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ABILITY OF A PROPIONATE-PRODUCING SYNTHETIC MICROBIAL CONSORTIUM TO RESTORE FUNCTIONALITY IN A DYSBIOSED HUMAN GUT MICROBIOME

Racha El Hage

Thesis submitted in fulfilment of the requirements for the degree of Doctor (PhD) in Applied Biological Sciences
Titel van het doctoraat in het Nederlands:
Vermogen van een propionaatproducerend synthetisch microbieel consortium om de functionaliteit te herstellen in een humaan darmmicrobioom in dysbiose

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Please refer to this work as:
ISBN 9789463572378

The work presented in this thesis was performed at the Center for Microbial Ecology and Technology, in the Faculty of Bioscience Engineering at Ghent University (Gent, Belgium). The research leading to these results has received funding from the People Program (Marie Curie Actions) of the European Union’s Seventh Framework Program FP7/2007–2013/under REA grant agreement n_ 606713.

Cover illustration: Reem El Hage
“Nothing in life is to be feared, it is only to be understood. Now is the time to understand more, so that we may fear less.”

Marie Curie
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Chapter 1
Introduction

This Chapter has been redrafted after
CHAPTER 1 - INTRODUCTION

1.1 - Importance of gut microbiota in human health.

The gut microbiota plays a significant role in human health, participating in several functions beneficial to the host (Patel and DuPont 2015; Kristensen et al. 2016). It has been implicated in preventing pathogen colonization (Hand 2016), shaping our immune system (Round and Mazmanian 2009; Patel and DuPont 2015; Macpherson, de Agüero and Ganal-Vonarburg 2017), stimulating the production of gastrointestinal hormones (Saulnier et al. 2013), and regulating brain behaviour (De Palma, Collins and Bercik 2014; De Palma et al. 2017) through production of neuroactive substances (Steenbergen et al. 2015; Kristensen et al. 2016).

The gut microbiota has been involved in the fermentation of nondigestible carbohydrates reaching the colon. This process leads to the production of short chain fatty acids (SCFA), which elicit health benefits (den Besten et al. 2013). The human gut microbiota can be manipulated through either passive or active processes. Passive factors include hygiene, lifestyle, and diet. For instance, primary colonizers of the gut involved in the immune development are shifted by sanitary practices (Zhou 2016). In addition, dietary constituents can promote phylogenetic variations in the microbiota (Graf et al. 2015). In this context, prebiotics are defined as “a substrate that is selectively utilized by host microorganisms conferring a health benefit” (Gibson et al. 2017). Prebiotics act as growth substrates (Patrascu et al. 2017) to enhance the activity of bacterial genera (Scott et al. 2015) such as bifidobacteria and butyrate-producing clostridia (Rivière et al. 2016). SCFA and vitamins resulting from the fermentation of these components are crucial for human health (Graf et al. 2015). In terms of lifestyle factors, physical activity is known to positively impact the diversity of gut microbiota. In fact, gut microbiota of athletes is more diverse than that of non-athletic subjects (Clarke et al. 2014). Amongst the active processes affecting microbiota composition are antibiotics and probiotics. Antibiotic use has been linked to dysbiosis (Langdon et al. 2016), even leading to low diversity, evenness, and taxonomic richness (Francino 2016; Dethlefsen and Relman 2011). These detrimental outcomes may lead to decreased SCFA, glycolysis, vitamin production, homeostasis of the immune system, and impaired protection against pathogens (Guarner and Malagelada 2003). As a result, antibiotic associated diarrhea (AAD) and recurrent infectious diseases like Clostridium difficile infection (CDI) may occur (Francino 2016).

On the other side of the spectrum are probiotics, which can affect the host either directly or through their products, or even influence the activity of resident bacteria in the host (Scott et al. 2015). Probiotics are defined as “live microorganisms which when administered in adequate amounts confer a health benefit on the host” (WHO/FAO 2006; Hill et al. 2014). The effect of probiotics in preventing metabolic syndromes such as obesity, type 2 diabetes (Kasińska and
Drzewoski 2015), and dyslipidemia has been reported (Asemi et al. 2013). For instance, administration of *Bifidobacterium* (Wang et al. 2014; Chen et al. 2011; Savcheniuk et al. 2014; Yin 2010; Reichold et al. 2014; Plaza-Diaz et al. 2014) and *Lactobacillus* species reduced body weight gain and adipose tissue in mice fed high-fat diet through stimulation of adiponectin production (Kim et al. 2013; Kobyliak et al. 2016). In addition, lactobacilli have been proven to have therapeutic effects in different pathologies (Di Cerbo et al. 2016). Moreover, probiotics regulate the mucosal immune response (Klaenhammer et al. 2012) by improving the activity of macrophages (Sang 2010) and changing the expression of the genes associated. Even though these outcomes depend on specific bacteria and strains, probiotics may interact with Toll-like receptors (TLR) and downregulate the expression of NF-κB and pro-inflammatory cytokines (Plaza-Diaz 2014; Ng et al. 2009). For instance, peptides of microbial anti-inflammatory molecules (MAM) that are found in the *F. prausnitzii* supernatant inhibit the NF-κB pathway *in vitro* and *in vivo* (Breyner et al. 2017), confirming the anti-inflammatory and therapeutic properties of *F. prausnitzii* (Martin et al. 2014). These properties and protective effects of *F. prausnitzii* were identified in different models such as dinitrobenzene sulfate (DNBS)-induced colitis model, dextran sodium sulfate (DSS)-induced colitis (Breyner et al. 2017), and 2,4,6-trinitrobenzenesulfonic acid (TNBS)-induced acute colitis in mice (Miquel et al. 2015). Additionally, levels of anti-inflammatory cytokines and immunoglobulins, immune cell proliferation, and production of proinflammatory cytokines produced by the T cells may be modulated following probiotic supplementation (Nazemian et al. 2016; Miettinen et al. 1996). Furthermore, probiotics can be alternative strategies for inflammatory disorders, as they upregulate the production of CD4+Foxp3+ regulatory T cells (Tregs) (Kwon et al. 2010; Yan and Polk 2011).

Different effects on the immune function may be species- and strain-related (Klaenhammer et al. 2012). It has been reported that probiotics have therapeutic effect on the central nervous system by reducing the intestinal inflammation. In this way, the regulation of HPA axis and the activity of the neurotransmitters may be improved (Wallace and Milev 2017). Probiotics from *Bifidobacterium* and *Lactobacillus* genera are usually delivered through fermented products such as yogurts, milk, and cheeses, or they can be delivered as food supplements (Besseling-van der Vaart et al. 2016).

1.2 - Manipulation of the gut microbiome using antibiotics

Antibiotics, which are used to prevent the growth or kill a microorganism that causes disease, have been considered the miracle drugs and are frequently prescribed in most countries (Quigley 2011; Nami et al. 2015). However, this is accompanied by antibiotic resistance, which is a major public-health threat, and which has been reported for almost every antibiotic
discovered (Nami et al. 2015). The common use of antibiotics in humans and the broad-spectrum activities of these antibiotics has led the human microbiome to take on substantial responsive changes to this therapy; this urges the need to investigate the different antibiotics to which the microbiome has developed resistance genes (Carlet 2012; Kazimierczak and Scott 2007; Nami et al. 2015). Indeed, this emphasizes the need to restrict antibiotic use to prevent treatment failure and the spread of antibiotic resistance (Jakobsson et al. 2010; Nami et al. 2015). Moreover, antibiotic use has been reported to impact the microbial intestinal metabolism, and potentially lead to obesity, type 2 diabetes and low-grade inflammation (Mikkelsen et al. 2016). A study by Perez-Cobas et al. (2013) verified that antibiotics targeting specific pathogens may drastically alter gut microbial ecology and interactions with host metabolism. In contrast, probiotics are considered living drugs that can enhance human health and reduce antibiotic use (Nami et al. 2015).

Microbial ecology research has proven the crosstalk between residing members of the gut microbiota and the host immune system. This tight interrelationship has been linked to the microbial metabolites responsible for the cell-to-cell communication through quorum-sensing, and through the activation of the eukaryotic cells by host defensins and modulation of cytokine profiles (Mikelsaar et al. 2011). As a result, such host functions can be positively influenced by the residing probiotic bacteria in our gut. However, these commensals require evaluation in human studies to elucidate their community composition and metabolic activities (Mikelsaar et al. 2011).

1.3 - Monostrain and Multistrain probiotics

Probiotics have been categorized into monostrain or multistrain/multispecies products (Timmerman et al. 2004). Different studies have confirmed positive effects on health when multistrain probiotics are used, due to the symbiosis among strains (Timmerman et al. 2004). Strains in multispecies probiotics can be from different genera. For instance, the efficacy of the multispecies probiotic consortium VSL#3 (Streptococcus thermophilus, Eubacterium faecium, Bifidobacterium breve, Bifidobacterium infantis, Bifidobacterium longum, Lactobacillus acidophilus, Lactobacillus plantarum, Lactobacillus casei and Lactobacillus delbrueckii subspecies bulgaricus) was proven for the treatment of ulcerative colitis (Timmerman et al. 2004; Venturi et al. 1999). Besides, VSL#3 supplementation in women with gestational diabetes mellitus (GDM) may help regulate inflammatory markers and positively influence glycemic control (Jafarnejad et al. 2016). In addition, Chapman, Gibson, and Rowland (2011) described that probiotic mixtures were more effective than single-strain probiotics in inhibiting pathogen growth and atopic dermatitis, suggesting further application on other diseases like Inflammatory bowel disease (IBD). Another multispecies probiotic called Ecologic®Tolerance/Syngut™ was developed using four different probiotic strains (Bifidobacterium lactis W51, Lactobacillus acidophilus W22, Lactobacillus plantarum W21 and
Lactococcus lactis W19). Strains of this consortium have been proven to strengthen the gut barrier function, have beneficial effects on post immunological induced stress, inhibit Th2, and stimulate IL-10 levels, thus providing beneficial effects in patients with food intolerance (Besseling-van der Vaart et al. 2016). Moreover, a multispecies probiotic consortium, Ecologic AAD (B. bifidum W23, B. lactis W18, B. longum W51, E. faecium W54, L. acidophilus W37 and W55, L. paracasei W72, L. plantarum W62, L. rhamnosus W71, and L. salivarius W24), reduced diarrhea-like bowel movements when administered in healthy volunteers taking amoxicillin (Koning et al. 2008). Multispecies probiotics also prevented rise in fasting plasma glucose (FPG), to decrease high sensitivity C-reactive protein (hs-CRP), and to increase plasma glutathione (GSH) in diabetic patients (Asemi et al. 2013). Van Minnen et al. (2007) provided evidence that manipulation of the intestinal microbiota with multispecies probiotics reduced bacterial translocation, morbidity and mortality in a rat model of acute pancreatitis. Furthermore, multispecies probiotics rapidly relieved IBS symptoms and shifted the microbiota composition (Yoon et al. 2013). According to these results, combining specific probiotic effects from diverse strains can lead to an additive and more synergetic multispecies probiotic consortium (Timmerman et al. 2007).

However, the phylogenetic origin of probiotics is currently limited to conventional formulations of Bifidobacterium, Lactobacillus species and other lactic acid bacteria (Govender et al. 2013) or yeast strains. This may decrease the probiotic effectiveness in the prevention or therapy of diseases entailing severe dysbiosis. Hence, a functionally and phylogenetically diverse probiotic product may be desirable when alterations in the gut microbiota composition are present (Marotz and Zarrinp 2016). For instance, CDI and recurrent CDI are major medical conditions that need urgent treatment when conventional antibiotics fail. As a result, development of complex communities with targeted functions is needed.

1.4 - The dilemma of Fecal Microbiota Transplant (FMT)

“Fecal microbiota transplantation (FMT) is the introduction of a fecal suspension derived from a healthy donor into the gastrointestinal (GI) tract of a diseased individual” (Borody, Paramsothy and Agrawal 2013). Fecal microbiota transplant (FMT) or fecal bacteriotherapy is an alternative strategy successfully used for the treatment of CDI (Kelly 2013). Severe antibiotic therapy and CDI trigger dysbiosis, reducing diversity and functionality of the gut endogenous microbiota (Brandt 2012). In this case, Clostridium difficile spores can germinate, colonize, and thrive in the gut. Treatment of CDI requires additional antibiotics, which increases the risk of recurrent CDI (rCDI) after cessation of treatment (Becattini, Taur and Pamer 2016; Francino 2016), due to infection with the original strain (Barbut et al. 2000; Marsh et al. 2012) or re-infection caused by a different strain (Johnson et al. 1989; Figueroa et al. 2012; Kelly 2009).
Poor colonization resistance from the gut microbiota and the patient’s poor immune response further contribute to CDI risk (Pérez-Cobas et al. 2015). Recurrent CDI risk is 10-20% after initial CDI (Surawicz et al. 2013), and it increases to 45% after a first relapse, and to 60% for those with 2 or more recurrences (Bartlett 1990). However, FMT can resolve both CDI and rCDI (Bakken 2009), with a success rate of 90% when further antibiotic treatments fail (Rao and Safdar 2015; Youngster et al. 2014). Given the success of FMT, it is now being considered as potential treatment for disorders such as ulcerative colitis (Shi et al. 2016), irritable bowel syndrome (Distrutti et al. 2016) and metabolic syndrome (Hartstra et al. 2015). For instance, FMT induced remission in patients with active ulcerative colitis (Moayyedi et al. 2015), potentially as a result of the introduction of normal microbiota and the subsequent correction of the imbalance in the microbiota caused by the disease (Bakken et al. 2011). The complexity of the fecal sample can be the key factor behind the positive shift in the microbiota composition generated by the FMT (Marotz and Zarrinpa 2016). Thus, diversity of the donor microbiome may be crucial (Leszczyszyn et al. 2016). Indeed, some patients do not respond to FMT, probably because only specific bacterial phylotypes can be therapeutic when effectively transferred (Vermeire et al. 2015). Hence, FMT efficacy for treating gastrointestinal disorders is controversial (Sbahi and Di Palma 2016). Adverse effects after FMT include nausea, vomit, fever, abdominal pain, and diarrhea (Pigneur and Sokol 2016; Vermeire et al. 2015). Data for long-term effects of FMT is lacking, but theoretically, certain disease phenotypes from the donor can be transferred to the patient (Sbahi and Di Palma 2016). This could be expected, as the uncharacterized nature of FMT may result in undetected or unmonitored risk factors such as viruses, pathogens or even allergens being passed to the FMT recipient, causing disease. To overcome this problem, Petrof et al. (2013) developed a characterized synthetic bacteria cocktail to substitute FMT. Alternatively, a thorough pre-screening should be performed on the donor before the actual FMT procedure. Thus, the French Group of Faecal microbiota Transplantation (FGFT) was created to secure and evaluate the practice in this field (Sokol et al. 2016). Despite having experience treating CDI, FMT is not yet the top treatment choice of physicians (Zipursky et al. 2014). However, the majority of gastroenterologists and physicians in metropolitan areas were supportive to the idea of creating a fecal transplantation center, and a high percentage of the physicians would refer their patients to those centers (Jiang et al. 2013).

1.5 - Alternatives for Fecal Microbiota Transplant

Additional microbiome therapeutics using characterized microbial communities of selected fecal bacteria could be developed to replace FMT and yield the desired outcome (Sbahi and Di Palma 2016). For instance, Petrof et al. (2013) described a stool substitute constituted by 33 different purified intestinal bacteria isolated from a healthy donor (Table 1.1), to treat rCDI. In this study, the synthetic bacterial mixture was infused through the colon of the infected
patient causing a change in the stool microbial profile. Major shifts reflecting the isolates of the synthetic mixture were still detectable six months after treatment. Thus, the concept of “RePOOPulate” the gut microbiome was coined. Authors of the study suggested that using a synthetic stool substitute may be an effective method to replace the use of FMT for treating rCDI. Although further validation is needed, complete resolution of the infection was achieved. Several advantages of this synthetic stool substitute can be highlighted. The composition of the administered bacterial cocktail is accurately characterized, facilitating registration. Further, assembly of the synthetic bacterial cocktail is highly reproducible, enabling standardization and upscaling. In addition, patient safety can be guaranteed, because the bacterial mixture can be rendered pathogen- and virus-free (Petrof et al. 2013). These data suggest that a multi-species community such as that in the RePOOPulate study, can be more effective than single-strain probiotics or mixed cultures of probiotic species. This can be because the RePOOPulate community preserved its structure and thus successfully colonized a new environment (Petrof et al. 2013). Moreover, RePOOPulate consisted of phylogenetically diverse community including strains with beneficial health effects that can be candidates for next generation probiotics.

Table 1.1: Strains composing the RePOOPulate consortium

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<tr>
<th>Composition of Stool Substitute (RePOOPulate)</th>
<th>Acidaminococcus intestinalis</th>
<th>Bacteroides ovatus</th>
<th>Bifidobacterium adolescentis (two different strains)</th>
<th>Bifidobacterium longum (two different strains)</th>
<th>Blautia producta</th>
<th>Clostridium cocleatum</th>
<th>Collinsella aerofaciens</th>
<th>Dorea longicatena (two different strains)</th>
<th>Escherichia coli</th>
<th>Eubacterium desmolans</th>
<th>Eubacterium eligens</th>
<th>Eubacterium limosum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acidaminococcus intestinalis</td>
<td>Eubacterium rectale (four different strains)</td>
<td>Eubacterium ventriosum</td>
<td>Faecalibacterium prausnitzii</td>
<td>Lachnospira pectinoshiza</td>
<td>Parabacteroides distasonis</td>
<td>Raoultella sp.</td>
<td>Roseburia faecalis</td>
<td>Roseburia intestinalis</td>
<td>Ruminococcus obeum (two different strains)</td>
<td>Ruminococcus torques (two different strains)</td>
<td>Streptococcus mitis</td>
<td></td>
</tr>
</tbody>
</table>

1.6 - Next Generation Probiotics

Looking at its internationally recognized definition, probiotics are live microorganisms that, when administered in adequate numbers, confer health benefits on the host. Probiotics are usually isolated from our commensal gut bacteria but cannot be given the definition of
probiotics until their stability, content, and health effect are characterized (Sanders 2008). Probiotics are thought to improve the balance in the host, prevent disturbances, and decrease the risk of pathogen colonization (Goldenberg et al. 2013). Probiotics may additionally impact the gut-brain axis. For example, *Bifidobacterium longum* NC3001 had beneficial effects on psychiatric comorbidities, which in turn could temporarily improve the quality of life in IBS patients, indicating that this probiotic reduces limbic reactivity (Pinto-Sanchez et al. 2017).

They have been referred to as functional foods or beneficial bacteria, and they have been considered for the prevention and treatment of *C. difficile*-associated diarrhea (CDAD) (Goldenberg et al. 2013). Probiotics can be found as capsules or food supplements in health food stores and supermarkets (Goldenberg et al. 2013). Pattani et al. (2013) reported that *Lactobacillus*-based formulations combined with antibiotics reduced the risk of antibiotic associated diarrhea (AAD) and CDI. They however suggested that larger studies are needed to decide on the use of probiotic/antibiotic combination as a therapy over the single species probiotic (Pattani et al. 2013). Furthermore, findings from randomized control trials (RCT) and meta analyses suggest that there is moderate evidence on the ability of probiotics to prevent primary CDI (people at risk of CDI), but there is no enough evidence suggesting the probiotics can prevent secondary CDI (recurrent CDI) (Evans and Johnson 2015). There are still some evidence gaps for the use of probiotics in the prevention of CDI such as the interaction between specific classes of antibiotics with the probiotics used on CDI risk, the bacterial taxa that provides the best efficacy in the prevention of CDI, and the use of probiotics in immunocompromised or critically ill patients (Rao and Young 2017). Hence, future RCT should consider these different concerns (Rao and Young 2017).

Overall, classical probiotics show limited effects on the human gut microbiota, seeking the need for a better selection and formulation of bacterial strains (Neef and Sanz 2013). Results from previous studies show promising outcomes in the treatment or prevention of diverse metabolic and inflammatory diseases by specific bacteria (Neef and Sanz 2013). Those probiotics encompass species different from *Lactobacillus* and *Bifidobacterium* (Cani and Van Hul 2015; Patel and DuPont 2015). Nevertheless, the gut microbiome is a complex community, which makes it difficult to define the host-microbe interaction.

The United Nations Food and Agriculture organization (FAO) definition of probiotics is broad, allowing flexibility in terms of the phylogenetic origin of probiotics. Information generated from previous studies assisted in the selection of next generation probiotics, which include members from *Clostridium* clusters IV, XIVa and XVIII, *Faecalibacterium prausnitzii*, *Akkermansia muciniphila*, *Bacteroides uniformis*, (Neef and Sanz 2013, Patel and Du Pont 2015), *Bacteroides fragilis* (Round et al. 2011), and *Eubacterium hallii* (Udayappan et al. 2016). These next generation probiotics were evaluated in preclinical trials and yielded positive outcomes for inflammatory and metabolic disorders (Neef and Sanz 2013; Patel and Du Pont 2015). In
addition, new techniques are required for the development of novel probiotic products containing strains from human origin. This is to say, these strains must come from the major groups of the intestinal microbiota, they must be defined to have a safe status and proven to have potential beneficial effects (Martin et al. 2017). In the following sections, we will discuss some of the most promising bacterial species that are currently under consideration for being used as next-generation probiotics.

1.6.1- *Faecalibacterium prausnitzii*

*F. prausnitzii* is an extreme oxygen sensitive (EOS) bacterium (Martin et al. 2017) belonging to the *Clostridium* cluster IV, and it accounts for 3-5% of the total fecal bacteria, and it is one of the predominant groups in the human feces (Breyner et al. 2017). Quévrain et al. (2016) reported low proportions of this species in the fecal and mucosa-associated microbiome in Crohn’s disease (CD). *F. prausnitzii* may possess *in vivo* and *in vitro* anti-inflammatory effects. Breyner et al. (2017) confirmed the anti-inflammatory properties of MAM, and their ability to reduce Th1 and Th17 pro-inflammatory cytokines in Mesenteric Lymphatic Node (MLN) and colon tissues in both DNBS and DSS colitis model. MAM also increased TGFβ cytokine production, which is involved in activation of NF-kB in DNBS model, thus protecting the host and decreasing intestinal inflammation (Breyner et al. 2017). In addition, *F. prausnitzii* can induce the *Clostridium*-specific IL-10-secreting regulatory T cell subset, present in several human colonic cells. Its capacity for lowering IL-12 and IFNγ production indicates that the interaction between *F. prausnitzii* and the host shape and maintain the gut barrier immune function (Quévrain et al. 2016). In this way, anti-inflammatory molecules from *F. prausnitzii* may be used as targeted anti-inflammatory drugs for CD. Moreover, MAM could function as a CD biomarker, predicting loss of *F. prausnitzii* functionality. However, further research should be conducted to elucidate the MAM production mechanisms, before considering it for CD management. Sokol et al. reported that low levels of *F. prausnitzii* on ileal Crohn’s mucosa were associated with CD recurrence after 6 months (Sokol et al. 2008). In addition, the oral administration of live *F. prausnitzii* or its supernatant in mice could reduce the severity of TNBS colitis and correct the associated dysbiosis (Sokol et al. 2008). The results from this study suggest that *F. prausnitzii* can be considered as a promising probiotic candidate for the treatment of pathologies characterized by chronic gut inflammation (Sokol et al. 2008). Besides, all *F. prausnitzii* strains have proven anti-inflammatory properties, which allows them to further be tested in murine models to determine their beneficial effects before moving to human trials (Martin et al. 2017).

1.6.2- *Akkermansia muciniphila*

Recent evidence shows that there is a link between the altered gut microbiota and metabolic diseases like obesity, diabetes mellitus, and cardiovascular disease (Schneeberger et al. 2015, Li et al. 2016, Dao et al. 2016). In contrast, higher abundance of *A. muciniphila*, a mucin
degrading microbe, was associated with healthier metabolic status. Schneeberger et al. (2015) and Everard et al. (2014) studied the effects of high fat diet on metabolic parameters and the human gut microbiota composition over time, and they found that *A. muciniphila* was decreased. The negative impact on *A. muciniphila* was associated with expression of lipid metabolism, inflammatory markers in adipose tissue, and different parameters like increased blood glucose, insulin resistance and plasma triglycerides (Schneeberger et al. 2015). This prompted the research towards investigating the putative positive role of *A. muciniphila* in adipose tissue homeostasis and metabolism. Dao et al. (2016) assessed clinical parameters and *A. muciniphila* abundance before and after a 6-week calorie restriction period, followed by stabilization diet. The results of this intervention study indicated that the higher abundance of *A. muciniphila* at baseline was associated with improvement in blood glucose homeostasis, lipid profile, and body fat distribution after the intervention. Thus, *A. muciniphila* can be used as a prognostic tool for the success of diet interventions (Dao at al. 2016). Moreover, Li et al. (2016) reported that administration of *A. muciniphila* could reverse atherosclerotic lesions, improve metabolic endotoxemia-induced inflammation, and ultimately restore gut barrier function (Li et al. 2016).

### 1.6.3- Bacteroides fragilis and Bacteroides uniformis

**Bacteroides** species are commensal bacteria that represent 25% of our gut bacterial population. They are gram negative, anaerobic, bile resistant, and non-spore forming bacteria. **Bacteroides** can be passed from the mother to the child during vaginal delivery, thus becoming primary colonizers of the gut. When retained in the gut, **Bacteroides** act as commensals and can be beneficial for the host (Wexler 2007). The most common isolate from the clinical specimens is *B. fragilis*, which is the most virulent **Bacteroides** species (Wexler 2007).

Bacterial colonization of the gut can greatly affect the immune system, either through direct host-bacteria interaction, or by molecules produced by our commensal bacteria. *B. fragilis* produces polysaccharide A (PSA), which is an immunomodulatory molecule that activates the T-cell dependent immune responses (Troy and Kasper 2011). Those responses are involved in the development and homeostasis of the host immune system (Troy and Kasper 2011). Furthermore, Round et al. (2011) demonstrated that *B. fragilis* activates TLR pathways. This occurs because PSA signals through TLR2 on forkhead box P3 (Foxp3+) regulatory T cells to boost immunologic tolerance. As a result, PSA can be considered as a model symbiosis factor, because it preserves the balance between T cell types and maintains the immune system homeostasis (Round et al. 2011).

As for **Bacteroides uniformis** (*B. uniformis*) CECT 7771, it is considered a potential probiotic strain originally isolated from the feces of healthy breastfed infants. Oral administration of this specific strain in high fat diet-fed mice improved lipid profile, reduced glucose insulin and leptin levels, increased TNF-α production by dendritic cells (DCs) in response to LPS stimulation,
and increased phagocytosis (Cano et al. 2012). Thus, administration of B. uniformis CECT 7771 can ameliorate metabolic disorder and immunological dysfunction related to intestinal dysbiosis in obese mice (Cano et al. 2012, Yang et al. 2016). Furthermore, acute administration of this strain to mice did not promote adverse effects on health status or food intake, and there was no bacteria translocation to blood, liver, or lymph nodes. This indicates that there are no safety concerns for this strain in mice, but further investigation should be completed in humans (Fernández-Murga and Sanz 2016).

1.6.4- Eubacterium hallii
E. hallii is an important anaerobic butyrate-producer resident in our gut, which influences the intestinal metabolic balance (Engels et al. 2016). Butyrate has been proposed to lower mucosal inflammation and oxidative status, strengthen the epithelial barrier function, modulate intestinal motility and being an energy source for colonocytes (Cani et al. 2011). E. hallii can also yield propionate from a broad range of substrates. This versatility may further support the host-gut microbiota homeostasis (Engels et al., 2016). Moreover, administration of E. hallii in obese and diabetic db/db mice increased energy metabolism and improved insulin sensitivity (Udayappan et al. 2016). However, increasing dosage of E. hallii did not impact body weight or food intake, indicating that this strain can be used to develop safe and effective alternatives for insulin sensitivity (Udayappan et al. 2016).

1.6.5- Single strain bio-therapeutics
Bacteroides thetaiotaomicron is a prevalent species within the Bacteroides genus of the human gut microbiota. It has previously been shown that this bacteria can increase mucosal barrier function and can limit pathogen invasion (Delday et al. 2018). Bacteroides thetaiotaomicron has been proven to have potent anti-inflammatory effects in vitro and in vivo as it modulates molecular signalling pathways of NF-κB (Kelly et al. 2004). This works by stopping the binding of the active component (RelA) of NF-κB to key genes in the nucleus, thus preventing the activation of pro-inflammatory pathways (Kelly et al. 2004). The full genome of B. thetaiotaomicron was sequenced and annotated by the Gordon Group (Washington University School of Medicine, USA) in 2003. As a result, Bacteroides thetaiotaomicron had been investigated in different studies and had been disclosed for its use in the prevention of inflammatory disorders. Moran (2014) discussed the possibilities for commercial manipulation of the microbiome, specifying the single bacterial species that act as a Live Biotherapeutic; for instance, Bacteroides thetaiotaomicron can act as a treatment for paediatric Crohn’s disease. Moreover, the patent WO 03/046580 disclosed the use of Bacteroides thetaiotaomicron as a medicine for the treatment of inflammatory disease. According to the background describing the efficacy of Bacteroides thetaiotaomicron against inflammatory disorders, a new patent for
Bacteroides thetaiotaomicron strain BT201 as a treatment of inflammatory disorder and/or an autoimmune disorder has been published (European Patent office 2017).

Another study by Delday et al. (2018) has investigated the effect of Bacteroides thetaiotaomicron type strain DSM 2079 in different rodent models of irritable bowel disease (IBD). The efficacy of the freeze-dried bacterial formulation and the purified recombinant protein of B. thetaiotaomicron was also investigated. The results revealed that Bacteroides thetaiotaomicron showed protective effects against the disease, and this has been demonstrated by the significant amelioration of weight loss, colon shortening, histopathological damage and immune activation. This efficacy has observed in both the actively growing cells and the freeze-dried cells of Bacteroides thetaiotaomicron. It was reported that the anti-inflammatory effect caused by this strain was due to the presence of pirin-like protein (PLP) identified by microarray analysis during the coculture of the bacterial strain with Caco-2 cells, and that reduced pro-inflammatory NF-κB signalling in these intestinal epithelial cells. This data indicate that this strain be may a novel alternative to current treatment options for Crohn’s disease (Delday et al. 2018).

Another single strain live biotherapeutic product that has been developed is BlautixTM (BHT). This product was developed for the treatment of irritable bowel syndrome (IBS). The active ingredient of this product is a strain of Blautia hydrogenotrophica (B. hydro) which is lyophilized and formulated in gastro-resistant enteric capsules for oral administration. This strain works by restoring the functional composition of the of the microbiota to a healthy state which is important in order to alleviate IBS-related symptoms (Weinberg et al. 2018).

A new study at Imperial College in London, has also reported a new single strain biotherapeutic. The strain of bacteria - Enterococcus gallinarum - known as to MRx0518 was given to treat up to 120 patients suffering from breast, prostate, ovarian, bladder, lung, head, neck and skin cancer in the weeks before surgery. The hypothesis of this study is that after the surgery the bacteria will help the body to increase its ability to fight off the cancer. Other studies will also be held to investigate the effect of this bacterium in asthma and in IBD patients. This bacterium has been isolated from the healthy human faeces and multiplied in the lab. It has then been freeze-dried to put it into a kind of suspended animation to be administered orally in a daily pill. The bacteria stay alive in the gut for two to three weeks before being flushed out. This strategy in safer than faecal transplants since not the entire microbiome is transferred, so there is no chance of involuntarily passing of other conditions (Knapton 2019).
1.7 – Cocktails of *Clostridium* cluster IV and XIVa members

As previously described, T<sub>regs</sub> can regulate immune homeostasis and serve as a therapeutic target for different gut inflammatory disorders. Induction of the colonic T<sub>regs</sub> is dependent on special properties of our commensal bacteria. *Clostridium* spp. belonging to clusters IV and XIVa (also known as *Clostridium leptum* and *coccoides* groups, respectively) are exceptional inducers of T<sub>regs</sub> in the colon and can be considered as therapeutic options for IBD and allergies (Atarashi et al. 2011). Previous work indicated that a cocktail of strains isolated for the human gut microbiota could be more effective than a single strain in preventing or treating disease. Thus, Atarashi et al. (2013) isolated 17 strains belonging to Clostridia clusters XIVa, IV and XVIII from a human faecal sample, which were effective in T<sub>reg</sub> cell differentiation and accumulation in mouse colon. Authors proposed that the short chain fatty acids produced by this community influenced the expression of Foxp3<sup>+</sup>, a key gene controlling T<sub>reg</sub> cell development (Atarashi et al. 2013). Incidentally, Clostridia clusters XIVa and IV are decreased in fecal samples from patients with inflammatory bowel disease, and thus the cocktail of the 17 strains could potentially reverse this dysbiosis (Atarashi et al. 2013).

