Effect of gut microbiota on intestinal integrity

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Summary

In the human gut the complex microbial community termed the gut microbiota reside. In the present work the bacterial part of the gut microbiota is in focus. It interacts with the host and is considered to have impact on host health. The gut microbiota specifically interacts directly and indirectly with the intestinal epithelial cells that together with a mucus layer functions as the final barrier between the luminal content and the underlying host tissue. Maintenance of this barrier is extremely important, as impairment may lead to inflammation and bacterial translocation resulting in adverse effects on host health. The intestinal integrity is here considered to be mainly maintained through the mucus layer covering the epithelial cells, and the epithelial cells, which forms the barrier through interactions by tight junction proteins. Alterations of the intestinal integrity may arise based on loss or altered proliferation of epithelial cells, alterations in the mucus layer as well as altered permeability at the tight junctions. This may lead to inflammation, hence causing more impairment of the barrier.

The gut microbiota is considered to be able to affect the intestinal integrity. Specifically some bacterial strains have been shown to affect barrier function both *in vitro* and *in vivo*, and some studies have correlated specific bacterial groups with markers for intestinal integrity. Therefore modulations of the gut bacterial composition may have an effect on the intestinal integrity. In general an increase in barrier function is considered beneficial, as it must limit translocation of lumen content to the underlying intestinal tissue. In the present work the effect of modulating the gut bacterial community on the intestinal integrity was evaluated by application of the *in vitro* setup the trans-epithelial electrical resistance (TER) assay and by determination of FITC-dextran permeability *in vivo* as well as gene expression analysis of genes relevant for intestinal integrity. Changes in bacterial community were determined using culture-independent methods as quantitative PCR and high-throughput sequencing of the V3-region in the 16S rRNA encoding gene.

Changes in the faecal bacterial composition of postmenopausal women following a dietary intervention with whole-grain or refined wheat and effects of faecal water on TER were initially examined (manuscript 1). Whole grain wheat was shown to increase the relative abundance of *Bifidobacterium* spp. during the intervention while refined wheat reduced *Bacteroides* spp. Faecal water collected from both dietary intervention groups increased TER, but no difference was found between the groups. However the effect of faecal water on TER tended to correlate negatively with relative abundance of *Bifidobacterium* spp.

Previous studies have connected modulation of the gut microbial composition by prebiotics to pathogen translocation, therefore the effect of modulating the gut bacterial composition with prebiotics and the subsequent effects on intestinal integrity were examined. Initially human faecal bacterial community was modulated by *in vitro* batch fermentations with prebiotics (manuscript 2). Effects of this modulation on TER were hence to be determined. This work is still ongoing. The effect of the putative prebiotic Xylo-oligosaccharides (XOS) on intestinal permeability of FITC-dextran was determined in rats (manuscript 3). In the same study the effects of increasing the abundance of commensal *Bifidobacterium* spp. by supplementation of such a commensal strain (*B. pseudolongum*) on intestinal permeability was examined. This was done as *Bifidobac-*

terium are stimulated by prebiotics, and based on the correlation previously determined in the work. Neither XOS nor *B. pseudolongum* affected intestinal permeability of 4 kDa FITC-dextran or the effect of caecal water on TER. In the study only gene expression of the tight junction protein occludin was affected by XOS supplementation. This was connected to only minor alterations of the gut microbial composition, potentially causing the lack of alterations in the intestinal integrity.

In order to modulate the gut microbial composition extensively rats were dosed with antibiotics and permeability for FITC-dextran was determined (manuscript 4). These treatments resulted in both increased as well as decreased intestinal permeability. Specifically cefotoximin and vancomycin decreased FITC-dextran permeability and modulated the gut bacterial composition. Metronidazole was not shown to modulate the gut bacterial composition, but it increased intestinal permeability. Finally, amoxicillin modulated the gut microbiota extensively but it did not affect FITC-dextran permeability.

Conclusively the present work shows that modulation of the gut microbiota may affect the intestinal integrity. However, it can, as of yet, not be concluded in which direction the gut microbiota should be modulated to increase or decrease intestinal integrity. In conclusion the present work has led to results that extend knowledge within the research field of intestinal integrity, but more research should be done in order to clarify which bacteria or community structure have an effect on intestinal integrity.

Resume

I menneskets tarmsystem er der et komplekst mikrobielt økosystem som betegnes tarmens mikrobiota. Her er den bakterielle del af tarmens mikrobiota i fokus. Det interagerer med værten og anses for at have indflydelse på værts helbred. Tarm mikrobiotaen interagerer direkte og indirekte med tarmens epitelceller, der sammen med et mucus lag fungerer som den sidste barriere mellem det luminale indhold og det underliggende væv. Vedligeholdelse af denne barriere er derfor yderst vigtig, da svækkelse af barrieren kan føre til betændelse og bakteriel translokation, hvilket kan resulterer i skadelige virkninger på værtens helbred. Tarmens integritet anses her for at være opretholdt primært gennem mucus laget der dækker epitelcellerne, og epitelcellerne, der danner barrieren gennem interaktioner ved tight junction. Ændringer i tarmens integritet kan opstå på grund af tab eller ændret proliferation af epitelceller, ændringer i mucus laget samt ændret gennemtrængelighed ved tight junctions. Dette kan føre til inflammation, og dermed forårsager mere svækkelse af barrieren.

Tarm mikrobiotaen anses for at kunne påvirke tarmens integritet. Konkret har det været vist at nogle bakteriestammer kan påvirke barriere funktion både *in vitro* og *in vivo*, og nogle undersøgelser har korreleret specifikke bakterielle grupper med markører for tarm integritet. Modulationer i sammensætninger af tarm mikrobiotaen kan have en effekt på tarmens integritet. I almindelighed er en stigning i barriere funktion anset som gavnlig, da det kan begrænse translokation af lumen indhold til det underliggende væv. I dette arbejde er effekten af at modulere tarm bakterie sammensætningen på tarmens integritet blevet vurderet ved anvendelse af *in vitro* metoden trans- epitel elektrisk modstand (TER) og ved bestemmelse af permeabilitet af FITC-dextran *in vivo* samt genekspressions analyse af gener, der er relevante for tarm integritet. Ændringer i sammensætningen af tarm bakterier blev bestemt ved anvendelse af kultur -uafhængige metoder som kvantitativ PCR og sekventering af V3-regionen i det 16S rRNA kodende gen.

Ændringer i bakterie sammensætningen i fækale prøver fra kvinder efter-overgangsalderen efter en kostintervention med fuldkorn- eller raffineret hvede og virkningerne af fækalt vand på TER blev undersøgt (manuscript 1). Fuldkornshvede viste sig at øge den relative mængde af *Bifidobacterium* spp. under interventionen, mens raffineret hvede reducerede *Bacteroides* spp. Fækalt vand indsamlet fra begge kostinterventions grupper øgede TER, men der var ingen forskel mellem grupperne. Effekten af fækalt vand på TER havde imidlertid en tendens til at korrelere negativt med den relative mængde af *Bifidobacterium* spp.

Tidligere undersøgelser har forbundet modulation af tarmens mikrobielle sammensætning af præbiotika med translokation af patogene bakterier, derfor blev virkning af at ændret tarm mikrobiota sammensætning på grund af prebiotika og de efterfølgende effekter på tarmens integritet undersøgt. Bakterie kompositionen i fækale prøver blev moduleret *in vitro* ved batch fermenteringer med præbiotika (manuscript 2). Effekter af inducerede ændringer på TER skulle efterfølgende undersøges. Dette arbejde er endnu ikke afsluttet. Effekten af det formodede præbiotika Xylo-oligosakkarider (XOS) på tarmens permeabilitet for FITC- dextran blev undersøgt i rotter (manuscript 3). I samme studie blev virkningerne af øget mængde af kommensale *Bifidobacterium* spp. på tarm integriteten undersøgt ved at dosere med en sådan kommensal stamme (*B.*

pseudolongum). Dette blev gjort da *Bifidobacterium* bliver stimuleret af præbiotika, og baseret på sammenhængen, der blev bestem tidligere i arbejdet. Hverken XOS eller *B. pseudolongum* påvirkede tarm permeabilitet for 4 kDa FITC-dextran eller effekten af caecal vand på TER. I studiet var det kun genudtryk for tight junction proteinet occludin som var påvirket af XOS doseringen. Dette var forbundet med mindre ændringer i tarmen mikrobiotaens sammensætning, hvilket kan have forårsaget den uændrede tarmens integritet.

For at modulere tarmen mikrobiotaens sammensætning meget blev rotter doseret med antibiotika og permeabiliteten for FITC-dextran blev bestemt (manuscript 4). Disse behandlinger resulterede i både øget samt nedsat tarm permeabilitet. Specifikt faldt FITC-dextran permeabilitet, og tarmens bakterie sammensætningen blev moduleret efter dosering med cefotoximin og vancomycin. Metronidazol modulerede ikke tarmens bakterie sammensætning, men det øgede tarm permeabiliteten. Endelig modulerede amoxicillin tarmens mikrobiota meget, men det påvirkede ikke FITC-dextran permeabiliteten.

Arbejdet viser, at modulering af tarmen mikrobiota kan påvirke tarmens integritet. Det kan dog endnu ikke konkluderes i hvilken retning tarmen mikrobiota bør ændres for at øge eller mindske tarm integritet. Det præsenterede arbejde har ført til resultater, der kan øge viden inden for emnet tarm integritet, men mere forskning er nødvendig for at klarlægge, hvilke bakterier, eller bakterielle samfunds struktur, der har en effekt på tarmens integritet.

Preface

This thesis was conducted in the Gut Ecology group at the Division of Food Microbiology (Division M) at DTU Food, National Food Institute at the Technical University of Denmark (DTU) from 1st of March 2011 to 28th of February 2014 under the supervision of Senior Scientist Martin Iain Bahl and Professor mso Tine Rask Licht. The PhD study included an external research visit to the Food & Bio-based Products group at AgResearch Grasslands, Palmerston North, New Zealand led by Dr. Nicole Roy in the fall 2012. The study was supported by the Gut, Grain & Greens (3G) Research Center supported by the Danish Strategic Research Council (11-116163). According to the requirements for obtaining a PhD degree at DTU the following thesis was handed in 28th of February 2014.

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A special thanks to my sister in-law Sille and to Kristian for proof reading the thesis.

Finally a great thanks to my family, my friends, and my boyfriend for all the support, love and help. I am you eternally grateful.

Ellen Gerd Christensen, February 2014.

Contents

List of manuscripts

Manuscript 1
Christensen, E. G., Licht, T .R., Kristensen, M., and Bahl, M. I., Bifidogenic effect of whole-grain wheat during a 12-week energy-restricted dietary intervention in postmenopausal woman. (2013). European Journal of Clinical Nutrition.

Manuscript 2
Christensen, E. G., Licht, T. R., Mohan, V., Roy, N., and Bahl, M. I., Effect of prebiotics on the faecal bacterial composition in *in vitro* fermentation and on trans-epithelial electrical resistance. (2014). Manuscript in preparation

Manuscript 3
Christensen, E. G., Licht, T. R., Leser, T. D., and Bahl, M. I., Effect of Xylo-oligosaccharides and commensal bifidobacteria on gut microbial composition and intestinal integrity in male Wistar rats. (2014). Manuscript submitted to BMC Microbiology Research Notes.

Manuscript 4
Tulstrup, M. V. -L., **Christensen, E. G.**, Licht, T. R., and Bahl, M. I., Antibiotic treatment affects intestinal permeability and gut microbial composition in female Wistar rats dependent on antibiotic class. (2014). Manuscript in preparation.

Manuscript not included in the thesis
Tougaard, P., Skov, S., Pedersen, A. E., Nielsen, D. S., Bahl, M. I., **Christensen, E. G.**, Licht, T. R., Metzdorff, S. B., Hansen, A. K., and Hansen, C. H. F., The impact of TL1A on gut homeostasis in mice. (2014). Submitted to Mucosal Immunology.

Abbreviations

CD	Crohn's disease
Cldn	Claudin
CM	Conditioned media
DP	Degree of polymerization
DSS	Dextran sodium sulfat
eCB	Endocannabinoid
FITC-dextran	Fluorescein isothiocyanate-dextran
FOS	Fructo-oligosaccharides
GALT	Gut-associated lymphoid tissue
GIT	Gastrointestinal tract
HF	High-fat
IBD	Inflammatory bowel disease
IEC	Intestinal epithelial cells
IFN-γ	Interferon-γ
JAM	Junctional adhesion molecule
LPS	Lipopolysaccahride
MAMPs	Microorganism-associated molecular patterns
MAPK	Mitogen-activated protein kinase
NEC	Necrotizing enterocolitis
NOD	Nuclotide-binding oligomerization domain
Ocln	Occludin
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PKC	Protein kinase C
qPCR	Quantitative PCR
SCFA	Short chain fatty acid
TER	Trans-epithelial electrical resistance
TGF-β	Transforming growth factor-β
TJ	Tight junction
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor-α
UC	Ulcerative colitis
ZO	Zona occludens
zot	Zonula occludens toxin
XOS	Xylo-oligosaccharides

Chapter 1

Objectives of the study

Every day we consume foods that pass through the digestive system. During this passage the food is processed by the different parts of the gastrointestinal tract (GIT), but also the large microbial ecosystem in the GIT termed the gut microbiota. It consists of among other fungi, viruses, and Archaea but predominantly bacteria, that are at focus in the present work. These are separated from the underlying host tissue by the intestinal wall, specifically by a layer of epithelial cells covered by a layer of mucus. The maintenance of this epithelial barrier is therefore extremely important. Based on the close contact between the bacterial community and the intestinal barrier, the bacteria may affect the intestinal integrity. Modulation of the gut microbiota by for example dietary change or antibiotic treatment may therefore affect the intestinal integrity, and in the end human health, see fig. 1.1.

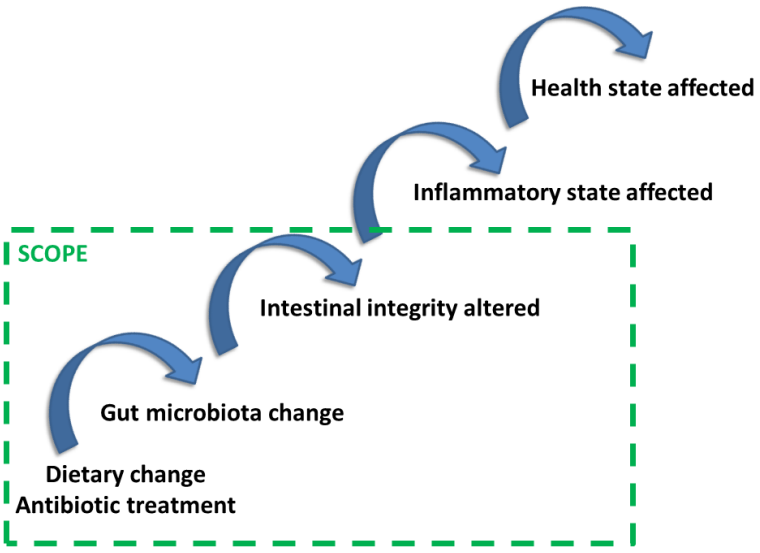


Figure 1.1: Hypothesis for interaction between gut microbiota and host health. The main focus for the present work is illustrated in the green box. The figure is a modified version of figure 8 in [1].

One of the objectives of the present study is to incorporate methods for determining intestinal integrity both *in vitro* and *in vivo* in the Gut Ecology group at DTU Food, National Food institute. Using these methods the effect of modulating the gut bacterial composition by different means as prebiotics, antibiotics, and whole-grain and refined

wheat dietary interventions on intestinal integrity was examined.

The first part of the thesis gives an introduction to the human digestive system, the gut microbiota, and the intestinal wall. Here the main focus is on the mucosa specifically the mucus layer and the epithelial cells, as they form the main barrier towards the luminary content. Epithelial cells interact through among other tight junctions. Modulation of protein interactions in the tight junctions are important for intestinal integrity, hence this complex is introduced. In addition intestinal integrity and modulation of such by bacteria, products from the bacteria as well as mechanisms that the host may use is described. Finally, modulation of the gut microbiota by prebiotics, putative prebiotics, antibiotics, and whole-grain products, and its effects on intestinal barrier integrity are presented. In the later parts of the thesis a section describes the methods used in the present work. Manuscripts prepared during the work are included, before outcomes and conclusions of the PhD study are discussed.

Chapter 2

Introduction

2.1 The human gastrointestinal tract

Humans consume food and drinks daily, most of which the body utilise for energy. But in and on these foods several different microorganisms resides, which are passed down the gastrointestinal tract (GIT) with the food. The food is consumed through the mouth, where it is chewed and swallowed, before passing through the eating tube down to the stomach, see fig. 2.1. In the stomach digestive enzymes as pepsin are released, as well as hydrochloride acid, which causes the pH to drop. The food content passes through the different sections of the small intestine; the duodenum, jejunum, and further on to the ileum, while pH increases (from 6.6 to 7.5 [2]) as bicarbonate is released. During this passage more digestive enzymes are released resulting in cleavage and absorption of carbohydrates, proteins, and lipids. Finally, the luminal content is passed to the colon (pH 6.4-7 [2]), which is divided into ascending, transcending, descending, and sigmonal parts. Here water and salts are absorbed. The remaining of the luminal content is transported out of the body at rectum as faeces [3].

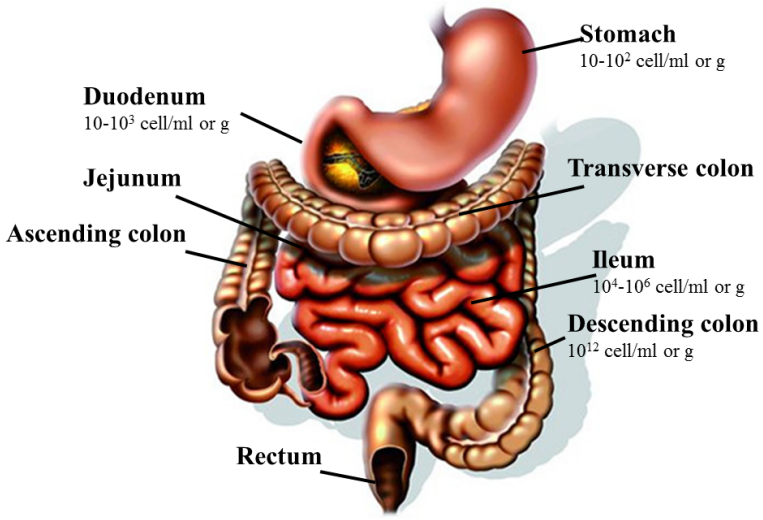


Figure 2.1: Overview of the human digestive system. The figure is a modified version of a figure from [4]. The stated number of cells is the approximately number of microorganisms per g or mL of content according to [5].

The environment in the GIT is generally anaerobic, but environmental conditions such as pH, transit times, and availability of nutrients or substrates change throughout the GIT. This has an effect on the microbial ecosystem in the gut leading to different numbers of microorganisms with an accumulation at the colon, see fig. 2.1.

2.2 Gut microbiota

The complex microbial ecosystem in the GIT is termed the gut microbiota. The gut microbiota consists of the entire microbial community in the human gut including bacteria, yeast, fungi, Archaea, and viruses, resulting in 10^{13} to 10^{14} microorganisms [6]. In this thesis the main focus is on the bacterial part of the gut microbiota, as this is predominant [6, 7, 8, 9]. This will accordingly be termed the gut microbiota or the gut bacterial community.

The bacteria in the gut reside there due to utilisation of the available substrates, but also based on natural selection from the host [9]. This has led to a relationship between host and the gut microbiota that gives mutual benefits [10]. Specifically the bacteria in the gut can form symbiotic and commensal relations with the host [11]. The host benefits from the bacterial community in the GIT, as it among other can degrade otherwise in-digestible substrates for the host. The host may subsequently use the produced metabolites. Additionally the gut microbiota protects the host from pathogenic bacteria by reducing availability of substrates and space [10].

Despite the complexity of the ecosystem it is mainly represented by a limited number of bacterial phyla, as Firmicutes, Bacteroidetes, Proteobacteria, Fusobacteria, Verrucomicrobia, and Actinobacteria, with Firmicutes and Bacteroidetes as dominant phyla, see fig. 2.2 [7, 8, 12]. Within the Firmicutes phyla the main genera found are *Faecalibacterium*, and *Roseburia*, see fig. 2.2 [8]. These genera include species that are able to produce butyrate, as they belong to specific *Clostridium* clusters [7]. For example *Faecalibacterium* and *Roseburia* belong to the *Clostridium* clusters IV and XIV, that are considered to produce butyrate [13], which at low concentrations have been shown to increase epithelial barrier function [14, 15]. Besides the butyrate producing bacteria the Firmicutes also include the *Lactobacillus* genus. Specific strains of *Lactobacillus* are used as probiotics. Probiotics was in 1998 defined as: "*living micro-organisms, which upon digestion in certain numbers exert health benefits beyond inherent basic nutrition*" according to [16]. Therefore are stimulation of these bacteria are considered to have beneficial effects on host health [17].

The other predominant phylum is Bacteroidetes. The main genera within the Bacteroidetes phyla are *Bacteroides*, *Alistipes* and *Prevotella* [8], while *Bifidobacterium* and *Collinsella* are the main genera representing the *Actinobacteria*, see fig. 2.2 [8]. *Bifidobacterium* strains are also used as probiotics. Overall the gut microbiota consists of different bacterial phyla and genera where some may affects the host.

The bacteria in the bacterial community do not only affect the host but also each other. This can occur through production of toxins or competition for available substrate but also through cross-feeding. In the colon the available substrate is mostly the parts of the diet, which the host has not or cannot degrade, such as dietary fibers and some proteins. These are degraded by saccharolytic and proteolytic fermentation, respectively. Proteolytic fermentation is mainly conducted by bacteria from the bac-

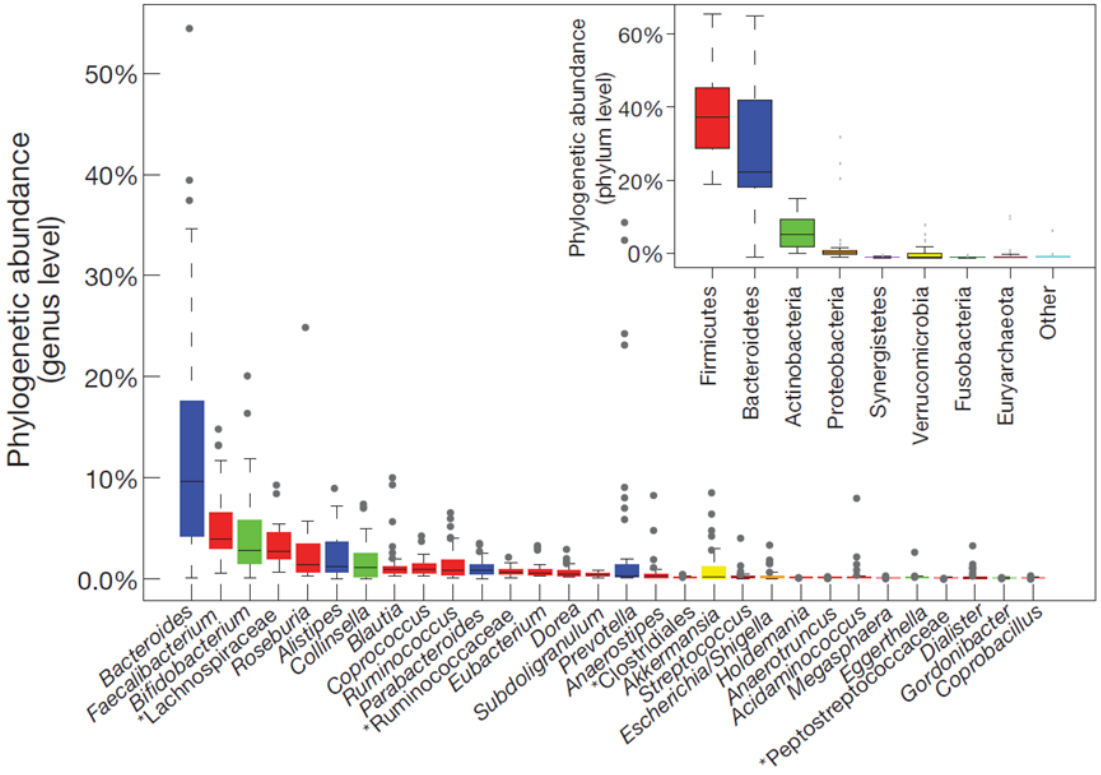


Figure 2.2: Diversity of bacterial genera in the human gut. The figure is a copy of figure 1B in [8]. *; indicates family level.

teroides and clostridia groups, while saccharolytic fermentation is mainly conducted by genera as *Bacteroides*, *Ruminococcus*, *Clostridium*, *Bifidobacterium*, and *Lactobacillus* leading to production of lactate and short chain fatty acids (SCFA) [5]. Some metabolites as lactate are subsequently utilised by other bacteria in the ecosystem, hence causing cross-feeding [18]. For example *Bifidobacterium* may degrade carbohydrates leading to lactate formation that other bacteria can utilise, hence potentially producing SCFA [5, 18]. Based on cross-feeding, and the inhibitory effects the bacteria may have on each other, dietary changes may modulate the abundance of specific bacterial groups that subsequently affect host health directly or indirectly by stimulating or reducing other bacterial groups, that then may affect host health.

There have been some discussions regarding the presence of a "core microbiome". Such a "core microbiome" would be highly relevant for evaluation of gastrointestinal disorders, and potentially increasing human health by modulating the microbiota in the direction of such a “core microbiome”. However, there is a high inter-individual difference between the gut microbiota of different humans, as the microbiota of an individual over time is more similar than the microbiota between relatives [19]. This makes the identification of a "core microbiome" difficult. Despite this Arumugam and coworkers in 2011 [8] published a study stating, that the human gut microbiome could be classified into three groups termed enterotypes. These enterotypes were driven by the bacterial genera *Bacteroides*, *Prevotella*, and *Ruminococcus* and seemed to be dependent on the

substrates used to generate energy [8]. Hence not a direct core microbiota, but distinct types of gut microbial compositions may exist. These may affect the possibility to modulate the microbiota, and use it a therapeutic target.

The mucus-associated bacterial community

The intestinal wall has an outer layer of mucus, see section 2.3.1, leading to a sub-environment within the intestinal lumen. Here bacteria can reside and utilize mucus for energy [20]. For example *Akkermansia muciniphila* have been found to utilize mucus as the sole energy source [21]. According to Van den Abbele bacteria residing in the mucus are not targeted by the host immune system, while bacteria residing in the intestinal lumen are [22]. Hence a specific bacterial community must reside in the mucus layer; here termed the mucus-associated bacterial community. The mucus-associated community is in close contact with the epithelial cells in the intestinal wall making these bacteria and their metabolites important for barrier function. Specifically these bacteria may produce SCFA, stimulate the immune system, and limit pathogen adhesion to the mucus layer [20]. This may all be important for maintaining barrier integrity.

In general, the mucus-associated bacterial community is considered to be different from the faecal bacterial community [7, 23, 24]. But of course the faecal bacterial community contains some of the bacteria found in the mucus, as these are transported out of the intestine with the faecal. In the stomach the mucus-associated community was found to include the phyla Proteobacteria, Firmicutes, Bacteroidetes, Actinobacteria, and Fusobacteria with a decreasing number of sequences in mucosal biopsies [25]. In mucosal biopsies from jejunum, distal ileum, ascending colon, and rectum Firmicutes and Bacteroidetes were the predominant phyla, but Proteobacteria, Fusobacteria, and Verrucomicrobia were also identified [26]. In distal ileum, ascending colon and rectum sequences belonging to the *Clostridium* clusters IV and XIVa were predominant [26]. Additionally in colon mucosal samples Firmicutes, Bacteroidetes, and Proteobacteria were predominant, but Fusobacteria, Cyanobacteria, Verrucomicrobia, Actinobacteria, Lentisphaera and TM7 have also been identified [27, 28]. In colonic mucosa genera such as *Clostridium*, *Faecalibacterium Ruminococcus*, *Lactobacillus*, and *Bacteroides* have been identified [24, 27]. Finally in rectal biopsies Firmicutes and Bacteroidetes were predominant, while Proteobacteria was present in lower levels in faeces than in rectal biopsies. Actinobacteria, Fusobacteria, Gemmatimonadetes, Lentisphaerae, Tenericutes and Verrucomicrobia were detected in the rectal biopsies, however lower levels were detected in faeces [23]. Hence in general the mucosal-associated microbiota consists of phyla and genera that can also be found in the intestinal lumen, hence the general gut microbiota. But the distribution or abundance of these is different than in faeces. The presence of among other Actinobacteria, *Clostridium*, *Ruminococcus* and *Lactobacillus* indicates that the mucus-associated community may affect the intestinal barrier through production of SCFA directly or through cross-feeding.

As the mucus-associated bacteria are in close contact with the epithelial cells, changes in the bacterial composition or dysbiosis in this niche may have direct effect on the epithelial cells, subsequently human health. For example patients with colorectal adenomas (tumor) have been shown to have a higher abundance of Proteobacteria and lower abundance of Bacteroidetes in the mucus-associated community than healthy participants [27]. The mucus-associated community has also been shown to be different in Crohn's dis-

ease (CD) and Ulcerative Colitis (UC) patients with a tendency for lower abundance of Firmicutes and higher abundance of Bacteroidetes than healthy individuals [28]. Additionally inflammatory bowel disease (IBD) patients had a lower bacterial diversity in colon mucus than healthy individuals [28]. As the stated disorders have been connected to impaired intestinal integrity [29], the dysbiosis in the mucus-associated community may have affected the barrier function.

2.3 The intestinal wall

The intestinal wall is the sole barrier between the luminal content including the gut microbiota and the underlying tissue. The intestinal wall is build up by four layers; the mucosa, submucosa, muscularis, and serosa, as illustrated in fig. 2.3. The serosa and the muscularis, consist of muscular tissue, and form the outer layers of the intestine. Closer to the lumen, the submucosa is found. It consists of connective tissue, blood vessels, and lymph vessels. The mucosa is in close contact with the gut microbiota and the luminal content [3], making it the main focus in the present work.

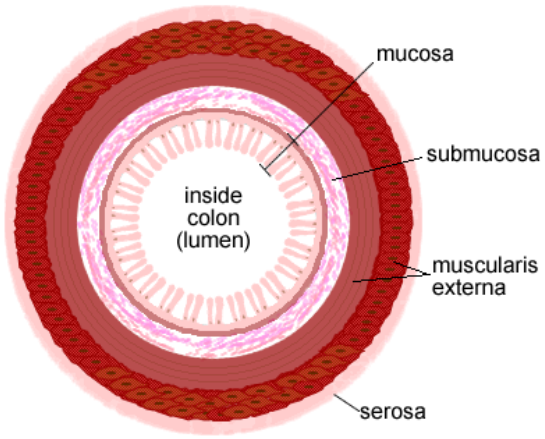


Figure 2.3: Cross section of the colon. The mucosa is at main focus here. It consists of a mucus layer covering the underlying layer of epithelial cells. Beneath the epithelial layer the lamina propria reside. The figure is copied from [30].

The mucosa has two purposes; to protect against luminal content and secondly to work as a selective barrier for nutrients, water, ions etc. Maintenance of the mucosal barrier is therefore extremely important. The mucosa is highly folded in the small intestine resulting in the formation of crypts and villi, while there are no villi in the colon [3, 31]. The mucosa consists of a layer of intestinal epithelial cells (IEC), which is covered by a mucus layer, see fig. 2.4. Under the epithelial cells in the Lamina propria the host immune cells reside in the gut-associated lymphoid tissue (GALT). The GALT is outside the main focus here, and is therefore not described. The mucus and IEC layer are described below.

2.3.1 The mucus layer

The IEC are covered by a mucus layer that protects IEC from the luminal content, including irritants, microbial attachment, and invasion [10, 32]. This layer consists of mucin, IgA, trefoil factor, antimicrobial peptides, water and ions, and has a gel-like structure, see fig. 2.4 [10, 32, 33, 34]. The antimicrobial peptides must help to limit microbial growth in the mucus layer, hence limiting direct contact between bacteria and IEC. Mucin, more precisely mucin-2 (MUC2), is the main component of the mucus layer in the intestine, and is secreted by goblet cells in the epithelial layer. When MUC2 is released from the goblet cells its volume increase, and it immediately becomes part of the mucus layer [34].

In the small intestine the mucus layer is not well defined, whereas it is well-defined and dense in the colon, see fig. 2.4 [34, 35]. It has been suggested, that the thickness of the mucus layer is correlated to the bacterial load [10]. In the colon, the mucus layer can be divide into two layers. A firm layer close to the IEC, where no bacteria reside, and a loose layer above, where bacteria can reside, see fig. 2.4 [36]. Accordingly the mucus-associated microbiota must be found in this looser part of the mucus layer. This loose top layer is considered to arise by proteolytic cleavage of MUC2 [36]. Despite the presence of bacteria in the loose part of the mucus layer, the IEC are still protected from the majority of the luminal content. As the mucus protects the IEC, the maintenance of the mucus layer is important for the intestinal integrity.

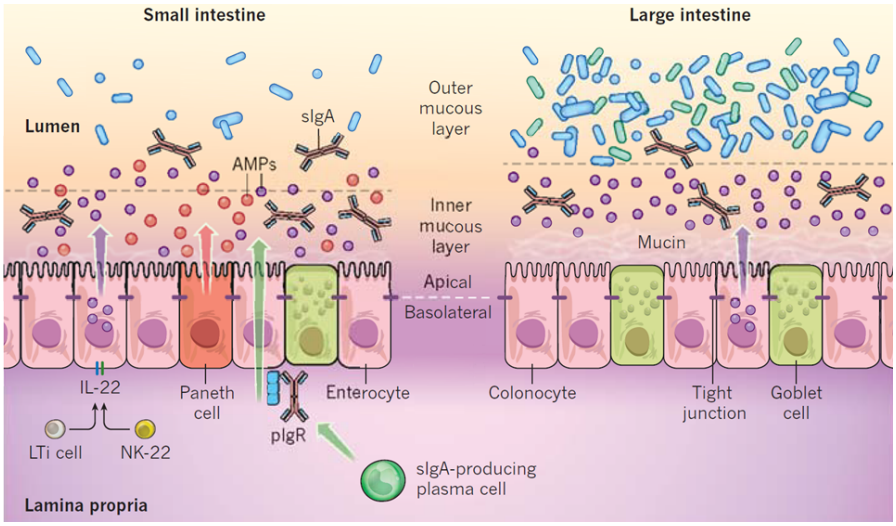


Figure 2.4: The epithelial layer in the small and colon (large intestine). The figures are copy of figure 2 in [10]. The blue rods represent the gut microbiota.

2.3.2 The epithelial layer

The only 20 µm thick epithelial layer is the outer cell layer of the intestinal wall, making it the final cell barrier between the luminal content and the host [37]. In general IEC are polarised cells with an apical surface towards the intestinal lumen with microvilli, while the basolateral surface, towards the underlying tissue, does not have villi, see

fig. 2.4. The selective barrier is constructed by the IEC and contact between adjacent cells. IEC have to initiate an immune response upon bacterial invasion, but also maintain homeostasis upon contact with the gut microbiota [37], as it otherwise could lead to a constant inflammatory state. In order to have this complex barrier function, as well as absorb nutrients from the lumen, the epithelial layer consists of several cell types each with their own specific task. These include M cells, goblet cells, Paneth cells, and absorptive cells termed enterocytes in the small intestine and colonocytes in colon, see fig. 2.4 [10, 31]. Also enteroendocrine cells, that secrete hormones are part of the IEC layer [31]. The IEC emerges from stem cells, that differentiate to the different cell types during the migration from the crypt to the villi[31, 38]. The different IEC cell types are shortly introduced.

Enterocytes

Enterocytes (and colonocytes) are highly polarised and are the main cell type in the epithelial barrier constituting up to 80 percent of IEC in the epithelial layer [31]. As the enterocytes are the predominate cells in the epithelial barrier, they and their interactions are mainly responsible for maintaining the barrier integrity. Besides this important task, the enterocytes absorb and transport nutrients from the intestinal lumen and into the blood stream [31], and secretes antimicrobial peptides as β-defensins and cathelicidins [37].

Goblet cells

Goblet cells are the main mucin producers in the epithelial layer. After differentiation they contain granules with mucin, which they release during migration from the crypt. Once reaching the villi the goblet cells have secreted the mucin granules, but also organelles trap between the granules, hence the goblet cell are released into the intestinal lumen, leading to a constant replacement of the goblet cells [32]. Goblet cells are found in the entire intestine, but the number of goblet cells increases during the passage through the GIT [32]. Subsequently the level of mucin and thickness of the mucus layer also increases leading to the thick mucus layer in the colon. In addition to secreting mucin goblet cells also release intestinal trefoil factor (TFF) and Resistin-Like Molecule-β (RELM-β) that is part of the innate immune system [39].

Paneth Cells

Paneth cells are a pyramid shape cell type that does not migrate from the crypt towards the villi but reside at the crypt in the small intestine, see fig. 2.4 [31, 38, 40]. In the crypt the Paneth cells take part in the innate immune system as they have granules containing lysozymes, α-defensins, and antimicrobial peptides that are released upon close contact with bacteria but also by cytokine and hormones stimuli [31, 37, 40]. These anti-microbial peptides are considered to be important for maintaining intestinal homeostasis, since they in several studies have been shown to be important for defense against inflammation [41]. In addition to the antimicrobial peptides, Paneth cells have been suggested to produce cytokines [40], making them important for communication between the epithelial layer and the underlying immune system.

M cells

In addition to the already stated cells, a cell type with a different structure termed M cells is found. In humans they do not have microvilli, but microfolds on the apical side of the membrane. These cells are mainly placed above the Peyer's patches in the small intestine, which are part of the GALT. But M cells are also found in the colon. M cells are filled with lymphocytes, as the basolateral side of the M cells has pockets that the lymphocytes can reside in [42]. Antigens are transported from the intestinal lumen across the M cells to the immune cells, resulting in antigen presentation and activation of immune cells e.g. B cells that then secrete IgA [42, 43]. M cells are therefore rather important for the communication between the luminal environment and the immune system.

In summary the main barrier between the luminal content and the underlying host tissue is a layer of IEC covered by a mucus layer. Maintenance of this barrier is therefore important for intestinal integrity. Impairment of the barrier may occur by changes in the thickness of the mucus layer, loss of IEC, or decreased interaction between the epithelial cells. The specific proteins involved in IEC interaction are described in the following paragraph.

2.3.3 Tight junction proteins

Interaction between IEC is important for maintaining intestinal barrier integrity. IEC interacts through tight junctions (TJ), desmosomes, adhesion junctions, and gap junctions, see fig. 2.5 [39, 44]. TJ are mainly responsible for controlling the paracellular flux between the epithelial cells, hence the permeability of the epithelial barrier, while desmosomes and adhesion junctions are important for communication and binding between the cells [44]. Therefore only the TJ are presented here.

TJ is a complex of several proteins that reside in the apical section of the epithelial cell, see fig. 2.5 [44]. These proteins includes both membrane bound and intracellular proteins that interact between adjacent cells, but also with the cytoskeleton in the cell [39, 44]. This helps stabilising the TJ, but also makes it possible for the cytoskeleton to regulate interactions between IEC [44]. The most relevant TJ proteins, for the present work, are introduced.

Occludin

Occludin (Ocln) is a transmembrane protein with four transmembrane regions, that are linked with two extracellular loops, and one intracellular loop, see fig. 2.5. The extracellular loops of occludin interact between adjacent IEC, forming a barrier between the cells. On the cytoplasmic side occludin has a short N-terminal and a longer C-terminal that interacts with the intracellular proteins in the TJ [44].

