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Towards understanding the modulation potential of dietary fibers on intestinal microbiota using human and a novel murine intestinal fermentation model

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Content

Abbreviations

AIEC	Adherent-invasive *E.coli*
AMPs	Antimicrobial peptides
ATG	Autophagy gene
ATP	Adenosine triphosphate
BCFA	Branched chain fatty acid
ButCoA	ButyrylCoA
CAT	Catalase
CD	Crohn's Disease
CH_4	Methane
CO_2	Carbon dioxide
COX	Cyclooxygenase
Cu	Copper
DF	Dietary fiber
DNA	Deoxyribonucleic acid
DP	Degree of polymerization
DSS	Dextran sodium sulfate
FA	Fatty acids
FAD	Flavin adenine dinucleotide
Fe	Iron
FMT	Fecal microbiota transplant
FOS	Fructooligosaccharide
FUT	Fucosyltransferase gene
GALT	Gut associated lymphoid tissue
GIT	Gastrointestinal tract
GOS	Galactooligosaccharide
GPR	G Protein-coupled receptor
GPX	Glutathione peroxidase
H_2	Hydrogen
HCl	Hydrochloric acid
HLA	Human leukocyte antigen
HMA	Human microbiota associated
$HO\cdot^-$	Hydroxyl radical
$HO_2\cdot$	Hydroperoxyl
HOCl	Hydrochlorus
HPLC	High performance liquid chromatography
H_2O_2	Hydrogen peroxide
H_2S	Hydrogen sulfide
IBD	Inflammatory bowel disease
IFN	Interferone
Ig	Immunoglobulin
IL-	Interleukin

IRGM	Immunity-related GTPase family M protein
MLN	Mesenteric lymph nodes
NADPH	Nicotinamide adenine dinucleotide phosphate
NF-κB	Necrosis factor KB
NH_3	Ammonia
NOD	Nitric oxide deoxygenase
NOX	NADPH oxidase
NSAID	Nonsteroidal anti-inflammatory drugs
OTU	Operational taxonomic unit
O_2	Oxygen
$O_2 \cdot^-$	Superoxide
O_3	Ozone
PEP	Phosphoenolpyruvate
PP	Peyer's Patch
PRR	Pattern recognition receptor
PTPN	Protein tyrosine phosphatase non-receptor type
PUFA	Polyunsaturated fatty acids
qPCR	Quantitative polymerase chain reaction
rDNA	Ribosomal deoxyribonucleic acid
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
RNHCl	Chloramine
RNS	Reactive nitrogen sepcies
RO·	Alcoxyl
RO_2·	Peroxyl
ROS	Reactive oxygen species
RT	Retention time
SCFA	Short chain fatty acid
SOD	Superoxide dismutase
spp.	Species
SRB	Sulphate-reducing bacteria
Th cell	T helper cell
TGF	Transforming growth factor
TLR	Toll-like receptor
TNF	Tumor necrosis factor
Cu	Copper
UC	Ulcerative colitis
WT	Wild type
XOS	Xylooligosaccharide

Summary

The mammalian gastrointestinal tract harbors a complex and diverse community of bacteria, called gut microbiota that exerts elemental physiological functions in health and disease. Diet shapes the gut microbiota composition and activity, which in turn modulates host metabolism and homeostasis. Non-digestible dietary fibers (DFs) for example, have beneficial effects on intestinal wellbeing by acting as a carbon source for growth and functionality of selective commensal bacteria within the gut. In the colon, microbial fermentation of complex carbohydrates generates short chain fatty acids (SCFAs), such as acetate, propionate and butyrate, which are absorbed by the intestinal epithelium conferring systemic health effects. The intake of DFs can lead to increased SCFA production due to promoted bacterial cross-feeding activities, a subsequent acidification of the lumen and thus homeostasis.

An adverse disruption of the structure or functionality of the gut microbiota, called microbial dysbiosis, induces significant alternations in the delicate microbe-host synergy and is associated with chronic inflammatory disorders, such as inflammatory bowel disease (IBD). There has been a rapid increase in IBD incidence, especially in Western style countries. Therefore, a Western-diet characterized by low DF intake might be a potential trigger for IBD. Besides diet, other environmental factors influence the intestinal microbiota and its activity and may induce microbial dysbiosis and trigger IBD. For example, oxidative stress caused by excessive reactive oxygen species and antibiotics are potential inducers of microbial dysbiosis.

The immediate impact of microbiota modulatory factors promoting health (dietary factors) or disease (oxidative stress and antibiotics) on intestinal microbial activity and structure is difficult to assess due to inaccessibility of human colon samples or end-point samples in mouse models. *In vitro* fermentation models mimicking the intestinal conditions offer the possibility of investigating the microbe-microbe interactions independent of the host. The main objective of this thesis was to investigate the potential modulating effects of different dietary fibers and direct adverse effects of oxidative and antibiotic stress on gut microbiota composition and functionality using an *in vitro* continuous fermentation model of the human colon and mouse cecum.

Study 1

In a first part, continuous *in vitro* colonic fermentation models using immobilized human faecal gut microbiota were set-up mimicking proximal colon condition to study the modulatory potential of four different dietary fibers. We immobilized two distinct faecal microbiota (D3 and D4) differing within the Bacteroidetes phylum with higher levels of *Bacteroidaceae* in D3 and higher levels of *Prevotellaceae* in D4, and we successfully maintained the two distinct microbiota, both on metabolic and phylogenetic levels in the PolyFermS model. We used three continuous *in vitro* fermentation PolyFermS models to study the modulating effects of inulin, β-glucan, xylo-oligosaccharide (XOS) and α-galacto-oligosaccharide (α-GOS) on two distinct gut microbiota, independently from host factors. The four dietary fibers (DF) were supplemented at a physiologic

concentration, equivalent to 9 g daily intake. DF treatments resulted in increased short-chain fatty acid production, evidencing fermentability of all tested fibers by the two microbiota, and led to consistent metabolic responses depending on the donor microbiota. Irrespectively of the DF, D3 *in vitro* microbiota responded by increased butyrate production, whereas D4 *in vitro* microbiota displayed higher propionate production. Moreover, supplementation of the two short-chain carbohydrates XOS and α-GOS resulted in high acetate production in all treatments for both microbiota. Interestingly, metabolic cross-feeding of butyrate-producers on acetate within both PolyFermS microbiota was microbiota-dependent and particularly observed upon inulin supplementation, with either increase in butyrate or acetate levels. At phylogenetic level, we also observed changes in abundance of specific bacterial taxa within one depending on the microbiota, which could explain the observed short-chain fatty acid profiles and possible cross-feeding interactions between the different functional bacterial populations.

Study 2

Next, a novel continuous fermentation model based on the PolyFermS platform inoculated with immobilized murine caecal microbiota was developed and validated. Murine models are the model of choice for studying the role of gut microbiota in health and disease. However, mice and human microbiota differ in species composition, and further investigation of the murine gut microbiota is important to improve current murine models and mechanistic understanding of the gut microbiota. Continuous *in vitro* fermentation models are powerful tools to investigate microbe-microbe interactions while circumventing animal testing and host factors. There is no model developed yet for murine intestinal microbiota fermentation.

In a first step, we determined the pH, bacterial composition and the metabolic profile in the caeca of C57BL/6 mice. To support growth and activity of the murine caecal bacterial populations, a complex murine nutritive growth medium was developed to mimic substrate conditions encountered in chyme entering the mouse caecum. Different factors of the fermentation model, including caecum sampling, fermentation starting mode, pH, retention time and growth medium composition were investigated in a sequential order to select conditions leading to microbial fermentation and composition akin to the caecum.

In the established model, high, representative and stable *in vitro* fermentation along continuous operation (up to 69 days) with main fermentation metabolites acetate, propionate and butyrate and ratios akin to the donor mouse caeca used to prepare the inoculum. The *in vitro* murine caecal microbiota was composed by mainly bacterial phyla Firmicutes and Bacteroidetes, and taxa belonging to important functional groups for intestinal fermentation (*Bacteroidaceae*, *Lachnospiraceae*, *Ruminococcaceae*, *Clostridiaceae*, *Lactobacillaceae* and *Verrucomicrobiaceae*). There was an overall decrease in bacterial diversity *in vitro* compared to caecal inoculum, but this did not affect microbial fermentation and stability. Adjustments of the components in the nutritive medium, of pH and retention time were carried out with the aim to control the high levels of

Enterobacteriaceae measured *in vitro*. We showed that long-term continuous cultivation of the caecal murine microbiota is possible while maintaining its functionality and phylogeny. This model could be used to study the impact of biotic and abiotic factors linked with health or disease on gut microbial functionality and structure prior to microbiota transplantation in mice to help elucidate mechanisms of host-microbiota interactions.

Study 3

In a last step, we used the novel developed murine caecum *in vitro* continuous fermentation models to study selected environmental factors leading to alternations of the gut microbiota. Thus, we assessed the direct adverse effects of oxidative stress (H_2O_2), metronidazole (used in IBD treatment, MTZ) and the beneficial modulating effects of inulin and XOS on two murine *in vitro* microbiota (F1 and F2). Our data reported that oxidative and antibiotic stresses led to a considerable dysbiosis of the gut microbiome. Oxidative stress resulted in immediate and consistent impairments of the microbial metabolism, increased facultative anaerobes (*Lactobacillus* and *Enterococcaceae*) and *Enterobacteriaceae*, and decreases in strict anaerobic butyrate-producers (i.e. *Coprococcus*) of both tested microbiota. Addition of MTZ caused a temporary inhibition of bacterial metabolism in both treatments of F1, which recovered after three days of antibiotic stress. The MTZ treatment also led to persistent increases in the abundance of facultative anaerobic bacterial taxa belonging to *Lactobacillus, Veillonella, Sutterella* and *Enterococcus*. Supplementation with XOS consistently increased total metabolite productions, mainly acetate, and increased abundances of bacterial taxa belonging to *Ruminococcus*, *Allobaculum* and [*Mogibacteriaceae*], whereas *Veillonella dispar* decreased. Inulin supplementation resulted in donor microbiota-dependent changes in the bacterial metabolism, promoting acetate and butyrate in F1 and propionate in F2. Interestingly, when the dosage of inulin was raised, the relative abundance of *Erysipelotrichaceae* taxa associated with butyrate production was specifically increased.

Conclusion

This thesis gives insights into the modulation potential of selected environmental factors on intestinal microbial fermentation using continuous *in vitro* models inoculated with murine caecal and human faecal immobilized microbiota. Our data suggests that the prebiotic mechanism through bacterial fermentation of the dietary fibers is not only determined by their physicochemical characteristics, but also highly depends on the baseline gut microbiota and addition level. A low-dosage supply of a dietary fiber only increased the metabolic activity of the *in vitro* microbiota, while supplementation of a high dose was additionally accompanied by changes in the intestinal microbiota composition. In addition, this thesis provided first insights on the detrimental effects of oxidative stress on *in vitro* gut microbiota structure and metabolism, thus possibly promoting microbial dysbiosis. Oxidative stress impaired carbohydrate fermentation and the

abundance of strict anaerobic butyrate producers, while it enhanced growth of Proteobacteria. Further, MTZ was also found to induce microbial dysbiosis by highly impairing intestinal bacterial composition and bacterial functionality. More studies are required to determine the exact influence of the initial gut microbiota composition on metabolic response upon prebiotic supplementation, and to identify the relationship between specific bacterial populations, metabolic response and dosage of dietary fibers. Furthermore, *in vivo* validation studies are required to elucidate the role of the gut microbiota and microbial shifts in health and chronic inflammatory diseases.

Zusammenfassung

Der Magen-Darm-Trakt von Säugetieren ist von einer sehr komplexen und vielfältigen Bakteriengemeinschaft besiedelt, die als Darmbakterien bezeichnet wird. Die Darmbakterien üben wesentliche physiologische Aufgaben bei Gesundheit und Krankheit aus. Die Ernährung beeinflusst die bakterielle Zusammensetzung und Aktivität, welche wiederum den Metabolismus und die Homöostase des Wirts modulieren. Unverdauliche Ballaststoffe, zum Beispiel, haben positive Auswirkungen auf das Wohlbefinden des Darms, indem sie als Kohlenstoffquelle für das Wachstum und die Funktionalität von spezifischen symbiontischen Bakterien dienen. Im Darm erzeugt der bakterielle Abbau von komplexen Kohlenhydraten kurzkettige Fettsäuren, wie etwa Acetat, Propionat und Butyrat, welche vom Darmepithel aufgenommen werden und folglich die Gesundheit beeinflussen. Die Aufnahme von Ballaststoffen kann zu einer erhöhten Erzeugung von kurzkettigen Fettsäuren führen, aufgrund von bakteriellen Kreuzfütterungen, anschliessendem Ansäuern und folglich Homöostase.

Eine unerwünschte Störung der Zusammensetzung und Funktionalität der Darmbakterien, auch mikrobielle Dysbiose genannt, kann erhebliche Veränderungen in der empfindlichen Wirt-Mikroben-Interaktion hervorrufen und kann mit chronisch-entzündlichen Erkrankungen, wie Inflammatory bowel disease (IBD), in Verbindung gebracht werden. Das Auftreten von IBD hat stark zugenommen, insbesondere in westlichen Ländern. Dieses westliche Ernährungsverhalten, welches durch eine geringe Aufnahme von Ballaststoffen gekennzeichnet ist, könnte einen möglichen Auslöser von IBD sein. Abgesehen von der Ernährung, können andere Umweltfaktoren die intestinale Mikrobiota und deren Aktivität beeinflussen, und eventuell zur mikrobiellen Dysbiose und IBD führen. Oxidativer Stress, welcher durch einen Überschuss von reaktiver Sauerstoff-Spezies entsteht, und Antibiotika sind mögliche Auslöser von mikrobieller Dysbiose.

Es ist schwierig die unmittelbaren Auswirkungen von modulierenden Faktoren, welche gesundheits- (Ballaststoffe) oder krankheitsfördernd (oxidativer Stress und Antibiotika) sind, auf die intestinale bakterielle Zusammensetzung und Aktivität abzuwägen, aufgrund der Unzugänglichkeit der humanen Darmproben oder den Endpunktproben bei Mäuseexperimenten. *In vitro* Fermentationsmodelle, welche die Bedingungen im Darm nachahmen, bieten die Möglichkeit, die Mikroben-Mikroben-Interaktionen zu untersuchen, unabhängig vom Wirt. Das Hauptziel dieser These war die Untersuchung des Modulierungspotentials von Ballaststoffen und ihre unmittelbare Auswirkung von oxidativem Stress und Antibiotikum auf die Darmbakterien und deren Funktionalität mittels kontinuierlichen *in vitro* Fermentationsmodellen des menschlichen Dickdarms und des Blinddarms (Caecum) der Maus.

1. Studie

In einem ersten Teil, wurden kontinuierliche *in vitro* Darmfermentationssysteme entwickelt, welche mit Fäkalproben von Erwachsenen angeimpft wurden, um das modulierende Potenzial von vier verschiedenen Ballaststoffen zu untersuchen. Zwei unterschiedliche fäkale Mikrobiota (D3 und D4) wurden immobilisiert, welche sich innerhalb der Bacteroidetes Abteilung unterschieden, dies mit erhöhter Abundanz von

Bacteroidaceae in D3 und erhöhter Abundanz von *Prevotellaceae* in D4. Die zwei verschiedenen Mikrobiota konnten auf metabolischer und phylogenetischer Ebene erfolgreich im PolyFermS Model erhalten werden. In dieser Studie, wurden drei verschiedene *in vitro* PolyFermS Fermentationsmodelle ausgeführt um die modulierende Wirkung von Inulin, β-glucan, Xylo-oligosaccharide (XOS) und α-Galacto-oligosaccharide (α-GOS) auf zwei verschiedene Darmmikrobiota zu untersuchen, unabhängig vom wirtsabhängigen Einfluss. Die Ergänzung der vier Ballaststoffe, welche einer täglichen Einnahme der physiologischen Konzentration von 9 Gramm entsprach. Die Behandlungen führten zu einem Anstieg der kurzkettigen Fettsäuren (SCFAs), welcher die Aufspaltung der Ballaststoffe durch die zwei Mikrobiota beweist, und zu einer Mikrobiota-spezifischen Stoffwechselantwort. Unabhängig vom Ballaststoff, reagierte *in vitro* Mikrobiota mit erhöhter Butyrat Erzeugung, während D4 *in vitro* Mikrobiota durch eine erhöhte Propionat Erzeugung auffiel. Ausserdem, führte die Supplementation der zwei kurzkettigen Kohlenhydrate XOS und α-GOS zu einer erhöhten Acetat Produktion in allen Tests unabhängig von der Mikrobiota. Interessanterweise konnte eine metabolische Kreuzfütterung von Acetat-Butyrate-Produzenten in beiden PolyFermS Mikrobiota beobachtet werden, welche insbesondere bei Inulin deutlich wurde, entweder mit einem Anstieg von Butyrat oder von Acetat. Auf phylogenetischer Ebene konnten wir Veränderungen in der Häufigkeit von spezifischen bakteriellen Taxa in der jeweiligen *in vitro* Mikrobiota feststellen, welche die beobachteten metabolischen Profile und die möglichen Kreuzfütterungen zwischen den verschiedenen funktionellen Bakterienkulturen erklären könnten.

2. Studie

Als Nächstes, wurde ein neuartiges kontinuierliches Fermentationsmodel, basierend auf der PolyFermS Plattform entwickelt und validiert, welches mit immobilisierten zäkalen Darmbakterien der Maus angeimpft wurde. Mausmodelle sind das Modell der Wahl um die Rolle der Darmbakterien in Gesundheit und Krankheit zu erläutern. Obwohl die Zusammensetzung der Darmbakterien von Maus und Mensch sich unterscheiden, sind weitere Untersuchungen der murinen Darm-Mikrobiota erforderlich, um die derzeitigen Mausmodelle zu verbessern und die Mechanismen der bakteriellen Interaktionen zu verstehen. Kontinuierliche *in vitro* Fermentationsmodelle sind wichtige Hilfsmittel um die Mikroben-Mikroben-Interaktionen zu studieren, indem man Tierversuche und Host-Faktoren umgeht. Bisher wurde kein Fermentationsmodell für die Darmbakterien der Maus entwickelt.

Zunächst bestimmten wir den pH, die bakterielle Zusammensetzung und das Stoffwechselprofil in den Blinddärmen (Caeca) von C57BL/6 Mäusen. Das Wachstum und die Aktivität der murinen zäkalen bakteriellen Population wurden gefördert durch die Entwicklung eines komplexen nährstoffreichen Mediums, um die Nährstoffverhältnisse im Chymus des Caecums nachzuahmen. Unterschiedliche Faktoren, einschliesslich die Probennahme des Caecums, der Fermentationsstart, der pH, die Retentionszeit oder der Nährmedien wurden der Reihe nach ermittelt um Konditionen zu wählen, welche zu einer bakteriellen Fermentation und Struktur ähnlich wie im Caecum führen.

Das etablierte Model wies ein hohes, repräsentatives und stabiles *in vitro* Fermentationsprofil auf (bis zu 69 Tagen), geprägt durch die Hauptmetaboliten Acetat, Propionat und Butyrat, und Metaboliten-Verhältnisse ähnlich wie im verwendeten Inokula des jeweiligen Modells. Die murine Mikrobiota im Fermentationsmodell bestand mehrheitlich aus den Hauptstämmen Firmicutes und Bacteroidetes, und Taxa, welche zu wichtigen funktionellen und fermentativen Gruppen gehören (*Bacteroidaceae*, *Lachnospiraceae*, *Ruminococcaceae*, *Clostridiaceae*, *Lactobacillaceae* und *Verrucomicrobiaceae*). Trotz einer allgemeinen Abnahme der bakteriellen Diversität im *in vitro* Model verglichen mit dem zäkalem Inokulum, wurde die bakterielle Fermentation nicht beeinträchtigt und erhalten. Die Anpassungen der Bestandteile des Nährmediums, des pHs und der Retentionszeit wurden vorgenommen um die hohen *in vitro* gemessenen Werte der *Enterobacteriaceae* zu kontrollieren. Insgesamt war eine Langzeitkultivierung der zäkalen murinen Darmbakterien möglich unter Wahrung der Funktionalität und Phylogenie. Dieses Modell könnte verwendet werden um die Auswirkungen von biotischen und abiotischen Faktoren, welche einen Zusammenhang mit Gesundheit und Krankheit besitzen zu untersuchen. Die genaue Untersuchung von deren direkten Effekt auf die bakterielle Struktur und Funktionalität ist nötig bevor man eine Transplantation der Mikrobiota durchfürht um die Mechanismen der Wirt-Mikroben-Interaktionen zu erläutern.

3. Studie

Im letzten Schritt haben wir dieses neuartiges *in vitro* Fermentationsmodell des murinen Blinddarms verwendet um ausgewählte Umweltfaktoren, welche Veränderungen der Darmbakterien hervorrufen, zu untersuchen. Daher untersuchten wir die direkten schädlichen Effekte von oxidativem Stress (H_2O_2), Metronidazole (wird in der IBD Behandlung verwendet, MTZ) und die positiven modulierenden Auswirkungen von inulin und XOS auf zwei murine *in vitro* Mikrobiota (F1 und F2). Aus unseren Ergebnissen geht hervor, dass oxidativer- und antibiotischer Stress zu einer erheblichen intestinalen Dysbiose führt. Unabhängig von der *in vitro* Mikrobiota resultierte oxidativer Stress in einer sofortigen und konsequenten Beeinträchtigung des mikrobiellen Stoffwechsels, einer Zunahme von fakultativ anaeroben Bakterien (*Lactobacillus* and *Enterococcaceae*) und *Enterobacteriaceae*, sowie einer Abnahme von streng anaeroben Butyrat-Produzenten (i.e. *Coprococcus*). Die Verabreichung von MTZ verursachte eine temporäre Hemmung des bakteriellen Stoffwechsels in beiden Behandlungen von F1, welches sich nach drei Tagen wieder erholte. Dennoch blieb eine erhöhte Häufigkeit von einigen fakultativ anaeroben Taxa, welche zu *Lactobacillus, Veillonella, Sutterella* und *Enterococcus* gehören, bestehen. Die Supplementation von XOS erhöhte durchgängig die Produktion von kurzkettigen Fettsäuren, vorwiegend Acetat, und die Abundanzen von bakteriellen Taxa gehörend zu *Ruminococcus*, *Allobaculum* und [*Mogibacteriaceae],* während *Veillonella dispar* abnahm. Die Zufuhr von inulin resultierte in einer spenderabhängigen Änderung des Stoffwechsels, mit erhöhten Produktionen von Acetat und Butyrat in F1 und Propionat in F2. Interessanterweise, als die Dosierung von Inulin erhöht wurde

führte dies zu einer erhöhten Häufigkeit von Taxa gehörend zu *Erysipelotrichaceae,* welche die erhöhten Butyrat-Konzentrationen erklären könnten.

Schlussfolgerung

Schlussfolgernd kann man sagen, dass diese These wichtige Einsichten über das Modulierungspotenzial von verschiedenen Umweltfaktoren auf die intestinale bakterielle Fermentation mithilfe kontinuierlicher *in vitro* Fermentationsmodelle, welche mit immobilisierter zäkaler Mikrobiota der Maus und fäkaler Mikrobiota von Erwachsenen inokuliert wurden. Unsere Daten weisen darauf hin, dass der präbiotische Effekt durch bakteriellen Abbau nicht nur von deren physikochemischen Eigenschaften bestimmt wird, sondern auch stark von der Ausgangszusammensetzung der Darmbakterien abhängig ist. Ausserdem konnten wir darlegen, dass eine geringe Dosis eines Ballaststoffes nur die Stoffwechselaktivität der *in vitro* Mikrobiota, während eine hohe Dosis zusätzlichauch noch die Zusammensetzung der Darmbakterien verursacht. Darüber hinaus, hat diese These auch erste Einblicke in die schädlichen Einwirkungen von oxidativem Stress auf die Struktur und den Metabolismus der intestinalen *in vitro* Mikrobiota geliefert, welche wahrscheinlich mikrobielle Dysbiosis fördern. Oxidativer Stress beeinträchtigte den Kohlenhydrat-Stoffwechsel und die Abundanz von den strikt anaeroben Butyrate-Produzenten, während er das Wachstum der Proteobacteria förderte. Es wurde weiterhin festgestellt, dass Metronidazole auch mikrobielle Dysbiose auslöst durch Veränderung der intestinalen Zusammensetzung und Aktivität. Mehr Studien sind notwendig um den genauen Einfluss der Ausgangszusammensetzung der Darmbakterien auf die Stoffwechselreaktion bei Supplementation von Präbiotika, und um die Zusammenhänge zwischen spezifischen bakteriellen Populationen, Stoffwechselreaktion und Ballaststoff-Dosis zu ermitteln. Nichtsdestotrotz sind *in vivo* Studien erforderlich um ferner die Auswirkungen dieser mibrobiellen Veränderungen auf die Gesundheit zu ermitteln.

Chapter 1

General introduction

This thesis studied the intestinal microbiota of both, human and mice, and for this reason, the introduction will focus on the gastrointestinal tract and their microbiota of both.

1. The gastrointestinal tract in humans and mice

1.1. Physiology and function of the gastrointestinal tract

The gastrointestinal tract (GIT) is a specialized, hollow, continuous and epithelium-lined tube that elongates from the mouth to the anus (**Figure 1.1**). The mammalian GIT is composed of the mouth, pharynx, esophagus, stomach, small intestine, large intestine and anus, having a length of about 5-7 meters in humans and on average 56 cm in mice. It is associated with accessory structures like salivary glands, which secrete fluids into the lumen (Seeley and VanPutte, 2014). The human GIT secretes up to 9 L of fluid per day, containing bile, ions, digestive enzymes, water and mucus (Greenwood-Van Meerveld et al., 2017).

The primary functions of the GIT are the digestion of food, absorption of nutrients and excretion. When food enters the mouth, it is physically broken down with the help of the teeth, and mixed with saliva by chewing. The saliva contains enzymes (amylases and lipases) that break down carbohydrates and fats, and therefore initiates the process of chemical digestion (DeSesso and Jacobson, 2001). The pre-digested material flows then from the mouth to the acidic stomach, where the stomach functions to churn, store and downgrade food into a substance called chyme (Healthcare, 2014; Treuting et al., 2018a; Treuting et al., 2018b). Here, digestion of fats and carbohydrates is ongoing while the chyme is mixed with proteases for protein degradation and gastric juice (including mucus, hydrochloric acid, gastrin, histamine, intrinsic factor and pepsin) through muscular contractions. The gastric juice not only contributes to the chemical degradation, but also kills most bacteria that were ingested with the food (Healthcare, 2014). This semi-fluid mixture is then transported to the small
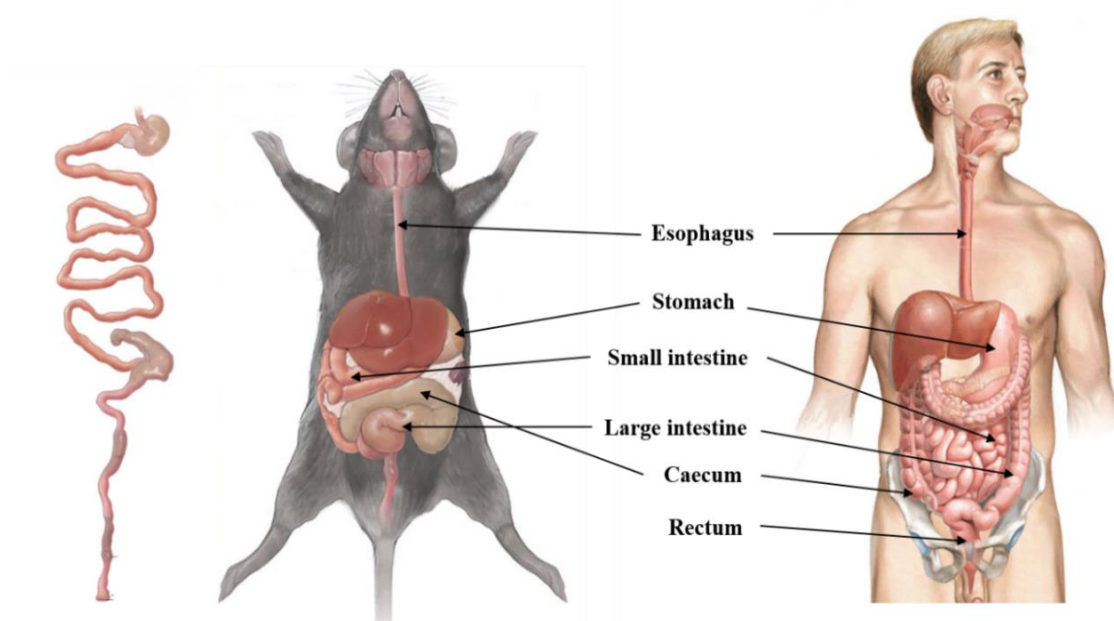

Figure 1.1: Human and mouse intestinal anatomy. Adapted from (Treuting and Dintzis, 2012; Treuting et al., 2012).

intestine, the major site for digestion and absorption of electrolytes, water and nutrients through passive and active absorption. Polysaccharides that escaped endogenous digestion in the small intestine, will enter and be broken down in the final section of the GIT, the large intestine (DeSesso and Jacobson, 2001). The large intestine in mice and humans is composed of the caecum, colon (ascending, transverse and descending) and rectum. It performs the vital tasks of absorbing vitamins, water and other many components from food and microbial metabolism, while changing the waste from liquid into feces. The human large intestine is the predominant fermentation section of indigestible carbohydrates, while breakdown is not seen in the appendix or caecum. In humans, the caecum is a relatively small anatomical structure with no clear function. Contrary to humans, in mice, the caecum constitutes up to one third of the large intestine and is the main fermentation site with the highest load of bacteria (Treuting and Dintzis, 2012). The murine caecum is large relative to the GIT, reflecting the adaptation to extract nutrients from indigestible plant-derived polysaccharides. The GIT of humans also includes an appendix (a vermiform organ attached to the caecum), which is absent in a mouse (Nguyen et al., 2015). After consumption of food, the overall intestinal transit time in a human requires 14 to 76 hours (highly dependent on the diet), while it takes only between 6 to 7 hours in a mouse (higher total metabolic rate) (Hugenholtz and de Vos, 2017).

1.2. Morphological structures

Even though the anatomy of the two digestive tracts is highly conserved, some major differences exist. Albeit both organisms have a comparable average ratio of intestinal body:area surface, the ratio highly differs in the different section of the GITs (Casteleyn et al., 2010; Treuting et al., 2018b; Treuting et al., 2012).

Generally, in order to perform the digestive process, the GIT is organized into four layers (from inside to outside): the mucosa, the submucosa, the surrounding muscles and the outmost layer serosa. To increase absorption surface, the mucosal and submucosal layers of the small and large intestine are composed of ridges and villi (Shen, 2009; Treuting et al., 2018a). The small intestinal villi in mice are taller than in humans, what increases the total surface area of the murine small intestine and probably compensating the lack of mucosal folds in the murine GIT. The human intestine harbors many sub-compartments, while the mouse intestine is smooth and has no division. Furthermore, the human colon is covered with a thicker mucosal layer (several 100 µm) compared to mice (~100 µm in large colon) (Hansson, 2012; Hugenholtz and de Vos, 2017; Johansson et al., 2015; Treuting and Dintzis, 2012). In mice, the distribution of the mucin-producing goblet cells is more abundant along the surface of intestinal crypts in the proximal colon, and decreases at the base in the distal colon and the rectum. In contrast, in humans, high numbers of goblet cells are rather seen from caecum to rectum. The human colonic mucosa has transverse folds, which are restricted to the caecum and proximal colon in mice. These additional folds possible create micro-compartmentalizations and ecological micro-niches for the bacterial communities. Another difference between the two organisms is the presence of Paneth cells,

which secrete antimicrobial substances into the gut lumen. In humans, these specific cells exist in the proximal colon and caecum, whereas in mice they are restricted to the caecum (Nguyen et al., 2015).

Intestinal immune response

The GIT, being one of the largest interfaces (250-400 m^2 in humans and around 1.42 m^2 in mice), functions as a protective barrier between the external and internal world, by comprising a huge variety of molecules (e.g. immunoglobulins) and protecting the body from microbial invasion (Bruneau, 2017; Casteleyn et al., 2010; Thursby and Juge, 2017; Urbanska et al., 2016). A well-tuned interplay and precise regulation between the intestinal epithelial cells and the innate and adaptive immune cells are necessary to maintain homeostasis, since they reside together with the commensal bacteria in the intestine (Peterson and Artis, 2014).

Intestinal epithelial cells form a physical - and a biochemical barrier that keep the segregation between luminal commensal bacteria and mucosal immune cells (Peterson and Artis, 2014). Intercellular junctions, consisting of adherent junctions, tight junctions and desmosomes maintain integrity of the single layered epithelium, and primarily restrict bacterial intrusion through the epithelium (Shimada et al., 2013; Venkatesh et al., 2014). The epithelial cells further include specialized enterocytes, which perform digestive functions or secrete hormones and mucus (Peterson and Artis, 2014). The viscous mucus is composed of mucin glycoproteins and is produced by goblet cells and Paneth cells (Birchenough et al., 2016; Kim and Ho, 2010; Knoop and Newberry, 2018). In case microbes succeed to penetrate the mucus, the complex network of carbohydrates of glycoproteins or – lipids, called gylcocalyx, hinders the invading bacteria. Further separation is achieved through the secretion of antimicrobial compounds/peptides (AMPs) or defensins by the intestinal epithelial cells. The secretion of these AMPs is regulated by the interaction of the enteric bacteria and the pattern recognition receptors (PRRs), like Toll like receptors (TLRs), which are found on the surface of Paneth cells and goblet cells (Vaishnava et al., 2011). The evolutionally conserved AMPs are small, amphiphilic, cationic peptides that interact with the microbial cell membranes, thus drive breakage through the formation of a pore (Reinhardt, 2017). Innate immune cells (macrophages, neutrophils, dendritic cells) have the ability of distinguishing between host- and bacterial cells through PRRs by recognizing conserved molecules on the surface of microorganisms (microbe-associated molecular patterns), such as lipopolysaccharide or flagellin. However, also intestinal epithelial cells can deliver antigens to enteric immune cells and thus induce adaptive immune response. The gut-associated lymphoid tissue (GALT), composed of Peyer's patches (PPs) and mesenteric lymph nodes (MLN), serves as antigen sampling and inductive sites of the mucosal immune system (Shi and Walker, 2015). Even though most bacteria are phagocytosed and killed by macrophages, some are sampled by M-cells or dendritic cells. M cells are found in the follicle-associated epithelia, which are specialized to ingest the antigens through phagocytosis and then deliver them to antigen-presenting cells (e.g. dendritic cells). Generally, recognition of bacteria leads to the activation of immune signaling pathways, which

upregulates the production of co-stimulatory molecules on antigen presenting cells and production of cytokines that activate other immune cells of the adaptive immunity (effector lymphocytes) (Purchiaroni et al., 2013). B cells residing in the lamia propria are stimulated by dendritic cells containing living bacteria, and differentiate into immunoglobulin A (IgA)-producing plasma cells. It is estimated that around 80 % of the antibody production happens in the gut lamina propria, and its binding to bacteria hinders their translocation across the intestinal epithelium (Purchiaroni et al., 2013; Robinson et al., 2015; Robles Alonso and Guarner, 2013).

2. Gut microbiota

The mammalian GIT harbors the largest collection of bacteria, eukarya and archaea, called gut microbiota, which has co-evolved with its host over thousands of years to elaborate a mutualistic partnership (Rooks and Garrett, 2016; Thursby and Juge, 2017).

2.1. The intestinal microbiota composition

2.1.1. The human intestinal microbiome

The human gut microbiota harbors an estimated number of 10^{14} bacteria, which belong to more than 1000 different species (Rajilic-Stojanovic et al., 2010; Thursby and Juge, 2017) and each individual possesses at least 160 bacterial species (Lloyd-Price et al., 2016; Qin et al., 2010). The varying environmental conditions in the different compartments of the GIT define the colonization and establishment of a defined bacterial community (**Figure 1.2**). Overall, the quantity and diversity of bacterial species increase longitudinally from the stomach to the colon. Due to low pH in the stomach, microbial counts are low (< 10^3 cells/g of intestinal content), but increase in the small intestine to 10^4-10^7 cells per gram, where growth is still limited by secretion of bile, pancreatic juice and rapid transit time. Highest bacterial counts (up to 10^{12} cells/g of intestinal content) are reached in the large intestine, where optimal bacterial growth conditions, such as a slow retention time, a high nutrient availability and a favorable pH can be found (Payne et al., 2012; Rooks and Garrett, 2016). Bacteria are not only subjected to the physiologic pressure from the host, but also to microbial competition within the niche, leading to a balance with some bacterial species present at high, others at low abundance (Arumugam et al., 2011; Bauer et al., 2018; Falony et al., 2016). Initially, most knowledge about the gut microbiota was obtained from intensive culture-based methods, while recently it is intensively studied using 16S rRNA sequencing approaches. Four bacterial phyla dominate the GIT: Firmicutes, Bacteroidetes, Proteobacteria and Actinobacteria. Other divisions found in faecal samples are Fusobacteria and Verrucomicrobia (Hug et al., 2016; Robles Alonso and Guarner, 2013; Thursby and Juge, 2017). A first main goal in gut microbiota research was to find bacterial populations that are present in the majority of individuals. Early microbiome research identified a "core" set of 33 bacterial taxa that are shared among healthy individuals (Human Microbiome Project, 2012; Xiao et al., 2015). In the large intestine, the families *Lachnospiraceae, Prevotellaceae* and *Rikenellaceae* are dominating the gut community (Arumugam et al., 2011; Donaldson et al., 2016; Gu et al., 2013). At genus level, the most prevalent anaerobic bacterial populations are *Blautia*, *Clostridium*, *Roseburia*, *Faecalibaterium, Eubacterium, Bacteroides* and *Bifidobacterium*, as well as facultative anaerobic taxa belonging to *Enterococcus, Klebsiella, Escherichia* and *Streptococcus* (Di Rienzi et al., 2013; Falony et al., 2016; Zhernakova et al., 2016). While large inter-individual differences in the taxonomic composition are observed, the metabolic pathways were found to be conserved

and stable across healthy individuals (namely core microbiome). These findings suggest that microbial communities might assemble depending on their coverage of a core set of metabolic genes during selection and colonization of microbial populations (see **section 2.3.1**) (Lloyd-Price et al., 2016). In addition, this metabolic redundancy probably permits stability and resilience to the intestinal bacterial community in case of environmental or dietary disturbances (Moya and Ferrer, 2016).

2.1.2. The murine intestinal microbiome

Even though the murine GIT is a continuous lumen, specific microenvironments in the different intestinal parts influence the bacterial composition. Longitudinal variations in physicochemical parameters highly influence the bacterial load and composition along the GIT. The oxygen availability, the fast transit time, the high concentrations of simple sugars and the ability of lactobacilli to adhere to the epithelium in the forestomach might explain its high prevalence in the stomach and small intestine (Tannock, 1995). Higher prevalence of bacilli and Proteobacteria are observed in the stomach and small intestine compared to the large intestine (Gu et al., 2013). Segmented filamentous bacteria, which are generally found in mice, are mainly located in the terminal ileum, where they interact with the intestinal epithelium (Farkas et al., 2015; Yin et al., 2013). Contrary to humans, the rodent caecum is the main fermentation site, containing the highest concentration of bacteria. In the murine caecal microbiota, seven different phyla, namely Actinobacteria, Bacteroidetes, Deferribacteres, Firmicutes, Proteobacteria, Tenericutes and Verrucomicrobia can be found, with Firmicutes, Bacteroidetes and Proteobacteria dominating the bacterial community. Within the Firmicutes phylum, the most common families are *Lachnospiraceae* and *Ruminococcaceae*, while *Porphyromonadaceae* is the dominating family within Bacteroidetes (Benson et al., 2010; Clavel et al., 2016; Gu et al., 2013). Further, members of the family S24-7 (Bacteroidetes) are also known to be prevalent in the murine intestinal microbiota, remaining largely «uncultivable» until today (Seedorf et al., 2014; Wang et al., 2007). Only recently, Lagkouvardos et al. succeeded to isolate and cultivate a first strain of this type in a medium containing meat and blood (Lagkouvardos et al., 2016). At genus level, the murine gut microbiota is characterized by high abundances of *Lactobacillus (Lactobacillaceae), Coprobacillus (Erysipelotrichaceae), Anaerotruncus (Ruminococcaceae), Marvinbryantia (Lachnospiraceae), Pseudoflavonifractor (*unclassified Clostridiales) and *Turcinibacter* (*Erysipelotrichaceae*), while *Faecalibacterium (Clostridiaceae), Prevotella (Prevotellaceae), Oscillobacter (Ruminococcaceae), Klebsiella (Enterobacteriaceae)* and *Ruminococcus* (*Ruminococcaceae*) are less prevalent in mice compared to human microbiota (Gu et al., 2013; Nguyen et al., 2015). Interestingly, the species *Mucispirillum schaedleri* within the phylum Deferribacteres has only been isolated from the murine intestinal microbiota and seems to be absent in humans (Clavel et al., 2016; Robertson et al., 2005).

In contrast, enrichments of the strict anaerobic families *Bacteroidaceae, Prevotellaceae, Rikenelleaceae, Lachnospiraceae* and *Ruminococcaceae*, along with higher abundances of the genera *Bacteroides, Alistipes*

and *Prevotella* can be found in the large intestine, where oxygen is less prevalent, transit time is slower and indigestible dietary fibers are available (Gu et al., 2013). These physiologic conditions and increased metabolic capacity results additionally in a higher bacterial diversity (Turnbaugh et al., 2009b). Within the mucosal layer lining the epithelium, mucin degraders, such as certain *Bacteroides* spp. and *Akkermanisa muciniphila* are present at high abundance (Berry et al., 2013; Yasuda et al., 2015). As with humans, there are significant variations in the bacterial populations among mice of the same or different breed (Hugenholtz and de Vos, 2017). Overall, 60 genera were found to be present in all mouse gut microbiota samples, thus termed as "core microbiota" (Xiao et al., 2015). Nevertheless, it should also be noted that more than 50 % of the taxa at family level are still unidentified. Possible explanations are for instance that the taxonomic classification of similar cultured bacteria remains obscure or these taxa do not have a representative strain in culture, yet. The same research group stated that approximatively 80 % of the samples contain such unknown taxa, which are present at a prevalence of around 20 % (Clavel et al., 2016). These facts highlight the need of characterization of this yet «unknown majority» that is a significant part of the murine intestinal microbiome (Clavel et al., 2016).

2.2.   From gut colonization to microbial stability over lifetime

Different phases of life are characterized by defined gut microbiota compositions, which are believed to have coevolved with the development of their host (Thaiss et al., 2015). Colonization of the gut of a newborn (by bacteria, fungi, viruses, eukaryotes and archaea) is a crucial process in the mammal's life cycle. Since the fetus is sterile *in utero*, the microbial colonization of the newborn commences during and after birth (Goldenberg et al., 2008; Lozupone et al., 2013; Rodriguez et al., 2015). A broad array of bacterial populations start to colonize at high numbers the intestine of mammals. In humans, important factors influencing the diversity of the colonizing bacteria are for instance the mode of delivery (vaginal vs caesarian) or the feeding (breastfeeding or formula fed) of infants. The colonization of the newborn mice is primarily influenced by the mother's vaginal community and subsequently by weaning (Pantoja-Feliciano et al., 2013). The postnatal environment is another crucial source of a significant diversified bacterial community, which colonizes successively the newborn's gut and may explain the high inter-individual diversity in the gut microbiota of newborns (Avershina et al., 2014; Milani et al., 2017; Rodriguez et al., 2015). Initially, the human infant intestinal microbiota is characterized by a low diversity and is mainly composed facultative anaerobes (Actinobacteria and Proteobacteria), which consume oxygen and create a suitable environment for strict anaerobes such as taxa belonging to *Clostridium, Bacteroides* and *Bifidobacterium* (Backhed et al., 2012; Milani et al., 2017; Qin et al., 2010). During the first year of life, the diversity increases progressively and an infant acquires an individual bacterial signature. At the age between 2 to 5, the bacterial composition and diversity of a child looks like that of an adult (Rodriguez et al., 2015; Thursby and Juge, 2017). In mice, high numbers of
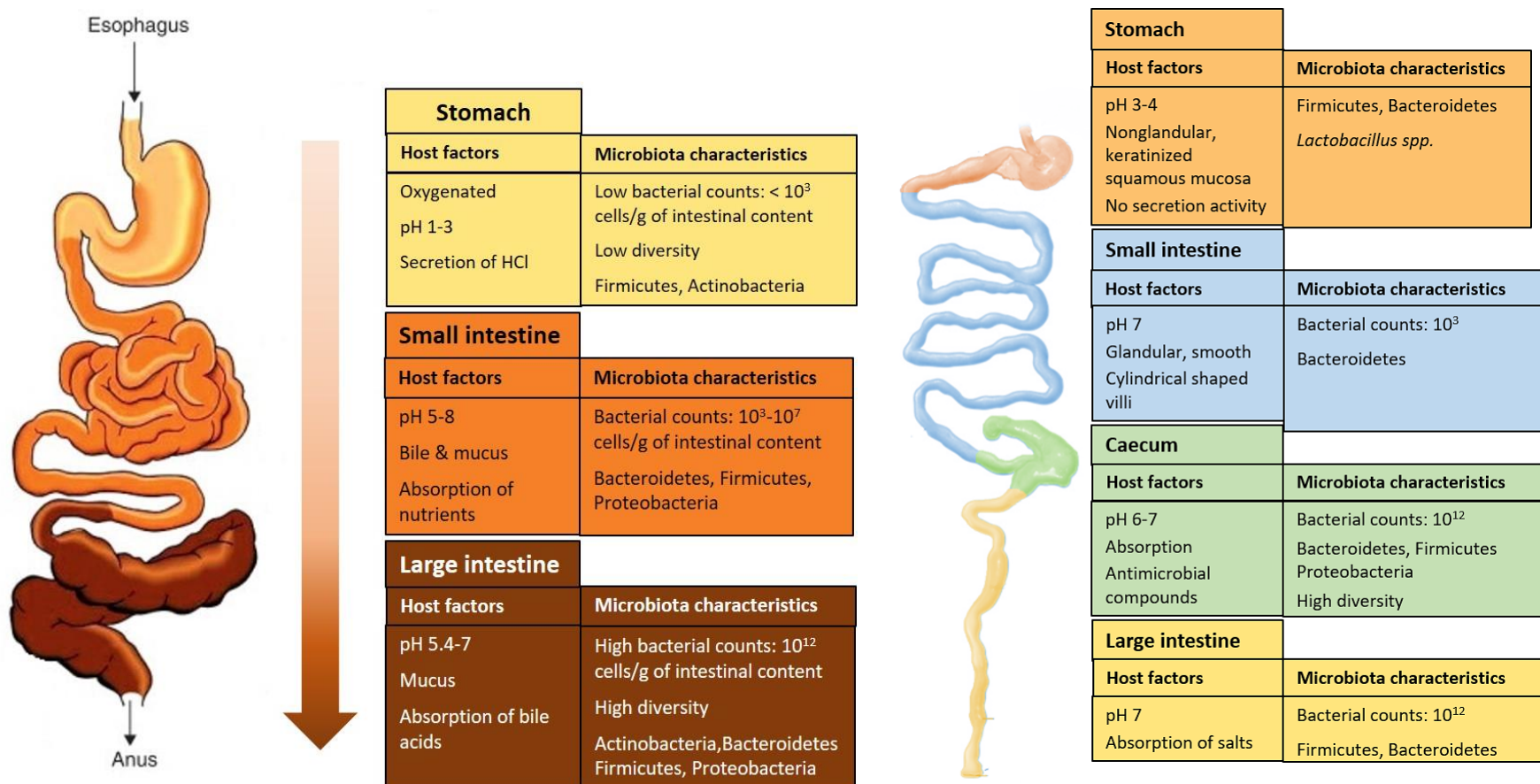
Figure 1.2: Abundance of bacteria in different segments of the human and murine gastrointestinal tract. Adapted from Payne et al. (2012), Tsabouri et al. (2014),Ohland and Jobin (2015), (Hugenholtz and de Vos, 2017), (Kohl et al., 2013) and (Engevik et al., 2013)

the facultative anaerobes *Lactobacillus* and *Streptococcus* species mark the initial colonization after birth, which produce high levels of lactate and thus acidifying the gut lumen (Angelakis et al., 2012; Kunji et al., 1996). When milk is slowly replaced by solid food, microbial diversity increases due to the colonization of strict anaerobes in the intestine and the availability of other metabolic pathways, which will lead to the production of metabolites and gases (Pantoja-Feliciano et al., 2013).

Life on earth is dictated by temporal changes in the environment, such as light, temperature and availability of nutrients, which affect the intrinsic rhythm and feeding behaviors of mammals. Advances in culture independent technologies revealed that the bacterial composition alters progressively over human and murine lifetime, but remains relatively stable after reaching adulthood, even when subjected to perturbations (such as antibiotics, disease or diet) (Dethlefsen and Relman, 2011; Gilbert et al., 2018). One study monitored the gut microbiota and daily lifestyle (diet, fitness, illness, travel) of two individuals over one year and found a long-term stability of the bacterial community. Travel (e.g. novel diet or environment) and enteric infections (such as food poisoning or diarrheal illness) were found to induce profound bacterial community disturbances. However, this study also highlighted the high inter-individual and qualitative different responses to perturbations: while one gut microbiota was able to restore the community to its original state within only 14 days, the second did not return to its initial state for more than 3 months (David et al., 2014a). Over the age of 65, the bacterial composition starts to change, with increased abundance of the phylum Bacteroidetes and the bacterial population of *Clostridium* cluster IV, and a significantly decreased diversity (Biagi et al., 2010; Claesson et al., 2011).

2.3. Different functions of the gut microbiota

The host has developed strategies to tolerate, coordinate and use bacterial metabolic pathways, immune modulation and microbial sensing to its benefits. A fine-tuned cross-talk between the microbiome and the host is necessary to maintain homeostasis. The main functions of the gut microbiota include structural and histological functions, protective functions and metabolic functions (**Figure 1.3**) (Rooks and Garrett, 2016).

2.3.1. Metabolic functions – expansion of the host metabolic capacity

The diverse gut microbiota comprises a gene set of about 3 million genes, which is about 150 times larger than the human genome (Qin et al., 2010; Ursell et al., 2014). This large metabolic repertoire of the gut microbiome allows expanding and complementing the metabolic activity by contributing enzymes that are not encoded in its host genome (Nicholson et al., 2012; Rowland et al., 2018). For example, human enzymes are unable to degrade most dietary fibers, since less than 20 glycosidases are found in the human genome (Martens et al., 2011). Endogenous enzymes can breakdown only a limited number of glycosidic linkages of a fraction of carbohydrates and disaccharides like sucrose and lactose (Cantarel et al., 2012). The colonic microbiota is

principally involved in the saccharolytic breakdown of complex polysaccharides or polyphenols, and the synthesis of essential vitamins, such as vitamin B12 (Rowland et al., 2018).
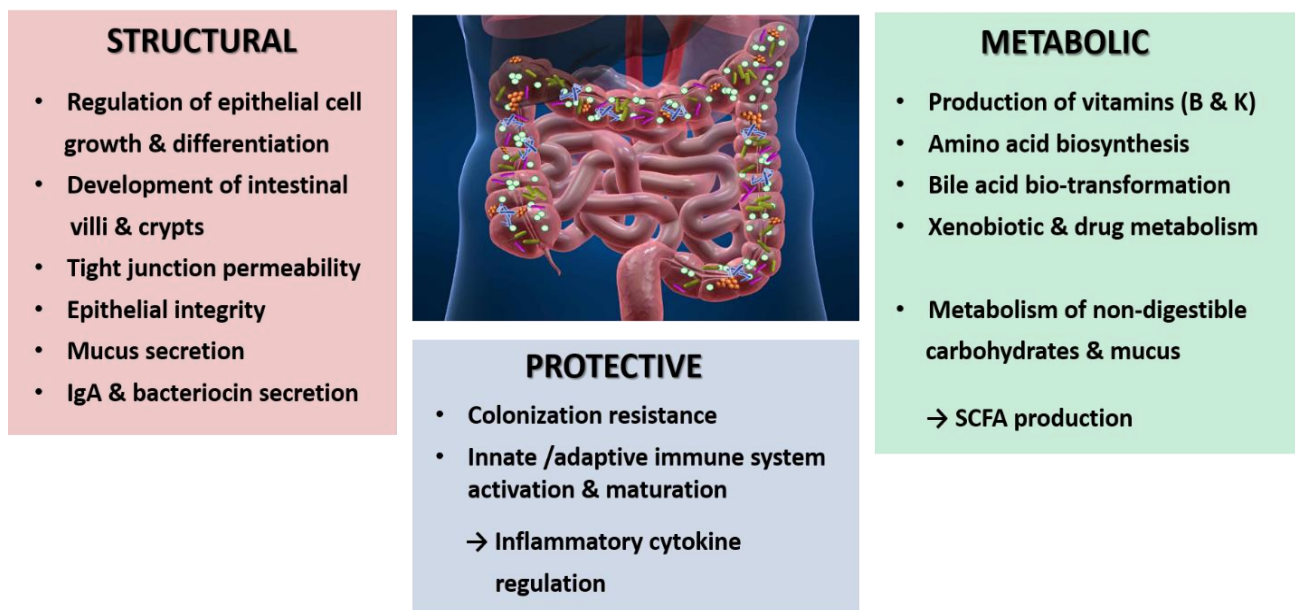

Figure 1.3: Different physiological functions of the gut microbiota, involved in metabolism, host defense and the development of the immune system. Adapted from (Prakash et al., 2011) and (Rajilic-Stojanovic et al., 2009)

Carbohydrate fermentation

When an individual consumes a rich plant-derived diet, various indigestible polysaccharides with different linkages will reach the lower GIT and become available for microbial fermentation. Between 20 to 60 grams of dietary carbohydrates reach the human colon per day (den Besten et al., 2014; Flint et al., 2012a). The bacterial fermentation of carbohydrates generates metabolites that are either taken up by the endogenous cells as an energy source or by other bacteria to be further metabolized into end products (**Figure 1.4**). The carbohydrate fermentation products are short-chain fatty acids (SCFA) and gases. Their productions are closely linked to the bacterial composition, retention time and diet (Rooks and Garrett, 2016). The metabolic interactions between the bacterial populations that simultaneously degrade the entering indigestible carbohydrates and endogenous mucus, is most likely the reason for the huge intestinal bacterial diversity (Hoek and Merks, 2017; Rabiu and Gibson, 2002). Substrate cross-feeding describes the production of a metabolic intermediate by one bacterium that can be used by another microorganism within the community (Seth and Taga, 2014). The commensal microbes differ broadly in their capacity to ferment the dietary fibers: while some bacteria have numerous enzymes that enable them to break down many different glycosidic linkages, others possess only a small range of enzymes (Martens et al., 2011; Scott et al., 2014). Only a subset of bacteria within the complex bacterial community have enzymes that can initiate the breakdown of

polysaccharides derived from plants, while most are non-cellulolytic bacteria that can exclusively use the released polysaccharides by primary degraders. From microscopic studies, it became evident that mucosa-adherent microorganisms within the community are more likely to be the primary degraders (Flint et al., 2008; Flint et al., 2012a). The most abundant SCFA, acetate, is produced by most commensals and acetogenic bacteria in the gut. Out of one molecule of glucose (C6), acetogenic bacteria can generate three molecules of acetate (C2). However, most commensals in the gut are non-acetogenic bacteria and ferment substrates by producing other metabolites, like propionate, butyrate, succinate, formate, lactate and ethanol. The generation of these various metabolites correlates with the microbiota structure, physiological conditions and nutrient availability (Louis et al., 2014; Rios-Covian et al., 2017). The following uttermost abundant metabolites in a healthy intestinal ecosystem are propionate (C3) and butyrate (C4), which are produced by different commensal bacterial populations (Koh et al., 2016; Reichardt et al., 2014). Propionate can be formed via three different pathways, namely succinate pathway, propanediol pathway and acrylate pathway. Bacteroidetes and some organisms of Firmicutes (e.g. *Dialister* spp. or *Veillonella* spp.) produce propionate by utilizing the metabolic intermediate succinate, which is an end-product for some bacteria (Macfarlane, 1997). Proteobacteria and some Firmicutes, such as *Roseburia inulinivorans* or *Ruminococcus obeum*, form propionate via the propanediol pathway, in which desoxyhexose sugars are used as intermediate substrates. The acrylate pathway, using lactate as an intermediate is the less commonly used pathway (Reichardt et al., 2014). Firmicutes mostly use the butyryl-CoA:acetate CoA-transferase (ButCoA) enzyme to generate butyrate,
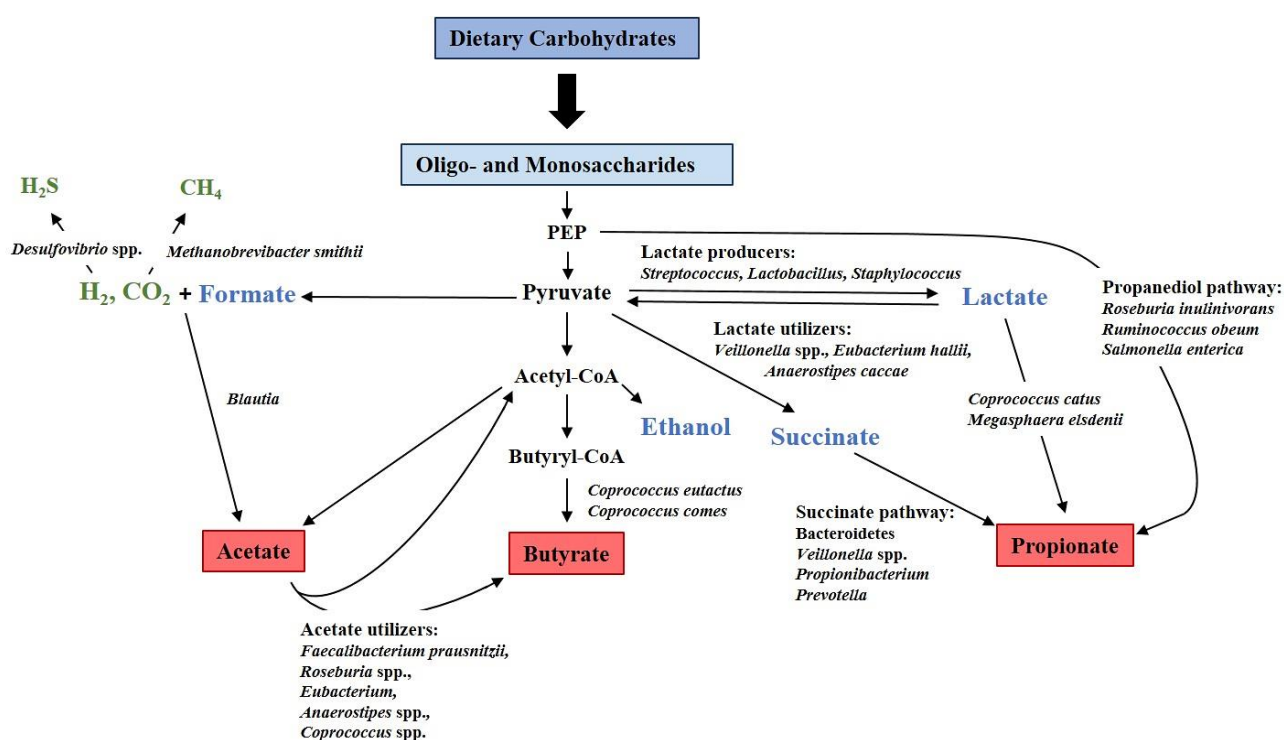

Figure 1.4: Main pathways of carbohydrate fermentation and bacterial cross-feeding in the gut. PEP, phosphoenolpyruvate. Adapted from Louis et al. (2014) and Blaut (2013).

followed by the less commonly used butyrate kinase and phosphotransbutyrylase (Stilling et al., 2016). In a healthy intestinal bacterial community, the most abundant bacteria belonging to *Roseburia* spp., *Eubacterium* spp*., Faecalibacterium prausnitzii* and *Anaerostipes* spp. use the ButCoA pathway by taking up acetate to generate butyrate (Flint et al., 2012a; Louis and Flint, 2017; Rios-Covian et al., 2017). Other bacteria belonging to family of *Lachnospiraceae* possess the ability to use lactate and acetate as intermediate metabolites to produce butyrate, and therefore hold an important role in the prevention of lactate accumulation in the gut (Belenguer et al., 2006; Louis and Flint, 2009). Colonic gases produced during carbohydrate fermentation comprise hydrogen (H_2), methane (CH_4), hydrogen sulphide (H_2S) and carbon dioxide (CO_2). The concentrations and accumulations of the gases depend on the interactions between the intestinal bacterial populations and endogenous physiology. Most of the CO_2, around half of the hydrogen and methane are absorbed by the colonocytes and introduced into the blood stream. H_2 production demands a well-tuned balance between H_2-producing (hydrogenogenic) bacteria and H_2-utilizing (hydrogenotrophic) bacteria. Prominent hydrogen-producing bacteria belong to *Roseburia* spp., *Anaerostipes caccae*, *Clostridium* spp., *Eubacterium rectale*, *Bacteroides* spp., *Victivallis vadensis* and *Ruminococcus* spp. Bacteria that can use hydrogen are divided into three different groups: reductive acetogens, methanogens and sulphate-reducing bacteria (SRB). Acetogens produce acetate by consuming CO_2 and H_2, methanogenic bacteria reduce CO_2 or methanol to CH_4 with the help of H_2 or formate as electron donor, and SRB produce H_2S by using H_2 and sulphate (Carbonero et al., 2012; den Besten et al., 2014; Koh et al., 2016; Nakamura et al., 2010; Rey et al., 2013; Rios-Covian et al., 2017; Zhernakova et al., 2016).

Protein fermentation

The intake of a protein-rich diet will lead to an increased fermentation of proteins in the colon with a rise of branched-chain fatty acids (BCFAs) and phenylacetic acid. Phenols, BCFAs and indoles are exclusively produced by the intestinal bacteria, since mammals do not possess enzymes for their synthesis (Louis et al., 2014; Ou et al., 2013). Fermentation of proteins is initiated in the colon, where hydrolysis into smaller peptides and amino acids by peptidases and proteases occurs. With the advancement towards the distal colon, where a more neutral to alkaline environment can be found, the enzymes become more active. While most of the SCFAs are the end-products of carbohydrate fermentation, reductive deamination of amino acid also yields some SCFAs. BCFAs are mainly products from branched amino acid breakdown, with isovalerate, isobutyrate and 2-methylbutyrate originating from the degradation of valine, leucine or isoleucine (Blachier et al., 2007; Smith and Macfarlane, 1997). Among Firmicutes and *Bacteroides* spp., some bacterial populations can metabolize aromatic amino acids and yield bioactive compounds, such as phenols, phenylacetic acids, indoles and p-cresol (Russell et al., 2013). Ammonia (NH_3) is produced as a result of deamination of amino acids or through urea hydrolysis via the bacterial enzyme urease (Blachier et al., 2007). Up to 4 grams of NH_3 are produced per day,

which are taken up either by intestinal bacteria for the own metabolism and protein synthesis, or by colonocytes for converting it to urea in the liver and excreting it in urine (Windey et al., 2012). The breakdown of sulfur amino acids (cysteine, cysteine, taurine, methionine) or sulfate deriving from diet or mucus generates the gas H_2S and its concentrations highly correlate with protein intake (Lewis and Cochrane, 2007; Magee et al., 2000).

Biology of SCFAs

The three main SCFAs acetate, propionate and butyrate are detected in ratios between 3:1:1 and 10:2:1 in healthy humans, with total SCFA concentrations in feces between 20 and 140 mM (Cummings et al., 1987; Macfarlane, 1997). In mice, SCFA concentrations produced by caecal microbiota not only depend on mice activity and feeding behavior, but also on the breed and diet. Generally, total SCFA concentrations in caecal contents up to 120 mM are observed, with ratios between 10:3:1 and 10:1:3 (den Besten et al., 2015; den Besten et al., 2014; Tahara et al., 2018). The three SCFAs hold important roles in the host. The most abundant SCFA acetate acts as an intermediate metabolite or co-factor for bacterial growth. Some bacteria (e.g. butyrate-producer *Faecalibacterium prausnitzii*) require the presence of acetate for growth (Duncan et al., 2004a). Further, the metabolite is also transported to peripheral tissues, is a necessary molecule in cholesterol metabolism, lipogenesis and is involved in central appetite regulation (Frost et al., 2014). Propionate can also be absorbed by the colonocytes as an energy source or is transported to the liver. Further, the metabolite is a key molecule involved in satiety by interacting with gut receptors (G protein-coupled receptor (GPR)), which activate gluconeogenesis. Propionate is transformed into glucose during intestinal gluconeogenesis and inhibits the hepatic glucose production (De Vadder et al., 2014). Butyrate plays a crucial role in human health as a main energy source for colonocytes, a gene expression regulator by inhibiting histone deacetylase, and an inductor of apoptosis of colonic cancer cells (Rooks and Garrett, 2016; Steliou et al., 2012). Recently it was observed that butyrate is also involved in the intestinal gluconeogenesis activation, thus in energy and glucose homeostasis (De Vadder et al., 2014).