1.8- Industrial applications and interests

1.8.1- Current Developments

Since the manipulation of the gut microbiota has been proven to be promising to prevent and treat different diseases, pharmaceutical and food industries are interested in the potential therapeutic approaches described before. For instance, Seres health and Rebiotix companies are working on developing a defined microbial cocktail and a standardized commercially-prepared FMT, respectively. These therapeutic approaches are intended to treat CDI, and can be used as an alternative for FMT. Synthetic microbial communities designed for transplants are expected to meet production, mode of action and safety standards (Orenstein et al. 2015; van der Lelie et al. 2017). For instance, Seres health developed SER-109, a novel biological agent proposed to restore the balance in the gut microbiome, promoting resistance to pathogenic invaders like *C. difficile* (Khanna et al. 2016). Seres health also developed SER-287 for the treatment of IBD and specifically ulcerative colitis ("Inflammatory Bowel Disease | Seres Therapeutics" 2017). Rebiotix commercially developed RBX2660, a mix of live human microbes for effective treatment of recurrent CDI (Ramesh et al. 2016). Moreover, other formulations including strains belonging to Clostridia classes IV and XIVa were designed to modulate the immune response (Atarashi et al. 2013). The original community of 17 strains (VE202) was developed by Vendanta Biosciences and Johnson & Johnson, and has provided an effective treatment for autoimmune disorders (Reardon 2014; Ratner 2015; van der Lelie et al. 2017).
1.8.2 – Technical Challenges

Several challenges concerning the stability of the probiotic during the commercial production are still unsolved. Microorganisms require strict conditions to grow, such as specific nutritional media and environmental conditions (suitable temperature, pH, water activity, oxygen content, among others). The product manufacturing and storage processes may impact the viability of bacterial strains, influencing probiotic potency and properties. In addition, it is fundamental to consider the viability of the probiotics after consumption. Bacterial strains should remain viable at sufficient numbers through the gastrointestinal tract (GIT) passage. Therefore, the selection of optimal culture medium and cell protectants is crucial to enhance the efficacy of the probiotic product. Moreover, as most probiotic strains are strict anaerobes or facultative anaerobes, oxygen permeation into carriers should be reduced, or oxygen scavengers should be introduced to reduce the redox potential (Shah et al. 2010). Probiotic bacteria can also be protected by microencapsulation, which has been proposed to improve the stability of the strains and can adapt to the GIT conditions (Heidebach et al. 2012). Nowadays, yogurts and fermented milk are the best-established vehicles for probiotics in the market. Fermented milk, typically contains three times the amount of probiotic cultures than yogurts (The Globe and Mail 2013). However, some probiotic strains are sensitive to the different conditions in fermented products, like oxygen and pH, which can, in turn, affect the stability of probiotics through post acidification during their storage in the fridge. To minimize this phenomenon, strains that lack the ability to post acidify should be selected (Damin et al., 2008). As a result, this can cause an economic burden for manufacturers, limiting the addition of probiotics in different products (Gueimonde and Sánchez 2012). Furthermore, manufacturing the probiotic product in a reproducible manner is a critical aspect (Silva and Freitas 2014). Shah et al. (2010) reported that changes in the number of viable probiotic bacteria during the storage of functional foods should be studied more extensively, as studies have shown poor viability of probiotic bacteria in functional foods (Shah et al. 2010).

1.8.3 – Regulatory Challenges

Probiotics are classified in different categories across countries. Their names and use as functional foods may vary according to different systems. For instance, probiotics fall in the Qualified presumption of safety (QPS) list provided by the European Food Safety Authority (EFSA) and referred to as functional foods since there was no legal definition for probiotics. The market for probiotics as functional foods expanded, as a result of probiotic food products like yogurts and fermented milk (Baldi and Arora 2015), containing conventional lactic acid bacteria (LAB). The QPS list is periodically updated according to the safety assessment of the biological products recommended to be added, and not all can be approved ("Scientific Opinion on The Maintenance of The List of QPS Biological Agents Intentionally Added to Food and Feed (2013 Update)” 2013; Ricci et al. 2017). A similar system applies in the USA as Generally
Recognized as Safe (GRAS) products should be approved by the FDA. However, if a probiotic is used as a dietary supplement in the USA, then it is considered as “food” and should be regulated by the Dietary Supplement Health and Education Act (DSHEA). If the probiotic was considered to have therapeutic purpose, the probiotic drug should be proven to be safe and effective to be approved by the FDA. Nevertheless, for both the FDA and EFSA, probiotics cannot be used in health claims. On the other hand, Japan acts as a global market leader, where probiotics are considered as both foods and drugs. According to the Japanese regulations, probiotic products are in a different category than foods and Foods for Specific Health Uses (FOSHU). Efficacy claims for probiotic products are prohibited on the labelling until the product gets the permission from the Ministry of Health and Welfare (MHLW) to be considered FOSHU, for which efficacy and safety validation is mandatory. FOSHU categorizes the food claims according to the scientific evidence and the strength of the supporting data provided. The government then divided the FOSHU health claims into subcategories, in which their effect could be in GIT, metabolism, cholesterol moderation, or bone health. Japanese regulations also approve new health claims on a regular basis (Baldi and Arora 2015).

As the definition and classification of probiotics by regulatory agents throughout the world is different, the status of probiotic products is still uncertain. Thus, reservations about probiotic products claims may arise among regulatory bodies, producers, and consumers. Since the probiotic concept is invading the world, further investigation for probiotic traits is needed. Moreover, most probiotics only include lactic acid bacteria, which possess limited phylogenetic diversity and functionality. Hence, critical update of the screenings required by regulatory agents is urgently needed.

1.8.4- Regulatory challenges for live bio-therapeutics

According to FDA, a live bio-therapeutic product (LBP), is a biological product that contains live organisms such as bacteria, that is able to prevent, treat or cure a certain disease or a condition in human, and that is not a vaccine. LBPs are not filterable viruses, oncolytic bacteria, or products intended as gene therapy agents, and they are not administered by injection (FDA 2016). An example of an LBP would be a lactobacilli strain that is administered orally to treat patients with ulcerative colitis. Other examples include Bifidobacteria, some Streptococcal species, Bacillus clausii, and the yeast Saccharomyces cerevisiae var boulardii. A recombinant LBP is composed of microorganisms that have been genetically modified through adding, deleting, or modifying their genetic material. A recombinant LBP is likely to raise additional considerations, which would require additional information to be submitted in an Investigational New Drug Application (IND). Prior to submission of their IND, the potential sponsors of an IND for a recombinant LBP are encouraged to contact FDA to attain additional guidance.” In 2008, FDA issued a guidance entitled, “Guidance for Industry: CGMP for Phase 1 Investigational
Drugs” dated July 2008 (Ref. 4). The guidance identifies that manufacturing controls needed to achieve appropriate product quality not only between investigational and commercial manufacture, but also among the various phases of clinical trials (FDA 2016). It has also been stated that during phase 1 of the studies, the emphasis should be placed on elements to assure the safety of the subjects. This includes the control of the raw materials and drug substance, assurance, stability, and where applicable, the non-clinical safety assessments. Quality control and quality assurance should be refined as product development proceeds. Sufficient information is required in each phase of the investigation to assure proper identification, quality, purity and strength of the investigational drug, and the amount of information differs between phases. For preparing an IND, one should refer to the FDA guidance for the content and format of the INDs for Phase 1 studies entitled, “Guidance for Industry: Content and Format of Investigational New Drug Applications (INDs) for Phase 1 Studies of Drugs, Including Well-Characterized, Therapeutic, Biotechnology-derived Products” dated November 1995 (Ref. 5). Drug Substance and Drug Product section of this guidance detail the information that should be included in an IND in order to support proceeding to clinical evaluation of an LBP’s safety in human subjects (FDA 2016). There are many LBPs available on the European market, however, until now, standards have not set out in the European Pharmacopoeia (Ph. Eur.) to ensure their quality. To address this issue, the European Pharmacopoeia Commission has ratified a general monograph laying down harmonized requirements for LBPs for human use and two general chapters addressing microbiological contamination of LBPs (EUROPEAN PHARMACOPOEIA PH. EUR. COMMISSION 2019). Methods for enumeration of contaminants and for detection of specified microorganisms were described in these two chapters. In addition, these chapters provided decision diagrams describing how to establish a suitable testing method and a supplementary tool to control the quality of LBPs (EUROPEAN PHARMACOPOEIA PH. EUR. COMMISSION 2019).

As the therapeutic characteristics are strain specific, requirements include a full morphological, biochemical, serological, and molecular characterization of the strains used. The tests aim to ensure the absence of antimicrobial resistance or any other virulence factors in LBPs. Other important requirements include verification of the potency by enumeration and microbial contamination detection (EUROPEAN PHARMACOPOEIA PH. EUR. COMMISSION 2019).

1.9 – Medical application

Despite the different studies and outcomes of FMT, FDA approval in North America has not been granted. At the beginning, FMT was considered as investigational new drugs (INDs), and FDA authorization was mandatory. Currently, patients unresponsive to standard antibiotic CDI therapies can opt for FMT after completing an informed consent, where they are notified that
FMT is still under investigation. However, SERES 109 and RBX2660 have been granted the Orphan Drug designation by the FDA (Rebiotix Media 2015; Seres Therapeutics 2015). As for the EMA in Europe, the use of FMT for the treatment of CDI has not been yet regulated (van Nood et al. 2014; Lowes 2016). Yet, FMT is regularly applied to curb infections across Europe, and it is considered in clinical trials for many other pathologies. In the search for safe FMT alternatives, research on microbiotic medicinal products (MMP) is in full development and novel applications are continuously being considered. These MMP developments require novel views and strategies from the scientific world, the industry, the medical field, and the regulatory bodies. In this context, platforms like the Pharmabiotic Research Institute have been created, to facilitate discussion between different stakeholders (“Pharmabiotic Research Institute” 2017). Overall, additional research needs to be conducted before using FMT alternatives containing characterized microbial communities and next generation probiotics, to guarantee their safety and reproducible efficacy.

1.10 – Conclusion
In conclusion, FMT may be replaced with a characterized multispecies bacterial mixture that can be safer, free of allergens or viruses, and capable of treating CDI. With the current in vitro and in vivo data, next generation probiotics hold promise to treat diverse medical conditions, and they can be more effective than single or multi strains of the commercial probiotics. Microdiversification is an important process by which a bacterial population can maximize its niche coverage, and thus ubiquity, in an ecosystem through modification of its trait space. Moreover, several different strains with proven health benefits can also be considered candidates for next generation probiotics and other microbiota-based drugs. However, additional research is required for an increased understanding of the interactions among those strains, aiming at producing a successful therapeutic formulation. Research should be conducted to demonstrate whether these probiotics can be applicable to humans, as safety assessments have only been completed in animals. Effective carriage of bacterial strains in food matrices is critical for survival. Thus, optimisation of the growing conditions, and even encapsulation must be considered to promote delivery and release of the live product in the colon. The development of next generation probiotics and microbiotic medicinal products hold promise for innovation in both the food/feed sector and the pharmaceutical industry. A close interaction between academia, industry and regulatory agencies is essential for developing safe and health-promoting products, as both prophylactic and therapeutic strategies.

1.11 - Research goal and outline.
The goal of this research is to introduce a synthetic microbial community that is composed of different commensal bacteria from the human gut, and that can act as a potential therapeutic for metabolic disorders. As propionate, a major short chain fatty acid in the gut, has been
proven to have adverse beneficial effects on metabolic disorder, enhancing its production in the gut would be an interesting approach for preventing associated risk factors. The established bacterial community is validated for its propionate production after co-culturing seven different commensal bacterial strains. The research was divided into two major sections that cover the establishment, optimization, and activity experiments, and subdivided in three research chapters, as presented in Figure 1.1.

The selection of the strains was performed by considering the different metabolic pathways for propionate production (acrylate pathway, succinate pathway, and propanediol pathway) (Chapter 2). The propionate-producing consortium was prepared in a fed-batch experiment by co-culturing seven different strains from different phylogenetic origin for 48 hours. As this consortium is supposed to be administered orally in the future, it had to survive the upper GI tract, so it was tested in “the smallest intestine” (TSI) model which represents the stomach and small intestine environments. The survival of the consortium was tested through its passage in the three different compartments of the small intestine, duodenum, jejunum, and ileum, and the results were presented in Chapter 2.

As the misuse of antibiotics have been associated with disruption in the gut microbiota and leads to different diseases like metabolic disorders, it is important to find a solution that can reverse those negative effects. In Chapter 3, the effect of the designed consortium on the functionality and community composition after antibiotic induced dysbiosis was tested in an in vitro mucosal simulator for the human intestine microbial ecosystem (M-SHIME).

Finally, in the second section of the research, the activity of the propionate-producing consortium was evaluated. In Chapter 4, the in vitro enterohepatic cell model of insulin resistance was used to assess the effects of the metabolites from the propionate-producing consortium. As Akkermansia muciniphila, a propionate-producing bacterium, has been reported to improve metabolic health, the effects of its metabolites on hepatic cells have also been assessed. Different markers were measured to understand the role of the gut metabolites on the gut-liver axis in a simulated scenario of subclinical metabolic inflammation.
Figure 1.1: Overview of the research chapters in this PhD thesis

Chapter 2: 
*In vitro* Co-culture 
*In vitro* Upper GIT

Chapter 3: 
*In vitro* colon simulation

Chapter 4: 
*In vitro* enterohepatic model of hyperglycemia
This Chapter has been redrafted after

CHAPTER 2: Establishment of a propionate-producing microbial consortium and its survival in “The Smallest Intestine” *in vitro* model

2.1 - Abstract.

Propionate is a major fermentation product in the gut with several health benefits towards improving metabolic disorder. In our research, we established a preadapted propionate producing microbial consortium from seven strains of the commensal human gut bacteria prepared in fed batch experiments. We tested the survival of our designed consortium before and after preadaptation in a low volume medium-throughput *in vitro* model simulating the human small intestine and including the ileal microbiota. Samples were collected from the different compartments of the small intestine (duodenum, jejunum and ileum). RNA extraction was performed to check which bacteria were active at different stages. The propionate producing consortium was tested in both fed and fasted conditions. The stringent environmental conditions of the small intestine exert selective pressure on commensal inhabitants of members of colonic communities. As a result, only *Lactobacillus* and *Blautia* survived all the three compartments of the small intestine in the fasted condition. Our results indicated that fed conditions were harsher on the bacteria. A protection method (eg. encapsulation) should be applied to the consortium to guarantee its survival in the upper intestine until it reaches the transverse colon where it is supposed to take action.
2.2 - Introduction.

Metabolic syndrome is an emerging public health problem that has been growing in the last decades. It has been linked to shifts in the gut microbiota, and thus changes in the short chain fatty acid (SCFA) production (Festi et al. 2014). One of the major SCFAs, propionate, has been proven to play a role in reducing obesity, a major risk factor for metabolic syndrome (Kasubuchi et al. 2015). Propionate stimulates anorexigenic hormones like PYY and GLP-1, which induce satiety, and reduce energy intake leading to weight loss (Chambers et al. 2014; Arora et al. 2011).

Given the microbial association with health status and even the putative role of microbiota in modulating disease risk and/or progression, the last years of scientific research have witnessed the evolution of next generation probiotics and microbial medicinal therapies or live biotherapeutic products (El Hage et al., 2017). Next generation probiotics are often originating from human commensals, and therefore often comprise strict anaerobes. These bacteria are different from the conventional probiotics that belong to lactic acid bacteria or yeasts (El Hage et al. 2017). To exemplify, studies with Akkermansia muciniphila demonstrated beneficial effects on metabolic disorders and several other pathologies (Ploveir et al. 2017). This mucin-degrading and propiogenic bacterium was therefore proposed as a next-generation probiotic candidate (Chia et al. 2018, Plovier et al. 2017). However, the greatest hurdles that candidate probiotic strains encounter, affecting their viability, are the nature of the food matrix, the highly acidic stomach conditions, membrane-disrupting bile salts and digestive enzymes in the small intestine (Mortazavian et al. 2012). Yet, as many of the next generation probiotic products are composed of human commensal strains, there is an increased chance of survival and colonization among the endogenous gut microbiota once they reach the colon in a viable state (Mortazavian et al., 2012). Studies have reported the harsh conditions of the upper GI tract and the difficulty for different beneficial strains to survive. However, gastrointestinal behavior and colonization of supplemented (probiotic) strains in the small intestine in the presence of a residing microbiota is scarce.

The small intestine consists of three distinct sections: duodenum, jejunum and ileum (Schneeman 2002). In the duodenum, bile salts and enzymes from the pancreas are secreted to break down and solubilize lipids, carbohydrates and proteins (Riethorst el al. 2016). The duodenal concentration of bile varies from around 4 mM (fasted state) to 10 mM (fed conditions) (Riethorst et al. 2016). The jejunum and ileum are mainly responsible for the absorption of nutrients and minerals (Kiela and Ghishan 2016). The ileum is populated with a community reaching $10^6$ – $10^8$ cells g$^{-1}$ (Booijink et al. 2007). The knowledge of the composition of the small intestinal microbiota is rather scarce, but recent studies indicate that Streptococcus, Veillonella, Haemophilus, Escherichia spp. among others are commonly found.
commensals in healthy subjects (Dlugosz et al. 2015; Chung et al. 2015). As for *Lactobacillus*, its presence in the small intestine community has been controversial among different studies. Studies using human subjects have always been the gold standard, but they are invasive and tedious and mechanisms of probiotic administration cannot be monitored on real-time. Animal models have been used as an alternative to humans to mimic all physiological aspects of the GI tract, but still those models can have limitations (Cieplak et al. 2018). *In vitro* static and dynamic models for the GI tract have been developed (Venema and van den Abbeele, 2013; Guerra et al. 2012; Hur et al. 2011). For instance, the INFOGEST working group recently published a standardized static *in vitro* digestion protocol (Minekus et al. 2014), which is now widely used. Most of the complex *in vitro* models reproduce processes occurring in the small intestine such as bile salts and small nutrient absorption, dynamic changes of pH, reproduction and simulation of enzymes secretion rates and realistic transit times. Nevertheless, none of the existing models mimic the small intestine microbiota, which has an important effect on health through its ability to metabolize nutrients and influence the absorption of bioactives (El Aidy et al. 2015) as well as being involved in energy metabolism (Patrascu et al. 2017; Zoetendal et al. 2012). Cieplak et al. (2018) developed The Smallest Intestine (TSI) *in vitro* model that includes duodenum, jejunum and ileum compartments with increased throughput, including the small intestine microbiota. This *in vitro* model could be used as a screening platform for studying microbial behavior and survival during small intestine passage.

We identified the ability of the gut microbiota to produce propionate as a possible target for improving health. Our aim was to establish a propionate-producing consortium capable of yielding sufficient propionate for prevention of risk factors associated to metabolic disease. Moreover, we aimed at testing the passage and survivability of the different bacterial members of our designed propionate-producing consortium in the TSI model, under simulated fasted and fed conditions.

2.3 - Materials and methods.

2.3.1 – Selection of strains for the propionate-producing consortium (PPC)

The strains used in the propionate producing consortium were selected according to the three different reported metabolic pathways for propionate production (succinate, acrylate, and propanediol pathways) (Reichardt et al. 2014) (Figure 5.2). *Veillonella parvula*, *Bacteroides vulgatus*, and *Bacteroides thetaiotaomicron* were reported to follow the succinate pathway in which they use succinate to produce propionate. *Coprococcus catus* has been reported to participate in the acrylate pathway for propionate production, in which lactate is consumed (Louis, Hold and Flint 2014). We supplied the consortium with *Lactobacillus plantarum*, a lactic acid bacteria, which produces lactate and acetate and can support the acrylate pathway for *Coprococcus catus*. Finally, *Ruminococcus obeum* uses the propanediol pathway (Louis, Hold
and Flint 2014) fermenting fucose for propionate production. Fucose can be produced from mucin degradation, and as *Akkermansia muciniphila* is a mucin-degrader producing propionate, we used this bacterium to initiate the fucose delivery for the propanediol pathway. The single strains produced between 0.4-3 mM of propionate (Figure 2.1), and the final propionate for the consortium was 34.5 mM on average (Figure 2.2).

![Figure 2.1: Short chain fatty acids concentrations produced by the seven individual strains used to design the propionate-producing consortium. The propionate concentrations of each of the seven strains was 0.4-3 mM. Cell counts for each strain have been obtained using the flow cytometry, and they were presented above each strain in CFU/ml.](image-url)
2.3.2 – Assembly of the preadapted propionate-producing consortium

Gut commensal strains were selected based on the reported metabolic pathways for propionate production (Reichardt et al. 2014; Table 2.1). Culturing of bacteria was performed in Reinforced Clostridium Medium (Oxoid Ltd, Basingstoke, Hampshire, UK), using the Hungate tube method and under anaerobic conditions (90% N₂/ 10% CO₂). Reinforced Clostridial Medium is a commonly used, nonselective enrichment medium where various anaerobic and facultative bacteria can be grown when incubated anaerobically. This medium was used to standardize the culture procedure because it has been demonstrated that this medium is highly successful in enabling growth from small inocula, in comparison to five other media tested. In a further comparison, the highest viable count obtainable was the criterion used, and again, RCM proved superior. All strains were incubated at 37°C for 48 h except for *L. plantarum*, which was incubated for 24 hours. All strains were collected for preadaptation when in stationary phase. At the end of the incubation, cell number was measured using flow cytometry, and standardised to 10⁸ cells ml⁻¹ (Hernandez-Sanabria et al. 2017).

In order to adapt the different bacterial strains to representative conditions of the human gut, the single strains were mixed and subjected to simulated colon conditions. Culturing medium
from the SHIME® model (UGent/Prodigest NV, Zwijnaarde, Belgium) was subjected to pre-digestion simulating the passage through the upper GI tract. Gastric digestion was mimicked by maintaining pH 2 for 2 h, followed by an intestinal digestion through addition of pancreatic juice [0.9 g L⁻¹ pancreatin (Sigma-Aldrich, St. Louis, USA), 6 g L⁻¹ oxgall bile salt (BD, Erembodegem, Belgium), and 25 g L⁻¹ NaHCO₃ (Carl Roth GmbH, Karlsruhe, Germany)]. pH was adjusted to 6.8, and medium was incubated for 2.5 h at 37⁰C to simulate the small intestine digestion. If pH had shifted at the end of the incubation, pH was again adjusted to 6.8 prior to addition of the bacterial cocktail.

Then, 5 ml of each strain were mixed under anaerobic conditions. The consortium was prepared by transferring 5 ml of the bacterial cocktail (10% v/v) to an anaerobic glass bottle containing 45 ml of pre-digested medium. Co-culturing occurred for 48 h, and 40% of the medium was replaced after 24h. The consortium was harvested after 48 hours, when the average propionate production was 34.5 mM (Figure 2.2). The viable cell count of the consortium was 10⁸ cfu mL⁻¹ when administered to the M-SHIME. The seven strains were cultured together for 48 hours in order to form the preadapted bacterial consortium in which they would act as a community after they have done the cross-feeding together.

Table 2.1: Bacterial strains used to prepare the propionate-producing consortium according to the different metabolic pathways for propionate production (Acrylate, Succinate, Propanediol).

<table>
<thead>
<tr>
<th>Bacterial species</th>
<th>Source</th>
<th>Pathway for propionate production</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacteroides vulgatus</td>
<td>LMG17767</td>
<td>Succinate pathway</td>
</tr>
<tr>
<td>Bacteroides thetaiotaomicron</td>
<td>DSM 2079</td>
<td>Succinate pathway</td>
</tr>
<tr>
<td>Coprococcus catus</td>
<td>ATCC 27761</td>
<td>Acrylate pathway</td>
</tr>
<tr>
<td>Veillonella parvula</td>
<td>DSM 2007</td>
<td>Succinate pathway</td>
</tr>
<tr>
<td>Ruminococcus obeum (Blautia)</td>
<td>DSM 25238</td>
<td>Propanediol pathway</td>
</tr>
<tr>
<td>Akkermansia muciniphila</td>
<td>DSM 22959</td>
<td>Propanediol pathway</td>
</tr>
<tr>
<td>Lactobacillus plantarum</td>
<td>LMG 9211</td>
<td>Acrylate pathway</td>
</tr>
</tbody>
</table>

2.3.3 - Assembly of freshly mixed bacterial cocktail
Similar to the preparation of the PPC, the same strains were cultured in Hungate tubes containing 10 ml of RCM under the same conditions. Then, 5 ml of each strain were mixed as described above.
2.3.4 - Difference between a preadapted and a freshly mixed bacteria cocktail
As described above, the preadapted bacterial consortium is prepared after growing each of the strains separately and co-culturing them in a new medium with the SHIME feed for 48 hours. During these 48 hours, the feed was replaced once a day, and the bacteria were cross-feeding to produce propionate. After the 48 hours, the consortium was harvested as a preadapted community in which all seven strains got adapted to each other during the fed batch. Preadaptation was important as it mimics the concept of a preadapted bacterial community in a fecal inoculum in which the bacteria were already fermenting together and formed a community. The freshly mixed consortium is the mixture of all the seven strains in which each strain was cultured separately. These seven were not co-cultured with each other, and in this context they do not form a community that has been preadapted. In fact, the freshly mixed consortium was used as the inoculum to start the fed batch that lead to the preadapted community.

2.3.5 - Semi-continuous incubation of the bacterial consortium
Bacterial consortium was inoculated in a total volume of 50 ml of SHIME nutritional medium (10 vol%) and incubated at 37°C. Feed replacement was performed twice a day (40% v/v), first exchange was completed before 16 h to prevent a strong pH drop and the next replacing was done after 24h. Oxygen was displaced from needles and syringes using nitrogen gas, to preserve the anaerobic atmosphere.

2.3.6 – Optimizing the semi-continuous fermentation for enhanced propionate production
Duration of the incubation for reaching the maximum amount of short chain fatty acids produced by our bacteria in SHIME medium was optimized. We ran the semi-continuous incubation for 10 days, and samples were collected twice a day in the morning and evening, and feed was replaced twice a day as well. Short chain fatty acids were determined from all samples to verify the exact time point at which SCFA production was stable and reached the maximum concentration. We concluded that the semi-continuous batch can run for 5 days and that the maximum amount of SCFAs was produced at day 4 and stabilized between days 5 and 7. However, since our fed batch experiments were inoculated only with our designed bacterial consortium of seven strains, this would not cause a drastic drop in pH after 16 hours as in the case of a fecal inoculum. For this reason, we validated the propionate production by our consortium by performing triplicate fed-batch incubations for 5 days, and replacing the feed only once a day. We sampled every 24 hours and measured the SCFAs produced during a 5-day incubation period. Our results indicated that the maximum production of SCFAs was obtained after 48 hours. Cell number and integrity was confirmed with a benchtop Accuri C6 cytometer (BD Biosciences, Erembodegem, Belgium). Samples were diluted and stained with 10 ul/ml combination of SYBR green I and propidium iodide (SGPI) as a viability indicator. The
samples were then measured in the flow cytometer as described by Hernandez-Sanabria et al. (2017).

2.3.7 – PCR-DGGE analysis of the propionate producing consortium
PCR amplifications of the V2-V3 region (200 bp) of the 16S rRNA gene of bacteria were performed with universal bacterial primers 338F-GC and 518R using the program outlined by Boon et al. (2003). PCR products were subjected to Denaturing Gradient Gel Electrophoresis (DGGE) using the DCode universal mutation detection system (Bio-Rad Laboratories, Inc., Hercules, CA). The procedure for DGGE was described by Hernandez-Sanabria et al. (2010). To effectively assign the band positions for each gel, a common ladder was included in each gel as an internal control. The ladder was generated by mixing PCR products of each of the single strains from the consortium, following the protocol described in Hernandez-Sanabria et al. (2010).

2.3.8– Selection and growth of the strains for the small intestine microbiota
The selection of the small intestine microbiota and the assembly of the TSI were developed by Cieplak et al. (2018). Seven bacterial strains representing prominent members of the ileal microbiota were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ) and mentioned by Cieplak et al. (2018). All strains selected were cultured anaerobically at 37°C in Gifu Anaerobic Medium (GAM Broth, NISSUI) and stored at -80 °C until use (Cieplak et al. 2018). Gifu Anaerobic Medium (GAM) is recommended as a general culture medium for cultivation and isolation of anaerobic bacteria. Prior to the experiments each strain from the small intestine microbiota was inoculated in 10 ml GAM broth and cultured in 37°C for the time appropriate for each strain (Cieplak et al. 2018). After strain growth, the tubes were centrifuged (5,000 g for 2 min), the supernatant was discarded, and the pellet was re-suspended in the volume of phosphate buffered saline (pH 7.4) appropriate to obtain a suspension of 10^8 CFU ml^-1 according to growth curves developed for each strain (OD vs. CFU ml^-1). All strains were mixed together before addition to the reactor (Cieplak et al. 2018).

2.3.9 – Assembly of The Smallest Intestine in vitro model
The Smallest Intestine in vitro model (TSI) simulates the passage through the human small intestine by an adjustment of pH and concentration of bile salts, pancreatic enzymes and dialysis to mimic absorption. Each TSI unit consist of 5 reactors, each with a working volume of 12 ml. Cieplek et al. (2018) depicts the experimental flow in which they display the system simulating passage of duodenum, jejunum and ileum. Parts of the model (outer cabinet, temperature and pH control) were adapted from the design of the recently developed CoMiniGut in vitro colon model (Wiese et al. 2018). During simulated small intestinal passage, temperature, pH levels and bile salt concentrations are controlled at physiologically relevant levels. The main unit of TSI is a composite climate box where temperature is controlled (37°C)
by flow of water from a circulating water bath (A10/AC150, ThermoScientific) through a copper/aluminium heat exchanger placed inside of the box. An external temperature sensor connected with the water bath and placed inside of the main unit constantly monitors the temperature. During the whole experiment, temperature was recorded by an independent temperature data logger (Temp 101A MadgeTech). Each box contained five stirred fused quartz glass reactors (AdValue Technology, USA), where each one represented the passage through the small intestine of one individual. Reactors were separately closed in PVC chambers and anaerobic conditions were achieved by using anaerobic sachets (Oxoid AnaeroGen, ThermoScientific). Anaerobic conditions were verified by color change of Oxoid Anaerobic Indicator (BR0055, Oxoid), which is impregnated with a resazurin redox indicator solution. Reactors were placed on a 5-unit magnetic stirrer plate (R05, IKA), which assured equal mixing of samples inside of the reactors. The reactors were closed with a septa lid (GR-2 rubber, Sigma-Aldrich) containing a pH probe inlet and needle inlets for the supply of enzymes and bile salts, as well as sampling, input and output for the dialysis chamber. Bile is absorbed by a dialysis process, which takes place in dialysis cassettes (Slide-A-Lyser G2, ThermoScientific). The dialysis chamber was filled with dialysis fluid (Milli-q water). A multichannel peristaltic pump (205S, Watson-Marlow) was connected to ensure constant flow of the chyme between the reactor and the dialysis cassettes (2.64 ml min-1). Beakers were stirred continuously (170 rpm) to facilitate effective dialysis. In addition, the dialysis process simulated the absorption of small nutrients and electrolytes from the chyme with a size smaller than the cut-off of the dialysis cassette specified as 10 kDa. The pH in each module was measured every 20 seconds using SP28X (Consort) pH electrodes. The reading was sent to the computer that adjusted the pH through acid and base pumps according to the pre-set pH value. In the TSI model, pH was controlled with 0.1M NaOH according to the pre-programmed experimental setup using syringe pumps (NE-500, WPI). The syringe pumps were automatically controlled by a computer using in-house made scripts in the 162 Matlab software (ver. R2015a, TheMathWorks, Inc.).

To simulate the electrolyte composition and osmotic pressure occurring in the human GIT - Simulated Gastric Fluid (SGF) and Simulated Intestinal Fluid (SIF) were used (Minekus et al. 2014). The SIF composition was modified by removal of sodium bicarbonate to allow more precise pH control during experiments. The TSI reactor was prepared by adding 4.1 ml of SGF, 3 ml or 4.5 ml of SIF depending if the experiment was conducted as simulating fed or fasted state, and 10 μl of 0.6M CaCl2. To simulate the duodenum, pancreatic juice, bile solution and feed/water were added. Pancreatic juice was added as a mixture (100 mg ml-1) of SIF and 8xUSP pancreatin from porcine pancreas (Sigma-Aldrich). This provides an enzyme composition close to what is found in humans (Minekus et al. 2014). The amount of pancreatic juice added was established according to the activity of trypsin which was at a level of 100 U
ml-1 in fed conditions (McConnell et al. 2008) and 40 U ml-1 in fasted state. A stock of 80 g l-1 was prepared and the exact concentration of bile salts was determined using a commercially available kit (Total Bile Acids Assay, Diazyme). To simulate conditions after ingestion of a meal, “fed state” was mimicked by the presence of 10 mM bile salts in the reactor and with addition of 1.4 ml of food replacement (Nutrison Energy Multi Fibre, Nutricia) as a source of nutrients (Cieplak et al. 2018). Small intestinal passage without the presence of food components was simulated by a “fasted state” by presence of 4 mM bile acids and 1.4 ml of water. Finally, pH was adjusted (by syringe) to 6.5 by titrating with 1 M NaOH (Cieplak et al. 2018). During the duodenal passage (2 hours) pH was elevated from 6.5 to 6.8 in steady increments (Cieplak et al. 2018). Next, the absorption of small nutrients and bile salts in the jejunum was simulated by continuously pumping the samples through the dialysis chambers with a 10 kDa membrane cut-off at a pumping rate of 2.64 ml min-1 for 4 hours, as specified above. During the 4-hour simulation of the jejunal passage pH was increased from 6.8 to 7.2. To simulate ileum, fresh SIF with a pH of 7.2 was added to the reactor to obtain a chyme to SIF ratio of 50:40 (v/v). Moreover, 1 ml of small intestine microbiota inoculum was added. The inoculum was adjusted to obtain 10^7 CFU ml-1 in the reactor. During simulated ileal passage (2 hours) pH was kept constant at 7.2 (Cieplak et al. 2018).

