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Influence of xylooligosaccharides (XOS) on gut microbiota composition and gut epithelial and neutrophilic gene expression.

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Preface

This study has been completed at the Institute of Basic Sciences and Environment at KU LIFE and at the National Food Institute at DTU. The thesis corresponds to 45 ECTS points and has been completed from November 2010 – June 2011.

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Finally and very importantly I would like to thank my family for supporting me all the way.
Jens (JGC Marketing) for helping me with the front page. Ditte and Mikkel for computer support, pictures, proofread etc. Special thanks to Klaus for your commitment and support. It has been invaluable, and I could not have done this without your help.

Signature Date

Resumé

Prebiotika defineres som en selektiv fermenteret ingrediens, der resulterer i specifikke ændringer i sammensætningen og/eller aktiviteten af tarmens microflora, hvilket fører til gavnlig effekt på værtens helbred. Især bifidobakterier er beskrevet som egnede til at vokse på prebiotika, hvilket er grunden til den brugte term "bifidogenisk effekt". Man mener, at prebiotika bliver nedbrudt i tyktarmen, men prebiotika kan måske også give øget bakteriavækst i tyndtarmen, da de bakterier der kan nedbryde prebiotika måske breder sig fra tyktarm til tyndtarm via deres forbedrede mulighed for føde her. Man mener også at prebiotika via den bifidogene effekt kan have positiv indvirkning på immunsystemet, men det vides ikke, om den bakterielle virkning kommer fra tyndtarm, tyktarm eller fra begge steder. Når prebiotika nedbrydes af bakterier, produceres der kortkædede fedtsyrer. Det vides ikke med sikkerhed, i hvor høj grad disse kortkædede fedtsyrer bliver absorberet fra tarmen og dermed har en effekt i bl.a. de neutrofile celler i blod. Xylooligosakkariders (XOS) er ikke klassificeret som et prebiotika - det er endnu kun en kandidat.

Design: Det har ikke været muligt at finde nogle *in vivo* undersøgelser af xylooligosakkariders (XOS) effekt på bakteriesammensætningen hele vejen igennem tarmen eller på gen ekspression. Formålet med dette speciale var at undersøge XOS's inflydelse på tarmens mikroflora og på gen ekspressionen i tarmepitelceller og i neutrofile celler i blod fra mus. Der er brugt B6 mus til undersøgelsen, og de blev delt i to grupper. Den ene fik almindeligt foder, den anden gruppe fik foder tilsat 10% XOS og diætperioden var 10 uger. Følgende kriterier blev benyttet til at måle den prebiotiske effekt: Ændringer I mikroflora sammensætningen af *Bacteriodetes* og *Firmicutes* phyla, *Lactobacilli*, *Bifodobacterium* og som reference *Eubacteria* phylum. Ændringer i det medfødte immun system blev målt ud fra ekspressionen af gener der koder for REGIIIγ, TLR2, TLR4, CD36, FFAR2, TNFα, CXCL1 og CXCL2 i tarmepitelcellerne, interleukin IL-1ß i blod og ß-actin blev benyttet som house keeper gen til alle undersøgelserne. Som undersøgelsesmetode blev brugt real time PCR (qPCR).

Resultater: Der blev fundet signifikant prebiotisk effekt af XOS behandlingen i forhold til antallet af *Bifidobacterium,* der blev øget igennem tarmen fra duodenum til colon og fæces. Gen ekspressionen af Cxcl1 var opreguleret i epitelcellerne fra duodenum, og gen ekspressionen af RegIIIγ var opreguleret i duodenum og ileum. I blod fandtes nedregulering af mRNA niveauet af Il-1β.

Konklusion: XOS har prebiotisk effekt: Indholdet af *Bifidobacterium* blev øget gennem hele tarmen, gen ekspressionen af RegIIIγ og Cxcl1 steg i tyndtarmen, og gen ekspressionen af Il-1β i blod blev nedreguleret.

Dette speciale har givet ny viden omkring effekten af XOS på genekspressionen i tamrepitelceller og i blod. Den signifikante stigning af indholdet af *Bifidobacterium* i tyndtarmen hos mus, der har fået XOS behandling, er også ny viden, da det primære sted for fermetering af de ikke fordøjelige kulhydrater med lav polymeriserings grad som XOS, er alment accepteret som værende i caecum og forreste del af colon.

Abstract

Prebiotic is defined as a selectively fermented ingredient that results in specific changes in the composition and/or activity of the gastrointestinal microflora, thus conferring benefit(s) upon host health. In particular bifidobacteria have been described as capable of growing on prebiotics, which is the reason for the used term 'bifidogenic effects' of prebiotics. It is claimed that prebiotics is digested in the large intestine, but the prebiotics might give rise to a higher number of bacteria in the small intestine, as those bacteria degrading prebiotics may expand from the large to the small intestine, due to their advantage for food here. It is also claimed, that prebiotics through their bifidogenic effect have a positive effect on the immune system, but it is not known whether the bacteria act from the small or the large intestine, or both. When prebiotics are digested by bacteria, short chain fatty acids (SCFAs) are produced. To which extend the SCFA are absorbed from the intestinal and act on e.g. the neutrophils in the blood is not fully established. Xylooligosaccharides (XOS) is not classified as a prebiotic yet - it is a candidate.

Design: No *in vivo* studies were found investigating XOS effects on bacterial content throughout the intestine and on gene expression. In this study the aim was to investigate the influence of XOS on the intestinal microbiota and on the gene expression in intestinal epithelial cells and in neutrophils in blood of mice. The animals used were B6 mice and they were split into two groups: 1 group on normal feed, 1 group on feed with 10% XOS and the diet period was 10 weeks. Prebiotic effects were measured as: Changes in the microbiota composition of the phyla *Bacteriodetes* and *Firmicutes*, *Lactobacilli*, *Bifodobacterium* and as reference the phylum *Eubacteria.* Changes in the innate immune system were measured as the expression of the genes encoding REGIIIγ, TLR2, TLR4, CD36, FFAR2, TNFα, CXCL1 and CXCL2 in intestinal epithelial cells, interleukin IL-1ß in blood and ß-actin as the overall house keeper gen. Real time PCR (qPCR) was used as the investigating method.

Results: Significant prebiotic effects of XOS treatment were seen in the content of *Bifidobacterium* which was increased throughout the intestine from duodenum to colon and feces. The expression of Cxcl1 was increased in epithelial cells from duodenum, the expression of RegIIIγ was increased in epithelial cells from dudenum and ileum, and the expression of Il-1β was decreased in blood.

Conclusion: XOS do have prebiotic effects: The content of *Bifidobacterium* increased throughout the intestine, gene expression of RegIIIγ and Cxcl1increased in the small intestine, and the gene expression of Il-1β decreased in blood.

This study presents new knowledge about the effects of XOS on gene expression in intestinal epithelial cells and in blood. The significant higher number of *Bifidobacterium* in the small intestine in mice fed with XOS is also new knowledge, since the primary site for fermentation of non-digestible dietary carbohydrates with low degree of polymerization such as XOS, are generally accepted to be in the cecum and the proximal colon.

List of Abbreviations

Cd36: Cluster of Differentiation 36

cDNA: Complementary DNA

CFU: Colony-forming unit

Ct: Cycle threshold

Cxcl: Chemokine (C-X-C motif) ligand

CXCR: CXC chemokine receptor

DC: Dendritic cell

DP: Degree of polymerization

FAE: Follicle-associated epithelial layer

Ffar: Free fatty acid receptor

FOS: Fructooligosaccharides

GALT: Gut-associated lymphoid tissue

GI: Gastro intestinal

GOS: Galacto-oligosaccharides

GPR: G-protein coupled receptor

HBSS: Hank's Balanced Salt Solution

IEC: Intestinal epithelial cell

Il-1ß: Interleukin 1 beta

IN: Inulin

LDL: low-density lipoprotein

LPS: Lipopolysaccharides

M cells: Microfold cells

MHC: Major histocompatibility complex

mRNA: Messenger RNA

MYD88: Myeloid differentiation primary-response protein 88

N0: Target molecules at start

NF-kB: Nuclear factor kappa-light-chain-enhancer of activated B cells

NS: No significant

OF: Oligofructose

OFI: Oligofructose and inulin

PAMP: Pathogens associated molecular patterns

PBS: Phosphate Buffered Saline

PP: Peyer’s patches

PRR: Pattern recognition receptor

qPCR: Quantitative polymerase chain reaction

Reg: Regenerating islet-derived protein

RQ: Relative quantification

rRNA: Ribosomal RNA

SCFAs: Short chain fatty acids

SDS: Sodium Dodecyl Sulfate

Tlr: Toll like receptors

Tnf: Tumor necrosis factor

TOS: Transgalactooligosaccharides

XOS: Xylooligosaccharider

1 Introduction

1.1 Prebiotic definition

ISAPP 6th Meeting of the International Scientific Association of Probiotics and Prebiotics, London, Ontario (2008) defined prebiotic as: A dietary prebiotic is a selectively fermented ingredient that results in specific changes in the composition and/or activity of the gastrointestinal microflora, thus conferring benefit(s) upon host health (Roberfroid, M. *et al.*, 2010).

1.2 Background

Prebiotics are fibers that can be degraded and exploited as a carbohydrate source by beneficial commensal bacteria. Bifidobacteria in particular have been described as capable of growing on prebiotics, which is the reason for the used term 'bifidogenic effects' of prebiotics. This property of prebiotics to promote growth of beneficial bacteria has resulted in a general belief in health promoting properties of prebiotics, and one often heard claim is; that prebiotics have 'positive effects on the immune system.

Prebiotic effect is now a well-established scientific fact (Roberfroid, M. *et al*., 2010).

The effects and the claimed mechanisms of actions are, however, not well documented, as may be evident from the following:

It is claimed that ingested prebiotics is digested in the large intestine.

But the prebiotic might give rise to a higher number of bacteria in the small intestine, as those bacteria that can degrade this carbohydrate source may expand from the large to the small intestine, due to their advantage for food here.

It is claimed that prebiotics increase the proportion of bifidobacteria and certain lactobacilli in the gut, and through their effects on the immune system, prebiotics have a positive effect on the immune system.

But it is not known how high a number of bacteria that is necessary for an effect on the immune system, nor whether the bacteria act from the small or the large intestine, or both. As an example, effects on the immune system have been demonstrated after ingestion of large numbers of lactobacilli (10^{10}); these bacteria move through the small intestine and are present in a vast number as compared to their appearance in the gut of individuals not taking probiotics. Thus, both the high number and the appearance in the small intestine may be important conditions that may not be achieved by administration of prebiotics.

When prebiotics are digested by bacteria, short chain fatty acids (SCFAs) are produced.
This has been demonstrated to affect the growth of the enterocytes as well as having anti-inflammatory effect on cells of the immune system. To which extend the SCFA are absorbed and act on e.g. the neutrophils in the blood is not fully established.
The hypothesis is that the XOS diet will increase the amount of SCFAs, and the increased amount of SCFAs will rapidly be transferred to the blood stream. In the blood the SCFAs will bind to the SCFAs receptors FFAR2 (GPR43) on the neutrophils, which will lead to a decrease expression of the pro-inflammatory interleukin IL-1β.

1.3 The thesis

The questions mentioned below will be addressed in a mouse experiment, in which various parameters (gut microbiota and epithelial gene expression in different part of the gut, and immune response in blood) will be analyzed. Two groups of mice, one on normal diet and one on XOS containing diet will be included.
The questions to be addressed in this thesis are:

- Can a higher number of bacteria be measured in the small intestine in mice fed with XOS ?
- Is bacterial content in duodenum, ileum, cecum, colon or feces the most representative descriptor for the microbial changes induced by the XOS administration ?
- Can changes in gene expression be measured in gut epithelium (in different parts of the gut), which can support a hypothesis about an immune stimulating and/or anti-inflammatory effect of XOS ?
- Can changes in the gene expression in blood be measured in the two groups of mice ?

XOS is not classified as a prebiotic - it is a candidate. In this study XOS has been chosen, inspired by a study by Petersen, A. et al. (2010) the National Food Institute, DTU, investigating the prebiotic effects of XOS and fructooligosaccharides (FOS) in mice. They found bifidogenic effect of both XOS and FOS but more pronounced for XOS.

Inulin-type fructans are the most widely studied prebiotics with regard to potential modulation of the microbiota, immune system and properties in food. Relatively little information is available on the properties of XOS, for which reason studies of prebiotic effects mentioned in this report, also are about inulin-type fructans and other prebiotic candidates.

Prebiotic effects in this study are measured as:

1) Changes in the microbiota composition of the phyla *Bacteriodetes* and *Firmicutes*, *Lactobacilli* , *Bifidobacterium* and as reference the phylum *Eubacteria.*
2) Changes in the innate immune system measuring the expression of the genes encoding REGIIIγ, bacteria receptors TLR2 and TLR4, fat receptors CD36 and FFAR2, cytokine TNFα, chemokine CXCL1 and CXCL2, interleukin IL-1ß in blood and ß-actin as the overall house keeper gen.

Bacteriodetes and *Firmicutes* were included to study changes in the overall composition of the microbiota, which is dominated by these two phyla. *Bifidobacterium* constitutes about 4 % , and *Lactobacillus* less than 2 % of the total microbiota. *Lactobacillus* and *Bifidobacterium* are included in the study because, as mentioned earlier, they are claimed to have beneficial effects on the host and are the target species of prebiotic treatment.
The genes included in the study, are chosen because they are suggested to respond to changes in the bacteria profile and changes of the fatty acid contents.

When investigating prebiotic effects, one way is to measure changes in content of SCFAs.
This has not been done in this study.

The immune system consists of both the innate- and adaptive immune system.
This report only deals with the innate immune system.

Prebiotic effects and their properties in food and food production are not a part of this study and will not be dealt with in depth. But because of my education in Gastronomy and Health it will be mentioned to perspective the possibilities of how prebiotic and their claimed beneficial effects could be part of a human diet.

2 Gut

The gut consists of the stomach and the small and large intestine. Duodenum is the first part, jejunum the middle, and ileum the lowest part of the small intestine. The large intestine starts at the cecum, colon is the middle part, and rectum the last part (figure 2.1).

Duodenum is the major site for the intestinal breakdown of food, and for the absorption of some minerals.

Jejunum/ileum moves food along, there is still some enzymatic digestion of food and a major absorption of nutrient from the gut contents.

Colon moves contents from the cecum to rectum and is hosting a large number of microorganisms, which contribute in the fermentation of unabsorbed materials, and here the absorption of water, some fat soluble vitamins and electrolytes takes places.

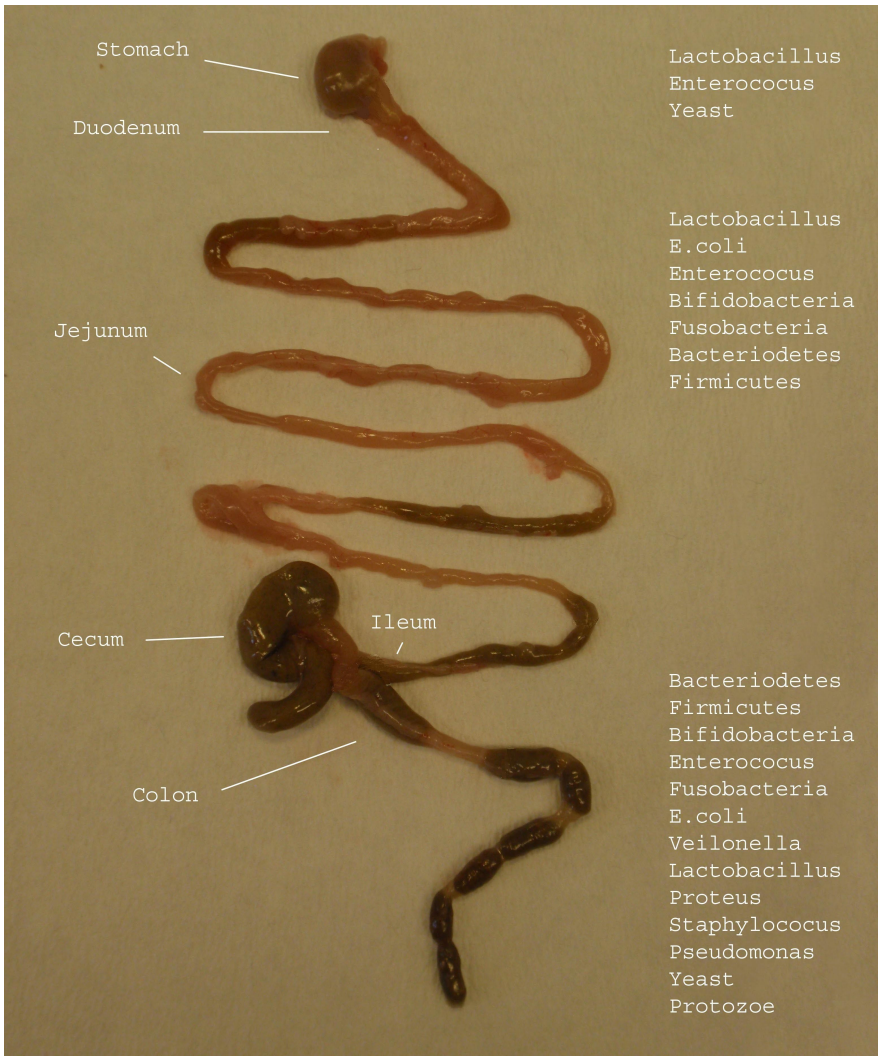


Figure 2.1 GI tract from a mouse with an overview of the bacterial content throughout the GI tract (Sangild, P. T. *et al*., 2010)

2.1 Mucosa Structures

Reference: Kato, T. & Owen, R. L. (2005)

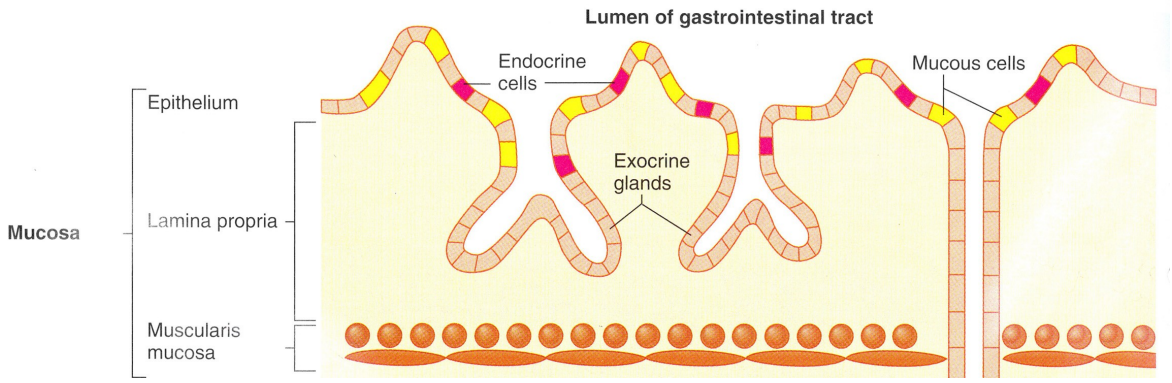


Figure 2.2 Mucosa structures of the gastroentestinal wall in longitudinal section (Widmaier, E. P. *et al.*, 2004).

Mucosa refers to the three layers of gastrointestinal tract wall nearest the lumen - epithelium, lamina propria and muscularis mucosa (figure 2.2). The total area of the mucosa surface of the human gastrointestinal tract is 300 m_2 , which makes it the largest surface area in the body that interacts with the external environment, and it therefore has an important role in maintaining good health.

The mucosa is the site for digestion and absorption of nutrients, and it also functions as a barrier against various harmful agents and infectious pathogens. Protection against such agents and infectious pathogens is executed by a physical barrier permeable to ions and molecules, and immunological barriers created and maintained by the immune defense system, which include the gut-associated lymphoid tissue (GALT) distributed throughout the intestinal tract and the systemic immune system. Mucos on the surface of the mucosa shields the mucosa epithelial cells from contact with the intestinal luminary environment. It is not digested because of its resistance to various enzymes.

The epithelial layer consists of different cell types such as absorptive enterocytes, goblet cells, enteroendocrine cells, Paneth cells and M cells (microfold cells). Goblet cells, enteroendocrine cells and Paneth cells are all secretory cells. Absorptive epithelial cells (enterocytes) constitute the majority of the mucosal epithelium.They have absorption functions but also induce innate inflammatory responses to protect the host against invasion (Dubert-Ferrandon A. *et al*, 2008.).

Their apical surface has numerous tightly packed microvilli. At their apices, the enterocytes are connected with adjacent epithelial cells mainly by junctional complexes. The tight junction plays a role in separating the external and internal environments and functions as a selective barrier.
Goblet cells, whose sole function is to secrete mucin, are present in both small and large instine, increase in number from proximal to the distal portion of the intestine, and are located on villi and in the crypt.
Beneath the mucosal epithelium is the connective and supportive tissue lamina propria. In this tissue immune cells, which form a functional unit with the mucosal epithelial cells, are present.

Characteristics for duodenum
The duodenum mucosa contains Brunner's glands, which secrete alkaline mucus.

Characteristics for ileum
The mucosa is made up of folds, each of which has villi on its surface, and the epithelial cells that line these villi possess even larger number of microvilli, therefore ileum has an extremely large surface area. Ileum has abundant of Peyer's patches (PP) located in the lamina propria as well as lymphoid nodes containing lymphocytes both belonging to the immune system.

Characteristics for cecum/colon
The surface is not convoluted and the mucosa lacks villi, so the surface is flat.

2.2 Mucosal immunity

The immune system is distributed throughout the body to provide defense of the host against a variety of pathogens.
The mucosa surface constitutes a part of the immune system, and it comprises barriers that allow exchange of selected compounds between the exterior and interior of the body, rendering them susceptible to infection. It has to inhibit pathogens adhesion and invasion by developing an immune response (a complex array of innate and adaptive mechanisms) and simultaneously permitting the uptake of dietary components (oral tolerance). To prevent a state of chronic inflammation, a complex balance between inflammatory responsiveness toward pathogens for protection, and uptake and transport of dietary components without harmful inflammatory responses, has to be achieved (Dubert-Ferrandon A. *et al*, 2008.).

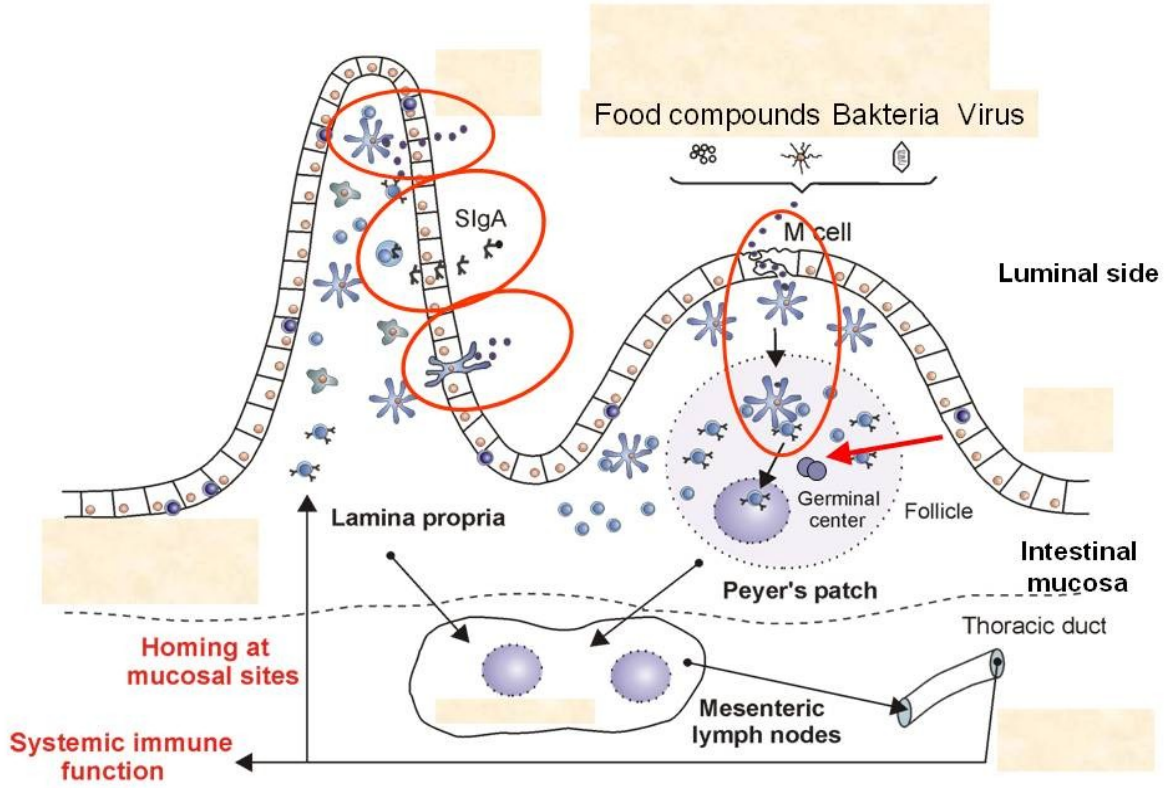


Figure 2.3 The gut associated lymphoid tissue (Frøkiær, H., 2009).