Vitamin synthesis

Mammals cannot synthesize all essential molecules (e.g. vitamins B or K) by themselves, and therefore rely on their presence in supplements, in our diet or on the capacity of the gut microbiota. The mammalian gut microbiome synthesizes some essential amino acids and vitamins, which are needed for the bacterial metabolism itself or as a precursor for the synthesis of some other compounds (e.g. enzymes) involved in the physiology of the host (LeBlanc et al., 2013). The group of B-vitamins (vitamin B12, biotin, folate or thiamin) are necessary for the synthesis of nucleotides and are involved in host-related processes, such as DNA synthesis or repair (LeBlanc et al., 2013). The intestinal bacterial community can also synthesize vitamin K (menaquinone). Genes involved in vitamin K synthesis were found in the genomes of 118 out of 254 gut

bacteria, comprising taxa belonging to *Prevotella, Lactobacillus, Akkermansia* and *Bacteroides* (Ravcheev and Thiele, 2016; Thursby and Juge, 2017).

2.3.2. Protective functions

An important function of the intestinal bacterial community is to form a protective barrier, thus restricting potential pathogens to attach and proliferate. This colonization resistance can be achieved via different mechanisms, such as mucus secretion stimulation, direct nutrient competition or through activation of the immune system (Tomasello et al., 2016). The produced SCFAs contribute to protection by acidifying the gastrointestinal lumen and consequently inhibiting growth of pathogens (Robles Alonso and Guarner, 2013). Further, the oxidation-reduction state in the gut has been suggested to be involved in the colonization resistance by creating an inhibitory environment to potential pathogens or non-commensals (Albenberg et al., 2014; Lind Due et al., 2003).

Recent gut microbiota research highlighted the crucial role of the bacteria themselves and their metabolism for the host immune system. The surface antigens and metabolites furnished by the gut microbiome are needed for the fine-tuning of the immune responses and the proper maturation of immune tissues. The single epithelial layer at the mucosal interface separating the host and microbiome permits the passage of metabolites and thus the subsequent interaction with host cells, such as with immune cells (Rooks and Garrett, 2016). The metabolites can inhibit histone deacetylases and promoting tolerogenic, anti-inflammatory cell lineages, which are important in the maintenance of immune homeostasis. Presence of SCFAs downregulate the production of pro-inflammatory cytokines (such as TNF) and activity of macrophages, dendritic cells and regulatory T cells through the NF-ᴋB pathway (Chang et al., 2014; Kendrick et al., 2010; Singh et al., 2010; Usami et al., 2008; Vinolo et al., 2011).

2.3.3. Trophic functions

Besides accomplishing protective and metabolic functions, bacteria and SCFAs also affect host intestinal structure and function as well as the central nervous system. The biochemical signaling between the GIT and the nervous system is crucial for normal and healthy brain function. In the gut, SCFA are essential for the fortification of the intestinal epithelial cell barrier function through the maintenance of the mucosa, the induction of goblet cell differentiation and the secretion of antimicrobial peptides. Some bacterial taxa of the microbiome were shown to reinforce the barrier via the tight junctions, which are intercellular adhesion complexes that control paracellular permeability (Gaudier et al., 2004; Prakash et al., 2011; Willemsen et al., 2003; Yarandi et al., 2016; Zihni et al., 2016). Besides reinforcing tight junctions, the gut bacteria play an elemental role in the proper development of intestinal cells and tissues. In particular butyrate regulates cell growth and differentiation, suppresses growth of transformed cells or even promotes the return from a

neoplastic to a non-neoplastic state (Salminen et al., 1998). There is good evidence that commensal bacteria affect the development of the villus microvasculature, since in germ-free mice, the villi are long and wide, while the colonic crypts are short and contain a reduced amount of cells compared to conventional mice (Hill and Artis, 2010; Prakash et al., 2011).

2.4. Factors affecting the microbiome

The bacterial composition along the GIT underlies intrinsic and extrinsic selective pressures, and is niche-specific due to different physiological properties, such as the chemical, immunological and nutritional gradients along the regions (Donaldson et al., 2016; Macpherson and McCoy, 2013).

2.4.1. Genetics

Generally, monozygotic twin have a more similar gut microbiome than dizygotic twins, and gut bacterial composition is more alike among family members, even though each individual harbors specific bacterial populations (Turnbaugh et al., 2009a; Yatsunenko et al., 2012). However, scientists found that some bacterial populations seem to be «inherited», since similar relative abundance of specific bacterial taxa can be detected in relatives (Walsh et al., 2014). The most heritable taxa are from the family *Christensenellaceae*, followed by the dominant microbial families *Lachnospiraceae, Ruminococcaceae* and *Bacteroidaceae* (Goodrich et al., 2014). A connection between host genetics and intestinal bacterial composition was demonstrated in murine models, and authors stated that the gut microbiome reacts as polygenic trait (cumulative effect of several genes). Leamy et al. (2014) revealed 42 microbiota-specific quantitative trait loci in 27 different genomic regions that seem to affect the relative abundance of approximatively 20 % of the bacterial taxa in the murine gut. Intriguingly, loci for *Lactococcus* and *Coriobacteriaceae* are closely located to genes involved in immune responses and regulations in mucosal surfaces, such as the TLR2 pathway, IFN-γ and IL-22 (Benson et al., 2010; Leamy et al., 2014).

2.4.2. Diet

Diet is one of the most important factors affecting the gut microbiota composition (Zmora et al., 2018). Carbohydrates, fat and proteins are the major components in a diet, and the amount of their consumption as well as their type were found to highly affect microbial composition (Koh et al., 2016). The ileal microbiota composition is mainly influenced by the ability of bacterial populations to break down simple sugars, reflecting its specific adaptation to the availability of microbiota-accessible carbohydrates (Hooper and Macpherson, 2010). Breakdown of proteins takes place in the distal colon, where most of the proteolytic bacteria are located. End-products of proteins metabolism are short-chain fatty acids, amino acids, amines or ammonia (Hamer et al., 2012; Louis et al., 2014). It was observed that mice and rats fed with a diet containing high concentrations of cysteine or threonine as well as proteins from whey cheese harbored higher levels of

bifidobacteria and lactobacilli, but decreased abundance of *Clostridiaceae* compared to mice with a standard diet (Guerville and Boudry, 2016; Sprong et al., 2010).

The high modulation effect of diet is especially observed when comparing the gut microbiota composition of people eating a Western (high in fat, refined carbohydrates and animal proteins) to that of an Eastern diet (carbohydrates derived from vegetables, rice, plants and fruits). A diet enriched in animal products is associated with higher prevalence of *Bacteroides* spp., whereas *Prevotella* spp. was strongly correlated with a more plant-based diet (Ananthakrishnan et al., 2012; Rajilic-Stojanovic et al., 2015). A diet rich in dietary fibers and vegetables was shown to decrease the pH in the gut, thus inhibiting growth of pathogenic bacteria like *Enterobacteriaceae* (Tomasello et al., 2014). No major influences of a short-term diet change can be observed on the ratios of main bacterial taxa (David et al., 2014b; Wu et al., 2011).

2.4.3. Other factors influencing the microbiome composition

The host's intestinal epithelium, peristalsis, antimicrobials and oxygen concentration influence as well the microbiome. Especially in the small intestine, high concentrations of antimicrobials, bile acids and oxygen, along with short transit times exist. For example, the diffusion of oxygen extends from the intestinal tissue throughout the mucosa into the lumen, and fast growing, aero-tolerant bacterial populations adherent to the mucosal surface consume the available oxygen and prevent oxygenation of the lumen. This leads to a gradual separation of the gut microbiota, which correlates with the oxygen gradient and the distribution of the mucus (Albenberg et al., 2014; Donaldson et al., 2016). A luminal oxygen gradient from host tissue into intestinal lumen may also influence aerotaxis of motile bacteria via energy sensing pathways (Taylor et al., 1999).

Acute host stresses, such as an acute trauma or burn injury affects the gut microbiota by decreasing functionality and abundance by up to 90 %. Since physiologic stress alters the stability of the bacterial structure and functionality, the host becomes consequently more susceptible to infections (Guyton and Alverdy, 2017; Shimizu et al., 2015). In addition, potential pathogenic and multidrug-resistant bacterial populations such as *Enterococcus* spp., *Enterobacteriaceae* or *Staphylococcus* spp. flourish in hospitalized patients being critically ill, which are normally low abundant in a healthy individual. One study even found that these patients comprise a very low bacterial diversity, comprising of only four microbial populations (Scheich et al., 2018; Zaborin et al., 2014). Patients subjected to an intestinal surgery often display significant shifted gut microbiomes, with high increases in *Enterococcus faecalis* and antibiotic resistant *Enterobacteriaceae* (Krezalek and Alverdy, 2016; Levesque et al., 2017; Shogan et al., 2014). One reason is the intake of antimicrobial drugs, like antibiotics, that often induce a large collapse of the bacterial community in the gut (Modi et al., 2014). Antibiotic therapy does not only affect a targeted pathogen, but the entire intestinal microbiome, inducing considerable short- and eventually long term changes of the intestinal microbiota structure and activity. Antimicrobial medications can influence the microbial composition in the gut in

different manners, depending on the dosage/duration, route of administration and the spectrum of the drug. However, high inter-individual response to the administration of one type of antibiotic is frequently observed, due to significant individual-to-individual variability in microbial composition. Nevertheless, antibiotic intake in early infancy has been correlated with several diseases, such as asthma, diabetes or obesity (Biedermann and Rogler, 2015; Jernberg et al., 2010; Turnbaugh et al., 2009a).

The ethnic background and the geographical place of residence are factors known to additionally influence bacterial composition and diversity (Cresci and Bawden, 2015). When comparing the microbial composition of Chinese and Caucasian people living in the United States and Hong Kong, the microbiota was not only distinct between countries, but also between ethnicities (Prideaux et al., 2013). Another study investigated more than 2000 residents of Amsterdam, being part of one of the six main ethnic groups. Indeed, each ethnic ancestry was characterized by some specific bacterial variations, but the authors also indicated that 21 bacterial taxa were shared among all individuals, regardless the ancestry (Deschasaux et al., 2018). A study conducted in China with more than 7000 individuals assessed the possible effect of geographic position on the gut microbiota. The authors found a significant correlation between geographic locations and variations in gut microbiota composition (He et al., 2018).

2.5. Techniques to analyze the microbial ecosystems

2.5.1. Culture dependent methods

Over the last decades, gut microbiota composition was explored by traditional bacterial culture methods, followed by phenotyping of the isolated bacteria through biochemical and morphological characteristics (Sarangi et al., 2018). However, these combined techniques allowed to isolate only up to 25 % of the bacteria of the anaerobic bacterial composition. With further optimizations of anaerobic culture procedures, e.g. the use of Hungate tubes, scientists succeeded to isolate dominant genera such as *Bacteroides, Clostridium* or *Bifidobacterium.* Disadvantages in using this method is the time consuming aspect, the difficulty of culturing and distinguishment of different bacterial populations on deeper phylogenetic level (Lagier et al., 2012; Lagier et al., 2015; Lozupone et al., 2012; Rajilic-Stojanovic and de Vos, 2014). Further, the inability of culturing most of the gut bacteria might be due to the burden of properly mimicking host-microbe or microbe-microbe interactions and human intestinal physiology, and additionally the lack of information about the essential growth requirements of the bacteria (Sekirov et al., 2010). Moreover, these techniques highly underestimate the bacterial diversity, since only viable cells can be cultured, and are consequently less useful for studying shifts in the bacterial community (Sarangi et al., 2018). Nevertheless, even if molecular techniques are most preferred nowadays, culture-dependent techniques made the foundation in the understanding of the bacterial functionality within the complex microbiota, and further phenotypic characterization of single

bacterial strains continues to be a necessary work to increase our knowledge and understanding of the findings (Blaut, 2013).

2.5.2. Culture independent methods

Next generation sequencing

The advancement and introduction of culture-independent techniques gave the researchers insights into the wide diversity of bacteria in the gut microbiota (Blaut, 2013). Especially the development of high-throughput DNA sequencing enables the characterization of microbiome composition without culturing. The principle of molecular biology techniques are based on the differences in the sequence of nucleotides of genes. First, genomic DNA is extracted from samples, followed by amplification and finally sequencing of 16S ribosomal RNA (rRNA) genes can be performed. 16S rRNA genes contain nine highly variable and conserved regions (V1-V9), allowing the taxonomic identification of species. 16S rRNA gene sequencing allows getting insights not only into the bacterial composition in a sample, but also into the diversity of species. While initially, sequencing was performed of the entire 16S rRNA gene, the focus was shifted to the sequencing of shorter sub regions of the gene, which are sufficient to provide a reliable identification and phylogenic classification of the bacterial taxa. The main advantages of high-throughput DNA sequencing technology are the ability to direct classify samples without pre-culturing and to analyze changes in composition over time (Robles Alonso and Guarner, 2013; Rooks and Garrett, 2016; Sarangi et al., 2018).

Other molecular approaches include metagenomics, metatranscriptomics, metaproteomics and metabolomics, which investigate DNA/RNA, proteins and metabolites, respectively. Metagenomics is based on the sequence of all genes within genomic DNA and is one of the most prominent analysis in gut microbiota research (Oulas et al., 2015). It provides the phylogenetic characterization and the biological functional capacity of the bacterial community. The ability to characterize the dynamics and regulation of a bacterial community as well as the expression profiles of host genes (metatranscriptomics) allows to investigate how a microbiota can influence its host, and how host genetics can affect the bacterial composition (Blekhman et al., 2015; Franzosa et al., 2015). Metaproteomics links the abundance and diversity of enzymes (proteins) to the phylogenetic origin. Metabolomics investigates the terminal products (metabolites) of the genome and gives an insight of the metabolic state (Robles Alonso and Guarner, 2013).

Quantitative PCR (qPCR)

Since the 1990s, the combination of extracted nucleic acids (DNA and RNA) and PCR enables first analyses of bacterial populations within a complex microbial community as well as to elucidate the microbiota structure and diversity (Giovannoni et al., 1990). Even though high-throughput sequencing remains the favorite technique in gut microbiota research, the ability of the PCR to specifically quantify target bacterial populations

or strains makes it a valuable method in microbial ecology (Smith and Osborn, 2009). The main principle of the qPCR method is amplification of 16S rRNA gene regions with specific primers for a target (from phylum to species level). The reaction mix contains a fluorescent dye (e.g. SYBR green) that emits fluorescence when it binds to double-stranded DNA, thus the DNA product. When the fluorescence intensity is plotted against the number of PCR cycles and using a logarithmic scale, the quantity of gene copy numbers present in a samples can be quantified (Adamski et al., 2014; Carey et al., 2007). The signal intensity is proportional to the amount of DNA. Advantages of qPCR method include the specific differentiation of target bacterial populations, speed and the ability to design primers for bacterial targets. Further, it is the most accurate culture-independent quantification of total bacterial amount. Another advantage is the worldwide availability of the qPCR kits. Major disadvantages include the PCR bias: the technique is unable to detect unknown species and is not occurring for some target-primer combinations (Fraher et al., 2012).

3. Inflammatory Bowel Disease

Inflammatory bowel diseases (IBD) are chronic and relapsing intestinal inflammation disorders, with the two main types ulcerative colitis (UC) and Crohn's disease (CD) (Matsuoka and Kanai, 2015). The two conditions have different clinical, histological and anatomical findings. While CD affects in a discontinuous transmural manner any region of the GIT (from ileum to colon), UC occurs exclusively in the colon and rectum with a continuous pattern affecting the mucosa. In addition, CD is also characterized by formation of granulomas, fistula and strictures, which are not present in UC (Tian et al., 2017; Zhu and Li, 2012). The disorder is often cyclical with periods of active inflammation and remission (Indriolo et al., 2011).

3.1. Etiology and pathology of IBD

Around 3.5 million people worldwide suffer from IBD, with around 70'000 new cases being diagnosed every year (**Figure 1.5**) (Ahuja and Tandon, 2010; Bernstein et al., 2010a). In the past, IBD was seen as a disease of Western countries, but the epidemiology of IBD has changed around the world at the turn of the 21st century. At the same time, little is known about the evolving incidence in non-western countries (Kaplan, 2015; Kaplan and Ng, 2017; Molodecky et al., 2012). However, latest reports highlighted the rising IBD incidence in South America, Eastern Europe, Africa and Asia. This incidence increase among nationalities and ethnicities among whom initially IBD was rarely observed, demonstrates the involvement of environmental triggers in IBD pathogenesis (Frolkis et al., 2013).

Until today, the exact cause of IBD remains unknown, but genetic background, gut microbiota, aberrant immune responses and environmental factors are considered to play an important role in the onset and evolution of IBD. Notably the rising incidence of IBD during the last decades proves that environmental factors must be crucial beside genetic determinants (Matsuoka and Kanai, 2015). In recent years, it became further evident that the gut microbiota is likewise involved in the pathophysiology of IBD. The most accepted hypothesis to date affirms that an inappropriate immune response against the gut commensals is triggered by environmental factors in a host with a genetic susceptibility (Goyette et al., 2007; Tian et al., 2017). The molecular mechanism of chronic inflammation includes the interaction of pro-inflammatory cytokines, chemokines, adhesion molecules, inflammatory mediators and oxygen free radicals (Kim et al., 2012). Typical symptoms of IBD are rectal bloody stool, diarrhea, abdominal pain and weight loss (Rigoli and Caruso, 2014; Zhu and Li, 2012). Due to the extended nature of the disease, high costs for therapy, surgery, hospitalization and social functioning occur (Ananthakrishnan, 2015).
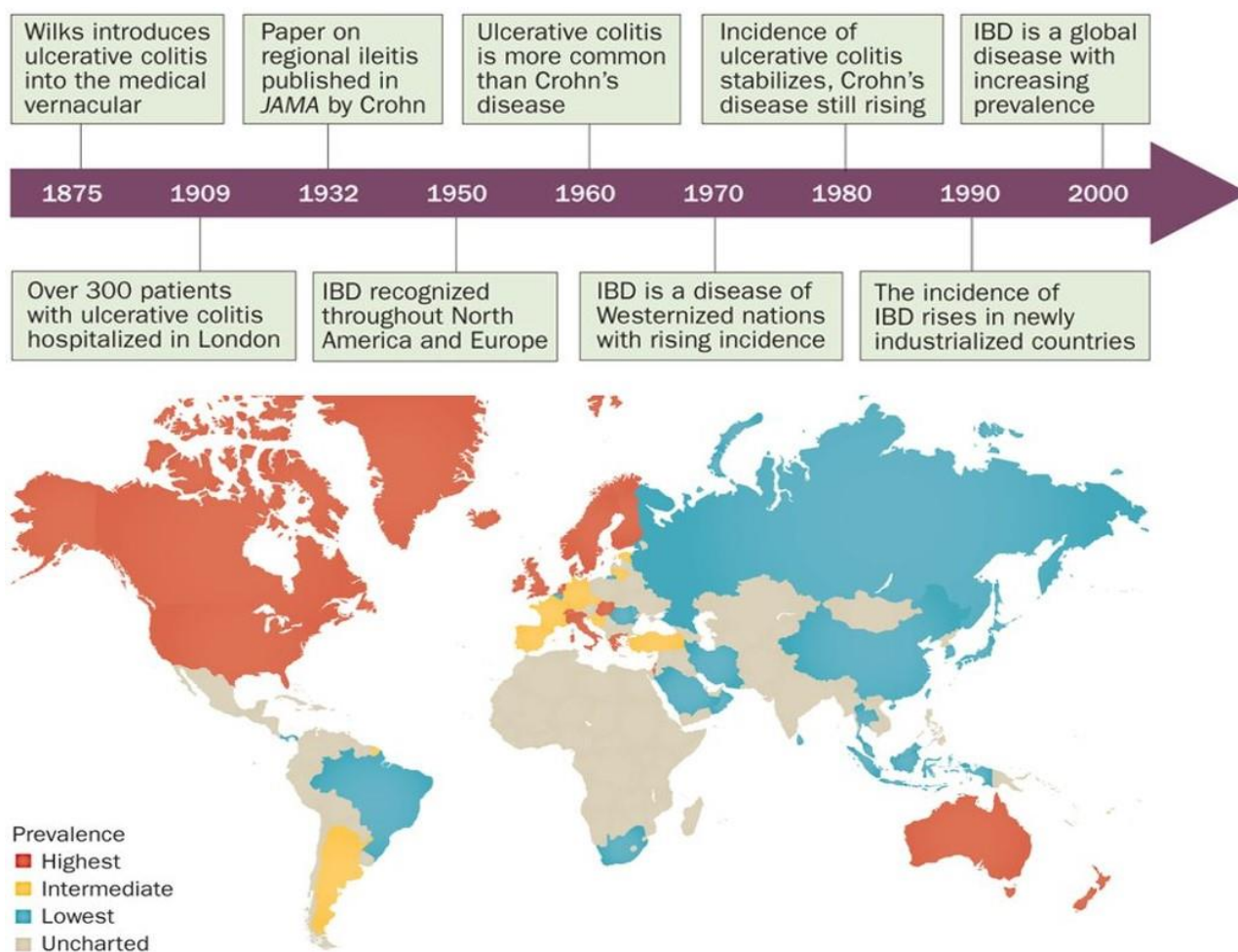
Figure 1.5: History and worldwide IBD incidence rates in 2015. Incidence rates were ranked into low (blue) to intermediate (yellow) to high (red). Adapted from Kaplan (2015).

3.1.1. Host factors and immune response

Genetic susceptibility

Familial aggregation and twin studies highlighted the role of genetics and hereditary components in the development of IBD, and up to 15 % of the CD patients have a family history (Abraham and Cho, 2009). When both parents suffer from the disease, the risk of developing it within the first 30 years of age is expected to be as high as one-in-three in the progeny (Gordon et al., 2015; Halme et al., 2006). Around 200 susceptibility loci are associated with IBD, and these loci are highly connected to pathways interacting with environmental factors and modulation of the intestinal homeostasis (Jostins et al., 2012; Khor et al., 2011; Uniken et al., 2017). Therefore, the loci might induce an impaired response to commensal bacteria in the disease. Even though CD and UC share common risk genes, both have disease-specific loci. UC-specific loci are for example

IL-10 and *HLA*, whereas *NOD2, ATG16L1* and *IRGM1* are specific for CD (Anderson et al., 2011; Festen et al., 2010; Fisher et al., 2008). *NOD2* was the first high-risk gene identified in CD patients and this gene is involved in the immune reaction stimulation by recognizing muramyl dipeptide, a cell wall peptidoglycan component of bacteria. Paneth cells, located at the base of intestinal crypts in the terminal ileum and producing antimicrobial defensins, also express *NOD2*. Thus, mutations in this gene cause significant alternations of the gut microbial composition. Patients suffering from IBD and carrying an altered *NOD2* allele have increased levels of mucosa-adherent microbes and decreased secretion of the anti-inflammatory IL-10 (Kostic et al., 2014; Philpott and Girardin, 2009; Stappenbeck et al., 2002). Significant changes in gut bacterial composition are observed when IBD patients carry the *NOD2* and *ATG16L1*, a gene involved in autophagy. Decreased numbers of *Faecalibacterium* and *Roseburia* and increased numbers of *Enterobacteriaceae* and *Bacteroides* are generally observed (Frank et al., 2011; Imhann et al., 2018). Subjects show increased risk for CD, when being homozygous for loss-of-function alleles of *FUT2*. Concerned individuals do not express ABO antigens on the mucosa in the GIT and show significant alternations in the mucosa-associated bacterial community (Rausch et al., 2011). Variants in the gene locus encoding protein tyrosine phosphatase non-receptor type 2 and 22 (PTPN2 and PTPN22), which negatively controls pro-inflammatory signaling cascades, has been positively correlated with IBD (Spalinger et al., 2018). A single nucleotide polymorphism in PTPN2 increases the risk factor for CD and UC, while a PTPN22 variant can protect from CD. Interestingly, the research group also observed that the variant alleles of both genes highly affect gut microbiota composition. The PTPN2 variant is positively correlated with increases in *Clostridiales* and *Lachnospiraceae* in CD patients, and decreases in *Roseburia* in UC patients. On the other hand, the variant in PTPN22 increased relative abundance of *Ruminococcus* (Yilmaz et al., 2018).

An interesting fact is that most people carrying IBD-associated risk gene variants will not develop IBD and remain healthy. This phenomenon suggest that other factors, such as environment or gene-gut microbiota interactions might be involved in the onset of the disease (Ye and McGovern, 2016).

Immune response

Since chronic inflammation in IBD is also due to an aberrant immune response, many research groups were investigating immune abnormalities in IBD (de Souza and Fiocchi, 2016). In IBD, production of IgG is increased against gut microbes, thus weakening self-tolerance. Especially in CD, innate immune cells contain mutations in their intracellular bacterial sensors, which recognize a microorganism's molecular pattern. A defect in the protein, which is a strong regulator and activator of inflammatory responses, will induce a chronic inflammatory response in the tissue (Borzutzky et al., 2010; Eckmann and Karin, 2005). Further, T-lymphocytes are excessively active against antigens of the commensal bacteria, because of an imbalance of cytokines secretions. The inadequate release of cytokines (TNF-α, TGF-β and IFN-γ) affects the extent of the

inflammation, and probably influencing onset of IBD (de Mattos et al., 2015; Feagins, 2010; Ghosh et al., 2006). It was previously widely accepted that CD is rather a Th1-mediated condition, while UC is a Th2-disease. This version was presently adjusted, assuming that both cytokines have various antagonistic effects (Muzes et al., 2012). In general, the aberrant immune response is not explicit against a single bacterial population, but rather against numerous bacterial populations of the gut community (Robles Alonso and Guarner, 2013).

Oxidative stress

Data from clinical and experimental animal models suggest that oxidative stress is involved in the development of IBD, since free radicals and reduced antioxidant are observed in inflammation. It is hypothesized that the increase of oxygen or oxygen stress in the gut might trigger IBD development (Zhu and Li, 2012).

Oxidative stress is defined as "an imbalance between the production of reactive oxygen species (ROS) and their removal by antioxidants" (Rezaie et al., 2007). ROS is a normal byproduct of oxygen metabolism, and is mainly produced by intracellular organelles like peroxisomes, endoplasmic reticulum, mitochondria or the nucleus (Novak and Mollen, 2015; Tian et al., 2017). Further, during inflammation, the gut microbiota can produce ROS (e.g *Helicobaterium pylori* and *Enterococcus faecalis*) and damaging directly infected cells (Irrazabal et al., 2014), and the gut bacteria can trigger ROS production through macrophage activation in response to an inflammatory state (Tian et al., 2017; Cao, 2018). *Enterococcus faecalis* can produce hydroxyl radicals and is for the high level of extracellular O_2^- that result in H_2O_2 and hydroxyl radicals (Irrazábal et al., 2014).

ROS are free radicals such as hydroxyl radicals (HO·$^-$), superoxide ($O_2\cdot^-$), alcoxyl (RO·), peroxyl (RO$_2$·) and hydroperoxyl (HO$_2$·); reactive nonradical substrates including oxygen (O_2), hydrochlorus acid (HOCl), hydrogen peroxide (H_2O_2), chloramines (RNHCl), ozone (O_3); and lipid hydroperoxidases (Tian et al., 2017). ROS are highly reactive due to the unpaired electrons and mediate intracellular damages of proteins, carbohydrate, nucleic acids and lipids. In mitochondria, excessive ROS production induces a lower ATP generation, and thus suppresses the intracellular electron transport chain and causes DNA damage (Chen and Gibson, 2008; Khodayari et al., 2013; Kryston et al., 2011). In addition, these reactive particles have the potential to upregulate expression of genes related to the innate and adaptive immune response in the GIT (Alzoghaibi, 2013). Interestingly, some enzymes involved in the endogenous ROS generation (mucosal NADPH oxidases (NOX)), have been reported as IBD risk factors (O'Neill et al., 2015). In healthy conditions, endogenous host cells possess an intricate antioxidant capacity and are able to cope with low concentrations of ROS. The defense mechanism consists of catalases (CAT), superoxide dismutases (SODs) and glutathione peroxidases (GPX) (Tian et al., 2017). Oxidative stress can further be triggered by other environmental stresses, like smoking, luminal antigens, chemotherapy, radiation, drugs, alcohol and the presence of the two metals, iron (Fe) and copper (Cu), in diet. Activated neutrophils and macrophages generate ROS and reactive nitrogen

species (RNS), and the accumulation of ROS drives damages of the mucosal layer, leading to bacterial invasion and thus to an immune response (Goyette et al., 2007; Seril et al., 2003). Until today, no studies investigated the direct adverse effect of oxidative stress on gut microbiota composition and functionality.

3.1.2. Environmental factors

In the last century, IBD incidence and prevalence increased remarkably globally, and particularly in countries where changes in diet, environment, hygiene and lifestyle happened. Due to the relatively short time period, this increase in IBD incidence cannot only be linked to a genetic drift, but rather might be the synergy with environmental factors, gut microbiota and genetic susceptibility (Molodecky et al., 2012).

Drugs

Some drugs have been associated with IBD through different pathophysiological mechanisms. Isotretinoin, a synthetic analog of a vitamin used for treating acne, has an inhibitory effect on the release of inflammatory mediators and prevents a neutrophil accumulation. This inhibitory effect may reduce the immune response to commensal bacterial stimulation and provoke amplified immune responses (Shale et al., 2009). A dosage dependent correlation between the amount of antibiotic supply and the risk of IBD was reported for antibiotics. Of all antibiotics tested, metronidazole has the strongest association, while penicillin the weakest association. Children who received an antibiotic in the first year of life, have an increased risk of IBD development compared to controls. However, the correlation is higher for CD than UC with the intake of the different classes of antibiotics (Aniwan et al., 2018; Shaw et al., 2011; Ungaro et al., 2014; Virta et al., 2012). NSAIDs (nonsteroidal anti-inflammatory drugs) are commonly used to treat arthritis; interestingly, 30 % of IBD patients suffer from arthritis and are treated by NSAIDs (Long et al., 2016; Vavricka et al., 2011). These drugs inhibit cyclooxygenase (COX), and consequently prostaglandins, which play an important role in mucosal defense mechanisms. Further, they damage membrane phospholipids, reduce ATP concentrations and induce a loss of membrane integrity or permeability (Bjarnason et al., 2018; Cipolla et al., 2002).

Smoking and stress

Since three decades, cigarette smoking is recognized as one of the major environmental factors that is highly associated with IBD pathogenesis. However, smoking cigarettes has different effects on both disease types: according to epidemiological data, smoking cigarettes is the most important risk factor for CD, whilst it positively influences UC progression (Lunney and Leong, 2012; Parkes et al., 2014). Until today, the exact mechanism how cigarette smoking is involved in the pathogenesis remains not understood. Tobacco smoke includes around 4500 different chemicals, among which a part is interfering directly with the individual, and others are highly toxic (Mehta et al., 2008). Among the most accepted assumptions are for instance changes

in cytokine concentrations, alternations in immune responses and changes in gut permeability (Perricone et al., 2016; Verschuere et al., 2012).

Observational cohort studies and mice experiments demonstrated a possible role of anxiety and depression in the development of IBD. Exposing mice to stressful stimuli showed reduced the overall diversity of the gut microbiota and enhanced levels of the pro-inflammatory cytokine IL-6 (Bailey et al., 2011; Bonaz and Bernstein, 2013; Goodhand et al., 2012). Further, severe colitis was observed in mice models in which depression was chemically triggered, but could be restored when an antidepressant was administered (Ananthakrishnan et al., 2013b; Ghia et al., 2009). Stress affects gut inflammation by stimulating the production of pro-inflammatory cytokines, alternating the intestinal permeability and bacterial composition or activating macrophages (Bernstein et al., 2010b; Bonaz and Bernstein, 2013).

Diet

Even if no diet could be directly linked to IBD, yet, it is a crucial factor that needs to be considered when studying the role of IBD (Kostic et al., 2014). In a cohort study, woman with long-term fiber intake had a 40 % reduction in risk of CD development, with an inverse correlation for fibers from fruits and vegetables, but not with whole grains (Ananthakrishnan et al., 2013a). Possible explanations are for instance the breakdown of soluble fibers (from fruits and vegetables) by the microbiota to SCFAs that restrict the expression of pro-inflammatory molecules (Andersen et al., 2012; Farooqui, 2015; Galvez et al., 2005) or the maintenance of integrity of the epithelial barrier and the consequent reduction of *E. coli* translocation (Roberts et al., 2010). Further, high consumption of n-6 polyunsaturated fatty acids (FAs) (omega-6 PUFA) and low intake of n-3 PUFA are correlated with increased risk and pathogenesis of IBD (Costea et al., 2014). In addition, an association of vitamin D with the pathogenesis of IBD was also identified (Limketkai et al., 2017). In mice experiments, a lack of vitamin D3 increased risk of colitis, and administration of the micronutrient alleviates the inflammation and inhibits pro-inflammatory gene expression or responsiveness of mononuclear cells to antigens (Froicu and Cantorna, 2007; Froicu et al., 2006; Limketkai et al., 2017). High dietary iron concentrations in drinking water was associated with higher risk of IBD, while zinc modulates the function of innate immune cells or inhibits the transcription of inflammatory compounds in the NF-$_k$B pathway (Cerasi et al., 2013; Haase and Rink, 2014; Lahiri and Abraham, 2014).

Other factors

There are three stages of IBD onset: an early peak of onset is occurring at younger than 10 years, a second onset is around 15 and 30 years and a late onset occurs around the age of 60 (Martin-de-Carpi et al., 2013). These phases correspond to moments in which the intestinal bacterial community alters in stability and diversity (Spor et al., 2011). The gut microbiota at young age has a low complexity and stability as a result of the mode of delivery, changes in the model of food intake (shift from breastfeeding to solid food), illness and

puberty (Dominguez-Bello et al., 2011). In elderly people, a decreased microbiota stability can be re-observed (Claesson et al., 2011).

It is assumed that environmental factors and inflammation contribute to microbial dysbiosis in early IBD onset, and therefore promoting IBD development. Further, there is also strong evidence that specific pathobionts (commensal bacteria that can cause IBD under particulate conditions (Chow et al., 2011).

3.1.3. Potential infectious- and commensal bacteria

In order to prove that the presence of a microorganism leads to a specific disease, the Koch's postulates need to be fulfilled. The determined bacterium must (i) occur in all cases of the same disease, (ii) be isolated from patients suffering from the disease, (iii) lead to the disease when introduced into a healthy host, and (iv) be re-isolated again of the newly infected host (Butto and Haller, 2016). However, IBD does not fulfil the Koch's postulates in the present scheme, since it is rather a polymicrobial disease than the result of a single microorganism (Singh et al., 2016). Since the last century, scientists made efforts to identify a single causal microorganism that can be linked to CD and UC.

Indeed, the obligate intracellular organism *Mycobacterium avium paratuberculosis* (MAP) was the first identified bacterium to be linked with CD (Sartor, 2008). In CD patients, a magnificent T cell response to MAP can be observed, along with a high secretion of TNF-α by macrophages infected by the pathogen (Nakase et al., 2011). Next, adhesive-invasive *E. coli* (AIEC) are frequently found in the ileal mucosa of CD patients, since the organism can adhere and subsequently penetrate intestinal epithelial cells. In addition, AIEC can replicate in macrophages and thus stimulate pro-inflammatory TNF-α production and trigger progression of IBD (Matsuoka and Kanai, 2015). These invasive strains are generally among the first *E. coli* strains that are isolated after a patient was diagnosed with IBD (O'Brien et al., 2017). Further, *Fusobacterium varium* was observed to attach to inflamed regions and to invade the mucosa at ulcers in UC, with increased anti- *F. varium* antibodies compared to healthy individuals (Ohkusa et al., 2002). Generally, infections are triggers of relapse in patients with established IBD, and one of the most prevalent triggers is *Clostridium difficile* infection in hospitalized patients (Ananthakrishnan, 2015). The microorganism *Helicobacter pylori* is generally acquired during childhood, and often detected in IBD patients (Fallone and Bitton, 2008; Luther et al., 2010). Interestingly, IBD incidences are small in countries with a high *H. pylori* infection rate, suggesting that the bacterium has an eventual protective role in IBD (Chen and Blaser, 2008; Luther et al., 2010; Sonnenberg and Genta, 2012; Zhang et al., 2011). A possible explanation might be the *H. pylori* increases Treg cell differentiation and production, what in turn decreases the following secretion of inflammatory cytokines (Rad et al., 2006).

Even though, some bacterial taxa of the gut community seem to have a protective action against IBD (Cammarota et al., 2015; Kitajima et al., 2001), recent studies have demonstrated that the complex gut microbiota is a crucial determinant in initiation and progress of IBD together with genetic alternations and

dysregulated immune response (Cammarota et al., 2015; Sartor, 2008). Gut bacteria participate in the pathogenesis by bacterial translocation from the intestinal lumen through the epithelial mucosa, and produce a necessary stimulus for immune-inflammatory responses, thus leading to mucosal injury (Robles Alonso and Guarner, 2013). Further, during inflammation, the gut microbiota can produce ROS and damaging directly infected cells (Irrazabal et al., 2014), and the gut bacteria can trigger ROS production through macrophage activation in response to an inflammatory state (Tian et al., 2017).

3.2. Microbial dysbiosis in IBD

Since the gut microbiome closely interacts with its host in many ways, a shift in its structure and functionality may exert significant consequences for the host's health (Linares et al., 2016). Dysbiosis is defined as "an imbalance of the bacterial composition and/or function that are normally found in a healthy host. It is generally associated with alternations of the immune function and increased susceptibility for inflammatory diseases, allergies and metabolic conditions" (Rooks and Garrett, 2016). A disruption of the eubiotic state is often marked by the bloom of bacteria belonging to the facultative anaerobic phylum Proteobacteria and a reduction of overall microbial diversity and the predominant strict anaerobic phyla Firmicutes and Bacteroidetes (Winter and Baumler, 2014).

The high improvements in DNA sequencing techniques and analysis enabled to reveal the considerable microbial signatures in the microbiome of IBD patients (Imhann et al., 2018; Kostic et al., 2014). The comparison of microbiota derived from stool or biopsy from healthy individuals and diseased patients highlighted a potential role of the gut microbiota in the etiology and progress of some gastrointestinal diseases, such as IBD (Marchesi et al., 2016). While the gut microbiota of healthy individuals displays a certain stability, the microbiome IBD patients is highly unstable. The microbial composition alternates during active and inactive periods of IBD (Martinez et al., 2008; Wills et al., 2014). It is important to mention that microbial dysbiosis differs when comparing fecal and mucosal samples of IBD patients, and can be explained that the gut microbiota consists of luminal and mucosa-associated bacteria. Some ecological studies pointed out that patients with IBD have lower microbial diversity in both, the mucosa- associated and faecal bacterial community (Mylonaki et al., 2005; Soko et al., 2009; Manichanh et al., 2006; Chen et al, 2014). In contrast to these findings, other studies did not observe significant differences in bacterial diversity and composition between inflamed and non-inflamed mucosa within IBD patients (Nishino et al., 2017; Forbes et al., 2016). Some studies reported that mucosa-associated microbes are increased in abundance compared to healthy controls, indicating that this location might be physiologically more relevant for mucosal samples of IBD patients. The authors suggested that the mucosa-associated bacteria directly affect the epithelial and mucosal functions and therefore play a more significant role in the pathophysiology of IBD (Lavelle et al., 2015; Sartor, 2015). Overall mucosa-associated and faecal bacterial communities in IBD have compared to healthy controls

decreased Firmicutes, *Clostridium coccoides, Bacteroides ovatus, B. vulgatus* and *Faecalibacterium prausnitzii*, while abundances of Proteobacteria and mucosa-associated aerobic and facultative anaerobic bacteria were increased (Mylonaki et al., 2005; Soko et al., 2009; Manichanh et al., 2006; Chen et al, 2014).
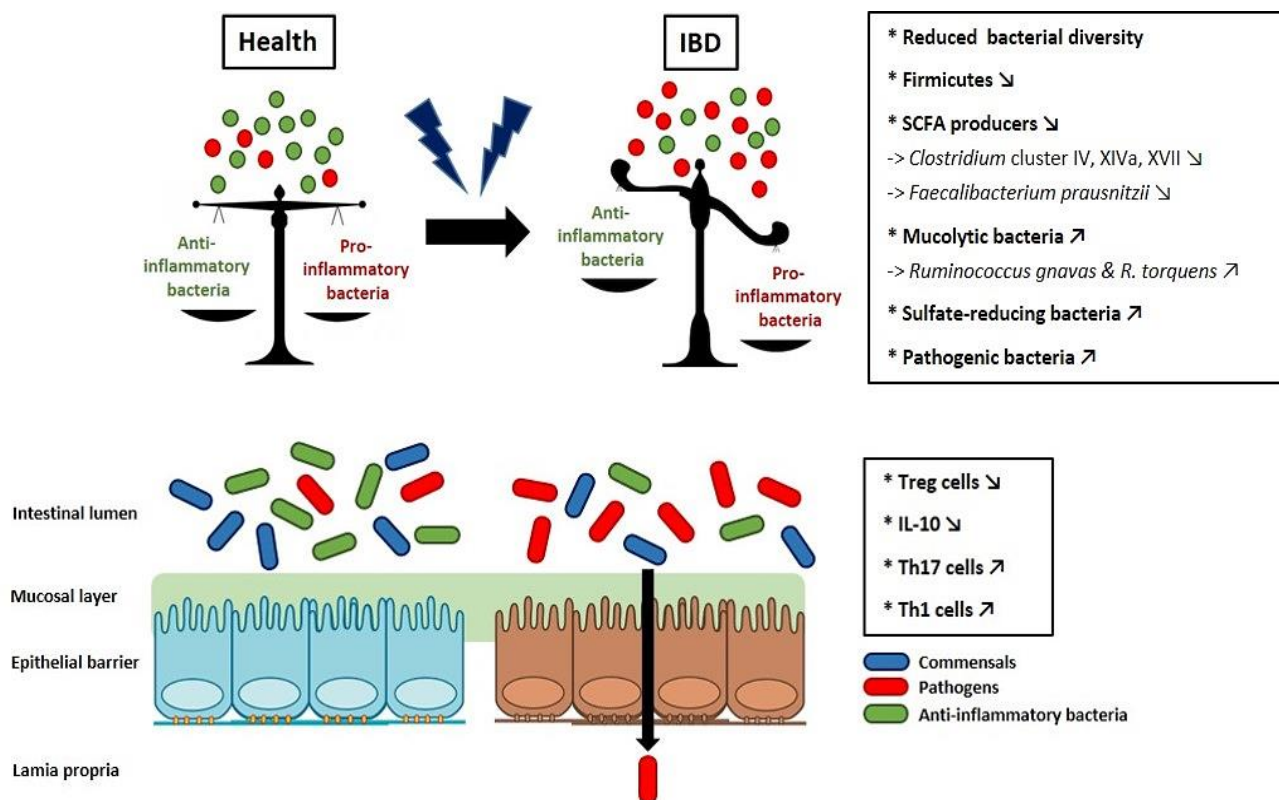
Figure 1.6: Microbial dysbiosis in IBD and uncontrolled immune responses leading to a defective epithelial barrier. Adapted from Zhang et al. (2017), Nagpal et al. (2014) and Nishida et al. (2018)

In comparison to healthy individuals, the gut microbiota of IBD patients is characterized by a decrease of Firmicutes, a bloom of Proteobacteria or Actinobacteria and a decrease in community diversity (**Figure 1.6**) (Gevers et al., 2017; Ho et al., 2015; Nagalingam and Lynch, 2012). The reduction in Firmicutes relative abundance and diversity is largely due to decreases in oxygen-sensitive butyrate producers belonging to *Ruminococcaceae* and *Lachnospiraceae* or other SCFA-producers such as *Odoribacter* and *Leuconostocaceae* (Matsuoka and Kanai, 2015; Morgan et al., 2012). Some publications suggested considering the phylum Firmicutes as a biomarker for defining IBD-related dysbiosis. In the IBD-microbiota, a decrease or even disappearance of the oxygen-sensitive and anti-inflammatory bacterium *Faecalibacterium prausnitzii* is often observed (Wang et al., 2014). IBD is characterized by a disruption of the epithelial barrier, which is suspected to lead to an increased intraluminal oxygen concentration, and thus favoring growth of facultative taxa belonging to Proteobacteria (Albenberg et al., 2014). The family *Enterobacteriaceae* is known to profit from compounds released during inflammation as terminal electron acceptors (Winter and Baumler, 2014). While some studies reported decreases in Bacteroidetes in IBD patients, others observed increased abundance of

this phylum (Frank et al., 2007; Gevers et al., 2017; Walker et al., 2011b; Wang et al., 2014). The genus *Fusobacterium* was also found at higher relative abundance in the colon of IBD patients compared to healthy individuals, and probably has a positive connection with IBD pathology. The phylum Fusobacteria is a group of adherent, anaerobic but invasive bacteria that are predominately colonizers of the oral cavity, but possess the ability to colonize the gut (Kostic et al., 2014). Furthermore, an increase in *Desulfovibrio*, a sulfate-reducing bacterial taxa is also observed in the bacterial community of IBD patients. These bacteria produce H_2S that damages the intestinal epithelial cells and thus triggers inflammation in the mucus (Lennon et al., 2014).

Along with the reduction of microbial diversity, metagenomics analysis revealed that an IBD microbiome has around 25 % fewer genes, due to a depletion of proteins and functional pathways (Erickson et al., 2012; Qin et al., 2010). The decrease in genes implies that around 12 % of the metabolic pathways differ from healthy individuals, with strong decreases in butanoate and propanoate pathways in diseased people (Morgan et al., 2012). Furthermore, significant alternations of the amino acid metabolism in IBD patients are observed: abundance of genes involved in the biosynthesis and metabolism of most amino acids were decreased, while those involved in the transport of histidine, arginine and lysine (cysteine-containing amino acids) are highly increased. During intestinal inflammation, mucin (rich in cysteine) secretion is upregulated and correlates with the upregulated genes for cysteine metabolism and transport (Morgan et al., 2012).

3.3. Therapies in IBD

Even though IBD has extensively been studied in the last years and the understanding of the patho-mechanisms has increased, no cure for the chronic inflammatory disorder is existing, yet (Tian et al., 2017). Different therapeutic approaches have shown that early beginning of a treatment is linked with a better response to therapy and less damage (Ananthakrishnan et al., 2012). In addition, diverse therapeutics are available for a personalized treatment, which in turn requires a prediction of the possible response to a therapy (Ben-Horin and Chowers, 2014; Siegel and Melmed, 2009). The IBD drug market reached 3.5 billion USD in 2009, and might grow to 5.6 billion USD by 2019. At this moment, numerous companies are generating new chemical substrates by altering the profile of a common drug or by neutralizing receptors, signaling molecules or cytokines, which are involved in the inflammatory response pathways. The main goals of the therapy are the initiation and maintenance of IBD remission. A standard therapy for IBD patients includes aminosalicylates, corticosteroids, immunomodulators and biological substrates. However the choice of pharmacological agents depends on the severity, location of the disease (Kondamudi et al., 2013).

3.3.1. Microbiota modulation strategies

Probiotics and prebiotics

Considering the involvement of the gut microbiota in IBD, the interest of treatments that positively affect the bacterial composition through probiotic and prebiotic supplementation increased in recent years. Probiotics (**section 4.2**), mostly lactobacilli, bifidobacteria and streptococci, are attractive alternatives, since IBD patients like the safety and nontoxicity of probiotics. VSL#3 administration (product contains four strains of lactobacilli, one strain of *Streptococcus* and three strains of bifidobacteria) was shown to have mild to moderate results in UC patients (Gionchetti et al., 2017). To date, only a limited number of studies investigated the effectiveness of probiotics in treating patients with CD. The efficacy of *Lactobacillus rhamnosus* intake solely or as adjuvant to the normal therapy for maintaining remission was compared to placebo in CD patients. In all studies, no significant effect compared to the placebo group could be found (Bousvaros et al., 2005; Schultz et al., 2004). Similar observations were seen for *Saccharomyces boulardii* (Doherty et al., 2009; Garcia Vilela et al., 2008; Guslandi et al., 2000). With increasing research and knowledge of the gut microbiome, more and more organisms were found to positively affect the host's health and are often referred to «next-generation probiotics» (O'Toole et al., 2017). Since the definition of probiotic by the United Nations Food and Agriculture organization is rather broad in terms of the phylogenetic origin of the probiotic, next generation probiotics comprise representative strains from *Clostridium* clusters IV, XIVa and XVIII, and the species *Akkermansia muciniphila, Bacteroides uniformis, Bacteroides fragilis, F. prausnitzii* and *Eubacterium hallii* (El Hage et al., 2017; Patel and DuPont, 2015; Udayappan et al., 2016). In subsequent clinical trials, the effectiveness of *Lactobacillus*, *E. coli* Nissle 1917 and VSL#3 were tested and positive effects were reported for metabolic and inflammatory diseases (Neef and Sanz, 2013; Patel and DuPont, 2015).

Prebiotics (**section 4.1**) are an attractive potential way of treatment for patients suffering from IBD, because it is safe, cost-effective and may considered as a long-term treatment. While the positive effect of prebiotics in IBD has been proven *in vitro* and in animal models for colitis, only a few human studies with IBD patients exist (Gionchetti et al., 2017) (**Section 4**). Even though there was no enhancement of the clinical symptoms, the patients presented reduced concentrations of pro-inflammatory dendritic cells in the lamia propria and increased anti-inflammatory (IL-10) dendritic cells (Benjamin et al., 2011).

Nutrition

In recent years, researchers started to assess the relationship between IBD and nutrition, since malnutrition can occur mainly in active phase and more in CD than in UC (Forbes et al., 2017). Malnutrition might be the result of decreased food intake, increased intestinal losses of nutrients and thus increased nutrient requirement (Goh and O'Morain, 2003). Therefore, nutritional care is essential in IBD management and includes prevention of malnutrition in patients (Forbes et al., 2017). The introduction of a specific diet in children, called enteral nutrition therapy, has proven to alleviate symptoms and intestinal inflammation in CD, and is the first line therapy to achieve remission (Gerasimidis et al., 2014). The enteral diet replaces the food

by a formula that is nutritionally complete, which is usually taken by mouth or via the nasogastric way. This specific diet is also proposed for IBD patients who are undernourished and need to compensate the nutritional requirements. In adults, the diet is mainly taken together with other medications in order to keep remission state in some patients (Gerasimidis et al., 2014; Ruemmele et al., 2014; Smith, 2008; Yamamoto et al., 2009).

Fecal microbiota transplantation

Fecal microbiota transplantation (FMT) is the standard last resort treatment for patients with recurrent and refractory *Clostridium difficile* infection (CDI), and has a success rate up to 90 % (Kassam et al., 2013). Patients with IBD usually have an increased predisposition for CDI (Syal et al., 2018). Some studies investigated the potential of FMT in treatment of CDI in IBD patients and found positive outcomes (Fischer et al., 2016; Hamilton et al., 2012; Kelly et al., 2014; Khoruts et al., 2016). Recent clinical trials have evaluated the efficacy of FMT in UC and found encouraging results when administration was performed using the lower gastrointestinal route (Costello et al., 2017; Moayyedi et al., 2015; Paramsothy et al., 2017a). No significant difference in remission rates was found when FMT was performed using the upper gastrointestinal route compared to the placebo group (Rossen et al., 2015). A recent meta-analysis demonstrated that the clinical remission and response rate with FMT in UC patients were 33 % and 52 %, respectively (Paramsothy et al., 2017b). Only a few studies were performed with CD patients and various results were observed (Suskind et al., 2015; Vaughn et al., 2016; Vermeire et al., 2016). FMT seems to be a potential method to induce remission in patients suffering from mild to moderate UC. However, the success rate of FMT in UC patients is highly dependent on the clinical and endoscopic severity of the disease (Paramsothy et al., 2017b). Challenges of treating CD patients with FMT include that gut dysbiosis is more pronounced compared to UC patients. In addition, patients with CD will need more intense and prolonged FMT due to its transmural nature of inflammation (Syal et al., 2018).

3.3.2. Last resort measure when therapy fails

During the course of IBD and despite the medications, up to 20 % of patients with UC and up to 80 % of those with CD will require surgery (Mowat et al., 2011). However, the main reasons for surgical interventions are emergency events or failure to respond to the medications (Annese et al., 2016). In UC patients, total surgical removal of the colon followed by ileoanal pouch anastomosis are the most common procedures, because they implement a durable cure for the patients (Annese et al., 2016; Sica and Biancone, 2013). The partial removal of the colon is rarely undertaken due to the high probability that the disease will persist in the remaining colonic parts. For CD, surgical removal of parts of the colon is not a definitive treatment and it is suggested for patients, who are impatient for medications. The surgical removal of scar tissues, which occur after repeated injury and healing, is less commonly performed in patients with narrow small intestine strictures (Andrews et al., 1989; Annese et al., 2016). However, decreasing surgery rates indicate that the administration of

immunosuppressant and biological medications alleviate symptoms and development of IBD (Annese et al., 2016).

In conclusion, intestinal resection should not become the ultimate treatment in IBD management, but remain an appropriate choice for CD and UC patients, who cannot bear the symptoms anymore and have a significant decrease in quality of life.

4. Strategies to modulate the gut microbiota and to improve health

The modulation of the rich and diverse gut microbiota is an emerging strategy to maintain or improve human health, or to treat disease (Gibson et al., 2017; Prescott et al., 2016). Indeed, many dietary studies demonstrated that the composition and functional properties of the bacterial ecosystem can be shifted through small non-dietary and dietary interventions, which include the supplementation of prebiotics or dietary fibers (DF) (Cani and Everard, 2016; Gibson, 2010). In contrast to probiotics, where live bacteria are added to the gut microbiome, prebiotics modify the gut functionality by shaping the beneficial bacteria of the endogenous commensal community (Sarbini and Rastall, 2011). DF intake varies across industrialized and unindustrialized countries, with an average intake of 12 to 30 grams per day in western countries (Holscher, 2017).

4.1. Prebiotics

4.1.1. The evolution of the definition «prebiotic»

Since over the last decades, the definition of a "prebiotic" substrate had been a matter of debate and was continuously reviewed based on the latest scientific findings and developments. Over 20 years ago, the first commercially available prebiotics fructans (fructooligosaccharides (FOS) and inulin) and galactans (galactooligosaccharides or GOS) were found to specifically enrich *Lactobacillus* and/or *Bifidobacterium* spp. within the gut microbiota, which initiated the prebiotic concept. Initially, based on culture methods, a compound was termed prebiotic, when it displayed «an ability to beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria already resident in the colon» (Gibson et al., 1995). About 10 years later, the definition of a prebiotic substrate was changed to «a selectively fermented ingredients that allow specific changes, both in composition and/or activity in the gastrointestinal microflora that confers benefits upon host well-being and health» (Gibson et al., 2004). This definition suggests that a prebiotic has to resist host digestion for being metabolized by intestinal bacteria, whose growth and/or activity are selectively stimulated. However, in the following years along, the criterion «selective fermentation» raised a problem to the scientists of the field, leading to its transitional removal for two years (Gibson, 2010). In 2015, a research group proposed to remove the specificity requirement, since multiple bacterial taxa, rather than single strains, are modulated by prebiotic substrates. This proposal give another definition of «a non-digestible compound that, through its metabolization by microorganisms in the gut, modulates the composition and/or activity of the gut microbiota, thus, conferring a beneficial physiological effect on the host» (Bindels et al., 2015). However, this definition needed to be further clarified on specificity, health attributes and mechanisms. The newest definition of a prebiotic substrate is that «a prebiotic is a

substrate that is selectively utilized by host microorganisms conferring a health benefit» (Gibson et al., 2017) (**Figure 1.7**). Considering the fact that multiple bacterial populations can metabolize a prebiotic, changes in SCFAs like acetate, propionate and butyrate are likely to occur, and which have a positive association with health (Koh et al., 2016).
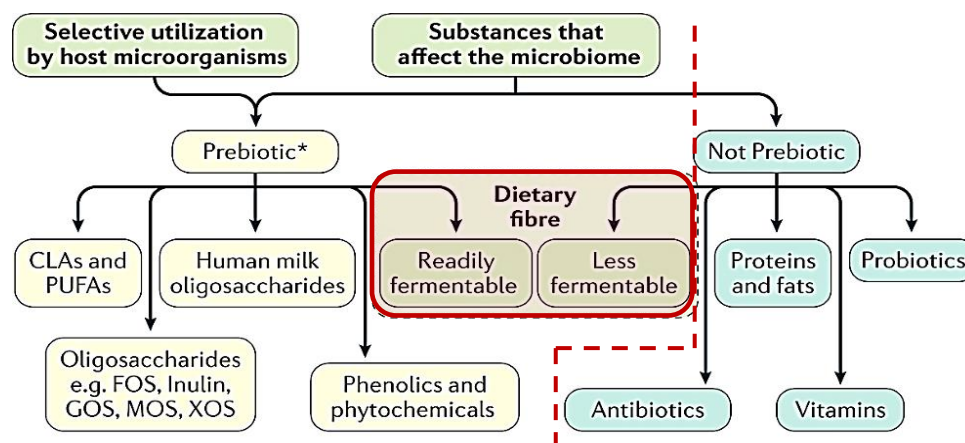
Figure 1.7: Differentiating a prebiotic compound based on the novel definition. CLA, conjugated linoleic acid; PUFA, polyunsaturated fatty acid; FOS, fructooligosaccharides; GOS, galactooligosaccharides; MOS, mannanoligosaccharide; XOS, xylooligosaccharide. Adapted from (Gibson et al., 2017).

4.1.2. Classifications, characteristics and origins of dietary fibers

Not all fibers can be called prebiotic, but most prebiotics can be classified as dietary fiber (DF) (Slavin, 2013). DF were defined by the Codex Alimentarius Commission in 2009, as «complex carbohydrate polymers with ten or more monomeric units, which are neither digested nor absorbed in the human small intestine and belong to the following categories: (i) edible carbohydrate polymers naturally occurring in foods as consumed, (ii) edible carbohydrate polymers which have been obtained from food raw materials by physical, enzymatic, or chemical means and which have a beneficial physiological effect demonstrated by generally accepted scientific evidence, and (iii) edible synthetic carbohydrate polymers which have a beneficial physiological effect demonstrated by generally accepted scientific evidence» (Codex Alimentarius Committee, 2010). However, in the research field and different countries, some flexibility exists in the definition of «dietary fiber» by including polysaccharides with three to nine sugar units in length (Jones, 2014).

Generally, since DFs are heterogeneous polysaccharides, their classification bases on origin, physiochemical properties and chemical composition and degree of polymerization (DP). Regarding origin, fiber originating from plants can be classified into fibers derived from grains, fruits, nuts, vegetable and legumes, and cereals. The origins from different plant types influence not only the physiochemical properties, but also the chemical compositions of the DFs (Elleuch et al., 2011; McRorie and Fahey, 2013). For instance, apples are an important source for pectin, while bananas include inulin-type fructans and resistant starch. Therefore, people

consuming a plant-rich diet are supplied with numerous different DFs and thus maintain a diverse gut microbiota (Bourquin et al., 1993). Fermentability, viscosity, binding and bulking ability and water solubility of a fiber are defined with the physicochemical properties. These properties not only influence its fermentation itself, but also provide first indications about its eventual manner of being metabolized by the bacterial community and its therapeutic effects for the host (Schieber et al., 2001; Slavin, 2013). Generally, the main health benefit of insoluble fibers (e.g. cellulose) is laxation. Intake of these fibers increase fecal bulking and stool water content, since the fibers possess a high water-binding ability, which consequently stimulates peristalsis (de Vries et al., 2015; Lawton et al., 2013; Vuksan et al., 2008). Bulking influences the structure of the food matrix, which affects the accessibility and availability of macronutrients for the gut microbiota (Grundy et al., 2016). Increased water availability and a decrease in macronutrient availability will further lead to modulation of the microbiota (Lattimer and Haub, 2010). Soluble and viscous fibers are readily fermentable (e.g. pectin or β-glucan), and are naturally found in whole grains (oats and barley) and fruits (Elleuch et al., 2011). When dissolved in water, these fibers form a gel, a feature that affects intestinal motility and absorption of cholesterol, triglycerides and glucose (Dikeman and Fahey, 2006). In fact, viscosity was demonstrated to beneficially and highly affect glucose homeostasis and appetite regulation by improving glycemic control and delaying gastrointestinal emptying (Schwartz et al., 1988; Wang et al., 2008). Other benefits of these fibers include, for instance the binding of bile acids and the high impact on bacterial composition. Resistant starches, fructans (e.g. inulin, FOS, GOS) and arabinoxylan oligosaccharides are soluble, non-viscous fibers that are easily fermented by the bacterial community (Fuller et al., 2016; Holscher, 2017). The intake of these fibers were demonstrated to strengthen gut barrier function, to improve mucosal immunity, to protect against pathogenic bacteria and to increase SCFA production (Roberfroid, 2007a). The underlying mechanisms include the modulation of SCFA and secondary bile acid production as well as the diminution of microbial components (e.g. LPS). Microbial metabolites are highly involved in energy homeostasis, fat accumulation, inflammatory and insulin signaling, and might also indirectly influence gastrointestinal transit (Delzenne et al., 2015; Gomes et al., 2017; Holscher, 2017; Kootte et al., 2012)

4.1.3. Complex carbohydrates that are prebiotics

Down to the present day, a number of carbohydrates have been demonstrated to display modulating effects, but FOS and GOS dominate the prebiotic field since the late 20th century (Gibson et al., 2017; Rastall and Gibson, 2015).

Inulin-type fructans and Fructooligosaccharides (FOS)

Inulin-type fructans and FOS have been studied extensively *in vivo* and *in vitro* for more than 20 years, but still some essential metabolic mechanisms remain unknown. Inulin is a long chain polysaccharide (between 10 and 65 units) comprising fructose monomers linked by β-(2,1) linkages, which is commonly consumed as a

functional ingredient due to its known modulating effects. In the small intestine, less than 10 % of the linkage bonds of the polymer are hydrolyzed by endogenous enzymes, and therefore becoming almost completely available for the colonic microbiota (Lattimer and Haub, 2010). Inulin is principally metabolized by *Bifidobacterium* populations, which have high expression of the enzymes β-fructanosidase and β-galactosidase (Gibson et al., 2017; Rastall, 2010; Roberfroid et al., 2010; Sawicki et al., 2017). However, it should be noted that not all *Bifidobacterium* strains possess the same capability to degrade this polymer (Huebner et al., 2007). It became evident that the modulating effect of inulin is not limited to bifidobacteria, but additionally stimulates other bacterial population within the intestinal community, like butyrate-producing bacteria (Ramirez-Farias et al., 2009; Scott et al., 2014). Bacteria belonging to *Bacteroides, Lactobacillus, Roseburia* and *Enterobacteriaceae* are able to metabolize inulin, when supplemented to a complex human intestinal microbiota (Falony et al., 2006; Goh and O'Morain, 2003; Van der Meulen et al., 2006b). FOS is a short-chain oligomer (between 3 and 5 units) consisting of nystose, kestose and 1-β-fructofuranosyl nystose monomers, and which are bound by β-(2,1) linkages (Ganaie et al., 2014). Similarly than inulin, FOS reaches the colon and is preferentially metabolized by bifidobacteria and lactobacilli (Gibson et al., 2017; Sawicki et al., 2017). FOS has a lower DP and is recovered through sucrose transgylcosylation by β-fructofuranosidases or by partial hydrolysis of the long chained inulin (Banuelos et al., 2008). FOS is generally fermented within 4 hours, while agave inulin (highly branched) begins to be fermented after 4 hours and has its peak after 6 hours, and chicory inulin, long chain linear fructan has a peak after 8 hours (Brighenti et al., 2006).

