Doctoral Thesis

Investigation of microbial interactions of Lactobacillus paracasei CNCM I-1518 with elderly gut microbiota and Clostridium difficile using advanced intestinal fermentation models

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INVESTIGATION OF MICROBIAL INTERACTIONS OF *LACTOBACILLUS PARACASEI* CNCM I-1518 WITH ELDERLY GUT MICROBIOTA AND *CLOSTRIDIUM DIFFICILE* USING ADVANCED INTESTINAL FERMENTATION MODELS

A thesis submitted to attain the degree of

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presented by

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2015
## Contents

Abbreviations  
Summary  
Zusammenfassung

### Chapter 1  
General introduction  
The human gastrointestinal tract and the important role  
of its microbiota  
*Clostridium difficile*  
Probiotics  
Intestinal models for studying bacterial infections  
Background and objectives of the thesis

### Chapter 2  
Design and investigation of PolyFermS *in vitro*  
continuous fermentation models inoculated with  
immobilized fecal microbiota mimicking the elderly colon

### Chapter 3  
*In vitro* investigation of *Clostridium difficile* colonization  
and antibiotic treatments using the PolyFermS model  
platform mimicking elderly intestinal fermentation

### Chapter 4  
*In vitro* ecological study of *Lactobacillus paracasei*  
CNCM I-1518 in healthy and *Clostridium difficile*  
colonized elderly gut microbiota

### Chapter 5  
General conclusions and perspectives

Bibliography  
Appendix  
Acknowledgments
## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAD</td>
<td>Antibiotic-associated diarrhea</td>
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<tr>
<td>AMPs</td>
<td>Antimicrobial peptides</td>
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<td>BCFA</td>
<td>Branched-chain fatty acid(s)</td>
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<tr>
<td>bp</td>
<td>base pairs</td>
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<tr>
<td>CD</td>
<td><em>Clostridium difficile</em></td>
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<td>CDAD</td>
<td><em>Clostridium difficile</em>-associated diarrhea</td>
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<td>CDI</td>
<td><em>Clostridium difficile</em> infection</td>
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<td>cDNA</td>
<td>complementary DNA</td>
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<tr>
<td>cfu</td>
<td>colony-forming units</td>
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<tr>
<td>CR</td>
<td>Control reactor</td>
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<tr>
<td>CWP(s)</td>
<td>Cell wall protein(s)</td>
</tr>
<tr>
<td>DC</td>
<td>Distal colon</td>
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<td>DGGE</td>
<td>Denaturing gradient gel electrophoresis</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<tr>
<td>EFSA</td>
<td>European Food Safety Authority</td>
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<tr>
<td>FAO</td>
<td>Food and Agriculture Organization of the United Nations</td>
</tr>
<tr>
<td>FISH</td>
<td>Fluorescent <em>in situ</em> hybridization</td>
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<td>FMT</td>
<td>Fecal microbiota transplantation</td>
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<tr>
<td>GALT</td>
<td>Gut-associated lymphoid tissue</td>
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<td>GIT</td>
<td>Gastrointestinal tract</td>
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<td>HIT</td>
<td>Human intestinal tract</td>
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<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
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<tr>
<td>IBD</td>
<td>Inflammatory bowel disease</td>
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<td>IgA</td>
<td>Immunoglobulin A</td>
</tr>
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<td>IR</td>
<td>Inoculum reactor</td>
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<td>LpC</td>
<td><em>Lactobacillus paracasei</em> CNCM I-1518</td>
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<tr>
<td>MAMPs</td>
<td>Microbial-associated molecular patterns</td>
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<tr>
<td>MLNs</td>
<td>Mesenteric lymph nodes</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>NCBI</td>
<td>National Center for Biotechnology Information</td>
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<td>NGS</td>
<td>Next generation sequencing</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
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<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>OTU</td>
<td>Operational taxonomic unit</td>
</tr>
<tr>
<td>PC</td>
<td>Proximal colon</td>
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<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PPs</td>
<td>Peyer’s patches</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative polymerase chain reaction</td>
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<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
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<tr>
<td>rRNA</td>
<td>ribosomal ribonucleic acid</td>
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<tr>
<td>RT</td>
<td>Retention time</td>
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<tr>
<td>SCFA</td>
<td>Short chain fatty acid(s)</td>
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<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SLP(s)</td>
<td>Surface layer protein(s)</td>
</tr>
<tr>
<td>TDC</td>
<td>Transverse-distal colon</td>
</tr>
<tr>
<td>TGGE</td>
<td>Temperature gradient gel electrophoresis</td>
</tr>
<tr>
<td>TLR(s)</td>
<td>Toll-like receptor(s)</td>
</tr>
<tr>
<td>T-RLFP</td>
<td>Terminal-restriction fragment length polymorphism</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
<tr>
<td>YCFA(-GSC)</td>
<td>Yeast extract-casein hydrolysate-fatty acid medium (supplemented with glucose, starch and cellobiose)</td>
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Summary
The human intestine is colonized by a dense and diverse microbial community, referred to as the gut microbiota that contributes to physiological processes important to the host, including the acquisition of nutrients and the development of the immune system. A healthy gut microbiota also protects against colonization of pathogens, hence antibiotic treatment or other environmental factors that disrupt the microbial community can result in overgrowth of potentially dangerous bacteria. One such bacterium is *Clostridium difficile* (CD) that can cause severe disease in association with broad-spectrum antibiotics, especially in the elderly population. CD is an obligate anaerobic, spore-forming and toxin-producing bacterium that belongs to the most important health-care associated pathogens worldwide and is the leading cause of antibiotic-associated diarrhea. Standard treatment of *C. difficile* infection (CDI) consists of the antibiotics vancomycin and metronidazole, however, they are associated with a high recurrence rate, and thus there is a need for alternative treatment. The use of probiotics as treatment or prevention of CDI could be of high potential since promising results were obtained in clinical trials. It was shown in one study that a probiotic drink containing *Lactobacillus paracasei* CNCM I-1518 (LpC) and conventional starter bacteria reduces the risk of *C. difficile*-associated diarrhea (CDAD) in elderly patients taking antibiotics. However, the mechanisms responsible for the protecting effect against CD remain to be clarified. Therefore, the central objective of this thesis was to investigate mechanisms and potential antagonistic effects of LpC against CD using *in vitro* models simulating the elderly colon.

To study microbial interactions in the complex environment of the elderly gut microbiota we developed and validated three different continuous fermentation models simulating the elderly colon. Each model was inoculated with immobilized fecal microbiota of healthy donors aged between 71 and 78 years. Model 1 was a “traditional” three-stage model of the proximal, transverse and distal colon. The design of models 2 and 3 was based on the novel PolyFermS platform that uses an inoculum reactor (IR) to continuously inoculate second stage reactors in parallel. Model 2 consisted of an IR inoculating two test systems each consisting of a proximal and distal colon reactor and in model 3 the IR was feeding five distal colon reactors connected in parallel. The donor of model 1 was less representative of the elderly population since no *Roseburia* spp. was detected in the fecal inoculum. The microbial profile in models 2 and 3 was analyzed with qPCR and 16S rRNA gene amplicon sequencing and was shown to be representative of the composition of the fecal donor and the elderly population.
population. Furthermore, a high reproducibility was demonstrated for control and test reactors of PolyFermS models regarding microbiota composition and activity.

We then applied the PolyFermS models with elderly microbiota to investigate CD colonization behavior. In model 2, CD (PCR ribotype 001) was inoculated as free vegetative cells. A few days after instillation, CD colonized the distal colon (pH 6.8) but not the proximal colon reactors (pH 5.7). In model 3, we increased the complexity of experimental design and tested the antibiotics ceftriaxone and metronidazole to induce germination of CD spores and for CD treatment, respectively, in reactors replicating the distal colon. We found that in our model ceftriaxone treatment was redundant for induction of spore germination since CD colonization was also observed in reactors free of antibiotic. Metronidazole challenge resulted in a substantial dysbiosis of the gut microbiota and a decrease in CD growth and toxin production to below the detection limit. However, shortly after cessation of the treatment, CD recovered reaching similar or even exceeding pre-metronidazole copy numbers and cytotoxin titers.

Since we demonstrated CD colonization in PolyFermS models with elderly microbiota we continued by investigating the potential of LpC to antagonize CD. LpC was added twice daily as free cells to test preventive treatment (model 2) and adjuvant therapy to metronidazole (model 3). LpC established transiently in reactors with average numbers of around log_{10} 8 copies per mL effluent. However, we did not observe an inhibitory effect of LpC on CD growth and toxin production in the continuous fermentation models. The direct interaction between LpC and CD (PCR ribotype 001) was also investigated in co-culture studies independent of the complex gut microbiota. Similar to colonic models, LpC had no inhibitory effect on CD growth but cytotoxin production was reduced by around 40% in co-culture with LpC compared to single culture. These findings suggest that the probiotic mechanism of LpC in CDI is not due to direct inhibition of growth. The inhibition of toxin production is a potential mechanism, however, no effect on toxin was observed in complex microbiota.

In a fourth continuous fermentation experiment we investigated the effect of LpC on the elderly gut microbiota independent of CD using 16S rRNA gene amplicon sequencing and metatranscriptomics. We demonstrated small changes in the gut microbiota composition associated with LpC, including increased abundances of the closely related genera *Lactobacillus* and *Enterococcus*, a shift in predominant butyrate producers and a decrease in
H₂ and CH₄ producing bacteria. On a functional level we found an increase in carbohydrate utilization activity with LpC. Our results indicate a contribution of LpC in the trophic interaction of dietary carbohydrate utilization with the commensal microbiota. As we observed an increase in abundance of *Faecalibacterium* we further investigated the interaction between LpC and *Faecalibacterium prausnitzii* in absence of complex microbiota and showed that cell lysis of *F. prausnitzii* is decreased in co-culture with LpC over extended incubation times of 72 h.

Taken together, we successfully applied continuous intestinal fermentation models with elderly immobilized gut microbiota to study CD colonization dynamics. We demonstrated CD colonization in reactors mimicking distal colon conditions without requiring the application of antibiotics. Metronidazole induced dysbiosis of the microbiota and reduced CD counts only temporarily. Using these models, we showed that the probiotic LpC strain had no inhibitory effects against CD growth or toxin production. However, a reducing effect of LpC on CD cytotoxin production was observed in co-culture studies independent of the complex microbiota. We further demonstrated *in vitro*, the potential of LpC to interact with a healthy gut microbiota on a structural and functional level. This thesis provided first insights on *in vitro* interaction of LpC and CD in complex gut microbiota. More studies, especially *in vivo* studies are needed to determine the role of host-related factors, including the immune system and the epithelial cell layer, in the possible protection of LpC against CD. Of particular interest is also the potential of LpC to modify the gut microbiota of elderly and how this *in vivo* might lead to protection against CD. Furthermore, additional clinical investigations would be required to show the efficacy of LpC single culture on the protection of CDAD, independent of other fermentative bacteria and fermented milk.
Zusammenfassung
Zusammenfassung


Das zentrale Ziel dieser Arbeit war deshalb die Mechanismen und die mögliche antagonistische Wirkung von LpC gegen CD zu untersuchen mittels in vitro Modellen die den Darm von älteren Menschen simulieren.

Um das mikrobielle Zusammenspiel im komplexen Umfeld der Darmflora von älteren Menschen zu untersuchen haben wir drei verschiedene kontinuierliche Fermentationsmodelle entwickelt und validiert. Jedes Modell wurde mit immobilisierter fäkaler Mikroflora von gesunden Spendern im Alter von 71 und 78 Jahren inokuliert. Modell 1 war ein traditionelles Drei-Stufen-Modell das den proximalen, querlaufenden und distalen Dickdarm darstellt. Das Design von Modell 2 und 3 basierte auf der neuartigen PolyFermS Plattform das einen Inokulum Reaktor (IR) verwendet für die kontinuierliche Inokulation von parallel angeschlossenen Reaktoren. Modell 2 bestand aus einem IR, der zwei Testsysteme inokulierte, die sich jeweils aus zwei Reaktoren zusammensetzten die den proximalen und
Zusammenfassung


Wir verwendetene die PolyFermS Modelle mit Darmflora von älteren Menschen um das Kolonisationsverhalten von CD zu untersuchen. Modell 2 wurde mit nicht immobilisierten und vegetativen CD Zellen (PCR Ribotyp 001) inokuliert. Ein paar Tage nach der Inokulation kolonisierte CD den distalen Reaktor (pH 6.8), aber nicht den proximalen Reaktor (pH 5.7). In Modell 3 erhöhten wir die Komplexität des Versuchs und untersuchten die Antibiotika Ceftriaxone und Metronidazole um entweder die Keiming der Sporen zu induzieren oder für die Behandlung von CD. Dieser Test hat uns gezeigt, dass in unserem Modell die Behandlung mit Ceftriaxone für die Induktion der Sporenkeimung überflüssig ist weil wir CD Kolonisation auch in Reaktoren ohne Antibiotika beobachtet haben. Die Behandlung der Darmflora mit Metronidazole führte zu einer Dysbiose der Darmflora und einer Abnahme des CD Wachstums und der Toxinproduktion bis unter die Nachweisgrenze. Doch kurz nach Absetzen der Behandlung erholte sich CD und verglichen mit vor der Behandlung wurden ähnliche oder sogar höhere CD Kopienzahlen sowie Zellgift Titer gemessen.

Zusammenfassung


Zusammenfassung

Chapter 1

General introduction
1. The human gastrointestinal tract and the important role of its microbiota

1.1. Function and physiology of the human gastrointestinal tract

1.1.1. Digestive function

The gastrointestinal tract (GIT) is a highly specialized organ that converts food into nutritional compounds that can be absorbed into the body. The GIT is composed of the mouth, esophagus, stomach, small intestine and large intestine (Figure 1.1). In addition, the pancreas and liver secrete into the small intestine and the GIT is connected to the vascular, lymphatic and nervous system for regulation of digestive response and food intake as well as delivery of absorbed compounds to the specific organs. Apart from making nutrients available for absorption, the GIT has also a role in metabolizing and eliminating non-nutrient and toxic compounds (Schneeman, 2002). The breakdown of food into absorbable compounds involves mechanical and biochemical processes. This digestive process begins at the mouth with the mechanical breakdown of food into smaller particles (mastication). At the same time the food particles are mixed with saliva in order to facilitate the passing through the esophagus to the stomach. In addition, the saliva is carrying enzymes that initiate the breakdown of carbohydrates and fats. Swallowing moves the bolus into the stomach where it is mixed with hydrochloride acid and digestive enzymes (i.e. pepsin, lingual and gastric lipase) by muscular contractions and thereby converting the bolus into chyme. The stomach is the most important site of absorption next to the intestinal tract; however, only monosaccharides and H₂O are absorbed. The chyme is then released into the small intestine which is the major site for digestion and absorption of nutrients. The small intestine is composed of the duodenum, jejunum and ileum. Digestive juices, including pancreatic enzymes and bile acids, induce the further digestion of fats, polysaccharides and peptides into absorbable nutrients. Through the peristaltic motor activity of the small intestine the chyme is passing through until it enters the large intestine (DeSesso & Jacobson, 2001, Schneeman, 2002), which is composed of the ascending, transverse and descending colon (Cummings & Macfarlane, 1991) and is the major site for absorption of water and electrolytes. Compounds that enter the large intestine are non-digestible polysaccharides, sloughed epithelial cells, mucins and proteins that are then used for growth of the gut microbiota and contribute to
stool formation and laxation. Products of the microbial fermentation include short chain fatty acids (SCFA), branched chain fatty acids (BCFA), gases and ammonia. SCFA are either absorbed from the colon for use as energy source in various tissues or for gluconeogenesis in the liver (Schneeman, 2002, Blaut, 2013).

Figure 1.1: The human gastrointestinal tract with pH and main digestive functions of the different compartments. Adapted from Cummings & Macfarlane (1991), McClements & Li (2010) and Lawley & Walker (2013).

1.1.1.1 Physiological changes in the elderly

Ageing is accompanied by physiological changes in the GI tract that are either induced by the ageing process itself as well as by a modified diet and a reduction of the functionality of the immune system. Masticatory dysfunction due to loss of teeth and muscle bulk, decreased sensitivity for taste and smell, delayed gastric emptying and changes in satiety peptide expression can result in an unbalanced energy intake in old age (Lovat, 1996, Tiikonen et al., 2010, Biagi et al., 2011). Reduced absorption or metabolism of nutrients may also decline with age as it was reported that calcium, iron and vitamin B12 absorption was reduced in the elderly. Slight changes in the gastrointestinal motility may occur in old age (Biagi et al., 2011). However, the effect of age on gastric emptying is controversial as in some studies ageing was associated with delayed gastric emptying (Horowitz et al., 1984, Clarkston et al.,
1997) whereas in the study of Madsen and Graff (2004) age had no effect on the gastric and small intestinal motility. Constipation is also often associated with old age and it was suggested that the reason is the reduced water and fiber intake (Tiihonen et al., 2010, Biagi et al., 2011). However, so far this was not clearly demonstrated as in several studies no differences in colonic or gastrointestinal transit time was tested between healthy adults and older subjects (Becker & Elsborg, 1979, Metcalf et al., 1987, Nagengast et al., 1988). On the other hand, colonic transit time was delayed in the elder group in the study of Madsen and Graff (2004). It is thus not yet elucidated if chronic constipation and colonic dysmotility are natural consequences of ageing.

In general the elderly are more susceptible to GI-related problems and illnesses, such as diverticulosis, upper gastrointestinal bleeding, gastric and colon cancers. Further, infections with *Helicobacter pylori* and *Clostridium difficile* is more common in the elderly (Tiihonen et al., 2010).

1.1.2. GIT barrier function

The intestinal tract is lined by the epithelium (Figure 1.2) that forms an important selective barrier against the external environment by allowing absorption of nutrients, electrolytes and water but providing protection against intraluminal compounds and bacteria (Round & Mazmanian, 2009). The epithelium is built of a single layer of cells organized in crypts and villi. The intestinal cells include the enterocytes responsible for absorptive and digestive functions as well as specialized intestinal cell lineages, including enteroendocrine, goblet and Paneth cells that maintain digestive and barrier functions (Peterson & Artis, 2014). Intercellular junctional complexes consisting of tight junctions, adherens junctions and desmosomes provide integrity of the cell layer (Lievin-Le Moal & Servin, 2006). The cell line is covered by a mucus layer which is mainly made up of gel-forming highly glycosylated proteins termed mucins secreted by the goblet and Paneth cells. The mucus of the colon consists of two layers; the outer one contains a large number of bacteria while the inner one resists bacterial penetration (Hooper et al., 2012).

The intestinal epithelial cells, together with macrophages, neutrophils and dendritic cells belong to the innate immune system that is responsible for protection of the host against infection. Cells of the innate immune system have pattern recognition receptors, such as toll-like receptors (TLRs) that enable the recognition of conserved molecules present on microorganisms termed microbial-associated molecular patterns (MAMPs), thereby distinguishing between commensal microbiota and pathogens (Purchiaroni et al., 2013). This
recognition induces a cascade of cellular responses that regulate epithelial cell production of antimicrobial proteins (AMPs) and chemokines as well as activation of the adaptive immune system (Cerf-Bensussan & Gaboriau-Routhiau, 2010, Purchiaroni et al., 2013).

The acquired immune response is primarily imprinted in the gut-associated lymphoid tissues (GALTs) that include the Peyer’s patches (PPs) and mesenteric lymph nodes (MLNs) and is characterized by a highly specific immune response that has been acquired over time (Cerf-Bensussan & Gaboriau-Routhiau, 2010, Purchiaroni et al., 2013). The lamina propria lies beneath the epithelial cell lining and contains a large number of macrophages, dendritic cells, T cells, and immunoglobulin A (IgA) secreting B cells (Purchiaroni et al., 2013). Macrophages in the lamina propria phagocytose and eliminate microbes that crossed the epithelium. However, the dendritic cells of the intestinal immune system may also sample some of the commensal bacteria and carry them to MLNs and interact with B and T cells in the PPs, inducing B cells to produce IgA. The secreted IgA are then transported across the epithelium where they can bind to luminal bacteria and prevent their translocation across the epithelium (Hooper et al., 2012).
Figure 1.2: The intestinal epithelium is covered by a mucus layer that consists of the outer and inner layer. The inner mucus layer and the epithelial cells build a barrier between the bacteria in the lumen and the immune system underlying the epithelial cells. Translocation of bacteria across the epithelial layer is prevented by secretion of antimicrobial peptides and production of IgA by B cells. Macrophages kill bacterial cells that crossed the epithelium. Dendritic cells sample live bacteria and induce specific IgA production (Hooper, 2009).

1.2. The GIT as bacterial ecosystem

The term ‘microbiota’ (‘small life’) describes the collective microbial community inhabiting a specific environment (Tremaroli & Backhed, 2012). The human GIT is colonized by a diverse microbiota consisting of around $10^{13}$ to $10^{14}$ bacterial cells composed of more than 1’000 different species. The human microbiota is thus estimated to contain 10-fold and 150-fold more cells and genes, respectively, than their host (Qin et al., 2010). The microbial community of the GIT is distributed throughout its length and the microbial density and composition varies greatly between the different segments (Figure 1.3), depending on nutrient availability, pH, transit, redox potential and host secretions. The stomach and
proximal small intestine is only sparsely populated due to acid, bile, pancreatic juice and short transit times that hinder colonization. The bacterial density increases in the ileum and the major concentration of microbes is reached in the colon with numbers between $10^{11}$ and $10^{12}$ cells per mL or g content (Isolauri et al., 2004, O’Hara & Shanahan, 2006, Power et al., 2014). The inaccessibility of the small and large intestine hamper studies of microbiota composition in these regions. Whereas much of our knowledge about the colonic microbiota derives from fecal material only limited data is available on the microbial composition in the small intestine. Effluent sampling from the ileum has however shown that the microbiota is less diverse and higher in relative abundances of *Veillonella*- and *Streptococcus*-related phylotypes as well as species belonging to *Clostridium* cluster I relative to fecal microbiota (Gerritsen et al., 2011).

**Figure 1.3:** Density and compositional differences in the microbiota of different regions of the human gastrointestinal tract. Adapted from Sekirov et al. (2010) and Cho & Blaser (2012).

### 1.3. The human gut microbiota

#### 1.3.1. Techniques for the analysis of gut microbiota composition and diversity

Technologies applied to study the human colonic microbiota can be divided into two major periods: the microscopic observation and culture-based methods and from 1995 on the
culture-independent methods (Lagier et al., 2012). Techniques applied in the study of gut microbial bacteria are described below and in Table 1.1.

It should be emphasized that sampling, transportation and storage can have major impacts on quality of the samples and are equally important than the selection of the analysis technique.

1.3.1.1 Culture-dependent methods

Culturing has been the first method used to characterize a bacterial ecosystem and with this method the number of bacterial species of the gastrointestinal microbiota was estimated between 400 and 500 (Mata et al., 1969, Moore & Holdeman, 1974, Finegold et al., 1977). However, around 80% of bacteria cannot be cultivated under standard laboratory conditions (Eckburg et al., 2005). This implicates that culture-dependent methods result in an incomplete characterization of the gut microbial diversity (Lagier et al., 2012). Using cultivation for microbial community analysis is laborious and time-consuming as culturing in several selective and non-selective media at anaerobic conditions are necessary. Furthermore, distinguishing at the species or strain-level becomes difficult, if not impossible using cultivation (Sekirov et al., 2010, Lagier et al., 2012). However, culture-based methods remain the only validated method to quantify viable bacteria and are important for characterization of new strains (e.g. probiotic strains) (Dethlefsen et al., 2006).

1.3.1.2 Culture-independent methods

The use of culture-independent methods allows for much more comprehensive studies of the composition and diversity of the gut microbiota and these methods are not limited by the typical issues of culturing. However, molecular methods have their own limitations such as PCR amplification and hybridization bias and most methods do not provide information on a functional level (Gong & Yang, 2012).

The 16S ribosomal RNA (16S rRNA) gene is a commonly used molecular marker for bacterial detection and enumeration. This gene was chosen due to its small size of around 1.5 kb and highly conserved but also variable regions that allow distinguishing between different species but provide enough similarity to identify members of the same larger phylogenetic group (Sekirov et al., 2010).

**PCR-based DNA profiling techniques.** PCR-based DNA fingerprinting methods that have typically been used for the characterization of the gut microbiota include denaturing gradient and temporal gradient gel electrophoresis (DGGE and TGGE) and terminal-restriction fragment length polymorphism (T-RLFP). These techniques create a DNA profile of the microbial community and can be used to study changes in bacterial populations by comparing
“fingerprints” of different samples of microbial communities thus they are mainly used for comparative purposes. DGGE and TGGE are based on PCR amplification of target genes (usually the 16S rRNA gene) using group- or species-specific primers. The amplified gene products are then separated using either a chemical (i.e. DGGE) or temperature (i.e. TGGE) gradient to denaturate the samples on a gel according to their guanine and cytosine content (Macfarlane & Macfarlane, 2004, Sekirov et al., 2010). T-RFLP is also based on PCR amplification of the 16S rRNA gene, however, the digestion of PCR amplicons generates fluorescently-labeled terminal restriction fragments and their length is then analyzed by electrophoresis (Gong & Yang, 2012).

Subsequent species identification of bands can be performed by excising and sequencing of the DNA fragments (Macfarlane & Macfarlane, 2004, Sekirov et al., 2010). The techniques are semi-quantitative as the relative intensity of a DNA band can give some indication on the abundance of the particular species (Gong & Yang, 2012). The main disadvantage of this method is that only a concentration of $10^6$ cells g$^{-1}$ feces are tested (Vael et al., 2011).

**Fluorescence in situ hybridization (FISH).** FISH quantifies bacteria using specific oligonucleotides probes that are labeled with fluorescent dyes. This method works without DNA extraction, instead, bacterial cells are treated with paraformaldehyde or ethanol to permeabilize them and allow the probe to directly enter the cell. In the cell the probe can hybridize to the 16S rRNA sequences that are unique to the targeted bacterial groups and cause them to fluorescence. Enumeration can be performed using a fluorescent microscope (Macfarlane & Macfarlane, 2004, Sekirov et al., 2010) or in combination with flow cytometry (Cinquin et al., 2006). FISH is especially suitable for microbial ecology studies such as the spatial organization of communities in the gut since bacteria can be detected in situ. The main disadvantages of this method are the laborious sample preparation and the high limit of detection ($10^6$ cells g$^{-1}$ feces) (Macfarlane & Macfarlane, 2004).

**DNA microarray.** The principle behind DNA microarrays is the same as for FISH, namely the use of fluorescently labeled oligonucleotides to hybridize with complementary nucleotide sequences, however, DNA microarray is a high-throughput method as multiple probes are applied and thousands of genes are analyzed in one experiment (Macfarlane & Macfarlane, 2004). Many studies have been performed using microarrays for quantitative and systemic studies of the gut microbiota (Gong & Yang, 2012, Paliy & Agans, 2012). The phylogenetic Human Intestinal Tract Chip (HITChip) targets the 16S rRNA gene of more than 1’000 bacterial species within the human gut (Rajilic-Stojanovic et al., 2009). DNA microarrays are
cost-effective and fast methods, however, hybridization bias is one of the main disadvantage (Sekirov et al., 2010).

**Quantitative real-time PCR (qPCR).** qPCR is often used to quantify bacterial populations occurring in the gut. This method determines the 16S rRNA gene copy numbers using genus- or species-specific primers and a fluorescent dye, such as SYBR® green, that can incorporate into the double-stranded DNA (Macfarlane & Macfarlane, 2004). In contrast to traditional PCR, the fluorescence emitted by accumulating PCR product is monitored following each cycle (i.e. real time). The target DNA concentration is proportional to the intensity of the fluorescent signal, thereby quantifying the amount of target bacteria. Absolute quantification requires a standard curve with known amounts of DNA or copy numbers of the target gene of the respective bacterial target strain (Gong & Yang, 2012).

This method has several advantages, such as the high sensitivity and accuracy that allows testing of subdominant populations (Matsuda et al., 2009). However, DNA extraction bias, issues with specificity of primers and the need for a reference strain as well as a target gene sequence are of disadvantage (Sekirov et al., 2010).

**DNA sequencing.** The Sanger method sequences cloned full-length 16S rRNA genes while two to three reads are necessary in order to cover the entire gene. This method maximizes the taxonomic resolution but it is limited due to its high costs and low efficiency (Gong & Yang, 2012, Weinstock, 2012).

In recent years the next generation sequencing (NGS) technologies were developed, which greatly enlarged our knowledge about the diversity of the human gut microbiome. Typically used NGS systems for gut microbiota studies are the 454 platform by Roche that uses pyrosequencing technology and the Illumina platform (HiSeq and MiSeq system) that is based on sequencing by synthesis. The NGS are high throughput technologies that are sensitive and thus allow deep sequencing at relatively low cost. However, the NGS technologies produce shorter sequence reads, meaning that only a portion of the 16S rRNA gene can be sampled (targeting between one and three of the hypervariable regions, like V2, V3 and V6), which leads to lower taxonomic certainty (Claesson et al., 2009, Gong & Yang, 2012, Lagier et al., 2012, Weinstock, 2012). 16S rRNA targeted sequencing is mainly used to compare community structure between individuals or to examine the effects of diet, antibiotic treatment and environmental factors on the gut microbiome (Weinstock, 2012). As example, the study of Dethlefsen et al. (2008) investigated the effect of a second-generation fluoroquinolone antibiotic (ciprofloxacin) on the gut microbiota using pyrosequencing of the V6 and V3 variable regions.
The NGS technologies require extensive bioinformatics analyses that involve quality check of reads, comparisons of sequences with either 16S rRNA databases or gene databases and production of ecological and statistical description plots that describe the community structure (Gong & Yang, 2012, Weinstock, 2012).

The third generation sequencing platforms, like PacBio by Pacific Biosciences or Oxford Nanopore, were recently introduced but are not yet widely used. These technologies provide the advantage of sequencing long reads, thereby reducing the effort needed for bioinformatics analysis (Gong & Yang, 2012).
### Table 1.1: Advantages and limitations of methods used to characterize the gut microbiota. Adapted from Zoetendal et al. (2004), Dethlefsen et al. (2006), Sekirov et al. (2010) and Weinstock (2012).

<table>
<thead>
<tr>
<th>Method</th>
<th>Advantages</th>
<th>Limitations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cultivation</td>
<td>Low cost, phenotypic identification, metabolic information</td>
<td>Labor intensive, underestimation of species diversity as only cultivable bacteria are tested, low throughput</td>
</tr>
<tr>
<td>Hybridization</td>
<td>FISH: Quantitative, spatial distribution of bacteria can be identified, high throughput, no PCR bias</td>
<td>Laborious sample preparation, high detection limit, unable to identify unknown species</td>
</tr>
<tr>
<td></td>
<td>DNA microarray: Quantitative, high throughput, fast, phylogenetic identification</td>
<td>Cross-hybridization, hybridization-bias, unable to identify unknown species, relatively high detection limit</td>
</tr>
<tr>
<td>Fingerprinting</td>
<td>DGGE/TGGE: Fast, comparative analysis, bands of interest can be sequenced</td>
<td>No phylogenetic identification, PCR bias, only relatively small fragments can be separated, not quantitative</td>
</tr>
<tr>
<td></td>
<td>TRFLP: Fast, low cost, comparative analysis</td>
<td>No phylogenetic identification, PCR bias, low taxonomic resolution, not quantitative</td>
</tr>
<tr>
<td></td>
<td>qPCR: High sensitivity, precise quantification, relatively fast</td>
<td>Need for reference strain, unable to identify unknown species, PCR bias</td>
</tr>
<tr>
<td></td>
<td>DNA sequencing: Phylogenetic identification, quantitative, high taxonomic resolution</td>
<td>Laborious, PCR and cloning bias, high cost</td>
</tr>
<tr>
<td></td>
<td>Full length sequencing (Sanger sequencing): Phylogenetic identification, quantitative, high taxonomic resolution</td>
<td>Laborious (bioinformatics), short read lengths</td>
</tr>
<tr>
<td></td>
<td>454 pyrosequencing &amp; Illumina sequencing: Phylogenetic identification, high taxonomic resolution, high throughput, no cloning bias, less PCR bias due to shorter amplicons</td>
<td>Laborious (bioinformatics), short read lengths</td>
</tr>
</tbody>
</table>

DGGE: Denaturing gradient gel electrophoresis; TGGE: Temperature gradient gel electrophoresis; TRFLP: Terminal restriction fragment length polymorphism

### 1.3.2. Techniques for the analysis of functional properties of the gut microbiota

The culture-independent methods targeting the 16S rRNA gene provide a large amount of information about community structure and diversity, however, these technologies do not tell much about the functional characteristics of the gut microbiota. The functional properties are, however, important to understand the contributions of particular microbial groups to the
ecology of the gut microbiota and thereby to host health (Sekirov et al., 2010, Gong & Yang, 2012).

Shotgun metagenomics is a comprehensive approach that sequences the total microbial community DNA and can be used for functional studies of the gut microbiota by comparing the sequences to known functional genes. However, metagenomics only predicts functional properties of communities as messenger RNA (mRNA), proteins or metabolites are not considered by this method (Lozupone et al., 2012). As example, the metagenomics approach was used to investigate how the consumption of a fermented milk product can change the human gut microbiome (Veiga et al., 2014). In addition, genes involved in butyrate synthesis were analyzed in order to assess the effect of the fermented milk product on butyrate producers.

Metatranscriptomics is similar to metagenomics as it is based on high-throughput sequencing of complex microbial populations, however, as the name implies, not DNA but RNA content is isolated and characterized. Thus, this method can distinguish between expressed and nonexpressed genes, thereby providing not only structural but also functional information. Metatranscriptomics can be used to study the effects of environmental factors and diet on the community-wide gene expression and may provide insights into the interactions between the host and the gut microbiota (Sekirov et al., 2010). Gosalbes et al. (2011) used metatranscriptomics to analyze the microbiota of healthy volunteers and found Lachnospiraceae, Ruminococcaceae, Bacteroidaceae, Prevotellaceae and Rikenellaceae as predominate active families and main functional roles were ascribed to carbohydrate metabolism, energy production and synthesis of cellular components.

Metaproteomics and metabolomics are other technologies for functional studies of the gut microbiota. In metaproteomics the extracted microbial proteins are fractionated and separated by gel electrophoresis. Target proteins are then identified using mass spectrometry and de novo sequencing. The main disadvantages of this method are the difficulties in detecting low abundance proteins and in purifying the proteins. Metabolomics relies on the analysis of multiple small metabolites, thereby creating a metabolic profile using analytical methods such as mass spectrometry (Gong & Yang, 2012). It is a sensitive method but due to the complexity of the sampling environment and presence of interfering compounds not all metabolites can be detected.
1.3.3. Gut microbiota diversity

1.3.3.1 Infant microbiota development

The human microbiota is established at birth with facultative anaerobes, such as *Enterobacteriaceae*, streptococci and staphylococci being the initial colonizers. These first colonizers consume the oxygen making the environment more suitable for obligate anaerobes. Over the first few weeks of life a microbial community dominated by anaerobes is established, including *Bifidobacterium* spp. and *Bacteroides* spp. (Nicholson et al., 2012, Nyangale et al., 2012, Power et al., 2014). The mode of delivery affects the establishment of the infant microbiota as the source of colonization differs. For vaginally born infants first inoculation occurs in the birth canal and the main colonization source is the maternal vaginal and intestinal microbiota. Infants born by Cesarean section are exposed to microbes coming directly from the environment (e.g. nursing staff and the air) (Isolauri et al., 2004, Power et al., 2014). Furthermore, feeding can affect the gut microbiota composition with *Bifidobacterium* and *Ruminococcus* dominating in breast-fed infants while the composition of formula-fed infants is more complex and can include *Streptococcus*, *Bacteroides* and *Clostridium* (Power et al., 2014). After weaning the microbiota becomes more diverse and approximately by the age of two an adult-like microbiota is established (Isolauri et al., 2004) (Figure 1.4).

**Figure 1.4:** Shaping of gut microbiota composition and activity during different life-stages. Adapted from Biagi et al. (2011), Duncan & Flint (2013) and Power et al. (2014).
1.3.3.2 Composition of adult microbiota

The diversity of the intestinal microbiota can be described according to its richness (‘who is present’ = numbers of species) and evenness (‘with how many are they present’ = relative abundance of each species) (Gerritsen et al., 2011). Our knowledge about bacterial diversity of the human gut microbiota has greatly increased with the development of molecular techniques, in particular high throughput sequencing methods. Each individual harbors a unique microbiota while most of the phylotypes belong to only five phyla: Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria and Verrucomicrobia (Figure 1.5) (Lozupone et al., 2012, Tremaroli & Backhed, 2012, Chassard & Lacroix, 2013). Arumugam et al. (2011) introduced the concept of a core microbiota that is common between individuals across different countries and that formed three clusters designated as ‘enterotypes’. Each enterotype is characterized by a relatively high representation of Bacteroides, Prevotella or Ruminococcus. However, later studies did not show segregation into enterotypes and it was suggested that due to the inter-individual variability it may not be easy to define a core microbiota which composition can be distinguished by ‘types’ (Lozupone et al., 2012).