2.2.1 GALT

In the gastrointestinal tract the cells of the immune system are present as single cells in the epithelial layer, but mostly in the lamina propia or in lymph nodes.

Components of the mucosal immune system, distributed within the monolayer of epithelial cells are: M cells specialized in sampling, Paneth cells specialized in microbial peptides release, and intraepithelial lymphocytes specialized in quick response to stimulus (figure 2.3) (Dubert-Ferrandon A. *et al*, 2008).

PP, the appendix, and solitary lymphoid nodules, are major components of the GALT. PP are important sites for the induction of immune responses. The surface of PP is covered by the follicle-associated epithelial layer (FAE). The FAE is enriched in the specialized antigen-sampling M cells. M cells can take up large antigens from the gut lumen and bring these antigens into direct contact with immune cells, thereby initiating protective mucosal immune responses. Each M cell forms a pocket, which contains T cells, B cells, macrophages, and dendritic cells (DC). In addition to the transport of luminal antigens, M cells serve as a port of entry for pathogens (Artis, D., 2008, Ishikawa, H. *et al*., 2005).

PP and mesenteric lymph nodes comprise the area in which the immune response is initiated.

Immune cells from PP and lamina propria migrate to the mesenteric lymph nodes and from here further on with the lymph to the bloodstream. Immune cells then make a systemic response or migrate back to the mucosa exerting a local response.

Lamina propria consists of single immune cells strayed in the connective tissue in villi, and is the area where the activated cells work (Sangild, P. T. *et al*., 2010).

Neutrophils comprise up to 70 % of the leukocytes, and circulate in bloodstream and tissues including epithelium. Neutrophils are very important immune cells comprising the first line of innate immunity. Activated neutrophils provide signals for the activation and maturation of macrophages as well as DC. Most of neutrophils are efficient phagocytes eliminating pathogens (Kumar, V. and Sharma, A., 2010). Lifespan of an activated neutrophil is 1-2 days, therefore stimulation to formation of new cells is important.

Innate immune recognition is mediated by germ-line encoded receptors. The strategy employed during this type of response of the immune system is based on the recognition of a few, highly conserved structures present on various types of microorganisms. Pathogens associated molecular patterns (PAMP) are usually essential for their survival, and these invariant structures are shared by entire classes of pathogens, but also of non-pathogenic microorganisms. (Gordon S., 2002).

2.2.2 Regenerating islet-derived protein IIIγ (REGIIIγ)

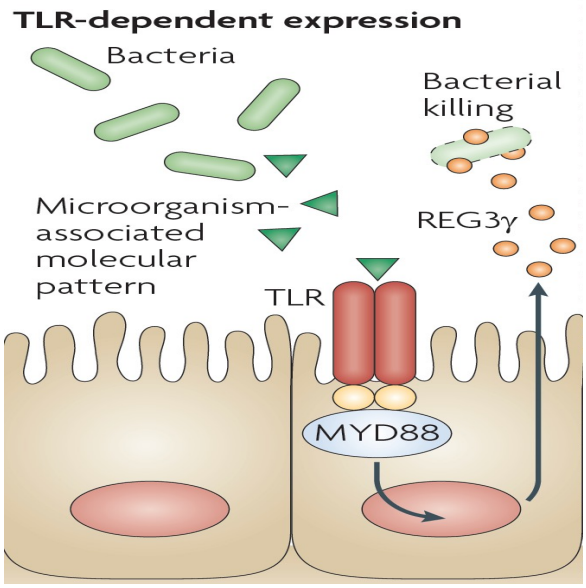


Figure 2.4 Regulation of REGIIIγ expression. The expression of REGIIIγ is controlled by microorganism-associated molecular patterns that activate TLRs and is dependent on the common TLR signaling adapter molecule myeloid differentiation primary-response protein 88 (MYD88). REGIIIγ expression is activated in both enterocytes and Paneth cells (Hooper, L. V. & Macpherson, A. J. , 2010).

REGIIIγ is secreted C-type lectins governed by epithelial TLRs (toll-like receptors), which kill Gram-positive bacteria and play a vital role in antimicrobial protection of the mammalian gut (figure 2.4). REGIIIγ bound bacterial targets via interactions with cell wall peptidoglycan and is expressed predominantly in the small intestine (Hooper, L. V. & Macpherson, A. J. , 2010). Inflammatory stimuli such as bacteria increase the gastroentestinal expression, and expression is suggested to reflect microbial colonization level in the small intestine. Cash, H. L. (2006) found increased expression in the distal region of ileum concomitant with increasing microbial densities. In contrast germfree mice showed minimal REGIIIγ expression throughout the small intestine. The expression is triggered by increased microbial epithelial contact at mucosa surfaces, to limited mucosal penetration by gut microbes. REGIIIγ is retained in the mucus layer and is virtually absent from the luminal content (Hooper, L. V. & Macpherson, A. J. , 2010, Lehotzky, R. E. *et al*., 2010, Cash, H. L. *et al.*, 2006).

2.2.3 Cytokines

Cytokines are small cell-signaling proteins, which are secreted by numerous cells of the immune system and are used extensively in intercellular communication. Each cytokine has a matching cell-surface receptor, and cascades of intracellular signaling then alter cell functions (Sartor, R. B. & Hoentjen, F., 2005).

The cytokine IL-1β (interleukin 1 beta) is an important mediator of the inflammatory response, and is involved in a variety of cellular activities, including cell proliferation, differentiation, and apoptosis (McGee, D. W., 1999, Bensi, G. *et al*. 1987).

TNF-α (tumor necrosis factor) is a multifunctional cytokine, which function overlaps with IL-1β. TNF-α stimulates the expression of acute phase proteins (Hirata, N. *et al.*, 2011, Sartor, R. B. & Hoentjen, F., 2005).

Chemokines are a subgroup of cytokines consisting of small structurally related molecules that regulate cell trafficking of various types of leukocytes. Chemokines also play fundamental roles in the development, homeostasis, and function of the immune system (Fujihashi, K. & Ernst, P. B., 1999).

CXCL1 and CXCL2 are small cytokines belonging to the CXC chemokine family. They are both pro-inflammatory cytokines (Pineau, I. *et al.* (2010).

2.2.4 Receptors

TLRs play a key role in the innate and adaptive immune system. They recognize structurally conserved molecules derived from microbes and activate immune cell responses. TLRs are a type of pattern recognition receptors (PRR) and recognize molecules that are broadly shared by pathogens but distinguishable from host molecules (PAMP). Activation of TLRs initiates a signaling cascade through the adapter molecules for induction of expression of various pro-inflammatory cytokines. The various TLRs exhibit different patterns of expression. There is abundant evidence that signaling through TLRs leading to pro-imflammatory gene expression. TLR1 to TLR9 are expressed by gut epithelial cells (Siednienko, J. & Miggin, S. M., 2009, MacDonald, T. T. & Monteleone, G., 2005).

TLR2 is a membrane protein which is expressed on the surface of certain cells. They recognize PAMPs and mediate production of cytokines. TLR2 mediates host response to Gram-positive bacteria and yeast (Siednienko, J. & Miggin, S. M., 2009, MacDonald, T. T., Monteleone, G., 2005).

TLR4 is a key regulator of both inflammation and epithelial homeostasis in the human intestine. TLR4 detects lipopolysaccharide (LPS) from Gram-negative bacterial cell wall. TLR4 is normally down-regulated in the gastrointestinal tract in order to prevent continuous activation of the innate immune system. Expression of TLR4 usually varies within the whole mucosa. Both intestinal epithelial cells (IEC) and infiltrating immune cells potentially express TLR4 (MacDonald, T. T. & Monteleone, G., 2005).

Scavenger receptors recognize modified low-density lipoprotein (LDL), and they widely recognize and uptake macromolecules having a negative charge as well as the modified LDL. Scavenger receptors are cell-surface proteins and exhibit distinctive ligan-binding properties, recognizing a wide range of ligands that include microbial surface constituents and intact microbes (Aung, K. M. *et al*., 2011, Hölzl, M. *et al*., 2011).

CD36 (Cluster of Differentiation 36) is a membrane protein found on the surface of many cell types, and is highly expressed in the intestine on the luminal surface of enterocytes. CD36 is a member of the class B scavenger receptor family. CD36 is expressed by macrophages and is considered important in innate immunity. CD36 binds many ligands including long chain fatty acids. Studies using genetically modified rodents have identified a role for CD36 in fatty acid uptake, glucose metabolism (Hajri, T. *et al*., 2002) and dietary fat processing in the intestine (Aung, K. M. et al., 2011, Drover, V. A. *et al.*, 2005,). CD36 has been shown to bind long-chain fatty acids and to facilitate their transfer into the cell. Deficiency or overexpression of the protein is associated with alterations in uptake and metabolism of long-chain fatty acids in rodents. In human Cd36 deficiency and polymorphisms in the Cd36 gene are associated with abnormalities in fatty acid clearance, insulin responsiveness and lipoprotein metabolism. The protein is highly expressed in the small intestine and localized to the apical membrane of villi enterocytes, and is absent from goblet cells (Nassir, F. *et al*., 2007). In small intestines of human and rodents CD36 expression is very high in proximal segments (duodenum, jejunum) and decrease from proximal to distal (Nassir, F. & Abomrad, N. A., 2009).
In a study by Nassir, F. *et al*., (2007) the role of intestinal CD36 in lipid uptake based on the hypothesis that it may have a primary role in proximal fatty acid absorption for chylomicron formation, whereas other mechanisms would play the major role in more distal parts of the intestine was examined. They found that CD36 expression measured in three equal-length segments (proximal to distal) was highest in proximal and lowest in distal intestine. The findings support the role of CD36 in proximal absorption of dietary fatty acid and cholesterol for optimal chylomicron formation, whereas CD36-independent mechanisms predominate in distal segments.

G-protein coupled receptor (GPR), have been identified as a family of receptors activated by SCFA (Maslowski, K. M. *et al.*, 2009). Recently the many G protein-coupled receptors have been renamed by the International Union Pharmacology as free fatty acid (FFA) receptors. Each of the FFA receptors is expressed differently. Now GPR43 is called FFAR2 or FFA2 (Karaki, S. & Kuwahara, A., 2010).

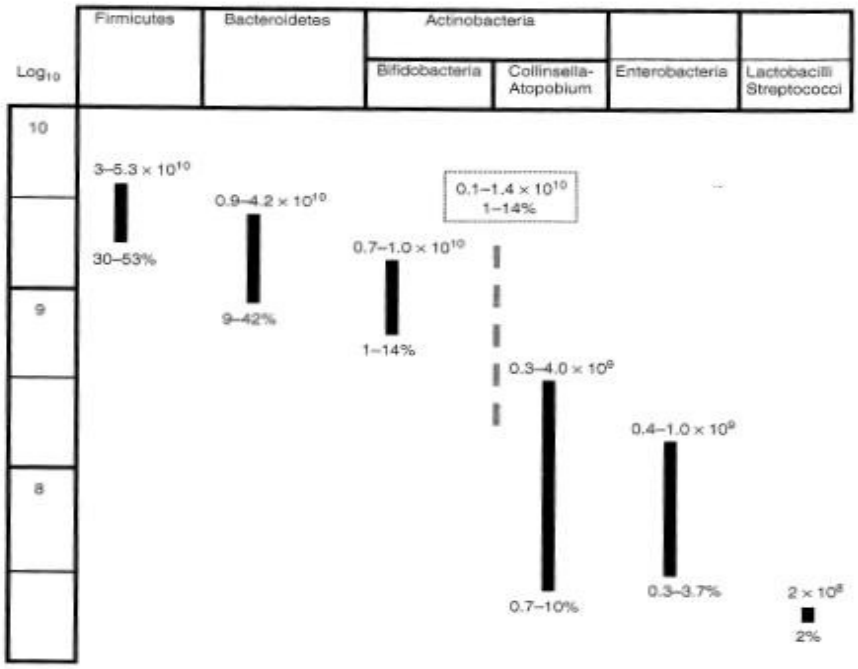
2.3 Microbiota

Reference: Roberfroid, M. B., 2008 and Wells, A. L. *et al*., 2008

The gut houses a complex and vast microbial community, with total estimates in the region of 10^{14} microorganisms, inhabiting the whole mammalian gastrointestinal tract. The concentration of microorganisms present varies according to location. Stomach contents (per gram) can be less than 10^3 CFU (colony-forming unit), in the small intestine content is about 10^4- 10^7, and 10^{10} - 10^{12} per gram in the colon, where the microbial numbers are highest. The whole microbiome is thought to contain approximately 100 times the number of genes in the human genome. There are four main microhabitats in the gastrointestinal tract; the epithelial surface, the mucus layer , the crypts of the ileum, cecum and colon, and the intestinal lumen (Sangild, P. T. *et al*., 2010).

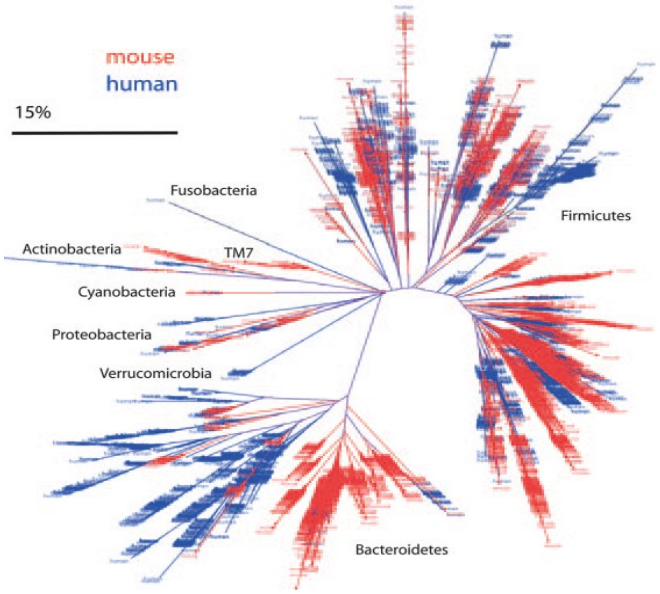
Implantation of the gut microbiota starts in the newborn intestine immediately after birth. During life the complexity of the microbiota increases from only a few groups in infants to a few hundreds of groups in later age. Therefore, the composition can be quantitatively and qualitatively highly individual.

The dominant human fecal flora is composed of 3 phyla: *Firmicutes*, *Bacteroides* and *Actinobacteria* (*Bifidobacterium, Collinsella-Atopobium*), which represent up to 75 % of the whole microbiota. Subdominant groups are *Enterobacteria*, streptococci and lactobacilli (figure 2.5).



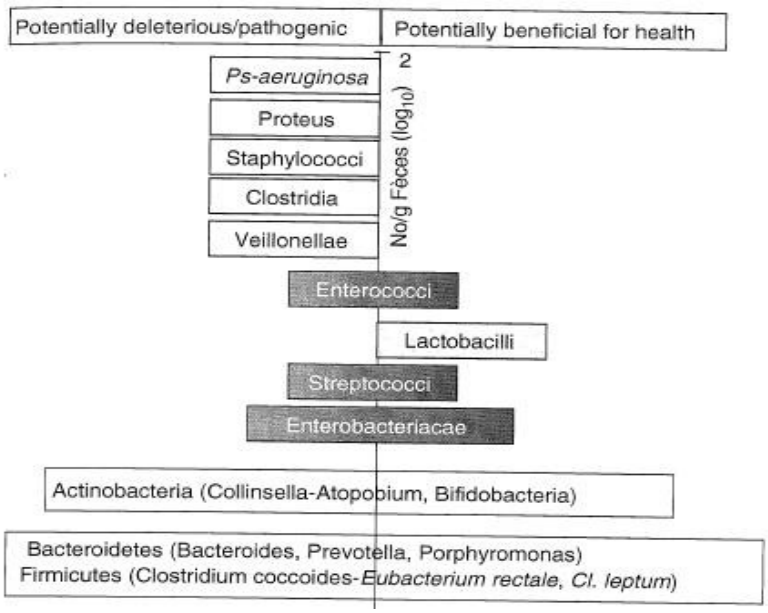
Figur 2.5 A quantitative overview of the predominant human microbiota resulting from phyla/groups analysis. The approximate number of bacteria in each phylum/group per gram feces is given either in absolute numbers or percentages (Roberfroid, M.,. B., 2008).

Ley, R. E. et *al*., (2005) found that 85% of the sequences from qPCR in mice represents genera that have not been detected in humans, so the majority of mouse gut species are unique, but they also found that mouse and human microbiotas are similar at the phyla level, with *Firmicutes* and *Bacteriodetes* dominating in the cecum (figure 2.6).



Figur 2.6 Bacterial diversity in the distal gut (cecum) of C57BL/6 mice. Phylogenetic tree of 5088 mouse cecum-associated 16S rRNA sequences and 11831 human colon-associated 16S rRNA sequences. Data were obtained by using the same 16S rRNA gene-directed primers and PCR cycle numbers. The bar represent 15% sequences divergence (Ley, R. E. et al., 2005).

The dominant phyla are not well classified in groups of potentially beneficial or deleterious bacteria, because of lack of the knowledge concerning activities in the different groups. *Bifidobacterium* and *Lactobacillus* are traditionally classified as beneficial for health (figure 2.7).



Figur 2.7 Schematic average distribution of dominant, subdominant, and minor components of human fecal microflora (Roberfroid, M. B., 2008).

2.3.1 Firmicutes

The phylum *Firmicutes* mostly consists of Gram-positive bacteria, many of them producing endospores. *Firmicutes* are typically divided into three groups: *Clostridia* (anaerobic), *Bacillus* (obligate or facultative aerobic) and *Mollicutes.*

Firmicutes are hightly diverse in phenotypic characteristics, but most of them can be detected by real-time PCR (qPCR) (Haakensen, M. *et al*., 2008).

2.3.2 Bacteriodetes

Bacteriodetes consist of Gram-negative bacteria not producing endospores. In the gut *Bacteriodetes* include *Bacteroides, Prevotella* and *Porthyromonas*. Some *Bacteriodes* produce toxin, but generally *Bacteroides* are classified as beneficial for the gut whereas *Prevotella* and *Porphyromonas* could be pathogenics (Roberfroid, M. *et al*., 2010, Casci, T. *et al*., 2006).

Ley, R. E. *et al.* (2005) found that obese humans and mice had intestinal flora with a lower percentage of *Bacteroides* and relatively more bacteria from the *Firmicutes* phylum than lean individuals.

2.3.3 Bifidobacteria

Bifidobacterium is a Gram-positive, non-motile anaerobic bacteria. Bifidobacteria are thought to have beneficial effects on human physiology and pathology. *Bifidobacterium* is considered as probiotics and used in the food industry to prevent and treat intestinal disorders. Bifidobacteria share some phenotypic features with lactic acid bacteria. *Bifidobacterium* represents up to 25% of the cultivable faecal bacteria in adults and 80 % in infants (Picard, C. *et al*., 2005). *Bifidobacterium* can utilize a wide range of substrates for fermentation, but only a few can ferment xylan. Some XOS are found to be poorly utilized by *Bifidobacterium* strains, but xylobiose has been reported to be bifinogenic (Fukuda, S. *et al*., 2011, Casci, T. *et al*., 2006,).

2.3.4 Lactobacillus

Lactobacilli are Gram-positive facultative anaerobic or microaerophilic bacteria. They are mostly nonsporulating, and are a part of the lactic acid bacteria group (convert lactose and other sugars to lactic acid). *Lactobacillus* is thought to have beneficial effects on humans, so lactic acid bacteria are important functionally but not predominant numerically in the gut. The most common intestinal *Lactobacillus* isolates are from the acidophilus group. Some *Lactobacillus* are used in food production and as probiotics (Casci, T. *et al.*, 2006, Mueller, S. *et al*. 2006).

2.4 Roles of the microbiota

The gut microbiota can communicate with itself (bacteria:bacteria) and with the host (bacteria:human). The microbiota is also a site of energy consumption, transformation and distribution. The composition of the microbiota appears to play important nutritional and physiopathological roles such as:
Prevention of gut colonization by potentially pathogenic microorganisms by out-competing invading pathogens for ecological niches and metabolic substrates. As an important source of energy for the cells of the gut wall through the fermentation of carbohydrates to SCFAs. Through modulation of the immune system, by education the naive infant immune system and serving as an important source of non-inflammatory immune simulators throughout life, and through modulation of gene expression and cell differentiation in the gut wall.
So the gastrointestinal tract and its microbiota form a symbiotic association and interact with each other to play a role not only in colonic function but also in whole body physiology. To best support interactions, the microbiota needs to have an appropriate composition, where species that are known or believed to be health promoting predominate over those that are or might become harmful pathogenic (Gibson *et al*. 1995).

2.4.1 Metabolic functions of the microbiota

Major roles of the microflora are to ferment dietary components to salvage of energy as SCFAs, produce vitamin K, and absorb ions (Guarder, F. & Malagelada, J. R., 2003).

The main fermentation products from non digestible carbohydrates (from e.g. some vegetables) are SCFAs . However, proteolytic fermentation from e.g. meat also generates potentially damaging compounds such as ammonia, amines and phenolic compounds. The most metabolic active area is cecum and the upper part of colon. Consequently this is the area of rapid bacterial growth, low pH (5-6) and high generation of SCFAs. The lower part of colon has less carbohydrate fermentation and pH is less acidic. The most relevant SCFAs are acetate, butyrate and propionate (Guarder, F. & Malagelada, J. R., 2003).

2.4.2 Trophic functions of the microbiota

The gut microbiota consumes, stores, and redistributes energy; it mediates physiologically important chemical transformations, and can maintain and repair itself through self replication (Bäckhed, F. *et al*. 2005).

The microbiota contribute to control of epithelial cell proliferation and differentiation, development and homoeostasis of the immune system.

Experiments have shown that the rate of production of crypt cells is reduced in the colon of rats bred in germ-free environment, and their crypts contain fewer cells than those of rats colonized by conventional flora, suggesting that intraluminal bacteria directly or indirectly affect cell proliferation in the colon. Differentiation of epithelia cells is greatly affected by interaction with resident microorganisms (Gordon JI., *et al*, 1997). An important role of SCFAs on colonic physiology is their trophic effect on the intestinal epithelium. All three major SCFAs stimulate epithelia cell proliferation in the large and small intestine *in vitro* (Guarder, F., Malagelada, J. R., 2003).

GALT comprises the largest pool of immune competent cells in the human body. The dialogue between host and bacteria at the mucosal interface seems to play a part in development of a competent immune system.

2.4.3 Protective functions of the microbiota

Protection against pathogens (the barrier effect).

Resident bacteria is a crucial line of resistance to colonization by exogenous microbes and therefore, is highly relevant in prevention of invasion of tissues by pathogens. There are several mechanisms thought to contribute to the colonization resistance, so it is a multifunctional defensive strategy. Germ free animals have shown that a lack of microflora leaves its host much more susceptible to infection.

Adhesion is an important factor of colonization resistance, as nonpathogenic organisms need not only to be able to adhere to the gut epithelia but also to proliferate on it, and bacterial strains with potential positive health benefits takes up invasion space. It is likely that colonization is rather temporary than permanent. Competitive environment is established because of the availability of nutrients between resident bacteria and pathogens. Bacteria can inhibit the growth of their competitors by producing antimicrobial substances (bacteriocins). The role of bacteriocins is mainly restricted to localized niches (Guarder, F., Malagelada, J. R., 2003).

3 Prebiotics

3.1 Criteria for classification

Not all dietary non digestible carbohydrates are prebiotics (definition in introduction). Any food that contains carbohydrates and in particular oligosaccharides, is potentially a prebiotic, but in order to be classified as such, it requires a scientific demonstration. Any dietary component that reaches the colon intact (or partly so) is a potential candidate for prebiotic attribute (Roberfroid, M. et al., 2010).
Criteria for classification as a prebiotic are:

- Resistance to gastric acidity, hydrolysis by mammalian digestive enzymes and GI absorption
- Fermentation by intestinal microflora
- Selective stimulation of the growth and/or activity(ies) of one or a limited number of intestinal bacteria beneficially associated with health and well-being

(Roberfroid, M. et al., 2010).

3.2 Prebiotic candidates

Name	Prebiotic status
Inulin-type fructans Cover all β(2-1) fructosyl-fructose molecules. Degree of polymerization (DP) from 2-60 units. The most common inulin-type frutans presently produced and used by the food industry is chicory inulin (IN). Fructooligosaccharides (FOS) is enzymatic synthesis from sucrose. Oligofructose (OF) is enzymatic partial hydrolysis of inulin	Classified as prebiotic *
Galacto-oligosaccharides, Trans-galacto-oligosaccharides (GOS and TOS) is an enzymatic transglycosylation of lactose which produce a mixture of oligosaccharides. Mixture of β(1→6);β(1→3);β(1→4) galactosyl-galactose	Classified as prebiotic.
Lactulose Disaccharide galactosyl β(1→4) fructose, produced from isomerization of lactose. (has not been used as a food ingredient or as a food supplement)	Classified as prebiotic.
Xylooligosaccharides (XOS). Oligomers from xylan linked by β –glycosidic bonds. Manufactured by enzymatic hydrolysis of xylan from corn cobs or wheat brain.	Cannot, presently, be classified as prebiotics.