Galactooligosaccharides (GOS)

GOS are short-chain carbohydrates that mainly comprise two to six residues of galactose units, which are obtained from lactose through the process of β-galactosidases by hydrolyzing lactose into glucose and galactose (Davis et al., 2011; Hernandez-Hernandez et al., 2011b). Depending on the enzyme, reaction conditions and substrate amount, different hydrolysis rates and transgalactosylation reactions occur and ending in distinct glycosidic linkages (Cardelle-Cobas et al., 2008a; Cardelle-Cobas et al., 2008b). However, they also occur naturally in the milk of humans or cows (Sangwan et al., 2011). The oligosaccharides are readily metabolized by gut commensals belonging to *Lactobacillus, Enterococcus* and *Bifidobacterium*, as well as other bacteria within the complex gut microbial community (Cardelle-Cobas et al., 2011; Davis et al., 2011; Sawicki et al., 2017; Scott et al., 2014). Even though it remains still not fully understood how bifidobacteria exert exactly their positive effects, it is known that these bacteria are able to enhance epithelial barrier activity and consequently decrease inflammatory antigens (Ohland and Macnaughton, 2010). Especially β-GOS was demonstrated to have significant lacto-bifidogenic effects *in vitro*, in animal and human studies, and being a suitable prebiotic compound to improve gastrointestinal structure, immunity and health (Bruno-Barcena and Azcarate-Peril, 2015; Maathuis et al., 2012). B-GOS specifically stimulates growth of two bifidobacterial strains, namely *B. bifidum* and *B. longum*, which have strong immunomodulatory characteristics (de Vrese et al., 2006;

Hasle et al., 2017; Schmidt et al., 2015). A second type, called α-GOS, derives from legumes and prebiotic activity has been demonstrated in humans, *in vivo* and *in vitro* (Bang et al., 2007; Hernandez-Hernandez et al., 2011a; Morel et al., 2015). Food supplemented with α-GOS was linked with a decrease in inflammatory markers (LPS and CRP). Further, GOS supplementations decrease the adhesion potential of pathogenic bacteria such as *Salmonella enterica* to host receptors, thus protecting the host from eventual illness (Searle et al., 2010).

Xylooligosaccharides (XOS)

The oligosaccharide has generally a polymerization degree of 2 or 7 xylose units linked with β-(1,4)-bonds. Different publications using *in vivo* (human and animal) or different *in vitro* approaches, studied the metabolism and effect of XOS on intestinal microbiota, observed that bifidobacteria are stimulated and preferentially ferment XOS (Childs et al., 2014; Jain et al., 2015; Makelainen et al., 2010). In humans, the administration of up to 5 g XOS per day did not only increase bifidobacteria counts in feces, but also induced total SCFA concentrations, reduced pH, enzyme activity and proteolytic metabolites (Lecerf et al., 2012). Next, it was shown that the consumption of the prebiotic XOS increases resistance against pathogenic bacteria like *Listeria monocytogenes* in animal models, and subsequent *in vitro* investigations revealed that XOS reduces adherence of pathogens to the epithelial layer in the intestine (Ebersbach et al., 2012; Ebersbach et al., 2010). Further, XOS is a valuable prebiotic ingredient, since it has the potency of reducing the cholesterol level or promoting the availability of calcium (Aachary and Prapulla, 2009; Sheu et al., 2008; Yang et al., 2015). Moreover, the carbohydrate manifests other important biological properties, such as antioxidant, antimicrobial, anti-inflammatory or immunoregulatory properties (Akpinar et al., 2009; Moure et al., 2006).

β-glucan

Cereal β-glucan, one of the most meaningful functional ingredient, is a long linear polysaccharide comprising glucose monomers, which are linked with β-(1.4), β-(1.6) and β-(1,3) linkages. This component is found in the endosperm of cereal grains, like oats and barley. The concentration of β-glucan in cereal fluctuates between 50 and 110 g/kg in barley and between 30 to 70 g/kg in oat (Bellato et al., 2011; Lam and Cheung, 2013; Skendi et al., 2003). The importance of β-glucan as a prebiotic ingredient has been indicated by numerous studies, demonstrating health promoting bioactive properties in hypercholesterolemia, insulin response or glycemia (Cai et al., 2012; Shen et al., 2011; Zhang et al., 2012). Moreover, β-glucan originating from cereals displays potential immunomodulating properties and antineoplastic (inhibition of a neoplasm) (Wood, 2007). The dietary fiber increases the response of the immune cells belonging to the innate and adaptive immunity by inducing and promoting functionality of macrophages, white cells and neutrophils (Vetvicka et al., 2007; Zekovic et al., 2005). *In vivo* experiments with β-glucan showed that upon pathogen challenge, the animals

Table 1.1: Structural characteristics of major prebiotics and their potential mechanisms of action. Adapted from Hughes et al. (2008), Carlson et al. (2017), Jain et al. (2015), Markowiak and Slizewska (2017), Vieira et al. (2013) and Gibson (2010).

Prebiotic/ Dietary fiber	Composition & structure	Mechanisms of action	Effects on gut microbiota	
Inulin	DP between 10 and 60 β-(2,1) linkages fructose monomers	Enhancement of immune response Decrease of endoscopic and histological inflammation Decrease in colonic pH	Bifidobacteria ↗ *Bacteroides* spp.↗ or ↘ *Lactobacillus* spp.↗ *Roseburia* spp.↗ *Enterococcus* spp. ↘ *Enterobacteriaceae* ↘ or ↗	Carlson et al., 2017; Lattimer and Haub, 2010; Gibson et al., 2017; Rastall, 2010; Sawicki et al., 2017; Roberfroid et al., 2010; Huebner et al., 2007; Ramirez-Farias t al., 2009 Scott et al., 2014; Falony et al., 2006
Fructo-oligosaccharide (FOS)	DP between 3 and 5 β-(2,1) linkages fructose monomers	Reduction in lipid accumulation Decrease in colonic pH Anti-inflammatory Local induction of ROS Reduction of body weight	Total anaerobes↗ Bifidobacteria↗ *Lactobacillus* spp.↗ *Enterobacteriaceae* ↗	Ganaie et al., 2014; Gibson et al., 2017; Sawicki et al., 2017; Banuelos et al., 2008
Galactooligosaccharide (GOS)	DP between 2 and 6 α-glucose-(1,4)-β-galactose	Promotion of immune responses Inhibition of intestinal bacterial overgrowth Enhancement of epithelial barrier Decrease of inflammatory markers Decrease of pathogen adhesion	Bifidobacteria↗ *Faecalibacterium* spp. ↗ *Lactobacillus* spp.↗ *Enterococcus* spp. ↗	Davis et al., 2011; Hernandez-Hernandez et al., 2011; Cardelle-Cobas et al., 2011; Sawicki et et al., 2017; Scott et al., 2014; Azcarate-Peril, 2015; Ohland and Macnaughton, 2010; Maathuis et al., 2012; de Vrese et al., 2006; Bang et al., 2007; Hasle et al, 2017; Morel et al., 2015; Schmidt et al
Xylooligosaccharide (XOS)	DP between 2 and 7 β-(1,4)-bonds xylose monomers	Immunostimulatory effects Anti-inflammation modulator Anticancer poperties Antioxidant properties Antimicrobial activity against pathogens	Bifidobacteria↗	Childs et al., 2014; Jain et al., 2015; Makelainen et al., 2010; Ebersbach et al., 2012; Ebersbach et al, 2010; Aachary and Prapulla et al., 2009; Yang et al., 2015; 2006
β-glucan	fiber β-(1.4) and β-(1,3) linkages glucose monomers	Immunomodulating properties Antineoplastic Promotion of immune responses Induction of functionality of immune cells Reduction of cholesterol Antioxidant properties	Bifidobacteria↗ *Lactobacillus* spp.↗ *Enterobacteriaceae* ↘ *Clostridium* spp.↗ Bacteroides spp.↗ Prevotella spp.↗	Bellato et al., 2011; Lam and Cheung, 2013; Skendi et al., 2003; Cai et al., 2012; Shen et al., 2011; Zhang et al., 2012; Wood, 2007; Vetvicka et al., 2007; Zekovic et al., 2005; Metzler-Zebeli et al., 2011; Mitsou et al., 2010; Nordlund et al., 2012; Kim and White, 2009 and 2010

displayed an enhanced immune response and faster bacterial clearance, thus reducing mortality of infected animals (Hetland et al., 2000; Saegusa et al., 2004). Beta-glucans can modulate directly or indirectly the gut bacterial community (Arena et al., 2016). *In vitro* and *in vivo* studies have shown that cereal β-glucan is metabolized by bacteria, and selectively promotes growth and functionality of a subset of microbial populations, namely *Bifidobacterium* and *Lactobacillus*, by inducing an increase in short-chain fatty acids. Most authors suggested that the viscosity of the fiber within the GIT (the gelation property) is the driving mechanism for the reduction of cholesterol and the promotion of the post prandial glucose metabolism (Metzler-Zebeli et al., 2011; Mitsou et al., 2010; Nordlund et al., 2012; Vasiljevic et al., 2007). Metabolism of β-glucan has been demonstrated to increase propionate productions upon supplementation in humans, a metabolite known to decrease cholesterol synthesis (Kim and White, 2009; Kim and White, 2010).

Arabinoxylan

Arabinoxylan is a component of hemicelluloses that contains a xylose backbone (β-1,4 linkages) with side chains made of arabinose. The fiber is the principal constituent in the cell wall of the endosperm and aleurone layer of whole grains (Fincher and Stone, 1986; Maes and Delcour, 2002). In the GIT, the fiber is highly viscous and is rapidly metabolized by the enteric bacterial community (Lattimer and Haub, 2010). Up to date, the prebiotic potential of high-molecular-weight arabinoxylans was extensively studied, while only sparse data exists for arabinoxylan oligosaccharides (Grootaert et al., 2009). Fermentation of arabinoxylan by the human microbiota induces butyrate and propionate productions, which have protective properties against colon cancer and hypercholesterolemia (Backhed et al., 2004; Grasten et al., 2003). When adults with type 2 diabetes consume around 15 g per day of arabinoxylan, they have lower blood glucose and insulin, thus an improved glucose tolerance. The exact mechanism how the fiber induces this state remains unknown, but it is assumed that the high viscosity inside the gut lumen reduces the proportion of how much glucose is absorbed. Another explanation is the low glycemic index of the fiber (Lu et al., 2004).

Cellulose

Cellulose is the most abundant polysaccharide on earth and prevalent in cell walls of green plants. The polymer is a linear chain, consisting of β-(1,4) linked glucose monomers. In humans, the polysaccharide is insoluble, but it can be fermented to some extend through anaerobic bacterial fermentation and induce high SCFA generation. Cellulose occurring in nature can be separated into two groups: amorphous and crystalline. The crystalline version includes non-covalent hydrogen bonds, what makes it insoluble in water. In order to use cellulose in food production, celluloses are modified by changing the degree of crystallization and hydrogen bonding. Cellulose becomes water-soluble as soon as these linkages are interrupted and crystallization is lost (Kim and Sungryul, 2006; Lattimer and Haub, 2010). The main bacterial populations that can degrade cellulose belong to Bacteroidetes or Firmicutes. However, the type of cellulose is critical for the rate of fermentation

(Slavin et al., 1981). Bacterial strains that can degrade cellulose include *Ruminococcus* spp., *Clostridium* spp., *Eubacterium* spp. and *Bacteroides* spp., but it has been indicated that the activity of these bacteria fluctuates depending on the methanogenic status of the community (Chassard et al., 2010; Flint et al., 2012a).

Pectin

Pectins are polysaccharides that are present in the cell walls of plants. Even though the fiber's structure is highly heterogeneous, depending on its origin and plant, it has a principal structural motif. It is a linear polysaccharide made of uronic acid residues connected with α-(1,4) linkages. This backbone can further be substituted by α-(1,2) linked rhamnose monomers. Moreover, side chains with different sugars, such as arabinose, glucose, xylose, mannose, fucose and galactose can be attached to the main backbone. Pectin is water soluble and is readily metabolized by the colonic bacterial community. The polymer can be found in citrus fruits at a concentration between 0.5 % and 3.5 %, with highest prevalence in the peel. Pectin has the property of forming a gel or thickening the chyme inside the GIT, and thus resulting in numerous health benefits, such as ameliorating lipid and cholesterol metabolism or for diabetes prevention (Lattimer and Haub, 2010; Muller-Maatsch et al., 2014). In human pediatric studies, it was observed that children that obtained pectin supplementations had a reduced diarrhea and less acute infections in the gut. An explanation might be that growth of pathogens, like Salmonella, *Shigella, Enterobacter, Klebsiella, Proteus* or *Citrobacter* is inhibited (Triplehorn and Millard, 2002). *In vitro*, pectin supplementation promotes growth of beneficial bacterial strains of *Bifidobacterium* and *Lactobacillus* (Olano-Martin et al., 2002). Further, the resulting stimulated acetate productions is thought to promote fibrin permeability and reduce fibrin tensile strength, thus decreasing coronary heart disease (Veldman et al., 1999).

4.2. Probiotics

A probiotic is defined as a «live microorganism that, when administered in adequate amounts, confer a health benefit on the host». This description includes a wide range of bacteria and usage, but discriminates live microbes applied as starter cultures from those originating of mixtures that are administered initially for their health benefit (Sanders, 2009). Even though probiotic strains often derive from the commensal gut community, they first need to be isolated, characterized and proven to exert health benefits prior being called a probiotic stain (Hill et al., 2014).

In literature, a number of probiotic bacterial strains have been described to confer a health benefit to the host, when administered at an adequate dose as a food supplement or as food. In Canada, for instance, a probiotic requires the intake of at least 10^9 colony forming units per portion of a *Bifidobacterium* (*adolescentis, animalis, bifidum, breve* and *longum*) and *Lactobacillus* (*acidophilus, casei, fermentum, gasseri, johnsonii, paracasei, plantarum, rhamnosus* and *salivarius*) strain (Health Canada, 2009). The support of the healthy

GIT and immune system ranges from the prevention of allergies to the inhibition of inflammation, which nevertheless remains strain-specific (Maidens et al., 2013). Other host benefits include the homeostasis and health of the oral cavity, the reproductive tract, lungs, gut-brain axis and skin (Hill et al., 2014).

5. Models in gut microbiota research

5.1. *In vitro* gut fermentation modeling

Animal models are suitable models for investigating host-microbe interactions due to their physiologic relevance, but they do not allow studying the bacterial processes occurring within the intestinal microbiome (Venema and van den Abbeele, 2013). The exploration of the complex microbe-microbe interactions upon for instance dietary supplementation are crucial to evaluate the functional potential of a bacterial community. *In vitro* intestinal fermentation models are powerful technological platforms that allow culturing the bacterial composition in spatial, environmental and temporal characteristics typical of the intestinal part of interest, even though the models lack physiological host features (immune system or water reabsorption) (McDonald et al., 2013; Payne et al., 2012; Venema and van den Abbeele, 2013). The fact that these models are host-free is often seen as a disadvantage, but the minimal approach allows to understand the dynamisms of the gut microbiota (Macfarlane and Macfarlane, 2007; Payne et al., 2012). The key advantage of *in vitro* models is their ability to culture a stable bacterial community under strictly controlled conditions and the ability of continuous sampling during fermentation time, which allows the close investigation of the microbial fermentation process. Another advantage is that there are no strict ethical considerations for the use of these models, so that *in vitro* modelling is optimal for investigating the effects of pathogens, lethal or radioactive substances on the gut microbiome (McDonald et al., 2013; Payne et al., 2012).

In vitro gut fermentation models generally comprise a single or multiple chemostats, inoculated with fecal microbiota (or a defined bacterial community), which are operated under anaerobic conditions, a nutritive growth medium mimicking the ileum chyme and constant physiologic temperature and pH. Batch fermentation models are closed systems (sealed reactors or flasks) that promote growth of a bacterial suspension without adding more nutrients under anaerobic conditions (Payne et al., 2012). Batch cultures are generally used to investigate SCFA production arising from active anaerobic bacterial fermentation of potential prebiotic dietary components, such as inulin-type fructans (Pompei et al., 2008). This model is generally easy to set up and inexpensive, and allows to test numerous substrates in different fecal microbiota in a short time (Macfarlane and Macfarlane, 2007; McDonald et al., 2013). A continuously change of the redox potential and pH occurs during bacterial breakdown of carbohydrates in the flasks, which have no pH control. This system can be operated only for a very limited time (several hours to 72 h), since changes in pH and redox potential and the substrate limitation can result in a non-typical bacterial composition, and further the batch cultures cannot establish a steady state. Other disadvantages are for instance that bacterial growth is highly dependent on the inoculation density and substrate consumption rate or the accumulation of toxic products and metabolites which might inhibit microbial growth (Payne et al., 2012).

In order to study the complex modulation of the bacterial community and functionality, the *in vitro* system needs continuous substrate supply to extend operation time and to reach steady-state conditions. In continuous fermentation models, growth of the intestinal microbiota in the reactors rely on additional fine-tuned experimental parameters, e.g physiologic retention time. The strict control of the operational parameters of *in vitro* models provides a robust technique for high reproducibility of a stable bacterial community (Payne et al., 2012; Venema and van den Abbeele, 2013). By connecting multiple chemostats, the complexity of the model increases, establishing a set-up with the capacity for simulating only one intestinal region or the entire colon. The development of a model that allows culturing a stable and reproducible microbiota in one or multiple vessels, is optimal for the evaluation of the effects of multiple compounds on the bacterial composition and functionality (Le Blay et al., 2009; Payne et al., 2012; Zihler Berner et al., 2013). Further, this system can be used to assess microbial mechanisms, such as the breakdown of protein and carbohydrates, the metabolism of steroids or bile acids or removal/production of hydrogen and mutagens (Macfarlane and Macfarlane, 2007). A major problem is the use of liquid fecal suspension for inoculating the model, resulting in an exclusively free-cell state of the bacterial populations and a fast washout of less competitive bacteria, thus limiting the operational time to less than 4 weeks (Cinquin et al., 2006; Macfarlane et al., 1998; Payne et al., 2012). Furthermore, these models cannot mimic the biofilm-associated microbes (sessile) living in the mucus layer (Van den Abbeele et al., 2011). To circumvent the problem of washout and the sessility, a method to immobilize the fecal microbiota was developed (Cinquin et al., 2004). The fecal microbiota is immobilized in a porous polysaccharide matrix, and fecal beads are transferred into a chemostat. This technique allows to circumvent the washout, to maintain high-cell densities and to protect the cells from shear forces of the strirrer (Cinquin et al., 2004, 2006; Doleyres et al., 2002). The development of the PolyFermS further bypassed the problems of reproducibility and biological replication, by permitting the investigation in parallel of different treatments on the same microbiota. This system using immobilized cells has been demonstrated to be run previously up to 90 days (Fehlbaum et al., 2015; Zihler Berner et al., 2013).

5.2. Studying the gut microbiota *in vivo*

Because of the complexity of the gut microbiota, single culture experiments of key bacterial populations supply only limited facts about their function within the community (McDonald et al., 2013). The gut microbiota can be directly studied, with the advantage of being derived from a biological host. Generally, the composition of the mammalian gut microbiota is influenced by numerous non-environmental and environmental factors, leading to high inter-individual variations and thus different results between control and treatment groups (Dave et al., 2012; Dethlefsen and Relman, 2011; Macfarlane and Macfarlane, 2007). Ethical restrictions generally limit for instance human studies to the examination of fecal samples, since invasive medical methods to access the intestine are prohibited. Consequently, standard investigations implicating humans require strict

research ethics approvals that limits experiments of unknown and unsafe materials. Other disadvantages are the amount of time, the expenses of human studies and the need of specialized equipment can restrict the study (Macfarlane and Macfarlane, 2007; Payne et al., 2012). The strict ethical limitations have driven researchers to find alternatives and to develop several animal models (Antonopoulos et al., 2009).

Laboratory mice are primary *in vivo* models in gut microbiota research to study functionality and role in health and diseases, mainly due to their high similarity in anatomy and physiology to humans (Clavel et al., 2016; Nguyen et al., 2015). Around ninety-nine percent of the murine genome is shared with humans, and key bacterial populations in both gut microbiomes are similar, making them powerful tools (Clavel et al., 2016; Nguyen et al., 2015; Spor et al., 2011). Other advantages are the possibility of experimenting under controlled environments, their small size, the low maintenance cost, the high reproduction rate, the short life cycles and the availability of numerous inbred strains (Andersen and Winter, 2017; Nguyen et al., 2015; Perlman, 2016; Uhl and Warner, 2015; Vandamme, 2014). Until today, an abundant collection of genetically-engineered mouse strains and models exist to assess host-microbe interactions (Eppig et al., 2015). It is then possible to study the functionality of the microbiota at different conditions (e.g. healthy vs. diseased or upon supplementation of a compound) and sacrifice the mice, which allows the access to contents of the different parts of the GIT, where SCFA are produced (Payne et al., 2012). Gnotobiotic mouse models include germ-free mice or mice with a defined bacterial community and are important models to study the role of the microbiota in health and disease. Generally, germ-free mice are colonized with a defined microbial community or consortia of microbes from a host. The use of these experimental models gave first prominent insights into the functionality of the gut microbiota in extra- and intra-intestinal immune-related diseases (Rooks and Garrett, 2016). Another example are Human microbiota-associated (HMA) mice are models that received an intestinal microbiota from a human donor, and allows to engraft very well this donor microbiota (Arrieta et al., 2016; Turnbaugh et al., 2009b).

5.2.1. Mouse models for IBD research

Until today, various experimental animal models for intestinal inflammation have helped to elucidate the molecular and cellular pathophysiology of IBD. Especially experiments with murine models helped to investigate the causality of an altered microbiome and pathological mechanisms in the development of IBD (Nguyen et al., 2015). The greater part of these models originate from chemical, immunological or gene manipulation, and for that reason do not completely simulate the multifactorial nature of human colitis (Kiesler et al., 2015). Five major classifications of murine IBD models exist (Mizoguchi, 2012) (**Figure 1.8**). Mouse models in which colitis is induced by noxious chemicals (dextran sodium sulfate (DSS), trinitrobenzene sulfate (TNBS), acetic acid oroxazolone) are the most commonly used in IBD research, because they are inexpensive, accessible and easy to handle. A disadvantage of these mouse models is that the inflammation is not fully representative of the immunohistopathology seen in IBD patients (Cominelli et al., 2017). Nevertheless, chemically induced models are important tools to investigate mechanisms of tissue injury and repair during acute colitis. In combination with genetically engineered mouse models, mouse models enable to elucidate the role of specific risk genes in the onset and development of colitis (Cominelli et al., 2017). In order to study the role of lymphocytes in IBD, researchers transfer immune cells in recipient immunodeficient
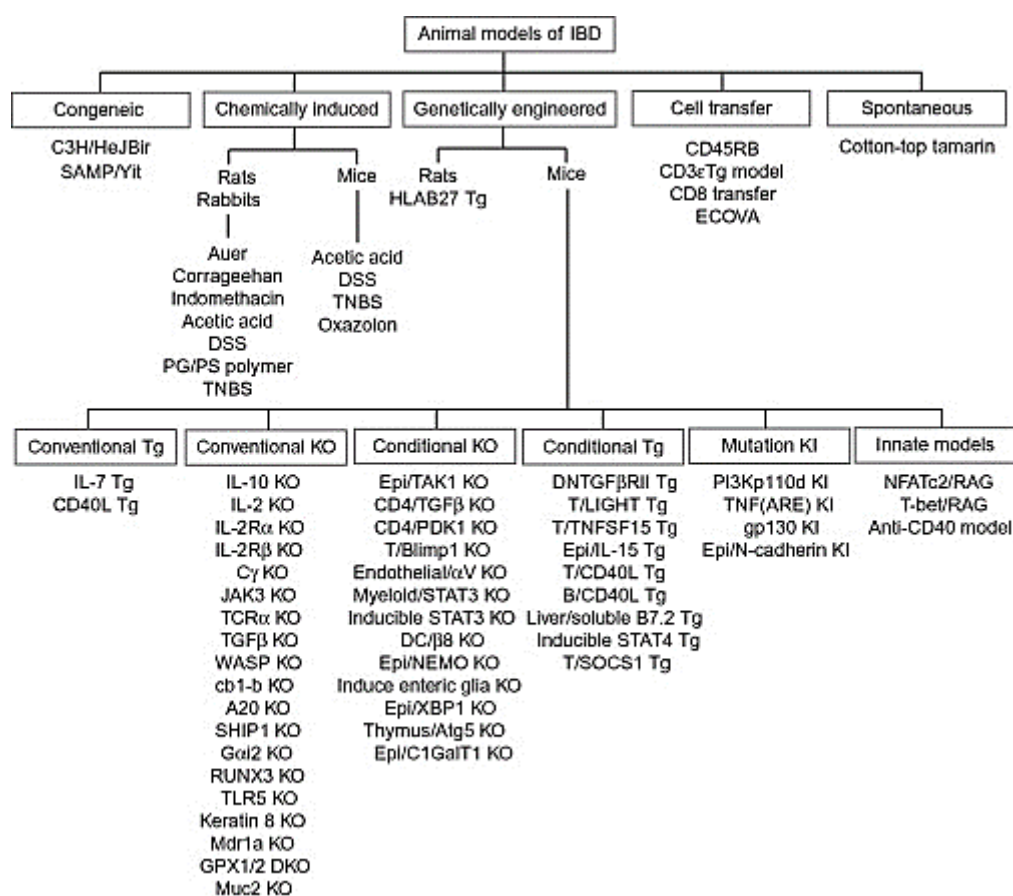
Figure 1.8: Classification of different mouse models of IBD research. Mizoguchi (2012)

mice, which are not producing B or T cells. These animal models are important to understand the role of specific immune cells in the disease. Bone marrow chimeric models are based on the irradiation of mice, which is followed by transfer procedures in order to localize compartments involved in the disease. These models are useful tools to elucidate the role of a specific gene and its product in the experimental colitis (Cominelli et al., 2017).

5.2.2. Challenges encountered with mouse models in microbiome research

Even though mice have many advantages, numerous significant differences exist between mice and humans (e.g. immune system, dietary habits, environment or gut physiology) that need to be considered when interpreting data (**Figure 1.9**). Germ-free mice, for instance, display a different metabolic phenotype, with the habit to excrete more lipids, consume more calories and weigh less than the conventional models, demonstrating that the colonization of microbes leads to an increased efficiency of energy extraction from diet (Rosenbaum et al., 2015; Wostmann et al., 1983). Further, the animals have severe immune defects and increased susceptibility to infections, mostly in the gut due to the underdevelopment of gut-associated lymphoid tissue, transformed crypt morphology and decreased mucus denseness (Deplancke and Gaskins, 2001; Rooks and Garrett, 2016). At cellular level, decreased levels of the mucosal antibody IgA can be observed, which influence the barrier integrity (Pabst, 2012). Since HMA-mice derive from germ-free mice, this model has also numerous biological and technical disadvantages: the initial missing microbiota impairs the normal maturation of the gut physiology and the host immune system (Chung et al., 2012; Tomas et al.,
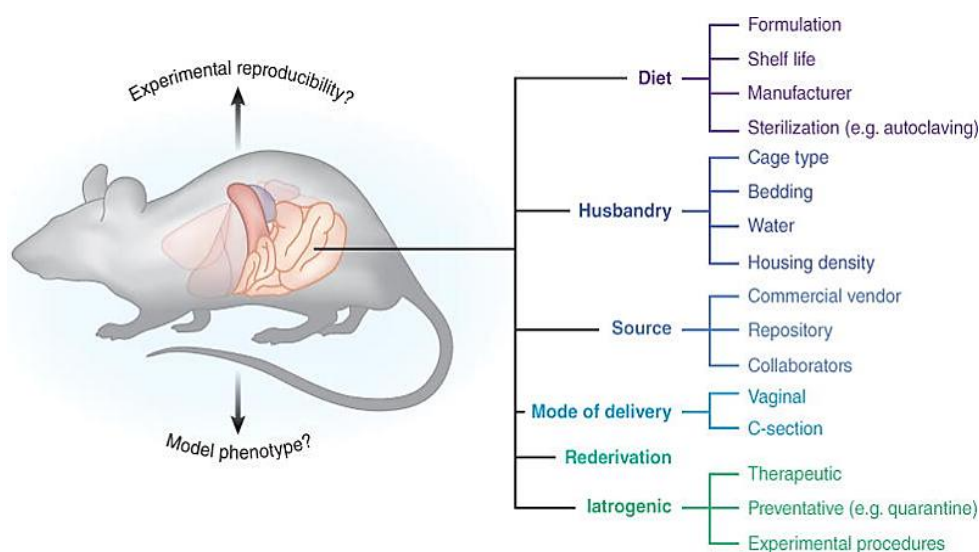
Figure 1.9: Different factors that are known to significantly affect the murine gut microbiota. (Franklin and Ericsson, 2017).

2013). In order to avoid these initial problems, other models have been established using conventional mice, in which the resident intestinal microbiota got depleted with antibiotics and inoculated with a human microbiota (Hintze et al., 2014; Staley et al., 2017). The most recent model is also based on conventional mice, that got humanized by fecal microbiota transplantation without preceding antibiotic intervention. This group used polyethylene glycol to clean the intestine before transplanting a human microbiota (Wrzosek et al., 2018). Further and as previously discussed in **section 2.4**, the gut microbiota in a mammal is influenced by factors like genotype, diet, drugs and maternal microbiota imprint. Latterly, more and more reviews started to report issues of reproducibility of results with murine models (Perrin, 2014). Husbandry-linked factors, for example the transfer of mice from one facility to another within the same building or the different methods of water decontamination can induce a small and temporal shift in the gut microbiota (Ma et al., 2012; Sasada et al., 2015; Sofi et al., 2014). Another determinant of variability is the label and composition of the diet. Recently, a label of one commercially available irradiated rodent chow extracted numerous bacterial spores from a commercially purchasable rodent chows, indicating that non-irradiated chow might be an early source of fungal and bacterial contamination (Franklin and Ericsson, 2017). The purchase of mice from different commercial vendors has been stated to be another source of early variation. Interestingly, mice purchased from most vendors comprise segmented filamentous bacteria, which is most likely acquired from contaminated food, which are not present in mice from the largest supplier of genetically engineered mice, The Jackson Laboratory (Ericsson et al., 2015; Hufeldt et al., 2010). Similar issues are observed in IBD research. Since not a single murine model is able to reproduce the exact pathophysiology of human IBD, each model provides important information of one aspect of the entire diseased condition. For example, the effectiveness of the most commonly used colitis model (DSS-induced colitis) in IBD research is also influenced by different factors, such as the molecular weight and concentration of DSS (generally between 1-5 %), time and repetitions of DSS supplementation, animal strains (some mice strains are more susceptible to chemically induced colitis) and environment (housing conditions or microbial state) (Eichele and Kharbanda, 2017).

6. Objectives of this thesis

The immediate impact of microbiota modulatory factors promoting health (dietary factors) or disease (oxidative stress and antibiotics) on intestinal microbial activity and structure is difficult to assess due to inaccessibility of human colon samples or end-point samples in mouse models. *In vitro* fermentation models mimicking the intestinal conditions offer many advantages, like the possibility of elucidate ecological questions, such as microbe-microbe interactions as a response to a variety of stimuli.

6.1. General objective

Therefore, the general objective of this thesis was to develop and operate *in vitro* models of different organisms to study the modulating effects of potential dietary fibers and the IBD-related environmental factors oxidative stress and antibiotic treatment (**Figure 1.10**).

6.2. Specific objectives

- To stepwise develop an *in vitro* continuous fermentation model of the murine caecal microbiota based on the PolyFermS platform

- To study the prebiotic potential of dietary fibers on human and murine gut microbiota using an *in vitro* continuous fermentation model

- To study *in vitro* the effect of oxidative and antibiotic stress on bacterial composition and metabolism using an *in vitro* continuous fermentation model simulating the murine caecum
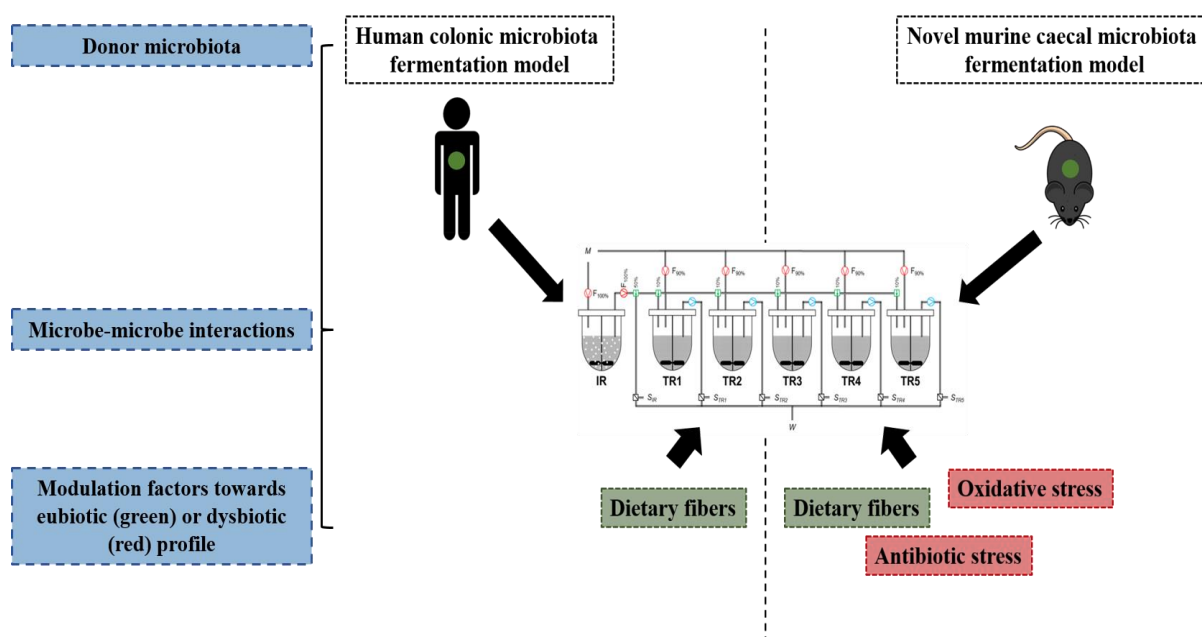
Figure 1.10: Schematic representation of research objectives.

Chapter 2

Understanding the prebiotic potential of different dietary fibers using an *in vitro* continuous adult fermentation model (PolyFermS)

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Abstract

Consumption of fermentable dietary fibers (DFs), which can induce growth and/or activity of specific beneficial populations, is suggested a promising strategy to modulate the gut microbiota and restore health in microbiota-linked diseases. Until today, inulin and fructo-oligosaccharides (FOS) are the best studied DFs, while little is known about the gut microbiota-modulating effects of β-glucan, α-galactooligosaccharide (α-GOS) and xylo-oligosaccharide (XOS). Here, we used three continuous *in vitro* fermentation PolyFermS model to study the modulating effect of these DFs on two distinct human adult proximal colon microbiota, independently from the host. Supplementation of DFs, equivalent to a 9 g daily intake, induced a consistent metabolic response depending on the donor microbiota. Irrespective to the DF supplemented, the *Bacteroidaceae-Ruminococcaceae* dominated microbiota produced more butyrate (up to 96 %), while the *Prevotellaceae-Ruminococcaceae* dominated microbiota produced more propionate (up to 40 %). Changes in abundance of specific bacterial taxa upon DF supplementation explained the observed changes in short-chain fatty acid profiles. Our data suggest that the metabolic profile of SCFA profile may be the most suitable and robust read-out to characterize microbiota-modulating effects of a DF and highlights importance to understand the inter-individual response to a prebiotic treatment for mechanistic understanding and human application.

Introduction

The human gut microbiota is composed of around 10^{14} bacterial cells that belong to more than 1000 species (Qin et al., 2010), dominated by members belonging to the two phyla, Firmicutes and Bacteroidetes (Qin et al., 2010; Turnbaugh et al., 2009a). Diet is known to strongly influence the composition of the gut microbiota as well as metabolites, dominated by the canonical short chain fatty acids (SCFAs) acetate, propionate and butyrate (De Filippo et al., 2010; Tremaroli and Backhed, 2012). Among healthy individuals, compositional and functional properties of the microbiome vary substantially, leading to highly variable responses to dietary interventions (Lampe et al., 2013; Louis et al., 2007; Lozupone et al., 2012; Walker et al., 2011a; Walker and Lawley, 2013). Baseline bacterial composition of the host microbiome has repeatedly been observed to be a key factor to explain responses of the gut microbiota to different dietary interventions (Bindels et al., 2015; Harmon et al., 2009; Korpela et al., 2014). Within the collective genome of several millions genes, the microbiome harbors the capacity of primary degradation of substrates by specialized bacteria, cross-feeding and competition, making the stratification of microbiota response profiles a major challenge in the field (Korpela et al., 2014; Qin et al., 2010).

Around 40 g of complex carbohydrates reach the colon each day after escaping breakdown by host enzymes (Cummings et al., 1992; Duncan et al., 2006; Walker et al., 2011a). Endogenous enzymes are unable to degrade numerous complex carbohydrates and plant polysaccharides (Tremaroli and Backhed, 2012). Non-digestible dietary fibers have been shown to have a beneficial effect on intestinal wellbeing acting as bulking agent and substrates for growth and activity of specific endogenous bacterial populations within the gastrointestinal tract (GIT) (Laparra and Sanz, 2010). The breakdown of the complex carbohydrates by the gut microbiota is a key factor for the stability and diversity of the intestinal ecosystem yielding energy not only for the host, but also for its microbiota. The presence of end metabolites such as the SCFAs acetate, propionate and butyrate, and absence of intermediate metabolite accumulation, such as for lactate, formate and succinate, are generally recognized as markers for a healthy microbiome (Flint et al., 2012b). Metabolism of fibers is occurring in the colon, especially in the proximal colon resulting in an increased production of organic acids and a decrease in luminal pH of 5.5-5.9 (Lawley and Walker, 2013). Acidification but more importantly the production of intermediate and end-metabolites have important consequences for the microbial composition, the establishment of key bacterial interactions and the proper functioning of host physiology (Duncan et al., 2009; Walker et al., 2005). Microbial SCFAs impact on gut health, as energy source for the intestinal epithelium and epigenetic factor influencing immune response, epithelial integrity, electrolytes re-absorption and gut motility. Butyrate is an energy substrate used by colonocytes, while acetate and propionate reach systemic circulation and affect metabolism and function of peripheral organs (e.g. liver, pancreas, brain, muscle) (Bergman, 1990; Morrison and Preston, 2016; Tremaroli and Backhed, 2012). Therefore, fiber modulation of microbiota composition and functions has crystallized as a promising strategy to promote gut and host health.

Dietary fibers (DFs) as main substrate for the gut microbiota are key factors of the microbial network in the gut. In particular inulin and fructo-oligosaccharides (FOS) have repeatedly been shown to selectively modulate the gut microbiota *in vitro* and *in vivo* with benefits for host health ("prebiotic effect") (Flint et al., 2012a; Grootaert et al., 2009; Juskiewicz et al., 2007; Roberfroid, 2007b; Rossi et al., 2005; Van den Abbeele et al., 2011). However, the lack of specific responses of bacterial groups has posed an important challenge in understanding the prebiotic mechanisms of most fibers (Bindels et al., 2015; Gibson et al., 2017). Multiple studies aiming at identification of novel prebiotics, such as β-glucans, galacto-oligosaccharides (GOS) and xylo-oligosaccharides (XOS), have encountered the same challenge of functional redundancy within phylogenetically diverse bacterial groups and limited understanding of the metabolite functions.

In vitro fermentation models are powerful approaches to investigate gut microbiota functionality without host effects in a highly controlled environment (Payne et al., 2012). These models allow the strict control of physiologic parameters, such as retention time, pH, temperature and anaerobiosis, and medium composition used to mimic the diet. Colonic models from simple short-term batch fermentations to multistage long-term continuous flow models were developed (Dostal et al., 2013; Tanner et al., 2014; Zihler Berner et al., 2013). Continuous models further control the medium flow rate, for culturing of microbiota in steady-state conditions, allowing a fiber to develop its full effect along the entire trophic chain, thereby increasing the physiological relevance of the experiment. In particular, continuous fermentation systems with immobilized gut microbiota were shown to simulate the high-cell density, biodiversity and long-term stability of the intestinal microbiota (Zihler Berner et al., 2013). This prevents washout of less competitive bacteria and ensures the repeated exposure of a single microbiota to different fibers (Payne et al., 2012; Tanner et al., 2014; Zihler Berner et al., 2013). The PolyFermS model allows the parallel testing of different treatments on singular microbiota.

The aim of this study was therefore to investigate the effects of novel potential prebiotics on healthy adult gut microbiota using PolyFermS *in vitro* continuous colonic fermentation model mimicking the adult proximal colon microbiota. Immobilization of two distinct fecal microbiota obtained from two healthy adult donors was performed. The two microbiota were propagated and stabilized in an inoculum reactor (IR) seeded with microbiota immobilized in polymer gel beads allowing continuous and prolonged culture of the microbiome (Dostal et al., 2013; Fehlbaum et al., 2015). The model design allowed parallel testing of four different dietary fiber supplementations (β-glucan, XOS, α-GOS and inulin) compared to a control reactor with no supplemented fiber, all inoculated with the same microbiota produced in IR. The effect of dietary fibers derived from plants was tested at a physiological concentration mimicking a daily intake of 9 g fibers in test reactors for 5-7 days to reach pseudo-steady state conditions. Microbiota composition and diversity was monitored with 16S rRNA gene amplicon sequencing, and SCFAs analysis by HPLC.

Material and Methods

Fecal bacteria immobilization

Fecal samples were donated by two healthy individuals (male, age 33 and 32), who did not receive antibiotic or probiotic supplementation for at least 3 months before donation. Fecal samples were collected in a sterile 50 mL Falcon tube in an airtight container together with one Anaerogen sachet (Oxoid) to obtain anaerobic conditions until transfer into an anaerobic chamber (10 % CO_2, 5 % H_2 and 85 % N_2) within 3 h (Coy Laboratories, Ann Arbor, MI, USA). Fecal bacteria were immobilized in 1-2 mm gel beads consisting of gellan gum (2.5%, w/v), xanthan (0.25%, w/v) and sodium citrate (0.2%, w/v) under anaerobic conditions as previously described in detail (Tanner et al., 2014; Zihler Berner et al., 2013). Sixty mL of freshly produced fecal beads were transferred in the IR bioreactor containing 140 mL of nutritive medium. For bead colonization, three consecutive fed-batch fermentations were carried out by replacing 100 mL fresh nutritive medium every 8-12 h. Bacteria, growing close to the bead surface are continuously released into the growth medium due to active cell growth in the high-biomass-density peripheral layer (Cinquin et al., 2004; Payne et al., 2012).

Nutritive medium

The nutritive medium was based on the composition described by Macfarlane *et al.* (1998) for simulation of the chyme in the adult human colon. It included (g L^{-1} of distilled water): pectin (citrus)(2), xylan (oat spelts)(2), arabinogalactan (larch) (2), guar gum (1), inulin (1), soluble potato starch (5), mucine (4), casein acid hydrolysate (3), peptone water (5), tryptone (5), yeast extract (4.5), L-cysteine HCl (0.8), bile salts (0.4), KH_2PO_4 (0.5), $NaHCO_3$ (1.5), NaCl (4.5), KCl (4.5), $MgSO_4$ anhydrated (0.61), $CaCl_2*2\ H_2O$ (0.1), $MnCl_2*4\ H_2O$ (0.2), $FeSO_4*7H_2O$ (0.005), hemin (0.05) and Tween 80 (1 mL). Prior sterilization (20 min, 120°C), the pH of the medium was adjusted to 5.7. One mL of a filter-sterilized (0.2 μm pore-size) vitamin solution (Michel et al. (1998)) was added to the sterilized and cooled down medium. All components of the fermentation medium were purchased from Sigma-Aldrich Chemie (Buchs, Switzerland), except for peptone water (Oxoid AG, Pratteln, Switzerland), inulin (Orafti®, RPN Food-technology AG, Sursee, Switzerland), bile salts (Oxoid AG), tryptone (Becton Dickinson AG, Allschwill, Switzerland), yeast extract (Merck, Darmstadt, Germany), KH_2PO_4 (VWR International AG), $NaHCO_3$ (Fluka, Buchs, Switzerland), NaCl (VWR international AG, Dietikon, Switzerland), KCl (Fluka, Buchs, Switzerland) and KH_2PO_4 (VWR International AG).

Four different dietary fibers (inulin-type fructan, β-glucan, XOS, α-GOS) were investigated (**Supplementary Table S2.1**) and supplemented to sterile nutritive medium at a concentration of 4 g L^{-1}, calculated for an estimated daily intake of 9 g L^{-1}, accounting for the reactor volume of 0.2 L compared to 0.75 L for the proximal colon volume, and a chime medium supply of 0.6 L medium per day, giving a mean retention time of 8 h. Complete hydration of dietary fibers was allowed for 24 h under high speed stirring at 4°C, as presented below.

Simulation of proximal colon microbiota and treatment with dietary fibers

A

B

F 1	Donor 3	Duration [days]	IR	CR	TR1	TR2	TR3	TR4
		12			Initial stabilization			
	Treatment period 1	7				inulin	B-glucan	XOS
		6			Wash-out & stabilization			
	Treatment period 2	7			XOS	α-GOS	inulin	B-glucan

F 2	Donor 4	Duration [days]	IR	CR	TR1	TR2	TR3	TR4
		15			Initial stabilization			
	Treatment period 1	7				B-glucan	α-GOS	XOS
		6			Wash-out & stabilization			
	Treatment period 2	7			XOS	inulin	B-glucan	α-GOS
		7			Wash-out & stabilization			
	Treatment period 3	7					inulin	

F 3	Donor 3	Duration [days]	IR	CR	TR1	TR2	TR3	TR4
		9			Initial stabilization			
	Treatment period	7			inulin	α-GOS		

Figure 2.1: Experimental set-up (A) and time schedule (B) of the continuous fermentation model with inoculum reactor (IR) and control (CR) and treatment (TR) reactors. CR and TR's were fed with effluent from IR and with nutritive medium during stabilization and washout periods. TRs were fed with effluent from IR and supplemented fermentation medium (4 g/L dietary fiber) during treatment periods.

Experimental set-up

The set-up of the PolyFermS model was adapted for the aim of the study and is schematized in **Figure 2.6 A**. The continuous fermentations were carried out for up to 42 days in a two-stage design with a total of six bioreactors (Sixfors, Infors, Bottmingen, Switzerland). Each reactor was operated with conditions selected to mimic the adult proximal colon (pH 5.7, stirring at 120 rpm, 37 °C, and mean retention time of 8 h). Anaerobiosis was maintained through continuous headspace flushing with CO_2, and a constant pH of 5.7 was

maintained by addition of 2.5 M NaOH. IR had an operation volume of 200 mL and was inoculated with 60 mL of fecal beads and connected via a peristaltic pump (Reglo, Ismatec, Glattbrugg, Switzerland) to all second stage reactors, one control reactor (CR) and four treatment reactors (TR1-4) operated in parallel. All reactors were operated at a working volume of 200 mL. Fresh sterile nutritive medium was continuously supplied to IR at a flow rate of 25 mL/h, and second stage reactors were inoculated with 5 % (v/v) (1.25 mL/h) IR effluent and supplied with 95 % (v/v) (23.75 mL/h) fresh fermentation medium.

Experimental procedure

An initial colonization and stabilization phase of up to 15 days, prior experimentation was done (**Figure 2.6 B**). Each treatment was performed for 7 days for reaching a stable state, monitored by metabolite analysis and base consumption. The treatment periods were alternated with re-stabilization phases of 5-7 days, aiming to washout effects of the previous applied treatment and re-establishment of a microbiota composition similar to that in IR. The DFs (**Supplementary Table S2.1**) were added to the medium (4 g/L) and connected to assigned treatment reactor.

Effluent samples were taken daily and separated into bacterial pellet (10 min of centrifugation at 14.000 g at 4 °C) and supernatant, and stored at -20 °C until further analysis. Stability of the reactor microbial communities was monitored by daily measurements of main fermentation metabolites concentrations in sample supernatant. Standard observed variations in the PolyFermS fermentation metabolites are normally lower than 10% and used to define functional microbial stability, before starting and analyzing samples of a treatment period.

Microbial metabolite analysis

High performance liquid chromatography (HPLC) analysis (Thermo Fisher Scientific Inc. Accela, Wohlen, Switzerland) was performed to determine the concentrations of SCFAs (acetate, butyrate and propionate), branched-chain fatty acids (BCFAs) (isobutyrate, valerate and isovalerate) and intermediate metabolites (lactate and formate) produced by the microbiota in the reactor effluents. Analyses were performed with a Hitachi LaChrome device (Merck, Dietikon, Switzerland) using a Cation-H refill cartridge (30 x 4.6 mm) connected to an Aminex® HPX-87H (300 x 7.8 mm) column. Due to biofilm formation, supernatants of IR and TR reactors were diluted in ultrapure water and filtered through a 0.22 or 0.45 µm nylon membrane (Infochroma AG, Zug, Switzerland) into glass vials and sealed with crimp-caps. Around 40 µL of the sample were injected into the HPLC with a flow rate of 0.4 mL min^{-1} and H_2SO_4 as an eluent.

Microbial community analysis

Genomic DNA extraction

The genomic DNA was extracted from 200 mg feces and pellet of 2 mL PolyFermS effluent using the FastDNA® SPIN Kit for Soil (MP Biomedicals, Illkirch, France). Total DNA concentration (ng/µL) and purity was determined by spectrophotometry using Nanodrop (Nanodrop ND 1000 Spectrophotometer, Thermo Scientific, Wilmington, USA).

Microbiota profiling with 16S rRNA gene amplicon sequencing

The bacterial composition of the fecal and PolyFermS samples was determined using tag-encoded 16S rRNA gene Miseq-based (Illumina, CA, USA) high throughput sequencing. DNA samples of the last day of each experimental period of each PolyFermS reactor were selected for assessing the bacterial composition and its stability in the PolyFermS model and the shifts after prebiotic treatments. The V4 region of the 16S rRNA gene was amplified with modified primers 515F (TATGGTAATTGTGTGNCAGCMGCCGCGGTAA) and 806R (AGTCAGTCAGCCGGACTACHVGGGTWTCTAAT). Library preparation and sequencing was performed by StarSEQ (Mainz, Germany) using for sequencing one MiSeq cell and the V2 2 x 250 bp paired end Next Tera chemistry supplemented with 20 % of PhiX.

The raw data set containing pair-ended reads with corresponding quality scores were merged using settings as previously mentioned (Zachariassen et al., 2017). The minimum length of merged reads was 200 bp. Quantitative Insight Into Microbial Ecology (QIIME) open source software package (1.7.0, 1.8.0 and 1.9.0) was used for subsequent analysis steps (Caporaso et al., 2010). Purging the dataset from chimeric reads and constructing de novo Operational Taxonomic Units (OUT) was conducted using the UPARSE pipeline (Edgar, 2013). The custom human intestinal 16S rRNA database (HITdb) was used as a reference database (Ritari et al., 2015). Alpha and beta diversity analysis was performed as previously described using iterative subsampling (36'000 reads/sample) (Krych et al., 2013). The raw sequence data has been submitted to European Nucleotide Archive (ENA) database with accession number PRJEB30418.

Statistical analysis

All statistical analysis for HPLC were carried out using the SigmaPlot 13.0 version, San Jose, California, USA. HPLC Data are expressed as means ± standard deviations (SD) of three days at the end of the stabilization and treatment period of each fermentation. HPLC data were compared between stabilization and treatment phase using the nonparametric Shapiro-Wilk test. *P*-values < 0.05 were considered significant. Brown-Forsythe test was used to determine differences between stabilization and treatment periods.

Results

Donor selection and transfer to PolyFermS

Fecal microbiota of 30 healthy individuals were screened using 16S rRNA gene amplicon sequencing to select two fecal donors with distinct taxonomic profile for dietary fiber supplementation study. Fecal microbiota variation across the donors was stratified into enterotypes *Ruminococcus*, *Bacteroides* and *Prevotella* as decribed by Arumugam et al. (2011) (**Figure 2.1 A**). Two donors, D3 and D4, were selected based on the difference in their dominant microbial taxa. The microbiota composition of both donors was representative for a healthy human microbiota (**Supplementary. Table S2.2**), with *Firmicutes* (D3: 51 %; D4: 57 %) and *Bacteroidetes* (D3: 26 %; D4: 32 %) as the dominant bacterial phyla. Donors differed from each other on family level with D3 fecal microbiota characterized by higher levels of *Bacteroidaceae* (D3: 17 %; D4: 8 %) and presence of *Verrucomicrobiaceae* (D3: 6 %; D4: < 0 %), and archaeal family *Methanobacteriaceae* (D3: 9 %; D4: 2 %). Whereas D4 fecal microbiota was characterized by higher levels of *Prevotellaceae* (D4: 17 %; D3: 0.1 %) and *Lachnospiraceae* (D4: 13 %; D3: 7 %) compared to D3 fecal microbiota (**Supplementary Table S2.2 B**). For assessing the α-diversity, the Shannon index for both fecal microbiota was calculated and both donors had a comparable Shannon index (D3: 5.8 ± 0.9; D4: 5.8 ± 0.9; **Supplementary Table S2.3**).

Fecal microbiota of both donors were studied in the PolyFermS continuous intestinal fermentation model operated with conditions mimicking the proximal colon microbiota. The model is composed of an inoculum reactor (IR), containing immobilized human adult fecal microbiota used to continuously inoculate several second stage control and experimental reactors mounted in parallel. The second stage reactors with the same complete microbiota allow comparing different treatments with a control. During 49 days of continuous operation of IRs inoculated with different donor microbiota, the microbial stability was assessed on phylogenetic and metabolic levels. The Shannon diversity, taking in account both abundance and evenness of species present in a sample, was reduced in the *in vitro* model compared with fecal microbiota (**Supplementary Table S.2.3**). The mean Shannon index of IR and second stage untreated microbiota (CR) was 4.3 ± 0.1 for F1, containing D3 microbiota (Shannon index 5.8 ± 0.9), and 3.6 ± 0.2 for F2, containing D4 microbiota (Shannon index 5.8 ± 0.9). On family level the microbiota composition within each fermentation reflected well its specific donor microbiota (**Supplementary Table S2.4 and Supplementary Table S2.5**) as previously observed in similar PolyFermS setup (Zihler Berner et al., 2013). Within F1 microbiota of IR and untreated control reactor, Firmicutes (56 %) and Bacteroidetes (26 %) were the predominant phyla. In correspondence with the fecal D3 microbiota, F1 microbiota was characterized by high levels of bacterial families *Bacteroidaceae* (IR: 23 ± 5 %; CR: 29 ± 6 %) and *Ruminococcaceae* (IR: 18 ± 3 %; CR: 11 ± 7 %). Whereas F2 microbiota was dominated by Bacteroidetes (58 %) over Firmicutes (36 %) and characterized by high levels of *Prevotellaceae* (IR: 53 ± 6 %; CR: 53 ± 4 %) and *Ruminococcaceae* (IR: 19 ± 4 %; CR: 18 ± 2 %), also in

correspondence with its donor fecal microbiota. Some bacterial families were detected at different abundances *in vitro* compared to the fecal sample. For example, in F1 increased abundances of *Acidaminococcaceae* (12 ± 1 %; 0.3 % in fecal sample) and *Enterobacteriaceae* (15 ± 4 %; undetected in fecal sample) and in F2 *Prevotellaceae* (36 ± 6 %; 17.2 % in fecal sample) and unclassified Lactobacillales (4 ± 1 %; undetectable in fecal sample) were detected (**Supplementary Table S2.2 and Supplementary Table S2.5**). A clear spatial separation and clustering of F1 and F2 microbiota was observed by Principle Coordinate Analysis (PCoA) on weighted UniFrac distance matrix on OTU level, indicating a distinct and stable microbial profile of the two donor microbiota *in vitro* (**Figure 2.1 B**).
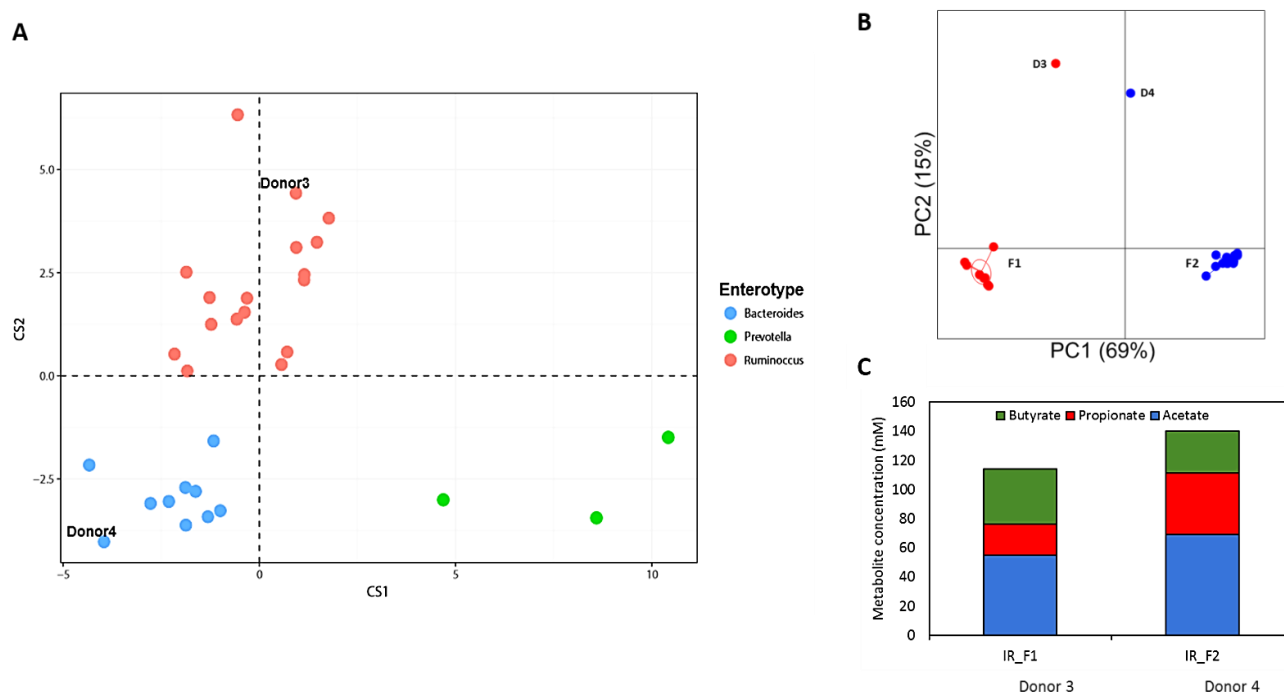
Figure 2.2: (A) Variation in fecal microbiota among the 30 healthy individuals represented in an enterotype plot for 16S rRNA gene amplicon sequence dataset generated as described on http://enterotyping.embl.de/. (B) Principle Coordinate Analysis based on the weighted UniFrac distance matrix on OTU level generated from fecal and non- treated PolyFermS microbiota of fermentation 1 (F1 with donor 3 microbiota) and fermentation 2 (F2 with donor 4 microbiota), showing conservation of the two distinct donor microbiota profiles in PolyFermS model. Microbiota included from inoculum reactor (IR) and control reactor (CR) from F1 on day 12, day 19, day 24 and day 31 and for F2 on day 8, day 14, day 19, day 25, day 31 and day 37. (C) Bar-plot representation of mean acetate (blue), propionate (red) and butyrate (green) concentrations (mM) of PolyFermS effluent samples of IR during stable operation phase and connection with experimental reactors (IR_F1: day 1 – day 36; IR_F2: day 1 –day 42).

Bacterial fermentation activity was monitored by SCFA analysis of fermentation effluents of PolyFermS reactors along the complete fermentation period. After the initial colonization-stabilization period of 12 and 15 days, total SCFA concentrations in IR effluents were stable with 153 (± 9) mM for 20 days in F1 and 123 (± 10) mM for 36 days in F2, respectively. Acetate was the predominant SCFA produced in both fermentations (F1: 55 ± 6 mM; F2: 69 ± 4 mM). Butyrate and propionate levels differed between both fermentations with F1 characterized by higher butyrate levels (F1: 38 ± 4 mM and F2: 29 ± 6 mM) and F2 characterized by higher propionate levels (F1: 21 ± 3 mM and F2: 42 ± 5 mM) (**Figure 2.1 C**). The metabolic profiles of IR over the whole

fermentation periods of 36 and 42 days for F1 and F2, respectively, showed stable concentrations of the main SCFAs (**Supplementary Figure S2.2**).

