University of Alberta

Prebiotic activity of isomalto-oligosaccharides

by

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Dedicated to my beloved parents

Abstract

Isomalto-oligosaccharides (IMO) with α(1→6) and α(1→4) glucosidic linkages are produced by enzymatic conversion of starch. Isomalto-oligosaccharides are partially digested in the intestine but little information is available regarding their metabolim *in vivo*. It was the aim of this study to investigate IMO metabolism by lactobacilli and bifidobacteria and to determine the effect of IMO diet on intestinal microbiota in a rodent model, and a rodent model for inflammatory bowel disease (IBD).

Different strains of bifidobacteria and lactobacilli were grown in de Man Rogosa Sharpe media with IMO as sources of carbohydrates. Substrates and metabolites of carbohydrates metabolism were analyzed. Lactobacilli metabolized isomaltose whereas isomalto-triose and higher degree of polymerisation (DP) oligosaccharides were metabolized by bifidobacteria first. To determine the modulating effects of IMO in the intestine, a group of six F344 rats were fed IMO diet for six weeks and compared to rats fed control diet. Assessment of intestinal microbiota and their metabolites was performed by PCR- denaturing gradient gel electrophoresis (PCR-DGGE), quantitative PCR (qPCR) and quantification of short chain fatty acids (SCFA). The *Lactobacillus* group was one of the dominant bacterial taxa in the fecal samples of rats. Isomalto-oligosaccharides selectively stimulated lactobacilli and increased their diversity in rats compared to those on control or inulin diet.

The potential health benefit of IMO was evaluated in a rodent model for IBD. Three groups of HLA-B27 rats were fed IMO, fructo-oligosaccharides (FOS) or

control diet for 12 weeks. The *Lactobacillus* group and bifidobacteria numbers were increased significantly in the fecal samples of rats fed IMO or FOS diet respectively. Numbers of *Enterobacteriaceae* family were significantly increased in the rats fed IMO or FOS diet compared to the control group. *Clostridium coccoides* group, *Clostridium leptum* group, *Clostridium* cluster XI and total number of bacteria were significantly decreased in the rats fed FOS diet compared to the rats fed control diet. Moreover, IMO and FOS diets showed a unique effect on intestinal microbiota compared to the control diet. Cecum histology scores showed a significant decrease of inflammation in the rats fed IMO or FOS diet compared to the rats fed control diet.

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List of symbols, nomenclatures, or abbreviations

NDO: Non- digestible oligosaccharides

SCFA: Short chain fatty acids

IMO: Isomalto-oligosaccharides

FOS: Fructo-oligosaccharides

GOS: Galacto-oligosaccharides

XOS: Xylo-oligosaccharides

HMO: Human milk oligosaccharides

MRS: de Man Rogosa Sharpe

IBD: Inflammatory bowel disease

UC: Ulcerative colitis

CD: Crohn's disease

DGGE: Denaturing gradient gel electrophoresis

QPCR: Quantitative polymerase chain reaction

UPGMA: Unweighted pair group method with arithmetic mean

ELISA: Enzyme linked immunosorbent assay

HPAEC-PAD: High performance anion exchange chromatography- pulsed amperometric detector

Chapter 1

Introduction

1. 1. Introduction

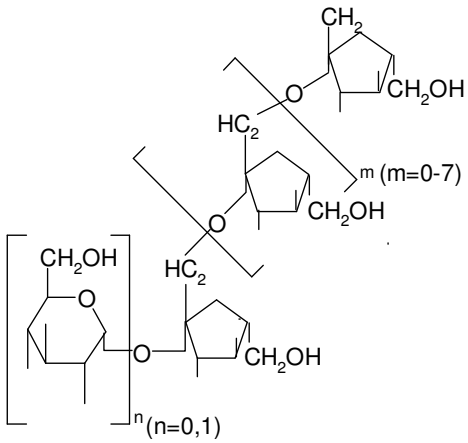
Plant cell-wall polysaccharides, oligosaccharides and storage polysaccharides (e.g. inulin) and resistant starch are the main components of fiber in the human diet (Flint et al, 2008). Based on their physiological fate, carbohydrates can be classified as digestible or non-digestible. Non-digestible oligosaccharides (NDO) and polysaccharides are carbohydrates that escape digestion in the stomach and small intestine and reach the colon largely intact. The specific configuration of monosaccharide units in some dietary oligosaccharides makes them non-digestible to hydrolytic enzymes of the human digestive system (Roberfroid and Slavin, 2000). The NDO differ in chain length, monosaccharide composition, degree of branching, and purity (Crittenden and Playne, 1996). Degree of polymerization is usually the indicator to separate oligosaccharides from polysaccharides (Cummings and Stephen, 2007).

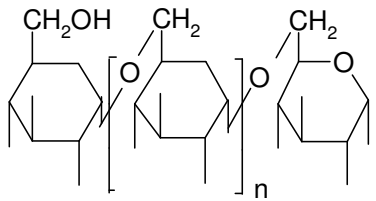
Animal and human digestive systems accommodate numerous bacteria specialized in fermentation of NDO with various structures and degree of polymerization. Microorganisms are active in different parts of the intestine and depending on the location produce various metabolites. Intestinal microbiota mainly rely on food content that escape the digestion and reach the intestine. Therefore, the type and amount of food that reaches the colon is very crucial to the microbiota (Flint et al., 2008). Saccharolytic and proteolytic fermentations usually take place in the proximal and the distal part of human colon, respectively (Macfarlane et al, 1992). Microorganisms in the gastro intestinal tract of herbivores are far more specialized in utilizing plant polysaccharides. In contrast,

microbiota of omnivores, notably humans, are less specialized in utilizing polysaccharides, and the contribution of fermentation to the host's energy balance is about 10% (Flint et al., 2008).

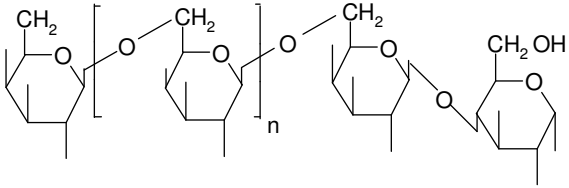
Prebiotics are non-digestible oligosaccharides and polysaccharides that are selectively fermented and allow specific changes, both in the composition and/or activity of gastrointestinal microbiota and confer benefits upon host well-being and health (Roberfroid, 2007). Probiotics are live microorganisms and when administered in adequate amount confer health benefits in the host (Sanders, 2008). As opposed to probiotics that have to compete with established microbiota, prebiotics stimulate the commensal bacteria already colonized in the large intestine (Flint et al., 2008). Previous studies were mainly focused on lactobacilli and bifidobacteria as beneficial bacteria in the colon. In this communication, metabolism of some of the NDO by lactobacilli and bifidobacteria is reviewed.

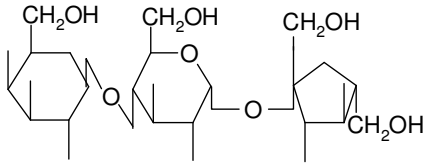
Fructo-oligosaccharides (FOS), galacto-oligosaccharides (GOS), soybean oligosaccharides, lactosucrose, isomalto-oligosaccharides (IMO) and xylo-oligosaccharides (XOS) are examples of NDO. Some of these are already used as prebiotics (Manning et al, 2004, Mussatto and Mancilha, 2007) (Figure 1.1 and Table 1.1). Fruits and vegetables such as onion, garlic, banana, leek, asparagus and Jerusalem artichoke are natural sources of fructo-oligosacchardes.





α (1→6) Isomalto-oligosaccharides

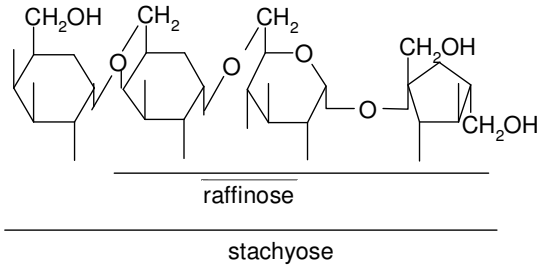


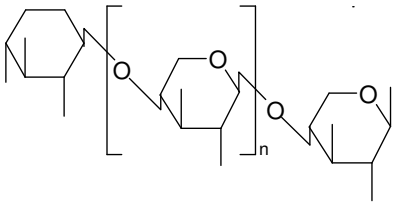


Fructo-oligosaccharides

β (1→6) galacto -oligosaccharides (b)

Lactosucrose





Soybean oligosaccharides

Xylo-oligosaccharides

Figure 1.1- Chemical structure of non-digestible oligosaccharides

Non-digestible oligosaccharides have several established health benefits for the host, including improvement of bowel function and relief from constipation and boosting production of short chain fatty acids (SCFA). Decrease or prevention of pathogenesis (colonization resistance), cancer protection, reduction of cholesterol, increasing of mineral absorption and amelioration of inflammatory bowel diseaese (IBD) are non-established health benefits of NDO (Adam et al, 2000, Dongowski et al., 2002, Govers et al., 1999, Hopkins and Macfarlane, 2003, Kleessen et al., 1997, Kleessen et al., 2001, Meyer and Stasse-Wolthuis, 2009, Perez-Conesa et al., 2007, Rowland et a., 1996, Wolin et al., 1999, Yen et al., 2011).

The largest effect of NDO is through the stimulation of microbiota in the colon, decreasing of the luminal pH and production of SCFA. Microbiota in the colon are influenced both by the host and the diet (Flint et al., 2008). Intestinal microbiota of infants originate from the mother and the environment at the time of birth. Shortly after birth, the microbiota start to colonize in the colon and are mainly dominated by bifidobacteria in breast-fed infants (Kleessen et al., 1995). Human milk oligosaccharides in breast-fed infants are the main source of energy for colonic microorganisms (Miller and McVeagh, 1999). Formula-fed infants often show a different microbial profile (Favier et al., 2002). After the weaning period, intestinal microbiota are changed according to the diet (Mitsuoka, 1990, LoCascio et al., 2010).

Commercial NDO are produced either (1) by extraction from natural sources e.g. inulin and soybean oligosaccharides, (2) partial enzymatic hydrolysis of polysaccharides e.g. xylo-oligosaccharides, or (3) by enzymatic synthesis from

one or a mixture of disaccharides using glycosyl hydrolase e.g. galacto-oligosaccharides from lactose or fructo-oligosaccharides from sucrose (Table 1.1). Fructans and GOS are the most studied NDO and are well established as prebiotics.

Isomalto-oligosaccharides are used in the food industry and are claimed to have bifidogenic properties (Kaneko et al., 1994, Kohomoto et al., 1991 and 1992). However, little information is available regarding the selectivity of IMO towards intestinal microbiota and their metabolites *in vitro* and in rodent models compared to FOS and GOS.

Table 1.1- Non-digestible oligosaccharides (NDO)*

Name	Structure
Fructo-oligosaccharides and Inulin	α-D-Glu-[(1→2)-β-D-Fru]$_n$ α-D-Fru-[(1→2)-β-D-Fru]$_n$
Galacto-oligosaccharides	[β-D-Gal (1→4/2,3,6)]$_n$-β-D-Gal(1→4)-D-Glu
Isomalto-oligosaccharides	α-D-Glu-[(1→6,4)-α-D-Glu]$_n$
Soybean oligosaccharides	[α-D-Gal-(1→6)]$_n$-α-D-Glu(1→2)-β-D-Fru
Xylo-oligosaccharides	β-D-Xyl[(1→ 4)-D-Xyl]$_n$
Lactosucrose	β-D-Gal-(1→4)-α-D-Glu-(1→2)β-D-Fru

* Glu: Glucose, Fru: Fructose, Gal: galactose, Xyl: Xylose

Many studies revealed the health benefits of NDO (Govers et al., 1999, Hopkins and Macfarlane, 2003, Kleessen et al., 1997, Kleessen et al., 2001, Yen et al., 2011). However, few studies connected the *in vitro* metabolism of NDO to their metabolism in animal models and humans. Studying the metabolism of NDO with different structures and degree of polymerization and elaborating on the metabolism of individual compounds by intestinal bacteria may predict their metabolite production and influence on the complex environment of the colon. Structure of FOS, GOS and IMO is briefly explained in the following sections.

1.2. Fructo-oligosaccharides (FOS)

A fructan is a carbohydrate mainly consisting of fructose residues. Fructans can be linear or branched. Commercial fructo-oligosaccharides (FOS) are oligosaccharides with fructose monomers (F) connected with β (2→1) with a terminal glucose (G), mainly composed of 1-kestose (GF2), nystose (GF3), and 1-fructosyl nystose (GF4). Fructo-oligosaccharides are either produced from sucrose by transfructosylation or partial hydrolysis of longer chain polysaccharides (Roberfroid and Delzenne, 1998). Inulin mostly contains β (2→1) fructosyl fructose linkages and glucose may or may not be present at the end of the molecule. The first monomer of either glucose or fructose has a pyranosyl configuration. Levan is a fructan mostly containing β (2→6) fructosyl fructose linkages. Glucose may or may not be present at the end of the molecule. Levans are mostly produced by bacteria (Tieking and Gänzle, 2005). Fructo-oligosaccharides have 30%-60% sweetness compared to sucrose (Belitz et al., 2004). Products referred to as inulin have degree of polymerization (DP) of 10- 60

whereas FOS products contain a DP of 3-10. Fructo-oligosaccharides are believed to be readily metabolized by bacteria compared to inulin with high degree of polymerization (Hernot et al., 2009) (Figure 1.1 and Table 1.1).

1.3. Galacto-oligosaccharides (GOS)

Transgalactosylation of lactose by β-galactosidase produce galacto-oligosaccharides with different bonds (Sako et al., 1999, Tzortzis and Vulevic, 2009). Synthetic GOS are commercially produced mainly from whey and have a degree of polymerization of 3-8. The linkages between the galactose molecules in GOS, the efficiency of transgalactosylation, and the components in the final products depend on the enzyme and conditions of the reaction (Sako et al., 1999). In addition to transgalactosylation, hydrolytic activity of galactosidase is very important and plays an important role in metabolism of colonic bacteria in the intestine (Van den Broek et al., 2008). More than 90% of GOS reaches the colon (van Loo et al., 1999). Microbial stimulation has been reported in human trials of GOS. (Alles et al., 1999, Bounik et al., 1997, Ito et al., 1990, Malinen et al., 2002, Moro et al., 2002). Commercially available GOS are mixtures of oligosaccharides of different DP, the unreacted lactose and monosaccharides. Specific physio-chemical and physiological properties vary depending on the mixture of GOS (Tzortzis and Vulevic, 2009) (Figure 1.1 and Table 1.1).

1.4. Isomalto-oligosaccharides (IMO)

Isomalto-oligosaccharides are used as low-calorie sweeteners in the food industry and contain α (1→6) glucosidic linkages (Kohomoto et al., 1991). Isomaltose, panose and isomalto-triose are usually present in commercial IMO. Isomalto-

oligosaccharides are present in fermented foods such as miso, sake and soy sauce. Isomaltose is a disaccharide present in honey (Kohomoto et al, 1988). Isomalto-oligosaccharides are moderately sweet (30%-60% compared to sucrose) (Belitz et al., 2004). They are partially hydrolyzed in the small intestine and ultimately reach the colon (Roberfroid and Slavin, 2000) (Figure 1.1 and Table 1.1).

1.5. Metabolism of non-digestible oligosaccharides by lactobacilli and bifidobacteria

The large intestine and its associated microbiota is a complex environment. It is estimated that hundreds of species of bacteria colonize and inhabit the colon. The study of intestinal microbiota is a challenging task. Access to the colon during fermentation is limited. The environment of colon is anaerobic. Moreover, inter-individual differences of microbiota exist in the colon (Benson et al, 2010). New techniques and application of improved molecular methods are all helpful in the *in vivo* study of microbiota in the intestinal environment. *In vitro* studies aiming to assess fermentability of pure oligosaccharides and their metabolites by a single bacterium strain in a small scale is an advantage compared to *in vivo* studies. Moreover, novel oligosaccharides need to be examined prior to animal or human trials. Bifidobacteria and lactobacilli have been mainly reviewed in the past for their health benefits and function in the physiology of colon (Meyer and Stasse-Wolthuis, 2009, van Loo et al., 1999). Metabolism of some of the NDO by lactobacilli and bifidobacteria is explained in the following sections.

1.5.1. Human milk oligosaccharides (HMO)

The components of milk vary across different species of mammals. The difference in milk components could be explained by adaptation to the environment, nutrients, growth conditions and requirements of mammalian infants (German et al, 2008). Human milk oligosaccharides contain sialic acid, N-acetylglucoseamine, L-fucose, D-glucose and D-galactose. Human milk oligosaccharides have lactose at the reducing end and are elongated by N-acetyl lactosamine units and extensive fucosylation or sialylation where fucose and sialic acid are located at the terminal position (German et al., 2008). Microorganisms capable of fermenting these oligosaccharides generally have intracellular glycosyl hydrolases such as fucosidase, ß-galactosidase, sialidase and ß-hexoaminidase. Their related transport genes are located in HMO utilization clusters within the genomes of these bacteria (Sela et al., 2008). Operons and metabolic pathways of the core HMO, lacto-N-biose (LNB), has been described recently (Nishimoto and Kitaoka, 2007). New genetic findings emphasize the role of diet in development of intestinal microbiota in addition to the environmental niche (Sela et al., 2008, Makarova et al., 2006). Human milk oligosaccharides benefit the infants through selective stimulation of microbiota and protect them from pathogens. Breast-fed infants have a greater population of *Bifidobacterium longum* subsp. *infantis* which is capable of metabolizing HMO. On the other hand, *B. longum* subsp. *longum,* particularly found in adults, is specialized for plant-derived carbon metabolism and cannot grow on HMO (Sela et al., 2008, LoCascio et al., 2010) (Table 1.2).

Table 1.2- Glycosyl hydrolase enzymes associated with metabolism of non-digestible oligosaccharides (NDO) in lactobacilli and bifidobacteria

NDO	Bifidobacteria	Lactobacilli	References
Fructans (FOS and inulin)	*B. breve* UCC2003 *B. lactis* DSM10140 *B. longum* NCIMB702259 ß- fructofuranosidase (intracellular) *B. longum* NCIMB702259, *B. lactis* Sucrose phosphorylase (intracellular)	*L. paracasei* 1195, ß- Fructosidase (extracellular), Fructofuranosidase (intracellular) *L. acidophillus* NCFM and *L. plantarum* WCFS1, ß- Fructosidase and Sucrose phosphorylase (intracellular)	Goh et al., 2006 & 2007 Kaplan and Hutkins, 2003, Barrangou et al., 2003 Saulnier et al., 2007 Ryan et al., 2005 Ehrmann et al., 2003 Janer et al., 2004 Kullin et al., 2006 Trindade et al., 2003
Soybean oligosaccharides		*L. curvatus* R08 *L. fermentum,* α-galactosidase (intracellular), *L. reuteri* LTH5448, *L. sanfranciscensis* TMW1.392 Levansucrase, (extracellular)	Yoon et al., 2008 Garro et al., 1993 Teixeira et al., 2009 Tieking et al., 2005
Isomalto-oligosaccharides (IMO), resistant strach (RS)	*B. breve* UCC2003, Amylopullulanase (extracellular)	Maltose phosphorylases in lactobacilli are all intracellular	Stolz et al., 1996 Nakai et al., 2009 O'Connell Motherway et al., 2008

NDO	Bifidobacteria	Lactobacilli	References
Galacto-oligosaccharides (GOS)	*B. longum* subs. *infantis* ATCC15697, ß- galactosidase (intracellular) *B. longum* NCC490, Endogalactanase (extracellular) *B. bifidum* DSM20215, ß- galactosidase (extracellular) *B. bifidum* DSM20215, *B. infantis* DSM20088, ß- galactosidase (intracellular)	ß- galactosidases in lactobacilli are all intracellular	Schwab et al., 2010 Sela et al., 2008 Hinz et al., 2005 Møller et al., 2001
Human milk oligosaccharides (HMO)	*B. longum* subs. *infantis* ATCC15697 Fucosidase, Sialidase, ß- hexosaminidase (all intracellular)	*L. plantarum* FUA3121 *L. mesenteroides* FUA3143 *L. reuteri* FUA3148 *L. fermentum* FUA3177 *L. acidophilus* FUA3191 *S. thermophilus* FUA3194 (barely grew on HMO but had growth on some monosaccharide components of HMO)	Sela et al., 2008 Nishimoto and Kitaoka, 2007 LoCascio et al., 2010 Schwab and Gänzle, 2010

1.5.2. Soybean oligosaccharides

Soybean oligosaccharides, raffinose and stachyose, are hydrolyzed by α-galactosidase cleaving the α(1→6) bonds. Mammalians do not have α-galactosidase in their intestine. However, some microorganisms in the intestine have the enzymes capable of cleaving α- galactoside bonds. Some lactobacilli and bifidobacteria hydrolyze α- galactosides in raffinose, stachyose and verbascose (Garro et al., 1994 and 1996, LeBlank et al., 2004). Alpha- galactosidase is generally intracellular and raffinose is the best substrate for this enzyme (LeBlank et al., 2004, Yoon et al., 2008). After separation of galactose from raffinose, fructosidases cleave the sucrose part of the oligosaccharide, releasing the fructose and make glucose accessible for the glycolysis pathway (Barrangou et al., 2006). Alternatively, sucrose phosphorylase plays this role. Kullin et al. (2006) reported activity of sucrose hydrolase and sucrose phosphorylase in *B. longum* exclusively involved in intracellular hydrolysis of sucrose. Tieking et al. (2005) confirmed transglucosylation and exopolysaccharide production of levansucrase in some lactobacilli but did not report any hydrolase activity. Recently the hydrolase activity of levansucrase was shown in strains of *Lactobacillus reuteri* LTH5448, *L. sanfranciscensis* TMW1.392 and their mutants (Teixeira et al., 2010, unpublished data). Possessing both levansucrase and α- galactosidase, *L. reuteri* showed faster growth on raffinose, stachyose and verbascose compared to its mutant strain and *L. sanfranciscensis*.

Intracellular enzymes particularly α- galactosidase, if excreted to the environment degrade the α-galactoside linkages and provide the carbon source for other

bacteria not able to grow on oligosaccharides with α- galactoside linkages (LeBlank et al., 2004) (Table 1.2).

