, absorption of water and electrolytes, and digestion of indigestible carbohydrates by the colonic microbiota (Vander *et al.*, 1998). Anatomically, the large intestine consists of the cecum, ascending colon, transverse colon, descending colon, sigmoid colon, and rectum (Guarner and Malagelada, 2003). The ascending colon is a saccharolytic environment where most bacterial metabolic activity and carbohydrate fermentation occur. The pH of the ascending colon is generally lower (approximately 5-6) than that of the distal colon. The reduced pH is considered to be the result of carbohydrate fermentation, which gives rise to the production of Short-Chain Fatty Acids (SCFAs) (Macfarlane *et al.*, 1992). Consequently, the carbohydrate availability decreases in the descending colon, which leads to a pH close to neutral. The rate of bacterial metabolism is lower, and protein and amino acids become a more dominant metabolic energy source for bacteria (Figure 2A) (Guarner and Malagelada, 2003;Vernazza *et al.*, 2006). Anaerobic fermentation of proteins by the microbiota produces branched SCFAs, however, it also generates a series of potentially toxic compounds such as ammonia, amines, and phenolic compounds (Vernazza *et al.*, 2006).

The wall of the colon consists of four tissue compartments (Figure 2B): mucosa, submucosa, muscularis externa, and serosa. The mucosa consists of a mucus layer, single layer of epithelium, the lamina propria, and a thin muscle layer (muscularis mucosae). The epithelium covering the mucosa consists of different types of cells, namely goblet cells (mucin secreting cells), enterocytes (absorptive cells), and endocrine cells (hormone secreting cells). These cells are linked together along the edges of their luminal surface by tight junctions. The submucosa is a connective tissue supporting the mucosa with blood vessels, lymphatic vessels, and nerves. The muscularis externa consists of two muscle layers: the longitudinal and the smooth muscle layer. Between the two muscle layers is a network of nerves (the myenteric nerve plexus). The serosa is the outer connective tissue layer, which connects the colon to the abdominal cavity (Vander *et al.*, 1998).

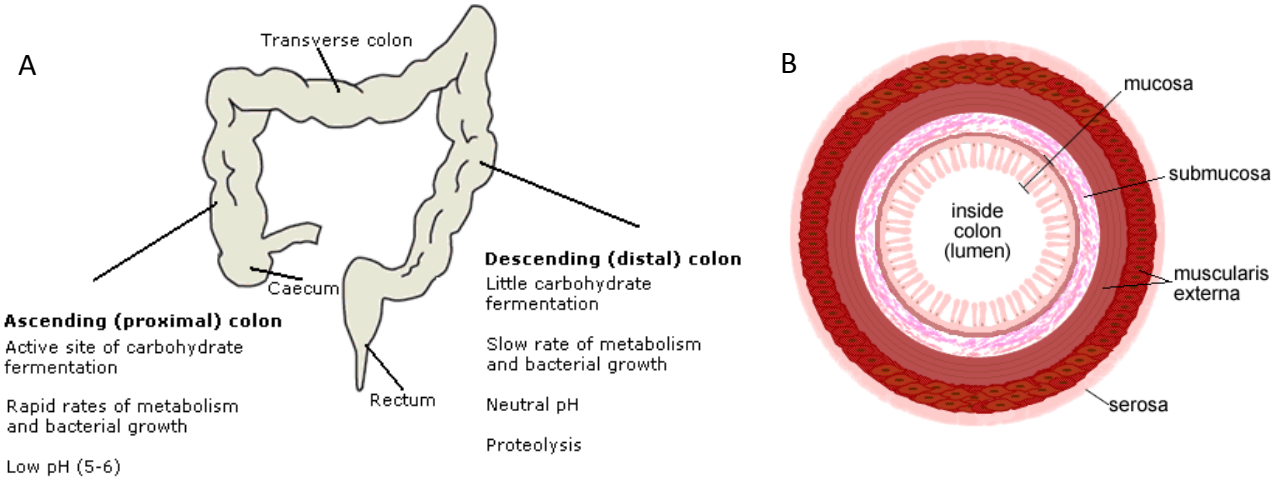

Figure 2: A) Anatomy of the colon with fermentation sites and metabolic activity. Adapted from Vernazza *et al.* (2006) and B) Cross-section of the colon wall illustrating lumen, mucosa, submucosa, muscolaris externa and serosa (Myers, 2007).

2. The colonic environment

The commensal bacteria who inhabit the colon are either located in the lumen or in the mucus. They are believed to play an important role in their specific environment, although it is not fully understood what selects the mucosal and luminal bacterial communities. A recent review by Van den Abbeele *et al.* (2011b) has suggested following hypothesis:

"*Microorganisms who are not targeted by host defense molecules upon colonization of the mucus layer, are able to reside in the mucosa associated microbial community, and commensals who are preferentially targeted by host defense molecules in the mucus layer, are restricted to the lumen*".

A description of the mucosal and luminal environment will be given in the following chapter and in addition, the role of the two microbial communities and how they interact with the host will be addressed.

2.1. The mucosal environment

The mucus layer covering the colonic epithelium results in a physical network that separates the lumen intestinal residents and pathogens from the host. The mucus layer can respond dynamically to infection, and is regulated by the underlying innate and adaptive immune system (McGuckin *et al.*, 2011). The colonic mucus constitutes a complex fluid, which is rich in gel-forming mucins. The mucins are large glycoproteins characterized by abundant and variable *O*-linked glycans attached to hydroxy amino acids clustered in PTS domains (high frequency of the amino acids proline, threonine, and serine). Sulfate residues and O-acetyl-substituted sialic acid can be found on the terminal oligosaccharides of the glycans (Lang *et al.*, 2007;Johansson *et al.*, 2011;McGuckin *et al.*, 2011). The glycans are an important energy source for mucosal bacteria and are used as adhesion sites for bacteria (Hansson and Johansson, 2010;Johansson *et al.*, 2011). However, they also serve a different role. They protect the protein core from proteases, preserving the integrity of the mucin polymer and due to their complex structure, decrease the number of bacteria able to grow on mucin (Moncada *et al.*, 2003).

The thickness of the colonic mucus is 700 µm and consists of two layers. The thin inner layer is a firmly adherent gel, which is physically difficult to dislodge and devoid of bacteria (Johansson *et al.*, 2008;Hansson and Johansson, 2010). The thick outer layer, on the other hand, is a loosely adherent gel, which is more easily dispersed, colonized and degraded by bacteria (Atuma *et al.*,

2001;Johansson *et al.*, 2008). The mucus gel also provides a matrix for the retention of anti-microbial molecules and secretory immunoglobulin A (IgA). The antimicrobial peptides are produced by the colonic epithelial cells, and include among others β-defensins and cathelicidin LL37 (Hase *et al.*, 2002;Tollin *et al.*, 2003), and the secretory IgA is synthesized by plasma cells in the lamina propria.

The commensal bacteria colonizing the outer mucus layer have evolved different microbial characteristics, which contribute to a specific selected mucosal community. Firstly, the bacteria have to carry adhesion molecules to bind to the mucus (Laparra and Sanz, 2009;Kankainen *et al.*, 2009;Johansson *et al.*, 2011). Cell surface components that promote the adherence of commensal bacteria to mucus include among others; fimbriae (Schell *et al.*, 2002;Kline *et al.*, 2009;Kankainen *et al.*, 2009), elongation factor Tu (EF-TU) (Granato *et al.*, 2004;Gilad *et al.*, 2011), extracellular mucus-binding protein (Mub, found in *Lactobacillus* spp.) (Roos and Jonsson, 2002) and lectin-like mannosespecific adhesin (Msa, found in *Lactobacillus* spp.) (Pretzer *et al.*, 2005). Secondly, the bacteria have to have the ability to gain nutrients from the host-derived mucins by synthesizing mucin-degrading enzymes. This often involves the combined action of bacterial proteases, glycosidases, sialidases and sulphatases, due to the complex structure of mucin (Killer and Marounek, 2011). A variety of bacteria are able to produce one or more of the mucin-degrading enzymes, which include *Akkermansia muciniphila* and species of *Bifidobacterium*, *Bacteroides, Prevotella*, and *Ruminococcus* (Bayliss and Houston, 1984;Wright *et al.*, 2000;Derrien *et al.*, 2004;Killer and Marounek, 2011). Thirdly, the bacteria have to be able to survive in the presence of an oxygen gradient along the mucus layer, as oxygen is continuously released from the blood (Van den Abbeele *et al.*, 2011b). Finally, the bacteria have to be resistant to antimicrobial peptides. Species of *Bacteroides* have shown to be resistant to several host defensins allowing their colonization of the outer layer mucus (Nuding *et al.*, 2009). Moreover, it has been suggested that the glycosylation pattern in mucins is an important factor for host selection of a specific colonic mucosal community (Hansson and Johansson, 2010). Recent study has shown that the glycosylation pattern is complex but relatively conserved in the human colon compared to other regions in the GI tract, hence specific adhesion molecules and mucin-degrading enzymes are needed suggesting selective advantages of the colonic commensal mucosal community (Larsson *et al.*, 2009).

2.2. The luminal environment

The high content and the long transit time of nutrients in the colon allow a sufficient reproduction rate and avoid wash out of the luminal bacteria; hence a high number of bacteria are able to colonize the lumen (10^{10} - 10^{11} CFU/ ml). These bacteria are either facultative or strict anaerobic and live in mutual relationship with their host helping extract energy by digesting indigestible polysaccharides (Holzapfel *et al.*, 1998;Backhed *et al.*, 2005). The range of indigestible dietary carbohydrates that reaches the colon include polysaccharides from components of plant cell walls (including cellulose, xylan, and pectin), as well as storage polysaccharides such as inulin and resistant starch (Hooper *et al.*, 2002). Recent metagenomic studies have revealed that the microbiome in the human gut is one of the richest sources of carbohydrate active enzymes with at least 81 families of glycoside-hydrolases (GH) with endo- and exo-activities (Gill *et al.*, 2006;Turnbaugh *et al.*, 2010). Of these families, GH13 (20%), GH3 (11%), GH2 (9%), GH1 (7%), and GH31 (5%) are the most abundant in the gut microbiome of human beings (Gill *et al.*, 2006;Li *et al.*, 2009). The main saccharolytic species in the colon belong to the genera *Bacteroides*, *Bifidobacterium*, *Ruminococcus*, *Eubacterium*, *Lactobacillus,* and *Clostridium* (Vernazza *et al.*, 2006).

Bacteroides, the most abundant genus in the colon (section 1.1), has shown to be able to degrade and ferment a wide variety of plant polysaccharides (Hooper *et al.*, 2002). A study by Tasse and colleagues (2010) demonstrated that *Bacteroides* has genes coding for glycosidic enzymes such as β-glucanases, xylanases, galactanases, and amylases. Additionally, the study showed that some of the genes coding for the carbohydrate active enzymes in *Bacteroides* were found in multiple clusters and could be involved in carbohydrate degradation, transport and/or binding. The high saccharolytic capacity of *Bacteroides* could partly explain why this genus is so abundant in the colon.

Bifidobacterium is another dominant genus in the human colon (section 1.1). To this date, nineteen genomes of strains from this genus have been completely sequenced. These include strains of *B. adolescentis*, *B. animalis* subsp. *lactis*, *B. angulatum*, *B. bifidum*, *B. breve*, *B. longum* subsp. *longum*, *B. longum* subsp. *infantis*, *B. pseudocatenulatum*, *B. catenulatum*, and *B. gallicum* (Pokusaeva *et al.*, 2011). Large numbers of the genes found in the *Bifidobacterium* genom have shown to encode proteins which are involved in carbohydrate metabolism and transport, such as GH, and sugar ABC transporters (Ventura *et al.*, 2007;Ventura *et al.*, 2010;Pokusaeva *et al.*, 2011).

As for *Bacteroides*, it has been shown that many of these genes are located in clusters (Schell *et al.*, 2002;Ventura *et al.*, 2007;Tasse *et al.*, 2010;Pokusaeva *et al.*, 2011). Genome sequencing of the different *Bifidobacterium* species has revealed that carbohydrate modifying enzymes can vary among species. Additionally, the affinity of the enzymes may vary depending on the length and complexity of the substrate (Warchol *et al.*, 2002;Margolles and de los Reyes-Gavilan, 2003;Janer *et al.*, 2004). Table 1 lists some of the GH found in *Bifidobacterium* spp.

Most of the biochemical characterized GH from *Bifidobacterium* spp. have shown to be active towards oligosaccharides, but only some GH are active towards polysaccharides (Van Den Broek and Voragen, 2008). Van Laere and colleagues (2000) studied the ability of four *Bifidobacterium* species (*B. breve*, *B. longum*, *B. infantis* and *B. adolescentis*) to ferment plant polysaccharides and their corresponding oligosaccharides *in vitro*. The results showed that the ability to ferment plant cell wall derived polysaccharides varied depending on *Bifidobacterium* species, but most of the species were able to ferment oligosaccharides. *B. breve* could ferment arabinogalactan, but not arabinan and arabinoxylan. This was in line with results from genome sequencing that has revealed the absence of arabinan- and arabinoxylan degrading enzymes in *B. breve* (Table 1). *B. infantis* was not able to ferment any of the polysaccharides even though genome sequencing has revealed that it may contain α-L-arabinofuranosidase (Table 1). Additionally, *B. infantis* was only able to utilize arabino-galactooligosaccharides. These results could indicate that the α-L-arabinofuranosidase found in *B. infantis* has a low affinity for plant polysaccharides but specificity for certain oligosaccharides. *B. longum* was able to utilize arabinogalactan, arabinan, and arabinoxylan. This is in agreement with previous work by Gueimonde *et al*. (2007), which showed that α-L-arabinofuranosidase activity in *B. longum* could be induced by arabinose- and/or xylose-containing polymers and/or their related oligosaccharides. Genome sequencing of *B. longum* has revealed that more than 8% of the genome is related to the catabolism of oligo- and polysaccharides. Among these genes, many were predicted to encode proteins involved in catalyzing the degradation of arabinose-containing saccharides such as intra- and extracellular α-L-arabinofuranosidases (Schell *et al.*, 2002). This provides *B. longum* with a competitive advantage in the utilization of different substrates in the gut (Van Den Broek and Voragen, 2008).

Table 1: The presence of glycoside-hydrolases in bifidobacteria.

Glycoside hydrolase (GH)	Glycoside hydrolase family	*Bifidobacterium* Species*	Catalyze reaction
Fructan β-(2,1)-fructosidase (EC 3.2.1.153)	GH32	*B. animalis* subsp. *lactis* *B. longum* subsp. *longum* *B. adolescentis*	Hydrolysis of terminal, non-reducing 2,1-linked β-D-fructofuranose residues in fructans.
α-L-arabinofuranosidase (EC 3.2.1.55)	GH3, GH43, GH51	*B. animalis* subsp. *lactis* *B. longum* subsp. *longum* *B. adolescentis* *B. longum* subsp. *infantis*	Hydrolysis of terminal non-reducing 1,3- or 1,5-linked α-L-arabinofuranoside residues in arabinan, arabinoxylan and arabinogalactan.
Endo-β-(1,4)-galactanase (EC 3.2.1.89)	GH53	*B. longum* subsp. *longum* *B. breve* *B. bifidum*	Hydrolysis of (1→4)-β-D-galactosidic linkages in arabinogalactans.
Endo-β-(1,4)-xylanase (EC 3.2.1.8)	GH5, GH8, GH43	*B. animalis* subsp. *lactis* *B. longum* subsp. *longum* *B. adolescentis* *B. longum* subsp. *infantis* *B. bifidum*	Hydrolysis of (1→4)-β-D-xylosidic linkages in xylans.
Oligo-α-(1,6)-glucosidase (EC 3.2.1.10)	GH13, GH31	*B. animalis* subsp. *lactis* *B. longum* subsp. *longum* *B. adolescentis*	Hydrolysis of (1→6)-α-D-glucosidic linkages in some oligosaccharides produced from starch and glycogen.
Glucan-β-(1,3)-glucosidase (EC 3.2.1.58)	GH3, GH5	*B. longum* subsp. *longum* *B. longum* subsp. *infantis*	Hydrolysis of β-D-glucose units from the non-reducing ends of (1→3)-β-D-glucans.
Pullulanase (EC 3.2.1.41)	GH13	*B. animalis* subsp. *lactis* *B. longum* subsp. *longum* *B. dentium* *B. adolescentis*	Hydrolysis of (1→6)-α-D-glucosidic linkages in pullulan, amylopectin and glycogen.

Data is based on database search (http://www.uniprot.org/uniprot and http://www.cazy.org, 2011-09-25)

*All bifidobacterial species listed have been detected in human feces (Turroni *et al.*, 2009;Tannock, 2010).

2.3. Difference in microbial community

Even though several species of the colon are able to inhabit both the mucosal and the luminal environment, the mucosal microbial community has shown to differ from the luminal community (Zoetendal *et al.*, 2002;Macfarlane *et al.*, 2005;Macfarlane, 2008;Van den Abbeele *et al.*, 2011a). Additionally, literature have revealed that dominant phylogenetic groups of mucosal microbial communities are similar in the ascending colon, descending colon, and rectum, but with high inter-individual variations (Zoetendal *et al.*, 2002;Lepage *et al.*, 2005;Eckburg *et al.*, 2005;Wang *et al.*, 2005a). In contrast, it has been demonstrated that luminal microbial communities differ depending on colonic region (Pochart *et al.*, 1993;Marteau *et al.*, 2001). This suggests that different environmental conditions found throughout the colonic lumen (described in section 1.2) may affect the composition of luminal bacteria, whereas the composition of mucosal bacteria is more influenced by host factors (described section 2.1). It is also plausible that the niches of the lumen and the mucus select a microbial population that may display different roles in the host. A recent study by Derrien *et al.* (2011) has demonstrated that germ-free mice mono-cultured with *Akkermansia muciniphila* (Gram-negative, strictly anaerobic mucin-degrading bacterium) or *Lactobacillus plantanum* (Gram- positive bacterium that utilize dietary carbohydrates but is incapable of utilizing mucin) led to different mucosal transcriptome changes depending on colonization of the different bacteria. *L. plantarum* was exclusively located in the colonic lumen and induced expression of genes involved in regulation of lipid and fatty acid metabolism, whereas *A. muciniphila* colonized the colonic mucus of the mice and induced expression of genes involved in regulatory immune processes. These results could imply that the mucosal microbiota are more involved in interaction with epithelial and immune cells compared to the luminal microbiota, since they reside closer to the IECs. Hence, the difference in bacterial community structure is likely driven by factors such as differential substrate availability (e.g. mucus versus undigested dietary residues) and host-microbe interactions. Figure 3 illustrates the mucosa and lumen of the colon.

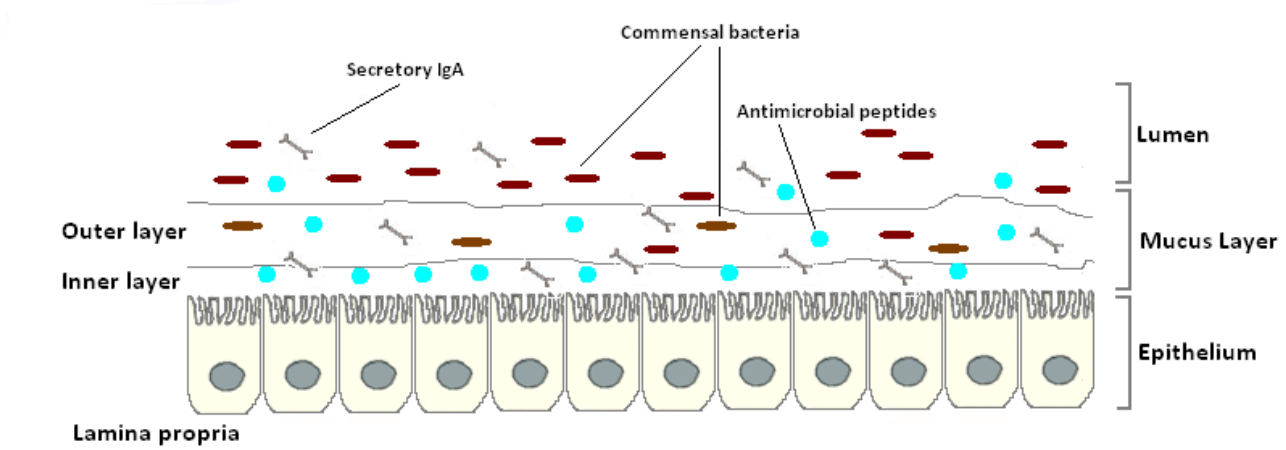

Figure 3: The colonic mucosa with epithelium and two mucus layers, and the lumen.

2.4. Interaction between host and commensal bacteria

The molecular mechanisms responsible for the host to tolerate the presence of commensals that being both the luminal and mucosal bacteria are not fully understood (Macpherson *et al.*, 2005), but the ability of the host's mucosal immune system to distinguish between commensal and pathogenic bacteria using a limited number of pattern-recognition receptors (PRRs) is believed to play a role (Sanz and De Palma, 2009;Van den Abbeele *et al.*, 2011b). PRRs are germline-encoded receptors, which can recognize conserved molecular patterns (pathogen-associated molecular patterns (PAMPs) or microorganism-associated molecular patterns (MAMPs)), which are essential for the survival of microorganism and therefore difficult for the microorganism to alter (Akira and Takeda, 2004;Van den Abbeele *et al.*, 2011b). PRRs are expressed by innate immune cells including macrophages and dendritic cells (DCs), by adaptive immune cells such as T cells, and by nonprofessional cells such as IECs, and are crucial for the initial recognition of microorganisms (Akira and Takeda, 2004;Round *et al.*, 2011). The best characterized class of PRRs is Toll-like receptors (TLRs). Eleven members of the TLR family have been identified in mammals, and different TLRs react with specific PAMPs or MAMPs, allowing the host to distinguish between self and foreign pathogens. Table 2 demonstrates the different TLRs and their ligands (Bacterial origin). As commensal and pathogenic bacteria produce similar ligands sensed by TLRs, epithelial and immune cells have to have mechanisms which avoid commensals to trigger hyper-responsiveness (Lebeer *et al.*, 2010). A wide variety of bacterial species residing within the human colon are Gram-positive bacteria (Section 1.1). In the cell wall of Gram-positive bacteria, lipoteichoic acids (LTA) are embedded in a thick layer of peptidoglycan (PG). As shown in Table 2, LTA are ligand for TLR2.

However, to avoid unnecessary detection of Gram-positive commensal bacteria, IECs have developed special mechanisms to tolerate the continuous presence of LTA molecules originating from commensals by expressing TLR2 and co-receptors at a limited level (Melmed *et al.*, 2003;Lebeer *et al.*, 2010). The same mechanism has been observed for TL4 on IECs, in which expression of TLR4 is down-regulated to avoid hyper-responsiveness to lipopolysaccharide (LPS) (Table 2) from commensal Gram-negative bacteria (Abreu *et al.*, 2001).

In literature, immune regulatory effects of commensal bacteria through TLRs have been observed. As an example, Hoarau *et al.* (2006) demonstrated that the supernatant of *B. breve* could cause maturation of DCs through TLR2 pathway. This effect led to high levels of interleukin (IL)-10 (inducer of regulatory T cells (Treg cells)) and low levels of IL-12, in contrast to DCs stimulated with LPS. Another *Bifidobacterium* species, *B. longum*, has shown to attenuate Tumor Necrosis Factor-alfa (TNF-α)-induced Nuclear factor kappa B (NF-κB) activation and NF-κB-mediated IL-8 expression through TLR9 (Ghadimi *et al.*, 2010). Finally, a study by Geuking *et al.* (2011) demonstrated that the colonization of mice with a completely benign commensal microbiota (altered Schaedler Flora) resulted in activation of colonic Treg cells in lamina propria through TLR signaling, which led to intestinal T cell homeostasis as reflected by the absence of T helper 1 cell or T helper 17 cell responses.

Table 2: TLR recognition of Microbial Components

Microbial Components	Species	TLR Usage	References
Triacyl lipopeptides	Bacteria and mycobacteria	TLR1	(Takeuchi *et al.*, 2002)
Peptidoglycan	Gram-positive bacteria	TLR2	(Takeuchi *et al.*, 1999;Schwandner *et al.*, 1999)
Fimbrillin	*Porphyromonas gingivalis*	TLR2/TLR4	(Davey *et al.*, 2008)
Polysaccharide A	*Bacteroides fragilis*	TLR2	(Round *et al.*, 2011)
Lipoarabinomannan	Mycobacteria	TLR2	(Means *et al.*, 1999)
Porins	*Neisseria*	TLR2	(Massari *et al.*, 2002)
Lipoteichoic acid	Gram-positive bacteria	TLR2/TLR6	(Schwandner *et al.*, 1999)
Lipopolysaccharide	Gram-negative bacteria	TLR4	(Poltorak *et al.*, 1998)
Flagellin	Flagellated bacteria	TLR5	(Hayashi *et al.*, 2001)
Diacyl lipopeptides	*Mycoplasma*	TLR6	(Takeuchi *et al.*, 2001)
CpG-containing DNA	Bacteria and mycobacteria	TLR9	(Hemmi *et al.*, 2000)
Not determined	Not determined	TLR10	-
Not determined	Uropathogenic bacteria	TLR11	(Zhang *et al.*, 2004)

3. Inflammatory Bowel disease

As described before, regulated interactions between commensal bacteria and host is important for colonic homeostasis, thus avoiding unnecessary reactions from the mucosal immune system. However, perturbation of this interaction can lead to colonic inflammation or worse diseases such as inflammatory bowel disease (IBD). This chapter will give a description of one of the IBDs, ulcerative colitis (UC), as well as elucidating some of the factors that may play a role for the pathogenesis of this disease.

3.1. Ulcerative colitis

UC is an idiopathic IBD, which is characterized by chronic inflammation of the colonic mucosa. UC is usually associated with recurrent attacks and complete remission of symptoms in the interim. Generally, UC has a highly unpredictable clinical course characterized by one or more years with inactive disease and relapses, which typically last 2-4 weeks provided that relevant treatment is given. The extent and severity of inflammation in the colon regions are variable. UC may be restricted to the distal colon and rectum (Proctitis, Proctosigmoiditis and Left-sided colitis), while in its most extended form, the entire colon is involved (Pancolitis) (Figure 4) (Ardizzone, 2003;Kornbluth and Sachar, 2010). The classical symptoms of patients in relapse are diarrhea with passage of blood or mucus, or both, occasional abdominal cramping and pain as well as systemic symptoms of fever and weight loss in severe cases (Farrell and Peppercorn, 2002;Ardizzone, 2003). The medical treatments of UC are antibiotics, anti-inflammatory drugs (e.g. mesalazine, olsalazine, sulfasalazine or corticosteroids), and/or immunosuppressive drugs (e.g. azathioprine, methotrexate or cyclosporine) (Kornbluth and Sachar, 2010). UC may affect any age group, although there is peak occurrence between ages 15 and 35. In Western Europe and USA, UC has an estimated prevalence of approximately 70 to 150 per 100.000 populations (Ardizzone, 2003;Kornbluth and Sachar, 2010).

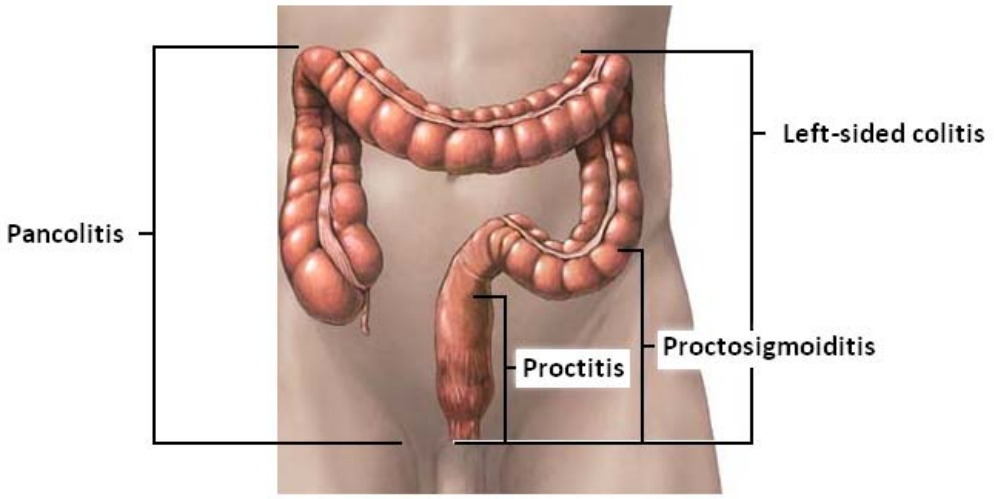

Figure 4: Location of UC in the different colon regions (Longstreth *et al.*, 2010)

The factors responsible for the initiation and perpetuation of UC are unknown (Loftus, 2004;Sartor, 2006). However, evidence suggests that the colonic host-bacterial interaction and microbial composition play a pivotal role in the pathogenesis of UC. Firstly, the colon is the area of the gut with the highest bacterial concentrations (section 1.1) and is also the site where inflammation occurs in UC patients. Secondly, genetically engineered germ-free mice, rats, and guinea pigs completely lack enterocolitis but develop intestinal inflammation within 1 – 4 weeks if they are colonized with conventional gut bacteria (Himmel *et al.*, 2008;Sartor, 2008). Thirdly, clinical studies have revealed that some PRRs have gene mutations (e.g. TRL9) or an increased expression on IECs (e.g. TLR2 and TLR4) in UC patients (De Jager *et al.*, 2007;Frolova *et al.*, 2008;Fuse *et al.*, 2010), and finally, studies have shown that UC patients in relapse have a colonic mucus layer that has an altered O-glycan profile and is significantly thinner compared to healthy subjects (Pullan *et al.*, 1994;Larsson *et al.*, 2011). Thus, gut host-bacterial interaction seems to be disturbed in UC, which may have consequences for the composition of the gut commensal bacteria.

3.2. Alteration in the bacterial community of ulcerative colitis patients

Compositional changes in the colonic microbiota have been observed when comparing fecal and mucosal samples from UC patients to that of healthy subjects (Lepage *et al.*, 2005;Frank *et al.*, 2007;Takaishi *et al.*, 2008;Sokol *et al.*, 2009;Qin *et al.*, 2010). Studies have demonstrated that the fecal and mucosal amount of *Firmicutes* was significantly lower in UC patients than in healthy subjects (Frank *et al.*, 2007;Sokol *et al.*, 2009). This was also applicable for the two dominant

clostridial groups within the *Firmicutes* phylum, *Clostridium coccoides* group and *Clostridium leptum* subgroup (Frank *et al.*, 2007;Takaishi *et al.*, 2008;Sokol *et al.*, 2009). Takiashi *et al*. (2008) have also shown that these two clostridial groups were significantly less abundant in fecal samples from UC patients in relapse compared to UC patients in remission. Studies have also demonstrated that the *Bacteroidetes* phylum was underrepresented in mucosal samples from UC patients compared to healthy subjects (Ott *et al.*, 2004;Frank *et al.*, 2007), and that the amount of *Bacteroides fragilis* group was low in fecal and mucosal samples from UC patients (Takaishi *et al.*, 2008). A common genus from the gut community, *Bifidobacterium* spp., has shown to be underrepresented in mucosal and fecal samples from UC patients compared to healthy subjects (Macfarlane *et al.*, 2004;Mylonaki *et al.*, 2005;Sokol *et al.*, 2009). Additionally, Mylonaki *et al*. (2005) revealed that bifidobacteria were significantly less abundant in mucosal samples from UC patients in remission and relapse than in healthy subjects. Bacteria that have shown to be increased in mucosal samples from UC patients than in healthy subjects include the sulphate-reducing bacteria *Desulfovibrio spp.* (belongs to the delta proteobacteria) (Rowan *et al.*, 2010), and the phylum *Proteobacteria* (Lepage *et al.*, 2005;Frank *et al.*, 2007).

4. Modulation of the gut microbiota

Selective stimulation of specific colonic bacteria with the promotion of their growth and metabolic activity could be a helpful approach in creating a benign gut microbial community (Vernazza *et al.*, 2006;Jacobs *et al.*, 2009). Because some bacteria are able to produce a high selection of carbohydrate active enzymes, they can grow on carbon sources, which may be less easily fermented by other members of the colonic community. Thus they have an advantage when competing with other bacterial species in a mixed community such as the human colon (Gibson and Roberfroid, 1995). In the following chapter, the ability of prebiotics or prebiotic candidates to modulate gut environment will be addressed. Additionally, the protective effects of prebiotics or prebiotic candidates on colitis in either human trials or animal studies will be described.

4.1. Prebiotics

Prebiotics are believed to have a putative health effect on the gut and are defined as, "*selectively fermented ingredients that cause specific changes in composition and/or activity in the gastrointestinal microbiota that confers benefits upon host well-being and health"* (Roberfroid, 2007).

The primary conditions for a food ingredient to be classified as a prebiotic depend on the compound to be resistant to gastric acidity and hydrolysis by mammalian enzymes, hence reaching the colon and stimulating a health promoting bacterial and/or metabolic environment. The colonic bacteria that are believed to promote host health include species belonging to the genera *Bifidobacterium* and *Lactobacillus,* as well as butyrate-producing species belonging to *C. coccoides* group and *C. leptum* subgroup (Rastall, 2007;Sokol *et al.*, 2008;Atarashi *et al.*, 2011). These species can prevent colonization of pathogenic bacteria by lowering colonic pH by producing SCFA during saccharolytic fermentation (Fooks and Gibson, 2002), producing antimicrobial compounds including bacteriocins (Saulnier *et al.*, 2011;Lee *et al.*, 2011), and competing for adhesion sites and nutrients (Gibson and Roberfroid, 1995;Lee *et al.*, 2003;Collado *et al.*, 2007;Chenoll *et al.*, 2011;Saulnier *et al.*, 2011). Additionally, they have shown to induce immune regulatory responses (Hoarau *et al.*, 2006;Sokol *et al.*, 2008;Zeuthen *et al.*, 2010;Atarashi *et al.*, 2011). Due to their health positive effects, strains of *Bifidobacterium* and *Lactobacillus* (probiotic bacteria) are also used as a supplement to the diet for modulation of the colonic environment to promote health;

hence the aim of pro- and prebiotic supplementation is similar. However, they achieve the effect in different ways: probiotics are introduced as exogenous species, whereas prebiotics stimulate indigenous bacteria (Vanhoutte *et al.*, 2006). The effects of probiotics (as supplement) on human health are, however, beyond the scope of this thesis.

4.2. The constitution of prebiotics

There is no consensus at this time regarding what constitutes a prebiotic, which means that it could be a non-digestible poly- or oligosaccharide, a bacterial exopolysaccharide, or a non-carbohydrate (Macfarlane *et al.*, 2010). The majority of prebiotic contenders are, however, non-digestible poly- and oligosaccharides, which are either extracted from plant tissue or synthesized using enzymes (Rastall, 2007). It is these compounds that this thesis will focus on.

Direct plant-extracted polysaccharides used as prebiotics (mainly inulin) often have a disperse molecular weight distribution with a degree of polymerization (DP) from three to around seventy (Sirisansaneeyakul *et al.*, 2007;Rastall, 2007). Non-digestible oligosaccharides used as prebiotics usually contain between three to ten sugar moieties. However, some disaccharides have also shown to posses similar properties to large sugars (Challa *et al.*, 1997;Rastall, 2007). Based on evidence available both from *in vitro* and *in vivo* experiments, only a small portion of non-digestible carbohydrates can be classified as prebiotics. These include: inulin, fructo-oligosaccharides (FOS), lactulose, and galacto-oligosaccharides (GOS) (Rastall, 2007). Other potential prebiotics will be addressed as prebiotic candidates or non-digestible carbohydrates throughout the rest of this thesis.

The chemical constituents and DP of prebiotic candidates are important factors for selective stimulation of a specific colonic microbiota profile during their fermentation. Different monosaccharide compositions and glycosidic linkages have shown to affect the fecal microbiota in different ways with linkages of α 1-2, β 1-4, and β 1-6 selectively stimulating the growth of bifidobacteria (Sanz *et al.*, 2005). Additionally, Sanz *et al.* (2006) demonstrated that synthesized oligosaccharides (maltose acceptor products) of DP3 showed high selectivity towards bifidobacteria in a fecal community, and oligosaccharides with a higher molecular weight (DP6-DP7) also resulted in selective fermentation.

Because chronic gut diseases such as UC arise in the descending colon and spread proximally (Section 3.1), the molecular weight could have an influence on the protective effect of prebiotics

on colitis, since the non-digestible carbohydrates should be able to reach the distal part of the colon and stimulate the activity of the microbiota in this colon region. Production of non-digestible carbohydrates with high molecular weight may help increase the persistence of the compound leading to slower fermentation, thus penetrating prebiotic effect all the way throughout the colon and not only affecting the microbiota in the ascending region (Vernazza *et al.*, 2006;Rastall, 2007). Langlands *et al.* (2004) examined the prebiotic effect of a mixture of inulin (DP range 6-60) and FOS (DP range 2-8). The study showed that the mixture could significantly increase the level of bifidobacteria and lactobacilli in both the ascending and descending colon of healthy human subjects. Approaches for slowly fermentation do not only include high chain length. Rose *et al.* (2010) demonstrated that starch-entrapped microspheres could be used to decrease the fermentation rate.

The ability of non-digestible carbohydrates to improve the colonic environment is not only restricted to the properties through fermentation. Other studies have shown that the exogenous structure of specific non-digestible carbohydrates can affect the presence of pathogenic bacteria. One of the features is the ability of some prebiotics (e.g. FOS and GOS) to act as a molecular receptor decoy, which can competitively inhibit bacterial adhesion (Kunz *et al.*, 2000;Shoaf *et al.*, 2006;Gibson *et al.*, 2006). Another feature is the ability of prebiotic candidates (e.g. XOS and cellobiose) to act as a repressor of virulence factors by repressing the gene expression in enteropathogens such as *Listeria monocytogenes* (Gilbreth *et al.*, 2004). In addition, a recent *in vitro* study using Caco2 cells has shown that FOS could initiate an anti-inflammatory response even in the absence of intestinal bacteria by inducing the nuclear receptor PPAR gamma (Zenhom *et al.*, 2011). Table 3 summarizes the desirable attributes of prebiotics.

Table 3: Desirable attributes of prebiotics

Action	Properties	Reference
Colonic fermentation	Selectively metabolized by bacteria with health promoting effects	(Fooks and Gibson, 2002;Rastall, 2007)
Persistence through the colon	Reaching both the proximal and to some extent the distal colon.	(Langlands *et al.*, 2004)
Inhibition of adhesion by pathogens	Possessing receptor like sequences.	(Kunz *et al.*, 2000;Shoaf *et al.*, 2006;Gibson *et al.*, 2006)
Repression of pathogenic bacteria	Down-regulating virulence factors.	(Gilbreth *et al.*, 2004)
Immune modulation	Regulating anti-inflammatory responses	(Zenhom *et al.*, 2011)

4.3. Fermentation metabolites

Metabolites produced by bacterial fermentation of non-digestible carbohydrates include SCFAs, primarily acetate, propionate, and butyrate, and a number of other intermediate metabolites such as lactate, pyruvate, ethanol, and succinate. In addition to this, gasses like hydrogen, carbon dioxide, and in some individuals methane are produced (Vernazza *et al.*, 2006). SCFAs are normally found as major products in the gut ecosystem compared to other metabolites (Macfarlane and Macfarlane, 2003).

SCFAs are produced within bacterial cells from monosaccharides generated from the hydrolysis of poly- and oligosaccharides (Topping and Clifton, 2001). The pathways for SCFA metabolism from dietary fibres are either through the glycolytic pathway, which is used by the majority of intestinal bacteria, or by the pentose phosphate pathway. Both pathways lead to the synthesis of pyruvate and acetyl-CoA (Macfarlane and Macfarlane, 2003). These metabolites are key control points in fermentative metabolism and can be converted into e.g. propionate, acetate and butyrate (Macfarlane and Macfarlane, 2003;Al-Lahham *et al.*, 2010).

Results from human sudden death victims have revealed that the SCFA molar ratios (%) of acetate:propionate:butyrate were similar in different regions of the large intestine, about 57:22:21 (Cummings *et al.*, 1987). However, SCFA concentrations are highest in the ascending colon, primarily because of high carbohydrate availability (section 1.2). Levels of SCFAs in the GI tract vary significantly depending on the amount of non-digestible poly- and oligosaccharides found in the diet and the composition of gut microbiota (Macfarlane and Macfarlane, 2003). *In vitro* fermentation studies have shown that acetate is the main product of arabinan and pectin hydrolysis (Ferguson and Jones, 2000;Al-Tamimi *et al.*, 2006), whereas large amounts of acetate and propionate are produced from arabinogalactan (Englyst *et al.*, 1987). Butyrate is formed in high amount from fructans (Karppinen *et al.*, 2000;Ferguson and Jones, 2000).

Around 95% of the SCFAs produced in the colon are absorbed and metabolized by IECs (primarily butyrate), liver, and muscles (Salminen *et al.*, 1998;Guilloteau *et al.*, 2010). SCFAs have local and systemic biological effects, which are proposed to be beneficial to human health (Karppinen *et al.*, 2001). Acetate seems to influence mechanisms responsible for inhibiting enteropathogenic bacteria with low pH as one of the antimicrobial factors (Fooks and Gibson, 2002) and propionate is believed to inhibit fatty acid and cholesterol biosynthesis in the liver (Nishina and Freedland, 1990;Cheng and Lai, 2000;Probert and Gibson, 2002). Butyrate is thought to play a role in

maintaining a normal IEC populations thereby preventing mutations (Topping and Clifton, 2001) and has shown to alter cytokine production towards a regulatory profile *in vitro* (Millard *et al.*, 2002). Both propionate and butyrate have shown to significantly decrease expression of invasion genes in *Salmonella typhimurium* (Lawhon *et al.*, 2002). It has recently been demonstrated that acetate, propionate, and butyrate are able to inhibit TNFα-mediated activation of the NF-κB pathway in human colon adenocarcinoma cell lines and induce anti-inflammatory activity in an *in vitro* model of murine experimental colitis (Tedelind *et al.*, 2007).

4.4. Metabolic cross-feeding

In a complex microbial community such as the gut extensive metabolic interactions occur. Metabolic products produced from non-digestible poly- or oligosaccharide by one bacterial species may provide substrates to support growth of another. This metabolic cross-feeding can have metabolic consequences, because the route of the substrate is not completely obvious (Belenguer *et al.*, 2006;Flint *et al.*, 2007). An example of inter-relatedness, which could benefit the host, is the formation of butyrate by metabolic cross-feeding. Fermentation of FOS seems to be mainly performed by lactate and acetate producing bacteria rather than butyrate producing bacteria (Damian *et al.*, 1999). However, Morrison *et al.* (2006) have shown that FOS can selectively stimulate the bacterial conversion of acetate and lactate to butyrate. In line with this, *in vitro* fermentation studies have demonstrated that butyrate-producing species such as *Eubacterium hallii* and *Anaerostipes caccae,* who are not able to grow on FOS in pure cultures, can utilize the fermentative products lactate and acetate to produce butyrate in co-culturing with FOS-degrading *Bifidobacterium* strains (Duncan *et al.*, 2003;Belenguer *et al.*, 2006). However, metabolic cross-feeding can also give rise to unfavourable stimulation of bacteria and metabolites. A predominant sulphate-reducing species, which inhabit the human colon, is *Desulfovibrio piger* (Fite *et al.*, 2004). *D. piger* utilize lactate as a preferred co-substrate, which is oxidized to acetate, while sulphate is reduced to sulphide (Loubinoux *et al.*, 2002). It has recently been demonstrated that *D. piger* could compete for lactate produced at relatively low concentrations by *B. adolescentis* in co-cultures with either *E. Hallii* or *A. caccae*. Production of butyrate by *E. Hallii* and *A. caccae* was inhibited by 50%, however, sulphide production by *D. piger* was unaffected by the presence of butyrate-producing bacteria (Marquet *et al.*, 2009). A high concentration of sulphide is believed to be toxic to IECs and may create cellular energy deficiency in IECs by inhibiting the β-oxidation of

butyrate. These side effects have implicated sulphate-reducing bacteria and sulphide in the pathogenesis of UC (Christl *et al.*, 1996;Rowan *et al.*, 2010). Hence, consumption of non-digestible carbohydrates can influence the host in different ways, and factors such as population dynamics and metabolite pathways should be considered when developing and validating new prebiotic candidates.

4.5. Protective effects of prebiotics or prebiotic candidates on inflammatory bowel disease

At the time of publication of this thesis, there have only been a small number of human trials examining prebiotic properties in IBD using non-digestible carbohydrates, and only a few studies have used microbiological and immunological analyses to validate the prebiotic effects (Table 4, based on literature search, 2011-09-14). Due to the small number of human trials found in literature examining prebiotic properties of non-digestible carbohydrates in UC, Table 4 lists trials using both UC and Crohn's disease (CD) patients. CD is another main type of IBD. As described previously, UC causes continuous mucosal inflammation, which is restricted to the colon (section 3.1). However, CD causes discontinuous transmural inflammation and affects any part of the GI tract but has a predilection for the terminal ileum (Blumberg and Strober, 2001). The classical symptoms of patients in relapse such as diarrhea, rectal bleeding, and abdominal pain are the same for both UC and CD (Farrell and Peppercorn, 2002;Ardizzone, 2003;Strober *et al.*, 2007). Human trials have shown protective effects of prebiotic candidates demonstrated by reduced disease activity, reduced levels of pro-inflammatory cytokines and increased levels of anti-inflammatory cytokines (Kanauchi *et al.*, 2003;Hanai *et al.*, 2004;Lindsay *et al.*, 2006;Casellas *et al.*, 2007;Faghfoori *et al.*, 2011). However conflicting results have been described, in which prebiotic consumption was unable to implement any protective effects (Hafer *et al.*, 2007;Benjamin *et al.*, 2011). However, the study by Hafer *et al.* (2007) did demonstrate that prebiotic consumption could increase the quality of life in the UC patients but not in CD patients. This suggests that the protective effect of prebiotics may differ depending on type of intestinal disease, hence site of inflammation.

The number of animal studies validating the protective effects of prebiotics in experimental colitis is somewhat higher than human trials. Some of them found in literature are listed in Table 5. Several of these *in vivo* studies using rats or mice with induced colitis have demonstrated that

consumption of prebiotics or prebiotic candidates decreased the severity of colonic damage, increased colonic levels of lactobacilli and bifidobacteria, and decreased levels of neutrophile infiltration and pro-inflammatory cytokines (Cherbut *et al.*, 2003;Rumi *et al.*, 2004;Hoentjen *et al.*, 2005;Camuesco *et al.*, 2005;Osman *et al.*, 2006;Lara-Villoslada *et al.*, 2006;Kanauchi *et al.*, 2008;Rodriguez-Cabezas *et al.*, 2010). However, discrepancies in results have been observed in the study by Geier *et al.* (2007), in which prebiotic consumption failed to protect rats from induced colitis, and even increased neutrophile infiltration.

Generally, little is still known regarding the protective effect of prebiotics or prebiotic candidates on IBD, and only a few different prebiotic candidates have been tested *in vivo* with some discrepancies in results, as can be seen in Table 4 and Table 5.

Table 4: Clinical trials on the prebiotic effect in inflammatory bowel disease (Ulcerative colitis (UC) and Crohn's disease (CD))

Subjects	Tiral Design*	Test Compound*	Duration time	Key finding	Reference
UC (relapse)	DBRPC	Treatment: OFI (12g/day), n=10 Placebo: Maltodextrose (12 g/day), n=9	2 weeks	*Compared with placebo, the prebiotic* Reduced disease activity Reduced fecal calprotectin	(Casellas *et al.*, 2007)
UC (remission)	Open label	Treatment: GBF (20 g/day), n=22 Control: No additive treatment, n=37	12 months	*Compared with control, the prebiotic* Reduced disease activity	(Hanai *et al.*, 2004)
UC (relapse)	Open label	Treatment: GBF (20-30 g/day), n=21 Control: No additive treatment, n=21	24 weeks	*Compared with control, the prebiotic* Reduced disease activity	(Kanauchi *et al.*, 2003)
UC (remission)	Open label	Treatment: GBF (30 g/day), n=20 Control: No additive treatment, n=21	2 months	*Compared with control, the prebiotic* Reduced levels of serum pro-inflammatory cytokines; TNF-α, IL-6 and IL-8	(Faghfoori *et al.*, 2011)
UC (active) CD (active)	RPC	Treatment: UC, lactulose (10 g/day), n=7 CD, lactulose (10 g/day, n=8 Control: UC, no additive treatment, n=7 CD, no additive treatment, n=9	4 months	*Compared with control, the prebiotic* Did not affect disease activity in either UC or CD Did not affect immunohistochemical parameters in either UC or CD Increased quality of life in UC	(Hafer *et al.*, 2007)
CD (active)	Open label	Treatment: OFI (15 g/day), n=10	3 weeks	*Compared with baseline, the prebiotic* Reduced disease activity Increased fecal bifidobacteria Increased dendritic cell IL-10 Increased dendritic cell TLR-2 and TLR-4 expression	(Lindsay *et al.*, 2006)
CD (active)	DBRPC	Treatment: OFI (15 g/day), n=54 Placebo: Maltodextrose (15 g/day), n=49	4 weeks	*Compared with placebo, the prebiotic* Did not affect disease activity Did not affect fecal bifidobacteria Increased dendritic cell IL-10 Reduced dendritic cell IL-6	(Benjamin *et al.*, 2011)

*Oligofructose-enriched inulin (OFI), Germinated barley foodstuff (GBF)
#Doubled-blind randomized, placebo controlled (DBRPC), randomized, placebo controlled (RPC)

Table 5: Animal studies used to demonstrate prebiotic effects in preventing colitis

Substrate*	Length of treatment *	Study design animals	Colitis inducer#	Key findings	Reference
OFI	14 days	Spraugue-Dawley rats (n=6 in each group)	DSS	*Compared with colitis control, the prebiotic* Decreased severity of colonic damage Increased cecal and colonic lactobacilli and bifidobacteria Decreased IL-1β Decreased neutrophile infiltration	(Osman *et al.*, 2006)
OFI	7 weeks	HLA B27 transgenic rats (n=8 in each group)	SPF	*Compared with colitis control, the prebiotic* Decreased severity of colonic damage Increased cecal and colonic lactobacilli and bifidobacteria Decreased IL-1β	(Hoentjen *et al.*, 2005)
FOS	14 days	Spraugue-Dawley rats (n=8 in each group)	DSS	*Compared with colitis control, the prebiotic* Increased neutrophile infiltration Did not result in greater reduction in colonic damage	(Geier *et al.*, 2007)
FOS	1 month	Wistar rats (n=20 in each group)	TNBS	*Compared with colitis control, the prebiotic* Decreased severity of colonic damage Increased cecal and colonic lactobacilli and bifidobacteria Decreased neutrophile infiltration	(Lara-Villoslada *et al.*, 2006)
FOS	14 days	Wistar rats (n=6 in each group)	TNBS	*Compared with colitis control, the prebiotic* Decreased severity of colonic damage Increased cecal and colonic lactobacilli and lactic acid bacteria Decreased neutrophile infiltration	(Cherbut *et al.*, 2003)
FOS	14 days	Wistar rats (n=10 in each group)	TNBS	*Compared with colitis control, the prebiotic* Decreased severity of colonic damage Decreased neutrophile infiltration Increased colonic lactobacilli and bifidobacteria Did not affect the level of IL-1β and TNF-α	(Rodriguez-Cabezas *et al.*, 2010)
Lactulose	14 days	Wistar rats (n=6 in each group)	DSS	*Compared with colitis control, the prebiotic* Decreased severity of colonic damage Decreased neutrophile infiltration	(Rumi *et al.*, 2004)
Lactulose	3 weeks	Wistar rats (n=10 in each group)	TNBS	*Compared with colitis control, the prebiotic* Decreased severity of colonic damage Decreased neutrophile infiltration Increased colonic lactobacilli and bifidobacteria Decreased colonic TNF-α	(Camuesco *et al.*, 2005)
GBF	9 weeks	SCID mice (n=8 in each group)	CD4+CD45RB^{high} T cells	*Compared with colitis control, the prebiotic* Decreased severity of colonic damage Increased colonic TGF-β Decreased colonic IL-6 and IFN-γ	(Kanauchi *et al.*, 2008)
GMO	14 days	Spraugue-Dawley rats (n=10 in each group)	DSS	*Compared with colitis control, the prebiotic* Decreased severity of colonic damage Decreased neutrophile infiltration	(Lara-Villostada *et al.*, 2006)

*Oligofructose-enriched inulin (OFI), fructo-oligosaccharide (FOS), Germinated barley foodstuff (GBF), Oligosaccharides from goat milk (GMO)
#Dextran sulfate sodium (DSS), pathogen-free conditions (SPF), trinitrobenzene sulfonic acid (TNBS)
Severe combined immunodeficiency (SCID)

5. Production of prebiotics and novel prebiotic candidates

Non-digestible poly- and oligosaccharides of various types are found as natural components in fruit, vegetables, milk and honey (Crittenden and Playne, 1996). With the exception of soybean oligosaccharides (produced by direct extraction from soybean whey) and lactulose (produced by alkaline isomerisation of lactose), most prebiotic oligosaccharides are manufactured using enzymatic processes (Crittenden and Playne, 1996;Rastall, 2007). They are either synthesized from monosaccharides (such as lactose and sucrose) by transglycosylation reactions or produced by controlled hydrolysis of polysaccharides, including starch, inulin or xylan (Crittenden and Playne, 1996;Grizard and Barthomeuf, 1999). In this chapter, the production of the classified prebiotic oligosaccharide, FOS, will be described, as well as the production of novel prebiotic candidates derived from pectin.