The importance of occludin in the formation and maintenance of TJ has been examined in occludin-deficient mice models and Caco-2 cells [45, 46, 47]. Occludin deficiency did not affect electrical resistance across intestinal sections, or mannitol permeability in mice [45, 47]. But the mice suffered from chronic inflammation in the gastric epithelium [47] and had decreased secretion of gastric acids [45]. Based on these results the authors considered occludin to have a regulatory function in the TJ or regulation of IEC differ-

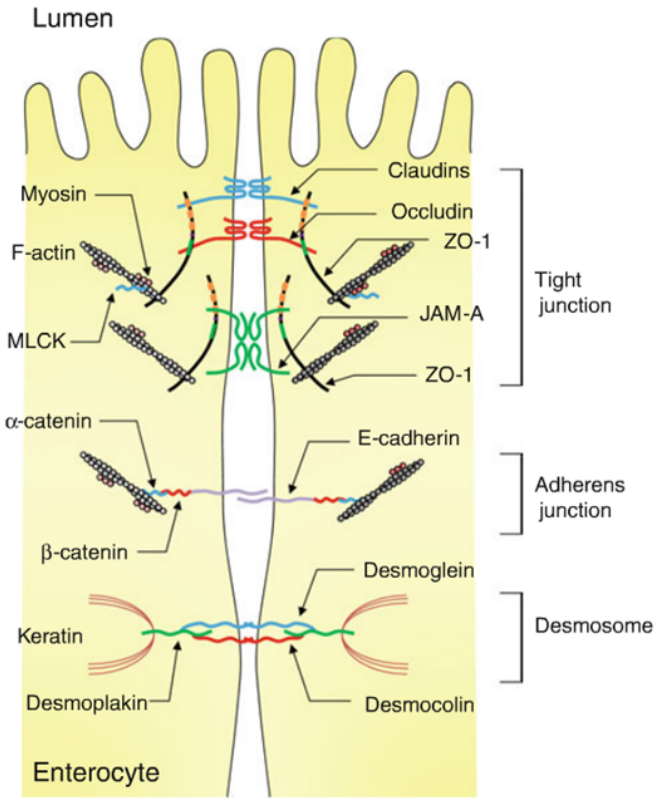


Figure 2.5: Interaction between epithelial cells through tight junction (TJ), adherens junction, and desmosome. The figure is a copy of figure 2 in [44].

entiation, but occludin was not considered to be essential for formation of TJ [45, 47]. Contradictory to this, Al-Sadi and co-workers found that depletion of occludin in mice and Caco-2 cells increased the flux of macromolecules, indicating that occludin is important for the paracellular flux of macromolecules [46]. However, this was not connected to a decrease in electrical resistance across the cell layer [46]. Since electrical resistance, must indicate the permeability of ions, which are be able to pass through the same areas as macromolecules, it is puzzling that Al-Sadi and co-works did not observe changes in this parameter.

Based on these studies it is difficult to clarify if occludin is essential for the formation of the TJ. However, as depletion of occludin affected the expression of another TJ protein claudin-2 at mRNA and protein level [46], and occludin-deficient mice had inflammation in the gastric epithelium [47], occludin may have importance for intestinal integrity.

Regulation of occludins interactions and localisation have been linked to phosphorylation [44]. For example some studies have shown that phosphorylation and de-phosphorylation of occludin by protein kinase C isoforms and the phosphatases PP2A and PPI regulates the assembly and disassembly of the TJ complex, respectively [48].

Claudins

In general the claudins (Cldn) are considered to be the backbone of the TJ complex [44], making these proteins very important for maintaining the intestinal integrity. The claudins are all membrane spanning with a short intracellular N-terminal and a long intracellular C-terminal, an intracellular loop, two extracellular loops, and domains spanning the membrane four times, see fig. 2.5 [49]. On the cytoplasmic side the claudins interact with zonula occludens (ZO) at the C-terminal, anchoring the claudins to the cytoskeleton, see fig. 2.5 [49, 50].

Through the extra cellular loops the claudins from adjacent cells interact with each other or other proteins, forming a barrier or pores [44, 50]. Specifically the first loop is considered to influence charge selectivity [49]. It seems that the claudins can be subdivided into two groups, based on whether they are part of forming a barrier or pores. The barrier-forming claudins are considered to be claudin-1, 3, 4, 5, 8, 9, 11, and 14. While the pore-forming claudins are claudin-2, 7, 12, and 15 [44]. The pore forming claudins may form a pore in the TJ, where molecules or ions of specific charge and size can pass. For example the number of pores that transport small molecules are increased by the induction of claudin-2, while mannitol flux is unaffected [51].

The expression level of claudins varies throughout the GIT [29, 44]. In duodenum and colon mainly the barrier forming claudins, as claudin-1, 3, 4, 5, and 8 are expressed while in ileum and jejunum the pore-forming claudins, as claudin-2, 7, and 12 are expressed more [29]. However, both pore- and barrier-forming claudins seem to be expressed in all the sections in the GIT [29]. This makes sense biologically, since nutrients are mainly absorbed in the small intestine, while the absorption is low in the colon.

The regulation of the claudins still needs to be elucidated. However phosphorylation of claudins is considered to affect localization and the interaction with other proteins, hence affect the permeability of the intestine [44, 50]. Claudins must be important for the intestinal integrity, as up-regulation of claudin-2 have in some studies been connected to CD and UC, while other claudins, as claudin-1 was down-regulated [29].

Zonula occludens

Zonula occludens (ZO) proteins are the intracellular proteins in the TJ, see fig. 2.5. There are three ZO proteins; ZO-1, ZO-2, and ZO-3, where ZO-1 have been studied most regarding effect on intestinal integrity. The ZO proteins have several domains all important for the regulation and maintenance of the TJ structure, see fig. 2.6.

The domains include three PDZ[1] domains, a SH3[2] domain, and a GUK[3] domain. Membrane proteins in the TJ interact with ZO through these domains, for example the claudins binds to ZO-1 at the PDZ1 domain, see fig. 2.6 [44]. Subsequently all the membrane proteins in the TJ can interact with one or more of the ZO proteins. Since ZO proteins also are able to bind to each other and the actin skeleton, see fig. 2.6 [44], the TJ proteins seem to interconnect with each other and the actin skeleton. The ZO proteins are therefore important for the assembly of the TJ complex, but also for the interaction between the different proteins in the TJ and the actin skeleton.

[1] Post-synaptic density 95/Drosophila discs large/zona-occludens 1
[2] Src homology-3
[3] region homolog to guanylate kinase

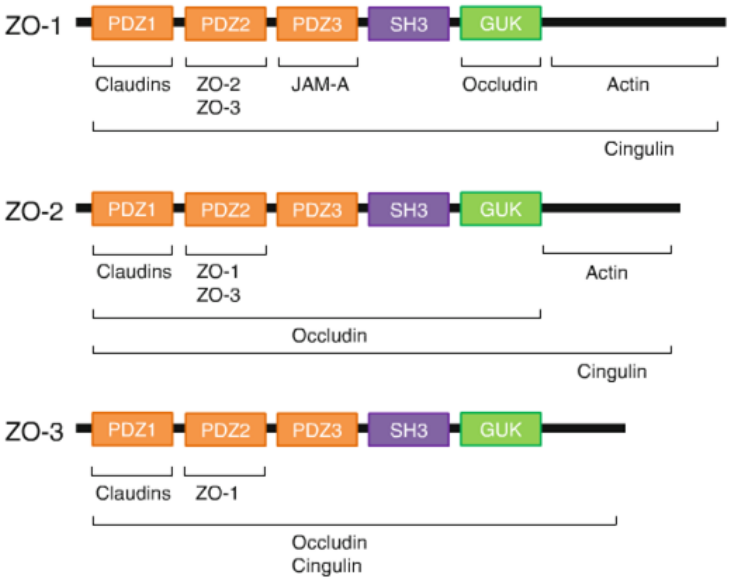


Figure 2.6: Structure of Zonula occludens proteins and which regions that interact with other proteins in the TJ. The figure is a copy of figure 3 in [44].

Other TJ proteins

In addition to the already mentioned TJ proteins, Junction Adhesion Molecule (JAM), tricellulin, and cingulin are also proteins part of the TJ. These are only mentioned here, as they either have not been examined in the present work, or their relevance for intestinal integrity have not been examined that intensively.

2.3.4 The selective barrier

The intestinal barrier needs to function as a selective barrier. Luminal bacteria should not cross the barrier, but nutrients, ions, vitamins etc. need to be absorbed or transported across the IEC barrier before utilisation by the host. This can occur through transcellular transport or paracellular transport across the epithelial layer, all leading to a selective barrier. Transcellular transport occurs through specific transport proteins or pores, while the paracellular transport occurs between the epithelial cells, hence across the TJ. Regarding intestinal integrity the paracellular transport is at main focus. Transport through the paracellular route is dependent on

- concentration gradient,
- surface area,
- time,
- the permeability of the barrier [52].

Regulation of the paracellular route is mainly through the interaction between adjacent cells so through the TJ. Alterations in TJ structure is therefore in main focus here.

Pore and *leak* pathway

There has been some evidence, that the TJ is not merely static, and that it may have different sizes of pores that molecules can pass through. This has led to the hypothesis of existence of two pathways within the TJ; the *pore* and the *leak* pathway.

In the 1970'es Claude [53] found an exponential relation between the number of TJ strands and electrical resistance across the epithelial layer. As the electrical resistance is a measure for the ion flux, the TJs across the epithelium had been considered to form a series of resistors across the epithelial layer. This would lead to a linear relation between number of TJ and resistance. But as the relation was shown to be exponential, Claude considered the TJ to contain pores for ions that could be opened or closed [53]. Later glucose was found to increase the flux of mannitol but not CrEDTA and inulin across intestinal tissue [54]. Hence the glucose must have affected pores of different sizes in the tissue. The authors therefore suggested a 2-3 pore system [54].

The existence of two pathways in the TJ was supported by determining permeability of probes or tracer molecules of different sizes simultaneously across cell lines as Caco-2 and T84 and pig ileum [51, 55, 56]. Tracers below 4 Å had a high flux, while larger molecules had a very low flux. Exposure to agents (EGTA, sodium caprate, and IFN-γ) that disturbed the TJ, showed that permeability for the different sizes probes, were affected differently by these agents [55]. EGTA and IFN-γ caused increased permeability for all size probes but especially larger probes, while sodium caprate caused an increase in flux for all probes [55, 56]. Based on these results, the authors supported the hypothesis of two pathways for permeation; one for small molecules, and one for larger molecules [55, 56]. Later the two types of pathways have been termed *pore* and *leak* pathway [57]. Whether the authors considered the two pathways to be based on presence of different types of TJ e.g. some that after stimuli results in flux of larger probes while other TJ would result in increased flux of smaller molecules, or if different agents caused different modulation of a general TJ complex leading to either of the pathways is unclear. In general a common TJ complex that is regulated by specific stimuli seems most preferable. However, as the expression of claudins changes through the GIT, this could indicate different structures of the TJ leading to the two pathways.

The presence of the pore and leak pathways in the TJ complex mean that changes in the TJ complex affects flux of different sized molecules. A change in the pore pathways will affect the ion conductance, hence uptake of e.g. calcium. It will also affect the electrical resistance across the epithelial layer. A change in the leak pathway would affect flux of larger molecules and electrical resistances [58]. In addition an increase in the leak pathway may increase translocation of luminal content, that may stimulate an immune response that could result in an inflammatory state. But the leak pathway is not large enough to let whole bacteria pass through the TJ [59]. However changes in the leak pathway may cause more adverse effects to the host, than changes in the pore pathway would, as an immune response may be induced, that potentially could lead inflammatory state and subsequent bacterial translocation.

2.4 Interaction between host and gut microbiota

The gut microbiota is in close contact with the mucosa. Therefore the gut microbiota and its products must affect the host, and vice versa. Of course the mucus-associated

Table 2.1: Ligand MAMPs for TLR and NOD-like receptors. The table is a modified version of table 1 in [60].

TLR	**MAMPs**	**Bacteria**	**Location on IEC**
TLR1	Triacyl lipopeptides	Mycobacteria	Surface membrane
TLR2	Peptidoglycan lipoteichoic acid	Gram-positive	Surface membrane
TLR4	LPS	Gram-negative	Basolateral membrane, endosomal membrane
TLR5	Flagellin	Bacteria	Basolateral membrane
TLR6	Diacyl lipopeptides	Mycobacteria	Surface membrane
TLR9	cytosine-phosphate-guanosine oligodeoxynucleotides (CpG-ODN)	Bacteria	Endosomal membrane
NOD-like receptor	**MAMPs**	**Bacteria**	**Location on IEC**
NOD1	Meso-lanthionine meso-diaminopimelic acid	Bacteria	Cytoplasm
NOD2	Muramyl dipeptide	Bacteria	Cytoplasm

bacteria must be in closer contact with IEC, than bacteria only residing in the lumen. Mucus-associated bacteria may therefore be very important for stimulation of the host immune system [22], but also intestinal integrity. However, bacteria residing in the lumen may also have an effect. Interaction between gut microbiota and IEC is therefore important for intestinal integrity. This interaction is based on a balanced system, where the host must not induce inflammation upon contact with commensal bacteria, as it would result in a constant inflammatory state, hence impaired intestinal integrity. But the host must induce an immune response upon bacterial invasion. Therefore the host cells need to differentiate between bacteria in the gut microbiota.

Epithelial cells and dendritic cells have receptors that can recognize microorganism-associated molecular patterns (MAMPs) [22]. MAMPs include peptidoglycans, lipoproteins, flagellins, lipopolysaccharides (LPS), and teichoic acids [22]. These comes from different types of bacteria, see table 2.1 [22]. Hence, the host cells distinguish between bacteria from the gut microbiota based on different compounds from the bacteria. The receptors on the epithelial cells, that recognize the MAMPs are the Toll-like receptors (TLR) and the nucleotide-binding oligomerization domain (NOD) like receptors, see table 2.1 [22]. TLR are situated on the outer surface of the epithelial cells, both on the apical and the basolateral side, but also on endosomal membranes, while NOD-like receptors are present on the cytoplasmic side of the cells, see table 2.1 [60]. Host cells can therefore identify MAMPs and/or bacteria that have translocated across the epithelial layer to the basolateral side of the epithelial cells, or have been engulfed by the host cell, as well as MAMPs or bacteria residing in the intestinal lumen [10]. Bacteria and/or MAMPs can make such translocations across the epithelial barrier through engulfment by immune cells, as dendritic cells, through M cells, and potentially through loose TJ [22].

In general, when MAMPs bind to TLR or NOD-like receptors it starts a signaling cascade involving NF-κB [22, 37]. The following response has been found to depend on what kind of MAMPs or bacteria that bind. Activation of TLR or NOD-like receptors by a pro-inflammatory stimuli leads to a cascade of phosphorylations, resulting in ubiquitination and degradation of IκB, so it disassociate from NF-κB. NF-κB then translocate to the nucleus where it functions as a transcription factor for pro-inflammatory genes [37]. This reaction may occur continuously in the intestine, as luminal bacteria may stimulate TLR on the epithelial cell apical surface constantly. Conditioned media from some commensal bacteria and/or the commensal bacteria itself have been found to stimulate NF-κB activity *in vitro*, an example being *Bacteroides uniformis* and *Clostridium sardiniensis* [61]. Other commensal bacteria as *Bifidobacterium breve*, *Bacteroides thetaiotaomicron*, and *Ruminococcus gnavus* did not affect NF-κB activity, while *Collinsella aerofaciens* inhibited NF-κB activity [61]. The commensal bacterium *Bacteroides thetaiotaomicron*, has also been found to induce an anti-inflammatory response by increasing the export of the NF-κB subunit (RelA) from the nucleus, so NF-κB had limited effect as a pro-inflammatory transcription factor [62]. Non-pathogenic *Salmonella* can also limit NF-κB activation by inhibiting degradation of IκB [63]. Based on this the commensal bacteria may modulate immune response in the epithelial layer through their interactions with the TLR, leading to an anti-inflammatory response, no response or a pro-inflammatory response, it is depending on the bacteria and MAMPs. This must limit the constant pro-inflammatory stimuli other bacteria in the gut microbiota have leading to homeostasis.

The homeostasis in the epithelial layer must also be affected by the placement of the TLR and NOD-like receptors, see table 2.1, as well as the expression of these. It has been suggested that epithelial cells have a lower response to TLR2 ligands (Gram-positive bacteria), see table 2.1, as TLR2 are expressed lower here [64]. Location of the receptors must also have an effect. For example TLR4 is located on the basolateral side of the epithelial cell, hence only bacteria or MAMPs reaching the basolateral side of the IEC may stimulate TLR4. While NOD-like receptors are activated by bacteria or MAMPs that reach the cytoplasm, see table 2.1. Therefore an immune response must be initiated if bacteria pass the epithelial barrier.

Conclusively the host can sense the bacterial community, this may in some cases cause a pro-inflammatory response, while an anti-inflammatory response is induced by other bacteria. However overall the host mucosa does not seem to be in a constant inflammatory state, hypothesising that the microbiota may not induce or counteracts pro-inflammatory stimuli, until a pro-inflammatory suppress this effect e.g. during pathogenic infection. The interaction between epithelial cells and the gut microbiota is of course important for intestinal integrity. If inflammation arises it may cause impairment of the epithelial barrier hence affecting intestinal integrity.

2.5 Intestinal integrity

The epithelial layer is the final barrier between the luminal content and the host. Maintenance of this barrier is therefore extremely important. Imbalance in barrier function has been connected to diseases as IBD, irritable bowel syndrome, and Celiac disease [29, 65]. Due to the close connection to gastrointestinal disorders, intestinal integrity may

become a very relevant research field within gut ecology, gastrointestinal disease, and effects of functional foods.

2.5.1 Definition of intestinal integrity

The word *"integrity"* is defined as

"*The state of being whole and undivided*" [66].

Considering the definition of the word *integrity*, intestinal integrity is here defined to deal with maintaining the intestinal barrier whole and assembled. This occurs through the mucus layer, the epithelial cells, and the connection between the IEC by TJ. Changes in intestinal integrity may therefore arise by

- changes in the mucus layer,
- changes in epithelial cell proliferation or cell death,
- changes in connection between adjacent cells by the TJ.

The term intestinal integrity is quite often used in connection with intestinal permeability. These two terms must be closely related, however they do not cover the same. Intestinal integrity is defined above, while intestinal permeability here is considered to reflect the passage of molecules across the IEC layer. Such a passage can be affected by the epithelial cells forming the barrier, but also interactions by TJs. However, changes in mucus layer alone may not affect the permeability. Hypothesizing that intestinal integrity is accomplished by the mucus layer, the epithelial layer, as well as TJs, while permeability does not cover the mucus layer. Based on this assumption, alterations in intestinal permeability must indicate altered intestinal integrity. One should however be aware that unaltered permeability for selected tracer molecules does not necessarily imply that intestinal integrity is not impaired, since the tracer molecule may be too large to pass across the impairment. Alterations of barrier functions are often measured by trans-epithelial electrical resistance (TER) *in vitro* that must be a measure of barrier integrity. Additionally as it measure both the leak and the pore pathway [58], alterations in this parameter must indicate alterations in the barrier integrity.

Bacterial translocations are by some also used to indicate impairment of intestinal integrity [67, 68, 69]. If bacteria should cross the intact epithelial layer through the TJ complex it should be through the leak pathway. But some state that the passage between the TJ is too narrow for bacteria to cross [59]. Therefore increased bacterial translocation is here not considered to initially occur through increased permeability at TJ. Additionally inflammation may lead to increased permeability as specific pro-inflammatory cytokines increases permeability (see section 2.5.3), this might result in complete disruption of the TJ or loss of epithelial cells, hence leading to bacterial translocation. In a recent study translocation of bacterial DNA and a labeled *E. coli* from the intestinal lumen into tissue was higher in mice fed a HF diet than mice fed a normal diet [67], hence considered to have impaired intestinal permeability. This support the hypothesis that impairment of intestinal integrity may lead to bacterial translocation. Conclusively bacterial translocation are here considered to result from decreased intestinal integrity, however the barrier impairment is not initially caused by transport of bacteria across the TJ.

2.5.2 Effect of changed intestinal permeability

Impairment of intestinal integrity is connected to inflammation and gastrointestinal disorders, hence impairment of intestinal integrity can cause adverse effects. Impaired intestinal integrity or altered intestinal permeability has been connected to among other CD, UC, Celiac disease, irritable bowel syndrome, necrotizing enterocolitis (NEC), and obesity [1, 29, 65, 70]. But what came first? The increased intestinal permeability or the disease? Patients with diseases such as CD have a barrier defect before clinical disease is initiated, while enteropathogenic *E. coli* infection leads to a defect barrier then disease according to [59]. Additionally some pathogenic bacteria have been shown to decrease TER, see table 2.2, indicating that increased permeability may be exploited by such pathogens, hence leading to bacterial translocation. In mice with induced NEC intestinal permeability was also increased before the disease onset [70]. This indicates that permeability may be increased before disease onset. Based on a literature review Turner [59] stated that disease may not be caused by only changes in the TJ, but such changes would increase immune response in the host, hence increasing the risk of disease. This occurs potentially by an increased inflammatory state. Others support this hypothesis. The permeability for fluorescein isothiocyanate-dextran (FITC-dextran) was positively correlated to plasma LPS [71], indicating that LPS can translocate together with FITC-dextran. This was by the authors in another paper suggested to lead to inflammation [1] and hence bacterial translocation.

The epithelial layer must however exploit the opening of the TJ complex during e.g. nutrient uptake. One may argue that increased permeability leading to increased flux of specific ions or nutrients across the epithelial layer may be beneficial. For example an increased flux of calcium may be beneficial as it would increase its uptake. It should however subsequently not result in an inflammatory stated, barrier impairment, and bacterial translocation. Changes in permeability up to a certain size or a specific duration may therefore be favorable. It is therefore relatively difficult to state if changes in intestinal permeability are beneficial or not; it depends on how much the integrity subsequently is changed. If the integrity is impaired leading to inflammation and disease the altered permeability is adverse, however if integrity is maintained following the altered permeability, host health may not be affected.

2.5.3 Alteration of intestinal integrity

The intestinal integrity can be modulated by affecting the interactions between TJ proteins, affecting the mucus layer thickness, and affecting IEC proliferation and death among other. The scope of the project is the effect of the bacteria on the intestinal permeability. In the intestinal lumen a wide range of bacteria may have an effect. However the host may also modulate the intestinal permeability. In the following paragraph the effect of bacteria on the intestinal integrity, as well as metabolites produced by the bacteria are described. Selected host mechanisms to regulate intestinal permeability are also briefly introduced, as these may be used by the bacteria.

Gut microbiota

The gut microbiota can affect the intestinal integrity [1]. This can occur by

- peptides or toxins,
- cellular structural components,
- metabolites [65].

Several studies have examined the effect of bacteria on the intestinal integrity both *in vitro* and *in vivo*. These studies have mainly been conducted with probiotic or potentially probiotic bacteria in connection to decreasing adverse effects of pathogens or induced intestinal disorders, see table 2.2 and 2.3. They do however indicate if and how bacteria may affect the intestinal integrity.

Several studies, see table 2.2 and 2.3, have examined the effect of the probiotic mixture VSL#3 that contains *Bifidobacterium longum*, *B. infantis*, *B. breve*, *Lactobacillus acidophilus*, *L. casei*, *L. delbrueckii* subsp. *L. bulgaricus*, *L. plantarum* and *Streptococcus salivarius* subsp. Thermophilus [72]. This mixture of bacterial strains and conditioned media (CM) from it have been shown to decrease permeability *in vitro* [72, 73]. This indicates that VSL#3 affects the epithelial cells by a soluble factor [72, 73]. The mechanism behind these effects have been shown to be connected to increased expression of mucin encoding genes, and reduce effects of pathogen exposure by limiting mammalian cell death and redistribution of ZO-1 introduced by the pathogen, see table 2.2 [73]. *In vivo*, see table 2.3, VSL#3 was shown to protect against increased intestinal permeability in gastrointestinal disease models [72, 74, 75]. This was connected to among other limiting apoptosis of epithelial cells and decreased levels of TJ proteins, that DDS-induced colitis had caused [75]. An *in vitro* study with mono-cultures of bacteria from the VSL#3 mixture has shown that conditioned media from *B. infantis* increased TER the most of the bacterial strains, as well as increased the protein expression of occludin and ZO-1 [76]. *B. infantis* has also been found to inhibit increased intestinal permeability in NEC animal models *in vivo* [70]. The probiotic VSL# 3 mixture may hence affect the intestinal integrity by limiting mammalian cell death induced by pathogens, increase the interaction between epithelial cells, affect mucin production, and limit adverse effects of induced NEC. All these effects seem to occur through soluble factors that may be produced by one or more of the bacteria in the mixture. Fermented dairy products with among other *B. lactis* and *L. lactis* can also decrease impairment of the epithelial barrier that was induced by stress in rats [77]. Hence different mixtures of bacteria may affect intestinal integrity. Overall the applied mixtures included *Bifidobacterium* spp. and *Lactobacillus* spp. are generally considered beneficial for host health.

Potential effects of bacterial strains of *Lactobacillus plantarum* have also been examined individually. This has led to indications of strain specific effects. For example mono-cultures of specific *L. plantarum* strains (MB452 and NF1298) were shown to increase TER *in vitro* [78, 79], while another strain (WCFS1) did exhibit this effect, see table 2.2 [80]. This effect on TER seemed to be dependent on the viability of the bacteria [78]. The different strain also seemed to affect the barrier function by different mechanisms. *L. plantarum* MB452 increased the staining ZO-1, ZO-2, occludin and cingulin [79], while *L. plantarum* WCFS1, which did not increase TER, moved ZO-1 closer to the TJ *in vitro* as well as increased the ZO-1 and occludin levels *in vivo* [80]. These studies indicate a strain difference for the effect of *L. plantarum* on the intestinal integrity. This may therefore also be valid for other bacterial species.

Table 2.2: *In vitro* studies determining the effect of bacteria on intestinal integrity.

Bacteria	Cell line	Method	Main results	Ref
VSL#3[4], *L. reuteri*, *Streptococcus bovi*, and *E. coli* DH5	T84	T84 was exposed to alive or heat-treated VSL#3 or condition media (CM) from such or mono-cultures of bacteria from VSL#3. Determined TER, electrical conductance, and mannitol permeability.	VSL#3 and CM from VSL#3 decreased permeability. Heat treated VSL#3 and CM had no effect. A soluble factor caused the effect. *L. reuteri* and *E.coli* increased permeability, hence one or more of the strains in VSL#3 may have an effect.	[72]
VSL#3, *E. coli* Nissle 1917 (probiotic), *Salmonella* Dublin (pathogen)	T84 and HT29	T84 were exposed to *E. coli* Nissle, VSL#3 and CM from VSL#3. TER, IL-8, and cell survival was determined with and without *Salmonella* Dublin challenge. Expression of mucin encoding genes was determined using HT29 cells.	VSL#3 and CM from VSL#3 increased TER while *Salmonella* Dublin decreased TER in T84. Pre-incubation with VSL#3 and CM from VSL#3 minimized adverse effects of *Salmonella* Dublin on TER. Water soluble proteins were considered to cause the effect. *Salmonella* Dublin redistributed ZO-1, this was decreased by VSL#3 and to some extent CM from VSL#3. Co-culture of VSL#3 and *E. coli* Nissle with *Salmonella* Dublin increased mammalian cell survival. VSL#3 increased gene expression of MUC2, MUC3 and MUC5AC. Inhibition of MAPK reduced effect of VSL#3 on TER and mucin expression.	[73]
Strains from VSL#3 (*B. breve*, *B. infantis*, *B. longum*, *L. acidophilus*, *L. delbrueckii* subsp. *bularicus*, *L. casei*, *L. plantarum*, *Streptococcus salvarius* subs. *thermophiles*) (Probiotics)	T84	Exposed cells to CM from VSL#3 strains. Determined TER during exposure to CM from *B. infantis* with and without TNF-α and IFN-γ. Determined TJ protein levels.	CM from *B. infantis* increased TER the most. It increased occludin and ZO-1 protein expression, while decreasing claudin-2 expression (data not significant). Pretreatment with CM limited adverse effects of the pro-inflammatory cytokines TNF-α and IFN-γ on TER.	[76]
L. plantarum MF1298 (potential probiotic)	Caco-2	Measured TER during exposure to the bacterium	Viable L. plantarum increased TER. Heat-treated bacteria, and CM had no effect on TER.	[78]

[4]Mixture of probiotic strains; *Bifidobacterium longum*, *B. infantis*, *B. breve*, *Lactobacillus acidophilus*, *L. casei*, *L. delbrueckii* subsp. *L. bulgaricus*, *L. plantarum* and *Streptococcus salivarius* subsp. Thermophilus [72].

Table 2.2: *In vitro* studies determining the effect of bacteria on intestinal integrity.

Bacteria	**Cell line**	**Method**	**Main results**	**Ref**
L. plantarum strain WCFS1 (probiotic)	Caco-2 BBE	Exposed cells to bacterium. Determined TER, FITC-dextran permeability, and cell viability.	*L. plantarum* but not bacterial growth medium increased fluorescent staining of ZO-1 close to the TJ complex. *L. plantarum* did not affect TER or FITC-dextran permeability. Considered the effect to be mediated by the TLR2 receptor.	[80]
L. plantarum MB452 (probiotic)	Caco-2	Exposed cells to the bacterium. Determined TER, gene expression and did fluorescent microscopy.	*L. plantarum* increased TER. *L. plantarum* increased protein and mRNA level of ZO-1, ZO-2, occludin, and cingulin.	[79]
L. rhamnosus GG, *L. plantarum*, *L. casie Shirota*, *B. Bb* 12, *Enterococcus faecium* strain S13, and *B. sp. 420* (probiotics)	Caco-2	Cultured the bacterial strains with different carbohydrates (Glucose, Raftilose, Raftaline, Fibersol-2E, and Frutafit IQ). Determined supernatants effect on TER.	Glucose: Increased TER with all strains. *L. casei Shirota* and *L. plantarum* fermentations had larger effects than non-fermented glucose. Raftilose: *L. rhamnosus* GG and *B. sp 420* fermentations had larger effect than non-fermented raftilose. Raftiline: All strains except *L. casei Shirota* fermentation increased TER more than non-fermented Raftaline. Fibersol-2E: No strains resulted in higher TER than non-fermented Fibersol-2E. Frutafit IQ: Non effect	[85]
B. lactis 420, *B. lactis* HN019, *L. acidophilus* NCFM, *L. salivarius* Ls-33 (protiotics) and *E. coli* O157:H7 (pathogen)	Caco-2	Determined TER after treatment with media from the bacterial strains.	*B. lactis* increased TER while *E. coli* O157:H7 decreased TER. *L. acidophilus* NCFM increased TER less than the control. Pretreatment with *B. lactis* decreased effects of *E. coli* O157:H7 on TER.	[81]
B. bifidum and *B. breve*	HT29	Determined TER and gene expression after exposure to UV killed bacteria	*B. bifidum* and *B. breve* both increased TER. No change in gene expression of TJ proteins were determined.	[86]

Table 2.2: *In vitro* studies determining the effect of bacteria on intestinal integrity.

Bacteria	Cell line	Method	Main results	Ref
E. coli Nissle 1917 (probiotic) and *E. coli* strain E2348/69 (EPEC) (pathogen)	T84	Determined gene expression, TER, and TJ proteins distribution in cells challenged with EPEC without, after, and before incubation with *E. coli* Nissle.	*E. coli* Nissle reduced adverse effect of EPEC on TER co-culturing and during post- challenge. EPEC reduced the mRNA expression of ZO-2, while *E. coli* Nissle increased the expression. Co-culture had the same effect as *E. coli* Nissle. EPEC relocated ZO-2 away from the TJ complex. *E.coli* Nissle had the opposite affect. Co-culture of the two strains did not affect ZO-2 location. *E. coli* Nissle counteracts the adverse effects caused by EPEC potentially by a mechanism involving PKC-ξ.	[82]

Table 2.3: *In vivo* studies determining the effect of bacteria on intestinal integrity.

Bacteria	Animal model	Method	Main results	Ref
VSL#3	129 Sv/Ev and IL-10 deficient 129 Sv/Ev mice.	Animals received VSL#3 for 4 weeks. Determined mannitiol flux, ion flux, and did histology.	VSL#3 increased ion transport in Il-10 deficient mice. Il-10 deficient mice had lower ion transport than control animals. VSL#3 decreased manntitol flux in both IL-10 deficient mice and controls.	[72]
VSL#3	DSS- induced colitis Balb/c mice	Determine inflammation, permeability by evans blue, TJ protein expression, and epithelial apoptosis in untreated mice, mice treated with DSS and placebo, and mice treated with DSS and VSL#3.	VSL#3 reduced development of colitis induced by DSS. DSS increased colonic permeability, and decreased levels of occludin, ZO-1, claudin-1, and -4, and increased epithelial apoptosis. VSL#3 administration counteracted effects of DSS.	[75]

Table 2.3: *In vivo* studies determining the effect of bacteria on intestinal integrity.

Bacteria	**Animal model**	**Method**	**Main results**	**Ref**
VSL#3	SAMP mice with chronic Crohn's disease-like ileitis	Administered VSL#3 to the animals at a low- and high-dose. Determined intestinal permeability, gene expression and did histology. Exposed ileum organ cultures to CM from VSL#3 and faecal extracts.	High-dose VSL#3 protected mucosal integrity according to histology. VSL#3 decreased small intestine permeability in SAMP mice. TNF-α mRNA was increased by high VSL#3 dose. Condition media from VSL#3 increased TNF-α secretion and activated the NF-κβ pathway in ileum organ cultures (*in vitro*).	[74]
Fermented dairy product with *B. lactis*, *L. delbrueckii*, *S. thermophilus*, and *L. lactis*	Female Wistar rats exposed to partial restrain stress.	Determined intestinal permeability for CrEDTA, blood endotoxin levels, and western blotting of JAM-A and occludin	Stress increased intestinal permeability, reduced JAM-A and occludin protein levels and increased LPS levels in blood. The fermented dairy product abolished this.	[77]
L. plantarum strain WCFS1	Healthy humans	*L. plantarum* was administered and biopsis from duodenum was collected 6 hours after. Did confocal microscopy.	*L. plantarum* increased the fluorescent staining of occludin and ZO-1 in the TJ.	[80]
E. coli Nissle 1917 (probiotic) and *K12 E. coli* strain MG1655 (pathogen)	Gnotobiotic BALB/c mice and BALB/c mice.	Gnotobiotic animals were coloniced with *E. coli* Nissle or *E. coli* MG1655. Induced colitis in BALBB/c mice with DSS. Determined effects of *E. coli* Nissle administration, changes in gene expression and protein level of ZO-1 and ZO-2, as well as colonic permeability and ion conductance.	*E. coli* Nissle increased ZO-1 expression in gnotobiotic mice and in DDS-treated animals. DSS-treated mice had a higher intestinal permeability but *E. coli* Nissle decreased this.	[84]

Table 2.3: *In vivo* studies determining the effect of bacteria on intestinal integrity.

Bacteria	**Animal model**	**Method**	**Main results**	**Ref**
B. infantis	IL-10 deficient mice (develop colitis spontaneously)	Determined intestinal integrity using the Ussinger chamber after 4 hours or 30 days of exposure to CM from *B. infantis*.	CM decreased intestinal permeability, increased mRNA levels of TGF-β, and decreased levels of IFN-γ.	[76]
B. longum subsp. longum JCM 1217, *B. adeolescentis* JCM 1275 (probiotic) and *E. coli* O157:H7 (pathogen)	Germ-free BALB/c mice	Infected germ-free and mice mono-colonized with probiotics with *E. coli*.	Mice colonized with *B. longum* survived infection with *E. coli*, while others did not. *E. coli* induced apoptosis in epithelium but *B. longum* inhibited this. *Bifidobacterium* species that prevented *E. coli* induced death produced more acetate than strains that could not.	[83]
B. infantis	Mice with induced NEC	NEC was induced and *B. infantis* administered. Determined changes in FITC-dextran permeability and expression and localization of TJ proteins.	NEC increase permeability before intestinal injury. *B. infanitis* inhibited adverse effects of NEC on permeability, occludin and claudin-4 localization.	[70]
B. animalis subsp. *lactis* 420	Mice with high-fat (HF) diet induced diabetes	Feed diabetic mice *B. animalis*. Determined translocation of labelled *E. coli* and expression of cytokines.	*B. animalis* reduced gene expression of pro-inflammaroty cytokines, and reduced translocation of *E. coli*	[67]
*Akkermansia muciniphil*a	C57BL/6 mice feed HF or control diet	Animals was dosed with *A. muciniphila*. Determined gut microbiota, serum LPS, and thickness of mucus layer.	Viable *A. muciniphila* reduced adverse effects HF diet had on mucus layer thickness, as well as reduced serum LPS.	[87]

The idea behind probiotics is that they should increase host health. Therefore some studies examine if probiotics can limit adverse effects of pathogens on barrier function, as shown by improvement of TER *in vitro* [73, 81, 82] and limiting epithelial cell apoptosis *in vivo* [83]. These probiotics have been suggested to introduce this effect by affecting gene expression of ZO-2, location of ZO-1 and ZO-2, as well as limiting mammalian cell death, and increased mucin gene expression, see table 2.2 and 2.3 [73, 82, 83]. Other probiotics have been examined for capability to reduce adverse effects of induced gastrointestinal disorders. An example is the probiotic *E. coli* Nissle 1917, which has been shown to increase the expression of ZO-1 and limit the effects of induced-colitis on permeability *in vivo* [84]. Additionally CM from *Bifidobacterium infantis* has also been found to decrease intestinal permeability in a colitis mode [76], while *B. animalis* reduced bacterial translocation [67].

It is however not only probiotics, that may affect intestinal integrity. Other bacteria, that are not considered probiotics, have also been found to affect intestinal integrity. For example administration of *Akkermansia muciniphila* to mice fed a high-fat (HF) diet resulted in an increase in mucus layer thickness, that the HF diet had reduced [87]. Additionally administration of this bacterium was also found to reduce serum LPS levels [87]. This was not linked to change in other bacterial groups than the specific bacteria [87]. Hence the mucin degrading *A. muciniphila* might stimulate mucus production, hence increasing intestinal integrity.

As the intestinal integrity may be affect by several bacteria or their interplay, it is relevant to determine effects of the complete community or bacterial groups on the intestinal integrity. A few studies have tried to correlate specific bacterial groups from the complex gut microbiota with markers for intestinal integrity. A negative correlation between bifidobacteria and plasma LPS levels in *ob/ob* mice have been shown [71]. Indicating that *Bifidobacterium* spp. may increase intestinal integrity, as also seen with mono-cultures, see table 2.2 and 2.3. Other studies have shown *Lactobacillus* positively and *Oscillibacter* negatively correlated with TER in the colon of diet-induced obese mice [88]. In fact *Oscillibacter* was also negatively correlated with the mRNA expression of ZO-1 [88]. As only a few studies have done such analysis, it is not possible to declare which bacteria in the complex gut microbiota that has an effect on the intestinal integrity. However these studies indicates that the gut microbiota indeed has an effect on the intestinal integrity.

The mechanism by which the gut microbiota affects the intestinal integrity has not been fully elucidated yet. However, LPS has been shown to cause metabolic endotoxemia in the same manner as HF diet in mice [89]. Additionally ZO-1 and occludin mRNA relative expression have been shown negatively correlated with FITC-dextran permeability [1, 71] indicating that altered expression of these TJ proteins may have an effect on permeability. It was suggested that the bacterial community caused the effect [1, 71]. It has later been suggested that the gut microbiota controls the intestinal permeability trough the endocannabinoid (eCB) system [90]. The gut microbiota is suggested to activate the eCB system, hence increasing intestinal permeability by alterations of localisation and distribution of ZO-1 and occludin [90]. This may however not be the sole mechanism for regulating intestinal integrity by bacteria in the gut microbiota, as mucin production and epithelial cell proliferation and death may be regulated by other mechanisms.

In conclusion, bacteria may affect the intestinal integrity directly or by production of specific compounds. This can affect the viability of the epithelial cells, the gene expression, protein level, and localization of TJ proteins, as well as expression of mucin genes. All factors that is relevant for intestinal integrity.

Metabolites

The studies represented above illustrate that some bacteria affect the intestinal integrity by direct interactions or through metabolites. Bacteria degrade substrates in the luminal content resulting in production of different metabolites and potentially cross-feeding. Commane and co-workers [85] examined the effect of supernatants from mono-cultures of probiotic strains and carbohydrates (putative prebiotics) on TER, see table 2.2. Here e.g. *L. rhamnosus* fermentations with some carbohydrates increased TER while other carbohydrates and bacterial strains had no effect [85]. This clearly illustrates that the effect of the bacteria metabolic compounds on the intestinal integrity is dependent on the available substrate. In addition the study indicated that the substrates themselves can affect intestinal integrity [85].