Table 3.1 Prebiotic overview (Roberfroid M.., 2010).

*The evidence available today both from *in vitro* and *in vivo* experiments supports the classification of inulin-type fructans as prebiotic, since they fulfill all three criteria. These compounds are now considered as the model prebiotics (Roberfroid M.B., 2008).

3.2.1 Xylooligosaccharides

XOS are found naturally in bamboo shoots, fruits, vegetables, milk and honey (Casci, T. *et al.*,2006).
XOS are usually produced from xylan by limited hydrolysis of endo-1,4- β-xylase.
Xylan are highly complex polysaccharides made from units of xylose, that are found in plant cell walls and some algae (Wu, Y. & Lin, K., 2011).
XOS are not hydrolyzed by the digestive enzymes in the small intestine, as the xylose molecules are linked by β(1-4) bonds which cannot be degraded by mammalian cells (Tuohy, K. M., 2005).
XOS mainly consist of xylobiose, xylothiose and xylotetraose (Tuohy, K. M., 2005). The commercial products are predominantly composed of the disaccharides xylobiose, with a small amount of higher oligosaccharides (Roberfroid M., 2008). Among XOS, di- and trisaccharides are the most activating components for the growth of *Bifidobacterium* (Wu, Y. & Lin, K., 2011).

Bifidogenic effects of XOS have been demonstrated *in vitro*, in a colon stimulator and as growth substrates for probiotics and intestinal strains (see prebiotic effects).
Studies based on pure cultures do not represent the conditions in the colon, and *in vitro* data do not demonstrate selective stimulation on bacterial growth in the GI tract, but bifidogenic effects have also been demonstrated *in vivo* (see prebiotic effects).
Most studies are carried out in fecal and cecal samples, and changes in the microflora in the small intestine lack investigating.

The parent molecule, xylan, is recognized as a dietary fiber indicating that it may reach the colon intact, but no data support this assumption. The ultimate test for prebiotic activity (i.e. human volunteer trials) is lacking for XOS, no experimental evidence to date certifying the nondigestibility of XOS in the upper gastrointestinal tract of human exist. Therefore the evidence for prebiotic status of XOS is still not sufficient, and XOS are not classified as a prebiotic (Roberfroid, M. et al., 2010, Roberfroid M. B., 2008).

3.3 Effects of prebiotics

The effect of a prebiotic is essentially indirect because it selectively feeds one or a limited number of microorganisms thus causing selective modification of the host's intestinal microflora. It is not the prebiotic itself but rather the changes induced in microflora composition that is responsible for its effects, as a result of intestinal fermentation and promotion of growth of beneficial members of the gut microbiota. Composition and metabolic activity of the intestinal flora are directly depending on dietary constitutions including prebiotics (Roberfroid, M. B., 2008).

There are varying levels of evidence and agreement, but the main areas of pathophysiological interest in which the effects of prebiotics have been investigated are: *Functional Effects* – intestinal /colonic functions (e.g. fecal, bulking, stool production), resistance to intestinal infections, bioavailability of minerals, especially Ca and Mg, immunomodulation, influence on gastrointestinal peptides especially glucagon-like peptide (GLP-1) and ghrelin, satiety and appetite. *Disease Risk Reduction* – Management of the infectious diarrhea, metabolic syndrome, obesity, osteoporosis, inflammatory bowel diseases and colon cancer. (Roberfroid, M. B., 2008).

Dose-response relationship, is a contentious issue of the *in vivo* human studies. The relationship between dose and magnitude of bifidogenic effect, although shown in certain studies with FOS supplementation, seems to be more relevant to baseline bifidobacteria levels rather than the dose ingested (Roberfroid M. B. 2008). There is currently no recommended daily intake of prebiotics. In human trials administrated doses have varied from 3-20 g per day. Some authors suggested 10 g per day as an optimum well-tolerated daily dose for adults (Tuohy, K. M., et al., 2005).

3.3.1 Prebiotic effects on microbiota composition

Prebiotics stimulate the growth of endogenous microbial population groups such as *Bifidobacterium* and *Lactobacillus.*

Bifidogenic effects have been described in rodents consuming XOS, most notably in animals presenting lower initial bifidobacterial numbers (Campbell, J. M. *et al*., 1997). But microorganisms belonging to *Bacteroides, Clostridium* and *Lactobacillus* genera are also able to metabolize XOS mixtures, mainly composed by xylobiose and xylotriose. However more complex XOS structures have presented a higher capability to be selectively fermented by bifidobacteria (Van Laere, K. M. J. *et al*., 2000).

Jaskari, J. *et al.* (1998) carried out a study on pure culture involving a range of bacteria. This indicates (not to a significant level) that XOS was metabolized by the majority of *Bifidobacterium* strains tested but also by *Bacteroides, Clostridium difficile*, and *E. coli.*
Bacteroides, Clostridium and *E. coli* were able to degrade XOS, but they were all far less efficient than any of the *Bifidobacterium* strains. *Lactobacillus* did not metabolize XOS.
Campbell, J. M. *et al*. (1997) carried out a study with rats feeding a XOS diet and examined fecal and cecal bacteria. They found significant increase in bifidobacterial growth.
Hsu, C. K. *et al*. (2004) evaluated the inhibitory effects of XOS on colon cancer. They found that XOS markedly decrease the cecal pH and increased *Bifidobacterium* population.
Petersen A. *et al*. (2010) reported microbial changes induced by XOS in the large intestine of mice challenged with *Salmonella*. Fecal and cecal samples from the mice were analyzed in order to study microbial changes potentially explaining observed effects on the pathogenesis of *Salmonella*. They saw a difference in the fecal samples when feeding with XOS, but not in the cecal. There was significant increase in *Bacteroides* group and in *Bifidobacterium* in mice fed with XOS. *Firmicutes* were reduced by XOS. An increased level of *Bifidobacterium* is thus not in itself preventive against *Salmonella* infection, since XOS fed mice were previously reported to be more severely affected by *Salmonella* than control animals. They found no increase in cecal concentration of SCFAs on XOS diets.
Chung, Y. C. *et al.* (2007) evaluated the effects of XOS on intestinal microbiota, GI function and nutritional parameters of elderly. They found that XOS supplementation (4 g /day for 3 weeks) significantly increased the population of bifidobacteria and decreased the fecal pH value.

In summary it seems that XOS are given bifidogenic effect in feces, but in cecum the bifidogenic effects give different results. *Lactobacillus, Bacteroides, Clostridium* and *E. coli* also seem to metabolize XOS, but not as efficient as *Bifidobacterium*. The content of *Clostridium* decrease when fed with XOS.

3.3.2 Prebiotic effects on SCFA production in GI tract

Fermentation of prebiotics results in the acidification of the colonic contents due to the formation of SCFAs which serve as fuel in different tissues and may play a role in the regulation of cellular processes. It has been estimated that SCFAs can contribute to about 5-15 % of the total caloric requirements of humans. However, the *in situ* production of total colonic SCFAs is difficult to determine because more than 90% of the SCFAs are rapidly absorbed and metabolized by the host (Karaki, S. & Kuwahara, A., 2010). Major SCFAs resulting from fermentation in the mammalian intestinal tract are acetate, propionate and butyrate, whereas lactate is an intermediate metabolite from the fermentation process (Macfarlane, S. & Macfarlane, G. T., 2003).

An *in vitro* study by Manisseri, C. & Gudipayi, M. (2010) suggested prebiotic nature of XOS. They carried out using *Bifidobacterium* and *Lactobacillus*, and the hydrolytic enzymes produced by the microorganisms assisted the digestions of XOS liberated from wheat bran. A slight increase in bacterial growth was observed for oligosaccharides having low degree of polymerization (xylobiose and xylotriose), and the utilization of the oligosaccharides by the bacteria strains was found to be strain specific. So it seems that *Bifidobacterium* had the enzymes for the degradation of XOS. Acetate was the main SCFA liberated due to *in vitro* fermentation of XOS.

Wang, J. *et al*., (2010) investigated the utilization of XOS from wheat bran by four *Bifidobacterium* strains (*B. adolscentis, B. longum, B. bifidum, B. breve*). *B. adolscentis* displayed the highest growth on XOS, whereas *B. breve* showed no growth. The SCFAs were predominantly acetate. During the first 16 h of the fermentation of XOS, acetic and lactic acids were formed rapidly, while butyric acid was formed slowly, and the concentration of propionic acid remained constant or even decreased slightly. Also Mäkeläinen, H. *et al.* (2010) found that fermentation of XOS and xylan *in vitro* increased the microbial production of SCFAs.

In summary, acetate is the main SCFA from the fermentation of XOS, but not all bifidobacteria can grow on XOS. In the beginning of the fermentation, acetate and lactate are formed rapidly, while butyrate is formed slowly. Propionate seems to be in a constant level.

3.3.3 Prebiotic effects on the immune system

No studies were found investigating XOS effect on the immune system for which reason inulin-type fructans and GOS will be the prebiotics mentioned in this part, to illustrate prebiotic effects on the innate immune system.

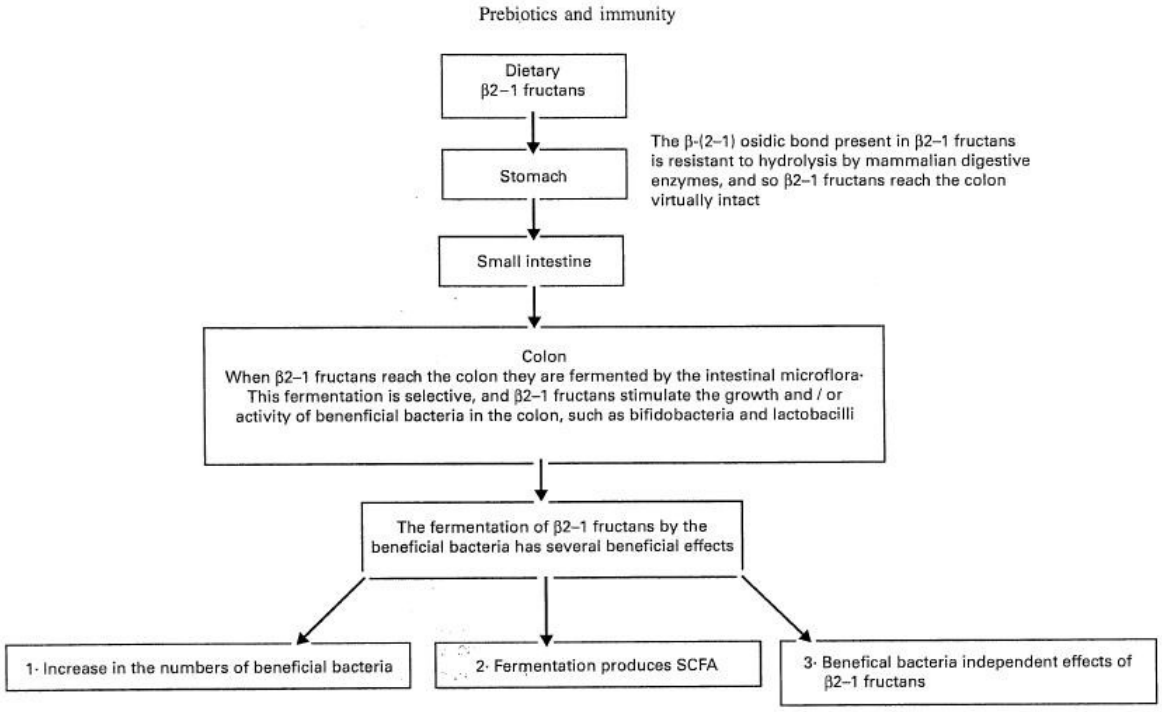


Figure 3.1 Mechanisms by which inulin-type fructans may influence host defense (Lomax A. R., Calder P. C., 2008).

A review by Lomax A. R. & Calder P. C. (2008) investigated the evidence of prebiotics on immune function, infection and inflammation, and found studies indicating that prebiotics (inulin-type fructans) have an impact on the immune system (figure 3.1).

Studies which investigate GALT and innate immune system, indicate improvement by inulin-type fructans intake (enhanced macrophage functions, increase MHC II expression) which could result in a beneficial effect on the host's primary response to infection. Trials involving infants and children mostly reported benefits as reduction in incidence or duration of infections. In adult trials, little effect was seen (decrease in relapse rate of diarrhoea and gastrointestinal infections, exert beneficial effects on infections in patient admitted to intensive care or surgery wards). Human studies report some benefits regarding inflammatory bowel disease (decrease disease activity, increased expression of Tlr4 on DC in lamina propria, decrease level of TNF-α in mucosal tissue) and atopic dermatitis, but findings in irritable bowel syndrome were inconsistent.

Taken together, the review suggested, that inulin-type fructans may improve the host's ability to respond successfully to certain intestinal infections and to modify some inflammatory conditions. But it seems that inulin-type fructans are most beneficial in those who are particularly susceptible to modifications of their immune systems like sick people, and especially children.

Investigations in rat models of inflammatory bowel disease demonstrated that prebiotics may alleviate acute inflammation. When rats received oligofructose (OF) and IN (OFI) before and after chemical colitis induction, lowered production of proinflammatory IL-1β in the colitis was reported. In addition, translocation of bacteria to mesenteric lymph nodes was significantly decreased (Osman M. *et al.*, 2006). In rats developing spontaneous colitis as response to the endogenous intestinal microflora, an intake of 5g IN/OF/kg body weight for 7 weeks resulted in reduced level of IL-1β in the cecal mucosa (Hoentjen *et al.*, 2005).
In a small randomized, double-blinded controlled human trial including subjects with ulcerative colitis, supplementation with *B. longum* and OF- enriched IN resulted in an improvement of the full clinical appearance of chronic inflammation. Furthermore, intestinal mRNA levels of the proinflammatory cytokines IL-1β and TNF-α were significantly reduced in synbiotic-treated subjects, while no significant differences were seen for the immunoregulatory cytokine IL-10 (Furrie E. *et al.*, 2005).

In summary, inulin-type fructans are able to modulate some aspects of the immune functions, to improve the host's ability to respond successfully to certain intestinal infections, and to modify some inflammatory conditions as in inflammatory bowel disease and ulcerative colitis. Inulin-type fructans seems to reduce the level of IL-1β and TNF-α and decrease the translocation of bacteria to the mesenteric lymph nodes. For children the beneficial effects seem to be on infection outcomes.

3.3.3.1 The mehanisms of prebiotic effects on the innate immune system

The exact underlying mechanisms of prebiotic induced alterations of the immune system are not completely known, but data suggest two ways of prebiotics induced immunological effects:

- Bacteria: Selective increase/decrease in specific bacteria that modulate expression of immune related genes, e.g. cytokine and Tlr
- Metabolites: Increase in intestinal SCFA production and enhanced binding of SCFA to FFA receptors on leukocytes

Effects on the immune system from the increase/decrease amount of bacteria

The prebiotic-induced shift in the intestinal microflora may change the presence of PAMP in the intestinal lumen. Through PRR such as the Toll-like receptors, local immune cells may respond to the molecular motifs (Akira *et al*., 2001).

In a study by Lindsay J. O. *et al,* (2006) with Crohn's disease patients, the daily intake of 15 g OF (70%) IN (30%) significantly decreased disease activity. They found a significant correlation between the effects of prebiotics on disease activity and its effects on the total concentration of mucosal bacteria. Patients who experienced clinical remission after prebiotic treatment had a significant increase in mucosal *Bifidobacterium* compared with those that had persistent disease activity. Fecal concentrations of *Bifidobacterium* increased in all participants. Thus the presence of *bifidobacterium* may have an anti-inflammatory effect.

Vulevic, J. *et al*. (2008) assessed the effect of GOS on immune function and fecal microflora composition in healthy elderly subjects. They found significant increases in phagocytosis and significant reduction in the production of proinflammatory cytokines IL-1ß and TNF-α in blood, showing that GOS treatment results in an overall antiinflammatory effect. They also found significantly increased numbers of beneficial bacteria in feces, especially *Bifidobacterium*, at the expense of less beneficial groups of bacteria.

Buddington K. K. *et al.* (2002) challenged mice fed IN diet with either *Listeria monocytogenes* or *Salmonella typhimurium*. None of the mice challenged with *Listeria monocytogenes* died, but when challenged with *Salmonella typhimurium* 60 % of them died, compaired to >80% for the control group. But the relation between changes in bacterial content and the immune function can not be drawn, since they did not investigate the intestinal microflora.

As mentioned earlier, Petersen, A. *et al*. (2010) found that an increased level of *Bifidobacterium* is not in itself preventive against *Salmonella* infection, since XOS or FOS fed mice were previously reported to be more severely affected by *Salmonella* than control animals.

In summary prebiotics increase fecal numbers of *Bifidobacterium* at the expense of less beneficial groups of bacteria. This leads to an increased phagocytosis, and a reduced IL-1ß and TNF-alpha production in blood. The prebiotic effects on pathogenic infections is not clear.

Effects on the immune system from the increased amount of SCFA

Enhanced SCFA production in the gut after prebiotic supplementation may increase the SCFA supply to immune cells located along the GALT and activate these cells with SCFA receptors. SCFAs are known to regulate proliferation and apoptosis of lymphocytes and monocytes and to inhibit NF-κB activity in colonic epithelia cells (Inan, M. S. *et al.*, 2000, Kurita-Ochiai, K *et al.,* 2003, Millard, A. L. *et al*., 2002).

A Study of pigs fed with a rye-based diet measured increase butyrate concentration in blood 8-10h following feeding (Bach Knudsen *et al*., 2005). *In vitro* butyrate is found to suppress lymphocyte proliferation and to up-regulate the anti-inflammatory cytokine IL-10 production of DC. (Cavaglieri *et al*., 2003, Kurita-Ochiai *et al.,* 2003, Millard *et al*. 2002, Säemann *et al*., 2000). Sanderson, I. R. (2007) found that butyrate may alter epithelial cells.

SCFA is known to exert cellular effects on blood leukocytes. How intraluminal SCFA are sensed by leukocytes are not completely known. Two FFA receptors FFAR2 (GPR43) and FFAR3 (GPR41) have been identified to bind and become activated by SCFA. Nilsson, N. E. *et al*. (2003) found the human gene to be predominantly expressed in blood leukocytes and, to a lesser extent, in spleenocytes. Acetate, propionate and butyrate represent the most capable SCFA in inducing calcium mobilization, which regulates leukocyte function in the immune system. For FFAR2, acetate and propionate have been found to be the most potent ligands (Ichimura, A. et al., 2009, Brown *et al*., 2003, Nilsson, N. E. *et al.*, 2003).

FFAR2 is highly expressed in various types of cells, including epithelial cells and mucosal mast cells in the rat ileum and colon (Karaki *et al.*, 2006), and have been reported to be highly expressed in human neutrophils and monocytes. The highly selective expression of FFAR2 in leukocytes, suggests a role in the recruitment of these cell populations toward sites of bacterial infection. A neutrophil specific SCFA receptor is potentially involved in the development of a variety of diseases characterized by either excessive or inefficient neutrophil recruitment and activation (Brown *et al*., 2003, Le Poul *et al.*, 2003).

Maslowski, M. K. *et al*. (2009) showed that SCFA-FFAR2 interactions profoundly affect inflammatory responses in mice chemically induced with colitis. They found that germ-free mice, which are devoid of bacteria and thus produce little or no SCFAs, like Grp43-/- mice showed dysregulation of certain inflammatory responses. They found that transcripts for human and mice FFAR2 exhibited enhanced expression in neutrophils. They also found that FFAR2 expression was closely regulated with receptors important for innate immunity, such as TLR2, TLR4, and CXCR2. Acetate induced a robust calcium flux in mouse (and human) neutrophils, but not in neutrophils from Gpr-/- mice, indicating that FFAR2 is the sole functional receptor for SCFAs on neutrophils. Acetate induced apoptosis in neutrophils in a dose-dependent and a FFAR2 dependent manner, and acetate stimulation of human neutrophils markedly reduced surface expression of pro-inflammatory receptors such as CXCR2. Taken together, this study could indicate that FFAR2 binding of SCFAs provides a molecular link between diet, gastrointestinal bacterial metabolism, and immune inflammatory responses.
In this context it is interesting again to mention the study by Wang, J. *et al*. (2010) finding that XOS fermentation results in SCFA production of predominantly acetate.
Karaki, S. & Kuwahara, A. (2010) also found that FFAR2 in the intestinal mucosa may be related to host defense mechanisms including innate immunity.

In summary prebiotic increase the production of SCFAs. In a pig study increased butyrate level in blood after prebiotic feeding was measured, and *in vitro* butyrate is found to have effects on the immune system.
FFAR2 is activated by SCFAs and acetate and propionate are the most potent ligands. FFAR2 is expressed in ileum and colon and there are indications that FFAR2 is the sole functional receptor for SCFAs on neutrophils. SCFA-FFAR2 interaction affects inflammatory responses and FFAR2 expression is closely regulated with receptors important for innate immunity, such as TLR2, TLR4 and CXCR2.

3.3.4 Where in the GI tract do the prebiotic effects take place ?

Prebiotic effect of inulin-type fructans in rodents and humans are well documented. But studies investigating changes in the intestinal microbiota, are often limited to feces or cecum contents. It is unclear whether the growth promotion effects only take place in the large intestine.
It is claimed that transit of residual foods through the stomach and small intestine is probably too rapid for the microbiota to exert a significant impact, which slows markedly in the colon. Due to the high residence time of colonic contents, as well as a diverse and profuse flora, the colonic microbiota is thought to play a more important role in the host health and well-being than is the case in the small intestine (Roberfroid, M. et al., 2010). Studies investigating the prebiotic effects throughout the GI tract are only few, but as the small intestine is long, has a large surface and a large amount of PP, the small intestine is an important place to strengthen the immune system and to investigate the prebiotic effects. Only one study investigating XOS was found, for which reason other prebiotics will be mentioned in this part, to illustrate where in the GI tract the prebiotic effects take place.

Moura, P. *et al*. (2008) compared the *in vitro* fermentation of XOS with different degree of polymerization (DP) by the intestinal digesta collected in ileum, caecum and distal colon of a piglet GI tract. The studied XOS were commercial short-chain XOS (DP 2-5, commercial XOS, comprising mainly xylobiose and xylotriose), medium-chain (DP 2-14, from corn cobs, and brewery's spent grain) and long-chain (DP 2-25, from eucalyptus wood). They measured populations of *Bacteroides/Prevotella, Bifodobacterium* and *Lactobacillus/Pediococcus* and concentrations of SCFAs. The decline of XOS concentration in the growth media and the increased SCFAs throughout the fermentation were indicative of extensive microbial fermentation by all inocula.
All tested XOS were fermented by the piglet ileal, caecum and colonic microbiota but fermentation of medium- and long-chain XOS was reduced by the ileal inoculum as compared to short-chain XOS, showing a lower fermentation efficiency of the small intestine microbiota towards longer molecules of XOS. All 3 XOS supported an enhancement of *Bifidobacterium* and *Lactobacillus* growth in the beginning of the fermentation. Growth of *Bacteroides/Prevotella* enhanced later on in the fermentation, so the stimulation was not selective for *Bifidobacterium* and *Lactobacillus.*
The inocula from caecum and colon fermented XOS faster and to a greater intent than the ileal inoculum. Acetate and butyrate were the major fermentation end-products, and acetate was detected in all sampling times. The reason that the long-chain XOS are not fermented efficiently in the small intestine could be because of the rapid transit time and because the population and variant of bacteria is

lower than in the large intestine.

Patterson, J. *et al.* (2010) determined the presence of luminal and adherent bacterial populations from 6 segments of the small and large intestine (jejunum, ileum, cecum, proximal colon, mid colon and distal colon) of pigson diets with short-chain inulin, long-chain inulin or a 50:50 mixture of both.
All 3 types of inulin enhanced the presence of adherent *Bifidobacterium* and *Lactobacillus* in the intestinal mucus of various gut segments. Changes were seen in the jejunum with short-chain inulin but did not appear until the distal ileum or cecum with long-chain inulin. They found similar effects of inulin on bacterial populations in the lumen contents, but inulin affects the growth of the adherent bacteria stronger and more consistently than in the luminal populations. All 3 types of inulin suppressed *Clostridium* and members of the *Enterobacteriaceae* in the lumen and mucosa of various gut segments. In conclusion all 3 types of inulin promoted a favorable intestinal microbial balance. Short-chain inulin seemed to be fermented to some extent in the jejunum and ileum by the resident microflora that were composed of more aerotolerant species such as *Lactobacillus*. In contrast, long-chain inulin was not degraded until reaching the distal ileum or the cecum where the bacterial population shift to more anaerobic species such as *Bifidobacterium*, *Bacteroides* and *Clostridium* that can contribute to the complete fermentation of the prebiotic in this region.
In an earlier study (Yasuda, K. *et al.*, 2009), the same group found that all 3 types of inulin decreased the mRNA level of the pro-inflammatory cytokine TNF in the intestinal mucosal tissues. These effects were more pronounced in the lower than the upper gut.
TNF is produced in response to LPS present in the outer membrane of Gram-negative bacteria. Inulin enhanced the abundance of Gram-positive bacteria. Because cell walls of Gram-positive bacteria do not contain LPS, there may be less exposure to these stimuli, resulting in the observed down-regulation of inflammatory-related genes.
Inulin is not hydrolyzed by mammalian digestive enzymes, but significant degradation of inulin was occurred in the ileum, indicating a bacterial fermentation of inulin in the upper part of the small intestine. No inulin was recovered in segments distal to the ileum in pigs fed diets containing supplemental inulin.