5.1. Fructo-oligosaccharides

FOS is found in several kinds of plants and vegetables such as banana, onion, asparagus roots, artichokes, shallot, and wheat (Sangeetha *et al.*, 2005). They are industrially produced in two ways leading to different products: either from inulin (commercially recovered from chicory root and Jerusalem artichoke) by hydrolysis or from transfructosylation of sucrose (Grizard and Barthomeuf, 1999).

In commercial inulin, both linear chains of α-D-glucopyranosyl-[β-D-fructofuranosyl (linked 2→1)]$_{n-1}$-D-fructofuranoside and β-D-fructopyranosyl-[β-D-fructofuranosyl (linked 2 →1)]$_{n-1}$-D-fructofuranoside residues are included. The number (n) of β-D-fructofuranose units varies from two to more than seventy units (Roberfroid and Delzenne, 1998). The production of FOS from inulin is a result of partial enzymic hydrolysis using inulinase (2,1-β-D-fructan fructanohydrolase (EC 3.2.1.7)). This enzyme is specific for inulin and catalyzes the breakdown of β-D-fructosidic linkages in inulin in an endo-type manner. The majority of the hydrolysates (FOS: DP2–10; average DP5) are fructofuranosyl residues, but a low proportion terminates in glycosyl residues (Roberfroid and Delzenne, 1998;Grizard and Barthomeuf, 1999).

The industrial production of FOS from sucrose uses mainly fructosyltransferase or *β*-fructofuranosidase from bacterial and fungal sources (Yun *et al.*, 1997;Kim *et al.*, 1998). FOS derived from sucrose all terminate in glucosyl residues, and are thus non-reducing. Additionally,

depending on the enzyme source, synthesized FOS has different linkages, which mainly compose of 1-kestose (α-D-glucopyranosyl-β-D-fructofuranosyl-β-D-fructofuranoside), nystose (α-D-glucopyranosyl-[β-D-fructofuranosyl]$_2$-β-D-fructofuranoside), and fructofuranosylnystose (α-D-glucopyranosyl-[β-D-fructofuranosyl]$_3$-β-D-fructofuranoside) (Yun *et al.*, 1997;Rastall, 2007). Formation of FOS from sucrose occurs through a set of reactions, which initiates with sucrose acting as both donor and acceptor of a fructosyl residue, yielding glucose and 1-kestose. Subsequently, 1-kestose can function as fructosyl donor, and either sucrose or another oligofructose can function as acceptor. As a result of repeated transfructosylations, linear FOS is synthesized (Figure 5) (Duchateau *et al.*, 1995;Kim *et al.*, 1998). Commercial synthesized FOS can contain high levels of glucose and sucrose, released as by-products. A previous study has compared the amount (%, w/w) of FOS produced by different enzyme forms and reaction modes, which led to mean values of 56% FOS, 27% glucose, and 17% sucrose (Yun *et al.*, 1997). Hence, removal of by-products using either membrane filtration or chromatographic procedures is important to produce products with high purity (Crittenden and Playne, 1996;Yun *et al.*, 1997).

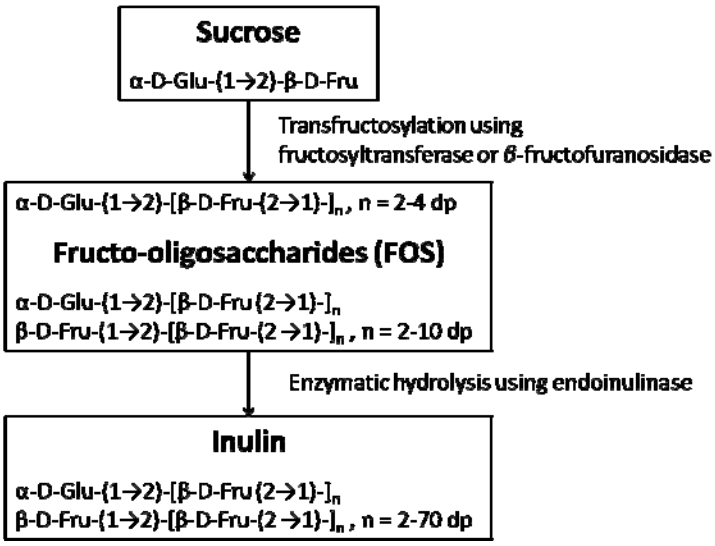

Figure 5: Production of commercial FOS from either sucrose or inulin. Adapted from Crittenden and Playne (1996)

5.2. Production of novel non-digestible carbohydrates from by-product streams

The use of by-product streams from industrial production of e.g. sugar and potato starch has become an area of interest, since by-products are rich sources of non-digestible plant fibers and

thus prospective starting material for obtaining novel prebiotic candidates. More than 4 million tons of sugar beet pulp and 1 million tons of potato pulp are produced annually in the European community. The main utilization of these low-valued by-products is as animal feed (Mayer and Hillebrandt, 1997;Holck *et al.*, 2011). Pectins are a major fraction of pulp and can be extracted from by-products, yielding commercially high-valued products (Buchholt *et al.*, 2004;Manderson *et al.*, 2005).

5.3. The structure of pectin

Pectin is a complex mixture of polysaccharides that make up about one third of the cell wall dry substance of higher plants (Grassin and Fauquembergue, 1996;Thakur *et al.*, 1997). Pectin is primarily polymers of galacturonic acid units (GalA) joined in linear chains by means of α-1,4 glycosidic linkages, which are organized in smooth regions (homogalacturonan, HG), and repeats of (1→2)-α-L-rhamnosyl-(1→4)-α-D-galactosyluronic acid disaccharide units (rhamnogalacturonan I, RGI), which is organized in hairy regions (Grassin and Fauquembergue, 1996;Thakur *et al.*, 1997). HG is partly methoxylated at the carboxyl group and O-acetylated on the O-2 and/or O-3. The side chains of the pectic backbone in RGI consist of neutral sugars such as arabinose and galactose, which are present in complex chains of considerable length. The neutral sugars carry ferulic acids. Ferulic acids esterify the O-2 position in arabinose residues and the O-6 position in galactose residues. The feruloyl substitutions can either be present as monomers or form dimers with other side chains (Levigne *et al.*, 2002). Side chains of arabinan, galactan, or arabinogalactan are often linked (1→4) to the rhamnose in RGI. Arabinan are mostly (1→5) linked arabinofuranosyl units, but arabinofuranosyl units can also be connected to each other at O-2, O-3, and O-5, forming a group of branched arabinan (Grassin and Fauquembergue, 1996). The galactose units in galactans are joined by (1→4) linkages, however, (1→3) and (1→6) linkages can also occur (Thakur *et al.*, 1997). Two types of arabinogalactans (AG) are found in the side chains of pectin. The first AG is composed of β-(1,4)-D-galactan chains with α-L-arabinofuranosyl units attached to the O-3 of the galactosyl units. The second AG is composed of a β-(1,3)-galactan chain. This chain is substituted by short β-(1,6)-D-galactan chains, which carry additional branches of (1,3)-and/or (1,5) linked α-L-arabinofuranosyl residues (Grassin and Fauquembergue, 1996;Perez *et al.*, 2000). The side chains of RGI can vary depending on plant source, from primarily branched arabinans in sugar beet (Oosterveld *et al.*, 2000) to mainly linear galactans in potato (Øbro *et al.*, 2004). Figure 6 illustrates

the structure of pectin. Another structural type of pectin is rhamnogalacturonan II (RGII), which is a less frequent complex, highly branched polysaccharide. The backbone of RGII consists of galacturonic acid units and its side chains consist of rare glycosyl residues like apiose methyl-fucose and 3-deoxy-D-manno-octuosonic acid (Perez *et al.*, 2003).

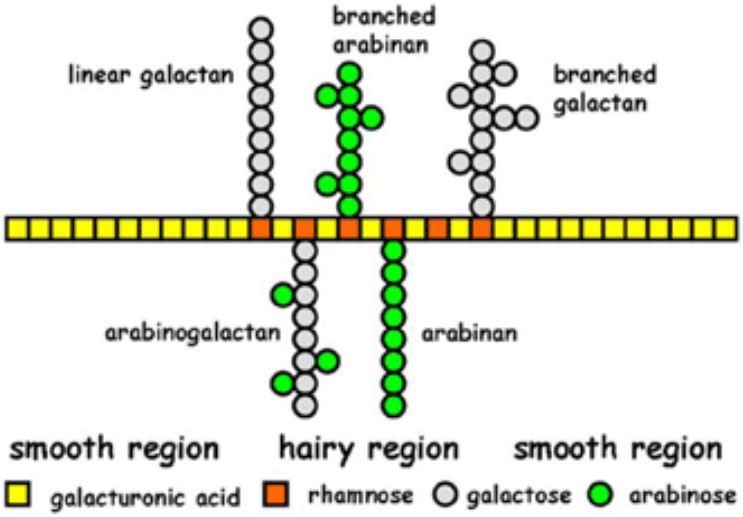

Figure 6: Structure of pectin demonstrating the smooth regions (homogalacturonan, HG) and hairy regions (rhamnogalacturonan I, RGI) with different side chains. Adapted from Gunning *et al.* (2009).

5.4. Production of poly- and oligosaccharides derived from pectin

Poly and- oligosaccharides can be made from pectins either by acid extraction or enzymatic hydrolysis (Rastall, 2007). Rhamnogalacturon and xylogalacturon pectic oligosaccharides and side chain arabino-oligosaccharides of pectin have previously been extracted from orange peel and sugar beet pulp, respectively, by acid extraction (Buchholt *et al.*, 2004;Rastall, 2007).

The hydrolysis of pectin can be performed using microbial enzymes which can catalyze the degradation of pectin structure. This degradation may give rise to differently structured oligosaccharides depending on the enzymes used. The backbone of pectin including HG and RGI can be hydrolyzed by a range of different enzymes. Polygalacturonase (EC 3.2.1.15) acts only on low-esterified pectins and catalyzes the hydrolysis of α-(1,4)-linkages of HG in an endo-acting manner (Grassin and Fauquembergue, 1996). Pectin lyase (EC 4.2.2.10) is a depolymerase of endo-type, which breaks bonds between methylated galacturonic residues in HG by β-elimination (Grassin and Fauquembergue, 1996). The backbone of RGI can be hydrolyzed by rhamnogalacturonan hydrolase (EC 3.2.1.-), which breaks the glycosidic bond of α-D-GalA-(1→2)-

α-L-Rhamnopyranosyl after acetyl groups have been removed (Schols *et al.*, 1990;Grassin and Fauquembergue, 1996). RGI can also be hydrolyzed by rhamnogalacturonan lyase (EC 4.2.2.-). The cleavage site and mechanism of the lyase differ from that of the hydrolase with cleavage of L-α-rhamnopyranosyl-(1→4)-α-D-GalA bonds by β-elimination (Mutter *et al.*, 1996).

The side chains of RGI can also be hydrolyzed by various enzymes. Arabinases (EC 3.2.1.99) catalyze the hydrolysis of α-(1,5)-linkages in linear arabinan in an endo-type manner, and arabinofuranosidases (EC 3.2.1.55) act in a exo-type manner, catalyzing the cleavage of α(1,2) and α(1,3) in arabinose residues (Grassin and Fauquembergue, 1996). Additionally, linear galactan can be degraded by galactanases (EC 3.2.1.89), which catalyze the endohydrolysis of (1→4)-β-D-galactosidic linkages, whereas β-galactosidases (EC 3.2.1.23) act on branched β(1,6) galactose residues (de Vries *et al.*, 2000).

A study by Al-Tamimi *et al.* (2006) has demonstrated that arabino-oligosaccharides from sugar beet pulp arabinan can be produced using the commercial enzyme Viscozyme® L (used as a cell wall lyase). Different fractions of oligosaccharides were obtained from the enzymatic hydrolysis. Each fraction contained a mixture of oligosaccharides with DP ranging from 2 to 8. Other studies have demonstrated production of pectic oligosaccharides from citrus (high methylated), apples (low methylated) (Olano-Martin *et al.*, 2001), and bergamot peel (Mandalari *et al.*, 2007) using endo-polygalacturonase. The produced oligosaccharides from citrus had DP ranging from 21-23 and 5-6, whereas the produced oligosaccharides from apples had DP of 20-21 (Olano-Martin *et al.*, 2001). The fraction of oligosaccharides obtained from bergamot peel had DP between 2 and 7 (Mandalari *et al.*, 2007).

So far, no human trials evaluating the prebiotic properties of poly- and oligosaccharides derived from pectin either in healthy subjects or UC patients exist (section 4.5), however, *in vitro* fermentation studies using fecal cultures from healthy humans have demonstrated that the oligosaccharides are able to show prebiotic properties by increasing the numbers of bifidobacteria, lactobacilli, and eubacteria (including *E. rectale*) (Olano-Martin *et al.*, 2002;Manderson *et al.*, 2005;Al-Tamimi *et al.*, 2006;Mandalari *et al.*, 2007). Additionally, Rhamnogalacturonans with a low degree of esterification and linear oligogalacturonids derived from sugar beet pectin have shown to have bioadhesive properties by adhering to porcine colonic

tissue. This was suggested to promote protection of epithelium against physical and microbial irritations in inflamed mucous membranes (Schmidgall and Hensel, 2002).

Methodology part

6. Methodology, considerations and choices

The methodologies used in the experimental part of this thesis were chosen in response to a number of circumstances and carefully balanced in order to meet the requirements of the overall project, this thesis is part of. The considerations and choices for the methods used are described in the following chapter.

6.1. *In vitro* fermentation systems

The preferred methods for testing prebiotic properties of novel carbohydrates are animals studies or best humans trials using microbiological and immunological analysis. However, if a high number of carbohydrates is to be tested both economically and ethically, *in vitro* experiments can be used for screening, giving first indications of prebiotic properties, even though the models have the limitations such as lack of host-bacterial interaction and absorptive processes (Rastall, 2007).

6.2. Batch static systems

In the present thesis, static batch systems were used to evaluate the prebiotic properties of novel carbohydrates derived from sugar beet and potato pulp pectin. However, several experimental parameters had to be determined prior to screening.

Reaction volume

The batch static system was chosen, since a large number of prebiotic candidates were to be tested and, additionally only a low amount of the developed compounds was available. This also meant that the reaction volume had to be scaled down to 2 ml (50 ml is often used (Barry *et al.*, 1995;Olano-Martin *et al.*, 2000;Karppinen *et al.*, 2000)), and hence non-pH controlled. Sanz *et al*. (2005) has previously developed a non-pH controlled microscaled batch system using 1 ml of reaction volume, which was validated against a pH-controlled batch system with larger reaction volume (150 ml). The down scaling of volume allowed testing of experimental saccharides, which were in limited supply.

Incubation time

An incubation time of 24 hours was used, based on preliminary growth experiments on FOS (5 g/l and 10 g/l) using pure cultures of strains from the genera *Lactobacillus*, *Bifidobacterium*, *Clostridium* and *Bacteroides*. The growth experiments showed that some of the species had a long lag-phase and needed a period of 24 hours to reach exponential growth. However, an incubation

time above 24 hours is not recommended, since fermentation products are not removed in batch systems, they can cause inhibition and result in a decline of microbial population and death of bacteria (Coles *et al.*, 2005).

Inocula

The samples used as inocula in *in vitro* fermentation systems are often fresh feces to insure viable bacteria cells. However, in the *in vitro* fermentation studies performed in this thesis, frozen fecal samples stored in glycerol (25%) were used (Paper 2-6). The use of frozen samples was due to different delivery days of samples from volunteers prior to the *in vitro* experiments. Use of frozen samples in *in vitro* fermentation studies has previously been validated by Rose *et al.* (2010). The study showed that during 150 hours of fermentation in a continuous system, the SCFA profiles were the same for samples originated from fresh or frozen feces. Furthermore, Rose *et al.* (2010) demonstrated that the composition of the microbiota in terms of viable cells was unaffected by freezing. This was in line with a previous study by Crowther (1971), who demonstrated that the viability of Gram-negative bacteria and Gram-positive bacteria was the same, no matter if the feces were frozen in glycerol or fresh.

Validation of the in vitro method

When using an *in vitro* system for screening, it has to be considered robust and repeatable, hence it needs to demonstrate reproducibility within reasonable limits of statistical variation (Coles *et al.*, 2005). Thus, repeatable *in vitro* experiments were conducted using the parameters mentioned above prior to the screening of prebiotic candidates described in the experimental part of this thesis. *In vitro* fermentations were carried out at five different occasions in an anaerobic cabinet (containing 10% H_2, 10% CO_2, and 80% N_2) using frozen fecal samples as inocula from three healthy subjects and FOS (5 g/l) as substrate in minimal medium. Each fermentation experiment of each subject was carried out in triplicates. After 24 hours of incubation, fermentation samples were taken out for DNA extraction, and levels of *Bacteroidetes*, *Firmicutes*, bifidobacteria and lactobacilli were quantified using quantitative Real-Time PCR (qPCR). The results demonstrated no significant difference in the levels of bacterial groups at the different occasions (Appendix 1). This implied that the fermentation set up was reproducible with a minimum of inocula from three different subjects. However, due to high inter-individual variation, additional subjects were added

to the system, if the amount of testing compound was sufficient. The *in vitro* fermentation procedure is further described in the following manuscripts; Paper 3 - 6.

6.3. Continues systems

Dynamic in vitro digestion methods are suited models to study the microbial ecology of the gastrointestinal tract. One of the most known physio-chemical models is the validated Simulator of the Human Intestinal Microbial Ecosystem (SHIME). This model is multi-compartmental and consists of a series of 5 vessels, simulating the stomach, small intestine and the three colon regions; ascending, transverse and descending (Molly *et al.*, 1993). Hence, measurements of fermentation activity and composition of the colon microbial community in the different colonic regions can be performed (Yoo and Chen, 2006). The SHIME is unable to simulate selective absorption of metabolites and fluids, and can only simulate the growth of the luminal bacteria, whereas the adhesion of bacteria to mucus is omitted. However, Abbeele *et al.* (2011a) have recently developed a dynamic *in vitro* gut model incorporating mucin-covered microcosms into the SHIME. This model named M-SHIME simulates both the lumen and mucus environment, and allows studying the mucosal microbiota and interaction between luminal and mucosal microbial communities. Other *in vitro* models have previously been used to screen the adhering potency of intestinal microbes. They include adhesion assays to various components of the intestinal surface: e.g. intestinal mucus (Ouwehand *et al.*, 2002b), mucins (Van den Abbeele *et al.*, 2009), colonic tissue (Ouwehand *et al.*, 2002a) or cell lines (Laparra and Sanz, 2009). However, they often only provide short-term information regarding axenic cultures and tend to ignore the microbial interaction between and within the luminal and mucosal microbiota.

The M-SHIME set up used in the experimental part of this thesis consisted of two vessels simulating the stomach and the small intestine, and six ascending colon vessels, which were run in parallel without the transverse and descending colon. All ascending colon vessels were modified by incorporating mucin-covered microcosms into the luminal suspension. The procedure for the M-SHIME is described in details in Paper 2.

6.4. DNA extraction

DNA extraction is a key factor affecting any approach for analyzing microbial diversity. Several methods have been reported for the isolation of microbial DNA from human stool samples (Machiels *et al.*, 2000;Clement and Kitts, 2000;Zhang *et al.*, 2006). However, problems can arise in

extraction methods and two of the major concerns important for accuracy of ecological studies are cell lysis efficiency and quality of DNA extracts (Li *et al.*, 2003). DNA from organisms, which cell walls are easily lysed (Gram-negative bacteria) can be sheared, if the extraction conditions are too rough. However, DNA can be difficult to recover from organisms with rigid cell walls (Gram-positive bacteria), if the cell lysis is insufficient. Additionally, it is important that extracted DNA is without unfavorable substances, because they may inhibit e.g. PCR amplification (Monteiro *et al.*, 1997;Lantz *et al.*, 1997). Mechanical disruption of human fecal samples prior extraction using QIAamp DNA stool MiniKit has previously shown to improve the amount and quality of DNA (Li *et al.*, 2007;Smith *et al.*, 2011). Thus, combination of these two methods allows effective lyses of bacteria cells and removal of organic contaminants leading to high quality DNA that can be further analyzed using down-stream molecular techniques (Li *et al.*, 2003). Prior to the *in vitro* studies performed in the experimental part of this thesis (Paper 2-6), the combined extraction method was validated by extracting DNA from both fermentation samples at 0 hours and fecal samples (n=6, healthy subjects) to ensure that this method could yield the same amount of DNA from the different sample types. Eight bacterial groups (four Gram-negative and four Gram-positive bacteria) were quantified by qPCR to compare DNA extracted from either fermentation or fecal samples. The results demonstrated no significant difference between the two sample types for the eight bacterial taxa (Appendix 2). Hence, the combined extraction method was used to extract DNA from both fecal and fermentation samples in the experiments of this thesis (Paper 2-6).

6.5. Quantitative Real-Time PCR

QPCR is a useful tool for quantitative detection of target sequences in e.g. bacterial community, due to its high sensitivity. In qPCR, the accumulation of product amplification is detected by the presence of a fluorescence reporter. The quantification is done by measuring the number of cycles required for fluorescent signal to reach a threshold level. The cycle number is proportional to the number of copies of DNA templates in a sample (Saunders, 2009). Different fluorescence reporters can be used in qPCR assay. One approached is the specific hybridization of a duallabelled TaqMan probe to PCR product. Another is based upon the binding of the fluorescent dye SYBR-Green into the PCR product (Ponchel *et al.*, 2003). SYBR-Green was used as fluorescent reporter in the qPCR assays in Paper 1-6.

Absolute or relative quantity can be used to calculate the level of bacterial target in a specific sample. Using absolute quantity, the amount of target bacterial nucleic acid is determined by an external standard, where it is important that the sequence of the standard is similar to the target sequence; hence the primer set binding site of the standard should be identical to that in the target sequence. Using relative quantity, the gene-of-interest is compared in relation to a reference gene. Hence, the target bacterial gene sequence is normalized against the total amount of bacterial gene sequences in a sample (Pfaffl *et al.*, 2009). This allows correction of differences in total DNA concentrations between individual samples. In the experiments of this thesis (Paper 1-6), we were interested in examining the difference in a given bacterial community between several samples rather than measuring the absolute quantity in each sample, consequently, relative quantities were used as calculation method for all qPCR data obtained in the experimental part. The procedure of qPCR and calculation of relative quantity are described in details in Paper 1-6.

Paper 1

Gram-negative bacteria account for differences in fecal microbiota between patients with ulcerative colitis and healthy controls.

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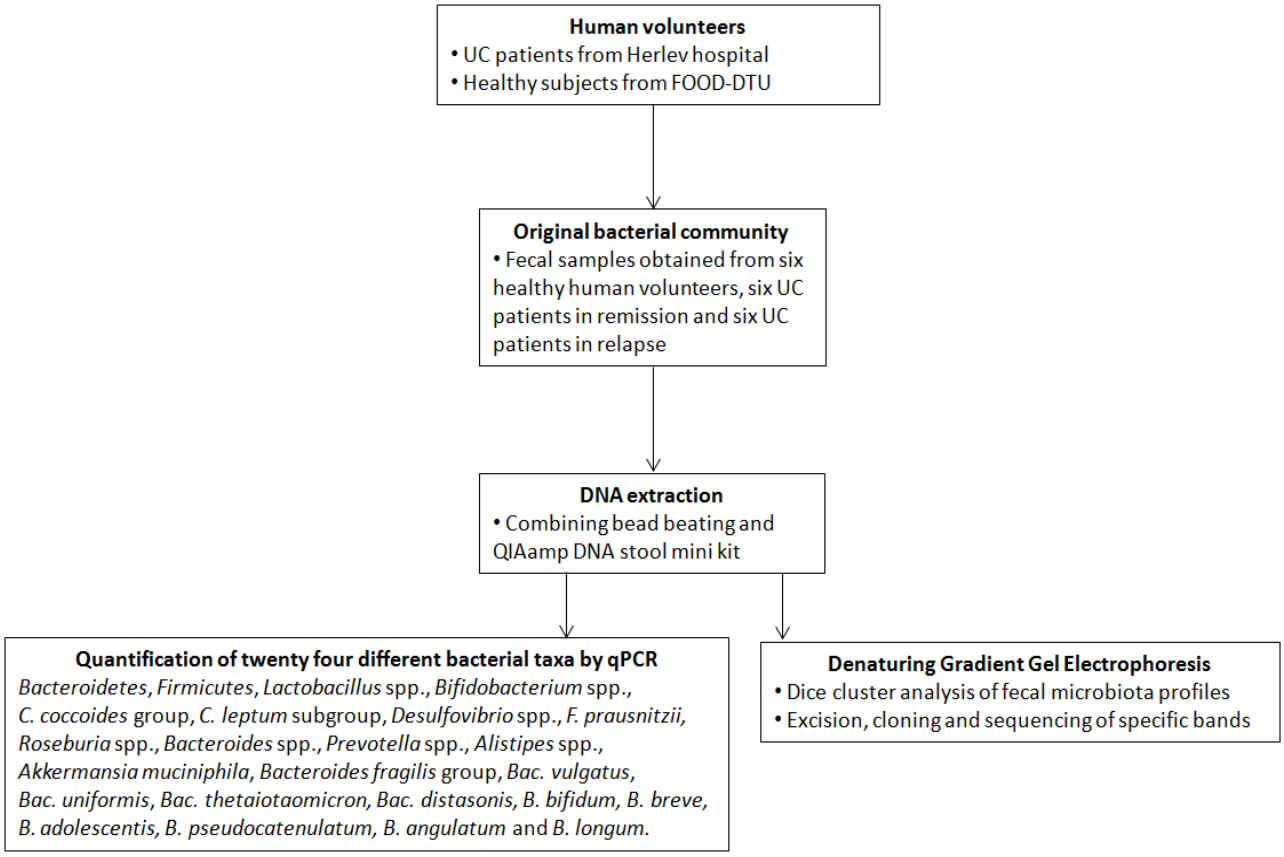
(Page numbers are relative to paper)

Introduction

The aim of this study was to compare the fecal microbiota derived from healthy controls, and UC patients in either remission or relapse.

Flow diagram



The author, Louise K. Vigsnæs, participated in the design of the study, conducted the experiments, performed the data analyzes and drafted the manuscript.

Gram-negative bacteria account for differences in fecal microbiota between patients with ulcerative colitis and healthy controls

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Key words: Ulcerative colitis, microbiota composition, Gram-negative bacteria, qPCR

Abstract

Background

Detailed knowledge about the composition of the intestinal microbiota may be critical to unravel the pathogenesis of ulcerative colitis (UC), a human chronic inflammatory bowel disease, since the intestinal microbes is thought to influence some of the key mechanisms that are involved in the inflammatory process of the gut mucosa. The aim of this study was to investigate the fecal microbiota in patients either with UC in remission, or with active disease, and in healthy controls. The composition of Gram-negative bacteria and Gram-positive bacteria respectively was examined, as antigenic structures of Gram-negative bacteria such as lipopolysaccharides have been related to the inflammatory responses and pathogenesis of inflammatory bowel disease.

Results

Dice cluster analysis and principal component analysis of fecal microbiota profiles obtained by Denaturing Gradient Gel Electrophoresis and quantitative PCR, respectively, revealed that the composition of fecal bacteria from UC patients differed from the healthy control group, and that this difference should be ascribed to Gram-negative bacteria, in particular members of the *Bacteroidetes* phylum, including the genera *Bacteroides* and *Prevotella*. The analysis did not show any clear grouping of the UC patients in remission with some samples resemble those of patients in relapse, while others resemble those of the healthy controls. *Lactobacillus* spp., *Akkermansia muciniphila* and *Bifidobacterium bifidum* were underrepresented in UC patients with clinically active disease compared to the healthy control group.

Conclusions

In line with previous communications, we have shown that the microbiota in UC patients differ from that in healthy controls. However, this is to our knowledge the first study to show a specific correlation of the composition of the Gram-negative population to this disease, and an underrepresentation of *Lactobacillus* spp., *Akkermansia muciniphila* and *Bifidobacterium bifidum* in UC patients. Additionally, the fact that some samples from patients in remission resembled those from healthy controls, while others resembled those from patients with active disease,

indicates that a change in the microbiota from 'healthy' to 'unhealthy' occurs during the remission period.

Background

Ulcerative colitis (UC) is an idiopathic inflammatory bowel disease (IBD) which is characterized by chronic inflammation of the colonic mucosa. UC is usually associated with recurrent attacks and complete remission of symptoms in the interim. The classical symptoms of patients in relapse are diarrhea with passage of blood or mucus, or both, occasional abdominal cramping and pain as well as systemic symptoms such as fever and weight loss in severe cases [2,13].

The aetiology of inflammatory bowel disease remains an enigma, and no known infectious agent has yet been demonstrated [30,44]. However, the intestinal microbiota seems to be involved in triggering inflammation, as observed in several animal models, where colitis could not be induced in the absence of a microbiota [45]. Hence, the intestinal microbes may influence some of the key mechanisms that are involved in the inflammatory processes of the gut mucosa [20,44]. Lipopolysaccharides (LPS) released by Gram- negative bacteria are able to trigger an inflammatory response through the nuclear factor κB (NF-κB) pathway leading to over-expression of pro-inflammatory cytokines [42]. However, certain Gram-positive bacteria, particularly bifidobacteria and lactobacilli, have been shown to have a beneficial therapeutic effect in inflammatory bowel disease [23,54]. Additionally, *in vitro* studies show that strains of lactobacilli and bifidobacteria do not possess any intrinsic pro-inflammatory characteristics but exert anti-inflammatory actions such as inhibition of NF-κB activation and IL-8 secretion [16,42]. Both Gram-negative and Gram-positive bacteria may therefore be involved in the pathogenesis of inflammatory bowel disease.

A recent study, including a large metagenomic sequencing approach, has shown that the fecal microbiota differs between subjects with UC and healthy controls [41]. However, only very few investigations [51,52] have so far examined if there are differences between the fecal microbiota from UC patients in relapse and in remission by quantitative methods.

The aim of our study was therefore to compare fecal microbiota derived from healthy controls, patients with UC in remission, and patients with active disease. Additionally, we wanted to investigate if the composition of Gram-negative bacteria and Gram-positive bacteria differed

depending the level of disease activity. Finally, and for the first time, the prevalences of seven selected species of *Bifidobacterium* in healthy controls and UC patients were under study. Denaturing Gradient Gel Electrophoresis (DGGE) and quantitative PCR (qPCR), both of which are culture-independent methods, were applied. Although not generating data as extensive as the costly metagenomic-analysis [41], these methods have recently proven very useful for analyzing the qualitative and quantitative diversity of human fecal microbial communities [8,56]. Additionally, qPCR is a useful tool for quantifying very low concentrations of bacterial targets [56,60]. The primers chosen for qPCR analysis targeted a broad range of selected bacteria taxa, presumed to play a role in the homeostasis of the colonic microbial ecosystem. Our finding should help elucidate compositional differences in the fecal microbiota in patients with ulcerative colitis and in healthy controls.

Methods

Subjects and fecal sampling

Fecal samples were obtained from 12 patients with UC and 6 healthy controls. Within the UC group, 6 patients were in clinical remission and 6 patients had active disease at the time of sampling according to clinical and endoscopical criteria [6]. The patients were previously diagnosed with UC according to standardised diagnostic criteria at the Department of Gastroenterology, Herlev Hospital [26]. The study was performed in accordance with the Second Helsinki Declaration, reported to the Danish Data Protection Agency and approved by the Regional Ethics Committee. Written, informed consent was obtained from each participant under a protocol approved by the Danish National Committee on Biomedical Research Ethics. Four of 6 patients with inactive UC received maintenance treatment with oral mesalazine in a dosage of 1.6-2.4 gram daily and one also azathioprine 100 mg daily. One patient received oral olsalazine (1 gram daily) and one no treatment. All six patients with active UC were treated with oral mesalazine in a dosage of 2.4-3.2 gram daily as well as topical mesalazine 1 gram daily either as an enema (n = 5) or as a suppository (n = 1). One patient also received azathioprine 100 mg daily. One patient had active extensive UC, one left sided colitis, and the rest either active proctitis or proctosigmoiditis. None of the participants had been treated with antibiotics for at least 2 months

before enrolment and there was no significant difference ($P > 0.10$) in the mean age of the participants comparing the 3 groups.

Sample collection and processing

The stool samples were collected at home by the participants in airtight containers and kept at 4 °C (limited storage time was encouraged [35]) until delivery to the laboratory, where they were processed immediately. 200 mg wet weight feces were collected in triplicates in the middle of each stool sample for DNA extraction.

Extraction of bacterial DNA from fecal samples

DNA was extracted from the feces samples using the QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) with an added bead-beater step as described previously [28]. The purified DNA was stored at – 20 °C until use.

PCR amplification for DGGE

Aliquots (10 µL) of purified DNA were applied to the following to give a 50 µL PCR reaction mixture: 20 µL of 5 PRIME MasterMix (2.5×) (VWR & Bie & Berntsen) and 10 pmol of each of the primers (Eurofins MWG Synthesis GmbH, Ebersberg, Germany). Universal bacterial primers HDA1-GC/HDA2 [55] targeting 16S rRNA genes were used in a touchdown PCR. Initial denaturation was at 96 °C for 5 min, amplification was carried out using 20 cycles including denaturation at 94 °C for 1 min, annealing at 65°C for 1 min decreased by 0.5°C for each cycle, and extension at 72°C for 1 min. This was followed by additional 5 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 1 min, and a final extension at 72°C for 5 min.

Analysis of fecal microbiota by DGGE

DGGE was carried out as described previously [5] using a DcodeTM Universal Mutation Detection System instrument and gradient former model 475 according to the manufacturer's instructions (Bio-Rad Labs, Hercules, California). The 9% polyamidegels were made with denaturing gradients ranging from 25% to 65%. The 100% denaturant solution contained 40% formamide and 7M urea. Thirteen microlitres PCR products were mixed with 3 µL loading dye before loading. Gels were run

in 1 x TAE at 60 °C for 16 h at 36 V, 28 mA, stained with ethidium bromide for 15 min, distained for 20 min, and viewed by UV-B trans illumination at 302 nm (Bio-Rad).

Analysis of DGGE patterns

The obtained DGGE patterns were analyzed using Bionumerics software version 5.0 (Applied Maths, Sint-Martens-Latem, Belgium). The triplicates taken from each fecal sample were analyzed to assess similarity of the three extractions. DGGE gels were normalized by an assigned marker (developed in our laboratory). A cluster analysis was performed based on Dice coefficient of similarity (weighted) with the unweighted pair group method with arithmetic averages clustering algorithm.

Excision, cloning and sequencing of selected bands from DGGE gels

Bands of specific interest from the human fecal microbiota profiles were excised from DGGE gels with a sterile razor, placed in 40 µL sterile water and incubated at 4 °C overnight for diffusion of DNA into the water. For sequencing of bands retrieved from the gels, the eluted DNA was used in a PCR with HDA1/2 primers without GC-clamp (4 min at 94°C, 20 cycles consisting of 30 s at 94°C, 30 s at 56°C, and 1 min at 68°C, and finally 7 min at 68°C). Subsequently, the PCR products were directly cloned into pCR® 4-TOPO (Invitrogen, Taastrup, Denmark) according to the manufacturer's instructions, and electroporated into electrocompetent *E. coli* TOP10 cells (Invitrogen) with a single pulse (2500 V, 400 Ω, 25 µF) by use of a Gene Pulser apparatus (Bio-Rad). Plasmid DNA was isolated from the cells using the Qiagen Mini Spin Prep kit, and subjected to PCR (HDA1/2-GC) as earlier described. The PCR product was run on a DGGE gel to check the purity and confirm the melting behaviour of the excised band. The inserts were sequenced by GATC (Konstanz, Germany) using primers T3 and T7. The obtained sequences were compared to known sequences in the Ribosomal Database (RDP, Michigan State University, Release 10), and aligned using BLAST (blastn) and the GenBank database.

Quantitative PCR assay conditions

Quantitative PCR was performed on an ABI Prism 7900 HT from Applied Biosystems. The amplification reactions were carried out in a total volume of 20 µL containing; 10 µL (EXPRESS SYBR® GreenER™ qPCR SuperMix, ROX, Invitrogen), primers (each at 200 nM concentration)

(Eurofins MWG Synthesis GmbH), 2 µL template DNA, and Nuclease-free water (Qiagen) purified for PCR. The amplification program consisted of one cycle at 50°C for 2 min; one cycle at 95°C for 10 min; 40 cycles at 95°C for 15 sec and 60°C for 1 min; and finally one cycle of melting curve analysis for amplicon specificity at 95°C for 15 sec, 60°C for 20 sec and increasing ramp rate by 2% until 95° for 15 sec.

Quantitative PCR primers

The primers specific to regions of the 16S rRNA genes of selected bacterial phyla- and groups, are listed in Table 1, while the species specific primers targeting either the 16S rRNA genes or the 16S-23S rRNA intergenic spacer region are listed in Table 2. Genus- and species specific primers for amplification of *Alistipes* spp. and *Bacteroides uniformis* were designed using 16S rRNA gene sequences from the EMBL-EBI database (http://www.ebi.ac.uk/ebisearch). Sequences were aligned by the BioEdit software (version 7.0.5.3; Ibis Biosciences, Carlsbad, CA) using ClusterW multiple alignment. The target-specific sites were assessed by FastPCR Software (version 6.1.9; Primer Digital Ltd., Helsinki, Finland). Specificity of the primers was evaluated *in silico* using the nucleotide BLAST, blastn algorithm (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The two primer sets were tested to confirm amplification of the 16S rRNA genes from the genomic DNA of *Bac. uniformis* (DSM 6597) and *Alistipes* spp., respectively. Additionally, the primer sets were tested to confirm that they did not amplify the 16S rRNA genes from species belonging to other phyla.

Quantitative PCR data handling

The relative quantities of gene targets encoding 16S rRNA gene sequences of the bacterial taxa were calculated using the $2^{\text{delta-delta Ct}}$ method according to Pfaffl [38], assuming primer efficiency at 1.0. The calculated results were analyzed as ratio of species specific 16S rRNA gene density relative to total bacterial 16S rRNA gene density in order to correct data for difference in total DNA concentration between individual samples. Standard curves were created using serial 10-fold dilutions of bacterial DNA extracted from one of the feces samples for all primer sets. Analysis of the standard curves allowed verification of PCR efficiency (limit 1.0±10%) for the chosen PCR conditions. All results were calculated as means of duplicate determinations, equal values required.

Statistics

PCA were generated by SAS JMP data analytical software (version 6.0.2; SAS Institute Inc., North Carolina, USA). Univariate ANOVA was used to analyze differences in ages and differences in DGGE bands of the three groups (version 5.03; GraphPad Software Inc., California, USA). Statistical analysis of the quantitative PCR data was performed with OriginPro software (version 8.1; OriginLab Corporation, Northampton, USA). Data was analyzed by univariate ANOVA. Where ANOVA indicated a significant difference multiple comparisons of means (Fisher's least significant different test) were used to determine significant differences among disease groups, assuming equal variances. The mean of the triplicate feces sample for each subject was used for the statistical calculations. PCR measurements were log-transformed before statistical analysis, if the data did not follow a normal distribution. Normal distribution was assessed using D'Agostino & Pearson omnibus normality test (GraphPad Prism version 5.03). Tests were considered statistically significant if P-values lower than 0.05 were obtained.

Results

DGGE analysis with universal primers and sequencing of DGGE bands

Microbial diversity of fecal samples was assessed by DGGE of 16S rRNA genes. The average number of bands (mean±SD) in the fecal profiles from the three groups (healthy 10.0±2.0, UC patients in remission 9.0±2.7 and UC patients in relapse 9.8±2.7) did not differ significantly ($P > 0.10$). Additionally, Dice cluster analysis revealed that the triplicates taken from each individual fecal sample were clustered together, demonstrating that the three extractions from each sample were similar (data not shown). Additionally, Dice cluster analysis was performed using one of the triplicate samples from each individual (See additional file 1). The dendrogram from this Dice cluster analysis showed two large clusters revealing that the DGGE profiles from the UC patients are more similar to each other than to the healthy controls. Cluster I contains ten samples all from UC patients, Cluster II contains five samples from healthy controls and two from UC patients, and a single (healthy) sample does not belong to either of the clusters. Part of this effect could be explained by the occurrence of three prominent bands present in either the profile of the healthy

controls or the UC patients. Band no. 1 was more prevalent in the profiles of the UC patients in relapse (5 out of 6 individuals) compared to the profiles of the healthy controls (2 out of 6 individuals) and the UC patients in remission (1 out of 6 individuals). Sequencing of the band revealed that it represented a species within the Gram-negative genus *Bacteroides* (See additional file 2). Band no. 2 was more prevalent in the healthy control profiles (4 out of 6 individuals) compared to the other two UC profiles (remission; 2 out of 6 individuals and relapse; 2 out of 6 individuals). Sequencing of the band revealed that it represented a species belonging to the gram-negative genus *Alistipes* (See additional file 2). Band no. 3 was more prevalent in the UC profiles (remission; 4 out of 6 individuals and relapse; 4 out of 6 individuals) compared to the profiles of the healthy controls (0 out of 6 individuals). The band was found to represent members of the Gram-negative genus *Prevotella* (See additional file 2).

Principal component analysis of the relative quantities of the fecal microbiota

QPCR was applied for measurement of 16S rRNA gene and 16S-23S intergenic spacer region content of selected bacterial taxa. The data were analysed using PCA. As seen on Figure 1, the PCA plots of UC patients in remission indicated only low levels of systematic variation, with two patients showing similar profile as the healthy controls, and four patients showing similar profile as the UC patients in relapse. However, a separation between the healthy controls and the UC patients in relapse could be observed from PC1 and PC2 (29.37% and 21.82% of explained variance, respectively). This was attributed to *Prevotella* spp. in the first direction (PC1) in combination with *Bacteroides* spp., *Bacteroides uniformis*, *Bacteroides fragilis* and *Bacteroides thetaiotaomicron* versus *Bacteroidetes*, *Bacteroides distasonis*, *Akk. muciniphila* and *Lactobacillus* spp. in the second direction (PC2).

PCA of the Gram-negative bacteria and Gram-positive bacteria showed a complete separation between the healthy control group and UC patients in relapse with respect to the composition of the Gram-negative bacteria (Figure 2). The variation was mainly attributed in the second direction (PC2) with a higher positive score for *Bacteroidetes*, *Akk. muciniphila*, *Desulfovibrio spp.*, and *Bac. distasonis* in the healthy controls, versus *Bacteroides* spp., *Prevotella* spp., *Bac. thetaiotaomicron* and *B. uniformis* in the UC patients in relapse. PCA of the UC patients in remission showed no clear grouping of the subjects.

The PCA of the Gram-positive bacteria showed no distinct clustering and no association of the UC patients group with particular bacterial genera was apparent (data not shown).

Statistical analysis of the relative quantities of the fecal microbiota

The relative quantities of the bacterial taxa obtained by qPCR (Table 3) revealed that the density of the *Lactobacillus* spp. was significantly lower in the UC patients in relapse than measured in the healthy controls ($P < 0.05$). The density of the butyrate-producing bacterial group, *Clostridium leptum* subgroup was not significantly different between the three groups. However, there was a trend showing lower densities of *Clostridium leptum* subgroup in the UC patients in remission and relapse than in the healthy controls ($P < 0.10$). The overall density of the bacteria belonging to the *Bacteroidetes* phylum was significantly lower in the UC patients in remission than in the healthy controls ($P < 0.05$) and there was a trend of lower levels of *Bacteroidetes* in the UC patients in relapse than in the healthy controls ($P < 0.10$).

Presence of *Akk*. *muciniphila* was detected only in three out of six fecal samples from UC patients in remission and three out of six fecal samples from UC patients in relapse, but in all the fecal samples (six out of six) of the healthy controls. Moreover, the average density of *Akk*. *muciniphila* in the three UC patients in relapse, in which it could be detected, was significantly lower than the average density of this species in the healthy controls ($P < 0.05$). No significant difference in *Akk*. *muciniphila* density was observed between the healthy controls and the UC patients in remission, in which it could be detected. The density of *Bac. distasonis* was not significantly different between the three groups. However, there was a trend showing lower densities in the UC patients in remission and relapse than in the healthy controls ($P < 0.10$).

In the UC patients in relapse the density of *Prevotella* spp. was slightly but not significantly increased compared to the healthy controls ($P < 0.10$). The relative quantities of *Bacteroides* spp., *Bac. uniformis,* and *Bac. thetaiotaomicron* were somewhat higher in the UC patients in relapse compared to the healthy controls, although no significant difference could be measured ($P > 0.10$).

Prevalence of selected *Bifidobacterium* species in fecal samples

The prevalence of six selected *Bifidobacterium* species, commonly detected in human feces, is shown in Figure 3. Interestingly, *Bifidobacterium bifidum* was present in 2/6 healthy controls, and

in 4/6 UC patients in remission, but was not detectable in any of the 6 UC patients in relapse. In contrast, prevalences of *Bifidobacterium adolescentis, Bifidobacterium catenulanum*, and *Bifidobacterium longum* did not appear different between the three groups of samples. *Bifidobacterium breve* and *Bifidobacterium angulatum* were not detected in any of the 18 samples investigated.

Discussion

Since the intestinal bacterial microbiota is believed to represent an important environmental factor playing a pivotal role in the pathogenesis of UC, a thorough understanding of the intestinal microbial composition is of major importance for unravelling mechanisms responsible for flare-ups of the disease. Cluster analysis of PCR-DGGE-based fingerprint and qPCR based PCA of the fecal communities revealed a different microbiota in UC patients compared to healthy controls, which is in good agreement with previous reports [15,41,51,52]. Differences in the DGGE-based profiles could be explained mainly by variations within the genera *Bacteroides*, *Prevotella* and *Alistipes* (See additional file 2). Additionally, PCA of the qPCR data revealed that the difference between UC patients in relapse and healthy controls was most apparent within the targeted Gram-negative bacteria and that *Bacteroidetes*, *Akk. muciniphila*, *Desulfovibrio spp.*, and *Bac. distasonis* versus *Bacteroides* spp., *Prevotella* spp., *Bac. thetaiotaomicron* and *Bac. uniformis* accounted for the separation (Figure 2). These data imply that the composition and abundance of Gram-negative bacteria, particularly those belonging to the *Bacteroidetes* phylum, may contribute to dysbiosis in colitis patients. A potent stimulator of inflammation from Gram-negative bacteria is LPS. Hence, an unfavourable composition of the Gram-negative bacteria may enhance abnormal LPS signaling and contribute to the sustained mucosal inflammation in UC patients.

The mucin-degrading bacterium *Akk. muciniphila*, which is a common member of the human microbiota has previously been reported as being involved in the pathogenesis of intestinal diseases [7,9,39]. In this study, a significantly lower density of *Akk. muciniphila* was found in UC patients in relapse than in the healthy control group. The reduced density of *Akk. muciniphila* could be a consequence of the reduced mucus layer in UC patients, since mucins constitute the primary carbon source of this species [53]. Pullan and coworkers [40] examined the colonic mucus

layer of UC patients and found that the mucus layer of the left colon and rectum was significantly thinner in the UC patients compared to the control group, and that areas of acute inflamed mucosa were denuded of mucus layer. It has previously been hypothesized that *Akk. muciniphila* could be a causal factor for intestinal inflammation, since its presence leads to degradation of the protective mucin layer [12,58]; however, our observations contradict this hypothesis.

Lactobacillus spp. was significantly less abundant in fecal microbiota of UC patients in relapse than in that of healthy controls (Table 3). To our knowledge, this has not previously been reported. Species of *Lactobacillus*, although constituting less than 2% of the total intestinal population, are considered to be beneficial commensal enteric bacteria and have, accordingly, been shown to exert anti-inflammatory effects [36,43,50,57]. Animal studies using probiotic strains have shown that certain lactobacilli down regulate pro-inflammatory cytokines, prevent chemical-induced colonic damage and reduce colonic permeability [25,36,37]. Additionally, a previous study has shown, that increased levels of lactobacilli can help maintain remission in UC patients [59]. Hence, the reduced content of *Lactobacillus* spp. in UC patients in relapse could have contributed to the flare-up, or alternatively these populations may have been reduced as a consequence of the inflammatory condition. To address this issue, long term studies, where patients are followed regularly in order to detect subtle changes in the intestinal microbiota prior to development of symptoms clinical activity, are needed.

A reduced level of the *C. leptum* subgroup (include potentially important butyrate producers) has previously been suggested to play a role in the onset of IBD flare-ups [51,52]. The protective role of *C. leptum* subgroup could be through their ability to induce regulatory T cells in the colon and through their metabolite, butyrate that can down regulate pro-inflammatory cytokine production via inhibition of NF-κβ. Both mechanisms stimulate an anti-inflammatory state in the colon [4,17,46]. However, possibly because of the relatively low number of patients investigated, we found no differences in the density of *C. leptum* subgroup between the three groups of subjects included in the present study. Nevertheless, there was a trend that the *C. leptum* subgroup was less abundant in UC patients in remission (P=0.08) as well as in those with relapse (P=0.09) compared to controls.

A recent metagenomic study has demonstrated the existence of enterotypes in the human gut microbiome and identified three of them that varied in species and functional composition. Each

of the enterotypes was formed by three distinct clusters. Two of these clusters are rich in *Bacteroides* and *Prevotella* (enterotype 1 and enterotype 2, respectively), whereas the third cluster is richer than enterotype 1 and enterotype 2 in *Ruminococcus* (Clostridiales order), *Alistipes* and *Akk. muciniphila* (enterotype 3) [3]. The present study demonstrated a predominance of *Bacteroides* spp. and *Prevotella* spp. in UC patients in relapse and a predominance of the *C. leptum* subgroup, *Akk. muciniphila* and *Lactobacillus* spp. in the healthy controls (Figure 1 and Table 2). This suggests that the enterotypes could be correlated with UC risk of individuals. *Bifidobacterium* spp. are believed to play an important role in maintaining intestinal health [24,31,34]. In our study, *B. bifidum* was completely absent in the UC patients in relapse, while frequently occurring in the other groups. Previous *in vitro* studies have shown that several strains of bifidobacteria, with *B. bifidum* being most effective, have an antagonistic potential on LPS-induced inflammatory responses by blocking NF-κB activation in intestinal epithelial cells [16,42,49]. Hence, a reduced level of *B. bifidum* and an altered composition of the Gram-negative bacteria in UC patients in relapse compared to healthy controls, as observed in this study, may favor intestinal inflammation.

