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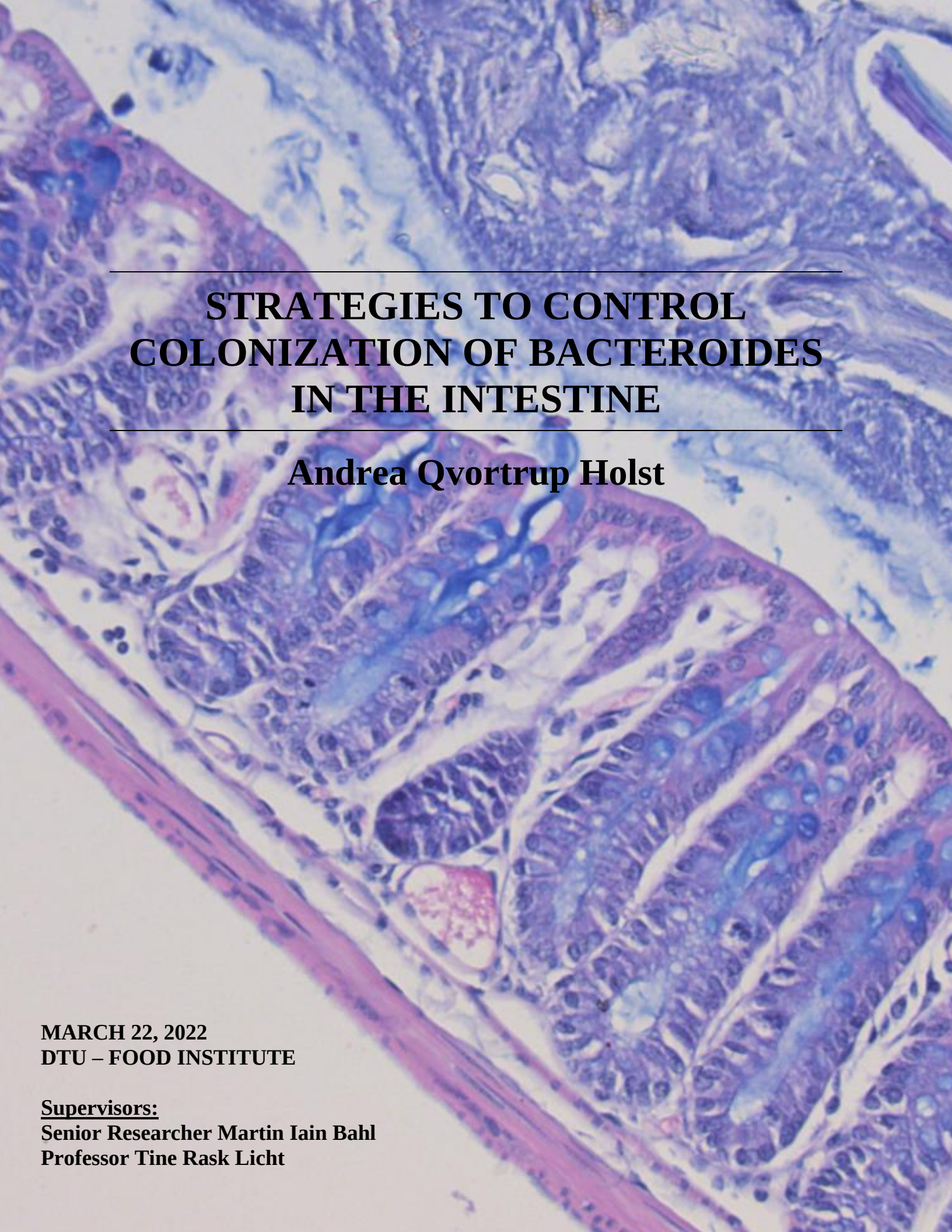

STRATEGIES TO CONTROL COLONIZATION OF BACTEROIDES IN THE INTESTINE

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DTU – FOOD INSTITUTE

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Strategies to control colonization of *Bacteroides* in the intestine

by Andrea Qvortrup Holst

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Cover: Colon section of HMO supplemented mouse. Stained with Hematoxylin and Alcian Blue.

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Abstract

The human gut contains a diverse microbial community known as the microbiota. The microbiota consists of several hundred to thousands of different bacterial species whereof the genus of *Bacteroides* is one of the most abundant. In most individuals, *Bacteroides* will become a substantial part of microbiota after infancy correlating with the introduction of dietary fibers and the cessation of breastfeeding. *Bacteroides* species are recognized for a large capacity of utilizing complex carbohydrates including host-derived glycans such as mucus and Human Milk Oligosaccharide (HMO). How the HMO-utilizing abilities of *Bacteroides* affect their abundance *in vivo* is not well understood. We investigated if an HMO supplement added to the drinking water of conventional mice affected their microbiota. We reported that the family of *Bacteroidaceae* and specifically the genus of *Phocaeicola* (formerly part of *Bacteroides*) increased in both absolute and relative abundance upon HMO supplementation and showed a concurrent decrease in the genus of the butyrate-producing *Lacrimispora*. The decrease in *Lacrmispora* correlated with a decrease in fecal levels of butyrate which might be important for gut health (**Manuscript I**).

Gut-adapted species such as *Bacteroides* are candidates for next-generation probiotics (NGP). We suggested that HMOs might be a means to selectively increase the abundance of specific *Bacteroides* species in the gut. We isolated Bacteroidales species from human donors to obtain a large and diverse strains collection and expand the potential for strain engineering. Out of this strain collection, we genome-sequenced four strains and reported the transfer of a 94.2kb mobile genetic element between four species of *Bacteroides* co-residing in the gut of a single human donor. The mobile element contained a Type VI secretion system (T6SS) found to confer antagonism between *Bacteroides* in the gut. We identified the T6SS as a possible target for *in situ* engineering of *Bacteroides.* Lastly, as a proof-of-concept study, we conferred tetracycline resistance in the T6SS of a *Bacteroides* strain and showed the *in vitro* transfer of the mobile element to two different species of *Bacteroides* recipients (**Manuscript II**). The studies reported in this Ph.D. contribute to a better understanding of the *Bacteroides* species in the gut ecosystem and further aid in the development of *Bacteroides* as a NGP.

Resumé

Vores tarme indeholder et mangfoldigt mikrobielt samfund kendt som vores mikrobiota. Vores mikrobiota består af flere hundrede til tusindvis af forskellige bakteriearter, hvoraf *Bacteroides* er en af de mest udbredte slægter. I de fleste mennesker vil *Bacteroide*s blive en væsentlig del af mikrobiotaen efter spædbarnsalderen, hvilket korrelerer med introduktionen af kostfibre og endvidere et ophør af amning. Arter indenfor *Bacteroides* er kendt for at have en stor kapacitet til at udnytte komplekse kulhydrater, herunder glykaner, såsom slim fra tarmceller og humane mælke-oligosakkarider (HMO). Hvordan metabolismen af HMO påvirker væksten af *Bacteroides* i tarmmiljøet er endnu ikke fuldt forstået. Vi undersøgte hvordan HMOer tilsat drikkevandet af konventionelle mus påvirker væksten af *Bacteroides* i mikrobiotaen. Vi rapporterede en tilvækst af familien *Bacteroidaceae* og specifikt af slægten *Phocaeicola* (tidligere en del af *Bacteroides*) i både absolut og relativ antal ved tilsætning af HMO og viste en samtidig nedgang i antallet af den butyrat-producerende *Lacrimispora*-slægt. Nedgangen i *Lacrimispora* korrelerede med et fald i fækale niveauer af butyrat, hvilket kan være vigtig for tarmsundheden (**Manuskript I**).

Bacteroides er en oplagt kandidat til næste generation af probiotika (NGP). Vi foreslog, at HMO'er kan være et middel til selektivt at øge mængden af specifikke *Bacteroides*-arter i tarmen. Vi isolerede Bacteroidales fra humane donorer for at lave en forskelligartet stammesamling og for at finde potentielle NGP-stammer. Vi genom-sekventerede fire stammer fra denne samling og rapporterede overførslen af et 94,2 kb mobilt genetisk element mellem fire arter af *Bacteroides* fra en enkelt human donor. Det mobile element indeholdt et Type VI-sekretionssystem (T6SS), der før har vist at kunne medføre antagonisme imellem *Bacteroides* i tarmen. Vi pegede på T6SS som et muligt mål for *in situ* gen-overførsel imellem *Bacteroides*. Til sidst tilføjede vi tetracyclinresistens i T6SS af en *Bacteroides*-stamme og viste *in vitro* overførsel af det mobile element til to forskellige arter af *Bacteroides*-recipienter (**Manuskript II**). Studierne rapporteret i denne Ph.d. bidrager til en bedre forståelse af *Bacteroides* i tarmsystemet og medvirker yderligere til udviklingen af *Bacteroides* som NGP.

List of manuscripts

Manuscript 1

Andrea Qvortrup Holst[1], Harshitha Jois[1,2], Martin Frederik Laursen[1], Morten O. A. Sommer[3], Tine Rask Licht[1] and Martin Iain Bahl[1]. *Human milk oligosaccharides induce acute yet reversible compositional changes in the gut microbiota of conventional mice linked to a reduction of butyrate levels.*

Affiliations: [1]National Food Institute, Technical University of Denmark, DK-2800 Kgs. Lyngby, Denmark, [2]Glycom / DSM, DK-2970 Hørsholm, Denmark, [3]Novo Nordisk Foundation Center for Biosustainability, Technical University of Denmark, DK 2800 Kgs. Lyngby, Denmark

In revision, FEMS µlife

Manuscript 2

Andrea Qvortrup Holst[1], Michael J. Coyne[2], Tine Rask Licht[1], Martin Iain Bahl[1], Laurie E. Comstock[2]. *Type VI secretion systems contained on almost identical 94.2 kb Integrative and Conjugative Elements indicate recent horizontal transfer events between four* Bacteroides *spp. co-residing in the gut.*

Affiliations: [1]National Food Institute, Technical University of Denmark, DK-2800 Kgs. Lyngby, Denmark, [2]Duchossois Family Institute, Department of Microbiology, University of Chicago, IL 60637, USA

In preparation.

<u>Manuscripts not included in this thesis</u>

Manuscript 3

Kira Sarup-Lytzen[1], Marcia Brinck[1], Andrea Qvortrup Holst[2], Martin Frederik Laursen[2], Felipe Lino[1], Tine Rask Licht[2], Martin Iain Bahl[2], Morten Otto Alexander Sommer[1]. *An Engineered Carbon Niche Increases Dosing Control of* Escherichia coli *Nissle 1917 in the Murine Gut.*

Affiliations: [1]Novo Nordisk Foundation Center for Biosustainability, Technical University of Denmark, DK 2800 Kgs. Lyngby, Denmark, [2]National Food Institute, Technical University of Denmark, DK-2800 Kgs. Lyngby, Denmark

In preparation

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Chapter I: Introduction

The human gut microbiota

Trillions of microbes collectively known as the gut microbiota colonize the gastro-intestinal tract of humans. The gut microbiota is composed of microorganisms such as archaea, viruses and protozoa but the vast majority of organisms belong to the domain of Bacteria. Adults reach about 10^{12} bacterial cells per gram of content towards the end of the gastro-intestinal tract with higher densities reached along the length of the intestines and along the mucus-lumen axis (Donaldson, Lee, and Mazmanian 2015). The diversity of the adult microbiota will typically be of a few hundred to a thousand different species while the vast majority belong to the two phyla of Firmicutes and Bacteroidetes (Huttenhower et al. 2012). The composition of the adult gut microbiota is individual and generally stable over time but may fluctuate in some cases (Lloyd-Price *et al.*. 2017). A fluctuating microbiota can also be a sign of dysbiosis, where the normal microbiota is characterized by an unbalance which can be due to e.g. a relative increase of harmful microbes, changes in metabolic pathways or immunological dysregulation (Sommer *et al.* 2017). The impact of the microbiota in health and disease has been increasingly appreciated within the past decade (Cho and Blaser 2012; Proctor *et al.* 2019). Microbial dysbiosis can be observed in, for example, inflammatory bowel disease, colon cancer, obesity and Type 1 as well as Type 2 diabetes (Figure 1.1) (Vatanen *et al.* 2018;

