Effects of selected non-digestible dietary carbohydrates on the composition of the large intestinal microbiota and susceptibility to salmonella infections

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EFFECTS OF SELECTED NON-DIGESTIBLE DIETARY CARBOHYDRATES ON THE COMPOSITION OF THE LARGE INTESTINAL MICROBIOTA AND SUSCEPTIBILITY TO *SALMONELLA* INFECTIONS

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PREFACE AND ACKNOWLEDGEMENTS

The present thesis presents the studies carried out during my three years enrolled as a Ph.D. student at the Division of Microbiology and Risk Assessment, National Food Institute, Technical University of Denmark (DTU).

The studies were carried out as a part of the PreGI project (Prebiotics for Prevention of Gut Infections) in the group "Applied microorganisms, diet and gut microbiology", led by Professor Tine R. Licht, in close collaboration with the Division of Toxicology and Risk Assessment, National Food Institute, DTU. Other collaborators were the Department of Systems Biology, DTU, the National Veterinary Institute, DTU and Danisco Health and Nutrition, Kantvik, Finland. Furthermore, a part of the study was carried out at the Commonwealth Scientific and Industrial Research Organisation (CSIRO), Food and Nutritional Sciences, Adelaide, Australia.

The main supervisor on the project was Professor Tine R. Licht, Division of Microbiology and Risk Assessment, and co-supervisor was Senior Scientist Morten Poulsen, Division of Toxicology and Risk Assessment. The project was funded by The Danish Council for Strategic Research

I would like to sincerely thank supervisor Tine R. Licht for guiding me through three years of research with encouraging enthusiasm and for guidance through the process of writing scientific papers. I thank co-supervisor Morten Poulsen for valuable help in planning and conduction the animal experiments. For scientific inputs a special thanks is given to Sampo J. Lahtinen, Health & Nutrition Platform Manager at Danisco and to Professor Hanne Frøkiær and Senior Scientist Andrea Wilcks.

Also, a grateful thanks to technicians Bodil Madsen and Kate Vibefeldt for their help through the experimental part of the study. Furthermore, I wish to thank all members of the group "Applied microorganisms, diet and gut microbiology" for creating a good working environment and all the staff at the Division of Microbiology and Risk Assessment for a warm and friendly atmosphere.

Last, but not least, a special thanks to Tine, Matilde, Louise, Lene and Karolina for their support during my years of studying and for keeping up the good spirit at the office.

Mørkhøj, April 2010
Anne Petersen

SUMMARY

The mammalian intestinal tract is a complex ecosystem colonised by a high and diverse number of commensal bacterial. Bacteria colonising the intestinal tract have a profound impact on host health e.g. by acting as a barrier against colonisation by pathogens and by contributing to digestion of complex food components. In this regard there is a considerable interest in dietary components that can modulate the gut microbiota and potentially improve gut health.

Some gut bacteria, known as probiotics, are belived to improve gut health upond ingestion, whereas non-digestible (ND) dietary carbohydrates, known as prebiotics, are food components aimed at selectively stimulating such beneficial bacteria already colonizing the intestinal tract. In this regard, prebiotics and other ND dietary carbohydrates may improve host resistance to intestinal infections by selectively modulating the composition of the gut microbiota or by stimulating the immune response.

Salmonella is a genus of Gram-negative bacteria that are a major cause of food-borne illness globally. Several studies with probiotics have demonstrated protective effects against murine *Salmonella* infections, while studies with prebiotics have shown conflicting results. Therefore the aim of the present thesis was to investigate the effect of selected ND dietary carbohydrates on the large intestinal microbiota and susceptibility to *Salmonella enterica* serovar Typhimurium SL1344 infection in mice.

The thesis contains an introduction to the digestive function of the gastrointestinal tract and the associated microbiota, followed by a description of dietary strategies for modulation of the intestinal microbiota with particular emphasis on effects on *Salmonella* infections. Subsequently, three manuscripts are presented based on the experimental studies performed.

Results presented in Manuscript I demonstrated no *in vivo* protective effect of the investigated carbohydrates against the *Salmonella* infection. In contrast, two of the investigated substrates (fructo-oligosaccharides and xylo-oligosaccharides) demonstrated an adverse rather than a protective effect against the infection.

Manuscript II investigated diet-induced changes in the large intestinal microbiota of mice exhibiting a reduced resistance to the *Salmonella* infection. Diets supplemented with fructo-oligosaccharides or xylo-oligosaccharides induced a number of microbial changes in the faecal microbiota including an increase in the *Bacteroidetes* phylum, the *Bacteroides fragilis* group and in *Bifidobacterium* spp., while reductions were observed in the *Firmicutes* phylum and the *Clostridium coccoides* group. The findings thus suggest that some microbial changes in the large intestine may increase the infectious potential of *Salmonella*.

The last study, presented in Manuscript III, was performed during a research stay at CSIRO Food and Nutritional Sciences, Australia. In this study a two-stage continuous fermenter was used to determine if incubating human faeces with xylo-oligosaccharides (XOS) lowers faecal water genotoxicity induced by protein fermentation. XOS fermentation was seen to reduce faecal water genotoxicity in vessel 1, but to increase the genotoxicity in vessel 2. Butyrate concentrations were significantly elevated in both vessels and could be related to an increase in the *C. coccoides* group. Other microbial changes observed, including a reduction in *Bifidobacterium* spp. and sulphate-reducing bacteria, suggest that quantities of some bacterial species are related to changes in faecal water genotoxicity.

Conclusively, the studies contribute to our knowledge of the effect of some ND dietary carbohydrates on the composition of the large intestinal microbiota and the effect such changes may have on the susceptibility to *Salmonella* infections or the risk of developing colon cancer.

SAMMENDRAG (DANISH SUMMARY)

Tarmkanalen hos pattedyr er et komplekst økosystem koloniseret af et højt og mangfoldigt antal naturligt forekommende bakterier. Bakterier, der koloniserer tarmkanalen har en afgørende betydning for værtens helbred f.eks. ved at fungere som en barriere mod kolonisering af patogene bakterier og ved at bidrage til fordøjelse af komplekse fødevarekomponenter. I denne forbindelse er der en betydelig interesse for fødevareingredienser, der kan ændre på sammensætningen af tarmmikrobiota og potentielt forbedre tarmmiljøet.

Indtagelse af nogle tarmbakterier, såkaldte probiotika, menes at have en gunstig effekt på tarmmiljøet, mens ufordøjelige kulhydrater, såkaldte præbiotika, er fødevareingredienser udviklet specielt til selektivt at stimulere sådanne gavnlige bakterie, som findes naturligt i tarmen. Præbiotika og andre ufordøjelige kulhydrater i kosten menes således at kunne reducere værtens modtagelighed for tarminfektioner ved selektivt at ændre på sammensætningen af tarmens mikrobiota eller ved at stimulere immunforsvaret.

Salmonella er en slægt af Gram-negative bakterier, der på verdensplan udgør en væsentlig årsag til fødevarerelaterede sygdomsudbrud. Flere studier har vist en forebyggende effekt af probiotika mod murine *Salmonella* infektioner, mens studier med præbiotika har vist modstridende resultater. Formålet med nærværende afhandling var således at undersøge effekter af udvalgte ufordøjelige kulhydrater på mikrobiotaen i tyktarmen og på modtageligheden for *Salmonella enterica* serovar Typhimurium SL1344 infektioner hos mus.

Afhandlingen omfatter en introduktion til mave-tarmkanalens fordøjelsesfunktion og den tilhørende mikrobiota. Efterfølgende gives en beskrivelse af kost-strategier udviklet med henblik på at ændre på sammensætningen af tarmmikrobiotaen med særligt fokus på studier omhandlende effekter på *Salmonella* infektioner. Afslutningsvist præsenteres resultaterne af de gennemførte eksperimentelle studier i tre manuskripter.

Resultater af fodringsforsøg med ufordøjelige kulhydrater (Manuskript I) viste ingen *in vivo* forebyggelse af *Salmonella* infektionen. Derimod resulterede fodring med to af de testede kulhydrater (frukto-oligosakkarider og xylo-oligosakkharider) i en øget modtagelighed for infektionen. Effekter af disse kulhydrater på sammensætningen af tarmmikrobiotaen blev undersøgt i Manuskript II. Foder indeholdende frukto-oligosakkarider eller xylo-oligosakkharider medførte en række ændringer i mikrobiotaen i fæces, herunder en stigning i *Bacteroidetes*, *Bacteroides fragilis* gruppen og i *Bifidobacterium* spp. samt en reduktion i *Firmicutes* og *Clostridium coccoides* gruppen. Resultaterne antyder således, at visse bakterielle ændringer i tyktarmens mikrobiota kan øge *Salmonellas*' infektionspotentiale.

Det sidste studie (Manuskript III) blev gennemført under forskningsopholdet ved CSIRO Food and Nutritional Sciences, Australien. Studiet omfattede anvendelsen af et to-trins fermenteringssystem til undersøgelse af, om xylo-oligosakkharider (XOS) ved inkubering med humant fæces kan reducere det genotoksiske potentiale af fækalt vand induceret af protein fermentering. XOS fermentering blev vist at reducere genotoksiciteten i den første fermentor, mens studiet viste en øget genotoksitet for den efterfølgende fermentor. Koncentrationen af butyrat var signifikant forøget i begge fermentorer og kunne relateres til en øget forekomst af *C. coccoides* gruppen. Andre ændringer i den bakterielle population, herunder en reduktion i *Bifidobacterium* spp. og sulfat-reducerende bakterier antydede, at forekomsten af visse bakteriearter kan relateres til ændringer i genotoksiteten af fækalt vand.

Samlet set, bidrager de gennemførte studier til viden om effekter af indtagelse af visse ufordøjelige kulhydrater på sammensætningen af mikrobiotaen i tyktarmen og hvilke konsekvenser dette kan have for modtageligheden for *Salmonella* infektioner eller risikoen for at udvikle tyktarmskræft.

ABBREVIATIONS

AGA	Apiogalacturonan
aps	Adenosine-5-phosphosulfate reductase gene
ATP	Adenosine triphosphate
CFU	Colony forming units
CSIRO	Commonwealth Scientific and Industrial Research Organisation
DGGE	Denaturing Gradient Gel Electrophoresis
dsr	Dissimilatory sulfite reductase gene
DP	Degree of polymerization
EPEC	Enteropathogenic *E. coli*
FAE	Follicle associated epithelium
FOS	Fructo-oligosaccharide
GalpA	Galacturonic acid
GI	Gastrointestinal
GOS	Galacto-oligosaccharide
Hb	Haemoglobin
HG	Homogalacturonan
IBD	Inflammatory bowel diseases
Ig	Immunoglobulin
LPF	Long polar fimbria
MLN	Mesenteric lymph nodes
NK cell	Natural killer cell
NKT cell	Natural kille T cell
ND	Non-digestible
PDX	Polydextrose
p.i.	Post-infection
RG	Rhamnogalacturonan
RNI	Reactive nitrogen intermediate
ROS	Reactive oxygen specie
RS	Resistant starch
SCFA	Short chain fatty acid
SCV	Salmonella containing vacuole
SD	Standard deviation
SEM	Standard error of the mean
***S.* Enteritidis**	*S. enterica* serovar Enteritidis
***S.* Paratyphi**	*S. enterica* serovar Paratyphi
SPI	Salmonella pathogenicity island
SRB	Sulphate-reducing bacteria
***S.* Typhi**	*S. enterica* serovar Typhi
***S.* Typhimurium**	*S. enterica* serovar Typhimurium
T3SS	Type-III-secretion-system
Th cell	T-helper cell
TOS	Transgalactosylated oligosaccharide
XGA	Xylogalacturonan
XOS	Xylo-oligosaccharide

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1. THE HUMAN GASTROINTESTINAL SYSTEM AND THE ASSOCIATED MICROBIOTA

Digestion of food and absorption of nutrients is a complex process performed by the gastrointestinal (GI) system (Figure 1). The system is composed of the GI tract, the saliva glands, the liver (producing bile), the gall bladder (stores and secretes bile) and the pancreas. The GI tract extents from the mouth to the anus covering the oral cavity, the esophagus, the stomach, the small intestine (the duodenum, the jejunum and the ileum), the cecum and the colon (the ascending colon, transverse colon, descending colon and sigmoid rectum) [1].

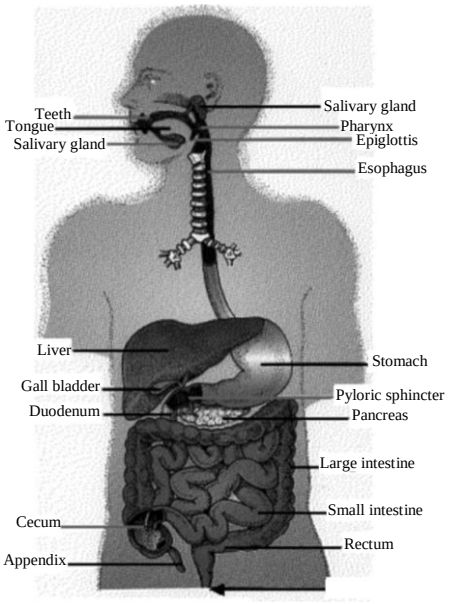

Figure 1. The human gastrointestinal system [1].

Microorganisms colonizing the intestinal tract have a profound impact on human health e.g. by acting as a barrier against pathogens and by contributing to degradation of complex food components resulting in the release of energy sources important for host health (e.g. short chain fatty acids) [2]. Some gut bacteria can be used to improve gut health. Probiotics (section 3.1) are microorganisms (most commonly bacteria of the genera *Lactobacillus* and *Bifidobacterium*) that upon ingestion exert beneficial effects on gut health, whereas non-digestible (ND) dietary carbohydrates known as prebiotics (section 3.2) are food components aimed at selectively stimulating beneficial bacteria already colonizing the intestinal tract [3].
The following section gives an introduction to the human digestive system and the associated microbiota. Subsequently, dietary strategies for modulation of the intestinal microbiota and possible benefits for host health are described with particular emphasis on effects on *Salmonella* infections.

1.1 THE DIGESTIVE FUNCTION OF THE GASTROINTESTINAL SYSTEM

The mechanical and enzymatic digestion of food starts in the mouth, where saliva acts as a solvent for solid foods. The secretion from the salivary glands contains enzymes, primarily α-amylase and lower amounts of lipase and ribonuclease that contribute to the initial hydrolysis

of the ingested food. Furthermore, saliva contains lysozyme with antibacterial activity and immunoglobulin A (IgA) that protects against food-borne antigens. From the mouth the moistened and partly hydrolysed food is passed on to the stomach through the oesophagus [1,4]. In the stomach the food is mixed with gastric juice producing the so-called chyme [4]. The low pH of the gastric juice kills most microorganisms and is an important defence mechanism against pathogens [5].

From the stomach the chyme is released into the upper part of the small intestine, the duodenum. The rate of chyme released depends on the composition of the processed food. Food with a high content of carbohydrates is released faster than protein-rich food, followed by release of chyme produced from a meal high in fat [1].

The small intestine is the part of the gastrointestinal tract, where most of the digestion and absorption of nutrients take place. The epithelial cells lining the small intestine contribute to the production of digestive enzymes and have a large surface area due to the presence of villi and microvilli [1]. In response to chyme passing into the duodenum, bile and pancreatic juice are secreted into the duodenal lumen. Bile is an alkaline solution containing bile acids, bile pigments and traces of cholesterol, fatty acids and phospholipids [1,4]. Bile acids are synthesized by the liver from cholesterol and are essential for digestion and absorption of dietary fat, cholesterol and fat-soluble vitamins [6,7].

The pancreatic secretion includes an electrolytic and an enzymatic secretion. The electrolytic secretion is alkaline with a pH of 7.5-9 and acts as a buffer of the acidic chyme making the pH optimal for the activity of the digestive enzymes in the enzymatic secretion. The enzymatic secretion contains a variety of enzymes for digestion of proteins (trypsin, chymotrypsin, carboxypeptidase and elastase), lipids (lipase, phospholipase, esterase), nucleic acids (ribonuclease, deoxyribonuclease) and carbohydrates (α-amylase) [1,8].

The pancreatic α-amylase has a higher activity compared to salivary α-amylase and the highest concentration of the enzyme is found in the duodenum. After entering the duodenum the majority of ingested starch is quickly hydrolysed to maltose, malto-oligosaccharides and α-limit dextrins by pancreatic α-amylase. Di- and oligosaccharides are further digested by enzymes produced by the epithelial cells of the duodenum and jejunum followed by absorption of monosaccharides. The most important enzymes are lactase (hydrolyses lactose), sucrase (hydrolyses sucrose), α-dextrinase (debranches α-limit dextrins) and glucoamylase (hydrolyses malto-oligosaccharides) [8].

Among polysaccharides, starch is the only one that is hydrolysed by digestive enzymes in the small intestine, since they are only capable of hydrolysing α-glycosidic linkages with the exception of lactase hydrolysing ß-bindings in lactose [1,9]. However, in the form of resistant starch (RS) parts of ingested starch can reach the colon undigested. Four major type of RS have been classified with RS_1 being physically inaccessible to digestion e.g. due to intact cells walls in grains and seeds, RS_2 comprises granular starch in e.g. potatoes, RS_3 is retrograded starch produced by cooking and cooling of starchy foods and RS_4 comprises chemically modified starch [10].

Oligo- and polysaccharides with ß-glycosidic linkages e.g. cellulose are not hydrolysed and pass the small intestine undigested [8]. Carbohydrates that resist digestion reach the colon chemically intact, where the digestive process is continued through bacterial fermentation [1]. The carbohydrates provide energy for bacterial growth with fermentation resulting in production of hydrogen, carbon dioxide and short chain fatty acids (SCFA) [4]. SCFAs function as the primary energy source for the colonic epithelium and are discussed further in section 2.1.

Dietary proteins are digested partly by pepsin in the stomach and partly by proteases secreted by the pancreas (trypsin, chymotrypsin, carboxypeptidase and elastase). These enzymes reduce the proteins to smaller peptides, that are further digested into amino acids and di-, tri- and tetra-peptides by membrane integrated peptidases produced by the epithelial cells of the duodenum and jejunum. The amino acids and small peptides are absorbed followed by hydrolysis of the peptides by cytosolic peptidases and release of the amino acids to the blood [1,8].

Digestion of dietary fats is a complex process involving emulsification and micelle formation [1]. The primary dietary lipids are triglycerids, which are emulsified in the small intestine with the help of bile acids. This produces emulsion droplets allowing the access of water-soluble lipolytic enzymes produced by the pancreas [8]. The digestion products (free fatty acids and mono-glycerides) need to form micelles in order to reach the epithelial surface [4]. Due to their amphipathic structure (hydrophilic and hydrophobic) bile acids are capable of forming micelles and to carry lipids to the surface of the epithelium. Here, the micelles disrupt and the lipids diffuse into the epithelial cells [4].

Throughout the digestive tract water is absorbed and the digestive process is terminated with undigested and non-fermented food residues, bacterial biomass, exfoliated cells and mucus stored in the rectum and finally excreted as faeces [1]. The time it takes a substance to travel through the entire gastrointestinal tract (the transit time) is on average 24-72 hours. Most of the time (18-64 hours) is in the colon, with the time in the stomach and small intestine only accounting for 4-8 hours [4].

1.2 COMPOSITION OF THE HUMAN GASTROINTESTINAL MICROBIOTA

The human gastrointestinal microbiota is a complex ecosystem containing all three domains of life: Bacteria, archaea, and eukarya [11]. Archaea and Eukarya only represent a single phylum each, whereas the composition of the bacterial microbiota is very diverse [12].

The intestinal microbiota is established shortly after birth with the mode of delivery (vaginal or caesarean) and the type of feeding (breast milk or infant formula) affecting the composition of bacteria initially colonising the gut. Faecal samples from infants delivered by caesarean section have been reported to contain lower numbers of bifidobacteria and bacteroides, but higher numbers of clostridia compared to vaginally delivered infants [13]. In addition, breast-fed and formula-fed infants have different microbiotas. Breast-fed infants are usually colonised by high numbers of bifidobacteria, whereas high numbers of bacteroides and clostridia colonise the intestinal tract of formula-fed infants compared to breast-fed [14,15].

The intestinal microbiota of children stabilizes at weaning and is comparable to the adult microbiota at around 2 years of age [4].

Several studies have attempted to describe the bacterial community of the intestinal tract in adult humans. Initially such studies were based on cultivation dependent methods, but more recent studies have include cultivation independent molecular methods based on analysis of 16S ribosomal DNA (rDNA) sequences [16]. Since molecular methods indicate that 60-80% of the human intestinal microbiota have not been cultivated [17], such methods have improved the ability to gain insight in the composition of the microbial community.

The current knowledge of the composition of the human gut microbiota has recently been reviewed. A total of nine bacterial phyla have been identified with the most dominating being the *Firmicutes*, *Bacteroidetes* and *Actinobacteria* followed by *Proteobacteria* (Figure 2) [12]. In a study by Eckburg *et al.* [18] investigating the composition of the human faecal microbiota, based on sequence analysis of 16S rDNA, seven bacterial phyla (*Firmicutes*, *Bacteroidetes*, *Proteobacteria*, *Actinobacteria*, *Verrucobacteria*, *Fusobacteria* and one unclassified phyla) were identified, as well as one archaeal phylotype. The dominating phyla were the *Firmicutes* (51% of the total bacterial sequences) and *Bacteroidetes* (48%). Among the *Firmicutes* 95% of the sequences belonged to the genera *Clostridium* with the majority belonging to the butyrate-producing cluster XIVa [18].

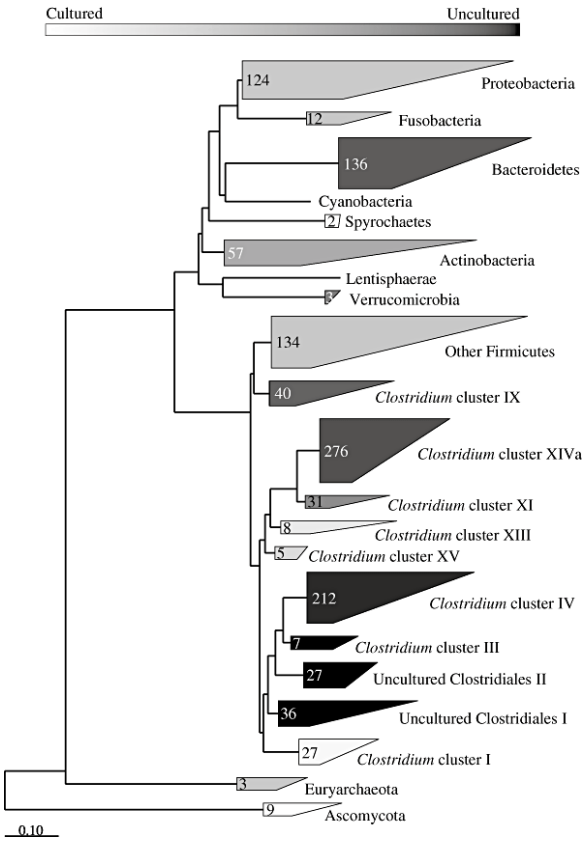

Figure 2. 16S rDNA-based phylogenetic tree of the microbial community present in the human intestinal tract. Differences in darkness indicate phylogenic groups corresponding to phylotypes detected in cultivation based studies (white) or in cultivation-independent studies (black). Numbers indicate distinct phylotypes within each group [12].

At genus level, based on cultivation dependent analysis of faecal samples, the microbiota is dominated by the genera *Bacteroides* (the *Bacteroides* phylum), *Clostridium*, *Eubacterium*, *Peptococcus*, *Peptostreptococcus*, *Ruminococcus* (the *Firmicutes* phylum) and *Bifidobacterium* (*Actinobacteria*). Sub-dominating genera are *Enterococcus* and *Lactobacillus*, *Escherichia*, *Enterobacter*, *Klebsiella* and *Proteus* [3].

Both the diversity and number of bacteria present varies according to the location in the GI tract (Figure 3) [19]. The total bacterial cell number has been estimated as 10^{14}, with numbers as high as 10^{12} cells/g recorded for the colon. Lower numbers are found in the stomach and duodenum ($<10^3$ cells/ml) and in the jejunum and ileum (10^4-10^6 cells/ml) [19,20]. Only few bacterial species can survive the acidic conditions in the stomach with *Heliobacter pylori* being the most well known "stomach bacterium" [5]. Other genera detected in the stomach and duodenum are lactobacilli and streptococci [4,19]. From the duodenum through the jejunum and ileum the bacterial diversity increases, as indicated by figure 3, with the most complex bacterial community present in the colon [19].

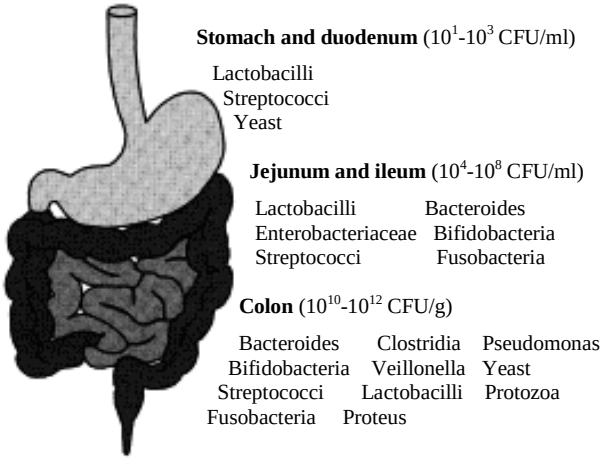

Figure 3. Microbial numbers and diversity throughout the human gastrointestinal tract [19].