The amount of base used to control pH was constantly recorded in order to compare inter and intra-variability between samples and experimental conditions (Cieplak et al. 2018). An illustration of the TSI model had been presented by Cieplak et al. (2018).

2.3.10 - Survival of single strains of the propionate-producing consortium in the TSI
Each strain (Table 2.1) was inoculated in 10 ml Reinforced Clostridium medium (RCM) broth medium and cultured for 24-48 hours. Cells were harvested by centrifugation (10,000 g for 2 min) after which the supernatant was discarded and the pellets resuspended in 10 ml of phosphate buffered saline (pH 7.4). The bacterial solution was plated on Gifu Anaerobic medium (GAM) plates to confirm the concentration at time zero. Four reactors were inoculated with 0.5 ml of the bacterial solution, while the fifth was kept to monitor contamination of any of the compartments used along the experiment. When checking the survival of the single strains, the small intestine microbiota was not added to the TSI. Survival was evaluated after the period of simulated digestion in the duodenum and ileum compartments via colony plate counts on GAM agar medium (37°C, 24-48 hours incubations at anaerobic conditions) after serial dilution using a saline solution (0.9% NaCl in water). Experiments were conducted separately for each strain in the fasted state.

2.3.11 - Survival of the propionate-producing consortium in the TSI
Preadapted propionate producing consortium and the freshly mixed bacterial cocktail were separately inoculated in the TSI in the fasted and fed conditions. Samples (0.5 ml) were collected at the end of each compartment (duodenum, jejunum, and ileum) and RNA protect
(Qiagen, Venlo, The Netherlands) was added following the manufacturer’s instructions and stored at -80°C for later RNA extraction with the RNeasy Mini Kit (Qiagen, Venlo, The Netherlands). Presence of transcriptionally active bacteria was assessed by transcribing the RNA using the Qiagen kit and performing 16S rRNA amplicon sequencing of the cDNA libraries using the Mi-Seq Illumina platform. Thus, we confirmed which of the strains of our consortium successfully survived the small intestine passage and stayed transcriptionally active. Each experiment was performed in duplicates (n = 2). Only cDNA sequencing was used in this experiment to determine the active strains. Flow cytometry analysis was not used in this experiment due to unavailability of a flowcytometer in the lab where in the experiment took place.

2.3.12 - Community composition and dynamics

Sequencing data was imported into R using phyloseq (McMurdie and Holmes 2013), and taxon abundances were rescaled by calculating the taxon proportions and multiplying them by the minimum sample size (n = 77893) present in the data set (McMurdie and Holmes 2014). Alpha diversity was estimated within each sample using inverse Simpson index. Pielou index was used as indicator of evenness in the community (Grunert et al. 2016). Differences in alpha diversity and evenness measures among compartments and between preadapted and fresh mix were compared using a repeated measures mixed model in GraphPad (GraphPad Prism 7.04, La Jolla, CA, USA). Comparisons between fasted and fed condition were performed using a 2-way Anova (Sidak’s method) and between different compartments using Tukey’s method (Annex I, Table S1 and S2).

Beta diversity estimates based on Chao and Bray-Curtis indices were used to examine dissimilarity and determine the impact of compartment on microbial community structure. Principal Coordinate Analysis (PCoA) was employed to visualize the differences among samples, using the vegan package in R (Oksanen et al. 2007) (Annex I, Figure S1). Stratified permutational multivariate analysis of variance (PERMANOVA) with 999 permutations was conducted to indicate the significance of condition and compartment on the microbial community differences.

2.4 - Results.

The main objective of this study was to test the survival of the bacterial strains composing the propionate-producing consortium in the TSI model under fasted and fed conditions. When reporting the results, we could see that some strains that do not belong to the consortium appeared suggesting the presence of contamination in the TSI model. The relative abundance of the strains resulting from contamination was not reported in our results.
2.4.1 - Optimization of the preadapted propionate-producing consortium
The consortium was harvested after 48 hours, when the average propionate concentration was 34.5 mM (Figure 2.2). Cell count was measured using flow cytometry at the end of the incubation, and standardised to $10^8$ cells ml$^{-1}$ (Figure 2.3). The PCR-DGGE results revealed that the DNA for all the strains was present in the samples from the preadapted propionate-producing consortium after 48 hours (Figure 2.4).

Figure 2.3: Flow cytometry revealed that the number of intact cells from the designed preadapted propionate-producing consortium is $10^8$ cfu mL$^{-1}$ (cfu g$^{-1}$).
Figure 2.4: Denaturing Gradient Gel Electrophoresis (DGGE) reveals the bands of each of the seven single strains in the preadapted propionate-producing consortium after generating the ladder belonging to the consortium. The last four columns are presenting the replicates from the PPC after the seven strains had been preadapted together. The samples from PPC show the bands from the different strains included in the consortium.

2.4.2- Feeding conditions affect survival of single strains in the TSI model
The survival of the seven strains involved in the propionate-producing consortium was tested in TSI model in two different feeding conditions: conditions mimicking a “fasted” small intestine (i.e. before food ingestion; bile salts = 4 mM; pancreatic juice = 40 U ml⁻¹) and a “fed” small intestine (i.e. after a meal; bile salts = 10 mM; pancreatic juice = 100 U ml⁻¹). The influence of the small intestinal microbiota consortium was tested in both feeding states. High concentrations of bile salts in the fed condition fostered harsh environment and differential survival of the different consortium members. Thus, survival of single strains was only tested under the fasted condition (Figure 2.5). Samples were collected before and after duodenal and after ileal passage. Plate cell counts revealed that *R. obeum* and *C. catus* did not grow before being introduced to the TSI model and did not survive the passage through any of the compartments of the TSI. *L. plantarum* and *B. thetaiotaomicron* were able to grow before introducing them to the TSI, and survived the duodenum with cell count of $2.45 \times 10^9$ and
1.74×10^9 cfu mL\(^{-1}\) (cfu g\(^{-1}\)) respectively, and through the ileum with cell count of 1.36×10^{10} and 2.62×10^8 cfu mL\(^{-1}\) (cfu g\(^{-1}\)) respectively. \textit{V. parvula, B. vulgatus,} and \textit{A. muciniphila} grew before administering to the TSI and survived duodenum compartment with cell counts of 2.66×10^8, 5.46×10^6, and 2.21×10^5 cfu mL\(^{-1}\) (cfu g\(^{-1}\)) respectively.

**Figure 2.5:** Survival of the seven strains after duodenum and ileum compartments during the passage in the simulated small intestine in the fasted state. Survival was evaluated by obtaining bacterial cell counts via colony plate counts after incubation period in each of duodenum and ileum compartments.

2.4.3- Fasted conditions present lower stress to the propionate-producing consortium than the fed conditions

At time 0, the abundant genera of the fresh mix inoculum included \textit{Lactobacillus, Bacteroides, Blautia, Clostridium,} and \textit{Veillonella} with \textit{Lactobacillus, Bacteroides,} and \textit{Blautia} having the highest relative abundances. Sequencing analysis revealed the active presence of additional strains in duodenum and jejunum, besides those initially introduced in the fresh mix (Figure 2.6). The presence of these additional genera indicate that additional colon commensals such as \textit{Clostridium sp.} were capable of invading the TSI model, as their spore-forming capacity may have enabled them to endure the stringent conditions of the small intestine and further colonise this environment. As the aim of this experiment was to test the survival of the active genera included in our propionate-producing consortium, all the results reported will only include the relative abundance of the genera included in our consortium. After the duodenum compartment, the relative abundance of \textit{Lactobacillus} and \textit{Veillonella} increased in both fasted
and fed conditions, while the relative abundance of *Blautia* remained higher in the fasted condition than in the fed. The relative abundance for *Bacteroides* decreased in both the fasted and fed conditions. In the jejunum compartment, we observed a decrease in most of the genera, where only *Lactobacillus* and *Blautia* appeared in the fed condition with *Lactobacillus* being the most dominant. As for the fasted state, there was a decrease in *Blautia* and *Bacteroides* and an increase in *Lactobacillus*. In the ileum compartment, we observed loss of most of the bacterial genera included in our fresh mix, and there was an increase in the relative abundance of the genera included in the ileal bacterial community like *Enterococcus*, *Enterobacteriacea*, and *Streptococcus* in both fasted and fed conditions. The relative abundance of genera related to the ileal community was higher in the fasted condition while the relative abundance of *Lactobacillus* decreased in this case, and the presence of *Blautia* was detected in this condition. *Blautia* disappeared in the fed condition, and only two genera related to the ileal bacterial community were observed: *Enterococcus* and *Enterobacteriacea* in addition to *Lactobacillus* from our consortium with *Lactobacillus* having the highest relative abundances.

According to the cDNA sequencing results, relative abundance plot revealed that more genera of our preadapted consortium were abundant in duodenum and jejunum compared to the ileum compartment (Figure 2.6). At time 0, the abundant genera of the preadapted consortium inoculum included *Lactobacillus* and *Bacteroides*, with higher relative abundance of *Lactobacillus*. After the duodenum compartment, the relative abundance of *Bacteroides* decreased in both fasted and fed conditions and the relative abundance for *Lactobacillus* increased in both fed and fasted conditions. In the jejunum compartment, we observed a decrease in *Bacteroides* and increase in *Lactobacillus* in the fasted condition. As for the fed state, there was loss of *Bacteroides* and increase in the relative abundance of *Lactobacillus*. In the ileum compartment, we observed loss of *Bacteroides* in fasted condition and dominant relative abundance of the genera included in the ileal bacterial community like *Enterococcus*, *Enterobacteriacea*, and *Streptococcus*. As for the fed condition in the ileum, the relative abundance of *Lactobacillus* was the most dominant, in addition to an increased relative abundance of *Enterococcus* and *Enterobacteriacea* genera related to the introduced ileal community.
Figure 2.6: Relative bacterial abundances of the transcriptionally active bacteria in different compartments of TSI (Inoculum: before entering the TSI, duodenum, jejunum, and ileum) and in fasted and fed conditions were determined after sequencing of cDNA. Different genera with the different relative abundances were observed in fresh mix and preadapted bacterial consortium. Two replicates per condition were averaged to get the results in each compartment.

2.4.4 - Community characteristics of the propionate-producing consortium changed among the different compartments of the TSI. Alpha diversity was significantly different between the community inoculated at time 0 and after the duodenum transit under fed conditions ($P<0.05$). There was a significant difference in the alpha diversity between the preadapted inoculum and duodenum in the fasted condition ($P<0.05$) (Figure 2.7) (Annex I, Table S1).
Figure 2.7: Differences in alpha diversity between the different compartments (Inoculum: before entering the TSI, duodenum, jejunum, and ileum) for Fresh mix and Preadapted bacterial consortium in both fasted and fed conditions.

There was a significant difference in evenness between the preadapted inoculum at time 0 and when the preadapted consortium reached the duodenum in the fasted condition ($P<0.05$). However, there was no significant difference in the evenness between the different compartments and conditions for the fresh mix ($P>0.05$) (Figure 2.8) (Annex I, Table S2).
Figure 2.8: Differences in evenness between the different compartments (Inoculum: before entering the TSI, duodenum, jejunum, and ileum) for Fresh mix and Preadapted bacterial consortium in both fasted and fed conditions.

2.5 - Discussion.

Several natural barriers challenge the viability of probiotics during their passage through the upper GI tract, from the stomach acidity to the length of exposure to bile salts, the level of bile salt activity, presence of oxygen, and other environmental characteristics (Bezkorovainy 2001; Lee and Salminen 2009). Bile salts are the most challenging obstacle in the small intestine (Bezkorovainy 2001). The most ideal case for a probiotic is to survive the passage through the GI tract and launch itself in the small intestine and the colon and exert its functionality that eventually results in its putative health benefits (Bezkorovainy 2001). Therefore, one of the crucial properties of the probiotic bacteria is the bile tolerance, since it determines the ability of the bacteria to survive the small intestine (Ruiz, Margolles and Sánchez 2013).

Although there is no common agreement on the minimum satisfactory level of viable probiotic cells per gram or millilitre of probiotic product, the concentrations of $10^6$ and $10^7$-$10^8$ cfu mL$^{-1}$ (cfu g$^{-1}$), have been generally acceptable. Our *in vitro* experiments using the TSI model showed that many variables can determine the degree to which bacteria survive passage through the upper gastrointestinal tract (Corcoran et al. 2005).
When testing the survival of the single strains of our consortium, we observed that *L. plantarum* could survive the duodenum and the ileum with a $1.36 \times 10^{10} \text{ cfu mL}^{-1} \text{ (cfu g}^{-1})$. *Lactobacillus* is intrinsically resistant to acid environments (Corcoran et al. 2005) and bile salts (Ruiz, Margolles and Sánchez 2013). Moreover, *L. plantarum* produces bile salt hydrolase enzyme which can also explain its survival through the different small intestine compartments (Begley, Hill and Gahan 2006). Additionally, *B. thetaiotaomicron* survived the duodenum and ileum compartments with $2.62 \times 10^8 \text{ cfu mL}^{-1} \text{ (cfu g}^{-1})$. *B. thetaiotaomicron* possesses selective bile-salt hydrolase (BSH) activity (Yao et al. 2018). BSHs are crucial in opposing the toxic effect of the glycoconjugated bile salts at low pH, and they are also important for having slight acidic pH optima (Begley, Hill and Gahan 2006). They are able to optimize the pH when bile enters the duodenum leading to acid reflux from the stomach or in specific microenvironments in the intestines when lactic acid bacteria cause a drop in the pH (Begley, Hill and Gahan 2006). Moreover, BSH-active strains may detoxify unconjugated bile acids (De Smet et al. 1995), or they could associate with 7- dehydroxylating bacteria that would dehydroxylate unconjugated bile acids (De Boever and Verstraete 1999; Begley, Hill and Gahan 2006). As a result, BSH activity can take a role in the survival and colonization of the strains within the GI tract (Moser and Savage 2001).

As for *V. parvula*, *B. vulgatus*, and *A. muciniphila*, we observed that those three strains survived the duodenum but did not survive the ileum compartment. Long-term exposure to bile salts through jejunum and ileum compartments may lead to strain death. Nevertheless, *Veillonella* sp. can hydrolyze conjugated bile salts (Wei et al. 2013) and *B. vulgatus* possesses BSH activity (Elkins and Savage 1998; Kawamoto et al. 1989). Therefore, oxygen toxicity may indeed play a role in viability and activity when the strains are tested in the suitable culture medium and at the optimal temperature (37°C) (Talwalkar and Kailasapathy 2004). However, the impact of oxygen on cell viability of probiotic bacteria is inconclusive (Talwalkar and Kailasapathy 2004). The abundance of *A. muciniphila* in presence of bile was positively correlated to circulating primary bile in mice (Pierre et al. 2016), and the addition of porcine bile extract in its growth medium led to increased growth (van der Ark et al. 2017). In contrast, addition of purified bile salts resulted in inhibited growth of *A. muciniphila* (van der Ark et al. 2018; Geerlings et al. 2018), which was not observed in our study.

pH values and different bile concentrations should be tested for several times for each strain to determine its survival in those conditions, and the results for viability are species and strain dependent (Lee and Salminen 2009). Cross-resistance between low pH and bile salts has been reported in some bile-adapted strains (Noriega et al. 2004) (Duwat et al. 2000). For instance, the strain may survive the bile in the duodenum when the stress response is already induced. Indeed, survival of candidate isolates can be guaranteed if tolerance, rather than direct resistance is observed (O’Sullivan 2006; Mortazavian et al. 2012).
Lower relative abundance and loss of some strains from the duodenum to the ileum under fed condition may be related to the higher concentration of bile salts during the fed condition. *Blautia* was persistent while *Bacteroides* decreased in all compartments in the case of the fresh mix in the fasted condition, probably because high levels of bile salts favour the proliferation of Gram-positive bacteria and reduction of the Gram-negative *Bacteroides* (Urdaneta and Casadesús 2017). However, the only genus that was consistently appearing was *Lactobacillus*, a facultative anaerobe resistant to bile salts. Moreover, the genera related to the ileal community were appearing in the ileum compartment since it was just introduced there as described by Cieplak et al. (2018) and did not have to pass through the duodenum and jejunum.

In addition, sequencing results revealed that only *Lactobacillus* and *Bacteroides* were abundant in the preadapted consortium at time 0, while other genera of the consortium were not active. This could suggest that the optimal propionate production (34.5mM) was achieved with only these two genera of the preadapted consortium being active after the cross-feeding and interacting during the 48 hours of incubation. In the fresh mix, *Bacteroides* decreased in the duodenum compartment, suggesting that we might not achieve the same amount of propionate production if the strains were supplied as a fresh bacterial cocktail. These observations propose the importance of having a protectant carrier to ensure the survival of the preadapted consortium in the upper GI tract.

This study highlights the importance of standardised administration guidelines for biotherapeutics. Our bacterial consortium was likely to have increased viability when administered under fasted conditions, which was also suggested in previous studies (Fredua-Agyeman and Gaisford, 2015). Microencapsulation has been applied to increase the survival of probiotics in food products like yogurts and other dairy products through protection from lyophilisation and rehydration, and from the acidity of the product (Dinakar and Mistry 1994; Kebary et al. 1998; Shah 2000; Talwalkar and Kailasapathy 2004). However, even if viability was ensured during the product formulation, it is crucial that live bacteria survive the upper GI tract (Mortazavian et al. 2012). Microencapsulation has been reported to protect probiotics from the detrimental factors during the passage in the GI tract like low PH (Sun and Griffiths 2000) bile salts (Lee and Heo 2000), and molecular oxygen in case of obligatory anaerobic microorganisms (Mortazavian et al. 2012).

Sufficient viable numbers must survive the upper GIT and reach the host colon to get beneficial effects (Bosnea et al. 2009). For this reason, *in vitro* experiments are essential to demonstrate their potency before piloting expensive and complex experiments (Millette et al. 2013). However, presence of food matrix (Millette et al. 2013) and interindividual differences in bile production and different foods can lead to contradictory outcomes (Millette et al. 2013). For instance, Begley et al. (2005) reported that bile acid levels in the intestine are not constant,
and they are relatively low until a fatty meal is ingested. The survival of bacteria is affected by the presence of food in the intestine as bacteria may not be exposed to bile in certain microenvironments created by the food matrix, or food constituents can even bind bile acids and prevent them from exerting toxicity. Furthermore, the tolerance of strains in bile broth in vitro systems may not truly imitate their ability to tolerate bile in vivo. Like any other physiological stresses, it is challenging to mimic exact in vivo conditions in a laboratory setting especially that all parameters that can affect survival are not being taken into account (Begley, Gahan and Hill 2005).

2.6 - Conclusions
The in vitro model revealed that a protectant is needed to help the consortium to reach the colon. Similar to probiotics, our propionate-producing consortium would be administrated orally and has to survive the passage through the harsh environment of the upper GIT and further exert its positive effect on the host. Microencapsulation may be an alternative to ensure integrity of the biotherapeutic, and future work is essential for establishing dosing in terms of frequency and viable cell number. In addition, presence of food matrix may impact the characteristics of live consortia, highlighting the urgency of optimising production pipelines for commercial and next-generation biotherapeutics.

2.7- Acknowledgements
The authors would like to thank the People Program (Marie Curie Actions) of the European Union’s Seventh Framework Program FP7/2007–2013/under REA grant agreement n_606713 for the research funding. The authors would also like to thank Basheer Yousef Aideh for his technical assistance during labwork at the University of Copenhagen.
Chapter 3

Propionate-producing consortium restores antibiotic-induced dysbiosis in a dynamic in vitro model of the human intestinal microbial ecosystem

This Chapter has been redrafted after

CHAPTER 3 - Propionate-producing consortium restores antibiotic-induced dysbiosis in a dynamic *in vitro* model of the human intestinal microbial ecosystem

3.1 - Abstract.

Metabolic syndrome is a growing public health concern. Efforts at searching for links with the gut microbiome have revealed that propionate is a major fermentation product in the gut with several health benefits towards energy homeostasis. For instance, propionate stimulates satiety-inducing hormones, leading to lower energy intake and reducing weight gain and associated risk factors. In (disease) scenarios where microbial dysbiosis is apparent, gut microbial production of propionate may be decreased. Here, we investigated the effect of a propionogenic bacterial consortium composed of *Lactobacillus plantarum*, *Bacteroides thetaiotaomicron*, *Ruminococcus obeum*, *Coprococcus catus*, *Bacteroides vulgatus*, *Akkermansia muciniphila*, and *Veillonella parvula* for its potential to restore *in vitro* propionate concentrations upon antibiotic-induced microbial dysbiosis. Using the mucosal simulator of the human intestinal microbial ecosystem (M-SHIME), we challenged the simulated colon microbiome with clindamycin. Addition of the propionogenic consortium resulted in successful colonization and subsequent restoration of propionate levels, while a positive effect on the mitochondrial membrane potential ($\Delta \Psi_m$) was observed in comparison with the controls. Our results support the development and application of next generation probiotics, which are composed of multiple bacterial strains with diverse functionality and phylogenetic background.
3.2 - Introduction

The human gut plays a major role in nutrition, metabolism, pathogen resistance, and regulation of immune response (Dethlefsen et al. 2008; Turroni et al. 2012). Microbial fermentation processes in the gut leading to production of short chain fatty acids (SCFA), are the result of metabolic interactions between different gut species (Dethlefsen et al. 2008). The main SCFAs (acetate, propionate, and butyrate) (McOrist et al. 2008) perform important physiological functions (Hosseini et al. 2011), occur in molar ratio of 3:1:1 in the colon (Hosseini et al. 2011) and are used by the microbiota for growth and maintenance of cellular functions of bacteria (Fernandes et al. 2014). Acetate is absorbed and transported to the liver for cholesterol and fatty acid synthesis in the host, playing a major role in enhancing ileal motility (Hosseini et al. 2011), while butyrate is the key energy source for colonocytes. Butyrate prevents proliferation of cancerous cells and stimulates differentiation of colon epithelial cells (Hosseini et al. 2011). The health effects of propionate go beyond the gut epithelium, as it lowers serum cholesterol levels, lipogenesis, and carcinogenesis risk (Fernandes et al. 2014). Propionate may also decrease obesity by promoting the secretion of PYY and GLP-1 hormones from human colonic cells (Chambers et al. 2014; Psichas et al. 2014; Morrison and Preston 2016; Tolhurst et al. 2011), inducing satiety and subsequently reducing energy intake and promoting weight loss (Arora et al. 2011). Propionate is a particularly interesting metabolite in the context of the aetiology and progress of metabolic disorder, which is becoming a public health issue. Metabolic syndrome is defined as a cluster of different biological conditions or metabolic disorders characterized by obesity, dyslipidemia, and type 2 diabetes (Alberti et al. 2009; Halcox and Quyyumi 2005; Moore et al. 2017; Nolan et al. 2017). This syndrome is linked to different comorbidities like cardiovascular disease, non-alcoholic fatty liver, arthritis, chronic kidney disease, and several types of cancers (Halcox and Quyyumi 2005; Moore et al. 2017; Dugas et al. 2016). Recent studies have reported an association between gut microbiota and metabolic syndrome, as the gut composition differs between healthy and diseased individuals. The gut microbiota is responsible for producing different regulatory peptide hormones (Silva and Bloom 2012), depending on the nutrient supply provided, as their interaction with receptors at different points in the gut-brain axis leads to satiety (Silva and Bloom 2012). Propionate has been reported to have the highest affinity for the free fatty acid receptor 2 (FFAR 2), involved in the regulation of metabolic homeostasis. In fact, long-term propionate delivery in the gut stimulates anorexigenic gut hormones, reducing intra-abdominal fat accretion, intrahepatocellular lipid content, and hepatic cholesterol synthesis in humans (Chambers et al. 2014; Arora et al. 2011). In addition, propionate is involved in activation of intestinal gluconeogenesis (IGN), thus regulating food intake and enhancing insulin sensitivity (Li et al. 2017).
As propionate production is associated with gut microbiome composition and functionality, different modulators such as antibiotics, prebiotics, and probiotics (El Hage et al. 2017) can impact this metabolite. Antibiotics may foster pathogenic opportunistic bacteria (Francino 2016), influencing human health (Jin et al. 2016). Long-term antibiotic use can lead to increased body mass index and weight gain in both humans and farm animals (Francino 2016), alter transcription of genes involved in liver lipid metabolism (Jin et al. 2016), increase insulin resistance, and steatosis in patients with fatty liver (Miquilena-Colina et al. 2011). Antibiotic use has also been reported to cause bacterial translocation, which may represent an additional inflammatory stimulus potentially promoting obesity (Knoop et al. 2015). Hence, antibiotic use is considered a risk factor for metabolic disorders (Francino 2016; Economopoulos et al. 2016). For instance, the Cl atom present in the clindamycin disrupts the mitochondrial membrane potential (Goldhill et al. 1996); similar disruption has been reported following oxidative damage in metabolic syndrome (Nicolson 2007). Thus, we applied clindamycin as an agent to simulate the conditions of oxidative stress occurring during metabolic syndrome.

Synthetic microbial communities have been proposed to prevent and treat disease and reverse gut dysbiosis more effectively than single strain approaches (El Hage et al. 2017). Because of its beneficial effects on the host metabolism, propionate in our gut could contribute to solve the metabolic syndrome puzzle. We therefore aimed at engineering a propionate-producing synthetic microbial community, with functional redundancy on the different metabolic pathways for propionate production (acrylate, succinate, propanediol) (Reichardt et al. 2014). We then evaluated its potential to restore gut functionality after antibiotic-associated dysbiosis, using the Simulator of the Human Intestinal Microbial Ecosystem (SHIME). Knowledge regarding successful engraftment of functional communities can be applied for developing preventative novel probiotic strategies to ameliorate microbiome imbalances associated with metabolic syndrome.

3.3 - Materials and methods.

3.3.1 - Selection of strains for the propionate-producing consortium (PPC)
Gut commensal strains were selected based on the reported metabolic pathways for propionate production (succinate, acrylate, and propanediol pathways) (Reichardt et al. 2014; Table 2.1). The selection of the strains composing the propionate-producing consortium (PPC) has been described in section 2.3.1 in chapter 2.

3.3.2 - Assembly of propionate-producing community
Culturing of bacteria was performed in Reinforced Clostridium Medium (Oxoid Ltd, Basingstoke, Hampshire, UK), using the Hungate tube method and under anaerobic conditions
(90% N₂/ 10% CO₂). All strains were incubated at 37°C for 48 h except for *L. plantarum*, which was incubated for 24 hours. At the end of the incubation, cell count was measured using flow cytometry, and standardised to $10^8$ cells ml$^{-1}$.

Following, the consortium was subjected to the environmental conditions of the colon. Thus, the consortium culturing medium (L-SHIME medium, Prodigest NV, Zwijnaarde, Belgium) was subjected to pre-digestion simulating the passage through the upper GI tract in the fed state. Gastric digestion was mimicked by maintaining pH 2 for 2 h, followed by addition of pancreatic juice [(0.9 g L$^{-1}$ pancreatin (Sigma-Aldrich, St. Louis, USA), 6 g L$^{-1}$ Oxgall (BD, Erembodegem, Belgium), and 25 g L$^{-1}$ NaHCO$_3$ (Carl Roth GmbH, Karlsruhe, Germany)]. pH was adjusted to 6.8, and medium was incubated for 2.5 h at 37°C to simulate the small intestine digestion. pH was adjusted to 6.8 prior addition of the bacterial cocktail.

Then, 5 ml of each strain were mixed under anaerobic conditions. The consortium was prepared by transferring 5 ml of the bacterial cocktail (10 % v/v) to an anaerobic glass bottle containing 45 ml of pre-digested medium. Co-culture occurred for 48 h, and 40% of the medium was replaced after 24h. The consortium was harvested after 48 hours, when the average propionate concentration was 34.5 mM (Annex II, Figure S1). The viable cell count of the consortium was $10^8$ cells ml$^{-1}$ when administered to the M-SHIME.

3.3.3 - Dynamic simulation of the colon environment

The mucosal simulator of the human intestinal microbial ecosystem (M-SHIME) is an *in vitro* model including both mucosal and luminal microbiota and simulating the digestive processes in the human intestinal tract (Van den Abbeele et al. 2012). We initially applied the model to evaluate the impact of a single dose of the consortium, in comparison with repeated doses, in separate SHIME runs. The experiments using single vs repeated doses were performed using fresh faecal material from one female (27 yo), and one male donor (29 yo). Validation of the repeated dosing required faecal samples from six more donors of the same age group (30 ± 5). All donors were healthy with a normal BMI and did not use antibiotics for the last 6 months. The M-SHIME setup consisted of double-jacketed reactors representing the stomach, small intestine and colon (Truchado et al. 2017). The optimal pH for propionate production is 6.4-6.5 (Belenguer et al. 2007; Zhuge et al. 2014), therefore, the transverse colon (pH 6.3-6.5) was selected where the PPC can optimally act. We simulated the environment of the transverse colon and thus, the pH was between 6.3-6.5, and the volume was 660 mL calculated upon retention time. Nutritional medium composition was described previously (Truchado et al. 2017), and each colon vessel had a mucosal environment consisting of 80 mucin agar-covered microcosms (AnoxKaldnes K1 carrier; AnoxKaldnes AB, Lund, Sweden), placed in a polyethylene netting (Zakkencentrale, Rotterdam, The Netherlands) (Van den Abbeele et al. 2012). Each M-SHIME vessel was inoculated with 8% (w/v) faecal slurry (Possemiers et al. 2004; Molly et al. 1993). Static incubation was completed for the first 16 h, to allow for initial
stabilization of the system. After 16 hours, the peristaltic pumps were started up to supply each colon vessel with 200 mL of pre-digested feed three times per day every 8 hours. Pre-digestion consisted of a 45-minute incubation in the stomach-small intestine compartment. All reactors were flushed with N₂ to ensure anaerobic conditions. A scheme of the M-SHIME is presented in supplementary material (Annex II, Figure S2).

After 10 days of stabilisation, 33.9 mg L⁻¹ of clindamycin (Sigma-Aldrich, St. Louis, USA) were added to all colon vessels twice per day for three days, to trigger dysbiosis. Four days after the last antibiotic treatment, a single dose (45 ml, 6.8% of volume) of the propionate-producing consortium was added to triplicate treatment vessels, while the other three reactors were kept as controls. Three days after the single dose, three consecutive doses of the treatment were added again for three consecutive days. The system was monitored for further four days to investigate the further effect of the consortium. The whole experiment ran for 27 days in case of the first 2 donors and 23 days for the six donors. Samples for SCFA analysis and for DNA extraction were collected every day before the first medium replacement. Samples that were sent for Illumina sequencing were samples from different days that present the end of the different phases of the SHIME run. Lumen samples were collected at days 11, 17, 20, and 23 representing stabilization phase, antibiotic treatment phase, propionate-producing consortium (PPC) treatment phase and washout phase respectively. The mucin samples were collected less frequently since mucin beads were changed every other day, so the samples collected were at days 9, 14 and 21 presenting stabilization phase, antibiotic treatment phase, and after PPC treatment phase respectively.

3.3.4 - Community functionality and composition

3.3.4.1 - SCFAs extraction

Short-chain fatty acids were used as benchmarks of community activity, and were collected from the luminal compartment, and extracted with diethyl ether (De Weirdt et al. 2017) and quantified as described by (De Weirdt et al. 2010). Total SCFA production was defined as the sum of the molar concentrations of acetate, propionate, butyrate, valerate, caproate, isobutyrate, isovalerate and isocaproate (De Weirdt et al. 2017). The detection limits according to CMET protocol were as follows: 30 mg/L Acetate, and 10mg/L propionate and longer VFAs. As iso-acids are indicative of proteolytic metabolism; we currently did not focus on shifts between carbohydrate – protein metabolism, but focused more on the restoration of propionate production in our study.

Differences in SCFA concentrations among treatments were compared using a repeated measures mixed model, with the lsmeans adjustment and Sidak correction for multiple comparisons (GraphPad Prism 7.04, La Jolla, CA, USA). Statistical significance was assumed at \( P < 0.05 \).
DNA extraction and Illumina library generation

Total DNA from luminal and mucosal samples was extracted using physical disruption with the bead beating method (Hernandez-Sanabria et al. 2010). Briefly, samples were thawed, manually homogenized, and centrifuged at 14,600 x g for 5 min at 4°C. The pellet was resuspended in 1 ml of lysis buffer (100 mM Tris pH8, 100mM Na EDTA pH8, 100 mM NaCl, 1% (w/v) polyvinylpyrrolidone, 1% PVP40, and 2% (w/v) sodium dodecyl sulphate) and transferred to a 2-ml microcentrifuge tube containing 0.3 g of zirconium beads (diameter, 0.1 mm). The cells were lysed in a Power Lyzer 24 (Mo Bio Laboratories, Carlsbad, CA, USA) for 3 minutes at 4800 rpm. DNA concentration and quality were verified based on the absorbance at 260 and 280 nm, using a DeNovix DS (Thermo Scientific, Waltham, USA).