Studies have also determined the effect of specific metabolites known to be found in the GIT on intestinal integrity. SCFA are produced during fermentation in the GIT. These are used as an energy source by the IEC [10]. Butyrate has been shown to decrease intestinal permeability in both *in vitro* [91] and *in vivo* setups by limiting increased intestinal permeability induced in disease models [92]. High concentrations of butyrate (8-10mM) have however resulted in increased permeability, see fig. 2.7 [14, 15]. This was caused by a dose-dependent induction of apoptosis in epithelial cells [15]. Therefore low concentrations of butyrate are considered beneficial, while high levels may have adverse effects. Specifically butyrate has been shown to increase ZO-1 and occludin staining as well as increasing claudin-1 expression [91], indicating increased integrity. Other SCFA such as acetate and propionate have also been found to increase TER *in vitro* [93]. Acetate was also reported to reduce the decrease in TER, that the pathogenic *E. coli* O157:H7 caused [83]. Finally, a mix of SCFA including acetate, propionate, and butyrate caused a decrease in intestinal permeability *in vivo* [93]. The mechanism behind the effect of SCFA on intestinal permeability still needs to be examined further. But as they are used for energy by the IEC, they must be beneficial, however the stated studies must indicate that it is only up to a certain concentration. Stimulation of SCFA producing bacteria, as bacteria belonging to the *Clostridium* clusters IV and XIV, as well as lactate producing bacteria as *Bifidobacterium* and *Lactobacillus*, that may cause cross-feeding leading to stimulation of SCFA producing bacteria may therefore be beneficial.

The bacterial community in the GIT also produces indole, see fig. 2.7. Indole is produced during catabolism of the amino acid tryptophan by both Gram-positive and Gram-negative bacteria [94]. Indole concentrations are lower in germ-free mice than specific pathogen free mice [94]. In connection the germ-free animals had a lower mRNA expression of claudin-7, occludin, and TJP1 (ZO-1) [94]. Additionally indole increased TER *in vitro* as well as increasing the expression of genes relevant for the organization of TJ proteins [95]. Hence the host seems to increase intestinal integrity by sensing this bacterial product.

The mentioned studies merely determine the effect of single metabolites or a relatively small mix of metabolites on the intestinal integrity in *in vitro* or *in vivo* setups. This

does not mimic the complex metabolite profile in the GIT. In order to have a mixture resembling the metabolite profile in the GIT it may be use full to apply faecal or caecal water in for example *in vitro* setups. This must include several compounds, as SCFA and indole, that could have effects on intestinal integrity. Previously a study has applied faecal water in the TER setup. This study showed that faecal water from elderly people increased the permeability *in vitro* while faecal water from adults had the opposite effect. Differences between the two types of faecal water were considered to potentially arise due to different composition in the bacterial community and hence metabolite profile [96]. Faecal or caecal water may hence be useful for mimicking the complex metabolite profile.

Overall metabolites formed in the GIT can affect the intestinal integrity. Modulation of the metabolite profile through modulation of the gut microbiota or administration of specific compounds known to increase integrity might therefore be used as a potential mode of action against intestinal disorders known to be associated with impaired intestinal integrity.

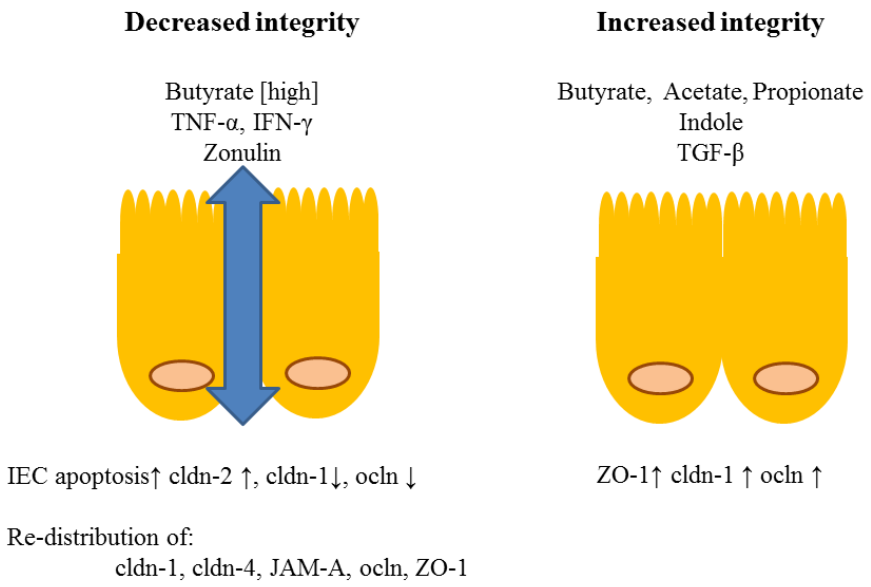


Figure 2.7: Overview of potential effects of metabolites, cytokines and zonulin on intestinal integrity as described in section 2.5.3. The arrow indicates increased(↑) or reduced (↓) expression. cldn; claudin, ocln; occludin.

Host

It is not only the gut microbiota and its metabolites that may affect the intestinal integrity. The host may also regulate the intestinal integrity. This can be as a response to the gut microbiota and its metabolites, as well as a response to inflammation. The reasoning for host regulation of intestinal permeability could be that immune cells then can sample from the intestinal lumen environment, or that more nutrients can be absorbed. Here the effect of cytokines, that bacteria may affect through interactions with

the epithelial cells, and zonulin are introduced, as these are often considered within the field of intestinal integrity.

Cytokines

The immune system and the epithelial layer communicate through among other cytokines making these molecules important for the regulation of intestinal integrity. Additionally epithelial cells sense bacteria and/or MAMPs through TLR and NOD-like receptors, that can lead to a pro-inflammatory or anti-inflammatory response, see section 2.4. Here effects of a limited number of cytokines on intestinal integrity are presented.

Several studies have examined effects of pro-inflammatory cytokines on intestinal permeability. Mainly tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) have been studied. The level of these cytokines are elevated in some gastrointestinal disorders [29], hence their effect on intestinal integrity may be important. TNF-α was shown to induced apoptosis, and decrease TER *in vitro*, see fig. 2.7 [76, 97, 98]. However a single study did not find any change in TER or permeability after exposure to TNF-α [99]. Changes in permeability due to TNF-α were connected to increased mRNA expression for claudin-2, caused by an increased promoter activity for the gene encoding claudin-2 [98], as well as redistribution of occludin and claudin-1 towards the cytoplasm [76]. IFN-γ was also found to decrease TER [56, 76, 99] as well as increasing the permeability of larger probes [56]. This indicates that IFN-γ may affect the leak pathway. The effects of IFN-γ on TJ protein levels are contradictory, as protein levels of claudin-1 and occludin were decreased in one study [56] and were not affected in another [99]. But claudin-1, claudin-4, JAM-1 (JAM-A), and occludin have been shown to be re-distributed towards the cytoplasm by IFN-γ exposure [76, 99]. IFN-γ may therefore affect the intestinal permeability by changing the localization of TJ proteins, hence decreasing the interaction between adjacent cells. Whether TJ protein levels are affected by these cytokines is unclear. When combining the two cytokines TER was found to decreased and the permeability was increased [99, 100]. Bruewer and co-workers [99] found that the combination of the two cytokines affected the distribution of TJ proteins in a similar manner as IFN-γ alone, where claudin-1, claudin-4, JAM-1 (JAM-A), and occludin were relocated towards the cytoplasm. Re-localization of occludin caused by combination of the two cytokines was also shown by Li and co-workers [100], here resulting in irregularities of the cell membrane. These studies support that the pro-inflammatory cytokines TNF-α and IFN-γ increases the intestinal permeability. These effects seem to occur through among other increased apoptosis in the epithelial cells, increased expression of claudin-2, decreased expression of occludin, and re-distribution of several TJ proteins away from the TJ complex. Redistribution of TJ proteins may be caused by activation of Myosin light chain kinase (MLCK) by the stated cytokines [59], potentially causing the cytoskeleton to pull in the TJ proteins, and relocate them.

It is not all cytokines, that result in increased permeability of the intestinal wall. The cytokine transforming growth factor-β (TGF-β) was shown to increase TER but not affect the permeability of a macromolecule [101]. In addition this cytokine could limit the adverse effects IFN-γ had on TER. This effect was however counteracted by increased levels of IFN-γ [101].

The outlined cytokines illustrates that cytokines produced by the host may have different effects on the intestinal permeability, potentially helping in maintaining intestinal

integrity. There are more cytokines, than the above mentioned, that can affect the intestinal integrity. These are not all included here, as it is outside the main scope of the thesis. But further information can be found in a paper by Al-Said and co-workers [102], where research regarding the effect of cytokines on the epithelial barrier is collected. Here it is stated that in general it seems that pro-inflammatory cytokines cause increased permeability, while anti-inflammatory cytokines have the opposite effect [102].

Zonulin

Zonulin is a human protein, that has evoked interest within the field of intestinal integrity, since it was identified as the human analog to zot (*Vibrio cholera* toxin), that is known to modulate permeability [103]. Zonulin has since been identified as the precursor for haptoglobulin-2 (HP2) [104]. Zonulin is considered to regulate the TJ, consequently being a modulator for the permeability of the epithelial barrier. Specifically zonulin has been connected to Celiac disease, where zonulin mRNA expression is higher in intestinal tissue in patients suffering from acute Celiac disease compared to healthy individuals [105].

The effect of zonulin on intestinal permeability has been examined *ex vivo* and *in vivo*. Initially zonulin was shown to decrease the electrical resistance in rhesus monkey jejunum and ileum tissue, while colon tissue was not affected, thus it was speculated that there is no specific receptors for zonulin in the colon [103]. *In vivo* zonulin was found to increase permeability in the small intestine as well as the gastroduodenal in mice [104]. Here merely the permeability in the stated sections were examined, making it difficult to exclude that zonulin may have an effect in the colon. It seems that the effect of zonulin on barrier integrity is reversible [106], hence removal or limitation of zonulin, may limit adverse effect on permeability. This could potentially be used for therapeutic means.

Zonulin may not be released constantly, but potentially under specific conditions, for example during bacterial exposure. The release of zonulin has indeed been shown to be stimulated by pathogenic and non-pathogenic bacteria in a range of animal tissue segments. A study was conducted with three *Escherichia coli* strains isolated from healthy infants and one pathogenic *Salmonella enterica* typhimurium strain. These are all Gram negative bacterial strains, hypothesizing that zonulin is released upon contact with Gram negative bacteria [106]. Indeed a Gram positive potential probiotic strain *L. plantarum* strain PP-217, was shown to decrease the release of zonulin in rabbit intestinal tissue stimulated by *E. coli*, in addition to limit the adverse effects on TER [107]. Based on the effect of bacteria on zonulin release it may be hypothesised, that bacteria can affect the intestinal permeability by inducing zonulin in the host. It has been suggested that zonulin may regulate bacterial colonization in the small intestine, as increased zonulin levels would open the TJ leading to water flowing into the lumen, hence flushing the bacterial community [107]. This hypothesis needs further examination.

The mechanism behind zonulins opening of the TJ has not been fully determined. Zonulin has been shown to redistribute ZO-1 away from the membrane in epithelial cells [106], as a result it reorganizes the TJ complex. The mechanism behind zonulins effect on permeability has been suggested to arise by activating PAR2 causing phosphorylation of the epidermal growth factor receptor (EGFR) [104]. The bacterial analog to zonulin, zot, has been suggested to activate proteinase-activated receptor 2 (PAR2) leading to phosphorylation of ZO-1 by PKC-α [108]. ZO-1 was subsequently found to disassociated

from claudin-1 and occludin causing the TJ complex to disassemble [108]. Zonulin have also been shown to induce disassembly between ZO-1 and the actin skeleton by inducing myosin phosphorylation, leading to disassociation of the TJ [108].

Conclusively, zonulin is produced by the host making it possible for the host to regulate intestinal permeability. Zonulin seems to regulate TJ by phosphorylation of ZO-1 and myosin. The cause of zonulin production may be bacterial contact, but further studies are needed to determine which bacteria, that may affect the zonulin production. However, it is clear that permeability of the TJ is not only regulated by zonulin, as TNF-α, that is known to reduce TER, does not induce zonulin production [106]. Therefore several mechanisms must be involved in TJ permeability regulation.

Collectively intestinal integrity can be modulated by the gut microbiota, its metabolites, cytokines and zonulin. It seems that the bacteria can modulate cytokines and zonulin, hence the intestinal integrity. The changes in intestinal integrity described here include redistribution of TJ proteins as well as altered expression of these at gene and protein level.

2.5.4 Application of different models for studying intestinal integrity

Studies examining the effects of e.g. bacteria, their metabolites, and cytokines on intestinal permeability and integrity apply both healthy and disease models, see table 2.2 and 2.3. Selection of which model to use depends on the aim of the study. Disease models have the clear advantaged that it can be determined if a treatment can counteract potential adverse effects the induced disease has on the intestinal integrity. But as the mechanisms behind most gastrointestinal disorders still needs to be elucidated, the disease models may not mimic the disease sufficiently [59]. Therefore, potential beneficial effects of a treatment found using a disease model, may not be functional in a patient. Additionally, disease models such as DSS-induced colitis may cause damages to epithelial cells leading to cell death, and not merely affect the TJ complex [59]. Hence the changed permeability in these models is a combination of epithelial cell death and changes in TJ and not merely changes in TJ [59]. Application of disease models is however useful as it can be elucidated if a treatment can limit adverse effect on intestinal permeability induced by disease.

Other studies apply healthy or non-challenge models, see table 2.2 and 2.3. Here it can be determined if e.g. a bacterial strain, specific compounds etc. affect intestinal permeability in a healthy gut. It can however not be excluded, that a treatment, with no effect in a healthy model, may have an effect in a disease model, as treatment may affect a state in a disease model, that is not present in the healthy model. The opposite can also be valid. In general it is difficult to determine a "more healthy state" in a healthy model, hence alterations leading to adverse effect or limiting induced adverse effects is easier to identify. The application of different models also means that they have different microbial composition. This may hence affect the outcome, potentially resulting in differences in experimental outcome determined between different models.

The scope of the current work was to determine if modulation of the "normal" gut microbiota had an effect on intestinal integrity, therefore healthy models were applied. Additionally alterations of the complete microbiota were used, and not just mono-cultures. This gives a more elaborate view of how this complex system affects the host intestinal integrity. Additionally the interplay between the bacteria in the gut microbiota is

included in this.

2.6 Modulation of the gut microbiota

Modulation of the gut microbial composition through intake of probiotics and prebiotics is considered to have beneficial effects on human health. The gut microbiota is however also affected by the consumed diet, dietary changes, antibiotic treatment etc. As the gut microbiota and its metabolites affect intestinal integrity, modulation of the gut microbiota may also affect the intestinal integrity. Modulation of the gut microbiota and intestinal integrity by antibiotics and selected prebiotics or putative prebiotics and whole-grain products are the main focus, as these have been applied in the study.

2.6.1 Antibiotics

Antibiotics are commonly used in treatment of bacterial infections in humans and animals. Antibiotics are compounds that kill or inhibit bacteria. They have different targets within the bacterial cell, see fig. 2.8, like

- DNA replication,
- transcription,
- protein synthesis,
- cell wall,
- cytoplasmic membrane [109].

Mechanisms behind the effects of antibiotics are outside the scope of the current project, however due to these different bacterial targets, antibiotics must modulate the gut microbiota differently. For example an antibiotic targeting Gram-positive bacteria may cause a decrease in the relative abundance of such, while potentially increasing the relative abundance of Gram-negative bacteria. Different antibiotics may therefore cause different effects on the gut microbiota and hence intestinal integrity.

Effect of antibiotics on intestinal integrity

The effect of antibiotic administration on the gut bacterial composition and intestinal integrity has been examined. Here beneficial effects of antibiotic treatment on intestinal integrity have been shown. For example antibiotic (ampicillin and neomycin) treatment was able to counteract a reduced mRNA expression of ZO-1, reduce plasma endotoxin, and increased intestinal permeability in HF diet mice [1]. A similar effect was seen in *ob/ob* mice following antibiotic treatment [1]. In the study, administration of antibiotics was shown to change the gut microbiota in mice, mice fed HF diet, and *ob/ob* mice resulting in a reduction in *Lactobacillus* spp., *Bifidobacterium* spp., and *Bacteroides-Prevotella* spp. compared to mice not administered antibiotics [1]. Hence the antibiotics reduced adverse effect on intestinal integrity potentially caused by obesity, but they also reduced bacteria that are considered health beneficial as *Bifidobacterium* spp. and *Lactobacillus*

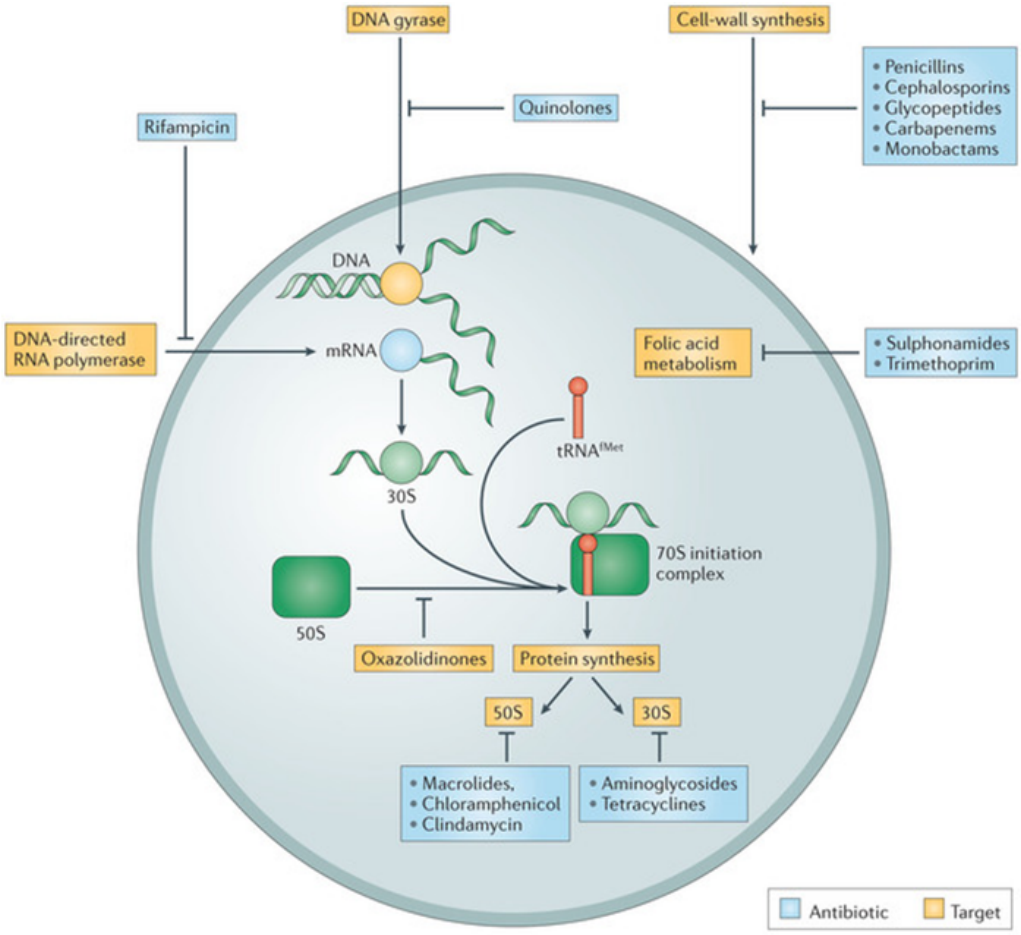


Figure 2.8: Antibiotic targets within the bacterial cell. The figure is a copy of figure 1 in [110].

spp.. Other studies have also examined effects of antibiotic administration. Administration of antibiotics ampicillin, neomycin and metronidazole has also been shown to reduce LPS levels in plasma in HF diet feed mice [111]. Increased levels of LPS in plasma have previously been considered to indicate increased intestinal permeability [1]. The antibiotics was shown to cause a change in the gut microbiota leading to an increase in *Proteobacteria* as well as a reduction in aerobic and anaerobic bacteria compared to the control [111]. Neomycin has also been found to increase *Proteobacteria* in water avoidance stressed rats, while rifaximin increased *Lactobacillus* [112]. Here Rifaximin was shown to reduce mucosal inflammation induced by the water avoidance stress, but neomycin did not have this effect [112]. Overall these stated studies indicate that antibiotics can modulate the gut microbiota leading to increases intestinal integrity.

Administration of antibiotics has also been found to have adverse effects on intestinal integrity. Administration of metronidazole to mice prior to infection with *Citrobacter rodentium* has been found to lead to impairment of intestinal integrity compared to streptomycin administration and no antibiotic treatment. Specifically metronidazole

pretreatment caused a reduction in mRNA expression of MUC2, TFF3 and Relmβ, that all take part in the mucus layer. This led to a thinner mucus layer, *Citrobacter rodentium* attached more to the mucosal layer, and inflammation was higher than in the control and streptomycin animals [113]. All indicating an impairment of intestinal integrity. The metronidazole was shown to stimulate *Lactobacilli*, while *Clostridium coccoides* group and *Bacteroidales* were reduced by the antibiotic [113].

Not all antibiotics seem to affect intestinal integrity. For example streptomycin did not affect mucus layer thickness in mice [113]. This may arise due to modulation of bacteria, that does not have an effect on the intestinal integrity, or that the induced modulations counteract each other, at the level of intestinal integrity.

Conclusively the application of antibiotics in treatment of bacterial infections may modulates the gut microbiota leading to increased, decreased or unaltered intestinal integrity, all dependent on the applied antibiotic.

2.6.2 Prebiotics

The gut microbiota may, in addition to antibiotic administration, be modulated by prebiotics, see fig. 2.9. Prebiotics are defined as *"selective fermented ingredients that cause specific changes in composition and/or activity in the gastrointestinal microbiota that confers benefits upon host well-being and health"* [114]. An increase in *Bifidobacterium* and *Lactobacillus* by such substrates are therefore considered to be a prebiotic effect [17].

In the present work a selection of prebiotics were used. These were selected based on previous studies linking these to altered intestinal integrity. These are only shortly introduced. The selected prebiotics are fructo-oligosaccharides (FOS) and inulin, as well as the putative prebiotics xylo-oligosaccharides (XOS). Inulin consists of β2,1-linked fructofuranose residues with a high degree of polymerization (DP). FOS has a quite similar structure but it has a lower DP (2-7 DP) [17]. While XOS are β1,4-linked xylo-oligosaccharides with a DP around 2-4 [17]. These are, due to their prebiotic or putative prebiotic status, considered to stimulate bifidobacteria and lactobacilli, but they may also affect other bacteria in the gut microbiota potentially through cross-feeding. Therefore prebiotic treatment may lead to effects on intestinal integrity.

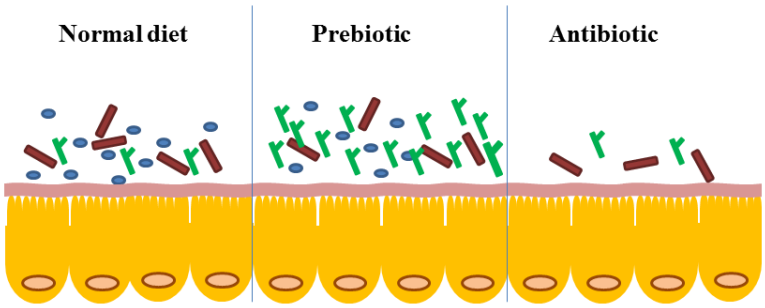


Figure 2.9: Overview of changes in the gut microbiota by prebiotic and antibiotic administration. The blue circles, red rods, and green y-shaped shapes illustrate bacteria. More specifically *Bifidobacterium* is illustrated by the green y-shaped bacteria.

Effect of prebiotics on intestinal integrity

Prebiotics and putative prebiotics can modulate the gut microbial composition, which may affect intestinal integrity. Both *in vitro* and *in vivo* studies have been conducted in order to determine if changes in the gut microbial composition due to such substrates could affect intestinal integrity.

In vitro studies have included the formally mentioned study by Commane and coworkers, see table 2.2 [85]. Others have also examined the effect of prebiotics on intestinal integrity by applying *in vitro* models. These studies have included fermentations with complex faecal bacterial communities, and subsequently applied supernatant from these batch *in vitro* faecal fermentations or continues systems with putative prebiotics or prebiotics. Here fermentation supernatant from the proximal and distal vessel in the Human Intestinal Microbial Ecosystem (SHIME) with branched fructans was shown to increased TER [115]. As were supernatant from batch fermentations with human faeces and resistant starches [116]. All illustrating that fermentation of prebiotics with a complex bacterial community may increase the intestinal integrity.

Other studies have examined if prebiotics affects the intestinal integrity *in vivo*. Several studies have determined if prebiotics affects infection by pathogenic bacteria, this can be used as an indicator for changes in intestinal integrity. It has led to contradicting results, where prebiotics and putative prebiotics have both increased and decreased pathogenic infection, in addition to some not having an effect on intestinal integrity.

Lactulose administration to rats has resulted in limited *Salmonella enteritidis* translocation, when lactulose was administered with a high calcium diet [117], while lactulose administration resulted in increased inflammation, when the diet was not high in calcium [69, 117]. Hypothesizing, that calcium may counter act or limit effects of lactulose [117]. Sole lactulose also resulted in increased cytotoxicity of faecal water and excretion of mucus [69, 117], indicating impaired intestinal integrity caused by lactulose administration. Lactulose was shown to stimulate *Enterobacteria*, *Bifidobacterium* and *Lactobacillus* [69]. This indicates, that stimulation of bacteria considered to be health promoting, as *Lactobacillus* and *Bifidobacterium*, may be connected to adverse effects. Stimulation of *Enterobacteria* could of course have resulted in the adverse effects, but *Enterobacteria* was also stimulated by another carbohydrate, that did not result in increased *S. enteritidis* translocation, mucus secretion, or faecal water cytotoxicity [69]. As the authors merely determined the effect of lactulose on selected bacterial groups, it cannot be excluded, that bacterial groups, which were not examined during the study, were affected by lactulose, leading to adverse effect, or that combination of stimulation of specific bacterial groups simultaneously causes the adverse effects.

The effect of other prebiotics on *Salmonella* translocation has also been examined. FOS was also shown to increase *S. enteritidis* translocation in rats [69, 118, 119] in a dose-dependent manner [118]. A similar effect was also determined in mice for *Salmonella* Typhimurium SL134 [68]. Indicating that FOS decrease intestinal integrity, as was confirmed by an increased permeability for CrEDTA following FOS administration in rats [120]. FOS stimulated bifidobacteria [69, 118, 120, 121], lactobacilli [69, 119, 120], *Enterobacteria* [69, 118, 119], *E. coli* [120], and *Bacteroides fragilis* group in the rodents. However it also reduced the Firmicutes in mice [121]. Stressing that stimulation of specific bacterial groups considered to be beneficial may not always result in beneficial effects. Besides stimulating these bacterial groups FOS also increased cytotoxicity of

faecal water, decreased faecal pH, and increased excretion of mucus [69, 118, 119, 120]. The increase in *Salmonella* translocation, faecal water cytotoxicity and decreased faecal pH may all cause adverse effects on the host, as it may irritate the mucosal barrier. An increase in mucin secretion is, however, not that straight forward. It may be indicating an increased mucin production, or an increased irritation of the mucosa leading to mucus release. Here the mucus concentration in caecum was increased by FOS [120], combined with the other outcomes it may indicating that the fermentation of FOS could irritate the mucosa resulting in increased mucus production. Administration of high calcium together with FOS resulted in no drop in the caecal pH, and limited faecal water cytotoxicity and mucin excretion [119]. Indicating that high calcium can limit adverse effects of FOS. It could however only reduce *Salmonella* translocation, not inhibit it [119].

Effects of FOS on intestinal permeability have also been examined in non-challenge studies. FOS was found to increase CrEDTA permeability in rats, but not permeability in the small intestine [122]. The effect was not due to faecal water composition, as faecal water from FOS feed animals was not found to affect the permeability for FITC-dextran across colonic tissue [122]. The lack of effect of the faecal water could be due to loss of volatile compounds during faecal water preparation, or that potential metabolites in faecal water was utilized or absorbed by host tissue before the faecal was transported out of the GIT at rectum.

The effect of FOS on intestinal integrity has also been found to be beneficial or have no effect. Contradictory to the adverse effects of FOS on intestinal integrity in rodents, FOS was not found to change CrEDTA permeability in humans even though it stimulated *Bifidobacterium* spp. and *Lactobacillus* spp., as well as mucin secretion [123]. The effect of FOS in humans was suggested to be caused by the high level of calcium present in the human gut that could counter act the effect of FOS, leading to no changes in intestinal permeability [123]. In addition to not affecting intestinal permeability in humans, administration of fermentable dietary fibers (oligo-fructose) to mice fed a HF diet was found to stimulate *Bifidobacterium* as well as decrease plasma endotoxin levels compared to mice fed HF diet [124], indicating increased intestinal integrity. Additionally *ob/ob* mice fed a HF diet supplemented with oligo-fructose had decreased permeability for FITC-dextran compared to *ob/ob* mice fed a HF diet, furthermore the LPS in blood decreased [71]. Hence FOS can limit effects caused by a HF diet. These beneficial effects were linked to stimulation of *Lactobacillus* spp. *Bifidobacterium* spp. and *C. coccoides-E. rectale* group, specifically *Bifidobacterium* spp. abundance was negatively correlated to plasma LPS levels [71]. Indicating, that stimulation of these bacterial groups, have beneficial effects, if the gut microbiota have been modulated by a HF diet before administration. Recently it have been suggested that the beneficial effects of prebiotic oligo-fructose in HF diet mice may be caused by stimulation of *A. muciniphila* normalising its abundance [87]. Hence potentially the FOS merely resulted in a gut microbial composition close to the normal microbial composition in mice, leading to normal intestinal permeability.

The mechanisms behind the effect of FOS on intestinal integrity are not elucidated yet. No changes were detected in the gene expression of TJ proteins, when FOS increased intestinal permeability in rats. An altered energy metabolism in the colon affecting the intestinal permeability was suggested to cause the effect [125]. The decrease in permeability in *ob/ob* mice fed a HF diet and FOS was connected to an increased mRNA expression of ZO-1 and occludin, as well as localization of these proteins closer to the

cell membrane [71]. The stated studies indicate that TJ may be affected by the prebiotic treatment. Differences between these studies may be caused by differences in the animal model, the diet, and the gut microbial composition before administration of FOS. Others have shown that FOS increased MUC2 gene expression in rats with experimental induced colitis compared rats not fed FOS [126]. Administration of *A. muciniphila* that is stimulated by oligo-fructose, was also shown to increase mucus layer thickness in HF diet mice [87]. Hence, FOS may stimulate specific bacterial groups that stimulate the mucin production.

The adverse effects of FOS on intestinal permeability have been suggested to arise based on a fast fermentation in the gut, leading to low pH that impaired the barrier [118]. This hypothesis was however disregarded as inulin that should be fermented at a lower rate than FOS, increased *Salmonella* levels in caecum after *Salmonella* challenge as well as increased *Salmonella* translocation, increased faecal cytotoxicity, and mucus secretion as FOS in rats [119]. Again high calcium dosing could limit the effect in rats [119]. Inulin has also increased *Listeria monocytogenes* infections in guinea pigs [127]. The bacterial community in rats was shown to be affected by inulin in a similar manner as FOS, since *Lactobacillus* and *Enterobacteria* were stimulated in the rats [119] . Based on these studies, it seems that the adverse effects of FOS were not due to fast fermentation. The adverse effect of inulin on intestinal permeability was not supported by a human intervention study. Here inulin-enriched pasta was found to decrease intestinal permeability in healthy humans and decreased zonulin in serum [128].

FOS, inulin and lactulose are not the only prebiotics that have been shown to increase pathogenic translocation. An increased bacterial translocation has also been reported in newborn rats fed galacto-oligosaccharide (GOS) and inulin together. This was connected to a reduced mRNA expression of ZO-1 [129]. In mice XOS increased *Salmonella* infection, while also reducing *Clostridium coccoides* group and the Firmicutes phyla, while the Bacteroidetes phyla, *Bacteroides fragilis* group and the *Bifidobacterium* spp. were stimulated by XOS [68, 121]. Contradictory to this XOS limited *Listeria monocytogenes* infections in guinea pigs [127]. It is therefore relevant to determine if the putative prebiotic XOS has an effect on intestinal integrity. This is to be evaluated during the present work.

In conclusion prebiotics modulate the gut microbial composition. This has in some animal models led to increased pathogen translocation that in some cases could be counteracted by high calcium, while it has limited pathogen translocation in other animal models. In humans it seems that prebiotics do not affect the intestinal permeability. It is therefore not possible to conclude if prebiotics have adverse or beneficial effects on intestinal integrity. An explanation for the different experimental outcomes could be different gut microbiota, or different effects of microbial changes on the epithelial cells in the different models. The studies do show, that impairment of the intestinal integrity can occur even when bacteria as *Bifidobacterium*, that are considered to be health promoting, are stimulated. Hence, an increase in such bacterial groups may not be linked to increased intestinal integrity.

2.6.3 Whole-grain products

In the entire world grains are consumed as part of the habitual diet from rice in China to rye in rye bread in Denmark. A grain consists of the outer layer called the bran,

the endosperm, and the germ, see fig. 2.10. The bran consists of fibers, minerals, vitamins, and phenolic compounds; the endosperm contains starch, proteins, minerals and vitamins, while the germ consists of vitamins, protein, minerals, and fat [130]. Grains are processed before consumption resulting in either whole grain or refined grains. According to the AACC whole grains are defined as "*Whole grains shall consist of the intact, ground, cracked or flaked caryopsis, whose principal anatomical components — the starchy endosperm, germ, and bran — are present in the same relative proportions as they exist in the intact caryopsis*" [131]. So whole grain contains all the vitamins, fibers, proteins etc. that are found in the complete grain. Whereas in refined grains the bran is removed, so the minerals, fibers, and vitamins from the bran are not present in refined grain products as e.g. white flour [130]. For several years whole-grain products

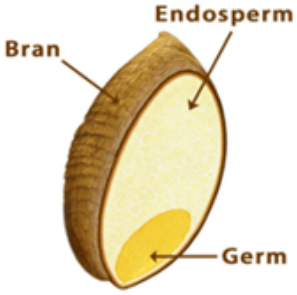


Figure 2.10: Structure of grain. Figure is modified from [132].

have been considered to have a beneficial effect on human health, by among other decreasing the risk of cardiovascular disease by unknown mechanisms. Due to the higher level of dietary fibers in whole-grain products, these may have a prebiotic potential, by stimulation of specific bacterial groups considered to have beneficial effects. It is therefore highly relevant to determine what effect whole grain products have on gut bacterial composition, and if these potentially can be linked to increased human health. In the following sections effects of whole grain products on the faecal bacterial composition in *in vitro* systems and human intervention studies, are described, as well as potential effects on intestinal integrity.

In vitro studies

There have been a limited number of *in vitro* studies examining the effect of whole-grain products on the gut microbial composition, see table 2.4. Connolly and coworkers did studies examining the effect of different types of oat, whole-grain cereals, and toasted wheat grains on the faecal bacterial composition in batch fermentations following stimulated human digestion, see table 2.4 [133, 134, 135]. These studies showed that whole-grain products in general increased the *Bifidobacterium* genus, while some also stimulated *Lactobacillus*. In addition to affecting the bacterial composition, also the SCFA level were increased, see table 2.4. This all support a potential prebiotic effect of whole-grain products. The flake size was found to affect the type and availability of fibers in the whole-grain products, as a large oat grain increased *Bifidobacterium* genus

more than a smaller oat flake did [133]. Hence less treatment or refinement of the grain may increase the prebiotic potential, stressing that whole-grain products may have a larger prebiotic potential than refined products.

The stated studies did not determine if there were any effects of these fermentation products on intestinal integrity. But as they modulated the bacterial composition, it is highly likely that they could affect intestinal integrity especially as some of the whole-grain products led to an increase in SCFA, see table 2.4.

Human intervention studies

The prebiotic potential of whole-grain products has also been determined in human dietary interventions, see table 2.5. Whole grain products seem to affect the bacterial composition differently. Whole grain wheat and maize based cereals have been found to increase *Bifidobacterium* spp. [137, 138] stressing a prebiotic potential of whole-grain products. Whole grain wheat also increased *Lactobacilli/Enteroccoi* as did wheat bran to a less extent [137]. Other whole-grain products did not affect *Bifidobacterium* and *Lactobacillus* [139, 140, 141] however an increase in butyrate producing bacteria was seen following whole-grain intake, see table 2.5 [140, 141].

The difference in experimental outcome between the human interventions described above may be due to use of different types of participants, (healthy versus metabolic disorder), different types of whole grains (different types of cereals), different study design (cross-over study versus parallel study), different durations (2 weeks versus 12-weeks) and use of different methods for bacterial composition determination (quantitative PCR versus HITchip). Hence, comparison between the different studies and their effect on bacterial composition needs to consider these differences between the studies. However, overall these studies, in connection to the *in vitro* studies, indicate a prebiotic potential of whole-grain product.

Effect of whole-grain products on intestinal integrity

As formally stated the effect of whole-grain products determined *in vitro* only considered effects on the bacterial community and SCFA, see table 2.4. But as the whole-grain products modulated the bacterial community as well as SCFA it may have effect on intestinal integrity. For the human intervention studies intestinal integrity was not determined. It is therefore relevant to determine if whole-grain products has an effect on intestinal integrity in addition to examining the effect of whole-grain products on the gut microbiota.

Table 2.4: *In vitro* studies examining the effect of whole-grain products on the microbial composition. ↑; increase, ↓;reduction.

Whole-grain product	**In vitro model**	**Effect on bacterial composition**	**Effect on SCFA**	**Ref**
Oat grain flakes of different size (oat 23's and oat 25's/26's)	Human digestive model and batch culture fermentation	Oat 25's/26's: *Bifidobacterium* genus ↑ Oat's 23's: *Bacteroides-Prevotella* group ↑	Oat 25's/26's: acetate, propionate and butyrate ↑ Oat 23's: acetate and propionate ↑	[133]
Cereals Jumbo porridge oat (JPO), 100% wholegrain aggregate (100% WGA), granola, 70% whole-grain loops (70% WGL), and instant porridge (IP)	Human digestive model and batch culture fermentation	JPO: *Bifidobacteium* genus ↑ and *Clostridium histolyticum* subgroup ↓ 100% WGA: *Bacteroides-Prevotella* group, *Bifidobacterium* genus, and *Lactobacillus –Enterococcus* group ↑, *Clostridium histolyticum* subgroup ↓ Granola: *Atopobium* cluster and most *Coriobacteriaceae* ssp., *Bacteroides-Prevotella* group, *Bifidobacterium* genus, *Clostridium histolyticum* sub group and *Lactobacillus-Enterococcous* group ↑ 70% WGL: *Bacteroides-Prevotella* group, *Bifidobacterium* genus, and *Lactobacillus-Enterococcus* group ↑	JPO: acetic acid and propionic acid ↑ IP: acetic acid, propionic acid and butyric acid ↑ 100% WGA: acetic acid, propionic acid and butyric acid ↑ Granola: acetic acid and propionic acid ↑ 70% WGL: acetic acid, propionic acid and butyric acid ↑	[135]
Toasted, partially toasted, and raw whole-grain wheat flakes	Human digestive model and batch culture fermentation	Raw wheat grains: *Bifidobacterium* genus, *C. histolyticum* subgroup, and *Lactobacillus-Enterococcus* group ↑ *Atopobium* cluster and most *Coriobacteriaceae* spp. ↓ Partially toasted wheat grains: *Bifidobacterium* genus and *C. histolyticum* subgroup ↑ Toasted wheat grains: *Atopobium* cluster and most *Coriobacteriaceae* spp., *Bacteroides-Prevotella* group, *Bifidobacterium* genus, and *C. historlyticum* subgroup ↑	All: acetate, propionate and butyrate ↑	[134]
Flours: Whole grain rye (WGR), Nutriwheat-whole-grain wheat (NW), pulses –chickpea and lentils (PF), and barly milled grains (BMG)	*In vitro* digestion and three-stage continuous fermentation system modeling the human colon	NW:Lactic acid bacteria, *Bifidobacterium* spp. and *Disulfovibrionales* spp. ↑ in vessel 1, 2, and 3 respectively. *Ruminococcus* spp. ↓ in all three vessels WGR: *Bifidobacterium* spp., lactic acid bacteria and *Disulfovibrionale*s spp. ↑ in all vessels and *Rosburia* subcluster ↑in vessel 1 BMG: *Rosburia* subcluster ↑ in vessel 2 and 3. *Disulfovibrionales* spp. ↑ in vessel 1 and 2. PF: *F. prausnitzii* ↓ in vessel 2 and 3. *Clostridium* cluster IX ↓ in vessel 2. *Clostridiu*m cluster XIVa and *Atropobiu*m cluster ↓ in vessel 1. *Roseburia/E. rectale* group ↓ in all vessels. *Bacteroides/Prevotella* group ↑ in the entire system. *Ruminococcus* spp. $<$ *uparrow* in vessel 3.	NW: butyrate ↓, propionate ↑ WGR: no significant effect BMG: acetate ↑ PF: acetate and propionate ↑ butyrate ↓	[136]

Table 2.5: Human intervention studies with whole-grain products. ↑; increase, ↓;reduction.

