Promotors:

Prof. dr. ir. Tom Van de Wiele

Center for Microbial Ecology and Technology (CMET)

Department of Biochemical and Microbial Technology

Faculty of Bioscience Engineering, Ghent University, Belgium

Members of the examination committee:

Prof. dr. ir. Frank Devlieghere (Chairman)

Laboratory of Food Microbiology and Food Preservation

Department of Food Safety and Food quality

Faculty of Bioscience Engineering, Ghent University, Belgium

Prof. dr. ir. John Van Camp (Secretary)

Research Group Food Chemistry and Human Nutrition

Department of Food Safety and Food quality

Faculty of Bioscience Engineering, Ghent University, Belgium

Prof. dr. Debby Laukens

Ghent Gut Inflammation Group

Department of Internal medicine

Faculty of Medicine and Health, Ghent University, Belgium

Dr. Clara Belzer

Laboratory of Microbiology

Department of Agrotechnology and Food Sciences

Wageningen University, The Netherlands

Dean of the Faculty of Bioscience engineering

Prof. dr. ir. Marc Van Meirvenne

Rector of Ghent University

Prof. dr. ir. Rik Van de Walle

IMPACT OF MUCIN AND MUCIN DEGRADING *AKKERMANSIA MUCINIPHILA* ON GUT MICROBIAL ECOLOGY AND MARKERS FOR GUT HEALTH

Ir. Florence Van Herreweghen

Thesis submitted in fulfillment of the requirements for the degree of Doctor (PhD) in Applied Biological Sciences at Ghent University

Dutch translation of the title:
De impact van mucine en mucine degaderende *Akkermansia muciniphila* op het microbiële ecosysteem en gezondheidsparameters van de darm.

Corver illustration by Margo Van Herreweghen

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Notation Index

AMP	Anti-microbial protein/peptide
AXOS	Arabinoxylan-oligosaccharides
CA	Corrspondance analysis
CD	Crohn’s disease
DGGE	Denaturing gradient gel electrophoresis
DMEM	Dulbecco’s Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EEC	Enteroendocrine cell
FAO	Food and Agriculture organization of the United Nations
FOS	Frusto-oligosaccharides
GLP-1	Glucagon-like peptide-1
HMO	Human milk oligosaccharides
IBD	Inflammatory bowel diseases
IFN	Interferon
(s)Ig	(secretory) Immunoglobulin
IL	Interleukin
LY	Lucifer yellow
MAMC	Mucosa-associated microbial community
MFA	Multiple Factor Analysis
NMDS	Non-metric multidimensional scaling
OTU	Operational taxonomic unit
Papp	Apparent permeability
PBS	Phosphate buffered solution
(q)PCR	(quantitative) Polymerase chain reaction
PCoA	Principle coordinate analysis
PYY	peptide tyrosine tyrosine
(r)RNA	(ribosomal) Ribonucleic acid
SCFA	Short-chain fatty acids

SHIME	Simulator of the human intestinal microbial ecosystem
SD	Standard deviation
Sus	Starch utilization system
SVD	Single Value Decomposition
TEER	Transepithelial electrical resistance
TJ	Tight junctions
TLR	Toll-like receptor
TNF	Tumor necrosis factor
UC	Ulcerative colitis
WHO	World Health Organization

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CHAPTER 1

Introduction

CHAPTER 1

Introduction

Scientific research in the last decades has revolutionized our insight in how microorganisms colonizing the human body correlate with and even impact our health. Microbiological research has come a long way from the 1680s when Antonie van Leeuwenhoek compared oral and fecal microbiota and found specific differences according to sample origin and even health status. From there, the field of microbiology developed slowly, until the late 1800s when Louis Pasteur and Robert Koch postulated and proved that microorganisms are the cause of infectious diseases. Further research was mainly focused on these pathogenic microorganisms and the treatment of infected people, which lead to the discovery of antibiotics. This discovery and the development of vaccines in the mid-1900s, strongly reduced the incidence of diseases like pneumonia, tuberculosis, meningitis, polio and so on. Parallel to the study of the pathogenic microorganisms in the late 1800s, the use of beneficial bacteria to improve health was introduced. Henry Tissier reported that acute gastroenteritis could be cured by intake of bifidobacteria and together with Elie Metchkinov they introduced the idea that the intake of probiotics is beneficial for human health (Ozen and Dinleyici, 2015). But it was only at the end of the twentieth century that research focus shifted from pathogens to the large amounts of commensal microorganisms living in and on the human body, without causing (direct) harm. These viral, archaeal, fungal and mainly bacterial communities are referred to as the human microbiota, and their collective genomes are called the microbiome.

These commensal bacteria are most densely populated in the human gastrointestinal tract, mainly in the colon. Research has shown how intricate the microbe-microbe and the host-microbe interactions are and how subtle imbalances in our microbial populations can cause disease. Previous studies show a correlation between gut microbiota composition and obesity (Ley *et al.*, 2006), inflammatory bowel diseases (Walker *et al.*, 2011), diabetes (Karlsson *et al.*, 2013),… The prevalence of these diseases has strongly increased over the past decades, initially solely observed in Western countries but more recently also in developing countries, and it is still increasing (Mosca *et al.*, 2016). In 2016, 650 million adults were obese (13%), and an estimated 422 million adults were suffering from diabetes (WHO, 2017). Inflammatory bowel disease (IBD), which comprises Crohn's disease and ulcerative colitis, affects more than 3.6 million people (Loftus, 2004). The possibility to remedy these conditions or mitigate their symptoms by interventions on the gut microbiota has inspired many researchers to investigate, unravel, and understand the complex microbial community and its interaction with the human host. Large scale human studies, such as the Human

Microbiome Project, MetaHIT, the Flemish Gut Flora Project… were set up to understand the links between gut bacteria, health and lifestyle.

Interestingly, scientific research has shown a negative correlation between the prevalence of above-mentioned diseases and the abundance of *Akkermansia muciniphila*, an important mucin degrading microbe in the human gut. While no causal relationship between *Akkermansia* and human health status has been established yet, several animal models for disease have demonstrated *Akkermansia's* (or *Akkermansia* derived products) potential to improve health markers. There are several knowledge gaps on the ecological integration of *Akkermansia* in the human gut. In light of these recent developments, this PhD research will focus on studying *Akkermansia muciniphila* and the mucin degradation niche in complex microbial ecosystems. This chapter will introduce the complexity of this microbial ecosystem, discussing both the host, the microbiota and their interaction, by giving a general informative overview and gradually zooming in on the PhD topic of *Akkermansia muciniphila* and mucin degradation.

1. Gut microbiome and health

The fact that we have a human microbiota, isn't surprising when we consider that bacteria have thrived on this planet for 3.5 billion years, animals for half a billion years and humans for just 200.000 years. So our entire evolution, from our oldest ancestor until now, has happened in the presence of bacteria. Many of the genes that were responsible for the evolutionary inventions that led to humans and certainly those involving signaling and immune regulation were in part driven by the interaction with the microbiome (Domazet-Loso and Tautz, 2008; McFall-Ngai *et al.*, 2012). Moreover, not only have we evolved with these bacteria, they have co-evolved with us. Studies have shown that free-living bacterial communities from very different environments, even extremes like acidic hot springs, are more similar to each other than the mammalian gut microbiota. This indicates a co-evolution over millions of years between vertebrates and their microbiota that has resulted in a community that thrives in the gut environment (Ley *et al.*, 2008; Pace, 1997).

The human microbiota is not confined to just the gut environment; also other outer and inner surfaces of the human body are colonized with bacteria (Figure 1.1). The skin hosts a maximal concentration of 10^{11} bacteria per m^2, saliva contains 10^9 bacteria per mL, dental plaque contains 10^{12} bacteria while the stomach is colonized by 10^3-10^4 bacteria per mL.

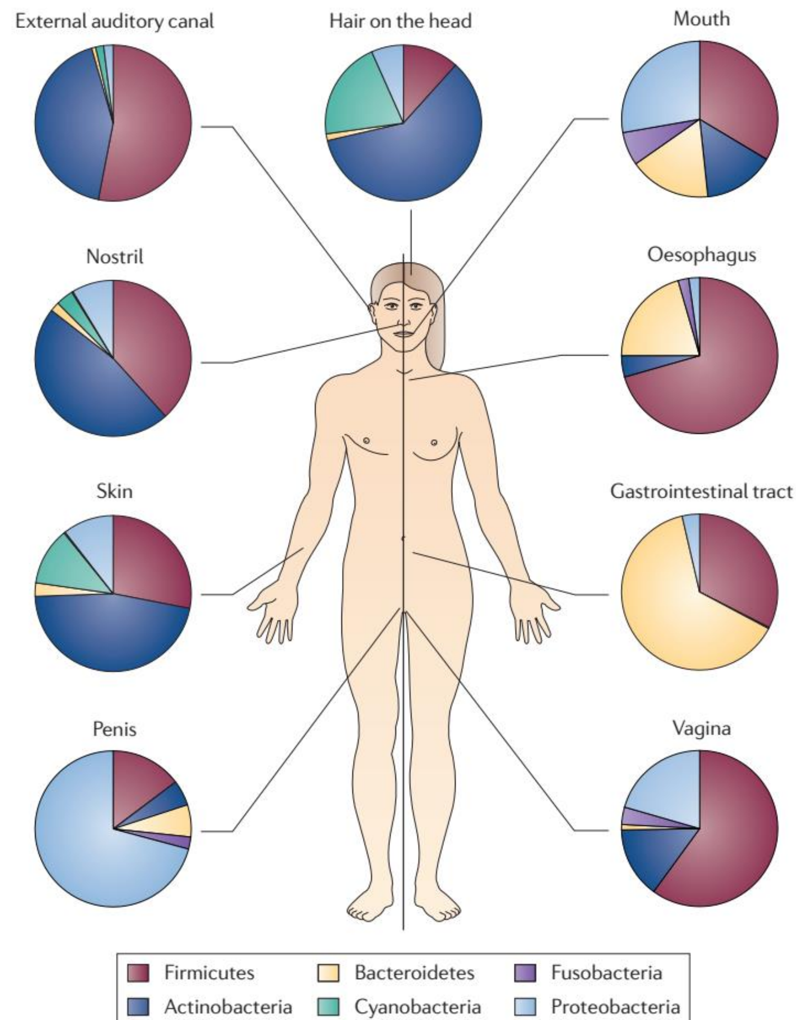

Figure 1. 1: Microbial community composition (relative abundances of the six dominant bacterial phyla) at different body sites in healthy people. Figure derived from (Spor *et al.*, 2011).

The most densely populated area of the human body is the large intestine (colon) with 10^{14} bacteria. Each body site (gut, skin, oral nasal, urogenital) is home to a unique community and also within body sites there are vast differences, for example different areas on the skin (armpits versus bellybutton), in the mouth (tongue versus buccal) and in the gut (stomach versus colon) (Costello *et al.*, 2009).

The total amount of bacterial cells equals that of the human cells, meaning that our body is 50% human and 50% bacterial (Sender *et al.*, 2016) and the microbiome contains around 3 million microbial genes, which is 100 times more than the 23.000 human genes in our own genome. The human genome, inherited from our parents, was generally thought to be stable during life compared to the dynamic microbiome that is influenced by environmental and host factors. However over the years, research has shown that our genome as well can be affected by environmental, so-called epigenetic factors, changing the epigenome (Simmons, 2008). The metabolic capacity of the microbiome is enormous, as well as its influence on the human host and is sometimes called 'our forgotten organ'. The

microbiome has the capacity to carry out more biochemical conversions than the liver, it protects against opportunistic pathogens, it manages the immune system, produces health beneficial compounds (vitamins, short chain fatty acids, ..) and breaks down indigestible food compounds (Thursby and Juge, 2017). SCFA, such as acetate, propionate and butyrate, are produced by bacterial fermentation activity. Propionate plays a role in gluconeogenesis in the liver, acetate is used in lipogenesis and butyrate has anti-carcinogenic effects and is the major energy source for colonocytes (Louis and Flint, 2017; Pryde *et al.*, 2002; Scott *et al.*, 2008).

It is important to remember that besides bacteria, the human microbiome also includes Archaea, viruses, and eukaryotes: *Methanobrevibacter smithii* is a member of the Archaea and involved in cross-feeding with bacteria (section 3.4); the virome is expected to be unique for each individual and an integral part of the healthy human ecosystem; eukaryotic members of the microbiome include fungi such as Candida and Saccharomyces. Insight in the functionality of these organisms within the microbiome is very limited since molecular-profiling techniques are mainly developed for bacteria (Lloyd-Price *et al.*, 2016). Knowledge about the taxonomy of the bacterial microorganisms and their abundance in different parts of the human body is increasing. However, knowledge is still lacking about how they function as a system: their interactions, which of these fulfill key functions, and how sensitive their community is (Jordan *et al.*, 2015). In a healthy, normal situation our microbiome provides us with health benefits, but when homeostasis is disturbed, it can have short- and long-term consequences for human health with several areas of host health that are compromised. The gut microbiota is for instance responsible for the production of essential vitamins such as folic acid, biotin and vitamin K, provides colonization resistance, it plays a role in host energy metabolism possibly contributing to overweight problems and it is considered an important modulator of our immune system (Thursby and Juge, 2017; Turnbaugh *et al.*, 2006).

1.1 Key health functions

Mucosal surfaces, like the colon, constitute the largest and most important interaction between the body and the outside environment and the mucosal epithelial cells are continuously exposed to pathogens. They are protected by both innate, such as the epithelial barrier, and adaptive immune systems, such as the mucosal immune system.

1.1.1 Epithelial barrier

The intestinal barrier is a multi-layered defense mechanism that protects our internal milieu against the harsh external environment in the gut lumen, preventing intrusion of antigens, while also allowing absorption of nutrients (Figure 1.2). In the lumen, commensal bacteria produce antimicrobial substances to inhibit colonization of pathogens. The mucus

layer between the lumen and the epithelial cells is a viscous gel matrix made up of mucin glycoproteins, trefoil peptides and surfactant lipids. It protects the epithelium from mechanical damage from food particles and due to its high concentration of sIgA and antimicrobial peptides (AMPs) it protects against bacterial invasion (Atuma *et al.*, 2001; Meyer-Hoffert *et al.*, 2008). Below the mucus layer lays the epithelium, consisting of epithelial cells separated by junction regions. These epithelial cells constitute the **epithelial barrier**, which is selective and facilitates transcellular transport of soluble or particulate matter through active transport proteins, diffusional processes or endocytosis and paracellular translocation across the junction regions (Bischoff *et al.*, 2014; Keita and Soderholm, 2012; Shen *et al.*, 2011). The junctions are regulated by the junctional complexes that consist of tight junctions, adherens junctions, gap junctions and desmosomes. Tight junctions form a seal between adjacent epithelial cells near the apical surface, thereby preventing paracellular diffusion of antigens or microorganisms across the epithelial barrier while allowing flow of water ions and small molecules (Farquhar and Palade, 1963; Zihni *et al.*, 2016). The adherens junctions are situated below the tight junctions and are involved in cell-cell adhesion, together with de desmosomes, and in and intracellular signaling, together with the gap junctions (Garrod and Chidgey, 2008; Perez-Moreno and Fuchs, 2006; Sosinsky and Nicholson, 2005). The tight junctions are complex structures containing over 50 proteins, including transmembrane proteins claudins and occludin, which interact with the actin cytoskeleton within the cell (Chiba *et al.*, 2008). Claudin-1,-3,-4,-5, and -8 tighten the tight junction whereas claudin-2 forms selective paracellular pores (Bucker *et al.*, 2010).The tight junctions are dynamic structures and are regulated by internal signaling and external stimuli from commensal bacteria and pathogens.

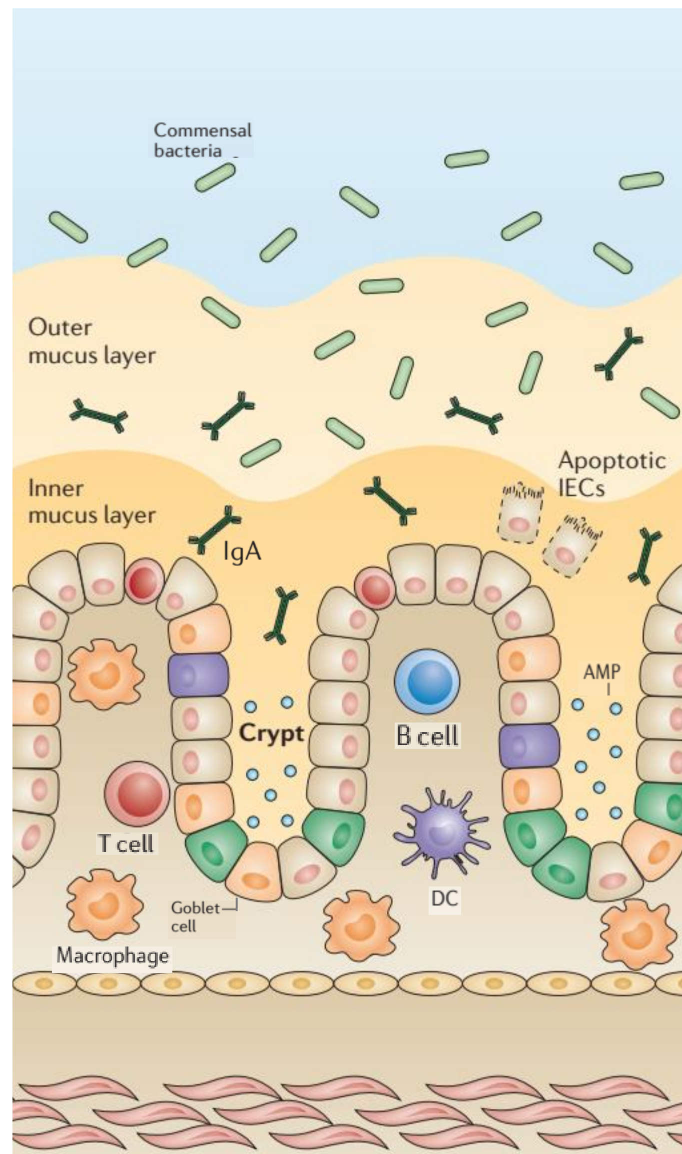

Figure 1. 2: The intestinal barrier: components of the multilayered defense system. Figure adapted from (Mowat and Agace, 2014). The epithelial cell layer is made up of intestinal epithelial cells (IECs, beige), goblet cells (orange), stem cells (green), intestinal endocrine cells (blue) and M-cells (red). IgA: Immunoglobulin A; DC: Dendritic cell; AMP: antimicrobial peptide.

A compromised intestinal barrier is characteristic for multiple diseases where the inflammation might be triggered by the translocation of luminal components into the host, such as IBD (Suenaert *et al.*, 2002), celiac disease (Vogelsang *et al.*, 1998) and obesity (Ley *et al.*, 2006). Intestinal barrier integrity also decreases with age and due to stress (Liu *et al.*, 2005; Saunders *et al.*, 1994). *In vitro* and *in vivo* experiments have shown that commensal bacteria and probiotics can increase intestinal barrier integrity. Patients suffering from Crohn's disease were treated with *Lactobacillus helveticus* and *L. rhamnosus*, and this treatment reduced intestinal permeability. Another study showed that *L. plantarum* could regulate tight junction proteins and provide protection against disruption of the epithelial

barrier (Ulluwishewa *et al.*, 2011). Several *in vivo* mice studies showed improvement of epithelial barrier function by *A. muciniphila* (Everard *et al.*, 2013; Li *et al.*, 2016; Shin *et al.*, 2014), including a study with obese mice where genes encoding tight junction proteins were affected by treatment with *A. muciniphila,* possibly through TLR2 activation (Plovier *et al.*, 2017). The commensal bacteria can regulate epithelial barrier function directly, by releasing metabolites such as acetate and butyrate or indirectly, by inducing the release of cytokines which can reduce (TNFα, IFNγ) and enhance (IL-10) barrier function (Arrieta *et al.*, 2006; Fukuda *et al.*, 2011; Hamer *et al.*, 2008).

1.1.2 Mucosal immune system

The gut-associated lymphoid tissue, representing the gut part of the total mucosal immune system, includes more than 70% of the total amount of immune cells in the human body, indicating the important role of intestinal immunity (Gaskins, 1997) (Figure 1.2).

The mucosal immune system can be partitioned into inductive and effector site. At the inductive site, antigens are taken up to initiate a proper immune response. In the epithelium at these inductive sites, Microfold cells or M cells are present and unlike their neighboring epithelial cells, they are specialized in antigen uptake from the lumen via transcytosis (Mowat and Agace, 2014). The antigen is released to cells of the immune system beneath the epithelium and presented by antigen presenting cells, to naïve T-cells. This causes and induction of activated antigen-specific T- and B-cells that (Hooper *et al.*, 2012) travel through the lymph vessels to the effector site, where the immune response is expressed. Immunoglobulin A (IgA) is the predominant antibody in the mucosa and can protect the mucosa in several ways (Herich, 2017). IgA binds pathogens and prevents their attachment to the epithelium, it may promote phagocytosis, it can enhance the entrapment of some bacteria in the mucus, it can neutralize toxins and interfere with pathogenic growth factors.

A very important aspect of the mucosal immune system is that the immune response must be carefully balanced between the inflammatory response required for pathogen eradication and a tolerant reaction towards self-tissue and commensal bacteria (Petersen and Round, 2014). This intricate balance is determined by multiple factors, such as the residing microbiota and the host itself (Mowat and Agace, 2014). Pattern recognition receptors (PRRs) are able to recognize pathogen/microbe associated molecular patterns (PAMPs and MAMPs) and thereby distinguish between self and non-self. These PRRs, such as Toll-like receptors (TLRs), are an important interface between the microbiota and the immune system. Their activation is a trigger than can impact T-cell differentiation to effector or suppressor T-cells, and this is a crucial aspect of immune homeostasis (Nutsch and Hsieh, 2012; Swiatczak and Cohen, 2015). For example, polysaccharide A (PSA) from *B.fragilis* can activate TLR2 and enhance T-cell differentiation towards the immune-

suppressive Treg cells (Nutsch and Hsieh, 2012; Round *et al.*, 2011). *A. muciniphila* induces both pro- and anti-inflammatory cytokines in peripheral blood mononuclear cells but its inflammation potential (TNF-α/IL-10) is lower than *F.prausnitzii* and *L.plantarum* and its cross-talk with the host could occur through activation of TLR2 and TLR4 (Ottman *et al.*, 2017d).

Bacteria play an essential role in immune system development, especially in early life. Improper immune modulation in infants can have long lasting effects that can cause unbalanced immune responses. A study by Cahenzli *et al.* (2013) showed that microbial diversity during early-life colonization shapes long-term IgE levels, which play an essential role in atopic allergic diseases.

1.1.3 Energy homeostasis

The main job of the intestinal cells is not just regulating the bacteria and protecting our internal areas from invading pathogens, but also the absorption of nutrients and energy. Energy homeostasis is an important well-controlled process that involves the regulation of food intake (energy inflow) and energy expenditure (energy outflow) (De Silva and Bloom, 2012). Multiple organs are involved in controlling energy homeostasis including the stomach, pancreas, intestine, brain (mainly hypothalamus) and liver, and they communicate using gut derived hormones, secreted by enteroendocrine cells (EEC) of the gastrointestinal tract and pancreas. A key function of these EECs is sensing the luminal content to modulate the hormone secretion that regulates food intake and energy storage (De Silva and Bloom, 2012; Greiner and Backhed, 2016; Spreckley and Murphy, 2015).

L-cells are EECs primarily present in the epithelium of the ileum and colon and they can sense specific macronutrients, which modulate the secretion of anorectic gut hormones glucagon-like peptide-1 (GLP-1) and peptide tyrosine tyrosine (PYY) (Cheung *et al.*, 2016). GLP-1 binds to specific receptors (GLP-1R) on β-cells of the pancreas, inducing the release of insulin. GLP-1 also increases insulin sensitivity of pancreatic α- and β-cells, liver cells and peripheral tissue and promotes β-cell proliferation while reducing apoptosis. By inhibiting gastric emptying, intestinal motility and glucagon secretion from pancreatic α-cells, GLP-1 can delay nutrient absorption (Burcelin *et al.*, 2007). PYY secretion occurs after nutrient ingestion and is proportionate to the caloric contact and macronutrient composition of the meal. PYY has been shown to reduce caloric intake and play a key role in regulating bodyweight by acting on appetite-regulating circuits in the brain. It also affects gut motility and leads to a sensation of fullness and satiety (Batterham *et al.*, 2002). Obesity is associated with lower circulating levels of PYY and GLP1 deficiency, while remaining responsive to the anorectic and glucoregulatory effects. Interestingly, germfree mice – having a completely sterile gut – have been shown to take up 30% more calories compared to

colonized mice, while having 40% less body fat. This shows a clear involvement of gut microbiota in energy homeostasis regulation (Burcelin *et al.*, 2007; De Silva and Bloom, 2012; Greiner and Backhed, 2016).

1.2 Microbial ecosystem homeostasis and dysbiosis

The gut microbiota is often described as a microbial ecosystem that functions as a microbial organ and that can, when in homeostasis, promote health (Tasnim et al., 2017). An ecosystem is viewed as the complex of living organisms (microorganisms) in a defined space (colon) and their interaction with each other and their environment (host). Diversity is an important measure for maintaining ecosystem homeostasis in the colon, as ecological theory predicts communities with high diversity to be more resilient to perturbations (Elmqvist *et al.*, 2003; Hautier *et al.*, 2009; Lozupone *et al.*, 2012). Since the beginning of the 21st century, studies have revealed significant perturbations in gut microbial communities in patients suffering from inflammatory bowel diseases (Frank *et al.*, 2011; Ni *et al.*, 2017; Sokol *et al.*, 2017), diabetes (Karlsson *et al.*, 2013; Kostic *et al.*, 2015; Marino *et al.*, 2017; Mullaney *et al.*, 2018), obesity (Cotillard *et al.*, 2013; Harakeh *et al.*, 2016; Le Chatelier *et al.*, 2013) and colorectal cancer (Gagniere *et al.*, 2016). Considering the enormous capacity of the gut microbiota, it is possible that these changes in microbial composition contribute to the initiation and/or persistence of above-mentioned diseases. Since many studies only provide an associative link between gut microbial composition and disease and few can prove actual cause-and-effect, there is a need for more causal evidence.

These perturbations in community structures are referred to as dysbiosis (dysbacteriosis). Dysbiosis is a term for microbial imbalance, qualitative and quantitative changes in the metabolic activity and local distribution of the gut microbiota (Holzapfel *et al.*, 1998). It can be characterized by the loss of beneficial microbes, the outgrowth of pathobionts and/or the loss of overall diversity (Petersen and Round, 2014). Some commensal bacteria can, for example, induce anti-inflammatory responses or reduce pro-inflammatory cytokines and loss of these beneficial bacteria would impact homeostasis and gut health (Atarashi and Honda, 2011; Round and Mazmanian, 2009). It has been shown that a more complex and diverse collection of microorganisms elicits maximal gut health benefits, and loss of diversity, especially early in life, might predispose for diseases (Abrahamsson *et al.*, 2013; Atarashi *et al.*, 2013; Cahenzli *et al.*, 2013). Dysbiosis may be caused by antibiotic use, metabolic alterations, psychological and physical stress, diet, and so on. The intestinal dysbiosis hypothesis proposes that the above-mentioned microbial imbalance is associated with and possibly caused by modern Western lifestyle and practices, which may thus, at least partly, underlie the increased risk for developing diseases such as IBD, Type-2 diabetes, colon cancer etc (Hawrelak and Myers, 2004; Mosca *et al.*, 2016).

The microbial contribution to an increased disease risk is thus not only coming from one ‘bad’ microbe prompting disease, but more associated with an imbalance in the entire endogenous microbiome from which opportunistic pathogens may or may not take benefit and proliferate and further aggravate disease. A more in-depth look into the normal microbiome composition and microbiome functionality is required.

2. Microbiome

2.1 Interindividual variability in microbiome composition

The bacterial colon community consists for 90% of bacteria belonging to the phyla Bacteroidetes and Firmicutes and the other 10% belong to Proteobacteria, Actinobacteria and Verrucomicrobia (Eckburg *et al.*, 2005). At the moment *A. muciniphila* is the only identified member of the Verrucomicrobia phylum in the gut (Fujio-Vejar *et al.*, 2017). The colon microbiota is characterized by high species richness, with more than 1500 species in total of which at least 160 species are shared among individuals (Lagier *et al.*, 2016; Qin *et al.*, 2010; Rajilic-Stojanovic and de Vos, 2014). True diversity is expected to be even higher since these numbers are obtained from relatively small cohorts (<1000 subjects) and it was estimated that for observing total richness around 45 000 individuals would require sampling (Falony *et al.*, 2016).

A variety of host and environmental factors influence gut microbial composition and establish inter-individual differences, such as age, diet, health status, gender and geography (Figure 1.3). An interesting study by De Filippo *et al.* (2010) showed substantial differences in the bacterial gut communities of children from Burkina Faso and Italy. The results point to diet being a driving factor with an increase in bacteria that can extract energy from the indigestible polysaccharide-rich diet in Burkinabe compared to Italian children. Ageing is typically linked with decreased microbial diversity and increased inflammatory status (Biagi *et al.*, 2010) and in the early life of preterm infants gender was shown to significantly contribute to gut microbiota development (Cong *et al.*, 2016). Of course, inter-individual variability is caused by the interaction of all these factors, making it a very complex issue that needs simplification. This was again confirmed in a cohort study (1106 individuals) where even with extensive metadata variables, such as medication, blood parameters, dietary and health information, only 16.4% of the microbiome variation could be explained (Falony *et al.*, 2016). Interestingly, stool consistency and medication were identified as the most explanatory covariates and are thus important metadata variables to be included in future studies.

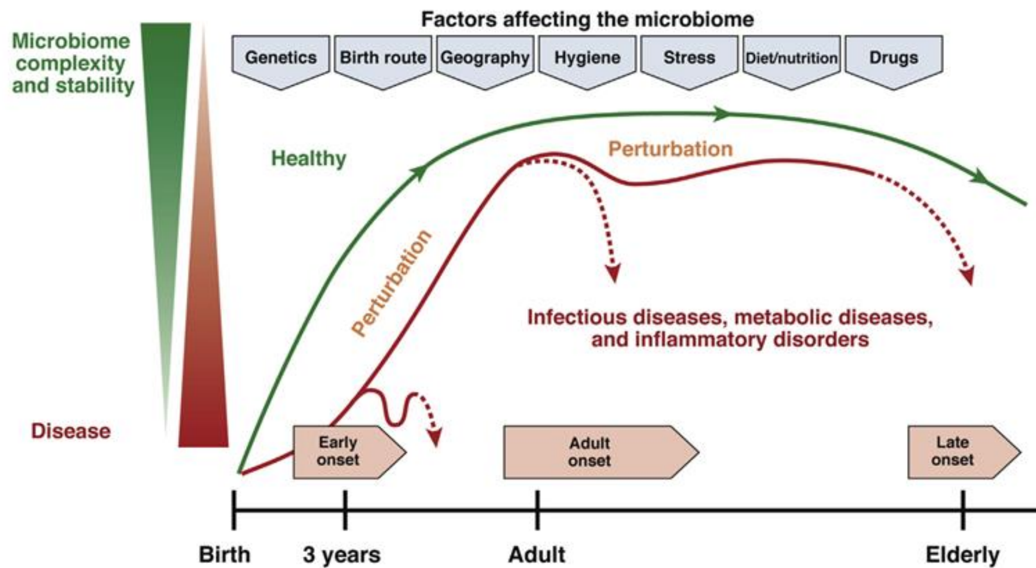

Figure 1. 3: Factors influencing the human gut microbiome in health and disease. Figure derived from (Kostic *et al.*, 2015).

The high complexity of the human microbiome and its interindividual variability make the development of modulation strategies, such as the pro- and prebiotics described in section 4.1, highly challenging. The success of such interventions, the fermentation of a prebiotic compound or the capacity of a probiotic, of course depends on the endogenous microbial community to which they are applied. The bacteria residing in the gut will determine how a compound is fermented and whether probiotic bacteria can fulfill their function and might get established in the gut. A study by Arumugam *et al.* (2011) suggested that the interindividual variability might manifest as a discrete amount of stable states, balanced communities. They identified 3 of those 'stable states', called enterotypes, characterized either by a predominance of *Bacteroides*, *Prevotella* or *Ruminococcus*. The concept of such enterotypes could be used as a predictive tool in treatments, for example establishing whether a certain enterotype associates with a positive outcome following drug or probiotic treatment. This could lead to a personalized microbiome-based diagnosis and therapy (Costea *et al.*, 2018). However, an individual's enterotype was demonstrated to be highly variable (Knights *et al.*, 2014) and other human microbiome studies support continuous gradients of dominant taxa rather than discrete enterotypes (Jeffery *et al.*, 2012). Furthermore, due to the functional redundancy it is more relevant to define microbiome subgroups based on functionality instead of composition.

2.2 Bacterial functions in the colon

The major bacterial functions in the colon are protective, metabolic and trophic (Figure 1.4). By competing for nutrients and preventing attachment the resident microbiota

offers **protection** from invasion by an incoming pathogen, known as colonization resistance. Also, the interaction between host and a healthy microbiota is critical for the development and homeostasis of the immune system (Macdonald and Monteleone, 2005). One of the **metabolic** functions is the synthesis of certain vitamins, such as vitamin K, by certain bacterial groups like *Bacteroides, Eubacterium* and *Propionibacterium.* A study showed that germ-free mice required supplementation of vitamin K and B12, since they did not have the bacteria to synthesize them (Canny and McCormick, 2008).

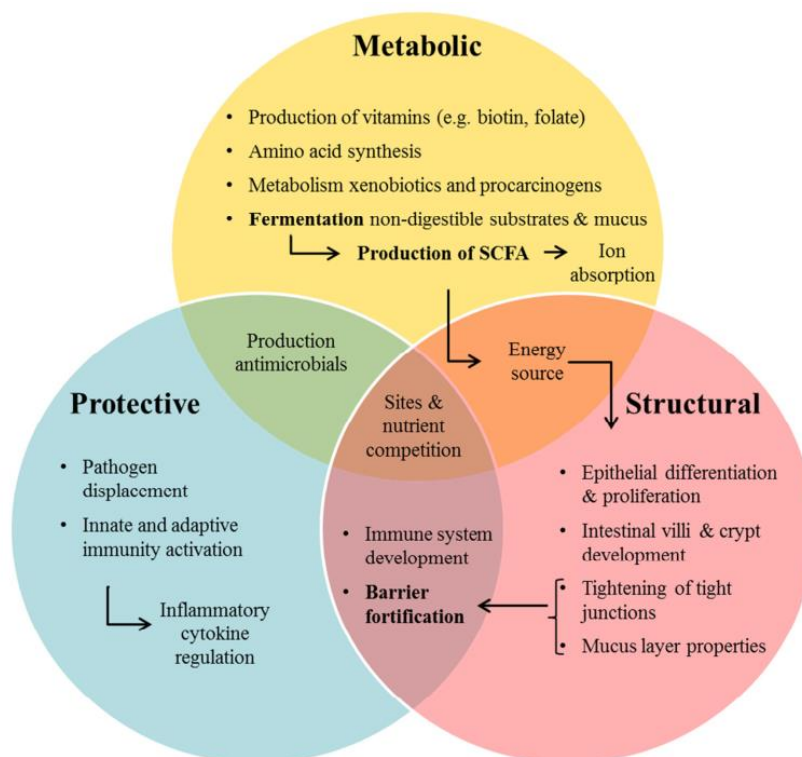

Figure 1. 4: Key health function of the human gut microbiota. Figure derived from (Geirnaert, 2015).

The major metabolic function of the bacterial community is probably its fermentative capacity. After the digestion of food in the stomach and small intestine, components that escaped digestion or were impervious to human enzymes, reach the large intestine with its 10^{14} bacteria. This anaerobic microbial community is able to ferment these components and produce a variety of metabolites, reflecting the impressive biochemical capacity of the microbiota (Flint *et al.*, 2012a; Flint *et al.*, 2012h; Louis *et al.*, 2007; Marcobal *et al.*, 2013a; Marcobal *et al.*, 2013d). The primary substrates for microbial fermentation are non-digestible carbohydrates such as resistant starch, plant cell walls and certain oligosaccharides. Since these non-digestible carbohydrates are diet-derived they vary widely in availability and structure and thus in the enzymatic capacity needed for degradation. In contrast, there is an almost constant supply of host-derived glycans, namely mucins, which differ less in structure

and availability. However not much is known yet on the nutritional aspect of these mucin glycans and further research is needed concerning the importance of mucin fermentation and the fermentation products for gut health. Major fermentation products of the saccharolytic fermentation are gasses and organic acids, especially short-chain fatty acids (SCFAs) acetate, propionate and butyrate (Bernalier-Donadille, 2010). They are typically formed in a 3/1/1 ratio at a combined concentration of 50-150 mM in the colon, depending on diet and microbial composition. The third bacterial function in the colon concerns the **trophic** effects exerted by these SCFAs on the intestinal epithelium as they play a role in controlling epithelial cell proliferation and differentiation (Frankel *et al.*, 1994).

Once the carbohydrates are fermented, undigested proteins remain and the bacterial metabolism turns to proteolytic fermentation. End products of proteolytic fermentation are for example branched SCFA, amines, phenols, indoles, thiols, CO_2, H_2, and H_2S, many of which have toxic properties. As digested material moves along the gut, carbohydrates become depleted and microbial metabolism of proteins and amino acids takes over, mainly in the distal colon. The latter process is thought to be linked with the increased prevalence of colonic disease at this site (Bernalier-Donadille, 2010; Nyangale *et al.*, 2012; Windey *et al.*, 2012).

The metabolic potency to carry out these fermentation processes, are redundantly present throughout the microbial community (Moya and Ferrer, 2016). For example, *Bacteroides* spp., *Prevotella* spp., *Bifidobacterium* spp., species belonging to Firmicutes *Clostridium* clusters IV and XIVa can degrade a variety of complex carbohydrates. So while qualitative and quantitative dietary changes or interindividual variability may lead to changes in community composition, they might not lead to functional changes. This phenomenon is known as functional redundancy and might protect the community from a dysbiosed state as it keeps the community functionally stable after perturbations. Stool samples of 242 individuals were analyzed for microbial composition profile, which showed immense diversity in community structure, and for functional profile, which showed immense similarity between individuals (Huttenhower *et al.*, 2012). Thus instead of looking for a core composition or compositional enterotypes, as described above, it is more relevant to define a functional core microbiome (Turnbaugh *et al.*, 2009).

2.3 Microenvironments and gradients in the colon

Within the environment of the gastrointestinal tract and even within the colon, there are microenvironments that select for a distinctive microbial community due to their specific properties. The stomach, with its very acidic pH (pH 2-5) and strong peristalsis, is minimally colonized (10^1-10^3 cells.mL^{-1}) and mainly by the pathobiont *Heliobacter pylori* and some oral

bacteria (Walter and Ley, 2011). The small intestine is characterized by secretion of bactericidal digestive enzymes, bile acids, short transit time (2-6h) and an active immune system, which restricts bacterial colonization and leads to a density of 10^3 -10^8 cells.mL^{-1}. Since the small intestine is difficult to access, microbiota studies in healthy individuals are rare, but *Streptococcus* and *Veillonella* could be described as core members of the small-intestinal community (van den Bogert *et al.*, 2013). The large intestine or colon is the most densely colonized environment of the human body, with 10^{11} cells.mL^{-1}. The conditions are optimal for bacterial growth and activity: less acidic pH (5-7), longer retention time (48-70h), low bile acid concentrations and a more tolerant immune system.

Within the colon, a longitudinal and axial gradient is observed. The longitudinal gradation, from the proximal colon to the distal colon, is characterized by differences in pH, fermentation activity and increasing mucus thickness (Ermund *et al.*, 2013). As described above, in the proximal colon saccharolytic fermentation takes place by bacteria such as *Bacteroides* spp., *Prevotella* spp., *Bifidobacterium* spp., *Clostridium* clusters IV and XIVa spp. These saccharolytic processes lead to the production of SCFA, which lower the pH. Autopsy samples from sudden death victims revealed SCFA concentration in the proximal colon (137-197 mmol/kg gut content) to be higher than in the distal colon (86-97 mmol/kg gut content) (Cummings and Englyst, 1987). In the distal colon, proteins and amino acids become the main energy source for the microbiota, since carbohydrates are depleted (Macfarlane *et al.*, 1992). The distal colon has a more neutral pH compared to the proximal colon, due to the higher amount of pH increasing proteolytic fermentation products, such as ammonia and the lower amounts of SCFA. *Bacteroides, Propionibacterium, Fusobacterium and Lactobacillus* are examples of genera producing proteases and hydrolyzing proteins (Kovatcheva-Datchary and Arora, 2013).

The axial gradient goes from the epithelial cells, through the mucus layer to the lumen and shows great differences in microbial diversity and density (Sommer and Backhed, 2013; Van den Abbeele *et al.*, 2011g). The colonic mucus layer consists of an dense, firmly attached inner layer and a loosely attached outer layer. The inner layer is mostly devoid of bacteria and is 100 µm thick while the outer mucus layer is 300-400 µm tick and is colonized by 10^5-10^6 bacteria/mL mucus (Johansson *et al.*, 2011; Johansson *et al.*, 2008). Interestingly, a recent study posits a new view, of a mucus layer that covers the fecal surface instead of the epithelium, and keeps the microbiota confined to the feces (Kamphuis *et al.*, 2017). The mucus environment differs from the lumen due to the high concentrations of host defense molecules (anti-microbial peptides and immunoglobulin-A) and an oxygen gradient, which required adaptation of the bacteria. Not only host factors but also microbial characteristics define microbial colonization of the mucus layer such as attachment to the mucus and the ability to gain nutrients from the host-derived mucins. This leads to a distinct mucus

associated microbial community (MAMC), that closely interacts with the host at the host-microbial interface (Jones *et al.*, 2018; Van den Abbeele *et al.*, 2011g), and is enriched in *Lachnospiraceae* and *Ruminococcaceae* of the *Firmicutes* phylum. This MAMC is hypothesized to be crucial for immunological priming whereas the luminal microorganisms would be more involved in nutrient digestion.

3. Bacterial glycan metabolism

As mentioned before, the main function of the gut microbiota is the fermentation of dietary or host-derived components, mainly glycans, which leads to the production of beneficial SCFA. Given the structural variety of these glycans, a number of enzymes are involved and so many bacteria are part of this degradation process or can profit from it.

3.1 Dietary glycans

The recommended fiber intake for adults is between the 28 and 35 grams per day, however the diet in industrialized nations generally falls well below this recommendation and this deficit has been linked to several diseases (Desai *et al.*, 2016; Sonnenburg and Sonnenburg, 2014). These undigested complex carbohydrates reach the colon, consisting of resistant starch, plant cell wall polysaccharides and fructans and oligosaccharides (Cummings *et al.*, 2001; Silvester *et al.*, 1995) (Figure 1.5). Dietary **starch** is mostly degraded by host amylases but a fraction is resistant due to either protection from plant cell wall polymers, its granular structure, retrogradation (which is caused by heating and cooling) or chemical cross-linking (Flint *et al.*, 2012h). This resistant starch is the main source of diet derived energy for the colonic bacteria (Lockyer and Nugent, 2017), for example *Bacteroides thetaiotaomicron*, *Bifidobacterium* spp., and *Roseburia intestinalis* possess the ability to bind to and degrade these starch granules (Louis *et al.*, 2007). Starch consists of a mixture of amylose and amylopectin, and a higher amylose content makes it more resistant to host degradation. Bacterial enzymes involved, are α amylases that hydrolyze α(1,4) bonds and type 1 pullulanases that hydrolyze α(1,6) bonds and amylopullulanases that do both (Flint *et al.*, 2012a).

Plant cell wall material includes cellulose, arbinoxylan, hemicellulose, lignin and pectins and they are degraded by variety of microbial hydrolases, esterases and lyases. Some of these structures, cellulose and lignin, cannot be fully degraded by human gut bacteria and these particles persist throughout the colon. Pectin and hemicellulose on the other hand are more fully degraded, in a two-step process. Primary degraders are able to degrade the pectin and hemicellulose structures present in cell wall matrices to soluble **oligosaccharides**. These include xylo-, galacto-, and manno-oligosaccharides, which can be

further metabolized by other bacteria (more details in 3.4). Primary degraders known to have the ability to degrade xylans, hemicellulose structure present in algae, are *Bacteroides ovatus* and *Roseburia intestinalis* (Flint *et al.*, 2012a).

Foods like onions, garlic, bananas and leek are rich in inulin type **fructans**, which are linear polymers of β-2,1 linked fructose residues, with a terminal glucose monomer and a degree of polymerisation (DP) between 3 and 60. Fructo-oligosaccharides (FOS) have the same structure but with a much lower DP (3-9). Both FOS and inulin are well studied prebiotics that stimulate the growth of Lactobacilli and Bifidobacteria and some *Clostridium* cluster XIVa bacteria like *Roseburia inulinivorans* (Eckburg *et al.*, 2005; Van Loo, 2004). Bacterial utilization of fructans depends on β-fructofuranidases enzymes, which vary, in a strain dependent manner, in their ability to cleave the β-2,1 bonds in sucrose, FOS and inulin.

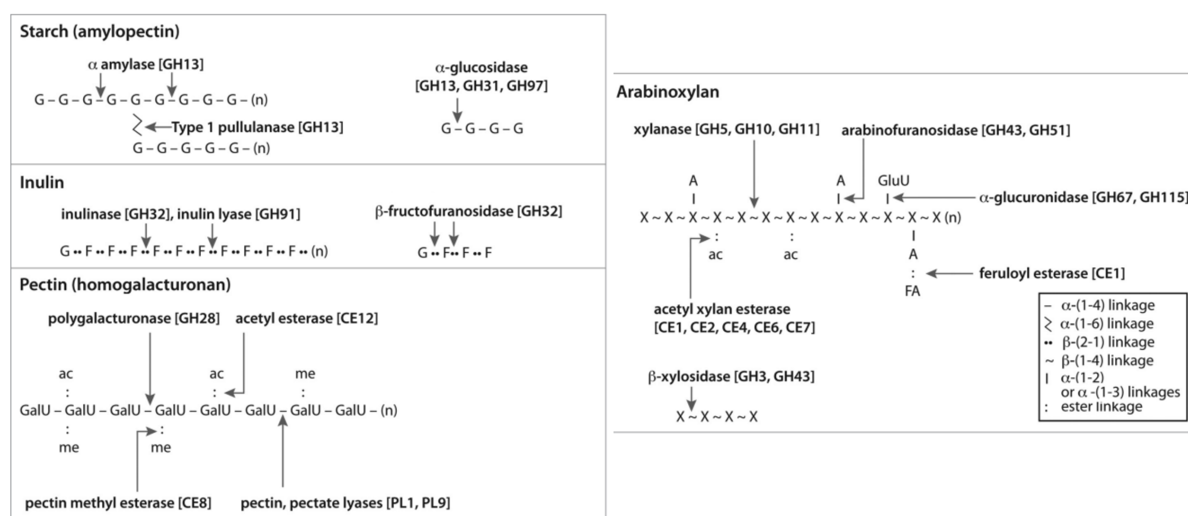

Figure 1. 5: Structure of diet-derived polysaccharides and microbial carbohydrate degrading enzyme activities. Enzyme families are indicated as follow: GH glycoside hydrolase; PL polysaccharide lyase; CE carbohydrate esterase. G, glucose; F, fructose; X, xylose; GalU, galacturonic acid; GlaU, glucuronic acid. Figure adapted from (Flint *et al.*, 2012a).

3.2 Host glycans

As opposed to dietary glycans that vary in composition and supply, the host-derived glycans from the mucus layer present a more continuous source of nutrients. Mucin glycoproteins are composed of O-glycosylated, and to a lesser extent N-glycosylated, protein backbones, with glycosyl chains of 2-12 monosaccharides, consisting of mainly galactose, fucose, N-acetylgalactosamine, N-acetylglucosamine and mannose (Figure 1.6) (Derrien, 2007; Lai *et al.*, 2009; Wilson, 2005).

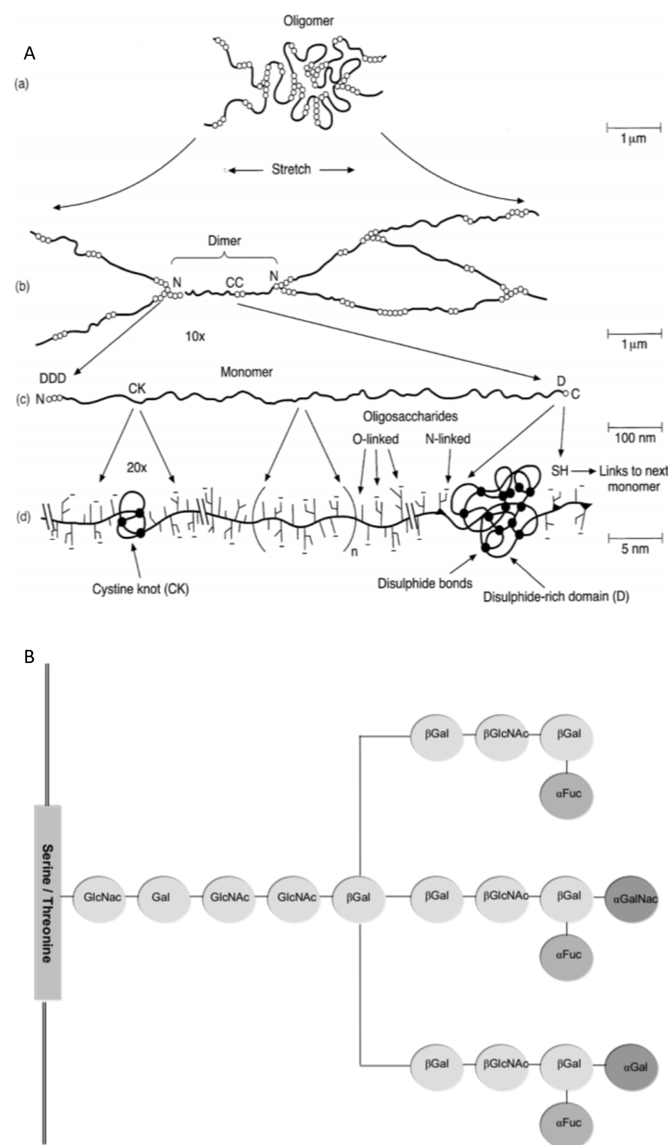

Figure 1. 6: (A) Monomeric and oligomeric structures of mucin: (a) mucin monomers (lines) linked together (circles) in an oligomeric gel, (b) linkage of the individual monomers is shown more clearly, (c) an individual monomer, with D domains which are involved in forming disulphide bonds between monomers, (d) more detailed structure of the monomer, containing many O- and N-linked oligosaccharides. Picture derived from (Wilson, 2005). (B) Mucin structure: composition of the glycosyl chains attached to the protein backbone. ((β)GlcNac: (β)N-acetylglucosamine; (β)Gal: (β)Galactose; (α)Fuc: (α)Fucose; (α)GalNac: (α)N-acetylgalactosamine) Figure derived from (Derrien, 2007).

The addition of sulphate and sialic acids on the terminal side of these glycosyl chains results in higher viscosity and better protection against bacterial and host enzymes (Robbe *et al.*, 2003). The protein backbone is rich in serine and threonine, and with proline, alanine and glycine they make up 80% of the total amino acid content (Schrager, 1970). Proline, serine and threonine make up the so called PTS domain, that occurs at least ones and is responsible for the variability in mucin length and extent of glycan attachment. The MUC gene family, with more than 20 genes is responsible for the production of mucins in humans

(Dekker *et al.*, 2002), of which MUC2 is the gel-forming mucin in colonic mucus (Johansson, 2012) (Figure 1.6). Continuous mucin production by the goblet cells and mucus desquamation contribute both to mucin presence in the mucus layer as in the lumen of the colon (Atuma *et al.*, 2001; Faure *et al.*, 2002; Johansson, 2012). Previously, it was thought that mucin degradation was detrimental for gut health but it is now clear that it is part of a normal turn-over process (Norin *et al.*, 1985). Due to the complexity of the mucin structure and the variation in glycosylation, a wide variety of specific enzymes are needed for its degradation, such as β-galactosidases, fucosidases, sialidases, fucosidases, N-acetylglucosaminidase, N-acetylgalactosaminidase and proteases (Tailford *et al.*, 2015a). This means that generally bacteria only possess a couple of these enzymes and complete degradation of these complex glycan structures requires cooperation of several species (Figure 1.7; Table 1.1) (Hoskins *et al.*, 1985; Marcobal *et al.*, 2013a; Marcobal *et al.*, 2013d; Png *et al.*, 2010; Ravcheev and Thiele, 2017).

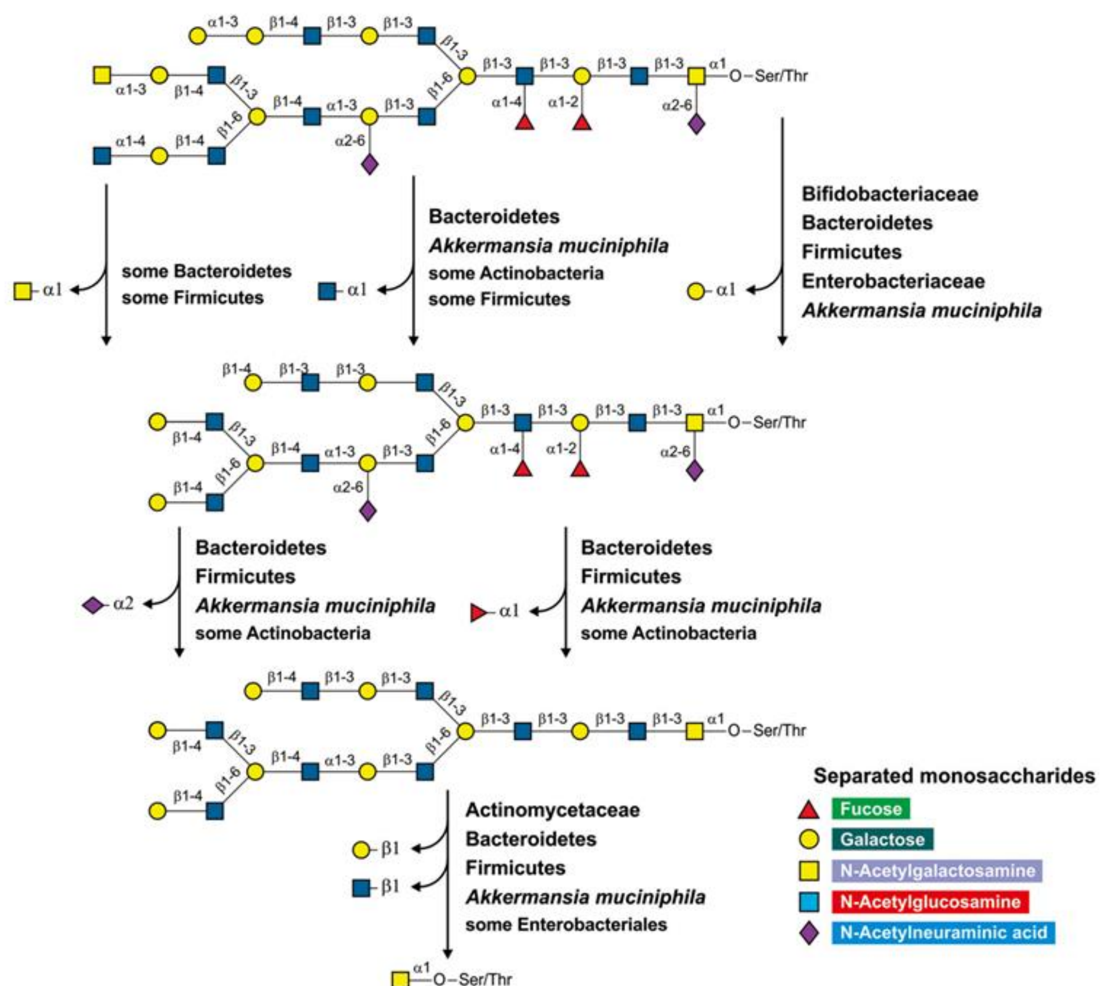

Figure 1. 7: Cleavage of a hypothetical mucin glycan by gut microbiota members. Figure adapted from (Ravcheev and Thiele, 2017).

Degradation of mucins leads to the release of less complex carbohydrates and the production of metabolites like acetate, lactate and propionate, some of which can be used by other bacteria, as part of a microbial food chain, to produce butyrate or other end products (Belzer and de Vos, 2012). The presence and the activity of mucin degrading species in the mucus layer, close to the host cells, may have strong effects, both positive and negative, on gut health.

The mucin degradation process involves several steps (Figure 1.7) (Ravcheev and Thiele, 2017; Tailford *et al.*, 2015a; Tailford *et al.*, 2015e), such as the release of **sialic acid (N-acetylneuraminic acid)** through sialidase activity, since these terminal sialic acid residues might prevent action from other glycoside hydrolases. There are bacteria that have the genes to release and metabolize sialic acid, such as *F. prausnitzii, R. gnavus* and *L. plantarum* (Almagro-Moreno and Boyd, 2009). *B. thetaiotaomicron* on the other hand can release but not consume the free sialic acid, so it becomes available for other bacteria. Pathogen *Salmonella thyphimurum* and pathobiont *Clostridium difficile* can use the free sialic acid but cannot release it themselves and so rely on others to profit from mucin degradation activity (Marcobal *et al.*, 2013d). A second step is the cleaving of **fucose** from galactose or N-acetylglucosamine residues in the O-glycosidic chain. This fucosidase activity of *B. longum* subsp *infantis*, *B. bifidum* and *R. gnavus* is a crucial element in their ability to derive energy from mucins. *B. thetaiotaomicron* encodes for multiple fucosidases which leads to high fucose availability in the lumen, benefiting other bacteria (Martens *et al.*, 2008). The third step, α-N-acetylgalactosaminidases cleave the **glycan core structure** from the serine/threonine amino acids in the protein backbone. These α-N-acetylgalactosaminidases vary in their specificity, for example the enzyme of *B. bifidum* is specific for the core 1 glycan while those of other bacteria have a broader spectrum (Katayama *et al.*, 2005). This leaves the oligosaccharide chains of these core structures and the **protein backbone** free for further degradation. As opposed to the bacteria described above who could carry out parts of the mucin degradation process, mucin degrading specialist *A. muciniphila* can use up to 85% of the total mucin structure (Table 1.1, Table 1.2). It has a an entire repertoire of enzymes involved in this process (α-D-galactosidase, β-D-galactosidase, β-D-fucosidase, N-acetyl-β-D-glucosaminidase, N-acetyl-α-D-galactosaminidase, N-acetyl-β-D-galactosaminidase, β-D-glucosidase, α-L-fucosidase, β -D-mannosidase) with both extracellular and intracellular activity (Table 1.2) (Derrien, 2007).

Table 1. 1: Bacterial mucin-degrading enzymes identified in the human gut. Table derived from (Derrien, 2007).

Organisms	Enzymes
Bacteroides fragilis	Neuraminidase, sulphatase, protease α- N-acetylgalactosaminidase, β-galactosidase, β -N-acetylglucosaminidase, α-fucosidases,
Bacteroides thetaiotaomicron	Sulphatase, neuraminidase α-fucosidase, β-galactosidase α- N-acetylgalactosaminidase β-N-acetylglucosaminidase
Bacteroides vulgatus	Neuraminidase, α and β-galactosidases, α-fucosidase β-N-acetylglucosaminidase, α and β -N-acetylgalactosaminidase
Bifidobacterium sp	α-L-Fucosidase, α-N-acetylgalactosaminidase
Bifidobacterium bifidum	Galactosyl-N-acetylhexosamine
Clostridium cocleatum	Neuraminidase, β-galactosidases, β -glucosidase, β-N-acetylglucosaminidase, α-N-acetylgalactosaminidase.
Clostridium septicum	β-Galactosidase,β-N-acetylglucosaminidase, glycosulphatase, neuraminidase
Prevotella sp RS2	Glycosulphatase
Ruminococcus torques	α-N-Acetylgalactosaminidase
Streptomyces sp	α-L-Fucosidase
Vibrio cholerae	Neuraminidase, endo-beta-N-acetylhexosaminidase, proteinases.

Table 1. 2:Activity of mucrin-degrading enzymes of *Akkermansia* muciniphila grown in mucin-based medium for 24h. (ND: no activity detected.) Tabe derived from (Derrien, 2007).

Enzyme assay	Enzyme activity (µmol/mg protein/min)	
	Intracellular	Extracellular
Glycosidases		
α-D-galactosidase	9.7	5.8
β-D-galactosidase	60.0	6.5
α-L-fucosidase	ND	4.0
β-D-fucosidase	3.3	ND
N-acetyl-α-D-galactosaminidase	23.3	ND
N-acetyl-β-D-galactosaminidase	25.0	ND
N-acetyl-β-D-glucosaminidase	216.7	ND
β-D-glucosidase	ND	5.8
α-D-mannosidase	ND	3.2
Sialidase	ND	ND
Sulphatase	11.8*	ND

*nmol release sulphate /h.

3.3 Glycan degrading bacteria

As can be seen from the chapters above ***Bacteroides*** species have a very versatile metabolic spectrum, which can explain their prevalence as dominant species in the colon. A Starch Utilization System (Sus), an organization of enzymes related to starch degradation, was identified and studied in *B. thetaiotaomicron* and this Sus complex appears to be a paradigm for glycan uptake in other *Bacteroides* species (Martens *et al.*, 2009) (Figure 1.8). Part of the Sus complex (SusCDEFG) is located at the cell surface where SusD, and likely also SusE and SusF, are responsible for the binding of starch molecules to the cell surface. SusG is an α-amylase that can hydrolyze the starch, after which the malto-oligosaccharides still bound to SusD, are translocated and released in the periplasm by SusC. There they are broken down by SusA and SusB to small saccharides, which are transported into the cytoplasm (Figure 1.8). This Sus-complex is a very efficient and selfish system that gives *B. thetaiotaomicron* an ecological advantage (Flint *et al.*, 2012a; Martens *et al.*, 2009). Also other Polysaccharide Utilization Loci (PULs), similar to the Sus complex, that are involved in the degradation of mucins, pectins and fructans have been discovered, making up 18% of the *B. thetaiotaomicron* genome. And in other *Bacteroides* species PULs have been

identified, involved in for example xylan and galactomannans degradation. Part of these Sus-like PULs is the ability to efficiently sense which substrate is available so the bacteria can adapt their glycan utilization to the nutrients present. It was shown that *B. thetaiotaomicron* changes its metabolism between dietary nutrient, host glycan or human milk oligosaccharide (HMO) degradation depending on the availability (Bjursell *et al.*, 2006; Mahowald *et al.*, 2009).

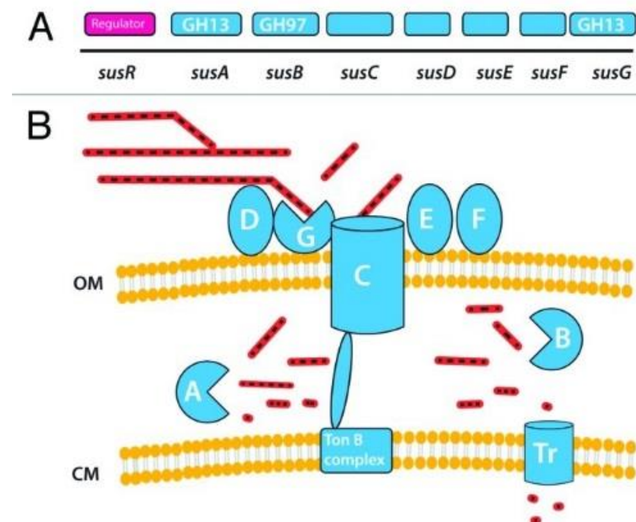

Figure 1. 8: *Bacteroides thetaiotaomicron sus* system. (A) shows order of genes in the *sus* cluster. (B) shows the organization and action of the gene products on or near the cell surface (OM outer membrane, CM cytoplasmic membrane). Starch molecules are shown as sugar chains, at various stages of hydrolysis. Figure derived from (Flint *et al.*, 2012a).

Within the ***Bifidobacterium*** genus, many genes are conserved between species, and of these conserved genes 6.5% was dedicated to carbohydrate metabolism (Bottacini *et al.*, 2010). But, despite this conserved bifido-genome, the substrate-specificity is species and strain dependent. *B. breve* and *B. adolescentis* are specialized in degradation of resistant starch while *B. bifidum* possesses specific enzymes related to degradation of galacto-oligosaccharides (Flint *et al.*, 2012a; Ryan *et al.*, 2006). Some bifidobacteria are unable to degrade any fructans (*B. bifidum* and *B. breve*), while others are able to use FOS or both FOS and short chain inulin (*B. adolescentis*). *B. longum* responded to a prebiotic treatment with long-chain arabinoxylans in humanized rats and the *B. bifidum* genome contains many genes dedicated to host glycan degradation (Turroni *et al.*, 2010; Van den Abbeele *et al.*, 2011a).

Within the **Firmicutes** phylum, *Lachnospiraceae* and *Rumminococcaceae* are the most abundant families accounting for 50-70% of the bacterial community in the fecal samples of healthy individuals. *Ruminococcus bromii* is a keystone species in the initial stages of particulate resistant starch degradation, demonstrating greater capabilities than even

B. thetaiotaomicron (Ze *et al.*, 2012) and *R. torques* possesses mucin-degrading capabilities (Hoskins, 1993). Within the *Lachnospiraceae* family, there are two important clusters of butyrate producing bacteria, *Clostridium* cluster IV and XIVa. Starch degradation is mostly achieved by member of the *Clostridium* cluster IV, for example *Roseburia inulinivorans*, which can also grow on FOS, inulin and on the sugar fucose, abundant in mucins (Scott *et al.*, 2008). Other inulin degraders are *Eubacterium rectale* (cluster IV) and *F. prausnitzii* (cluster XIVa) (Ramirez-Farias *et al.*, 2009). *F. prausnitzii* is also able to utilize N-acetyl glucosamine for growth, so it might be involved in the host glycan degradation food chain, together with *R. inulinivorans* (Lopez-Siles *et al.*, 2012).

However, these bacteria are not alone but part of a complex microbial community and their behavior will be different and dependent on the presence and activity of other bacteria.

3.4 Cross-feeding interactions

In the chapters above, mainly the **'primary degraders'** have been mentioned, the bacteria with the ability to degrade a wide range (*Bacteroides* spp.) or a more select range (*Bifidobacterium* spp.) of complex carbohydrates. However, there are many species that are not able to metabolize complex carbohydrate structures and grow on the fermentation products of primary degraders, and this is termed cross-feeding. Cross-feeding has been mainly described as the metabolic interactions between bifidobacterial species and butyrate producing species to explain the unexpected butyrogenic effects of some prebiotics observed *in vivo* (Riviere *et al.*, 2016) (Figure 1.9). To study this, bacteria were grown in co-culture on prebiotic substances and degradation and production activity was monitored. A first type of cross-feeding is based on the requirement of butyrate producing species like *R. intestinalis, R. inulinivorans* and *F. prausnitzii* for exogenous **acetate** to degrade oligofructose and produce butyrate. In co-culture experiments, acetate was provided by bifidobacterial growth on oligofructose, after which the oligofructose was further degraded by the combined efforts of both bifidobacterial and butyrate producing species (Falony *et al.*, 2009; Falony *et al.*, 2006; Moens *et al.*, 2016). Of course, in the more complex environment of the gut the exogenous acetate can be provided by many more species.

Another type of cross-feeding takes place when butyrate producing species not only need acetate but are also unable to degrade the available substrate. Bifidobacterial growth on oligofructose was shown to provide short chain **oligosaccharides**, like fructose, and **lactate** which were consumed by *R. hominis* and *Anaerostipes caccae*, respectively, and led to growth and butyrate production (Belenguer *et al.*, 2006). A third type of cross-feeding has been described for growth on arabinoxylan-oligosaccharides (AXOS), similar to the first type of cross-feeding where both bifidobacterial and butyrate producing strains are capable of degrading the substrate. But in this case the bifidobacterial strain is additionally stimulated by

consumption of the monosaccharides released by the metabolism of an *E. rectale* strain, which benefits from the acetate produced by *B. longum.*

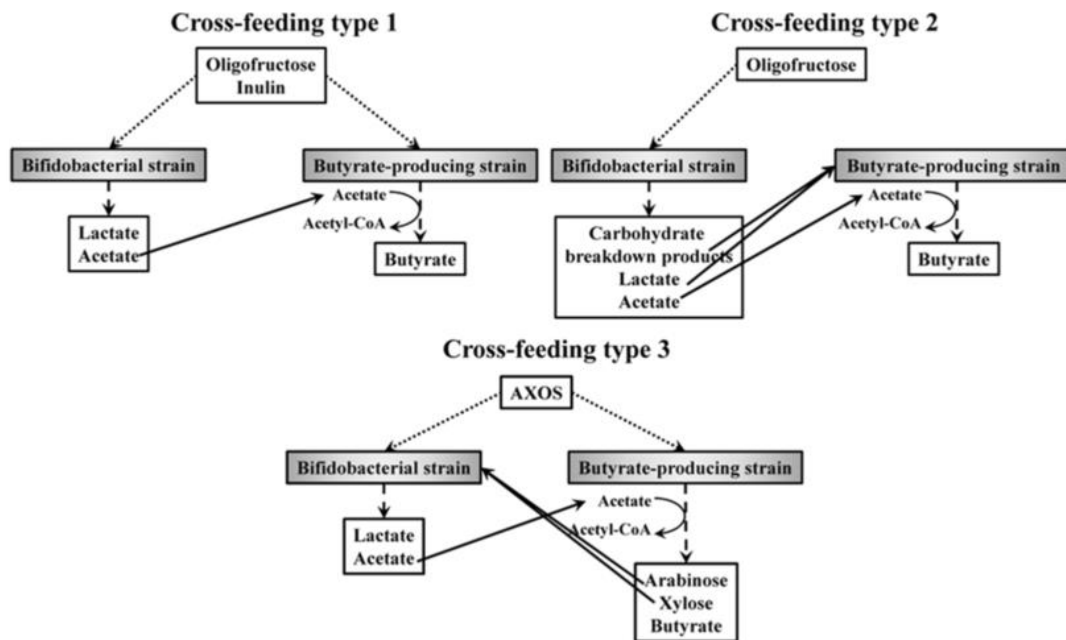

Figure 1. 9: Different types of cross-feeding that can take place between *Bifidobacterium* spp. and species of butyrate producing colon bacteria in the human colon. (….) indicate consumption of oligofructose, inulin and AXOS; (- - -) indicate production of carbohydrate breakdown products and/or metabolic end products; (–) indicate cross-feeding interaction between bifidobacterial strain and butyrate producing strains. Figure derived from (Riviere *et al.*, 2016).