Dietary fiber supplementation induces different metabolic and microbial responses *in vitro* depending on donor microbiota

The effects of dietary fibers β-glucan, XOS, α-GOS and inulin on metabolic and microbial responses of stable PolyFermS microbiota of F1 and F2 were tested at a concentration of 4 g/L in the fermentation medium, mimicking an estimated daily intake of 9 g/day, for 7 days alternated by re-stabilization phases of 5 days, and two repetitions (noted I and II) were done for dietary fiber application within F1 and F2, except for α-GOS. Supplementation was performed through addition of sterile, non-heated fiber to the complex medium. Metabolic response of both microbiota was assessed by measuring SCFA production at the end of treatment (3 day sampling) and comparing to the production measured during the stabilization period before a fiber treatment is applied. Addition of dietary fibers yielded an overall increase in total SCFA production for both microbiota in F1 and F2 compared to stabilization, with mean increase ranging from 3 to 54 mM (F1) and 16 to 38 mM (F2), indicating fermentation of all supplemented fibers (**Supplementary Figure S2.3**). Acetate production was significantly enhanced for both microbiota upon supplementation with XOS and α-GOS, whereas during β-glucan treatment acetate levels remained stable. Butyrate and propionate productions were also increased by fiber supplementation but with microbiota-dependent response. Butyrate production increased (between 2 and 96 %) in F1 (D3), while propionate production was enhanced (between 3 and 40 %) in F2 (D4) microbiota upon β-glucan, XOS, α-GOS and inulin supplementation (**Figure 2.2 A and B and Supplementary Table S2.6 A**). In addition, inulin also raised butyrate production in F2 (D4). Metabolic interactions within the PolyFermS microbiota upon fiber treatment were observed when inulin was supplemented. This is demonstrated by an increase in butyrate, when acetate levels remained stable or decreased (F1 treatment 2 and F2), but when acetate concentrations increased, butyrate levels remained stable (F1 treatment 1), suggesting cross-feeding of butyrate-producers on acetate. Interestingly we did not observe a consistent change of a single OTU upon fiber supplementation, confirming the current hypothesis of high functional redundancy within the gut microbiota and suggesting a multi-strain response causing observed changes in SCFA production. Principle coordinate analysis (PCoA) on weighted UniFrac distances of the fiber-supplemented PolyFermS microbiota allowed us to identify taxa that stratify the microbiota of F1 and F2 in relation to the dietary fiber supplementation and their abundances (**Figure 2.3 A and B**). Due to the different microbiota of F1 (*Bacteroidaceae-Ruminococcaceae* dominated) and F2 (*Prevotellaceae-Ruminococcaceae* dominated), the response on OTU level upon a dietary fiber was in some cases different. For example, α-GOS supplementation resulted in consistent higher levels compared to stabilization period of *Lachnospiraceae* in F2 microbiota during both treatment periods (I: 6 to 12 % and II: 5 to 10 %; **Supplementary Table S2.7 B**), also reflected by a *Blautia* (6) and *Eubacterium rectale* (25) OTU in the PCoA. Whereas in F1
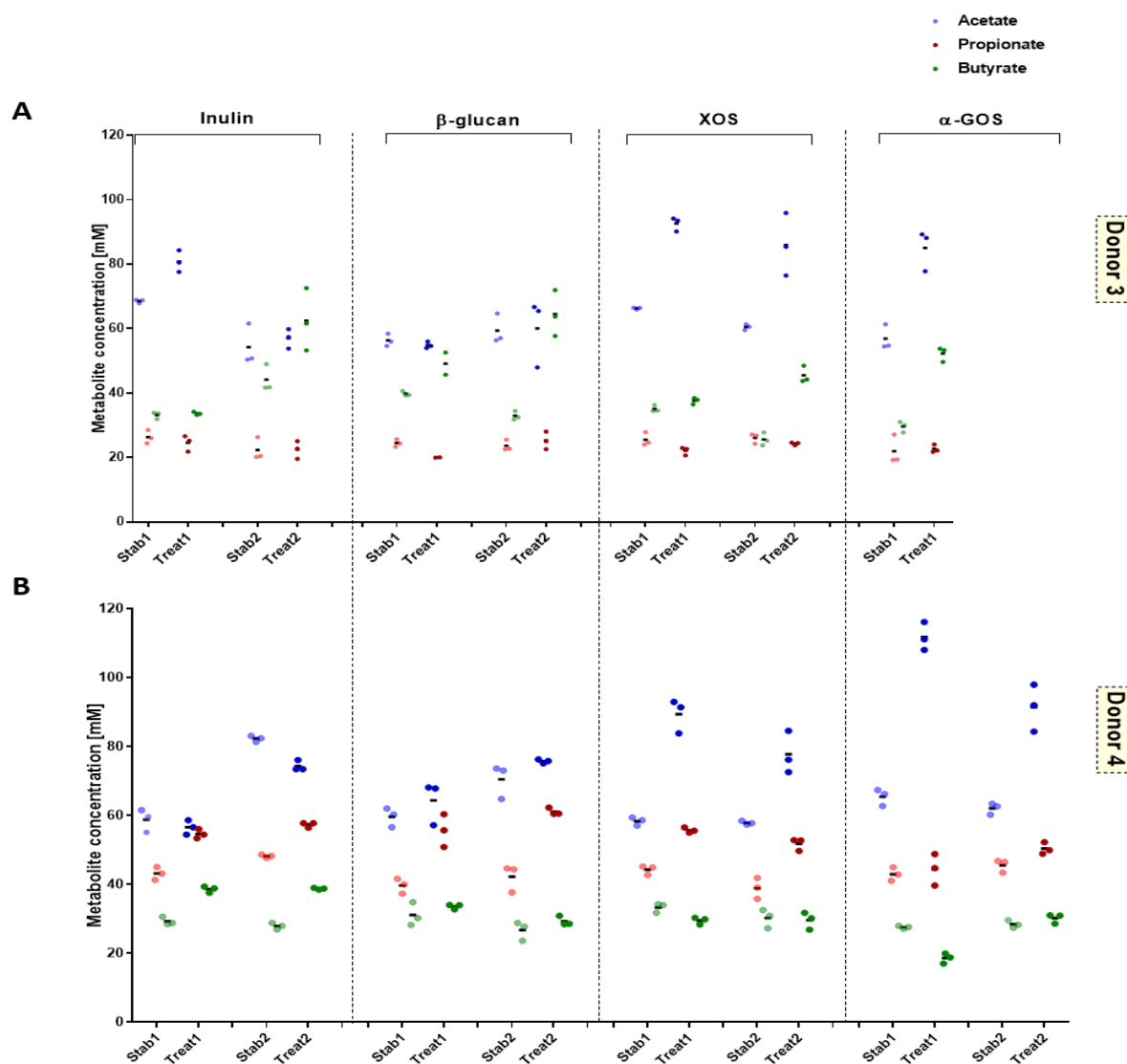
Figure 2.3: Effect of dietary fiber supplementation on fermentation metabolite concentration (A and B) and microbiota of fermentation 1 (F1) (donor 3) and fermentation 2 (F2) (donor 4). Mean (black horizontal line) from 3 consecutive measurements of acetate, propionate and butyrate concentrations (mM) in the respective reactor at end of stabilization and treatment phase for F1 (D3 microbiota) (A) and F2 (D4 microbiota) (B).Two replicates are shown for each dietary fiber treatment.

microbiota *Lachnospiraceae* levels decreased upon α-GOS supplementation (18 to 13 %, **Supplementary Table S2.7 A**). In PCoA, β-glucan supplemented F1 microbiota was determined by *Eubacteriaceae* OTUs (*E. siraeum* (7) and *E. rectale* (6)), in accordance with higher *Eubacteriaceae* levels compared to stabilization periods (I: 10 to 23 % and II: 7 to 20 %; **Supplementary Table S2.7 A**). On the other hand, β-glucan supplemented F2 microbiota was separated in the PCoA by a *Sutterella wadsworthensis* OTU (28). An increased relative abundance of *Prevotellaceae* in both repetitions of β-glucan supplementation was also observed (I: 26 to 61 % and II: 54 to 58 %; **Supplementary Table S2.7 B**) compared to the previous stabilization period. Interestingly, inulin showed consistent changes in relative abundance upon supplementation, independent of the donor microbiota. After inulin supplementation, the relative abundance of *Ruminococcaceae* increased in both

fermentations (F1: 22 to 28 % and F2: I: 13 to 19 % and II: 16 to 22 %; **Supplementary Table S2.7 B**). No consistent changes in relative abundance of main bacterial families could be detected upon XOS supplementation in both microbiota (**Supplementary Table 2.7**). However, in the PCoA the XOS supplemented microbiota of F2 were both determined by an *E. rectale* OTU (10 and 25; **Figure 2.3 B**), which indicates a higher abundance of *E. rectale* in XOS supplemented F2 microbiota.
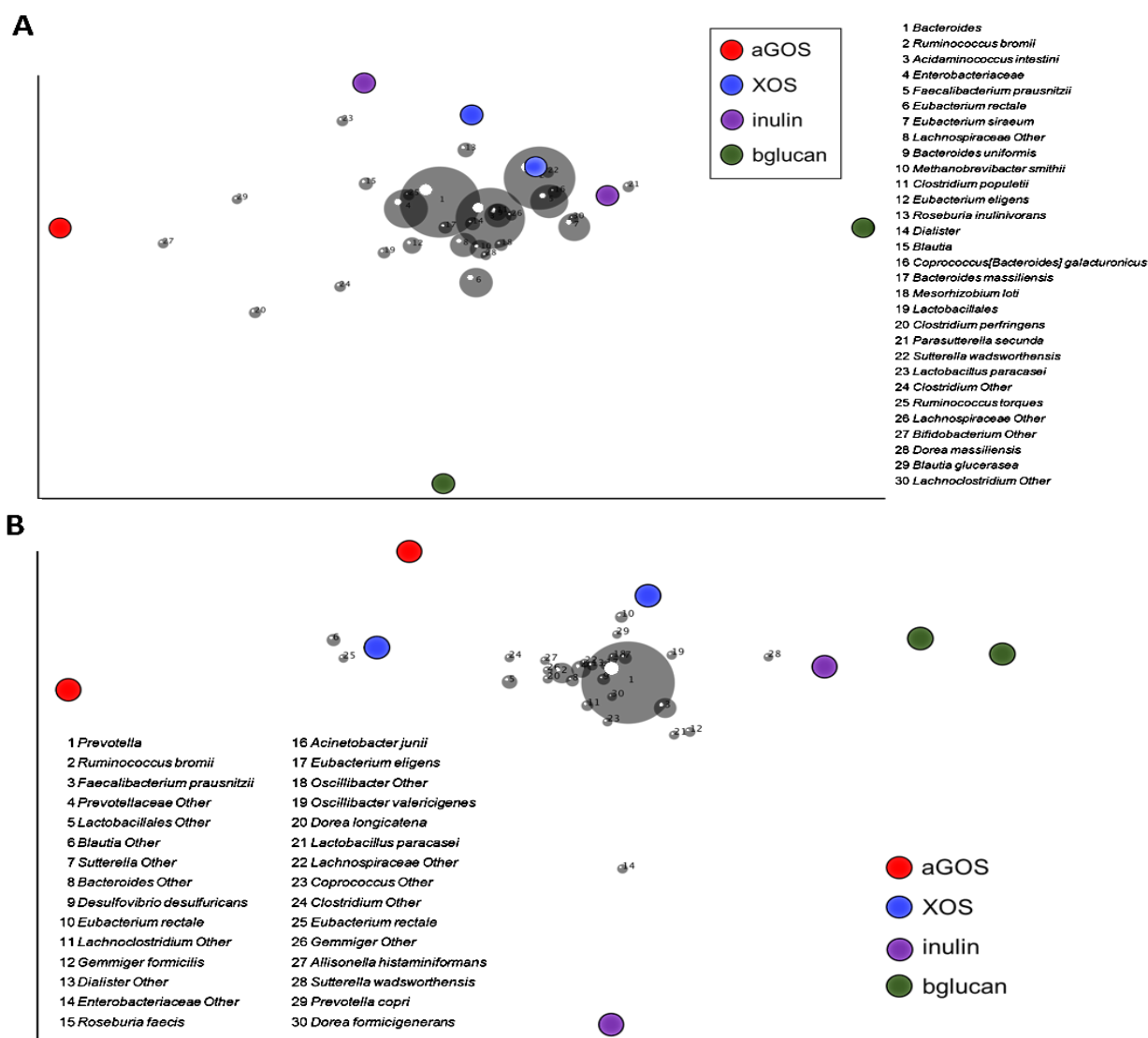
Figure 2.3: Effect of dietary fiber supplementation on microbiota (A and B) of fermentation 1 (F1; donor 3) and fermentation 2 (F2; donor 4). Principle components analysis (PCA) biplot showing variation among the PolyFermS microbiota of F1 (B) and F2 (D) after supplementation with dietary fibers XOS, a GOS, b glucan and inulin. Variables included in the PCA were relative abundance of OTUs (> 0,05%) and OTUs are represented as numbers.

Reproducible metabolic response to dietary fiber supplementation in PolyFermS model with same microbiota

Advances in research are based on the reproducibility of previously published data or investigated results and findings (Freedman et al., 2015). In this study, we performed a third fermentation with D3 microbiota 15

months after F1 with treatments inulin and α-GOS. In order to assess the microbial composition similarities or eventual differences after one year, the relative abundances (V4 region of 16S rRNA gene) of the different phyla and families were compared (**Supplementary Table S2.8**). At phylum level, fecal microbiota of donor D3 at both time points used to inoculate F1 and F3 was dominated by *Firmicutes* (F1 D3: 51 % and F3 D3: 50 %) and *Bacteroidetes* (F1 D3: 25 % and F3 D3: 31 %). The key bacterial families defining D3 microbiota remained stable with dominance of *Bacteroidaceae* (F1 D3: 17 % and F3 D3: 22 %) and *Ruminococcaceae* (F1 D3: 21 % and F3 D3: 16 %) and presence of *Verrucomicrobiaceae* (F1 D3: 6 % and F3 D3: 4 %) and *Methanobacteriaceae* (F1 D3: 9 % and F3 D3: 6 %).
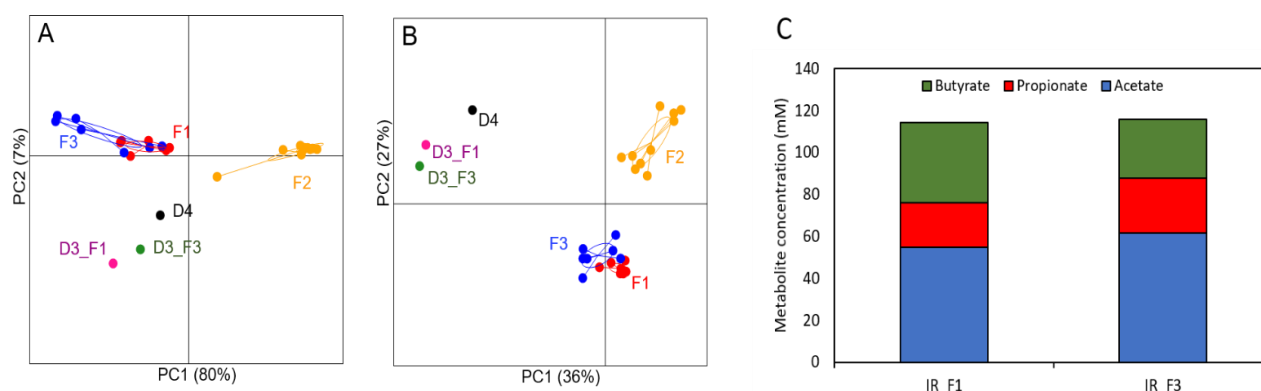
Figure 2.4: Reproducability over time of PolyFermS model inoculated with same healthy fecal donor (D3); F3 was operated 15 months after F1. PCoA plots of weighted (A) and unweighted (B) were performed based on the UniFrac distance matrix generated from sequencing V4 region of 16S rRNA genes in samples from donor's feces and fermentation effluents of F1 (D3), F2 (D4) and F3 (D3). Each circle represents a sample from feces and effluent samples from F1 (D3) (red), F2 (D4) (orange) and F3 (D3) (purple). Bar-plot representation of the mean acetate (blue), propionate (red) and butyrate (green) concentrations (mM) (C) of PolyFermS effluent samples of inoculum reactor (IR) during stable operation phase and connection with experimental reactors (IR_F1: day 1 – day 36; IR_F3: day 1 –day 23); D3, donor 3; F1,

In order to assess reproducibility and stability of the D3 microbiota in our PolyFermS model, the fermentation effluent microbiota of the non-treated reactors were compared to fecal donor and F1 and F2 microbiota. PCoA of UniFrac distances (**Figure 2.4 A and B**) showed F1 and F3 microbiota (D3) were more similar to each other on OTU composition (unweighted) and abundance (weighted) compared to F2 microbiota (D4), demonstrating the reproducible conservation of donor microbiota profile *in vitro*. The abundance of the dominant bacterial families was also comparable between F3 and F1 microbiota in inoculum reactors with *Bacteroidaceae* (F3: 29 %; F1: 26 %) and *Ruminococcaceae* (F3: 19 %; F1: 19 %) as predominant families (**Supplementary Table S2.8 B**).Total SCFA production was also comparable between F3 and F1 with 129 ± 7 mM and 123 ± 10 mM, respectively (**Figure 2.4 C**). There was a slight difference in SCFA profile in the IR effluents, with lower butyrate levels in F3 (22 %) compared to F1 (31 %) but comparable acetate (F3: 48 %; F1: 45 %) and propionate (F3: 20 %; F1: 17 %) concentrations (**Figure 2.4 C**). After reaching stable SCFA profile in reactors during stabilization, supplementation with inulin or α-GOS resulted in significant increases of total SCFAs (**Figure 2.5 A; Supplementary Table S2.6 B**). Similar to F1, addition of α-GOS (from 28 ± 2 to 44 ± 1 mM, during stabilization

and treatment, respectively) or inulin (from 30 ± 2 to 41 ± 3 mM) enhanced butyrate production, while inulin stimulated acetate production (from 61 ± 2 to 66 ± 2 mM). α-GOS supplementation resulted in increased levels of *Ruminococcaceae* (from 24 to 31 % during stabilization and treatment, respectively) and *Bacteroidaceae* (from 22 to 26 %), (**Supplementary Table S2.9**). Simultaneously a decrease in *Eubacteriaceae* (17 to 0 %) was observed. The relative abundance of *Ruminococcaceae* was increased by inulin supplementation (from 41to 48 %), while abundance of the *Eubacteriaceae* decreased (from 15 to 9 %). 10 %) was observed. The relative abundance *o*f *Ruminococcaceae* was increased by inulin supplementation (from 41 to 48 %), while abundance of the *Eubacteriaceae* decreased (from 15 to 9 %).
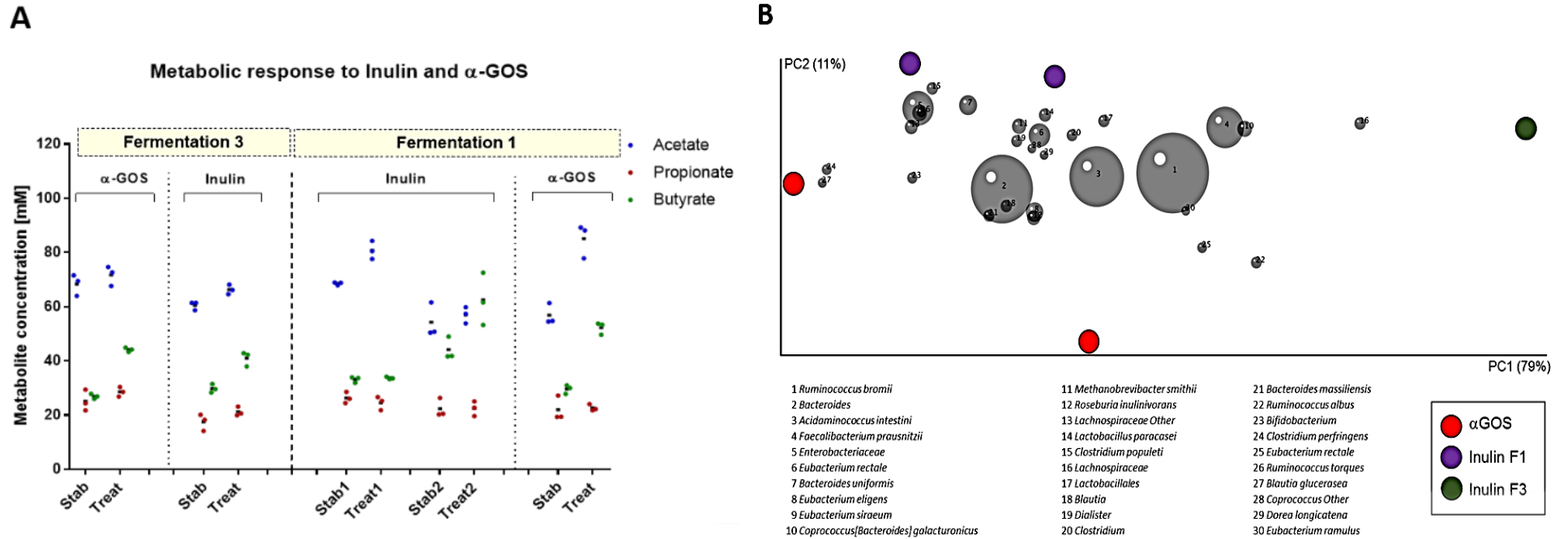
Figure 2.4: Repetition of α-GOS and inulin supplementation to D3 microbiota in PolyFermS resulted in comparable higher butyrate productions and stimulates specific OTUs (PCA). (A) Mean (black horizontal line) from 3 consecutive measurements of acetate, propionate and butyrate concentrations (mM) in the respective reactor at end of stabilization and treatment phase for fermentation 1(F1) and fermentation 3 (F3) (donor 3).Two replicates are shown for each dietary fiber treatment. (B) Principle components analysis (PCA) biplot showing variation in the PolyFermS microbiota of F1 and F3 after supplementation with dietary fibers a GOS and inulin. Variables included in the PCA were relative abundance of OTUs (> 0,05%) and OTUs are represented as numbers.

Discussion

In this study the effect of supplementation of four different dietary fibers on human gut microbiota was investigated at the levels of metabolic and bacterial composition using a continuous *in vitro* fermentation system, modeling adult proximal colon conditions. By using two distinct fecal microbiota composition we could demonstrate a consistent and donor-dependent butyrogenic or propionigenic response towards the fiber treatments.

Both on metabolic and phylogenetic levels we successfully maintained two distinct colon microbial communities in the PolyFermS reactors reflecting the corresponding fecal microbiota donor. Both fecal microbiota were dominated by Firmicutes and Bacteroidetes species and differed mainly on family level within the Bacteroidetes phylum with higher levels of *Bacteroidaceae* species in D3, low in D4, and high levels of *Prevotellaceae* in D4, low in D3. Several other reports observed that individuals with high levels of *Prevotella* have low levels of *Bacteroides* and vice versa (Arumugam et al., 2011; Koren et al., 2013; Wu et al., 2011), suggesting niche competition within the human gut microbiota. Abundance of *Bacteroides* has been associated with animal/fat-rich diets (Koren et al., 2013; Wu et al., 2011), while high *Prevotella* abundance has been associated with plant-rich and vegetarian diets (Martinez et al., 2015; Wu et al., 2011). Difference in abundance of Bacteroidetes phylum between the two donor microbiota was preserved and even enhanced in our PolyFermS model for proximal colon microbiota, despite identical chime-simulating fermentation medium composition. The PolyFermS abundance levels fall within the range reported in a recently published large cohort of 1106 fecal microbiota of Western European individuals with *Bacteroides*: 0.1-72 % and *Prevotella*: 0-56 % (Falony et al., 2016). Stable SCFA profiles were obtained in the untreated control reactors of F1 and F2, reflecting maintenance of functional stability of bacterial community during continuous operation of 36 and 43 days, respectively. The *Prevotellaceae*-dominated microbiota (F2) was characterized by high propionate production and can be explained by propionate –producing capacity of *Prevotella* species (Louis and Flint, 2017). It was earlier reported that higher fecal propionate levels are associated with *Prevotella* species (De Filippis et al., 2016; Salonen and de Vos, 2014). In another cohort it was shown that individuals with more than 20 % *Prevotella* have higher levels of methylmalonyl-CoA mutase, a key enzyme involved in propionate production, in their fecal metaproteome (Kolmeder et al., 2016). The high butyrate production in F1 *(Bacteroidaceae*-dominated) microbiota is likely due butyrate-producing *Lachnospiraceae* and *Ruminococcaceae* species, which were the second and third dominating families within F1. *Bacteroidaceae* species do not produce butyrate within the human gut (Louis and Flint, 2017; Tanca et al., 2017), but may contribute to the butyrate pool by their acetate production, used as co-substrate during butyryl-CoA-transferase route in gut bacteria (Louis et al., 2004).

Our data showed that all dietary fibers supplemented in the PolyFermS microbiota increased SCFA production, displaying fermentability of all tested substrates by both microbiota. Overall, F1 microbiota responded to all dietary fibers by increased butyrate production, whereas F2 microbiota showed increased propionate production. This specific response was consistent among the different treatments and the three fermentations. Similar inter-individual differences in butyrogenic or propionigenic response were observed in static batch experiments with fecal microbiota supplemented with wheat bran particles or inulin (De Paepe et al., 2017), and with FOS and two arabinoxylan variants (Chen et al., 2017). Comparable to our observations, Chen et al. (Chen et al., 2017) showed that *Prevotella*-dominated microbiota responded by higher propionate production upon fiber supplementation *in vitro*. Both in F1 and F2, XOS and αGOS resulted in strong increase in acetate levels, which is produced by almost all heterotrophic gut bacteria (Luc, 2009). Both dietary fibers were short-chain types, which makes them easier fermentable (Sanchez et al., 2009) and it was shown *in vitro* that various intestinal bacteria can use GOS and XOS (Scott et al., 2014). This broad-range utilization may explain the different effects measured on microbiota composition upon XOS supplementation. Metabolic cross-feeding between acetate- and butyrate- producers resulting in higher butyrate levels was observed with inulin in both PolyFermS microbiota, and repetitions. Inulin can be degraded by different *Bifidobacterium* spp., *Lactobacillus* spp. (Riviere et al., 2016) and some butyrate-producing *Roseburia* spp. (Falony et al., 2009), *F. prausnitzii* and *Eubacterium rectale* (De Vuyst et al., 2014). Bifidobacteria and lactobacilli produce acetate and lactate, which can in turn be utilized by butyrate-producing bacteria. During these cross-feeding interactions on inulin-type fructans both commensalism (cross-feeding on acetate and lactate) and substrate competition occurs between both bacterial groups in co-culture experiments (Moens et al., 2016). We observed a consistent increase in *Ruminococcaceae*, but no increase of *Actinobacteriaceae* (bifidobacteria), which suggests that in our set-up the inulin-degrading *Ruminococcaceae* (e. g. *F. prausnitzii*) produced butyrate while consuming the available acetate in the mixed microbial environment. Indeed, increased *Ruminococcaceae* levels upon inulin treatment was linked with an increase in a *F. prausnitzii* OTU, which is in accordance with *in vitro* (Chung et al., 2016) and human observations (Louis et al., 2010; Ramirez-Farias et al., 2009). B-glucan supplementation resulted in increased butyrate and *Eubacteriaceae* levels in F1 microbiota, and increased propionate and *Prevotellaceae* levels in F2. Both changes in microbial composition explain the change in metabolic profile as *Eubacteriaceae* species produce butyrate and acetate, which becomes available for cross-feeding interactions, while *Prevotellaceae* produce propionate (Louis and Flint, 2017). The increase in *Bacteroides-Prevotella* group and propionate production was also observed *in vitro* with oat β-glucan (Hughes et al., 2008). *Prevotella* can better ferment complex polysaccharides from the diet than *Bacteroides* (Rampelli et al., 2015), which may explain their competitive advantage upon β-glucan supplementation.

Overall, we did not detect consistent or systematic changes in the microbiota composition upon dietary fiber supplementation. It appears that the microbiota modulation by dietary fibers occurs at species level as

demonstrated by Chung et al. (Chung et al., 2016) in a continuous *in vitro* fermentation with three different fecal microbiota and dietary fibers as sole carbohydrate source. Due to the higher inter-individual variation at species level within the human gut microbiota, it can be expected that a single fiber will not induce a strong specific modification of the microbiota at species level in different individuals. Indeed, human intervention studies with dietary fibers showed marked inter-individual microbiota changes, which were depended on the individuals' dominant microbiota composition (Walker et al., 2011a).

Compared with *in vivo* and human studies, there are limitations of the *in vitro* approach, since the models do not replicate all the conditions that occur in the colon, resulting in enriching and diminishing of bacterial populations. However, our data correspond well with dietary fiber fermentation *in vivo* and allowed insight into the complex cross-feeding mechanisms. In particular, we managed to show that dietary fibers induce dynamic responses depending on an individual's specific microbiota. Main strength of *in vitro* fermentation models for prebiotic research is that one can follow the *in situ* SCFA production upon treatment, whereas in human intervention studies fecal SCFA concentrations are only a proxy for colonic fermentation and mainly a result of absorption of the SCFA in the intestine and lack thereof. Our approach of repeated dietary fiber supplementation to a stable and active gut microbiota in a continuous fermentation model allowed elucidating direct and indirect metabolic and compositional shifts that can occur in the human gut using long-term supplementation. This is in contrast to often used static batch incubations with fecal microbiota and dietary fibers, which reflect a direct effect of a fiber on the growth and activity of fast substrate utilizers and often neglects the indirect effects or long term shifts that might have the most profound effect on microbiome composition.

To summarize, our study showed that two distinct fecal microbial consortia maintained *in vitro* in the PolyFermS continuous intestinal fermentation model inoculated with immobilized adult fecal microbiota responded differently to dietary fiber supplementation on metabolic and compositional level. Irrespective to the dietary fiber supplemented the *Bacteroidaceae-Ruminococcaceae* dominated microbiota produced more butyrate while the *Prevotellaceae-Ruminococcaceae* dominated microbiota produced more propionate. No fiber-specific change on phylogenetic level was observed, but changes in abundance of specific families or species level OTUs within a microbiota together with cross-feeding interactions between the different functional groups could explain the observed changes in SCFA profiles. Our data suggest that the metabolic profile of SCFA may be the most suitable and robust read-out to characterize the microbiota-modulating effect of a fiber and emphasize on the importance to understand inter-individual responses to a prebiotic treatment for mechanistic understanding and human application.

Acknowledgements
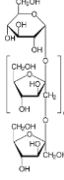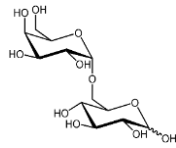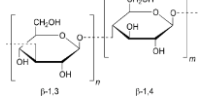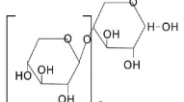
Competing interests

The authors declare no competing interests.

Supporting Material

Supplementary Table S2.1: Characteristics of the dietary fibers used for medium supplementation during treatment periods.

Dietary fiber	Trade name, Supplier	Full name	Sugar moieties	Linkage	Degree of polymerization	Structure	Source	Purity
Inulin-type fructan	Fibrulose F97 (Cosucra Group, Warcoing, Belgium)	Fructo-oligosaccharide	fructose, glucose	β-(2,1) terminal Glc: α–(1,2)	2-20		chicory roots	DM: 96 ± 1 % DP≤ 10: 70 ± 5% on DM DP≤ 20: 94 ± 5% on DM DP >20: 5 ± 5% on DM
α-GOS	Cravingz'Gone® P (Olygose, Compiégne Cedex, France)	α-galacto-oligosaccharide	galactose, glucose	α–(1,6)	2 (melibiose) 3 (manninotriose)		peas	99%
β-glucan	Beta Glucan Powder (CEPARO, Edmonton, Canada)	β-(1,3)-(1,4)-D-glucan	glucose	β-(1,3) β-(1,4)	fiber		oats	90%
XOS	XOS 95P (Longlive, Shandong, China)	Xylo-oligosaccharide	D-xylose	β-(1,4)	2-7		ligno-cellulosic wastes	≥95% DP 2-7

Supplementary Table S2.2: Taxonomic summary of fecal microbiota of selected healthy human donors for PolyFermS experiments. Abundance of phyla (**Table 2. A**) and families (**Table 2. B**) obtained by 16S amplicon sequencing of V4 region.

A

Phylum	D3	D4
Firmicutes	51%	57%
Bacteroidetes	25%	32%
Euryarchaeota	9%	2%
Actinobacteria	6%	6%
Verrucomicrobia	6%	-
Proteobacteria	3%	1%
Tenericutes	-	3%

B

Phylum	Family	D3	D4
Firmicutes	*Ruminococcaceae*	20.9%	19.7%
Firmicutes	*Lachnospiraceae*	7.3%	13.4%
Bacteroidetes	*Bacteroidaceae*	16.9%	8.4%
Bacteroidetes	*Prevotellaceae*	0.1%	17.2%
Euryarchaeota	*Methanobacteriaceae*	8.7%	1.6%
Firmicutes	*Oscillospiraceae*	6.8%	4.0%
Bacteroidetes	*Rikenellaceae*	6.0%	3.4%
Verrucomicrobia	*Verrucomicrobiaceae*	6.2%	0.0%
Firmicutes	Unclassified Clostridiales	2.7%	5.3%
Firmicutes	*Eubacteriaceae*	4.3%	7.1%
Actinobacteria	*Bifidobacteriaceae*	3.6%	4.1%
Firmicutes	*Clostridiaceae*	2.9%	1.5%
Firmicutes	unclassified	0.2%	2.4%
Proteobacteria	RF32; unclassified	0.1%	-
Bacteroidetes	*Porphyromonadaceae*	2.3%	3.6%
Firmicutes	*Peptostreptococcaceae*	2.2%	1.2%
Firmicutes	*Erysipelotrichaceae*	0.8%	2.1%
Proteobacteria	*Desulfovibrionaceae*	2.0%	0.2%
Bacteroidetes	*Odoribacteriaceae*	-	-
Actinobacteria	*Coriobacteriaceae*	1.5%	1.3%
Bacteroidetes	*Paraprevotellaceae*	-	-
Firmicutes	*Acidaminococcaceae*	0.3%	-
Firmicutes	*Christenellaceae*	0.9%	0.3%
Firmicutes	*Erysipelotrichaceae*	0.8%	2.1%
Firmicutes	*Veillonellaceae*	0.7%	0.6%
	Sum	**98.2%**	**99.5%**

Supplementary Table S2.3*: Mean Shannon diversity index of inoculum reactor (IR) and control reactor (CR) samples for different time points along the fermentation periods. Data shown are means ± SD obtained of 11 replicates per sample.

Shannon index					
Fermentation 1		**Fermentation 2**		**Fermentation 3**	
D3	5.77 ± 0.92	D4	5.82 ± 0.89	D3	3.84 ± 0.39
IR_D12	4.52 ± 0.01	IR_D8	3.58 ± 0.41	IR_D8	4.07 ± 0.01
IR_D19	4.33 ± 0.52	IR_D14	3.35 ± 0.38	IR_D14	4.02 ± 0.01
IR_D24	4.39 ± 0.51	IR_D19	3.37 ± 0.31	CR_D8	3.89 ± 0.43
TR_D31	4.32 ± 0.54	IR_D25	3.37 ± 0.36	CR_D14	3.83 ± 0.39
CR_D12	4.08 ± 0.53	IR_D31	3.91 ± 0.41		
CR_D19	4.30 ± 0.45	IR_D37	3.86 ± 0.49		
CR_D24	4.15 ± 0.47	CR_D8	3.61 ± 0.39		
CR_D31	4.35 ± 0.52	CR_D14	3.60 ± 0.39		
		CR_D14	3.49 ± 0.39		
		CR_D25	3.34 ± 0.40		
		CR_D31	3.77 ± 0.37		
		CR_D37	3.73 ± 0.42		

Supplementary Table S2.4: Summary microbial phyla obtained by V4 region 16S amplicon sequencing within non-treated PolyFermS reactors. Inoculum reactor (IR) and control reactor (CR) with donor 3 microbiota (Fermentation 1) on day 12, day 19, day 24 and day 31. Inoculum reactor (IR) and control reactor (CR) with donor 4 microbiota (Fermentation 2) on day 8, day 14, day 19, day 25, day 31 and day 37.

Phylum	Fermentation 1							
	IR_d12	IR_d19	IR_d24	IR_d31	CR_d12	CR_d19	CR_d24	CR_d31
Firmicutes	58%	56%	54%	62%	65%	55%	42%	57%
Bacteroidetes	27%	25%	22%	16%	24%	28%	37%	28%
Proteobacteria	14%	18%	22%	15%	11%	15%	21%	13%
Euryarchaeota	1%	1%	1%	2%	0%	2%	0%	2%
Actinobacteria	0%	0%	0%	5%	0%	0%	0%	0%
Verrucomicrobia	0%	0%	0%	0%	0%	0%	0%	0%

Phylum	Fermentation 2											
	IR_d8	IR_d14	IR_d19	IR_d25	IR_d31	IR_d37	CR_d8	CR_d14	CR_d19	CR_d25	CR_d31	CR_d37
Bacteroidetes	56%	60%	60%	61%	50%	52%	61%	62%	62%	62%	52%	56%
Firmicutes	40%	34%	34%	35%	41%	33%	35%	33%	32%	34%	40%	36%
Proteobacteria	4%	6%	6%	3%	9%	14%	4%	5%	5%	3%	7%	7%
Actinobacteria	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
Euryarchaeota	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
Verrucomicrobia	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%

Supplementary Table S2.5: Summary of most abundant (>5%) microbial families obtained by 16S V4 region amplicon sequencing within non-treated PolyFermS reactors. Inoculum reactor (IR) and control reactor (CR) with donor 3 microbiota (Fermentation 1) on day 12, day 19, day 24 and day 31. Inoculum reactor (IR) and control reactor (CR) with donor 4 microbiota (Fermentation 2) on day 8, day 14, day 19, day 25, day 31 and day 37.

Phylum	Family	Fermentation 1							
		IR_d12	IR_d19	IR_d24	IR_d31	CR_d12	CR_d19	CR_d24	CR_d31
Bacteroidetes	*Bacteroidaceae*	27.0%	25.4%	22.3%	16.2%	23.4%	28.3%	37.1%	27.7%
Firmicutes	*Lachnospiraceae*	13.4%	13.1%	12.0%	8.4%	14.0%	14.8%	9.8%	19.3%
Firmicutes	*Acidaminococcaceae*	12.6%	10.5%	11.6%	11.2%	15.1%	11.8%	10.4%	11.5%
Firmicutes	*Ruminococcaceae*	18.4%	19.4%	13.8%	21.8%	20.7%	6.7%	5.6%	11.4%
Proteobacteria	*Enterobacteriaceae*	9.6%	14.9%	19.6%	14.4%	5.5%	8.8%	14.0%	9.9%
Firmicutes	*Eubacteriaceae*	8.6%	7.6%	10.5%	15.1%	4.9%	16.3%	12.7%	8.2%

Phylum	Family	Fermentation 2											
		IR_d8	IR_d14	IR_d19	IR_d25	IR_d31	IR_d37	CR_d8	CR_d14	CR_d19	CR_d25	CR_d31	CR_d37
Bacteroidetes	*Prevotellaceae*	52.8%	58.4%	58.2%	58.1%	44.3%	48.6%	50.6%	52.7%	57.9%	58.2%	48.4%	52.3%
Firmicutes	*Ruminococcaceae*	19.7%	19.6%	18.0%	18.9%	25.0%	14.0%	17.1%	17.0%	14.3%	20.1%	21.0%	18.9%
Firmicutes	*Lachnospiraceae*	8.5%	5.9%	6.8%	8.1%	6.6%	9.8%	8.0%	7.3%	5.5%	5.9%	5.5%	6.8%
Firmicutes	unclassified Lactobacillales	3.6%	2.9%	3.8%	3.3%	4.0%	3.7%	3.6%	3.5%	7.1%	4.0%	7.1%	4.4%
Bacteroidetes	*Bacteroidaceae*	2.6%	1.8%	1.8%	2.5%	4.8%	2.5%	10.1%	9.2%	3.7%	3.4%	3.6%	2.8%

Supplementary Table S2.6: Mean metabolite concentrations (mM) measured by HPLC in effluent samples of stable stabilization and treatment phases in test reactors. **(A)** Mean metabolite concentrations with standard error of effluent sample in Fermentation 1 (donor 3) and 2 (donor 4) during stable stabilization and fiber treatment phases (applying the stability criterion of 20 % variation on 3 consecutive days of a period). **(B)** Mean metabolite concentrations with standard error of effluent sample in Fermentation 3 (D3) (applying the stability criterion of 20 % variation on 3 consecutive days of a period). Significant differences between mean concentration before and at end of treatment period on 0.05 (a), 0.01 (b) and 0.001 (c) level indicated.

A

Ferm	Total SCFAs	Inulin	b-glucan	XOS	α-GOS
1	Stabilization I	138.54 ± 3.35	131.24 ± 2.84	136.82 ± 2.75	113.93 ± 6.39
	Treatment I	143.68 ± 6.11	133.91 ± 6.53	161.64 ± 7.13	167.79 ± 5.54^c
	Stabilization II	125.91 ± 13.84	120.94 ± 7.93	116.25 ± 4.96	
	Treatment II	150.11 ± 6.35	157.71 ± 20.83	162.35 ± 12.09^b	
2	Stabilization I	133.67 ± 7.62	138.87 ± 10.12	133.77 ± 3.63	142.38 ± 2.83
	Treatment I	154.40 ± 2.84^a	154.83 ± 12.85	160.48 ± 5.29^b	175.48 ± 9.37^b
	Stabilization II	166.89 ± 3.17	145.75 ± 14.44	139.69 ± 7.05	138.92 ± 6.81
	Treatment II	175.84 ± 2.45^a	172.62 ± 1.88^a	178.03 ± 3.56^b	174.76 ± 3.89^b
	Acetate	**Inulin**	**b-glucan**	**XOS**	**α-GOS**
1	Stabilization I	68.56 ± 0.59	56.37 ± 1.96	56.37 ± 1.76	56.88 ± 3.88
	Treatment I	80.82 ± 3.37^b	54.90 ± 1.01	92.62 ± 1.76^b	85.10 ± 6.30^b
	Stabilization II	54.26 ± 6.38	59.41 ± 4.60	66.32 ± 0.28	
	Treatment II	57.02 ± 3.01	60.08 ± 10.48	92.62 ± 2.15^c	
2	Stabilization I	58.76 ± 3.31	59.63 ± 2.77	57.88 ± 0.55	65.44 ± 2.44
	Treatment I	56.56 ± 2.11	64.38 ± 6.23	77.78 ± 6.14^b	111.78 ± 4.10^c
	Stabilization II	82.31 ± 0.87	70.48 ± 4.94	58.38 ± 1.19	62.11 ± 1.67
	Treatment II	74.32 ± 1.52^a	75.74 ± 0.58	89.39 ± 4.87^c	91.43 ± 6.82^b
	Propionate	**Inulin**	**b-glucan**	**XOS**	**α-GOS**
1	Stabilization I	26.36 ± 2.10	24.51 ± 1.17	24.51 ± 0.93	21.96 ± 4.53
	Treatment I	24.57 ± 2.47	18.78 ± 2.16^a	22.10 ± 0.22	22.70 ± 1.20
	Stabilization II	22.37 ± 3.47	42.22 ± 3.95	25.56 ± 2.02	
	Treatment II	22.47 ± 2.73	61.12 ± 1.00^b	22.10 ± 1.27	
2	Stabilization I	43.17 ± 1.88	39.66 ± 2.18	38.92 ± 3.04	43.00 ± 1.94
	Treatment I	54.62 ± 1.33^c	55.64 ± 4.75^b	51.78 ± 1.79^b	44.39 ± 4.57
	Stabilization II	48.20 ± 0.46	43.66 ± 3.66	44.28 ± 1.33	45.67 ± 1.87
	Treatment II	57.34 ± 0.78^b	61.11 ± 10^a	55.72 ± 0.75^c	50.37 ± 1.73^a
	Butyrate	**Inulin**	**b-glucan**	**XOS**	**α-GOS**
1	Stabilization I	33.20 ± 1.10	39.82 ± 0.73	39.82 ± 0.05	29.63 ± 1.65
	Treatment I	33.68 ± 0.45	49.91 ± 4.87^b	37.62 ± 0.35^a	52.26 ± 2.26^c
	Stabilization II	44.19 ± 4.15	32.95 ± 1.37	35.12 ± 1.00	
	Treatment II	62.49 ± 9.70^a	64.50 ± 7.17^b	37.62 ± 1.00^b	
2	Stabilization I	29.28 ± 1.17	31.10 ± 3.40	30.24 ± 2.69	27.56 ± 0.42
	Treatment I	38.59 ± 0.91^c	33.58 ± 0.74	29.59 ± 2.48	18.6 ± 1.46^c
	Stabilization II	27.92 ± 0.95	26.71 ± 2.73	33.31 ± 1.35	28.43 ± 1.10
	Treatment II	38.76 ± 0.27^b	29.30 ± 1.36	29.52 ± 1.00^a	30.18 ± 1.36

Mean and standard error reported (*n* = 3).
a Significantly different from Stabilization (p < 0.05)
b Significantly different from Stabilization (p < 0.01)
c Significantly different from Stabilization (p < 0.001)

B

Fermentation 3		
Total SCFAs	**α-GOS**	**Inulin**
Stabilization	134.21 ± 1.44	114.10 ± 4.25
Treatment	154.97 ± 5.93^a	138.98 ± 6.51^b
Acetate	**α-GOS**	**Inulin**
Stabilization	71.98 ± 2.73	60.51 ± 1.60
Treatment	71.67 ± 3.65	66.33 ± 1.75^a
Propionate	**α-GOS**	**Inulin**
Stabilization	22.36 ± 1.74	17.50 ± 3.06
Treatment	28.60 ± 1.75^a	21.24 ± 1.69
Butyrate	**α-GOS**	**Inulin**
Stabilization	27.64 ± 1.52	29.79 ± 1.58
Treatment	44.12 ± 0.79^c	41.03 ± 2.66^b

Mean and standard error reported (*n* = 3).
a Significantly different from Stabilization (p < 0.05)
b Significantly different from Stabilization (p < 0.01)
c Significantly different from Stabilization (p < 0.001)

Supplementary Table S2.7A*:* Abundance on family level in fermentation samples before (stab) and at end of dietary fiber suplementation in fermentation 1 with donor 3 (D3) microbiota. For inulin, b glucan and XOS there are two repetitions performed.

A

Phylum	Family	Fermentation 1													
		Stab	Inulin_I	Stab	Inulin_II	Stab	β glucan_I	Stab	β glucan_II	Stab	XOS_I	Stab	XOS_II	Stab	α GOS_I
Firmicutes	Ruminococcaceae	22%	28%	28%	20%	19%	26%	21%	10%	24%	22%	19%	22%	19%	9%
Bacteroidetes	Bacteroidaceae	20%	19%	20%	20%	18%	14%	22%	18%	22%	21%	27%	22%	22%	23%
Firmicutes	Eubacteriaceae	18%	8%	12%	11%	10%	23%	7%	20%	7%	8%	6%	6%	10%	14%
Firmicutes	Lachnospiraceae	13%	13%	14%	13%	19%	9%	16%	15%	19%	20%	14%	19%	18%	13%
Firmicutes	Acidaminococcaceae	18%	11%	12%	10%	19%	16%	10%	13%	13%	10%	13%	15%	12%	14%
Proteobacteria	Enterobacteriaceae	0%	10%	5%	12%	1%	2%	11%	7%	1%	7%	7%	3%	11%	11%
Proteobacteria	Alphaproteobacteria	1%	1%	0%	1%	2%	0%	3%	2%	2%	3%	7%	0%	2%	0%
Euryarchaeota	Methanobacteriaceae	2%	3%	2%	2%	2%	2%	2%	5%	2%	2%	1%	2%	1%	2%
Proteobacteria	Sutterellaceae	4%	1%	3%	2%	3%	3%	2%	0%	4%	3%	1%	2%	1%	0%
Firmicutes	Clostridiaceae	0%	2%	0%	1%	4%	0%	1%	4%	1%	0%	1%	0%	0%	5%
Firmicutes	Veillonellaceae	1%	1%	2%	1%	1%	1%	1%	2%	2%	1%	0%	2%	2%	1%
Firmicutes	Alphaproteobacteria	1%	0%	0%	0%	1%	1%	1%	1%	2%	1%	0%	0%	0%	1%
Firmicutes	Lactobacillales	0%	0%	0%	2%	0%	1%	1%	2%	0%	0%	1%	0%	0%	1%
Firmicutes	Lactobacillaceae	0%	0%	0%	5%	0%	0%	0%	0%	0%	0%	0%	3%	0%	1%

Supplementary Table S2.7B: Abundance on family level in fermentation samples before (stab) and at end of dietary fiber suplementation in fermentation 2 with donor 4 (D4) microbiota. For all dietary fibers there are two repetitions performed

B

Phylum	Family	Fermentation 2															
		Stab	Inulin_I	Stab	Inulin_II	Stab	β glucan_I	Stab	β glucan_II	Stab	XOS_I	Stab	XOS_II	Stab	α GOS_I	Stab	α GOS_II
Bacteroidetes	Prevotellaceae	57%	52%	53%	58%	26%	61%	54%	58%	55%	57%	54%	52%	49%	49%	58%	55%
Firmicutes	Ruminococcaceae	13%	19%	16%	22%	22%	18%	22%	16%	16%	16%	13%	14%	13%	19%	17%	14%
Bacteroidetes	Bacteroidaceae	3%	1%	3%	2%	14%	2%	2%	4%	8%	1%	4%	3%	14%	4%	3%	2%
Firmicutes	Lachnospiraceae	7%	7%	4%	4%	9%	4%	6%	4%	6%	5%	6%	11%	6%	12%	5%	10%
Proteobacteria	Desulfovibrionaceae	2%	2%	4%	2%	6%	1%	2%	2%	3%	3%	3%	2%	3%	2%	2%	2%
Firmicutes	Eubacteriaceae	2%	1%	1%	1%	3%	5%	2%	3%	3%	6%	4%	3%	3%	2%	1%	7%
Firmicutes	Lactobacillales	3%	4%	6%	2%	6%	1%	2%	3%	2%	5%	6%	6%	3%	5%	6%	4%
Proteobacteria	Sutterellaceae	2%	1%	2%	3%	4%	3%	4%	3%	2%	3%	4%	3%	3%	2%	4%	2%
Firmicutes	Oscillospiraceae	1%	0%	3%	1%	3%	1%	2%	3%	2%	1%	2%	2%	2%	1%	2%	1%
Firmicutes	Clostridiales	0%	0%	1%	0%	3%	0%	0%	0%	1%	0%	1%	0%	1%	0%	0%	0%
Firmicutes	Veillonellaceae	1%	1%	1%	1%	1%	1%	2%	1%	1%	2%	1%	1%	1%	1%	1%	2%
Firmicutes	Lactobacillaceae	1%	1%	1%	1%	0%	0%	0%	1%	1%	1%	0%	0%	0%	0%	1%	0%
Proteobacteria	Enterobacteriaceae	4%	4%	3%	1%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	1%
Proteobacteria	Moraxellaceae	1%	6%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%

Supplementary Table S2.8: Summary microbial phyla obtained by V4 region 16S amplicon sequencing within non-treated PolyFermS reactors and their respective fecal inoculum of donor 3. **B**:Summary of most abundant (>5%) microbial families obtained by 16S V4 region amplicon sequencing within non-treated PolyFermS reactors with donor 3 (D3) microbiota. Inoculum reactor (IR) and control reactor (CR) of fermentation 1 on day 12 and day 19 and of fermentation 3 on day 8 and day 14.

A

Phylum	Fermentation 3				Fermentation 1				
	Feces_D3	IR_d8	IR_d14	CR_d8	CR_d14	Feces_D3	IR_d12	IR_d19	CR_d12	CR_d19
Firmicutes	50%	67%	68%	82%	84%	51%	58%	56%	65%	55%
Bacteroidetes	31%	29%	28%	10%	6%	25%	27%	26%	23%	28%
Proteobacteria	5%	1%	2%	7%	7%	3%	14%	18%	11%	15%
Euryarchaeota	6%	2%	1%	1%	1%	9%	1%	1%	0%	1%
Actinobacteria	4%	2%	1%	1%	1%	6%	0%	0%	0%	0%
Verrucomicrobia	4%	0%	0%	0%	0%	6%	0%	0%	0%	0%

B

Phylum	Family	Fermentation 3					Fermentation 1				
		Feces_D3	IR_d8	IR_d14	CR_d8	CR_d14	Feces_D3	IR_d12	IR_d19	CR_d12	CR_d19
Firmicutes	*Ruminococcaceae*	16.4%	16.0%	22.5%	38.7%	43.2%	20.9%	18.8%	19.0%	20.8%	6.6%
Firmicutes	*Acidaminococcaceae*	0.6%	22.9%	15.7%	11.8%	18.8%	0.3%	12.6%	10.3%	15.4%	11.9%
Firmicutes	*Eubacteriaceae*	3.4%	14.1%	17.0%	19.1%	9.6%	4.3%	8.5%	7.7%	4.9%	16.2%
Proteobacteria	*Enterobacteriaceae*	0.3%	0.0%	0.0%	6.1%	7.1%	0.1%	9.7%	15.0%	5.3%	8.9%
Firmicutes	*Lachnospiraceae*	11.0%	8.7%	8.6%	8.7%	6.5%	7.3%	13.2%	13.0%	13.9%	14.6%
Bacteroidetes	*Bacteroidaceae*	21.7%	29.0%	28.3%	9.9%	6.4%	16.9%	26.9%	25.6%	23.3%	28.3%
Euryarchaeota	*Methanobacteriaceae*	6.0%	1.9%	0.6%	0.8%	1.3%	8.7%	0.7%	0.6%	0.1%	1.4%
Firmicutes	*Oscillospiraceae*	8.9%	1.0%	0.6%	0.6%	0.5%	6.8%	0.9%	0.7%	0.2%	0.6%
Verrucomicrobia	*Verrucomicrobiaceae*	4.2%	0.0%	0.0%	0.0%	0.0%	6.1%	0.0%	0.0%	0.0%	0.0%
Bacteroidetes	*Rikenellaceae*	4.4%	0.0%	0.0%	0.0%	0.0%	6.1%	0.0%	0.1%	0.0%	0.1%

Supplementary Table S2.9: Abundance on family level in fermentation samples before (stab) and at end of dietary fiber suplementation in fermentation 1 and 3 (donor 3).

Phylum	Family	Fermentation 3				Fermentation 1					
		Stab	Inulin_I	Stab	a GOS_I	Stab	Inulin_I	Stab	Inulin_II	Stab	a GOS_I
Firmicutes	*Ruminococcaceae*	41%	48%	24%	31%	22%	29%	28%	19%	19%	9%
Bacteroidetes	*Bacteroidaceae*	6%	6%	22%	26%	20%	19%	21%	21%	21%	23%
Firmicutes	*Acidaminococcaceae*	16%	15%	17%	16%	17%	11%	12%	10%	13%	13%
Firmicutes	*Lachnospiraceae*	14%	12%	9%	10%	14%	14%	14%	13%	18%	13%
Firmicutes	*Eubacteriaceae*	15%	9%	17%	10%	18%	8%	12%	11%	10%	14%
Firmicutes	*Veillonellaceae*	2%	2%	2%	1%	1%	2%	2%	1%	2%	1%
Firmicutes	*Oscillospiraceae*	1%	1%	1%	1%	0%	0%	1%	0%	0%	0%
Firmicutes	*Lactobacillaceae*	1%	2%	1%	1%	0%	0%	0%	4%	0%	1%
Euryarchaeota	*Methanobacteriaceae*	1%	1%	2%	1%	2%	3%	2%	2%	1%	2%
Actinobacteria	*Bifidobacteriaceae*	1%	1%	0%	0%	0%	0%	0%	0%	0%	3%
Firmicutes	*Clostridiaceae*	1%	1%	1%	0%	0%	2%	0%	1%	0%	5%
Firmicutes	*Other*	0%	2%	0%	0%	0%	0%	0%	2%	0%	1%
Proteobacteria	unclassified	0%	0%	0%	0%	1%	1%	0%	1%	2%	0%
Proteobacteria	*Sutterellaceae*	0%	0%	1%	0%	4%	1%	3%	2%	1%	0%
Proteobacteria	*Enterobacteriaceae*	0%	0%	0%	0%	0%	10%	4%	12%	11%	11%
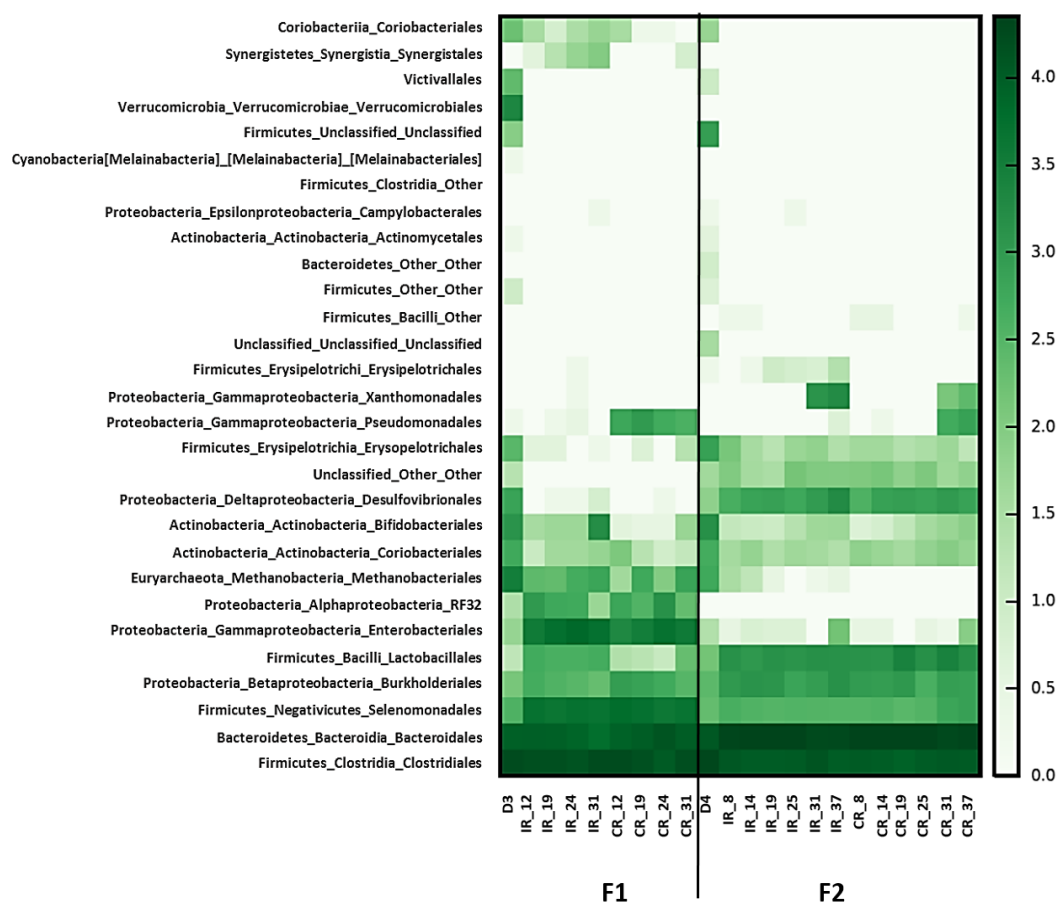
Supplementary Figure S2.1: Heatmap depicting the most abundant OTUs (> 1 %) at order level in the fecal (donor 3 and donor 4) and PolyFermS microbiota of inoculum reactor (IR) and control reactor (CR) along both fermentations. Heatmap was constructed using normalized abundance of each OTU
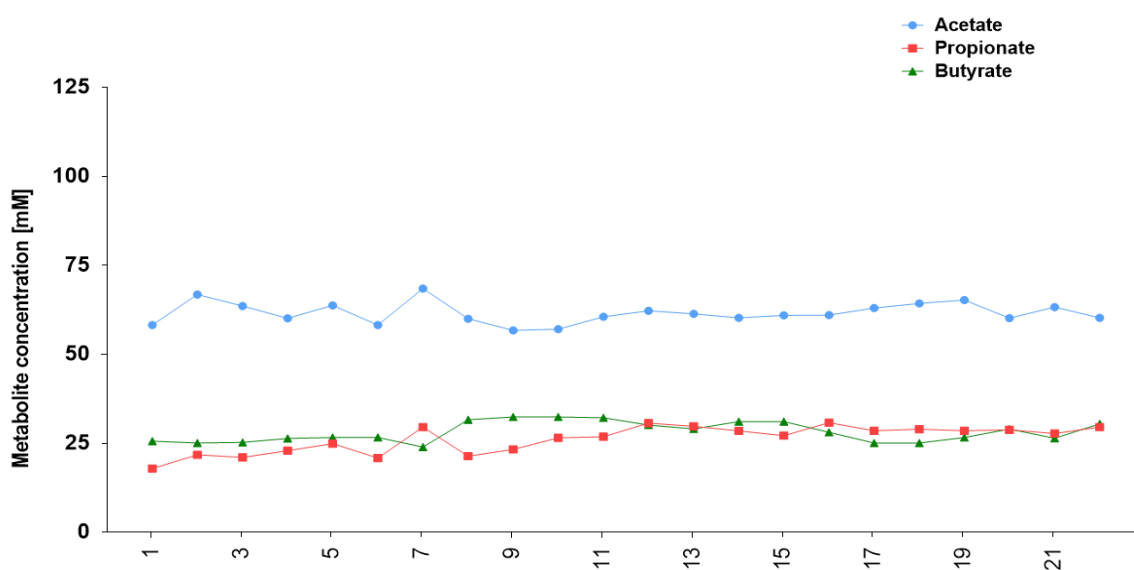
Supplementary Figure S2.4: Daily SCFA concentrations in fermentation effluents of inoculum reactor (IR) in Fermentation 3 (donor 3) measured by HPLC.
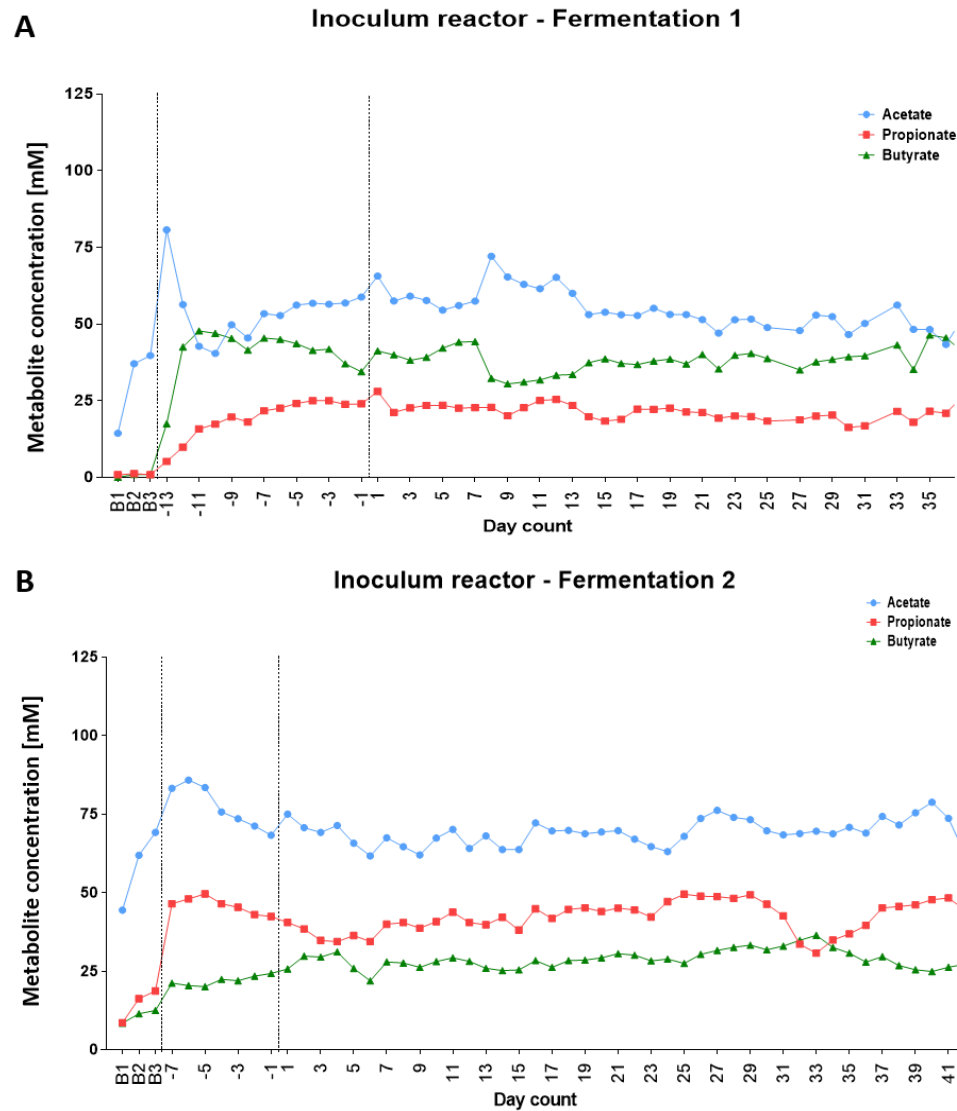
Supplementary Figure S2.2: Daily SCFA concentrations in fermentation effluents of inoculum reactor (IR) (**A & C**) of both PolyFermS fermentations measured by HPLC. Colonization: three consecutive batch fermentations for bead colonization. IR stabilization: stabilization period in continuous mode to reach steady-state
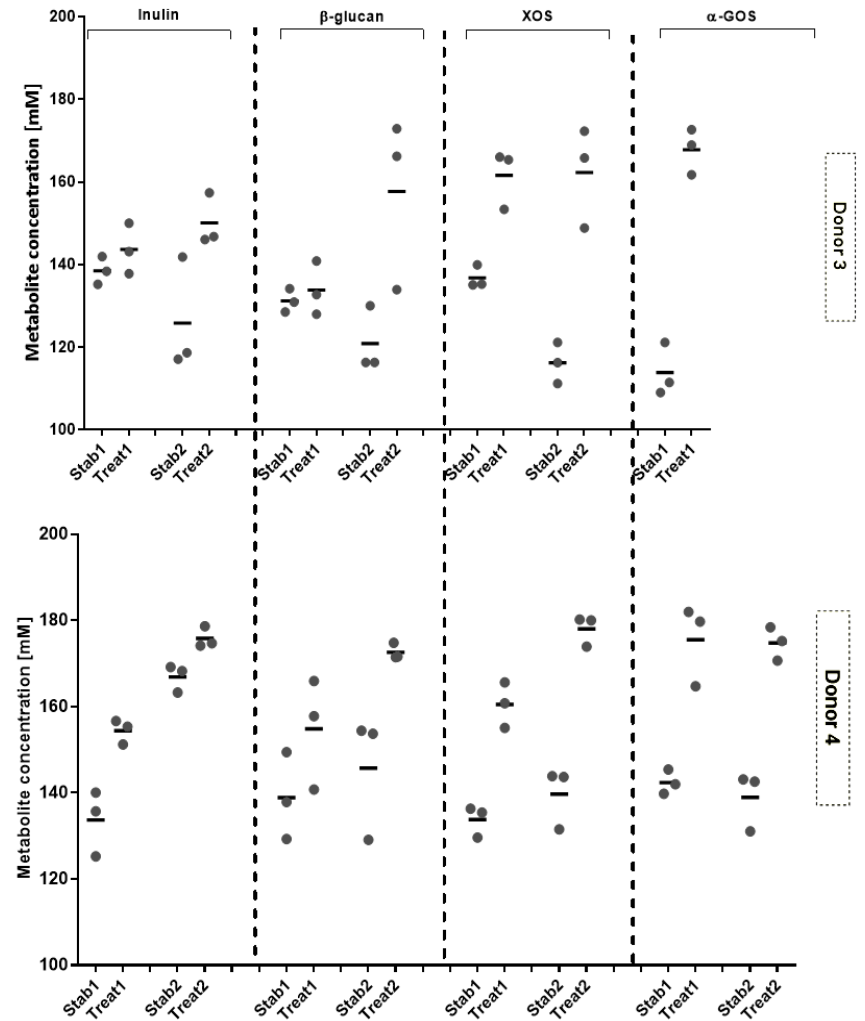
Supplementary Figure S2.3: Treatments with dietary fibers result in higher Total SCFA productions. Mean (black horizontal line) from three consecutive measurements of total SCFAs (mM) in the respective reactor at end of stabilization and treatment phase for fermentation 1 (Donor 3) and fermentation 2 (Donor 4). Two replicates are shown for each dietary fiber treatment, except α-GOS in fermentation
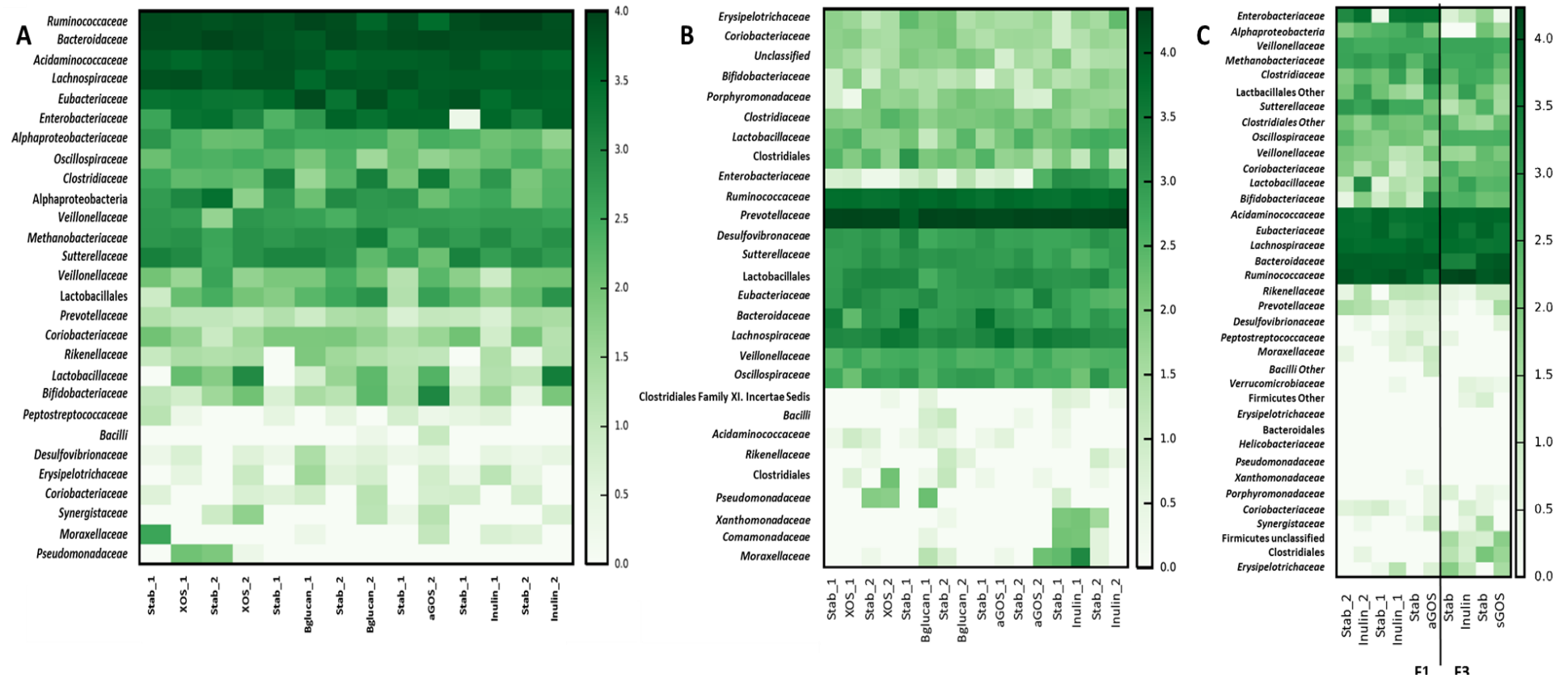
Supplementary Figure S2.5: Heatmap depicting abundance of the most abundant OTUs (> 1 %) on family level in fermentation microbiota at end of stabilization and treatment periods. Heatmap was constructed using normalized abundance of each OTU. A: F1 (D3), B: F2 (D4), C: F1+F3 (D3); F, fermentation; D, donor.