1.2.1 THE HUMAN VERSUS THE MOUSE INTESTINAL MICROBIOTA

In the present thesis the effects of dietary interventions were studied using mice. Even though mice are an often used model in the study of dietary effects on the intestinal microbiota, the composition of the mouse gut microbiota have not been studied as intensively as for humans. Still, a considerable similarity between the mouse and human gut microbiota have been reported by Ley et al. [21] based on analysis of caecal 16S rDNA sequences from mice. Ley and co-workers found that the dominating phyla in mice were the *Firmicutes* and *Bacteroidetes* as reported for humans [18]. *Firmicutes* accounted for ~60% of a total of 5.088 sequences isolated from lean C57BL/6J mice and *Bacteroidetes* for ~40%. More than 75% of the *Firmicutes* belonged in the *Clostridium* cluster XIVa also reported as a dominating cluster in humans [18].

2. FERMENTATION OF NON-DIGESTIBLE DIETARY CARBOHYDRATES

Non-digestible (ND) dietary carbohydrates, that escape digestion in the small intestine, become available as growth substrates for the colonic microbiota. In humans, the majority of ND dietary carbohydrates that reach the colon are plant cell wall polysaccharides (e.g. cellulose, arabinoxylan, xyloglucan, ß-glucan, mannan, pectins and lignin) and resistant starch [22,23]. In addition, commercial products of ND dietary carbohydrates known as prebiotics e.g. inulin and fructo-oligosaccharides are also consumed as a way of selectively manipulating the composition of the gut microbiota [24].

Degradation of undigested carbohydrates in the colon initially involves bacterial groups capable of degrading complex polysaccharides [23]. In humans, bacterial species capable of degrading dietary fibres (e.g. cellulose, inulin and xylan) have been identified within the genera *Bacteroides*, *Roseburia*, *Ruminococcus* and *Bifidobacterium* [23,25-29]. Since *Bacteroides* is one of the numerically dominating bacterial genera in the human large intestine [30], they are likely to play a central role in degradation of complex carbohydrates entering the colon. This has been confirmed by the capability of *Bacteroides* spp. to utilizing a variety of plant polysaccharides [31]. In addition, a large proportion of the genome of *B. thetaiotaomicron* encodes genes involved in harvesting and metabolizing polysaccharides [32]. According to Xu *et al.* [32] the representation of glycosylhydrolases (e.g. α- and ß-galactosidases, α- and ß-glucosidases, ß-glucuronidases, ß-fructofuranosidases, α-mannosidases, amylases and xylanases) in the genome of *B. thetaiotaomicron* exceeds that of any other sequenced bacteria.

Hydrolysis of polysaccharides into smaller fragments (oligosaccharides) makes the substrates available for fermentation by other members of the bacterial community [33]. Non-digestible oligosaccharides such as fructo-oligosaccharides (FOS) and inulin (a mixture of oligo- and polysaccharides) occur naturally in a variety of vegetables and fruits as a source of carbohydrate storage [1] and are used to selectively stimulate the growth of bifidobacteria in the human large intestine [24]. The ability of bifidobacteria to ferment FOS has been ascribed to the activity of β-fructofuranosidases identified in several bifidobacterial species including *B. lactis* [34], *B. breve* [35], *B. infantis* [36] and *B. longum* [37]. The genome of *B. longum* has been fully sequenced and was found to dedicate more than 8% of the genome to metabolism of oligosaccharides. Besides encoding many enzymes for fermentation of mono- and disaccharides, the genome encoded a β-fructofuranosidase as well as proteins with homology towards xylanases, arabinosidases, α-galactosidases, β-galactosidases, β-glucosidases and hexoaminidases. Based on these findings *B. longum* is capable of fermenting a wide selection of carbohydrates. In addition, eight high-affinity oligosaccharide transporters were identified likely to provide *B. longum* with a competitive advantage in the uptake of oligosaccharides [37].

Studies on the activity of the β-fructofuranosidases (all intracellular) encoded by *B. lactis*, *B. breve* and *B. infantis* demonstrated that the affinity of the enzyme may vary between species and that the ability to cleave β-1.2 bounds in inulin and FOS is affected by the complexity of the substrate. In *B. lactis* the strongest affinity was observed for terminal β-1.2 bounds

between fructose units, while β-fructofuranosidases produced by *B. breve* only cleaved β-1.2 glucose-fructose links. In all three species a high activity was recorded towards degradation of FOS, whereas only low activities were recorded towards inulin [34-36]. Differences in the enzyme activity towards FOS and inulin are in agreement with results from the *in vitro* fermentation studies by Rossi *et al.* [29] investigating the relationship between chain length of fructans (inulin and FOS) and the ability of bifidobacteria to ferment them. In this study only few bifidobacterial strains (8 of 55) were capable of fermenting inulin and the fermentation was related to production of extracellular β-fructofuranosidases.

2.1 SHORT CHAIN FATTY ACIDS

The major end-products of bacterial fermentation of dietary carbohydrates are short chain fatty acids (SCFA) and gasses including H_2, CO_2 and CH_4 [30]. The primary SCFAs produced in the human large intestine are acetate, propionate and butyrate with the majority of SCFAs absorbed in the colon and excretion of only 5-10% in faeces [38]. In humans, the total SCFA concentration in the proximal colon is ~70-140 mM depending on diets and decreases to ~20-70 mM in the distal colon [10]. The faecal molar ratio (%) of the three dominating SCFAs is approximately 60:20:20 (acetate:propionate:butyrate), although the ratio may be affected by dietary changes [10,38]. From *in vitro* studies, simulating the conditions in the human colon, the molar ratio (%) of the three acids have been recorded as 60-80 for acetate, 14-22 for propionate and 8-23 for butyrate [39].

SCFAs are produced within the bacterial cells from monosaccharides generated from the breakdown of oligo- and polysaccharides. In the cells metabolism of monosaccharides result in the release of SCFAs along with a net production of 4 adenosine triphosphate (ATP) molecules [40]. All three SCFAs are rapidly absorbed by host tissue and are primarily metabolized by the gut epithelium, liver and muscles [39].

Acetate is absorbed by the gut epithelium, passes through the liver via portal blood to peripheral tissues, where it provides energy to muscles (e.g. skeletal and cardiac muscles and the brain) [39,41].

Propionate is transported to the liver through the portal vein and is an important precursor for gluconeogenesis in ruminants [40]. In humans, the role of propionate metabolism is less clear, but it is suggested to be involved in the cholesterol lowering effect of dietary fibres [42]. In rats, studies on the effect of propionate on cholesterol metabolism have demonstrated reduced cholesterol levels in the liver and blood [43-45]. In a study with human volunteers serum propionate was negatively correlated to total serum cholesterol in men but not in women [46], whereas other studies have reported no effect of propionate on serum cholesterol levels in humans [47-49].

Of the three SCFAs most studies have dealt with the effect of butyrate on colonic health. Besides being the preferred energy sources for colonic epithelial cells [50], butyrate functions as a signalling molecule involved in cell cycle arrest and induction of apoptosis in cancer cells [10,51-53] and has been implicated in protection against colon cancer [54-56]. Furthermore, butyrate may reduce the infectious potential of *Salmonella*. In a study by Van Immerseel *et al.*

[57] pretreatment of *S. enterica* serovar Enteritidis (*S.* Enteritidis) with butyrate significantly reduced pathogen invasion of chicken caecal epithelial cells *in vitro* and feed supplemented with butyrate reduced numbers of the pathogen in the ceaca of chickens [58]. A reduced invasion of epithelial cells after exposure to butyrate may be explained by a down-regulation of expression of genes important for *Salmonella* invasion as demonstrated by Gantois *et al.* [59]. Growth of *S.* Enteritidis and *S. enterica* serovar Typhimurium (*S.* Typhimurium) in media supplemented with butyrate was seen to reduce invasion of HeLa cells for both serovars and to down-regulate expression of invasion-associated genes encoded by the *Salmonella* pathogenicity island (SPI-1) (described further in section 4.1) [59].

Degradation of dietary fibres in the large intestine is a complex metabolic pathway involving several bacterial species and creates the opportunity for cross-feeding. This may in turn affect the release of metabolic end-products such as SCFAs (Figure 4) [60]. Differences in SCFA production may also be a reflection of different dietary carbohydrates yielding different amounts and types of SCFAs [41]. Starch fermentation generally yield high ratios of butyrate, whereas pectin is a poor source of butyrate [41,61]. In contrast, pectin is a good source of acetate production [41]. Hence, by feeding different fiber sources it is possible to manipulate the types and amounts of SCFAs produced [62].

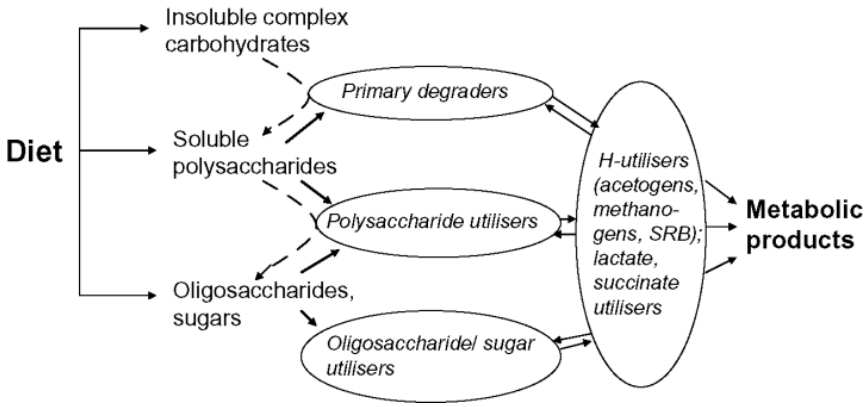

Figure 4. Schematic presentation of cross-feeding in relation to microbial degradation of complex carbohydrates in the large intestine [60].

Examples of cross-feeding affecting the production of SCFAs have been demonstrated *in vitro*. Co-culture of butyrate-producing strains of *Eubacterium*, *Anaerostipes* (both lactate-utilizers) and *Roseburia* with *Bifidobacterium adolescentis* demonstrated two routes of metabolic cross-feeding [63]. All three butyrate-producing strains were unable to ferment the growth substrates (starch and FOS) in mono-cultures but produced butyrate in co-culture with *B. adolescentis*. Hence, the results indicate cross-feeding of either lactate produced by *B. adolescentis* (*Eubacterium* and *Anaerostipes*) or of partially degraded carbohydrates released by *B. adolescentis* (*Roseburia*) [63].

The occurrence of cross-feeding between strains of bifidobacteria has also been suggested. In the *in vitro* study by Rossi *et al.* [29] only few strains of bifidobacteria were capable of fermenting inulin in mono-cultures. However, no difference in the growth of bifidobacteria on FOS and inulin were observed in faecal cultures. The results thus suggest, that stimulation of bifidobacteria in mixed cultures and *in vivo* may partly be explained by cross-feeding of oligosaccharides released by inulin-degrading strains, demonstrating the nutritional dependence among bacteria colonizing the large intestine [29,60].

3. DIETARY STRATEGIES FOR MODULATING THE INTESTINAL MICROBIOTA

Some intestinal bacteria are regarded as beneficial for gut health [64]. In general, the gut microbiota have been divided into genera of either potentially harmful/pathogenic or potentially health promoting bacteria [30,64]. The genera *Bifidobacterium* and *Lactobacillus* do not contain any known pathogens [64] and are classified as potentially health promoting with positive effects on 1) inhibition of growth of harmful bacteria, 2) stimulation of immune functions, 3) digestion and absorption of food ingredients/minerals and 4) synthesis of vitamins [30]. At least three strategies exist within modulation of the gut microbiota. Probiotics are the concept of consuming microorganisms with known beneficial effects on gut health [65], prebiotics are non-digestible dietary carbohydrates claimed to be specifically fermented by beneficial gut bacteria [30] and finally, synbiotics are a combination of pro- and prebiotics with the idea, that probiotics travel to the colon, while prebiotics support their growth combined with a stimulation of already present beneficial bacteria [16].

3.1 PROBIOTICS

The concept of probiotics was initially defined by Fuller [65] as ”a live microbial feed supplement that beneficially affects the host animal by improving its intestinal microbial balance”. In order to do so probiotics need to fulfil four criteria: 1) probiotics must be capable of being prepared in a viable manner and in large scale, 2) probiotics need to remain viable and stable during storage and use, 3) probiotics should be able to survive the conditions in the intestinal tract and 4) the host should gain beneficially from ingesting the probiotics [30]. More recently probiotics have been defined by Salminen *et al.* [66] as “ microbial cell preparations or components of microbial cells that have a beneficial effect on the health and well-being of the host”. According to this definition probiotics do not need to be viable.

Probiotics have traditionally been used for years in the production of fermented food products such as yoghurt due to their production of lactic acid [16]. Several bacterial genera have been used as probiotics (lactobacilli, streptococci, enterococci, lactococci, bifidobacteria and bacillus), but also fungi such as *Aspergillus* spp. and *Saccharomyces* spp. have been used. Still, the most frequently used probiotics are species of the genera *Lactobacillus* and *Bifidobacterium* (e.g. *Lactobacillus acidophilus, L. ca*sei, *L. rhamnosus, L. reuteri, L. plantarum* and *L. johnsonii* and *Bifidobacterium bifidum, B. lactis, B. longum B. infantis* and *B. breve*) [4,30].

A considerable number of benefits for human health have been postulated to result from the intake of probiotics. These include 1) prevention of diarrheal illnesses, 2) prevention of common infections (e.g. colds and fever), 3) prevention of allergic disorders, 4) prevention of inflammatory bowel disease and 5) prevention of colon cancer (Table 1). Furthermore, protection against pathogenic infections has been investigated *in vitro* [67-71] and *in vivo* [72-85] using animal models, including studies on prevention of *Salmonella* infections [78-85].

Table 1. Potential health benefits of probiotic consumption studied in humans

Effect	**References**
Prevention of diarrheal illnesses	[86-89]
Prevention of common infections (e.g. colds and fever)	[88,90-93]
Prevention of allergic disorders	[94-99]
Prevention of inflammatory bowel disease	[100-104]
Prevention of colon cancer	[105,106]

3.1.1 ANTAGONISTIC EFFECTS OF PROBIOTICS

One way by which probiotics may contribute to gut health is by improving the colonization resistance [107], being the mechanism whereby the intestinal microbiota limits colonization of exogenous and potentially pathogenic microorganisms [108]. Several factors may contribute to an improved colonization resistance, with one being the release of acidic metabolic end-products such as lactic acid and other SCFAs that lower the gut pH to levels below those optimal for growth and competition by the pathogen. Other factors contributing to the colonization resistance are competitive exclusion of adherence of the pathogen, competition for nutrients, production of antimicrobial substances and immune modulation [107]. For a detailed review on the antagonistic activities of *Lactobacillus* and *Bifidobacterium* the reader is referred to the review by Servin [109], with some examples given below.

Competitive exclusion of pathogen adherence *in vitro* by 12 commercial probiotic strains has been investigated by Collado *et al.* [71]. All probiotic strains were able to inhibit and displace adhesion of *Bacteroides vulgatus*, *Clostridium histolyticum*, *Clostridium difficile*, *Staphylococcus aureus* and *Enterobacter aerogens*, but most could not inhibit adhesion of *Escherichia coli* (12/12), *Listeria monocytogenes* (7/12) and *S.* Typhimurium (9/12) as well as displace adhesion of *E. coli* (12/12) and *S.* Typhimurium (7/12). By competitive exclusion adhesion of four pathogens cold be inhibited by nearly all probiotics (*C. difficile* (12/12), *S. aureus* (12/12), *E. aerogens* (12/12) and *B. vulgatus* (11/12)). Based on these results some probiotics can successfully be used as inhibitors of pathogen adhesion, but results are in particular affected by the pathogens tested [71].

Production of antimicrobial substances is another mechanism by which probiotics may protect against infections. Production of acidic metabolites such as lactic and acetic acid and the pH reductive effect is one example [109]. Acid production by *Bifidobacterium infantis* has been observed to inhibit growth of *E. coli* O157 and *S.* Typhimurium [110] and several studies with

Lactobacillus spp. have demonstrated inhibition of growth of human pathogens due to acid production [109].

Anti-bacterial substances distinct from lactic and acetic acid are also produced by lactobacilli and bifidobacteria [109]. An example of such is the non-bacteriocin antibacterial component produced by *L. acidophilus* strain LA1 with demonstrated *in vitro* activity against a range of Gram-positive and Gram-negative pathogens including *S. aureus, L. monocytogenes, Pseudomonas aeruginosa* and *S.* Typhimurium [111]. For bifidobacteria antimicrobial activity of two strains isolated from infant stools was demonstrated with viability of selected strains of *Klebsiella pneumoniae, Yersinia pseudotuberculosis, E. coli, S. aureus, P. aeruginosa* and *S.* Typhimurium greatly reduced after 1 or 3 hours of incubation with culture supernatants from either of two bifidobacterial strains [112].

The last antagonistic mechanism of probiotics described here is the ability to stimulate the immune system. The use of probiotics has been implicated in the maturation of the immune system in infants, in regulation of the Th1/Th2 balance and in prevention of immune-mediated diseases, such as allergies [113-115]. However, these topics are beyond the scope of this thesis and here focus will be on examples of immune modulation with positive effects on protection against *Salmonella* infections.

In studies investigating prevention of *Salmonella* infections probiotics have been shown to influence a number of immune effects that improve host resistance to the pathogen e.g. the activity of phagocytic cells, cytokine production and levels of immunoglobulins (Ig) [113].

Increased phagocytic activity of blood and peritoneal cell preparations was observed in studies with mice fed *L. rhamnosus* HN001 or *B. lactis* HN019 prior to *S.* Typhimurium infection [78,79]. These findings were accompanied by enhanced survival rates, reduced numbers of *Salmonella* in the liver and spleen and increased titers of *Salmonella*-specific antibodies in serum, mucosa and intestinal fluids.

A heat-killed multi-strain mixture of *L. acidophilus* was seen *in vivo* to reduce serum TNF-α levels and to protect against *S.* Typhimurium infection in mice. *In vitro*, the probiotic mixture stimulated the phagocytic activity of murine macrophage cells. [84]. Furthermore, oral administration of *B. longum* to mice prior to *S.* Typhimurium infection increased the survival rate and reduced the production of IFN-γ by the spleen, suggesting a reduced inflammatory response as the protective effect of the probiotic administration [81].

The effect of probiotics on the immune response towards an attenuated *S. enterica* serovar Typhi (*S.* Typhi) vaccine in humans was investigated by Link-Amster *et al.* [116]. Human volunteers consumed fermented milk containing *L. acidophilus* La1 and bifidobacteria B12 (a commercial mixed culture) for three weeks or were restricted from consuming fresh fermented products (control group). When an attenuated *S.* Typhi was given to the volunteers, to mimic an enteropathogenic infection, the titer of specific serum IgA to *S.* Typhi was 4-fold increased in the probiotic group compared to 2.5-fold in the control group, indicating that probiotics may enhance the effectiveness of oral a *Salmonella* vaccine in humans [116].

3.2 NON-DIGESTIBLE DIETARY CARBOHYDRATES

Non-digestible dietary carbohydrates are another approach to obtain health benefits of intestinal beneficial bacteria already present in the intestinal tract and a healthy and balanced gut microbiota has been described as one that is predominantly saccharolytic with significant numbers of bifidobacteria and lactobacilli [4].

The concept of prebiotics was initially defined by Gibson & Roberfroid [30] as "non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon [30]. Since then the definition has been refined by Gibson *et al.* [24] and prebiotics are now defined as " selectively fermented ingredients that allow specific changes, both in the composition and/or activity of the gastrointestinal microbiota that confers benefits upon host well-being and health".

According to Gibson and Roberfroid [24] a food ingredient has to fulfil three criteria to be classified as prebiotic:

A prebiotic should:

1) Resist gastric acidity, hydrolysis by mammalian enzymes and gastrointestinal absorption.
2) Be fermented by the intestinal microbiota.
3) Selectively stimulate the growth and/or activity of intestinal bacteria associated with health and wellbeing.

Referring to these three criteria only inulin and FOS, galacto-oligosaccharides (GOS) and lactulose have been classified as prebiotic substrates [24]. Among other prebiotic candidates evaluated in the study by Gibson and Roberfroid [24] are isomalto-oligosaccharides, lactosucrose, xylo-oligosaccharides (XOS), soyabean oligosaccharides and gluco-oligosaccharides.

One aspect of critical importance to the prebiotic concept is the selective stimulation of bifidobacteria and lactobacilli at the expense of other bacterial groups [117,118]. The selectivity may be affected by characteristics such as the type of glycosidic linkage, degree of branching and the degree of polymerization (DP), being the number of repeat monomer units in a polymer chain [119]. The DP influences where in the large intestine fermentation occurs. Non-digestible carbohydrates with a low DP reach the proximal colon, where substrate availability and bacterial growth is generally high and the pH is low (5-6) as a result of intense acid production. In contrast, carbohydrates with a higher DP e.g. inulin may be available for fermentation in the distal colon [3,120].

The indigestibility of prebiotics and other ND dietary carbohydrates is a result of the ß-configuration of the glycosidic bound between monosaccharides, whereas human gastrointestinal digestive enzymes are specific for α-glycosidic bounds [8,20]. However, ND dietary carbohydrates with α-configuration also exists e.g. polydextrose and pectins. In principle, these can be degraded by human digestive enzymes, but reach the colon largely undigested due to their high molecular weight [121,122].

3.2.1. HEALTH BENEFITS OF NON-DIGESTIBLE DIETARY CARBOHYDRATES

ND dietary carbohydrates have the potential to modulate intestinal bacterial fermentation patterns, which may in turn affect several physiological functions [123]. A large number of health-promoting effects of prebiotics/ND dietary carbohydrates have been hypothesized (Table 2). These are generally the same as suggested for probiotics, since the primary effect of prebiotics is through the interaction with the intestinal microbiota [20,123,124]. Although far from all postulated effects of prebiotics have been fully demonstrated [20,124], a bifidogenic effect of prebiotics and other potential prebiotic carbohydrates have been demonstrated as described in section 3.2.2 for the substrates investigated in the present thesis.

Table 2. Potential health-benefits of prebiotics/non-digestible dietary carbohydrates

Effects	**References**
Prevention of diarrhoea (traveller's and antibiotic-associated)	[125,126]
Treatment of inflammatory bowel diseases	[127,128]
Prevention of allergic disorders	[129,130]
Immune modulation	[131,132]
Improved mineral absorption (mainly Ca and Mg)	[133-135]
Regulation of lipid metabolism	[136,137]
Improved bowel habit	[117,138-140]
Reduced risk of colon cancer development	[141-144]

One potential beneficial effect of consuming ND dietary carbohydrates is improved bowel habit with relief of constipation, reduced transit time, and increased faecal bulking [117,138-140]. Reduced transit time, as a result of increased bacterial biomass and hence increased stool frequency, may thus decrease the exposure time of the gut epithelium to potential carcinogens of dietary origin [20]. Diet and bacterial metabolism are factors, in addition to genetic susceptibility, that plays an important role in the risk of developing colon cancer [118] and consumption of diets high in red meat have been associated with this type of cancer [145-147]. In contrast, an inverse association between intake of dietary fibre and incidences of colon cancer have been demonstrated [148,149]. Release and accumulation of potential carcinogen by-products from protein degradation such as ammonia, phenols, indoles and amines [150] may thus be reduced by diets rich in fibre and hence, by changes in the microbial composition towards primarily saccharolytic bacteria (e.g. bifidobacteria and lactobacilli) [106,150].

A number of *in vivo* studies have investigated the potential of prebiotics on prevention of *Salmonella* infections in rodents [83,85,151-157] (discussed in section 4.2). Besides the potential protective effect of prebiotics exerted through modulation of the gut microbiota, prebiotics and other ND dietary carbohydrates may also protect against pathogen adhesion and invasion by receptor mimicry [158,159]. Attachment to epithelial cell surface receptors is

often the first step in the pathogenesis of entero-pathogens and prebiotics acting as receptor analogues might inhibit infection, with pathogen binding to soluble oligosaccharides rather than to host cell receptors [107,158,160]. For example, GOS have been shown *in vitro* to reduce adherence of enteropathogenic *E. coli* (EPEC) to HEp-2 and Caco-2 cells, and the anti-adhesive activity of GOS was more effective than of both FOS and inulin [160]. Similarily, GOS was found to reduce the invasion of *S.* Typhimurium SL1344 and LT2 to HT29 cells lines [155]. Furthermore, pectins and pectic oligosaccharids reduced the activity of *E. coli* O157:H7 produced shiga toxin, likely by inhibiting binding of the toxin [161].

3.2.2. APPLICATION AND BIFIDOGENIC EFFECT OF INVESTIGATED CARBOHYDRATES

Besides the potential for modulating the gut microbiota, ND dietary carbohydrates are used in the food industry as bulging agents and as fat and sugar replacers [119]. The chemical structure, natural sources and functional properties of the ND dietary carbohydrates investigated in the present thesis are described below, along with studies on their effects on the composition of the gut microbiota from human studies, if possible, or from animal or *in vitro* studies. An overview of all tested carbohydrates is given in Table 3.

INULIN AND FRUCTO-OLIGOSACCHARIDES

Inulin and fructo-oligosaccharide (FOS) are by far the best studied prebiotics. Both compounds are polymers of D-fructose units linked by β-2.1-glycosidic bounds often with an α-1.2-linked D-glucose at the terminal end of the molecule (Figure 5) [1,118].

Inulin occurs naturally in a variety of vegetables and fruits as a source of carbohydrate storage with onion, banana, garlic and leek being the most common natural sources of inulin [1]. Commercial inulin is essentially produced from chicory roots. Chicory inulin is a mixture of oligomers and polymers with a DP ranging from 2-60 and an average DP of 12.

FOS is prepared from inulin by enzymatic hydrolysis yielding oligomers with a DP of ~2-7 and an average DP of 4 [1]. Alternatively, FOS can be synthesized using fungal β-fructosidases by transfructosylation. In this process fructose units are added to sucrose molecules by β-2.1-linkages typically yielding oligomers with a DP of 2-4 [120].