Conclusions

Based on the results presented within this study, we conclude that UC patients in relapse appear to have a different fecal microbiota than healthy controls and this difference could be ascribed to the Gram-negative bacteria, especially members of the *Bacteroidetes* phylum and *Akk. muciniphila*. Some samples from patients in remission resemble those of patients in relapse, while others resemble those of the healthy controls, indicating that the microbiota is unstable and perhaps changes during the remission period, which, in theory, may contribute to provoke a new flare-up. Our findings indicate that an alteration in the composition of the Gram-negative bacteria and a reduction of the beneficial Gram-positive bacteria lactobacilli and *B. bifidum* plays a role in provoking flare-ups in UC, but do not prove whether the observed changes are causal to or rather results from the relapse condition. Further longitudinal studies are warranted to extend the present findings and address this issue.

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Table 1 - 16S rRNA primers used in this study

Target taxon	Primer	Sequence (5'-3')	Fragment size (bp)	Reference
Bifidobacterium spp.	F-bifido	cgc gtc ygg tgt gaa ag	244	[10]
	R-bifido	ccc cac atc cag cat cca		
Lactobacillus spp.	Lacto-F	agc agt agg gaa tct tcc a	341	[21,55]
	Lacto-R	cac cgc tac aca tgg ag		
Clostridium leptum subgroup	sg-Clept-F	gca caa gca gtg gag t	239	[32,48]
	sg-Clept-R	ctt cct ccg ttt tgt caa		
Clostridium coccoides group	g-Ccoc-F	aaa tga cgg tac ctg act aa	440	[32]
	g-Ccoc-R	ctt tga gtt tca ttc ttg cga a		
Roseburia spp.	RosF	tac tgc att gga aac tgt cg	230	[27]
	RosR	cgg cac cga aga gca at		
Firmicutes phylum	Firm934F	gga gya tgt ggt tta att cga agc a	126	[18]
	Firm1060R	agc tga cga caa cca tgc ac		
Bacteroidetes phylum	Bact934F	gga rca tgt ggt tta att cga tga t	126	[18]
	Bact1060R	agc tga cga caa cca tgc ag		
Bacteroides spp.	BacAll_2F	gtc agt tgt gaa agt ttg c	127	[1]
	BacAll_2R	caa tcg gag ttc ttc gtg		
Prevotella spp.	PrevF	cac caa ggc gac gat ca	283	[27]
	PrevR	gga taa cgc cyg gac ct		
Alistipes spp.	Alis F1-124	tta gag atg ggc atg cgt tgt	320	This study
	Alis R1-423	tga atc ctc cgt att acc gcg		
Bac. fragilis group	Bfr -F	ctg aac cag cca agt agc g	230	[29]
	Bfr -R	ccg caa act ttc aca act gac tta		
Desulfovibrio spp.	DSV691-F	ccg tag ata tct gga gga aca tca g	136	[14]
	DSV826-R	aca tct agc atc cat cgt tta cag c		
V2-V3 16S rRNA region*	HDA1	act cct acg gga ggc agc agt	200	[55]
	HDA2	gta tta ccg cgg ctg ctg gca c		
16S rRNA region*	TBA-F	cgg caa cga gcg caa ccc	130	[11]
	TBA-R	cca ttg tag cac gtg tgt agc c		

*The HDA and TBA primer were used as total bacteria DNA targets in order to normalize, hence correcting differences in total DNA concentration between individual samples.

Table 2 - 16S rRNA gene and 16S-23S rRNA intergenic spacer region of species specific primers used in this study

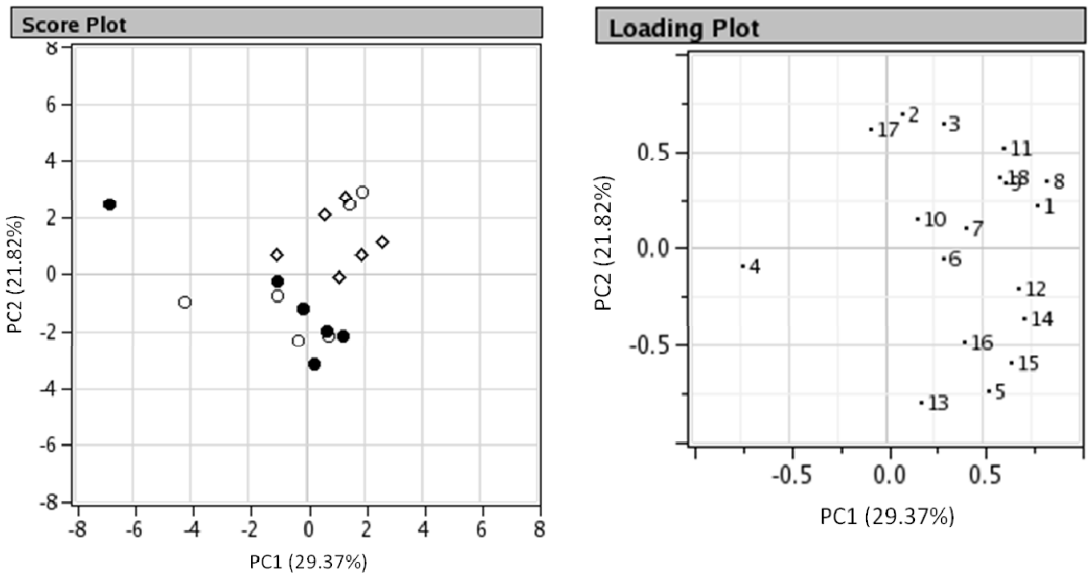
Target taxon	Primer	Sequence (5'-3')	Fragment size (bp)	Reference
Faecalibacterium prausnitzii *	Fprau 07	cca tga att gcc ttc aaa act gtt	140	[51]
	Fprau 02	gag cct cag cgt cag ttg gt		
*Akkermansia muciniphila**	AM1	cag cac gtg aag gtg ggg ac	327	[9]
	AM2	cct tgc ggt tgg ctt cag at		
*Bifidobacterium bifidum**	BiBIF-1	cca cat gat cgc atg tga ttg	278	[33]
	BiBIF-2	ccg aag gct tgc tcc caa a		
*Bifidobacterium breve**	BiBRE-1	ccg gat gct cca tca cac	288	[33]
	BiBRE-2	aca aag tgc ctt gct ccc t		
*Bifidobacterium adolescentis**	BiADO-1	ctc cag ttg gat gca tgt c	279	[33]
	BiADO-2	cga agg ctt gct ccc agt		
*Bifidobacterium angulatum**	BiANG-1	cag tcc atc gca tgg tgg t	275	[33]
	BiANG-2	gaa ggc ttg ctc ccc aac		
*Bifidobacterium pseudocatenulatum/B. catenulatum**	BiCATg-1	cgg atg ctc cga ctc ct	289	[33]
	BiCATg-2	cga agg ctt gct ccc gat		
Bifidobacterium longum#	F_long_IS	tgg aag acg tcg ttg gct tt	109	[19]
	R_long_IS	atc gcg cca ggc aaa a		
*Bacteroides vulgatus**	B.vulga-F	aag gga gcg tag atg gat gtt ta	192	[22]
	B.vulga-R	cga gcc tca atg tca gtt gc		
*Bacteroides uniformis**	B.uni F2	ata acg agc gca acc ctt atc	190	This study
	B.uni R2	tta ggg att agc atc acg tcg		
*Bacteroides thetaiotaomicron**	B_theta F	cgt tcc att agg cag ttg gt	110	[47]
	B_theta R	aca cgg tcc aaa ctc cta cg		
Bacteroides distasonis#	Bdis-F	tga tcc ctt gtg ctg ct	220	[29]
	Bdis-R	atc ccc ctc att cgg a		

*Primers targeting the 16S rRNA gene
#Primers targeting the 16S-23S intergenic spacer region

Table 3 - Relative fold change of bacteria target from UC patients compared to healthy controls (Set to 1).

Bacteria taxa	Remission	Relapse
Firmicutes phylum	1,08±0,42	0,79±0,15
Bacteroidetes phylum	0,61±0,12*	0,65±0,11#
Bacteroides spp.	1,38±0,45	1,71±0,49
Prevotella spp.	1,35±0,43	1,96±0,79#
Alistipes spp.	0,94±0,49	0,97±0,48
C. leptum subgroup	0,55±0,17#	0,56±0,13#
C. coccoides group	1,40±0,47	0,90±0,19
Roseburia	0,77±0,16	0,71±0,23
Desulfovibrio spp.	0,84±0,15	0,73±0,08
Lactobacillus spp.	0,95±0,08	0,79±0,04*
Bifidobacterium spp.	1,07±0,05	0,88±0,15
F. prausnitzii	0,87±0,12	0,84±0,11
Akk. muciniphila	0,88±0,13	0,69±0,18*
Bac. fragilis group	0,86±0,35	1,11±0,32
Bac. vulgates	0,89±0,09	0,91±0,12
Bac. uniformis	0,90±0,26	1,54±0,45
Bac. thetaiotaomicron	0,85±0,15	1,25±0,24
Bac. distasonis	0,61±0,13#	0,57±0,19#

All numbers are average ± SEM of the six samples in each group.
Asterisks (*) designates a significant difference from the healthy control group ($P < 0.05$), while pound signs (#) designates a trend suggesting difference from the healthy control group ($0.05 < P < 0.10$).



1. *Firmicutes* phylum
2. *Bacteroidetes* phylum
3. *Akkermansia muciniphila*
4. *Prevotella* spp.
5. *Bacteroides* spp.
6. *Alistipes* spp.
7. *Clostridium coccoides* group
8. *Clostridium leptum* subgroup
9. *Desulfovibrio* spp.
10. *Roseburia* spp.
11. *Lactobacillus* spp.
12. *Bifidobacterium* spp.
13. *Bacteroides uniformis*
14. *Bacteroides vulgatus*
15. *Bacteroides fragilis group*
16. *Bacteroides thetaiotaomicron*
17. *Bacteroides distasonis*
18. *Faecalibacterium prausnitzii*

Figure 1. Principal component analysis of the quantitative PCR measurements using the first and second principal component (PC1and PC2). The sources of the communities are indicated by diamond for healthy subjects, open circles for UC patients in remission, and closed circles for UC patients in relapse. Numbers in the loading plot indicate the listed bacterial taxa used in this analysis.

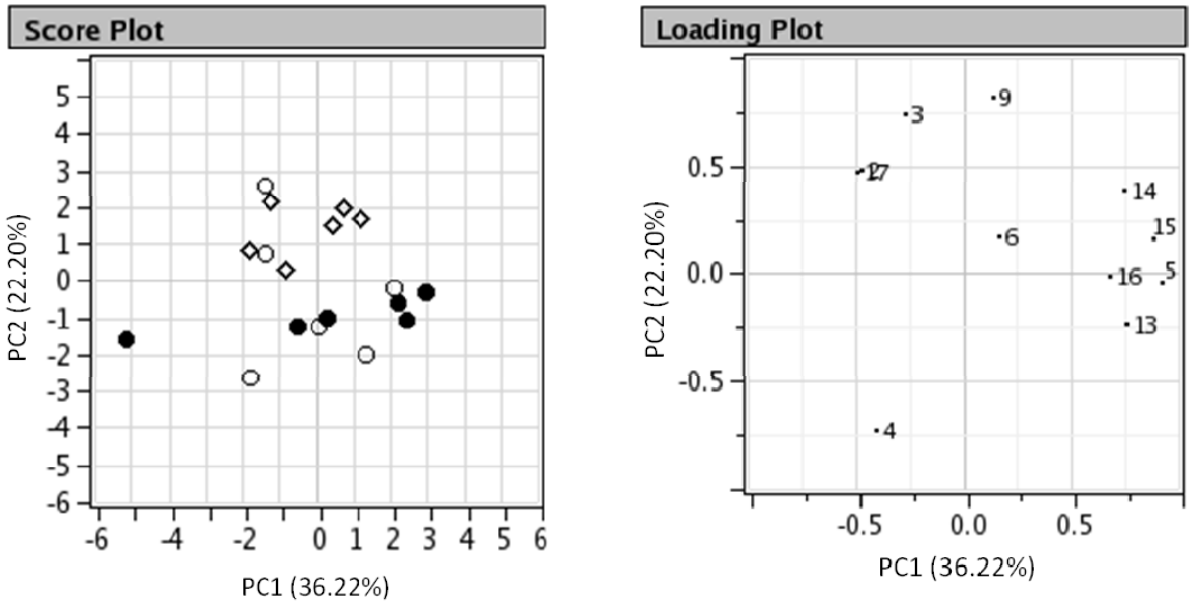


Figure 2: Principal component analysis of the quantitative PCR measurements for Gram-negative bacteria. The amount of variability accounted for by PC136.22% and PC2 22.20%. The sources of the communities are indicated by diamond for healthy subjects, open circles for UC patients in remission, and closed circles for UC patients in relapse. Numbers in the loading plot indicate the bacterial taxa listed in Figure 1.

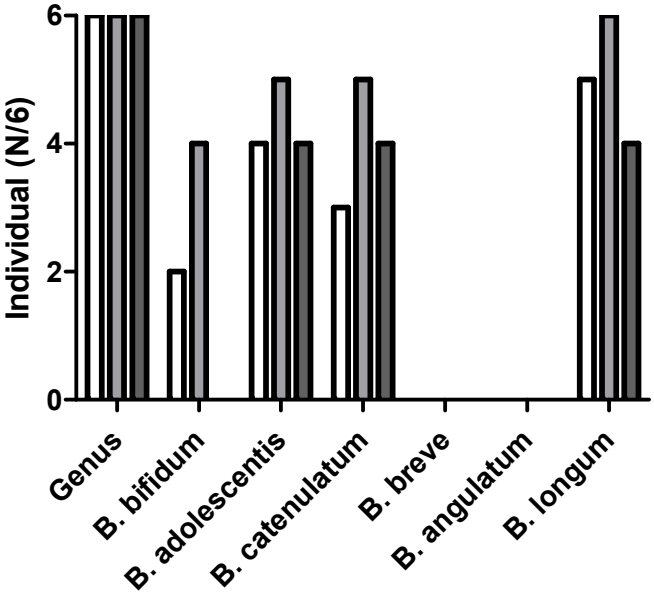


Figure 3: Prevalence of six selected *Bifidobacterium* species in the individual faecal samples from healthy controls (white), UC patients in remission (light grey) and UC patients in relapse (dark grey). *B. catenulatum* is both *B. catenulatum* and *B. pseudocatenulatum*

Supplementary

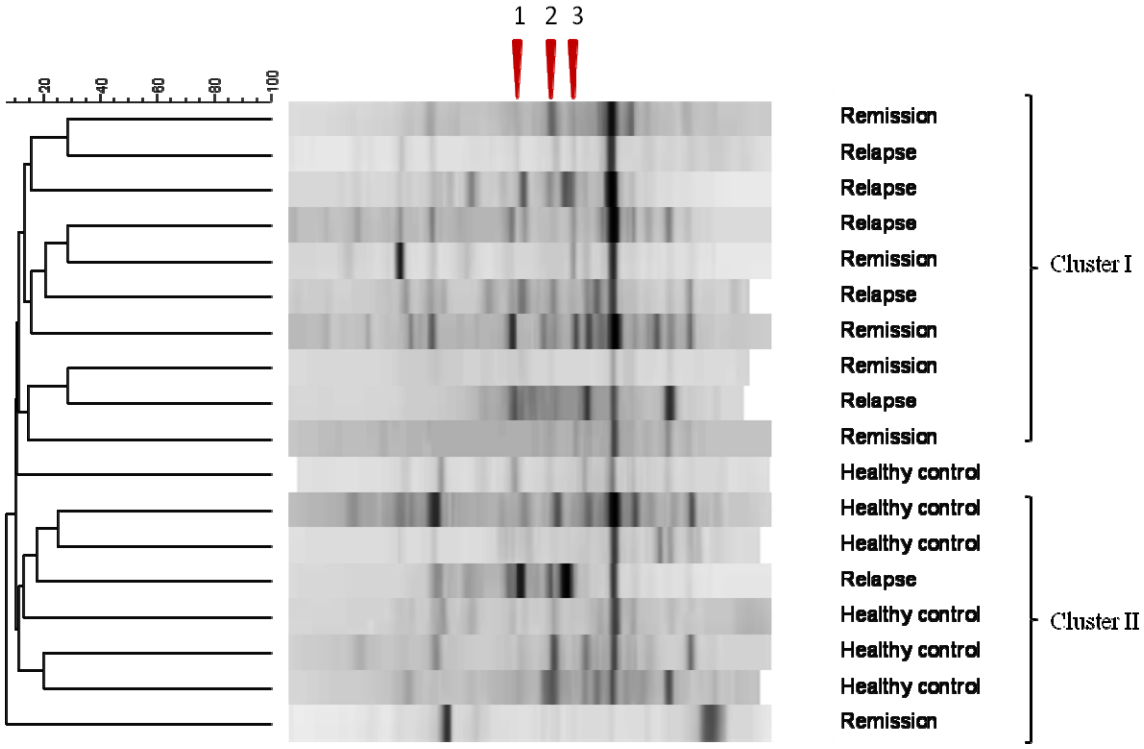


Figure S1. Dice cluster analysis of universal DGGE gel profiles from faecal content of healthy controls, UC patients in remission or relapse. Bands indicate by red arrows represents *Bacteroides* (1), *Alistpes* (2), and *Prevotella* (3). Metric scale indicates degree of similarity in percentage.

Table S1 - Results of sequence analysis of bands excised from DGGE and their closes related bacterial sequences based on the RDP database

Band no.	Fragment size (bp)	Presence of band in individuals of each group		Phylum	Genus	Sequence identity (%)*
1	169	Healthy	2/6	*Bacteroidetes*	*Bacteroides*	94
		Remission	1/6			
		Relapse	5/6			
2	169	Healthy	4/6	*Bacteroidetes*	*Alistipes*	97
		Remission	2/6			
		Relapse	2/6			
3	169	Healthy	0/6	*Bacteroidetes*	*Prevotella*	97
		Remission	4/6			
		Relapse	4/6			

*Percentage of identity between the excised bands sequences and sequences found on the RDP database

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Paper 2

Fecal lactobacilli and bifidobacteria from ulcerative colitis patients display reduced ability to colonize mucus in the M-SHIME.

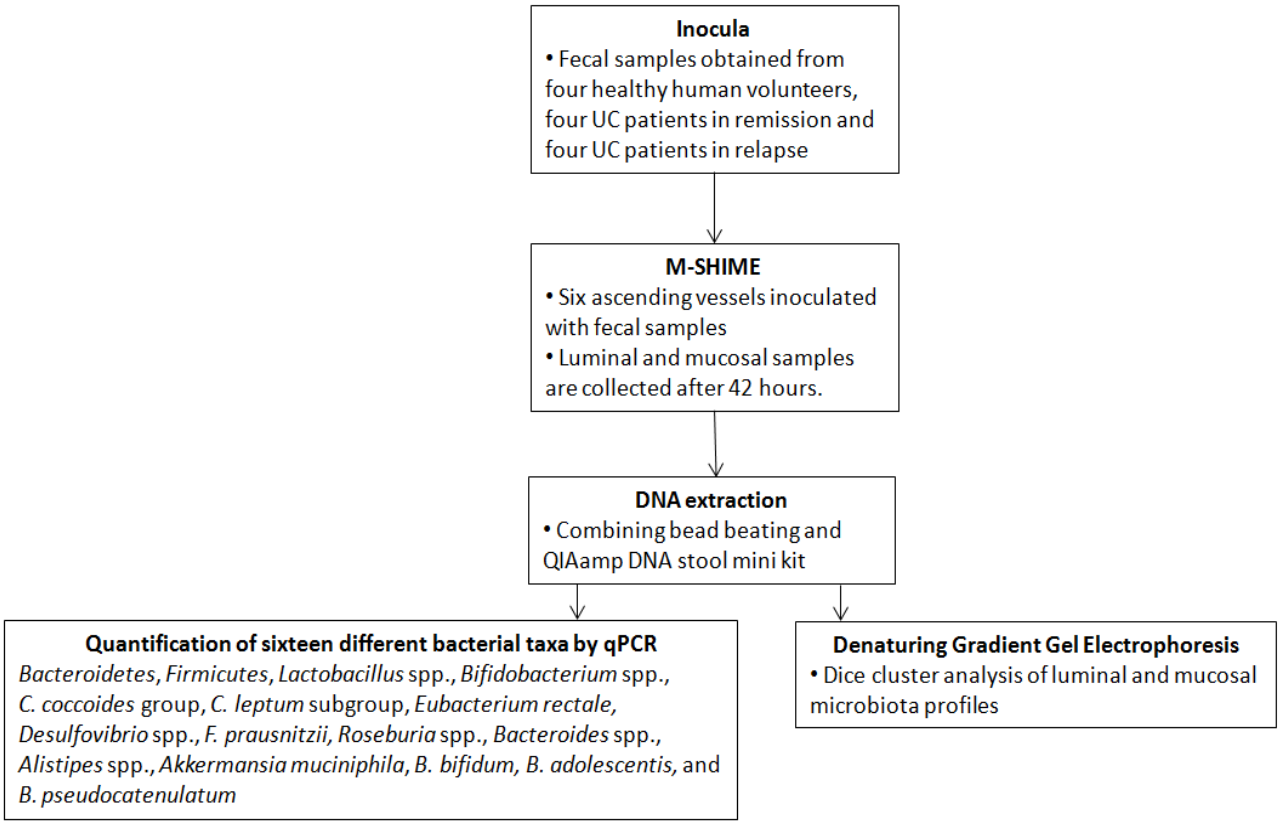
Paper in preparation

(Page numbers are relative to paper)

Introduction

The aim of this study was to examine the ability of fecal microbiota derived from UC patients in either remission or relapse, and healthy subjects to colonize an artificial mucus layer using a dynamic *in vitro* gut model.

Flow diagram



The author, Louise K. Vigsnæs, participated in the design of the study, conducted the experiments, performed the data analyzes and drafted the manuscript.

The manuscript is still in a preliminary state. The following analyzes and data treatments will be added in the final version:

- Growth curves for sixteen different bacterial taxa (see flow diagram): Samples were taken out at 0, 18, 26 and 42 hours from the lumen. Louise K. Vigsnæs is conducting these analyzes.
- SCFA analysis for acetate, butyrate and propionate: Samples were taken out at 0, 1.5, 3, 5, 18, 20, 22, 23.5, 25, and 42 hours. Louise K. Vigsnæs and Pieter Van den Abbeele are conducting these analyzes.
- Mass spectrometry (MS) on samples from the lumen and mucus (42 hours). Karolina Sulek is conducting the MS.

Fecal lactobacilli and bifidobacteria from ulcerative colitis patients display reduced ability to colonize mucus in the M-SHIME.

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Key word: Ulcerative colitis, mucus colonization, qPCR, bifidobacteria, lactobacilli, butyrate-producing bacteria, dynamic gut model.

Abstract

Mucus is secreted by goblet cells in the colon and is rich in gel-forming glycoproteins such as mucins. The mucus layer serves as a defense barrier, which separates the luminal bacterial residents and pathogens from the underlying epithelium. The aim of our study was to elucidate the ability of fecal bacteria derived from UC patients in remission (n=4) or relapse (n=4) and from healthy subjects (n=4), to colonize the mucus layer. For this purpose, we used a novel dynamic *in vitro* gut model (M-SHIME), adapted from the validated Simulator of the Human Intestinal Microbial Ecosystem (SHIME) by incorporation of mucin-covered microcosms. Denaturing Gradient Gel Electrophoresis (DGGE) and quantitative Real-Time PCR (qPCR) were used to analyze the composition of the 'luminal' and 'mucosal' microbiota after 42 hours colonization in the dynamic gut model. Cluster analysis of PCR-DGGE-based fingerprints as well as Principal Component Analysis (PCA) of qPCR data revealed that the microbiota of the mucus largely differed from that of the lumen. This difference was mainly explained by differences occurring within the groups of lactic acid bacteria and butyrate-producing bacteria. Additionally, qPCR data revealed that lactobacilli and bifidobacteria from UC patients (especially in relapse) had a significantly decreased capacity to colonize intestinal mucus compared to those from healthy subjects. Our results thus suggest that the ability of certain fecal bacteria to colonize the mucosal environment is reduced in UC patients in relapse but only to some extent in UC patients in remission, which implies that the inflammatory state may have an influence on microbial adhesion capacity or vice versa.

Introduction

The mucus layer lining the epithelium of the gastrointestinal tract is important for the protection of the epithelium in humans. The colonic mucus constitutes a defense barrier, which separates the intestinal bacterial residents and pathogens from the underlying epithelium. The mucus layer is rich in gel-forming mucins, glycoproteins, which provides a matrix for retention of antimicrobial peptides and immunoglobulins including IgA secreted by the host [29,42] Commensal bacteria have been found to colonize the colonic mucus layer. However, bacteria are most abundant in the outer layer of the mucus at the luminal side [22,23]. Previous studies have shown that the microbial community found in the colonic mucus differs from that of the luminal community [13,67]. Several microbial characteristics have contributed to the evolvement of this specifically selected mucosal community. These include the ability of the bacteria to utilize mucin glycans as energy source, and resistance to nonspecific antimicrobial peptides and specific antimicrobial immunoglobulins produced by the host [23,25]. Additionally, some bacteria express adhesion molecules enabling them to bind to the mucin. Many of the adhesion molecules expressed by the colonic bacteria have mucin glycans as specific epitopes [27,49]. It has previously been suggested that the glycosylation pattern in mucin, hence the attachment site and energy source for the colonic bacteria, is an important factor for host selection of a specific mucosal community [23]. Lack or defects in this pattern may allow bacteria to reach the epithelium, and trigger colonic inflammation.

Ulcerative colitis (UC) is an idiopathic inflammatory bowel disease, which is characterized by chronic inflammation of the colonic mucosa. UC is usually associated with recurrent attacks and complete remission of symptoms in the interim [2,28]. The etiology of inflammatory bowel disease remains an enigma, and no known infectious agent has been demonstrated [37,53]. Human studies have revealed that UC patients have a colonic mucus layer that has an altered O-glycan profile and is significantly thinner than that of healthy subjects, which may select for a different mucosal microbial profile [33,51]. Consistently, several studies have shown that patients with UC have an altered bacterial microbiota [16,52,57,58]. The host-bacterial interaction may thus play a pivotal role in the pathogenesis of UC.

In vitro models are well-suited to screen the adhering potency of intestinal microbes. They include adhesion assays to various components of the intestinal surface: e.g. intestinal mucus [47], mucins

[60], colonic tissue [46] or cell lines [31]. However, they often only provide short-term information regarding axenic cultures and tend to ignore the microbial interaction between and within the luminal and mucosal microbiota.

Only recently, a dynamic *in vitro* gut model has been developed which simulates both the luminal and mucosal environment [61]. This model, named the M-SHIME was adapted from the validated Simulator of the Human Intestinal Microbial Ecosystem (SHIME) [59], and hence allows studying the mucosal microbiota and interaction between luminal and mucosal microbial communities.

The aim of this study was to investigate the ability of fecal microbiota from healthy subjects and UC patients in either remission or relapse to colonize the artificial mucus layer of the M-SHIME. Denaturing Gradient Gel Electrophoresis (DGGE) and quantitative Real-Time PCR (qPCR), both of which are culture-independent methods, were applied. DGGE was used to obtain fingerprints of the total microbiota of the three groups after colonization in the M-SHIME, while qPCR was used to quantify a broad range of selected bacteria taxa presumed to play a role in the homeostasis of the colonic microbial ecosystem.

Materials and Methods

Human volunteers and clinical characteristics of the UC patients

Fecal samples were obtained from 8 patients with UC and 4 healthy controls. Within the UC group, 4 patients were in clinical remission and 4 patients had active disease at the time of sampling according to clinical and endoscopical criteria [6]. The patients were previously diagnosed with UC according to standardized diagnostic criteria at the Department of Gastroenterology, Herlev Hospital [30]. The study was performed in accordance with the Second Helsinki Declaration, reported to the Danish Data Protection Agency and approved by the Regional Ethics Committee. Written, informed consent was obtained from each participant under a protocol approved by the Danish National Committee on Biomedical Research Ethics. UC patients received mesalazine (anti-inflammatory drug) and azathioprine (immunosuppressive drug). One of the patients had active pancolitis, one other had active left-sided colitis and the rest had either active proctitis or proctosigmoiditis. None of the participants had been treated with antibiotics for at least 2 months

before enrolment and there was no significant difference (P = 0.32) in the mean age of the participants comparing the 3 groups.

Sample collection and processing

Stool samples were collected at the home of the participant in airtight containers and stored at 4°C (limited storage time was encouraged [44]) until delivery to the laboratory, where they were processed immediately. Feces were homogenized in glycerol to give a 25% feces/glycerol slurry. This was performed in an anaerobic cabinet (Macs Work Station, Don Whitley, containing 10% H2, 10% CO2, and 80% N2). The processed samples were stored at -80°C until further analysis.

Growth medium and chemicals

Unless stated otherwise, chemicals were obtained from Sigma (Bornem, Belgium). The M-SHIME feed contained 1.0 g/l arabinogalactan, 2.0 g/l pectin, 1.0 g/l xylan, 3.0 g/l starch, 0.4 g/l glucose, 3.0 g/l yeast extract, 1.0 g/l peptone, 4.0 g/l mucin, and 0.5 g/l cystein. Pancreatic juice contained 12.5 g/l NaHCO3, 6.0 g/l bile salts (Difco, Bierbeek, Belgium) and 0.9 g/l pancreatin. Mucin agar was prepared by boiling autoclaved distilled H_2O containing 5% porcine mucin type II and 1% agar. The pH was adjusted to 6.8 with 10 M NaOH.

M-SHIME

A dynamic *in vitro* model for the human intestinal tract, which accounts for both the luminal and mucosal microbiota, was designed by Van den Abbeele *et al.* [61]. The M-SHIME set up consisted of two vessels simulating the stomach and the small intestine, and six ascending colon vessels, which were run in parallel without the transverse and descending colon (Figure 1). All ascending colon vessels were modified by incorporating mucin-covered microsms into the luminal suspension. 80 mucin-covered microcosms were added per 500 ml luminal suspension. The microcosms (length = 7 mm, diameter = 9 mm, total surface area = 800 m2/m3, AnoxKaldnes K1 carrier, AnoxKaldnes AB, Lund, Sweden) were coated by submerging them in mucin agar.

Each ascending compartment (500 ml) was inoculated with 27 ml of a 1:10 dilution of the feces/glycerol slurry. Inoculum preparation was done as described previously [48]. Three times per day, 140 ml SHIME feed and 60 ml pancreatic juice were added to the stomach and small intestine,

respectively [61]. The system was flushed with nitrogen twice a day to keep anaerobic conditions. After 42 h of incubation, samples were collected from the luminal content of the ascending vessels (luminal samples) for DNA extraction. The microcosms covered in mucin agar were washed 3 times in autoclaved phosphate buffer; subsequently, the mucin agar (mucosal samples) was collected for DNA extraction.

The M-SHIME was carried out at two different occasions with six participants for each run (two healthy, two UC patients in remission and two UC patients in relapse). The position of the inocula from either healthy subjects or UC patients was changed for each run.

Extraction of bacterial DNA

Before extraction of the mucosal samples, the samples were heated for 15 min at 55^{o}C to make the agar soluble. Subsequently, DNA was extracted from both luminal and mucosal samples using the QIAamp DNA Stool mini kit (Qiagen, Hilden, Germany) with a bead beater step in advance, as previously described [36]. For each luminal and mucosal sample, DNA was extracted in duplicates. The purified DNA was stored at -20°C until use.

PCR amplification for DGGE

Aliquots (10 µL) of purified DNA (5 ng/µl of pooled DNA from the duplicate DNA extractions) were applied to the following to give a 50 µL PCR reaction mixture: 20 µL of 5 PRIME MasterMix (2.5×) (VWR & Bie & Berntsen) and 10 pmol of each of the primers (Eurofins MWG Synthesis GmbH, Ebersberg, Germany). Primers HDA1-GC/HDA2 [65] targeting 16S rRNA genes from all bacteria were used in a touchdown PCR. Initial denaturation was at 96 °C for 5 min, amplification was carried out using 20 cycles including denaturation at 94 °C for 1 min, annealing at 65°C for 1 min decreased by 0.5°C for each cycle, and extension at 72°C for 1 min. This was followed by additional 5 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 1 min, and a final extension at 72°C for 5 min.

Analysis of luminal and mucosal microbiota by DGGE

DGGE was carried out as described previously [5] using a DcodeTM Universal Mutation Detection System instrument and gradient former model 475 according to the manufacturer's instructions

(Bio-Rad Labs, Hercules, California). The 9% polyamide gels were made with denaturing gradients ranging from 25% to 65%. The 100% denaturant solution contained 40% formamide and 7M urea. Thirteen microlitres PCR products were mixed with 3 µL loading dye before loading. Gels were run in 1 x TAE at 60 °C for 16 h at 36 V, 28 mA, stained with ethidium bromide for 15 min, destained for 20 min, and viewed by UV-B trans illumination at 302 nm (Bio-Rad). The BioNumerics software, version 4.60 (Applied Maths, Sint-Martens-Latem, Belgium) was used for identification of bands and normalization of band patterns from DGGE gels. DGGE gels were normalized by an assigned marker (developed in our laboratory). A cluster analysis was performed based on Dice coefficient of similarity (weighted) with the unweighted pair group method with arithmetic averages clustering algorithm.

Quantitative PCR assay conditions

QPCR was performed on an ABI Prism 7900 HT from Applied Biosystems. The amplification reactions were carried out in a total volume of 11 µL containing; 5.50 µL (EXPRESS SYBR® GreenER™ qPCR SuperMix, ROX, Invitrogen), primers (each at 200 nM concentration) (Eurofins MWG Synthesis GmbH), 2 µL template DNA, and Nuclease-free water (Qiagen) purified for PCR. The amplification program consisted of one cycle at 50°C for 2 min; one cycle at 95°C for 10 min; 40 cycles at 95°C for 15 sec and 60°C for 1 min; and finally one cycle of melting curve analysis for amplicon specificity at 95°C for 15 sec, 60°C for 20 sec and increasing ramp rate by 1.92°C/min until 95° for 15 sec. DNA (5 ng/µl) from the duplicate DNA extractions of each sample was used for the qPCR.

Quantitative PCR primer and data handling

The primers specific to regions of the 16S rRNA genes of selected bacterial taxa are listed in Table 1. The relative quantities of gene targets encoding gene sequences of the bacterial taxa were calculated using $2^{DeltaCt}$, assuming primer efficiency at 1.0. Delta Ct is the Ct-values of the bacterial target normalized against Ct-values of the total bacterial population in a sample. Ct is the threshold cycle calculated by the ABI software (SDS version 2.2; Applied Biosystems, Foster City, California, USA) as the PCR cycle, where amplifications signal exceeds the selected threshold value, also set by the ABI software. Prior to the quantification of the M-SHIME samples, standard curves were created using serial 10-fold dilutions of bacterial DNA extracted from one of the M-SHIME

samples for all primer sets. Analysis of the standard curves allowed verification of PCR efficiency for the chosen PCR conditions. Additionally, all primers were tested to confirm sensitivity and specificity using DNA from pure bacterial species.

Statistical analysis

Statistical analysis of the qPCR data was performed with SAS JMP software (version 6.0.2; SAS Institute Inc., Cary, North Carolina, USA). Data was analyzed by a mixed model ANOVA, where microbiota source (healthy, UC patients in remission and UC patients in relapse) and community (lumen and mucus) were used as fixed effects, while individual subjects were entered as random effects. The ANOVA was used to determine significant differences among the two communities. Pair-wise t-test was used to compare significant difference between the two communities for each microbiota source. The PCR measurements were log transformed before statistical analysis to obtain normal distribution of the data. Normal distribution was assessed using D'Agostino & Pearson omnibus normality test. Univariate ANOVA was used to analyze difference in the age of the three groups using GraphPad Prism software (version 5.03; GraphPad Software Inc., La Jolla, California, USA). Tests were considered statistically significant if P-values lower than 0.05 were obtained. Principal Component Analysis (PCA) was carried out using SAS JMP software (version 6.0.2) on the qPCR data to investigate the difference between luminal and mucosal community and to address which bacterial taxa may be important for colonization.

Results

Microbial community analysis using DGGE

Comparison of DGGE profiles containing 16S ribosomal genes amplified from luminal and mucosal samples of healthy subjects and UC patients after 42h colonization revealed a distinct difference between the luminal and mucosal environment in terms of bacterial composition (Figure 2). The dendrogram from the Dice cluster analysis showed three clusters with five luminal samples in cluster I (53.89% similarity), all mucosal samples in cluster II (54.61% similarity) and seven luminal samples in cluster III (41.15% similarity). Clustering did not occur according to the health status of the human subjects (healthy, UC in remission and UC in relapse).

Microbial community analysis using qPCR

After colonization (42 hours) of the luminal or the mucosal environment by fecal microbes from healthy subjects and UC patients (in remission or relapse), qPCR was applied to measure the density of gene targets encoding 16S rRNA gene content of selected bacterial taxonomic units. Principal component analysis (PCA) was performed on the relative quantities of bacterial levels to examine the main differences between all samples. While no obvious difference based on subject health status was observed, luminal and mucosal samples were separated from one another (Figure 3). The separation among all samples was explained by PC1 and PC2 (25.29% and 15.77% of explained variance, respectively). Especially PC2 explained the difference between the luminal and mucosal environment. The relative quantities of following bacterial taxa were higher in the luminal content: *Bifidobacterium* spp., *B. bifidum, B. pseudocatenulatum, B. adolescentis*, *Lactobacillus* spp., *C. leptum* subgroup, *C. coccoides* group, *F. prausnitzii*, *Akk. muciniphila* and *Alistipes* spp.. In contrast, *Roseburia* spp. and *E. rectale* rather correlated with the mucosal environment (Table 2).

The preference of specific bacterial groups to colonize the mucosal and/or luminal compartment was different depending on the three microbiota sources (healthy subjects, UC patients in remission or UC patients in relapse) (Table 2). In the vessels with communities derived from UC patients in relapse, mucus was colonized by significantly lower levels of bifidobacteria, *B. adolescentis*, lactobacilli, *C. coccoides* group, *C. leptum* subgroup, and *Alistipes* spp.($P<0.01$, $P<0.05$, $P<0.05$, $P<0.05$, $P<0.05$, and $P<0.05$, respectively). Additionally, lower level of *B. bifidum* and *Bacteroides* spp. derived from UC patients in relapse were measured in mucus, although it was only borderline significant ($P=0.071$ and $P=0.066$, respectively). The densities of bifidobacteria, *B. adolescentis*, B*. pseudocatenulatum*, lactobacilli, *C. leptum* subgroup, *Faecalibacterium prausnitzii*, and *Alistipes* spp. derived from UC patients in remission were significantly lower in mucus compared to lumen ($P<0.01$, $P<0.01$, $P<0.05$, $P<0.01$, $P<0.05$, $P<0.05$, and $P<0.05$, respectively). However, higher contents of *E. rectale* and *Roseburia* spp. derived from UC patients in remission were measured in mucus than in lumen. The difference was statistically significant for *E. rectale* ($P<0.05$), but only borderline significant for *Roseburia* spp. ($P=0.066$).

Statistical analysis on the relative quantities in the luminal or mucosal compartment from the three microbiota sources revealed only differences among mucosal samples and not among

luminal samples (Table 3). Relative quantities of *Bifidobacterium* spp. and *Lactobacillus* spp. in mucus was significantly lower ($P<0.05$ and $p<0.01$, respectively) when the communities derived from UC patients in relapse than from healthy subjects (Table 3). Moreover, the UC-relapse community had lower relative levels of *Lactobacillus* spp. ($p<0.01$) and *E. rectale* ($p = 0.09$) compared to the UC remission-community. No statistical difference in the colonization of mucus comparing the three microbiota sources was seen for the rest of the bacterial taxa.

Discussion

In this study, we applied a recently developed dynamic *in vitro* gut model, the M-SHIME to investigate the microbiota differences between healthy subjects and UC patients, either in relapse or in remission. The advantages of this model are that it allows investigating such differences during long-term incubations and not only in the luminal content but also at the intestinal mucosal surface. Further, the impact of the host on the microbial composition is excluded so that it allows focusing on microbe-microbe interactions and the intrinsic colonization ability of gut microbes. By doing so, we observed a distinct difference between the luminal and mucosal microbiota which was greater than the inter-individual variability. Moreover, important differences were observed within the mucosal microbiota of UC patients in relapse including depletion in the level of lactobacilli, bifidobacteria, *Clostridium coccoides* and *Clostridium leptum*.

In line with previous communications [38,67], the present study demonstrates that the *in vitro* mucosal microbial community differs from the luminal one, independent of whether the microbiota was derived from healthy subjects, UC patients in remission, or UC patients in relapse (Figure 2 and Figure 3). Our data imply that the difference between the mucosal and luminal microbiota dominates the individual sample variation and that the butyrate-producing bacteria and lactic acid bacteria are important bacterial groups in respect to colonic colonization of either lumen or mucus. The luminal and mucosal bacteria have previously been demonstrated to display different roles in the host, and it has been proposed that the mucosal microbiota is more involved in interaction with the epithelial and immune cells than the luminal microbiota, since it resides closer to the intestinal epithelial cells [12,62]. Hence, an altered mucosal microbial community may play an important role in dys-regulated immune responses. Given its importance and its vast

difference from the luminal microbiota, it is crucial to apply an *in vitro* model that accounts for the study of the mucosal microbiota.

We have demonstrated that the ability to colonize the mucus was significantly lower for bifidobacteria and lactobacilli originating from UC patients when compared to those derived from healthy subjects (Table 3). In consistence with our observations, lower levels of bifidobacteria in the mucus layer of biopsy specimens from UC patients have previously been described [39,43]. Bifidobacteria and lactobacilli are believed to play an important role in promoting intestinal health, due to their ability to inhibit colonization of pathogenic bacteria by lowering of colonic pH [15], produce antimicrobial compounds [34,54], and compete for adhesion sites [7,9,35,54]. Additionally, they have shown to stimulate immune regulatory responses [21,66]. Hence, a depletion of bifidobacteria and lactobacilli in UC patients could have a consequence for colonic health and favor inflammation. Stimulation of these groups through pre- or probiotics could be an approach in prevention of flare ups in UC.

In previous literature, the glycosylation patterns in UC patients in relapse has shown to differ compared to control subjects and UC patients in remission, however, the aberrant profile is reversible upon remission [33] and the microbiota has shown to be altered in UC patients in relapse compared to controls but only to some extent in UC patients in remission [57,58,64]. It is not well understood how changes in mucus composition can affect adhesion and colonization of gut microorganisms, however, a changed mucus niche in UC patients in relapse may select a different microbiota community and upon remission the level may not be reversed for all bacteria, even though the mucus structure is back to “normal” [33].

Species or strain specific mucus adhesion promoting proteins have been found in several bifidobacteria and lactobacilli. This includes among others fimbriae (*L. rhamnosus* GG, *L. Johnsonii* NCC533, *B. animalis* subsp. *lactis*, *B. bifidum*)[17,24,50], Msa, mannose-specific adhesin protein (*L. plantarum*) [49], MucBP domain containing proteins (lactic acid bacteria) [26], and adhesion-like factor EF-Tu (*L. plantarum, B. animalis* subsp. *lactis*) [17,63]. The expression of adhesion molecules may be changed in the lactic acid bacteria from UC patients, hence their inability to colonize the mucus *in vitro,* however, to our knowledge, no species of bifidobacteria or lactobacilli have be isolated from UC patients to identify adhesion molecules.

The composition of the species of lactobacilli and bifidobacteria derived from UC patients could also be the reason for the lower level found in the mucus *in vitro*, since expression of adhesion molecules and mucus-degrading enzymes is likely to be species specific. In the present study, the content of three *Bifidobacterium* species in the M-SHIME vessels for the three microbiota (healthy subjects, UC patients in remission or relapse) sources was measured. The ability to colonize the mucus was reduced for *B. bifidum* and *B. adolescentis* originating from UC patients in relapse while *B. adolescentis* and *B. pseudocatenulatum* was reduced in the mucus compared to the lumen in the vessels with microbiota derived from UC patients in remission. No significant difference in the lumen and mucus content of the three *Bifidobacterium* species derived from healthy subjects could be observed. This could imply that the *Bifidobacterium* species that are able to colonize the mucus may differ depending on disease state origin, resulting in different species composition. In agreement with this, previous study has shown that the ability of different lactic acid bacteria strains to adhere to intestinal mucus was disease-specific; hence the inflammatory state in UC may influence mucosal adhesion [45].

In the present study, the content of *C. coccoides* group and *C. leptum* subgroup derived from UC patients in relapse was significantly lower in the mucus than in the lumen. This was also true for the *C. leptum* group and *F. prausnitzii* derived from UC patients in remission (Table 2). Depletion of the two clostridial groups including *F. prausnitzii* in UC patients has previously been described in fecal samples [57,58]. In contrast, *E. rectale* demonstrated high capacity for adhesion to mucus in the present study, particularly when derived from UC patients in remission or from healthy subjects. This was also the case for *Roseburia* spp. derived from UC patients in remission (Table 3). Little is known regarding the ability of the species from clostridial groups to colonize the mucus. However, our results imply that some species have high capacity for adhesion depending on microbiota sources. Clostridial groups such as *C. coccoides* group and *C. leptum* subgroup are believed to be important for colon health and immune homeostasis. This was demonstrated by Atarashi *et al.* [3], who showed that *C. coccoides* group and *C. leptum* subgroup can stimulate mucosal immune regulatory responses through activation of regulatory T cells. Additionally, *C. coccoides* group and *C. leptum* subgroup include several bacteria that are able to produce butyrate, which has shown to down regulate pro-inflammatory cytokine production via inhibition of NF-κβ and as a result stimulate an anti-inflammatory state in the colon [18,55]. This suggests

that *C. coccoides* group and *C. leptum* subgroup are important commensal bacteria that if depleted in the mucus could have a consequence for colonic health.

We conclude that the mucosal bacterial community differs from that of the luminal. The difference was ascribed to butyrate-producing bacteria and lactic acid bacteria and was dominating the inter-individual sample variation. Bifidobacteria and lactobacilli derived from UC patients had decreased capacity to colonize the mucus, which may either be due to changed expression of adhesion molecules and/or to the composition of species within the genera *Bifidobacterium* and *Lactobacillus*. Our findings indicates that the ability of fecal bacteria to colonize the mucus is reduced in UC patients in relapse but only to some extent in UC patients in remission, which implies that the inflammatory state may have an influence on microbial adhesion capacity. However, further *in vivo* studies are needed to confirm these findings.

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Table 1 - 16S rRNA gene of phylum, group and species specific primers

Target taxon	Primer	Sequence (5'-3')	Fragment size (bp)	Reference
Bifidobacterium spp.	F-bifido	cgc gtc ygg tgt gaa ag	244	[10]
	R-bifido	ccc cac atc cag cat cca		
Lactobacillus spp.	Lacto-F	agc agt agg gaa tct tcc a	341	[20,65]
	Lacto-R	cac cgc tac aca tgg ag		
Clostridium leptum subgroup	sg-Clept-F	gca caa gca gtg gag t	239	[40,56]
	sg-Clept-R	ctt cct ccg ttt tgt caa		
Clostridium coccoides group	g-Ccoc-F	aaa tga cgg tac ctg act aa	440	[40]
	g-Ccoc-R	ctt tga gtt tca ttc ttg cga a		
Roseburia spp.	RosF	tac tgc att gga aac tgt cg	230	[32]
	RosR	cgg cac cga aga gca at		
Firmicutes phylum	Firm934F	gga gya tgt ggt tta att cga agc a	126	[19]
	Firm1060R	agc tga cga caa cca tgc ac		
Bacteroidetes phylum	Bact934F	gga rca tgt ggt tta att cga tga t	126	[19]
	Bact1060R	agc tga cga caa cca tgc ag		
Bacteroides spp.	BacAll_2F	gtc agt tgt gaa agt ttg c	127	[1]
	BacAll_2R	caa tcg gag ttc ttc gtg		
Alistipes spp.	Alis F1-124	tta gag atg ggc atg cgt tgt	320	[64]
	Alis R1-423	tga atc ctc cgt att acc gcg		
Desulfovibrio spp.	DSV691-F	ccg tag ata tct gga gga aca tca g	136	[14]
	DSV826-R	aca tct agc atc cat cgt tta cag c		
V2-V3 16S rRNA region*	HDA1	act cct acg gga ggc agc agt	200	[65]
	HDA2	gta tta ccg cgg ctg ctg gca c		
16S rRNA region*	TBA-F	cgg caa cga gcg caa ccc	130	[11]
	TBA-R	cca ttg tag cac gtg tgt agc c		
Faecalibacterium prausnitzii	Fprau 07	cca tga att gcc ttc aaa act gtt	140	[57]
	Fprau 02	gag cct cag cgt cag ttg gt		

Table 1 continues

Eubacterium rectale	Eu-rec_1F	aag gga agc aac gct gtg aa	200	[4]
	Eu-rec_2R	Cgg tta ggt cac tgg ctt c		
Akkermansia muciniphila	AM1	cag cac gtg aag gtg ggg ac	327	[8]
	AM2	cct tgc ggt tgg ctt cag at		
Bifidobacterium bifidum	BiBIF-1	cca cat gat cgc atg tga ttg	278	[41]
	BiBIF-2	ccg aag gct tgc tcc caa a		
Bifidobacterium adolescentis	BiADO-1	ctc cag ttg gat gca tgt c	279	[41]
	BiADO-2	cga agg ctt gct ccc agt		
Bifidobacterium pseudocatenulatum/ Bifidobacterium catenulatum	BiCATg-1	cgg atg ctc cga ctc ct	289	[41]
	BiCATg-2	cga agg ctt gct ccc gat		

*The HDA and TBA primer were used as total bacteria DNA targets in order to normalize, hence correcting differences in total DNA concentration between individual samples.

Table 2 - Preference of bacterial phylum/group/species to colonize mucosal over the luminal compartment, expressed as ratio of the relative quantities in the mucosal and luminal compartment (%)

	Log (average)[a]		Ratios (%)[b]		
Bacterial taxa	**Lumen**	**Mucus**	**Healthy subjects**	**UC remission**	**UC relapse**
Firmicutes	7.70 (±0.07)	7.71 (±0.03)	100.36 (±1.23)	100.46 (±1.64)	99.85 (±0.89)
• *Clostridium leptum* subgroup	6.27 (±0.30)	5.41 (±0.21)	96.33 (±8.43)	83.81 (±5.12)*	82.39 (±3.35)*
○ *Faecalibacterium prausnitzii*	5.08 (±0.34)	4.61 (±0.26)	102.62 (±13.35)	83.75 (±4.22)*	93.91 (±4.97)
• *Clostridium coccoides* group	5.53 (±0.33)	4.29 (±0.29)	88.47 (±10.94)	74.10 (±11.74)	66.81 (±10.73)*
○ *Roseburia* spp.	3.39 (±0.24)	4.31 (±0.47)	108.01 (±7.70)	137.83 (±12.28)#	146.06 (±41.24)
○ *Eubacterium rectal*	2.55 (±0.21)	3.21 (±0.28)	128.02 (±11.17)#	149.25 (±15.99)*	104.60 (±9.57)
• *Lactobacillus* spp.	6.39 (±0.40)	5.20 (±0.40)	94.70 (±8.85)	81.32 (±2.77)**	68.59 (±7.64)*
Bacteroidetes	5.65 (±0.23)	5.58 (±0.15)	99.98 (±2.84)	106.52 (±9.69)	94.36 (±7.57)
• *Bacteroides* spp.	5.46 (±0.24)	5.33 (±0.19)	98.44 (±2.87)	112.93 (±9.33)	86.17 (±4.66)#
• *Alistipes* spp.	4.62 (±0.21)	3.22 (±0.37)	65.05 (17.58)	68.12 (±11.76)*	71.91 (±8.47)*
Actinobacteria					
• *Bifidobacterium* spp.	7.35 (±0.11)	6.67 (±0.18)	94.60 (±3.12)	91.24 (±1.53)**	86.17 (±2.24)**
○ *B. bifidum*	4.48 (±0.55)	3.99 (±0.55)	90.82 (±5.05)	100.69 (±5.15)	73.86 (±7.58)#
○ *B. adolescentis*	5.43 (±0.47)	4.65 (±0.27)	85.99 (±6.14)	78.43 (±1.88)**	58.66 (±22.92)*
○ *B. pseudocatenulatum*	5.31 (±0.55)	4.54 (±0.45)	101.27 (±4.68)	79.79 (±3.55)*	94.60 (±15.00)
Proteobacteria					
• *Desulfovibrio* spp.	3.56 (±0.24)	3.49 (±0.23)	101.19 (±1.75)	101.22 (±4.53)	92.74 (±3.89)
Verrucomicrobia					
• *Akkermansia muciniphila*	3.68 (±0.36)	2.48 (±0.38)	71.03 (±15.02)	73.49 (±10.30)	69.81 (±10.46)

[a] Logarithmic average of relative quantities for healthy subjects and UC patients from luminal or mucosal bacteria levels.