Whole-grain product	**Study design**	**Duration of intervention**	**Participants**	**Effect on bacterial composition**	**Ref**
Whole grain wheat and wheat bran (placebo) cereal	Double-blind, placebo-controlled, crossover study	3 weeks with 2 weeks wash out period between the two diets	31 healthy participants between 20 and 42 years old	Whole grain wheat: Bifidobacterium spp. and Lactobacilli/enteroccci ↑ Wheat bran: Lactobacilli/enterococci ↑	[137]
Maize-derived whole-grain and non-whole-grain cereal	Double-blind, placebo-controlled, crossover study	2 weeks run-in period, 3 weeks intervention period, 3-weeks wash out period followed by a 3-weeks intervention period	33 healthy participants	Whole-grain: *Bifidobacterium* spp. *Atopobium* cluster spp. ↑ Non-whole-grain: *Atopobium* cluster spp., REC cluster ↑	[138]
Whole grain cereal rich diet and refined grain cereal diet	Crossover study	Loop of 1 week lead in, 2 weeks intervention diet, and 2 weeks post intervention period	22 healthy participants between 20 and 50 years old	Whole grain increased *Enterococcus* and *Clostridium leptum* compared to refined grain	[141]
Whole-grain barely diet, whole-grain brown rice diet and whole-grain barely and rice diet	Randomized cross-over study	4 weeks for each dietary intervention with 2 weeks washout period in between	28 participants, where 13 participants were overweight	Brown rice: *Firmicutes* ↑ *Bacteroidetes* ↓ Whole-grain barely: *Firmicutes* phyla, *Incertae* sedis XIV family, *Blautia* genus ↑ *Bacteroidetes* phylum, *Bacteroidaceae* family, *Bacteroides* genus ↓ Brown rice and whole-grain barely: *Firmicutes* phylum, *Incertae* sedis XIV family ↑ *Bacteroidetes* phylum, *Porphyromonadaceae* family, *Blautia familyodoribacter* familiy ↓	[140]
Rye bread diet and refined white wheat bread diet	Parallel, controlled	12 weeks	51 participants with metabolic syndrome	White wheat bread diet:*Bacteroidetes* spp. ↓, *Clostridium* cluster IV, *Collinsella* and *Atropobium* spp. ↑ Rye bread diet: No change	[139]

Chapter 3

Methodology part

Methods applied in the present PhD study were selected based on application of methods previously applied in our research group, establishment of new methods, as well as utilization of available samples from collaborators. The applied methods are introduced and discussed here.

3.1 Modulation of the gut microbiota

The gut microbiota can be modulated in *in vitro* and *in vivo* models, and in human interventions. *In vitro* models can be used for initial screening of for example putative prebiotics, while *in vivo* studies including animal models and human interventions, are used to determine effects of the host on the gut microbiota and vice versa.

3.1.1 *In vitro* models

Several studies have examined the effect of whole-grain products on the microbial composition with *in vitro* fermentations [133, 134, 135, 136], as well as the effect of prebiotics and prebiotic candidates [142, 143, 144].

In the present work *in vitro* anaerobic batch fermentations with commercial and putative prebiotics were conducted for 24 hours (manuscript 2). Faeces samples collected from healthy humans were used as inoculum. The *in vitro* batch fermentation was applied as it has been used for several studies in our research group [142, 143, 144]. Here the reaction volume was increased 10-fold (from 2 ml to 20 ml) compared to previously in order to have sufficient supernatant for the downstream experiments. Fermentations were, due to the larger volume, rocked to have sufficient mixing. The pH was not controlled during the fermentation, as it reached levels similar to the small-scale fermentations (manuscript 2) [142].

Application of *in vitro* batch fermentations merely mimics potential changes in the microbial community. It does not take into account effects of host response as absorption of e.g. SCFA, buffering, or immune responses. However by applying such fermentations it is possible to determine production of e.g. SCFA that would be absorbed, and therefore difficult to determine, *in vivo*. *In vitro* fermentations in general are highly useful when initially testing the capability of substrates to alter the bacterial community.

3.1.2 *In vivo* models

Animal models are often used following *in vitro* models, as the interaction between host and microbial community can be determined. Animal models can be subdivided

into disease and healthy models. In the present work healthy animal models were used in order to determine effects of changes in the gut microbiota on intestinal integrity during healthy conditions (manuscript 3 and 4). Wistar rats were selected, as changes in intestinal permeability and bacterial translocation following prebiotic treatment have previously been determined in rats [69, 117, 118, 119, 120]. Furthermore we were able to isolate *Bifidobacterium* spp. from faeces from Wistar rats (manuscript 3).

Use of animal models has the great advantage, that the interplay between host and gut microbiota is determined. *In vivo* studies are however more expensive than *in vitro* studies. Additionally from an ethical point of view *in vivo* studies should be limited and only conducted if necessary.

3.1.3 Human dietary interventions

Within the field of gut microbiology human dietary interventions are often used to link effects of diet on the gut microbiota and host health. Here the dietary interventions quite often include alterations of the commensal diet with a commonly consumed food product e.g. whole-grain products [145].

There are mainly used two setups for human dietary intervention studies; cross-over and parallel. In cross-over studies the same person receives for example two types of diet in two subsequent periods with e.g. a wash out period between them. Potential inter-individual effects are therefore minimized. In a parallel study design two groups of participants receives different types of diet, and then groups are compared. Inter-individual effects can therefore have an effect on the experimental outcome. An obstacle with cross-over studies is the longer duration of the intervention, leading to risk of drop outs. Limiting the intervention period may increase risk of limited or no response to the treatment.

The application of human intervention studies in gut microbiota examinations have the clear advantage that effects of host and bacterial interaction on the gut microbiota is included. However often only faecal samples are collected during these studies, as this does not require invasive methods. Samples of the mucus-associated community may also be relevant, but as it require invasive methods these are often not included. A clear disadvantage of human dietary intervention studies are the need for a large number of participants', based on the large inter-individual variation in the gut microbiota and as there is a high risk of drop outs. This leads to increased costs. Furthermore participants habitual diet differs, which affect the results.

3.2 Studying the gut microbiota

Initially bacterial ecosystems were explored by culturing on selective and non-selective media. However it has become clear, that most of the habitants of the gut microbiota are not culturable, therefore culture-independent methods were used in the present work. Initially DNA was extracted from the complex environmental samples. Subsequently, the relative abundance of bacterial families was determined by amplicon sequencing of the 16S rRNA encoding gene. Additionally the relative abundance of specific bacterial groups were determined by quantitative PCR.

3.2.1 DNA extraction

There is a range of commercial kits as well as non-commercial methods for extracting DNA. In the present work all DNA extractions from faecal and caecal samples were conducted with the commercial kit PowerSoil ®DNA Isolation Kit from MoBio (manuscript 1 to 4). This kit was applied as it gives a high yield [146] in addition to being column based, as is recommended for DNA extractions for downstream sequencing.

3.2.2 Quantitative real-time PCR

The relative abundance of selected bacteria groups was determined using quantitative PCR (qPCR) (manuscript 1 to 3). Quantitative PCR is based on PCR, but the level of PCR product is monitored throughout the reaction, in the present work by the double-stranded DNA binding dye SYBR green. Following the qPCR reaction the amplification curve, see fig. 3.1, is applied to determine the initial DNA concentration (N_0) based on

$$N_q = N_0 * (1 + E)^{C_q} \tag{3.1}$$

Here N_q is the amount of DNA at threshold, while E is the efficiency of the reaction, and C_q is the cycle number at the threshold, see fig. 3.1.

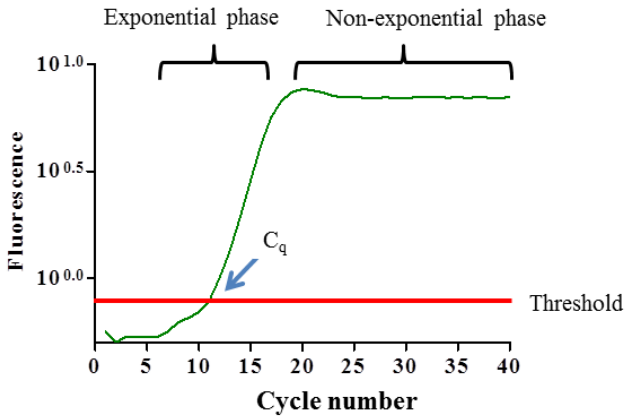


Figure 3.1: Quantitative PCR amplification curve.

In order to automate data analysis the LinRegPCR software was applied. LinRegPCR estimates the mean PCR efficiency (E_{mean}) for each amplicon group (primer set), and applies this to calculate N_0 for each reaction [147]. Relative abundance for each bacterial group was subsequently determined by comparison to the N_0 for total bacteria;

$$\frac{N_{0,\text{ bacterial group}}}{N_{0,\text{ total bacteria}}} \tag{3.2}$$

Application of qPCR to determine the relative abundance of bacterial groups has the advantage that bacterial groups with low abundance (down to 0.001 percent of total bacteria) can be determined [148]. A clear disadvantage is, that only differences or changes in selected bacterial groups are identified, causing a potential bias in the

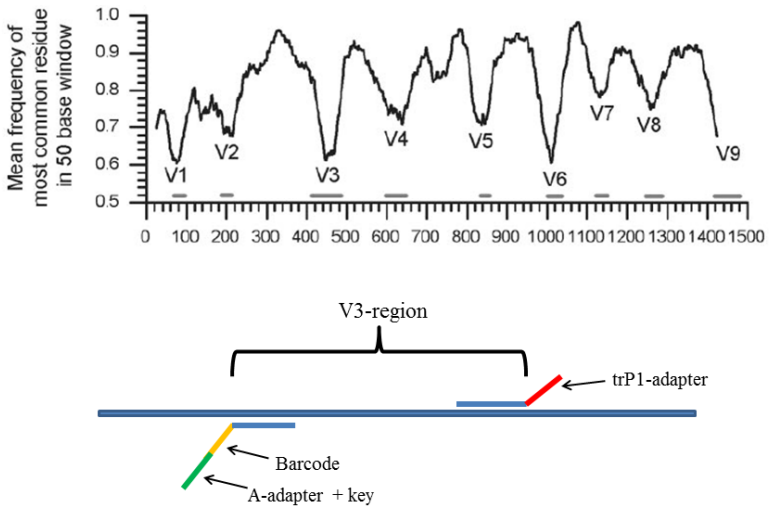


Figure 3.2: The 16S rRNA encoding gene and it variable regions are illustrated(top). The figure is a copy of figure 3B from [149]. Bottom: The applied setup for targeting the V3-region in the 16S rRNA encoding gene with primers for Ion Torrent^{TM} sequencing. Blue; primers, green; A-adapter + key, yellow; barcode, red; trP1-adaptor

experimental outcome. Additionally only the bacterial community is examined, hence alterations in Archaea, fungi, yeast, and viruses are not determined in this set up.

3.2.3 Amplicon sequencing

Compositions of the microbiota in faecal and caecal samples were determined using amplicon sequencing in the V3-region of the 16S rRNA encoding gene (manuscript 3 and 4). All bacteria encode the 16S rRNA gene, and it contains both conserved and variable regions, see fig. 3.2. Amplification and subsequent sequencing of variable regions are therefore used for identifying the bacterial composition in a complex environment.

Preparation of samples

Initially DNA was extracted from faecal samples. The V3-region of the 16S rRNA encoding gene, see fig. 3.2, was selected as targets for amplification and sequencing using the Ion Torrent^{TM} platform, as done by others [150]. Universal bacterial primers targeting the flanking conserved regions of the V3-region were applied to amplify the V3-region in each sample with PCR. The applied forward primer contained a A-adaptor + key as well as a 10-12 bp barcode, while the reverse primer had a truncated P1-adapter (trP1-adapter), see fig. 3.2. These are used for the down-stream processing during sequencing as well as data analysis. After PCR amplification the products were run on an agarose gel, and products (approximately 260 bp), were extracted for each sample.

The products from the samples were mixed, so there were equal levels of DNA from each sample.

Sequencing using the Ion Torrent™ Platform

Initially DNA is hybridized to beads based on the adapter added during PCR amplification. Using emulsion PCR each PCR product is amplified, and the formed products attach to the specific bead [151]. All the beads are subsequently distributed into individual wells in a chip. Here an Ion 318™ chip for the Ion Torrent platform was used. The Ion Torrent™ platform is a semiconductor platform, that utilizes the release of protons upon nucleotide binding. Sequencing is initiated by flushing the chip with one nucleotide at a time. If a nucleotide bind to the DNA sequence on the bead a proton is released resulting in a voltage change. If two identical bases occur next to each other two protons are released leading to a higher voltage changes. These voltage changes are detected for each well in the chip by the Ion Torrent™ platform. Hence during the DNA synthesis the sequence is monitored [152].

Data analysis

Sequencing data is initially analysed in the CLC bio genomic workbench (Qiagen). Sequences are di-multiclassified by sorting based on barcodes, hence sequences from the same sample is collected together. The barcode, primer sequence, and adapter are removed. The data is trimmed, so it only includes sequences with a length of 110-180 bp. Furthermore the confidential interval on correct base parring during sequencing is chosen to 95 percent corresponding to a Phred quality score above $Q = 13$ ($Q = -10 \cdot \log 0.05$) and ambiguous nucleotide is set to 2. The sequences for each sample are then classified to a bacterial taxa using the Multiclassifier tool from the Ribosomal Database Project [153, 154]. A bootstrap cut-off above 0.5 is chosen, since such classifications based on the 16S rRNA encoding V3-region have resulted in correct classification for 95 percent of the sequences previously [155]. Finally based on the sequence length (110-180 bp) the sequences can be classified accurately down to family level [153]. Unclassified and other bacteria are calculated as the difference between total number of bacterial reads and classified reads. The relative abundance of the bacterial families is then determined compared to the number of total bacterial reads, as

$$\frac{Reads_{\text{bacterial family}}}{Reads_{\text{total bacteria}}} \tag{3.3}$$

Determination of the microbiota by amplicon sequencing has the same limitation as the qPCR based method, since only the bacterial community is determined, hence changes in Archaea, fungi, and viruses are not determined. It does however have the advantage over qPCR, that the whole bacterial community is examined. The depth of the analysis is however lower, than the qPCR based method, as sequences cannot be classified below family level based on the length of the sequence [153].

Determination of the gut microbiota by amplicon sequencing and qPCR are both based on prior knowledge of bacterial genome sequences, as primers are applied as well as sequences are classified based on comparison to known sequenced bacteria or 16S rRNA encoding regions. Therefore the large proportion of unidentified bacteria, that

Figure 3.3: Instruments used to determine trans-epithelial resistance. On the left the Millicell ERS-2 Volt-Ohm Meter [156] on the right the cellZscope® from nano Analytics [157]

has not been cultured or sequenced yet, cannot be identified by either method. These may have impact on host health, it is therefore relevant to identify these.

3.3 Studying intestinal integrity

Intestinal integrity can be determined both *in vitro* and *in vivo*. As for modulation of the intestinal microbiota, the *in vitro* models can be used as a measure for screening potential effects on intestinal integrity. While the use of *in vivo* models gives a more elaborate view of how the entire system is affected. Here the applied *in vitro* model and *in vivo* model are described.

3.3.1 *In vitro* model

Trans-epithelial resistance

Trans-epithelial resistance (TER) is determination of electrical resistance across a monolayer of epithelial cells. TER indicates the flux of ions, hence it measures both the leak and pore pathway, see section 2.3.4, as ions can pass the epithelial lining by both pathways [58]. Basically it means that an increase in TER indicates, that the epithelial layer is more tight, but it cannot be concluded, which pathway is affected.

Practically TER is determined using a voltmeter. In the current work two types of voltmeters have been used; the manual Millicell ERS-2 Volt-Ohm meter (Merck Millipore) and the cellZscope® (nano Analytics), see fig. 3.3, (manuscript 1 to 4). The Millicell ERS-2 Volt-Ohm meter requires, that cell culture plates are removed from the incubator while measuring, potentially affecting the mammalian cells. Additionally the electrode is held manually which may cause a bias in the measurements. Instead the cellZscope® measures TER automatically in the incubator. Hence a large number of samples can be examined at the same time. Both setups gives the same type of data, but the cellZscope® is less time consuming for the user and affects the cells to a less extent.

TER have been used in a range of studies to examine the effect of probiotics, pathogenic bacteria, CM from bacterial cultures, mucin etc. on intestinal integrity *in vitro* [72, 73, 76, 78, 79, 81, 82, 85, 96, 158, 159]. These studies have mainly used the mammalian cell line Caco-2, but also T84 and HT29. As Caco-2 is the mostly used cell line for TER measurements it was also used in the present work. Here changes in TER were determined following exposure to faecal water and caecal water (manuscript 1 and 3). Others have also applied such substances [96] or supernatant from *in vitro* fermentation models [115, 116].

TER is a good method for determining intestinal integrity *in vitro*, but it does have clear disadvantages. Culturing of the mammalian cells is rather time consuming, as the cells are seeded 3 weeks before use and medium has to be changed often. Furthermore, the cells are seeded and cultured on Transwell membranes ®, that are rather expensive. Collectively this means that the TER assay is rather expensive and time consuming. Additionally it requires an automatic system, as the CellZscope, to have a high throughput. It may however be a good method for initial screening of effects of bacterial strains or substrates on intestinal integrity, as well as a measure for effects of changes in metabolite profile in faeces on intestinal integrity following an *in vivo* experiment if this has not been determined *in vivo*.

3.3.2 *In vivo* model

Determination of intestinal integrity *in vivo* may be achieved by determining the permeability of tracer molecules. Some studies do however also use the Ussinger chamber, where the electrical resistance across an intestinal section *ex vivo* is determined. In the present work the permeability of a tracer molecule has been determined (manuscript 3 and 4).

Transfer of tracer molecules

Tracer molecules can be used both in humans and in animals, but also in *in vitro* models. In general the used tracer molecules should be small, water soluble, non-toxic, non-charged, not metabolized after absorption, and not degraded by the gut [52]. In addition to this the tracer molecule should be cleared by the kidney if the tracer molecule is to be measured in the urine [52]. The application of different tracer molecules results in permeability measures of different regions of the intestinal sections [52]. For example sucrose, lactulose, mannitol, rhamnosus, and cellobiose are degraded by the luminal content, hence they can only be used to determine the permeability of the small intestine, while CrEDTA and sucrolase are not degraded, so they can be used for measure of permeability in the colon [52].

In the present study intestinal permeability have been determined in an animal model. Previous animal studies have applied tracer molecules as CrEDTA [120, 160] and FITC-dextran [1, 71]. FITC-dextran was selected as a tracer for intestinal permeability in this work, as FITC-dextran is measured in the plasma. Collection of blood was considered more favorable than collection of urine samples from the animals. Animals were dosed with 600 mg/kg by oral gavage two hours before euthanasation. The dosing level has previously been applied by Cani and co-workers [1, 71]. In the present work FITC-dextran levels were determined in the plasma two hours after administration, while Cani

and co-workers determined the levels one and four hours after administration [1, 71]. Plasma FITC-dextran concentrations were hence determined by comparison to a standard curve. Increased levels of FITC-dextran in plasma compared to the control group were considered to be a result of increased intestinal permeability.

FITC-dextran is available in different sizes dependent on the branching on the dextran. Subsequently, use of different sizes molecules illustrates differences in pore size forming the intestinal permeability. Here 4 KDa FITC-dextran was applied as in [1, 71]. This is a relatively large molecule, so changes in permeability for this molecule must illustrate a change in the "leak" pathway [58]. Changes in intestinal integrity merely affecting the permeability for ions are therefore not identified by such assays. Hence it can, based solely on a FITC-dextran permeability assay, not be excluded that permeability of smaller molecules or ions has changed. It may therefore be favorable to use a combination of several assays e.g. FITC-dextran assay and TER to determine changed intestinal integrity for a range of different size molecules. Others have used PEG (polyethylene glycol) oligomers of different sizes and hence determined permeability of tracer molecules using liquid chromatography-mass spectrometry [55, 56].

Application of FITC-coupled molecules is rather expensive, as the molecule itself is expensive. Additionally in the present work animals were housed in pairs. One animal from each cage were used in the FITC-dextran assay, while standard curves were prepared with plasma and tissue collected form the other animal. It may therefore be necessary to include more animals in each group causing an increase in expenses.

3.3.3 Gene expression

Gene expression analysis of selected TJ proteins and mucin was conducted in order to determine if changes in gut microbiota affected intestinal integrity by modulating gene expression of these genes. Gene expression analyses using quantitative reverse transcription PCR were selected as our research group have had good experiences with this setup [161]. Primers were selected based on a literature review. Each primer set was verified by blasting, and the size of the amplicon was verified by running on an agarose gel.

A limited number of relevant genes were selected for analysis. The mucin MUC2 encoding gene *Muc2* was selected as it is the main mucin producing gene. Genes encoding ZO-1 (*ZO-1*) and occludin (*Ocln*) were selected as ZO-1 and occludin are major components of the TJ protein complex. Finally, claudin-1 (*Cldn-1*) was selected as a representative for the barrier forming claudins. The relative expression was compared to the two reference genes encoding glyceraldehyd-3-phosphate (*Gapdh*) and beta-actin (*Actb*). These have been used as single reference genes in other studies [91, 162, 163, 164]. Here two reference genes were used to limit potential biases due to effects from the experimental setup on the expression of the reference genes.

The use of gene expression analysis to examine effects on the TJ, does not reveal if changes in the distribution or the TJ proteins has occurred or if the protein level of specific proteins have changed. Such changes could have been examined with Western-blotting and fluorescent labeling of the specific proteins followed by microscopy.

Manuscript 1

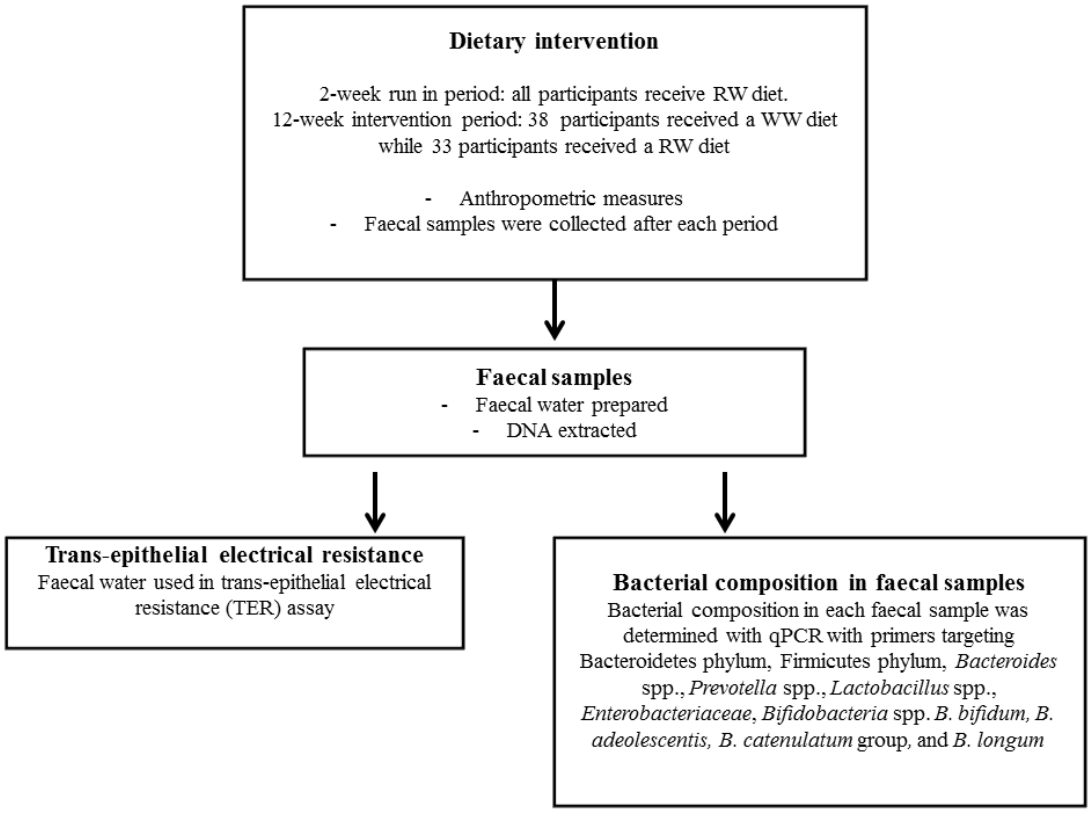
Bifidogenic effect of whole-grain wheat during a 12-week energy-restricted dietary intervention in postmenopausal woman

Published in European Journal of Clinical Nutrition
(Page numbers are relative to the paper)

Introduction

The aim of this study was to determine if refined wheat (RW) and whole-grain wheat (WW) modulated the gut microbial composition in postmenopausal women during a 12-week dietary intervention following a 2-week run-in period. In addition the effect of faecal water on trans-epithelial electrical resistances (TER) was determined.

Flowsheet



European Journal of Clinical Nutrition (2013) **67**, 1316–1321

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ORIGINAL ARTICLE

Bifidogenic effect of whole-grain wheat during a 12-week energy-restricted dietary intervention in postmenopausal women

EG Christensen[1], TR Licht[1], M Kristensen[2] and MI Bahl[1]

BACKGROUND/OBJECTIVES: Consumption of whole-grain products is known to have beneficial effects on human health. The effects of whole-grain products on the intestinal microbiota and intestinal integrity have, however, only been studied limitedly. We investigate changes of the human gut microbiota composition after consumption of whole-grain (WW) or refined wheat (RW) and further study effects on gut wall integrity.

SUBJECTS/METHODS: Quantitative PCR was used to determine changes in the gut bacterial composition in postmenopausal women following a 12-week energy-restricted dietary intervention with WW ($N = 38$) or RW ($N = 34$). Intestinal integrity was determined by measuring trans-epithelial resistance (TER) across a Caco-2 cell monolayer, following exposure to faecal water.

RESULTS: No significant differences in microbiota composition were observed between the two dietary groups; however, the whole-grain intervention increased the relative abundance of *Bifidobacterium* compared to baseline, supporting a prebiotic effect of whole-grain wheat. Faecal water increased TER independent of dietary intervention, indicating that commensal bacteria produce metabolites that generally provide a positive effect on intestinal integrity. Combining microbiota composition data from the run-in period with its effect on TER revealed a tendency for a negative correlation between the relative abundance of *Bifidobacterium* and TER ($P = 0.09$). This contradicts previous findings but supports observations of increased *Salmonella* infection in animal models following treatment with bifidogenic prebiotics.

CONCLUSIONS: The present study shows that whole-grain wheat consumption increases the abundance of bifidobacteria compared to baseline and may have indirect effects on the integrity of the intestinal wall.

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Keywords: whole grain; gut microbiota; intestinal integrity; Bifidobacteria.

INTRODUCTION

Consumption of whole-grain products is considered to have a beneficial effect on human health, by reducing the risk of developing cardiovascular disease, obesity, type II diabetes and specific types of cancer.[1,2] The observed beneficial effects of whole-grain products on human health may in part be caused by modulation of the gut bacterial composition. Intake of whole-grain products provides substrate to specific groups of naturally colonizing bacteria, including groups generally considered to be positively associated with a healthy intestinal environment, such as bifidobacteria and lactobacilli.[3] Bifidobacteria have been shown to have beneficial effects on intestinal homeostasis either through direct contact with the epithelium or by production of specific metabolites. For example, conditioned media from *B. infantis* has been shown to decrease intestinal permeability in both *in vitro* and *in vivo* models, and the effect was attributed to peptide bioactive factors produced by bifidobacteria.[4] A bifidogenic effect is thus commonly interpreted as a prebiotic effect. In humans, use of whole-grain products as prebiotics has only been examined in a limited number of controlled dietary intervention studies. Increased numbers of faecal bifidobacteria and lactobacilli associated with consumption of whole-grain wheat (WW) cereals compared to wheat bran have been reported[5] and also a bifidogenic effect of maize-based whole-grain breakfast cereal has also been found.[6] Other studies do however not find any effect of whole-grain products on levels of bifidobacteria and lactobacilli, including a recent study of obese participants (BMI of 26–39 kg/m^2) with metabolic syndrome.[7,8] A number of *in vitro* fermentation and colonic model studies also indicate a prebiotic effect of whole-grain products.[9–12] Further investigations are needed to verify the potential prebiotic effect of whole-grain products compared to refined grain products. The effect of WW is especially important, as wheat is one of the most consumed grains world-wide.[2]

Changes in bacterial composition in the gut may directly affect the host through interaction with the cells in the intestinal epithelium. Additionally, modulation of gut bacterial composition will result in changes in the metabolite profile in the intestine, which may also affect the intestinal epithelial cells and, potentially, intestinal integrity.[13] The epithelial cells in the intestinal wall and their lateral connection mediated by tight junction proteins provide a selective barrier. Permeability has been suggested to be determined by a pore pathway, which allows ions and uncharged solutes to cross the epithelial barrier and a leak pathway through which larger molecules may pass.[14] An increase in the leak pathway may thus increase translocation of macro-molecules and

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Contributors: All authors contributed to the design of the study. EGC performed laboratory work. Data analysis was performed by EGC together with MIB and TRL. Anthropometric and clinical data were provided by MK. The manuscript was written by EGC and MIB and all authors contributed to the final revision of the manuscript.
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pathogens, resulting in adverse effects. Consumption of the bifidogenic prebiotic fructo-oligosaccharide is known to increase calcium uptake, but has also been connected to increased *Salmonella* translocation and/or infection in rodent models.[15–18] Also, electrical conductance across rat caecum and colon has been positively correlated to calcium flux.[19]

Previously, we reported the findings on the primary endpoints (adiposity and cardiovascular risk markers) of a randomized energy-restricted dietary intervention (refined vs whole-grain wheat[20]). In the present study, we explored changes in gut bacterial composition caused by the energy-restricted diet enriched with WW or RW. In addition, we investigated correlations between abundances of specific bacterial taxa with anthropometric and clinical measures and evaluated effects of faecal water on intestinal integrity using trans-epithelial resistance (TER).

MATERIALS AND METHODS

Study design

The study was conducted subsequent to an open-label parallel intervention study of 12-weeks duration.[20] Briefly, 79 overweight or obese postmenopausal women (BMI, 27–37 kg/m^2, age 45–70 years) were randomly allocated to consume either WW or RW for 12 weeks after a 2-week run-in period, during which all participants were provided with RW. In total, 72 participants completed the study (WW; $N = 38$, RW; $N = 34$), with no difference between the groups in any of the determined baseline characteristics.[20] During the study, all participants consumed an energy-restricted diet (deficit of at least 1250 kJ/day). The intervention foods were designed to replace approximately 2 MJ of the participant's habitual diet and consisted of bread, pasta and biscuits providing 105 g of whole grain daily (WW) or no whole grain (RW). This study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving human subjects were approved by the Ethical Committee of the Capital Region of Denmark (KF 01 290502). Written informed consent was obtained from all participants.

Collection and handling of faecal samples

Participants collected one or two faecal samples in 48 h during the last week of the run-in period and the intervention period. Samples were homogenized 1:1 in water and stored at −80 °C until analysis.[20] This study includes the analysis of the first sample from both time points for 37 participants from the WW group and 33 participants from the RW group. Faecal homogenates were thawed and centrifuged (18 500 *g*, 15 min, 4 °C). Pellets were used for DNA extraction using Mobio PowerLyzer PowerSoil DNA isolation Kit (MO BIO Laboratories, Carlsbad, CA, USA). Faecal water was obtained by combining faecal water from the same time point and following a second centrifugation of the supernatants (13 000 *g*, 15 min, 4 °C), sterile filtration and frozen at −20 °C. Faecal water from 14 (WW) and 11 (RW) participants were used for further analysis.

Quantitative PCR

Relative abundances, prevalence and fold changes were determined for 11 bacterial taxa (Table 1) by use of quantitative PCR (qPCR) targeting regions within the 16 S rRNA genes as described for the Gut low-density array.[21] Seven primer sets were selected to determine the overall composition of the bacterial community. Four species-specific primer sets targeting bifidobacterial species were included to determined prevalences within this genus. The primers Prev-F and Prev-R were designed based on alignments of 16 S rRNA genes from type species within the *Prevotella* genus and other genera within the *Bacteroidetes* phylum by use of the CLC Main Workbench software (CLC bio, Aarhus, Denmark). Primers were tested for specificity both *in silico* by use of Primer-BLAST[22] and by qPCR. Data obtained from qPCR were analysed as described[21] by use of the SDS 2.2 (Applied Biosystems, Foster City, CA, USA) and LinRegPCR version 11.1 software using default settings.[23,24] Average N_0-values were determined for two technical replicates. The relative abundance for each specific bacterial group was calculated by division with the average N_0-value obtained with a universal bacterial primer set on the same community DNA target. A calculated relative abundance of 0.001% was used as detection limit. For fold change calculations and correlations the relative abundance was set to 0.0005% for bacterial groups not reaching the limit of detection. Fold changes were not calculated if both samples were below the detection limit.

Cell culture

The Caco-2 cells (passage 29–45) were routinely cultured in MEM media (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Lonza, Basel, Switzerland), 1% non-essential amino acids (Invitrogen) and 100 μg/ml gentamicin in 5% CO_2 atmosphere at 37 °C with media change every second or third day and passed when above 70% confluence. For TER, Caco-2 cells were trypsinized, centrifuged (800 *g*, 10 min) and diluted to 2×10^5 cells/ml in media without gentamicin. A volume of 500 μl cell suspension was seeded in the apical compartments of Transwell permeable supports (Corning, New York, NY, USA, 3460, 0.4 μm, diameter 12 mm)

Table 1. Primers used for quantitative PCR

Target	*Primer*	*Primer sequence (5′–3′)*	*Amplicon size (bp)*	*Reference*
All bacteria (V3 16S rRNA region)	HDA1	5′-ACTCCTACGGGAGGCAGCAGT-3′	174–199	Walter J *et al.*[36]
	HDA2	5′-GTATTACCGCGGCTGCTGGCAC-3′		
Bacteroidetes phylum	Bact943F	5′-GGARCATGTGGTTTAATTCGATGAT-3′	126	Guo X *et al.*[37]
	Bact1060R	5′-AGCTGACGACAACCATGCAG-3′		
Firmicutes phylum	Firm934F	5′-GGAGYATGTGGTTTAATTCGAAGCA-3′	126	Guo X *et al.*[37]
	Firm1060R	5′-AGCTGACGACAACCATGCAC-3′		
Bacteroides spp.	B3F	5′-CGATGGATAGGGGTTCTGAGAGGA-3′	238	Bergström *et al.*[21]
	B3R	5′-GCTGGCACGGAGTTAGCCGA-3′		
Prevotella spp.	Prev-F	5′-GATGGGGATGCGTCTGATTAG-3′	290	This study
	Prev-R	5′-CTGGCACGGAATTAGCCGG-3′		
Lactobacillus spp.	Lacto-F	5′-AGCAGTAGGGAATCTTCCA-3′	341	Walter J *et al.*[38]
	Lacto-R	5′-CACCGCTACACATGGAG-3′		Heilig HG *et al.*[39]
Enterobacteriaceae	Eco1457F	5′-CATTGACGTTACCCGCAGAAGAAGC-3′	194	Bartosch S *et al.*[40]
	Eco1652R	5′-CTCTACGAGACTCAAGCTTG-3′		(Slightly modified)
Bifidobacteria spp.	F-bifido	5′-CGCGTCYGGTGTGAAAG-3′	244	Delroisse JM *et al.*[41]
	R-bifido	5′-CCCCACATCCAGCATCCA-3′		
B. bifidum	BiBIF-1	5′-CCACATGATCGCATGTGATTG-3′	278	Matsuki T *et al.*[42]
	BiBIF-2	5′-CCGAAGGCTTGCTCCCAAA-3′		
B. adeolescentis	BiADO-1	5′-CTCCAGTTGGATGCATGTC-3′	279	Matsuki T *et al.*[42]
	BiADO-2	5′-CGAAGGCTTGCTCCCAGT-3′		
B. catenulatum group	BiCATg-1	5′-CGGATGCTCCGACTCCT-3′	289	Matsuki T *et al.*[42]
	BiCATg-2	5′-CGAAGGCTTGCTCCCGAT-3′		
B. longum	A5F	5′-GGATGTTCCAGTTGATCGCATGGTC-3′	286	Bergström *et al.*[21]
	A5R	5′-TCACTCSCGCTTGCTCCCCGAT-3′		

with 1.5 ml cell free growth media in the basolateral compartments. Two inserts in each plate, not seeded with cells, were used for background resistance. Cells were cultured for 21 days, allowing cell differentiation. The day before, the TER assay media was changed and 400 µl growth media was added to the apical compartment.

Trans-epithelial resistance assay

Differentiation of the Caco-2 cells was monitored in one well by measuring TER using a Millicell ERS-2 volt-ohm meter (Millipore, Billerica, MA, USA) following the manufacture's instructions but with pre-heating of the electrode. Wells with absolute TER > 900 Ω were deemed fully confluent and differentiated and thus included in the experiments. TER was measured at time 0 and 24 h, following addition of 100 µl pre-heated faecal water or sterile MilliQ water (control). Faecal water collected from the same person was used in the same plate. Experiments were conducted in duplicate or triplicate in different plates. The measured TER for each sample was corrected by subtracting the mean resistance in the two blank wells in the plate. The unit area TER was determined by multiplying with the membrane surface area (1.12 cm^2). Fold changes in TER over 24 h were subsequently determined and normalized to the changes in TER of the controls. For each collection time and each individual an average of the normalized change in TER was calculated.

Anthropometric and clinical measures

Anthropometric and clinical measures were all obtained as previously described.[20]

Statistical methods

Statistical analysis was conducted in GraphPad Prism 5.0. D'Agostino & Pearson omnibus normality test were used. Differences in fold changes for bacterial groups between the groups were evaluated with one-way ANOVA with Kruskal–Wallis test and Dunns post-test. Changes in the relative abundance of bacterial groups during the intervention were assessed with the Wilcoxon Signed Rank test, by comparing the mean log 2 transformed fold changes to a hypothetical value of zero (indicating no change). Changes in TER were assessed by one-sample *t*-test comparing with a hypothetical mean of 1. Differences in TER between dietary groups were determined by unpaired *t*-test. Correlations between relative abundances of bacterial groups and TER, faecal pH and cardiovascular risk markers were determined using Pearson's correlations. Correlations with $P<0.05$ were considered significant and $P<0.10$ a trend.

RESULTS

Faecal bacterial composition

Fold changes of the seven bacterial groups were determined for each individual and mean log 2 transformed fold changes were calculated (Figure 1). No significant differences in microbiota composition were observed between the two dietary groups. The WW intervention, but not the RW intervention resulted in an increase in abundance of *Bifidobacterium* ($P=0.04$) compared to baseline. A decrease in abundance of *Bacteroides* ($P=0.04$) compared with baseline was found in the RW group. None of the other five bacterial groups showed significant changes in abundance following the 12-week intervention; however, a decrease in abundance was observed for *Firmicutes* ($P=0.02$), when data for all individuals were analysed collectively.