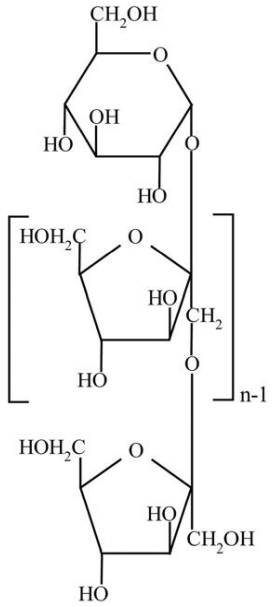

Figure 5. Chemical structure of inulin and fructo-oligosaccharide. n equals the number of fructose units [20].

In the food industry inulin is used as gelating agent and as a fat replacer, whereas FOS is used as a sugar replacer with a sweetness of ~35% compared to sucrose and a low caloric value of 1.5 kcal g^{-1} (sucrose 4 kcal g^{-1}) [1,162].

The inulin (Orafti ST-gel) used in the present thesis is a white, odourless, soluble powder extracted from chicory roots. The inulin content is ~92% with the remaining 8% being a mixture of glucose, fructose and sucrose. It has an average DP of ≥10 and a sweetness of 10%

compared to sucrose. The fructo-oligosaccharides (Orafti P95) used in the thesis has an oligo-fructose content of 95%, a 30% sweetness compared to sucrose and a DP of 2-8 (www.orafti.com).

Both inulin and FOS are regarded as bifidogenic in infants and adults, although variations in the bifidogenic effect have been observed [64,120]. In healthy adults the lowest dosage of inulin and FOS with a demonstrated bifidogenic effect is 5 g/day based on analysis of faecal samples [64,163-165]. In infants a daily dosage of as low as 1.25 g inulin and 1.7 g of an inulin/FOS mixture has been reported as bifidogenic [64,166,167]. In addition, several studies report an increase in lactobacilli in infant stools [64]. Besides the reported effects on the faecal microbiota Langlands *et al.* [168] found, that a mixture of inulin and FOS (7.5 g/day of each substrate) supplemented to adults also increased numbers of bifidobacteria and lactobacilli in the mucosa-associated microbiota of the large intestine, with the largest effect observed in the distal colon.
Although inulin and FOS are intended to selectively promote growth of bifidobacteria and lactobacilli, they may also affect growth of other gut bacteria. In some infant or adult studies a reduction in potentially harmful bacteria such as *Bacteroides* spp. and *Clostridium* spp. was observed [139,169,170], while these bacterial genera were stimulated in other studies [163,164,171,172] demonstrating that inulin and FOS can also enhance non-target bacteria.

GALACTO-OLIGOSACCHARIDES

Galacto-oligosaccharides (GOS) are chains of D-galactose monomers linked by β-1.4 or β-1.6 bounds with a terminal α-1.4 bound D-glucose molecule (Figure 6). GOS is naturally present in both human milk, particularly in colostrum, and cow's milk and often has a DP of ~2-5 [162,173]. For commercial products GOS is usually produced by β-galactosidase treatment of whey-derived lactose, which is formed as a by-product from the dairy industry [162]. For GOS production β–galactosidases from various fungi, yeast and bacteria are used resulting in differences in the glycosidic linkages in the final product e.g. β-1.4 or β-1.6 [162,173].
GOS preparations have a caloric value of only 1.7 kcal g^{-1} and a third of the sweetness of sucrose making the oligomer useful as a sweetener in the production of foods and beverages. Furthermore, GOS is used to increase the texture and mouth feel of a variety foods as well as a bulking agent [162]. The GOS used in the present thesis was provided by Danisco Health and Nutrition, Kantvik, Finland. The oligomer had a DP of ~2-6, but may contain traces of the starting material lactose and monomers of glucose and galactose.

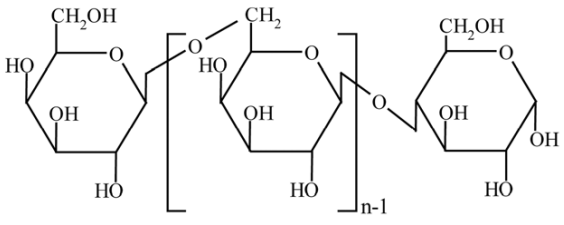

Figure 6. Chemical structure (β-1.6) of galacto-oligosaccharides. n equals the number of galactose units [20].

The prebiotic properties of GOS have in particular been investigated in infants fed formulas supplemented with oligosaccharides. A mixture of 10% FOS and 90% GOS has been developed to simulate the carbohydrate composition of human milk with the intention of using the mixture in infant formulas [162]. Bifidobacteria dominate the gut microbiota of breast fed babies, which is believed to result from their utilization of milk oligosaccharides, including GOS [14,15,162]. Infants fed formulas supplemented with the FOS/GOS mixture (4-8 g/L) was seen to develop a faecal microbiota that resembles that of breast fed babies, with increased numbers of bifidobacteria and lactobacilli compared to infants fed standard formulas [174-176].

In studies with adults a bifidogenic effect of GOS has also been observed [177,178], with a daily dosage of 10 g GOS recommended to obtain such an effect [162,173]. In addition to the increase in faecal bifidobacteria GOS stimulated the growth of lactobacilli in one study [177]. *In vitro*, microbial changes induced by GOS fermentation were seen to alter the fermentative activity of a human faecal inoculum resulting in a reduction in pH and in an increase in SCFA concentrations as compared to fermentation without GOS [178].

XYLO-OLIGOSACCHARIDES

Xylo-oligosaccharides (XOS) are oligomers of xylose units linked by ß-1.4 linkages (Figure 7) [118]. XOS can be produced at industrial scale by chemical/enzymatic treatment of xylan-rich materials. Typical raw materials for XOS production are hardwood, corn cobs, straws, bagasses, hulls and bran [179]. The resulting XOS products typically have a DP of ~2-4 [117]. The sweetness of xylobiose (DP=2) is about 30% compared to sucrose making is useful as a low calorie-sweetener. In addition, the oligomer is commercially used as a food ingredient in Japan in FOSHU foods (Food for Specified Health Use) [179].

The XOS used in the present thesis (provided by Danisco Health and Nutrition, Kantvik, Finland) was prepared from xylan and had a purity of >92% with xylose compounds ranging from DP2 to DP10 (majority of DP2 and DP3).

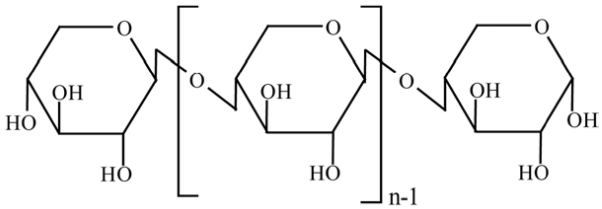

Figure 7. Chemical structure of xylo-oligosaccharide, n equals the number of xylose units [20].

Xylo-oligosaccharides are considered as promising prebiotic candidates [24], that have been shown to be effectively fermented by several bifidobacterial species (*B. bifidum, B. infantis, B. longum, B. adolescentis, B. angulatum, B. catenulatum* and *B. lactis*) in *in vitro* mono-cultures. In contrast, utilization of XOS by strains of *Lactobacillus* was less efficient [180,181]. In mixed faecal batch cultures and semi-continuous fermentation systems,

inoculated with a human faecal microbiota, stimulation of bifidobacteria by XOS has also been demonstrated [182-184].

In vivo, studies with rats fed diets supplemented with 6% XOS have reported increased numbers of caecal and faecal bifidobacteria [142,185], while numbers of lactobacilli were unaffected [185]. In the study by Campbell *et al.* [185] the bifidogenic effect was accompanied by a reduced faecal and caecal pH and increased concentrations of caecal SCFAs. In both studies the bifidogenic effect of XOS was greater that observed for FOS [142,185].

In humans consumption of XOS for three weeks by elderly aged ≥65 increased faecal numbers of bifidobacteria, decreased faecal pH and increased faecal moisture [186].

Growth of other intestinal bacteria (*Enterococcus* spp., *Bacteroides* spp., *Clostridium* spp. and *E. coli*) was generally very limited or absent in the *in vitro* mono-culture studies. Only few strains of *Enterococcus*, *Bacteroides* and *Clostridium* grew on XOS [180,181].

CEREAL BETA-GLUCAN

Beta-glucan (ß-glucan) is a major component of the cell wall of commercially important cereals including oat, barley, rye and wheat [187]. The structure of cereal ß-glucans are linear chains of D-glucose units linked by ß-1.3 and ß-1.4 glycosidic bounds. The structure consists of two main building blocks of three (cellotriosyl) or four (cellotetraosyl) ß-1.4 bound D-glucose units separated by a single ß-1.3 binding (Figure 8). The two blocks make up more than 90% of the ß-glucan structure. The remaining part of the polymer is mainly composed of longer cellulosic sequences of 5-14 D-glucose units [187,188]. The DP of ß-glucans is variable and may be >500 [132].

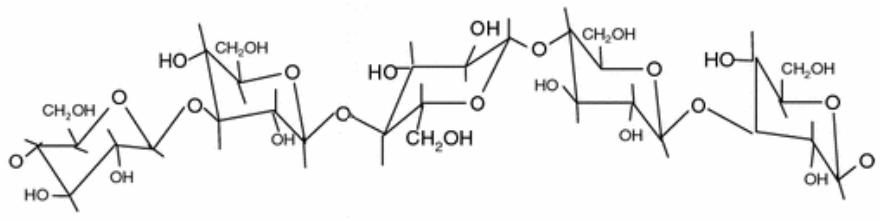

Figure 8. Chemical structure of cereal beta-glucan presented as a cellotriosyl unit. Modified from [188].

The use of ß-glucans in the food industry is mainly due to their gelling capacity and ability to increase the viscosity of aqueous solutions. ß-glucans may also be used as a fat replacer in calorie-reduced foods. The polymer has successfully been used in the manufacture of several food products including cereals, pasta, noodles, bakery products, dairy products and meat products [187]. The ß-glucan used in the present thesis was the high purity (75%) barley ß-glucan Glucagel™ purchased from GraceLinc Ltd.

In vitro mono-culture studies with barley ß-glucan demonstrated that none of the selected strains of bifidobacteria and lactobacilli were able to ferment the polysaccharide. Among

other tested gut bacteria all *Bacteroides* isolates and *Clostridium beijerinckii* fermented ß-glucan, whereas growth of *E. coli* and *Enterococcus* isolates was not supported [180].
Degradation of ß-glucooligomers, prepared by enzymatic hydrolysis of ß-glucan, was shown to support the growth of *L. rhamnosus* GG, whereas utilization of the oligomers by strains of *L. acidophilus*, *Bifidobacterium* spp., *Bacteroides* spp., *C. difficile* and *E. coli* was generally poor [181]. Based on these results ß-glucan is unlikely to directly promote growth of bifidobacteria and lactobacilli in the gut, but it may support growth of *Bacteriodes* spp.

PECTINS

Pectins are complex polysaccharides present in plant cell walls and are mainly composed of a backbone of α-1.4-linked galacturonic acid units [189]. The pectin polysaccharides are divided into five structural classes designated homogalacturonan (HG), xylogalacturonan (XGA), apiogalacturonan (AGA) and rhamnogalacturonan I (RG-I) and II (RG-II) [122].
HG is a polymer of α-1.4-linked D-galacturonic acid (GalpA) that can account for more than 60% of the pectins in the plant cell wall. The galacturonic acid units may be partly methylated at C-6 or acetylated at O-2 or O-3 (Figure 9). Based on HP extracted from apple, beet and citrus the DP of HP ranges from approximately 70-100 [122].
The remaining pectin classes are HG substituted with side chains or with differences in the GalpA backbone. XGA is HG substituted with D-xylose at C-3 of the GalpA units. AGA is substituted with D-apiose at C-2 or C-3 and is found in aquatic plants. RG-I has a backbone of repeating units of galacturonic acid and rhamnose $[\rightarrow\alpha\text{-D-GalpA-1.2-}\alpha\text{-L-Rhap-1.4}\rightarrow]_n$ with side chains of α-arabinan, ß-galactan and type-I arabinogalactan. RG-II is an even more complex structure consisting of a HG backbone (7-9 residues long) with four side-chains (designated A-D) incorporating another ten different monosaccharides into the structure [122].

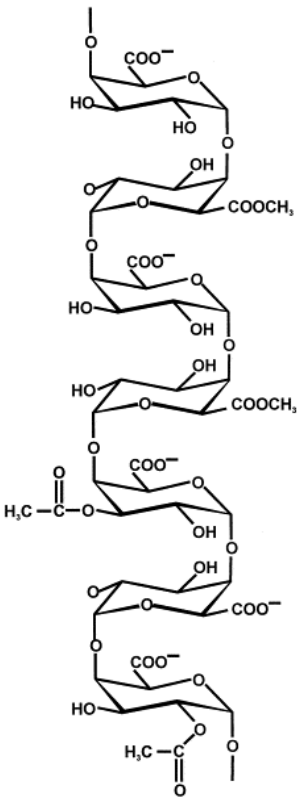

Figure 9. Chemical structure of pectin belonging to the structural class homo-galacturonan (HG) [122].

For commercial production pectins are extracted from citrus peel and apple pomace. The most important function of pectins is in the production of jams and jellies due to its gel forming and water holding capacity [189,190]. Examples of other useful applications of pectin in the food industry are as thickener and stabiliser in dairy products, as texturizer in low calorie soft drinks that lacks the mouth feel provided by sucrose and in the control of the size of ice crystals in ice [189]. The pectin used in the present thesis was raw apple pectin purchased from Obipektin AG with galacturonic acid constituting ~75% of the polymer.

The bifidogenic effect of pectins and pectic-oligosaccharides have been studied by Olano-Martin *et al.* [191]. The pectins investigated were citrus pectin and apple pectin and their derived oligosaccharides. In mono-cultures growth rates of selected gut bacteria (*Bifidobacterium* spp., *Lactobacillus* spp., *Bacteroides* spp., *Clostridium* spp., *Enterococcus faecalis* and *E. coli*) were generally higher on apple pectin and apple pectin oligosaccharides compared to citrus pectin/oligosaccharides. The pectins and the oligosaccharides did not particularly promote growth of bifidobacteria or lactobacilli in mono-cultures. However, in mixed cultures, inoculated with a human faecal microbiota, a significant increase in bifidobacteria was observed for both pectins and oligosaccharides, but the bifidogenic effect was more pronounced for the oligosaccharides compared to their parent pectins. Still, when compared to fructo-oligosaccharides the pectic-oligosaccharides were not a particularly effective prebiotic candidate [191].
Other studies investigating the ability of bifidobacteria to ferment pectin or pectic-oligosaccharides *in vitro* found that the majority of 229 investigated strains (29 species) did not ferment pectin [192], whereas Mandalari *et al.* [190] demonstrated a bifidogenic effect of an extract from citrus peel rich in pectic-oligosaccharides. The observed bifidogenic effect was stronger than observed for fructo-oligosaccharides. However, the extract also contained small amounts of other carbohydrates (rhamnose, arabiose, xylose, mannose, galactose, and glucose) that might contribute to the growth of bifidobacteria.

POLYDEXTROSE

Polydextrose (PDX) is a water-souble polymer of glucose with a low caloric value (approx. 1 kcal g^{-1}). In the food industry PDX is used as a bulking agent, texturizer and thickener and as a sugar and fat replacer. It is synthesized by random polymerization of glucose molecules (α-1.6 glycosidic bounds predominate) and has a complex structure with a DP ranging from 1-100 with an average of ~10 [119,121,193].
The PDX used in the present thesis was provided by Danisco Health and Nutrition, Kantvik, Finland, with the majority (~90%) of the polymer having a DP of 3-30 and an average DP of 12.

Effects of polydextrose intake on physiological functions in human volunteers have been investigated by Zhong *et al.* [194]. Volunteers were assigned to groups consuming 4, 8 or 12 g PDX a day for a period of four weeks or to a control group with no intake of PDX. All concentrations of polydextrose improved bowel function, increased faecal weights and decreased faecal pH proportionally with PDX intake. A daily intake of 8 and 12 g PDX increased faecal concentrations of acetate, butyrate and isobutyrate. For all groups consuming polydextrose a decrease in faecal *Bacteroides* spp. (*B. fragilis*, *B. vulgatus* and *B. intermedius*) was observed, whereas *Lactobacillus* spp. and *Bifidobacterium* spp. increased relatively to polydextrose intake. Based on these results a daily intake of 4-12 g/day polydextrose has beneficial effects on gut health with proliferation of favourable groups of gut bacteria and an acidification of the gut environment.

Table 3. Chemical structure, natural source and method of manufacture of non-digestible carbohydrates

Carbohydrate	**Chemical structure**	**DP[1]**	**Natural source/methods of manufacture**
Inulin and FOS[2]	D-fructose units linked by β-2.1 bounds. Terminal α-1.2-linked D-glucose.	Inulin 2-60 FOS ~2-7	Onion, banana, garlic, leek and chicory root Inulin: Extraction from chicory root. FOS: Hydrolysis of chicory inulin or enzymatic synthesis.
GOS[3]	D-galactose units linked by β-1.4 or β-1.6 bounds. Terminal α-1.4 bound D-glucose unit.	~2-5	Human and cow's milk. Enzymatic synthesis from lactose.
XOS[4]	Xylose units linked by ß-1.4 bounds.	~2-4	Bamboo shoots. Produced by chemical/enzymatic treatment of xylan-rich material.
Cereal ß-glucan	Linear chains of D-glucose units linked by ß-1.4 or ß-1.3 bounds.	Variable, >500	Oat, barley, rye and wheat. Extraction from natural sources.
Pectins	Largely composed of a backbone of α-1.4-linked galacturonic acid. Five structural groups with variation in side chains and backbone.	Variable, 70-100	Plant cell walls. Commercially produced from citrus peel and apple pomace.
Polydextrose	Composed of glucose units, α-1.6 bounds predominate	Variable, 1-100	Chemical synthesis by random polymerization of glucose.

[1]Degree of polymerization, [2]Fructo-oligosaccharide, [3]Galacto-oligosaccharide, [4]Xylo-oligosaccharide

4. THE FOOD-BORNE PATHOGEN *SALMONELLA* TYPHIMURIUM

Salmonella is a genus of Gram negative bacteria that are a major cause of food-borne illness globally [195]. In 2007, a total of 151.995 confirmed cases of human salmonellosis were reported in the EU (31.1 cases per population of 100.000). The specific age distribution revealed that the majority of reported cases were within the group of 0-4 year old children (125.4 cases per population of 100.000). This was approximately three times higher than the rate of cases in the age group 5-14 years and six to nine times higher than the incidence of cases reported for those aged ≥15 [196].

The two most common reported *Salmonella* serovars in 2007 in the EU were *S.* Enteritidis and *S.* Typhimurium. Together the two serovars represented 81% of known types in human cases, with *S.* Enteritidis accounting for 64.5% and *S.* Typhimurium for 16.5%. *Salmonella* was most often isolated from fresh meat and meat products, particularly of poultry origin, followed by pig meat. Other food products less frequently associated with *Salmonella* were eggs, fishery products, vegetables and fruit [196].

4.1 INTESTINAL PATHOGENESIS OF *S.* TYPHIMURIUM

S. Typhimurium infection in susceptible mice provides a well-characterized model for *S.* Typhi pathogenesis in humans. After infection with *S.* Typhimurium mice develop a systemic disease similar to human typhoide fever [197].

After oral exposure, a proportion of ingested *S.* Typhimurium cells survive the acidic environment in the stomach and arrive in the small intestine, where the pathogen translocates through the epithelial cell layer [198]. The primary site of *S.* Typhimurium invasion is believed to be M cells located in the follicle associated epithelium (FAE) of Peyer's patches in the distal small intestine [197,199]. Following M cell invasion *Salmonella* infect phagocytes, preferentially macrophages, in the lamina propia whereby the pathogen gains access to the lymphatic system and bloodstream and subsequently the liver and spleen [200].

M cell invasion by *Salmonella* is believed to be mediated, at least partly, by a specific adhesin - the long polar fimbria (LPF) [199]. This has been observed with *lpf* mutants recovered in lower numbers from Peyer's patches, mesenteric lymph nodes, liver and spleen of mice compared to recovery of wild-type *Salmonella* [201]. Other fimbria, besides LPF, suggested to also contribute to M cell targeting is the type 1 fimbria, capable of binding to mannose oligosaccharide receptors on host cells [202].

M cells are specialised in delivering antigens from the gut lumen to phagocytic cells via transepithelial vesicular transport [198,203] and are characterised by a reduced number of microvilli and a thin glycocalyx compared to enterocytes. These characteristics, combined with reduced quantities of secretory IgA at the FAE surface, makes M cells vulnerable to infection by pathogens [199]. In addition to M cell invasion *S.* Typhimurium infection has been associated with subsequent M cell and FAE destruction providing easy and less restricted translocation across the epithelial surface [204].

Invasion of M cells by *Salmonella* involves effector proteins encoded by the "*Salmonella* pathogenicity islands" (SPI), including SPI-1 and SPI-2. Both SPI-1 and SPI-2 encodes "type-III-secretion-systems" (T3SS), of which T3SS encoded by SPI-1 is important for invasion of M cells and SPI-2 T3SS for intracellular survival in host macrophages [197,205]. Following contact of *Salmonella* with M cells the SPI-1 T3SS forms a needle-like structure, through which bacterial proteins are injected into the cytosol of host cells [205]. Some of these proteins have actin-binding actitivites, resulting in cytoskeletal rearrangements leading to bacterial internalization [197,205]. The importance of SPI-1 in the virulence of *Salmonella* has been demonstrated with *Salmonella* SPI-1 mutants showing reduced abilities to invade M cells [206-208].

The ability of *Salmonella* to survie in macrophages is required for development of a systemic infection [200]. Intracellulary within macrophages *Salmonella* reside in unique membrane bound vacuoles, termed the *Salmonella* containing vacuole (SCV), which permit intracellular survival and replication [195,209]. Within the vacuole *Salmonella* escapes contact with some of the antimicrobial components produced by macrophages. Examples are reactive oxygen species (ROS) and reactive nitrogen intermediates (RNI) produced in response to *Salmonella* infection. *Salmonella* is capable of inhibiting expression or delivery of the enzymes involved in the production of ROS and RNI to the SCV in an SPI-2 dependent manner [210]. Other examples of SPI-2 functions are the role of SPI-2 effector proteins in the control of the intracellular position of the SCV [209] and in inhibition of maturation of the SCV into a phagolysosome [211].

The liver and spleen are the two main sites of *Salmonella* proliferation within macrophages. The precise mechanisms involved in spread of *Salmonella* to new cells are not fully clear, but may invole *Salmonella* residing within the macrophage for the lifetime of the hoste cell followed by infection of new macrophages [200]. Furthermore, *Salmonella* is capable of inducing macrophage cell-death by at least two mechanisms. Either a rapid macrophage cell death is induced in a SPI-1 dependent manner or a delayed macrophage cell death is induced in a SPI-2 dependent manner [200,212]. Dead or dying macrophages infected by *Salmonella* may then be phagocytosed by new macrophages providing a new site of survival and replication of the pathogen [200].

The bacterial distribution and the lesions in the liver and spleen of mice infected with *S.* Typhimurium resemble those observed in humans suffering of typhoid fever [213]. In murine infections extensive growth of *Salmonella* in the organs leads to death with a lethal load of 10^8 viable bacteria per. organ [214]. Death is believed to result from organ failure with pathological signs of infection being enlarged Peyer's patches, liver and spleen and with organs appearing pale and friable [213,214].

Alternatives to the classical M cell dependent route of *Salmonella* invasion have also been described suggesting enterocytes as potential targets for invasion [215]. Moreover, Vazquez-Torres *et al.* [216] found that a non-invasive SPI-1-deficient *S.* Typhimurium strain can disseminate from the gut lumen of mice via CD18-expressing phagocytic cells. The CD-18

mediated route was proposed based on the observation that levels of the SPI-1-deficient *Salmonella* strain in the liver and spleen of CD-18 deficient mice were reduced compared to wild-type mice. In addition, dendritic cells can penetrate epithelial cells and take up *Salmonella* from the gut lumen [217]. Furthermore, *S.* Enteritidis infection in rats have demonstrated that the infection affects gene expression in the rat colon, suggesting that in addition to the ileum, the colon is also a target for *Salmonella* invasion [218].

4.2 EFFECTS OF NON-DIGESTIBLE CARBOHYDRATES ON *SALMONELLA* INFECTIONS

Several studies with probiotics have demonstrated protective effects against murine *Salmonella* infections [78-85]. Similarly, studies with prebiotics have investigated the effect on *Salmonella* infections in broilers [219,220], swine [221-223], rats and mice [83,85,151-157]. In the following section focus will be on the effects of prebiotics and other ND dietary carbohydrates on *Salmonella* infections in rodents.

Studies with mice and rats on prevention of *Salmonella* infections have demonstrated conflicting results. Some studies have demonstrated a protective effect of either prebiotics in combination with probiotics (synbiotics) [83,85] or of prebiotics alone [83,85,155-157], while others have demonstrated adverse effects of prebiotic consumption on *Salmonella* infections [151-154].

Preventive effects of either prebiotics alone (inulin), probiotics (*Lactobacillus acidophilus*) or synbiotics (inulin and *L. acidophilus*) against *Salmonella*-induced liver damage in orally challenged mice (single dose of $5x10^6$ cfu) was investigated by Rishi *et al.* [85]. Both prebiotic (2 mg/day), probiotic (10^{10} cfu/day) and synbiotic administration resulted in decreased pathogen translocation to the liver seven days post-infection (p.i.). Furthermore, histology of liver sections showed that signs of liver damage were reduced by all three treatments as compared with non-supplemented challenged mice. However, generally a greater protection was observed for probiotics than for prebiotics and results did not indicate a synergistic effect of synbiotic administration.