[b] Ratios (%) calculated as 100*mucosal samples/luminal samples, hence below 100%, low adherence capacity to mucus and above 100%, high adherence capacity to mucus. *B. pseudocatenulatum* is both *B. catenulatum* and *B. pseudocatenulatum*

Asterisks (*) indicate significant differences between mucosal and luminal samples (*P<0.05 and **P<0.01), while pound signs (#) designates a trend suggesting difference between mucosal and luminal samples (0.05< P < 0.10).

Table 3 - Relative fold change of bacteria derived from UC patients compared to healthy subjects, both in the mucosal and luminal environment.

	Lumen		Mucus	
Bacterial taxa	**UC remission**	**UC relapse**	**UC remission**	**UC relapse**
Firmicutes	98.99 (±2.34)	101.92 (±0.52)	99.02 (±0.96)	101.43 (±0.40)
• *Clostridium leptum* subgroup	105.94 (±9.04)	120.45 (±3.06)	95.21 (±10.49)	106.03 (±4.53)
○ *Faecalibacterium prausnitzii*	120.28 (±7.78)	125.79 (±3.57)	103.39 (±2.32)	122.12 (±5.42)
• *Clostridium coccoides* group	97.68 (±14.02)	119.86 (±6.22)	69.89 (±23.95)	94.02 (±16.97)
○ *Roseburia* spp.	103.12 (±7.65)	72.29 (±11.66)	133.15 (±19.83)	89.96 (±24.74)
○ *Eubacterium rectale*	134.55 (±19.10)	135.28 (±21.79)	151.34 (±13.81)*	112.20 (±20.97)$^{\$}$
• *Lactobacillus* spp.	109.74 (±3.52)	80.06 (±12.88)	96.27 (±4.42)	57.99 (±8.93)**.$^{\#\#}$
Bacteroidetes	95.33 (±11.68)	107.10 (±4.71)	98.92 (±6.41)	100.04 (±4.25)
• *Bacteroides* spp.	93.35 (±11.66)	108.68 (±5.75)	104.27 (±6.83)	94.82 (±6.63)
• *Alistipes* spp.	100.97 (±11.46)	111.57 (±6.01)	100.60 (±19.04)	118.90 (±18.49)
Actinobacteria				
• *Bifidobacterium* spp.	99.97 (±3.72)	97.65 (±2.65)	96.53 (±4.95)	89.04 (±4.22)*
○ *B. bifidum*	90.81 (±20.29)	71.53 (±16.65)	72.64 (±28.17)	59.51 (±16.52)
○ *B. adolescentis*	117.66 (±6.59)	85.99 (±20.90)	109.46 (±7.42)	73.06 (±26.13)
○ *B. pseudocatenulatum*	113.94 (±7.96)	96.16 (±22.55)	99.55 (±8.11)	90.74 (±18.72)
Proteobacteria				
• *Desulfovibrio* spp.	117.09 (±17.88)	120.72 (±5.61)	117.92 (±18.33)	111.03 (±4.90)
Verrucomicrobia				
○ *Akkermansia muciniphila*	118.41 (±8.00)	85.11 (±20.53)	88.42 (±33.35)	62.23 (±15.78)

All numbers are average ± SEM of the four samples in each UC group of either lumen or mucus. The relative fold change values (%) are compared to healthy controls (set to 100). *B. pseudocatenulatum* is both *B. catenulatum* and *B. pseudocatenulatum*
Asterisks (*) designates a significant difference from the healthy control group (*P < 0.05 and **P<0.01), while pound signs (#) designates a significant difference from UC patients in remission (#P < 0.05 and ##P<0.01). Dollar sign ($) designates a trend suggesting difference from the UC patients in remission (0.05< P < 0.10).

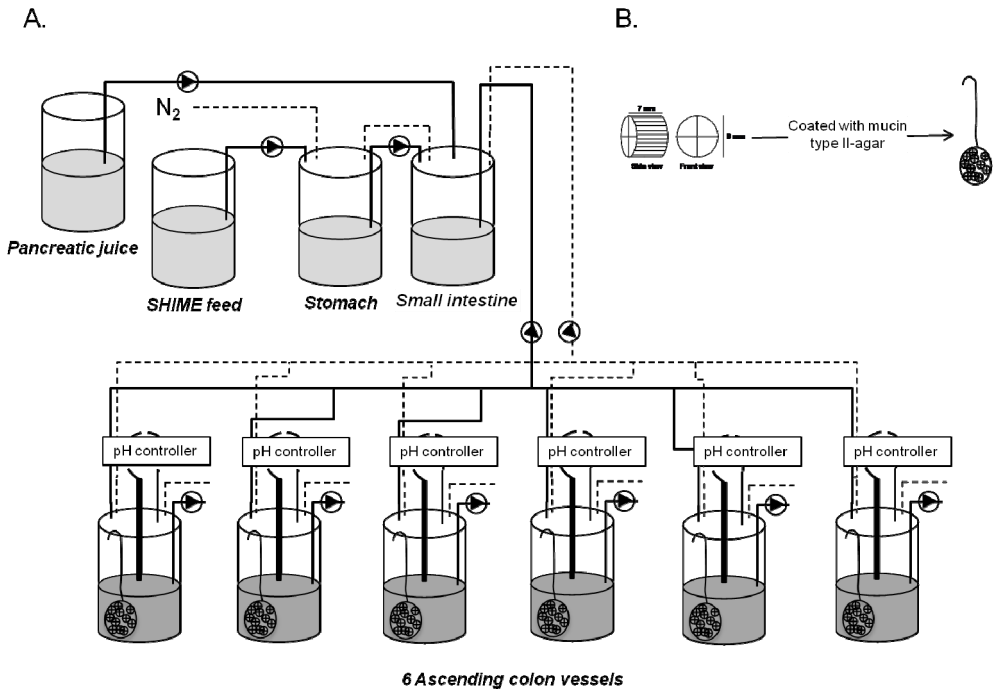


Figure 1. A) Schematic overview of the M-SHIME with 6 ascending colon vessels; B) Detailed scheme of mucin type II agar-covered microcosms. - - - -: headspace connection; ——: liquid connection; ⊛: pump.

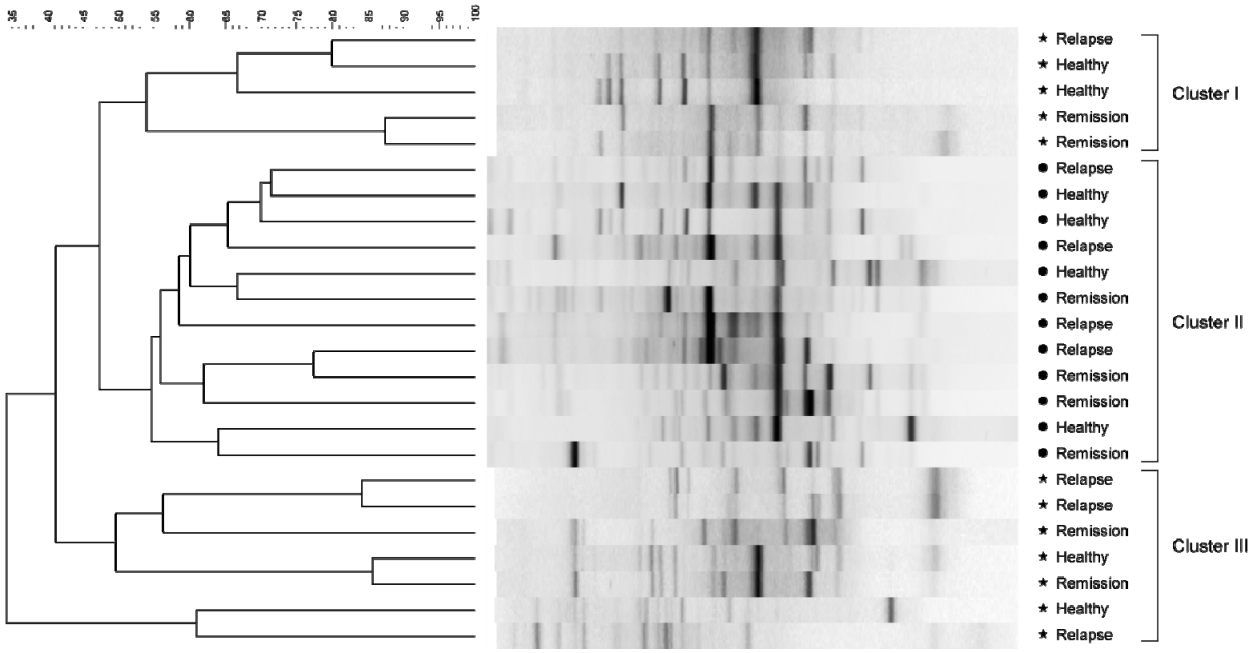
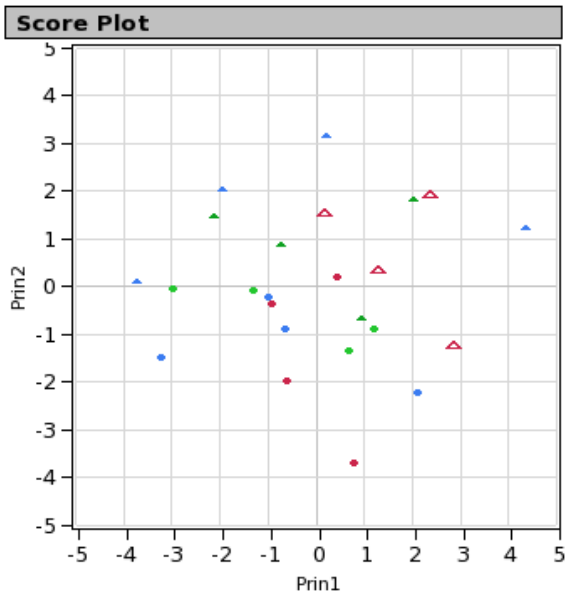


Figure 2. Dice cluster analysis of universal DGGE gel profiles from *in vitro* luminal and mucosal communities derived from healthy subjects, UC patients in remission or relapse. The luminal samples are indicated by stars (*) and the mucosal samples are indicated by full circle (•). The dendrogram can be divided into three clusters: Cluster I, luminal samples (53.89% similarity). Cluster II, mucosal samples (54.61% similarity). Cluster III, luminal samples (41.15% similarity). Metric scale indicates degree of similarity in percentage.

A.



B.

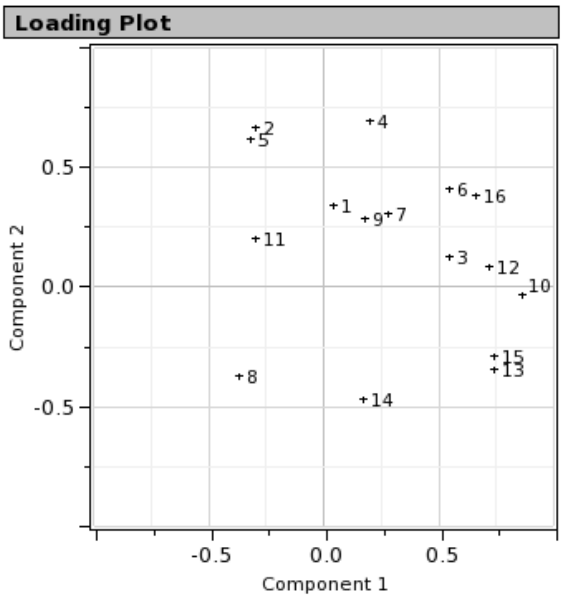


Figure 3. Principal component analysis of the quantitative PCR measurements using the first and second principal component (PC1: 25.29% and 15.77%). A) Score plot showing the luminal community indicated by triangle (Δ) and mucosal community indicated by circle (•). Sources of the communities are indicated by green for healthy subjects, blue for UC patients in remission, and red for UC patients in relapse. B) Loading plot, indicating each of the measured bacterial taxa as determined by quantitative Real-Time PCR. 1. *Bifidobacterium bifidum*, 2. *Bifidobacterium adolescentis*, 3. *Bifidobacterium pseudocatenulatum*, 4. *Bifidobacterium* spp., 5. *Lactobacillus* spp., 6. *Clostridium leptum* subgroup, 7. *Clostridium coccoides* group, 8. *Eubacterium rectale*, 9. *Faecalibacterium prausnitzii*, 10. *Desulfovibrio* spp., 11. *Akkermansia muciniphila*, 12. *Firmicutes*, 13. *Bacteroidetes*, 14. *Roseburia* spp., 15. *Bacteroides* spp., 16. *Alistipes* spp.

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Paper 3

In vitro Fermentation of Sugar Beet Arabino-Oligosaccharides by Fecal Microbiota Obtained from Patients with Ulcerative Colitis Selectively Stimulates the Growth of *Bifidobacterium* spp. and *Lactobacillus* spp.

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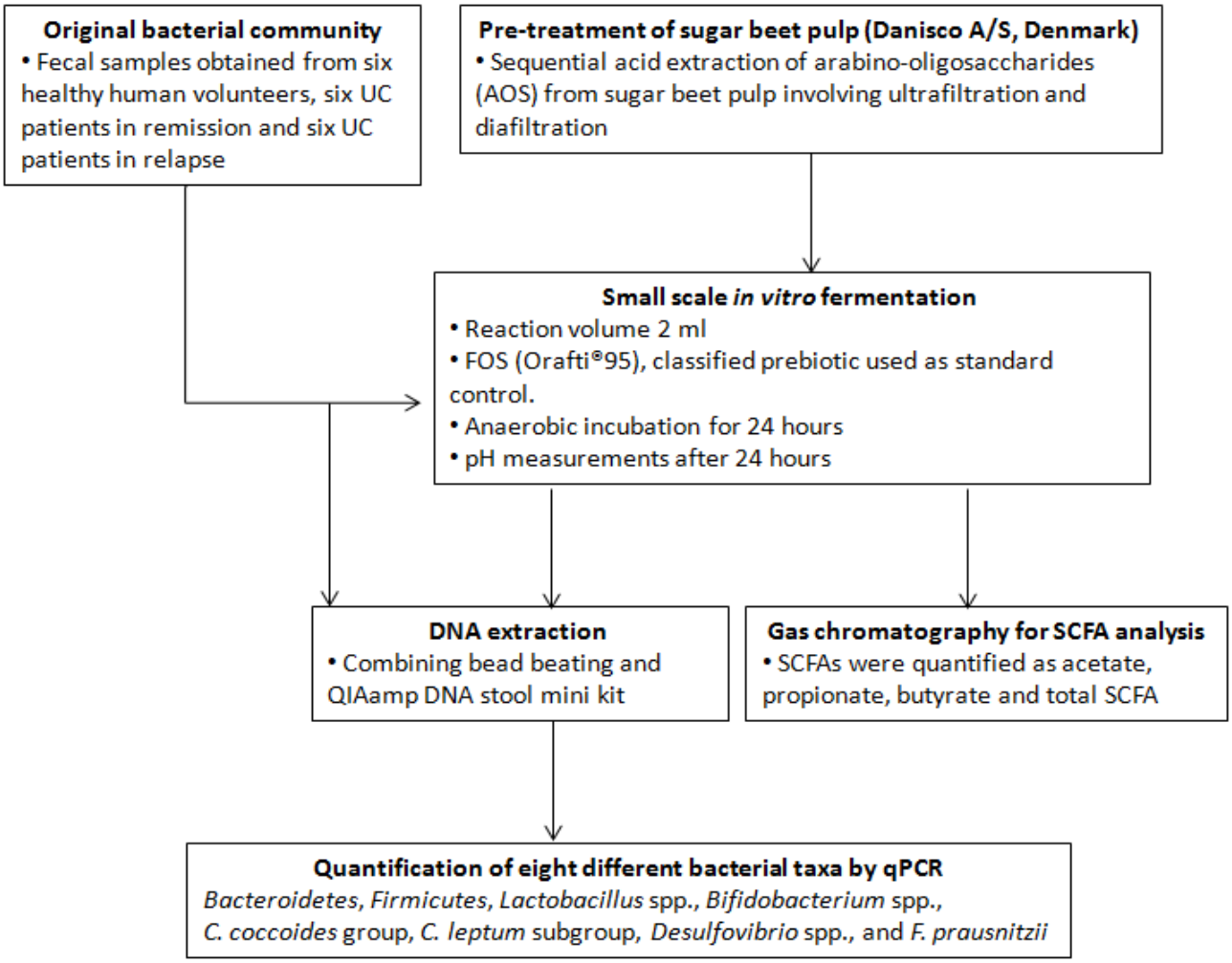
Introduction

The aim of this study was to examine potential prebiotic properties of arabino-oligosaccharides from sugar beet pulp using mixed cultures of human fecal bacteria from healthy subjects, and UC patients in either remission or relapse in a small scale *in vitro* model.

Flow diagram



The author, Louise K. Vigsnæs, participated in the design of the study, conducted the experiments, performed the data analyzes and drafted the manuscript.

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In Vitro Fermentation of Sugar Beet Arabino-Oligosaccharides by Fecal Microbiota Obtained from Patients with Ulcerative Colitis To Selectively Stimulate the Growth of *Bifidobacterium* spp. and *Lactobacillus* spp.▽†

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The potential prebiotic properties of arabino-oligosaccharides (AOS) derived from sugar beet pulp was studied using mixed cultures of human fecal bacteria from patients with ulcerative colitis (UC), in remission or with active disease, and in healthy controls. These results were compared to those for fructo-oligosaccharides (FOS), which are known to have a prebiotic effect. Fermentation studies were carried out using a small-scale static batch system, and changes in the fecal microbial communities and metabolites were monitored after 24 h by quantitative real-time PCR and short-chain fatty acid analysis. With a few minor exceptions, AOS affected the communities similarly to what was seen for FOS. Quantitative real-time PCR revealed that *Bifidobacterium* spp. and *Lactobacillus* spp. were selectively increased after fermentation of AOS or FOS by fecal microbiota derived from UC patients. The stimulation of growth of *Lactobacillus* spp. and *Bifidobacterium* spp. was accompanied by a high production of acetate and hence a decrease of pH. The fermentation of AOS may help improve the inflammatory conditions in UC patients through stimulation of bacteria eliciting anti-inflammatory responses and through production of acetate. AOS may therefore represent a new prebiotic candidate for reduction of the risk of flare-ups in UC patients. However, human trials are needed to confirm a health-promoting effect.

During the twentieth century a significant increase in the incidence of inflammatory bowel diseases has occurred in Western Europe and North America (33). Ulcerative colitis (UC) is an idiopathic inflammatory bowel disease characterized by chronic inflammation of the colonic mucosa. UC is usually associated with chronic remissions, which are periods in which patients are completely symptom free, and relapse periods, characterized by diarrhea with passage of blood or mucus, occasional abdominal cramping, and pain as well as, in severe cases, systemic symptoms, including fever and weight loss (3, 4). The etiology of inflammatory bowel disease remains unclear, and no causal infectious agent has been identified. The commensal bacterial intestinal community represents the environmental factor most frequently implicated in the development of UC (4, 60). Evidence for this implication is provided by the complete lack of enterocolitis in genetically engineered germfree mice, rats, and guinea pigs, which reproducibly develop intestinal inflammation within 1 to 4 weeks if they are colonized with conventional gut bacteria (61). Thus, a dysbiosis in the composition of the gut microbiota may influence key mechanisms involved in the inflammatory process of the intestinal mucosa (4, 21, 60). Earlier studies have shown differences between the intestinal microbiotas of UC patients and those of healthy subjects (45, 64). In particular, the patients with UC in relapse are reported to have a small amount of bifidobacteria (64) and we have observed that the prevalence of lactobacilli in UC patients is also low compared to that in healthy subjects (L. K. Vigsnæs et al., submitted for publication). Bifidobacteria and lactobacilli are believed to play an important role in maintaining intestinal health due to their effect on maturation and balancing of the immune system (43, 59, 71) and to their inhibition of pathogens (8, 17, 53). Hence, an underrepresentation of bifidobacteria and lactobacilli may compromise the colon health and contribute to a higher risk of flare-up in patients with UC. Maintenance of a healthy gut microbiota and homeostasis can be promoted by the consumption of indigestible carbohydrates or dietary fibers (11, 27, 37). However, a sufficient fiber intake is required for the desired effect (14). Prebiotics are defined as "selectively fermented ingredients that cause specific changes in composition and/or activity in the gastointestinal microbiota, which confer benefits upon host well-being and health" (47). The selective stimulation of specific colonic bacteria is explained by the capability of these bacteria to break down the glycosidic linkages in the prebiotic carbohydrates. These bacteria are able to grow on particular carbon sources, which are less easily fermented by other members of the intestinal community. This provides them with a selective advantage when competing with other bacterial species in a mixed bacterial community such as the human colon (58). Thus, the monosaccharide composition, glycosidic linkage, and length of the prebiotics contribute to the relative increase of beneficial bacteria, including bifidobacteria and lactobacilli (29, 46, 57). Metabolites produced by bacterial

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fermentation of prebiotics include the short-chain fatty acids (SCFA) acetate, propionate, and butyrate. SCFA have local and systemic biological effects that are proposed to be beneficial to human health (69). Acetate is important for lowering the pH of the intestine, thereby inhibiting enteropathogenic bacteria (16, 17). Butyrate is the preferred energy source for colonocytes, and studies have shown that butyrate can act as an anticarcinogenic agent through selective induction of apoptosis in colon cancer cells (39, 52, 54). With respect to inflammatory bowel diseases, butyrate is important because it stimulates an anti-inflammatory state in the colon by downregulation of proinflammatory cytokine production (62). Ingestion of prebiotic compounds is considered an alternative approach for treatment of UC, since an alteration of the colonic microenvironment toward homeostasis may help decrease the risk of flare-ups.

Sugar beet pulp, originating from industrial production of sugar, is rich in pectins, which are heterogeneous molecules containing regions of homogalacturonan and rhamnogalacturonan I. In sugar beet pectin, the side chains of rhamnogalacturonan I are often rich in arabinan composed of α-1,5-linked backbone with a high degree of α-1,2- and/or 1,3-arabinofuranosyl substitutions along with some β-1,4-linked galactan (40).

Significant potential exists for converting such by-product streams from industrial bioprocesses into new value-added products. Several types of nondigestible fibers and oligosaccharides from plant cell walls have shown the capability to modulate the human gut microbial composition in *in vitro* studies. Some arabino-oligosaccharides (AOS) from sugar beet pectin (1) and AOS derived from lemon peel (26) are reported to stimulate the growth of bifidobacteria to the same extent as the "gold standard" prebiotic fructo-oligosaccharides (FOS) and inulin, respectively (42, 55, 57). High-molecular-weight rhamnogalacturonan I from potato pulp was superior to FOS in stimulating bifidogenic growth (66).

The aim of this work was to examine the potential prebiotic properties of AOS released from rhamnogalacturonan I during sequential acid extraction of pectin from sugar beet pulp. Fermentation-induced changes in fecal microbial communities obtained from either healthy subjects or patients with UC in remission or relapse were investigated to establish whether fermentation of AOS influenced the fecal communities from UC patients in the same way as known prebiotic FOS.

MATERIALS AND METHODS

Carbohydrates and chemicals. Sugar beet AOS were obtained from Danisco A/S (Nakskov, Denmark). The AOS were identical with the substrate described as "starting material" by Holck and coworkers (24) and was found as linear and branched arabino-oligosaccharides mainly of 2 to 10 degrees of polymerization (DP). The content of monosaccharides (mainly arabinose, glucose, and fructose) contributed to 125 mg/g dry matter. The arabinose moiety in the total sample accounted for 85 mol%. The ferulic acid content was 36 μg/g dry matter (24). Fructo-oligosaccharides (FOS) (2 to 8 DP) (OraftiR95) were obtained from Beneo-Orafti (Tienen, Belgium). Unless stated otherwise, chemicals were obtained from Sigma–Aldrich (Steinheim, Germany).

Human volunteers and clinical characteristics of the UC patients. Fecal samples were obtained from 12 patients with UC and six healthy controls (four women and two men). Within the UC group, six patients (three women and three men) were in clinical remission and six patients (three women and three men) had active disease at the time of sampling according to clinical and endoscopic criteria (5). The patients were previously diagnosed with UC according to standardized diagnostic criteria at the Department of Medical Gastroenterology, Herlev Hospital (28). The study was performed in accordance with the Second Helsinki Declaration, reported to the Danish Data Protection Agency, and approved by the Regional Ethics Committee. Four of the six patients with inactive UC received maintenance treatment with oral mesalazine (anti-inflammatory bowel-specific aminosalicylate drug) in a dosage of 1.6 to 2.4 g daily, and one also received azathioprine (purine analogue immunosuppressive drug) 100 mg daily. One patient received oral olsalazine (anti-inflammatory bowel-specific azodisalicylate drug) (1 g daily), and one received no treatment. All six patients with active UC were treated with oral mesalazine in a dosage of 2.4 to 3.2 g daily as well as topical mesalazine 1 g daily either as an enema ($n = 5$) or as a suppository ($n = 1$). One patient also received azathioprine 100 mg daily. One patient had active extensive UC, one left-sided colitis, and the rest either active proctitis or proctosigmoiditis. One patient with well-established proctitis refused a new sigmoidoscopy. None of the participants had been treated with antibiotics for at least 2 months before enrollment, and there was no significant difference ($P = 0.57$) in the mean age of the participants between the three groups (healthy control, 41 ± 9 years; UC patients in remission, 41 ± 8 years; and UC patients in relapse, 45 ± 8 years).

Sample collection and processing. The stool samples were collected at the home of the participant in airtight containers and stored at 4°C (limited storage time was encouraged [41]) until delivery to the laboratory, where they were processed immediately. Feces (200 mg, wet weight) were collected in triplicates in the middle of each stool sample for DNA extraction and used for the measurement of original bacterial communities. At the same time, feces were prepared for *in vitro* fermentation in an anaerobic cabinet (Macs Work Station; Don Whitley), containing 10% H_2, 10% CO_2, and 80% N_2. The fecal samples for *in vitro* fermentation were homogenized in 50% glycerol (1:1 dilution) and stored at −80°C until further analysis, as described below. The use of frozen samples compared to fresh samples in fermentation experiments has been validated previously (49).

Small-scale *in vitro* fermentation. A small-scale *in vitro* fermentation method was used to assess the effect of AOS on the microbial composition in human fecal samples. FOS was applied as a standard with known bifidogenic effect (50). AOS and FOS were sterilely filtrated and added to an autoclaved minimal basal medium to give a final concentration of 5 g/liter in a reaction volume of 2 ml. The minimal basal medium contained 2 g/liter of peptone water, 1 g/liter of yeast extract, 0.1 g/liter of NaCl, 0.04 g/liter of K_2HPO_4, 0.04 g/liter of KH_2PO_4, 0.01 g/liter of $MgSO_4 \cdot 7H_2O$, 0.01 g/liter of $CaCl_2 \cdot 2H_2O$, 2 g/liter of $NaHCO_3$, 0.5 g/liter of bile salts, 0.5 g/liter of L-cysteine hydrochloride, 50 mg/liter of hemin, 10 μl/liter of vitamin K1, 2 ml/liter of Tween 80 (VWR, Darmstadt, Germany), and 0.05‰ (wt/vol) resazurin solution. The pH of the minimal medium with added FOS or AOS was adjusted to 7, and the solutions were reduced overnight in an anaerobic cabinet. Fecal samples were defrosted in an anaerobic cabinet, and 10% (wt/vol) fecal slurry was prepared by mixing of the samples with anoxic PBS (Oxoid, Greve, Denmark). The reduced minimal medium with added FOS or AOS was distributed into 3.8-ml sterile screw-cap vials (Nunc) and inoculated to a final concentration of 1% (wt/vol) feces. Caps were loosely placed on the vials to allow gas exchange but avoid evaporation. Each fermentation experiment for the fecal sample of each subject was carried out in triplicates for each carbohydrate source. A parallel incubation with no added substrate was used as a control for the SCFA analysis and pH measurements. The fermentation was non-pH controlled and nonstirred, due to the low reaction volume, and was carried out in an anaerobic cabinet at 37°C. After 24 h of fermentation, the pH of the 2-ml fermentation samples was measured (pH indicator strips, pH 2.0 to 9.0; Merck) in the anaerobic cabinet, and subsequently a 1-ml fermentation sample was taken out for extraction of bacterial DNA and SCFA analysis, respectively.

Extraction of bacterial DNA. DNA was extracted from each of the triplicate fecal samples and each of the triplicate fermentation samples using the QIAamp DNA stool minikit (Qiagen, Hilden, Germany) with a bead beater step in advance, as previously described (31). The purified DNA was stored at −20°C until use.

Real-time PCR assay conditions. Amplification and detection of purified bacterial DNA by real-time PCR was performed with the ABI228 Prism 7900 HT from Applied Biosystems using optical grade 384-well plates. Each amplification reaction was done in duplicate for each of the triplicate fecal and triplicate fermentation samples in a final volume of 11 μl containing 5.50 μl EXPRESS SYBR GreenER qPCR SuperMix (Invitrogen A/S, Taastrup, Denmark), 200 nM each of the primers (Eurofins MWG Synthesis GmbH, Ebersberg, Germany), 2 μl template DNA (5 ng/μl), and nuclease-free water purified for PCR (Qiagen). The amplification program consisted of one cycle at 50°C for 2 min, one cycle at 95°C for 10 min, 40 cycles at 95°C for 15 s and 60°C for 1 min, and finally one

TABLE 1. 16S rRNA gene primers used in this study

Target taxon	Primer	Sequence (5′–3′)	PCR product (bp)	Efficiency (E)[b]	Reference
Faecalibacterium prausnitzii	Fprau 07 Fprau 02	CCA TGA ATT GCC TTC AAA ACT GTT GAG CCT CAG CGT CAG TTG GT	140	1.90	64
Bifidobacterium spp.	F-bifido R-bifido	CGC GTC YGG TGT GAA AG CCC CAC ATC CAG CAT CCA	244	2.04	9
Lactobacillus spp.	Lacto-F Lacto-R	AGC AGT AGG GAA TCT TCC A CAC CGC TAC ACA TGG AG	341	1.98	22, 70
Clostridium leptum subgroup	sg-Clept-F sg-Clept-R	GCA CAA GCA GTG GAG T CTT CCT CCG TTT TGT CAA	239	2.03	38, 63
Clostridium coccoides group	g-Ccoc-F g-Ccoc-R	AAA TGA CGG TAC CTG ACT AA CTT TGA GTT TCA TTC TTG CGA A	440	2.02	38
Firmicutes phylum	Firm934F Firm1060R	GGA GYA TGT GGT TTA ATT CGA AGC A AGC TGA CGA CAA CCA TGC AC	126	2.01	20
Bacteroidetes phylum	Bact934F Bact1060R	GGA RCA TGT GGT TTA ATT CGA TGA T AGC TGA CGA CAA CCA TGC AG	126	1.98	20
Desulfovibrio spp.	DSV691-F DSV826-R	CCG TAG ATA TCT GGA GGA ACA TCA G ACA TCT AGC ATC CAT CGT TTA CAG C	136	1.90	15
V2-V3 16S rRNA region[a]	HDA1 HDA2	ACT CCT ACG GGA GGC AGC AGT GTA TTA CCG CGG CTG CTG GCA C	200	1.98	70
16S rRNA region[a]	TBA-F TBA-R	CGG CAA CGA GCG CAA CCC CCA TTG TAG CAC GTG TGT AGC C	130	2.04	10

[a] The HDA and TBA primers were used as total bacterial DNA targets to normalize for differences in total DNA concentrations between individual samples.
[b] Primer efficiency is calculated from the slope of the standard curve for each primer set, $E = 10^{-1/\text{slope}}$.

cycle of melting curve analysis for amplicon specificity at 95°C for 15 s and 60°C for 20 s, increasing the ramp rate by 1.92°C/min until maintaining at 95°C for 15 s. The 16S rRNA-targeting primers used in this study are listed in Table 1. The primers were tested to confirm sensitivity and specificity (see Table S1 in the supplemental material).

Real-time PCR data handling. The relative quantities and relative ratios of gene targets encoding 16S rRNA sequences of the bacterial taxa were calculated using $E^{\Delta CT}$ and $E^{\Delta CT\ (\text{fermentation sample})}/E^{\Delta CT\ (\text{original bacterial community})}$, respectively, where E is the efficiency of the primer calculated from the slope of the standard curve ($E = 10^{-1/\text{slope}}$) and ΔCT is the C_T value of the bacterial target normalized against the C_T value of the total bacterial population in the sample (23). C_T is the threshold cycle calculated by the ABI software (SDS version 2.2; Applied Biosystems, Foster City, CA) as the PCR cycle, where the amplification signal exceeds the selected threshold value, also set by the ABI software. Standard curves were created using serial 10-fold dilutions of bacterial DNA extracted from one of the fermentation samples for all primer sets. Analysis of the standard curves allowed verification of PCR efficiency for the chosen PCR conditions (Table 1). All results were calculated as the means of duplicate determinations, equal values required.

Gas chromatography for SCFA analysis. Prior to quantification of SCFA, the samples were acidified to pH <2 with 17% H_3PO_4 and centrifuged at 10,000 × *g* for 10 min at room temperature to remove particle material. The supernatants were collected and filtered through a 0.45-μm-pore-size filter (Milipore, Copenhagen, Denmark) to remove bacterial cells. SCFA were analyzed using gas chromatography (Perkin Elmer 400 Clarus) with a flame ionization detector and a capillary column (Agilent Technologies, Horsholm, Denmark). The temperatures of the injector and detector were 175°C and 200°C, respectively. The temperature gradient was as follows: 70°C for 1 min, ramped at 8°C/min to 150°C, and after that ramped at 45°C/min to 230°C in 3 min (total time, 15.78 min). The injection volume of the sample was 0.5 μl, and the nitrogen flow was 12 ml/min. The quantified SCFA were analyzed as acids: acetic, propionic, and butyric. The standards used in the SCFA analysis were the same acids as mentioned above with concentrations of 1 to 40 mM.

Statistics. Statistical analysis of the quantitative real-time PCR (qPCR) data was performed with the SAS JMP software (version 6.0.2; SAS Institute Inc., Cary, NC). Data were analyzed by a mixed-model analysis of variance (ANOVA), where the microbiota source (healthy controls, UC patients in remission, and UC patients in relapse) and the incubations (inoculum, FOS, and AOS) were used as the fixed effects, while the individual subjects were entered as random effects. When ANOVA indicated a significant difference, a pairwise multiple comparison of means (Student's *t* test) was used to determine significant differences among treatment combinations, assuming equal variances. The PCR measurements were log transformed before statistical analysis to obtain normal distribution of the data. Normal distribution was assessed using a D'Agostino and Pearson omnibus normality test with the GraphPad Prism software (version 5.03; GraphPad Software Inc., La Jolla, CA). Statistical analysis of the SCFA data was performed with the SAS JMP software (version 6.0.2) using a two-way ANOVA. Univariate ANOVA was used to analyze differences in the ages of the three groups (GraphPad Prism software, version 5.03). Tests were considered statistically significant if P values lower than 0.05 were obtained. Principal component analysis (PCA) was carried out using the SAS JMP software (version 6.0.2) on the qPCR data to investigate the difference between the carbohydrate incubations and, additionally, which bacterial taxa may be important for fermentation of the tested carbohydrates.

RESULTS

Bacterial communities in the fecal samples. The relative quantification of gene targets encoding 16S rRNA gene sequences present in the original bacterial communities derived from fecal samples (Fig. 1) revealed that densities of *Lactobacillus* spp. were significantly lower in the UC patients in relapse than in healthy controls ($P < 0.05$). No significant difference between densities of *Lactobacillus* spp. present in UC patients

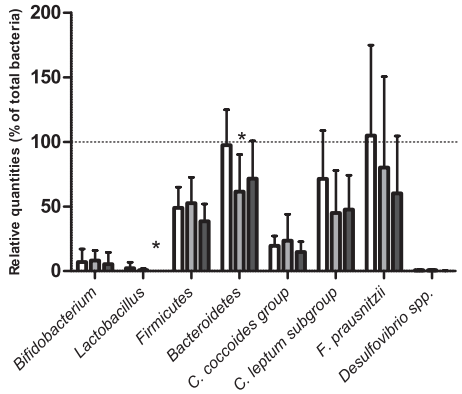

FIG. 1. Relative quantities of gene targets of the original bacterial community in fecal samples from healthy controls (white), ulcerative colitis patients in remission (gray), and ulcerative colitis patients in relapse (black). Target genes encoded 16S rRNA from *Lactobacillus* spp., *Bifidobacterium* spp., *Firmicutes*, *Bacteroidetes*, *Clostridium coccoides* group, *Clostridium leptum* subgroup, *Faecalibacterium prausnitzii*, and *Desulfovibrio* spp. The relative quantities (RQ) were calculated as $RQ = E^{\Delta CT}$, where E represents the primer efficiency and ΔCT was the C_T value of the bacterial target normalized against the C_T value of the total bacterial population in the sample. Total bacteria were set to 100% (indicated by dotted line). Error bars represent averages ± standard errors of the means of relative quantities in target species in each of the six samples in each group. Asterisks indicate a significant difference among groups ($P < 0.05$).

in remission and relapse, respectively, was observed. However, there was a trend of lower densities of lactobacilli in the relapse than in the remission group ($0.05 < P < 0.10$). Densities of the *Bacteroidetes* phylum were significantly lower in the UC patients in remission than in healthy controls ($P < 0.05$). The mean relative quantity of *Bifidobacterium* spp. was lower in the UC patients in relapse than in the other groups, although this was not statistically significant, probably due to high interindividual variation in the three groups. There were no significant differences in the densities of the *Firmicutes*, the *Clostridium coccoides* group, the *Clostridium leptum* subgroup, *Faecalibacterium prausnitzii*, and *Desulfovibrio* spp. between the three groups.

Changes in fecal microbial communities after *in vitro* fermentation. After fermentation of FOS and AOS in fecal slurries obtained from healthy subjects and UC patients in either remission or relapse, qPCR was applied to measure the density of gene targets encoding 16S rRNA genes of selected bacterial taxonomic units (Fig. 2). In fecal communities derived from healthy subjects and UC patients in remission, fermentation of FOS resulted in a significant increase in the content of *Bifidobacterium* spp. ($P < 0.05$). However, fermentation of AOS did not similarly increase the proportion of bifidobacteria in these samples, although a trend for higher levels of bifidobacteria in the communities from healthy subjects was observed ($P = 0.078$). The communities derived from patients with UC in relapse showed a significant increase in the density of bifidobacteria after fermentation with FOS as well as with AOS ($P < 0.05$). The relative abundances of *Lactobacillus* spp. in fecal microbiota derived from patients with UC in remission and relapse were significantly increased after fermentation with either FOS or AOS ($P < 0.05$). The fecal communities from healthy subjects contained a significantly higher density of lactobacilli after fermentation with FOS ($P < 0.05$). Although this was only borderline significant for the samples fermented with AOS, there was a strong trend of a similar effect ($P = 0.057$).

A significant reduction of the *Firmicutes* phylum and of taxa belonging to the *Firmicutes* (*C. coccoides* group, *C. leptum* subgroup, and *F. prausnitzii*) was measured in all three fecal microbiota groups after fermentation with AOS ($P < 0.05$). *Firmicutes* phylum levels in the samples fermented with FOS were unaffected, although levels of the *C. coccoides* group, the *C. leptum* subgroup, and *F. prausnitzii* were significantly reduced ($P < 0.05$). FOS and AOS fermentation both resulted in a decrease of the relative abundance of *Bacteroidetes* phylum and *Desulfovibrio* spp. (genus belonging to the *Proteobacteria* phylum) after fermentation of all three fecal microbiota groups ($P < 0.05$).

No significant differences between the effects of the two carbohydrate sources AOS and FOS were observed, except in the case of *Bifidobacterium* spp., which were more abundant ($P < 0.05$) in the samples from healthy subjects after incubation with FOS than in those incubated with AOS, and *Desulfovibrio* spp., where the density was significantly lower ($P < 0.05$) in the samples from UC patients in remission after incubation with FOS than after incubation with AOS.

SCFA analysis and pH measurements after fermentation. Amounts of SCFA (acetate, propionate, butyrate) produced by fermentation of FOS and AOS and pH measurements are shown in Table 2. For all types of samples (healthy, remission, and relapse), the pH was lower in samples incubated with FOS and AOS than in the samples incubated without added substrate. The amount of acetate was significantly higher ($P < 0.05$) in all types of samples fermented with FOS than in the samples with no substrate added. Additionally, acetate was higher in samples from patients in relapse incubated with AOS. A significantly lower ($P < 0.05$) amount of propionate was measured in samples from healthy subjects incubated with FOS than in samples incubated with no substrate added. No significant difference was observed in the amount of butyrate after fermentation, independent of substrate and origin of the microbial community.

Principal component analysis (PCA) of bacterial composition. The different bacterial taxonomic units as a function of disease status and incubation with FOS or AOS were subjected to PCA to generate an overview of the variation in the ability of the microbial communities from either healthy subjects, UC patients in remission, or UC patients in relapse to ferment the two carbohydrate sources. Data were visualized in two dimensions using a principal component score and loading plot (Fig. 3A and B, respectively). Two principal components were calculated for the model with a total of 73.60% of variance expressed. The samples before (original bacterial communities) and after incubation with or without either FOS or AOS for all three stages of disease were clearly differentiated by the PC1, with the fermented samples clustering to the left of the PCA score plot (Fig. 3A). However, differentiation between samples incubated with FOS and AOS was not seen. The loading plot revealed that the primary bacterial taxa influencing the differentiation between the original bacterial communities and samples incubated with added substrate were the *Bifidobacterium* spp. and the *Lactobacillus* spp. (Fig. 3B).

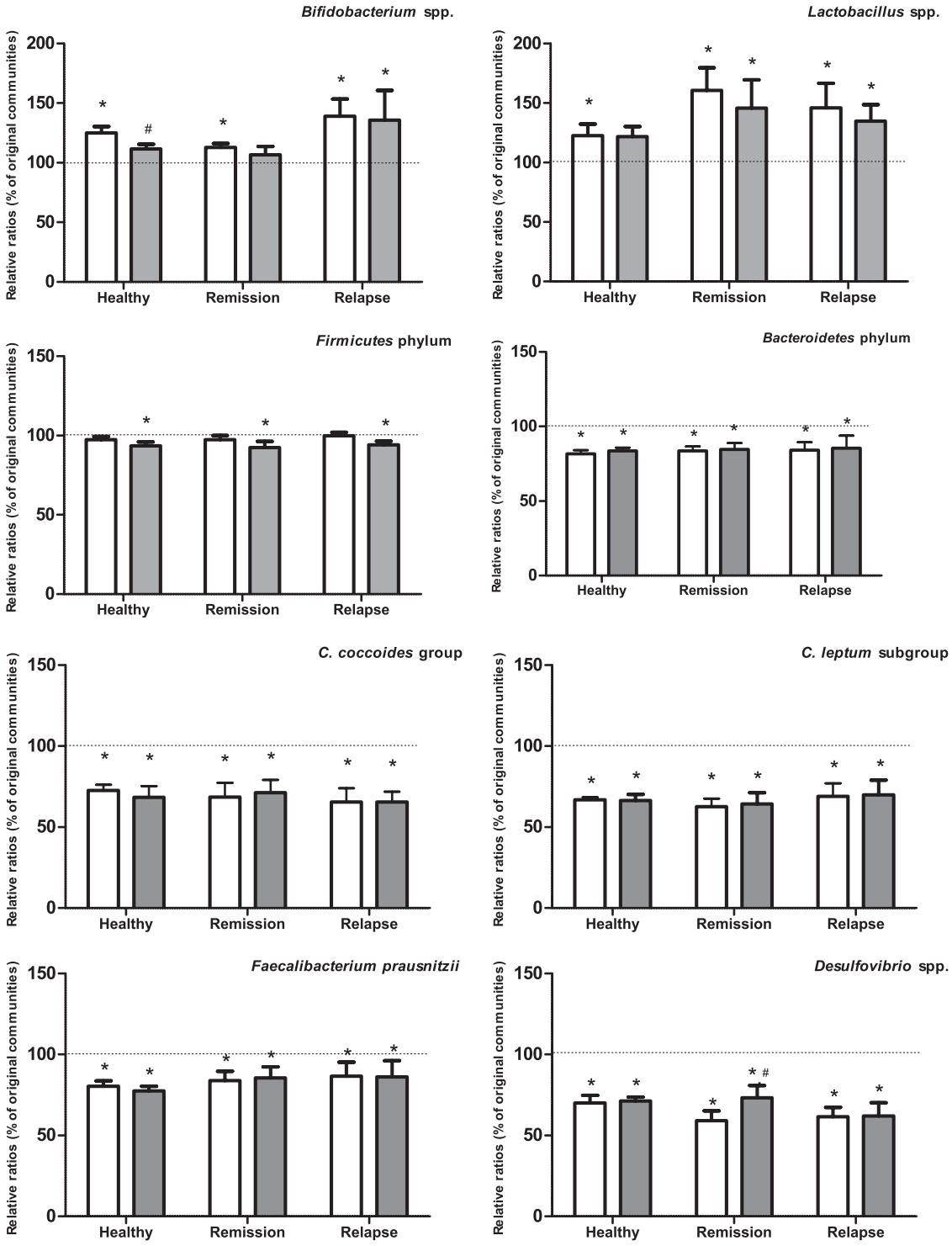

FIG. 2. Relative ratios (RR) of target genes in samples incubated with FOS (white) and AOS (gray) compared to original bacterial communities, calculated as $RR = E^{\Delta CT\ (\text{fermentation samples})}/E^{\Delta CT\ (\text{original bacterial community})}$. Original communities were set to 100% for each group (indicated by dotted lines). Error bars represent averages ± standard errors of the means of relative changes in target species in each of the six samples in each group. Asterisks (*) indicate significant differences from the original bacterial communities, while pound signs (#) indicate significant differences from FOS-fermented samples.

TABLE 2. pH measurements and short chain fatty acids liberated during fermentation

Parameter	Result for patient group[a]								
	Healthy			Remission			Relapse		
	NS	FOS	AOS	NS	FOS	AOS	NS	FOS	AOS
pH range[b]	6.5–7.5	4.5–5.5	5–5.5	6.5–7.5	4.5–5.5	4.5–6	6.5–7.5	4.5–5.5	4.5–5.5
Total SCFA	36.34 (± 3.5)	40.97 (± 5.9)	33.77 (± 4.4)	24.53 (± 2.6)	32.79 (± 4.5)	22.35 (± 2.8)	14.95 (± 1.2)	28.95 (± 3.6)	21.25 (± 2.7)
Acetate	23.06^{A} (± 2.7)	36.49^{B} (± 5.8)	27.73^{A} (± 3.3)	16.96^{A} (± 2.0)	27.93^{B} (± 1.9)	17.67^{A} (± 1.8)	7.74^{A} (± 2.1)	22.78^{B} (± 4.6)	17.08^{B} (± 1.6)
Propionate	5.91^{A} (± 2.1)	1.61^{B} (± 0.18)	2.54^{AB} (± 1.1)	2.59 (± 0.82)	2.24 (± 1.1)	0.78 (± 0.16)	2.31 (± 0.7)	2.40 (± 0.9)	2.38 (± 1.5)
Butyrate	6.03 (± 1.5)	1.46 (± 0.41)	2.97 (± 2.4)	4.11 (± 1.2)	2.21 (± 0.9)	2.16 (± 1.5)	3.90 (± 1.5)	3.40 (± 1.8)	1.51 (± 1.0)

[a] NS, no substrate added; FOS, fructo-oligosaccharide; AOS, arabino-oligosaccharide. Total SCFA is acetate, butyrate, propionate, iso-butyrate, iso-valate, and valate. All numbers are means of six samples ± standard errors of the means and are expressed as nM. Unlike superscript letters are significantly different ($P < 0.05$), comparing each disease group.

[b] pH was measured using a pH indicator stick. Due to the uncertainty in the method, a range of pH for the different fermentations is used.

DISCUSSION

In this study, the mean relative quantity of *Bifidobacterium* spp. in the original bacterial community was lower in UC patients in relapse than in healthy controls (Fig. 1), but this was not statistically significant. A previous study by Sokol and coworkers (64) has shown significantly lower levels of *Bifidobacterium* spp. in fecal samples of UC patients. Additionally, *Lactobacillus* spp. was significantly less abundant in the original bacterial community of UC patients in relapse than in that of healthy controls.

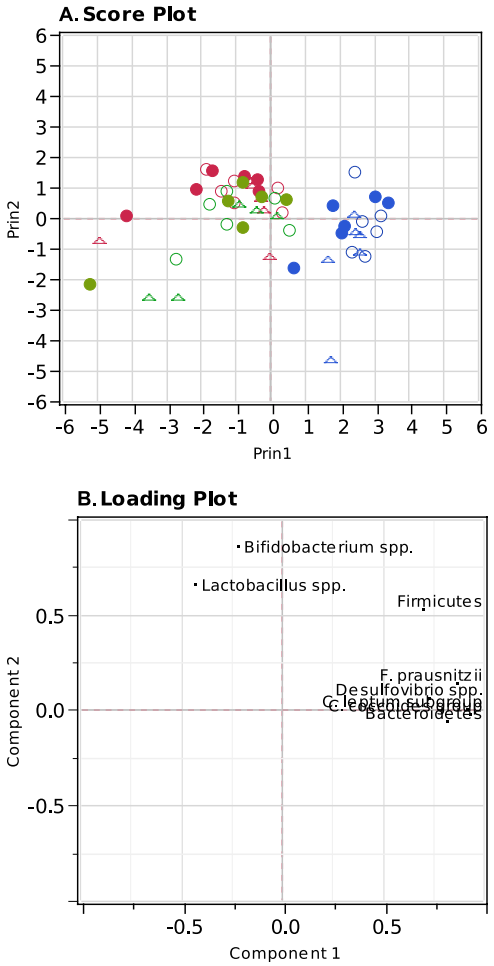

FIG. 3. Principal component analysis of the quantitative PCR measurements using the first and second principal component (PC1, 54.94%; PC2, 18.66%). (A) Score plot showing the original bacterial communities (blue), samples incubated with FOS (red) and samples incubated with AOS (green). Sources of the communities are indicated by open circles (○) for healthy subjects, closed circles (●) for UC patients in remission, and triangles (Δ) for UC patients in relapse. (B) Loading plot, indicating each of the measured bacterial taxa as determined by quantitative real-time PCR.