In addition to bacterial phyla, the gut also harbors methanogenic archaea, eukaryotes and viruses. Presence of archaea (Methanobrevibacter smithii and Methanobrevibacter stadtmanae) is variable between subjects and it is suggested that prevalence increases with age (Lagier et al., 2012, Lozupone et al., 2012).

The gut microbiota composition of each individual is influenced by diet, host genotype, disease and medications, such as antibiotics (Dethlefsen et al., 2006, Lozupone et al., 2012), yet during adulthood, the major bacterial groups remain relatively stable over time.
1.3.3.3 Gut microbiota in the elderly

A reduction in microbial stability (O’Toole & Claesson, 2010, Tiihonen et al., 2010) and structural changes of the gut microbiota of elderly (usually defined as people over 65 years) were reported (Table 1.2). Culture dependent methods have shown a decrease in total numbers and species diversity of bifidobacteria in elderly (Hopkins et al., 2001, Hopkins & Macfarlane, 2002, Woodmansey et al., 2004). In later studies using FISH or qPCR bifidobacteria levels were similar in adults and elderly (Tiihonen et al., 2008, Mariat et al., 2009). However, bifidobacteria abundance was also decreased in the elderly in the study of Zwielehner et al. (2009) using qPCR. In centenarians the bifidobacteria levels were lower compared to adults (Biagi et al., 2010). Some of the variability in findings regarding the bifidobacteria numbers might be explained by the notable age-related temporal instability in Actinobacteria found by Rajilic-Stojanovic et al. (2009). In many studies aging was associated with an increase in facultative anaerobes, including enterococci, enterobacteria and staphylococci (Woodmansey et al., 2004, Mariat et al., 2009, Rajilic-Stojanovic et al., 2009, Biagi et al., 2010). Increased Lactobacillus/Enterococcus levels were found in the elderly compared to the young in the study of Tiihonen et al. using FISH whereas the Clostridium coccoides-Eubacterium rectale group was significantly decreased in the elderly (Tiihonen et
al., 2008). Conflicting results on the effect of age on the two major phylogenetic groups were reported in various studies. In a large-scale study with 161 elderly Irish subjects it was found that *Bacteroides* was the dominant genus among the microbiota of the elderly and the proportion of the Firmicutes phylum was lower in the elderly compared to the younger adults using pyrosequencing (Claesson et al., 2011). An extreme variability in the microbiota composition of the elderly was noted in the study of Claesson et al. with Bacteroidetes and Firmicutes abundances ranging from 3% to 94%. A decreased Firmicutes:Bacteroidetes ratio in old age compared to young adults was also found in the study of Mariat et al. (2009) using qPCR. However, compared to infants no significant difference was found in the Firmicutes:Bacteroidetes ratio. The effect of age on *Bacteroides* was country specific in the study of Mueller et al. (2006) using FISH as e.g. in Italian subjects *Bacteroides* levels were decreased in the elderly. Using the HIT chip species within Bacteroidetes, including *Alistipes* and several *Bacteroides* species, were significantly less abundant in the elderly compared to adults (Rajilic-Stojanovic et al., 2009). Within the Firmicutes phylum a distinct abundance pattern of *Clostridium* groups was found in the Irish elderly (Claesson et al., 2011) with cluster IV being the predominant *Clostridium* cluster in the elderly compared to cluster XIVa in the younger group. In contrast, *Clostridium* cluster IV abundance and diversity was significantly higher in the young compared to the elderly in the study of Zwielehner et al. (2009) using qPCR and fingerprinting methods.

In summary, studies have demonstrated that the composition of the gut microbiota changes with age. However, a common core in the composition of the elderly microbiota has not been identified and requires further investigations. Physiological changes, diet and need for more medication are clearly factors that affect the gut microbial composition and activity in old age and should be addressed in future studies, similar to the study by Claesson et al. (2012).
<table>
<thead>
<tr>
<th>Subjects and sample size</th>
<th>Main findings</th>
<th>Method</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy elderly (n=35), hospitalized elderly (n=38) and hospitalized elderly receiving antibiotics (n=21); 63-103 yrs</td>
<td>Decreased total bacteria numbers in hospitalized elderly and further decrease in elderly taking antibiotics</td>
<td>qPCR</td>
<td>(Bartosch et al., 2004)</td>
</tr>
<tr>
<td>Healthy adults (20-50 yrs; n=85) and healthy elderly (&gt; 60 yrs; n=145) from five European countries</td>
<td>Increased enterobacteria levels in elderly</td>
<td>FISH</td>
<td>(Mueller et al., 2006)</td>
</tr>
<tr>
<td>Healthy elderly (average age of 71 yrs; n=5) and adults (average age of 33 yrs; n=5), 30 fecal samples collected at 3 time points over a period of 2 months</td>
<td>Abundance of groups within <em>Bacilli</em> increased and within <em>Bacteroidetes</em> decreased in elderly</td>
<td>HITChip</td>
<td>(Rajilic-Stojanovic et al., 2009)</td>
</tr>
<tr>
<td>Hospitalized elderly (86 ± 8 yrs; n=17) and healthy adults (24 ± 2.5 yrs; n=17)</td>
<td>Decreased bifidobacteria and <em>Clostridium</em> cluster IV levels in hospitalized elderly subjects</td>
<td>DGGE, qPCR</td>
<td>(Zwielehner et al., 2009)</td>
</tr>
<tr>
<td>Healthy elderly (70-90 yrs; n=20), healthy adults (25-45 yrs; n=21) and healthy infants (3 weeks to 10 months; n=21)</td>
<td>Higher levels of <em>E. coli</em> and <em>Firmicutes</em>:Bacteroidetes ratio much lower in elderly compared to adults</td>
<td>qPCR</td>
<td>(Mariat et al., 2009)</td>
</tr>
<tr>
<td>Elderly NSAID users (70-85 yrs; n=9) healthy elderly non-users (70-85 yrs; n=9) and healthy adults (21-39 yrs; n=9), 96 fecal samples from three time points 1 week apart</td>
<td>Lower levels of <em>Firmicutes</em> while higher levels of <em>Bacteroidetes</em> in elderly</td>
<td>Cloned-based full-length 16S rRNA sequencing</td>
<td>(Makivuokko et al., 2010)</td>
</tr>
<tr>
<td>Centenarians (99-104 yrs; n=21), elderly offspring of centenarians (59-78 yrs; n=21), elderly (63-76 yrs; n=22), and adults (25-40 yrs; n=20)</td>
<td>Decrease in bifidobacteria and <em>Faecalibacterium prausnitzii</em> levels in centenarians</td>
<td>HITChip and qPCR</td>
<td>(Biagi et al., 2010)</td>
</tr>
<tr>
<td>Hospitalized elderly (&gt;65 yrs; n=161) and young healthy subjects (28-46 yrs; n=9)</td>
<td>Greater abundance of <em>Bacteroides</em> spp. and <em>Clostridium</em> cluster IV in elderly</td>
<td>Pyrosequencing</td>
<td>(Claesson et al., 2011)</td>
</tr>
<tr>
<td>Elderly (77-95 yrs; n=38) and adults (57-67 yrs; n=38)</td>
<td>Lower levels of <em>Bacteroides</em>, <em>Clostridium</em> XIVa group and <em>Faecalibacterium</em> while increased numbers of <em>Lactobacillus</em> in elderly compared to adults</td>
<td>qPCR</td>
<td>(Salazar et al., 2013)</td>
</tr>
</tbody>
</table>

HITChip: Human intestinal tract chip; FISH: Fluorescent in situ hybridization; DGGE: Denaturing gradient gel electrophoresis; NSAID: Non-steroidal anti-inflammatory drugs
1.3.4. Gut microbiota metabolism

1.3.4.1 Carbohydrate fermentation

The gut microbiota has an important metabolic function that includes the extraction of energy of otherwise indigestible food components (e.g., plant cell wall polysaccharides, resistant starch, some oligosaccharides and disaccharides), the degradation of potentially toxic food compounds and the synthesis of certain vitamins and amino acids.

Dietary carbohydrates that reach the colon (around 20-60 g per day) are predominately metabolized by microbial fermentation to short-chain fatty acids (SCFAs) and gases (CO\textsubscript{2} and H\textsubscript{2}) (Flint \textit{et al.}, 2012, den Besten \textit{et al.}, 2013). The proximal colon is the major site of fermentation of complex carbohydrates (Lawley & Walker, 2013). The main SCFA produced are acetate, propionate and butyrate and their molar ratio in the colon and in stool was estimated to be around 60:20:20. SCFA are well absorbed by the colonocytes and total concentrations of SCFA decrease from 70-140 mM in the proximal colon to 20-70 mM in the distal colon and only around 5% of the SCFA are secreted in the feces (den Besten \textit{et al.}, 2013).

Production of SCFA by the microbiota involves several steps and formation of intermediates such as lactate, succinate, ethanol and formate (Blaut, 2013). Products formed by primary degraders of complex carbohydrates can further be metabolized by other bacteria by a process called cross-feeding (Scott \textit{et al.}, 2011) as displayed in Figure 1.6. Acetate is formed by many bacteria as primary fermentation or from H\textsubscript{2} and CO\textsubscript{2} by acetogenic bacteria. Propionate can either be directly formed from carbohydrates or via cross-feeding from succinate and lactate. Formation of butyrate is mainly performed by members of the \textit{Clostridium} clusters XIVa and IV either via the enzyme butyrate kinase or acyl transferase that converts butyryl-CoA to butyrate (Hoyles & Wallace, 2010). The acyl transferase is used in the formation of butyrate from acetate by cross-feeding (Duncan \textit{et al.}, 2002b, Duncan \textit{et al.}, 2004). Butyrate may also be formed from lactate by transforming lactate to pyruvate as it was shown for \textit{Eubacterium hallii} (Belenguer \textit{et al.}, 2006, Belenguer \textit{et al.}, 2007). Accumulation of lactate in the colon is rare since lactate is used as a substrate via cross-feeding. Increased levels of colonic lactate concentrations were linked to intestinal dysbiosis (Hoyles & Wallace, 2010, Scott \textit{et al.}, 2011).

Gases produced by colonic microbes include hydrogen, carbon dioxide, methane and hydrogen sulfide and their concentrations in the colon depend on the activity of H\textsubscript{2}-producing (hydrogenogenic) and H\textsubscript{2}-using (hydrogenotrophic) microbes. Species belonging to
Ruminococcus, Roseburia, Clostridium and Bacteroides are known to produce H₂. Hydrogenothropic microbes can be divided in three groups, the methanogens, the acetogens and the sulphate-reducing bacteria. The methanogens (predominantly Methanobrevibacter smithii and Methanospaera stadtmannae) are archae that metabolize CO₂ with the aid of H₂ or formate to CH₄. Acetogens, predominantly Blautia, use H₂ to produce acetate as described above and sulphate reducing bacteria, such as Desulfovibrio spp., produce H₂S by metabolizing H₂ (Carbonero et al., 2012).

SCFA serve as energy source in various tissues, including in colonocytes that derive 60-70% of their energy supply from SCFA, mainly from butyrate and acetate. Acetate is also taken up by the liver and used as energy source and as substrate e.g. for cholesterol synthesis or as co-substrate for amino acid synthesis. Other tissues that are involved in the metabolism of acetate include the heart, adipose tissue, kidney, and muscle tissue. Remaining butyrate that is not used as energy source by colonocytes is mostly oxidized in the liver, thus preventing toxic systemic concentrations (den Besten et al., 2013). Butyrate production in the colon is of particular interest due to its protective role in colonic diseases (Pryde et al., 2002). Propionate is also metabolized by the liver, however, to which extent propionate serves as energy source in humans is unknown (den Besten et al., 2013).
1.3.4.2 Protein fermentation

Colonic fermentation of proteins, also termed putrefaction, occurs mainly in the distal part of the colon as a result of carbohydrate depletion in the proximal and transverse part. Proteins that reach the colon (around 3-12 g per day) derive from dietary sources that are highly digestible but escape digestion in the small intestine. Moreover, endogenous protein can also be metabolized by colonic bacteria (Blaut, 2013). Protein degradation starts with the hydrolysis of protein into oligopeptides and amino acids that may serve as carbon, nitrogen and energy source (Figure 1.7). Amino acids can then be further metabolized into SCFA, BCFA, ammonia, amines, polyamines, hydrogen sulfide, thiols, phenols, indoles and gases (Nyangale et al., 2012, Blaut, 2013). In vitro studies with human cells have shown that some products of protein fermentation, such as H₂S and NH₃ are potentially toxic (Windey et al., 2012). Proteolytic bacteria include species of Bacteroides and Clostridium (Scott et al., 2011), however, knowledge about bacterial fermentation of proteins and the respective bacterial groups is limited compared to carbohydrates.
1.3.4.3 Metabolism of host-derived substances

Primary bile acids (cholic and chenodeoxycholic acid) are synthesized in the liver from cholesterol and are excreted into the gut lumen to facilitate absorption of dietary fat, fat-soluble vitamins and cholesterol. Primary bile acids are conjugated to glycine or taurine; however, in the distal part of the ileum primary bile salts can also be metabolized by bacteria into secondary metabolites. These bacteria include species of the genera *Bacteroides*, *Clostridium*, *Eubacterium*, *Lactobacillus*, and *Escherichia* (Nicholson et al., 2012, Blaut, 2013). Choline can be obtained from food (red meat and eggs) but can also be synthesized by the host. It is primarily metabolized in the liver; however, bacteria can also convert choline to toxic methylamines (trimethylamine), which is then further metabolized in the liver to trimethylamine-N-oxide. These transformations were shown to trigger non-alcoholic fatty liver disease in mice (Nicholson et al., 2012, Tremaroli & Backhed, 2012).

1.3.4.4 Changes in microbial activity in old age

Changes in the gut microbiota composition in old age may also impact gut microbial activity. Woodmansey *et al.* (2004) and Tiihonen *et al.* (2008) found a decrease in fecal SCFA concentrations in elderly compared to adults. In addition, Tiihonen *et al.* found increased BCFA and lactate concentrations in the elderly. This may indicate a shift from saccharolytic fermentation to putrefactive microbial metabolism in old age (Woodmansey, 2007). Possibly, the reduced consumption of fiber and a slower gastrointestinal transit time lead to a decrease in SCFA concentrations. This may further lead to an increase in luminal pH and thus
decreased absorption of minerals and increased risk of infections. Moreover, toxic compounds formed by proteolytic fermentation may lead to an increased risk of colon cancer (Tiihonen et al., 2010, Duncan & Flint, 2013).

1.3.5. Intestinal dysbiosis

Intestinal homeostasis depends on many complex factors including the composition and diversity of the gut microbiota as well as interactions between the microbiota and the host (Maloy & Powrie, 2011). An imbalance of the normal gut microbiota composition is termed dysbiosis. A number of diseases have been associated with dysbiosis, such as inflammatory bowel disease (IBD) that is suggested to be caused by an abnormal communication between the gut microbial communities and the mucosal immune system (Robles Alonso & Guarner, 2013). One important factor that can cause intestinal dysbiosis is antibiotic treatment that is often accompanied by antibiotic-associated diarrhea (AAD). Antibiotics usually induce short-term changes to the microbiota composition but in some cases the alterations can be long-lasting (Ferreyra et al., 2014). Using TTGE resilience of dominant fecal microbiota upon short-course antibiotic challenge was shown in patients receiving amoxicillin, however, in one of the six patients major modifications were demonstrated even two months after discontinuation of antibiotic treatment (De La Cochaniere et al., 2005). In a recent metatranscriptomics study with elderly subjects it was shown that different antibiotic classes (β-lactam antibiotics and fluoroquinolones) affected the total gut microbiota differently but the effect at the RNA level was similar which may explains why various antibiotics cause the same intestinal diseases (Knecht et al., 2014). In another multi-omics study with one patient undergoing β-lactam therapy it was suggested that antibiotics alter the gut microbial ecology as well as interactions with the host metabolism at a high level (Perez-Cobas et al., 2012). An overall decrease in diversity due to antibiotic treatment was observed in the studies of Dethlefsen et al. (2008) and Dubourg et al. (2014) using pyrosequencing as well as in the study of Perez-Cobas et al. (2012).

A disturbed gut microbiota can result in a loss of colonization resistance, a term that describes the inhibition of colonization of enteropathogens by the gut microbiota (Lawley & Walker, 2013). As example, infection with the enteropathogenic bacterium Clostridium difficile is often associated with broad-spectrum antibiotic treatment, thus endorsing that the effect of the antibiotic treatment on the bacterial community structure and diversity resulted in the loss of colonization resistance and an overgrowth of this pathogen.
2. **Clostridium difficile**

2.1. **Microbiology of C. difficile**

*C. difficile* is an obligate anaerobic, gram-positive and rod-shaped bacterium. It was first isolated in 1935 and received its name due to the difficulties with isolation and culturing that the researchers encountered (Hall & O'Toole, 1935). *C. difficile* is saccharolytic and proteolytic and it can be found in most soils and freshwaters (Noren, 2010, Carroll & Bartlett, 2011). In addition it was recently reported that *C. difficile* 630, a clinical isolate and model strain, is able to grow autotrophically on gases (CO₂ and H₂) as sole energy source (Kopke et al., 2013). *C. difficile* forms spores that are metabolically dormant, survive for long periods and are highly resistant to desiccation, chemicals and extreme temperatures. Spores contribute to the survival in the host and contaminate the environment such as hospitals, where they can persist for months or years (Rupnik et al., 2009, Carroll & Bartlett, 2011). Signals that trigger *C. difficile* sporulation have not been identified so far but they could be related to stress factors, such as nutrient starvations as well as to quorum sensing (Paredes-Sabja et al., 2014).

Typing is important to monitor *C. difficile* infections and epidemic spreads. The most frequently used molecular typing methods are toxino typing based on tcdA/tcdB sequence polymorphisms, PCR ribotyping based on the variability of the intergenic regions separating the 16S and 23S rRNA genes, whole genome restriction pattern polymorphism (pulsed-field typing and restriction endonuclease analysis typing), multi-locus repeat- or non-repeat-based sequence variations (MLST, MLVA), and surface-protein variations (SLP typing) (Carroll & Bartlett, 2011, Vedantam et al., 2012, Knetsch et al., 2013).

2.2. **C. difficile virulence factors**

2.2.1. **Toxins**

Two toxins, toxin A (TcdA) and toxin B (TcdB) are the key virulence factors produced by *C. difficile*. Although toxigenic as well as non-toxigenic strains can colonize their host only toxin-producing strains are associated with disease. TcdA (308 kDa) and TcdB (269 kDa) belong to the family of large clostridial toxins (LCTs), which are monoglycosyltransferases that inactivate Rho family GTPases (Rho, Rac, Ras, Ral and Cdc42). The Rho proteins are responsible for several cellular processes, such as cell-cell adhesion and maintenance of the cytoskeleton. Inactivation of the GTPases occurs through glycosylation and results in
alterations of the actin cytoskeleton, disruption of the barrier function and apoptosis (Carter et al., 2012, Pruitt & Lacy, 2012, Vedantam et al., 2012). TcdA and TcdB are encoded by the genes tcdA and tcdB on the pathogenicity locus (PaLoc, 19.6 kb) which is conserved in toxigenic strains (Figure 1.8). Additional to tcdA and tcdB three more genes are encoded on the PaLoc: tcdR, tcdC and tcdE. The two genes tcdR and tcdC are major positive and negative regulators of toxin production, respectively and tcdE encodes a protein that facilitates the release of the toxins from the cell (Carroll & Bartlett, 2011, Carter et al., 2012).

The structure of the toxins can be divided into two components: an A and B subunit. The A subunit is an N-terminal glucosyltransferase domain (GTD) that inactivates host GTPases by glucosylation. The B subunit is involved in the delivery of the A subunit into the target cell. The mechanism of cellular intoxication can by divided into four steps: (1) binding of toxins to the surface of the cell and internalization by receptor-mediated endocytosis; (2) formation of pores in the endosome triggered by low pH that then leads to translocation of GTD; (3) autoproteolysis of the GTD leading to the release of this domain into the cytosol; (4) glucosylation of the Rho family GTPases by the GTD that leads to the loss of structural integrity of the target cell (Pruitt & Lacy, 2012).

The toxins induce cell rounding in many cell types, such as Vero (African green monkey kidney) cells while TcdB is 100 – 10’000 times more potent than TcdA (Pruitt & Lacy, 2012). In animal models it was shown that TcdA causes fluid accumulation and inflammation in the intestinal tract while TcdB caused no or only minimal intestinal pathology. Consequently, TcdA was referred to as enterotoxin and TcdB as cytotoxin that is only effective after TcdA has caused prior tissue damage (Pruitt & Lacy, 2012). More recently, however, it was demonstrated that TcdA^-TcdB^+ strains are pathogenic in animals and the importance of toxin B in disease was re-established (Lyras et al., 2009, Kuehne et al., 2010). Further, all natural occurring strains that cause disease produce TcdB. However, naturally
occurring TcdA\(^+\) TcdB\(^-\) have not been reported yet (Vedantam et al., 2012). Potentially a third toxin, the binary toxin or \textit{C. difficile} transferase (CDT) also contributes to the virulence of \textit{C. difficile} (Young & Hanna, 2014). The binary toxin is produced by some strains, including hypervirulent strains and acts by ADP-ribosylating actin leading to disruption or rearrangement of the host cell cytoskeleton. It is encoded on the \textit{C. difficile} chromosome on a region called CdtLoc by two genes cdtA and cdtB together with a positive regulator of the binary toxin, the cdtR gene. The role of CDT in disease remains unclear, but recent studies suggest that CDT could be an important colonization factor as it may enhance bacterial attachment to host cells (Carter et al., 2012, Vedantam et al., 2012).

\textit{In vitro} toxin production is usually detected at the end of the exponential growth phase and maximal yields are obtained after four to five days of fermentation. How environmental factors affect toxin production remains largely unknown, however, there are indications that toxin production is enhanced by stress, such as sub-inhibitory levels of antibiotics. It was also shown that toxin production was reduced when glucose or other rapidly metabolizable sugars were available in the medium (Dupuy & Sonenshein, 1998).

\subsection*{2.2.2. Other virulence factors}

Apart from toxins \textit{C. difficile} also has other virulence factors important in disease (Vedantam et al., 2012):

\textbf{Fibronectin-binding proteins.} The \textit{C. difficile} genome encodes for several proteins with putative and demonstrated abilities to bind host extracellular matrix components. How these fibronectin-binding proteins are involved in colonization still requires further studies (Vedantam et al., 2012).

\textbf{Surface layer proteins (SLPs).} \textit{C. difficile} possesses a cell surface layer (S-layer) that is composed of many proteins arranged in a crystalline lattice. SLPs bind \textit{in vitro} to Vero cells and human epithelial cell lines and induce inflammation and antibody responses in the host. Best known is the SlpA which has been shown to be the major contributor to \textit{C. difficile} attachment \textit{in vitro}. The SLPs belong to a larger class of proteins known as cell-wall proteins (CWPs), including the two cysteine proteases Cwp84 and Cwp13 that have been shown to be involved in the assembly of the S-layer (Madan & Petri Jr, 2012, Vedantam et al., 2012).

\textbf{Flagella.} Flagella expression by \textit{C. difficile} enhances motility in mucus-rich environments such as the gut. However, it is still unknown how the flagella contribute to disease since in
hamster models it was shown that flagella are unnecessary for virulence (Vedantam et al., 2012).

**Cell-surface polysaccharides.** Two cell-surface polysaccharides, PS-I and PS-II, were found in *C. difficile* strains. PS-I was only found in the ribotype 027, while PS-II was found in all strains tested so far. Both polysaccharides serve as potential vaccines against *C. difficile*. Peptidoglycan is another polysaccharide found in *C. difficile* and possibly important for virulence since it was shown in other pathogenic bacteria that variations in peptidoglycan structure can provide resistance to β-lactam antibiotics and lysozyme (Vedantam et al., 2012).

### 2.3. *C. difficile* infection

*C. difficile* is abundant in newborns and it was believed that this bacterium is part of the normal intestinal microbiota until in 1978 first associations between *C. difficile* and pseudomembranous colitis, an inflammation of the colon usually related to antibiotic usage, were made (Bartlett et al., 1978). Since 2001, *C. difficile* is emerging as the leading cause of nosocomial and antibiotic-associated diarrhea and due to high and severe incidences has become one of the most important health-care associated pathogens worldwide (Zucca et al., 2013, Zanella Terrier et al., 2014).

#### 2.3.1. Epidemiology

Broad-spectrum antibiotics are the main risk factor for CDI with recent antibiotic usage identified in over 90% of hospitalized patients (Dubberke et al., 2011). Another important risk factor is hospitalization or contact with the healthcare system, especially with *C. difficile* case-associated areas. However, a significant amount of cases (estimated around 28%) are community-acquired with particular high numbers in long term care facilities where there are many elderly (Heinlen & Ballard, 2010, Oldfield et al., 2014). In general, advanced age (>65 years) is another important risk factor for CDI. Sharp increases in incidences of around 10-fold have been shown for this age group and 90% of all deaths due to CDI are accounted for the elderly (Carroll & Bartlett, 2011, Oldfield et al., 2014). Other factors associated with CDI are treatment with proton-pump inhibitors, H₂ antagonists, antineoplastic agents and presence of other gastrointestinal diseases such as IBD (Deneve et al., 2009, Heinlen & Ballard, 2010, Oldfield et al., 2014).

Increases in incidences and severity of CDI have been reported in North America and Europe since 2000. A five-fold increase in incidences in the whole populations and an eight-fold
increase in the elderly have been reported in North America (Deneve et al., 2009). In 2008, the surveillance system of the European Center for Disease Control estimated a mean incidence of 5.5 CDI cases per 10’000 patients (Carroll & Bartlett, 2011). C. difficile strains that are associated with severe and epidemic diseases in North America and Europe belong to the PCR ribotypes 001, 017, 027, 078 and 106 (Vedantam et al., 2012). The rising incidences have been especially attributed to ribotype 027 (restriction endonuclease type B1 and pulsed field type NAP1), although in Europe ribotype 027 accounted for only 5% of 89 typed strains according to the 2008 European surveillance system (Rupnik et al., 2009, Carroll & Bartlett, 2011).

2.3.2. Pathogenesis

C. difficile spores are usually transmitted between hospitalized patients or environmental contamination via the fecal-oral route (Figure 1.9). Following ingestion and survival of the gastric acid barrier, spores germinate in the small intestine and the vegetative form proliferates in the large intestine if conditions are preferably for C. difficile which is often the case in immunocompromised or antibiotic treated individuals (Deneve et al., 2009, Vedantam et al., 2012). Spore germination can be induced by small molecule germinants, primarily bile salts (Paredes-Sabja et al., 2014). The vegetative C. difficile cells start colonizing the host mucosal surface by the help of virulence factors. Once the C. difficile cells adhere to the enterocytes toxin production begins that result in clinical manifestations (Deneve et al., 2009, Vedantam et al., 2012). Typical is diarrhea and abdominal cramps that are accompanied by fever, abdominal pain, nausea, vomiting and dehydration. In addition, elevated white blood cell count and neutrophilic predominance are observed. In severe cases patients develop fulminant colitis that can lead to toxic megacolon (Dubberke et al., 2011).
2.3.3. Diagnosis

Diagnosis of *C. difficile* is based on demonstration of either toxigenic strains or toxins themselves in diarrheal stool. However, there is controversy about the state of the art technique for diagnosis of CDI (Ananthakrishnan, 2011, Oldfield et al., 2014). For many years the cell-culture cytotoxicity assay used to be the gold standard. This test is performed by inoculation of a monolayer of a particular cell line (e.g. Vero cells) with a stool filtrate and examined for toxin-induced cytopathic effect (rounding of cells). When a cytopathic effect is observed, confirmation by neutralization with antitoxins of either *C. sordelli* or *C. difficile* is performed. However, the test is expensive, results are delayed and it is technically complex (Bartlett, 2010, Oldfield et al., 2014). More recently, toxigenic culturing was considered as the method of choice. Toxigenic culture requires inoculation of selective media for *C. difficile* (cycloserine, cefoxitin, fructose agar or CCFA) with stool specimen. As toxigenic and non-toxigenic strains are recovered another test is needed for analysis of toxin production (Bartlett, 2010, Carroll & Bartlett, 2011, Oldfield et al., 2014). Enzyme immunoassays for toxin A/B are currently widely used in clinical laboratories as fast, inexpensive and technically convenient but low sensitivity tests. Another enzyme immunoassay test is available that is directed against glutamine dehydrogenase, an enzyme almost exclusively produced by *C. difficile*. The glutamine dehydrogenase test is more sensitive than the
immunoassay for toxins. However, this test has to be combined with an additional analysis for toxin production. PCR for detection of the toxin B gene is a promising stand-alone test that is fast and sensitive. However, this test shares the same problematic with toxigenic culturing which is that only the toxin gene is tested but not actual toxin presence in the stool (Bartlett, 2010, Oldfield et al., 2014). Another promising method for CDI diagnosis is the non-PCR based gene amplification method that detects the pathogenicity locus of toxigenic *C. difficile* strains by loop-mediated isothermal amplification. As for the PCR test presence of toxin in stool is not detected (Oldfield et al., 2014). It is usually recommended to use a testing algorithm for detection of toxin producing *C. difficile* in stool. A 3-step algorithm can consist of an initial glutamate dehydrogenase test followed by an enzyme immunoassay for toxin A/B if the first test is positive. If both tests are positive the patient is positive for CDI. If the enzyme immunoassay for toxin A/B is negative the result will be confirmed with a PCR. Depending on the laboratory and national standards different testing algorithms are applied (Carroll & Bartlett, 2011, Oldfield et al., 2014).

2.4. *C. difficile* and the gut microbiota

It is suggested that alterations of the composition or diversity of the gut microbiota and thus the breakdown of colonization resistance are essential for development of CDI. A healthy and functional gut microbiota usually protects against colonization by pathogens. Possible mechanisms in colonization resistance include competitive metabolic interactions, niche exclusion and induction of host immune responses (Kamada et al., 2013). Asymptomatic carriage of *C. difficile* is common and accounts for around 7 to 20% among hospitalized patients (Dubberke et al., 2011, Zanella Terrier et al., 2014). Interestingly 25 to 80% of infants harbor *C. difficile* probably due to the low capacity of the infant gut microbiota to suppress growth of *C. difficile* (Adlerberth et al., 2014). Although a significant amount of *C. difficile* strains found in infants are toxin producers they rarely develop disease. Possible reasons are the lack of toxin receptors necessary for disease development, the poorly developed cellular signaling pathways in the gut mucosa or protective factors in the infant gut or from the human breast milk (Adlerberth et al., 2014, Seekatz & Young, 2014).

The gut microbiota may take an important part in each step of *C. difficile* pathogenesis, including the germination of spores, colonization in the gut and disease development through toxin production (Seekatz & Young, 2014).
2.4.1. CDI development and the metabolic environment of the gut

The first step in pathogenesis of \textit{C. difficile} is the ingestion of spores and germination into vegetative cells that can colonize the gastrointestinal tract. However, for germination to occur favorable gastrointestinal conditions are required (Seekatz & Young, 2014). One such condition is the ratio of primary to secondary bile acids in the gut. Bile acids are secreted by humans into the small intestine during digestion. Primary bile acids (cholic acid and chenodeoxycholic acid), which are conjugated to an amino acid (glycine or taurine) as well as glycine itself were shown to stimulate germination of \textit{C. difficile} spores \textit{in vitro}. Secondary bile salts (deoxycholate and lithocholate), on the other hand, were shown to inhibit spore germination (Sorg & Sonenshein, 2008). 400 to 800 mg of bile passes daily from the ileum into the cecum where it becomes available for biotransformation by bacteria. Many different species of bacteria are able to transform primary bile acids into secondary bile acids via the enzyme 7-dehydroxylase. Hence, the gut microbiota plays an important role in the bile acid composition in the intestine and alterations in the microbial communities can also impact metabolite availability (Sorg & Sonenshein, 2008, Britton & Young, 2012).

A connection between antibiotic treatment and changed bile acid availability, thereby enhancing \textit{C. difficile} spore germination was suggested in animal studies (Giel et al., 2010, Miezeiewski et al., 2014). In a clinical study high concentrations of primary bile acids in patients suffering from recurrent CDI were demonstrated while after fecal microbiota transplantation (FMT) therapy secondary bile acids were again increased with similar concentrations to that of the healthy donors (Weingarden et al., 2014).

The metabolism of the gut microbiota may also impact \textit{C. difficile} toxin production as it was shown that toxin production is induced when nutrients are limited. In particular for proline, cysteine, butanol and glucose suppressed toxin production was observed. On the other hand, butyrate supplementation and media limited in biotin induced toxin production (Theriot & Young, 2014).

2.4.2. Antibiotic use and CDI development

\textit{C. difficile}-associated diarrhea (CDAD) accounts for around 10 – 25% of all antibiotic-associated diarrhea (AAD) cases and it can occur up to eight weeks after antibiotic therapy (Lenoir-Wijnkoop et al., 2014, Power et al., 2014). Although not all antibiotics have the same
risk of predisposing CDI it was shown that cumulative antibiotic exposure over time increases the risk of developing CDI. It is thus suggested that the total ‘collateral damage’ due to the alteration of the intestinal microbiota is the main cause of decreasing colonization resistance in CDI (Britton & Young, 2012). The effect of antibiotics on the gut microbiota and subsequent development of CDI was studied in several murine models. Lawley et al. (2009) observed that *C. difficile* asymptomatically colonized intestines of immunocompetent mice but when the mice were treated with clindamycin a contagious supershedder state was triggered. The gut microbiota composition of the treated mice was reduced in overall microbial diversity and dominated by enterococci and Proteobacteria. A reduction in intestinal microbial diversity in mice upon clindamycin treatment was also observed by Buffie et al. (2012) with a concomitant shift towards phylotypes of *Enterobacteriaceae, Akkermansia*, and *Mollicutes*. Mice remained susceptible to *C. difficile* colonization for at least 10 days after clindamycin treatment. Mice treated with tigecycline were also susceptible to *C. difficile* challenge and changes in microbiota structure due to antibiotic treatment included large decreases and increases in Bacteroidetes and Proteobacteria levels, respectively (Bassis et al., 2014).

The mechanisms by which antibiotic administration leads to CDI are not entirely clear and the relative risk is not yet predictable from the characteristic effect that specific antibiotics have on the gut microbiota structure (Britton & Young, 2012).

### 2.4.3. Colonization resistance against *C. difficile*

Niche exclusion could be a mechanism in the gut microbiota resistance against *C. difficile* as it was shown that pretreatment of hamsters with non-toxigenic *C. difficile* strains was protective against challenge with toxigenic *C. difficile* strains possibly due to competition for a limiting carbon source (Britton & Young, 2012). Another mechanism could be the production of bacteriocins by the gut microbiota with activity against *C. difficile*. Thuricin CD, a bacteriocin produced by a *Bacillus thuringensis* strain, was as effective against *C. difficile* as metronidazole tested in an *in vitro* colonic fermentation model (Rea et al., 2011b).

The identification of specific members of the gut microbiota that contribute to protection from CDI is difficult and there are only few studies that analyzed the effect of CDI or asymptomatic colonization on the human gut microbiota and even less studies with samples prior to CDI development (Seekatz & Young, 2014, Theriot & Young, 2014).

In three studies the changes in the gut microbiota composition in response to *C. difficile* was investigated in elderly subjects (Hopkins & Macfarlane, 2002, Manges et al., 2010, Rea et
Using cellular fatty acid profiles, Hopkins and Macfarlane (2002) observed compositional changes in the gut microbiota of elderly subjects with CDAD compared to healthy elderly. The ratio of anaerobic:facultative anaerobic species isolated per subject was lower in CDAD patients compared to the healthy elderly group. Further, reduced numbers of *Bacteroides*, *Prevotella* and bifidobacteria were found in the CDAD group. Using a microarray Manges et al. (2010) found that probe intensities were higher for Firmicutes and lower for Bacteroidetes in patients with CDAD compared to the control subjects. In a study with elderly Irish subjects the microbiota between subjects that were culture negative for *C. difficile*, asymptomatic carriers of *C. difficile* and subjects that developed CDAD was compared using pyrosequencing. Whereas there was no dramatic difference between culture negative and asymptomatic carrier samples a reduced microbial diversity was demonstrated in subjects that developed CDAD (Rea et al., 2011a). In another study 39 subject with CDI, 36 subject with *C. difficile* negative nosocomial diarrhea and 40 healthy control subjects were compared using pyrosequencing (Antharam et al., 2013). In the CDI and *C. difficile* negative diarrhea groups a marked decrease in diversity and species richness was measured in particular of phylotypes within the Firmicutes phylum. Depletion was also observed for the families *Ruminococcaceae* and *Lachnospiraceae*. Increased abundances of *Enterococcus*, *Veillonella* and *Lactobacillus* as well as members from the *Gammaproteobacteria* class were found in the CDI group but not in the *C. difficile* negative diarrhea group. Reductions in the family of *Clostridiales Incertae Sedis* XI was associated with an increased risk of nosocomial CDI in a study with 50 subjects analyzed by pyrosequencing (Vincent et al., 2013). In another study it was reported that antibiotic treated mice susceptible to CDI were dominant in Proteobacteria whereas in resistant mice return of Firmicutes, in particular of the family *Lachnospiraceae* was observed (Reeves et al., 2011). The same group demonstrated that a *Lachnospiraceae* murine isolate was protective against CDI development as researchers found that pre-colonizing germfree mice with the *Lachnospiraceae* isolate significantly decreased *C. difficile* colonization and disease development compared to pre-colonization with an *E. coli* murine isolate (Reeves et al., 2012). In a recent study the changes in metabolically active species (16S rRNA amplicons generated from cDNA) was analyzed in relation to CDAD (Knecht et al., 2014). However, no significant differences were found in the metabolically active microbiota associated with CDI.