Improved protection of synbiotics was demonstrated in the study by Asahara *et al.* [83], where administration of *B. breve* (10^8 cfu/day) combined with transgalactosylated oligosaccharides (TOS) (10 mg/day) improved the preventive effect of *B. breve* during a 7-day period post oral *S.* Typhimurium challenge of mice (single dose of 10^2 cfu), while TOS alone had no preventive effect on the infection.

Studies on the preventive effect of prebiotics alone have demonstrated an increased survival rate of mice fed inulin during a 2-week period post *S.* Typhimurium challenge (single dose of 10^3 cfu). Still, a mortality rate of 60% was observed in the group fed a diet containing 10% inulin compared to >80% in the control group, whereas FOS supplementation did not increase the survival rate significantly [157]. Since infection by intraperitoneal injection of the pathogen was used in the study, the model does not mimic a food-borne infection and the reduced mortality rate may be a result of immune modulation rather that protection exerted by the mucosal barrier, although this was not investigated in the study.

The ability of prebiotics to stimulate the immune response towards *Salmonella* infections was investigated by Benyacoub *et al.* [156]. In an initial study, feeding mice a diet containing 5% of a FOS:inulin mixture (70:30%) for one week prior to oral immunization with an attenuated *S.* Typhimurium vaccine enhanced the specific antibody response towards *Salmonella*, stimulated the phagocytic activity of peritoneal macrophages, and enhanced cytokine production (IFN-γ and IL-12) by spleen cells. Whether the observed immunological effects were sufficient in providing protection against oral challenge with wild-type *S.* Typhimurium SL1344 (single dose of $3x10^7$ cfu) was investigated in a second study. Alone (without vaccination) the prebiotic mixture did not provide any protection against the infection. However, the survival rate of mice fed prebiotics prior to vaccination was increased to 73% compared to 40% in vaccinated control-fed mice, suggesting that fructo-oligosaccharides can enhance the efficiency of *Salmonella* vaccines.

Besides inulin and FOS, GOS have been demonstrated to provide protection against murine *S.* Typhimurium infection [155]. In this study GOS was administrated to mice (2.5 g/kg) 30 minutes prior to oral challenge with 10^7 cfu *S.* Typhimurium SL1344. All mice dosed with GOS did not show clinical signs of infection throughout the study period (5 days). Furthermore, GOS reduced pathogen invasion of the liver and spleen (Day 3 and 4 p.i.) and reduced numbers of *Salmonella* in the ileum (Day 3 and 4 p.i.), colon (Day 3 p.i.) and cecum (Day 3 and 5 p.i.). However, the model chosen with GOS administration just prior to pathogen challenge does not mimic continuous ingestion of the prebiotic and it is likely, that the observed protective effect is an effect of blocking of pathogen adhesion rather than an effect of microbial changes induced by GOS [155].

Based on the studies above, there are indications of protective effects of prebiotics against *Salmonella* infections, although the studies by Benyacoub *et al.* [156] and Asahara *et al.* [83] demonstrate, that prebiotics alone are not sufficient in providing protection. In contrast, studies by a single group of researchers [151-154] have demonstrated increased, rather than decreased, translocation of *Salmonella* to extra-intestinal sites in prebiotic-fed rats. Diets containing 3-6% FOS or inulin were seen to increase translocation of *S.* Enteritidis (single dose of 10^8-10^{10} cfu) measured as increased urinary excretion of nitrates and nitrites (NO_x). Furthermore, prebiotic feeding increased the cytotoxicity of faecal water and faecal mucin excretion indicating mucosal irritation [151-154]. An important aspect of these studies was, that they were all based on low-calcium diets (0.80-1.20 g Ca/kg) and that a diet higher in calcium (4.0 g Ca/kg) could counteract most of the observed adverse effects [153]. The exact mechanism behind the FOS and inulin-induced adverse effects on intestinal permeability in these studies is unclear. However, it is suggested that the adverse effects is caused by increased production of lactic acid and other short chain fatty acids leading to irritation of the mucosal barrier [153,154] and that dietary calcium can counteract this effect by reducing the acidity of the gut environment [224].

5. AIM OF THE STUDY

Based on studies with probiotics [78-84], demonstrating preventive effects on murine *S.* Typhimurium infections, the aim of the present thesis was to investigate whether similar protective effects could be demonstrated with prebiotics and other potential prebiotic dietary carbohydrates.

Prebiotics are expected to improve gut health in a manner similar to probiotics. Still, they overcome some of the possible limitation of incorporating live bacteria in the diet. With prebiotics, problems associated with microbial survival during passage through the digestive tract is not a concern [118] and in contrast to probiotics, where introduced bacteria have to compete with the established microbiota, prebiotics target bacteria already colonising the gut. For these reasons, prebiotics may be a more efficient way of manipulate the gut microbiota [162].

The initial focus of the project was prevention of *S.* Typhimurium infection with the idea of identifying new prebiotic substrates with preventive effects against *S.* Typhimurium SL1344 infection. The studies were carried out using the BALB/c mouse model providing a model of human typhoid fever (Manuscript I). Subsequently, the intention of the thesis was to investigate effects of the carbohydrates with the best potential for pathogen inhibition on the composition of the gut microbiota, production of short chain fatty acids (SCFA) and immune modulation in host animals. However, results obtained from the animal studies demonstrated adverse rather that protective effects of prebiotic administration. Based on these results samples from animals showing reduced resistance to the *Salmonella* infection were chosen for further analysis with the aim of investigating changes in the intestinal microbiota and SCFA production which could potentially explain the observed differences in the infection susceptibility (Manuscript II).

Since the carbohydrates investigated were shown not to be efficient in providing protection against the *Salmonella* infection, the research stay at CSIRO Food and Nutritional Sciences was an opportunity to investigate another potential health benefit of prebiotic consumption. Research at the division has previously demonstrated that resistant starch fed to rats attenuates protein-induced colonic DNA damage - an initial sign of colon cancer [54,225-228]. Thus, the aim of the study performed at CSIRO was to investigate whether a similar preventive effect could be demonstrated by XOS and inulin *in vitro* using a two-stage continuous fermenter. At present samples from fermentation of inulin is still being analysed, wherefore only results from fermentation of XOS are included in the thesis (Manuscript III).

6. MANUSCRIPT I

SOME PUTATIVE PREBIOTICS INCREASE THE SEVERITY OF *SALMONELLA ENTERICA* SEROVAR TYPHIMURIUM INFECTION IN MICE

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Some putative prebiotics increase the severity of *Salmonella enterica* serovar Typhimurium infection in mice

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Abstract

Background: Prebiotics are non-digestible food ingredients believed to beneficially affect host health by selectively stimulating the growth of the beneficial bacteria residing in the gut. Such beneficial bacteria have been reported to protect against pathogenic infections. However, contradicting results on prevention of *Salmonella* infections with prebiotics have been published. The aim of the present study was to examine whether S. Typhimurium SL1344 infection in mice could be prevented by administration of dietary carbohydrates with different structures and digestibility profiles. BALB/c mice were fed a diet containing 10% of either of the following carbohydrates: inulin, fructo-oligosaccharide, xylo-oligosaccharide, galacto-oligosaccharide, apple pectin, polydextrose or beta-glucan for three weeks prior to oral *Salmonella* challenge (10^7 CFU) and compared to mice fed a cornstarch-based control diet.

Results: The mice fed with diets containing fructo-oligosaccharide (FOS) or xylo-oligosaccharide (XOS) had significantly higher ($P < 0.01$ and $P < 0.05$) numbers of S. Typhimurium SL1344 in liver, spleen and mesenteric lymph nodes when compared to the mice fed with the cornstarch-based control diet. Significantly increased amounts ($P < 0.01$) of *Salmonella* were detected in ileal and fecal contents of mice fed with diets supplemented with apple pectin, however these mice did not show significantly higher numbers of S. Typhimyrium in liver, spleen and lymph nodes than animals from the control group ($P < 0.20$).

The acute-phase protein haptoglobin was a good marker for translocation of S. Typhimurium in mice. In accordance with the increased counts of *Salmonella* in the organs, serum concentrations of haptoglobin were significantly increased in the mice fed with FOS or XOS ($P < 0.001$). Caecum weight was increased in the mice fed with FOS ($P < 0.01$), XOS ($P < 0.01$), or polydextrose (P <

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0.001), and caecal pH was reduced in the mice fed with polydextrose ($P < 0.001$). *In vitro* fermentation in monocultures revealed that *S.* Typhimurium SL1344 is capable of fermenting FOS, beta-glucan and GOS with a corresponding decline in pH.

Conclusion: Supplementing a cornstarch-based rodent diet with 10% FOS or XOS was found to increase the translocation of *S.* Typhimurium SL1344 to internal organs in mice, while 10% apple pectin was found to increase the numbers of S. Typhimurium in intestinal content and feces.

Background

One of the basic physiological functions of the resident microbiota is that it functions as a microbial barrier against pathogens [1]. A healthy, balanced microbiota has been suggested to be predominantly saccharolytic, with significant numbers of bifidobacteria and lactobacilli [2]. The use of pre- and probiotics has thus been suggested as approaches to prevent *Salmonella* infections and infections by enteric pathogens in general [3-5].

Prebiotics were originally defined as "non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improve host health" [6]. The main candidates that meet the required criteria for classification of a food ingredient as a prebiotic are fructo-oligosaccharides, including inulin, galacto-oligosaccharides and lactulose [7]. Numerous studies have shown that prebiotics stimulate the growth of bifidobacteria and lactobacilli *in vivo* [8-12] and specific strains from these genera have been shown to suppress bacterial infections including those caused by ingestion of *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) [13-17]. Mechanisms proposed to explain the enhanced resistance to pathogens induced by lactobacilli and bifidobacteria include (i) competitive inhibition of the epithelial and mucosal adherence of pathogens, (ii) production of antimicrobial substances, (iii) immune modulation, and (iv) production of short chain fatty acids which can reduce the growth of acid-sensitive pathogens like *Salmonella* [1,18,19].

Salmonella infections are a global problem with *Salmonella enterica* serovar Typhi (*S.* Typhi) and serovar Paratyphi (*S.* Paratyphi) causing epidemics of severe systemic infections in developing countries [20,21]. *S.* Typhi and *S.* Paratyphi do not cause systemic infections in other mammalian hosts than humans, but the BALB/c mouse model used in the present study provides a murine model of human typhoid fever [22]. In the EU, *Salmonella enterica* serovar Enteritidis (*S.* Enteritidis) and *S.* Typhimurium are the most frequently reported serovars causing human salmonellosis. A total number of 160.649 cases of human salmonellosis were reported in the EU in 2006 [23].

Despite the promising effects of probiotics on the prevention of *Salmonella* infections in mice [13,14,17,24], studies with prebiotics have shown conflicting results. Inulin has been found to reduce the mortality of mice challenged with *S.* Typhimurium [25] and in rats fed an inulin-oligofructose diet, numbers of *S.* Typhimurium in the content of ileum and caecum were reduced [26]. Additionally, increased resistance to *S.* Typhimurium infection in mice was reported with combined administration of bifidobacteria and galacto-oligosaccharides [15]. Finally, a recent study showed that oral administration of galacto-oligosaccharides to mice immediately prior to *S.* Typhimurium SL1344 infection reduced the clinical signs of infection, significantly reduced the organ counts of *S.* Typhimurium, and reduced the pathology associated with murine salmonellosis [27]. In contrast to these findings, a number of papers reporting an increased translocation of *S.* Enteritidis in rats fed inulin, fructo-oligosaccharides or lactulose have been published by one group of investigators [28-31]. However, these studies were all based on low calcium-diets and the adverse effect could be reversed by oral administration of calcium [31].

The aim of the present study was to examine if mouse susceptibility to *S.* Typhimurium SL1344 infection was affected by ingestion of carbohydrates with different structures and digestibility profiles. Effects of diets containing inulin, fructo-oligosaccharide (FOS), xylo-oligosaccharide (XOS), galacto-oligosaccharide (GOS), apple pectin, polydextrose or beta-glucan on murine *S.* Typhimurium infection were compared to a cornstarch-based control diet. This is, to our knowledge, the first study comparing the effects of non-digestible carbohydrates with different structures on *Salmonella* infection.

Results

Body weight and euthanisation

To monitor the effect of feeding with different potentially prebiotic carbohydrates on the susceptibility to infection with S. Typhimurium, groups of mice were fed with diets containing either of the seven abovementioned carbohydrates for three weeks prior to challenge with *Salmonella*.

During the three weeks of feeding on the experimental diets, no significant differences in mean body weights were recorded between the dietary groups. Following the

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Salmonella challenge, the mice were monitored and euthanized before schedule in case of adverse signs of infection due to ethical considerations.

Only mice euthanised as scheduled on Day 5 were included in the analysis. These constituted five mice in the group fed polydextrose, six mice in the groups fed apple pectin, beta-glucan and GOS, seven mice in the groups fed XOS and control diet (study B), and all mice in the remaining groups (inulin, FOS and control diet in study A+C).

Caecum weight and pH

The weight of caecum was significantly increased in mice fed diets containing FOS ($P < 0.01$), XOS ($P < 0.01$) or polydextrose ($P < 0.001$) when compared to groups fed the control diet (Table 1). Polydextrose ingestion was found to decrease ($P < 0.001$) the caecal pH (Table 1).

Salmonella *cultivated from faecal samples and distal part of ileum*

There was a trend (Figure 1), though not statistically significant, indicating that faecal counts of *S*. Typhimurium cultivated from faecal samples were higher on Day 3 after challenge in the groups fed FOS ($P = 0.068$) and XOS ($P = 0.066$) when compared to the group fed the control diet. (Data not shown). In mice fed apple pectin, faecal counts of *S*. Typhimurium were significantly higher on Day 3 ($P < 0.01$) and Day 5 ($P < 0.01$) (Figure 1C). The increased faecal counts in the apple pectin group corresponded to a significantly higher number of *S*. Typhimurium in the content of the distal part of ileum at euthanisation on Day 5 ($P < 0.01$). Also in the FOS and XOS group, there was a trend that ileal *Salmonella* counts were elevated ($P = 0.182$ and $P = 0.242$, respectively), though this was not statistically significant (Figure 1A).

Table 1: Weight and pH of caecum five days post challenge[a]

	N[b]	Caecum weight incl. content (mg)	pH of caecal content
Study A:			
Control	7	198.96 ± 14.15	7.52 ± 0.06
FOS	10	355.32 ± 32.09**	7.72 ± 0.19
XOS	7	358.74 ± 44.66**	7.45 ± 0.25
Study B:			
Control	7	181.70 ± 10.60	7.08 ± 0.12
Beta-glucan	6	206.40 ± 76.03	6.85 ± 0.17
GOS	6	174.83 ± 38.95	7.07 ± 0.15
Study C:			
Control	8	205.36 ± 20.93	7.17 ± 0.05
Inulin	8	263.24 ± 24.05	7.07 ± 0.09
Apple pectin	6	216.68 ± 18.20	7.02 ± 0.14
Polydextrose	5	637.74 ± 61.11***	6.60 ± 0.05***

[a]Values represent means ± SEM. [b]Group size on Day 5 post challenge. One mouse died during the acclimatisation period in the control group in study A. **$P < 0.01$; ***$P < 0.001$.

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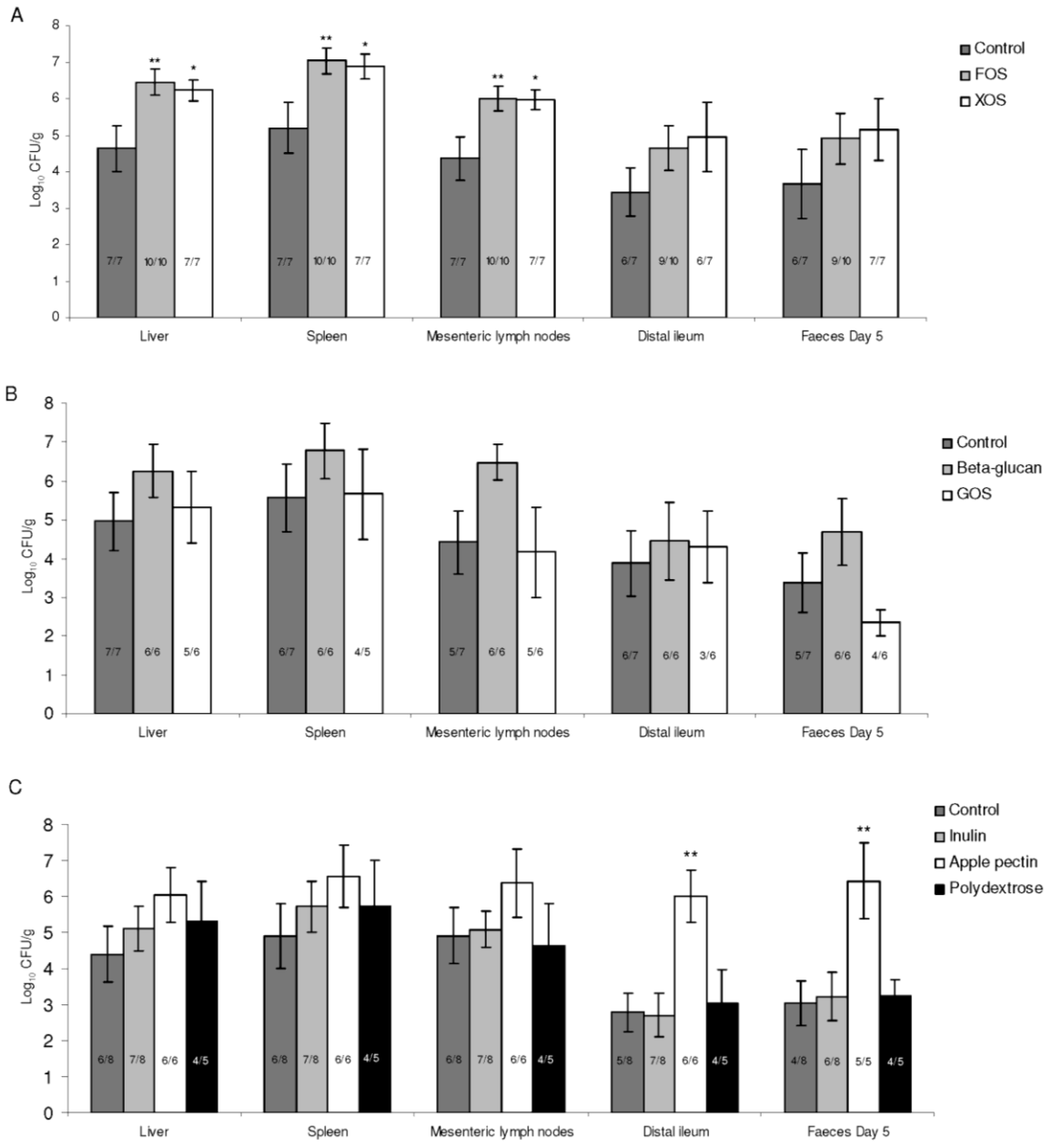

Figure 1

***Salmonella* counts in organs, distal ileum, and faeces**. Enumeration of *S.* Typhimurium SL1344 from the liver, spleen, mesenteric lymph nodes, distal part of ileum and faeces from mice five days post challenge. A: Control, FOS and XOS; B: Control, beta-glucan and GOS; C: Control, inulin, apple pectin and polydextrose. Values represent means ± SEM. Prevalences of mice with detectable numbers of *Salmonella* in the organs are shown on the columns. *P < 0.05; **P < 0.01

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Feeding with beta-glucan and GOS did not significantly affect the ileal and fecal numbers of *Salmonella* when compared to the control (Figure 1B).

Salmonella *cultivated from liver, spleen and mesenteric lymph nodes*

Numbers of *S*. Typhimurium cultivated from the liver, spleen and mesenteric lymph nodes were significantly higher in mice fed FOS ($P < 0.01$) or XOS ($P < 0.05$) with an increase in the mean CFU counts of approximately 1.6 to1.8 logs (Figure 1A). In animals fed with apple pectin, a similar trend showing increased counts of *Salmonella* in liver ($P = 0.154$) and spleen ($P = 0.198$) was observed.

Feeding with beta-glucan and GOS did not significantly affect the numbers of *Salmonella* in the investigated organs when compared to the control (Figure 1B).

Serum levels of haptoglobin

In all dietary groups the concentration of serum haptoglobin was markedly and significantly elevated by *Salmonella* challenge (Table 2). The mean haptoglobin concentration was between 1 and 25 μg/ml for all groups before infection. By contrast infection caused haptoglobin concentrations to rise to between approximately 500 to 2500 μg/ml at Day 5 post infection, which was a significant ($P < 0.05$) increase for all infected groups with the exception of the control group in study C, where only a trend was observed ($P = 0.112$).

When comparing infected groups fed putative prebiotics with infected control groups, it was seen that for mice fed FOS and XOS, serum haptoglobin concentrations were significantly higher, $P < 0.01$ and $P < 0.05$ respectively, when compared to the control group. In the other parts of the study, it was also seen that prebiotic groups generally did not cause a lower and in most cases caused a higher haptoglobin concentration after infection compared to the control group, with the notable exception of GOS where the trend was a lower level.

Cellular Composition of the Spleen of mice from Study C

To further explore the action of the immune system on *Salmonella* infection in Study C, the composition of immune cells ($CD4^+$ and $CD8^+$ T cells, NK and NKT cells, B cells, dendritic cells and neutrophils) within the spleen of non-infected as well as infected mice was analysed by flow cytometry. No significant effects of the different prebiotic feeds were demonstrated, however, a significant increase in the percentage of neutrophils ($P < 0.01$) within the spleen of infected mice was found, compared to non-infected controls (Figure 2A). This increase positively correlated with the numbers of *S*. Typhimurium cultivated five days post challenge from liver ($P < 0.001$), spleen ($P < 0.001$) and mesenteric lymph nodes ($P < 0.01$) (Figure 2B), but not from ileum (data not shown). Furthermore, a positive correlation between the percentages of $CD4^+$ T cells within the spleen of infected mice and the numbers of *S*. Typhimurium cultivated from liver ($P < 0.05$), spleen ($P < 0.05$) and mesenteric lymph nodes ($P < 0.05$) five days post challenge was established (Figure 2C), although the increase in $CD4^+$ T cells in infected mice was not significant.

Table 2: Serum haptoglobin concentrations (μg/ml) in mice before and after *Salmonella* challenge[a]

	N[b]	Unifected	Infected
Study A:			
Control	5	5.96 ± 2.37	514.97 ± 258.32*
FOS	9	1.42 ± 0.49+	1796.93 ± 268.37***++
XOS	7	4.05 ± 2.87	1584.67 ± 346.58***+
Study B:			
Control	7	25.52 ± 12.20	1469.57 ± 455.12*
Beta-glucan	6	1.56 ± 0.49	1704.18 ± 368.97***
GOS	6	7.54 ± 5.44	966.68 ± 283.58**
Study C:			
Control	7	17.03 ± 6.39	1384.38 ± 515.84
Inulin	7	9.64 ± 7.38	2369.71 ± 862.14**
Apple pectin	5	3.55 ± 2.83	1993.22 ± 673.85***
Polydextrose	5	14.82 ± 10.47	1477.68 ± 512.44*

[a]Values represent means ± SEM. [b]Numbers of mice where serum haptoglobin was measured in uninfected and infected mice. *Significantly different from the corresponding concentration measured in uninfected mice. *P < 0.05; **P < 0.01; ***P < 0.001. +Significantly different from the concentration measured in infected mice fed the control diet. +P < 0.05; ++P < 0.01.

In vitro fermentation study

By *in vitro* fermentation using monocultures of *S*. Typhimurium, this strain was seen to utilise FOS ($P < 0.01$), beta-glucan ($P < 0.05$) and GOS ($P < 0.001$), but not XOS, Inulin, apple pectin or polydextrose. In accordance with these results, a lowering of the culture pH was seen after fermentation with FOS ($P < 0.01$), beta-glucan ($P < 0.001$), and GOS ($P < 0.001$). A significant decrease in the pH was also recorded in the culture with polydextrose ($P < 0.001$) even though this carbohydrate was not found to support growth of the *Salmonella* strain (data not shown).

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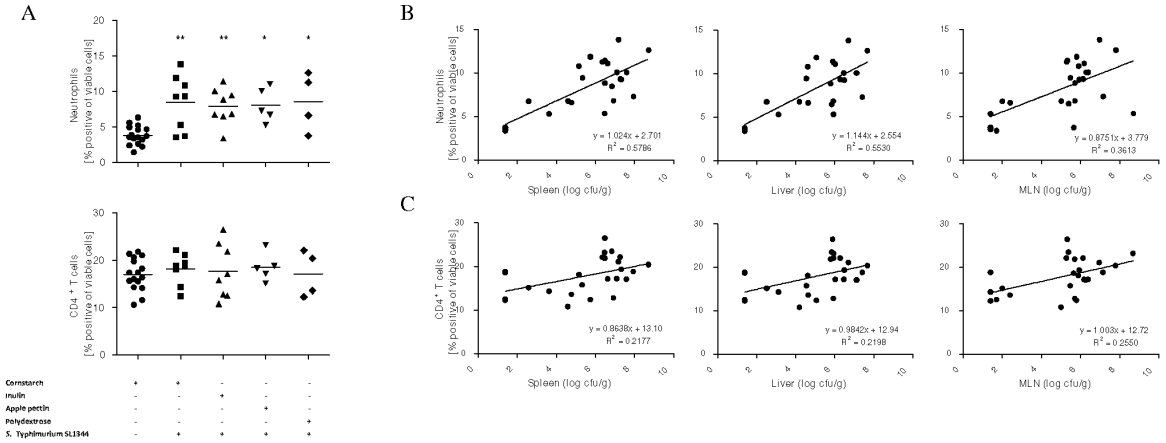

Figure 2
Prevalence and linear correlations of immune cells in spleen after Salmonella challenge. A: The percentages of neutrophils and CD4+ T cells within the spleen of infected versus non-infected mice. * P < 0.05; **P < 0.01. Linear correlations between numbers of cultivated *Salmonella* from spleen, liver and mesenteric lymph nodes and prevalence of B: neutrophils and C: CD4+ T cells.