In this last case there is actual cross-feeding, with both strains benefitting from products the other produced (Riviere *et al.*, 2015; Riviere *et al.*, 2016). Another example of such a bidirectional feeding interaction was observed between *A. muciniphila* and *Eubacterium hallii* when grown on mucin (Figure 1.10). The liberation of mucin derived oligosaccharides by *A. muciniphila* metabolism, could be used to sustain *E. hallii* growth and butyrate production. Besides butyrate, *E. hallii* also produced vitamin B12 which resulted in propionate production by *A. muciniphila.* Cross-feeding with *A. muciniphila* on mucin was also shown for *A. caccae* and *F. prausnitzii*, benefitting from both the released oligosaccharides and the produced acetate (Belzer *et al.*, 2017).

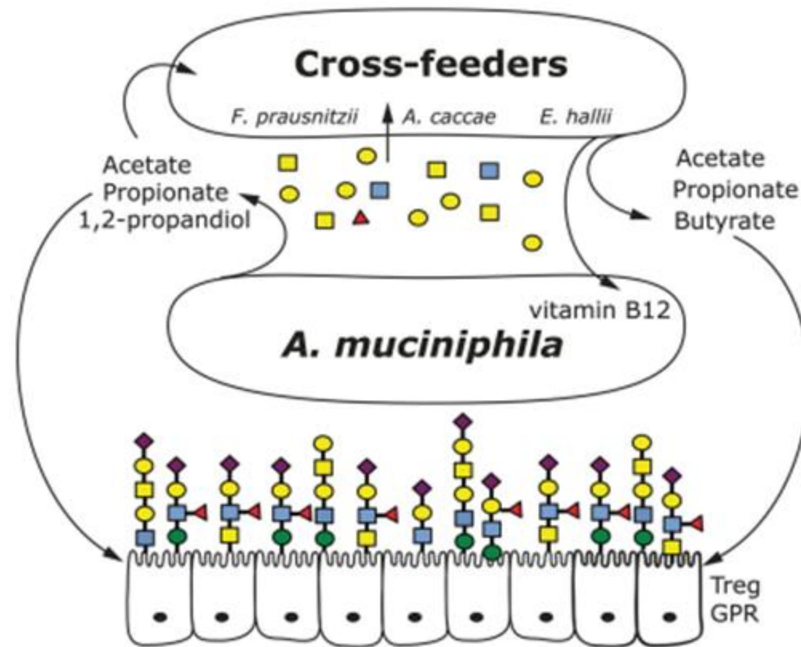

Figure 1. 10: Schematic overview of mucus-dependent cross-feeding network. Keystone mucolytic bacteria, such as *A. muciniphila*, degrade mucin glycans resulting in oligosaccharides (mainly, galactose, fucose, mannose and N-acetylglucosamine) and SCFA (acetate, propionate and 1,2-propanediol) that can be used for growth, as well as propionate, butyrate and vitamin B_{12} production by cross-feeding partners. Figure derived from (Belzer *et al.*, 2017).

Besides oligosaccharides and SCFA, also H_2 gas is used in important metabolic interactions. Hydrogenotrophic microorganisms are not abundant in the colon, but they perform a fundamental task for maintaining efficient microbial fermentation: the removal of H_2 gas, which is a byproduct of fermentation activity (Rios-Covian *et al.*, 2016; Stilling *et al.*, 2016). Acetogens, like *Blautia* spp., convert hydrogen into acetate by using CO_2. Methanogenesis, performed by archaea *Methanibrevibacter smithii*, is the conversion of H_2 into methane using CO_2 (Bik *et al.*, 2017). A third group of H_2 removers are the sulfate reducing bacteria (SRB), in the human gut represented by *Desulfovibrio* species. They convert H_2 to H_2S by using free sulfate as an electron acceptor and depend on other bacteria for both the H_2 and the free sulfate, which can come from mucins or food derived products (Rey *et al.*, 2013).

The above mentioned examples of cross-feeding, and many more, describe important metabolic interactions between gastrointestinal bacteria and indicate the difficulty of predicting the effect of a dietary treatment in a complex microbial community; because changes in activity or metabolism in one species will affect many others.

4. Maintaining host-microbe homeostasis

4.1 Modulation of the microbiome

Considering the various and far-reaching consequences of changes in microbial community composition and activity, there is a need to investigate ways to modulate the microbial community. A well-known way is by using antibiotics, but these are very disruptive and can have long lasting effects on the community homeostasis.

The most straightforward approach to modulate the gut microbiota is through **prebiotics**, which are defined as *'substrates that are selectively utilized by host microorganisms conferring a health benefit'* (Gibson *et al.*, 2017). Well-documented examples are fructo- and galactooligosaccharides, inulin and long-chain arabinoxylans that because of their difference in structure require different bacteria or bacterial consortia for degradation. Besides these well-studies carbohydrates, also other compounds such as polyphenols have been described to display prebiotic properties (Bindels *et al.*, 2015). The impact of prebiotic consumption has, until now, been mainly described for *Bifidobacterium* and *Lactobacillus* species (Riviere *et al.*, 2016). More recent studies however demonstrate that prebiotics stimulate many other bacteria as well and may not be as selective as previously assumed (Bindels *et al.*, 2015), since inulin type fructans can be consumed by some butyrate producing colon bacteria as well (Falony *et al.*, 2009; Falony *et al.*, 2006; Moens *et al.*, 2016; Riviere *et al.*, 2016) (As described above). Also cross-feeding interactions on these complex carbohydrate structures would lead to the involvement of many more species than initially thought. The fermentation of prebiotic compounds leads to production of specific SCFA, vitamins and other products (Graf *et al.*, 2015), which confer their own health effects toward the host. Due to their structural complexity, fermentation goes slowly and this prolongs the saccharolytic activities into to the distal colon, thereby reducing the production of toxic metabolites from protein and lipid metabolism (Grootaert *et al.*, 2009; Neyrinck *et al.*, 2011).

While prebiotics target the endogenous microbiota, it is possible that a dysbiosed community lacks the microorganisms that need to be targeted by prebiotics. An alternative way to modulate the microbiome is therefore a probiotic strategy. **Probiotics** are defined as '*live microorganisms which, when administered in adequate amounts, confer a health benefit on the host*' (Hill *et al.*, 2014; WHO/FAO, 2006). These probiotic bacteria can benefit the host through their presence, through their fermentation products or by affecting the resident bacteria (Scott *et al.*, 2015). At the moment, probiotic formulations are limited to *Bifidobacterium*, *Lactobacillus* species and other lactic acid bacteria or yeasts, usually delivered in a yoghurt, milk or cheese matrix (Besseling-van der Vaart *et al.*, 2016). The

evaluation of the "health benefit that is conferred to the host", as mentioned in the definition, is the responsibility of the European Food Safety Authority (EFSA). Since the implementation of EU legislation on health claims in 2009, only one claim has been approved: the benefit on lactose digestion when consuming live *Lactobacillus delbrueckii* subsp. *Bulgaricus* and *Streptococcus thermophiles* strains present in yogurt or fermented milk (El Hage *et al.*, 2017). More than 400 claim have been discarded since 2008, so while these products are available for consumption, no reference to the claimed health effect is allowed. Since the definition of a probiotic itself inherently suggests a health benefit, the term probiotic is banned for commercial purposes since 2012, to avoid the misleading of consumers. However in the scientific community, this research included, the term probiotic is still used, without the restrictions on the aspect of health benefits.

Instead of modulating the endogenous microbial community, it is also a possibility to remove the endogenous community and replace it with the fecal microbial suspension from a healthy donor, i.e. fecal microbial transplantation (FMT). This was already used in fourth-century China where this "yellow soup" was used to treat diarrhea. FMT has regained interest as is has proven to be effective in treating recurrent *Clostridium difficile* infections (van Nood *et al.*, 2013). However, in other pathologies with a more complicated etiology, like IBD, FMT has not yet been as successful (Colman and Rubin, 2014; Geirnaert, 2015).

4.2 Microbes with health promoting potential

The probiotic definition does not exclude microbial species coming from a more diverse phylogenetic background. Scientists have been searching for microbes that support the host-microbiome homeostasis either by supporting the normal microbial ecosystem, preventing it from tipping over towards a dysbiosed state or by fulfilling key functions for the host. Such microorganisms with key functionality and from phylogenetic origin other than conventional lactic acid producing microbes are being considered as next-generation probiotics. *Akkermansia muciniphila, Faecalibacterium prausnitzii*, *Butyricicoccus pullicaecorum, Eubacterium hallii, Bacteroides fragilis* and several others are being proposed. However, these next-generation probiotic candidates require a rigorous safety assessment and elucidation of their mode of action to persuade regulatory bodies to approve these bacteria or bacterial mixes as biotherapeutic agents. Two species, *Faecalibacterium prausnitzii* and *Akkermansia muciniphila*, both correlating with human health and being considered as the next generation probiotic, are discussed in more detail.

4.2.1 Faecalibacterium prausnitzii

Faecalibacterium prausnitzii is a gut bacterium, member of the Firmicutes phylum, *Clostridium leptum* group. It is abundantly present in healthy people, making up approximately 5% of the microbial community. It has been proposed as a sensor and actor in human intestinal health, due to its anti-inflammatory effects and its inverse correlation with IBD symptoms (Miquel *et al.*, 2013). Not only is it less abundantly present in patients suffering from inflammatory bowel diseases (IBD) but also irritable bowel syndrome (IBS), colorectal cancer (CRC), coeliac disease (CD) and obesity (Balamurugan *et al.*, 2010; De Palma *et al.*, 2010; Furet *et al.*, 2010; Neish, 2009; Rajilic-Stojanovic *et al.*, 2011; Sokol *et al.*, 2008b). Administration of *F. prausnitzii* or its culture supernatant was shown to be protective against chemically induced colitis in different rodent models (Miquel *et al.*, 2013; Sokol *et al.*, 2008b). The anti-inflammatory effects can be partly explained by the production of certain metabolites that could i) inhibit NF-kB activation and IL-8 production, ii) upregulate anti-inflammatory cytokine and Treg cell production and iii) improve intestinal barrier integrity. *F. prausnitzii* is able to produce acetate, D-lactate and formate but is mainly known for its high production of butyrate. Due to the inhibitory effect of butyrate on histon deacetylases in colonocytes and immune cells and butyrate interacting with G-protein coupled receptor 109A (GPR109A) on the surface of the colonocytes, butyrate exerts anti-inflammatory and anti-carcinogenic effects. It leads to a downregulation of pro-inflammatory cytokines, promotion Treg cells and IL-10 producing T-cells and selective induction of cell apoptosis (Guilloteau *et al.*, 2010).

Butyrate production by *F. prausnitzii* thus explains some of its protective effects in colitis, but also other factors have been identified that can contribute to its anti-inflammatory effects. A microbial anti-inflammatory molecule (MAM) protein (15 kDa) produced by *F. prausnitzii*, is able to inhibit the Nf-kB pathway and exert anti-inflammatory effects in DSS- and DNBS- induced colitis in mice (Breyner *et al.*, 2017; Quevrain *et al.*, 2016a; Quevrain *et al.*, 2016b). Additionally, the extra-polysaccharide matrix (EPM) produced by the biofilm producing *F. prausnitzii* HTF-F strain could reduce the production of pro-inflammatory IL-12 and this strain was shown to be more protective to DSS-induced colitis in mice compared to the type strain (Rossi *et al.*, 2015). *F. prausnitzii* or one of its secreted bioactive compounds may thus lead to the development of innovative therapeutic strategies for preventing or treating colitis.

4.2.2 Akkermansia muciniphila

Akkermansia muciniphila is an abundant member of the human gut microbiota (1-4%), using mucin as its sole carbon, nitrogen and energy source (Collado *et al.*, 2007; Tailford *et*

al., 2015a). It is the only intestinal member of the Verrucomicrobia phylum and its abundance has been linked with gut health in several human *in vivo* correlation studies (Collado *et al.*, 2007; Png *et al.*, 2010; Wang *et al.*, 2011; Zhang *et al.*, 2013).

A study of its genome shows 61 proteins predicted to be involved in mucin degradation (2.8% of all proteins) and its high mucin-degrading capacity in an *in vivo* mice study (Berry *et al.*, 2013; van Passel *et al.*, 2011). Mucins are the preferred substrate of *A. muciniphila*, yielding acetate and propionate as by-products of their fermentation (Derrien *et al.*, 2004). Acetate and propionate enter the portal vein more efficiently than butyrate, and exert their effects partly outside the gut. Acetate can be used as a substrate for fatty acid synthesis and cholesterol in the liver, increases colonic blood flow and oxygen uptake, and it is important to protect from enteric infections (Fukuda *et al.*, 2011). In contrast, propionate inhibits the incorporation of acetate into fatty acids and cholesterol and has been related with specific health benefits. Propionate induces satiety and it may therefore play an important role in energy homeostasis (Hosseini *et al.*, 2011; Nishina and Freedland, 1990; Ruijschop *et al.*, 2008). In monoculture, *A. muciniphila* produces acetate and propionate (60:40 molar ratio) from mucins, subsequently impacting the host genes involved in lipid metabolism (Hnf4α) and proliferation (Tp53 and Tp73) (Derrien *et al.*, 2004; Lukovac *et al.*, 2014).

A. muciniphila abundance has been negatively correlated with many diseases, but only for diabetes and obesity have there been studies showing a beneficial/protective effect of *A. muciniphila.* In a study with obese mice on a high-fat diet, it was shown that administration of *A. muciniphila* reversed insulin resistance, dyslipidemia, metabolic endotoxemia and fat mass gain (Everard *et al.*, 2013). Plovier *et al.* (2017) discovered that the beneficial effects are at least partly due to a specific outer membrane protein, Amuc_1100, which is involved in the formation of pili by *A. muciniphila*. This protein could be involved in the interaction between *A. muciniphila* and Toll-Like Receptor 2, which is an immunoregulatory protein that modulates intestinal homeostasis and host metabolism (Cani and de Vos, 2017). A study with CaCo-2 cell lines showed that *A. muciniphila* improved the integrity of the epithelial cell layer, suggesting its ability to strengthen an impaired gut barrier (Reunanen *et al.*, 2015). Metabolic endotoxemia results from impaired barrier function and by strengthening the gut barrier, *A. muciniphila* could reverse metabolic endotoxemia as described above (Everard *et al.*, 2013). Apart from oral administration of *A. muciniphila,* uptake of dietary compounds such as fish oil and cranberry extract also increased *A. muciniphila* abundances and led to healthier mice (Anhe *et al.*, 2015; Caesar *et al.*, 2015). However, other studies have shown increased abundance of *A. muciniphila* to be correlated with colon cancer and DSS-induced colitis, which might be explained by the overexpression of certain mucin types in colon cancer and DSS colitis and the reduced food intake in colon cancer (Berry *et al.*, 2012; Berry *et al.*, 2013; Borges-Canha *et al.*, 2015; Weir *et al.*, 2013a).

A. muciniphila shows great promise for use as a next generation probiotic or in obesity and diabetes therapies. However, questions remain regarding the effect of *A. muciniphila* administration on the resident microbial community, its dependency on mucin or is susceptibility to environmental changes in the colon environment.

4.3 Potential role of mucus in maintaining homeostasis

Besides diet derived substrates, also host derived substrates such as colonic mucins are to be considered as an opportunity for microbiota modulation. Given the complexity of the general mucin structure only about 1% of the gut microbiota is able to degrade mucin, but many more species may be indirectly involved since 90% of the mucin structure consists of specific carbohydrates, making it amenable to microbial fermentation and cross-feeding interactions. As mucin breakdown and fermentation will at least in part take place in proximity of the epithelial layer, putative health effects can be expected. Prebiotic action of mucins could potentially stimulate endogenous *A. muciniphila* or aid in the establishment of exogenous *A. muciniphila*, increasing the health promoting impact of this bacterium and inducing cross-feeding interactions with butyrate producing bacteria. However, the prebiotic potential of mucin in stimulating endogenous or probiotic *A. muciniphila* and the impact on the microbial ecosystem in the gut is poorly understood. The ecological function of host-glycan degradation and its position together with *A. muciniphila* in the microbial gut ecosystem remains to be investigated.

5. Objectives and outline of this research

Studies linking changes in the gut microbiota composition to human health status have reported an inverse correlation between *Akkermansia muciniphila* and disorders such as IBD, obesity and diabetes, while it is present at high abundances (1-4%) in the healthy human population. Key characteristic of *A. muciniphila* is its mucin degradation capacity, which leads to the production of acetate and propionate and may be part of cross-feeding networks resulting in butyrate production. *A. muciniphila* has been positioned as a health biomarker and is currently explored as a therapeutic agent for obesity or a new generation probiotic. However, more information is required about its behavior in the complex microbial ecosystem in the colon, about the potential role of mucins to influence *A. muciniphila* behavior and the impact of its probiotic administration on the microbial ecosystem and the host, which is the aim of this PhD research. *In vitro* technology used in this research, such as the simulator or the human intestinal ecosystem (SHIME®) and the transwell co-culture cell model, allowed for mechanistic research and helped to overcome some confounding elements of *in vivo* studies, such as variations in mucin availability.

Chapter 2 evaluated the colonization behavior of *A. muciniphila* in a mucin rich environment in the presence of a complex microbial community. To ensure efficient and abundant colonization of *A. muciniphila* a donor was selected with high amounts of *A. muciniphila* previously shown to successfully colonize the SHIME. Using this inoculum guaranteed that we could dynamically monitor its ecological behavior and investigate the impact of variable conditions on *A. muciniphila*, such as the stabilization period, differences in colon pH, prebiotic supplementation and variable mucin supply. Since this study explored the effect of mucin and pH towards *A. muciniphila* in one microbial background, **Chapter 3** aimed at testing the biological reproducibility of our previous findings. This enabled us to elucidate whether the gut microbial response and *A. muciniphila* sensitivity to changes in host-glycans and pH is dependent on the microbial background or not.

To study microbial cross-feeding and competition interactions of *A. muciniphila* more in detail, **Chapter 4** investigated different primary degraders for host or dietary glycan degradation and their effect on butyrate production. These interactions are difficult to study in a complex bacterial community and so a synthetic microbial community was used, with *A. muciniphila* and *B. thetaiotaomicron* as the primary glycan degraders.

Chapter 5 aimed at investigating the impact of *A. muciniphila* administration on the endogenous community and taking into account its nutritional specificity, treatment was investigated with and without addition of mucin. This allowed us to elucidate the importance of mucin presence to modulate the efficiency of the probiotic supplementation with *A. muciniphila*. At the end of these treatments, an antibiotic pulse was administered after

which the microbial community was allowed to recover. The goal was to establish whether treatment with either *A. muciniphila* and/or mucin would lend resilience towards an antibiotic induced disturbance or mediate a faster ecosystem recovery. Before this antibiotic pulse, supernatant samples were taken from these microbial communities shaped by the pro-, pre- and synbiotic treatments, to study their effect on the intestinal epithelium and the underlying immune cells in **Chapter 6**. By combining Caco-2 epithelial cell line with activated THP-1 cells (macrophages) this co-culture cell model offered the possibility to study exposure effect on epithelial barrier function and pro-or anti-inflammatory responses of the epithelium. The goal was to evaluate whether *A. muciniphila* addition and/or the presence of a host-glycan degradation niche changed the communities in such a way that would impact gut barrier function and immune response.

In **Chapter 7** an overview of the obtained research outcomes is given in combination with a general discussion of the research topic.

CHAPTER 2

In vitro colonization of the distal colon by *Akkermansia muciniphila* is largely mucin and pH dependent

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This chapter has been redrafted after

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CHAPTER 2

In vitro colonization of the distal colon by *Akkermansia muciniphila* is largely mucin and pH dependent.

Abstract

Host mucin is the main constituent of the mucus layer that covers the gut epithelium of the host, and an important source of glycans for the bacteria colonizing the intestine. *Akkermansia muciniphila* is a mucin-degrading bacterium, abundant in the human gut, that is able to produce acetate and propionate during this degradation process. *A. muciniphila* has been correlated with human health in previous studies, but a mechanistic explanation is lacking. In this study, the main site of colonization was characterized alongside additional conditions, such as differences in colon pH, prebiotic supplementation and variable mucin supply. A dynamic *in vitro* gut model, the Simulator of the Human Intestinal Microbial Ecosystem (SHIME®) was used to perform this in-depth exploration of the ecological behavior of *A. muciniphila* in one biological environment and to overcome the limitations of in vivo studies. *A. muciniphila* was found to colonize the distal colon (±8 log copies mL^{-1}) more abundantly than the proximal colon (±4 log copies mL^{-1}) and this colonization pattern was pH-dependent. The addition of mucin caused a specific increase of *A. muciniphila* (±4,5 log increase over two days), far exceeding the response of any other bacteria present, together with an increase in propionate. Our results indicate the preference of *A. muciniphila* for the distal colon environment due to its higher pH and uncovered the quick and stable response of *A. muciniphila* to mucin supplementation.

1. Introduction

Over recent decades, multiple correlations have been established between human health and the composition of the gut microbiota (Round and Mazmanian, 2009). *Akkermansia muciniphila* is a commensal gut bacterium that represents 1-3% of the total microbiota (Derrien *et al.*, 2008) and has been associated with beneficial health effects in several studies. It was shown to be absent in patients suffering from inflammatory bowel disease (IBD), while being abundantly present in healthy individuals (Png *et al.*, 2010). Lower *A. muciniphila* numbers have been encountered in patients with obesity, autism and type 2 diabetes (Wang *et al.*, 2011; Zhang *et al.*, 2009; Zhang *et al.*, 2013), and the initial correlation with type 2 diabetes (Qin *et al.*, 2012) could be attributed to the confounding effect of metformin, which stimulates the growth of *A. muciniphila* (Forslund *et al.*, 2015; Lee and Ko, 2014; Shin *et al.*, 2014). *A. muciniphila* supplementation reversed fat mass gain, metabolic endotoxemia, adipose tissue inflammation, and insulin resistance in obese mice that received a high-fat diet (Everard *et al.*, 2013). Not only probiotic treatment with *A. muciniphila* but also treatment of mice with dietary compounds such as cranberry extract and fish oil, rich in polyphenols and omega-3 fatty acids respectively, resulted in higher abundances of *A. muciniphila* and metabolically healthier mice/phenotypes (Anhe *et al.*, 2015; Caesar *et al.*, 2015). The direct impact of *A. muciniphila* on the host, its interactions with other beneficial intestinal microbes and its susceptibility to prebiotics remain to be elucidated.

Mucins are the preferred substrate of *A. muciniphila*, yielding acetate and propionate as by-products of their fermentation (Derrien *et al.*, 2004). Mucin glycans constitute 80% of the dry weight of the mucus layer that covers the intestinal epithelium (Johansson *et al.*, 2008). They are composed of O-glycosylated protein backbones, with chains of 2 to 12 monosaccharides, mostly galactose, fucose, N-acetylgalactosamine, N-acetylglucosamine, mannose and sialic acid. They may also be N-glycosylated with the same compounds to a lesser extent (Lai *et al.*, 2009). Mucins are not only confined to the mucus layer but are also present in the luminal content, due to the continuous mucin production, roughly between 6 and 15 g per day, and the constant mucus desquamation (Atuma *et al.*, 2001; Faure *et al.*, 2002; Johansson, 2012; Wilson, 2005). The degradation of host- and diet-derived glycans results in production of short-chain fatty acids (SCFA), mainly acetate, propionate and butyrate. Butyrate serves as an energy source for colonocytes, while it may also protect from inflammatory disorders and suppress the growth of colonic tumors (Guilloteau *et al.*, 2010). Acetate and propionate enter the portal vein more efficiently than butyrate. Acetate can be used as a substrate for fatty acid synthesis and cholesterol in the liver, increases colonic blood flow and oxygen uptake, and it is important to protect from enteric infections (Fukuda *et al.*, 2011). In contrast, propionate inhibits the incorporation of acetate into fatty acids and

cholesterol and has been related with specific health benefits. Propionate induces satiety and it may therefore play an important role in obesity (Hosseini *et al.*, 2011; Nishina and Freedland, 1990; Ruijschop *et al.*, 2008). In monoculture, *A. muciniphila* produces acetate and propionate (60:40 molar ratio) from mucins, which impacts the host genes involved in lipid metabolism (Hnf4α) and proliferation (Tp53 and Tp73) (Derrien *et al.*, 2004; Lukovac *et al.*, 2014). Although multiple studies associate *A. muciniphila* with human health indicators (Anhe *et al.*, 2015; Belzer and de Vos, 2012; Png *et al.*, 2010; Santacruz *et al.*, 2010; Schneeberger *et al.*, 2015; Swidsinski *et al.*, 2011; Wang *et al.*, 2011; Zhang *et al.*, 2009), the specific factors that govern *A. muciniphila* colonization within a mixed community and its contribution to the overall acetate, propionate and/or butyrate production have not been addressed.

Production of mucin by the host epithelium is dependent on specific food ingredients (Van den Abbeele *et al.*, 2011a), drugs (Wlodarska *et al.*, 2011) or disease states (Fyderek *et al.*, 2009), thus interfering with the abundance of mucin degraders. For example, consumption of prebiotics was shown to shift mucin degradation to the distal colon (Van den Abbeele *et al.*, 2011a). Therefore, sampling along different sites of the intestine becomes essential for understanding the ecological behavior and colonization potential of mucin degraders in the gut. Also, in patients with Crohn's disease and ulcerative colitis the mucus layer is three times thinner than in healthy people (Fyderek *et al.*, 2009) and antibiotic treatment with metronidazole resulted in a thinner inner mucus layer (Wlodarska *et al.*, 2011). These are confounding factors that can be overcome with dynamic *in vitro* gut models, like the SHIME®, that exclude the host environment and allow simulating different sites of the intestine. Such *in vitro* models provide a great opportunity for mechanistic research that aims at unraveling the ecology of mucin degraders, such as *Bacteroides* sp. and *A. muciniphila*, which have been shown to thrive in these models (Van den Abbeele *et al.*, 2010).

The aims of this study were to evaluate the colonization behavior of *A. muciniphila* in a mucin rich environment in presence of a complex microbial community. Since this research serves as a start to characterize *A. muciniphila,* a donor was selected with high amounts of *A. muciniphila* previously shown to successfully colonize the SHIME (Van den Abbeele *et al.*, 2013; Van den Abbeele *et al.*, 2010; Van den Abbeele *et al.*, 2011g). Hence this inoculum guaranteed that we could see the effects of the variable conditions, such as the stabilization period, differences in colon pH, prebiotic supplementation and variable mucin supply, on *A. muciniphila* and dynamically monitor its ecological behavior. However, this only provides us with results from one microbial background and further research considering multiple donors, will need to be performed to investigate the effect of the microbial parameter on

A. muciniphila. We assessed the overall microbial and metabolic changes associated with evolving numbers of *A. muciniphila.*

2. Materials and methods

2.1 Chemicals and growth media

Chemicals were obtained from Sigma (Bornem, Belgium), unless stated otherwise. The nutritional medium for the SHIME consisted of (in g L^{-1}) arabinogalactan (1.0), arabinoxylan (2.0) (BioActor, Maastricht, The Netherlands), starch (2) (Anco, Roeselare, Belgium), xylan (1.0), pectin (2.0), D-(+)-glucose (0.4), yeast extract (3.0), peptone (1.0), cysteine (0.5) and commercial pig gastric mucin (4.0). Composition of pig gastric mucin is typically around 20% hexosamine, 18% total hexose, 48% protein, and 9% sialic acid. The monosaccharide composition of mucin was determined (in g 100 g^{-1} DM): L-arabinose (0.05), D-xylose (0.04), D-mannose (0.28), D-galactose (7.47) and D-glucose (1.95). This medium was autoclaved and acidified to pH 2.0. The pancreatic juice contained (in g/L) $NaHCO_3$ (12.5), bile salts (6.0) (Difco, Bierbeek, Belgium) and pancreatin (0.9).

Our previous experience with a fecal microbial inoculum from a 28-year old male individual (Van den Abbeele *et al.*, 2013; Van den Abbeele *et al.*, 2010; Van den Abbeele *et al.*, 2011g) showed that *A. muciniphila* colonization was consistently abundant and efficient. Hence, this was an ideal inoculum to subject *A. muciniphila* to variable conditions and dynamically monitor its ecological behavior. Fecal samples were collected and prepared within 1h according to standard procedures (Molly *et al.*, 1993). In short, aliquots (20 g) of freshly voided fecal samples were diluted and homogenized with 100 mL 0.1 M phosphate buffer (8.8 g K2HPO4.L^{-1} and 6.8 g KH2PO4.L^{-1}, pH 6.8) containing 1 g.L^{-1} sodium thioglycolate as reducing agent. After removal of the particulate material by centrifugation (2 min, 500 g) the fecal suspension was used as inoculum.

2.2 Long term dynamic *in vitro* gut model for the luminal colon microbiota (SHIME)

The long-term colonization of *A. muciniphila* within a mixed human gut microbiota was assessed in the dynamic *in vitro* gut model, SHIME® (ProDigest-Ghent University, Ghent, Belgium). The model consists of five compartments that simulate the stomach, the small intestine and three or two colon regions (namely ascending, transverse and descending colon or proximal and distal colon, respectively) (Van den Abbeele *et al.*, 2010). Each anaerobic compartment was continuously stirred at 37°C and flushed with N_2 (15 min/day) to ensure anaerobic conditions after sampling. On day 0, the colon compartments were filled with nutritional medium and inoculated with 40 mL of 20% (w/v) fecal slurry. Following an

overnight static incubation of the colon compartments (16 h), the stomach and small intestine compartments operate on the fill and draw principle, with peristaltic pumps adding 140 mL nutritional medium and 60 mL pancreatic juice three times a day and gradually emptying the small intestine compartment into the colon compartments after gastro-intestinal digestion. The volume in the colon compartments is kept constant by the simultaneous fluid flow in and out of compartments (Possemiers *et al.*, 2004). Samples were taken from the vessels, daily (10h00) before new feed entered the colon compartments.

By applying relevant environmental conditions (retention time, nutrition and pH) to each colon region, the fecal microbiota evolves to establish region-specific microbial communities. This stabilization process requires two weeks and it is reproducible for SHIME-units that are run simultaneously (Van den Abbeele *et al.*, 2010).

Samples from a previous experiment were analyzed to provide the preliminary data for the experiments performed in this study (Van den Abbeele *et al.*, 2010). *A. muciniphila* colonization was studied in the three colon regions of the SHIME, using *A. muciniphila*-specific primers and qPCR on samples of a published SHIME-study using the same donor (Van den Abbeele *et al.*, 2010). Samples were collected on day 19 and 26 after inoculation from two stable SHIME-units run simultaneously. The net SCFA production was determined to link the SCFA production to the numbers of *A. muciniphila* in specific colon regions. As steady conditions prevailed upon stabilization, the net SCFA production equals to the difference between the concentrations in subsequent colon regions.

Three SHIME experiments were performed in this study. For the purpose of the first one, the "**stabilization experiment"**, investigating the stabilization of *A. muciniphila*, the SHIME set-up consisted of two colon compartments, proximal and distal (Supplementary Figure 2.1A). The second one, the "**pH and inulin experiment",** focusing on pH and prebiotics, consisted of three parallel distal colon vessels at different pH intervals: 6.6 to 6.9 (Distal-high pH, Dh), 6.15 to 6.4 (Distal-medium pH, Dm) and 5.6 to 5.9 (Distal-low pH, Dl) (Figure 2.1A). The same proximal colon vessel (PC) was used to feed all three to ensure identical nutritional conditions in the different distal colon vessels. After 11 days of normal nutritional feeding, 5 g L^{-1} of inulin were supplemented to the feed. This allowed the investigation of the prebiotic effect of inulin in the proximal colon and in the distal colon at different pH intervals. For the third one, the "**Mucin experiment"** evaluating the effect of mucin supplementation, the set-up of the experiment consisted of one proximal (PC) and three parallel distal colon vessels (Distal 1-3) (Figure 2.6A). After supplementing a mucin-free nutritional medium during the first 10 days, 8 g L^{-1} of mucin were delivered to the proximal colon vessel on day 10, and from day 12 to 15, 4 g L^{-1} mucin were supplied.

A. muciniphila numbers were quantified with qPCR and mucin and SCFA concentrations were determined (cfr. supra). Based on qPCR results of the pH and mucin

experiment, respectively 32 and 20 samples of the most important time points , were characterized using next-generation sequencing (cfr. supra).

2.3 Microbial community analysis

The DNA extraction procedure was adapted from Boon *et al.* (2003) with modifications to increase the release of DNA from microbial cells. 1% SDS was added during the first extraction step and mechanic lysis was performed. Copy number of the16S rRNA gene of *A. muciniphila* was estimated by quantitative PCR on 10-or 100-fold diluted DNA, using specific primers for *A. muciniphila* (AM1 and AM2) (Collado *et al.*, 2007). Primer concentration was 300 nM. Standard curves were constructed with serial dilutions (10^2 to 10^8 copies.μL^{-1}) of plasmid DNA from clones of *A. muciniphila*. PCR was performed to amplify the plasmid containing the sequence insert using described protocols (Collado *et al.*, 2007). The specificity of all primers was verified by the amplification of amplicons of the correct size from the target products in all samples. Quantitative PCR was performed in a StepOnePlus Real-Time PCR system (Applied Biosystems, Ghent, Belgium) using *Power* SYBR® Green PCR 2X Master Mix (Applied Biosystems, Ghent, Belgium) according to the manufacturer's instructions. The results were expressed as log copies mL^{-1} of initial sample.

Biodiversity was analyzed using Illumina high throughput sequencing (MiSeq, Illumina, Hayward, CA,USA). The V5-V6 hypervariable region of the 16S rRNA gene was amplified using primers 807F and 1050R (Bohorquez *et al.*, 2012). Libraries were prepared by pooling equimolar ratios of amplicons (200 ng of each sample), and tagged with a unique barcode (Camarinha-Silva *et al.*, 2014). Resulting libraries were sequenced on a Miseq (Illumina, Hayward, CA, USA) using 250 bp single-end sequencing chemistry. Single reads were trimmed to 120 nucleotides and quality filters were performed as previously described (Camarinha-Silva *et al.*, 2014). Samples from both experiments were analyzed separately. From the pH experiment (32 samples) and the mucin experiment (20 samples), 905284 and 544294 operational taxonomic units (OTUs), respectively, were retrieved and were clustered into 132 unique taxa. Sequence composition was compared using the RDP Classifier tool (Wang *et al.*, 2007)(Wang *et al.*, 2007) and SILVA database (Pruesse *et al.*, 2007). Data were randomly subsampled to the sequence count of the sample with the lowest sequence count using the function rarefy_even_depth from the phyloseq package from R (McMurdie and Holmes, 2013); relative abundances of the top twelve taxa, with their deepest possible RDP classification up to the family level were determined (Kerckhof *et al.*, 2014). Rarefaction curves, richness and biodiversity indices were obtained with the vegan package in R (Oksanen *et al.*, 2011). Sequences are deposited and publically available in the European Nucleotides Archives under the accession numbers LN832064-LN832188.

2.4 Metabolic activity analysis

For mucin quantification, SHIME samples were diluted in PBS in a 1:20 ratio and determined fluorimetrically, as described by Bovee-Oudenhoven *et al.* (1997). Briefly, oligosaccharides were liberated from mucin via β-elimination with dilute alkali and subsequently reducing ends were derivatized with 2-cyanoacetamide. Mucin levels are expressed as mM oligosaccharide equivalents using standard solutions of N-acetylgalactosamine.

Acetate, propionate, butyrate, valerate, caproate, isobutyrate, isovalerate and isocaproate were measured as described previously (Andersen *et al.*, 2014). Data were analysed using the SPSS 19 software (SPSS Inc., Chicago, USA). Normality was determined with a Kolmogorov-Smirnov test before investigating probability of intergroup differences. Significant differences between treatments were detected using one-way ANOVA or the non-parametric Mann-Whitney U test in case of non-normality. Post hoc analysis was performed using the Bonferroni correction for equal variances or Dunnett test when variances were assumed to be different., Pearson correlation coefficients were calculated to investigate the relation between the SCFA and *A. muciniphila*. Significance was set at 0.05.

2.5 Multivariate statistical analysis

Multiple Factor Analysis (MFA) was employed to assess the variations on the relative bacterial abundances in the pH experiment on each SHIME vessel at different pH, following inulin supplementation. MFA was employed to simplify the data by reducing the dimensionality of the dataset encompassing the bacterial families and the qualitative descriptors (pH and sampling time). In this way, MFA allowed for balancing the influence of each group of bacterial families, for investigating the associations between time and pH, and to produce a representation of the individual samples grouped according to their similarities regarding the relative bacterial abundances. Bacterial abundances were weighted on a global PCA and results were explained in a factor map (de Tayrac *et al.*, 2009), where the value of the abundance of each bacterial family (vector) for the corresponding pH (factor) was plotted (Grunert *et al.*, 2016). The function MFA from the FactoMineR package (Le *et al.*, 2008) was performed in R. Parametric bootstrapping was applied to construct confidence ellipses around the barycentre of the abundances on each SHIME vessel to visualize whether the bacterial abundances were significantly different among colon vessels. Confidence intervals of the average coordinates of the bacterial abundances were represented by the ellipses, indicating 95% of similarity among bacterial abundances. If the ellipses were not overlapping, the bacterial abundances were significantly different;

incomplete overlap indicated that bacterial abundances were significantly different in the samples outside the ellipse (Dehlholm *et al.*, 2012). Hence, non-overlapping confidence "ellipses" denoted that two vessels differed at significant level ($P > 0.05$).

Correspondence analysis (CA) using the Single Value Decomposition (SVD) highlighted the variations in relative bacterial abundance in the mucin experiment of the 3 distal colon vessel on the SHIME pre- and post-mucin treatment. Computations were performed in the R language, using the ca package (Nenadic and Greenacre, 2007). The percentages of explained variance (inertias) for the bacterial abundances (rows) and time point (columns) on each dimension of the CA were determined. Total variance was explained in 4 factors or dimensions. The graphical display of the CA showed relationships among the relative abundances of specific bacterial groups (Hernandez-Sanabria *et al.*, 2013). The area of the point symbols plotted in the CA indicates the mass. Masses are the marginal proportions of the relative bacterial abundances (row variable). These proportions are used to weight all the bacterial abundances when computing the distance between the abundance of an "x" bacterial family to the centroid of the abundance of all bacterial families. This weighting has the effect of compensating for unequal numbers of cases (Nenadic and Greenacre, 2007). Therefore, the area of a point in the graph will indicate the relative contribution of a particular bacterial family to the total variance. The color intensity of the point symbol and that of the arrow are proportional to the absolute contribution of the points in terms of a percentage of explained variance. Darker colors of the arrows indicate higher percentages of variance attributed to a particular time point. Point symbols with darker color represent the bacterial abundances that contributed more to the variance.

3. Results

3.1 Stabilization of *A. muciniphila* in the colon environment

We previously demonstrated an 8-fold increase of *A. muciniphila* in the complex microbial community of the distal colon regions (transverse and descending) compared to the proximal colon by applying a phylogenetic microarray (HITChip) on consecutive colon regions of a stabilized SHIME-model (19-26 days after inoculation with a fecal sample) (Supplementary Table 2.1) (Van den Abbeele *et al.*, 2010). QPCR analysis of *A. muciniphila* confirmed that *A. muciniphila* increased in the distal colon regions (>10^5–fold increase), with no difference between transverse and descending colon compartments (P=0.102) (Supplementary Table 2.1). During the stabilization experiment, we monitored *A. muciniphila* copy number and mucin degradation upon inoculation of the *in vitro* gut model with a human fecal sample to gain insight in the temporal patterns of *A. muciniphila* colonization in the distal colon (Supplementary Figure 2.1B-C). Initially (day 0-3), *A. muciniphila* increased in both the proximal and the distal colon region, coinciding with efficient mucin degradation in both compartments. Starting from day 3 after inoculation, *A. muciniphila* washed out from the proximal colon, as measured with qPCR and mucin started to accumulate in this colon region. At the same time, *A. muciniphila* abundantly colonized the distal colon, which was characterized by almost complete mucin degradation. *A. muciniphila* numbers stabilized 6 days after inoculation.

3.2 Effect of pH and inulin on *A. muciniphila* in the distal colon

As previous experiments showed that *A. muciniphila* proliferates in the distal but not the proximal colon compartment of the SHIME (Van den Abbeele *et al.*, 2010), we focused on the distal colon during the pH experiment to evaluate the effect of pH on *A. muciniphila* colonization (Figure 2.1A). Quantitative PCR analysis confirmed that *A. muciniphila* was present the proximal colon compartment, yet at abundances close to the detection limit (Figure 2.1B). Moreover, a strong pH-dependent colonization of the distal colon was observed; the average abundance over time was measured at 7.98 ± 0.07 log copies ml^{-1} with pH between 6.15-6.4 (Dm) and 8.35 ± 0.05 log copies ml^{-1} at pH 6.6-6.9 (Dh). Despite the identical nutritional conditions in the distal colon region with low pH (5.6-5.9), *A. muciniphila* numbers decreased dramatically to around the detection limit (= 4 log copies ml^{-1}), similar to the abundances in the proximal colon. *A. muciniphila* thrived at neutral pH values. Further, prebiotic treatment with inulin at day 11 did not significantly affect *A. muciniphila* numbers (Supplementary Table 2.2).

SCFA analysis revealed that the distal colon regions at higher pH (Dh/Dm) contained high acetate, propionate and initially also valerate/branched SCFA levels and low butyrate levels (Figure 2.2). Administration of inulin resulted in increased acetate and butyrate in comparison with levels of branched SCFA in the proximal colon and a slight increase in butyrate and propionate levels in the distal colon vessels.

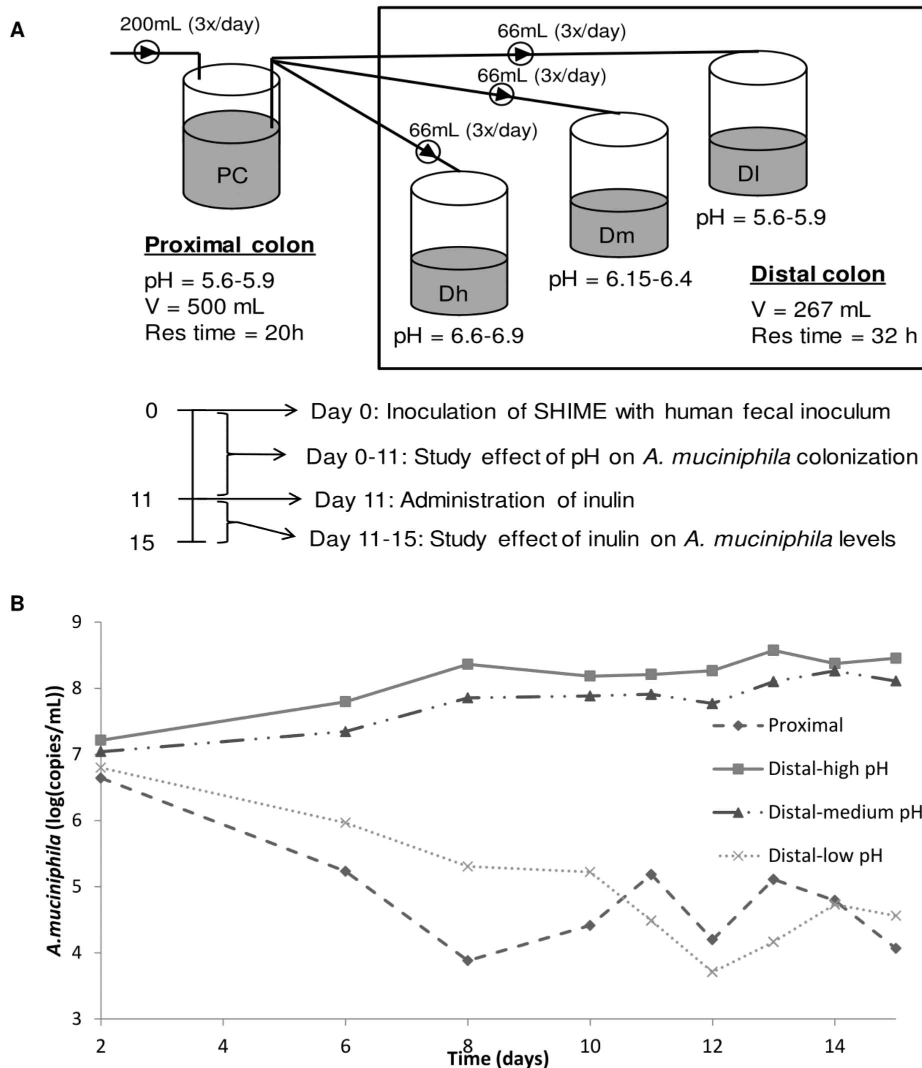


Figure 2.1: pH and inulin experiment. (A) Experimental set-up of the SHIME run to investigate the effect of pH on the colonization of *A. muciniphila.* After receiving normal nutritional medium during the first 11 days, 5g L^{-1} inulin was supplemented to the feed from day 11 until day 15. (B) *A. muciniphila* abundance (log (copies ml^{-1})) measured with qPCR as a function of time after inoculation (days) for the proximal colon (PC) and the three distal colon regions on different pH values: Distal-high pH (6.6-6.9), Distal-medium pH (6.15-6.4) and Distal-low pH (5.6-5.9). Technical variation was never higher than 3%.

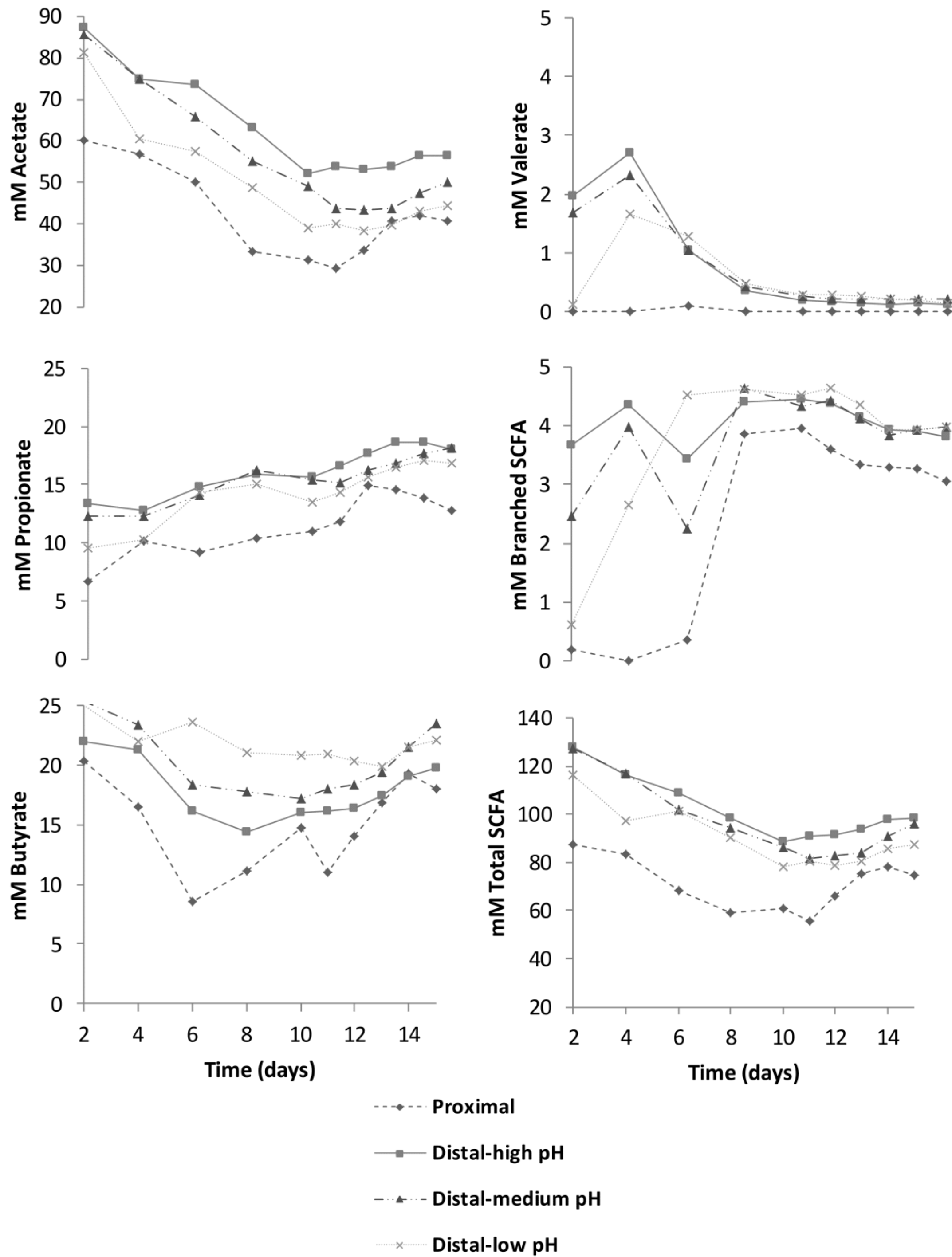


Figure 2.2: Short chain fatty acid (SCFA) concentration (mM) as a function of time after inoculation (days) in the different colon regions, proximal and distal with high, medium and low pH. Inulin (5g L^{-1}) was supplemented to the feed from day 11 until day 15. Distal-high pH (6.6-6.9), Distal-medium pH (6.15-6.4) and Distal-low pH (5.6-5.9).

3.3 Microbial composition analysis of the colon environment

Illumina sequencing showed high relative abundance of *A. muciniphila* in the distal colon with medium and high pH, compared to the distal colon with low pH and the proximal colon, confirming the qPCR results (Figure 2.3A). This increase of *A. muciniphila* is at the expense of several other species like *Clostridium* sp. and *Bacteroides* sp. and coincides with higher relative abundances of *Alistipes*, *Parabacteroides* and *Bilophila* species (Figure 2.3). It is remarkable that the differences in the pH lead to such a strong response in one species, *A. muciniphila,* compared to the other species in this complex microbial community.

Multiple factor analysis was employed to interpret how the differences in pH impacted the bacterial abundances in each region of the colon and whether the relative abundances remained stable before and after the supplementation with inulin (Figure 2.3B). The goal of this analysis was to discriminate whether the relative abundances of each colon fraction were similar among them. In the figure the colored symbols, representing the bacterial abundances in the different environments at different time points, were plotted in a two dimensional space. The variables described on each dimension are included in Supplementary Table 2.3. The first dimension comprised of the relative bacterial abundances associated with day 6 in particular, and in the distal colon with high pH, while the second dimension explained the relative abundances in the distal colon on day 10 (Supplementary Table 2.3). These two dimensions accounted for 54% of the differences among relative abundances on the different locations and time points. The third dimension included the relative abundances that are correlated with days 14 and 15 and it accounts for 14% of the total variance. It may be suggested that the first dimension describes the initial colonizers, because it is significantly correlated with day 6, whereas the second dimension explains the genus correlated with high pH (distal colon) and the third dimension describes the genera correlated with the inulin treatment, because day 14 and 15 are comprised in this dimension. Genera that are positively correlated with the second dimension are *Alistipes, Bilophila* and *Akkermansia* and with the third dimension are *Lactobacillus, Succiniclasticum, Propionispora* and *Lachnoclostridium* whereas *Bacteroides* is negatively correlated with this dimension (Supplementary Table 2.3). The fourth dimension included the genera correlated with location with low pH (distal colon).

At day 6, the bacterial abundances are different between locations, which can be due to the initial adaptation to pH and other environmental conditions (Figure 2.3B). Proximal and distal colon (with low pH) partially overlap, which indicates that the relative bacterial abundances are similar in the time points inside the overlapping area. The partial overlap may be explained by the pH, which is low in both locations. The area of the confidence ellipse is smaller in the high pH, indicating that the relative abundances in high pH tended to

be not significantly different (Figure 2.3B). MFA showed that in the distal colon with higher pH, the relative abundances on day 8 and 10 (pre-inulin) are significantly different from the relative abundances on day 14 (post-inulin). However, the effect was not consistent when pH was low.

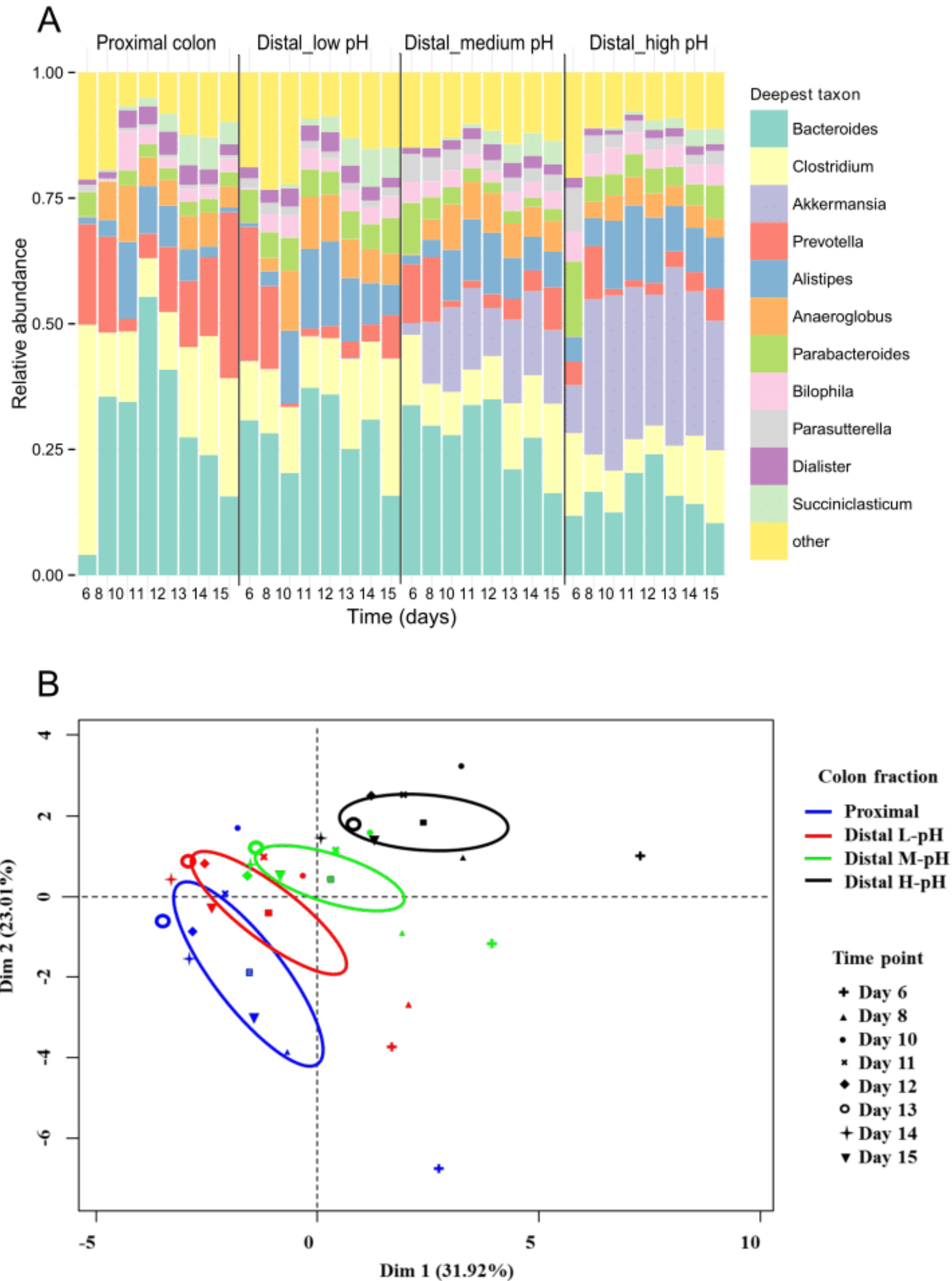


Figure 2. 3: Illumina sequencing results of the pH and inulin experiment. Inulin (5g/L) was supplemented to the feed from day 11 until day 15. Distal-high pH (6.6-6.9), Distal-medium pH (6.15-6.4) and Distal-low pH (5.6-5.9). (A) An overview of the relative abundance (% of the total community) of the 11 most abundant genera in the different colon vessels for the different time points (days). (B) Multiple Factor Analysis (MFA) was employed to assess the relative bacterial abundances detected in each SHIME vessel and for the different time points based on the Illumina data from the different samples ($p<0.05$).

3.4 The effect of variable mucin concentration in the colon environment on *A. muciniphila* abundance

To further unravel the behavior of *A. muciniphila* in a complex microbial community, the effect of mucin presence was investigated. In this mucin experiment, the aim was to wash out *A. muciniphila* from the distal colon compartment by feeding mucin-free nutritional medium to the SHIME (Figure 2.4A). Based on qPCR, *A. muciniphila* decreased during administration of mucin-free feed but did not wash out entirely from the distal colon at day 10 (Figure 2.4B). The initial high numbers are probably caused by growth on mucins present in the inoculum. Upon the supplementation of mucin, *A. muciniphila* numbers increased (3.03 ± 0.13 log copies mL^{-1} from day 10 to 11) in all distal colon regions (P=0.0006) but not in the proximal colon (P=0.3) (Figure 2.4B). There was a distal increase of acetate, propionate, butyrate, valerate and branched SCFA upon addition of mucins (Figure 2.5). Only acetate, valerate and especially propionate increased in the distal colon compartment when accounting for the proximal SCFA production, while butyrate and branched SCFA were already produced in the proximal colon compartment. The *A. muciniphila* increase in the distal colon on day 11 thus correlates with increased propionate levels. As can be seen from the qPCR and SCFA results, the three distal colon vessels were stable throughout the experimental period.

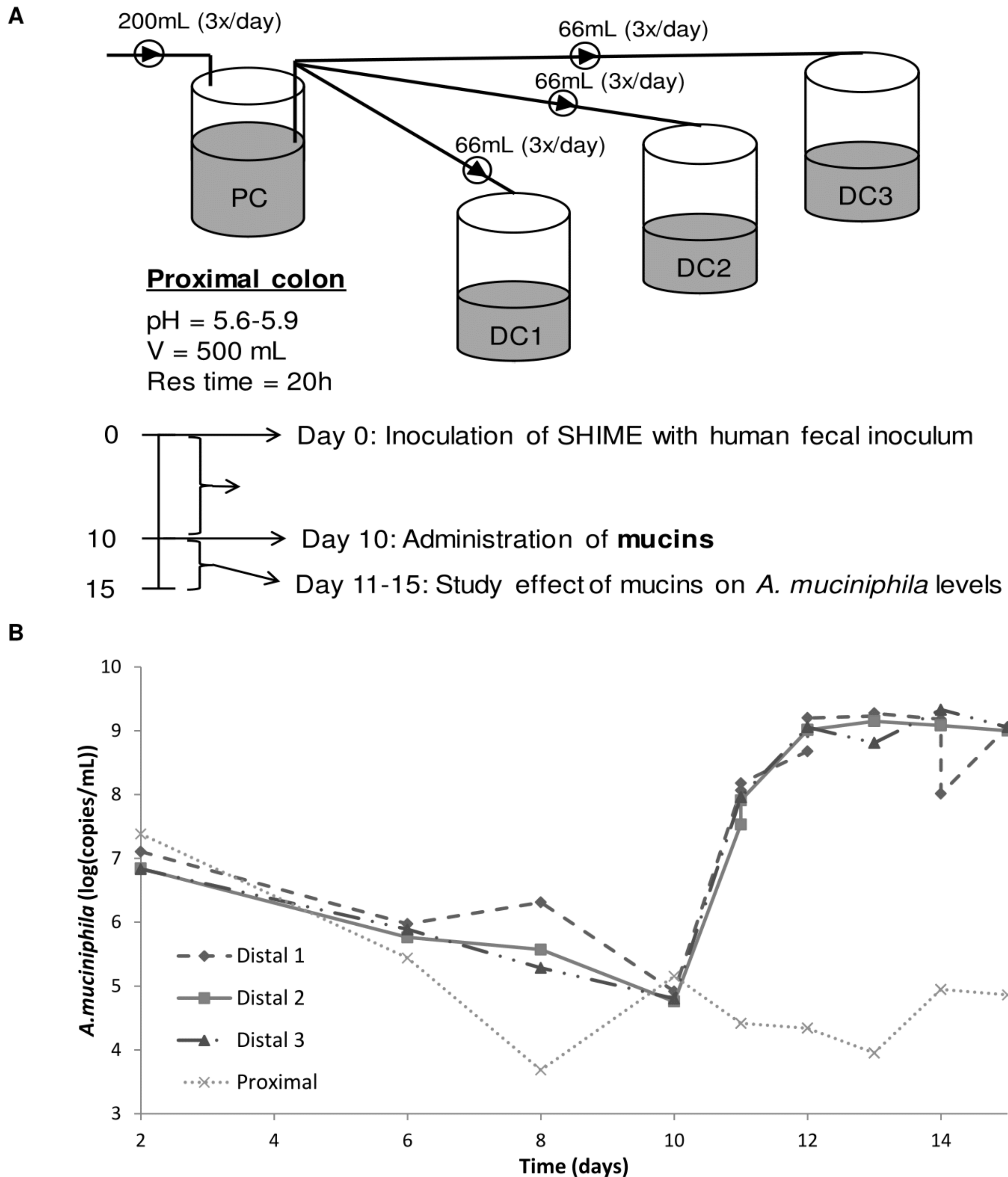


Figure 2. 4: Mucin experiment. (A) Experimental set-up of the SHIME run to investigate the effect of mucin depletion and administration on the colonization of *A. muciniphila.* After receiving mucin depleted nutritional medium during the first 10 days, 8g/ L^{-1} was dosed to the proximal colon on day 10, while 4g L^{-1} was dosed from day 12-15. (B) *A. muciniphila* abundance (log (copies mL^{-1})) measured with qPCR as a function of time (days) after inoculation for the proximal colon and the three replicate distal colon regions (Distal 1-3). Technical variation was never higher than 3%.

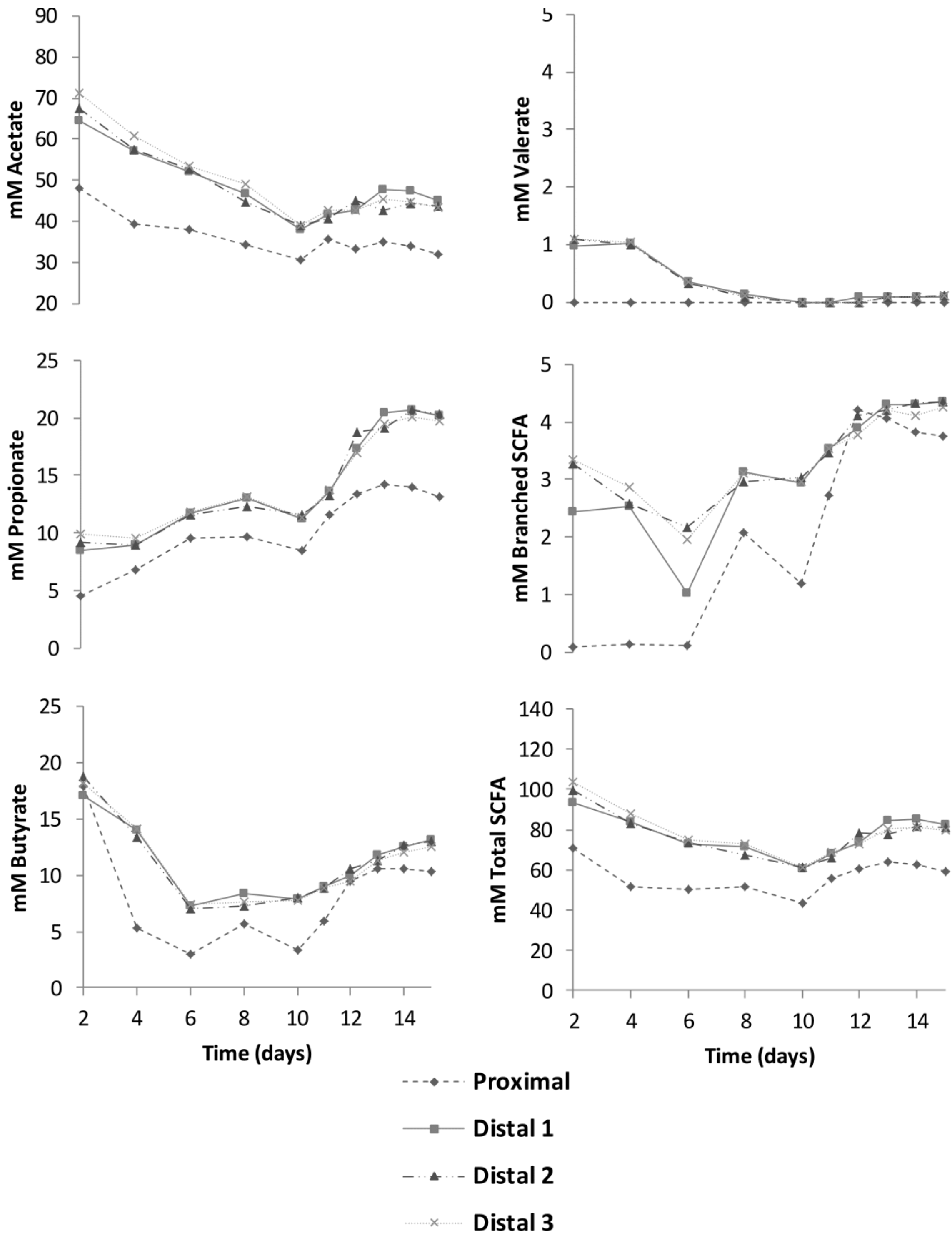


Figure 2. 5: SCFA levels (mM) as a function of time (days) after inoculation for the proximal colon and the three distal colon regions that were fed a mucin-free feed until day 10 after which 8g L^{-1} (day 10-12) and 4g L^{-1} mucin (day 12-15) was administered.

3.5 Microbial composition analysis of the colon environment with variable mucin supply

Illumina sequencing data showed a strong increase in relative abundance of *A. muciniphila* after mucin addition, confirming the qPCR results (Figure 2.6A). This increase coincides with a decrease of almost all the other genera, except for *Parabacteroides*, which increase together with *A. muciniphila. Lactobacillus* and some *Clostridium* species are significantly more abundant after mucin supplementation (Supplementary Table 2.4). Correspondence analysis was used to further explain the variations in relative abundances (Figure 2.6B). The area of the points in the figure indicates the relative frequency of a particular genus and darker colors of the arrows indicate higher percentages of variance attributed to a particular time point. Point symbols with darker color represents the bacterial abundances that contributed more to the variance among time points (Supplementary Table 2.5). The presence of a distinctive microbial community before and after the supplementation with mucin was validated based on the relative abundances of the bacteria associated with each time point. It is clear that dimension 1, which accounted for 87% of the total variance among samples, shows the distinction between the community before and after mucin supplementation. Day 8 and 10 are characterized by *Lachnoclostridium, Bacteroides* and *Parasutterella* whereas day 12 and 15 are characterized by *Akkermansia*, *Parabacteroides* and *Lactobacillus* (Supplementary Table 2.5). Changes in the abundance of *Akkermansia* alone explain 53.76% of total variance. Dimension 2 accounts for 9% of the total variance and is mostly characterized by the community in transition.