Chapter 3

Stepwise development of an *in vitro* continuous fermentation model for the murine caecal microbiota.

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Abstract

Murine models are valuable tools to study the role of gut microbiota in health or disease. However, murine and human microbiota differ in species composition, so further investigation of the murine gut microbiota is important to gain a better mechanistic understanding. Continuous *in vitro* fermentation models are powerful tools to investigate microbe-microbe interactions while circumventing animal testing and host confounding factors, but are lacking for murine gut microbiota. We therefore developed a novel continuous fermentation model based on the PolyFermS platform adapted to the murine caecum and inoculated with immobilized caecal microbiota. We followed a stepwise model development approach by adjusting parameters [pH, retention time (RT), growth medium] to reach fermentation metabolite profiles and marker bacterial levels similar to the inoculum. The final model had a stable and inoculum-alike fermentation profile during continuous operation. A lower pH during startup and continuous operation stimulated bacterial fermentation (115 mM short-chain fatty acids at pH 7 to 159 mM at pH 6.5). Adjustments to nutritive medium, a decreased pH and increased RT helped control the *in vitro Enterobacteriaceae* levels, which often bloom in fermentation models, to 6.6 log gene copies/mL in final model. In parallel, the *Lactobacillus*, *Lachnospiraceae*, and *Ruminococcaceae* levels were better maintained *in vitro* with concentrations of 8.5 log gene copies/mL, 8.8 log gene copies/mL and 7.5 log gene copies/mL, respectively, in the final model. An independent repetition with final model parameters showed reproducible results in maintaining the inoculum fermentation metabolite profile and its marker bacterial levels. Microbiota community analysis of the final model showed a decreased bacterial diversity and compositional differences compared to caecal inoculum microbiota. Most of the caecal bacterial families were represented *in vitro*, but taxa of the *Muribaculaceae* family were not maintained. Functional metagenomics prediction showed conserved metabolic and functional KEGG pathways between *in vitro* and caecal inoculum microbiota. To conclude, we showed that a rational and stepwise approach allowed us to model *in vitro* the murine caecal microbiota and functions. Our model is a first step to develop murine microbiota model systems and offers the potential to study microbiota functionality and structure *ex vivo*.

Introduction

The gastrointestinal tract of the mammalian host is inhabited by a dense, complex and diverse bacterial community, termed the gut microbiota (Robles Alonso and Guarner, 2013). The gut microbiota is involved in key processes beneficial for the host such as dietary compound metabolism, pathogen displacement, or immune system development (Robles Alonso and Guarner, 2013; Round and Mazmanian, 2009). A large proportion of the vast amount of evidence that the gut microbiota influences many physiological and pathological processes in the host comes from murine studies (Round and Palm, 2018). Mouse models are a valuable tool for human biology, disease and pharmaceutical research due to the high similarity in physiology, anatomy, and genetics (Nguyen et al., 2015). However, mice and humans differ in gastrointestinal tract anatomy, dietary habits, circadian rhythm, and environmental microbes and pathogens, which leads to differences in their gut microbiota composition and activity (Hugenholtz and de Vos, 2017; Nguyen et al., 2015; Uhl and Warner, 2015).

In humans, the large intestine is the main site of fermentation, and contains the highest microbial load and activity, whereas in mice, intestinal fermentation mainly takes place in the caecum, a "bag-like" link between the small intestine and the colon that is nearly absent in humans (Nguyen et al. 2015). Morphological differences in compartmentalization, retention times (RTs) and mixing conditions likely influences composition, richness and diversity of the gut microbiota in humans and mice. In both humans and mice, fermentation of indigestible food compounds produces the short-chain fatty acids (SCFAs) acetate, propionate and butyrate (Burokas et al., 2017; Flint et al., 2012b; Herrmann et al., 2018). The bacterial composition influences the fermentation capacity and end microbial metabolite profiles (Flint et al. 2012). At phylum level, the murine and human intestinal microbiota are similar with the two dominating phyla Firmicutes and Bacteroidetes (Clavel et al., 2016; Hugenholtz and de Vos, 2017; Ley et al., 2006). However, when comparing the bacterial composition at a deeper phylogenetic level, mice and humans show clear differences in genera composition and abundance. First comparisons showed that 85% of detected bacterial genera in the mouse gut are not present in that of humans (Ley et al., 2005) and further studies identified mouse- and human-specific taxa (Clavel et al., 2016; Nguyen et al., 2015; Xiao et al., 2015). Genera like *Lactobacillus, Turicibacter & Coprobacillus* (*Erysipelotrichaceae*)*, Anaerotruncus* (*Ruminococcaceae*)*, Marvinbryantia* (*Lachnospiraceae*)*, and *Pseudoflavonifractor* (unclassified *Clostridiales*) are present at higher levels in the murine intestinal microbiota, while *Prevotella, Faecalibacterium, Ruminococcus, Oscillibacter* and *Klebsiella* are more abundant in human (Nguyen et al. 2015; Xiao et al. 2015). Deep metagenome sequencing revealed that only 4% of microbial genes in the mouse gut were shared with human gut samples, but almost 80% of their annotated functions were common (Xiao et al. 2015). This indicates a similar functionality of mouse and human gut microbiota, but performed by different species and strains.

Recently, efforts were made to isolate the murine gut bacteria (Lagkouvardos et al., 2016) to formulate minimal microbial consortia for studying microbiota-derived functions in gnotobiotic mouse models (Clavel et al., 2017). Simplified defined consortia may not recapitulate all functions of complex microbiota. Continuous *in vitro* fermentation models that contained the complex gut microbial communities mimicking the conditions of the modeled host were successful developed for ecological and mechanistic studies of human (Dostal et al., 2015; Geirnaert et al., 2015; Lacroix et al., 2015; McDonald et al., 2015; Zihler Berner et al., 2013) and monogastric animal (Tanner et al., 2014) gut microbiota, which avoided ethical concerns and host confounding factors. However, to our knowledge no complex murine gut microbiota model has been developed, apart from simple, closed and short-term batch cultures (Herrmann et al., 2017; Li et al., 2018; Salyer et al., 2013) or continuous flow cultures without pH control and taxonomic validation (Freter et al., 1983). In contrast to batch cultures, continuous fermentation models offer the advantage of continuous substrate supply and removal of toxic fermentation products, which are required for growth and establishment of balanced and representative ecosystems. Physiological parameters like RT, temperature, pH, and redox potential are highly controlled in continuous fermentation, which facilitates the establishment of steady-state conditions (Payne et al. 2012). In addition, *in vitro* continuous gut fermentation models may allow simulation of the spatial, environmental and temporal features of a specific gut environment. Challenges of *in vitro* fermentation models include the large amount of gut microbiota sample (mainly feces) required for inoculation and the loss of less competitive bacteria by rapid washout. To address these challenges, fecal microbiota can be immobilized in porous gel beads and run in continuous culture, which allows models to operate at high cell density akin to the gut, prevents washout of slow growing bacteria, and reproduces both planktonic and sessile states of the gut microbiota (Fehlbaum et al., 2015; Poeker et al., 2018; Tanner et al., 2014; Zihler Berner et al., 2013). In addition, only very small amounts of donor material (1–2 g) are required to establish a fermentation, therefore enabling modeling of small animal gut microbiota.

In this study, we developed a continuous fermentation model of the murine caecal microbiota based on the PolyFermS platform inoculated with immobilized caecal microbiota of C57BL/6 mice. In a first step, we determined the pH, the bacterial composition and the metabolic profile in the caeca of C57BL/6 mice. To support growth of the murine caecal bacterial populations and activity, a complex murine nutritive growth medium was developed to mimic substrate conditions encountered in mouse caecum chyme. Different factors of the fermentation model, including caecum sampling, fermentation starting mode, pH, RT and adjustment of growth medium were investigated and adjusted in a sequential order, using five different models inoculated with different pooled fresh immobilized caecum microbiota from 4-5 mice and operated up to 69 days. The microbiota composition in the reactor effluents was analyzed by quantitative real time PCR (qPCR) and 16S rRNA amplicon sequencing and compared with the caecal inoculum. Metabolic activity and functional stability of the microbiota was monitored by SCFA analysis.

Material and Methods

Caecal microbiota collection

Healthy female WT C57BL/6J mice aged 9–13 weeks were obtained from Charles River (Lyon, France) and housed in groups at University Hospital of Zurich. Housing conditions were controlled at 22°C, room humidity and 12 h light/dark cycle. Mice were provided with mouse/rat maintenance chow (Kliba Nafag, Kaiseraugst, Switzerland) and *ad libitum* drinking water. Mice were housed in different cages and were sacrificed on the same day in the morning by cervical dislocation, after which the caecum was immediately removed and placed on a sterile petri plate. The pH of the fresh caecal content in the intact caecal pouch was immediately determined using a probe pre-calibrated pH meter (Metrohm 744 pH Meter, Metrohm Ltd., Herisau, Switzerland). The caecal content was collected into DNAase-free tubes, immediately snap frozen in liquid nitrogen, and then stored at -20°C until DNA and SCFA extraction. Mouse experiments were conducted according to Swiss animal welfare legislation, and the local veterinary office approved all procedures (Veterinäramt des Kantons Zürich; Nr. ZH220/2016).

In vitro fermentation model

Nutritive medium

The nutritive medium was formulated from the validated bacterial growth medium described by Macfarlane et al. (1998) for cultivating the human gut microbiota *in vitro*. It was modified to approximate the carbohydrate and protein ratio in murine caecum chyme. Standard mouse chow is composed of approximately 18% (w/w) protein, 54% (w/w) soluble carbohydrate and 4.5% (w/w) crude fiber (**Supplementary Table S3.1 B**). Considering an average daily chow intake of 3.5 g per C57BL/6J mouse (Champy et al., 2008),), a daily carbohydrate intake of 1.9 g, 0.6 g protein and 0.14 g crude fibers was considered. To calculate the amount of carbohydrates (excluding crude fibers) and proteins reaching the caecum, upper gastrointestinal digestibility indices of 95% for carbohydrates (Dahlqvist and Thomson, 1963a, b; Lee et al., 2011) and 90 % for proteins (Kerr et al., 2014) were applied. This resulted in 0.09 g of dietary carbohydrates and 0.06 g of dietary proteins reaching the caecum each day or a dietary carbohydrate:protein ratio of 60:40. Macfarlane medium was adapted accordingly (**Supplementary Table S3.1 A**). To simulate the mouse chow more closely, we excluded guar gum and inulin, and replaced soluble potato starch with soluble corn starch. Concentrations of the protein sources casein, peptone, tryptone and yeast extract were increased to meet the calculated murine caecum chyme protein concentration. The mucin concentration was kept at 4 g/L. The pectin concentration was kept at 2 g/L to avoid flow disturbance in tubes from increased viscosity. The carbohydrate:protein ratio was approximately 60:40 in medium 1 and 55:45 in medium 2 (**Supplementary Table S3.1 A**), which did not include

the contribution of yeast extract and mucin used for simulating the endogenous protein and carbohydrate sources in the gastrointestinal tract (Cornick et al., 2015). After sterilization (20 min, 120°C) and cooling to 4°C, 1 mL of a filter-sterilized (0.2 μm pore-size) vitamin solution (Michel et al. 1998) was added to the medium. For initial bead colonization, the nutritive growth medium was supplemented with 20% (v/v) effluent from a previous fermentation (acetate:propionate:butyrate 2.5:1.5:1) or 20% (v/v) rumen fluid (4.5:3:1) and 0.1% (m/v) cellobiose (**Table 1 A**). All components were purchased from Sigma-Aldrich Chemie (Buchs, Switzerland), except for xylane (Angene, London, United Kingdom), peptone water (Oxoid AG, Pratteln, Switzerland), bile salts (Oxbile, Oxoid AG), tryptone (BD, Sparks, United States), yeast extract (Merck, Darmstadt, Germany), $NaHCO_3$ (Fischer Scientific, Pittsburgh, PA, United States), NaCl and KH_2PO_4 (VWR International AG, Dietikon, Switzerland), $MgSO_4$·anhydrous (Acros Organics, Geel, Belgium) and $MnCl_2$·$4H_2O$ (Fluka, Buchs, Switzerland).

Caecal microbiota immobilization and bead colonization

For each immobilization procedure, four to five caeca from healthy 9- to 13- weeks old female C57BL/6 mice were collected to prepare the inoculum. All caecal contents were collected in the morning shortly (models 1 and 2) or a couple of hours (models 3–5) after the dark period. In model 1, caecal contents were dissected aerobically from the remaining gastrointestinal tract with surgical scissors, placed onto a sterile Petri dish and immediately transferred to an anaerobic box until further processing. In models 2–5, the caeca were tied off with surgical threads to avoid oxygen stress to the microbiota, placed onto a sterile Petri dish and immediately transferred to an anaerobic jar, and transferred into an anaerobic chamber (10% CO_2, 5% H_2, and 85% N_2) (Coy Laboratories, Ann Arbor, MI, United States), for dissection and pooling contents. In models 3–5, caecal bacteria were washed with pre-reduced peptone water (0.1 %, pH 7, Thermo Fisher Diagnostics AG, Pratteln, Switzerland) before immobilization to remove potential interfering endogenous enzymes, salts and other cellular products that hinder proper gel bead formation. Caecal bacteria were immobilized in 1–2 mm gel beads consisting of gellan gum (2.5%, m/v), xanthan (0.25%, m/v), and sodium citrate (0.2%, m/v) in an anaerobic chamber as previously described (Poeker et al. 2018). Sixty milliliters of freshly produced caecal beads were transferred to a glass bioreactor containing 140 mL of sterile murine nutritive medium. For initial bead colonization, two or three consecutive fed-batch fermentations were carried out by replacing 100 mL of the spent medium by fresh nutritive medium every 6–24 h, depending on the model (**Table 3.1 A**). The reactor headspace was continuously flushed with CO_2, to ensure anaerobiosis in the system. The temperature was controlled at 37°C and the pH at selected values (depending on the model and fermentation step) by automatic addition of 2.5 M NaOH.

Continuous caecum microbiota fermentation and treatments

After batch fermentations, the reactor containing caecal beads was switched to continuous mode by continuously supplying fresh, sterile and anaerobic nutritive medium and removing an equivalent volume of

fermented medium with peristaltic pumps (Reglo, Ismatec, Glattbrugg, Switzerland) (Table 3.1 B). For all bioreactors, stirring speed was carried out at 180 rpm, working volume was 200 mL, temperature was set to 37°C and anaerobiosis was maintained by continuously flushing the headspace of bioreactors. In models 1–3, bioreactors were operated with constant conditions of pH 7 and a continuous flow rate of 22.2 mL/h (RT of 9 h) of medium 1 (6.8 g/L starch). Bioreactor in model 4 was maintained at pH 6.5 and supplied with fresh nutritive medium 2 (2 g/L starch) at a RT of 9 h. In model 5 and model 5*, reactors were operated with constant conditions of pH 6.5 and a continuous flow rate of 16.7 mL/h (corresponds to a RT of 12 h) of medium 2. Effluent samples were taken daily and separated into bacterial pellets (10 min at 14,000 × g at 4°C) and supernatant, and stored at -20°C until further analysis. Stability of *in vitro* microbiota was monitored by daily measurements of the main fermentation metabolite concentrations in sample supernatants.

Table 3.1: Conditions of initial bead batch colonization (A) and continuous fermentation (B) for the different tested models of mouse caecal fermentation.

(A) Batch conditions						
	Model 1	**Model 2**	**Model 3**	**Model 4**	**Model 5**	**Models 5***
Nutritive medium*	6.8 g/L starch	6.8 g/L starch	6.8 g/L starch	2 g/L starch	2 g/L starch	2 g/L starch
Supplementations	20% effluent	10 mM lactate	0.1%cellobiose 20% rumen fluid	0.1%cellobiose 20% rumen fluid	0.1%cellobiose (Batch 1 and 2) 20% rumen fluid (Batch 1)	0.1%cellobiose (Batch 1 and 2) 20% rumen fluid (Batch 1)
PH	7	7	7	5.8	6	6
Fed-batch times						
Batch 1	24 h	24 h	16 h	20 h	20 h	20 h
Batch 2	24 h	24 h	5 h	6 h,	8 h	8 h
Batch 3	6 h	6 h				
(B) Continuous fermentation conditions						
	Model 1	**Model 2**	**Model 3**	**Model 4**	**Model 5**	**Models 5***
Nutritive medium*	6.8 g/L starch	6.8 g/L starch	6.8 g/L starch	2 g/L starch	2 g/L starch	2 g/L starch
pH	7	7	7	6.5	6.5	6.5
Retention time	9 h	9 h	9 h	9 h	12 h	12 h
Total fermentation time (days)	13	44	32	53	69	42

Microbial metabolite analysis

Caecal samples were mixed with 300 μL 0.15 mM H_2SO_4, homogenized and centrifuged at 4°C at 9000 × g for 20 min. Supernatant was filtered directly into HPLC vials through a 0.45 μm nylon membrane (Infochroma AG, Zug, Switzerland). Fermentation samples were centrifuged at 4°C at 14,000 × g for 10 min. The pellet was used for DNA extraction and the supernatant was filtered into glass vials through a 0.45 μm nylon membrane. HPLC analysis (Thermo Fisher Scientific Inc. Accela, Wohlen, Switzerland) was performed to determine the concentrations of SCFAs (formate, propionate, acetate, butyrate, and valerate), branched-chain fatty acids (BCFAs) (isobutyrate and isovalerate) and intermediate metabolites (lactate and succinate). Analyses were performed with an Accela Chromatography System and RI-detector (Thermo Fisher Scientific Inc., Reinach,

Switzerland), equipped with a Security Guard Carbo-H cartridge (4 × 3.0 mm) and a Rezex ROA-Organic Acid H⁺ column (300 × 7.8 mm). Volumes of 40 μL were injected into the HPLC with a flow rate of 0.4 mL/min and H_2SO_4 as an eluent (Poeker et al. 2018).

Microbial community analysis

Genomic DNA extraction

Total genomic DNA was extracted from 100 to 200 mg of caecal contents and the pellet of 2 mL of fermentation effluent using the FastDNA® SPIN Kit for Soil (MP Biomedicals, Illkirch Cedex, France) according to the manufacturer's instructions. Total DNA concentration was measured by spectrophotometry using a Nanodrop® ND-1000 Spectrophotometer (Wiltec AG, Littau, Switzerland) and samples were stored at -20°C until analysis.

Quantitative PCR analysis

DNA extracts were used for qPCR to enumerate the gene copy numbers of total bacteria and the specific marker bacterial groups: Firmicutes, *Ruminococcaceae*, *Lachnospiraceae*, *Lactobacillus-Leuconostoc-Pediococcus*, Bacteroidetes, *Bacteroides*, *Enterobacteriaceae*, *Akkermansia* (**Supplementary Table S3.2**). qPCR assays were performed using 1 μL of 10- or 100- fold diluted genomic DNA, 2x SYBR Green qPCR Mastermix (Life Technologies, Labgene Scientific Instruments, Zug, Switzerland), 100 μM of each forward and reverse primer, resulting in a total volume of 25 μL in a 96-well plate. The analysis was performed in an ABI PRISM 7500-PCR -sequence detection system (Applied Biosystems, Zug, Switzerland). Each reaction was run in duplicate. For quantification, a dilution series of standards was obtained by amplification of the linearized plasmids containing the gene of a representative bacterial species belonging to the target group, and included in each run (Pham et al., 2017). Primer specificity and verification of the presence of the desired amplicon was determined by melting curve analysis. PCR efficiency (%) was calculated from the slope of the standard curve of each qPCR assay. Assays with an efficiency of 80–110% (slope of 3.2–3.9) were included in data analysis.

Microbiota profiling with 16S amplicon sequencing

The bacterial community in caecal samples was analyzed using the primers 341F (5'-CCTAYGGGRBGCASCAG-3') and 806R (5'-GGACTACNNGGGTATCTAAT-3'), which flank the V3-V4 region. MiSeq adaptors were added by PCR. Sequencing of caecum microbiota of different mice was performed with Illumina MiSeq (Genotoul, Castanet-Tolosan Cedex Mainz, France). For the microbiota analysis of caecal inocula and fermentation samples of the final models 5 and 5*, the V4 region of the 16S rRNA gene was amplified with primers 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3'). Sequencing was performed with Illumina MiSeq (Genomic Diversity Centre, ETH Zurich, Zurich, Switzerland) with V2 reagent kit for 2 × 250 bp paired end Next Tera chemistry supplemented with 20% of PhiX. The raw sequence data has been submitted

to European Nucleotide Archive (ENA) database with accession number PRJEB30419. The open-source bioinformatics pipeline Quantitative Insight Into Microbial Ecology (QIIME) (Caporaso et al., 2010) was used to process the raw 16S rRNA gene sequencing data. The raw dataset containing pair-ended reads with corresponding quality scores were merged and trimmed using fastq_mergepairs and fastq_filter scripts implemented in the UPARSE pipeline (Edgar, 2013) as previously described (Krych et al., 2018). The minimum overlap length of trimmed reads was set to 50 bp (V4) or 20 bp (V3V4). The minimum length of merged reads was 180 bp (V4) or 200 bp (V3V4), the max expected error E = 2.0, and first truncating position with quality score $N \leq 4$. Purging the dataset from chimeric reads and constructing *de novo* Operational Taxonomic Units (OTU) were conducted using the UPARSE pipeline (Edgar 2013). The green genes 16S rRNA gene collection (version 13.8) was used as a reference database (Werner et al., 2012) and an OTU count table including taxonomy was generated. The OTUs assigned to the S24-7 family were reported in the manuscript as taxa of the *Muribaculaceae* family (Lagkouvardos et al. 2016). QIIME open source software package (1.8.0 and 1.9.0) was used for subsequent analysis, including alpha and beta diversity, and the PICRUSt (phylogenetic investigation of communities by reconstruction of unobserved states) (Langille et al., 2013) analysis to predict the KEGG metabolic pathways and COG functional groups from microbiota samples.

Statistical analysis

To compare SCFA concentrations between fermentation models, statistical tests were carried out using SigmaPlot 13.0 (SigmaPlot Software, La Jolla, CA, United States). Significance level was set at 0.05. Normality of the data set was tested with the Kolmogorov–Smirnov test. In case of normality, mean values of two different groups were compared with an independent samples *t*-test. In case of non-normality, differences were tested with non-parametric Mann–Whitney *U* test. Data are expressed as means ± standard deviations (SD) of 3 days of the stabilization and treatment periods with standard observed variations of metabolites lower than 10% in the same reactor in each fermentation. To assess if there were significant differences in taxa abundance between caecal inoculum and PolyFermS microbiota, or between different PolyFermS microbiota, the DESeq2 method (Love et al., 2014) was used on the unnormalised OTU count data as previously suggested by McMurdie and Holmes (McMurdie and Holmes, 2014).

Results

In the first step, caecal physiological parameters such as pH, bacterial fermentation metabolites and composition were investigated for the C57BL/6 mouse strain. This breed was selected to inoculate the fermenters because it is the most widely used strain in biomedical and gut microbiota research (Bryant, 2011). To establish the *in vitro* murine caecal microbiota model, we analyzed fermentation metabolites by HPLC and quantified specific bacterial populations, particularly the stress- and oxygen-sensitive families *Lachnospiraceae* and *Ruminococcaceae*, and the genus *Lactobacillus* by qPCR as main markers for representative bacterial groups of murine gut microbiota. Overgrowth of the *Enterobacteriaceae* family was also monitored to detect a potential lack of control of oxygen stress during sampling and start-up of fermentation. These microbial indicators were used to suggest subsequent changes during the sampling, bead colonization and continuous fermentation process to achieve a balance akin to the mouse caecum content used for immobilization and inoculation of the continuous fermentation model. The final conditions set for model 5 were repeated with two caecal inocula prepared from different mice (model 5*).

Composition of caecal WT C57BL/6 mouse microbiota and metabolites

Caecal pH in WT C57BL/6 mice (n = 15) ranged from 6.2 to 6.9 with a mean of 6.5 ± 0.2 (**Figure 3.1 A**). Caecal fermentation metabolites; the SCFAs acetate, propionate and butyrate; the intermediates succinate and lactate; and the BCFA isobutyrate, were detected in all murine caeca (**Figure 3.1 B)**. Important individual variations of total metabolite concentration (17.7–74.1 µmol/g; average of 56.7 ± 14.6 µmol/g), and of the main end metabolites acetate (28.5 ± 5.0 µmol/g), propionate (5.1 ± 1.4 µmol/g) and butyrate (23.5 ± 5.9 µmol/g) were measured, while succinate (between 1 and 11 µmol/g), lactate (1–2 µmol/g), and isobutyrate (1–2 µmol/g) were detected at lower concentrations. The average acetate:propionate:butyrate ratio was 50:9:41, which was in the range of previous reports in same mouse strain on similar chow (Krautkramer et al., 2016). Caecal microbiota were dominated by bacterial phyla Firmicutes (56.0 ± 8.0%) and Bacteroidetes (38.9 ± 7.3%), and also harbored Proteobacteria at lower levels (4.6 ± 1.3%) (**Figure 3.1 C**). Within Bacteroidetes, *Muribaculaceae* (S24-7) and *Rikenellaceae* were the most abundant families, and within the Firmicutes an unclassified Clostridiales family, *Lachnospiraceae* and *Ruminococcaceae* (**Figure 3.1 D**). The α-diversity Shannon index (H) ranged from 5.6 to 6.2 (average 5.9 ± 0.9) (data not shown). Principal Coordinate Analysis (PCoA) was used to analyze β-diversity to characterize the degree of individual variations among the caecal murine microbial communities. Mice of the same age co-housed within the same cage for 21 days or in different cages were analyzed. The caecal microbiota of mice housed in the same cage did not cluster and individual mouse microbiota scattered in both unweighted and weighted Unifrac distance PCoA (**Supplementary Figure S3.1**), indicating a large inter-individual variation in caecal microbiota composition.
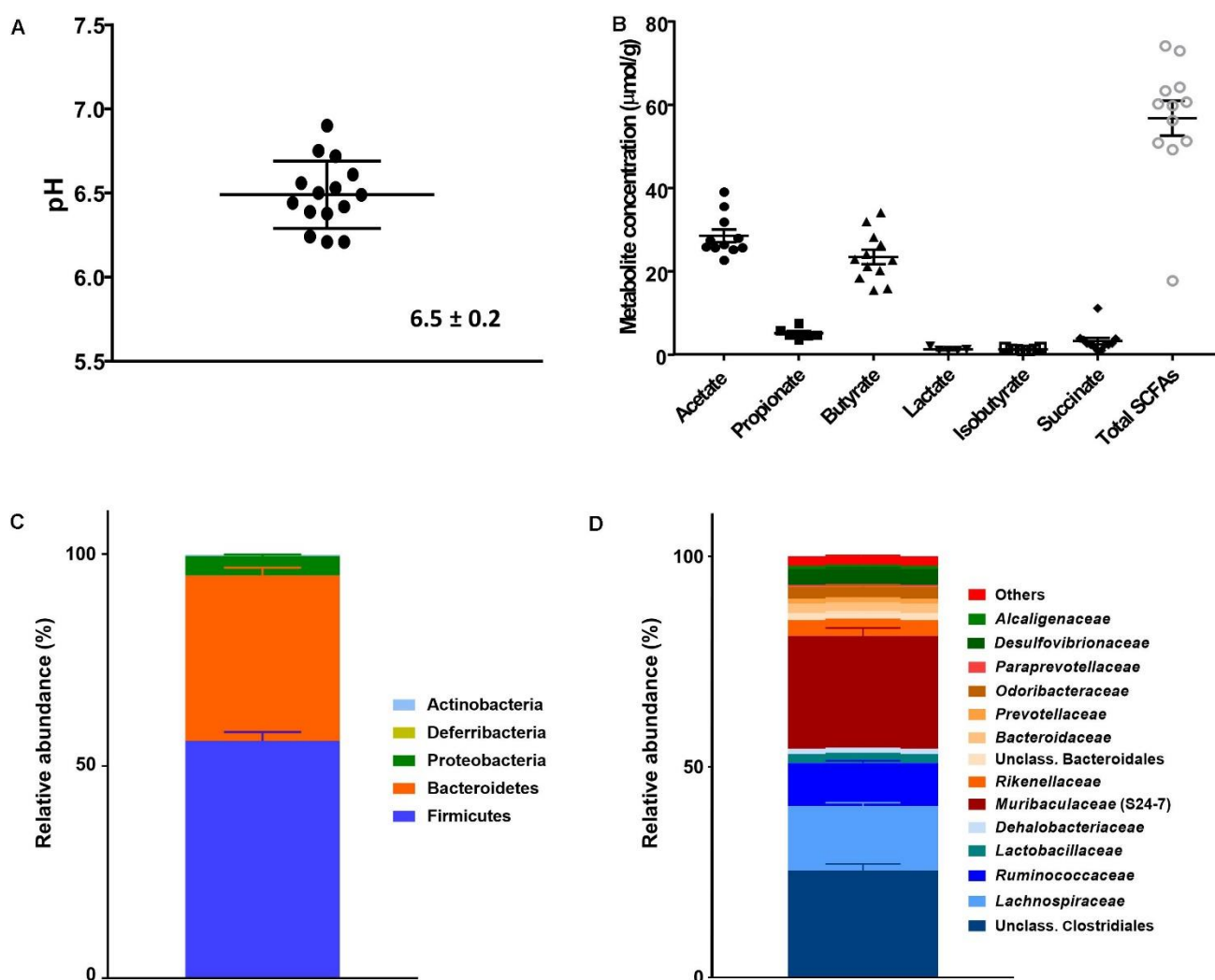
Figure 3.1: Analysis of caecal contents of WT C57BL/6 mice (means ± SEM; n = 15).
(A) pH; **(B)** Metabolite concentrations (μmol/g); **(C)** Microbial composition obtained by 16S rRNA amplicon sequencing and expressed as relative abundance at phylum and **(D)** family level. Data are mean ± SD. Values < 1% are summarized in the group «Others».

Development of *in vitro* model for murine caecal microbiota fermentation

During bead colonization, the effects of pH in combination with different nutritive media as well as initial batch fermentations conditions for bead colonization were assessed. During continuous operation, the impact of pH in combination with different RTs were investigated to improve maintenance of activity and composition of the murine caecal microbiota *in vitro* and compare to *in vivo* caecum data.

In model 1, caecal content sampling and processing was performed rapidly but under aerobic conditions and bead colonization was done at pH 7 in nutritive medium 1 supplemented with 20% (v/v) fermentation effluent during three batch cultures (**Table 3.1 A**). During continuous operation, constant conditions of pH 7 and RT 9 h were used. Metabolic stability was observed after 4 days of continuous fermentation with a mean total metabolite concentration of 115 ± 10 mM from day 4 to day 12 (**Figure 3.2 A** and **Supplementary**

Figure 3.2: Bacterial activity and composition of caecal inocula and reactor effluents of different models. **(A)** Concentrations of metabolites (mM) in caecal incolula and reactor effluents of stabilization phases expressed as mean metabolite concentrations with standard error. **(B)** Quantification of key bacterial populations in caecal inocula and fermenation samples of different models. Data obtained by qPCR and expressed as means ± SD log gene copies/g or mL when n > 1. BDL, below detection limit of 4 log10 gene copies.

Figure S3.2 A). Acetate (65 ± 4 mM) was the main produced metabolite, followed by butyrate (14 ± 3 mM) and propionate (15 ± 4 mM) (**Supplementary Table S3.3**). We observed higher levels of lactate, an intermediate fermentation metabolite, in the caecal inoculum compared to the first sampled caecal contents **(Figure 3.1 B)** and compared to *in vitro* levels **(Figure 3.2 A).** This might be because caecal contents for model 1 were sampled in the morning shortly after dark period and it was previously observed that lactate levels were then highest (Hamaguchi et al., 2015; Tahara et al., 2018). At the bacterial compositional level, a decrease in butyrate-producing families *Ruminococcaceae* (-1.2 log gene copies) and *Lachnospiraceae* (-0.6 log gene copies) was observed compared to the corresponding caecal inoculum (**Figure 3.2 B; Supplementary Table S3.4**). *Lactobacillus* spp. were also decreased *in vitro* compared to the caecal inoculum (-2.4 log gene copies vs. *in vivo*), while *Enterobacteriaceae* were increased (+1.5 log gene copies vs. *in vivo*).

We assumed that the short exposure to oxygen during sample collection favored the growth of facultative anaerobes, including *Enterobacteriaceae* and was detrimental to strict anaerobes such as bacterial taxa within Clostridiales. Therefore, in model 2 the sampling procedure was adapted to better protect the microbiota from oxygen. In addition, the batch fermentation medium was supplemented with lactate to enhance the growth and activity of lactate-consuming butyrate-producers within the bacterial order Clostridiales (Bourriaud et al., 2005; Tao et al., 2016). Metabolic stability of model 2 was reached after 12 days of continuous operation (**Figure 3.2 A; Supplementary Figure S3.2 B**) with mean total metabolite production of 114 ± 3 mM. In model 2, an acetate:propionate:butyrate (AA:PA:BA) ratio of 61:24:15 was observed, with slightly increased pr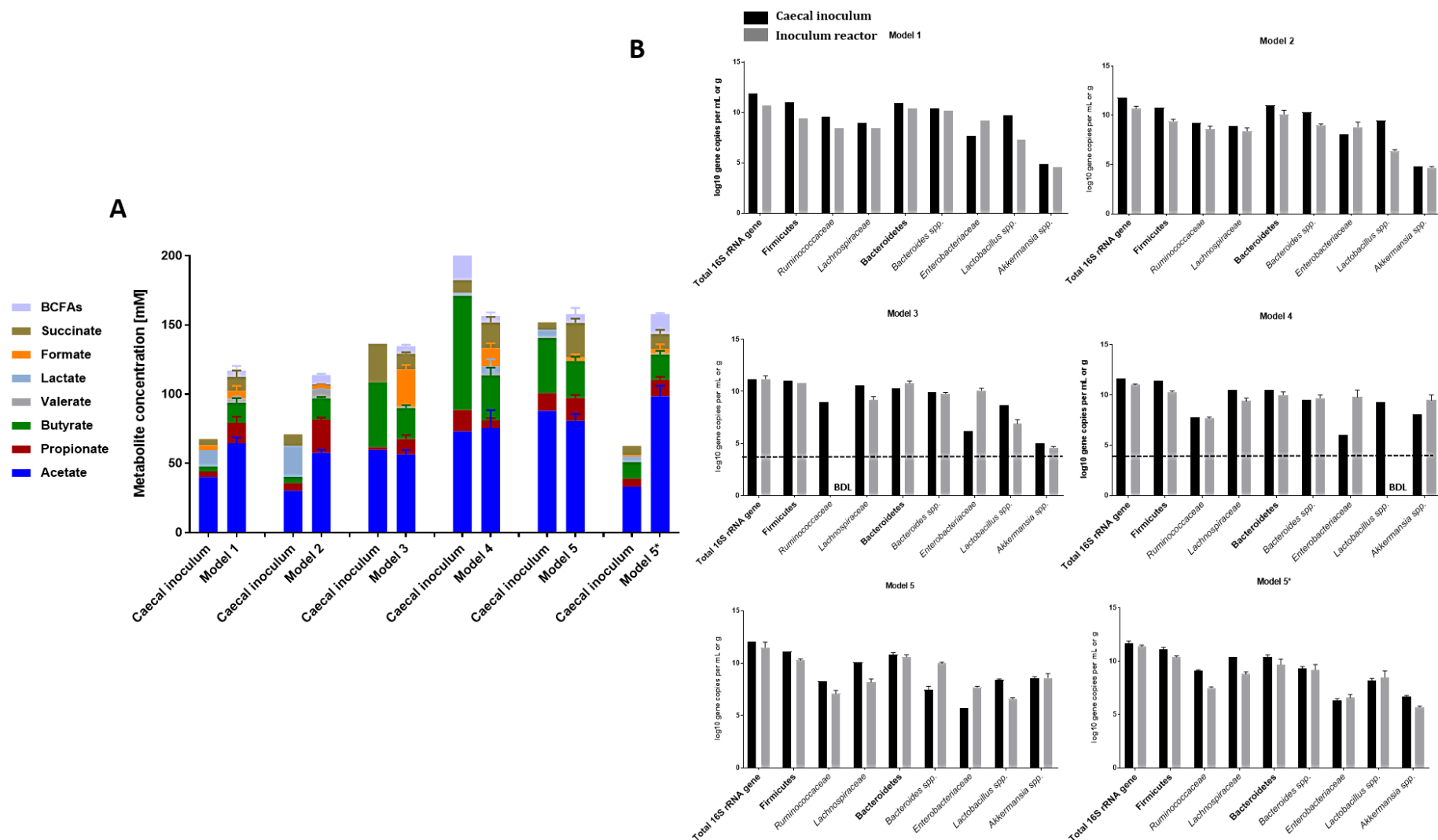opionate levels compared to the caecal inoculum (73:15:12) (**Supplementary Table S3.3**). Compared to the caecal microbiota inoculum, *Enterobacteriaceae* (+0.7 log gene copies) were increased in model 2 effluents, while *Lactobacillus* spp. (-3.1 log gene copies) and butyrate-producers (*Lachnospiraceae*: -0.5 log gene copies; *Ruminococcaceae*: -0.6 log gene copies) were decreased (**Figure 3.2 B; Supplementary Table S3.4**).

The medium of model 3 was supplemented with both cellobiose and rumen fluid to improve bead colonization of butyrate-producing bacteria and to enhance the growth of *Lactobacillus* spp. Rumen fluid contains primary nutrients for cross-feeding and microbial growth factors (Bryant, 1959; Kamada et al., 2013; van Zanten et al., 2014). In addition, the number of bead colonization batch fermentations was reduced to two, and the duration for the first batch was extended to 16 h to decrease the growth advantage of fast-growing *Enterobacteriaceae*, promote growth and activity of butyrate-producing bacteria (Clostridia), and achieve a more complete carbohydrate fermentation with high re-utilization of intermediate metabolites (lactate, formate, acetate, succinate). Metabolic stability of model 3 was reached after 15 days, with a higher mean total metabolite concentration (135 ± 4 mM) compared to model 2 ($p < 0.05$) and model 1 ($p < 0.05$) and a AA:PA:BA molar ratio of 63:12:25 (**Figure 3.2 A; Supplementary Table S3.3; Supplementary Figure S3.3 A**). However, concentrations of the butyrate-producing families *Lachnospiraceae* (-1.1 log gene copies vs. *in vivo*) and *Ruminococcaceae* (below detection limit) remained low compared to caecal inoculum. Further, lower levels

of *Lactobacillus* spp. (-1.8 log gene copies) and higher levels of *Enterobacteriaceae* (+3.9 log gene copies) were detected in the effluent samples compared to the caecal inoculum (**Figure 3.2 B; Supplementary Table S3.4**).

Other possible strategies to prevent the outgrowth of *Enterobacteriaceae* are reducing the concentration of simple carbohydrates in the nutritive medium (here: corn starch), and decreasing the pH (from pH 7 to 5.8 during batch fermentation and from pH 7 to 6.5 during continuous operation), since optimal growth pH of these bacteria is close to neutrality (**Supplementary Table S3.1; Table 3.1**). After 19 days of continuous culture, steady metabolite production was reached with higher total metabolite production (154 ± 13 mM) compared to previous model 3 ($p < 0.05$) and a AA:PA:BA ratio of 66:4:29 (**Figure 3.2 A; Supplementary Table S3.3; Supplementary Figure S3.3 B**). High butyrate levels were observed in caecal inoculum (83 mM) and reactor effluent (33 mM), which can be associated with high and comparable levels of butyrate-producing families *Ruminococcaceae* (7.7 ± 0.1 log gene copies/mL and 7.5 log gene copies/g, respectively) and *Lachnospiraceae* (9.4 ± 0.3 log gene copies/mL and 10.5 log gene copies/g, respectively (**Figure 3.2 B; Supplementary Table 3.S4**). However, the concentration of *Enterobacteriaceae* remained high (+3.8 log gene copies/mL compared to caecal inoculum) and *Lactobacillus* spp. remained below the detection limit.

A recent study found that some bacterial populations within Clostridiales order are positively associated with long RTs in humans, which may also promote growth conditions for the slow-growing bacterial populations in our model (Roager et al., 2016). Therefore, in model 5 we assessed whether an increase in RT from 9 h (model 4) to 12 h can prevent overgrowth of fast-growing *Enterobacteriaceae*. A high total metabolite production was obtained (158 ± 9 mM) with higher levels of propionate ($p < 0.05$) and lower levels of the intermediate metabolite formate ($p < 0.05$) compared to model 4 (**Figure 3.2 A; Supplementary Figure S3.4; Supplementary Table S3.3**). The AA:PA:BA ratio *in vitro* (65:14:22) was comparable with the ratio detected in the caecal inoculum (62:9:28). Concentrations of potential butyrate-producing bacterial markers *Lachnospiraceae* (8.2 ± 0.3 log gene copies/mL vs. 10.1 ± 0.0 log gene copies/g) and *Ruminococcaceae* (7.1 ± 0.3 log gene copies/mL vs. 8.3 ± 0.0 log gene 372 copies/g) were lower in effluent samples compared with caecal inoculum levels (**Figure 3.2 B; Supplementary Table S3.4**). Despite this, increased retention resulted in less overgrowth of *Enterobacteriaceae* (5.7 ± 0.1 log gene copies/g caecal content vs. 7.7 ± 0.1 log gene copies/mL model 5 effluent) and no severe loss of *Lactobacillus* spp. (8.4 ± 0.1 log gene copies/g caecal content vs. 6.6 ± 0.1 log gene copies/mL model 5 effluent) compared to model 4.

As model 5 conditions reflected the metabolic and bacterial concentrations of the mouse caecum adequately, an independent repetition of model 5 was performed (starting from another caecal microbiota inoculum; referred to as model 5*). The total fermentation metabolite production (161 ± 7 mM) was comparable to levels in model 5 (**Figure 3.2 A; Supplementary Table S3.3** Overall, the metabolite production in both model 5 and 5* was stable during continuous operation of 69 and 43 days, respectively (**Supplementary Figures S3.4 &**

S3.5). At the bacterial marker level, comparable concentrations of *Bacteroides* spp. and *Akkermansia* spp. were detected in model 5* effluent samples compared to its caecal inoculum (**Supplementary Table S3.4**).). Interestingly, the *in vitro Akkermansia* spp. levels (5.7 ± 0.1 log gene copies/mL) reflected the lower concentrations present in caecal inoculum (6.7 ± 0.1 log gene copies/g in caecal inoculum 5* vs. 8.6 ± 0.1 log gene copies/g in caecal inoculum 5). *Enterobacteriaceae* spp. (6.6 ± 0.3 log gene copies/mL) and *Lactobacillus* spp. (8.5 ± 0.6 log gene copies/mL) established at comparable levels to those detected in the caecal inoculum (6.3 ± 0.1 log gene copies/g and 8.2 ± 0.2 log gene copies/g, respectively).

Microbiota analysis of final *in vitro* continuous fermentation murine caecum model

To assess the overall microbiota composition and diversity in comparison to the caecal inocula, reactor effluent microbiota of model 5 and 5* were further analyzed by 16S rRNA gene sequencing. The composition of reactor microbiota differed from the caecal inoculum microbiota as indicated in a PCoA-biplot on weighted and unweighted UniFrac distance (**Figure 3.3 A & B**). Furthermore, model 5 microbiota differed from model 5* microbiota as shown by a spatial separation and clustering in both unweighted and weighted UniFrac PCoA biplots. The two caecal inocula microbiota showed scattering in the unweighted UniFrac PCoA, indicating qualitative differences in their composition.

The bacterial diversity within the reactor microbiota was lower compared to the caecal inocula microbiota (**Figure 3.3 C**). Both the Shannon diversity-index that takes into account the number of observed OTUs and their relative evenness and the observed species diversity decreased from 5.2 for caecal inocula to 2.7 ± 0.6 for reactor effluent samples and from 130 to 35, respectively.

Overall abundance shifts from *in vivo* to *in vitro* caecal microbial communities occurred (**Figure 3.3 D & E**). Compared to the caecal inocula, the Bacteroidetes phylum abundance increased while Firmicutes phylum abundance decreased in both *in vitro* microbiota; this shift was most pronounced in model 5* (**Figure 3.3 D, Supplementary Table S3.5**). Certain genera and species from the caecal inoculum flourished *in vitro*, while others became established at a lower abundance or below the detection limit. This resulted in significant and large log2 fold changes in abundance when comparing caecal inoculum to corresponding fermentation samples using DeSeq2 analysis (**Supplementary Figure S3.6 & S3.7**). Taxa from *Bacteroides, Anaerococcus* and unclassified *Clostridiaceae* genera were enriched *in vitro* in both models with a log2 fold change exceeding 4. Some abundant (>1%) taxa in the caecal inocula established at a lower abundance (unclassified Clostridiales and *Lactobacillus* genera) or were not detected (taxa from *Muribaculaceae* (S24-7) family).

Compositional differences between the two *in vitro* microbiota were observed, which were in line with the quantitative differences detected by qPCR (**Supplementary Figure S3.8**). Model 5 *in vitro* microbiota was characterized by taxa belonging to *Akkermansia*, *Enterobacteriaceae*, *Parabacteroides*, and *Clostridiaceae*,

while in model 5∗, *in vitro* microbiota taxa belonging to *Lactobacillus*, *Peptostreptococcaceae, Blautia*, and *Anaerofilum* established better compared to model 5.

We next predicted the gene content from the 16S rRNA sequence data by PICRUSt (phylogenetic investigation of communities by reconstruction of unobserved states) for revealing potential functional differences between both *in vitro*- and caecal inocula microbiota. Conserved metabolic and functional KEGG pathways were observed in both microbiota types (**Supplementary Figure S3.9**), indicating a similar microbial functional potential between *in vitro*- and caecal inocula microbiota.
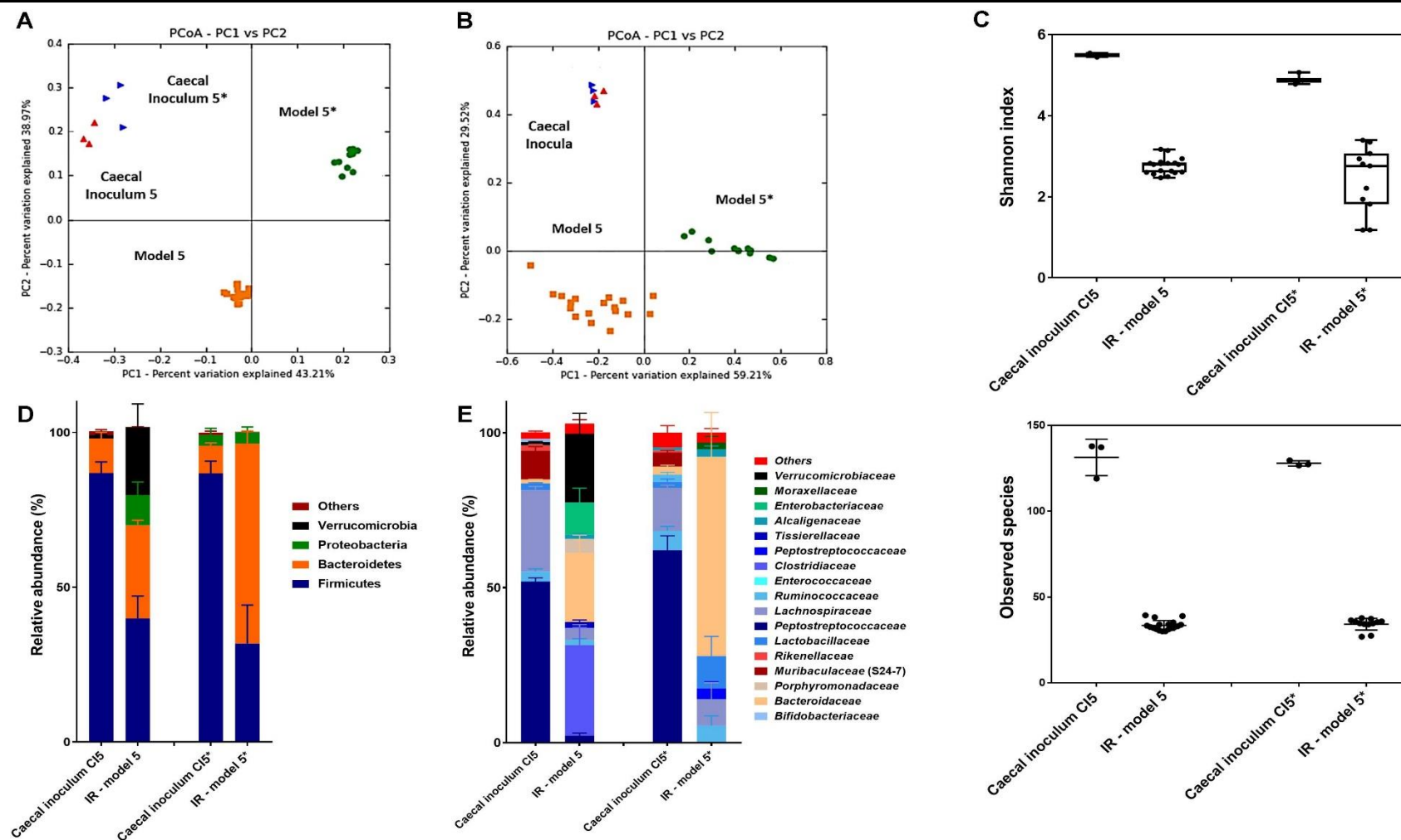
Figure 3.3: Microbial composition and diversity analysis in caeal inocula and reactor effluents of model 5 and 5*. (**A and B**) Principle Coordinate Analysis (PCoA) of caecal inocula and reactor microbiota based on unweighted (**A**) and weighted (**B**) UniFrac analysis matrix on OTU level. Each point represents a microbiota sample from murine caecal content used as inoculum for model 5 (red Δ) and model 5* (blue Δ) or from stabilized reactor effluents samples of model 5 (orange □) and model 5* (green ○). (**C and D**) Alpha diversity measured by Shannon diversity index and observed species. Boxes represent the interquartile range (IQR) between the 5th and 95th percentiles, respectively. (D and E) Microbial composition in caecal samples of WT C57BL/6 mice obtained by 16S rRNA amplicon sequencing. Relative abundance at phylum and family level of caecal inocula and fermentation samples of model 5 and 5*. Data are mean ± SD. Values < 1% are summarized in the group «Others».

Discussion

In this study, we aimed to develop a continuous *in vitro* fermentation model that reflects the metabolic activity and phylogeny of healthy WT mouse caecal microbiota. An important prerequisite for *in vitro* studies is the rational selection of models and conditions, while keeping in mind that these models can never completely represent reality (Lacroix et al., 2015). Therefore, we followed a stepwise approach for model development by adjusting parameters to reach fermentation metabolite profiles and marker bacterial levels similar to the *in vivo* situation.

During continuous operation a stable *in vitro* fermentation with main fermentation metabolites acetate, propionate and butyrate was reached, which is in line with *in vivo* measurements in this and other studies reporting ratios of murine caecal microbial fermentation metabolites (Burokas et al., 2017; Herrmann et al., 2018; Krautkramer et al., 2016). *In vivo*, the SCFA are continuous and efficiently absorbed by the intestinal epithelium (Morrison and Preston, 2016), resulting in an underestimation of the actual caecal fermentation capacity based on caecal SCFA measurements. Accordingly, the total SCFA concentrations *in vitro* were higher than *in vivo* because absorption is not simulated. Hence, model values reflect the total fermentation capacity of the modeled caecal microbiota. First models showed a limited fermentation capacity, but thanks to the optimization steps the total metabolite production increased from 115 mM in model 1 to 159 mM and 161 mM in model 5 and 5*, respectively. Along with the improved fermentation capacity, a simultaneous longer stabilization time of the models was observed; this might be explained by the growth and balance of a more complex microbiota reliant on cross-feeding mechanisms. A lower pH during startup and continuous operation (models 3–4) stimulated bacterial fermentation as previously observed in human microbiota fermentation models (Walker et al., 2005; Zihler Berner et al., 2013). Increased RT (model 4–5) resulted in decreased accumulation of the intermediate metabolites formate and lactate, and higher levels of branched SCFA, which are specific markers for protein fermentation and associated with long RTs (Davila et al. 2013; Tottey et al. 2017). Succinate was after acetate, propionate and butyrate the microbial metabolite detected at the highest concentrations in all caecal murine fermentation models and their respective caecal inocula. In humans, succinate is considered an intermediate metabolite in the global intestinal microbiota fermentation process (Macfarlane and Macfarlane, 2003) , since several gut bacteria can convert succinate to propionate or butyrate (Louis and Flint, 2017). In mice, high levels of caecal succinate were reported in response to dietary fiber treatment (Everard et al., 2014) and high succinate levels have been demonstrated to improve glucose metabolism via intestinal gluconeogenesis (De Vadder et al., 2016). When sufficient carbohydrates are present, Bacteroides taxa show reduced need to decarboxylate succinate; thus succinate accumulates instead of propionate (Macy et al., 1978). Furthermore, in Bifidobacteria succinate production is associated with growth (Van der Meulen et al., 2006a), which may explain the high levels detected in our continuous fermentation model due to continuous supply of carbohydrates and therefore growth of these bacteria. The

qualitative assessment of the predicted microbial functions by PICRUSt indicated that the gene contents of most pathways were maintained in our model, despite changes in abundances of bacterial populations. These results also suggest that the reactor microbiota as a whole did not change its functional fermentation potential, such as metabolic cross-feeding pathways, from *in vivo* (caecal inoculum) to *in vitro* (reactor).

The *in vitro* murine caecal microbiota was mainly composed of the bacterial phyla Firmicutes and Bacteroidetes, both in the range of *in vivo* caecal microbiota compositions, previously reported (Clavel et al., 2016; Hugenholtz and de Vos, 2017; Ley et al., 2006). However, there was a shift toward higher Bacteroidetes levels compared to caecal inocula; similar shifts were reported for *in vitro* human intestinal microbiota models (Fehlbaum et al., 2015; McDonald et al., 2015; Rajilic-Stojanovic et al., 2010; Van den Abbeele et al., 2010). The high levels of complex carbohydrates in nutritive media used for intestinal fermentation models may favor the growth of Bacteroidetes taxa, since they have a higher glycan-degrading capacity compared to Firmicutes species (Mahowald et al., 2009). The taxa within murine bacterial families that were maintained in the *in vitro* fermentations belong to important functional groups for intestinal fermentation such as primary fibrolytic (*Bacteroidaceae*, *Ruminococcaceae*, *Bifidobacteriaceae*), glycolytic (*Lactobacillaceae, Enterococcaceae, Enterobacteriaceae)* and mucolytic *(Verrucomicrobiaceae, Bacteroidaceae)* bacteria*;* and secondary butyrate- and propionate-producing bacteria (*Lachnospiraceae*, *Ruminococcaceae*, *Erysipelotrichaceae, Rikenellaceae, Verrucomicrobiaceae*) (Chassard and Lacroix, 2013). The prevalent murine intestinal bacterial family *Muribaculaceae* (S24-7) was not maintained in our *In vitro* model. Only recently, Lagkouvardos et al. (2016) succeeded to isolate and cultivate the first strain of this family in a medium containing meat and blood. Interestingly, a recent study reported that this *Muribaculaceae* (S24-7) strain is extremely sensitive to high osmolality (Tropini et al., 2018) and the higher osmolality in our reactors compared to caecum may also explain its low establishment *in vitro*. Further characterization of the physiology and nutritional requirements of strains from the *Muribaculaceae* family and other taxa that were not maintained *in vitro* will be important to further optimize our murine nutritive fermentation medium. Adjustments to nutritive medium, pH and RT helped to control the *in vitro* levels of *Enterobacteriaceae*, which often bloom in fermentation models (McDonald et al. 2015; Fehlbaum et al. 2015) due to their competitive advantage during initial colonization and balancing of the fermentation model.

There was an important overall decrease in bacterial diversity *in vitro* compared to the high caecal inoculum diversity, which were in the range of published Shannon diversity indices ranging from 4.5 to 6 (Holm et al., 2015; Hu et al., 2017; Nagpal et al., 2018). Such effects were also observed in other *in vitro* fermentation models inoculated with high diverse intestinal microbial communities from humans (infants, adult and elderly) and swine (Feria-Gervasio et al., 2014; McDonald et al., 2015; Poeker et al., 2018; Rajilic-Stojanovic et al., 2010; Van den Abbeele et al., 2010; Tanner et al., 2014). *In vitro* models cannot simulate all the conditions occurring in the host, which are not well-known or cannot be mimicked such as immune response, variation in feed rates

and composition, hormonal and digestive secretions (e.g., bile), feedback mechanisms, absorption and peristaltic movements, all of which influence microbial diversity and can only be an approximation of realistic conditions (Lacroix et al., 2015). The well-controlled conditions *in vitro* may result in a loss of redundant species or species thriving on specific host secretions. Murine intestinal bacterial isolation efforts (Lagkouvardos et al. 2016) offer the opportunity to identify species-specific growth requirements and will allow adaptation of fermentation conditions for their improved establishment *in vitro*. Finally, the presence of transient bacteria, i.e., from upper gastrointestinal tract, diet and ingested microbes due to coprophagy, in the caecal inocula may also overestimate the bacterial diversity of the resident caecal community. By mimicking the murine caecal conditions, we obtained a microbial community composed of caecal murine-derived bacteria, with reduced complexity but resulting in a stable and functional caecal fermentation.

Mouse experiments have gained attention as tool to study the gut microbiota in health and disease. However, their poor reproducibility within and between facilities was associated with high variability among the mouse gut microbiota, which makes it hard to draw robust conclusions (Laukens et al., 2016).). Factors contributing to the heterogeneity of the mouse gut microbiota within a facility include differences in food intake (Zarrinpar et al., 2014) , maternal effects (Hufeldt et al., 2010), hormones (Org et al., 2015), cage (Hildebrand et al., 2013), presence of surrounding animals (Rausch et al., 2016) and other stressors (Bangsgaard, 2012) and environmental factors. To control for these factors, our strategy is to uncouple the microbiota from the host and study it under very well-controlled conditions (e.g., fermenter). In contrast to *in vivo* models, *in vitro* models allow study of the dynamics of the complex microbiota following manipulations, particularly to follow production of *in situ* fermentation metabolites, which are partly absorbed *in vivo*. Identified microbes or metabolites that drive microbiota functionality can then be validated further *in vivo*. Moreover, the continuous cultivated *in vitro* murine caecal microbiota can be used as transplantation material to study host–microbiota interactions in murine models of health and disease. This approach is currently performed with pooled murine fecal material, which is less controlled and available, or with bacterial consortia of human- or mouse-derived strains (Clavel et al., 2016). Recently the Oligo-Mouse-Microbiota (Brugiroux et al., 2016), a mixture of up to 15 intestinal murine-derived strains from the mouse intestinal bacterial collection (Lagkouvardos et al. 2016), was established following a bottom-up approach of rational strain selection. Alternatively, with our novel murine caecal fermentation model we can follow a top–down approach of creating functional different murine-derived bacterial communities with higher diversity compared to current consortia.

We showed that it is feasible to maintain a stable and simplified, but yet representative *in vitro* murine microbiota in a continuous murine caecal fermentation model inoculated with immobilized caecal microbiota. Our simplified, yet representative *in vitro* murine bacterial community showed a similar functionality to inoculum microbiota. We demonstrated that it is feasible to continuously cultivate caecal murine microbiota while maintaining its overall functionality over a long time period. Our model is a first step in the development

of a mouse microbiota model system. With the expected increased knowledge of mouse gut isolates, further improvements of our murine *in vitro* model can be carried out by fine-tuning operational or nutritional requirements, and hence to increase preservation of microbial diversity in our model. In addition, our model can be expanded further with second-stage reactors, continuously inoculated with reactor effluent to allow parallel testing of different manipulations on the same microbiota. Hence, our novel *in vitro* model is a promising tool for studying the murine microbe–microbe interactions in response to biotic or abiotic factors that are linked to gut microbial functionality and structure.

Acknowledgments

Supporting Material

Supplementary Table S3.1: Composition of murine nutritive growth medium (g/L) (A) and of three nutritive mouse chows (B).