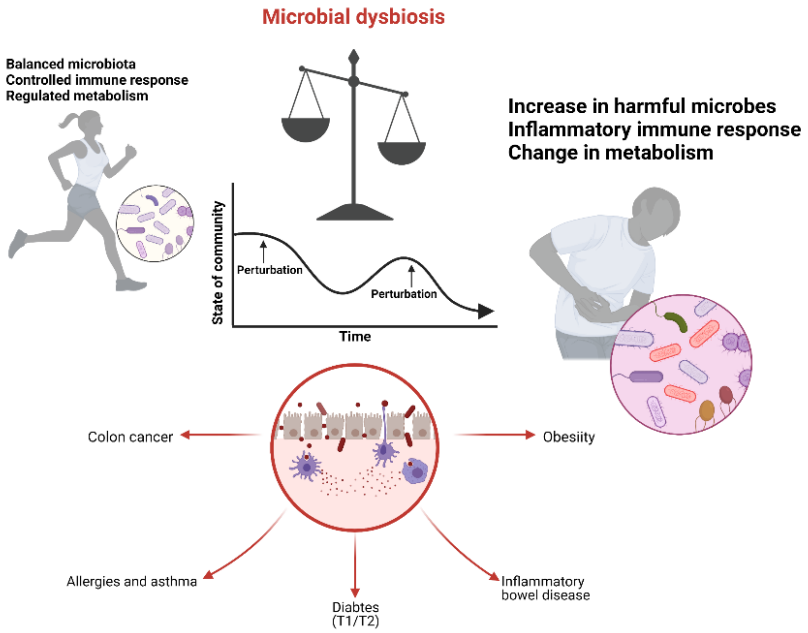


Figure 1.1 Microbial dysbiosis is the trajectory away from the balanced microbiota and is associated with many disease states. The graph indicates the timeline with different perturbation events on the path towards microbial dysbiosis. Created with Biorender.com

Zhou *et al.* 2019; Hsiao *et al.* 2013; Erickson *et al.* 2012; Lloyd-Price *et al.* 2019; Frank *et al*. 2011; Lewis *et al*. 2015; P. M. Smith *et a.*. 2013). Vatanen *et al.* showed that the onset of Type 1 diabetes was preceded by a change in microbial composition while Zhou *et al.* identified earlier markers of Type 2 diabetes associated with pro-inflammatory changes of the microbiota. Lloyd-Price *et al.* characterized the dysregulation associated with inflammatory bowel diseases and found a significant decrease of obligate anaerobic bacteria with a concurrent decrease of short-chain fatty acids (SCFA). However, the causalities between dysbiosis and disease are in many cases blurry and the disentanglement of cause and effect is an important area of research.

The Human Microbiome Project launched in 2007 has aided in creating the needed baseline of the healthy (no overt diseases) human microbiota (Huttenhower *et al.* 2012; Methé *et al.* 2012). The process was further accelerated by the technological advances obtained within the same period and today, research in the interphase of human health and microbiota continues to grow (Knight *et al.* 2018; Surana and Kasper 2017; Lloyd-Price *et al*. 2017). The microbiota is important for gut function, metabolic degradation and immune development and some of these aspects will be discussed in the coming sections. However, the fundamental relationship between host and the microbiota is maybe best simplified by observing the extensive developmental and metabolic defects experienced by germ-free animals lacking a microbiota (Turnbaugh *et al*. 2006; Bouskra *et al.* 2008; Ding *et al.* 2004; Mazmanian *et al.* 2005; Abrams, Bauer, and Sprinz 1963; Olszak *et al.* 2012).

Microbiota in infancy

Infants will during and shortly after birth be colonized by bacteria that compose the first microbiota (Shao *et al*. 2019). The first microbes are acquired from the birth canal and gut microbiota of the mother and possibly from various environmental sources but will soon track onto the mainly conserved succession towards the adult-like microbiota (Stewart *et al*. 2018). Breastmilk is also a source of microbial seeding and is further supplementing the infant with human milk oligosaccharides (HMOs) (Martin Frederik

Laursen *et al*. 2021). HMOs are glycans with prebiotic properties and are expressed by the nursing mothers in the amount of 5-25g/L making them the 3rd largest solid component of the milk (Bode 2012; Chen 2015). However, HMOs constitute no direct nutritional value for the child, which is unable to degrade them, why the majority of HMOs pass indigested to the distal intestines, where they benefit specific bacterial species (Figure 1.2) (Bode 2015; Gnoth *et al.* 2000; Brand-Miller *et al.* 1998). About 200 structurally different human HMOs are known and they can be classified into sialylated, fucosyllated and neutral structures dependent on the sidechains decorating the core component of a lactose group (Bode 2012, 2015). Mothers will express unique combinations of the different HMO but generally, the three HMOs 2'-fucosyllactose (2'FL), 3-fucosyllactose (3FL) and Lacto-N-Tetraose (LNT) will be found in large majorities (Chen 2015). The main degraders of HMOs in early life are the *Bifidobacteria* group that are also shown to dominate the microbiota of most breastfeed infants (Martin Frederik Laursen *et al.* 2021; Tsukuda *et al.* 2021). The *Bifidobacteria* genus has been studied intensively the past few years to elucidate their role in the developing microbiota and concurrent infant health. Recently, it has been confirmed that specific *Bifidobacteria* species pose unique adaptations related to uptake of fucosylated HMOs which proposedly is a host-specific adaptation (Sakanaka, Hansen, *et al.* 2019; Sakanaka, Gotoh, *et al.* 2019). Further, specific tryptophan-derived metabolites produced by infant-like *Bifidobacteria*, including indole-3-lactate, have been shown to obtain immune-modularly and anti-inflammatory functions and thus promote healthy immune development (Martin F. Laursen *et al.* 2021; Ehrlich *et al.* 2020) These results together indicates an intimate mutualistic relationship between *Bifidobacteria* and the host. *Bacteroides* is another genera of bacteria with species identified as HMO

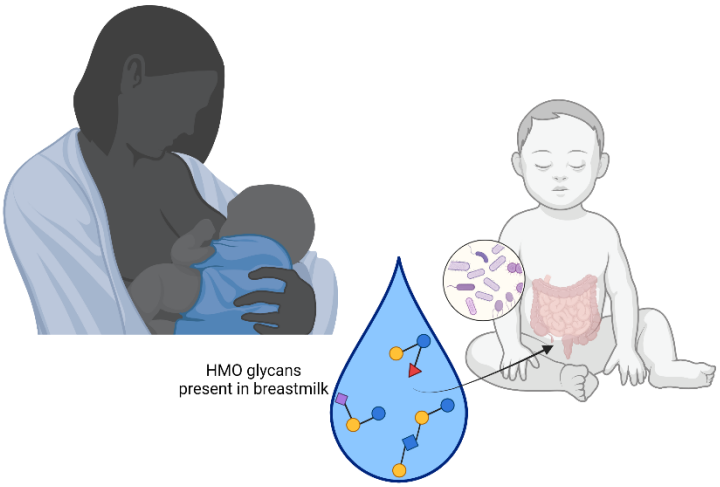


Figure 1.2 The newborn child will soon after birth begin nursing and thereby ingest HMOs which selectively shapes the developing microbiota. Created with Biorender.com

utilizers. *In vitro* studies have confirmed that specific *Bacteroidaceae* species grow well in minimal media supplemented with a HMO as the sole carbon source while their role for HMO degradation in the ecological system of the gut is not well understood for this family (Salli *et al.* 2021; Yu, Chen, and Newburg 2013). *Bacteroidaceae* species are also found in the microbiota of some breastfed children and are actually in few individuals found in higher abundance than the *Bifidobacteria* (Stewart *et al.* 2018; Bäckhed *et al.* 2015). The dynamics between *Bacteroidaceae* and *Bifidobacteria* have been studied in animal models under HMO supplementation (Pruss *et al.* 2020; Marcobal *et al.* 2011). Marcobal *et al.* found that *Bifidobacteria* has the advantage in settings with HMO as the source of carbohydrates as they grow efficiently on HMOs alone while *Bacteroidaceae* uses a mucin-utilization pathway for HMO degradation and thus obtains the advantages in settings with additional complex carbohydrates available. Likewise, Pruss *et al.* recently found that *Bacteroidaceae* can utilize different host mucus-glycan efficiently when derived from other carbohydrates while *Bifidobacteria* rely on HMOs alone (Pruss *et al.* 2020). Thus, *Bacteroidaceae* can be described as a mucus-utilization generalist while *Bifidobacteria* can be described as a HMO-utilization specialist.

Microbial development from infancy to toddlerhood

When the child starts weaning, also known as the phase of complementary feeding, complex dietary fibers will be introduced and thus available for the microbiota (Martin Frederik Laursen *et al*. 2016). This phase is also a transitional phase for the microbiota where large structural changes of the microbial composition will occur. Usually, the *Bifidobacteria* dominated microbiota will in a relatively short temporal window develop to a more adult-like biota dominated by Firmicutes and Bacteroidetes (Stewart *et al.* 2018). Interestingly, recent studies have suggested that it is not the introduction of more dietary fibers but the cessation of breastfeeding that constitutes the turning-point for the microbiota (Tsukuda *et al.* 2021). Tsukuda *et al.* monitored the microbial development of a cohort of children by dense sampling throughout the first two years of life while correlating with data on gut metabolites and dietary habits. The sampling revealed a specific increase in the phyla of Bacteroidetes and Firmicutes only after breastfeeding was

terminated which further correlated with a change in bacterial-produced metabolites. The SCFA propionate and butyrate, generally associated with gut health, was found in significant amounts only after reaching the adult-like microbial composition and correlated with an increase of Clostridiales species (Louis and Flint 2017). While breastfeeding is certainly an important factor for microbial development, other factors are found to influence the microbiota in the transitional period. For example, it is found that the presence of older siblings can accelerate the acquisition of some commensal bacterial strains such as *Faecalibacterium prausnitzii*, likely by the fecal-oral route (Martin Frederik Laursen *et al.* 2017). Also, the choice of diet for the child can influence the microbial composition while the use of antibiotics in childhood most certainly leads to acute changes in the microbiota (Nobel *et al.* 2015; Reyman *et al.* 2022; Galazzo *et al.* 2020; De Filippo *et al.* 2010). How these factors may interact on different levels and which determining power they provide for the microbial composition in adulthood is a complex question. However, the microbial composition in childhood and certain metabolic markers has been identified as precursors for diseases in later life and future studies might elucidate the different trajectories of the developing microbiota (Vatanen *et al.* 2018; Depner *et al.* 2020; De Filippis *et al.* 2021; Stokholm *et al.* 2018).

Effect of diet and dietary supplementations

The microbiota modify the diet we ingest and the dietary components are instrumental for the microbial composition (Sonnenburg and Bäckhed 2016; Turnbaugh *et al.* 2009). Early studies by Turnbaugh *et al* demonstrated that an obese phenotype could be transferred between mice by fecal transplantation. When lean mice were transplanted with the fecal content of the obese mice an acute increase in weight was observed of mice on a high-fat diet. The weight gain was determined to be due to an increased capacity for energy harvest of the obese-type microbiota (Turnbaugh *et al.* 2006). Later it was shown that also a human metabolic phenotype could be transferred directly to mice by fecal transplantation experiments (Ridaura *et al*. 2013). The microbiota is indeed important for energy-harvest from the diet and the human enzymatic capacity for degrading the different dietary components is remarkably limited (Kaoutari *et al.* 2013). The collected genomic capacity of the microbiome is calculated to exceed that of the human approximately by

1000 fold (Li *et al*. 2014). The microbiota is especially rich in Carbohydrate-Active Enzymes (CAZymes) used for degradation of the more complex carbohydrates that pass through the proximal intestinal system undigested and reach the distal parts of intestines (Grabitske and Slavin 2008). CAZymes catalyse the breakdown of glycoconjugates, oligosaccharides and polysaccharides to monosaccharides that the different members of the microbiota can metabolize for energy. The fermentation of these complex carbohydrates will result in the net production of SCFA, mainly butyrate, propionate and acetate (Topping and Clifton 2001). The major producers of SCFA are the obligate anaerobic species from the phylum of Firmicutes with the class of Clostridia containing important members such as the family of *Lachnospiraceae* and the species *Faecalibacterium prausnitzii* (Louis and Flint 2009; Vacca *et al*. 2020; Haas and Blanchard 2019). The SCFAs and butyrate, in particular, are found to promote gut health why manipulation of the microbial composition with diet or prebiotics is proposed as a strategy to optimize gut health or treat diseases such as inflammatory bowel disease (Yadav *et al*. 2022; Joossens *et al*. 2011; Geirnaert *et al*. 2017).