The prevalences of the genus *Bifidobacterium* and four bifidobacterial species were determined (Figure 2). Individuals found to harbour specific bifidobacterial species before the intervention period mostly harboured the same species after the 12-week intervention (data not shown).

Trans-epithelial resistance

The exposure of a Caco-2 cell monolayer to faecal water resulted in a significant mean increase in TER for WW and RW samples obtained at run-in and after the intervention (Figure 3). There was no difference in the effect of faecal water between the dietary groups after the intervention ($P=0.76$). Overall, a 7.5% increase in

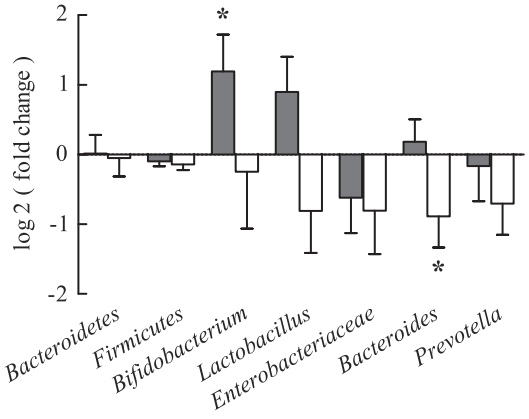

Figure 1. Observed fold changes in bacterial groups following intervention with whole-grain wheat (WW; grey) and refined wheat (RW; white). Mean log 2 transformed fold changes for the individuals in the two dietary groups are shown with error bars for s.e.m. No differences between dietary groups were found. * significant differences from a hypothetical value of 0 (change compared with baseline) according to the Wilcoxon Signed Rank test ($P=0.04$ for *Bifidobacterium* (WW) and $P=0.04$ for *Bacteroides* (RW)).

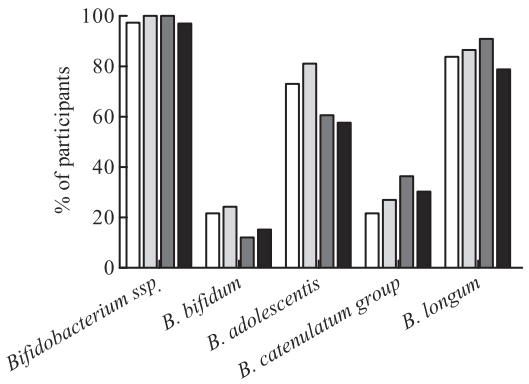

Figure 2. Prevalence of *Bifidobacterium* ssp. (%) for the whole-grain wheat group (WW; $N=37$) at run-in (white) and after the intervention (light grey) and for the refined wheat group (RW; $N=33$) at run-in (dark grey) and after the intervention (black).

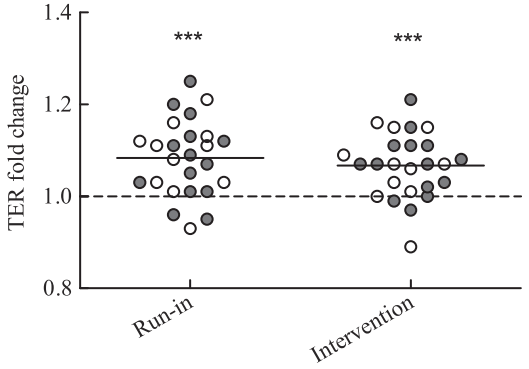

Figure 3. Trans-epithelial resistance fold change after 24 h of exposure to faecal water compared to the control. Mean values for individuals at run-in ($N=25$) and following the intervention ($N=25$) are illustrated collectively for the whole-grain wheat (WW; grey) and the refined wheat (RW; white). *** significant differences from a hypothetical value of 1 (no change), $P<0.0001$.

TER was found following addition of faecal water as compared to water. TER changes measured for two samples from different individuals were considered outliers due to their extreme values (0.51 and 1.44) and were excluded from the data analysis, resulting in normal distribution of the data.

The effect of faecal bacterial composition on TER

The relative abundance of *Bifidobacterium* showed a tendency to correlate negatively with TER ($P = 0.09$, Pearson's $r = -0.342$) in samples obtained during run-in (Figure 4). No other significant correlations were found between the microbial composition and fold change in TER. Faecal pH of the original sample did not correlate with TER ($P = 0.6$, Pearson's $r = -0.122$), but does correlate positively with the relative abundance of *Bacteroidetes* ($P = 0.02$, Pearson's $r = 0.285$) (Supplementary Figure 1).

Correlations between gut bacterial composition and cardiovascular risk markers

A significant negative correlation was observed between the relative abundance of the phylum *Bacteroidetes* and fat mass percentage ($P = 0.04$, Pearson's $r = -0.244$) and trunk fat percentage ($P = 0.01$, Pearson's $r = -0.299$) (Figure 5). Conversely, relative abundance of *Bifidobacterium* was positively correlated with fat mass percentage ($P = 0.02$, Pearson's $r = 0.282$) and trunk fat percentage ($P = 0.009$, Pearson's $r = 0.312$), with a trend observed for fat mass ($P = 0.07$, Pearson's $r = 0.220$) (Figure 5), sagittal abdominal diameter ($P = 0.05$, Pearson's $r = 0.245$) and BMI ($P = 0.1$, Pearson's $r = 0.201$) (Supplementary Figure 1). Both the systolic and diastolic blood pressure was found to be negatively correlated with the relative abundance of *Firmicutes* ($P = 0.02$, Pearson's $r = -0.281$ and $P = 0.002$, Pearson's $r = -0.367$, respectively) and also *Firmicutes* correlated negatively with Il-6 ($P = 0.01$, Pearson's $r = -0.296$) (Supplementary Figure 1). None of the other bacterial groups showed significant correlations with the determined anthropometric measures.

DISCUSSION

In the present study, intake of the WW diet, but not the RW diet, was shown to increase the mean relative abundance of bifidobacteria compared to baseline after the 12-week dietary intervention study, indicating a prebiotic effect of WW (Figure 1). This finding is consistent with a recent cross-over study ($N = 31$) in humans receiving wheat bran or WW breakfast cereals for 3 weeks[5] and further supported by *in vitro* studies.[11,12] Together with our findings this supports the prebiotic potential of WW products.

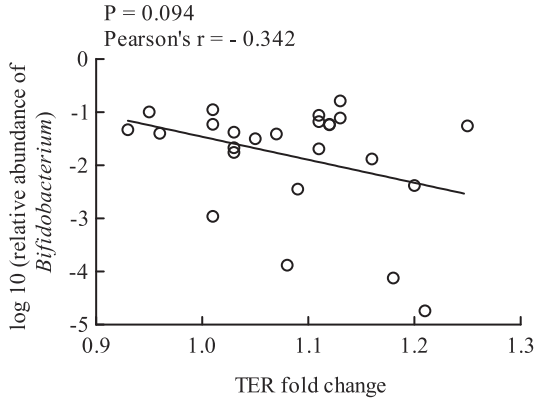

Figure 4. Correlation between trans-epithelial resistance (TER) fold change and relative abundance of *Bifidobacterium* ($P = 0.09$, Pearson's $r = -0.342$). The linear regression line is included to illustrate the correlation.

In the present study, the abundance of the *Bacteroides* genus was reduced in the RW group following the intervention but did not change in the WW group. This is consistent with another recent study, which reports decrease in abundance of *Bacteroidetes* spp. following intake of white wheat bread but not wholegrain and fiber-rich rye bread.[7] The authors suggest this to be caused by removal of rye bread from the diet. The decrease in relative abundance of *Bacteroides* indicates that the two-week run-in period was not sufficient to allow a steady state for the gut microbiota potentially due to the energy-restricted diet. The observed changes in bacterial composition in this study may thus be caused by the different types of wheat, the energy restriction or their combination. Combining fold-change data of bacterial groups for all participants irrespectively of diet showed a decrease in the relative abundance of *Firmicutes* compared with baseline, which could be attributed to the energy-restricted diet and subsequent weight loss in both groups.[20] This finding is consistent with the previous finding that ob/ob mice had higher levels of *Firmicutes* compared to lean mice[25] and that obese humans have a higher abundance of *Firmicutes* than lean humans.[26] The overall prevalence of the four bifidobacterial species (Figure 2) was not altered by the dietary intervention and showed that *B. longum* and *B. adolescentis* were the most prevalent, which is consistent with previous reports for the adult populations.[27,28]

Faecal water on average increased TER by 7.5% irrespectively of intervention diet, indicating that the complex community in the gut may in general cause increased intestinal integrity. Faecal water recovered from adults and elderly (both males and females) have in a recent study been assessed in a very similar assay, revealing an average increased TER by 4% in adults (39 ± 9.7 years) and decrease by 5% in elderly (76 ± 7.5 years).[29] Here, subjects included in the faecal water analysis had an average age of 60.0 ± 5.6 years, placing them between the two mentioned age groups, indicating that the previously reported negative effect of faecal water may be associated with age above 60 years. Higher concentrations of acetic- and propionic acid and total SCFA in adults compared to the elderly have previously been found, which may result from an age-dependent change in gut bacterial composition.[29]

A tendency for a negative correlation between TER and relative abundance of *Bifidobacterium* was shown in the present study. This contradicts previous *in vitro* studies, which have shown a positive effect of whole-cell *Bifidobacterium* and conditioned media on TER in cell assays.[4,30,31] Other studies have attributed increased levels of bifidobacteria in the gut to increased gut integrity. One example of this is a study showing that an increase in *Bifidobacterium* in ob/ob mice due to prebiotic treatment resulted in a decrease in gut permeability.[32] However, several studies[15–18,33,34] also observe increased *Salmonella* translocation and/or infection in rodents *in vivo* following intake of bifidogenic oligosaccharides, which could be caused by a reduction in epithelial integrity in the gut, as shown by Ten Bruggencate *et al.*[17] We speculate that any negative correlation between *Bifidobacterium* and TER may be connected to metabolic cross-feeding between different bacterial groups, resulting in changed abundances of other bacteria that may influence TER. Such effects will not be revealed in *in vitro* fermentation models based on bifidobacterial monocultures. Links between fiber-induced increased levels of *Bifidobacterium* and intestinal permeability in healthy human males have been studied previously, but no alterations of intestinal permeability were reported.[35] It should be noted that increased intestinal permeability may in some circumstances be beneficial to the host organism, as, for instance, calcium flux has been shown to be positively correlated to the conductance across intestinal sections from rats.[19]

We looked for correlations between anthropometric and biochemical measures and relative abundances of the seven bacterial groups quantified from faecal samples collected during the run-in period and thus occurring independently of diet. The relative

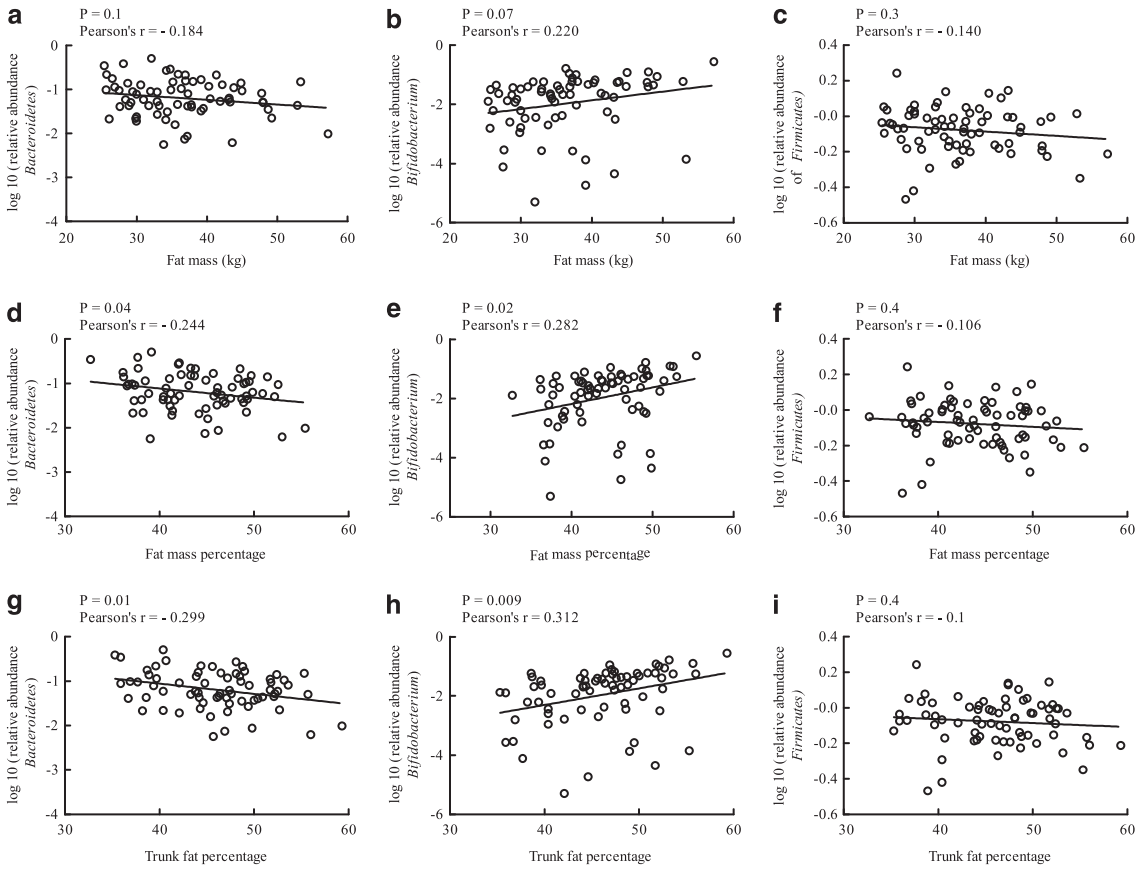

Figure 5. Pearson's correlation between fat mass (kg) (a, b, c), fat mass percentage (d, e, f), trunk fat percentage (g, h, i) and log 10 transformed relative abundance of *Bacteroidetes*, *Bifidobacterium* and *Firmicutes*. Linear regression lines are included to illustrate the correlations.

abundance of *Bifidobacterium* was found to be positively correlated to body fat mass percentage and trunk fat percentage, and a similar tendency was observed for fat mass (Figure 5). These findings suggest that high numbers of bifidobacteria are associated with adiposity, which could be explained by better utilization of energy due to metabolism of, for example, dietary fibres. Additionally, we found a negative correlation of the relative abundance of *Bacteroidetes* to body fat mass percentage as well as to trunk fat percentage (Figure 5). Consistently, we found a reduction of *Bacteroides* in the RW group, which is previously reported to have a smaller reduction in body fat percentage than seen in the WW group during the intervention.[20] Lastly, we observed a negative correlation between *Firmicutes* and both systolic and diastolic blood pressure, which warrants further study. Overall, the identified correlations between members of the gut microbiota and host anthropometric and biochemical measures provide interesting new insights into bacterial/host interactions; however, more focused studies are needed to address issues of causality.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

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Manuscript 2

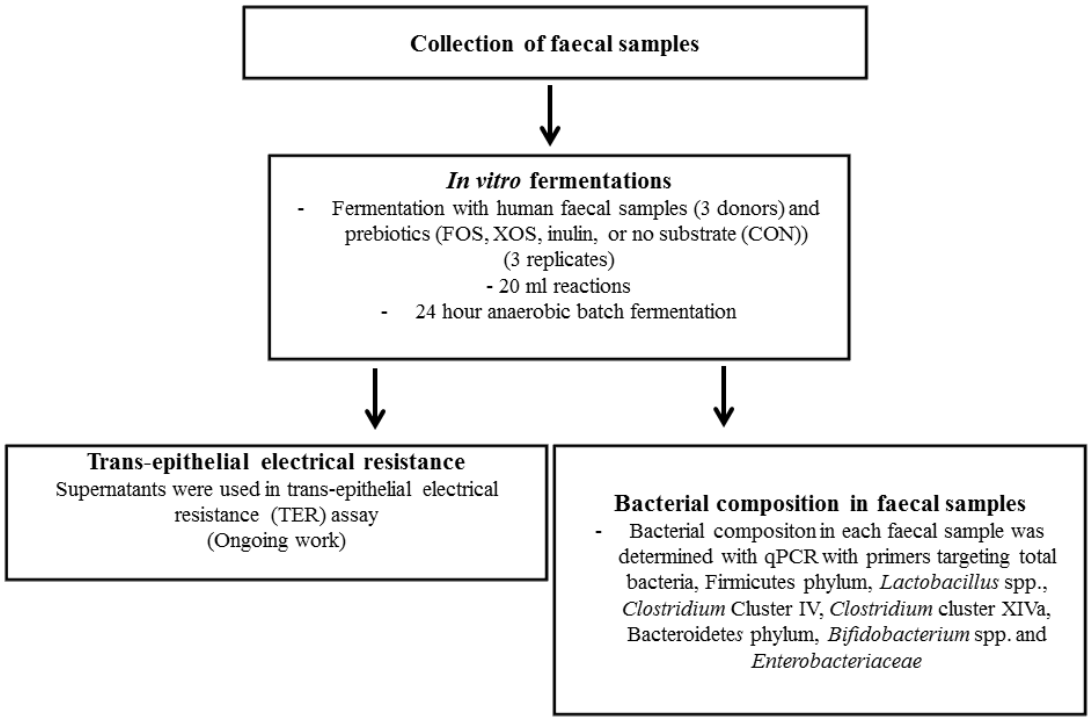
Effect of prebiotics on the faecal bacterial composition in *in vitro* fermentation and on trans-epithelial electrical resistance

Manuscript in preparation
(Page numbers are relative to the manuscript)

Introduction

The aim of the present study was to determine if prebiotics and putative prebiotics changed the bacterial composition in *in vitro* fermentations. Subsequently the effect of such changes on intestinal integrity was to be determined using the trans-epithelial electrical resistance (TER) assay.

Flowsheet



Effect of prebiotics on the faecal bacterial composition in *in vitro* fermentation and on trans-epithelial electrical resistance.

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Key words: Prebiotic, microbiota, trans-epithelial electrical resistance, batch fermentation

Running title: Effect of prebiotics on bacterial composition and TER

Abstract

The present study was conducted to determine if prebiotics and putative prebiotics modulated the bacterial composition in *in vitro* batch fermentations and if such modulations would affect intestinal integrity *in vitro* determined by the trans-epithelial resistance (TER) assay. This could subsequently be applied in future studies when testing prebiotic candidates.

Initially *in vitro* batch fermentations with Fructooligosaccharide (FOS), Xylooligosaccharide (XOS), inulin, and no substrate controls (CON) were conducted with inoculum of human faeces from three healthy donors. Following 24 hours, changes in bacterial composition in the fermentations were determined by quantitative PCR. FOS significantly increased the relative abundance of *Lactobacillus* ssp. and *Bifidobacterium* ssp. compared to the CON and *Lactobacillus* ssp. more than XOS and inulin, while XOS increased the abundance of *Lactobacillus* ssp. compared to the CON. Supernatants were obtained from *in vitro* fermentation vessels and stored for future use in TER assays with Caco-2 cells. This experimental work is still in progress due to unforeseen delays and therefore results of TER analysis are not included here. As the experimental work with the TER assay has not been completed, it cannot be concluded if supernatants from *in vitro* fermentations can be used for investigating effects of prebiotic-mediated bacterial modulation on TER.

Introduction

The gut microbiota has in recent years become acknowledged to affect human health (reviewed by [1]). Modulation of the gut microbiota can occur through dietary changes. For example a high-fat diet is considered to modulate the gut microbiota, hence increasing intestinal permeability and cause inflammation [2]. Other dietary interventions are considered to have beneficial effects on host health including consumption of probiotics and prebiotics. They may increase the abundance of certain bacterial taxa that are considered to have beneficial effects on human health including *Lactobacillus* spp. and *Bifidobacterium* spp. [1]. In addition to this an increase in butyrate producing bacteria such as bacteria belonging to *Clostridium* clusters IV and XIVa [3] may also cause beneficial effects, as butyrate has been shown to increase barrier function [4]. In general, any change in the microbial profile has a direct effect on the metabolite profile in the gut. Collectively, these affect the epithelial cells in the intestinal wall, the immune system, and subsequently human health[5]. It is therefore highly relevant to determine if prebiotic-mediated modulation of the gut microbiota has an effect on health.

In the present study the main focus is on the effect of prebiotic treatment on intestinal integrity. Prebiotics are defined as "*selective fermented ingredients that cause specific changes in composition and/or activity in the gastrointestinal microbiota that confers benefits upon host well-being and health*" [6]. During the initial screening of prebiotics stimulation of growth of specific bacteria are examined [1], [6]. But it is also highly relevant to determine if the modulations of the bacterial community and its metabolites affect the host. Here effects of these on the epithelial barrier integrity are to be examined. Alterations in the barrier function of the epithelial cells in the mucosa are important as impairment of the epithelial barrier is connected to gastrointestinal disorders such as inflammatory bowel diseases[7]. Furthermore if prebiotic treatment can modulate the gut microbiota leading to an increase in barrier function it may be used for therapeutic means for such disorders.

The epithelial lining in the intestine is the main physical barrier between the luminal bacteria and the host. Epithelial cells interact with each other through tight junction proteins, hence forming a selective barrier [8], [9]. The intestinal integrity is mainly determined by the tight junctions between the epithelial cells. Paracellular transport can occur though the tight junction complex. It has been suggested that there are two pathways, where molecules can cross the tight junction complex; the *leak* pathway, where larger molecules pass through, and the *pore* pathway, where small molecules and ions pass at a high capacity (reviewed by [10]). Changes in either pathway affect barrier integrity and can be determined by measuring the trans-epithelial electrical resistance (TER). In general an increase in TER is considered to be beneficial [11]–[14], as it indicates increased interaction between

epithelial cells, hence limiting potential translocation of bacterial compounds such as LPS. Here we want to implement the TER assay when screening prebiotics and prebiotic candidates for their effect on microbial communities in *in vitro* batch fermentations. Previously supernatants from *in vitro* fermentation models have been used in the TER assay [12], [15], [16].

The aim of the present study was to determine if prebiotics (FOS and inulin) and a putative prebiotic (XOS) changed the bacterial composition in *in vitro* batch fermentation of human faecal bacterial communities. This was determined by quantitative PCR. Subsequently, it was to be determined if supernatants from such *in vitro* fermentations had an effect on epithelial integrity in an *in vitro* assay.

Materials and methods

Collection of faecal samples

Faecal samples were collected from three healthy Danish volunteers, a 27 year old male, a 42 year old male, and a 22 year old female. None of the volunteers had received antibiotics for about two months prior to the collection time. Samples were collected in plastic containers, and transferred as fast as possible to an anaerobic chamber (Macs Work Station; Don Whitley, 10% H_2, 10% CO_2, and 80% N_2). The outer layers of the faecal samples were scraped away with a sterile scalpel or spoon to remove parts that had been exposed to oxygen. The remaining parts of the faecal samples were collected in pre-weighed tubes. These were tightly sealed and rapidly weighed outside the anaerobic chamber, before transport back into the chamber. Here an equal volume of 50% glycerol supplemented with 0.5 µg/mL resazurin was added to each sample and homogenised. This resulted in a 50% faecal solution that was aliquotet out with 6 mL in 50mL Falcon tubes. The tubes were sealed tightly and rapidly transported to -80˚C for storage.

In vitro fermentation

In vitro fermentations were conducted in minimal media with final concentrations of 2g/l buffered peptone water (Oxoid CM0509), 1 g/l yeast extract (Oxoid, LP0021), 0.1 g/l NaCl, 0.04 g/l K_2HPO_4, 0.04 g/l KH_2PO_4, 0.01 g/l $MgSO_4 \bullet 7H_2O$, 0.01 g/L $CaCl_2 \bullet 2H_2O$, 2 g/l $NaHCO_3$, 0.5 g/l bile salt (Oxoid, LP0055), 2 mL/l Tween 80, and 0.5 mg/l Resazurin (sterile filtered solution). The pH was adjusted to 7 and autoclaved. Solutions of Hemin (sigma, H-55533), L-cystein HCL (Sigma, C7477), and Vitamin K1 (Sigma, V3501) were prepared in sterile milliQ water and 96% ethanol, respectively, and sterile filtered. The solutions were added to the minimal media resulting in a final concentration in fermentations of 5 mg/l Hemin, 0.5 g/l L-cystein HCL, and 10 µl/l Vitamin K1.

Solutions of FOS (Orafti ®P95, bene Orafti) and XOS (Shandong Longlive bio-technology Co., LTD.) were prepared in sterile MilliQ water (100 mg/mL), sterile filtered, aliquoted, and stored at 5˚C. FOS

solution, XOS solution, and sterile milliQ water (CON) was added to minimal media in equal volumes. For FOS, XOS and CON four 50mL tubes with 18mL of media were reduced in the anaerobic chamber for 26-27 hours. Inulin solution (dahlia tubers, Sigma, DP36) was made on the day of the fermentation and added to the minimal media in the same manner as the FOS, XOS and CON solution, but in milliQ water that has been reduced in the anaerobic cabinet. The inulin was not dissolvable in water therefore mixing was conducted every time inulin solution or media with inulin was transferred. Four Falcon tubes with 18 mL of media with inulin were prepared.

Faecal samples were thawed in the anaerobic chamber and diluted in reduced PBS (Oxoid, Br0014G) supplemented with 0.5 mg/L Resazurin resulting in 10% w/v faeces inoculums. 2 mL of inoculums were added to 18 mL of minimal media with substrate or no substrate (CON), resulting in 1% w/v faeces and 10 mg/mL prebiotic. All fermentations were conducted in triplicates. Additionally 2 mL of reduced PBS supplemented with Resazurin was added to one tube with 18 mL of minimal media with substrate for each prebiotic and the CON (no faeces added to fermentation (N.F.)). The pH was determined with pH strips (range 1-14). All fermentations were kept in the anaerobic chamber for 24 hours with constant shaking on a rocking table (Biosan rocking table).

After 24 hours all tubes were sealed tightly, stored on ice and centrifuged (18.500 g, 15 min, 4˚C). 200 µl supernatant was used for pH determinations, while the remaining supernatant was sterile filtered (0.2 µm) before storage at -20˚C. Supernatants from replicate fermentations were later defrosted, mixed in equal volumes, aliquoted, and stored at -20^{o}C. Pellets were used for DNA extraction.

DNA extraction

DNA was extracted from the pellet using Mobio PowerLyzer PowerSoil DNA following the manufacture's recommendations with minor modifications. Bead-beating was conducted at highest speed (on a MM 300 Mixer mill (Retsch)) for 4 minutes. The DNA concentrations were determined fluorometrically (Qubit ®dsDNA HS assay, Invitrogen). DNA was stored at -20˚C until further use.

Quantitative PCR

Relative abundance of bacterial taxa in the *in vitro* fermentations was determined for 7 bacterial taxa (Table 1) using quantitative real-time PCR (qPCR) targeting the 16S rRNA genes as described by [17]. Briefly, qPCR was performed on an ABI prism 7900HT (Applied Biosystems) using 384-well MicroAmp® Optical reaction plates and SYBR-green chemistry. The qPCR reactions contained 5.5 µL of 2x SYBR Green PCR Master Mix (Applied Biosystems), 0.2pmol/µl of both the forward and reverse primer (Eurofins, mwg operon), and 2 ng of template DNA in a total volume of 11 µL reaction mix. Liquid handling was performed with an epMotion 5075 robot (Eppendorf, Hørsholm, Denmark). The

thermo-cycling program consisted of initial heat-treatment and denaturation at 50°C for 2 minutes and 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute and finally dissociation curve analysis for assessing amplicon specificity (95°C for 15 seconds, 60°C for 15 seconds, then slowly increasing to 95°C (ramp rate 1.9°C/min)). Each reaction was conducted in duplicates. For each donor the quantification of all bacterial taxa for all fermentations were conducted in the same reaction plate.

Initially amplification curves and melting curves were evaluated in the SDS 2.2 software. Non-baseline corrected data was then exported to excel before data analysis in LinRegPCR using default settings [18], [19](dr. J.M. Ruijter, Heart Failure Research Center Acadmic Medical Center, Amsterdam, The Netherlands) as previously published [17]. Here baseline correction was conducted for the individual reactions, before a window of linearity was determined. Following individual PCR efficiencies for each reaction was calculated. Based on this a mean PCR efficiency for the amplicon group was calculated and used together with the Cq value to determine N_0 for each reaction [19]. For each fermentation and amplicon average N_0 values were calculated for the duplicates. N_0 values below 0.001% of the calculated N_0 for total bacteria (universal primer), was set to 0.0005% of N_0 for the total bacteria. Replicates were allowed to differ by maximally 2 C_t values. The relative abundance for each amplification group for each of the fermentations was calculated by division with average N_0 for total bacteria. For each donor an average of the relative abundance for the three replicate fermentations were calculated. Fold changes compared to the CON were calculated for each donor and log 2 transformed. Changes in the bacterial taxa due to fermentation with the specific substrate were evaluated with a one sample t-test compared to a hypothetical mean of 0 corresponding to no change. Differences between treatments were evaluated with one-way ANOVA with Bonferroni's Multiple Comparison Test.

Results

In vitro fermentations

Triplicate fermentations were conducted for CON and for each of the three substrates FOS, XOS, and inulin for each donor. Additionally a single fermentation without faeces was conducted for CON, FOS, XOS and inulin. After 24 hours all fermentations with faeces had cloudy supernatants, while fermentations without faeces did not. At inoculation all fermentations had a pH of 7. After 24 hours of incubation the fermentations without faeces maintained a pH of 7, as did CON (Table 2). All fermentations with substrate and faeces had lower pH (Table 2).

DNA extraction

DNA was extracted from each of the fermentations and used downstream for quantitative PCR. In general the DNA yield was relatively lower for donor B compared to donor A and C (Table 3).

Bacterial composition

Changes in the relative abundance of bacterial taxa were determined by qPCR targeting the Firmicutes phyla, *Lactobacillus* spp., *Clostridium* cluster IV*, Clostridium* cluster XIV, Bacteroidetes phyla, *Bifidobacterium* spp., and the *Enterobacteriaceae* familiy. To limit the effect of differences in bacterial composition between the three donors, log2 transformed fold changes (compared to CON) were used for comparison. *Lactobacillus* spp. was significantly increased by FOS (P=0.03) and XOS (P=0.02) compared to the CON (see figure 1). For *Lactobacillus* spp. FOS resulted in a higher fold change than XOS and Inulin compared to CON (P<0.05). *Bifidobacterium* spp. was also significantly increased by FOS (P=0.005) compared to CON, while XOS tended to have an effect (P=0.05) (Figure 1). The remaining bacterial taxa did not show any significant changes.

Trans-epithelial resistance

Work with determining the effect of supernatants from the *in vitro* batch fermentations on TER is still ongoing. Results can therefore not be included. However preliminary data indicated that the application of the supernatants in the TER assay gave useful results (data not shown).

Discussion

The present study was conducted to determine if *in vitro* batch fermentations with the prebiotics FOS and inulin, and the putative prebiotics XOS affected the bacterial composition in human faeces. Subsequently effects of supernatants from such fermentations on barrier function were to be determined in the TER assay.

During the fermentation with faeces and all substrates the pH dropped from 7 to between 4 and 5 for all donors. The drop in pH indicates fermentation and acid production by the bacterial communities. Negative controls without faeces and CON (no substrate) did not have any change in pH indicating no bacterial growth. It is therefore proposed to be valid to use CON for the individual donors as controls. As the fermentations with substrate are compared to the 24-hour fermentation without supplementation of substrate (CON), changes in the microbial composition must arise due to utilization of the applied substrate. Hence the determined changes in bacterial composition are due to the substrate.

Concentrations of DNA seemed to differ between the donors and between the fermentations with and without substrate (Table 3). The differences in DNA concentration may be due to differences in compounds that can inhibit the DNA extraction process, or different numbers of bacteria in faeces

from the different donors. However, as the relative abundance of bacterial taxa was determined by comparison to the total bacteria, as previously described [17], the differences in DNA concentration was not considered to be a problem.

Significant increases in both *Lactobacillus* ssp. and *Bifidobacterium* spp. were found for FOS, and *Lactobacillus* spp. was stimulated by XOS. No change in relative abundance was found after inulin fermentation for these two bacterial taxa. FOS and inulin are prebiotics, while XOS has not been declared as such yet, but is considered to be a putative prebiotic [6], [20]. These substrates are therefore all expected to stimulate *Bifidobacterium* spp. and *Lactobacillus* spp. [1]. The lack of a stimulation of these bacterial taxa by inulin may be caused by the specific community composition of the relative few donors included in the present work.

For the remaining bacterial taxa it is difficult to make any conclusions on the effect of the three substrates, as the effect highly differs between the three donors. For example it seems that the *Enterobacteriacae* family is decreased for donor A and C by all three substrates, while no effect was found for donor B (see Figure 1). Differences in the bacterial composition within the *Enterobacteriacae* family between the three donors could have caused this effect. Another example is donor C that showed an increase in *Clostridium* cluster XIVa, while donor A and donor B had a decrease for all substrates. This highlights the need for including a higher number of donors. Others that used *in vitro* fermentations to determine effects of substrates on bacterial composition in our research group have usually included a higher number of donors [21], [22], but three donors have also been used successfully [23]. As the aim of the present study merely was an initial screening only three donors were included.

The work with the TER assay is still in progress. Initial work with the supernatants in the TER assay did show that the supernatants could be applied in the system (data not shown). However issues with the cell cultures resulted in the need to run the experimental work again. Consequently, it can at the present state not be concluded if fermentation media from *in vitro* batch fermentations can be used to assess the effect of prebiotics in the TER assay. Commane and co-workers have previously applied the TER assay to determine effects of probiotics and prebiotics on intestinal integrity [12]. Additionally others have shown that supernatants from 24 and 48 hours of *in vitro* batch faecal fermentations with resistant starches increased TER [16]. Here supernatants from continuous fermentation model were toxic for the Caco-2 cells, while supernatants from batch system were not [16]. Supernatants from a continuous fermentation system; the Simulator of Human Intestinal Microbial Ecosystem (SHIME), have also been applied successfully in the TER system [15]. It indicates that the applied fermentation model might affect the downstream TER assay and mammalian cell viability. Based on these former studies we consider it likely that combination of *in vitro* batch

fermentations with human faeces followed by use of the TER assay can be applied for screening for the effect of prebiotics and putative prebiotics on intestinal integrity. This would be highly relevant in the initial screenings of such substrates.

Acknowledgment

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Tables

Table 1. Primer sets used for determination of relative abundance of bacterial taxa. Primer name, sequence, bacterial target, product size, and references are stated.

Name	Bacterial target	Primer sequence (5'-3')	Amplicon size (bp)	Reference
116	All bacteria	ACTCCTACGGGAGGCAGCAGT	174-199	[24]
		GTATTACCGCGGCTGCTGGCAC		
164	*Firmicutes* phylum	TGAAACTYAAAGGAATTGACG	157	[25]
		ACCATGCACCACCTGTC		
32	*Lactobacillus* ssp.	AGCAGTAGGGAATCTTCCA	341	[26]
		CACCGCTACACATGGAG		[27]
60	*Clostridium* Cluster IV	GCACAAGCAGTGGAGT	239	[28]
	(*C. leptum* group)	CTTCCTCCGTTTTGTCAA		
69	*Clostridium* Cluster XIVa	AAATGACGGTACCTGACTAA	438-441	[29]
	(*C. coccoides)*	CTTTGAGTTTCATTCTTGCGAA		
114	*Bacteroidetes* phylum	GGARCATGTGGTTTAATTCGATGAT	126	[30]
		AGCTGACGACAACCATGCAG		
140	*Bifidobacterium* ssp	GCGTGCTTAACACATGCAAGTC	126	[31]
		CACCCGTTTCCAGGAGCTATT		
134	*Enterobacteriaceae* family	CATTGACGTTACCCGCAGAAGAAGC	195	[32]
		CTCTACGAGACTCAAGCTTGC		

Table 2. pH of supernatants after 24 hours of fermentation (24 h). All replicate fermentations had the same pH. CON; no substrate added. The pH was determined using pH strips (range 1-14). N.F.: No faeces added to this fermentation.

	Donor			
Substrate	**A**	**B**	**C**	**N.F.**
CON	7	7	7	7
FOS	4	4	4	7
XOS	4	4	4	7
Inulin	4	4	4-5	7

Table 3. DNA concentrations (ng/µl) following extraction from fermentations.

		DNA (ng/µl)		
		Donor		
Substrate	**Fermentation No.**	**A**	**B**	**C**
CON	1	40.1	6.23	29.2
	2	38	6.01	19.1
	3	10.4	7.27	18.9
FOS	1	2.96	1.31	6.03
	2	2.02	0.83	5.07
	3	2.17	0.78	5.57
XOS	1	2.83	0.973	19.2
	2	2.41	1.26	6.62
	3	2.59	0.91	7.53
Inulin	1	6.13	1.16	6.32
	2	8	0.94	30.1
	3	5.17	2.49	26.9

Figures

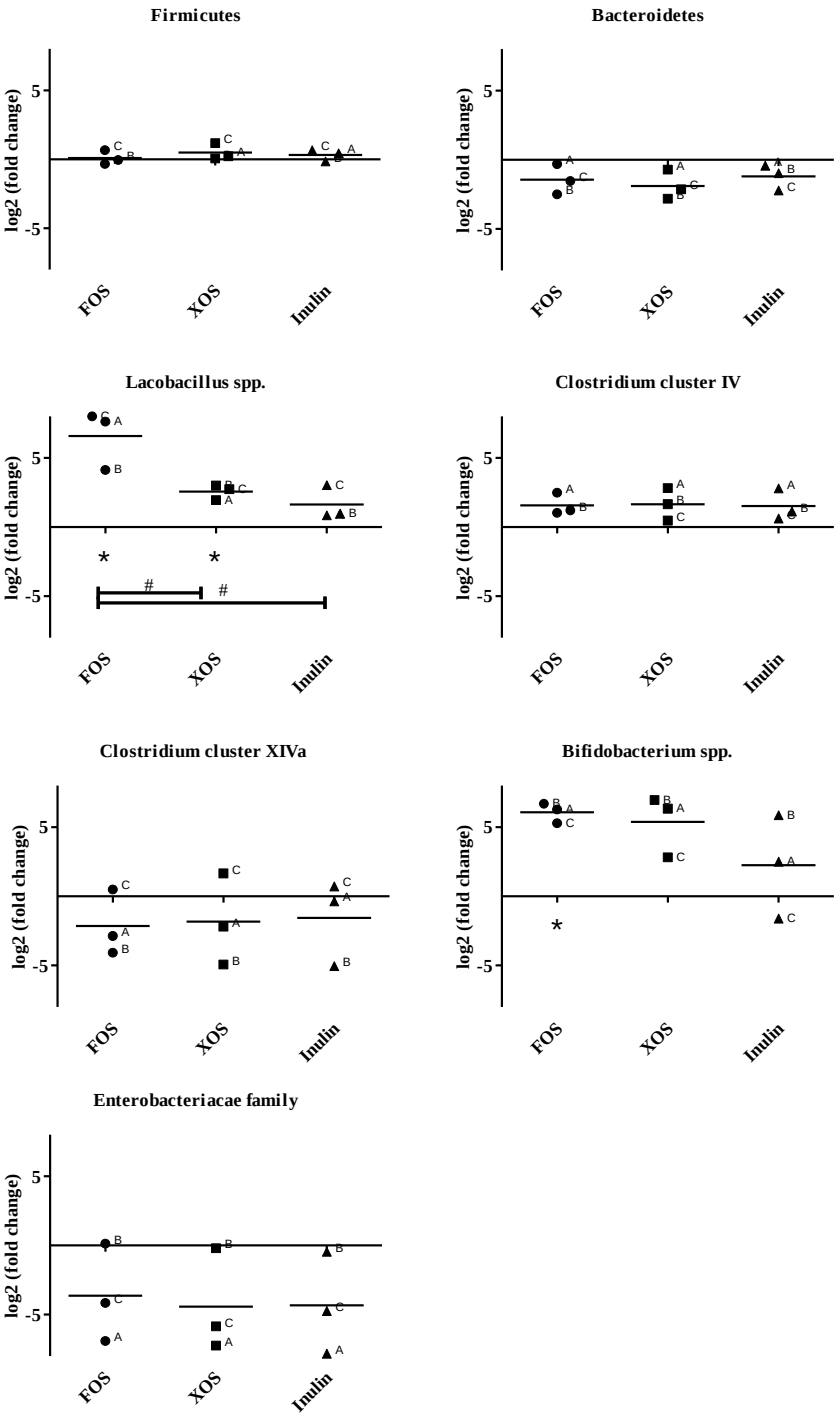

Figure 1. Log 2 (fold change) in bacterial taxa. Mean for the three donors are illustrated. The change for each donor is labelled with the donor name *; significant different from hypothetical mean of 0 according to one sample t-test (P<0.05), # significant difference based on one-way ANOVA (P <0.05)

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Manuscript 3

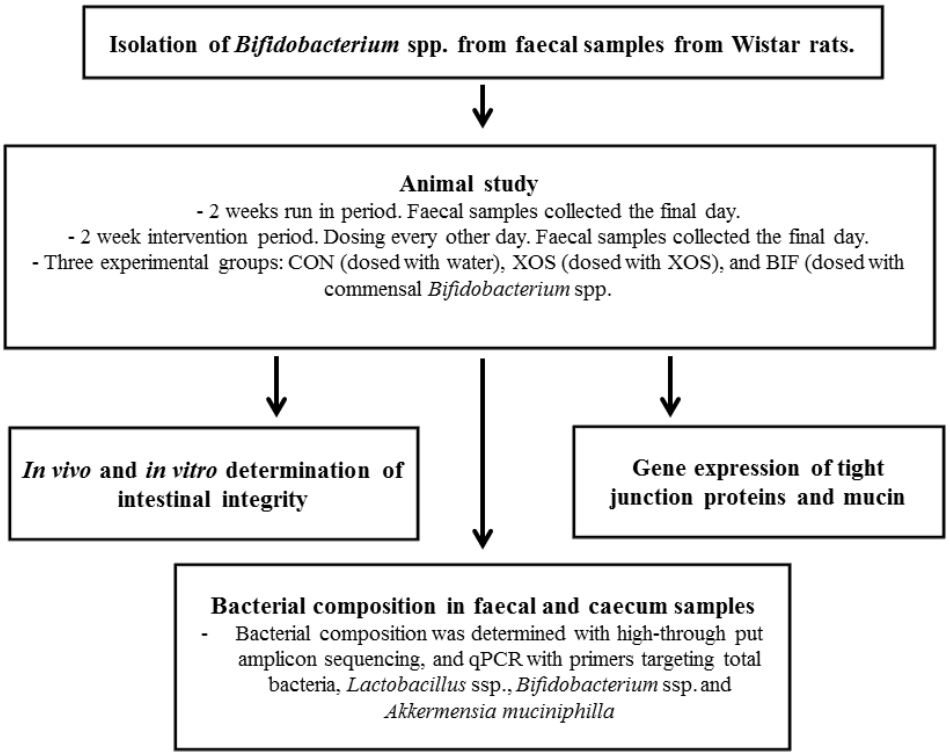
Effect of Xylo-oligosaccharides and commensal bifidobacteria on gut microbial composition and intestinal integrity in male Wistar rats

Manuscript submitted to BMC Microbiology Research Notes February 2014
(Page numbers are relative to the manuscript)

Introduction

The aim of the present study was to determine if the commercial putative prebiotic XOS affected the intestinal integrity in rats. As XOS is considered to increase the abundance of *Bifidobacterium* it was also examined whether increased abundance of commensal *Bifidobacterium* affected the intestinal integrity.