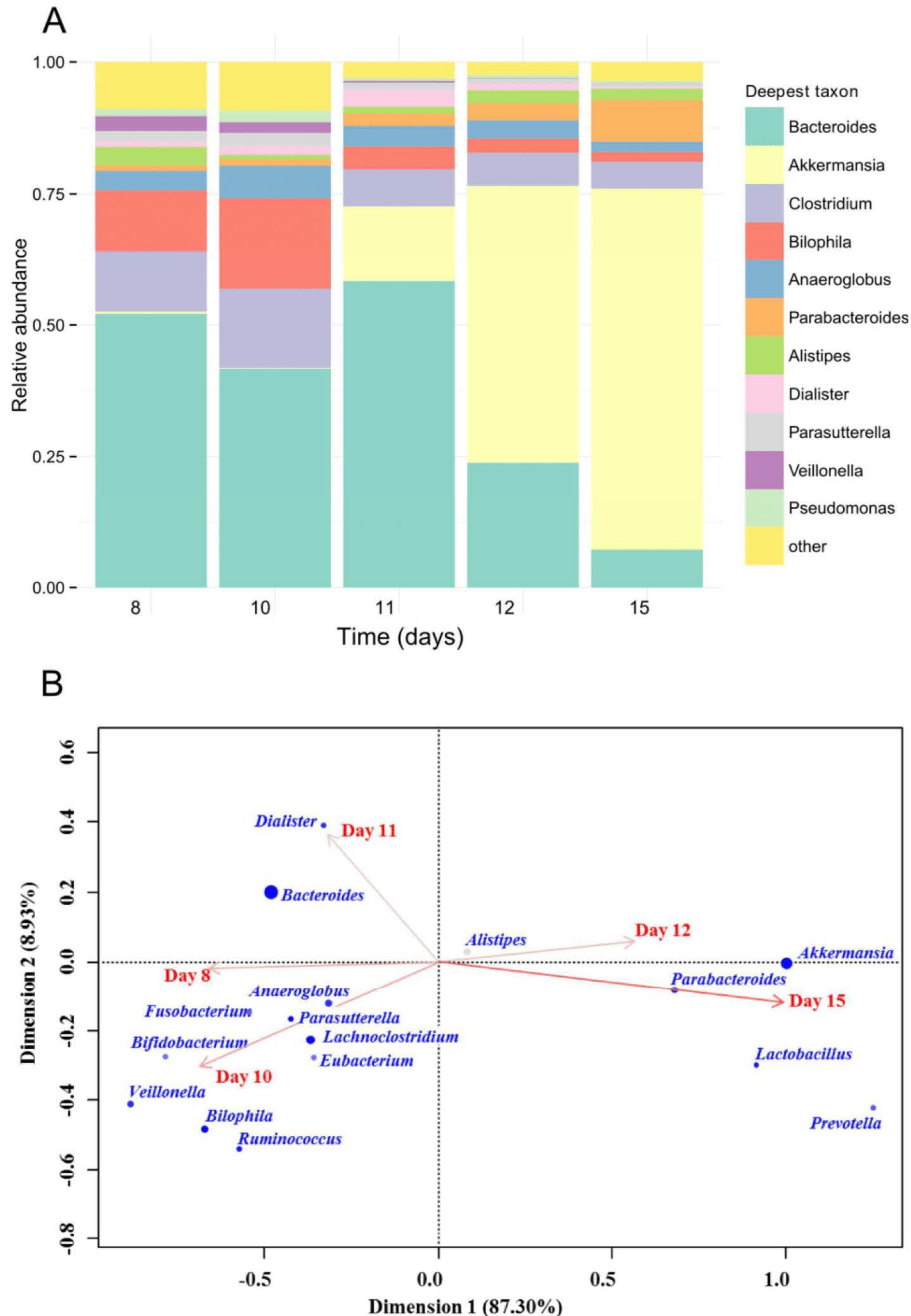


Figure 2. 6: Illumina sequencing results of the mucin experiment where a mucin-free feed was fed until day 10 after which 8g L^{-1} (day 10-12) and 4g L^{-1} mucin (day 12-15) was administered. (A) An overview of the relative abundance (% of the total community) of the 11 most abundant genera in the distal colon vessels (averaged) for the different time points (days). (B) Correspondence analysis (CA) using the Single Value Decomposition (SVD) was employed to highlight the variations in relative bacterial abundance of the distal colon vessel on the SHIME pre- and post-mucin treatment ($p<0.05$).

4. Discussion

We used a dynamic *in vitro* gut model (SHIME) to gain insight in the colonization and metabolic performance of the mucin-degrading gut symbiont *A. muciniphila* within the three regions of the human colon and in the presence of a complex gut microbiota. This overcame limitations that are inherently associated with human *in vivo* studies, such as the difficult access to the different regions of the colon and confounding effects of processes that may indirectly impact host mucin production and fitness of mucin degraders. Due to the successful and abundant colonization of *A. muciniphila*, we created an ideal biological environment to study the ecological behavior of *A. muciniphila* and the impact of pH and mucin.

Using the *in vitro* SHIME model and a specific inoculum with high abundances of *A. muciniphila,* the combination of qPCR analysis, HITChip and Illumina sequencing demonstrated the preferential colonization of the distal colon ecosystem by *A. muciniphila*. The preference of *A. muciniphila* for distal colon colonization was shown, yet partial mucin degradation also took place in the proximal colon. This process was probably performed by *Bacteroides* species, which are colonizers of the proximal colon compartment (Figure 2.3A, Supplementary Figure 2.2) and which are known as versatile glycan-degrading microbes (Martens *et al.*, 2009; Salyers *et al.*, 1977). In contrast, our results suggested that *A. muciniphila* was the specialist mucin degrader in the distal colon compartment, because its abundant colonization significantly correlates with mucin concentrations (Supplementary Figure 2.1, Figure 4, Figure 6) and mucin degradation (Supplementary Figure 2.1). Propionate is a major end product of *A. muciniphila* metabolism, hence the increase in propionate after mucin supplementation indicated that *A. muciniphila* was degrading mucin in the distal colon (Derrien *et al.*, 2004) (Figure 2.5). When the food bolus arrives in the distal colon, the carbohydrates are already fermented and the proteolytic fermentation starts. This leads to the formation of phenols, branched short chain fatty acids, ammines and other end products, many of which have toxic effects (Macfarlane and Macfarlane, 2012; Nyangale *et al.*, 2012). The presence of *A. muciniphila* in the distal colon and its production of beneficial short chain fatty acids from mucins can thus be a protective strategy by prolonging the saccharolytic fermentation and maybe counteracting some of the deleterious effects of the proteolytic fermentation.. This location preference of *A. muciniphila* for the distal colon compartment of the SHIME can derive from parameters, such as pH, nutrient availability, residence time, and the microbial community, which differ between colon regions.

Further research into these different parameters, shows that the preferential colonization of *A. muciniphila* seems to be determined by the pH, because *A. muciniphila*

numbers were different between distal colon vessels that only differ in pH. This confirms results from *A. muciniphila* grown in monoculture, where the optimum pH for growth was 6.5 and no growth was observed below pH 5.5 or above pH 8 (Derrien *et al.*, 2004). So *A. muciniphila* thrives at the more neutral pH (6.6 to 6.9) of the distal colon compartment and is less competitive in the proximal colon with lower pH (5.6 to 5.9) (Cummings, 1997). *A. muciniphila* might be locally outcompeted by *Bacteroides* species, which have the ability to grow and compete across a broad pH range (Macfarlane *et al.*, 1995). *A. muciniphila* prefers colon environment with high pH and is correlated with higher acetate and propionate production but lower butyrate concentration. In the vessels with pH 6.6 to 6.9, more acetate and propionate were produced but less butyrate, which has been previously reported (Walker *et al.*, 2005). The decrease in butyrate may be a direct effect of pH as butyrate producers proliferate at lower pH (Duncan *et al.*, 2009). The increase in propionate may be attributed to higher *A. muciniphila* abundances, as other genera correlated with higher pH in this experiment are not known for their high propionate production. *Bilophila* and *Alistipes* have been previously associated with undesirable gut health traits, like most species that thrive at more neutral pH in the distal colon (Cummings, 1997; da Silva *et al.*, 2008; Nyangale *et al.*, 2012; Rautio *et al.*, 2003). However, it cannot be ruled out that other factors play a role. For instance, the pH may have an effect on the mucin structure or its enzymatic accessibility and also the microbial background may play a role since we only tested it in one microbial environment.

Not only was the effect of pH and inulin investigated, but also the effect of variable mucin availability. The most obvious result of the administration of mucin after mucin depletion was the increase in *A. muciniphila* numbers in the distal colon compartment. Mucin supplementation also produced an increase in butyrate production in the proximal colon (Figure 2.5). This increased butyrate production was potentially established via cross-feeding with *Bacteroides* and other species that can degrade mucin (Martens *et al.*, 2008; Martens *et al.*, 2009; Salyers *et al.*, 1977). An increase in the relative abundance of *Bifidobacterium* species was observed in the proximal colon at day 15. This may indicate that those species are capable of partial mucin degradation and they could also contribute to the increase in butyrate (Hoskins *et al.*, 1985; Killer and Marounek, 2011; Png *et al.*, 2010) (Supplementary Figure 2.2). In the distal colon compartment, the mucin administration caused a significant increase in propionate production that can be attributed to *A. muciniphila* and *Parabacteroides* species, which both characterize the new mucin-associated community (Figure 2.6). *Parabacteroides* sp. produce acetate and succinate, which can be used by other species to produce propionate, for example *Phascolarctobacterium,* which relies for its carbon-source on succinate (Deldot *et al.*, 1993; Sakamoto and Benno, 2006; Watanabe *et*

al., 2012). Increase in propionate production in a mucin degrading environment might thus be a biological marker for *A muciniphila.*

We observed that colonization of *A. muciniphila* using this specific inoculum in our *in vitro* system is dependent on pH and mucin, but not on inulin.. In a rat study (Van den Abbeele *et al.*, 2011a) it was shown that inulin shifted *A. muciniphila* from the caecum to more distal regions and increased the fecal numbers of *A. muciniphila*. As mentioned above, the colonization of *A. muciniphila* is very pH dependent. Therefore, as inulin supplementation is known to lower pH (Van den Abbeele *et al.*, 2011a; Welters *et al.*, 2002), it might force *A. muciniphila* to colonize more distal regions in the rats. Inulin does not only affect pH but it also stimulates mucin secretion by the host (Barcelo *et al.*, 2000; Schmidt-Wittig *et al.*, 1996; Shimotoyodome *et al.*, 2000). Since *A. muciniphila* is a known mucin degrader and susceptible to changes in mucin availability, this is another way by which inulin can exert an indirect effect on *A. muciniphila* numbers. In our *in vitro* study however, the pH and the mucin concentration were maintained constant and no effect of inulin on *A. muciniphila* could be observed. Inulin treatment did stimulate *Lactobacillus* species, which has been described in many other studies (Kleessen *et al.*, 2001; Macfarlane *et al.*, 2006; Makras *et al.*, 2005; Sghir *et al.*, 1998). Also propionate producing bacteria *Succiniclasticum* and *Propionispora* increased after inulin treatment, which can explain the higher propionate concentration (Abou-Zeid *et al.*, 2004; van Gylswyk, 1995). The increased butyrate concentration however could not be linked to higher abundances of butyrate producing bacteria (Supplementary Table 2.2).

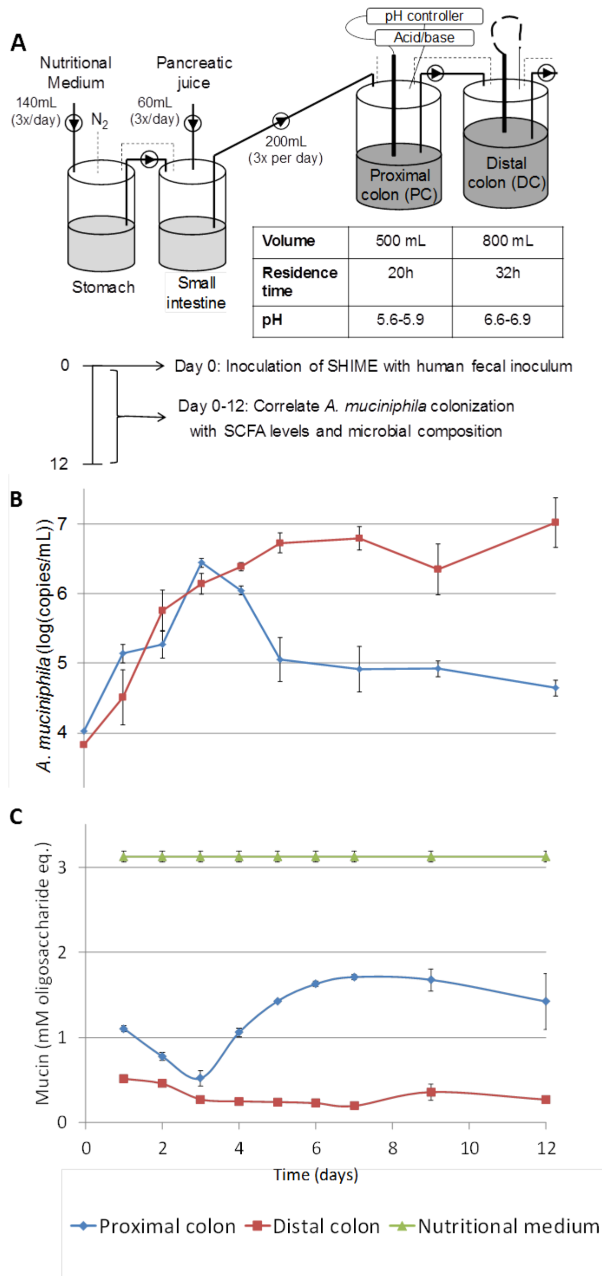
Our experiments showed the mechanism behind the effect of inulin, pH and mucin on the colonization of *A. muciniphila* and confirmed our hypothesis. In this way, prebiotics like inulin may exert a beneficial effect along the entire length of the colon, by stimulating mucin secretion and shifting the mucin degradation to distal regions. This process may yield higher propionate and acetate production. Other dietary compounds that can favor *A. muciniphila* are for example cranberries, which are rich in polyphenols and have been shown to increase the *A. muciniphila* population in diet-induced obese mice (Anhe *et al.*, 2015). Also in mice fed with fish-oil, rich in omega-3 fatty acids, *A. muciniphila* was enriched (Caesar *et al.*, 2015). The manner in which these products stimulate *A. muciniphila* is not yet clear and, like with inulin, the effect might not be the same in our *in vitro* system. Human milk oligosaccharides (HMO) resemble mucins in their structure and *A. muciniphila* has the capacity to grow on these HMOs. So treatment with human milk oligosaccharides might directly affect *A. muciniphila*, just like mucin treatment, and thus be useful in our *in vitro* SHIME model.

For the experiments performed in this paper a microbial environment was created with pronounced *A. muciniphila* colonization to ensure the study of its ecological behavior. The experiments were carried out in the *in vitro* SHIME model that is perfect for these mechanistic studies. Although there were hundreds of other species present, only *A. muciniphila* reacted that strongly to the changes in the SHIME environment, i.e. different pH and mucin concentration. Further research considering multiple donors, providing different microbial environments with different *A. muciniphila* abundances, should be performed to elucidate whether the sensitivity of *A. muciniphila* is dependent on the microbial background or not. The results shown in this paper are indicative of a high sensitivity of *A. muciniphila* to environmental changes. This high sensitivity could explain why its abundance changes drastically *in vivo* when the conditions in the colon change, for example due to certain disease states (Everard *et al.*, 2011; Png *et al.*, 2010; Wang *et al.*, 2011; Zhang *et al.*, 2009). This could clarify why *A. muciniphila* is so often correlated with diseases and might be an indicator species for gut health.

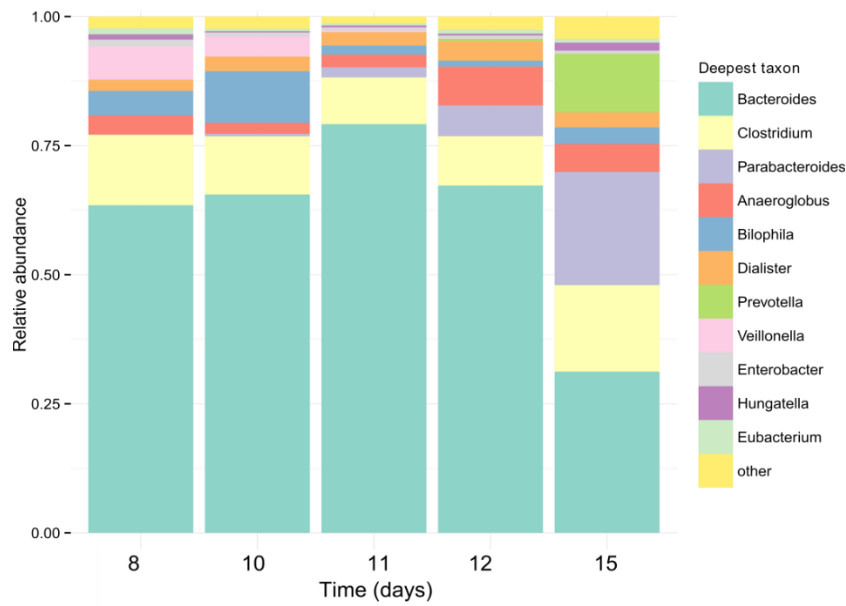
5. Acknowledgements

F.V.H. is a doctoral research fellow supported by the Agency for Innovation by Science and Technology (Grant number 131774).

6. Supplementary information



Supplementary Figure 2.1: Stabilization experiment. (A) Set-up of experiment in which the dynamic colonization of *A. muciniphila* during stabilization of a faecal sample in the SHIME was investigated. As earlier studies demonstrated that the three colon regions of the a conventional L-SHIME can be distinguished in a proximal (ascending) and distal (transverse and descending) colon, the setup was simplified to a 2-compartment simulation. On day 0, colon vessels are filled with nutritional medium and inoculated with a faecal slurry. By applying relevant conditions (retention time, nutrition, pH), the faecal microbiota evolve reproducibly to colon region-specific microbial communities. (B) Average (± SEM) A. muciniphila abundance (log (copies/mL)) measured with qPCR as a function of time after inoculation (days) for the proximal colon and the distal colon region. (C)Average (± SEM) mucin concentration, expressed as mM oligosaccharide equivalents, in the nutritional medium (feed) coming in, the proximal and distal colon as a function of time (days) after inoculation



Supplementary Figure 2. 2: An overview of the relative abundance (% of the total community) of the 11 most abundant genera in the proximal colon vessel for the different time points (days) with mucin depletion from day 0 until day 10 and mucin supplementation from day 10 until day 15.

Supplementary Table 2. 1: The average (± SEM) abundance (%) of A. muciniphila based on the HITChip analysis, as reported by Van den Abbeele et al. (2010), and the average (± SEM) absolute numbers (log copies/mL reactor volume) based on A. muciniphila-specific qPCR in the proximal and distal colon regions of a stabilized SHIME (day 19 and 26 after inoculation) (n = 4). Average (± SEM) absolute (mM) and proportional (mol %) net SCFA production in the proximal and distal colon regions of a stabilized SHIME (day 19, 21, 23 and 26 after inoculation), based on the absolute SCFA concentrations reported by Van den Abbeele et al. (2010). Values indicated with a different superscript are significantly different (a or b).

		Proximal colon	Distal colon	
		Ascending	Transverse	Descending
HITChip (%) (Van den Abbeele, et al., 2010)		0.05 ± 0.01^{a}	0.41 ± 0.13^{b}	0.27 ± 0.07^{b}
qPCR (log copies/mL) (this study)		3.36 ± 0.54^{a}	8.43 ± 0.12^{b}	8.15 ± 0.17^{b}
Absolute values (mM)	Acetate	31.7 ± 0.2^{a}	$+ 2.9 \pm 0.3^{a}$	$+ 3.9 \pm 0.3^{a}$
	Propionate	11.6 ± 1.3^{a}	$+ 2.5 \pm 0.1^{b}$	$+ 0.5 \pm 0.1^{a}$
	Butyrate	3.0 ± 0.8^{a}	$+ 1.3 \pm 0.1^{b}$	$+ 1.0 \pm 0.1^{b}$
Proportional values of net production (mol%)	Acetate	68.5	43.3	72.1
	Propionate	25.1	37.7	9.8
	Butyrate	6.5	19.0	18.1

Supplementary Table 2.2: Table of the significant increased or decreased OTUs after inulin supplementation. OTUs belonging to the *Akkermansia* genus are not included in this table since *Akkermansia* numbers were not significantly changed with inulin supplementation. P-value is the result of a two tailed T-test with unequal variance and the level of change is the abundance after inulin supplementation compared with the abundance before (α= 0.05).

Location	Genus	OTU.identity	P.Value	level.of.change
Proximal				
	Faecalibacterium	OTU26 and 37	0.02	0.07
	Fusicatenibacter	OTU53, 86 and 114	0.04	5.40
	Parabacteroides	OTU10 and 21	0.00	12.94
	Propionispora	OTU24	0.01	8.14
	Succiniclasticum	OTU12	0.01	10.70
	Veillonella	OTU13	0.03	0.01
Distal-low pH				
	Achromobacter	OTU42	0.05	9.00
	Bacteroides	OTU15	0.02	0.00
	Bacteroides	OTU58	0.05	10.25
	Bacteroides	OTU127	0.02	2.88
	Bacteroides	OTU36	0.02	0.00
	Bacteroides	OTU1	0.02	2.02
	Bacteroides	OTU43	0.02	0.10
	Bacteroides	OTU16	0.04	0.03
	Bifidobacterium	OTU19 and 105	0.00	0.11
	Blautia	OTU91	0.03	0.24
	Clostridium	OTU50	0.01	0.14
	Collinsella	OTU41	0.02	0.13
	Coprococcus	OTU62	0.01	0.09
	Eubacterium	OTU51	0.02	0.23
	Faecalibacterium	OTU26 and 37	0.01	0.09
	Hungatella	OTU23, 32, and 124	0.01	0.36
	Lactobacillus	OTU25	0.05	0.03
	Megasphaera	OTU22	0.02	0.04
	Oscillobacter	OTU70	0.02	0.03
	Propionispora	OTU24	0.01	13.10
	Ruminococcus obeum	OTU68	0.01	0.55
	Succiniclasticum	OTU12	0.02	13.89
	Veillonella	OTU13	0.00	0.06
Distal-medium pH				
	Bacteroides	OTU130	0.05	0.14
	Bifidobacterium	OTU19 and 105	0.02	0.12
	Clostridium	OTU101	0.01	13.80
	Clostridium	OTU76	0.04	4.08
	Collinsella	OTU41	0.02	0.16
	Dialister	OTU8	0.04	1.49
	Faecalibacterium	OTU26 and 37	0.01	0.35
	Lactobacillus	OTU25	0.02	7.73
	Parasutterella	OTU123, 125, 128, 129, 18, 27, 69, 72, 115, 117 and 118	0.02	0.60
	Propionispora	OTU24	0.01	13.85
	Succiniclasticum	OTU12	0.01	12.84
	Veillonella	OTU13	0.02	0.07
Distal-high pH				
	Bifidobacterium	OTU19 and 105	0.03	0.06
	Clostridium	OTU56	0.04	0.23
	Clostridium	OTU76	0.04	2.22
	Eubacterium	OTU51	0.01	0.28
	Faecalibacterium	OTU26 and 37	0.01	0.25
	Lactobacillus	OTU25	0.03	7.88
	Propionispora	OTU24	0.01	10.66
	Pseudobutyrivibrio	OTU111	0.02	0.11
	Succiniclasticum	OTU12	0.01	10.49
	Veillonella	OTU13	0.00	0.10

Supplementary Table 2.3: Correlations between relative bacterial abundances and dimension of the Multiple Factor Analysis. For the descriptors, a one-way analysis of variance was performed using the coordinates of the bacterial abundances by pH, by time point and by location. Student T-test was used to compare the average of the relative bacterial abundances of the pH/time point/location with the total average of all bacterial abundances. The value of the correlation of the with the dimension is indicated (*, P < 0.0001; **, P < 0.05; *, P < 0.1). Negative values indicate negative correlations.**

Dimension	Variance (%)	Descriptor	Estimate	*P value*	*Genus*	Correlation
					Parasutterella	0,758***
					Bifidobacterium	0,718***
					Ruminococcus	0,717***
					Eisenbergiella	0,696***
					Parabacteroides	0,610***
		Day 6	3,9	0,0003	*Faecalibacterium*	0,508**
		High pH	2,38	0,0009	*Pseudomonas*	0,502**
1	31,9	Distal colon	1,94	0,0009	*Akkermansia*	0,430**
		Low pH	-1,78	0,001	*Veillonella*	0,239**
		Proximal colon	-1,55	0,04	*Bilophila*	0,164**
					Lachnoclostridium	-0,086**
					Alistipes	-0,481**
					Bacteroides	-0,624***
					Lactobacillus	-0,675***
					Anaeroglobus	-0,719***
					Dialister	-0,749***
					Succiniclasticum	-0,769***
					Alistipes	0,752***
					Bilophila	0,737***
					Akkermansia	0,608***
					Pseudomonas	0,486**
					Ruminococcus	0,479**
		Distal colon	1,83	0,003	*Anaeroglobus*	0,410**
		High pH	1,46	0,003	*Eisenbergiella*	0,280**
		Day 10	1,74	0,07	*Parasutterella*	0,248**
2	23	Low pH	-1,51	0,001	*Lactobacillus*	0,224**
		Proximal colon	-1,87	0,002	*Propionispora*	0,103**
		Day 6	-2,67	0,005	*Succiniclasticum*	0,068**
					Parabacteroides	0,064**
					Dialister	0,05**
					Faecalibacterium	-0,173**
					Bifidobacterium	-0,553**
					Lachnoclostridium	-0,698***
					Veillonella	-0,721***
					Prevotella	-0,839***
					Lactobacillus	0,642***
					Succiniclasticum	0,607***
					Propionispora	0,558***
					Lachnoclostridium	0,503***
					Parabacteroides	0,416**
					Eisebergiella	0,409**
		Day 15	2,19	0,002	*Ruminococcus*	0,241**
		Day 14	1,57	0,04	*Parasutterella*	0,205**
3	14,1	Day 11	-1,69	0,03	*Bilophila*	0,203**
		Day 8	-1,49	0,05	*Prevotella*	0,133**
		Day 10	-1,47	0,05	*Akkermansia*	0,100**
					Dialister	-0,185**
					Veillonella	-0,221**
					Pseudomonas	-0,249**
					Alistipes	-0,315**
					Anaeroglobus	-0,333**
					Faecalibacterium	-0,346**
					Bacteroides	-0,588***
					Parabacteroides	0,579**
					Dialister	0,518**
4	10	Distal low	1,14	0,005	*Faecalibacterium*	0,499**
		Distal high	-0,762	0,07	*Bacteroides*	0,394**
					Akkermansia	-0,574**
					Pseudomonas	-0,418**

Supplementary Table 2.4: Table of the significant increased or decreased OTUs in the distal colon compartment after mucin supplementation. P-value is the result of a two tailed T-test with unequal variance and the level of change is the abundance after mucin supplementation compared with the abundance before (α= 0.05).

Genus	OTU.identity	P.value	level.of.change
Alistipes	OTU39	0,010670229	0,56022409
Achromobacter	OTU42	0,038095805	0,293877551
Akkermansia	OTU2	0,000604632	167,0666667
Anaeroglobus	OTU4 and 103	0,018926045	0,621920563
Bacteroides	OTU130	0,001607348	?
Bifidobacterium	OTU19	0,008738608	0,066079295
Bilophila	OTU6	0,000521956	0,207740239
Blautia	OTU28	0,002104784	0,169289962
Collinsella	OTU41	0,004417081	0,282051282
Clostridium	OTU46	0,004367185	0,21969697
Clostridium	OTU107	0,027220304	2,024509804
Clostridium	OTU 113	0,036193876	0
Clostridium	OTU106	0,000253595	0,438645276
Clostridium	OTU56	0,010313308	0,320610687
Clostridium	OTU104	0,0136114	0,152380952
Clostridium	OTU76	0,00548313	0,273015873
Clostridium	OTU92	0,010534356	0,256410256
Clostridium	OTU71	0,011394601	0,28125
Clostridium	OTU35	0,017257706	0,206751055
Clostridium	OTU89	0,013896494	0,47826087
Clostridium	OTU40	9,16704E-06	0,187242798
Parabacteroides	OTU10 and 21	0,004815559	3,933333333
Enterococcus	OTU61	0,0304484	0,140350877
Ethanoligenens	OTU81	0,006439547	0,193333333
Eubacterium	OTU51	0,0260356	0,181818182
Eubacterium	OTU17	0,01771176	0,265432099
Fusobacterium	OTU34	0,034105022	0,300405954
Faecalibacterium	OTU26 and 37	0,001370181	0,45014245
Hungatella	OTU32, 23 and 124	0,012552626	0,226415094
Lactobacillus	OTU25	0,022205942	5,015873016
Parasutterella	OTU123, 125, 128, 129, 18, 27, 69, 72, 115, 117 and 118	0,00061688	0,48
Ruminococcus	OTU57	0,015788228	0,208333333
Ruminococcus	OTU116	0,028513763	0,108333333
Terrisporobacter	OTU95	0,016929752	0,111111111
Veillonella	OTU13	0,000582873	0,077294686
Victivallaceae	OTU83	0,0132379	0,315789474

Supplementary Table 2. 5: Correspondence analysis (CA) using the Single Value Decomposition (SVD) was employed to highlight the variations in relative bacterial abundance of the distal colon vessel on the SHIME pre- and post-mucin treatment. (upper) Relative bacterial abundances in distal colon vessels supplemented with mucin. The value of the correlation of the with the dimension is indicated (*, P < 0.0001; ; **, P < 0.05; *, P < 0.1). Negative values indicate negative correlations. (lower) Relative bacterial abundances and their contribution to the total variance among time points.**

Dimension	Variance	Variable	Correlation	P value
1	87.3%	*Akkermansia*	0.999	<0.0001
		Parabacteroides	0.825	0.05
		Lactobacillus	0.815	0.05
		Day 15	0.976	0.05
		Day 12	0.928	0.05
		Bacteroides	-0.849	0.05
		Parasutterella	-0.825	0.05
		Lachnoclostridium	-0.720	0.05
2	8.9%	*Dialister*	0.452	0.05
		Bacteroides	0.150	0.05
		Day 11	0.544	0.05
		Ruminococcus	-0.425	0.05
		Bilophila	-0.338	0.05
		Day 10	-0.156	0.05
3	2.5%	*Eubacterium*	0.536	0.05
		Alistipes	0.900	0.05
		Bifidobacterium	0.490	0.05
		Fusobacterium	0.386	0.05
		Veillonella	0.182	0.05
		Day 8	0.207	0.05
		Dialister	-0.218	0.05

Genus	Variance (% of the total)	Time point	Variance (% of the total)
Lachnoclostridium	3.23	Day 8	17.40
Eubacterium	0.26	Day 10	20.65
Ruminococcus	1.13	Day 11	9.25
Dialister	0.99	Day 12	13.23
Veillonella	2.37	Day 15	39.47
Anaeroglobus	1.10		
Alistipes	0.77		
Akkermansia	53.76		
Bifidobacterium	0.71		
Bacteroides	19.37		
Bilophila	10.10		
Fusobacterium	0.55		
Lactobacillus	0.18		
Prevotella	1.56		
Parabacteroides	3.30		
Parasutterella	0.63		

CHAPTER 3

A host glycan degradation niche in a dynamic gut model increases *Akkermansia muciniphila* abundance and changes microbiome composition in a donor independent fashion

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This chapter has been redrafted after

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CHAPTER 3

A host glycan degradation niche in a dynamic gut model increases *Akkermansia muciniphila* abundance and changes microbiome composition in a donor independent fashion

Abstract

Akkermansia muciniphila, an abundant mucolytic colon microorganism, has been correlated with human health in various studies. To identify the optimal conditions for successful *in vivo* application as a potential probiotic, the *in vitro* SHIME model was used to reach a mechanistic understanding of *A. muciniphila's* colonization preferences and its response to environmental parameters such as colon pH and host glycans. After a period of mucin deprivation, we found that mucin supplementation results in significantly different microbial communities, with more *Akkermansia, Bacteroides* and *Ruminococcus,* compared to the mucin-deprived communities. Mucin treatment accounted for 26% of the observed variation in the microbial community at OTU level (p=0.001), whereas the donor effect was limited (8%) (p=0.035), indicating host glycans to constitute an important ecological niche shaping the microbiota composition. The effect of colonic pH had a less profound impact on the microbiome with both pH and donor origin explaining around 10% of the variability in the dataset. Yet, higher simulated colonic pH had a positive impact on *Akkermansia* abundance while SCFA analysis displayed a preference for propionate production with higher colonic pH. Our results show that host glycans as nutritional resource are a more important modulator of the gut microbiome than colon pH as environmental factor.

1. Introduction

Akkermansia muciniphila is an abundant mucin-degrading member of the human gut microbiota and its abundance has been linked with gut health in several studies over the past decade (Collado *et al.*, 2007; Png *et al.*, 2010; Wang *et al.*, 2011; Zhang *et al.*, 2013). In a study with obese mice on a high-fat diet, it was shown that administration of *A. muciniphila* reversed insulin resistance, dyslipidemia, metabolic endotoxemia and fat mass gain (Everard *et al.*, 2013). Plovier *et al.* (2017) discovered that the beneficial effects are at least partly due to a specific outer membrane protein. Apart from oral administration of *A. muciniphila*, uptake of dietary compounds such as fish oil and cranberry extract also increased *A. muciniphila* abundances and led to healthier mice (Anhe *et al.*, 2015; Caesar *et al.*, 2015). Other studies have shown an increased abundance of *A. muciniphila* in subjects with colon cancer, which might be explained by the overexpression of certain mucin types in colon cancer (Borges-Canha *et al.*, 2015; Weir *et al.*, 2013b). Considering the various and far-reaching consequences of changes in microbial community composition and activity, there is a need to investigate the many forces that shape the microbial community, including various nutrient sources, antimicrobial compounds, ionic conditions and gut pH (Duncan *et al.*, 2009).

One dominant factor that influences the gut microbial community composition is the influx of glycans into the colon, both from diet and host mucosal secretions. As opposed to dietary glycans that vary in composition and supply, the host derived glycans from the mucus layer present a more continuous source of nutrients. Mucin glycans are composed of O-glycosylated, and to a lesser extent N-glycosylated, protein backbones, with glycosyl chains of 2-12 monosaccharides, consisting of mainly galactose, fucose, N-acetylgalactosamine, N-acetylglucosamine, mannose and sialic acid (Lai *et al.*, 2009). Continuous mucin production by the goblet cells and mucus desquamation contribute both to mucin presence in the mucus layer as in the lumen of the colon (Atuma *et al.*, 2001; Faure *et al.*, 2002; Johansson, 2012). Previously, it was thought that mucin degradation was detrimental for gut health but it is now clear that it is part of a normal turn-over process (Norin *et al.*, 1985). Due to the complexity of the mucin structure and the variation in glycosylation, a wide variety of specific enzymes are needed for its degradation, such as galactosidase, sialidase, fucosidase and N-acetylgalactosaminidase (Tailford *et al.*, 2015a). This means that only few species have the enzymatic capacity for initiating partial or full mucin degradation, including *A. muciniphila, Bacteroides thetaiotaomicron, B. fragilis, Ruminococcus gnavus, R. torques, Bifidobacterium bifidum*, … (Hoskins *et al.*, 1985; Marcobal *et al.*, 2011; Martens *et al.*, 2008; Png *et al.*, 2010). Degradation of mucins leads to the release of less complex carbohydrates and the production of organic acids like acetate, lactate and propionate, some of which can be used by other bacteria, as part of a microbial food chain, to produce butyrate or other end

products. The presence and the activity of host glycan degrading species in the mucus layer, close to the host cells, may have strong effects, both positive and negative, on gut health. The role of host glycans in microbial community dynamics and host-microbe interactions therefore requires further study.

Using SHIME as a dynamic model of the human gut and a human microbial inoculum with high reported *Akkermansia* abundance (Van den Abbeele *et al.*, 2013; Van den Abbeele *et al.*, 2012; Van den Abbeele *et al.*, 2011g), we previously showed that mucin is a profound parameter impacting colonization ability of *Akkermansia*. Additionally, its ability to colonize different *in vitro* colon regions was also highly dependent on the prevailing pH (Chapter 2).

Colonic pH is determined by host secretions and microbial fermentation products, such as pH-lowering by SCFA synthesis. In the proximal colon, pH is slightly lower compared to the distal colon, due to active carbohydrate fermentation leading to high amounts of SCFA (Macfarlane *et al.*, 1992). Besides the effect on the microbiota, pH also influences bile acid solubility and cation availability (Scholz-Ahrens and Schrezenmeir, 2007). Information about the impact of colonic pH towards the residing microbiota is rare and in the context of determining growth optimum mostly focused on the effect towards single species (Duncan *et al.*, 2009; Walker *et al.*, 2005).

As our previous work only explored the effect of mucin and pH towards *A. muciniphila* in one microbial background (=microbial inoculum from 1 human donor) (Chapter 2), the current study aims at testing the biological reproducibility of our previous findings. SHIME experiments were performed to study the effect of pH and the presence or absence of a host-glycan degradation niche in colon compartments separately inoculated with the microbiota from eight donors. This enabled us to elucidate whether the gut microbial response and *A. muciniphila* colonization sensitivity to changes in host-glycans and pH is dependent on the microbial background or not.

2. Materials and methods

2.1 Long-term dynamic *in vitro* gut model for the luminal colon microbiota (SHIME)

The long-term colonization of *A. muciniphila* within a mixed human gut microbiota was assessed in the dynamic *in vitro* gut model, SHIME® (ProDigest-Ghent University, Ghent, Belgium). The model and its nutritional medium is described in Chapter 2. Fecal samples were collected from healthy donors between the age of 25-35 and prepared within 1h according to standard procedures (Molly *et al.*, 1993) (Chapter 2) and used for inoculation.

Two SHIME experiments were performed in this study. The set-up of the **mucin experiment**, which evaluates the effect of mucin deprivation and subsequent supplementation, is shown in Figure 3.1. Eight colon vessels, with a retention time of 40 hours and a pH between 6.6-6.9 (distal colon pH), were inoculated with the fecal suspension of eight donors. After supplementing a mucin-free nutritional medium during the first 8 days, of mucin (8 g L^{-1}) was delivered to the proximal colon vessel on day 8, and from day 9 to 11, 4 g L^{-1} mucin were supplied. The set-up of the second experiment, the **pH experiment**, is shown in Figure 3.2. Here eight colon vessels were inoculated with the fecal suspension of four donors, with four colon vessels being kept at pH 6.6-6.9 (high pH, distal colon pH) and four at pH 5.6-5.9 (low pH, proximal colon pH). All colon vessels had the same retention time (40h) and were fed normal nutritional medium during 11 days. This experiment was repeated with four different donors so that in total the pH experiment was carried out with eight fecal inocula.

Samples were taken for SCFA analysis, as described previously (Andersen *et al.*, 2014) and for DNA extraction (Geirnaert, 2015) so 16S rRNA gene amplicon sequencing (Illumina MiSeq) (De Paepe *et al.*, 2017) and *A. muciniphila* qPCR quantification (Collado *et al.*, 2007) could be performed.

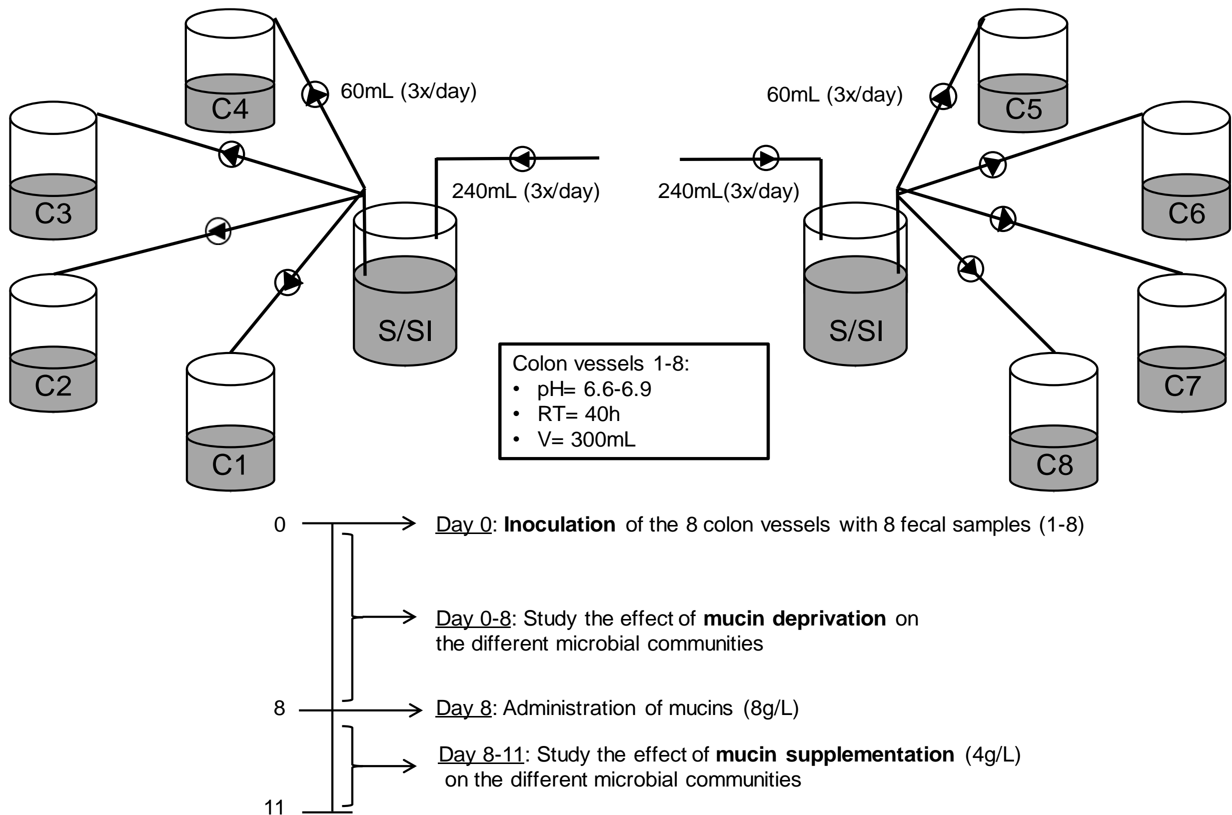

Figure 3.1: Experimental set-up of the Simulator of the Human Intestinal Microbial Ecosystem (SHIME) system for the mucin experiment.

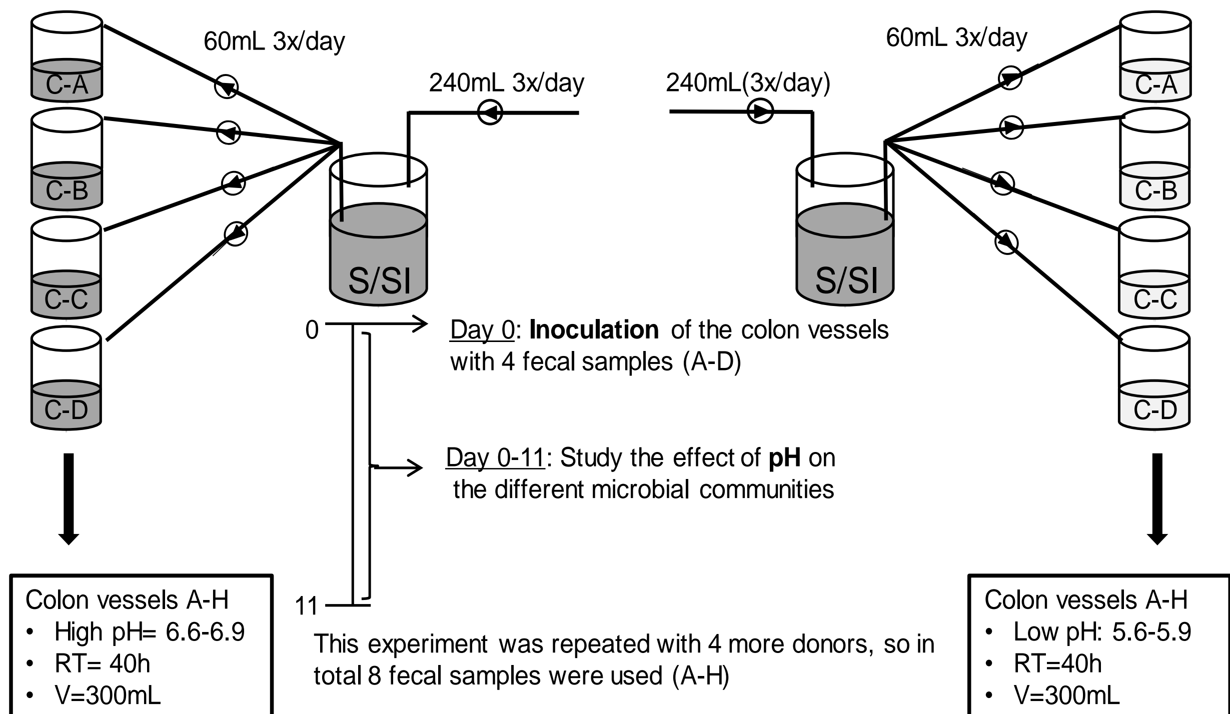

Figure 3. 2: Experimental set-up of the SHIME system for the pH experiment.

2.2 Microbial community analysis

DNA-extraction was performed by a combination of chemical and mechanical lysis through a beat beating step as reported by (Geirnaert, 2015). As starting material, the pellet obtained after centrifuging 1 mL of luminal sample at 5,000 g for 10 min was used. The DNA quality was verified on a 1.5 % (w/v) agarose gel.

Total bacterial 16S rRNA gene and the species-specific 16S rRNA gene of *A. muciniphila* was quantified with qPCR on 100- and 10- fold diluted DNA extracts, respectively, using a StepOnePlus Real-Time PCR system (Applied Biosystems, Carlsbad, CA). Primers for total bacteria (338F ACTCCTACGGGAGGCAGCAG, 518R ATTACCGCGGCTGCTGG) were used with the following cycling program: 3 min at 95°C followed by 40 cycles of 1 min at 95°C, 40 s at 56°C and 40 s at 72°C (Ovreas *et al.*, 1997). *A. muciniphila* specific primers (AM1 GAGCACGTGAAGGTGGGGAC, AM2 CCTTGCGGTTGGCTTCAGAT) were used with the following cycling program: 5 min at 95°C followed by 40 cycles of 15 s at 95°C, 40 s at 60°C and 30 s at 72°C and a final extension at 72°C for 5 min (Collado *et al.*, 2007). The qPCR mix consisted of 14.19 µL sterile nuclease-free water (Sigma-aldrich, St. Louis, MO, US) and 2.5 µL Taq buffer (10x, with KCl) containing 0.025 units Recombinant Taq DNA-polymerase μL^{-1}, 0.2 mM dNTP Mix, 1.5 mM MgCl2 (Fermentas Molecular Biology Tools, Waltham, MA, US), 0.2 µM Primer F, 0.2 µM Primer R, 0.05 $\mu g\ \mu L^{-1}$ BSA (Roche Applied Science, Penzberg, Germany) and 0.125 µL 20 x SYBR green (1:500 diluted from a 10 000 x SYBR green I nucleic acid stain concentrate in DMSO, Sigma Aldrich, St. Louis, MO, US). For each sample, 5 µL diluted DNA extract was added to 20 µL PCR-mix in technical triplicate in a qPCR plate and for each qPCR assay, standard curves were created by a 10-fold dilution series of DNA of a plasmid containing the targeted 16S rRNA gene fragment.

The bacterial community after 4, 8 and 11 days of incubation in the mucin experiment and after 4 and 11 days of the pH experiment was assessed using amplicon sequencing of the 16S rRNA gene (De Paepe *et al.*, 2017). DNA samples were sent out to LGC Genomics (Teddington, Middlesex, UK) for library preparation and sequencing on an Illumina Miseq platform, as described by De Paepe *et al.* (2017). The V3-V4 region of the 16S rRNA gene was amplified by PCR using primers (341F CCTACGGGNGGCWGCAG, 785R GACTACHVGGGTATCTAAKCC) derived from Klindworth *et al.* (2013), with a slight modification to the reverse primer by introducing another degenerated position (K) to make it more universal. The sequencing data has been submitted to the NCBI (National Center for Biotechnology Information) database under accession code SRP126579. The mothur software package (v.1.39.5) and guidelines were used to process the amplicon data

generated by LGC Genomics, as described in detail by De Paepe *et al.* (2017); (Kozich *et al.*, 2013).

2.3 Statistical analysis

All statistical analysis were performed in R, version 3.2.2.

To visualize differences in microbial community composition between donors and conditions (mucin deprived vs. rich and high vs. low pH), the most abundant genera were visualized in bargraphs (Figures 3.4 and 3.9) and ordination and clustering techniques were applied. For these purposes, the shared file was further processed to remove OTU's with too low abundance according to the arbitrary cut-off's described by McMurdie and Holmes (2014). An OTU is defined in this manuscript as a collection of sequences, that are found to be more than 97% similar to one another in the V3-V4 region of their 16S rRNA gene after applying hierarchical clustering (Chen *et al.*, 2013; Schloss and Westcott, 2011; Schloss *et al.*, 2009; Wang *et al.*, 2012). To deal with differences in sampling depth, proportional data transformed on the common scale to the lowest number of reads was used (McMurdie and Holmes, 2014). A table with the most abundant OTUs classified to the species level using both RDP Seqmatch tool and NCBI BLAST is given in Supplementary Table 3.1 and a table with the first 300 OTUs classified to the deepest taxonomic level is given in Supplementary Table 3.4.

Principle Coordinate Analysis (PCoA; package stats) was conducted based on the abundance-based Jaccard dissimilarity matrix (package vegan and visualized with ggplot2 (Anderson *et al.*, 2006; Cox, 2001; Oksanen, 2016; Ramette, 2007) (Figures 3.6 and 3.11). This procedure was repeated on OTU and genus level focusing on both the comparison between the donors and between the applied conditions. On the genus level, weighed averages of genera abundances were *a posteriori* added to the ordination plot using the wascores function in vegan (Oksanen, 2016). To confirm the trends, observed data was clustered by means of an Unweighed Pair-Grouped Method using arithmetic Averages (UPGMA) clustering method (Maechler, 2016). The significance of observed group separations was assessed with a Permutational Multivariate Analysis of Variance (PERMANOVA) using distance matrixes (package vegan) (Oksanen, 2016). Prior to this formal hypothesis testing, the assumption of similar multivariate dispersions was evaluated, using betadisper function (package vegan).

Interpretation of the results is preceded by a permutation test of the db RDA results to confirm that a relationship exists between the response data and the exploratory variables. Using the same principle, the significance of the first two constrained axis was evaluated. The constrained fraction of the variance, explained by the exploratory variables is adjusted by applying a subtractive procedure (Borcard *et al.*, 2011; Peres-Neto *et al.*, 2006). The

results of the db RDA were visualized in a type 2 scaling correlation triplot (Supplementary Figures 3.3 and 3.9; Supplementary Tables 3.2 and 3.3). The two first canonical axes were annotated with the proportional constrained eigenvalues. Site scores were displayed as weighed sums of species and the factor levels of the habitat explanatory variable were represented as centroids. In order to improve readability of the graph, the number of OTUs represented as vectors in the triplot were limited to the most relevant taxa.

In order to find statistically significant differences in species abundance between the different conditions (mucin deprived vs. rich and high vs. low pH) the DESeq package was applied (α=0.01) as suggested by McMurdie and Holmes (2014) and results are shown in Figures 3.5 and 3.10 (Love *et al.*, 2014). The factors mucin and pH were used in the design formula. More information on the DESeq methodology is given in Figure 3.3.

The abundance data of bacterial community at operational taxonomic unit (OTU) level was used to construct the co-occurrence networks in a similar fashion as described in De Vrieze *et al.* (2016). Co-occurrence networks were built with similarity-based techniques, using Pearson correlation for OTU absolute abundance data in a pair-wise manner. The threshold of p-values was set at 0.05 for all networks construction. The threshold of coefficient correlation coefficients (r) for all network was set to 0.70 (positive interactions) and -0.70 (negative interactions). The data from the mucin experiment (eight donors) at day 4, 8 and 11 as well as from the pH experiment (eight donors, high and low) at day 11 were used to construct the five co-occurrence networks (Supplementary Figures 3.4-3.6 and 3.10-3.11) to reveal the succession patterns of the interaction among microorganisms over time in presence of mucin or through value of pH.

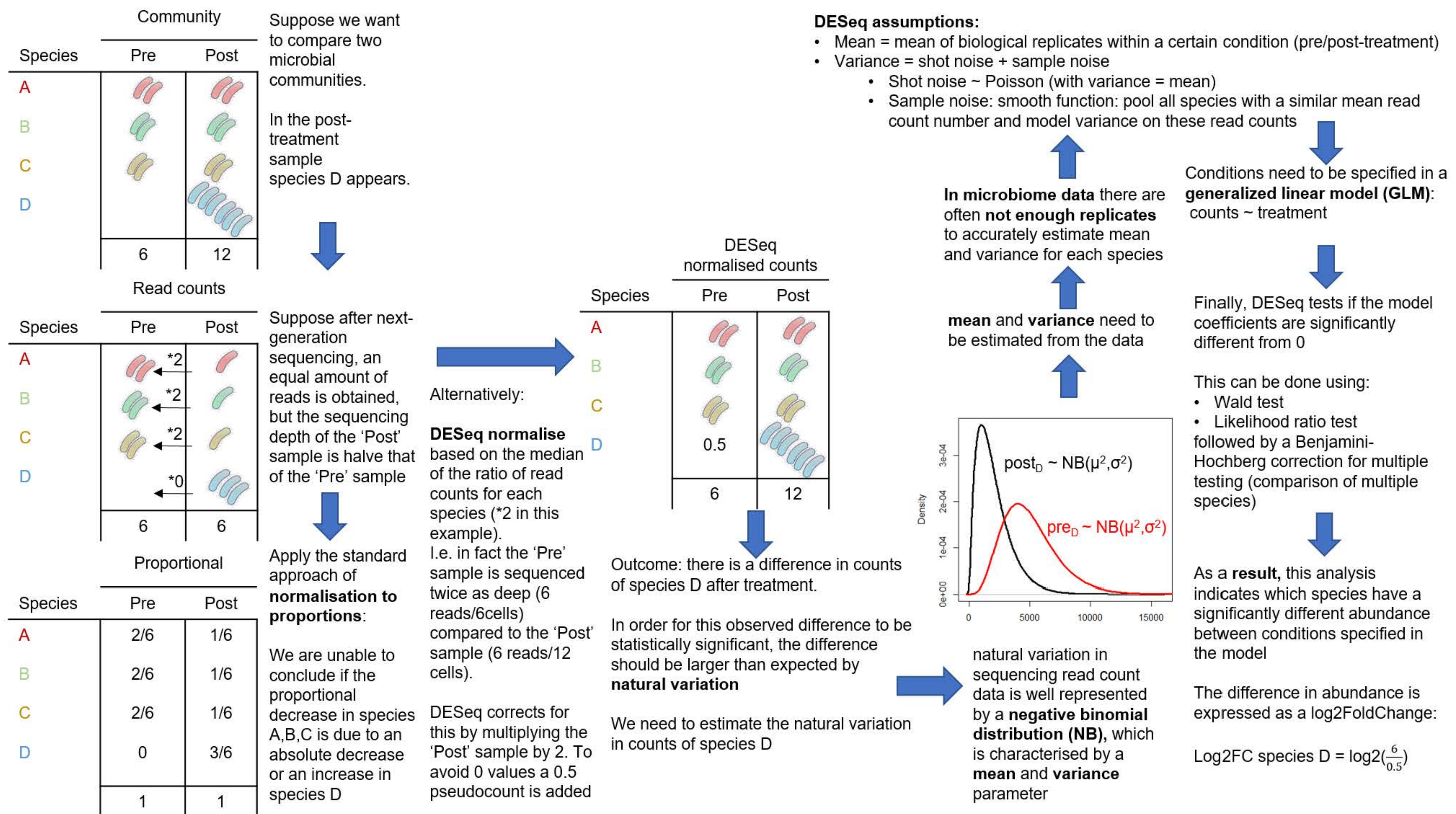

Figure 3. 3: DESeq methodology Figure derived from (De Paepe, 2018).

3. Results

3.1 Impact of mucin addition on the bacterial community structure

The mucin experiment studies the effect of mucin deprivation and mucin supplementation on the bacterial community of eight donors, as shown in the experimental set up (Figure 3.1).

Amplicon sequencing of the 16S rRNA gene was performed on samples taken at the end of the mucin deprivation period (day 8) and at the end of the mucin supplementation period (day 11). The relative abundances of the most abundant genera are shown in Figure 3.4. The addition of mucin to the feed clearly induced changes to the bacterial community and increased the microbial diversity ($p<0.01$) (Supplementary Figure 3.1), but total bacterial count, as measured by qPCR, remained stable (Supplementary Figure 3.2). For some genera the shifts in relative abundance were apparent for all eight donors, such as the increase the relative abundance of the *Bacteroides, Parabacteroides* and *Ruminococcus* genera and a decrease in the relative abundance of the genera *Escherichia*/*Shigella* (Figure 3.4). Other observed shifts were more dependent on donor, emphasizing the importance of studying inter-individual variability. For example, the relative abundance of *Clostridium* cluster XIVa was increased in donors 1, 6,7, and 8 but decreased in donors 4 and 5 (Figure 3.4). DESeq hypothesis testing was performed, which shows the significantly ($p<0.01$) affected OTUs over all eight donors by supplementation of mucin (Figure 3.5). This analysis showed that for example the DESeq normalized abundance of *Akkermansia, Bacteroides, Ruminocuccus* and *Sutterella* were significantly increased in the mucin rich bacterial community whereas the abundance of the *Escherichia/Shigella* and *Roseburia* genera was significantly decreased by the addition of mucin (Figure 3.5).

PCoA analyses at the genus level (Figure 3.6) revealed that the bacterial communities clustered together based on mucin enrichment or mucin deprivation, and not based on donor. The cluster of mucin-deprived and mucin-rich bacterial communities were significantly different from each other, as confirmed by Permutational Multivariate Analysis of Variance ($p=0.001$). The mucin-deprived bacterial community is characterized by, among others, representatives of the genera *Roseburia, Alistipes* and *Esherichia/Shigella* while the mucin-rich bacterial community is characterized by a higher relative abundance of representatives of the genera *Ruminococcus, Bacteroides* and *Akkermansia* (Figure 3.6).

To quantify and distinguish between donor and treatment effects a partial distance based rda analysis was performed, showing that mucin treatment accounted for 26% of the observed variation in the microbial community at OTU level ($p=0.001$), whereas the donor effect was limited (8%) ($p=0.035$) (Supplementary Table 3.2; Supplementary Figure 3.3).

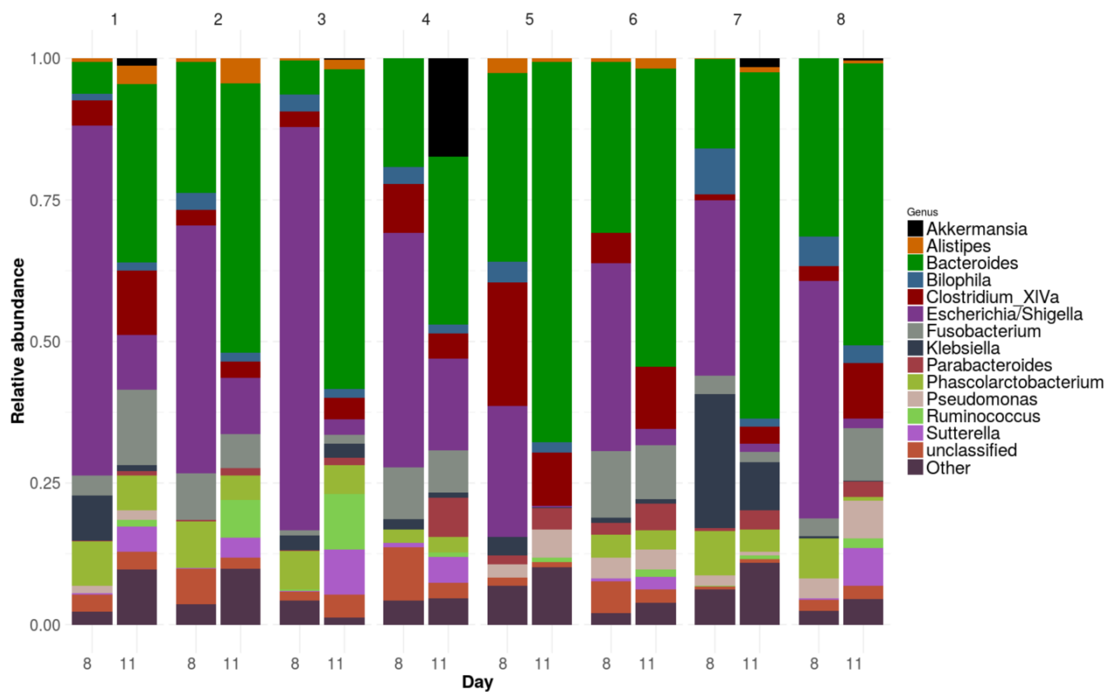

Figure 3. 4: 16S rRNA sequencing results of the mucin experiment where mucin-free feed was fed until day 8, after which 8g/L (day 8) and 4g/L (day 9-11) mucin was added to the feed. Overview of the relative abundance (%) of the total bacterial community of the 13 most abundant genera in the SHIME colon vessels inoculated with the fecal sample of 8 different donors at the end of the mucin deprivation (day 8) and mucin supplementation (day 11). "Other" refers to the remainder of relative abundance at the genus level which are summed together.

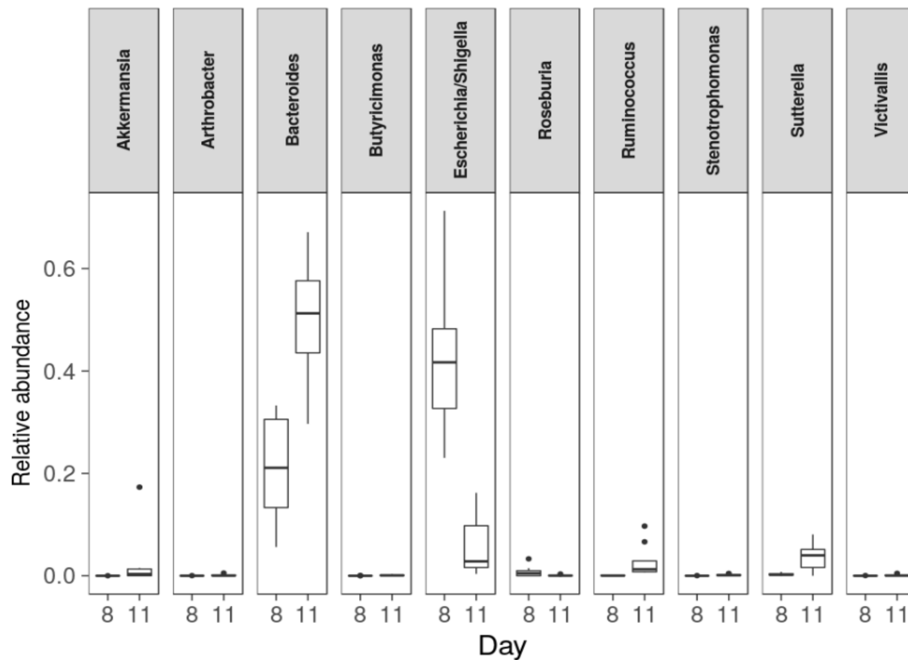

Figure 3. 5: Boxplots of the relative abundance of bacterial genera that are significantly different, as determined with DESeq hypothesis testing, between the mucin deprived community (day 8) and the mucin rich community (day 11) over all eight donors (P<0.01).

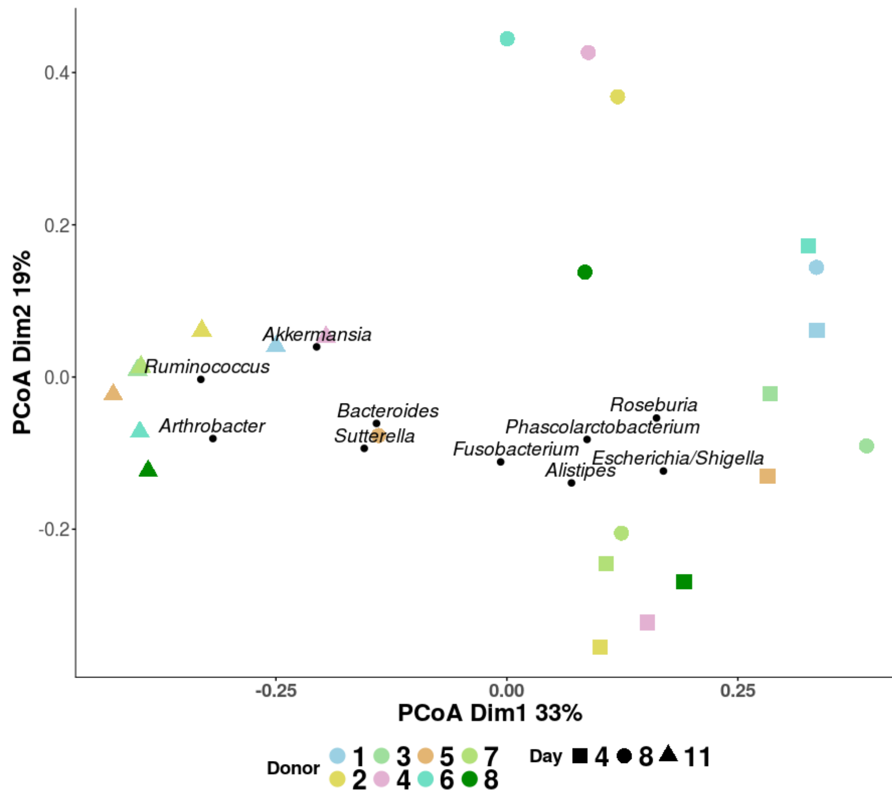

Figure 3. 6: A PCoA biplot revealed a distinct mucin rich (triangles) and mucin deprived (circles) microbial community for all 8 donors (colors). Squared represent bacterial communities at day 4 (mid-mucin deprivation). Weighted average scores of the most abundant genera characteristic of the mucin deprived and mucin rich bacterial community were a posteriori projected.

Co-occurrence network analysis was performed on the bacterial communities at day 4, day 8 and at day 11 (Supplementary Figure 3.4, 3.5, 3.6). Comparing the networks at day 4 and day 8 shows a reduction in network complexity during mucin deprivation, with a decrease in edge to node ratio from 2.22 at day 4 to 1.34 at day 8 and a decrease in interaction clusters. Upon mucin administration, the network complexity increases with an edge to node ratio of 1.34 at day 8 to 1.87 at day 11 and an increase in interaction clusters is observed. Also, *A. muciniphila* appears in center of an interaction cluster after mucin supplementation, which might indicate that it become a 'hub' or keystone species in the mucin rich bacterial community.

Akkermansia muciniphila is a known mucin degradation specialist and was in previous experiments shown to strongly respond to mucin supplementation (Ottman *et al.*, 2017a) (Chapter 2). In this study, qPCR analysis revealed that for six out of the eight donors (1,3,4,5,7,8), *A. muciniphila* abundances decreased during mucin deprivation and increased rapidly as soon as mucin was supplemented to the feed, with an average increase of $1.2*10^4$ (± $2*10^3$) (n=6) (Figure 3.7). This response was independent of the abundance of *A. muciniphila* at the beginning of the experiment, since in the case of donor 3, *A. muciniphila*

abundance was below the quantification limit at the start the experiment, but upon mucin supplementation, its relative abundance increased ten thousand fold. Donor 2, on the other hand, started with a higher initial abundance of *A. muciniphila* but did not respond to the mucin treatment. The relative abundance of *A. muciniphila* in donors 2 and 6 were close or under the qPCR detection limit and also with 16S rRNA gene sequencing no *A. muciniphila* was detected (Figure 3.7).

The relative abundance of the SCFA produced during the mucin experiment did not vary much between the donors, with an average of 70% (± 3%) acetate, 21% propionate (±3%) and 7% (±2%) butyrate (Figure 3.8). During mucin deprivation, there is a significant decrease in proportional and absolute concentrations of acetate ($p<0.01$) and butyrate ($p<0.01$) levels but addition of mucin to the feed did not significantly impact the SCFA composition ($p>0.05$). No difference could be observed between the SCFA profile for donors with an *A. muciniphila* response to the mucin treatment (1,3,4,5,7,8) compared to the donors without this response (2 and 6), so the increase in *A. muciniphila* did not affect the SCFA profiles of these responsive donors in the *in vitro* gut model.

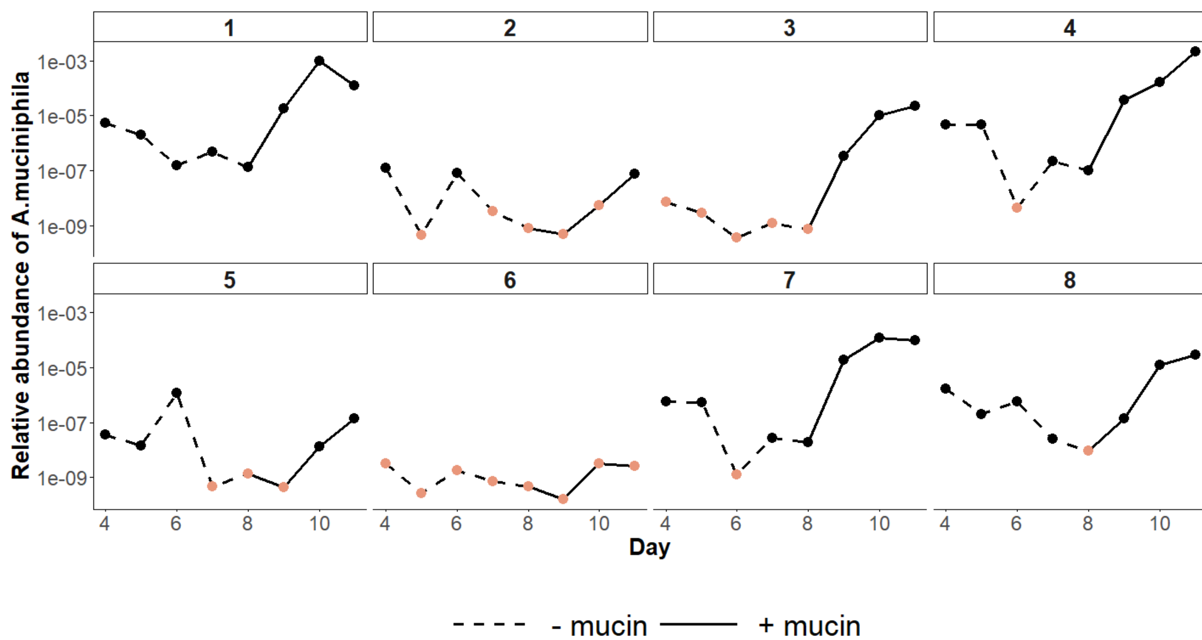

Figure 3. 7: Log (base 10) scaled relative abundance of *A. muciniphila* over total bacteria over time (days), measured with qPCR. SHIME vessels (inoculated with fecal inoculum from donors 1-8) were fed mucin free SHIME feed during 8 days (dotted line), after which mucin was supplemented to the feed (4g L^{-1}) (full line). Light red dots indicate values below the quantification limit.