Long-term dietary habits seem to be the determining factor for gut microbiota composition (Wu *et al*. 2011). The human gut can be seen as a large bioreactor and the nutritional input will directly modulate the microbial composition in this simplified understanding (Sonnenburg and Bäckhed 2016). In humans, unraveling the direct effect of diet on microbial composition is a challenging task though, as many unknown variables are introduced both concerning human genetics and the environment (Turnbaugh *et al*. 2009). However, also large clinical trials investigating specific dietary interventions support the observation that the form and amount of fibers ingested most likely has the largest effect on microbial composition (Hansen *et al*. 2018).

Prebiotics has been suggested as a method to increase the abundance of beneficial bacteria in a more specific way. Prebiotics are compounds that are indigestible for humans but can be fermented by specific microbes or groups of microbes to stimulate their growth with health benefits for the host (Roberfroid *et al*. 2010). The most studied prebiotics are probably the compounds inulin, fructooligosaccharides (FOS) and Galactooligosaccharides (GOS) which have been shown to selectively increase the abundance of

Lactobacillus and *Bifidobacteria* (Yadav *et al*. 2022). Further, GOS and FOS have been suggested as a treatment for different gastrointestinal symptoms. For example, FOS has been pursued as a treatment of constipation in children (Souza *et al*. 2018; Scholtens, Goossens, and Staiano 2014) while FOS and a mixture of four different pro- and prebiotic were prospected to relieve Chrohn's disease symptoms (Benjamin *et al*. 2011; Chermesh *et al*. 2007). However, the clinical studies have generally shown none or very limited effects.

Human milk oligosaccharides have recently entered the commercial market as a prebiotic supplementation in infant formula (Puccio *et al*. 2017). Clinical trials of infants supplemented with the HMOs 2'FL and 3FL in the formula demonstrated no adverse effects and found an increase in the relative abundance of *Bifidobacteria* by fecal sampling (Marriage *et al*. 2015; Puccio *et al*. 2017). Further, HMOs have been considered as a prebiotic supplementation for older children and adults to benefit gut health (Elison *et al*. 2016; Palsson *et al*. 2020). Palsson *et al.* performed a placebo-controlled clinical study with 2'FL and LNT in 75 overweight children. No adverse effects were observed after HMO supplementation and a significant increase in *Bifidobacteria* was found in fecal samples. A recent clinical trial investigated the effect of HMOs in adults suffering from inflammatory bowel disease (IBS). In the study, 60 patients were supplemented with different doses of 2'FL and LNT or placebo and supplemented patients ended the treatment period with a relative fecal increase of *Bifidobacteria*. However, no symptom relief was observed in any of the groups (Iribarren *et al*. 2021).

Bacteroidetes of the human gut

Bacteroidetes is one of the dominating phyla of the human microbiota and the gram-negative order of Bacteroidales contains some of the most prevalent gut species such as *Bacteroides fragilis* and *Phocaeicola vulgatus* (Lee *et al*. 2013). Some species from the genus of *Bacteroides* have recently been taxonomically reclassified based on data-driven analysis of available genomes. The new genus *Phocaeicola* was created and now contains the species formerly known as *B. dorei* and *B. vulgatus*, while most other prevalent *Bacteroides* species remain in the order of *Bacteroides* (García-López *et al*. 2019).

The phylum of Bacteroidetes is recognized for being mainly commensal and can obtain stable long-term colonization in the human gut (Lloyd-Price *et al*. 2017; Wu *et al*. 2011). In the study by Wu *et al*., it was suggested that the gut microbiota classifies to two different enterotypes dependent on the ratio of the two Bacteroidales genera *Bacteroides* to *Prevotella* (Arumugam *et al*. 2011). The *Bacteroides* enterotype was associated with a diet high in animal protein and saturated fats while the *Prevotella* enterotype was associated with a diet high in polysaccharides. The influence of diet on Bacteroidetes species can also be observed when comparing western and non-western civilizations. A study by Schnorr *et al.* investigated the microbial difference between a rural African population with a Paleolithic diet, rich in plant fibers and an urban European population with a diet richer in animal products and processed food. Schnorr *et al.* observed marked microbial distinctions between the two populations. The rural microbiota-type obtained a higher alpha diversity, less prevalence of *Clostridiales* species, a diverse Bacteroidetes community while no *Bifidobacteria* was observed. This distinct community was proposed to form an equilibrium of a symbiotic microbiota to the distinct diet. (Schnorr *et al*. 2014).

Bacteroidetes species indeed obtain a large capacity for degrading diverse carbohydrates including complex polysaccharides (Rogers *et al*. 2013). Genome-sequencing of the human symbiont *B. thetaiotaomicron* revealed a remarkable diversity of carbohydrate degradation genes. Nearly one-fifth of the 6.26 Mbp genome of the strain was dedicated to the degradation of polysaccharides (Xu *et al*. 2003; Martens, Chiang, and Gordon 2008). The unusual ability of *Bacteroides* species to recognize and metabolize the many different carbohydrates is related to the unique structure of gene clusters termed polysaccharide utilization loci (PULs) (Salyers *et al.* 1977; Lapébie *et al.* 2019). These PULs are tightly regulated and only transcribed when the corresponding polysaccharide or polysaccharide intermediate are available in the nearby environment (Cameron *et al.* 2014; Lynch and Sonnenburg 2012; Martens *et al.* 2009). Further, niche differentiation of *Bacteroides* species has shown to be a direct consequence of PULs and PULs even seems to determine the biogeographical location of species within the intestinal lumen (Lee *et al*. 2013; Donaldson, Lee, and Mazmanian 2015). PULs transcribe the capability of utilizing both dietary and host-

derived carbohydrate sources. As touched upon in earlier sections, *Bacteroides* species also pose the ability to utilize many different glycans such as HMOs and mucus-glycans (Sonnenburg *et al.* 2005; Martens, Chiang, and Gordon 2008; Ndeh *et al*. 2020; Marcobal *et al*. 2011).

Glycan metabolism in *Bacteroides* species

Since 1977, it has been known that *Bacteroides* species can degrade an unusual array of carbohydrates including host-derived glycans (Salyers *et al*. 1977). Mucus-glycan's create the spatial distance from the epithelial cell layer and are secreted in large amounts from goblet cells in the colon (Johansson *et al.* 2008; Paone and Cani 2020). Mucus-glycan's are also an important reservoir of feeding that can be utilized by some *Bacteroides* species under conditions with less nutritional inflow and thereby sustain a temporal population (Sonnenburg *et al*. 2005; Martens, Chiang, and Gordon 2008; Desai *et al*. 2016). Desai *et al.* showed how specific *Bacteroides* species would utilize host mucus when derived of dietary fibers by using a mice model with a synthetic microbiota. They further showed that the mucus erosion caused by specific *Bacteroides* species would allow for pathogen invasion in the epithelia (Desai *et al*. 2016). In the human counterpart, the ability of *Bacteroides* to utilize host-derived mucus is generally seen as a symbiotic trait (Wexler and Goodman 2017; Pruss *et al*. 2020). Underlining this, patients suffering from inflammatory bowel disease often show reduced relative abundance of *Bacteroides* species in the gut and this might be linked to an increased inflammatory response in the intestines (Round and Mazmanian 2009; De Filippis *et al*. 2021).

HMOs are structurally similar to the mucus-glycans and *Bacteroides* species can utilize different HMOs by mucin-utilization pathways (Bode 2015; Marcobal *et al*. 2011). Research groups have by culture-experiments investigated the ability of several species within the order of Bacterodiales to grow on some of the major HMOs as the sole carbon source (Yu, Chen, and Newburg 2013; Salli *et al*. 2021). A detailed list of the HMO degrading profiles of gut-related strains can be obtained in the articles referred, but generally the species *P. vulgatus, B. fragilis* and *B. thetaiotaomicron* are able to utilize HMOs.

Pruss *et al*. showed that humanized mice supplemented with either HMOs or mucus-derived glycans obtained similar changes to the microbial composition. Further, mucus-derived glycans protected the humanized mouse against a *Clostridium difficile* infection and accelerated post-antibiotic recovery time. Based on these studies the authors proposed that the host selects for a mucin-consuming microbial community to promote stability and symbiosis in the gut ecosystem (Pruss *et al*. 2020). In a recent review article, the connection between glycan-utilization and the gut microbiota was discussed and the ability to utilize glycans was hypothesized to increase the diversity and the niche specialization of the microbiota (Briggs, Grondin, and Brumer 2021)

Competition within Bacteroidales and the mechanism of antagonism

The gut ecosystem contains hundreds of different bacterial strains that compete for resources and space (Donaldson, Lee, and Mazmanian 2015). Competition can be based on indirect competition for nutrients and niches and direct competition by an array of antagonistic systems (Roelofs *et al*. 2016; Gallegos-Monterrosa and Coulthurst 2021; Leth *et al*. 2018; Patnode *et al*. 2019). The nutritional input will influence the competition between the species and only those able to utilize one or a few limiting nutrients more efficiently will survive (Pereira and Berry 2017). This concept should be seen in the broader perspective as "efficient utilization" may differ in time and space in the fluctuating environment of the gut and as bacterial co-existence may take many forms (Faust and Raes 2012; Lloyd-Price *et al*. 2017). Patnode *et al*. have investigated competition for nutrients by using a gnotobiotic mouse model colonized with a consortium of different *Bacteroides* species. The mice were fed a range of different fiber preparations and the growth characteristics of the consortium were identified by proteomics and genetic screening. The study revealed general catabolic flexibility of the *Bacteroides* species to avoid direct competition when alternative glycans were present but also that key species could finely tune their metabolism to obtain a survival advantage under limiting conditions (Patnode *et al*. 2019). Competition for nutrients have also been observed between species from the two main gut phyla Firmicutes and Bacteroidetes in a series of studies by Mahowald *et al*. They inoculated the phyla-representative strains of *Eubacterium rectale* and *Bacteroides thetaiotaomicron*,

respectively, in a 1:1 competition assay using a gnotobiotic mouse model and identified the key co-colonization responses by analyzing proteomics and transcriptomics data. Interestingly, *B. thetaiotaomicron* stimulated host production of glycans and upregulated glycan-utilization genes only when co-inoculated with *E. rectale* which created a nutritional niche that was inaccessible for *E. rectale* (Mahowald *et al*. 2009). Generally, the ecosystem of the gut should be perceived as a complex network of bacterial interactions with cross-feeding and exclusion, competition and co-existence mechanisms. While some of the mechanism of competition and co-existence is slowly being unraveled by the reconstructive approaches described above, they most certainly have a great impact on the ecosystem of the microbiota (Faust and Raes 2012; Rakoff-Nahoum, Foster, and Comstock 2016).

Bacteroidales species possess several potent systems for direct antagonism. Generally, these systems require close proximity to target cells and are thus effective in the dense ecosystem of the gut (A. G. Wexler and Goodman 2017). Bacteriocins are antimicrobial toxins secreted from bacterial cells by diffusion. Bacteriocins typically target closely related species or strains and the toxins act by pore formation, nucleic acid degradation or inhibition of cell wall synthesis (Cotter, Hill, and Ross 2005). In the order of Bacteroidales the antimicrobial peptide toxins, termed Bacteroidetocins are widespread among gut isolates (Coyne *et al*. 2019; Chatzidaki-Livanis, Coyne, and Comstock 2014). In a study by Roelofs *et al* the secretion of specific Bacteroidetocins (termed BSAP) was shown to mediate direct competition between the two *Bacteroides* species. Isolates of *B. fragilis* and *B. uniformis* each produced a distinct type of BSAP (BSAP-1 and BSAP-2) with a target to outer surface molecules on sensitive cells. Immunity to the BSAP was conferred by expressing an orthologue version of the surface molecules. Roelofs *et al* further showed that the BSAP-producing isolates antagonized and outcompeted an isogenic non-producing isolate in a gnotobiotic mouse model (Roelofs *et al*. 2016).