The studies mentioned above show that up to now no specific compositional profile has been associated with decreased colonization resistance against *C. difficile*. The inherent inter-
individual variability of the human microbiota and the lack of prospective human samples before CDI complicate the search of specific community members (Seekatz & Young, 2014, Theriot & Young, 2014). Some of the specific alterations of the gut microbiota related to CDI were common between the studies, such as a general decrease in bacterial diversity, decrease in members of the Bacteroidetes phylum and an increase in members of the Proteobacteria phylum.

2.4.3.1 The gut microbiota in CDI recurrence

There are only few studies that investigated changes in gut microbiota composition that could lead to recurrent CDI. In one study with 10 elderly individuals it was shown that the diversity of the microbiota was reduced in patients with recurrent CDI compared to patients that suffered only from a single episode of CDI (Chang et al., 2008). Most of the knowledge about the microbiota composition during recurrent CDI comes from studies investigating the efficacy of FMT therapy. FMT profoundly changes the fecal microbiota of recipients but long-term changes of the microbiota seem to be recipient–specific (Seekatz & Young, 2014). However, in one recent study it was also suggested that the impact of donor’s microbiota on the long-term composition of the recipient is donor-dependent (Fuentes et al., 2014).

In general an increase in microbial diversity was reported for fecal samples following FMT and members of the Firmicutes and Bacteroidetes phyla recovered while levels of Proteobacteria were decreased (Hamilton et al., 2013, van Nood et al., 2013, Fuentes et al., 2014). In the study by Fuentes et al. (2014) species were identified that may trigger the development of CDI. Species within the Bacteroidetes and Firmicutes, in particular members of the Clostridium clusters IV and XIVa, as well as butyrate-producing species related to Megasphaera elsdenii (Clostridium cluster IX) were higher in non-CDI patients compared to CDI patients.

2.4.4. Other risk factors of CDI that cause disruption of the gut microbiota

Many risk factors associated with CDI can cause a disrupted gut microbiota. One such risk factor is old age that was associated with changes in the gut microbiota composition as well in a general degradation of the immune system. Compositional changes of the gut microbiota associated with old age were presented before (1.3.3.3). The use of proton pump inhibitors, another risk factor of CDI, is increasing the pH of the stomach and could thus also affect the
microbiota in the intestine. Other gastrointestinal diseases can also increase the risk of CDI, such as IBD which was associated with a modulated intestinal microbial composition (Berg et al., 2013).

### 2.5. Host immune response to CDI

In addition to the colonization resistance of the gut microbiota against *C. difficile* there are other factors that contribute to the protection from CDI or influence the severity of the disease, such as the immune response.

#### 2.5.1. Innate immune response

The secretion of antimicrobial peptides, including defensins and cathelicidins, by the intestinal epithelial cells reduce tissue damage and inflammation caused by *C. difficile* toxins A and B and inflammatory cytokine production. Another host defense mechanism that was recently reported is the S-nitrosylation that inhibits toxin cleavage and cell entry (Solomon, 2013, Sun & Hirota, 2015). Once the intestinal barrier is crossed immune cells within the mucosa are exposed to TcdA and TcdB that trigger inflammation and tissue damage. At this step the host immune response has an important role in protection against symptomatic disease. Intoxicated epithelial cells activate the release of proinflammatory mediators, including interleukin 8 (IL-8) and macrophage inflammatory protein 2 (Solomon, 2013). *C. difficile* toxins also activate the TLR signaling cascade, including TLR4 and TLR5 dependent signaling pathways. It was reported that TLR5 activation by flagellin (from *Salmonella*) was sufficient to prevent CDI after antibiotic treatment thus suggesting that the TLR signaling may be important in host protection against disease (Britton & Young, 2012, Madan & Petri Jr, 2012). The release of cytokine and chemokine mediators is followed by an intense neutrophil infiltration. The neutrophils play a major role in disease pathogenesis and in severe cases the neutrophils can move into the lumen and contribute to the formation of pseudomembranes as observed in pseudomembranous colitis (Madan & Petri Jr, 2012, Solomon, 2013).

#### 2.5.2. Adaptive immune response

It is estimated that approx. 60% of healthy adults and older children have detectable serum IgG and IgA antibodies to *C. difficile* toxins. In mice and hamsters it was shown that passive immunization with these antibodies to *C. difficile* toxins can be protective against disease (Madan & Petri Jr, 2012, Solomon, 2013). IgA is important for mucosal immunity and low
levels of fecal IgA was associated with prolonged CDI symptoms and recurrence of disease. Further, a high IgG response to *C. difficile* toxins and surface proteins during colonization was associated with protection against CDI development (Solomon, 2013).

### 2.6. Treatment of CDI

The initial step in treating *C. difficile* is, if possible, the cessation of current antibiotic treatment or to change the treatment to an antibiotic with a narrower target spectrum (Noren, 2010, Ananthakrishnan, 2011).

#### 2.6.1. Antibiotic treatment

**First episode CDI.** The drug of choice for CDI remains controversial. According to the guidelines of clinical infectious diseases (CID) and of the infectious diseases society of America (IDSA) it is recommended to treat mild to moderate CDI with 500 mg orally metronidazole three times daily and to treat severe cases with 125 mg orally vancomycin four times daily (Bartlett, 2010, Oldfield *et al.*, 2014). In severe and complicated CDI cases, such as ileus or toxic megacolon, a combination of vancomycin at a dose of 500 mg four times daily (nasogastric tube if necessary) and intravenous metronidazole 500 – 750 mg three times daily is recommended (Bartlett, 2010, Chaparro-Rojas & Mullane, 2013, Oldfield *et al.*, 2014). Metronidazole is less expensive than vancomycin and does not involve the risk of development of vancomycin resistant enterococci (Bartlett, 2010, Chaparro-Rojas & Mullane, 2013). The response rate to metronidazole and vancomycin were shown to be similar in mild to moderate diarrhea but increased for vancomycin in severe CDI (Chaparro-Rojas & Mullane, 2013, Oldfield *et al.*, 2014). Vancomycin is also superior in terms of pharmacology as high intraluminal concentrations and low levels of systemic absorption are reported for orally given vancomycin. Metronidazole, on the other hand has a high absorption rate with only around 14% excreted in stool. Although metronidazole levels are rather low, it was shown *in vitro* that metronidazole is very rapidly bactericidal at 8-times the minimum inhibitory concentration (Oldfield *et al.*, 2014).

Other more novel antibiotics investigated for CDI are fidaxomicin, nitazoxanide, rifaximin, teicoplanin and tigecycline. Except for fidaxomicin none of the antibiotics listed are approved for CDI treatment. Fidaxomicin is taken orally and was shown to be similarly effective as vancomycin but has less disturbing effect on the gut microbiota due to a narrower spectrum of activity. However, its main disadvantage is the high cost (Zucca *et al.*, 2013).
**Recurrent CDI.** A major issue with antibiotic treatment of CDI is the high recurrence rate after an initial treatment since antibiotics do not eradicate *C. difficile* spores and can reduce colonization resistance by killing commensal bacteria (Greathouse *et al.*, 2015). Around 20-30% of the patients will experience a first recurrence of CDI and after each incidence, the chance for recurrence increases to up to 65%. Risk factors associated with recurrent CDI are antibiotics, old age, antacid use and the presence of comorbidities (Ananthakrishnan, 2011, Oldfield *et al.*, 2014). Initial treatment of mild recurrent CDI is analogous to first episode CDI. Patients with severe recurrent CDI may benefit from a high dose of vancomycin (500 mg four times daily for two weeks) or tapered dosing of vancomycin over longer periods of up to six weeks. More recently, rifaxomicin is used in some cases after initial vancomycin treatment, as ‘chaser’ antibiotic (Ananthakrishnan, 2011, Oldfield *et al.*, 2014).

### 2.6.2. Alternative treatments

In small series and case reports good results have been obtained for therapy with intravenous immunoglobulin targeting toxin A and B and could be prescribed as adjunctive therapy with vancomycin in recurrent CDI. However, more studies are needed for proof of efficacy of immunoglobulin treatment (Zucca *et al.*, 2013, Oldfield *et al.*, 2014). Toxin-binding agents (or anion binding resins) have also been used to treat CDI but in *in vitro* human intestinal models and in Phase III clinical trials only poor efficacy was obtained (Baines *et al.*, 2009a, Zucca *et al.*, 2013) and concerns have raised as these agents also bind to vancomycin (Oldfield *et al.*, 2014).

The use of indigenous intestinal microbiota from a healthy donor for restoration of the intestinal microbiota of the patient suffering from recurrent CDI is sometimes performed in severe cases. Donor specimen delivery is performed by nasogastric tube, colonoscope or enema. FMT is a promising treatment for recurrent CDI with reported success rates in 92% of patients from overall 317 patients across 27 cases (Gough *et al.*, 2011), indicating that re-enforcing the gut microbiota barrier is a better approach to treat CDI compared to killing bacteria with antibiotics. The major drawbacks of FMT are the unappealing nature it is associated with for most patients, the lack of consensus protocol for FMT procedure and the lack of ideal source of the donor samples. Usually, the donor feces derive from spouses, intimate partners or close family members. According to the American Food and Drug Administration (FDA) FMT treatment requires an informed consent of the patient including the statement that “the use of FMT products to treat *C. difficile* is investigational and a discussion of its potential risks” (Oldfield *et al.*, 2014).
3. **Probiotics**

The term ‘probiotic’ derives from the Greek and means ‘for life’. It was first introduced by Lilly and Stillwell (1965) although scientific suggestions that certain bacteria might benefit the host were already noted before including in the work of Metchnikoff (1907). The nowadays commonly accepted definition of probiotics is: ‘live microorganisms which when administered in adequate amounts confer a health benefit on the host’ (FAO/WHO, 2002).

Probiotics may be used by healthy subjects to maintain a healthy state and decrease the risk of illness or as a therapeutic to treat particular diseases. This difference is important since depending on the use in healthy or ill people the probiotic product will either be applied to food and supplements or is confined to drugs (Sanders et al., 2013). The minimum requirements needed to call a microbial strain ‘probiotic’ were generated by the joint FAO/WHO working group and include: (1) the assessment of strain identity using phenotypic and genotypic techniques (2) *in vitro* tests demonstrating functional characteristics including resistance to gastric acidity, bile salts and digestive enzymes, immune stimulation, suppression of enteropathogenic colonization, etc. (3) assessment of safety and (4) *in vivo* studies that demonstrate health effects in the target host (FAO/WHO, 2002, Huys et al., 2013). The use of health claims on probiotic products in the European Union are only allowed after a scientific assessment has been carried out by the by the Panel on Dietetic Products, Nutrition, and Allergies (NDA) of the European Food Safety Authority (EFSA) (Huys et al., 2013). So far no specific health claim for a probiotic has been approved by the EFSA (Sanders et al., 2013).

Probiotics can be found in a range of products, including yoghurts, drinks, capsules and dietary supplements (O'Toole & Cooney, 2008) with varying cell concentrations whereas the minimum required therapeutic dose for each strain is mostly not specified. Microorganisms used as probiotics are often members of the genera *Lactobacillus* and *Bifidobacterium* and less frequently *Streptococcus, Enterococcus, Bacillus* and *Propionibacterium* species. Gram-negative bacteria, such as *Enterococcus faecium* and *E. coli* (*E. coli* Nissle 1971), and the yeast *Saccharomyces boulardii* are other well-known probiotics (Gerritsen et al., 2011).

Many microorganisms that are considered probiotic have been traditionally used in the food industry, including strains belonging to lactic acid bacteria and bifidobacteria and are often referred as GRAS (Generally Recognized As Safe) due to their long history of safe use. In 2007, the EFSA released the concept of qualified presumption of safety (QPS) that, similar to the GRAS status, lists microorganisms that can be considered as safe (Huys et al., 2013).
Combinations of probiotic strains or species, multistrain or multispecies probiotics, have gained interest since there may be an additive or synergistic effect. However, studies comparing efficacy of probiotic mixtures and single-strain probiotics are limited and thus opposite effects between the probiotic strains could also be possible (Gerritsen et al., 2011).

The effect of probiotics has been studied in a number of gastrointestinal disorders, including IBD, constipation and diarrhea as well as for colon cancer, allergic diseases, obesity and metabolic disorders (Gerritsen et al., 2011). In particular for the prevention or treatment of AAD robust clinical data can be found on the efficacy of probiotics as suggested in several reviews and meta-analyses (Allen et al., 2010, Avadhani & Miley, 2011, Hempel et al., 2012).

It has to be emphasized that probiotic properties are strain specific and that there is no single mechanisms of action of probiotics, thus the efficacy of one strain cannot be extrapolated to other probiotics.

### 3.1. Mechanisms of action of probiotics

A number of mechanism of action of probiotics have been described, including direct interactions with bacteria in the gut lumen, improvement of the epithelial barrier function and the enhancement of the immune response (Gerritsen et al., 2011).

**Stimulation of host immune response:** Consumption of probiotics can result in an innate and/or adaptive immune response, including interactions with the intestinal epithelial cells and dendritic cells as well as with macrophages and lymphocytes (Howarth & Wang, 2013). One mechanism of probiotic effects on immunity is via the production of IgA. As example, up-regulation of specific anti-toxin A secretory IgA expression in animal models of CDAD was shown for *S. boulardii* (Qamar et al., 2001). In general, *S. boulardii* has the so far best described immunomodulatory actions with respect to CDI (Pothoulakis, 2009). *Bacillus coagulans* GBI-30, 6086 attenuated chemokine release that led to reduced neutrophil influx and colonic inflammation in *C. difficile*-induced colitis in mice (Fitzpatrick et al., 2011, Fitzpatrick et al., 2012). Two *Lactobacillus* isolates from infant feces suppressed IL-8 production in *C. difficile*-stimulated colonic epithelial cells by inhibiting activation of transcription factors for IL-8 gene expression. The decrease in release of IL-8 may decrease the influx of neutrophils into the colonic mucosa (Boonma et al., 2014).

**Competitive exclusion, stimulation of commensal microbiota and strengthening of the epithelial barrier:** Probiotics may also act by suppressing the colonization of the lumen by
pathogens. This suppression can be mediated by the production of acids, including SCFA that lower luminal pH and thus inhibit growth of pathogens or by depletion of essential nutrients (Parkes et al., 2009, Hell et al., 2013). Hindrance of bacterial adhesion to mucosal surfaces and invasion of epithelial cells is another important mechanism of action of probiotics. \textit{In vitro} it was demonstrated for many probiotic strains that they have the ability to adhere to human epithelial cell lines and thereby also inhibit adhesion of pathogenic strains such as \textit{Salmonella typhimurium} and \textit{E. coli} (Parkes et al., 2009). The probiotic mixture VSL#3 (consisting of nine strains of bifidobacteria, lactobacilli and \textit{Streptococcus thermophilus}) was shown to improve the epithelial barrier by up-regulating the MUC2 gene and thus inducing colonic mucin production (Parkes et al., 2009). The same multispecies probiotic was also shown to attenuate intestinal permeability by regulating tight junction proteins (Howarth & Wang, 2013). Probiotics may enhance colonization resistance of the gut microbiota by restoring or maintaining the gut microbiota composition associated with a healthy status. This is of particular interest during antibiotic therapy that can result in dysbiosis of the gut microbiota but also in the elderly as aging is associated with an altered gut microbiota composition that may affect host health (Reid et al., 2011, Rea et al., 2013). However, probiotic bacteria are often transiently passing through the gastrointestinal tract and thus the microbiota will likely turn to the previous state once the probiotic therapy is stopped (O’Toole & Cooney, 2008, Howarth & Wang, 2013).

\textbf{Secretion of antimicrobial substances and acids:} The production of antimicrobials by probiotics, such as proteinaceous antimicrobial substances, known as bacteriocins, hydrogen peroxide, organic acids and conjugated bile acids is another potential mechanism by which bacteria can directly fight other bacteria and thus preventing colonization of pathogens (Rea et al., 2013).

\textbf{Inhibition of toxin production:} Toxins are important bacterial virulence factors. In particular in diarrhea one important mode of action of probiotics is the protection of the host against toxins (Oelschlaeger, 2010). In rats it was demonstrated that the yeast \textit{Saccharomyces boulardii} is effective against \textit{C. difficile} by reducing binding of toxin A to the intestinal brush border, intestinal fluid secretion and intestinal permeability (Pothoulakis et al., 1993). In rat and human colonic cells it was then shown that \textit{S. boulardii} produces a protease that hydrolyses \textit{C. difficile} toxins A and B and thus reduces enterotoxic and cytotoxic effects of \textit{C. difficile} (Castagliuolo et al., 1996, Castagliuolo et al., 1999).
Many potential mechanisms of action have been ascribed to probiotics; however, most of the studies were performed in vitro or by using animal models and these studies do not necessarily correspond to efficacy in humans. Probiotic studies in humans usually assess clinical outcome only and in few studies the effect of the probiotic on gut microbiota composition and function was analyzed. However, mostly the fecal microbiota is studied while the effect of probiotics on other areas of the GIT is neglected.

### 3.2. Probiotics as prevention or treatment of CDI investigated in clinical trials

There is a great interest in the use of probiotics for prevention and treatment of CDI. Probiotics may be used in preventing CDI (primary prophylaxis) or as adjuvant therapy during or after antibiotic treatment to prevent recurrent CDI (secondary prophylaxis) (Parkes et al., 2009). In a recent Cochrane review and meta-analysis of 23 randomized controlled trials on the effect of probiotics on CDAD (Goldenberg et al., 2013) it was concluded that probiotics significantly reduce the risk of CDAD by 64% as the incidence was 2.0% in the probiotic group compared to 5.5% in the placebo control group. However, in the same review the effect of probiotics on the incidence of CDI was analyzed from 13 trials and no statistically significant reduction could be seen.

In the clinical trials several probiotics, including *Lactobacillus* and *Bifidobacterium* strains as well as the yeast *S. boulardii*, were investigated either as single probiotic agent or in combination with other probiotics (Table 1.3). *S. boulardii* is one of the best characterized probiotic for use in CDI. In two human studies it was shown that *S. boulardii* in combination with vancomycin was superior in the prevention of recurrent CDAD compared to placebo in combination with vancomycin (McFarland et al., 1994, Surawicz et al., 2000). Promising results were obtained in three clinical trials testing the probiotic combination Bio-K+ (*Lactobacillus acidophilus* CL1285 and *Lactobacillus casei* LBC80R) for prevention of first-time CDAD incidence. In first studies the combination of the two strains was provided in fermented milk (Beausoleil et al., 2007, Sampalis et al., 2010) and in another study patients received a capsule containing the two probiotics strains at different concentrations to evaluate dose-response (Gao et al., 2010). The PLACIDE trial is the most recent and so far largest (n=2941) multicenter and randomized controlled clinical trial investigating the effect of probiotics on AAD and CDAD (Allen et al., 2013). The probiotic was a multistrain preparation of lactobacilli and bifidobacteria prepared in a capsule and study subjects were
elderly taking antibiotics. The study found no significant reduction for AAD and CDAD in the probiotic group. However, CDAD outcome was very low with only 0.8% in the probiotic group and 1.2% in the placebo group.

The most interesting clinical trial with regard to this thesis is the one by Hickson et al. (2007). In this study the effect of a fermented drink containing *Lactobacillus casei* DN-114001, *Lactobacillus bulgaricus* and *Streptococcus thermophilus* (Actimel®) was tested in elderly hospitalized patients taking antibiotics on the primary outcome AAD and the secondary outcome CDAD. The authors found an absolute risk reduction of 17% for CDAD in the probiotic group. However, the Hickson study shows some limitations and bias. The patients were highly selected as e.g. patients on high-risk antibiotics (clindamycin, cephalosporins, aminopenicillins) were not included in the study. Then, the placebo group received a sterile (not fermented) milk drink thus not testing for the effect of fermented milk itself independent of the probiotic strain. Moreover, the milk could have caused more episodes of diarrhea which were then tested for *C. difficile* toxins since it is unclear if both groups were equally tested for toxins. Finally, the healthcare staff was not sufficiently blinded since the probiotic drink was in a 100 mL compared to 200 mL bottle for the placebo (Miller, 2009, Goldenberg et al., 2013). Further studies are needed to confirm the results obtained from the Hickson study as well as *in vitro* or animal studies for investigation of the probiotic mechanism in CDI prevention or treatment.
Table 1.3: Randomized controlled trials of probiotics for primary CDI prevention in patients receiving antibiotics. Adapted from Johnson et al. (2012).

<table>
<thead>
<tr>
<th>Probiotic (daily dose, cfu)</th>
<th>Subjects (number)</th>
<th>Treatment duration (follow-up duration)</th>
<th>CDI rate</th>
<th>Comments</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Saccharomyces boulardii</em> 2 x 10^10</td>
<td>Adult inpatients (180)</td>
<td>Duration of antibiotics + 14 days (no f/up)</td>
<td>5/64 (7.8%)</td>
<td>3/116 (2.6%)</td>
<td>1 USA hospital</td>
</tr>
<tr>
<td><em>S. boulardii</em> 3 x 10^10</td>
<td>Adult inpatients (193)</td>
<td>Duration of antibiotics + 3 days (7 weeks f/up)</td>
<td>4/96 (4.2%)</td>
<td>3/97 (3.1%)</td>
<td>4 USA hospitals, patients on beta-lactam antibiotics</td>
</tr>
<tr>
<td><em>S. boulardii</em> 4.5 x 10^9</td>
<td>Elderly patients (69)</td>
<td>Duration of antibiotics (no f/up)</td>
<td>3/36 (8.3%)</td>
<td>5/33 (15.2%)</td>
<td>1 UK hospital</td>
</tr>
<tr>
<td><em>S. boulardii</em> 1 x 10^9</td>
<td>Adult inpatients (151)</td>
<td>Duration of antibiotics (4 weeks f/up)</td>
<td>2/78 (2.6%)</td>
<td>0/73 (0%)</td>
<td>1 Turkish hospital</td>
</tr>
<tr>
<td><em>Lactobacillus acidophilus, Bifidobacterium bifidum</em> 2 x 10^10</td>
<td>Elderly (138)</td>
<td>20 days (no f/up)</td>
<td>5/69 (7.2%)</td>
<td>2/69 (2.9%)</td>
<td>1 UK hospital</td>
</tr>
<tr>
<td><em>Lactobacillus rhamnosus GG</em> 2 x 10^10</td>
<td>Adult inpatients (267)</td>
<td>14 days (7 days f/up)</td>
<td>3/134 (2.2%)</td>
<td>2/133 (1.5%)</td>
<td>1 USA hospital</td>
</tr>
<tr>
<td><em>Lactobacillus casei</em> DN-114001, <em>Lactobacillus bulgaricus, Streptococcus thermophilus</em> 4.2 x 10^10</td>
<td>Elderly inpatients (109)</td>
<td>Duration of antibiotics + 7 days (f/up 4 weeks after discharge)</td>
<td>9/53 (17%)</td>
<td>0/56 (0%)</td>
<td>3 UK hospitals, patients on high risk antibiotics excluded (Actimel)</td>
</tr>
<tr>
<td><em>L. acidophilus</em> CL1285, <em>L. casei</em> LBC80R 5 x 10^10</td>
<td>Adult inpatients (89)</td>
<td>Duration of antibiotics (additional 21 days f/up)</td>
<td>7/45 (15.6%)</td>
<td>1/44 (2.3%)</td>
<td>1 Canadian hospital, 9 month study (Bio-K+, fermented milk)</td>
</tr>
<tr>
<td><em>L. acidophilus</em> CL1285, <em>L. casei</em> LBC80R 5 x 10^10</td>
<td>Adult (437)</td>
<td>Duration of antibiotics + 5 days (additional 21 days f/up)</td>
<td>4/221 (1.8%)</td>
<td>1/216 (0.5%)</td>
<td>8 Canadian hospital, patients on antibiotics for 3-14 days (Bio-K+, fermented milk)</td>
</tr>
<tr>
<td><em>L. acidophilus</em> CL1285, <em>L. casei</em> LBC80R Low dose: 5 x 10^10 High dose: 10 x 10^10</td>
<td>Adult inpatients (255)</td>
<td>Duration of antibiotics + 5 days (additional 21 days f/up)</td>
<td>20/84 (23.8%)</td>
<td>Low dose: 8/85 (9.4%) High dose: 1/86 (1.2%)</td>
<td>1 Chinese hospital, 3 month study (Bio-K+, capsules)</td>
</tr>
<tr>
<td><em>L. acidophilus</em> CUL60 and CUL21, <em>B. bifidum, Bifidobacterium lactis</em> 6 x 10^10</td>
<td>Elderly inpatients (2941)</td>
<td>21 days (12 weeks f/up)</td>
<td>1471/17 (1.2%)</td>
<td>1470/12 (0.8%)</td>
<td>5 UK hospitals</td>
</tr>
</tbody>
</table>
3.3. *L. paracasei* CNCM I-1518

The genus *Lactobacillus* is a large and diverse group of Gram-positive, non-sporulating bacteria belonging to the lactic acid bacteria that produce lactic acid as their main fermentation end product (Salminen *et al.*, 2004). *Lactobacillus* species are commonly found in the gastrointestinal tract and other body cavities of humans and animals as well as in a variety of food, such as fermented milk, meat and plant (Hammes & Vogel, 1995). One of this species is *Lactobacillus paracasei* that has been used as starter cultures for dairy products and also as probiotic bacteria. The classification and nomenclature of the *Lactobacillus casei* and *L. paracasei* group has been a matter of debate. However, the majority of *L. casei* and *L. paracasei* strains are members of the same species which should be named *Lactobacillus paracasei* subsp. *paracasei* according to the current nomenclature. Many publications, however, refer to both species names (*L. casei* and *L. paracasei*) (Smokvina *et al.*, 2013). The taxonomic relationship between the *L. paracasei/casei* species with *Lactobacillus zea* and *Lactobacillus rhamnosus*, which all together are known as the *L. casei* group have also been debated (Desai *et al.*, 2006, Diancourt *et al.*, 2007). However, sequencing of the 50S ribosomal protein L2 (rplB) of 52 *L. casei* strains showed that all strains analyzed had a similar sequence to that of the *L. casei* reference strain (ATCC 334) and formed a cluster that was clearly distinct from *L. zeae* and *L. rhamnosus* as well as from current taxonomic type strain of *L. casei* ATCC 393^T^ (Diancourt *et al.*, 2007) (Figure 1.10).
Included in the study of Diancourt was also the strain *L. casei* DN-114001, which was renamed to *L. paracasei* CNCM I-1518 (LpC) according to the current nomenclature and is the probiotic strain of the fermented milk drink marketed globally by Danone and called Actimel® (or DanActive in the United States and Canada). The concentration of the probiotic strain in Actimel is approximately $10^8$ cfu mL$^{-1}$ (Rochet *et al.*, 2006). In addition, Actimel contains the traditional yoghurt starter cultures *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus salivarius* subsp. *thermophilus*. The capacity of LpC to survive the transit through the digestive tract of humans was demonstrated previously with high survival rates of 28% in the feces (Oozeer *et al.*, 2006, Rochet *et al.*, 2008). In human microbiota-associated mice it was demonstrated that LpC survives but does not multiple in the GI tract (Oozeer *et al.*, 2002) and that the probiotic bacterium initiates protein synthesis during GI transit (Oozeer *et al.*, 2005). Moreover, in several *in vivo* studies the consumption of Actimel was associated with health benefits. In two studies the effect of Actimel on incidence and duration...
of diarrhea in healthy children of 6 – 24 months was assessed. The first study showed decreased duration of diarrhea in children consuming Actimel compared to jellied milk but not to standard yoghurt or milk fermented with yoghurt cultures (Pedone et al., 1999). In the second study, incidence of diarrhea was decreased in children consuming Actimel (15.9%) compared to standard yoghurt (22%) (Pedone et al., 2000). In two multicenter randomized control trials Actimel consumption was associated with decreased incidence of common infectious diseases, including GIT infections, in children (Merenstein et al., 2010) and elderly (Guillemard et al., 2010). In another randomized control study an increase in specific antibody titers after vaccination against influenza was observed in elderly subjects consuming Actimel (Boge et al., 2009). Actimel was also suggested to enhance the rate of eradication of *Helicobacter pylori* in children treated with gastritis receiving antibiotics (Sykora et al., 2005).

Several studies investigated *in vitro* the capability of the probiotic LpC strain itself to reinforce the intestinal barrier functions. LpC expressed inhibitory effects on adhesion of adherent-invasive *E. coli* isolated from Crohn’s disease patients to intestinal epithelial cells (Ingrassia et al., 2005) but did not inhibit adhesion of enteropathogenic *E. coli* strain (Parassol et al., 2005). However, the study of Parassol demonstrated the ability of LpC to reinforce the intestinal barrier function by inhibiting the increase in enteropathogenic *E. coli*-induced paracellular permeability. Bactericidal activity of LpC against *Salmonella enterica* serovar *Typhimurium* was shown by Fayol-Messaoudi et al. (2005). LpC was also shown to trigger the down-regulation of transcription genes encoding the proinflammatory effectors and adherence molecules in *Shigella* infected intestinal Caco-2 cells (Tien et al., 2006) and significantly reduced the release of tumor necrosis factor-α from mucosal explants of Crohn’s disease patients (Borruel et al., 2002). However, in an *in vivo* study no difference could be demonstrated in stimulation of pro-inflammatory cytokines between a yoghurt with LpC and a conventional yoghurt (Meyer et al., 2007) In germ-free rats infected with rotavirus LpC was associated with reduced clinical signs of diarrhea and prevention of rotavirus infection (Guerin-Danan et al., 2001) and soluble factors of LpC blocked infection of mucus-secreting HT29-MTX cells by rotavirus strains (Varyukhina et al., 2012).

The effect of LpC on CDI is of particular interest for this thesis. In 2010, the EFSA Panel on Dietic Products evaluated a health claim by Danone Produits Frais France that reads as follows: ‘Actimel® decreases the presence of *C. difficile* toxins, in the intestinal tract and reduces the incidence of acute diarrhea associated with their presence in the gut of susceptible
ageing people’. In total seven publications on human studies, three unpublished human studies, eight published and one unpublished non-human studies were submitted to the Panel. Of the human studies only three referred to CDAD and of these three only the study by Hickson et al. (2007) showed a statistically significant risk reduction for CDAD. However, the Hickson study had considerable limitations according to the Panel. Five human studies investigated only the kinetics, metabolism and survival of LpC. One in vitro study showed inhibitory effect of LpC on C. difficile; however, according to the Panel this does not predict an effect against C. difficile in humans. In conclusion, the evidence provided was not sufficient to establish a cause and effect relationship between the consumption of Actimel and risk reduction of CDAD by reducing C. difficile toxins (EFSA, 2011).
4. **Intestinal models for studying bacterial infections**

Studying the complex bacterial ecosystem of the gut as well interactions with the host in humans can be restricted due to hindered accessibility of the GIT and the large variability in the colonic microbiota composition between individuals. Therefore, intestinal *in vivo* and *in vitro* models are required for mechanistic studies as they allow the systematic manipulation of different factors with an increased level of experimental control.

4.1. **Animal models**

Animal models are especially interesting for studying host-microbe interactions. Main studied animals are rodents, in particular mice and rats. However, differences in microbiota composition between rodents and humans have been reported (Krych *et al.*, 2013) and thus gnotobiotic animal models have been developed, which are germ-free animals that are inoculated with specific microorganisms or even a complete human microbiota (=human gut microbiota associated animals) (Lacroix *et al.*, 2015). However, for studying infections usually conventional murine models are used. A well-established model is the murine model that uses *Citrobacter rodentium* to induce infection. Several of the pathogenic mechanisms of this murine pathogen are similar to enteropathogenic and enterohaemorrhagic *E. coli*. Mice pre-treated with antibiotics and then infected with *Salmonella typhimurium* is another prominent infection model (Papadimitriou *et al.*, 2015).

4.1.1. **Animal models for CDI**

Various animals have been used to study CDI, including hamsters, mice, rats, rabbits, hares, guinea pigs, prairie dogs and quails. In few studies the use of larger animals, including foals piglets and monkeys was described (Best *et al.*, 2012). Most animal models for the study of CDI require pre-treatment with antibiotics as - similar to humans - untreated animals are relative resistant to disease. The most widely used animal models to study CDI are the hamster and mouse models (Dubberke *et al.*, 2011, Best *et al.*, 2012, Hutton *et al.*, 2014).

Hamster models, in particular Syrian hamsters, were applied in a number of studies of clindamycin-induced enterocolitis. The pathology observed in hamsters is regarded as closest to human antibiotic-associated enterocolitis (Dubberke *et al.*, 2011). However, there are also key differences. In hamsters, infection occurs in caecum and not in the colon as for humans. Further, if left untreated the disease is rapidly fatal in hamsters and the outcome of the
Intestinal models for studying infections

The hamster model is used to study many aspects of disease, including colonization, transmission, virulence factors, vaccine development, testing of treatments and virulence of various C. difficile isolates (Best et al., 2012, Hutton et al., 2014). Hamster models were also used to study the relative contributions of C. difficile toxins (Lyras et al., 2009). One of the main limitations in hamster models is the lack of immunological reagents to study host responses to CDI (Best et al., 2012, Hutton et al., 2014).

Increasing use of mouse models is mainly due to the greater availability of mouse-specific reagents to perform host-tissue analysis (Hutton et al., 2014). Various models were developed to induce susceptibility to C. difficile as healthy mice are relatively resistant to CDI. These models include the gnotobiotic/germ-free mice, mice treated with a cocktail of antibiotics or a single antibiotic as well as human microbiota-associated mouse models (Best et al., 2012, Hutton et al., 2014). The advantage of germ-free mice is that no pre-treatment with antibiotics is required; however, not all of the normal immune and mucosal responses are developed in this model. Mice pre-treated with antibiotics before C. difficile challenge develop diarrhea, weight loss and histological damage similar to the disease pathology in humans. These mouse models have also been used to study the role of the gut microbiota in CDI development, C. difficile colonization and transmission, efficacy of treatments such as FMT as well as disease recurrence. In general, extrapolating results from animal to human has its limitations as there are notable inter-species differences in susceptibility to CDI (Hutton et al., 2014).

4.2. In vitro models

In vitro models are usually less expensive, not restricted by ethical concerns and provide an even higher degree of experimental control compared to animal models. They are especially useful for first investigations or screenings of novel treatments against infections before application in in vivo models and human studies.

Common to in vitro gut fermentation modeling is the inoculation of a single or multiple reactors with fecal microbiota and anaerobic operation conditions at physiological temperature and pH. The models range from simple batch fermentation in serum bottles to more sophisticated pH-controlled multi-stage continuous fermentations (Payne et al., 2012a). Static batch fermentation models are closed systems containing a limited amount of substrate; thus these systems are restricted to short time course fermentations that usually last between 24 and 48 h. Furthermore, in case of absence of pH control, metabolite production reduces
pH which can also prevent further microbial activity. Bacteria in batch fermentations follow a typical growth curve due to substrate depletion over time. However, growth can be limited in batch fermentation systems if they contain high cell densities similar to the colon. Batch fermentations are, nonetheless, a convenient method for screening of a large number of substrates or fecal samples (Gibson & Fuller, 2000, Payne et al., 2012a).

Continuous fermentation models facilitate long-term studies and allow investigations of the gut microbiota under steady-state conditions. These models work efficiently at high cell densities and bacterial growth and metabolism can be tightly controlled by adjusting pH and nutrient feed thus simulating intestinal conditions more closely than batch studies. Single-stage continuous fermentation models are usually used at proximal colon conditions. Multi-stage fermentation models are of interest for simulation of the different regions of the colon (Macfarlane & Macfarlane, 2007, Payne et al., 2012a). Macfarlane et al. (1998) developed a three-stage model replicating the proximal, transverse and distal regions of the colon. The model was validated using measurements from gut contents of sudden death victims. The first reactor (proximal colon) is rich in nutrients and operated at acidic conditions with shorter retention time compared to the third reactor (distal colon) that is low in substrate and runs at neutral pH.