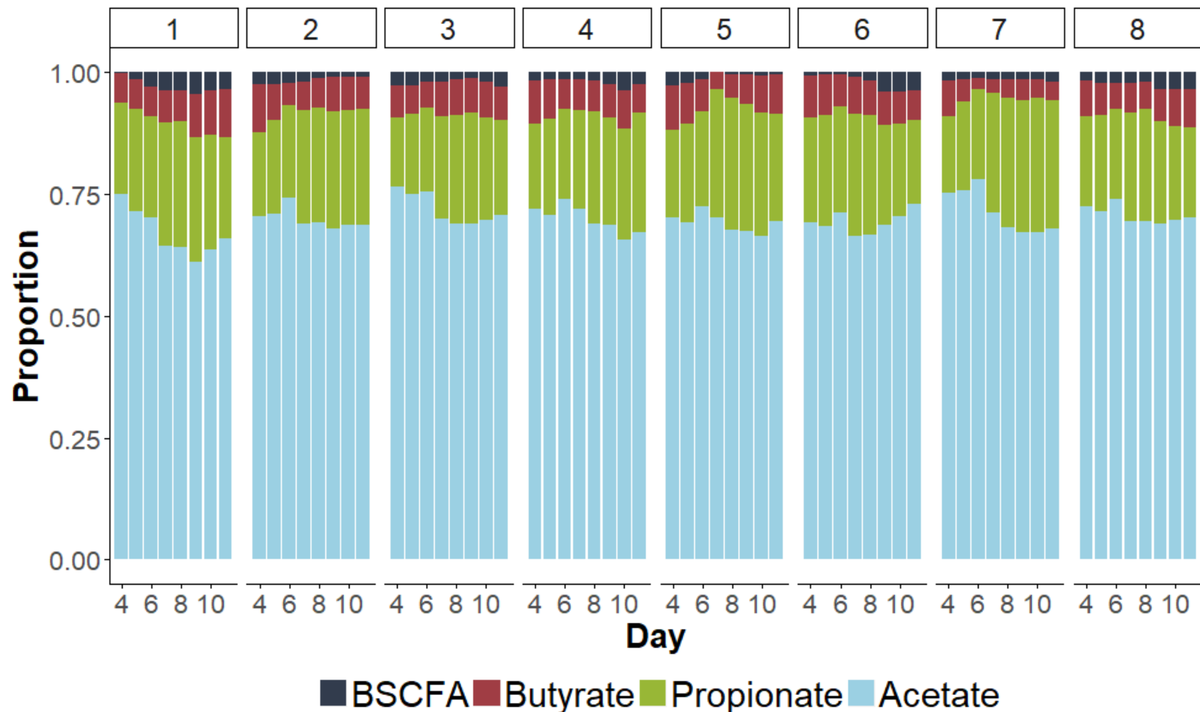

Figure 3. 8: Proportional values of short chain fatty acids acetate, propionate, butyrate and branched SCFA (BSCFA), produced overt time (days). SHIME vessels (inoculated with fecal inoculum from donors 1-8) were fed mucin free SHIME feed during 8 days, after which mucin was supplemented to the feed (4g L^{-1}).

3.2 Impact of pH on the bacterial community structure

The pH experiment (Figure 3.2) was set up to investigate whether the colonization preference of *A. muciniphila* for the distal colon due to its high pH, is dependent on initial *A. muciniphila* abundances and/or on the composition of the bacterial community.

16S rRNA gene amplicon sequencing showed that the differences between the bacterial communities at high (6.6-6.9) and low pH (5.6-5.9) are dependent on the donor (Figure 3.9). *Bacteroides* spp. was more abundant at high pH in donor 4, more abundant at low pH in donor 5 and 7 and equally abundant at both pH ranges in the other donors. *Clostridium* cluster XIVa species were less abundant at low pH in donors 1,3,4 and 6 but more abundant at high pH in donors 2 and 5 (Figure 3.9). *A. muciniphila* on the other hand, is, when detected, always more abundant at high pH (Figure 3.9). Microbial diversity in the communities was similar at high and low pH ($p>0.05$) as was the total bacterial count (Supplementary Figure 3.7 and 3.8) Using DESeq hypothesis testing, genera were identified that were significantly different in abundance between low and high pH over all eight donors (Figure 3.10). Most pronounced differences were higher relative abundances of *Akkermansia, Escherichia/Shigella* and *Ruminococcus* at high pH (Figure 3.10). A PCoA biplot showed both donor and pH act as determining factors for bacterial community

composition, with clustering of the communities according to pH, while maintaining high variability between donors (Figure 3.11). To quantify and distinguish between donor and pH effects a partial distance based rda analysis was performed, showing that pH (10%) and donor (9%) effects were limited but significant (p=0.006, resp. p=0.046) (Supplementary Table 3.3; Supplementary Figure 3.9).

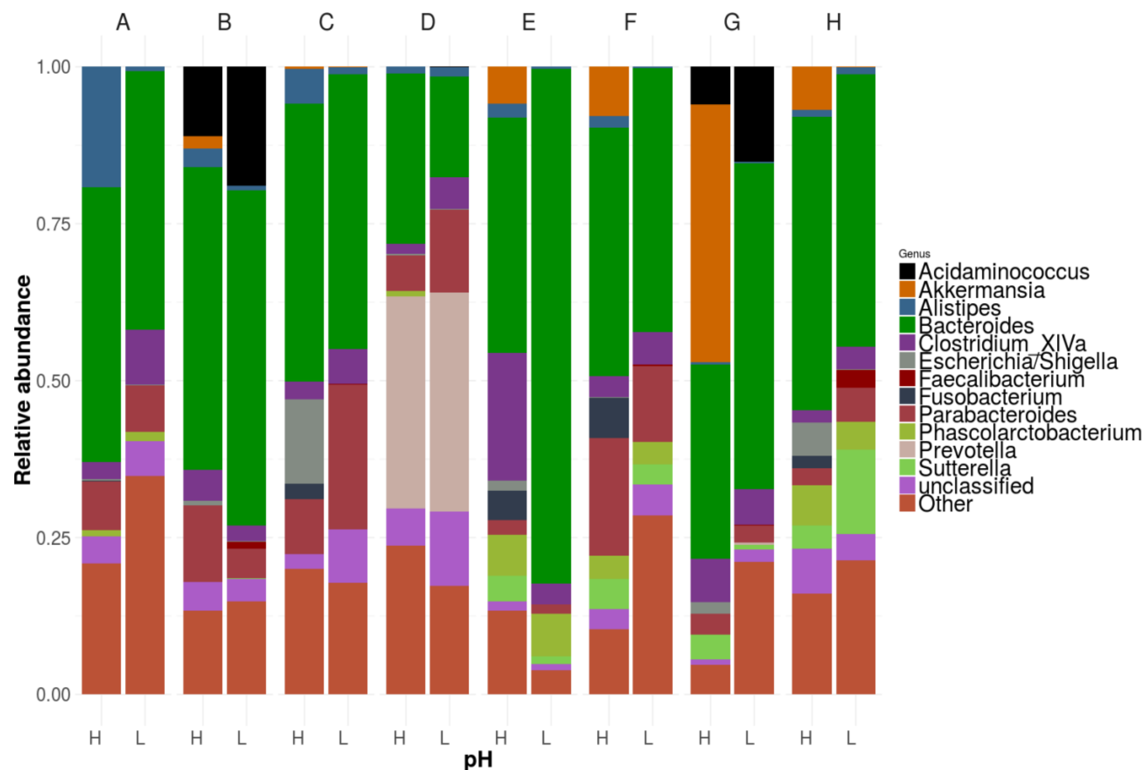

Figure 3. 9: Relative abundances at the genus level of the pH experiment at day 11. Colon vessels, inoculated with fecal inoculum from donors A-H, were kept at high pH (6.6-6.9) or at low pH (5.6-5.9) for 11 days after inoculation. Overview of the relative abundance (%) of the total community of the 13 most abundant genera in the SHIME colon vessels at day 11.). "Other" refers to the remainder of relative abundance at the genus level which are summed together.

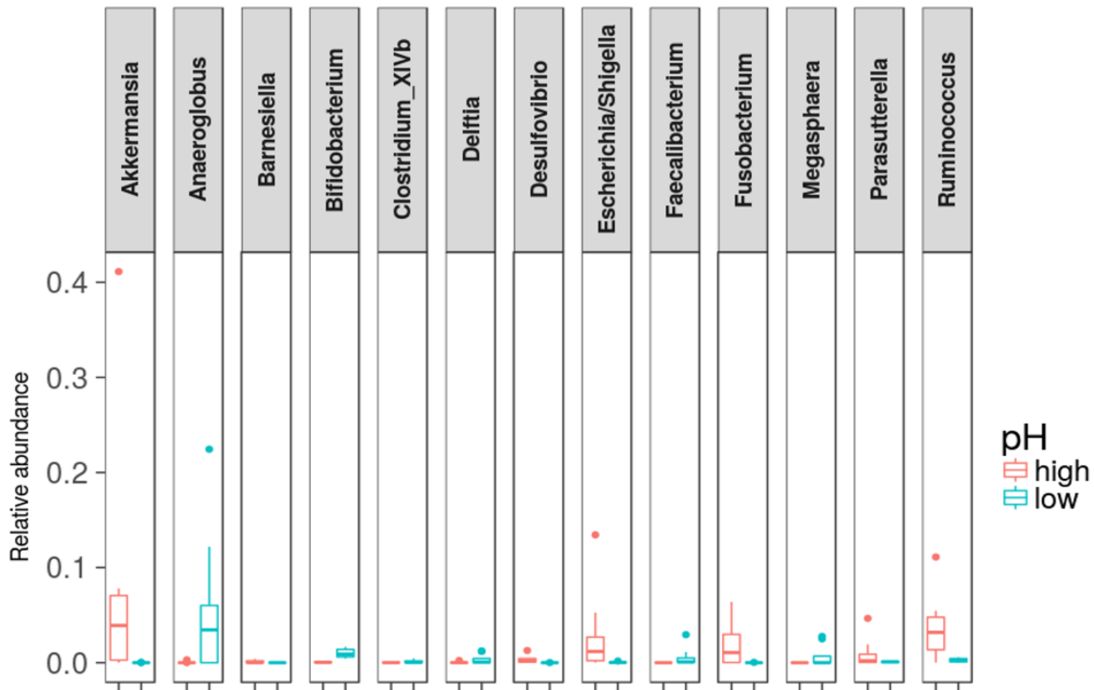

Figure 3. 10: Boxplots of the relative abundance of genera that are significantly different, determined with DeSEQ hypothesis testing, between the colon vessel at high and low pH at day 11 over all eight donors (α=0.01).

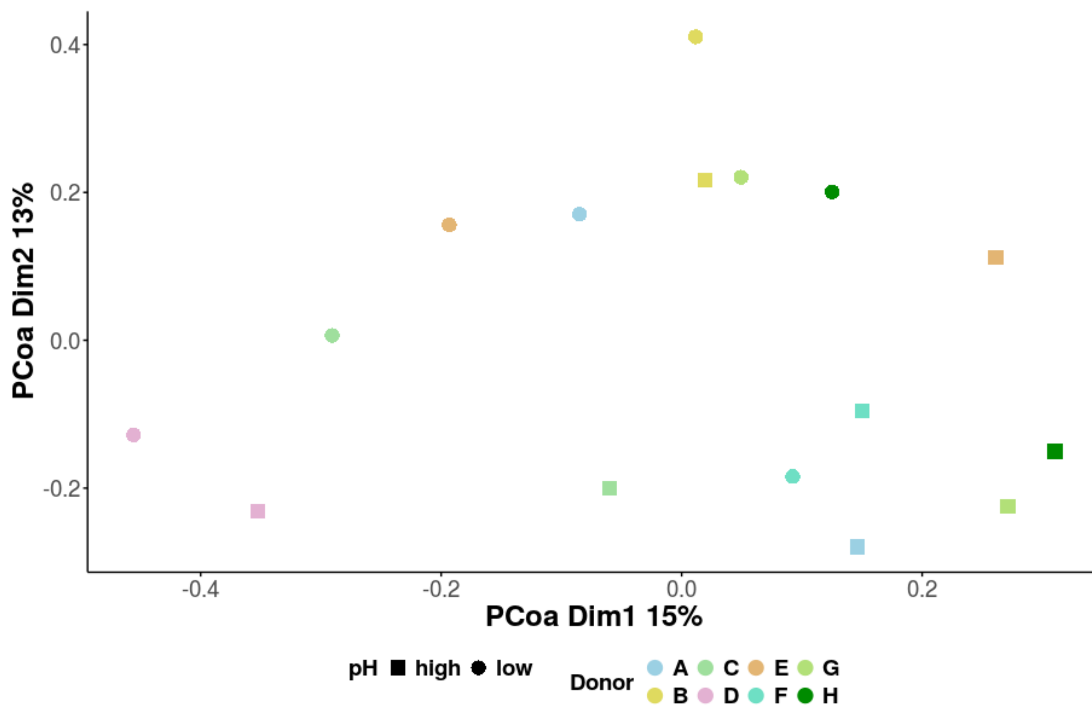

Figure 3. 11: A PCoA biplot of the microbial community at high pH (squares) and low pH (circles) for all 8 donors (colours) at day 11.

Co-occurrence network analysis at day 11 resulted in a high and low pH network (Supplementary Figure 3.10, 3.11). Both networks are of similar complexity with an edge to node ratio of 1.49 for the low pH co-occurrence network and of 1.56 for the high pH network.

qPCR analysis of *A. muciniphila* relative abundances over time, shows that for all donors except one, *A. muciniphila* colonizes the distal colon compartment at high pH (6.6-6.9) more abundantly than at low pH (5.6-5.9) (p=0.02), with average relative abundances of $2.33*10^{-2}$ (± $2.2*10^{-2}$) and $6.76*10^{-6}$ (±$1.28*10^{-5}$) respectively (Figure 3.12). This difference was not observed for donor A, where *A. muciniphila* abundances were very low at both low and high pH (Figure 3.12). pH also impacted the fermentation activity, with high pH values resulting in significantly more acetate and propionate and low pH values resulting in more butyrate (p<0.01). Yet, no significant differences in branched SCFA were observed (Figure 3.13). Only for donor A at low pH a proportionally higher propionate level was observed than at high pH. Overall, there was a higher total SCFA production at high pH compared to low pH (P<0.01).

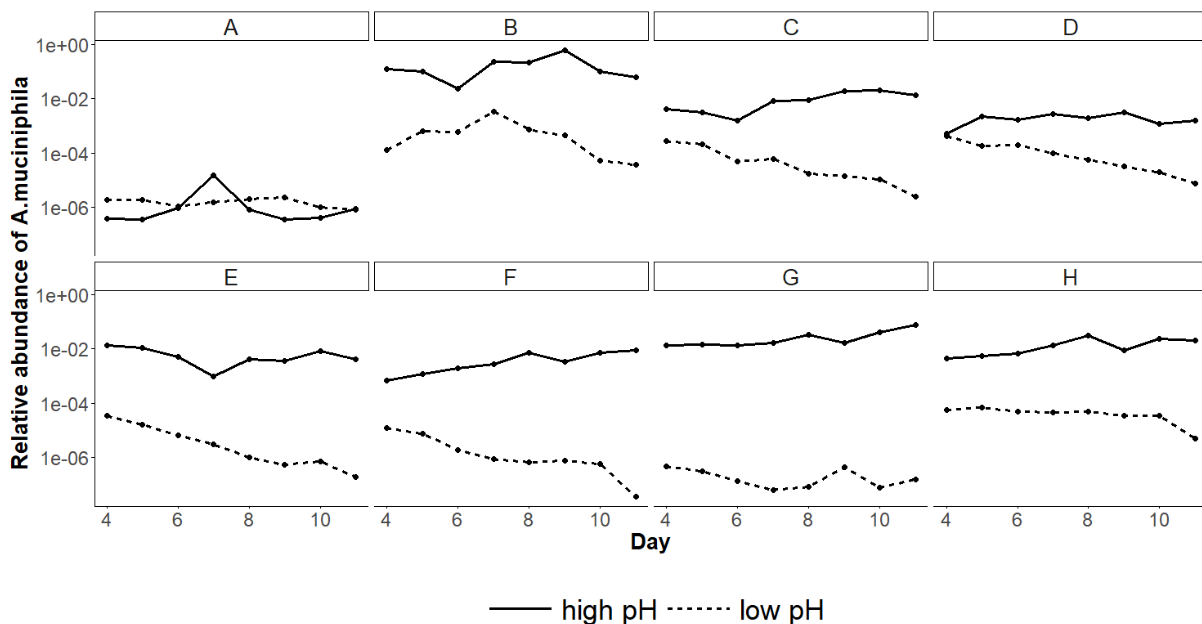

Figure 3. 12: Log (base 10) scaled Relative abundance of *A. muciniphila* compared to total bacteria over time (days), measured with qPCR. SHIME vessels (inoculated with fecal inoculum from donors A-H) were kept at either high pH (6.6-6.9), shown as the full line, or at low pH (5.6-5.9), shown as the dotted line.

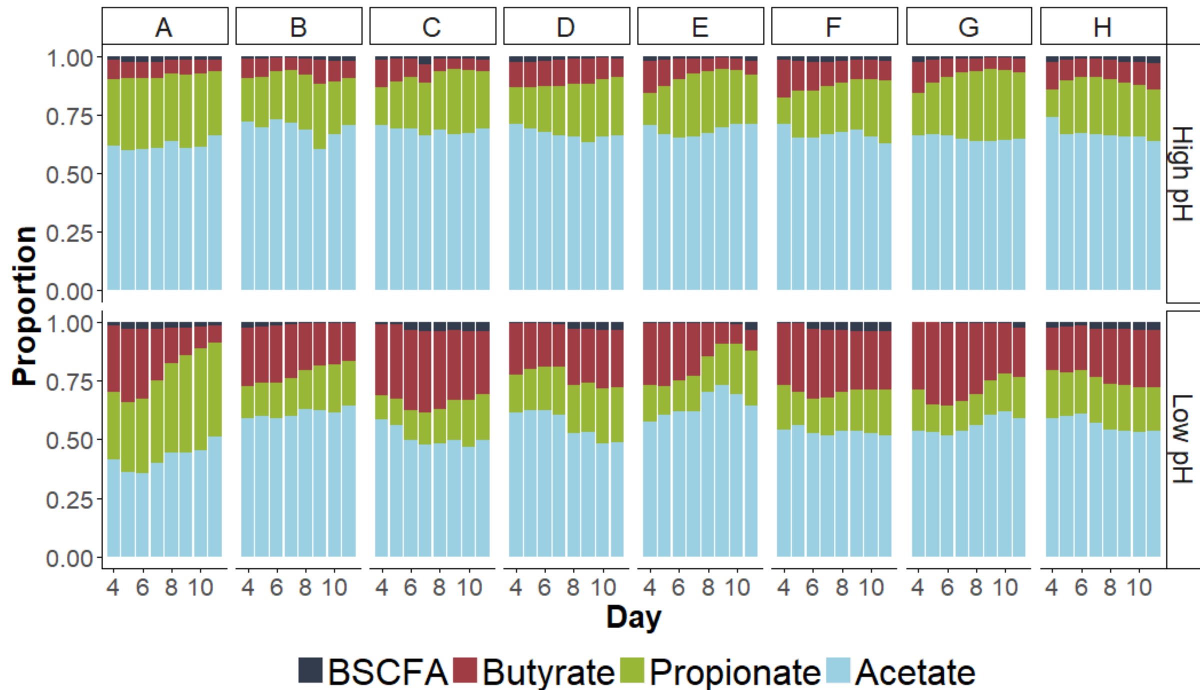

Figure 3. 13: Proportional values of short chain fatty acids acetate, propionate, butyrate and branched SCFA, produced over time (days). SHIME vessels (inoculated with fecal inoculum from donors A-H) were kept at either high pH (6.6-6.9) or low pH (5.6-5.9).

4. Discussion

Our finding that mucins as nutritional resource are a more important modulator of the gut microbiome than colon pH as environmental factor indicates that host glycan degradation represents a relevant ecological niche shaping the composition of the simulated colon microbiota. With (relatively) higher levels of *Akkermansia, Bacteroides, Ruminococcus, Sutterella and Arthrobacter,* the cluster of mucin-rich bacterial communities was significantly different from the mucin-deprived communities. Microbiome variation explained by host glycan presence (26%) exceeded the variability in microbiome composition that is explained by donor (8%), whereas the variance explained by the environmental factor (pH) did not (10% resp., 9%). This finding was characterized by a high biological reproducibility across the microbiota from 8 human donors. In spite of the increased nutritional value of the feed (addition of a host glycan) the total bacterial count remained constant, indicating that the carrying capacity of the *in vitro* colon ecosystem had been reached. This was also illustrated by the increase in above-mentioned genera corresponding with a decrease of for example *Roseburia, Alistipes* and *Escherichia/Shigella.* The addition of mucin and the change it caused in the bacterial community composition did not coincide with a change in SCFA production profile. Rather than an increase in microbial metabolic activity this could indicate that mucin supplementation induces a shift in microbial metabolism from fiber degradation

during the mucin deprivation period to proportionally more host glycan degradation during the mucin supplementation period. Changing the profile of substrate degradation is not a common trait across all members of the microbiota, but primarily reserved for 'glycan-generalists' like *Bacteroides* species. These have broad glycan-degradation abilities, both diet- and host-derived, and they can change their metabolism upon changing nutrient availability (Koropatkin *et al.*, 2012; Marcobal *et al.*, 2011; Marcobal *et al.*, 2013d; Salyers *et al.*, 1977). It was already shown in germfree mice, fed a high fat/low fiber diet and colonized with *E. rectale* and *B. thetaiotaomicron,* that *B. thetaiotaomicron* changes its metabolism to host glycan degradation (Mahowald *et al.*, 2009). In our study however, the fiber content remained constant and mucin was supplemented as an additional energy source. This indicates some *Bacteroides* spp. to have a preference for mucin degradation compared to fiber degradation. The higher abundances of *Akkermansia* and *Ruminococcus* in the mucin rich community are expected since *A. muciniphila* specialized in mucin degradation and several *Ruminococcus* spp. are known to degrade mucin, although the extent of the degradation varies between species and strains (Crost *et al.*, 2013; Png *et al.*, 2010).

A. muciniphila has been mentioned in multiple studies as correlated with human health and a potential probiotic, with recently promising results for the treatment of diabetes and obesity (Plovier *et al.*, 2017). In this study, the abundance of *Akkermansia* spp. was specifically influenced by the mucin treatment, as demonstrated with a specific qPCR assay and 16S rRNA gene sequencing, with a decrease in abundance during mucin deprivation and a sharp increase during mucin supplementation (Figure 3.4, 3.7). *Akkermansia* spp. are known as mucin degrading specialists, as reflected by the high percentage of mucin-consumption related functions encoded in the small genome of *Akkermansia*, as opposed to *Bacteroides* (Marcobal *et al.*, 2013d). Due to its specialization in mucin degradation it is expected that *Akkermansia* abundances would decrease when no mucin in available and respond rapidly to mucin supplementation. This was already shown for one donor with specifically high *Akkermansia* abundance in a previous study (Chapter 2). In the present study we show a similar response of *Akkermansia* to the mucin treatment in 6 out of 8 donors. Importantly, this response is independent of *Akkermansia's* initial abundance (Figure 3.7). However, the strength of the increase in abundance does vary between donors and this might be due to the presence of other bacteria that can compete for the mucin degradation. Moreover, several studies have suggested that bacteria adjust their metabolism depending on the identity of other bacteria in the environment (Mahowald *et al.*, 2009; Sonnenburg *et al.*, 2006). This was exemplified by Png *et al.* (2010) who showed that *A. muciniphila* grown on mucins in co-culture with non-mucolytic *B. fragilis*, was less abundant and degraded less mucins compared to monoculture. So it is possible that some species have the ability to discourage *A. muciniphila* from thriving on mucins, without actually competing for the mucins.

Our results show the dependence of *A. muciniphila* on mucin and thus future *in vivo* (probiotic) applications of *A. muciniphila* might benefit from prebiotics that show resemblance to mucin structures or compounds that can increase the mucin concentration, to ensure abundant colonization. Promising results regarding the former strategy have already been obtained by (Ottman, 2015) who demonstrated *Akkermansia's* capacity to grow on human milk oligosaccharides.

Co-occurrence networks showed a decreased complexity in network structure during mucin deprivation, which increased again after mucin supplementation. This increased complexity in co-occurrence network clusters may be an indication of mucin structural complexity requiring enhanced cross-feeding interactions between bacteria in order to consume it as a nutritional resource. In contrast, the co-occurrence networks of the pH experiment, showed no difference in complexity between high pH (6.6-6.9) and low pH (5.6-5.9). In contrast to mucin, the effect of colonic pH had a less profound impact in the microbiome with donor origin and pH explaining the variability in the dataset equally; among others *Bacteroides, Clostridium* cluster XIVa and *Parabacteroides* preference for high or low pH varied between the donors (Figure 3.9). To our knowledge, previous studies did not address these inter-individual differences in response to pH, although not many studies have been done regarding the impact of pH variation on specific bacteria within different complex microbial communities.

Some interesting observations were noted: *Bacteroides* species typically display lower growth rates at pH values lower than 6.5 and even become undetectable at pH lower than 5.5 (Duncan *et al.*, 2009; Walker *et al.*, 2005). However, this study showed *Bacteroides* species from donors 5 and 7 to have a preference for pH 5.6-5.9 over pH 6.6-6.9 . Secondly, members from the *Clostridium* cluster XIVa were previously shown to better tolerate lower pH and to profit from the decrease in *Bacteroides* by exploiting the available nutrients and increasing in numbers (Duncan *et al.*, 2009). In our study, this trade-off between *Bacteroides* and *Clostridium* cluster XIVa was not visible, but at the phylum level, *Firmicutes* abundance was higher at low pH and *Bacteroidetes* at high pH for all donors except donor 5. Thirdly, some differences in community composition between high and low pH were consistent for all donors. The increased relative abundance of *Escherichia/Shigella*, *Ruminococcus* and *Akkermansia* at high pH and of *Bifidobacterium* at low pH are supportive of previous findings (Duncan *et al.*, 2009; Roe *et al.*, 1998; Van Herreweghen *et al.*, 2017; Walker *et al.*, 2005). The metabolic differences (SCFA profile) between high and low pH were apparent for all the donors and show more butyrate at low pH and more propionate and acetate at high pH. Butyrate production by human gut microbes typically occurs at the expense of acetate. Our findings are in line with previous studies (Walker *et al.*, 2005) (Chapter 2) but the metabolic profile can only partially be linked to the bacterial composition. For example, the increased

propionate production at high pH may be due to higher amounts of propionate-producing *Akkermansia* and *Ruminococcus* species (Crost *et al.*, 2013; Derrien *et al.*, 2004). *Faecalibacterium* might contribute to the higher butyrate concentration at low pH, but butyrate producing *Clostridium* cluster XIVa was not more abundant at low pH in every donor. Thus the pH might affect the metabolic activity of certain species more than their actual abundance. Indeed, while the metabolic profile shows clear differences between low and high pH and almost no variations between donors, the clustering of the bacterial communities according to pH is less clear (Figure 3.11), and much more subjected to donor variability.

Inter-individual variability is a hallmark of microbiome composition in the human gut. While many factors shape the microbiome over time, our study demonstrates that host glycan presence, rather than colonic pH is a more important driver of microbiome composition. Microbiome variation explained by host glycan presence even exceeds the variability in microbiome composition that is commonly observed between individuals. The addition of host-glycans results in microbial communities with higher abundance of *Akkermansia,* while simultaneously increasing the complexity of the co-occurrence networks and potentially making *A. muciniphila* a key player. Our previous findings of *Akkermansia* taking clear benefit from mucin presence and higher colonic pH (Chapter 2), holds true for different biological backgrounds from different donors.

5. Acknowledgements

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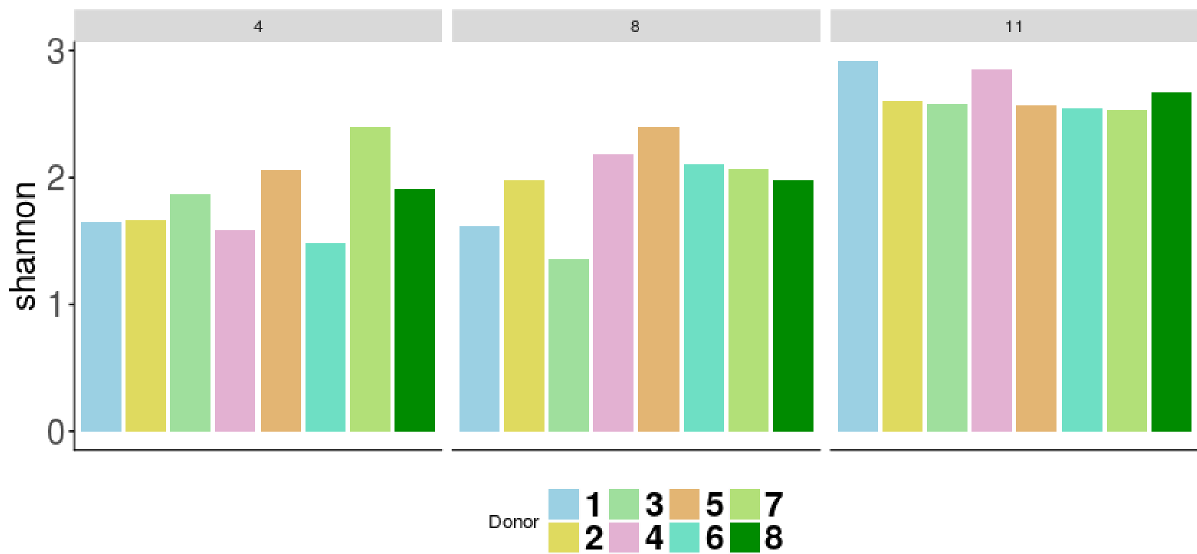
The authors would like to thank Jana De Bodt and Tim Lacoere for the technical support, Kim de Paepe for the support in analyzing the Illumina data and for the statistical support.

6. Supplementary information

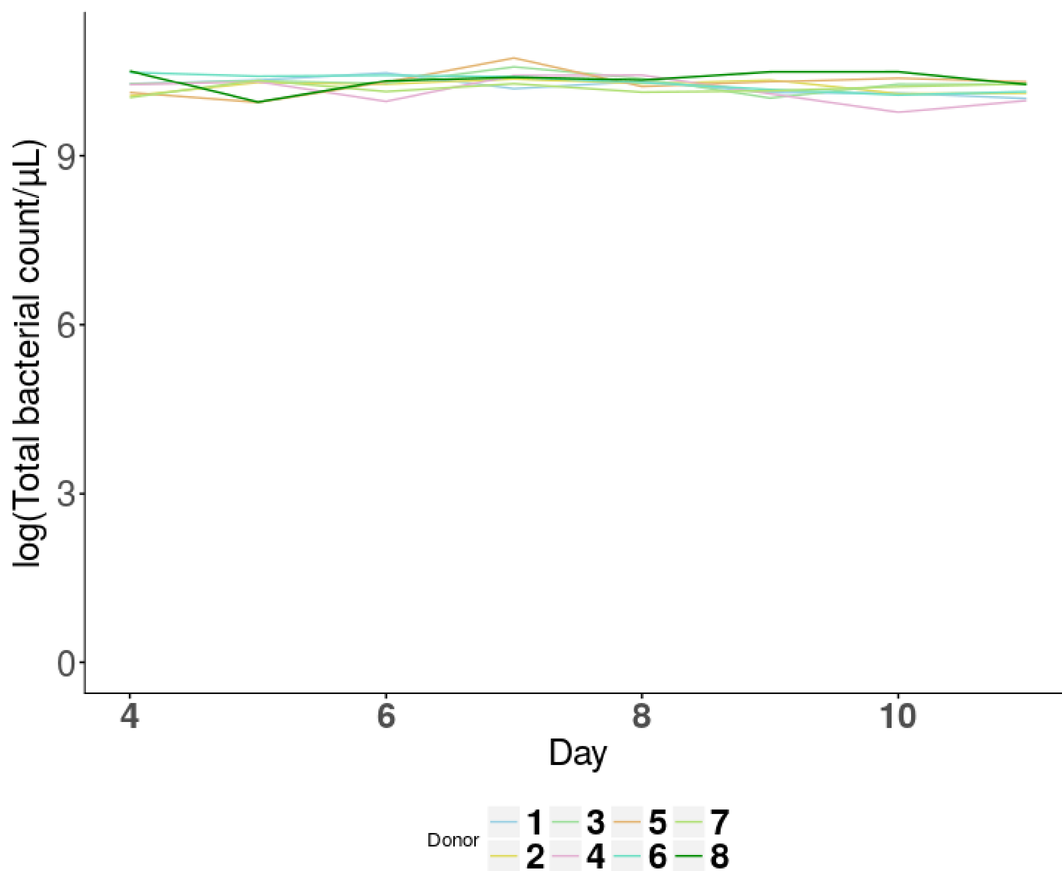
Supplementary Table 3. 1:RDP Seqmatch and NCBI BLAST results for the most abundant species in the microbial communities, as determined by amplicon sequencing. The similarity score (Sab) as calculated by RDP, and the NCBI BLAST output for the best hit and the next best hit(s) are shown.

		RDP	NCBI BLAST		
		Sab	**Query coverage (%)**	**E-value**	**Identity (%)**
OTU1	*Escherichia coli*	1	100	0	100
	Shigella sonnei	1	100	0	100
OTU2	*Bacteroides ovatus*	0.966	100	0	99
	Bacteroides xylanisolvens	0.877	100	0	98
	Bacteroides thetaiotaomicron	0.857	100	0	97
OTU3	*Bacteroides dorei*	1	100	0	100
	Bacteroides vulgatus	0.951	100	0	99
OTU4	*Bacteroides uniformis*	1	100	0	100
	Bacteroides rodentium	0.906	100	0	97
OTU5	*Clostridium boltaea*	1	100	0	100
	Clostridium clostridioforme	0.977	100	0	100
	Clostridium citroniae	0.964	100	0	99
OTU6	*Phascolarctobacterium faecium*	1	100	0	100
	Phascolarctobacterium succinatutens	0.734	100	0	93
OTU7	*Fusobacterium mortiferum*	0.995	100	0	100
	Fusobacterium nucleatum	0.964	100	0	95
OTU8	*Fusobacterium nucleatum*	1	100	0	100
	Fusobacterium simiae	0.946	100	0	99
OTU9	*Bilophila wadsworthia*	0.973			
	Desulfovibiro simplex	0.701	100	0	92
OTU10	*Bacteroides thetaiotamicron*	1	100	0	100
	Bacteroides faecichinchillae	0.947	100	0	99
OTU11	*Alistipes onderdonkii*	1	100	0	100
	Alistipes shahii	0.882	100	0	97
OTU12	*Bacteroides fragilis*	1	100	0	100
	Bacteroides thetaiotaomicron	0.827	100	0	94
OTU13	*Akkermansia muciniphila*	1	100	0	100
	Verrucomicrobium spinosum	0.567	99	0	84
OTU14	*Parabacteroides distasonis*	0.939	100	0	99
	Parabacteroides gordonii	0.696	100	2E-147	93
OTU15	*Klebsiella variicola*	1	100	0	100
	Klebsiella quasipneumoniae	0.968	100	0	99
OTU16	*Faecalibacterium prausnitzii*	0.918	100	0	99
	Subdoligranulum variabile	0.735	100	1E-165	93
OTU17	*Bacteroides cellulosilyticus*	0.966	100	0	100
	Bacteroides intestinalis	0.934	100	0	99
OTU18	*Bacteroides massiliensis*	1	100	0	100
	Bacteroides finegoldii	0.797	100	0	95

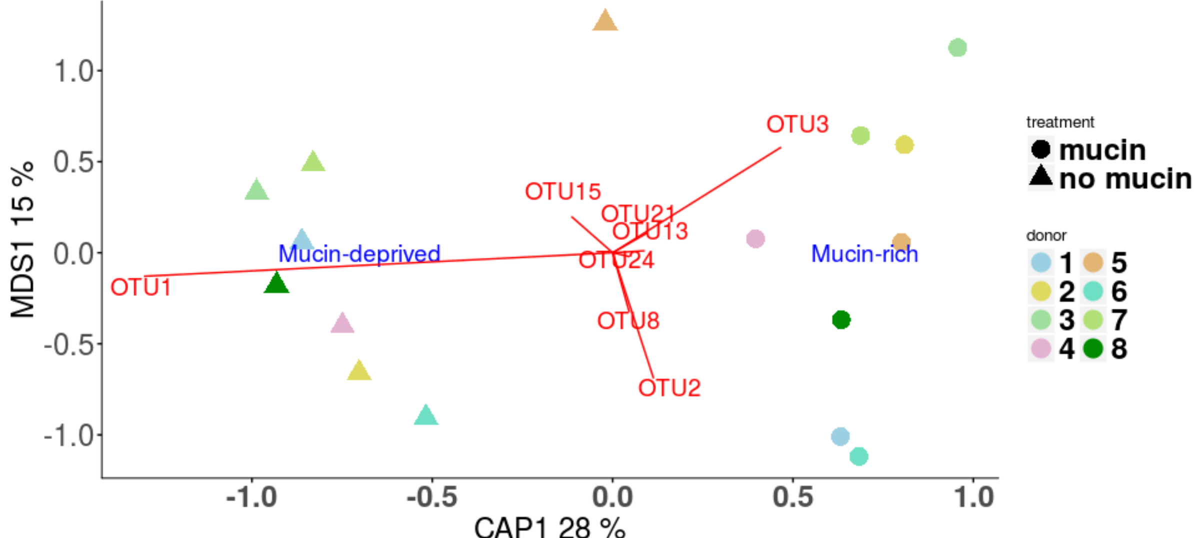
OTU19	*Sutterella wadsworthensis*	1	100	0	100
	Sutterella stercoricanis	0.793	100	0	94
OTU20	*Prevotella copri*	0.84	100	0	97
	Prevotella albensis	0.714	100	5E-175	93
OTU21	*Rumiococcus torques*	0.98	100	0	99
	Ruminococcus faecis	0.89	100	0	98
OTU22	*Pseudomonas aeruginosa*	1	100	0	100
	Pseudomonas otitidis	0.959	100	0	99
OTU23	*Bacteroides caccae*	0.983	100	0	99
	Bacteroides faecis	0.867	100	0	98
	Bacteroides finegoldii	0.86	100	0	98
OTU24	*Parabacteroides merdae*	1	100	0	100
	Parabacteroides johnsonii	0.896	100	0	98
OTU25	*Sutterella stercoricanis*	0.928	100	0	99
	Sutterella parvirubra	0.763	100	0	94
OTU26	*Parasuttelrella excrementihominis*	1	100	0	100
	Parasutterella secunda	0.603	100	4E-166	91
OTU27	*Phascolarctobacterium succinatutens*	1	100	0	100
	Phascolarctobacterium faecium	0.72	100	0	93
OTU28	*Megamonas funiformis*	0.93	100	0	98
	Megamonas rupellensis	0.908	100	0	98
OTU29	*Cloacibacillus evryensis*	1	100	0	100
	Cloacibacillus porcorum	0.832	100	0	97
OTU30	*Clostridium saccharolyticum*	0.849	100	0	97
	Clostridium xylanolyticum	0.849	100	0	97
	Clostridium asparagiforme	0.838	100	0	97



Supplementary Figure 3. 1: Alpha-diversity for the bacterial communities at OTU level for the different days. Alpha-diversity was significantly higher in the mucin-rich compared to the mucin deprived communities ($p<0.01$)



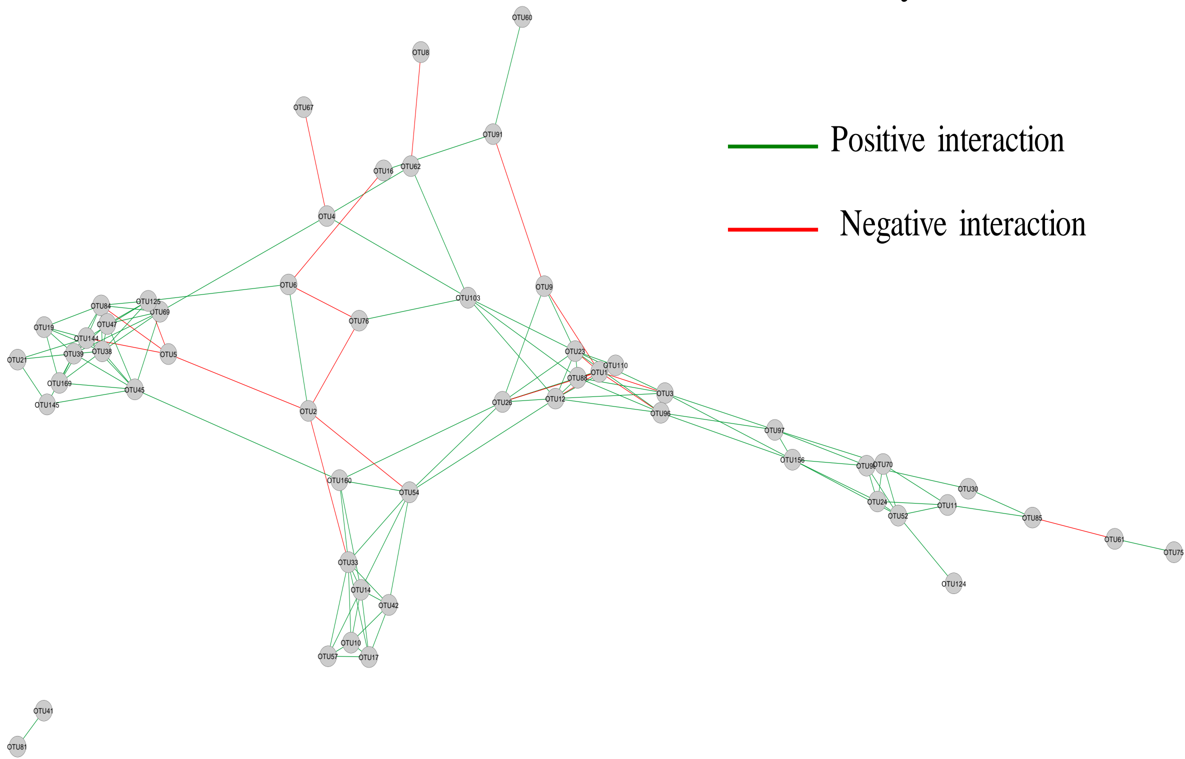
Supplementary Figure 3. 2: Log (base 10) scale of total bacterial counts over time (days), measured with qPCR. SHIME vessels (inoculated with fecal inoculum from donors 1-8) were fed mucin free SHIME feed during 8 days, after which mucin was supplemented to the feed (4g L^{-1}).



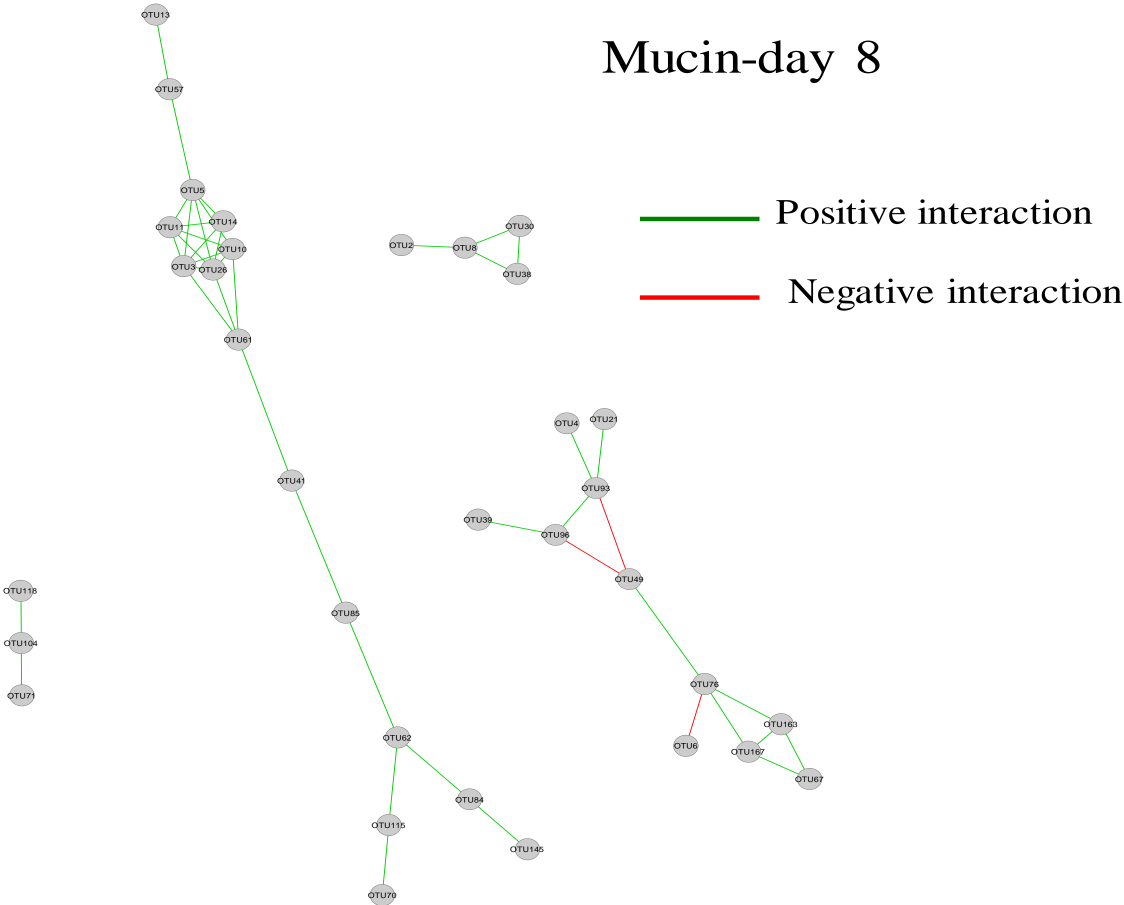
Supplementary Figure 3. 3: Partial distance based redundancy analysis correlation triplot. Treatment significantly (p=0.001) contributes to variation in species community composition. (OTU1~*Eschericia/Shigella*; OTU2, OTU3~*Bacteroides*; OTU8~*Fusobacterium*; OTU13~*Akkermansia*; OTU15~*Klebsiella*; OTU21~*Ruminococcus*; OTU24~*Parabacteroides*)

Supplementary Table 3. 2: Partial distance based redundancy analysis. The contribution of the different factors (mucin treatment and donor), and their significance level, to the variation in species level community composition.

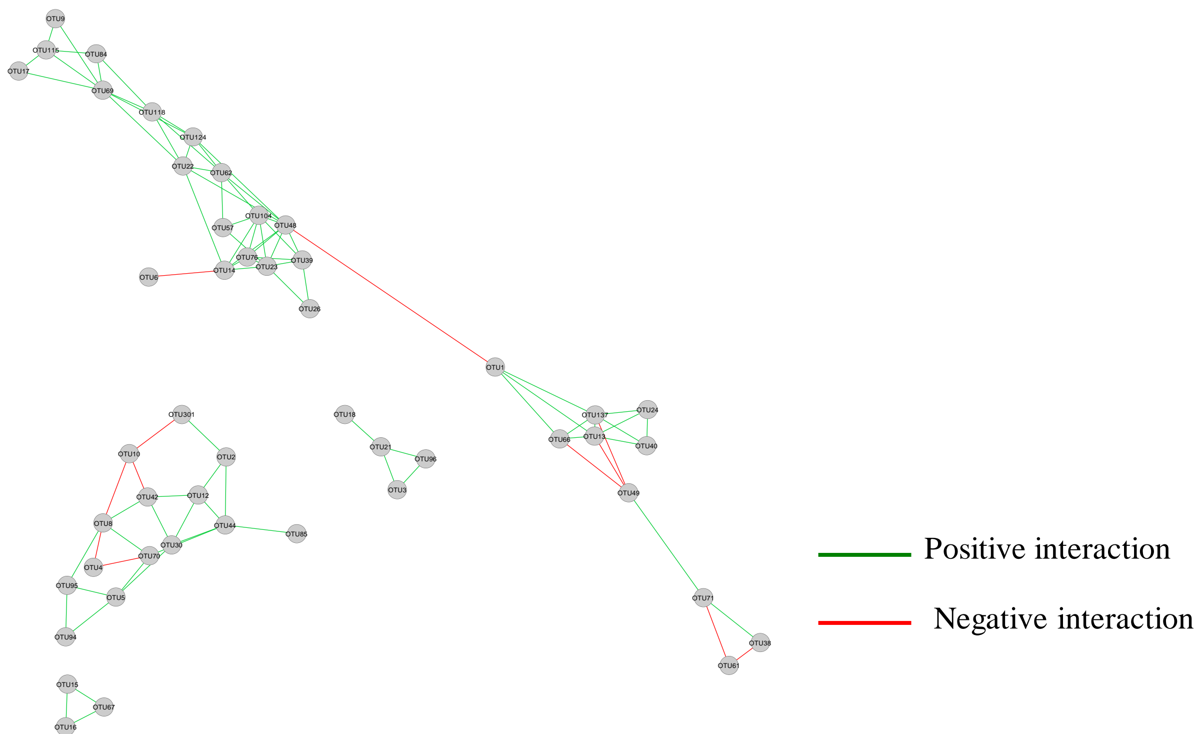
	% variance explained	p-value
Mucin	26%	0.001
Donor	8%	0.035



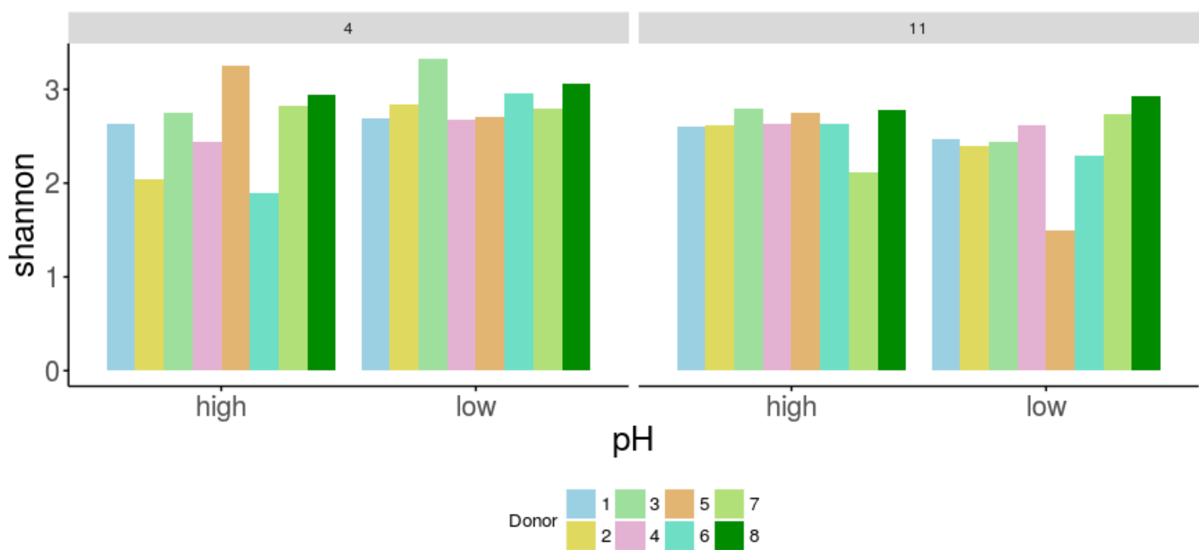
Supplementary Figure 3. 4: Co-occurrence network at OTU level for the eight donors after 4 days of mucin deprivation. (α=0.05, |r|>0.7)



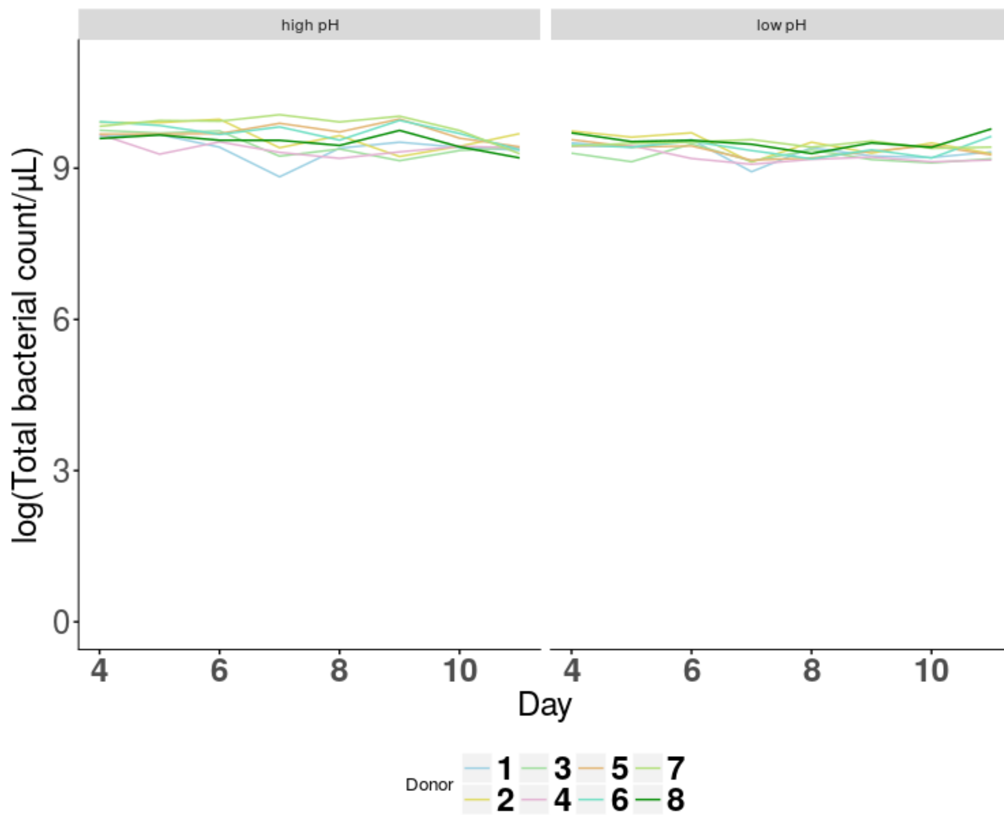
Supplementary Figure 3. 5: Co-occurrence network at OUT level for the eight donors after 8 days of mucin deprivation. (α=0.05, |r|>0.7)



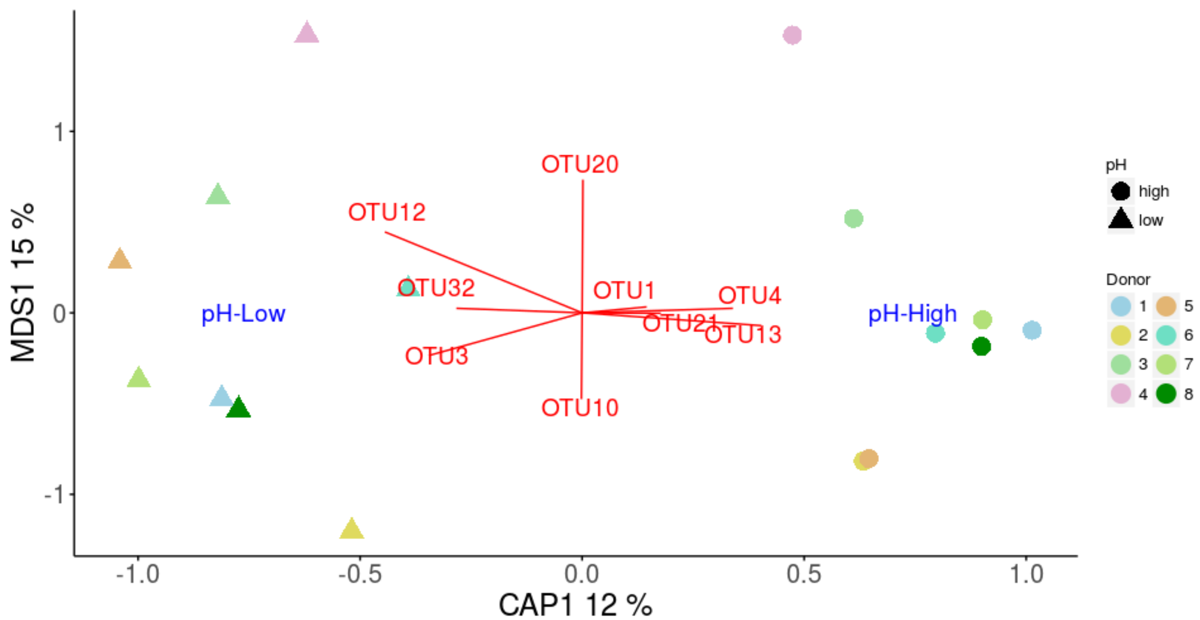
Supplementary Figure 3. 6: Co-occurrence network at OUT level for the eight donors after 4 days of mucin supplementation. (α=0.05, |r|>0.7)



Supplementary Figure 3. 7: Alpha-diversity for the bacterial communities at OTU level for the different days and at high (6.6-6.9) and low (5.6-5.9) pH. Alpha-diversity was not significantly different, at neither day 4 nor day 11, between high or low pH (p>0.05).



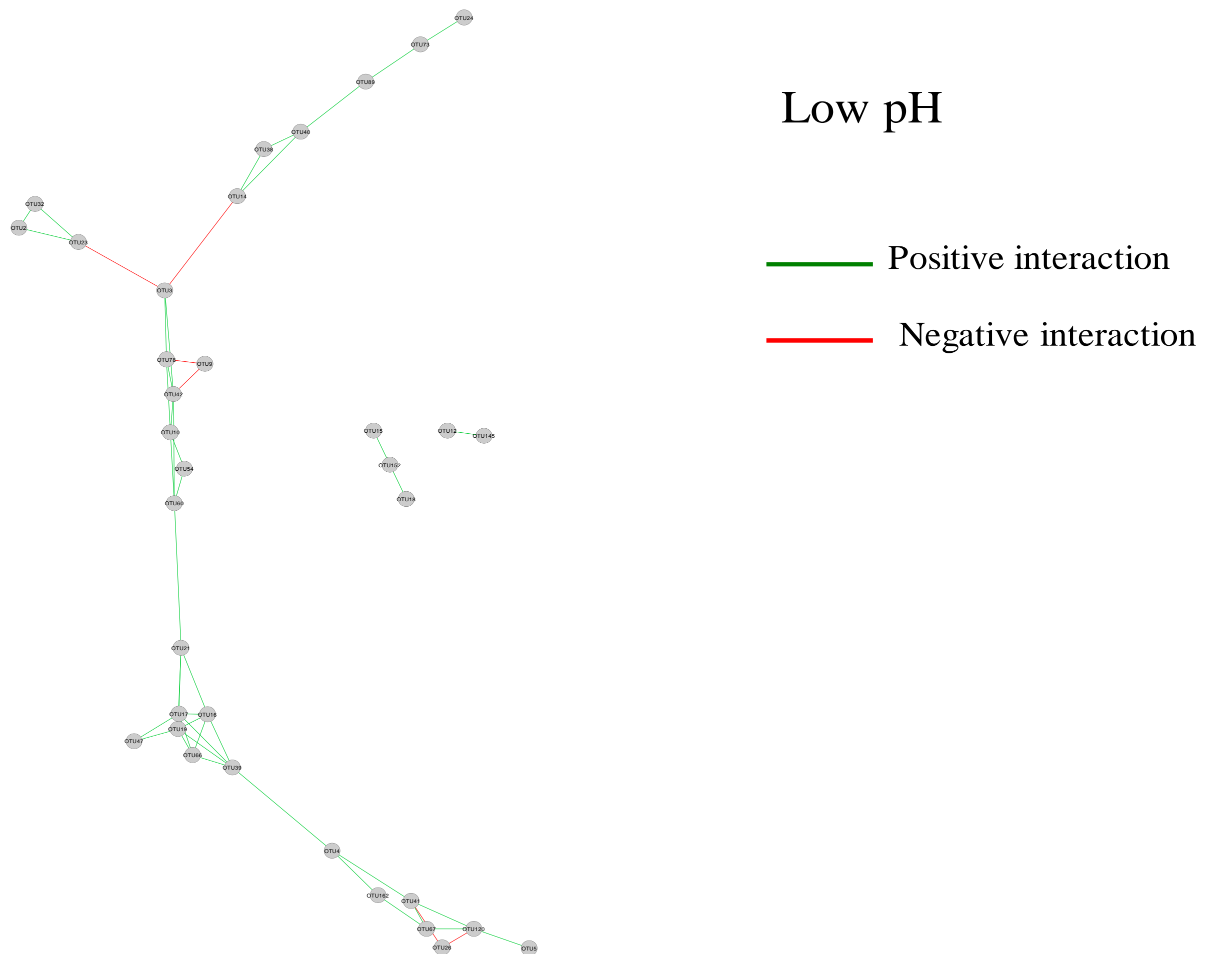
Supplementary Figure 3. 8: Log (base 10) scale of total bacterial counts over time (days), measured with qPCR. SHIME vessels (inoculated with fecal inoculum from donors 1-8) were kept at either low (5.6-5.9) or high (6.6-6.9) pH.



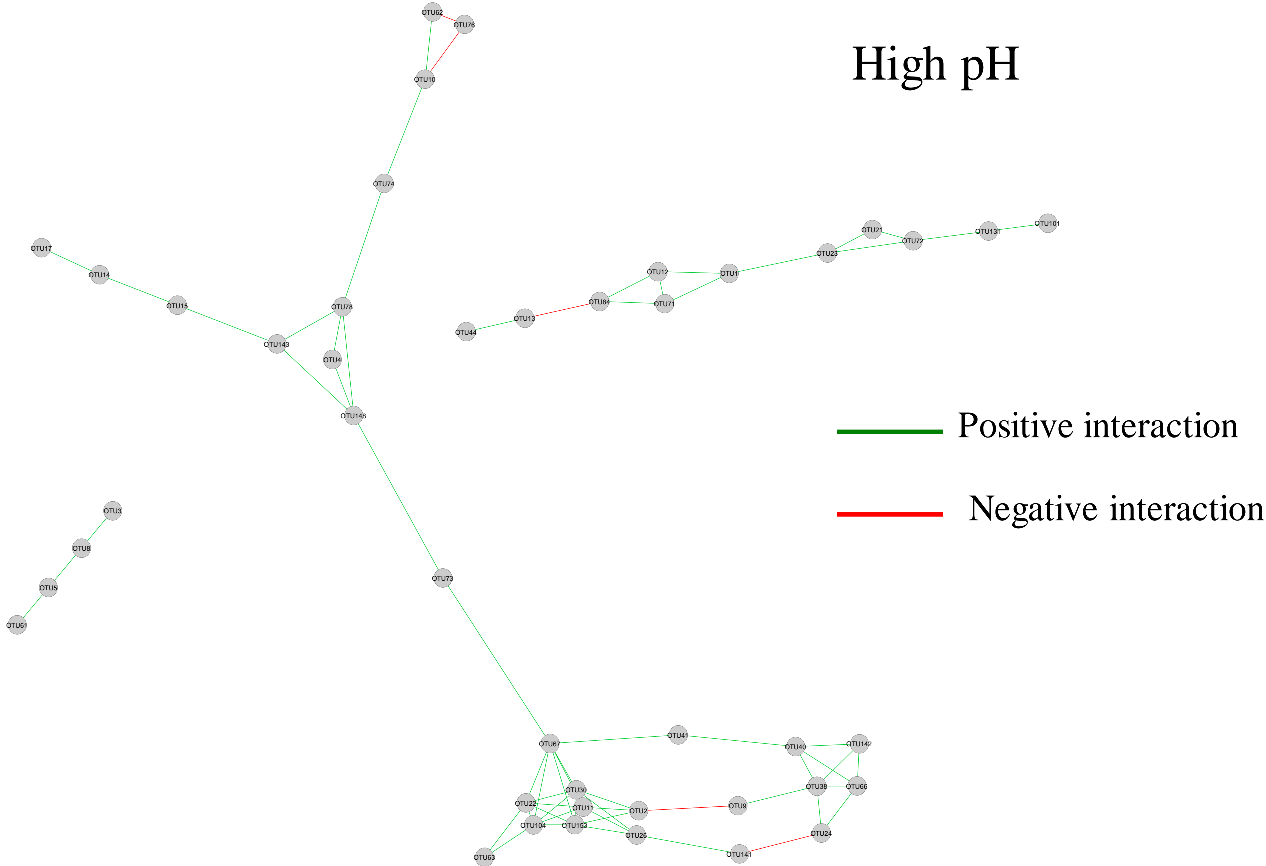
Supplementary Figure 3. 9: Partial distance based redundancy analysis correlation triplot. pH and donor display limited effects on variation in species community composition. (OTU1~*Eschericia/Shigella*; OTU3,OTU4,OTU10,OTU12~*Bacteroides*;OTU13~*Akkermansia*;OTU20~*Prevotella*; OTU21~*Ruminococcus*; OTU32~*Anaeroglobus*)

Supplementary Table 3. 3: Partial distance based redundancy analysis. The contribution of the different factors (pH and donor), and their significance level, to the variation in species level community composition.

	% variance explained	p-value
pH	10%	0.006
Donor	9%	0.046



Supplementary Figure 3. 10: Co-occurrence network at OTU level for the eight donors after 11 days stabilization at low pH (5.6-5.9). (α=0.05, |r|>0.7)



Supplementary Figure 3. 11: Co-occurrence network at OTU level for the eight donors after 11 days stabilization at high pH (6.6-6.9). (α=0.05, |r|>0.7)

Supplementary Table 3.4: Deepest level of taxonomic classification of first 300 OTUs.