Type VI Secretion Systems of Bacteroidales

A form of antagonism that has only recently been identified in Bacteroidales species is the Type VI Secretions System (T6SS) (Coyne et al. 2014). T6SS is an antagonism system known from diverse gram-negative bacteria and is recognized by a distinct structure of core genes (Cianfanelli, Monlezun, and Coulthurst 2016). The system is characterized by a conserved structural part and a more variable toxin and immunity component. The structural part consists of a retractile needle-like structure that is anchored in the bacterial cell wall by a baseplates. The needle-like structure is used for puncturing neighboring cells with toxins loaded at top of the needle (Figure 1.3). The toxin component is variable and can be from different classes of toxins but will often be a type of RNAase or cell-wall disrupting toxin. The toxic component is followed by an immunity element that protects the cell and the sister-cells from its own toxins. The target of T6SS is generally closely related species or strains of same species carrying a different T6SS toxin-immunity pair (Cianfanelli, Monlezun, and Coulthurst 2016). In Bacteroidales, the T6SS is classified into three different Genetic Architectures (GA) based on how the genes of the core elements are structured (Coyne, Roelofs, and Comstock 2016). The GA3 has so far been found only in *Bacteroides fragilis* and can carry potent toxins with target to a range of different species (Chatzidaki-Livanis et al. 2016).

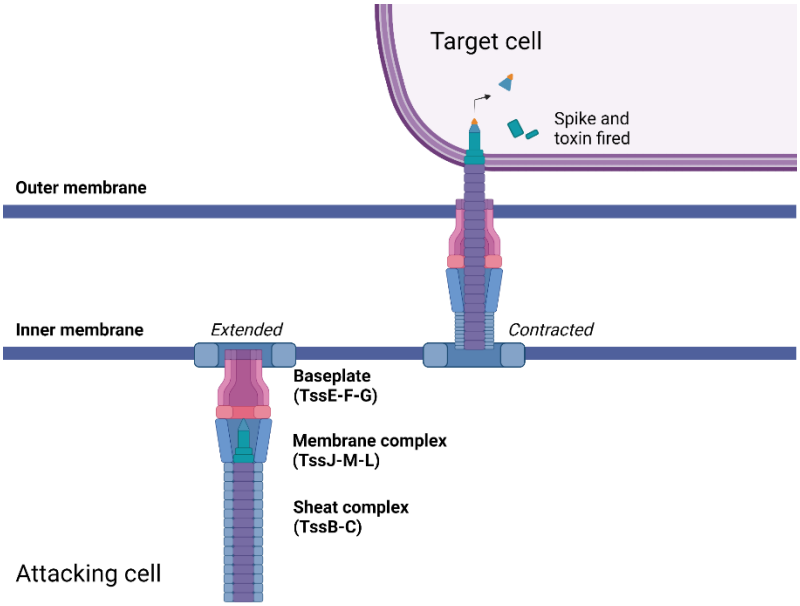


Figure 1.3. Graphic representation of the T6SS of *Bacteroides* species. The core structure of the needle is assembled in the cell cytoplasma with the sheat-complex (TssB/TssC) anchored to the baseplate (TssE/TssF/TssG). The membrane complex (TssJ/TssM/ TssL) will guide the needle through the membrane upon the attack by collapsing the sheat complex. When the target cell is penetrated the toxin and spike are fired. Created with Biorender.com

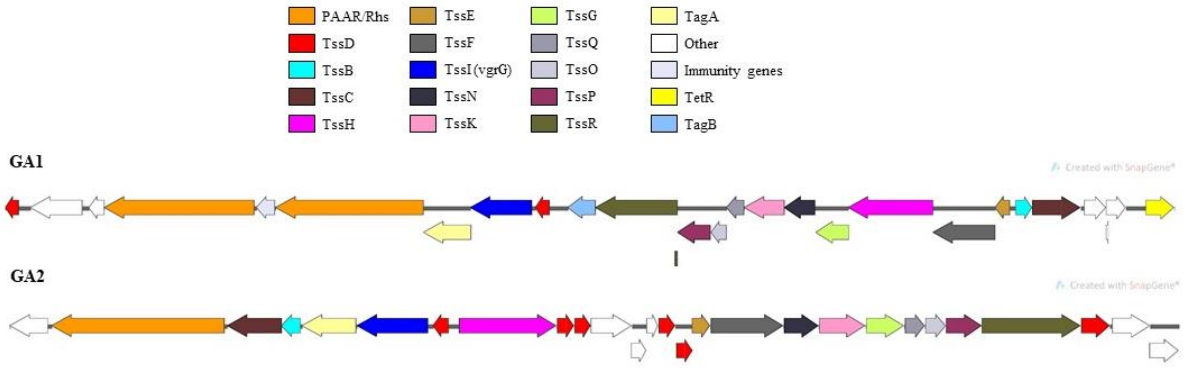


Figure 1.4. Schematic representation of the genetic structure of GA1 and GA2. The GA1 has a distinct structure with two variable regions (In white) while GA2 has three variable regions and several TssD genes (In red) spread across the T6SS. Modified from **Manuscript II**.

The GA1 and GA2 are found on several different Bacteroidales species and are prevalent in the genera of *Bacteroides*, *Phocaeicola* and *Parabacteroides.* The GA1 and GA2 are carried on Integrative and Conjugative Elements (ICE) and can be transferred horizontally between species in the human gut (García-Bayona, Coyne, and Comstock 2021). The structural components of GA1 and GA2 are termed from TssB to TssR and the toxin component is found downstream from the PAAR/Rhs spike genes. Further, GA1 contains two variables regions while GA2 contains three variable regions (Figure 1.4) (Coyne, Roelofs, and Comstock 2016).

Probiotics and next generation probiotics

Probiotic bacteria were early defined as "live microorganisms that when administered in adequate amounts confer a health benefit on the host" (World Health Organization; Food and Agriculture Organization of the United Nations 2001). This definition has recently been refined to better reflect current research in the area to include (i) sufficiently characterized; (ii) safe for the intended use; (iii) supported by one positive human clinical trial; (iv) alive at an efficacious dose (Binda *et al*. 2020). Probiotic bacteria are legally characterized as food or dietary supplements and are readily available on the commercial market as added to food or as a direct supplement (Suez *et al*. 2019). Probiotic bacteria are also applied as modulatory agents for different diseases such as inflammatory bowel disease, antibiotic-associated diarrhea and atopic dermatitis although none of the probiotic products has fulfilled the regulations of the European Food Association (EFSA) to obtain an actual health claim. Traditionally, probiotic research has been based on the milk-associated bacterial groups of *Lactobacillus* and *Bifidobacteria* (Ouwehand and Salminen 1998). These strains are aerotolerant and thus possible to handle without special equipment such as anaerobic chambers. The beneficial effects of probiotics are best established in relation to relief of acute diarrhea in children and to some degree inflammation and antibiotic-associated diarrhea of adults (Canani *et al*. 2007; Goldenberg *et al.* 2015). A global overview of clinical studies concerning the effects of probiotics show a general increase in number of studies over the years (Dronkers,

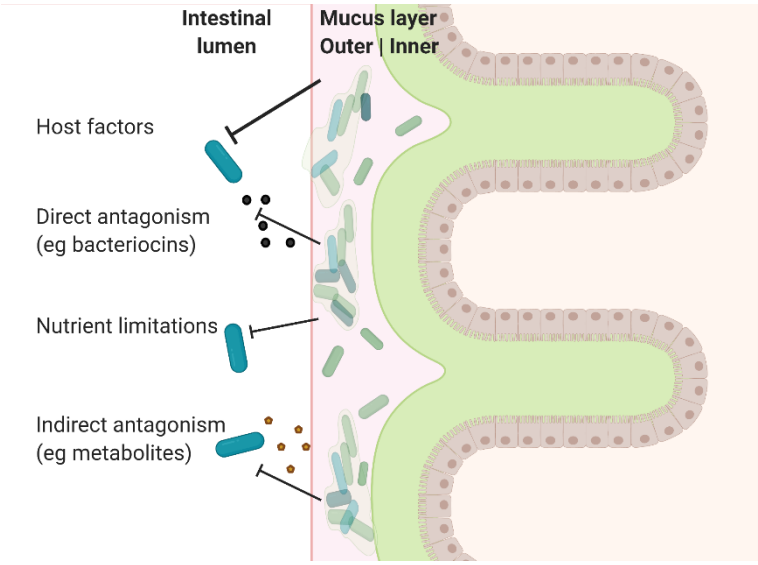


Figure 1.5 The microbiota is resilient to colonization by transient bacteria in the intestinal tract. Bacteria passing through the intestines competes for nutrients and space and might be exposed to direct competition such as secreted antimicrobial toxins. Created with Biorender.com

Ouwehand, and Rijkers 2020). A few studies have addressed the possible risk related to intake of probiotics and found that a compromised microbiota, e.g. caused by a course of antibiotics, might even be harmed or the restoration impaired by the intake of probiotics (Suez *et al.* 2018). Further, it has been debated that the reason for pursuing traditional probiotics such as *Lactobacillus* and *Bifidobacteria* is reflecting the ease of handling these strains in the process of manufacturing and is less related to the relevance of the species for benefitting human health (Torp *et al.* 2022). This subject has been reviewed in detail elsewhere and generally, the association between probiotics and human health is highly debated (Suez *et al*. 2019; Veiga *et al*. 2020).

The gut microbiota obtains a great resilience towards perturbations, including invasion of transient microbes, which is also a symbiotic response for avoiding pathogens near the mucus lining and for protecting the integrity of the epithelia (Figure 1.5) (Lawley and Walker 2013; Zhang *et al*. 2016; Zmora *et al*. 2018). The most studied probiotics, *Lactobacillus* and *Bifidobacteria*, are not naturally present in significant numbers in the adult human microbiota (Lloyd-Price et al. 2017). This has started the search for new candidate probiotics isolated among the gut-adapted species. Next-generation probiotics (NGP) are species better adapted to the intestinal system which might influence their performance when present in the ecosystem of the host. The potential candidates for NGP are also less studied in the laboratory as they generally do not thrive under standard laboratory conditions (OToole, Marchesi, and Hill 2017).

Species from the genus *Bacteroides* have been suggested as candidates for NGP. For example, *B. acidofaciens* has been found to prevent obesity and improve insulin sensitivity in a knockout mouse model (Yang *et al*. 2017). Also, the well-studied *B. thetaiotaomicron* has received increasing attention as a possible NGP candidate through modulatory effects on the microbiota (Chia et al. 2020; Wrzosek et al. 2013).

Other interesting candidates for NGP are the butyrate-producing *Faecalibacterium prausnitzii* and the mucin-degrading *Akkermansia muciniphila* (Sokol et al. 2008; Everard et al. 2013). Both species have on

several occasions been associated with gut health but are extremely aerosensitive why new methods of manufacturing and delivery are under development (Kim et al. 2020; Plovier et al. 2017).

An interesting development is a possible synergy created between NGP and the synthetic biology approach. The use of genetic engineering techniques is prospected to greatly expand the application for probiotics or the so-called "live biotherapeutic products" (Mimee, Citorik, and Lu 2016; Riglar and Silver 2018). Though, molecular methods have traditionally been performed on *Escherichia coli* and are developed for this particular use and are thus not readily available for the vast majority of gut-related isolates. Generally, *E. coli* is not a prominent member of the adult microbiota and does not colonize well in animal models (Lloyd-Price *et al*. 2017; Yatsunenko *et al*. 2012; Freter *et al*. 1983). Thus, *E. coli* is not considered a NGP candidate *per se*, leaving a gap between which isolates might be relevant for human health by bacterial community-related research and which isolates have the needed molecular toolbox readily available.