There are also in vitro models that simulate the complete GIT (including digestive functions). The SHIME (simulator of the human intestinal microbial ecosystem) model consists either of five reactors which are connected in series and simulate the duodenum/jejunum, the ileum and the three regions of the large intestine (Molly et al., 1993) or of six reactors including a reactor simulating the stomach (Alander et al., 1999). This system was used for instance for probiotic and prebiotic investigations (Gmeiner et al., 2000, Sivieri et al., 2013, Terpend et al., 2013). The combination of the TIM-1 (simulating digestive functions) and TIM-2 (simulation of colon conditions) represents another gastrointestinal model that was used for different studies such as pharmaceutical investigations (Minekus et al., 1995, Minekus et al., 1999, Souliman et al., 2007).

Most of these in vitro continuous fermentation models use fecal suspensions to inoculate the reactor system resulting in a rapid wash-out of less competitive bacteria and thus short operation times. In addition, these systems only mimic the planktonic state of the gut microbiota and lack to reproduce the biofilm-associated (sessile) bacteria residing in the colon. In this regard, the process of immobilization has been developed in which freshly collected feces is immobilized in polysaccharide beads and continuously fermented at proximal colon conditions (Cinquín et al., 2004). High bacterial cell densities and continuous
release of cells from the peripheral layer due to active growth and substrate limitation as well as toxic product diffusion was demonstrated in beads (Cinquin et al., 2004, Payne et al., 2012a). These models using immobilized microbiota have been successfully applied for continuous fermentations of up 71 days (Le Blay et al., 2010, Payne et al., 2012a) including for investigations of antibiotic treatment and Salmonella infection in the child gut (Le Blay et al., 2009) and probiotic treatments against Salmonella infection (Zihler et al., 2010).

One of the main challenges in in vitro gut fermentation modeling is the lack of true replication (Payne et al., 2012a). This problem was recently addressed by the development of the PolyFermS model, that allows the testing of different environmental factors in several reactors connected in parallel and inoculated with the same child microbiota (Zihler Berner et al., 2013). The PolyFermS model was also successfully applied to study the effect of probiotics on Salmonella infection in the swine proximal colon (Tanner et al., 2014a).

Mechanistic studies of the elderly gut microbiota are of major interest due to the changes in gastrointestinal physiology and gut microbial diversity in old age which may be directly linked to health (Biagi et al., 2013). However, in vitro continuous models have so far only scarcely been used for studies of the elderly colon. A three-stage fermentation model inoculated with pooled feces of multiple elder donors was developed and validated for studying the effect of antibiotics on CDI (Baines et al., 2005, Freeman et al., 2007, Baines et al., 2009a) and in a similar three-stage model but inoculated with feces from a single donor the effect of pro-, pre- and synbiotics on the elderly gut microbiota was investigated (Likotrafiti et al., 2014).

A main disadvantage of the in vitro intestinal fermentation models is that microbiota interactions with the host are not investigated. Thus, studies were conducted where intestinal models were combined with human cell models (Zihler et al., 2011, Dostal et al., 2014b). Another model that allows studies of bacteria-host interactions cultures bacterial with human epithelial cells on a chip, termed gut-on-a-chip (Kim et al., 2012).

4.2.1. In vitro models for CDI

In vitro fermentation models have been used to study the role of antibiotics in C. difficile colonization, the dynamics of the gut microbiota and in the evaluation of therapeutics for CDI.
Batch fermentation models inoculated with fecal slurries and run at pH typical for distal colon conditions were used to study the potential of a bacteriophage (Meader et al., 2010) or bacteriocin (Rea et al., 2011b) for treatment of *C. difficile*. However, batch fermentation models are not well suited to investigate CDI and treatment possibilities since experiments are usually limited to 48 h and thus do not allow to study colonization of the pathogen and potential recurrence after treatment.

A triple-stage continuous intestinal model for investigations of antibiotics and *C. difficile* was applied in a series of studies (Baines et al., 2005, Freeman et al., 2005, Baines et al., 2006, Freeman et al., 2007, Baines et al., 2008, Baines et al., 2009a, Baines et al., 2009b, Saxton et al., 2009, Baines et al., 2011, Baines et al., 2013, Chilton et al., 2013, Crowther et al., 2013, Chilton et al., 2014). The intestinal model was adapted from the three-stage model described by Macfarlane et al. (1998). The three vessels are connected in a weir cascade and simulate the proximal (pH 5.5), transverse (pH 6.2) and distal colon (pH 6.8). The first reactor is inoculated with fecal slurry that derived from pooled feces from healthy elderly volunteers. Following a two-week stabilization period the experimental period is carried out for six to eight weeks. *C. difficile* is introduced as spores at a concentration of $10^7$ cfu. *C. difficile* vegetative cells, spores and gut microbial populations are assessed by plating on selective agar. In addition, toxin and antimicrobial concentrations are assessed. The main experiments carried out in this model were the examination of antimicrobials in their capacity to induce *C. difficile* germination, growth and toxin production and in their efficacy to treat CDI. *C. difficile* ribotypes tested include 027, 001 and 106. The experimental procedure in studies investigating the propensity of antimicrobials to induce *C. difficile* germination was as follows: *C. difficile* spores were introduced once the system was stabilized and no treatment was performed for the following seven days. After this week another inoculum of *C. difficile* spores was introduced and at the same time the antimicrobial was added to the system. The first *C. difficile* period without antibiotics serves as control period with the rationale that spores do not germinate when lacking antibiotic pressure. Antimicrobials investigated for induction of CDI include cefotaxime, fluoroquinolones and pipercaillin/tazobactam (Freeman et al., 2003, Baines et al., 2005, Saxton et al., 2009). Clindamycin was used to induce spore germination in studies investigating antimicrobials for treatment of CDI since it was observed that *C. difficile* spores remained quiescent without antimicrobial stimulation. Metronidazole and vancomycin were investigated as treatments (Freeman et al., 2007, Baines et al., 2009b) as well as novel antibiotics including ramoplanin, oritavancin, linezolid, tolevamer and surotomycin (Freeman et al., 2005, Baines et al., 2008, Baines et al., 2009a, Baines et al., 2009b).
2011, Chilton et al., 2014) and other investigational compounds (Crowther et al., 2013). Recently the model was adapted for the investigation of biofilm-associated bacteria by integrating glass-rods into the reactors that allow the formation of biofilms (Crowther et al., 2014).

Interesting results were obtained with the intestinal model for *C. difficile* by Baines et al. regarding novel antibiotic treatments. However, the microbiota diversity, which is an important factor in CDI regarding colonization as well as recurrence after treatment, is not well investigated in these studies since only plating is performed to assess microbial composition. Therefore, *in vitro* intestinal models for ecological studies of the microbiota and *C. difficile* are lacking thus far.
5. Background and objectives of the thesis

In recent years *C. difficile* has become one of the most important health-care associated pathogens worldwide and the leading cause of antibiotic-associated diarrhea (Zucca et al., 2013). Development of CDI is associated with usage of broad-spectrum antibiotics and the consequential disruption of the gut microbiota homeostasis allowing germination of *C. difficile* spores and subsequent colonization in the colon (Bartlett et al., 1978, Oldfield et al., 2014). The risk of developing CDI is especially high in old age, with colonization rates of up to 73% in hospitalized elderly (Deneve et al., 2009, Carroll & Bartlett, 2011). The standard antibiotic treatments for CDI include vancomycin and metronidazole; however, a high recurrence rate of up to 35% is associated with these antibiotics (Gough et al., 2011), thus there is a need for alternative treatments.

The use of probiotics as treatment or prevention of CDI could represent a high potential strategy. Hickson et al. (2007) demonstrated that a fermented milk drink containing the probiotic *Lactobacillus paracasei* CNCM I-1518 strain (Actimel®) can reduce the incidence of CDAD in elderly patients taking antibiotics. However, probiotic mechanisms explaining the protecting effect of Actimel against *C. difficile* remain to be clarified. In this regard, *in vitro* models simulating the colon are well-suited for mechanistic studies of probiotic-pathogen interactions while circumventing ethical and practical issues of *in vivo* studies (Payne et al., 2012a).

It was previously demonstrated that the elderly microbiota differs from adult microbiota (Claesson et al., 2011, Biagi et al., 2013); however continuous fermentation models simulating the colonic microbiota in old age are limited to few three-stage models (Baines et al., 2005, Likotrafiti et al., 2014). Similar, *in vitro* models to investigate potential new antimicrobials for *C. difficile* in complex intestinal microbiota are restricted to batch fermentation studies (Meader et al., 2010, Rea et al., 2011b) and one three-stage continuous model (Baines et al., 2005).

5.1. General objective

The general objective of this thesis was to develop and use *in vitro* models simulating the elderly colon to study potential antagonistic effects of *Lactobacillus paracasei* CNCM I-1518 (LpC) against *Clostridium difficile*. 
5.2. **Specific objectives**

The research strategy of this thesis included the development of several *in vitro* fermentation models with elderly gut microbiota for the investigations of LpC effects on *C. difficile* colonization and on the microbiota (Figure 1.11). Furthermore, co-culture studies were performed to investigate in-depth the direct interactions between LpC and either *C. difficile* or specific commensal gut microbiota members. The specific objectives of this thesis were defined as follows:

1. To develop *in vitro* continuous fermentation models simulating the elderly colon using the novel PolyFermS platform
2. To implement the *in vitro* continuous fermentation models of the elderly colon for investigations of *C. difficile* colonization behavior and antibiotic treatments
3. To study *in vitro* the effect of LpC on growth and toxin production of *C. difficile*
4. To assess the potential of LpC to modulate the gut microbiota structure and function by studying the transcriptome response in continuous colonic fermentation models simulating the elderly colon

![Figure 1.11: Schematic illustration of research objectives.](image)
Chapter 2

Design and investigation of PolyFermS *in vitro* continuous fermentation models inoculated with immobilized fecal microbiota mimicking the elderly colon

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Abstract

*In vitro* gut modeling is a useful approach to investigate some factors and mechanisms of the gut microbiota independent of the effects of the host. This study tested the use of immobilized fecal microbiota to develop different designs of continuous colonic fermentation models mimicking elderly gut fermentation. Model 1 was a three-stage fermentation mimicking the proximal, transverse and distal colon. Models 2 and 3 were based on the new PolyFermS platform composed of an inoculum reactor seeded with immobilized fecal microbiota and used to continuously inoculate with the same microbiota different second-stage reactors mounted in parallel. The main gut bacterial groups, microbial diversity and metabolite production were monitored in effluents of all reactors using quantitative PCR, 16S rRNA gene 454-pyrosequencing, and HPLC, respectively. In all models, a diverse microbiota resembling the one tested in donor’s fecal sample was established. Metabolic stability in inoculum reactors seeded with immobilized fecal microbiota was shown for operation times of up to 80 days. A high microbial and metabolic reproducibility was demonstrated for downstream control and experimental reactors of a PolyFermS model. The PolyFermS models tested here are particularly suited to investigate the effects of environmental factors, such as diet and drugs, in a controlled setting with the same microbiota source.
Introduction

The human colon harbors a large number of microbes forming a complex ecosystem responsible for various processes in the host. Under normal conditions, the gut microbiota acts as a barrier against enteropathogens, contributes to the development of the immune system and exerts important metabolic functions; which includes the production of short chain fatty acids (SCFA; such as acetate, propionate and butyrate) by breaking down complex carbohydrates that provide energy to epithelial cells and to the host (Chassard & Lacroix, 2013, Robles Alonso & Guarner, 2013). Each human harbors a unique gut microbiota composition consisting of bacteria belonging mainly to the phyla Firmicutes or Bacteroidetes and, to a lesser extent, to Actinobacteria, Proteobacteria and Verrucomicrobia (Duncan & Flint, 2013, Power et al., 2014). Colonization of the gut occurs first during birth, and throughout the first 2–3 years of life the microbial composition becomes established towards an adult-like microbiota. Recent studies indicate that the gut microbiota remains stable in adulthood, except for temporary alterations due to diet, disease and antibiotic treatment. However, an important shift in the microbial composition occurs during old age that is associated with a reduction in stability and often in biodiversity (O'Toole & Claesson, 2010, Ottman et al., 2012, Biagi et al., 2013, Duncan & Flint, 2013, Power et al., 2014). Additionally, a large inter-individual variability of the gut microbiota composition was reported for elderly Irish subjects of a large-scale in vivo study, with pyrosequencing reads assigned to the phyla Bacteroidetes and Firmicutes ranging from 3 to 94% (Claesson et al., 2011). To date, no common core microbiota for the elderly was defined, partly due to the various physiological factors, including lifestyle, diet and need for medications that change in old age. Thus, establishing the changes in the composition with ageing still require further investigations (Ottman et al., 2012, Power et al., 2014).

Intestinal fermentation models allow the in vitro cultivation of gut microbiota to study their composition and function, uncoupled from the host. As such, models provide greater control, easier manipulation, and no ethical restrictions relative to in vivo studies, and are very complementary to in vivo strategies for elucidating mechanisms of gut microbiota (Lacroix et al., 2015). Intestinal models have developed from batch for short-term fermentation studies to single or multistage continuous culture models that allow long-term studies due to substrate replenishment and toxic product removal (Payne et al., 2012a). However, one of the main challenges of the continuous models is the reproduction of the biofilm-associated microbes of the gut that is important to prevent washout of the less competitive bacteria. Immobilization
of gut microbiota in gellan-xanthan gel beads has shown to reproduce the free and biofilm associated states of bacterial populations and to maintain the bacterial diversity at high cell densities in continuous intestinal reactors over periods of up to 71 days (Cinquín et al., 2004, Cinquin et al., 2006, Payne et al., 2012a). Furthermore, reproducibility and biological replication of continuous intestinal models was recently improved by the introduction of the PolyFermS model that allows the parallel testing of treatments with the same gut microbiota, and which has been validated for the child and the swine proximal colon (Zihler Berner et al., 2013, Tanner et al., 2014b).

In vitro intestinal fermentations models have been developed and validated (Payne et al., 2012a) to investigate factors of microbiota composition and metabolism of infants to adults while the elderly gut microbiota was only scarcely analyzed. Several studies were performed in continuous three-stage models for investigating the effects of antibiotics on Clostridium difficile infection. For these studies C. difficile was inoculated with mixed fecal samples from multiple elder donors and the system was challenged with antibiotics to promote the germination and growth of the sporulated bacteria, while microbiota analysis was only done with cultivation (Baines et al., 2005, Freeman et al., 2007, Baines et al., 2009b, Crowther et al., 2014). In a recent study batch cultures and continuous three-stage models inoculated with microbiota from single fecal samples of elder donors were used to investigate probiotics, prebiotics and synbiotics. Fluorescent in situ hybridization methods were used to monitor gut microbiota composition (Likotrafiti et al., 2014). To date, no study has reported a detailed analysis of gut microbiota establishment and diversity in in vitro fermentation models reproducing the gut of aged (over 65 years) people.

The aim of this study was to investigate the use of immobilized fecal microbiota to develop different designs of continuous colonic fermentation models mimicking elderly gut fermentation. Immobilization of fecal microbiota obtained from three different donors was performed independently. Fecal beads were used to inoculate an immobilized cell reactor operated with conditions selected to mimic the proximal colon section of an elder. Three model designs, all starting with an IR, were tested for different experimental questions to mimic proximal and distal colon conditions. Model 1 was based on the three-stage design, with immobilized microbiota inoculated in a first proximal colon reactor connected to a transverse and a distal colon reactor, previously validated for infant and child microbiota (Cinquín et al., 2006, Zihler et al., 2010, Payne et al., 2012b). Models 2 and 3 were developed based on the PolyFermS platform, which recently has been validated with child (Zihler Berner et al., 2013) and swine (Tanner et al., 2014b) microbiota, with adjusted
conditions for the elderly microbiota. In model 2 the inoculum reactor containing immobilized fecal microbiota was connected with two parallel sets of 2-stage reactors mimicking the proximal and distal colon. In model 3, five reactors were connected in parallel to the inoculum reactor, with all five test reactors mimicking conditions of the distal colon. The microbiota composition in reactor effluents was monitored and compared to that of the corresponding fecal donor, and temporal stability of the models and reproducibility of downstream reactors within a PolyFermS model were demonstrated. Microbiota composition, diversity (qPCR and pyrosequencing) and activity (HPLC) were monitored in reactor effluents over operation periods of up to 80 days.
Materials and Methods

Ethics Statement
The Ethics Committee of ETH Zurich exempted this study from review because sample collection was not in terms of intervention. An informed written consent was, however, obtained from the fecal donors.

Fecal inoculum and immobilization
For each fermentation experiment a fresh fecal sample from a different donor was used for the immobilization procedure. Fecal samples were collected from three healthy women, aged 71 (fermentation 1), 72 (fermentation 2) and 78 years (fermentation 3), who did not receive antibiotic treatment for at least three months prior to sample collection, and who did not consume probiotics on a regular basis. Immediately after defecating, the fecal sample was transferred to a tube containing 5 mL of sterile, pre-reduced peptone water (0.1%, pH 7), placed in an anaerobic jar (Anaerojar, Oxoid, Hampshire, England), and transported and processed within three hours. Handling and encapsulation of the fecal microbiota into 1-2 mm gel beads composed of gellan (2.5% w/v), xanthan (0.25% w/v), and sodium citrate (0.2% w/v, Sigma-Aldrich Chemie GmbH, Buchs, Switzerland) was performed in an anaerobic chamber as previously described (Zihler et al., 2010).

Fermentation medium
The fermentation medium was based on the composition described by MacFarlane et al. (1998) for simulation of adult chyme entering the colon. It contained (g L⁻¹ of distilled water): pectin from citrus (2), xylan from oat spelt (2), arabinogalactan from larch wood (2), guar gum (1), inulin (1), soluble potato starch (5), mucin (4), casein acid hydrolysate (3), peptone water (5), tryptone (5), yeast extract (4.5), cysteine (0.8), bile salts (0.4), KH₂PO₄ (0.5), NaHCO₃ (1.5), NaCl (4.5), KCl (4.5), MgSO₄ anhydrous (0.6), CaCl₂ x 2H₂O (0.1), MnCl₂ x 4H₂O (0.2), FeSO₄ x 7H₂O (0.005), hemin (0.05) and Tween 80 (1). One mL of a filter-sterilized (0.2 µm pore-size) vitamin solution (Michel et al., 1998) was added to 1 L of autoclaved (20 min, 120°C) and cooled medium. All components of the nutritive medium were purchased from Sigma-Aldrich Chemie, except for inulin (Orafti®, BENEKO kindly provided by RPN Foodtechnology AG, Sursee, Switzerland), peptone water (Oxoid AG, Pratteln, Switzerland), bile salts (Oxoid AG), tryptone (Becton Dickinson AG, Allschwil, Switzerland) and KH₂PO₄ (VWR International AG).
Experimental setup
Various reactor set-ups were applied for the three continuous intestinal fermentation experiments (Figure 2.1). The inoculum reactor (IR) seeded with donor’s microbiota immobilized in polysaccharide gel beads, and operated with proximal colon conditions, was common to all models. Model 1 was a classical three-stage system consisting of three reactors placed in series and operated under conditions of the proximal (PC corresponding to IR), transverse (TC) and distal colon (DC) (Zihler et al., 2010). Model 2 was based on the recently developed PolyFermS platform (Zihler Berner et al., 2013, Tanner et al., 2014b) and consisted of an IR with immobilized fecal microbiota in proximal colon conditions used to continuously inoculate two parallel systems (10% v/v of the feed), each composed of a proximal (PC1 and PC2) and a transverse-distal reactor (DC1 and DC2). For model 3, the chyme medium fermented in IR with immobilized microbiota and operated with proximal colon conditions was used to continuously feed (100% v/v of the feed) five reactors mounted in parallel and mimicking conditions of a transverse-distal colon. In models 2 and 3, one system or reactor downstream to IR was used as control while the other system (PC2-DC2) or reactors (TR1-TR4) were used to comparatively test treatments, respectively.

Fermentation procedures
The IR inoculated with 30% v/v gel beads was used to first colonize the beads with repeated-batch fermentations. The fresh medium was replaced every 12 h, for a total fermentation time of 60 to 72 h, depending on the model. Temperature was set at 37°C, stirring speed at 120 rpm and the pH was controlled at 5.5 or 5.7 by addition of 2.5 M NaOH. Sterile nutritive medium (4°C) was pumped continuously via a peristaltic pump (Reglo analog, Ismatec, Glattbrugg, Switzerland). Total mean retention times between 28 to 38.5 h were used in models 1 - 3, which is in the range of previously measured values for total colonic transit time in healthy elderly subjects that were between 25 and 66 h (Nagengast et al., 1988, Madsen, 1992, Merkel et al., 1993, Evans et al., 1998, Madsen & Graff, 2004).

The reactors and nutritive media were continuously flushed with a low flow of CO₂ to maintain anaerobic conditions during fermentation. The conditions of the models along with the model specific design and trials are briefly presented below and summarized in Figure 2.1.

Model 1. Six repeated-batch fermentations (72 h) were performed to colonize beads in PC (fermentation volume of 200 mL, pH of 5.5). Then two reactors mimicking conditions of transverse (TC) and distal colon (DC) (400 mL, pH 6.2 and 6.8, respectively) were connected in series to PC. The feed flow rate of the nutritive medium was set at 26 mL h⁻¹, giving mean
retention times of 7.7 h for IR and 15.4 h for TC and DC, for a total system retention time of 38.5 h. The model was stabilized for 14 days.

**Model 2.** Five repeated-batch fermentations (60 h) were performed to colonize beads in IR (225 mL and pH 5.7). The model was then switched to continuous mode with a medium flow rate of 25 mL h\(^{-1}\) for an additional five days. Two proximal colon reactors (PC1 and PC2, 250 mL and pH 5.7) were attached to IR, and each PC was connected to distal colon reactors (DC1 and DC2, 450 mL and pH 6.8) used to mimic transverse and distal colon conditions. Compared with model 1, the pH control set-point was increased from 5.5 to 5.7. This modification was implemented in order to better match the pH of the proximal colon section *in vivo*, which has been reported to be in the range from 5.5 to 5.9 (Lawley & Walker, 2013) and to enhance metabolic activity in IR and PC’s. PC1 and PC2 were continuously inoculated with 2.5 mL h\(^{-1}\) (10%) fermented medium from IR and 22.5 mL h\(^{-1}\) (90%) fresh nutritive medium, for total flow rate of 25 mL h\(^{-1}\). Mean retention times in PC and DC reactors were 10 and 18 h, respectively, for a total retention time of 28 h (PC1 and DC1; PC2 and DC2). The model was continuously operated for an additional 18 days to reach stability and used for testing, for a total fermentation time of 55 days.

**Model 3.** Beads colonization was carried out in five repeated-batch cultures (60 h) in IR (450 mL and pH 5.7). Five DC reactors (225 mL and pH 6.8) mounted in parallel and mimicking conditions of transverse-distal colon were connected to IR. The fresh nutritive medium flow rate in IR was set at 45 mL h\(^{-1}\), while each DC reactors was fed with 9 mL h\(^{-1}\) medium fermented in IR. The mean retention times in IR and DC reactors of model 3 were 10 and 25 h, respectively, for a total retention time of 35 h. The model was stabilized for 14 days and used for testing, for a total fermentation of 80 days.
Materials and Methods

Figure 2.1: Set-up of the continuous fermentation models with immobilized gut microbiota (A) Model 1. 3-stage model consisting of a proximal, transverse and distal colon reactor (B) Model 2. 2-stage model with an inoculum reactor connected to two parallel test systems consisting of a proximal and distal colon reactor (C) Model 3. 2-stage model with an inoculum reactor feeding five distal colon reactors connected in parallel; RT: Retention time, V: Volume

Sampling and analysis
During continuous fermentations, effluent samples (10 mL) were collected daily from each reactor. Because the different models were also used for experimental trials with Clostridium difficile and antibiotic and probiotic treatments, only samples obtained during periods of
control conditions are reported for model assessment. Analyses of microbial composition by quantitative polymerase chain reaction (qPCR) and pyrosequencing (model 2 and 3) were performed on samples from three days at the end of stabilization: days 9, 11 and 13 of model 1, days 16, 17 and 18 of model 2 and days 10, 11 and 12 of model 3. Metabolite concentrations in effluents of all reactors were tested daily during the entire fermentation by high-performance liquid chromatography (HPLC). Data corresponding to control condition periods (no treatment applied) are reported. Long term temporal stability was tested with IR data from models 2 and 3 since these reactors was not subjected to any manipulation over the entire culture period.

**DNA extraction**

For qPCR and pyrosequencing analyses total microbial DNA of 200 mg feces and 2 mL effluent samples was extracted using the FastDNA® SPIN Kit for Soil (MP Biomedicals, Illkirch, France) and a final elution volume of 100 µL. DNA concentrations were determined using a Nanodrop® ND-1000 Spectrophotometer (Witec AG, Littau, Switzerland).

**qPCR analysis**

Total bacteria and predominant bacterial groups were enumerated using specific primers (Table S2.1). One µL of 10- or 100-fold diluted DNA was amplified in a total volume of 25 µL as described in (Zihler *et al.*, 2010), using 2 x SYBR Green PCR Master Mix (Applied Biosystems, Zug, Switzerland). Each reaction was run in duplicate on an ABI PRISM 7500-PCR sequence detection system (Applied Biosystems). For quantification, standard curves were produced by amplification of the DNA of the reference strain of the respective target group (Dostal *et al.*, 2013).

**HPLC analysis**

SCFA (acetate, propionate, butyrate and valerate), formate, lactate and branched-chain fatty acids (BCFA; isobutyrate and isovalerate) in fermentation effluent samples from all reactors were determined by HPLC analysis (Thermo Fisher Scientific Inc. Accela, Wohlen, Switzerland) in duplicate (Cleusix *et al.*, 2008). Effluent supernatants were 2-fold diluted with sterile ultra-pure water and filtered directly into vials through a 0.45 µm nylon HPLC filter (Infochroma AG, Zug, Switzerland). The analysis was run at a flow rate of 0.4 mL min\(^{-1}\) using an Aminex HPX-87H column (Bio-Rad Laboratories AG, Reinach, Switzerland) and 10 mM H\(_2\)SO\(_4\) as eluent.

**Microbiota profiling by 454 pyrosequencing**

454-pyrosequencing analysis of total genomic DNA of fecal and effluent samples was carried out at DNAVision (Gosselies, Belgium). The V5-V6 hypervariable 16S rRNA region was
amplified using specific primers 784F (5’- AGGATTAGATACCTKGTA-3’) and 1061R (5’-CRRCACGAGCTGACGAC-3’) (Andersson et al., 2008). The forward primer contained the sequence of the Titanium A adaptor and a unique barcode sequence. Pyrosequencing was carried out using primer A on a 454 Life Sciences Genome Sequencer FLX instrument (Roche Applied Science, Vilvoorde, Belgium) following Titanium chemistry. The data obtained was analyzed using the open source software package Quantitative Insights Into Microbial Ecology (QIIME), v1.7 (Caporaso et al., 2010). Raw sequencing reads were filtered based on selected quality criteria such as: (1) no mismatch with the primer sequences and barcode tags; (2) no ambiguous bases (Ns); (3) read-lengths not shorter than 200 base pairs (bp) or longer than 1000 bp; (4) the average quality score in a sliding window of 50 bp not to fall below 25; (5) excluding homopolymer runs higher than 6 nt. Sequences that passed quality filtering were clustered into OTUs at 97% identity level using cd-hit (Li & Godzik, 2006). Representative sequences (the most abundant) for each OTU were aligned using PyNAST and taxonomically assigned using Greengenes v_13_08 database. Chimera were removed using ChimeraSlayer (Haas et al., 2011) leading to 8245 +/- 1924 (mean +/- SD) reads per sample. These phylogenies were combined with absence/presence or abundance information for each OTU to calculate unweighted or weighted UniFrac distances, respectively, using rarefaction of 7000 sequences per samples. Principal coordinates analysis (PCoA) was applied to the distance matrices for visualization. Alpha diversity (diversity within sample) was calculated using Shannon (evenness) indexes. All 454-pyrosequencing files have been deposited to the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) under accession number SRP053000.

**Statistical analysis**

Statistical analyses of HPLC and qPCR data (log10-transformed) were performed using JMP 8.0 (SAS Institute Inc., Cary, NC). Data are expressed as means ± SD of three days at the end of the stabilization period of each fermentation model. For every model the qPCR and HPLC data were compared between the reactors using the nonparametric Kruskal-Wallis test. p-values < 0.05 were considered significant. Monte Carlo permutation procedure was used to determine difference between proximal and distal colon using 999 permutations. Correlation between genus-level phylotypes and metabolites (acetate, propionate, butyrate, isobutyrate, isovalerate and valerate) were done in fermentation models 2 and 3. Analysis was done using R package “Microbiome” (Lahti et al., 2013) using Spearman correlation. p-values were corrected for multiple testing using Benjamini–Hochberg. Resulting q-values < 0.05 were considered as significant.
**Results**

**Microbial diversity of fecal microbiota**

The composition of dominant bacterial groups in fecal donor samples was assessed by analyzing the 16S rRNA gene copy numbers of total and selected bacterial groups using qPCR. All bacterial populations tested were detected in fecal samples, except for the *Roseburia* spp./*E. rectale* group and *Methanobacteriales* that were below the detection limit in the fecal inoculum of model 1 (Table 2.1). Predominant bacterial groups of all three fecal samples were *Bacteroides* spp. and *Clostridium* cluster IV within which *Faecalibacterium prausnitzii* was dominant. *Enterobacteriaceae*, *Lactobacillus* spp. and *Bifidobacterium* spp. belonged to the subdominant populations in all three fecal inocula.

The microbial profile of fecal inocula of model 2 and 3 was additionally analyzed by pyrosequencing (Figure 2.2). Phyla of both fecal samples were mainly assigned to Firmicutes and Bacteroidetes, with a Bacteroidetes:Firmicutes ratio of 0.15 and 0.30 for donor 2 and 3, respectively, and followed by Actinobacteria, Proteobacteria and Tenericutes (Figure 2.2A). The dominant families, *Lachnospiraceae*, *Bacteroidaceae* and *Ruminococcaceae* were similar for both microbiota (Figure 2.2B). At the genus level an unassigned genus belonging to the family of *Lachnospiraceae* was most abundant in fecal sample 2, followed by *Blautia* and *Bacteroides* (Figure 2.2C). In fecal sample 3 the same unassigned genus at similar abundance (∼ 20 %) to fecal sample 2 was most abundant, closely followed by *Bacteroides*.

**Microbial composition of effluents determined by qPCR**

Reactor effluents at the end of stabilization of all models were analyzed by qPCR to compare the microbial composition to the fecal donor and between reactors of a model (Table 2.1). Total bacterial numbers were, in general, high (> 10 log\(_{10}\) gene copies mL\(^{-1}\)) but between 0.3 to 1.0 log\(_{10}\) lower compared to the corresponding donor’s fecal sample while no differences were observed between reactors within a model. In effluent samples of model 1, the *Roseburia* spp./*E. rectale* group and *Methanobacteriales* were not detected which was consistent with the lack of these groups in the corresponding fecal donor. *Bacteroides* spp. and *Enterobacteriaceae* were predominant in the PC and DC reactors of model 1, respectively. However copy numbers of specific population groups were significantly different between PC and DC effluents of model 1, with the exception of the total 16S rRNA gene and *Bifidobacterium* spp. The microbial composition of the DC reactor was more similar to the fecal donor than for the PC reactor, except for total bacteria, *Lactobacillus* spp. and *Bifidobacterium* spp.
Similar to the corresponding fecal samples, the predominant bacterial groups in effluents from model 2 and 3 comprised *Bacteroides* spp., *Clostridium* cluster IV and *Faecalibacterium prausnitzii*. In model 2, no significant difference among all reactors was found for copy numbers of total 16S rRNA gene, *Bacteroides* spp., *Faecalibacterium prausnitzii, Clostridium* cluster IV and *Roseburia* spp./*E. rectale* group. Only small (less than 0.4 log_{10}) but significant differences were detected in DC2 for *Enterobacteriaceae* compared to IR and PC1, and for *Lactobacillus* spp. compared to IR, PC1 and PC2. *Bifidobacterium* spp. gene copy numbers were approx. 1 log higher in test system 1 compared to test system 2 and IR of model 2. *Methanobacteriales* numbers were significantly lower at PC conditions compared to IR and DC reactors.

In model 3, no significant difference was observed between reactors for most tested populations. Only small (≤ 0.3 log_{10} gene copies mL^{-1}), but significant differences were measured for *Bifidobacterium* spp. numbers in TR4 compared to TR1, for *Bacteroides* and *Lactobacillus* spp. between IR and test reactors or CR, and for *Roseburia* spp. in CR and TR3 compared to IR.
Table 2.1: qPCR enumeration of bacterial groups in fecal inocula and effluent samples of models’ reactors at the end of the stabilization period.

<table>
<thead>
<tr>
<th></th>
<th>Total 16S rRNA gene</th>
<th>Bacteroides spp.</th>
<th>Enterobacteriaceae</th>
<th>Lactobacillus spp.</th>
<th>Bifidobacterium spp.</th>
<th>F. prausnitzii</th>
<th>Clostridium Cluster IV</th>
<th>Roseburia spp./E. rectale</th>
<th>Methanobacteriales</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Model 1</strong></td>
<td></td>
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<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Donor 1</td>
<td>11.4</td>
<td>9.3</td>
<td>7.7</td>
<td>7.6</td>
<td>6.6</td>
<td>8.7</td>
<td>8.7</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>PC 1</td>
<td>10.6 ± 0.02</td>
<td>7.1 ± 0.1</td>
<td>9.4 ± 0.1</td>
<td>7.5 ± 0.2</td>
<td>8.2 ± 0.1</td>
<td>5.4 ± 0.2</td>
<td>6.7 ± 0.4</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>DC 1</td>
<td>10.5 ± 0.1</td>
<td>9.5 ± 0.01</td>
<td>8.2 ± 0.1</td>
<td>8.0 ± 0.2</td>
<td>8.3 ± 0.1</td>
<td>7.4 ± 0.1</td>
<td>8.1 ± 0.1</td>
<td>ND</td>
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<td><strong>Model 2</strong></td>
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<tr>
<td>Donor 2</td>
<td>11.1</td>
<td>9.6</td>
<td>7.2</td>
<td>8.3</td>
<td>8.5</td>
<td>10.1</td>
<td>10.3</td>
<td>9.4</td>
<td>8.6</td>
</tr>
<tr>
<td>IR 2</td>
<td>10.2 ± 0.2</td>
<td>10.0 ± 0.2</td>
<td>8.9 ± 0.1</td>
<td>6.1 ± 0.1</td>
<td>6.7 ± 0.1</td>
<td>9.1 ± 0.6</td>
<td>10.1 ± 0.2</td>
<td>8.6 ± 0.3</td>
<td>8.8 ± 0.1</td>
</tr>
<tr>
<td>PC1 2</td>
<td>10.3 ± 0.05</td>
<td>10.0 ± 0.2</td>
<td>9.1 ± 0.3</td>
<td>6.1 ± 0.02</td>
<td>7.7 ± 0.1</td>
<td>9.2 ± 0.4</td>
<td>10.1 ± 0.1</td>
<td>8.6 ± 0.3</td>
<td>7.0 ± 0.4</td>
</tr>
<tr>
<td>DC1 2</td>
<td>10.3 ± 0.2</td>
<td>9.9 ± 0.1</td>
<td>8.9 ± 0.1</td>
<td>6.2 ± 0.1</td>
<td>7.6 ± 0.1</td>
<td>9.2 ± 0.2</td>
<td>9.9 ± 0.1</td>
<td>8.6 ± 0.2</td>
<td>8.9 ± 0.04</td>
</tr>
<tr>
<td>PC2 2</td>
<td>10.3 ± 0.1</td>
<td>10.0 ± 0.1</td>
<td>8.8 ± 0.3</td>
<td>6.2 ± 0.1</td>
<td>6.6 ± 0.1</td>
<td>9.0 ± 0.6</td>
<td>10.0 ± 0.2</td>
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<td>7.3 ± 0.3</td>
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<td>10.1 ± 0.2</td>
<td>9.8 ± 0.2</td>
<td>8.7 ± 0.1</td>
<td>6.3 ± 0.001</td>
<td>6.8 ± 0.2</td>
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<td>9.9 ± 0.1</td>
<td>8.3 ± 0.2</td>
<td>8.9 ± 0.1</td>
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<tr>
<td><strong>Model 3</strong></td>
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<tr>
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<td>10.6</td>
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<td>10.6</td>
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<td>8.8 ± 0.1</td>
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</tr>
<tr>
<td>CR 2</td>
<td>11.1 ± 0.1</td>
<td>10.0 ± 0.1</td>
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<td>6.9 ± 0.1</td>
<td>7.6 ± 0.1</td>
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<td>8.3 ± 0.04</td>
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<tr>
<td>TR1 2</td>
<td>11.2 ± 0.2</td>
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<td>8.3 ± 0.04</td>
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<td>9.9 ± 0.02</td>
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<tr>
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<td>11.2 ± 0.1</td>
<td>10.0 ± 0.2</td>
<td>9.5 ± 0.1</td>
<td>7.0 ± 0.2</td>
<td>7.5 ± 0.2</td>
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<tr>
<td>TR4 2</td>
<td>11.2 ± 0.1</td>
<td>10.0 ± 0.1</td>
<td>9.5 ± 0.1</td>
<td>6.8 ± 0.4</td>
<td>7.7 ± 0.2</td>
<td>10.2 ± 0.1</td>
<td>9.9 ± 0.2</td>
<td>8.6 ± 0.3</td>
<td>7.9 ± 1.0</td>
</tr>
</tbody>
</table>

PC, proximal colon reactor; DC, distal colon reactor; IR, inoculum reactor; CR, control reactor; TR, test reactor

1Data are mean log$_{10}$ copies 16S rRNA gene g$^{-1}$ feces; samples were analyzed in duplicate. ND, not detected

2Data are mean log$_{10}$ copies 16S rRNA gene mL$^{-1}$ fermentation effluent ± SD of three last days at the end of the stabilization period; samples were analyzed in duplicate. ND, not detected.