Smiricky-Tjardes, M. R. *et al*. (2003) evaluated TOS addition on swine ileal and fecal bacteria populations, and ileal SCFAs production. Feces and ileal digesta were analyzed for *Bifidobacterium* and *Lactobacillus* populations. Ileal digesta samples were analyzed for SCFAs.
TOS increased concentrations of *Bifidobacterium* and *Lactobacillus* and concentrations of SCFA. Fecal amount of *Bifidobacterium* and *Lactobacillus* were increased, more pronounced for *Bifidobacterium* than for *Lactobacillus*. There were no significant differences in ileal bacteria population.
Ileal digestibility was 100 % for pigs consuming the TOS diet. Ileal propionate and butyrate concentration was greater for pigs fed TOS, but no affect on acetate concentrations in ileal effluent was seen. The authors suggested that the value of SCFA in the ileal digesta indicates that fermentation of TOS starts before the large intestine, often considered as the only site of fermentation of oligosaccharides. They referred to a study by Houdijk (1998) which indicates that oligosaccharides fermentation might actually start as early as the stomach. That study reported lower pH values of gastric contents when 1,5 % TOS was fed to pigs.

Loh, G. *et al*. (2006) assessed the effect of inulin on the intestinal microbiota by fluorescent *in situ* hybridization in growing pigs. They found that inulin affected intestinal SCFA, and enhanced the number of pigs with colonic bifidobacteria. Up to 50 % of IN was degraded in the jejunum, with lactate as the main fermentation product followed by acetate. In colon acetate was the major bacterial metabolite followed by propionate and butyrate, with lactate scarcely detected.

Kaji, I. *et al*. (2011) Investigated the effects of supplementation of FOS on the density distribution of FFAR2-expressing and GLP-1- producing enteroendocrine L cell in colon, cecum and terminal ileum of rats. The FFAR2-positive enteroendocrine cells were quantified immunohistochemically. The same analysis was performed in surgical specimens from human lower intestine. They hypothesized that SCFAs produced by bacterial fermentation are involved in enteroendocrine cell proliferation through FFAR2 in the large intestine.
For the control group the density of the FFAR2-positive enteroendocrine cells showed a statistically significant increasing gradient across the three segments of the colon, from the proximal to the distal colon. The value for the terminal ileum was intermediate between the values for the middle and distal colons. For the FOS group the densities of FFAR2- positive enteroendocrine cells were significantly increased by two-fold in the proximal colon leading to a suspension of the increasing gradient seeing in the control group. In terminal ileum the value of FFAR2 was increased to a level a little lower than the

proximal colon. In cecum no changes was seen when treated with FOS.
The authors suggested that the fermentation and absorption of FOS is complete in the proximal colon, and therefore the increased densities of FFAR2- positive enteroendocrine L-cells is restricted to the proximal colon, where the concentration of luminal SCFAs is high.
Their results indicated that expression of FFAR2 is inducible by ligand-stimulation.

In summary, prebiotic affects the intestinal microflora. It seems that XOS and other oligosaccharides can be fermented all over the GI tract, leading to changes in microbial composition and SCFA production. In the proximal part of the GI tract it is mostly the short-chain oligosaccharides which are fermented, and further throughout the gut medium- and longer chain (more complex) oligosaccharides are fermented.
XOS supported an enhancement of *Bifidobacterium* and *Lactobacillus* growth, but this stimulation was not selective, as growth of *Bacteroides/Prevotella* increased too. Acetate and butyrate was the major fermentation end-products. Prebiotic enhanced the abundance of *Bifidobacterium* and *Lactobacilllus* and suppressed the growth of *Clostridium* and *Enterobacteriaceae* which favorable intestinal microbial balance.
Prebiotic lead to a decrease in the mRNA level of TNF, with more pronounced effect in colon than the small intestine. Prebiotic affected intestinal SCFA, with lactate and acetate as the main fermented products in the small intestine, and acetate followed by propionate and butyrate in the colon, and SCFAs affects immune cells functions in the small intestine.
The density of FFAR2-positive enteroendocrine cells showed a statistically significant increased gradient across the colon, from the proximal to the distal colon. When treated with prebiotic the densities of FFAR2- positive enteroendocrine cells were significantly increased by two-fold in the proximal colon leading to a suspension of the increasing gradient. Prebiotic increased the FFAR2 expression in the terminal ileum and proximal colon.

4 Food and prebiotics

Several prebiotics are found in vegetables and fruits. Food however, contain only trace level of prebiotics, so developments have been taken to approach removing of the active ingredients and adding them to more frequently consumed products in order to attain levels, whereby a prebiotic effect may occur (Roberfroid, M. et al., 2010).

Oligosaccharides are linear or branched carbohydrates consisting primarily of 2-20 sugar units. From a nutritional point of view, oligomeric structures with DP up to 50 are defined as resistant short- chain carbohydrates which cannot be hydrolyzed by mammalian endogenous enzymes of the small intestine (Englyst, K. N. & Englyst, H. N., 2005).

Oligosaccharides are present in human breast milk and can be found in concentrations up to 12g/ liter comprising the third largest component of breast milk (Newburg, D.S. *et al*, 2004).

As mentioned earlier, XOS are found naturally in bamboo shoots, fruits, vegetables, milk and honey (Casci, T. *et al*. ,2006).

4.1 Incorporating of prebiotics in food

Some examples of products that have taken advantage of prebiotics are: Functional beverages, cereals with an increased amount of dietary fibers, infant food, nutraceuticals, where prebiotics are made into tablets or capsules (some also containing probiotics) and weight management products where sugar is replaced by prebiotics (Thammarutwasik, P. *et al*., 2009).

Special care must be taken when using prebiotics as ingredients in food products. The compatibility of these ingredients with the products in terms of physical (particle size, solubility, viscosity), sensory (color, taste, flavor) and nutritional (health benefits, potential hazards, dosage, stability) must be well understood. But properly used they may be an additional tool to combat diseases and to improve the health and well being.

In food, prebiotic can change organoleptic characteristics and upgrading sensory properties as taste and mouth feel. Oligosaccharides have mouth feel similar to that of table sugar but only 20-70 % of sweetness (Wu, Y. & Lin, K., 2011). Long-chain inulin has a fat-like mouthfeel, which has been reported to have a successful benefit in replacing fat in baked goods, table spread, fillings, dressing, and frozen dessert. Therefore inulin could be an attractive fat mimetic as it improves mouthfeel in most food systems (Arayana, K. J. *et al*., 2007).

Sensory attributes can have different levels of importance depending upon the type of food. Texture makes a significant contribution to the overall food quality, contributing with both flavor and

appearance.

Only one study was found incorporating XOS in food, for which reason other oligosaccharides will also be mentioned in this part.

4.1.1 Meat product

In meat products which are cooked and frozen, it is important to protect protein from denaturation. There has been demonstrated a protective effect of XOS on myofibrillar protein gels during heating and frozen conditions. Wu, Y. & Lin, KI. (2011) investigated the quality attributes of chinese-style meat-ball (kung-wan) with partial substitution of sucrose with XOS. Kung-wan is an emulsified meat product which has been water-cooked and stored at -18 °C or below. The textural quality is paramount in Chinese meatballs. Various combinations of XOS and sucrose were investigated. Sensory springiness, hardness, chewiness, and overall acceptability of all treatments were not found significantly different. Water- soluble protein and total extractable protein concentrations of XOS 4% containing treatments were higher than other treatments at any frozen storage period.

In summary addition of XOS or sucrose single or in combination resulted in meatballs with comparable quality.

4.1.2 Dairy products

Milk and dairy products have been at the forefront in the functional food area and continue to be the main vehicle for administration of probiotics and prebiotics through the diet.

Yoghurt

Arayana, K.J. e*t al* (2007) found that short-chain inulin containing yoghurt had a significantly lower pH than remaining yoghurt and higher flavor scores than the control. The yoghurt containing long-chain inulin had a better body and texture. Inulins of various chain lengths did not affect viscosity, color and product appearance. Taken together, prebiotics affected some quality attributes of probiotic yoghurts. Allgeyer, L.C. e*t al*. (2010) found that for overall acceptance inulin was liked most (compared to soluble corn fiber and polydextrose), they were characterized by a medium level of sweetness and high viscosity. From this study it was determined that a more viscous yoghurt drink with medium sweetness was preferred by the consumers. Such products can be achieved through the addition of prebiotics. Results from Arcia, P. L: *et al.* (2010) indicated that flow and viscoelastic parameters varied among samples depending on inulin and sucrose concentration. For liquid and semisolid products, thickness is the textural attribute that most influence consumer response, though smoothness and creaminess are also influential. Long-chain inulin can modify texture because it crystallizes in presence of water forming particle network structure. Consequently long-chain inulin has been used in low-fat products to

improve creaminess and consistency, mimicking those of full-fat products.

Gonzalez, N. J. *et al*. (2011) determined the effect of FOS. Data indicated that the differences between the samples were either because of the fat content or the presence of synbiotics and prebiotics. Yoghurt with prebiotic was not significantly different from controls, indicating that prebiotics can be added without impacting acceptance. Samples containing the synbiotic had a negative impact on acceptance. The sour aroma was higher in the samples containing FOS. The overall results showed that whole milk drink containing FOS was the most acceptable sample.

Castro F. P. (2008) found that addition of oligofructose in fermented lactic beverage was preferred in relation to control, but did not adversely alter their sensory acceptability. Also Staffolo, M. D. (2004) found that the sensory properties for yoghurt containing inulin were not significantly different from the control. On the other hand Guven M. *et al.* (2005) found that organoleptic quality of yoghurt decreased when increasing inulin concentration.

Dessert

Criscio T. D. *et al.* (2010) investigated effects of inulin in ice cream. Most of the ice creams showed good nutritional and sensory properties, but 10 % inulin altered the sensory and physical properties to much. The best results were obtained with 2,5 % inulin. The addition of inulin altered the texture of the ice cream. It could be related to changes in freezing points because of higher solute concentrations together with the gelling properties of inulin and the increased water binding, which improve viscosity and modify the rheology.

Tarrega, A. *et al.* (2010) assessed to what extent low-fat custards were affected by adding different blends of two inulins with different average chain length (short and long). They found that inulin blends enhanced product flavor intensity and thickness. The use of 50:50 blend, which affords certain nutritional advantages, could also act as a fat replacer. Inulin addition increased both sweetness and vanilla intensity, most remarkable in samples with high amount of short-chain inulin. In general the low-fat samples were perceived to have significantly more flavor and greater sweetness intensity than the full-fat control samples.

Cardarelli, H. R. *et al*. (2008) evaluated the effect of inulin on a functional chocolate mousse during storage at 4°C for up to 28 days. The addition of prebiotic resulted in a firmer and more adhesive (adhesiveness is defined as the force required to remove the material that adheres to the mouth during the normal eating process) chocolate mousse. This effect was intensified with the presence of inulin. Taken together, the use of inulin was advantageous, conferring to favorable texture and sensory properties.

In summary prebiotics affected some quality attributes of yoghurts. Short-chain inulin lowered pH and increased sweetness flavor. Long-chain inulin enhanced body texture. The prebiotic did not affect color and product appearance. Prebiotics can be used as fat replacement, as the creaminess is improved. Products containing prebiotics did not differ a lot from controls, meaning that prebiotics can be added without impacting acceptance, but higher concentrations of inulin decrease organonoleptic quality.
In ice cream inulin affected texture. In low-fat custards prebiotic enhanced thichness and flavor intensity as short-chain inulin increases sweetness and vanilla. In chocolate mousse prebiotics gave a more adhesive mousse and favorable texture and sensory properties.

4.1.3 Cereals

Insoluble fiber ingredients, such as bran, have traditionally been used in products such as cereal bars, breads and breakfast cereals, but the palatability has limited the level that can be incorporated into different systems. Soluble fiber ingredients are currently of greater interest in the formulation of healthy foods, because they are more palatable. In addition, some can be used in food to add viscosity (Dutcosky, S. D. *et al*., 2006). XOS can function as water-soluble fiber (Manisseri, C. & Gudipati, M., 2010)

Inulin, oligofructose and gum acacia are soluble fibers. They offer a variety of technological functional properties as water retention, enhanced viscosity for improving binding and texture, stability at different temperature levels and a stable pH.
Dutcosky, S. D. et al. (2006) wanted to develop tasty cereal bars with prebiotic properties. Inulin , oligofructose and gum acacia were the ingredients added. Improved effects were found on dryness appearance, hardness, chewiness, rightness, sweetness and crunchiness with added prebiotics. The added fibers influenced more the texture and appearance than the aroma and flavor. The optimized formulations showed, that blends of fibers imparted to the bars showed better textural characteristics than each fiber alone. The selected formulations aimed at reduction of 40% sugar and 18-20% caloric value, while providing an average increase of 200% in total fiber.

Bread is a solid-like porous matrix whose quality is multifactor dependent.
In a study by Angioloni, A. and Collar, C. (2011) locust bean gum and carboxymethylcellulose singly and in binary with FOS and GOS were used to replace wheat flour at a 10 % substitution level in bread formulation. The largest particle size was observed for GOS, while FOS was the smallest one. Dietary fibers with larger particle size resulted in highly sensory acceptable breads. Fibers exhibiting high viscoelasticity and complex viscosity in concentrated solutions yielded breads with better sensory perception. Overall acceptability depended more on organoleptic characteristics than on visual characteristics. Few sensory properties (firmness and overall acceptability) and most nutritional bread properties were found to depend on dietary fiber molecular characteristics.

Taylor, T. P. *et al.* (2008) replaced sucrose by tagatose in cookies at various levels from 25-100%. The tagatose containing cookies were harder and darker with a lower spread than the control. The brown color of the 100 % tagatose cookies was liked better than the control's, but their sweetness was disliked. When sucrose was partially replaced with tagatose, a softening effect on the cookie dough was generally observed. They found that tagatose appears to be suitable as partial replacement for sucrose. Using tagatose to replace sucrose in cookies would reduce the amount of metabolizeable sugars in the diet as well as providing the desirable prebiotic effect.

In summary different kinds of soluble fibers altered dryness appearance, hardness, chewiness, brightness, sweetness and crunchiness in cereal bars. The optimal blend of soluble fibers gave a better texture, reduced sugar content and calorie value and increased fiber content. In bread, prebiotics could partial replace wheat flour. Prebiotics with large particle size resulted in higher sensory acceptable breads. Prebiotics can influence viscosity, leading to better sensory perception and texture features. In cookies prebiotic lead to a harder, darker and more sweet cookie, and prebiotic could be suitable as partial replacement of sucrose.

5 Materials and Methods

5.1 Experimental design

The animals used were 24 B6 mice C57BL/6NTac (Taconic, Lille Skensved, Denmark), all males and 3½ weeks old upon arrival. They were split into two groups – 12 mice in each, 1 group got normal feed and 1 group got feed with XOS (appendix 13). The Feed was from Altromin in Holland, and the test feed was added 10 % XOS, Dp 2-6 (Danisco Health & Nutrition, Kantwik, Finland). The 6 mice were co housed. Diet period was 10 weeks with feed ad libitum, and the mice were killed when 14 weeks old. When killed mice were weighed and stool samples were collected if possible, then the mice were anesthetized and killed by cervical dislocation. Then immediate sampling was done.

Gene expressions of Tnf-α, Cxcl1,Cxcl2, Tlr2, Tlr4, Cd36, Ffar2, RegIIIg and the housekeeper gene β-actin, were measured in epithelial cells from duodenum, ileum and colon. In blood gene expression of Il-1β and again β-actinin were measured.

Bacterial content were measured in tissue from duodenum, ileum, and colon and from cecum content and stool. The bacteria measured were *Bacteriodetes* phylym, *Firmicutes* phylym, *Lactobacillus* and *Bifidobacterium*.

5.2 Sample collection

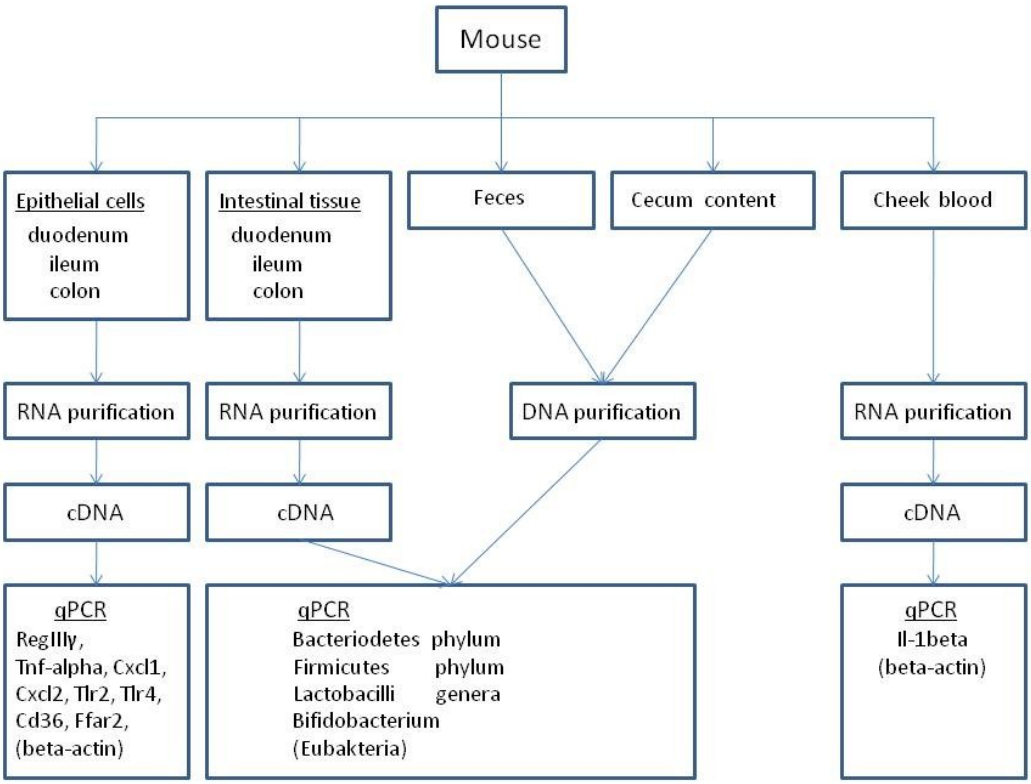


Figure 5.1 Overview of sampling collections and methods

5.2.1 Blood

Blood from the cheek was collected in epp. tubes for gene expression. RNA was isolated, then synthesized of cDNA and finally gene expression were quantified by qPCR (figure 5.1).

5.2.2 Intestinal: duodenum, ileum, colon

Immediately after killing samples were collected as following (figure 5.1): The first 4 cm of the duodenum after the stomach, the last 4 cm of ileum before cecum and the first 4 cm of colon after the cecum (figur 5.2).

½-1 cm of each intestinal segment was placed in RNA-later for bacteria profile, and stored in -20°C until RNA isolation. The samples were homogenized, RNA isolated then synthesized of cDNA, and finally bacterial profile was quantified by qPCR.

The rest of the 4 cm of the intestinal segments were cleaned and cut for measuring the gene expression. Immediately after, epithelial cells were purified. RNA isolated, then synthesized of cDNA, and finally gene expression was quantified by qPCR.

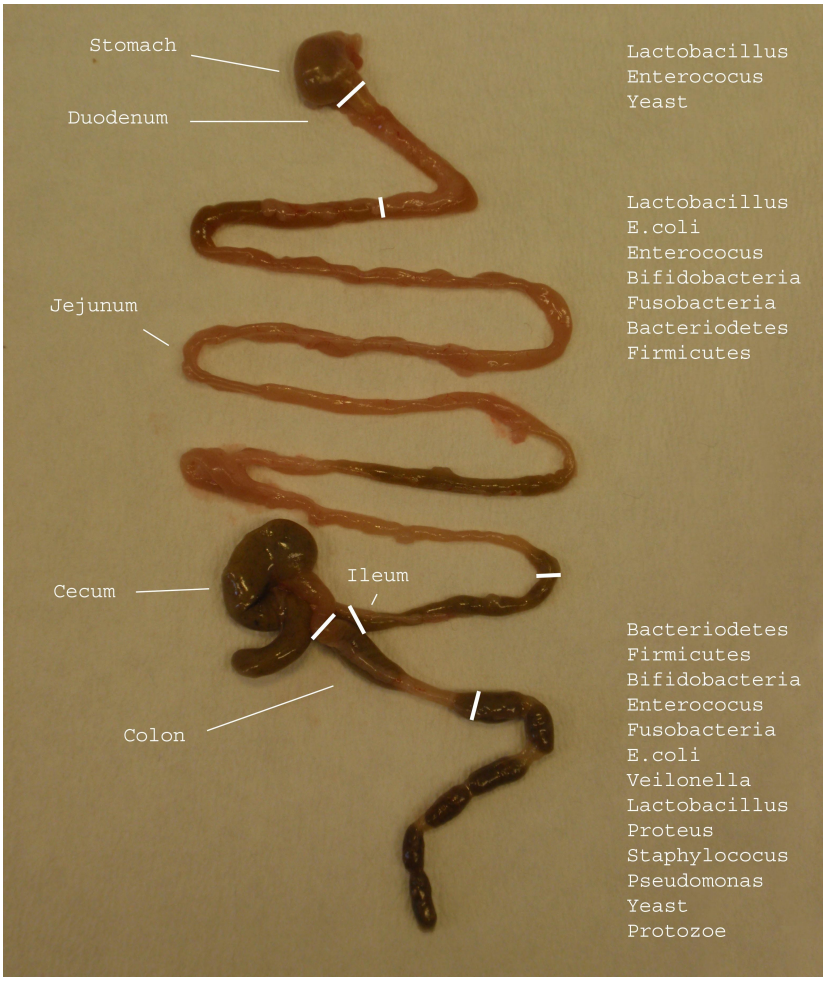


Figure 5.2 GI tract of a mouse. The white marks indicate where the samples of 4 cm have been taken.

5.2.3 Stool/cecum contents

Stool and cecum content were collected immediately after killing the mice for measuring of bacterial profile. The samples were stored at -80°C until DNA isolation, and finally bacterial profile was quantified by qPCR (figure 5.1).

5.3 Purification of total DNA from stool and cecum contents

5.3.1 Qiagen DNA Stool Mini kit 50 cat no:51504.

This kit is designed for rapid purification of total DNA. The procedure comprised of following steps: Lysis of stool samples in buffer ASL, absorption of impurities to InhibitorEX matrix, and purification of DNA on QIAamp Mini spin column.

Protein was digested and degraded under denaturing conditions during 70°C. incubated with proteinase K. Buffering conditions were then adjusted to allow optimal binding of DNA to QIAamp membrane, and the sample was loaded onto the QIAamp spin column. DNA was absorbed onto the membrane during a brief centrifugation step. Optimized salt concentration and pH conditions in the lysate ensure that remains of impurities, which could inhibit PCR, were not retained on the membrane. DNA bound to the membrane was washed in two centrifugation steps, ensuring complete removal of any residual impurities without affecting DNA binding. Purified concentrated DNA was eluted from the membrane in low-salt buffer. The eluted DNA was suitable for direct use in PCR. All centrifugation steps should be carried out at room temperature(QIAamp DNA stool Handbook, 2010).

5.3.2 Protocol (for more details see appendix 1)

Lysis of samples: 200 mg stool/cecum content was weighed and added 800 µl Tebuffer 10:1, then centrifuged and pellet was discarded in 1,2 ml ASL buffer. SDS treatment: 500 µl Zirconia beads and 30 µl SDS were added and shaken on high speed. Then centrifuged and pellet was used for further treatment. Absorption of impurities:1 inhibitorEX tablet was added and incubate, 1 min at room temperature. Centrifuged 2 times. 200 µl supernatant, 200 µl AL buffer and 15 µl Proteinase K were mixed and incubated in 70°C for 10 min. 200 µl 96 % ethanol was added. Purification of DNA on QIAamp Mini colums: The lysate was applied to the column, then centrifuged, and filtrate was discarded , 500 µl wash solution was added, then centrifuged, and filtrate was discarded, 500 µl wash solution was added, then centrifuged, and filtrate was discarded, and 200 µl AE buffer was added to the column, incubated at room temperature and centrifuged to elute DNA. Purified DNA was stored at –20°C.