Engineering of probiotics

Engineered probiotics or the development of "live biotherapeutic products" are prospected to have great potential for the treatment of diseases (Mimee, Citorik, and Lu 2016; Riglar and Silver 2018; OToole, Marchesi, and Hill 2017). In the field of synthetic biology, research within the past decades has evolved from the design of simple functional constructs to the design of complex and responding circuits that can act by decision-making in the gut environment (Figure 1.6) (Gardner, Cantor, and Collins 2000; Elowitz, Leibier, and Leibler 2000;

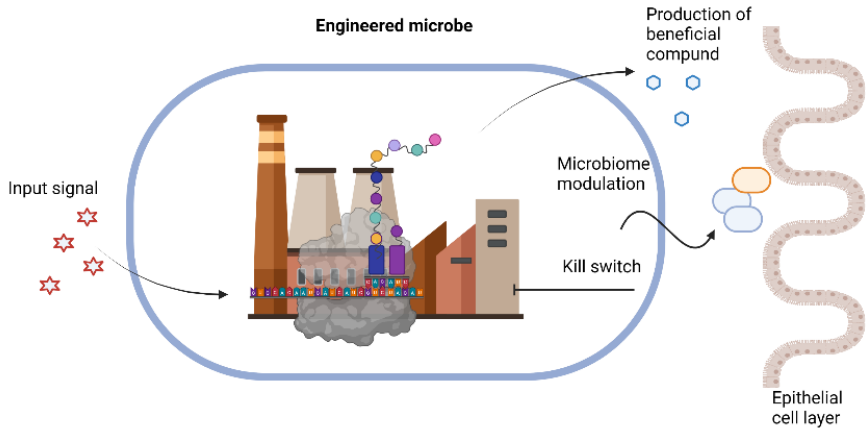


Figure 1.6. Genetic engineering of probiotic bacteria enables targeted functions and sense and response control. An input signal can initiate the production of a beneficial compound that is secreted from the cell in the local environment of the gut. Created with Biorender.com

Riglar et al. 2017; Isabella et al. 2018; Praveschotinunt et al. 2019).

Most studies have focused on *E.coli* Nissle 1917 as the chassis strain for engineering, however, also *Lactococcus lactis* and *Bacteroides thetaiotaomicron* have gained been studied as potential candidates (Mimee et al. 2015; Steidler et al. 2000; Braat et al. 2006). For example, *L. lactis* was the first engineered strain to be applied in a human clinical trial with the treatment of Crohn's disease by delivery of a human interleukin-10 (Braat et al. 2006). The clinical study showed no adverse effects of patients treated with the engineered *L. lactis* but also limited therapeutic effects. Further, an engineered *E.coli* Nissle 1917 was applied in a clinical trial for the treatment of the human metabolic disease phenylketonuria and research with this strain is ongoing (Isabella et al. 2018).

The molecular toolbox for *Bacteroides* species is currently expanding and more tools are becoming commercially available (García-Bayona and Comstock 2019). Studies of *B. thetaiotaomicron* have identified a set of tunable promotors that by encoding unique fluorescent profiles can enable single-strain distinction in the mouse gut (Whitaker, Shepherd, and Sonnenburg 2017). Engraftment studies have also been successfully conducted with *B. thetaiotaomicron* in a humanized mouse model and showed a high level of dosing control by the utilization of a niche carbon source (Shepherd *et al*. 2018).

Engineered probiotic bacteria are starting to enter the commercial market by companies such as Synlogic ® and Novome Biotechnologies ®, which both have on-going clinical trials for several of their product based on engineered *E.coli* Nissle 1917 (clinicaltrials.gov identification NCT04909723 and NCT04534842). The commercial market is expected to enter a new avenue with the advancement of molecular techniques for NGP and a better overall understanding of possible targets in the different human diseases (Veiga et al. 2020; OToole, Marchesi, and Hill 2017).

Chapter II: Objectives of the study and methodological considerations

Objectives and aims

1) Develop strategies for specific and controlled colonization of *Bacteroides* species in the gut
2) Explore the effects on the microbial community when specifically increasing *Bacteroides* species
3) Identify new methods for the engineering of *Bacteroides* species including *in situ* engineering by transfer of mobile elements

We aimed to develop a method for acute and controlled increase of *Bacteroides* species in the context of a mature microbiota and specifically chose to further investigate the *Bacteroides*-enriching capacities of HMOs based on a pilot experiment in conventional mice. We aimed to investigate the overall effects on the microbial community upon supplementation of the mice as other studies have suggested that microbial manipulation might have an unforeseen effect on the community. After the successful experiments with a specific increase of *Bacteroides* species in mice, we next isolated *Bacteroides* species from human donors to obtain a large and diverse strains collection and expand the potential for strain engineering. We aimed to identify targets for strains engineering and especially *in situ* engineering with mobile elements.

Summary of results

Bacteroidetes is a dominating phylum in the human gut microbiota and contains the host-adapted genera of *Bacteroides*. *Bacteroides* species are recognized for a large capacity of utilizing complex carbohydrates including host-derived glycans and HMOs. Even though *Bacteroides* species are well studied *in vitro* less is known about their functions *in vivo*. Human milk oligosaccharides have recently entered the commercial market as a prebiotic substance and we decided to investigate the effects of three structurally different HMOs, namely 6'-sialyllactose, Lacto-*N*-Tertraose and 3-fucosyllactose in a conventional mice model fed a conventional fiber-rich diet. The results presented in **Manuscript I** demonstrate that the family of *Bacteroidaceae* and specifically the genus of *Phocaeicola* increased in both absolute and relative abundance upon HMO supplementation in the context of the mature microbiota of conventional mice. We further show

a concurrent decrease in the genus of the butyrate-producing *Lacrimispora* and that this decrease is coupled with a decrease in fecal amounts of butyrate which was significant for the 3FL supplemented animals. We show that the increase of *Bacteroidaceae* is acutely inducible following just one day of HMO supplementation and further that the increase can be reversed after just one day without HMO supplementation.

Bacteroides species contain potent mechanisms of competition such as Type VI secretion systems (T6SS) and interestingly, some types of T6SS are contained on mobile elements. In **Manuscript II** we obtain a diverse collection of Bacteroidales species from six healthy human donors and screen for the different types of T6SS. We report the transfer of a 94.2kb mobile genetic element (ICE) containing the T6SS between four different species of *Bacteroides* co-residing in the gut of a single human donor. We confer tetracycline-resistance in the mobile element containing the T6SS of a genome-sequenced strain and show transfer of the mobile element to two different species of *Bacteroides* recipients.

The development of next-generation probiotics requires tools to control colonization of human-relevant strains, such as *Bacteroides*, in the gut. Further, a better understanding of the overall effect on microbial composition when manipulating specific species will enable a more targeted approach to strain engineering. We suggest that HMOs might be a means to selectively increase the abundance of specific *Bacteroides* species in the gut. We do, however, also advocate for considerations to the overall effects on the community upon selective *Bacteroides* increase. We report a proof-of-concept study by conferring tetracycline resistance in a mobile element of a *Bacteroides* strain that could enable *in situ* engineering of relevant *Bacteroides* species in the future.

Methodological considerations and pilot studies

Bacterial community analysis

The analysis of bacterial communities can be performed by an array of methods of either quantitative or qualitative nature and be based on sequencing or culturing (Knight *et al.* 2018). Culturing of bacterial isolates is not able to represent the total diversity of the gut microbiota so bacterial community analysis using 16s rRNA gene sequencing is still considered part of the best practice for analyzing microbiota data (Methé *et al.* 2012; Huttenhower *et al.* 2012). The bacterial species definition of 97.5% homology across the full-length 16s rRNA gene has been challenged and suggested increased to 98.5% (Knight et al. 2018). Whole-genome sequencing of bacterial strains is becoming a more assessable tool in microbiota research and strain definitions might be based on genomes in the future. (Lloyd-Price *et al*. 2017; Coyne and Comstock 2019; Lapébie *et al*. 2019). The use of 16s rRNA genes for phylogenetic analysis can sometimes cause confusions of taxonomic relationships and recently, several of these confusions have been corrected with data-driven genome analysis (Haas and Blanchard 2019; García-López *et al.* 2019). Further, techniques such as transcriptomic, proteomics and metagenomics analysis are enabling a better understanding of both diversity and function of the microbiota and coupling of these tools are already providing new insights into the microbiota (Patnode et al. 2019; García-Bayona et al. 2020; Bolyen et al. 2019). The technique used for microbial community analysis during the present studies only allowed for partial16s rRNA gene sequencing so any compositional definitions below genus level were refrained. We decided to use a standardized sequence analysis pipeline to increase the reproducibility of the study and allow ease in comparisons to other studies (Caporaso *et al*. 2010). The QIIME 2 platform, including the Divisive Amplicon Denoising Algorithm (DADA2) to generate amplicon sequence variants, was used for data-analysis in the studies. This platform was recently updated and is generally advised for compositional analysis and provides many build-in function such as calculations of alpha- and beta diversity indexes and generation of statistical measures (Bolyen *et al.* 2019).

Design of animal experiments and pilot studies

The first phase of planning an animal experiment should focus on ethical and legal considerations and this important subject is discussed in later sections of this chapter. In the following paragraph, the methodological consideration is focused on the animal experiments conducted for the 3-fucosyllactose (3FL) supplementation studies (Figure 2.1). The studies were performed in concurrence with the introduction of two different engineered *E.coli* Nissle 1917 strains that were enabled to utilize the prebiotics melezitose and 3-fucosyllactose (Appendix I). The melezitose engraftment pilot study with the engineered *E.coli* Nissle 1917 was conducted to assess colonization of the strain after a streptomycin pre-treatment (0.5% w/v) under melezitose supplementation (5% w/v) (Appendix II S2). The studies concerning engraftment of the engineered *E.coli* Nissle 1917 are the main focus of **Manuscript III** and will not be discussed in detail here.

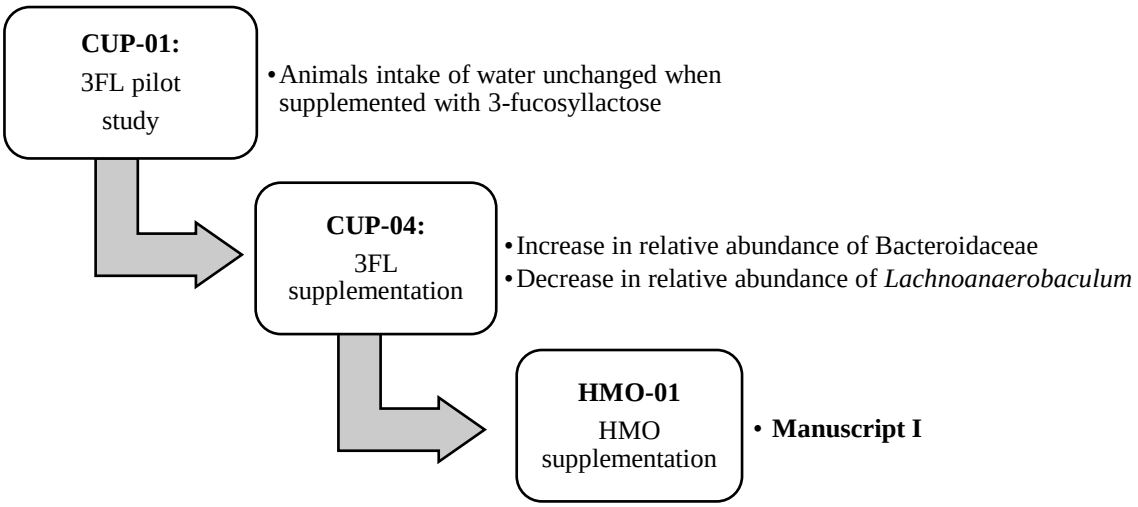
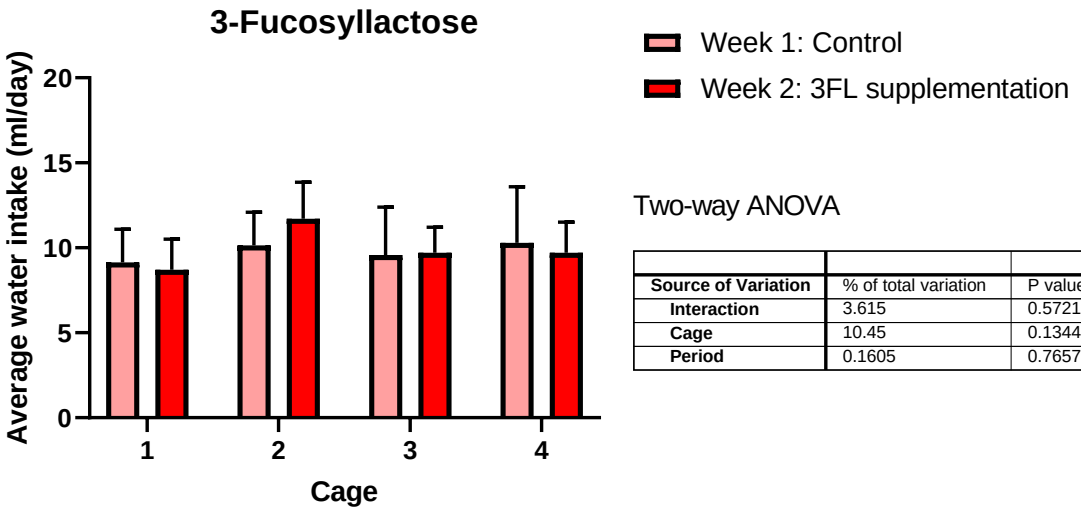


Figure 2.1. Flow diagram of studies. In the pilot study CUP-01 animals (conventional NMRI mice) intake of drinking water was measured to compare 3FL supplemented and control groups. In the study CUP-04 half of the animals received 3FL supplementation (n=24) and an increase in *Bacteroidaceae* in concurrence with a decrease in *Lacnhoanaerobaculum* was observed when comparing to control animals (n=24). In the study HMO-01 three different HMOs were supplemented to groups of NMRI mice (n=8 per group) and a similar increase in *Bacteroidaceae* in concurrence with a decrease in *Lacrimispora* was observed and reported in **Manuscript I.**

Introductions of novel compounds in an animal experiment can inflict unaccounted variables such as animals refraining from eating or drinking (A. J. Smith *et al*. 2018). Thus, we performed the pilot study CUP-01 to assess whether supplementation of the animals with 3FL at 5% (w/v) added in drinking water would affect the intake of water or general wellbeing of the animals (Figure 2.2).