	Taxonomy
OTU31	Acidaminococcus
OTU32	Anaeroglobus
OTU33	Gemmiger
OTU34	Bacteroides
OTU35	Bacteroidales
OTU36	Burkholderiales
OTU37	Acidaminococcus
OTU38	Lachnospiraceae
OTU39	Roseburia
OTU40	Stenotrophomonas
OTU41	Bifidobacterium
OTU42	Collinsella
OTU43	Dialister
OTU44	Clostridium_XlVa
OTU45	Oscillibacter
OTU46	Mitsuokella
OTU47	Bifidobacterium
OTU48	Alcaligenes
OTU49	Ochrobactrum
OTU50	Alphaproteobacteria
OTU51	Bacteroides
OTU52	Lachnospiraceae
OTU53	Dialister
OTU54	Ruminococcaceae
OTU55	Prevotella
OTU56	Megasphaera
OTU57	Clostridium_XlVa
OTU58	Dialister
OTU59	Lachnospiraceae
OTU60	Blautia
OTU61	Clostridium_XlVa
OTU62	Clostridium_XlVa
OTU63	Ruminococcaceae
OTU64	Parasutterella
OTU65	Desulfovibrio
OTU66	Achromobacter
OTU67	Bacteria
OTU68	Bacteria
OTU69	Clostridium_XlVa
OTU70	Ruminococcaceae
OTU71	Desulfovibrio
OTU72	Bacteroides
OTU73	Alistipes
OTU74	Clostridium_XlVa
OTU75	Veillonella
OTU76	Flavonifractor
OTU77	Burkholderia
OTU78	Lachnospiraceae
OTU79	Desulfovibrionaceae
OTU80	Megasphaera
OTU81	Streptococcus
OTU82	Burkholderiales
OTU83	Dialister
OTU84	Blautia
OTU85	Lachnospiraceae
OTU86	Bacteroides
OTU87	Pseudomonas
OTU88	Enterococcus
OTU89	Delftia
OTU90	Veillonella
OTU91	Ruminococcaceae
OTU92	Veillonellaceae
OTU93	Ruminococcus
OTU94	Dorea
OTU95	Allisonella
OTU96	Eubacteriaceae
OTU97	Victivallis
OTU98	Ruminococcaceae
OTU99	Alphaproteobacteria
OTU100	Ruminococcaceae
OTU101	Victivallis
OTU102	Coprococcus
OTU103	Firmicutes
OTU104	Lachnospiraceae
OTU105	Alphaproteobacteria
OTU106	Sutterella
OTU107	Ruminococcus
OTU108	Providencia
OTU109	Comamonas
OTU110	Veillonella
OTU111	Prevotella
OTU112	Barnesiella
OTU113	Proteobacteria
OTU114	Aeromonas
OTU115	Clostridium_XlVa
OTU116	Oscillibacter
OTU117	Ruminococcaceae
OTU118	Arthrobacter
OTU119	Lachnospiraceae
OTU120	Lachnospiraceae
OTU121	Morganella
OTU122	Ruminococcaceae
OTU123	Parabacteroides
OTU124	Bacteria
OTU125	Lachnospiraceae
OTU126	Clostridium_XlVb
OTU127	Parabacteroides
OTU128	Lysinibacillus
OTU129	Actinomycetales
OTU130	Clostridium_XlVa
OTU131	Alistipes
OTU132	Clostridium_XlVa
OTU133	Blautia
OTU134	Anaerostipes
OTU135	Enterobacteriaceae
OTU136	Lachnospiraceae
OTU137	Butyricicoccus
OTU138	Parasutterella
OTU139	Lachnospiraceae
OTU140	Mitsuokella
OTU141	Lachnospiraceae
OTU142	Butyricimonas
OTU143	Lachnospiraceae
OTU144	Dorea
OTU145	Pelomonas
OTU146	Alistipes
OTU147	Butyricicoccus
OTU148	Odoribacter
OTU149	Ruminococcaceae
OTU150	Oscillibacter
OTU151	Burkholderiales
OTU152	Alistipes
OTU153	Butyricimonas
OTU154	Burkholderiales
OTU155	Dialister
OTU156	Coprococcus
OTU157	Ruminococcaceae
OTU158	Oscillibacter
OTU159	Clostridium_XVIII
OTU160	Clostridiales
OTU161	Veillonellaceae
OTU162	Clostridium_XlVb
OTU163	Ralstonia
OTU164	Lachnospiraceae
OTU165	Alistipes
OTU166	Lachnospiraceae
OTU167	Clostridium_sensu_stricto
OTU168	Lachnospiraceae
OTU169	Oscillibacter
OTU170	Clostridiales
OTU171	Clostridium_XlVa
OTU172	Bacteroides
OTU173	Oscillibacter
OTU174	Acidaminococcaceae
OTU175	Collinsella
OTU176	Blautia
OTU177	Clostridiales
OTU178	Ruminococcaceae
OTU179	Lachnospiraceae
OTU180	Flavonifractor
OTU181	Lachnospiraceae
OTU182	Lachnospira
OTU183	Bacteria
OTU184	Firmicutes
OTU185	Paraprevotella
OTU186	Eggerthella
OTU187	Bacteria
OTU188	Dialister
OTU189	Ruminococcaceae
OTU190	Clostridium_XlVa
OTU191	Butyricimonas
OTU192	Bacteria
OTU193	Lachnospiraceae
OTU194	Clostridiales
OTU195	Ruminococcaceae
OTU196	Coriobacteriaceae
OTU197	Enterobacteriaceae
OTU198	Sediminibacterium
OTU199	Bacteria
OTU200	Lachnospiraceae
OTU201	Ruminococcaceae
OTU202	Clostridium_IV
OTU203	Clostridiales
OTU204	Bifidobacterium
OTU205	Blautia
OTU206	Bifidobacterium
OTU207	Alistipes
OTU208	Clostridium_IV
OTU209	Butyricimonas
OTU210	Bradyrhizobium
OTU211	Clostridium_IV
OTU212	Alloprevotella

OTU213	Proteus
OTU214	Erysipelotrichaceae
OTU215	Streptococcus
OTU216	Clostridium_IV
OTU217	Bacteroides
OTU218	Clostridium_IV
OTU219	Anaerofilum
OTU220	Clostridiales
OTU221	Ruminococcaceae
OTU222	Lachnospiraceae
OTU223	Clostridium_XlVa
OTU224	Bacteria
OTU225	Clostridium_IV
OTU226	Bacteroides
OTU227	Oscillibacter
OTU228	Erysipelotrichaceae
OTU229	Clostridium_XlVb
OTU230	Clostridium_XVIII
OTU231	Dialister
OTU232	Anaeroglobus
OTU233	Holdemania
OTU234	Lachnospiraceae
OTU235	Clostridiales
OTU236	Ruminococcaceae
OTU237	Ruminococcaceae
OTU238	Lachnospiraceae
OTU239	Ruminococcaceae
OTU240	Prevotellaceae
OTU241	Bacteroides
OTU242	Ruminococcaceae
OTU243	Oxalobacteraceae
OTU244	Dialister
OTU245	Propionibacterium
OTU246	Bacteroides
OTU247	Clostridium_IV
OTU248	Clostridiales
OTU249	Collinsella
OTU250	Firmicutes
OTU251	Lentisphaerae
OTU252	Ruminococcaceae
OTU253	Ruminococcaceae
OTU254	Cloacibacillus
OTU255	Bacteroidales
OTU256	Anaerostipes
OTU257	Firmicutes
OTU258	Ruminococcaceae
OTU259	Clostridiales
OTU260	Bacteroides
OTU261	Alistipes
OTU262	Streptococcus
OTU263	Bilophila
OTU264	Streptococcus
OTU265	Bacteroidetes
OTU266	Bacteria
OTU267	Clostridiales
OTU268	Ruminococcaceae
OTU269	Ruminococcaceae
OTU270	Sutterella
OTU271	Proteobacteria
OTU272	Bacteria
OTU273	Clostridiales
OTU274	Lachnospiraceae
OTU275	Betaproteobacteria
OTU276	Butyricicoccus
OTU277	Sphingomonas
OTU278	Lachnospiraceae
OTU279	Bacteria
OTU280	Firmicutes
OTU281	Enterobacteriaceae
OTU282	Desulfomicrobium
OTU283	Bacteroides
OTU284	Enterobacteriaceae
OTU285	Lachnospiraceae
OTU286	Megamonas
OTU287	Ruminococcaceae
OTU288	Ruminococcaceae
OTU289	Ruminococcaceae
OTU290	Collinsella
OTU291	Bacteria
OTU292	Ruminococcaceae
OTU293	Clostridiales
OTU294	Pleomorphomonas
OTU295	Roseburia
OTU296	Bacteroides
OTU297	Firmicutes
OTU298	Bilophila
OTU299	Flavonifractor
OTU300	Klebsiella

CHAPTER 4

A synthetic gut ecosystem demonstrates that variable ratios of primary degraders do not impact butyrate producing functionality despite phylogenetic shifts

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This chapter has been redrafted after

Van Herreweghen, F., Rotsaert, C., Van de Wiele, T. A synthetic gut ecosystem demonstrates that variable ratios of primary degraders do not impact butyrate producing functionality despite phylogenetic shifts. *Manuscirpt in preparation.*

CHAPTER 4
A synthetic gut ecosystem demonstrates that variable ratios of primary degraders do not impact butyrate producing functionality despite phylogenetic shifts

Abstract

Glycan degradation is considered an important driver of microbial metabolic networks in the human colon, facilitating the production of for instance butyrate, a fermentation product with health-modulatory potential. Using a synthetic microbial community, the competitiveness of the primary degraders *A. muciniphila* and *B. thetaiotaomicron* to occupy different glycan degrading functional niches (dietary vs. host glycans) under altered physicochemical (neutral vs. slightly acidic pH) conditions and the subsequent impact on a population of cross-feeding butyrate producers was evaluated. Trimming down ecosystem complexity to eight-species consortia allowed us to monitor the individual microbial species. Joint presence of both primary degraders did not lead to a competitive exclusion in the presence of mucin and *A. muciniphila* was not outcompeted. Shifts in pH and primary degrader abundance was selective for butyrate producers (*A. caccae* as opposed to *F. prausnitzii*) while the butyrate producing functionality was maintained. This indicates that functional redundancy facilitating functional stability is an important feature of gut microbial ecosystems even at a miniaturized scale.

1. Introduction

The human gut microbiome is characterized by enormous species richness and diverse functionality, determining ecosystem dynamics, interaction with the human host and eventually impacting human health. A hallmark feature of the gut microbiome is its functional redundancy, putatively facilitating functional stability and ecosystem resilience during periods of stress (Moya and Ferrer, 2016), but also allowing specific microorganisms to respond in a versatile manner during changing nutrient conditions. A dominant factor influencing community composition and functionality is the influx of glycans, both from dietary as host origin into the colon.

On the one hand, dietary fiber presents a functional niche that can be occupied by several gut microorganisms and that can trigger the proliferation of primary and secondary carbohydrate degraders, subsequently stimulating cross-feeding microorganisms that become part of a larger microbial metabolic network. The recommended daily intake of dietary fiber is 28-35 grams but in many cases – especially in industrialized countries – the actual uptake is much lower (Burkitt, 1987; Sonnenburg and Sonnenburg, 2014). Dietary fiber can be composed of complex carbohydrates like resistant starch, pectins and xylans. As these compounds are mostly indigestible in the upper digestive tract, they reach the colon in an intact manner (Cummings and Englyst, 1987; Silvester *et al.*, 1995). Their complex structure typically requires a variety of bacterial enzymes to be fully degraded and this glycan degrading capacity is typically determined at species and strain level. Bacteria with limited enzymatic capacity are therefore susceptible to dietary changes or dependent on other bacteria for cross-feeding. Other bacteria display a much broader glycan degrading capacity with *Bacteroides thetaiotaomicron* being the best-studied species. It was shown that *B. thetaiotaomicron* can change its metabolism between dietary nutrient, host glycan or human milk oligosaccharide (HMO) degradation depending on glycan availability (Bjursell *et al.*, 2006; Mahowald *et al.*, 2009). With respect to the functional niche of glycan degradation *Bacteroides* species are generally considered highly versatile microorganisms, possibly explaining their prevalence as dominant species in the colon.

On the other hand, host glycans derived from the mucus layer present a more continuous source of nutrients to the gut microbiota, unlike dietary glycans that are more variable in supply and composition. Mucin glycans are composed of O-glycosylated, and to a lesser extent N-glycosylated, protein backbones, with glycosyl chains of 2-12 monosaccharides, mainly consisting of galactose, fucose, N-acetylgalactosamine, N-acetylglucosamine, mannose and sialic acid (Lai *et al.*, 2009). Only few species have the enzymatic capacity for initiating partial or full mucin degradation, including *A. muciniphila, B. thetaiotaomicron, B. fragilis, Ruminococcus gnavus, R. torques, and Bifidobacterium*

bifidum (Hoskins *et al.*, 1985; Marcobal *et al.*, 2013d; Martens *et al.*, 2008; Png *et al.*, 2010). *A. muciniphila* is considered a mucin-degrading specialist and its high mucin-degrading capacity was shown in an *in vivo* mice study (Berry *et al.*, 2013; Ottman *et al.*, 2017a). Other *in vitro* studies, studying *A. muciniphila* in a complex microbial community, have shown its high dependency on mucin availability and its sensitivity to pH changes (Chapter 2, Chapter 3). So as opposed to *B. thetaiotaomicron*, which is flexible in its glycan degrading portfolio and tolerates a wider pH range (Duncan *et al.*, 2009), *A. muciniphila* has more stringent growth conditions. Interestingly, research over the last decade has seen a remarkable correlation between *A. muciniphila* abundance and gut health (Collado *et al.*, 2007; Png *et al.*, 2010; Wang *et al.*, 2011; Zhang *et al.*, 2009). In a study with obese mice on a high-fat diet, it was shown that administration of *A. muciniphila* reversed insulin resistance, dyslipidemia, metabolic endotoxemia and fat mass gain (Everard *et al.*, 2013). This biotherapeutic potential of *Akkermansia* has triggered questions around its interaction with other bacteria, its response to changing nutritional conditions and its competitiveness with bacteria that can occupy the functional niche of mucin metabolism.

Glycan degradation is considered an important driver of microbial metabolic networks facilitating the production of for instance butyrate, a fermentation product with health-modulatory potential (Brahe *et al.*, 2013; Guilloteau *et al.*, 2010). Following up on our previous findings (Chapter 2, Chapter 3) we studied the competitiveness of the primary degraders *Akkermansia* and *Bacteroides* to occupy different glycan degrading functional niches (dietary vs. host glycans) under altered physicochemical (neutral vs. slightly acidic pH) conditions. The subsequent impact on a population of cross-feeding butyrate producers was also evaluated. As one-on-one microbial interactions are often difficult to discern in the complex background of human gut microbiota, we used a synthetic ecosystem approach, similar to the approach of Desai *et al.* (2016). Trimming down ecosystem complexity to eight-species consortia allowed us to monitor the individual microbial species.

2. Materials and methods

2.1 Growth media and bacterial strains

Chemicals were obtained from Sigma (Bornem, Belgium), unless stated otherwise. The nutritional medium for the experiment, in which the synthetic community was grown, consisted of (in g L^{-1}) arabic gum* (1.0), starch (1) (Anco, Roeselare, Belgium), xylan* (1.0) (Carl Roth, Germany), pectin* (2.0), D-(+)-glucose (0.4), yeast extract (3.0) (Oxoid Ltd., Basingstoke, Hampshire, UK), peptone (1.0) (Oxoid Ltd., Basingstoke, Hampshire, UK), and commercial pig gastric mucin° Type II (4.0).

Depending on the imposed nutritional conditions of the experiment either mucin° alone, fibers* alone or both°* were added. Depending on the imposed pH either 8.66 g L^{-1} KH2PO4 and 13.67 g L^{-1} Na2HPO4 (High pH, pH 7.0) or 18.91 g L^{-1} KH2PO4, 2.98 g L^{-1} Na2HPO4 and 4.2 g L^{-1} NaHCO3 (Low pH, pH 6.1) were added. The bacteria were grown as pure cultures in anaerobic M2GSC medium at pH 6 prepared as described by Miyazaki *et al.* (1997) using 15 % (v/v) of clarified rumen fluid instead of 30 % (v/v).

The synthetic community consisted of 5 species as a standard inoculum, being butyrate producers *Butyricicoccus pullicaecorum* (LMG 24109), *Anaerostipes caccae* (DSMZ 14662) and *Faecalibacterium prausnitzii* (DSMZ 17677) and lactate producers *Bifidobacterium longum* (LMG 11047) and *Lactobacillus plantarum* (LMG 9211). To this standard inoculum, different ratios of mucin degrader *Akkermansia muciniphila* (DSMZ 22959) and versatile glycan degrader *Bacteroides thetaiotaomicron* (LMG 11262) were added.

After 24h growth the pure cultures were washed with anaerobic PBS (0.8 g L^{-1} NaCl and 0.2 g L^{-1} KCl) in an anaerobic (10 % CO_2 and 90 % N_2) workstation (GP Campus, Jacomex, Dagneux, France). Using flow cytometry (cfr. infra), the bacteria were quantified and $1*10^6$ bacteria mL^{-1} of each species was inoculated at the start of the experiment. For the inoculum A:B(1000:1) (cfr. infra) 10^6 bacteria mL^{-1} of *A.muciniphila* and 10^3 bacteria mL^{-1} of *B.thetaiotaomicron* were added.

2.2 Experimental set-up

To study the competition and cross-feeding interactions between the bacteria in this synthetic bacterial community, several environmental conditions and different primary degraders were tested (Table 4.1). Three media with different carbohydrate sources (fibers, mucin or both), at low (6.1) and high (7) pH, were inoculated with a synthetic microbial community. This community consists of several butyrate producing bacteria, such as

A. caccae, which can use lactate to produce butyrate; *F. prausnitzii,* which is capable of degrading more complex carbohydrate structures; and *B. pullicaecorum*, which has been shown to be a very successful colonizer. Besides the butyrate producing bacteria, two lactic acid bacteria where present, *B. longum* and *L. plantarum* to enable the butyrate production pathway over lactate. This microbial community was supplemented with primary degraders *Akkermansia muciniphila*, a mucin degradation specialist and *B. thetaiotaomicron*, a versatile glycan degrader. To inoculum A:B(1:1), both primary degraders were added in the same concentration and in inoculum B only *B. thetaiotaomicron* was added. Inoculum A:B(1000:1) is the inoculum to which *A. muciniphila* and *B. thetaiotaomicron* are added in a different ratio, with 1000 times less *B. thetaiotaomicron* than *A. muciniphila.*

Table 4. 1: Experimental conditions: media, inoculum and pH.

	Standard inoculum + *A. muciniphila* + *B. thetaiotaomicron* Unequal amounts (A:B(1000:1))	**Standard inoculum + *A. muciniphila* + *B. thetaiotaomicron* Equal amounts (A:B(1:1))**	**Standard inoculum + *B. thetaiotaomicron* (B)**
Fiber + mucin	3 x pH 6,1 3 x pH 7	3 x pH 6,1 3 x pH 7	3 x pH 6,1 3 x pH 7
Fiber	3 x pH 6,1 3 x pH 7	3 x pH 6,1 3 x pH 7	3 x pH 6,1 3 x pH 7
Mucin	3 x pH 6,1 3 x pH 7	3 x pH 6,1 3 x pH 7	3 x pH 6,1 3 x pH 7

The experiment lasted for 58h and samples were taken for short chain fatty acid (SCFA) analysis, pH measurement, denaturating gradient gel electrophoresis (DGGE) and quantitative polymerase chain reaction (qPCR). Sampling for SCFA analysis, as described previously (Andersen *et al.*, 2014), and pH occurred 0,10,23,24,30,34,47,48,54 and 58h after inoculation and for DNA extraction (qPCR and DGGE) at 0 and 58h. At 24h and 48h fresh growth medium was supplemented to replace the volume taken by sampling.

2.3 Analysis

DNA-extraction was performed by a combination of chemical and mechanical lysis through a bead beating step as reported by (Geirnaert, 2015). As starting material, the pellet obtained after centrifuging 1 mL of luminal sample at 5,000 g for 10 min was used. The DNA quality was verified on a 1.5 % (w/v) agarose gel.

2.3.1 PCR-DGGE

To analyze the composition of the synthetic bacterial community at time 0 and after 58h of growth in different environmental conditions, PCR was performed with general bacterial primers with GC-clamp to amplify a 16S rRNA gene fragment (338F-GC and 518R). PCR amplicons were separated by denaturing gradient gel electrophoresis (DGGE) using an Ingeny phorU2X2 DGGE-system (Ingeny, Goes, the Netherlands). After electrophoresis, gels were stained for 20 min in dark in a 33x SYBR Green (Life Technologies, Invitrogen) 1x Tris-Acetate-EDTA buffer (Applichem). Stained gels were immediately photographed on a UV-transillumination table with camera (OptiGo 600, Isogen) and software ProXima AQ-4 (Isogen Life Sciences, the Netherlands). Normalization and further analysis of the gels was carried out using BioNumerics software version 5.10 (Applied Maths, Sint-Martens-Latem, Belgium).

2.3.2 qPCR

Total bacterial 16S rRNA gene and the species-specific 16S rRNA gene of *A.°muciniphila*, *F.prausnitzii, A.caccae and B.pullicaecorum,* genus-specific *Lactobacillus* and phylum-specific *Bacteroidetes* was quantified with qPCR on 10- fold diluted DNA extracts, using a StepOnePlus Real-Time PCR system (Applied Biosystems, Carlsbad, CA). Relative and absolute abundances of community members are shown in Figures 4.3, 4.5, 4.7 and Supplementary Figures 4.3, 4.4, 4.5. The primers (Table 4.2) were used with the following cycling program: 5 min at 95°C followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. The amplification reactions were carried out in triplicate in a volume of 20 µL which contained 18 µL of mastermix and 2 µL of DNA template. The mastermix (per mL) consisted of 555 µL iTaq™ Universal SYBR® Green Supermix, 0.44 µM of each primer and 356 µL of PCR water. For each qPCR assay, standard curves were created by a 10-fold dilution series of DNA of a plasmid containing the targeted 16S rRNA gene fragment. qPCR with *B. pullicaecorum* primers showed some amplification of *L. plantarum* DNA as well, however this only amounted to 0.001%-0.01% of amplified DNA with *B. pullicaecorum* primers that might be *L. plantarum*.

Table 4. 2: Primer sequences used for the qPCR analysis.

Total bacteria	338F ACTCCTACGGGAGGCAGCAG 518R ATTACCGCGGCTGCTGG	(Ovreas *et al.*, 1997)
A.muciniphila	AM1F GAGCACGTGAAGGTGGGGAC AM2R CCTTGCGGTTGGCTTCAGAT	(Collado *et al.*, 2007)
Bacteroidetes	934F GGARCATGTGGTTTAATTCGATGAT 1060R AGCTGACGACAACCATGCAG	(Guo *et al.*, 2008)
Lactobacillus	Lacto05F AGCAGTAGGGAATCTTCCA Lacto04R CGCCACTGGTGTTCYTCCATATA	(Sokol *et al.*, 2008b)
F.prausnitzii	Fprau07F CCATGAATTGCCTTCAAAACTGTT Fprau02R GAGCCTCAGCGTCAGTTGGT	(Sokol *et al.*, 2008b)
A.caccae	AnaerF GTTTTCGGATGGATTTCCTATAT AnaerR CTTTTCACACTGAATCATGCGATT	(Kurakawa *et al.*, 2015)
B.pullicaecorum	BpF GAGGCAGCAGTGGGGAA BpR TCTTCAGGTACCGTCATTTGTT	(Geirnaert, 2015)

2.3.3 Flow cytometry

Bacterial counts were measured by flow cytometry as described by Van Nevel *et al.* (2013). Samples of the pure cultures were diluted in a filter sterile phosphate buffered solution to obtain cell numbers within the detection range (10^3-10^6 cells/mL). Next, the samples were stained with SYBR Green I (10000x diluted from stock, Invitrogen) and incubated for 13 min at 37°C before measurement. The flow cytometer (BD Accuri C6 flow cytometer, BD, Erembodegem, Belgium) was equipped with a 488 nm solid-state laser and Milli-Q was used as sheath fluid. Cell counts were done by measuring the number of particles in a set volume and quality control of cell counting was done with standardized beads. Background was monitored by measuring a filtered sample, equally diluted as the test samples. Each sample was performed in triplicate.

3. Results

Different glycan sources (fiber as dietary glycan, mucin as host glycan) were presented to a synthetic community of primary degraders (*A. muciniphila* and/or *B. thetaiotaomicron*), butyrate producers (*Anaerostipes caccae*, *Butyricicoccus pullicaecorum* and *Faecalibacterium prausnitzii*) and lactate producers (*Lactobacillus plantarum* and *Bifidobacterium longum*). To discern the response from the primary degraders to differential glycan availability different ratios of primary degraders were studied: either *B. thetaiotaomicron* (B) alone, *A. muciniphila* and *B. thetaiotaomicron* in equal amounts (A:B(1:1)) or *A. muciniphila* and *B. thetaiotaomicron*, added in unequal amounts (A:B(1000:1)).

While all incubations were pH-buffered, slight acidification was observed depending on medium and synthetic community (Supplementary Figure 4.2). Incubations at high pH started from pH 6.9-7 and dropped to 6.7-6.5, while incubations at low pH conditions started at pH 6.1-6.2 and dropped to 6.1-5.5. Incubations with both fiber as mucin displayed the highest acidification (pH drop to 5.5), corresponding with the highest production of short chain fatty acids (SCFA) (Figure 4.1). These altered nutrient and pH conditions also impacted community composition, as revealed by DGGE profiles and qPCR analyses (Figure 4.2, Figure 4.3, Figure 4.5, Figure 4.7). *F. prausnitzii* and *A. muciniphila* showed a preference for a high pH environment while *A. caccae* thrived better at low pH. In contrast *B. pullicaecorum* did not display any profound environmental preferences, thriving at all pH and medium conditions or growing in the presence of any primary degrader. Although *B. longum* was added at the start of the experiment, it was detected in none of the samples after 58 hours.

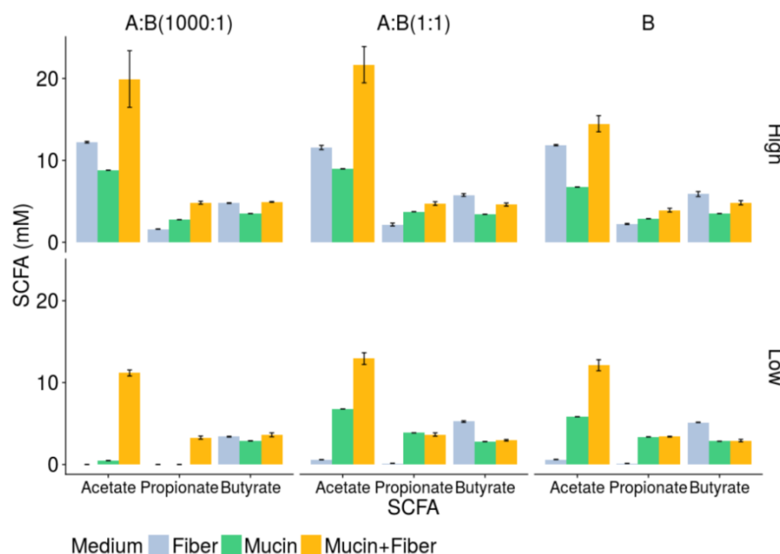

Figure 4. 1: Short chain fatty acids (SCFA) produced after 58h growth in mucin, fiber of mucin+fiber medium, at high or low pH, by the different communities.

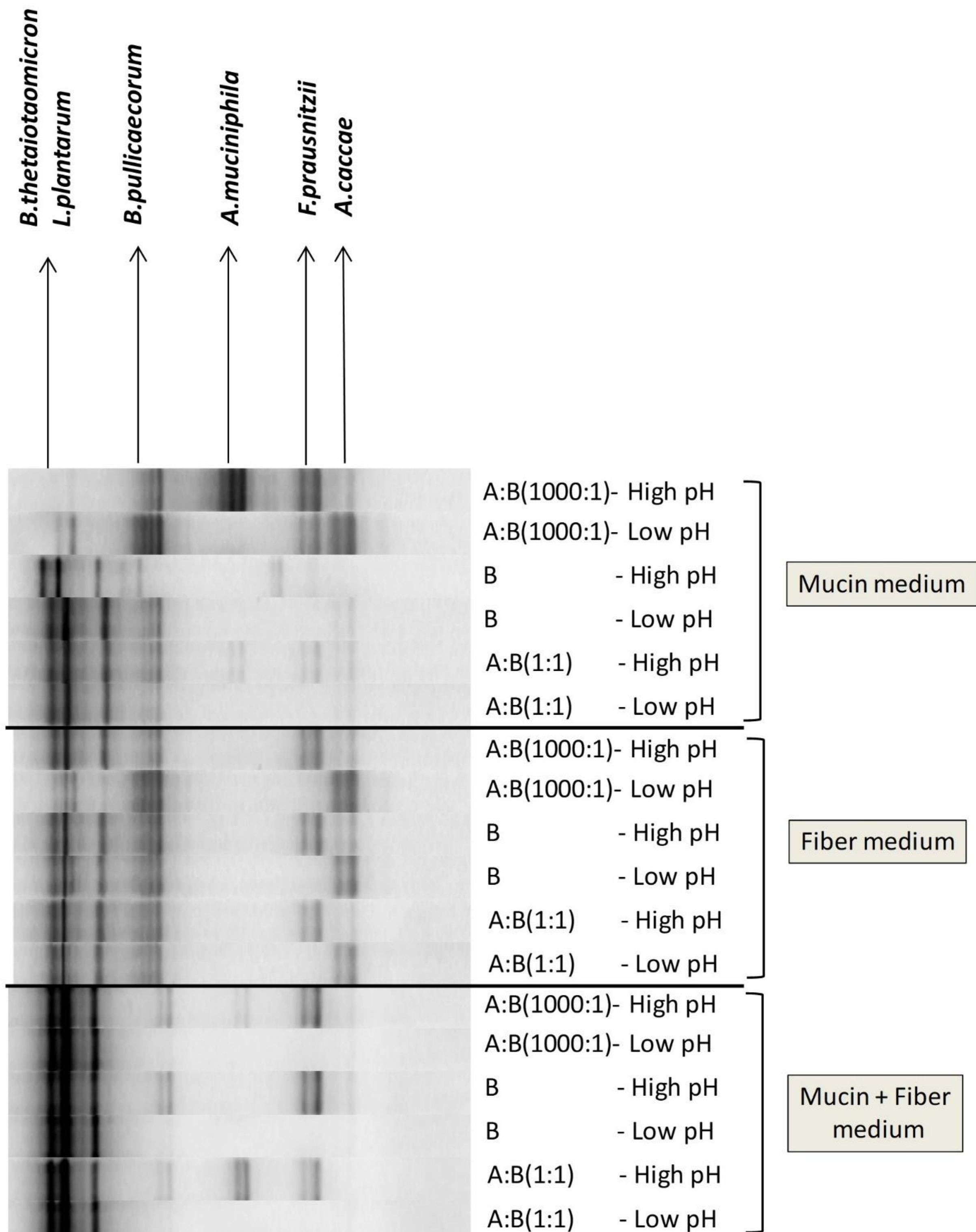

Figure 4. 2: DGGE profile of different synthetic communities after 58h growth in mucin, fiber of mucin+fiber medium, at high or low pH. By comparing the profiles with that of the monocultures (Supplementary Figure 4.1), bands could be assigned to species used in the synthetic community. The bands of *B. thetaiotaomicron* and *L. plantarum* overlap and are difficult to distinguish from each other.

3.1 Cross-feeding and competition of mucin-rich medium

The main goal of this set of incubations was to see whether *B. thetaiotaomicron* could thrive with mucin as main carbon source and compete with or even displace the mucin-degrading specialist *A. muciniphila*. In absence of *A. muciniphila*, *B. thetaiotaomicron* indeed occupied the functional niche of mucin degradation (Figure 4.3, Supplementary Figure 4.3). When inoculated at equal amounts, no real competition was observed at high pH as *Akkermansia* and *Bacteroides* both thrived very well in the community. *Akkermansia* did get outcompeted at low pH, yet this was primarily attributed to *Akkermansia's* intrinsic sensitivity to acidic pH as it was not able to grow either at low pH when inoculated as initially dominant primary degrader (A:B (1000:1)). At high pH *A. muciniphila* dominated the entire population. Interestingly, *Bacteroides* did not benefit from the lack of *A. muciniphila* at low pH conditions. At low *Bacteroides* inoculum density it was even *Butyricicoccus pullicaecorum* that became the most dominant species in the community.

Conditions with joint presence of *B. thetaiotaomicron* and *A. muciniphila* resulted in more acetate production compared to when *Bacteroides* was the sole primary degrader (Figure 4.4). *B. thetaiotaomicron* and *A. muciniphila* can both produce propionate: this was confirmed by consistent propionate levels, independently of the initial primary degrader abundance. However, absence of propionate at high *A. muciniphila* inoculation and at low pH was again indicative of *Akkermansia's* sensitivity to slightly acidic environments. Finally, butyrate production was highly consistent throughout all incubations. As none of the supplemented butyrate producers are able to grow on mucin (Belzer *et al.*, 2017; Desai *et al.*, 2016), the observed butyrate production must be the result from cross-feeding, either via acetate or lactate or the consumption of oligosaccharides released upon mucin degradation. Interestingly, DGGE and qPCR showed a high abundance of *A. caccae,* which produces butyrate via lactate consumption. This coincided nicely with more intense DGGE bands of lactate-producer *L. plantarum,* which increased with 2.5 log units/µL during growth on mucin (qPCR) (Figure 4.3, Supplementary Figure 4.3). Butyrate production in this community may thus in part be the result of cross-feeding interactions between *L. plantarum* and *A.* caccae.

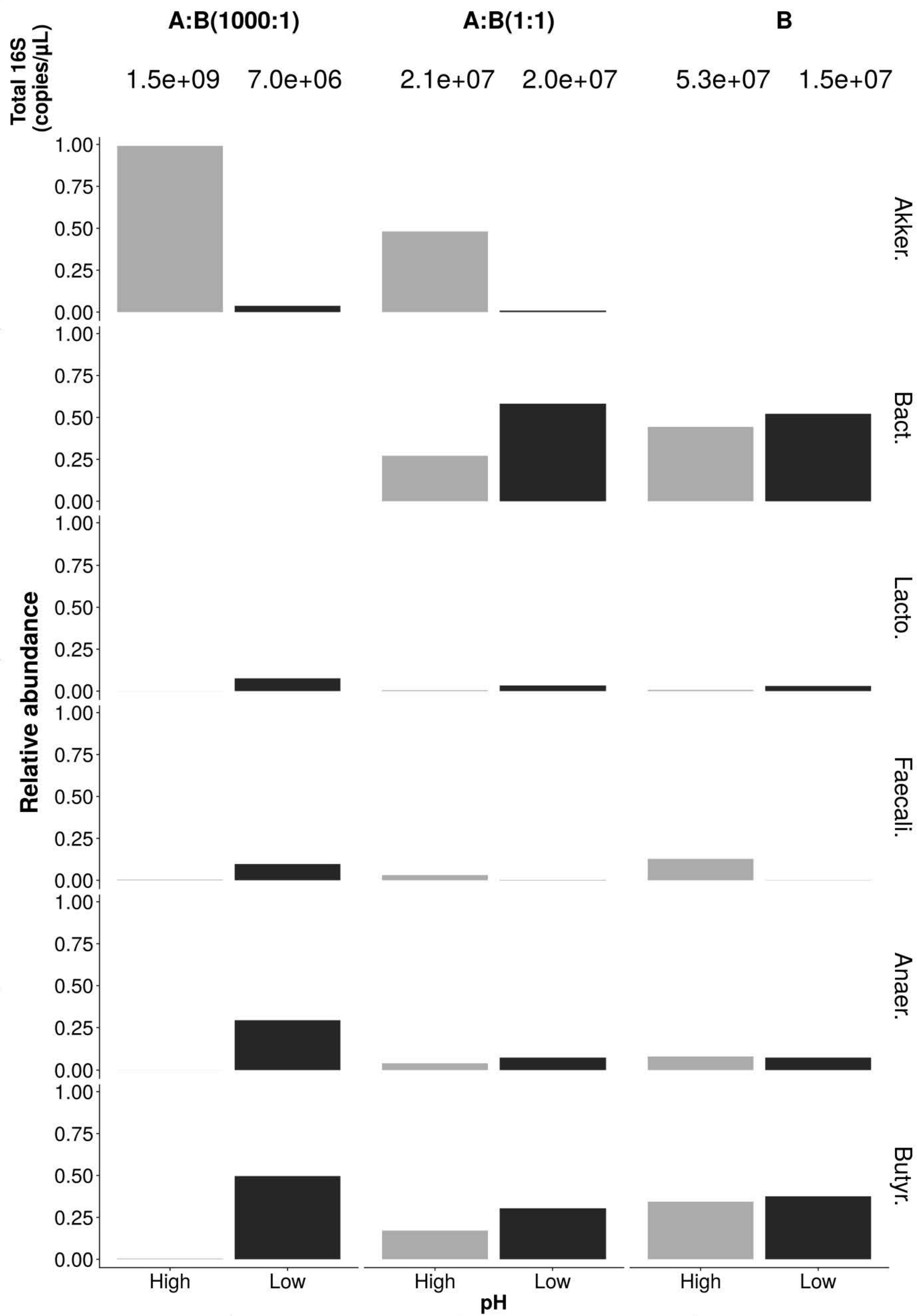

Figure 4. 3:qPCR results of total bacteria (copies/µL) and of the members of the synthetic community (relative abundance) for the experiment in mucin-rich medium. (Akker= *A.muciniphila*, Bact= *B.thetaiotaomicron*, Lacto= *L.plantarum*, Faecali= *F.prausnitzii*, Anaer= *A.caccae*, Butyr= *B.pullicaecorum*)

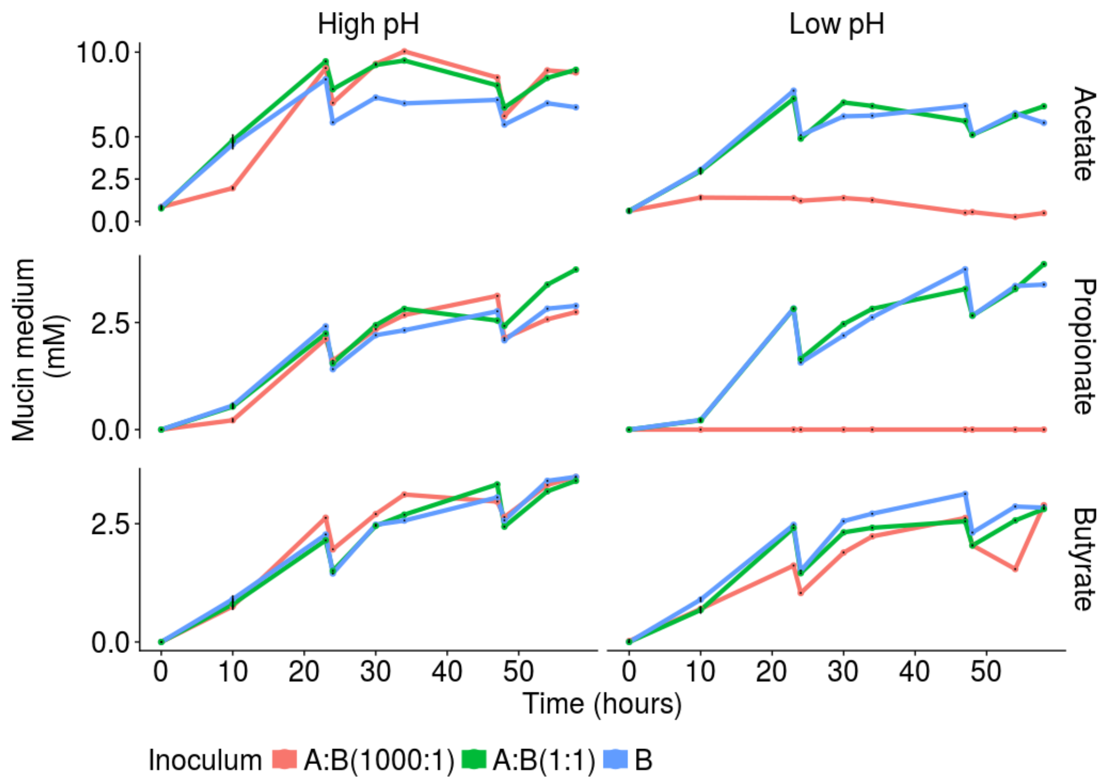

Figure 4. 4:SCFA (mM) during growth on mucin-rich medium (n=3).

3.2 Cross-feeding and competition of fiber-rich medium

Presence of fiber as main carbon source should confirm *Bacteroides's* functional niche occupation as main fiber degrader. Indeed, no growth of *A. muciniphila* could be detected in the fiber-rich medium (Figure 4.5, Supplementary Figure 4.4). *B. thetaiotaomicron* on the other hand, dominated at high pH, independent from initial concentrations. Interestingly, *L. plantarum*, *A. caccae and B. pullicaecorum* abundances increased significantly during all incubations at low pH (Supplementary Figure 4.4), and especially when *B. thetaiotaomicron* started from lower concentration *B. pullicaecorum* became the most abundant species (A:B(1000:1)) (Figure 4.5).

Acetate and butyrate were produced at high pH, but production was slower when *B. thetaiotaomicron* was inoculated at lower abundance and this difference was overcome by the end of the experiment (Figure 4.6). Interestingly, the fiber-rich medium did not result in pronounced propionate production, certainly not at low pH. With respect to butyrate producers, consistent *Butyricicoccus* abundance was noted from DGGE and qCPCR analysis; interestingly, *F. prausnitzii* was abundant at high pH while *A. caccae* was more abundant at low pH (Figure 4.5, Supplementary Figure 4.4). This is indicative of *F. prausnitzii's* butyrate production benefitting from acetate and *A. caccae's* butyrate

production using lactate via cross-feeding. Indeed low pH incubations did not reveal any acetate production while butyrate levels were similar compared with the high pH incubations. Butyrate production may thus be the result from lactate production (not measured), which is also confirmed by above-mentioned increase of *L. plantarum* at all low pH incubations, or from acetate being directly consumed for butyrate production by *B. pullicaecorum*, that dominated at low pH and low *B. thetaiotaomicron* inoculation.

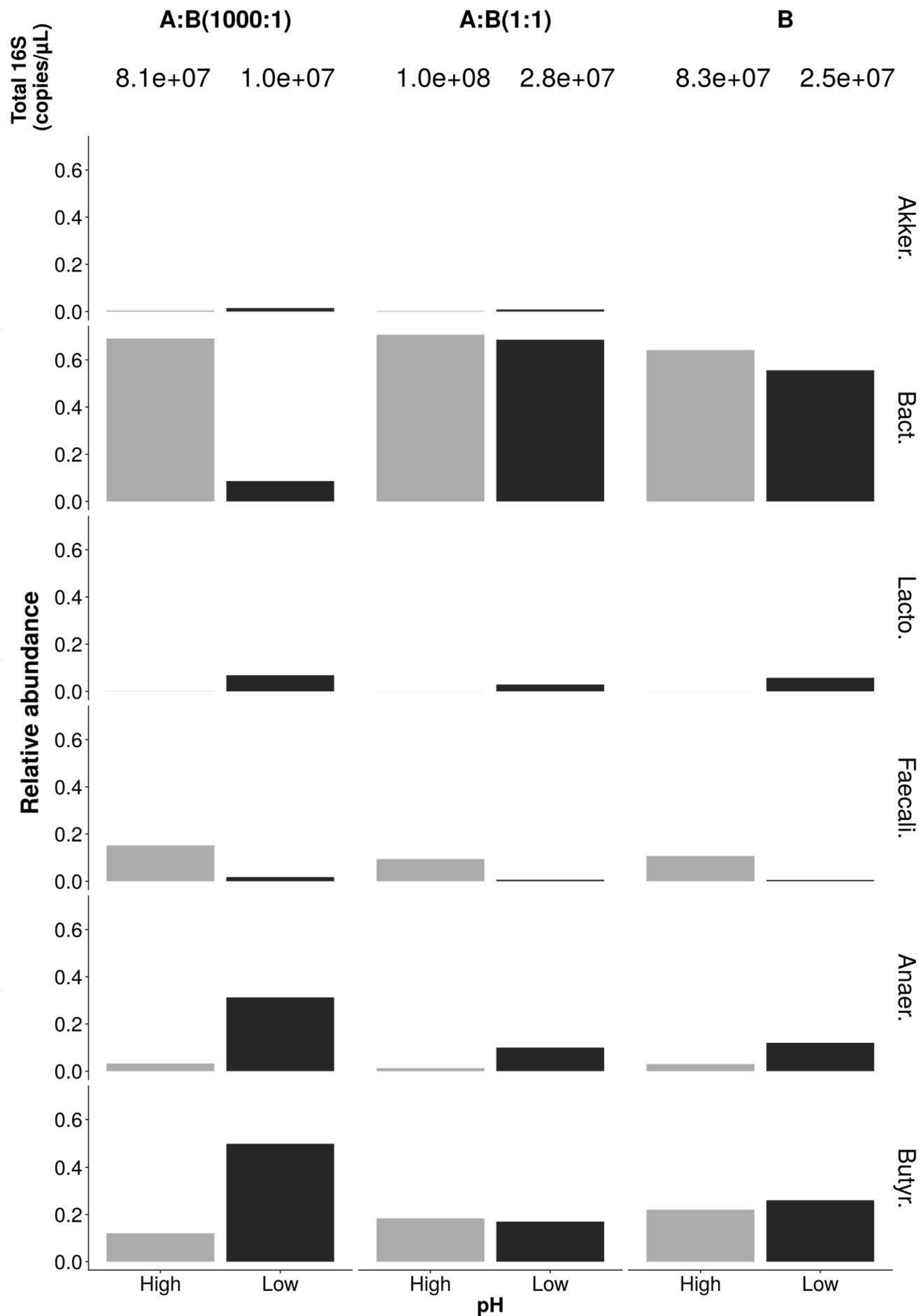

Figure 4. 5: qPCR results of total bacteria (copies/µL) and of the members of the synthetic community (relative abundance) for the experiment in fiber-rich medium. (Akker= *A.muciniphila*, Bact= *B.thetaiotaomicron*, Lacto= *L.plantarum*, Faecali= *F.prausnitzii*, Anaer= *A.caccae*, Butyr= *B.pullicaecorum*)

.

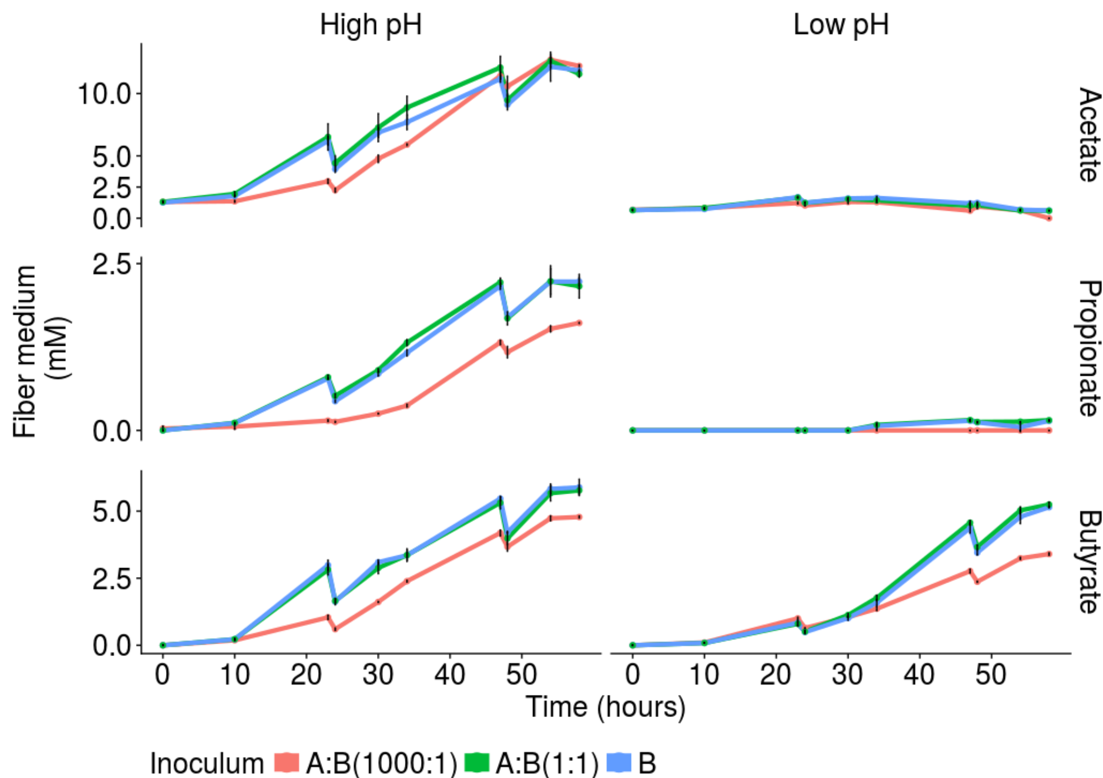

Figure 4. 6:SCFA (mM) during growth on fiber-rich medium (n=3).

3.3 Cross-feeding and competition of fiber- and mucin-rich medium

When both fibers and mucin were present, *B. thetaiotaomicron* and *A. muciniphila* were both abundant at high pH, but at low pH was *B. thetaiotaomicron* the only dominant one. In this medium, *B. thetaiotaomicron* had no problem overcoming its disadvantage at inoculation (A:B(10000:1)) and showed no pH preference. *L. plantarum* did not grow well, probably due to competition with *B. thetaiotaomicron* and/or *A. muciniphila* (Figure 4.7, Supplementary Figure 4.5).

There was more propionate and acetate produced when both *B. thetaiotaomicron* and *A. muciniphila* were abundant compared to *B. thetaiotaomicron* dominance, indicating that additional fermentation takes place and no competition between the two species occurs (Figure 4.8). This additional fermentation might in part explain the slightly higher butyrate concentrations at high pH (both species abundant) compared to low pH (only *B. thetaiotaomicron* abundant). However, when only *B. thetaiotaomicron* was inoculated (B), also more butyrate was produced at high pH.

In the DGGE profile no bands were detected for any butyrate producer at low pH but qPCR analyses showed an increase in abundance of *A.caccae* and *B.pullicaecorum*, and

butyrate was produced (Figure 4.2, Figure 4.7, Figure 4.8, Supplementary Figure 4.5). The lower abundance of butyrate producers in these sample might be explained by the bigger drop in pH, to pH 5.5, as more SCFA were formed during growth in this medium. Bands of less abundant species are difficult to detect, especially when other species (*B. thetaiotaomicron*) are very abundant and give high intensity bands.

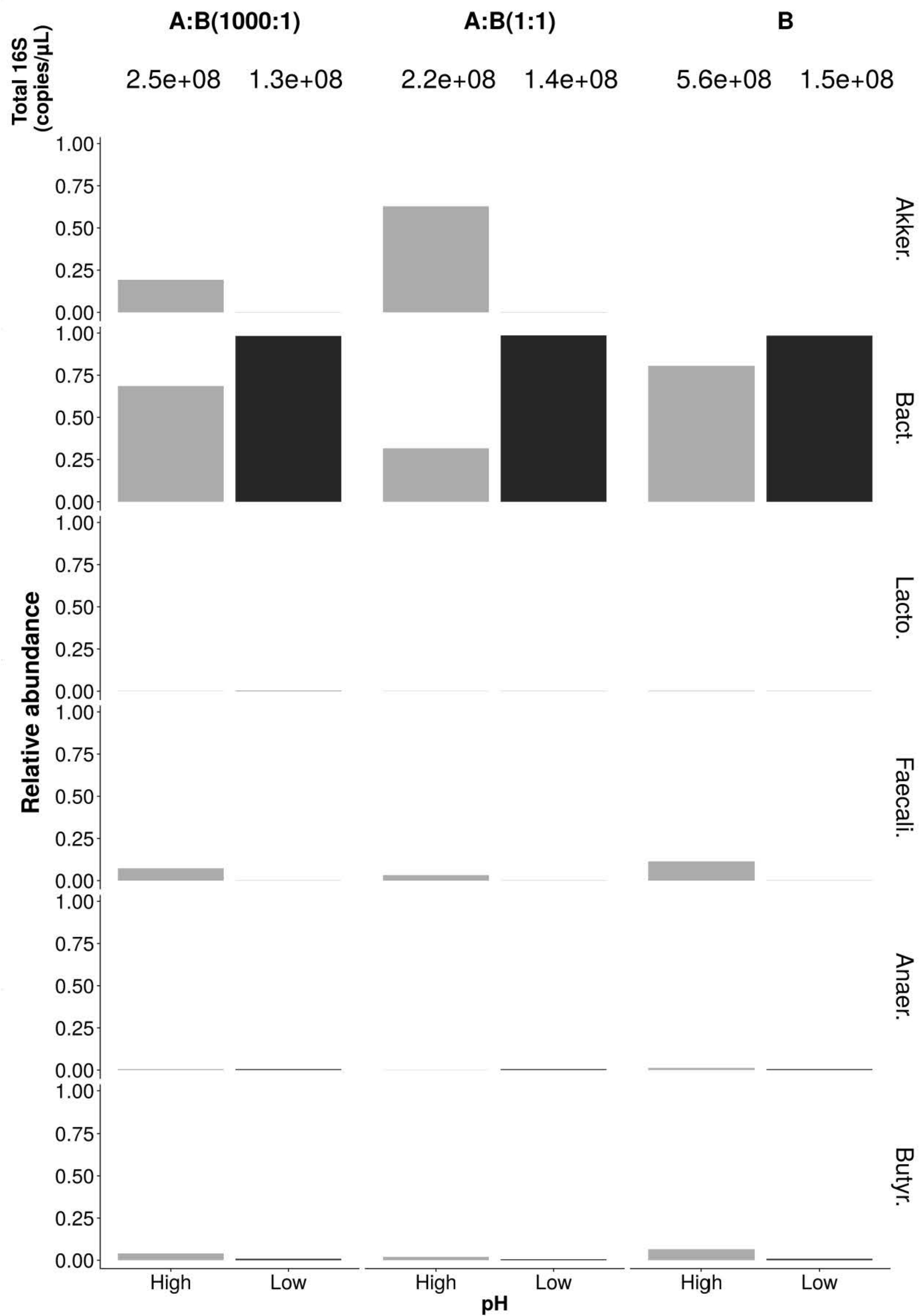

Figure 4. 7: qPCR results of total bacteria (copies/µL) and of the members of the synthetic community (relative abundance) for the experiment in mucin- and fiber-rich medium. (Akker= *A.muciniphila*, Bact= *B.thetaiotaomicron*, Lacto= *L.plantarum*, Faecali= *F.prausnitzii*, Anaer= *A.caccae*, Butyr= *B.pullicaecorum*)

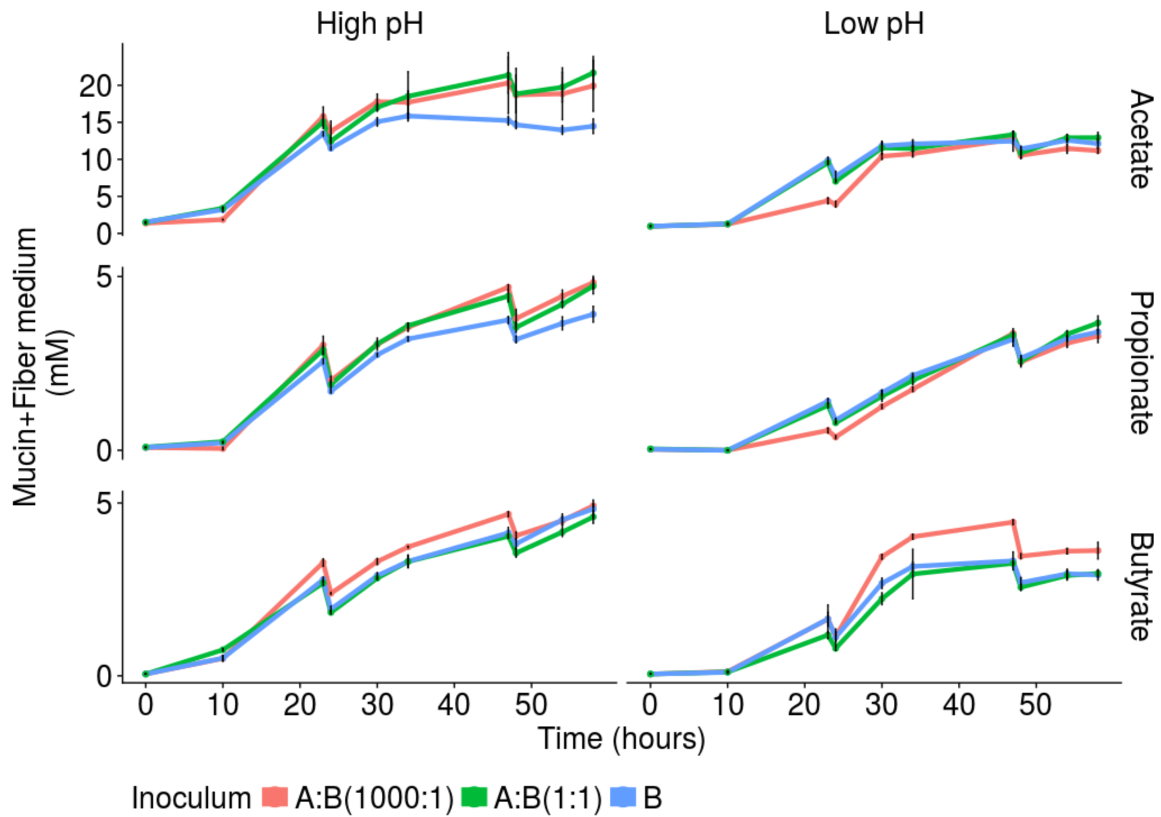

Figure 4. 8:SCFA(mM) during growth on fiber- and mucin-rich medium (n=3).

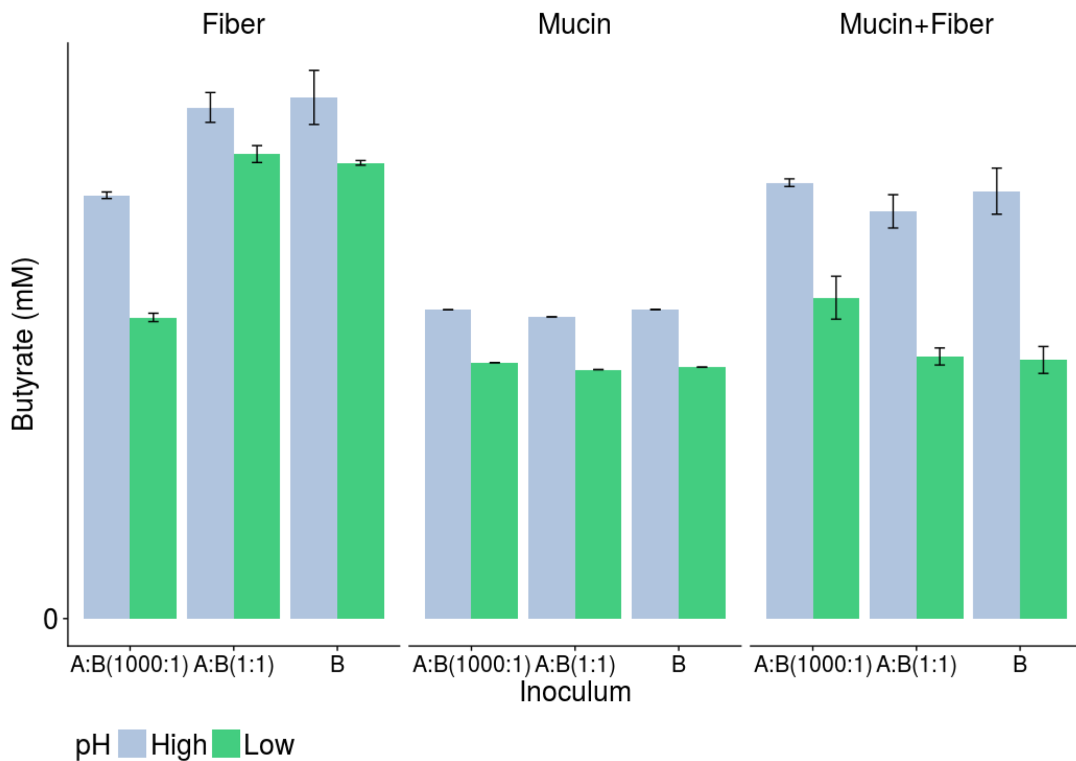

Figure 4. 9: Butyrate concentration (mM) at the end (T58) of the experiment.

4. Discussion

This study again confirmed *Akkermansia muciniphila's* status as mucin degrading specialist and its sensitivity to slightly acidic environments. *Bacteroides thetaiotaomicron* on the other hand proved a versatile organism, increasing its growth both on fiber as mucin. It did however display a larger affinity for fiber since it could overcome its initial disadvantage (A:B(1000:1)) when grown on fibers but not on mucin. In the latter case it was overgrown by *A. muciniphila* at high pH and by *B. pullicaecorum, A. caccae and L. plantarum* at low pH. Neither *B. pullicaecorum* nor *A. caccae* are known to have the ability to, even partly, degrade mucin and since they grow mostly on simple sugars, their observed growth in this medium might indicate cross-feeding (Belenguer *et al.*, 2007; Eeckhaut *et al.*, 2008; Geirnaert, 2015; Moens *et al.*, 2016). *L. plantarum* encodes a cluster of genes involved in sialic acid metabolism, whereby the sialic acid is released from the mucin structure and used as a carbon and nitrogen source, which can explain its growth in the mucin-rich medium (Almagro-Moreno and Boyd, 2009). We thus conclude that occupation of the mucin-degrading functional niche depends on initial primary degrader abundance and pH environment.

Interestingly, the joint presence of *Akkermansia* and *Bacteroides* under mucin rich conditions and similar levels of *Bacteroides* in mucin or mucin+fiber medium indicates no competition between the two, at least not when mucin is sufficiently present. The production of acetate from the mucin-rich medium in presence of *A. muciniphila* was independent of *B. thetaiotaomicron* presence and higher than the incubations with *B. thetaiotaomicron* as sole degrader. This indicates *A. muciniphila* to be a more efficient mucin degrader than *B. thetaiotaomicron.* In medium with fibers and mucin, more acetate was produced when both *Akkermansia* and *Bacteroides* were abundant compared to *Bacteroides* alone. This shows that degradation of mucin and fibers is complementary when different primary degraders are present.

In the fiber-rich medium, no growth of *A. muciniphila* was detected and we expected dominance of *B. thetaiotaomicron* at both high and low pH, independent from the inoculum. This was the case at high pH, but at low pH *B. thetaiotaomicron* had to compete with *L. plantarum* and the latter even became equally abundant when *B. thetaiotaomicron* was inoculated at a lower density (A:B(1000:1)). Interestingly, *B. pullicaecorum* dominated the synthetic community at low pH, in both fiber-rich and mucin-rich medium when *B. thetaiotaomicron* was inoculated at lower density. Previous studies indicate that it relies on other species to degrade complex substrates to simple sugars, so *B. pullicaecorum* dominance in these conditions probably results from cross-feeding (Eeckhaut *et al.*, 2008; Geirnaert, 2015; Moens *et al.*, 2016). *L. plantarum* can metabolize a large diversity of carbon

sources, including all major types of oligosaccharides (Ganzle and Follador, 2012) and it was suggested that it might have its own extracellular enzyme system for breakdown of complex carbohydrates (Siezen *et al.*, 2006). Despite the fact that *B. thetaiotaomicron* is a very efficient fiber degrader, the higher competition with *L. plantarum* could confirm previous findings that *Bacteroides* may suffer from growth inhibition due to lactate at pH values closer to its pKa (Duncan *et al.*, 2009).

We hypothesized that the different ratios in primary degraders would also affect cross-feeding towards butyrate. While differences in butyrate production were noticed between different growth media (mucin vs. fiber vs. mucin+fiber), butyrate producing functionality for the same medium remained constant under variable primary degraders ratios. Nevertheless some shifts in the butyrate producing community were observed. *A. caccae* abundance consistently coincided with increased growth of *L. plantarum*. The latter benefitted from the lack of *A. muciniphila* growth at low pH in mucin-rich medium and *Lactobacillus* got enriched at low pH in the fiber-rich medium for all primary degrader ratios. The initial degradation activity by *L. plantarum*, will result in the production of lactate and release of monomers, and could thus deliver the ideal substrates for butyrate producing *A. caccae.* No such specific interaction between primary degrader and butyrate producer was observed for the other bacteria. *F. prausnitzii* showed preference for conditions at high pH, but no specific response towards nutrients or primary degrader. In contrast to *A. caccae*, which has a narrow metabolic range, *F. prausnitzii* can metabolize a variety of oligosaccharides, such as fructose, arabinose, galactose and N-acetylglucosamine, and needs acetate for the production of butyrate (Desai *et al.*, 2016; Rios-Covian *et al.*, 2016). These carbohydrates are released by both fiber and mucin degradation and the acetate can be provided by both *A. muciniphila* and *B. thetaiotaomicron*, which explains why *F. prausnitzii* does not seem to be affected by medium or primary degrader. The same was observed for *B. pullicaecorum* which proved to be an even more efficient colonizer displaying no particular pH preference. Interestingly, butyrate production was generally higher at high pH which seems to contrast with other studies (Walker *et al.*, 2005) (Chapter 2, Chapter 3). The fact that these studies were carried out with complex microbial communities may explain this discrepancy. In our synthetic consortium primary degraders are more abundant and seemingly more active at high pH, resulting in more acetate and release of less complex substrates, which facilitates cross-feeding to butyrate. In contrast to what was expected, most butyrate was formed in fiber-rich medium and not in the medium with most nutrients (fiber+mucin). Butyrate production was lowest for incubations with mucin-rich medium. These observations demonstrate that nutritional conditions can selectively facilitate butyrate production although it was not reflected in abundance of butyrate producers (Figure 4.9, Figure 4.1).

In conclusion, the use of a synthetic bacterial community composed of primary and secondary glycan degraders, producers of acetate, propionate, butyrate and lactate, bacteria with a narrow as well as with a wide metabolic range, lead us to draw some interesting conclusions. *A. muciniphila* relies on the presence of a mucin-degrading functional niche while *B. thetaiotaomicron* is a more versatile microorganism occupying different glycan degrading niches. Yet, joint presence of both primary degraders did not lead to a competitive exclusion in the presence of mucin; *A. muciniphila* was not even overgrown by *B. thetaiotaomicron* when additional dietary glycans were available. Surprisingly, in scenarios of low *Bacteroides* abundance and at low pH *L. plantarum* can sometimes outcompete *Bacteroides*, indicating that probiotic supplementation of *Lactobacillus* could become successful when circumstances are appropriate. Finally, shifts in pH and consequence for primary degrader abundance was selective for butyrate producers (*A. caccae* as opposed to *F. prausnitzii*) while the butyrate producing functionality was maintained. This indicates that functional redundancy facilitating functional stability is an important feature of gut microbial ecosystems even at a miniaturized scale.

5. Acknowledgements

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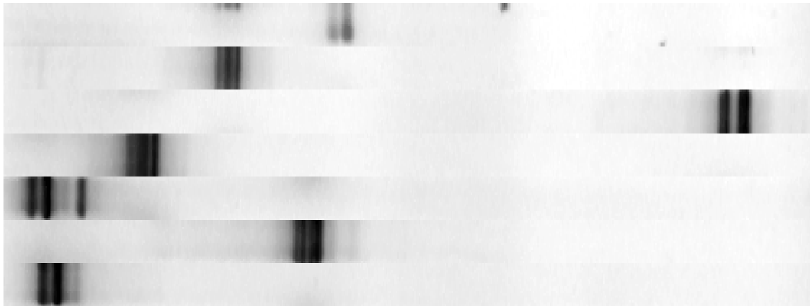
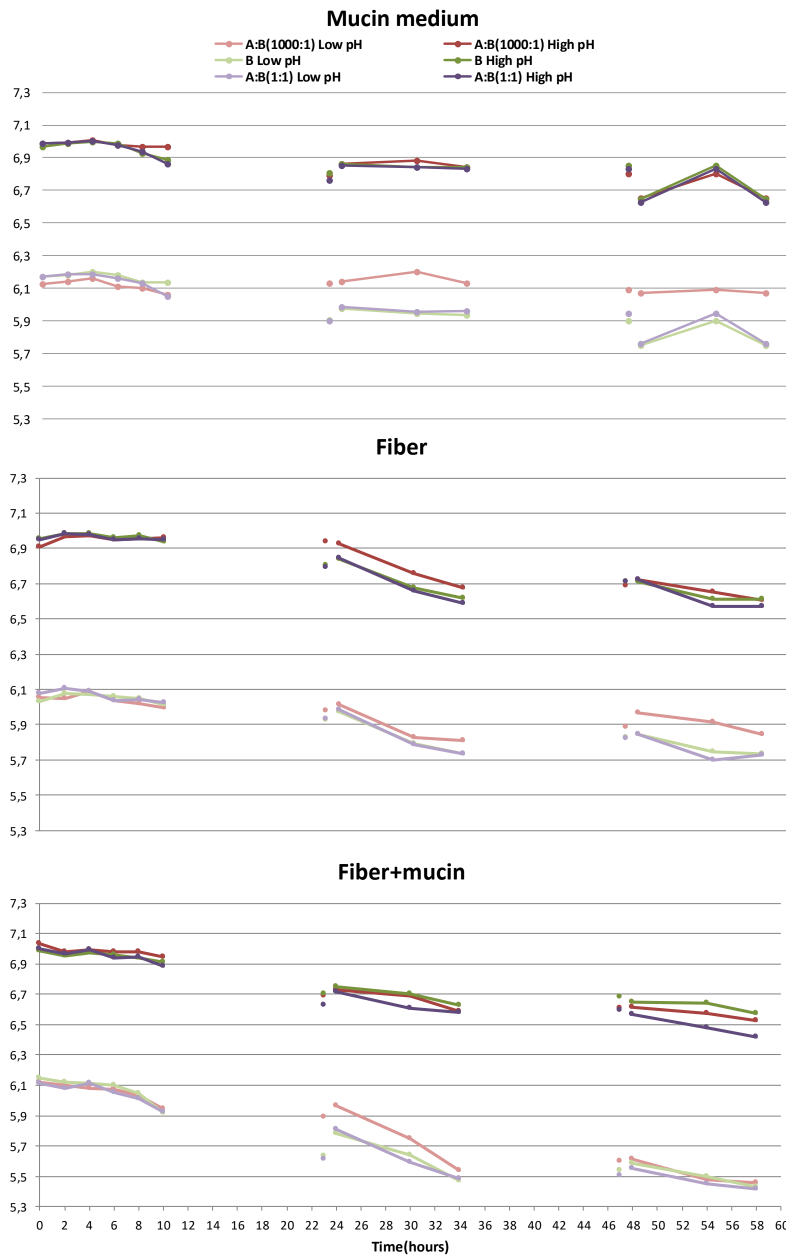
The authors would like to thank Jana De Bodt and Chloë Rotsaert for the technical support.

6. Supplementary information

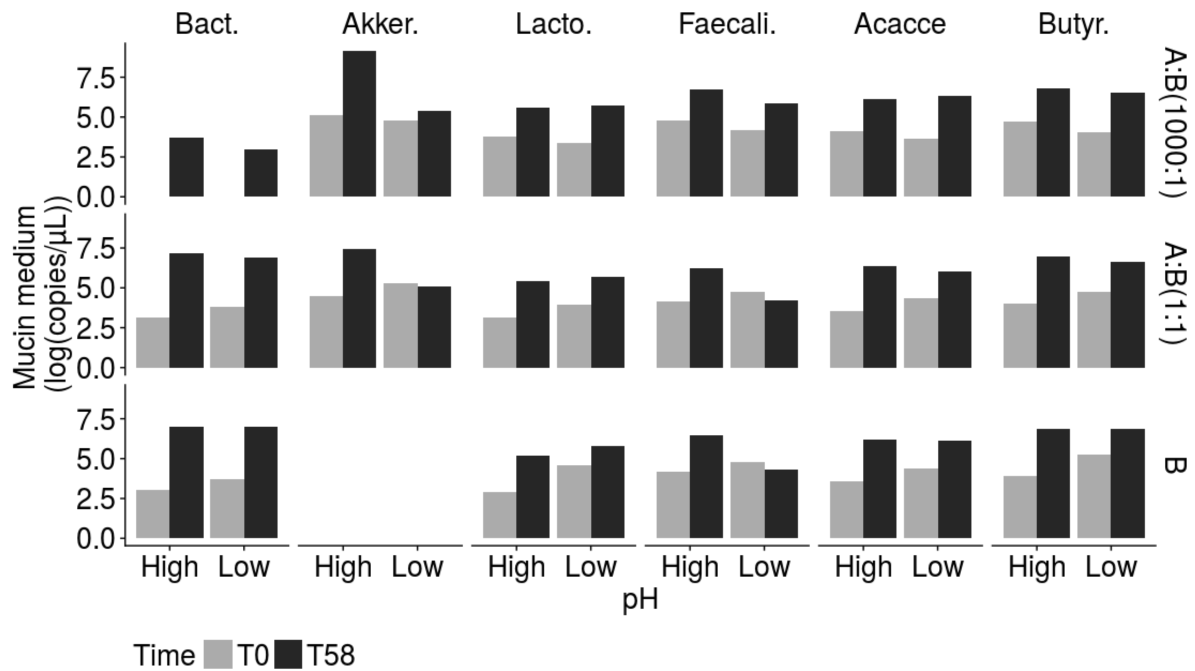


. A.caccae
. A.muciniphila
. B.longum
. B.pullicaecorum
. B.thetaiotaomicron
. F.prausnitzii
. L.plantarum

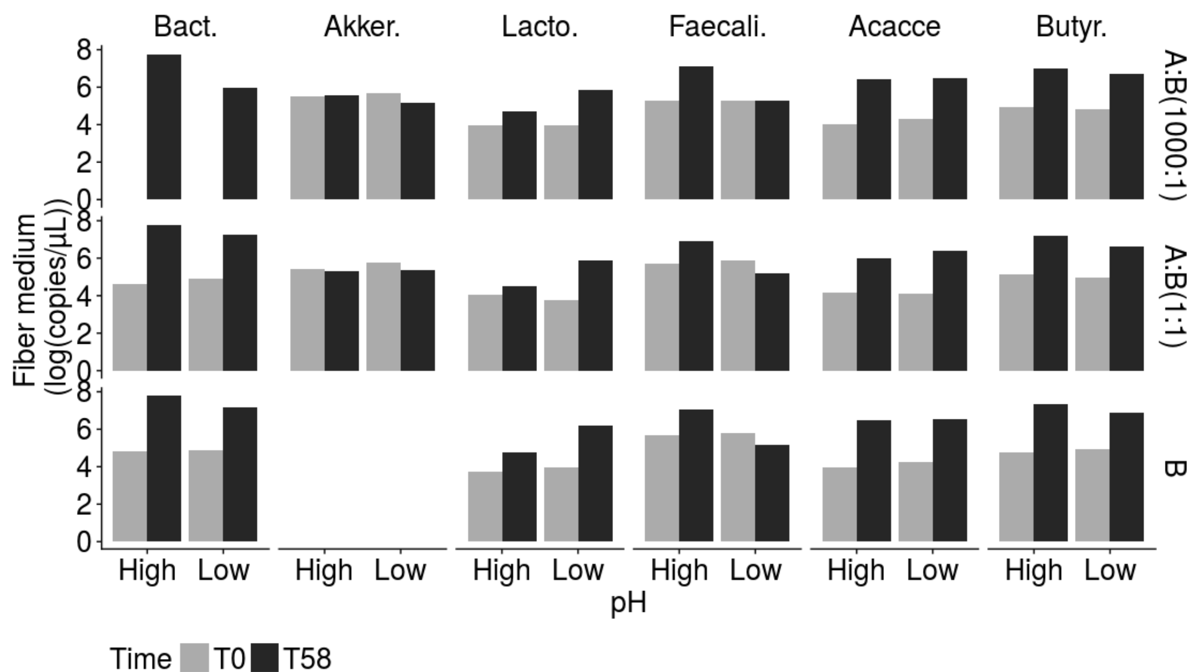
Supplementary Figure 4. 1: DGGE profile of the pure cultures.