It should be acknowledged that differences between the microbial communities may partly be the consequence of the use of anti-inflammatory drugs, since a recent study showed that melsalazine affected the fecal bacterial community composition in patients with inflammatory bowel syndrome (2). In the present study, four out of six UC patients in remission received melsalazine, whereas all the UC patients in relapse received the drug.

Fermentation of FOS resulted in significantly higher levels of bifidobacteria in all types of microbial communities originating from either healthy subjects or UC patients. The bifidogenic effect of FOS using fecal samples from healthy subjects is in accordance with a number of previous *in vitro* studies (24, 50, 55, 58). In contrast to these observations, a recent study did not reveal any significant difference in the level of bifidobacteria after incubation of fecal microbial communities derived from healthy subjects with FOS and even demonstrated a significant decrease in this bacterial group after FOS incubation of fecal communities from patients with inactive Crohn's disease or active UC (49). The discrepancy in results between the present study and the results reported by Rose and coworkers (49) might be explained by the use of different incubation methods. In the present study, a static batch system was used for each individual fecal community, while Rose and coworkers (49) pooled the individual fecal samples and used the dynamic TIM-2 system. In agreement with the present approach, many *in vivo* trials suggest a bifidogenic effect of FOS in subjects with inflammatory bowel disease. A recent trial using rats induced with colitis by 2,4,6-trinitrobenzenesulfonic acid (TNBS) showed that administration of FOS had an anti-inflammatory effect and significantly increased concentrations of bifidobacteria in cecum and colon (48). Additionally, a small trial with patients suffering from active Crohn's disease showed that administration of FOS significantly increased the mucosal and fecal counts of bifidobacteria. Furthermore, an increase in the percentage of lamina propria dendritic cells expressing the anti-inflammatory cytokine interleukin 10 (IL-10) as well as a significant upregulation of expression of the

Toll-like receptors (TLRs) TLR2 and TLR4 was observed. The authors of reference 32 suggest that this was caused by the bifidogenic effect of FOS and that an increase of bifidobacteria can act via TLRs or other pattern recognition receptors on dendritic cells to stimulate IL-10 production.

Incubation with AOS gave rise to a significantly higher level of bifidobacteria in fecal microbial communities derived from UC patients in relapse, while no difference was seen after AOS incubation of fecal communities from healthy subjects or UC patients in remission. It was surprising to us that no statistically significant increase of bifidobacteria upon AOS fermentation by microbial communities derived from healthy subjects was observed, since previous experiments from our lab have shown that AOS caused a significantly higher level of bifidobacteria in fecal microbial communities derived from healthy subjects (24). However, there was a similar bifidogenic trend in the present study ($P = 0.078$), and we speculate that the lack of significance is explained by a larger variation between the individual samples used. A previous *in vitro* fermentation study using feces from healthy subjects reported that *Bifidobacterium* spp. varied in their ability to hydrolyze arabino-oligosaccharides and that they grew better on low-molecular-weight fractions than on high-molecular-weight fractions of the oligosaccharides (1). Genome sequencing has revealed that carbohydrate-modifying enzymes can vary between species. *Bifidobacterium longum*, *Bifidobacterium adolescentis*, *Bifidobacterium animalis* subsp. *lactis*, *Bifidobacterium bifidum*, *Bifidobacterium pseudocatenulatum*, and *Bifidobacterium breve*, which are all commonly detected in human adult feces (67), have genes encoding β-fructofuranosidase and α-L-arabinofuranosidase, enabling the bacteria to utilize FOS and AOS, respectively (UniProtKB database, http://www.uniprot.org/uniprot). α-L-Arabinofuranosidase catalyzes the hydrolysis of the glycosidic bond between an arabinose moiety and the backbone, but sequence analysis does not necessarily reveal if the specificity is toward branched arabinan or arabinoxylan. However, *B. breve* and *B. bifidum* do not harbor α-L-arabinanase, which catalyzes the hydrolysis of AOS backbone (UniProtKB database, http://www.uniprot.org/uniprot). However, it has been demonstrated that *B. adolescentis* and *B. longum* are able to degrade linear arabino-oligosaccharides (8 DP) to the same extent as FOS, whereas *B. breve* is able only to hydrolyze FOS and *B. bifidum* is not able to degrade either FOS or AOS. In the same study, *B. adolescentis* utilized neutral oligosaccharides derived from apple pectin (65). In another study, also based on pure cultures, it was observed that *B. breve*, *B. longum*, and *B. adolescentis* were able to partly ferment a mixture of linear arabino-oligosaccharides (2 to 5 DP) derived from sugar beet pectin; however, FOS was more efficiently fermented (68). The observed differences in the response from the bifidobacterial communities reported here may thus be explained by a different intragenic composition of bifidobacteria in the individual samples. Densities of lactobacilli in all fermentation samples were significantly increased after incubation with FOS of microbial communities from healthy subjects and UC patients. The same was seen after incubation with AOS, although it did not reach statistical significance in healthy subjects ($P = 0.057$). The importance of the presence of *Lactobacillus* spp. in the gut was previously highlighted by a study with IL-10-deficient mice with colitis and reduced *Lactobacillus* spp. levels, which showed that restoration of *Lactobacillus* spp. to a normal level reduced the development of colitis (35). Additionally, a human trial showed that ingestion of *Lactobacillus* GG aided the maintenance of remission in patients with UC (72). Our *in vitro* observations thus suggest that AOS as well as FOS consumption might help patients suffering from UC by increasing their levels of intestinal lactobacilli.

Bacteroidetes and *Firmicutes* (including *C. coccoides* group, *C. leptum* subgroup, *F. prausnitzii* and *Lactobacillus* spp.) represent the two dominant bacterial phyla in the human gut and together comprise about 90% of the large intestinal microbiota (13, 45). A changed ratio between these groups has been suggested to affect intestinal inflammation (64). In the present study, no effect on the ratio between the *Bacteroidetes* and *Firmicutes* was caused by incubation with either FOS or AOS. Nevertheless, the relative quantity of members of the phylum *Bacteroidetes* was significantly decreased in samples incubated with FOS and AOS for all fecal communities tested, probably due to the increase in bifidobacteria, which belong to the phylum *Actinobacteria*. It may, however, be speculated that the observed decrease was the result of the lower level of pH observed after the 24 h of fermentation (Table 2) rather than the inability of species of this phylum to utilize the carbohydrates, since responses to pH vary considerably between different phylogenetic groups of human colonic anaerobes and variation in pH tolerance among the *Bacteroidetes* phylum has been observed (12). However, in agreement with an effect of the oligosaccharides added, Van Laere and coworkers (68) demonstrated that several species of *Bacteroides*, including *Bacteroides vulgatus*, *Bacteroides ovatus*, and *Bacteroides thetaiotaomicron*, are not able to utilize a mixture of linear arabino-oligosaccharides (2 to 5 DP) and can only partly degrade FOS. Additionally, pH-controlled *in vitro* studies also have reported a significant decrease in the population size of *Bacteroides* after incubation of FOS with fecal communities from UC patients (49) and healthy subjects (36). Taken together, these findings indicate that even though the *Bacteriodetes* phylum contains genera that can degrade and ferment a wide variety of plant polysaccharides and oligosaccharides (25), they may not be able to utilize FOS and AOS and hence may not compete in the fermentor community when these are the primary carbohydrate sources.

The butyrate-producing species *F. prausnitzii* and the butyrate-producing bacterial groups *C. coccoides* (cluster XIVa) and *C. leptum* (cluster IV) are important for colonic health and have an anti-inflammatory effect due to their production of butyrate (34). However, in the present study their relative abundance was decreased after incubation with FOS as well as AOS in all three types of fecal communities. A similar effect of FOS consumption on the abundance of the *C. coccoides* group has been observed in mouse trials (44).

The relative density of *Desulfovibrio* spp. was significantly decreased by incubation with FOS as well as AOS in all three types of samples. *Desulfovibrio* spp. is the predominant sulfate-reducing bacteria in the human colon, and the principal by-product of sulfate-reducing bacteria is hydrogen sulfide (51). The pH plays an important role for the growth of sulfate-reducing bacteria and their production of hydrogen sulfate. These bacteria prefer a growth environment that is neutral or slightly alkaline (18), hence the low pH in the FOS- and AOS-

incubated samples is likely to have caused the relative decrease of *Desulfovibrio* spp. A similar reduction in pH has been demonstrated in the cecum and colon of animals given FOS compared to control animals (6, 30, 48).

Sulfate-reducing bacteria have previously been linked to UC development, because high concentrations of sulfides inhibit butyrate metabolism in colonocytes and induce hyper-proliferation and metabolic abnormalities in epithelial cells, similar to those observed with UC (7, 15). Additionally, Rowan and coworkers (51) found that higher levels of *Desulfovibrio* spp. were detected in the colonic mucus of patients with active UC than in healthy controls, indicating a role of this bacterium in the pathogenesis of UC. However, in the present study, no significant difference in the abundance of *Desulfovibrio* spp. was observed between the bacterial communities originating from each of the three groups, which may be explained by the use of fecal samples as opposed to mucosal samples (73). Still, this does not exclude that a reduction in abundance of *Desulfovibrio* spp. as caused by FOS and AOS in the present study may be beneficial in the prevention of colitis.

The SCFA profile reflects microbial activity (55). Acetate is the predominant acid produced by bacterial fermentation in fecal communities, and in consistence with the observed increase in the acetate-producing bifidobacteria and lactobacilli, our study revealed a significantly larger amount of acetate in the samples incubated with either FOS or AOS. The higher production of acetate may be the main cause of the observed decrease in pH resulting from FOS and AOS fermentation. Significantly lower levels of propionate were observed after incubation of bacterial communities from healthy subjects with FOS. Similar results have previously been reported by other studies, where FOS fermentation resulted in lower levels of propionate than other substrates, including, e.g., gentio-oligosaccharides (56) and AOS (1). One of the major intestinal producers of propionate is *Propionibacterium* (19), and we speculate that the population size of *Propionibacterium* species may be higher (and more easily reduced after fermentation) in healthy subjects than in UC patients, since no difference in propionate level was observed after FOS incubation using fecal samples from UC patients.

PCA of the qPCR data revealed that the bacterial groups separating the fermented samples from the original bacterial communities were *Bifidobacterium* spp. and *Lactobacillus* spp., which were both significantly more abundant in the microbiota after fermentation of either FOS or AOS by communities derived from UC patients (Fig. 2 and 3). Communities incubated with FOS and AOS were not differentiated by the PCA (Fig. 3), suggesting that these two oligosaccharides affected the microbiotas similarly.

Our results suggest that the positive effects already observed in human and animal trials using FOS may be applicable also for AOS, which opens the possibility of exploration of, e.g., by-product streams of sugar production for prebiotic preparations. Hence, the presence and fermentation of AOS may help prevent proinflammatory conditions and enhance the resistance to colonization of opportunistic pathogens, e.g., through increased production of acetate (17) and reduced pH in the colonic environment. Considering the reduced levels of *Lactobacillus* spp. and *Bifidobacterium* spp. present in UC patients in relapse, a stimulation of these bacterial groups may prevent flare-ups. However, human trials are needed to confirm a health-promoting effect of AOS in UC patients.

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Paper 4

Feruloylated and non-feruloylated arabino-oligosaccharides from sugar beet pectin selectively stimulate the growth of *Bifidobacterium* spp. in human fecal *in vitro* fermentation.

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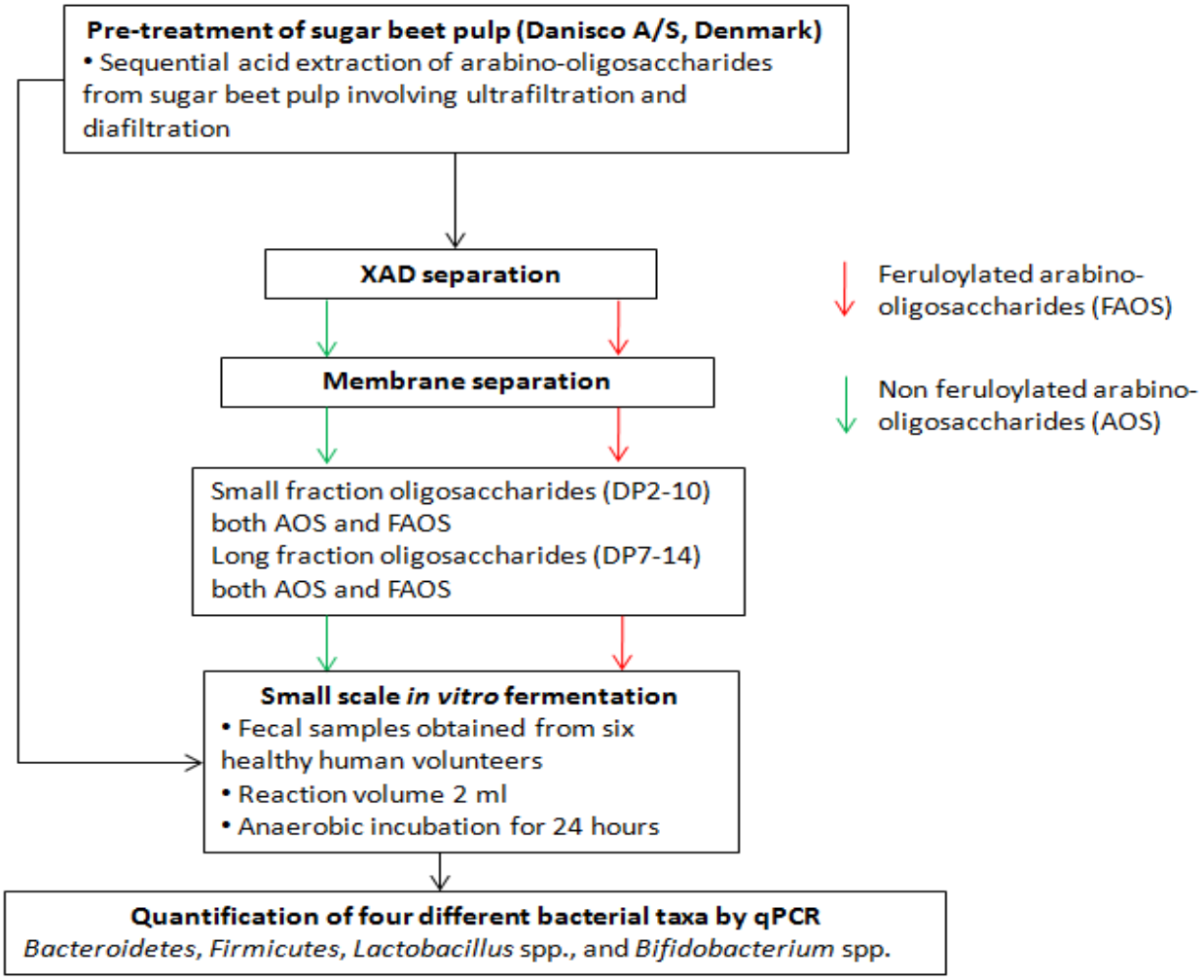
Introduction

The aim of this study was to prepare fractions of arabino-oligosaccharides with different chain length and feruloyl substitutions. The different oligosaccharides were assessed for prebiotic properties using human fecal *in vitro* fermentations.

Flow diagram



The author, Louise K. Vigsnæs, performed the small scale *in vitro* fermentations, and conducted the quantification of the bacterial taxa after fermentation. Louise K. Vigsnæs took part in the evaluation of the results and in preparation of manuscript regarding the *in vitro* fermentation study.

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ARTICLE

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Feruloylated and Nonferuloylated Arabino-oligosaccharides from Sugar Beet Pectin Selectively Stimulate the Growth of *Bifidobacterium* spp. in Human Fecal in Vitro Fermentations

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ABSTRACT: The side chains of the rhamnogalacturonan I fraction in sugar beet pectin are particularly rich in arabinan moieties, which may be substituted with feruloyl groups. In this work the arabinan-rich fraction resulting from sugar beet pulp based pectin production was separated by Amberlite XAD hydrophobic interaction and membrane separation into four fractions based on feruloyl substitution and arabino-oligosaccharide chain length: short-chain (DP 2−10) and long-chain (DP 7−14) feruloylated and nonferuloylated arabino-oligosaccharides, respectively. HPAEC, SEC, and MALDI-TOF/TOF analyses of the fractions confirmed the presence of singly and doubly substituted feruloylated arabino-oligosaccharides in the feruloyl-substituted fractions. In vitro microbial fermentation by human fecal samples ($n = 6$ healthy human volunteers) showed a selective stimulation of bifidobacteria by both the feruloylated and the nonferuloylated long-chain arabino-oligosaccharides to the same extent as the prebiotic fructo-oligosaccharides control. None of the fractions stimulated the growth of the potential pathogen *Clostridium difficile* in monocultures. This work provides a first report on the separation of potentially bioactive feruloylated arabino-oligosaccharides from sugar beet pulp and an initial indication of the potentially larger bifidogenic effect of relatively long-chain arabino-oligosaccharides as opposed to short-chain arabino-oligosaccharides.

KEYWORDS: prebiotics, arabino-oligosaccharides, feruloyl substitution, hydrophilic interaction chromatography, *Clostridium difficile*

INTRODUCTION

Sugar beet pulp is a large side stream from the industrial production of sugar. Currently, the main utilization of sugar beet pulp is for cattle feed, but pectin can be extracted for a limited number of applications. In sugar beet pectin the side chains of rhamnogalacturonan I (RGI) are especially rich in arabinan composed of α-(1,5)-linked backbones with a high degree of α-(1,2) and/or α-(1,3) arabinofuranosyl substitutions[1] along with some β-(1,4)-linked galactan. The side chains of sugar beet RGI can be feruloyl substituted either on O-2 in the main backbone of α-(1,5)-linked arabinan, on O-5 in the terminal arabinose,[2] or on O-6 in the main backbone of galactan.[3] The content of ferulic acid can be up to 8.3 mg/g pectin.[4] The feruloyl substitutions can either be present as monomers or form dimers with other side chains through oxidative coupling reactions.[5] Diferulic cross-linking plays an important role in plant texture.[6]

Sugar beet pectin-derived arabino-oligosaccharides with DP 2−6 and DP < 8 have been reported to selectively stimulate bifidobacteria over clostridia, lactobacilli, and bacteroides in in vitro fecal fermentations.[7,8] Moreover, in single-culture studies it has been shown that linear arabino-oligosaccharides may be utilized by *Bifidobacterium adolescentis*, *Bifidobacterium longum*, and *Bacteroides vulgatus*, but not by other bifidobacteria or the tested lactobacilli.[9]

The biological effect of oligosaccharide feruloyl substitution on gut microbiota has so far mainly been investigated for arabino-xylo-oligosaccharides. Feruloylated arabino-xylo-oligosaccharides were shown to stimulate the growth of *Bifidobacterium bifidum* in a single-culture study,[10] xylo-feruloyl-arabinose from grass cell walls has been reported to be metabolized by rat gut microbiota,[11] and recently ferulic acid was found to be released from durum wheat oligosaccharides during digestion by human intestinal microbiota.[12] Moderate feruloyl substitution was shown not to impede the degradation of maize cell wall by human intestinal microbiota.[13]

Probiotic bacteria such as bifidobacteria and lactobacilli have been shown to be able to specifically express feruloyl esterase activity in the presence of feruloylated substrates. *Bifidobacterium* sp., including *B. longum*, thus express feruloyl esterase activity in the presence of wheat bran, rye bran, barley spent grain, and larchwood arabinogalactan,[14] and *Lactobacillus acidophilus* expresses feruloyl esterase activity in the presence of destarched wheat bran.[15] These studies on arabinoxylan-derived oligosaccharides are not directly comparable to feruloyl-substituted arabino-oligosaccharides, because feruloyl substitution in

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arabinoxylan is O-5 linked and feruloyl substitutions in arabino-oligosaccharides are mainly O-2 linked. Therefore, these two types of feruloyl-substituted substrates require different types of feruloyl esterases for enzyme-catalyzed ferulic acid removal. It is known that type B feruloyl esterases are active on pectin-derived substrates,[16] but little has been reported with respect to bifidogenic or other prebiotic effects of pectin-derived O-2 ferulic acid—arabinose conjugates. We hypothesized that O-2 feruloyl substitutions on arabino-oligosaccharides derived from sugar beet pectin might exhibit prebiotic effects and, secondly, that the arabino-oligosaccharide backbone chain length might influence the outcome. The purpose of the present study was therefore to first prepare fractions of different arabino-oligosaccharide chain lengths enriched in feruloyl substitutions, and the corresponding nonferulated counterparts, both from sugar beet pectin and then to evaluate the in vitro fermentability of the fractions by human intestinal microbiota.

MATERIALS AND METHODS

Substrate. Sugar beet arabino-oligosaccharides were obtained from Danisco A/S (Nakskov, Denmark). These arabino-oligosaccharides, in the following referred to as "starting material", were supplied as a liquid side stream from the ultrafiltration and diafiltration step in the sequential acid extraction of pectin with nitric acid from sugar beet pulp, involving removal of insoluble cellulose, ultrafiltration, and diafiltration with a 50 kDa cutoff.[4]

Chemicals. Ferulic acid, cinnamic acid, sodium hydroxide, sodium acetate, D-glucuronic acid, D-galactose, D-arabinose, D-fucose, L-rhamnose monohydrate, D-galacturonic acid monohydrate, and 2,5-dihydroxybenzoic acid were obtained from Sigma-Aldrich (Steinheim, Germany). D-Glucose and D-xylose were obtained from Merck (Darmstadt, Germany). Trifluoroacetic acid was from Riedel-deHaën (Seelze, Germany). Arabino-oligosaccharide standards with DP from 2 to 5 were obtained from Megazyme (Bray, Co. Wicklow, Ireland). Acetonitrile was obtained from LGC Promochem (Middlesex, U.K.). Amberlite XAD-2 was obtained from Rohm and Haas Denmark (Copenhagen, Denmark). Fructo-oligosaccharides (FOS) (DP 2−8; OraftiP95) were obtained from Beneo-Orafti (Tienen, Belgium).

XAD Separation. XAD separation was performed at room temperature in a 200 mL stirred membrane reactor model 8200 (Millipore, Billerica, MA) equipped with a 100 kDa regenerated cellulose membrane (Millipore), connected to compressed nitrogen for flux regulation. The reactor contents were mixed by magnetic stirring. The starting material was added to the XAD material (washed in 2 volumes of ethanol and 2 volumes of water prior to use) in a 1:1 volume ratio. The suspension was stirred for 1 h, and unbound material was then removed by filtration. In each round, the contact time between the eluent and the XAD was 30 min before filtration. Putative nonferuloylated arabino-oligosaccharides (denoted AOS) were eluted in 4 sample volumes of water, and feruloylated arabino-oligosaccharides (denoted FAOS) were eluted in 5 sample volumes of methanol/water 1:1 (v/v). The methanol/water fraction was dried on a rotary evaporator and resolubilized in deionized water. The AOS and FAOS fractions were subsequently separated according to molecular weight (see below). Residual material was eluted in 3 sample volumes of 100% methanol. This fraction was not used further.

Membrane Separation. Separation according to size for both the AOS fraction and the FAOS fraction was performed in a 200 mL stirred membrane reactor model 8200 (Millipore) equipped with a 1 kDa MWCO regenerated cellulose membrane (Millipore), connected to compressed nitrogen for flux regulation. The reactor contents were mixed by magnetic stirring using an RCT basic magnetic stirrer (IKA, Germany). The temperature was maintained at 45 °C. Filtration was performed until the retentate volume was 10% of the sample volume, followed by diafiltration in 1 sample volume of deionized water. After ultra- and diafiltration, all permeates and retentates were concentrated on a rotary evaporator, lyophilized, resolubilized in water, and stored at 4 °C until further use. The permeates enriched in low molecular weight (small) oligosaccharides were denoted SAOS and SFAOS, respectively, and the retentates enriched in high molecular weight (long) oligosaccharides were denoted LAOS and LFAOS, respectively.

Acid Hydrolysis. The monosaccharide composition of all fractions was determined by acid hydrolysis with 2 g/L substrate concentration and 2 M trifluoroacetic acid at 121 °C for 2 h followed by lyophilization and resolubilization in deionized water, and the recovery of monosaccharides was determined by performing the same hydrolysis on D-fucose, L-rhamnose, D-arabinose, D-galactose, D-glucose, D-xylose, D-galacturonic acid, and D-glucuronic acid.[17] Relative standard deviation values for all measurements were determined for at least three measurements and ranged from 0.5 to 10%.

Ionic Exchange Chromatography (HPAEC). Monosaccharide composition and AOS concentrations were analyzed by high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) using a Dionex BioLC system (Dionex Corp., Sunnyvale, CA) equipped with a Dionex CarboPac PA1 analytical column (4 × 250 mm). For analysis of monosaccharides a two-eluent system comprising deionized water and a 500 mM NaOH aqueous solution was used. The monosaccharides were separated by isocratic elution with 25 mM NaOH for 4 min, 10 mM NaOH for 14 min, and 500 mM NaOH for 12 min, followed by regeneration with 25 mM NaOH for 5 min. For analysis of oligosaccharides, a three-eluent system comprising deionized water, a 500 mM NaOH aqueous solution, and a 500 mM sodium acetate aqueous solution was used (modified from ref 18). AOS were eluted isocratically with 25 mM NaOAc for 5 min followed by a linear gradient from 25 to 400 mM NaOAc for 20 min and then isocratically with 400 mM NaOAc for 10 min. The NaOH concentration was maintained at 100 mM throughout the run. The eluent flow was always kept at 1.0 mL/min. Quantification was carried out using external monosaccharide standards: D-fucose, L-rhamnose, D-arabinose, D-galactose, D-glucose, D-xylose, and D-galacturonic acid or di-, tri-, tetra-, and pentamers of arabino-oligosaccharides. Amounts were expressed as milligrams per gram of dry matter (mg/g) or mole percentage (mol %). The following pulse potentials and durations were used for detection: E1 = 0.1 V, t1 = 400 ms; E2 = −2 V, t2 = 20 ms; E3 = 0.6 V, m3 =10 ms; E4 = −0.1 V, t4 = 60 ms. Data were collected and analyzed with the program Chromeleon 6.80 SP4 Build 2361 software (Dionex Corp., Sunnyvale, CA).

Size Exclusion Chromatography. HPSEC was performed using a P680 HPLC pump, an ASI-100 automated sample injector, and an RI-101 refractive index detector (Dionex). Samples were separated on a Shodex SB-806HQGPC column (300 × 8 mm) with a Shodex SB-G guard column (50 × 6 mm) from Showa Denko K.K. (Tokyo, Japan) with 100 mM sodium acetate, pH 6, as mobile phase at a flow rate of 0.5 mL/min; the temperature was maintained at 40 °C. Prior to injection, the sample was diluted in 100 mM sodium acetate to avoid large buffer peaks.[19]

Phenolics Analysis. Ferulic acid was released from arabinan structures by saponification by adding NaOH to a final concentration of 1 M followed by incubation at room temperature for 1 h. Subsequently, the pH was adjusted to <3 by adding HCl. Ferulic acid and vanillin were analyzed using RP-HPLC with DAD, Chemstation 1100 series, Hewlett-Packard, and an ODS-L Optimal (250 × 4.6 mm, 5 μm) column from Capital HPLC. The chromatographic profile consisted of buffer A (5% acetonitrile, 1 mM TFA) and acetonitrile starting with 20% acetonitrile. Running gradient was up to 40% acetonitrile for 35 min and further up to 100% for another 3 min, followed by regeneration to 20% acetonitrile for 2 min. Column temperature was maintained at 40 °C.[20] The amount of *trans*-ferulic acid was detected and quantified at 316 nm

Table 1. 16S rRNA Primers for Real Time PCR

target gene	forward primer (5′−3′)	reverse primer (5′−3′)	efficiency (%)	ref
Bifidobacterium spp.	cgc gtc ygg tgt gaa ag	ccc cac atc cag cat cca	101	32
Lactobacillus spp.	agc agt agg gaa tct tcc a	cac cgc tac aca tgg ag	95	33, 34
Firmicutes phylum	gga gya tgt ggt tta att cga agc a	agc tga cga caa cca tgc ac	99	35
Bacteroidetes phylum	gga rca tgt ggt tta att cga tga t	agc tga cga caa cca tgc ag	100	35
total bacteria	act cct acg gga ggc agc agt	gta tta ccg cgg ctg ctg gca c	101	34

Table 2. Mass and Ferulic Acid Distributions in Fractions from XAD Separation

	dry matter (mass %)	ferulic acid (μmol/g)	ferulic acid (mol %)
starting material	100.0	36.2	100.0
unbound	47.9	6.1	8.1
water	28.7	0.7	0.5
MeOH/water	21.3	143.9	84.8
MeOH	1.8	12.0	0.6
loss	0.4		6.1

using an authentic external standard and expressed as micromoles per gram of dry matter (μmol/g). *cis*-Ferulic acid and vanillin were detected and recognized at 316 nm but quantified at 280 nm according to response factors as described by Waldron et al.[21]

HILIC Chromatography. Separation of feruloylated AOS based on hydrophilic interaction was performed using an ÄKTA purifier 100 workstation equipped with a UV-900 monitor, a pH/C monitor, and a Frac-950 fraction collector, equipped with an TSK-GEL Amide-80 column (250 × 4.6 mm i.d., 5 μm, Tosoh Bioscience), all controlled by UNICORN software. The temperature was maintained at 55 °C using a Thermasphere HPLC column heater/chiller (Phenomenex). The elution system consisted of acetonitrile and deionized water. The column was equilibrated with 80% acetonitrile for 2 column volumes (CV), isocratic elution at 80% for 2 CV, followed by a linear gradient at 2% acetonitrile/CV. Feruloylated compounds were detected and quantified by UV absorption at 316 nm using ferulic acid as external standard. Prior to injection, the sample was adjusted to 80% acetonitrile, centrifuged briefly for removal of precipitate, and filtered through a 0.22 μm nylon filter.

Mass Spectrometry. One microliter of sample followed by 0.5 μL of matrix solution (20 mg/mL 2,5-dihydroxybenzoic acid 70% acetonitrile and 0.1% trifluoroacetic acid/water) was added to an Opti-TOF 384 well plate. For fast crystallization, the sample was dried under a lamp and an additional 0.5 μL of matrix solution added. The samples were analyzed on a 4800 Plus MALDI TOF/TOF (AB Sciex) mass spectrometer. The instrument was operated in reflectron, positive ion mode. Acceleration voltage was 20 kV. Depending on the sample analyzed, laser intensity and number of laser shots were varied to obtain optimal spectra. The mass range was set to 100−3500 Da. For all MS/MS data, air was used as collision gas. The MS and MS/MS data were exported as a text file using DataExplorer (version 4.6), and each spectrum was smoothed, labeled, and analyzed manually employing M/Z (Genomic Solutions). For annotation of MS/MS spectra, the nomenclature suggested by Doman and Costello[22] was applied.

Subjects and Fecal Samples. Fecal samples were obtained from six healthy volunteers (mean age = 40.7 ± 8.5 years). None of the participants had been treated with antibiotics for at least 3 months before participation and had no history of gastrointestinal disorder. Whole stools were collected in airtight containers, immediately stored at 4 °C, and processed within 12 h. Feces (200 mg wet weight) were collected for DNA extraction, and additional fecal samples for in vitro fermentation were prepared by homogenization in 50% glycerol (1:1 dilution in deionized water) in an anaerobic cabinet (containing 10% H_2, 10% CO_2, and 80% N_2) and stored at −80 °C until further analysis.

In Vitro Fermentation. A small-scale in vitro fermentation method was used to assess the fermentability of the oligosaccharides on human fecal samples principally as reported previously.[23] FOS was applied as a standard with known bifidogenic effect. Oligosaccharides or FOS were added to an autoclaved minimal basal medium to give a final concentration of 5 g (dry matter)/L in a reaction volume of 2 mL. The solutions were reduced in an anaerobic cabinet overnight. The minimal basal medium contained, per liter, 2 g of peptone water, 1 g of yeast extract, 0.1 g of NaCl, 0.04 g of K_2HPO_4, 0.04 g of KH_2PO_4, 0.01 g of $MgSO_4 \cdot 7H_2O$, 0.01 g of $CaCl_2 \cdot 2H_2O$, 2 g of $NaHCO_3$, 0.5 g of bile salts, 0.5 g of L-cysteine hydrochloride, 0.005 g of hemin, 10 μL of vitamin K_1 (0.02 mM), 2 mL of Tween 80, and 1 mL of 0.05% (w/v) resazurin solution. A 10% (w/v) fecal slurry was prepared by mixing the feces stored in 50% glycerol with degassed PBS. The reduced minimal medium samples with added oligosaccharides were inoculated at a final concentration of 1% (w/v) feces. Each fermentation experiment for the fecal sample of each healthy volunteer was carried out in triplicate to give 6 × 3 fermentations that were incubated at 37 °C for 24 h in an anaerobic cabinet.

Extraction of Bacterial DNA. DNA was extracted from fecal and fermentation samples using the QIAamp Stool DNA Mini Kit (Qiagen, Hilden, Germany) with a bead-beater step in advance, as described by Leser et al.[24] The purified DNA was stored at −20 °C until use.

Real-Time PCR Assay Conditions. Amplification and detection of purified bacterial DNA by real-time PCR were performed with the ABI-Prism 7900 HT from Applied Biosystems using optical grade 384-well plates. Primers specifically targeting 16S rRNA gene sequences of the *Bacteroidetes* and *Firmicutes* phyla and the *Lactobacillus* and *Bifidobacterium* genera were included in the qPCR analysis (Table 1). The amplification reactions were carried out in a total volume of 11 μL containing 50 μL of EXPRESS SYBR GreenER qPCR SuperMix (Invitrogen A/S, Taastrup, Denmark), 400 nM of each of the primers (Eurofins MWG Synthesis GmbH, Ebersberg, Germany), 2 μL of template DNA, and nuclease-free water (Qiagen) purified for PCR. The amplification program consisted of one cycle at 50 °C for 2 min; one cycle at 95 °C for 10 min; 40 cycles at 95 °C for 15 s and 60 °C for 1 min; and finally one cycle of melting curve analysis for amplicon specificity at 95 °C for 15 s and 60 °C for 20 s with increasing ramp rate by 2% until 95 °C for 15 s.

Real-Time PCR Data Handling. The relative quantity of gene targets encoding 16S rRNA of the bacterial taxa were calculated using the approximation 2^{-Ct}. Ct is the threshold cycle calculated by the ABI software as the PCR cycle, where the amplifications signal exceeds the selected threshold value, also set by the software. The amounts of bacterial specific DNA targets were normalized to total bacteria DNA targets to correct for differences in total DNA concentration between individual samples. Standard curves were created using serial 10-fold dilutions of bacterial DNA extracted from one of the fermentation samples for all primer sets. Analysis of the standard curves allowed verification of PCR efficiency for the chosen PCR conditions (Table 1). All results were calculated as means of duplicate determinations.

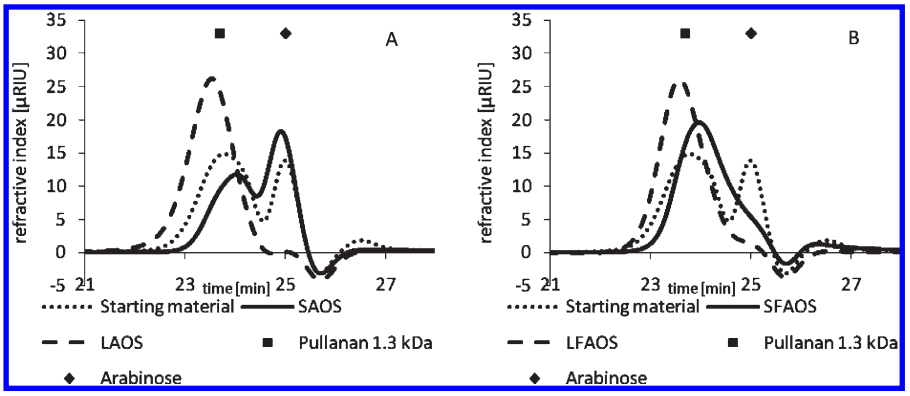

Figure 1. Size exclusion chromatography SAOS and LAOS (A) and SFAOS and LFAOS (B), both in comparison with the original starting material, arabinose, and 1.3 kDa pullanan standard.

Statistics. Statistical analysis was performed using GraphPad PRISM v5.03. One-way ANOVA and Tukey's multiple-comparison test were used to determine significant differences among bacteria populations for the in vitro fermentation. The quantitative PCR measurements were log-transformed before statistical analysis to obtain normal distribution of the data. Tests were considered to be statistically significant if P values lower than 0.05 were obtained.

Bioscreen. The ability of the different fractions to function as a sole carbon source for the potential pathogen *Clostridium difficile* was assessed in a pure culture fermentation study. *C. difficile* was obtained from Deutche Sammlung Microorganismes and grown in reinforced clostridial media for preculturing and tryptic soy broth without dextrose (both from Bacto, BD, Franklin Lakes, NJ) for the experiment. Bacterial growth in a 1% w/v substrate solution was measured with the automatic Bioscreen C system as described by Mäkeläinen et al.[25] and measured as the area under the growth curve (OD_{600} × min) obtained from the Bioscreen data. This method also takes the speed of growth initiation into account, compared to just evaluating the end absorbance.

RESULTS AND DISCUSSION

Separation Based on Feruloyl Substitution. The original starting material, which comprised a ferulic acid content of 36 μmol/g (Table 2), was separated into feruloylated (FAOS) and nonferuloylated (AOS) oligosaccharides by the XAD separation. The unbound fraction (representing the part of the starting material unable to bind to the XAD material) represented 48% of total sample load and had a ferulic acid concentration of 6 μmol/g. The recovered water fraction, containing the AOS fraction, accounted for 29% of total sample load and had a ferulic acid concentration of 0.7 μmol/g, whereas 21% of total sample load was found in the MeOH/water fraction, which had a ferulic acid concentration of 144 μmol/g. The MeOH/water fraction accounted for 85% of the total ferulic acid load, and thus contained the FAOS fraction. The remaining 2% of sample load was found in the MeOH fraction with a ferulic acid concentration of 12 μmol/g and trace amounts of vanillin. No further analysis of this fraction was conducted.

Separation Based on Molecular Weight. The AOS and FAOS fractions were both separated according to size and subsequently analyzed with respect to average molecular weight, content of monosaccharides and short oligosaccharides, monosaccharide composition, and ferulic acid content. Size exclusion chromatography (Figure 1) showed the original starting material to have a dual distribution, with one peak at 25 min corresponding to monomers and a larger broader peak around 1.0 kDa. For the nonferulated fractions (Figure 1A) LAOS showed one homogeneous peak above 1.0 kDa and SAOS showed two peaks, both below 1.0 kDa. For the feruloylated fractions, the larger fraction LFAOS had a shape and size similar to those of LAOS, whereas SFAOS showed a broader distribution and had only a shoulder that corresponds to the presence of lower amounts of monomers, compared to the starting material (Figure 1B).

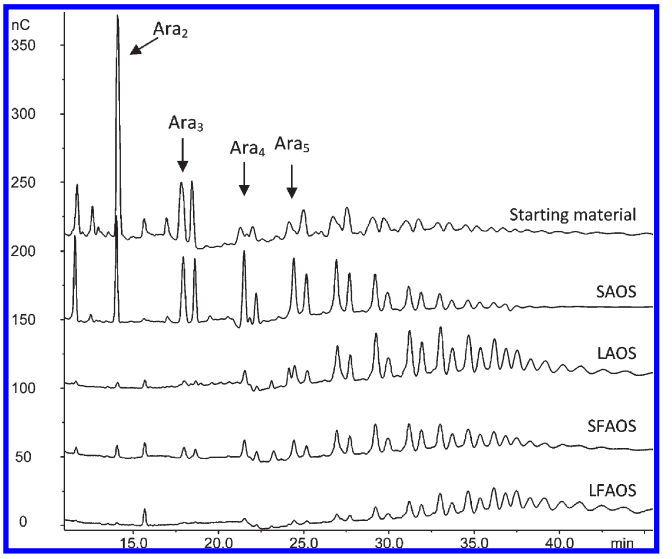

Figure 2. HPAEC elution profiles with electrochemical detection. Elution of monomers is not shown. Linear α-1,5 arabinooligosaccharides DP 2−5 are marked.

Carbohydrate Composition. The starting material and the four final fractions, SAOS, LAOS, SFAOS, and LFAOS, were investigated with respect to content of monosaccharides, shorter linear arabino-oligosaccharides, and monosaccharide composition using HPAEC directly on the fractions and on fractions subjected to acid hydrolysis. HPAEC analysis of the original starting material showed that approximately 125 mg/g was monosaccharides (mainly arabinose, glucose, and fructose) and 18 mg/g was made up of linear α-1,5 arabinooligosaccharides DP 2−5. For SAOS the amount of monosaccharides was 15 mg/g, and linear arabinooligosaccharides constituted 17 mg/g. For LAOS, SFAOS, and LFAOS the amounts of monosaccharides were significantly lower. These results indicated that the majority of monomers did not bind to the XAD and therefore could be assumed to be in the unbound fraction.

HPAEC (Figure 2) revealed pairs of peaks, which may be indicative of branched arabino-oligosaccharides because branched arabino-oligosaccharides do not coelute with linear arabino-oligosaccharides.[26] HPAEC of SFAOS showed elution of a broad peak with a retention time comparable to that of monomers, but no identification was possible (Figure 3). The broad peak with heavy tailing indicated coelution of various smaller compounds, presumably feruloylated arabinose and arabinobiose. The monosaccharide analysis after acid hydrolysis revealed that besides at least 80% AOS, all of the samples contained traces of galactose, rhamnose, and galacturonic acid normally found in RGI (Table 3). Glucose was found mainly in the SAOS fraction. Fucose, xylose, and glucuronic acid were detected in the starting material and the high molecular weight fractions (LAOS and LFAOS) in trace amounts (<0.1 mol %, data not shown). The presence of these sugars could indicate the presence of trace amounts of RGII and/or xyloglucan.

Ferulic Acid Distribution. Analysis of the *trans*-ferulic acid content of each fraction revealed that the ferulic acid present in the water fraction from XAD separation, after the membrane separation, primarily was found in the high molecular fraction, whereas the distribution of ferulic acid in the SFAOS and LFAOS fractions was in the same order of magnitude (Table 3). Besides *trans*-ferulic acid, minor amounts of *cis*-ferulic acid were found in the range of 0.2−2.8 μmol/g in all fractions. Also, minor amounts of vanillin were detected, mainly in the SFAOS fraction.

Feruloylated arabino-oligosaccharides SFAOS and LFAOS were further analyzed using hydrophilic separation (Figure 4). Each peak was collected and analyzed by MALDI-TOF and TOF/TOF. Series of arabino-oligosaccharides with a single feruloyl substitution with DP 2−10 and series with double feruloyl substitutions DP 7−14 were detected (Table 4). No ferulic dimers were detected in the analyzed fractions, even though reverse phase analysis indicated the presence of putative dimers (data not shown). No feruloyl substitutions on galactose residues were detected. MS/MS analysis confirmed the presence of monosubstituted and double-substituted arabino-oligosaccharides, but MS/MS data did not give sufficient results for complete determination of the degree of branching or the exact point of feruloyl substitution on each type of molecule, because molecules with different substitution patterns would have the same fragmentation pattern. As exemplified by MS/MS fragmentation of a single feruloylated DP 5 (Figure 5) from fraction V (Figure 4; Table 4), at least two different molecules were present in the fraction. In the fragmentation nomenclature the suffix "F" describes whether a feruloyl substitution is present on the ion or not. Arabinose moieties are numbered from the reducing end. Because MS/MS analysis was performed in positive mode, sodium adducts were formed. The fragmentation pattern lacked any trace of ferulated Y_1, Z_1, and X_0 ions, indicating that feruloyl substitution did not occur at the reducing end. The presence of both feruloylated and nonferuloylated Y_2 and $^{1,4}X_2$ ions (m/z 481, 509, 305, and 333, respectively) indicated that feruloyl substitution occurred at arabinose 2 in one of the molecule types. The presence of $^{1,4}X_4F$ (m/z 773) would indicate that no O-2 or O-3 feruloyl substitutions were present at the nonreducing end, but the presence of $^{0,3}X_4$ (m/z 641) indicated feruloyl substitution at O-5 at the nonreducing end. It was not possible to obtain ions confirming any feruloyl substitution at arabinose 3 or 4. The fragmentation pattern thus clearly indicated the presence of at least two different molecules, one type with feruloyl substitution at the O-5 at the nonreducing end and one type with feruloyl substitution at O-2 at arabinose 2. For simplicity, only linear structures were considered, and the presumably branched nature of sugar beet arabinan is not considered. Besides arabino-oligosaccharides singly substituted with ferulic acid, also arabino-oligosaccharides doubly substituted with ferulic acid were observed. The exact structures could not be elucidated by MS/MS due to an even higher complexity and diversity than the singly substituted arabino-oligosaccharides (data not shown).

The sample preparation prior to HILIC separation might have favored the recovery of lower DPs, because the adjustment of acetonitrile concentration to 80% caused some of the sample to precipitate. MALDI-TOF analysis of the SFAOS and LFAOS precipitates showed traces of feruloylated arabino-oligosaccharides with sizes up to DP 22 and 30, respectively (data not shown). Still, the HILIC separation gave information about the relative abundance of each size of oligomers based on the area of each peak. SFAOS consisted mainly of oligosaccharides with DP 1−5, whereas LFAOS had a broader distribution, with some oligosaccharides below 1 kDa being present based on both HPSEC and HILIC data.

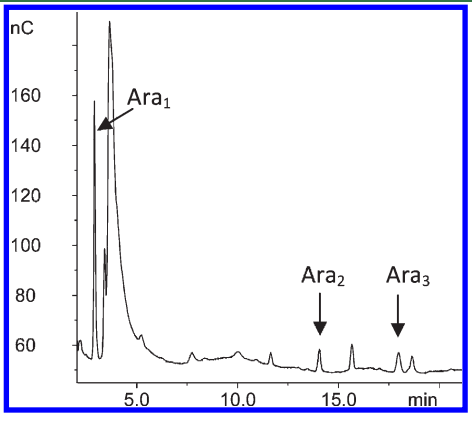

Figure 3. HPAEC elution profile of SFAOS with electrochemical detection. Arabinose and linear α-1,5 arabinooligosaccharides DP 2−3 are marked.

Table 3. Mass Distribution, Ferulic Acid Content, and Monosaccharide Composition of Fractions from Membrane Separation

			mol %					
	mass distribution (mg/g starting material)	ferulic acid content (μmol/g)	rhamnose	arabinose	galactose	glucose	galA	fructose
starting material	1000	36.2	1.13	85.16	1.95	6.78	1.63	2.77
SAOS	196.9	1.7	1.75	82.15	1.62	13.18	0.70	0.61
LAOS	40.1	66.0	0.99	86.68	3.44	0.75	7.07	0.09
SFAOS	67.2	186.5	1.47	92.63	3.14	0.69	1.17	0.25
LFAOS	148.7	247.3	0.72	95.98	1.77	0.29	0.82	0.00

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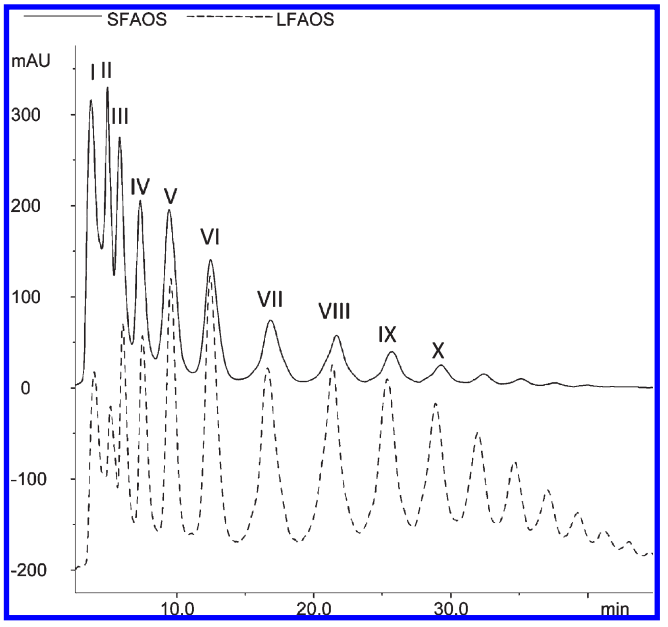

Figure 4. HILIC separation profile with UV detection at 316 nm. For peak identities refer to Table 4.

The HILIC separation method is capable of detection of ferulated arabino-oligosaccharides from DP 1 to at least DP 15−20. Analysis of LAOS did not show any molecules within this size frame (data not shown), despite this fraction containing significant amounts of ferulic acid (Table 3). According to the HPAEC analysis, LAOS did contain oligosaccharides with an approximate size of DP 5−10, but apparently the feruloyl substitution was present on oligosaccharides with higher DP than DP 20. (The term "oligosaccharides" is used for consistency.) The fact that only the relatively larger oligosaccharides were feruloyl substituted justifies the appearance of ferulated molecules in the water fraction, because the overall hydrophilicity of, for example, an arabino-oligosaccharide with DP 20 and a single feruloyl substitution would still fractionate into the water fraction.

In Vitro Fermentation. Quantitative real-time PCR from in vitro fermentations showed that fermentation on SAOS, LAOS, LFAOS, and the starting material selectively increased the density of *Bifidobacterium* spp. significantly ($P < 0.05$, $P < 0.01$ $P < 0.001$, and $P < 0.05$, respectively) when compared to the original fecal sample (Figure 6). The densities of bifidobacteria after fermentation of the high molecular weight fractions, LAOS and LFAOS, were not significantly different from the densities obtained by fermentation of FOS, which is considered to be the "golden standard" within the field of prebiotics. This result confirmed that the induced growth was due to the arabino-oligosaccharides and not a result of the presence of monomers. The finding that arabino-oligosaccharides are bifidogenic is in agreement with a recently patented discovery stating that branched arabino-oligosaccharides with DP 2−15 are bifidogenic.[27] However, the patent[27] stated that sugar beet derived arabinose-rich pectin oligosaccharides do not exert prebiotic effects in vitro. This statement may be due to the fact that mainly homogalacturonan sugar beet pectin oligosaccharides were evaluated in the patent as opposed to the arabino-oligosaccharides described in the present work, which consisted of only 1−7% galacturonic acid and 87−96% arabinose (Table 3). Fermentation of the LAOS and LFAOS fractions did not yield significantly different results,

Table 4. Peak Identities from HILIC Separation (Fig 4)[a]

peak	ara_nFA	ara_nFA_2
I		
II	DP2	
III	DP3	
IV	DP4	
V	DP5	DP7
VI	DP6	DP8
VII	DP6, DP7	DP9
VIII	DP7, DP8	DP10
IX	DP8, DP9	DP11, DP12
X	DP9, DP10	DP13, DP14

[a] Underscore indicates the mass with highest intensity in the MALDI-TOF analysis (MALDI-TOF data not shown).

indicating that the size of the oligosaccharides was more important for selective bacterial stimulation than the amount of feruloyl substitutions. The findings that the high molecular weight fractions were more bifidogenic than the low molecular weight fractions further elaborates on the findings reported by Al-Tamimi et al.[8] which indicated that the low molecular weight fractions were more selective for bifidobacteria than arabinan. In that study[8] only arabino-oligosaccharides up to DP 8 were tested, and some fractions appeared to contain mainly monosaccharides. The fact that feruloyl substitution was no hindrance to bifidogenic metabolism was in good correlation with the results by Funk et al.[13] They found that human intestinal microbial communities were able to degrade maize cell wall material regardless of ferulate levels.