Figure 2.2



Two-way ANOVA

Source of Variation	% of total variation	P value
Interaction	3.615	0.5721
Cage	10.45	0.1344
Period	0.1605	0.7657

Figure 2.2. Pilot study CUP-01. Measures of water intake across control period and 3FL (5% w/v) supplementation period. In four cages with two animals each the daily water intake was measured by weighing of animals drinking bottles. A control period of 7 days with autoclaved tap water was followed by a 7 days 3FL supplementation period. A two-way ANOVA test was performed to assess if any differences in water intake within each cage and between the four cages were found when comparing week 1 and week 2. ANOVA results can be obtained in the table.

We found no differences in drinking water intake when comparing week 1 and week 2 of the 3FL (5% w/v) supplemented groups and observed no effects on animals' well-being (Appendix II S1). We decided to continue with a larger study investigating differences in microbial composition comparing 3FL supplemented and control animals.

The second animal study CUP-04 with 3FL supplementation (5% w/v) was performed in concurrence with an engraftment study using the strain *E.coli* Nissle 1917 FU2.3ng. The engraftment studies of the

engineered *E.coli* Nissle 1917 is the main focus of **Manuscript III**, which is not included but will briefly be explained in the following to provide the context of the animal experiments. In short, *E.coli* was genetically modified to utilize 3FL by the incorporation of a gene-cluster from *Bifidobacterium infantis* and the engineered strain named *E.coli* Nissle 1917 FU2.3ng (*E. coli* FU2.3ng). A control strain of *E.coli* Nissle 1917 was made to contain an empty vector and named *E.coli* Nissle 1917 MUTng (*E. coli* MUTng). We hypothesized that *E. coli* FU2.3ng could utilize the 3FL as a niche carbon source for engraftment in the murine gut (Shepherd *et al*. 2018).

The CUP04 study was designed with four groups of 12 mice (n=48) whereof groups one and two were inoculated with the *E.coli* MUTng empty vector control and groups three and four were inoculated with *E. coli* FU2.3ng. Animals from groups two and four received 3FL (5% w/v) supplementation from day -1 and all animals were inoculated with the respective *E.coli* strain by oral gavage on Day 0. Fecal samples were obtained from Day 0 to Day 9 where animals were euthanized and gut content collected (Figure 2.3 A).

Bacterial DNA was extracted from fecal samples obtained on Day 0 and Day 8 and 16s rRNA gene sequencing performed as described in **Manuscript I**. Differences in microbial composition between the experimental groups were analyzed with the Bray-Curtis dissimilarity matrix (Clarke 1993). An ANCOM test comparing 3FL supplemented and un-supplemented animals was performed on samples from Day 8 (Mandal et al. 2015). Principal coordinate analysis based on Bray-Curtis dissimilarity matrices showed that animals supplemented with 3FL obtained a significantly different microbial composition compared to un-supplemented animals irrespective of the inoculation with *E.coli* MUTng or *E. coli* FU2.3ng. We found that the difference was present on Day 0, one day after 3FL supplementation started, and that the difference was more pronounced on Day 8 (Figure 2.3 B). The compositional changes observed on Day 8 were investigated by an ANCOM test comparing 3FL supplemented and unsupplemented animals to identify which bacterial genera contributed to the observed changes. Using this stringent test we found the family of *Bacteroidaceae* was significantly increased in supplemented animals and the genus of

Lachnoanaerobaculum (sister genus of *Lacrimispora* in the family of *Lachnospiraceae*) was significantly decreased (Figure 2.3 C).

Figure 2.3

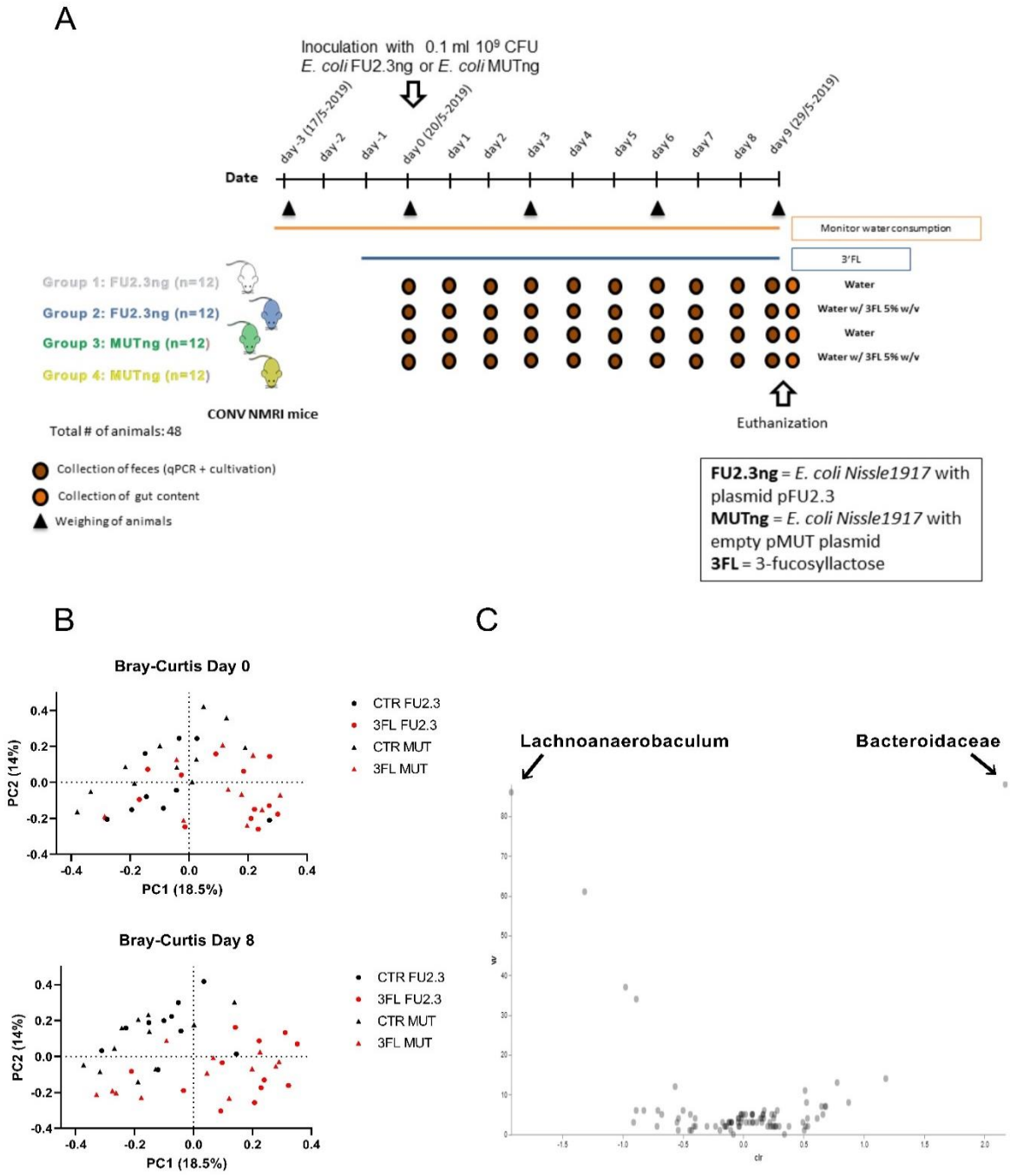


Figure 2.3 Supplementation with 3FL (5% w/v) and engraftment of *E. coli* FU2.3ng and the effects on microbial composition. (A) The experimental design with indications of 3FL supplementation period and E.coli *MUTng* or E. coli *FU2.3ng* inoculum. (B) Principal coordinate analysis based on Bray-Curtis dissimilarity on Day 0 and Day 8. Colored by supplementation/un-supplemented status and shaped according to E.coli *MUTng* or E. coli *FU2.3ng* inoculum. (C) Results of ANCOM analysis indicated in the graph by ctr-value (effect size difference) and W-statistic (number of genera that a single genus is tested to be significantly different against) comparing supplemented un-supplemented animals on Day 8.

Bacterial growth and isolation

Culturing bacteria in agar media and bacterial growth in batch cultures are fundamental techniques in microbiology. Obtaining growth curves of single isolates in batch cultures can provide information on the basic physiology of the strain in the chosen media such as the growth rate, lag phase and carrying capacity (Eley, Greenwood, and O'Grady 1985). Today, bacterial growth curves can be obtained high-throughput by the use of microtiter plates, automated optical density measures and computer programs to transfer data into results (Sprouffske and Wagner 2016). Bacterial cultivation is still an important tool in microbiota research and culturing of isolates can be combined with advanced molecular techniques to obtain new knowledge of e.g. bacterial metabolism or antagonism (Rettedal, Gumpert, and Sommer 2014; Liu et al. 2021; García-Bayona et al. 2020).

During the present studies, bacterial isolates were obtained by culturing methods for several experimental purposes. In animal experiment HMO-01 (**Manuscript I)** an important dataset was the CFU counts obtained for *Bacteroidaceae* species with and without HMO supplementation. For this purpose, the *Bacteroidaceae* selective Brucella-based agar media with Hemin, Vitamin K1 and defibrinated sheep blood added 50 mg/L kanamycin and 10 mg/L vancomycin (BrLa+Kan+Van) was applied. This media is optimized for the nutritional requirements of *Bacteroidaceae* and further contains the antibiotics vancomycin and kanamycin that this family of bacteria is naturally resistant to (Sheppard, Cammarata, and Martin 1990; Bacic and Smith 2008). *Bacteroidaceae* bacteria are obligate anaerobic but aerotolerant and can thus be handled under atmospheric oxygen levels but are restricted to growth only under anaerobic

conditions (H. M. Wexler 2007). The BrLa+Kan+Van media provided an effective incubation of *Bacteroidaceae* isolates and only a few isolates obtained on this media proved to be of other bacterial families (**Manuscript I**). The BrLa+Kan+Van media was further applied to obtain an isolate collection of diverse *Bacteroidaceae* species from fecal and gut samples of mice under the HMO-01 study (**Manuscript I**) and from fecal samples of healthy human donors (**Manuscript II**). The two isolate collections provided the starting point for in-depth analysis of strains in both studies.