1.5.3. Fructans with different degree of polymerization

Fructan is comprised of FOS with DP of 3-10 and inulin with DP up to 60. The ability of lactobacilli and bifidobacteria to utilize FOS is well established (Kaplan and Hutkins, 2000, Goh et al, 2006 and 2007). The genome sequence of bifidobacteria in particular shows that they have several regions for transport and metabolism of oligosaccharides (Schell et al., 2002). Metabolism of levan for example was confirmed in bifidobacteria (Korakli et al., 2002). However, in some cases poor growth was reported on inulin with high DP compared to FOS with low DP (Ehrmann et al., 2003, Janer et al., 2004, Rossi et al., 2005). Intracellular activity of ß-fructofuranosidase was reported in *B. lactis* DSM10140 (Ehrmann et al., 2003) and *B. breve* UCC2003 (Ryan et al., 2005). Fructofuranosidase is an exo-enzyme separating one molecule of glucose from the end of fructo-oligosaccharides (Ehrmann et al., 2003, Ryan et al., 2005). Falony et al. (2009) reported metabolism of fructans in different strains of bifidobacteria. Four major phenotypes was distinguished. The first group did not utilize inulin or FOS independent of the presence or absence of ß-fructofuranosidase gene. The second group had preferential growth on FOS but did not grow on inulin. The third category partially degraded inulin and had non-preferential growth on FOS. The beta-fructofuranosidase gene was present in both group 2 and 3. Group 4 of bifidobacteria rapidly utilized FOS and had similar phenotypic properties as group 3 in consumption of inulin. Fermentation of less-readily available fructans in

bifidobacteria seem to have occurred simultaneously intra and extracellularly rather than primarily outside the cell or in different stages (Falony et al., 2009). Bifidobacteria might also be assisted by other microorganisms in metabolizing the NDO which are not readily available for fermentation (Flint et al, 2008). Both extracellular and intracellular metabolism of fructans were reported by ß-fructosidase and ß-fructofuranosidase in *L. paracasei* 1195. Monosaccharide components of FOS are transferred inside the cell shortly after cleavage by extracellular enzymes (Goh et al., 2006 and 2007, Kaplan and Hutkins, 2003). However, most of the enzymes characterized in lactobacilli were intracellular.

Genome analysis of *L. acidophillus* NCFM showed the intracellular activity of the exo-enzyme fructosidase, on raffinose, levan and inulin (Barrangou et al., 2003). Similarly, *L. plantarum* WCFS1 preferentially grew on short chain FOS (1-kestose) and the enzyme involved in FOS metabolism was intracellular. Moreover, a number of genes expressed in *L. plantarum* WCFS1 on FOS showed a significant difference compared to glucose where it was mostly related to carbohydrate metabolism or transport binding proteins (Saulnier et al., 2007). It has been suggested that ß-fructofuranosidase activity of *L. plantarum* WCFS1 is closely associated with sucrose phosphorolysis and it is likely that FOS are transferred into the cells after phosphorylation. Preferential consumption of FOS by *L. plantarum* WCFS1 could be likely explained by limitation in sucrose phosphorolysis activity (Saulnier et al., 2007). A similar operon containing sucrose phosphorylase and ß-fructofuranosidase was reported in *B. breve* (Ryan et al., 2005).

Taken all together, inulin-type fructans with high DP are metabolized if the strain possesses extracellular ß-fructosidase (Makras et al., 2005, Rossi et al., 2005). However, the contradictory bifidogenic properties of fructans with high DP *in vivo* could be explained by cross feeding of bifidobacteria by other colonic bacteria (Falony et al., 2006, Flint et al., 2008, Rossi et al., 2005). It seems that all fructan degrading enzymes with different nomenclatures could be placed in one large ß-fructosidase group (Barrangou et al., 2003) (Table 1.2).

1.5.4. Galacto-oligosaccharides (GOS)

Beta- galactosidases are intracellular in lactobacilli. ß- galactosidases are also diverse in particular strains of bacteria. However, the exact reason behind this diversity is unknown. Two genes of *lac*M and *lac*L encoding ß-galactosidase were described in some strains of lactobacilli (Nguyen et al., 2007, Obst et al., 1995) e.g. ß-galactosidase in *L. acidophilus* R22 (Nguyen et al., 2007). Møller et al., characterized three ß- galactosidases in *B. bifidum* DSM20215, one being extracellular and two others intracellular. They also reported the intracellular activity of a ß- galactosidase in *B.infantis* DSM20088 (Møller et al., 2001). The extracellular activity of an endogalactanase was documented in *B. longum* NCC490. This enzyme shows endo- activity first and then exo- activity towards the reducing ends of GOS (Hinz et al., 2005) (Table 1.2).

1.5.5. Resistant starch (RS) and Isomalto-oligosaccharides (IMO)

Different types of starch which are resistant to digestion in the human intestine due to physical accessibility, granular form, reterogradation or modification in the original structure, are called resistant starch. Resistant starch reaches the colon

and is used as a source of energy by different microorganisms to produce SCFA (Laurentin and Edwards, 2005). Commercial IMO are produced by enzymatic liquefaction following by enzymatic conversion of starch. Isomalto-oligosaccharides are partially digestible oligosaccharides with DP of 2-10. Isomalto-oligosaccharides stimulate intestinal bifidobacteria and lactobacilli (Kaneko et al., 1994, Kohomoto et al., 1991 and 1992, Mizbukhi et al, 2005). Bifidobacteria metabolize IMO with high DP first through their glycoside hydrolyze enzymes belonging to the amylopullulanase family (Ryan et al., 2006). The *ApuB* gene coding for amylopullulanase was characterized in *B. breve* UCC2003. The pullulanase domain of amylopullulanase was recognized as an extracellular enzyme excised maltotriose and polymers of that from pullulan *in vitro*. Amylopullulanase is the only enzyme in *B. bereve* UCC2003 to metabolize starch, pullulan and glycogen (O'Connell Motherway et al., 2008). Amylopullulanse likely plays an important role in the colon environment particularly in the weaning period when infants do not chew the food properly and their enzymatic system has not been developed completely. Bifidobacteria with amylolytic activity compete with other bacteria in utilizing the RS-type carbohydrates (O'Connell Motherway et al., 2008). Lactobacilli generally possess intracellular enzymes mostly involved in hydrolysis of disaccharides and oligosaccharides with lower DP for example maltose or sucrose phosphorylase are two enzymes involved in metabolizing maltose and sucrose, the preferred source of carbohydrates, in *L. reuteri* (Ehrmann et al., 1998, Stolz et al., 1996). Maltose phosphorylase of *L. acidophilus* NCFM have maltose/maltodextrin transporters

and metabolize maltodextrins intracellularly by exo-hydrolase activity providing the substrate for maltose phosphorylase and phospho glucomutase. Maltose phosphorylase of *L. acidophilus* NCFM was not active on isomaltose (Nakai et al., 2009). Maltose phosphorylase has a narrow specificity and does not cleave malto-triose or malto-tetraose (Ehrmann and Vogel, 1998). In brief, chain length or DP of oligosaccharides has a direct effect on fermentability of carbohydrates (Hernot et al., 2009, Stewart et al., 2008). Moreover, some lactobacilli and bifidobacteria utilize oligosaccharides preferentially (Table 1.2).

Overall, using NDO is very strain dependent (Rossi et al., 2005). Metabolism of NDO is more challenging for lactobacilli compared to common gram positive or gram negative bacteria present in the intestine. For example 2.9% of the annotated genes in *L. plantarum* WCFS1 are coded for carbohydrate metabolizing enzymes (Kleerebezem et al., 2003). *L. plantarum* has the largest genome size reported among all lactobacilli. Moreover, metabolic simplification and loss of ancestral genes are the common trend in evolution of LAB (Makarova et al., 2006). However, in *B. longum* about 8% of total annotated genes are involved in carbohydrate metabolism (Schell et al., 2002). In addition, more complex transporters are present in bifidobacteria compared to simple dominant phospho transferase systems in less abundant species of the colon (Parche et al., 2007).

1.6. Successful microorganisms in metabolism of NDO in the large intestine

In the complex environment of colon, the transport system, close proximity to NDO and enzymatic system are important factors in the growth of microorganisms and their competition. Nutritionally versatile microbiota that are

highly equipped with different glycoside hydrolase enzymes are adaptable to changing diet and the environment of colon (Macfarlane et al., 2008). Among NDO, Fructo-oligosaccharides, XOS or GOS with low DP are the preferable substrates for lactobacilli, whereas bifidobacteria prefer NDO with high DP (Degnan and Macfarlane et al., 1995, Gopal et al., 2001, Moura et al., 2007, Kaplan and Hutkins, 2000, Wang et al., 2010). Among different bacteria present in the large inetstine, *Bacteroides* is an example of a group of bacteria highly equipped with glycoside hydrolases to degrade plant polysaccharides and oligosaccharides competing with lactobacilli and bifidobacteria. *Bacteroides* genome sequence showed approximately 5 times more glycoside hydrolases compared to bifidobacteria (Flint et al., 2008). Selective stimulation of bifidobacteria or lactobacilli with NDO in the colon indicates that they are successful candidates in competing with *Bacteroides* (Flint et al., 2008). Bifidobacteria have high-affinity transport systems that make them competitive and persistent in the complex environment of the colon compared to other bacteria (Schell et al., 2002).

1.7. Overview and objectives

To predict the effect of IMO in the large intestine of humans, metabolism of IMO was investigated *in vitro* and in rodent models. It was the overall hypothesis of this study that IMO selectively stimulate one or a limited group of bacteria in the colon and decrease the inflammation in an ulcerative colitis rodent model. It was the first aim of this study to investigate the metabolism of IMO in lactobacilli and bifidobacteria. To further confirm the *in vitro* metabolism of IMO in lactobacilli

and bifidobacteria the second study targeted metabolism of IMO in a rodent model, to examine the effects of IMO on intestinal microbiota and SCFA production in the colon. To study the health effects of IMO and to validate the modulating effect of IMO, an IBD rodent model was employed to investigate the effects of IMO on intestinal microbiota, SCFA production and reduction of colitis.

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Chapter 2

Metabolism of isomalto-oligosaccharides by lactobacilli and bifidobacteria

Introduction

Bifidobacteria and lactobacilli utilize polysaccharides and oligosaccharides as carbohydrate sources. More than 8% of total annotated genes of bifidobacteria are involved in carbohydrate metabolism (Van den Broek et al., 2008). In lactobacilli, there are more limited genes involved in metabolism of carbohydrates. For example, 2.9% of the genes in *Lactobacillus plantarum* WCFS1 dedicated to carbohydrate modifying enzymes (Kleerebezem et al., 2003). Kaplan et al. (2000) reported utilization of fructo-oligosaccharides (FOS) especially low molecular weight oligosaccharides by some lactobacilli. Specific growth rate and cell yields were higher on oligosaccharides compared to monosaccharides in many cases. However, inter-species and inter-strain differences were observed (Hopkins et al., 1998). Lactobacilli also reported to have a preference for xylo-oligosaccharides (XOS) with low degree of polymerisation (DP) (Moura et al., 2007). *Lactobacillus sanfranciscensis* metabolizes maltose and exports glucose outside the cell, which is then used after maltose is completely utilized (Stolz et al., 1993). Most of the carbohydrate modifying enzymes produced by lactobacilli are considered to be intracellular. Few extracellular enzymes, such as levansucrase or fructosidase have been documented in lactobacilli (Goh et al., 2007, Tieking et al., 2005). *L. rhamnosus* DR20 showed preference towards monosaccharides and disaccharides in a mixture of galacto-oligosaccharides (GOS) whereas *Bifidobacterium lactis* DR10 used trisaccharides and tetrasaccharides (Gopal et al., 2001). A similar preference was observed in *Bifidobacterium adolescentis* (Amaretti et al., 2006). Ehrmann et al. (2003) confirmed fructofuranosidase

activity of a probiotic strain of bifidobacteria on FOS, inulin and sucrose. Preferential utilization of XOS by *Bifidobacterium* spp. was observed towards higher DP in many studies (Stolz et al., 1993, Wang et al., 2010). Strains of bifidobacteria hydrolyze oligosaacharides into different DP by glycoside hydrolase enzymes. These enzymes are classified as type II or III of pullulanase family (Ryan et al., 2006, Van den Broek et al, 2008). Bifidobacteria possessing extracellular enzymes, preferentially metabolize large oligosaccharides. In addition, chain length seems to influence the fermentability of non-digestible oligosaccharides (NDO) (Stewart et al., 2008).

Prebiotics are selectively fermentable compounds which allow specific changes, both in the composition and/or activity in the gastrointestinal microbiota that confers benefits upon host well-being and health (Roberfroid, 2007). Prebiotics have numerous health benefits for the host such as improving the constipation, alteration of microbiota, usually referring to as bifidogenic properties, and production of short chain fatty acids (SCFA). Non-digestible oligosaccharides such as FOS and GOS are well recognized as prebiotics (Gibson et al., 2010, Meyer et al., 2009). Metabolism of FOS and GOS by lactobacilli and bifidobacteria has been dedescribed before. However, there is little information regarding the metabolim of isomalto-oligosaccharides (IMO) by lactobacilli and bifidobacteria. Commercial IMO are partially digestible and contain α(1→6) and α(1→4) linkages. Isomaltose, iosomaltotriose, panose and isomalto-tetraose are major compounds in IMO. Previous studies showed that IMO have bifidogenic properties (Kaneko et al., 1994, Kohomoto et al., 1988, 1991 and 1992). It was the

aim of this study to compare metabolism of IMO in lactobacilli and bifidobacteria and to explore possible pathway(s) involved in utilization of isomaltose in lactobacilli.

Materials and methods

Culture conditions

Lactobacilli and bifidobacteria were grown in modified de Man Rogosa Sharpe media (mMRS). Components of mMRS were as follows per liter of H_2O: tryptone, 10 g; yeast extract, 5 g; meat extract, 5 g; K_2HPO_4, 2.6 g; KH_2PO_4, 4 g; $FeSO_4$, 0.03 g; $MgSO_4$, 0.1 g; $MnSO_4$, 0.05 g; cysteine-HCl, 0.5 g; Tween 80, 1 mL (All obtained from Sigma or Difco). The medium was sterilised and 1 mL of a vitamin mix containing folic acid, pyridoxal phosphate, thiamine, riboflavin, cobalamin, and pantothenic acid (0.2 g L^{-1} each) was added per litter of sterilized medium and 10 g L^{-1} of IMO (BioNeutra Inc., Edmonton, Canada), glucose or maltose were added to the media (IMO-MRS, Mal-MRS, Glu-MRS) as indicated for vitamins.

Pure cultures were plated on mMRS agar, transferred to mMRS broth and one percent of overnight culture was used to inoculate and propagate the culture. Lactobacilli and bifidobacteria strains used in this study (Table 1) were all incubated at 37ºC in mMRS. Lactobacilli were incubated in anaerobic jars under modified atmosphere conditions (5% CO_2, 4% O_2, 10% H_2 and balance of N_2). Bifidobacteria were incubated in anaerobic chamber (the same gas combination as above). Cells were centrifuged at 17000 x *g* and supernatants were either directly used to analyze the carbohydrates and organic acids or diluted and then analyzed

where it was applicable. Maltose and glucose were applied as control carbohydrates compared to metabolism of IMO by bifidobacteria and glucose was used as a control where metabolism of IMO was tested by lactobacilli.

Table 2.1- Strains used for growth of different bacteria in current study

Strain name and number	Origin of microorganism
L. reuteri LTH5795	Human intestine
L. reuteri 100-23	Rodent intestine
L. johnsoni FUA3040	Rat intestine
L. reuteri FUA3042	Rat intestine
L. reuteri LTH5448	Sourdough
L. reuteri TMW1.106	Sourdough
B. breve ATCC15700	Human intestine
B. longum ATCC15707	Human intestine
B. longum subsp. *infantis* ATCC15697	Human intestine

Maltose phosphorylase activity of lactobacilli

L. reuteri 100-23 was grown on maltose-MRS agar, transferred to maltose-MRS broth, then subcultured in IMO-MRS broth and incubated at 37ºC. Media containing fully grown cells were centrifuged. Cells were washed two times with citrate sodium hydroxide solution (pH=6) and then resuspended in the same buffer in a tube containing silica beads at the bottom. Cells were transferred on ice and

disrupted with mini bead beater (Biospec Products, Bartlesville, USA) for three 1 min cycles (for total of 3 min). Disrupted cells were centrifuged at 17000 x *g* for 5 to 8 min. Supernatant was collected (20 µL) as a source of crude cell extract to measure phosphorylase activity. Citrate buffer of 100 mmol L^{-1} (pH=6) and potassium phosphate of 10 mmol L^{-1} (pH=6) and 100 mmol L^{-1} (705 µL) were used where it was applicable. Fifty µL of 20 mmol L^{-1} magnesium sulphate was added to all samples. Twenty five µL of 4 mg mL^{-1} glucose 6-Phosphate dehydrogenase (from *Saccharomyces cerviseae*), 100 µL of 10 mmol L^{-1} NADP and 100 µL of 0.5 mmol L^{-1} maltose or IMO containing isomaltose were employed to direct the reaction to the final product of 6-phospho gluconate (all chemicals obtained from Sigma). Enzymatic reaction carried out at 37ºC and production of NADPH was measured at 340 nm. Enzyme activity was then calculated as 1 unit of maltose phosphorylase catalyzed the reduction of 1 µmol NADP to NADPH mg min^{-1} protein (Stolz et al., 1996). To report the enzyme activity in a standard approach, Bradford test was performed and commassie brilliant blue G-250 (Bio-Rad, USA) was used as a dye reagent. Bovine serum albumin (BSA) (Invitrogen, Canada) with working concentration of 1 mg mL^{-1} was employed to prepare the standard curve. Crude cell extracts and Bradford dye solution were incubated at room temperature for 5 min and absorbance was measured at 600 nm.

Quantification of substrates and metabolites of carbohydrate metabolism

Cultures were centrifuged at 17000 x *g* for 5 min. Supernatant were diluted 1000 times where it was applicable. Oligosaccharides in the supernatant then were

analyzed by high performance chromatography with pulsed amperometric detection (HPAEC-PAD) with a Carbopac PA20 column at 25°C combined with an ED40 chemical detector (Dionex, Oakville, Canada). Water (A), 200 mM NaOH (B) and 1 M Na-acetate (C) were used as solvents at a flow rate of 0.25 mL min^{-1}. The gradient used in this study was as follows: 0 min 30.4% B, 1.3% C, 22 min 30.4% B, and 11.34% C followed by washing and regeneration. The total run time was 50 min for each sample. The concentrations of isomaltose, isomaltotriose, maltose and glucose (Sigma, Canada) were determined with an external standard calibration curve (Galle et al., 2010). To analyze organic acids and simple sugars, samples were centrifuged to separate the cells. 50 µL of perchloric acid (70%) was added to supernatants and incubated at 4°C overnight. Samples were centrifuged at 17000 x *g* for 5 min and supernatants were collected and injected on to the HLPC. Substrates and end products of fermentation were separated by HPLC (1200 series, Agilent technologies, USA) equipped with an Aminex HPX 87H column (300 x 7.8 mm) (BioRad; Mississauga, Canada). The column temperature was set to 70ºC. Refractive index and UV detectors were attached to the column and a wavelength of 210 nm was used to detect the signals. The mobile phase was 5 mM H_2SO_4 at a flow rate of 0.4 mL min^{-1} (Dlusskaya et al., 2008). Acetic acid, lactic acid and glucose were used as external standards where it was applicable. All standards were obtained from Sigma (Canada).

Results

Metabolism of IMO by lactobacilli and bifidobacteria

To determine whether lactobacilli and bifidobacteria metabolize IMO differently, pure cultures of bifidobacteria and lactobacilli were grown in IMO-MRS medium. The oligosaccharides were analysed with HPLC. Isomaltose, isomalto-triose and a peak tentatively identified as panose were detected as major components of IMO based on known external standards available in the market.

Lactobacilli reuteri LTH5795 metabolised isomaltose. Isomalto-triose and glucose concentrations, however, remained unchanged after 24 hours of fermentation (Figure 2.1.B). Other strains of lactobacilli generated comparable peaks to *Lactobacilli reuteri* LTH5795. Isomaltose and isomalto-triose, two major compounds in IMO, and metabolites of lactobacilli were quantified. Both homo and heterofermentative lactobacilli metabolized IMO in mMRS medium. *L. reuteri* LTH5795, *L. reuteri* FUA3042, *L. reuteri* LTH5448 and *L. reuteri* TMW1.106 used isomaltose and produced mainly lactic acid and ethanol, whereas *L. johnsoni* FUA3040 produced only lactic acid (Figure 2.2). Isomalto-triose was not metabolized by lactobacilli and remained in the media. In heterofermentative lactobacilli 1 mol of isomaltose approximately yielded 2 moles of lactate and 2 moles of ethanol. Isomaltose consumption (~4 mmol/L) thus accounts for formation of ~8 mmol/L of lactic acid and ethanol. Accumulation of more than 20 mmol/L of lactic acid and ethanol indicates conversion of other substrates to extra mols of lactic acid and ethanol. Glucose was used in the media as a positive control (data not shown).