No significant changes were seen in the densities of *Lactobacillus* spp. and *Firmicutes* after fermentation. The relative amount of *Bacteroidetes* decreased significantly ($P < 0.001$) for all tested fractions, including FOS, as compared to the inoculum (Figure 6). This decrease was most likely due to an increase in other types of bacteria. The relative balance between *Firmicutes* and *Bacteroidetes* is believed to play a role in obesity risk, both with respect to developing obesity as shown by introducing "obese microbiota" in germ-free mice[28] and by the observation that the relative levels of *Firmicutes* and *Bacteroidetes* change, that is, the relative levels of *Bacteroidetes* increase even though the levels of *Firmicutes* are still dominant, when the diet for obese humans is restricted.[29] In the study presented in this paper the relative level of *Firmicutes* remained unchanged and the relative level of *Bacteroidetes* decreased significantly, thereby altering the relative balance toward a higher *Firmicutes*/*Bacteroidetes* ratio. These data suggest that all of the oligosaccharide fractions, including FOS, a commercial prebiotic, may increase the risk of obesity and maybe, in turn, the risk of developing the metabolic syndrome associated with obesity. However, it is not clear if the extent of change observed after the in vitro fermentations has any significance, as the available in vivo evidence of an effect of change in the *Firmicutes*/*Bacteroidetes* ratio was recorded over a longer period, namely 1 year, and the change was accompanied by a change in body weight (in humans).[29] Clearly, the data therefore require further investigation.

Besides the positive bifidogenic effects of the feruloylated arabino-oligosaccharides, it was investigated whether these substrates could be fermented by an opportunistic intestinal pathogen, *C. difficile*. *C. difficile* infection is linked to consumption of antibiotics, which disrupt the normal intestinal microbiota, allowing *C. difficile*

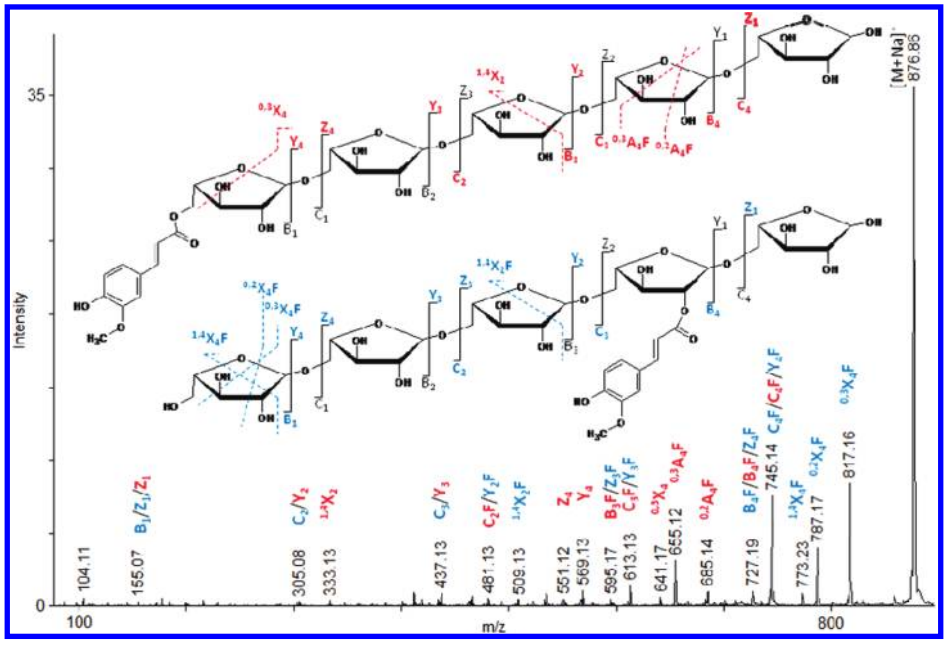

Figure 5. MS/MS high-energy CID spectrum of a sodium adduct of ara_5FA (fraction V, Figure 4), illustrating the fragmentation pattern and nomenclature, and two different proposed structures.

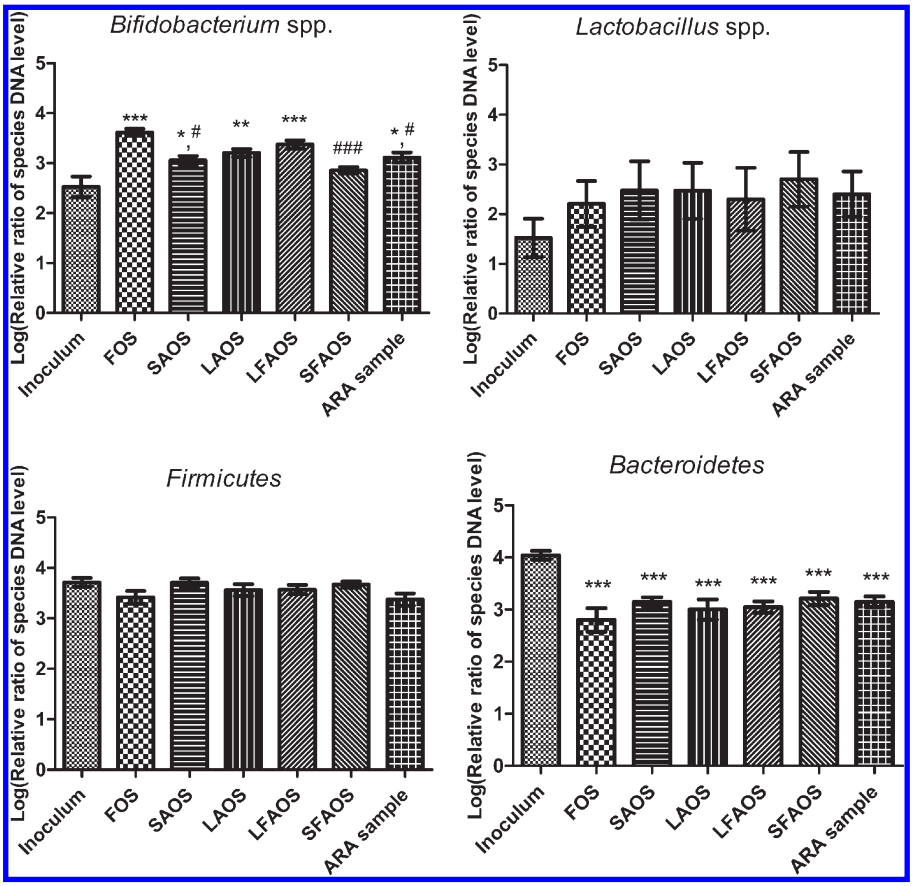

Figure 6. Relative quantities of target genes in samples from original fecal bacterial communities and after fermentation of oligosaccharides by these communities. Target genes encoded 16S rRNA from *Bifidobacterium* spp., *Lactobacillus* spp., *Bacteroidetes*, and *Firmicutes*. Fecal samples were obtained from the six healthy volunteers. The bars represent the average ± SEM of the response from six volunteers. Asterisks indicate a significant difference between target density in the original community and in the fermented samples: $P < 0.05$, ∗; $P < 0.01$, ∗∗; $P < 0.001$, ∗∗∗. Pound signs indicate a significant difference between target density after fermentation of FOS and after fermentation of the oligosaccharides: $P < 0.05$, #; $P < 0.01$, ##; $P < 0.001$, ###.

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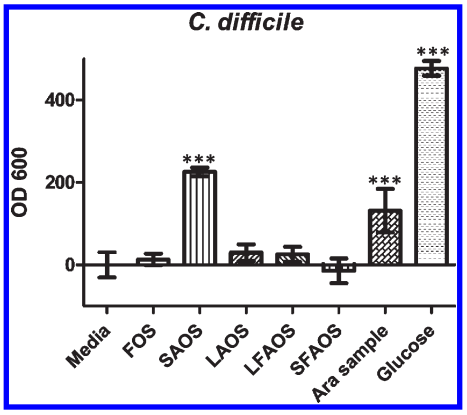

Figure 7. Induced growth of *Clostridium difficile* expressed as area under the curve. The bars represent the average ± SEM of 10 fermentations. Asterisks indicate a significant difference between media and the fermentation of the oligosaccharides: $P < 0.05$, ∗; $P < 0.01$, ∗∗; $P < 0.001$, ∗∗∗.

to establish itself and induce disease.[30] Over the past decade an increase in cases of *C. difficile*-associated diarrhea has been observed. Only a limited number of antibiotics are available for treatment of *C. difficile* infections. Currently, vancomycin or metronidazole is recommended for treatment, and many patients suffer from relapse following infections.[31]

Single-culture fermentations of *C. difficile* showed that glucose (positive control), starting material, and SAOS were able to support bacterial growth, but FOS, LAOS, SFAOS, and LFAOS did not sustain growth (Figure 7). Similar to what was seen in the mixed fermentations, it was not possible to distinguish between the effects of the feruloylated and the nonferuloylated high molecular weight fraction in the monoculture fermentations. It may be speculated that the starting material and SAOS supported growth of *C. difficile* due to the high concentrations of monosaccharides such as arabinose and glucose relative to oligosaccharides present in these samples. It should be noted that single-culture studies do not take substrate competition and possible positive or negative effects of secondary metabolites from competing microbiota into account. The data obtained deserve further investigation in mixed fecal fermentations. Nevertheless, the lack of induced growth of *C. difficile* provides a good indication that the tested compounds will not stimulate this species in vivo. Both fecal fermentations and single-culture experiments showed similar results for both LAOS and LFAOS. Although the LFAOS tended to elicit a higher selective stimulation of bifidobacteria than LAOS, the results from these two fractions were not significantly different. The effects of these two high molecular weight fractions differed significantly from their low molecular weight counterparts. The membrane separation procedure applied in this experimental setup provided a fast, but crude, method for an initial separation based on size. As we reported recently,[23] even a single DP change in oligosaccharide chain length (of homogalacturonides) may elicit a differential response in fecal fermentations. The data therefore provide an incentive to evaluate the effects of even more defined structures with respect to the role of feruloyl substitution and chain length for prebiotic response. The HILIC separation procedure showed great potential in separating the feruloylated arabino-oligosaccharides according to chain length. Post treatment with feruloyl esterases could finally generate similar structures (or oligosaccharide mixtures having similarly narrow DP profiles) with and without feruloyl substitutions. The availability of more well-defined structures, or of oligosaccharide mixtures having very narrow chain-length profiles and substitutions, could make it possible to examine and develop an improved understanding of the functionality and possible bioactive role of feruloyl-substituted arabino-oligosaccharides and potentially provide a new base for upgrading of sugar beet pulp to valuable functional food ingredients.

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ABBREVIATIONS USED

DP, degree of polymerization; DM, dry matter; SAOS, small arabino-oligosaccharides; LAOS, long arabino-oligosaccharides; SFAOS, small ferulated arabino-oligosaccharides; LFAOS, long ferulated arabino-oligosaccharides.

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Paper 5

Maximal release of highly bifidogenic soluble dietary fibers from industrial potato pulp by minimal enzymatic treatment.

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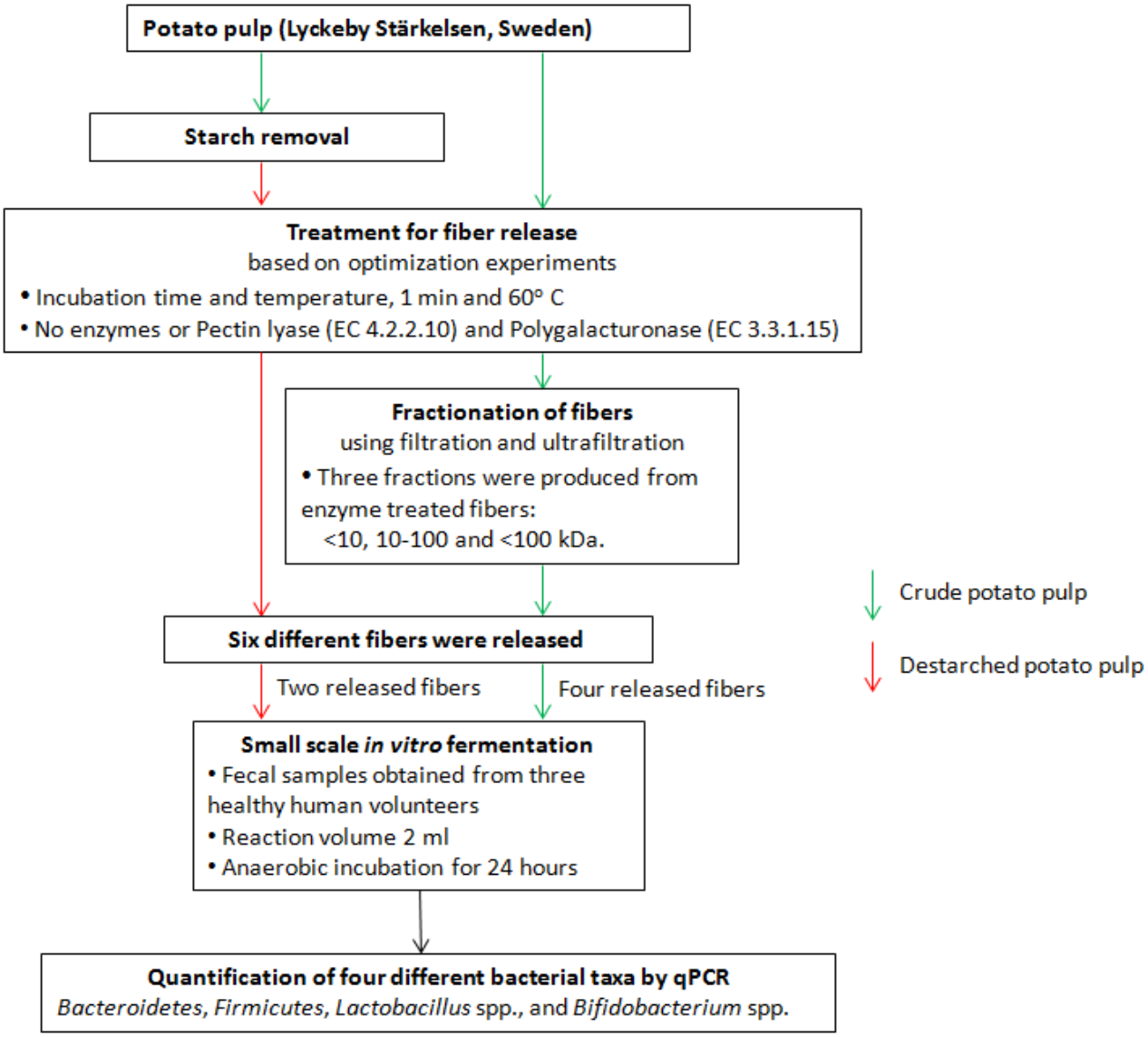
Introduction

The aim of this study was to solubilize dietary fibers from potato pulp by a one-step minimal treatment procedure, and additionally evaluate the prebiotic properties of the released fibers using human fecal *in vitro* fermentations.

Flow diagram



The author, Louise K. Vigsnæs, performed the small scale *in vitro* fermentations, and conducted the quantification of the bacterial taxa after fermentation. Louise K. Vigsnæs took part in the evaluation of the results and in preparation of manuscript regarding the *in vitro* fermentation study.

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Maximal release of highly bifidogenic soluble dietary fibers from industrial potato pulp by minimal enzymatic treatment

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Abstract Potato pulp is a poorly utilized, high-volume co-processing product resulting from industrial potato starch manufacturing. Potato pulp mainly consists of the tuber plant cell wall material and is particularly rich in pectin, notably galactan branched rhamnogalacturonan I type pectin which has previously been shown to exhibit promising properties as dietary fiber. The objective of this study was to solubilize dietary fibers from potato pulp by a one-step minimal treatment procedure and evaluate the prebiotic potential of the fibers. Statistically designed experiments were conducted to investigate the influence of enzyme type, dosage, substrate level, incubation time, and temperature on the enzyme catalyzed solubilization to define the optimal minimal enzyme treatment for maximal fiber solubilization. The result was a method that within 1 min released 75% [weight/weight (w/w)] dry matter from 1% (w/w) potato pulp treated with 1.0% (w/w) [enzyme/substrate (E/S)] pectin lyase from *Aspergillus nidulans* and 1.0% (w/w) E/S polygalacturonase from *Aspergillus aculeatus* at pH 6.0 and 60 °C. Molecular size fractionation of the solubilized fibers revealed two major fractions: one fraction rich in galacturonic acid of 10–100 kDa indicating mainly homogalacturonan, and a fraction >100 kDa rich in galactose, presumably mainly made up of β-1,4-galactan chains of rhamnogalacturonan I. When fermented *in vitro* by microbial communities derived from fecal samples from three healthy human volunteers, both of the solubilized fiber fractions were more bifidogenic than fructo-oligosaccharides (FOS). Notably the fibers having molecular masses of >100 kDa selectively increased the densities of *Bifidobacterium* spp. and *Lactobacillus* spp. 2–3 times more than FOS.

Keywords Potato pulp · Dietary fiber · Pectin lyase · Polygalacturonase · Galactan · *Bifidobacterium*

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Introduction

Potato pulp resulting as a co-processing product from industrial potato starch production is made up of the cell walls of the potato tuber and is mainly composed of pectin, cellulose, hemicelluloses, in addition to containing residual starch (Meyer et al. 2009). The monomeric composition of destarched potato pulp indicates that the potato pulp cell wall polysaccharides are mainly composed of galactose, galacturonic acid, arabinose, and rhamnose indicating most of the cell wall material is made up of homogalacturonan and rhamnogalacturonan I with long galactan side chains (Thomassen and Meyer 2010). The fermentability of enzymatically solubilized fibers from potato pulp was studied already in 1998 in a small intervention trial in which seven healthy human volunteers consumed the potato fiber in different ways (raw, baked, etc.; Olesen et

al. 1998). Consumption of the enzymatically solubilized potato fibers increased the end-expiratory H2 and delayed the oro-cecal transit time and the study therefore concluded that the soluble potato fibers were fermentable dietary fibers (Olesen et al. 1998). When rats consumed similar enzymatically solubilized fibers from potato pulp, they exhibited a significantly lower weight gain than control groups fed with cellulose fibers or insoluble potato pulp fibers (Lærke et al. 2007). In these studies, the solubilized potato fibers were mixtures of different molecular structures having different molecular masses. A first step in obtaining at least a provisional understanding, and a direction for designing the enzymatic solubilization to produce fibers exerting maximal biological benefits, would be to map the possible relationship between the solubilized polysaccharide structures and their putative biological effects by assessing the influence of the molecular size and composition on the growth of human intestinal bacteria.

Pectin can be solubilized from plant material by different enzymes which has been shown by, e.g., Ishii (1981; 1982). Previously in our lab, multicomponent plant cell wall degrading enzyme preparations were used to solubilize pectinaceous fibers from potato pulp (Meyer et al. 2009). However, the use of monocomponent enzymes for solubilization entails the possibility of targeting the enzymatic attack of the substrate, avoiding undesirable enzyme catalyzed degradation of the released fibers, and may furthermore provide knowledge about the accessibility of the pectin in the plant cell wall material. Based on the available compositional data, the soluble potato fibers may be hypothesized to be mainly made up of rhamnogalacturonan I fragments having extensive galactan side chains (Meyer et al. 2009). Pectin lyase, polygalacturonase, and presumably pectin methyl esterase would therefore be relevant enzyme candidates for solubilization of such dietary fibers from potato pulp. Hence, the hypothesis behind this study was that it should be possible to solubilize the potential galactan-rhamnogalacturonan I dietary fiber fraction from potato pulp by use of a few selected enzyme activities attacking the pectin homogalacturonan backbone, and the objective of the work was to test this hypothesis. The action of selected pectinolytic enzymes on potato pulp was examined in statistically designed experiments in which the separate and interactive effects of different reaction factors were also evaluated in order to design a minimal procedure by which the maximal amount of fiber could be released. The solubilized fibers were subsequently fractionated into two large fractions according to molecular mass and the effect of the potential dietary fibers on the composition of human intestinal bacterial ecosystems was evaluated by small scale *in vitro* fermentation.

Materials and methods

Chemicals

Pectin from citrus fruits, isopropanol, pullulan, polygalacturonic acid, D-galactose, L-arabinose, L-rhamnose monohydrate, D-fucose, D-mannose, D-galacturonic acid monohydrate, and vitamin K_1 were purchased from Sigma-Aldrich (Steinhein, Germany). Dextran was from Pharmacia (Uppsala, Sweden) and D-xylose and D-glucose from Merck (Darmstadt, Germany). Trifluoroacetic acid was from Riedel-deHaën (Seelze, Germany), anoxic phosphate buffered saline (PBS) was from Oxoid (Greve, Denmark), FOS (DP 2–8; Orafti®P95) were obtained from Beneo-Orafti (Tienen, Belgium), and Tween80 from VWR (Darmstadt, Germany). All chemicals used were analytical grade.

Pulp and enzymes

Fresh potato pulp was supplied by Lyckeby Stärkelsen (Kristianstad, Sweden). The potato pulp was stored at −21 °C until use. The enzymes used are listed in Table 1. The pectin lyase (PL1), the polygalacturonase (PG1), and the pectin methyl esterase (PME), all from *Aspergillus nidulans*, were produced in fermentations essentially as described by Stratton et al. (1998). The *Pichia pastoris* clones transformed with the pectin lyase gene AN2569.2, the polygalacturonase gene AN4372.2, and the pectin methyl esterase gene AN3390.2 were obtained from the Fungal Genetic Stock Center as described by Bauer et al. (2005). The pectin lyase (PL2) from *Aspergillus niger* was supplied by Danisco (Brabrand, Denmark), and the polygalacturonase (PG2) from *A. aculeatus* and the Viscozyme® L preparation were from Novozymes (Bagsværd, Denmark).

Protein concentration in enzyme solutions

Protein was determined by bicinchoninic acid protein assay with bovine serum albumin as standard (Thermo Fisher Scientific, Rockford, IL).

Reducing ends

The amount of reducing ends was determined by a modified down-scaled method described by Lever (1972, 1977). In brief, 1 M bismuth, 1 M potassium sodium tartrate, and 3 M sodium hydroxide were mixed with 0.5 M sodium hydroxide and 5% [weight/volume (w/v)] 4-hydroxybenzoic acid hydrazide in 0.5 M hydrochloric acid in the ratio 1:899:100. The reagent was mixed with a sample and incubated at 70 °C for 10 min, cooled to room temperature, and the absorbance was measured at 410 nm in an Infinite200 microplate reader (Tecan, Salzburg,

Table 1 Origin, classification and properties of the enzymes employed in this study

Enzyme	Source	Molecular mass (kDa)	Protein concentration (g/l)	Activity at pH 6, 60 °C (U/mg protein)	Family	EC no.	Reference
Pectin lyase (PL1)	*A. nidulans*	40	27.5±0.6	0.8± 0.1	PL1	4.2.2.10	Bauer et al. (2006)
Pectin lyase (PL2)	*A. niger*	38	17.2±1.4	0.2±0.0	PL1	4.2.2.10	Limberg et al. (2000)
Polygalacturonase (PG1)	*A. nidulans*	38	56.4±1.7	107±15	GH28	3.2.1.15	Bauer et al. (2006)
Polygalacturonase (PG2)[a]	*A. aculeatus*	39	–	11±0.8	GH28	3.2.1.15	–
Pectin methyl esterase (PME)	*A. nidulans*	36	55.5±1.3	n.d.	CE8	3.1.1.11	Bauer et al. (2006)

The protein levels were used to dose the enzymes at equivalent protein/substrate (w/w) ratios in the fiber release experiments

n.d. Not determined

[a] Solid enzyme preparation

Austria). Data collection was controlled by the program Tecan i-control version 1.5.14.0 (Tecan). Galacturonic acid was applied as standard.

Acid hydrolysis and high-performance anion-exchange chromatography

Hydrolysis of polysaccharides and separation and quantification of the monosaccharides by high-performance anion-exchange chromatography were done as described by Thomassen and Meyer (2010).

Protein determination in fiber

The protein content was determined as total amino acid assessment after 6 M hydrochloric acid hydrolysis followed by separation by ion exchange chromatography (Barkholt and Jensen 1989).

High-performance size-exclusion chromatography

High-performance size-exclusion chromatography was performed as described by Rasmussen and Meyer (2010) with the following modifications: The mobile phase and the sample solution matrix were 0.1 M acetate buffer pH 6.0. The injected volume was 25 µl and the analysis was carried out at 30 °C. Molecular markers were pullulan standards with a mass of 1.3, 10, and 400 kDa, respectively, and dextran with a mass of 110 kDa.

Enzyme activities

Pectin lyase activity was measured on 1 g/l pectin from citrus fruits by incubating 1% (w/w) E/S in Mcilvaine buffer (mixture of 0.1 M citric acid and 0.2 M disodium hydrogen phosphate) at pH 6.0 and 60 °C. The increase in absorbance was determined at 235 nm during 4 min in an Infinite200 microplate reader (Tecan, Salzburg, Austria); the data collection was controlled by the program Tecan i-control version 1.5.14.0 (Tecan). The extinction coefficient used was 5.5 $mM^{-1} \cdot 1^{-1}$ (Van den Broek et al. 1997). The polygalacturonase activity was measured on 2 g/l polygalacturonic acid by incubating 0.01–0.5% (w/w) E/S in Mcilvaine buffer at pH 6.0 and 60 °C. Samples were collected every minute for 5 min. The enzyme was inactivated by adding 50 mM NaOH and the amount of reducing ends was measured as described above.

Removal of starch from potato pulp

The starch was removed from the potato pulp using the rationalized one-step method described by Thomassen and Meyer (2010).

Release of fiber from potato pulp

Definition of the required enzyme activities

The statistically designed experiments were randomized, quadratic modified simplex centroid designs. The tree factors were: PL1 dose, PG1 dose, PME dose [each 0–5% (w/w) E/S] in all runs. 1% (w/w) destarched potato pulp in 0.1 M phosphate buffer pH 6.0 was preheated to 40 °C for 5 min and the enzymes added. The samples were incubated in a heating shaker (Eppendorf, Hauppauge, NY) at 750 rpm for 15 min at 40 °C followed by incubation at 100 °C for 10 min before centrifugation (15,000×*g* for 10 min). The supernatant was filtered using a 0.2 µm syringe tip filter (Phenomenex, Torrance, CA) and precipitated with 70% isopropanol for 30 min at room temperature. After centrifugation (5,000×g for 5 min) remaining liquid was removed by incubation over night at 105 °C and the dry matter determined.

Definition of the optimal experimental conditions

The experiments were randomized, quadratic central composite designs. Each design contained 26 different combinations of the five factors: dry matter [1–6% (w/w)], temperature (40–60 °C), time (1–15 min), PL1 dose [0.2–

2.5% (w/w) E/S], and PG1 dose [0.2–2.5% (w/w) E/S] with three center points. Destarched potato pulp [1% (w/w)] in 0.1 M phosphate buffer pH 6.0 was preheated to 40–60 °C for 5 min, and the enzymes were added. The samples were incubated in a heating shaker at 750 rpm for 1–15 min at 40–60 °C then at 100 °C for 10 min before centrifugation (15,000×*g* for 10 min). The supernatant was then filtered and the solubilized polysaccharides precipitated in isopropanol as described above.

Definition of the right enzyme combination and dose

Destarched potato pulp [1% (w/w)] was preheated at 60 °C in 0.1 M phosphate buffer pH 6.0 for 5 min. PL1, PL2, PG1, and PG2 [0–2.5% (w/w)] were added in different combinations, and the samples were incubated in a heating shaker at 750 rpm at 60 °C for 0–1 min and at 100 °C for 10 min followed by centrifugation, filtration, and precipitation as described above.

Minimal procedure on destarched or crude potato pulp

Destarched potato pulp [1% (w/w)] or crude potato pulp corresponding to 1% (w/w) destarched potato pulp was preheated at 60 °C in 0.1 M phosphate buffer pH 6.0 for 5 min. PL1 [1.0% (w/w) E/S] and PG2 [1.0% (w/w) E/S] were added and the samples were incubated at 750 rpm at 60 °C for 1, 15, 30, or 60 min and 100 °C for 10 min followed by centrifugation, filtration, and precipitation as described above. Samples where no enzyme was added were treated by incubating the samples at 60 °C for 1 min.

Solubilization of dry matter by Viscozyme® L

Destarched potato pulp [1% (w/w)] or crude potato pulp corresponding to 1% (w/w) destarched potato pulp was treated by the optimized procedure described by Meyer et al. (2009).

Fiber production and fractionation: 3 L process

Crude potato pulp was preheated at 60 °C in 0.1 M phosphate buffer pH 6.0 for 5 min. PL1 [1% (w/w) E/S] and PG2 [1% (w/w) E/S] was added and incubated at 60 °C for 1 min followed by heat treatment at 100 °C for 10 min. The supernatant was filtered through Celite® 545 followed by ultrafiltration. First, the supernatant was filtered using a 100 kDa polyethersulfone spiral wound ultrafiltration module (Millipore, Ballerica, MA). Then the permeate was filtered through a 10 kDa polyethersulfone spiral wound ultrafiltration module (Millipore). The ultrafiltration lead to three fractions: <10, 10–100, and <100 kDa. The fractions were concentrated and precipitated by isopropanol. The precipitated material was collected after centrifugation (2,000×*g*, 15 min) and dried over night at 40 °C. The fractions were ground to <2 mm in an Ultra Centrifugal Mill ZM 200 (Retsch, Haan, Germany). The protein content, monosaccharide composition, and size distribution were analyzed as described above.

Analysis of biological activity

Subjects and fecal sampling

Fecal samples were obtained from three healthy volunteers (mean age 42.7±10.0 years). None of the participants had been treated with antibiotics for at least 3 months before attendance and had no history of gastrointestinal disorder. Whole stools were collected in airtight containers, immediately stored at 4 °C, and processed within 12 h. The fresh feces samples were homogenized in 50% glycerol (1:1 dilution) in an anerobic cabinet (containing 10% H_2, 10% CO_2, and 80% N_2) and stored at −80 °C until further analysis as described below.

In vitro fermentation

Fibers or FOS were added to an autoclaved minimal basal medium to a final concentration of 5 g (dry matter)/l in a reaction volume of 2 ml. FOS was applied as a standard with known bifidogenic effect. The minimal basal medium contained 2 g/l peptone water, 1 g/l yeast extract, 0.1 g/l NaCl (1.71 mM), 0.04 g/l (0.23 mM) K_2HPO_4, 0.04 g/l (0.29 mM) KH_2PO_4, 0.01 g/l (0.04 mM) $MgSO_4 \cdot 7H_2O$, 0.01 g/l (0.07 mM) $CaCl_2 \cdot 2H_2O$, 2 g/l (23.81 mM) $NaHCO_3$, 0.5 g/l bile salts, 0.5 g/l L-cysteine hydrochloride, 0.005 g/l hemin, 10 µl/l vitamin K_1 (0.02 mM), 2 ml/l Tween 80, and 0.05‰ (w/v) resazurin solution. A 10% (w/v) fecal slurry was prepared by mixing the feces stored in 50% glycerol with anoxic PBS. The fiber samples were sterilized by ultraviolet light for 3 min and reduced for 24 h in an anerobic cabinet. After the reduction, the samples were checked to ensure that no contamination was present. The reduced minimal medium with added fibers was inoculated to a final concentration of 1% (w/v) feces. Fermentations of each fecal sample of each healthy volunteer was done in triplicate to give 3×3 fermentations that were incubated at 37 °C for 24 h in an anerobic cabinet.

Extraction of bacterial DNA from fermentation samples

Deoxyribonucleic acid (DNA) was extracted from fermentation samples using the QIAamp DNA Stool mini kit (Qiagen, Hilden, Germany) with a bead beater step in advance, as previously described (Leser et al. 2000). The purified DNA was stored at −20 °C until use.

Real-time PCR assay conditions

Amplification and detection of purified bacterial DNA by real-time polymerase chain reaction (PCR) was performed with the ABI-Prism 7900 HT (Applied Biosystems, Carlsbad, CA) using optical grade 384-well plates. Each amplification reaction was done in duplicate in a final volume of 20 μl containing; 10 μl EXPRESS SYBR® GreenER™ qPCR SuperMix (Invitrogen, Taastrup, Denmark), 10 pmol of each of the primers (Eurofins MWG Synthesis, Ebersberg, Germany), 2 μl template DNA, and nuclease-free water purified for PCR. The amplification program consisted of 1 cycle at 50 °C for 2 min; 1 cycle at 95 °C for 10 min; 40 cycles at 95 °C for 15 s, 60 °C for 1 min; and finally 1 cycle of melting curve analysis for amplicon specificity at 95 °C for 15 s, 60 °C for 20 s, increasing ramp rate by 2% until 95 °C for 15 s. The 16 S rRNA primers used are listed in Table 2. Standard curves were created for each primer set using serial tenfold dilutions of bacterial DNA extracted from one of the fermentation samples with the threshold cycle (Ct) values calculated by the ABI software (SDS 2.2). The analysis of the standard curves allowed verification of PCR efficiency for the chosen PCR conditions (Table 2). The DNA level of the bacterial target groups for each reaction was calculated from the standard curves. All results were calculated relatively as ratios of species DNA levels to total bacteria expression levels in order to correct data for differences in total DNA concentration between individual samples.

Statistics

Calculation of means and standard deviations were done in Excel (Microsoft). The program used for design of the experimental templates and the evaluation of the effects and the interactions by multiple linear regression was MODDE version 7.0.0.1 (Umetrics, Umeå, Sweden). Statistical analysis of the bioactivity data was performed using GraphPad PRISM v5.03 for Windows. One-way analysis of variance (ANOVA) and Dunnett's posthoc test were used to determine significant differences among bacteria populations using the different applied fibers compared to FOS. Differences were considered to be significant when $P<0.05$. All reported data are given as average values determined after minimum duplicate determination.

Results

Enzyme concentration and activity

The activities of the pectin lyases ranged from 0.2 to 0.8 U/mg, and the activity of the two polygalacturonases were 11 and 107 U/mg, respectively, lowest for PG2, the enzyme from *A. aculeatus* (Table 1).

Release of fibers from potato pulp

Enzyme activities needed

The response contour plot prepared from the data indicated that it was possible to release at least 70% of the dry matter by employing only PL1 and PG1 (Fig. 1). Furthermore, the data indicated that some dry matter was released from the pulp even without addition of PL1, PG1 or PME (Fig. 1).

Optimal experimental conditions

Since PME was not needed to release >70% of the dry matter from destarched potato pulp, only the temperature, dry matter, incubation time, and enzyme dose of PL1 and PG1 were varied. It was possible to release 74–75% of the dry matter using 1% (w/w) potato pulp, and 2.5% (w/w) E/S of both enzymes at 40 °C for 15 min or at 60 °C for 1 min (data not shown). The response surface plot also indicated that 70% (w/w) of the dry matter could be released from destarched potato pulp by enzymatic treat-

Table 2 16S rRNA primers for real-time PCR used to quantify the level of the four different bacterial taxa in the in vitro fermentations of the fecal samples with different potato fibers

Target gene	Forward primer (5′–3′)	Reverse primer (5′–3′)	Product size (bp)	Correlation coefficient (R^2)	Efficiency (%)	Reference
Bifidobacterium spp.	cgc gtc ygg tgt gaa ag	ccc cac atc cag cat cca	244	0.991	94	Delroisse et al. (2008)
Lactobacillus spp.	agc agt agg gaa tct tcc a	cac cgc tac aca tgg ag	341	0.992	102	Heilig et al. (2002), Walter et al. (2000)
Firmicutes phylum	gga gya tgt ggt tta att cga agc a	agc tga cga caa cca tgc ac	126	0.999	100	Guo et al. (2008)
Bacteroidetes phylum	gga rca tgt ggt tta att cga tga t	agc tga cga caa cca tgc ag	126	0.999	97	Guo et al. (2008)
Total bacteria[a]	cgg caa cga gcg caa ccc	cca ttg tag cac gtg tgt agc c	130	0.999	97	Denman and McSweeney (2006)

[a] PCR for the total bacteria primer set was run in parallel for each set of primers for all samples

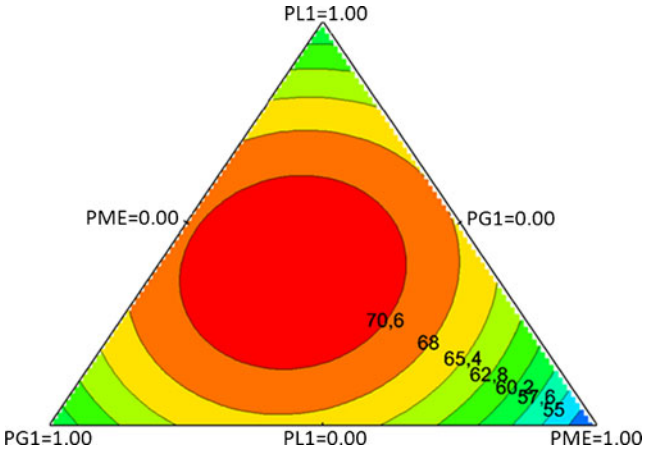

Fig. 1 A response contour plot showing the amount of released dry matter from potato pulp using PL1, PG1, and PME at pH 6, 40 °C. PL1=1.00 means that PL1 was present in the highest dose [5% (w/w) E/S] and no other enzymes were added, and PL1=0.00 means that PL1 was not present and that the full dose of enzyme was split equally between PME [2.5% (w/w) E/S] and PG1 [2.5% (w/w) E/S], etc

ment for 1 min at 60 °C, 1% (w/w) dry matter using either of three different treatments: 2.5% (w/w) E/S PL1 and 0.2% (w/w) E/S PG1, or 0.7% (w/w) E/S PL1 and 2.5% (w/w) E/S PG1, or 1.5% (w/w) E/S of both enzymes (Fig. 2). The initial dry matter level had a significant effect on the amount of released dry matter (Fig. 3). The response surface plots indicated that to have a yield of at least 70%, the amount of dry matter should be maximum 1.3% (w/w). Multiple linear regression analysis of the data showed that an increase in PL1 and PG1 dose would significantly

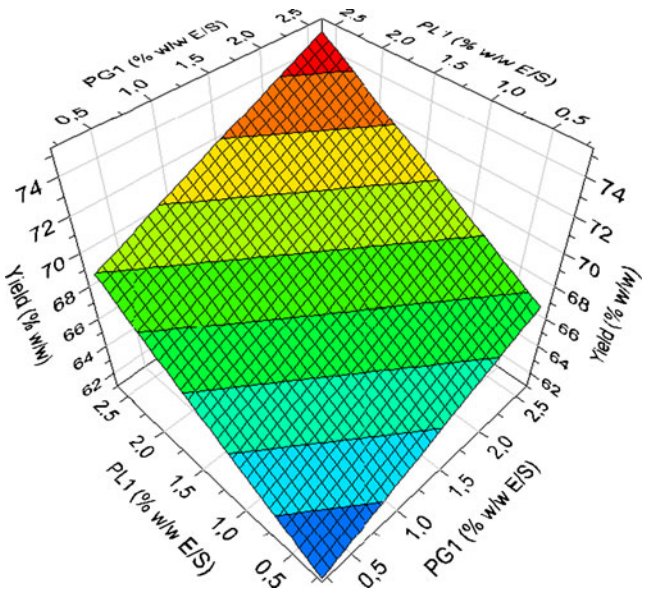

Fig. 2 A three-dimensional response surface showing the amount of released dry matter [% (w/w)] as function of PL1 [% (w/w) E/S] and PG1 [% (w/w) E/S]. Q^2=0.883, R^2=0.991; constant factor: temperature=60 °C, incubation time=1 min, dry matter=1% w/w

increase the amount of released dry matter whereas a decrease in dry matter also increased the release of dry matter (Table 3). In addition, pectin lyase and dry matter interaction had a significant effect on the amount of released dry matter.

Enzyme combination and dose

To assess the optimal procedure for release of minimum 70% (w/w) dry matter from destarched potato pulp, experiments were made using PL1, PL2, PG1, and PG2 in different doses and combinations under the conditions found above. Of the dry matter, 15% (w/w) was released during the 5 min of preheating with no enzyme present and around 41% (w/w) after additional 1 min of heating (Fig. 4a). Incubation with 0.8 and/or 1.6% (w/w) E/S PL1, PL2, PG1, or PG2 released 41–68% (w/w) dry matter (Fig. 4a).

The yield increased in response to PL1 and PG1 dosage; dry matter yield started from 59%, when 0.2% (w/w) E/S PL1 and PG1 was added and seemed to reach a maximum at 74–75% (w/w) dry matter, when 1.0% or 2.5% (w/w) of each enzyme were added (Fig. 4a).

Treatments with a combination of 0.2–0.8% (w/w) E/S PL1 and PG2 released 60–70% (w/w) of the dry matter, whereas 1.0–2.5% (w/w) E/S of PL1 and PG2 released 73–76% of the dry matter (Fig. 4b).

Similar experiments were made with PL2+PG1 and PL2+PG2, but the general yields obtained were lower than those obtained with PL1+PG1 and PL1+PG2 (data not shown).

Based on the results presented above, the best minimal procedure for releasing 75% of the dry matter was: 1.0% (w/w) E/S PL1 and 1.0% (w/w) E/S PG2 incubated with 1% (w/w) destarched potato pulp for 1 min at pH 6, 60 °C. If the objective was to use only one enzyme, it was possible to release 68% dry matter by applying 0.8% (w/w) E/S PG2 (Fig. 4a). The minimal enzyme dose for releasing 70% by weight of dry matter was 0.8% (w/w) E/S PL1 and 0.8% (w/w) E/S PG2 (Fig. 4b). It may be possible to use lower enzyme dosage, but most likely, it requires the incubation time to be extended.

Fractionation of released fibers

The minimal procedure using PL1+PG2 was scaled up and the released fibers were fractionated into three fractions: >10, 10–100, and >100 kDa. The distribution of the dry matter in fraction 10–100 and >100 kDa was 17% and 71%, respectively. The fraction containing molecules with a mass< 10 kDa was not possible to precipitate with isopropanol and was therefore considered to be the mass not present in the two other fractions (11%).

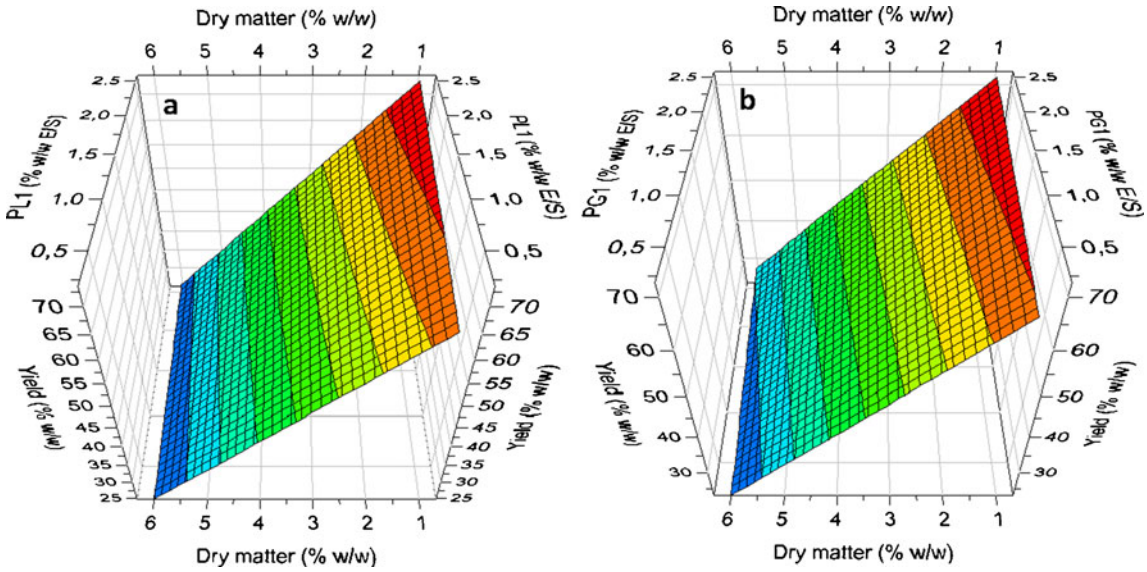

Fig. 3 Three-dimensional response surface showing the amount of released dry matter [yield,% (w/w)] as function of *a* PL1 [% (w/w) E/S] and initial dry matter [% (w/w)] and *b* PG1 [% (w/w) E/S] and initial dry matter [% (w/w)]. Q^2=0.883, R^2=0.991. The constant factors are set to the center values

Fiber from destarched versus from crude potato pulp

Extended treatment of crude and destarched potato pulp for up to 1 h under minimal conditions neither increase nor decrease the yield of released fibers; neither did the yield of dry matter released during extended treatment without addition of enzyme change (data not shown). Incubation of crude and destarched potato pulp with Viscozyme® L (as described by Meyer et al. 2009) released significantly less dry matter than incubation with the optimal procedure (data not shown).

Table 3 Multiple linear regression results on the released dry matter obtained after optimization of experimental conditions for fiber release (Q^2=0.883, R^2=0.991)

Parameters and interactions (×)	Percent by weight of dry matter	
	Coefficient	P^*
Temperature	−0.13	No effect
PL1	2.05	1.05×10^{-5}
PG1	2.41	1.07×10^{-6}
Dry matter	−17.46	7.80×10^{-24}
Time	0.71	No effect
PL1×dry matter	−0.92	0.006
Constant	47.66	1.47×10^{-33}

P^* =0.05 indicates significance at the 95% level. The validity of the model was confirmed by the value of the center points [47.71±1.25% (w/w)] being close to the coefficient of the constant

Characterization of the released fibers

Monosaccharide composition of released fibers

The monosaccharide composition in the samples DNE, CNE, DPP, CPP, and CPP>100 (Table 4) were generally similar, containing approximately 55–66% galactose, 9–10% arabinose, and 1.3–1.7% rhamnose, but with some variation in the glucose levels (Table 5). In contrast, the main component in CPP10–100 was galacturonic acid (47.7±5.6%), but the rhamnose level was approximately the same as in the other fibers (1.4±0.1%; Table 5). No fucose or xylose was detected in any of the fiber samples.

Molecular size of released fibers

Size exclusion chromatograms of the DNE showed that the majority of the released dry matter had a molecular mass of approximately 0.4 and 1.0 kDa (Fig. 5). A limited part of the sample was made up of molecules having a higher molecular mass. A similar chromatogram was obtained for CNE (data not shown). The chromatogram for CPP contained four main peaks: 0.4, 1.0, 4.8, and >400 kDa. A similar chromatogram was obtained for DPP (data not shown). The molecular mass of CPP10–100 was 5.4 kDa at the highest point of molecular mass distributed almost equally on each side of the chromatogram top point. Furthermore, there was a minor peak at molecular mass of around 0.4 kDa (Fig. 5). In CPP>100, the majority of the dry matter had molecular mass>110 kDa with maximum >400 kDa (Fig. 5).

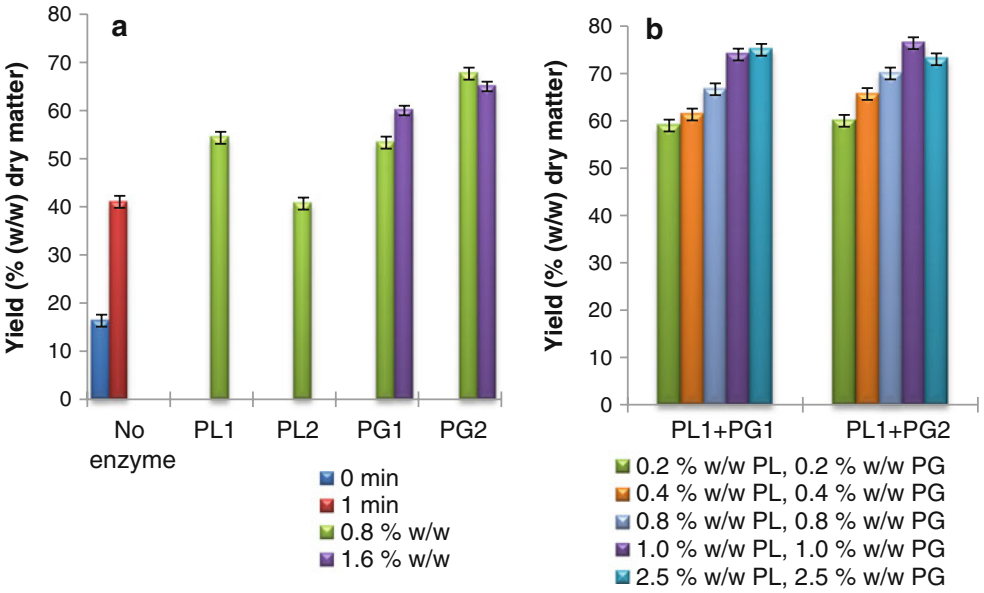

Fig. 4 Released dry matter from destarched potato pulp at pH 6.0 and 60 °C by applying *a* no enzyme or one enzyme or *b* different combinations of different enzymes

Biological activity

After fermentation in fecal slurries obtained from three different subjects, quantitative real-time PCR was applied to measure the density of gene targets encoding 16 S rRNA of selected bacterial taxonomic units (Fig. 6). The ability of the released fibers (Table 4) to selectively stimulate the growth of the given bacterial taxa was compared to that of FOS. The fecal communities fermented on CPP>100 had a significantly higher content of *Bifidobacterium* than the same fecal communities fermented on FOS ($P<0.05$; Fig. 6a). A similar trend was found for the samples fermented on CPP10–100, but the difference to FOS did not reach statistical significance ($P<0.10$). The *Bacteroidetes* 16 S rRNA gene content was significantly higher in the fecal communities fermented on CNE than those fermented on FOS ($P<0.05$; Fig. 6c). There were no differences in the *Lactobacillus* and *Firmicutes* content between the fermentation samples containing the applied fibers and FOS (Fig. 6b, d, respectively).

Discussion

When evaluated in a tertiary mixture design using PME, PL1 and PG1, it was possible to release >70% of the dry matter from destarched potato pulp by a combination of PL1 and PG1; the combination released more dry matter than either enzyme separately (Fig. 1). This confirmed that homogalacturonan contained galacturonic acid units both with and without methyl group substitutions and that bonds between both substituted and unsubstituted galacturonic acid moieties should be hydrolyzed in order to release the fibers. PME was expected to catalyze the removal of the methyl groups, reducing the need for the pectin lyase. However, the direct effect of PL treatment was higher in this experimental setup.

Optimization of fiber release

The incubation time and temperature did not significantly change the amount of dry matter released and therefore 1 min and 60 °C was chosen as optimal (Table 3). The

Table 4 The abbreviated name codes of the released fibers

Sample	Abbreviated name code
DNE	**D**estarched potato pulp, fiber released by addition of **N**o **E**nzyme
DPP	**D**estarched potato pulp, fiber released by **P**ectin lyase and **P**olygalacturonase
CNE	**C**rude potato pulp, fiber released by addition of **N**o **E**nzyme
CPP	**C**rude potato pulp, fiber released by **P**ectin lyase and **P**olygalacturonase
CPP10–100	**C**rude potato pulp, fiber released by **P**ectin lyase and **P**olygalacturonase, fraction **10–100** kDa
CPP>100	**C**rude potato pulp, fiber released by **P**ectin lyase and **P**olygalacturonase, fraction **>100** kDa

Table 5 Carbohydrate and protein content in the fiber released from potato pulp by the minimal procedure: 1% (w/w) potato pulp treated with 1.0% (w/w) E/S PL1 and 1% (w/w) E/S PG2 at pH 6.0 and 60 °C for 1 min

% (mole)	DNE	DPP	CNE	CPP	CPP10–100	CPP>100
Glucose	18.1±0.4	10.0±0.5	11.7±0.7	4.8±0.1	10.1±0.5	7.1±1.0
Rhamnose	1.4±0.1	1.3±0.1	1.5±0.1	1.3±0.1	1.4±0.1	1.7±0.3
Galacturonic acid	16.7±0.4	18.5±2.4	14.7±2.8	22.7±1.3	47.7±5.6	13.6±5.0
Galactose	54.8±2.7	58.3±1.2	62.7±3.4	59.1±0.3	32.4±0.4	65.5±0.7
Arabinose	9.0±1.9	9.6±1.2	9.5±1.3	8.6±1.2	3.7±0.7	8.5±0.8
Mannose	0.0±0.0	0.0±0.0	0.0±0.0	1.1±0.1	3.3±0.2	2.3±0.3
Protein	n.d.	2.3	n.d.	1.7	1.5	1.4

higher temperature was chosen to minimize the risk of microbial growth. Approximately 21–22% by weight of the crude (dry) potato pulp is starch (Thomassen and Meyer 2010). The optimization experiments were carried out on destarched potato pulp to avoid interference from starch on the enzymatic release and the *in vitro* fermentation. The same amount of dry matter was released from destarched and crude potato pulp by applying the minimal procedure, indicating that the remaining starch polymers in crude potato pulp were insoluble.