5.4 Purification of total RNA from blood

5.4.1 MagMAX-96 Blood RNA Isolation Kit Part no: AM 1837

This kit is designed for rapid isolation of total and viral RNA from mammalian whole blood and milk in 96-well plates. After preparation and homogenization samples were mixed with magnetic beads that have a nucleic acid binding surface. The beads and the bound nucleic acid were then magnetically captured and washed to remove cell debris, protein, and other contaminants. Then the nucleic acids were treated with DNase and purified from the reaction mixture. Finally RNA was eluted in 50 µl of low salt buffer (MagMAX-96 Blood RNA Isolation Kit instruction manual, 2008).

5.4.2 Preparation of blood samples

20 µl blood was placed into epp. tube with 30 µl PBS (Appendix 2), mixed and added 10 µl Lysis/binding Enhancer. Incubated at room temperature on a shaking board for 10 min.

5.4.3 Protocol KingFisher MagMAX-96 Blood RNA Isolation Procedure Overview

(for more details see appendix 1)

Pipet MagMAX-96 Blood RNA Isolation Kit reagents into a KingFisher 200 µl plate. To each well in *row A:* 130 µl Lysis/bindings Solution, 60 µl sample, 20 µl Beads mix. *Row B*:150 µl Wash, Solution 1. *Row C*: 150 µl Wash Solution 2. *Row D*: 50 µl Diluted TURBO Dnase (Appendix 2).*Row E*: 150 µl Wash Solution 1. *Row F*: 150 µl Wash Solution 2, and finally r*ow G*: 50 µl Elution Buffer. Insert the plate into the KingFisher 96 machine and use program 1837 (see below). After the Dnase treatment 130 µl of rebinding solution is added to each well in row D before the program is continued. The purified RNA is then transferred to epp. tube and stored at -20°C.

5.4.4 MagMAX Total program 1837

A: Lysis Binding, 5 min.
B: 1st Wash 1, 1 min.
C: 1st Wash 2, 1 min.
C: Dry, 30 sec.
D: Turbo Dnase, 5 min.
D: Pause
D: Rebind RNA, 3 min.
E: 1st Wash 2, 30 sec.
F: 2nd Wash 2, 30 sec.
F: Dry, 1 min.
G: Elution, 3 min.

5.5 Preparation of intestinal epithelial cells from duodenum, ileum and colon

Epithelial-cells are connected by junctional complexes, the tight junction. Calcium is an important component in the junction bindings. When treating the epithelial-cells with EDTA, which binds calcium, epithelial-cells are loosened from the mucosa and become single cells. After EDTA treatment the mucosa / epithelial-cells mix is placed on a filter, and the single epithelial-cells are filtered throughout the filter, while the rest of the mucosa is staying on top of the filter.

5.5.1 Protocol(for more details see appendix 1)

4 cm duodenum, ileum or colon is placed in HBSS (Appendix 2) in a petri dish (on ice). The intestinal pieces are cut open lengthwise, cleaned for contents, and cut into small pieces (on ice). The intestinal pieces are transferred into tubes with 10 ml 37°C HBSS with 2 mM EDTA (Appendix 2). They are shaken vigorously and placed at a 37°C water-bath in 10 min – shaken vigorously every 3-4 min. Then the intestinal suspensions are transferred for filtering to a 70µm filter place on a tube. 15 ml cold PBS (Appendix 2) is added onto the filter and the tube are filled up to 40 ml with cold PBS. From here cells are kept on ice. Centrifuge and pellet are re suspended in 1 ml cold PBS. Centrifuge and the pellet is re-suspended in 300µl lysisbuffer (MacMAX-96 RNA Isolation Kit Am1830) with 0,5 µl β-mercaptoethanol (14,3 M.) From here cells are kept at room temperature. Vortex until homogenized and samples are ready for RNA purification.

5.6 Preparation and homogenization of tissue from duodenum, ileum and colon

FastPreb treating is a mechanic homogenization which smashes the tissue. The tissue is added lysisbuffer, which inhibited the RNAses and lysis the cells.

5.6.1 Protocol (for more details see appendix 1)

0,3-0,5 g glass beads are transferred to a FastPrep tube. 300 µl lysis buffer (MacMAX-96 RNA Isolation Kit Am1830) with 0,5 µl β-mercaptoethanol (14,3 M.) is added. The intestinal tissues from RNAlater are transferred to a piece of paper to remove RNAlater crystals, and the intestinal tissues are added to lysis buffer. Samples are shaken in FastPrep machine (FP120, Bio 101, Thermo Savant) at speed 6,5 in 45 second and then centrifuged. *For duodenum and ileum*: 100µl sample are transferred into 60µl isopropanol (in a 96-well plate, row A). *For colon*: 150µl sample are transferred into 90µl isopropanol (in a 96-well plate, row A).Then the procedure for KingFisher MagMax-96 Total RNA Isolation Protocol is followed.

5.7 Purification of total RNA from intestinal tract

5.7.1 MagMAX-96 Total RNA Isolation Kit cat no: AM1830

This kit is designed for rapid purification of total RNA in 96-well plates. After preparation and homogenization samples are mixed with magnetic beads that have a nucleic acid binding surface. The beads and the bound nucleic acid are then magnetically captured and washed to remove cell debris, protein, and other contaminants. Then the nucleic acids are treated with DNase and purified from the reaction mixture. Finally RNA is eluted in 50 µl of low salt buffer (MagMAX-96 Total RNA Isolation Kit instruction manual, 2007).

5.7.2 Protocol: KingFisher MagMax-96 Total RNA Isolation Protocol Overview

(for more details see appendix 4)

The KingFisher processors automate the nucleic acid isolation.

Pipet MagMAX-96 Total RNA Isolation Kit reagents into a KingFisher 200 µl plate. To each well in *row A:* 300 µl samples and 20 µl Beads mix. *Row B*:150 µl Wash, Solution 1. *Row C*: 150 µl Wash Solution 2. *Row D*: 50 µl Diluted TURBO Dnase (Appendix). *Row E*: 150 µl Wash Solution 1. *Row F*: 150 µl Wash Solution 2, and finally *row G*: 50 µl Elution Buffer. Insert the plate into the KingFisher 96 machine and use program 1830 (see below). After the Dnase treatment 100 µl of rebinding solution is added to each well in row D before the program is continued. The purified RNA is transferred to epp. tube and stored at -20°C.

5.7.3 MagMAX Total program 1830

A: Lysis Binding, 5 min.
B: 1st Wash 1, 30 sec.
C: 1st Wash 2, 30 sec.
D: Turbo Dnase, 10 min.
D: Pause
D: Rebind RNA, 3 min.
E: 1st Wash 2, 30 sec.
F: 2nd Wash 2, 30 sec.
F: Dry, 1 min.
G: Elution, 3 min.

5.8 Assessing RNA and DNA yield and purification

(for more details see appendix 1)

The yield and purification of RNA or DNA were measured by using a NanoDrop Thermo Scientific NanoDrop 2000, Spectrophotometer (RNA) or NanoDrop, ND-1000 Spectrophotometer (DNA). The technology combines fiber optics and surface tension to hold and measure small amounts of sample. 2

µl droplet is loaded onto the optical pedestal, and the sample is drawn into a column and measured (http://nanodrop.com/Default.aspx). The Nanodrop scans absorbances from 200 nm to 350 nm to asses both the concentration of nucleic acids as well as the purity of the sample. Nucleic acid has an absorption maximum at 260 nm (http://www.biomedicalgenomics.org/RNA_quality_control.html)

5.9 Complementary DNA synthesis

Reference: "High-Capacity cDNA Reverse Transcription Kits For 200 and 1000 reactions", protocol, AB Applied Biosystems.

Complementary DNA (cDNA) is synthesized as single stranded DNA from the extracted RNA in a reaction catalyzed by the enzymes' reverse transcriptase and by using random hexamer primers.

5.9.1 High-Capacity Reverse Transcribtase kit, part. no: 4368814

This kit synthesize single-stranded cDNA from total RNA, using random hexaminer primers and a reaction volume of 20 µl.

5.9.2 Protocol (appendix 5)

About 500 ng of total RNA per 20 µl reaction were used.

Preparation of RT Master mix: Per sample: 2,0 µl 10 x RT buffer, 0,8 µl 25 x dNTP mix, 2,0 µl 10 x Random Primers, 1,0 µl MultiScribe RT, 0,2 µl Nuclease-free H_2O. Kept on ice until use.

A 200µl PCR tube per sample is prepared with 6 µl RT Master mix and 14 µl template, starting with water, then RT Master Mix and finally total RNA solution.

Reverse transcription is performed in a GeneAMP PCR System 9700

Program:

Step	Temp	Time
1	25°C	10 min. - primer annealing
2	37°C	120 min.- enzyme activities
3	85°C	5 sec. - heat inactivating
4	4°C	∞

Table 5.1 Program for the cDNA synthesis

After cDNA synthesize, samples are diluted with nuclease-free H_2O to a cDNA concentration at 3ng/µl. The cDNA is now suitable for quantitative PCR applications. Store at -20°C until use.

5.10 Quantitative PCR (qPCR)/ gene expression

References:

Bergström, A. (2011), Real-Time PCR Vs. Traditional PCR, Applied Biosystems Part Number 431001 Rev. A, TagMan One-Step RT-PCR Master Mix Reagents Kit, Applied Biosystems, Part Number 4310299 Rev. E, (2010), Krabbe I. D. (2007).

Real-time PCR or quantitative PCR (qPCR is the name used in the rest of the study) is designed to collect data as the reaction is proceeding. qPCR detects PCR amplification during the early (exponential) phase of the reaction, and can detect as little as a two-fold change.
A basic PCR amplification have three phases – exponential,linear and plateau (figure 5.3) In the exponential phase there is a doubling of the product, accumulating at every cycle, assuming very specific and precise reaction with 100 % reaction efficiency. In the linear phase the reaction components are being consumed, the reaction is slowing down, and maybe products have started degradation. The depletion of reagents will occur at different rates for each replicate. In the last phase, the plateau phase, the reaction has stopped and no more products are being made. Each reaction will plateau at a different point, due to the different reaction kinetics for each sample.

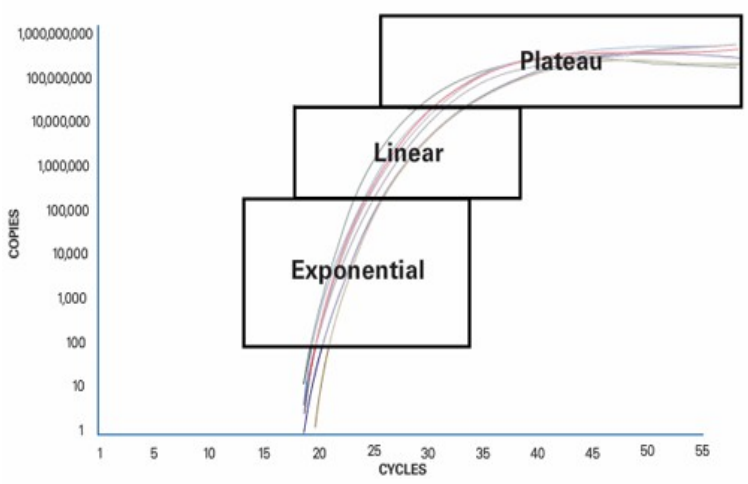


Figure 5.3 The three phases during PCR amplification – the exponential phase, the linear phase and the plateau phase. http://www.appliedbiosystems.com/absite/us/en/home/applications-technologies/real-time-pcr/real-time-pcr-vs-traditional-pcr.html?ICID=EDI-Lrn2

During the exponential phase two values are important for further analysis – the threshold line and the cycle threshold (C_T) (figure 5.4). The threshold line is the level of detection, or the point at which a reaction reaches a fluorescent intensity above the background (see below). The threshold line is set in the exponential phase of the amplification for the most accurate reading. The cycle at which the sample

reaches this level is called the cycle threshold (C_T). C_T is inversed proportional to the logarithm of the concentration of DNA at the reaction's start.

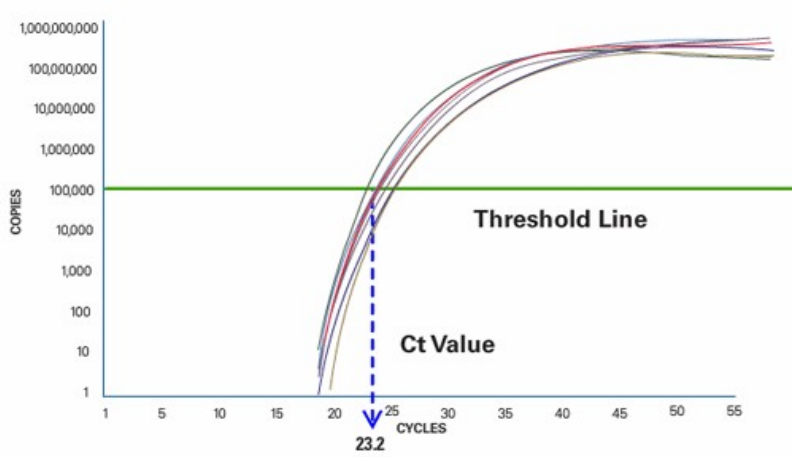


Figure 5.4 C_T value and threshold line during the exponential phase. The PCR cycle at which the sample reaches a fluorescent intensity above background is the Cycle Threshold or Ct http://www.appliedbiosystems.com/absite/us/en/home/applications-technologies/real-time-pcr/real-time-pcr-vs-traditional-pcr.html?ICID=EDI-Lrn2

PCR can be used for quantitative as well as qualitative analysis. A qualitative analysis can determine whether or not a specific DNA sequence is present in the sample (yes/no answer). A quantitative analysis can measure DNA concentration in the sample at the start of the PCR reaction.
Quantification can be measured as absolute or relative. Absolute quantification is used when a precise concentration in the sample is wanted (not used in this study). Relative quantification is used to compare amplification level with a control.
C_T values for the controls and the samples are compared to determine if the unknown samples are up or down regulated in relation to the control sample (used in this study). The C_T value is used in a relative quantification by comparison to an reference sample (housekeeping gene).
A housekeeping gene is a gene which is known to have a constitutive expression level in the cell, and its expression is not affected by stimuli. β-actin is the housekeeping gene used in this study.
There is a quantitative relationship between the amount of starting sample and the amount of PCR product at any given cycle number. qPCR detects the accumulation of amplicons during the reaction, making quantification of DNA. The quantity of DNA theoretically doubles every cycle during the exponential phase, and relative amounts of DNA can be calculated.

In this study, qPCR exploits the 5'nuclease activity of DNA polymerase to cleave an oligonucleotide (TagMan probe) during PCR. The TagMan probe contains a reporter dye at the 5' end of the probe and a quencher dye at the 3' end of the probe. During the reaction, if the target of interest is present, the probe has been designed to specifically anneal between the forward and the reverse primer sites. When the enzyme reaches the annealed probe it starts to cleave it. Cleavage of the probe separates the reporter dye from the quencher dye, resulting in an increased fluorescence of the reporter due (figure 5.5). Accumulation of PCR products is detected directly by monitoring the increase in fluorescence of the reporter dye. The amount of reporter signal increases proportional to the amount of product being produced for a given sample. When the fluorescent signal reporter increases to a detectable level it can be captured and displayed as an amplification plot.

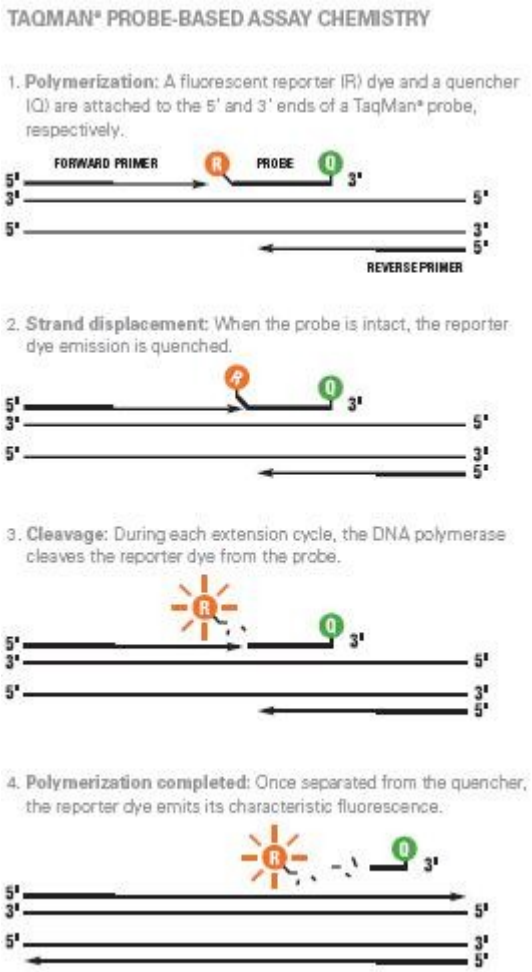


Figure 5.5 The TaqMan probe reactions (http://www.appliedbiosystems.com/absite/us/en/home.html)

The amplification plot contains information for the quantitative measurement of DNA. It is a plot of fluorescent signals versus cycle numbers. The higher the starting copy number of the nucleic acid target, the sooner a significant increase in the fluorescence is observed.

5.10. 1 Delta-Delta C_T quantification method (appendix 12)

Delta-Delta C_T method is an approximation method. Relative quantification RQ (fold increase) can be calculated by this comparative C_T method.

C_T expression is normalized to the expression of a reference gene (β-actin). For calculation of the ΔC_T value this formula is used: $\Delta C_T = C_T$ (target) – C_T (reference). This ensures normalization of the different samples independent of the amount of starting material.

Comparative gene expression is calculated as $\Delta\Delta C_T = \Delta C_T$ (target) – ΔC_T (control). In this study the ΔC_T (control) is mean ΔC_T from ileum without XOS. When an untreated sample is used as control sample, the RQ value is indicative of the gene fold change of the target gene caused by the XOS treatment compared to the untreated sample.

Fold changes values RQ = $(2^{-\Delta\Delta CT})$ is plotted.

TagMan Gene expression Assay (20X) from Applied Biosystem	
Genes	numbers
Actin-b	Mm 00607939_s1
Cd36	Mm 01135198_m1
Ffar2	Mm 02620654_s1
Tlr2	Mm 00442346_m1
Tlr4	Mm 00445273_m1
Tnf-alpha	Mm 00443258_m1
RegIIIγ	Mm 00441127_m1
Cxcl1	Mm 00433859_m1
Cxcl2	Mm 00436450_m1
Il-1ß	Mm 01336189_m1

Table 5.2 Primers for gen-expression.

5.10.2 Protocol (for more details see appendix 9)

Mix per sample: 0,5µl TagMan Gene expression Assay (20X), 5µl TagMan Fast Universal PCR Master Mix, No UNG (2X) and 2,5µl dest. nucelase free H_2O.

In a 96 well plate each well is loaded with: 8µl mix and 2µl sample. The plate is measured in the AB Applied Biosystems StepOnePlus Real-Time PCR system. Volume is set to 10µl, and the program as seen below (40 cycles). After 40 min. data is ready for analyzing.

5.10.3 QPCR cycling program:

Time	**Temp.**	
2 min.	95°C	
1 sec.	95°C	→↓
20 sec.	60°C	↑←

Table 5.3 QPCR cycling program for gen-expression

5.11 Quantitative PCR (qPCR)/ bacterial profile

References:

Real-Time PCR Vs. Traditional PCR, Applied Biosystems Part Number 431001 Rev. A,
Krabbe, I. D. (2007)
Bergström, A. (2011)

Bacterial qPCR is typically 16s rRNA quantification of levels and/or concentrations of specific bacteria (genera/phyla).

Here the fluorescent reporter molecule is the DNA binding dye SYBR Green. TagMan probe is specific to a pre-determined target, SYBR Green binds un-specifically to all double stranded DNA. It doesn't bind to single stranded DNA, and in solutions, free unbound SYBR Green only makes very little fluorescence. When SYBR Green binds to double stranded DNA, the intensity of the fluorescent emission increases about 1000 x. As more double stranded amplicons are produced , SYBR Green dye signal will increase in direct proportion to the DNA concentration (figure 5.6).

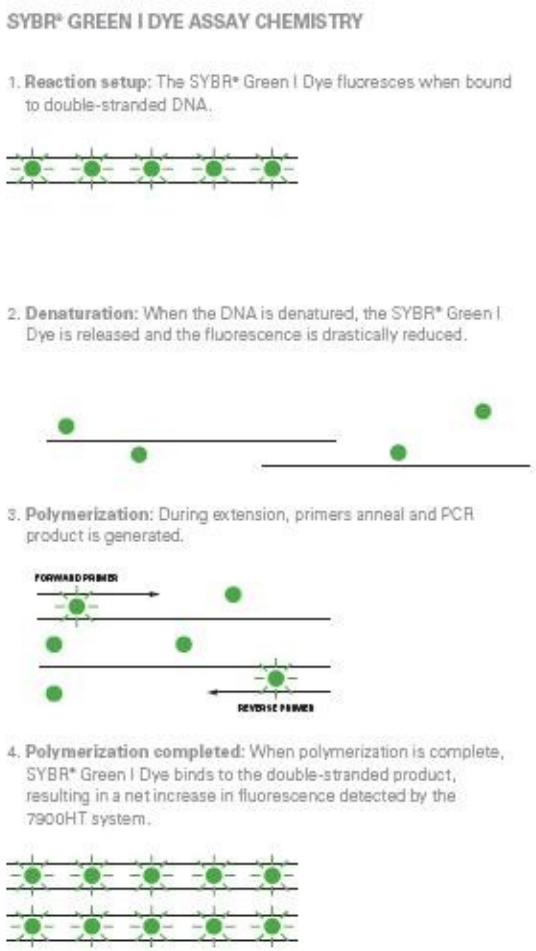


Figure 5.6 The SybrGreen reactions (http://www.appliedbiosystems.com/absite/us/en/home.html)

When using SYBR Green the limitation is, that it binds un-specifically to all DNA, and the specificity is determined exclusively by the primers. The temperature at which DNA is melting is determined by length of the molecule. If there are different lengths of the PCR amplicons, it can be shown in a Dissociation Curve, which shows if more than 1 PCR product is amplified. E.g. primer dimer (primer molecules that have attached hybridizes to each other because of strings of complementary bases in the primers) will have a melting point lower than PCR products, because they are normally shorter than amplicon. After the last cycle, a melting curve analysis of dsDNA is performed, making the Dissociation Curve after each run.

5.11.1 LinReg PCR quantification method (appendix 10)

References:
http://ebookbrowse.com/linregpcr-help-manual-v11-0-pdf-d18727191
Bergström, A. (2011)

LinReg PCR is an approximation method measuring relative quantification. It is a free software for qPCR calculations, working on raw data exported from qPCR software. LinReg PCR calculates individual samples efficiencies and mean efficiency based on all individual efficiencies in the actual experiment = $E_{mean.}$ Target molecules at start = N0. Output is given as: N0 = threshold/ $E_{meanCt.}$
LinReg PCR permits the omission of efficiency outliers, failed samples, samples with baseline errors, and no plateau and noisy samples. It also does baseline correction of data set. Data can be directly exported to Excel.
Data from the N0 column is used for the further analysis of the data. N0 of the target has to be normalized to the reference, which in this study is *Eubacteria*, to take into account the differences in the purification step and individual differences, to find the real output. Two primers of *Eubacteria* were used in this study, and the geometric mean (Vandesompele, J. *et al., 2002)* of N0 is used as the reference. Real output is calculated as N0 target/geometric mean of N0 reference.
At last N0 (appendix 11) of samples from each group of samples (duodenum, ileum, colon, feces or cecum content) are normalized to 100 in relation to mean of samples without XOS (N0= (target /mean target) x 100 or N0= (Target $_{XOS}$ / mean target) x 100.
The calculated results were analyzed as a ratio of species specific 16s rRNA levels relative to total bacterial 16s rRNA levels in order to correct data for differences in total DNA concentration and DNA quality between individual samples. All samples were analyzed in duplicates.