The V3-V4 hypervariable region of the 16S rRNA gene was amplified using primers 341F and 785R. Illumina sequencing adapters and dual-index barcodes were added to the amplicon, using a limited-cycle PCR that included an initial denaturation step at 95 ºC for 3 min, 15 cycles of a denaturation step at 95ºC for 30 s, an annealing step at 55 ºC for 10s, an extension step at 72 ºC for 45 s, and a final extension at 72 ºC for 5 min. Following, a clean-up step was performed using the AMPure XP beads (Beckman-Coulter, Krefeld, Germany) to remove free primers and primer-dimer species from amplicons. A second PCR to attach the specific Illumina multiplexing sequencing primers and index primers, was performed. Thermal cycling included an initial denaturation step at 95 ºC for 3 min, 8 cycles of a denaturation step at 95ºC for 30 s, an annealing step at 55 ºC for 30 s, an extension step at 72 ºC for 30 s, and a final extension at 72 ºC for 5 min.

These PCR products were verified by gel electrophoresis, purified using the Promega Wizard PCR clean-up kit (Promega, Madison, WI, USA) following the manufacturer’s instructions and quantified with the Quantifluor dsDNA System kit (Promega, Leiden, The Netherlands). High-throughput amplicon sequencing of the V3 – V4 hypervariable region (Klindworth et al. 2013) was performed with the Illumina MiSeq platform according to the manufacturer’s guidelines at LGC Genomics GmbH (Berlin, Germany). Contigs were created by merging paired-end reads based on the Phred quality score (of both reads) heuristic as described by Kozich et al. (2013) (Kozich et al. 2013 in Mothur (Schloss et al. 2009) (v.1.33.3). Contigs were aligned to the SILVA database and filtered from those with (i) ambiguous bases, (ii) more than 10 homopolymers, and (iii) those not corresponding to the V3 – V4 region.

Chimera removal and operational taxonomic unit (OTU) clustering of the sequencing reads was performed using UCHIME, with the nearest neighbour clustering algorithm implemented in mothur, at 0.03 distance (Edgar 2010). Phylotype representatives were then generated by clustering at 97% similarity (1 mismatch), with a confidence level of at least 80 with Cyanobacteria, Eukaryota, and Archaea lineages removed. For taxonomic classification, sequence composition of the dataset was compared using the RDP Classifier tool (Wang et
al. 2007), and the RDP trainset (Cole et al. 2009) version 9. Quality of the sequencing and post-processing pipeline was verified by incorporating mock samples (n = 12 species) in triplicate into the same sequencing run. After examining read counts, if any OTU was not classified up to genus level, the consensus sequence was blasted using the SILVA database (Pruesse et al. 2012) to obtain the taxonomic classification. In addition, all OTU sequences were aligned with those obtained from the sequencing of the 16S rRNA genes of each species, using Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/).

3.3.5 - Community composition and dynamics

Data was imported into R using phyloseq (McMurdie et al. 2013), and taxon abundances were rescaled by calculating the taxon proportions and multiplying them by the minimum sample size (n = 24789) present in the data set (McMurdie et al. 2014). Alpha diversity was initially estimated within each sample using Richness, Fisher’s diversity, Shannon, Simpson, and inverse Simpson indices. Inverse Simpson was the metric used for final assessment. Pielou index was used as indicator of evenness in the community (Grunert et al. 2016). Differences in alpha diversity and evenness measures among treatments were compared using a repeated measures mixed model in SAS, using the lsmeans adjustments and Bonferroni correction for multiple comparisons (version 9.4, SAS Institute, Cary, USA). To confirm these results, comparisons between control and treatment were performed using a 2-way Anova (Sidak’s method) and between and within time points using Tukey’s method in GraphPad (GraphPad Prism 7.04, La Jolla, CA, USA) (Annex II, Tables S1 and S2).

Beta diversity estimates based on Chao and Bray-Curtis indices were used to examine dissimilarity and determine the impact of treatment and time on microbial community structure. Principal Coordinate Analysis (PCoA) was employed to visualize the differences among samples, using the vegan package in R (Oksanen et al. 2007) (Annex II, Figure S3). Stratified permutational multivariate analysis of variance (PERMANOVA) with 999 permutations was conducted to indicate the significance of time and treatment on the microbial community differences. ANOVA was applied to reveal whether the distribution of the genera was different between treatments over time (Oksanen et al. 2007). Because of the over-dispersion in the OTU counts data, a zero-inflated count model was used to assess the effect of time and treatment and the interactions between time*treatment on each individual genus. Zero-inflated models explain the excess of zeros by modelling the data as a mixture of a Poisson distribution or a negative binomial distribution. When a zero count is observed there is the zero-inflation probability, because the observation came from the always-zero distribution. When the underlying count distribution is a Poisson distribution, the model is called a zero-inflated Poisson distribution and if the count distribution is a negative binomial distribution, the mixture is called a zero-inflated negative binomial distribution. The final model was selected based on the Akaike Information Criterion (AIC). Differences among library size sample were accounted
for with the offset option in proc GLIMMIX in SAS (Paschold et al. 2012). P values for each comparison were converted to q-values that were then used to identify differences in relative abundances of bacterial genera while controlling false discovery rate (FDR) at the 5% level (Storey and Tibshirani 2003).

Bipartite networks were inferred to highlight functional associations among bacterial genera and metabolites, using a pair-wise similarity matrix obtained from a Regularized Canonical Correlation Analysis (Lê Cao et al. 2011). Values of the similarity matrix were computed as the correlation between the relative abundances of bacterial genera and the metabolic variables, projected onto the space spanned by the first components retained in the analysis. Three relevant components were obtained setting a threshold of $r \geq 0.7$ and genera were disseminated in the plot, in close relation with the variables correlated (De Weirdt et al. 2017).

3.3.6 - Enumeration of microbial cells using flow cytometry

To assess variation in cell counts in lumen and mucin compartments, we analysed SHIME samples collected at different time points from different phases of the SHIME run (stabilization phase, after antibiotic phase, after treatment phase and washout phase).

**Cell counts from lumen and mucin samples.** Samples used for cell counts were frozen at -20°C. All samples were diluted 1:1 in filter-sterilized PBS, and vortexed for 1 minute at maximum speed. Mucin samples were disrupted for 40 seconds at 1800 rpm (Power Lyser 24, MO BIO Laboratories, Carlsbad, USA), and centrifuged at 500 × g for 4 minutes, while lumen samples were centrifuged at 500 × g for 2 minutes. Supernatants were collected and passed through 20µm filters (Filcon, BD Medimachine, Erembodegem, Belgium) to remove particulate matter. Filtered samples were then diluted 5000 times in filter-sterilized PBS, and 198µl of the diluted sample was stained with 2µl of SYBR Green (SG) (10,000× diluted from stock; Invitrogen, Carlsbad, USA) in 96 flat-bottom well plate. The plate with the stained samples was incubated for 20 minutes at 37°C. Flow cytometric analysis of the microbial cells present in the suspension was performed using a C6 plus Accuri flow cytometer (BD Biosciences, Erembodegem, Belgium) equipped with a 488 nm laser, following previously described methods (Props et al. 2017). Fluorescence events were monitored using the FL1 533/30 nm and FL3 > 670 nm optical detectors. Forward and sideways-scattered light was also collected. The BD Accuri CSampler software was used to gate and separate the microbial fluorescence events on the SSC-A and FITC-A density plot from the lumen and mucin SHIME sample background. Gating was evaluated using a 0.2µm filter. A threshold value of 1,000 was applied on the FL1 channel. The gated fluorescence events were evaluated on the SSC-FL1 density plot, to exclude remaining background events and to obtain an accurate microbial cell count. Instrument and gating settings were kept identical for all samples.

For quantification of absolute numbers of each taxon, samples were rescaled by multiplying the relative abundance of each genus by the flow cytometry cell counts.
3.3.7 - Assessment of the mitochondrial membrane potential

Mitochondrial membrane potential ($\Delta \Psi_m$) is a central intermediate in oxidative energy metabolism (Gerencser et al. 2012), and it is associated to the metabolic activity of the eukaryotic cells. Mitochondrial membrane potential of epithelial cells is fundamental for gut motility as well as for cell division, as it modulates the distribution of several conserved cell division proteins (Strahl and Hamoen 2010). Propionate promotes flux of Cl$^-$ to the mucosa, increasing the short-circuit current, hence stimulating colonic contractions through a change in potential difference (Yajima 1988). As reduced intestinal motility and low-grade inflammation are markers of metabolic syndrome (Müller et al. 2018), we assessed the capability of the propionate-producing consortium to change the mitochondrial membrane potential ($\Delta \Psi_m$). We used an in vitro model of the gut epithelium to reveal whether the supplementation of the consortium could potentially restore disrupted membrane potential, as observed in metabolic syndrome. Caco-2 cells were seeded onto opaque clear bottom 96-well plates (Corning, NY, USA) at a density of 20000 cells/well and maintained for 72 hours. Then, cell culture media was removed, and cells were exposed to the treatments in table 3.1.

Table 3.1: Treatments added to epithelial cell model to assess changes on membrane potential. A negative control was used in which the cells did not get any treatment or disruption. CCCP was used as a positive control for disruption of membrane potential. The metabolites from the propionate-producing consortium (PPC) were added above the cells after being filter-sterilized. To assess the ability of the consortium (PPC) to restore the disruption caused by clindamycin (CLN), its metabolites were added together with clindamycin above the epithelial cells. P/A/B, propionate/acetate/butyrate mixture at the ratios produced by the consortium.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Concentration</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clindamycin hydrochloride</td>
<td>33.9 mg/L</td>
<td>24 h</td>
</tr>
<tr>
<td>Filter-sterilized PPC (1:5 v/v in DMEM)</td>
<td>2:1 Acetate:Propionate ratio</td>
<td>24 h</td>
</tr>
<tr>
<td>CLN + PPC</td>
<td>33.9 mg/L + P/A/B</td>
<td>24h</td>
</tr>
<tr>
<td>Cell culture medium (negative control)</td>
<td>-</td>
<td>24h</td>
</tr>
<tr>
<td>CCCP (positive control for disrupted $\Delta \Psi_m$)</td>
<td>50µM</td>
<td>2 h</td>
</tr>
<tr>
<td>DMSO (control for CCCP vehicle)</td>
<td>&lt; 0.01%</td>
<td>2h</td>
</tr>
</tbody>
</table>
All the compounds were diluted from the stock solution in DMSO to the corresponding working solution in DMEM without supplementation. Filter-sterilized consortium was diluted 1:5 (v/v) in DMEM without supplementation. DMSO was used as a control.

After exposing the cells to the treatments for 24 h at 37°C, (95% humidity, 10% CO2), cells were washed once with 200 µL of PBS with Ca++ and Mg++ (Sigma) and 100 µL of JC-1 stain (10 µM in DMEM) (Cayman Chemical, Michigan, USA) were added to the wells and incubated for 20 min. Following, wells were washed with PBS supplied with Ca++ and Mg++ and 10% FBS, and 100 µL of DMEM were added to each well before measuring with a SpectraMax Plus Microplate Reader (Molecular Devices, LLC). Excitation/emission wavelength pairs were set at 475/530 nm and 475/590 nm, for JC-1 monomer and aggregate detection, respectively. 5,5,6,6'-tetrachloro-1,1',3,3' tetraethylbenzimidazoylcarbocyanine iodide (JC-1) dye has been developed to detect ΔΨM in healthy and apoptotic cells across multiple cell types (Sivandzade, Bhalerao and Cucullo 2019). In healthy cells, the dye localizes in the mitochondria where it forms aggregate the fluoresce red. When the mitochondria is disrupted and the mitochondrial membrane collapses, the dye diffuses in the cytosol in monomeric form which fluoresces green. Thus, the ratio red to green fluorescence (aggregate/monomer) reflects mitochondrial membrane integrity (McGill et al. 2011). As a result, a low aggregate/monomer ratio indicates mitochondrial membrane disruption.

The background (A590 of non-stained cells) was subtracted from the test signals. Results were expressed as the ratio between aggregate/monomer. All Pairwise Multiple Comparison Procedures and Statistical analysis was done using Holm-Sidak method in GraphPad (GraphPad Prism 7.04, La Jolla, CA, USA).

3.4 - Results.

We assessed the reproduciability of the simulated clindamycin-induced dysbiosis using the Mucosal Simulator of the Human Intestinal Ecosystem (M-SHIME), before evaluating the potential of the propionate-producing consortium to restore functionality. Two M-SHIME runs were separately conducted with faecal microbiota from two volunteers, and treatments were supplied to triplicate reactors. Concentrations and profiles of SCFA in the luminal content of the proximal colon compartments across the three technical replicate reactors were found to be reproducible (Annex II, Figures S4 and S5).

3.4.1 - Gut microbiome functionality is improved when a propionate-producing consortium is supplied after antibiotic use in single donor experiments

Altered production of SCFA is considered one of the hallmarks of dysbiosis, and thus, we validated that clindamycin disrupted the fermentation pattern of the simulated gut ecosystem. Upon antibiotic supplementation, propionate and butyrate decreased by approximately 57%
and 95% respectively and remained consistently and significantly low ($P < 0.05$) across all triplicate experiments for both donors 1 and 2 (Annex II, Figures S4 and S5). In the single donor experiments, we found that one single dose of the propionate-producing consortium did not promote functional recovery on either of the donors (Annex II, Figures S4 and S5), whereas three consecutive doses of the consortium triggered a significant increase in propionate production only in donor 1 ($14.84 \pm 1.06\text{mM}; P < 0.05$, Annex II, Figure S4). This positive outcome remained consistent across the three replicates until the end of the experiment (Annex II, Figure S4, Table S3). As the impact of one single dose of the consortium seemed to be marginal, we decided to use three consecutive doses in a multiple-donor experiment. We conducted an extra M-SHIME run with faecal samples from six different donors of the same age group ($30 \pm 5\text{yo}$). Functional recovery and inter-individual variability were assessed, and all the following results presented were from the multiple-donor experiment.

3.4.2 - Modulation of microbiota functionality by a propionate-producing consortium upon antibiotic induced dysbiosis

After disruption with clindamycin, there was a significant and consistent decrease in butyrate, propionate, and acetate by 88%, 46% and 16% respectively ($P < 0.05$) (Figure 3.1). Higher decrease was observed in propionate and butyrate due to the decrease in *Akkermansia, Blautia, and Lachnospiraceae* than are main producers for propionate or butyrate. Lower decrease in acetate might be due the fact that acetate production pathways are widely distributed among bacterial groups while propionate, butyrate and lactate pathways are more highly conserved and substrate specific (Morrison and Preston 2016). Inter-individual differences in SCFA production were observed upon dosing the propionate-producing consortium. Repeated supplementation of the consortium promoted significant increase in propionate production ($12.47 \pm 0.88\text{mM}; P < 0.05$), compared with the control ($7.57 \pm 0.37\text{mM}$), resulting in nearly 100% recovery of the initial propionate concentrations. Restoration of butyrate and acetate was variable across the 6 different donors and no significant recovery was observed (Figure 3.1). These observations confirm that our designed microbial consortium led to effective functional recovery of propionate production following dysbiosis.
Figure 3.1: Addition of the propionate-producing consortium (PPC) promotes recovery of propionate production after antibiotic-associated disruption. CTR: Control, TRT: Treatment. (A) Short chain fatty acid production during the 3 different phases of the experiment: Stabilization, post-antibiotic disruption, and post-addition of the propionate-producing consortium. Days in which antibiotics and PPC were added are indicated by arrows. Antibiotic treatment was added on days 11, 12, and 13. Treatment was added on days 17, 18, and 19. (B) Propionate was the main short chain fatty acid impacted after 2 doses, as propionate levels significantly increased from day 19 in the treatment reactors (P < 0.05). No significant difference was detected for acetate (C) and butyrate (D) after the treatment was added (P>0.05).

3.4.3 - Propionate-producing consortium supports the partial recovery of the epithelial mitochondrial membrane potential after antibiotic disruption

Mitochondrial membrane potential of epithelial cells is fundamental for gut motility as well as for cell division, as it modulates the distribution of several conserved cell division proteins (Strahl and Hamoen 2010). Carbonyl cyanide m-chlorophenylhydrazone (CCCP) was used as a positive control for the disruption of the membrane potential, because it rapidly disperses the proton motive force (pmf) or membrane potential (Strahl and Hamoen 2010). The concentration used for triggering a membrane disruption was set high (50 uM) to ensure the desired effect. The CCCP dose was adjusted based on the response of Caco-2 cells, within the range of concentrations and times previously reported in literature (Rainbolt, Saunders and Wiseman 2014; Yang et al. 2001; Ikeda et al. 2015; Sgarbi et al. 2014; Wang et al. 2017; Liu et al. 2017; Panina et al. 2019). Lower doses could be used in the future as described by Ruas
et al. (2016). A significant decrease in membrane potential was observed when CCCP was added to our cell model, confirming the negative effect of the CCCP on the membrane potential (Annex II, Table S4, \( P < 0.05 \)), (Figure 3.2). Clindamycin decreased the membrane potential by approximately 80% \( (P < 0.05) \), confirming the negative effect of the antibiotic towards epithelial cells. When clindamycin was added together with the propionate-producing consortium, the ratio of the aggregate monomer of the JC1 was increased by 40% \( (P < 0.05) \), indicating partial recovery of the membrane potential after clindamycin disruption.

![Figure 3.2](image)

Figure 3.2: Propionate-producing consortium triggered a partial recovery for the membrane potential after clindamycin disruption. Following clindamycin (CLN) supplementation, membrane potential tended to be higher when one dose of the propionate-producing consortium (PPC) was provided, in comparison with exposure to clindamycin alone. Protonophore carbonyl cyanide m-chlorophenylhydrazone (CCCP) was used as a positive control to disrupt membrane potential. All Pairwise Multiple Comparisons between treatments were assessed using Holm-Sidak method with overall significance level equal to 0.05. Significant differences were indicated using different superscripts. Presence of the same letter in the superscript indicates that samples are not significantly different \( (P>0.05) \).

3.4.4 - Antibiotic use significantly decreased the bacterial cell load
A workflow for the quantitative microbiome profiling can be built through parallelization of amplicon sequencing and flow cytometric enumeration of microbial cells (Vandeputte et al. 2017). Flow cytometry revealed a significant decrease in the cell count after antibiotic use in both lumen and mucin compartments \( (P<0.05) \). Administration of the propionate-producing
consortium did not impact the total cell load ($P > 0.05$). However, cell count decreased in the reactors that were not supplemented (Annex II, Figure S6).

3.4.5 - Propionate-producing consortium shaped the bacterial community based on number of doses and host influence

As shifts in bacterial taxa and decrease in community diversity are benchmarks of dysbiosis, we dynamically monitored both luminal and mucosal communities using the M-SHIME. Alpha diversity was unchanged in control and treatment reactors in the multi-donor experiment upon consortium supplementation (Figure 3.3A).

Clindamycin significantly decreased richness in all luminal compartments (Figure 3.3B) (Annex II, Table S1), but consecutive doses of the propionate-producing consortium triggered a significant recovery of this metric in the lumen compartment ($P < 0.05$). The difference in richness between the control and treatment was not significant until the end of the washout period, when the treatment reactors had significantly higher total number of species than the control reactors ($P < 0.05$, Figure 3.3B) (Annex II, Table S1). Total number of species tended to be higher in the mucin compartment as well. As the mucosal communities are in close contact with the host, successful function transfer in the mucosal compartment may be relevant for possible host effects (Figure 3.3E) (Annex II, Table S2). Antibiotics have stronger effect on the activity of mucosal microbial communities, as the washout of clindamycin is somewhat delayed compared to washout from the luminal environment. This is due to the fact that the mucin beads were changed every other day, in which only half of the mucin beads were changed during every replacement to mimic the in vivo mucus layer turnover. In contrast, the luminal compartment experiences a washout that is far more frequent as the feed is being replaced 3 times per day. This results in a longer persistence of antibiotics in the mucin compartment, and this is also a confirmation of previous in vivo observations studies (Rashid et al. 2015; Brismar et al. 1990). In addition, evenness of mucosal communities following antibiotic-induced dysbiosis tended to be lower in comparison with that at the end of the stabilization period (Figure 3F, Table S2). In contrast, evenness in the lumen community after dysbiosis remained constant, suggesting that mucus bacteria are more sensitive to antibiotics than bacteria in the lumen environment (Figure 3.3C) (Annex II, Table S1). We observed that antibiotic supplementation promoted both loss of bacterial richness and increased relative abundance of Enterobacteriaceae and Escherichia-Shigella, albeit not significant for the latter in the mucin (Figure 3.4) (Annex II, Table S5D). Importantly, relative abundance of Escherichia-Shigella was significantly decreased in the lumen when the consortium was supplied (Annex II, Table S5C). Pathologies characterized by microbial dysbiosis have in common a decrease in the community composition complexity, as well as an increase in aero-tolerant genera such as Enterobacteriaceae (Vonaesch et al. 2018). Although microbial community can be deeply
disrupted upon antibiotic use, our results indicate that the propionate-producing consortium shaped the community based on the number of treatment doses and donor.

Figure 3.3: Propionate-producing consortium triggered an increase of total number of species after the washout period only in the lumen compartment. Relative abundance was quantified at the end of stabilization phase, antibiotic treatment phase, propionate-producing consortium phase, and washout phase. Control and treatment compartments were indicated with orange and blue respectively. The propionate producing consortium was added only in the treatment compartments. No effect of the consortium on the alpha diversity of lumen (A) and the mucin (D) were detected. Antibiotic supplementation triggered significant decrease in the total number of species ($P < 0.05$) only in the lumen compartment (B), but not in mucin compartment (E). The propionate-producing consortium significantly increased the total number of species at the end of the washout (B). No effect of the consortium on the evenness of the community in the lumen (C) and the mucin (F) was observed.

Repeated doses of the consortium confirmed a potential recovery of the community, both through direct engraftment and through indirect reinforcement of other propionate producers. For instance, unclassified Lachnospiraceae, Akkermansia and Bacteroides increased their relative abundance in both compartments, indicating that some members of our consortium (Coprococcus catus, Akkermansia muciniphila, Bacteroides thetaiotaomicron and B. vulgatus)
may have been successfully engrafted in the mucin compartment (Figure 3.4) (Annex II, Table S5B). Moreover, the presence of these genera even after the washout may suggest that indirect positive reinforcement of the overall community may have ensued. Long-lasting increased relative abundance of *Akkermansia* species seem to be consistent across individuals, as indicated by the community composition at the end of the washout period (Figure 3.4). Moreover, the relative abundance of other species, such as *Veillonella* (Annex II, Table S5A), indicate that the intra-individual engraftment and indirect reinforcement of the consortium may be enduring.

Figure 3.4: Relative bacterial abundances on the luminal and mucosal compartments shifted among end of stabilization, after antibiotic use, after 3 doses of PPC and after washout. Genera with the highest relative abundances across time points were uncovered in the (A) lumen compartment and (B) mucosal compartment. Six replicates (donors) were averaged to highlight the inter-individual effect of the propionate-producing consortium.

3.4.6 - Addition of the propionate-producing consortium amends community metabolic networks following environmental disruption

Relevance networks analyses indicated that SCFA-production networks shifted with antibiotic treatment. As metabolic products were significantly decreased when the environmental
disruption happened, larger networks including bacteria associated with acetate production were observed (Annex II, Figure S7A). Increased acetate concentrations seemed to correlate with increased relative abundances of *Bifidobacterium, Faecalibacterium, Flavonifractor* and *Anaerotruncus* (Annex II, Figure S7A and Table S5C). With respect to butyrate, a positive association was only found with increased relative abundance of *Bacteroides*. No genera were significantly associated with high concentrations of propionate, after antibiotic was provided (Annex II, Figure S7A) \((P < 0.05)\).

The relevance network for the control reactors displayed intermingled propionate and butyrate networks (Figure 3.5B). This may suggest that the community competes for the substrate available, preventing from significantly increasing propionate production. Upon repeated dosage of the consortium, the propionate network showed that higher relative abundances of Unclassified Lactobacillaceae, *Morganella, Hungatella, Erysipelatoclostridium*, Unclassified Lachnospiraceae and *Bilophila* were associated with higher concentrations of propionate (Figure 3.5A) (Annex II, Table S5C). The increased relative abundance of these and other genera such as *Parabacteroides* in the mucin (succinate producer) may confirm the indirect positive reinforcement of the propionate-producing consortium on the overall community (Annex II, Table S5C). Moreover, *Hungatella* has been reported to thrive on medium used to produce probiotic bacteria (Kaur et al. 2013).

Finally, the enduring indirect impact of the consortium was validated on the propionate network at the end of the washout period (Annex II, Figure S7B). We observed that higher relative abundance of Unclassified Lactobacillaceae was positively associated with increased concentrations of propionate \((P < 0.05)\), potentially revealing that acetate-producing bacteria belonging to this family \((Lactobacillus\) sp.) actively participate in the functional recovery of the community. The lasting effect of the consortium may as well indicate the successful engraftment of one of the genera comprised in this community. Higher abundances of *Akkermansia* were associated with higher concentrations of acetate at the end of the washout (Annex II, Figure S7B). This could be an additional indicator of the successful adaptation and operational efficacy of the consortium.
Figure 3.5: Bacterial interactions networks influenced the production of the major short chain fatty acids over time. (A) Treatment reactors at day 20 after adding 3 doses of the
propionate consortium, (B) Control vessels at day 20. These bipartite networks are based on the regularised canonical correlations between relative bacterial abundances and relative concentrations of the main SCFA. Interactions have been filtered for an absolute correlation above 0.8 and are coloured following the key shown. Significant interactions are shorter lines, and genera with similar abundances within SHIME compartment tend to cluster closely.

3.4.7 - Variations in cell densities confirmed the impact of the propionate-producing consortium in the compositional dynamics of the community

We performed absolute quantification of the taxa detected in our study, to comprehensively explain the differences observed in the relative abundances. Our results confirmed the direct engraftment of one of the members of our consortium, as the absolute abundance of *Veillonella* significantly increased in both luminal and mucosal compartments of the treatment reactors after providing 3 doses of the consortium (*P* < 0.05) (Figure 3.6) (Annex II, Tables S6 and S7). As for the indirect reinforcement, relevance networks initially suggested that some genera were linked to propionate production. We observed a significant increase in absolute abundance of unclassified Lactobacillaceae after adding the 3 doses of the consortium in both lumen and mucin (*P* < 0.05) (Figure 3.7) (Annex II, Tables S8 and S9). Moreover, unclassified Lachnospiraceae significantly increased in the treatment vessels after the washout period in the lumen compartment (*P* < 0.05) (Figure 3.7) (Annex II, Table S8). Although *Bilophila* showed increased relative abundance after adding the 3 doses of the consortium, absolute abundance was not increased (Figure 3.7) (Annex II, Tables S8 and S9). The combined approach of relative and absolute abundances assisted to elucidate the course of action of the propionate-producing consortium. Thus, the observed positive effect of the consortium may be explained as a synergic impact on the cell counts and on the relative abundances of taxa involved in propionate production pathways.
Figure 3.6: Absolute abundance of the seven genera included in the propionate-producing consortium. Quantification of absolute abundances revealed the direct
engraftment of specific genera in the lumen (A) and mucin (B) compartments. *Veillonella* showed a significant increase \((P < 0.05)\) in both lumen and mucin compartments after the administration of the propionate-producing consortium.
Figure 3.7: Absolute abundance of genera involved propionate production upon consortium supplementation. Quantification of absolute abundance validated the
indirect reinforcement of specific genera in the lumen (A) and mucin (B) compartments. Lactobacillaceae was significantly increased in both lumen and mucin compartments ($P < 0.05$) upon administration of the propionate-producing consortium. Lachnospiraceae showed a significant increase only in the lumen compartment at the end of the washout phase ($P < 0.05$).

3.5 - Discussion

In our study, we aimed at engineering our propionate-producing consortium considering the three major pathways for propionate production (acrylate, succinate, and propanediol pathways) (Reichardt et al. 2014). The consortium positively impacted functionality and composition of the microbial community and supported the partial recovery of membrane potential after clindamycin disruption.

SCFA analysis from the luminal SHIME samples revealed a significant drop in bacterial metabolic activity after clindamycin-induced dysbiosis. Previous studies reported the ability of antibiotics to significantly influence taxonomic richness, diversity and evenness (Dethlefsen et al. 2008; Francino 2016; Rojo et al. 2017; Dethlefsen and Relman 2011), as observed in the lumen and mucosal communities of our experiment. In addition, flow cytometry analysis confirmed the negative effect of dysbiosis on bacterial cell counts, as they significantly decreased upon antibiotic administration. We used clindamycin since the Cl atom present in clindamycin disrupts the mitochondrial membrane potential (Goldhill et al. 1996); similar disruption has been reported following oxidative damage in metabolic syndrome (Nicolson 2007). Thus, we applied clindamycin as an agent to stimulate the conditions of oxidative stress occurring during metabolic syndrome. We did not aim for a therapeutic treatment as the protocol applied for a C. difficile infection. We aimed only for a disruption of the microbial community without complete suppression of the bacterial population. In the single donor experiment, one single dose of the propionate-producing consortium did not impact neither functionality measured by SCFA production nor microbial community. The dose we provided may have not been enough for a beneficial effect, as in the case of probiotic strategies that can only confer a positive effect when administered in adequate amounts (Ouwehand 2017). Repeated doses of our consortium showed a significant effect on the functionality in one of the 2 donors of the single-donor experiments, and in all six donors in the multiple-donor experiment, with full recovery of propionate production. Resident bacteria potentially interacted with the supplied propionate-producing consortium, as those administered may have provided metabolites like acetate, lactate, and propionate. Thus, resident bacteria cross-feeding on these metabolites eventually led to the production of propionate (Derrien and van Hylckama...
Vlieg 2015). Our results suggest that restoration of propionate production by our engineered consortium was successful. Despite the positive impact of the propionate-producing consortium, some elements of dysbiosis remained unchanged. For instance, the consistent relative abundance of Proteobacteria like *Escherichia-Shigella* and *Bilophila* before and after treatment, indicates that those bacteria can act as opportunistic pathobionts in cases of dysbiosis. *Bilophila* may have utilized mucin-degradation products from our consortium to produce propionate. The positive correlation between *Bilophila* and propionate may be an indicator of the inflammatory status of the environment that resulted after antibiotics (Feng et al. 2017). However, absolute abundance of this genus was not increased when the consortium was provided. Instead, members from our propionate-producing consortium like *Bacteroides* and *Akkermansia* may have occupied the niche of primary carbohydrate degradation and subsequently promoted fermentation by resident bacteria, as is the case for mucin metabolism. Mucin degradation can liberate sugars, amino acids, sialic acids, and sulphate that can be consumed as substrates by the resident commensals (Derrien and van Hylckama Vlieg 2015) or even by the provided bacteria. Our consortium contained a strain of *Akkermansia muciniphila*, a specialized mucin-degrading bacterium (Chia et al. 2018), which could have provided sugar monomers from mucin upon degradation. Mucin-derived sugars like fucose could be utilized by *Akkermansia muciniphila* (Ottman et al. 2017) or by *Ruminococcus obeum* (Lachnospiraceae) to produce propionate through the propanediol pathway (Reichardt et al. 2014; Flint et al. 2014). This could explain the increase in the relative abundance in *Akkermansia* and Lachnospiraceae after administering the three doses of our consortium.

Ingested bacteria can impact resident communities through at least three different mechanisms: through trophic interactions, a direct alteration in fitness, or an indirect alteration in fitness through altered production of host-derived molecules (Derrien and van Hylckama Vlieg 2015). One of the markers considered for successful colonization from biotherapeutics is engraftment (Smillie et al. 2018). Engraftment originally refers to “incorporation of grafted tissue into the body of the host” (Miller et al. 2005), and it has been applied to explain the stable establishment of a bacterial strain in the human gut (Maldonado-Gómez et al. 2016). In our SHIME model, the complexity of the simulated colonic ecosystem allowed for analysing the impact of the administered bacterial community on microbial interaction networks, independently of host inputs. To ensure engraftment, long-term persistence of the different species of the live microbes in the consortium should be monitored in different donors, as described by Maldonado-Gómez et al. (Maldonado-Gómez et al. 2016). The beads coated with mucin in our model provided a comprehensive overview of the bacterial colonization process. For instance, we observed an increase in the relative abundance of unclassified Lachnospiraceae, *Akkermansia* and *Bacteroides* in the mucosal compartment, after repeated
doses. This indicated that some members of the consortium (*Akkermansia muciniphila*, *Coprococcus catus*, *Ruminococcus obeum* and *Bacteroides thetaiotaomicron* and *Bacteroides vulgatus*) may have been successfully engrafted in the mucin compartment. In our experiment, we attained long-lasting increased relative abundance of *Akkermansia* species across individuals, as indicated by the community composition at the end of the washout period. Previous reports suggest that species traits such as functionality are major drivers of bacterial colonisation (Smillie et al. 2018). In this way, the functional redundancy of our consortium may have ensured prevalence in the lumen following repeated dosage, even after four days. *Akkermansia* is considered a common member of the autochthonous human gut microbiome, which may guarantee permanent colonization as opposed to commercial probiotics belonging to lactic-acid producing bacteria (Maldonado-Gómez et al. 2016). Nevertheless, whether higher engraftment success is a general attribute of autochthonous members of the microbiome, or whether it is specific for certain probiotic strains needs to be elucidated (Maldonado-Gómez et al. 2016).