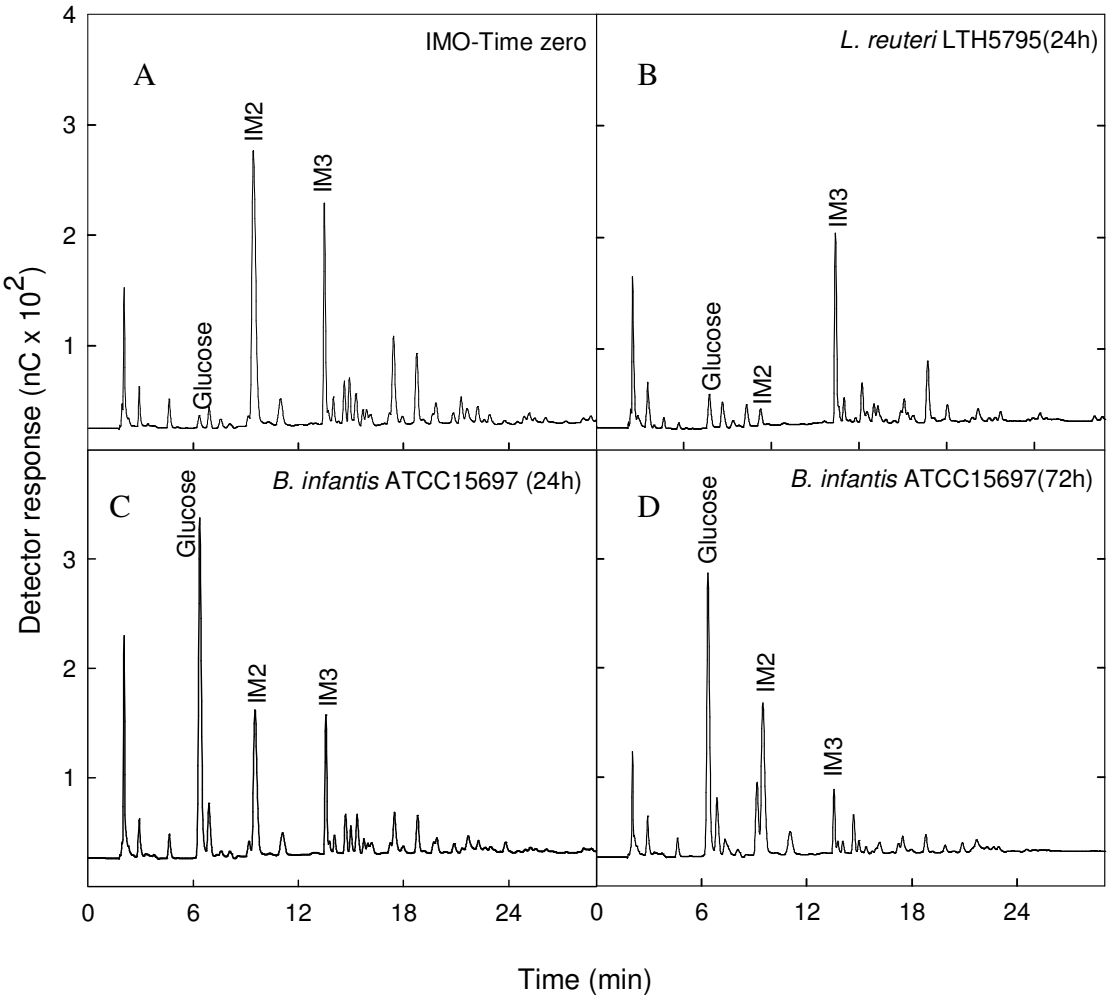

Figure 2.1- HPLC chromatogram showing oligosaccharides profile of IMO before (A) and after fermentation by *L. reuteri* LTH5795 after 24h (B) and *B. infantis* ATCC 15697 after 24 h (C) and 72 h (D) in mMRS respectively

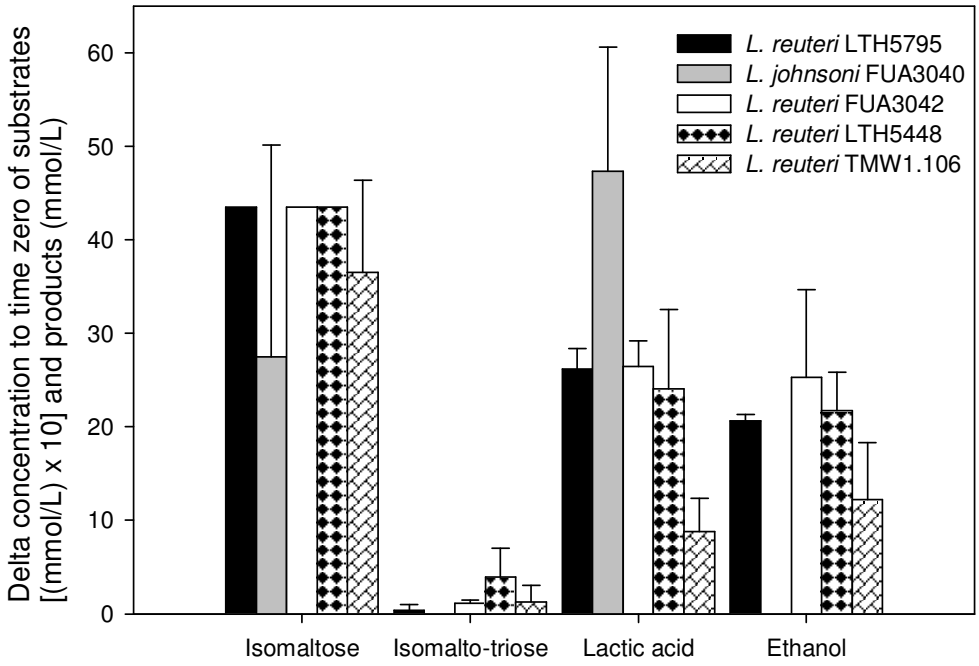

Figure 2.2- Metabolism of IMO in mMRS by *L. reuteri* LTH5795, *L. johnsonii* FUA3040, *L. reuteri* FUA3042, *L. reuteri* LTH5448 and *L. reuteri* TMW 1.106. The initial concentration of isomaltose and isomalto-triose was determined as 4.35 ± 1.91 mmol/L and 1.49 ± 0.05 mmol/L respectively.

Bifidobacteria either metabolised isomalto-triose first rather than isomaltose or isomalto-triose and isomaltose were consumed and produced at the same rate. Glucose was not a preferred substrate for bifidobacteria. *Bifidobacterium longum* subs. *infantis* ATCC15697 accumulated glucose in the media after 24 hours as oligosaccharides were cleaved to lower molecular weight compounds (Figure 2.1.C). After 72 hours, isomalto-triose was almost completely metabolized whereas isomaltose was still present in the media. The concentration of

isomaltose was lower than time zero (Figure 2.1.D). Two strains of *B. longum* ATCC15707 and *B. breve* ATCC15700 generated similar HPLC peaks. Growth kinetics for different strains of bifidobacteria indicates isomalto-triose was consumed completely after 24 or 48 hours of fermentation in all strains of bifidobacteria tested whereas isomaltose was still present in the media after 72 hours (Figure 2.3). Glucose was increased in the first 24 hours of fermentation by *B. longum* subs. *infantis* ATCC15697 and *B. longum* ATCC15707 and then stayed constant for the next 48 hours (Figure 2.3.A, B). In fermentation of IMO by *B. breve* ATCC15700, however, glucose level was slightly increased after 24 hours and then was completely consumed after 48 hours (Figure 2.3.C). Bifidobacteria produced lactic and acetic acid and amount of acetic acid was 1.8-2 times as lactic acid (Figure 2.3). This is in agreement with previous studies showing metabolism of carbohydrates by bifidobacteria and production of acetic and lactic acids with a ratio of 1.5 and up to 2.9, depending on the strain and substrate (Korakli et al., 2002). Metabolism of IMO compared with maltose and glucose as control carbohydrates and similar results obtained with glucose and maltose (data not shown).

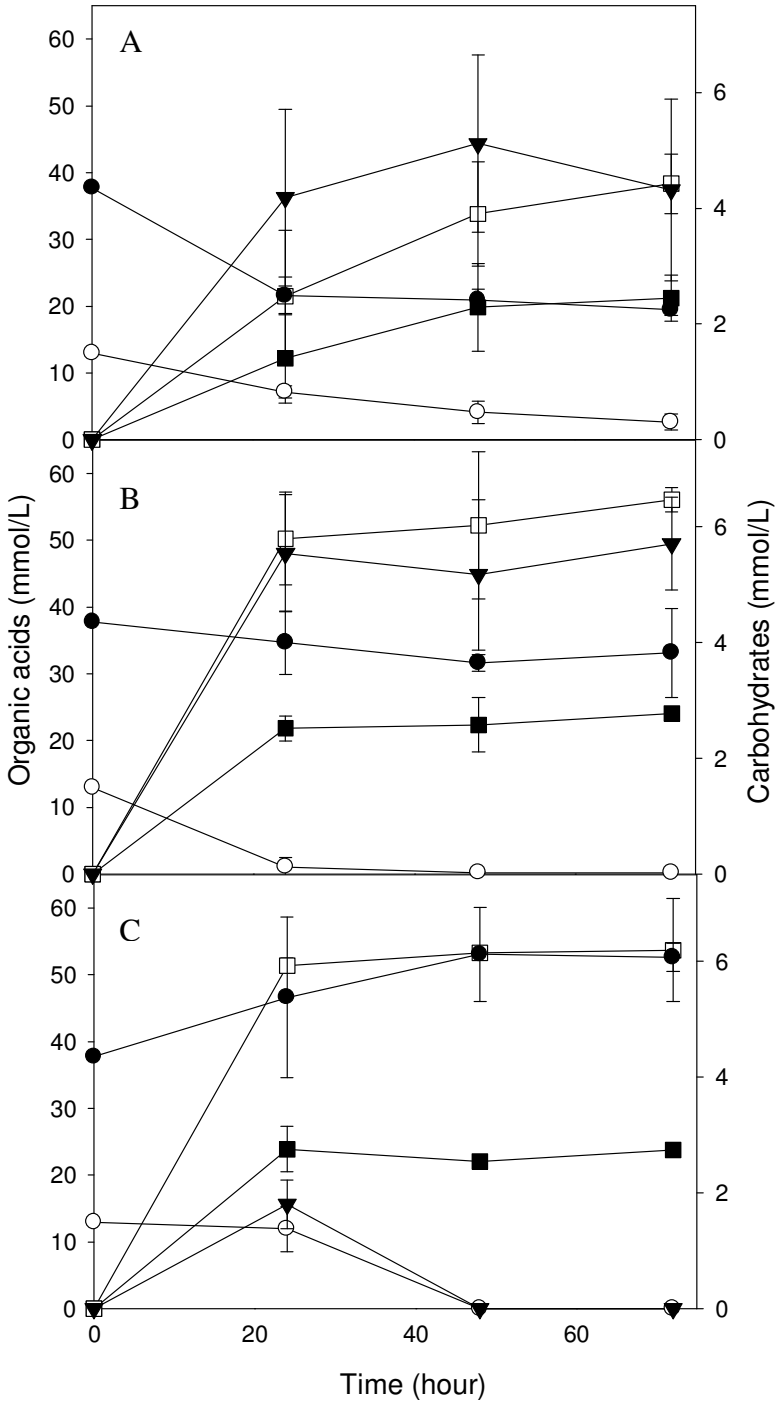

Figure 2.3- Metabolism of IMO in mMRS and kinetics of growth by *B. infantis* ATCC15697 (A), *B. longum* ATCC15707(B) and *B. breve* ATCC15700 (C). Lactic acid (■), acetic acid (□), isomaltose(●), isomaltotriose (○), glucose (▼)

Maltose phosphorylase activity of lactobacilli

To compare phosphorylase activity of lactobacilli when maltose or isomaltose were added to the reaction, *L. reuteri* 100-23 was selected with its complete genome sequence available. Phosphorylase activity of *L. reuteri* 100-23 was measured for maltose or isomaltose in three buffers containing 0, 10 or 100 mmol L^{-1} of phosphate. Crude cell extract of *L. reuteri* 100-23 grown on IMO-MRS was added to the reaction. Increasing phosphate from 10 to 100 mmol L^{-1} in the reaction significantly increased phosphorylase activity independent of maltose or isomaltose addition (Figure 2.4). There was no significant difference observed in maltose phosphorylase activity of *L. reuteri* 100-23 where either maltose or isomaltose added as a substrate at 37 ºC. Similar results were obtained for strain *L. reuteri* LTH5795 in the same conditions (data not shown).

Discussion

Compared to lactobacilli, metabolism of IMO by bifidobacteria showed that bifidobacteria prefer IMO with higher DP. Moreover, they accumulated glucose in the media. Isomalto-triose was completely metabolized after 24 or 48 hours of whereas isomaltose was still available in the media. In a similar study, 25% of the bifidobacteria tested possessed external enzymes and were involved in cleavage of oligosaccharides with α(1→4) and α(1→6) linkages (Wang et al., 2010). Lower molecular weight oligosaccharides and monosaccharides are transferred through the membrane and used in cytoplasm by bifidobacteria. Lactobacilli preferentially metabolize isomaltose compared to isomalto-triose during the fermentation of IMO.

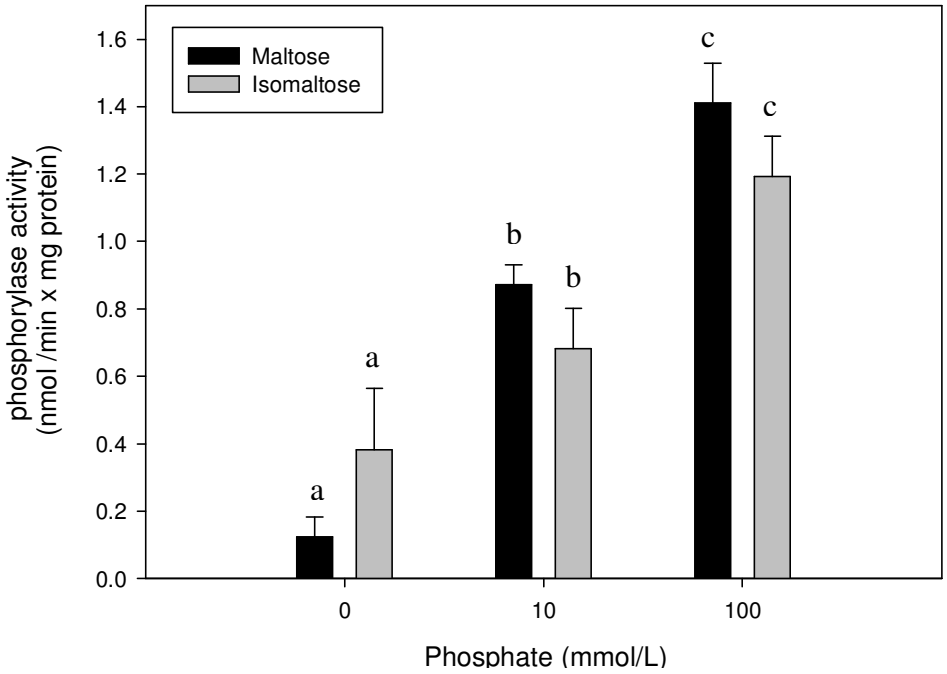

Figure 2.4- Phosphorylase activity of crude cell extract of *L. reuteri* 100-23 grown in IMO-MRS where maltose (black) or isomaltose (grey) added as a substrate in the reaction. Non similar letters are significantly different.

In bifidobacteria, isomalto-triose was almost completely metabolized after 72 hours whereas isomaltose was still present in the media during the same period (Figure 2.1). Goh et al. (2007) suggested a similar exo-hydrolase activity for ß-fructosidase in a medium that contained FOS. Lactobacilli and bifidobacteria behaved differently in the metabolism of IMO and is likely related to the different enzymes they possess. Maltose phosphorylase was likely involved in the metabolism of isomaltose in lactobacilli. However, other phosphorylase enzymes might be present in lactobacilli. In bifidobacteria, extracellular enzymes from

pullulanase family were involved in metabolism of IMO. Phosphorolysis is a common mechanism for uptake of maltose in lactobacilli (Stolz et al., 1996). Maltose phosphorylase is an intracellular enzyme located in the cytoplasm of *L. reuteri* and functions in the presence of inorganic phosphate by attaching one phosphate molecule to glucose resulting in the production of glucose 6-phosphate without expending any ATP (Stolz et al., 1996). Maltose phopshoryalse has a narrow specificity and does not cleave malto-trisoe or malto-tetraose (Ehrmann et al., 1998). Isomalto-oligosaccharides are partially digestible. Low DP IMO are metabolized by lactobacilli in the forestomach of rats whereas in humans the undigested portion of IMO reach the colon and fermented by colonic microbiota.

Maltose and isomaltose were both metabolized by intracellular phosphorylase enzymes. There was no significant difference between enzyme activities where maltose or isomaltose was added to the reaction. Increasing phosphate level from 10 mmol L^{-1} to 100 mmol L^{-1} significantly altered the activity of enzyme. In brief, a phosphorylase enzyme was likely involved in cleavage and metabolism of isomaltose.

Maltose phosphoryalse and kojibiose phosphorylase in *L. reuteri* were often referred to enzymes involved in maltose and kojibiose metabolism. Only one gene in the entire genome sequence of *L. reuteri* 100-23 was found for kojibiose phosphorylase. Moreover, the kojibiose phosphorylase gene was 98% identical to the maltose phosphorylase gene in *L. reuteri* DSM 20016 and *L. reuteri* JCM1112. Among maltose phosphorylases and kojibiose phoshporylases that have been cloned and characterized, kojibiose phosphorylase of *L. reuteri* 100-23

is very identical to *L. sanfranciscensis* and it is not identical to *Thermoanaerobacter brockii* ATCC35047 (Ehrmann et al., 1998, Yamamoto et al., 2004). Maltose phosphorylase of *L. acidophilus* NCFM and kojibiose phosphorylase of *L. reuteri* 100-23 have 72% identity at the nucleotide level (Nakai et al., 2009). We propose that maltose phosphoryalse and kojibiose phosphorylase are one enzyme, with two names and are involved in metabolism of maltose, isomaltose and kojibiose.

Lactobacilli are mostly found on plants and upper intestine of pigs, chickens and rodents whereas bifidobacteria are primarily inhabit the human and some animal's intestine. Some bifidobacteria have limited host distribution and age specifications (Biavati et al., 2000, Lamendella et al., 2008, Turroni et al., 2009). Bifdobacteria and lactobacilli preference of carbohydrates with different DP supports the speculation that ecological niches might play an important role in prevalence of bacteria in one environment versus the other (Walter, 2008). Taken all together, lactobacilli are capable of using NDO with low DP whereas some bifidobacteria prefer NDO with high DP, such as resistant starch (RS) or inulin (Rodríguez-Cabezas et al., 2010). However, preference of bacteria towards NDO is very strain-dependent relying on the enzymes possessed by bacteria (Falony et al., 2009). Bifidobacteria, generally have a variety of glycoside hydrolases and that makes them versatile compared to lactobacilli. For example bifidobacteria in current study, tended to utilize isomalto-triose first compared to isomaltose. This is in agreement with the metabolism of FOS and RS by bifidobacteria and lactobacilli in rats. Rodriguez-Cabezas et al. (2010) reported increase of

lactobacilli and bifidobacteria numbers both by FOS and by mixture of FOS and RS (2g day^{-1}) whereas RS alone did not stimulate growth of lactobacilli.

Bifidobacteria and lactobacilli are present in the gastrointestinal system of many hosts and may benefit the host and other groups of bacteria through cross-feeding (Falony et al., 2006). However, other groups of bacteria having rich carbohydrate modifying enzymes do co-exist with lactobacilli and bifidobacteria in the gastrointestinal system and compete for carbohydrates (Van den Broek et al., 2008). Selection of NDO targeting a specific genus, species or even a strain of bacteria is very critical when prebiotics are recommended to improve well being of the host. This is the first study which compared metabolism of IMO in bifidobacteria and lactobacilli. Isomalto-oligosaccharides are potential candidates to selectively stimulate bifidobacteria in the colon. Moreover, Short chain fatty acids produced by different species of lactobacilli and bifidobacteria in the colon might benefit the host through maintenance of the intestinal system and increase well being of the host. In conclusion, with the possibility of modifying the structure of IMO it is quite feasible to make products with different DP. Isomalto-oligosaccharides with higher DP is expected to target bifidobacteria.

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Chapter 3

Metabolism of isomalto-oligosaccharides in rats*

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The panose series standards in Figure 3.1 was kindly provided by Dr. Ying Hu.

Introduction

Dietary non-digestible oligosaccharides (NDO) modulate the composition and activity of intestinal microbiota and they may also exert health benefits in the host. They improve bowel function, may prevent overgrowth of pathogenic bacteria through selective stimulatation of non-pathogenic members of intestinal microbiota, and increase production of short chain fatty acids (SCFA). SCFA reduce the luminal pH and provide energy for colonocytes (Topping and Clifton, 2001; Meyer and Stasse-Wolthuis, 2009). Intestinal fermentation and health benefits of fructooligosaccharides and galactooligosaccharides have been well documented in animal and human studies (Gibson et al, 2010, Meyer and Stasse-Wolthuis, 2009). However, information regarding intestinal fermentation of other oligosaccharides that are present in food or are used as food additives such as isomaltooligosaccharides (IMO), soybean oligosaccharides, and lactulose, is limited (Kolida and Gibson, 2008). The diverse structure, monomer composition, and degree of polymerization of dietary oligosaccharides influences not only intestinal fermentation and SCFA production, (Kleessen et al., 2001, Nilsson and Nyman, 2005) but also affects their technological properties in food.

IMO with α (1→6) and α (1→4) glucosidic linkages are used as alternative low-calorie sweeteners in food products (Kohomoto et al., 1992). IMO are only partially digested and the undigested portion is fermented in the colon. The caloric content of a commercial IMO preparation was about 75% when compared to maltose (Kohomoto et al., 1992). IMO improve constipation similar to other fibers (Wang et al., 2001). At high doses, NDO and polysaccharides usually have

a laxative effect. However, IMO are generally tolerated at higher dosages compared to other NDO (Kaneko et al., 1994). Commercial IMO preparations consist of isomaltose, isomalto-triose, panose, and isomalto-tetraose as major compounds. Differnet products differ substantially in their composition, particularly the proportion of digestible carbohydrates (maltose and glucose), the proportion of α (1→4) linkages, and the degree of polymerization (Kohmoto et al., 1991 and 1992, Yen et al., 2010), and these differences in composition likely influence digestibility, caloric content, and their effect on intestinal microbiota.

Several human studies indicate that IMO are bifidogenic (Kaneko et al., 1994, Kohomoto et al., 1988, Kohomoto et al., 1991 and 1992). However, the majority of these studies relied on culture-dependent methods that targeted only a few bacterial groups in the colon. Information regarding the *in vivo* effect of IMO on intestinal microbiota using culture-independent methods is limited (Yen et al., 2010). The aim of this study was to investigate the effect of IMO on microbiota composition and short chain fatty acids (SCFA) production in the intestine of rats using culture-independent molecular techniques.

Materials and methods

Determination of IMO components with high performance anion exchange chromatography – pulsed amperometric detection (HPAEC-PAD)

IMO were obtained from BioNeutra Inc. (Edmonton, Canada). The composition of the IMO preparation was specified by the supplier as IMO with predominantly α (1→6) linkages and a degree of polymerization (DP) of 2 (18-25%), DP 3 (15-

23 %), DP 4 (14-22%), DP 5 (8-10%), DP 6 (6-8%), DP 7 (2-4%) and DP 8 (2-3%). Isomalto-oligosaccharides were analyzed by HPAEC-PAD with a Carbopac PA20 column coupled to an ED40 chemical detector (Dionex, Oakville, Canada) using water (A), 200 mmol L^{-1} NaOH (B) and 1 mol L^{-1} Na-acetate (C) as solvents at a flow rate of 0.25 mL min^{-1} and a temperature of 25°C. The gradient was as follows: 0 min 30.4% B, 1.3% C, 22 min 30.4% B, and 11.34% C followed by washing and regeneration. Isomaltose, isomalto-triose and panose were identified and quantified by use of external standards (all obtained from Sigma, ON, Canada). Other peaks were tentatively identified by synthesizing oligosaccharides of the panose series with dextransucrase of *Weissella minor* ATCC35912 (Galle et al., 2010). In brief, *W. minor* was grown for 24 h in modified MRS containing 230 mmol L^{-1} sucrose as glucosyl-donor and 55 mmol L^{-1} maltose as glucosyl-acceptor for synthesis of oligosaccharides. Bacterial cells were removed by centrifugation and oligosaccharides in the supernatant were analyzed by HPAEC-PAD as described above.