The recommended routine culturing media for *Bacteroides* isolates is Brain Heart Infusion media supplemented with Vitamin K1 (10 µg/L) and Hemin (5mg/L) (BHIS) (Bacic and Smith 2008). This media was applied for growth curve analysis of *Phocaeicola vulgatus* isolates obtained during the HMO-01 mice study (Appendix III) and a *Phocaeicola vulgatus* type strain (NCBI ATCC 8482) (Schoch 2020). We performed several growth-analysis of the selected *P. vulgatus* strains to test the hypothesis that isolates collected from communities with the larger relative increase in *Phocaeicola* would show higher growth rates under 3FL supplementation. We found that the isolates incubated in BHIS media supplemented with 2% (w/v) 3-fucosyllactose obtained the expected growth physiology and showed decent similarity between the technical replicates (Figure 2.4 A). The doubling-time and carrying capacity of the isolates was calculated by use of the R-code "Growthcurver" as recommended by Sprouffske and Wagner (Sprouffske and Wagner 2016). We identified a significantly longer doubling time for isolate 25 while isolate 33.1, 33.2, 34, 40 and the reference strain obtained similar doubling times (Figure 2.4 B). The carrying capacity was significantly reduced for isolate 25 and the reference strain while isolates 33.1, 33.2, 34 and 40 obtained similar carrying capacities (Figure 2.4 C). We could not confirm the stated hypothesis on the available data and further investigation is needed. We did not succeed in obtaining representative incubation of the isolates in minimal media supplemented with 3FL as the carbon source even though other research groups have reported performing this assay successfully (Salli et al. 2021; Yu, Chen, and Newburg 2013).

Figure 2.4

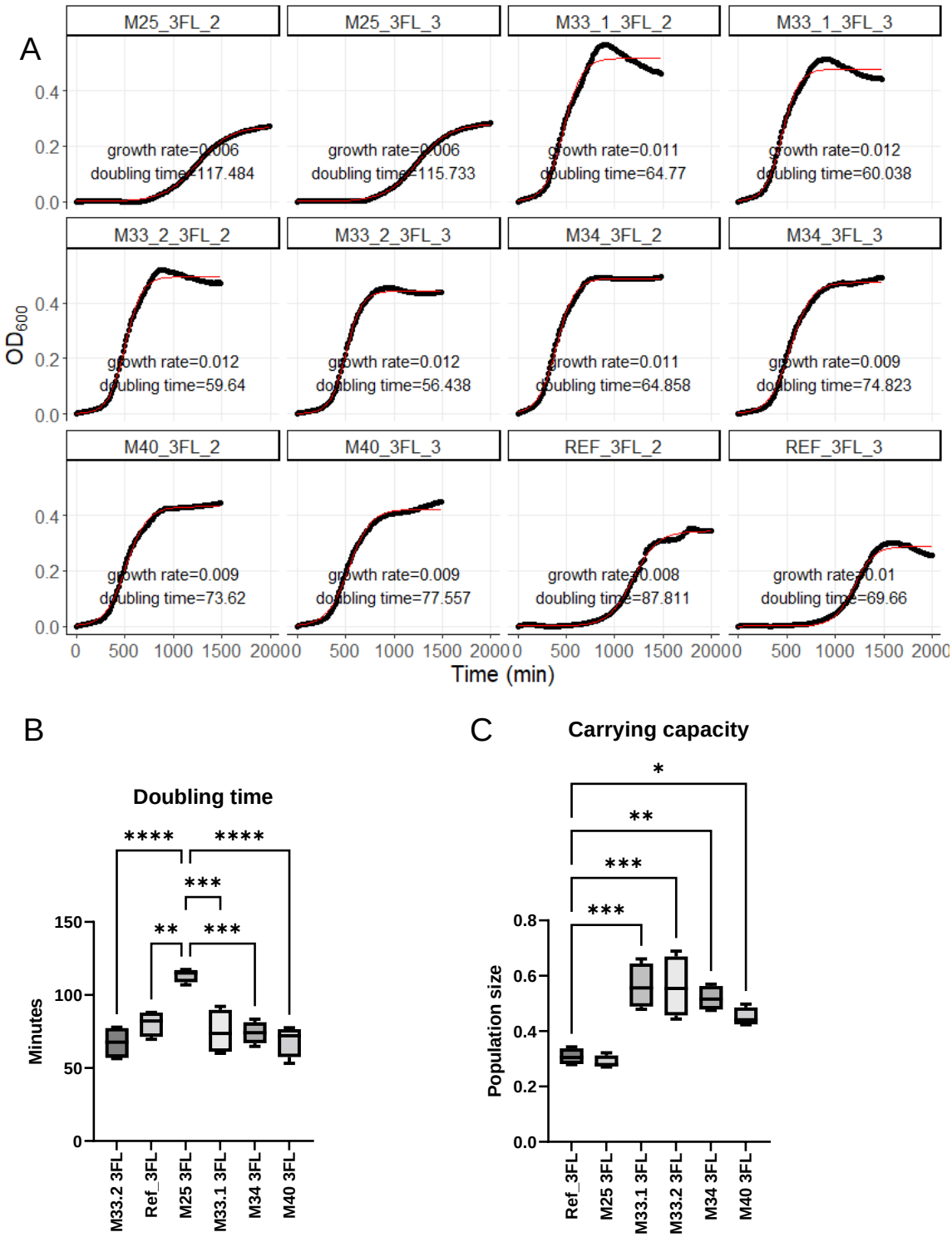

A
M25_3FL_2
M25_3FL_3
M33_1_3FL_2
M33_1_3FL_3
growth rate=0.006
doubling time=117.484
growth rate=0.006
doubling time=115.733
growth rate=0.011
doubling time=64.77
growth rate=0.012
doubling time=60.038
M33_2_3FL_2
M33_2_3FL_3
M34_3FL_2
M34_3FL_3
growth rate=0.012
doubling time=59.64
growth rate=0.012
doubling time=56.438
growth rate=0.011
doubling time=64.858
growth rate=0.009
doubling time=74.823
M40_3FL_2
M40_3FL_3
REF_3FL_2
REF_3FL_3
growth rate=0.009
doubling time=73.62
growth rate=0.009
doubling time=77.557
growth rate=0.008
doubling time=87.811
growth rate=0.01
doubling time=69.66
OD600
Time (min)
B
Doubling time
Minutes
M33.2 3FL
Ref_3FL
M25 3FL
M33.1 3FL
M34 3FL
M40 3FL
C
Carrying capacity
Population size
Ref_3FL
M25 3FL
M33.1 3FL
M33.2 3FL
M34 3FL
M40 3FL

Figure 2.4. Growth curves were obtained from selected *P. vulgatus* isolates inoculated in BHIS media added 2 % (w/v) 3-fucosyllactose. Data were obtained as OD595 measures every 15 min for 48 hours by use of an Infinite F50 plate reader under anaerobic conditions. (A) Growth curves of each isolate were performed as four technical replicates and individually fitted to an exponential curve. Two replications of each isolate are shown. (B) Isolates doubling time was calculated and averaged across the technical replicates and compared between the isolates. (C) Carrying capacity of isolates was calculated and averaged across the technical replicates and compared to the reference strain (NCBI ATCC 8482). Calculations are based on the formula by Sprouffske and Wagner (Sprouffske and Wagner 2016). Identification is according to StrainName_replicate number (Appendix III).

Histology

Sampling intestinal tissue for histological staining in animal studies for quantitative or qualitative analysis is a technically challenging task. The process of obtaining high-quality data is cumbersome and involves several steps that each can be performed by different methodologies (Williams *et al.* 2016; Johansson and Hansson 2012). The mouse intestines are fragile and the mucosa delicate why a strategy for quick fixation after euthanizing the animal is needed. Further, the embedment and sectioning of the samples should be performed by experienced personnel and the staining of the samples after developed protocols (Alturkistani, Tashkandi, and Mohammedsaleh 2016). To obtain quantitative data, scoring schemes can be applied to assess inflammation and morphology of the intestinal section after predetermined parameters (Erben *et al.* 2014). Sections can also be applied for more qualitative measures sometimes in combination with advanced staining and imaging techniques such as fluorescent probes coupled with binding of specific antibodies (Whitaker, Shepherd, and Sonnenburg 2017). The reproducibility of histological data has been debated and while a majority of the method for data acquisitions still requires a partly subjective interpretation, computer-based imaging analysis concepts are currently being developed (Crissman *et al.* 2004; Percie du Sert *et al.* 2020; Nanes 2015; Desai *et al.* 2016). Thus, the researcher should be aware of the possible limitations, and publishing of results based on sections of questionable quality should be refrained from (Blick *et al*. 2019).

We decided to obtain histological sections from the HMO01 animal experiment (Appendix III). We hypothesized that differences in mucosal thickness between experimental groups could be observed based on the shown differences of microbial composition (Desai *et al.* 2016; Paone and Cani 2020). The samples were obtained from mid-colon sections containing a fecal pellet to protect the mucus layer during handling. In short, fixation of the sample was performed with Carnoy's solution for optimal mucus preservation before repeated ethanol/xylene-washing and embedding by an Excelsior AS tissue processor (Johansson and Hansson 2012). A selection (n=10) of the embedded samples were cut at 5µM in 5 replicates followed by hematoxylin and alcian blue staining (Henwood 2017).

The sections were imaged with a Zeiss LSM 700 microscope with 20x opticals and the staining was found to be successful with epithelial cells seen in purple and the mucus layer in blue. We did not succeed in observing an inner mucus layer firmly attached to the epithelia of the colon. The mucosa was clearly visible in the sections but detached from the epithelia and in several occurrences fractured (Figure 2.5). We concluded the quality was not adequate to obtain measures of mucosal thickness after the prepared protocol (Johansson *et al*. 2008).

Figure 2.5

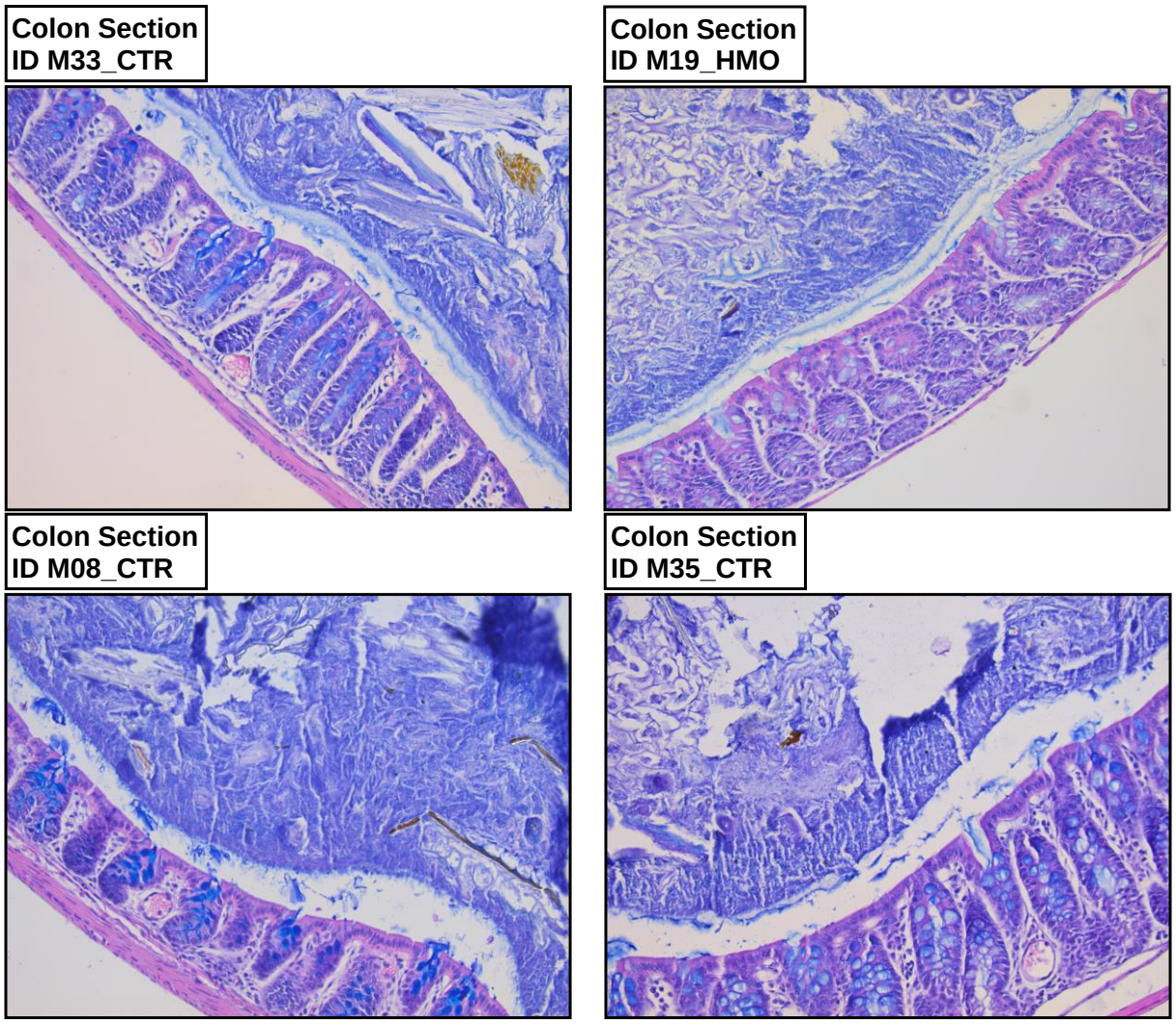


Figure 2.5. Imaging of mouse colon sections. Examples obtained from animal experiment HMO01 (see appendix III for further details). Fecal-filled sections from mid-colon were obtained from each animal (n=40) and stored in Carnoy's solution before ethanol/xylene-washing and embedding. The sections (n=10) were cut at 5µM and stained with alcian blue and hematoxylin and imaged with 20x opticals. The epithelial cells are seen in purple and the mucus layer in light blue. Top images (ID M33_CTR and ID M19_HMO) shows a clearly defined mucosal layer detached from the epithelia. Bottom images (ID M08_CTR and ID M35_CTR) shows a detached and fractures mucosal layer. Identification (ID) is according to mouse number_experimental group (Appendix III).