Values with different letters are significantly different within one model (p<0.05)
Microbiota profile and diversity in effluents determined by pyrosequencing

To assess the microbial diversity sequencing of the V5-V6 region of 16S rRNA gene was performed by 454 FLX pyrosequencing of all reactors effluent samples of model 2 and selected reactors (IR, CR, TR3 and TR4) of model 3 (Figure 2.2) at the end of stabilization phase and compared to diversity of corresponding feces. The main phyla in IR of model 2 and 3 were Firmicutes and Bacteroidetes followed by Actinobacteria, Proteobacteria and Tenericutes (Figure 2.2A). The ratio Bacteroidetes:Firmicutes was increased in IR and distal colon reactors of model 2 (ratios of 1.7 in IR and 1.2 in DC1) and model 3 (ratios of 0.8 in IR and 1.4 in CR) relative to the corresponding fecal inoculum (ratios 0.2 and 0.3, respectively). Bacteroidetes:Firmicutes ratios in DC2 of model 2 and TR3 and TR4 of model 3 were similar to DC1 and CR, respectively (data not shown). On the family and genus levels Bacteroidaceae and Bacteroides were dominant in all reactors of both models 2 and 3 (Figure 2.2B and C). Bacteroidaceae abundances increased from 10% to approx. 60% and from 36% to approx. 53% in the effluent samples of model 2 and 3, respectively, compared to the fecal donor samples. In general, very similar microbial patterns (family and genus level) were obtained for all reactors within a model. In both DC reactors of model 2 the abundance of Bacteroidaceae (54.8% for DC1 and 52.0% for DC2, Figure 2.2) decreased compared to PC reactors (61.2% for PC1 and 70.1% for PC2). Other small differences at family and genus levels between the PC and DC reactors of each test system were observed. In model 3, minor differences between composition in IR and DC reactors were observed while microbial patterns were highly comparable between the DC reactors.

Beta diversity of bacterial populations at the end of stabilization phase of model 2 and 3 was analyzed using Principal coordinate analysis (PCoA) (Figure 2.3). Significant differences between DC and PC were observed using both Unifrac distances ($p<0.005$). In models 2 and 3 a clear separation of reactors operated with proximal colon conditions (IRs, PC1 and PC2) and distal colon conditions (DC1-DC2, CR-TR3-TR4, respectively) was observed.

The Shannon diversity index was assessed for fecal donor samples and effluent samples of models 2 and 3 (Figure 2.4). A lower diversity was measured in model 2 (mean Shannon index of 5.3 ± 0.4 calculated for all reactors) and model 3 reactors (mean Shannon index of 6.4 ± 0.1 calculated for IR, CR, TR3 and TR4) compared to that of the corresponding fecal samples (Shannon index of 7.5 and 7.4 for model 2 and 3, respectively). In model 2, a higher diversity was obtained for DC (5.6 ± 0.2) compared to PC reactors (4.9 ± 0.2); while in model 3 the Shannon diversity was similar for all tested reactors with values between 6.2 and 6.6.
Figure 2.2: Microbial composition of fecal samples and reactors of PolyFermS models measured by 454 pyrosequencing. Relative abundance at (A) phylum level of fecal samples of donors 2 and 3, IR and DC1 of model 2 and IR and CR of model 3 (B) family level and (C) genus level of fecal samples of donors 2 and 3, all reactors of model 2 and reactors IR, CR, TR3 and TR4 of model 3 identified by pyrosequencing of the V5-V6 hypervariable regions of the 16S rRNA gene. Effluent samples are average values of three last days at the end of the stabilization period. Parentheses indicate an unknown family belonging to an order or an unknown genus belonging to a family or order. Values < 1 % are summarized in the group “others”.

A

B

C
Figure 2.3: PCoA analysis of PolyFermS models based on weighted and unweighted UniFrac analysis. Each symbol is representing a different reactor. (A) Three last days at the end of the stabilization period of all reactors of model 2 (IR, PC1, DC1, PC2 and DC2) and (B) three last days at the end of the stabilization period of model 3 (IR, CR, TR3 and TR4)
Figure 2.4: Shannon diversity index of fecal samples and reactors of PolyFermS models. The Shannon index was assessed in fecal donors 2 and 3, all reactors of model 2 and reactors IR, CR, TR3 and TR4 of model 3 of three last days at the end of stabilization phase. A higher Shannon index reflects a more diverse community (in abundance and evenness).

Metabolite analysis

Metabolites were measured by HPLC in fermentation effluents at the end of the stabilization period of all reactors of models 1, 2 and 3 to assess the metabolic activity and intra model stability (Table 2.2). After the initial stabilization periods, high and stable metabolic activities were measured over the entire fermentation in IR’s of models 2 and 3 which were operated under constant conditions and used to demonstrate temporal stability of the PolyFermS models (Figure 2.5).

The concentrations of SCFA tested in reactors were model and reactor (different colon sections) dependent, while intermediate products lactate and formate remained undetected in fermentation effluents. Acetate was the main metabolite in reactor effluents of all models, followed by butyrate and propionate which were generally produced at similar levels within a reactor. Butyrate concentrations were around 10 mM higher than propionate in CR and TR reactors of model 3. The molar ratios of acetate, butyrate and propionate in IR of the three models were different. In IR of model 1, operated at pH 5.5, a higher acetate fraction was produced (87/6/6) compared to IR of model 2 (60/23/17) and 3 (47/33/20) which were operated at a higher pH of 5.7. Higher concentrations of acetate and propionate were measured in TC and DC reactors of model 1, and DC reactors of models 2 and 3 compared to reactors IR and PCs of the same models, operated with proximal colon conditions. Butyrate concentrations increased along the reactors of the 3-stage model 1, but remained unchanged between PC and DC reactors of models 2 and 3. The mean concentrations of acetate, butyrate
Results

and propionate in the five distal reactors of model 3, were 76.5 ± 6.5, 41.7 ± 6.0 and 30.2 ± 3.4 mM, respectively, with small (less than 8 mM) but significant differences among reactors for acetate and propionate. Valerate was only detected in DC reactors from model 2. BCFA were not detected in any PC reactors. Isovalerate and isobutyrate were present in effluents samples from DC reactors of model 1 and model 2, at higher concentration in the latter. For model 3 isovalerate was only measured at low concentrations (≤ 1.0 mM) close to the detection limit in some DC reactors.

Figure 2.5: Daily mean SCFA concentrations in fermentation effluents of IR of PolyFermS models measured by HPLC. Initial stabilization: stabilization period in continuous mode to reach pseudo steady-state.; stabilized operation mode: continuous operation mode during pseudo steady-state conditions. (A) Model 2 and (B) model 3; (♦) acetate, (●) butyrate, and (■) propionate
### Table 2.2: Metabolites concentration (mM) and ratios (%) measured by HPLC in effluent samples of models’ reactors at the end of the stabilization period.

<table>
<thead>
<tr>
<th></th>
<th>Concentrations (mM)</th>
<th>Ratios (%)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Acetate</td>
<td>Butyrate</td>
<td>Propionate</td>
</tr>
<tr>
<td>Model 1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PC 1</td>
<td>74.1 ± 2.4&lt;sup&gt;A&lt;/sup&gt;</td>
<td>5.4 ± 1.0&lt;sup&gt;A&lt;/sup&gt;</td>
<td>5.4 ± 0.7&lt;sup&gt;A&lt;/sup&gt;</td>
</tr>
<tr>
<td>TC 1</td>
<td>108.6 ± 7.1&lt;sup&gt;B&lt;/sup&gt;</td>
<td>15.4 ± 2.1&lt;sup&gt;B&lt;/sup&gt;</td>
<td>15.3 ± 0.3&lt;sup&gt;B&lt;/sup&gt;</td>
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<tr>
<td>DC 1</td>
<td>110.7 ± 7.8&lt;sup&gt;B&lt;/sup&gt;</td>
<td>15.4 ± 0.8&lt;sup&gt;B&lt;/sup&gt;</td>
<td>15.3 ± 2.0&lt;sup&gt;B&lt;/sup&gt;</td>
</tr>
<tr>
<td>Model 2</td>
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<td>IR 1</td>
<td>66.3 ± 2.7&lt;sup&gt;A&lt;/sup&gt;</td>
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<td>19.1 ± 0.8&lt;sup&gt;A&lt;/sup&gt;</td>
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<td>PC1</td>
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<td>20.8 ± 1.4&lt;sup&gt;AB&lt;/sup&gt;</td>
<td>19.9 ± 0.6&lt;sup&gt;A&lt;/sup&gt;</td>
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<td>DC1</td>
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<td>24.6 ± 0.2&lt;sup&gt;B&lt;/sup&gt;</td>
</tr>
<tr>
<td>PC2</td>
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<td>20.2 ± 3.3&lt;sup&gt;BC&lt;/sup&gt;</td>
<td>26.5 ± 3.3&lt;sup&gt;BC&lt;/sup&gt;</td>
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<td>IR 2</td>
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<td>CR 1</td>
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<td>42.8 ± 1.8&lt;sup&gt;B&lt;/sup&gt;</td>
<td>31.5 ± 2.4&lt;sup&gt;B&lt;/sup&gt;</td>
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<tr>
<td>TR1</td>
<td>76.8 ± 4.1&lt;sup&gt;BC&lt;/sup&gt;</td>
<td>42.7 ± 5.0&lt;sup&gt;BC&lt;/sup&gt;</td>
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<tr>
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<tr>
<td>TR3</td>
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<td>39.7 ± 1.4&lt;sup&gt;B&lt;/sup&gt;</td>
<td>30.9 ± 0.9&lt;sup&gt;B&lt;/sup&gt;</td>
</tr>
<tr>
<td>TR4</td>
<td>73.7 ± 0.7&lt;sup&gt;C&lt;/sup&gt;</td>
<td>42.4 ± 1.9&lt;sup&gt;C&lt;/sup&gt;</td>
<td>28.8 ± 1.1&lt;sup&gt;B&lt;/sup&gt;</td>
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</table>

PC, proximal colon reactor; DC, distal colon reactor; IR, inoculum reactor; CR, control reactor; TR, test reactor.

Data are means ± SD of three last days at the end of the stabilization period; samples were analyzed in duplicate. ND, not detected; BCFA, Branched-chain fatty acids.

Values with different letters are significantly different within one model (p<0.05).
Results

Correlations between microbiota and metabolite production

Pyrosequencing data on the genus level and metabolite concentrations measured by HPLC were investigated to test significant correlations between phylogenetic groups and metabolic activity. For model 2, significant negative correlations were calculated between isobutyrate, isovalerate and valerate concentrations and the dominant genera Ruminococcus and Bacteroides (Figure 2.6). Butyrate was positively correlated with the dominant genus Roseburia and unclassified members of Ruminococcaceae and Lachnospiraceae. In contrast, a dominant unclassified member of Enterococcaceae was negatively correlated with butyrate and positively correlated with all other metabolites detected. Furthermore, many genera detected at less than 1% (Dialister, Anaerococcus and unclassified genera of Rikenellaceae and Mogibacteriaceae) showed positive correlations with isobutyrate, isovalerate and valerate with the exception of Oscillospira, Peptoniphilus and an unclassified genus of Peptostreptococcaceae (with abundances above 1% but in distal colon reactors only).

Correlations between metabolites (only acetate and propionate) and phylogenetic groups were also found for model 3 (Figure S2.1)

![Figure 2.6: Correlations between genus-level phylogenetic groups and metabolites (SCFA, BCFA) of three last days at the end of stabilization period of model 2. The correlations, assessed by Spearman are indicated by either red (positive) and blue (negative), the significant correlations ($q < 0.05$) are indicated by ‘+’. Only genus related phylotypes > 0.1% and with at least one significant correlation with metabolites are depicted. Parentheses indicate an unknown genus belonging to a family or order.](image-url)
Discussion

Colonic fermentation models are useful tools to investigate factors that can influence the composition and metabolism of the gut microbiota, such as diet, antibiotic treatment, and bacterial infections in vitro and independent of the host (Payne et al., 2012a, Lacroix et al., 2015). An important aspect for in vitro studies is the rational design of models and conditions, considering host target, model characteristics and limits, and the recognition that models are not perfect representation of reality.

In the present study, we report the first-time investigation of continuous fermentation models with fecal microbiota obtained from different healthy volunteers aged between 71 and 78 years using in-depth characterization methods of the microbial diversity. We immobilized the fecal microbiota and inoculated the fecal biocatalysts in the inoculum reactor of the tested models with different designs. A dense and diverse microbiota could be established in PolyFermS models, with reproducible microbial composition and metabolic activity for downstream test and control reactors within a model.

During collection and immobilization of fecal microbiota from each elderly donor special attention was paid to keep anaerobic conditions from donor to reactor, in order to reproduce both the planktonic and sessile forms of bacteria in the colon, as previously suggested (Payne et al., 2012a). Gel beads can provide a protective microenvironment for the bacteria and allow the growth of complex and stable gut ecosystems at high cell densities of up to ca. log 11 cells per mL effluent as observed in the present study with elder gut microbiota, preventing the loss of slow growing bacteria. As expected from the lack of water reabsorption total bacteria numbers in reactor effluents of models were up to 1.0 log₁₀ lower compared to the corresponding donor’s fecal sample. All bacterial groups tested in the fecal inoculum with qPCR were present in the corresponding models. The main differences in the bacterial composition and metabolic activity amongst models can be assigned to the different fecal inoculum used. In particular, *Roseburia* spp. was not detected in the feces and effluents of model 1 and this may explain the high acetate and low butyrate concentrations in this model (Table 2.2) since *Roseburia* is a main contributor for the conversion of acetate into butyrate (Duncan et al., 2002b, Belenguer et al., 2006).

The pH of IR of model 1 was set to 5.5 in order to replicate the pH set in previous elder gut fermentation models (Baines et al., 2005, Freeman et al., 2007). However, this pH is in the low range for the human proximal colon in vivo (Nugent et al., 2001, Lawley & Walker, 2013), and for models 2 and 3, the pH in IR and PCs was set to 5.7. This pH elevation
induced an increase in total metabolites by approximately 20%, in agreement with previous observations made in the PolyFermS model with child microbiota (Zihler Berner et al., 2013). In contrast, butyrate concentrations did not increase in model 1 (pH 5.5) relative to model 2 and 3 (pH 5.7), as would have been expected from the stimulation of butyrate production at the lower pH, as observed in the previous study (Zihler Berner et al., 2013). This is likely due to the lack of Roseburia spp. and the low F. prausnitzii numbers in model 1 which are the main butyrate producers in the human gut microbiota (Walker et al., 2005). In models 2 and 3, the microbiota composition tested with qPCR was very similar between IR or PC reactors and distal reactors, while some limited changes were measured with pyrosequencing. In contrast, most targeted populations by qPCR significantly increased from PC (IR) to DC of model 1, suggesting that the low pH of 5.5 limited the growth of the targeted groups.

With qPCR we detected high Enterobacteraeae copy numbers in reactor effluents compared to feces for all three models. This was observed in previous gut fermentation models (Zihler et al., 2010, Tanner et al., 2014b) and may be due to competitive advantage of these fast growing and robust bacteria that allows them to occupy niches during the immobilization process and the succeeding batch fermentation. The low levels of SCFA in the beginning of batch fermentation may further explain the increase in Enterobacteraeae in reactors, as SCFA have inhibitory effects against Enterobacteraeae, such as Escherichia coli (Duncan et al., 2009).

The microbial composition of models 2 and 3 and corresponding fecal inocula may be considered more representative of the elderly population than the fecal inoculum of model 1, which did not harbor Roseburia spp., although the genus Roseburia was assigned at approximately 3% in fecal samples from elderly Irish subjects (Claesson et al., 2011). 454 pyrosequencing was performed using the V5-V6 hypervariable region that was previously used to profile gut microbiota (De Filippo et al., 2010, Montassier et al., 2014, Zhang et al., 2015). In general, similar microbial profiles between effluent samples of model 2 and 3 and corresponding fecal donors were obtained. However, in both models the ratio of Firmicutes:Bacteroidetes was decreased when compared to the fecal sample, likely due to host-related factors including water and metabolite absorption and intestinal cells and host interaction, both of which are lacking in the fermentation models (Van den Abbeele et al., 2010, Zihler Berner et al., 2013). Furthermore, the strictly controlled environmental factors, such as pH, transit time and medium composition in the in vitro models do no fully represent the specific donor conditions, thereby further contributing to in vitro and in vivo variations.
Despite the increase in Bacteroidetes in models 2 and 3, the Firmicutes:Bacteroidetes ratios of the fecal donor and the models were all in the range of previously reported data recorded during a large-scale *in vivo* study with elderly Irish people (Claesson *et al.*, 2011). Indeed large inter-individual variations were observed in this study; however, the Bacteroidetes:Firmicutes ratio was shown to be higher in the elder, relative to the adult population. The majority of the reads was assigned to Firmicutes and Bacteroidetes while only low levels of Proteobacteria and Actinobacteria were detected in concordance with *in vivo* findings (Biagi *et al.*, 2010, Claesson *et al.*, 2011). Many of the predominant genera (abundance > 1%) including *Bacteroides*, *Faecalibacterium*, *Roseburia* and *Ruminococcus* were also found above 1% in elderly Irish subjects (Claesson *et al.*, 2011). No in-depth characterization of the microbiota in donor and effluent samples was reported in previous investigations of fermentation models of the elderly microbiota, in which only traditional plating methods (Baines *et al.*, 2005, Crowther *et al.*, 2014) or FISH (Likotrafiti *et al.*, 2014) were used.

SCFA are mainly produced from carbohydrate fermentation and to a lesser extent via degradation of proteins and amino acids; the effects of SCFA on the host are well documented (Chassard & Lacroix, 2013). It was previously found that the major SCFA found in stools of healthy volunteers between 14 – 74 years of age were: acetate, propionate and butyrate at an approx. ratio of 3:1:1 (Schwiertz *et al.*, 2010). In our study, similar ratios were found in distal colon reactors of models 2 (4:1.5:1) and 3 (3:1.5:2), whereas in model 1 the acetate fraction was considerably higher (7:1:1), likely due to the low pH of 5.5 in IR and the fecal microbiota used in this model as discussed above. *In vivo* investigations are, however, hampered by the continuous absorption of metabolic products, which results in less than 5% of total production excreted in the feces (den Besten *et al.*, 2013) along with the difficulty associated with obtaining samples from different regions of the colon. Therefore, metabolite concentrations and ratios in feces are not indicative for the colonic microbiota activity. In contrast *in vitro* modeling allows accurate measuring of the metabolic activity of the gut microbiota for the tested model conditions. Stable SCFA concentrations were obtained throughout the fermentation in the untreated IR’s of the PolyFermS models demonstrating maintenance of gut microbial activity over the entire fermentation of 55 and 80 days for model 2 and 3, respectively (Figure 2.5).

BCFA are products of protein and amino acid fermentation but the formation of BCFA and associated species is not well studied (Nyangale *et al.*, 2012). In the colon of elders, an
increase in proteolytic activity and a decrease in concentrations of SCFA were reported (Woodmansey, 2007, Tiihonen et al., 2008). Metabolites of amino acid fermentation can have toxic effects on the colonic lumen and were associated with several gut disorders (Nyangale et al., 2012). In the tested models, BCFA were solely detected in significant levels within the distal reactors of model 1 and 2. This observation is consistent with the understanding that the distal colon is the major site for proteolysis whereas carbohydrate fermentation is the main energy yielding process in the proximal colon, resulting in a lower pH in this section (Cummings & Macfarlane, 1991, Duncan et al., 2009). In model 2, genera with abundances of less than 1% were positively correlated with isobutyrate, valerate and isovalerate, suggesting that the dominant bacteria were mainly responsible for saccharolytic fermentation while proteins were degraded by the subdominant populations. Very low or no BCFA were detected in CR and TR reactors of model 3 which was set to mimic fermentation of transverse-distal colon sections within one reactor. This may be explained by the microbiota composition of model 3 that was different from model 1 and 2. The microbiota-dependent production of BCFA suggests the importance of using individual microbiota for inoculating intestinal fermentation models instead of pooling microbiota from different donors, as done in many studies for inoculation of gut fermentation models.

A major feature of the PolyFermS models over the three-stage model (model 1) is that several treatments can be investigated simultaneously and compared to a control inoculated with the same microbiota, thus generating reproducible and accurate data rather than when treatments are applied during consecutive periods. In both PolyFermS models microbial diversity and metabolic activity was very similar between control and test reactors. Model 2 built with two sets of proximal and distal colon reactors can be used for a broad range of studies, in proximal and distal colon conditions, such as the effect of an altered diet and administration of antibiotics on the gut microbiota in old age. Furthermore, the model is applicable for the in vitro investigation of the elderly microbiota in combination with health-related questions such as the manipulation of the gut microbiota using pro- and prebiotics (Tanner et al., 2014a). PolyFermS model 3 built with multiple parallel distal colon reactors can be especially useful to study the effect of factors related to age on microbial metabolism in the lower colon, such as promotion of putrefaction due to low fiber intake. The PolyFermS intestinal platform has potential to be scaled down and adapted with multi-reactors to enhance screening efficiency.
To conclude, in the present study we showed the stability and reproducibility of PolyFermS continuous colonic fermentation models inoculated with immobilized elderly microbiota. Immobilization requires only small amounts of high quality fecal material to prime a gut model that can be stably operated over several months for testing parallel treatments in consecutive blocks (Tanner et al., 2014a). The PolyFermS platform should be suitable for a range of in vitro gut microbiota investigations, from classical microbe interaction studies to complex ecological studies of the elderly gut microbiome investigated by in-depth analysis of the microbial diversity.

Acknowledgments

We thank Liselotte Wallquist, Lukas Meile, Simon Galenda and Markus Reichlin for technical assistance. The research presented in this paper was funded by Danone Research (Palaiseau, France).
# Supporting information

**Table S 2.1:** Primers used for enumeration of bacterial groups by qPCR.

<table>
<thead>
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<th>Name</th>
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<td>Total 16S rRNA genes</td>
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<td>Bacteroides-Prevotella group</td>
<td>(Ramirez-Farias et al., 2009)</td>
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<td>Roseburia spp./E. rectale</td>
<td>(Ramirez-Farias et al., 2009)</td>
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<tr>
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<td>Clostridium cluster IV</td>
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<td>(Tymensen &amp; McAllister, 2012)</td>
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**Figure S 2.1:** Correlations between genus-level phylogenetic groups and acetate and propionate of three last days at the end of the stabilization period of model 3. The correlations, assessed by Spearman are indicated by either red (positive) and blue (negative), the significant correlations ($q < 0.05$) are indicated by ‘+’. Only genus related phylotypes > 0.1% and with at least one significant correlation with metabolites are depicted.
Chapter 3

**In vitro** investigation of *Clostridium difficile* colonization and antibiotic treatments using the PolyFermS model platform mimicking elderly intestinal fermentation

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Manuscript to be submitted.
Abstract

*Clostridium difficile* (CD), a spore-forming and toxin-producing bacterium, is the main cause for antibiotic-associated diarrhea in the elderly. Here we investigated the colonization of CD in novel *in vitro* intestinal fermentation models inoculated with immobilized elderly fecal microbiota and the effects of antibiotic treatments.

Two PolyFermS model setups were applied each inoculated with microbiota from a different elderly donor and either designed to mimic proximal and transverse-distal colon conditions (model 1) or for multiple parallel treatment investigations at transverse-distal colon conditions (model 2). The colonization of two CD strains of different PCR ribotypes, and supplied as vegetative cells (ribotype 001 in model 1) or spores (ribotypes 001 and 012 in model 2) was tested. Additionally treatments with ceftriaxone (daily 150 mg L\(^{-1}\)) and metronidazole (twice 333 mg L\(^{-1}\) daily) were investigated (model 2) for their effects on gut microbiota composition (qPCR, 16S pyrosequencing) and activity (HPLC), CD spore germination and colonization, and cytotoxin titer (Vero cell assay).

CD remained undetected after inoculating vegetative cells in proximal colon of model 1, but was shown to colonize transverse-distal reactors of both models, reaching copy numbers of up to \(\log_{10} 8\) per mL effluent with stable production of toxin correlating with cell numbers. Ceftriaxone treatment in transverse-distal colon showed only small effects on gut microbiota composition and activity and did not promote CD colonization compared to antibiotic-free control reactors. In contrast treatment with metronidazole after colonization of CD induced large modifications in the microbiota and decreased CD numbers below the detection limit of the specific qPCR. However, a fast CD recurrence was measured only two days after cessation of metronidazole treatment.

We showed that in our *in vitro* models, independent of host factors, stable CD colonization was induced by CD vegetative cells or spores but was limited to the transverse-distal section, and did not require the application of ceftriaxone. Furthermore treatment with metronidazole induced dysbiosis of the gut microbiota, and only reduced temporarily the counts of CD, in agreement with recurrence of CD infection *in vivo*. This study suggests that an undisturbed microbiota alone does not inhibit CD colonization thus indicating an important contribution of host-related factors in the protection against CD infection.
**Introduction**

*Clostridium difficile* (CD) was first identified in the 1970s as the causative agent of antibiotic-associated pseudomembranous colitis and is now the leading cause of hospital-acquired diarrhea (Zucca *et al.*, 2013). CD is a gram-positive anaerobic bacterium harboring several virulence factors such as the ability to form spores and produce toxins (Rupnik *et al.*, 2009). Interestingly, CD is part of the normal gut microbiota in 25 to 80% of infants but usually does not cause disease although a significant fraction of the CD strains were found to be toxin producers (Adlerberth *et al.*, 2014). One of the main functions of an undisturbed gut microbiota is resistance against colonization of pathogens (Kamada *et al.*, 2013). The disruption of the microbiota and thus the colonization resistance is usually the first step in the pathogenesis of CD infection (CDI). Following ingestion of CD spores, germination into the vegetative form is necessary for colonization in the gut, with subsequent toxin production leading to clinical manifestations (Vedantam *et al.*, 2012). Treatment with broad-spectrum antibiotics, such as ampicillin, clindamycin and third-generation cephalosporins (ceftriaxone, cefotaxime and ceftazidime) are considered as main risk factor for CDI (Oldfield *et al.*, 2014). The elderly population is especially at risk to develop CDI, with CD colonization rates of up to 73% in hospital patients above 65 years (Deneve *et al.*, 2009). Standard therapy for CDI is antibiotic treatment with metronidazole or vancomycin. However, efficacy of these antibiotics is limited, with recurrence observed in 20 - 40% of the cases, mainly due to development of antibiotic resistances and a loss of gut barrier function that allows residual CD to re-colonize the colon after the antibiotic treatment is cancelled (Zucca *et al.*, 2013). Indeed the important role of an undisturbed microbiota that provides colonization resistance is reflected in the high success rate of fecal microbiota transplantation of up to circa 92% that is used for severe CDI cases (Gough *et al.*, 2011).

Various animal models have been used to study CDI mechanisms and investigate antibiotic treatments. However, the use of animals is restricted due to ethical and practical reasons and notable inter-species differences in susceptibility to CDI. *In vitro* gut fermentation models represent an innovative technological platform consisting of multiple model designs which permit investigations, including survival or mechanistic studies on commensal–pathogen interactions and drug testing (Payne *et al.*, 2012a, Lacroix *et al.*, 2015). Different *in vitro* intestinal models with CDI, from simple batch to multistage continuous systems, have also been reported (Best *et al.*, 2012). Mainly batch culture systems were applied to study CD interactions with fecal microbiota with the aim to test alternative treatments to antibiotics.
(Hopkins & Macfarlane, 2003, Meader et al., 2010, Rea et al., 2011b, Tejero-Sarinena et al., 2013). A three-stage intestinal model which may be more representative for colonic conditions, inoculated with pooled fecal microbiota from elderly donors was applied in a series of studies with different CD ribotypes and antibiotic treatments, as reviewed by Best et al. (2012). With this model CD spores were inoculated while the antibiotic clindamycin was used to induce CD spore germination (Freeman et al., 2005, Freeman et al., 2007, Baines et al., 2009b). However monitoring of intestinal microbiota composition was limited to cultivation methods targeting a restricted range of gut bacteria and no metabolic assessment was reported. Furthermore no study has yet reported in-depth analysis of CD colonization ability in in vitro continuous fermentation models, using molecular methods and next generation sequencing to describe microbiota effects.

We have recently setup and validated a new PolyFermS continuous model platform, inoculated with immobilized fecal microbiota, mimicking different sections of the elderly colon (chapter 2). We showed that the models closely reproduce the gut microbiota composition, density and activity of the fecal donor. Furthermore the PolyFermS platform allows testing simultaneously several treatments compared to a control in parallel reactors inoculated with the same fecal microbiota generated in the upstream inoculum reactor (IR) seeded with immobilized fecal microbiota (Zihler Berner et al., 2013, Tanner et al., 2014b).

The aim of this study was to establish a robust in vitro continuous colonic fermentation for CDI in the elderly, based on the recently developed elderly PolyFermS platform (chapter 2). We investigated different modes of CD colonization (vegetative cells and spores) of a clinical isolate of PCR ribotype 012 and a type strain of the epidemic PCR ribotype 001, as well as the effects of two antibiotics (with ceftriaxone or metronidazole) known to promote or used to treat CDI.
Materials and Methods

C. difficile strains

C. difficile DSM 1296 (PCR ribotype 001) and C. difficile NCTC 13307 (PCR ribotype 012) were purchased from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany) and the National Collection of Type Cultures (NCTC, Salisbury, United Kingdom), respectively. Vegetative cells of CD DSM 1296 and NCTC 13307 were cultured from glycerol stocks (33%, -80°C) and routinely grown in BHI supplemented with 0.05% (w/v) L-cysteine (BHIS) in anaerobic Hungate tubes. Vegetative CD cells for inoculation of reactors were grown in serum flasks containing chyme fermentation medium at 37°C for 15 h. The medium composition was based on that described by MacFarlane et al. (1998) for simulation of adult ileal chyme, and prepared according to chapter 2. Anaerobic conditions in serum flasks were achieved by flushing the headspace with N₂ and CO₂ at 3:1 ratio. Spores of CD DSM 1296 and NCTC 13307 were prepared according to Sorg and Dineen (2009). Shortly, several large petri dishes were spread with each 150 µL of an overnight culture of CD and incubated in an anaerobic chamber at 37°C for 10 days. Growth on the agar was collected by flooding the agar with sterile ice-cold water. The suspension of vegetative cells and spores was kept at 4°C overnight in order to enhance the release of spores from mother cells. Then, the suspension was centrifuged (14’000 g, 1 min) and the pellet was washed several times with ice-cold water. Histodenz (Sigma-Aldrich) was used to separate the free spores from vegetative bacteria and cell debris. The spore pellet was washed several times with ice-cold water and finally resuspended in 200 µL sterile water. For spore enumeration a sample was plated on BHI agar containing 0.1% taurocholate (Sigma) and incubated for 48 h in an anaerobic chamber at 37°C. Spore suspensions were adjusted to a concentration of 10⁷ cfu mL⁻¹ using sterile water, and kept at 4°C until further use.

Antibiotics

Rocephin® (ceftriaxone sodium, >90% purity, Roche, Pharma AG, Reinach, Switzerland) was diluted in sterile ultra-pure water (1 g in 10 mL) and 340 µL of the solution was added once a day, or in two separate doses, to the reactors corresponding to a total added amount of 150 mg L⁻¹. This concentration was adapted from Baines et al. (2011) and is based on fecal ceftriaxone levels found in feces of volunteers with a mean concentration of 152 mg kg⁻¹ on day four of treatment (Pletz et al., 2004). Crystalline metronidazole (Sigma-Aldrich, Buchs, Switzerland) was added twice daily directly to reactors to provide a final concentration of 333
mg L\(^{-1}\) with each addition. Usually only low concentrations of metronidazole are measured in stool because metronidazole is partially metabolized by gut bacteria (Newton et al., 2013). It was therefore difficult to select the physiological antibiotic concentration as in the gut. Concentrations used in other \textit{in vitro} studies ranged from 9.3 to 330 mg L\(^{-1}\) (Freeman et al., 2007, Meader et al., 2010, Rea et al., 2011b). In this study we applied a rather high metronidazole concentration to account for the high numbers of CD in the reactors.

**Fermentation medium**

The fermentation medium was based on the composition described by MacFarlane et al. (1998) for simulation of adult ileal chyme. Ingredients and preparation were described previously (chapter 2).

**Fecal inoculum and fermentation setup**

The experimental setup of the two continuous \textit{in vitro} fermentation models (PolyFermS models) is schematically illustrated in Figure 3.1, and a detailed description of the experimental procedure is presented in chapter 2. Briefly, fresh fecal samples were obtained from two healthy women, aged 72 (model 1) and 78 (model 2) who did not receive antibiotic treatment at least during the previous three months and did not consume probiotics on a regular basis. The fecal microbiota was processed and immobilized in gellan-xanthan beads as described previously (chapter 2).

The two models were composed of a first inoculum reactor (IR) seeded with 30\% (v/v) polysaccharide fecal beads, operated with conditions selected for the elder proximal colon (37°C, retention time of 9 h, pH 5.7). In model 1 the IR was used to continuously inoculate (10\% v/v of the feed) reactors operated with proximal (PC, retention time of 9 h, pH 5.7) and transverse-distal colon conditions (TDC, 37°C, retention time of 18 h, pH 6.8). The PC reactor was continuously fed with 10\% effluent from IR and 90\% fresh medium to mimic the human chyme (see above). For model 2 the IR was directly connected to five downstream reactors mounted in parallel (one control reactor (CR) and four test reactors (TR1–4)), 100\% fed with effluent produced in IR and operated with conditions mimicking fermentation in the transverse-distal colon section (37°C, retention time of 25 h, pH 6.8).

**Experimental design**

Establishment and detailed characterization of the colonization and stability of the two continuous colonic fermentation models was described in chapter 2.

Model 1 was used to test colonization of vegetative cells CD 1296 in PC and TDC colon conditions. CD cells (log\(_{10}\) 9.8 cfu) were spiked into PC (250 mL fermentation volume) after
18 days of model stabilization, indicated as day 1 (Figure 3.1). Effluents of PC and TDC reactors were analyzed after 6 h and daily over a total period of 16 days.

Model 2 was used to test the germination of spores and colonization of vegetative cells of two CD ribotypes, with and without simultaneous treatment with antibiotics (ceftiraxone or metronidazole). Treatments were applied in TR1-4 operated at TDC conditions during six consecutive periods over 40 days (Figure 3.1). CR served as control reactor during the entire experiment, with no treatment applied. During period A, CD spores (10^7 cfu) were added once in TR1 and TR2 (strain 1296) and TR3 and TR4 (strain 13307) after 14 days of model stabilization (indicated as day 1). Rocephin® was supplied twice daily for five days in TR1 and TR3 to supply a concentration of 75 mg L^{-1} per injection in the fermentation volume [Period A]. However no effect of antibiotic was observed on the microbiota composition and activity. Therefore reactors were disconnected from IR during the treatments [Period B-F] and fed with 100% fresh fermentation medium to prevent the continuous microbiota supply from IR that could mask the effects of antibiotics. During period B, CD spores (10^7 cfu, strain 1296 in TR1 and TR2, and strain 13307 in TR3 and TR4) were inoculated on two consecutive days (days 6 and 7) and Rocephin® was added once daily during five days (150 mg L^{-1}). During period C spores of strain 13307 were added to TR1 and TR2 previously treated with spores of strain 1296, but in TR1 no CD cells were detected. CD was allowed to colonize reactors TR1 and TR4 during another five days [Period D] while TR2 and TR3 were used to test probiotic treatments which are not shown in this study. During period E addition of metronidazole (333 mg L^{-1} twice daily) was tested in TR1 and TR4 during 10 days, followed by 10 days recovery [Period F] without antibiotic addition.
Figure 3.1: Experimental design of the continuous fermentation models. (A) Model 1. Vegetative CD cells of strain DSM 1296 were added to PC on day 1 and growth was observed in PC and TDC during 16 days. (B) Model 2. CR and TR’s were fed with effluent from IR during period A and with fermentation medium during period B-F. Addition of CD spores (10^7 cfu) of strain 1296 or 13307 was performed in TR’s as indicated. TR1 and TR4 were treated with metronidazole during period E.