Experimental design

Five weeks old F344 rats were housed in specific pathogen free conditions (SPF). Rats in three treatment groups were fed either IMO (BioNeutra), commercial inulin (Raftiline HP, Raffinerie Tirlemontoise, Tienen, Belgium) or a control diet. Each treatment group consisted of six rats and one or two animals were housed per cage. The base diet was a commercial laboratory rodent diet (5001, Lab Diet, USA) with the following composition: 23% crude protein; 4.5% crude fat; 6% fiber; 8% ash. Inulin or IMO were added to this base diet at 8 g (kg body weight)$^{-1}$

for 6 weeks. Rats were given free access to water. Sampling of stool from individual animals was performed at 5, 8 and 11 weeks of age. Fecal samples were immediately snap-frozen at -80°C for analysis of intestinal microbiota and SCFA. Experiments were approved by the University of Alberta Animal Policy and Welfare Committee (UAPWC) in accordance with the Canadian Council on Animal Care (CCAC) guidelines.

PCR-denaturing gradient gel electrophoresis (DGGE)

PCR-DGGE analysis with universal primers was performed as previously described (Tannock et al., 2000). In brief, DNA from fecal samples was extracted using the Qiagen DNA extraction kit and the DNA concentration was adjusted to 50-70 mg L^{-1}. Universal primers HAD1-GC (5'-CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GAC TCC TAC GGG AGG CAG CAG T-3'and HAD2 (5'-GTA TTA CCTG CGG CTG CTG GCA C-3') were used to amplify bacterial rDNA. DGGE was performed by using a DCode™ Universal Mutation Detection System (Bio-Rad, Hercules, CA, USA) in 6% acrylamide gels with a denaturing gradient of 30–55%. Electrophoresis was performed at 150 V and 60 °C for about 3 hours. Gels were stained with ethidium bromide and viewed by UV trans-illumination. Patterns were normalized by including PCR products from one sample on all gels. Cluster analysis was performed by unweighted pair group method with arithmetic mean (UPGMA) based on the dice correlation coefficient using an optimization coefficient of 1% (Bionumerics software, version 3, Applied Maths, Sint-Martens-Latem, Belgium).

DGGE analysis of the diversity in *Lactobacillus-Pediococcus-Leuconostoc-Weissella* species was performed using the following primers:

LAC1 (5'- AGC AGT AGG GAA TCT TCC A-3'and LAC2- GC(5'- CGC CCG GGG CGC GCC CCG GGC GGC CCG GGG GCA CCG GGG GAT TYC ACC GCT ACA CAT G-3') with subsequent separation of amplicons by DGGE, as described (Walter et al., 2001). Bands from *Lactobacillus* group-specific DGGE were excised from the gel, used as template for PCR amplification with primers Lac1 and Lac2, and sequenced in the MBSU facility of Department of Biological Sciences at the University of Alberta (Accession numbers: *L. animalis*, HM765476; *L. reuteri*, HM765477, HM765478 and HQ658983). Sequences were matched to type strain sequences available on RNA database project (http://rdp.cme.msu.edu/seqmatch/seqmatch_intro.jsp).

Quantification of microbiota by quantitative PCR (qPCR).

Group-specific primers were used to quantify 16S rRNA gene copy numbers (rDNA) of *Lactobacillus-Pediococcus-Leuconostoc-Weissella* species (*Lactobacillus* group), *Bifidobacterium* spp., *Bacteroides-Prevotella-Porphyromonas* spp. (*Bacteroides* group), clostridal clusters I (*C. perfringens* group), IV (*C. leptum* group), XI (*C. difficile* group), and XIV (*C. coccoides* group), *Enterobacteriaceae*, and total bacteria. Genes coding for butyrate CoA-CoA transferase and butyrate kinase were quantified using degenerate primers (Table 3.1). PCR reactions were carried out on a fast real time PCR unit (Applied Biosystems, Streetsville, Canada), PCR cycles were set to 95 °C for 5 min initial denaturation, followed by 40 cycles at 95 °C for 15 s; annealing for 30 s (Table

3.1), and 72 °C for 30 s extension. The master mix (25 µL) contained 12.5 µL Applied Biosystems Fast SYBR Green master mix, 1 µL template DNA and 0.05 pM primer. To establish standard curves, DNA of one of the samples was used as PCR template. The respective amplicons were purified, and serial 10-fold dilutions of PCR products were used as templates for standard curves in qPCR. Samples from individual animals were analysed in at least duplicate. Results from samples obtained from the same treatment group and time point were averaged and reported as log (gene copy number g^{-1}).

Analysis of short chain fatty acids in stool samples with gas chromatography (GC).

SCFA were extracted from 100 mg stool samples by adding 200 µL of 5% phosphoric acid. Solids were removed by centrifugation at 17000 x *g* and supernatants were injected on a Stabilwax-DA column (30 m, 0.53 mm ID, 0.5 µm df). The head pressure was 7.5 psi, split vent flow was set to 20 mL min^{-1} or adjusted as required. Injector temperature was 170 ºC. The column temperature was held at 90ºC for 0.1 min, increased to 170 ºC at 10 ºC min^{-1} and held for 2 min. The detector temperature was 190 ºC. Concentrations of acetate, propionate, butyrate, iso-butyrate, iso-valerate, valerate, and caproate was determined used external standards (obtained from Sigma, ON, Canada). Iso-caproate was used as internal standard.

Statistical analysis.

A mixed procedure with repeated measures was used to analyze the effect of diet, time and the interaction of time and diet using SAS software. Data from five

weeks old rats were used as co-variates. Results were reported as mean values and their standard errors. The least significant difference test was used to identify differences between treatments. Differences were considered to be significant if $P< 0.05$.

Table 3.1- Oligonucleotide primers used in qPCR of fecal samples

Bacterial group (amplicon size)	Olionucleotide sequence (5`->3`)	A_T*	Reference
Lactobacillus group (341 bp)	F: AGCAGTAGGGAATCTTCCA R: CACCGCTACACATGGAG	62	Walter et al., 2001 Heilig et al., 2002
Bifidobacterium spp. (243 bp)	F: TCGCGTCYGGTGTGAAAG R: CCACATCCAGCRTCCAC	60	Rinttilä et al., 2004
Cluster XIV (438-441 bp)	F: AAATGACGGTACCTGACTAA R: CTTTGAGTTTCATTCTTGCGAA	60	Matsuki et al., 2002
Cluster IV (230 bp)	F: GCACAAGCAGTGGAGT R: CTTCCTCCGTTTTGTCAA	60	Matsuki et al., 2004
Cluster I (120 bp)	F: ATGCAAGTCGAGCGAKG R: TATGCGGTATTAATCTYCCTTT	60	Rintillä et al., 2004
Bacteroides-group (140 bp)	F: GGTGTCGGCTTAAGTGCCAT R: CGGAYGTAAGGGCCGTGC	60	Rintillä et al., 2004
Enterobacteriaceae family (195 bp)	F: CATTGACGTTACCCGCAGAAGAAGC R: CTCTACGAGACTCAAGCTTGC	53	Bartosch et al., 2004
Butyryl CoA-CoA transferase (530 bp)	F: CIGAICATTTCACITGGAAYWSITGGCAYATG R: CCTGCCTTTGCAATRTCIACRAANGC	53	Louis and Flint, 2007
Cluster XI (bp) (~180 bp)	F: ACG CTA CTT GAG GAG GA R: GAG CCG TAG CCT TTC ACT	58	Song et al., 2004
Butyrate kinase (301 bp)	F: GTA TAG ATT ACT IRY IAT HAA YCC NGG R: CAA GCT CRT CIA CIA CIA CNG GRT CAN C	53	Louis and Flint, 2007
Total bacteria (200 bp)	F: CGG YCC AGA CTC CTA CGG G R: TTA CCG CGG CTG CTG GCA C	60	Rintillä et al., 2004

Results

Characterization of the IMO preparation.

Carbohydrates in the IMO preparation were separated and quantified by HPAEC-PAD (Figure 3.1). Isomaltose, isomaltotriose, and panose accounted for 11.3 ± 2.9, 5.8 ± 1.2 and 5.6 ± 2.2 % (w/w) of the IMO preparation, respectively; glucose and maltose were essentially absent. 6'Glucosylpanose and 6'6'diglucosylpanose were also identified using enzymatically synthesized standards (Figure 3.1). Dextransucrase from *W. minor* produces oligodextran from sucrose and maltose, a homologous series of linear oligosaccharides composed of α (1→6) linked glucose moieties and a maltose residue at the reducing end. These oligosaccharides elute with increasing degrees of polymerization (Galle et al., 2010, Dols et al., 1998). Other disaccharides and higher oligosaccharides were additional components of the IMO preparation, but these could not be identified with external or enzymatically synthesized standards.

Qualitative analysis of fecal microbiota with DGGE.

PCR-DGGE was employed to initially assess qualitative effects of IMO or inulin on the fecal microbiota. Cluster analysis of PCR-DGGE at 8 weeks of age showed that almost all fecal samples of rats fed inulin were separated from those fed control or IMO diets. There was no clear separation between treatment groups at 11 weeks of age (Figure 3.2.A, B).

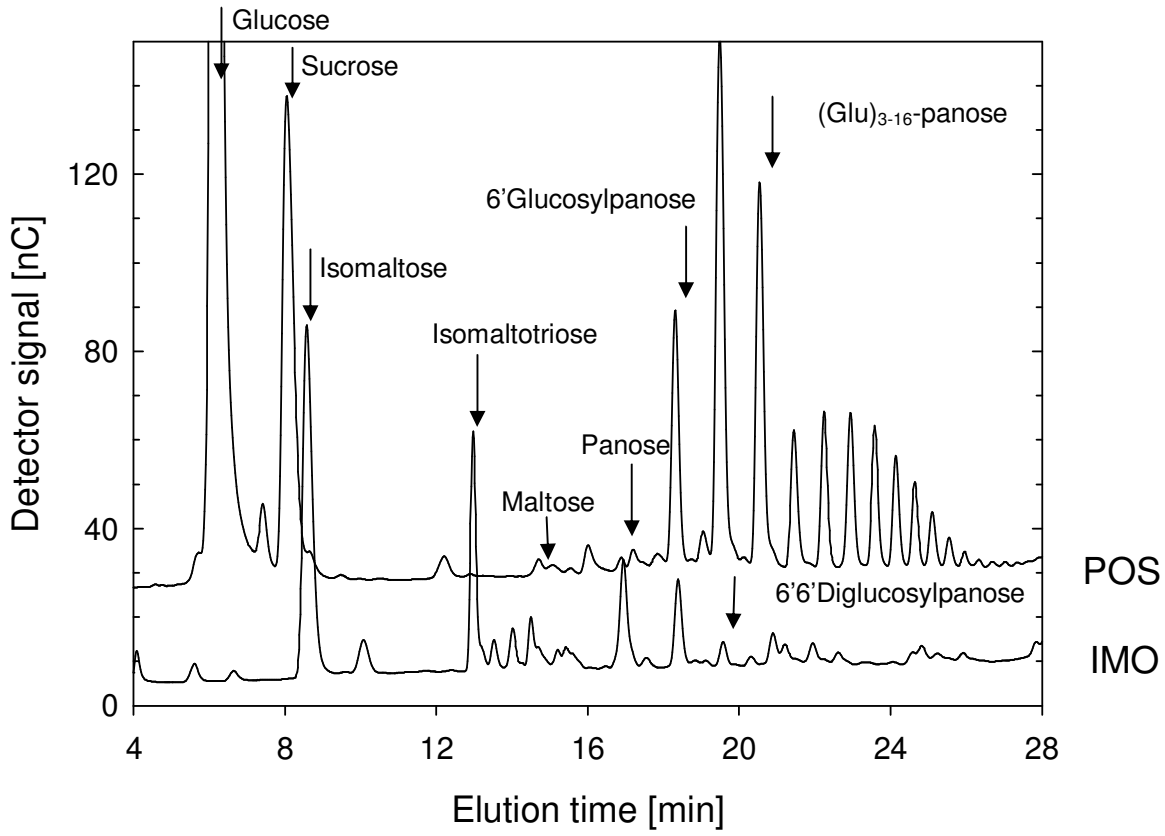


Figure 3.1- HPAEC-PAD separation of IMO (lower trace), and of oligosaccharides of the panose-series (POS) synthesized with dextransucrase of *Weissella minor* ATCC35912 with maltose as acceptor carbohydrate. Glucose, sucrose, isomaltose, isomaltotriose, maltose, and panose were identified and quantified by use of external standards; 6'glucosylpanose and 6'6'diglucosylpanose were tentatively identified by enzymatic synthesis of oligosaccharides of the panose series

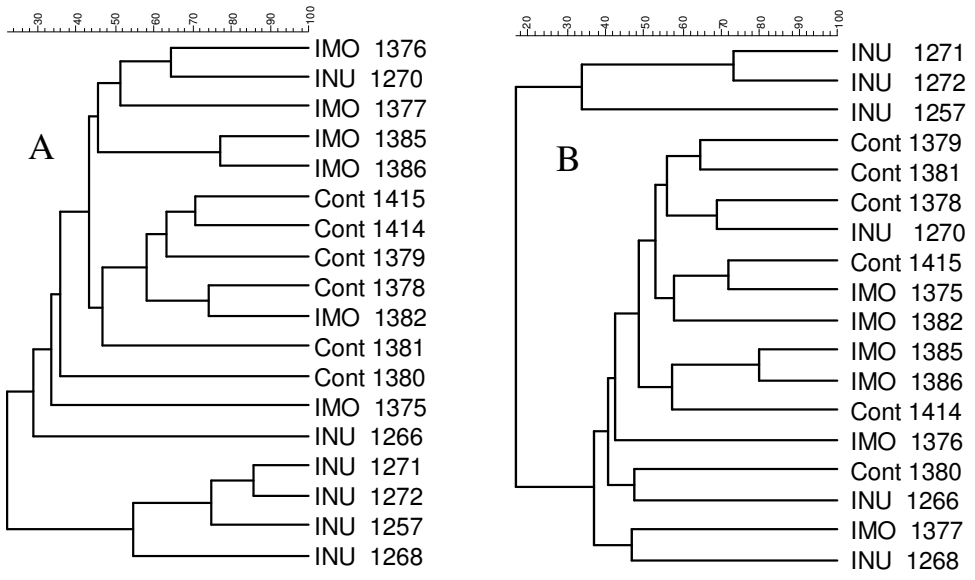


Figure 3.2- PCR- DGGE of fecal samples of rats at 8 weeks of age (Panel A) and at 11 weeks of age (Panel B) fed IMO, inulin or control diet with universal primers (Dice correlation coefficient, Tol: position tolerance 1%, Opt: optimization 0.5%). IMO: Isomalto-oligosaccharides, INU: inulin, Cont: control, #: rat number

Quantitative analysis of fecal microbiota with qPCR.

Quantitative differences between bacterial taxa in fecal samples from IMO-fed and control animals were assessed using qPCR and group-specific primers (Table 3.2). The *Lactobacillus*-group was one of the dominant bacterial taxa in the samples; feeding IMO significantly increased rDNA copy numbers of fecal organisms in the *Lactobacillus* group compared to rats on control diet (Table 3.2). In contrast, the number of bifidobacteria in fecal samples from rats fed IMO was low and significantly different from animals fed the control diet at eleven weeks of age. Fecal clostridial cluster XI and *Enterobacteriacea*e decreased over time in

both treatment groups. The *Bacteroides* group as well as clostridial clusters I, IV, and XIV were not affected by diet or time. Total number of fecal bacteria was increased in rats fed IMO compared to control diet.

Quantitative analysis of genes encoding key enzymes of bacterial butyrate metabolism demonstrated that genes encoding butyrate kinase were below the detection limit of 10^4 gene copies g^{-1} in all samples (data not shown). Copy numbers of genes encoding butyrate CoA-CoA transferase were unaffected by diet or time.

Table 3.2- Effect of diet, time and interaction of diet and time on DNA copy numbers of fecal bacteria

Bacterial group diet	Weeks				Statistical significance of effect: *P*		
					Diet	Time	Diet x Time
	5 wk	8wk	11wk	Pooled SEM			
Lactobacillus group							
Control	7.82	7.74	7.46	0.17	*	ns	*
IMO	8	8.14	8.57	0.17			
Bifidobacteria							
Control	6.07	6.36	6.08	0.09	*	*	*
IMO	6.13	5.84	5.51	0.09			
Cluster XIV							
Control	8.18	8.45	8.13	0.14	ns	ns	ns
IMO	8.48	8.26	8.29	0.14			
Cluster IV							
Control	8.10	8.30	8.11	0.14	ns	ns	ns
IMO	8.08	8.33	8.30	0.14			
Bacteroides							
Control	9.84	9.99	9.61	0.17	ns	ns	ns
IMO	9.82	9.61	9.58	0.17			
Cluster I							
Control	8.69	8.81	8.58	0.16	ns	ns	ns
IMO	8.34	8.59	8.35	0.16			
Enterobacteriacea							
Control	8.02	8.03	7.34	0.16	ns	ns	*
IMO	7.94	7.98	7.54	0.16			
Cluster XI							
Control	7.43	7.18	6.93	0.15	ns	ns	*
IMO	7.60	6.99	7.04	0.15			
But CoA- CoA transferase							
Control	6.64	6.89	6.59	0.19	ns	ns	ns
IMO	6.77	6.53	6.54	0.19			
Total bacteria							
Control	10.10	10.15	9.85	0.14	*	ns	ns
IMO	10.44	10.28	10.28	0.14			

* IMO: isomalto-oligosaccharides diet, Significant (P< 0.05), ns= not significant

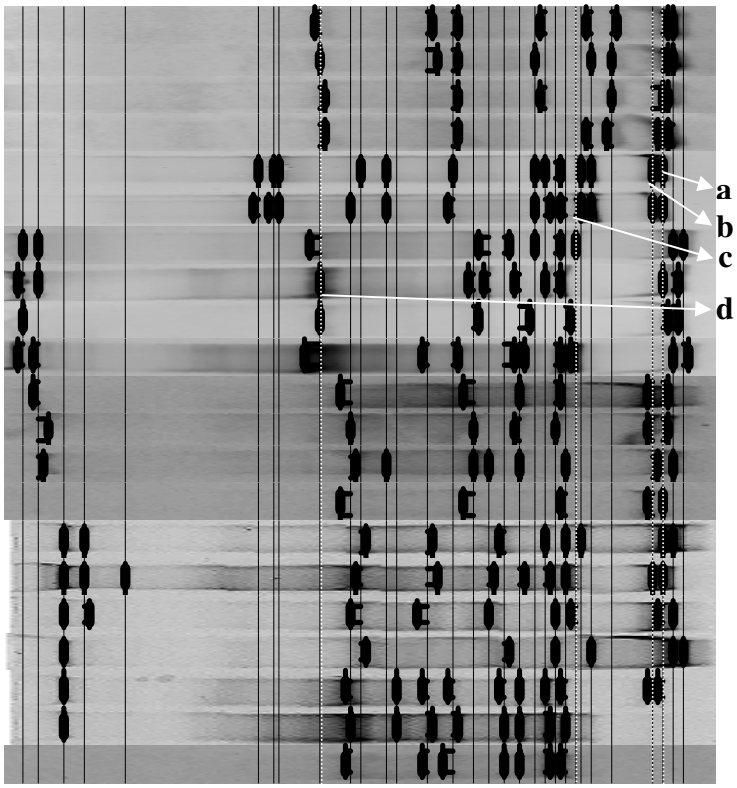
Qualitative assessment of organisms in the *Lactobacillus* group by PCR-DGGE with group-specific primers.

To determine if the increased abundance of organisms in the *Lactobacillus* group in rats fed IMO was associated with an increased biodiversity, PCR-DGGE analysis with primers specific for the *Lactobacillus* group was performed (Figure 3.3). The number of bands in fecal samples of rats fed IMO diet after 8 or 11 weeks of age were not significantly increased compared to those from the same time points in rats fed control diets, and cluster analysis did not clearly separate the banding patterns according to the diet. However, one specific band was observed almost exclusively in rats fed IMO. Four bands differing in their abundance in IMO-fed and control animals were subjected to sequence analysis. Band a, identified as *L. reuteri,* was present in most animals. Band b, also identified as *L. reuteri,* was present in most samples from rats fed IMO but was essentially absent in rats fed the control diet. Band c, attributed to *L. animalis*, was present in few animals fed either IMO or control diets. Band d, also identified as *L. reuteri,* was present in most of the control rats but was absent in rats fed IMO diet (Figure 3.3).

Short chain fatty acids (SCFA).

SCFA were analysed in the fecal samples of rats fed IMO or inulin diet to determine the effect of non-digestible carbohydrates in colonic carbohydrate fermentation. Acetate, butyrate and propionate were the dominant end products of bacterial fermentation in fecal samples, whereas iso-butyrate, iso-valerate, valerate and caproate were minor components of SCFA. Inulin did not change

fecal SCFA concentrations compared to rats on a control diet. However, IMO significantly decreased fecal acetate compared to rats fed control diet at 11 weeks of age. Total SCFA was also decreased in rats fed IMO diet, compared to control and inulin treatments. Propionate, butyrate, iso-butyrate, iso-valerate, valerate and caproate were not affected by dietary intervention treatments (Figure 3.4.A, B).