Flowsheet



Effect of Xylo-oligosaccharides and commensal bifidobacteria on gut microbial composition and intestinal integrity in male Wistar rats.

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Key words: Xylooligosaccharides, Bifidobacterium, gut microbiota, intestinal integrity

Running title: XOS and Bifidobacterium; effect on gut microbiota and intestinal integrity

Abstract

Background: Consumption of prebiotics modulates the gut microbiota, subsequently affecting the bacterial composition, metabolite profile, and human health. Previous studies indicate that also changes in intestinal integrity may occur. In order to explore this further we have investigated the effect of the putative prebiotic xylo-oligosaccharides (XOS) on the gut microbiota and intestinal integrity in male Wistar rats. As changes in intestinal integrity may be related to the expected bifidogenic effect of XOS, we additionally addressed effects of supplementation with a commensal *Bifidobacterium pseudolongum* (BIF) isolated from the same breed of laboratory rats.

Results: Changes in faecal and caecal bacterial composition determined by 16S rRNA gene sequencing and quantitative PCR for selected bacterial groups revealed that the overall bacterial composition did not differ markedly between the control (CON), XOS, and BIF groups, when correcting for multiple comparisons. However as hypothesised, the relative abundance of *Bifidobacterium* spp. was increased in XOS-fed rats as compared to CON in faecal samples after the intervention. Also *Lactobacillus* spp. was increased in both the XOS and BIF groups in caecum content compared to CON. Intestinal permeability determined *in vivo* by FITC-dextran permeability and *in vitro* using extracted caecum water in trans-epithelial resistance (TER) assay showed no effect on intestinal integrity in either the XOS or the BIF groups. However, the expression of occludin, which is part of the tight junction complex, was increased in the XOS group compared to the CON group.

Conclusions: Supplementation of XOS and a commensal *Bifidobacterium pseudolongum* did not have conclusive effects on intestinal integrity in rats as only significant change in expression of a single tight junction protein gene was found for the XOS group.

Background

The complex microbial community of the gut environment is thought to interact with the host organism and to affect human health [1]. Modulation of the gut microbial composition by consumption of specific substances such as prebiotics and probiotics may therefore affect intestinal and systemic health. Previous studies of the modulatory effect of established prebiotics as well as putative prebiotics have mainly focused on *Bifidobacterium* ssp. and *Lactobacillus* ssp. in the microbiota [2-5] as these are considered to have beneficial effects on health [6]. Effects on other bacterial groups, potentially with adverse effects on health, may thus have been overlooked. The development of high-throughput sequencing techniques now makes it feasible to survey the entire microbiota. In addition to determining the effect of pre- and probiotics on the complete gut microbiota, it is important to understand how such effects influence host health. An important marker for health is intestinal integrity, as increased intestinal gut permeability previously has been connected to intestinal disorders including inflammatory bowel diseases and celiac disease [7,8]. Gut wall permeability can be determined *in vivo* by examining the permeability of molecules with a defined size, such as FITC-dextran [9] and CrEDTA [10]. In addition, effects on intestinal integrity can be estimated by determining the expression and localization of tight-junction proteins. Effects of gut content on intestinal integrity may also be assessed *in vitro* by examining the effect of metabolites from the community found in e.g. faecal water on trans-epithelial resistance (TER) in epithelial cell monolayers [11,12].

Several previous studies have examined the effect of prebiotic supplementation on pathogen invasion in animal challenge studies. Prebiotic fructo-oligosaccharides (FOS) and the putative prebiotic xylo-oligosaccharides (XOS) [13] have previously been found to stimulate translocation of *Salmonella* in rats [14,15] and mice [16]. Here the prebiotics also stimulated increase in *Bifidobacterium* spp. [14,17] and *Lactobacillus* spp. [14,15], which are both considered to have a

beneficial effect on host health. In connection to this, FOS has been found to increase permeability of CrEDTA in rats, while also stimulating these two groups of bacteria [18]. Also, we have recently shown a trend for an inverse association between the relative abundance of *Bifidobacterium* spp. in human faeces and the effect of faecal water on trans-epithelial resistance (TER) [11]. This however does not necessarily implicate that bifidobacteria or lactobacilli are involved in the observed adverse effects, but the effects could be attributed to other factors, such as changes in non-investigated bacterial groups. The modulation of the microbiota as whole by prebiotics may thus result in adverse effects on the intestinal integrity. Also *in vitro* studies show that *B. infantis* produce compounds that increase TER [19] and that UV-killed *B. bifidum* and *B. breve* increase TER [20]. Furthermore *in vivo* studies show that bifidobacteria increase intestinal integrity in animal disease models [21,22]. We hypothesize, that an increase in *Bifidobacterium* ssp. caused by e.g. consumption of prebiotics may affect the intestinal permeability indirectly through the effect on other bacteria, causing conditions that allow increase in *Salmonella* translocation upon challenge. The aim of the present study is thus to determine effects of XOS and commensal bifidobacteria on the gut microbiota and the intestinal integrity in healthy, unchallenged rats using high throughput 16S rRNA gene sequencing quantitative PCR and three different methods to determine intestinal permeability. The study provides new insights into understanding interactions between gut bacterial community composition and intestinal integrity.

Methods

Isolation of a commensal *Bifidobacterium* spp. from rats

Faecal samples from Wistar rats were obtained prior to the animal studies from the same facility (Taconic, Lille Skensved, Denmark). Bifidobacteria were isolated from the faecal samples by plating on Bifidus Selective Medium (BSM) agar (Fluka), incubation anaerobically at 37^{o}C for three days, selection for correct colony morphology (pink or dark brown colonies) and verification

by PCR using bifidobacteria-specific primers BifF/BifR (Table 1). Universal primers 27F (5'-AGA GTT TGA TYM TGG CTC AG-3') and 907R (5'- CCG TCA ATT CMT TTG AGT TT-3') were used for sequencing. The PCR products obtained with the universal primers were purified by gel-electrophoresis and the 16S rRNA gene partially sequenced using the same primers. Four isolates were found to be identical and have 99.4 % sequence homology over 726 bp to *Bifidobacterium pseudolongum* subsp. *globosum* strain JCM 5820 by BLAST search [23]. Since the four isolated strains were identical, we chose a single strain, designated *B. pseudolongum* TR2_39 for this study. Aliquots of TR2_39 (1 ml) were frozen in 7.5% glycerol and stored at -80^{o}C.

Animals and housing

6 week-old male Wistar rats were purchased from Taconic (Lille Skensved, Denmark) and originated from the same stable where faecal samples used to isolate TR2_39 were collected. On arrival the animals were housed in pairs and had *ad libitum* access to chow (Altromin 1324) and drinking water throughout the experiment. The environment was controlled with 12-hour light/dark cycles, temperature at 22 ± 1°C, relative humidity at 55±5% and 8-10 air changes per hour. Animals were observed twice a day. The animal experiment was carried out under the supervision of the Danish National Agency for Protection of Experimental Animals.

Experimental design

Four days after arrival the animals were weighed and cages were allocated randomly to the three experimental groups, namely CON (dosed with sterile water), XOS (dosed with XOS), and BIF (dosed with *B. pseudolongum* TR2_39) with 16 animals (8 cages) in each group. The XOS was obtained from Shandong Longlive Bio-Technology CO. Ltd, China as 95% pure powder extracted from corncob (zea). To limit potential effects of co-housing and coprophagia on the gut microbial composition, the animals were housed together for additionally 2 weeks before the dosing period

was initiated. During the acclimatization period the weight of the animals, and the water and feed intake was monitored as intake per cage per day.

During the intervention period the animals were given oral gavage with 1ml milliQ water (CON), 2 ml 500mg/ml XOS (XOS) or 1 ml *B. pseudolungum* TR2_39, approximately 2.2-6.2*10^8CFU/ml (BIF) every second day for 14-16 days. The inoculum was prepared fresh for each dosing day from one aliquot of glycerol-frozen TR2_39, by anaerobic cultivation in four tubes with 45 ml BSM broth for approximately 48 hours followed by wash in reduced PBS and resuspension in PBS. The optical density was adjusted to OD_{600} = 10. Half of the animals were euthanized (CO_2 chamber and decapitation) on day 14 and the remaining on day 16 after the initial dosing. Animals in the same cage were euthainised sequentially. Weight, water, and feed intake was monitored during the intervention period, as described for the acclimation period. Faecal samples were collected on Day 0 prior to initial dosage, and the day before euthanisation (Day 13 or 15) by collecting defecate directly in tubes. Samples were stored at -80°C until analysis.

***In vivo* intestinal permeability assay**

On the day of euthanisation, intestinal integrity was determined by measuring the permeability of FITC-dextran, using a similar approach as previously described [9]. Animals were fasted for at least 9 hours before the assay. From each cage, one animal was orally dosed with 0.5 ml 120 mg/ml FITC-dextran (4 kDa, Sigma-aldrich FD-4) per 100 g (corresponding to 600 mg/kg animal) bodyweight while the other was dosed with 0.5 ml PBS per 100 g bodyweight. Two hours after dosage, animals were euthanized and blood was collected from the neck directly into 50 ml Falcon tubes with 100 µl EDTA (0.5M, pH 8, Ambion). Blood samples were immediately centrifuged (3800 rpm, 5 min) to collect plasma. Plasma was centrifuged again, diluted 1:1 in PBS and stored at 5°C until analysis on the same day. Analysis of each sample was done in triplicate by transferring

volumes of 60 µl plasma-PBS solution to a black 96-well microtiter plate (Proxiplate-96 F, Perkin Elmer) and measuring the florescence at excitation 485 nm / emission 535 nm (Victor TM X4, Perkin Elmer). Standard curves were prepared for each of the euthanisation days, by adding fixed concentrations of FITC-dextran to plasma-PBS prepared from animals dosed with PBS.

Dissection of animals

Only animals not dosed with FITC-dextran were dissected to exclude potential effects of FITC-dextran in the down-stream analysis. Abdomens were rinsed in 70% ethanol and dried with a paper towel before the incision. Approximately 2.5-4 cm from the caecum, an ileal section (0.5-1.0 cm) was removed and rinsed in PBS before storage in 1 ml RNAlater® (Life Technologies). Colonic sections were taken where the first pellet of content was visible (often 4-5 cm from caecum), and treated the same way as ileal samples. Finally, contents from the caecum were collected, where after the ceacal tissues were washed in PBS and stored in RNAlater®. Caecal contents were stored at -80°C, while tissues in RNAlater® were stored at 5°C overnight, and then transferred to -80°C.

Collection of caecal content and cecal water

Caecal contents were weighed and homogenized 1:1 in MillliQ water. Slurries were centrifuged (11.000g, 15min) and the pellets stored at -80°C in aliquots of approximately 250mg. Supernatants were centrifuged again and the pH was determined (Thermo, Orion star) before sterile filtration (0.2µm pore size, Sarstedt) and storage at -20°C.

Extraction of bacterial DNA

DNA was extracted from faecal samples collected before the initial dosing (Day 0), the day before euthanisation (Day 13 or Day 15), as well as from caecal samples using the MoBio PowerLyzer® PowerSoil® DNA isolation kit (Mobio) following the recommendations of the manufacturer. DNA

concentrations were determined using Qubit ds DNA HS assay kit (Invitrogen). DNA was stored at -20°C until further analysis.

Ion Torrent sequencing

The bacterial composition was determined by sequencing of the V3-region of the 16S rRNA gene in bacterial DNA extracted from caecal contents, and from faecal samples collected before (Day 0) and after the intervention (Day 13 and Day 15) originating from animals not used for the FITC-dextran permeability assay (i.e. total of 24 animals). Amplification of the V3-region and subsequent sequencing was performed using the Ion Torrent PGM platform essentially as previously published [24]. Briefly, the V3-region of the 16S rRNA gene was amplified using a universal forward primer (PBU 5'-A-adapter-TCAG-barcode-CCTACGGGAGGCAGCAG-3') with a unique 10-12 bp barcode for each bacterial community (IonXpress barcode as suggested by the supplier, Life Technologies) and a universal reverse primer (PBR 5'-trP1-adapter-ATTACCGCGGCTGCTGG-3'). PCR reactions were conducted with 4µl HF-buffer, 0.4µl dNTP (10mM of each base), 1µM forward primer, 1µM reverse primer, 5ng template DNA, and 0.2µl Phusion High-Fidelity DNA polymerase (Thermo Scientific) in a reaction volume of 20µl. Reactions were run at 98°C for 30 seconds followed by 24 cycles of 98°C for 15 seconds and 72°C for 30 seconds, before 72°C for 5 minutes and cooling at 4°C. Products were separated on a 1.5% agarose gel with SYBR-safe at 100V for 90 minutes, visualized with the Safe Imager™ 2.0 (Invitrogen) and bands of expected size (approximately 260bp) were excised from the gel. DNA was extracted using MinElute Gel extraction kit (Qiagen) following the recommendations of the manufacturer. DNA concentrations were determined with Qubit HS assay and a library constructed by mixing an equal amount of PCR products from each original community. Sequencing was performed on a 318-chip for Ion Torrent sequencing using the Ion OneTouch^{TM} 200 Template Kit v2 DL. Sequence data were obtained in

FASTQ format and further processed using CLC bio genomic workbench (Qiagen) in order to de-multiplex and remove sequencing primers. Further quality trimming using default settings (quality score = 0.05, trim ambiguous nucleotides=2) and selection of reads with a final length between 110bp – 180bp was performed before exporting reads in FASTA format. The number of good quality reads used for taxonomical assignment ranged from 46,877 to 100,000. All sequence reads were taxonomically classified using the Ribosomal Database Project Multiclassifier tool [25]. A bootstrap cut-off ≥ 50%, was chosen as recommended for fragments below 250bp and previously shown to be effective [26]. Relative abundance of bacterial taxa (family level) were determined for each community by comparing the number of reads assigned to a specific family to total number of reads assigned to the bacterial root. To limit variation between animals, the fold-change during the intervention was determined by calculating relative abundance before divided by relative abundance after, and log 2 transformations of these data. Bacterial taxa that were detected either before or after the intervention, but not in the corresponding before/after-sample from the same animal were set to 0.0005% analogous to 1 read in 200,000 reads.

Quantitative PCR

The relative abundances of *Bifidobacterium* spp., *Lactobacillus* spp., and *Akkermensia muciniphila* in faecal samples from all animals as well as caecal samples were determined using quantitative PCR in a total reaction volume of 11 µl in 384-well microtiter plates using a LightCycler 480 II (Roche Applied Science). Each reaction contained 1X SYBR green mix (Roche Applied Science), 0,2 pmol/µl of each primer (Table 1), and 2 µl template DNA (1ng/µl) and setup in four technical replicates with DNA from faecal samples collected before and after the intervention run on the same plate. Reaction conditions were: 95°C for 5 min, 40 cycles of 95°C for 10 sec, 60°C for 15 sec, and 72°C for 45sec, followed by melting curve generation (95°C for 5 sec, 65 for 1 min and

increasing the temperature to 98°C with a rate of 0.11°C/sec with continuous fluorescence detection). Data was initially analysed in the LightCycler® 480 software. Noise band and threshold was set automatically using the LightCycler® 480 software. Average C_q-values of the four technical replicates calculated by the software were used for data analysis. Single Cq values differing by more than 2 cycles were considered outliers. The relative abundances of each gene target normalized to the total number of 16S rRNA genes (universal bacterial primer) were calculated as $(1+E_{universal})^{Cq\_universal}/(1+E_{target})^{Cq\_target}$. Mean PCR efficiency (E) for each primer set was calculated by use of the LinRegPCR software [27]. If the relative abundance was calculated to be below 0.001% of the total bacteria (corresponding to the ratio being below 10^{-5}), it was set to half this value.

RNA extraction and cDNA preparation.

Total RNA was extracted from approximately 20mg of ileum, caecum, and colon tissue using the RNeasy mini kit (Qiagen) following the suppliers recommendations. RNA concentration and purity was determined using Nanodrop Spectrophotometer ND-1000 (Thermo Scientific). Samples with A260/A280 between 1.8 and 2.1 were used in the further analysis. RNA was stored at -80°C. The cDNA was prepared immediately from 500ng RNA in 20µl reactions using the SuperScript VILO cDNA Synthesis Kit (Life technologies) following the suppliers recommendations and stored at -20°C until further use.

Gene expression analysis

The relative expression of the tight junction proteins claudin-1 (*CLDN1*), ZO-1, and occludin (*OCLN*), and mucin 2 (*MUC2*), involved in mucin production, were determined with quantitative PCR using actin beta (*ACTB*) and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) as reference genes (table 1). Reaction conditions were as above and the reactions run under the

following conditions; 95°C for 5min, 40 cycles of 95°C for 10 sec, 60°C for 10 sec, and 72°C for 30sec, followed by melting curve preparation 95°C for 5sec, 65 for 1min and 98°C continually. As template, 2µl 10-fold diluted cDNA was used. The relative expression was calculated using the geometric mean of the two reference genes.

Trans-epithelial resistance

The mammalian cell line Caco-2 (passage 15-25) were cultured in DMEM (Gibco) supplemented with 20% heat inactivated fetal bovine serum (Gibco), 1X Non-essential amino acids (Thermo Scientific), and 1X Pen/strep (Biological industries) at 37°C and 5% CO_2. Cells were trypsinized when 60-80% confluent. A cell suspension of 10^5 cells/ml was prepared and 500µl was seeded in the apical compartment of 12mm, 0.4µm pore size Transwell® polyester membrane inserts (Corning, USA), while 1.5ml medium was added to the basolateral compartment. Cells were cultured on the inserts for 21 days with change of medium twice a week. At day 21 the cells were moved to the cellZscope ® (nanoAnalytics, Germany). Culture medium was changed, and accordingly 760µl and 1.65ml medium was added to the apical and basolateral compartment, respectively. TER was monitored for 20-23 hours. 76µl medium was then replaced with caecal water, sterile milliQ water (water) (control of the dilution of the cell culture media), or standard cell culture media (cell media control) (control of the cells), resulting in exposure to 5% caecal water. TER was subsequently measured every hour for 24 hours. All treatments were conducted in three replicates. All caecal water samples obtained from a given animal were analysed on the same day. Caecal water from the animals were used randomly, and placed randomly in the cellZscope ®. The percentage changes in TER were determined based on the last measured TER before exposing the cells (t = 0). In most cases an average of the three replicates was calculated; however for a few samples only two replicates were used.

Statistics

All data analysis was conducted in GraphPad Prims version 5.0 for Windows (GraphPad Software, CA, USA.) if not otherwise stated. Differences in animal weight, water intake, feed intake, FITC-dextran plasma concentrations, and caecal water pH between groups were assessed by one-way ANOVA with Bonferroni post-test or Kruskal-Wallis Dunns post-test for non-normally distributed data. The Metastats tool [28] was used for 16S rRNA gene sequence analysis using non-parametric t-tests based on 1000 permutations and setting the false discovery rate $q = 0.05$ as significant. For selected bacterial groups the relative abundances and fold-changes, determined by both 16S rRNA gene sequencing and qPCR, were also compared between CON and both XOS and BIF using Mann-Whitney U-test. Log 2 transformed fold changes were compared to a hypothetical median of zero using the Wilcoxon signed rank test. Differences in gene expression of tight junction proteins and MUC2 between different types of tissue were determined for the CON group by one-way ANOVA with Bonferroni post-test or Kruskal Wallis test with Dunns post-test (not normally distributed data). Differences between CON and XOS or BIF for the individual tissues were determined using Mann-Whitney U-test. Correlation analysis was determined using the Spearman correlation, considering $P < 0.05$ to be significant. The X^2-test was used to compare the number of observed differences between faecal and caecal samples in the three groups.

Results

Animal growth, feed, and water intake.

There were no significant differences in animal weight gain between the three groups (Figure 1). Additionally, no significant differences in water and feed intake between the three groups were recorded (data not shown).

Bacterial composition

Bacterial community analysis at phylum level based on 16S rRNA sequencing of 24 animals (one from each cage) before intervention revealed variation in the relative abundance (Figure 2A), and markedly *Actinobacteria* varied approximately 100-fold from 0.085% to 10.9% and *Bifidobacteriaceae* 10,000-fold from 0.001% to 10.7% between individual animals (Figure 2B). Significant negative correlations were found between *Bacteroidetes* and *Firmicutes* ($P < 0.0001$, $R = -0.82$) and *Firmicutes* and *Actinobacteria* ($P = 0.019$, $R = -0.48$) and also a negative correlation between *Bacteroidetes* and *Actinobacteria* ($P = 0.023$, $R = 0.46$). No significant differences in relative abundances before and after intervention were found between any of the detected bacterial families in faecal samples from the two intervention groups as compared to the CON group after correction for multiple testing (Figure 3). Neither did principal component analysis of sequencing data at the family-level show any clustering of samples according to intervention group (data not shown). Additionally, no differences in the fold-change (after/before) of any of the detected bacterial families were found between the groups after correction for multiple testing (data not shown). We did however observe differences in the mean relative abundances of several bacterial families between faecal samples and caecal content samples (Table 2).

Analyses of relative abundance and fold-change during the intervention for bacteria belonging to the *Bifidobacteriaceae* and *Lactobacilliaceae* were conducted separately as we hypothesized these groups to be affected and also included qPCR-based assessment of the relative abundance of *Bifidobacterium* spp., *Lactobacillus* spp., and *Akkermansia muciniphila* (Figure 4). Taken together, results obtained by qPCR (Figure 4B, D, and F) appeared very similar to the sequencing data (Figure 4A, C, and E). Fold-change data show that *Lactobacillus* ssp. increased in the CON group ($P = 0.014$) and the BIF group ($P = 0.0018$) compared to baseline (qPCR data). In addition, *A. muciniphila* significantly increased compared to baseline in the XOS intervention group ($P =$

0.014). There were no significant differences in fold-change for either of the bacterial taxa between the control and the two treatment groups. Sequencing data revealed a trend for a larger fold-change of *Bifidobacterium* spp. in the XOS group than in the CON group (P = 0.10), however this was not confirmed by qPCR (P = 0.19). Nevertheless, qPCR showed that the relative abundance of *Bifidobacterium* spp. in faeces (Figure 4D) was higher in the XOS group than in the CON group (P = 0.044), while this was not confirmed by sequencing data (Figure 4C, P = 0.23).

In caecal content (Figure 4E and 4F) both the XOS and BIF groups had higher relative abundance of *Lactobacillus* spp. than the CON group (XOS; P = 0.04, BIF; P = 0.03) according to qPCR, while a tendency for this was confirmed by sequencing analysis (XOS; P = 0.08, BIF; P = 0.08). Additionally, XOS tended to increase *Bifidobacteriacae* in caecum content (P = 0.10) detected by sequencing.

Intestinal permeability

No differences in FITC-dextran concentration in the plasma were observed between the three groups (Figure 5A). The results from two animals, one from the CON group, and one from the BIF group, were excluded due to technical errors.

The average caecal water pH was 7.53 ± 0.15 (SD), 7.48 ± 0.23, and 7.58 ± 0.20 for the XOS group, BIF group, and CON group, respectively with no significant differences between the groups. Caecal water from all three groups on average increased TER as compared to the controls exposed to diluted cell media or normal cell media (Figure 5B), but no significant differences were found between the three experimental groups after 24 hours of exposure (Figure 5C), although the TER was consistently lower in all time points between 12 and 24 hours after exposure to caecal water from either of the treatment groups as compared to CON (Figure 5B).

Gene expression

Differences in gene expression between tissue types were determined for the CON group (figure 6). Expression of *MUC2* was higher in the colonic tissue than in ileal ($P < 0.01$) and caecal tissue ($P < 0.001$), and also expression of ZO-1 was higher in colon than ileum ($P < 0.001$). The expression of claudin-1 and occludin did not differ between the intestinal sections. The relative expression of occludin in colon was higher ($P = 0.04$) in the XOS group than in the CON group (Figure 6C). No other significant differences between the groups were found.

Correlations between gene expression of epithelial cells, measures of intestinal integrity and relative abundance of selected bacterial groups.

No significant correlations were found between the relative abundance of *Bifidobacterium* spp., *Lactobacillus* spp. or *Akkermansia muciniphilla* in caecal content and faecal samples (qPCR), and relative expression of CLDN-1, ZO-1, MUC2, and OCLN in ileal, caecal and colonic tissue, as well as plasma FITC-dextran concentrations and TER, irrespective of experimental group (data not shown).

Discussion

Changes in the gut microbial composition have been proposed to affect intestinal integrity [9]. The present study was designed to address this issue further by focusing on the effects of bifidobacterial abundance on microbial community composition and intestinal integrity in male Wistar rats. Two different approaches were used to increase levels of bifidobacteria, namely (i) oral dosage with live cultures of an endogenously isolated strain (probiotic approach) and (ii) oral dosage with XOS, which has previously been shown to stimulate bifidobacterial growth in a mouse model [17] (prebiotic approach).

Experimental animals bred and treated under standardized conditions are generally expected to exhibit less inter-individual variation than a free-living human population and consequently it should require fewer individuals to find effects in dietary intervention studies. Comparison of the animals at base-line (Figure 2A) revealed less variation within the two most abundant phyla, *Firmicutes* and *Bacteroidetes*, than reported in human studies [29], but interestingly, for bacteria belonging to the *Actinobacteria*, a more than 100-fold difference in relative abundance was observed between animals. For the *Bifidobacteriaceae* family, belonging to the *Actinobacteria*, we observed approximately 10,000-fold difference in relative abundance before the intervention commenced (Figure 2B). The high initial level of variation within the *Bifidobacteriaceae* in this study may impede detection of the expected XOS or BIF driven increase in relative abundance of this bacterial group during the intervention, as such an increase was only detectable by qPCR, and not by sequencing of community-derived 16S genes. The increased relative abundance of bifidobacteria following intake of XOS is consistent with a previous study in male Sprague-Dawley rats, which showed increase in both faecal and caecal levels of bifidobacteria following a 14-day intervention with XOS added to feed at 6% [30] and also an increase is reported in XOS-fed mice [17]. Animals in the BIF group received approximately 2.2-6.2*10^8 *B. pseudolungum* cells every second day during the intervention. This did however not result in higher levels of bifidobacteria in either caecum content or faecal samples at termination. In spite of the fact that the bifidobacterial strain applied was isolated from similar rats, we speculate that the strain did not colonize and/or proliferate in the rat gut, resulting in washout before faecal samples were obtained approximately 24 hours after the last dosage. A study addressing intestinal transit of *B. bifidum* following gavage in mice showed a peak in the abundance of this strain in faeces at around 6 hours after dosage and subsequently a significant reduction after 18 hours [31]. Alternatively, the dosing level was too low

to have an effect or bifidobacterial cells may not have survived passage through the acidic environment of the rat stomach.

Quantitative PCR as well as 16S rRNA amplicon sequencing revealed higher caecal levels of *Lactobacillus* spp. in both the XOS and BIF groups compared to the CON groups after intervention (Figure 4E-F). This is consistent with a prebiotic effect of XOS [6] and confirms that increasing the abundance of one bacterial group may influence the abundance of another through e.g. metabolic cross-feeding processes [32] or by changing environmental conditions such as pH. Detection of significant differences in the relative abundance of *Lactobacillus* spp. between the groups was facilitated by a relatively low initial variation of *Lactobacilliaceae* (approximately 70-fold) compared to *Bifidobacteriaceae* (Figure 2B). Quantatative PCR is anticipated to result in better quantification than amplicon sequencing, especially for low-abundant bacterial groups, due to the low absolute number of sequence reads in the latter. In the present study we observe only slightly more significant differences by the qPCR approach compared to the sequencing approach (Fig. 4) indicating only marginally higher power.

The mucin degrading species *A. muciniphila* was included in the qPCR analysis, due to its status as potential marker for intestinal health (reviewed by [33]). An increase in levels of *A. muciniphila* after the intervention compared to baseline was found only in the XOS group (Figure 4B). This may be explained by a XOS-induced increased production of mucin, as *A. mucinphilla* is capable of degrading mucin as sole carbon source [34]. Also *A. mucinphilla* is reported to be reduced in patients suffering from disruption of the gut mucus layer due to mucosal inflammation [35] as well as in ob/ob mice [36]. Prebiotics have previously been shown to normalize, hence increase, *A. muciniphila* abundance in obese and type 2 diabetic mice and also administration of viable *A. muciniphila* was connected to improvement of metabolic disorders in mice fed a high-fat diet, potentially due to reestablishment of the mucus layer [36]. Nevertheless, we observed no

differences in expression of the mucin gene (*MUC2*) between the three experimental groups in any of the intestinal segments (Figure 6B). However, as the actual amount of mucus was not determined, this does not exclude the possibility of increased mucin levels in the XOS group due to post-transcriptional alterations and/or increased expression of other mucin encoding genes. Previously increased levels of mucin secretion were reported in animals fed FOS [14,18,37]. Mucins secretion was also increased in humans, but this was not connected to altered permeability for CrEDTA [38].

The overall mean gut microbiota composition in faecal samples was very similar in all three groups before the intervention and remained so during the intervention (Figure 3). No differences in microbiota composition after the interventions were observed between treatment groups after correction for multiple comparisons (Figure 3). We observed several bacterial families which differed in mean relative abundance in cecum content compared to faecal samples, including higher levels of *Actinobacteria* and lower levels of *Peptostreptococcaceae* and *Veillonellaceae* associated with faecal samples in all three intervention groups (Table 2). We observed fewer families that differed in relative abundance between faces and cecum content in the XOS and BIF groups than in the control group but this was not significant (X^2-test).

Measures of rat gut integrity were obtained by three independent measures namely (i) permeability of FITC-dextran molecules across the epithelial barrier (Figure 5A), (ii) trans-epithelial resistance of Caco-2 cells after exposure to caecal water (Figure 5B-C), and (iii) relative expression of genes encoding tight junctions proteins or mucin (Figure 6). These measures were selected to collectively cover different aspects of gut permeability. Intestinal permeability is mainly determined by paracellular transport between epithelial cells, which has been suggested to be divided into two pathways: The high-capacity “pore pathway” where small molecules (below 4Å) can pass, and the low-capacity “leak pathway” where larger molecules may pass (reviewed by [39]). Changes in

FITC-dextran permeability indicate a change in the leak-pathway, while changes in TER may indicate changes in both pathways [39]. We found no statistically significant effect on either FITC-dextran permeability or TER after 24 hours between treatment groups and the CON group of animals (Figure 5A and C). Nevertheless, TER was observed to be consistently higher in the CON than both the XOS and BIF groups from around 12 hours until termination at 24 hours, indicating an increase in permeability in the Caco-2 monolayer during exposure to caecal water from XOS and BIF (Figure 5B). This is consistent with a previously observed trend for a negative correlation between TER and relative abundance of bifidobacteria [11]. Caecal-water collected from CON, XOS of BIF animals increased TER during 24-hours significantly more than water, which was used as control. This suggests that caecal water positively affects tight-junction interaction, which is consistent with similar observations on faecal-water [11]. Expression levels of occludin genes in colonic tissue were significantly higher in the XOS group than in the CON group. Changes in expression of ZO-1 and occludin in ob/ob mice after consumption of prebiotics have previously been studied showing that prebiotic treatment increased levels of *Bifidobacterium* spp. as well as occludin and ZO-1 expression in jejunum, and also decrease FITC-dextran (4kDa) permeability [40]. Additionally, high-fat feeding was reported to decrease *Bifidobacterium* spp., increase intestinal permeability and decrease the expression of ZO-1 and occludin [9]. It should be noted that specific strains of bifidobacteria may have varying effects on markers of intestinal integrity [19,41,42], which could explain the relatively low effect of the *B. pseudolongum* isolate in the current study.

CONCLUSION

The present study was designed to address the hypothesis that increased levels of bifidobacteria are linked to decreased intestinal integrity caused by modulation of the microbiota, as indicated by previous studies showing increased *Salmonella* translocation following intake of prebiotics in

rodents [14-17]. However, this hypothesis was not confirmed, perhaps because the limited effects of XOS and dosage of bifidobacteria on intestinal bifidobacterial loads were insufficient to induce measurable changes in intestinal integrity. Our observations of increased occludin expression after XOS consumption seem to contradict the hypothesis, while the consistent decrease in TER caused by caecal water from BIF and XOS rats, although not significant, points in a confirmatory direction.

COMPETING INTERESTS

Authors have no conflicts of interest to declare.

AUTHOR CONTRIBUTIONS

EGC, MIB and TRL planned the study. EGC performed experimental work, data analysis, and first drafting of the manuscript. TDL coordinated TER analysis. All authors contributed to interpretation of data and final revision of the manuscript.

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Tables

Table 1. Primers used for PCR and quantitative PCR

Target	Primer	Primer sequence (5'-3')	Size (bp)	Ref
Bifidobacterium spp.	BifF	GCGTGCTTAACACATGCAAGTC	126	[43]
	BifR	CACCCGTTTCCAGGAGCTATT		
Lactobacillus spp.	LactoAll_1F	AGCAGTAGGGAATCTTCCA	341	[44]
	LactoAll_1R	CACCGCTACACATGGAG		[45]
Akkermansia muciniphila	AM1	CAGCACGTGAAGGTGGGGAC	327	[46]
	AM2	CCTTGCGGTTGGCTTCAGAT		
Universal bacteria	HDA1	ACTCCTACGGGAGGCAGCAGT	200	[47]
	HDA2	GTATTACCGCGGCTGCTGGCAC		
Beta-actin (Actb)	ACTB_A	CACCCGCGA GTACAACCTT	207	[48]
	ACTB_B	CCCATACCCACCATCACACC		
Glyceraldehyd-3-phosphate (*Gadph*)	GAPDH2_A	CAAGTTCAACGGCACAGTCAAG	123	[49]
	GAPDH2_B	ACATACTCAGCACCAGCATCAC		
Mucin 2 (*MUC2*)	MUC2_A	TCCCTCTTACAAGGGCAATG	123	[50]
	MUC2_B	TTCCAGCTGTTCCCAAAGTC		
Claudin-1(*CLDN-1*)	CLDN-1_A	TGTCCACCATTGGCATGAAG	118	[51]
	CLDN-1_B	GCCACTAATGTCGCCAGACC		
Occludin (*OCLN*)	OCLN_A	GCCTTTTGCTTCATCGCTTC	125	[49]
	OCLN_B	AACACCATGATGCCCAGGAT		
Zonula occludens-1 (ZO-1)	ZO-1_A	AAGCCAGTCACGATCTCCCG	106	[49]
	ZO-1_B	GCGCTCTTCCTCTCTGCTCC		

Table 2. Differences detected between caecal and faecal samples.