IR, inoculum reactor; PC, proximal colon; TDC, transverse-distal colon; CR, control reactor; TR, test reactor. CRO I: 75 mg L^-1 ceftriaxone addition twice daily; CRO II + III: 150 mg L^-1 ceftriaxone addition once daily; CD, C. difficile; MTZ, metronidazole; REC, Recovery.
Materials and Methods

qPCR analysis

Genomic DNA was extracted from fermentation effluent samples (2 mL) using the FastDNA® SPIN Kit for Soil (MP Biomedicals, Illkirch, France) and a final elution volume of 100 µL. Total bacteria and specific bacterial groups prevalent in the gut were enumerated using previously described primers (chapter 2). CD was quantified using primers (forward, 5’-TTG AGC GAT TTA CTT CGG TAA AGA-3’ and reverse, 5’- CCA TCC TGT ACT GGC TCA CCT -3’) and conditions described previously (Rinttila et al., 2004). One µL of 10- or 100 fold diluted DNA was amplified in a total volume of 25 µL as described in Zihler et al. (2010) using 2 x SYBR Green PCR Master Mix (Applied Biosystems, Zug, Switzerland). Each reaction was run in duplicate on an ABI PRISM 7500-PCR sequence detection system (Applied Biosystems). For quantification, standard curves were produced by amplification of the DNA of the reference strain of the respective target group.

HPLC analysis

Short chain fatty acids (SCFA; acetate, propionate, butyrate and valerate), lactate, formate and branched-chain fatty acids (BCFA; isobutyrate and isovalerate) in fermentation effluent samples from all reactors were determined daily by HPLC analysis in duplicate (Thermo Fisher Scientific Inc. Accela, Wohlen, Switzerland) (Tanner et al., 2014b). Effluent supernatants (500 µL) were 2-fold diluted with sterile ultra-pure water and filtered directly into vials through a 0.45 µm nylon HPLC filter (Infochroma AG, Zug, Switzerland). The analysis was run at a flow rate of 0.4 mL min⁻¹ using an Aminex HPX-87H column (Bio-Rad Laboratories AG, Reinach, Switzerland) and 10 mM H₂SO₄ as eluent.

Microbiota profiling by 454 pyrosequencing

454-pyrosequencing analysis of total genomic DNA was carried out at DNAVision (Gosselies, Belgium). The V5-V6 hypervariable 16S rRNA region was amplified using specific primers 784F (5’- AGGATTAGATAACCCTKGTAGA-3’) and 1061R (5’- CRRCACGAGCTGACGACGAC-3’) (Andersson et al., 2008). The forward primer contained the sequence of the Titanium A adaptor and a unique barcode sequence. Pyrosequencing was carried out using primer A on a 454 Life Sciences Genome Sequencer FLX instrument (Roche Applied Science, Vilvoorde, Belgium) following Titanium chemistry. The obtained data was analyzed using the open source software package Quantitative Insights Into Microbial Ecology (QIIME), v1.7 (Caporaso et al., 2010). Briefly, raw sequencing reads were filtered based on selected quality criteria such as: (1) no mismatch with the primer sequences and barcode tags, (2) no ambiguous bases (Ns), (3) read-lengths not shorter than 200 base pairs (bp) or longer than 1000 bp, (4) the average quality score in a sliding window
of 50 bp not to fall below 25) excluding homopolymer runs higher than 6 nt. Sequences that passed quality filtering were clustered into OTUs at 97% identity level using usearch (Edgar, 2010). Representative sequences (the most abundant) for each OTU were aligned using PyNAST and taxonomically assigned using Silva database (108). These phylogenies were combined with absence/presence or abundance information for each OTU to calculate unweighted or weighted UniFrac distances, respectively using rarefaction of 7000 sequences per samples. Principal coordinates analysis (PCoA) was applied to the distance matrices for visualization. Alpha diversity (diversity within sample) was calculated using Shannon (evenness) indexes.

**Vero cell analysis**

CD toxin production in reactor effluents of model 2 was estimated using Vero cell cytotoxicity assays as described previously (Freeman et al., 2003, Vohra & Poxton, 2011). Briefly, 500 µL reactor effluent samples were centrifuged (10’000 g, 10 min) and the supernatant was filtered through 0.45 µm membrane filter (Infochroma AG, Zug, Switzerland) and subsequently serially diluted in peptone buffered saline (PBS) to 10⁻⁷. Twenty µL thereof was then mixed with 30 µL of cell media Dulbecco’s Modified Eagle’s medium (DMEM, Life Technologies) and added in duplicate to Vero cell culture monolayers prepared in 96-well microtiter plates. The cell culture toxin assay trays were incubated at 37°C in 5% CO₂ atmosphere and read after 48 h under an inverted microscope. A positive cytotoxin activity was indicated by cell rounding, with the end-point titer defined as the last dilution at which 50% rounded cells were measured. The action of CD cytotoxin in the samples was confirmed by neutralization with CD antitoxin (Alere Health BV, Tilburg, NL).

**Statistical analysis**

Statistical analysis of HPLC and qPCR data was performed using JMP 8.0 (SAS Institute Inc., Cary, NC). HPLC and qPCR data are expressed as means ± SD of the last three days of each treatment period. qPCR data were log₁₀-transformed. qPCR and HPLC data among reactors were compared using the nonparametric Kruskal-Wallis test. p-values < 0.05 were considered significant.
Results

Colonization of CD vegetative cells

To evaluate the colonization of CD vegetative cells in both proximal and distal colon reactors of model 1, strain 1296 cells were inoculated in PC ($\log_{10} 9.8$ cfu in 250 mL), and colonization was monitored by qPCR during 16 days continuous fermentation (Table 3.1). The gene copy number of CD was 7.4 and 7.3 $\log_{10}$ copies mL$^{-1}$ in PC and TDC reactors, respectively, 6 h post spiking. In both reactors a wash-out was observed until day 3, after which CD numbers reached below detection limit of the qPCR test ($\log_{10} 4.4$ copy numbers). However, CD was detected again after day 9 continuous culture in the distal reactor TDC, reaching high copy numbers of $\log_{10} 7.8$ per mL on day 16 post-spiking, while CD did not recover in PC. Therefore for further experiments model 2 was designed to investigate CD colonization in TDC treatment reactors.

Table 3.1: qPCR enumeration of CD strain 1296 in effluent samples of PC and TDC of model 1.

<table>
<thead>
<tr>
<th>Days post CD instillation</th>
<th>PC (gene copies mL$^{-1}$)</th>
<th>TDC (gene copies mL$^{-1}$)</th>
</tr>
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<tr>
<td>0.25</td>
<td>7.4</td>
<td>7.3</td>
</tr>
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</tr>
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<td>11</td>
<td>ND</td>
<td>6.4</td>
</tr>
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</tr>
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<td>ND</td>
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</tr>
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<td>7.8</td>
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<tr>
<td>16</td>
<td>ND</td>
<td>7.8</td>
</tr>
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</table>

Data are mean $\log_{10}$ copies 16S rRNA gene mL$^{-1}$ fermentation effluent from 6 h after CD vegetative cells inoculation ($\log_{10} 10$ copy numbers) and during the following 16 days. Samples were analyzed in duplicate. The detection limit of qPCR of CD was $\log_{10} 4.4$ copy numbers. PC, proximal colon; TDC, transverse-distal colon; CD, C. difficile; ND, not detected.
Colonization of CD spores and susceptibility to ceftriaxone

The effect of ceftriaxone treatment on spore outgrowth and colonization of CD 1296 and 13307 was investigated in model 2 reactors mimicking TDC conditions (Figure 3.2). In period A CD spores (10⁷ cfu) were added in TR1 and TR2 (strain 1296), and in TR3 and TR4 (strain 13307), while treatment with ceftriaxone (75 mg L⁻¹, twice daily) was performed simultaneously in TR1 and TR3. In none of the TR’s CD growth was detected after five days. In addition no significant differences in the bacterial groups concentration or metabolite concentrations (SCFA, BCFA and lactate) in effluent samples of both reactors was found compared to CR, using qPCR and HPLC, respectively (see below). Therefore during subsequent test periods CR and TR’s were disconnected from IR and fed with fresh medium only in order to prevent a continuous supply of cells from IR that could have prevented the effect of the antibiotic. In period B a second inoculation of CD spores (10⁷ cfu), similar to period A, was applied on two consecutive days and ceftriaxone treatment (150 mg L⁻¹, once daily) was also applied in TR1 and TR3 for five days. CD growth was detected after two to five days (corresponding to seven to 10 days after first inoculation of spores) in TDC reactors with (TR3) and without (TR4) antibiotic for strain 13307, and in TDC reactor with no antibiotic (TR2) for strain 1296, but not in antibiotic treated reactor TR1 (Figure 3.2A). For period C, CD spores (10⁷ cfu) of strain 13307 were instilled twice in TR1 (with ceftriaxone) and TR2 (without) to confirm the better colonization of spores of strain 13307. Only small effects were measured for the additional spore inoculation in TR2, while CD growth was not detected after five days in TR1. When antibiotic application in TR1 was discontinued, CD growth in TR1 was detected after four days in period D and reached log₁₀ 8.8 mL⁻¹ at the end of this period.

Similar high CD numbers of 8.3 to 8.7 log₁₀ copies mL⁻¹ were determined in TR2, TR3 and TR4 15 days after the first inoculation of spores (Figure 3.2A). Cytotoxin activity in TR2 – TR4 was identified shortly after first detection of CD cell growth and ranged between 2.9 and 3.9 log₁₀ cytotoxin titer mL⁻¹, with highest cytoxin titers measured in TR2 (no antibiotic and inoculation of both CD strains).

Growth and toxin production of CD during metronidazole treatment

Treatment with metronidazole was investigated in TR1 and TR4, colonized with both CD strains (log₁₀ 8.8 copy numbers mL⁻¹) or with strain 13307 (log₁₀ 8.5 copy numbers mL⁻¹), respectively (Period E, from day 21 to day 30, Figure 3.2B). CD gene copy numbers decreased rapidly after one day reaching the detection limit of the qPCR test after three and
Results

six days of metronidazole treatment in TR1 and TR4, respectively. Toxin production steadily decreased from 2.8 and 3.6 \( \log_{10} \) cytotoxin titer mL\(^{-1}\) before starting the antibiotic to non-detectable levels after one and two days metronidazole treatment in TR1 and TR4, respectively. CD was detected again at low numbers of \( \log_{10} \) 6.3 gene copies mL\(^{-1}\) in TR4 on day 9 of antibiotic treatment but not in TR1.

The recovery of CD growth and toxin production after cessation of metronidazole treatment was monitored for 10 days (Period F, Figure 3.2B). After only two days, CD was determined at high numbers of around \( \log_{10} \) 9 copies mL\(^{-1}\) in both TR1 and TR4. The toxin titer in TR1 during the first four days increased to up to \( 5 \log_{10} \) cytotoxin titer mL\(^{-1}\), and steadily decreased thereafter to reach \( 3.7 \log_{10} \) cytotoxin titer mL\(^{-1}\) after 10 days. In contrast toxin production was detected after two days in TR4 with constant titers until the end of the period (average values of 3.34 ± 0.09).
Figure 3.2: CD copy numbers and cytotoxin titers in TR’s of model 2. (A) Period B and C in TR2-4. CD growth was first detected seven days post initial CD spores instillation and copy numbers and cytotoxin titers were monitored during remaining days of period B and the entire period C in TR2 - TR4 (distal colon) of model 2. TR3 was additionally treated with ceftriaxone (=CRO) during period B. TR1 was treated with CRO during period B and C but no CD growth was detected in this reactor during these periods. (B) Period D-F in TR1 and TR4. In TR1 CD was first detected 19 days post initial CD spores instillation and copy numbers were continuously monitored during the remaining fermentation days. 21 days after spores instillation metronidazole treatment [E] was performed in TR1 and TR4 for 10 days and followed by a recovery period [F] of 10 days.

CD copy numbers and cytotoxin titers were determined by qPCR and Vero cell assays, respectively. (---) CD detection limit of 4.4 copies mL⁻¹.
Effect of ceftriaxone on gut microbiota composition, diversity and metabolites

Abundance of selected bacterial groups in ceftriaxone treated reactor TR1 during period A and B compared to CR is shown in Figure 3.3A and mean copy numbers during the three last days of period A and B of all tested groups are listed in Table 3.2 for CR and both antibiotic treated reactors TR1 and TR3.

Treatment with ceftriaxone was investigated in model 2 using different approaches. During the first period [A] CR and all TR’s were connected to IR and continuously fed with 100% effluent from IR. No significant difference in the bacterial groups concentration in effluent samples of both reactors was observed compared to CR using qPCR (Period A, Table 3.2 and Figure 3.3A). As a consequence a second treatment period with ceftriaxone was initiated, with CR and TR’s disconnected from IR and instead supplied with 100% fresh fermentation medium. A significant decrease of $1.5 \log_{10}$ in Bifidobacterium spp. was observed in both TR1 and TR3 compared to CR. Small but significant lower copy numbers were also observed for Clostridium cluster IV and Roseburia spp. (only TR3) compared to CR (Period B, Table 3.2). The microbial composition and diversity of effluent samples of TR3 was determined with pyrosequencing and compared to CR during the last three days of the 2nd ceftriaxone treatment [Period B]. A similar composition was measured at the genus level in TR3 and CR (Figure S3.1), with overlapping clusters based on UniFrac distances (Figure 3.5). The average Shannon diversity index measured in effluent samples of the three last days of period B in TR3 and CR were not different, with $3.9 \pm 0.2$ and $3.8 \pm 0.2$, respectively.

Metabolite concentration profiles during the entire fermentation periods A and B are shown for TR1 and compared to CR (Figure 3.3B). Mean metabolite concentrations during the three last days are shown for CR, TR1 and TR3 in Table 3.3. Metabolite concentrations were similar in ceftriaxone treated reactors TR1 and TR3 compared to CR during both periods A and B when reactors were connected or disconnected from IR, respectively, except for slightly higher acetate concentration in TR3 during period B.
Figure 3.3: Effect of antibiotic treatment on gut microbial groups and metabolites. (A) Abundance of selected microbial groups tested with qPCR in ceftriaxone (=CRO) treated reactor TR1 relative to CR during period CRO I [A] and CRO II [B]. (B) Daily mean metabolites concentrations in TR1 and CR during period CRO I [A] and CRO II [B] assessed with HPLC. (C) Abundance of selected microbial groups tested with qPCR in metronidazole treated reactor TR4 relative to CR during metronidazole (=MTZ) [E] and recovery (=REC) period [F]. (D) Daily mean metabolites concentrations in CR and metronidazole treated reactor TR4 during MTZ [E] and REC period [F].
### Table 3.2: qPCR enumeration of bacterial groups in effluent samples of antibiotic treatment [A, B and E] and recovery [F] periods in TRs compared to CR of model 2.

<table>
<thead>
<tr>
<th></th>
<th>Total 16S rRNA gene</th>
<th>Bacteroides spp.</th>
<th>Enterobacteriaceae</th>
<th>Lactobacillus spp.</th>
<th>Bifidobacterium spp.</th>
<th>F. prausnitzii</th>
<th>Clostridium cluster IV</th>
<th>Roseburia spp./E. rectale</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Period A (CRO I)</strong></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CR</td>
<td>11.3 ± 0.2</td>
<td>10.3 ± 0.1</td>
<td>9.8 ± 0.2</td>
<td>7.3 ± 0.3</td>
<td>8.4 ± 0.3</td>
<td>9.0 ± 0.1</td>
<td>9.8 ± 0.2</td>
<td>7.4 ± 0.1</td>
</tr>
<tr>
<td>TR1</td>
<td>11.2 ± 0.2</td>
<td>10.1 ± 0.1</td>
<td>9.7 ± 0.1</td>
<td>7.3 ± 0.2</td>
<td>8.3 ± 0.4</td>
<td>8.9 ± 0.1</td>
<td>9.7 ± 0.2</td>
<td>7.4 ± 0.1</td>
</tr>
<tr>
<td>TR3</td>
<td>11.4 ± 0.07</td>
<td>10.1 ± 0.1</td>
<td>9.8 ± 0.3</td>
<td>7.5 ± 0.1</td>
<td>8.2 ± 0.1</td>
<td>8.9 ± 0.2</td>
<td>9.5 ± 0.5</td>
<td>7.4 ± 0.1</td>
</tr>
<tr>
<td><strong>Period B (CRO II)</strong></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CR</td>
<td>11.3 ± 0.4</td>
<td>10.3 ± 0.5</td>
<td>9.6 ± 0.3</td>
<td>8.3 ± 0.3</td>
<td>8.2 ± 0.2</td>
<td>8.9 ± 0.3</td>
<td>9.1 ± 0.2</td>
<td>6.5 ± 0.1</td>
</tr>
<tr>
<td>TR1</td>
<td>11.3 ± 0.04</td>
<td>10.6 ± 0.3</td>
<td>9.7 ± 0.2</td>
<td>8.3 ± 0.1</td>
<td>6.7 ± 0.2*</td>
<td>8.6 ± 0.4</td>
<td>8.8 ± 0.1*</td>
<td>6.0 ± 0.4</td>
</tr>
<tr>
<td>TR3</td>
<td>11.4 ± 0.1</td>
<td>10.5 ± 0.1</td>
<td>9.5 ± 0.1</td>
<td>8.5 ± 0.2</td>
<td>6.7 ± 0.3*</td>
<td>8.6 ± 0.5</td>
<td>8.8 ± 0.1*</td>
<td>6.1 ± 0.2*</td>
</tr>
<tr>
<td><strong>Period E (MTZ)</strong></td>
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</tr>
<tr>
<td>CR</td>
<td>10.9 ± 0.2</td>
<td>9.6 ± 0.2</td>
<td>10.4 ± 0.2</td>
<td>7.6 ± 0.2</td>
<td>7.1 ± 0.3</td>
<td>9.1 ± 0.1</td>
<td>9.7 ± 0.1</td>
<td>5.8 ± 0.1</td>
</tr>
<tr>
<td>TR1</td>
<td>11.2 ± 0.2</td>
<td>9.5 ± 0.2</td>
<td>9.4 ± 0.5*</td>
<td>7.7 ± 0.3</td>
<td>9.5 ± 0.2*</td>
<td>5.7 ± 0.1*</td>
<td>5.9 ± 0.2*</td>
<td>5.1 ± 0.2*</td>
</tr>
<tr>
<td>TR4</td>
<td>11.1 ± 0.1</td>
<td>9.7 ± 0.2</td>
<td>9.3 ± 0.3*</td>
<td>8.1 ± 0.3</td>
<td>9.4 ± 0.3*</td>
<td>6.7 ± 0.1*</td>
<td>6.7 ± 0.2*</td>
<td>5.0 ± 0.2*</td>
</tr>
<tr>
<td><strong>Period F (REC)</strong></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CR</td>
<td>11.3 ± 0.2</td>
<td>10.2 ± 0.1</td>
<td>10.1 ± 0.4</td>
<td>7.2 ± 0.5</td>
<td>7.1 ± 0.2</td>
<td>8.3 ± 0.2</td>
<td>9.7 ± 0.2</td>
<td>6.4 ± 0.1</td>
</tr>
<tr>
<td>TR1</td>
<td>11.7 ± 0.3</td>
<td>10.3 ± 0.1</td>
<td>10.1 ± 0.3</td>
<td>7.4 ± 0.4</td>
<td>7.6 ± 0.3*</td>
<td>8.2 ± 0.3</td>
<td>8.5 ± 0.03*</td>
<td>6.3 ± 0.1</td>
</tr>
<tr>
<td>TR4</td>
<td>11.3 ± 0.1</td>
<td>10.1 ± 0.2</td>
<td>10.3 ± 0.4</td>
<td>7.4 ± 0.1</td>
<td>7.1 ± 0.1</td>
<td>7.2 ± 0.2*</td>
<td>8.6 ± 0.2*</td>
<td>5.5 ± 0.02*</td>
</tr>
</tbody>
</table>

CR, control reactor; TR, test reactor; CRO, ceftriaxone; MTZ, metronidazole; REC, recovery.

Data are mean log_{10} copies 16S rRNA gene mL^{-1} fermentation effluent ± SD of the three last days of treatment periods; samples were analyzed in duplicate. Means with an asterisk (*) differ significantly from the control reactor within the same bacterial group (p<0.05).
Table 3.3: Metabolites concentration (mM) and SCFA ratios (%) measured by HPLC in effluent samples of antibiotic treatment [A, B and E] and recovery [F] periods in TRs compared to CR of model 2.

<table>
<thead>
<tr>
<th></th>
<th>Acetate</th>
<th>Propionate</th>
<th>Butyrate</th>
<th>Formate</th>
<th>Total metabolites</th>
<th>SCFA Ratios (%)</th>
<th></th>
<th></th>
<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Acetate</td>
<td>Propionate</td>
<td>Butyrate</td>
<td></td>
</tr>
<tr>
<td>Period A (CRO I)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>CR</td>
<td>75.7 ± 4.8</td>
<td>33.4 ± 2.5</td>
<td>38.4 ± 2.2</td>
<td>ND</td>
<td>147.5 ± 5.8</td>
<td>51.3</td>
<td>22.6</td>
<td>26.0</td>
<td></td>
</tr>
<tr>
<td>TR1</td>
<td>75.0 ± 3.0</td>
<td>36.7 ± 4.0</td>
<td>37.4 ± 0.9</td>
<td>ND</td>
<td>148.4 ± 6.4</td>
<td>20.5</td>
<td>24.7</td>
<td>24.7</td>
<td></td>
</tr>
<tr>
<td>TR3</td>
<td>76.5 ± 4.1</td>
<td>33.5 ± 2.0</td>
<td>35.8 ± 1.3</td>
<td>ND</td>
<td>145.8 ± 4.7</td>
<td>52.5</td>
<td>23.0</td>
<td>24.6</td>
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</tr>
<tr>
<td>Period B (CRO II)</td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>CR</td>
<td>91.2 ± 0.9</td>
<td>43.6 ± 2.2</td>
<td>18.4 ± 4.6</td>
<td>ND</td>
<td>153.2 ± 5.2</td>
<td>59.5</td>
<td>28.5</td>
<td>12.0</td>
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<tr>
<td>TR1</td>
<td>93.5 ± 7.4</td>
<td>47.2 ± 4.3</td>
<td>19.5 ± 2.6</td>
<td>ND</td>
<td>160.2 ± 8.9</td>
<td>58.4</td>
<td>29.5</td>
<td>12.2</td>
<td></td>
</tr>
<tr>
<td>TR3</td>
<td>97.0 ± 5.1*</td>
<td>45.6 ± 4.3</td>
<td>19.6 ± 2.1</td>
<td>ND</td>
<td>162.2 ± 7.0</td>
<td>97</td>
<td>45.6</td>
<td>19.6</td>
<td></td>
</tr>
<tr>
<td>Period E (MTZ)</td>
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</tr>
<tr>
<td>CR</td>
<td>99.2 ± 2.4</td>
<td>37.0 ± 1.2</td>
<td>13.3 ± 0.5</td>
<td>ND</td>
<td>149.5 ± 2.7</td>
<td>66.4</td>
<td>24.7</td>
<td>8.9</td>
<td></td>
</tr>
<tr>
<td>TR1</td>
<td>44.1 ± 0.7*</td>
<td>35.9 ± 1.8</td>
<td>ND</td>
<td>10.6 ± 1.0</td>
<td>90.6 ± 2.2*</td>
<td>55.1</td>
<td>44.9</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>TR4</td>
<td>45.6 ± 2.5*</td>
<td>37.0 ± 1.3</td>
<td>ND</td>
<td>11.3 ± 1.6</td>
<td>93.9 ± 3.2*</td>
<td>55.2</td>
<td>44.8</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Period F (REC)</td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CR</td>
<td>92.6 ± 10.9</td>
<td>39.9 ± 6.2</td>
<td>13.4 ± 0.2</td>
<td>ND</td>
<td>145.9 ± 12.5</td>
<td>63.5</td>
<td>27.3</td>
<td>9.2</td>
<td></td>
</tr>
<tr>
<td>TR1</td>
<td>83.7 ± 0.4</td>
<td>43.8 ± 2.4</td>
<td>13.4 ± 0.7</td>
<td>ND</td>
<td>140.9 ± 2.5</td>
<td>59.4</td>
<td>31.1</td>
<td>9.5</td>
<td></td>
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<tr>
<td>TR4</td>
<td>88.4 ± 8.6</td>
<td>44.8 ± 9.8</td>
<td>13.5 ± 1.6</td>
<td>ND</td>
<td>146.7 ± 13.1</td>
<td>60.3</td>
<td>30.5</td>
<td>9.2</td>
<td></td>
</tr>
</tbody>
</table>

CR, control reactor; TR, test reactor; SCFA, short-chain fatty acids; CRO, ceftriaxone; MTZ, metronidazole; REC, recovery; ND, not detected.

Data are means ± SD of the three last days of treatment period; samples were analyzed in duplicate.

Means with an asterisk (*) differ significantly from the control reactor within the same metabolite (p<0.05).
Effect of metronidazole on gut microbiota composition, diversity and metabolites

The effect of metronidazole (333 mg L\(^{-1}\) added twice daily) on gut microbiota composition and metabolites in TR1 and TR4 was investigated in period E during the three last days and compared with CR (Table 3.2 and 3.3). The abundance of selected bacterial groups and the metabolite concentrations were additionally determined during the entire fermentation period in TR4 and compared to CR (Figure 3.3C and 3.3D). Microbial diversity was assessed with pyrosequencing during the three last days in CR and TR4 (Figure 3.4 and 3.5). Metronidazole considerably affected the microbial composition determined with qPCR. A decrease in copy numbers of *Enterobacteriaceae*, *F. prausnitzii*, *Clostridium* cluster IV and *Roseburia* spp. of up to 3.8 log\(_{10}\) was measured in TR1 and TR4 during the last three days of metronidazole treatment compared to CR (Period E, Table 3.2). In contrast, an increase for *Bifidobacterium* spp. (2.4 and 2.3 log\(_{10}\) in TR1 and TR4, respectively) was measured during metronidazole treatment compared to CR. Pyrosequencing of TR4 effluent samples revealed a profoundly altered microbial composition during the last three days of metronidazole compared to CR. At the phylum level Bacteroidetes decreased from average values of 61% in CR to 21% in TR4, Actinobacteria increased from less than 1% in CR to 39% in TR4, and Firmicutes and Proteobacteria exhibited less pronounced changes within 5% (Figure S3.2). At the genus level, high abundance of up to 21% were observed in TR4 for *Trueperella*, *Actinomyces* and *Enterococcus* that were at low abundance of less than 1% or undetected in CR (Figure 3.4). The mean Shannon diversity index measured during the last three days of metronidazole treatment was 3.8 ± 0.03 in TR4 compared to 4.3 ± 0.1 in CR (Figure 3.4). In addition PCoA analysis showed a clear separation between samples from TR4 and CR during metronidazole period (Figure 3.5).

Metronidazole also largely impacted gut microbiota metabolic activity, with a 50% decrease of acetate concentrations in TR1 and TR4 compared to CR, a complete inhibition of butyrate production, and significant formate accumulation (Table 3.3 and Figure 3.3D). Propionate concentrations first decreased from 25 mM to around 4 mM in TR4 but recovered during the last days of metronidazole treatment to reach concentrations of around 36 mM.

Recovery of gut microbiota after metronidazole treatment

The recovery of the intestinal fermentation in TR1 and TR4 was monitored over 10 days [Period F] after the end of the metronidazole period. All bacterial populations analyzed with qPCR showed recovery trend towards CR composition upon cessation of metronidazole treatment (Figure 3.3C and Table 3.2). However, the populations of *F. prausnitzii* and
**Clostridium** cluster IV were still significantly lower in TRs compared to CR, while *Bifidobacterium* spp. was higher in TR1 compared to CR at the end of the period (**Table 3.2**). Pyrosequencing data at the phylum level showed similar Bacteroidetes abundance of 57% in TR4 compared to 64% in CR, but Actinobacteria abundance was still higher in TR4 than in CR (10% versus below 1%, **Figure S 3.2**). Most genera that increased and became dominant during metronidazole treatment decreased or became undetectable during the three last days of recovery period (**Figure 3.4**). The Shannon diversity index determined at the end of the recovery period was similar in TR4 (4.5 ± 0.07) and CR (4.2 ± 0.2) (**Figure 3.4**). In addition PCoA analysis showed that microbial composition of the three last days of recovery period in TR4 clustered close to samples from CR of the same treatment period (**Figure 3.5**). Metabolites in TR4 converged towards CR concentrations during the 10 days of recovery period and at the end metabolites concentrations in TR1 and TR4 were not significantly different from that in CR (**Table 3.3** and **Figure 3.3D**).
Figure 3.4: Microbial composition and diversity in reactors CR and TR4 of model 2 during metronidazole (=MTZ) [E] and recovery (=REC) [F] period measured by 454-pyrosequencing. The microbiota profile in reactor effluents of the last three days of period E and F was analyzed by 454-pyrosequencing of the V5-V6 hypervariable regions of the 16S rRNA gene. (A) Relative abundance at genus level. Values < 1% are summarized in the group “others”; uc, unclassified. (B) Diversity measured by the Shannon diversity index. A higher Shannon diversity index reflects a more diverse community (in abundance and evenness).
Figure 3.5: PCoA analysis of CR, TR3 and TR4 of model 2 during ceftriaxone [B], metronidazole [E] and recovery [F] periods based on weighted UniFrac distance matrix. The three last days of period B (circles), period E (squares) and period F (pentagons) are presented for TR3 (period B) and TR4 (period E and F) and compared to CR (during period B, E and F) that was not treated.
Discussion

*In vitro* intestinal fermentation models are useful tools to investigate CD colonization and interactions with the human gut microbiota, independent of the effects of the host. The human colon can be approximated to continuous fermenter, divided in regions (ascending, transverse and descending colonic) that are different in metabolic activity and microbial composition. A major advancement of *in vitro* gut fermentation models was the development of multistage continuous fermentation models which enable the simulation of horizontal colon processes (Macfarlane *et al.*, 1998). Continuous colonic fermentation models for the investigation of CDI are scarce and restricted to mainly three-stage models, inoculated with a liquid fecal suspension from pooled donors. Furthermore studies have mainly been done with efficacy of antibiotics on CD infected models using only cultivation methods for microbiota composition characterization (Freeman *et al.*, 2005, Baines *et al.*, 2009b).

In a recent study we investigated different *in vitro* continuous fermentation models inoculated with immobilized fecal microbiota from single elderly donors and characterized microbiota composition, diversity and activity, using qPCR and pyrosequencing, and HPLC, respectively (chapter 2). Immobilization of fresh gut microbiota under strict anaerobiosis was used to reproduce both the planktonic (free-cell) and sessile (biofilm-associated) states of bacterial populations in the colon, and to address problems associated with inoculum washout. This approach was shown to yield self-contained continuous fermentation system of high cell density, diversity and population stability, close to the human GI tract (Payne *et al.*, 2012a).

In this study, we used two PolyFermS models each inoculated with a single microbiota of different elderly donors to investigate colonization dynamics of CD inoculated as vegetative cells or spores.

It is suggested that the small intestine is the site of CD spores germination into the metabolically active vegetative cells (Koenigsknecht *et al.*, 2014, Carlson *et al.*, 2015), which then proliferate in the large intestine and cause symptoms typical for CDI (Paredes-Sabja *et al.*, 2008, Vedantam *et al.*, 2012). Inoculation of vegetative cells in our model of the proximal and transverse-distal colon revealed that CD was able to colonize only in transverse-distal colon conditions (pH 6.8) while it was not detected in the proximal colon reactor (pH 5.7), suggesting that the lower pH of the proximal colon (Lawley & Walker, 2013) prevented CD growth. Our data are in agreement with previous studies that showed no colonization of CD...
added as spores in the proximal colon section of a three stage colonic fermentation model (Baines et al., 2011, Baines et al., 2013).

In model 1 the CD concentration (up to $\log_{10} 8$ copies mL$^{-1}$) determined in TDC reactor was in the range of qPCR data for feces of antibiotic-associated diarrhea patients (aged 3 to 89 years), that ranged from 5.6 to 11.2 cell equivalents $\log_{10} g^{-1}$ (Naaber et al., 2011). Growth of CD in gut models upon addition of vegetative cells was tested in batch fermentations only and using plating cell counts of up to $8 \log_{10} \text{cfu mL}^{-1}$ were determined after 24 h (Hopkins & Macfarlane, 2003, Rea et al., 2011b). Although we used a high inoculum of vegetative cells ($\log_{10} 9.8 \text{ cfu}$), CD cells were washed-out and remained undetected during a period of six days before colonizing in TDC.

The PolyFermS design of model 2 was set to mimic TDC conditions in experimental and control reactors inoculated with the same elderly microbiota. We investigated the colonization of two PCR ribotypes of CD when instilled as spores, similar to the previous three-stage model (Freeman et al., 2005, Baines et al., 2009b) and the effects of two antibiotics, ceftriaxone and metronidazole that are known to respectively promote and treat the infection. CD remained undetected in all TRs for five days after which a repeated inoculation of spores was performed. Since we did not quantify CD spores it is not known if growth was below the detection limit of the qPCR method or if spores remained quiescent during the days before growth was detected, however, in a continuous fermentation system its rationale to assume that spores are washed out of the system according to the transit time. Nonetheless, a quiescent state of CD spores for a period of at least seven days was previously reported in a three-stage model, and tentatively explained by the absence of antibiotic treatment (Baines et al., 2011, Baines et al., 2013). In contrast, our data demonstrate that application of antibiotic is not necessary for CD colonization in the in vitro colonic fermentation model. This is in contrast to in vivo data in humans and animal models suggesting that CD infection is promoted by a disturbed microbiota mainly due to antibiotic treatment (Hutton et al., 2014, Oldfield et al., 2014). This difference between in vitro and in vivo may be due to specific mechanisms of the host including immunoprotection and interactions with the epithelial cell layer, that are lacking in vitro.

Ceftriaxone is a broad-spectrum antibiotic that is associated with a high risk of developing diarrhea due to CD acquisition (Privitera et al., 1991, Bartlett & Gerding, 2008). The fecal microbiota was reported to be affected during ceftriaxone treatment, with a marked decrease
in lactobacilli, bifidobacteria, clostridia and *Bacteroides* while enterococci were increased (Pletz et al., 2004). Here, we observed only minor effects on the gut microbiota during ceftriaxone, with *Bifidobacterium* spp. being most affected while diversity and metabolic activity was not changed compared to control reactors. The effect of ceftriaxone on *Bifidobacterium* spp. was consistent with Baines (2011), however, the authors only used plating for gut microbiota enumeration, thus diversity and also metabolic activity were not tested.

Metronidazole is used in the treatment of anaerobic infections (Lofmark et al., 2010), including in cases of mild to moderate CDI (Surawicz et al., 2013). In our intestinal model, we measured an extreme shift of the gut microbiota composition during metronidazole treatment, with a very large increase of Actinobacteria at the expense of Bacteroidetes and inhibition of butyrate production. A detrimental effect of metronidazole on Bacteroidetes was also observed in a previous distal colon model, however, the antibiotic mainly promoted growth of bacteria belonging to the phylum Proteobacteria (Rea et al., 2011b). A higher concentration of metronidazole (333 mg L\(^{-1}\), twice daily) was applied compared to previous fermentation models with concentrations varying greatly between 9.3 mg L\(^{-1}\) every 8 hours (Freeman et al., 2007) to 330 mg L\(^{-1}\) daily (Meader et al., 2010). However, with the lower concentration no effect was observed on CD counts and toxin production (Freeman et al., 2007). In our distal colon model, we observed a strong reduction in CD numbers and toxin production to below the detection limits. An important issue with treatment of CDI is the high recurrence rate after antibiotic treatment, with around 27% of patients being affected after metronidazole treatment (Vardakas et al., 2012). Our data showed that a fast recurrence of CD occurred only two days after cessation of metronidazole treatment, while gut bacterial groups that were mainly affected by metronidazole (*Clostridium* cluster IV, *F. prausnitzii* and *Roseburia* spp.) showed only partial recovery to levels of the control after 10 days. To our knowledge this is the first gut fermentation model that monitored recovery of CD after metronidazole treatment.

In conclusion, our study shows that *in vitro* continuous intestinal models can be used for investigating CD colonization dynamics and antibiotic treatments on gut microbiota independent of host factors. We demonstrated high competitiveness of CD in the ecosystem of the gut microbiota, inoculated either as vegetative cells or spores, in reactors mimicking TDC conditions, with no requirement of antibiotic treatment. Treatment with metronidazole induced deleterious effects on the gut microbiota composition and activity, and a temporary
inhibition of CD while fast recurrence after cessation of treatment was observed. Immobilization of fecal microbiota in gel beads allowed the investigation of a high bacterial cell density similar to in vivo gut microbiota. The design of the PolyFermS models proved well-suited for comparison of treatment effect directly to control conditions in reactors inoculated with the same microbiota. Our PolyFermS models for CD can be used to test new antimicrobial treatments for their activity against CD and to assess their overall effect on the gut microbiota composition and activity using in-depth analysis of microbial diversity.