Diet #(time)	a	b	c	d
Cont 5(11)	+	-	-	+
Cont 5(8)	+	-	-	+
Cont 4(11)	+	-	-	+
Cont 4(8)	+	-	-	+
IMO 1(11)	+	+	+	-
IMO 1(8)	+	+	+	-
Cont 1(11)	-	-	+	+
Cont 2(8)	+	-	-	+
Cont 2(11)	+	-	-	+
Cont 1(8)	-	-	+	-
IMO 5(11)	+	+	-	-
IMO 6(11)	+	+	-	-
IMO 6(8)	-	+	-	-
IMO 4(8)	+	+	-	-
IMO 2(11)	+	-	+	-
IMO 3(11)	+	+	-	-
IMO 3(8)	+	-	+	-
IMO 2(8)	-	-	-	-
Cont 3(11)	+	+	-	-
Cont 3(8)	-	-	-	-
IMO 4(11)	-	-	-	-

Figure 3.3- DGGE of fecal samples at 8 and 11 weeks of age fed IMO or control diets run with lactobacilli- specific primers. Band assignment was carried out with BioNumerics with a 1% tolerance for the position of the band in the gels; presence or absence of bands that were identified by sequencing is shown to the right of the gel. IMO: Isomalto-oligosaccharides, Cont: control, #: rat number, a: *Lactobacillus reuteri* b: *Lactobacillus reuteri* and c: *Lactobacillus animalis,* excised from the gel and sequenced. Numbers in brackets are ages of rats in weeks. (+): band present, (-): band absent

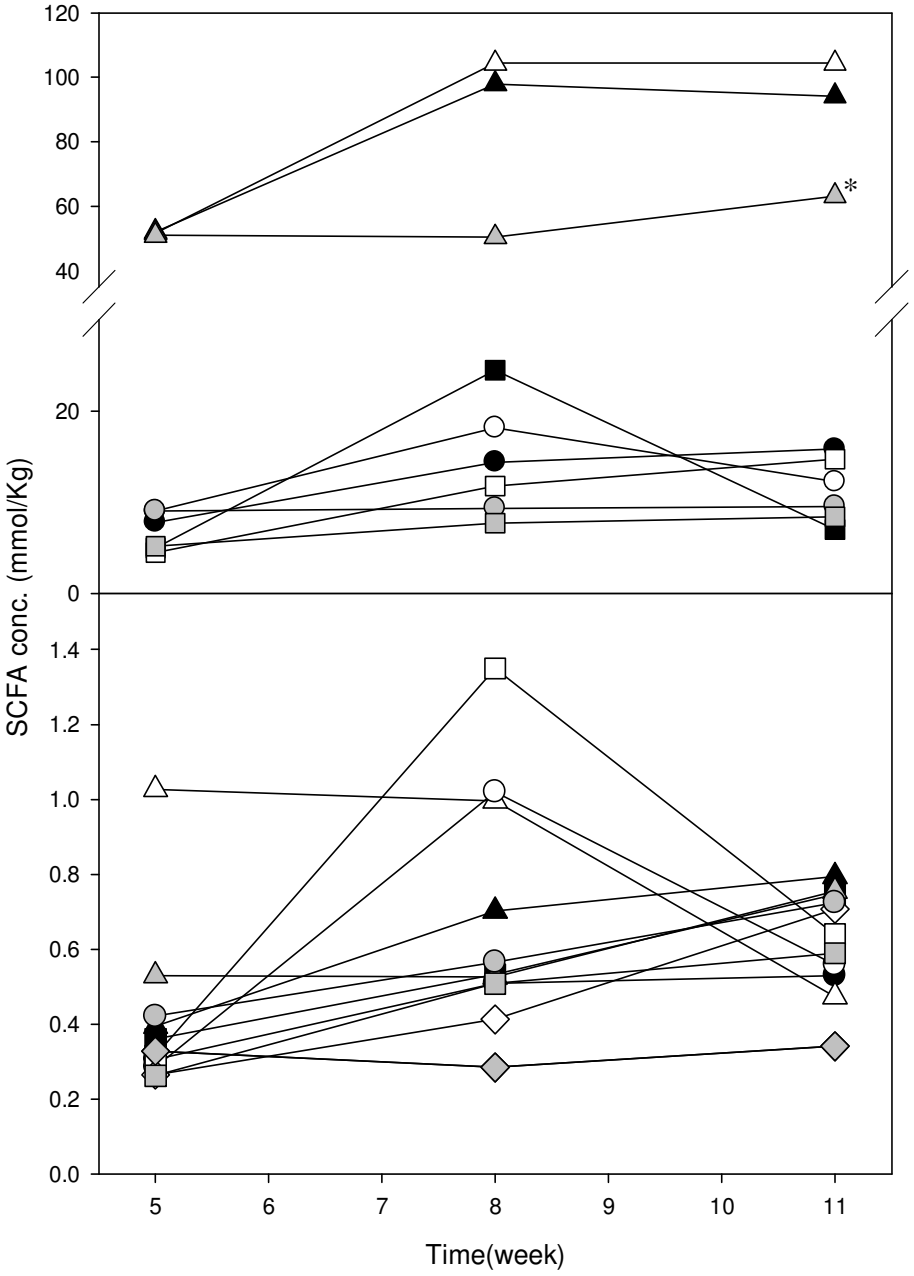


Figure 3.4- SCFA concentration in the fecal samples of rats fed commercial inulin (black symbols), IMO (grey symbols) or control diet (white symbols). Panel A: acetate (▲), propionate (●), butyrate (■), Panel B: Iso-butyrate (▲), iso-valerate (●), valerate (■), caproate (◆), *: significantly different, $p<0.05$ (n=6)

Discussion

Isomaltooligosaccharides are produced commercially by transglucosylation of maltodextrins obtained by starch hydrolysis (Pan and Lee, 2005). Starch hydrolysis and transglycosylation activity determine the degree of polymerization as well as the ratio of α (1→4) to α (1→6) linkages of IMO. The composition of commercial IMO preparations differs substantially; products containing up to 40% disaccharides and a substantial proportion of maltose and glucose (Kohomoto et al., 1992, Kaneko et al., 1994, Yen et al., 2010). The IMO preparation employed in this study did not contain maltose and glucose, whereas 6'glucosylpanose and 6'6'diglucosylpanose were identified by enzymatic synthesis of oligosaccharides of the panose series (Dols et al., 1997, Galle et al., 2010). Isomaltose is hydrolysed by brush border enzymes in the intestinal epithelium, the digestibility of isomaltotriose and panose is unclear, and longer chain oligosaccharides are considered non-digestible (Kohomoto et al., 1992, Kaneko et al., 1995). The composition of commercial IMO preparations thus affects digestibility and their effect on the composition of intestinal microbiota.

This study assessed the influence of an IMO preparation on intestinal microbiota of rats by PCR-DGGE and qPCR targeting dominant bacterial groups of the rodent intestine (Benson et al., 2010). The effect of inulin on intestinal microbiota of rodents is well established (Kleessen et al., 2001, Gibson et al., 2010, Meyer and Stasse-Wolthuis, 2009), and samples from rats fed inulin were therefore analysed using DGGE, and qPCR quantification of bifidobacteria and lactobacilli only. In keeping with previous studies, inulin significantly increased numbers of

bifidobacteria from 5.8 to 6.5 log copy numbers, whereas the abundance of the *Lactobacillus* group remained unchanged (data not shown, Kleessen et al., 2001). Dietary IMO exhibited a remarkable specificity towards the stimulation of the *Lactobacillus* group. *Lactobacillus* species colonise the rodent forestomach (Walter, 2008), and are a dominant bacterial groups in fecal microbiota of rodents (Benson et al., 2010). Previous studies in rodent models also reported increased numbers of lactobacilli as a result of dietary intervention with IMO (Kaneko et al., 1990). *L. animalis*, *L. johnsonii,* and *L. reuteri* are dominant *Lactobacillus* species in the rodent intestine. Other *Lactobacillus* spp., pediococci, *Leuconostoc* spp., and *Weissella* spp., which are also detected by the *Lactobacillus*-group primers, are substantially less abundant (Benson et al., 2010, Walter, 2008). Analysis of PCR-DGGE patterns generated with primers specific for the *Lactobacillus* group indicates that a strain of *L. reuteri* was specifically stimulated by IMO.

The number of bifidobacteria decreased in rats fed an IMO diet. However, previous reports indicated that IMO increased fecal bifidobacteria in BALB/c mice (Kaneko et al., 1990). This discrepancy can be attributed to the low numbers of bifidobacteria in rodent intestines, in contrast to lactobacilli (Walter, 2008). *Bifidobacterium* spp. have extracellular enzymes hydrolysing polymeric α (1→4) and α (1→6)-linked glucans (Ryan et al., 2006). In contrast, enzymes for IMO metabolism in lactobacilli are unknown, however lactobacilli have only few, if any, extracellular glycosyl hydrolases and preferentially metabolise disaccharides using intracellular hydrolases or phosphorylases (Gänzle et al., 2007). Lactobacilli are thus expected to preferentially metabolise low molecular weight IMO,

whereas bifidobacteria are capable of hydrolysis of larger polymeric glucans. Similarly, lactobacilli and bifidobacteria exhibited preference towards metabolism of low and high molecular weight galacto-oligosaccharides, respectively (Gopal et al., 2001).

The total number of fecal bacteria increased in rats fed IMO diet compared to control diet. Other groups of bacteria were not affected by the IMO diet. Fecal *Enterobacteriacea* decreased at 11 weeks of age in all animals irrespective of the diet. Inulin or fructo-oligosaccharides altered the numbers of organisms in the *Clostridium coccoides* cluster as well as enterococci in the rat intestine. (Kleessen et al., 2001).

Differences between the microbiota of rats and humans relate to the physiology of the digestive tract. Rats have a forestomach with non-secretory epithelium which is absent in humans, moreover, fibre fermentation occurs in the cecum in rats and in the colon in humans (Tiihonen et al., 2008). Bifidobacteria occupy narrow environmental niches compared to lactobacilli, belong to the dominant bacteria in humans, and colonise the intestine of infants shortly after birth (Biavati et al., 2000, Lamendella, et al., 2008). In contrast, lactobacilli are abundant throughout the rodent digestive tract, but are much less abundant in human intestines (Walter, 2008, Walter et al., 2008). Initial numbers of lactobacilli and bifidobacteria seem to be an important factor in stimulation of bacteria by NDO regardless of the host (Tiihonen et al., 2008). Despite these differences between rodent models and humans, the bifidogenic effect of fructans in rats (Kleessen et al, 2001, Rodriguez-Cabezas et al.) matches results in human studies (Bouhnik et al., 2004,

Tuohy et al., 2001, Whelan et al., 2005). Studies in humans to determine the effect of dietary IMO on intestinal microbiota relied predominantly on culture-dependent methods. Isomalto-oligosaccharides were bifidogenic at a dose of 10 g day^{-1}, and stimulated lactobacilli in a dose-dependent fashion (Kaneko et al., 1994, Kohomoto et al., 1991). However, cultivation media for enumeration of bifidobacteria and lactobacilli have a questionable selectivity (Simpson et al., 2004, Mikkelsen et al., 2003) and do not allow the quantification of other major bacterial groups in the fecal samples. The use of fluorescent-in-situ-hybridisation with four group-specific probes indicated that dietary IMO stimulated bifidobacteria and particularly lactobacilli in elderly, constipated patients, whereas the abundance of *Bacteroides* spp. and *Clostridium* spp. decreased (Yen at al., 2010). In summary, an increased abundance of lactobacilli as observed in this study corresponds to human studies whereas the effect of IMO on the abundance of bifidobacteria appears to differ between rodent models and human studies.

Although studies in humans demonstrated increased SCFA concentrations after consumption of up to 10 g day^{-1} of IMO (Chen et al., 2001, Yen et al., 2010), this study found decreased acetate and total SCFA in rats fed IMO. Ninety five percent of SCFA produced by intestinal bacteria are rapidly absorbed by the colon, only unabsorbed SCFA are detected in the feces (Topping and Clifton, 2001, Wong et al., 2006). IMO likely stimulates lactate and SCFA production in the upper intestine of rats, resulting in SCFA absorption in the intestine and decreased concentrations in feces.

In conclusion, IMO exhibited a remarkable selectivity and particularly increased the abundance and biodiversity of lactobacilli. Structural differences in non-digestible carbohydrates substantially influence their effect on the intestinal microbiota (Kleessen et al., 2001, Nilsson and Nyman, 2005, Metzler-Zebeli et al., 2010). Studies in humans indicated that the dose required to achieve bifidogenic effects with IMO with a DP of 3 – 4 is lower when compared to IMO with a DP of 2 – 3 (Kaneko et al., 1994). The effect of polymers with mixed α–(1→4) and α (1→6) linkages analogous to IMO, reuteran (Kralj et al., 1999), on intestinal microbiota have not been studied. Resistant starch with α (1→4) linkages has specific bifidogenic properties both in rats and humans (Bouhnik et al., 2004). It may thus be possible to alter the specificity of IMO by modifying their degree of polymerization.

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Chapter 4

Metabolism of isomalto-oligosaccharides in a rodent model of ulcerative colitis*

* This paper will be submitted for publication.

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Introduction

Inflammatory bowel diseaes (IBD) are defined as spontaneous relapsing immune-mediated disorder of the intestinal tract. Two forms of this disease are recognized as ulcerative colitis (UC) and crohn's disease (CD) (Lakatos, 2006). There are similarities between UC and CD. For example, abnormal composition of musoca-associated bacteria was reported in patients with UC or CD (Sartor and Muehlbauer, 2007). Moreover, the microbiota of mucosa associated samples were significantly different in IBD patients compared to control patients (Frank et al., 2007). However, the microbiota profiles were differed in individuals with UC or CD (Bibiloni et al., 2006). The role of genetic background and intestinal microbiota in initiation and perpetuation of intestinal inflammation has been well studied in rat models (Madsen et al., 2000, Mitsuyama et al., 2008, Schultz et al., 2004, Sellon et al., 1998, Rath et al., 2001). There are different rat models for study of IBD. HLA-B27 is a transgenic model which develops colitis, arthritis and gasteritis with presence of microorganisms, in a specific pathogen free (SPF) environment (Hammer et al., 1990).

The function of non-digestible oligosaccharides (NDO) and polysaccharides in the reduction of colitis has been studied previously (Hakansson et al., 2009, Nishimura et al., 2010). Prebiotics are non digestible oligosaccharides and polysaccharides which are selectively fermented and allow specific changes, both in the composition and/or activity of the gastrointestinal microbiota that confers benefits upon host well-being and health (Roberfroid, 2007). Prior studies suggested that plant polysaccharides inhibit colitis in the rat models (Koetzner at

al., 2010). HLA-B27 model of colitis has been used successfully in the past to study the effect of prebiotics (in particular fructans) and probiotics on population of bacteria and reduction of colitis (Dieleman et al, 2003 and 2004, Hoentjen et al., 2005, Schultz et al., 2004). Commercial IMO containing α(1→6) and α(1→4) glucosidic linkages have some modulating effect on intestinal microbiota in humans (Kaneko et al., 1994, Kohomoto et al., 1991 and 1992). However, there is no evidence of any effect of IMO on intestinal microbiota in the rodent models of colitis and prevention or amelioration of colitis. It was the aim of this study to investigate the modulating effect of IMO on intestinal microbiota, SCFA and possible reduction of colitis compared to FOS and a control diet in a rodent model of ulcerative colitis.

Materials and methods

Experimental design

A group of 7 HLA-B27 rats (3 male and 4 females) fed IMO (Bio Neutra, Edmonton, Canada), 6 rats (3 male and 3 females) fed commercial FOS (Raffinerie Tirlemontoise, Tienen, Belgium) and 8 rats (5 males and 3 females) fed control diet without any oligosaccharides. Rats were kept in a pathogen free environment and fed the experimental diet from 4 weeks of age. They usually develop colitis at 6-8 weeks of age. The composition of diet was explained in chapter 3. Fructo-oligosaccharides and IMO were added to the diet at 8 g/kg body weight and day over a 12 week period. Rat chow without any oligosaccharide was used as the sole source of feed for control group. Stool sampling was performed at 5, 12 and 16 weeks of age and necropsy at 16 weeks of age. All fecal samples

were immediately frozen at -80ºC for further analysis of bacteria and SCFA. Approval for these experiments was obtained from the University of Alberta Animal Policy and Welfare committee (UAPWC) which was in accordance with the Canadian Council on Animal Care (CCAC) guidelines.

Histology

Colon and cecum tissues were fixed and stained as described previously (Dieleman et al., 2003, Rath et al., 1996). Colon and cecum samples were evaluated blindly with a validated histologic inflammatory score ranging from zero to four (Rath et al., 1996).

Cecal cytokine analysis

Cecal tissues were thawed and lysed in PBS buffer containing a mixture of protease inhibitors (Cominelli et al., 1990). Tissue was homogenized after and the homogenate was assayed for IL-1ß as described elsewhere (Dieleman et al., 2003). IL-1ß was measured by ELISA (National Institute for Biologic Standards and Controls, UK).

Quantification of microbiota by real time PCR

DNA of fecal samples extracted with Qiagen DNA extration kit and analyzed with culture independent analysis at 5 (before feeding the oligosaccharide diet), 12 and 16 weeks of age. PCR conditions, primers, preparation of standard curves, and reaction condition were explained elsewhere (Metzler-Zebeli et al., 2010).

PCR-denaturing gradient gel electrophoresis (DGGE)

The effect of IMO and FOS diet on intestinal microbiota was determined by PCR-DGGE. DNA concentrations of fecal samples adjusted to 50-70 mg/L. Primers HAD1-GC and HAD2 were used for PCR amplification (sequences are available in chapter 3). DGGE was performed as described elsewhere (Tannock et al., 2000, Hoentjen et al., 2005). Cluster analysis was performed by UPGMA algorithm based on the dice correlation coefficient using an optimization coefficient of 1% (Bionumerics software, version 3, Applied Maths, Sint-Martens-Latem, Belgium).

Analysis of short chain fatty acids (SCFA) in the stool samples with High Performance Liquid Chromatography (HPLC)

For sample preparation, 300 µL water was added to 100 mg of the stool samples. After centrifugation at 17000 x *g* for 15 min, supernatant was diluted 3 times with 7% perchloric acid and was incubated overnight at 4 °C. Clear supernatant was collected after centrifugation and injected to HPLC. Short chain fatty acids were determined by HPLC with an Aminex HPX 87H column (300 x 7.8mm, Agilent, USA) at a temperature of 70 °C and a flow rate of 0.4 mL min^{-1} with 5 mM H_2SO_4 as the eluent. Refractive index and UV detectors were used at 210 nm. The injection volume was 20 µL. Concentration of acetate, propionate and butyrate were determined using external standards.

Statistical analysis

Mixed procedure (Proc Mixed) with repeated measures was used to analyze the effect of diet, time, sex, interaction of time and diet, and interaction of diet and

sex. Five weeks data were used as a covariate in the analysis. Results were reported as mean values and their standard errors. The least significant difference test was used to locate the differences between treatments. Differences were considered to be significant if $P< 0.05$. Student t-test and Mann-Whitney rank sum test were applied to evaluate gross cecal scores and histology scores. The discrimination model was developed by linear discriminant analysis in the JMP software (version 8.0.1, SAS Institute Inc., NC) to examine the potential correlation of copy numbers and diets and of inflammation and diets. Principal component analysis (PCA) was performed to observe the potential grouping of gene copies of bacterial groups, SCFA, gross gut scores, colon and cecum histology scores according to the diets.

Results

Histology and measurement of proinflammatory cytokine (1L-1β)

To estimate the inflammation, the thickness of the cecum wall was visually scored between 0 to 4. Gross gut scores did not show any significant difference between rats fed control diet, FOS or IMO diets (Table 4.1). Histology scores however, showed a significant difference between rats fed FOS or IMO diet compared to the rats fed a control diet. Cecum of rats fed a control diet showed inflammation whereas those fed IMO or FOS diet had non-inflammed status. The inflammation was less pronounced in the colon of rats compared to the cecum. Inflammation was significantly decreased in the colon of rats fed IMO or FOS diet compared to control rats (Table 4.1). Linear discriminant analysis of inflammation scores against different diets showed clear distinction of rats fed IMO or FOS diet, from

rats fed control diet (Figure 4.1.b). Pro-inflammatory cytokines such as IL-1β are indicators of inflammation. IL-1β was measured in milligram of protein in the cecum and colon tissues of rats to validate the modulating effect of diet on intestinal inflammation. There was no significant difference of IL-1β in the rats fed IMO compared to the control samples whereas FOS diet significantly changed the IL-1β concentration in the cecum compared to the rats fed control diet (Table 4.1). However, IL-1β was significantly decreased in the cecum tissues of female rats fed IMO (920 ± 307) or FOS diet (760 ± 108) compared to the female rats fed a control diet (2150 ± 380) if rats were divided according to the sex. There was only a significant decrease in the IL-1β level, in the male cecal samples fed FOS diet (410 ± 154) compared to the cecum of male rats fed a control diet (1600 ± 309).

Quantitative analysis of fecal microbiota with qPCR

To investigate the modulating effect of NDO on intestinal microbiota different groups of bacteria were enumerated by qPCR. The average of the effect of diet was compared for all groups of bacteria. The average of *Lactobacillus-Pediococcus- Leuconostoc-Weissella* (*Lactobacillus* group) was significantly higher in the fecal samples of rats fed IMO compared to the samples of rats fed control or FOS diet. However lactobacilli, group was decreased by aging. Bifidobacteria numbers were increased in the rats fed FOS diet compared to the rats fed control or IMO diet. Cluster XIVa (*C. coccoides- Eubacterium rectale* group), Cluster IV *(C. leptum*), Cluster XI and total number of bacteria were

Table 4.1- Histology scores and IL-1β concentration in the cecum and colon of rats fed IMO, FOS or a control diet

Diet	Cecum histology scores	Colon histology scores	Cecum tissue IL-1β	Colon tissue IL-1β
control	2.78 ± 0.07 [a]	1.89 ± 0.09 [a]	1940 ± 270 [a]	1460 ± 500 [a]
IMO	1.64 ± 0.18 [b]	1.58 ± 0.06 [b]	1280 ± 320 [ab]	710 ± 60 [a]
FOS	1.95 ± 0.18 [b]	1.37 ± 0.05 [c]	590 ± 115 [b]	530 ± 140 [a]

significantly decreased in the rats fed FOS diet compared to the rats fed control diet. Moreover, Cluster XIVa numbers were also showed a significant difference compared to the rats fed IMO diet. Aging decreased Cluster XIVa, cluster I (*C. perfringens* group), *Bacteroides* (*Bacteroides* group) and total number of bacteria irrespective of diet treatment, Cluster IV in rats fed IMO or FOS diet and *Enterobacteriaceae* numbers in rats fed control or FOS diet. There was a significant difference between *Bacteroides* group numbers in the fecal samples of rats fed FOS diet compared to the rats fed IMO diet. Numbers of *Enterobacteriaceae* family were significantly increased in the rats fed IMO or FOS diet compared to rats fed control diet. DNA copy numbers in the fecal samples of rats having bacterial butyrate COA acetyl COA transferase gene showed significantly lower numbers in the rats fed FOS or IMO diet. Copy numbers of bacteria carrying butyrate kinase gene were below the detection limit (Table 4.2). Linear discriminant analysis of diets versus gene copy numbers for different bacterial groups showed distinct effect of NDO on intestinal microbiota and clear separation of IMO and FOS diet from each other and control diet. Overall, each treatment (diet) had its unique influence on intestinal microbiota (Figure 4.1.a). Principal component analysis showed that *Lactobacillus* group and *Enterobacteriaceae* were negatively correlated to inflammation whereas other groups of bacteria showed neither positive nor negative correlation to inflammation (Figure 4.2). Effect of sex on different groups of bacteria was not significant (Table 4.2).