A

Constituent	Medium 1 g/l	Medium 2 g/l
Pectine (citrus)	2	2
Xylane (oat spelts)	4.8	4.8
Arabinogalactan (larch wood)	4.8	4.8
Soluble starch (corn)	6.8	2
Mucine	4	4
Casein acid hydrolysate	3.6	3.6
Peptone water	6	6
Bacto™ Tryptone	6	6
Yeast extract	5.4	5.4
L-Cystein HCl	0.8	0.8
Bile salts (adult formulation: 0.4)	0.4	0.4
KH_2PO_4	0.5	0.5
$NaHCO_3$	1.5	1.5
NaCl	4.5	4.5
KCl	4.5	4.5
$MgSO_4$ anhydrous (M: 120.37)	0.61	0.61
$CaCl_2 \cdot 2H_2O$ (M: 147.02)	0.1	0.1
$MnCl_2 \cdot 4H_2O$ (M: 197.91)	0.2	0.2
$FeSO_4 \cdot 7H_2O$ (M: 278.02)	0.005	0.005
Hemin solution	0.05	0.05
Tween 80	1 ml	1 ml
Vitamin solution	1 ml	1 ml

B

KLIBA NAFAG 3430 Mouse and Rat		
Carbohydrates	58.7%	approx 68 % fibers
Proteins	18.5%	
Fat/ash	10.8%	

Supplementary Table S3.2: Primers used for the enumeration of specific bacterial populations in caecal and effluent samples by qPCR analysis

Name	Sequence 5'-3'	Target gene	Reference
Eub 338F	ACT CCT ACG GGA GGC AGC AG	Total bacteria	Guo et al., 2008
Eub 518R	ATT ACC GCG GCT GCT GG		
Firm 934F	GGA GYA TGT GGT TTA ATT CGA AGC A	Firmicutes	Guo et al., 2008
Firm 1060R	AGC TGA CGA CAA CCA TGC AC		
Clep 866mF	TTA ACA CAA TAA GTW ATC CAC CTG G	*Clostridium* Cluster IV	Ramirez-Farias et al., 2009
Clep 1240mR	ACC TTC CTC CGT TTT GTC AAC		
Clep 14aF	AAA TGA CGG TAC CTG ACT AA	*Clostridium* Cluster XIVa	Matsuki et al., 2002
Clep 14aR	CTT TGA GTT TCA TTC TTG CGA A		
Bac 303F	GAA GGT CCC CCA CAT TG	Bacteroidetes	Bartosch et al., 2004
Bact Pre-rev	CTT TGA GTT TCA CCG TTG CCG G		
Bac 303F	GAA GGT CCC CCA CAT TG	*Bacteroides* spp.	Ramirez-Farias et al., 2009
Bfr-Femrev	CGC KAC TTG GCT GGT TCA G		
Eco 1457F	CAT TGA CGT TAC CCG CAG AAG AAG C	*Enterobacteriaceae*	Bartosch et al., 2004
Eco 1652R	CTC TAC GAG ACT CAA GCT TGC		
F_Lacto 05	AGC AGT AGG GAA TCT TCC A	*Lactobacillus | Leuconostoc | Pediococcus* spp.	Furet et al., 2009
R_Lacto 04	CGC CAC TGG TGT TCY TCC ATA TA		
AM1	CAG CAC GTG AAG GTG GGG AC	*Akkermanisa* spp.	Collado et al., 2007
AM2	CCT TGC GGT TGG CTT CAG AT		
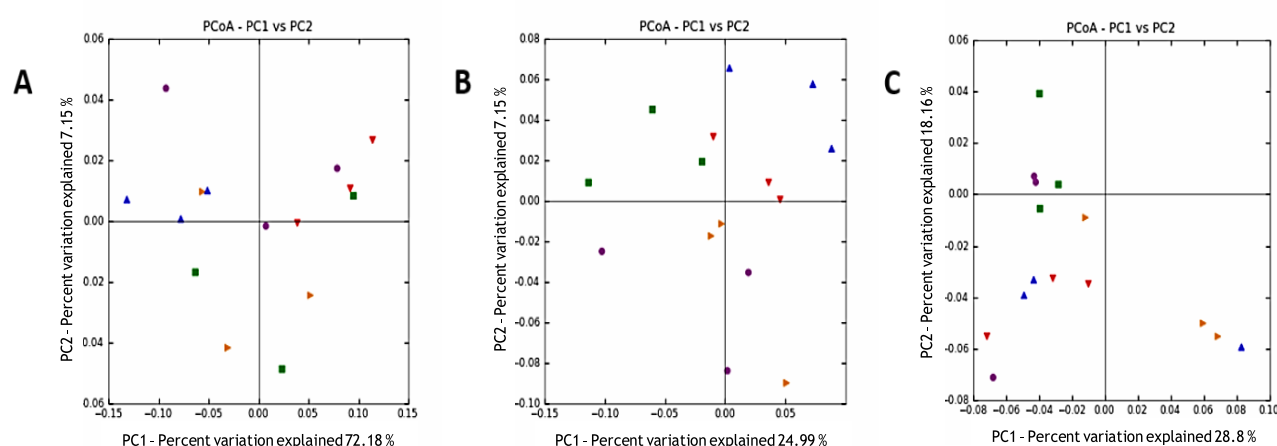
Supplementary Figure S3.1: Individual variations among the caecal murine microbial communities. PCoA showing caecal microbiota from WT C57BL/6 mice housed in different cages on weighted (**A**), unweighteg (**B**) and generalized (**C**) UniFrac distance matrix. N=3 per cage; cage 1: red; cage 2: blue; cage 3: orange; cage 4:green; cage 5: pruple.

Supplementary Table S3.5: Summary microbial phyla and most abundant (>1 %) bacterial families obtained by V4 region 16S amplicon sequencing within PolyFermS reactors of model 5 and 5*. Values < 1% are summarized in the group «Others».

Taxon	Caecal inoculum 5	IR Model 5	Caecal inoculum 5*	IR Model 5*
Actinobacteria	**0.7% ± 0.5%**	**0.1% ± 0.1%**	**0.3% ± 0.1%**	**0.4% ± 0.9%**
Actinomycetaceae	0.0% ± 0.0%	0.0% ± 0.0%	0.0% ± 0.0%	0.3% ± 0.7%
Microbacteriaceae	0.0% ± 0.0%	0.0% ± 0.0%	0.0% ± 0.0%	0.0% ± 0.0%
Bifidobacteriaceae	0.3% ± 0.6%	0.0% ± 0.0%	0.1% ± 0.0%	0.1% ± 0.1%
Coriobacteriaceae	0.4% ± 0.1%	0.1% ± 0.1%	0.3% ± 0.0%	0.1% ± 0.1%
Bacteroidetes	**11.1% ± 3.1%**	**29.1% ± 6.4%**	**9.1% ± 1.7%**	**64.7% ± 14.2%**
UC Bacteroidales	0.1% ± 0.1%	0.0% ± 0.0%	0.0% ± 0.0%	0.0% ± 0.0%
Bacteroidaceae	0.9% ± 0.5%	24.4% ± 7.3%	2.6% ± 0.5%	64.3% ± 14.3%
Porphyromonadaceae	0.0% ± 0.0%	4.7% ± 2.1%	0.4% ± 0.2%	0.2% ± 0.1%
Prevotellaceae	0.2% ± 0.2%	0.0% ± 0.0%	0.2% ± 0.1%	0.0% ± 0.0%
Rikenellaceae	1.4% ± 0.4%	0.0% ± 0.0%	0.6% ± 0.7%	0.2% ± 0.2%
Muribaculaceae	8.2% ± 2.1%	0.0% ± 0.0%	4.7% ± 1.0%	0.0% ± 0.0%
Odoribacteraceae	0.1% ± 0.1%	0.0% ± 0.0%	0.0% ± 0.0%	0.0% ± 0.0%
Paraprevotellaceae	0.1% ± 0.1%	0.0% ± 0.0%	0.6% ± 0.4%	0.0% ± 0.0%
Deferribacteraceae	0.1% ± 0.1%	0.0% ± 0.0%	0.3% ± 0.3%	0.0% ± 0.0%
Firmicutes	**86.9% ± 3.3%**	**40.0% ± 7.5%**	**86.7% ± 4.0%**	**31.8% ± 12.5%**
Planococcaceae	0.0% ± 0.0%	0.0% ± 0.0%	0.0% ± 0.0%	0.1% ± 0.2%
Enterococcaceae	0.0% ± 0.0%	0.8% ± 0.3%	0.0% ± 0.0%	0.1% ± 0.1%
Lactobacillaceae	5.6% ± 5.9%	0.2% ± 0.2%	2.0% ± 0.8%	11.5% ± 4.8%
Leuconostocaceae	0.0% ± 0.0%	0.0% ± 0.0%	0.0% ± 0.0%	0.8% ± 0.5%
Streptococcaceae	0.0% ± 0.0%	0.0% ± 0.0%	0.0% ± 0.0%	0.4% ± 0.5%
UC Clostridiales	57.8% ± 10.9%	2.0% ± 1.3%	62.1% ± 4.6%	0.0% ± 0.0%
Clostridiaceae	0.1% ± 0.0%	28.7% ± 8.0%	0.0% ± 0.0%	0.9% ± 0.8%
Dehalobacteriaceae	0.4% ± 0.3%	0.0% ± 0.0%	0.2% ± 0.1%	0.0% ± 0.0%
Eubacteriaceae	0.0% ± 0.0%	0.0% ± 0.0%	0.0% ± 0.0%	0.0% ± 0.0%
Lachnospiraceae	19.6% ± 12.4%	3.6% ± 1.3%	13.7% ± 1.2%	8.5% ± 5.3%
Peptostreptococcaceae	0.0% ± 0.0%	0.0% ± 0.0%	0.0% ± 0.0%	3.3% ± 2.2%
Ruminococcaceae	3.3% ± 0.9%	1.4% ± 0.7%	6.4% ± 1.5%	5.5% ± 3.1%
Veillonellaceae	0.0% ± 0.0%	0.8% ± 0.5%	0.0% ± 0.0%	0.0% ± 0.0%
Mogibacteriaceae	0.1% ± 0.0%	0.0% ± 0.0%	0.0% ± 0.0%	0.0% ± 0.0%
Tissierellaceae	0.0% ± 0.0%	2.0% ± 1.1%	0.0% ± 0.0%	0.4% ± 0.4%
Erysipelotrichaceae	0.1% ± 0.1%	0.6% ± 0.4%	2.2% ± 0.6%	0.3% ± 0.2%
Proteobacteria	**0.2% ± 0.0%**	**10.5% ± 6.7%**	**3.5% ± 1.9%**	**3.1% ± 2.0%**
Alcaligenaceae	0.1% ± 0.0%	0.5% ± 0.6%	0.5% ± 0.3%	1.8% ± 1.3%
Desulfovibrionaceae	0.1% ± 0.0%	0.0% ± 0.0%	1.1% ± 0.3%	0.0% ± 0.0%
Helicobacteraceae	0.0% ± 0.0%	0.0% ± 0.0%	1.8% ± 1.4%	0.0% ± 0.0%
Enterobacteriaceae	0.0% ± 0.0%	10.0% ± 6.6%	0.0% ± 0.0%	0.3% ± 0.3%
Moraxellaceae	0.0% ± 0.0%	0.0% ± 0.0%	0.0% ± 0.0%	1.1% ± 1.5%
Verrucomicrobia	**0.8% ± 0.7%**	**20.3% ± 7.8%**	**0.0% ± 0.0%**	**0.0% ± 0.0%**
Verrucomicrobiaceae	0.8% ± 0.7%	20.3% ± 7.8%	0.0% ± 0.0%	0.0% ± 0.0%

Supplementary Table S3.3: Concentrations of metabolites (mM) and ratios measured by HPLC in fermentation effluents of stabilized fermentation periody and caecal contents. Mean metabolite concentrations with standard error of effluent samples in reactor effluents during stable stabilization phase and caecal contents (n=15).

	Concentrations (mM)									Ratios (%)			Days
	Formate	Acetate	Propionate	Butyrate	Valerate	Lactate	Succinate	BCFAs	Total metabolites	Acetate	Propionate	Butyrate	
Model 1													
Caecal content[1]	3.5	40.2	3.9	3.6	ND	12.0	4.5	ND	55.7	83.3	8.3	8.3	
IR[2]	5.4±3.6	64.6±4.3	14.9±4.4	14.4±3.4	2.4±2.3	0.8±0.1	10.1±4.6	4.6±3.3	114.8±9.7	69.1	16.0	14.9	d4-12
Model 2													
Caecal content[1]	ND	30.2	5.6	4.6	ND	22.2	8.4	ND	48.8	73.2	14.6	12.2	
IR[2]	2.6±0.7	57.6±2.4	24.1±1.4	15.3±0.9	5.8±0.2	1.0±0.1	0.8±0.2	7.0±0.9	114.0±3.2	60.6	24.2	15.2	d12-28
Model 3													
Caecal content[1]	ND	59.8	ND	46.7	ND	ND	28.0	0.8	88.6	56.1	ND	43.9	
IR[2]	17.8±9.0	56.5±3.2	11.2±2.8	22.5±1.9	ND	0.5±0.2	11.3±1.0	5.5±1.1	134.8±4.4	62.6	12.1	25.3	d15-21
Model 4													
Caecal content[1]	ND	73.2	15.4	82.5	0.7	1.5	9.1	152.6	335.0	42.7	8.8	48.5	
IR[2]	12.7±1.8	74.8±9.6	5.1±1.9	33.4±2.2	1.0±0.0	3.8±1.0	19.7±1.7	2.5±0.4	154.3±13.4	66.4	4.4	29.2	d19-34
Model 5													
Caecal inoculum[1]	ND	88.0	12.8	39.9	0.8	5.2	5.3	ND	152.0	62.4	9.2	28.4	
IR[2]	2.7±2.1	80.4±5.1	16.8±2.1	26.6±3.0	ND	ND	25.1±3.4	3.5±2.2	158.8±8.9	64.5	13.7	21.8	d26-69
Model 5*													
Caecal inoculum[1]	1.2	33.2	5.9	11.8	0.5	3.5	6.5	ND	59.1	64.7	11.8	23.5	
IR[2]	3.8±3.1	98.5±7.5	12.0±1.4	18.2±2.7	ND	1.3±0.5	10.5±3.1	14.4±1.1	160.9±7.4	76.7	9.3	14.0	d13-42

[1] extracted from 100 mg of caecal content

[2] Mean and standard error reported over whole stabilized fermenation time

Supplementary Table S3.4: qPCR quantification of bacterial populations in caecal inocula and fermentation samples of different models at the end of the stabilization phase.

	Total 16S rRNA gene	Firmicutes	*Ruminococcaceae*	*Lachnospiraceae*	Bacteroidetes	Bacteroides spp.	*Enterobacteriaceae*	*Lactobacillus spp.*	*Akkermansia spp.*	Days
Model 1										
Caecal inoculum[1]	11.9	11.0	9.6	9.0	10.9	10.4	7.7	9.7	4.9	
IR[3]	10.7	9.4	8.4	8.4	10.4	10.2	9.2	7.3	4.6	8
Model 2										
Caecal inoculum[1]	11.8	10.8	9.2	8.9	11.0	10.3	8.1	9.5	4.8	
IR[4]	10.7 ± 0.2	9.4 ± 0.2	8.6 ± 0.3	8.4 ± 0.3	10.1 ± 0.4	9.0 ± 0.3	8.8 ± 0.5	6.4 ± 0.1	4.7 ± 0.1	26-28
Model 3										
Caecal inoculum[1]	11.2	11.0	9.0	10.6	10.3	9.9	6.2	8.7	5.0	
IR[4]	11.2 ± 0.3	10.8 ± 0.3	BDL	9.2 ± 0.3	10.8 ± 0.2	9.8 ± 0.1	10.1 ± 0.2	6.9 ± 0.4	4.6 ± 0.1	17-19
Model 4										
Caecal inoculum[1]	11.6	11.4	7.8	10.5	10.5	9.5	6.0	9.3	8.1	
IR[4]	11 ± 0.1	10.3 ± 0.1	7.7 ± 0.1	9.4 ± 0.3	10.0 ± 0.3	9.7 ± 0.3	9.8 ± 0.7	BDL	9.5 ± 0.5	27-29
Model 5										
Caecal inoculum[2]	12.1 ± 0.0	11.1 ± 0.0	8.3 ± 0.0	10.1 ± 0.0	10.8 ± 0.2	7.5 ± 0.3	5.7 ± 0.0	8.4 ± 0.1	8.6 ± 0.1	
IR[4]	11.5 ± 0.5	10.3 ± 0.1	7.1 ± 0.3	8.2 ± 0.3	10.6 ± 0.2	10.0 ± 0.1	7.7 ± 0.1	6.6 ± 0.1	8.6 ± 0.4	29, 30 & 36
Model 5*										
Caecal inoculum[2]	11.7 ± 0.2	11.1 ± 0.2	9.1 ± 0.1	10.4 ± 0.0	10.4 ± 0.2	9.3 ± 0.1	6.3 ± 0.2	8.2 ± 0.2	6.7 ± 0.1	
IR[4]	11.4 ± 0.1	10.4 ± 0.1	7.5 ± 0.1	8.8 ± 0.2	9.7 ± 0.5	9.2 ± 0.5	6.6 ± 0.3	8.5 ± 0.6	5.7 ± 0.1	18-20

[1] Data are mean $\log_{10}$ copies 16S rRNA gene g^{-1} of caecal inoculum used for fermentation

[2] Data are mean $\log_{10}$ copies 16S rRNA gene g^{-1} ± SD of caecal inoculum (extracted twice) used for fermentation

[3] Data are mean log10 copies 16S rRNA gene mL^{-1} of one day at the end of the stabilization period

[4] Data are mean log10 copies 16S rRNA gene mL^{-1} ± SD of one day at the end of the stabilization period extracted in duplicate; samples were analyzed in duplicate

BDL of 4.0 $\log_{10}$ copies per mL
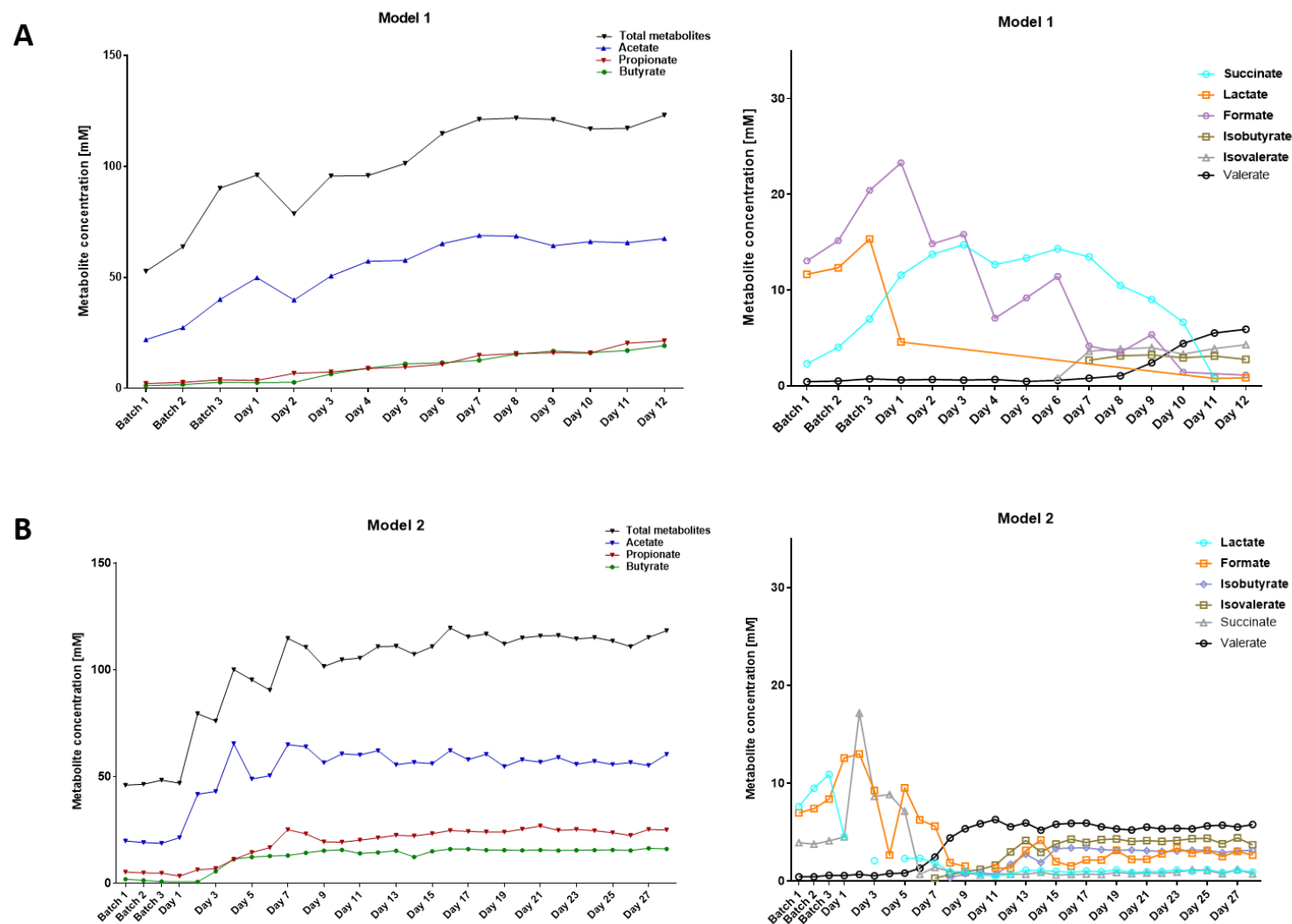
Supplementary Figure S3.2: Daily fermentation metabolite concentrations in reactor effluents of models 1 and 2 (**A & B**) measured by HPLC. Left the end metabolites (acetate, propionate and butyrate), and right the intermediate metabolites (formate, lactate andsuccinate) and branched-chain fatty acids (isobutyrate and isovalerate) and valerate. Colonization: three consecutive fed-batch fermentations for bead colonization.
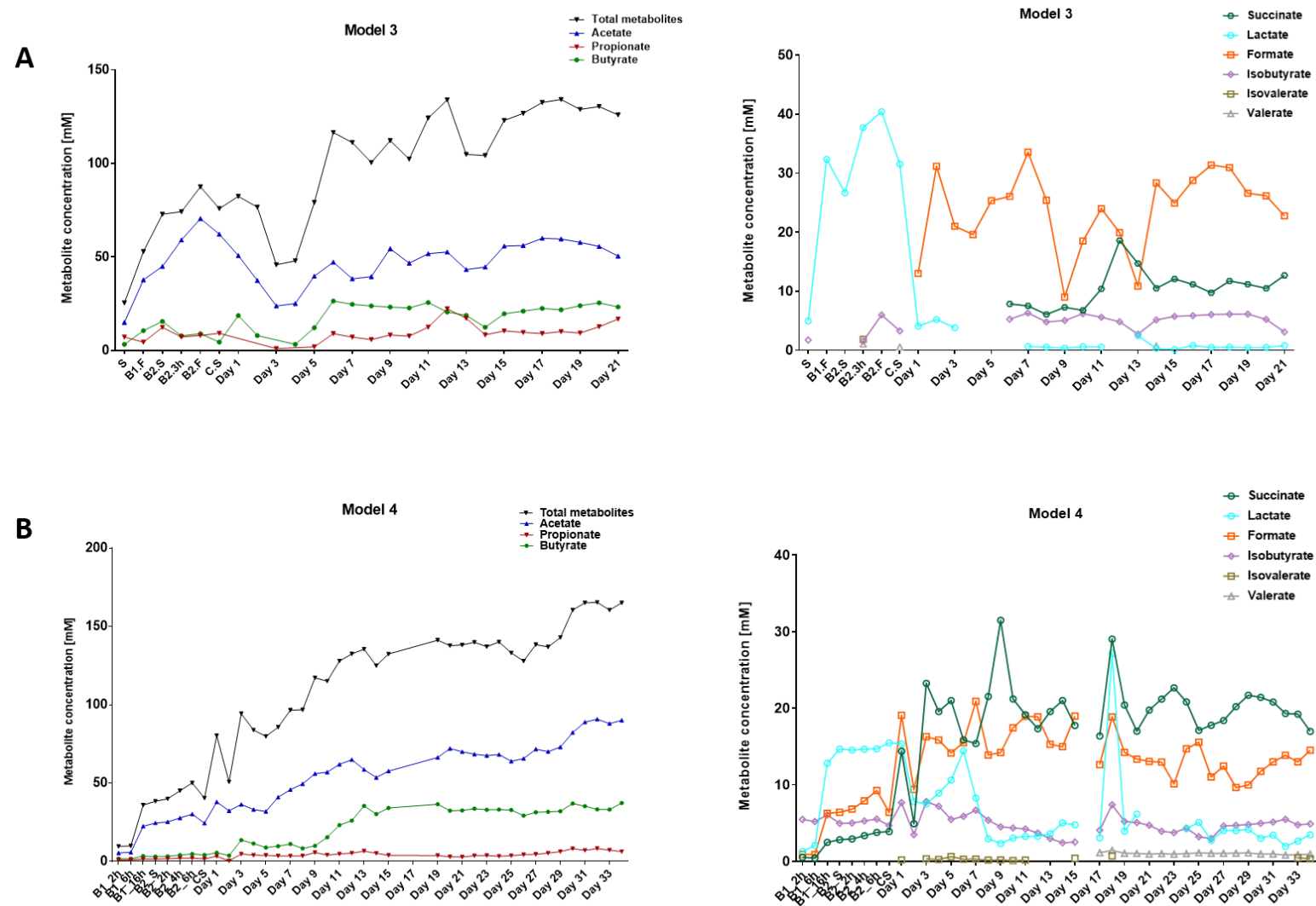
Supplementary Figure S3.3: Daily fermentation metabolite concentrations in reactor effluents of models 3 and 4 (**A & B**) measured by HPLC. Left the end metabolites (acetate, propionate and butyrate), and right the intermediate metabolites (formate, lactate andsuccinate) and branched-chain fatty acids (isobutyrate and isovalerate) and valerate. Colonization: two consecutive fed-batch fermentations for bead colonization.
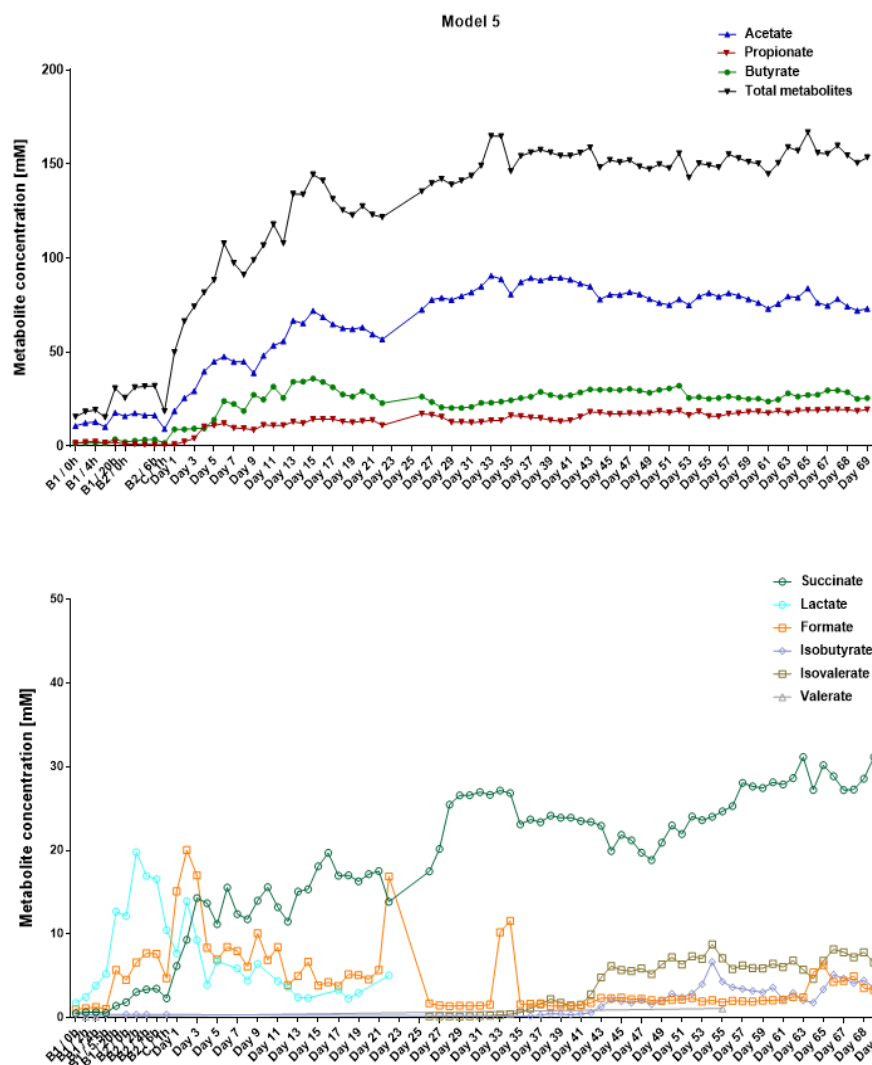
Supplementary Figure S3.4: Daily fermentation metabolite concentrations in reactor effluents of models 5 measured by HPLC. Top: end metabolites (acetate, propionate and butyrate), and right the intermediate metabolites (formate, lactate andsuccinate) and branched-chain fatty acids (isobutyrate and isovalerate) and valerate. Colonization: two consecutive fed-batch fermentations for bead colonization.
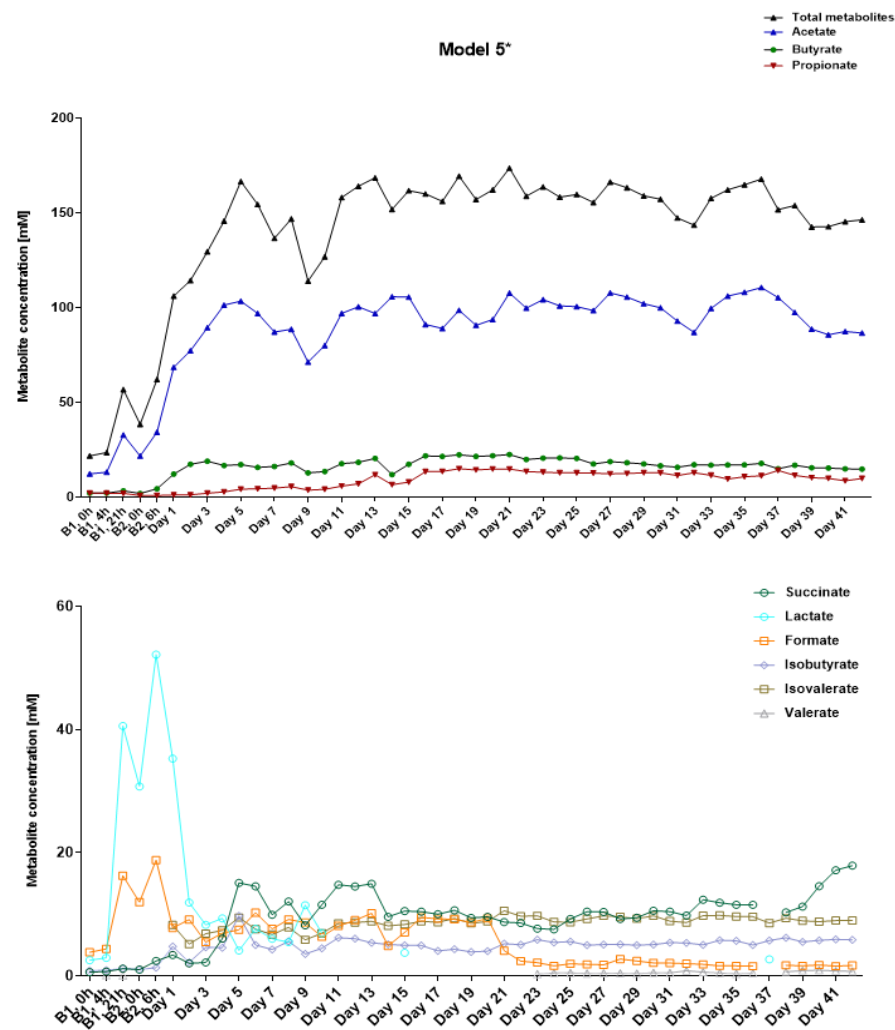
Supplementary Figure S3.5: Daily fermentation metabolite concentrations in reactor effluents of models 5* measured by HPLC. Top: end metabolites (acetate, propionate and butyrate), and right the intermediate metabolites (formate, lactate andsuccinate) and branched-chain fatty acids (isobutyrate and isovalerate) and valerate. Colonization: two consecutive fed-batch fermentations for bead colonization.

Cecal inoculum versus Model 5
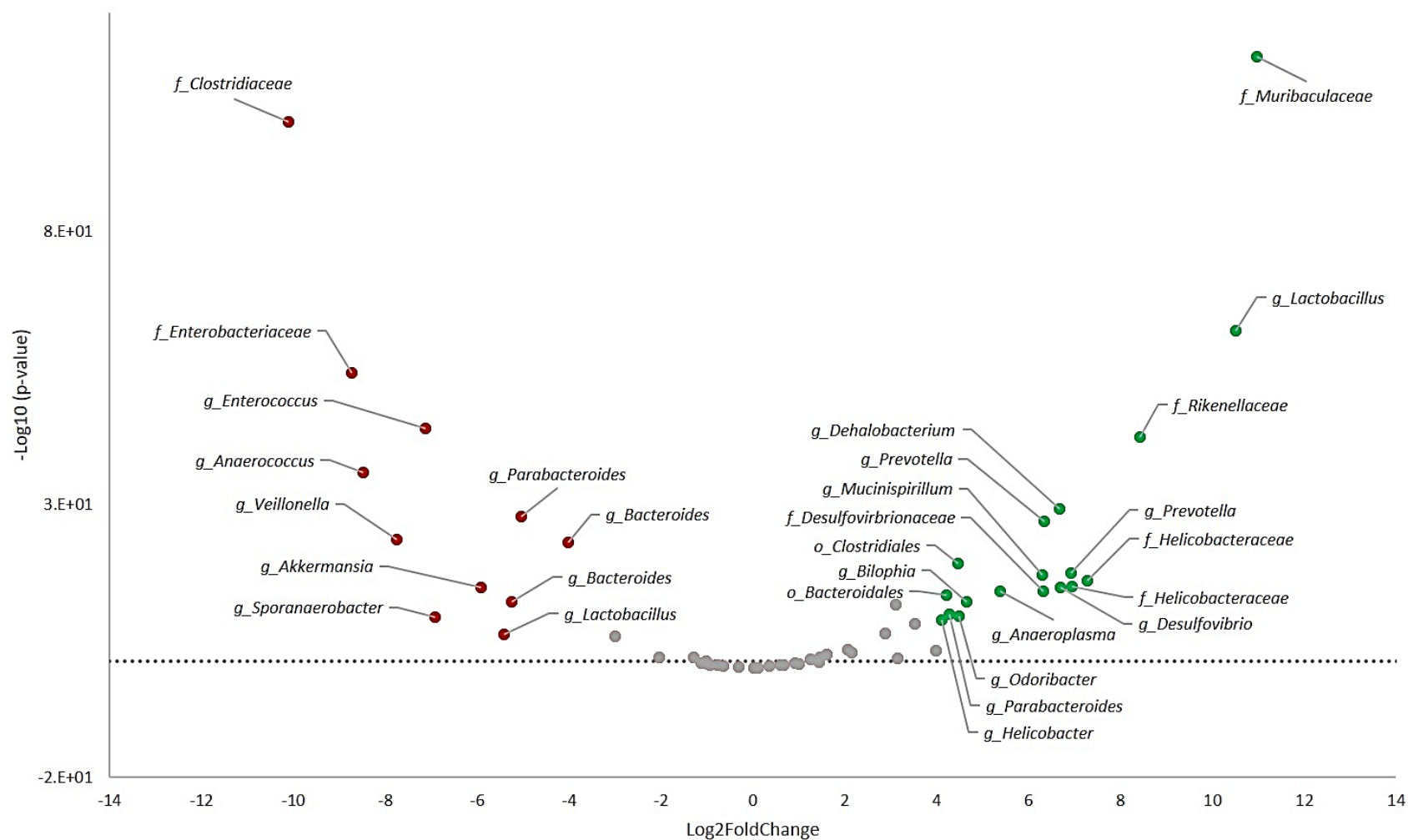
Supplementary Figure S3.6: Microbiota plots illustrating OTUs that were significantly enriched (green) and reduced (red) in reactor effluent of model 5 compared to the caecal inoculum as determined by differential abundance analysis. Each point represents an individual OUT, and the Y-axis indicates the Log2 fold change of relative abundance. The dashed line represents the statistically p-value of 0.05.

Cecal inoculum versus Model 5*
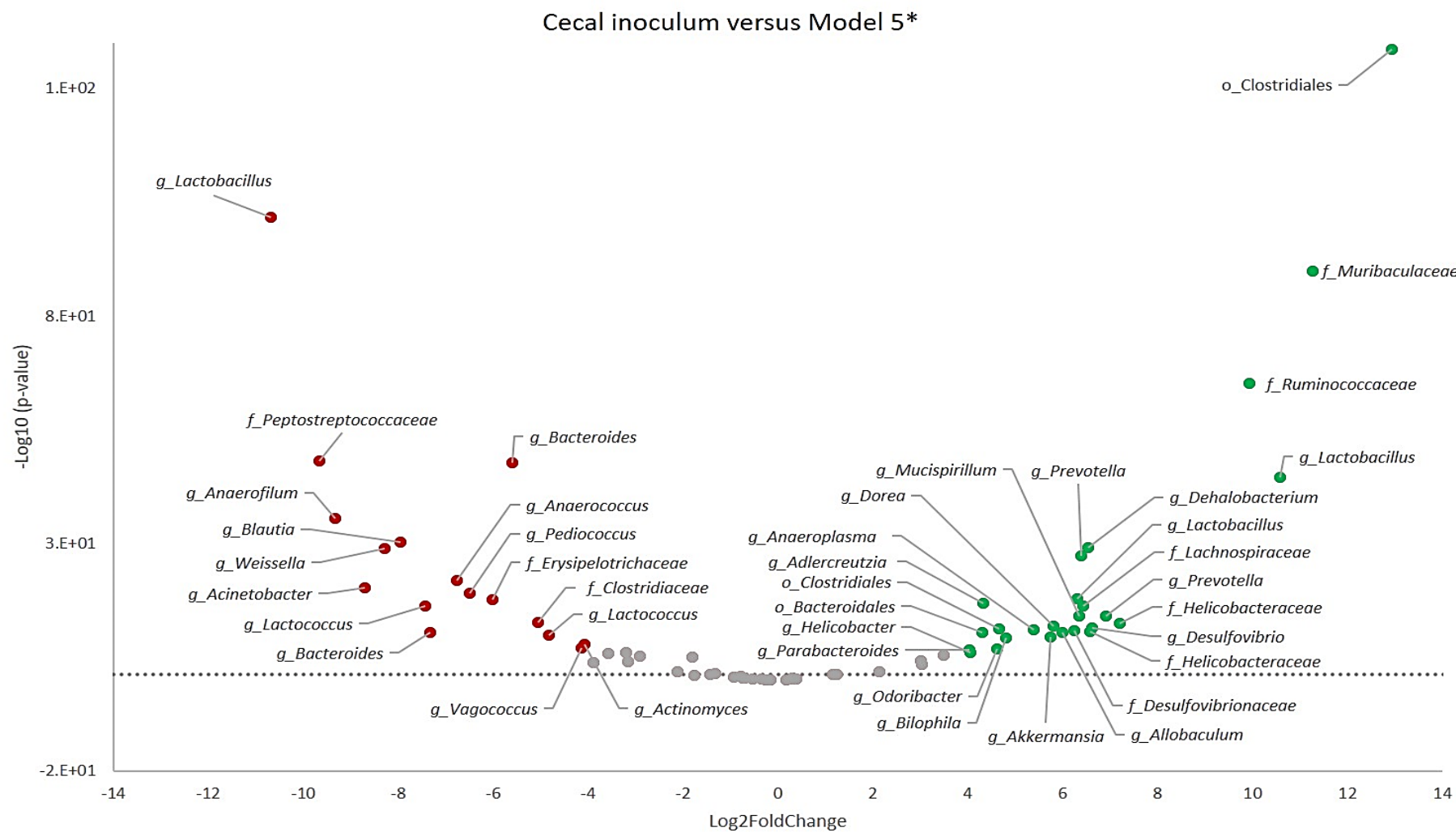
Supplementary Figure S3.7: Microbiota plots illustrating OTUs that were significantly enriched (green) and reduced (red) in reactor effluent of model 5* compared to the caecal inoculum as determined by differential abundance analysis. Each point represents an individual OUT, and the Y-axis indicates the Log2 fold change of relative abundance. The dashed line represents the statistically p-value of 0.05.
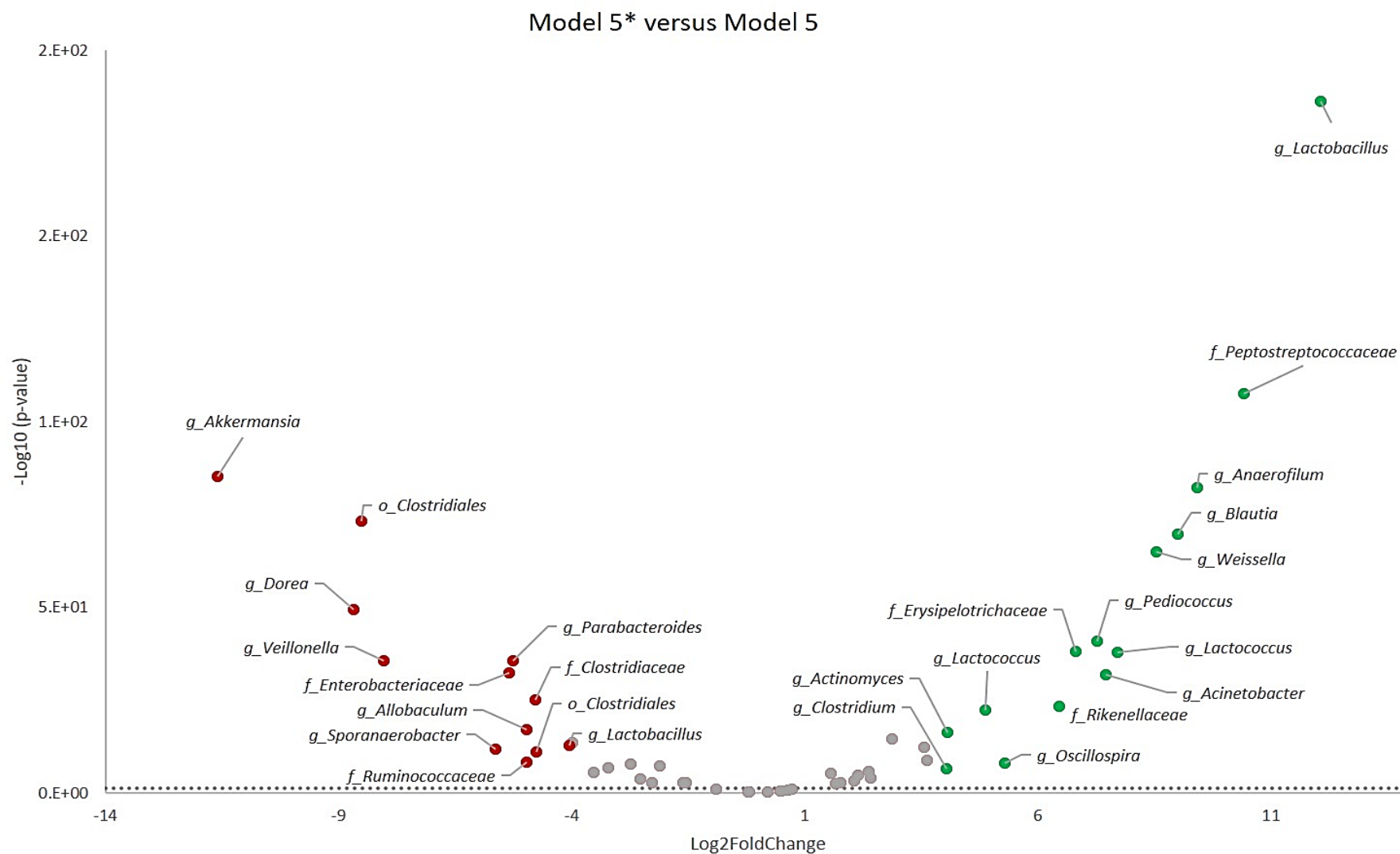
Supplementary Figure S3.8: Microbiota plots illustrating OTUs that were significantly enriched (green) and reduced (red) in reactor effluent of model 5 vs reactor effluent of model 5* as determined by differential abundance analysis. Each point represents an individual OUT, and the Y-axis indicates the Log2 fold change of relative abundance. The dashed line represents the statistically p-value of 0.05.
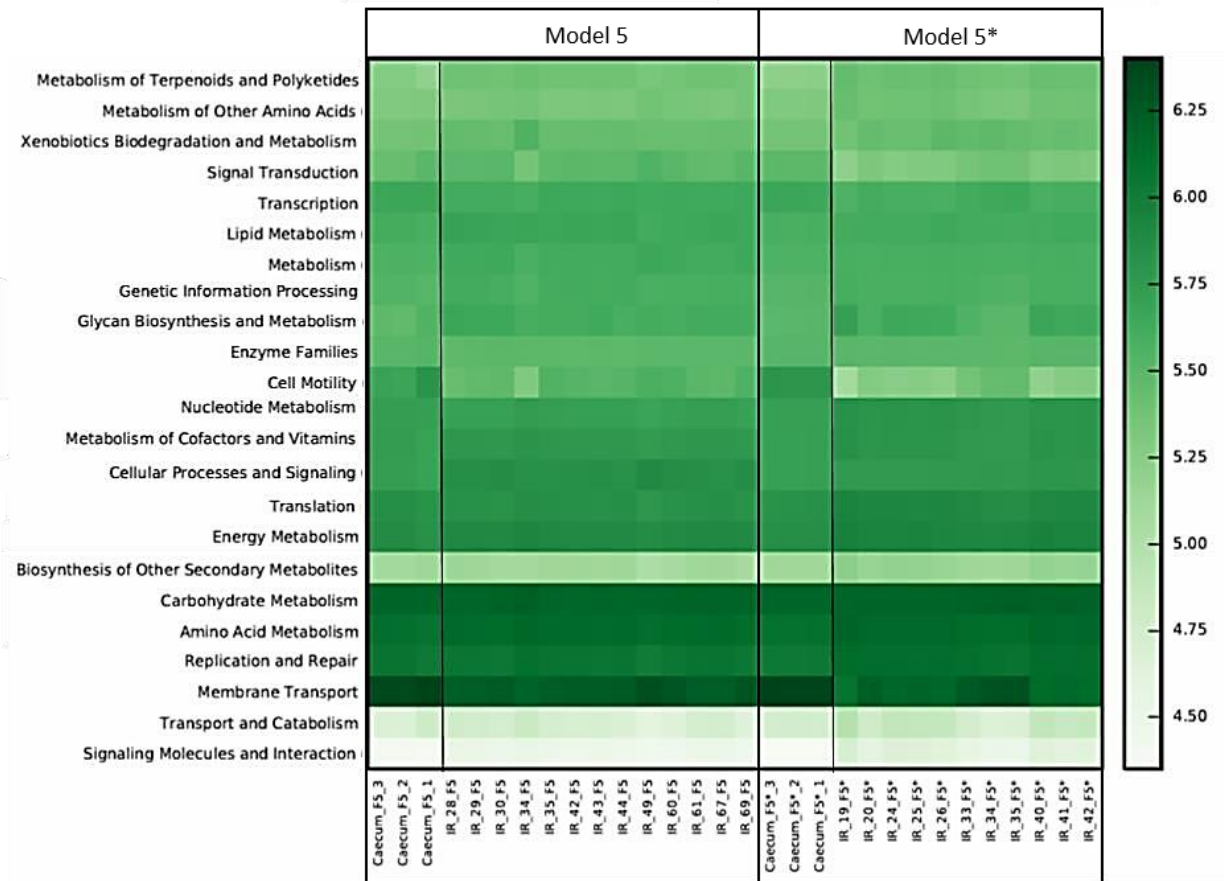
Supplementary Figure S3.9: Predictive functional profiling of microbial communities of caecal inocula and model 5 and 5* (during stabilization) by PICRUSt. Heatmap depicting the log transformed gene abundance of microbiota-associated predicted KEGG pathways. Numbers in scale represent log range of gene abundance for this dataset.

Chapter 4

In vitro modulation of intestinal microbiota towards eubiosis and dysbiosis by dietary fibers, oxidative stress and antibiotic treatment

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Abstract

Inflammatory bowel disease (IBD) is a chronic and relapsing inflammatory disease of the gastrointestinal tract that is characterized by an inappropriate immune response against the gut commensals, and triggered by environmental factors in a host with a genetic susceptibility. IBD risk factors including diet, antibiotic exposure and oxidative stress, were proposed to be major contributing factors in the IBD development through modulation of the gut microbiome. *In vitro* models of intestinal fermentation are powerful tools to investigate the effects of environmental factors on gut microbiota, independently of the host. The aim of our study was to assess *in vitro* the adverse effects of oxidative stress by hydrogen peroxide (associated with gut tissue inflammation), and metronidazole (used in IBD treatment), and potential beneficial modulating effects of dietary fibers (inulin and xylo-oligosaccharide, XOS) on mouse microbiota, using a novel continuous *in vitro* fermentation PolyFermS models inoculated with immobilized microbiota and mimicking mouse caecum conditions, repeated twice.

Oxidative stress provided by 2.5 mM hydrogen peroxide highly impaired bacterial fermentation in both microbiota, along with pronounced reduced abundances of strict anaerobic butyrate producers and enrichment of facultative anaerobes and the phylum Proteobacteria. Metronidazole decreased fermentation activity, but only short-term, and resulted in persistent increases in the abundance of the facultative anaerobic bacterial taxa belonging to *Lactobacillus, Veillonella* and *Enterococcus*. In contrast, supplementation with inulin and XOS (3.4 g/L and 5 g/L) increased short chain fatty acid productions, mainly acetate, propionate and butyrate. Inulin selectively increased relative abundance of *Erysipelotrichaceae* taxa (5 g/L) associated with butyrate production. This study, for the first time demonstrated the direct effects of oxidative stress, metronidazole, XOS and inulin on the murine *in vitro* gut microbiota, independently of the host, inducing immediate modulations of the microbiota functionality. The findings of this study are consistent with *in vivo* observation of factors effect on human microbiota, suggesting that the new mouse PolyFermS model is a valid tool to characterize the modulation potential of various environmental and dietary factors on the gut microbiota.

Introduction

Inflammatory bowel disease (IBD) is a chronic and relapsing intestinal inflammation disorder, comprising the two main conditions, ulcerative colitis (UC) and Crohn's disease (CD) (Matsuoka and Kanai, 2015). Even though the main etiology and pathogenesis of IBD remains unknown, it became evident that an aberrant immune response to the commensal microbiota occurs in a genetically susceptible individual that is driven by environmental factors (Ananthakrishnan, 2015; Ek et al., 2014; Matsuoka and Kanai, 2015; Ni et al., 2017). In the last two decades, mice models helped to elucidate the molecular and cellular pathophysiology of IBD, and highlighted the strong association between gut microbiota and disease (King and Sarvetnick, 2011; Kostic et al., 2014). Advances in microbiota analysis revealed strong alternations in gut bacterial composition between individuals suffering from IBD and healthy individuals, characterized by a lower microbial diversity and a bloom of potential pathogens within the Proteobacteria phylum, such as *Enterobacteriaceae*. Moreover, a decrease in the relative abundance of the phyla Bacteroidetes and Firmicutes including butyrate-producing bacteria belonging to *Ruminococcaceae* and *Lachnospiraceae* was reported (Ananthakrishnan et al., 2018; Rehman et al., 2016; Sokol et al., 2008). Metagenomic and metabolomic approaches revealed high alternations in the functional potential of dysbiotic microbiota, characterized by upregulated pathways and changed expression profiles of some bacterial species (Schirmer et al., 2018). Expression of genes in the biosynthesis of siderophores and branched-chain amino acids was found to be increased in dysbiotic communities, likely in response to the changed environment in the gut lumen (Ananthakrishnan et al., 2017; Lewis et al., 2015). Interestingly, some butyrate-producing bacterial species, such as *F. prausnitzii* or *B. vulgatus* that are generally found to be highly reduced in colitis, often dominate pathway transcriptions in IBD (Schirmer et al., 2018). Butyrate is a crucial metabolite for maintaining intestinal homeostasis and improving epithelial barrier function or reducing inflammation (Hamer et al., 2008). For some time, the central role of the gut microbiome in chronic intestinal inflammation has been assumed and numerous studies have tried to elucidate whether microbial dysbiosis is a cause or consequence of inflammation. First alternations of the gut microbiota might happen during early onset of IBD and are eventually partly responsible for the progression of the disease, but environmental factors like inflammation further contribute to the dysbiotic microbiota. Microbial dysbiosis probably indicates the reaction of the gut microbiota to the inflammatory stress that is characterized by changes in the oxidative environment in the gut. In addition, alternation in the microbiota structure leads to alternations of the bacterial metabolism, which in consequence also might contribute to the pathogenesis of IBD (Ni et al., 2017). In the DSS colitis mouse model, the most common used experimental model of acute colonic inflammation, comparable reduction in microbial diversity and increase of normally underrepresented bacteria are observed (De Fazio et al., 2014; Eichele and Kharbanda, 2017). Similar to human IBD situation, the acute colitis mouse microbiota is characterized by increased abundance of pro-inflammatory families such as

Enterobacteriaceae, Enterococaceae and *Bacillaceae*, and decreased abundance in *Prevotella, Clostridium* and *Lactobacillus* (De Fazio et al., 2014; Hakansson et al., 2015; Winter and Baumler, 2014).

Recent findings highlight the critical impact of intrinsic and extrinsic environmental risk factors on the gut microbiota, thus on the development and propagation of the inflammatory state in IBD (Ananthakrishnan, 2015; Ananthakrishnan et al., 2018). Increasing data from experimental models and clinical studies demonstrate that oxidative stress is highly associated with IBD pathogenesis and in driving mucosal layer damages, and thus bacterial invasion and stimulation of the immune response (Ananthakrishnan et al., 2018; Goyette et al., 2007). Oxidative stress is an imbalance between the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS), and their removal by antioxidants (Rezaie et al., 2007). During periods with active inflammation in IBD patients, macrophages release high concentrations of ROS as part of the host's defense mechanism (Collins, 2005). Recent findings suggest that this stress signaling plays an important role in mucosal homeostasis in the gut (Bhattacharyya et al., 2014).

Some antibiotics may trigger IBD by different pathophysiological mechanisms and a dosage-dependent correlation between the amount of antibiotic supply and the risk of IBD was reported (Shaw et al., 2011). In young healthy adults, the gut microbiota was shown to be resilient to a short broad-spectrum antibiotic treatment with recovery of most of the bacterial populations (Palleja et al., 2018). Other studies reported that antibiotic treatments can change the gut microbial composition for up to several months (Jernberg et al., 2007; Lindgren et al., 2009). The first interconnection between antibiotics and IBD was indicated by Card et al. (2004), noting that people with an IBD-diagnosis often underwent antibiotic treatment a few years before. Further, children who received antibiotics in the first year of life have a threefold higher risk of developing IBD later in life (Shaw et al., 2010, 2011, 2013).

Generally, changes in the diet are frequent in our contemporary society, inducing strong shifts in the gut microbial composition and eventually causing an aberrant immune response (David et al., 2014; Lewis and Abreu, 2017). Despite, no diet was demonstrated to be directly correlated to IBD, yet, it is an important environmental factor that needs to be considered when studying the role of diet in IBD (Kostic et al., 2014). Two case-control studies revealed that the consumption of dietary fibers originating from fruits and vegetables reduces the risk of CD in children and woman, while fibers of whole grains, bran or cereal did not (Amre et al., 2007; Ananthakrishnan et al., 2013). Non-digestible dietary fibers are energy substrates for growth and activity of specific commensal bacterial populations within the intestine, which in turn have a beneficial effect for the host's health (Laparra and Sanz, 2010).

Despite the fact that the microbiome is dynamic, most human and animal studies in IBD research have primarily monitored the intestinal microbiota composition and functionality on single time points analysis. Further, the assessment of the immediate impact of IBD risk factors on human gut microbiota composition

and metabolism is limited by the inaccessibility of the gastrointestinal tract, leading to the almost exclusive analysis of faecal microbiota. During mice experiments, most studies analyzed the faecal material, while the mucosal and caecal microbiota become only accessible *post mortem*. Moreover, in order to obtain a more complete understanding of the gut microbiota dynamics upon response to IBD risk factors, it is of high interest to include microbial metabolite analysis. The use of *in vitro* intestinal fermentation models allows monitoring metabolite production and gut microbiota dynamics over time and upon controlled environmental conditions treatment independent of host factors.

In vitro gut fermentation models are useful technological platforms that allow stable cultivation of complex bacterial communities with highly controlled environmental conditions, like pH, temperature or retention time (Payne et al., 2012). Continuous fermentation models are powerful tools to study the effect of different exogenous factors on the microbial community and functionality, independent of the host (Payne et al., 2012). Recently, a novel continuous fermentation model based on PolyFermS platform was developed and validated for the murine caecal microbiota. This model was shown to maintain key bacterial populations of a murine caecal microbiota, allowing investigation of different treatments. *In vitro* models are useful tools to study environmental factors contributing to alternations in the gut microbiota away from the host and they can provide mechanistic insights that could help to identify new or fine-tuned current therapeutic methods (Ananthakrishnan et al., 2018). To gain insight into factors promoting or correcting microbiota dysbiosis in IBD, this study used a novel developed *in vitro* continuous fermentation model (PolyFermS) for the murine caecum to assess the effects of oxidative stress, metronidazole (antibiotic used in IBD) and prebiotics (inulin and XOS) on murine caecal microbiota and metabolites (**chapter 3**). Two independent caecal microbiota were immobilized in caecal beads and cultivated in an inoculum reactor (IR) to produce a constant artificial caecal microbiota. Five additional reactors were operated in parallel and continuously inoculated with the same microbiota produced in IR. The overall goal was to investigate the effect of selected treatments, oxidative and antibiotic stresses as well as prebiotics, and second further validate the mouse caecum model by comparing *in vitro* responses with published data obtained with healthy and acute inflammation mouse models.

Material and Methods

Nutritive medium

The nutritive medium, designed to simulate the murine caecal chyme included (g L^{-1} of distilled water): pectin (citrus) (2), xylan (oat spelts) (4.8), arabinogalactan (larch wood) (4.8), soluble corn starch (2), mucin (4), casein acid hydrolysate (3.6), peptone water (6), tryptone (6), yeast extract (5.4), L-cysteine HCL (0.8), bile salts (0.4), KH_2PO_4 (0.5), $NaHCO_3$ (1.5), NaCl (4.5), KCl (4.5), $MgSO_4$ anhydrated (0.61), $CaCl_2*2\ H_2O$ (0.1), $MnCl_2*4\ H_2O$ (0.2), $FeSO_4*7H_2O$ (0.005), hemin (0.05) and Tween 80 (1 mL) (**chapter 3**). Prior sterilization (120°C, 20 min), the pH of the nutritive medium was adjusted to 6.5. After sterilization, one mL of a filter-sterilized (0.2 µm pore-size) vitamin solution was added to the sterilized and cooled down medium (Michel et al., 1998). All components were purchased from Sigma-Aldrich Chemie (Buchs, Switzerland), except for xylane (Angene, London, England), peptone water (Oxoid AG, Pratteln, Switzerland), inulin (Orafti®, RPN Food-technology AG, Sursee, Switzerland), XOS (Longlive, Shandong, China), bile salts (Oxoid AG), tryptone (BD, Sparks, USA), yeast extract (Merck, Darmstadt, Germany), KH_2PO_4 (VWR International AG), $NaHCO_3$ (Fischer Scientific, Pittsburgh, USA), NaCl (VWR international AG, Dietikon, Switzerland), $MgSO_4$·anhydrous (Acros Organics, Geel, Belgium), $MnCl_2$·$4H_2O$ (Fluka, Buchs, Switzerland) and KH_2PO_4 (VWR International AG).

Two dietary fibers (inulin-type fructan and XOS), the antibiotic metronidazole (Sigma-Aldrich, Buchs, Switzerland) and hydrogen peroxide (H_2O_2) treatments were investigated. The dietary fibers (3.4 g L^{-1} and 5 g L^{-1}) and metronidazole (112.8 mg L^{-1}) were supplemented to sterile nutritive fermentation medium in a laminar flow bench. Complete hydration of dietary fibers was allowed for 24 h under high speed stirring at 4°C. Metronidazole (112.8 mg L^{-1}) was added to sterile fermentation medium before connection to treatment reactor. Hydrogen peroxide at 0.5 M (H_2O_2 30% (Perhydrol) for analysis; EMSURE ISO, Merck KGaA, Darmstadt, Germany) was continuously dropped into an assigned reactor at a speed of 1 mL/h with the help of a peristaltic pump (Ismatec, Wertheim, Germany) to achieve a final concentration of 2.5 mM in the reactor. The peroxide solution was protected from light by lagging the tubings with tinfoil.

Caecal bacteria immobilization

Caecal contents of five healthy 12 weeks old female C57BL/6 mice were collected for immobilization as described before (**chapter 3**). Caeca were tied off with surgical threads prior dissection with surgical scissors, placed onto a sterile Petri dish and transported in anaerobic jar to the laboratory. Caeca were transferred into an anaerobic chamber (10 % CO_2, 5 % H_2 and 85 % N_2) (Coy Laboratories, Ann Arbor, MI, USA) and pooled together. The caecal bacteria were washed with pre-reduced peptone water (0.1 %, pH 7, Thermo Fisher Diagnostics AG, Pratteln, Switzerland) and immobilized in 1-2 mm diameter gel beads (gellan gum (2.5%, w/v), xanthan (0.25%, w/v) and sodium citrate (0.2%, w/v)) under anaerobic conditions as previously described

(**chapter 3**). Sixty mL of freshly produced caecal microbiota beads were transferred in the inoculum reactor (IR) containing 140 mL of nutritive medium (pH 6), supplemented with 20 % rumen fluid and 0.1 % cellobiose. For bead colonization, two consecutive batch fermentations were carried out, replacing 100 mL spent medium with fresh nutritive medium (0.1 % cellobiose; pH 6) after 20 and 6 hours, respectively.

Experimental set-up

The *in vitro* fermentation model for cultivation of murine caecal microbiota was presented in detail in **chapter 3** and the set-up was adapted for the aim of this study (**Figure 4.1 A**). The fermentations were carried out for up to 69 days, using a two-stage design with six bioreactors (Sixfors, Infors, Bottmingen, Switzerland). The reactors were operated under conditions of the murine caecum (pH 6.5, stirring at 180 rpm, 37°C and a retention time of 12h). Anaerobiosis in the bioreactors was maintained by continuous CO_2 headspace flushing, and the pH of 6.5 was kept by addition of 2.5 M NaOH. IR had working volume of 200 mL and was inoculated with 60 mL (30 %, v/v) caecal beads from the respective caecal inocula and connected via a peristaltic pump (Reglo, Ismatec, Glattbrugg, Switzerland) to five second stage test reactors (TRs). Fresh medium was continuously supplied to IR at a flow rate of 16.7 mL/h. All TRs (200 mL working volume) were continuously inoculated with 5 % fermentation effluent from the IR and 95 % fresh, sterile, anaerobic nutritive medium.

After an initial stabilization period of IR for up to 23 days in continuous mode, TRs were connected to IR in parallel and operated in continuous mode as presented above. An initial stabilization of second stage TRs for up to 10 days was carried out prior treatments application period (**Figure 4.1 B**). Each treatment was performed for 6 or 7 days for reaching pseudo-steady state, monitored by fermentation metabolite production. Between each treatment period, TRs were disconnected from model, washed and re-connected to the IR, aiming to avoid memory effects of the previous applied treatment and re-establish bacterial composition balance. Within each experimental period, one randomly selected TR reactor was used as a control, with the same fermentation medium and conditions as used for IR. Dietary fibers (3.4 g/L and 5 g/L) and metronidazole (MTZ) (112.8 mg/L) were added to the growth medium of the corresponding treatment reactor. Hydrogen peroxide (0.5 M) was continuously dropped into the bioreactor at a flow rate of 1 mL/h, for a final concentration of 2.5 mM in the reactor.