Discussion

In the present study we report for the first time that changes in the carbohydrate composition of the diet impair the resistance of BALB/c mice to severe *S*. Typhimurium SL1344 challenge. Mice fed with a diet containing 10% FOS or XOS had significantly higher numbers of *S*. Typhimurium in liver (P = 0.006 and P = 0.023, respectively), spleen (P = 0.010 and P = 0.025, respectively) and mesenteric lymph nodes (P = 0.009 and P = 0.017, respectively) when compared to mice fed with the control diet. Additionally, a similar trend was observed for the mice fed with apple pectin, which also had elevated numbers of *Salmonella* in liver (P = 0.154) and spleen (P = 0.198).

The haptoglobin concentrations seen in the infected mice quite closely correlated with the degree of translocation of *Salmonella*, scored as the numbers of CFU of *Salmonella* in liver, spleen and mesenteric lymph nodes in the dietary groups of each of the three experiments. Thus in Study A, the significantly increased number of *Salmonella* in the organs of the FOS and XOS groups compared to the group fed the control diet (Figure 1) correlated with haptoglobin concentrations that were significantly increased in the same groups compared to the control group (Table 2). In Study B and C, no statistically significant differences after infection were detected in either haptoglobin concentration or organ counts between the dietary groups and the control group of each experiments. Still, there was a trend for correlation between high haptoglobin concentrations and high organ counts, as seen for example for the apple pectin group of Study C (in which the haptoglobin level was most significantly increased compared to the level observed before infection) while low haptoglobin levels correlated with low organ counts as observed for the GOS group in study B.

To further explore the mechanism behind the increase in haptoglobin concentration observed post challenge with *Salmonella* in study A and B, in study C we included flow cytometric analysis of the cellular composition of the spleen. Of all the cell subsets analysed, only the proportions of neutrophils were significantly increased upon infection. We also found a positive correlation between the number of neutrophils in the spleen and the CFU of *Salmonella* in the organs of the infected mice, but not the CFU of *Salmonella* in the ileum, indicating that the neutrophil number and thus the haptoglobin concentration reflects an immune response towards the bacteria translocated to the organs rather than the *Salmonella* present in the gastrointestinal tract. This is in accordance with earlier findings demonstrating that neutrophils are important for host survival during the primary response to *Salmonella* infection, primarily due to control of bacterial replication [32]. Other investigators have reported changes in other cell subsets in the spleen post infection, e.g. a decrease in T, NK and NKT cells [33], but although there was a posi-

tive correlation between organ CFU and T cell numbers, we did not find other significant changes in the cell numbers of the different cell populations analysed.

Studies reporting adverse effects of FOS and inulin on *S.* Enteritidis infections in rats have been published [28-31]. In these studies it is hypothesised that the increased translocation of *S.* Enteritidis, measured as increased urinary excretion of nitrates and nitrites, is caused by fermentation of the prebiotics producing high concentrations of lactic acid and short chain fatty acids. This was found to impair the mucosal barrier, measured as faecal mucin excretion [28-31]. However, the studies were all based on low calcium diets (0.80-1.20 g Ca/kg) and the adverse effect could be reversed by oral administration of calcium [31]. Acidification of the gut content has been shown to be counteracted by dietary calcium, suggesting that the increased translocation could be connected to low pH [34,35]. However, the diets used in our study contained the amount of calcium recommended for rodents (5 g/kg) [36], and our results thus contradict that the observed increased translocation occurs only when the diet is low in calcium. Additionally, our results contradict that acidification *per se* should mediate the increased translocation, since no drop in cecal pH was observed in animals fed with FOS or XOS in the present study (Table 1).

The major effects of prebiotic fermentation are typically seen in the large intestine, however according to the refined definition of prebiotics [7], as well to the results presented here, the effects are not restricted to the colon. *Salmonella* translocates primarily through M cells located in the ileal Peyer's patches [37], and an increased concentration would be likely to result in an increased number of phagocytosed *S.* Typhimurium. However, even though the trends in our data indicated that a high ileal content of the pathogen was accompanied by a high amount of *Salmonella* in internal organs (Figure 1), it should be noted that consumption FOS and XOS, leading to significantly increased amounts of *Salmonella* in liver and spleen was *not* accompanied by significantly increased ileal counts of the pathogen ($P > 0.20$), and that apple pectin, which significantly increased ileal Salmonella counts did not lead to significantly increased numbers of this pathogen in the internal organs ($P = 0.154$ and $P = 0.198$, respectively).

With the notable exception of GOS, our data suggest that small-molecule prebiotics increase *Salmonella* translocation more than larger molecules (Figure 1). Ten Bruggencate *et al.* [31] studied the effect of FOS and inulin on *S.* Enteritidis infection in rats and reported an increase in *S.* Enteritidis translocation in rats fed a low calcium diet with FOS as well as with inulin. However, in the present study, no increased translocation of *S.* Typhimurium was observed in mice fed inulin (Figure 1C). We speculate that the effect of prebiotics on bacterial translocation may be different in rats and mice, and may also depend on the *Salmonella* serovar used, and on other dietary or environmental factors than calcium.

A recent study demonstrated that oral administration of a mixture of GOS can reduce numbers of *S.* Typhimurium SL1344 in the liver and spleen of BALB/c mice when given just prior to infection [27]. This is in contradiction to the results reported in the present paper, which show no protective effect of GOS against *Salmonella* (Figure 1). The differences may be explained by the fact that oral delivery of GOS (2500 mg/kg) was given to mice just 30 minutes prior to *Salmonella* challenge [27], as opposed to the approach chosen in the present study, which was designed to mimic how continuous ingestion of non-digestible carbohydrates (e.g. as part of a regular diet) affects susceptibility to infection.

Our findings of increased caecum weight (Table 1) in mice fed FOS, XOS or polydextrose indicate increased fermentation in caecum. However, the increase was only accompanied by a decline in caecal pH in the group fed polydextrose. In accordance with our findings, polydextrose has been reported to increase the weight of caecal dry matter, to decrease caecal pH and to change the composition of the caecal microbial community in rats [38]. Similar changes have been reported for FOS and XOS in rats with increased numbers of caecal bifidobacteria [11].

Our *in vitro* fermentation experiment showed that *S.* Typhimurium SL1344 is capable of fermenting FOS, beta-glucan, GOS and glucose with a corresponding decline in pH. Polydextrose was not found to support growth of the *Salmonella* strain, but a significant reduction in pH was recorded, indicating metabolic activity. In accordance with our observation, Ten Bruggencate *et al.* 2003 [29] stated that *Salmonella* can use FOS as a substrate for growth. Additionally, Fooks & Gibson [18] reported growth of *S.* Enteritidis on inulin, FOS and XOS, however generally with a lower specific growth rate than selected probiotic strains. In co-culture with probiotics growth of the *Salmonella* strains was significantly reduced by FOS and XOS.

The results obtained from the *in vitro* studies did not explain our *in vivo* observations. While e.g. apple pectin was not fermented by *Salmonella in vitro*, highly increased levels of ileal *S.* Typhimurium was observed in animals fed with this carbohydrate (Figure 1C). This may reflect the growth of *Salmonella* on by-products from fermentation of apple pectin or XOS by other gut bacteria. Additionally, *in vivo*, *Salmonella* competes for nutrients with the resident microbiota, of which some bacteria may be more efficient in fermenting the various carbohydrate

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sources than what we see for *Salmonella in vitro*. Factors such as the chain length, branching, and the type of bond linking the monomers, in view of specific enzymes required for fermentation, are likely to contribute to the *in vivo* competition. Our results thus further highlight that laboratory monocultures are not adequate for prediction of bacterial growth (or absence of growth) in the complex intestinal ecosystem.

Conclusion

Based on the results presented within this study we conclude that changes in the carbohydrate composition of diets fed to mice alter the resistance to *S*. Typhimurium infections. This raises important doubts about the potential use of certain prebiotics for prevention of *Salmonella* infections. However, it should be kept in mind that our observations do not contradict the proposed beneficial effects of prebiotics in prevention of life-style related diseases such as colon cancer, inflammatory bowel disease and cardiovascular disease, which are likely to be affected by completely different mechanisms than those important for protection against pathogens.

Methods

Animals and housing

4 week-old conventional male BALB/c mice were purchased from Taconic Europe (Lille Skensved, Denmark) and housed individually in standard cages in an environmentally controlled facility with a 12-h light/dark cycle. During the study the temperature was kept at 22 ± 1°C, relative humidity at 55 ± 5% and air was changed 8-10 times per hour. Animal experiments were carried out under the supervision of the Danish National Agency for Protection of Experimental Animals.

Salmonella *strain*

A *gfp*+ tagged *S*. Typhimurium SL1344 strain resistant to nalidixic acid and chloramphenicol was constructed and used throughout this study in order to facilitate enumeration and verification of *Salmonella* in un-sterile samples. To construct this strain, a spontaneous nalidixic acid resistant mutant of *S*. Typhimurium SL1344 (designated JB371) was initially selected. Next, the genetic element P*rpsM*'-*gfp*$^+$-*cat* of strain JH3016 [33] was introduced into the chromosomally located *putPA* region of strain JB371 by P22 transduction using a P22 lysate of strain JH3016 (kindly provided by Isabelle Hautefort, Norwich, UK). The resulting *gfp*+ tagged *S*. Typhimurium SL1344 strain resistant to nalidixic acid and chloramphenicol was designated JB400 (designated *S*. Typhimurium throughout the paper).

Dietary Carbohydrates

Inulin, DP 2-60 (Orafti ST-Gel, Beneo-Orafti, Tienen, Belgium) and FOS, DP 2-8 (Orafti P95, Beneo-Orafti, Tienen, Belgium) were purchased from Alsiano, Birkeroed, Denmark. XOS, DP 2-6, GOS, DP 2-6, and polydextrose with an average DP of 12 were kindly provided by Danisco Health & Nutrition, Kantvik, Finland. Apple pectin was purchased from Obipektin AG, Bischofszell, Switzerland and beta-glucan (Glucagel™ 75) was purchased from GraceLinc Limited, Christchurch, New Zealand.

Challenge protocol

S. Typhimurium SL1344 was grown in closed 50 ml tubes at 37°C, 200 rpm overnight in 20 ml LB broth supplemented with 10 μg/ml chloramphenicol. Overnight cultures were diluted to 10^8 CFU/ml in saline and animals were orally infected with 0.1 ml (10^7 CFU) by gastric gavage. The number of CFU in the inoculum was determined by plating on LB-agar plates supplemented with 10 μg/ml chloramphenicol. The inoculum size was chosen based on a series of pilot-experiments determining the dose-response of this particular strain in the animal model.

Diets and experimental design

For an acclimatisation period of 1-2 weeks prior to commencement of the feeding experiments the mice were fed a standard mouse diet produced in house as previously described [39] based on the rodent diet AIN-93 [36] containing cornstarch as the major carbohydrate source. Subsequently, the mice were randomised to 8 dietary groups with 8 mice per group (10 in the FOS group). The experimental diets based on AIN-93 were supplemented with 10% of either of the following carbohydrates: fructo-oligosaccharide (FOS), xylo-oligosaccharide (XOS), beta-glucan, galacto-oligosaccharide (GOS), inulin, apple pectin or polydextrose in place of an equal amount (w/w) of cornstarch. Three independent studies were carried out with a cornstarch-based diet as control: Study A: Control, FOS and XOS; study B: Control, beta-glucan and GOS; study C: Control, inulin, apple pectin and polydextrose). Diets and water acidified with citric acid to pH 3.0 to prevent growth of microorganisms were provided ad libitum.

Mice were fed the respective diets for three weeks prior to *Salmonella* challenge and body weight was recorded weekly. Following the three weeks all mice were challenged with 10^7 CFU *S*. Typhimurium SL1344 and scheduled for euthanisation on Day 5 after challenge. The mice were kept on their respective diets and observed twice a day. If symptoms of severe disease (ruffled fur, changed behaviour) developed, the mice were euthanised immediately due to ethical considerations.

On the day of euthanisation the mice were dissected and *S*. Typhimurium SL1344 was cultivated from the liver, spleen, mesenteric lymph nodes and content of the distal part of ileum. The weight (with content) and pH of caecum were recorded for each mouse. In the study with FOS

and XOS the caecal content was diluted 3× in sterile water before pH was measured.

Salmonella *cultivated from organs, content of distal ileum and faecal samples*

Liver, spleen, mesenteric lymph nodes and content of the distal part of ileum were 10-fold diluted in saline and homogenised. Serial dilutions of the homogenates were plated on LB-agar plates containing 10 μg/ml chloramphenicol. The plates were incubated aerobically at 37°C overnight. Faecal samples (wet weight) were collected from mice on Days 1, 3 and 5 after *Salmonella* challenge and cultivated as described for the organ samples.

Measurement of serum haptoglobin concentrations

Blood samples were taken from all mice one week prior to *Salmonella* challenge and on the day of euthanisation for analysis of the acute phase protein haptoglobin. Haptoglobin has been described as a highly reactive acute phase protein in mice [40] whereas for example C-reactive protein is not a prominent acute phase protein in the mouse [41]. The samples were stored overnight at 5°C and centrifuged at 3000 rpm for 20 minutes for isolation of serum. Serum samples were stored at -20°C. Buffers used for the haptoglobin determination were PBS/T (0.05% (v/v) Tween 20 in PBS) and PBS/T/BSA (0.05% (v/v) Tween 20 in PBS, 1% BSA (Sigma-Aldrich A2153)). All chemicals were from Sigma-Aldrich, all incubation volumes were 100 μl/well and incubations were at room temperature, unless otherwise indicated. ELISA plates (NUNC MaxiSorp) were coated with rabbit anti human haptoglobin (DAKO A030) diluted 1:10000 in 0.1 M sodium hydrogencarbonate pH 9.6 and stored overnight at 5°C. Plates were washed four times in PBS/T, blocked with PBS/T/BSA (200 μl/well) and incubated for 30 minutes. Plates were then washed as before and loaded with a mouse haptoglobin standard (RS-90HPT, Gentaur Molecular Products, Belgium) diluted 1:2000 in PBS/T/BSA and applied in six 2-fold dilutions (each dilutions applied in two wells). Serum samples were also determined in duplicate, and diluted in PBS/T/BSA. After incubation for one hour, plates were washed as above and then incubated with biotinylated A030 diluted in PBS/T/BSA for one hour followed by washing as before. A030 was biotinylated by incubation at pH 8.2 with biotin-N-hydroxysuccinimide (approximately 100 μg/mg immunoglobulin), followed by dialysis against PBS. Finally, plates were incubated with peroxidase-conjugated streptavidin (DAKO P397) diluted 1:5000 in PBS/T/BSA for one hour, washed as before and stained with tetramethyl benzidine/peroxide substrate (TMB PLUS from Kem-En-Tec, Denmark). The reaction was stopped by adding 100 μl 0.5 M H_2SO_4 to each well and the optical density at 450 nm corrected for background optical density at 650 nm was recorded using a dedicated ELISA reader (Thermo Multiskan Ex spectrophotometer, Thermo Scientific, Waltham, MA, USA). All samples including standards were determined in duplicate. Sample values were calculated from the curve fitted to the readings of the standard (using Ascent software v. 2.6, Thermo Scientific). The detection limit of the assay was 0. 5 μg/ml.

Immunocytostaining and Flow Cytometry

Single-cell suspensions were prepared from spleens and transferred to round-bottomed 96-well polystyrene plates (NUNC, Roskilde, Denmark) with 3×10^5 cells/well. Fcγ III/II (3 μg/ml, 50 μg/ml; BD Biosciences) was added for 10 minutes to block non-specific binding of antibodies. An additional 50 μl/well PBS-Az containing fluorochrome-conjugated antibodies at various concentrations was added and the cells were incubated for 45 minutes. The cells were then washed and resuspended in 200 μl/well PBS-Az containing 2% formaldehyde for flow cytometric analyses. All stainings were carried out at or below 4°C. The antibodies used in this study were APC-conjugated anti-mouse CD4, clone RM4-5 (rat IgG2a,κ); PE-conjugated anti-mouse CD3e, clone 145-2C11 (Armenian hamster IgG); APC-conjugated anti-mouse CD8a (Ly2), clone 53-6.7 (rat IgG2a, κ); APC-conjugated anti-mouse CD49b, clone DX5 (rat IgM, κ); PE-conjugated anti-mouse CD19, clone 1D3 (rat IgG2a, κ); APC-conjugated anti-mouse CD11c, clone N418 (Armenian hamster IgG); APC-conjugated anti-mouse Ly-6G (Gr-1), clone RB6-8C5 (rat IgG2b, κ) and isotype controls for rat IgG2a, κ; rat IgG2b, κ; Armenian hamster IgG1, clone eBio299Arm; rat IgM, κ, all purchased from eBioscience. Stained cells were analysed on a BD FACSArray flow cytometer (BD Biosciences) and data was analysed using FCS Express 3.0 software (De Novo Software, CA).

In vitro *fermentation of non-digestible dietary carbohydrates*

The fermentation study was performed using a basal medium containing: peptone water (2 g/L, Oxoid), yeast extract (2 g/L, Oxoid), NaCl (0.1 g/L, Merck), K_2HPO_4 (0.04 g/L, Merck), KH_2PO_4 (0.04 g/L, Merck), $MgSO_4 \cdot 7H_2O$ (0.01 g/L, Merck), $CaCl_2 \cdot 6H_2O$ (0.01 g/L, Sigma-Aldrich), $NaHCO_3$ (2 g/L, Merck), haemin (0.005 g/L, Sigma-Aldrich), L-cystein HCL (0.5 g/L, Sigma-Aldrich), bile salts (0.5 g/L, Oxoid), Tween 80 (2 ml/L, Merck), vitamin K_1 (10 μl/L, Sigma-Aldrich), resazurin (0.001 g/L, Sigma-Aldrich) and 1% (wt/vol) test carbohydrate (inulin, FOS, XOS, GOS, beta-glucan, apple pectin, polydextrose and glucose) [42].

Stock solutions of peptone water, NaCl, K_2HPO_4, KH_2PO_4, $CaCl_2 \cdot 6H_2O$, $MgSO_4 \cdot 7H_2O$ and $NaHCO_3$ were prepared and autoclaved (121°C, 15 min.). Appropriate volumes of the stock solutions were mixed, autoclaved and supplemented with sterile filtered (0.2 μm) solutions

of bile salts, L-cystein HCL, resazurin and yeast extract. Furthermore, haemin, Tween 80 and vitamin K_1 were added. Stock solutions of the test carbohydrates were prepared by autoclaving (XOS, beta-glucan and apple pectin) or by sterile filtration (inulin, FOS, GOS, polydextrose and glucose).

An overnight culture of *S.* Typhimurium SL1344 (cultivated in 20 ml LB broth supplemented with 100 μg/ml nalidixic acid) was centrifuged at 1500 g for 30 minutes at 5°C and re-suspended in basal medium. The culture was inoculated in basal medium supplemented with test carbohydrates to an initial OD_{600} of 0.01. The fermentation study was performed under anaerobic conditions at 37°C, 200 rpm for 24 hours with recording of the initial and 24 h OD_{600} and pH values. A positive control (glucose) and a blank control with no additional carbon source added were included in the study. The sterility of the basal medium and carbohydrates was tested by incubation without bacterial inoculation. pH was measured before and after fermentation. Growth on a given carbohydrate was defined as significant difference from the OD600 measured in the blank sample after fermentation. All fermentations were performed in triplicate.

Statistical analysis

All parameters were analysed using a one-way analysis of variance (ANOVA). Where ANOVA indicated a significant difference Student's t-test was used to compare dietary groups with control. All statistical analyses were carried out using SAS JMP 6.0.2. *P* values of < 0.05 were considered statistically significant.

Authors' contributions

All authors were part of a project group, which continuously followed and discussed the progress of the experiments. AP designed and carried out the animal studies, performed the statistical analysis and drafted the manuscript. TRL and HF conceived of the study and participated in its design and coordination as well as in the preparation of the manuscript. ALP carried out the *in vitro* fermentation study, PMHH carried out the haptoglobin determination, JBA performed the fluorescent tagging of the *Salmonella* strain, RBS performed the immunocytostaining and flow cytometry, and MP contributed to feed design and statistical analysis. SJL and AO contributed significantly to the interpretation of data and the preparation of the manuscript. All authors read and approved the final manuscript.

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7. MANUSCRIPT II

ANALYSIS OF THE INTESTINAL MICROBIOTA OF OLIGOSACCHARIDE FED MICE EXHIBITING REDUCED RESISTANCE TO *SALMONELLA* INFECTION

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SUBMITTED TO BENEFICIAL MICROBES

Analysis of the intestinal microbiota of oligosaccharide fed mice exhibiting reduced resistance to *Salmonella* infection

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Abstract

Certain indigestible carbohydrates, known as prebiotics, are claimed to be beneficial for gut health through a selective stimulation of beneficial gut microbes including *Bifidobacterium*. However, stimulation of beneficial microbes does not necessarily imply a preventive effect against pathogen infection. We recently demonstrated a reduced resistance to *Salmonella* infection in mice fed diets containing fructo-oligosaccharides (FOS) or xylo-oligosaccharides (XOS). In the present study, faecal and caecal samples from the same mice were analysed in order to study microbial changes potentially explaining the observed effects on the pathogenesis of *Salmonella*.

Denaturing gradient gel electrophoresis revealed that the microbiota in faecal samples from mice fed FOS or XOS was different from faecal samples collected before the feeding trial as well as from faecal profiles generated from control animals. This difference was not seen for caecal profiles. Further analysis of faecal samples by real-time PCR demonstrated a significant increase in the *Bacteroidetes* phylum, the *Bacteroides fragilis* group and in *Bifidobacterium* spp. in mice fed FOS or XOS. The observed bifidogenic effect was more pronounced for XOS than for FOS. The *Firmicutes* phylum and the *Clostridium coccoides* group were reduced by both FOS and XOS. Surprisingly, no significant differences were detected between faecal samples collected before and after pathogen challenge in any of the groups. Furthermore, no effect of diets on caecal concentrations of short chain fatty acids was recorded.

In conclusion, diets supplemented with FOS or XOS induced a number of microbial changes in the faecal microbiota of mice. The observed effects of XOS were qualitatively similar to those of FOS, but the most prominent bifidogenic effect was seen for XOS. An increased level of bifidobacteria is thus not in itself preventive against *Salmonella* infections, since the same XOS or FOS-fed mice were previously reported to be more severely affected by *Salmonella* challenge than control animals.

Keywords: Prebiotics, fructo-oligosaccharides, xylo-oligosaccharides, beneficial microbes, bifidobacteria.

Introduction

Prebiotic carbohydrates were originally defined by Gibson and Roberfroid (1995) as "non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon" and in many contexts, dietary carbohydrates are considered prebiotic if they increase the concentration of bifidobacteria in the intestine.

Here, we report microbial changes induced by fructo-oligosaccharides (FOS) and xylo-oligosaccharides (XOS) in the large intestine of mice challenged with *Salmonella*. Many studies of prebiotic effects have focused on consumption of FOS, which is an established prebiotic substrate with a demonstrated bifidogenic effect (Roberfroid *et al*., 1998). XOS, on the other hand, is regarded as an emerging prebiotic candidate (Roberfroid, 2007; Tuohy *et al*., 2005) of which a bifidogenic effect has been demonstrated *in vitro* (Crittenden *et al*., 2002; Jaskari *et al*., 1998; Mäkeläinen *et al*., 2010) and *in vivo* (Campbell *et al*., 1997; Chung *et al*., 2007; Hsu *et al*., 2004).

Both FOS and XOS are oligomers linked by ß-glycosidic bounds that are not hydrolysed by digestive enzymes produced in the small intestine (Swennen *et al*., 2006). Hence, they pass this part of the gut undigested and may function as a substrate for the large intestinal microbiota (Swennen *et al*., 2006; Tuohy *et al*., 2005). FOS are composed of monomers of fructose units linked by ß-2.1 bounds (Roberfroid, 2005; Tuohy *et al*., 2005), whereas the monomers in XOS are xylose units linked by ß-1.4 bounds (Tuohy *et al*., 2005).

Even though several health benefits have been associated with the microbial effects of prebiotic consumption including protection against gastrointestinal pathogens (Asahara *et al*., 2001; Buddington *et al*., 2002), studies from our lab (Petersen *et al*., 2009) as well as from Ten Bruggencate and co-workers (Bovee-Oudenhoven *et al*., 2003; Ten Bruggencate *et al*., 2003; Ten Bruggencate *et al*., 2004; Ten Bruggencate *et al*., 2005) have demonstrated adverse effects on the susceptibility to *Salmonella* infections in mice and rats. More specifically, we found a markedly reduced resistance to infection by *Salmonella enterica* serovar Typhimurium SL1344 in mice fed diets supplemented with 10% FOS or 10% XOS (Petersen *et al*., 2009). Based on these results, the aim of the present study was to investigate changes in the faecal and caecal microbiota of the same mice, which could potentially explain the reduced resistance to pathogen challenge. Additionally, caecal short chain fatty acids (SCFA) were measured to determine whether their concentration was affected by changes in the microbiota. This is, to our knowledge, the first study describing diet-induced changes in the intestinal microbiota of mice exhibiting impaired resistance to *Salmonella* infections.