Table 4.2- Effect of diet, time, sex and interaction of them on DNA copy numbers of bacteria*

Bacterial group diets	Time						Statistical significance of effect: *P*				
	5 wk	SEM	12 wk	SEM	16 wk	SEM*	Diet	Time	sex	Diet x Time	Diet x sex
Lactobacillus group							*	*	ns	ns	ns
Control	8.15[a]	0.16	8.03[a]	0.16	7.89[a]	0.16					
IMO	8.86[a]	0.16	8.35[a]	0.16	8.35[a]	0.16					
FOS	8.56[a]	0.15	7.85[a]	0.17	8.07[a]	0.15					
Bifidobacteria							*	ns	ns	*	*
Control	7.39[a]	0.10	7.43[ac]	0.10	7.16[a]	0.10					
IMO	7.49[a]	0.11	6.97[a]	0.11	6.84[a]	0.11					
FOS	7.27[a]	0.12	7.74[bc]	0.13	8.02[b]	0.12					
Clostridium cluster XIVa							*	*	ns	ns	ns
Control	9.25[a]	0.15	8.74[a]	0.15	8.70[a]	0.15					
IMO	9.32[a]	0.16	8.87[a]	0.16	8.46[ac]	0.16					
FOS	9.15[a]	0.17	8.43[a]	0.19	7.81[bc]	0.17					
Clostridium cluster IV							ns	*	ns	*	ns
Control	8.33[a]	0.16	8.38[a]	0.16	8.27[a]	0.16					
IMO	8.72[a]	0.17	8.08[a]	0.17	8.04[ac]	0.17					
FOS	8.57[a]	0.17	8.28[a]	0.19	7.29[bc]	0.17					
Bacteroides group							*	*	ns	*	ns
Control	10.22[a]	0.11	9.94[a]	0.14	9.74[a]	0.30					
IMO	10.60[a]	0.11	10.0[a]	0.15	9.88[a]	0.32					
FOS	10.38[a]	0.12	9.55[a]	0.17	9.01[a]	0.30					
Clostridial cluster I							ns	*	ns	*	ns
Control	8.67[a]	0.14	8.65[a]	0.14	8.24[a]	0.28					
IMO	9.06[a]	0.15	8.38[a]	0.15	8.41[a]	0.29					
FOS	9.05[a]	0.16	8.03[a]	0.18	7.56[a]	0.31					
Enterobacteriaceae							*	*	ns	ns	ns
Control	6.90[a]	0.20	7.08[a]	0.20	6.46[a]	0.20					
IMO	8.05[b]	0.17	7.72[a]	0.17	7.83[bc]	0.17					
FOS	7.98[b]	0.17	7.79[a]	0.19	7.28[ac]	0.17					
Clostridial cluster XI							*	ns	ns	ns	ns
Control	7.08[a]	0.22	6.94[a]	0.22	6.76[a]	0.22					
IMO	6.76[a]	0.23	6.75[a]	0.23	6.79[a]	0.23					
FOS	6.36[a]	0.25	6.91[a]	0.27	5.77[a]	0.25					
But CoA- CoA transferase							*	*	ns	*	ns
Control	6.74[a]	0.21	6.54[a]	0.21	6.28[a]	0.21					
IMO	5.41[bc]	0.20	4.49[bc]	0.20	4.2[bc]	0.20					
FOS	5.59[bc]	0.20	4.81[bc]	0.22	4.59[bc]	0.20					
Total bacteria							*	*	ns	ns	ns
Control	10.79[a]	0.09	10.47[a]	0.08	10.33[a]	0.20					
IMO	10.77[a]	0.09	10.18[a]	0.08	10.06[a]	0.21					
FOS	10.62[a]	0.10	10.05[a]	0.10	9.50[a]	0.23					

* Non-similar letters in one bacterial group and column are significantly different, SEM: pooled SEM

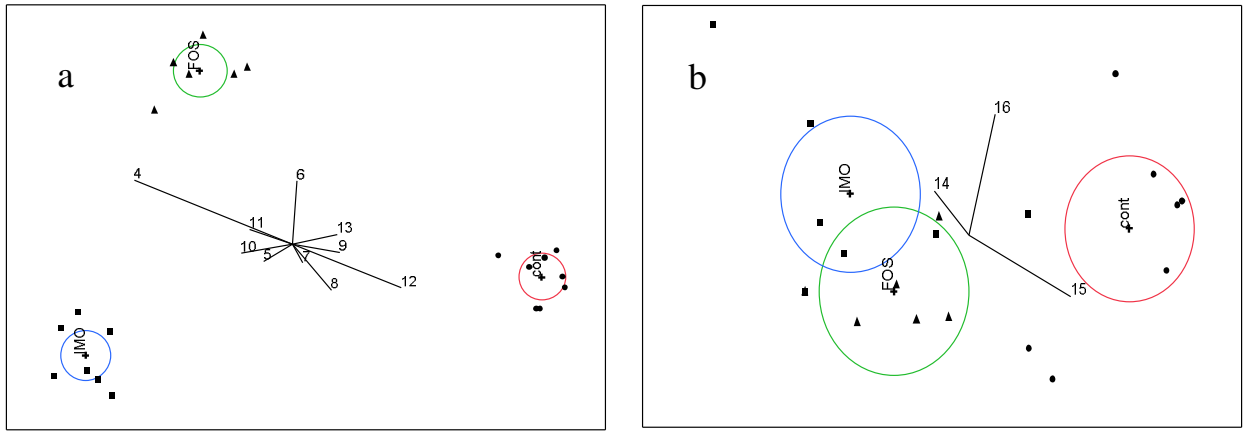

Figure 4.1- (a) Linear discriminant analysis of control, IMO and FOS diets versus gene copies for bacterial groups and (b) inflammation scores. IMO: Isomalto-oligosaccharides, FOS: Fructooligosaccharides. 1. propionate, 2. butyrate, 3. acetate, 4. *Bacteroides* group, 5. Cluster IV (*C. leptum* group), 6. bifidobacteria, 7. Cluster XIV (*C. coccoides group*), 8. Cluster I (*C. perfringens* group), 9. Cluster XI (*C. dificile* group), 10. *Enterobacteriacea*e, 11. *Lactobacillus* group, 12. total bacteria, 13. butyrate CoA CoA transferase, 14. gross gut scores, 15. cecum histology scores, 16. colon histology scores.

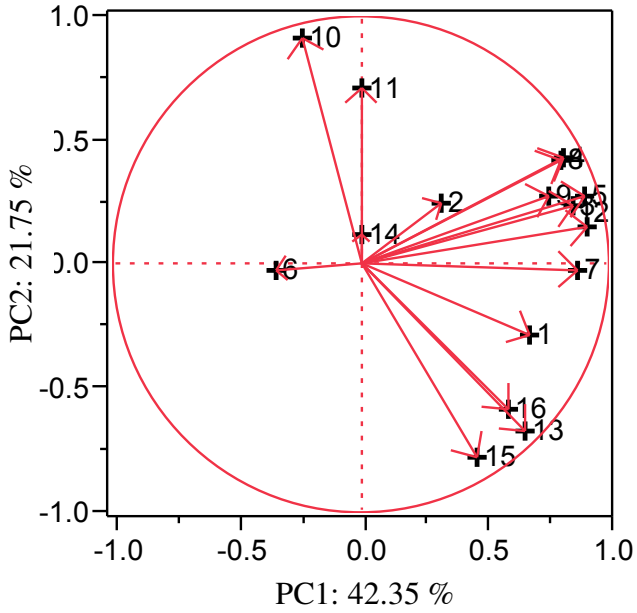

Figure 4.2- Loading plot showing the gene copy numbers of bacteria groups, SCFA, gross gut scores, cecum and colon histology scores (PC1: 42.35%, PC2: 21.75%). 1. propionate, 2. butyrate, 3. acetate, 4. *Bacteroides* group, 5. Cluster IV (*C. leptum* group), 6. bifidobacteria, 7. Cluster XIV (*C. coccoides group*), 8. Cluster I (*C. perfringens* group), 9. Cluster XI (*C. dificile* group), 10. *Enterobacteriacea*e, 11. *Lactobacillus* group, 12. total bacteria, 13. butyrate CoA CoA transferase, 14. gross gut scores, 15. cecum histology scores, 16. colon histology scores.

Qualitative analysis of fecal microbiota with DGGE

To establish the effect of diet on change of intestinal microbiota in HLA-B27 transgenic rats DNA of fecal samples were amplified with 16S RNA universal primers and DGGE was performed. Cluster analysis of the gels showed that the fecal DNA samples of rats were separated according to the diet at 12 weeks of age. At 16 weeks of age samples of rats fed IMO or FOS diet were only separated from control samples and not from each other (Figure 4.3). Effect of diet and inflammation were clearly observed with DGGE.

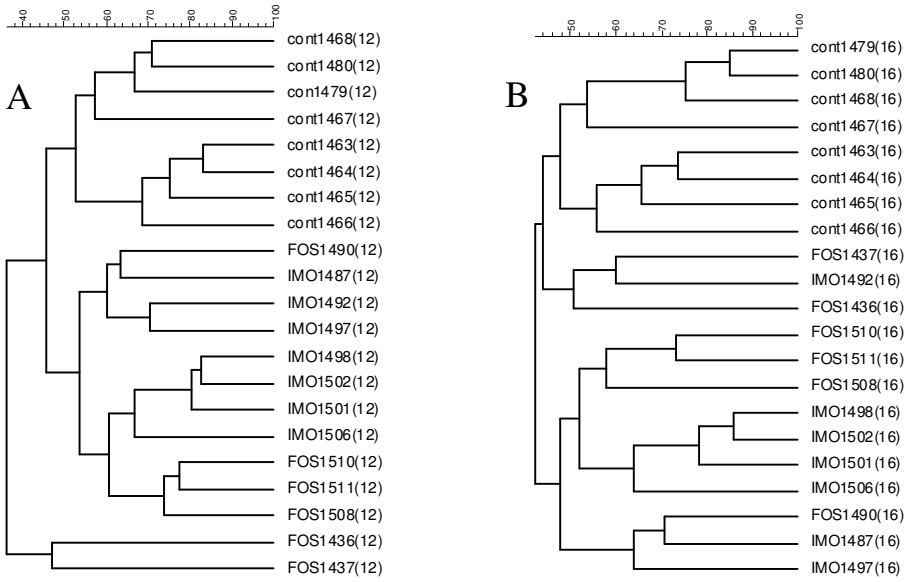

Figure 4.3- Dendrogram of DGGE of fecal samples obtained from rats fed IMO, FOS or control diets at 12 (A) and 16 (B) weeks of age normalized on different gels. For cluster analysis, UPGMA algorithm was used based on dice correlation coefficient with an optimization coefficient of 1%. IMO: Isomalto-oligosaccharides diet, FOS: FOS diet, Cont: control diet, numbers indicating rat numbers

Short chain fatty acids (SCFA)

To determine the effect of IMO or FOS treatment on intestinal microbiota, SCFA the direct metabolites of gastrointestinal microbiota, were measured by HPLC. Acetate, butyrate, isobutyrate, propionate, isovalerate and valerate were quantified. Acetate was increased by aging significantly, regardless of diet. Acetate was also decreased significantly in the rats fed FOS compared to the rats fed IMO or control diet. Butyrate and propionate were not affected by IMO or FOS diet compared to control diet (Figure 4.4). Isobutyrate, isovalerate and

valerate were below the detection limit (data not shown). The SCFA in the cecum contents were compared to the SCFA in the fecal samples at 16 weeks of age to determine the effect of fermentation site on the concentration of SCFA. Butyrate and propionate were differed significantly; having higher concentration in the cecal samples compared to fecal samples at 16 weeks of age, irrespective of diet.

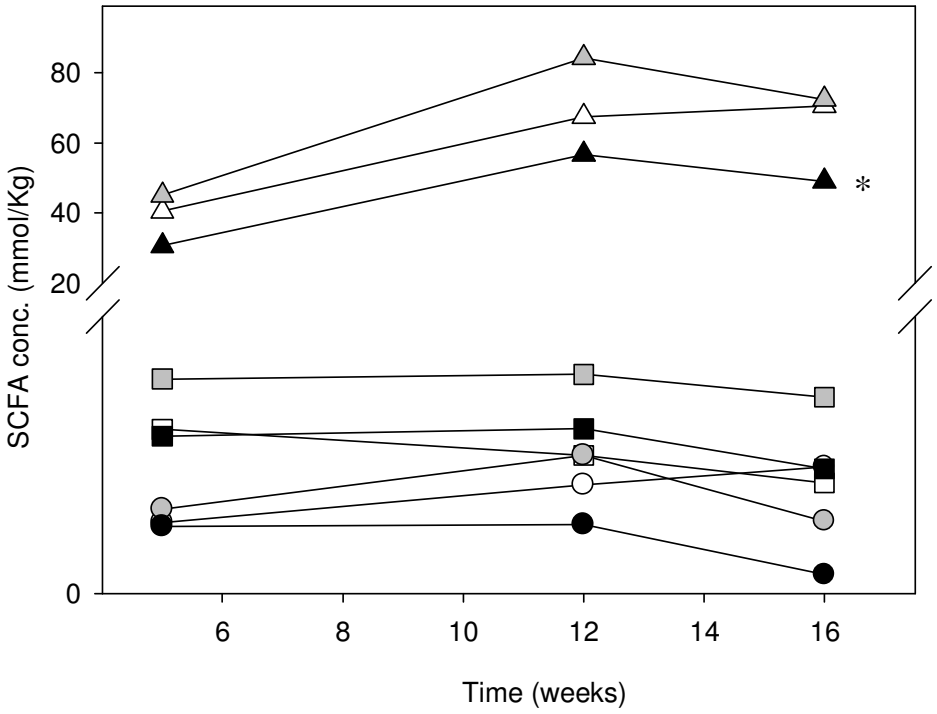

Figure 4.4- SCFA concentration in the fecal samples of HLA-B27 rats fed IMO, FOS or a control diet at 5, 12 and 16 weeks of age, acetate (▲), propionate (●), butyrate (■), Cont: white, IMO, grey, FOS: black, *: significantly different

Discussion

To determine the level of inflammation in rats, different parameters are to be measured. Histology scores and IL-1β are two important estimates of inflammation in the rat model of colitis. Histology scores in both cecum and colon of rats fed IMO were decreased significantly compared to the cecum and colon of rats fed control diet, demonstrating the effect of IMO on improvement of colitis. However, measurement of IL-1β by ELISA showed that inflammation was not significantly different in rats fed IMO diet compared to control diet. Surprisingly, separation of samples according to the sex demonstrated significant decrease of IL-1β in the cecum tissues of female rats fed IMO or FOS diet. Concentration of IL-1β in the cecum tissues of male rats fed FOS was also significantly different compared to the rats fed control diet. The IL-1β values should be interpreted carefully due to the low number of rats in each group. Further experiment with appropriate number of rats in each group is recommended to validate these results. Non digestible oligosaccharides and polysaccharides such as FOS, inulin and resistant starch with different DP decreased or prevented inflammation and damage to the colon in different rat models of colitis (Cherbut et al., 2003, Hoentjen et al., 2005, Koetzner at al, Lara- Villoslada et al, 2006, 2010, Moreau et al., 2003, Schultz et al, 2004, Videla et al., 2001). The effect of diet in reduction of colitis might be through the stimulation of inhabitant microbiota and likely immunological effects on the host (Sartor and Muehlbauer, 2007).

Effect of diet on change of microbiota was well pronounced with PCR-DGGE. Rats fed IMO or FOS were distinctly separated from rats fed a control diet.

Diversity of bacteria was shown to be decreased in IL-10 deficient mice and HLA-B27 transgenic rats compared to wild type and control rats respectively (McBurney et al., 2006, Wohlgemuth et al., 2009). Addition of inulin to the diet of HLA-B27 transgenic rats increased the diversity, stimulated a strain of bifidobacteria and reduced the severity of colitis (Schultz et al., 2004).

Quantitative PCR in current study, showed increase of *Lactobacillus* group in the rats fed IMO compared to the rats fed FOS or a control diet. *Lactobacillus* group was also increased in a non-transgenic rat model fed IMO compared to the rats fed control diet (Ketabi et al., 2010). Bifidobacteria numbers were decreased in the rats fed IMO compared to the rats fed control diet whereas they were increased in the fecal samples of rats fed FOS diet. Lactobacilli have a high population in the gastrointestinal system of rats but their population is low in humans. However, bifidobacteria numbers are low and variable in rats whereas they comprise up to 10% of total microbiota in the human large intestine (Walter, 2008). Decreasing of lactobacilli in the neonatal period in IL-10 deficient mice and other models of colitis has been reported before (Kataoka et al., 2008). Fructans with different DP increased bifidobacteria and lactobacilli (Hoentjen et al., 2005, Lara- Villoslada et al, 2006).

Cluster XIVa (*C. coccoides- Eubacterium rectale* group), cluster IV (*C. leptum* group), Cluster XI and total number of bacteria were decreased in the rats fed FOS compared to the rats fed IMO or control diet at 16 weeks of age. *Clostridium* Cluster XIVa and cluster IV are dominant gram positive and major butyrate producing bacteria in the large intestine of humans (Barcellina et al, 2000). Zhang

et al. (2007) reported unchanged composition of dominant bacteria in UC patients. *C. leptum* group, however, was decreased in the biopsy samples of two different regions with or without ulcer. Decreasing of cluster XI numbers (containing several pathogenic bacteria), by FOS diet might decrease the risk of *C. difficile*, a member of this group, that cause nosocomial diarrhoea (Heinlen et al., 2010). Patients with UC are susceptible to *C. difficile* infection (Musa et al., 2010). Kleesen et al. (2002) showed that *C. histolyticum*/ *C. lituseburense* group (belong to cluster XI and cluster II respectively) made up approximately 21% of total bacteria in patients with UC. These two groups harbour potentially pathogenic bacteria.

In current study, the *Enterobacteriaceae* numbers were increased in the rats fed either IMO or FOS diet compared to the rats fed control diet whereas histology scores and concentrations of IL-1β confirmed the reduction of inflammation. Contrary results were observed for *Enterobacteriaceae* family in IBD rodent models. Severity of disease was positively correlated to increase of *Enterobacteriaceae* in some models of colitis (Kataoka et al., 2008, Wohlgemuth et al., 2009) whereas in other models, *Enterobacteriaceae* spp. were decreased (Ott et al., 2004) or remained unchanged in the patients (Frank et al., 2007). Author's previous study showed that *Enterobacteriaceae* numbers was not affected by IMO or inulin diet in a non-transgenic rat model (Ketabi et al., 2010). Rats mono-associated with *Escherichia coli* did not develop colitis (Rath et al., 1999). Moreover, *E. coli* Nissle 1917 was shown to be protective in patients at the remission state (Kruis et al., 1997). Although *Lactobacillus* group and

Enterobacteriaceae family were increased in the fecal samples of rats fed IMO or FOS diet, they were negatively correlated to the colon and cecum histology scores. Koleva et al. (2010) observed similar correlation where FOS fed to the same rat model.

Rats fed FOS had a significantly lower number of *Bacteroides* group compared to the rats fed IMO diet. However, *Bacteroides* group numbers in rats fed IMO or FOS was not significantly different from control group. *Bacteroides* group is a dominant group in humans' microbiota (Hayashi et al., 2002). However, there are contradictory reports on *Bacteroides* group stating the pathogenesis or protective role of this group in patients and rats with UC. Rath et al. (1996 and 1999) observed the pathogenic effect of *B. vulgatus* in mono-associated rats. Contrary, a mild inflammation was observed in *B. vulgatus* mono-associated mice compared to germ free mice transferred to a SPF environment (Sellon et al., 1998). High abundance of *Bacteroides* and *Prevotella* spp. were detected in the mucosal or colon contents of patients with UC compared to control patients (Bamba et al., 1995, Lucke et al., 2006, Swidsinski et al, 2009). Takaishi et al. (2008) reported decrease of *B. fragilis* group in the fecal samples of patients with IBD and also dominance of *B. vulgatus* in the healthy patients. *Bacteroidetes* and *Lachnospiraceae* subgroups of *Firmicutes* (comprises of clostridial clusters XIVa and IV) were decreased in colonic samples of patients with UC and CD compared to control samples (Frank et al., 2007). The protective effect of intestinal microbiota in a group of children in a rural area of west Africa was also linked to

the dominant *Bacteroidetes* phylum (versus *Firmicutes*) compared to a group of children in Europe (De Filippo et al., 2010).

Previous study on IMO in non-transgenic rats showed exclusive increase of lactobacilli (Ketabi et al, 2010). However, feeding HLA-B27 rats by IMO showed changes in population of different groups of bacteria in addition to lactobacilli. It seems that inflammation rather than diet was responsible for change of bacteria. Stimulation of commensal bacteria in the gastrointestinal system of transgenic rats may affect the balance of microbiota and eventually cause pathogenesis and inflammation (Rath et al., 1999, Takaishi et al., 2008). Mono-associated models of colitis proved the hostility of some bacteria in the gastrointestinal tract whereas others are protective or play a minor role in mediating the inflammation (Rath et al., 1999). In other words, different representatives of the commensal microbiota do not have the same mechanism for induction of IBD (Kim et al, 2007).

The modulating effect of IMO and FOS diets on microbiota was also determined quantitatively through SCFA in the fecal samples. Acetate was the main SCFA in the fecal samples and was significantly decreased in the fecal samples of rats fed FOS compared to the rats fed IMO or control diet.

Isomalto-oligosaccharides and FOS altered the population of intestinal microbiota, reduced the inflammation and ameliorated the colitis in transgenic rats. Similar effects of NDO in decreasing of inflammation may seem to be contrary to the structure-function relation of NDO. However, inter-individual differences and particular stimulatory effect of NDO on intestinal microbiota may increase the chance of using NDO on the individual basis in the future.

Isomalto-oligosaccharides seem to be more selective compared to FOS diet. Addition of NDO to the diet of patients has explicit effects on intestinal microbiota. Altering the balance of bacteria towards the non-pathogenic or butyrate producing bacteria may ameliorate the inflammation and reduce the severity of colitis. Butyrate provides energy for colonocytes and disrupts the cytokine production pathway that triggers the inflammation (Videla et al., 2001). In conclusion, decreasing of inflammation by NDO in transgenic rats cannot be attributed to a specific group of bacteria. Taken all together, intestinal microbiota play an important role in pathogenesis and induction of colitis. However, it seems that combination of microbiota involved in pathogenesis and aetiology of colitis rather than a single pathogen.

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Chapter 5

General discussion and conclusions

General discussion and conclusions

Isomalto-oligosaccharides have been referred to as NDO and as potential prebiotics and may selectively stimulate intestinal microbiota. However, little information is available regarding the metabolism and *in vivo* effect of IMO on intestinal microbiota. Understanding how metabolism of novel oligosaccharides occurs *in vitro* may be helpful in predicting how they may behave *in vivo* in animals or humans. Metabolism of IMO by lactobacilli and bifidobacteria confirmed the fermentability of these oligosaccharides. Lactobacilli preferred isomaltose whereas bifidobacteria consumed isomalto-triose and higher DP of oligosaccharides first. Selectivity of lactobacilli towards isomaltose showed that they likely have a less complex enzymatic system to metabolize IMO compared to bifidobacteria. Current, but limited, knowledge about lactobacilli confirms that they metabolize short chain oligosaccharides e.g. GOS or soybean oligosaccharides intra cellularly (Garro et al., 1993, Schwab et al., 2010, Yoon et al., 2008) whereas bifidobacteria possess extracellular enzymes cable of utilizing longer chain oligosaccharides such as inulin (Falony et al., 2009). Although metabolizing long chain carbohydrates is challenging for lactobacilli, bifidobacteria are successful in tackling the diet change and versatility of carbohydrates in the large intestine (Kleerebezem et al., 2003, Broek et al., 2008). Successful competition of bifidobacteria with other intestinal microbiota could be explained by their high-affinity transport system that makes bifidobacteria competitive and persistent in the gastrointestinal tract (Schell et al., 2002). Lactobacilli inhabit plants and are also present in the upper-intestine of chickens,

pigs and rodents whereas their population in the colon of humans is very low and variable (Walter, 2008). Genomic analysis confirmed the dedication of lactobacilli species to specific environmental niches, e.g. dairy products, plants or colonic environment (O'Sullivan et al., 2009, Walter, 2008). However, bifidobacteria mostly inhabit the large intestine of humans and some animals (Biavati et al., 2000); where they have a higher population in humans compared to lactobacilli.