Release of fiber from potato pulp by Viscozyme® L

It has been shown that it is possible to release 28% by weight of dry matter from destarched potato pulp by and optimized Viscozyme® L treatment (Meyer et al. 2009). The experiment was repeated during this study, but the amount of released dry matter was insignificant as compared to the amount released by the minimal procedure. The difference might be due to the Viscozyme® preparation comprising a number of pectinolytic activities vatalyzing the degradation of the fibers to a low molecular size, not able to be precipitated by isopropanol and thereby determined by the present method.

Release of fibers from potato pulp without addition of enzyme

When destarched potato pulp was incubated at pH 6 and 60 °C for 1 min 41% by weight of the dry matter was released (Fig. 4b). A similar amount of dry matter was released from crude potato pulp (data not shown) eliminating the possibility that the enzyme used for starch removal caused the increased amount of released dry matter. The possible influence of divalent cations viz. chelation might play a role, but this hypothesis requires further experimental substantiation.

Characterization of the released fibers

The monosaccharide compositions indicated that CPP10–100 and CPP>100 were mainly made up of homogalacturonan and rhamnogalacturonan I polysaccharides, respectively. The monosaccharide composition and size-exclusion chromatogram indicated that CPP>100 mainly consisted of polysaccharides of homogalacturonan and rhamnogalacturonan I with large galactan side chains. This is in complete accordance with the data obtained with Viscozyme® L treatment on destarched potato pulp (Meyer et al. 2009) and the tentative conclusion that the enzymatically released high molecular weight fraction contained relatively large galactan side chains (Meyer et al. 2009).

Biological activity

The bioactivity of the released fibers (Table 5) was tested by fermentation in fecal bacterial communities obtained from three healthy human subjects. The effect of the fibers on the content of *Bifidobacterium* and *Lactobacillus* was compared to the effect of FOS. FOS was used as a standard

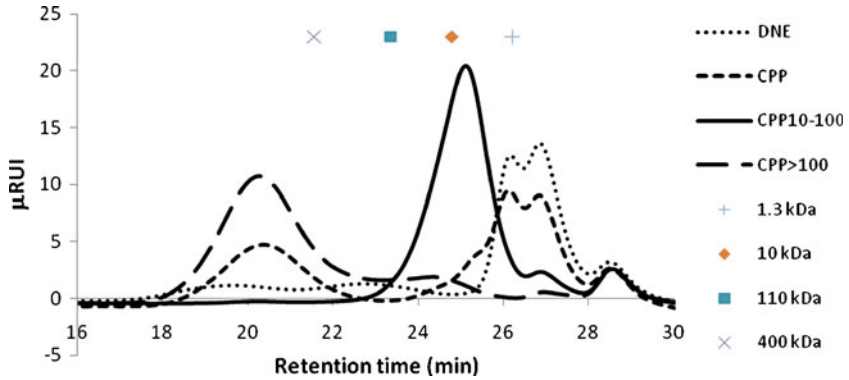

Fig. 5 Size exclusion chromatogram of the fractions released from potato pulp

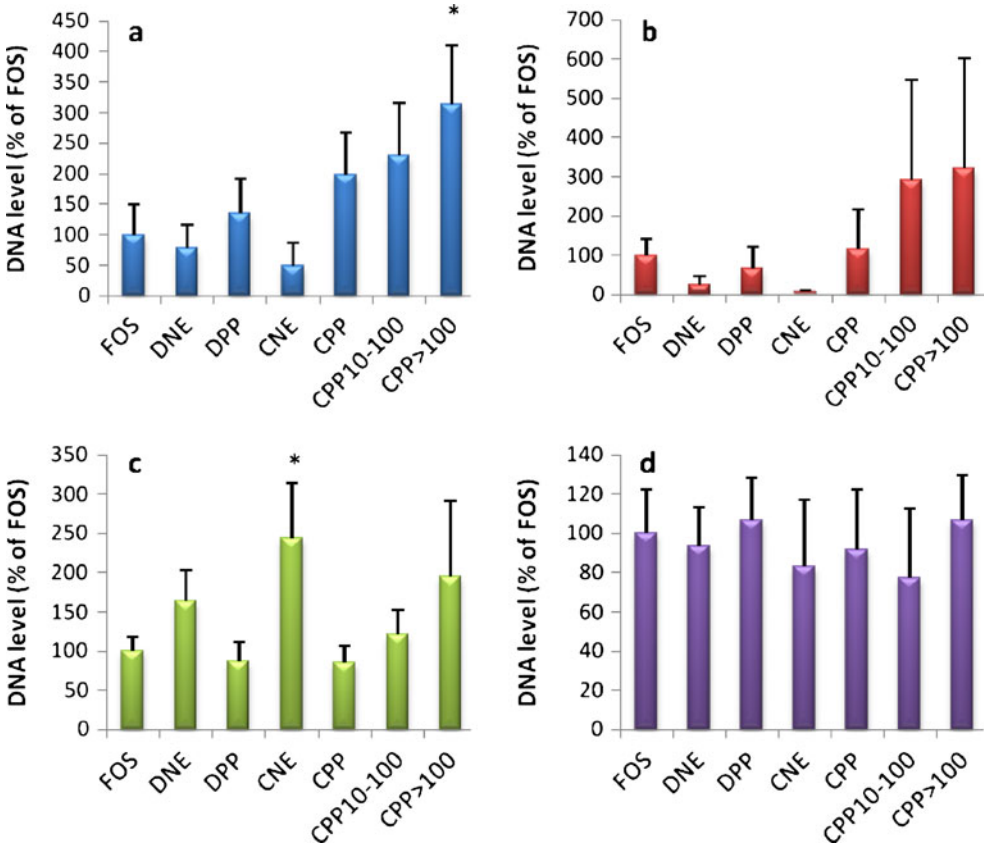

Fig. 6 The biological activity of the fibers on *a Bifidobacterium*, *b Lactobacillus*, *c Bacteroidetes*, and *d Firmicutes*. The *bars* represent the average±SEM of triplicate fermentations. DNA amount in the fermentation samples of FOS was set to 100%. *Asterisks* indicate a significant difference from the fermentation samples containing FOS; **P*<0.05

based on its known selective effect on the content of *Bifidobacterium* and *Lactobacillus* (Palframan et al. 2002; Rycroft et al. 2001; Sanz et al. 2005). *Bifidobacterium* and *Lactobacillus* (in adults consisting of about 4% and less than 2% of the total microbiota, respectively) were included in the analysis, since they are associated with various health benefits in the colon and hence target for prebiotic treatment (Kleerebezem and Vaughan 2009). The *Bacteroidetes* and *Firmicutes* phyla were included in the qPCR analysis to study changes in the overall composition of the intestinal microbiota, which in humans is dominated by these two phyla. The balance between them is believed to play a role, e.g., in development of obesity and intestinal inflammation (Guo et al. 2008; Sokol et al. 2009; Turnbaugh et al. 2006).

The density of *Bifidobacterium* was higher after fermentation on four out of six applied fibers compared to FOS (Fig. 6a). The stimulation of Bifidobacterial growth by CPP>100 was significantly higher than FOS ($P<0.05$), and a similar trend was observed for CPP10–100. Additionally, CPP10–100 and CPP>100 also stimulated the growth of *Lactobacillus* equally well as FOS. These results indicate that CPP10–100 and CPP>100 have beneficial effects on the fecal microbiota composition. When considering the differences in the monosaccharide compositions of the CPP10–100 and CPP>100 fibers, described above, the data also indicate that especially long galactan side chains and presumably also homogalacturonan could be the reason for the selective stimulation of the growth of *Bifidobacterium* and *Lactobacillus*. Van Laere et al. (2000) tested the fermentability of arabinogalactan-enriched polysaccharides from soy on pure cultures of *Bifidobacterium* and *Lactobacillus* and showed that both *Bifidobacterium* and *Lactobacillus* species were able to partly degrade these fibers. The level of *Bacteroidetes* was significantly higher in the microbial communities fermented on CNE than on FOS. DNE and CNE have similar monosaccharide composition as some of the other fibers but lower average molecular mass (mainly <1 kDa). Van Laere et al. (2000) showed that *Bacteroides* strains (belonging to the *Bacteroidetes* phylum) were able to degrade arabinogalactooligosaccharides which could cause the effect shown in Fig. 6c, where CNE containing mainly oligosaccharides have predominant ability to selectively increase the abundancy of the *Bacteroidetes*. It should be noted that the amount of dry matter added in the *in vitro* fermentation was the same for all fibers and since the amount of dry matter released without addition of enzymes was less than when enzymes were added, the relative amount of added starch/glucose in fermentation with CNE and DNE was higher. Pure culture experiments have been conducted in previews studies to reveal differences in the growth and degradation kinetics of specific bacteria (Olano-Martin et al.

2002; Rossi et al. 2005). However, the use of human feces as inocula, as done in this study, gives the ability to examine simultaneous effects on growth of more bacterial groups at the same time and to address selective stimulation of given bacterial taxa in the complex ecosystem constituted by the fecal population. However, one cannot draw conclusions from *in vitro* fermentation on the efficiency of the prebiotic capacity of the fiber fractions but the prebiotic (bifidogenic) effect must be considered as promising with regard to enzymatic released potato fiber as a prebiotic candidate.

In potato pulp galactan, the galactose units are expected to be mainly β-1,4-linked. By use of the CAZY database (Cantarel et al. 2009) different species within *Bifidobacterium*, *Bacteroidetes*, and *Firmicutes* were all found to contain both β-galactosidase (EC 3.2.1.23), endo-β-1,6-galactanase (EC 3.2.1.-), β-1,3-galactosidase (EC 3.2.1.145), and endo-β-1,4-galactanase (EC 3.2.1.89). *Lactobacillus* species contain the same enzyme activities except endo-β-1,6-galactanase (EC 3.2.1.-). The response in Fig. 6a for CPP>100 might indicate that bifidobacteria express particularly high levels of β-galactanase activity that catalyze the degradation of β-1,4-galactan allowing the bacteria to utilize the β-1,4-galactan as a carbon source. Further research is, however, required to confirm this. In any case, the results obtained in this study indicate that the enzymatically produced β-1,4-galactan rich potato fibers, especially those with high molecular weights, may have potential as functional food ingredients with bifidogenic properties.

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Paper 6

Tailored enzymatic production of oligosaccharides from sugar beet pectin and evidence of differential effects of a single DP chain length difference human fecal microbiota composition after *in vitro* fermentation.

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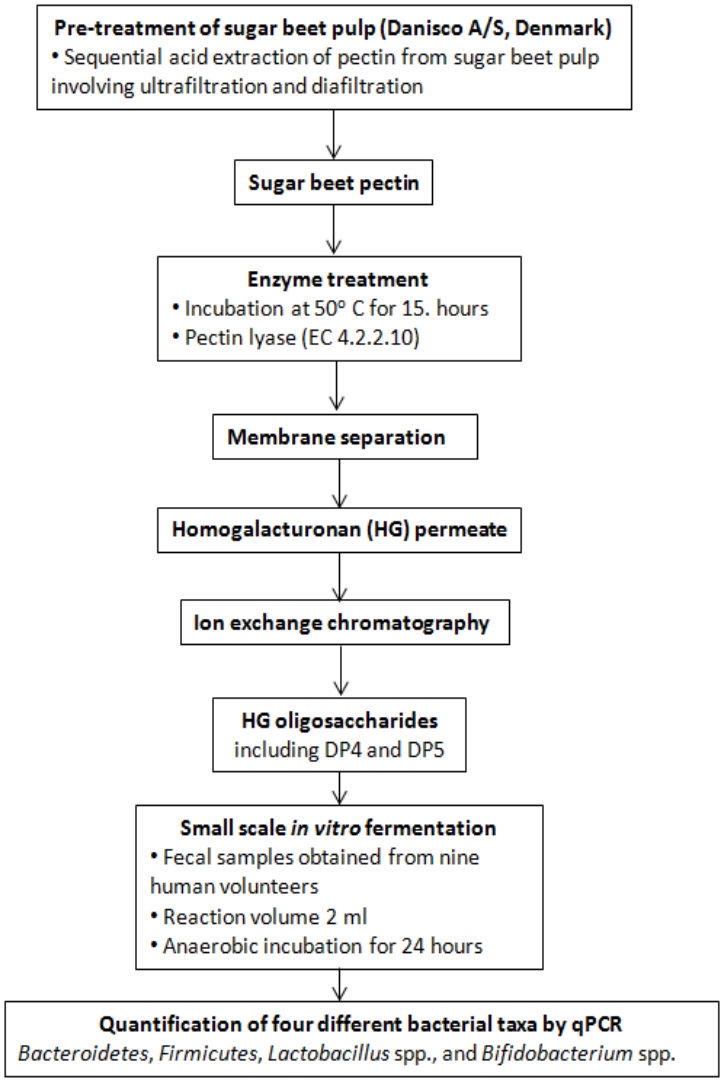
Introduction

The aim of this study was to produce homogalacturonan and rhamnogalacturonan oligosaccharides with a defined degree of polymerization from sugar beet pectin. The ability of different chain lengths of HG oligosaccharides to alter the ratio between *Bacteroidetes* and *Firmicutes* was assessed by *in vitro* fermentation using human fecal samples.

Flow diagram



Quantification of *Lactobacillus* spp. and *Bifidobacterium* spp. was not included in the paper, but is described in Appendix 3.

The author, Louise K. Vigsnæs, performed the small scale *in vitro* fermentations, and conducted the quantification of the bacterial taxa after fermentation. Louise K. Vigsnæs took part in the evaluation of the results and in preparation of manuscript regarding the *in vitro* fermentation study.

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Tailored enzymatic production of oligosaccharides from sugar beet pectin and evidence of differential effects of a single DP chain length difference on human faecal microbiota composition after *in vitro* fermentation

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ABSTRACT

Sugar beet pectin was degraded enzymatically and separated by ion exchange chromatography into series of highly purified homogalacturonides and rhamnogalacturonides. MALDI-TOF/TOF mass-spectrometry was used to determine sizes and structural features. The methodology was based on the sequential use of monocomponent enzymes that were selected to target specific substructures in the sugar beet pectin. Notably pectin lyase and rhamnogalacturonan I lyase were used, which allowed detection of the resulting cleavage products by UV spectroscopy. Seven different homogalacturonides (HG) with degrees of polymerization (DP) from 2 to 8 and six different rhamnogalacturonide (RGI) structures, ranging from DP4 to 6 with defined galactose substitutions were purified. Total recoveries of 200 mg homogalacturonides and 67 mg rhamnogalacturonides per gram sugar beet pectin were obtained. This integrated biorefining method provides an option for advanced upgrading of sugar beet pectin into HG and RGI oligosaccharides of defined size and structure. *In vitro* microbial fermentation by human faecal samples ($n = 9$) showed a different response to the DP4 and DP5 HG structures on the ratio between *Bacteroidetes* and *Firmicutes*. This indicates that pectic oligosaccharides with only slightly different structures have significantly different biological effects. This is the first report of pectic oligosaccharide activity on gut bacterial populations related to the metabolic syndrome associated with obesity.

1. Introduction

Side-streams from agricultural industries are the subject of a number of studies to generate added-value products. From the sugar industry, sugar beet pulp comprises more than 4 million tons/year in the European Community. Sugar beet pectin is a major fraction of this renewable resource, but has so far only been utilized commercially for a limited number of applications [1]. Pectin, including sugar beet pectin, is one of the most structurally complex types of polysaccharides in nature. Pectin is generally defined as a heteropolysaccharide predominantly containing galacturonic acid (GalA) residues that are organized in a pattern of "smooth" homogalacturonan (HG) regions and ramified "hairy" rhamnogalacturonan I (RGI) regions in which neutral sugars are present as side chains. HG consists of linear stretches of α-1,4-linked galacturonic acid residues, which are partially methoxylated at the carboxyl group and *O*-acetylated on *O*-2 and/or *O*-3. The length of HG in sugar beet pectin is relatively shorter than the HG from citrus and apple [1]. The acetylation in sugar beet pectin can be as high 50% as reported by Levigne et al. [2]. The backbone of RGI is composed of the repeating disaccharide [→2)-α-L-Rha*p*–(1→4)-α-D-Gal*p*A-(1→], and, depending on the source [3], 20–80% of the rhamnose moieties may be substituted at the *O*-4 position with arabinan (α-1,5-linked arabinose with some α-L-ara*f* substitutions at C-2 or C-3), galactan (β-1,4-linked galactose) and/or arabinogalactan (Fig. 1A). RGI can also be *O*-acetylated at the *O*-3 position of galacturonic acid. The amount of RGI in sugar beet pectin is relatively higher compared to apple and citrus pectin, comprising up to 25% of the dry weight [4]. Furthermore RGI side chains in sugar beet pectin are particularly rich in arabinose moieties [5] which are substituted with ferulic acid (approximately 1% (w/w). Most of the ferulic acids are bound as monomers, but some as various types of dimers [6].

Abbreviations: HG, homogalacturonan; RGI, rhamnogalacturonan I; SB, sugar beet.

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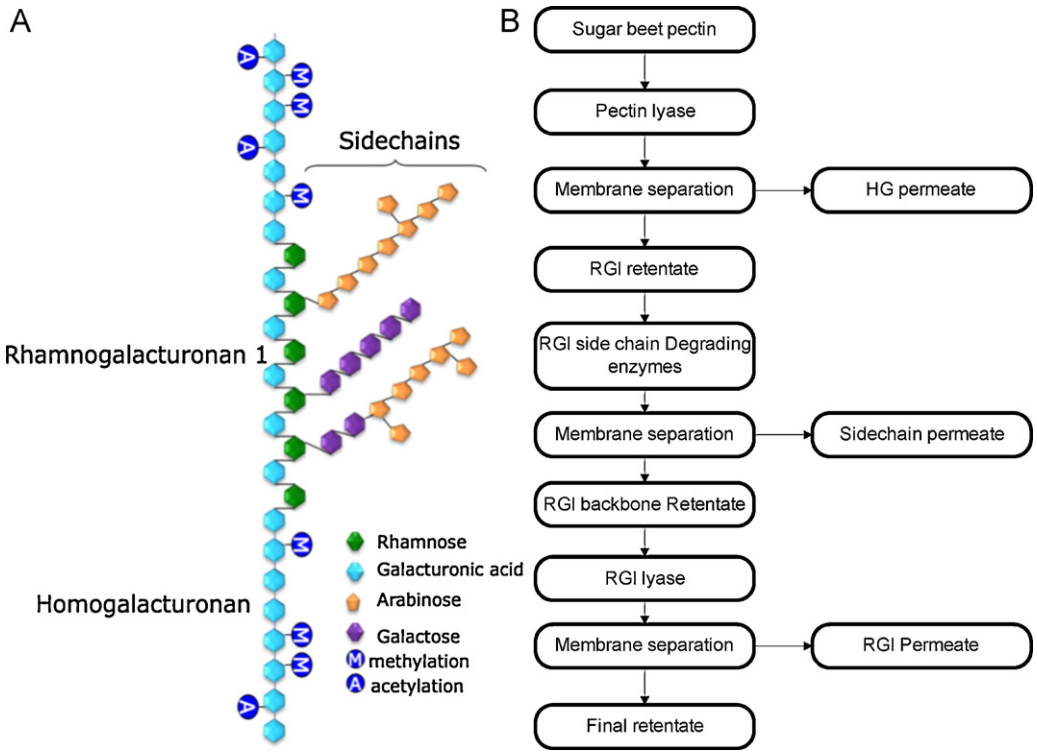

Fig. 1. (A) Schematic illustration of sugar beet pectin structural elements. (B) Schematic flow sheet for biorefining strategy indicating the enzymatic treatments and membrane separation steps.

Finally sugar beet pectin contains minor amounts of rhamnogalacturonan II (RGII), that has a backbone similar to HG substituted with side chains of a complex structure and composition [7]. The combination of shorter HG chain length, high degree of acetylation and the large amount of side chains contributes to the poor gelling properties of sugar beet pectin [1].

Several studies have indicated health promoting effects of pectin derived compounds. These effects include (i) prebiotic effects that selectively increase growth and/or activity of bacteria considered to be probiotic such as *Bifidobacterium* and *Lactobacillus* strains [8–12], (ii) modification of the ratio between subgroups of the phyla *Bacteroidetes* and *Firmicutes* [13], and (iii) a decrease in the growth of potentially pathogenic bacteria like clostridium [11]. It has also been reported that certain pectin derived oligosaccharides reduce the binding of *E. coli* Shiga-like toxin in human colonic adenocarcinoma cell receptors [14], induce apoptosis in human colonic adenocarcinoma cells [15] and prostate cancer cells [18], inhibit proliferation of several types of cancer along with antioxidant effects [16], and increase urinary excretion of toxic metals [17]. A common theme in numerous papers regarding health promoting effects of pectin is the dependency of size of the applied poly- and oligosaccharides [12,14,16]. Nevertheless, the studies assessing the putative bioactivity of pectin have been performed using mixtures of heterogeneous pectin derived fractions with a relatively broad size distribution [9–11,18,19]. Several studies based on the pectic oligosaccharides generated by Olano-Martin et al. [20] revealed that a low methoxyl fraction with a binominal molecular weight distribution of approximately 3.8 kDa and 0.97 kDa (roughly corresponding to DP21–23 and DP5–6) showed better potential as a health promoting candidate than a high methoxyl fraction with a molecular weight of approximately 3.5 kDa (corresponding to DP20–21). The work by Manderson et al. [8] and Mandalari et al. [9] indicated that pectic oligosaccharides with DP <10 had optimal bifidogenic effects. The same pectic oligosaccharide fraction used by Manderson et al. [8], which contained methyl-esterified HG and RGI oligosaccharides, also inhibited the adhesion of *E. coli* to human colonic adenocarcinoma cells [21]. However, a pectic oligosaccharide fraction enriched in RGI oligosaccharides inhibited the invasion of Caco-2 cells by *Campylobacter jejuni* but did not influence bacterial adhesion to these cells [22]. Subtle differences in pectic oligosaccharide structure thus appear to significantly alter the health-promoting biological activity. The use of pectic oligosaccharide mixtures can therefore obscure the results, and prevent a detailed understanding of possible structure function relationships of pectin derived oligosaccharides in relation to health promoting effects.

Pectic oligogalacturonides have previously been successfully prepared at the preparative scale by use of Aminopropyl-silica, Q-sepharose, CarboPac PA1 or PA100 chromatography [23–26]. Notably, gram quantities of saturated and unsaturated HG oligosaccharides up to DP7 were prepared following polygalacturonase and pectate lyase degradation of citrus polygalacturonic acid [23] and milligram quantities of homogalacturonides up to DP20 from autoclaved, hydrolyzed citrus polygalacturonic acid were also prepared [25]. Similarly, apple pectin derived rhamnogalacturonides have been isolated by a combination of preparative scale size-exclusion and ion exchange chromatography with CarboPac PA1 or PA100 columns [26,27]. The homogalacturonide separation method described by Hotchkiss et al. [23] was fast, had high sample load capacity and a good isolation efficiency range of 10–136 mg/peak h, whereas the work done by Mutter et al. and Schols et al. [26,27] was mainly designed to examine specific enzymatic action and degradation of pectic hairy regions, with less emphasis on achieving high recoveries and quantification of the oligomers.

This article introduces a method for integrated production of homogalacturonan and rhamnogalacturonan oligosaccharides with a defined degree of polymerization. The method builds on sequential dissection of sugar beet pectin using monocomponent enzymes, membrane separations, and ion exchange chromatogra-

Table 1
List of enzymes.

Enzyme	Source	pH optimum	Temperature optimum	EC number	Family	Reference
Pectin lyase	*Aspergillus nidulans*	7.0	n.a.	4.2.2.10	PL 1	[29]
β-Galactosidase-1	*Aspergillus niger*	4.0–4.5	60 °C	3.2.1.23	GH 35	Megazyme
β-Galactosidase-2	*Kluyveromyces lactis*	n.a.	n.a.	3.2.1.23	GH 2	n.a.
β-Galactanase	*Aspergillus niger*	4.0–4.5	50 °C	3.2.1.89	GH 53	Megazyme
α-Arabinofuranosidase	*Aspergillus aculeatus*	4.0–4.5	30 °C	3.2.1.55	–	[50]
α-Arabinanase	*Aspergillus aculeatus*	5.5	30 °C	3.2.1.99	GH 43	[50]
RGI lyase	*Bacillus licheniformis*	8.0–8.5	60–65 °C	4.2.2.–	–	Unpublished data

phy to obtain the structural elements and avoids the use of organic solvents. To assess the possible effect of different chain length, the study includes evaluation of the biological activity of homogalacturonan oligosaccharides DP4 and DP5 based on their capacity to alter the ratio between *Bacteroidetes* and *Firmicutes*, which represent the dominant bacterial phyla in the human intestine. This is assessed by *in vitro* bacterial fermentation using human faecal samples.

2. Materials and methods

2.1. Substrate

Sugar beet pectin was obtained from Danisco A/S (Nakskov, Denmark). The pectin had been prepared by sequential acid extraction with nitric acid from sugar beet pulp, involving removal of insoluble cellulose, ultrafiltration, and diafiltration with a 50 kDa cutoff essentially as described by Buchholt et al. [1], except that precipitation in isopropanol was replaced by spray drying. Degree of methoxylation and acetylation were determined to be 59% and 20%, respectively.

2.2. Chemicals

Ammonium formate, Trizma-HCl, sodium acetate, acetic acid, hepes, manganese chloride, D-glucuronic acid, D-galactose, D-arabinose, D-fucose, L-rhamnose monohydrate, D-galacturonic acid monohydrate, and α-cyano-4-hydroxy-cinnamic acid were purchased from Sigma–Aldrich (Steinheim, Germany) D-glucose and D-xylose were purchased from Merck (Darmstadt, Germany). Trifluoroacetic acid was from Riedel-de Haën (Seelze, Germany). High resolution Source 15Q anion exchange resin and chromatography columns were obtained from GE Healthcare Biosciences (Uppsala, Sweden). All chemicals used were analytical grade.

2.3. Enzymes

Pectin lyase was produced by fermentation essentially as described by Stratton et al. [28]. The *Pichia pastoris* clone transformed with the pectin lyase gene AN2569.2 was obtained from the Fungal Genetic Stock Center as described by Bauer et al. [29]. Rhamnogalacturonase I from *Bacillus licheniformis* was cloned and expressed in *P. pastoris* (unpublished results). Galactanase and β-galactosidase-1 were purchased from Megazyme (Bray, Co. Wicklow, Ireland), β-galactosidase-2 was purchased from Sigma–Aldrich (Steinheim, Germany). 1 and 2 denotes the origin of gene, either *Aspergillus niger* (1) or *Kluyveromyces lactis* (2). Arabinanase and α-L-arabinofuranosidase were obtained from Novozymes (Bagsvaerd, Denmark) (Table 1).

2.4. Biorefining strategy

The sugar beet pectin was separated into homogalacturonan (HG) and rhamnogalacturonan I (RGI) by sequentially applying monocomponent enzymes addressed towards certain structures in the pectin molecule, followed by removal of the released oligomers by membrane separation (Fig. 1B). Pectin lyase was employed to catalyze the cleavage of methoxylated homogalacturonan by β-elimination. This gave rise to a double bond in between carbon 4 and 5 of galacturonic acid in the non-reducing end of one of the cleavage products. In turn, this allowed the assessment of the molar level of released products in the HG permeate by UV spectroscopy. Next, enzymes active towards arabinan and galactan side chains of the RGI were added to catalyze the release of arabinose and galactose oligomers and monomers. These products were separated into the side chain (SC) permeate (Fig. 1). Finally RGI lyase was added to the retentate to catalyze the cleavage of the remaining RGI backbone by β-elimination (Fig. 1) again introducing a detectable double bond. The released RGI oligomers were separated into the RGI permeate. Any unprocessed material was found in the final retentate.

2.5. Membrane reactions and separation

All reactions and separations were performed in a 200 mL stirred membrane reactor model 8200 (Millipore, Billerica, MA) equipped with a 3 kDa regenerated cellulose membrane (Millipore, Billerica, MA), connected to compressed nitrogen for flux regulation. The reactor contents were mixed by magnetic stirring using a RCT basic magnetic stirrer (IKA, Germany). Temperature control was maintained using a Julabo ED5 water bath (Julabo, Germany).

2.6. Pectin lyase treatment

A 3% (w/w) SB pectin solution in 50 mM Tris buffer pH 8 was treated with 0.5% E/S (enzyme/substrate) pectin lyase and incubated at 50 °C for 15 h in a stirred and thermostatically controlled reactor. The reaction mixture was transferred to the membrane reactor and filtration was performed at 50 °C and 2 bars. The retentate was diafiltrated twice in 0.7 sample volumes of water. All permeate was pooled and stored at 5 °C until further use.

2.7. Side chain degrading treatment

A 1.4% (w/w) RGI retentate solution in 50 mM acetate buffer pH 5 was treated with a mixture of 0.2% β-galactosidase-1, 4.7% β-galactosidase-2, 0.2% galactanase, 0.8% arabinofuranosidase, and 0.8% arabinanase (all percentages in % E/S) and incubated in a membrane reactor at 45 °C for 16 h. The enzymes were dosed according to a tentative estimate of the available concentration of the specific structural substrate element and the individual enzyme activity. Filtration was performed at 50 °C and 2 bars. The retentate was diafiltrated once in 1 sample volume of water. All permeate was pooled and stored at 5 °C until further use.

2.8. RGI lyase treatment

Prior to the enzymatic treatment, RGI lyase was incubated in 100 mM $MnCl_2$ for 0.5 h for activation [30]. A 0.75% (w/w) RGI backbone solution in 50 mM Hepes buffer pH 7.8 was treated with 0.5% E/S RGI lyase and incubated in a membrane reactor at 60 °C for 2 h. Filtration was performed at 60 °C and 2 bars. The retentate was diafiltrated twice in 1 sample volume of water. All permeate was pooled and stored at 5 °C until further use.

2.9. Alkaline deesterification

Prior to ion exchange chromatography, methoxyl- and acetyl groups were removed from pectic substances using cold alkaline conditions. The low temperature was used in order to prevent spontaneous β-elimination [31]. Sample temperature was lowered to approx. 5 °C and cold 2 M NaOH was added under stirring until pH 13 and a final concentration of 40 mM NaOH was reached. Samples were incubated for 24 h. After incubation pH was adjusted using 1 M acetate buffer pH 6.5 and 2 M HCl. Samples were diluted to conductivity under or equal to the conductivity of the ion exchange buffer.

2.10. Ion exchange chromatography

Anion separation was performed at room temperature with an ÄKTA purifier 100 work station equipped with a P-900 pump, UV-900 monitor, pH/C monitor, and Frac-950 fraction collector, all controlled by UNICORN software. A HR16/10 column with a 23 mL packed bed of high resolution Source 15Q was used for separation. The elution of the unsaturated oligosaccharides was monitored by the UV absorption at 235 nm and subsequently quantified by use of the molar extinction coefficient 4600 M^{-1} cm^{-1} [32]. Baseline correction was made with a blank run. Elution was performed at a flow rate of 10 mL/min with water and ammonium formate as eluents.

Before injection the column was equilibrated with 20 mM ammonium formate for two column volumes (CV). After injection the column was washed with 20 mM ammonium formate for three CV, followed by elution with a gradient specified below. After elution the column was regenerated with 1000 mM ammonium formate for two CV and with water for three CV. To obtain the oligomers in the HG permeate, a sample was injected on the column and eluted using a linear gradient

Table 2
16S rRNA primers for real time PCR.

Target gene	Forward primer (5′–3′)	Reverse primer (5′–3′)	Product size (bp)	Correlation coefficient (R^2)	Efficiency (%)	Reference
Firmicutes phylum	gga gya tgt ggt tta att cga agc a	agc tga cga caa cca tgc ac	126	0.999	101	[51]
Bacteroidetes phylum	gga rca tgt ggt tta att cga tga t	agc tga cga caa cca tgc ag	126	0.999	99	[51]
Total bacteria[a]	cgg caa cga gcg caa ccc	cca ttg tag cac gtg tgt agc c	130	0.999	99	[52]

[a] PCR for the total bacteria primer set was run in parallel for each set of primers for all samples.

from 80 to 550 mM ammonium formate for 20.25 CV. Collection of peaks of interest was performed using a level fractionation parameter. To obtain the oligomers in the RGI permeate, a sample was injected on the column and eluted using a linear gradient from 20 to 400 mM ammonium formate for 30 CV. Fractionation was performed using an elution volume as the fractionation parameter. The collected fractions for both types of oligomers were lyophilized to remove water and ammonium formate [33,34] and analyzed by MALDI-MS and MS/MS.

2.11. MALDI-MS

All mass-spectrometric analysis of the oligosaccharides was performed on a 4800 Plus MALDI TOF/TOF™ (AB SCIEX) mass spectrometer. 0.5 μl of the sample was applied to a stainless steel MALDI target and mixed with 0.5 μl of the matrix, α-cyano-4-hydroxy-cinnamic acid dissolved at a concentration of 10 mg/mL in 70%ACN/0.1% TFA/water. The instrument was operated in negative ion reflector mode and the *m*/*z* range from 100 to 3500 was monitored. A total of around 1000 laser shots were applied to each sample in MS mode. In MS/MS mode around 2000 shots were obtained. The laser intensity was manually varied in order to obtain the best possible spectra (MS: from 4500 to 4800. MS/MS: from 4800 to 5100). MALDI-TOF-MS and MS/MS data were exported as text files using DataExplorer (version 4.0) and each spectrum were smoothed, labeled and analyzed manually in *m*/*z* (Genomic Solutions®). For annotation of MS/MS spectra, the nomenclature suggested in [35] was applied.

2.12. Combined acid and enzymatic hydrolysis

The monosaccharide composition of all fractions were determined by a combined acid and enzymatic hydrolysis modified from Garna et al. [36]. Each substrate was treated in 4 mL final volume of 0.2 M trifluoroacetic acid having a substrate concentration of 2.5 g/L for 72 h at 80 °C. After acid hydrolysis, pH was adjusted to 5 using 500 mM acetate buffer pH 5, followed by 5 M NaOH. The acid hydrolysate was treated with Viscozyme (Novozymes A/S, Bagsvaerd, Denmark) for 24 h at 50 °C as described in [36]. Samples were filtered through a 0.2 μm filter prior to HPAEC-PAD analysis. The recovery of monosaccharides was determined by performing the same hydrolysis on D-fucose, L-rhamnose, D-arabinose, D-galactose, D-glucose, D-xylose, D-galacturonic acid and D-glucuronic acid. The recovery factors were used to quantify the monosaccharides. Relative standard deviation values were determined for at least 3 measurements and ranged from 2 to 14%. The amount of fucose was below the quantification limit in all fractions.

2.13. HPAEC analysis

Quantification of the monosaccharides was performed by HPAEC-PAD using an ICS-3000 system consisting of a gradient pump (model DP-1), an electrochemical detector/chromatography module (model DC-1) coupled to an autosampler (Dionex Corp., Sunnyvale, CA). The separation was made using a CarboPac™ PA20 (3 mm × 150 mm) analytical column based on a method of [37] with the following modifications: a two-eluent system comprising deionized water and a 0.5 M NaOH aqueous solution was used. Before injection of each sample (10 μl) the column was reequilibrated with 15 mM NaOH for 10 min. After injection of the sample a linear gradient from 15 mM NaOH to 5 mM NaOH from 0 to 1.5 min was applied, followed by isocratic elution with 5 mM NaOH from 1.5 to 3 min, another linear gradient from 5 mM NaOH to 2.5 mM NaOH from 3 to 7 min, another isocratic elution with 2.5 mM NaOH from 7 to 12 min, and a final isocratic elution with 500 mM NaOH from 12 to 25 min. The eluent flow rate was always kept at 0.5 mL/min. The quantification was carried out using the external monosaccharide standards: D-fucose, L-rhamnose, D-arabinose, D-galactose, D-glucose, D-xylose and D-galacturonic acid. The following pulse potentials and durations were used for detection: $E1 = 0.1$ V, $t1 = 400$ ms; $E2 = -2$ V, $t2 = 20$ ms; $E3 = 0.6$ V, $m3 = 10$ ms; $E4 = -0.1$ V, $t4 = 70$ ms. Data were collected and analyzed with the program Chromeleon 6.80 SP4 Build 2361 software (Dionex Corp., Sunnyvale, CA).

2.14. Subjects and faecal samples

Faecal samples were obtained from 6 patients with ulcerative colitis (UC) and 3 healthy volunteers. Within the UC group, 3 patients were in remission and 3 patients were in relapse at the time of sampling. None of the participants had been treated with antibiotics for at least 2 months before enrolment, and the 3 healthy volunteers had no history of gastrointestinal disorder. There was no significant difference in the mean age of the participants comparing the 3 groups (healthy control: 42.7 ± 10.0 years, UC patients in remission: 36.0 ± 5.0 years and UC patients in relapse: 44.3 ± 5.5 years). Whole stools were collected in airtight containers, immediately stored at 4 °C and processed within 12 h. 200 mg wet weight faeces were collected for DNA extraction, and at the same time, faeces were prepared for *in vitro* fermentation. The faecal samples for *in vitro* fermentation were homogenised in 50% glycerol (1:1 dilution) in an anaerobic cabinet (containing 10% H_2, 10% CO_2, and 80% N_2) and stored at −80 °C until further analysis.

2.15. In vitro fermentation

A small scale *in vitro* fermentation method was developed to assess the fermentability of selected oligosaccharides on human faecal samples. Sterile filtered homogalacturonan oligosaccharides DP4 and DP5 were added to autoclaved minimal basal medium to give a final concentration of 5 g/L in a total reaction volume of 2 mL. The solutions were reduced in an anaerobic cabinet over night. The minimal basal medium contained, per liter, 2 g of peptone water, 1 g of yeast extract, 0.1 g of NaCl, 0.04 g of K_2HPO_4, 0.04 g of KH_2PO_4, 0.01 g of $MgSO_4 \cdot 7H_2O$, 0.01 g of $CaCl_2 \cdot 2H_2O$, 2 g of $NaHCO_3$, 0.5 g of bile salts, 0.5 g of L-cysteine hydrochloride, 0.005 g of hemin, 10 μl of vitamin K_1 (0.02 mM), 2 ml of Tween 80, and 1 mL of 0.05% (w/v) resazurin solution. A 10% (w/v) faecal slurry was prepared by mixing the faeces diluted in 50% glycerol with anoxic PBS (Oxoid, Greve, Denmark). The reduced minimal medium with added oligosaccharide was inoculated with the faecal slurry to a final concentration of 1% (w/v) faeces. The fermentations were performed at 37 °C for 24 h in an anaerobic cabinet.

2.16. Extraction of bacterial DNA

DNA was extracted from faecal and fermentation samples using the QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) with a bead-beater step in advance, as described by Leser et al. [38]. The purified DNA was stored at −20 °C until use.

2.17. Real-time PCR assay conditions

Amplification and detection of purified bacterial DNA by real-time PCR were performed with the ABI-Prism 7900 HT from Applied Biosystems using optical grade 384-well plates. Primers specifically targeting 16S rRNA gene sequences of the *Bacteroidetes* and *Firmicutes* phyla, respectively, were included in the qPCR analysis (Table 2). The amplification reactions were carried out in a total volume of 10 μl containing; 5 μl EXPRESS SYBR® GreenER™ qPCR SuperMix (Invitrogen A/S, Taastrup, Denmark), 10 pmol of each of the primers (Eurofins MWG Synthesis GmbH, Ebersberg, Germany), 2 μl template DNA, and Nuclease-free water (Qiagen) purified for PCR. The amplification program consisted of one cycle at 50 °C for 2 min; one cycle at 95 °C for 10 min; 40 cycles at 95 °C for 15 sec and 60 °C for 1 min; and finally one cycle of melting curve analysis for amplicon specificity at 95 °C for 15 s, 60 °C for 20 s and increasing ramp rate by 2% until 95° for 15 s.

2.18. Realtime PCR data handling

The threshold cycle (Ct) values and baseline settings were determined by automatic analysis settings set by the ABI software. The relative changes in the abundancy of gene targets encoding 16S rRNA of *Bacteroidetes* and *Firmicutes* were calculated using the approximation 2^{-Ct}. Ct is the threshold cycle calculated by the ABI software as the PCR cycle, where amplifications signal exceeds the selected threshold value, also set by the software. The amounts of phylum specific DNA targets were normalized to total bacteria DNA targets in order to correct for differences in total DNA concentration between individual samples [39]. Standard curves were created using serial 10-fold dilutions of bacterial DNA extracted from one of the fermentation samples for all primer sets. Analysis of the standard curves allowed verification of PCR efficiency for the chosen PCR conditions (Table 2). All results were calculated as means of duplicate determinations.

2.19. Statistical analysis

Statistical analysis of quantitative PCR data was done using the GraphPad Prism (version 5.03). Data was analyzed by one-way ANOVA followed by a pair-wise multiple comparison of means (Student's *t*). Tests were considered statistically significant if P values <0.05 were obtained.

Table 3
Mass balance in percentage based on dry matter content vs. theoretical mass. The molar distribution of monosaccharides is based on the quantification of each main monosaccharide (without considering methylation and acetylation) in each fraction compared to the total amount in 1 g of the starting material. Data for glucose and glucuronic acid is not shown.

	Observed mass	Theoretical mass	Rhamnose		Arabinose		Galactose		Galacturonic acid	
			mmol	%	mmol	%	mmol	%	mmol	%
SB pectin	100	100	0.35	100	0.91	100	0.68	100	3.53	100
HG permeate	63	56	0.00	0	0.44	49	0.14	21	2.74	78
SC permeate	19	23	0.10	29	0.35	38	0.29	44	0.35	10
RGI permeate	11	11	0.12	34	0.00	0	0.13	19	0.15	4
Final retentate	2	0	0.01	1	0.00	0	0.01	2	0.02	1
Other	5	4	0.13	35	0.12	13	0.11	15	0.30	8

3. Results and discussion

3.1. Mass distribution

Based on the monosaccharide composition of each fraction (without considering the degrees of methyl and acetyl esterification) an overall mass balance and the molar distribution of each of the major monosaccharides were calculated. Assuming an ideal distribution of the enzymatically cleaved pectin products, the theoretical masses of each fraction was calculated and compared to the observed masses for the dry matter mass distribution (Table 3). As expected from the general pectin structure, the highest mass (63%) was found in the HG permeate (Table 3). The experimental mass of the HG permeate was slightly higher than the expected theoretical mass, whereas the experimental SC permeate mass (19%) was correspondingly smaller than the expected theoretical mass. This slight discrepancy was presumably due to the recovery of some arabinose and galactose in the HG permeate (Table 3) (discussed further below).

3.2. Molar distribution

On a molar basis, the HG permeate following the pectin lyase treatment contained 78% of the total galacturonic acid from SB pectin and the composition of HG permeate was dominated by galacturonic acid (Table 3), suggesting that the HG permeate, as expected, was mainly made up of galacturonides. Relatively high levels of arabinose and some galactose were found in the HG permeate (Table 3), but since no rhamnose was found in this fraction, presumably the arabinose and galactose must have existed as monomers or oligosaccharides in the SB pectin prior to the first enzymatic treatment. The pectin in native sugar beet pulp is rich in arabinan present as side chains on the RGI [5]. Since these arabinan side chains are particularly acid labile [1], the amounts (molar levels) of free arabino-oligomers and even arabinose monomers were higher than the levels of galacto-oligomers and galactose in the pectin (Table 3). During the industrial preparation, the pectin had been purified via combined diafiltration and concentration via ultrafiltration, but the arabino-oligomers and any monomeric arabinose were apparently not liberated from the pectin during this procedure, presumably because of the high viscosity. Hence, when the pectin backbone was degraded the arabinose and arabino-oligomers were apparently liberated. Regardless, the presences of arabino-oligomers and/or arabinose in the HG permeate did not affect the purity of the final HG oligomers obtained by ion-exchange chromatography (see below). Similarly some glucose was found mainly in the HG permeate (data not shown), indicating that also glucose was present at mono- or oligomers prior to enzymatic treatment. As for arabinose and galactose, some glucose might be present in the pectin since the diafiltration step in the industrial pectin isolation process cannot completely remove all monomers and very low DP oligosaccharides. The SC permeate contained 10% of total galacturonic acid, and 38% and 44% of the total molar level of arabinose and galactose respectively. The SC permeate also contained 29% of total rhamnose. The occurrence of galacturonic acid and rhamnose in the SC permeate might be due to liberation of smaller RGI molecules during the pectin lyase treatment. These molecules were not small enough to pass through the 3K MWCO membrane along with the HG permeate, but after applying the side chain degrading enzymes, the naked RGI backbones were small enough to pass through the membrane. A molecule consisting of alternating galacturonic acid and rhamnose with a DP of 10 will be small enough to pass through this membrane. The employment of a membrane with a lower cut-off would have increased the retention of RGI-like molecules, but would concurrently have decreased the flux significantly. The presence of non-equimolar amounts of rhamnose and galacturonic acid was presumably due to insufficient washing of the RGI retentate during the removal of the HG permeate. In the RGI permeate, the finding of equimolar amounts of galacturonic acid (0.15 mmol) and rhamnose (0.12 mmol) indicated a RGI backbone structure of alternating rhamnose and galacturonic acid residues. Relatively high levels of galactose, almost equimolar to the levels of rhamnose and galacturonic acid, were also found in the RGI permeate. Despite the use of high doses of two different β-galactosidases, the obtained data indicated an insufficient enzymatic treatment with β-galactosidase – the galactanase alone is unable to catalyze the removal of all galactose from the RGI backbone [40]. The incomplete removal of galactose from the RGI backbone was reported previously [41] where smaller RGI backbone oligomers apparently were a more suitable substrate for β-galactosidase than the longer RGI polymers. In contrast, no detectable amounts of arabinose were found in the RGI permeate, which indicated an efficient arabinose removal in the side chain degrading step.

3.3. Purification of homogalacturonan oligosaccharides

After alkaline deesterfication, the HG permeate was purified by anion exchange chromatography and the peaks were detected by UV monitoring at 235 nm (Fig. 2). Fractions were collected, lyophilized and analyzed by MALDI-TOF MS for structural verification. Each fraction resulted in a mass spectrum containing one or two major components together with minor impurities (primarily matrix peaks). The MS spectra clearly showed that the procedure for deesterification was successful. The structure of the oligogalacturonides was determined by MS/MS analysis. As exemplified in Fig. 3, the MS/MS spectra of the component in fraction H7 revealed several C type fragments resulting from sequential losses of 176. For the pure oligogalacturonides these were found to be equivalent in mass to the Z type fragments, in accordance with the fragmentation nomenclature suggested by Domon and Costello [35]. The m/z value of the C1-ion is only 175 instead of 193 due to the β-elimination action of pectin lyase. The fractionation pattern with C and Z type ions in abundance and the presence of a -60 Da ion is in complete accordance with the results obtained for unsaturated unmethylated oligogalacturonides previ-

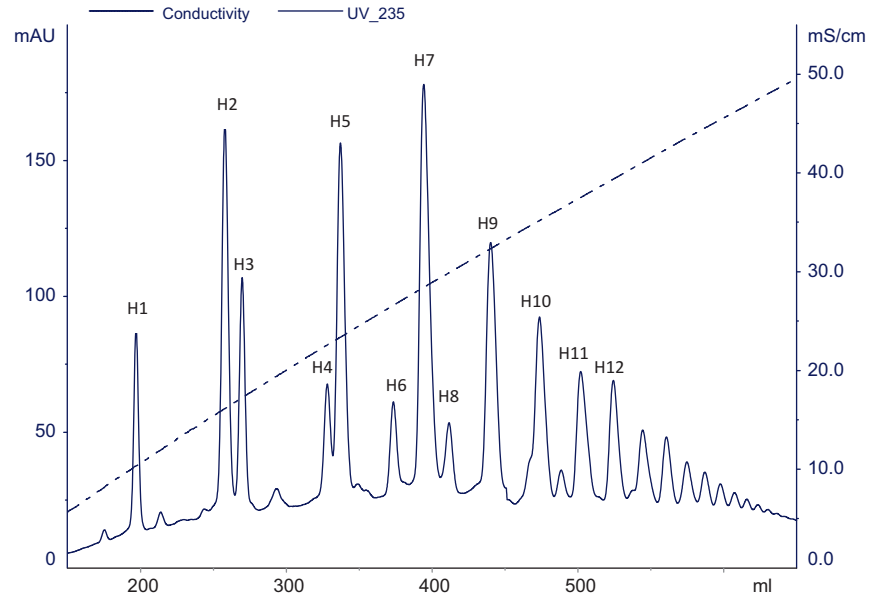

Fig. 2. Elution profile of deesterified HG permeate during ionic exchange chromatography on a Source 15Q packed HR16/10 column using a linear gradient of ammonium formate and UV detection at 235 nm. For peak codes, refer to Table 4.

ously described by Korner et al. [42]. Hence fractions H2, H5, H7, and H9-12 (Fig. 2) were confirmed to contain unsaturated oligogalacturonides with a degree of polymerization (DP) of 2–8 respectively (Table 4).

Fraction H1 contained DP2 with some impurities. The faster elution of the DP2 molecules in H1 compared to H2 (Fig. 2) might be due to interference of the binding to the quaternary ammonium ion in the SourceQ matrix by the impurities. Fraction H3, H4, H6, and H8 all contained compounds with masses corresponding to the weight of unsaturated oligogalacturonides +103. MS/MS data on these fractions also revealed several fragments with sequential losses of 176, i.e. consistent with oligogalacturonide fragments. MS/MS analysis revealed both C and Z type of ions (Fig. 4), but the definite structures were not obtainable. These types of compounds were also found in fraction H9-H11, but only in minor amounts. Further studies are needed to determine the exact structure of these sugars, but the spectrum clearly reveals the existence of a homogalacturonan as the backbone of the structure.