Materials and methods

Experimental design and sample collection

Faecal and caecal samples were obtained during a previously described feeding study with mice fed a selection of seven dietary carbohydrates (Petersen *et al*., 2009). The experimental diets based on the AIN-93 rodent diet (Reeves *et al*., 1993) were supplemented with 10% (w/w) dietary carbohydrates at the expense of cornstarch (Poulsen *et al*., 2002). The samples analysed were from mice fed diets supplemented with 10% fructo-oligosaccharides (FOS) or 10% xylo-oligosaccharides (XOS) or from mice fed the cornstarch based control diet. FOS, DP 2-8 (Orafti P95, Beneo-Orafti, Tienen, Belgium) were purchased from Alsiano, Birkeroed, Denmark and XOS, DP 2-6 were kindly provided by Danisco Health & Nutrition, Kantvik, Finland. Briefly, 4 week-old conventional male BALB/c mice were purchased from Taconic Europe (Lille Skensved, Denmark) and housed individually for an acclimatisation period of 1-2 weeks prior to onset of the feeding experiments. The mice were randomized (by weight) to groups of 8 animals (10 in the FOS group) and fed the experimental diets for three weeks prior to oral challenge with 10^7 CFU *Salmonella enterica* serovar Typhimurium SL1344 (*S.* Typhimurium SL1344). For analysis of changes in the microbial composition fresh faecal samples were collected on the day prior to onset of the feeding study (start), after 3 weeks of feeding mice the experimental diets (before challenge, BC) and on Day 4 after *Salmonella* challenge (after challenge, AC). The contents of caeca were collected at euthanization on Day 5 (Control N=7, FOS N=10, XOS N=7). Animal experiments were carried out under the supervision of the Danish National Agency for Protection of Experimental Animals.

DNA extraction from faecal and caecal samples

Either approximately 100 mg fresh faecal samples or half of the caecal content (approx. 10-170 mg) was dissolved in 1 ml TE-buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8) and centrifuged at 2100 rpm for 2 min. The supernatants were centrifuged for 5 min. at 13.000 rpm and pellets were dissolved in 1.2 ml TE-buffer. Samples were transferred to tubes containing 0.5 ml zirconia-silica beads (0.1 mm, Biospec Products) and 30 µl 10% sodium dodecyl sulphate (SDS). Bacterial cells were lysed by shaking for 4 min. on a bead-beater (Retsch MM300, VWR International) and centrifuged at 4500 rpm for 1 min. Supernatants were kept at -20 ºC until further treatment. DNA was extracted using the QIAamp DNA stool Mini Kit (Qiagen) according to the manufacturer's instructions and stored in 200 µl elution buffer at -20 ºC until use. DNA extraction failed for one faecal sample and two caecal samples.

PCR amplification for DGGE

PCR amplifications were performed in a total volume of 50 µl containing 10 µl DNA (diluted to ≤5 ng/µl from extraction), 20 µl 2.5x master mix (5Prime) and 40 pmol of each of the universal primers HDA1-GC and HDA2 targeting the V2-V3 region of the 16S rRNA gene (Walter *et al*., 2000). Amplification was performed on a Peltier Thermal Cycler model Tetrad2 (MJ Research) as a touchdown PCR. Initial denaturation was at 96 ºC for 5 min.,

amplification was carried out in 20 cycles of denaturation at 94 ºC for 1 min., annealing at 65 ºC 1 min. decreased by 0.5 ºC for each cycle, and elongation at 72 ºC for 1 min. This was followed by additionally 5 cycles of denaturation at 94 ºC 1 min., annealing at 55 ºC for 1 min. and elongation at 72 ºC for 1 min. followed by a final elongation at 72 ºC for 5 min. The products (200 bp) were verified by gel electrophoresis before proceeding to the DGGE analysis

Analysis of faecal and caecal microbiota by DGGE

DGGE was carried out using a Dcode™ Universal Mutation Detection System instrument and a gradient former model 475 according to the manufacturer's instructions (Bio-Rad). The gels were prepared from two 9% acrylamide (acrylamide-bis 37.5:1, Bio-Rad) stock solutions (0% and 100% in respect to urea and formamide concentrations) in 1xTAE (20 mM Tris, 10 mM acetate, 0.5 M EDTA, pH 7.4). The 100% stock solution corresponded to 7 M urea and 40% formamide. The gels were made with a denaturing gradient of 25-65%. 13 µl PCR product was mixed with 3 µl loading dye before loading. Gels were run in 1xTAE at 60 ºC for 16 hours at 36 V, 28 mA, stained with ethidium bromide (Bio-Rad) for 15 min., and washed for 20 min. Pictures of gels were taken by UV illumination using a Gel Doc apparatus (Bio-Rad). The BioNumerics software, version 3.0 (Applied Maths), was used for detection of bands and normalization of band patterns from the DGGE gels based on a marker loaded in every 5. lane. Cluster analyses were performed based on common and different bands using the binary coefficient Dice.

Cloning and sequencing of selected bands from DGGE gels

Bands of interest from the faecal DGGE profile of animals fed FOS or XOS were excised from the gels, placed in 40 µl sterile nuclease-free water (Ambion) and kept at 4 ºC for at least 24 hours for diffusion of the DNA into the water. 8 µl of the DNA-containing water was used in a PCR with the HDA1/HDA2 primers without GC-clamp (94 ºC for 4 min., 20 cycles of 94 ºC for 30 sec., 56 ºC for 30 sec. and 68 ºC for 1 min. followed by a final elongation at 68 ºC for 7 min.). Fresh PCR products were cloned using the TOPO TA Cloning kit for Sequencing (Invitrogen) according the manufacturer's instructions. Briefly, PCR products were cloned into pCR 4-TOPO vectors and electroporated (2500 V, 400 Ω, 25 µF) into One Shot TOP10 electrocompetent *E. coli* cells by use of a MicroPulser Electroporation apparatus (Bio-Rad). Colonies of *E. coli* cells cultured on selective Luria-Bertani plates (LB + 100 µg/ml ampicillin) were inoculated in LB broth (LB + 100 µg/ml ampicillin) overnight and plasmid DNA was isolated using the Qiagen Mini Spin Prep kit. PCR amplification with the HDA1-GC and HDA2 primers was performed on the isolated plasmid DNA as described above. The PCR products were run on a DGGE gel along with the original DNA profile to confirm the melting behaviour of the excised band. From the isolated plasmid DNA the inserts were sequenced by GATC Biotech (Konstanz, Germany) using the primer T3. The obtained sequences were compared to existing sequences in the Ribosomal Database (RDP, Michigan State University, Release 10) and in the NCBI GenBank database using nucleotide blast.

Real-time PCR analysis

Real-time PCR was performed on DNA extracted from faecal and caecal samples using the primers and amplifications conditions listed in Table 1. Amplifications were performed at 50 ºC 2 min., 95 ºC 10 min. and 40 cycles of 95 ºC 15 sec. and 56-60 ºC 1 min. (Table 1) on an Applied Biosystems 7900HT instrument in a total volume of 20 µl containing 2 µl template DNA, 10 µl SYBR Green Supermix with premixed ROX (Bio-Rad), 200 nM primers and nuclease free water (USB Corporation) to a final volume of 20 µl. All results were calculated from a standard curve based on DNA from one animal with the threshold cycle (Ct) calculated by the ABI software as the PCR cycle, where amplification signals exceed the selected threshold value, also set by the software. Analysis of the standard curve allowed verification of PCR efficiencies close to 100% for the chosen PCR conditions. The calculated results were analyzed as ratios of species specific 16S rRNA levels relative to total bacterial 16S rRNA levels in order to correct data for differences in total DNA concentration between individual samples. All samples were analyzed in duplicates. The specificity of the primers was verified by post-PCR melting curve analysis (56-95 ºC) and 2% agarose gels. In the analysis of the caecal samples animal no. 21 (FOS) and no. 27 (XOS) were excluded due to low DNA concentrations.

Table 1. Primers and amplification conditions used for real-time PCR analysis

Target group	Primer	Annealing/elongation temperature	Reference
Total bacteria	1114F 1275R	60 ºC	Denman and McSweeney, 2006
Bacteroidetes phylum	Bact934F Bact1060R	60 ºC	Guo *et al*., 2008
Firmicutes phylum	Firm934F Firm1060R	60 ºC	Guo *et al*., 2008
B. fragilis group	Bfr-F Bfr-R	56 ºC	Liu *et al*., 2003
C. coccoides group	g-Ccoc-F g-Ccoc-R	58 ºC	Matsuki *et al*., 2004
Lactobacillus spp.	Lacto-F Lacto-R	58 ºC	Rinttila *et al*., 2004
Bifidobacterium spp.	F-bifido R-bifido	60 ºC	Delroisse *et al*., 2008

Analysis of SCFA composition in caecal contents

Acetate, propionate, and butyrate in the caecal contents were analyzed using capillary electrophoresis and indirect UV detection by a method modified from Westergaard *et al.* (Westergaard *et al*., 1998). Half of the caecal content (approx. 10-170 mg) was diluted 30x (w/vol): initially 3x diluted in sterile water to gain a volume large enough for pH measurement followed by 10x dilution in an alkaline buffer (0.1 M Tris with 100 µM malonic acid as internal standard, pH 8.7). Samples were centrifuged (14000 g, 10 min., 4 ºC) and supernatants were sterile filtered by centrifugation (12000 g, 5 min., 4 ºC) using 0.45 µm Ultrafree-MC Centrifuge filter devices (Millipore). Samples were kept at -80 ºC until further analysis. A running buffer (2 mM 1.2.4-benzenetricarboxylic acid, 8 mM Tris, 0.3 mM

tetradecyl-trimethyl-ammonium bromide, pH 7.6) was prepared and sterile filtered (0.45 μm). Prior to analysis samples were diluted 2x in ½ running buffer (running buffer diluted 1:1 in sterile water). A standard containing 0-800 μM acetic, propionic and butyric acid diluted in ½ running buffer with 50.8 μM malonic acid was included in each run. Samples and standards were measured in duplicates. The analysis was performed using a fused-silica capillary with an id. of 75 μm and a length of 72/80.5 cm (72 cm to the detector and 80.5 cm total length) (Aligent Technologies). Prior to each run the capillary was pre-treated with 1 M NaOH for 30 sec., 0.1 M NaOH for 1 min., water for 30 sec. and running buffer for 5 min. Samples were injected by pressure (35 mbar) and run at -30 kV for 15 min. at 20 ºC on a G1600A ^{3D}Capillary electrophoresis instrument (Hewlett-Packard). Measurement of SCFA failed in animal no. 15 (FOS) and animal no. 27 (XOS) likely due to the degree of dilution (30x) of the caecal content at euthanization.

Statistics

Statistical analysis was performed using a one-way ANOVA on data with diet as the only variable (SCFA analysis and real-time PCR analysis of caecal samples). A two-way ANOVA was performed on data with time and diet as variables (number of DGGE bands and real-time PCR analysis of faecal samples). When ANOVA indicated a significant difference, Student's t-test was used to compare means of treatments. All statistical analyses were carried out using SAS JMP 7.0. P-values of <0.05 were considered statistically significant.

Results

Denaturing Gradient Gel Electrophoresis (DGGE) profiling

Microbial diversity of the faecal and caecal samples was assessed by DGGE of 16S ribosomal genes amplified by universal bacterial primers. The number of bands did not differ significantly between dietary groups or between time points. The average number of bands (mean±SEM) in the faecal and caecal DGGE profiles from the control group were 15.6±0.9 (start), 18.1±1.0 (before challenge, BC), 18.1±0.5 (after challenge, AC) and 17.0±2.0 (caecum). In the FOS-fed group, the numbers were 16.7±1.3 (start), 15.6±1.4 (BC), 16.1±1.3 (AC) and 15.0±1.9 (caecum), while the number of bands measured in profiles from the XOS-fed group was 14.1±1.5 (start), 15.7±0.9 (BC), 16.8±1.2 (AC) and 17.3±1.1 (caecum).

Dice cluster analysis of DGGE profiles revealed that feeding mice with FOS (Figure 1A) or XOS (Figure 1B) for three weeks induced changes in the composition of the faecal and caecal microbiota as compared to the start faecal profiles obtained before onset of the prebiotic feeding. Only in one animal in the XOS group (animal no. 29) the start profile did not cluster with the remaining start samples (Figure 1B). For most animals BC and AC profiles clustered next to each other, indicating that *Salmonella* challenge did not affect the profiles (Figure 1A and 1B). In mice fed the control diet, DGGE profiles from start, BC and AC samples clustered together, while the caecal profiles clustered separately from the faecal profiles (Figure 1C), indicating that the faecal microbiota was different from the caecal microbiota in these animals.

The faecal DGGE profiles from mice fed FOS or XOS at sampling time BC (Figure 2A) or AC (Figure 2B) were different from the control group, whereas the FOS and XOS profiles clustered together, indicating that the two oligosaccharides had comparable effects on the composition of the faecal microbiota. In contrast, DGGE performed on DNA extracted from the caecal contents did not cluster into feeding groups (Figure 2C). Profiles from faecal samples collected prior to feeding the prebiotic or control diets were similar between groups (data not shown). Three prominent bands present in most faecal BC and AC samples from FOS or XOS fed mice, but absent or weak in all samples collected before prebiotic feeding and in the control group were identified (marked 1, 2 and 3 in Figure 2A and 2B). Sequencing of two bands from BC profiles belonging to band class no. 1 or no. 2 revealed a similarity of ≥95% to species within the genus *Bacteroides*. Band class no. 3 was found to represent members of the family *Lachnospiraceae* belonging to the order *Clostridiales* within the *Firmicutes* phylum. However, determining the identity of band no. 3 to genus level was not possible, probably due to the limited length (200bp) of the cloned PCR-product.

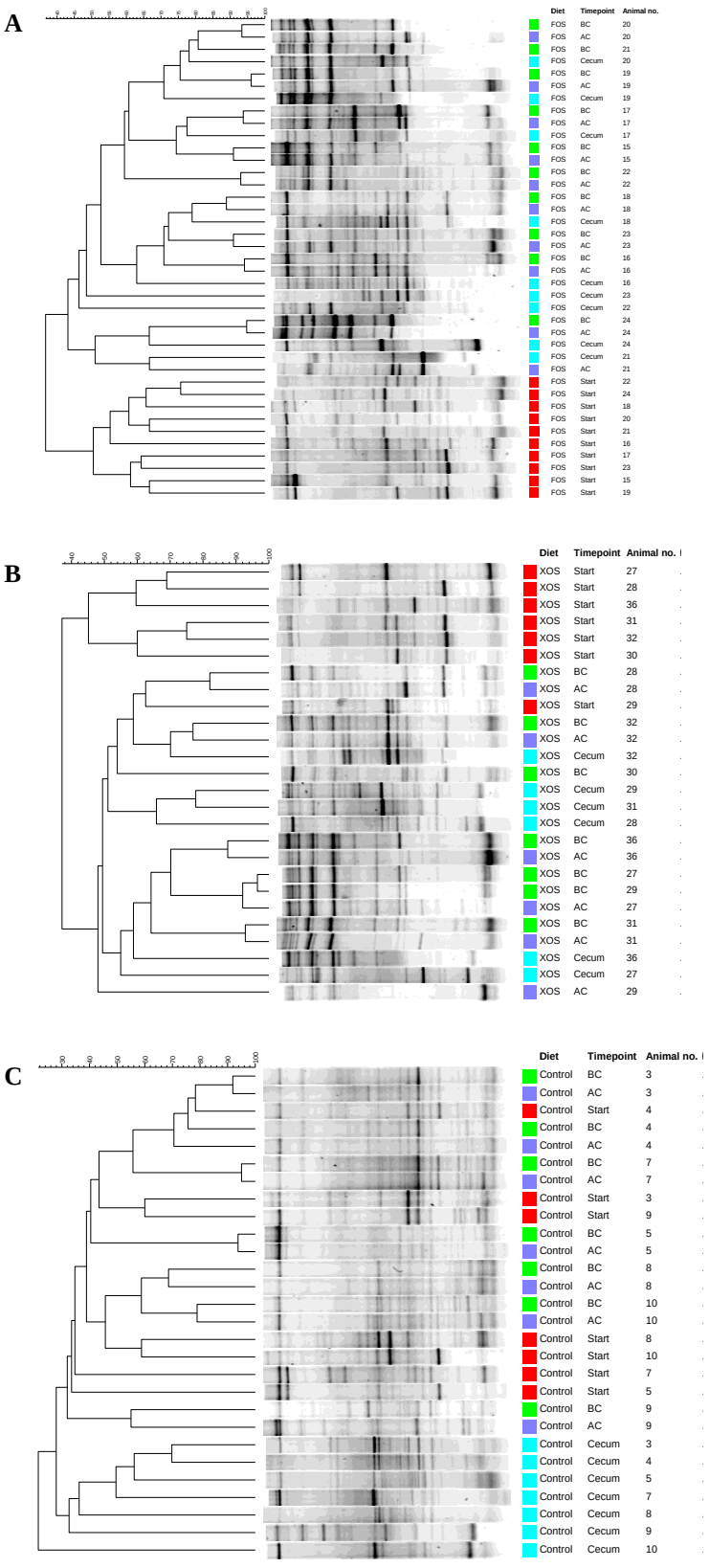

Figure 1. Faecal and caecal DGGE profiles of mice fed FOS, XOS or the control diet.
Dice cluster analysis (optimization = 0.42) of faecal and caecal DGGE profiles of mice fed 10% FOS (A), 10% XOS (B) or the control diet (C). Faecal samples were collected on the day prior to onset of the feeding trial (start), after 3 weeks of feeding mice the experimental diets (before challenge, BC) and on Day 4 after *S.* Typhimurium SL1344 challenge (after challenge, AC). Caecal samples were collected at euthanization on Day 5 after *Salmonella* challenge.

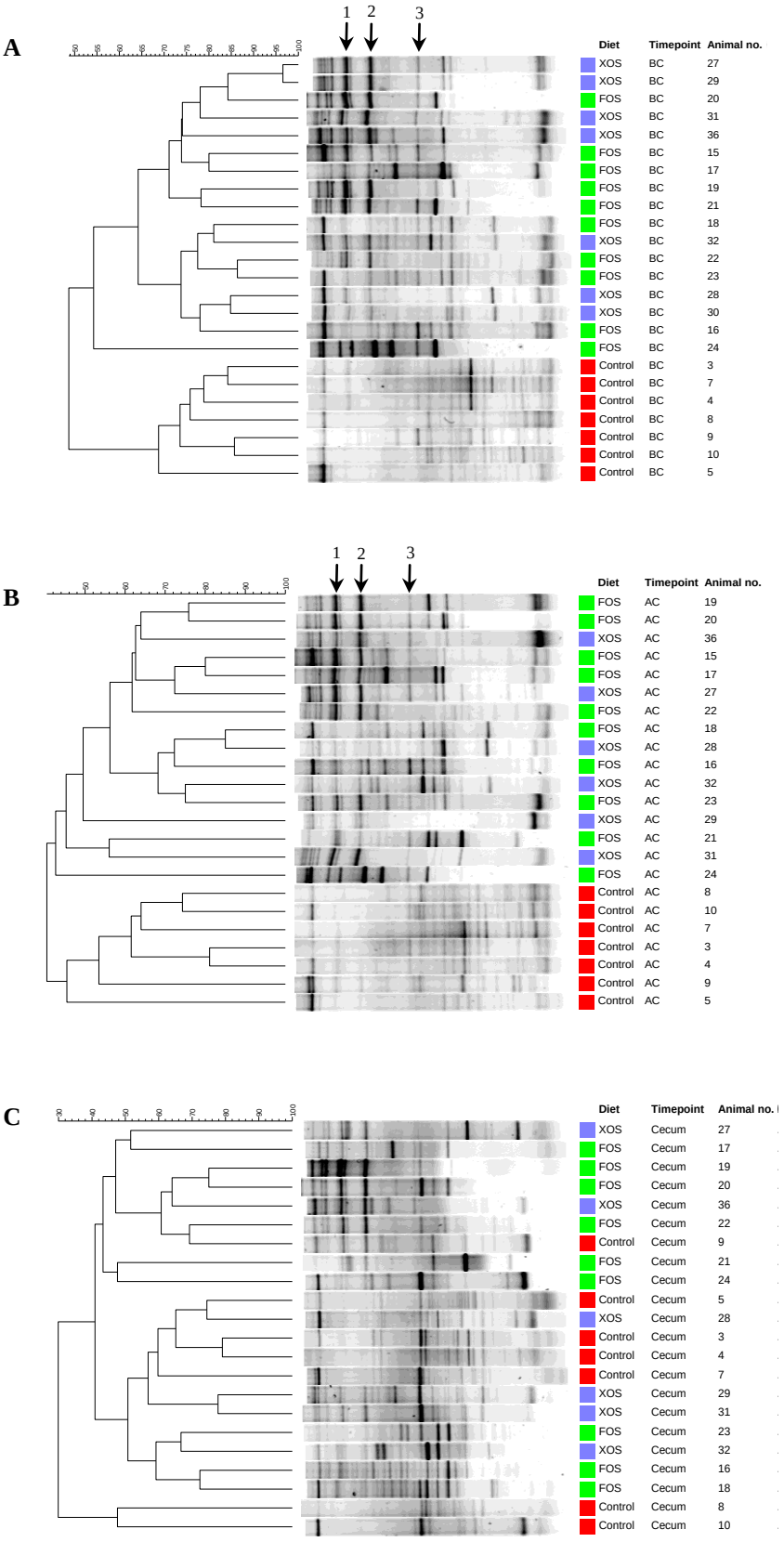

Figure 2. Comparison of DGGE profiles from FOS, XOS or control-fed mice. Dice cluster analysis (optimisation = 0.42) of DGGE profiles of A) faecal samples collected before challenge (BC); B) faecal samples collected on Day 4 after *S.* Typhimurium SL1344 challenge (AC); C) caecal samples collected at euthanization on Day 5 after *Salmonella* challenge. Arrows indicate band classes present in BC and AC profiles from FOS or XOS fed mice, but absent or weak in start profiles and profiles from the control group.

Real-time PCR analysis of the faecal and caecal microbiota

Real-time PCR was performed to study quantitative changes in the faecal and caecal microbiota induced by the experimental diets and by *Salmonella* challenge. Within the control group, comparison of faecal samples collected before challenge (BC) and after challenge (AC) with start samples demonstrated that only the *Clostridium coccoides* group was changed over time. Compared to start samples set to 100, the *C. coccoides* group was reduced to 44.6±9.0 (mean±SEM) in BC samples (P=0.0128) (Figure 3A).

The prevalence of bacteria belonging to the *Bacteriodetes* phylum or to the *Bacteroides fragilis* group was significantly increased in faecal samples from mice fed FOS or XOS. Compared to start samples set to 100, the *Bacteriodetes* phylum was increased approximately 2-fold by FOS feeding (BC: 237.3±33.9, P<0.0001; AC: 202.3±36.3, P=0.0034), while the *B. fragilis* group was increased from 2.5- to 3-fold (BC: 289.8±23.8; AC: 263.7±29.1; both P<0.0001). XOS feeding increased the *Bacteriodetes* phylum approximately 2-fold in BC samples (195.4±21.5, P=0.0473) and the *B. fragilis* group by 3- to 4-fold (BC: 406.9±29.2, P<0.0001; AC: 292.5±77.8, P=0.0032) (Figure 3A).

The prevalence of bacteria belonging to the *Firmicutes* phylum or the *C. coccoides* group was significantly reduced in faeces by FOS or XOS feeding (Figure 3A). Compared to start samples set to 100, FOS feeding reduced both groups by approximately 2-fold (*Firmicutes*: BC 47.6±9.0; AC 41.1±5.3, both P<0.0001; *C. coccoides* group: BC 52.1±15.0, P=0.0038; AC 40.4±7.8, P=0.0004). XOS feeding reduced *Firmicutes* by approximately 2-fold (BC: 44.0±11.4, P=0.0012; AC: 44.0±14.7, P=0.0019) and the *C. coccoides* group by 3- to 6-fold (BC: 16.1±4.1, P=0.0002; AC: 32.7±18.0, P=0.0028) (Figure 3A).

The abundance of *Bifidobacterium* spp. in faecal samples was markedly increased by FOS and XOS feeding. Compared to start samples set to 100, a more than 100-fold induction in *Bifidobacterium* was seen for mice fed FOS (BC 13613.7±3952.7, P=0.0291; AC 16694.8±6675.8 P=0.0079). The bifidogenic effect of XOS was even stronger. Compared to start samples set to 100, XOS increased the abundance of faecal *Bifidobacterium* by approximately 800-fold (BC 83115.6±17728.8, P<0.0001; AC 76544.9±27556.3, P=0.0001), which was up to 6-fold more than observed for FOS (BC P=0.0038; AC P=0.0445) (Figure 3B). The levels of *Lactobacillus* spp. were unaffected in all groups (data not shown).

No significant differences were detected between BC and AC samples in any of the groups demonstrating that *Salmonella* infection did not affect the abundance of the studied bacterial groups. Analysis of the caecal samples collected at euthanization on Day 5 after *Salmonella* challenge revealed that only the *B. fragilis* group in mice fed FOS was significantly affected by the dietary changes. Compared to the control group set to 100 the abundance of the *B. fragilis* group in the caecum of FOS fed animals was increased to 243.0±40.1 (P=0.0212) (data not shown).