WoL: common points in WoL: 4		Chemistry: DNA binding dyes Input: ds-DNA	N0 = threshold /(Eff_mean^Cq)			LEGEND
Threshold: common threshold	mean_PCR_eff	Cq	N0	Sample_Use	Quality_checks	Sample Use: 1: used for W-o-L setting
0.202	1.759	33.005	1.62E-09	1 2 3	0----------	2: contributes to mean PCR efficiency
0.202	1.759	28.695	1.85E-08	1 2 3	0----------	3: N0 value calculated
0.202	1.759	27.186	4.35E-08	1 2 3	0----------	0: not used / calculated
0.202	1.759	39.823	3.46E-11	0 0 3	---3--6-------	
0.202	1.759	25.835	9.32E-08	1 2 3	0----------	Quality Checks:
0.202	1.759	21.593	1.02E-06	1 2 3	0----------	0: passed all checks
0.202	1.759	30.989	5.07E-09	1 2 3	0----------	1: no amplification
0.202	1.759	22.252	7.05E-07	1 2 3	0----------	2: baseline error
0.202	1.759	25.925	8.86E-08	1 2 3	0----------	3: no plateau
0.202	1.759	17.900	8.23E-06	1 2 3	0----------	4: noisy sample
0.202	1.759	20.054	2.44E-06	1 2 3	0----------	5: PCR efficiency outside 10%
0.202	1.759	18.875	4.74E-06	1 2 3	0----------	6: excluded from mean Eff
0.202	1.759	20.190	2.26E-06	1 2 3	0----------	7: excluded by user
0.202	1.759	15.956	2.47E-05	1 2 3	0----------	8: included by user
0.202	1.759	19.674	3.02E-06	1 2 3	0----------	9: manual baseline
0.202	1.759	23.305	3.89E-07	1 2 3	0----------	
0.202	1.759	33.852	1.01E-09	0 0 3	---3--6-------	if amplicon groups are defined the rules are applied per group
0.202	1.759	28.978	1.58E-08	1 0 3	-----56-------	
0.202	1.759	27.834	3.01E-08	1 2 3	0----------	User Choices:
0.202	1.759	39.227	4.84E-11	0 0 3	---3--6-------	
0.202	1.759	26.308	7.13E-08	1 2 3	0----------	Calculation of Mean Efficiency:
0.202	1.759	21.909	8.55E-07	1 2 3	0----------	exclude <no plateau> samples
0.202	1.759	31.352	4.13E-09	1 2 3	0----------	exclude <efficiency outlier> samples
0.202	1.759	22.338	6.71E-07	1 2 3	0----------	efficiency outlier defined as >10% from group median
0.202	1.759	26.208	7.55E-08	1 2 3	0----------	Baseline Estimation:
0.202	1.759	18.001	7.77E-06	1 2 3	0----------	log-linear phase criterion: strictly continuous Log-linear phase

Figur 5.7 Example of output from LinReg PCR to Excel showing the quality check output (Cq = C_T).

Quality control of output can be seen in the last four columns of the output, which gives information on the quality of the samples. Each samples get a 3-digit code showing in the Sample_Use column. It tells whether the sample is used for (1) setting the window-of-linearity, (2) calculating the mean efficiency, and (3) whether a starting concentration (N0) is calculated. 123 shows the perfect choice.

The quality_checks column contains 0 when the sample is OK. The other 9 positions can contain values of 1 through 9 of which the explanation is given in the legend column under quality checks. Based on these values the decision to include or exclude the N0 values from further analysis can be done.

Primers			
Target	Primer sequences	Amplicon site	Reference
Bifidobacteria H2930 33-5326-3/8, Bifido 243 bp F H2930 33-5326-4/18, Bifido 243bp R	Forward: 5' TCG CGT CYG GTG TGA AAG-3' Revers: 5' CCA CAT CCA GCR TCC AC-3'	243	Rinttila *et al.*, 2004
Lactobacilli H001 43-2506-1/6 Lactobacillus F H001 43-2506-2/6, Lactobacillus R	Forward:5' AGC AGT AGG GAA TCT TCC A-3' Revers:5' CAC CGC TAC ACA TGG AG-3'	341	Walter *et al.*, 2001, Heilig *et al.,* 2002
Firmicutes H11754$_4$-2467-5/50, Firm 934F H11754$_4$-2467-6/50, Firm 1060R	Forward: 5' GGA GYA TGT GGT TTA ATT CGA AGC A -3' Revers:5' AGC TGA CGA CAA CCA TGC AC-3'	126	Guo *et al.,* 2008
Bacteriodetes H11754$_4$-2467 9/50, Bacteriodetes F H11754$_4$-2467 10/50, Bacteriodetes R	Forward:5' GGA RCA TGT GCT TTA ATT CGA TGA T-3' Revers:5' AGC TCA CGA CAA CAA TGC AG-3'	126	Guo *et al.,* 2008
Eubacteria H11754$_4$-2467-11/50, Eubac_GUOF, Eub 338f H11754$_4$-2467-12/50, Eubac_GUOR, Eub 518R	Forward: 5' ACT CCT ACG GGA GGC AGC AG-3' Revers: 5'ATT ACC GCG GCT GCT GG-3'	180	Guo *et al.,* 2008
Eubacteria Eu Anne F Eu Anne R	Forward: 5'CGG CAA CGA GCG CAA CCC-3' Revers: 5'CCA TTG TAG CAC GTG TGT AGC C-3'	161	Denman & McSweeney, 2006

Table 5.4 primers for bacterial profile

5.11.2 Protocol (for more details see appendix 6,7,8)

Mastermix per sample: 5,5 µl SYBR Green (AB Applied Biosystems, SYBR Green, PCR Master Mix), 0,22 µl primer forward, 0,22 µl primer revers, 3,06 µl nuclease free water = total amount 9 µl. DNA from bacteria controls, stool samples, and cecum contents are diluted to 3 ng/µl. All samples: cDNA, DNA, bacteria controls, and nuclease free water are transferred to two 96 well plates. Working in a clean room with reagents on ice, primers are diluted from 100 pmol/µl to 10 pmol/µl in nuclease free water. Using a robot (Eppendorf, epMotion 5075), 2µl samples and 9µl mastermix are transferred to 384 well plates (one plate for each primer). The plate is measured in the AB Applied Biosystems Science 7900HT Fast Real-Time PCR System. Program as seen below. After 1 hour and 50 min. data is ready for analyzing.

5.11.3 QPCR cycling program:

Step description	Temp. and time	Numbers of cycles
Stage 1	50°C 2 min.	1
Stage 2	95°C 10 min.	1
Stage 3	95°C 15 sec.	40
	60°C 1 min.	
Stage 4	95°C 15 sec.	1
	60°C 20 sec.	
	95°C 15 sec.	

Table 5.5 qPCR cycling program for bacterial profile

5. 12 Statistics

In this study two groups were compared, that were samples with or without XOS treatment in each part of the GI tract. A statistical analysis was performed using unpaired two tailed t-test to compare means of treatments.

All statistical analysis and generation of graphs were carried out by using GraphPad Prism 5 (GraphPad Software, San Diego, California, USA).

P values $\leq 0,05$ were considered statistically significant and asterisks will indicate statistical significance as follows: $P \leq 0,05$:*, $P \leq 0,01$:**, $P \leq 0,001$:***.

6 Results

6.1 Bacterial profile

qPCR was performed to study quantitative changes in the microbiota induced by the experimental diet with XOS.

6.1.1 *Bacteriodetes* and *Firmicutes.*

The *Bacteriodetes* and *Firmicutes* phyla were included to study changes in the overall composition of the small and large intestinal microbiota, which, as mentioned earlier, are dominated by these two phyla.

The relative amount (N0 *Bacteriodetes*/N0 *Eubacterium*) of the *Bacteriodetes* phylum is shown in table 6.1 and figure 6.1. Taken together it seems that XOS in this study had no significant effect on the amount of *Bacteriodetes* phylum in the GI tract.

***Bacteriodetes* phylum**				
Sample	Relative amount (N0 *bacteriodetes*/N0 *Eubacterium*) Normalized to 100 in relation to mean of sample without XOS		P-value	
Duodenum	↑	156	0.3237	
Ileum	↑	132	0.4081	
Cecum content	↑	123	0.2205	
Colon	↓	62	0.2538	
Feces	↑	233	0.2052	

Table 6.1 Results from qPCR measuring *Bacteriodetes* phylum.

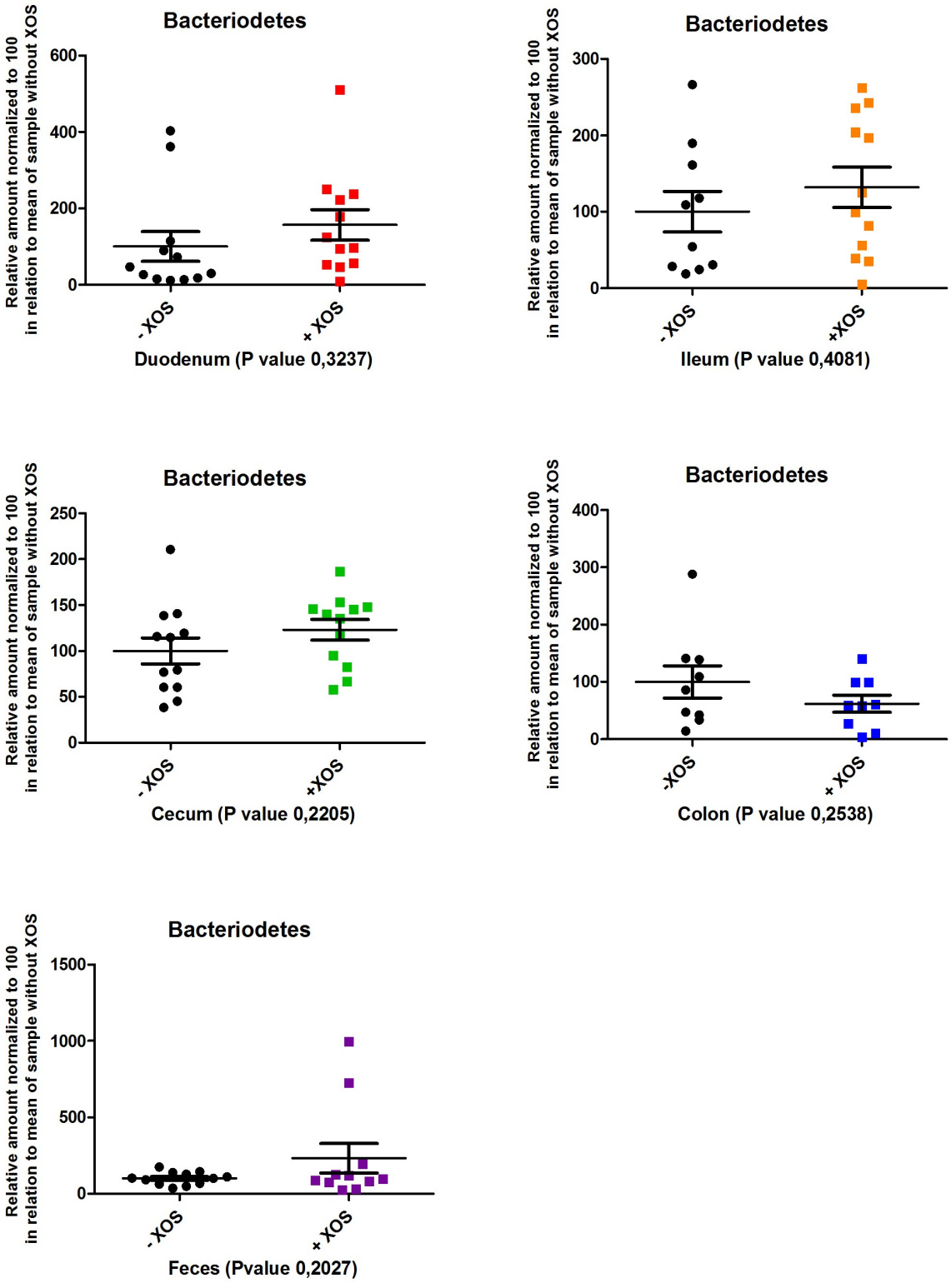


Figure 6.1 The results of the qPCR analysis of *Bacteriodetes* phylum in duodenum, ileum, cecum, colon and feces. The calculated results were analyzed as a ratio of species specific 16s rRNA levels relative to total bacterial 16s rRNA levels. The calculation method used is LinReg PCR. Target molecules at start are normalized to the reference *Eubacteria* and are normalized to 100 in relation to mean of samples without XOS. Data are presented as mean ± SEM. No significant differences between the control group and the XOS treated group from each segment of the gut were seen.

The relative amount (N0 *Firmicutes*/N0 *Eubacterium*) of the *Firmicutes* phylum is shown in table 6.2 and figure 6.2. In cecum content there was a significant small increase in the relative amount of *Firmicutes c*ompared to sample without XOS. Taken together the XOS diet did not seem to have great effects on the relative amount of *Firmicutes* in the GI tract, even though there is a little significant increase in the sample from the cecum content.

***Firmicutes* phyla**				
Sample	Relative amount (N0 *Firmicutes*/N0 *Eubacterium*) Normalized to 100 in relation to mean of sample without XOS		P-value	
Duodenum	↑	119	0.7979	
Ileum	↓	81	0.3672	
Cecum content	↑	134	0.0238	*
Colon	↓	92	0.8198	
Feces	↑	138	0.5302	

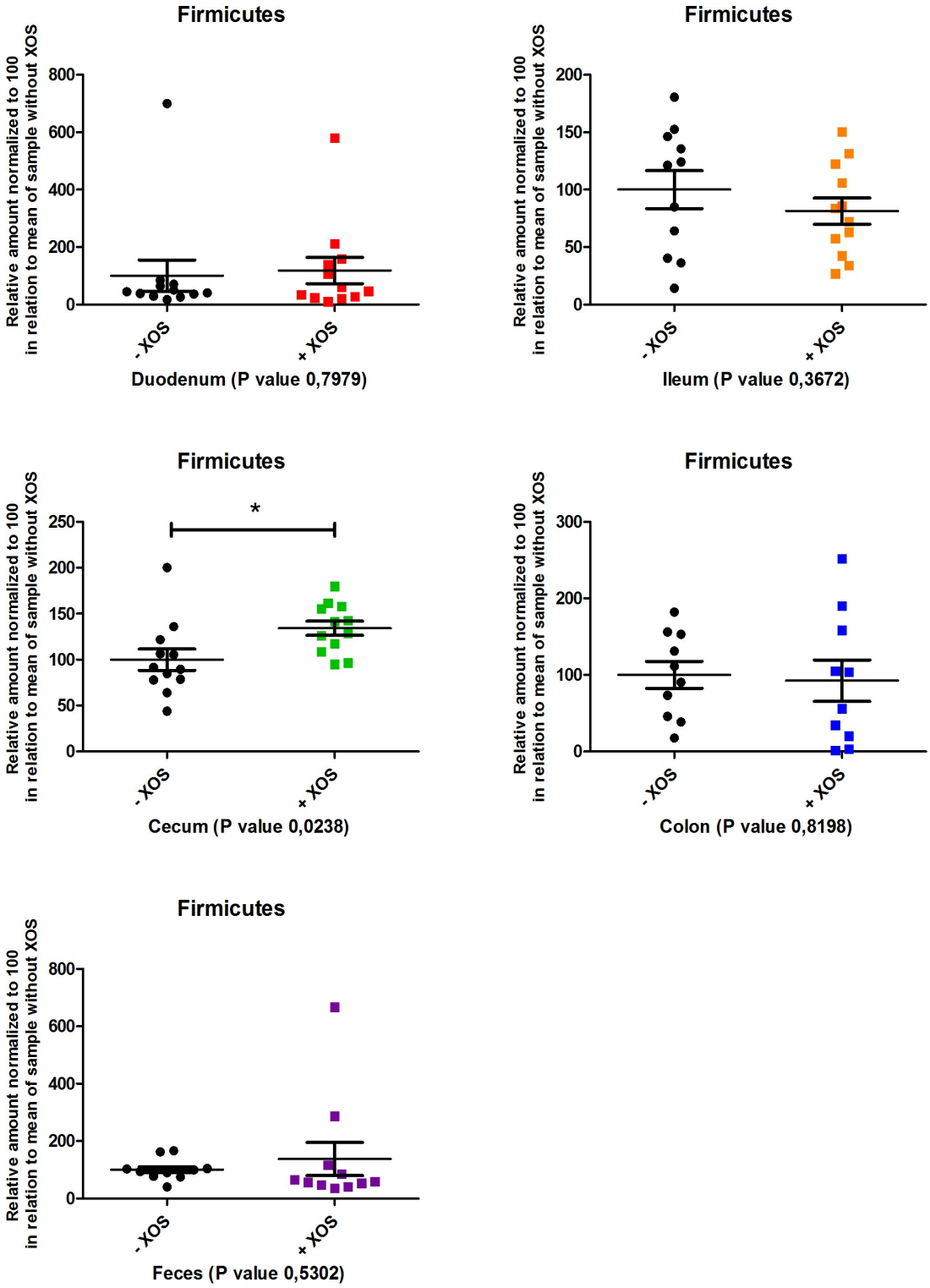


Table 6.2 Results from qPCR measuring *Firmicutes* phylum.

Figure 6.2 The results of the qPCR analysis of Firmicutes phylum in duodenum, ileum, cecum, colon and feces. The calculated results were analyzed as a ratio of species specific16s rRNA levels relative to total bacterial 16s rRNA levels. The calculation method used is LinReg PCR. Target molecules at start are normalized to the reference Eubacteria and are normalized to 100 in relation to mean of samples without XOS. Data are presented as mean ± SEM. In cecum content there was a significant (P value 0.0238) small increase in the relative amount of *Firmicutes*. No significant differences between the control group and the XOS treated group were seen for the other segments of the gut.

6.1.2 *Bifidobacterium* and *Lactobacillus*

The *Bifidobacterium* and *Lactobacillus* were included in the study in order to measure changes in the different sections of the gut.

Lactobacillus and *Bifidobakterium* normally constitute a little proportion of the total microbiota, but they were included in the analysis as they are thought to have beneficial effects on the host and are the target species of prebiotic treatment.

The relative amount (N0 *bifidobacterium*/N0 *Eubacterium*) of the *Bifidobacterium* is shown in table 6.3 and figure 6.3.

There is significant increase in the relative amount of *Bifidobacterium* in every part of the GI tract. Compared to sample without XOS, XOS diet increased *Bifidobacterium* approximate 4½-fold in duodenum, 20-fold in ileum, 45-fold in cecum content and colon, and 12½-fold in feces. Taken together XOS diet significantly increases the relative amount of *Bifidobacterium* in duodenum, ileum, cecum content, colon and feces, and it seems as there is a gradient from duodenum to cecum content, and from colon the amount decreases towards the distal end of the large intestine.

Bifidobacterium.				
Sample	Relative amount (N0 *bifidobacterium*/N0 *Eubacterium*) Normalized to 100 in relation to mean of sample without XOS		P-value	
Duodenum	↑	465	0.0305	*
Ileum	↑	2015	0.0001	* * *
Cecum content	↑	4697	0.0016	* *
Colon	↑	4374	0.0464	*
Feces	↑	1258	0.0035	* *

Table 6.3 Results from qPCR measuring *Bifidobacterium*.

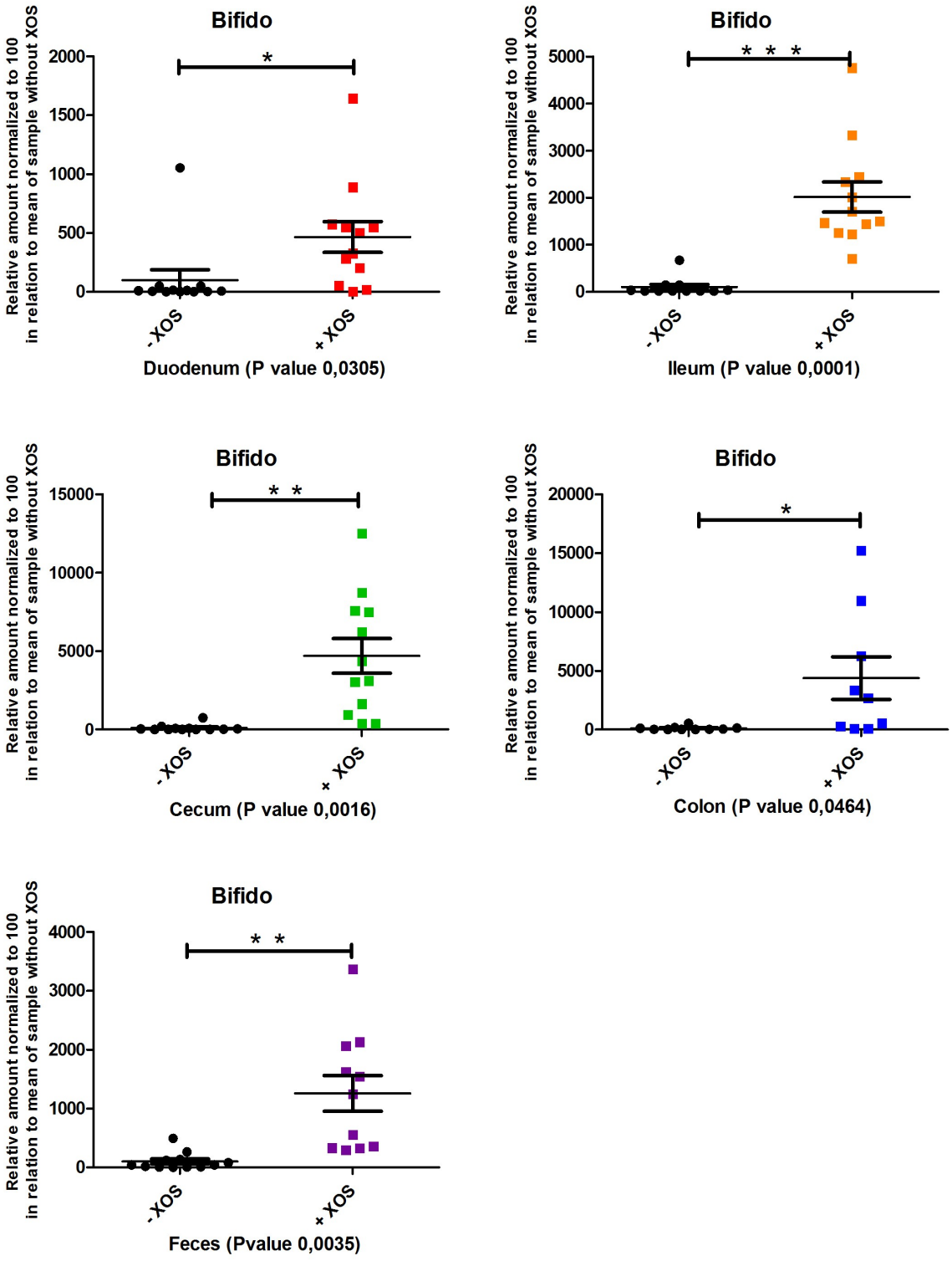


Figure 6.3 The results of the qPCR analysis of *Bifidobacterium* in duodenum, ileum, cecum, colon and feces. The calculated results were analyzed as a ratio of species specific16s rRNA levels relative to total bacterial 16s rRNA levels. The calculation method used is LinReg PCR. Target molecules at start are normalized to the reference *Eubacteria* and are normalized to 100 in relation to mean of samples without XOS. Data are presented as mean ± SEM. A significant increase were seen in duodenum (p value 0.0305), ileum (p value 0.0001), cecum content (p value 0.0016), colon (p value 0.0464) and feces (p value 0.0036).

The relative amount (N0 *Lactobacillus*/N0 *Eubacterium*)of the *Lactobacillus* is shown in table 6.4 and figure 6.4. Taken together it seems that XOS in this study had no significant effect on the amount of *Lactobacillus* in the GI tract. In the analysis of *Lactobacillus,* there could be a problem with the sensitiveness of the method. Since the amount of *Lactobacillus* is low, the detection of signal first occurred in the end of the analysis, meaning uncertainty with the results.