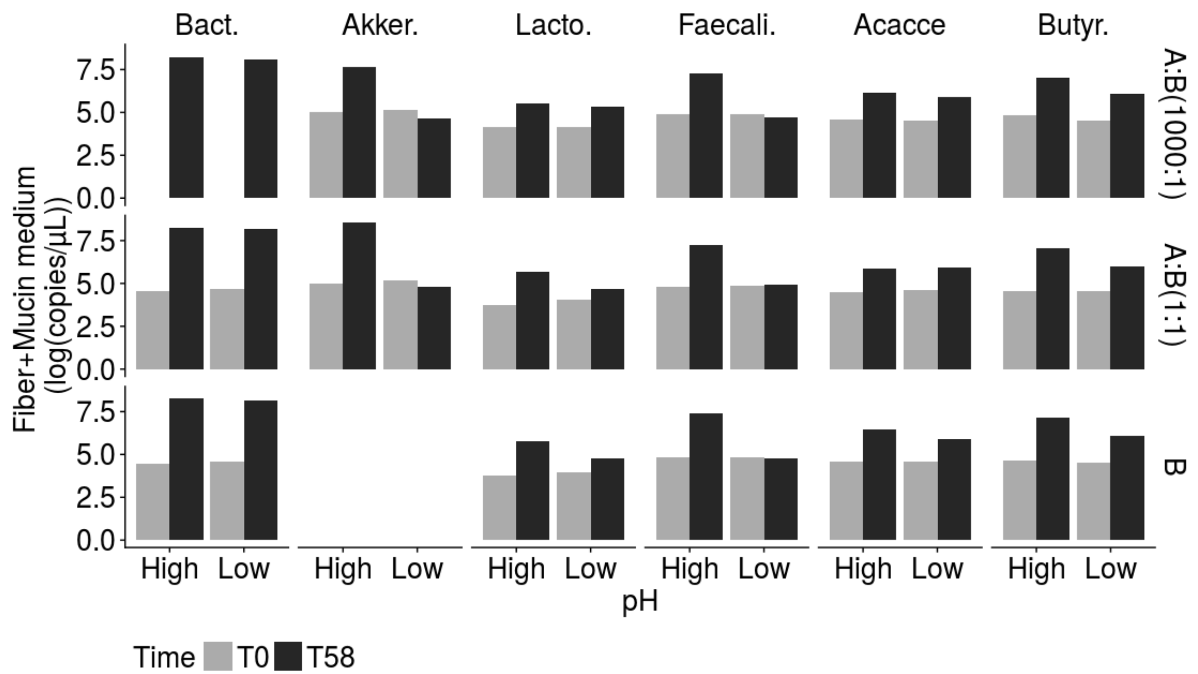
Supplementary Figure 4. 2:pH measurements during the growth of the synthetic community.



Supplementary Figure 4. 3: Abundance of the members of the synthetic community (log(copies/µL)) in the mucin-rich medium at the beginning (T0) and end (T58) of the experiment as measured by qPCR analyses. (Akker= *A.muciniphila*, Bact= *B.thetaiotaomicron*, Lacto= *L.plantarum*, Faecali= *F.prausnitzii*, Anaer= *A.caccae*, Butyr= *B.pullicaecorum*).



Supplementary Figure 4. 4: Abundance of the members of the synthetic community (log(copies/µL)) in the fiber-rich medium at the beginning (T0) and end (T58) of the experiment as measured by qPCR analyses. (Akker= *A.muciniphila*, Bact= *B.thetaiotaomicron*, Lacto= *L.plantarum*, Faecali= *F.prausnitzii*, Anaer= *A.caccae*, Butyr= *B.pullicaecorum*)



Supplementary Figure 4. 5: Abundance of the members of the synthetic community (log(copies/µL)) in the fibre- and mucin-rich medium at the beginning (T0) and end (T58) of the experiment as measured by qPCR analyses. (Akker= *A.muciniphila*, Bact= *B.thetaiotaomicron*, Lacto= *L.plantarum*, Faecali= *F.prausnitzii*, Anaer= *A.caccae*, Butyr= *B.pullicaecorum*)

CHAPTER 5

In vitro supplementation of *Akkermansia muciniphila* rather than stimulation of endogenous *Akkermansia muciniphila* results in a higher dominance in the host glycan degradation niche

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This chapter has been redrafted after

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CHAPTER 5

In vitro supplementation of *Akkermansia muciniphila* rather than stimulation of endogenous *Akkermansia muciniphila* results in a higher dominance in the host glycan degradation niche

Abstract

A. muciniphila is an abundantly present commensal mucin degrading gut bacterium (1 – 4%), widely distributed among healthy individuals. It has been positioned as a health biomarker and is currently explored as a biotherapeutic agent and next generation probiotic. Preliminary and ongoing research is mostly based on *in vivo* mouse models and human intervention trials. While these allow the assessment of physiologically relevant end markers, the analysis of fecal samples presents limitations with respect to the in-depth mechanistic characterization of *Akkermansia* effects at the level of the microbiome. We aimed to evaluate the effect of *A. muciniphila* treatment on the endogenous community from four different donors in a validated, controlled *in vitro* model of the gut microbial ecosystem (SHIME®). Taking into account the nutritional specificity and sensitivity of *A. muciniphila* to mucin deprivation and supplementation (Chapter 2, Chapter 3, Chapter 4), and the prebiotic-like action of mucins in the colon environment, the interplay between mucin, *A. muciniphila* and the endogenous community was investigated. Effects of *A. muciniphila* on the microbial community composition were limited and functional changes were primarily attributed to mucin addition. Indeed, mucin addition resulted in significantly higher acetate, propionate and butyrate production for all four donors, independent from *A. muciniphila* addition. This study revealed that the supplementation of *A. muciniphila* together with mucin limited the prebiotic-like effect in inducing compositional changes that was observed for mucin.

1. Introduction

Akkermansia muciniphila was isolated as a mucin degrading bacterium in 2004 (Derrien *et al.*, 2004). Since its discovery *A. muciniphila* has been reported by many studies as its abundance is inversely correlated with disorders such as IBD, obesity, autism, appendicitis and diabetes (Png *et al.*, 2010; Santacruz *et al.*, 2010; Swidsinski *et al.*, 2011; Wang *et al.*, 2011; Zhang *et al.*, 2009). A study with obese mice showed that *A. muciniphila* can exert therapeutic effects since its supplementation reversed high-fat diet induced insulin resistance, dyslipidemia, metabolic endotoxemia and fat mass gain (Everard *et al.*, 2013). Plovier *et al.* (2017) showed that pasteurization of *A. muciniphila* before treatment enhanced its beneficial impact and that the beneficial effects were, at least partly, due to a specific outer membrane protein (Amuc_1100). An ongoing clinical study by the university of Leuven is investigating the effects associated with the administration of *A. muciniphila* on the metabolic disorders related to overweight and obesity in humans.

A. muciniphila has been referred to as a possible next-generation probiotic (Belzer and de Vos, 2012; Cani and de Vos, 2017; Zhou, 2017), a broad term that conforms to the normal definition of a probiotic and comprises micro-organisms with potential health benefits, which do not necessarily have a Qualified Presumption of Safety (QPS) or Generally Regarded as Safe (GRAS) status. Some of these next generation probiotics are likely to be used in a pharmaceutical context, which makes them fit well within the emerging concept of live biotherapeutic products: “a biological product that: (1) contains live organisms, such as bacteria; (2) is applicable to the prevention, treatment, or cure of a disease or condition of human beings; and (3) is not a vaccine” (O'Toole *et al.*, 2017). Since there is no consensus on the correct terminology yet, we consider *A. muciniphila* to be a live biotherapeutic product, thereby avoiding confusion with established probiotic products. The mode of action of *A. muciniphila* can be by directly interacting with the host, for example through the Amuc_1100 protein, of by indirect interplay with the endogenous microbial community.

This established community, together with the high turn-over in the gastrointestinal tract, however, presents a challenge for the stable introduction and maintenance of biotherapeutics. In that respect, the availability of nutrients, selectively sustaining the growth of a biotherapeutic agent, could be an important factor in determining the success rate of future therapies. In case of *A. muciniphila*, mucins have been identified as a major determinant of its colonization capacity (Berry *et al.*, 2013; Ottman *et al.*, 2017a). In the Simulator of the Human Intestinal Microbial Ecosystem (SHIME®), a dynamic model of the colonic microbial ecosystem; mucin deprivation and supplementation was shown to

specifically affect *A. muciniphila* abundances, more than any other species present (Chapter 2, Chapter 3).

This reflects the superior ability of *A. muciniphila* to use up to 85% of the complex mucin structure, which is composed of O-glycosylated and to a lesser extent N-glycosylated protein backbones, with chains of 2 to 12 monosaccharides, mostly galactose, fucose, N-acetylgalactosamine, N-acetylglucosamine, mannose and sialic acid (Derrien, 2007; Lai *et al.*, 2009). *In vivo* mice trials have demonstrated that *A. muciniphila* efficiently degrades mucins (Berry *et al.*, 2013; van Passel *et al.*, 2011). To this end, it possesses an entire repertoire of enzymes with both extracellular and intracellular activity (Derrien, 2007). A study of its genome showed the presence of 61 proteins predicted to be involved in mucin degradation (11% of all proteins). Mucin degradation by *A. muciniphila* leads to the release of oligosaccharides and the production of acetate and propionate, both of which can stimulate microbial metabolic interactions, as well as, a host response (Derrien *et al.*, 2004; Reunanen *et al.*, 2015). Other bacteria in close proximity could profit from the mucolytic activity by using the oligosaccharides and acetate for growth and metabolic conversions, such as butyrate production (Belzer *et al.*, 2017; Chia *et al.*, 2018). It has been hypothesized that the presence and activity of these cross-feeding bacteria co-existing with *A. muciniphila* at the mucus layer, might provide additional colonization resistance against pathogens and could impact host response due to their proximity to the epithelial cells (Belzer and de Vos, 2012; Cani and de Vos, 2017).

Only a few other species have the enzymatic capacity for initiating partial or full mucin degradation, including *Bacteroides thetaiotaomicron*, *Ruminococcus gnavus*, *Ruminococcus torques and Bifidobacterium bifidum* (Hoskins *et al.*, 1985; Marcobal *et al.*, 2013d; Martens *et al.*, 2008; Png *et al.*, 2010). Considering the limited number of species that can degrade the complex mucin structure and described health effect conferred by its degradation, mucins fit the definition of prebiotic substances: ‘substrates that are selectively utilized by host microorganisms conferring a health benefit’ (Cani and de Vos, 2017; Gibson *et al.*, 2017; Ouwehand *et al.*, 2005). As mucin glycans constitute 80% of the dry weight of the mucus layer covering the intestinal epithelium and are present in the luminal content as a consequence of the continuous mucus desquamation, the human body can be described as producing its own prebiotic (Johansson *et al.*, 2011; Johansson, 2012; Johansson *et al.*, 2008). Mucin thus plays an important role in the interaction between *A. muciniphila,* the microbial community and the host.

Considering the ongoing studies and future perspective for *A. muciniphila* as a biotherapeutic agent, we aimed at investigating the effect of *A. muciniphila* treatment on the endogenous community. For this purpose, the *in vitro* SHIME model was used, with colon compartments separately inoculated with the microbiota from four human donors. Taking into

account its nutritional specificity, treatment of *A. muciniphila* was investigated with and without addition of mucin. This allowed us to elucidate the importance of mucin presence to modulate the efficiency of the supplementation with *A. muciniphila*. At the end of these treatments, an antibiotic pulse was administered after which the microbial community was allowed to recover. The goal was to establish whether the interplay between *A. muciniphila,* mucin and the microbial community would lend resilience towards an antibiotic-induced disturbance or mediate a faster ecosystem recovery.

2. Materials and methods

2.1 Bacterial strains

Akkermansia muciniphila (DSMZ 22959, Type strain) was cultured in reinforced clostridial medium (RCM) medium with mucin for 24h, prior to the daily treatment of the colon vessels (day 10-20). After 24h growth the pure culture was washed with anaerobic PBS (0.8 g L^{-1} NaCl and 0.2 g L^{-1} KCl) in an anaerobic (10 % CO_2 and 90 % N_2) workstation (GP Campus, Jacomex, Dagneux, France). Using flowcytometry (as described in Chapter 4), the *A. muciniphila* concentration was quantified and was standardized to $2,5*10^8 \pm 5*10^7$ cells mL^{-1} before supplementation to the SHIME colon compartments (Van Nevel *et al.*, 2013).

2.2 Long-term dynamic *in vitro* gut model for the luminal colon microbiota (SHIME)

The dynamic *in vitro* SHIME® model (ProDigest-Ghent University, Ghent, Belgium) was used to study the impact of supplementation of live *A. muciniphila*, with or without the presence of a host glycan degradation niche, in different microbial communities. The model and its nutritional medium is described in Chapter 2. Fecal samples were collected from healthy donors between the age of 25-35 and prepared within 1h according to standard procedures (Molly *et al.*, 1993) (Chapter 2) and used for inoculation.

The set-up of this experiment is shown in Figure 5.1. Fecal suspension of 4 donors was used to inoculate the colon vessels (4 colon vessels/donor), with a retention time (RT) of 40h and a pH between 6.6-6.9 (distal colon pH). During the mucin deprivation period (day 0-10), a mucin-free nutritional medium was fed to the colon vessels. From day 10-20 onwards (treatment period), 4 different treatments were applied to the 4 colon vessels/donor: "**+Akk+Muc**" where *A. muciniphila* was daily administered to the colon vessels after sampling and mucin (4 g L^{-1}) was added to the feed; "**+Akk-Muc**" where only *A. muciniphila* was added; "**-Akk+Muc**" where only mucin (4 g L^{-1}) was added; and **"-Akk-Muc**" which is

identical to the medium provided during the mucin deprivation period. After this ten-day treatment period, an antibiotic mix, containing ciprofloxacin, amoxicillin and tetracycline at respectively 40, 40 and 10 mg L^{-1} final colonic concentration, was supplemented directly into every colon vessel to induce an acute stress (Marzorati *et al.*, 2017).

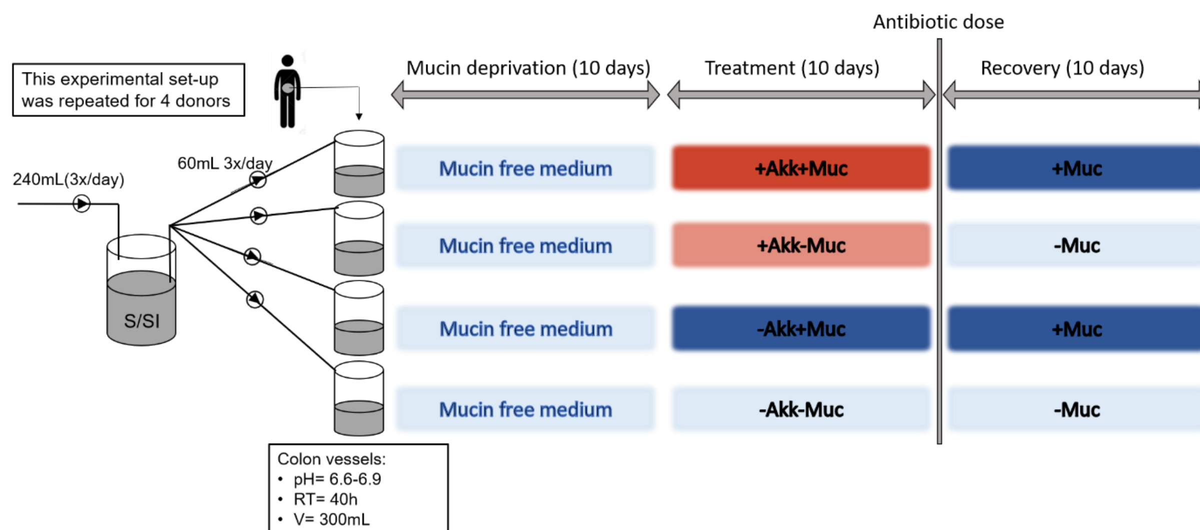


Figure 5. 1: Experimental set-up of the SHIME experiment. Akk: *A. muciniphila.* Muc: mucin. RT: retention time.

Samples were taken daily for SCFA analysis, as described previously (Andersen *et al.*, 2014) and every two days for DNA extraction (Geirnaert, 2015), followed by 16S rRNA gene amplicon sequencing (Illumina MiSeq) (De Paepe *et al.*, 2017) and *A. muciniphila* qPCR quantification (Collado *et al.*, 2007).

2.3 Microbial community analysis

DNA extraction was performed by a combination of chemical and mechanical lysis through a bead beating step as reported by Geirnaert *et al.* (2015). As starting material, the pellet obtained after centrifuging 1 mL of luminal sample at 5,000 x g for 10 min was used. The DNA quality was verified on a 1.5 % (w/v) agarose gel.

Total bacterial and *Akkermansia*-specific 16S rRNA gene copy number was quantified with qPCR on 100- and 10- fold diluted DNA extracts, respectively, using a StepOnePlus Real-Time PCR system (Applied Biosystems, Carlsbad, CA), as described in Chapter 3.

The bacterial community on various timepoints during the experiment was assessed using amplicon sequencing of the 16S rRNA gene (De Paepe *et al.*, 2017). DNA samples were sent out to LGC Genomics (Teddington, Middlesex, UK) for library preparation and sequencing on an Illumina Miseq platform, as described by De Paepe *et al.* (2017). The V3-V4 region of the 16S rRNA gene was amplified by PCR using primers (341F CCTACGGGNGGCWGCAG, 785R GACTACHVGGGTATCTAAKCC) derived from

Klindworth *et al.* (2013), with a slight modification to the reverse primer by introducing another degenerated position (K) to make it more universal. The sequencing data has been submitted to the NCBI (National Center for Biotechnology Information) database under accession code (SRP126579). The mothur software package (v.1.39.5) and guidelines were used to process the amplicon data generated by LGC Genomics, as described in detail by De Paepe *et al.* (2017)(Kozich *et al.*, 2013).

2.4 Statistical analysis

All statistical analysis were performed in R, version 3.4.3.

2.4.1 Functional data

Non-parametric, rank based longitudinal data analysis of the SCFA production (measured acetate, propionate, butyrate and branched SCFA concentrations) over time was conducted using the R package nparLD (nparLD_2.1). Wald and ANOVA type statistics were used to assess the significance of the combined mucin and *A. muciniphila* treatment in function of time (f1-ld-f1 design). A significant time effect was observed, which was expected as the treatment was applied after an initial stabilisation period of ten days and the system was disturbed after 20 days by an antibiotic pulse. The longitudinal data analysis was therefore repeated on the subsetted data (stabilisation, treatment prior to antibiotic pulse and treatment post antibiotic pulse). The relative treatment effects obtained by nparLD were verified by a partial redundancy analysis, followed by a PCA (package vegan_2.4-4).

Acetate, propionate, butyrate and branched SCFA levels were modelled in function of the treatment (with *A. muciniphila* and mucin), conditional on the period (stabilisation, treatment prior to antibiotic pulse and treatment post antibiotic pulse) and inter-individual differences (factor donor). Similarly, donor and period were considered as main effects, conditional on the other factors. Permutation tests were applied to assess the statistical significance of the global model and the individual canonical axes (Legendre *et al.*, 2011). The RDA results were plotted in a type II scaling correlation triplot, displaying the constrained canonical (labelled RDA1/2) and in case of the *A. muciniphila* or mucin effect the first unconstrained residual (labelled PC1) axis. Both axes were annotated with the proportional eigenvalues representing their contribution to the total (both constrained and unconstrained) variance. The coordinates of the sites were derived from the weighed sums of the scores of the response variables. Next to the absolute metabolite concentrations, the relative proportion of the metabolites is an important marker. Therefore the above outlined procedure was repeated using the metabolite ratios. Additionally, in order to assess if significant interactions occurred between the explanatory variables, a global RDA was performed based on a regression model including interaction terms in addition to each of the main effects.

2.4.2 Microbial community data

To visualize differences in microbial community composition between donors, treatments and antibiotic response, ordination and clustering techniques were applied. For these purposes, the shared file was further processed to remove OTU's with too low abundance according to the arbitrary cut-off's described by McMurdie and Holmes (2014). An OTU is defined in this manuscript as a collection of sequences, that are found to be more than 97% similar to one another in the V3-V4 region of their 16S rRNA gene after applying hierarchical clustering (Chen *et al.*, 2013; Schloss and Westcott, 2011; Schloss *et al.*, 2009; Wang *et al.*, 2012). To deal with differences in sampling depth, proportional data transformed on the common scale to the lowest number of reads was used (McMurdie and Holmes, 2014). A table with the most abundant OTUs classified to the species level using both RDP Seqmatch tool and NCBI BLAST is given in Supplementary Table 5.1.

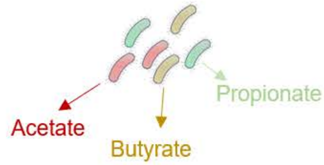
Principle Coordinate Analysis (**PCoA**; package stats) was conducted based on the abundance-based Jaccard dissimilarity matrix (package vegan and visualized with ggplot2 (Anderson *et al.*, 2006; Cox, 2001; Oksanen, 2016; Ramette, 2007). This procedure was repeated on OTU and genus level focusing on both the comparison between the donors and between the applied treatments. On the genus level, weighed averages of genera abundances were *a posteriori* added to the ordination plot using the wascores function in vegan (Oksanen, 2016). Donor and treatment both influenced the grouping of samples, which was further explored using a partial distance based redundancy analysis at species level (db RDA) (Vardakou *et al.*, 2007). The scores obtained by a PCoA were modelled in function of the treatment, with the effects of the inter-individual variability and treatment period being partialled out using the capscale function of the package vegan (package vegan_2.4-4) (Oksanen, 2016; Ramette, 2007). Interpretation of the results is preceded by a permutation test of the **db RDA** results to confirm that a relationship exists between the response data and the exploratory variables. Using the same principle, the significance of the first two constrained axis was evaluated. The constrained fraction of the variance, explained by the exploratory variables is adjusted by applying a subtractive procedure (Borcard *et al.*, 2011; Peres-Neto *et al.*, 2006). The fraction of the variance explained by the exploratory variables and its significance are given in Supplementary Table 5.2.

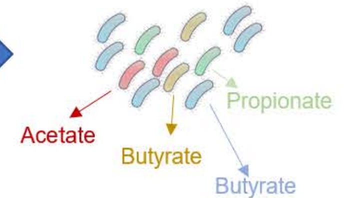
In a next step, Sparse Partial Least Squares Discriminant Analysis (**sPLS-DA**) (mixOmics_6.3.1) was performed to select the taxonomic features most predictive of the treatment ("+Akk+Muc","-Akk+Muc","+Akk-Muc","-Akk-Muc") (Figure 5.2). Hereto, a factorial response variable was created, indicating the treatment condition of each sample. The filtered proportional OTU level abundances were used as predictors. The number of components and OTUs or genera to include in the sPLS-DA model was assessed based on

the classification error rates obtained after a five-fold Cross-validation. The final sPLS-DA model, with an optimum of 3 components was displayed (Supplementary Figure 5.2) and the proportional abundances of the most predictive and most abundant OTUs and genera were represented in a heatmap (Figure 5.6).

Finally, in order to find statistically significant differences in species and genus level abundance between the different treatments, the **DESeq** package was applied on the filtered, unnormalized data at the end of the treatment period (Day 20) (α=0.05) as suggested by (Love *et al.*, 2014); McMurdie and Holmes (2014) (Figure 3.3). The factors Treatment and Donor were used in the design formula and the effect of the treatment was determined by a likelihood ratio test on the difference in deviance between a full and reduced model formula. An empirical Bayes shrinkage correction was employed for low counts (Love *et al.*, 2014). Pairwise significant differences were obtained using Wald tests, specifying all pairwise combinations of treatments as the contrast argument. Results from the pairwise comparisons were visualized in a volcanoplot, showing the -log10 (adjusted p-value) as a function of the shrunken log2 FoldChange. Species with an absolute shrunken log2FoldChange exceeding 2, were annotated in the plot (Quackenbush, 2002). The most pronounced significant differences at species level were shown in side-by-side boxplots comparing the normalized counts (plus a 0.5 pseudocount) during treatments.

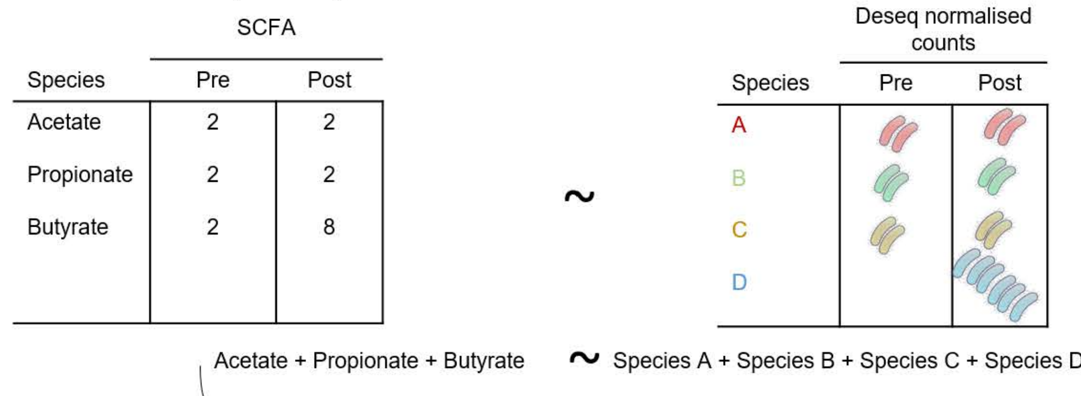
To assess the effect of the antibiotic pulse on the microbial community, the DESeq package was again applied. The effect of the antibiotic pulse was determined by a likelihood ratio test on the difference in deviance between a full and reduced model formula. An empirical Bayes shrinkage correction was employed for low counts (Love *et al.*, 2014). Results from the pairwise comparisons, for each treatment comparing before and after antibiotic pulse, were visualized in a volcanoplot, showing the -log10 (adjusted p-value) as a function of the shrunken log2 FoldChange. Genera with an absolute shrunken log2FoldChange exceeding 1, were annotated in the plot (Quackenbush, 2002) (Supplementary Figure 5.4). Also alpha-diversity was calculated using the Shannon coefficient (vegan package) an visualized in Supplementary Figure 5.5.





A treatment induces related shifts in:
- The microbial community composition
- The microbial metabolic acitivity (e.g. acetate, propionate and butyrate production)

Partial Least Squares Regression can be used to relate the two data matrixes:



	SCFA	
Species	Pre	Post
Acetate	2	2
Propionate	2	2
Butyrate	2	8

~

	Deseq normalised counts	
Species	Pre	Post
A		
B		
C		
D		

The **linear regression model** is obtained by finding a linear combination of species abundances that best explain the variance in the SCFA data.

Using variable selection techniques, the model can be simplified by reducing the number of response (individual SCFA) and independent (species) variables. This variation on the technique is known as **sparse Partial Least Squares Regression.**

In case of qualitative response variables (eg. a factor variable encoding treatment or control), the method can be applied by recoding the qualitative factor as a quantitative dummy variable (eg. 1 for treatment and 0 for control). This variation on the method is termend **Partial Least Squares Discrimant analysis.**

Another related technique is **distance based redundancy analysis.** In this case, the response variables are transformed into a dissimaliry matrix, which is used to construct Principle Coordinates, acting as a measure of the variance in the response data.

Figure 5. 2:Regression based analyes.

3. Results

During the 10 day **mucin deprivation period**, mucin free medium was fed to the SHIME system, creating mucin deprived communities, derived from the fecal samples of four different donors. These communities were characterized by a reduction of *A. muciniphila* abundances (Figure 5.5) and similar short chain fatty acid (SCFA) profiles (Figure 5.3) with 36.32 ±6.85 mM acetate, 9.41±0.81 mM propionate and 4.19±1.42 mM butyrate (n=16). The *A. muciniphila* abundance of around 0.01% in donors 1,2,3 decreased a 1000 fold due to mucin deprivation for donors 1 and 2 and was close to the quantification limit in donor 3 (Figure 5.5). In the case of donor 4, *A. muciniphila* abundance levels remained close to the quantification limit during the entire mucin deprivation period (Figure 5.5).

From day 10 onwards, *A. muciniphila* and/or mucin were added to the SHIME. Partial redundancy analysis indicates that mucin treatment accounted for 17% of the observed variation in SCFA concentrations (p=0.001) (Figure 5.4). Treatments with mucin (-Akk+Muc, +Akk+Muc) caused an increase in acetate, propionate and butyrate production in all donors, whereas the addition of *A. muciniphila* did not have a significant effect, contributing to only 0.5% of variation in SCFA concentrations (p=0.11) (Figures 5.3 and 5.4). Besides mucin treatment, inter-individual differences were significant but less pronounced (2%) (Figure 5.4). The donor effect was most visible during the combined *A. muciniphila* and mucin treatment (Figure 5.4). In donor 2 the response to the addition of mucin depended on *A. muciniphila* co-administration: when treated with both *A. muciniphila* and mucin, propionate production increased but when treated with only mucin, butyrate production increased (Figure 5.3). Interestingly, the endogenous *A. muciniphila* in this donor did not respond to the mucin treatment (Figure 5.5), whereas it did in the other donors; mucin addition in donor 1 resulted in a delayed increase of endogenous *A. muciniphila* abundances after four days; in donor 4, the delay lasted longer (six days) and levels remained lower compared to the *A. muciniphila* and mucin combination; for donor 3, a fast increase was observed followed by a decrease after 4 days. Daily supplementation of *A. muciniphila* resulted in a fast increase in *A. muciniphila* abundances resulting in a stable high population density throughout the treatment period (Figure 5.5). Mucin further stimulated growth of endogenous or supplemented *A. muciniphila*. Unlike previous observations (Chapter 2, Chapter 3) when no *A. muciniphila* and mucin were added (-Akk-Muc), *A. muciniphila* was not entirely washed out during the 30 day experiment (Figure 5.5).

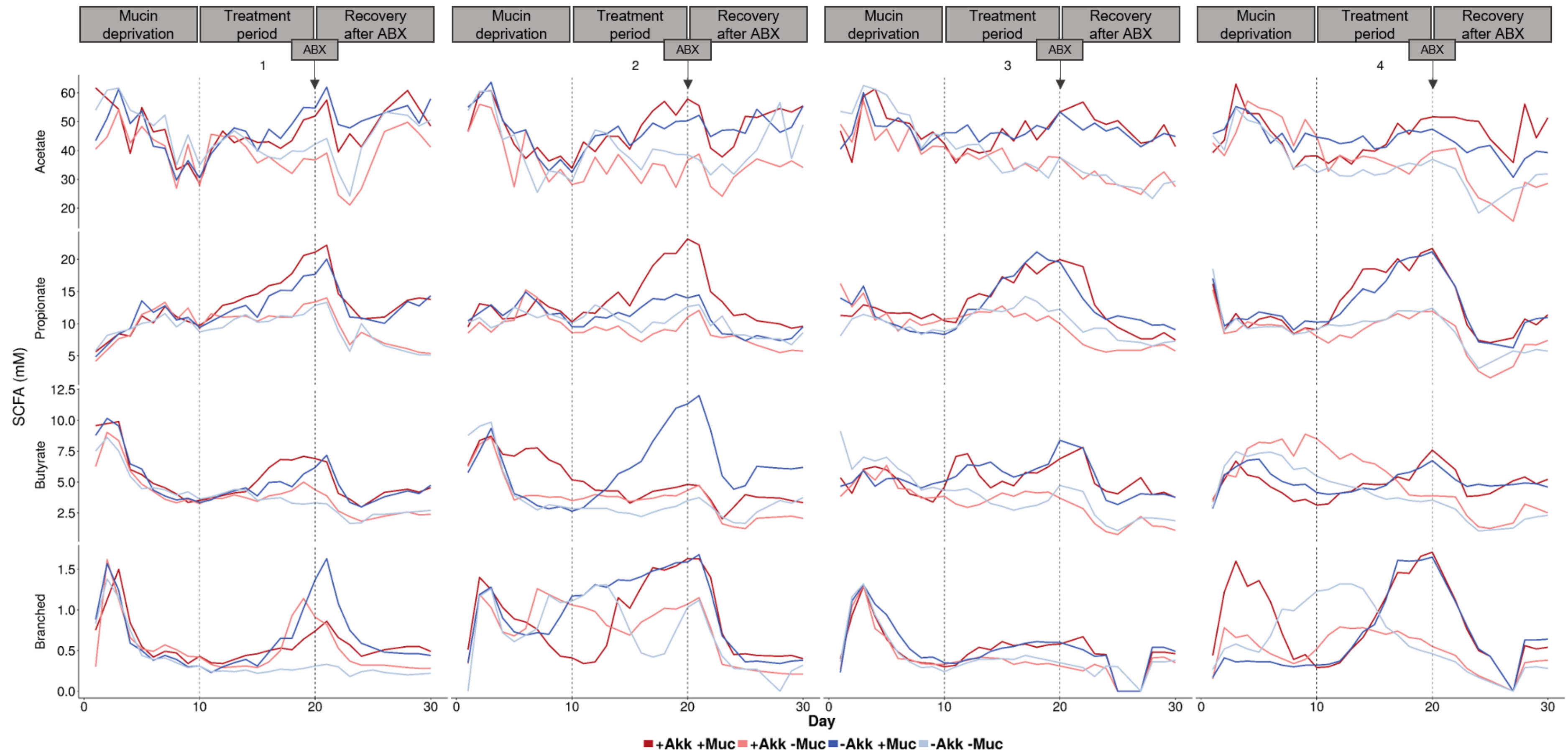


Figure 5. 3 : Short chain fatty acid concentration (mM) measured in the colon vessels inoculated with fecal samples of donors 1-4. From day 0-10 mucin-free feed was administered. From day 10-20 onwards, different treatments were applied:vessels were treated with either *A.muciniphila* (+Akk-Muc), mucin (-Akk+Muc) (4g L^{-1}), a combination of both (+Akk+Muc) or no treatment(-Akk-Muc). At day 20 all vessels were treated with an antibiotic mix (ABX), after which *A. muciniphila* treatments, in contrast to the mucin treatments were discontinued.

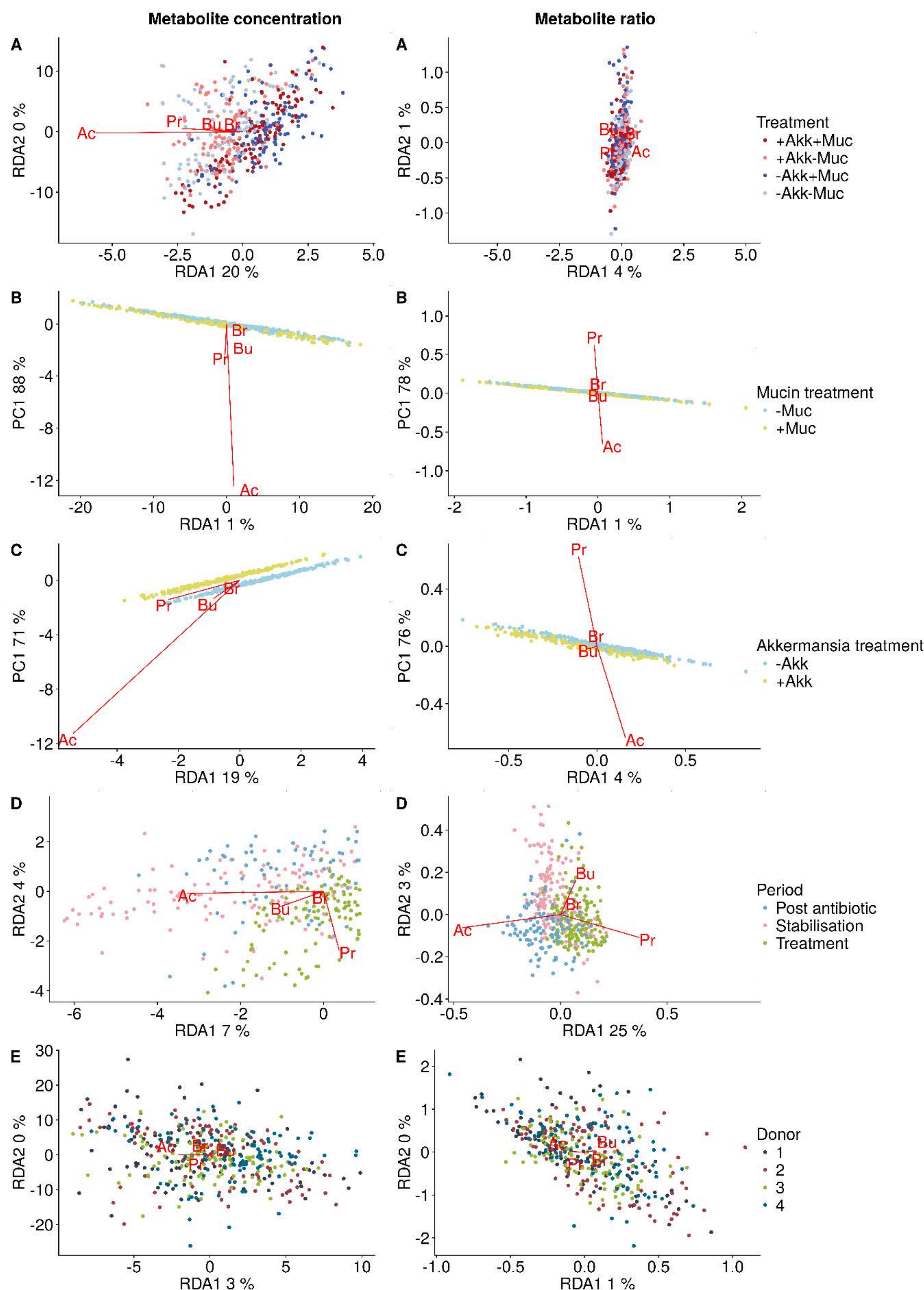


Figure 5. 4: Partial redundance analysis correlation triplot with the response variables (Ac=acetate, Pr=propionate, Bu=butyrate, Br=branched SCFA) indicated in red and the different factors represented in the legends.

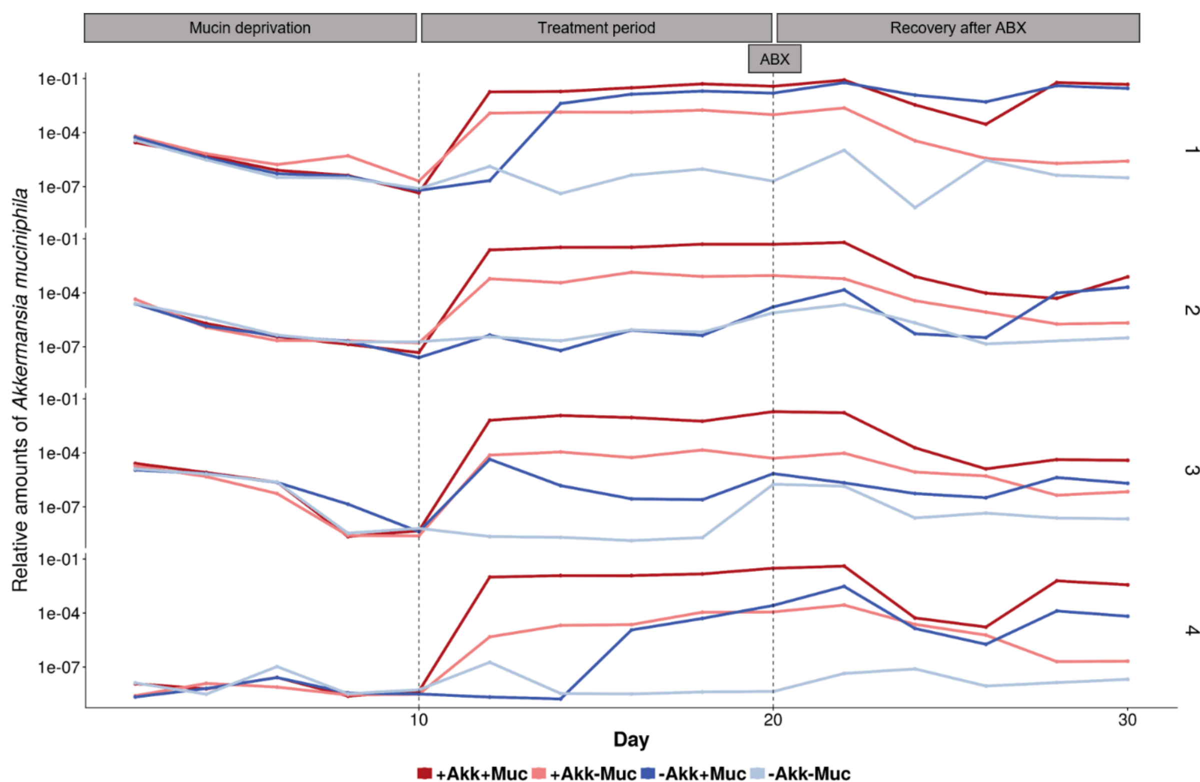


Figure 5. 5: Log (base 10) scaled relative abundance of *A. muciniphila* over total bacteria, measured with qPCR. Colon vessels were inoculated with fecal samples of donors 1-4. From day 0-10 mucin-free feed was added. From day 10-20 different treatments were imposed: vessels were treated with either *A. muciniphila* (+Akk-Muc), mucin (-Akk+Muc) (4g L^{-1}), a combination of both (+Akk+Muc) or no treatment(-Akk-Muc). At day 20 all vessels were treated with an antibiotic mix (ABX), after which *A. muciniphila treatments*, in contrast to the mucin treatments were discontinued.

Besides *A. muciniphila* (OTU9), other members of the microbial community were affected by the different treatment combinations. An unsupervised principle coordinates ordination displayed no clear donor or treatment dependent clustering, illustrating the individuality of the response to mucin and *A. muciniphila* (Figure 5.6). To quantify and distinguish between donor and treatment effects a partial distance based rda analysis was performed, showing that mucin (8%) and *A. muciniphila* (7%) effects were limited and non-significant (Supplementary Table 5.2). In order to select the taxonomic entities which were most discriminative for each of the different treatments a sPLS-DA was performed. The final model, retaining only the 75 most predictive OTUs, showed a clustering by treatment (3D plot Supplementary Figure 5.2). *Clostridium* cluster XIVa OTU26 and *Veillonella* OTUs 44 and 46 were characteristic of the control treatment without mucin or *A. muciniphila* (Figure 5.7). The addition of *A. muciniphila* had little effects on the microbial community, whereas mucin supplementation resulted in proportional increases of *A. muciniphila* (OTU9), OTU20, OTU21, OTU24, OTU48, OTU43, OTU32 and OTU37. Interestingly, co-administration of *A. muciniphila* restricted the effect of mucin to these OTUs and amplified the *A. muciniphila*

upsurge (Figure 5.7). This was also reflected at the genus level (Figure 5.7) and in the DESeq2 analysis, comparing the different conditions after ten days of treatment (Figure 5.8). Mucin treatment (-Akk+Muc vs -Akk-Muc), significantly stimulated *A. muciniphila* (OTU9), OTU32-OTU63 (~ *Clostridium cluster XIVa*) and OTU48 (~*Ruminococcus torques*) (Figure 5.6). OTU46 (~Veilonellaceae), OTU41 (~*Enterobacter*) and OTU26 (~Clostridium cluster XIVa), on the other hand, were characteristic of mucin-deprived communities (Figure 5.8). In the presence of added *A. muciniphila* (+Akk+Muc vs +Akk-Muc) less OTUs were significantly affected, comprising OTU9 and OTU32 increasing in abundance and OTU62 (~Enterobacteriaceae) and OTU78 (~Lachnospiraceae) decreasing in abundance (Figure 5.8).

In line with the spls-DA, the effect of *A. muciniphila* supplementation on the community was very limited, with only 0.33% of the community at OTU level significantly affected. *A. muciniphila* adversely affected OTU48 (~*R.torques*) abundances in the presence of mucin (+Akk+Muc) and OTU41 (~*Enterobacter*) in the treatment without mucin (+Akk-Muc).

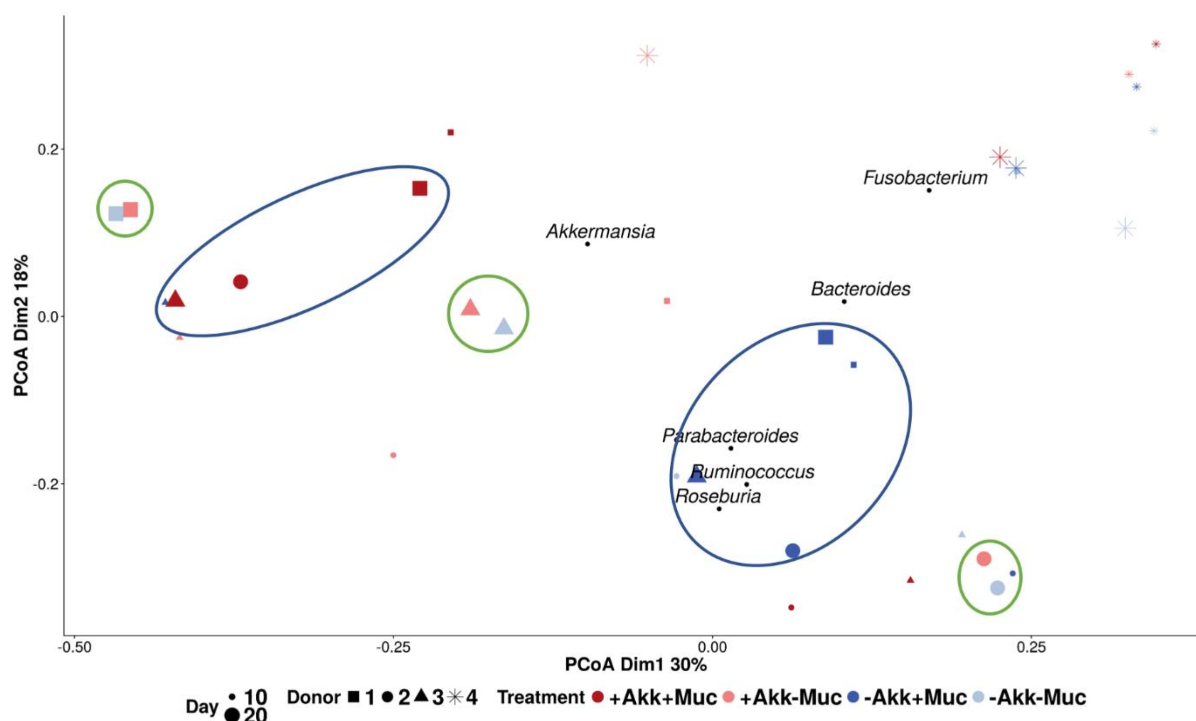


Figure 5. 6: A PCoA biplot revealed the effect of treatment (colors) on the bacterial communities of the different donors (shapes) comparing day 10 and 20 (size). Blue ellipses show clustering of donors 1-3 with mucin; with or without *A. muciniphila*. Green ellipses show clustering according to donor without mucin, independent from *A. muciniphila* treatment. Weighted average scores of genera characteristic of treatments were *a posteriori* projected.

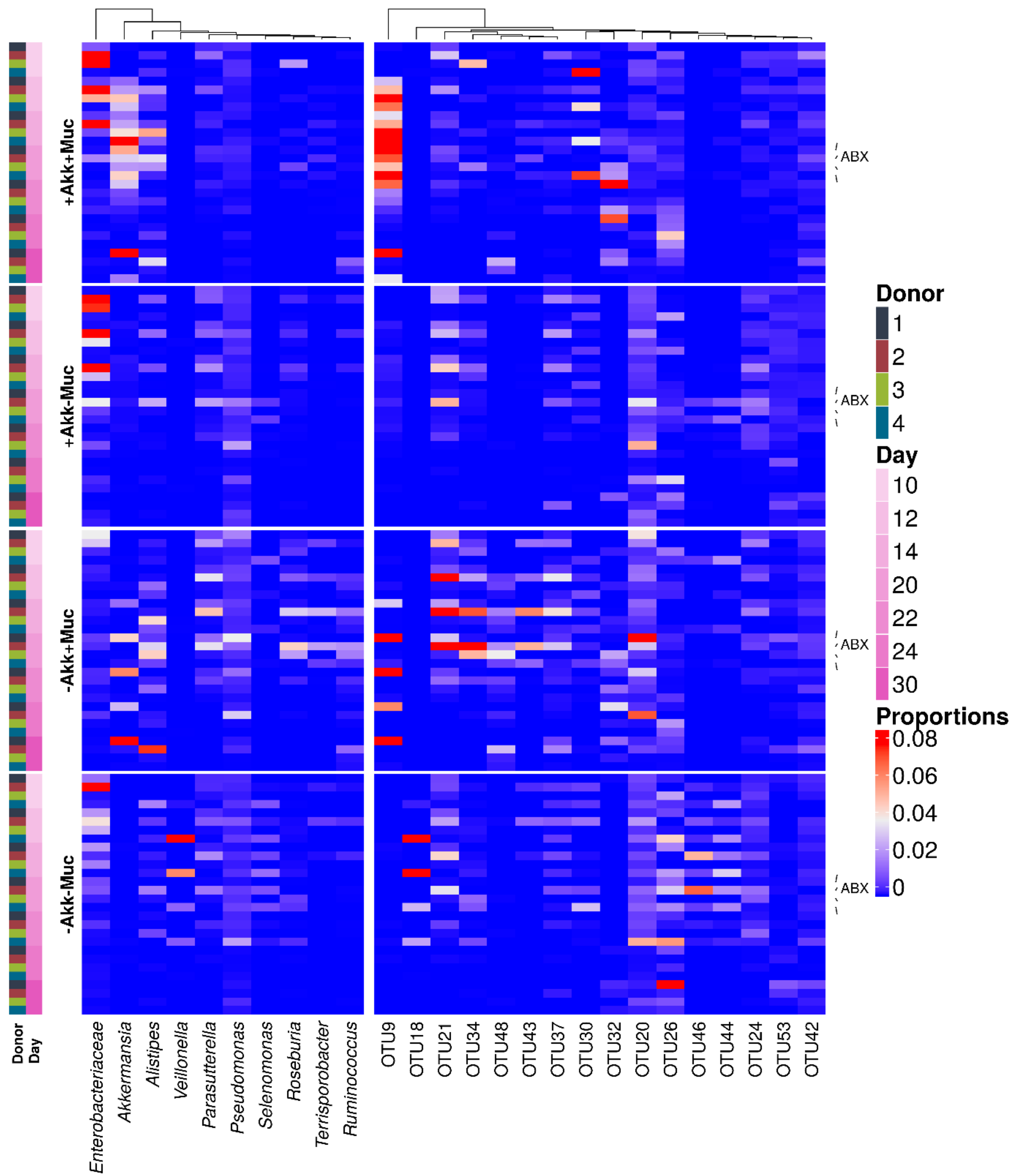


Figure 5. 7: Heatmap representation of the most predictive genera (left side) and OTUs (right side) for the different treatments as determined by sPLS-DA regression analysis.

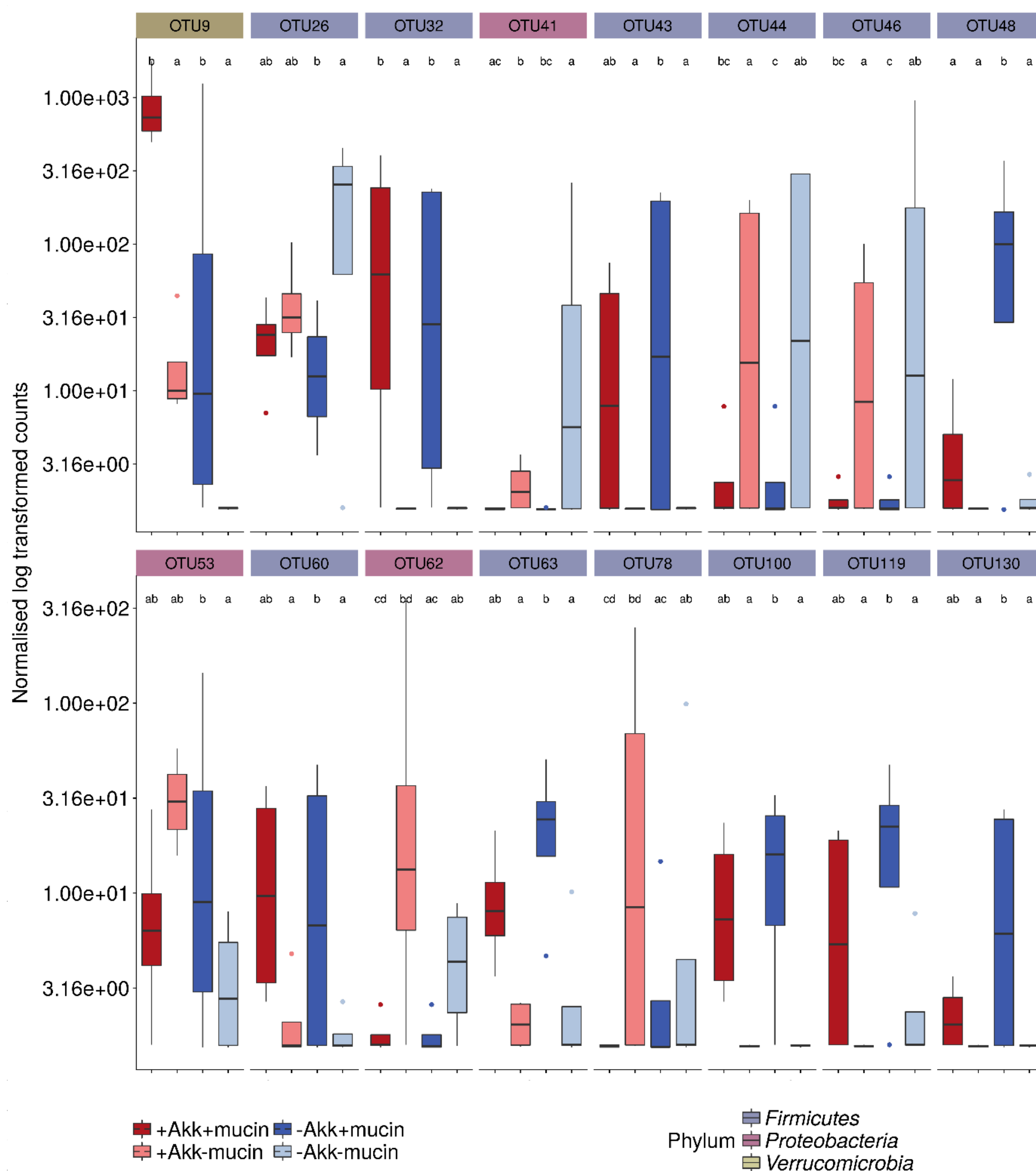


Figure 5. 8: Boxplots of OTUs that were significantly different in abundance between treatments (day 20) over all four donors as determined by DESeq2 analysis (α=0.05). Color of the boxplots represents the different treatments and facet labels are colored according to the phylum level classification. Letter codes show significance.

The DeSeq2 procedure resulted in few significant OTUs across all four donors. These inter-individual differences are apparent from the PCoA analyses at the genus level before (day 10) and after treatment (day 20) (Figure 5.6). Samples from donor 4 clustered separately, partly due to the higher relative abundance of *Fusobacterium* spp. For the other donors, different clusters could be distinguished in response to the treatments. Communities after treatment with mucin are characterized by *Ruminococcus, Roseburia* and *Parabacteroides* presence. Conditions without mucin on the other hand clustered according to donor, independent from *A. muciniphila* treatment (green ellipses). So the mucin effect on the community composition is influenced by addition of *A. muciniphila* whereas *A. muciniphila* had no effect without mucin.

After 10 days of treatment (day 20), an **antibiotic** pulse, containing ciprofloxacin, tetracycline and amoxicillin was applied to the colon vessels, after which *A. muciniphila* treatment ceased but mucin treatment continued. The effect of this antibiotic disturbance was followed up to investigate whether preceding treatment with mucin and/or *A muciniphila* would have protective effects. At the functional level as well no protective effects were observed as the drop in SCFA production after the antibiotic pulse resulted in more similar SCFA profiles across treatments (Figure 5.3). The decrease in propionate and butyrate after the antibiotic pulse was significantly larger ($p<0.05$) in the presence of mucin, off-setting the initial positive effects of mucin addition. Acetate almost fully recovered to the levels before antibiotic disturbance within ten days. Propionate and butyrate levels remained significantly lower throughout the antibiotic wash-out period ($p<0.01$). Four to six days after the disturbance, *A. muciniphila* abundance was lowest, after which it increased again in conditions with mucin and after ten days recovery a clear difference, although not significant, between conditions with and without mucin was visible (Figure 5.5). Also at the community level, no protective effects were observed from the treatments and the disturbance persisted after 10 days. The antibiotic pulse marginally reduced total bacterial count (Supplementary Figure 5.3) and affected the same genera, such as *Roseburia, Bifidobacterium, Alistipes, Butyricicoccus, Enterobacteriaceae, …,* independent of the preceding treatment, as was determined by DESeq analysis (Supplementary Figure 5.4). Alpha-diversity was significantly reduced after antibiotic treatment and did not recover within 10 days ($p<0.01$) (Supplementary Figure 5.5).

An interesting effect of the treatments and antibiotic disturbance was observed for **donor 2**. Endogenous *A. muciniphila* did not increase with mucin addition during the treatment period, but suddenly responded to mucin after the antibiotic disturbance (Figure 5.5). Interestingly, this mucin treatment, without response of *A. muciniphila,* caused an increase in butyrate, significantly larger than in any other donor or for any other treatment, and induced no response in propionate (Figure 5.3). A detailed inspection of the time course

of the relative abundances from species that were significantly affected solely by the mucin treatment, revealed an interesting response of *Ruminococcus* species OTU48 and OTU65, together with butyrate producing *Roseburia* OTU34 to the mucin treatment in donor 2 (Figure 5.9). These species might be involved in the observed difference in butyrate between those two treatments (Figure 5.2). *Ruminococcus* species OTU48 and OTU65 responded to mucin treatment without *A. muciniphila supplementation*, but not to other treatments, together with butyrate producing *Roseburia* species (OTU34) which increased greatly. After antibiotic disturbance, OTU65 and OTU34 did not recover, whereas OTU48 did. OTU34 and OTU48 displayed a similar response to mucin treatment in donor 3, which was characterized by a less pronounced *A. muciniphila* response (Figure 5.5, Figure 5.9).

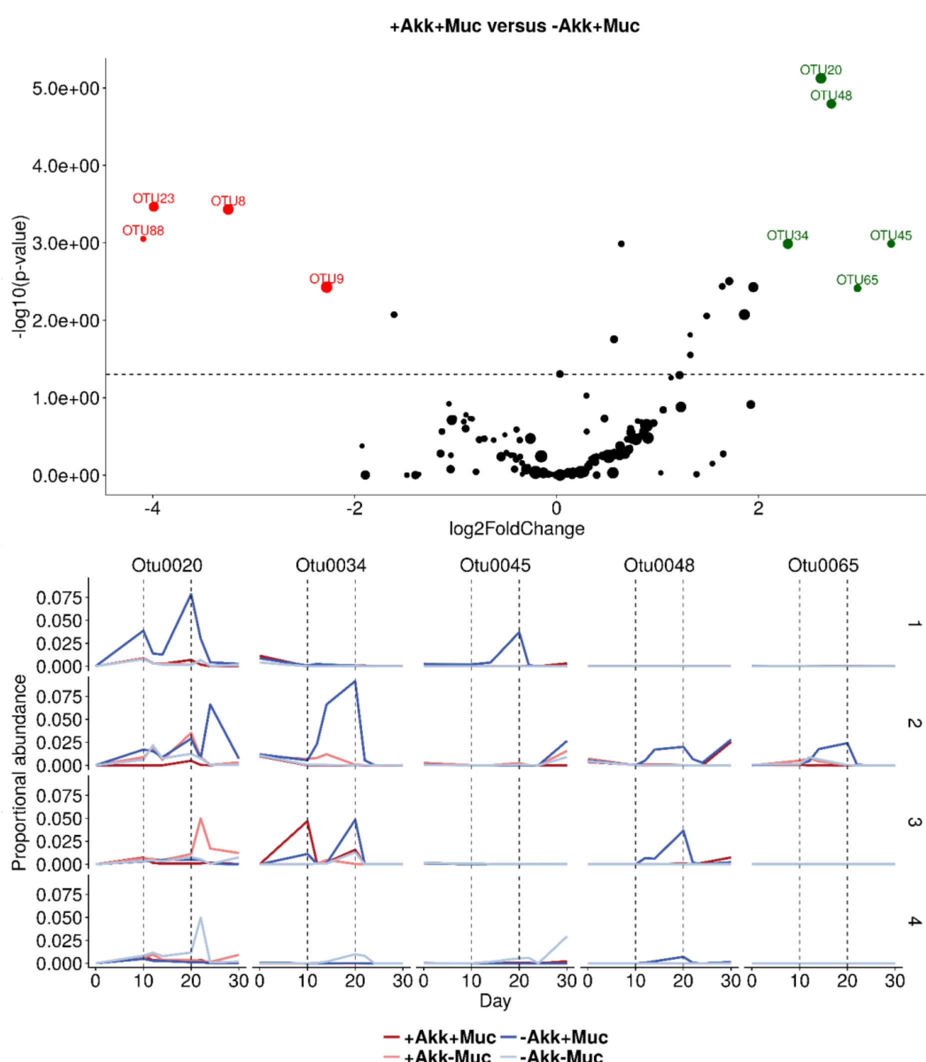


Figure 5. 9: (upper) Volcano plot showing results from DESeq2 analysis between treatments "+Akk+Muc" and "-Akk+Muc" in donor 2. Green and red dots represent OTUs more abundant in "-Akk+Muc", respectively,"+Akk+Muc" and the size indicates the relative abundance of the OTU in the community. (lower) Relative abundance of OTUs stimulated by mucin treatment"-Akk+Muc" in donor 2. As a comparison, relative abundances for the other donors are shown too.

4. Discussion

Synbiotics are the combination of a probiotic/live biotherapeutic and prebiotic with the potential advantage of the prebiotic compound increasing the survival and activity of the probiotic (Gibson and Roberfroid, 1995; Schrezenmeir and de Vrese, 2001). Studies combining *Lactobacillus* or *Bifidobacterium* species with inulin or fructooligosaccharides, observed a superior functionality of the synbiotic, as it was more effective at modulating the gut microbiota than the prebiotic or probiotic alone (Markowiak and Slizewska, 2017; Paturi *et al.*, 2015; Saulnier *et al.*, 2008). We hypothesized that the same might be true for the combination of biotherapeutic *A. muciniphila* and prebiotic-like mucin: *A. muciniphila*, being a specialist mucin degrader, would use the mucin, producing acetate and propionate and releasing mucin-derived oligosaccharides and thus have a greater impact on the community composition and functionality. For example by stimulating cross-feeding on acetate by butyrate producing species (Belzer *et al.*, 2017; Chia *et al.*, 2018) (Chapter 4). Therefore, we set out to investigate the ecological effect of supplementation of live *A. muciniphila*, the prebiotic-like action of mucin and the interplay between the two on complex microbial communities of four donors in the *in vitro* SHIME model.

Mucin addition had the largest impact on the microbial community composition and functionality. Mucin enriched communities, without addition of exogenous *A. muciniphila,* were characterized by higher endogenous *Akkermansia, Roseburia, Ruminococcus* and *Parabacteroides* proportions. Similar community shifts upon mucin addition were observed in previous studies (Chapter 2, Chapter 3). Mucin addition resulted in significant increases in acetate, propionate and butyrate production ($p<0.01$) for all donors. This increase was independent of *A. muciniphila* addition, except for donor 2, where the butyrate increase was three times higher in the absence of *A. muciniphila* supplementation. Interestingly endogenous *A. muciniphila*, although present, did not increase upon mucin supplementation in this donor. In contrast with the mucin treatment, the addition of *A. muciniphila* hardly affected the community. Abundances of OTU41 (~Enterobacter) and OTU48 (~*R.torques*) decreased and no changes in SCFA production were induced by the *Akkermansia* treatment.

When comparing the effect of *A muciniphila* with and without mucin on community level, only two species were significantly increased by the combined supplementation of *A. muciniphila* and mucin: *A. muciniphila* for obvious reasons and a *Clostridium* cluster XIVa species (OTU32). It is not clear whether the latter would benefit from putative cross-feeding interactions. *Ruminococcus torques,* a known mucin degrader (Png *et al.*, 2010), on the other hand, significantly decreased by co-administered *A. muciniphila* and mucin compared to the condition supplemented with only mucin. This suggests that co-administration of 8 log units of *A. muciniphila* gives an initial numerical advantage over other species, resulting in a more

efficient occupation of the mucin-degradation niche, thereby outcompeting endogenous community members like *R. torques*. In contrast, if mucin is administered alone, the endogenous microbiota can probably compete more efficiently with the endogenous *A. muciniphila*, eventually resulting in a bigger community change.

These findings confirm earlier findings with a synthetic microbial community investigating competition for mucin degradation between *A. muciniphila* and *B. thetaiotaomicron* (Chapter 4). When both were added as primary degraders, at the same concentration on a mucin rich medium, they became equally abundant, whereas when *A. muciniphila* was added in 1.000 times higher amounts compared to *B. thetaiotaomicron*, *A. muciniphila* outcompeted *B. thetaiotaomicron.* It would be interesting to repeat this study with other mucin-degrading bacteria, such as *Ruminococcus* species.

To conclude, our initial hypothesis, stating that joint supplementation of *A. muciniphila* and mucin more effectively induces cross-feeding to for instance butyrate compared to mucin alone, does not seem to hold. The highest increase in butyrate was induced by mucin at low *A. muciniphila* abundance (Donor 2). OTU32, belonging to the butyrate-producing genus *Roseburia*, was specifically increased by mucin treatment in donor 2 and to a lesser extent in donor 3. However, no *Roseburia* species have been identified to degrade mucin. Butyrate production would thus be the result of cross-feeding, with for example OTU48 and OTU65, both belonging to *Ruminococcus* and increased by the mucin treatment. Species like *R. gnavus* and *R. torques* are known mucin degraders and might thus deliver acetate and mucin-derived oligosaccharides to *Roseburia* and other butyrate producing species (Hoskins, 1993; Hoskins *et al.*, 1985; Marcobal *et al.*, 2013d; Png *et al.*, 2010). It is a possibility that this cross-feeding consortium prevented endogenous *A. muciniphila* from benefitting from the mucin. In support of this hypothesis, we observed that *A. muciniphila* abundance increased upon mucin treatment after disturbance of the community with antibiotics, together with OTU48 (~*R. torques*), while OTU32 (~*Roseburia*) and OTU65 (~*Ruminococcus*) did not recover.

Yet the combined addition of *A. muciniphila* and mucin may still provide a protective advantage in case of an acute stress. We chose antibiotic administration as a relevant stress factor for the gut microbiota and the mix of amoxicillin, tetracycline and ciprofloxacin was previously found to display a broad antimicrobial spectrum (Marzorati *et al.*, 2017). Antibiotic disruption of the microbial community ten days after the mucin and/or *Akkermansia* treatment caused a profound decrease in SCFA production, in line with results from *in vivo* and *in vitro* studies (Gustafsson *et al.*, 1998; Marzorati *et al.*, 2017; Van den Abbeele *et al.*, 2012); yet the profiles of SCFA were not altered. In addition, community composition was heavily affected and it did not recover within the ten day recovery period. This antibiotic stress

abolished the functional and compositional changes induced by the different treatments. Thus, no protective effects from mucin and/or *Akkermansia* treatment were observed.

In contrast with previous studies, *A. muciniphila* was not washed out of the system when no mucin was added to the feed during 30 days (Chapter 2, Chapter 3). Its abundance decreased due to the mucin deprivation in the first 10-14 days, but stabilized afterwards. Plovier *et al.* (2017) previously obtained dense *A. muciniphila* cultures on a mucin-free medium, containing peptone, glucose; N-acetylglucosamine and threonine. All compounds of this mucin-free medium were also present in our mucin-free SHIME feed, possibly explaining why *A. muciniphila* did not completely disappear.