Non-digestible oligosaccharides and polysaccharides play an important role in intestinal physiology and health. Investigating the metabolism of NDO by intestinal microbiota helps to better understand the complex environment of the colon and to explain the adjustment of microbiota to the diet. Moreover, few studies connected metabolism of individual NDO *in vitro*, to their metabolism in the colon. In the current study, metabolism of IMO in lactobacilli and bifidobacteria was investigated and further examined in a rodent model to quantitatively study the change of microbiota associated with the diet. Isomalto-oligosaccharides metabolism in the rodent model clearly confirmed the selectivity of IMO and exclusive effect of isomaltose on lactobacilli compared to the control diet. Lactobacilli in rats have a stable population and form a biofilm in the forestomach of rats whereas bifidobacteria have a low and variable numbers (Walter, 2008). An isomalto-oligosaccharide with low degree of polymerisation (DP), isomaltose, stimulated lactobacilli in the upper gastrointestinal tract of rats and continued its journey towards the colon.

To further examine the health benefits of IMO, a diet containing IMO was tested in a rodent model of inflammatory bowel disease (IBD). Non-digestible

oligosaccharides have been used for treatment or keeping the IBD a state of remission (Håkansson et al., 2009, Hoentjen et al., 2005, Nishimura et al., 2010). However, this is the first report on the application of IMO in a rodent model of ulcerative colitis (UC). Although inter-individual differences exist in rats (Benson et al., 2010) and inter-subject variabilities are present among fecal and colonoscopy samples (Eckburg et al., 2005), IBD rat models are good candidates to study immunological responses of intestinal inflammation and the pathogenesis or the protective role of microorganisms in the intestine (Nell et al., 2010). Addition of IMO and FOS to the diet of transgenic rats confirmed selectivity of IMO for lactobacilli and FOS for bifidobacteria. In addition to lactobacilli and bifidobacteria, IMO increased *Enterobacteriaceae* groups significantly whereas FOS stimulated *Enterobacteriaceae* group and decreased some other clustridial clusters including butyrate producing bacteria and clusters comprising of potentially pathogenic bacteria. Moreover, both IMO and FOS diets reduced the inflammation.

Structure differences in NDO play an important role in stimulation and growth of different groups of bacteria in the colon of rats and might have various health effects for the host. Bacteria have the specific ability to degrade certain bonds in carbohydrates through their glycosyl hydrolases. The effect of diet on intestinal microbiota has been confirmed by new genetic approaches and sequencing of the whole genome of bacteria, in addition to environmental factors and ecological niche (Sela at al., 2008). In the rodent model of UC effects of IMO and FOS diets on intestinal microbiota were unique compared to control diet. Moreover, IMO

were more selective in stimulation of intestinal microbiota compared to FOS. Quantification of different groups of bacteria in the gastrointestinal tract and not only lactobacilli and bifidobacteria should be considered for any inclusive study of intestinal microbiota. For example, in a broad study at the phylum level a few subgroups of *Firmicutes* and *Bacteroidetes* were decreased whereas *Atcinobacteria* and *Proteobacteria* were increased in IBD patients (Frank et al., 2007).

Lactobacilli and bifidobacteria are less equipped with glycoside hydrolase enzymes compared to other intestinal species such as *Bacteroides*, carrying numerous glycoside hydrolases. Preferential growth of lactobacilli and bifidobacteria on IMO was demonstrated (chapter 2). The modulating effect of IMO in non-transgenic rats, also proved their selectivity (chapter 3). Moreover, the selective stimulation of lactobacilli in transgenic rats was similar to that of non-transgenic rats (chapter 4). In brief, NDO such as IMO are selective towards specific group of bacteria.

It has been suggested that different microbiota are involved in pathogenesis of UC (Frank et al., 2007). In addition, amelioration of colitis and decreasing of inflammation with NDO cannot be attributed only to specific group of bacteria. More research needs to be done to explore different aspects of using NDO in treatment of UC such as selectivity of NDO or permeability and translocation of bacteria in the colon. Further application of NDO in humans (Looijer-van Langen and Dieleman, 2009) and other rodent models such as tumour bearing rats will

likely help understand the role of diet on intestinal microbiota and their interactions with the host.

Current research and similar studies with different NDO confirms the correlation between structure and function of NDO in the intestine. In brief, with the possibility of producing IMO with different DP in the industry, it is likely to target bifidobacteria or lactobacilli in the human intestine according to specific DP and diet. Stimulation of a particular group of bacteria in the colon may increase production of SCFA, ameliorate the inflammation in patients suffering from colitis and provide other health benefits to the host. However, similar effects of NDO in the intestine such as decreasing of inflammation in the rodent models may seem to be contrary to the structure-function relation of NDO. Specific stimulatory effect of NDO on different groups of bacteria and inter-individual differences increases the speculation that health effects of NDO e.g. decreasing of inflammation in UC, could be quite individual-specific. It is likely that in the future, different NDO may be produced for particular disease or individual group of patients.

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Appendices.

The first draft of the following manuscript prepared to be submitted for publication in 2011. The study related to the effect of CPT-11 chemotherapy and oral glutamine on intestinal microbiota in tumor bearing rats.

Title: Irinotecan (CPT-11) chemotherapy and oral glutamine alter colonic microbiota in tumor bearing rats

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Abstract

Irinotecan (CPT-11) treatment for cancer produces toxic side effects, especially late-onset diarrhea. Intestinal microbiota mediate this toxicity and are agents causing systemic infection after CPT-11-induced loss of barrier function. This study aimed to analyse changes in intestinal microbiota induced by CPT-11 chemotherapy. We used a combination of qualitative and quantitative taxonomic and functional analysis to characterize the responses of intestinal microbiota to two CPT-11-based regimens, in presence or absence of oral bolus glutamine, a treatment we previously showed to mitigate CPT-11 toxicity. In a dose-intensive regimen, tumor-bearing rats received CPT-11 (125 mg/kg × 3 days), with or without oral glutamine bolus (0.75 g/kg). In a clinically-oriented regimen rats received two cycles of CPT-11 (50 mg/kg) followed by 5-flurouracil (5-FU) (50 mg/kg). The numbers of colonic *Clostridium* cluster XI and *Enterobacteriaceae* spp. were increased with both regimens, with changes of a larger magnitude using the dose-intensive therapy. The dose intensive regimen also reduced the abundance of bifidobacteria and the *Lactobacillus* group. In addition, CPT-11 therapy induced bacterial translocation to the mesenteric lymph nodes, notably species of the *Enterobacteriaceae* family, enterococci and staphylococci. Virulence factor/toxin genes of enteric pathogenic *Escherichia coli* and *Clostridium difficile* were not detected in the cecal microbiota and translocated bacteria. Glutamine partially mitigated changes to intestinal microbiota 6 h after CPT-11 administration, but those effects were no longer significant after 7 days. CPT-11-based chemotherapies caused major disruption in intestinal microbiota, and bacterial translocation. Glutamine partially mitigated the early changes of CPT-11-based chemotherapy.

Introduction

Irinotecan (CPT-11, 7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxy-camptothecin) is used to treat colorectal and other cancers. *In vivo*, CPT-11 is converted to SN-38, the pharmacologically active form responsible for both anti-tumor activity and dose-limiting toxicity. SN-38 undergoes hepatic glucuronidation and is secreted into the bile as its inactive glucuronide derivative SN-38G[1]. Local deconjugation of SN-38G catalyzed by β-glucuronidase produced by colonic microbiota intensifies the epithelial exposure to SN-38 and is believed to play a critical role in mediating gut toxicity[2, 3]. Diarrhea is one of the most clinically significant toxicities of CPT-11, which is experienced by more than 80 % of the patients to various extents[4]. Patients with diarrhea undergo changes in their chemotherapy, including dose reductions (45 %), delays in therapy (71 %), reduction in dose intensity (64 %), and discontinuation of therapy (3 %) [5]. Therefore, the diarrhea caused by CPT-11 is a key restriction, limiting CPT-11's utility and efficacy in colorectal cancer treatment.

The implication of intestinal microbiota in CPT-11 toxicity was shown by the observation that germ-free mice had lower intestinal damage scores and less diarrhea than holoxenic mice when treated with CPT-11[6]. Due to their role in localized SN-38 production, bacteria with β-glucuronidase activity have been considered the main inducers of CPT-11 toxicity in colon[3], where diffuse mucosal damage due to this agent has been observed[7]. Prophylaxis with antibiotics reduced SN-38 concentration and/or diarrhea in both animal models and patients[8, 9, 10]. Both the number and relative proportion of individual bacterial groups are important for maintaining the homeostasis of the intestine. Moreover, specific bacterial organisms translocate from the intestine and cause systemic illness and sepsis[11].

Glutamine protects the gut during a variety of stress conditions including trauma[12], sepsis[13], and cancer chemotherapy[14]. Oral bolus glutamine reduced the incidence and severity of late-onset diarrhea following CPT-11 treatment in a rat model[15]. Glutamine led to potentially protective responses, including heat

shock protein induction, increase in the ratio of reduced to oxidized glutathione, and increased proportions of CD3+CD8+ lymphocytes and memory CD8+ in mesenteric lymph nodes. Glutamine also prevented the CPT-11-induced increase of β-glucuronidase activity in the cecum[15], suggesting a potential effect on intestinal microbiota.

Although the role of intestinal microbiota in CPT-11 toxicity is well established, studies documenting CPT-11-induced changes of the intestinal microbiota did not use clinically relevant dosages[16, 17]. Information on effects of interaction between diet and CPT-11 on microbiota as a basis for dietary intervention to mitigate CPT-11 toxicity is essentially lacking.

This study aimed to employ a rat model for CPT-11 chemotherapy for colorectal cancer[18] to investigate the responses of intestinal microbiota to CPT-11-based regimens. A dose-intensive monotherapy regimen as well as regimen designed to recapitulate clinical therapy of colorectal cancer were applied. Cecal and fecal microbiota were evaluated with qualitative and quantitative molecular methods using primers targeting 16S rRNA genes of major bacterial species, quantification of genes encoding virulence factors and toxins, and the characterization of translocated bacterial species.

Materials and methods

Animals and treatments. Experimental conditions and diets are described in detail elsewhere[15, 18]. Animal use was approved by the Institutional Animal Care Committee and conducted in accordance with the Guidelines of the Canadian Council on Animal Care. In brief, female Fisher 344 rats (body weight, 150–180 g), 11-12 weeks of age, were obtained from Charles River (QC, Canada). Rats were housed 2 per cage in a temperature (22°C) and light controlled (12 h light) room; water and food were available *ad libitum*. One week before chemotherapy rats were separated into individual housing in wire-bottom cages. The Ward colorectal carcinoma was provided by Dr. Y. Rustum, Roswell Park Institute. Tumor pieces (0.05 g) were transplanted subcutaneously on the flank via trocar under slight isoflurane anesthesia. Tumor volume was estimated as described

(15). CPT-11 was provided by Pfizer as a ready-to-use clinical formulation. Atropine (0.6 g/L) was a clinical injectable formulation.

Diet. The diets used in this study are described elsewhere (18). Briefly, semi-purified diet was based on AIN-76 basal diet, with a modified fat component similar to a North American dietary pattern with respect to energy % as fat and levels of n-3, n-6, saturated and polyunsaturated fatty acids. Rats were initially fed Rodent Laboratory Chow (Harlan Teklad, Madison, WI). During the adaptation period, this non-purified diet was mixed with study diet (50/50, w/w) for one week, followed by transition to 100% semi-purified diet starting 2 weeks prior to tumor implantation.

Chemotherapy regimens and glutamine administration. Two regimens were used (Figure 1). In both regimens chemotherapy by intravenous route was initiated when tumor volume reached ~2 cm^3, and atropine (1 mg/kg s.c.) was administered immediately before each CPT-11 injection to alleviate early-onset cholinergic symptoms. Sample size was 6 animals per treatment and time point.

In a dose-intensive regimen designed to test the specific effects of CPT-11 and glutamine, tumor-bearing rats received CPT-11 (125 mg/kg × 3 days) (Figure 1A), with or without glutamine bolus[15]. Glutamine was administered by oral gavage (0.75 g/kg) 30 min before each daily CPT-11 injection. The sham treatment group received an equal volume of sterile water. Glutamine (Sigma-Aldrich, Canada) was made as a 3% wt/v solution immediately before use, and filtered with a 0.45-µm filter. The day before CPT-11 administration was designated day 0. Rats were killed on day 0, on day 3 (6 hours after the 3rd injection of CPT-11) to capture early responses, and on day 7.

In a second regimen designed to recapitulate clinical therapy of colorectal cancer, rats received two cycles of CPT-11/5-FU treatment (Figure 1B). The day before first CPT-11 injection was designated day 0. Animals received weekly CPT-11 (50 mg/kg) and 5-FU (50 mg/kg) injections on day 1 and 8 and on day 2 and 9, respectively. Animals were killed on day 0, day 7 (prior to the second treatment cycle), and days 10 and 11 (one and two days after the 2nd treatment cycle) to follow changes in microbiota after each cycle.

Sample collection and DNA isolation. Rats were killed by CO_2 asphyxiation. Feces, mesenteric lymph nodes and cecal contents were collected under aseptic conditions. DNA was extracted from cecal or fecal samples using the QIAamp DNA Stool Mini Kit (Qiagen, Canada).

PCR-denaturing gradient gel electrophoresis (DGGE). PCR-DGGE was done on fecal samples from the dose-intensive regimen as described by Tannock et al.[19].

Quantification of major bacterial groups, virulence factors, and translocated species by quantitative PCR (qPCR). Quantitative PCR was performed as described by Metzler-Zebeli et al.[20]. Major bacterial groups in cecal and fecal microbiota were quantified using group-specific primers targeting total bacteria[21], *Bacteroides-Prevotella-Porphyromonas* (*Bacteroides* group, [22]), *Lactobacillus-Pediococcus-Leuconostoc-Weissella (Lactobacillus* group,[23, 24]), *Bifidobacterium* spp.[22], *Clostridium* clusters I[22], IV[25], XI[26], and XIV[27], and *Enterobacteriacaea* spp.[28]. Bacterial species present in mesenteric lymph nodes were quantified in cecal samples by qPCR using genus or species-specific primers targeting *Citrobacter freundii*[29], *Klebsielle oxytoca*[30], *Proteus mirabilis*[31], *Morganella morganii*[32], *Staphylococcus* spp.[33], *Escherichia coli*[34] and *Enterococcus* spp.[35]. Diarrhea- and enteric infection-associated virulence factors in cecal microbiota were quantified using primers targeting virulence factor/toxin genes of *Clostridium difficile*[36], enterohemorrhagic *E. coli* (EHEC), enterotoxic *E. coli* (ETEC), and enteroaggregative *E. coli* (EAEC)[37]. The presence of these virulence factors was also determined in *E. coli* strains isolated from mesenteric lymph nodes.

Isolation and identification of bacteria from mesenteric lymph nodes. Bacterial translocation in CPT-11-treated rats was determined in mesenteric lymph nodes adjacent to the cecum in both regimens. Cell counts were determined by surface plating of serial dilutions on sheep blood agar plates after incubation at 30°C for 48 h under aerobic conditions. Isolates were purified by serial dilution streaking on sheep blood agar until uniform colony morphology was obtained. Subsequent cultivation of cultures was performed in Brain Heart Infusion medium

at 30°C. DNA was extracted from these overnight cultures using DNeasy Blood & Tissue Kit (Qiagen, USA). Bacterial 16S rRNA genes were amplified using 616V (5' –AGA GTT TGA TYM TGG CTC-3') and 630R (5'-CAK AAA GGA GGT GAT CC-3') universal primers, and sequenced at Macrogen Corp. (MD, USA). The sequences were analyzed using the Ribosomal Database Project (rdp.cme.msu.edu). Virulence factors of pathogenic *E. coli* (EHEC, ETEC, and EAEC) in *E. coli* isolates were detected by PCR using primers listed in Table 1 of the online supplementary material.

Antimicrobial activity of CPT-11 and SN-38 *in vitro*. The minimal inhibitory concentration (MIC) of CPT-11 and SN-38 was determined using a critical dilution assay. Four organisms of intestinal origin, *Lactobacillus reuteri* FUA3041, *Lactobacillus johnsonii* FUA3040 (both isolated from rodents), *E. coli* FUA1170 (isolated from cow rectum) and *Bifidobacterium animalis* DSM 10140 were used to represent Gram-negative and Gram-positive intestinal bacteria. CPT-11 and SN-38 concentrations ranging from 0.016 to 8 g/L and from 0.004 to 2 g/L, respectively, were tested to match or exceed concentrations found in the lumen of the colon *in vivo*[8]. Positive and negative controls (with and without inoculation of indicator strains) were used to compare the growth of bacteria in the wells.

Statistics. Data analysis was performed using the Statistical Analysis Systems (SAS Institute Inc., Cary, NC). Differences in DNA copy numbers of cecal samples were evaluated using one-way ANOVA followed by least significant difference (LSD). Differences in DNA copy numbers of fecal samples from the same animal over time were analyzed using repeated measures ANOVA and LSD. Non parametric data were analyzed with Kruskal-Wallis k-sample test. Data were expressed as mean ± SEM. A P value of ≤ 0.05 was considered statistically significant.

Results

Effects of chemotherapy regimens on the hosts. In the dose-intensive regimen, diarrhea occurred in both sham- and glutamine-treated groups. Glutamine gavage decreased the incidence of severe diarrhea[15]. Relative food intake and relative

body weight of both groups dropped immediately after CPT-11 treatment but showed a trend of recovery by day 7. In the CPT-11/5-FU regimen, diarrhea was absent in animals at all time points. Both the relative body weight and the relative food intake showed little change after the 1st cycle of treatment, but were significantly reduced after the 2nd cycle (data not shown). Analysis of samples obtained in this regimen aimed to characterize microbiota after the 2nd cycle of chemotherapy, as it was at this time that adverse effects were observed.

Qualitative analysis of fecal microbiota using DGGE. To determine the effect of CPT-11 and glutamine gavage on intestinal microbiota of animals treated with a high dose of CPT-11, PCR-DGGE analysis was applied to compare the microbial profile of fecal samples in the dose-intensive regimen (Figure 2). DGGE patterns from samples obtained at day 7 clustered together independent of glutamine gavage, indicating that CPT-11 by itself had a major influence on the composition of intestinal microbiota. However, most samples from glutamine-treated animals taken 6 h after the 3rd chemotherapy dose clustered separate from samples from sham-treated animals at the same time point.

Quantitative analysis of cecal and fecal microbiota using qPCR. The abundance of bacterial taxa in cecal samples in the dose-intensive treatment with CPT-11 is shown in Figure 3. Data are reported as gene copy numbers on a log scale. *Clostridium* cluster I was below detection limit at all time points. In sham-treated animals, the total bacteria number decreased by ~1 log 6h after chemotherapy, and all bacterial groups except the *Clostridium* cluster XI were significantly lower compared to day 0. The *Bacteroides* group and *Clostridium* clusters IV and XIV decreased by 1-3 logs. These changes were partially restored by day 7. However, the abundance of *Clostridium* cluster IV and XIV, the *Lactobacillus* group, and *Bifidobacterium* spp. remained significantly lower than at day 0. *Clostridium* cluster XI and *Enterobacteriaceae* remained ~0.5 and ~1.5 log higher than day 0, respectively.

Bolus glutamine modified some bacterial groups at 6h (Figure 3). The reductions in *Bacteroides* group, *Lactobacillus* group, *Clostridium* cluster IV, and *Enterobacteriaceae* spp. were not as pronounced as in the sham-treated group. By

day 7, total bacteria, *Bacteroides* group, *Clostridium* clusters IV and XIV were restored to the same level as day 0. However, 7 days after glutamine and CPT 11 administration, changes in bacterial groups were not different from the sham-treated animals.

The abundance of bacterial taxa in cecal samples in two cycles of CPT-11/5-FU therapy is shown in Figure 4A. The most pronounced changes were observed after administration of the second cycle, i.e. two or three days after the second CPT-11 application. At day 11, numbers of *Clostridium* cluster XI increased by ~2 logs, and *Clostridium* cluster XIV and *Enterobacteriaceae* spp. increased by ~0.5 log. The *Clostridium* cluster IV decreased by ~0.5 log. No significant change was detected for other bacterial groups.

To assess if microflora changes induced by chemotherapy were also observed in the colon, fecal microbiota of animals treated with the CPT-11/5-FU regimen were characterized using qPCR. Microbiota in fecal samples (Figure 4B) were more stable over time than cecal microbiota. The most pronounced changes, such as an increase of *Clostridium* clusters I and XI, occurred between day 0 and day 7.

Quantification of diarrhea-associated virulence factors in cecal samples. To determine whether the increased abundance of *Enterobacteriaceae* spp. and *Clostridium* cluster XI after chemotherapy was associated with increased abundance of pathogenic or toxinogenic organisms in these groups, virulence factor/toxin genes of pathogenic *C. difficile* and *E. coli* in cecum were quantified by qPCR in cecal samples in the CPT-11/5-FU regimen. Gene copy numbers of all virulence factor/toxin genes assayed were below detection limit at all time points.

Identification of translocated bacteria from mesenteric lymph nodes. Mesenteric lymph nodes were collected aseptically, bacteria infecting the mesenteric lymph nodes were isolated by surface plating, and bacterial isolates were identified by sequencing of 16S rRNA genes (Table 1). Bacterial isolates obtained from mesenteric lymph nodes of CPT-11 treated animals belong to the family *Enterobacteriaceae* and the genera *Enterococcus* and *Staphylococcus*. *E.*

coli isolates were also characterized with respect to the presence of virulence factors of enteric pathogenic *E. coli*. However, none of these virulence factors were detected in any of the isolates.

Quantification of translocated bacteria in cecum To determine whether bacterial translocation to mesenteric lymph nodes is attributable to an increased abundance of opportunistic pathogens in cecal microbiota, the abundance of translocated bacterial species or genera in cecal samples from CPT-11/5-FU treated animals was quantified by qPCR (Figure 5). *Morganella morganii* was below detection limit in all animals at all time points. An increased abundance of *Citrobacter freundii, Klebsiella oxytoca,* and *Enterococcus* spp. was observed, but numbers of other translocated organisms remained unchanged by chemotherapy.