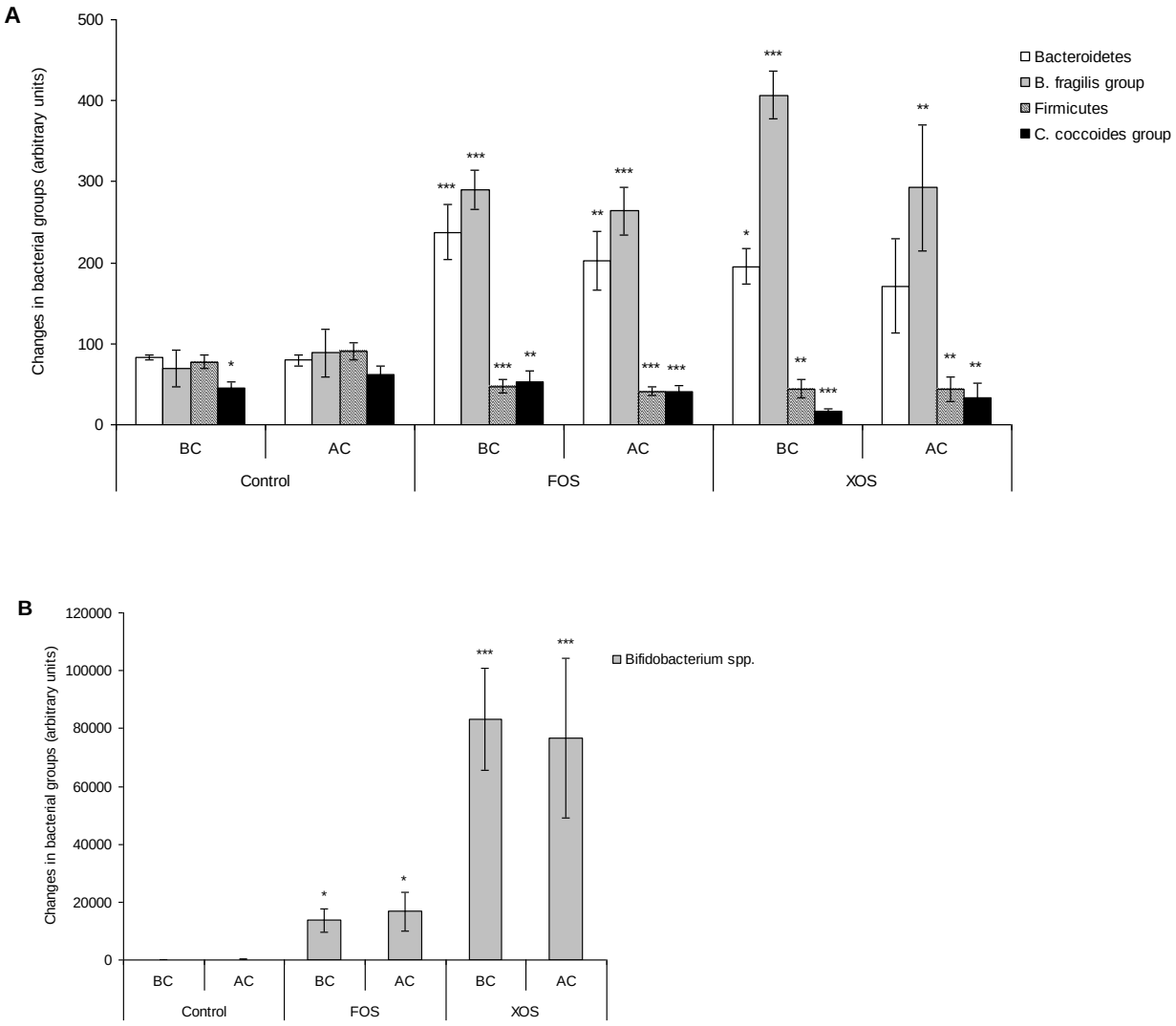

Figure 3. Changes in bacterial groups in faecal samples analysed by real-time PCR. Real-time PCR analysis of faecal samples collected after 3 weeks of feeding mice the experimental diets (before challenge, BC) and on Day 4 after *S.* Typhimurium SL1344 challenge (after challenge, AC) compared to start samples set to 100. A) Changes in the *Bacteroidetes* phylum, the *Bacteroides fragilis* group, the *Firmicutes* phylum and the *Clostridium coccoides* group. B) Changes in *Bifidobacterium* spp. Changes in bacterial groups are presented as mean±SEM. *P<0.05; **P<0.01; ***P<0.001.

Effects of diets on caecal SCFA concentrations

Of the three fatty acids measured, the caecal SCFA concentrations were dominated by acetic acid followed by propionic and butyric acid. The concentrations of acetic acid, propionic acid and butyric acid were (mean±SEM) 59.1±5.6, 9.3±0.8, 6.4±1.4 mM in control mice, 50.6±8.9, 12.9±2.4, 4.3±1.3 mM in FOS fed mice, and 60.7±24.5, 13.5±3.5, 4.4±0.8 mM in XOS fed mice, respectively. Consumption of FOS and XOS had no significant effect on the concentration of the three acids measured.

Discussion

In the present study we report how diets supplemented with 10% FOS or 10% XOS affect the faecal and caecal microbiota of mice challenged with *Salmonella*. We have previously shown that FOS or XOS supplemented diets impair the resistance of mice to *Salmonella* infection resulting in significantly higher numbers of *Salmonella* in the liver, spleen and mesenteric lymph nodes, as well as increased levels of acute-phase proteins in the blood when compared to a control group (Petersen *et al*., 2009). In the present study, DGGE, real-time PCR and SCFA analyses were performed on samples collected from the same mice.

DGGE analysis revealed that both FOS and XOS consumption induced changes in the faecal microbiota compared to either start samples (Figure 1A and 1B) or control animals (Figure 2A and 2B). In contrast, caecal profiles were not different from control animals (Figure 2C). Our data thus suggests, that changes induced by FOS or XOS fermentation are more pronounced in faeces than in the caecum. Consumption of the control diet did not induce changes in the DGGE profiles generated from faecal samples. However, the caecal profiles from control-fed animals clustered separately from the faecal profiles (Figure 1C). This is in agreement with other studies showing that the caecal microbiota differs from the microbiota of faecal samples (Marteau *et al*., 2001).

In faecal samples a significant increase in bacteria belonging to the *Bacteroidetes* phylum and the *B. fragilis* group was recorded by real-time PCR for mice fed FOS or XOS compared to start samples - with the sole exception of *Bacteroidetes* in AC samples from the XOS group (Figure 3A). This is in agreement with the DGGE profiling showing prominent bands representing *Bacteroides* spp. in the XOS and FOS groups. *Bacteroides* spp. have been described as the most numerous and versatile polysaccharide utilizers in the colon and have been shown to degrade a variety of plant oligo- and polysaccharides (Gibson and Roberfroid, 1995; Salyers *et al*., 1977; Van Laere *et al*., 2000). Furthermore, the genome of *Bacteroides thetaiotaomicron*, isolated from human faeces, encodes several glycosylhydrolases including ß-fructofuranosidases involved in the breakdown of fructo-oligosaccharides (Xu *et al*., 2003) and *Bacteroides* spp. have been shown, at least to some extent, to ferment FOS *in vitro* (Van Laere *et al*., 2000). In addition, *in vitro* studies have demonstrated growth of *Bacteroides* spp. on XOS (Crittenden *et al*., 2002; Jaskari *et al*., 1998; Van Laere *et al*., 2000). However, utilization of XOS by *Bacteroides* was less efficient compared to bifidobacteria in the study by Jaskari *et al.* (1998).

Bifidobacterium spp. were markedly increased in the faecal microbiota by feeding on both FOS or XOS. However, the increase was larger for the XOS group compared to the group fed FOS (Figure 3B). The observed bifidogenic effect is consistent with results from other studies on the effect of FOS and XOS on the gut microbiota of mice (Santos *et al*., 2006) and rats (Campbell *et al*., 1997; Hsu *et al*., 2004). Furthermore, β-fructofuranosidases involved in the breakdown of FOS have been identified in several species of bifidobaceria (Janer *et al*., 2004; Ryan *et al*., 2005; Schell *et al*., 2002; Warchol *et al*., 2002) and growth of mono-cultures of bifidobacteria on both FOS and XOS have been demonstrated *in vitro* (Crittenden *et al*., 2002; Jaskari *et al*., 1998; Van Laere *et al*., 2000). A selective stimulation of bifidobacteria is one of

the claimed health benefits of prebiotic consumption and is believed to play an important role in maintaining colonization resistance and inhibition of growth of intestinal pathogens (Gibson *et al*., 2005; Gibson and Roberfroid, 1995). However, the bifidogenic effect observed for FOS and XOS in the present study obviously did not result in protection against the *Salmonella* infection.

The lack of effect on *Lactobacillus* spp. reported within this paper is in agreement with the study by Campbell *et al.* (1997), where FOS and XOS were found to have no effect on either faecal and caecal numbers of *Lactobacillus* in rats. *In vitro*, XOS was found not to support the growth of *Lactobacillus* spp. in the study by Jaskari *et al.* (1998), whereas only a limited growth of *Lactobacillus* spp. on FOS and XOS has been reported in other *in vitro* studies (Crittenden *et al*., 2002; Van Laere *et al*., 2000).

Concentrations of bacteria belonging to the *Firmicutes* phylum and the *C. coccoides* group were significantly reduced in faeces by both FOS and XOS feeding (Figure 3A). This reduction and the increase in *Bacteroidetes* and the *B. fragilis* group demonstrate comparable effects of FOS and XOS fermentation, as also observed in the DGGE analysis. In the caecal samples, only the *B. fragilis* group was significantly increased in the FOS group supporting the lack of clustering in DGGE profiles from the caecum (Figure 2C).

None of the dietary interventions had any effect on the concentration of short chain fatty acids in the caeca of the mice. In this context it should be noted that a reduction in the *C. coccoides* group, comprising important butyrate producing strains (Louis and Flint, 2009), was observed in faecal samples but not in the caecum. Thus, differences in the infection susceptibility can not be explained by differences in butyrate levels. In a series of studies reporting adverse effects of FOS on S. *enteritidis* infections in rats (Bovee-Oudenhoven *et al*., 2003; Ten Bruggencate *et al*., 2003; Ten Bruggencate *et al*., 2005) production of lactic acid and other short chain fatty acids were hypothesised to cause the increased translocation of *Salmonella*. Since no increase in SCFA production was reported in this study our results do not support the hypothesis.

We speculate that the observed reduction in the *Firmicutes* phylum and the *C. coccoides* group in faecal samples may partly explain the reduced resistance to the *Salmonella* infection as seen for FOS or XOS fed mice. The *C. coccoides* group constitutes a significant part of the *Firmicutes* phylum in mice (Ley *et al*., 2005) and are considered to be important for colonic health due to the production of butyrate (Louis and Flint, 2009). However, it should be noted that changes in the colonic microbiota might only have limited effects on the *Salmonella* infection since M cells located in the ileal Peyer's patches are believed to be the primary site of pathogen translocation (Santos *et al*., 2003). On the other hand, alternative routes of intestinal translocation of *Salmonella* have been described, suggesting enterocytes as potential targets for invasion (van Asten *et al*., 2005) as well as uptake of the pathogen from the gut lumen by CD18-expressing phagocytes (Vazquez-Torres *et al*., 1999). Furthermore, *Salmonella* infection in rats has been shown to affect colonic mucosal gene expression, suggesting that both the ileum and colon are targets for *Salmonella* invasion (Rodenburg *et al*., 2007).

In conclusion, results from our study suggest that previously published adverse effects of FOS and XOS on *Salmonella* infections in mice (Petersen *et al*., 2009) might be associated with diet-induced changes in the intestinal microbiota and even though a significant bifidogenic effect was seen, a protective effect against *Salmonella* infection was not observed. We conclude that while bifidobacteria may have beneficial effects on some aspects of colonic health (Gibson and Roberfroid, 1995), a similar beneficial effect against intestinal infections does not necessarily occur.

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8. MANUSCRIPT III

XYLO-OLIGOSACCHARIDES ALTER BACTERIAL POPULATIONS IN A TWO-STAGE CONTINUOUS FERMENTER, INCREASE BUTYRATE PRODUCTION AND AFFECT PROTEIN-INDUCED FAECAL WATER GENOTOXICITY

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IN PREPARATION FOR PUBLICATION

Xylo-oligosaccharides alter bacterial populations in a two-stage continuous fermenter, increase butyrate production and affect protein-induced faecal water genotoxicity

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Abstract

Diets high in meat, but low in fibre, have been associated with increased risk of developing colon cancer, while a reduced risk has been linked to diets high in fibre. Studies in rats also show that dietary fibre as resistant starch can attenuate colonic DNA damage induced by high levels of dietary protein, including a soy protein isolate. In the present study, we examine whether xylo-oligosaccharides (XOS) can reduce protein-induced faecal water genotoxicity. Substrates were fermented *in vitro* by a human faecal microbiota using a two-stage continuous fermenter simulating the conditions of the proximal colon (vessel 1, pH 5.5) and of the distal colon (vessel 2, pH 6.8). As an inducer of genetic damage 3% soy protein was added to a basal media along with either 1% cornstarch or 1% XOS. Ten days of cornstarch fermentation with soy protein followed by ten days of XOS fermentation with soy protein significantly reduced faecal water genotoxicity in vessel 1, while an increased genotoxicity was observed for vessel 2. In both vessels XOS fermentation significantly increased the average butyrate concentration. Relative to cornstarch fermentation XOS increased numbers of the *Clostridium coccoides* group in both vessels. Furthermore, the *Bacteroides fragilis* group, *Lactobacillus* spp. and sulphate-reducing bacteria (SRB) were increased in vessel 1. In both vessels a reduction in *Faecalibacterium prausnitzii* was observed in addition to reductions in *Bifidobacterium* spp., the *Clostridium leptum* group and SRB in vessel 2. Based on these results XOS fermentation is capable of stimulating butyrate producing bacteria (the *C. coccoides group*), to increase butyrate concentrations and to alter protein-induced faecal water genotoxicity with a potential to protect against genetic damage in the proximal colon.

Introduction

During recent years there has been a considerable interest in dietary components that can modulate the gut microbiota and potentially improve gut health [1]. In this regard the concept of prebiotic carbohydrates has been developed to selectively stimulate the growth and activity of beneficial bacteria (predominately bifidobacteria and lactobacilli) in the intestinal tract [2,3]. The most well studied prebiotics are inulin and fructo-oligosaccharides (FOS), while xylo-oligosaccharides (XOS) are considered as promising prebiotic candidates [3,4]. XOS oligomeres are composed of xylose units linked by ß-1.4 linkages [5,6]. Due to their ß-configuration the oligomeres are not degraded by human digestive enzymes and reach the large intestine chemically intact, where they act as a substrate for the colonic microbiota [6]. XOS has been shown to support the growth of several species of bifidobacteria in *in vitro* mono-cultures [7,8] and in mixed cultures [9-11]. *In vivo*, studies have demonstrated a bifidogenic effect in rats [12,13] and humans [14]. Effects on lactobacilli are more diverse with some *in vitro* and *in vivo* studies reporting no significant change in this bacterial genus [7,9,11,12], while others report growth of at least some *Lactobacillus* spp. on XOS [8,10,15].

Several health-benefits of prebiotic consumption have been postulated including a potential reduced risk of colon cancer [16,17]. The possibility of dietary modification of colon cancer risk have been investigated by Hsu *et al.* [13] by administration of XOS and FOS to 1,2-dimethylhydrazine (DMH) treated rats. In the study both prebiotics reduced numbers of pre-cancerous lesions (aberrant crypt foci) in the distal colon, with the largest reduction observed for XOS.

Epidemiological studies indicate that the risk of developing colon cancer is increased by consumption of high-protein diets [18-20]. In contrast, consumption of complex carbohydrates may provide protection against development of colon cancer [5,21-27]. Mechanisms responsible for the protective effect include reduced transit time, as a result of increased bacterial biomass and hence increased stool frequency [28], increased short chain fatty acid (SCFA) production, in particular butyrate [29,30], and changes in the microbial composition towards primarily saccharolytic bacteria (eg. bifidobacteria and lactobacilli) [16]. Butyrate is the preferred energy source for colonic epithelial cells [31], and is believed to be an important mediator of the protective effect of dietary fibres against colon cancer [23,32,33]. From *in vitro* studies proposed mechanisms responsible for the anti-carcinogenic effect of butyrate is induction of cell cycle arrest and apoptosis in cancer cells promoting a normal phenotype of colonocytes [34-37].

Using a continuous two-stage fermenter system the aim of the present study was to investigate the genotoxic potential of faecal water from XOS fermentation using the comet assay to elucidate whether protein-induced genetic damage on human HT-29 colonocytes could be reduced by fermentation of XOS as compared to cornstarch, and to relate these changes to the effect of XOS fermentation on the composition of a human faecal microbiota and SCFA production.

Materials and Methods

Faecal inoculum

Faecal samples from five healthy adult volunteers, who had not received antibiotics or experienced episodes of diarrhoea for 4 weeks prior to the study, were collected and kept on ice at 5 ºC in airtight plastic bags until use (a maximum of two hours on ice was allowed). In an anaerobic chamber samples were homogenized, pooled and diluted in anaerobic PBS (0.1 M, pH 7.2) to produce a 20% (w/v) faecal slurry. The slurry was prepared just prior to inoculation of the fermenter.

Fermentation media

The fermentation study was preformed using a basal media prepared according to Bruck *et al.* [38] with minor changes: NaCl (0.05 g/L, Sigma-Aldrich), K_2HPO4 (0.02 g/L, BDH Laboratory Supplies), KH_2PO (0.02 g/L, Ajax Finechem), $MgSO_4 \cdot 7H_2O$ (0.005 g/L, Sigma-Aldrich), $CaCl_2 \cdot 2H_2O$ (0.0034 g/L, BDH Laboratory Supplies), $NaHCO_3$ (1 g/L, Sigma-Aldrich), haemin (0.0025 g/L, Sigma-Aldrich), Cystein HCL (0.25 g/L, Sigma-Aldrich), bile salts (0.25 g/L, Oxoid), Tween 80 (1 ml/L, Sigma-Aldrich) and vitamin K1 (5 µl/L, Sigma-Aldrich). The media was prepared in 10x stock solutions with pH adjusted to 7.0, sterilized by autoclaving at 121 ºC for 20 min. and kept at 5 ºC. Media, ready for use in the fermenter, was prepared from stocks in a volume of 3 L just prior to connecting the media reservoir to the fermenter. As an inducer of DNA damage the media was supplemented with 3% (wt/v) soy protein (Morlife Pty. Ltd., Labrador, Australia) previously shown to induce DNA damage *in vivo* [24]. Carbohydrate sources were 1% (wt/v) of either a highly digestible low amylose cornstarch (3401C, The National Starch and Chemical Company, Australia) or xylo-oligosacharides (XOS), DP 2-6 (Danisco Health & Nutrition, Kantvik, Finland).

Two- stage continuous fermenter system

The fermenter was set up as described by Bruck *et al.* [38] with minor changes. The fermenter consisted of two glass vessels, vessel 1 (V1) and vessel 2 (V2), with an operating volume of 220 ml and 320 ml, respectively. Vessel temperature was kept at 37 ºC and pH was automatically controlled with 0.1 M NaOH. V1's pH was kept at 5.5, representing the low pH environment of the proximal colon and 6.8 in V2, representing the more neutral pH in the distal colon. Both vessels and media reservoir were magnetically stirred and kept anaerobic by continuous gassing with sterile filtered oxygen-free nitrogen.

The fermenter was set up on the day prior to inoculation (Day -1). Basal media supplemented with 3% soy protein and 1% cornstarch was added to each vessel to allow the temperature to reach 37 ºC and anaerobic conditions to develop. On the following day (Day 0) the faecal slurry was added to the vessels to a final concentration of 2% and the first sample (7 ml) was taken. On Day 1 the media reservoir and the pump were connected to V1 with a flow rate of 0.03 L/hour (total transit time of 18 hours). V1 subsequently supplied V2. Samples (7 ml) were taken daily in the morning from each vessel and kept at -80 ºC until further analysis. The

media reservoir was changed every third day with cornstarch as the carbohydrate source for the first 10 days (Days 1-10) followed by 10 days with XOS fermentation (Days 11-20).

Growth and maintenance of HT29 cells

Human HT29 colonocytes were cultured in Dulbecco's Modified Eagel Medium (DMEM) supplemented with 10% fetal bovine serum, 0.37% $NaHCO_3$, 0.60% HEPES (Sigma-Aldrich) and 1% Antibiotic/Antimycotic at 37 ºC in an atmosphere of 5% CO_2. All chemicals were from Invitrogen unless otherwise stated. pH of the media was adjusted to 7.3. Cells were grown in 20 ml supplemented DMEM in 75 cm^3 tissue culture flasks (Grenier Bio-One) and passaged once weekly with cells being ~90% confluent.

Faecal water assay

Faecal water was isolated from samples collected on Days 8, 9 and 10 (cornstarch) and Days 18, 19 and 20 (XOS) from vessel 1 and 2 by centrifugation at 2000 g for 45 min, 4 ºC and stored at -80 ºC. HT29 cells were treated with a homogenous sample of faecal water from Days 8-10 or Days 18-20. Media from a flask of ~90% confluent HT29 cells was carefully removed. Cells were washed twice in 10 ml pre-warm (37 ºC) PBS and incubated with 1 ml Trypsin-EDTA (Invitrogen) for 5 min. at 37 ºC, 5% CO_2. Cells were disaggregated in 10 ml pre-warm (37 ºC) supplemented DMEM, counted and diluted to a concentration of ~8.500 cells/ml. Cell suspensions (2 ml/well) were added to 6-well tissue culture plates (BD Biosciences) and incubated overnight at 37 ºC, 5% CO_2.

For treatment of cells with faecal water a 20% dilution in PBS was chosen based on comet assays performed with 0.1-100% faecal water. Media was removed from cells growing overnight and cells were washed twice in 1 ml PBS. Faecal water (1 ml) was added to each well and cells were incubated for 30 min. at 37 ºC, 5% CO_2. Control cells were incubated with 1 ml 50 µM H_2O_2 (positive control) (Sigma-Aldrich) and with 1 ml PBS (negative control). After 30 min. solutions were removed and cells were washed twice in 1 ml PBS. Trypsin-EDTA (100 µl) was added to each well and plates were incubated for 5 min. at 37 ºC, 5% CO_2. Pre-warm supplemented DMEM (2 ml) was added to each well and cells were carefully disaggregated. From each well 500 µl aliquots were transferred to microcentrifuge tubes, and centrifuged at 4000 rpm for 5 min. Media was discharged and cells were used for the comet assay. Trypan blue was added to 20 µl cell suspension from each well to determine the viability of cells.

Comet assay

Single-strand DNA breaks induced by the faecal water treatments were investigated using the single-cell gel electrophoresis assay (comet assay) [39,40]. Cells isolated from the faecal water assay were resuspended in 200 µl pre-warm low melting agarose (LMA) (Trevigen) and 45 µl were pipetted onto the 1st well of two comet assay glass slides (Trevigen). Another 45 µl LMA was added to the remaining cells, mixed briefly and 45 µl were pipetted onto the 2nd well of the two slides. The cell suspension was spread evenly across the surface of the glass

slide, covered with a cover slip and kept on ice for 30 min. to allow the agarose to solidify. The cover slips were removed and the slides were immersed in a cold lysis buffer (Trevigen) at 4 ºC for 1 hour. Slides were placed in an electrophoresis tank containing alkaline electrophoresis buffer (300 mM NaOH, 1 mM Na_2EDTA, pH>13) kept at 4 ºC. Slides were submerged in the buffer for 20 min. before electrophoresis were conducted at 25 V, 300 mM for 20 min. Slides were removed from the alkaline buffer and placed in a pH neutralizing buffer (400 mM Tris-HCl, pH 7.5) for 3x5 min., fixed in 96% ethanol for 5 min. and left to dry at 37 ºC for 5 min. Slides were stained with propidium iodide (Sigma-Aldrich) and images were captured using an Olympus BX-41 fluorescent microscope and the software Image Pro Plus (Media Cybernetics Inc.). Tail length, % DNA in tail and comet tail moment (the product of tail length and the fraction of DNA in the tail) were calculated for ~50 cells pr. slide using CometScore™ v1.5 (TriTek Corp.). Apoptotic cells were excluded from the analysis based on their morphology.

SCFA analysis

Fermentation samples (1 ml) from Days 1-20 were diluted 1:3 in an internal standard (1.68 mM Heptanoic acid, pH 7) and left for sedimentation of particulate material. Supernatants (150 µl) were distilled by vacuum distillation as described by Patten *et al.* [41]. Distillates (60 µl) were analysed for total acids and SCFAs (acetate, butyrate and propionate), in duplicates, using Agilent Technologies 6890N Network Gas Chromatograph System fitted with a Zebron ZB-FFAP capillary GC column (Dimension: 30m x 0.53mm I.D) (Phenomenex) as previously described by McOrist *et al.* [42]. A standard mixture of acetic, propionic, isobutyric, butyric, isovaleric, valeric, caproic and heptanoic acids was used to calibrate the GC.

DNA extraction and quantification

DNA was extracted from 0.5 ml of the fermentation samples collected on Days 8-10 (cornstarch) and Days 18-20 (XOS). Samples were centrifuged at 13.000 g for 5 min. and pellets were resuspended in 1.2 ml TE Buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8) and transferred to 2 ml microcentrifuge tubes containing 0.5 ml zirconia-silica beads (0.1 mm, Biospec Products) and 30 µl 10% SDS. Bacteria cells were lysed by shaking for 5 min. on a minibead beater on high speed and centrifuged at 4500 g for 1 min. DNA was extracted from supernatants using the QIAamp DNA Stool Mini Kit (Quiagen) according to the manufacturer's instructions and stored at -20 ºC until use. DNA concentrations were quantified using Quanti-iT Pico Green (Invitrogen) with fluorescence measured using a PTC-200 Peltier Thermal Cycler (MJ Research).