To conclude, this *in vitro* study with four donors revealed that the joint supplementation of *A. muciniphila* with mucin limited the prebiotic-like effect that was observed for mucin in inducing compositional changes. While cross-feeding on mucin has been shown for butyrate-producing bacteria and *A. muciniphila* in co-culture experiments (Belzer *et al.*, 2017) (Chapter 4), *A. muciniphila* does not seem to enhance cross-feeding in a complex microbial background. Addition of both mucin and *A. muciniphila* might lead to *A. muciniphila,* dominating the mucin degradation niche, while sole mucin addition leads to involvement of several species, including *A. muciniphila, Ruminococcus, Clostridium* cluster *XIVa,* and *Lachnospiraceae.* When aiming at the modulation of (mucus-associated) microbiota, stimulation of endogenous *A. muciniphila* might thus be more successful compared to its administration as a live biotherapeutic product.

5. Acknowledgements

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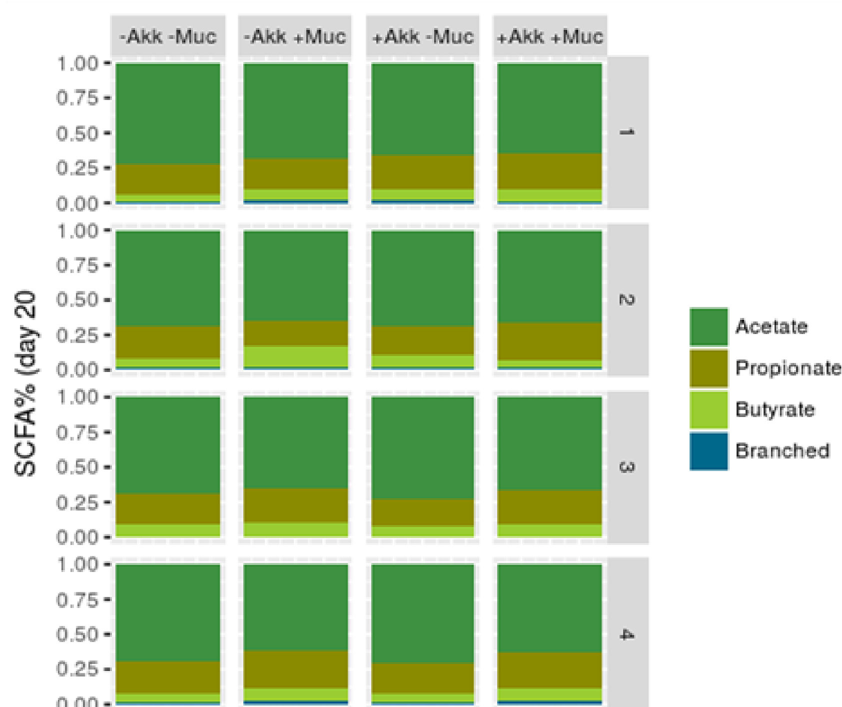
The authors would like to thank Jana De Bodt and Chloë Rotsaert for the technical support and Kim de Paepe for statistical support.

6. Supplementary information



Supplementary Figure 5. 1: Proportions of acetate, propionate, butyrate and branched SCFA for the different donors and treatments at day 20.

Supplementary Table 5. 1: RDP Seqmatch and NCBI BLAST results for the most abundant and relevant species in the microbial communities, as determined by amplicon sequencing. The similarity score (Sab) as calculated by RDP, and the NCBI BLAST output for the best hit and the next best hit(s) are shown.

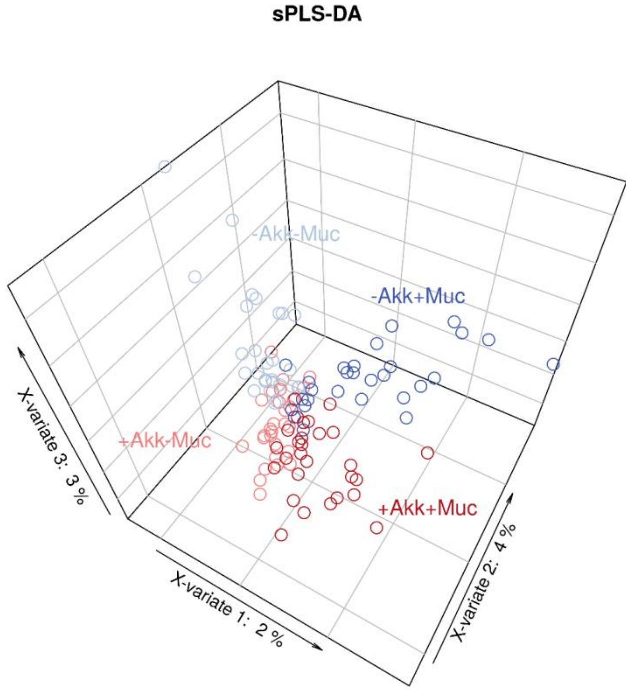
		RDP	NCBI BLAST		
		Sab	Query coverage (%)	E-score	Identity (%)
OTU1	*Escherichia/Shigella fergusonii*	1	100	0	100
	Escherichia/Shigella flexneri	1	100	0	100
	Shigella sonnei	1	100	0	100
OTU2	*Clostridium bolteae*	1	100	0	100
	Clostridium clostridioforme	0,977	100	0	100
	Clostridium citroniae	0,964	100	0	99
OTU3	*Bacteroides ovatus*	0,961	100	0	99
	Bacteroides xylanisolvens	0,891	100	0	98
OTU4	*Fusobacterium varium*	0,995	100	0	100
	Fusobacterium ulcerans	0,928	100	0	99
OTU5	*Bacteroides uniformis*	1	100	0	100
	Bacteroides rodentium	0,906	100	0	97
OTU6	*Bacteroides dorei*	1	100	0	100
	Bacteroides vulgatus	0,954	100	0	99

OTU7	*Bilophila wadsworthia*	0,973			
	Desulfovibrio simplex	0,701	100	9E-173	92
OTU8	*Kluyvera cryocrescens*	0,983	100	0	99
	Enterobacter aerogenes	0,947	100	0	99
OTU9	*Akkermansia muciniphila*	1	100	0	100
	Verrucomicrobium spinosum	0,567	99	3E-107	84
OTU10	*Phascolarctobacterium succinatutens*	0,985	100	0	99
	Phascolarctobacterium faecium	0,723	100	0	94
OTU11	*Bacteroides thetaiotaomicron*	1	100	0	100
	Bacteroides faecichinchillae	0,947	100	0	99
OTU12	*Fusobacterium nucleatum*	0,982	100	0	99
	Fusobacterium simiae	0,946	100	0	99
OTU13	*Cloacibacillus porcorum*	0,929	100	0	99
	Cloacibacillus evryensis	0,837	100	0	96
OTU14	*Alistipes onderdonkii*	1	100	0	100
	Alistipes shahii	0,882	100	0	97
OTU15	*Bacteroides xylanisolvens*	1	100	0	100
	Bacteroides acidifaciens	0,959	100	0	99
OTU16	*Clostridium aldenense*	0,964	100	0	99
	Clostridium saccharolyticum	0,869	100	0	98
OTU17	*Veillonella tobetsuensis*	0,978	100	0	99
	Veillonella rogosae	0,971	100	0	99
OTU18	*Veillonella atypica*	0,956	100	0	99
	Veillonella dispar	0,932	100	0	98
OTU19	*Blautia coccoides*	1	100	0	100
	Blautia schinkii	0,985	100	0	98
OTU20	*Pseudomonas aeruginosa*	1	100	0	100
	Pseudomonas otitidis	959	100	0	99
OTU21	*Parasutterella excrementihominis*	1	100	0	100
	Parasutterella secunda	0,603	100	4E-166	91
OTU22	*Parabacteroides distasonis*	0,956	100	0	99
	Parabacteroides gordonii	0,664	100	4E-171	92
OTU23	*Citrobacter freundii*	1	100	0	100
	Raoultella terrigena	0,964	100	0	99
OTU24	*Bifidobacterium adolescentis*	1	100	0	100
	Bifidobacterium faecale	1	100	0	100
OTU25	*Bacteroides cellulosilyticus*	0,951	100	0	99
	Bacteroides intestinalis	0,92	100	0	99
OTU26	*Clostridium hathewayi*	1	100	0	100
	Clostridium xylanolyticum	0,879	100	0	97
OTU27	*Dialister invisus*	1	100	0	100
	Dialister propionicifaciens	0,839	100	0	95
OTU28	*Insolitispirillum peregrinum*	0,525	99	2E-127	87
	Novispirillum itersonii	0,525	99	9E-128	87
OTU29	*Enterobacter asburiae*	1	100	0	100
	Enterobacter xiangfangensis	1	100	0	100
OTU30	*Bacteroides acidifaciens*	0,889	100	0	96
	Bacteroides thetaiotaomicron	0,843	100	0	95

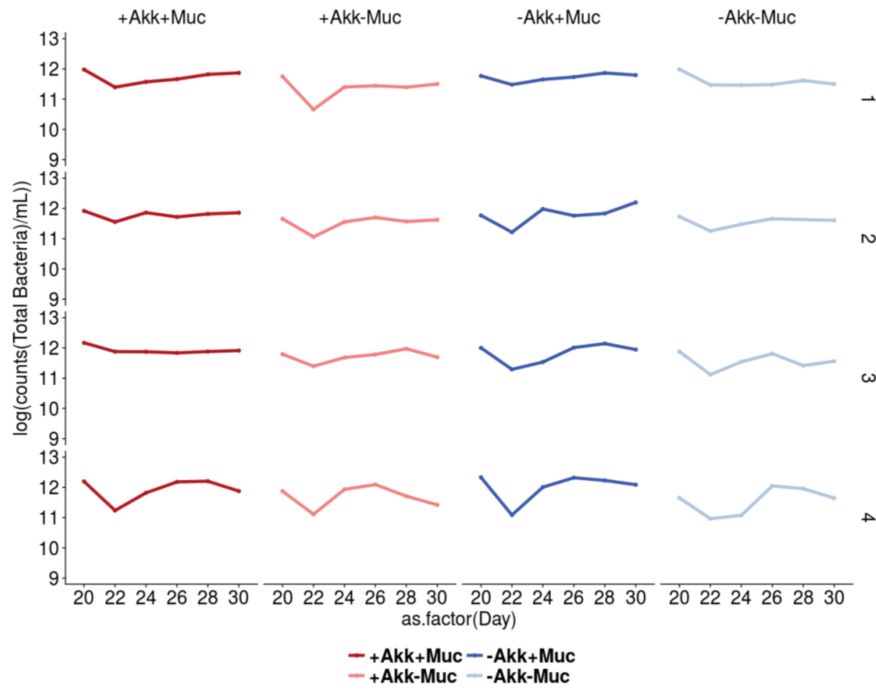
OTU32	*Clostridium hylemonae*	0,896	100	0	98
	Ruminococcus gnavus	0,83	100	0	96
OTU34	*Roseburia faecis*	0,949	100	0	99
	Eubacterium rectale	0,949	100	0	100
OTU37	*Clostridium xylanolyticum*	0,849	100	0	97
	Clostridium saccharolyticum	0,849	100	0	97
OTU41	*Enterobacter kobei*	0,983	100	0	99
	Enterobacter cloacae	0,966	100	0	99
OTU42	*Clostridium scindens*	1	100	0	100
	Clostridium hylemonae	0,843	100	0	96
OTU43	*Terrisporobacter glycolicus*	0,956	100	0	99
	Terrisporobacter mayombei	0,951	100	0	99
OTU44	*Selenomonas infelix*	0,983	100	0	99
	Selenomonas noxia	0,896	100	0	97
OTU45	*Blautia faecis*	1	100	0	100
	Blautia glucerasea	0,926	100	0	99
OTU46	*Selenomonas infelix*	0,923	100	0	98
	Selenomonas noxia	0,897	100	0	97
OTU48	*Ruminococcus torques*	0,98	100	0	99
	Ruminococcus faecis	0,89	100	0	98
OTU53	*Stenotrophomonas maltophilia*	0,978	100	0	98
	Stenotrophomonas pavanii	0,894	100	0	98
OTU60	*Murimonas intestini*	1	100	0	100
	Ruminococcus lactaris	0,877	100	0	97
OTU65	*Ruminococcus torques*	0,87	100	0	96
	Ruminococcus lactaris	0,826	100	0	97
OTU78	*Clostridium colinum*	0,83	100	0	96
	Eubacterium ventriosum	0,638	100	2E-149	90
OTU100	*Lactonifactor longoviformis*	0,811	100	0	96
	Roseburia intestinalis	0,749	100	4E-176	94
OTU119	*Anaerofilum pentosovorans*	0,861	100	0	97
	Anaerofilum agile	0,843	100	0	97
OTU130	*Clostridium lactatifermentans*	0,741	100	3E-167	93
	Clostridium propionicum	0,723	100	6E-179	95

Supplementary Table 5. 2: Partial distance based redundancy analysis. The contribution of the different factors, and significance level, to the variation in species level community composition.

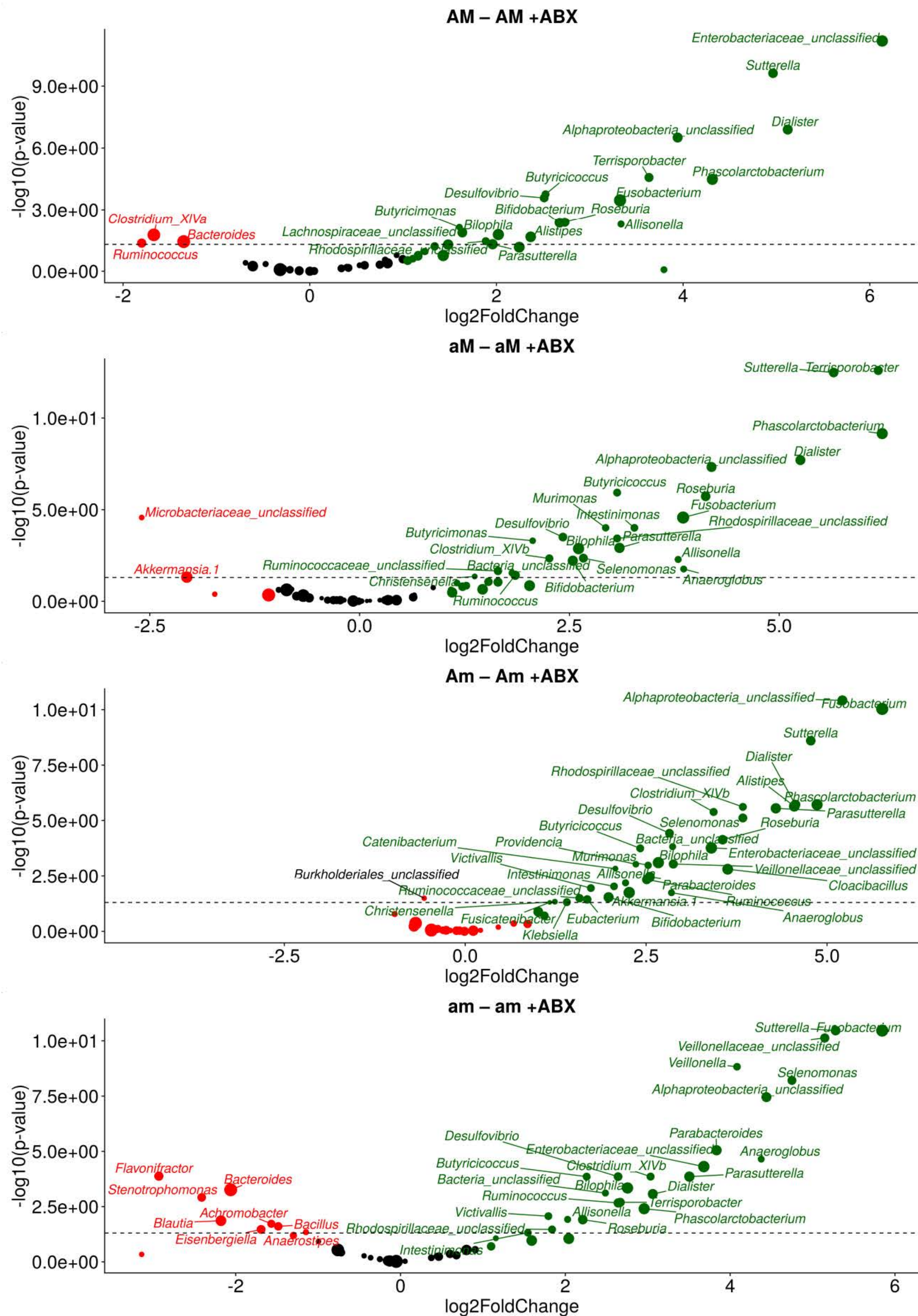
	% variance explained	**p-value**
Donor	14%	0.041
Treatment	20%	0.346
Mucin	8%	0.197
A. muciniphila	7%	0.278



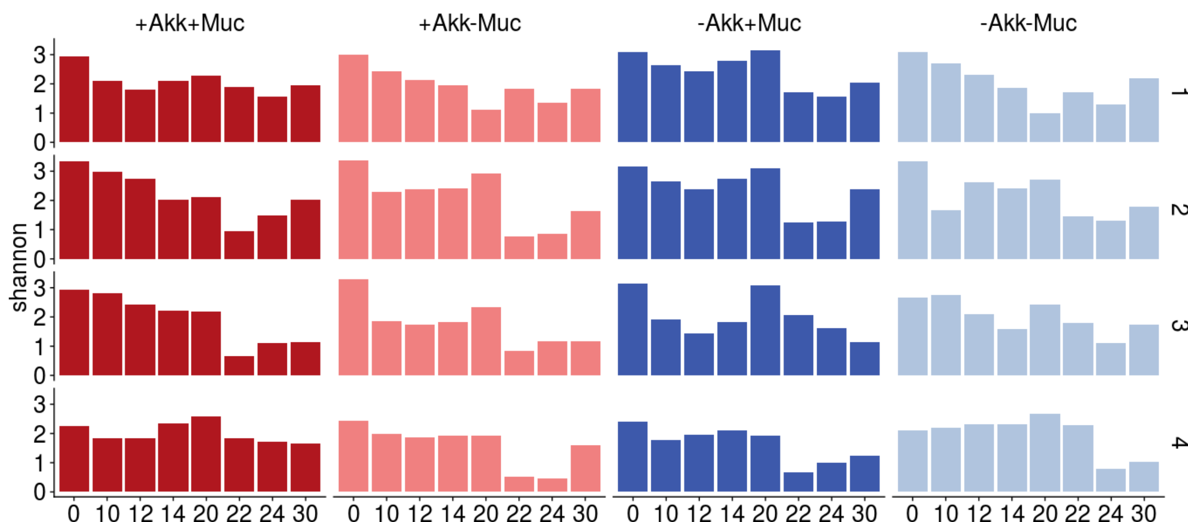
Supplementary Figure 5. 2: Optimal sPLS-DA model, as determined by fivefold cross-validation, retaining the species most predictive of the different treatments (+Akk+Muc; +Akk-Muc; -Akk+Muc; -Akk-Muc).



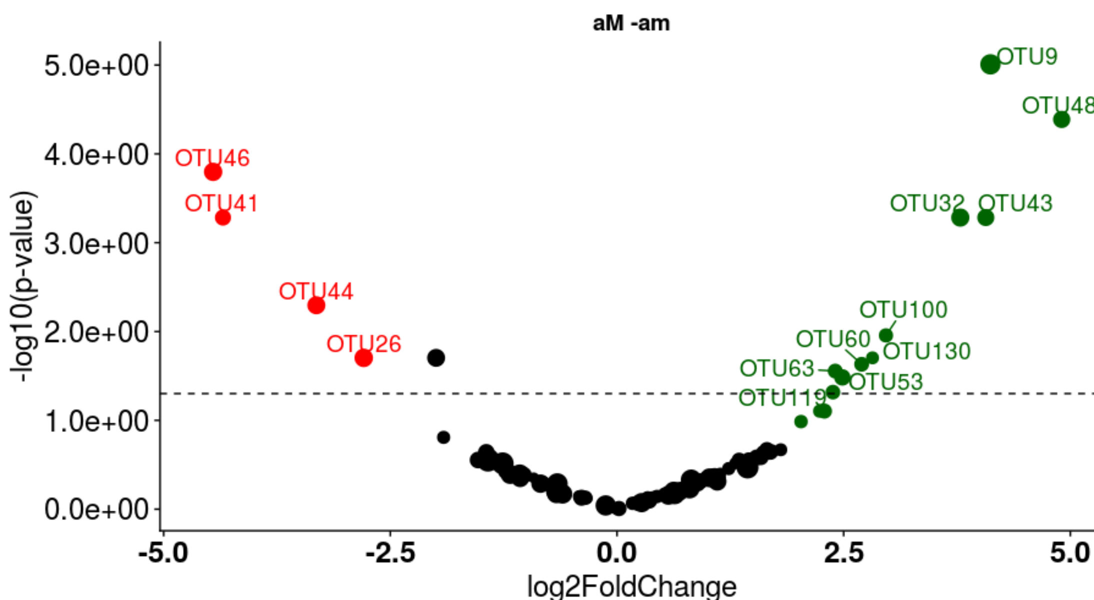
Supplementary Figure 5. 3:qPCR analysis of 16S rRNA gene showing the response of the total acterial counts to the antibiotic pulse for the different donors and treatments.



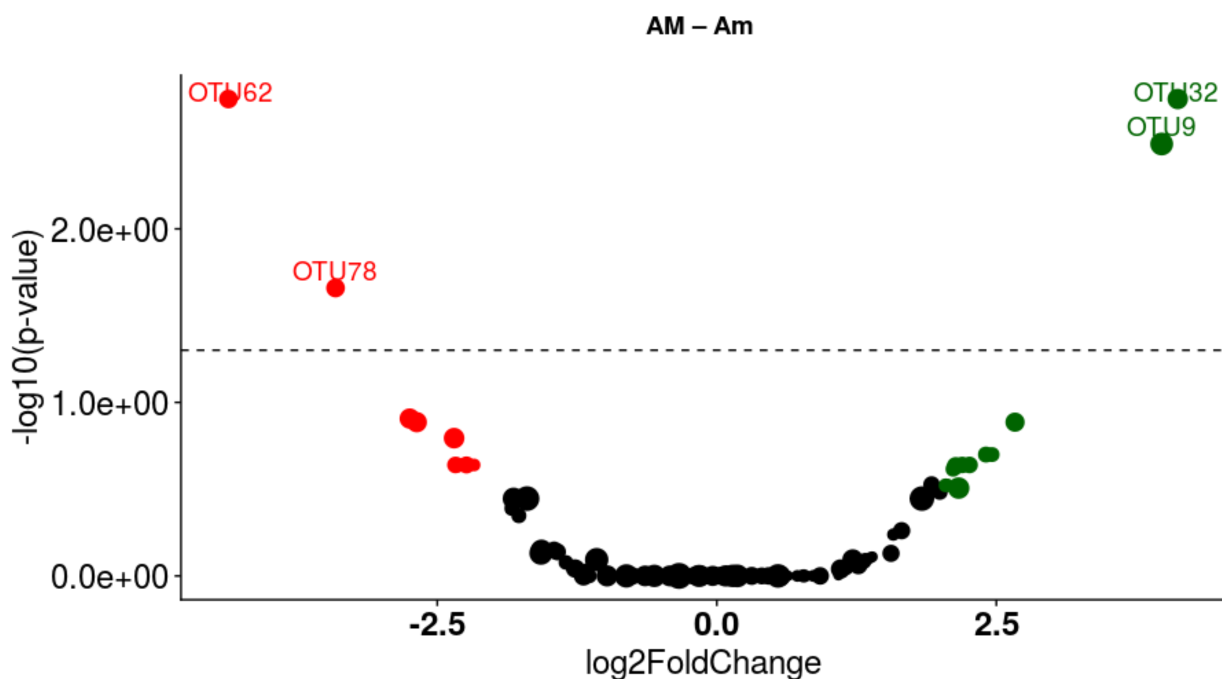
Supplementary Figure 5. 4: Volcano plot showing results from the DESeq2 analysis showing the significantly (p<0.01) affected genera by antibiotic disturbance for each treatment (AM=+Akk+Muc; aM=-Akk+Muc; Am=+Akk-Muc; am=-Akk-Muc). Green and red dots show OTUs more abundant before and after antibiotic disturbance, respectively, and the size indicates the relative abundance of the OTU in the community.



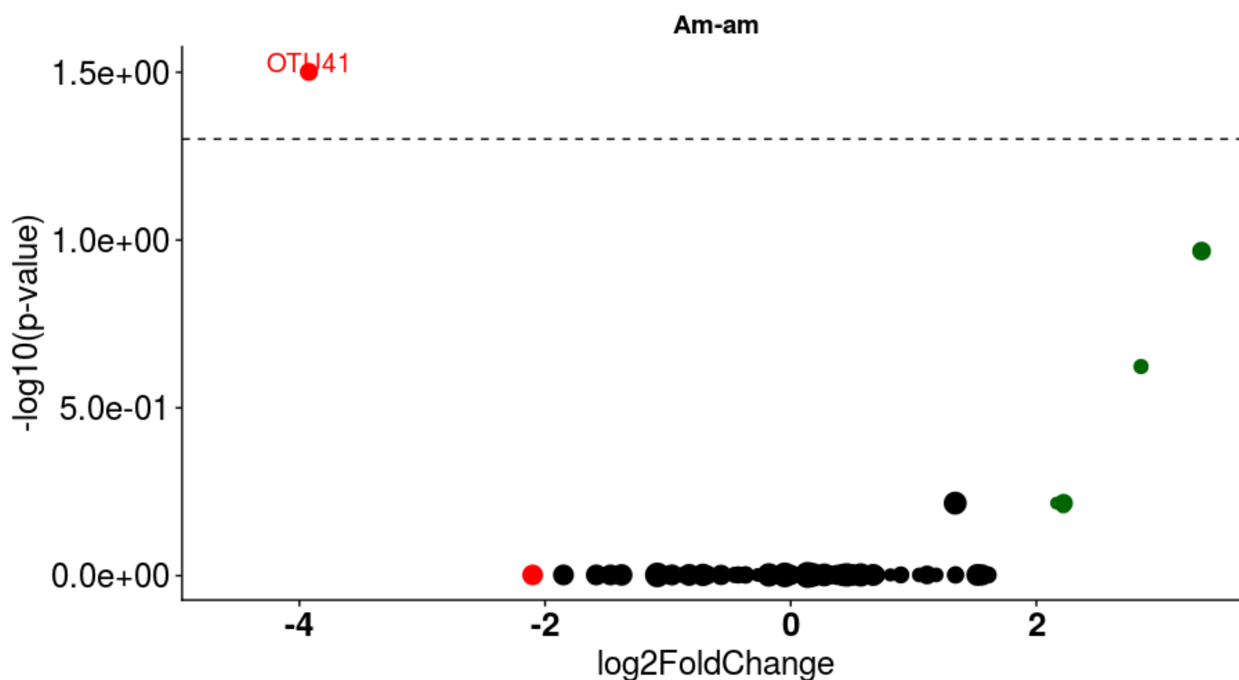
Supplementary Figure 5. 5: Alpha-diversity, measured by Shannon coefficient, over time (Days) for the different donors and treatments.



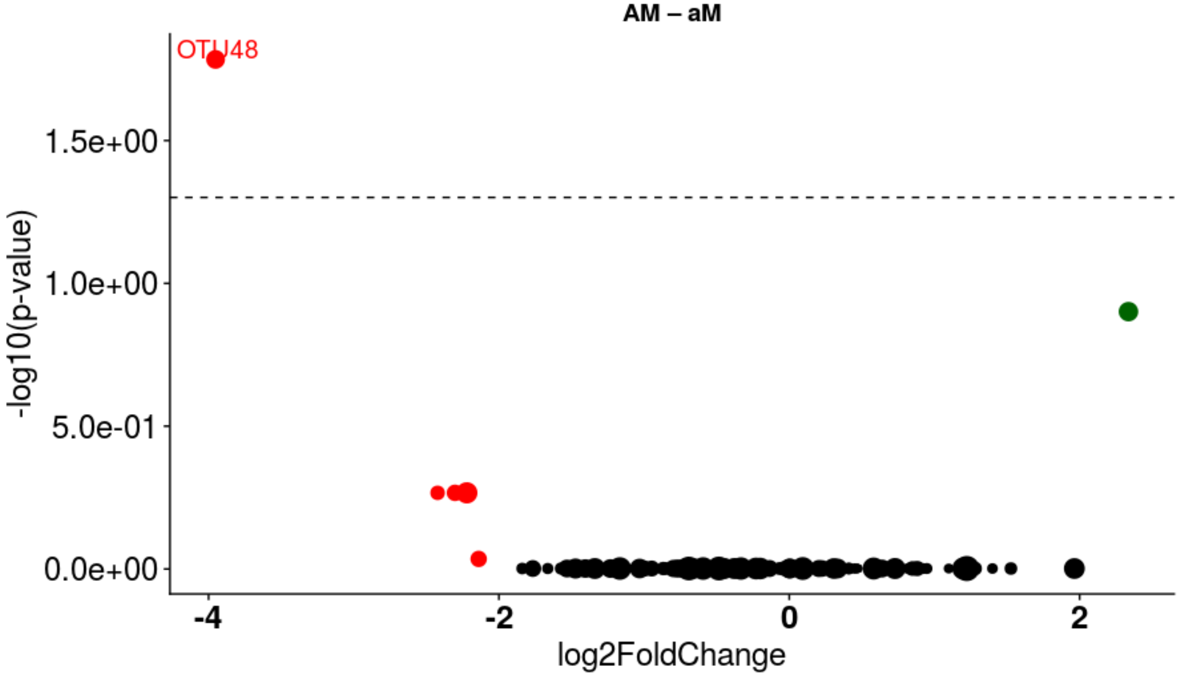
Supplementary Figure 5.6: Volcano plot showing results from the DESeq2 analysis comparing treatments " –Akk+Muc" and "-Akk-Muc". Green and red dots show OTUs more abundant in "-Akk+Muc", respectively, "- Akk- Muc" and the size indicates the relative abundance of the OTU in the community.



Supplementary Figure 5. 7: Volcano plot showing results from DESeq2 analysis between treatments " + Akk+Muc" and "+Akk-Muc". Green dot show OTUs more abundant in "+Akk+Muc", red dots for "+ Akk- Muc" and the size indicates relative abundance of the OTU in the community.



Supplementary Figure 5. 8: Volcano plot showing results from DESeq2 analysis between treatments " + Akk-Muc" and "-Akk-Muc". Green dot show OTUs more abundant in "+Akk-Muc", red dots for "- Akk-Muc" and the size indicates relative abundance of the OTU in the community.



Supplementary Figure 5. 9: Volcano plot showing results from DESeq2 analysis between treatments " + Akk+Muc" and "-Akk+Muc". Green dot show OTUs more abundant in "+Akk+Muc", red dots for "-Akk+ Muc" and the size indicates relative abundance of the OTU in the community.

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CHAPTER 6

Mucin and mucin degrading *Akkermansia muciniphila* display differentially protective effects towards *in vitro* epithelial barrier functioning and immune modulation

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This chapter has been redrafted after

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CHAPTER 6

Mucin and mucin degrading *Akkermansia muciniphila* display differentially protective effects towards *in vitro* epithelial barrier functioning and immune modulation

Abstract

The colonic mucus layer, a viscous gel matrix made up of mucin glycoproteins, separates the gut lumen from the epithelial cells and provides the interface for host-microbe interactions. The presence and the activity of mucin degrading consortia in the mucus layer, close to the host cells, induces host response and may play a relevant role on gut health. The aim of this study was to evaluate the differential effects *in vitro* of the gut microbiota modulation by mucin and/or *A. muciniphila* on the epithelial barrier function and immune response. Fecal microbial communities from three healthy donors were stabilized in the simulator of the human intestinal microbial ecosystem (SHIME) and the microbial communities were shaped through supplementation of *A. muciniphila* as live biotherapeutic or by introduction of mucin, representing a host glycan degradation niche. The effect of filtered-sterilized SHIME supernatants on intestinal barrier and cytokine (IL-6 and TNF-α) production was evaluated in a co-culture model of Caco-2 cells with differentiated macrophage-like THP-1 cells using a bi-compartmental system. Mucin and *Akkermansia*-modulated communities induced the most beneficial response by increasing the trans-epithelial resistance (TEER) and reducing TNF-α and IL-6 production.

1. Introduction

The human colon hosts a complex and diverse microbial community that is able to impact host health through intricate host-microbe interactions, which have to be carefully regulated to maintain homeostasis (Backhed *et al.*, 2012). The mucus layer is a viscous gel matrix made up of mucin glycoproteins that separates the gut lumen from the epithelial cells and thus provides the interface between the host and the gut microbiota. Besides acting as a barrier, the mucus layer, and specifically the mucin glycans, also serves as a growth substrate for colonic bacteria, an aspect that has gained more attention recently (De Weirdt and Van de Wiele, 2015). It has recently been established that mucin degradation, which was previously thought of as detrimental for gut health, is part of a normal turn-over process (Norin *et al.*, 1985). Due to the complexity of the mucin structure and the variation in glycosylation, a wide variety of specific enzymes are required for its degradation, and as a consequence few bacteria possess the enzymatic capacity to grow on mucins (Marcobal *et al.*, 2013a; Marcobal *et al.*, 2013d; Martens *et al.*, 2008; Png *et al.*, 2010; Tailford *et al.*, 2015a). Degradation of mucins leads to the release of less complex carbohydrates and the production of metabolites like acetate, lactate, and propionate, which can be used by other bacteria to produce butyrate or other end products (Belzer and de Vos, 2012). The presence and the activity of mucin degrading consortia in the mucus layer, close to the host cells, is a key element in the host-microbiome crosstalk affecting gut health in a positive or detrimental way.

Akkermansia muciniphila is regarded as a mucin degrading specialist as it can use up to 85% of the total mucin structure and has a an entire repertoire of intra-and extracellular enzymes involved in this process with both extracellular and intracellular activity (Derrien, 2007). A study of its genome revealed 61 proteins predicted to be involved in mucin degradation (11% of all proteins) and its high mucin-degrading capacity was shown in an *in vivo* mice study (Berry *et al.*, 2013; van Passel *et al.*, 2011). Metabolic activity of *A. muciniphila* on mucins can stimulate microbial metabolic interactions and induce a host response (Belzer *et al.*, 2017; Chia *et al.*, 2018; Derrien *et al.*, 2004; Reunanen *et al.*, 2015). Recently, *A. muciniphila* has been proposed as a key bacterial modulator in the cross-talk between host and gut microbiota, in which a specific outer membrane protein (Amuc_1100) played an important role. *A. muciniphila* and Amuc_1100 induced both anti-and pro-inflammatory cytokine response in human derived peripheral blood mononuclear cells and both increased epithelial cell-layer integrity of Caco-2 monolayer (Ottman *et al.*, 2017d), indicating a complex immunomodulatory role, affecting the dialogue with the host. The improvement of epithelial barrier function by *A. muciniphila* has been shown in several *in vivo* mice studies (Everard *et al.*, 2013; Li *et al.*, 2016; Shin *et al.*, 2014), including a study with

obese mice showing that genes encoding tight junction proteins were affected by treatment with *A. muciniphila* and Amuc_1100, possibly through TLR2 activation (Plovier *et al.*, 2017).

The epithelial barrier is constituted by intestinal epithelial cells that are firmly attached to each other by tight junctions and regulate translocation to underlying immune effector cells (Groschwitz and Hogan, 2009). Decreased epithelial barrier functioning can increase gut permeability which leads to low grade inflammation and is observed for a variety of human diseases such as IBD, diabetes and obesity (Bischoff *et al.*, 2014). The commensal bacteria can regulate epithelial barrier function, by releasing metabolites such as acetate and butyrate or by inducing the release of cytokines which can reduce (TNAα, IFNγ) and enhance (IL-10) barrier function (Arrieta *et al.*, 2006; Fukuda *et al.*, 2011; Hamer *et al.*, 2008). Modulation of the microbiota, by pre-or probiotics, may thus provide therapeutic options for maintaining epithelial barrier functioning and gut homeostasis.

The aim of this study was to assess the *in vitro* effect of gut microbiota modulation by supplementation of live *A. muciniphila* and/or introduction of a mucin niche on the epithelial barrier function and immune response. A combination of the simulator of the human intestinal microbial ecosystem (SHIME) with a co-culture of enterocyte-like (Caco-2) and macrophage-like (THP-1) showed that microbial communities modulated by mucin and *A. muciniphila* had a significant impact on gut barrier function and immune response.

2. Materials and methods

2.1 Cell cultures

2.1.1 Caco-2 cells

Caco-2 is the most widely used immortalized cell line for developing human GI tract in *in vitro* models. This cell line spontaneously differentiates into polarized cells with distinct mucosal (apical) and serosal (basolateral) cell membrane domains, brush border enzymes and polarized expression of transporters (Artursson *et al.*, 2012). The Caco-2 cells were obtained from the European Collection of Authenticated Cell Cultures (Caco-2 ECACC 86010202, Public Health England, UK). Cell maintenance was carried out in 25 cm^2 flasks to which 4 mL of Dulbecco's Modified Eagle Medium (DMEM) with high glucose (4.5 g L^{-1}) and GlutaMAX™ (Gibco, Langley, OK, USA) was added. The DMEM was supplemented with: 10% (v/v) heat-inactivated fetal bovine serum (FBS) (FBS, Greiner Bio-One, Wemmel, Belgium), 1% non-essential amino acids and 2% penicillin/streptomycin (Life Technologies, Merelbeke, Belgium) to obtain complete cell growth medium (DMEMc). Medium was refreshed every two days and cells were subcultured when they reached 70-80% confluence. Briefly, Caco-2 cells were detached with a pre-wash with 10 mL of PBS without calcium and magnesium (PBS, Gibco, Langley, OK, USA), trypsinized for 5-8 min with 1 mL of trypsin solution (2.5 g L^{-1}) and EDTA (0.2 g L^{-1}) (Gibco, Langley, OK, USA) and neutralized by the addition of supplemented medium, followed by reseeding at a density of 5 x 10^4 cells cm^{-2}. The cells were incubated at 37°C in an atmosphere with 95% relative humidity and a CO_2 flow of 10%. All the cell cultures were used between passages 43 and 47.

2.1.2 THP-1 cells

The THP-1 cells were obtained from the European Collection of Authenticated Cell Cultures (THP-1 ECACC 88081201). Cell maintenance was carried out in 75 cm^2 flasks containing 20 mL of Roswell Park Memorial Institute (RPMI) 1640 medium, supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) (FBS, Greiner Bio-One, Wemmel, Belgium), 1% sodium pyruvate, and 2% penicillin/streptomycin (Life Technologies, Merelbeke, Belgium). Medium was refreshed every two days and cells were subcultured after reaching 1 x 10^6 cells mL^{-1}. Briefly, THP-1 cells were centrifuged (1000 rpm; 5 min), suspended in 5 mL of media and diluted 1/5 in a new cell culture flask. The cells were incubated at 37°C in an atmosphere with 95% relative humidity and a CO_2 flow of 10%. All the cell cultures were used between the passage 71 and 73.

The cell morphology was analyzed by phase-contrast microscopy (Motic AE31, VWR, Leuven, Belgium).

2.1.3 Co-culture in Transwell plates

Caco-2 cell differentiation and the posterior tests were carried out in double chamber wells (Corning® HTS Transwell®-24 well, pore size 0.4 µm; Costar, NY) equipped with separate apical and basolateral compartments and a porous support on which the Caco-2 cells form a monolayer. The Caco-2 cells were seeded at a density of 7.5 x 10^4 cells cm^{-2} on top of the semipermeable filter and maintained with DMEMc without antibiotic/antifungal solution, until differentiation (15 days). Refreshments of the apical and basal media were done every 2 days.

At day 16 post-seeding, THP-1 cells were added to the basal compartment of the Transwells in a density of $1x10^5$ cells cm^{-2} in RPMI 1640 media without antibiotic/antifungal solution, and maintained in co-culture for 24 hours. Subsequently, the THP-1 were differentiated to macrophages by adding phorbol 12-myristate 12-acetate (PMA) (25 nM) (Sigma, Belgium) for two days. Thereafter, the cells were refreshed and maintained 24 hours in absence of PMA. Then, SHIME supernatants were added to the apical compartment (1.5 mL), simultaneously to LPS (10 ng mL^{-1}). As control conditions, also cell culture medium was added to the co-culture, with and without LPS. All conditions were tested in triplicate and the exposure lasted 24 hours.

2.2 SHIME supernatant

The dynamic in vitro SHIME® model (ProDigest-Ghent University, Ghent, Belgium) was used to study the impact of a probiotic treatment of *A. muciniphila*, with or without the presence of a host glycan degradation niche, in four microbial communities from healthy donors. The set-up is described in detail in Chapter 4. Briefly, fecal suspension of four donors was used to inoculate the colon vessels (4 colon vessels/donor). During the stabilization period (day 0-10), a mucin-free nutritional medium was delivered to the colon vessels. From day 10-20 onwards (treatment period), 4 different treatments were applied: "**+Akk+Muc**" where *A. muciniphila* was daily administered to the colon vessels and mucin was added to the feed; "**+Akk-Muc**" where only *A. muciniphila* was added to the colon vessels; "**-Akk+Muc**" where only mucin was added to the feed; and **"-Akk-Muc**", which was not different from the stabilization period where neither *A. muciniphila* nor mucin were added. At the end of the treatment period, samples were taken from the established communities from donors 1,2 and 4 (further mentioned as donors A, B, C) to use for co-culture experiments with Caco-2 and THP-1 cells. These three donors were selected for following reasons: the experiment with inoculum from donor 2 showed an interesting reaction of *Akkermansia muciniphila* and butyrate/propionate to the mucin supplementation (Chapter 5);

donor 4 was most dissimilar from the other donors with respect to his microbiome composition (Supplementary figure 6.3); and the experiment with inoculum from donor 1 had the most expected response in *Akkermansia* abundance: delayed response to mucin treatment, but after 10 days of treatment reaching equal levels as after mucin+*Akkermansia* treatment (Chapter 5).

Samples were centrifuged for 10 min. at 1500g, the supernatant was collected and filter-sterilized over a 0.22 µm PVDF syringe filter (Merck Millipore, Darmstadt, Germany) and immediately stored at -80°C in 1 mL aliquots.

2.3 Analysis

2.3.1 Measurements of epithelial barrier function: transepithelial electrical resistance (TEER) and apparent permeability coefficient (P_{app}) of paracellular marker

The monolayer integrity was assessed by measuring the transepithelial electrical resistance (TEER) and the apparent permeability coefficient (Papp) of the paracellular transport marker lucifer yellow (LY, Sigma-Aldrich, Belgium). A Millicel-ERS (Millipore Corporation, Belgium) was used for the TEER measurements. Measurements of the TEER were performed every 3-4 days after Caco-2 seeding. In addition, TEER was measured at day 15 post- seeding, at the moment of co-culturing with THP-1 cells, after THP-1 differentiation, before supernatant addition, and at the end of the assay. TEER values were expressed as increase/decrease of TEER values at the end of the assay (24h) relative to the beginning: ΔTEER (%) = $\frac{TEER_{24h} - TEER_{0h}}{TEER_{0h}} \times 100$

Papp of LY, which is mainly transported via the paracellular route, was used to assess the integrity of the epithelial cell monolayer. Papp of LY was measured by adding the marker (100 µM) to the apical compartment of the wells. After 15, 30, 60 and 120 min, 100 µL of medium was removed from the basolateral compartment and replaced with an equal volume of fresh medium (DMEM high glucose supplemented with 20% FBS). LY fluorescence was measured at an excitation/emission wavelength of 485/520 nm in black 96 plates (Greiner), using a microplate fluorescence reader (Spectramax Gemini XS Microplate Reader, Molecular devices, Orleans, CA). A calibration curve (0, 5, 10, 25, 50 and 100 µM) for LY quantification was included in duplicate in each reading. The Papp was calculated as previously described (Calatayud *et al.*, 2010).

2.3.2 Western blot analysis

Cells were lysed in radio-immunoprecipitation assay (RIPA) buffer supplemented with phosphatase and a protease inhibitor cocktail (Sigma-Aldrich). The concentrations of protein lysates were determined (Bio-Rad Laboratories), and 30 µg of each sample was separated on a 4-20% Criterion Stain Free gradient gel (Bio-Rad Laboratories). Next, the gel was activated by UV exposure for 1 min using the Chemidoc MP Imaging system (Bio-Rad Laboratories), and proteins were transferred to a nitrocellulose membrane (Bio-Rad Laboratories). Membranes were blocked with 5% skim milk in Tris buffered saline with 0.1% Tween-20 (TBST) (Sigma-Aldrich) and incubated overnight at 4°C with primary antibodies (1/1000 dilution) in 5% BSA/TBST [anti-ZO-1 (Cell Signaling), anti-E-cadherin (Cell Signaling) or anti-occludin (Abcam)]. Next, blots were incubated for 1 hour at room temperature with horseradish peroxidase-conjugated secondary antibodies (1/10 000 dilution, Cell Signaling). Bands were visualized using chemiluminescence (Bio-Rad Laboratories) and imaged on a Chemidoc MP Imager.

2.3.3 Cytokine quantification

Protein levels of IL6 and TNFα in the supernatant were determined using Luminex technology according to the manufacturer's guidelines (Bio-Rad Laboratories).

2.4 Statistical analysis

Statistically significant differences were determined by Pairwise Wilcoxon Rank Sum Tests with Holm correction (α=0.05).

Donor and treatment were explored as explanatory variables in a partial redundancy analysis, with as response variables the epithelial barrier function parameters (TEER and Papp of LY), inflammatory markers (IL-6 and TNFα) and SCFA concentration in the supernatant (to which the co-culture model was exposed) (package vegan_2.4-4). The statistical significance of the global model and the individual canonical axes was assessed using Permutation tests (Legendre *et al.*, 2011). The RDA results were plotted in a type II scaling correlation triplot, displaying the constrained canonical (labelled RDA1/2) axes, annotated with the proportional eigenvalues representing their contribution to the total variance. The coordinates of the sites were derived from the weighed sums of the scores of the response variables and explanatory variables are represented by centroids denoting the donor or treatment factor levels.

In order to relate the microbiome composition to the functional response, sparse partial least squares regression was performed (mixOmics_6.3.1) (Le Cao *et al.*, 2016). A smart feature selection procedure was applied to identify genera associated with the cell response.

The initial model was built in regression mode with the TEER, LY, TNFα and IL-6 values as response (Y) variables as a function of the proportional microbial community composition at genus level (X). 2 Dimensions and 50 X variables were selected after tuning based on a Leave One Out (LOO) validation (Le Cao *et al.*, 2016; Le Cao *et al.*, 2008). Results were represented in a clustered image map, visualizing the correlations between the X and Y variables.

3. Results

Characteristics of the SHIME supernatants amended or not with *A. muciniphila* and/or mucin are shown in Supplementary Figure 6.1-6.3. These supernatants were used to study whether the different amendments would affect the epithelial cells and elicit a response from immune cells (Figure 6.1). The **epithelial barrier function** was evaluated by measuring TEER and Papp of LY, and the inflammatory response was measured by quantifying IL-6 and TNF-α in apical and basal compartments. LPS was administered as a stressor to the differentiated Caco-2 cells to enhance the effect of the applied treatments. LPS administration, without SHIME supernatant, did not affect TEER values, but significantly increased the Papp of LY (~380%, $p < 0.05$) and induced the production of IL-6 and TNFα production in both apical and basal compartments (Supplementary Figure 6.4).

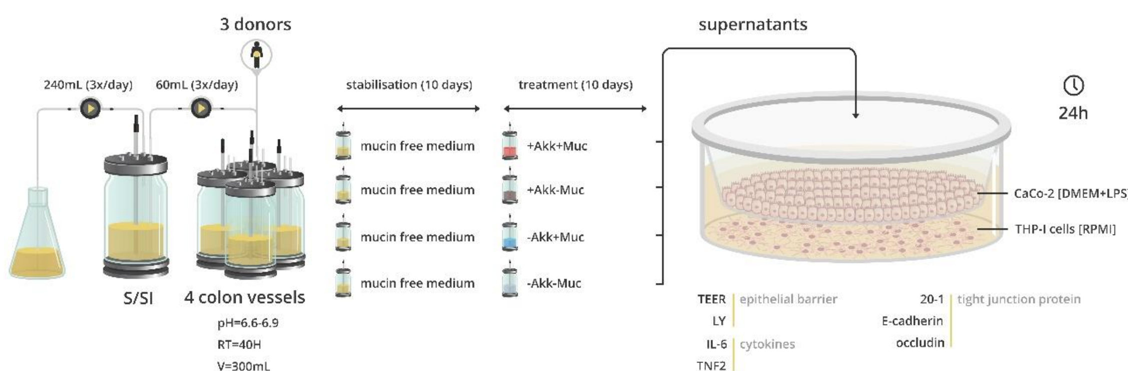

Figure 6. 1: Experimental set-up of the co-culture experiment with Caco-2 and THP-1 cells that were exposed to SHIME supernatant (in triplicate), previously modulated by different treatments.

The SHIME supernatants were derived from microbial communities with different donor origin, and this inter-individual variability impacted the cellular response. The ΔTEER in cells exposed to SHIME supernatants without any amendment ("-Akk-Muc" : controls) was similar and close to 0 in donors B and C. Cells exposed to control supernatant from donor A were characterized by decreased TEER values after 24h (Figure 6.2). Papp of LY also showed interindividual variability, with Papp values around 10^{-5} cm s^{-1} in donor C and lower LY Papp values in donor A and B (donor C > donor B > donor A (~4 x 10^{-6} cm s^{-1}). Despite the donor-related differences, epithelial barrier parameters also responded to the treatments. Supernatant from microbial community amended with mucin (from donor A and C) caused a significantly ($p < 0.05$) higher increase in TEER value compared to the supernatants without mucin. For donor A, TEER increase was higher with the addition of *A. muciniphila,* and for donor C it was higher without *A .muciniphila.* Supernatant from amended microbial communities of donor B caused no significant change in TEER value. Interestingly, the

response of the Papp of LY, also a parameter for epithelial barrier function, showed a consistent trend for the three donors. Contrary to the TEER values, which responded more to mucin-amended samples than to *A. muciniphila* treatment, the Papp of LY was significantly ($p < 0.05$) reduced by *A. muciniphila*-amended supernatants, independent from mucin presence (Figure 6.2). Papp values of LY from cells exposed to cell culture media (control) were 1.2×10^{-7} cm s^{-1} (Supplementary Figure 6.4).

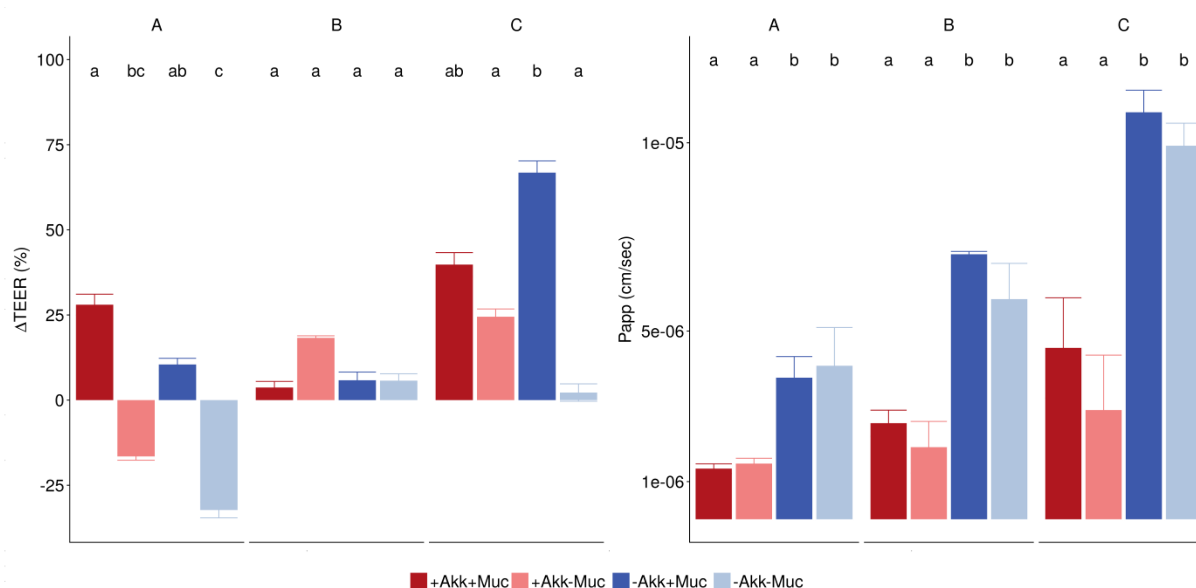

Figure 6. 2: Epithelial barrier function in the co-culture model with Caco-2 and THP-1 cells exposed (24h) to SHIME supernatant samples (3 donors A-C), amended with *A. muciniphila* (+Akk-Muc), mucin (-Akk+Muc), both (+Akk+Muc) or untreated (-Akk-Muc) (n=3). Statistically significant differences between treatments (α=0.05) are denoted by the letters a, b and c. Identical letters indicate no significant differences (p>0.05). The trans-epithelial electrical resistance (TEER) is expressed as the proportional change in TEER values after 24h exposure (%); Apparent permeability coefficient (Papp) of LY is expressed in cm/sec.

Next, the tight junction proteins (ZO-1, E-cadherin, and occludin) of the Caco-2 cells exposed to supernatants from donors A and C were qualitatively assessed by western blot (Figure 6.3). Donors A and C were selected because they induced the largest response in TEER and Papp of LY. The response of the tight junction proteins was variable between the donors.

Samples exposed to supernatant from donor A did not show differences in protein expression between treatments, or between treatments and control cells exposed to cell culture media. By contracts, an apparent reduced protein expression of ZO-1, E-cadherin and increased occludin expression was observed for the cells exposed to SHIME supernatant from donor C without amendment (-Akk-Muc) compared to the control. The

presence of mucin, *A. muciniphila* or both induced an apparent increase in E-cadherin expression, while occludin was apparently reduced in -Akk+Muc condition, when compared to the non-amended samples (-Akk-Muc).

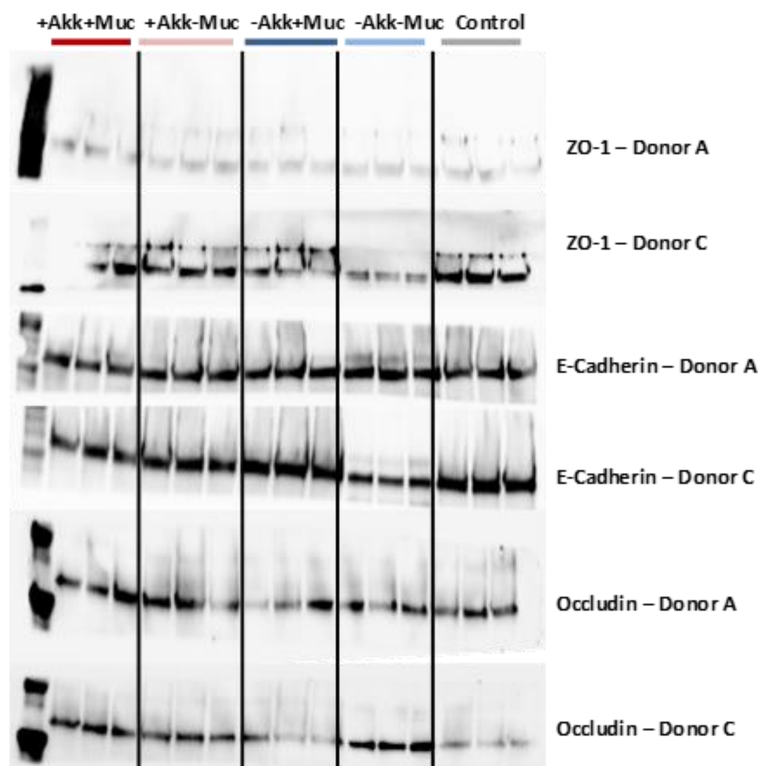

Figure 6. 3: Expression of tight junction (TJ) proteins : Zonula occludens-1 (ZO-1), E-Cadherin and Occludin in the Caco-2 cells assessed by Western blotting. Co-culture model with Caco-2 and THP-1 cells was exposed (24h) to SHIME supernatant samples, amended with A. muciniphila (+Akk-Muc), mucin (-Akk+Muc), both (+Akk+Muc) or not treated (-Akk-Muc). Control condition is referred to the Caco-2 cells exposed to cell culture media (DMEMc).

The **inflammatory response** of the Caco-2 and THP-1 cells was evaluated by measuring IL-6 and TNFα production in both the apical and the basal part of the co-culture model (Figure 6.4). The inter-individual variability induced no specific trends, but one outlier was observed in the apical part: supernatant from the microbial community of donor A without amendment increased IL-6 threefold and TNFα fourfold, while other supernatant evoked a more moderate response. For donor C, IL-6 production was significantly decreased (~65%, $p < 0.05$) when cells were exposed to mucin-amended supernatants compared to conditions without mucin. For donor B, this trend was only observed in cells exposed to supernatant of the mucin treatment without *A. muciniphila.* TNFα production was significantly higher when exposed to supernatant of mucin with *A. muciniphila* treatment from donor B, but otherwise there were no difference in response to treatments. Concentration of IL-6 in the basal part was significantly higher when treated with supernatant without mucin, independent

of *A. muciniphila* treatment, from donors B and C, but for donor A there seemed to be an additional effect of *A. muciniphila* treatment. TNFα was only significantly reduced (~80%. $p < 0.05$) in cells exposed to mucin-amendment supernatants from donor C (Figure 6.4).

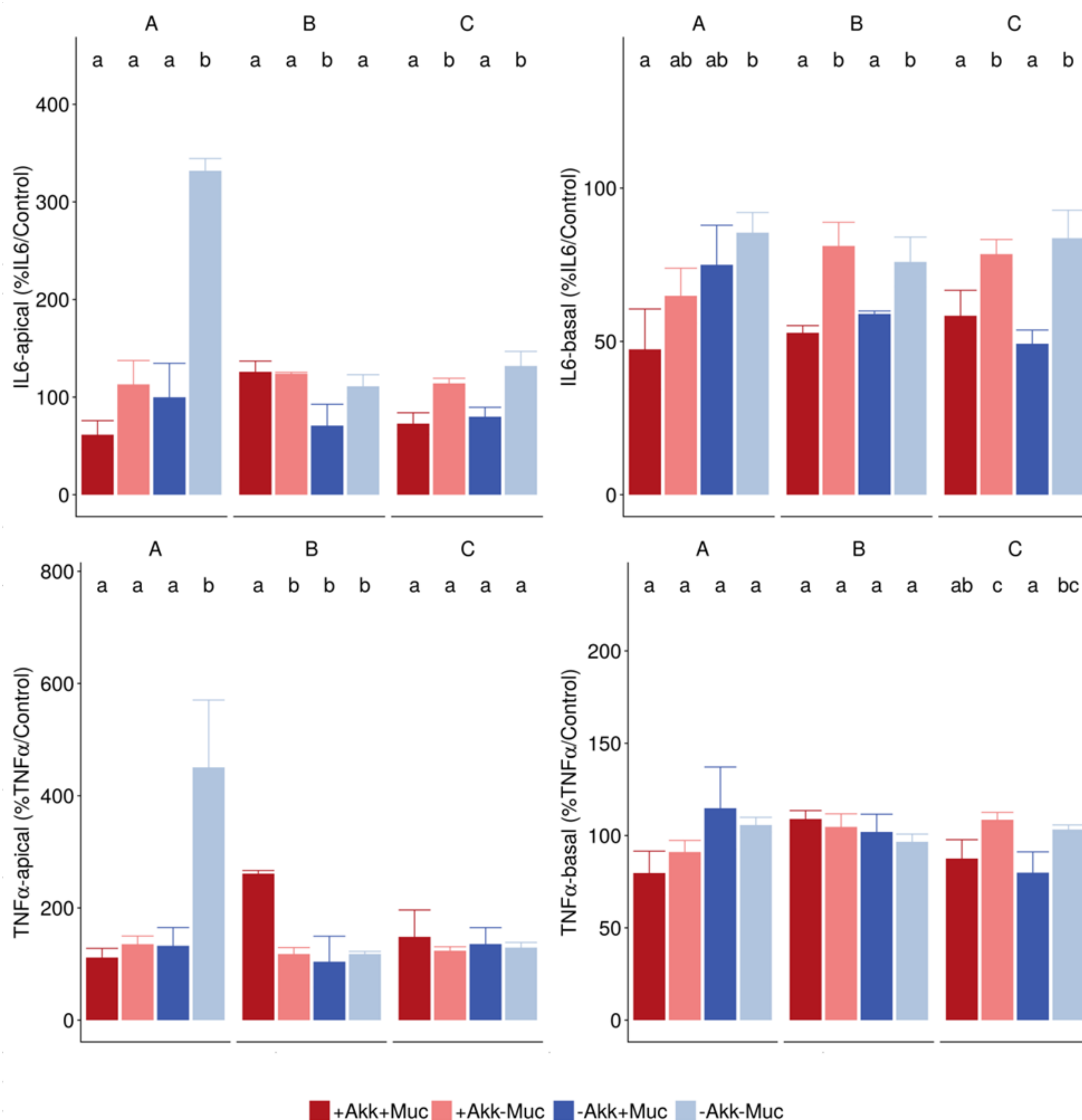

Figure 6. 4: Inflammatory response in the co-culture model with Caco-2 and THP-1 cells exposed (24h) to SHIME supernatant samples (3 donors A-C), amended with *A. muciniphila* (+Akk-Muc), mucin (-Akk+Muc), both (+Akk+Muc) or not treated (-Akk-Muc). Statistically significant differences between treatments (α=0.05) are denoted by the letters a, b and c. Identical letters indicate no statistical differences (p>0.05). Cytokines IL-6 and TNFα in both apical and basal part: relative to concentration produced in control (cells exposed to cell culture media) condition (%).

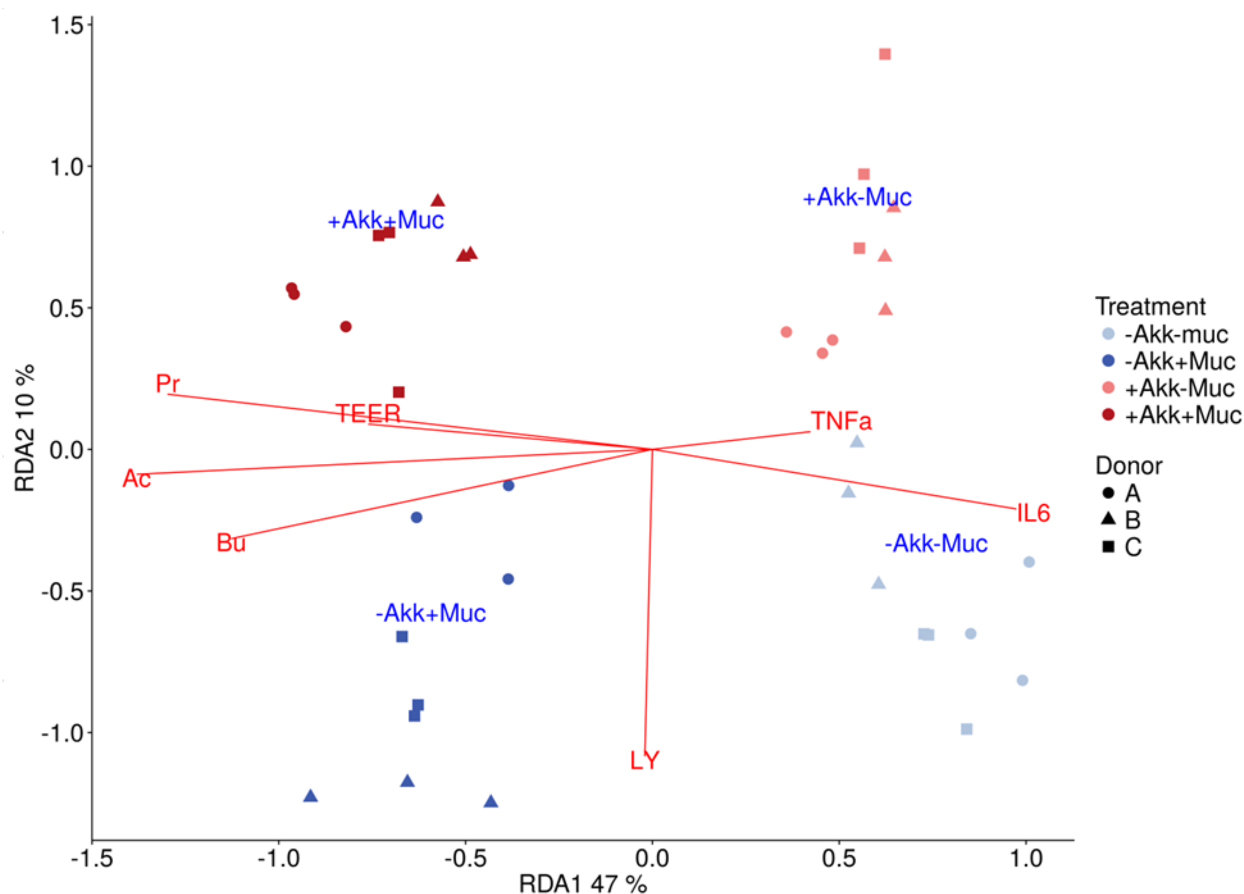

Figure 6. 5: Redundancy analysis (RDA) with donor and treatment explored as explanatory variables and the epithelial barrier function parameters (TEER and Papp of LY), inflammatory response (IL-6 and TNFα) and SCFA concentration (to which the co-culture model was exposed) as response variables.

Besides a donor-dependent effect, redundancy analysis revealed a clear treatment effect on the epithelial barrier function and the inflammatory response, as observed from the treatment based grouping of samples (Figure 6.5). Treatment had a higher impact than inter-individual variability on the cellular response. The different grouping is mainly based on the presence or absence of a mucin degradation niche. Mucin increased microbial SCFA production and induced higher TEER values. In the absence of mucin, TNFα and IL-6 production was stimulated to a higher extent. *A. muciniphila* treatment had less impact on the cellular response, but was associated with lower paracellular transport of LY. Sparse partial least squares regression analysis, visualized as a correlation heatmap (Figure 6.6), correlates the cellular response with the relative abundance of the most predictive genera in the SHIME samples, from which the supernatants were derived. Higher pro-inflammatory cytokine production (TNFa and IL6) was correlated with an increased abundance of some genera known to include opportunistic pathogens, such as *Escherichia/Shigella* and *Klebsiella* but also with *Bifidobacterium* and *Anaerostipes*. Genera correlated with better epithelial barrier functioning include *Lactonifactor* (~Papp), *Bacteroides and Anaerofilum*

(~TEER) and members of the *Ruminococcaceae* (~TEER), *Alphaproteobacteria* and *Clostridiales* (~Papp). Some genera were correlated with both TEER and Papp like *Akkermansia, Murimonas* and *Eubacterium* (Figure 6.6).

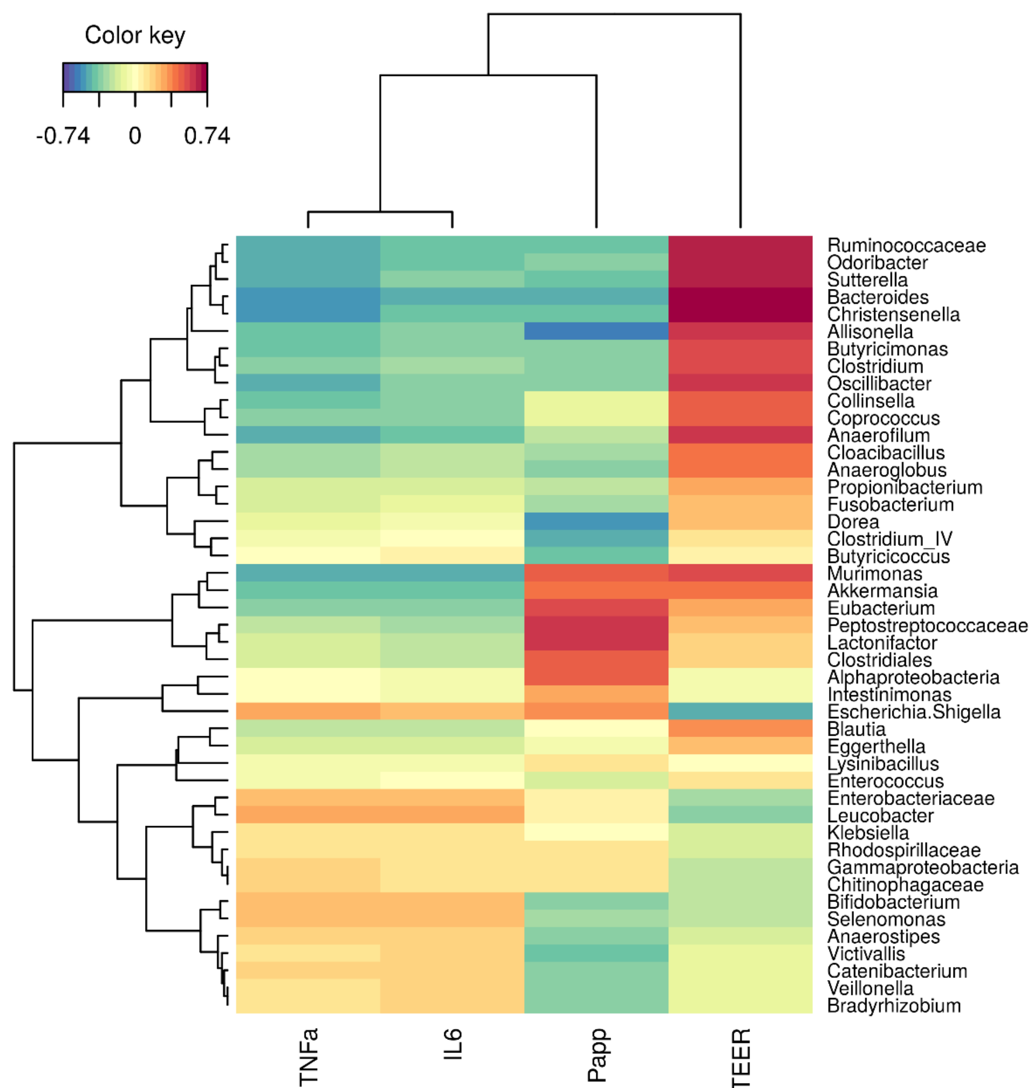

Figure 6. 6: Heatmap visualizing the correlation between the response variables (TNFα, IL6, Papp^{-1} and TEER) and the 50 most predictive genera as determined by sparse partial least squares regression analysis (the inverse of the Papp values was used to calculate the correlations with Papp of LY. A higher correlation indicates a similar trend in genus abundance and epithelial barrier function.