			CON			XOS			BIF		
Phylum	**Family**	**Sample**	**mean**	**±**	**SEM**	**mean**	**±**	**SEM**	**mean**	**±**	**SEM**
Firmicutes	*Lachnospiraceae*	Faecal	3.6E-01	±	3.6E-02	2.9E-01	±	3.7E-02	3.2E-01	±	4.5E-02
		Caecal	4.3E-01	±	4.4E-02	4.5E-01	±	3.0E-02	4.4E-01	±	2.8E-02
	Peptostreptococcaceae	Faecal	7.7E-03	±	2.2E-03	8.2E-03	±	3.0E-03	7.0E-03	±	2.0E-03
		Caecal	2.4E-02	±	8.7E-03	2.4E-02	±	4.9E-03	2.6E-02	±	5.0E-03
	Erysipelotrichaceae	Faecal	1.2E-02	±	3.6E-03	1.1E-02	±	2.9E-03	1.1E-02	±	2.5E-03
		Caecal	3.9E-03	±	8.9E-04	6.1E-03	±	2.7E-03	3.8E-03	±	6.3E-04
	Lactobacillaceae	Faecal	3.4E-02	±	8.0E-03	8.5E-02	±	2.7E-02	9.7E-02	±	3.4E-02
		Caecal	5.9E-03	±	3.4E-03	6.0E-03	±	8.0E-04	1.5E-02	±	6.6E-03
	Streptococcaceae	Faecal	3.6E-04	±	6.9E-05	3.8E-04	±	1.1E-04	2.2E-04	±	5.2E-05
		Caecal	1.3E-04	±	3.6E-05	2.1E-04	±	8.3E-05	1.9E-04	±	7.1E-05
	Staphylococcaceae	Faecal	1.2E-04	±	3.3E-05	8.0E-05	±	1.2E-05	1.2E-04	±	1.4E-05
		Caecal	3.6E-05	±	9.0E-06	4.0E-05	±	1.1E-05	6.2E-05	±	2.2E-05
	Veillonellaceae	Faecal	N.D.			N.D.			N.D.		
		Caecal	2.6E-04	±	1.6E-04	9.5E-04	±	7.5E-04	4.8E-04	±	3.2E-04
Bacteroidetes	*Rikenellaceae*	Faecal	3.0E-02	±	5.6E-03	2.4E-02	±	5.7E-03	1.9E-02	±	4.6E-03
		Caecal	8.6E-02	±	2.1E-02	5.3E-02	±	1.2E-02	4.0E-02	±	9.4E-03
Actinobacteria	*Micrococcaceae*	Faecal	2.6E-04	±	5.4E-05	1.4E-04	±	3.1E-05	1.7E-04	±	2.3E-05
		Caecal	3.1E-05	±	1.1E-05	2.8E-05	±	1.0E-05	6.5E-05	±	1.7E-05
	Corynebacteriaceae	Faecal	6.6E-05	±	1.5E-05	5.9E-05	±	2.0E-05	5.4E-05	±	1.1E-05
		Caecal	1.9E-05	±	6.3E-06	1.0E-05	±	4.6E-06	5.8E-05	±	3.9E-05
	Coriobacteriaceae	Faecal	1.5E-03	±	3.2E-04	1.7E-03	±	1.9E-04	1.9E-03	±	4.6E-04
		Caecal	2.4E-04	±	6.1E-05	2.5E-04	±	4.8E-05	5.5E-04	±	1.4E-04
Proteobacteria	*Desulfovibrionaceae*	Faecal	1.3E-03	±	4.9E-04	9.9E-04	±	5.6E-04	1.1E-03	±	5.6E-04
		Caecal	5.9E-03	±	1.1E-03	5.7E-03	±	3.1E-03	6.5E-03	±	2.2E-03
	**Hyphomicrobiaceae*	Faecal	N.D.			N.D.			1.9E-06	±	1.9E-06
		Caecal	3.2E-05	±	9.8E-06	4.2E-05	±	1.9E-05	3.9E-05	±	2.1E-05
Deferribacteres	*Deferribacteraceae*	Faecal	1.0E-04	±	2.4E-05	1.3E-04	±	6.0E-05	1.1E-04	±	2.0E-05
		Caecal	4.1E-04	±	9.2E-05	4.1E-04	±	1.3E-04	3.5E-04	±	8.3E-05

Mean ± SEM are shown and coloured for those families with significant differences after correction for False Discovery Rate ($q < 0.05$). Green: Mean higher in faecal sample and Red: Mean higher in caecal sample.

*Note that the family *Hyphomicrobiaceae* contains the genera *Gemmiger*, which shows high 16S rRNA gene sequence homology to members of the *Ruminococcaceae* family (*Firmicutes*), and may thus be taxonomically misplaced.

Figure Legends

Figure 1. Animal weight gain during the study. Mean with SD is illustrated for each treatment group; CON (circles), XOS (triangles), and BIF (squares). The arrow indicates initiation of the dosing period.

Figure 2: Bacterial community composition of individual animals before intervention based on 16S rRNA gene sequencing. A: Bacterial community composition at phylum level for one animal from each of the 24 separate cages. Columns are ordered with increasing relative abundance of *Firmicutes*. B: The relative abundance for selected phyla and families are shown as dot-plots with geometric average indicated by a horizontal line.

Figure 3. Bacterial community composition in faecal and caecum content samples based on 16S rRNA gene sequencing. The mean bacterial composition is shown at the family level for faecal samples obtained before intervention (A-C), after intervention (D-F) and caecal content samples (G-I) for animal in CON, XOS and BIF groups. Differences in mean relative abundances were only observed between faecal and caecal samples as detailed in table 2.

Figure 4. Fold changes and relative abundances determined by 16S rRNA gene sequencing and qPCR. Columns show means with SEM (A-B) or box and whisker plots with full range (C-F) for *Bifidobacteriaceae* (dark grey) and *Lactobacillacae* (light gray) determined by high through-put sequencing (A, C & E) and for *Bifidobacterium* spp. (dark grey), *Lactobacillus* spp. (light gray) and *Akkermansia muciniphilla* (white), determined by qPCR (B,D and F). Akkermansia muciniphilla was not included in 16S sequencing due to low abundance. Analysis were performed on community DNA extracted from faecal samples (A-D) or cecum content (E-F). In panels A and B, significant differences from baseline are indicated with asterisks ($p<0.05$). Observed differences between groups are indicated with P-values.

Figure 5. Gut integrity as determined by FITC-dextran permeability and Trans-Epithelial Resistance (TER). FITC-dextran concentrations in plasma (A) and relative TER across Caco-2 cells exposed to caecal water during the 24-hour exposure period (B) and at 24 hours after exposure (C). Dot plots with means indicated by horizontal lines (A and C) and mean values with SEM for groups CON (blue circles), XOS (green squares), BIF (red triangles) as well as water (grey circle) and cell media control (black squares) are shown (B).

Figure 6. Relative expression of *MUC2, CLDN-1, OCLN*, and ZO-1. Mean relative gene expression compared to the geometric mean of the reference genes GAPDH and ACTB in tissue samples obtained from ileum, ceceum and colon from animals in CON (dark gray), XOS (light gray) and BIF (white) groups. Observed differences between groups are indicated with p-values. Error bars indicate SEM.

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Figure 1

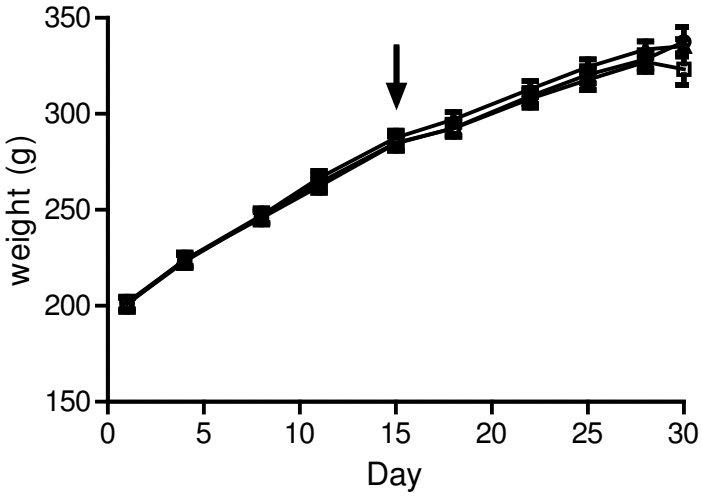
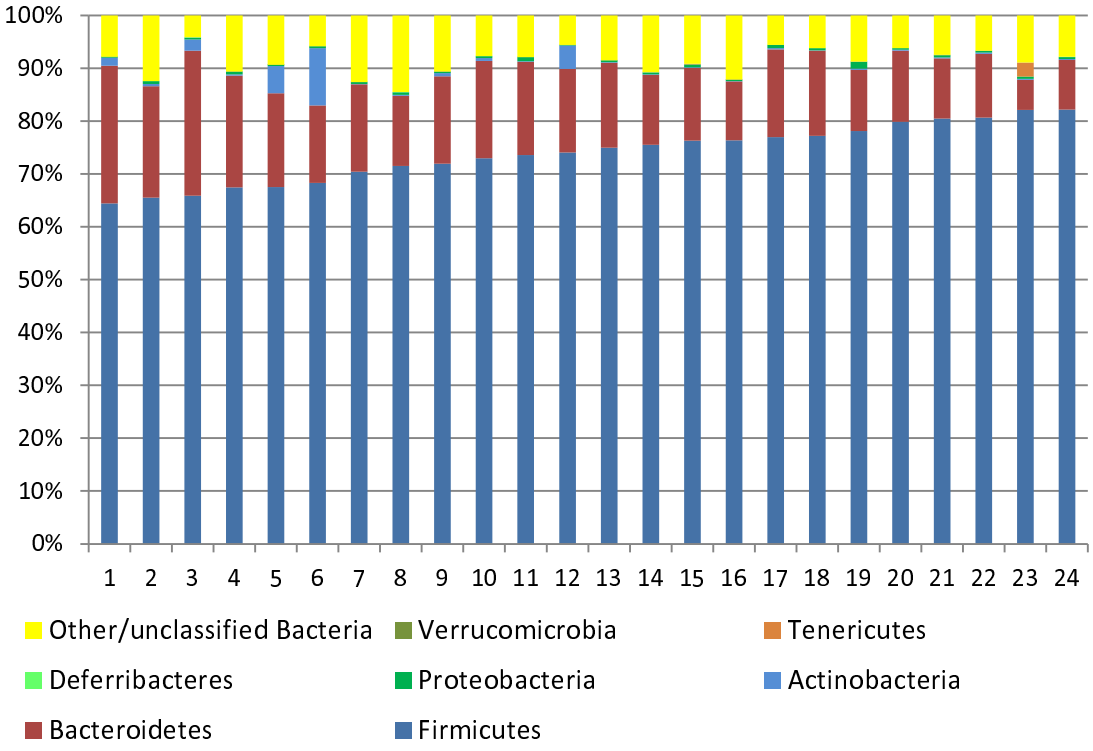


Figure 2

A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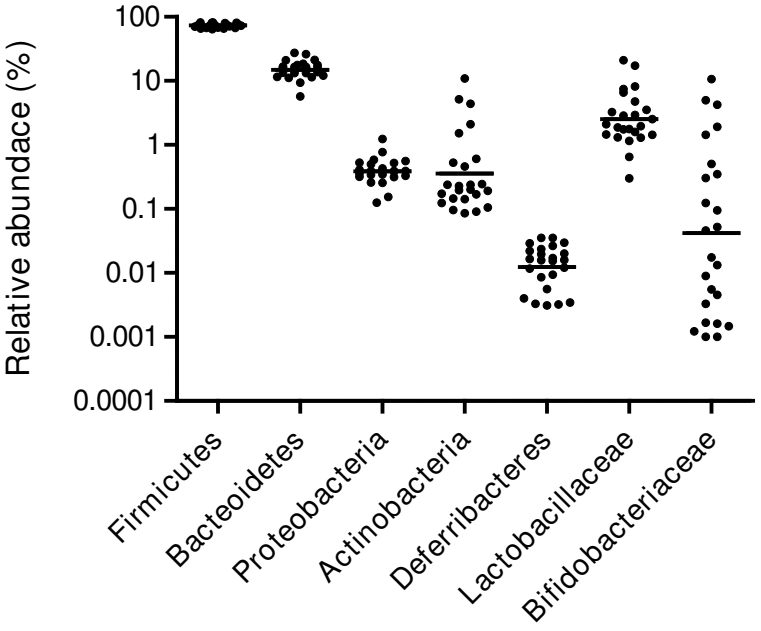
Proteobacteria
Actinobacteria
Bacteroidetes
Firmicutes

B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10
1
0.1
0.01
0.001
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Proteobacteria
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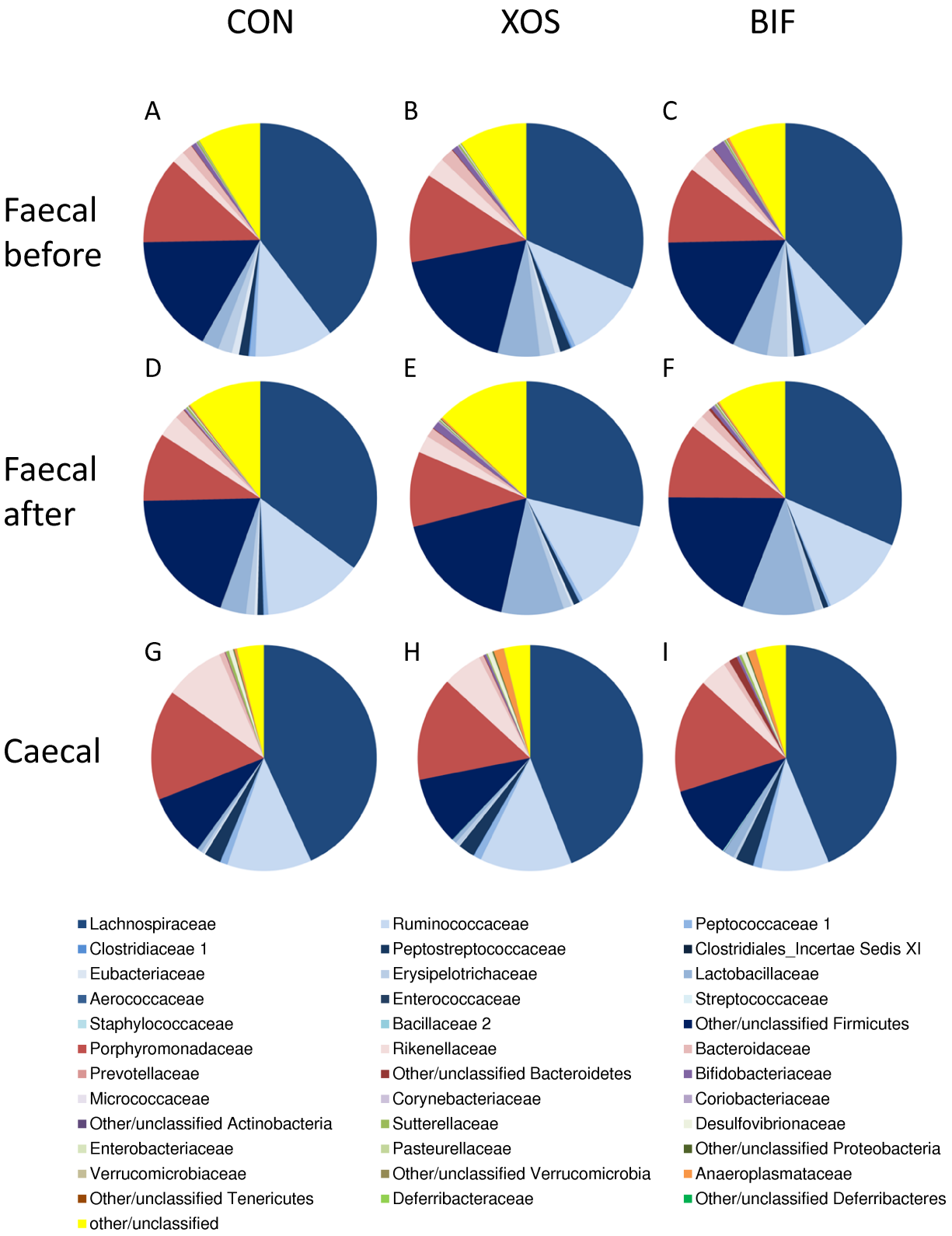
Deferribacteres
Lactobacillaceae
Bifidobacteriaceae

Figure 3


CON
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BIF
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B
C
Faecal before
D
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Faecal after
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Caecal
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Ruminococcaceae
Peptococcaceae 1
Clostridiaceae 1
Peptostreptococcaceae
Clostridiales_Incertae Sedis XI
Eubacteriaceae
Erysipelotrichaceae
Lactobacillaceae
Aerococcaceae
Enterococcaceae
Streptococcaceae
Staphylococcaceae
Bacillaceae 2
Other/unclassified Firmicutes
Porphyromonadaceae
Rikenellaceae
Bacteroidaceae
Prevotellaceae
Other/unclassified Bacteroidetes
Bifidobacteriaceae
Micrococcaceae
Corynebacteriaceae
Coriobacteriaceae
Other/unclassified Actinobacteria
Sutterellaceae
Desulfovibrionaceae
Enterobacteriaceae
Pasteurellaceae
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Verrucomicrobiaceae
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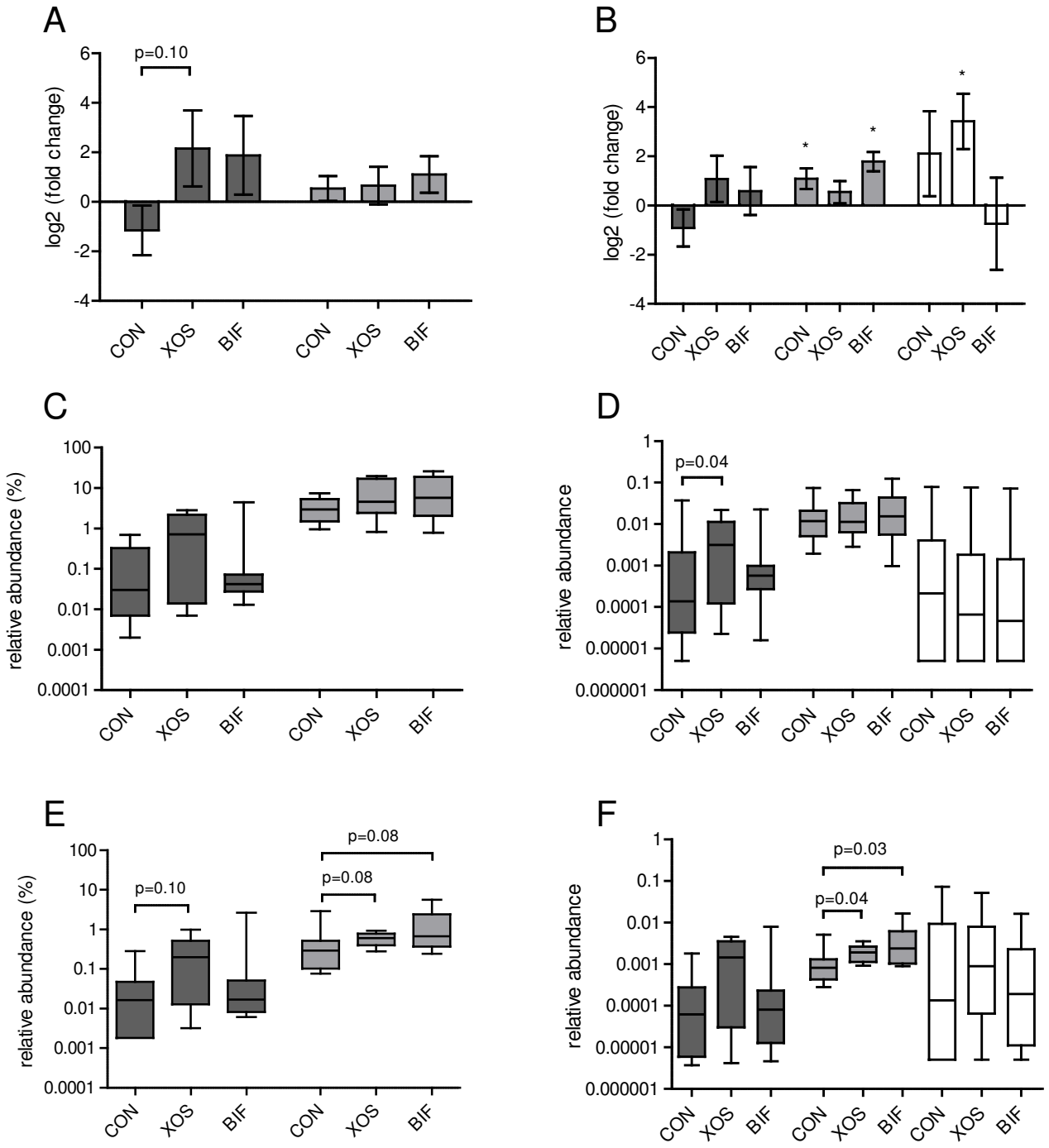
other/unclassified

Figure 4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0
-2
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CON
XOS
BIF
CON
XOS
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B
log2 (fold change)
*
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CON
XOS
BIF
CON
XOS
BIF
CON
XOS
BIF
C
relative abundance (%)
100
10
1
0.1
0.01
0.001
0.0001
CON
XOS
BIF
CON
XOS
BIF
D
p=0.04
relative abundance
1
0.1
0.01
0.001
0.0001
0.00001
0.000001
CON
XOS
BIF
CON
XOS
BIF
CON
XOS
BIF
E
p=0.08
p=0.08
p=0.10
relative abundance (%)
100
10
1
0.1
0.01
0.001
0.0001
CON
XOS
BIF
CON
XOS
BIF
F
p=0.03
p=0.04
relative abundance
1
0.1
0.01
0.001
0.0001
0.00001
0.000001
CON
XOS
BIF
CON
XOS
BIF
CON
XOS
BIF

Figure 5

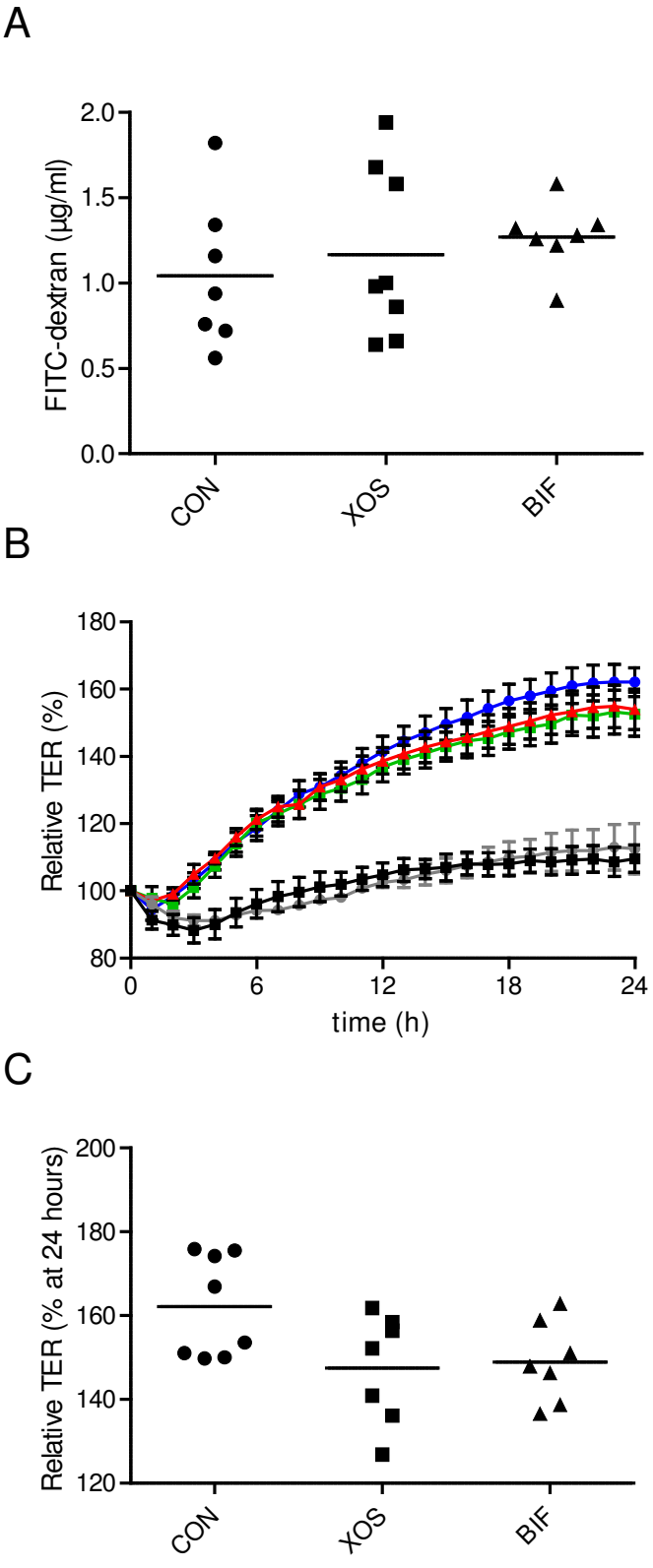
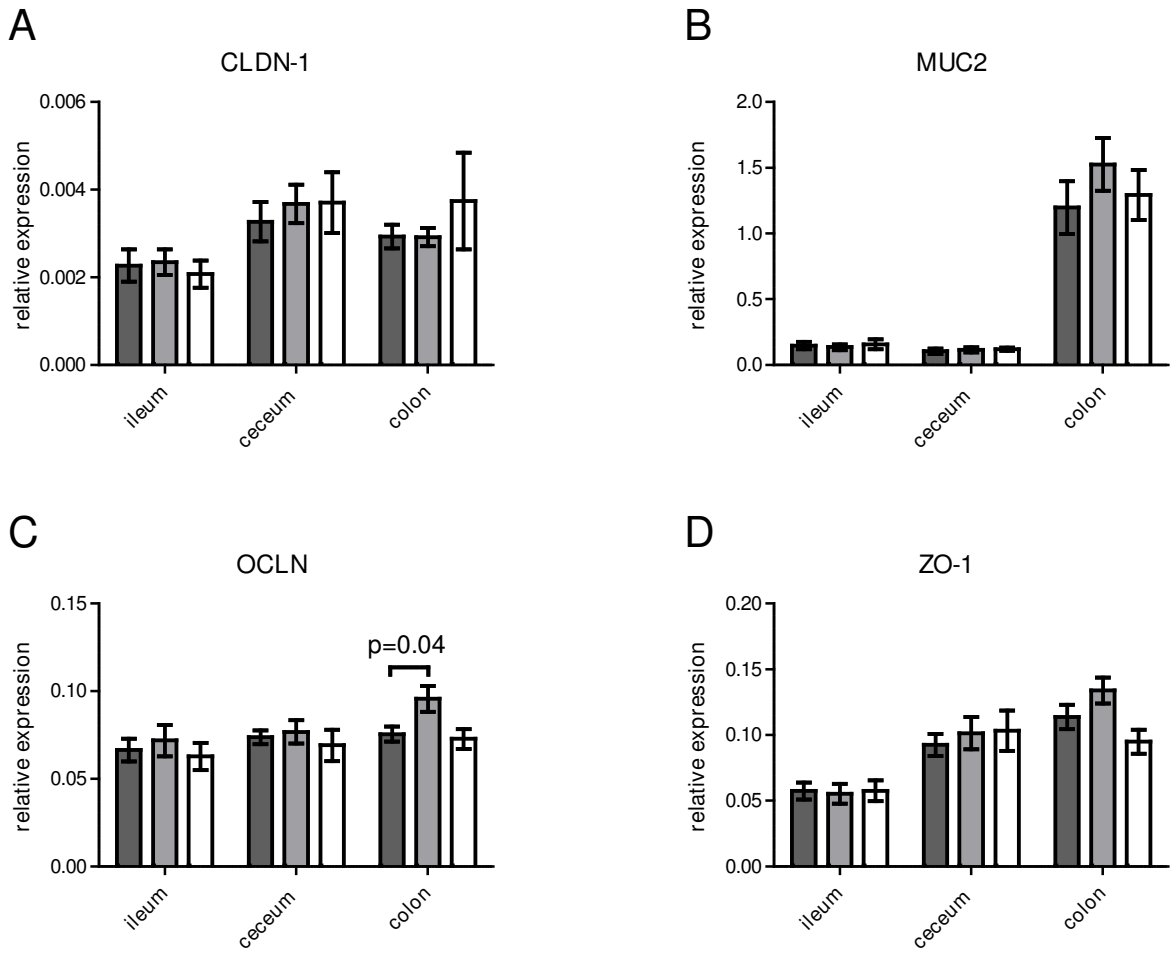

A
2.0
1.5
1.0
0.5
0.0
FITC-dextran (µg/ml)
CON
XOS
BIF
B
180
160
140
120
100
80
Relative TER (%)
0
6
12
18
24
time (h)
C
200
180
160
140
120
Relative TER (% at 24 hours)
CON
XOS
BIF

Figure 6


A
CLDN-1
relative expression
0.006
0.004
0.002
0.000
ileum
ceceum
colon
B
MUC2
relative expression
2.0
1.5
1.0
0.5
0.0
ileum
ceceum
colon
C
OCLN
relative expression
0.15
0.10
0.05
0.00
p=0.04
ileum
ceceum
colon
D
ZO-1
relative expression
0.20
0.15
0.10
0.05
0.00
ileum
ceceum
colon

Manuscript 4

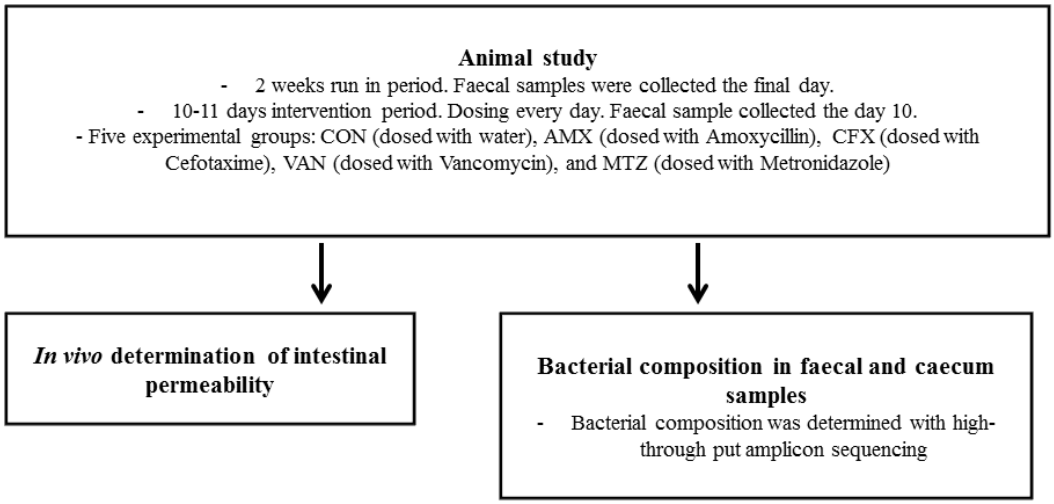
Antibiotic treatment affects intestinal permeability and gut microbial composition in female Wistar rats dependent on antibiotic class

Manuscript in preparation
(Page numbers are relative to the manuscript)

Introduction

The aim of the study was to determine the effects of antibiotics on the gut microbial composition and intestinal permeability.

Flowsheet



Antibiotic treatment affects intestinal permeability and gut microbial composition in female Wistar rats dependent on antibiotic class.

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Key words: Antibiotics, gut microbiota, intestinal integrity

Running title: Antibiotics affect intestinal integrity and gut microbiota

Abstract

Introduction: Antibiotics are frequently adminitered orally to treat both systemic and localized bacterial infections in almost any body location. As a consequence of this the commensal gut microbiota is very often affected as well. This may disrupt the normal balance and subsequently affect intestinal integrity and host health.

Methods: Female Wistar rats (n=60) were dosed with amoxicillin (AMX), cefotaxime (CTX), vancomycin (VAN), metronidazole (MTZ), or water (CON) every day for 10-11 days (n=12 in each group). Changes in bacterial composition in faecal and caecal content were determined by partial sequencing of the 16S rRNA gene. Intestinal permeability was determined *in vivo* by measuring permeability of 4kDa FITC-dextran.

Results: Intestinal permeability was increased by administration of MTZ, while CTX and VAN decreased intestinal permeability. Bacterial composition was significantly influenced by AMX, CTX and VAN but not by MTZ. In all groups with significant changes compared to CON, Firmicutes was reduced while Bacteroidetes and Proteobacteria were increased. For CTX abundance of *Bifidobacteriaceae* in the caecum content increased significantly while in the VAN group *Lactobacillaceae* increased in both caecal and faecal samples. Administration of AMX, CTX and MTZ resulted in increased water intake, while only AMX affected feed intake. Caecum weight was increased by AMX and VAN and the latter also increased caecum pH.

Conclusion: Specific antibiotics were shown to affect intestinal permeability in either a positive or negative direction dependent on the class of antibiotic. Changes in gut microbial composition, which were also observed, could be linked to intestinal permeability, although changes in permeability did not always result from major changes in microbiota and v*ice versa*.

Introduction

The gut microbiota is considered to have great impact on host health through either direct interaction with host cells or through production of metabolites such as short chain fatty acids (1). Modulation of the gut microbiota can therefore potentially affect host health, which may occur through alterations of intestinal integrity (2). At present bacterial infections in human are very often treated with orally administered antibiotics irrespective of the actual location of the infection. These

antibiotics will inevitably affect the complex and finely tuned microbial ecosystem residing in the gut (3). At present numerous studies have examined effects of different antibiotics on the gut microbiota (4–7), but very few have focused on effects on intestinal integrity and the cause of any such effects (8). Intestinal permeability is an frequently employed maker for intestinal health, as increased intestinal permeability may lead to inflammation caused by bacterial components, such as lipopolysaccharide (LPS), crossing the epithelial barrier (8). Intestinal permeability is mainly controlled by the interaction between tight junction proteins in epithelial cells. Small molecules, such as ions, are considered to pass through a high conducting 'pore' pathway in the tight junctions, while larger molecules, including LPS, may pass through the 'leak' pathway as previously defined (2, 9). Intestinal permeability, by the 'leak' pathway, can be assessed by determining the permeability of FITC-dextran with a defined molecular size (2, 8, 9).

The effect of antibiotics on intestinal integrity has previously been studied in connection with high fat diet (8). Here high-fat diet increased intestinal permeability, but subsequent antibiotic treatment was shown to reduce the intestinal permeability again. Others have shown that antibiotic treatment in childhood is associated with Crohn's disease (10), which is one of the gastrointestinal disorders that is connected to increased intestinal permeability (11). Hence antibiotics may affect intestinal permeability and host health. The effect of antibiotics on the bacterial composition and the intestinal permeability must however be dependent on the specific targets of the antibiotics, therefore different classes of antibiotics must have different effects on intestinal health.

In the present study we examined how four antibiotics, namely; amoxicillin (AMX), cefotaxime (CTX), vancomycin (VAN), and metronidazole (MTZ) affected the gut microbial composition and intestinal integrity in female Wistar rats. These antibiotics represent different classes and were chosen due to their common oral use in humans and varying bacterial targets (Table 1). Changes in bacterial composition was determined using high-through put sequencing of the V3-region of the 16S rRNA encoding gene, while changes in intestinal permeability were determined *in vivo* by FITC-dextran permeability assay.

Materials and methods

Animals and housing

8-week old specific pathogen free female Wistar Hannover rats (n =60) were purchased from Taconic (Lille Skensved, Denmark). Animals were housed in pairs under controlled environmental

conditions (12-hours light/dark cycles, temperature 21.5 ± 0.3^{0}C, relative humidity 51.3 ± 3.1%, 8-10 air changes per hour). Animals had access to *ad libitum* water and feed (Altromin 1324) throughout the experiment. Animal weight as well as feed and water intake was monitored weekly during the invention period. Animals were monitored twice a day. The animal experiment was carried out under the supervision of the Danish National Agency for Protection of Experimental Animals.

Experimental design

Upon arrival animals were housed in pairs. The following day animals were weighed and the cages were randomly allocated into five groups (with six cages in each group) based on weight. Animals were housed together for 2 weeks, before the experimental period was initiated to limit effects of co-housing and coprophagia. During the experimental period animals received a daily dosage of 0.5 mL of antibiotic solution (Table 1) AMX; 60 mg/mL amoxicillin (Sigma-Aldrich, A8523), CTX; 8 mg/mL cefotaxime (Sigma-Aldrich, C7912), VAN; 8 mg/mL vancomycin (Sigma-Aldrich, 861987), MTZ; 8 mg/mL Metronidazole (Sigma-Aldrich, M1547) or water (CON) by oral gavage for 10 or 11 days. Faecal pellets were collected directly from the individual rats before the initial dosing (day 0) and the day before euthanization of the first animals (day 10) and immediately frozen at -20^{o}C.

***In vivo* intestinal permeability assay**

Intestinal permeability was determined on the day of euthanization by measuring the permeability of FITC-dextran by an approach similar to previous studies (8, 12). Briefly, animals were fasted for at least 9 hours before the assay. For each cage one animal was dosed with 0.5 mL 120 mg/mL FITC-dextran (4kDa, Sigma-Aldrich, FD-4) per 100 g body weight (corresponding to 600 mg/kg animal), while the other animal was dosed with a corresponding dose of phosphate buffered saline (PBS). Exactly two hours after dosing animals were euthanized (CO_2 and decapitation), and blood was collected from the neck into 50 ml Falcon tubes containing 100µl EDTA (0.5M, pH 8, Ambion). Blood was centrifuged (1500 G, 10 min, 4^{o}C), and plasma collected. Plasma was centrifuged again (5 min) before mixing 1:1 with PBS. Plasma-PBS solutions were stored dark at 5^{0}C, until analysis the same day. Fluorescence was measured in three replicate wells for each sample (75 µl) in black 96-well microtiter plates (Proxiplate-96 F, Perkin Elmer) using a Victor TM X4 Plate reader (Perkin Elmer) with excitation at 485 nm and emission at 535 nm. FITC-dextran concentrations in plasma were calculated using a standard curve.

Dissection of animals

Only animals that had not received FITC-dextran during the permeability assay were dissected (n =30). The caecum was removed, weighed, and caecal content was collected. Finally, pH was measured directly in the caecum content (Thermo Scientific, Orion 3 Star).

Extraction of bacterial community DNA

Gut microbiota composition was determined for animals, that had not been dosed with FITC-dextran (n =30). DNA was extracted from faecal samples collected on the initial day of dosing (day 0), and the day before the euthanization of the first animals (day 10), as well as from caecal content using the MoBio PowerLyzer® Power Soil® DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA) according to the manufacturer's recommendations with minor modifications. A maximum of 200 mg samples was used for extraction and samples were heated to 65°C for 10 min after addition of the C1 solution. Bead beating was conducted at 30 cycles/s for 4 min (Retsch MM 300 mixer mill). DNA concentrations were measured with the Qubit dsDNA HS kit (Life Technologies).

Amplicon sequencing of the 16S rRNA encoding gene

The bacterial composition was determined by sequencing of the V3-region of the 16S rRNA gene in bacterial community DNA. Amplification of the V3-region and subsequent sequencing was performed using the Ion Torrent PGM platform (Life Technologies) essentially as previously published (13). Briefly, the V3-region of the 16S rRNA gene was amplified using a universal forward primer (PBU 5'-A-adapter-TCAG-barcode-CCTACGGGAGGCAGCAG-3') with a unique 10-12 bp barcode for each bacterial community (IonXpress barcode as suggested by the supplier, Life Technologies) and a universal reverse primer (PBR 5'-trP1-adapter-ATTACCGCGGCTGCTGG-3'). PCR reactions were conducted with 4µl HF-buffer, 0.4µl dNTP (10mM of each base), 1µM forward primer, 1µM reverse primer, 5ng template DNA, and 0.2µl Phusion High-Fidelity DNA polymerase (Thermo Scientific) in a reaction volume of 20µl. Reactions were run at 98^0C for 30 seconds followed by 24 cycles of 98^0C for 15 seconds and 72^0C for 30 seconds, before 72^0C for 5 minutes and cooling at 4^0C. Products were purified by gel electrophoresis and DNA concentrations were determined with Qubit HS assay, and a library was constructed by mixing an equal amount of PCR products from each original community. Sequencing was performed on a 318-chip for Ion Torrent sequencing using the Ion OneTouch™ 200 Template Kit v2 DL. Sequence data were obtained in FASTQ format and further processed using CLC bio genomic workbench (Qiagen) in order to de-multiplex and remove sequencing

primers. Further quality trimming using default settings (quality score = 0.05, trim ambiguous nucleotides = 2) and selection of reads with a final length between 110bp – 180bp was performed before exporting reads in FASTA format. The number of good quality reads used for taxonomical assignment ranged from 17,205 to 96,897 (except for one faecal sample, from before the intervention, that was represented by only 2,555 reads). All sequence reads were taxonomically classified using the Ribosomal Database Project Multiclassifier tool (14). A bootstrap cut-off ≥ 50%, was chosen as recommended for fragments below 250bp and previously shown to be effective (15). Relative abundance of bacterial taxa (family level) were determined for each community by comparing the number of reads assigned to a specific family to total number of reads assigned to the bacterial root. For log transformation of data a relative abundance of 0.0005% analogous to 1 read in 200.000 reads was applied as a minimum.

Principal component analysis and statistics

Principal component analysis was performed on $\log_{10}$-tranforemd relative abundance data using Latentix version 2.11. All Statistical analysis was conducted in GraphPad Prism 5 unless stated otherwise. Animal weight was compared using two-way ANOVA with Bonferroni post-test comparing each of the treatment groups with CON. Average feed and water intake per day during the intervention was calculated for each cage. Differences in FITC-dextran plasma concentrations, caecum weight, and pH between treatment groups and CON were determined using non-parametric Mann-Withney tests. Statistical analysis of the 16S rRNA gene sequencing data were done with the online version of the Metastats tool for detection of differentially abundant features (http://metastats.cbcb.umd.edu/detection.html) based on 1000 permutations and a q-value significance level of 0.05 (16).