Real-time PCR

Real-time PCR was performed on DNA extracted from fermentation samples collected on Days 8-10 (cornstarch) and Days 18-20 (XOS). Primers and amplification conditions for quantification of specific bacterial groups and species are listed in Table 1 and Table 2. All reactions were performed in 10 µl reactions with 1 µl template DNA, except *Akkermansia*

municiphila and sulphate-reducing bacteria. For these assays 3 µl template DNA was used in a 20 µl reaction. Each reaction contained template DNA, Ssofast Evagreen Supermix (2x) (Bio-Rad), primers (Table 1), 0.4 µl BSA (Promega) or 1 µl Dimethyl Sulfoxide (sulfate-reducing bacteria only) (Sigma-Aldrich) and Milli-Q to a final volume of 10 or 20 µl. Each sample was analysed in triplicates per PCR run. Amplifications were performed with an initial denaturation at 98 ºC for 2 min. followed by 35-40 cycles of 98 ºC for 5 sec., 52-65 ºC for 15-60 sec. and 72 ºC for 30-45 sec (Table 2). A final melting-curve analysis was performed after completion of all cycles with fluorescence collected at 0.5-1 ºC intervals between 55 and 95 ºC. A series of 10-fold dilutions of control template were analysed in parallel with the fermentation samples. All reactions were run on a PTC-200 Peltier Thermal Cycler (MJ Research) and analysed using MJ Opticon Monitor Analysis Software Version 3.1 (Bio-Rad) and qBase (Biogazelle). In order to correct data for differences in total DNA concentrations between samples results were analysed relative to total bacterial amplification.

Table 1. Primers used for real-time PCR assays

Target	Primer	Sequence (5'-3')	nM	Reference
Total bacteria	1114F	CGGCAACGAGCGCAACCC	150	[43]
	1275R	CCATTGTAGCACGTGTGTAGCC		
Akkermansia municiphila	AM1	CAGCACGTGAAGGTGGGGAC	350	[44]
	AM2	CCTTGCGGTTGGCTTCAGAT		
Bacteroides fragilis group	Bfr-F	CTGAACCAGCCAAGTAGCG	500	[45]
	Bfr-R	CCGCAAACTTTCACAACTGACTTA		
Bifidobacterium spp.	Bif-F	TCGCGTC(C/T)GGTGTGAAAG	600	[46]
	Bif-R	CCACATCCAGC(A/G)TCCAC		
Clostridium coccoides group	g-Ccoc-F	AAATGACGGTACCTGACTAA	250	[47]
	g-Ccoc-R	CTTTGAGTTTCATTCTTGCGA A		
Clostridium leptum group	sg-Clept-F	CTTTGAGTTTCATTCTTGCGAA	250	[47]
	sg-Clept-R	GCACAAGCAGTGGAGT		
Escherichia coli	E.coli F	CATGCCGCGTGTATGAAGAA	375	[48]
	E.coli R	CGGGTAACGTCAATGAGCAAA		
Faecalibacterium praunitzii	FPR-1F	AGATGGCCTCGCGTCCGA	500	[49]
	FPR-2R	CCGAAGACCTTCTTCCTCC		
Lactobacillus group	Lacto-F	AGCAGTAGGGAATCTTCCA	500	[46]
	Lacto-R	CACCGCTACACATGGAG		
SRB[1]*_aps*[2]	APS3F	TGGCAGATCATGWTYAAYGG	400	Modified from [50]
	APS2R	GGGCCGTAACCRTCYTTRAA		
SRB*_dsr*[3]	DSR1F+	ACSCACTGGAAGCACGGCGG	400	[51]
	DSR-R	GTGGMRCCGTGCAKRTTGG		
Desulfovibrio spp (SRB)	DSV691-F	CCGTAGATATCTGGAGGAACATCAG	300	[52]
	DSV826-R	ACATCTAGCATCCATCGTTTACAGC		

[1]Sulfate-reducing bacteria, [2]Adenosine-5-phosphosulfate reductase gene, [3]Dissimilatory sulfite reductase gene.

Table 2. Amplification conditions for real-time PCR assays

Target	**Annealing**		**Elongation**	
	ºC	Time (sec.)	ºC	Time (sec.)
Total bacteria	60	20	72	45
Akkermansia municiphila	63	30	72	30
Bacteroides fragilis group	58	60	72	30
Bifidobacterium spp.	58	20	72	30
Clostridium coccoides group	58	20	72	45
Clostridium leptum group	58	20	72	45
Escherichia coli	60	20	72	45
Faecalibacterium praunitzii	62	20	72	40
Lactobacillus group	58	30	72	30
SRB_*aps*	58	30	72	60
SRB_*dsr*	65	15	72	30
Desulfovibrio spp. (SRB)	62	30	72	30

Statistics

Data was tested for normal distribution using the Shapiro-Wilk test. Normal distributed data was analysed using a two-way analysis of variance (ANOVA) with fermenter and carbohydrate as variables. Where ANOVA indicated a significant difference Student's t-test was used to compare means of treatments. Data that did not meet the criteria of normal distribution was analysed using the non-parametric Kruskal-Wallis test. All statistical analyses were carried out using SAS JMP version 7. P values of <0.05 were considered statistically significant.

Results

Faecal water genotoxicity

Viability of the HT29 cells used for the comet assay was assessed by the trypan blue exclusion method and was shown to be greater than 95% (data not shown). Single-stranded DNA breaks were assessed by the comet assay performed with a homogenous sample of faecal water from Days 8-10 (cornstarch) or Days 18-20 (XOS). Results are presented as tail length, % DNA in the tail and tail moment (Table 3).

Table 3. Genetic damage induced by faecal water samples from fermentation of cornstarch or XOS

DNA damage (arbitrary units)				
Vessel	Carbohydrate	Tail length	% DNA in tail	Tail moment
V1 (pH 5.5)	Cornstarch, Days 8-10	44.08±2.94^{b}	14.71±0.85^{bc}	7.62±0.74^{b}
	XOS, Days 18-20	**33.32±2.36^{c}**	13.98±0.62^{c}	**5.37±0.51^{c}**
V2 (pH 6.8)	Cornstarch, Days 8-10	34.54±2.04^{c}	16.51±0.79^{ab}	6.70±0.56^{bc}
	XOS, Days 18-20	**62.01±3.77^{a}**	17.82±0.79^{a}	**12.79±1.01^{a}**

Values (mean±SEM) within each column with unlike superscript letters were significantly different (P<0.05).

Results from the comet assay revealed differences in the effect of XOS fermentation on the genotoxicity of faecal water samples from the two vessels. In vessel 1, tail length (P=0.0097) and tail moment (P=0.0293) was reduced by XOS fermentation indicating a protective effect of XOS against protein-induced genetic damage. In contrast, an increase in tail length and tail moment was observed in vessel 2, both P<0.0001. No significant effect on % DNA in the tail was observed within the vessels.

Short chain fatty acids

SCFA production confirmed the occurrence of bacterial fermentation in both vessels. The average concentration of total acids and SCFAs was significantly higher in vessel 2 compared to vessel 1 (P<0.0001), indicating a higher level of fermentation in vessel 2 (Table 4). In vessel 1, the average concentration of acetate from fermentation of cornstarch (Days 1-10) was significantly higher relative to XOS fermentation (Days 11-20) (P=0.0411). In contrast, the concentration of propionate was increased by XOS (P=0.0284) in vessel 1. In both vessels, the average concentration of butyrate was significantly increase by XOS fermentation compared to fermentation of cornstarch (V1: P=0.0186, V2: P<0.0001).

Table 4. Average SCFA concentration from fermentation of cornstarch or XOS

Average SCFA concentration (mM)					
Vessel	Carbohydrate	Acetate	Butyrate	Propionate	Total acids
V1 (pH 5.5)	Cornstarch, Days 1-10	**71.6±11.1^{b}**	15.6±5.0^{d}	2.5±2.0^{c}	90.4±13.5^{b}
	XOS, Days 11-20	62.4±18.4^{c}	**22.5±8.4^{c}**	**2.8±1.5^{b}**	88.3±22.4^{b}
V2 (pH 6.8)	Cornstarch, Days 1-10	107.0±15.8^{a}	34.5±6.8^{b}	24.7±10.5^{a}	181.8±37.9^{a}
	XOS, Days 11-20	99.0±7.8^{a}	**44.7±5.8^{a}**	27.2±6.2^{a}	179.0±13.3^{a}

Values (mean±SD) within each column with unlike superscript letters were significantly different (P<0.05).

Changes in bacterial population induced by XOS fermentation

Among the analysed bacterial groups only the *C. coccoides* group was significantly stimulated by XOS fermentation in both vessels relative to cornstarch fermentation (V1: P=0.015; V2: P=0.007) (Table 5). Furthermore, the *B. fragilis* group (P=0.024), *Lactobacillus* spp. (P=0.042) and sulphate-reducing bacteria (SRB) (*aps*: P=0.004; *dsr*: P=0.008; *Desulfovibrio* spp. P=0.015) were increased in vessel 1. Bacterial groups reduced by XOS fermentation were *F. prausnitzii* in both vessels (V1: P=0.001; V2: P=0.003) as well as *Bifidobacterium* spp. (P=0.01), the *C. leptum* group (P=0.001), SRB_*aps* (P=0.039) and SRB_*dsr* (P=0.018) in vessel 2. Levels of *Akkermansia municiphila* and *E. coli* were unaffected by the change in carbohydrate in both vessels.

Table 5. Relative quantification of specific bacterial groups from fermentation of cornstarch or XOS

Bacterial group	**Vessel 1 (pH 5.5)**		**Vessel 2 (pH 6.8)**	
	Cornstarch	XOS	Cornstarch	XOS
	Days 8-10	Days 18-20	Days 8-10	Days 18-20
A. municiphila	0.028±0.042	0.018±0.010	0.013±0.009	0.008±0.003
B. fragilis group	0.014±0.009[b]	**0.524±0.344[a]**	6.437±0.968	7.045±0.641
Bifidobacterium spp.	0.638±0.111	0.671±0.127	1.019±0.285[a]	**0.338±0.082[b]**
C. coccoides group	0.739±0.252[b]	**4.268±1.845[a]**	0.936±0.403[b]	**2.597±0.278[a]**
C. leptum group	0.065±0.008	0.116±0.055	1.087±0.103[a]	**0.433±0.054[b]**
E. coli	0.006±0.005	0.289±0.400	0.025±0.025	0.053±0.026
F. prausnitzii	0.468±0.067[a]	**0.119±0.026[b]**	1.004±0.284[a]	**0.179±0.065[b]**
Lactobacillus spp.	0.059±0.019[b]	**0.122±0.032[a]**	0.029±0.007	0.023±0.003
SRB[2]_*aps*[3]	0.014±0.007[b]	**0.281±0.122[a]**	0.626±0.220[a]	**0.301±0.019[b]**
SRB_*dsr*[4]	0.009±0.009[b]	**0.348±0.184[a]**	0.806±0.309[a]	**0.218±0.055[b]**
Desulfovibrio spp. (SRB)	0.012±0.007[b]	**0.429± 0.297[a]**	2.469± 0.672	1.531±0.468

[1]Values (mean±SD) were calculated relative to total bacteria. From each vessel, values within each row with unlike superscript letters were significantly different (P<0.05). [2]Sulfate-reducing bacteria, [3]Adenosine-5-phosphosulfate reductase gene, [4]Dissimilatory sulfite reductase gene.

Discussion

The present study investigated the effect of *in vitro* fermentation of XOS on the genotoxicity of faecal water samples, SCFA production and on the composition of a human faecal microbiota. XOS was selected based on its potential use as a prebiotic substrate believed to promote gut health via e.g. SCFA production and an altered gut microbiota.

Differences in the genotoxic potential of faecal water samples after XOS fermentation were observed for the two vessels, suggesting that XOS could protect against protein-induced DNA damage in the colon, but that the protection is restricted to the proximal colon. Several animal studies have implicated dietary fibres and production of butyrate in protection against colon cancer [23,32,33]. In the present study a significantly elevated concentration of butyrate was seen for both vessels, but a reduction in genotoxicity was only observed for vessel 1. Thus, from our results an increased butyrate concentration is not in itself preventive against protein-induced genetic damage.

In humans butyrate is mainly produced by *Roseburia* spp. and *Eubacterium rectale*, both members of the *C. coccoides* group (clostridial cluster XIVa), and to a lesser extent by *F. prausnitzii* belonging to the *C. leptum* group (clostridial cluster IV) [53]. Hence, the increase in butyrate concentrations seen in the present study is consistent with the increase in the *C. coccoides* group in both vessels.

The differences observed in the genotoxicity between the two vessels might be explained by XOS induced changes in the composition of the bacterial population. Both *Lactobacillus* spp., the *B. fragilis* group and sulphate-reducing bacteria were stimulated by XOS fermentation in vessel 1 (pH 5.5), but not in vessel 2 (pH 6.8). Growth of *Lactobacillus* on XOS have previously been shown *in vitro* [10] and a recent study from our lab demonstrated increased levels of the *B. fragilis* group in the faecal microbiota of mice fed XOS (Manuscript II). The increase in these bacterial groups in vessel 1 may result from the higher level of carbohydrate availability in this vessel compared to vessel 2. This would be consistent with *in vivo* conditions where the primary site of fermentation of non-digestible carbohydrates is the proximal colon [54], particularly with carbohydrates with a low degree of polymerization (DP) such as XOS [55]. Thus, it is possible that the reduction in faecal water genotoxicity observed for vessel 1, but not for vessel 2, may at least partly result from differences in the level of XOS fermentation between the two vessels. *In vivo*, a consequence of the intense fermentation of carbohydrates in the proximal colon is that less is available for fermentation in the distal colon making metabolism of proteins quantitatively more dominating [54]. In addition to production of SCFAs, degradation of proteins also generates potential genotoxic substrates such as ammonia, phenols, indoles, and amines [56,57], which may increase the genotoxic potential of food residues entering the distal colon.

The role of sulphate-reducing bacteria (SRB) in gastrointestinal health is poorly understood [58]. In the present study an increase in SRB was observed for vessel 1 (decreased genotoxicity), whereas a decrease in SRB was observed for vessel 2 (increased genotoxicity). Our results thus indicate a positive role of SRB in the gut.

SRB are a diverse bacterial group of which the genus *Desulfovibrio* is the most common SRB isolated from animal and human faeces. Within the intestinal tract SRB are the terminal oxidizers in the anaerobic degradation of organic matter from which they reduce sulphur-containing compounds to hydrogen sulphide (H_2S) [58]. H_2S produced by SRB have been suggested as a potential toxin to the gut epithelium [59,60] implicating SRB in the pathogenesis of IBD [61,62] and colon cancer [63]. In contrast, other studies have reported no increase in colonic *Desulfovibrio* spp. and no elevated faecal H_2S concentration in ulcerative colitis patients [52,64,65]. Studies investigating the presence of faecal *Desulfovibrio* spp. in colon cancer patients either found no difference or reported a decrease (potentially caused by colectomy procedures) in *Desulfovibrio* spp. [58,66]. Thus, collectively these studies question the specific role of H_2S and SRB in the gut.

In a recent study by Wallace *et al.* [67], H_2S, produced by colonic tissue, was demonstrated as preventive to experimentally induced colitis in rats suggesting an anti-inflammatory effect of H_2S. Furthermore, inhibition of H_2S synthesis significantly increased mortality rates of rats suffering from colitis [67]. From these results it is possible that also bacterial derived H_2S is beneficial for the colonic epithelium suggesting a predominantly positive role of sulphate-reducing bacteria in the gut as also indicated by our study.

The present study did not demonstrate a bifidogenic effect of XOS, and in fact a decrease in bifidobacteria was observed for vessel 2. Other *in vitro* studies investigating the effect of XOS on the composition of a human faecal microbiota have reported an increase in bifidobacteria [9-11]. *In vivo* studies of the effect of XOS on the human gut microbiota are limited, but a recent study by Chung *et al.* [14] demonstrated a bifidogenic effect of XOS consumption in elderly aged ≥65. Furthermore, studies with rodents have demonstrated an increase in faecal and caecal numbers of bifidobacteria in response to XOS feeding [12,13]. Since bifidobacteria have been suggested to play a role in protection of the colonic epithelium [68], a reduction in this bacterial genus might increase the risk of intestinal disorders. Hence, the decrease in *Bifidobacterium* spp. as well as the decrease in SRB observed for vessel 2 may both be factors contributing to the increased genotoxicity. Furthermore, a reduction in *F. prausnitzii*, as observed for both vessels, may be associated with reduced protection of the gut mucosa, since this bacterium has been shown to exhibit anti-inflammatory effects and is present in low numbers in the faecal microbiota of humans suffering from inflammatory bowel diseases (IBD) [69,70].

In conclusion, the present study demonstrated the ability of XOS to reduce protein-induced genetic damage in vessel 1, to stimulate numbers of butyrate producing bacteria (the *C. coccoides* group) as well as butyrate production. Butyrate alone was not protective against genetic damage induced by protein fermentation as observed for vessel 2, whereas quantities of some bacterial groups and species were related to changes in the genotoxicity of faecal water. Reductions in *Bifidobacterium* spp. and sulphate-reducing bacteria (SRB) were related to an increase in genetic damage, suggesting a potential beneficial role of SRB in gut health rather than relating this bacterial group to intestinal disorders as previously suggested.

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9. SUMMARISING DISCUSSION AND CONCLUSION

The concept of prebiotics was introduced as an approach of selectively stimulating the growth and/or activity of beneficial bacteria indigenously present in the intestinal tract [24,30]. The aim of Manuscript I was to investigate whether consumption of prebiotic dietary carbohydrates and other potential prebiotics could improve the resistance of mice to *S.* Typhimurium SL1344 infection. Despite studies with probiotics demonstrating protective effects against murine *S.* Typhimurium infections [78-85], such an effect was not observed for the carbohydrates investigated in Manuscript I. None of the carbohydrates exhibited any protective effect against the *Salmonella* infection and in fact, two of the tested carbohydrates, FOS and XOS, increased numbers of the pathogen in the liver, spleen and mesenteric lymph nodes relative to control-fed mice. In mice fed apple pectin a markedly increased number of the pathogen was observed in the content of the distal ileum and in faecal samples. Additionally, a trend, though not statistically significant (P=0.18-0.29), indicating increased pathogen numbers in the content of the distal ileum and faecal samples was observed for mice fed FOS and XOS. Similarly, a trend towards increased numbers of *Salmonella* in the investigated organs was observed for the group fed apple pectin (P=0.15-0.21). Based on these results the hypothesis was that a high ileal level of *Salmonella* was accompanied by a high content of the pathogen in internal organs even though this was only indicated by trends in our data.

In accordance with the increased organ counts of *Salmonella* in mice fed FOS or XOS the concentration of the acute phase protein haptoglobin, measured in serum samples, was significantly elevated in these two dietary groups relative to infected mice fed the control diet. Haptoglobin is produced by the liver in response to tissue damage and inflammation with interleukin 6 thought to be the major regulator of the acute phase protein response [229,230]. Binding of free plasma haemoglobin (Hb) is generally accepted as the primary function of haptoglobin, whereby the host is protected against oxidative damage mediated by free Hb, renal damage as a result of Hb accumulation and Hb loss (and thus loss of iron) [231]. Thus, the increase in serum haptoglobin concentrations as well as the positive correlation between neutrophils in the spleen and numbers of *Salmonella* in the organs, but not in the distal ileum (Manuscript I, study C), indicate an immune response towards bacteria translocated to the organs rather than *Salmonella* present in the ileum.

To further investigate potential explanations for the increase in pathogen translocation observed for FOS or XOS fed mice, changes in the faecal and caecal microbiota of these mice were analysed in Manuscript II. In this study, the faecal microbiota of FOS or XOS fed mice was seen to differ from the control group by DGGE and real-time PCR analysis. Among the investigated bacterial groups the changes induced by FOS or XOS were comparable and constituted a significant increase in the *Bacteroidetes* phylum, the *Bacteroides fragilis* group and in *Bifidobacterium* spp. in faecal samples as well as a reduction in the *Firmicutes* phylum and *Clostridium coccoides* group. Assuming that these changes were, at least partly, the cause of the increased translocation of *Salmonella* in FOS or XOS fed mice, changes in the large

intestinal microbiota can affect the pathogenesis of the pathogen even though ileal M cells are described as the classical route of *Salmonella* invasion [197]. In this context it should be noted that FOS-fed rats challenged with *S.* Enteritidis in the study by Bovee-Oudenhoven *et al.* [152] did not show sings of intestinal inflammation, measured as myeloperoxidase activity, in the ileal mucosa. In contrast, a significantly increased enzyme activity was observed for the caecal and colonic mucosa suggesting pathogen translocation through the large intestinal epithelium.

Since samples from the ileum were not available for analysis in Manuscript II, it is unknown whether microbial changes in this part of the gut were a factor contributing to the increased level of translocation. However, the DGGE and real-time PCR analysis performed on faecal and caecal samples demonstrated that the effects of the experimental diets were largely restricted to the faecal microbiota, suggesting that diet-induced microbial changes in the ileum would be limited.

Increased numbers of bifidobacteria are generally regarded as beneficial to gut health [30]. Manuscript II revealed a strong bifidogenic effect of both FOS and XOS, but this was not seen to provide protection against the *Salmonella* infection. Similar results have been published by Ten Bruggencate and co-workers [151,152,154], where FOS feeding of rats increased faecal numbers of bifidobacteria prior to *S.* Enteritidis infection. Despite the bifidogenic effect, FOS administration was seen to increase the intestinal permeability and to increase pathogen translocation. Thus collectively, these and our results do not support the assumption that increased numbers of bifidobacteria improve host resistance to infections by gut pathogens. Still, studies with orally applied strains of bifidobacteria (*B. longum*, *B. lactis* and *B. breve*) have demonstrated protective effects against murine *Salmonella* infections [78,81,83]. Within other areas of research related to gut health such as treatment and/or prevention of inflammatory bowel disease, allergies, diarrhoea and colon cancer, clinical investigations have shown promising results of probiotic administration of *Bifidobacterium* spp., but at present these are generally not sufficient for any final conclusions to be drawn [86,87,232-235].

Among studies with prebiotics, with a demonstrated bifidogenic effect, Kleessen *et al.* [236] studied the effect of inulin and FOS on the intestinal mucosal morphology (height of villi, depth of crypts, number of goblet cells) and on the thickness of the epithelial mucus layer by comparing germ-free rats and rats colonised with a human faecal microbiota. The thickness of the epithelial mucus layer, villus height, crypth depth and numbers of goblet cells were higher in rats with a human faecal microbiota compared to germ-free rats, and the morphological features were significantly enhanced in these animals by the prebiotic diet. Additionally, numbers of mucosa-associated bifidobacteria in the distal colon were stimulated by the experimental diet. Together these findings suggest a role of the prebiotics in stabilizing the mucosal barrier of the gut and indicate that bifidobacteria are involved in protecting the mucosal epithelium. However, these results contradict our findings of a reduced resistance to the *Salmonella* infection, as observed in Manuscript I, despite the bifidogenic effect of both FOS and XOS.

The last study included in this thesis (Manuscript III) addressed the effect of *in vitro* fermentation of XOS on the composition of a human faecal microbiota and whether fermentation of this carbohydrate could provide protection against protein-induced genetic damage. Using a two-stage fermenter XOS was seen to increase butyrate production in both vessels as compared to fermentation of cornstarch. The increase in butyrate concentrations was consistent with an increased level of the *C. coccoides* group, comprising important butyrate producing bacteria [237], in both vessels, but only with a reduced faecal water genotoxicity in vessel 1 (pH 5.5). In contrast, the genotoxicity was increased in vessel 2 (pH 6.8). Microbial changes suggested to contribute to the increase in genotoxicity were the reductions in *Bifidobacterium* spp. and sulphate-reducing bacteria (SRB) observed for vessel 2. In contrast, an increase in SRB was observed for vessel 1 (decreased genotoxicity), suggesting a beneficial effect of SRB relative to protection against protein-induced genetic damage. Other microbial changes potentially associated with the reduced genotoxicity in vessel 1 were the increase in *Lactobacillus* spp. and the *B. fragilis* group, both previously shown to be stimulated by XOS [182, Manuscript II]. The increase in these bacterial groups may thus indicate a higher level of XOS fermentation in vessel 1 relative to vessel 2, potentially reducing the release of genotoxic by-products from protein degradation in vessel 1 and thus reducing the genotoxic potential of faecal water samples from this vessel.

In vitro models are associated with some limitations such as the lack of a host immune system and the absorptive processes exerted by the gut epithelium [238]. Hence, some of the inconsistency observed between the *in vitro* fermentation study and the *in vivo* study (Manuscript I and II) might be explained by the models used. For example, the lack of absorption of SCFAs in the *in vitro* model may reveal changes in the production of acids that would not have been seen in an *in vivo* model. Concerning the decrease in bifidobacteria seen *in vitro* it should be note that XOS was seen to stimulate bifidobacteria in Manuscript II, as also observed in other *in vivo* studies [142,185,186]. Other contradicting results were the increase in the *C. coccoides* group seen *in vitro* versus the decrease in this bacterial group observed *in vivo* in faecal samples from mice fed XOS. Thus, even though *in vitro* modelling provides a means of investigating effects related to otherwise inaccessible gut regions in humans [238] some inconsistency between results obtained might arise from the models chosen. In addition, some of the inconsistency between the *in vivo* and *in vitro* studies presented in this thesis may also reflect differences in the mouse vs. the human microbiota.

In conclusion, the experimental studies included in this thesis add to our understanding of effects of non-digestible dietary carbohydrates on the composition of the large intestinal microbiota and how dietary interventions with such substrates may affect the susceptibility to *Salmonella* infections or the risk of developing colon cancer. The new knowledge gained includes (I) a demonstrated reduced resistance of mice to *S.* Typhimurium infection in response to FOS or XOS feeding, (II) the ability of FOS and XOS to change the overall composition of the murine faecal microbiota, (III) the ability of XOS to stimulate numbers of butyrate producing bacteria (the *C. coccoides* group) and the production of butyrate *in vitro*

and (IV) an altered human faecal water genotoxicity as a result of XOS fermentation demonstrating distinct effects relative to fermentation vessels.

10. REFERENCES

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