Antimicrobial activity of CPT-11 and SN-38 *in vitro*. The altered abundance of total bacteria and of several specific taxa after dose-intensive CPT-11 suggested possible antimicrobial activity of CPT-11 or its metabolite SN-38. The MICs of these compounds were determined using intestinal isolates of *Lactobacillus* spp., *E. coli*, and *Bifidobacterium animalis* as indicator strains. The MICs of CPT-11 and SN-38 were more than 8 g/L and 2 g/L, respectively, indicating neither compound had direct inhibitory effect on intestinal microbiota.

Discussion

We characterized the effect of two CPT-11-based chemotherapy regimens on intestinal microbiota of tumor-bearing rats through a combination of qualitative taxonomic, quantitative taxonomic, and functional analysis. Qualitative analysis using DGGE revealed that CPT-11 induced major shifts of the intestinal microbiota. Quantitative PCR established that particularly the abundance of colonic *Enterobacteriaceae* spp. and *Clostridium* cluster XI, two bacterial groups that contain opportunistic pathogens, were consistently increased after CPT-11 chemotherapy. Translocated bacteria particularly *Enterobacteriaceae*, although these organisms were only a minor component of cecal microbiota. Finally, analysis of bacterial virulence factors/toxins associated with diarrhea and enteric infections suggested that virulence factors of enteropathogenic *E. coli* and *Clostridium* cluster XI were not a direct cause of

diarrhea. To our knowledge, this is the first time that the changes of intestinal microbiota during CPT-11-based chemotherapies have been characterized in such depth.

Regimens were performed to characterize microbial changes in response to two different CPT-11-based regimens. The severity of CPT-11-induced diarrhea increased with both total and everyday dosages[18, 38]. The dose-intensive CPT-11 induced major shifts in the intestinal microbiota of rats. To determine whether similar changes in microbiota also occur in a less intense treatment, a second regimen was conducted using two cycles of low-dose CPT-11 and 5-FU to simulate dosing regimens more commonly used in clinical settings. This protocol induced changes to intestinal microbiota that were similar to the dose-intensive CPT-11, but these changes were much less pronounced.

Previous studies on the effect of CPT-11 on intestinal microbiota[16, 17] were based on fecal samples. However, gut injury induced by CPT-11 chemotherapy was observed mostly in the cecum[3, 39]. Changes in fecal microbiota were observed at an earlier time point, but with less magnitude after chemotherapy compared to changes in cecal microbiota (Figures 4A and 4B). The most pronounced change in bacterial groups was an increased abundance of the *Clostridium* cluster XI and *Enterobacteriaceae* spp., which was seen in both cecal and fecal samples, and after both chemotherapy regimens. These changes were thus not only closely associated with CPT-11 treatment, but they also affected the entire large bowel.

The reduction of total bacteria at 6h in the dose-intensive regimen can be attributed to a diluting effect of diarrhea observed at 6h[15]. Of interest, the gene copy number of *Clostridium* cluster XI did not decrease but increased about tenfold, indicating a dramatic increase in the proportion of this group within the total bacteria. The *Clostridium* cluster XI accounts for less than 1 % of the total intestinal microbiota but includes important pathogenic species such as *C. difficile*. The proportion of *Clostridium* cluster XI is very limited in healthy individuals[40]. Increase of *Clostridium* cluster XI, especially *C. difficile,* is observed only when the composition of resident microbiota is severely altered,

such as in patients with chronic idiopathic diarrhea[41] and inflammatory bowel disease[42]. Hence, an increase of *Clostridium* cluster XI indicates a major disruption of cecal microbiota.

Enterobacteriaceae harbor several pathogenic or opportunistic pathogenic species, e.g. pathogenic *E. coli*, which are implicated in various diseases and infections[43]. On the other hand, specific probiotic strains of *E. coli* may exert a beneficial effect by inhibiting pathogenic *E. coli* such as enterohaemorrhagic *E. coli*[44]. We therefore quantified virulence factors of pathogenic *E. coli* as well as *C. difficile,* the most common gastrointestinal pathogens in *Enterobacteriaceae* spp. and the *Clostridium* cluster XI, respectively[43, 45]. Virulence factors or toxin genes were not detected in cecal samples or in translocated isolates, indicating that the increase of *Clostridium* cluster XI and *Enterobacteriaceae* virulence factors did not contribute to CPT-11 induced diarrhea.

Gastrointestinal mucositis and the accompanying microbiota alteration are common side effects of chemotherapies, however their mechanisms are still unknown [46]. This study showed that the changes caused by a combination of CPT-11 and 5-FU were distinctive from those caused by CPT-11 or 5-FU alone. Dose intensive CPT-11 alone caused decreased the abundance of the *Lactobacillus* group and bifidobacteria, and increased organisms in the *Bacteroides* group as well as *Enterobacteriaceae* (this study). 5-FU alone led to increased the *Bacteroides* group, clostridia, *E. coli* and *Staphylococcus* spp., and decreased *Lactobacillus* spp. and *Enterococcus* spp.[47]. Because microflora changes induced by the various chemotherapies only partially overlap, the microbiota alteration appears to be specific not only to the dosing regimen, but also to the chemotherapeutic agent(s) used.

Glutamine had beneficial effects on high dose CPT-11 induced gut injury[15]. Glutamine is able to improve gastrointestinal health by stimulating proliferation of enterocytes, and by preserving integrity and permeability of the epithelium[48, 49]. DGGE and qPCR results consistently showed that glutamine had only a temporary effect on microbiota at 6h after dose-intensive regimen. In contrast to the effect of CPT-11, which persisted for 7 days after chemotherapy,

the effect of glutamine gavage on intestinal microbiota was no longer apparent at day 7. However, 6h post chemotherapy was a critical time point in the dose-intensive regimen, as apoptosis of enterocytes and diarrhea were observed at this time point[15]. It is thus likely that translocation also started at this time point. It remains unclear whether the effect of glutamine on intestinal microbiota is a direct effect of glutamine metabolism by intestinal microbiota, or results from improved integrity of the small intestinal epithelium by glutamine. Since glutamine is absorbed and metabolized before digesta reach the cecum[50], the latter explanation seems more likely.

CPT-11 increased the risk of bacterial translocation, which is consistent with other studies[6, 11]. Bacterial translocation is defined as the passage of viable bacteria from the gastrointestinal tract to extraintestinal sites. Conditions that promote bacterial translocation from the GI tract include intestinal bacterial overgrowth, immunodeficiency, and increased intestinal permeability by mucosal injury. These mechanisms can act synergistically to promote translocation[51]. The last two conditions were met in our animals: CPT-11-based regimens resulted in impaired immune functions exhibited by cytotoxic T cell depletion, and damage to the intestinal mucosa[15]. In the CPT-11/5-FU regimen, CPT-11-based chemotherapy induced increased abundance in of 3 out of 6 translocated species or genera. However, the abundance of all translocated organisms in cecal digesta remained below 0.001% of total bacteria. The translocated bacteria (Table 1) are generally considered opportunistic pathogens, i.e. they are unlikely to infect a healthy host, but can induce systemic infections if the mucosal barrier is breached.

Gut toxicity of CPT-11 is dependent on β-glucuronidase activity of intestinal bacteria, which recycles SN-38G to the cycotoxic SN-38[3, 6]. Several intestinal bacteria, including clostridia, bifidobacteria, *Bacteroides* spp., *Eubacterium* spp., *Ruminococcus* spp., and *Enterobacteriaciae* spp. exhibit β-glucuronidase activity[52, 53, 54]. However, it remains unknown which bacterial groups are major contributors to intestinal β-glucuronidase activity[55]. Among isolates that are representative for the dominant bacterial groups in human feces, β-glucuronidase activity was found in organisms of the *Clostridium* clusters XIVa

393 and IV[56].

394 **Conclusion**

395 CPT-11-based chemotherapies altered the composition of intestinal
396 microbiota and induced bacterial translocation of resident intestinal bacteria in a
397 time-and dose-dependent manner. Dietary intervention with concomitant
398 glutamine, previously shown to be beneficial, showed only a temporary effect on
399 the composition of intestinal microbiota. Dysbiosis induced by CPT-11-based
400 chemotherapies must be taken into account for the pathogenesis of mucositis and
401 sepsis. The use of antibiotics alleviated the toxicity of CPT-11 chemotherapy in
402 the same animal model as employed here[57] as well as in patients[10]. However,
403 modulation of intestinal microbiota by appropriate dietary intervention can also
404 attenuate mucositis by influencing inflammatory processes, intestinal
405 permeability, mucus composition, and by improving epithelial repair[46]. This
406 study forms an important basis for future investigations related a protective role of
407 intestinal microbiota in CPT-11-induced mucositis.

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Figure legends.

Figure 1. Experimental design of dose-intensive CPT-11 regimen (A) and CPT-11/5-FU regimen (B). Black arrows represent treatment at different time points. White arrows represent the time points at which cecal and fecal samples were taken. Grey arrows represent the time points at which translocated organisms were isolated from mesenteric lymph nodes (n=6/time point). In the dose-intensive regimen, occurrence of death began at day 5. In order to catch potential bacterial translocation right before death, we repeated the dose-intensive regimen on another group of animals (n=10). The animals were killed at day 4 and mesenteric lymph nodes were collected.

Figure 2. DGGE profiles of the fecal microbiota of sham- and glutamine-treated rats at day 0, 6h, and day 7 in the dose-intensive regimen.

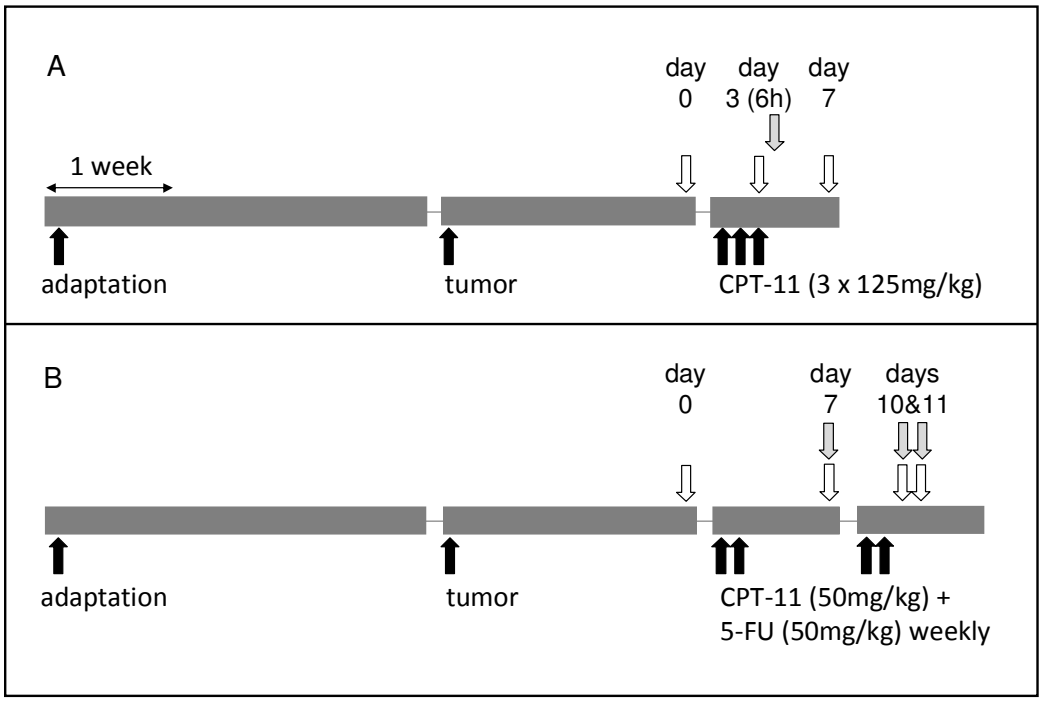
Figure 3. Gene copy numbers for major bacterial groups per gram of cecal digesta of sham-treated rats at day 0 (black bars), 6h (doted bars), day 7 (white bars), and glutamine-treated rats at 6h (light grey bars) and day 7 (dark grey bars) in the dose-intensive regimen.

Figure 4. Gene copy numbers for major bacterial groups in the cecum (Panel A) and in feces (Panel B) of rats in the in the CPT-11/5-FU regimen. Gene copy numbers were determined at day 0 (black bars), day 7 (light grey bars), day 10 (white bars), and day 11 (dark grey bars)

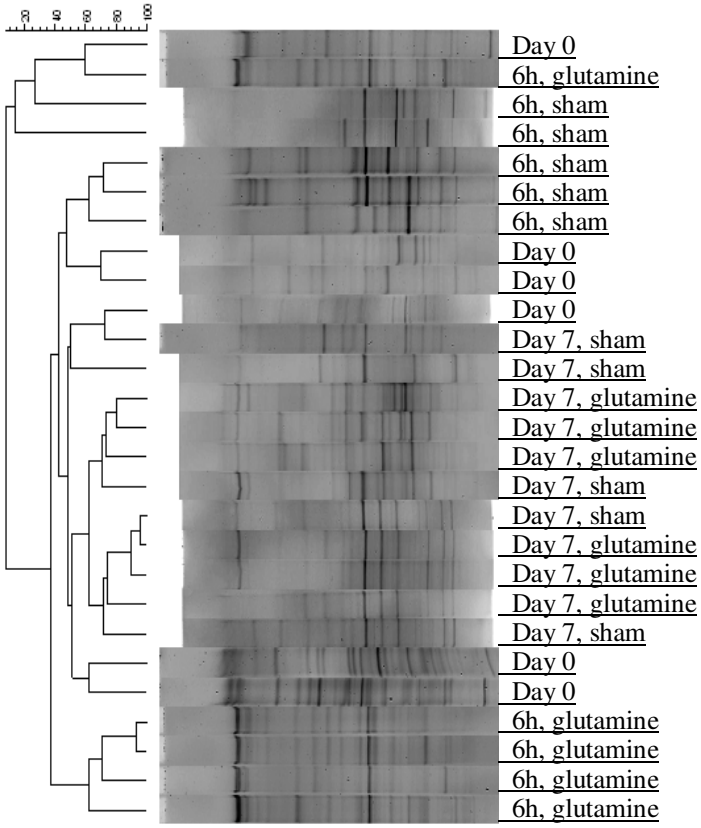
Figure 5. Gene copy numbers for bacterial species or genera in the cecal digesta of rats in the CPT-11/5-FU regimen at day 0 (black bars), day 7 (light grey bars), day 10 (white bars), and day 11 (dark grey bars). Primers targeted those bacterial taxa found to translocate to the mesenteric lymphnodes (see Table 1).

Table 1. Taxonomic identification of translocated bacteria isolated from mesenteric lymphnodes in two CPT-11-based regimens.

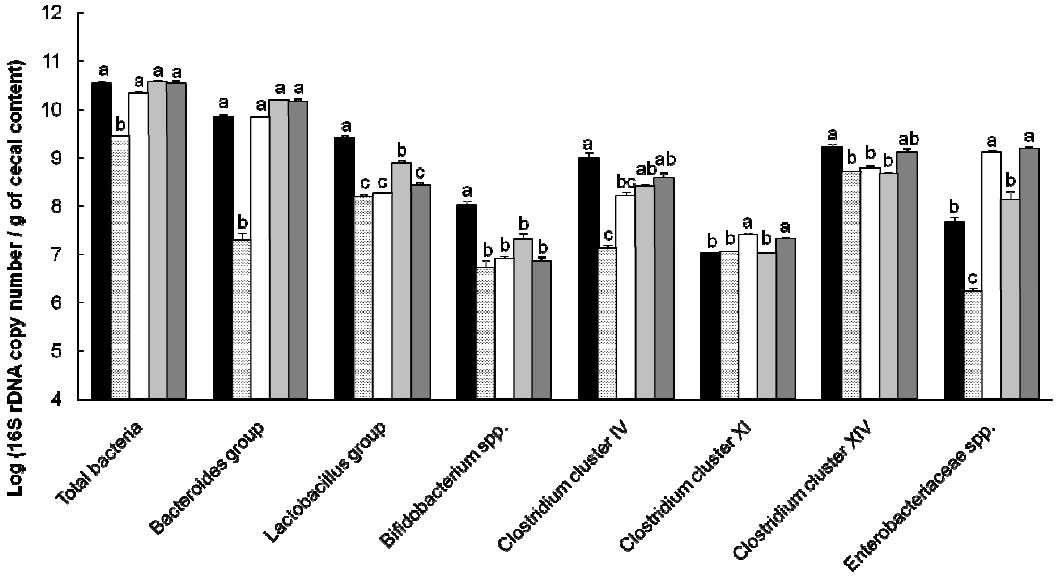
	Sequence Accession number	# of Base Pairs Sequenced	Homology to type strain
Dose-intensive CPT-11 regimen			
Morganella morganii FUA1235	HQ169114	1455	0.971
Escherichia coli FUA1236	HQ169115	612	1.000
Proteus mirabilis FUA1237	HQ169116	1454	0.982
Proteus mirabilis FUA1239	HQ169117	1455	0.983
Proteus mirabilis FUA1240	HQ169118	1454	0.979
Staphylococcus epidermidis FUA2058	HQ169119	1464	0.999
Enterococcus avium FUA3332	HQ169120	1471	1.000
Staphylococcus cohnii FUA2059	HQ169121	1464	0.981
Escherichia coli FUA1241	HQ169122	1455	1.000
Enterococcus faecalis FUA3333	HQ169123	653	1.000
Escherichia coli FUA1242	HQ169124	1454	0.998
Morganella morganii FUA1243	HQ169125	1454	0.989
Enterococcus faecalis FUA 3334	HQ184922	1475	0.976
Morganella morganii FUA1245	HQ169126	1455	0.983
Two-cycle CPT-11/5-FU regimen			
Citrobacter freundii	HQ694731	1408	0.976
Klebsiella oxytoca	HQ694732	1403	0.983
Proteus mirabilis	HQ694733	1267	0.992
Staphylococcus warneri	HQ694734	1301	1.000



Lin et al. Figure 1.



Lin et al. Figure 2.



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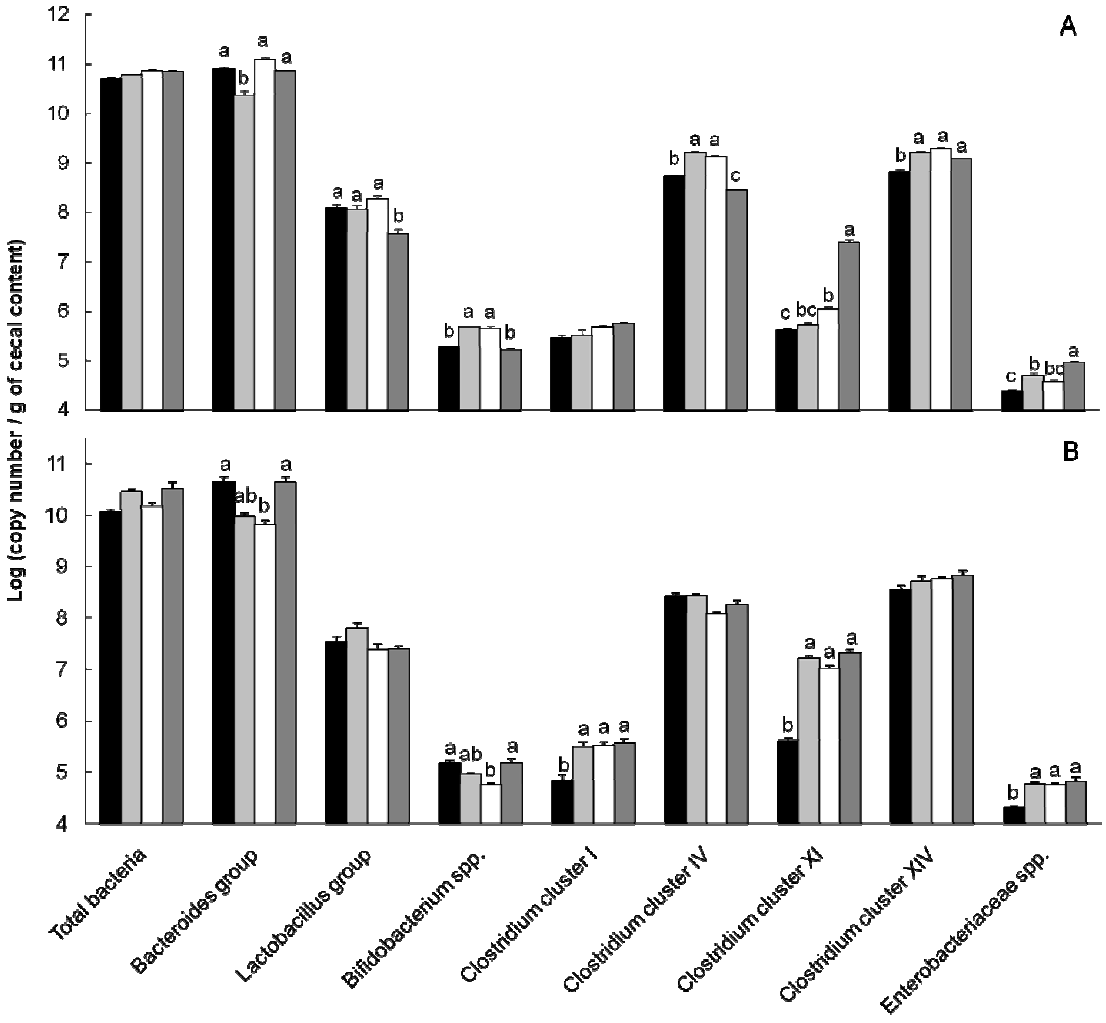

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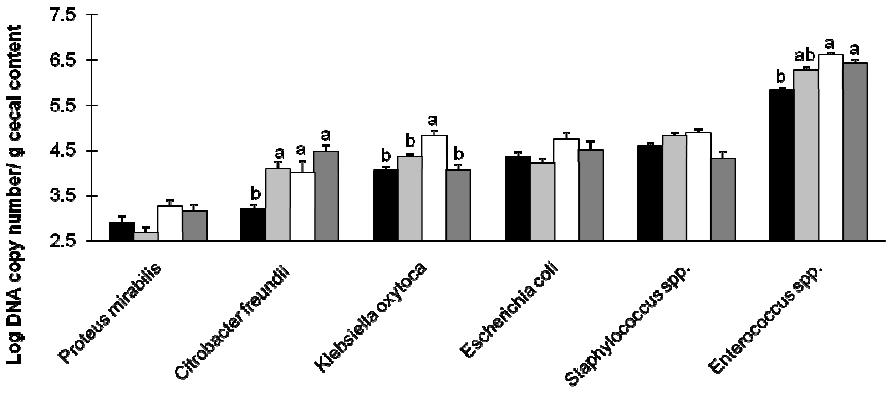

Lin et al. Figure 5.

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