Results

Animal weight, feed and water intake

Animal weight did not differ between the CON group and any of the four treatment groups at any of the three time-points during the experiment (Figure 1A). Neither did weight gain during the intervention period (Fig 1B). Animals in the AMX group had a significantly lower average feed intake per day during the intervention period compared to CON (P = 0.017), while MTZ tended to have a higher feed intake (P = 0.054) (Figure 1C). Animals in the AMX, CTX, and MTZ groups had a higher average water intake per day, than animals in the CON group (P = 0.002, P = 0.0043, P = 0.0037, respectively) (Figure 1D).

Caecum weight and pH

Administration of AMX and VAN resulted in increased caecum weight compared to CON (P = 0.041 and P = 0.0022) (Figure 2A and 3). Additionally pH was higher in the caecum of the VAN group compared to the CON group (P = 0.0022).

Intestinal permeability

The intestinal permeability was determined *in vivo* by determining FITC-dextran permeability (Figure 4). Antibiotics CTX and VAN both resulted in a lower FITC-dextran plasma concentrations compared to CON (P = 0.041, and P = 0.045, respectively), hence decreased intestinal permeability. Administration of MTZ increased permeability compared to CON (P = 0.015).

Gut microbial composition

The bacterial composition in the AMX, CTX, and VAN groups differed significantly from the CON in both caecum content and faeces samples, while MTZ did not affect the bacterial composition significantly (Figure 5 and Table 2). Principal component analysis (PCA) (Figure 6) showed that animals dosed with AMX, CTX, and VAN each formed a separate cluster with faecal and caecal samples clustering together (Figure 6a). Only a slight difference was observed for MTZ compared to CON. In the CON group faecal and caecal samples clearly clustered separately, with fecal samples having a lower PC#1 score. The PCA loading plot (Figure 6b) indicated that Proteobacteria and specifically *Enterobacteriaceae* may be causing the shift towards higher PC#1 score for antibiotics AMX, VAN and CTX.

Significant differences in bacterial families, in faeces and caecum between treatment groups and CON, are calculated as $\log_2$ (fold-changes) in Table 2. AMX overall decreased the relative abundance of Firmicutes families while Bacteroidetes families were increased (Figure 5), specifically *Bacteroidaceae*. Families belonging to Proteobacteria were also increased; here *Enterobacteriaceae* was increased in both caecum and faeces while *Desulfovibrionaceae* was only increased in caecum (Table 2). For CTX Bacteroidetes families were increased in both caecum and faeces, especially *Bacteroidaceae*, while *Rikenellaceae* was only increased in caecum. In the caecum content the relative abundance of *Enterococcaceae* and *Bifidobacteriaceae* increased significantly while especially the *Ruminococcaceae* were decrease by CTX. In the VAN group *Lactobacillaceae* increased in both caecal and fecal samples and *Peptococcaceae* in caecum, while the Firmicutes overall decreased. Proteobacteria families were significantly increased by VAN. Here *Desulfovibrionaceae*, *Sutterellaceae* and *Enterococcaceae* in both faeces and caecum

increased. Additionally *Verrucomicobria* were increased significantly. Overall faeces and caecum samples within the same treatment group show similar changes in bacterial composition compared to CON (Figure 6). In all the groups with significant changes the Firmicutes were reduced while Bacteroidetes and Proteobacteria were increased (Figure 5).

Discussion

Our study showed that both VAN and CTX caused a decrease in intestinal permeability of 4 kDa FITC-dextran, thus indicating a strengthening of integrity. This was correlated to changes in bacterial community composition in both faecal and caecum samples, which were very similar within an antibiotic group, but different between groups as shown by principal component analysis (Fig. 6). As expected VAN, known to target Gram positive bacteria, reduced the relative abundance of several bacterial families within the Firmicutes and Actinobacteria phyla and increased the relative abundance of several Gram negative bacterial families within the Proteobacteria as well as *Verrucomicrobiaceae* consistent with previous studies (17). A reduction of the Gram negative *Porphyromonadaceae* and notably also an increase in relative abundance of *Lactobacillaceae* was also observed (Table 2). The latter of these is consistent with the observed decrease in intestinal permeability as *Lactobacillu*s spp. have previously been shown to increase intestinal integrity in *in vitro* models (18–20). For animals dosed with CTX we observed fewer significant changes in relative abundance of bacterial families than for VAN, however an increase in *Bifidobacterium* spp. was seen, which again is consistent with the observed positive effect on permeability, as *Bifidobacterium* spp. have also been shown to increase integrity in both *in vitro* and *in vivo* models (21–25). The relative abundance of Proteobacteria was also generally increased by VAN, which could lead to increased levels of lipopolysaccharide (LPS) crossing the intestinal barrier thus causing inflammation (8). Since increased intestinal permeability is considered to initiate this cascade, the increased abundance of Proteobacteria found here, may not affect the intestinal permeability. Finally, bacterial families within the Firmicutes were reduced by VAN including *Rumincoccaceae* and *Lachnospiraceae*, which belong to the butyrate producing *Clostridium* clusters IV and XIVa (26). This reduction could explain the increase in pH in the caecum. Previously butyrate has been shown to decrease intestinal permeability in *in vitro* and animal disease models (27, 28). The reduction of butyrate producing bacteria did, however not seem to increase the intestinal permeability in VAN in the present study.

The antibiotic MTZ caused an increase in the intestinal permeability, but very little change in the microbiota composition was seen in both cecum and colon compared to CON. The observed lack of effect on the microbiota is supported by the literature, and is due to low concentrations of active metronidazole reaching the cecum and colon because the agent is generally well absorbed (29). This indicates that MTZ may cause alterations to the intestinal permeability independent of the gut microbiota. However, in the present study the bacterial community was only examined in the caecum and in faecal samples, hence alterations in bacterial composition in the small intestine were not determined. Thus, MTZ could potentially affect the bacterial composition and the intestinal integrity in the small intestine. Others have also shown that MTZ can affect intestinal integrity in mice, showing that it caused a decrease in the mucus thickness, down regulation of *Muc2, TFF3*, and *Relmβ* gene expression and interestingly also changes in the colonic microbiota (30). This could lead to an impairment of the intestinal epithelial barrier, based on a reduced mucus layer, leading to the increased intestinal permeability found in the present study.

Of the four antibiotics investigated in the present study, only AMX was found not to affect the intestinal permeability. This is interesting because AMX resulted in major changes in the bacterial composition, including reduction of butyrate producing bacteria (*Ruminococcaceae* and *Lachnospiraceae*) as well as increase of *Enterobacteriaceae* (Table 2 and Fig 6) and also enlargement of the caecum was observed similar to VAN, which may be caused by depletion of bacteria as seen in germ-free mice (31). Finally, opposite VAN, AMX caused a reduction of *Lactobacillacae* in faeces, which as previously mentioned could affect intestinal integrity.

In the present work MTZ, that did not affect the gut microbiota, had the highest impact on the intestinal permeability, while AMX, that modulated the gut microbiota significantly, had no effect on the intestinal permeability. This may contradict the hypothesis, that changes in the gut microbiota modulate the intestinal integrity. It should however be noted, that in this study intestinal integrity was only evaluated by the permeability of 4 kDa FITC-dextran. This is a relatively large molecule that is considered to pass through the ‘leak’ pathway in the tight junction protein complex (9). Alterations in the ‘pore’ pathway, where smaller molecules and ions can pass are therefore not identified in the current work. Amoxicillin could therefore potentially alter the intestinal permeability for molecules smaller than FITC-dextran. Additionally, the remaining antibiotics could also affect the permeability for such molecules in addition to the observed effects on FITC-dextran.

In conclusion, the present study shows, that antibiotic treatment has a major effect on the gut microbiota composition, which is very similar for separately housed rats within the same treatment group but varies between antibiotics. The observed varying effects of different classes of antibiotics on intestinal integrity warrants further investigation and could be considered during selection of appropriate treatment for bacterial infections.

Acknowledgments

The present work was funded by a grant from the Danish Council for Independent Research, Technology and Production Sciences (DFF-1335-00092). We thank Anne Ørngreen and her department for handling of animals, Li Zhang and Louise K. Vigsnæs for help during the animal dissections and Bodil Madsen for excellent technical support.

Tables

Table 1. Characteristics of the applied antibiotics.

Antibiotic	**Abbr.**	**Class**	**Bacterial targets** (3)
Amoxicillin	AMX	Penicillin	Moderate spectrum, Gram-positives
Cefotaxime	CTX	Cephalosporin (3^{rd} gen.)	Broad-spectrum, Gram-positives and Gram-negatives
Vancomycin	VAN	Glycopeptide	Gram-positives
Metronidazole	MTZ	Nitroimidazole	Broad-spectrum anaerobes.

Table 2. Log_2 (Fold change) of bacterial families for treatment groups compared to CON in caecum and faeces.

Phylum	Family	AMX		CTX		VAN		MTZ	
		C	F	C	F	C	F	C	F
Bacteroidetes	*Bacteroidaceae*	6.5*	5.3*	5.1*	4.2*	4.4	2.8	0.5	-0.3
	Rikenellaceae	-1.5	-5.3*	2.6*	1.9	-0.3	-0.3	-0.6	-1.6
	Porphyromonadaceae	-6.5*	-11.8*	-1.2	-2.2*	-11.5*	-12.6*	0.2	0.0
Firmicutes	*Ruminococcaceae*	-10.5*	-12.1*	-2.1*	-3.6*	-9.9*	-10.8*	0.1	0.1
	Lachnospiraceae	-3.5*	-8.4*	-0.6	0.3	-6.6*	-1.1*	-0.1	0.0
	Eubacteriaceae	≤-8.4	≤-9.3	1.8	-1.4	≤-8.4	≤-9.3*	0.6	-0.1
	Peptostreptococcaceae	1.7	1.6	-0.3	2.6	-5.4*	-0.5	-1.1	-2.3
	Peptococcaceae 1	-10.5*	-9.4*	-1.4	-3.5*	-11.0*	≤-9.2*	0.6	1.0
	Cl. Incertae Sedis XI	≤-1.4	≤-2.7*	-1.3	-1.7	≤-1.4	≤-2.7*	1.7	-3.1
	Erysipelotrichaceae	-1.9	-4.9*	2.6	2.9	2.9	2.8	0.7	0.5
	Lactobacillaceae	0.8	-2.3*	0.5	1.5	4.4*	3.2*	0.0	1.7
	Enterococcaceae	8.5*	3.0	8.3*	4.8	2.1	-1.7	0.8	-1.2
	Streptococcaceae	≤-2.3	-4.7*	1.4	-1.0	0.4	-1.3	0.5	0.6
Proteobacteria	*Desulfovibrionaceae*	4.7*	1.2	3.6	1.7	5.5*	3.4*	0.9	-0.8
	Sutterellaceae	2.5	-0.3	1.2	0.3	5.8*	3.2*	1.5	0.4
	Enterobacteriaceae	9.9*	8.8*	7.9	4.5	10.6*	9.2*	2.5	2.0
Actinobacteria	*Coriobacteriaceae*	-2.6*	≤-8.6*	0.2	-2.5*	≤-5.6*	≤-8.6*	2.2	0.5
	Bifidobacteriaceae	-1.0	1.2	4.8*	9.6	-2.2	1.5	≤-2.8	0.5
	Micrococcaceae	-1.3	≤-5.5*	2.7	-1.3	1.9	-2.1	-0.9	-0.7
	Deferribacteraceae	≤-6.4	-5.2*	≤-6.4	≤-5.0*	4.3	2.1	-2.1	-0.9
Verrucomicobria	*Verrucomicrobiaceae*	≤-1.2	≤-2.2	2.7	1.7	9.1*	6.0*	4.3	1.9
Other	Other/Unclassified	-2.1*	-4.9*	-2.3*	-3.8*	0.9	-3.5*	-0.3	-0.3

Values show log_2(fold-change) in relative abundance of bacterial families in caecum (C) and faeces (F) of antibiotic treated groups (ABX) compared to the same bacterial groups in the CON group (log_2(ABX/CON)). Intensity of green/red shading indicates level of increase/decrease and asterisks indicate significant differences ($q<0.05$). When no reads were observed for specific families a value of 0.0005% was applied as a lower detection limit for calculations (fold-changes indicated with ≤ or ≥).

Figures

Figure 1

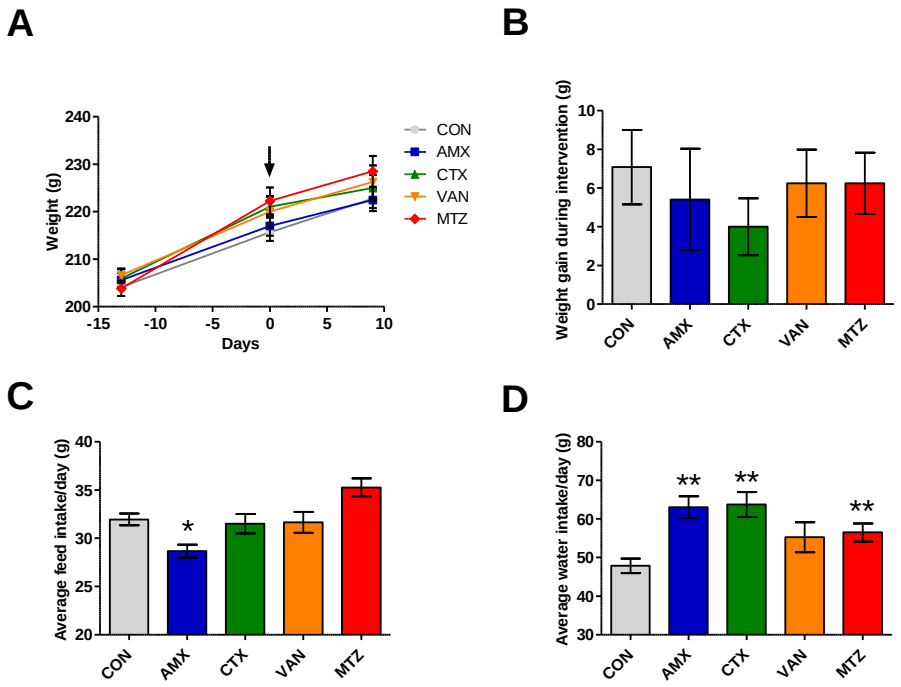

Figure 1. Animal weight, food and water intake. (A) Mean animal weight during the acclimation and intervention period. The arrow indicates the initial dosing. No significant differences were determined between the CON and either of the treatments at day -13, 0 and 9 according to a two-way ANOVA. (B) Mean weight gain during the intervention with antibiotics (day 0 to 9). (C) Mean feed intake per day during the intervention period and (D) mean water intake per day during the intervention. Bars show averages for each group and error bars show SEM. Significant differences from CON group are indicated by asterisks (*; $P < 0.05$, **; $P < 0.01$).

Figure 2

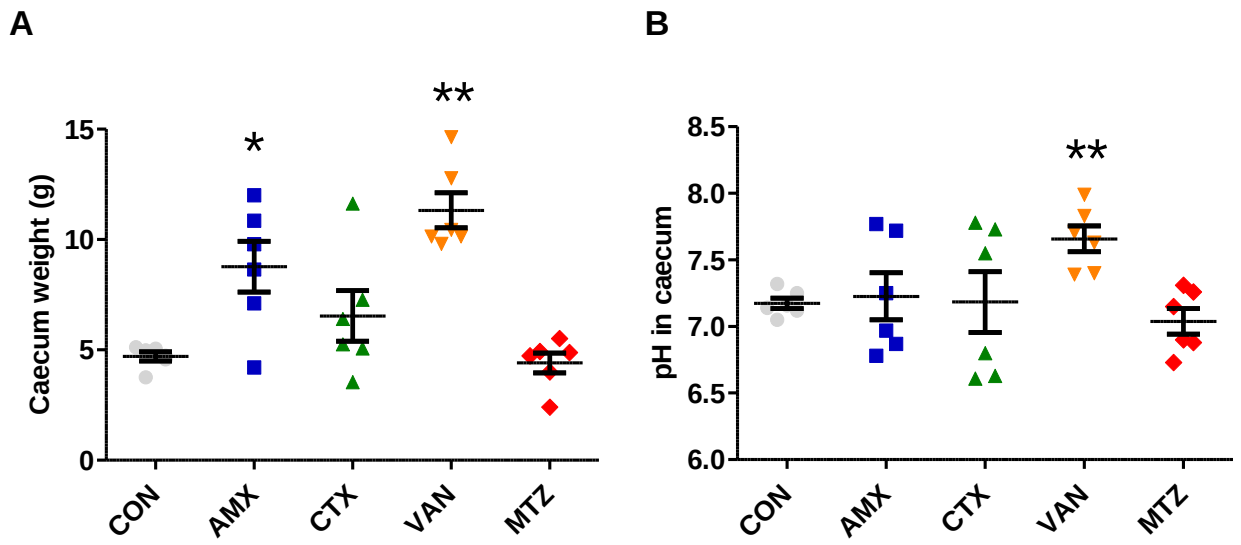

Figure 2: Characteristics of cecum. (A) Weight of caecum and (B) pH in caecum. Each point represents an individual animal. Horizontal lines and error bars show means and SEM, respectively. Significant differences from CON group are indicated by asterisks (*; $P < 0.05$, **; $P < 0.01$).

Figure 3

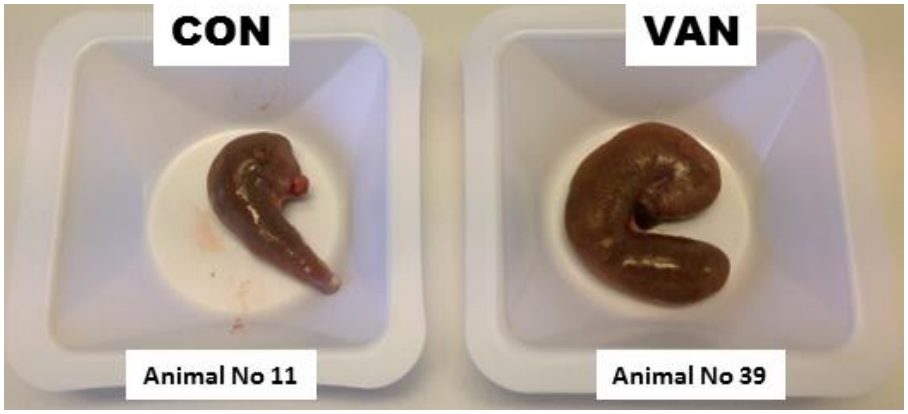

Figure 3. Size of caecum. Representative photographs of a caecum from an animal in the CON group (5.06 g) and from the VAN group (10.14 g).

Figure 4

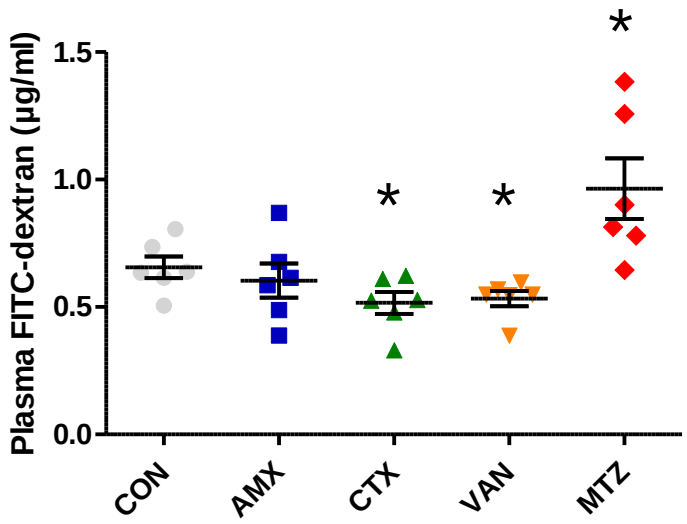

Figure 4: Plasma FITC-dextran concentrations. Each point represents an individual animal. Horizontal lines and error bars show means and SEM, respectively. Significant differences from CON group are indicated by asterisks (*; $P < 0.05$).

Figure 5

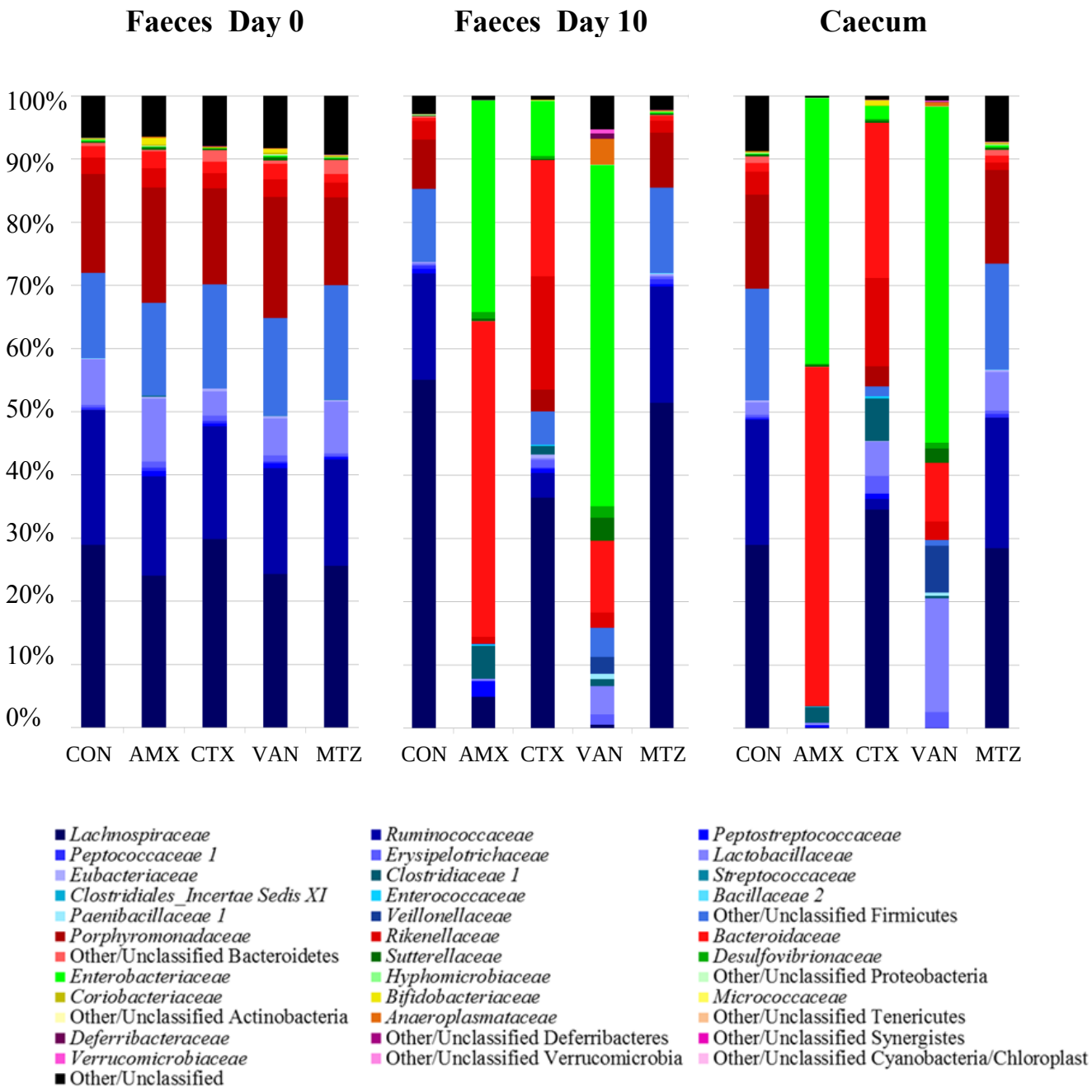

Figure 5. Bacterial community composition in faeces and caecum content samples based on 16S rRNA gene sequencing. The mean bacterial composition is shown at the family level for faecal samples (day 0 and 10) and caecal content samples for animal in CON, AMX, CTX, VAN and MTZ groups. The most abundant bacterial phyla are represented by Firmicutes (blue colors), Bacteroidetes (red colors), Proteobacteria (green colors), Actinobacteria (yellow colors), and unclassified bacteria (black).

Figure 6

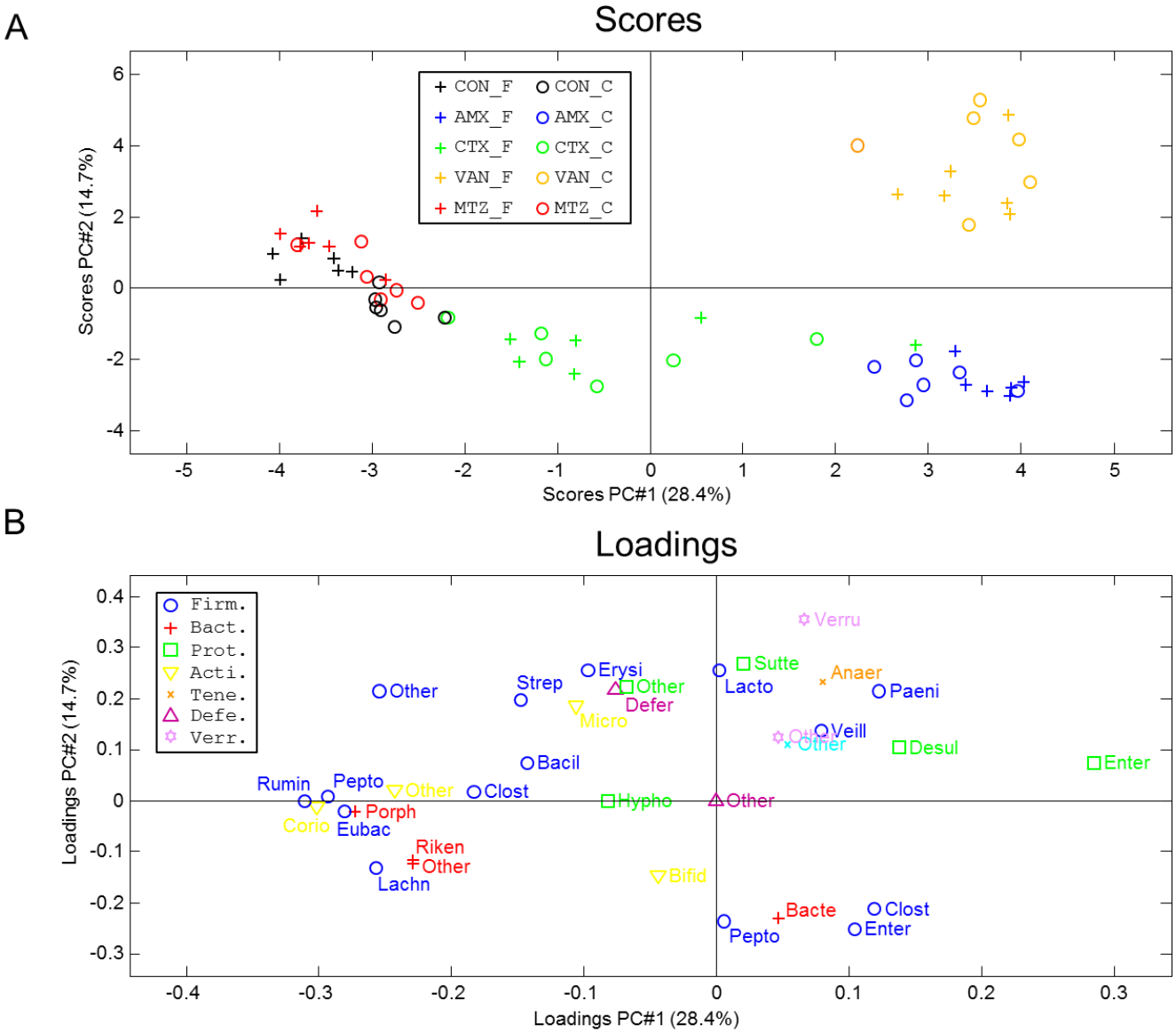

Figure 6: Principal component analysis (PCA) of the relative abundances of detected bacterial families in faecal and caecal samples. (A) The score plot shows samples grouped according to treatment groups CON (black), AMX (blue), CTX (green), VAN (yellow) and MTZ (red) groups, with six animals in each group. ○: Cecal samples; +: Feces samples. (B) Loading plot indicating each of the bacterial families colored according to phylum. Firm, Firmicutes (blue); Bact, Bacteroidetes (red); Prot, Proteobacteria (green), Acto, Actinobacteria (yellow); Tene, Tenericutes (orange); Defe, deferribacteres (pink) and Verr, Verrucomicrobia (light pink).

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460

Chapter 4

Discussion

The gut microbiota is important for host health. In the present work the interaction between the gut microbiota and intestinal integrity has been studied, specifically during the experimental work the effect of modulating the gut microbiota and the subsequent effects on intestinal integrity have been studied.

4.1 Effect of modulating the gut microbiota on intestinal integrity

In order to determine if the gut microbiota affects the intestinal integrity it is helpful to modulate the gut microbiota in a given direction, and determine if this has an effect on intestinal integrity. In the present work the gut microbiota was modulated by three different means namely; dietary whole-grain or refined wheat intake, prebiotics and supplementation with commensal bacterial strain, and antibiotics. These modulations were further conducted using different models. Specifically:

- Supplementation of whole-grain and refined wheat products to the diet of post-menopausal women (manuscript 1).
- Modulation of human faecal bacterial composition by prebiotic and putative prebiotics (manuscript 2).
- Modulation of the rat gut microbiota with XOS and a commensal *B. pseudolongum* (manuscript 3).
- Modulation of the rat gut microbiota with antibiotics (manuscript 4).

Modulation of the gut microbiota with whole-grain and refined wheat products were only identified to affect *Bifidobacterium* spp. and *Bacteroides* spp. during the intervention period, respectively, whereas there was no difference in effect of faecal water on TER between the two dietary groups (manuscript 1). Therefore dietary intervention with whole-grain products may not be sufficient to alter the intestinal integrity. This can however not be excluded as determinations of effects on intestinal integrity was determined *ex vivo*. More clear conclusions could have been made, if the intestinal permeability had been determined during the dietary intervention, but such analysis were not conducted. To the best of my knowledge the effect of whole-grain products on intestinal integrity has not been examined previously. However whole-grain products have been shown to increase SCFA among other butyrate *in vitro*, see table 2.4 and as butyrate has been found to increase barrier function in some studies [91, 92] whole-grain products

may increase intestinal integrity. Therefore the effects of intake of whole-grain products need further investigation.

Others have shown, that administration of prebiotics and putative prebiotics may cause both increased and decreased pathogen translocation in rodents [68, 69, 118, 119, 127], as well as altered intestinal permeability [120, 122]. This indicates an impairment of the intestinal barrier when some prebiotics are applied. In some of these studies the prebiotic administration resulted in a stimulation of bifidobacteria [69, 118, 120, 121]. Since we also showed a negative trend between *Bifidobacterium* spp. and TER (manuscript 1) it was examined, if stimulation of this bacterial group by a bifidogenic putative prebiotic XOS or administration of a commensal *Bifidobacterium* spp. had an effect on intestinal integrity (manuscript 3). This potential effects may arise not due to the bifidobacteria them self, but based on cross-feeding leading to stimulation of other non-determined bacteria that could affect intestinal integrity. The modulations introduced in this study did not alter permeability for FITC-dextran *in vivo* and caecal water from the different experimental groups did not have different effect on TER *in vitro* (manuscript 3). But only slight modulations of the gut microbial composition were identified, potentially resulting in the limited effects on intestinal integrity. Based on these stated studies it does not seem that XOS or administration of a commensal bifidobacteria affected intestinal integrity. This contradicts the observed effects of XOS on *Salmonella* translocation in mice [68], but is supported by the limited *Listeria monocytogenes* inflammation in guinea pigs [127]. The different experimental outcomes may be dependent on the applied animal model, its gut microbiota and to what extend the gut microbiota is modulated.

In addition to the stated study, the effect of modulation of the gut microbiota with prebiotics and putative prebiotics, using batch *in vitro* fermentations, were conducted (manuscript 2). Supernatants from these fermentations were to be applied in the TER assay, to determine if the modulation of the gut microbiota had an effect on TER. The work is however still ongoing, hence this is not discussed further.

The final method applied for modulating the gut microbiota was antibiotic administration that resulted in much larger modulations of the gut microbiota and intestinal permeability, than the other studies conducted in the present work. Here the intestinal permeability for FITC-dextran was decreased following cefotaxime and vancomycin (manuscript 4). This was connected to an increase in bacterial families belonging to the Proteobacteria and a reduction in bacterial families belonging to the Firmicutes, specifically the *Lactobacillaceae* for vancomycin (manuscript 4). While cefotoxime administration led to an increase in relative abundance of families belonging to the *Bacteroidaceae* bacterial family in faeces and caecum, and *Bifidobacteriaceae* in caecum (manuscript 4). Increased relative abundance of *Bifidobacteriaceae* and *Lactobacillaceae* could potentially explain the decreased intestinal permeability for FITC-dextran, as these previously have been shown to increase barrier function, see table 2.2 and table 2.3. Other studies have also shown an increased intestinal integrity following antibiotic administration. Previously a mixture of ampicillin and neomycin have been shown to reduce increased intestinal permeability in HF diet mice and *ob/ob* mice [1]. This was connected to a reduction in *Bifidobacterium* spp., *Lactobacillus* spp. and *Bacteroides-Prevotella* spp. [1] Additionally a mixture of ampicillin, neomycin, and metronidazole has been shown to reduce plasma LPS levels in HF diet mice, as well as increasing the level of Proteobacteria and decreasing aerobic and anaerobic bacteria [111]. This collectively indicate, that some an-

tibiotics may modulate the gut microbiota and subsequent increase intestinal integrity. However not all the applied antibiotics decreased intestinal permeability. One antibiotic, that modulated the gut microbiota extensively, more specifically amoxicillin, did not alter FITC-dextran permeability. Additionally metronidazole, that increased FITC-dextran permeability did not have an effect on the gut microbiota at bacterial family level (manuscript 4). The results for amoxicillin and metronidazole could indicate, that changes in intestinal permeability are not always connected to the gut microbiota. However, it cannot be excluded that amoxicillin altered permeability for molecules smaller than FITC-dextran, see section 4.2, or that metronidazole altered the gut microbiota in other sections in the GIT e.g. the small intestine, than investigated in the study, or that changes occurred at lower bacterial order than family level. This would not be identified by the applied methods. Previously metronidazole has been shown to stimulate *Lactobacillus* and reduce *Clostridium coccoides* group and *Bacteroidales* [113]. Furthermore this antibiotic caused reduced mucus layer thickness as well as increased inflammation [113]. Collectively this indicates that methronidazole may cause impaired intestinal integrity, while vancomycin and cefotoximin increased barrier function (manuscript 4).

In conclusion these studies and literature show that the intestinal integrity may be affected by modulations of the gut microbiota through dietary changes, prebiotics, and antibiotics. The outcome is dependent on the extend that the gut microbiota is altered, but potentially also if only a limited number of bacterial groups are affected. On the basis of these studies it is hypothesised, that intestinal integrity may be affected when the gut microbiota is highly modulated away from the stable microbiota. This may explain why intestinal integrity is altered in gastrointestinal disorders, where the gut microbiota may be in dysbiosis.

4.2 Means of determining the intestinal integrity

Intestinal integrity was in the present study considered to be defined as "maintaining the intestinal barrier whole and assembled", see section 2.5. Changes in intestinal integrity was on this basis considered to arise due to changes in IEC apoptosis and proliferation, alterations of the protective mucus layer, or altered interaction between adjacent IEC through the TJ. The main focus was here on the intestinal permeability for FITC-dextran, but also barrier function based on TER determinations was considered. Alterations in FITC-dextran permeability may arise due to altered interactions in TJ, specifically in the leak pathway [58]. Therefore it can not be excluded, that a treatment affect intestinal permeability of smaller molecules, despite of the permeability of 4kDa FITC-dextran being unaltered. Loss of epithelial cells may also cause increased FITC-dextran flux, hence altered FITC-dextran permeability can indicate alterations in both the leak pathway in the TJ and loss of IEC. However, TER, that measure electrical resistance, hence ion conductance, must determine both the pore and the leak pathway [58], as well as loss of IEC. It is therefore relevant to combine these two methods as done in the present work (manuscript 3). In other studies the permeability of molecules of different size have been determined simultaneously [55, 56]. This would be useful in future work, as changes in both the pore and leak pathway in the TJ may be identified.

In order to have an additional measure for intestinal integrity the gene expression of genes encoding TJ proteins and the mucin MUC2 encoding gene were determined

(manuscript 3). Connected to no alterations in FITC-dextran permeability only minor changes as decreased occludin gene expression by XOS was determined (manuscript 3). Previously FITC-dextran plasma concentrations have been correlated with ZO-1 and occludin mRNA expression in mice fed a HF diet [1, 71]. This was not seen in the present study (manuscript 3). As FITC-dextran is to cross at the leak-pathway it seems valid that alterations of TJ proteins that make up the major structure of the TJs are linked to permeability of this molecule. However changes in permeability at the TJs may also arise by re-localisation of TJ proteins or altered levels of these proteins. Hence further work within this field would lead to additional knowledge by including such measures. In addition to this it could also be recommended to include analysis of several of the claudins, both at mRNA expression and protein level, as they can be barrier and pore forming, see section 2.3.3. Additionally they are considered to be the backbone of the TJ. Alterations of the pore forming claudins may therefore potentially be used as a measure of the pore pathway.

Intestinal integrity is also dependent on the mucus layer, as this protects the IEC against hazardous substances. Alterations in the mucus layer thickness as well as mucus secretion could therefore be relevant to determine. Increased mucus secretion may indicate an impairment of the epithelial barrier, as it has been connected to increased *Salmonella* translocation [69, 118] and intestinal permeability in rats [120]. However, increased mucus secretion could also indicate an increased mucus production, hence a potential increase in the mucus layer that could be beneficial.

Overall intestinal integrity is the result of interaction between the gut microbiota, the consumed diet, the IEC, and the host immune system. This is a very intertwined and complex system. It is therefore very difficult to determine a single measure that determined the overall intestinal integrity. Therefore several measures for intestinal integrity, as well as determination of permeability of different size molecules should be included in further work regarding intestinal integrity.

4.3 Impact of altered intestinal integrity

Altered intestinal permeability has been connected to gastrointestinal disorders [29]. Therefore an impairment of intestinal integrity may cause inflammation and subsequent bacterial translocation leading to adverse effects for the host. In literature one hypothesis is, that permeability of the IEC barrier is first altered and then the inflammation arise [59]. On this basis an increase in intestinal permeability can cause adverse effects. However this must depend on for how long the permeability is altered and to what extent. Large impairments caused by loss of TJ function or IEC may of course result in luminal content crossing the IEC barrier and lead to inflammation. Alterations for shorter time spans, which only led to minor molecules or less luminal crossing the IEC barrier, may only cause adverse effects if it results in a massive inflammatory response leading to further impairment. In fact permeability of specific molecules, for example ions, may be beneficial, as it would increase the uptake. Hence the consequence of altered intestinal integrity is dependent on the severity, the duration, and the following host response.

Chapter 5

Conclusion

The work in this thesis leads to further knowledge regarding the interaction between gut microbiota and intestinal integrity. Specifically effects of modulations of the gut microbiota on intestinal integrity have been examined, leading to these main conclusions:

- Whole-grain wheat products increase the relative abundance of *Bifidobacterium* spp. while refined wheat products reduce the relative abundance of *Bacteroides* spp. in post-menopausal women during the intervention. This did not change effects of faecal water on TER (manuscript 1).
- Relative abundance of *Bifidobacterium* spp. tended to correlate negatively with TER, indicating that stimulation of *Bifidobacterium* spp. may be connected to decreased intestinal barrier function (manuscript 1).
- XOS and a commensal *B. pseudolongum* did not have an effect on the gut microbiota or intestinal permeability, and caecal waters effect on TER in male Wistar rats (manuscript 3).
- Antibiotic administration modulated both gut microbial composition and intestinal permeability in female Wistar rats (manuscript 4).
- Relatively large modulations of the gut microbiota may have an effect on intestinal integrity, while minor modulations may not have an effect.

Intestinal integrity is a very complex field, since it is affected by the connection between the host, the bacterial community and the diet. Therefore the field still needs more examination. During this work it would be highly recommended to include several markers for intestinal permeability, examine the mucus layer, and extensively evaluate the gut microbiota, as this would highly extend the current knowledge within this field.

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