Effluent samples from all reactors were taken daily. The supernatant (10 min centrifugation at 14.000 g at 4°C) and bacterial pellet were stored at -20°C until further analysis. Metabolic stability of the reactors was monitored by daily measurements of main metabolites in supernatants. Standard observed variations in the PolyFermS fermentation metabolites are normally less than 10%. Functional microbial stability of a reactor during stabilization and treatment periods was, before starting and analyzing samples of a treatment period was defined by the coefficient of variation.
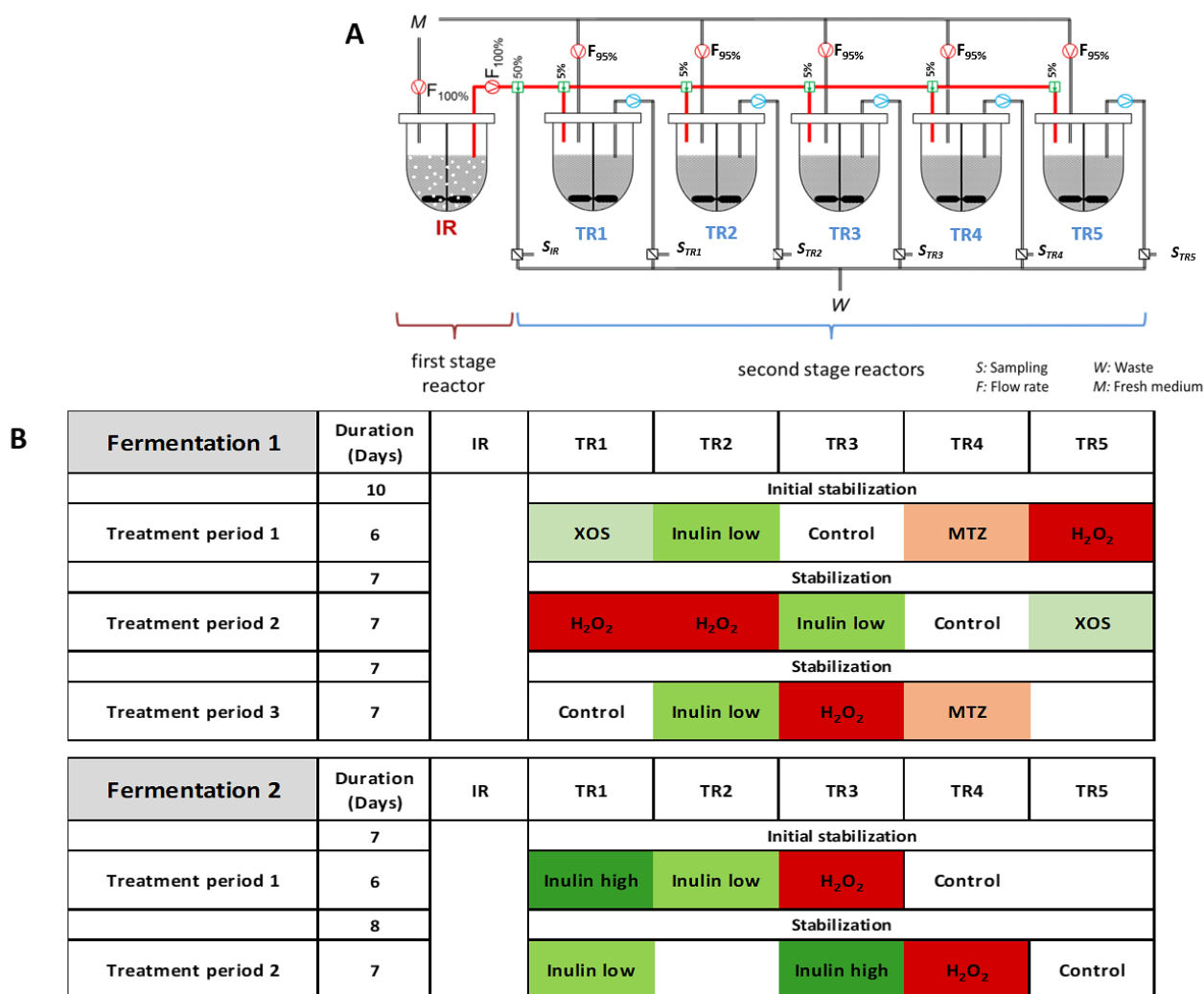
Figure 4.1: (A) Experimental set-up and treatments tested in the PolyFermS model mimicking the murine caecal microbiota. Test reactors (TRs) were fed with fermentation effluent from inoculum reactor (IR) and with nutritive medium during stabilization periods. (B) During treatment periods with dietary fiber treatments, TRs were fed with effluent from IR and supplemented fermentation medium (3.4 g/L or 5 g/L dietary fiber). A feed rate of 1 ml/h of 0.5 M H_2O_2 was continuously applied into the assigned TRs. TRs were fed with 112.8 mg/L metronidazole supplemented growth medium during antibiotic treatment periods

Microbial metabolite analysis

Concentrations of SCFAs (acetate, butyrate and propionate), branched-chain fatty acids (BCFAs) (isobutyrate, valerate and isovalerate) and intermediate substrates (lactate and formate) produced by the microbiota in the fermentation effluents was determined using a high performance liquid chromatography (HPLC) analysis (Thermo Fisher Scientific Inc. Accela, Wohlen, Switzerland). Analysis were performed with an Accela Chromatography System and Accela RI-detector (Thermo Fisher Scientific Inc, Reinach, Switzerland) with a Security Guard Carbo-H cartridge (4 x 3.0 mm) and a Rezex ROA-Organic Acid H^+ column (300 x 7.8 mm). Around 40 µL of the sample was injected into the HPLC with a flow rate of 0.4 mL min^{-1} and H_2SO_4 as an eluent (**chapter 2**).

Microbial community analysis

Genomic DNA extraction

Total genomic DNA was extracted from the pellet of 2 mL fermentation effluent using the FastDNA® SPIN Kit for Soil (MP Biomedicals, Illkirch Cedex, France) according to the manufacturer's instructions. Total DNA concentration (ng/µL) and purity was measured by spectrophotometry using Nanodrop®ND-1000 Spectrophotometer (Nanodrop ND 1000 Spectrophotometer, Therma Scientific, Wilmington, USA) and samples were stored at –20°C until further molecular analysis.

Microbiota profiling with 16S amplicon sequencing

For the microbiota analysis of PolyFermS effluent samples, the V4 region of the 16S rRNA gene was amplified with primers 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3'). Sequencing was performed with MiSeq (GDC, Zurich, Switzerland) using V2 reagent kit for 2 x 250 bp paired end Next Tera chemistry supplemented with 20 % of PhiX. The raw data with quality scores were merged and trimmed using settings as previously described (Zachariassen et al., 2017), with a minimum lengths of reads of 200 bp (V4) and 250 bp (V3-V4), using the fastq_mergepairs and fastq_filter scripts in the UPARSE pipeline. Quantitative Insight Into Microbial Ecology (QIIME) open source software (1.8.0 and 1.9.0) was used for analysis (Caporaso et al., 2010). The dataset was purged from chimeric reads and *de novo* operational taxonomic units (OTUs) were constructed using the UPARSE pipeline (Edgar, 2013). The Green genes database (version 13.8) was used as a reference database (Werner et al., 2012). Alpha and beta diversity analysis was performed as previously mentioned, with the script alpha_beta_diversity.pV8.sh (Krych et al., 2013).

Bioinformatics & statistical analysis

Bacterial diversity was assessed within samples (α-diversity) or between samples (β-diversity) using QIIME v.1.9. Alpha diversity was measured with Shannon index (OTU-based diversity) and observed species. Beta diversity was assessed with UniFrac, a community dissimilarity metric, which is based on the fraction of unique branch length observed in different bacterial communities in a common phylogenetic tree. The phylogenetic distances are calculated as the fraction of unshared branch lengths between the pair of bacterial communities. Unweighted UniFrac distances compare microbial communities based on the presence/absence of members, while weighted UniFrac also incorporates relative abundance information. UniFrac-based Principal Coordinate Analysis (PCoA) helps in the exploration of potential factors (such as treatment) that might explain the groupings of similar communities (Lozupone et al., 2005, 2007). The generalized UniFrac distance contains an extra parameter controlling the weight on abundant lineages so the distance is not dominated by highly abundant lineages (Chen et al., 2012). Statistical analysis of sequencing data of three days at the end of the stabilization and treatment period of each fermentation was performed using QIIME. We used DESeq2 package to perform differential abundance analysis on raw OTU-level count data between two assessed

groups (here: last three days of stabilization and treatment period) (Love et al., 2014; McMurdie et al., 2014). In brief, the differential abundance and richness analyses is done with a generalized linear model of counts following a negative binomial distribution. It is scaled by a normalization factor that determines differences in sequencing depth between samples. Differential OTU abundances are assessed using the Wald tests and p-values are adjusted by false discovery rate (p-adj < 0.05) (Noguera et al., 2018). Canonical correspondence analysis (CCA) explores linear combination of selected variables correlating with linear combination of OTUs. This analysis allows finding a strong correlation between two datasets. Location of bacterial OTUs in the plot indicate its dependence on the environmental factor. The length of a metabolite arrow illustrates the strength of the link of the metabolite to bacterial community composition. CCA analysis does not only provide insight how a treatment influenced a bacterial community, but also displays potential interactions occurring between bacterial taxa and a variable. All statistical analyses for HPLC data were performed using one way ANOVA (SigmaPlot 13.0, San Jose, California, USA). Data are expressed as means ± standard deviations (SD) of three days of the stabilization and treatment periods with standard observed variations of metabolites lower than 10% in the same reactor in each fermentation. P values < 0.05 were considered significant.

Results

Impact of oxidative and antibiotic stress on murine intestinal microbiota

The effect of H_2O_2 at 2.5 mM was tested on bacterial and metabolic response of *in vitro* murine caecal microbiota of fermentation 1 (F1) and fermentation 2 (F2). Four repetitions of the treatment were performed within F1 and two repetitions within F2. Metronidazole supplementation (112.8 mg/L) was tested twice during F1. Metabolic and bacterial response of both microbiota were compared to the reactor's stabilization period before the treatment (3 days sampling), due to slight metabolic differences from one reactor to the other.

Oxidative stress induced decrease microbial fermentation

Oxidative stress (H_2O_2) resulted in an overall decrease in total metabolite production in both models F1 and F2 compared to stabilization, with mean decrease ranging from 22 and 50 mM in F1 and 45 and 63 mM in F2, indicating impaired bacterial fermentation (**Figure S4.2 A and B**; **Supplementary Table S4.9; Supplementary Table S4.11**). Significant decreases in acetate production (averaging 21 % in F1 and 25 % in F2, respectively), propionate production (25 and 52 %, respectively) and butyrate production (64 and 62 %, respectively) were observed for both fermentations. Metabolite production levels in F2 microbiota started to recover at day 4 of treatment, but not in F1. Simultaneously, intermediate metabolites formate (average of 7 % in F1 and 1 % in F2, respectively) and lactate (5 % and 5 %, respectively) were higher in reactor effluents than during corresponding stabilization periods. Succinate (average of 64 % in F1 and 53 % in F2, respectively) and BCFAs (average of 91 % in F1 and 41 % in F2, respectively) production decreased upon peroxide supplementation.

Oxidative stress significantly inhibited butyrate-producers and increased relative abundance of *Enterobacteriaceae in vitro*

Oxidative stress with 2.5 mM with H_2O_2 caused specific compositional shifts, according to PCoA plots based on weighted and unweighted UniFrac metrics generated from sequencing 16S rRNA gene (**Figure 4.2 C and D**). Compared to the previous stabilization period, oxidative stress led to a decrease in abundance of the phylum Bacteroidetes while Firmicutes and Proteobacteria phyla increased in both microbiota (**Supplementary Table S4.1; Supplementary Table S4.2**). DESeq2 analysis (Log_2 fold change > 2; $p < 0.05$) was applied to detect OTUs that show significant differential relative abundance between the stabilization and treatment period (last three days) (**Supplementary Figure S4.1; Supplementary Figure S4.2**). Oxidative stress induced consistent shifts of certain taxa in both fermentations, resulting in statistical significance and high fold-change compared to stabilization. Within the phylum Firmicutes, the species *Lactobacillus zeae* and taxa belonging to the genera *Vagococcus* and *Enterococcus* were highly enriched in both *in vitro* murine microbiota, while taxa within *Lachnospiraceae* spp., *Anaerococcus* spp., *Coprococcus* spp. and the species *Ruminococcus gnavus* were

decreased. Within the phylum Proteobacteria, a pronounced increase in abundance was seen in taxa belonging to *Enterobacteriaceae*. Additionally, decreased abundance of the phylum Bacteroidetes can be linked to decreased abundance of taxa belonging to *Bacteroides*. Likely due to different microbiota compositions of F1 and F2, oxidative stress induced additionally distinct phylogenetic responses (**Supplementary Table S4.1; Supplementary Table S4.2**). F1 microbiota, taxa that were found to be significantly decreased belonged to *Akkermansia, Ruminococcus, Dorea, Allobaculum* and unclassified Clostridiales, while in F2 microbiota increases in taxa belonging to *Streptococcus, Lactococcus* and unclassified *Microbacteriaceae* genera were observed.

Specific microbial taxa correlate with metabolites upon oxidative stress

Canonical correspondence analysis (CCA) was used to test correlations between individual metabolites and overall microbial community or individual OTUs (**Figure 4.2 E**). We observed a clear separation between the bacterial communities corresponding to the stabilization period compared with the peroxide-treated samples, which can be associated to consistent increased relative abundances of taxa belonging to *Enterococcaceae, Lactobacillaceae* and *Enterobacteriaceae* during oxidative stress (**Supplementary Table S4.1**). Further, lactate appeared to be highly associated with oxidative stress, while main SCFAs acetate, propionate and butyrate were negatively associated with the treatment. At phylogenetic level, the lactate-producing taxa *Enterococcaceae* and *Lactobacillaceae* were positively correlated with lactate in F1, while *Enterobacteriaceae* and the lactate-consuming taxa *Veillonellaceae* in F2.

Metronidazole caused a temporal but strong decrease in bacterial metabolism and shift composition

The effect of metronidazole (MTZ) was tested during F1 at a concentration of 112.8 mg/L with two repetitions (noted I and II). An initial rapid decrease in total metabolites (16 and 19 mM) during the first days of MTZ supplementation was observed, followed by recovery of metabolic levels after only 3 days (repetition I) to 4 days (repetition II) of treatment (**Figure 4.3 A**; **Supplementary Table S4.10**). Furthermore, MTZ had a less marked effect on bacterial metabolite production in repetition I compared to repetition II, during which acetate, propionate and butyrate production decreased until day 4. Formate production increased (13 mM in repetition I and 2 mM in repetition II, respectively). PCoA plots based on unweighted UniFrac distance matrices (presence/absence matric) showed that MTZ samples differed from stabilization samples, while no differences were observed on weighted (presence/absence/abundance matrix) and generalized UniFrac distance matrices (considering semi-abundant bacterial groups) (**Figure 4.3 C-E**). To assess overall differences in bacterial community structure, we performed DeSeq2 analysis (Log_2 fold change > 1; $p < 0.05$) (**Supplementary Table S4.3**).At phylum level, changes in relative abundances in Firmicutes, Bacteroidetes and Proteobacteria were

observed. Antibiotic shifts resulted in increased relative abundances of *Veillonella dispar*, *Lactobacillus reuteri* and taxa belonging to *Enterococcus* and *Sutterella*.

A

B
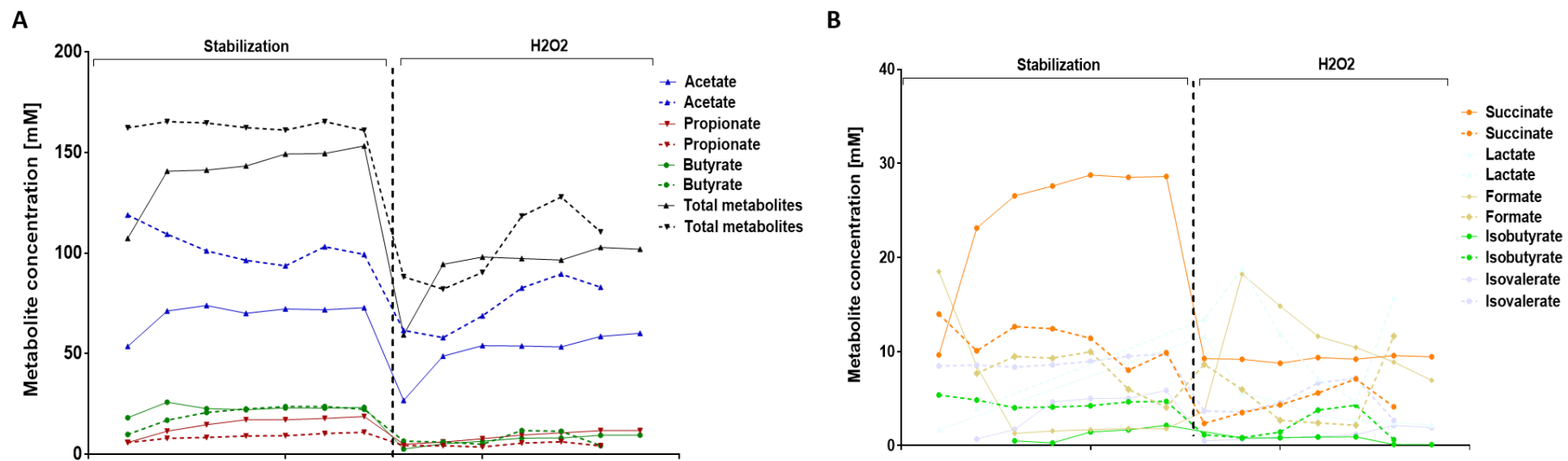
Figure 4.2: (A) SCFA concentrations in reactor effluent and (B) intermediate metabolites (succinate, lactate and formate) and BCFA (isobutyrate and isovalerate) concentrations. Continuous line = treatment 2-Fermentation 1; dashed line = treatment 2- Fermentation 2.
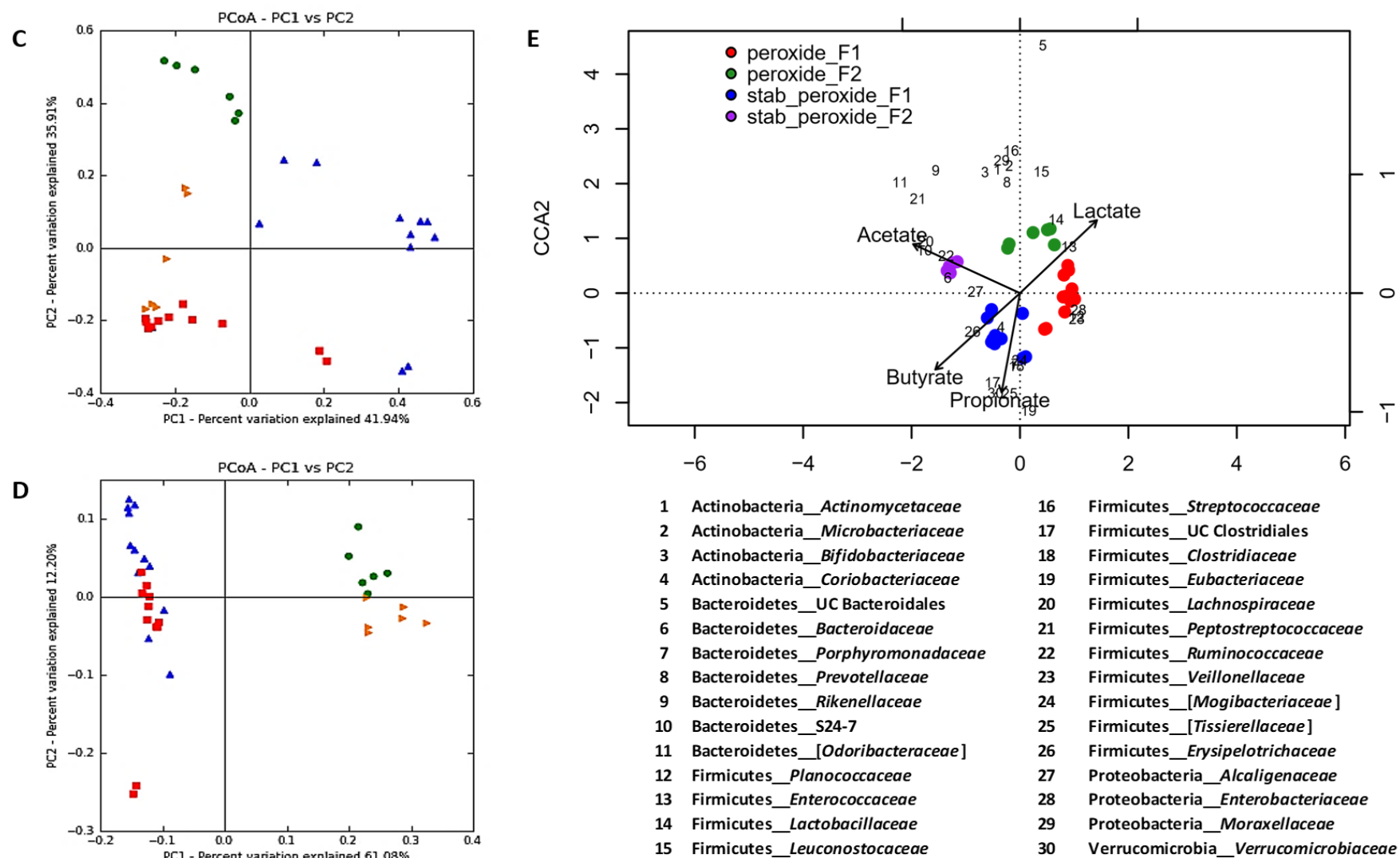
Figure 4.2: PCoA plot based on weighted (C) and unweighted (D) UniFrac distance matrix on OTU level generated from stabilization and 0.5 M peroxide treated PolyFermS reactor effluents of fermentation 1 (stabilization: blue; treated: red) and fermentation 2 (stabilization: green; treated: orange). Each circle represents a sample (E) Canonical Correspondance Analysis (CCA) showed significant effects of 0.5 M peroxide on metabolite production and microbiota composition
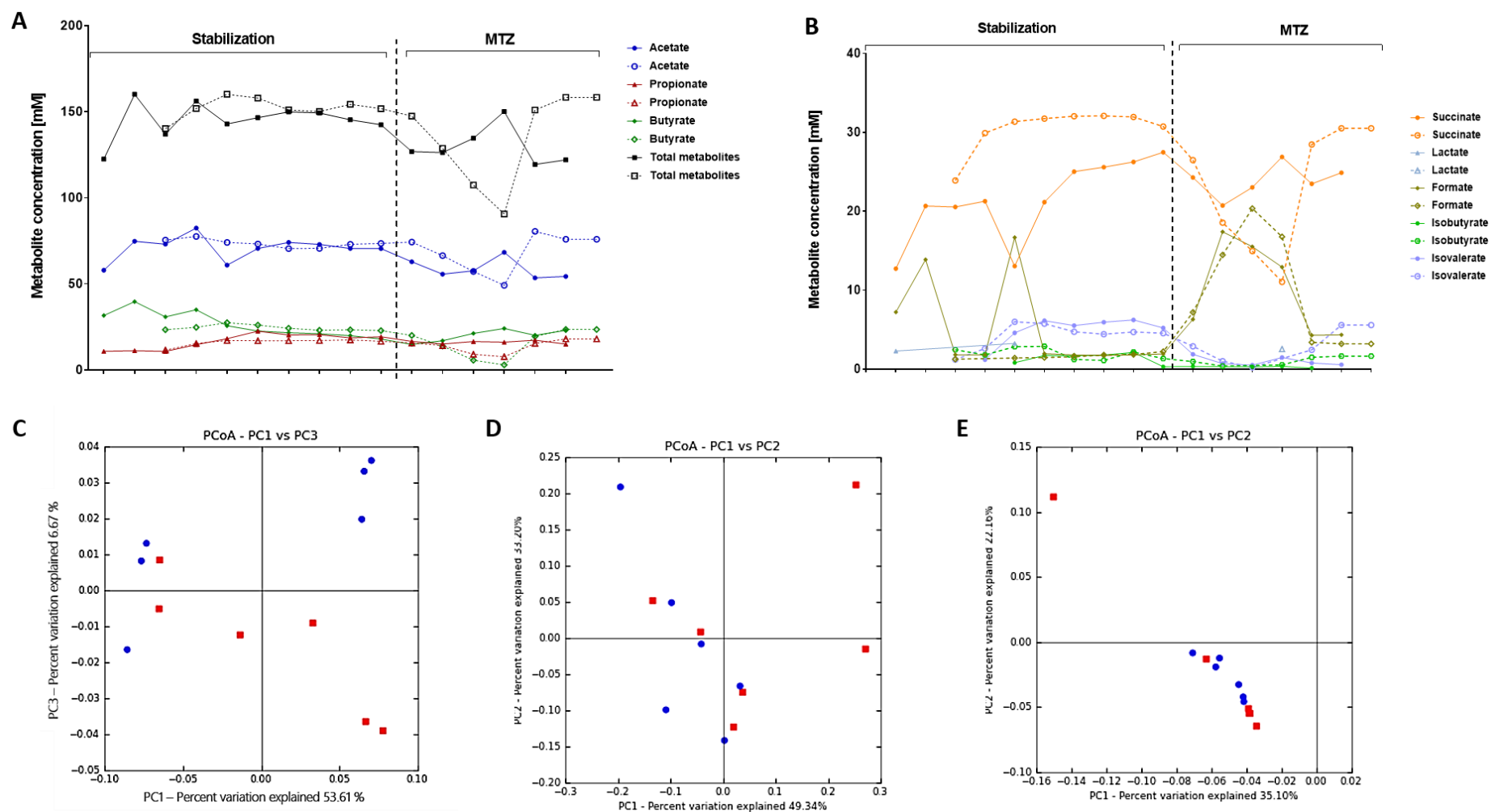
Figure 4.3: (A and B) Daily metabolite concentrations in fermentation effluents of treatment reactors during stabilization and MTZ treatment tested in F1. PCoA plot based on unweighted (C), weighted (D) and generalized € UniFrac distance matrix on OTU level generated from non-treated and 112.8 mg metronidazole (MTZ) treated PolyFermS reactor effluents of fermentation 1 (untreated: blue; treated: red). Each circle represents a sample.

Effects of dietary fiber supplementation on caecal microbiota

The effects of inulin on the bacterial and metabolic responses of PolyFermS microbiota of F1 and F2 were studied at a concentration of 3.4 g/L, with three repetitions within F1 and two repetitions within F2 were performed. XOS supplementation (3.4 g/L) was only tested on F1 microbiota with two repetitions. Inulin as the fiber inducing most effects was also tested at a higher concentration of 5 g/L during F2 (two repetitions). Metabolic and bacterial response of both microbiota were compared to the reactor's stabilization period before the treatment (3 days sampling), due to slight metabolic and microbial differences from one reactor to the other that might lead to false negative outcomes.

Inulin supplementation altered metabolite production *in vitro* depending on microbiota

Inulin supplementation induced different metabolic responses depending on microbiota and dose. Addition of 3.4 g/L inulin yielded an increase of total metabolite production in F1 (mean increases between 17 and 35 mM for the three repetitions), but not in F2 microbiota. However, supplementation of 5 g/L inulin during F2 increased total metabolite production, by 18 and 30 mM for the two repetitions (**Supplementary Table S4.9; Supplementary Table S4.10; Supplementary Table S4.11**). In F1, inulin (3.4 g/L) significantly enhanced acetate (between 10 and 23 % for three repetitions) and butyrate (21 to 34 %) production (**Figure S.4 A and B; Figure S4.4 A and B**). In F2 microbiota, low inulin dosage increased propionate production (by 133 and 207 % for two repetitions), but significantly decreased butyrate levels (**Figure S.4 A and B; Figure S4.4 A and B**). Simultaneously, formate and succinate productions were enhanced in F1 microbiota, but decreased in F2 microbiota. Acetate production was enhanced upon high dose inulin treatment (**Figure S4.4 B and Supplementary Table S4.11**), along with butyrate (41 and 55 %) and propionate (12 and 22 %). In addition, succinate accumulated (33 and 27 %) in reactor effluents during intervention.

Inulin supplementation effects mouse microbiota composition dose-dependently

Low dosage of inulin did not change the murine caecal microbiota composition in F1 and F2, with no consistent changes at phylum level. DESeq2 analysis (Log$_2$ fold change > 2; $p < 0.05$) was performed to detect OTUs that show significant differential relative abundance between the stabilization and treatment period (last three days) (**Figure 4.4**; **Supplementary Figure S4.3; Supplementary Table S4.4; Supplementary Table S4.5 Supplementary Table S4.6; Supplementary Table 4.7**). Interestingly, the two different microbiota responded differently upon inulin intervention. Inulin (3.4 g/L) resulted in consistent higher levels of taxa belonging to *Allobaculum spp.* and unclassified Clostridiales in F1 compared to the stabilization period, whereas a pronounced increase in taxa belonging to *Pedicoccus*. and *Lysinibacillus* was observed in F2.

High inulin supplementation profoundly altered microbiome compared to stabilization period in F2 (**Figure 4.4; Supplementary Figure S4.4; Figure 4.4**; **Supplementary Table 4.4; Supplementary Table S4.6;**

Supplementary Table 4.7). Shannon index of treated reactor effluents of the second treatment was increased compared to stabilization (2.8 ± 0.3 for stabilization and 3.7 ± 0.5 for treatment), while no increase was observed during the first intervention. Within the phylum Firmicutes, high inulin intervention resulted in higher relative abundances of *Lactobacillus zeae, Lactobacillus brevis* and taxa belonging to *Pediococcus, Eubacterium, Lactococcus, Allobaculum* and RFN20 (*Erysipelotrichaceae*) (Log$_2$ fold change > 2; p < 0.05). Within the phylum Actinobacteria, increases in abundance were seen in taxa belonging to *Actinomyces, Actinomycetaceae* and *Coriobacteriaceae*.

Taxa of butyrate producers correlate with metabolic outcomes

The CCA biplot was applied to visualize how bacterial taxonomy composition was associated with the metabolite productions, for acetate, propionate, butyrate and succinate. (**Figure 4.4 C**). For low inulin supplementation no separate clustering of stabilization and treatment samples was observed. In contrast, samples treated with 5 g/L inulin were differentiated from stabilization samples, and correlated positively with butyrate levels, and at phylogenetic level, with two butyrate-producing taxa *Lachnospiraceae* and *Ruminococcaceae*.

XOS supplementation induced acetate production *in vitro*

Similar to inulin treatments, XOS supplementation increased total metabolite production in F1 microbiota, with increases of 17 and 29 mM for the two repetitions (**Figure 4.4 A; Supplementary Table 4.10**). Acetate (18 and 11 %, respectively) and propionate (15 and 29 %, respectively) levels were significantly enhanced upon supplementation with XOS. In addition, XOS also raised butyrate production in repetition 2. For assessing overall differences in bacterial community structure during dietary supplementation and stabilization period, we performed DeSeq2 analysis (Log$_2$ fold change > 1; p < 0.05) (**Supplementary Table 4.8**). XOS supplementation resulted in increased relative abundances of taxa belonging to *Ruminococcus, Allobaculum* and *Mogibacteriaceae,* while decreased levels of the species *Veillonella dispar* were observed. CCA biplot analysis did not show any separation between the samples from stabilization and treatment and no consistent OTU could be positively associated with the treatment.
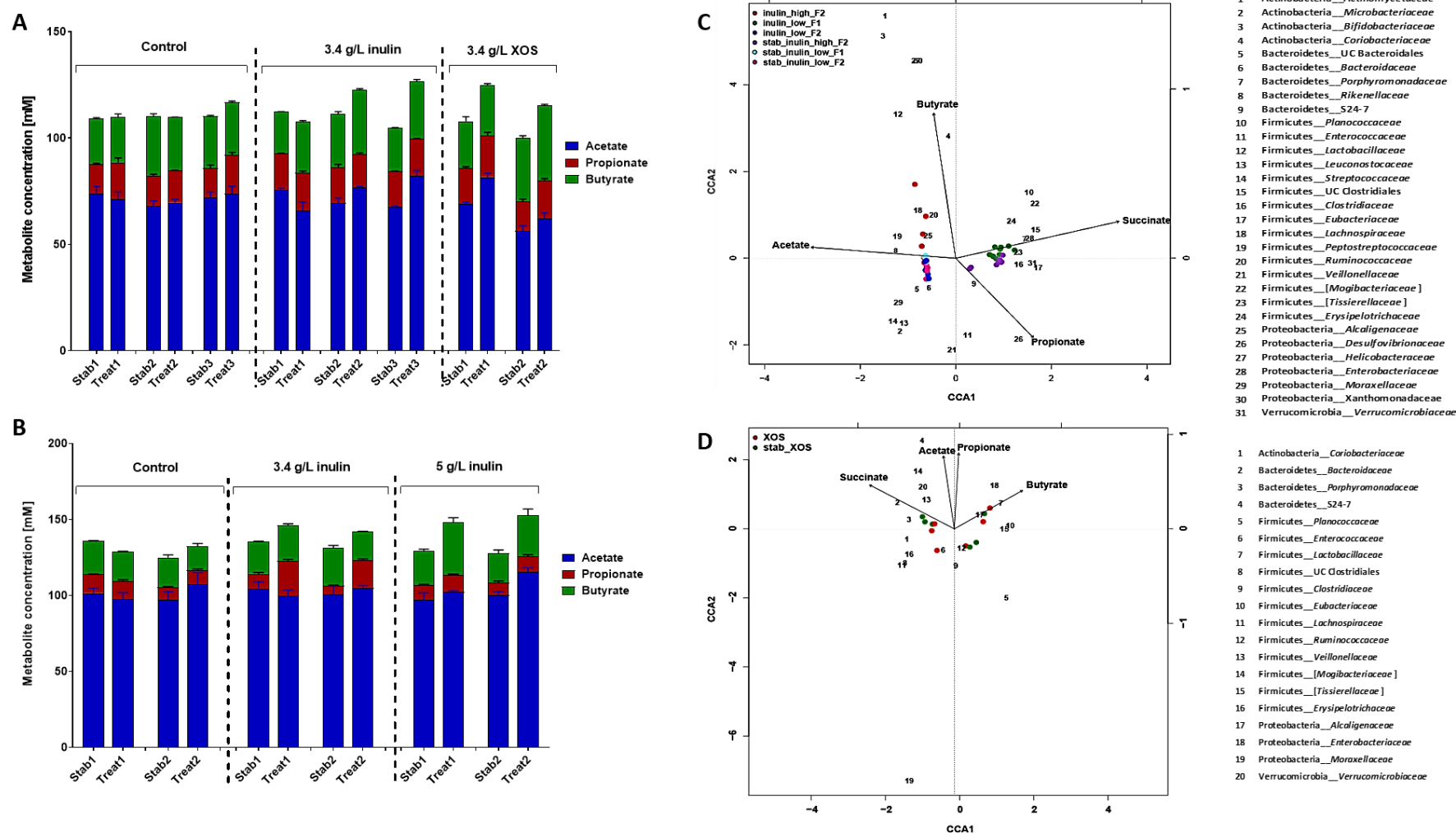
Figure 4.4: (A and B) Effect of dietary fiber supplementation on fermentation metabolite concentrations in fermentation 1 (A) and fermentation 2 (B). Mean from 3 consecutive measurements of acetate, propionate and butyrate (mM) in the treatement reactors with less than 10 % variation during stabilization and treatment phase. (C) Canonical Correspondance Analysis (CCA) showed effects of inulin intervention on metabolite production and microbiota composition of fermentation 1. A high dose (5 g/L) and a low (3.4 g/L) dose of inulin were applied to fermentation 2 microbiota. (D) CCA showing the effect of XOSintervention on fermentation 1 microbiota. F1, fermentation 1; F2, fermentation 2; stab, stabilization; treat, treatment.

Discussion

In vitro colonic fermentation models are powerful platforms for studying the impact of environmental factors (e.g. stresses), dietary supplements or medications (e.g. antibiotics) on the gut microbiota and functionality, independent of the host (Zihler Berner et al., 2013). Here, we investigated the effect of inulin, XOS and metronidazole supplementation on mouse caecal microbiota *in vitro*, and to our knowledge, this is the first study investigating the direct effect of oxidative stress on gut microbiota composition and functionality *in vitro*. Responses to modulations by dietary fibers and metronidazole treatments were compared with published data on the effects of the same treatments in mouse models to confirm the validity of the novel PolyFermS mouse continuous caecum fermentation model.

Indigestible plant-derived dietary fibers can have the ability to beneficially affect host's health (Gibson et al., 2017; Laparra and Sanz, 2010). The complex polysaccharides reach the colon, where the commensal gut bacteria can break them down into SCFAs (Flint et al., 2012). Dietary fibers, such as inulin and XOS are well studied prebiotic supplements that have shown beneficial effects for humans and mice (Christensen et al., 2014; Gibson et al., 2017; Hansen et al., 2013; Hoving et al., 2018). In the present study, supplementation of inulin (two different concentrations) and XOS significantly attenuated bacterial metabolism. Except for low dose of inulin in F2 microbiota, all treatments with the dietary fibers resulted in increased metabolite productions, demonstrating fermentability of the complex carbohydrates. In both treatments, XOS, a short-chain carbohydrate, resulted in increased acetate levels, which can be produced my most heterotrophic microbes in the intestinal microbiome (Luc, 2009). *In vitro* studies showed that diverse commensal gut bacteria can utilize XOS, what might explain the various effects on microbial composition (Poeker et al., 2018; Scott et al., 2014). Inulin can be readily metabolized by taxa belonging to *Lactobacillus* and *Bifidobacterium* and *Eubacterium* (De Vuyst and Leroy, 2011; Riviere et al., 2016). Inulin supplementation is generally correlated to increased abundance of bifidobacteria and lactobacilli in clinical studies and animal models (Loh et al., 2006; Riviere et al., 2016; Salazar et al., 2015). Our data showed that the low tested concentration of both dietary fibers did not affect *in vitro* microbiota structure and in particular did not induce a bifidogenic effect, but increased the production of main SCFAs. In F2, we observed a consistent increase of abundance of taxa belonging to *Pediococcus* (*Lactobacillaceae*), a genus that also produces lactate and whose growth is highly stimulated at the presence of inulin (de Souza de Azevedo et al., 2017). Inulin increased consistently the relative abundance of the genus *Allobacullum*, in agreement with recent mouse studies (Catry et al., 2018; Hoving et al., 2018). The higher inulin concentration altered significantly the microbiome structure compared to the lower concentration. Similar dosage-dependent responses upon dietary fiber supplementations were observed in other *in vivo* and *in vitro* experiments with different human fecal microbiota (Sasaki et al., 2018; Singh et al., 2018; Fehlbaum et al., 2018). Fiber metabolism also indirectly alters bacterial populations that are

not able break down a specific complex dietary fiber, but which can generally consume the produced acetate and lactate by primary degraders, in a mechanism of cross-feeding, and convert acetate or lactate to butyrate and propionate (Duncan et al., 2004a; Duncan et al., 2004b; Zoetendal et al., 2012). Low inulin supplementation induced a butyrogenic response in F1 and a propionigenic response in F2, while in F2 butyrate levels increased only upon high inulin supplementation. Consistently, human intervention and *in vitro* studies with dietary fibers showed significant inter-individual responses, which were depended on the dominant and responsive bacterial populations within the microbiota (Poeker et al., 2018; Walker et al., 2011). A possible explanation for the butyrogenic response is that the accessibility of the fiber increases for other bacterial populations or the availability of intermediates for lactate- and acetate-dependent colon bacteria increases (Calame et al., 2008). We observed consistent increases in taxa belonging to the family *Erysipelotrichaceae* upon inulin treatments, which were demonstrated to express genes involved in acetyl-coA to butyryl-coA conversion and might contribute to the butyrate production seen in our set-up (Seedorf et al., 2008). Overall, the observed increases in taxa belonging to *Erysipelotrichaceae* and Clostridiales suggest being the most active bacterial community members for the acetate and butyrate production.

Metronidazole induced a transient decrease in SCFA production, followed by a recovery period after 3 days, which is in accordance with *in vitro* and *in vivo* studies (Fehlbaum et al., 2016). During metronidazole treatment, BCFA and succinate levels decreased simultaneously with main SCFA production, while formate highly accumulated to high levels during the first days of treatment. Most lactate-utilizers forming butyrate are strict anaerobic bacteria, which are negatively affected by the antibiotic stress, and thus explaining the decrease in butyrate (Flint et al., 2015). The weak effect of the antibiotic on propionate production may be explained by the highly abundant species *Veillonella dispar*, which is known to be able to convert the intermediate lactate to propionate or fumarate to succinate (Flint et al., 2015). Further, only a moderate impact on the phylum Bacteroidetes was observed, which includes many aerotolerant and propionate-producing bacterial populations (Rooks et al., 2014). Marked reductions in Clostridiales and *Lachnospiraceae* spp after metronidazole treatments were previously reported (Becker et al., 2017; Jakobsson et al., 2010), while no change was observed in our study. Similarly, supplementation of metronidazole, which is highly effective against anaerobic bacteria (Lofmark et al., 2010), induced increases in *Veillonella dispar*, *Lactobacillus* and *Enterococcus.* Indeed, similar increased levels of *Enterococcus* spp. upon metronidazole administration were previously reported in clinical and animal models (Becker et al., 2017; Jakobsson et al., 2010).

Functional alternation of the gut microbiota occurs as a result of microbial dysbiosis and a dysbiotic microbiota generally displays a decrease in genes that are involved in carbohydrate fermentation (Morgan et al., 2012; Qin et al., 2010). Indeed, our data showed that oxidative stress decreased total metabolite production, displaying functional imparity of the *in vitro* microbiota. Further, levels of the anti-inflammatory metabolite butyrate have been shown to be decreased in feces of IBD patients and it is assumed that the low occurrence

of butyrate in the intestine might be involved in the development of inflammation (Matsuoka and Kanai, 2015; Takaishi et al., 2008). Our data also showed reduced levels of butyrate upon oxidative stress, which can be explained by significantly decreased abundances of the butyrate-producing members *Lachnospiraceae, Anaerococcus* and *Ruminococcus*. In our study, the decrease in main SCFAs and the increase of intermediates, such as lactate and formate, correlate with the observed shifts within strict anaerobes upon hydrogen peroxide treatment. Fecal lactate level is shown to be increased in individuals suffering from intestinal inflammation, including IBD, and is considered to be a biomarker to detect dysbiosis (Franzosa et al., 2018; Hove et al., 1994; Mayeur et al., 2013). Disturbance of a normal gut microbiota composition can bring along an accumulation of intermediates, because intermediate consumers are negatively affected. Decreases in intermediate-consuming taxa belonging to Bacteroidetes, which are mainly acetate and propionate producers (den Besten et al., 2014; Reichardt et al., 2014), and butyrate producers belonging to the clostridial clusters IV and XIVa within the Firmicutes phylum might explain the accumulation of lactate and formate (Riviere et al., 2016; Van den Abbeele et al., 2013). Oxidative stress highly disrupted gut microbiota composition by promoting growth of Proteobacteria and facultative anaerobes, which is in accordance with human observations (Winter and Baumler, 2014; Zhu and Li, 2012). Treatments with hydrogen peroxide disrupts strict anaerobiosis in the microbial ecosystem and provides a selective growth advantage to facultative anaerobes (Rigottier-Gois, 2013), such as the observed members of *Lactobacillaceae* (Zevin et al., 2017*), Enterococcaceae* (Arboleya et al., 2012), and *Enterobacteriaceae* (Zeng et al., 2017). A study found that individuals suffering from IBD display a decrease in *Lactobacillus* and *Bacteroides* species abundance (Loh and Blaut, 2012). During oxidative stress, a similar consistent reduction in taxa belonging to *Bacteroides* was observed, while the level of one *Lactobacillus* species (*Lactobacillus zeae*) was increased. A dysbiotic gut microbiota is further characterized by a reduction of the predominant strict anaerobic phylum Firmicutes, which includes the main butyrate-producers (Winter and Baumler, 2014). Upon hydrogen peroxide treatments, we observed a consistent decrease in the butyrate producer *Coprococcus* spp., a genus found to occur at low levels in IBD patients and that was taken as a screening biomarker for CD (Santoru et al., 2017).

In summary, we were able to modulate *ex vivo* gut microbiota towards an improved or a dysbiotic profile by applying selected environmental conditions in the *in vitro* mouse caecum fermentation model. Our results demonstrated a fast metabolic modulation of the intestinal microbiota upon dietary fiber supplementation, while the metabolic response was dependent on the type fiber, on the dosage of inulin, and the phylogenetic characteristic of the bacterial community. Metronidazole was found to induce a transient impairment of bacterial fermentation, and was associated with persistent changes in the bacterial community. This study provides first insights on the direct detrimental effects of oxidative stress by hydrogen peroxide on gut microbiota structure and functionality *in vitro*, with adverse effects on the abundance of strict anaerobic butyrate-producer and on microbial fermentation, and enhanced growth of facultative anaerobes (e.g.

Proteobacteria). Our data support the validity of the PolyFermS caecum fermentation model for investigating modulation of microbiota by environmental and dietary factors. Our *ex-vivo* modified intestinal microbiota has further the potential to be used as donor material for microbiota transplantation in mouse models for studying the role and mechanisms of profiled microbiota in health and disease.

Supplementary material
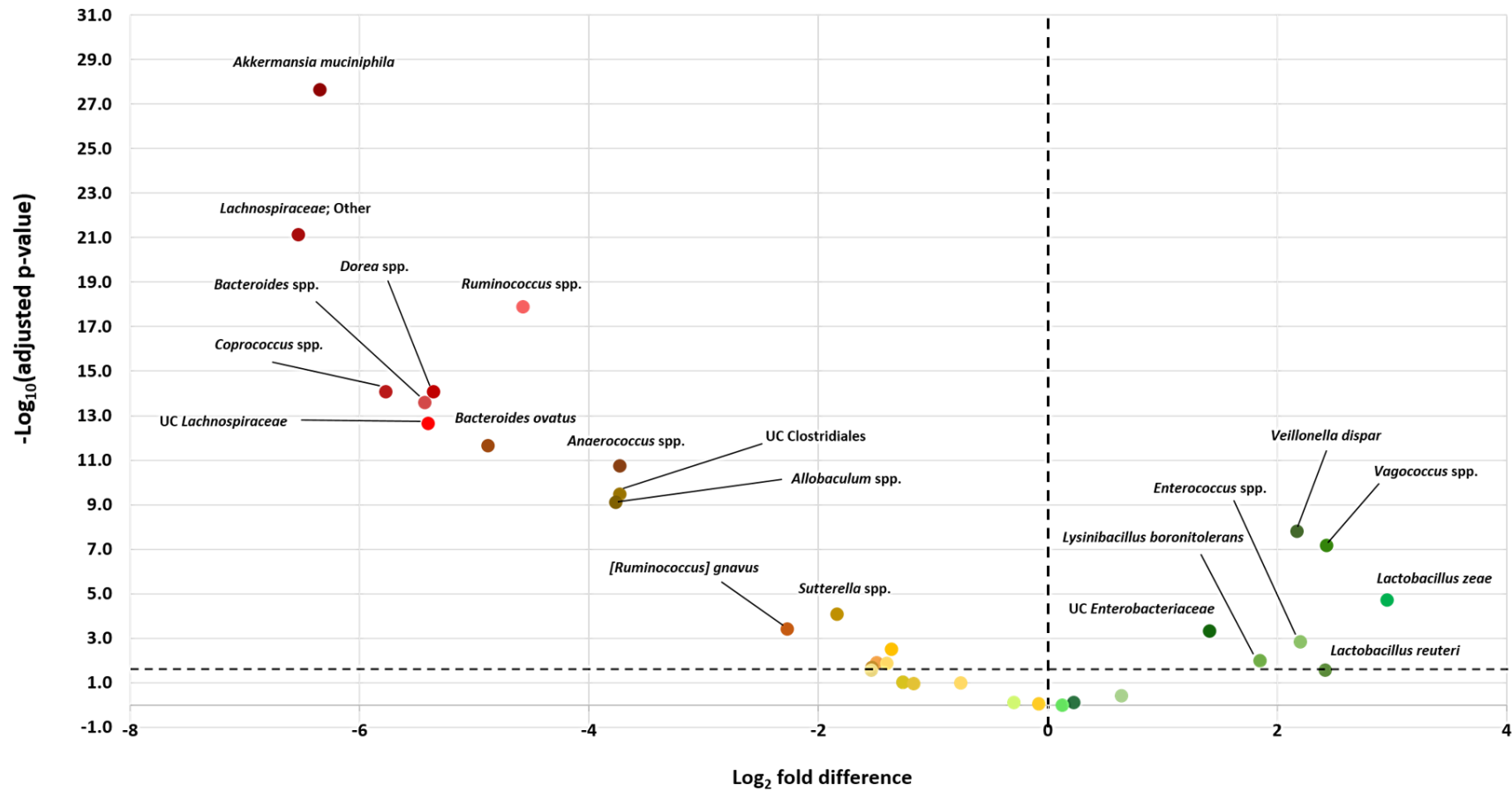
Supplementary Figure S4.1 Microbiota plots illustrating OTUs that were significantly enriched (green) and reduced (red) in reactor effluent during peroxide supplementation in fermentation 1 as determined by differential abundance analysis. Each point represents an individual OUT, and the Y-axis indicates the Log2 fold change of relative abundance. The dashed line represents the statistically p-value of 0.05.
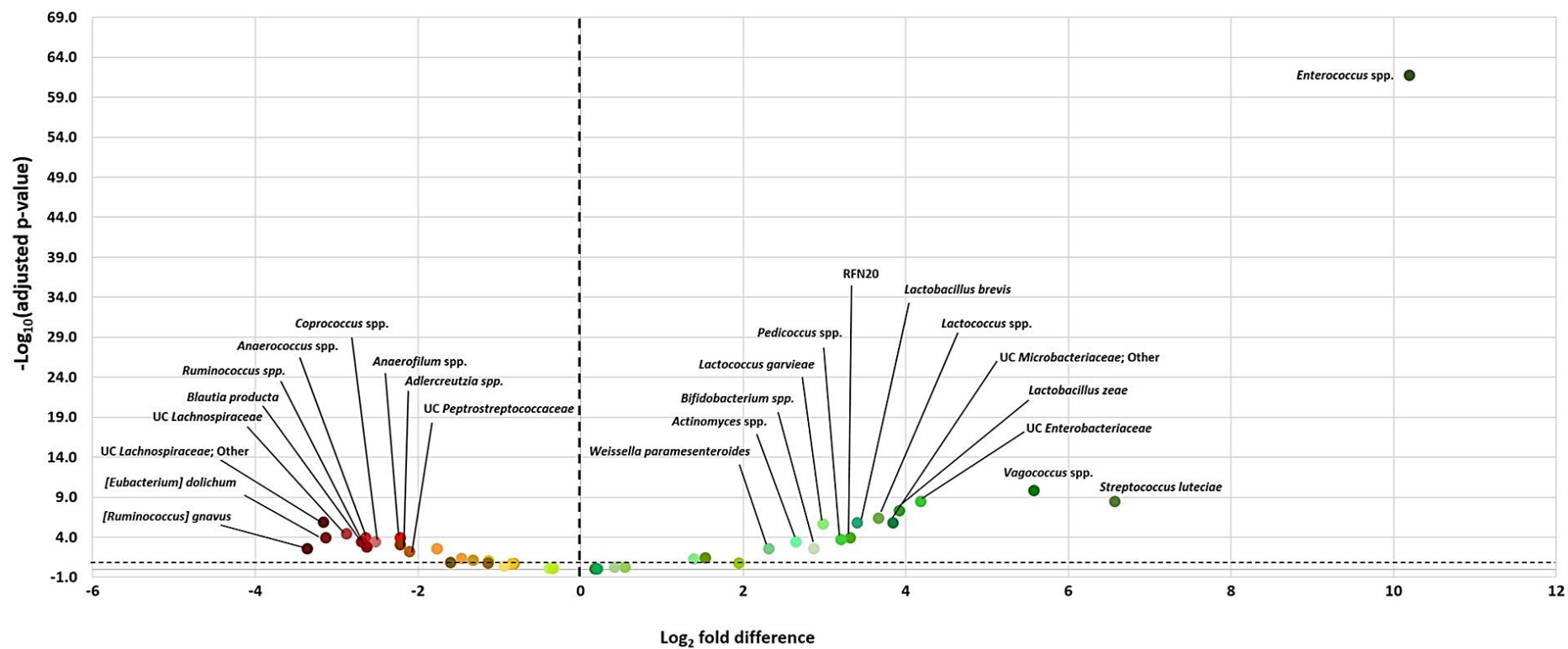
Supplementary Figure S4.2: Microbiota plots illustrating OTUs that were significantly enriched (green) and reduced (red) in reactor effluent during peroxide supplementation in fermentation 2 as determined by differential abundance analysis. Each point represents an individual OUT, and the Y-axis indicates the Log2 fold change of relative abundance. The dashed line represents the statistically p-value of 0.05.
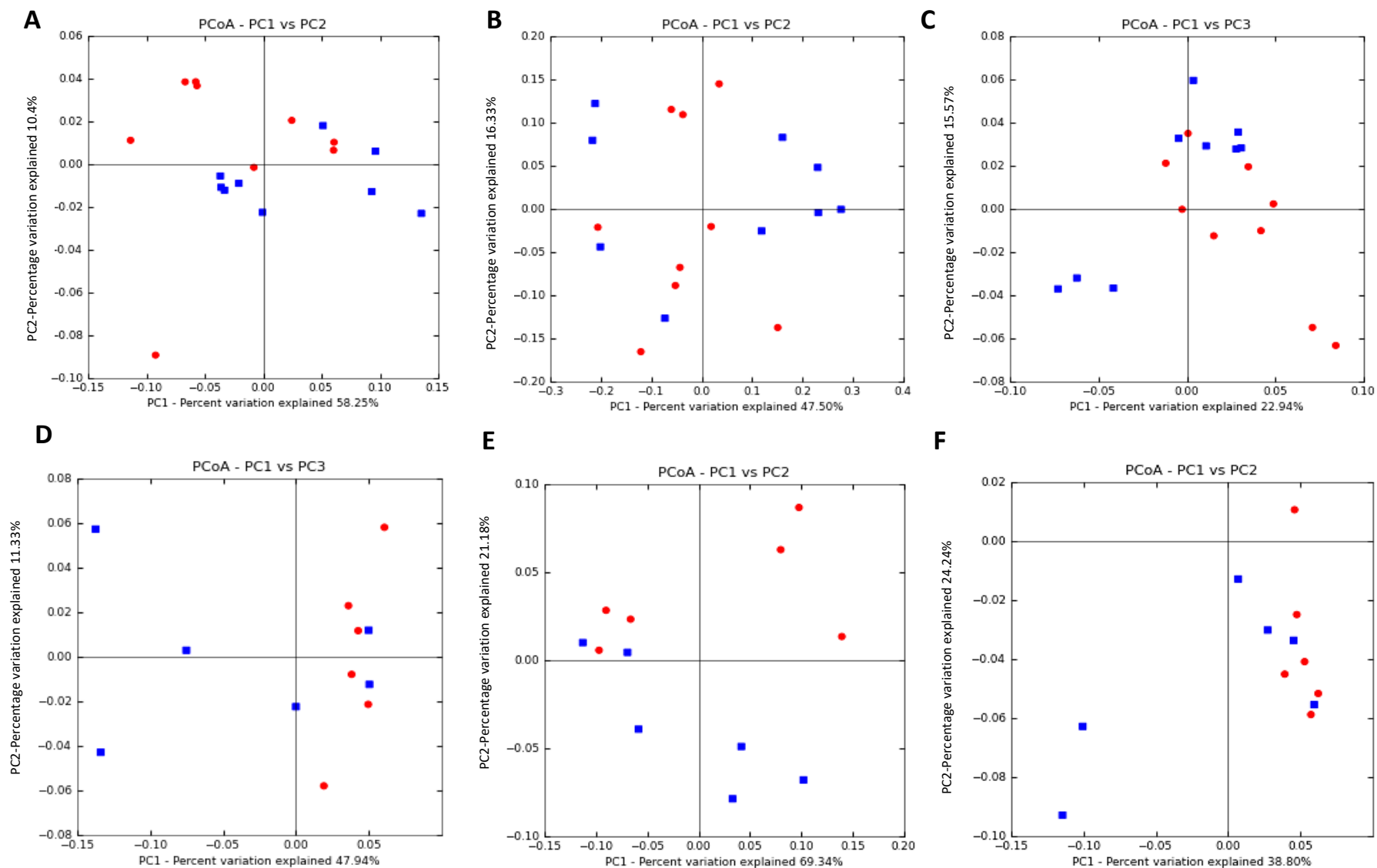
Supplementary Figure S4.3: PCoA plot based on unweighted (A), weighted (B) and generalized (C) UniFrac distance matrix on OTU level generated from non-treated and 3.4 g/L inulin treated PolyFermS reactor effluents of fermentation1 (untreated: blue; treated: red). PCoA plot based on unweighted (D), weighted (E) and generalized (F) UniFrac distance matrix on OTU level generated from non-treated and 3.4 g/L inulin treated PolyFermS reactor effluents of fermentation2 (untreated: blue; treated: red). Each circle represents a sample.
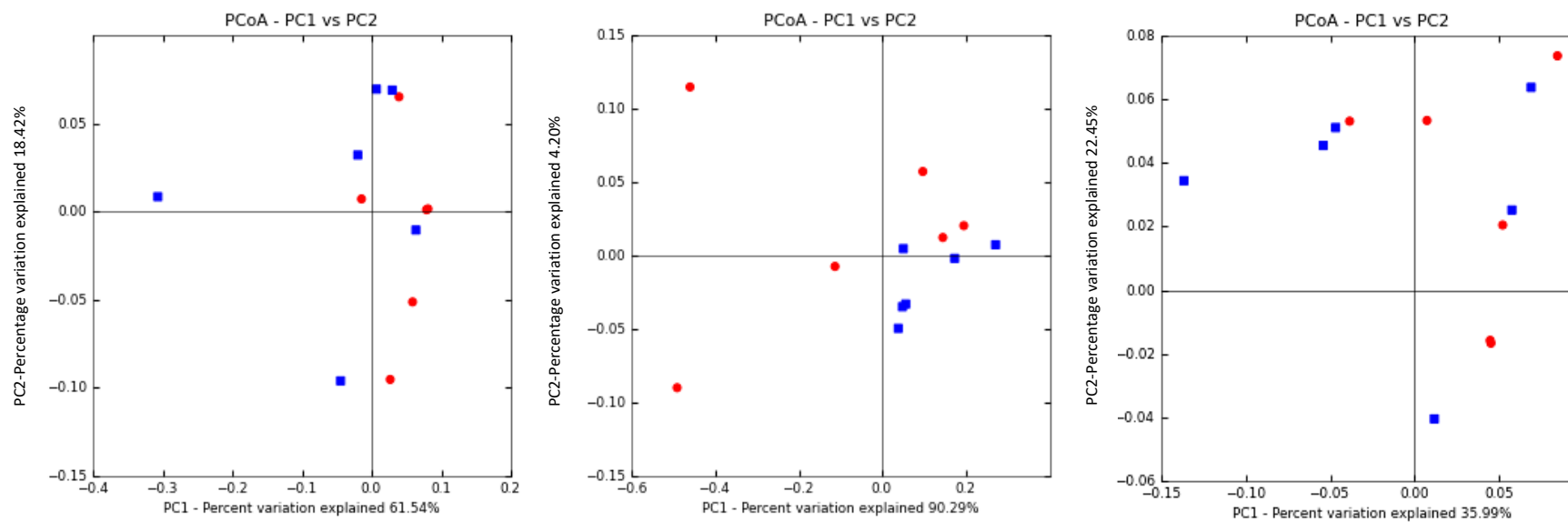
Supplementary Figure S4.4: PCoA plot based on unweighted (A), weighted (B) and generalized (C) UniFrac distance matrix on OTU level generated from non-treated and 5 g/L inulin treated PolyFermS reactor effluents of fermentation 2 (untreated: blue; treated: red). Each circle represents a sample
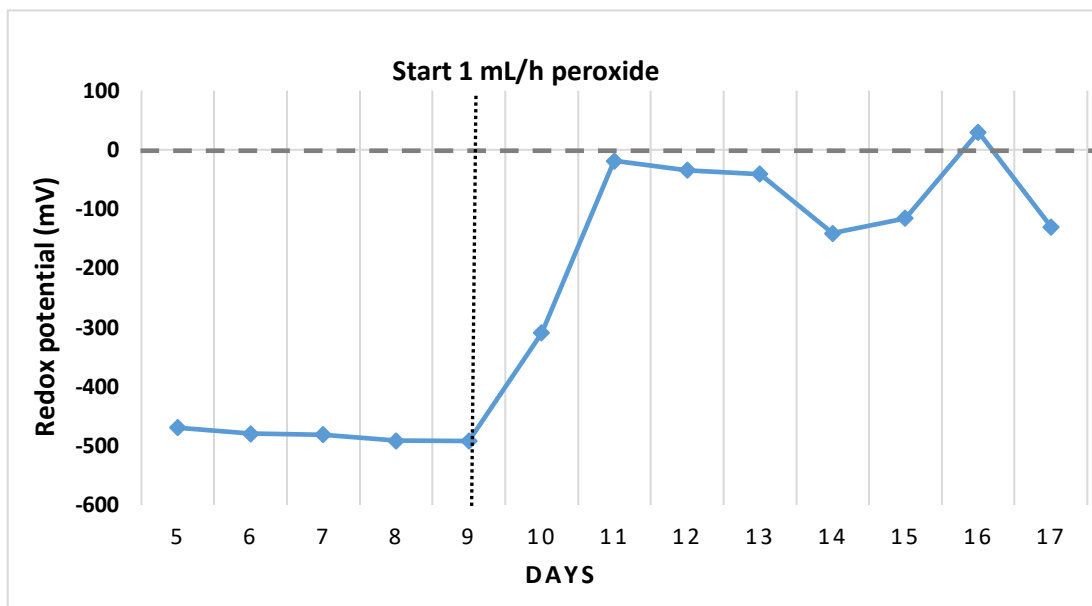
Supplementary Figure S4.5: Redox potential (mV) in reactor effluent in treatment 3 of fermentation 1
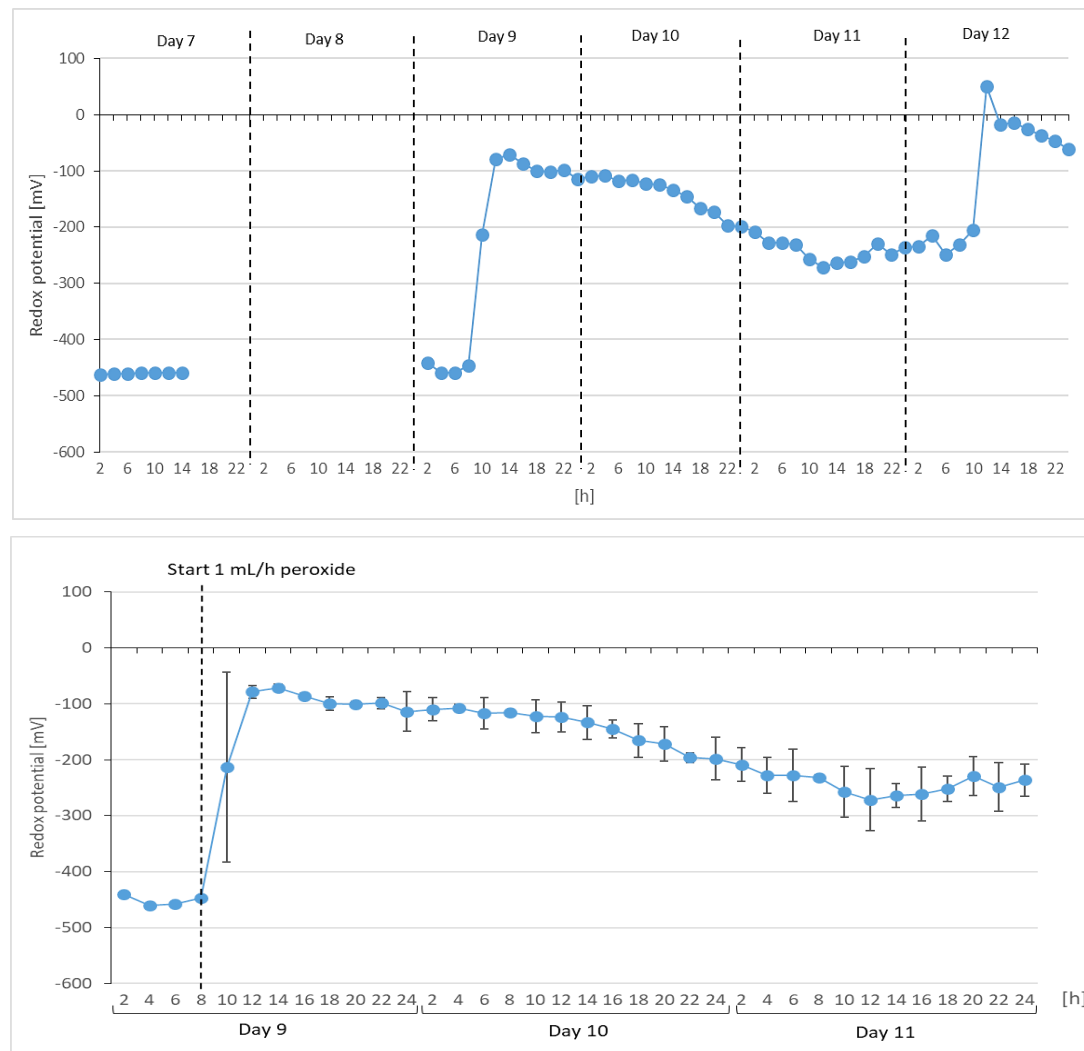
Supplementary Figure S4.6: Redox potential (mV) in reactor effluent in treatment 1 of fermentation 2

Supplementary Table S4.1: Summary microbial phyla and most abundant (> 1 %) bacterial families obtained by V4 region 16S amplicon sequencing within PolyFermS reactors before (stabilization) and during peroxide treatment in fermentation 1. Values < 1 % are summarized in the group «Others». Data are means ± SD of three consecutive days.