Acknowledgements

We thank Simon Galenda and Markus Reichlin for technical assistance. The research described in this paper was funded by Danone Research (Palaiseau, France).
Supporting information

Figure S 3.1: Microbial composition in reactors CR and TR3 of model 2 during the three last days of the 2nd ceftriaxone period [B] measured by 454-pyrosequencing on genus level. The microbiota profile in effluents of CR and TR3 of model 2 during the three last days of period B was analyzed by 454-pyrosequencing of the V5-V6 hypervariable regions of the 16S rRNA gene. TR3 was treated with ceftriaxone during the entire five days of period B. Values < 1% are summarized in the group “others”; uc, unclassified.
Figure S 3.2: Microbial composition in reactors CR and TR4 of model 2 during metronidazole [E] and recovery [F] period measured by 454-pyrosequencing. The microbiota profile in reactor effluents of the last three days of period D and E was analyzed by 454-pyrosequencing of the V5-V6 hypervariable regions of the 16S rRNA gene. (A) Relative abundance at phylum level and (B) relative abundance at family level. Values < 1% are summarized in the group “others”; uc, unclassified.
Chapter 4

*In vitro* ecological study of *Lactobacillus paracasei* CNCM I-1518 in healthy and *Clostridium difficile* colonized elderly gut microbiota

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Abstract

Consumption of probiotic bacteria can result in a transient colonization of the human gut and thereby in potential interactions with the commensal microbiota. In this study, we used novel PolyFermS continuous fermentation models to investigate interactions of the probiotic *Lactobacillus paracasei* CNCM I-1518 (LpC) with the structure and function of healthy elderly gut microbiota using 16S rRNA gene amplicon sequencing and metatranscriptomics and with *Clostridium difficile* (CD) – a pathogen prevalent in the elderly population.

Small changes in microbiota composition were detected upon daily addition of LpC, including increased abundances of closely related genera *Lactobacillus* and *Enterococcus* and of the butyrate producer *Faecalibacterium*. Microbiota gene expression was also modulated by LpC with distinct response to the *Faecalibacterium* transcriptome and an increase in carbohydrate utilization. However, no inhibitory effect of LpC was observed on CD colonization in the intestinal models, under the tested conditions.

Our data suggest that LpC has modulatory effects on composition and function of elderly gut microbiota *in vitro*, but does not affect CD growth and toxin production within a complex microbiota.
Introduction

The large intestine is the most densely populated site of the human body with over $10^{14}$ microbial cells that belong mainly to the domain Bacteria. This diverse microbial community exerts functions that are important to maintain host health, including energy and nutrients supply by fermentation of otherwise indigestible food components, development of a balanced immune system and the protection against pathogens, termed colonization resistance (Vollaard & Clasener, 1994, Robles Alonso & Guarner, 2013). Different diseases have been associated with compositional changes in intestinal communities (Robles Alonso & Guarner, 2013) and a disruption of the healthy microbial communities (dysbiosis) can result in the loss of colonization resistance and an overgrowth of pathogens, such as *Clostridium difficile* (CD) (Kamada et al., 2013).

Apart from disease, diet and medications are important modulators of the gut microbiota. However, the gut microbiota also changes throughout the different stages of human life (O'Toole & Claesson, 2010, Duncan & Flint, 2013). Old age was associated with a decrease in potentially beneficial bacteria, including bifidobacteria (Woodmansey et al., 2004) and *Faecalibacterium praunitzii* and an increase in facultative anaerobes, such as enterobacteria (Mueller et al., 2006, Biagi et al., 2010). Furthermore, the risk of CD infection is elevated in old age (Oldfield et al., 2014). Decreases in short chain fatty acids (SCFA) production have also been described for the elderly (Woodmansey et al., 2004, Tiihonen et al., 2008). How these compositional and metabolic changes are linked to host health in old age is not well known (Biagi et al., 2013).

Probiotics are defined as “live microorganisms which, when administered in adequate amounts, confer a health benefit on the host” (FAO/WHO, 2002). Probiotics are of interest to maintain a balanced microbiota in the healthy host but also as alternative treatment for gastrointestinal infections (de LeBlanc & LeBlanc, 2014). Mechanisms of probiotic action include the direct interaction with the commensal gut microbiota, inhibition of enteric pathogens or their metabolites and modulation of the immune system (Oelschlaeger, 2010). Probiotics have been associated with improved clinical outcome in several studies but the effect of probiotics on gut microbiota composition and especially on the functional activity is not well known. *Lactobacillus* strains are often used as probiotics due to their technological properties and the general assumption that they are safe as they have been traditionally used in fermented dairy products (Ouwehand et al., 2002). *Lactobacillus paracasei* CNCM I-1518
(LpC) is a probiotic that belongs to the *Lactobacillus casei* group consisting of *L. casei, L. paracasei* subspecies *paracasei* and *L. rhamnosus*. This strain is able to survive gastrointestinal transit and to modulate immune function (Borruel *et al.*, 2002, Oozeer *et al.*, 2006, Tien *et al.*, 2006, Rochet *et al.*, 2008). Further, fermented milk containing LpC was associated with decreased duration of common gastrointestinal and respiratory infections (Guillemard *et al.*, 2010), consumption of fermented milk containing LpC also reduced the incidence of antibiotic-associated and CD-associated diarrhea in elderly patients taking antibiotics (Hickson *et al.*, 2007). Assessing the effect of probiotics on the gut microbiota composition and activity can be difficult due to the hindered accessibility of the gastrointestinal tract. *In vitro* models simulating the human colon represent a useful tool for mechanistic studies of interactions of probiotics with the gut microbiota and pathogens (Payne *et al.*, 2012a, Lacroix *et al.*, 2015).

We recently developed *in vitro* fermentation models on the novel PolyFermS platform (Zihler Berner *et al.*, 2013) with elderly immobilized fecal microbiota for investigations of CD colonization and antibiotic treatment testing (chapter 2 and 3). Here we assessed the potential of LpC to alter the elderly gut microbiota reproduced in these *in vitro* colonic continuous fermentation models of the PolyFermS platform. The microbiota was investigated for changes in abundances of phylotypes and in the active microbial communities and their functional properties using 16S rRNA gene amplicon sequencing and metatranscriptomics. Additionally, co-cultures of LpC with members of the commensal microbiota or the enteric pathogen CD were investigated in batch fermentations. The potential of LpC to counteract CD was further tested *in vitro* in the environment of the complex gut microbiota.
Materials and Methods

Bacterial strains

*Lactobacillus paracasei* CNCM I-1518 (LpC) was provided by Danone Research (Palaiseau, France). *Faecalibacterium prausnitzii* DSM 17677 was purchased from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ). *Clostridium difficile* DSM 1296 (PCR ribotype 001) and *Clostridium difficile* NCTC 13307 (PCR ribotype 012) were purchased from DSMZ and the National Collection of Type Cultures (NCTC), respectively. For inoculation of colonic fermentation studies LpC and vegetative cells of CD DSM 1296 were cultured from glycerol stocks (33%, -80°C) at 37°C in serum flasks flushed with N\textsubscript{2} and CO\textsubscript{2} at 3:1 ratio or anaerobic Hungate tubes using the Hungate culturing technique (Bryant, 1972) for LpC and CD DSM 1296, respectively, containing fermentation medium simulating human chyme. Spores of CD DSM 1296 and NCTC 13307 were prepared according to Sorg and Dineen (2009) as described before (chapter 3).

Yeast extract-casein hydrolysate-fatty acids (YCFA) medium (Duncan et al., 2002a) was used to routinely culture the bacterial strains in anaerobic Hungate tubes at 37°C for coculture studies of LpC with either *F. prausnitzii* or CD DSM 1296. YCFA was supplemented with glucose, soluble starch and cellobiose (Sigma-Aldrich Chemie GmbH, Buchs, Switzerland), each at a concentration of 2 g L\textsuperscript{-1} (YCFA-GSC).

Continuous colonic fermentation setup

All three continuous *in vitro* fermentation models investigated in this study were based on the PolyFermS design (Zihler Berner et al., 2013) and are displayed in Figure 4.1. Common to all three models was an inoculum reactor (IR, 37°C, retention time of 9 h, pH 5.7) inoculated with fecal microbiota immobilized in gellan-xanthan beads. Model 1 and 3 were inoculated with fecal microbiota obtained from the same elderly donor (female, 78 years) with a seven month interval. Model 2 was inoculated with fecal microbiota from a different female (72 years). Fecal donors did not receive antibiotic treatment for at least three months prior to sample collection and did not consume probiotics on a regular basis. An informed written consent was obtained from both donors. A fermentation medium simulating human chyme was used in all three models, as presented previously (chapter 2). In model 1 the IR was connected in parallel to a control reactor (PC_CR) and test reactor treated with LpC (PC_LpC) that were run at conditions mimicking the proximal colon (PC, 37°C, retention time 9 h, pH 5.7). PC_CR and PC_LpC were continuously inoculated with 10% effluent from IR and 90% fresh fermentation medium. The IR of model 2 was connected in parallel to two
sets of two-stage reactors (control (PC_CR + DC_CR) and LpC test reactors (PC_LpC + DC_LpC)) mimicking the proximal (PC, 37°C, retention time of 9 h, pH 5.7) and distal colon (DC, 37°C, retention time of 18 h, pH 6.8) and were continuously fed with 10% effluent from IR and 90% fresh fermentation medium. The IR of the third fermentation model was connected to a control (DC_CR) and LpC test reactor (DC_LpC) operated at distal colon conditions (37°C, retention time of 25 h, pH 6.8) and fed with 100% of the effluent from IR. During antibiotic treatment and recovery period in model 3 control and test reactors were fed with fresh fermentation medium to avoid the inflow of untreated microbiota.

**Experimental design of colonic models**

In model 1 the effect of LpC on the healthy elderly proximal colon microbiota was investigated. A stabilization period of 14 days was performed before PC_LpC was inoculated with LpC twice daily for 10 days (Figure 4.1). LpC was prepared from an overnight culture which was centrifuged (6'000 g, 5 min). The pellet was resuspended in fresh fermentation medium and inoculated with a syringe to obtain final concentrations of around log_{10} 7.5 copy numbers mL^{-1} that corresponds to the approximate number of living LpC cells that were detected in stool samples following ingestion (Oozeer et al., 2006). Microbiota composition was analyzed with 16S rRNA gene amplicon sequencing on four selected days at the beginning and at the end of LpC treatment (days 16, 17, 23 and 24, Figure 4.1). qPCR was performed during the last days of stabilization period and throughout the treatment period of selected bacterial groups that were impacted by LpC according to 16S rRNA amplicon sequencing or metatranscriptomics. The metatranscriptome was analyzed on three days corresponding to the beginning, middle and end of LpC treatment (days 16, 18 and 24). The metabolic activity was assessed with HPLC during the three last days of stabilization period and throughout the LpC treatment period.

Model 2 was previously described for development of elderly microbiota models and for CD colonization investigations (chapter 2 and 3). After an initial stabilization phase of 18 days (chapter 2) and treatment periods for CD investigations (chapter 3), control and test reactors were exchanged with new reactors, that were connected to IR for a stabilization phase of five days before treatment with LpC was started (Figure 4.1). LpC was inoculated twice daily into test system 2 (PC_LpC + DC_LpC) during the entire treatment period of 11 days (days 6-16). On day 11, DC_CR and DC_LpC were inoculated once with vegetative cells of CD DSM 1296. The CD cells were prepared from an overnight culture which was centrifuged (6'000 g, 5 min). The pellet was resuspended in fresh fermentation medium and inoculated with a syringe to obtain final concentrations of approximately log_{10} 7 copy numbers mL^{-1}. 

134
Reactor effluents of the DC reactors were collected 6 h post CD inoculation and afterwards daily to determine gene copies of LpC and CD as well as cytotoxin titers.

In model 3 which mimicked the distal colon segment, the effect of LpC on CD NCTC 13307 was investigated before, during and after metronidazole treatment (Figure 4.1). The CD NCTC 13307 strain was chosen due to better colonization of reactors upon spore inoculation as described before (chapter 3). Both DC reactors were instilled with CD spores at a concentration of $10^7$ cfu, which were added once on two consecutive days in beginning of CD colonization period. LpC treatment was performed in reactor DC_LpC from day 10 of fermentation with twice daily addition of LpC cells throughout the remaining days of fermentation as described above for model 1. Metronidazole (Sigma-Aldrich) treatment was performed twice daily at a final concentration of 333 mg L$^{-1}$ at days 16 – 25 in DC_CR and DC_LpC. Reactor effluents of DC’s were collected daily for qPCR analysis of LpC and CD, and for Vero cell analysis.

The effect of ceftriaxone and metronidazole on CD spore germination and colonization, respectively, as well as the general effect of these antibiotics on the gut microbiota was presented earlier (chapter 3).
Figure 4.1: Experimental timeline of continuous colonic fermentation models. (A) Model 1. PC_LpC was treated with LpC twice daily at days 15-24. (B) Model 2. Test system 2 (PC_LpC + DC_LpC) was treated with LpC twice daily at days 6-16; CD DSM 1296 vegetative cells were inoculated on day 11 into DC_CR and DC_LpC. (C) Model 3. CD NCTC 13307 spores were inoculated on day 1 and 2 into DC_CR and DC_LpC; DC_LpC was treated with LpC twice daily at days 11-35; DC_CR and DC_LpC were treated with metronidazole (days 16-25) and recovery was observed at days 26-35.

IR, inoculum reactor; PC, proximal colon; DC, distal colon; CR, control reactor; LpC, L. paracasei CNCM I-1518; CD, C. difficile
Co-culture of LpC and F. prausnitzii

Growth of F. prausnitzii was investigated in co-culture with LpC because an increase in relative abundance of the genus Faecalibacterium was observed during LpC treatment in PC_LpC reactor of model 1 by 16S rRNA gene amplicon sequencing. Culturing was performed in Hungate tubes containing 10 mL YCFA-GSC medium. For each measurement point, individual tubes were inoculated in triplicate with 2% of three overnight cultures. Optical density (OD$_{600nm}$), pH and metabolites were analyzed at 0, 8, 48 and 72 h of incubation. qPCR analysis was performed to determine CRISPR or 16S rRNA gene copies of LpC and F. prausnitzii, respectively as described below. Metabolite concentrations were assessed from culture supernatants using HPLC analysis. Strains were also grown individually for comparison. The co-culture test was performed four times with three replicates each time and average values of the four tests are presented.

Co-culture of LpC and CD DSM 1296

LpC and CD DSM 1296 were investigated in co-culture to assess the effect of LpC on CD growth and toxin production in the absence of complex microbiota. Strains were grown in Hungate tubes containing 10 mL YCFA-GSC medium and for each intended measurement time point separate tubes were inoculated in triplicate with 2% or 4%, of CD and LpC, respectively, of overnight cultures. Because LpC grew slower than CD, LpC was inoculated first and CD was added after 5 h. OD, pH and metabolites were determined at 0, 5, 10, 13 and 25 h. Cell counts were additionally determined by plating on Wilkins-Chalgren agar (Oxoid AG, Pratteln, Switzerland) supplemented with cysteine-HCL and resazurin (Sigma-Aldrich). Serial dilutions were prepared in an anaerobic chamber and plates were incubated at 37°C in anaerobic jars (BioMérieux Suisse SA). Colonies of LpC and CD were distinguished by different colony morphology. Toxin production was assessed after 13 and 25 h incubation in co-cultures and compared to single culture using the Vero cell assay test. The co-culture test was performed twice with three replicates each time and average values of the two tests are presented.

DNA extraction and qPCR analysis

Genomic DNA was extracted from 2 mL fermentation effluent and co-cultures using the FastDNA® SPIN Kit for Soil (MP Biomedicals, Illkirch, France). Specific bacterial groups and species were measured in duplicate on an ABI PRISM 7500-PCR (Applied Biosystems, Zug, Switzerland) using a reaction volume of 25 µL as described before (Zihler et al., 2010). All assays were carried out using the 2 x SYBR Green PCR Master Mix (Applied Biosystems). Specific primers were used for enumeration of bacterial groups, LpC and CD.
Standard curves preparation and reaction conditions were described previously (Dostal et al., 2013).

**16S rRNA gene amplicon sequencing**

16S rRNA gene amplicon sequencing of effluent samples of colonic model 1 was carried out at DNAVision (Gosselies, Belgium) on a 454 Life Sciences Genome Sequencer FLX instrument (Roche Applied Science, Vilvoorde, Belgium). Amplification of the V5-V6 hypervariable 16S rRNA region was performed using primers 784F and 1061R (Andersson et al., 2008). Data was analyzed using the open source software package Quantitative Insights Into Microbial Ecology (QIIME), v1.7 (Caporaso et al., 2010) as described before (chapter 3).

**RNA isolation and metatranscriptome sequencing**

One mL effluent samples of colonic model 1 was collected directly from reactors and mixed with 1 mL 60% glycerol at -40°C, kept on ice for 20 min and centrifuged for 15 min (3220 x g, 4°C). The supernatant was discarded and the pellet was shock-frozen in liquid nitrogen and stored at -80°C. For total RNA isolation, pellets were resuspended in 400 µL cold MRS-C and transferred to a screw cap tube containing 500 µL chlorophorm/phenol (1:1, v/v), 30 µL SDS 10% (Stevens et al., 2008), 30 µL 3 M Na-acetate and 400 mg zirconium beads (0.1 mm). The mixture was disrupted in a bead beater (4 x 40 sec, 5 m s⁻¹) with cooling on ice between cycles and centrifuged for 12 min (12’000 x g, 4°C). The supernatant was added to 200 µL ice cold chloroform, centrifuged again as before and from the resulting supernatants RNA was isolated using the High Pure RNA Isolation Kit (Roche Diagnostics, Switzerland) according to the manufacturer’s instructions. RNA concentrations and quality were determined on a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Washington, USA) and on an Agilent 2100 Bioanalyzer (Agilent, Basel, Switzerland), respectively. Double-stranded cDNA libraries were paired-end sequenced using an Illumina HiSeq (Functional Genomic Center Zurich, Switzerland).

For bioinformatics analysis, a pipeline consisting of SortMeRNA (Kopylova et al., 2012) for separation of rRNA and mRNA, and FLASH (Magoc & Salzberg, 2011) for overlapping the paired-end sequences were used. rRNA sequences (100 000) were compared to the modified SILVA database provided by CREST (Lanzen et al., 2012). Putative mRNA reads were compared to the NCBI RefSeq database using MALT (http://ab.inf.uni-tuebingen.de/software/malt/) which is based on DIAMOND (Buchfink et al., 2015). Transcripts were taxonomically classified using MEGAN (Huson et al., 2007). Putative
mRNA reads were also uploaded to MG-RAST for functional classification according to the SEED Subsystem scheme using default settings.

**HPLC analysis**

Acetate, butyrate, propionate, formate and lactate were determined in fermentation effluent and co-culture samples by HPLC in duplicate (Thermo Fisher Scientific Inc. Accela, Wohlen, Switzerland). Sample supernatants were filtered into vials through a 0.45 µm nylon HPLC filter (Infochroma AG, Zug, Switzerland). The analysis was run at a flow rate of 0.4 mL min\(^{-1}\) using an Aminex HPX-87H (Bio-Rad Laboratories AG, Reinach, Switzerland) or Rezex ROA-Organic Acid column, for effluent and co-culture samples, respectively and 10 mM H\(_2\)SO\(_4\) as eluent. A refractive index detector was used for detection.

**Vero cell analysis**

CD cytotoxin production was monitored in effluent samples of colonic models 2 and 3 and in samples of co-culture test of LpC with CD DSM 1296 using a Vero cell cytotoxicity assay as described before (chapter 3).

**Statistical analysis**

Statistical analyses of co-culture studies were done using JMP 10.0 (SAS Institute, USA). All data are expressed as mean ± SD of several co-culture tests performed in triplicate in batch fermentation studies. Growth (log\(_{10}\)-transformed), pH values, metabolites and toxin production were compared between pure and co-cultures using the non-parametric Kruskal-Wallis test. Statistical analyses of metatranscriptomics data were done using an one-tailed Student’s t-test for relative abundance comparisons between control and LpC reactor.
Results

Effect of LpC on the gut microbiota structure in proximal colon conditions (model 1)

16S rRNA gene libraries were used to determine the impact of the addition of LpC on taxon abundance. In PC_CR Firmicutes and Bacteroidetes were the dominant phyla, Proteobacteria and Actinobacteria represented less than 6% of the community. Clostridiales were the dominant bacterial order with Lachnospiraceae and Ruminococcaceae contributing the majority of reads (Figure S4.1). Lactobacillaceae represented between 0.9 and 2.7% of effluent microbiota; a mean relative abundance of 2.4% of Lactobacillus spp. relative to total bacterial 16S rRNA genes was determined using qPCR. During treatment period, an increase of Bacteroidetes (Bacteroidaceae and Bacteroidales) was noted. Therefore we calculated shifts in ratios of CR relative to LpC to identify a treatment effect of LpC inoculation.

LpC was not detected during stabilization phase in PC_CR and PC_LpC, and in PC_CR during treatment using strain specific qPCR. In PC_LpC, addition of LpC at log_{10} 8 cfu mL^{-1} led to an average relative abundance of 0.18 % CRISPR gene copies vs 16S rRNA genes of total bacteria. During treatment, the abundance of LpC increased five-fold (Figure 4.2A).

The addition of LpC had little impact on the abundance of major phyla, however, analysis of days 17, 18, 23 and 24 during treatment showed an increase of unclassified Bacilli and Ruminococcaceae as well as Enterococcus, Lactobacillus, Blautia, Lachnospiraceae Incertae Sedis and Faecalibacterium in PC_LpC compared to PC_CR (Table 4.1). At the same time, the addition of LpC was associated with decreased abundance of Bacteroides, Paraprevotella, Peptoniphilus, Coprococcus, Roseburia and Ruminococcus. Using qPCR a mean relative increase of Faecalibacterium and a decrease in Bacteroides spp. and Roseburia spp./E. rectale after the addition of LpC was confirmed (Figure 4.2B).
Figure 4.2: Abundance of selected bacterial groups in proximal colon section of model 1 assessed with qPCR (A) LpC abundance (CRISPR gene copies) compared to total bacteria (16S rRNA gene copies) in PC_LpC during treatment period. (B) Relative abundance of *Faecalibacterium*, *Bacteroides* spp., *Roseburia* spp./*Eubacterium rectale* and *Methanobrevibacter* in PC_LpC (16S rRNA genes target taxon relative to 16S rRNA genes total bacteria) normalized to relative abundance in PC_CR during last three days of stabilization period (days 12-14, □) and during treatment period (days 15-24, ♦).
Table 4.1: Relative abundance of phylogenetic groups in fermentation effluent of PC_LpC compared to PC_CR in proximal colon conditions (model 1) assessed with 16S rRNA gene amplicon sequencing. Only taxonomic groups that differed in abundance between PC_CR and PC_LpC are shown.

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<td>0.68</td>
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</table>

uc: unclassified
Effect of LpC on metatranscriptome and metabolite formation in proximal colon conditions (model 1)

Metatranscriptomics analysis was performed in model 1 during the treatment period to assess the impact of LpC on the functional activity of the microbiota. RNA sequencing of fermentation effluents yielded between 4.5 and 12.8 Mio overlapped reads (average size 153±31 bp), between 7.4 and 10.7% of those reads were identified as putative mRNA transcripts (225 000 and 1.57 Mio reads) (Table S4.2).

Microbiota composition of the metatranscriptome. A subset of 100 000 rRNA reads per samples was used for taxonomic classification. Bacteria and archaea were detected in the effluents of the in vitro fermentation model (Table 4.2 and Table S4.3). Clostridiales were the dominant bacterial order (78-89% of rRNA reads and 66-73% of mRNA reads, Tables S4.3 and S4.4) with unclassified Clostridiales (14-17%), Lachnospiraceae (23-27%) and Ruminococcaceae (38-49%) contributing the majority of transcripts, confirming results obtained by 16S rRNA gene amplicon sequencing. Unclassified Veillonellaceae and Veillonella were increased in the presence of LpC while Paraprevotella, Erysipelotrichaceae Incertae Sedis and Blautia decreased (Table 4.2). Methanobacteriales contributed between 0.01 and 0.04% of 16S rRNA transcripts. Unclassified Methanobrevibacteriaceae and Methanobrevibacter also decreased in reactors LpC had been added. This decrease after LpC addition could be confirmed by qPCR (Figure 4.2B). Taxonomic classification of mRNA reads was consistent with the community structure revealed by rRNA analysis (Table S4.3).

Functional activity of the microbiota. Between 74.166 and 1.7 Mio reads were assigned to SEED categories using MG-RAST. Almost 40% of all transcripts belonged to SEED categories ‘Carbohydrate metabolism’ and ‘Protein metabolism’ indicating high metabolic activity of the proximal colon microbiota.

The addition of LpC had little impact on the relative abundance of most SEED categories (Table 4.3). However, relative abundance of transcripts assigned to SEED category ‘Membrane transport’ was significantly (p<0.05) increased when LpC was present in the fermentation vessel due to the enhanced transcription of fructose and mannose, galactose and sucrose specific sugar phosphotransferase systems ((PTS) Tables 4.3 and 4.4). As these categories were all related to sugar transport, we further investigated SEED category ‘Carbohydrate Metabolism’. Subcategories ‘Di- and Oligosaccharides’ and ‘Fermentation’ were consistently slightly enhanced on all three days tested when LpC was added compared to controls. Within ‘Di- and Oligosaccharides’, sucrose phosphorylase (EC2.4.1.7), sucrose-
6-phosphate hydrolase (EC3.2.1.26) and ‘Alpha-galactosidase’ were 1.2-5 fold more abundant compared to controls. Within category ‘Fermentation’, ‘Butanol biosynthesis’ (3-hydroxybutyryl-CoA dehydrogenase (EC 1.1.1.157), 1.1-1.2 fold; and Pyruvate formate-lyase (EC 2.3.1.54), 1.2-1.8 fold) and ‘Mixed Acid Fermentation’ (also Pyruvate formate-lyase) were 1.1-1.2 fold increased.

**Metabolic activity of the gut microbiota.** Metabolic activity at proximal colon conditions was assessed using HPLC. The main metabolite was acetate with concentrations around 55 mM followed by butyrate (25 mM) and propionate (8 mM). Metabolite concentrations were similar for PC_CR and PC_LpC throughout the treatment period ([Figure S4.2](#)).
Table 4.2: Relative abundance of taxonomic groups in the proximal colon section (model 1) assessed with metatranscriptomics. Only taxonomic groups that differed in abundance between PC_CR and PC_LpC are shown.

<table>
<thead>
<tr>
<th></th>
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<td>0.48</td>
<td>0.95</td>
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<tr>
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uc: unclassified
Table 4.3: Relative abundance of SEED categories Level 1 in proximal colon section (model 1). In bold: SEED categories that are increased in PC_LpC relative to PC_CR ($p<0.05$).

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<tr>
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<td>0.89</td>
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<td>14.05</td>
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<td>0.97</td>
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</tr>
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<td>0.90</td>
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<td>0.97</td>
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<td>0.40</td>
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<td>1.06</td>
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<td>1.12</td>
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<td>1.22</td>
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Table 4.4: Relative abundance of transcripts assigned to PTS systems that are increased in PC_LpC relative to PC_CR (SEED subcategories of ‘Membrane Transport (SEED L1)’ in proximal colon section (model 1) (p≤0.05).

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<td>1.43</td>
<td>0.18</td>
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PTS: phosphotransferase systems

The *Faecalibacterium* transcriptome

We observed an increase in relative abundance of *Faecalibacterium* after the addition of LpC both in 16S rRNA amplicon sequencing and qPCR. Therefore, we extracted transcripts assigned to *Faecalibacterium* from the metatranscriptome (33,000-75,000 transcripts) to investigate whether there was an impact of LpC on the *Faecalibacterium* transcriptome.

Most transcripts were assigned to ‘Carbohydrates’ (approx. 25%), ‘Protein metabolism’ (approx. 16%) and ‘Clustering-based subsystems’ (approx. 14%) ([Table S4.5](#)). Addition of LpC had little impact on relative abundance of most SEED categories L1, but ‘Clustering-based subsystems’ and ‘Metabolism of aromatic compounds’ were significantly (p<0.05) reduced and increased, respectively. Increase of the latter was due to increased abundance of transcripts (1.2-1.3fold) of acetyl-CoA acetyltransferase in PC_LpC ([Table S4.6](#)). Acetyl-CoA acetyltransferase is involved in several pathways, therefore we also observed enhanced relative abundance of SEED subcategories level 2 ‘Anaerobic degradation of aromatic compounds’ (subcategory level L3 ‘Anaerobic benzoate metabolism’), ‘Fermentation’ (‘Butanol biosynthesis’) and ‘Lysine, threonine, methionine, and cysteine’ (‘Lysine fermentation’) in PC_LpC. Also the transcription of acetate kinase was enhanced 1.1-1.8fold in PC_LpC. Acetate kinase is involved in pathways represented by SEED categories related to ‘Fermentations’ (‘Fermentations: Lactate’, ‘Fermentations: Mixed acid’), Lysine, threonine, methionine, and cysteine (‘Lysine fermentation’), ‘Central carbohydrate metabolism’ (‘Pyruvate metabolism II: acetyl-CoA, acetogenesis from pyruvate’), and ‘Sugar Alcohols’ (‘Ethanolamine utilization’).

In contrast, 4-alpha-glucanotransferase (amylomaltsase) (EC 2.4.1.25) and Oligopeptide ABC transporter, periplasmic oligopeptide-binding protein OppA (TC 3.A.1.5.1) were 1.3-1.7 fold and 1.5-1.7 fold higher expressed in control fermentations (PC_CR), respectively.
**F. prausnitzii** in co-culture with LpC

The increase in *Faecalibacterium* abundance associated with LpC in proximal colon conditions (model 1) led us to further investigate interactions of the two species in batch fermentations (Figure 4.3). In single culture, *F. prausnitzii* reached maximum OD of 0.4 ± 0.1 after 8 h that decreased to 0.1 ± 0.1 after 72 h. Within 8 h, log 16S rRNA gene copies increased by 1.3, a decrease of *F. prausnitzii* 16S rRNA gene copies after 48 and 72 h was also observed by qPCR. The pH dropped from 6.8 ± 0.04 to 6.2 ± 0.1 within 48 h and remained stable thereafter.

In co-culture, maximum OD values for LpC and *F. prausnitzii* were measured after 48 h (1.3 ± 0.1), they were similar to OD values recorded when LpC was grown alone (1.2 ± 0.1). Also, pH values were similar in co-culture and in LpC single culture and were significantly lower than of *F. prausnitzii* single culture. LpC CRISPR copy numbers increased up to 48 h of incubation in single culture as well as co-culture. In co-culture, *F. prausnitzii* log 16S rRNA gene copies increased by 1.3 similar to single culture, however, log gene copies decreased significantly less in co-culture. Butyrate concentrations were not significantly different in *F. prausnitzii* single culture (5.3 ± 0.9 mM) compared to co-culture (5.9 ± 2.2 mM) at all tested time points (Table S4.7).
Figure 4.3: Co-culture study of LpC and *F. prausnitzii*. (A) OD\textsubscript{600} values of co-culture and single cultures of LpC and *F. prausnitzii*. (B) Changes in log\textsubscript{10} copy numbers mL\textsuperscript{-1} of LpC and *F. prausnitzii* in co- and single cultures compared to 0 h. (C) pH values in co-culture and single cultures of LpC and *F. prausnitzii*.

Values are means ± SD of triplicate analysis of four separate experiments (n=12), except for time point 72 h that was tested in three different experiments (n=9); Values with an asterisk (*) indicate significant difference between growth conditions (p<0.05).
CD growth and toxin production in co-cultures with LpC

Infection with CD often occurs after treatment with broad-spectrum antibiotics and incidences are increased in the elderly population. To investigate the potential of LpC to attenuate growth and toxin production of CD we first performed batch cultures of LpC co-cultivated with CD DSM 1296. Due to slower growth LpC was inoculated 5 h ahead of CD to yield a balanced growth of both strains (Figure 4.4A). Significantly reduced viable cell numbers were measured for CD after 10 and 13 h of fermentation in co-culture (7.4 ± 0.1 and 7.3 ± 0.2 log\(_{10}\) cfu mL\(^{-1}\)) compared to pure culture (7.8 ± 0.1 and 7.9 ± 0.1 log\(_{10}\) cfu mL\(^{-1}\)). After 25 h cell counts decreased to 5.3 ± 0.3 and 6.5 ± 0.2 in pure and co-cultures, respectively. Cytotoxin titers were also significantly lower in co-culture compared to single culture after 13 and 25 h fermentation (Figure 4.4B).

Lactate and formate formation was significantly higher in single cultures compared to co-cultures for LpC and CD, respectively, while acetate formation in CD single culture was similar to co-culture (Table S4.9).
Figure 4.4: Growth and cytotoxin production in co-culture study of LpC with CD DSM 1296. (A) log_{10} cfu mL^{-1} of CD and LpC in co-culture and respective single cultures. (B) cytotoxin titers of CD single culture compared to co-culture after 13 and 25 h of incubation calculated relative to CD cell counts.

Values are means ± SD of triplicate analysis of two separate experiments (n=6). Values with an asterisk (*) correspond to growth or cytotoxin titers in single cultures that are significantly different from co-culture (p<0.05).

**LpC impact on CD growth and toxin production in distal colon conditions (models 2 and 3)**

The interaction between LpC and CD was further investigated in complex microbiota at distal colon conditions. The impact of preventive LpC treatment on CD DSM 1296 colonization was determined in model 2. The effect of LpC treatment in combination with metronidazole
on CD NCTC 13307 colonization was investigated in model 3. Interaction of LpC and CD was investigated in the distal colon section as we previously observed that CD did not grow in the proximal colon section of our colonic fermentation models (chapter 3).

In model 2, LpC remained stable during days 6 – 11 in DC_LpC ($10^7.7 \pm 0.3$ CRISPR copies mL$^{-1}$ effluent, Figure 4.5A). However, after CD inoculation copy numbers of LpC increased by more than one log during the first days and remained constant thereafter, averaging $10^{8.8 \pm 0.1}$ CRISPR copies mL$^{-1}$ effluent. On day 11, CD DSM 1296 ($10^9.5$ copies) was added into both distal colon reactors of the model. During the first 24 h, CD copy numbers decreased by approximately 1 log, and then steadily increased from day 13 to 16 to reach a similar concentration of $10^{7.3}$ and 7.6 copies mL$^{-1}$ in DC_CR and DC_LpC, respectively. Toxin titers were similar in control and treatment reactor throughout the fermentation period, with average titers of $10^{2.9 \pm 0.2}$ and $2.8 \pm 0.2$ per mL effluent in DC_CR and DC_LpC, respectively.

In model 3, the effect of LpC on CD was assessed during five days after colonization of CD NCTC and then during 10 days of metronidazole treatment followed by 10 days of post antibiotic recovery (Figure 4.5B). CD copy numbers were similar in DC_CR and DC_LpC before the addition of LpC with average values of $10^{8.6 \pm 0.2}$ and $10^{8.4 \pm 0.2}$ 16S rRNA gene copies mL$^{-1}$ effluent (day 8 to 10), respectively. After addition, LpC CRISPR copies averaged at $10^{7.0 \pm 0.3}$ per mL and slightly increased during metronidazole treatment ($10^{7.3 \pm 0.3}$ copy numbers mL$^{-1}$). During the first three days of recovery LpC decreased by around 1 log and reached final abundance of $10^{6.0 \pm 0.3}$ CRISPR copies mL$^{-1}$ at day 35. CD NCTC 13307 growth continuously decreased in both reactors during metronidazole treatment and reached the detection limit after four days in DC_LpC compared to six days in DC_CR. In agreement, toxin production was below detection limit shortly after start of metronidazole treatment. CD reappeared at 6.3 copy numbers mL$^{-1}$ in DC_CR at days 24 and 25 of metronidazole treatment. A fast recovery of CD was observed in both DC’s with a slight delay for DC_LpC. After two days of recovery, CD 16S rRNA gene copies were stable in both reactors ($10^{8.7 \pm 0.2}$ and $8.8 \pm 0.2$ mL$^{-1}$ in DC_CR and DC_LpC, respectively) until the end of the period. Cytotoxin titers were similar in both reactors during the recovery period.
Results

Figure 4.5: LpC and CD log_{10} copy numbers and cytotoxin titers in continuous colonic fermentation models. (A) Model 2. LpC cells were inoculated twice daily from day 6 on into test system 2 (PC_LpC + DC_LpC) and on day 11 CD DSM 1296 vegetative cells were inoculated into distal colon reactors of both test systems (DC_CR and DC_LpC). (B) Model 3. CD NCTC 13307 log_{10} copy numbers were monitored from first growth detected on day 5 and 2 in DC_CR and DC_LpC, respectively. LpC was inoculated twice daily in DC_LpC starting from day 11. Both reactors were treated with metronidazole from day 16 - 25 and recovery was monitored during days 26 - 35.