Metabolic syndrome leads to excess cellular oxidative stress and oxidative damage of mitochondrial components, impacting mitochondrial membrane potential (Nicolson 2007). Clindamycin is a chlorinated analogue of lincomycin and inhibits basal epithelial transport (Goldhill et al. 1996), impacting electrical field stimulation (EFS) (Goldhill et al. 1996) and mitochondrial membrane potential (Goldhill et al. 1996). Cl is an atom that is chemically bound to the molecule and replaces the hydroxyl group at position 7 in the original lincomycin molecule (Swayze, Griffey and Bennett 2007). In our epithelial cell model, the presence of a Cl atom in the molecule of the drug reduced the basal short circuit current (Goldhill et al. 1996) and disrupted the mitochondrial membrane potential upon exposure to clindamycin. Proper mitochondrial membrane potential is a requirement for oxidative phosphorylation (Nicolson 2007; Mitchell 1966) and impaired mitochondrial oxidative phosphorylation is one of the contributors to the development of the metabolic syndrome (Ren et al. 2010). Hence, the partial recovery of the membrane potential following the addition of the propionate-producing consortium suggests that application of our functional community may be a promising strategy to amend microbial dysbiosis and confer beneficial effects towards host epithelium.

Nowadays, next-generation probiotics and live biotherapeutics are being developed based on core members of the microbiome (Olle 2013), as in the case of our propionate-producing consortium. Endogenous core bacterial strains included in these biotherapeutics may have higher ecological fitness when administered to humans compared to the exogenous strains, such as commercial probiotics. However, the concept of ecological performance related to probiotic functionality is yet to be elucidated (Maldonado-Gómez et al. 2016).
3.6 – Conclusion

In conclusion, members of our gut microbiome can be used as new generation probiotics for targeting different health aspects. We confirmed that the established propionate-producing consortium can impact functionality by restoring the propionate production after antibiotic-induced dysbiosis. The key question would be if the propionate-producing consortium can impact beyond the ecology of the gut microbiome and influence host health. Importantly, as such consortia are to be administered orally, developing carrier matrices to ensure survival through the harsh conditions of the upper GI tract should be earnestly considered. Further research to determine dose-response outcome and long-term benefits will foster our knowledge on novel probiotic consortia. Indeed, understanding strain selection and the metabolic pathways for producing different SCFA will aid in the development of functional consortia targeted for prevention and management of major health concerns, such as metabolic syndrome, or even for personalized nutrition strategies.

3.7 - Acknowledgements.

The authors would like to thank the People Program (Marie Curie Actions) of the European Union’s Seventh Framework Program FP7/2007–2013/under REA grant agreement n_606713 for the research funding. The authors acknowledge Dr. Davide Gottardi for his scientific advice on the preparation of the bacterial consortium. The authors also thank Brecht Hoflack, Jana De Bodt, Tim Lacoere for their technical assistance, and Dr. Stephen Anderson for his support with English grammar and style.
Chapter 4

Supplementation of propionate-producing consortium improves markers of insulin resistance in an *in vitro* model of gut-liver axis

This Chapter has been redrafted after

CHAPTER 4 - Supplementation of a propionate-producing consortium improves markers of insulin resistance in an in vitro model of gut-liver axis

4.1 - Abstract.

Microbe-host research from recent years has revealed the gut-liver crosstalk to be an important determinant of human health with effects, amongst others, on energy homeostasis. While gut microbes produce a huge range of metabolites, specific compounds such as short chain fatty acids (SCFA) can enter the portal circulation and reach the liver, which is involved in glucose homeostasis and diabetes control. Propionate, a major SCFA that goes in the portal circulation, is involved in activation of intestinal gluconeogenesis (IGN), thereby regulating food intake and enhancing insulin sensitivity leading to metabolic homeostasis. While microbiome modulating strategies may target the increased microbial production of propionate, it is not clear whether such effects also trickles through to the hepatic cellular level. Here, we designed a propionate-producing consortium using a selection of commensal gut bacteria, and we investigated the impact of their delivered metabolites in an in vitro enterohepatic model of insulin resistance. Liver glycogen storage and different inflammatory markers were evaluated to understand the role of the gut metabolites on the gut-liver crosstalk in a simulated scenario of subclinical metabolic inflammation. The metabolites produced by our designed consortium were able to increase liver glycogen by approximately 57% and decrease pro-inflammatory markers such as IL-8 by 12%, thus elucidating the positive effect of our consortium on metabolic function and low-grade inflammation. Our results suggest that microbiota-derived products can be a promising strategy due to their multipurpose and adaptability, and their potential ability to manage metabolic diseases.
4.2 - Introduction.

The gut and the liver are interconnected organs from the digestive system, with key absorptive and metabolic functions. While the liver is exposed to gut bacteria metabolites and ingested nutrients (Brandl and Schnabl 2017) via the portal circulation (Das and Makharia 2014; Chen et al. 2017), the liver is also crucial for controlling glucose homeostasis and diabetes. When excess glucose is diverted to the liver, insulin is unable to control gluconeogenesis and activate glycogen synthesis. However, lipogenesis continues occurring (Samuel and Schulman 2012) and re-esterification in the liver may further increase insulin resistance, ultimately leading to hyperglycemia and metabolic disorders (Savage et al. 2007). Due to its low expression in adult liver, little attention has been paid to lipoprotein lipase (LPL). Liu et al. revealed that hepatic LPL is involved in the regulation of plasma LPL activity and lipid homeostasis (Liu et al. 2016). Moreover, studies have shown that insulin activates LPL promoting hydrolysis of chylomicrons, and LPL activity is reduced in patients with type 2 diabetes (Vergès 2015). Insulin resistance has also been associated with increased levels of ApoC-III, an inhibitor of LPL (Vergès 2015).

Short chain fatty acids (SCFAs) are important energy sources for our body and may go into the portal circulation. One the major SCFA is propionate; it is involved in activation of intestinal gluconeogenesis (IGN) (Vadder et al. 2014) and acts as a direct gluconeogenic substrate leading to regulation in food intake and glucose metabolism (Weitkunat et al. 2016). In addition high ratio of propionate reduces hepatic triglycerides and improves insulin sensitivity (Weitkunat et al. 2016) which results in metabolic homeostasis (Todesco et al. 1991; Aspey, Chambers and Frost 2018). Moreover, propionate stimulates the gut hormones PYY and GLP-1 leading to satiety (Chambers et al. 2014; Psichas et al. 2014; Morrison and Preston 2016; Tolhurst et al. 2011). In fact, long-term propionate delivery in the gut reduces intra-abdominal fat accretion, intrahepatocellular lipid content, and hepatic cholesterol synthesis in humans (Chambers et al. 2014; Arora et al. 2011). Indeed, protective effects against inflammatory and oxidative stimuli in endothelial cells have been attributed to propionate (Aspey et al. 2018). Gut microbial fermentation of non-digestible carbohydrates is the main source of circulating propionate in humans (Reichardt et al. 2014; Vogt and Wolever 2003). The above pro-homeostatic actions of propionate emphasize the contribution of SCFAs in maintaining normal physiological functions (Hoyles et al. 2018). Hence, propionate represents a model for mutually beneficial interactions between the host and microbiota (Hoyles et al. 2018). In fact, absence of gut bacteria, and the subsequent lack of SCFA have been associated with increased inflammatory response (Maslowski et al. 2009). Evidence of SCFA to counteract cardiometabolic risk factors such as insulin resistance has been uncovered. Previous studies have reported the impact of synthetic propionate (Canfora et al. 2015) or have sought to shift propionate through dietary interventions (Chambers et al. 2015). Moreover, Akkermansia
muciniphila, a mucin degrading gut bacterium that produces propionate and acetate (Derrien et al. 2011), was able to reverse the effects of the high fat diet that led to metabolic disorders like fat mass gain, metabolic endotoxemia, adipose tissue inflammation, and insulin resistance (Everard et al. 2013). These findings suggest that A. muciniphila is a promising candidate of the next generation beneficial microbes that can be used for the treatment or prevention of metabolic disorders (Cani and de Vos 2017). However, assessing the outcome of bacteria-derived propionate at cellular level has been scarcely described. In this research, we engineered a consortium of gut commensal bacteria for propionate production and we investigated the impact of their delivered metabolites and metabolites from A. muciniphila in an in vitro enterohepatic model of insulin resistance. We evaluated liver glycogen storage and inflammatory markers to elucidate the role of the microbiome metabolites on the gut-liver crosstalk during a state of subclinical metabolic inflammation. Modulating the gut microbiome-host links through relatively simple and targeted interventions represents a tantalizing therapeutic prospect. Our results suggest that microbiota-derived products are versatile messengers with wide applicability and potentially positive impact on the management of metabolic diseases.

4.3 - Materials and Methods.
All the reagents and chemicals used in this research for cell work were obtained from Merck KGaA (Darmstadt, Germany), unless otherwise stated.

4.3.1 - Selection of strains for the propionate-producing consortium (PPC)
Gut commensal strains were selected based on the reported metabolic pathways for propionate production (succinate, acrylate, and propanediol pathways) (Reichardt et al. 2014; Table 2.1) and as described in Chapter 2 section 2.3.1.

4.3.2 - Assembly of propionate producing community (PPC)
The propionate-producing consortium was assembled as described in Chapter 3 section 3.3.2. It was harvested after 48h incubation at 37°C.

4.3.3 - Metabolic activity of the propionate producing community (PPC)
Short-chain fatty acids were collected from the co-culture, extracted with diethyl ether and used as benchmarks of community activity (De Weirdt et al. 2017). Total SCFA production was defined as the sum of the molar concentrations of acetate, propionate, butyrate, valerate, caproate, isobutyrate, isovalerate and isocaproate (De Weirdt et al. 2017).

4.3.4 - Construction of the insulin resistance model using Caco-2, HT29-MTX and HepG2
The Caco-2, HT29-MTX and HepG2 cells were obtained from the European Collection of Authenticated Cell Cultures (Caco-2 ECACC 86010202; HT29-MTX-E12 ECACC 12040401,
HepG2 85011430; Public Health England, UK). Routine cell maintenance was carried out as previously described in Geirnaert et al. (2017). All the cultures were used between passages 50 and 60. 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).

In the apical compartment, the Caco-2/HT29-MTX cells (90/10) were seeded at a density of 7.5 x 10⁴ cells/cm² on the semipermeable membrane and maintained with Dulbecco’s Modified Eagle Medium with high glucose (4.5 g/L) (DMEM) and GlutaMAX, supplemented with 10% (v/v) heat-inactivated Fetal Bovine Serum (iFBS, Greiner Bio-One, Wemmel, Belgium) and 1% (v/v) penicillin/streptomycin (Life Technologies, Merelbeke, Belgium) until differentiation (15 days). Refreshments of the apical and basal media were performed every 2 days.

At day 15 post-seeding, HepG2 cells (5 x 10⁴ cells/cm²) were seeded into the basal compartment in Minimum Essential Medium Eagle (MEM) supplemented with 10% (v/v) iFBS and 1% (v/v) GlutaMAX™. The co-culture was maintained for 5 days, refreshing the apical and basal compartments every 2 days with respectively DMEM-HG or MEM without antibiotic/antifungal the apical and basal compartment, respectively. Cells were maintained at 37°C in a humidified atmosphere and 10% CO₂.

4.3.5 - Cell treatments

HepG2 cells were maintained with two different D-glucose concentrations: 5mM, referred as low-glucose or 30mM, referred to as high-glucose (Weil et al. 2009), added to the basolateral compartment of the Transwell system. In order to develop a model of insulin resistance, HepG2 cells were exposed to high glucose treatments and maintained for 24 hours, followed with 100 nM insulin for 15 min at the end of the incubation as described by Cordero-Herrera et al. (2014). High glucose condition was used in the model to mimic a case of insulin resistance that is considered one of the parameters of diabetes. Additionally, filter-sterilized supernatants from the treatments described in Annex III, Table S1, were diluted 1:5 (v/v) in DMEM without supplementation and added to the apical compartment. This dilution ratio was used to avoid the cytotoxicity from the bacterial supernatants on the cells. SCFAs from a 48-hour culture of the PPC and of A. muciniphila were determined, and used to prepare a synthetic mixture without the bacterial background and having identical concentrations of acetate, propionate and butyrate (Annex III, Table S1). These mixtures were prepared from their corresponding sodium salts, resuspended in DMEM without supplementation and added to the apical compartment. Sampled cells were washed twice with PBS, scrapped, snap frozen and stored at -80°C for further analyses.

DMEM without supplementation (control 1), and sterile bacterial medium diluted 1:5 in DMEM without supplementation (control 2) were used as controls. When no differences between control 1 and control 2 were observed, only control 2 was reported.
4.3.6 - Assessment of epithelial barrier function and cellular viability
The monolayer integrity was assessed by measuring the transepithelial electrical resistance (TEER) and the apparent permeability (P\text{app}) of the paracellular transport marker lucifer yellow (LY), as previously described in Geirnaert et al. (2017).
At the end of the assays, the cellular viability was assessed by Trypan Blue staining and manual cell count using a Neubauer chamber and a PrimoVert Zeiss Microscope (Zeiss, Belgium). All treatments revealed that viable cells were above 80% of the total.

4.3.7 - Quantification of inflammatory markers
IL-8, IL-6, and TNF-α were quantified under high and low glucose treatments using the Human IL-8 (CXCL8), IL-6, TNF-α and Mini ABTS ELISA Development Kit (PeproTech, Rocky Hill, USA). The detection limits according to the manufacturer were as follows 31 pg/ml TNF-α, for 24 pg/ml IL-6 and 8 pg/ml IL-8.

4.3.8 - Glycogen quantification and Lipoprotein Lipase (LPL) activity following insulin resistance challenge
Glycogen quantification was performed in HepG2 cells for all treated and control samples following the protocol for Glycogen Assay Kit (Cayman Chemical, Item No. 700480).
LPL Assay Kit was performed for all treated samples and controls following the manufacturer instructions (Cell Biolabs, catalog No.STA-610). The detection limits according to the manufacturer were as follows 2.5ug/ml (±0.5ug/ml) glycogen, and ~1 mUnits/mL LPL.

4.3.9 - Statistical analysis
All multiple pairwise comparisons were done in GraphPad (GraphPad Prism 7.04, La Jolla, CA, USA) using one-way Anova and Tukey's comparison. Statistical significance was assumed at $P < 0.05$.

4.4 - Results.
4.4.1 - Metabolites from the propionate-producing microbial consortium increased glycogen storage in hepatic cells
The enterohepatic model was exposed to different treatments under the low and high glucose conditions. Treatments included metabolites from the PPC, from A. muciniphila, from PPC-Akk, and the synthetic SCFA mixture that was prepared without bacterial background and that contained the same amounts of the main SCFAs produced by the PPC and by A. muciniphila.
Control condition was considered when none of the mentioned treatments were added.
Under control conditions, glycogen levels in the enterohepatic model were 9.4 ± 0.9 µg/mL, and no significant differences between low and high glucose treatments were observed (Figure 4.1) (Annex III, Table S2). HepG2 cells alone, without contact with Caco-2/HT29-MTX cells stored significantly lower glycogen when low (3.2 ± 0.1 µg/mL) and high glucose (6.4 ± 0.5
µg/mL) were supplied (Annex III, Table S2). This result reveals active cross-talk between cell types, which could promote glycogen synthesis or insulin-response in hepatic cells.

When the enterohepatic model was exposed to PPC treatment and high glucose, HepG2 cells stored significantly higher levels of glycogen (22.6 ± 4.3 µg/ml) than the control (9.4 ± 0.9 µg/ml) \( (P < 0.001) \), A. muciniphila (9.4 ± 0.8 µg/ml) \( (P < 0.001) \) or PPC-Akk (9.8 ± 0.5 µg/ml) \( (P < 0.01) \) treatments, which was not observed when low glucose concentrations were supplied (Figure 4.1) (Annex III, Table S2). After the exposure of the enterohepatic model to SCFA levels comparable to PPC and Akk, the glycogen content in HepG2 varied between 7.7 and 11.4 µg/ml, without significant differences between high or low glucose treatments (Figure 4.1) (Annex III, Table S2).
Figure 4.1: Glycogen levels were significantly higher when the metabolites from the PPC were added to the enterohepatic cell model in high glucose treatments. Other treatments did not have any significant effect on the glycogen levels neither on the low nor in the high glucose treatments. PPC, propionate-producing consortium; PPC-Akk, propionate-producing consortium without *A. muciniphila*; Akk, *A. muciniphila* alone; SCFA PPC, synthetic mix of SCFA in identical concentrations to those determined in the PPC; SCFA Akk, synthetic mix of SCFA in identical concentrations to those determined in a pure culture of *A. muciniphila*. Different letters above the bars indicate significant difference ($P < 0.05$).

4.4.2 - Metabolites from PPC promote LPL activity in the hepatic cells

LPL is a crucial enzyme for catalyzing the hydrolysis of triglycerides (TG) in circulation and plays a critical role in regulating plasma LPL activity and lipid metabolism (Liu et al. 2016). However, little attention has been paid to LPL because of its low expression in adult liver.

LPL levels were not significantly different from the control when the metabolites from *A. muciniphila* alone, and the PPC-Akk were added to the enterohepatic model. In contrast, when we added the metabolites of the PPC to the enterohepatic cell model, we observed a significant increase on LPL levels ($21.191 \pm 0.658$, $P<0.05$) when cells were exposed to high glucose concentrations (Figure 4.2) (Annex III, Table S3). This indicates that the metabolites produced by our PPC enhance LPL levels, which in turn may regulate plasma LPL activity and lipid homeostasis.
4.4.3 - Metabolites from PPC tended to decrease inflammation in liver cells without cytotoxic effects

IL-8 levels in the control treatment varied from 3047 ± 36 pg/mL when low glucose was supplied to 3532 ± 135 pg/mL in the high glucose treatment (*P < 0.01*). IL-8 produced by Caco-2/HT29-MTX alone was not significantly different between low and high glucose treatments, but it was lower than that in the enterohepatic model (1256 ± 263 pg/mL, *P < 0.001*) (Figure 4.3) (Annex III, Table S4). A lowering effect on IL-8 was only observed in the high glucose treatment following addition of the metabolites of the PPC (PPC: 2802 ± 219 pg/mL; Akk: 3034 ± 61 pg/mL) while no change was observed in the low glucose condition (Figure 4.3) (Annex III, Table S4). In contrast, the metabolites of the PPC, PPC-Akk or Akk did not significantly impact IL-6 levels in the basolateral compartment when compared to the control treatment (Figure 4.4) (Annex III, Table S5). As for the inflammatory marker TNF-α, we observed that the metabolites from *A. muciniphila* significantly increased the TNF-α levels in the basolateral compartment compared to all other treatments (Figure 4.5) (Annex III, Table S6). Our results
indicate that metabolites from the PPC have the tendency to decrease inflammation markers like IL-8 and TNF-α associated to insulin resistance and diabetes, while counteracting cytotoxicity as IL-6 remained unchanged. However, results from the supernatant of *A. muciniphila* indicated that the bacteria can induce inflammation in case of insulin resistance.

**Figure 4.3:** Metabolites from the PPC tended to decrease IL-8 levels when added on the enterohepatic cell model with insulin resistance. Data were compared using one-way ANOVA following by Tukeys test to test the difference between the different treatments. No significant difference was observed between treatments (*P* > 0.05).
Figure 4.4: Metabolites from the PPC did not have any effect on IL-6 levels. Data were compared using one-way ANOVA following by Tukeys test to test the difference between the different treatments. No significant difference was observed between treatments ($P>0.05$).

Figure 4.5: Metabolites from *Akkermansia* significantly increased TNF-α in the insulin resistance enterohepatic model. Metabolites from the PPC and other treatments did not cause an increase in TNF-α levels. Data were compared using one-way ANOVA.
following by Tukey's test to test the difference between the different treatments. Different letters above the bars indicate significant difference ($P<0.05$).

4.4.4 - Epithelial barrier function was maintained upon the addition of bacterial consortia-derived metabolites

The epithelial barrier integrity of the enterohepatic model was evaluated by two functional measures of the tight junctions: TEER and Papp of LY. Prior to the treatments, the enterohepatic model had average TEER values ($n=6$) of 682 ± 46 Ω, without significant differences between plates. TEER values of Caco-2/HT29-MTX alone were significantly lower (483 ± 22 Ω; $n=6$, $P<0.01$). TEER values were maintained above 80% of the initial TEER during the 24 hours of exposure of the cells to the different treatments, indicating that the epithelial barrier function was not disrupted during the assay (Annex III, Table S7).

Low and high glucose levels influenced the TEER values in models not exposed to microbial metabolites (630 ± 32 Ω and 575 ± 49 Ω, respectively, $P<0.05$). On the contrary, glucose concentration did not impact TEER values in the other treatments. However, TEER was significantly improved when the PPC metabolites were supplied, in comparison with the unexposed controls challenged with low (PPC: 707 ± 33; control: 630 ± 32 Ω) or high (PPC: 677 ± 16; control: 575 ± 49 Ω) glucose concentrations. The same trend was observed in the synthetic mixture of SCFA mimicking the PPC-derived SCFA, with values significantly higher than the controls in the low (676 ± 49 Ω) and high (663 ± 12 Ω) glucose levels ($P<0.05$). However, in the case of *A. muciniphila*, there was a significant improvement only in the case of high glucose (Akk: 639 ± 24; control: 575 ± 49 Ω) ($P<0.05$), compared to low glucose (Akk: 607 ± 11; control: 630 ± 32 Ω). No significant effect on TEER values for the synthetic mixture of SCFAs from Akk was observed neither in low nor high glucose conditions.

In addition, the percentage of LY transport in the enterohepatic model was below the threshold for considering damage in the epithelial barrier (1.7 ± 0.3%), but LY transport was significantly higher in the Caco-2/HT29-MTX alone (1.2 ± 0.04%, $P<0.01$). Small but significant differences were observed between the enterohepatic model exposed to low (1.2 ± 0.04 %) and high (1.5 ± 0.1 %) glucose levels in the control treatment ($P<0.05$). LY transport in the high glucose treatment after exposure to the PPC (1.32 ± 0.09 %) or *Akkermansia* (1.29 ± 0.06%), was significantly decreased ($P<0.01$), but not when the PPC-Akk metabolites were added (1.51 ± 0.05%) (Annex III, Table S8).

We only consider the epithelial barrier assessment as a proof-of-concept of the impact on the epithelial barrier integrity during the assay.
4.5 - Discussion

The gut-liver axis communicates organs of the digestive system through the biliary tract, portal vein and systemic cross-talk, hence gut factors can regulate liver glucose and lipid metabolism (Tripathi et al. 2018). Our in vitro model may possess some limitations associated to the cancer origin of the cell lines, because some differences in protein expression between the in vitro models and in vivo samples may be present. However, the stable phenotype, unlimited life span and high availability increase the reproducibility and repeatability of the assays. In addition, the co-culture of multiple cell lines may change the phenotype and cell behavior, generating unique and representative in vitro models. We observed significant differences between previously reported cell models like Caco-2/HT29-MTX alone and HepG2 alone and our enterohepatic model in terms of epithelial barrier function and functionality. In addition, the model was developed by the combination of simulated epithelial layer of Caco-2 (enterocyte-like) and HT29-MTX cells (goblet-like) cells, in basolateral contact with HepG2 (hepatocyte-like). For this reason, the use of our model as an improved tool for mimicking the enterohepatic system is representative.

Previous research demonstrated the relationship between gut microbiome and metabolic diseases. Abnormal shifts in the composition of gut microbiota contribute to metabolic diseases, including obesity, type 2 diabetes (T2DM) and cardiometabolic diseases (Cani and de Vos 2017, Zhao et al. 2018). The main characteristics of obesity and type 2 diabetes are altered gut microbiota, gut barrier disruption, and inflammation (Zhao et al. 2017). A key element in the gut-liver crosstalk is the intestinal permeability, mainly maintained by tight junction complexes (Tripathi et al. 2018). Hyperglycemia and insulin resistance have been associated with several inflammatory factors which may lead in reduced epithelial barrier function (König et al. 2016; Thaiss et al. 2018). Glucose causes disruption for tight junctions increasing insulin resistance. New research published in Science demonstrates that hyperglycaemia is linked to and drives intestinal barrier dysfunction and the resultant risk of systemic dissemination of enteric infection in animal models (Thaiss et al. 2018). The findings pinpoint glucose levels as having a key role in influencing intestinal barrier function, and highlight a potential mechanism for the altered intestinal epithelial integrity observed in the metabolic syndrome (Thaiss et al. 2018). As a result, there was a disruption in the epithelial barrier function that led to its reduction. Therefore, lost of epithelial barrier may allow the passage of bacterial metabolites (e.g SCFA) to the basolateral compartment, thus increasing the contact of HepG2 cells with bacterial compounds.

When the enterohepatic model was exposed to glucose levels and insulin mimicking insulin resistance (30 mmol/L), the epithelial barrier function was significantly reduced. However, supplementation with PPC- or Akk-derived metabolites restored this parameter, indicating that other bacterial metabolites besides SCFA may be relevant for regulating the intestinal barrier.
function. Pili-like proteins from *A. muciniphila* can modulate the gut barrier function and strengthen the integrity of the epithelial Caco-2 cell layer (Ottman et al. 2017). We observed that the metabolites derived from the PPC-Akk did not support the recovery of the intestinal barrier, suggesting that *A. muciniphila* may be a key element in regulating intestinal permeability. Indeed, *A. muciniphila* has been proposed as a promising candidate for the treatment or prevention of metabolic disorders (Cani and de Vos 2017). However, the metabolites from *A. muciniphila* alone or the consortium without *A. muciniphila* were not able to significantly increase glycogen storage or LPL levels in hepatic cells in the case of enterohepatic model of insulin resistance. This effect may be caused by the different composition of SCFA in the different treatments. This mainly suggests that *A. muciniphila* is not able to accomplish all the jobs the PPC was able to do, signifying the *A. muciniphila* requires the presence of all the other members of our designed consortium in order to achieve fully. Moreover, this implies that there is a syntrophic relationship between all the seven members of the PPC that lead to their optimal effect.

We revealed the ability of the metabolites from our PPC to increase glycogen storage, while a synthetic mixture of SCFA failed to replicate such effect. This finding confirms that other bacterial metabolites beyond SCFA may act as signaling factors in glycogen synthesis. Studies have shown that liver glycogen accumulation can protect against the negative effects of high fat diet, such as glucose intolerance (López-Soldado et al. 2014). Accumulation of hepatic glycogen contributes to decreased food intake and lower body weight and adiposity (Ritter et al. 1994; Grill et al. 1995; López-Soldado et al. 2014). For this reason, hepatic glycogen can act as a potential target for pharmacological manipulation of diabetes and obesity (López-Soldado et al. 2014). Our findings stress the importance of the role that microbiota-derived metabolites have on the gut-liver crosstalk and ultimately on the glycogen regulation and energetic metabolism. Gut-derived SCFAs play important roles as substrates for glucose, cholesterol and lipid metabolism (den Besten et al. 2013). Propionate can enhance the lipid buffering capacity of adipose tissue by increasing LPL-mediated triglyceride extraction, resulting in reduced lipid overflow to the adipose tissue (Canfora et al. 2015). We observed that only the metabolites from the PPC significantly contributed to increased LPL levels in hepatic cells. While *A. muciniphila* alone mainly produced acetate, the PPC contained a mixture of propionate and acetate. Acetate has been reported to act as lipogenic substrate, but propionate suppresses lipogenesis through decreased expression of fatty acid synthase (Canfora et al. 2015). Thus, the optimal effect of the PPC was obtained when all seven members of the consortium including *A. muciniphila* were present. Successful cross-feeding interactions between gut microbes may be fundamental for *de novo* gluconeogenesis (den Besten at al. 2013) and attenuation of lipogenesis.
IL-8 levels in the enterohepatic model were higher than in the Caco-2/HT29-MTX co-culture, indicating an active production of IL-8 by HepG2 cells (Gómez-Quiroz et al. 2003). The supernatant from our designed PPC tended to decrease IL-8 levels, implying the presence of potential anti-inflammatory properties. IL-8 is a pro-inflammatory adipocytokine linked to insulin resistance, and its reduction may prevent diabetes (Kobashi et al. 2009). TNF-α has also been reported as key molecule contributing to metabolic syndrome features such as obesity and insulin resistance (Hotamisligil et al. 1993, Miyazaki et al. 2003). Similarly, IL-6 is another cytokine that may contribute to the pathogenesis of obesity and insulin resistance (Jung and Choi 2014) but this correlation is still controversial (Di Gregorio et al. 2004). Propionate supplementation has been reported to reduce mRNA expression and secretion of TNF-α (Canfora et al. 2015). Studies have also reported that TNF-α is increased during insulin resistance, and Akkermansia is able reduce TNF-α levels (Zhang et al. 2018). Surprisingly, we could observe that supernant of A. muciniphila increased TNF-α levels in the insulin resistance enterohepatic model indicating a pro-inflammatory property. Ottman et al. (2017) previously reported that A. muciniphila induced TNF-α in human derived peripheral blood mononuclear cells (PBMCs), indicating that A. muciniphila cannot be strictly defined as anti- or pro-inflammatory, but may instead play a major role in preserving the balance of the gut ecosystem. In addition, We uncovered that IL-6 levels were not affected by any of our treatments. As IL-6 is an important inducer of the acute phase response and infection defense, our results indicate that the PPC- and the A. muciniphila-derived metabolites are not cytotoxic and do not produce liver injury.

4.6 - Conclusion.
In conclusion, our findings provide a novel overview on the gut microbiome-liver crosstalk and its positive effect on metabolic function and low-grade inflammation. The optimal effect of the consortium was obtained when all members were present and successfully cross-feeding, but longer supplementation periods and higher number of doses may be required for a long-term effect. Targeted interventions for managing metabolic diseases have become relevant on the past years. Nevertheless, using members of our native microbiome may represent a promising strategy, as their adaptability and functional versatility can be sourced for engineering multipurpose consortia to prevent and counteract risk factors of metabolic disease.

4.7 – Acknowledgements
The authors would like to thank the People Program (Marie Curie Actions) of the European Union’s Seventh Framework Program FP7/2007–2013/under REA grant agreement n_606713 for the research funding.
Chapter 5
General Discussion
CHAPTER 5 - GENERAL DISCUSSION.

5.1 - Positioning the research

Metabolic syndrome is defined by the WHO as a pathologic condition characterized by abdominal obesity, hypertension, insulin resistance, and hyperlipidemia (Saklayen 2018). This syndrome is associated with other risk factors such as type 2 diabetes, cardiovascular disease, stroke and other comorbidities (Alberti et al. 2006; Saklayen 2018). It has been reported by the International Diabetes Federation (IDF) that the prevalence of metabolic syndrome is around 25% of the world’s adult population (O’Neill and O’Driscoll 2015) and this percentage can vary with ethnicity, age, gender, and race (Kaur 2014). Higher prevalence of metabolic syndrome leads to higher morbidity and mortality (Moreira et al. 2014). Moreover, patients with metabolic syndrome have a fivefold higher risk to develop diabetes than people without metabolic syndrome (Alberti et al. 2006). As a result, this non-communicable disease has become a major public health issue (Saklayen 2018).

Studies in animals and humans have confirmed that the gut microbiota exerts a significant role in the pathogenesis of the metabolic syndrome (Festi et al. 2014). Research has provided evidence that the gut microbiota can impact host metabolic balance by modulating energy absorption, appetite, gut motility, glucose and lipid metabolism, in addition to hepatic fatty storage (Festi et al. 2014). Gut microbial composition and functions are influenced by different factors such as diet, use of antibiotics, mode of delivery at birth, genetics and other environmental factors (Wen and Duffy 2017). Therefore, dysbiosis in the human gut microbiota caused by any of those factors impacts the host energy balance leading to metabolic disorders (He et al. 2018). Studies have shown that the manipulation of the gut microbiota by the administration of probiotics can reduce intestinal low-grade inflammation and improve gut barrier integrity thereby enhancing metabolic balance and endorsing weight loss (Festi et al. 2014). However, the phylogenetic origin of probiotics is currently limited to conventional formulations of Bifidobacterium, Lactobacillus species and other lactic acid bacteria (LAB) (Govender et al., 2013) or yeast strains. This may decrease the probiotic effectiveness in the prevention or therapy of diseases entailing severe dysbiosis. Hence, a functionally and phylogenetically diverse probiotic product may be desirable when alterations in the gut microbiota composition are present (Marotz and Zarrinpar 2016; El Hage et al. 2017).