4. Discussion

Supernatant from SHIME microbial communities treated with *A. muciniphila* and /or mucin induced a response of the co-culture model of epithelial cells and macrophages that was dependent on treatment and maintained some of the inter-donor variability. Both mucin and *A. muciniphila* treatment strengthened the epithelial barrier but displayed differential effects on TEER and Papp of LY, two common measures for epithelial barrier functioning. Supernatant from microbiota treated with mucin increased TEER, except from donor B, but did not impact paracellular permeability, which was decreased by supernatant from *A. muciniphila* treatment, independent from mucin presence. The samples from mucin treatment were characterized by a higher concentration of acetate, propionate, and butyrate compared to the samples of treatment without mucin (Supplementary Figure 6.1). The effect of short chain fatty acids, especially butyrate, on epithelial barrier function is well studied in both *in vitro* and *in vivo* studies (Mariadason *et al.*, 1997; Ploger *et al.*, 2012), showing that butyrate, propionate and acetate increased barrier integrity, in a dose dependent manner. At concentrations similar to those of our mucin treatment samples (1.5-2 mM), butyrate increased TEER, decreased paracellular permeability of LY, promoted expression of tight junction proteins and increased the relocation of ZO-1 and occludin which resulted in an increased barrier integrity of Caco-2 monolayer (Mariadason *et al.*, 1997; Peng *et al.*, 2007; Peng *et al.*, 2009). This explains the observed increase in TEER after exposure of Caco-2 cells to the supernatants of microbiota treated with mucin from donors A and C, but fails to explain why these changes were not observed with samples from donor B, having a similar SCFA profile. This deviating response is likely due to other bacterial metabolites, such as N-acyl amides, which also affect the host cells (Ray, 2017).

The supernatant from mucin treatment induced no response in paracellular permeability of LY but the permeability was decreased by supernatant of microbiota treated with A. muciniphila. The tight junctions are composed by, at least, two functional pathways: 1) high-capacity and charge selective pore pathways allowing passage of non-charged, small ions; 2) low-capacity leak pathway allowing the flux of larger ions and molecules, independently of their charge (Keita and Soderholm, 2012; Shen *et al.*, 2011; Ulluwishewa *et al.*, 2011). It is possible that mucin, A. muciniphila or combination of both alters the paracellular flux of the co-culture model in a different way. The field of host-microbe interaction is still developing and there is not yet clear answer for this finding. Most of the permeability and drug transport assays have been performed using well defined media composition, but in this study the supernatant of complex microbial communities were used. The incorporation of complex and more realistic matrices when studying host-microbe

interactions can improve our understanding of pathophysiological processes occurring in the gut and can change our understanding of routine tests of intestinal permeability.

A. muciniphila has been described to improve epithelial barrier in many studies, both *in vitro* as well as *in vivo*. Mice studies showed that increased abundance of *A. muciniphila,* by prebiotic (polyphenols or oligofructose) or probiotic treatment, improved gut barrier function by increasing the expression of tight junction proteins ZO-1 and occludin, by increasing mucus thickness, and impacting GLP-2 secretion from L-cells (Everard *et al.*, 2013; Everard *et al.*, 2011; Li *et al.*, 2016; Roopchand *et al.*, 2015). *In vitro* cell culture studies described that exposing differentiated Caco-2 monolayers to *A. muciniphila* or its supernatant improved the enterocyte monolayer integrity, as measured by TEER (Ottman *et al.*, 2017d; Reunanen *et al.*, 2015). These protective effects of *A. muciniphila* have been attributed to a highly abundant outer membrane protein, Amuc_1100, which may have been liberated and could be present in our supernatant (Ottman *et al.*, 2017d; Plovier *et al.*, 2017). The changes in paracellular permeability in this research were observed for supernatant of microbiota to which *A. muciniphila* was added (+Akk+Muc, +Akk-Muc). However endogenous *A. muciniphila* increased to similar levels upon treatment with mucin (-Akk+Muc), questioning the role of *A. muciniphila* (Supplementary Figure 6.2 and 6.3). This might be an indication of a different host response to endogenous and externally added *A. muciniphila.* However, this was not indicated in mice studies where endogenous *A. muciniphila* was first increased through prebiotic treatment and in a second study externally added (Everard *et al.*, 2013; Everard *et al.*, 2011).

The selection of the cell lines used in this research was based on previous literature. Caco-2 cells spontaneously differentiate into an enterocyte-like phenotype and display a good functional correlation to human intestinal tissue (Rubas *et al.*, 1996; Rubas *et al.*, 1993). It is the standard model for studying epithelial barrier function since these cells develop functional tight junction complexes in a higher degree compared to other monolayer of cell lines (e.g. HT29-MTX) (Geirnaert *et al.*, 2017; Lea, 2015). The differentiation of Caco-2 cells induce a small intestine-like phenotype, but the colon origin is still retained in the cells, as demonstrated by high TEER values (up to 1500 Ω cm^{-2}) (Artursson *et al.*, 2012; Artursson *et al.*, 1993). Recent research has showed that the T84 cell line could be a better model to resemble the colonocyte phenotype (Devriese *et al.*, 2017), however it Is less characterized than the Caco-2 cells.

The co-culture of Caco-2 with PMA-differentiated THP-1 cells (macrophage-like) offers the possibility to study the exposure effect on health status and pro-or anti-inflammatory responses of the epithelium (Kampfer *et al.*, 2017; Kanzato *et al.*, 2001). Supernatant from microbiota treated with mucin was more successful at reducing the inflammatory response (TNF-α and IL-6) caused by LPS applied to the apical part. This might be due to their higher

SCFA concentrations, as mentioned above, since butyrate has been shown to reduce production of pro-inflammatory mediators, such as TNF-α and IL-6, by macrophages (Fukae *et al.*, 2005; Saemann *et al.*, 2000; Vinolo *et al.*, 2011). It may also be an indirect effect as these samples increased the TEER and thus strengthened the epithelial barriers such that the THP-1 cells were less exposed to the LPS or other pro-inflammatory antigens in the supernatant. Supernatant from untreated microbiota (-Akk-Muc) from donor A induced a specific decrease in TEER and an increase in paracellular permeability, indicating an impaired epithelial barrier which may have led to the three- and fourfold increase in the pro-inflammatory cytokines IL-6, respectively, TNF-α compared to the control). This increase was undone by *A. muciniphila* supplementation, which is surprising given the finding that *A. muciniphila* supernatant exposure resulted in higher IL-10 and TNF-α production compared to supernatant from *F. prausnitzii* and *L. plantarum* in a study measuring cytokine production in human derived peripheral blood mononuclear cells (PBMCs) (Ottman *et al.*, 2017d). The latter study, however, was limited to pure cultures, while supernatants in our experiment originated from a complex microbial SHIME community, which is more representative of the complex colonic environment

Despite the limitation of immortalized cell lines for *in vitro* modelling, the use of well characterized and stable cell lines also offers advantages as repeatability, reproducibility, and low cost. Our results suggest that further tests using more representative models of the human colonic epithelium, such as cell lines originated from normal tissues or organoids are desirable.

To conclude, the goal of this experiment was to evaluate whether modulation of complex microbial communities by the biotherapeutic treatment with *A. muciniphila*, prebiotic-like mucin or the combination of *A. muciniphila* with mucin, would differentially impact gut barrier function and immune response. Thereby, our set-up differs from most *in vitro* studies applying probiotic candidates (culture supernatant of) the pure culture probiotic candidate to epithelial cells (Ohland and Macnaughton, 2010). By using the complex microbial supernatant to treat the co-culture model more realistic treatments effects including microbe-microbe interactions are considered. This experiment showed that treatments applied to a SHIME system, inoculated with microbiota from different donors, resulted in microbial communities whose supernatant could differentially impact the intestinal epithelial barrier and the underlying immune cells, hence preserving inter-individual differences. The supernatant of the treatment with both mucin and *A. muciniphila* induced the most beneficial response, with the mucin responsible for increased trans-epithelial resistance (TEER) and reduced TNF-α and IL-6 production, and *A. muciniphila* responsible for decreased epithelial permeability.

5. Acknowledgements

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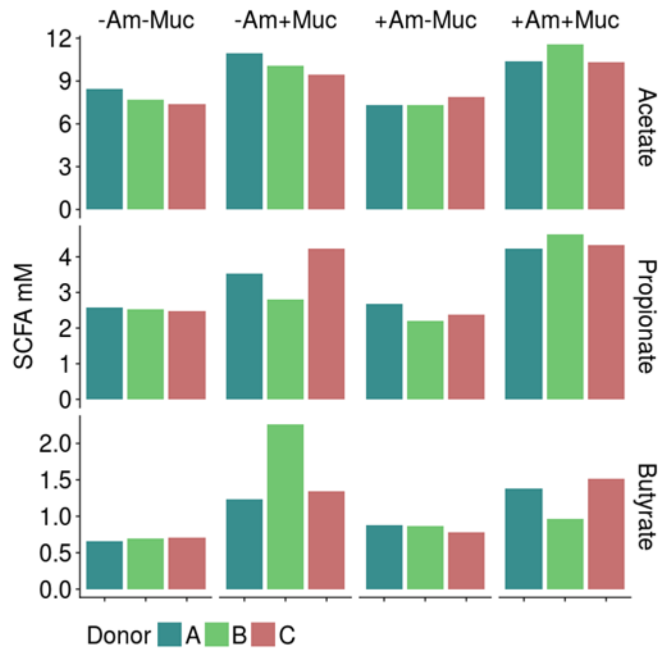
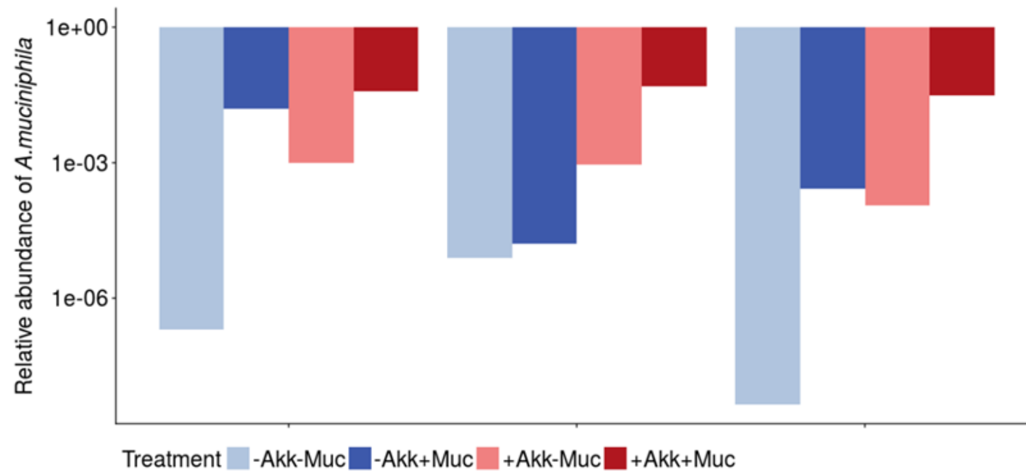
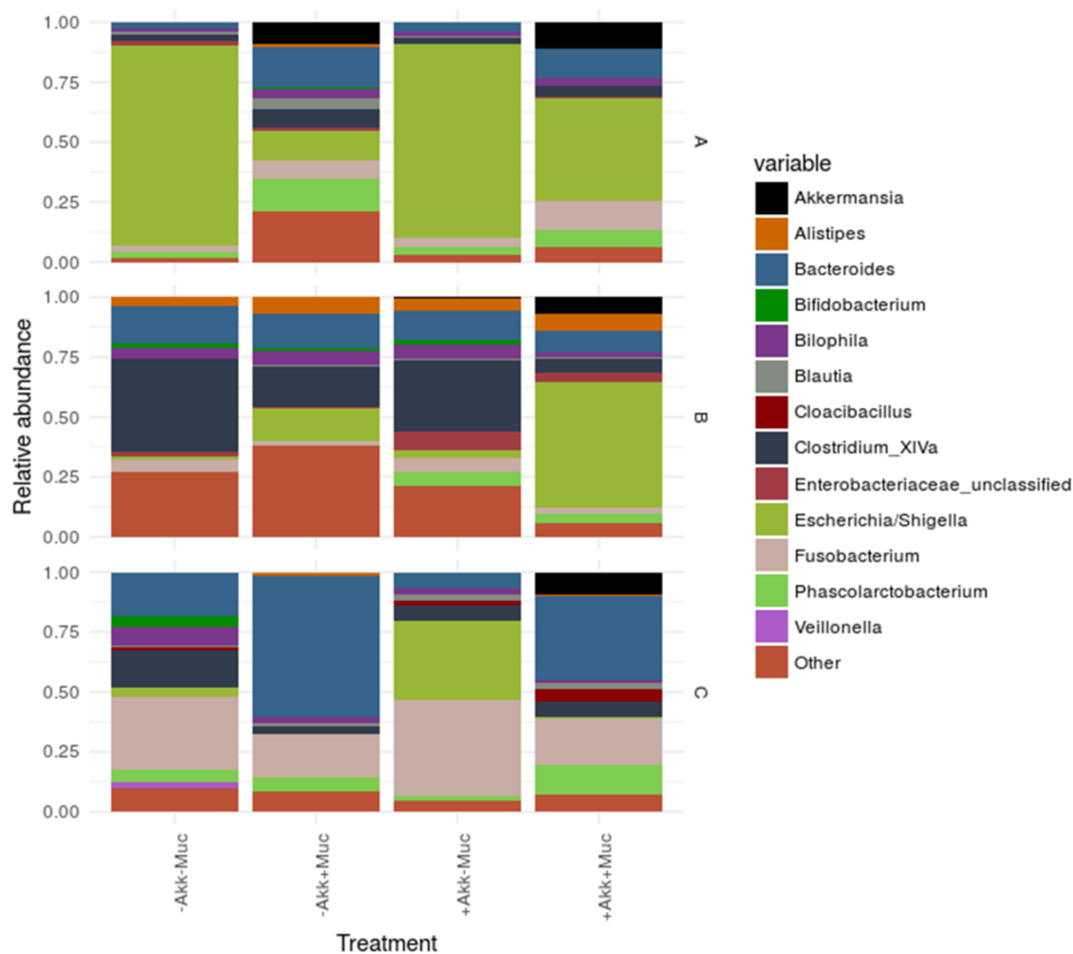
6. Supplementary information



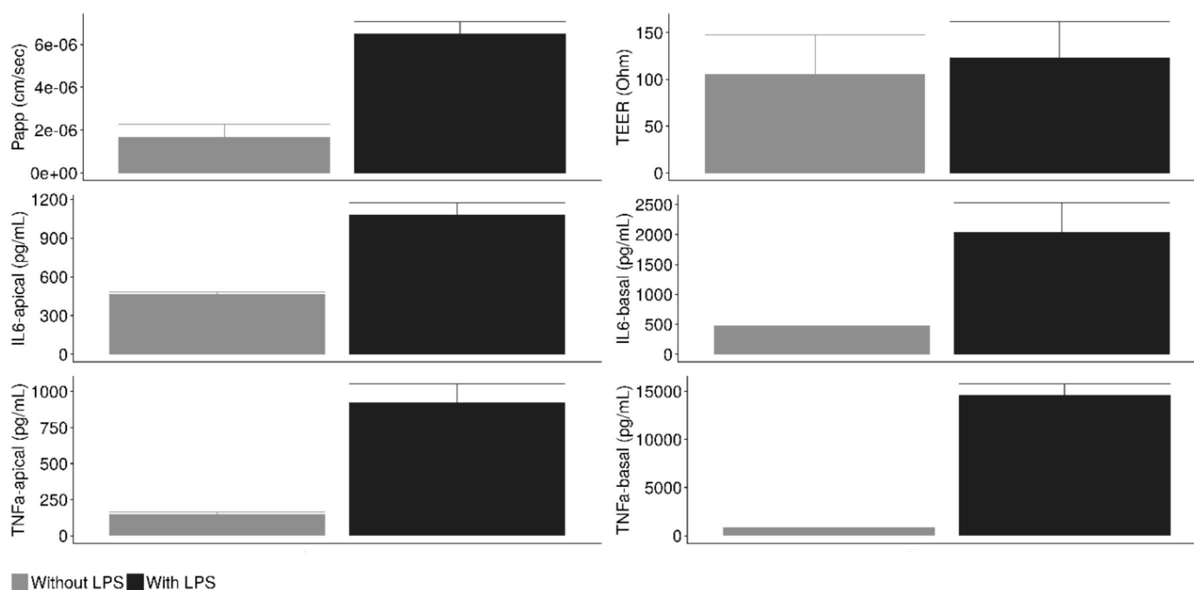
Supplementary Figure 6. 1: Short chain fatty acids concentration (mM) applied to the co-culture cell-model in the amended supernatants.



Supplementary Figure 6. 2: Relative abundance of *A. muciniphila* in the microbial communities from the different donors after treatment.



Supplementary Figure 6. 3: Relative abundance of the 13 most abundant genera in the microbial communities from the different donors after treatment.



Supplementary Figure 6. 4: Effect of cell culture medium without (grey) and with (black) LPS on the co-culture cell model.

CHAPTER 7

General discussion

CHAPTER 7

General discussion

1. Positioning of the research and main results

The notion that our gut microbiota correlates with and even impacts our health has inspired many researchers to investigate, unravel, and understand the complex microbial community and its interaction with the human host. Research into identification of biomarkers for gut health and ways to modulate the microbiota composition and activity to improve health, has put *A. muciniphila* in the spotlight. Its abundance is decreased in patients suffering from IBD, obesity, diabetes and autism, but it occurs in high abundance and with high prevalence in healthy people. Studies with high-fat diet fed mice showed that treatment with *A. muciniphila* reversed metabolic endotoxemia and had preventive effects on obesity and diabetes development. As a mucin degrader, *A. muciniphila* colonizes an interesting but not-fully described niche, being host-glycan degradation. Given the diversity and complexity of host glycan structures, strategies for degradation to free sugars rely on the action of a panel of enzymes, produced by only 1% of the microbial community. The release of oligosaccharides and fermentation products during mucin degradation can be used by other bacteria, thereby expanding the host glycan degradation niche. The ability of these microorganisms to profit both directly and indirectly from endogenous glycans can facilitate their close location to the host epithelium. The presence and the activity of mucin degrading consortia in the mucus layer, close to the host cells, is a key element in the host-microbiome crosstalk affecting gut health in a positive or detrimental way. Plenty of research concerning *A. muciniphila* has been done, but little is known about its behavior in the complex microbial ecosystem in the colon, about the potential role of mucins to influence *A. muciniphila* behavior and the impact of its probiotic administration on the microbial ecosystem and the host (Figure 7.1).

To gain more insight into the **role of *A. muciniphila* in host glycan degradation and the importance of this niche for the microbial ecosystem** and for gut health, several *in vitro* models were used (Figure 7.1): the SHIME system in chapters 2,3 and 5 to study *A. muciniphila* and host glycans in different complex microbial communities, a fed-batch system with a synthetic microbial community in chapter 4 to examine bacterial interactions in different nutritional environments and a co-culture cell model with epithelial cells and macrophages in chapter 6 to study the effect of *A. muciniphila* and host glycan treated communities on epithelial barrier function and pro-or anti-inflammatory responses.

The SHIME experiment in **Chapter 2**, inoculated with a fecal sample from a donor with high *A. muciniphila* abundance, revealed that *A. muciniphila* preferentially colonized the

distal colon and that this preference was due to pH, which is more neutral compared to the proximal colon. Mucin deprivation decreased *A. muciniphila* abundances and subsequent mucin supplementation caused a specific increase of *A. muciniphila*, far exceeding the response of other bacteria present, together with an increase in propionate. Since this research served as a start to characterize *A. muciniphila,* a donor was selected with high amounts of *A. muciniphila* previously shown to successfully colonize the SHIME (Van den Abbeele *et al.*, 2013; Van den Abbeele *et al.*, 2011a; Van den Abbeele *et al.*, 2010). To study the biological reproducibility of these findings, the effect of pH and the presence or absence of a host-glycan degradation niche was investigated in colon compartments separately inoculated with the microbiota from eight donors in **Chapter 3**. pH specificity and nutrient sensitivity of *A. muciniphila* was confirmed in these different microbial communities with variable *A. muciniphila* concentrations. We found that mucin supplementation resulted in more similar microbial communities for the eight donors, indicating that host glycans constitute an important ecological niche shaping the microbiota composition. With higher levels of *Akkermansia*, *Bacteroides*, and *Ruminococcus*, the cluster of mucin-enriched bacterial communities was significantly different from the mucin-deprived communities, yet no differences at the functional level were observed. The effect of colonic pH had a less profound impact on the microbiome with donor origin explaining most of the variability.

Chapter 4 studied the competitiveness of *A. muciniphila* in different nutritional and environmental conditions and how this affected cross-feeding to butyrate in a synthetic microbial community. *A. muciniphila* was equally abundant as *B. thetaiotaomicron* at high pH when mucin was present, even when *B. thetaiotaomicron* had additional fibers to grow on. However, when *B. thetaiotaomicron* was inoculated at lower abundances the host glycan degradation niche was dominated by *A. muciniphila.* Butyrate concentration was affected by pH and by medium and highest in fiber-rich medium at high pH. Butyrate producing species responded differentially to pH or cross-feeding partner, with *F. prausnitzii* preferring high pH and *A. caccae* responding to *L. plantarum.* Cross-feeding on mucin between butyrate producing species and *A. muciniphila* was shown in this synthetic community experiment. However in complex microbial communities, in **Chapter 5,** *A. muciniphila* supplementation did not seem to enhance cross-feeding on mucin to butyrate, in contrast to other mucin degrading bacteria. Addition of both mucin and *A. muciniphila* may lead to *A. muciniphila* dominating the mucin degradation niche, while sole mucin addition led to involvement of several species, including A*. muciniphila, Ruminococcus, Clostridium* cluster *XIVa,* and *Lachnospiraceae.* This study revealed that mucin was more effective in inducing compositional changes and equally effective on the functional level compared to the combination of *A. muciniphila* and mucin. Supernatant samples were taken from these microbial communities shaped by the treatment with *A. muciniphila*, mucin or both, to study

their effect on the intestinal epithelium and the underlying immune cells in **Chapter 6**. The response of the co-culture model of epithelial-like cells (Caco-2) and macrophage-like cells (THP-1) was dependent on treatment and maintained some of the inter-donor variability. The supernatant of the treatment with both mucin and *A. muciniphila* induced the most beneficial response, with the mucin responsible for increased trans-epithelial resistance (TEER) and reduced TNF-α and IL-6 production, and *A. muciniphila* responsible for decreased epithelial permeability and increased expression of tight junction protein occludin.

Overall, this research, using the complex microbial communities from several donors, showed the nutrient specificity of *A. muciniphila* and its sensitivity to changes in the colon environment, and provided valuable information about the prebiotic action of host derived glycans. However, inter-individual differences remain an important factor of variability in research results, requiring further elucidation.

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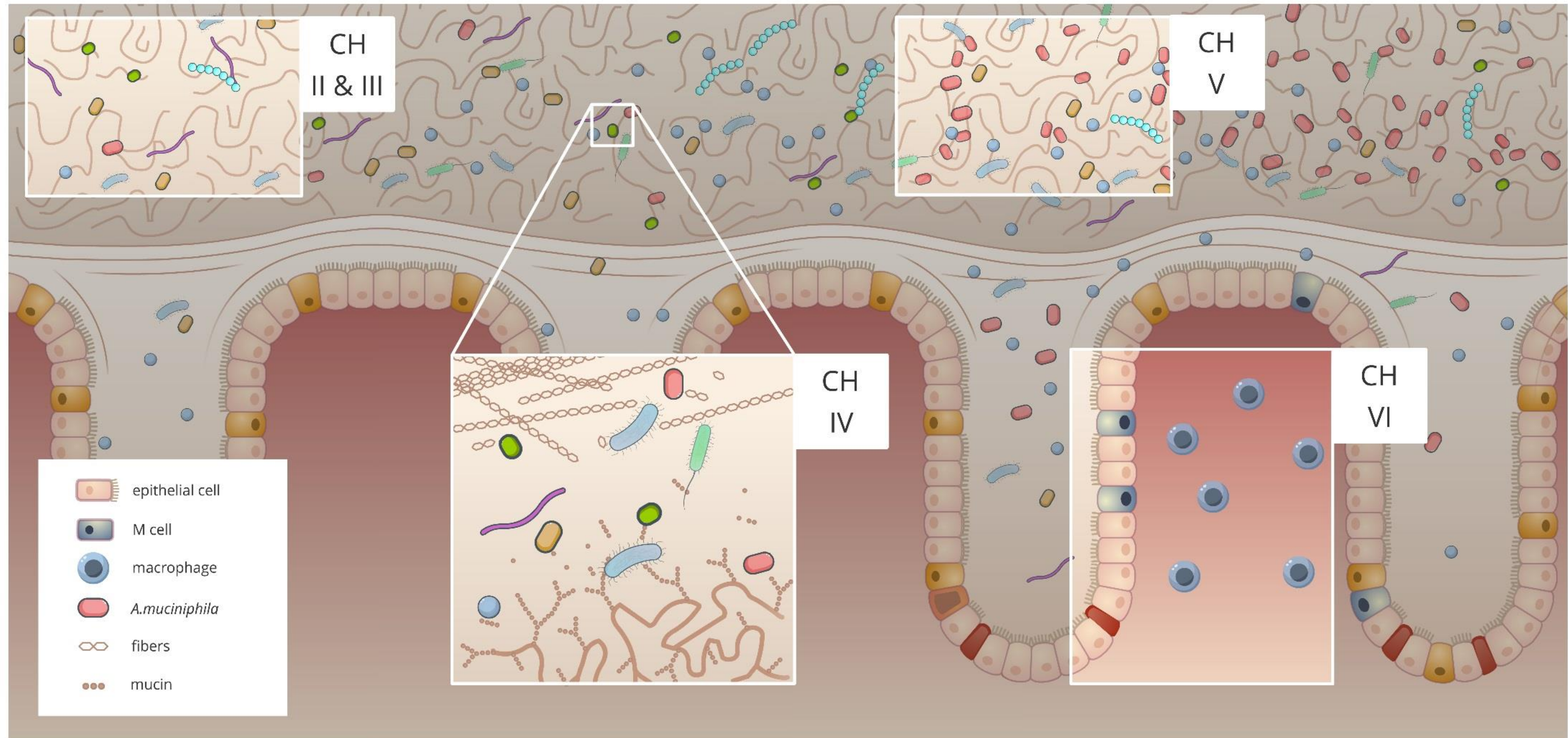

Figure 7. 1: Overview of the experiments in this PhD research. CH II &III: Study of the response of microbial communities, and in specific *A. muciniphila*, to environmental changes in the colon; CH IV: synthetic community experiment, with a selection of microorganisms, to study the cross-feeding and competition interaction on mucins and fibers; CH V: Modulation of microbial communities by addition of exogenous *A. muciniphila* and/or mucin; CH VI: impact of the supernatant of the modulated microbial communities (CH V) on epithelial barrier function and immune response. Main research outcomes are described in the text.

2. Possible future applications

2.1 *Akkermansia muciniphila* as a biomarker for gut health

Considering the impact of the microbial gut communities on host health and association between dysbiosis and many diseases (Chapter 1), the gut microbiota provides a huge potential of new biomarkers. Biomarkers are measurable biological indicators that can be used to assess health status, for (early) detection or diagnosis of diseases or to evaluate the efficacy of treatments. A panel of biomarkers, including SCFAs produced by and cytokines induced by the microbial community, has proven useful to discriminate clinically diagnosed IBS patients from healthy controls (Mujagic *et al.*, 2016). Not just compounds produced or induced by the microbial community can serve as biomarkers, in some instances the microbial community composition itself could be used to detect gut related diseases before conventional diagnostics can (Marchesi *et al.*, 2016; Tedjo *et al.*, 2016). The microbial community in the gut of Parkinson's patients differs from that of healthy people even at a very early stage of the disease, and so using this biomarker can lead to early treatment of the disease (Heintz-Buschart *et al.*, 2018). By better understanding the role of the microbiota in the development of a disease, it might be possible in the future to use the absence or presence of certain specific key species as a biomarker.

The abundance of *A. muciniphila* in the gut has been reported by many studies to be inversely correlated to health, with lower numbers of *A. muciniphila* in patients suffering from IBD, obesity, diabetes and autism (Chapter 1). In IBD the decrease in *A. muciniphila* corresponds with an increase of *Ruminococcus* species (*R. gnavus* and *R. torques),* and this shift in mucolytic species has been proposed to be a suitable biomarker for mucosal integrity in IBD (Berry and Reinisch, 2013). Abundance of *A. muciniphila* was also indicated as a subclinical biomarker for increased risk of obesity and type 2 diabetes (Yassour *et al.*, 2016). *A. muciniphila* was shown to be associated with a healthier metabolic status and might thus be useful as a biomarker for metabolic syndrome (Dao *et al.*, 2016; Everard *et al.*, 2013; Everard *et al.*, 2011). Our results show *A. muciniphila* to be sensitive to certain changes in the colon environment, which is a requisite for a potential bacterial biomarker. It is highly dependent on mucin as a nutrient source, and so changes in the mucus layer in IBD or other patients, would impact *A. muciniphila* abundance (Berry *et al.*, 2013). *A. muciniphila* also showed sensitivity toward decreasing pH, which is dependent on microbial activity and host regulation. Given the sensitivity of *A. muciniphila* to changes in important environmental factors, regulated by both microbial activity and the host itself, and given its correlation with several diseased states, *A. muciniphila* might indeed be a very interesting biomarker

candidate. Also *Clostridium* clusters IV and XIVa, containing butyrate producing species, have gained a lot of attention in the last years due to their contribution to gut health (Velasquez-Manoff, 2015). The abundance of *B. pullicaecorum*, *F. prausnitzii*, and *E. rectale* is markedly decreased in IBD patients compared to healthy individuals (Riviere *et al.*, 2016) and an association has been suggested between *F. prausnitzii* and disease activity in CD patients (Tedjo *et al.*, 2016).

Though still at the research stage, constructing specific gut biomarker panels comprising several key species, like *A. muciniphila* and *F. prausnitzii,* host and bacterial metabolites might be a future approach to assess health status, diagnose certain diseases or evaluate the efficacy of a therapy. To eventually reach the point where we could use these intestinal biomarkers, several hurdles need to be overcome, like the validation of candidate biomarkers an the standardization of their measurement. A critical hurdle is the extensive inter-individual differences. In the studies mentioned above, groups of patients were compared to groups of healthy individuals, which partly cancelled out the inter-individual differences. However, when interpreting observations of one person, the question remains how to define baseline or healthy levels for our indicators, since it is still unclear how a healthy gut microbiome should be defined

2.2 *Akkermansia muciniphila* as a (live) biotherapeutic product

Currently a clinical trial is ongoing to evaluate the effects associated with the administration of live or heat-killed *A. muciniphila* on the metabolic disorders related to overweight and obesity in humans. Administration of *Akkermansia muciniphila* was already found to reduce body weight gain, fat mass gain, glycaemia and inflammatory markers in diet-induced obese mice and its effect could partly be explained by the action of an abundant outer membrane protein Amuc_1100 (Everard *et al.*, 2013; Plovier *et al.*, 2017). The unexpected finding that pasteurized *A. muciniphila* exerted stronger effects than the live bacterium and the identification of Amuc_1100, demonstrated that *A. muciniphila* doesn't have to be alive and metabolically active for its effects. Chapter 6 showed indeed a decrease in intestinal permeability induced by supernatant of microbial communities treated with *A. muciniphila*, independent of its metabolic activity. This is highly significant from a possible application point of view since it circumvents the problems associated with the large scale production and application of a strict anaerobic bacterium. Beside pasteurized *A. muciniphila*, also treatment with isolated bacterial proteins, like Amuc_1100, is a future possibility. The data from the study by Plovier *et al.* (2017) indicate that other pasteurization-resistant outer membrane proteins might be involved in the beneficial interaction between *A. muciniphila*

and the host. Further research into identifying and isolating these proteins is needed and might lead to the application of a mix of bacterial proteins in the treatment of obesity and related metabolic disorders.

The discovery of the potential role and use of *A. muciniphila* in obesity occurred after a dietary intervention study with obese mice that increased the levels of *A. muciniphila* and improved host health (Everard *et al.*, 2011). Various other animal studies reported that treatment with a specific nutritional component, like polyphenols, conjugated linoleic acid and betacyanins, improved host health (e.g. by inducing weight loss) and increased *A. muciniphila* abundances (Anhe *et al.*, 2015; Chaplin *et al.*, 2015; Gomez-Gallego *et al.*, 2014; Roopchand *et al.*, 2015; Song *et al.*, 2016; Tachon *et al.*, 2013). However this positive correlation between *A. muciniphila* and healthier phenotypes is mainly described after high fat diets and in obese animals. Despite the studies observing a negative correlation between *A. muciniphila* abundance and several diseases, the opposite has only been reported for obesity and related metabolic disorders. An intervention study showed that obese and overweight individuals with high abundance of *A. muciniphila* displayed greater improvement in insulin sensitivity markers and other clinical parameters after calorie restriction (Dao *et al.*, 2016).

Future research should explore the possible application of *A. muciniphila* in other diseases, like IBD. The defects in the mucus layer, characteristic in IBD, allow bacteria to reach the epithelium and induce an immune response, which could result in the development of intestinal inflammation. Microbial dysbiosis is important in IBD pathogenesis and given the reduced protection of the mucus layer and the enhanced interaction with the epithelium, modulation of the microbiota to restore gut homeostasis can be of interest (Frank *et al.*, 2011; Larsson *et al.*, 2011; Swidsinski *et al.*, 2002). Due to lacking efficacy of current probiotic formulations, research focus is shifting towards novel species that can be used as next generation probiotics or live biotherapeutic product (Geirnaert, 2015). As mentioned above, *A. muciniphila* abundance is inversely correlated with IBD while other mucolytic bacteria, *Ruminococcus* spp., are more abundant (Png *et al.*, 2010). Interestingly, treatment with *A. muciniphila*, live or pasteurized, was found to stimulate mucin production and *A. muciniphila* increased goblet cell abundance (Plovier *et al.*, 2017; Shin *et al.*, 2014). An *in vitro* study with colonic cell lines (HT-29 and Caco2) showed that *A. muciniphila* adhered to the epithelium, strengthened the intestinal barrier and demonstrated low inflammatory potential (Reunanen *et al.*, 2015). In human derived peripheral blood mononuclear cells, *A. muciniphila* was found to induce both anti-and pro-inflammatory cytokine response, indicating a more complex role in immunomodulation. Live *A. muciniphila* induced production of anti-inflammatory IL-10 to the same extent as *F. prausnitzii* and *L. plantarum*, while *A. muciniphila* supernatant induced higher IL-10 production (Ottman *et al.*, 2017d).

F. prausnitzii has been correlated with remission in IBD and its ability to induce high levels of IL-10 is one of the mechanisms behind its anti-inflammatory effect (Sokol *et al*., 2008a; Sokol *et al*., 2008b). In conclusion, both *in vitro* and *in vivo* studies demonstrate that *A. muciniphila* can improve intestinal barrier functioning, both epithelial barrier and the mucus layer, and can communicate with the host, with potential anti-inflammatory responses. This makes *A. muciniphila* an interesting target for further research into biotherapeutic products for IBD.

However, the effects of biotherapeutic application of *A. muciniphila* have not yet been studied in immunocompromised conditions and additional research is certainly needed. Indications of a beneficial effect of *A. muciniphila* in IBD can be derived from a study by Kang *et al.* (2013) where extracellular vesicles from *A. muciniphila* were found to protect against DSS-induced colitis.

Careful attention has to be paid to the risks involved in administering high doses of live bacteria to immunocompromised patients, since this could lead to infection, bacterial translocation and sepsis. The work by Duparc *et al.* (2017) leads us to wonder whether the pasteurization of other bacteria could enhance their beneficial effects on inflammation and epithelial barrier integrity as it did for *A. muciniphila*. The use of pasteurized bacteria could already significantly reduce the risk of using biotherapeutic products in susceptible patients. Butyrate producing species, specifically *F. prausnitzii* and *B. pullicaecorum,* have been proposed as potential live biotherapeutic products for use in IBD and future research should investigate whether pasteurization influences their beneficial effects. Given the high interindividual variability in response to probiotic treatments, the use of a multi-species mix that possesses different beneficial properties would be more effective and result in a wider spectrum of action. Besides the effect of pasteurization, the combination of butyrate producing species with *A. muciniphila* as a multi-species mix is worth further studying.

3. A protective role for *Akkermansia muciniphila* in the host glycan degradation niche?

Given its specialized mucin degradation activity *A. muciniphila* has been proposed to be a keystone species supporting other bacteria through cross-feeding interactions and shaping the microbial community at the mucosal interface (Belzer and de Vos, 2012; Chia *et al*., 2018). However, very little is known about the importance of *A. muciniphila* in the mucin degradation niche and whether it is interchangeable with other mucolytic bacteria. Due to its specific response to mucin, as shown throughout this research, and its high prevalence in humans (Collado *et al*., 2007), the study of a mucin degradation niche without *A. muciniphila* is very difficult when working with fecal samples. In Chapter 5, one donor provided us with a

microbial community where mucin supplementation did not lead to the increase of endogenous *A. muciniphila* abundance as was observed for the others donors and thus allowed us to study mucin degradation without dominance of *A. muciniphila.* A higher abundance of *Ruminococcus* and *Roseburia* species was observed, which might be responsible for the higher concentration of butyrate.

A way to avoid the mentioned drawbacks of a fecal inoculum is by using a synthetic community. By trimming down ecosystem complexity it becomes more feasible to discern one-on-one microbial interactions which is often difficult in the complex background of human gut microbiota. The ability to select and compare different community compositions can give an indication about the role specific bacteria play in the community. In Chapter 4 we observed that changing the primary degrader, be it *A. muciniphila* or *B. thetaiotaomicron*, did not impact butyrate producing functionality. More research is needed to discern whether the identity of the mucolytic species affects the functionality and composition of the bacterial community of the mucin degradation niche. This further research should focus on: (i) Expanding the synthetic community by including mucolytic *Ruminococcus* species, more butyrate producing bacteria and other cross-feeders; (ii) assessing functionality broader than SCFA production and including other parameters such as stability and resilience of the community and colonization resistance; (iii) investigating the effect on the host by combining these *in vitro* studies with cell cultures or by introducing the synthetic communities into germfree mice, as was done by Desai *et al.* (2016). The use of *in vivo* models introduces an important factor, being the cross-talk between the bacteria and the host, into this research. *A. muciniphila* might differentiate itself from other mucolytic bacteria in this aspect, since it has shown to have the ability to stimulate mucus production, modulate host response and is commonly associated with a healthy gut. In a study by Png *et al.* (2010) an interesting hypothesis was posited of a negative feedback loop in which *A. muciniphila* determines the abundance of the mucosa-associated bacteria in health: mucolytic activity of *A. muciniphila* increases substrate availability, thereby sustaining the mucosa-associated community. When this community becomes too abundant it will inhibit growth of *A. muciniphila*, thus limiting the substrate availability and controlling the abundance of mucosa-associated bacteria. However, this assumes that no other mucolytic bacteria would be present in the community that could take over mucin degradation, even if *A. muciniphila* was inhibited as hypothesized. The mice study by Desai *et al.* (2016) showed that the presence of *A. muciniphila* in a synthetic community could not protect against excessive mucolytic action during dietary fiber deprivation, which shaped the synthetic community into a mucus-eroding microbiota and promoted epithelial access. Repeating this experiment with different synthetic communities could reveal whether *Akkermansia* or other bacteria or bacterial consortia could exert

protective effects, shielding the host from excessive mucolytic action and keeping the mucus layer healthy.

4. Prebiotic-like properties of host glycans

The mucus layer plays an important role in the intricate interactions between the bacteria that colonize our colon and the host. Besides being an essential part of the intestinal barrier, the mucus layer provides an important source of nutrients in the form of mucins, whose complex structure requires the interaction of specific bacteria for its degradation and can involve many others through cross-feeding interactions. This nutritional aspect of the mucus layer is especially important during periods of fasting or dietary fiber deprivation (Desai *et al.*, 2016; Kohl *et al.*, 2014; Sonoyama *et al.*, 2009). Due to the proximity to the epithelium, the metabolites of the bacteria involved in this mucin degradation niche might be more readily taken up by the epithelial cells, thereby enhancing their influence on the host. Considering the limited number of species that can degrade the complex mucin structure and the described and proposed health effects conferred by its degradation, mucins fit in the definition of prebiotic substances, ‘substrates that are selectively utilized by host microorganisms conferring a health benefit’, and the human body can be described as producing its own prebiotic-like substance (Belzer and de Vos, 2012; Derrien *et al.*, 2004; Gibson *et al.*, 2017; Ouwehand *et al.*, 2005; Reunanen *et al.*, 2015).

Mucin degrading capacities have been described for several species (Chapter 1), but few studies have focused on trophic interactions during growth on mucin or the importance of this host-glycan degradation niche for the microbial ecosystem. The *in vitro* models used throughout this research allowed the study of introducing host glycans and observing the prebiotic-like effect on microbial communities coming from different donors. Overall, mucin supplementation made different microbial communities more similar to each other, increased *A. muciniphila* and *Ruminococcus* abundance and increased SCFA production (Chapter 2, Chapter 3, Chapter 5). The presence of a host-glycan degradation niche induced strengthening of the epithelial barrier and modulated cytokine production (Chapter 6). Our results indicate that host-glycans deserve further attention for their prebiotic-like properties in modulating gut microbiota and possibly host health.

Certain fibers are capable of altering the secretion dynamic of colonic mucus and could increase mucus turnover (Brownlee *et al.*, 2007). Besides better protective properties of the mucus layer by enhanced exclusion of trapped bacteria, this would increase mucin released by desquamation. We posit here that this increase in mucin could be an endogenous prebiotic-like treatment, stimulating mucin degradation without harming the mucus layer. In a

rat study, prebiotic treatment with inulin was posited to increase levels of ceacal mucin, which were degraded along the colon and increased fecal *A. muciniphila* abundance (Van den Abbeele *et al.*, 2011a). Future research should identify more mucogenic compounds and assess whether the induced increase in mucin production and degradation is indeed not harmful to the mucus layer and consequently host health. Due to its animal origin, prebiotic treatment with mucin glycans is impossible and highly impractical. However, biotechnological production of (fucosylated) human milk oligosaccharides is already a possibility (Petschacher and Nidetzky, 2016; Soetaert, 2016) and given the structural similarity biotechnological production of mucin glycans might not be too far in the future? Of course, more research is necessary to assess the effect of prebiotic treatment with synthetic mucin glycans. The expected increase in mucolytic bacteria might be a risk for mucus layer structure and gut homeostasis.

5. Advantages and disadvantages of the *in vitro* models for the study of the host glycan degradation

During this research we used *in vitro* models to study the interactions between mucin glycans, *A. muciniphila*, and the microbial community and their ability to elicit a host response. *In vitro* models of the colon microbial community are designed to simulate the physiological conditions of the colon and are ideal for mechanistic studies because of the ability to control and vary several parameters and to take samples at regular timepoints and from different, *in vivo* difficult to reach, regions (Marzorati *et al.*, 2011; Marzorati *et al.*, 2014). The *in vitro* models used here include a semi-continuous system (fed-batch; Chapter 4) and a continuous model (SHIME; Chapter 2, 3 and 5). The SHIME model allows for preservation of inter-individual differences in microbial composition and functionality and makes it possible to differentiate between responders and non-responders to a certain treatment (Van den Abbeele *et al.*, 2010; Van den Abbeele *et al.*, 2012), as was observed in Chapters 3 and 5. The ability to control the supply of mucin glycan (varying the concentration but not the structure) necessitated the use of *in vitro* models. Besides, the nutritional role of mucins cannot be separated from the protective role of the mucus layer *in vivo*, and attachment to the mucus layer or the anti-microbial peptides in the mucus layer would have confounding effects. The *in vitro* model (SHIME) used in the research thus provided essential advantages to study the impact of mucin degradation on the community. However, there still remain many aspects of the mucin degradation niche that should be addressed in future research, such as variability in mucin structure and supply influenced by inter-individual variability, host health and the cross-talk with the microbial community.

Compared to dietary glycans, the composition and structure of mucin glycans are often described as less variable, making them a more consistent source of nutrients. However, mucin glycans have been described to be susceptible to certain **bacterial** factors. Probiotic *Lactobacillus* species are able to increase the secretion of mucin by stimulating the production of MUC2, *B. thetaiotaomicron* has been shown to increase the differentiation of goblet cells, thereby influencing mucin production (Caballero-Franco *et al.*, 2007; Mattar *et al.*, 2002; Wrzosek *et al.*, 2013) and *A. muciniphila*, live or pasteurized, was found to stimulate mucin production increase goblet cell abundance (Plovier *et al.*, 2017; Shin *et al.*, 2014). More indirectly, bacterial fermentation products such as butyrate and propionate can increase the mucin production by goblet cells in the colon (Barcelo *et al.*, 2000). Besides these bacterial factors, also host **genetics** influence mucin structure through the α1–2 fucosyltransferase (FUT2) gene. This FUT2 gene encodes for the addition of a L-fucose residue to the terminal galactose residue of mucin glycans and is not functional in approximately 20% of the people. Due to the terminal position of fucose, this sugar plays an important role at the interface of bacterial interactions with the mucus. The absence of fucose significantly impacts gut microbial composition and recent studies have found non-secretors to have increased susceptibility to chronic inflammatory conditions linked to the gut microbiota, such as Crohn’s disease (Rausch *et al.*, 2011; Wacklin *et al.*, 2014).

Mucus thickness and structure is highly involved in many gut related **diseases**, amongst others ulcerative colitis (UC) and Crohn disease (CD). Under normal conditions, bacterial mucin degradation is in balance with new production by the goblet cells. Changes in the structure of the mucin glycans, for example due to altered glycosylation, might shift the balance toward faster degradation (Larsson *et al.*, 2011). These changes in glycosylation, resulting in shorter glycan structures, have been observed in patients with active UC, but seemed to be caused by inflammation and not UC per se (Johansson, 2014; Johansson *et al.*, 2014). Reduced sulfation, which makes the mucin glycans more susceptible to degradation (as described in Chapter 1), has been observed for UC patients and leads to increased susceptibility to induced colitis in mice (Corfield, 2015; Dawson *et al.*, 2009). UC involves increased immune reactions towards the microbiota due to increased bacterial exposure, which might be the result to reduced thickness and increased permeability of the mucus layer (Johansson *et al.*, 2014). Without the intact (inner) mucus layer providing separation between bacteria and epithelium, bacteria can reach the epithelium and stimulate an immune system response that can lead to the development of intestinal inflammation. Unlike UC, a thicker mucus layer has been observed in CD patients. However, CD involves defects in the secretion of antimicrobial molecules, which allows bacteria to penetrate the crypts inducing intestinal inflammation (Geirnaert, 2015).

A study by Schroeder *et al.* (2018) identified **diet** to be an important factor indirectly influencing the intestinal mucus layer. Mice fed a western style diet had an altered gut microbiota composition, increased permeability and reduced growth rate of the inner mucus layer. After microbial transplantation with microbiota from chow fed mice, the barrier defects were reversed. Prebiotic treatment with inulin prevented the increase in mucus permeability and administration of *Bifidobacterium longum* could increase mucus growth rate. Another mice study, mentioned before (Desai *et al.*, 2016) with a synthetic human microbiota observed that during dietary fiber deprivation, the microbial community uses the host derived mucin glycans as nutrients. This led to erosion of the mucus barrier leaving the epithelium vulnerable for colitis by the mucosal pathogen, *Citrobacter rodentium*.

Overall, the effects of these environmental and host factors result in differences in the trophic interactions between the mucus layer and the gut microbiota, and impact the role of the mucin degradation niche in the gut ecosystem. At the moment, neither *in vivo* nor *in vitro* models provide suitable possibilities to take these factors into account, due to the intricate interplay between bacteria, mucins and host. To overcome these problems, more *in vivo* studies are needed to study the variances in mucin glycans and mucus layer structure more in detail. With more knowledge gathered from the *in vivo* studies, it might be possible to use this for *in vitro* simulation. As mentioned above, biotechnological production of mucin glycans might be possible in the future and with more detailed knowledge about the variability in mucin glycan structure, we would be able to produce different types of mucin glycans. However, at the moment this biotechnological production of mucins is still something of the future and the bottleneck for more detailed *in vivo* studies is the difficulty to access and study the mucus layer. This is exemplified by the recent study by (Kamphuis *et al.*, 2017) where the commonly accepted organization of the mucus layer is put into question. Given the involvement of the mucus layer in gut health and the undefined role of the mucin degradation in the ecosystem, which has been extensively described in this research study, it is of paramount importance that further research should focus on overcoming the abovementioned obstacles.

6. Conclusions

Studies linking changes in the gut microbiota composition to human health status have reported an inverse correlation between *Akkermansia muciniphila* and disorders such as IBD, obesity and diabetes. *A.muciniphila* has been positioned as a health biomarker and is currently explored as a therapeutic agent for obesity. The key characteristic of *A. muciniphila* is its mucin degrading capacity, by which it may contribute to cross-feeding networks

enhancing its effect on the microbiota and the host. The focus of this PhD research was to unravel the behavior of *A. muciniphila* in the complex microbial ecosystem of the colon and the potential role of mucins to influence *A. muciniphila* and its impact on the microbial community and the host. By using *in vitro* technologies, including the SHIME® model and a co-culture cell model, we have shown that:

- *A. muciniphila* is sensitive to slightly acidic pH and therefore preferentially colonizes the distal colon, where the pH is closer to neutral;
    - Biotherapeutic application of *A. muciniphila* may need to consider protection from pH until arrival at more neutral pH.
- *A. muciniphila* shows high nutrient specificity: mucin deprivation leads to decreased abundance of *A. muciniphila* and mucin supplementation induces a specific and strong increase in *A. muciniphila* abundance;
    - Stimulation of endogenous *A. muciniphila* might be achieved by stimulation of mucin production or by prebiotic-like supplementation of biotechnologically produced mucin. The latter could be used as well for synbiotic formulations with *A. muciniphila.*
- Cross-feeding on mucin to butyrate is not enhanced with increased *A. muciniphila* abundance as primary degraders do not impact butyrate producing functionality but might induce phylogenetic shifts;
    - Strategies with primary degraders to modulate the microbiota's functionality may depend on an individual's microbiome composition and response. This indicates that effects on the microbiome cannot be generalized across human individuals and it is a plea for a more personalized approach.
- Host glycans constitute an important ecological niche shaping the microbiota composition more effectively, exceeding donor variability, compared to the environmental modulator pH;
    - Administration of biotechnologically produced compounds with mucin-like molecular structures may in that sense be a possible prebiotic treatment.
- Mucin and *A. muciniphila* display differentially protective effects towards *in vitro* epithelial barrier and immune modulation.
    - Incorporating complex and more realistic matrices when studying host-microbe interactions is needed to improve our understanding of routine tests of intestinal permeability and our insight in (patho)physiological processes in the gut

This research gained more insight into the positioning of *A. muciniphila* in host glycan degradation and the importance of this niche for the microbial ecosystem and its possible role for gut health markers. The obtained findings substantiate its further use as biotherapeutic agent.

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Summary - Samenvatting

Summary

Scientific research in the last decades has revolutionized our insight in how microorganisms colonizing the human body correlate with and even impact our health and how intricate the microbe-microbe and the host-microbe interactions are. Subtle imbalances in our microbial populations can cause disease and studies have shown correlations between gut microbiota composition and obesity, inflammatory bowel diseases, diabetes, cancer, acute appendicitis, colon cancer, … Research into identification of biomarkers for gut health and ways to modulate the microbiota composition and activity to improve health, has put *A. muciniphila* in the spotlight. Its abundance is decreased in patients suffering from IBD, obesity, diabetes and autism, but it occurs in high abundance and with high prevalence in healthy people. As a mucin degrader, *A. muciniphila* colonizes an interesting but not-fully described niche, being host-glycan degradation. These mucin glycans make up the protective mucus layer that separates the epithelial cells from the gut lumen. Besides acting as a barrier, the mucus layer, and specifically the mucin glycans, also serve as a substrate for growth for colonic bacteria, an aspect that has gained more attention recently. Previously, it was thought that mucin degradation was detrimental for gut health but it is now clear that it is part of a normal turnover process. Given the diversity and complexity of host glycan structures, strategies for degradation to free sugars rely on the action of a panel of enzymes, produced by only 1% of the microbial community. The release of oligosaccharides and fermentation products during mucin degradation can be used by other bacteria, thereby expanding the host glycan degradation niche. The ability of these microorganisms to profit; both directly and indirectly, from endogenous glycans can facilitate their close location to the host epithelium, where they may exert a disproportionate effect on human health. Plenty of research concerning *A. muciniphila* has been done, but more information is required concerning its behaviour in the complex microbial ecosystem in the colon, the potential role of mucins to influence *A. muciniphila* behaviour and the impact of its probiotic administration on the microbial ecosystem and the host, which was the focus of this PhD research. *In vitro* technology used in this research, such as the simulator or the human intestinal ecosystem (SHIME®) and the transwell co-culture cell model, allowed for mechanistic research that aimed at unravelling the ecology of mucin degraders and helped to overcome some confounding elements of *in vivo* studies, such as variations in mucin production by the host.

The first part of this PhD research focused on the role of *A. muciniphila* in host glycan degradation and the importance of this niche for the microbial ecosystem. **Chapter 2** studied the colonization behaviour of *A. muciniphila* under variable conditions and revealed that

A. muciniphila preferentially colonized the distal colon and that this preference was due to pH, which is more neutral compared to the proximal colon. Mucin deprivation decreased *A. muciniphila* abundances and subsequent mucin supplementation caused a specific increase of *A. muciniphila*, far exceeding the response of other bacteria present. To study the biological reproducibility of these findings, the effect of pH and the presence or absence of a host-glycan degradation niche was investigated in colon compartments separately inoculated with the microbiota from eight donors in **Chapter 3**. pH specificity and nutrient sensitivity of *A. muciniphila* was confirmed in these different microbial communities. Mucin supplementation resulted in more similar microbial communities for the eight donors, indicating host glycans to constitute an important ecological niche shaping the microbiota composition. The effect of colonic pH had a less profound impact on the microbiome with donor origin explaining most of the variability.

To asses microbial cross-feeding and competition interactions of *A. muciniphila* more in detail, **Chapter 4** investigated different primary degraders for host or dietary glycan degradation and their effect on butyrate production. These interactions are difficult to study in a complex bacterial community and so a synthetic microbial community was used, with *A. muciniphila* and *B. thetaiotaomicron* as the primary glycan degraders. Joint presence of both primary degraders did not lead to a competitive exclusion in the presence of mucin; *A. muciniphila* was not even overgrown by *B. thetaiotaomicron* when additional dietary glycans were available. Shifts in pH and primary degrader abundance was selective for butyrate producers while the butyrate producing functionality was maintained.

The second part of this PhD research focused on the modulation of the microbial community by administration of *A. muciniphila* and the presence/absence of a host-glycan degradation niche, and its impact gut barrier function and immune response. Addition of both mucin and *A. muciniphila* to microbial gut communities from different donors (**Chapter 5**) might lead to *A. muciniphila* dominating the mucin degradation niche, while sole mucin addition led to involvement of several species, including *A. muciniphila, Ruminococcus, Clostridium cluster XIVa,* and *Lachnospiraceae.* Supernatant samples were taken from the microbial communities shaped by these treatments, to study their effect on the intestinal epithelium and the underlying immune cells in **Chapter 6**. The supernatant of the treatment with both mucin and *A. muciniphila* induced the most beneficial response, with the mucin responsible for increased trans-epithelial resistance (TEER) and reduced TNF-α and IL-6 production, and *A. muciniphila* responsible for decreased epithelial permeability

Overall, this research, using the complex microbial communities from several donors, showed the nutrient specificity of *A. muciniphila* and its sensitivity to changes in the colon environment, and provided valuable information about the prebiotic-like action of host derived

glycans. However, the inter-individual differences impacting our results need further elucidation.

Samenvatting

De laatste decennia heeft wetenschappelijk onderzoek ons inzicht in de bacteriën die ons lichaam koloniseren gerevolutioneerd: hoe deze een impact hebben op onze gezondheid en hoe complex de interacties tussen de bacteriën onderling en tussen de bacteriën en de gastheer zijn. Subtiele verschillen in onze microbiële gemeenschap kunnen ziektes veroorzaken en studies tonen een correlatie tussen de darmmicrobiota en obesitas, inflammatoire darmziekten (IBD), diabetes, kanker, … Onderzoek naar het identificeren van biomarkers voor intestinale gezondheid en naar manieren om de samenstelling en functionaliteit van de microbiota te beïnvloeden, heeft de darmbacterie *Akkermansia muciniphila* in de kijker gezet. Deze bacterie is minder abundant aanwezig in patiënten die lijden aan IBD, obesitas, diabetes en autisme, maar komt abundant voor in gezonde mensen. *A. muciniphila* wordt gekarakteriseerd door zijn capaciteit om mucines af te breken. Deze mucines zijn een belangrijk onderdeel van de mucuslaag, een beschermende slijmlaag die de darmcellen beschermt tegen darmbacteriën. De mucuslaag en de mucines die hem opbouwen, hebben niet enkel een barrièrefunctie maar dienen ook als voedingsbron voor darmbacteriën, een aspect dat meer en meer aandacht krijgt. Waar er voorheen gedacht werd dat mucine-afbraak schadelijk zou zijn, is het ondertussen duidelijk dat de degradatie bijdraagt tot een gezonde mucuslaag en mucineproductie door de gastheer stimuleert. Omwille van de complexiteit van de mucinestructuur, is er voor de afbraak een resem aan enzymen nodig, die slechts door 1% van de microbiota geproduceerd worden. Hoewel mucine-afbraak door slechts enkele bacteriën kan worden uitgevoerd, kunnen andere bacteriën hier ook indirect van profiteren: ze kunnen gebruik maken van de minder complexe suikers en omzettingsproducten die vrijgesteld worden tijdens de afbraak. Vanwege de nabijheid van de mucuslaag tot de darmcellen, hebben de bacteriën die betrokken zijn bij de mucine-afbraak, zowel direct als indirect, een groter effect op de menselijke gezondheid. Hoewel er reeds veel onderzoek gevoerd is naar *A. muciniphila*, is er nog informatie nodig over hoe deze bacterie zich gedraagt in de microbiële gemeenschap in het colon, hoe mucine dit gedrag kan beïnvloeden en hoe het toedienen van *A. muciniphila* de microbiële gemeenschap en de gastheer zal beïnvloeden. Het gebruik van *in vitro* technologie in dit onderzoek, zoals de simulator van het humaan intestinaal ecosysteem (SHIME) en het co-cultuur celmodel, ondersteunde mechanistisch onderzoek dat doelde op het ontrafelen van de ecologie van mucine-degraderende bacteriën en liet toe dat bepaalde nadelige kenmerken van *in vivo* studies vermeden werden, zoals variaties in de mucineproductie door de gastheer.

In het eerste deel van dit doctoraatsonderzoek lag de focus op de rol van *A. muciniphila* in de mucinedegradatie en de betekenis van deze niche voor het microbieel ecosysteem. **Hoofdstuk 2** bestudeerde het kolonisatiegedrag van *A. muciniphila* in het colon in variërende omstandigheden en toonde aan dat *A. muciniphila* de distale colonregio prefereert omwille van de zuurtegraad (pH), die hier neutraler is dan in de proximale regio. Mucinedeprivatie verminderde de aanwezigheid van *A. muciniphila* en de daaropvolgende toevoeging van mucine veroorzaakte een specifieke toename van *A. muciniphila* bacteriën die de respons van de andere bacteriën ver oversteeg. Om de biologische herhaalbaarheid van deze bevindingen te testen, werd in **Hoofdstuk 3** onderzocht of gelijkaardige effecten van pH en mucine op de microbiële gemeenschappen van acht donoren werden waargenomen. De pH- en mucinespecificiteit van *A. muciniphila* werd bevestigd in deze acht verschillende microbiële gemeenschappen en het toedienen van mucine maakte deze gemeenschappen meer gelijkend op elkaar, erop wijzend dat mucines een belangrijke ecologische niche zijn die de microbiële gemeenschap vormgeeft. Het effect van pH was minder diepgaand en meer onderhevig aan interindividuele verschillen.

Om de nutritionele interacties van *A. muciniphila* meer in detail te kunnen bestuderen, werd in **Hoofdstuk 4** gebruik gemaakt van een minder complexe, synthetisch samengestelde microbiële gemeenschap in medium met mucine en/of vezels als voedingsbron. Met *A. muciniphila* en *Bacteroides thetaiotaomicron* als primaire degradeerders, werd de competitie tussen beiden onderzocht. Daarnaast werd er ook gekeken naar de syntrofische interacties met butyraatproducerende bacteriën. Groei van beide primaire degradeerders zonder competitieve uitsluiting was mogelijk op medium met mucine, zelfs wanneer *B. thetaiotaomicron* daarbovenop gebruik kon maken van vezels. Veranderingen in pH en primaire degradeerder selecteerden specifieke butyraatproducenten, maar de productie van butyraat werd gehandhaafd.

In het tweede deel van dit doctoraatsonderzoek lag de focus op het wijzigen van de microbiële gemeenschap door toediening van *A. muciniphila* en de aan- of afwezigheid van mucine en de impact hiervan op de epitheliale barrière en het immuunsysteem. Toediening van zowel mucine als *A. muciniphila* aan microbiële gemeenschappen van verschillende donoren, kan leiden tot de dominantie van *A. muciniphila* in de mucinedegradatie niche. Toediening van enkel mucine daarentegen, leidde tot betrokkenheid van verschillende bacteriën waaronder *A. muciniphila, Ruminococcus, Clostridium cluster XIVa,* and *Lachnospiraceae.* Stalen werden genomen van de microbiële gemeenschappen na deze behandelingen om hun effect op de epitheliale cellen en de onderliggende immuuncellen te bestuderen in **Hoofdstuk 6.** Het supernatans van de behandeling met zowel mucine als *A. muciniphila* induceerde de meest voordelige respons, met mucine verantwoordelijk voor

een stijging in trans-epitheliale resistentie (TEER) en verminderde TNF-α en IL-6 productie, en *A. muciniphila* verantwoordelijk voor verminderde epitheliale permeabiliteit.

Tijdens dit doctoraatsonderzoek, gebruik makende van de complexe microbiële gemeenschappen van meerdere donoren, werd de nutriëntspecificiteit van *A. muciniphila* voor mucine aangetoond, de gevoeligheid van deze bacterie voor veranderingen in de colonomgeving, en werd er waardevolle informatie verschaft over de prebiotisch-achtige werking van mucine. Niettemin is er nood aan verder onderzoek om de impact van interindividuele verschillen uit te klaren.

Scientific Curriculum Vitae

Personal information

Name:	Florence Van Herreweghen
Date and place of birth:	26th of March 1989, Vilvoorde, Belgium
Nationality:	Belgian
Email:	floorvh@gmail.com; Florence.VanHerreweghen@ugent.be
Phone:	+32486 612 905
Affiliation:	Ghent University – Faculty of Bioscience Engineering Center for Microbial Ecology and Technology (CMET, www.cmet.ugent.be)
Workplace address:	Coupure Links 653, 9000 Ghent, Belgium

Education

Jan 2014 – present	**PhD Candidate in Applied Biological Sciences** Ghent University, CMET Supervisor: Prof. dr. ir. Tom Van de Wiele Title: Impact of mucin and mucin degrading *Akkermansia muciniphila* on gut microbial ecology and markers for gut health
2011-2013	**Master of Science in Bioscience Engineering, Cell and Gene Biotechnology** Ghent University. Graduated with distinction Master thesis: Cross-feeding between the mucin degrading gut commensal *Akkermansia muciniphila* and butyrate producing gut bacteria. (Prof. dr. ir. Tom Van de Wiele)
2007-2011	**Bachelor of Science in Bioscience Engineering, Cell and Gene Biotechnology** Ghent University.

Publications

International peer reviewed publications (A1)

1. **Van Herreweghen F.** and Van den Abbeele P., De Mulder T., De Weirdt R., Geirnaert A., Hernandez-Sanabria E., Vilchez-Vargas R., Jauregui R., Pieper D.H., Belzer C., De Vos W.M. and Van de Wiele T. (2017), In vitro colonisation of the distal colon by Akkermansia muciniphila is largely mucin and pH dependent. Benef Microbes 8: 81-96.

2. De Weirdt, R., Hernandez-Sanabria, E., Fievez, V., Mees, E., Geirnaert, A., **Van Herreweghen, F**., Vilchez-Vargas, R., Van den Abbeele, P., Jauregui, R., Pieper, D. H., Vlaeminck, B. and Van de Wiele, T. (2017), Mucosa-associated biohydrogenating microbes protect the simulated colon microbiome from stress associated with high concentrations of poly-unsaturated fat. Environ Microbiol, 19: 722–739. doi:10.1111/1462-2920.13622

National publications with editorial review (A3)

1. De Paepe, K., and **Van Herreweghen, F**., Van den Abbeele, P., Geirnaert, A., Marzorati, M., Van de Wiele, T. (2016) Opportuniteiten voor volgende generatie probiotica. Food, Science and Law

Articles intended for peer review

1. **Van Herreweghen, F.,** De Paepe, K., Roume, H., Kerckhof, FM., Van de Wiele, T**.** A host glycan degradation niche in a dynamic gut model increases *Akkermansia muciniphila* abundance and changes microbiome composition in a donor independent fashion. *Submitted at FEMS Microbial Ecology.*

2. **Van Herreweghen, F.,** Rotsaert, C., Van de Wiele, T. A synthetic gut ecosystem demonstrates that variable ratios of primary degraders do not impact butyrate producing functionality despite phylogenetic shifts. *In preparation*
3. **Van Herreweghen, F.,** De Paepe, K., Marzorati, M., Van de Wiele, T. *In vitro* supplementation of *Akkermansia muciniphila* rather than stimulation of endogenous *A.muciniphila* results in a higher dominance in the host glycan degradation niche. *In preparation*
4. **Van Herreweghen, F.,** De Paepe, K., Calatayud, M.& Van de Wiele, T. Mucin and mucin degrading *Akkermansia muciniphila* display differentially protective effects towards *in vitro* epithelial barrier functioning and immune modulation. *In preparation*

Participation at international conferences

Oral presentations

1. **Van Herreweghen F.,** Van den Abbeele P., De Paepe, K., Hernandez-Sanabria E., Van de Wiele T. (2017), A host glycan degradation niche in a dynamic gut model increases *Akkermansia muciniphila* abundance and changes microbiome composition in a donor independent fashion. Presented at: 22nd National Symposium For Applied Biological Sciences (Leuven, Belgium, February 7th, 2017)
2. **Van Herreweghen F.,** Van den Abbeele P., De Paepe, K., Hernandez-Sanabria E., Van de Wiele T. (2017), A host glycan degradation niche in a dynamic gut model increases *Akkermansia muciniphila* abundance and changes microbiome composition in a donor independent fashion. Presented at: 11th International Scientific Conference On Probiotics, Prebiotics, Gut Microbiota And Health (Budapest, Hungary, June 20th 2017)

Poster presentations

1. **Van Herreweghen F.** and Van den Abbeele P., De Mulder T., De Weirdt R., Geirnaert A., Hernandez-Sanabria E., Vilchez-Vargas R., Jauregui R., Pieper D.H., Belzer C., De Vos W.M. and Van de Wiele T. Unraveling the behavior of *Akkermansia muciniphila* in a complex microbial gut community. Presented at: 16th Gut Day Symposium (Amsterdam, The Netherlands, November 27th, 2014).
2. **Van Herreweghen F.** and Van den Abbeele P., De Mulder T., De Weirdt R., Geirnaert A., Hernandez-Sanabria E., Vilchez-Vargas R., Jauregui R., Pieper D.H., Belzer C., De Vos W.M. and Van de Wiele T. *Akkermansia muciniphila* colonization of an in vitro distal colon is mucin dependent. Presented at: 18th Gut Day Symposium (Venlo, The Netherlands, November 27th, 2016).

Teaching

1. Responsible for practical exercises for the course ‘Host-microbe interactions’ (Prof. Tom Van de Wiele) (2014-2015)
2. Tutor of 3 bachelor and 1 master students during their thesis (2014-2017)
3. Teaching STEM project for Atheneum Zottegem (2017)

Awards

1. Best oral presentation, 22nd National Symposium For Applied Biological Sciences, Leuven, Belgium, February 7th, 2017

Acknowledgements - Dankwoord

Acknowledgements-Dankwoord

A PhD trajectory is typically one with a few bumps in the road, and mine wasn't any different. The work in the lab can lead to great highs when everything is working fine, results keep on coming in and are confirming hypotheses; or when you find something completely unexpected. But it can also lead to deep sinkholes when you have no idea what is going wrong or how you can fix it, when the bacteria just don't want to listen and behave or when staring at a blank page and forcing your mind to make a perfect sentence. Well, during my bumpy road I got to share my highs with -and got pulled out of some sinkholes by- quite a few extraordinary people and in this chapter I will attempt to thank them adequately.

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