Lactobacillus				
Sample	Relative amount (N0 *Lactobacillus*/N0 *Eubacterium*) Normalized to 100 in relation to mean of sample without XOS		P-value	
Duodenum	↓	78	0.8150	
Ileum	↑	204	0.1071	
Cecum content	↑	518	0.0709	
Colon	↑	1253	0.2436	
Feces	↓	85	0.6203	

Table 6.4 Results from qPCR measuring *Lactobacillus.*

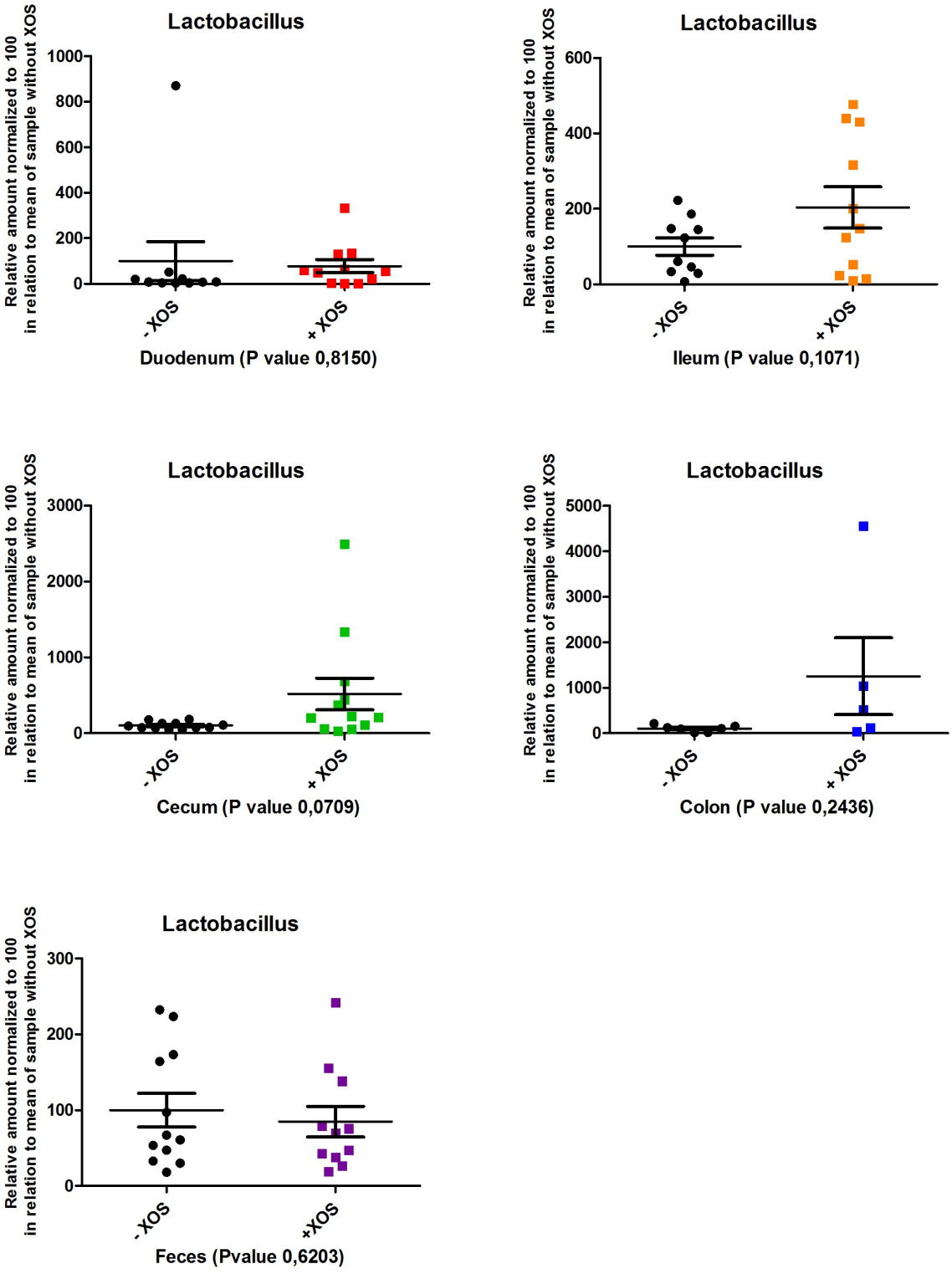


Figure 6.4 The results of the qPCR analysis of *Lactobacillus* in duodenum, ileum, cecum, colon and feces. The calculated results were analyzed as a ratio of species specific16s rRNA levels relative to total bacterial 16s rRNA levels. The calculation method used is LinReg PCR. Target molecules at start are normalized to the reference *Eubacteria* and are normalized to 100 in relation to mean of samples without XOS. Data are presented as mean ± SEM. No significant differences between the control group and the XOS treated group from each segment of the gut were seen.

A summary of analysis of the intestinal microbiota done in this study is shown in table 6.5 The analysis of *Bacteriodetes* phylum showed no significant (NS) fold changes when treated with XOS. *Firmicutes* had a significant fold change in the cecum content, but it was very low, so the overall impression is, that XOS diet did not have a significant effect on the *Firmicutes* phylum either.
Fold changes for *Lactobacillus* were not significant.
XOS diet had a significant effect on *Bifidobacterium* with an increase amount through the small and large intestinal. There was a gradient from proximal duodenum into colon and then a decrease in feces .

sample	*Bacteriodetes*	*Firmicutes*	*Lactobacillus*	*Bifidobacterium*
Duodenum	NS	NS	NS	4,5 x (p 0,0305)
Ileum	NS	NS	NS	20 x (p 0,0001)
Cecum content	NS	1,3 x (p 0,0238)	NS	45 x (p 0,0016)
Colon	NS	NS	NS	45 x (p 0,0464)
Faeces	NS	NS	NS	12 x (p 0,0035)

Table 6.5 Over view of the fold changes when treated with XOS for the analyzed bacteria.

Figure 6.5 shows the relative content of *Bifidobacterium, Lactobacillus, Bacteriodetes* and *Lactobacillus* for each sample locations in relation to the reference (*Eubacterium*).
It seems that for *Bifidobacterium,* ileum is the main location for changes in total bacterial content, in relation to reference (*Eubacterium) w*hen treated with XOS.

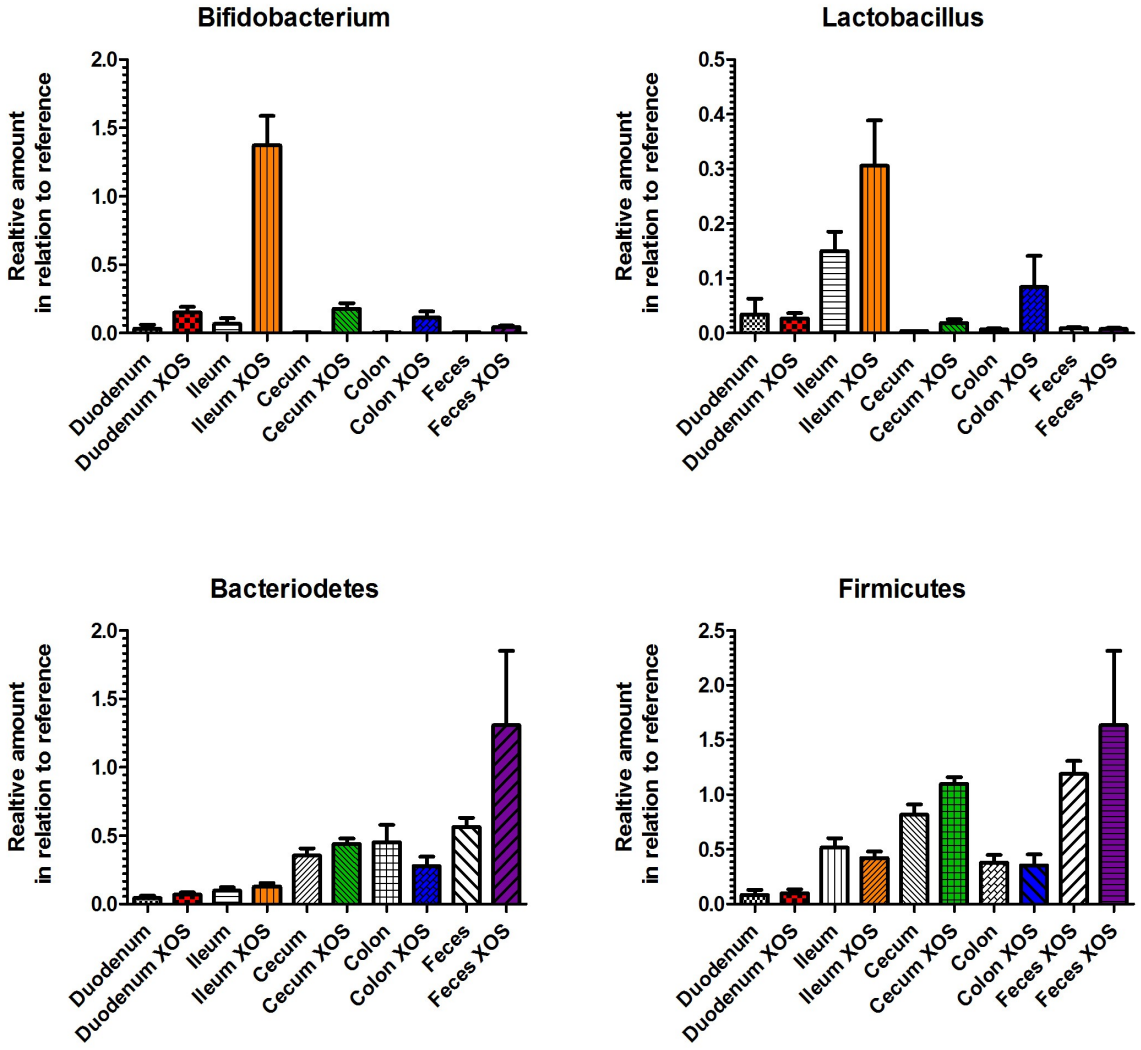


Figure 6.5 Relative bacterial content for each sample sites (duodenum, ileum, cecum content, colon and feces).
The calculated results were analyzed as a ratio of species specific16s rRNA levels relative to total bacterial 16s rRNA levels. Target molecules at start are normalized to the reference *Eubacteria.* Data are presented as mean ± SEM. For *Bifidobacterium,* ileum is the main location for changes in total bacterial content when treated with XOS.

6.2 gene expression

qPCR was performed to study changes in gene expression in blood and the GI tract, induced by the experimental diet with XOS. The genes included in the study are chosen because they are suggested to respond to changes in the bacteria profile and changes of the fatty acids contents.

6.2.1Epithelial-cells

mRNA levels in epithelial cells from duodenum, ileum and colon were investigated after 10 weeks of treatment with XOS. The genes measured were RegIIIγ, Tlr2, Tlr4, Tnf-α, Cxcl1, Cxcl2, Cd36 and Ffar2, and the purpose was to investigate if XOS had influence on their expression profile.

6.2.1.1 Tlr2 and Tlr4

Toll-like receptors recognize structurally conserved molecules derived from microbes and activate immune cell responses.

Tlr's here represented as Tlr2 and Tlr4 (figure 6.6) are lowest expressed in duodenum, middle expressed in ileum and highest expressed in colon. No significant differences between the control group and the XOS treated group from each segment of the gut were seen.

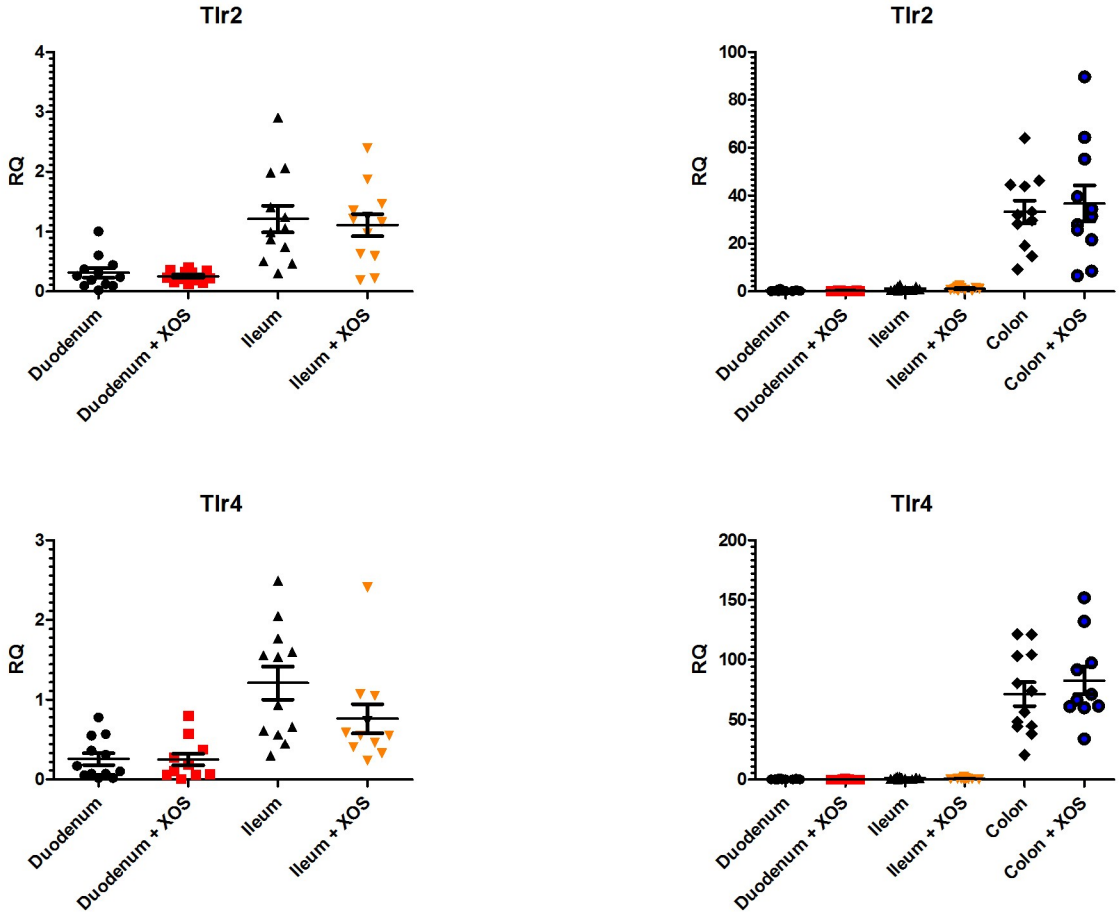


Figure 6.6 The results of the qPCR analysis of Tlr2 and Tlr4 expression in epithelial-cells from duodenum, ileum and colon. The calculation method used is Delta-Delta C_T method. C_T expression is normalized to the expression of the reference gene β-actin. The ΔC_T (control) is mean ΔC_T from ileum without XOS. RQ (Relative Quantification) value is indicative of the gene fold change of the target gene caused by the XOS treatment compared to the untreated sample. Data are presented as mean ± SEM. No significant differences between the control group and the XOS treated group from each segment of the gut were seen.

6.2.1.2 Cxcl1,Cxcl2 and Tnf-α

TNF-α, CXCL1 and CXCL2 are involved in attraction of neutrophiles as they are pro-inflammatory cytokines and stimulate the expression of acute phase proteins. CXCL1 and CXCL2 are chemokines playing roles in the development, homeostasis and function of the immune system.

Tnf-α is expressed lowest in duodenum and highest in colon. Cxcl2 is expressed equally throughout the small and large intestinal (figure 6.7). Expression of Tnf-α and Cxcl2 showed no significant differences between the control group and the XOS treated group from each segment of the gut.

Cxcl1 is expressed equally throughout the small intestine. There is a significant (P value 0,0488) increase in the Cxcl1 expression in duodenum with XOS treatment, but no significant changes in ileum, revealing that XOS is affecting Cxcl1 expression in duodenum. Cxcl1 was only investigated in duodenum and ileum.

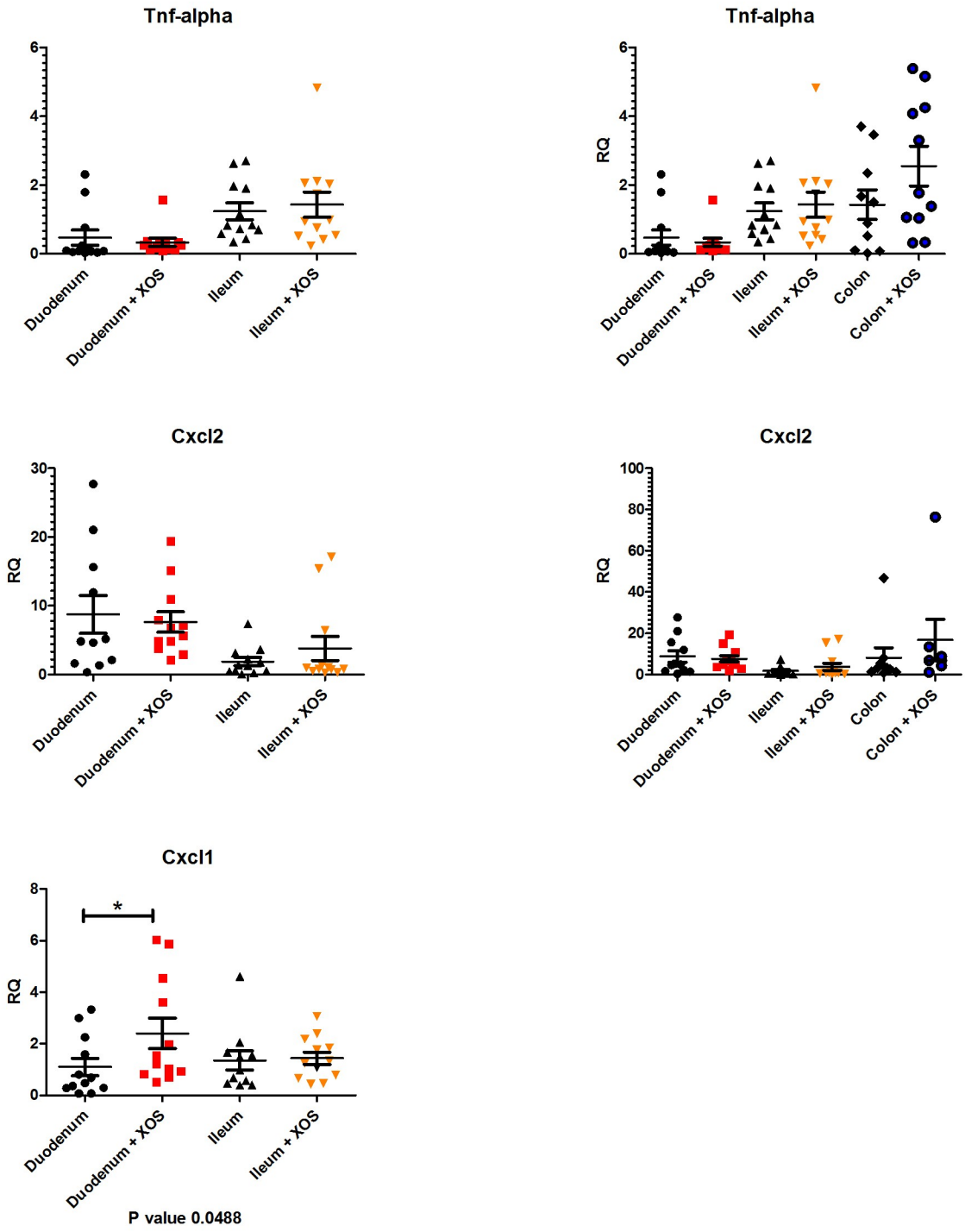


Figure 6.7 The results of the qPCR analysis of Tnf-α, Cxcl1 and Cxcl2 expression in epithelial-cells from duodenum, ileum and colon. The calculation method used is Delta-Delta C_T method. C_T expression is normalized to the expression of the reference gene β-actin. The ΔC_T (control) is mean ΔC_T from ileum without XOS. RQ (Relative Quantification) value is indicative of the gene fold change of the target gene caused by the XOS treatment compared to the untreated sample. Data are presented as mean ± SEM. No significant differences between the control group and the XOS treated group from each segment of the gut were seen for Tnf-α and Cxcl2. There is a significant (P value 0,0488) increase in the Cxcl1 expression in duodenum with XOS treatment.

6.2.1.3 RegIIIγ

REGIIIγ prevents Gram-positive bacteria from entering the mucosa and thereby plays a vital role in antimicrobial protection of the mammalian gut. REGIIIγ may reflect microbial colonization level, and its expression is triggered by increased microbial epithelial contact at mucosa surface.

RegIIIγ is expressed predominantly in the small intestine when compared with the three segments of the GI tract (figure 6.8), and there are significant differences between the control group and the XOS treated group in both duodenum (p value= 0.0001) and ileum (p value= 0.0114).

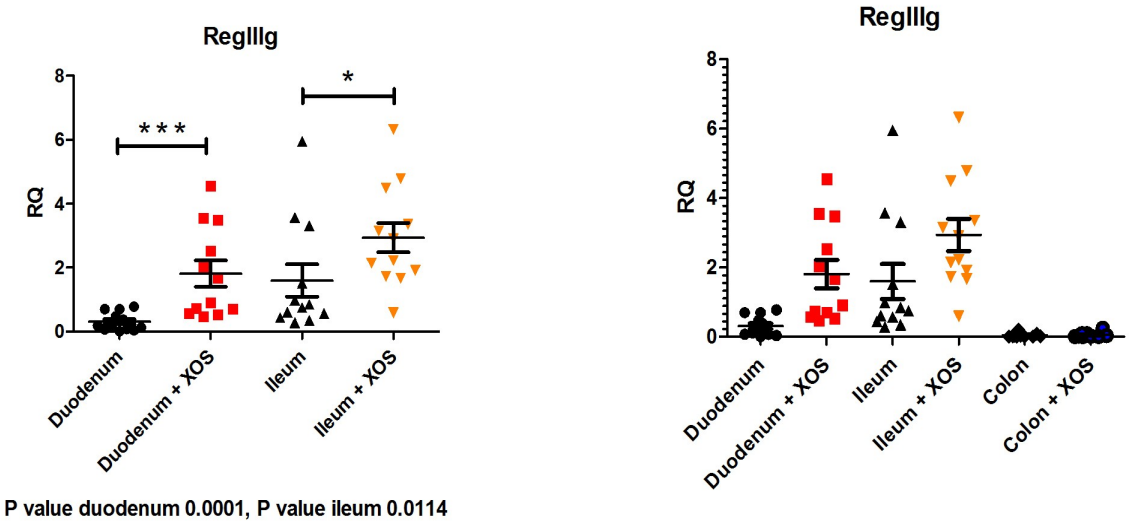


Figure 6.8 The results of the qPCR analysis of RegIIIγ expression in epithelial-cells from duodenum, ileum and colon. The calculation method used is Delta-Delta C_T method. C_T expression is normalized to the expression of the reference gene β-actin. The ΔC_T (control) is mean ΔC_T from ileum without XOS. RQ (Relative Quantification) value is indicative of the gene fold change of the target gene caused by the XOS treatment compared to the untreated sample. Data are presented as mean ± SEM . Significant differences between the control group and the XOS treated group in both duodenum (p value= 0.0001) and ileum (p value= 0.0114) were seen.

6.2.1.4 Cd36and Ffar2

CD36 plays a role in fatty acid uptake and glucose metabolism. CD36 binds long chain fatty acids and thereby playing a role in dietary fat processing in the intestine.

In this study mRNA levels of CD36 are highly expressed in duodenum and decrease from proximal to distal of the intestine (figure 6.9), and there are no significant differences between the control group and the XOS treated group from each segment of the gut.

FFAR2 is highly expressed in various types of cells, including epithelial cells. FFAR2 is activated by SCFAs especially acetate and propionate and suggested inducible by SCFA stimulation. SCFA-FFAR2 interactions have been shown to affect inflammatory response.

In this study Ffar2 is expressed equally throughout the small and large intestinal (figure 6.9). Expression of Ffar2 showed no significant differences between the control group and the XOS treated group from each segment of the gut.

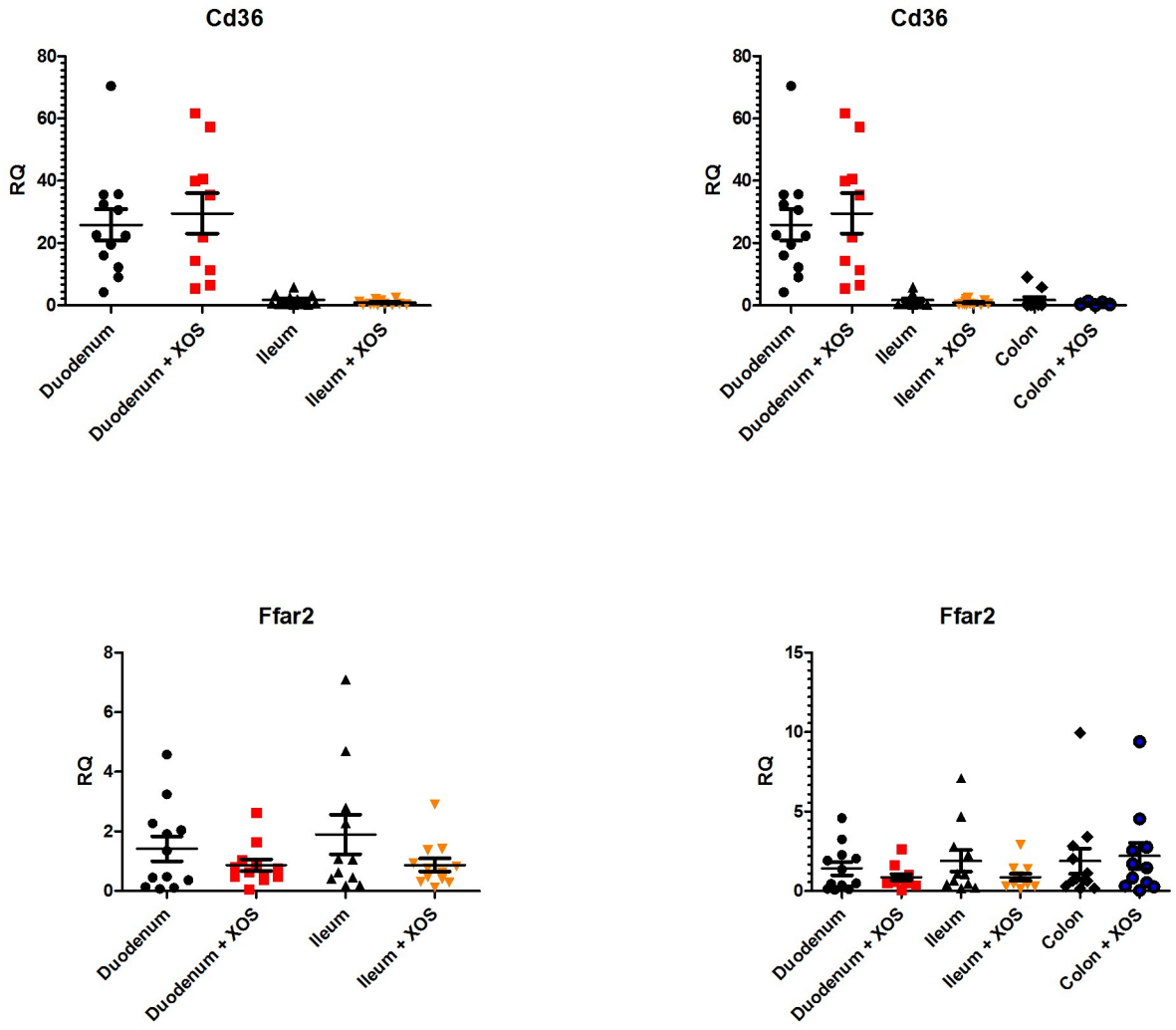


Figure 6.9 The results of the qPCR analysis of Cd36 and Ffar2 expression in epithelial-cells from duodenum, ileum and colon. The calculation method used is Delta-Delta C_T method. C_T expression is normalized to the expression of the reference gene β-actin. The ΔC_T (control) is mean ΔC_T from ileum without XOS. RQ (Relative Quantification) value is indicative of the gene fold change of the target gene caused by the XOS treatment compared to the untreated sample. Data are presented as mean ± SEM. No significant differences between the control group and the XOS treated group from each segment of the gut were seen

6.2.2 Blood

Gene expression in blood was investigated after 9-10 weeks of XOS treatment. mRNA levels of the proinflammatory cytokine Il-1β were analyzed in order to investigate if XOS had influence on Il-1β expression in neutrophiles.