Propionate, a major short chain fatty acid in the gut that has been less studied compared to the other metabolites, provides different health-promoting effects (Hosseini et al. 2011). Propionate has anti-lipogenic, cholesterol-lowering, glucose-controlling (Kasubuchi et al. 2015), anti-inflammatory and anti-carcinogenic effects (Hosseini et al. 2011; Vinolo et al. 2011). Moreover, propionate can enhance satiety as it can upregulate the postprandial plasma peptide YY (PYY) and glucagon-like peptide-1 (GLP-1) hormones from colonic cells and
induce leptin production (Hosseini et al. 2011) which could be very interesting in the time in which the incidence of obesity is increasing around the world due to different factors. Studies that involved proteomic work suggested that the effects of propionate at the cellular level can differ from the action of butyrate (Kilner et al. 2012; Reichardt et al. 2014). The SCFA receptors FFA2 and FFA3 can mediate some of the actions of propionate, but still more investigation is needed to determine the exact mechanisms (Ulven 2012). Evidence showed that propionate can activate FFAR3 leading to increase in heart rate and energy expenditure through sympathetic activation. This sympathetic activation by FFAR3 leads to the release of noradrenalin from the sympathetic neurons (Inoue et al. 2012) indicating that FFAR3 regulates sympathetic activity by sensing the nutritional state, thus maintaining body energy homeostasis (Kasubuchi et al. 2015). For this reason, enhancing propionate production in the gut is an fascinating approach to increase satiety and maintain metabolic health (Reichardt et al. 2014).

Our research aimed at establishing a propionate-producing consortium composed of seven different strains of the human commensal gut bacteria. This designed consortium could be used as synthetic community that can boost propionate production in the gut and improve metabolic health.

Our research questions were:

1) Would our designed propionate-producing bacterial consortium restore the propionate production after the antibiotic-induced dysbiosis in the colon?

2) How do metabolites from our designed consortium act on an enterohepatic cell model of hyperglycaemia?

To unravel this puzzle, we tested the effect of our engineered propionate-producing consortium on an antibiotic-dybiosed bacterial community in vitro, and we investigated the effect of the metabolites from our consortium on an enterohepatic in vitro cell model of hyperglycaemia. Moreover, we studied the survival of our consortium in an in vitro model of the upper GIT under its harsh conditions. Three research chapters elaborate on the different outcomes of our research.

5.2 – Main research outcomes

The main research outcomes are summarized in figure 5.1.

In Chapter 2, we discussed the establishment of the propionate-producing consortium (PPC) and investigated in vitro survival through the upper gastrointestinal tract. Our candidates for the consortium were selected to cover all the different metabolic pathways that lead to propionate production. All the strains were grown separately and then co-cultured together in a fed batch experiment for 48 hours to perform the possible cross-feeding leading to high propionate production (average of 34.5 mM). The PPC was always freshly prepared and
harvested after 48 hours to perform all the different tests throughout this research. After testing the survival in the upper GIT *in vitro* (TSI model), we observed that not all the strains survived the harsh conditions of the upper GIT, especially with the different concentrations of bile salts throughout the small intestine. This suggests the importance of having a protectant carrier, such as an encapsulation technique, to protect the consortium through its passage in the upper GIT and release it in the colon, where the consortium should take action.

**Chapter 3** investigated the functionality of our propionate-producing consortium following antibiotic induced dysbiosis in the M-SHIME system. Our designed consortium has proven to restore functionality in terms of propionate production for the dysbiosed microbial community *in vitro*. Moreover, the PPC restored functionality by either direct engraftment of some of its members or indirect reinforcement for other genera involved in propionate production. In addition, our designed consortium supported the partial recovery of the epithelial mitochondrial membrane potential after antibiotic disruption.

The gut-liver crosstalk was investigated in **Chapter 4**, where the supernatant from our designed consortium was added on an enterohepatic cell model of hyperglycaemia. Liver glycogen storage and inflammatory markers were evaluated to assess the role of the gut microbiota metabolites on the gut-liver crosstalk under subclinical metabolic inflammation. Glycogen storage and LPL activity in the hepatic cells were increased when the supernatant from the PPC was added. Furthermore, the metabolites from the PPC decreased inflammation in the hepatic cells. The effects of the supernatant from the PPC were compared to those of *Akkermansia muciniphila*, and the results indicated that the PPC without *A. muciniphila* or *A. muciniphila* alone lacked the same positive effects. This indicates that *A. muciniphila* can only effectively act when combined together with all the other members of the consortium, and the optimal effect of the consortium is only obtained when all members are present and successfully cross-feeding. For long-term effect, longer supplementation periods and higher number of doses may be required.
5.3 – Connecting antibiotic-associated dysbiosis to metabolic syndrome

The intestinal microbiota plays a critical role in the aetiology, development and modulation of several diseases. Amongst the important global and public health diseases are obesity and metabolic syndrome (Economopoulos et al. 2016). The role of gut microbiota in metabolic syndrome includes several mechanisms such as increased energy harvest, production of toxic metabolites, and an increase in intestinal permeability leading to high levels of lipopolysaccharides (LPS) in the systemic circulation, and thereby low-grade inflammation (Economopoulos et al. 2016). Metabolic syndrome in adults can be caused by a disturbance in the gut community as a result of excessive and misuse of antibiotics in early life (Economopoulos et al. 2016). Antibiotics can improve human lives by treating or preventing diseases and they are prescribed in most countries (Quigley 2011). However, antibiotic consumption can alter gut microbial ecology and the interaction with the host metabolism (Perez-Cobas et al. 2013). In Chapter 3,
the negative effects on the gut microbiota upon antibiotic addition were evaluated, and we observed a decrease in diversity in the colon community in addition to decrease in cell count. Moreover, we detected that addition of clindamycin caused disruption of the mitochondrial membrane potential when it was added to the caco-2 cells.

5.4 - Restoration of dysbiosis with “microbial-based” products

The intestinal microbial community contributes to important functions in the body such as nutrient metabolism, calibration of metabolic functions, education of the immune system, and host defence against pathogens (Flint et al. 2012; Maranduba et al. 2015). For this reason, gut dysbiosis, or the disturbance in the gut microbiome balance can lead to several pathologies. During dysbiosis, there is loss of overall microbial diversity and parallel overgrowth of pathobionts (Schippa et al. 2012; Petersen and Round 2014; Carding et al. 2015; Gagliardi et al. 2018) as we could observe in our case upon addition of clindamycin in Chapter 3. To maintain the eubiotic state of the gut microbiome, several strategies have been followed including administration of probiotics, prebiotics, symbiotic, faecal microbial transplant, and bacterial consortium transplant (Gagliardi et al. 2018). All of these strategies aim to replace the harmful microbes with favourable ones to restore the balance.

Preclinical studies have been conducted and proven that there are promising results with strains that are different from the classic Lactobacillus and Bifidobacterium strains (Neef and Sanz 2013; Patel and DuPont 2015). These strains could be related to next generation probiotics including Akkermansia muciniphila, members of Clostridium clusters IV, XIVa, and XVIII, and F. prausnitzii (Schneeberger et al. 2015). As next-generation probiotics must include strains belonging to major gut microbiota groups, should be safe and possess potential beneficial effects (Martin et al. 2017), more strains with the mentioned characteristics could be considered potential candidates for next generation probiotics (Gagliardi et al. 2018). Since the cooperating nature of microbiomes seems to be an indispensable trait of the gut microbiota in health and disease, studies should consider developing multi-species consortia impacting this system of cooperating organisms and that ensures the strong and long-term restoring effect. For instance, a live biotherapeutic was developed by selecting 100 different commensal strains with a range of abundant numbers in the microbiota and successfully modulated the intestinal microbiota of elderly people (Le 2017). In addition to this successful biotherapeutic, other studies were conducted with multi-strain bacteria and showed promising results. These studies included the combination of 17 Clostridium strains of human origin, which reduced the severity of colitis in rodents, and the mixture of VSL#3 (composed of Lactobacillus casei, Lactobacillus plantarum, Lactobacillus bulgaricus, and Lactobacillus acidophilus; Bifidobacterium longum, Bifidobacterium breve, Bifidobacterium infantis; and S. thermophilus) that showed positive effects in ulcerative colitis treatment (Madsen 2001). Moreover, other multi-species consortia showed positive effects on IBS and other diseases (Gagliardi et al. 2018).
This indeed emphasizes the importance of having a multi-species biotherapeutic that can actually modulate the gut microbiome and not to be restricted to using bacteria with incomplete functionality and single phylogenetic origin. In addition, omics technologies could assist to establish networks of bacterial consortia that work jointly to influence human physiological processes (Gagliardi et al. 2018).

5.5 – Importance of tailored therapies to restore gut dysbioysis

Bacterial consortium transplant can modulate the gut microbial ecosystem, and studies have confirmed a complete recovery from antibiotic-induced intestinal dysbiosis with either FMT or bacterial consortium transplant, indicating that the effects of bacterial consortium transplant were similar to those of the FMT (Li et al. 2015; Petrof and Khoruts 2014). Therefore, a characterized community developed from faecal bacteria can be used to substitute FMT (Sbahi and Di Palma 2016). RePOOPulate, is a bacterial consortium established from 33 different gut bacteria isolates from a healthy human donor, and it was used to treat recurrent CDI (Petrof et al. 2013). As a result, biotherapeutics or synthetic microbial consortia for treatment or prevention of disease can be assembled from native commensal gut bacteria with beneficial properties (Olle 2013; Ding et al. 2016). Bacterial consortia are usually well defined and characterized and may guarantee standardization when the proportions of each bacterium in the consortium are defined. They could also be tailored to the type and level of dysbiosis (Gagliardi et al. 2018). Furthermore, patients’ safety could be ensured with an in-depth characterized community such as the bacterial consortium transplant, rather than the uncharacterized nature of the FMT. Several studies have been conducted to determine personalised therapies to rebalance the intestinal ecosystem; however, the challenge is that there is no universal cure due to different factors impacting the gut microbiome homeostasis. Prescription of tailored microbial therapies specific for different types of dysbioises should carefully consider the gut microbiota in each patient by assessing data from “omics” technologies (Gagliardi et al. 2018). These platforms could allow for characterizing the microbial community in each patient and its functions and examining the genetic potential of the community (Gagliardi et al. 2018).

The low-diversity dysbiosis in the gut is characterized by the changes in bacterial metabolic flux, and decreased relative abundance of many species including Lachnospiraceae and Ruminococcaceae (Kriss et al. 2018). Similar scenario was observed upon the addition of clindamycin during our SHIME run. Our tailored propionate-producing consortium restored the in vitro propionate production altered upon the clindamycin-induced gut dysbiosis, and it was also able increase the relative abundance of Lachnospiraceae (Chapter 3). However, diversity and richness of the adult microbiome may be a poor marker of dysbiosis, as this could also be caused by different factors such as transit time (Bajaj et al. 2017). Nevertheless, dysbiosis can also come in many different forms, some of which have no change or increase in diversity, so
efforts to reverse the low-diversity dysbiosis must consider the different attributes of the specific diseases detected (Kriss et al. 2018). For this reason, considering tailored bacterial consortia for specific diseases is also crucial.

5.6 – Practical considerations for microbial-based products

5.6.1- Interactions between bacteria in the synthetic microbial consortium

The human microbiota refers to a microbial ecosystem living in the human body that has been linked to human health and disease status (Wang et al. 2017). The development of biotherapeutics is rapidly evolving, and their potential benefits have been recognized for some time although their effectiveness is not completely demonstrated yet (Chapman, Gibson and Rowland 2011). In fact, synthetic microbial consortia could perform better than monocultures, thus providing a new frontier for synthetic biology (Brenner, You and Arnold 2008; Gerchman and Weiss 2004; Purnick and Weiss 2009). The design principles for the synthetic microbial consortia are based on the interactions between microbes including the cell-to-cell communication and metabolites exchange (Ding et al. 2016). It is important to focus on the key signal molecules, on the production, diffusion, absorption, consumption, and response of metabolites in order establish and improve the synthetic microbial consortium (Ding et al. 2016). In addition, selecting phylogenetically distant species for co-culturing leads the consortium to gain novel functionality and higher production efficiency (Lindemann et al. 2016). Thus, fitness mechanisms and cross-feeding interactions are the main considerations to obtain a stable, efficient and controlled synthetic microbial consortium (Ding et al. 2016). As a result, preadaptation of the seven strains in our consortium was important as it mimics the community characteristics in terms of the different competitive and cooperative interactions that occur between bacteria in communities. Ecological studies have reported that organisms in nature interact concurrently by competing for some resources while exchanging others. Over time, these microbial behaviors create interspecies dependencies which is established by differing specialized phenotypes across various microbes. These facts cause a key challenge for engineering consortia with stable interactions as functions across a microbial population has to be understood in order to achieve desirable population-level behaviors (Johns et al. 2016). When designing our propionate producing consortium, the different metabolic pathways for propionate production were considered. The selected seven different strains (Figure 5.2) from different phylogenetic origin were supposed to cross-feed using the metabolites described in Chapter 2. Moreover, to optimize and get a reproducible propionate-producing consortium, different incubation durations and feeding patterns were assessed to obtain the optimal propionate concentrations.
Figure 5.2: The different pathways for propionate production (Acrylate, Succinate, and Propanediol). The seven different bacteria that we selected are specified in each pathway they take to produce propionate. This figure has been adapted from Louis et al. (2014).

5.6.2 – Required dosing
The doses of probiotics needed to achieve the clinical efficacy is variable. Recommendation for the products containing probiotics is a minimum viable cell number of $10^6$ and $10^8$ CFU/g of the product final or $10^8$–$10^{10}$ CFU/day (Champagne et al. 2011). Although probiotics are generally labelled as safe, they could also cause unfavourable events in some cases, such as systemic infections, altered metabolic pathways, intensified immune stimulation, gene transfer, and gastrointestinal disorders. Thus, additional studies are needed to specify the occurrence and severity (Doron and Snydman 2015; Gagliardi et al. 2018). Additionally, the minimal dose or numbers required to achieve a probiotic effect have not been described. The dose required
to treat an acute illness with a specific probiotic can be lower or higher in order of 10-100 folds or more in terms of CFU (Minelli and Benini 2008). For instance, in acute infectious diarrhoea, higher doses for short term seem to be more effective than low doses, while in chronic or immunological disease, the effect also counts on the durations of the treatment (Minelli and Benini 2008). As a result, it can be deduced that probiotic effects seem to be dose-dependent. Nevertheless, since most of the studies are done in vitro, the dose effect stays controversial. It has also been reported that the usual effective dose for probiotics in humans is $10^7-10^9$ CFU/mg per day (Minelli and Benini 2008). Studies have indicated that dose, timing and duration of the treatment are important to determine the optimal effect of the probiotic (Minelli and Benini 2008). It could also be possible that the probiotic colonization is impacted by the quantity of the native microbiome present (BC Dairy 2019) that can differ between individuals. In this context methods for quantification of numbers coupled to beneficial bacterial activity are needed. In Chapter 3, we described how we quantified the absolute numbers of each taxon, upon rescaling the relative abundance of each genus based on flow cytometry cell counts, as described by Props et al. (2017). Moreover, Vandeputte et al. also reported that a workflow for the quantitative microbiome profiling can be built through parallelization of amplicon sequencing and flow cytometric enumeration of microbial cells (Vandeputte et al. 2017). However, as our study has been conducted in vitro, no final conclusion can be made about the required dose of the PPC that is needed to reach the long-term positive effect. Yet, the positive effect on the mitochondrial membrane potential with only one dose of PPC supernatant (Chapter 3), suggests that a full recovery may be achieved with more doses. This proposal of multiple doses could also apply to the hyperglycaemia-induced cell model, where the supernatant from only one dose of PPC promoted positive effects (Chapter 4).

5.6.3 - How to protect the biotherapeutic from the harsh conditions during the passage in the GI tract?
Overall, biotherapeutics or synthetic consortia need to maximize its accumulation in the targeted compartment as any administered drug. This would require four key components: the delivery vehicle, the necessary stability to reach the target site, retention within the intended site, and proper timing for the release of the consortium to function effectively (Bae and Park 2011).
To reach its target site, which is the colon, the biotherapeutic or the synthetic microbial consortium has to pass the harsh conditions of the upper GI tract including gastric acid and bile salts. In the case of the propionate-producing consortium, the strains used were anaerobic and cannot survive the acidic conditions, so they will need a protectant carrier. Microencapsulation is a protectant technique that has been reported to protect probiotics during their passage in the gastrointestinal tract; it also allows the diffusion of substrates and metabolites in and out of the capsule (Mortazavian et al. 2012). Microencapsulation also aims
to make the probiotic reach the target site in the gastrointestinal tract, and it guarantees immunomodulation, thus there will be no interaction between the immune system and the entrapped bacteria (Prakash and Jones 2005; Prakash and Martoni 2006). It has been reported that microencapsulation can preserve probiotic cells from detrimental conditions such as low pH and high acidity (Sun and Griffiths 2000), bile salts (Lee and Heo 2000), molecular oxygen in case of obligatory anaerobic microorganisms, and heat and cold shocks during processing techniques (Mortazavian et al. 2012). This enhances the survival of bacteria in foods and dairy products (Solanki et al. 2013). In our study, the testing took place in the transverse compartment, as this consortium is supposed to be protected by a certain capsule that releases its content in the transverse colon where the consortium is supposed to take action.

5.7 – Postbiotics: New concept for gut health

Studies have delivered evidence of several mechanisms leading to the health-promoting effects of selected gut bacteria. These mechanisms include, the modification of gut microbiota, the competitive adherence of epithelium and the mucosa, enhancement of epithelial barrier function, and the modulation of the immune system (Bermúdez-Brito et al. 2012; Vyas and Ranganathan 2012). Although these mechanisms have been linked to bacterial viability (Sanders 2009), recent evidence suggests that viability is not essential to accomplish the health-promoting effects, as not all the mechanistic or clinical benefits are related to this characteristic (Aguilar-Toalá et al. 2018). As a result, the term of paraprobiotic and postbiotic emerged, indicating that viability is not necessity for the health benefits, and this would offer a potential for their use in functional foods (Aguilar-Toalá et al. 2018). Parabiotics, also referred to as non-viable probiotics or ghost probiotics, can confer health benefits when administered in adequate amounts (Taverniti and Guglielmetti 2011; Tsilingiri and Rescigno 2013). Bacterial cell inactivation leads bacteria to be unable of growing, and thus they can retain the beneficial health effects that their viable form provided (de Almada et al. 2016). The term postbiotics can also refer to the metabiotics, biogenics, or metabolites like cell-free supernatants that are considered soluble factors or metabolic by-products secreted by live bacteria or released after bacterial lysis (Aguilar-Toalá et al. 2018). These by-products can provide physiological benefits to the host by offering supplementary bioactivity (Aguilar-Toalá et al. 2018). For instance, bacterial metabolites such SCFAs are considered pivotal mediators of the host-microbiota communication and can elicit several modulatory effects on the host (De Vadder et al. 2014; Vieira, Fukumori and Ferreira 2016). Other soluble by-products resulting from postbiotics are enzymes, peptides, teichoic acids, organic acids, cell surface proteins, peptidoglycan-derived muropeptides, vitamins, endo- and exo-polysaccharides, and plasmalogens (Konstantinov et al. 2013; Oberg et al. 2011; Tsilingiri and Rescigno 2013). Although the health benefits of postbiotics are not fully clarified, scientific records have provided evidence that postbiotics
have different functional properties such as antioxidants, antimicrobial and immunomodulatory, yet not limited to those only. These properties can have a positive impact of microbiota homeostasis or the host signalling and metabolic pathways which impact the physiological, immunological, regulatory and metabolic reactions (Sharma and Shukla 2016; Shenderov 2013). Few studies have reported the findings on postbiotics mainly from Lactic acid bacteria (Cicenia et al. 2014; Konstantinov et al. 2013; Patel and Denning 2013). Aguilar-Toalá et al. (2018) reported some new outcomes on postbiotics coming from next-generation probiotics such as Faecalibacterium prausnitzii. Postbiotics are differentiated by their elemental products such as lipids (e.g. butyrate, propionate, dimethyl acetyl-derived plasmalogen), proteins (e.g. lactocepin, p40 molecule), carbohydrates (e.g. galactose-rich polysaccharides, and teichoic acids), vitamins/co-factors (e.g., B-group vitamins), organic acids (e.g., propionic and 3-phenyllactic acid) and complexes molecules such as peptidoglycan- derived muropeptides, lipoteichoic acids (Konstantinov et al. 2013; Tsilingiri & Rescigno 2013). They are also differentiated by their physiological functions which include immunomodulation, anti-inflammatory, hypocholesterolemic, anti-obesogenic, anti-hypertensive, anti-proliferative, and antioxidant effects (Nakamura et al. 2016; Shin et al. 2010). Shenderov (2013) reported that postbiotics have favourable absorption, metabolism, and excretion abilities, which could indicate high capacity to signal several organs and tissues in the host thereby provoking biological responses. Studies have stated that SCFAs produced by gut microbiota can act as signalling molecules to improve glucose homeostasis and insulin sensitivity and regulate lipid metabolism. These can be achieved through the activation of G protein-coupled receptors (GPRs) which leads to regulation of energy balance thus metabolic homeostasis (Canfora, Jocken, and Blaak 2015; Kimura et al. 2013). The bioactive properties revealed in postbiotics propose that these compounds contribute to human health by enhancing particular physiological effects. The combined effects of postbiotics with other biological metabolites and live microorganisms can result in the protective effect (Thanh et al. 2010). As the exact mechanisms have not been fully elucidated, additional studies and research are needed to explore and characterize new postbiotics, which may contribute to the understanding of the modulation of signalling pathways (Aguilar-Toalá et al. 2018).

In our research (Chapter 3), when the supernatant from the PPC was added together with the clindamycin to the Caco-2 cells, it caused partial recovery to the epithelial mitochondrial membrane potential disrupted when clindamycin was added alone. In addition, in Chapter 4, we detected positive effects of the PPC supernatant when it was added to the enterohepatic cell model of hyperglycaemia. As postbiotics are also referred to as metabolites or cell free supernatants with beneficial health effects, then the supernatant resulting from our propionate-producing consortium has an indication of postbiotic structure. However, in this case the
metabolites are resulting from a designed bacterial consortium that involved seven strains of the commensal gut bacteria and not from conventional probiotic strains.

5.8 – Necessity of Akkermansia muciniphila in the propionate-producing consortium

Studies have provided evidence indicating that the lack of liver glycogen increases liver fat accumulation and leads to insulin resistance, while increased liver glycogen improves glucose tolerance (Ros et al. 2010) independently of insulin signalling (Irimia et al. 2017). Gut microbiota has been reported to be involved since different microbial communities can contribute to the pathogenesis and progression of liver diseases (Wu et al. 2017). Thus, modulating the composition and the function of gut microbiota can be a potential intervention for liver injury.

*A. muciniphila*, a candidate for next generation probiotic, has been reported to have a cross-talk with host metabolism due to its beneficial roles in metabolic and immunological disorders (Shin et al. 2014; Plovier et al. 2017). This bacterium has been proven to improve glucose tolerance, insulin sensitivity, and ameliorate metabolic system inflammation (Everard et al. 2013; Shin et al., 2014; Plovier et al. 2017; Zhao et al. 2017). In addition, pre-treatment with *A. muciniphila* could alleviate liver damage through altering the transaminase activities and histologic injuries (Wu et al. 2017). Studies reported that supplementation of *A. muciniphila* reduced inflammation signalling in the muscle and liver and decreased metabolic endotoxemia (Zhao et al. 2017). Thus, *A. muciniphila* can reshape the gut microbial community and induce a protective profile (Wu et al. 2017) and could be a promising probiotic candidate with beneficial effects on liver diseases. Furthermore, Zhang et al. (2018) reported that *A. muciniphila* could improve liver function, reduce oxidative stress, alleviate gluco/lipotoxicity, inhibit inflammation, and regulate intestinal microbiota, thus managing type 2 diabetes (Zhang et al. 2018). *A. muciniphila* has a mucolytic activity, and it can produce oligosaccharides, vitamins and SCFAs (Lopez-Siles et al. 2018). The metabolites from *A. muciniphila* could be used by other commensal gut bacteria as a source of energy and may change the relative abundance of other gut bacteria and regulate commensal interactions (Belzer and de Vos 2012, Wu et al. 2017; Lopez-Siles et al. 2018).

In our study, we observed a significant positive effect on glycogen and LPL activity in hepatic cells when the metabolites from the PPC were added. However, we could not detect a similar result when metabolites from *A. muciniphila* alone or from our consortium without *A. muciniphila* were added to the enterohepatic cell model of hyperglycaemia. This indicates that the propionate-producing consortium needs all the seven bacterial members to be included to fully perform its positive effect. *A. muciniphila* potentially provided different metabolites to cross-feed with the seven strains. As reported in a previous study, a syntrophic relationship
between *A. muciniphila* and *F. prausnitzii* was revealed, and the enrichment or depletion of one could imply the same effect in the other (Lopez-Siles et al. 2018). That study also stated that *F. prausnitzii* consumed oligosaccharides derived from the mucin degraded by *A. muciniphila*, and its growth was also stimulated by acetate and other metabolites in the medium, which may have been provided by *A. muciniphila* (Duncan et al. 2002; Lopez-Siles et al. 2012; Lopez-Siles et al. 2018). Another co-culture study between *A. muciniphila* and *A. caccae* showed the ecological dependency between a mucin degrader and a butyrate producer (Chia et al. 2018). This study used metatranscriptomics as an explorative approach to decipher bacterial interactions in the mucosal environment as both strains were mucosa-associated (Chia et al. 2018). These results ensure that there was a positive correlation between all the seven strains of the PPC, leading to the optimal results discussed in Chapter 4. To further understand the in-depth mechanism of the bacterial interactions that happened between the seven strains of our designed PPC, metatranscriptome approaches may be needed in the future.

5.9- Risk-benefit analysis for propionate

Propionate has been proven to promote satiety by stimulating the gut anorectic hormones, the PYY and the GLP-1, and prevent long-term weight gain in healthy humans (Chambers et al. 2014). Chambers et al. (2014) demonstrated that 10g inulin-propionate ester ingestion leads to a 2.5-fold increase in daily colonic propionate production, and it also leads to increase in plasma propionate levels, and this in turn leads to greater release of PYY and GLP-1 hormones and decrease in caloric intake.

Propionate has also been reported to have the highest affinity for the free fatty acid receptor 2 (FFAR 2), involved in the regulation of metabolic homeostasis. In fact, long-term propionate delivery in the gut stimulates anorexigenic gut hormones, reducing intra-abdominal fat accretion, intrahepatocellular lipid content, and hepatic cholesterol synthesis in humans (Chambers et al. 2014; Arora et al. 2011). In addition, propionate is involved in activation of intestinal gluconeogenesis (IGN), thus regulating food intake and enhancing insulin sensitivity (Li et al. 2017).

In our study we observed that the metabolites from the propionate-producing consortium were able to increase glycogen and LPL levels in a insulin resistance-induced enterohepatic model. Studies have shown that liver glycogen accumulation can protect against the negative effects of high fat diet, such as glucose intolerance (López-Soldado et al. 2014). Accumulation of hepatic glycogen contributes to decreased food intake and lower body weight and adiposity (Ritter et al. 1994; Grill et al. 1995; López-Soldado et al. 2014). For this reason, hepatic glycogen can act as a potential target for pharmacological manipulation of diabetes and obesity (López-Soldado et al. 2014). Our findings stress the importance of propionate on the gut-liver
crosstalk and ultimately on the glycogen regulation and energetic metabolism. Moreover, propionate can enhance the lipid buffering capacity of adipose tissue by increasing LPL-mediated triglyceride extraction, resulting in reduced lipid overflow to the adipose tissue (Canfora et al. 2015).

On the other hand, propionate has been investigated as a food additive that prevents molds and preserves food (Tirosh et al. 2019). The research involved mice and humans used as subjects for propionate consumption. In contrast to the previous findings we reported, the study in mice found that, the short-term consumption of propionate has led to high blood sugar levels, and the long-term consumption led to weigh gain and insulin resistance. In this study, after the researchers gave the propionate to the mice, they found that this additive led to an increase in several hormones. These hormones were glucagon, that is responsible for giving an order to the liver to release glucose in the blood stream, norepinephrine, that is responsible for regulating blood pressure and raising blood sugar, and fatty acid-binding protein 4 or (FABP4), that is involved in fatty acid metabolism. This rush in hormones led to hyperglycemia, or high blood glucose levels, in the mice. For the long-term trial, the researchers gave the mice drinking water with low doses of propionate which was equivalent to the amount in the processed-food based diet for humans for 20 weeks. This chronic propionate exposure led animals to gain more weight and showed increased insulin resistance, as compared to the mice that did not consume propionate.

To determine how those results can translate to humans, the researchers held a small trial that involved 14 healthy and lean individuals with no diabetes. The participants that consumed propionate experienced temporary increase in insulin resistance over a period of few hours compared to those who did not consume this food additive. The amount of propionate that was given to subjects in this study was 1 gram which was the typical amount added in a single meal of processed food. The participants had blood samples before the meal and after the meal at regular intervals for 4 hours.

After one week of washout, the participants switched meals in which the ones who had originally the propionate meal had the placebo and vice versa. This study was double-blinded. The results showed that the individuals who received the propionate experienced an increase in norepinephrine, glucagon and FABP4 hormone levels similar to what happened in the mice study. The participants that received propionate also showed increased levels of insulin and insulin resistance. In fact, both groups showed similar blood sugar peaks after the meals, but those that received propionate took slightly longer return to baseline.

However, the authors of this study reported that the current literature suggests that orally ingested propionate does not mimic the beneficial metabolic effects attributed to SCFAs derived from bacteria in the colon, and that may result in adverse metabolic effects, including insulin resistance and glucose intolerance. This divergence may be due to the different doses
and routes of administration, and the local effects of propionate on proximal enterocytes versus distal colonocytes, as has been recently suggested for acetate (Van de Beek et al. 2016). The authors also suggested that this discrepancy may be explained by the interactions of propionate and the colonic mucosa with other SCFAs and other gut metabolites. The reported results in this study showing that oral propionate in processed foods leads to a potential increase in endogenous glucose production is still of a concern, especially that chronic hyperinsulinemia can drive obesity and metabolic abnormalities. Nevertheless, this study only found only an association and did not exclusively prove that propionate causes insulin resistance or diabetes. Those results cannot be applied for general humans as the study was conducted in mice and only on 14 healthy individuals. In addition, this suggests that propionate might have different effects depending where it enters the body and the site of action. For this reason, additional studies in larger human populations, with longer exposure to various doses of propionate, are needed to better elucidate the various metabolic effects of propionate in humans.

5.10 – Safety and regulatory considerations
5.10.1 – Safety considerations
As several types of microbes are used as probiotics, the safety of probiotics is tied to the nature of the specific microbe and its intended use, and this requires several considerations (Sanders et al. 2010). As probiotics are administered when they are alive, unlike other food products or drugs components, they possess the potential for infectivity or in situ toxin production (Sanders et al. 2010). Safety considerations should include the potential vulnerability of the patient or consumer, the dose and duration of intake, and both the frequency and manner of administration (Sanders et al. 2010; Samtiya et al. 2019). Moreover, the bacteria used should be completely safe, so the horizontal gene transfer that can result in the acquisition of virulence genes or antibiotic resistance genes which comprises a theoretical risk of transfer for less innocuous member of the gut microbial community, must also be considered (Sanders et al. 2010; Wassenaar and Klein 2008). Genetic stability of the probiotic overtime, the potential for pathogenicity or toxicogenecity, and the detrimental metabolic activities must also be evaluated to the characteristics of the genus and species of the bacteria used (Sanders et al. 2010). Immunological effects should also be considered especially in specific vulnerable populations like infants with undeveloped immune function (Sanders et al. 2010). The use of genomic sequencing technologies to obtain the complete bacterial genome can both resolve and create safety issues (Wassenaar and Klein 2008). In addition, to address the safety concern, the World Health Organization has developed guidelines for the assessment of probiotics in food, which has designated parameters for preclinical testing, clinical trials, and
labelling that includes strains identification, safety evaluation, and efficacy testing in vitro and in animal models (Samtiya et al. 2019). For this reason, safety assessment has been procured as an important criterion and a primary step for the selection of probiotics strains prior to the incorporation in the food chain (Samtiya et al. 2019).

5.10.2 – Regulatory considerations

For a bacterium to be classified as a “probiotic” or “functional food” that is safe to be administered for humans, it should fall in the Qualified Presumption of Safety (QPS) list provided by the European Food Safety Authority (EFSA) (Baldi and Arora 2015) or Generally Recognized as Safe (GRAS) products that are approved by the FDA in the United States. The market for probiotics as functional foods expanded as they have been incorporated in food products like yogurts and fermented milk (Baldi and Arora 2015) that mainly contain conventional LAB. The QPS list is updated periodically according to the safety assessment of the biological products recommended to be added, but not all can be approved (Scientific Opinion on The Maintenance of The List of QPS Biological Agents Intentionally Added to EFSA Panel on Biological Hazards (BIOHAZ), 2013; Ricci et al. 2017). However, in the United States, when the probiotic is used as a dietary supplement, it is considered as “food” and should be approved by the Dietary Supplement Health and Education Act (DSHEA). On the other hand, if the probiotic was used for therapeutic purpose then it needs to be approved by the FDA (El Hage et al. 2017). Both the EFSA and the FDA do not approve probiotics to be used in health claims.