Target locus of LpC was the CRISPR gene compared to 16S rRNA for CD. (---) CD detection limit of 4.4 log_{10} copy numbers mL^{-1}.
Discussion

Probiotics can transiently colonize the human colon, leading to an alteration of composition and activity of the commensal microbiota (Derrien & van Hylckama Vlieg, 2015). It was suggested that these changes could enhance general homeostasis of the gut microbiota, thereby preventing overgrowth of enteric pathogens such as *C. difficile* (Hell *et al.*, 2013). The hindered accessibility of the gastrointestinal tract hampers clinical studies on the effect of probiotics on gut microbiota of different colonic sections. In the current work we applied different *in vitro* colonic fermentation models operated with controlled conditions to test the response of the commensal elderly gut microbiota to the probiotic strain *L. paracasei* CNCM I-1518 and to investigate the probiotic-pathogen interaction with *C. difficile*, independently of host factors such as the epithelial cell layer and immune response.

Levels of LpC applied in the *in vitro* model were in the range of fecal concentrations of the same strain assessed *in vivo* (Oozeer *et al.*, 2006, Collins *et al.*, 2014). Upon daily addition, numbers of LpC remained stable or even increased indicating the possibility of temporary persistence. Colonization of LpC in the reactors was nevertheless transient since cessation of probiotic addition was accompanied by a rapid wash-out within three days (data not shown). Transient properties were ascribed to many other *Lactobacillus* species used as probiotics (Derrien & van Hylckama Vlieg, 2015).

Despite low abundance relative to commensal bacteria and transient properties, probiotics impact elderly microbiota composition. Consumption of probiotics led to an increase in bifidobacteria and lactobacilli (Ouwehand *et al.*, 2008, Lahtinen *et al.*, 2012) and decreased the abundance of opportunistic pathogens (Rampelli *et al.*, 2013). Here, we demonstrated increases in abundances of phylotypes belonging to the Lactobacillales, including *Lactobacillus* and *Enterococcus*, suggesting that LpC enhances niche colonization of closely related genera. An increase in fecal concentrations of the *Lactobacillus/Enterococcus* group was previously observed upon consumption of fermented milk containing a *Lactobacillus salivarius* strain (Collins *et al.*, 2002) or *Lactobacillus rhamnosus* (Tannock *et al.*, 2000) by healthy adults.

It was previously reported that Bacteroidetes were more abundant in elderly compared to adults (Claesson *et al.*, 2011). Here, LpC supplementation was associated with decreased abundances of phylotypes within the Bacteroidetes, suggesting a possible conversion to more adult-like microbiota with LpC. Compositional modifications observed after addition of LpC might be related to changes in trophic interactions.
In a recent study no effect on fecal microbiota composition but community-wide transcriptional changes were observed (Eloe-Fadrosh et al., 2015). Here we determined the impact of a probiotic on the proximal colon microbiota. The gut microbiota functional profile varied little during the test period confirming stability of the fermentation model. The high proportion of transcripts assigned to ‘Carbohydrates’ and ‘Protein metabolism’ indicated bacterial growth, carbohydrate fermentation and SCFA formation in the presence of high substrate concentrations successfully mimicking the scenario in the human proximal colon. Despite shifts in microbiota composition, we only observed minor alterations of relative abundance of microbial functions upon addition of LpC possibly due to functional redundancy of the intestinal microbiota. For example, a shift in butyrate producing phylotypes after addition of LpC with decreased abundance of Roseburia, Peptoniphilus, Ruminococcus and Coprococcus, and increased abundance of Faecalibacterium was observed, nevertheless, relative abundance of the SEED category ‘Fermentation’ including transcripts related to butyrate formation was not impacted. In agreement, the SCFA profile in both reactors was highly similar despite the presence of LpC. A positive correlation between L. paracasei supplementation and Faecalibacterium was reported earlier for healthy adults (Zhang et al., 2014). The addition of LpC was associated with an increased relative abundance of genes transcribing enzymes involved in several pathways within the Faecalibacterium transcriptome, including carbohydrate metabolism and degradation of aromatic compounds.

In co-cultures, LpC did not negatively impact growth of F. prausnitzii, indicating that both strains do not compete for substrates or are inhibited by metabolites formed. However, LpC enhanced survival of F. prausnitzii in the stationary growth phase. A possible reason could be the lower pH in co-culture compared to pure F. prausnitzii culture that may have protected cells from autolysis as it has been observed in several bacterial species (Rice & Bayles, 2008).

Probiotics were suggested as alternative treatment for gastrointestinal diseases including antibiotic-related infections, such as CD infection (Sanders et al., 2013). Several studies reported a reduction in CD associated diarrhea with probiotics, including Saccharomyces boulardii (Pothoulakis, 2009) and Lactobacillus acidophilus in combination with L. casei (Gao et al., 2010) as well as a milk drink with yoghurt starter bacteria and LpC (Hickson et al., 2007). S. boulardii acts by secreting a protease which is able to cleave toxin A and possesses enzymatic activity against CD toxin B (Castagliuolo et al., 1999). However, for
most probiotics the mechanism of action in CD infection remains unknown. Here, we found reduced CD cytotoxin titers when co-cultivated with LpC compared to pure culture indicating a possible proteolytic activity of LpC similar to S. boulardii. However, no reduction in CD cytotoxin titers was observed in continuous fermentation studies with LpC tested as preventive treatment (model 2) or as adjuvant therapy to metronidazole treatment (model 3). It could be that the complex microbiota had inhibitory effects on the LpC activity against CD toxins.

To conclude, this is the first-time investigation of the effect of a probiotic strain on the transcriptome of elderly gut microbiota using in vitro intestinal fermentation models. We demonstrated a compositional and functional response of the microbiota on LpC, with an enhancing effect on Faecalibacterium abundance and activity, a decrease in abundance of \( \text{H}_2 \) and \( \text{CH}_4 \) fermentative bacteria, and an increase in carbohydrate utilization, indicating a possible contribution of LpC in the trophic interaction of dietary carbohydrate utilization with the commensal microbiota. We thus showed that the probiotic LpC strain directly interacts with the human gut microbiota independent of the host. In contrast, no effect of LpC was observed on CD in complex microbiota uncoupled from the host when tested as preventive treatment or concomitantly to metronidazole. Thus, host-microbiota interaction studies should be conducted for further investigations of the mechanism of LpC in treatment or prevention of CD infection.

**Acknowledgements**

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Supporting information

Figure S 4.1: Composition of fermentation effluent samples in PC_CR of model 1 assessed with 16S rRNA gene amplicon sequencing. (A) Phylum level and (B) family level; uc, unclassified.

Figure S 4.2: Metabolite concentrations in PC_LpC and PC_CR of colonic model 1 assessed with HPLC. LpC was added twice daily to PC_LpC starting from day 15. Total metabolites (squares), acetate (diamonds), butyrate (triangles) and propionate (circles).
**Table S 4.1:** Primers used for enumeration of specific bacterial groups with qPCR.

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<td>GTTAGCACCCTTAAAGACG</td>
<td><em>Lactobacillus paracasei</em> CNCM I-1518</td>
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<td>(Collins et al., 2014)</td>
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<td><em>Bacteroides-Prevotella</em> group</td>
<td>16S rRNA</td>
<td>(Ramirez-Farias et al., 2009)</td>
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<td><em>Lactobacillus/Pediococcus/Leuconostoc spp.</em></td>
<td>16S rRNA</td>
<td>(Furet et al., 2009)</td>
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<td>Butyryl-CoA CoA transferase</td>
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### Table S 4.2: RNA sequencing information colonic model 1.

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<td>641.164</td>
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<td>135.390</td>
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<td>5.934.633</td>
<td>640.993</td>
<td>9.7</td>
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<td>7.204.870</td>
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### Table S 4.3: Relative abundance of bacterial orders (%) (rRNA reads). In bold: orders that are decreased in PC_LpC compared to PC_CR (p<0.05).

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<tbody>
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<td>0.69</td>
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uc: unclassified
Table S 4.4: Taxonomic assignment mRNA reads in PC_CR or in PC_LpC (order level). In bold: orders that are decreased in PC_LpC relative to PC_CR ($p<0.05$).

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<td>0.85</td>
</tr>
<tr>
<td>24</td>
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<td>1.01</td>
<td>72.33</td>
<td>72.01</td>
<td>1.00</td>
<td>65.67</td>
<td>67.63</td>
<td>1.03</td>
</tr>
</tbody>
</table>

uc: unclassified
**Table S 4.5**: Relative abundance of SEED categories of transcripts assigned to *Faecalibacterium* in proximal colon section (model 1). In bold: SEED categories that are increased or decreased in PC_LpC relative to PC_CR ($p<0.05$).

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
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<tbody>
<tr>
<td>16</td>
<td>Amino Acids and Derivatives</td>
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<td>7.78</td>
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<td>8.31</td>
<td>8.84</td>
<td>1.06</td>
<td>8.55</td>
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<td>Carbohydrates</td>
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<td>25.13</td>
<td>24.80</td>
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</tr>
<tr>
<td>24</td>
<td>Cell Division and Cell Cycle</td>
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<td>0.88</td>
<td>0.91</td>
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<td>1.12</td>
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<tr>
<td></td>
<td>Cell Wall and Capsule</td>
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<tr>
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<tr>
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<td>Metabolism of Aromatic Compounds</td>
<td>1.08</td>
<td>1.19</td>
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<td>Miscellaneous</td>
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<td>3.93</td>
<td>0.99</td>
<td>4.88</td>
<td>4.21</td>
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<tr>
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<td>Motility and Chemotaxis</td>
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<td>0.50</td>
<td>1.70</td>
<td>0.95</td>
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<td>0.33</td>
<td>0.58</td>
<td>0.31</td>
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<tr>
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<td>Nitrogen Metabolism</td>
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<td>0.17</td>
<td>0.19</td>
<td>1.12</td>
<td>0.18</td>
<td>0.08</td>
<td>0.42</td>
</tr>
<tr>
<td></td>
<td>Nucleosides and Nucleotides</td>
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<td>3.13</td>
<td>0.91</td>
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<td>3.23</td>
<td>1.14</td>
<td>2.10</td>
<td>3.05</td>
<td>1.45</td>
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<td>Phages, Prophages, Transposable elements, Plasmids</td>
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<td>0.18</td>
<td>0.82</td>
<td>0.20</td>
<td>0.08</td>
<td>0.40</td>
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<td>0.18</td>
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<td>0.92</td>
<td>2.35</td>
<td>2.50</td>
<td>1.06</td>
<td>2.21</td>
<td>2.76</td>
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<td>Potassium metabolism</td>
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<td>2.08</td>
<td>1.86</td>
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<tr>
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<td>Protein Metabolism</td>
<td>0.14</td>
<td>0.16</td>
<td>1.15</td>
<td>0.17</td>
<td>0.19</td>
<td>1.12</td>
<td>0.18</td>
<td>0.08</td>
<td>0.42</td>
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<tr>
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<td>Protein Metabolism</td>
<td>2.17</td>
<td>1.90</td>
<td>0.87</td>
<td>2.60</td>
<td>2.82</td>
<td>1.09</td>
<td>3.47</td>
<td>2.98</td>
<td>0.86</td>
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<tr>
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<td>0.89</td>
<td>0.46</td>
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<td>0.57</td>
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<tr>
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<td>Regulation and Cell signaling</td>
<td>0.21</td>
<td>0.22</td>
<td>1.05</td>
<td>0.11</td>
<td>0.12</td>
<td>1.11</td>
<td>0.16</td>
<td>0.13</td>
<td>0.82</td>
</tr>
<tr>
<td></td>
<td>Respiration</td>
<td>0.02</td>
<td>0.02</td>
<td>0.90</td>
<td>0.01</td>
<td>0.02</td>
<td>2.28</td>
<td>0.02</td>
<td>0.03</td>
<td>1.50</td>
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<td>Secondary Metabolism</td>
<td>0.08</td>
<td>0.06</td>
<td>0.84</td>
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<td>0.08</td>
<td>1.40</td>
<td>0.05</td>
<td>0.05</td>
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<td>Stress Response</td>
<td>1.03</td>
<td>0.94</td>
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<td>0.91</td>
<td>0.96</td>
<td>1.05</td>
<td>1.17</td>
<td>1.00</td>
<td>0.85</td>
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<tr>
<td></td>
<td>Sulfur Metabolism</td>
<td>16.64</td>
<td>16.28</td>
<td>0.98</td>
<td>16.16</td>
<td>15.35</td>
<td>0.95</td>
<td>14.71</td>
<td>16.56</td>
<td>1.13</td>
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<tr>
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<td>Virulence, Disease and Defense</td>
<td>4.04</td>
<td>3.29</td>
<td>0.81</td>
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<td>4.36</td>
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<td>4.51</td>
<td>4.28</td>
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</tr>
<tr>
<td></td>
<td>Virulence, Disease and Defense</td>
<td>1.47</td>
<td>1.55</td>
<td>1.05</td>
<td>1.79</td>
<td>1.89</td>
<td>1.06</td>
<td>1.51</td>
<td>1.69</td>
<td>1.12</td>
</tr>
</tbody>
</table>
Table S 4.6: Relative abundance of transcripts that were different in PC_LpC relative to PC_CR in proximal colon section (model 1).

<table>
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<tr>
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<th></th>
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<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetyl-CoA acetyltransferase (EC 2.3.1.9)</td>
<td>0.70</td>
<td>0.89</td>
<td>1.27</td>
<td>0.89</td>
<td>1.03</td>
<td>1.15</td>
<td>0.73</td>
<td>0.87</td>
<td>1.20</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acetate kinase (EC 2.7.2.1)</td>
<td>0.31</td>
<td>0.48</td>
<td>1.53</td>
<td>0.35</td>
<td>0.64</td>
<td>1.84</td>
<td>0.72</td>
<td>0.77</td>
<td>1.07</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4-alpha-glucanotransferase (amylomaltase) (EC 2.4.1.25)</td>
<td>1.11</td>
<td>0.80</td>
<td>0.72</td>
<td>1.18</td>
<td>0.92</td>
<td>0.78</td>
<td>1.24</td>
<td>0.75</td>
<td>0.60</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oligopeptide ABC transporter, periplasmic oligopeptide-binding protein OppA (TC 3.A.1.5.1)</td>
<td>0.22</td>
<td>0.15</td>
<td>0.65</td>
<td>0.30</td>
<td>0.19</td>
<td>0.61</td>
<td>0.32</td>
<td>0.18</td>
<td>0.57</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table S 4.7: Production of metabolites (mM) in co-culture and single cultures of LpC and *F. prausnitzii* of all time points analyzed in co-culture study.

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Culture</th>
<th>Lactate</th>
<th>Formate</th>
<th>Acetate</th>
<th>Butyrate</th>
<th>Propionate</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Co-culture</td>
<td>ND</td>
<td>ND</td>
<td>26.4 ± 7.2</td>
<td>ND</td>
<td>8.5 ± 1.1</td>
</tr>
<tr>
<td></td>
<td>LpC</td>
<td>ND</td>
<td>ND</td>
<td>28.0 ± 0.9</td>
<td>ND</td>
<td>9.5 ± 0.8</td>
</tr>
<tr>
<td></td>
<td><em>F. prausnitzii</em></td>
<td>ND</td>
<td>0.5 ± 0.4</td>
<td>27.7 ± 3.0</td>
<td>ND</td>
<td>9.8 ± 1.3</td>
</tr>
<tr>
<td>8</td>
<td>Co-culture</td>
<td>8.3 ± 1.5</td>
<td>4.2 ± 1.5</td>
<td>27.6 ± 1.3</td>
<td>1.9 ± 1.2</td>
<td>8.1 ± 0.7</td>
</tr>
<tr>
<td></td>
<td>LpC</td>
<td>8.3 ± 1.6</td>
<td>ND</td>
<td>27.1 ± 5.8</td>
<td>ND</td>
<td>9.0 ± 1.5</td>
</tr>
<tr>
<td></td>
<td><em>F. prausnitzii</em></td>
<td>ND</td>
<td>4.3 ± 1.0</td>
<td>28.9 ± 1.4</td>
<td>2.2 ± 0.8</td>
<td>8.8 ± 3.8</td>
</tr>
<tr>
<td>48</td>
<td>Co-culture</td>
<td>21.9 ± 1.0</td>
<td>9.8 ± 1.6</td>
<td>26.3 ± 2.2</td>
<td>6.2 ± 1.2</td>
<td>7.5 ± 3.1</td>
</tr>
<tr>
<td></td>
<td>LpC</td>
<td>31.9 ± 2.8*</td>
<td>2.3 ± 2.2*</td>
<td>28.7 ± 2.6*</td>
<td>ND</td>
<td>8.8 ± 0.4</td>
</tr>
<tr>
<td></td>
<td><em>F. prausnitzii</em></td>
<td>ND</td>
<td>11.0 ± 2.0</td>
<td>28.5 ± 3.1*</td>
<td>5.2 ± 1.6</td>
<td>9.7 ± 0.6*</td>
</tr>
<tr>
<td>72</td>
<td>Co-culture</td>
<td>21.6 ± 1.7</td>
<td>8.3 ± 2.3</td>
<td>26.2 ± 2.8</td>
<td>5.9 ± 2.2</td>
<td>8.3 ± 1.2</td>
</tr>
<tr>
<td></td>
<td>LpC</td>
<td>33.6 ± 3.1*</td>
<td>1.5 ± 2.1*</td>
<td>29.8 ± 1.3*</td>
<td>ND</td>
<td>9.7 ± 0.5</td>
</tr>
<tr>
<td></td>
<td><em>F. prausnitzii</em></td>
<td>ND</td>
<td>10.3 ± 1.8</td>
<td>29.6 ± 1.9*</td>
<td>5.3 ± 0.9</td>
<td>9.7 ± 0.6</td>
</tr>
</tbody>
</table>

Data are means ± SD of three replicates tested in four different experiments (n=12), except for time point 72 h that was tested in three different experiments (n=9); samples were analyzed in duplicate. ND, not detected. Means with an asterisk (*) are significantly different from the co-culture (p<0.05).
Table S 4.8: Changes in pH during incubation of co-culture and the respective single culture CD DSM 1296 and LpC in co-culture study.

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Co-culture</th>
<th>CD</th>
<th>LpC</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>6.46 ± 0.07</td>
<td>NA</td>
<td>6.42 ± 0.04</td>
</tr>
<tr>
<td>5</td>
<td>6.32 ± 0.05</td>
<td>6.40 ± 0.11</td>
<td>6.35 ± 0.07</td>
</tr>
<tr>
<td>10</td>
<td>5.62 ± 0.02</td>
<td>6.01 ± 0.01*</td>
<td>5.81 ± 0.03*</td>
</tr>
<tr>
<td>13</td>
<td>5.54 ± 0.06</td>
<td>5.67 ± 0.04*</td>
<td>5.63 ± 0.05</td>
</tr>
<tr>
<td>25</td>
<td>5.66 ± 0.02</td>
<td>5.75 ± 0.02*</td>
<td>5.70 ± 0.02*</td>
</tr>
</tbody>
</table>

Values represent means ± SD of three replicates tested in two separate experiments (n=6). Means with an asterisk (*) are significantly different from the co-culture (p<0.05). NA, not analyzed.
**Table S 4.9:** Production of metabolites (mM) in co-culture and single cultures of LpC and CD DSM 1296 of all time points analyzed in co-culture study.

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Culture</th>
<th>Lactate</th>
<th>Formate</th>
<th>Acetate</th>
<th>Butyrate</th>
<th>Propionate</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Co-culture</td>
<td>1.7 ± 0.3</td>
<td>0.8 ± 0.2</td>
<td>9.4 ± 0.6</td>
<td>ND</td>
<td>2.3 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>LpC</td>
<td>1.7 ± 0.3</td>
<td>1.6 ± 3.0</td>
<td>9.5 ± 3.1</td>
<td>ND</td>
<td>2.2 ± 0.9</td>
</tr>
<tr>
<td></td>
<td>CD</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>ND</td>
<td>NA</td>
</tr>
<tr>
<td>5</td>
<td>Co-culture</td>
<td>6.2 ± 0.5</td>
<td>0.7 ± 0.3</td>
<td>9.8 ± 0.6</td>
<td>ND</td>
<td>2.1 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>LpC</td>
<td>6.6 ± 0.6</td>
<td>0.7 ± 0.3</td>
<td>10.0 ± 0.5</td>
<td>ND</td>
<td>2.3 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>CD</td>
<td>0.3 ± 0.1*</td>
<td>1.5 ± 3.1</td>
<td>10.1 ± 1.9</td>
<td>ND</td>
<td>2.3 ± 0.9</td>
</tr>
<tr>
<td>10</td>
<td>Co-culture</td>
<td>20.2 ± 1.5</td>
<td>4.0 ± 1.8</td>
<td>16.1 ± 1.7</td>
<td>0.9 ± 0.5</td>
<td>2.1 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>LpC</td>
<td>20.8 ± 5.5</td>
<td>1.0 ± 0.2</td>
<td>10.5 ± 0.4*</td>
<td>ND</td>
<td>2.2 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>CD</td>
<td>1.3 ± 0.1*</td>
<td>7.3 ± 0.5</td>
<td>18.3 ± 0.7</td>
<td>0.7 ± 0.3</td>
<td>2.0 ± 0.3</td>
</tr>
<tr>
<td>13</td>
<td>Co-culture</td>
<td>22.1 ± 0.7</td>
<td>4.2 ± 0.9</td>
<td>16.5 ± 0.7</td>
<td>1.2 ± 0.3</td>
<td>2.1 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>LpC</td>
<td>28.7 ± 1.4*</td>
<td>0.9 ± 0.4*</td>
<td>10.7 ± 0.6*</td>
<td>ND</td>
<td>2.3 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>CD</td>
<td>3.6 ± 0.4*</td>
<td>7.7 ± 1.0*</td>
<td>20.0 ± 0.4</td>
<td>2.5 ± 0.9*</td>
<td>1.6 ± 0.4*</td>
</tr>
<tr>
<td>25</td>
<td>Co-culture</td>
<td>22.1 ± 0.9</td>
<td>5.1 ± 0.5</td>
<td>18.7 ± 1.8</td>
<td>1.7 ± 0.5</td>
<td>2.3 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>LpC</td>
<td>28.7 ± 0.6*</td>
<td>1.2 ± 0.1*</td>
<td>10.9 ± 0.9*</td>
<td>ND</td>
<td>2.3 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>CD</td>
<td>3.8 ± 0.4*</td>
<td>7.8 ± 0.1*</td>
<td>21.2 ± 0.7</td>
<td>2.9 ± 0.6*</td>
<td>1.7 ± 0.2*</td>
</tr>
</tbody>
</table>

Data are means ± SD of three replicates tested in two different experiments (n=6); samples were analyzed in duplicate. ND, not detected; NA, not analyzed. Means with an asterisk (*) are significantly different from the co-culture (p<0.05).
Chapter 5

Conclusions and perspectives
General conclusions

In the framework of this thesis we investigated \textit{in vitro} the potential of the probiotic strain \textit{Lactobacillus paracasei} CNCM I-1518 (LpC) to antagonize the pathogen \textit{Clostridium difficile} (CD) in complex elderly microbiota. An intestinal model for the investigation of CD and potential treatments has been developed by Baines \textit{et al.} (2005). However, the three-stage model of Baines \textit{et al.} has some limitations: (1) the model is inoculated with a fecal suspension, and does not account for the investigation of biofilm-associated bacteria; (2) the microbiota is analyzed with plating only, therefore does not assess the complete microbial diversity and (3) the design of the model restricts the integration of a control reactor for direct comparison to the treatment. The research strategy of this thesis addressed the limitations of the current CD \textit{in vitro} models by including the development of a new intestinal model for CD. This model was further used to test the potential of LpC to fight CD.

\textit{In vitro} intestinal fermentation models have been developed to investigate the gut microbiota of infants and adults while studies of the elderly gut microbiota are limited to three-stage models, applying culture based (Baines \textit{et al.}, 2005, Crowther \textit{et al.}, 2014) or FISH methods (Likotrafiti \textit{et al.}, 2014) for microbiota monitoring. The first step of this thesis was therefore to develop new models of the elderly microbiota using qPCR and 16S rRNA gene amplicon sequencing for in-depth characterization of the elderly microbiota (chapter 2). We developed three different models with microbiota from donors aged between 71 and 78 years. The fecal microbiota was immobilized in gel beads that provided a protective microenvironment for the bacteria and allowed the growth of a stable gut ecosystem at high cell densities of up to log 11 cells per mL effluent. The main differences in the bacterial composition and metabolic activity between the models may be assigned to the different donor fecal inocula and pH applied in IR of 5.5 (model 1) and 5.7 (model 2 and 3). The pH of 5.5 of model 1 was selected according to pH set in previous elderly gut fermentation models (Baines \textit{et al.}, 2005) and was increased to pH 5.7 in model 2 and 3 to account for a low production of butyrate and propionate measured in IR of model 1. Because no \textit{Roseburia} spp. was detected in fecal donor 1, the microbial composition in this sample and in the effluent of model 1 was less representative of the elderly population compared to the two other donors and the corresponding fermentations. Similar microbial profiles between effluent samples of model 2 and 3 and the corresponding fecal donors were obtained. However, the ratio of Firmicutes:Bacteroidetes was decreased in both models, likely due to variations between \textit{in}
environmental conditions and specific donor conditions as well as host interactions that are not mimicked in in vitro models.

Model 1 was built according to the “traditional” three-stage design while model 2 and 3 were based on the new flexible PolyFermS platform that was recently developed and validated with child and swine microbiota (Zihler Berner et al., 2013, Tanner et al., 2014b). With the PolyFermS models several treatments can be investigated simultaneously and compared to a control inoculated with the same microbiota, thus generating more accurate data for comparison and detection of treatments effects than applying treatments during consecutive periods, such as in model 1, or independent runs. In both PolyFermS models microbial diversity and metabolic activity was highly similar between control and test reactors, suggesting that these in vitro models are well-suited for ecological studies of the elderly gut microbiome.

Intestinal models for CD are limited to batch fermentation studies (Meader et al., 2010, Rea et al., 2011b) and the three-stage continuous fermentation model developed by Baines et al. (2005). We therefore applied the PolyFermS models with elderly gut microbiota to develop a new model for CD investigations (chapter 3). First, we tested free vegetative CD cells of PCR ribotype 001 in the in vitro model mimicking proximal and transverse-distal conditions (model 2), where we observed colonization after a few days in the transverse-distal (pH 6.8) but not the proximal (pH 5.7) colon reactor, suggesting that the pH of 5.7 is too low for competitive CD growth. In a next approach (model 3) we applied CD spores (PCR ribotypes 001 and 012) and investigated the broad-spectrum antibiotic ceftriaxone for induction of spore germination similar to Baines et al. (2011) however, in contrast to Baines et al. we simultaneously analyzed CD growth following spore inoculation independent of antibiotic pressure. Interestingly, CD of ribotype 012 colonized reactors in both conditions (with and without antibiotic) with short delay of three days in the reactor free of antibiotics. Colonization of ribotype 001 was also tested in the same conditions and the opposite was observed since growth was detected first in the reactor free of antibiotics. Our findings suggest that uncoupled from the host no antibiotics are needed for disruption of colonization resistance to see CD colonization. Model 3 was then challenged with metronidazole to assess the applicability for antibiotic treatments testing. Shortly after introduction of metronidazole CD growth and toxin production was not detectable anymore. However, only one day after cessation of metronidazole CD growth and toxin production were detected again and even exceeded pre-metronidazole levels.
With ceftriaxone we observed only slight effects on microbiota composition and metabolic activity. In contrast, metronidazole induced a strong dysbiosis and inhibition of butyrate production. Most of the bacterial groups recovered 10 days after cessation of metronidazole but their recovery was delayed compared to CD.

The potential of LpC as probiotic treatment in CDI was analyzed in co-culture studies independent of the complex microbiota and with regard to the elderly microbiota using the *in vitro* fermentation models developed for CD (chapter 4). The co-culture tests were performed in batch fermentations of 24 h at the same initial pH of 6.8 that was also set in the transverse-distal colon reactors of the continuous fermentation experiments. LpC cells were inoculated five hours prior to CD and at a twofold inoculation rate to account for the faster growth of the pathogen. During exponential growth viable cell count of CD (ribotype 001) was slightly lower in single cultures compared to co-cultures. However, at the end of the fermentation the cell number was higher in the co-cultures compared to single cultures. Although no effect on CD growth was observed at the end of the fermentation we demonstrated around 40% decreased toxin production in co-culture with LpC compared to single CD cultures during exponential phase and at the end of the fermentation.

In intestinal models we investigated LpC as preventive treatment (model 2) and complementary therapy to metronidazole (model 3). LpC colonized the reactors transiently with copy numbers of around $\log_{10} 7.5$ per mL effluent which is similar to *in vivo* (Oozeer et al., 2006, Collins et al., 2014). In neither model CD growth nor toxin production was reduced in presence of LpC compared to control and CD growth was observed at high numbers of up to $\log_{10} 9$ gene copy numbers per mL effluent. Thus, in complex microbiota we could not replicate the effect of LpC on toxin production from co-culture studies.

In a last step, we investigated how the transient colonization of LpC might result in interactions with the gut microbiota. The study was performed in a fourth fermentation experiment using the PolyFermS design with control and test reactors run at proximal colon conditions. 16S rRNA gene amplicon sequencing and metatranscriptomics were used to assess microbiota composition and activity. Although no major alteration of the microbiota was observed with LpC, which is expected from probiotic interventions, we demonstrated some changes in abundance of specific bacterial groups. We observed an increase in the closely related genera *Enterococcus* and *Lactobacillus*, a shift in the butyrate producing bacteria and a decrease in Bacteroidetes members. Furthermore, the abundance of $H_2$ and
CH₄ producing microbes was reduced with LpC, thus the probiotic may be effective in alleviating gastrointestinal complaints related to increased colonic gas production. On a functional level we observed an increase in carbohydrate utilization activity suggesting that LpC induced changes in trophic interactions between gut microbes. The increase in *Faecalibacterium* that was observed in the continuous fermentation was further investigated in co-culture tests and it was shown that LpC can prevent the cell lysis of *Faecalibacterium prausnitzii* to a certain extent during 72 h incubation. The mechanism behind this interaction remains to be elucidated.

In this last fermentation experiment we showed that a healthy elder gut microbiota responded to LpC intervention. We also showed that the PolyFermS models are well-suited for microbe interaction studies since environmental conditions can be highly controlled. In human studies high interindividual differences exist, thus it can be difficult to detect probiotic effects on microbiota regarding their relatively low number compared to predominant gut microbes.

In the framework of this doctoral thesis, we successfully developed continuous colonic fermentation models with elderly microbiota for CD investigations, probiotic treatment testing and interaction studies with commensal gut microbes. We found that LpC had no direct inhibitory effects against CD in complex microbiota and co-culture studies. However, a compositional and functional response of elderly microbiota on LpC was demonstrated.
Perspectives

The research in this thesis is based on the outcome of one clinical trial that showed reduced CDAD incidence in elderly patients consuming a fermented milk drink that contained LpC and conventional starter bacteria (*Streptococcus thermophilus* and *Lactobacillus bulgaricus*) (Hickson *et al.*, 2007), while the individual contribution of the different bacterial strains is not known thus far. Promising results were obtained in the first clinical trial but the study had some limitations that were criticized by the EFSA Panel (EFSA, 2011), including the selection of study participants since patients on high-risk antibiotics were excluded and the choice of placebo drink, which was a sterile milk drink. It would be recommended to conduct further clinical trials for testing the efficacy of LpC in prevention of CDAD. An increased number of subjects should be investigated for the effect of LpC without starter bacteria, for example as probiotic capsule and compared to a suitable placebo control. The repetition of the clinical trial is important to prove the effect of LpC against CD.

In complex microbiota but uncoupled from the host, no antagonistic effect of LpC on CD was observed in the framework of this thesis as illustrated in Figure 5.1. We thus concluded that LpC does not directly antagonize CD e.g. by secretion of antimicrobial substances or by competitive inhibition. Other potential mechanisms described for probiotics involve host-related factors, such as the epithelial cell layer and the immune system. Thus, for further *in vitro* evaluations of the potential of LpC in treatment or prevention of CDI, immune and human epithelial cells should be considered (Figure 5.1). A possible strategy is the combination of the elderly intestinal models with cellular models (e.g. human cell lines Caco-2 and mucus secreting HT29-MTX), an approach that was previously used for evaluating probiotic-pathogen interactions (Zihler *et al.*, 2011). In order to consider the immune response, peripheral blood mononuclear cells can be included as a third component besides human epithelial and bacterial cells (Dostal *et al.*, 2014a). The cellular models can be used to investigate the competition between LpC and CD for adhesion sites, mucin production and also the potential of LpC to inhibit binding of CD toxins to the epithelium, a property that was described for the yeast *Saccharomyces boulardii* (Pothoulakis *et al.*, 1993). Studies with cellular models are usually performed in aerobic conditions, however CD is a strict anaerobic bacterium and therefore modifications of the current methods are required.
Revealing data with respect to efficacy of LpC in CDI could be obtained when performing animal studies. In particular, mouse models are well-suited to investigate the immune response in CDI. As example, in a mouse model it was shown that the probiotic *S. boulardii* enhances IgA immune response to CD toxin A (Qamar et al., 2001). Furthermore, studies could be performed in human-microbiota-associated mouse models for investigations of host-microbiota interactions, an important factor regarding CD colonization and thus interesting for investigations with LpC. The effect of aging on immune response to CDI during LpC treatment could be addressed by using aged gnotobiotic mice as shown by Pawlowski et al. (2010). A first limitation of these *in vivo* models is that animals differ in the pathology of CDI compared to humans and it can be difficult to extrapolate findings from animal models to humans. Furthermore, high concentrations and combinations of antibiotics are required for pretreatment of mice or hamsters in order for CD to colonize their intestine, at which point their microbiota and immune response is more predisposed to infections compared to the case of a human that develops CDI.

An important finding of this thesis is that CD colonized the intestinal models with elderly microbiota without prior antibiotic treatment and uncoupled from the host. This finding is in contradiction with the general assumption that a healthy microbiota provides resistance against CD and that antibiotics are promoters for CDI. It could however be that the microbiota in old age is in general more susceptible to CD compared to adult microbiota. It would therefore be of interest to test if CD colonization occurs as well if the fermentation model is inoculated with a fecal microbiota from an adult host. If differences are observed between colonization resistance of adult and elderly microbiota, then further studies could be performed to investigate key bacterial groups in colonization prevention.

In the last fermentation trial we demonstrated the ability of LpC to interact with the elderly gut microbiota, uncoupled from the host. The PolyFermS design proved as an effective model for this study due to controlled environmental conditions that allows detecting small but significant effects. An even increased reproducibility could be achieved by adding another fermentation test period with the same treatment but control and test reactors are switched for observation of repetition of treatment effect, thus applying randomization, similar to *in vivo* trials.
Chapter 5

The interaction of probiotics with the colonic microbiota could result in improvement of colonization resistance against the opportunistic growth of pathogens, such as CD or also in general enhancement of gut health. In this thesis we demonstrated associations of LpC with butyrate (Faecalibacterium and Roseburia) and H₂ and CH₄ producing microbes (Bacteroides, Ruminococcus and methanogens). Butyrate and colonic gas production is interesting regarding host health because butyrate has beneficial effects on gut health while an overproduction of H₂ and CH₄ is associated with gastrointestinal diseases. Thus, co-culture or mixed culture studies of LpC with these commensal gut microbes could be performed to investigate growth behavior and functional interactions using transcriptomics analysis. These in vitro interactions studies may elucidate how LpC affects the trophic relations in complex microbiota and how they are linked to gut health, in particular in the elderly, thereby addressing the application of LpC in gastrointestinal complaints unrelated to CD, such as IBD or constipation.

In this thesis we investigated the effect of LpC on microbiota derived from healthy elderly individuals. However, CDI is associated with broad-spectrum antibiotherapy. Therefore, the potential of LpC to interact with the microbiota should also be investigated in antibiotic treated microbiota. We used ceftriaxone in reactors during investigation of CD colonization. However, only small changes on the microbiota were observed with this antibiotic. Other broad-spectrum antibiotics should therefore be investigated for LpC effects on microbiota and studies should be performed at proximal colon conditions since activity of the antibiotic is increased in this colonic section compared to distal colon conditions.
Figure 5.1: Schematic overview of the obtained results and future research perspectives


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186


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Figure A 1: Overview on fermentation models and experiments performed. CD, *Clostridium difficile*; LpC, *Lactobacillus paracasei* CNCM I-1518.
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