Taxon	Stabilization 1			Treatment 1			Stabilization 2			Treatment 2			Stabilization 3			Treatment 3			Stabilization 4			Treatment 4		
Bacteroidetes	3.5%	±	1.7%	0.3%	±	0.1%	32.8%	±	1.8%	6.4%	±	2.0%	31.0%	±	3.0%	1.2%	±	1.6%	40.7%	±	11.1%	1.3%	±	0.4%
Bacteroidaceae	0.3%	±	0.0%	0.1%	±	0.0%	25.0%	±	2.4%	1.1%	±	0.2%	26.0%	±	2.4%	0.8%	±	1.1%	38.7%	±	10.7%	0.4%	±	0.3%
Porphyromonadaceae	3.2%	±	1.7%	0.2%	±	0.1%	7.8%	±	0.6%	5.4%	±	1.8%	4.2%	±	0.6%	0.4%	±	0.4%	2.0%	±	0.6%	0.9%	±	0.3%
Firmicutes	55.2%	±	0.7%	48.8%	±	25.6%	43.0%	±	3.8%	59.0%	±	8.7%	40.5%	±	3.2%	53.4%	±	14.0%	15.6%	±	2.5%	35.9%	±	13.6%
Planococcaceae	3.6%	±	1.1%	7.1%	±	3.5%	0.1%	±	0.1%	10.4%	±	4.0%	0.9%	±	0.9%	7.1%	±	3.9%	1.0%	±	0.8%	4.9%	±	4.9%
Enterococcaceae	1.0%	±	0.1%	16.6%	±	9.9%	0.2%	±	0.1%	7.0%	±	3.9%	0.6%	±	0.3%	11.5%	±	8.2%	0.8%	±	0.3%	11.0%	±	4.4%
Lactobacillaceae	3.0%	±	1.0%	23.3%	±	11.7%	0.1%	±	0.0%	8.8%	±	5.4%	0.0%	±	0.0%	30.6%	±	8.9%	0.1%	±	0.0%	7.5%	±	3.5%
UC Clostridiales	0.0%	±	0.0%	0.0%	±	0.0%	1.3%	±	0.3%	0.1%	±	0.0%	0.8%	±	0.3%	0.0%	±	0.0%	1.4%	±	0.3%	0.1%	±	0.1%
Clostridiaceae	32.5%	±	0.5%	0.6%	±	0.0%	35.4%	±	3.3%	30.2%	±	22.2%	32.6%	±	3.7%	0.4%	±	0.3%	5.2%	±	0.8%	10.5%	±	5.9%
Eubacteriaceae	2.7%	±	0.4%	0.2%	±	0.1%	0.0%	±	0.0%	0.0%	±	0.0%	0.0%	±	0.0%	0.0%	±	0.0%	0.0%	±	0.0%	0.0%	±	0.0%
Lachnospiraceae	0.0%	±	0.0%	0.0%	±	0.0%	3.2%	±	0.3%	0.1%	±	0.0%	2.3%	±	0.3%	0.0%	±	0.0%	3.0%	±	0.5%	0.1%	±	0.0%
Ruminococcaceae	0.0%	±	0.0%	0.0%	±	0.0%	0.0%	±	0.0%	0.0%	±	0.0%	0.0%	±	0.0%	0.0%	±	0.0%	1.8%	±	0.5%	0.0%	±	0.0%
Veillonellaceae	0.0%	±	0.0%	0.0%	±	0.0%	0.2%	±	0.1%	2.6%	±	0.8%	0.3%	±	0.1%	2.8%	±	2.1%	0.3%	±	0.1%	1.6%	±	0.4%
[Tissierellaceae]	11.3%	±	0.4%	0.1%	±	0.0%	0.0%	±	0.0%	0.0%	±	0.0%	1.0%	±	0.3%	0.1%	±	0.1%	1.7%	±	0.2%	0.1%	±	0.0%
Erysipelotrichaceae	0.0%	±	0.0%	0.0%	±	0.0%	0.0%	±	0.0%	0.0%	±	0.0%	1.0%	±	0.3%	0.1%	±	0.1%	0.0%	±	0.0%	0.0%	±	0.0%
Proteobacteria	18.4%	±	2.4%	50.6%	±	25.4%	3.9%	±	0.8%	33.9%	±	6.7%	10.0%	±	1.1%	45.1%	±	14.6%	28.3%	±	17.4%	62.4%	±	14.3%
Alcaligenaceae	1.7%	±	0.1%	0.1%	±	0.0%		±			±		0.8%	±	0.2%	0.4%	±	0.3%	0.0%	±	0.0%	0.0%	±	0.0%
Enterobacteriaceae	16.7%	±	2.6%	50.8%	±	25.4%	3.5%	±	0.7%	33.8%	±	6.7%	9.2%	±	1.2%	44.7%	±	14.8%	27.8%	±	17.5%	62.2%	±	14.3%
Verrucomicrobia	22.9%	±	0.0%	0.1%	±	0.1%	20.2%	±	2.3%	0.2%	±	0.1%	18.5%	±	1.7%	0.2%	±	0.2%	15.4%	±	5.4%	0.4%	±	0.4%
Verrucomicrobiaceae	22.9%	±	0.0%	0.1%	±	0.1%	20.2%	±	2.3%	0.2%	±	0.1%	18.5%	±	1.7%	0.2%	±	0.2%	15.4%	±	5.4%	0.4%	±	0.4%
Others	2.0%	±	0.3%	1.4%	±	0.5%	2.4%	±	0.8%	1.1%	±	0.7%	3.5%	±	0.5%	1.5%	±	0.9%	2.1%	±	0.5%	1.9%	±	1.2%

Supplementary Table S4.2: Summary microbial phyla and most abundant (> 1 %) bacterial families obtained by V4 region 16S amplicon sequencing within PolyFermS reactors before (stabilization) and during peroxide treatment in fermentation 2. Values < 1 % are summarized in the group «Others». Data are means ± SD of three consecutive days.

Taxon	Stabilization 1			Treatment 1			Stabilization 2			Treatment 2		
Actinobacteria	**0.0%**	**±**	**0.0%**	**1.1%**	**±**	**0.9%**	**0.2%**	**±**	**0.0%**	**0.2%**	**±**	**0.2%**
Bifidobacteriaceae	0.0%	±	0.0%	0.8%	±	0.8%	0.1%	±	0.0%	0.1%	±	0.1%
Actinomycetaceae	0.0%	±	0.0%	0.2%	±	0.1%	0.1%	±	0.0%	0.1%	±	0.1%
Microbacteriaceae	0.0%	±	0.0%	0.1%	±	0.1%	0.0%	±	0.0%	0.0%	±	0.0%
Bacteroidetes	**82.0%**	**±**	**4.2%**	**25.7%**	**±**	**18.1%**	**59.1%**	**±**	**4.0%**	**10.1%**	**±**	**5.9%**
Bacteroidaceae	81.9%	±	4.1%	25.5%	±	18.1%	59.1%	±	4.0%	10.1%	±	5.9%
Firmicutes	**16.7%**	**±**	**4.3%**	**69.2%**	**±**	**18.9%**	**38.4%**	**±**	**4.0%**	**81.1%**	**±**	**2.3%**
Planococcaceae	0.3%	±	0.1%	1.4%	±	0.9%	0.0%	±	0.0%	0.0%	±	0.0%
Enterococcaceae	0.0%	±	0.0%	16.7%	±	6.5%	0.1%	±	0.0%	18.7%	±	13.5%
Lactobacillaceae	0.9%	±	0.6%	40.0%	±	19.6%	7.9%	±	3.8%	54.9%	±	11.8%
Leuconostocaceae	1.1%	±	0.6%	1.7%	±	0.9%	0.1%	±	0.0%	4.0%	±	4.0%
Streptococcaceae	0.2%	±	0.1%	4.7%	±	1.7%	0.1%	±	0.0%	1.0%	±	0.6%
Clostridiaceae	0.2%	±	0.1%	0.4%	±	0.2%	1.2%	±	0.3%	0.3%	±	0.2%
Lachnospiraceae	9.8%	±	4.5%	2.0%	±	1.5%	20.1%	±	1.0%	1.4%	±	1.2%
Peptostreptococcaceae	1.4%	±	0.1%	0.9%	±	0.7%	3.8%	±	1.1%	0.2%	±	0.1%
Ruminococcaceae	2.3%	±	0.7%	1.3%	±	0.9%	4.3%	±	0.4%	0.7%	±	0.5%
Proteobacteria	**1.2%**	**±**	**0.2%**	**4.1%**	**±**	**1.7%**	**2.3%**	**±**	**0.1%**	**8.6%**	**±**	**7.3%**
Alcaligenaceae	0.9%	±	0.2%	0.4%	±	0.2%	1.7%	±	0.1%	0.2%	±	0.1%
Enterobacteriaceae	0.3%	±	0.1%	3.0%	±	1.5%	0.3%	±	0.1%	8.0%	±	7.2%
Others	2.8%	±	0.9%	3.0%	±	0.9%	2.7%	±	0.8%	2.4%	±	0.7%

Supplementary Table 4.3: (A) Summary microbial phyla and most abundant (> 1 %) bacterial families obtained by V4 region 16S amplicon sequencing within PolyFermS reactors before (stabilization) and during metronidazole treatment in fermentation 1. Values < 1 % are summarized in the group «Others». Data are means ± SD of three consecutive days. (B) Table displaying OTUs that significantly enriched (positive Log2 fold change) or reduced (negative Log2 fold change) in reactor effluent during metronidazole supplementation.

A

Taxon	Stabilization 1			Treatment 1			Stabilization 2			Treatment 2		
Actinobacteria	0.1%	±	0.1%	0.0%	±	0.0%	0.0%	±	0.0%	0.1%	±	0.0%
Bacteroidetes	23.3%	±	6.9%	26.0%	±	4.0%	34.7%	±	7.0%	24.2%	±	12.1%
Bacteroidaceae	16.3%	±	5.7%	19.0%	±	6.3%	28.2%	±	6.4%	18.3%	±	11.3%
Porphyromonadaceae	7.0%	±	2.6%	6.5%	±	3.2%	6.5%	±	1.6%	5.3%	±	2.7%
Firmicutes	38.9%	±	6.7%	29.3%	±	12.9%	34.2%	±	1.4%	24.3%	±	6.1%
Planococcaceae												
Enterococcaceae	0.9%	±	0.2%	3.5%	±	3.5%	2.1%	±	2.0%	0.9%	±	0.2%
Lactobacillaceae	0.5%	±	0.5%	1.2%	±	1.2%	1.0%	±	0.6%	1.1%	±	1.3%
UC Clostridiales	1.2%	±	0.9%	1.9%	±	1.6%	1.0%	±	1.0%	1.2%	±	1.0%
Clostridiaceae	28.8%	±	8.7%	11.8%	±	9.6%	22.4%	±	5.4%	12.8%	±	10.2%
Eubacteriaceae												
Lachnospiraceae	2.3%	±	1.6%	2.4%	±	1.9%	1.1%	±	0.7%	2.3%	±	2.0%
Ruminococcaceae	1.1%	±	1.0%	2.0%	±	1.4%	0.5%	±	0.5%	1.3%	±	1.1%
Veillonellaceae	0.4%	±	0.1%	2.3%	±	2.1%	0.8%	±	0.8%	0.6%	±	0.3%
[Tissierellaceae]	1.2%	±	0.1%	2.1%	±	0.7%	1.4%	±	0.2%	1.6%	±	0.2%
Erysipelotrichaceae	2.5%	±	2.5%	0.3%	±	0.2%	3.9%	±	3.3%	2.2%	±	2.2%
Proteobacteria	14.5%	±	1.8%	23.4%	±	8.5%	16.1%	±	8.2%	28.4%	±	5.3%
Alcaligenaceae	0.4%	±	0.2%	0.8%	±	0.4%	0.7%	±	0.1%	0.6%	±	0.4%
Enterobacteriaceae	14.1%	±	1.7%	22.6%	±	8.1%	15.4%	±	7.9%	27.8%	±	6.7%
Verrucomicrobia	23.2%	±	2.0%	21.3%	±	3.5%	15.0%	±	4.6%	23.0%	±	14.3%
Verrucomicrobiaceae	23.2%	±	2.0%	21.3%	±	3.5%	15.0%	±	4.6%	23.0%	±	14.3%
Others	1.9%	±	0.7%	1.7%	±	0.6%	2.0%	±	0.4%	1.5%	±	0.6%

B

OTU	baseMean	log2FoldChange	pvalue	padj	taxonomy		
					Phylum	Family	Genus/Species
OTU21	989	1.47	0.01	0.26	Firmicutes	Enterococcaceae	Enterococcus spp.
OTU52	568	1.60	0.01	0.26	Firmicutes	Veillonellaceae	Veillonella dispar
OTU64	326	1.01	0.01	0.36	Proteobacteria	Alcaligenaceae	Sutterella spp.
OTU25	411	1.39	0.04	0.73	Firmicutes	Lactobacillaceae	Lactobacillus reuteri
OTU14	2	-1.00	0.08	0.88	Bacteroidetes	Rikenellaceae	
OTU40	150	-1.16	0.08	0.88	Firmicutes	Lachnospiraceae	
OTU59	5	-1.09	0.06	0.88	Firmicutes	Erysipelotrichaceae	

Supplementary Table S4.4: Summary microbial phyla and most abundant (> 1 %) bacterial families obtained by V4 region 16S amplicon sequencing within PolyFermS reactors before (stabilization) and during low dose inulin treatment in fermentation 1. Values < 1 % are summarized in the group «Others». Data are means ± SD of three consecutive days.

Taxon	Stabilization 1			Treatment 1			Stabilization 2			Treatment 2			Stabilization 3			Treatment 3		
Actinobacteria	0.1%	±	0.0%	0.1%	±	0.0%	0.0%	±	0.0%	0.1%	±	0.1%	0.1%	±	0.0%	0.1%	±	0.1%
Bacteroidetes	21.8%	±	2.2%	26.3%	±	4.2%	28.3%	±	3.2%	31.4%	±	5.5%	43.8%	±	12.3%	15.6%	±	6.0%
Bacteroidaceae	17.5%	±	0.8%	22.0%	±	4.7%	21.7%	±	1.9%	21.5%	±	8.0%	41.5%	±	12.1%	7.2%	±	5.4%
Porphyromonadaceae	4.2%	±	1.6%	4.3%	±	1.0%	6.6%	±	1.3%	9.8%	±	5.6%	2.3%	±	0.2%	8.3%	±	2.4%
Firmicutes	43.1%	±	4.4%	33.5%	±	7.5%	31.6%	±	8.3%	38.1%	±	14.4%	16.6%	±	1.9%	37.8%	±	7.3%
Planococcaceae	0.0%	±	0.0%	0.0%	±	0.0%	2.3%	±	2.3%	4.3%	±	1.8%	0.9%	±	0.3%	1.3%	±	0.6%
Enterococcaceae	0.4%	±	0.2%	1.8%	±	1.0%	0.3%	±	0.1%	0.3%	±	0.2%	0.8%	±	0.1%	1.3%	±	0.4%
UC Clostridiales	0.7%	±	0.2%	4.9%	±	1.0%	0.9%	±	0.1%	4.1%	±	1.5%	1.4%	±	0.4%	5.9%	±	3.0%
Clostridiaceae	34.0%	±	4.8%	4.2%	±	0.2%	23.4%	±	7.0%	10.3%	±	6.0%	4.4%	±	0.0%	12.3%	±	8.3%
Lachnospiraceae	3.2%	±	0.5%	3.9%	±	0.8%	2.6%	±	0.4%	5.2%	±	1.2%	3.7%	±	0.9%	5.7%	±	1.8%
[Tissierellaceae]	1.6%	±	0.4%	1.8%	±	0.2%	1.0%	±	0.2%	2.6%	±	0.9%	2.1%	±	0.2%	5.0%	±	3.6%
Erysipelotrichaceae	0.6%	±	0.3%	14.7%	±	6.0%	0.3%	±	0.2%	9.4%	±	7.0%	0.2%	±	0.1%	2.4%	±	2.0%
Ruminococcaceae	2.3%	±	0.3%	1.6%	±	0.5%	0.7%	±	0.3%	1.4%	±	0.4%	2.5%	±	0.6%	2.8%	±	0.7%
Proteobacteria	8.6%	±	0.9%	9.4%	±	5.3%	17.7%	±	16.9%	19.7%	±	7.2%	20.9%	±	16.7%	28.4%	±	13.5%
Alcaligenaceae	0.4%	±	0.2%	0.1%	±	0.0%	0.4%	±	0.1%	0.9%	±	0.8%	0.6%	±	0.0%	0.6%	±	0.2%
Enterobacteriaceae	8.3%	±	0.7%	9.4%	±	5.3%	17.3%	±	16.9%	18.8%	±	7.4%	20.3%	±	16.8%	27.9%	±	13.5%
Verrucomicrobia	26.4%	±	3.5%	30.7%	±	5.0%	22.4%	±	7.9%	10.8%	±	5.9%	18.6%	±	2.9%	18.1%	±	3.3%
Others	2.5%	±	0.5%	0.9%	±	0.4%	2.2%	±	1.1%	1.2%	±	0.1%	2.8%	±	0.7%	2.3%	±	1.0%

Supplementary Table S4.5: Summary microbial phyla and most abundant (> 1 %) bacterial families obtained by V4 region 16S amplicon sequencing within PolyFermS reactors before (stabilization) and during low dose inulin treatment in fermentation 2. Values < 1 % are summarized in the group «Others». Data are means ± SD of three consecutive days.

Taxon	Stabilization 1			Treatment 1			Stabilization 2			Treatment 2		
Actinobacteria	0.0%	±	0.0%	0.1%	±	0.0%	0.1%	±	0.0%	0.1%	±	0.0%
Bacteroidetes	78.9%	±	2.5%	79.5%	±	1.6%	67.7%	±	3.1%	61.6%	±	2.8%
Bacteroidaceae	78.9%	±	2.5%	79.5%	±	1.6%	67.7%	±	3.1%	61.6%	±	2.8%
Firmicutes	19.9%	±	2.4%	19.4%	±	1.8%	30.9%	±	3.0%	36.1%	±	2.7%
Planococcaceae	0.0%	±	0.0%	0.0%	±	0.0%	2.3%	±	2.3%	4.3%	±	1.8%
Enterococcaceae	0.0%	±	0.0%	0.0%	±	0.0%	0.1%	±	0.1%	4.2%	±	3.2%
Leuconostocaceae	0.7%	±	0.7%	0.7%	±	0.7%	0.3%	±	0.3%	0.3%	±	0.3%
Streptococcaceae	1.0%	±	0.9%	0.4%	±	0.2%	1.5%	±	1.1%	4.3%	±	1.8%
Lactobacillaceae	2.2%	±	1.4%	4.9%	±	2.3%	0.1%	±	0.1%	7.2%	±	3.9%
Clostridiaceae	0.2%	±	0.1%	0.3%	±	0.1%	1.1%	±	0.2%	1.1%	±	0.2%
Lachnospiraceae	11.7%	±	3.3%	8.6%	±	0.8%	20.5%	±	4.2%	8.2%	±	5.4%
Peptostreptococcaceae	1.2%	±	0.3%	0.8%	±	0.1%	2.1%	±	0.6%	3.1%	±	0.3%
Erysipelotrichaceae	0.1%	±	0.0%	0.5%	±	0.2%	0.9%	±	0.2%	1.1%	±	0.1%
Ruminococcaceae	2.5%	±	1.0%	2.3%	±	0.8%	3.8%	±	0.5%	4.7%	±	0.8%
Veillonellaceae	0.0%	±	0.0%	0.4%	±	0.0%	0.4%	±	0.4%	1.8%	±	0.2%
Proteobacteria	1.1%	±	0.7%	1.0%	±	0.4%	1.2%	±	0.2%	2.1%	±	0.3%
Alcaligenaceae	0.6%	±	0.0%	0.8%	±	0.3%	0.8%	±	0.1%	1.4%	±	0.1%
Others	2.6%	±	1.2%	3.8%	±	0.2%	2.9%	±	0.7%	2.2%	±	0.8%

Supplementary Table S4.6: Summary microbial phyla and most abundant (> 1 %) bacterial families obtained by V4 region 16S amplicon sequencing within PolyFermS reactors before (stabilization) and during high dose inulin treatment in fermentation 2. Values < 1 % are summarized in the group «Others». Data are means ± SD of three consecutive days.

Taxon	Stabilization 1			Treatment 1			Stabilization 2			Treatment 2		
Actinobacteria	**0.0%**	**±**	**0.0%**	**0.1%**	**±**	**0.0%**	**0.1%**	**±**	**0.0%**	**0.1%**	**±**	**0.0%**
Bacteroidetes	**78.9%**	**±**	**2.5%**	**79.5%**	**±**	**1.6%**	**67.7%**	**±**	**3.1%**	**61.6%**	**±**	**2.8%**
Bacteroidaceae	78.9%	±	2.5%	79.5%	±	1.6%	67.7%	±	3.1%	61.6%	±	2.8%
Firmicutes	**19.9%**	**±**	**2.4%**	**19.4%**	**±**	**1.8%**	**30.9%**	**±**	**3.0%**	**36.1%**	**±**	**2.7%**
Planococcaceae	0.0%	±	0.0%	0.0%	±	0.0%	2.3%	±	2.3%	4.3%	±	1.8%
Enterococcaceae	0.0%	±	0.0%	0.0%	±	0.0%	0.1%	±	0.1%	4.2%	±	3.2%
Leuconostocaceae	0.7%	±	0.7%	0.7%	±	0.7%	0.3%	±	0.3%	0.3%	±	0.3%
Streptococcaceae	1.0%	±	0.9%	0.4%	±	0.2%	1.5%	±	1.1%	4.3%	±	1.8%
Lactobacillaceae	2.2%	±	1.4%	4.9%	±	2.3%	0.1%	±	0.1%	7.2%	±	3.9%
Clostridiaceae	0.2%	±	0.1%	0.3%	±	0.1%	1.1%	±	0.2%	1.1%	±	0.2%
Lachnospiraceae	11.7%	±	3.3%	8.6%	±	0.8%	20.5%	±	4.2%	8.2%	±	5.4%
Peptostreptococcaceae	1.2%	±	0.3%	0.8%	±	0.1%	2.1%	±	0.6%	3.1%	±	0.3%
Erysipelotrichaceae	0.1%	±	0.0%	0.5%	±	0.2%	0.9%	±	0.2%	1.1%	±	0.1%
Ruminococcaceae	2.5%	±	1.0%	2.3%	±	0.8%	3.8%	±	0.5%	4.7%	±	0.8%
Veillonellaceae	0.0%	±	0.0%	0.4%	±	0.0%	0.4%	±	0.4%	1.8%	±	0.2%
Proteobacteria	**1.1%**	**±**	**0.7%**	**1.0%**	**±**	**0.4%**	**1.2%**	**±**	**0.2%**	**2.1%**	**±**	**0.3%**
Alcaligenaceae	0.6%	±	0.0%	0.8%	±	0.3%	0.8%	±	0.1%	1.4%	±	0.1%
Others	2.6%	±	1.2%	3.8%	±	0.2%	2.9%	±	0.7%	2.2%	±	0.8%

Supplementary Table S4.7: (A & B) Tables displaying OTUs that significantly enriched (positive Log2 fold change) or reduced (negative Log2 fold change) in reactor effluent during low dose inulin treatment in fermentation 1 and 2. (C) Table displaying OTUs that significantly enriched or reduced in reactor effluent during high dose inulin tretment in fermentation 2.

A

Fermentation 1- low inulin

OTU	baseMean	log2FoldChange	pvalue	padj	Phylum	Family	Genus/Species
OTU52	1589	3.96	5.69E-13	3.98E-11	Firmicutes	Erysipelotrichaceae	Allobaculum spp.
OTU26	1250	1.95	3.55E-08	1.24E-06	Firmicutes	UC Clostridiales	
OTU27	6345	-1.38	0.00	0.08	Firmicutes	Clostridiaceae	
OTU8	9897	-1.08	0.01	0.14	Bacteroidetes	Bacteroidaceae	Bacteroides spp.
OTU12	2	-1.33	0.05	0.63	Bacteroidetes	Rikenellaceae	
OTU45	10	1.38	0.05	0.63	Firmicutes	[Mogibacteriaceae]	
OTU5	9	-0.93	0.07	0.65	Actinobacteria	Coriobacteriaceae	
OTU70	8904	-0.54	0.07	0.65	Verrucomicrobia	Verrucomicrobiaceae	Akkermansia spp.
OTU10	2473	0.53	0.13	0.83	Bacteroidetes	Porphyromonadaceae	Parabacteroides spp.
OTU19	324	0.68	0.14	0.83	Firmicutes	Enterococcaceae	Enterococcus spp.
OTU32	230	1.03	0.14	0.83	Firmicutes	Lachnospiraceae	
OTU37	50	0.54	0.17	0.83	Firmicutes	Lachnospiraceae	[Ruminococcus]
OTU39	25	1.18	0.16	0.83	Firmicutes	Ruminococcaceae	
OTU51	7	-1.17	0.13	0.83	Firmicutes	Erysipelotrichaceae	
OTU20	2	0.84	0.19	0.91	Firmicutes	Enterococcaceae	Vagococcus

B

Fermentation 2 -low inulin

OTU	baseMean	log2FoldChange	pvalue	padj	Phylum	Family	Genus/Species
OTU27	302	4.82	1.54E-15	1.20E-13	Firmicutes	Lactobacillaceae	Pediococcus spp.
OTU20	16	3.20	3.22E-05	0.001	Firmicutes	Planococcaceae	Lysinibacillus boronitolerans
OTU50	83	1.89	0.002	0.060	Firmicutes	Ruminococcaceae	Ruminococcus spp.
OTU10	6	-1.71	0.004	0.079	Bacteroidetes	Porphyromonadaceae	
OTU61	3	-1.73	0.008	0.123	Firmicutes	Erysipelotrichaceae	Catenibacterium spp.
OTU62	17	1.96	0.010	0.125	Firmicutes	Erysipelotrichaceae	RFN20 spp.
OTU40	5215	-0.81	0.031	0.347	Firmicutes	Lachnospiraceae	
OTU32	4	-1.02	0.043	0.417	Firmicutes	UC Clostridiales	
OTU1	7	1.13	0.061	0.525	Actinobacteria	Actinomycetaceae	
OTU2	6	0.76	0.113	0.597	Actinobacteria	Actinomycetaceae	
OTU11	95	0.77	0.117	0.597	Bacteroidetes	Porphyromonadaceae	Parabacteroides spp.
OTU22	66	1.20	0.125	0.597	Firmicutes	Enterococcaceae	Vagococcus spp.
OTU24	1386	1.21	0.099	0.597	Firmicutes	Lactobacillaceae	Lactobacillus brevis
OTU34	51	1.46	0.077	0.597	Firmicutes	Clostridiaceae	Clostridium
OTU41	616	-0.63	0.118	0.597	Firmicutes	Lachnospiraceae	Blautia producta
OTU42	72	0.67	0.138	0.597	Firmicutes	Lachnospiraceae	Coprococcus spp.
OTU48	280	0.69	0.132	0.597	Firmicutes	Ruminococcaceae	Anaerofilum spp.
OTU72	156	-0.93	0.105	0.597	Proteobacteria	Enterobacteriaceae	

C

Fermentation 2- inulin high

OTU	baseMean	log2FoldChange	pvalue	padj	Phylum	Family	Genus/Species
OTU27	1291	4.07	3.06E-08	1.68E-06	Firmicutes	Lactobacillaceae	Pediococcus spp.
OTU2	28	3.50	4.10E-07	1.13E-05	Actinobacteria	Actinomycetaceae	Actinomyces spp.
OTU62	28	4.32	9.04E-07	1.66E-05	Firmicutes	Erysipelotrichaceae	RFN20 spp.
OTU60	41	3.84	2.41E-06	3.32E-05	Firmicutes	Erysipelotrichaceae	Allobaculum spp.
OTU29	279	2.09	3.67E-05	0.0004	Firmicutes	Streptococcaceae	Lactococcus spp.
OTU1	22	2.79	5.25E-05	0.0005	Actinobacteria	Actinomycetaceae	
OTU5	53	2.54	0.0003	0.0020	Actinobacteria	Coriobacteriaceae	
OTU26	8	2.17	0.0004	0.0026	Firmicutes	Lactobacillaceae	Lactobacillus zeae
OTU24	4701	1.78	0.0023	0.0139	Firmicutes	Lactobacillaceae	Lactobacillus brevis
OTU48	552	1.46	0.0058	0.0301	Firmicutes	Ruminococcaceae	Anaerofilum spp.
OTU63	425	1.73	0.0060	0.0301	Firmicutes	Erysipelotrichaceae	[Eubacterium] dolichum
OTU32	5	-1.43	0.0123	0.0564	Firmicutes	UC Clostridiales	
OTU28	267	-1.54	0.0177	0.0750	Firmicutes	Leuconostocaceae	Weissella paramesenteroides
OTU9	127	-1.93	0.0195	0.0766	Bacteroidetes	Bacteroidaceae	Bacteroides ovatus
OTU3	4	1.41	0.0265	0.0874	Actinobacteria	Microbacteriaceae	Other
OTU42	275	1.08	0.0270	0.0874	Firmicutes	Lachnospiraceae	Coprococcus spp.
OTU61	4	-1.40	0.0270	0.0874	Firmicutes	Erysipelotrichaceae	Catenibacterium spp.
OTU72	139	-1.10	0.0555	0.1697	Proteobacteria	Enterobacteriaceae	
OTU4	64	1.48	0.0781	0.2209	Actinobacteria	Bifidobacteriaceae	Bifidobacterium thermacidophilum
OTU54	151	-0.72	0.0842	0.2209	Firmicutes	[Tissierellaceae]	Anaerococcus spp.
OTU79	8	-1.11	0.0843	0.2209	Verrucomicrobia	Verrucomicrobiaceae	Akkermansia muciniphila
OTU74	2	-1.01	0.1381	0.3454	Proteobacteria	Moraxellaceae	Acinetobacter spp.
OTU11	121	0.91	0.1858	0.4220	Bacteroidetes	Porphyromonadaceae	Parabacteroides spp.
OTU22	15	-0.95	0.1836	0.4220	Firmicutes	Enterococcaceae	Vagococcus spp.
OTU25	3	0.79	0.1938	0.4220	Firmicutes	Lactobacillaceae	Lactobacillus reuteri
OTU30	48	0.78	0.1995	0.4220	Firmicutes	Streptococcaceae	Lactococcus garvieae
OTU64	1053	-0.74	0.2429	0.4947	Proteobacteria	Alcaligenaceae	Sutterella spp.
OTU15	2	-0.71	0.2817	0.5342	Bacteroidetes	S24-7	
OTU34	197	1.17	0.2720	0.5342	Firmicutes	Clostridiaceae	Clostridium spp.
OTU8	34107	-0.52	0.3219	0.5901	Bacteroidetes	Bacteroidaceae	Bacteroides spp.
OTU52	1	-0.67	0.3374	0.5985	Firmicutes	Veillonellaceae	Veillonella dispar
OTU33	226	-0.44	0.4249	0.7187	Firmicutes	Clostridiaceae	
OTU75	46	0.56	0.4312	0.7187	Proteobacteria	Moraxellaceae	Acinetobacter rhizosphaerae

Supplementary Table S4.8: Summary microbial phyla and most abundant (> 1 %) bacterial families obtained by V4 region 16S amplicon sequencing within PolyFermS reactors before (stabilization) and during XOS treatment in fermentation 2. Values < 1 % are summarized in the group «Others». Data are means ± SD of three consecutive days. (B) Table displaying OTUs that significantly enriched (positive Log2 fold change) or reduced (negative Log2 fold change) in reactor effluent during inulin supplementation.

A

Taxon	Stabilization 1			Treatment 1			Stabilization 2			Treatment 2		
Actinobacteria	0.1%	±	0.1%	0.1%	±	0.1%	0.0%	±	0.0%	0.0%	±	0.0%
Bacteroidetes	41.6%	±	7.4%	27.3%	±	6.3%	1.2%	±	0.1%	1.2%	±	0.8%
Bacteroidaceae	33.6%	±	7.3%	15.6%	±	5.8%	0.1%	±	0.0%	0.1%	±	0.1%
Porphyromonadaceae	8.0%	±	0.7%	11.7%	±	0.9%	1.1%	±	0.1%	1.0%	±	0.1%
Firmicutes	33.4%	±	6.3%	54.1%	±	18.3%	66.9%	±	23.4%	51.4%	±	12.4%
Planococcaceae	0.0%	±	0.0%	0.0%	±	0.0%	4.3%	±	4.3%	2.6%	±	2.6%
Lactobacillaceae	0.3%	±	0.3%	0.1%	±	0.0%	0.8%	±	0.1%	2.2%	±	1.1%
UC Clostridiales	1.1%	±	0.1%	1.7%	±	0.8%	0.1%	±	0.0%	0.1%	±	0.0%
Clostridiaceae	26.6%	±	7.2%	37.8%	±	14.3%	42.1%	±	20.9%	10.4%	±	4.7%
Lachnospiraceae	2.4%	±	1.1%	6.1%	±	2.2%	0.1%	±	0.0%	9.9%	±	3.5%
Eubacteriaceae	0.0%	±	0.0%	0.0%	±	0.0%	2.6%	±	0.8%	9.9%	±	3.5%
Peptostreptococcaceae	1.2%	±	0.3%	0.8%	±	0.1%	2.1%	±	0.6%	3.1%	±	0.3%
Erysipelotrichaceae	0.6%	±	0.2%	5.1%	±	1.2%	0.3%	±	0.1%	0.1%	±	0.0%
Ruminococcaceae	0.3%	±	0.1%	1.1%	±	0.5%	0.5%	±	0.2%	1.3%	±	0.3%
[Tissierellaceae]	1.2%	±	0.2%	1.2%	±	0.1%	15.5%	±	2.0%	24.2%	±	2.3%
Proteobacteria	6.7%	±	0.9%	8.7%	±	5.7%	30.6%	±	22.6%	40.6%	±	17.2%
Alcaligenaceae	0.7%	±	0.1%	0.0%	±	0.0%	0.4%	±	0.4%	2.1%	±	0.8%
Enterobacteriaceae	6.0%	±	1.0%	8.6%	±	5.6%	30.2%	±	23.0%	38.5%	±	18.0%
Verrucomicrobia	18.2%	±	3.2%	9.8%	±	8.2%	1.4%	±	0.6%	6.8%	±	4.0%
Others	3.8%	±	1.0%	1.3%	±	0.6%	3.2%	±	0.8%	1.6%	±	0.6%

B

OTU	baseMean	log2FoldChange	pvalue	padj	taxonomy		
					Phylum	**Family**	**Genus/Species**
OTU52	71.91666667	-1.303689805	0.010700854	0.763587963	Firmicutes	*Veillonellaceae*	*Veillonella dispar*
OTU53	3.083333333	1.019233611	0.028997011	0.763587963	Firmicutes	*[Mogibacteriaceae]*	
OTU60	423.4166667	1.156217329	0.025127082	0.763587963	Firmicutes	*Erysipelotrichaceae*	*Allobaculum* spp.
OTU50	244.3333333	0.855390024	0.041908774	0.827698283	Firmicutes	*Ruminococcaceae*	*Ruminococcus* spp.

Fermentation 1 - XOS

Supplementary Table 9: Metabolite concentrations (mM) and ratios measured by HPLC in fermentation effluents of stabilized fermentation periods and treatment periods (control, oxidative stress and inulin) in fermentation 1. Mean metabolite concentrations with standard error of effluent samples in reactor effluents.

| | Concentrations (mM) | | | | | | | | | Days |
	Acetate	Butyrate	Propionate	Lactate	Formate	Succinate	BCFAs	Total metabolites		
Control										
Stabilization 1	73.9 ± 3.7	21.7 ± 0.4	13.7 ± 0.5	ND	1.8 ± 0.1	28.3 ± 0.6	0.7 ± 0.6	140.1 ± 3.3		10-12
Treatment 1	71.4 ± 3.4	21.5 ± 1.4	17.2 ± 2.1	ND	4.9 ± 2.5	27.4 ± 1.2	3.5 ± 1.2	145.8 ± 7.4		14-16
P value	0.409	0.838	0.049		0.095	0.400	0.026	0.286		
Stabilization 2	67.9 ± 2.5	28.1 ± 1.1	14.3 ± 0.8	ND	2.5 ± 0.1	27.3 ± 0.2	2.1 ± 0.4	142.2 ± 2.8		21-23
Treatment 2	69.6 ± 1.5	25.1 ± 0.1	15.1 ± 0.2	ND	2.0 ± 0.1	27.0 ± 0.2	2.8 ± 0.6	141.6 ± 1.2		29-31
P value	0.386	0.009	0.188		0.008	0.400	0.168	0.759		
Stabilization 3	71.9 ± 2.8	24.5 ± 0.4	14.0 ± 1.4	ND	4.5 ± 1.2	26.1 ± 0.7	3.8 ± 2.3	144.8 ± 6.1		39-41
Treatment 3	73.9 ± 3.4	24.7 ± 0.6	18.2 ± 1.2	ND	7.9 ± 0.3	26.5 ± 0.3	11.0 ± 1.6	162.2 ± 4.6		46-48
P value	0.469	0.518	0.017		0.011	0.611	0.012	0.017		
0.5 M H2O2										
Stabilization 1	59.0 ± 0.9	23.6 ± 1.2	13.8 ± 1.3	1.6 ± 0.1	5.8 ± 0.1	2.5 ± 0.4	2.8 ± 0.2	109.0 ± 2.7		8-10
Treatment 1	48.8 ± 3.2	7.6 ± 1.4	13.0 ± 2.4	5.9 ± 3.0	7.7 ± 4.5	4.4 ± 2.0	0.1 ± 0.1	87.7 ± 6.2		14-16
P value	0.006	< 0.001	0.652	0.100	0.498	0.179	< 0.001	0.005		
Stabilization 2	72.3 ± 0.5	23.1 ± 0.2	17.9 ± 0.8	ND	1.8 ± 0.1	28.6 ± 0.1	7.1 ± 0.8	150.7 ± 2.2		22-24
Treatment 2	57.4 ± 3.5	9.0 ± 0.8	11.4 ± 0.6	2.5 ± 0.2	8.8 ± 1.8	9.4 ± 0.2	2.2 ± 0.1	100.5 ± 3.4		29-31
P value	0.002	< 0.001	< 0.001	< 0.001	0.100	< 0.001	< 0.001	< 0.001		
Stabilization 3	71.7 ± 2.4	24.6 ± 0.8	15.0 ± 0.9	ND	2.0 ± 0.2	28.6 ± 0.7	2.4 ± 0.4	144.2 ± 4.2		22-24
Treatment 3	55.2 ± 8.7	9.5 ± 2.9	15.8 ± 4.6	6.3 ± 5.8	3.3 ± 1.0	9.9 ± 1.3	3.5 ± 2.3	103.5 ± 12.8		28-30
P value	0.033	< 0.001	0.777	0.134	0.081	< 0.001	0.490	0.006		
Stabilization 4	67.6 ± 0.6	19.7 ± 0.3	16.5 ± 0.5	ND	1.9 ± 0.2	27.3 ± 0.3	6.6 ± 0.4	139.7 ± 1.9		40-42
Treatment 4	51.5 ± 4.0	6.5 ± 0.6	6.4 ± 1.1	4.9 ± 2.8	15.8 ± 2.5	10.8 ± 1.0	1.3 ± 1.0	97.2 ± 5.3		47-49
P value	0.002	< 0.001	< 0.001	0.039	< 0.001	< 0.001	0.001	< 0.001		
3.4 g/L inulin										
Stabilization 1	75.6 ± 0.7	19.7 ± 0.1	17.0 ± 0.3	ND	ND	27.5 ± 0.2	3.3 ± 0.4	143.2 ± 0.8		8-10
Treatment 1	65.9 ± 4.0	24.0 ± 0.5	17.8 ± 0.8	ND	8.8 ± 5.2	41.1 ± 2.7	4.3 ± 1.6	161.8 ± 4.0		14-16
P value	0.014	< 0.001	0.190		0.042	< 0.001	0.364	0.001		
Stabilization 2	69.4 ± 2.2	25.0 ± 1.1	16.9 ± 1.3	ND	2.0 ± 0.1	27.9 ± 0.6	6.3 ± 0.1	147.4 ± 5.0		22-24
Treatment 2	76.6 ± 0.6	30.3 ± 0.6	15.8 ± 0.7	ND	3.2 ± 0.5	29.3 ± 5.3	7.5 ± 1.6	164.0 ± 1.5		28-30
P value	0.006	0.002	0.256		0.011	0.66	0.269	0.005		
Stabilization 3	67.2 ± 0.5	20.3 ± 0.3	16.9 ± 0.2	ND	1.7 ± 0.2	28.4 ± 0.4	6.2 ± 0.5	141.0 ± 0.8		40-42
Treatment 3	82.3 ± 2.4	27.1 ± 1.0	17.2 ± 0.4	ND	4.4 ± 0.1	36.9 ± 1.2	7.6 ± 1.0	175.6 ± 2.6		47-49
P value	< 0.001	< 0.001	0.242		< 0.001	< 0.001	0.095	< 0.001		

Supplementary Table S4.10: Metabolite concentrations (mM) and ratios measured by HPLC in fermentation effluents of stabilized fermentation periods and treatment periods (Control, XOS and Metronidazole) in fermentation 1. Mean metabolite concentrations with standard error of effluent samples in reactor effluents.

	Concentrations (mM)								Days
	Acetate	Butyrate	Propionate	Lactate	Formate	Succinate	BCFAs	Total metabolites	
Control									
Stabilization 1	73.9 ± 3.7	21.7 ± 0.4	13.7 ± 0.5	ND	1.8 ± 0.1	28.3 ± 0.6	0.7 ± 0.6	140.1 ± 3.3	10-12
Treatment 1	71.4 ± 3.4	21.5 ± 1.4	17.2 ± 2.1	ND	4.9 ± 2.5	27.4 ± 1.2	3.5 ± 1.2	145.8 ± 7.4	14-16
P value	0.409	0.838	0.049		0.095	0.400	0.026	0.286	
Stabilization 2	67.9 ± 2.5	28.1 ± 1.1	14.3 ± 0.8	ND	2.5 ± 0.1	27.3 ± 0.2	2.1 ± 0.4	142.2 ± 2.8	21-23
Treatment 2	69.6 ± 1.5	25.1 ± 0.1	15.1 ± 0.2	ND	2.0 ± 0.1	27.0 ± 0.2	2.8 ± 0.6	141.6 ± 1.2	29-31
P value	0.386	0.009	0.188		0.008	0.400	0.168	0.759	
Stabilization 3	71.9 ± 2.8	24.5 ± 0.4	14.0 ± 1.4	ND	4.5 ± 1.2	26.1 ± 0.7	3.8 ± 2.3	144.8 ± 6.1	39-41
Treatment 3	73.9 ± 3.4	24.7 ± 0.6	18.2 ± 1.2	ND	7.9 ± 0.3	26.5 ± 0.3	11.0 ± 1.6	162.2 ± 4.6	46-48
P value	0.469	0.518	0.017		0.011	0.611	0.012	0.017	
3.4 g/L XOS									
Stabilization 1	69.0 ± 0.9	22.0 ± 2.2	16.9 ± 0.6	ND	2.0 ± 0.5	27.9 ± 2.7	0.5 ± 0.4	138.3 ± 1.9	8-10
Treatment 1	81.7 ± 1.8	23.6 ± 0.9	19.4 ± 1.7	ND	2.0 ± 0.4	36.2 ± 2.2	3.9 ± 1.7	166.8 ± 3.8	13-15
P value	< 0.001	0.299	0.077		0.457	0.015	0.100	< 0.001	
Stabilization 2	56.3 ± 2.4	29.7 ± 1.2	13.9 ± 1.0	1.5 ± 0.1	8.3 ± 1.6	2.1 ± 0.7	5.0 ± 1.4	116.8 ± 3.9	22-24
Treatment 2	62.2 ± 2.7	35.3 ± 0.6	17.9 ± 0.9	2.0 ± 1.3	8.9 ± 0.8	1.5 ± 0.2	6.0 ± 1.8	133.7 ± 5.1	29-31
P value	0.048	0.008	0.002	0.486	0.608	0.204	0.344	0.010	
112.8 mg/L MTZ									
Stabilization 1	71.3 ± 0.7	19.6 ± 1.6	19.4 ± 1.0	ND	1.8 ± 0.1	26.4 ± 1.0	7.2 ± 1.5	145.7 ± 3.5	8-10
Treatment 1	60.5 ± 6.8	20.7 ± 3.0	15.8 ± 0.8	ND	15.3 ± 2.2	23.5 ± 3.1	1.1 ± 0.6	137.0 ± 12.1	12-14
P value	0.055	0.637	0.007		0.100	0.200	0.003	0.297	
Stabilization 2	54.3 ± 1.5	17.3 ± 0.2	12.8 ± 0.4	ND	1.5 ± 0.2	23.7 ± 0.7	4.6 ± 0.7	114.1 ± 2.1	40-42
Treatment 2	77.5 ± 2.6	22.1 ± 2.4	17.1 ± 1.5	ND	3.3 ± 0.1	29.8 ± 1.2	6.1 ± 1.9	155.9 ± 4.2	47-49
P value	0.045	0.525	0.928		< 0.001	0.096	0.979	0.237	

Supplementary Table S4.11: Metabolite concentrations (mM) and ratios measured by HPLC in fermentation effluents of stabilized fermentation periods and treatment periods (Control, oxidative stress and low/high dose of inulin) in fermentation 2. Mean metabolite concentrations with standard error of effluent samples in reactor effluents.

	Concentrations (mM)								Days
	Acetate	Butyrate	Propionate	Lactate	Formate	Succinate	BCFAs	Total metabolites	
Control									
Stabilization 1	101.4 ± 3.3	22.1 ± 0.3	12.4 ± 0.3	ND	7.6 ± 2.2	10.6 ± 0.9	4.8 ± 0.8	168.3 ± 2.3	6-8
Treatment 1	97.5 ± 4.4	19.6 ± 0.2	11.9 ± 0.9	ND	1.9 ± 0.3	8.3 ± 2.9	6.0 ± 0.2	154.1 ± 6.7	11-13
P value	0.281	< 0.001	0.431		0.011	0.276	0.085	0.025	
Stabilization 2	97.3 ± 5.3	19.3 ± 1.8	8.0 ± 0.6	ND	ND	13.8 ± 1.9	14.2 ± 0.5	152.9 ± 5.6	8-10
Treatment 2	107.5 ± 7.5	15.9 ± 1.9	9.0 ± 0.9	ND	ND	12.2 ± 1.9	15.0 ± 0.9	159.7 ± 6.1	14-16
P value	0.127	0.066	0.186			0.375	0.288	0.227	
0.5 M H2O2									
Stabilization 1	98.7 ± 4.8	23.2 ± 0.8	10.1 ± 0.9	ND	6.7 ± 3.0	9.8 ± 1.7	6.0 ± 1.1	162.5 ± 2.5	5-7
Treatment 1	85.1 ± 3.9	9.2 ± 4.2	5.3 ± 1.0	9.2 ± 5.8	5.4 ± 5.4	5.6 ± 1.5	4.7 ± 0.9	118.0 ± 8.7	11-13
P value	0.018	0.005	0.003	0.052	0.74	0.033	0.100	0.001	
Stabilization 2	111.5 ± 1.9	16.0 ± 0.3	8.4 ± 0.7	ND	ND	15.2 ± 0.6	4.9 ± 0.1	165.3 ± 2.0	21-23
Treatment 2	71.4 ± 12.4	5.8 ± 3.0	3.7 ± 1.6	8.4 ± 5.0	2.9 ± 2.5	5.6 ± 1.5	2.0 ± 1.7	101.9 ± 16.0	29-31
P value	0.100	0.004	0.009	0.044	0.119	< 0.001	0.046	0.100	
3.4 g/L inulin									
Stabilization 1	104.2 ± 4.8	21.3 ± 0.4	9.9 ± 1.0	ND	5.3 ± 2.5	10.4 ± 3.0	14.1 ± 1.1	165.2 ± 8.0	5-7
Treatment 1	99.5 ± 4.1	23.4 ± 1.2	23.1 ± 1.1	2.6 ± 0.4	2.0 ± 0.2	0.6 ± 0.4	15.0 ± 0.4	164.3 ± 6.1	11-13
P value	0.267	0.100	< 0.001	< 0.001	0.090	0.005	0.231	0.884	
Stabilization 2	100.5 ± 5.7	24.8 ± 1.8	5.9 ± 0.5	2.1 ± 0.2	3.9 ± 0.1	3.7 ± 0.2	14.1 ± 0.1	152.9 ± 4.4	16-18
Treatment 2	104.8 ± 1.5	18.9 ± 0.4	18.1 ± 1.1	2.5 ± 0.6	ND	0.4 ± 0.1	14.6 ± 0.3	159.7 ± 2.3	27-29
P value	0.277	0.006	< 0.001		< 0.001	0.100	0.051	0.038	
5 g/L inulin									
Stabilization 1	96.9 ± 4.8	22.2 ± 1.3	10.1 ± 0.4	ND	5.8 ± 2.6	9.8 ± 1.3	13.3 ± 0.6	158.1 ± 0.5	5-7
Treatment 1	102.3 ± 0.8	34.3 ± 3.3	11.3 ± 0.5	ND	1.9 ± 0.1	13.0 ± 0.7	13.6 ± 0.6	176.5 ± 5.5	11-13
P value	0.131	0.004	0.027		0.062	0.023	0.400	0.100	
Stabilization 2	100.1 ± 1.1	19.3 ± 2.2	8.4 ± 1.1	ND	ND	14.3 ± 0.7	9.1 ± 0.3	156.0 ± 3.0	20-22
Treatment 2	115.5 ± 2.8	27.2 ± 4.1	10.2 ± 1.2	ND	ND	18.1 ± 0.7	9.8 ± 0.3	185.8 ± 3.6	24-26
P value	0.002	0.041	0.200			0.003	0.06	< 0.001	

Supplementary Table S4.12*: Mean Shannon diversity index and observed species HPLC in fermentation effluents of stabilized fermentation periods and treatment periods (metronidazole, oxidative stress and low/high dose of inulin) in fermentation 1 and 2.. Data shown are means (n=3) ± SD obtained of 11 measurments.

Fermentation 1	Shannon index	Observed species	Fermentation 2	Shannon index	Observed species
Inulin 3.4 g/L			**Inulin 3.4 g/L**		
Stabilization 1	2.6 ± 0.2	28.1 ± 8.4	Stabilization 1	1.6 ± 0.2	28.9 ± 9.2
Treatment 1	2.9 ± 0.3	33.9 ± 10.4	Treatment 1	1.7 ± 0.3	35.6 ± 11.5
Stabilization 2	2.7 ± 0.2	31.7 ± 9.3	Stabilization 2	2.5 ± 0.3	37.6 ± 11.3
Treatment 2	3.3 ± 0.4	36.8 ± 10.5	Treatment 2	3.1 ± 0.4	39.8 ± 11.4
Stabilization 3	2.5 ± 0.2	34.4 ± 10.2			
Treatment 3	3.2 ± 0.4	38.4 ± 11.4			
XOS 3.4 g/L			**Inulin 5 g/L**		
Stabilization 1	2.6 ± 0.2	31.9 ± 9.8	Stabilization 1	1.7 ± 0.4	30.7 ± 9.8
Treatment 1	2.5 ± 0.2	32.9 ± 10.0	Treatment 1	2.2 ± 0.3	33.7 ± 10.6
Stabilization 2	2.0 ± 0.2	23.4 ± 7.3	Stabilization 2	2.8 ± 0.3	37.5 ± 11.0
Treatment 2	2.3 ± 0.3	21.1 ± 6.1	Treatment 2	3.7 ± 0.5	39.4 ± 11.0
MTZ 112.8 mg/L			**0.5 M H_2O_2**		
Stabilization 1	2.7 ± 0.2	31.3 ± 10.2	Stabilization 1	1.5 ± 0.2	31.1 ± 9.8
Treatment 1	2.6 ± 0.3	25.0 ± 9.0	Treatment 1	2.5 ± 0.4	35.0 ± 10.7
Stabilization 2	2.8 ± 0.3	34.8 ± 10.4	Stabilization 2	2.7 ± 0.3	36.9 ± 10.8
Treatment 2	3.0 ± 0.3	35.2 ± 0.3	Treatment 2	2.4 ± 0.2	33.8 ± 10.3
0.5 M H_2O_2					
Stabilization 1	2.5 ± 0.4	1.5 ± 0.5			
Treatment 1	21.4 ± 6.3	16.7 ± 5.1			
Stabilization 2	2.6 ± 0.2	32.1 ± 9.7			
Treatment 2	2.5 ± 0.3	21.8 ± 6.7			
Stabilization 3	2.6 ± 0.2	1.9 ± 0.3			
Treatment 3	31.1 ± 9.3	18.4 ± 6.6			
Stabilization 4	2.5 ± 0.3	33.9 ± 10.1			
Treatment 4	1.9 ± 0.4	21.7 ± 7.2			

Chapter 5

General conclusion and perspectives

5.1. Conclusions

Gut microbiota research is a rapidly progressing field and it became evident that the intestinal bacterial community plays an important role in host health. Since the gut microbiota is involved in metabolism, immune function and digestion, an alternation of the microbial composition and functionality will affect host health and eventually lead to disease development. In order to promote health and to prevent or manage disease, it is therefore important to increase our understanding how gut microbiota is modulated by diet, medication or environmental factors, and further to understand their role in various diseases. Until today, it is less known about the metabolic adaptability of the gut microbiota in response to different modulations, such as dietary fibers, oxidative stress and antibiotics. In this context, the overall aim of this thesis was to gain new mechanistic insights into the dynamic response of the gut microbiota to potential modulating effects of dietary fiber supplementation and to adverse effects of oxidative stress and antibiotics using *in vitro* continuous fermentation models.

In vitro **characterization of the modulatory impact of dietary fibers on bacterial functionality and composition of gut microbiota from healthy adult gut microbiota**

During the last decades, modulation of the gut microbiome has arisen great interest to confer health benefits to the host. In human studies, the prebiotic effect of a dietary fiber relies mainly on the quantification of faecal microbial populations (Carlson et al., 2017), thus not allowing to study the immediate responsiveness of the bacterial populations and the production of beneficial SCFA upon supplementation. However, characterizing the modulations of the gut microbiota and functionality to specific prebiotic interventions is mandatory for determining the association between key bacterial species, bacterial interactions and activity in relation and a given dietary fiber. Continuous *in vitro* fermentation models using immobilized faecal microbiota are powerful tools for measuring metabolite production upon a perturbation, and therefore understanding the kinetics of intestinal fermentation (Cinquin et al., 2004, 2006; Dostal et al., 2013; Fehlbaum et al., 2016). Consequently, in order to gain more insights into the complex bacterial interactions during carbohydrate metabolism, we aimed to investigate the modulation potential of the dietary fibers inulin, β-glucan, α-GOS and XOS on two divergent human proximal colon microbiota using three continuous *in vitro* fermentation systems (PolyFermS) (**Chapter 2;** (Poeker et al., 2018)). Our data demonstrated that we were able to maintain *in vitro* the specific metabolic and phylogenetic characteristics of both distinct colon microbiota, and therefore proving the robustness of the PolyFermS model for gut microbiota research. The most important finding was that fermentation of each dietary fiber is correlated with increased SCFA productions, while the physicochemical properties of the fiber and the baseline intestinal bacterial composition dictate the metabolic response offering specific health benefits. Irrespective to the dietary fiber, the two *in vitro* microbiota responded in a specific and consistent manner: the *Bacteroidaceae-Ruminococcaceae* dominated microbiota

produced more butyrate, while the *Prevotellaceae-Ruminococcaceae* dominated microbiota produced more propionate. Also, the different carbohydrate structures, presenting unique carbon sources for selective stimulation of divergent bacterial populations, had a large impact on specific SCFA production (Carlson et al., 2017). For example, addition of the short-chain carbohydrates XOS and α-GOS resulted in an accumulation of acetate, likely due to their nature of being readily accessible and fermentable by various commensal bacteria. Moreover, we were able to observe cross-feeding interactions between acetate-or lactate- and butyrate producers in both *in vitro* microbiota, especially upon inulin supplementation. Interestingly, the impact of dietary fiber supplementation was only observed at metabolic but not at phylogenetic level, suggesting that the metabolic adaptability of gut microbiota to dietary fiber supplementation is rapid, due to an induction of the polysaccharide-degrading enzymes of microbes possessing this specific enzymatic activity (McIntosh et al., 2012). Furthermore, the repeatability of the effects of the different dietary fibers on *in vitro* microbiota activity manifests the sensitivity of the PolyFermS model as a potential tool for investigating the modulating effects of novel prebiotic compounds on gut microbiota.

To conclude, our data raised awareness that there are significant differences in the fermentation capability of dietary fibers depending on the phylogenetic characteristics of the gut microbiota. The rate and extent of beneficial metabolite production clearly depend on the structure of the complex dietary fibers and the inter-individual differences of the intestinal microbial composition.

Development of an *in vitro* continuous fermentation model to culture the murine caecal gut microbiota

Murine models are the model of choice for studying functionality and role of the gut microbiota in human health and disease. However, mouse models do not allow studying the complex bacterial interactions occurring within the gut microbiome upon, for instance, supplementation of prebiotics or antibiotics. *In vitro* fermentation models are a powerful alternative to study the complex microbe-microbe interactions without host factors. Further, most of the murine *in vitro* fermentation models are simple batch or continuous flow cultures, which present important limitations, such as no pH control, loss of less competitive bacteria by rapid washout or limitation of experimental duration. Therefore, we aimed to develop a novel *in vitro* continuous fermentation model based on the PolyFermS platform for the murine caecal microbiota (**Chapter 3**). For the development of the novel murine *in vitro* model, we quantified fermentation metabolites and defined the facultative anaerobic *Enterobacteriaceae* and the highly oxygen-sensitive butyrate-producers as marker bacterial populations for detecting eventual suboptimal conditions in each model. We showed that rational and stepwise adjustments of cecum sampling, fermentation starting mode, pH, retention time and growth medium composition allowed to maintain *in vitro* metabolite profiles and marker bacterial populations similar to the murine caecal inoculum. For instance, control of *in vitro* levels of fastidious *Enterobacteriaceae,* which

generally bloom in *in vitro* fermentation models, was achieved by reducing pH and simple carbohydrate concentrations in the nutritive medium as well as increasing of retention time in our final models. In our final model, the adjusted nutritive medium (low starch concentration) and operation parameters (pH 6.5, 37°C and retention time 12 h) allowed cultivating a simplified *in vitro* murine bacterial community, but yet maintaining the relevant metabolic interactions and key bacterial populations for the *in vivo* murine caecum.

In conclusion, we showed that it is feasible to maintain a stable and simplified, but yet representative *in vitro* murine microbiota. Nevertheless, our novel *in vitro* model has the potential to be expanded, allowing us to conduct modulation experiments towards different profiles (eubiotic or dysbiotic) using stimuli known to alter the structure of gut communities *in vitro* and *in vivo*. The model will allow elucidating microbe-microbe interactions upon modulations independent of the host.

In vitro modulation of the murine gut microbiota by dietary fibers, oxidative stress and antibiotics

Murine models have been widely used to study the effects of modified diets and prebiotic dietary fiber supplementation or deprivation on gut microbiota and host's health. Further, microbial dysbiosis has been associated to chronic intestinal inflammatory diseases, such as IBD, but whether an altered microbiome is a cause or a consequence in the development of IBD, remains ambiguous (Nguyen et al., 2015). Oxidative stress and antibiotics, have been suggested to be highly involved in the development and propagation of the inflammatory state in IBD. Our novel *in vitro* model mimicking murine caecal intestinal microbiota has the potential to modulate gut microbiota towards different states and to elucidate, whether specific perturbations lead to compositional and metabolic alternations comparable to those seen in the host. Therefore, we explored the modulating effects of oxidative stress and metronidazole towards dysbiosis and dietary fibers towards eubiosis on two divergent murine caecal microbiota using *in vitro* fermentation model for mice (**Chapter 4**).

Firstly, we aimed to study whether oxidative stress and antibiotics affect the murine caecal microbiota composition and functionality using two *in vitro* fermentation models. The impact of oxidative stress (H_2O_2) was investigated on two murine caecal microbiota and metabolites. We demonstrated that the disruption of strict anaerobiosis had an immediate and detrimental effect on strict anaerobic butyrate producers (i.e. *Coprococcus*), and at the same time enhanced the growth of facultative anaerobes (i.e. *Enterobacteriaceae*, *Lactobacillaceae* and *Enterococcaceae*). Furthermore, oxidative stress highly impaired bacterial metabolism in the intestinal ecosystem, as a subsequent result of microbial dysbiosis and of the decrease in functional genes involved in carbohydrate fermentation. Commensal bacteria and their produced metabolites, particularly butyrate, are highly involved in reinforcing intestinal permeability and immune homeostasis (Chang et al., 2014; Yarandi et al., 2016; Zihni et al., 2016). We assume that the detrimental impact of oxidative stress on intestinal bacterial community and metabolism might play an important role in inflammatory diseases of the

gut. The repeatability of our results showed that the continuous *in vitro* fermentation model is a robust approach for studying the direct response of the gut microbiota upon hydrogen peroxide. Then, we investigated the adverse impact of metronidazole, an antibiotic administered to treat anaerobic infections, on the murine gut microbiota composition and metabolism. Our data showed that the intestinal microbiota shows a pronounced resilience upon antibiotic treatment, inducing a short-term impairment of bacterial functionality and persistent shifts in the bacterial community.

In conclusion, this study provided first insights on the direct adverse effects of oxidative stress on intestinal microbiota composition and functionality by highly impairing microbial fermentation and composition. Metronidazole induced temporal, but strong decreases in metabolite production and alternations of the bacterial composition.

Similar to our previous human *in vitro* study, inulin supplementations led to a microbiota-dependent response, with promoted acetate and butyrate productions in fermentation 1 and propionate production in fermentation 2. Nevertheless, the most important finding in this study is the effect of dosage upon microbiota modulation. As with the human *in vitro* microbiota, inulin supplementation at a low dosage led to immediate modulations of the microbial activity, while no alternations at phylogenetic level were observed. However, increasing the dosage of inulin from 3.4 g /L to 5 g/L. stimulated main SCFA productions and increased relative abundances in butyrate-producers (*Erysipelotrichaceae* and Clostridiales), which might explain the promoted butyrate formation. Supplementation of the prebiotic XOS (low dosage) resulted in increased acetate formation, thus confirming a positive association between XOS and the SCFA acetate. XOS supplementation further led to decreased levels of *Veillonella dispar* and increases of bacterial taxa belonging to Firmicutes, which may explain the high accumulation of acetate in the ecosystem.

In summary of these investigations, our data suggest that prebiotic dietary fibers at lower dosages are efficient of stimulating the functionality of the intestinal bacterial community, while higher amounts of prebiotics are necessary to activate growth of specific bacterial populations. Further, the results of this study confirmed the fast metabolic modulation of the gut microbiota upon prebiotic supplementation, while the metabolic response correlates with the physicochemical properties of the fiber as well as the phylogenetic characteristic of the gut microbiota.

5.2. Perspectives

The research in chapter 2 highlighted the strong association of the baseline intestinal microbial composition and the metabolic response upon dietary supplementation. Inter-individual differences have to be considered for a full assessment of the impact of certain dietary compounds on the microbiota. Further studies are required to elucidate the influence of the baseline intestinal microbiota on metabolic response upon prebiotic dietary fiber supplementation and to identify the causal relationship between key bacterial species and metabolic responses *in vivo*. Therefore, in future, *in vivo* human studies are necessary to explore the beneficial

health effects of the potential novel prebiotics and to investigate the modulation potential at phylogenetic level. It is evident that the proper characterization of a gut microbiota is elementary to set-up personalized dietary therapies for improving human health and interventions in clinical settings for treating disorders by targeting the intestinal bacterial community.

In chapter 3, we demonstrated that with stepwise and rational adjustment of fermentation conditions, we were able to establish a novel murine *in vitro* continuous fermentation model inoculated with caecal gut microbiota. Our model is a preliminary step in the development of an appropriate and final mouse model. In order to increase preservation of bacterial diversity in our murine *in vitro* model, it is necessary to isolate more murine intestinal bacterial species and to assess their specific growth requirements for the subsequent fine-tuning of the operational conditions. In future, it is possible to expand our model with second-stage reactors, mounted in parallel and continuously inoculated with IR effluent.

For this reason, we applied this expanded fermentation set-up to study the modulation effect of biotic and abiotic factors linked with health or disease on gut microbial functionality and structure. In chapter 4, we were able to modulate the gut microbiota in a highly controlled manner towards an improved (prebiotics) or a dysbiotic (hydrogen peroxide or antibiotics) microbiota. Further studies should also investigate the effect of oxidative stress on human *in vitro* bacterial communities. Since we observed a strong correlation between the baseline gut microbiota and metabolic response upon dietary fiber supplementation, further studies should also assess whether each baseline gut microbiota is equally resilient to adverse environmental factors. Prebiotic dietary fibers have the ability to modulate the gut microbiota in a positive manner, indicating a potential strategy as a dietary treatment in disease by reintroducing high amount of substrates aiming to restore the gut microbiome. Further, it is mandatory to elucidate the mechanism by which the bacterial populations respond to the stresses (resistance mechanisms) and may influence the risk of intestinal inflammatory diseases. The careful evaluation of the specific effect of oxidative stress or an antibiotic on the intestinal microbiota will supply important information for directing and developing treatment strategies that re-modulate the bacterial composition. Our *ex-vivo* modified gut microbiota has the potential to be used as donor material for microbiota transplantation studies and a tool for the mechanistic understanding, whether microbial dysbiosis is a cause or a consequence in the development of colitis. In this context, it would be interesting to perform FMT with *in vitro* produced and modulated gut microbiota in colitis mouse models to alleviate or exacerbate inflammation state.

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