Since the definitions and classifications for probiotics differ by regulatory agents among the world, this keeps the status of probiotics uncertain. Hence, doubts about probiotic products among regulatory bodies, producers and consumers might arise (El Hage et al. 2017). As most probiotics include only LAB which possess limited phylogenetic diversity and functionality, critical update of the screenings by required regulatory agents is urgently needed.

Safety and regulatory assessments on our propionate-producing consortium must be completed prior to consideration for incorporation in food products.

5.11 – Future Perspectives

Several considerations are fundamental to establish a microbial consortium for human use. These include safety, means and purpose of administration, manufacturing process and survival of the strains. As we have established, our propionate-producing consortium from commensal gut bacteria including one probiotic (L. plantarum), and one next-generation probiotic (A. muciniphila), the other strains still need to be tested for their safety before commercial use. In addition, since propionate has been reported to stimulate the production of the PYY and the GLP-1 gut hormones, measuring these hormones upon addition of the PPC in vivo must be tested to ensure their activity on the gut-liver crosstalk. Metagenomics and
genomics approaches could be used to determine the different pathways that strains follow to produce propionate, as described by Richardt et al. (2014). Moreover, metatranscriptomics are needed to understand in-depth the mechanistic interactions between bacteria. Metatranscriptomics revealed the trophic interactions between mucosal keystone species *A. muciniphila* and *A. cacaee* during a co-culture experiment (Chia et al. 2018). By testing the cross-talk between all systems, many new inducible systems for versatile control of engineered communities could be characterized (Scott and Hasty 2016). The characterization of each biotherapeutic should be completed considering the relative benefits to the target disease. As the PPC supernatant positively reversed insulin resistance effects in the *in vitro* cell model, the PPC must be tested *in vivo* for safety measurements and also in an obese-induced or diabetes-induced mice to fully comprehend its effects. Persistence of the outcomes must be further assessed *in vitro*, as well as survival and viability following microencapsulation. As for postbiotics, optimized models of the upper GI tract simulator and the SHIME will allow assessing their stability in the manufactured products. These considerations highlight the urgency of developing representative *in vitro* model mimicking the whole human gastrointestinal tract (Aguilar-Toalá et al. 2018). Special attention should be paid to develop uniform and defined culturing procedures to eliminate the variability of postbiotic production, as environmental factors may influence bacterial metabolism and lead to transient variability. Similar to probiotics, metabolomics and genomic studies could be implemented to support the health claims of postbiotics (Aguilar-Toalá et al. 2018). In addition extra cell work with extra parameters could be interesting to investigate in the future. For instance, pancreatic cells would be of great value to test the effect of the PPC in case of insulin resistance. The pancreas is able to maintain blood glucose levels within a very narrow range through its various hormones, particularly glucagon and insulin. This could be accomplished by the opposing and balanced actions of glucagon and insulin, referred to as glucose homeostasis (Röder et al. 2016). The effect of the PPC can be tested on these cells to check if it could be aiding in the prevention of diabetes. Another cell type that could be tested would be the enteroendocrine cells. Enteroendocrine cells (EEC) have been reported to have a major role in regulating gut motility, secretion, and production of peptide hormones that control insulin secretion and food intake. Intestinal enteroendocrine cells such as L-cells are responsible for the production of the GLP-1 and the PYY gut hormones that are responsible for satiety (Covasa et al. 2019). L-cells strategically detect the presence of nutrients, microbiota and their metabolites. The microbiota controls enteroendocrine cells differentiation and the number of GLP-1 and PYY-secreting L-cells (Covasa et al. 2019). For this reason, it would be of a great value to test the effect of the PPC supernatant on the L-cells and measure the secretion of PYY and GLP-1 hormones. Besides, lipid accumulation may be a relevant parameter to test in the proposed cell model, enterohepatic model in our case, as de novo lipogenesis may exert impact on glycerol
accumulation (Saponaro et al. 2015). Techniques such as red Nile will shed light on the role of lipid droplets stored in HepG2 cells and their effect on hyperglycaemia, so this can be considered for future studies. Furthermore, the use of metagenomics, metatranscriptomics and metabolomics, can reveal the possible secondary metabolites that microorganisms produce, indicating the silent genes that can be activated within microbial consortia (Zhang et al. 2018). This could also be important for future commercial production of the biotherapeutic.

5.11.1- HepG2 vs HepRG

Studies have reported that primary human hepatocytes are considered the gold standard of hepatic culture models due to their close resemblance to in vivo functionality; however, these cells have drawbacks. They are limited in availability, and due to the fact that they are from different donors, their response to drugs could vary (McGill et al. 2011). In addition, these cells have a limited lifespan, have a decreasing functionality in long-term culture (Ragol et al. 2019), and they undergo phenotypic changes and display variability in CYP450 expression over time. Liver cell lines that are widely used are either obtained from tumor tissue or by genetic engineering of primary human hepatocytes (PHH). HepG2 and HepaRG, the presumably most prominent hepatic cell lines, and are both generated from human hepatoma (Ragol et al. 2019). In contrast to PHH, most hepatoma cells are stable, largely available with low cost, and easy to work with, nearly unlimited lifetime, and stable phenotype that does not depend on donor characteristics (Donato et al. 2015), but their high proliferative potential is accompanied by functional losses (Rogal et al. 2019). To start with, HepaRG cells were isolated from a hepatoma in a female patient with cirrhosis subsequent to hepatitis C virus infection (HCV). HepaRG cells are bipotent progenitors in which two morphologically distinct populations become apparent upon differentiation: hepatocyte-like cells and biliary epithelial-like cells. Moreover, studies have demonstrated that this cell line is able to highly express xenobiotic metabolizing enzymes, which is comparable to primary human hepatocytes, thus suggesting their use in drug studies (McGill et al. 2011). As for HepG2, this cell line has extensively been used in cell-based metabolic studies; however, as this cell line is of malignant origin, they have the tendency to present abnormal metabolic phenotype compared to human primary cells. For instance, HepG2 cells have increased rates of de novo lipogenesis and glycolysis (Pereira da Silva et al. 2009; Daniels et al. 2014; Nikolaou et al. 2016). In addition, it has been reported that HepG2 is best used to model hepatic cancer and its treatment strategies, but it is not ideal for studying hepatic biotransformation due to atypical quantities of drug metabolism enzymes and deficient metabolic activities (Rogal et al. 2019). In addition, HepG2 express many differentiated hepatic functions, like synthesis and secretion of plasma proteins, lipoprotein metabolism and transport, bile acid synthesis, glycogen synthesis or insulin signaling, and
cholesterol and triglyceride metabolism (Donato et al. 2014). However, HepG2 cells have a major drawback that is their limited ability to express drug metabolizing enzymes and transporters (Donato et al. 2014). Furthermore, what makes HepG2 different from liver tissue or primary hepatocytes is in particular are the characteristic hepatic transporters sodium-taurocholate cotransporting polypeptide, organic anion transporting polypeptide C, and bile salt export pump that are absent or poorly expressed in HepG2 (Donato et al. 2014). Contrary to HepG2, the HepaRG cell line is an alternative to PHHs in the context of metabolic or toxicological studies; as a result of its higher grade of differentiation, HepaRG cells retain more hepatic metabolic functions, including drug metabolizing cytochrome P450 (CYP) enzyme activities (Rogal et al. 2019). Moreover, Richter et al. has reported that the HepaRG provided better results than HepG2 regarding their total number and abundance of metabolites. Despite that HepG2 were easier to handle, required less complicate cultivation media, and they were cost effective, they could not be compared to PHH (Richter et al. 2017). For this reason, the metabolites from our PPC could also be tested on HepaRG cell line in the future to have clearer overview and extra information that could relate to the primary hepatic cells.

5.12 – Conclusion
Beneficial modulation of the gut microbiota using biotherapeutics has gained attention and importance over time. Biotherapeutics are characterized bacterial communities from gut commensal bacteria and can be used for the prevention or treatment of disease. These consortia include different bacterial strains with different phylogenetic origin not limited to a narrow selection, as opposed to conventional probiotics belonging to lactic acid bacteria. In vitro human gut models such as the TSI and SHIME and the developed cell models allowed us to reveal that:

- The PPC could not survive the three different compartments of the small intestine due to either the high concentration of the bile salts or some oxygen diffusion in the system
- The PPC recovered the functionality of propionate production in the colon after antibiotic-induced dysbiosis
- The administration of PPC led to direct engraftment of some of its members in the microbial community in the simulated gut microbial ecosystem, in addition to the indirect reinforcement of other bacterial genera involved in propionate production
- The supernatant from one dose of PPC caused partial recovery of the mitochondrial membrane potential of caco-2 cells after being disrupted with clindamycin
- The supernatant from one dose of PPC increased glycogen storage and LPL activity in hepatic cells, and decreased IL-8 which is an inflammatory marker in hepatic cells
• The optimal effect of the PPC can only be reached when all the seven members of the consortium are interacting together

These findings indicate that the PPC could be a promising therapeutic approach, yet more investigation is to be performed. Considering the bacterial metabolic pathways involved in the desired outcome is utmost crucial to ensure the exchange of metabolites among the members and thus gain higher production efficiency.
6- Summary- Samenvatting

Summary

The gut microbiota has been known to be associated with human health and the onset of different diseases. Several factors can lead to the manipulation of the gut microbiota, including antibiotics, probiotics, prebiotics and other environmental factors. The excessive and misuse of antibiotics causes a disruption for the gut microbiota and thereby a disorder in short chain fatty acids production. Dysbiosis has been linked to diseases such as metabolic disorder. Studies have shown that propionate, a major short chain fatty acid, is able to stimulate the production of PYY and the GLP-1 gut hormones that are able to control satiety thus decreasing energy intake and causing weight loss. Propionate can also contribute to metabolic health by activating the intestinal gluconeogenesis, which in turn can maintain energy homeostasis by regulation of food intake and improvement of insulin sensitivity. As a result, the gut microbiota can serve as a therapeutic target for metabolic syndrome. Some studies have shown the positive effect of the administration of probiotics and fecal microbial transplant on metabolic syndrome. However, the probiotics used are of limited phylogenetic origin mainly belonging to lactic acid bacteria, and fecal microbial transplant has an uncharacterized nature of bacteria that need several considerations before being administered to humans. As a result, a more characterized bacterial consortium including different beneficial strains might be needed as a therapeutic for metabolic disorders.

In the first part of our research, we have worked on developing a multispecies bacterial consortium that is able to produce propionate, the beneficial short chain fatty acid. In Chapter 2, we have described how we designed the propionate-producing bacterial consortium by considering the three different metabolic pathways that lead to propionate production (acrylate pathway, succinate pathway, and propanediol pathway). Seven different strains were selected taking into account the possible cross-feeding mechanisms that could occur leading to propionate production. The seven strains were co-cultured together in a fed-batch experiment for 48 hours yielding a propionate concentration of 34.5mM. The aim of the co-culturing was to have a preadapted bacterial consortium that can be harvested as a bacterial community in which strains have already done their different cross-feeding. Moreover, since the propionate-producing consortium is supposed to be administered orally and take action in the colon, it has to pass the harsh conditions of the upper gastrointestinal tract. For this reason, it was tested in the TSI model that represents “The Smallest Intestine” model and that included a consortium of the ileal bacteria. The results showed that the propionate-producing consortium could not survive the different parts of the small intestine, and that fed conditions were more harsh than fasted conditions. This indicated that a carrier is needed to protect the consortium from the
harsh conditions of the bile salts in the small intestine, and that it is recommended that our designed consortium is administered in the fasted state.

To assess the effect of the propionate-producing consortium on the microbial functionality upon antibiotic-induced dysbiosis, we used the M-SHIME model, an \textit{in vitro} simulator for the human intestinal microbial ecosystem (Chapter 3). The impact of addition of the propionate-producing consortium was tested for six different donors after which the antibiotic was added to induce a case of dysbiosis. After adding antibiotics, the concentrations of short chain fatty acids produced in the SHIME decreased drastically. The administration of the consortium for three consecutive days caused a full recovery for propionate production reaching the normal levels before the antibiotic disruption. Moreover, the community composition analysis showed that the addition of the propionate-producing consortium was able to cause direct engraftment for some bacterial genera related to the introduced strains in addition to the indirect reinforcement of other genera associated with propionate production. This revealed that the administration of our consortium restored functionality and caused a shift to the microbial community towards enhancing propionate production. Furthermore, the effect of the supernatant from the propionate-producing consortium was assessed on a caco-2 cell model in which addition of clindamycin caused disruption of the mitochondrial membrane potential. The metabolites from the consortium were able to cause partial recovery of the mitochondrial membrane potential following antibiotic disruption.

In the second part of the research, the gut-liver crosstalk was studied. In Chapter 4, we investigated the impact of the metabolites from the propionate-producing consortium and \textit{Akkermanis\textit{ia muciniphila}}, which is also known to be a propiogenic bacterium, in an \textit{in vitro} enterohepatic model of insulin resistance. Liver glycogen storage, lipoprotein lipase activity, and different inflammatory markers in hepatic cells were evaluated to understand the role of the metabolites from gut bacteria on the gut-liver crosstalk in a simulated scenario of subclinical metabolic inflammation. Only the metabolites produced by our designed consortium of seven strains were able to increase hepatic glycogen levels and lipoprotein lipase activity and decrease pro-inflammatory markers such as IL-8. These results elucidated the positive effect of the propionate-producing consortium on metabolic function and low-grade inflammation. Moreover, to observe the optimal effect of the consortium, all seven strains need to be present to do the necessary cross-feeding and yield the beneficial metabolites.

In conclusion, this PhD research showed that the designed propionate-producing consortium is a promising strategy to restore dysbiosis and manage metabolic disorders, thanks to its versatile mode of action and production of beneficial metabolites. To achieve a long-term effect of the designed consortium, more doses and longer periods of administration might be needed. Next to this, more advanced techniques such as metagenomics, metatranscriptomics and
metabolomics are needed to reveal the bacterial interactions and the possible secondary metabolites that microorganisms can produce.

Sumenvatting
Het is gekend dat de darmmicrobiota verband houdt met de gezondheid van de mens en de aanvang van verschillende ziekten. Verscheidene factoren, zoals antibiotica, probiotica, prebiotica en andere omgevingsfactoren, kunnen de darmmicrobiota beïnvloeden. Het overmatig gebruik en misbruik van antibiotica heeft geleid tot een verstoring van de darmmicrobiota en daardoor tot een stoornis in de aanmaak van korte keten vetzuren. Dysbiose is gelinkt aan ziekten, waaronder stofwisselingsziekten. Onderzoek heeft aangetoond dat propionaat, een belangrijk korte keten vetzuur, de PYY en GLP-1 hormonen in de darm kan stimuleren. Deze hormonen dragen bij aan de controle van het vermogen van de energie-inname en gewichtsverlies. Propionaat kan ook bijdragen aan de metabole gezondheid door activering van de gluconeogenese, wat op zijn beurt de energiehomeostase in stand houdt door het regelen van de voedselinname en het verbeteren van insulinengevoeligheid. Hierdoor kan de darmmicrobiota dienen als een doelwit voor therapie. Sommige onderzoeken hebben het positief effect van propionaat in de behandeling van proctitis en fecale microbiële transplantaties op stofwisselingsziekten. De gebruikte probiotica hebben echter een beperkte fyllogenetische oorsprong en behoren voornamelijk tot de lactobacillen, en fecale microbiële transplantaties zijn weinig gekarakteriseerd op vlak van bacteriën die goed in overweging genomen moeten worden vooraleer ze worden toegediend aan mensen. Om deze redenen kan een beter gekarakteriseerd bacterieel consortium nodig zijn als therapie voor metabole ziekten.

In het eerste deel van ons onderzoek hebben we gewerkt rond het ontwikkelen van een multispecies bacterieel consortium dat propionaat, het gezonde korte keten vetzuur, kan produceren. In hoofdstuk 2 is beschreven hoe we het propionaatproducerende consortium ontworpen hebben door de drie verschillende metabolische pathways te beschouwen die tot propionaatproductie leiden (de acrylaat pathway, de succinaat pathway, en de propaandiol pathway). Zeven verschillende bacteriële stammen werden geselecteerd met inachtneming van mogelijke crossfeeding mechanismen die zouden kunnen voorkomen en tot propionaatproductie zouden kunnen leiden. De zeven stammen werden voor 48 uur gecocultiveerd in een fed-batch experiment, wat een propionaatconcentratie van 34,5 mM opleverde. De cocultivering had als doel een vooraf aangepast consortium te bekomen dat geoogst kan worden als een bacteriële gemeenschap waarin de verschillende stammen reeds een crossfeeding netwerk hebben opgezet. Daarenboven, gezien het propionaatproducerende
consortium bedoeld is voor orale inname, maar zijn functie dient uit te oefenen in de colon, moet het de moeilijke omstandigheden van het bovenste deel van het gastro-intestinaal kanaal zien te overleven. Om deze reden werd het consortium getest in het TSI model, “het kleinste darm model”, dat een consortium van ileale bacteriën bevat. De resultaten toonden aan dat het propionaatproducerende consortium de passage door de verschillende delen van de dunne darm niet kon overleven, waarbij de gevoede toestand moeilijker was dan de gevaste toestand. Dit toonde aan dat er een drager nodig is om het consortium te beschermen tegen de schadelijke effecten van galzouten in de dunne darm, en dat het aangewezen is om het consortium toe te dienen in de gevaste toestand.

Om het effect na te gaan van het propionaatproducerende consortium op de microbiële functionaliteit gedurende antibioticum-geïnduceerde dysbiose, werd het M-SHIME model gebruikt, een *in vitro* simulator voor het humaan intestinaal microbioel ecosysteem (hoofdstuk 3). De impact van het toedienen van het propionaatproducerende consortium werd getest voor 6 verschillende donoren, waarna antibiotica werden toegediend om een dysbiotische toestand te induceren. Na het toedienen van de antibiotica daalden de concentraties van korte keten vetzuren geproduceerd in de SHIME drastisch. Toedienen van het consortium voor drie opeenvolgende dagen zorgde voor een volledig herstel van de propionaatproductie, waarbij het normale niveau van voor de verstoring door antibioticabeerkt werd. Daarenboven toonde kwantificatie van de microbiële gemeenschap aan dat het toedienen van het propionaatproducerende consortium zorgde voor implanatatie van enkele bacteriële genera gerelateerd aan de geïntroduceerde stammen, naast de indirecte versterking van andere genera geassocieerd met propionaatproductie. Dit toonde aan dat het toedienen van ons consortium zorgde voor herstel van de functionaliteit en zorgde voor een verschuiving van de microbiële gemeenschap die leidde tot een verhoogde propionaatproductie. Het effect van supernatans van het propionaatproducerende consortium werd eveneens getest in het caco-2 cel model, waarbij de mitochondriële membraanpotentiaal verstoord werd door clindamycine. De metabolieten van het consortium zorgden voor gedeeltelijk herstel van de mitochondriële membraanpotentiaal na de verstoring door antibioticabeerkt.

In het tweede deel van dit onderzoek werd de darm-lever crosstalk onderzocht. In hoofdstuk 4 hebben we de impact van metabolieten van het propionaatproducerende consortium en *Akkermansia muciniphila*, eveneens een propiogene bacterie, bestudeerd in een *in vitro* enterohepatisch model van hyperglycemie. Glycogeenopslag in de lever, lipoproteïne lipase activiteit, en verschillende inflammatoire merkers in levercellen werden beoordeeld om inzicht te krijgen in de rol van metabolieten van darmbacteriën op darm-lever crosstalk in een gesimuleerde toestand van subklinische metabole ontsteking. Enkel de metabolieten geproduceerd door het door ons ontworpen consortium van zeven stammen veroorzaakten
een stijging van de glycogeenlevels in de lever en van lipoproteïne lipase activiteit, en een daling van inflammatoire merkers, waaronder IL-8. Deze resultaten tonen het positieve effect aan van het propionaatproducerende consortium op stofwisselingsfuncties en lichte ontsteking. Daarnaast bleek dat, om het optimale effect van het consortium te bekomen, alle zeven stammen aanwezig moeten zijn om de nodige crossfeeding en productie van gezonde metaboliën te bewerkstelligen.

Samenvattend heeft dit doctoraatsonderzoek aangetoond dat het ontworpen propionaatproducerende consortium een veelbelovende strategie is vanwege zijn multifunctionaliteit, en potentiële mogelijkheid van zijn metaboliën om stofwisselingsziekten te beheersen. Om een lange-termijn effect van het ontworpen consortium te bekomen, zouden meerdere doses en langere periodes van toediening noodzakelijk kunnen zijn. Meer gespecialiseerde technieken zoals metagenomics, metatranscriptomics en metabolomics zijn echter vereist om bacteriële interacties en mogelijke secundaire metaboliën die micro-organismen kunnen produceren aan te tonen.


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ANNEX I

Table S1: Community structure indices for Fresh mix of bacterial cocktail. Inverse simpson indicates Alpha diversity, and Pielou indicates Evenness. Comparisons between Fasted and Fed conditions in the same compartment were performed using 2-way Anova test (Sidak’s method). Differences between the same condition (Fasted or Fed) in different compartments were assessed using 2-way Anova (Tukey’s method), and superscripts indicate significance (*P<0.05*).

<table>
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<th>Compartment</th>
<th>Condition</th>
<th>P value</th>
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</thead>
<tbody>
<tr>
<td>Inverse Simpson</td>
<td>Inoculum</td>
<td>4.15 ± 0.54&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.15 ± 0.54&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td></td>
<td>Duodenum</td>
<td>8.84 ± 4.16&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.36 ± 0.54&lt;sup&gt;c&lt;/sup&gt;</td>
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<td></td>
<td>Jejunum</td>
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<td>2.59 ± 0.01&lt;sup&gt;bc&lt;/sup&gt;</td>
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<tr>
<td></td>
<td>Ileum</td>
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<td>3.95 ± 0.12&lt;sup&gt;bc&lt;/sup&gt;</td>
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<td>Pielou</td>
<td>Inoculum</td>
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<td>0.46 ± 0.04&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>Duodenum</td>
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<td>0.44 ± 0.01&lt;sup&gt;b&lt;/sup&gt;</td>
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Table S2: Community structure indices for Preadapted bacterial consortium. Inverse simpson indicates Alpha diversity, and Pielou indicates Evenness. Comparisons between Fasted and Fed conditions in the same compartment were performed using 2-way Anova test (Sidak’s method). Differences between the same condition (Fasted or Fed) in different compartments were assessed using 2-way Anova (Tukey’s method), and superscripts indicate significance ($P<0.05$).

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<td>Fed (Mean % ± SEM)</td>
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<td>Inoculum</td>
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<tr>
<td>Duodenum</td>
<td>13.09 ± 0.44$^a$</td>
<td>8.83 ± 3.21$^b$</td>
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<tr>
<td>Jejunum</td>
<td>9.87 ± 2.39$^a$</td>
<td>3.84 ± 1.32$^b$</td>
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Table S3: Community structure indices for Fresh Mix and Preadapted bacterial consortium in the fasted condition. Inverse simpson indicates Alpha diversity, and Pielou indicates Evenness. Comparisons between Fresh Mix and Preadapted consortium in the same compartment were performed using 2-way Anova test (Sidak’s method). Significance was considered at ($P<0.05$).

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<td>Preadapted (Mean % ± SEM)</td>
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<td>Jejunum</td>
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Table S4: Community structure indices for Fresh Mix and Preadapted bacterial consortium in the fed condition. Inverse simpson indicates Alpha diversity, and Pielou indicates Evenness. Comparisons between Fresh Mix and Preadapted consortium in the same compartment were performed using 2-way Anova test (Sidak’s method). Significance was considered at ($P<0.05$).

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<tr>
<td>Pielou</td>
<td>Inoculum</td>
<td>0.46 ± 0.04</td>
<td>0.50 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>Duodenum</td>
<td>0.43 ± 0.06</td>
<td>0.60 ± 0.06</td>
</tr>
<tr>
<td></td>
<td>Jejunum</td>
<td>0.31 ± 0.02</td>
<td>0.38 ± 0.10</td>
</tr>
<tr>
<td></td>
<td>Ileum</td>
<td>0.44 ± 0.01</td>
<td>0.42 ± 0.12</td>
</tr>
</tbody>
</table>
Figure S1: PCoA analysis showing the dispersion of the microbial community by fasted and fed conditions for the fresh mix and preadapted microbial consortium in the different TSI compartments (Inoculum: before entering the TSI, duodenum, jejunum, and ileum).
ANNEX II

Table S1: Community structure indices in the lumen compartment. 1: End of Stabilization phase, 2: After Antibiotic use, 3: After 3 doses of propionate-producing consortium, and 4: washout phase. Inverse simpson indicates Alpha diversity, Pielou indicates Evenness, and Total Species indicates the total number of Species or richness. Comparisons between control (CTR) and Treatment (TRT) at the same time point were performed using 2-way Anova test (Sidak’s method). Differences between the same treatment at different time points were assessed using 2-way Anova (Tukey’s method), and superscripts indicate significance ($P<0.05$).

<table>
<thead>
<tr>
<th>Index</th>
<th>TPT</th>
<th>Condition</th>
<th>$P$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inverse Simpson</td>
<td></td>
<td>CTR (Mean % ± SEM)</td>
<td>TRT(Mean % ± SEM)</td>
</tr>
<tr>
<td>1</td>
<td></td>
<td>3.94 ± 0.61$^a$</td>
<td>3.52 ± 0.36$^b$</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>3.65 ± 0.31$^a$</td>
<td>3.74 ± 0.46$^b$</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>3.65 ± 0.24$^a$</td>
<td>3.63 ± 0.39$^b$</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>3.41 ± 0.16$^a$</td>
<td>3.76 ± 0.41$^b$</td>
</tr>
<tr>
<td>Pielou</td>
<td></td>
<td>0.46 ± 0.03$^a$</td>
<td>0.46 ± 0.02$^b$</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>0.48 ± 0.02$^a$</td>
<td>0.47 ± 0.04$^b$</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>0.47 ± 0.02$^a$</td>
<td>0.46 ± 0.02$^b$</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>0.45 ± 0.01$^a$</td>
<td>0.46 ± 0.02$^b$</td>
</tr>
<tr>
<td>Total species</td>
<td>1</td>
<td>51.33 ± 3.16$^a$</td>
<td>53.67 ± 3.21$^d$</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>26.00 ± 1.26$^b$</td>
<td>28.33 ± 2.04$^e$</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>29.17 ± 1.85$^{bc}$</td>
<td>36.50 ± 2.08$^f$</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>33.50 ± 1.98$^{bc}$</td>
<td>42.17 ± 1.42$^f$</td>
</tr>
</tbody>
</table>


Table S2: Community structure indices in the mucin compartment. 1: End of Stabilization phase, 2: After Antibiotic use, and 3: After 3 doses of propionate-producing consortium. Inverse simpson indicates Alpha diversity, Pielou indicates Evenness, and Total Species indicates the total number of Species or richness. Comparisons between control (CTR) and Treatment (TRT) at the same time point were performed using 2-way Anova test (Sidak’s method). Differences between the same treatment at different time points were assessed using 2-way Anova (Tukey’s method), and superscripts indicate significance ($P<0.05$).

<table>
<thead>
<tr>
<th>Index</th>
<th>TPT</th>
<th>Condition</th>
<th>$P$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inverse Simpson</td>
<td></td>
<td>CTR (Mean % ± SEM)</td>
<td>TRT (Mean % ± SEM)</td>
</tr>
<tr>
<td>1</td>
<td></td>
<td>5.09 ± 0.81$^a$</td>
<td>6.22 ± 1.09$^b$</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>4.31 ± 0.47$^a$</td>
<td>5.82 ± 1.09$^b$</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>4.69 ± 0.60$^a$</td>
<td>5.45 ± 0.35$^b$</td>
</tr>
<tr>
<td>Pielou</td>
<td></td>
<td>0.52 ± 0.03$^a$</td>
<td>0.55 ± 0.03$^b$</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>0.46 ± 0.03$^a$</td>
<td>0.52 ± 0.03$^b$</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>0.50 ± 0.03$^a$</td>
<td>0.55 ± 0.02$^b$</td>
</tr>
<tr>
<td>Total species</td>
<td></td>
<td>64.00 ± 7.10$^a$</td>
<td>68.33 ± 3.08$^c$</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>54.50 ± 3.10$^a$</td>
<td>59.67 ± 4.69$^c$</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>39.33 ± 3.68$^b$</td>
<td>44.17 ± 2.79$^d$</td>
</tr>
</tbody>
</table>


Table S3: Differences in major short chain fatty acid concentrations between control and treatment in the lumen at different time points (TPT) in Donor 1. Time point (TPT) is expressed in days. Days selected were day 9 (before antibiotic treatment), day 17 (after antibiotics), day 18 (after single dose of propionate producing consortium), day 24 (after 3 doses of propionate producing treatment) and day 27 (end of the washout period).

<table>
<thead>
<tr>
<th>SCFA</th>
<th>TPT</th>
<th>Condition</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>CTR (Mean % ± SEM)</td>
<td>TRT(Mean % ± SEM)</td>
</tr>
<tr>
<td>Acetate</td>
<td>9</td>
<td>30.65 ± 5.32</td>
<td>32.59 ± 2.80</td>
</tr>
<tr>
<td></td>
<td>17</td>
<td>30.08 ± 1.04</td>
<td>31.24 ± 2.20</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>29.88 ± 1.20</td>
<td>31.25 ± 0.99</td>
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<tr>
<td></td>
<td>24</td>
<td>34.08 ± 2.95</td>
<td>35.69 ± 1.76</td>
</tr>
<tr>
<td></td>
<td>27</td>
<td>31.90 ± 2.94</td>
<td>34.3 ± 1.74</td>
</tr>
<tr>
<td>Propionate</td>
<td>9</td>
<td>12.87 ± 1.38</td>
<td>15.09 ± 0.22</td>
</tr>
<tr>
<td></td>
<td>17</td>
<td>5.10 ± 0.35</td>
<td>5.75 ± 0.15</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>4.81 ± 0.13</td>
<td>5.85 ± 0.12</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>9.62 ± 1.58</td>
<td>14.84 ± 1.06</td>
</tr>
<tr>
<td></td>
<td>27</td>
<td>10.14 ± 1.44</td>
<td>14.44 ±0.89</td>
</tr>
<tr>
<td>Butyrate</td>
<td>9</td>
<td>9.93 ± 2.46</td>
<td>6.40 ± 1.27</td>
</tr>
<tr>
<td></td>
<td>17</td>
<td>0.33 ± 0.12</td>
<td>0.24 ± 0.05</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>0.67 ± 0.09</td>
<td>0.43 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>2.52 ± 0.23</td>
<td>2.48 ± 0.22</td>
</tr>
<tr>
<td></td>
<td>27</td>
<td>2.29 ± 0.11</td>
<td>2.82 ± 0.32</td>
</tr>
</tbody>
</table>
Table S4: Ratios of the aggregate monomer of the JC1 values (indicative for membrane potential) after the different treatments applied on the epithelial cells. Overall significance level = 0.05. Clindamycin treatment (CLN) showed a similar effect to CCCP which is the positive control for mitochondrial membrane potential disruption. Propionate producing consortium (PPC) showed a significant effect on membrane potential when added together with CLN, showing significant difference (P<0.05) with CLN treatment alone. Significant differences were indicated by different superscripts.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean% ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.793 ± 0.035&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>CCCP</td>
<td>0.189 ± 0.008&lt;sup&gt;bc&lt;/sup&gt;</td>
</tr>
<tr>
<td>CLN</td>
<td>0.205 ± 0.011&lt;sup&gt;bc&lt;/sup&gt;</td>
</tr>
<tr>
<td>CLN+PPC</td>
<td>0.461 ± 0.086&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>PPC</td>
<td>0.628 ± 0.083&lt;sup&gt;ad&lt;/sup&gt;</td>
</tr>
</tbody>
</table>
Table S5A: Bacterial relative abundances of taxa belonging to the Propionate-producing consortium (PPC) in the luminal compartment of the M-SHIME at the end of the stabilisation phase (time point 1), after clindamycin supplementation (time point 2), after 3 days of PPC administration (time point 3) and after 4 days of washout (time point 4). NS= not significantly different. Different superscripts indicate significantly different means.