6.2.2.1 Il-1β

Like TNF-α, CXCL1 and CXCL2, the cytokine IL-1β is involved in attraction of neutrophiles and is an important mediator of the inflammatory response, stimulating the expression of acute phase proteins.

Il-1ß expression is seen in figure 6.10. There was a significant (p value 0,0199) decreased mRNA level of Il-1ß after XOS treatment, indicating that XOS had an effect on the Il-1ß expression in blood.

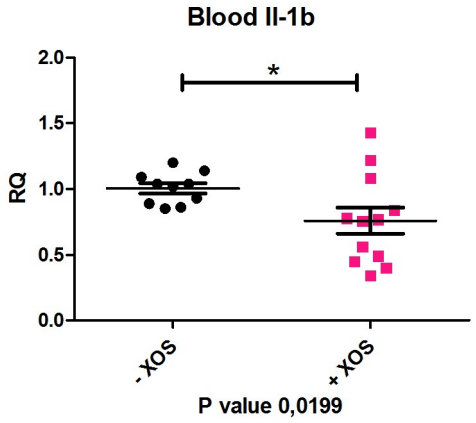


Figure 6.10 The results of the qPCR analysis of Il-1ß expression in blood. The calculation method used is Delta-Delta C_T method. C_T expression is normalized to the expression of the reference gene β-actin. The ΔC_T (control) is mean ΔC_T from ileum without XOS. RQ (Relative Quantification) value is indicative of the gene fold change of the target gene caused by the XOS. Data are presented as mean ± SEM. A significant (p value 0,0199) decreased gene expression of Il-1ß after XOS treatment were seen.

6.2.3 Bifidobacterium and gen-expression

The relationship between the amount of *Bifidobacterium* and the gen-expression for each mouse was investigated to see if a higher amount of *Bifidobacterium* lead to an alteration in gene expression. No correlation between the amount of *Bifidobacterium* and the gene expression was found (figure 6.11)

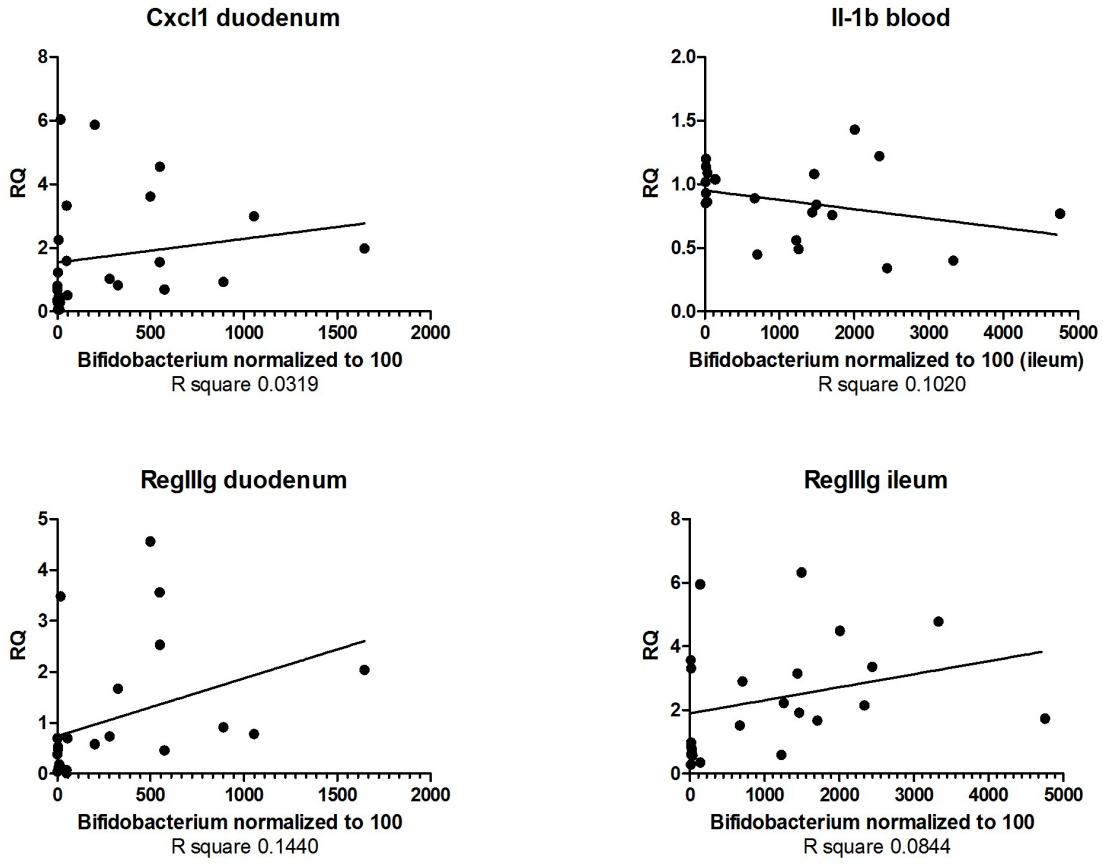


Figure 6.11 Amount of *Bifidobacterium* in relation to gen-expression of Cxcl1, RegIIIγ and Il-1β. For *Bifidobacterium* The calculated results were analyzed as a ratio of species specific16s rRNA levels relative to total bacterial 16s rRNA levels. Target molecules at start are normalized to the reference *Eubacteria* and are normalized to 100 in relation to mean of samples without XOS. RQ (Relative Quantification) value is indicative of the gene fold changes, and expression is normalized to the expression of the reference gene β-actin. The ΔC_T (control) is mean ΔC_T from ileum without XOS. Linear regression analysis showed no correlation between amount of *Bifidobacterium* and gen-expression.

7 Discussion

The purpose of the present study was to investigate how diet supplemented with 10% XOS affected the microbiota and the expression of selected genes in the small and large intestine and in blood of mice.

In this study no significant changes in the amount of *Bacteriodetes* and *Firmicutes* were found when feeding with XOS diet. Microorganisms belonging to *Bacteroides* able to degrade XOS, even though they are all far less efficient than *Bifidobacterium* strains (Jaskari, J. et al.1998, Campbell, J. M. et al.,1997), and therefor an increase of those two phyla could have been expected.
Petersen A. *et al*. (2010) saw a significant increase in the *Bacteroides* group and a decrease in *Firmicutes* group in mice feeding with a diet with the same XOS supplementation as used in this study.

Prebiotics are generally claimed to affect the growth of *Lactobacillus.* But as regards to XOS, the findings are not in agreement. Van Laere, K. M. J. *et al*., 2000 *in vitro* found that *Lactobacillus* is able to metabolise XOS , whereas Jaskari, J. *et al.*1998 *in vitro* found *Lactobacillus* not to metabolize XOS. Petersen A. *et al*. (2010) and Campbell, J. M. *et al*., (1997) found no effect of XOS treatment on *Lactobacillus* growth in rodents, whereas Moura, P. *et al*. (2008) found that all tested XOS fermented by the piglet ileal, caecum and colonic microbiota *in vitro* supported an enhancement of *Lactobacillus* in the beginning of the fermentation.
This study found no significant changes in the amount of *Lactobacillus* when treated with XOS diet. But in ileum, cecum content and colon there was a tendency of an increased gradient against the distal end of the GI tract. It could be speculated that there could be problems with the sensitivity of the method. Since the amount of *Lactobacillus* in the GI tract is low, high C_T values were obtained, meaning uncertainty with the results.

XOS have been found to have great bifidogenic effect, and the observed bifidogenic effect in this study is consistent with results from other *in vivo* studies (Petersen A. *et al*. 2010, Chung, Y. C**.** *et al.* 2007**,** Hsu, C. K. *et al*. 2004, Campbell, J. M. *et al*., 1997), and growth of pure cultures of bifidobacteria on XOS has also been demonstrated *in vitro.* (Mäkeläinen, H. *et al.* 2010, Moura, P. *et al*. 2008, Van Laere, K. M. J. *et al*., 2000, Jaskari, J. *et al.* 1998,)

This study suggested that *Bifidobacterium* can grow *in vivo* on XOS and is fermented in all part of the GI tract, since the XOS diet significantly increase the relative amount of *Bifidobacterium* in duodenum,

ileum, cecum content, colon and faeces. The XOS used in this study was a short-chain XOS with a Dp 2-6, and short-chain XOS fermentation in all part of the GI tract, is in agreement with the *in vitro* study by Moura, P. *et al*. (2008). They found the rate of consumption of medium- and long-chain XOS was reduced in the fermentation by the ileal inoculum as compared to short-chain XOS (Dp 2-5). The slower fermentation rate of medium- and long- chain XOS by the ileal inoculum denotes a lower fermentation efficiency of the small intestine microbiota towards longer molecules of XOS, when compared to short-chain XOS. XOS with a DP up to 25 was fermented by ileal, caecal and colonic microbiota in piglet. The same tendency has been seen in studies with other prebiotics (Patterson, J. *et al.* 2010, Yasuda, K. *et al.*, 2009, Smiricky-Tjardes, M. R. *et al*. 2003).
Manisseri, C. & Gudipayi, M. (2010) found a slight increase in growth of *Bifidobacterium* on oligosaccharides having low degree of polymerization (xylobiose and xylotriose) *in vitro*. On the other hand Van Laere, K. M. J. *et al*., (2000) found that more complex XOS structures presented a high capability to be selectively fermented by bifidobacteria.

In this study there is an increased gradient of bifidobacteria growth from duodenum to cecum content, and from colon the amount decreased against the distal end of the large intestinal when treated with XOS. XOS diet increased *Bifidobacterium* approximately 4½-fold in duodenum, 20-fold in ileum, 45-fold in cecum content and colon, and 12½-fold in feces, compared to control diet.
No studies fully support this result, since normally only fecal and cecal bacterial content are examined when investigating prebiotic effects. Thus, fold-changes throughout the GI tract when treated with XOS has not been investigated or published before.
It could be speculated that the gradient in the increased *Bifidobacterium* content in the small intestine is caused by an immigration from the increased amount in cecum, or if it is caused only by the increase of the local amount of *Bifidobacterium,* or maybe a combination is most likely.
The bifidogenic degradation of XOS starts in duodenum, and XOS, segments of XOS and some *Bifidobacterium* are traveling all the way out with feces. This could also be an explanation for the increased gradient of *Bifidobacterium* content from the small to the large intestine.
One could argue that because short-chain XOS is highly fermentable, no XOS substrate for bacterial growth would be left when reaching the distal intestine. However, because of the continuous supply of XOS by ad libitum feeding the substrate may still be present in the distal intestine.

As mentioned, *Bifidobacterium* was increased 45- fold in the cecum content and in colon, but when related to total bacterial content (figure 6.5), this 45-fold increase only seems to have minor influence

on the total microbiota content in these regions. In ileum on the other hand, the 20-fold increase of *Bifidobacterium* seems to have great influence on the total bacterial content, as the total amount of other species than *Bifidobacterium* is lower in the small intestine.

This study suggested that XOS are fermented in all parts of the GI tract, since the XOS diet significantly increased the relative amount of *Bifidobacterium* in duodenum, ileum, cecum content, colon and feces. Since SCFAs are liberated due to the fermentation of XOS, SCFAs are often measured to determine effects of prebiotics, and whether their concentrations are affected by changes in the microbiota. Manisseri, C. & Gudipayi, M. (2010) and Wang, J. *et al*., (2010) found acetate to be the main SCFA liberated due to *in vitro* fermentation of XOS. Petersen A. *et al*. (2010) found no significant effects on the SCFA concentration when treated with XOS.
Protein level of genes and SCFA content were not investigated in this study, but measuring SCFAs could have given this study stronger evidence about the effects of XOS.

Cd36 was found to be highest expressed in duodenum. This is in agreement with Nassir, F. & Abomrad, N. A., (2009) who found that CD36 protein level is very high in proximal segments (duodenum, jejunum) and decreasing proximal to distal in small intestines of human and rodents. Their hypothesis was that CD36 has a primary role in proximal fatty acid absorption, whereas other CD36-independent mechanisms predominate in distal segments.
No significant differences were found in this study between the control group and the XOS treated group from each segment of the gut. Maybe it is because CD36 is involved in fatty acid absorption for chylomicron, which means long-chain fatty acids, and thereby CD36 is not affected by changes in the amount of SCFAs.

At protein level FFAR2 is found expressed in ileum and colon. In rats, Kaji, I. *et al*. (2011) found FFAR2 expressed as an increasing gradient across segments of colon, from the proximal to the distal colon. The value for the terminal ileum was intermediate between the values for the middle and distal colons. When treated with prebiotic (FOS) the protein level of FFAR2 was increased by two-fold in the proximal colon reaching the level of the distal colon, and the increasing gradient through the colon was abolished. FFAR2 levels in terminal ileum were increased to levels slightly lower than in the proximal colon. In cecum no changes in FFAR2 levels were seen.
Gene expression of Ffar2 in this study did not correlate with the Kaji, I. *et al*. (2011) results, as no

changes were found in Ffar2 expression throughout the intestine when treated with XOS. An expression gradient from small to large intestinal was not seen either. Maybe the expression gradient and influence of prebiotic can only be measured at the protein level.

SCFA-FFAR2 interaction affects inflammatory response. FFAR2 could be the sole functional receptor for SCFAs on neutrophils, and it is activated by SCFAs - acetate and propionate are the most potent ligands. FFAR2 binding of SCFAs maybe provides a molecular link between diet, gastrointestinal bacterial metabolism, and immune inflammatory responses (Karaki, S. & Kuwahara, A., 2010, Maslowski, M. K. *et al*. 2009).

IL-1β is an important mediator of the inflammatory response and it is expressed in a variety of cell types e.g. neutrophils.

The gen-expression of Il-1β in blood in this study was significant decreased when treated with XOS. This was in agreement with the study's hypothesis that the XOS diet would increase the amount of SCFAs in the blood. SCFAs would be bound to the SCFAs receptors (FFAR2) on the neutrophils, which would down regulate expression of the pro-inflammatory interleukin IL-1β.

The decreased Il-1β expression in blood when treated with XOS is also in agreement with the study by Vulevic, J. *et al*. (2008) who found decreased IL-1β protein level in blood when treated with GOS. The decrease in the m-RNA level of Il-1β could cause an enhancement of the immune system especially in elderly and in relation to chronic inflammatory diseases.

Changes in gene expression in the small intestine were found in this study, and these changes were in agreement with other studies which have found changes in gene expression when treating with prebiotics other than XOS (Yasuda, K. *et al.*, 2009,Vulevic, J. *et al*. ,2008, Osman M. *et al,* 2006, Furrie E. *et al*, 2005). To my knowledge, no results have been published showing effects of XOS treatment on gene expression.

Studies investigating prebiotics effects on gene expression are normally done when the immune system is challenged in one way or another - infection, inflammation, cancer etc. In those studies prebiotics are able to modulate some aspects of the immune functions, to improve the host's ability to respond successfully to certain intestinal infections or to modify some inflammatory conditions like reducing

the level of IL-1β and TNF-α.

In this study, the normal balance of the immune system was not challenged, and maybe that is why only a few of the measured genes showed significant changes.

A second explanation could be that just a few genes are measured, and maybe these genes are not the ones, on which XOS has effect on the expression.

A third explanation could be, that a higher amount of *Bifidobacterium* is needed to cause changes in the gene expression. An increased amount of *Bifidobacterium* was seen, but if this increased amount was big enough to change gene expression of Tlr2, Tlr4, Tnf-α, Cxcl2, Cd36 and Ffar2 is not known.

The relationship of the microbiota and the intestinal immune system is described as homeostatic, as the microbiota-stimulated immune mechanisms maintain gut homeostasis, and changes in the microbiota composition are a challenge to the homeostasis. Immune mechanisms limit the direct bacterial contact with epithelial cell surface, promote rapid detection and killing of penetrate bacteria and minimize exposure of resident bacteria to the systemic immune system (Hooper, L. V. & Macpherson, A. J., 2010).

A key element of the mammalian intestinal strategy for maintaining homeostasis with the microbiota is to minimize contact between luminal microorganisms and the intestinal epithelial cell surface. Here the secretion of the antimicrobial C-type lectin REGIIIγ is an important factor. In this study the content of the Gram-positive *Bifidobacterium* increased when treated with XOS, which lead to a significantly upregulation of the Gram-positive killing RegIIIγ in duodenum and ileum. This increased expression of RegIIIγ could cause a better host protection of Gram-positive pathogens e.g. Listeria.

CXCL1 is a pro-inflammatory cytokine and also a part of the microbiota-stimulated immune system. CXCL1 is a potent neutrophil chemo-attractant, and Shea-Donohue, T. et al. (2008) found that neutrophil infiltration induced by CXCL1 is an essential component of the intestinal response to inflammatory stimuli as well as the ability of the intestine to restore mucosal barrier integrity, as production of CXCL1 has a protective role. Relatively little is known about the regulation of CXCL1 expression or its role in inflammation, but LPS is suggested to be a very potent inducer. Despite that CXCL1 has been observed to be strongly up-regulated in both *in vivo* and *in vitro* systems, its role in the induction of a protective versus pathological response remains unclear (Shea-Donohue, T. et al., 2008).

This study found a significant increase in expression of Cxcl1 in duodenum when treated with XOS. This increase is suggested to be caused by the increased amount of *Bifidobacterium.* The content of *Bifidobacterium* was increased throughout the intestine with lowest increase in duodenum, but the

expression of Cxcl1 is only up regulated in duodenum. Maybe the expression is more aggressive in this part of the gut, because the bacterial content normally is low. The influence of the up regulated Cxcl1 expression for the homeostasis is not known, maybe it has a protection role or maybe it could lead to a more sensitive region for inflammation.

One could argue that XOS is resistant to digestive processes in the upper part of the GI tract, as mammalian cells do not have the enzymes to break down ß (1-4) bonds, and the degradation is thus dependent on the microflora. And since the other two criteria (fermentation by intestinal microflora and selective stimulation of the growth and/or activity(ies) of one or a limited number of intestinal bacteria beneficially associated with health and well-being) are fulfilled in relation to the results of this study, maybe XOS can be classified as prebiotics ?

8 Conclusion

Prebiotic effects were seen for XOS after 10 weeks treatment:

- Content of *Bifidobacterium* was increased throughout the intestine from duodenum to colon and feces.
- Expression of Cxcl1 was increased in epithelial cells from duodenum.
- Expression of RegIIIγ was increased in epithelial cells from dudenum and ileum.
- Expression of Il-1β was decreased in blood.

Prebitoic effects of XOS treatment were not seen in:

- Changes in content of *Bacteriodetes* phylum, *Firmicutes* phylum and *Lactobacilli.*
- Changes in epithelial gene expression of Tlr2, Tlr4, Tnf-α, Cxcl2, Cd36 and Ffar2.

The questions to be addressed in this thesis were:

Can a higher number of bacteria be measured in the small intestine in mice fed with XOS?

Bifidobacterium was increased in all segments of the intestine. There is a significant higher number of *Bifidobacterium* in the small intestine in mice fed with XOS. This is new knowledge since the primary sites for fermentation of non-digestible dietary carbohydrates with low degree of polymerization such as XOS are accepted to be the cecum and the proximal colon (Roberfroid, M. et al., 2010). No significant changes were seen for *Bacteriodetes* and *Firmicutes phyla.*

Is bacterial content in duodenum, ileum, cecum, colon or feces the most representative descriptor for the microbial changes induced by the XOS administration?

In this study XOS treatment did have an effect on the *Bifidobacterium* content. *Bifidobacterium* in cecum and colon are the most representative descriptors if an indication of the absolute amount is wanted, whereas ileum is the most representative descriptor for the largest relative increase of *Bifidobacterium* in relation to changes in the total microbial content.

Can changes in gene expression be measured in gut epithelium (in different parts of the gut), which can support a hypothesis about an immune stimulating and/or anti-inflammatory effect of XOS ?

When treated with XOS changes in epithelial gene expression were found for Cxcl1 in duodenum and RegIIIγ in duodenum and ileum. REGIIIγ prevents Gram-positive bacteria from entering the mucosa and thereby plays a vital role in antimicrobial protection of the small intestine. Cxcl1 is a pro-inflammatory cytokine, which plays roles in the development, homeostasis and function of the immune

system.

Can changes in gene expression in blood be measured in the two groups of mice?

XOS treatment showed significant decrease in IL-1β expression in relation to the control group.

9 Perspective

This study found bifidogenic effect of XOS but only a few changes in the expression of genes included in the innate immune system. Further studies could help getting more knowledge and evidence about effects of XOS.

This study investigated nine genes (RegIIIγ, Tlr2, Tlr4, Cd36, Ffar2, Cxcl1,Cxcl2, Tnf-alpha and Il-1β). In further studies more genes could be investigated, as the genes used in this study are not necessarily the most appropriate to use when investigating prebiotic effects.

In this study the normal balance of the immune system was not challenged. Future experiments should challenge the immune system when/after treating with XOS or maybe use fat mice, as they are already in an inflammatory condition. Maybe that would show more changes in the innate immune response.

SCFAs are liberated due to the fermentation of XOS, and SCFAs are often measured to determine microbial changes and prebiotics effects. An experiment measuring SCFAs in different parts of the gut and in the blood could be a way to prove beneficial effects of XOS.

In vitro fermentation of XOS with different degrees of polymerization (DP) by the intestinal digesta from different parts of the gut has been compared (Moura, P. *et al*.,2008). XOS with a DP up to 25 were fermented by ileal, caecal and colonic microbiota, but as short-chain XOS were fermented from proximal small intestine, the longer chain XOS reduced the rate of fermentation in ileum.
In this study XOS with Dp 2-6 were used. Future *in vivo* experiments could investigate XOS with different Dp and their degradation and effects throughout the GI tract.

In this study the prebiotic effects of XOS have been shown to associate with biomarkers of the immune system e.g. decrease of Il-1β in blood. Blood sampling is an easy test, and measuring of m-RNA level of Il-1β in blood could be used as a bio marker for prebiotic effect and maybe as an indicating of the SCFA level.

XOS are naturally found in bamboo shoots, fruits, vegetables, milk and honey (Casci, T. *et al*. ,2006, and can be a part of the normal human Danish food. Health benefits from prebiotics may also be obtained by incorporating them into products. Special care must be taken when using prebiotics as ingredients in food products. But properly used they may be an additional tool to combat diseases and

to improve the health and well being.

In food, prebiotic can change organoleptic characteristics and upgrade sensory properties as taste and mouth feel. Some oligosaccharides have mouth feel similar to that of table sugar (Wu, Y. & Lin, K., 2011) and some have a fat-like mouth feel, which has been reported successfully to be used as replacement of fat (Arayana, K. J. *et al*., 2007). Prebiotic can change texture which makes a significant contribution to the overall food quality.

If future studies support a beneficial effect of XOS in human as found in this study in mice, incorporation of XOS in food products could be a good idea. Beyond health benefits caused by bifidogenic effect, incorporation of XOS could give better texture and mouthfeel, reduced sugar and fat content, reduce calorie value, and increase fiber content.

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