The yields of each type of homogalacturonide ranged from approximately 6 to 46 mg for each g of initial SB pectin, with a total recovery of approximately 200 mg HG oligomers/g SB pectin (Table 4). The yield of each type of compound per run ranged from 9 to 73 mg/g sample load. With the column capacity and flow rate employed this was equivalent to an isolation efficiency of 0.6–4.2 mg/peak h. This recovery range was not as high as that reported by Hotchkiss et al. [23], but somewhat better than that reported by Hotchkiss et al. [25], albeit they isolated longer oligogalacturonides (DP8–20). Since the HG permeate accounted for 0.6 g of 1 g initial pectin, as indicated in the mass balance (Table 3), one third of this mass was thus recovered as pure and defined homogalacturonides and is equivalent to a recovery of 20% from initial sugar beet pectin. It can be assumed that peaks eluted later

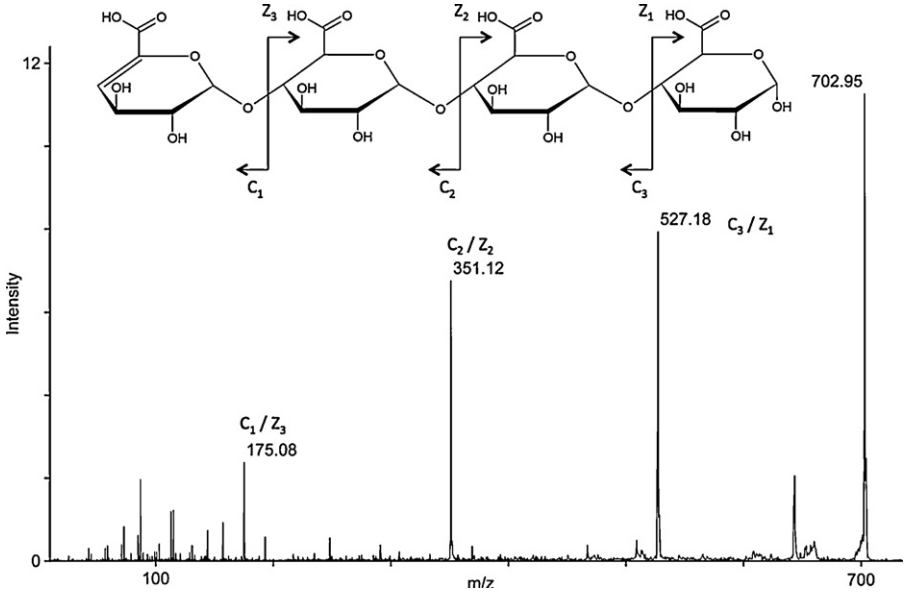

Fig. 3. MS/MS high energy CID spectrum of galA$_4$ (H7, Fig. 2) illustrating the fragmentation pattern and nomenclature, and the proposed structure for the unsaturated galA$_4$.

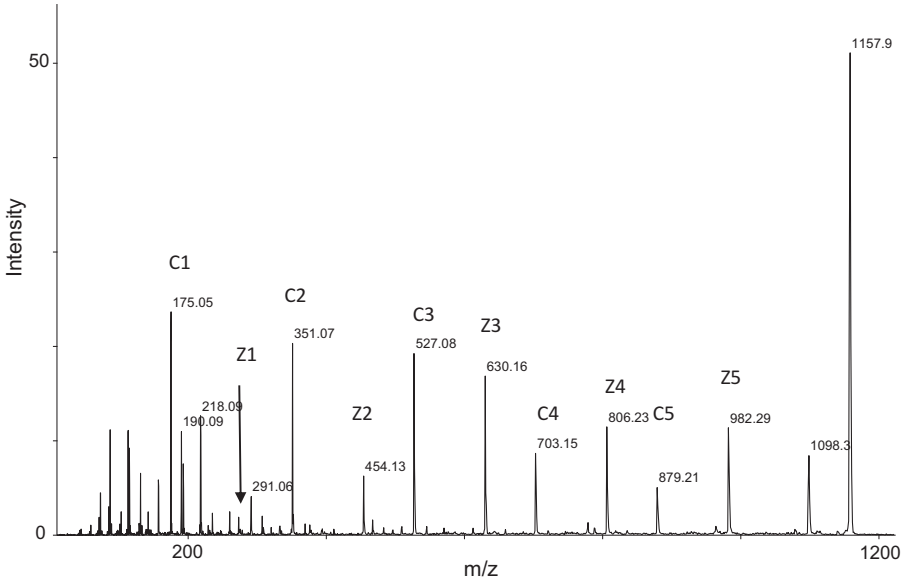

Fig. 4. MS/MS high energy CID spectrum of the MS peak at 1157.9, H8 (Fig. 2). The mass is found to be 103 higher than DP6 (or 73 less than DP7). The spectrum is annotated assuming that the extra 103 Da is located in the reducing end. In case it is located in the non-reduced end, the C and Z ions should be reversed.

Table 4
Identified compounds, peak identity and yields from HG permeate. Yield in mg/g initial pectin. For peak identities, refer to (Fig. 2). Yields are given as means and coefficient of variance were in the range of 1.3–3.1%.

Peak ID	Structure	Yield mg/g pectin
H1	$galA_2$	5.8
H2	$galA_2$	15.2
H5	$galA_3$	24.0
H7	$galA_4$	46.2
H9	$galA_5$	37.1
H10	$galA_6$	32.6
H11	$galA_7$	25.7
H12	$galA_8$	23.3
Total		201.1

than H12 will contain homogalacturonides with a DP above 8, and thereby could add approximately 50 mg/g pectin. However, since no verification by MS/MS was performed, these putative homogalacturonides could not be taken into account in the total recovery. Likewise it can be assumed, based on MS/MS data, that H3-4, 6, 8 (Fig. 2) roughly could add additional 50 mg/g pectin to the overall yield, since these peaks also contained some galacturonic acid.

3.4. Purification of rhamnogalacturonan I oligosaccharides

Analogously to the procedure used for the HG permeate, the RGI permeate was de-esterified by alkaline treatment and purified by anion exchange chromatography (Fig. 5). Fractions were collected, lyophilized and analyzed by MALDI-TOF MS and MS/MS for structural determination. The major compound of peak R1 was found to be $galA_2rha_2gal_2$ and the MS/MS data proved that the two galactose molecules were evenly distributed, one on each rhamnose (Fig. 6). The MS/MS spectrum illustrated a characteristic loss involving an A type cross-ring cleavage of the rhamnose as discussed in [43] (Fig. 7). No further studies were done to determine which of the bonds were cleaved as this was not relevant for the study. The residual ion, a cross ring fragment, is denoted CRF_x, x denoting the number of the cleaved monomer. These characteristic CRF ions were seen in all the examined MS/MS spectra of the RGI like compounds. Fraction R1 also contained trace amounts of $galA_2rha_2gal_3$. Contrary to the major compound, which was found

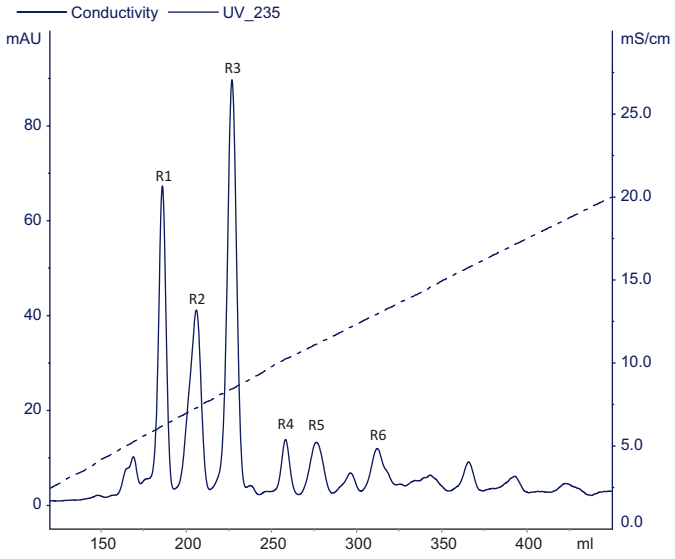

Fig. 5. Elution profile of deesterified RGI permeate during ionic exchange chromatography on a Source 15Q packed HR16/10 column using a linear gradient of ammonium formate and UV detection at 235 nm. For peak codes, refer to Table 5.

to have one galactose on each rhamnose, the MS/MS spectrum showed all 3 galactose units to be localized on a single rhamnose, either the one located in the reducing end or the one next to the non-reduced unsaturated galacturonic acid (the spectrum revealed a mixture of both) (data not shown). R2 contained a $galA_2rha_2gal$ molecule. MS/MS data showed that this peak comprised of a mixture of two kinds of structures with the galactose on either the reducing-end rhamnose or on the internal rhamnose. R3 was pure $galA_2rha_2$ with no traces of other compounds. R4 contained mainly $galA_3rha_3gal_3$ with one galactose on each rhamnose. Also trace amounts of putative $galA_2rha_2gal_2$ were observed, but this structure was not confirmed by MS/MS analysis. R5 contained $galA_3rha_3gal_2$ in a mixture of compounds with the unsubstituted rhamnose in either the reducing end, the middle position, or next to the non-reducing unsaturated galacturonic acid. The MS spectrum of peak R6 revealed two different components. One of them being $galA_3rha_3$, the other one being $galA_3rha_2gal_2$. MS/MS anal-

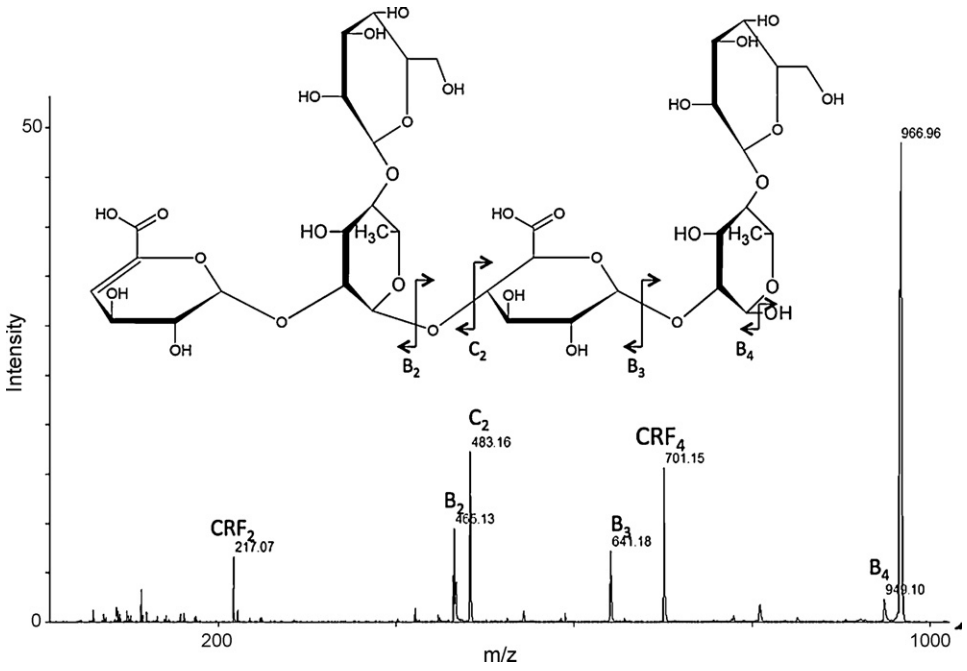

Fig. 6. MS/MS high energy CID spectrum of $galA_2rha_2gal_2$ (R1, Fig. 5) illustrating the fragmentation pattern and nomenclature, and the proposed structure.

ysis of $galA_3rha_2gal_2$ revealed the co-existence of two different structures (Fig. 8A and B). One of the structures consisted of two galacturonic acids, the non-reducing being unsaturated, followed by a rha-galA-rha segment with both rhamnoses substituted with a single galactose (Fig. 8A). This molecule corresponded well with the previous findings of the connecting linkage between homo- and rhamnogalacturonan I in apple pectin [44] but this is the first report of this connecting linkage in sugar beet pectin. The second structure showed an alternating galacturonic acid-rhamnose backbone structure, but with a galacturonic acid in both the reducing and the non-reducing end, the latter being unsaturated. Galactose was distributed evenly (Fig. 8B). These two structures indicated that rhamnogalacturonan I is covalently linked to homogalacturonan in both the reducing and the non-reducing end in sugar beet pectin.

Particularly the finding of the two consecutive galacturonic acid moieties in R6 supports the comprehension of pectin being built of consecutive homogalacturonan and rhamnogalacturonan I structural elements. In turn, this finding rejects the alternative model structure proposed by Vincken [45] with homogalacturonan being a side chain of the rhamnogalacturonan I backbone. While the finding of the molecule with a galacturonic acid in both the reducing and non-reducing end also supports the classic pectin model (Fig. 1) it cannot rule out the alternative model.

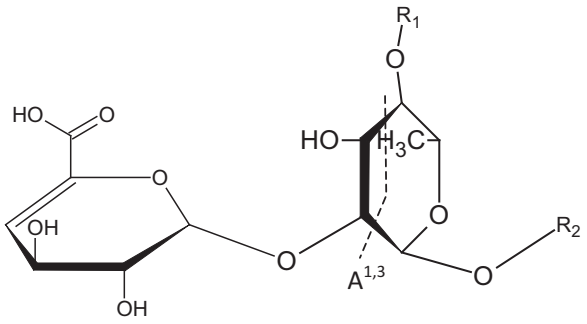

Fig. 7. Type A cross-ring cleavage of rhamnose in a RGI subfragment (CRF_x). As an example of a potential cleavage pattern an $A^{1,3}$ cleavage is indicated by a dotted line. Principle adapted from [43].

Table 5
Identified compounds, peak identity and yields from RGI permeate. Yield in mg/g initial pectin. For peak identities, refer to (Fig. 5).

Peak ID	Structure	Yield mg/g pectin
R1	$galA_2rha_2gal_2$	16.5
R2	$galA_2rha_2gal$	13.9
R3	$galA_2rha_2$	20.2
R4	$galA_3rha_3gal_3$	5.1
R5	$galA_3rha_3gal_2$	6.7
R6	$galA_3rha_3$ $galA_3rha_2gal_2$	5.2
Total		67.6

The yield for each RGI like oligosaccharide ranged from 5 to 20 mg/g pectin with a total of 67.6 mg/g pectin (Table 5). The yield of each type of compound per run ranged from 47 to 184 mg/g sample load. This yield per sample load is larger than reported for homogalacturonides due to a smaller population of different molecules in the sample. With the employed chromatography conditions, this yield was equivalent to an isolation efficiency of 0.1–0.4 mg/peak h, which was much lower than for the homogalacturonides, but the sample load was never increased in order to increase the efficiency. The yield of R6 was based on an average molecular weight of the two compounds described, since no quantification was possible. According to the mass balance, the RGI permeate accounted for 110 mg/g pectin. Out of this, approximately 60% was thus recovered as defined RGI like molecules. As seen for the homogalacturonides, the chromatographic elution profile of RGI permeate (Fig. 5) indicated the presence of small amounts of RGI like molecules with higher DP than the one observed in R6, but the lack of MS/MS verification prevented these peaks from further investigation and yield calculations.

3.5. *In vitro* fermentation

Quantitative RealTime PCR from *in vitro* fermentations showed differences in the relative ratio of *Bacteroidetes* and *Firmicutes* dependent on the type of substrate. The density of *Bacteroidetes* was significantly lower in both fermentations compared to the

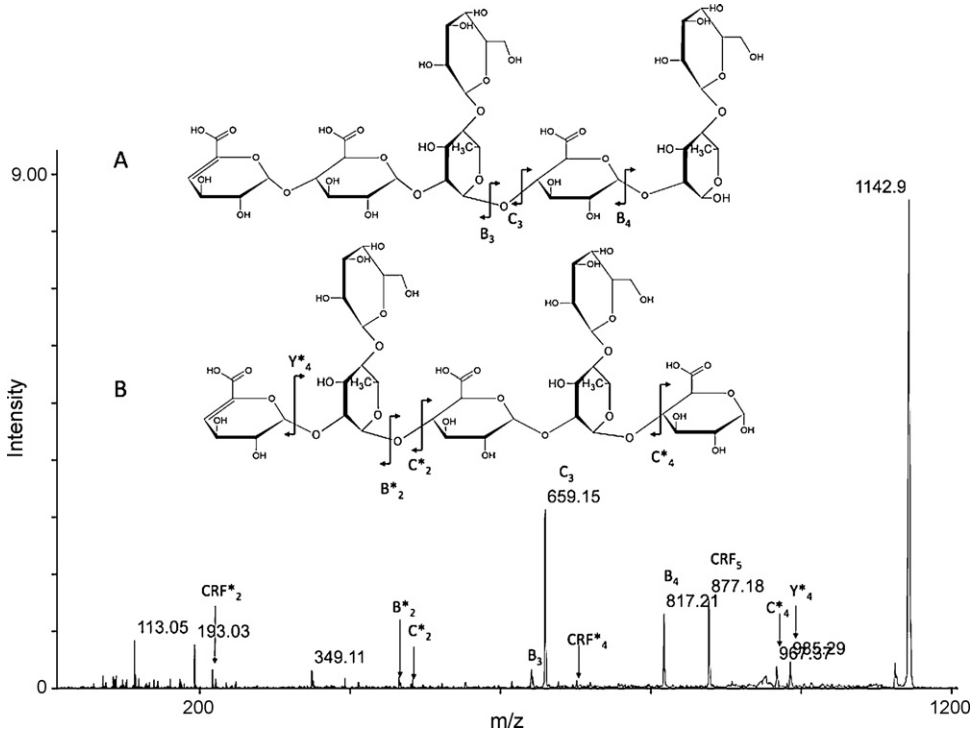

Fig. 8. MS/MS High energy CID spectrum of the two identified $galA_3rha_2gal_2$ structures illustrating the fragmentation pattern and nomenclature, and the proposed structures of the compound with two galacturonic acids in the non-reducing end (A) and the molecule with galacturonic acid on both reducing and non-reducing end (B). The fragments interpreted as resulting from CID of component B is marked with an asterisk.

inocula (DP4 and DP5 compared to inocula; $P < 0.001$ and $P < 0.01$, respectively). The density of *Firmicutes* was significantly higher in the faecal samples fermented on DP4 compared to the inoculum ($P < 0.001$). Surprisingly, fermentation of homogalacturonan oligosaccharides with DP5 yielded a density of *Bacteroidetes* which was significantly higher than obtained by fermentation of DP4 ($P < 0.05$), while conversely, fermentation of the DP4 substrate resulted in densities of *Firmicutes* significantly higher than obtained after fermentation of DP5 ($P < 0.01$) (Fig. 9). No differences between the effects on microbial communities obtained from healthy subjects, UC patients in remission, and UC patients in relapse were detectable.

The two bacterial phyla *Firmicutes* and *Bacteroidetes* together constitute the major part of the intestinal microbiota. The relative increase in one of these phyla may therefore be accompanied by a relative decrease in the other one. It is nevertheless still the balance between the *Bacteroidetes* and *Firmicutes* phyla that is believed to play a role in risk of obesity development [46]. Hence, the potential biological effect of a modified ratio of *Bacteroidetes* and *Firmicutes* might be beneficial with respect to treatment of intestinal inflammation, but needs further investigation.

3.6. Biological evaluation of homogalacturonan oligosaccharides

The literature about prebiotic activities of pectic oligosaccharides is mainly focused on hydrolase-produced oligosaccharides. In this study oligosaccharides with unsaturated non-reducing ends were used. Metabolism of the unsaturated galacturonic acid

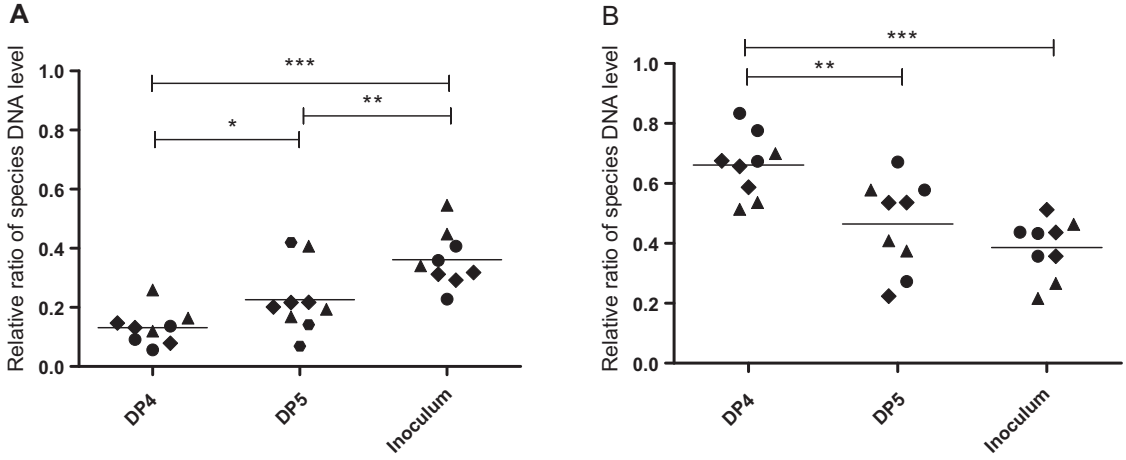

Fig. 9. Relative amounts of target genes in samples from original bacterial communities, and after fermentation of DP4 and DP5 by these microbial communities. Target genes encoded 16S rRNA from *Bacteroidetes* (A) and *Firmicutes* (B). Faecal samples were obtained from three healthy subjects (▲), three ulcerative colitis patients in remission (♦) and three ulcerative colitis patients in relapse (●). The horizontal lines show the mean of the nine observations. Asterisks indicate a significant difference among groups $P < 0.05$ (*); $P < 0.01$ (**); $P < 0.001$ (***).

requires a different pathway than metabolism of saturated galacturonic acid, as described for *Erwinia* and *Pseudonomas* species [47,48], but previous studies by Dongowski and Anger [49] showed that unsaturated pectic oligosaccharides were formed as intermediate metabolites in *in vitro* fermentation of pectin by human faecal microbiota. This indicates that the human gut microbiota is able to metabolize unsaturated galacturonic acid residuesas the data obtained in the present study also suggests. The homogalacturonan oligosaccharides described in this paper (DP2–8, without methyl esterification) are candidates for *in vitro* prebiotic activity based on the literature and will be the subject of our future investigation.

4. Conclusions

The present work provided a new lean method for biorefining of pectin involving the sequential targeted enzyme catalyzed dissection to produce homogalacturonides and rhamnogalacturonides of defined molecular size. The results showed that it was possible to separate individual HG oligomers with DP from 2 to 8, as well as short RGI oligomers by one integrated procedure. The RGI oligomers were separated with respect to the number of repeating backbone units, and also with respect to galactose substitutions. The detailed MS/MS analysis revealed the presence of RGI molecules containing either two consecutive galacturonic acid moieties in the non-reducing end or a galacturonic acid in both reducing and non-reducing end, indicating the covalent link between homogalacturonan and rhamnogalacturonan I in sugar beet pectin. An interesting spin-off result was the finding of the molecular structure containing two consecutive galacturonic acid attached to a rhamnose, which rejects the alternative pectin structure proposed by Vincken et al. [45]. This integrated method provides an option for production of at least milligram quantities of high purity HG and RGI oligosaccharides of defined size and structure. The production of larger amounts and/or industrial scale production by use of this methodological strategy may require further optimization with respect to the cost of the chromatographic downstream processing. The availability of well defined structures makes it possible to examine and develop an improved understanding of the functionality and possible bioactivity of pectin derived structural elements. An important further direction may also be the comparison of the putative prebiotic activity of unsaturated vs. saturated pectic oligosaccharides. *In vitro* assessment of the influence of DP4 and DP5 homogalacturonides on the relative abundance of two different bacterial phyla in faecal microbial communities revealed a significantly different effect of the two slightly different structures, which opens a range of possibilities for further research addressing the effect of oligosaccharide length on the metabolic syndrome associated with obesity, inflammatory bowel disease and prebiotic potential.

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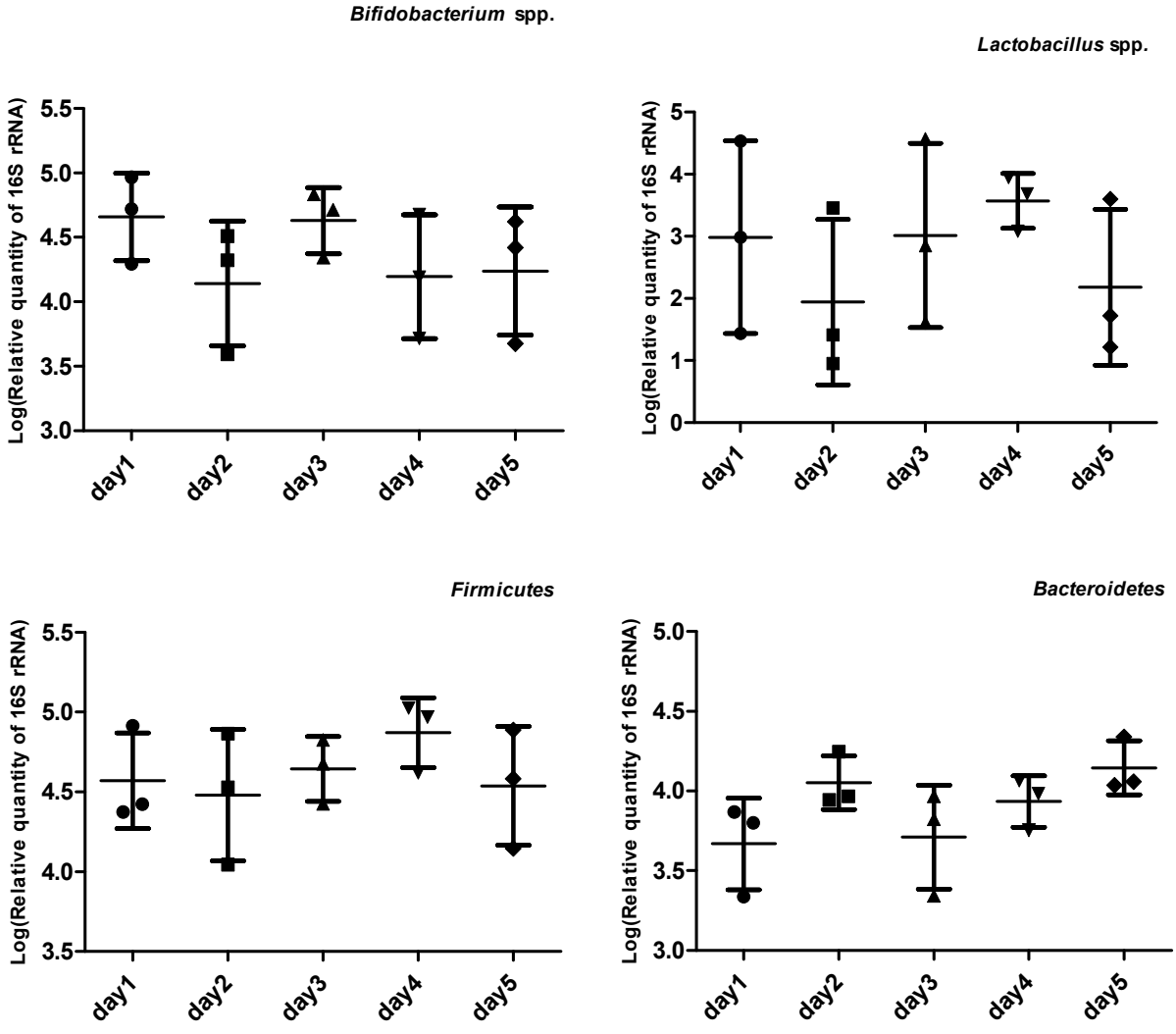
Methodology appendixes

Appendix 1

Quantification of bacterial taxa at five different occasions

Quantitative Real-Time PCR was performed using primers targeting *Firmicutes* (Guo *et al.*, 2008), *Bacteroidetes* (Guo et al., 2008)*, Bifidobacterium* spp. (Delroisse *et al.*, 2008) and *Lactobacillus* spp. (Walter *et al.*, 2000;Heilig *et al.*, 2002). DNA extraction and qPCR procedure are performed as described in Paper 2-6.



Relative quantities of gene targets encoding 16S rRNA from *Bifidobacterium* spp., *Lactobacillus* spp., *Firmicutes* and *Bacteroidetes* in fermentation samples incubated with FOS for 24 hours at five different occasions (day1-day5). Fecal samples were obtained from three healthy subjects. The

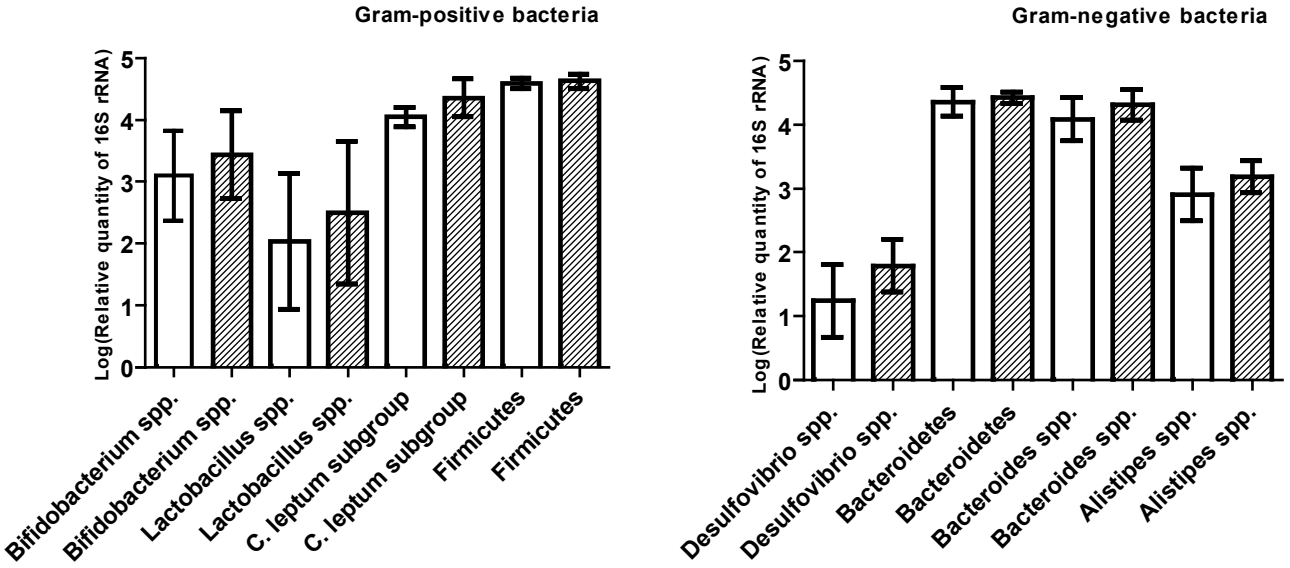
horizontal lines show the mean of the three observations and the error bars represent standard deviation for the three healthy subjects.

The data was log-transformed before statistical analysis to obtain normal distribution. Univariate ANOVA was used to analyze difference in days. Tests were considered statistically significant if P-values lower than 0.05 were obtained.

Appendix 2

Quantification of eight bacterial taxa to validate the combined DNA extraction method

Quantitative Real-Time PCR was performed using primers targeting *Bifidobacterium* spp. (Delroisse *et al.*, 2008), *Lactobacillus* spp. (Walter *et al.*, 2000;Heilig *et al.*, 2002), *Coccoides leptum* subgroup (Walter *et al.*, 2000;Matsuki *et al.*, 2004;Shen *et al.*, 2006), *Firmicutes* (Guo *et al.*, 2008), *Desulfovibrio* spp. (Fite *et al.*, 2004), *Bacteroidetes* (Guo et al., 2008), *Bacteroides* spp. (Ahmed *et al.*, 2007), *Alistipes* spp. (Paper 1) and. DNA extraction and qPCR procedure are performed as described in Paper 2-6.



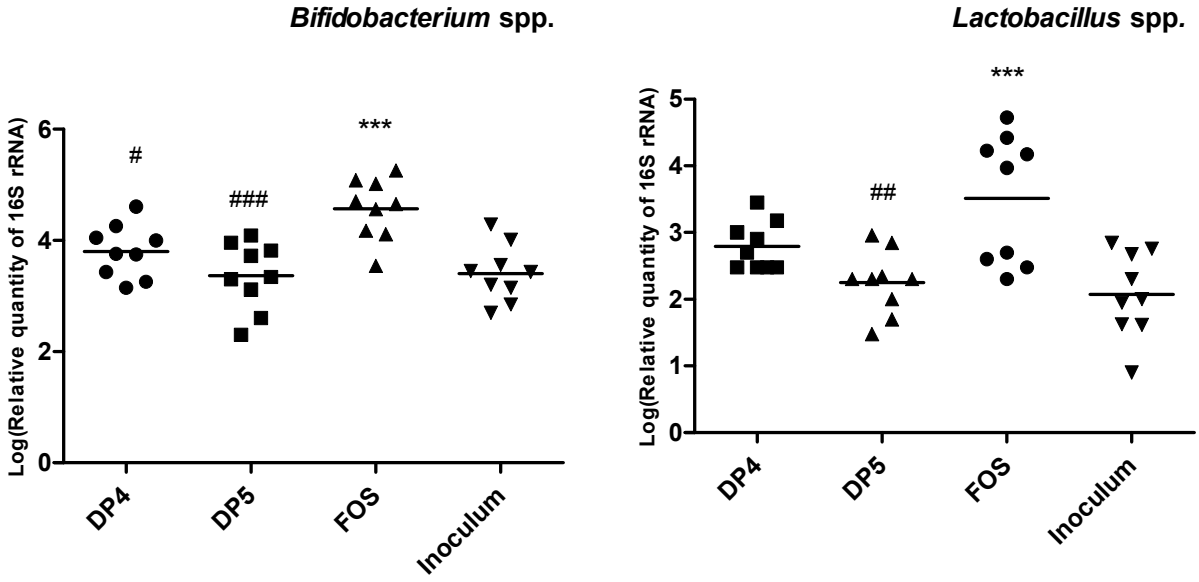
Relative quantities of gene targets encoding 16S rRNA from four Gram-positive bacteria and four Gram-negative bacteria in either 0 hours fermentation samples (white) or fecal samples (hatched). Samples were obtained from three healthy subjects. Error bars represent averages ± standard deviation of the three different subjects.

The data was log-transformed before statistical analysis to obtain normal distribution. Two-tailed pair wise t-test was used to analyze difference in samples. Tests were considered statistically significant if P-values lower than 0.05 were obtained.

Appendix 3

Additional results for Paper 6: Quantification of *Lactobacillus* spp. and *Bifidobacterium* spp. from fermentation samples incubated with DP4, DP5 and FOS.

Quantitative Real-Time PCR was performed using group specific primers targeting *Bifidobacterium* spp. (Delroisse *et al.*, 2008) and *Lactobacillus* spp. (Walter *et al.*, 2000;Heilig *et al.*, 2002). DNA extraction and qPCR procedure are performed as described in Paper 6.



Relative quantities of gene targets encoding 16S rRNA from *Bifidobacterium* spp., *Lactobacillus* spp. in inoculum and fermentation samples incubated with DP4, DP5 and FOS for 24 hours. Fecal samples were obtained from three healthy subjects, three ulcerative colitis patients in remission and three in relapse. The horizontal lines show the mean of the nine observations. Asterisks indicate a significant difference from inoculum: P<0.05 (*), P<0.01 (**), P<0.001 (***), while pound signs (#) indicate significant differences from FOS fermented samples: P<0.05 (#), P<0.01 (##), P<0.001 (###).

For statistical analysis, the nine samples were analyzed independent of health status. The data was log-transformed before statistical analysis to obtain normal distribution. Univariate ANOVA was used to analyze difference in treatment. When ANOVA indicated a significant difference, a post

hoc test, Tukey's multiple comparison test was performed. Tests were considered statistically significant if P-values lower than 0.05 were obtained.

The results revealed that DP4 and DP5 were unable to significantly stimulate the growth of bifidobacteria and lactobacilli after 24 hours of incubation. However, incubation with FOS significantly increased the levels of bifidobacteria and lactobacilli. The level of bifidobacteria was significantly lower after DP4 and DP5 fermentation compared to FOS. This was also applicable for the level of lactobacilli, but only for DP5.

Appendix 3

Discussion and conclusion

7. Discussion and perspectives

7.1. Compositional changes of the fecal microbiota and their capacity to colonize mucus

The commensal bacteria found in the human gut are important for host health, and maintenance of tolerance toward the intestinal bacteria by the host is critical to preserve this health. It is believed that the interaction between host and commensal bacteria is disturbed in UC and this may have a consequence for the microbiota composition of the gut (Sartor, 2006). Several studies have previously examined the microbial composition in UC patients and found that it differs from that of healthy subjects (Ott *et al.*, 2004;Mylonaki *et al.*, 2005;Frank *et al.*, 2007;Takaishi *et al.*, 2008;Sokol *et al.*, 2009;Qin *et al.*, 2010). Among these studies, only a few have used samples from both UC patients in remission and relapse for comparison (Mylonaki *et al.*, 2005;Takaishi *et al.*, 2008;Sokol *et al.*, 2009).

In Paper 1, fecal samples from UC patients in remission or relapse, and healthy subjects were analyzed by PCR-DGGE fingerprint and qPCR based principal component analysis (PCA). The results revealed that UC patients in relapse appeared to have a different fecal microbiota than healthy subjects and this difference could be ascribed to the Gram-negative bacteria. A potent stimulator of inflammation from Gram-negative bacteria is LPS, hence an altered composition of Gram-negative bacteria could add to abnormal LPS signaling enhancing mucosal inflammation in UC. Adding to this, the overall bacterial changes observed in UC patients in relapse described both in Paper 1 and in literature (Mylonaki *et al.*, 2005;Takaishi *et al.*, 2008;Sokol *et al.*, 2009) could further promote intestinal inflammation, since the changed composition of commensal bacteria may be unable to sufficiently induce regulatory immune responses, hence incapable of terminating inflammation (Geuking *et al.*, 2011). It should however be noted that the bacterial changes could be a consequence of intestinal inflammation and not the cause, due to hyper-responsiveness of the mucosal immune system towards commensals through mutations and/or over-expression of TLRs (De Jager *et al.*, 2007;Frolova *et al.*, 2008;Fuse *et al.*, 2010).

The results from Paper 1 also revealed that some samples from patients in remission resembled those of patients in relapse, while others resembled those of healthy subjects indicating that the level of some bacterial groups derived from UC patients in remission is similar to healthy subjects, while others is similar to UC patients in relapse. This is in accordance with previous

communications (Takaishi *et al.*, 2008;Sokol *et al.*, 2009), and could indicate that the commensal microbiota in remission is unstable and unfavorable events could contribute to microbial compositional changes provoking flare-ups. However, further longitudinal studies are needed to obtain sufficient information both microbiological and immunological on this area, hence helping elucidate if the microbial compositional changes are causal to or consequence of disease.

The ability of the fecal microbiota derived from UC patients in either remission or relapse and healthy subjects to colonize an artificial mucus layer was examined in Paper 2. The results from this study demonstrated that the mucosal microbial community differs from that of the lumen, whether the microbiota was derived from healthy subjects, UC patients in remission or relapse. These findings are in agreement with previous *in vivo* (Zoetendal *et al.*, 2002;Macfarlane, 2008) and *in vitro* studies (Macfarlane *et al.*, 2005;Van den Abbeele *et al.*, 2011a). Additionally, the results from Paper 2 revealed that bifidobacteria and lactobacilli from UC patients in relapse were found in significantly lower levels in mucus than when derived from healthy subjects. However, the question is, whether the low levels of adhered lactic acid bacteria from UC patients in relapse are a result of low baseline levels in feces or decreased capacity of the bacteria to adhere? Based on results from Paper 1 (using some of the same individuals as in Paper 2), it was demonstrated that the level of fecal *Lactobacillus* spp. was significantly lower in UC patients in relapse than in healthy subjects. However, no significant difference was observed for the fecal levels of *Bifidobacterium* spp.. This could imply that lactic acid bacteria derived from UC patients in relapse have decreased capacity to colonize the mucus independent of baseline levels in feces. The decreased capacity of bifidobacteria and lactobacilli to colonize the mucus could be due to changed expression of adhesion molecules. Adhesion-promoting molecules have been observed in species of *Lactobacillus* and *Bifidobacterium* including fimbriae (Pridmore *et al.*, 2004;Kankainen *et al.*, 2009;Gilad *et al.*, 2011), Msa, mannose-specific adhesin protein (Pretzer *et al.*, 2005), MucBP domain containing proteins (Kleerebezem *et al.*, 2010), and elongation factor (EF-Tu) (Granato *et al.*, 2004;Gilad *et al.*, 2011). However, proteomic analyses of bifidobacteria or lactobacilli isolated from UC patients have to my knowledge not previously been conducted to reveal, if changes in bacterial phenotypes occur during mucosal inflammation.

The decreased capacity to colonize mucus could also be due to intra-species variations within the lactic acid bacteria genera. In Paper 1 and 2, different species of *Bifidobacterium* were quantified

using SYBR-Green based assays with primers targeting either 16S rRNA gene sequences or 16S-23S rRNA intergenic spacer regions. The primers were selected based on sensitivity and specificity, which led to a limited number of *Bifidobacterium* species target. However, quantification of numerous species of lactic acid bacteria found in the human gut is needed to draw any conclusions on intragenic compositional changes from individual samples. The design of primers may give some problems when targeting species within a genus, since primer specificity and sensitivity can be difficult to obtain. Even though 16S rRNA gene sequences can be used for species identification and quantification ((Matsuki *et al.*, 1998;Byun *et al.*, 2004;Matsuki *et al.*, 2004) and Paper 1 – 2), they have some limitations in discriminating species of related taxa or within the same genus, due to high 16S rRNA sequence similarities (Fox *et al.*, 1992). Protein-coding genes or 16-23S rRNA intergenic spacer regions may be a better choice, since both exhibit higher sequence variations than 16S rRNA gene sequences (Saikaly *et al.*, 2007). Previous studies have used 16S-23S rRNA intergenic spacer regions (Haarman and Knol, 2005), as well as Paper 1 or *recA* (Masco *et al.*, 2007) to quantify species within *Bifidobacterium* showing high specificity.

The low levels of lactic acid bacteria derived from UC patients found in the artificial mucus layer in Paper 2 have previously been described for bifidobacteria from mucus layer of biopsy specimens from UC patients (Macfarlane *et al.*, 2004;Mylonaki *et al.*, 2005). As mentioned above, the results from Paper 1 demonstrated low abundances of fecal lactobacilli in UC patients in relapse than in healthy subjects, but no significant difference was observed for the fecal bifidobacteria. Low levels of fecal bifidobacteria in UC patients in relapse have previously been described by Sokol *et al.* (2009). The discrepancy in results could be due to differences in the amount of participants in the two studies: Paper 1 is based upon six participants in each group whereas the study by Sokol *et al.* (2009) covers thirteen UC patients in replase and twenty seven healthy controls. The low amount of participants in Paper 1 is a weakness in the study, since it gives a low confidence interval (high margin of error); hence is not as representative for a true population, as when using a high amount of participants. Additionally, due to high inter-individual variations significant power can be difficult to reach.

However, when comparing Paper 1 to previous communications (Takaishi *et al.*, 2008;Qin *et al.*, 2010;Arumugam *et al.*, 2011), several similar tendencies can be found, which justifies the results of the study.

The use of fecal samples to explore the colonic bacterial community in humans has been widely used, since these samples are easy to collect without invasive procedures compared to colonic biopsies (Takaishi *et al.*, 2008;Sokol *et al.*, 2009;Qin *et al.*, 2010;Larsen *et al.*, 2010). Nevertheless, the fecal microbiota may not be representative for the colonic mucosal microbiota, given that literature has revealed that the two microbiota communities differ from that of the others (Zoetendal *et al.*, 2002;Eckburg *et al.*, 2005;Turroni *et al.*, 2009). In view of examining involvement of the intestinal microbiota in UC pathogenesis, the mucosal microbiota could be more relevant to study, due to their closer interaction with the epithelium (Derrien *et al.*, 2011;Van den Abbeele *et al.*, 2011b). However, owing to sampling restrictions, feces were analyzed in paper 1 to compare microbiota derived from healthy subjects and UC patients.

7.2. Modulation of the fecal microbial community from UC patients

Bifidobacteria and lactobacilli are believed to play an important role in promoting intestinal health and species of both genera have shown to offer anti-inflammatory effects by down-regulation of pro-inflammatory cytokines and induction of Treg cells (Hoarau *et al.*, 2006;Peran *et al.*, 2007;Ghadimi *et al.*, 2010). A reduced level of lactobacilli and bifidobacteria could compromise the colon health and favor intestinal inflammation. Hence selective stimulation of lactic acid bacteria by prebiotics could be an approach to help decrease risk of flare-ups in UC patients. In Paper 3, fermentation-induced changes in fecal microbial communities obtained from either healthy subjects and UC patients in remission or relapse were investigated using arabino-oligosaccharides (AOS; DP2-10) derived from sugar beet pulp. The potential prebiotic properties of AOS was compared to FOS (classified prebiotic (Rastall, 2007)). Incubation with AOS and FOS gave rise to selective stimulation of bifidobacteria and lactobacilli in fecal microbial communities derived from UC patients. The ability of *Bifidobacterium* spp. and *Lactobacillus* spp. to utilize AOS or FOS and compete for substrate could be ascribed the presence of GH such as α-L-arabinofuranosidases (EC 3.2.1.55), and/or fructan β-(2,1)-fructosidase (EC 3.2.1.153) in species of these genera (http://www.cazy.org/ and http://www.uniprot.org/uniprot/, 2011-10-11). No human trials or *in vivo* studies have been reported examining the protective effect of AOS against colitis. However, the protective effect of FOS has been demonstrated in animals with induced colonic inflammation. The studies showed that consumption of FOS resulted in decreased severity of colonic damage and increased amount of colonic lactobacilli and bifidobacteria (Cherbut *et al.*, 2003;Lara-

Villoslada *et al.*, 2006;Rodriguez-Cabezas *et al.*, 2010). Additionally, Casellas *et al.* (2007) demonstrated that a mixture of FOS and inulin reduced disease activity in humans with UC after prebiotic consumption.

The decreased levels of *C. coccoides* group, *C. leptum* subgroup and *Desulfovibrio* spp. after AOS and FOS incubation implied that these bacterial groups are not able to utilize AOS or FOS (paper 3). This is in agreement with results from database search of gene sequences that revealed that only few species of *C. coccoides* group, *C. leptum* subgroup and *Desulfovibrio* spp. contain α-L-arabinofuranosidases (EC 3.2.1.55), and/or fructan β-(2,1)-fructosidase (EC 3.2.1.153) (http://www.cazy.org/, 2011-10-11). Though, it was expected based on cross-feeding studies (Duncan *et al.*, 2003;Belenguer *et al.*, 2006;Marquet *et al.*, 2009) that metabolites produced (e.g. acetate and lactate) during the fermentation of FOS and AOS could stimulate the growth of *C. coccoides* group, *C. leptum* subgroup and *Desulfovibrio* spp., however that was not observed in the study of paper 3. Hence, *C. coccoides* group, *C. leptum* subgroup and *Desulfovibrio* spp. are not able to compete in a fermentor community with AOS and FOS as primary carbohydrate sources. In addition, the inability of competing in the fermentor community with FOS and AOS was observed for *Bacteroidetes.* This was in line with previous communication, where species of *Bacteroides* have shown to be unable to utilize AOS (DP2-5) and could only partly degrade FOS (Van Laere *et al.*, 2000), even though genome sequencing has reveal that species of *Bacteroides* contain GH such as α-L-arabinofuranosidases (EC 3.2.1.55), arabinases (EC 3.2.1.99) and/or fructan β-(2,1)-fructosidase (EC 3.2.1.153) (http://www.cazy.org/, 2011-10-22).

Since feces is suggested to mainly consist of bacteria shed from the mucus or washed out from the lumen (Kurokawa *et al.*, 2007;Booijink *et al.*, 2007), both luminal and mucosal communities of lactobacilli and bifidobacteria may have been stimulated after incubation of AOS and FOS in Paper 3. Previous work by Van den Abbeele *et al.* (2011c) has shown that prebiotic candidates such as long-chain arabinoxylans can stimulate bifidobacteria both in the mucus and lumen of humanized rats with *Bifidobacterium longum* being the most dominant *Bifidobacterium* species of both communities after prebiotic treatment.

Collectively, the results from Paper 3 suggest that the positive effect already observed in human and animal trials using FOS may be applicable also for AOS. Hence presence and fermentation of AOS may help prevent pro-inflammatory conditions and enhance resistance to colonization of

opportunistic pathogens; however *in vivo* studies or human trials are needed to confirm the ability of AOS to stimulate the growth of beneficial bacteria both in the lumen and mucus and through this able to promote health effects against colitis.

7.3. Validation of prebiotic properties of novel carbohydrates

In Paper 4 and 5, novel carbohydrate preparations were tested in human fecal *in vitro* systems for prebiotic properties using FOS as prebiotic standard. The studies revealed that membrane separated AOS (DP7-14) either feruloylated or nonferuloylated from sugar beet pectin (paper 4) and enzymatically produced β-1,4-galactan from potato pulp (paper 5) could stimulate the growth of bifidobacteria to the same extent or even higher than FOS after 24 hours of fermentation.
In line with the results from Paper 4, bifidogenic effect of AOS (different chain length, DP2-10) has been described *in vitro* in previous communications (fecal samples from healthy subjects) (Al-Tamimi *et al.*, 2006;Hotchkiss *et al.*, 2010) and Paper 3 (fecal samples from UC patients). It was expected that the feruloylated AOS from Paper 4 could selectively increase the growth of lactobacilli and bifidobacteria, because species of these genera have shown to be able to specifically express feruloyl esterase (Wang *et al.*, 2005b;Szwajgier and Dmowska, 2010). However, the data revealed that the presence of feruloyl substitutions did not seem to be of significant importance for the bifidogenic effect of AOS, but rather the molecular weight with high molecular weight AOS inducing growth of bifidobacteria.
The results from Paper 5 demonstrated selective stimulation of bifidobacterial growth on high molecular weight β-1,4-galactan from potato pulp. Literature has previous described that pure culture species of *Bacteroides*, *Bifidobacterium* and *Lactobacillus* are able to utilize arabinogalactan-enriched polysaccharides (Van Laere *et al.*, 2000) and in agreement with this, database search (http://www.cazy.org/, 2011-10-22) has revealed that species of *Bacteroides*, *Bifidobacterium* and *Lactobacillus* contain β-1,4-galactanase (EC 3.2.1.89); an enzyme that catalyzes the degradation of β-1,4-galactan. This indicates that even though several genera are able to utilize and grow on galactan, they may not be able to compete in mixed culture systems to the same extent as bifidobacteria.
Paper 6 demonstrated that unsaturated oligogalacturonides (DP4 and DP5) from sugar beet HG were able to significantly alter the relative abundance of *Firmicutes* and *Bacteroidetes* using human fecal *in vitro* systems. *Firmicutes* and *Bacterioidetes* constitute the major part of the

intestinal microbiota (Eckburg *et al.*, 2005;Arumugam *et al.*, 2011) and dysbiosis in the *Firmicutes/Bacteroidetes* ratio has been associated with intestinal colitis (Sokol *et al.*, 2009). Thus, it could be speculated that a modified ratio of *Firmicutes* and *Bacterioidetes* could be beneficial with respect to mucosal inflammation. However, in addition to stimulate bifidobacteria and lactobacilli, the oligogalacturonides were unable to significantly induce growth (Appendix 3), which meant that short chains of HG lack prebiotic properties.

Collectively, the results from Paper 4-6 demonstrate that novel carbohydrate preparations derived from sugar beet and potato pulp may have prebiotic properties depending on monosaccharide composition and molecular weight by selectively stimulating the growth of bifidobacteria. The fact that it was the high molecular weight carbohydrates that stimulated bifidobacteria *in vitro* is a desirable attribute *in vivo*, since long-chain carbohydrates may be slowly fermented by beneficial bacteria in the colon, thus allowing penetration of prebiotic effect all the way throughout the colon. However, further *in vivo* studies are needed to confirm prebiotic properties of these carbohydrate preparations and their persistence throughout the colon.

8. Conclusion

The studies of this thesis add to our understanding of the intestinal microbial differences in healthy subjects and UC patients in respect to microbiota composition and which role it may play in colonic inflammation and in addition, the prebiotic properties of novel carbohydrate preparations on beneficial changes of gut microbiota.

The knowledge gained in this thesis helps to lead to these major conclusions:

- The microbiota in UC patients in relapse differs from that of healthy subjects.
- UC can be correlated with the composition of Gram-negative bacteria.
- The adhesion capacity of beneficial Gram-positive bacteria such as lactobacilli and bifidobacteria is disease dependent.
- AOS derived from sugar beet pulp may represent a new prebiotic candidate for helping UC patients to maintain remission.
- Poly- and oligosaccharides derived from sugar beet or potato pulp display prebiotic properties depending on monosaccharide composition with high molecular weight fractions of galactose (potato) or arabinose (sugar beet) chains being most bifidogenic.

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