<table>
<thead>
<tr>
<th>Taxon</th>
<th>Time point</th>
<th>Treatment (Mean ± SEM)</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>PPC</td>
<td>Control</td>
</tr>
<tr>
<td>Akkermansia</td>
<td>1</td>
<td>0.0045 ± 0.002^{ab}</td>
<td>0.0018 ± 0.0007^a</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.0021 ± 0.009^{ab}</td>
<td>0.00008 ± 0.00003^b</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0.0065 ± 0.004^{ab}</td>
<td>0.0018 ± 0.0006^b</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>0.0123 ± 0.005^{ab}</td>
<td>0.0071 ± 0.0044^c</td>
</tr>
<tr>
<td>Bacteroides</td>
<td>1</td>
<td>0.0153 ± 0.001^{a}</td>
<td>0.0143 ± 0.002^a</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.0136 ± 0.0651^a</td>
<td>0.0134 ± 0.001^a</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0.0125 ± 0.001^b</td>
<td>0.0104 ± 0.0009^b</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>0.0126 ± 0.001^a</td>
<td>0.0112 ± 0.002^b</td>
</tr>
<tr>
<td>Blautia</td>
<td>1</td>
<td>0.0012 ± 0.005^{a}</td>
<td>0.0014 ± 0.0006^a</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.0008 ± 0.0003^{b}</td>
<td>0.0003 ± 0.0001^a</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0.0008 ± 0.0002^{b}</td>
<td>0.0003 ± 0.0001^a</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>0.0021 ± 0.0007^{c}</td>
<td>0.0011 ± 0.0004^a</td>
</tr>
<tr>
<td>Coprococcus</td>
<td>1</td>
<td>0.000001 ± 4E-6^{a}</td>
<td>0.00001 ± 5.4E-6^a</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>2.6E-8 ± 0.0106^b</td>
<td>3.1E-6 ± 2.86E-6^a</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>2.2E-8 ± 9.0E-9^c</td>
<td>1.9E-6 ± 1.8E-6^b</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>1.2E-7 ± 4.8E-8^d</td>
<td>6.9E-6 ± 3.9E-6^a</td>
</tr>
<tr>
<td>Lactobacillus</td>
<td>1</td>
<td>Below detection limit</td>
<td>Below detection limit</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Below detection limit</td>
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<td>Below detection limit</td>
<td>Below detection limit</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>Below detection limit</td>
<td>Below detection limit</td>
</tr>
<tr>
<td>Veillonella</td>
<td>1</td>
<td>0.00029 ± 0.0003^{a}</td>
<td>0.00019 ± 0.0002^a</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.00091 ± 0.0008^{b}</td>
<td>0.0044 ± 0.0004^b</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0.0074 ± 0.0007^{c}</td>
<td>0.0043 ± 0.0039^c</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>0.017 ± 0.012^{d}</td>
<td>0.0028 ± 0.0025^d</td>
</tr>
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Table S5B: Bacterial relative abundances of taxa belonging to the Propionate-producing consortium (PPC) in the mucosal compartment of the M-SHIME at the end of the stabilisation phase (time point 1), after clindamycin supplementation (time point 2), after 3 days of PPC administration (time point 3). NS= not significantly different. Different superscripts indicate significantly different means.

<table>
<thead>
<tr>
<th>Taxon</th>
<th>Time point</th>
<th>Treatment (Mean ± SEM)</th>
<th>Effect</th>
<th>Time</th>
<th>Treatment</th>
<th>Time*Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>PPC</td>
<td>Control</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Akkermansia</strong></td>
<td>1</td>
<td>0.0156 ± 0.005a</td>
<td>0.0078 ± 0.0003a</td>
<td>0.001</td>
<td>0.09</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.0141 ± 0.009a</td>
<td>0.0036 ± 0.0001b</td>
<td>0.002</td>
<td>0.01</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0.051 ± 0.004b</td>
<td>0.0023 ± 0.0001b</td>
<td>0.001</td>
<td>0.09</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Bacteroides</strong></td>
<td>1</td>
<td>0.0060 ± 0.0014</td>
<td>0.0064 ± 0.0014</td>
<td>0.06</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.0055 ± 0.0018</td>
<td>0.0049 ± 0.0007</td>
<td>0.008</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0.0039 ± 0.0011</td>
<td>0.0032 ± 0.0006</td>
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<td></td>
</tr>
<tr>
<td><strong>Blautia</strong></td>
<td>1</td>
<td>0.0007 ± 0.0002a</td>
<td>0.0007 ± 0.0002a</td>
<td>0.008</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.0003 ± 0.001b</td>
<td>0.0002 ± 0.00008b</td>
<td>&lt;0.0001</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0.0003 ± 0.00007b</td>
<td>0.0002 ± 0.00006b</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Coprococcus</strong></td>
<td>1</td>
<td>0.00004 ± 3E-6a</td>
<td>0.00002 ± 5.8E-6a</td>
<td>0.008</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
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<td>2</td>
<td>0.00003 ± 0.00002a</td>
<td>4.7E-6 ± 2.01E-6b</td>
<td>0.001</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>3.66E-6 ± 1.3E-6b</td>
<td>4.6E-6 ± 5.5E-6ab</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Lactobacillus</strong></td>
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<td>Below detection limit</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
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<td>Below detection limit</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Below detection limit</td>
<td>Below detection limit</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>Veillonella</strong></td>
<td>1</td>
<td>0.00048 ± 0.0007a</td>
<td>0.00031 ± 0.0004a</td>
<td>&lt;0.0001</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.000115 ± 0.00002b</td>
<td>0.00015 ± 0.00002b</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0.0076 ± 0.0001c</td>
<td>0.0013 ± 0.0019c</td>
<td></td>
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</tbody>
</table>
Table S5C: Taxa whose relative abundance was impacted through supplementation of the Propionate-producing consortium (PPC) in the luminal compartment of the M-SHIME at the end of the stabilisation phase (time point 1), after clindamycin supplementation (time point 2), after 3 days of PPC administration (time point 3) and after 4 days of washout (time point 4). NS= not significantly different. Different superscripts indicate significantly different means across time points within a treatment.

<table>
<thead>
<tr>
<th>Taxon</th>
<th>Time point</th>
<th>Treatment (Mean ± SEM)</th>
<th>Effect</th>
<th>Time</th>
<th>Treatment</th>
<th>Time*Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Anaerostipes</strong></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>5.8E-7 ± 3.3E-7a</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>2.4E-9 ± 1.1E-9b</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>2.2E-9 ± 9.2E-10b</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>1.7E-6 ± 1.3E-6b</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.2E-6 ± 7.8E-7a</td>
<td></td>
<td></td>
<td></td>
<td>&lt;0.0001 NS NS</td>
</tr>
<tr>
<td><strong>Bifidobacterium</strong></td>
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<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>0.0002 ± 0.00004a</td>
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</tr>
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<td>0.00006 ± 0.00003b</td>
<td></td>
<td></td>
<td></td>
<td>&lt;0.0001 NS NS</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0.00005 ± 0.00004c</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td>0.0001 ± 0.0002b</td>
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<td>4.9E-6 ± 2.9E-6b</td>
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Table S5D: Taxa whose relative abundance was impacted through the supplementation of the Propionate-producing consortium (PPC) in the mucosal compartment of the M-SHIME, at the end of the stabilisation phase (time point 1), after clindamycin supplementation (time point 2), after 3 days of PPC administration (time point 3). NS= not significantly different. Different superscripts indicate significantly different means across time points within a treatment.

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<th>Taxon</th>
<th>Time point</th>
<th>Treatment (Mean ± SEM)</th>
<th>Effect</th>
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<td>Control</td>
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<td>0.0044 ± 0.0003b</td>
<td>0.00146 ± 0.0007a</td>
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<td>0.0002 ± 0.00006b</td>
<td>0.00006 ± 0.0002b</td>
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<td>0.00014 ± 0.0005b</td>
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<td>0.0017 ± 0.0004</td>
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<tr>
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<td>0.00003 ± 0.00004</td>
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<tr>
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<td>0.0006 ± 0.0001</td>
<td>0.0006 ± 0.0002</td>
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<td>0.0007 ± 0.0001</td>
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<td>0.0050 ± 0.0009</td>
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<td>0.0009 ± 0.0002</td>
</tr>
<tr>
<td><strong>Parabacteroides</strong></td>
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<td>0.0007 ± 0.0001</td>
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<td>0.0050 ± 0.0009</td>
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<td>0.0009 ± 0.0002</td>
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<td>0.0010 ± 0.0002</td>
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<td>0.0007 ± 0.0003</td>
<td>0.0004 ± 0.00005</td>
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<td>0.0002 ± 0.00005</td>
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<td>1.6E-8 ± 6.1E-9</td>
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Table S6: Differences in absolute abundances of the certain genera included in the propionate-producing consortium between control and treatment vessels in the lumen compartment. The significant increase in Veillonella in the treatment vessels indicates its successful direct engraftment (P<0.05). The differences were assessed by 2-way Anova test using Holm-sidak method with significance at P<0.05. Time point (TPT) indicates the different phases at which the samples were analysed (1: End of stabilization phase, 2: After antibiotic treatment, 3: After 3 doses of propionate producing consortium, and 4: Washout phase).

<table>
<thead>
<tr>
<th>Genus</th>
<th>TPT</th>
<th>CTR (Mean % ± SEM)</th>
<th>TRT(Mean % ± SEM)</th>
<th>P value</th>
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<td>0</td>
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</tr>
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<td>0</td>
<td>0</td>
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<td>4.09e+04 ± 1.98e+04</td>
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<td>1.21e+08 ±7.71e+07</td>
<td>1.45e+08 ± 7.67e+07</td>
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Table S7: Differences in absolute abundances of the certain genera included in the propionate-producing consortium between control and treatment vessels in the mucin compartment. The significant increase in Veillonella in the treatment vessels indicates its successful direct engraftment (P<0.05). The differences were assessed by 2-way Anova test using Holm-sidak method with significance at P<0.05. Time point (TPT) indicates the different phases at which the samples were analysed (1: End of stabilization phase, 2: After antibiotic treatment, and 3: After 3 doses of propionate producing consortium).

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<th>TRT (Mean % ± SEM)</th>
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Table S8: Differences in absolute abundances of the certain genera that were involved in propionate production after adding the propionate-producing consortium in the lumen compartment. The significant increase in unclassified Lactobacillacea and unclassified Lachnospiraceae in the treatment vessels indicates successful indirect reinforcement of those genera (P<0.05). The differences were assessed by 2-way Anova test using Holm-sidak method with significance at P<0.05. Time point (TPT) indicates the different phases at which the samples were analysed (1: End of stabilization phase, 2: After antibiotic treatment, 3: After 3 doses of propionate producing consortium, and 4: Washout phase).

<table>
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<th>TRT (Mean % ± SEM)</th>
<th>P value</th>
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<td>NS</td>
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Table S9: Differences in absolute abundances of the certain genera that were involved in propionate production after adding the propionate-producing consortium in the mucin compartment. The significant increase in unclassified Lactobacillacea in the treatment vessels indicates successful indirect reinforcement of this genus in the mucin (P<0.05). The differences were assessed by 2-way Anova test using Holm-sidak method with significance at P<0.05. Time point (TPT) indicates the different phases at which the samples were analysed (1: End of stabilization phase, 2: After antibiotic treatment, and 3: After 3 doses of propionate producing consortium).

<table>
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<th>Genus</th>
<th>TPT</th>
<th>CTR (Mean % ± SEM)</th>
<th>TRT (Mean % ± SEM)</th>
<th>P value</th>
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<tbody>
<tr>
<td>unclassified_Lactobacillacea</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>NS</td>
</tr>
<tr>
<td></td>
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<td>0</td>
<td>0</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0</td>
<td>7.27e+05 ± 1.91e+05</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>unclassified Lachnospiraceae</td>
<td>1</td>
<td>4.21e+07 ± 8.90e+06</td>
<td>5.93e+07 ± 1.69e+07</td>
<td>NS</td>
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<tr>
<td></td>
<td>2</td>
<td>1.01e+07 ± 2.32e+06</td>
<td>2.37e+07 ± 8.12e+06</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>9.44e+06 ± 3.30e+06</td>
<td>1.85e+07 ± 8.62e+06</td>
<td>NS</td>
</tr>
<tr>
<td>Bilophila</td>
<td>1</td>
<td>5.37e+07 ± 1.39e+07</td>
<td>6.35e+07 ± 1.48e+07</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>8.73e+06 ± 2.38e+06</td>
<td>1.91e+07 ± 7.24e+06</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>1.70e+06 ± 6.18e+06</td>
<td>1.67e+07 ± 4.23e+06</td>
<td>NS</td>
</tr>
</tbody>
</table>
Figure S1: Production of SCFA after 48h of a fed batch for the 7 strains of the propionate producing consortium. One dose of the consortium was prepared fresh on a daily basis for each day of the treatment on the SHIME run. Four replicates of the fed batch are presented with an average of 34.5 mM.
Figure S2: Scheme of SHIME setups used in our experiments with the timeline of each experiment. M-SHIME setup for the single-donor experiment in which the fecal sample from one donor was tested at a time. Control and treatment vessels were in triplicates (A). A timeline is provided under the SHIME setup showing the different phases of the SHIME run at different days for the total of 27 days (A). M-SHIME setup for the multiple-donor experiment in which the fecal samples from six different donors were inoculated in separate vessels considering control and treatment condition as shown in (B). A timeline is provided under the SHIME scheme showing the different phases of the SHIME run at different days for the total of 23 days (B).
Figure S3: PCoA analysis showing the dispersion of the microbial community by treatment for the 6 donors in the lumen (A) and mucin (B) compartments.
Figure S4: Addition of the propionate producing consortium (PPC) promotes recovery of propionate production after antibiotic-associated disruption in donor 1. (A) Short chain fatty acid production during the 4 different phases of the experiment: Stabilization, post-antibiotic disruption, post-addition of a single dose of the propionate producing consortium, and post-addition of 3 doses of the propionate producing consortium. Single dose of treatment was added on day 17, and the 3 doses were added on days 20, 21, and 22. (B) Propionate was the main short chain fatty acid impacted already after 2 doses (Days 21-27) in the treatment reactors ($P < 0.05$). No significant difference was detected for acetate (C) and butyrate (D) after the treatment was added.
Figure S5: Addition of the propionate producing consortium (PPC) did not have any significant effect propionate production after antibiotic-associated disruption in donor 2. (A) Short chain fatty acid production during the 4 different phases of the experiment: Stabilization, post-antibiotic disruption, post-addition of a single dose of the propionate producing consortium, and post-addition of 3 doses of the propionate producing consortium. Single dose of treatment was added on day 17, and the 3 doses were added on days 20, 21, and 22. (B) Propionate was the main short chain fatty acid was not impacted after any of the treatment doses (Days 21-27) in the treatment reactors ($P >0.05$). No significant difference was detected for acetate (C) and butyrate (D) after the treatment was added.
Figure S6: Cell counts were significantly lower after antibiotic use in both lumen and mucin compartments (P<0.05). Cell counts for lumen SHIME samples significantly decreased after antibiotic treatment (P<0.05). Cell counts for lumen samples were compared at 4 different time points for 4 different SHIME phases: 1-Stabilization phase, 2-after antibiotic phase, 3-after PPC treatment phase, and 4-the last day of the SHIME run that is the washout phase. There was no significant increase for the cell load after PPC treatment (A). Cell counts for mucin SHIME samples significantly decreased after antibiotic treatment (P<0.05). Cell counts for mucin samples were compared at 4 different time points for 3 different SHIME phases: 1-Stabilization phase, 2-after antibiotic phase, and 3-after PPC treatment phase. There was no significant increase for the cell load after PPC treatment (B).
Figure S7: Network of bacterial interactions influencing the production of the major short chain fatty acids over time. (A) Treatment reactors after antibiotics treatment and before adding 3 doses of the propionate consortium, (B) Treatment reactors after 3 days period of washout after the propionate consortium dosing. These bipartite networks are based on the regularised canonical correlations between relative bacterial abundances and relative concentrations of the main SCFA. Interactions have been filtered for an absolute correlation above 0.8 and are coloured following the key shown. Significant interactions are shorter lines, and genera with similar abundances within SHIME.
compartment tend to cluster closely. Networks depict genera potentially performing roles in propionate production.
ANNEX III

### Table S1: Differences in glycogen storage in the hepatic cells between different treatments and between high and low glucose conditions.

The significant increase in glycogen when the supernatant from the PPC was added indicates positive effect of PPC towards increasing glycogen storage in the case of high glucose (P<0.05). The differences between treatments were assessed by one-way Anova test using Tukey’s method with significance at P<0.05. Values are presented in ug/ml.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Condition</th>
<th>High Glucose (Mean % ± SEM)</th>
<th>Low Glucose (Mean % ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPC</td>
<td></td>
<td>22.60 ± 2.18&lt;sup&gt;a&lt;/sup&gt;</td>
<td>9.55 ± 0.12&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>SCFA PPC</td>
<td></td>
<td>8.53 ± 0.19&lt;sup&gt;b&lt;/sup&gt;</td>
<td>7.70 ± 0.22&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>SCFA Akk</td>
<td></td>
<td>10.93 ± 0.15&lt;sup&gt;b&lt;/sup&gt;</td>
<td>11.40 ± 0.24&lt;sup&gt;bd&lt;/sup&gt;</td>
</tr>
<tr>
<td>Akk</td>
<td></td>
<td>9.38 ± 0.42&lt;sup&gt;b&lt;/sup&gt;</td>
<td>8.13 ± 0.10&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>PPC-Akk</td>
<td></td>
<td>9.83 ± 0.23&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.98 ± 0.13&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td>9.30 ± 0.45&lt;sup&gt;b&lt;/sup&gt;</td>
<td>9.48 ± 0.64&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>HepG2</td>
<td></td>
<td>6.45 ± 0.23&lt;sup&gt;be&lt;/sup&gt;</td>
<td>3.18 ± 0.05&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Table S1: Different treatments with different SCFA amounts that were added on the enterohepatic cell model. Treatments were diluted 5 fold in DMEM before adding to the cell model.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Acetate (mM)</th>
<th>Propionate (mM)</th>
<th>Butyrate (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPC</td>
<td>37.65</td>
<td>34.49</td>
<td>0</td>
</tr>
<tr>
<td>PPC-Akk</td>
<td>35.73</td>
<td>33.64</td>
<td>0</td>
</tr>
<tr>
<td>Akk</td>
<td>26.66</td>
<td>0.80</td>
<td>0.38</td>
</tr>
<tr>
<td>SCFA PPC</td>
<td>37</td>
<td>34</td>
<td>0</td>
</tr>
<tr>
<td>SCFA Akk</td>
<td>27</td>
<td>0.8</td>
<td>0.4</td>
</tr>
</tbody>
</table>

Table S2: Differences in glycogen storage in the hepatic cells between different treatments and between high and low glucose conditions. The significant increase in glycogen when the supernatant from the PPC was added indicates positive effect of PPC towards increasing glycogen storage in the case of high glucose (P<0.05). The differences between treatments were assessed by one-way Anova test using Tukey’s method with significance at P<0.05. Values are presented in ug/ml.
Table S3: Differences in lipoprotein lipase (LPL) activity levels in the hepatic cells between different treatments and between high and low glucose conditions. The significant increase in LPL when the supernatant from the PPC was added indicates positive effect of PPC towards increasing LPL activity storage in the case of high glucose (P<0.05). The differences between treatments were assessed by one-way Anova test using Tukey’s method with significance at P<0.05. Values are presented in mU/mL.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Condition</th>
<th>High Glucose (Mean % ± SEM)</th>
<th>Low Glucose (Mean % ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPC</td>
<td></td>
<td>21.67 ± 0.58(^a)</td>
<td>16.06 ± 0.80(^{ab})</td>
</tr>
<tr>
<td>SCFA PPC</td>
<td></td>
<td>15.73 ± 1.29(^{bc})</td>
<td>10.65 ± 0.90(^{bc})</td>
</tr>
<tr>
<td>SCFA Akk</td>
<td></td>
<td>18.38 ± 2.76(^{abc})</td>
<td>10.40 ± 0.71(^{cd})</td>
</tr>
<tr>
<td>Akk</td>
<td></td>
<td>13.25 ± 0.38(^{bd})</td>
<td>10.57 ± 0.74(^{bd})</td>
</tr>
<tr>
<td>PPC-Akk</td>
<td></td>
<td>16.47 ± 1.04(^{abce})</td>
<td>13.52 ± 0.70(^{bcde})</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td>17.69 ± 1.17(^{abce})</td>
<td>11.11 ± 0.73(^{bcde})</td>
</tr>
</tbody>
</table>

Table S4: Differences in IL-8 levels in the hepatic cells between different treatments and between high and low glucose conditions. The decrease in IL-8 when the supernatant from the PPC was added indicates the potential positive effect of PPC towards decreasing inflammatory markers in the case of high glucose. The differences between treatments were assessed by one-way Anova test using Tukey’s method with significance at P<0.05. Values are presented in pg/ml.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Condition</th>
<th>High Glucose (Mean % ± SEM)</th>
<th>Low Glucose (Mean % ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPC</td>
<td></td>
<td>2802.67 ± 219.83(^a)</td>
<td>2478.89 ± 203.32(^a)</td>
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<tr>
<td>SCFA PPC</td>
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<td>3213.33 ± 461.96(^a)</td>
<td>2930.00 ± 380.79(^a)</td>
</tr>
<tr>
<td>SCFA Akk</td>
<td></td>
<td>2872.89 ± 348.43(^a)</td>
<td>2586.00 ± 270.41(^a)</td>
</tr>
<tr>
<td>Akk</td>
<td></td>
<td>3034.00 ± 61.23(^a)</td>
<td>2819.33 ± 86.43(^a)</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td>3532.00 ± 135.57(^a)</td>
<td>3047.33 ± 36.56(^a)</td>
</tr>
<tr>
<td>Caco2</td>
<td></td>
<td>1327.94 ± 224.06(^c)</td>
<td>1183.37 ± 48.28(^c)</td>
</tr>
</tbody>
</table>
Table S5: Differences in IL-6 levels in the hepatic cells between different treatments and between high and low glucose conditions. There was no significant difference between treatments ($P>0.05$) indicating no effect of any treatment on IL-6 levels. The differences between treatments were assessed by one-way Anova test using Tukey’s method with significance at $P<0.05$. Values are presented in pg/ml.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Condition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>High Glucose (Mean % ± SEM)</td>
</tr>
<tr>
<td>PPC</td>
<td>628.22 ± 26.51</td>
</tr>
<tr>
<td>SCFA PPC</td>
<td>612.44 ± 27.26</td>
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<tr>
<td>SCFA Akk</td>
<td>651.11 ± 41.20</td>
</tr>
<tr>
<td>Akk</td>
<td>585.56 ± 22.82</td>
</tr>
<tr>
<td>Control</td>
<td>604.00 ± 18.58</td>
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</table>

Table S6: Differences in TNF-α levels in the hepatic cells between different treatments and between high and low glucose conditions. There was no significant difference between treatments ($P>0.05$) indicating no effect of any treatment on TNF-α levels. The differences between treatments were assessed by one-way Anova test using Tukey’s method with significance at $P<0.05$. Values are presented in pg/ml.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Condition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>High Glucose (Mean % ± SEM)</td>
</tr>
<tr>
<td>PPC</td>
<td>170.63 ± 10.72\textsuperscript{bc}</td>
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<tr>
<td>SCFA PPC</td>
<td>175.44 ± 7.88\textsuperscript{bc}</td>
</tr>
<tr>
<td>SCFA Akk</td>
<td>159.19 ± 8.81\textsuperscript{bc}</td>
</tr>
<tr>
<td>Akk</td>
<td>226.41 ± 12.04\textsuperscript{a}</td>
</tr>
<tr>
<td>Control</td>
<td>190.44 ± 4.90\textsuperscript{bc}</td>
</tr>
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</table>
Table S7: TEER values (Ohms) are expressed as mean ± SEM (n = 6).

<table>
<thead>
<tr>
<th></th>
<th>Low glucose</th>
<th>High glucose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>630 ± 32</td>
<td>575 ± 49</td>
</tr>
<tr>
<td>Caco-2</td>
<td>566 ± 32</td>
<td>563 ± 36</td>
</tr>
<tr>
<td>PPC</td>
<td>707 ± 33</td>
<td>677 ± 16</td>
</tr>
<tr>
<td>PPC-Akk</td>
<td>643 ± 27</td>
<td>548 ± 62</td>
</tr>
<tr>
<td>Akk</td>
<td>607 ± 11</td>
<td>639 ± 24</td>
</tr>
<tr>
<td>SCFA PPC</td>
<td>676 ± 25</td>
<td>663 ± 12</td>
</tr>
<tr>
<td>SCFA Akk</td>
<td>576 ± 10</td>
<td>579 ± 8</td>
</tr>
</tbody>
</table>

Table S8: Values of LY transport (%) to the basolateral compartment of the Transwell system are expressed as mean ± SEM (n = 6).

<table>
<thead>
<tr>
<th></th>
<th>Low glucose</th>
<th>High glucose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1,46 ± 0,10</td>
<td>1,14 ± 0,20</td>
</tr>
<tr>
<td>Caco-2/HT29-MTX</td>
<td>1,44 ± 0,30</td>
<td>1,84 ± 0,15</td>
</tr>
<tr>
<td>PPC</td>
<td>1,37 ± 0,03</td>
<td>1,32 ± 0,09</td>
</tr>
<tr>
<td>PPC-Akk</td>
<td>1,42 ± 0,07</td>
<td>1,51 ± 0,05</td>
</tr>
<tr>
<td>Akk</td>
<td>1,35 ± 0,08</td>
<td>1,50 ± 0,23</td>
</tr>
<tr>
<td>SCFA PPC</td>
<td>1,34 ± 0,04</td>
<td>1,57 ± 0,11</td>
</tr>
<tr>
<td>SCFA Akk</td>
<td>1,33 ± 0,08</td>
<td>1,27 ± 0,07</td>
</tr>
</tbody>
</table>
EDUCATION

**Ghent University** - PhD candidate in Applied Biological Sciences
Ability of a propionate producing synthetic microbial consortium to restore functionality in a dysbiosed human gut microbiome

**University of Aberdeen** - MSc in Molecular Nutrition
Ability of isolated human gut bacteria to utilize oligosaccharides from human milk

**American University of Beirut** - BSc in Nutrition and Dietetics
Concentration: Therapeutic Nutrition

EXPERIENCE

**PhD researcher** - Ghent University
Center for microbial ecology and Technology (Cmet)
Establishment of a multispecies propionate producing synthetic microbial community able to restore antibiotic associated gut dysbiosis

Laboratory experience:
- Building bioreactors like the in vitro model system (Simulator of the human intestinal microbial ecosystem (SHIME))
- Plating and MIC determination (minimum inhibitory determination)
- Aerobic and Anaerobic culturing
- Molecular techniques (DNA and RNA extractions, PCR, DGGE)
- VFA extraction techniques

**Academic- secondment at University of Copenhagen**
Testing the passage of the members of our designed propionate-producing microbial consortium in the upper GI tract specifically in The smallest Intestine (TSI) model
Industry Secondment-ProDigest
Lyophilization the propionate-producing bacterial consortium and testing its survival afterwards in fed batch experiments that mimic the SHIME 2013-2014

Research Assistant – American University of Beirut
Department of Pediatrics and Adolescent Medicine

Six Month Research at Rowett Institute-University of Aberdeen 2013
Microbiology Lab
Anaerobic culturing of gut bacteria – thin layer chromatography (TLC) – Microscopy

Six Month Training in Dietetics field-Mount Lebanon Hospital 2011-2012
Covered topics:
• Imbalance of body weight
• Diabetes and Endocrinology
• Diseases of the circulatory system and Anemia
• Gastroenterology
• Kidney Disorders
• Pulmonary Diseases
• Infancy and Childhood
• Tube Feeding

PUBLICATIONS


under stress conditions, as shown in a gastrointestinal simulator. *Molecular Nutrition & Food Research, 61*(1), p.1600150.

**PRESENTATIONS**

**International Yakult Symposium-Gent**  
Flash talk and poster presentation: Ability of a propionate producing consortium to promote recovery from antibiotic-associated gut dysbiosis  
**Sep. 2017**

**9th Probiotics, Prebiotics & New foods - Rome**  
Poster presentation: Ability of a propionate producing consortium to promote recovery from antibiotic-associated gut dysbiosis  
**Nov. 2016**

**18th Gut Day Symposium – Venlo**  
Oral presentation: Potential of a probiotic propionate producing community to recover from the disorder caused by antibiotics  
**Oct. 2016**

**4th World Congress on targeting Microbiota - Paris**  
Poster presentation: Potency of a propionate-producing microbial consortium for restoring gut microbial functionality in gut dysbiosis  
**Sep. 2017**

**Research Training**

**Doctoral school courses and workshops completed:**

- Interphase processes of host associated microorganisms-2015
- Leadership Foundation Course-2015
- Effective Graphical Displays -November 2015

**Awards**

**Poster Award Winner**-International Yakult Symposium (April 2018)

**PhD Scholarship**- Marie Curie PhD Fellowship (Oct. 2014)

**SKILLS**

**Teaching:** mentoring and supervision of a masters student for one year during his dissertation, in addition to teaching assistant in practical lab work in Host-microbe interaction course

**Computer programs:** Microsoft office: Excel, PowerPoint, and Word

**Statistical software:** GraphPad and Sigma Plot

**Languages:** Full professional proficiency of English and scientific writing, Arabic (mother tongue), Fair in French
Acknowledgements

Achieving a PhD is not easy without the support of people surrounding you. In this occasion, I would like to take the opportunity to thank several people whom having their support has led me for this great achievement which is an important milestone in my life. First, I would like to start thanking my supervisor Prof. Tom Van De Wiele who has given me the chance to be part of his team and who supported me continuously throughout my research. Thank you for your continuous feedbacks on the different experimental and theoretical work that I was doing, and thank you for taking your time to read my work on and on. I thank you also for giving me from your time whenever needed despite your busy schedule. I would also like to thank you for criticizing my work in a way that helped improve it to reach this stage, and thank you for guiding me throughout my PhD.

I would like to take the chance to thank Dr. Emma Hernandez-Sanabria who was my supervisor and mentor throughout my PhD. I am so grateful for all your help and support throughout this journey. I would like to thank you for teaching me a lot of new lab techniques and the way of thinking and looking at things that helped me achieve the most reliable results. I would like to thank you for your continuous advice and for the theoretical and practical help and for reading my work several times and always giving me feedback. I would also like to thank you for the great figures generated in R that I wouldn’t have achieved without your help. Thank you for believing in me and my potentials, and for always supporting me. Thank you for not only being a mentor but also for being a great friend and sister.

I would like to thank my examination committee members Prof. dr. Abdul Mouazen, Prof. dr. Andreja Rajkovic, Prof. dr. ir. John Van Camp, Prof. dr. Filip Van Immerseel, and Dr. Kristof Van Emelen for their time and revisions, and for their valuable feedback that helped improve the quality of my PhD thesis significantly.

I would also like to express my gratitude for the administrative and technical assistance I have received from the staff members at CMET. I would like to specifically thank the people who had a considerable contribution during my PhD from paper work to lab assistance. Thank you Christine Graveel, Regine Haspeslagh and Sarah Steenbeke for the help in all the paper and administrative work, and again thanks to Regine for helping me in arranging for my public defence. Thank you Jana De Bodt, Tim Lacoere, Renee Graveel, Siska Maertens, Greet Van De Velde, and Mike Taghon for the technical assistance.

I would also like to thank colleagues in CMET and in specific colleagues in the HAM cluster for their inputs and knowledge sharing during the clusters and meetings. Special thanks to my officemates Ruben Props, Chris Callewaert, Frederiek Maarten Kerckhof, Giovanni Ganendra, Yusuf Cagatay Ersan, Ralph Lindeboom, Nicole Hahn, Xiaona Hu, and Funda Torun for their friendship, advice and the good times we had spent in the office.
Special thanks to Dr. Marta Calatayud Arroyo for her help in all cell work experiments, for her continuous support and advice, and her feedback on my work. I would also like to thank Charlotte De Rudder for the sweet conversations, and for her help in writing the summary of my PhD thesis in Dutch. Thanks to Tomasz Cieplak for his assistance during my experiments in the University of Copenhagen.

My very special thanks and gratitude goes to my very close friends in Gent whom I consider family away from home. Thank you Emma Hernandez-Sanabria, Chiara Ilgrande, Davide Gottardi, Nayaret Acosta and of course my favourite Matti, Elham Ehsani and lovely Elsa, Janet Molina, Lorenzo Cimmino, and Annalucia Stanisci for the good times we have spent together and for all your support and love throughout this long journey.

I am extremely thankful for my family members including my grandfather who was one of the major reasons that I planned to go for a PhD, my uncles, aunts, and my cousins for their continuous support. I am so grateful and thankful for my sisters Lulu, Reem, Aya, and my lovely nephews Hammoudy and Alloushi who were a huge source of support to me in my long journey away from home, and who were always proud of me in every step I achieved throughout my PhD and throughout my whole educational path. Thank you for your continuous support and love. I would also like to express a special thanks and gratitude for my twin sister, Reem, for drawing the image on the cover of my thesis and who had to repeat the drawing several times in order to make it that perfect!

My supportive husband Mohamad, thank you for being there in every step of my PhD. Thank you for always supporting me and believing in me, and thank you for always spreading the positive vibes and love around me. Thank you for always listening to me and showing me how proud you are. You have been a major support in this long journey in every single way from inviting me to dinners when my papers were accepted to getting me flowers whenever I accomplished an achievement. Thank you for reading my work when I needed another opinion and thank you for accompanying me to check on the shime on the weekends despite the not very pleasant smell. Thank you for everything.

Last but not least, I would like to deeply thank my Mom and Dad whom without their support I wouldn’t have made it to this stage. Thank you for always believing in me and making me believe that my limit is the sky. Thank you for your continuous support throughout all my educational journey and in every step I attempt to do. Thank you for always being proud of me which pushed me to finish this stage successfully as I never wanted to put you or your hopes down. I love you endlessly.