Ethical and legal considerations

Animals experiments continue to be essential parts of the research area of human health and the conduct of studies that involve the sacrifices of animals are not expected to be discontinued soon (A. J. Smith 2020). Animal studies are still mandatory by regulatory directives when generating data used for the safety assessment of new chemicals or in drug development before entering clinical trials (European Parliament Council of the European Union 2010). Further, in biomedical sciences, animal experiments are often the key part of the methodologies when the study involves interactions with host interphase such as studies of the microbiota (Smith *et al.* 2013; Saeidi *et al.* 2011; Desai *et al.* 2016; Zmora *et al.* 2018).

The sacrifice of an animal life should be well justified. Further, it should be performed under the legal responsibilities deemed by the regulatory directives of the place of the sacrifice. The latter part is decided by legislation and is mandatory to consult before performing an animal experiment (European Parliament Council of the European Union 2010; Bekendtgørelse af lov om dyreforsøg 2014). To justify an animal experiment one can refer to the 3R-concept which is now a national and internationally accepted standard (Danish 3R center (https://en.3rcenter.dk) UK-based 3R (https://nc3rs.org.uk), FELASA (https://felasa.eu)). The 3R's stand for Replace, Reduce and Redefine. "Replacements" is defined as seeking out all possible non-animal options, which might include cell cultures, gut-on a chip and *in vitro* fermenters before referring to animals (Sambuy *et al.* 2005; Ashammakhi *et al.* 2020; Tsamandouras *et al.* 2017; Cinquin *et al.* 2006). Animals should only be considered when no other option is expected to provide the needed validity of the experiments. "Reduction" is defined as using the minimum number of animals for a valid result. This could be achieved by obtaining comparable information from fewer animals or by obtaining more information from the same number of animals (A. J. Smith 2020). To reach the goal of reduction careful planning of the animal experiment must take place. First, a power calculation should be performed to know the minimum number of animals needed in the experimental group to detect the effect by a statistical test. Careful planning of the experiment should allow for minimizing variables and avoiding artifacts. Finally, appropriate statistical methods should be applied and reporting of the study design should

be adequate (Percie du Sert *et al.* 2020). Comprehensive guidance for achieving the well-planned animal experiment can be found in the PREPARE guidelines (A. J. Smith *et al.* 2018).

The animal experiment has suffered from a lack of reproducibility and has been critiqued for not providing the scientific value needed to justify the many lives taken (Frye *et al.* 2015; Perrin 2014). To overcome this challenge a working group created the ARRIVE guidelines to improve the transparency of studies involving animals (Kilkenny *et al.* 2010). This guideline was recently revised and updated with current best practices when publishing work involving animal experiments (Percie du Sert *et al.* 2020). The ARRIVE guidelines encourage better reporting of studies with consideration to the many details that could influence the study outcome. The list includes grouping and randomization of animals, inclusion/exclusion criteria, power calculations and statistical methods and results handling. Better reporting should enable a solid knowledge base that would help the researcher in decision-making and improve the reproducibility of the research.

The last R "Redefine" is related to minimizing the discomfort of the animals, which could be reflected in the procedures, handling and housing of the animals. Minimal discomfort could be obtained by better training of the caretakers handling the animals and by performing the needed procedures with optimized techniques and tools (A. J. Smith 2020). Recently, also the general wellbeing of the animals has been included in "Redefine" as research has shown that animals provide better experimental data if they are allowed to better express their natural behaviors (Poole 1997; Ratuski and Weary 2022). One example is the handling of animals where studies have found that picking a mouse by the tails is a stressful handling technique that should be avoided. Instead, a "cupping" or "tunnel" technique should be used to optimize the wellbeing of the animals (Hurst and West 2010; Gouveia and Hurst 2019). In the end, animal welfare is a question about making the better science.

Chapter III: Discussion

The use of animal models for human-related research

Animal models are an important tool for investigations in the field of human health and is especially useful for the more mechanistic experiments that a human clinical trial cannot accommodate. The microbiota has been shown to affect the different disease parameters and should be accounted for in the different animal models of human diseases. The microbial compositions or even the presence of specific species can change the phenotype of the animal (A. K. Hansen *et al*. 2014). Heterogeneity between animals in the experimental setup should be accounted for and the introduction of confounding factors e.g. by manipulations of animal's microbiota in developmental windows or by pre-study use of antibiotics should be avoided (Laukens *et al.* 2015). Most human gut species are not found in mice but at higher taxonomic levels humans and mice microbiota are strikingly similar (Ley *et al*. 2005). The legislation for approving new products for human use e.g. probiotics is founded on animal trials for dosing control and to detect any adverse effects (Bekendtgørelse af lov om dyreforsøg 2014; European Parliament Council of the European Union 2010). The studies conducted for the HMO supplementation in **Manuscript I** might be a good example of how animal models can provide new insight on microbiota-related effects of a compound. In the animal experiment, we reported the HMO-induced increase in *Bacteroidaceae* species and a concurrent decrease in *Lachnospiraceae* species and fecal levels of butyrate. The question remains if the finding is translational. How representative the animal model used in **Manuscript I** may be is unsettled. As discussed above, the type of animal model and even the breeder or the specific litter can influence the outcome of the experiments. During the HMO supplementation studies great care was taken to apply animal experimental guidelines in order to avoid bias and increase the reproducibility of the studies (Percie du Sert *et al*. 2020; A. J. Smith 2020). We repeated the studies of HMO supplementation in two experimental set-ups and used conventional mice without any possible confounding pretreatment. A next step to substantiate the finding could be to use another conventional animal model, a humanized mouse model or to perform a human clinical trial (Park and Im 2020).

Prebiotics, probiotics and gut homeostasis

The use of HMOs as a prebiotic supplementation in infant formula has in several studies been confirmed to cause a relative increase in *Bifidobacteria* in fecal samples of the infant (Marriage *et al*. 2015; Puccio *et al*. 2017). However, the motives of using HMO as a prebiotic supplementation for older children and adults to benefit gut health is not fully explainable, as *Bifidobacteria* are not normally present in substantial amounts after weaning of the child (Tsukuda *et al*. 2021).

We show that HMOs constitute an effective prebiotic of the *Bacteroides* species in the conventional mouse model with a specific effect on the *Phocaeicola* genus. *Phocaeicola* contains the species of *P. vulgatus* and *P. dorei* (formerly *Bacteroides vulgatus* and *B. dorei*) that are both important members of the human microbiota (Zitomersky, Coyne, and Comstock 2011). A similar prebiotic effect on *Phocaeicola* could be hypothesized in the human counterpart even though the low levels of *Bifidobacteria* that many adults contain could drive the competition in another direction (Pruss *et al*. 2020; Marcobal *et al*. 2011; Elison *et al*. 2016)

We further explored *Bacteroides* as a potential candidate for next-generation probiotic (NGP). So far, research concerning the health benefits of probiotic bacteria has in most cases been unconvincing (Zmora *et al.* 2018; Suez *et al.* 2018). The lack of substantial findings in probiotic research could be related to the general gap in knowledge of causalities in microbiota research (Rosen and Palm 2018; Surana and Kasper 2017). The synthetic biology platform explored for NGP allows for a more targeted approach in the use of probiotic bacteria. By utilizing gene engineering techniques the probiotic bacteria can be applied for the treatment of specific diseases or symptoms (Mimee, Citorik, and Lu 2016; Riglar and Silver 2018). In diseases like Ulcerative colitis or Crohn's, it is evident that microbial dysbiosis is contributing to the disease (Lloyd-Price *et al*. 2019; Frank *et al*. 2011; Geirnaert *et al*. 2017). However, several prebiotic and probiotic formulations have failed to obtain a significant effect in clinical trials (Palsson *et al*. 2020; Schultz 2008;

Chermesh *et al*. 2007). Interventions with an engineered NGP with a specific target when dysbiosis is first observed might enable restoration of the microbial community before the trajectory away from gut homeostasis is inevitable (Figure 3.1).

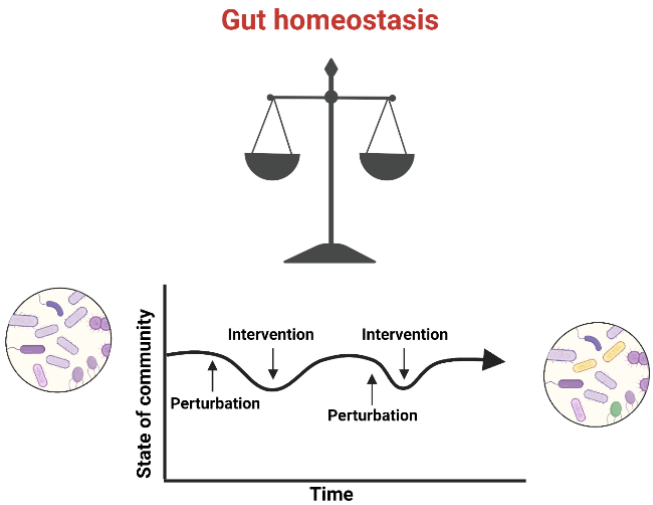


Figure 3.1. Gut homeostasis might be restored by interventions with pre- or probiotics and thereby avoid a disease state. The graph shows a timeline with different perturbation events followed by an intervention event. Created with Biorender.com

How modulations or manipulations of the microbiota could better gut health, without an evident disease state, is more questionable. Natural selection has optimized the microbiota in the human gut through hundred-thousands of years of host-microbiota interactions (Donaldson, Lee, and Mazmanian 2015; Lee et al. 2013). One possible target of NGP modulations could be related to the "loss of old friends" hypothesis linked to the significant increase of chronic diseases seen in the westernized cultures (Dominguez Bello et al. 2018; Blaser 2018). For example, the microbial composition in childhood and certain metabolic markers has been identified as preceding the onset of asthma and Type 1 diabetes in later life (Vatanen *et al.* 2018; Depner *et al.* 2020; De Filippis *et al.* 2021; Stokholm *et al.* 2018). When we know more about the microbial factors to the disease, we can initiate an intervention upon the first indications of disease and modulate the microbiota in the desired direction (Figure 3.1)

The genetic engineering of NGP may hold great potential but many fundamental questions need to be answered. How will the strain interact with the surroundings? Will it colonize or be washed through? Will the strain cause an antagonistic response of the microbiota or be able to occupy a niche? If the niche is occupied, who will be outcompeted and what will that mean for the overall microbiota, function and metabolic output? We would need to understand fundamental aspects of the microbial ecology in-depth to allow a targeted approach involving engineered NGP (**Manuscript I**).

Bacteroides as next generation probiotic

Bacteroides are interesting candidates for NGP as they are among the most abundant bacterial genera in the human gut (Lloyd-Price et al. 2017). Generally, they are also symbiotic and are natural colonizers of different niches in the intestinal system (A. G. Wexler and Goodman 2017). New methods for genetic engineering in *Bacteroides* are being developed and studies have identified tunable promoters to control the expression of specific genes and shown strategies of *Bacteroides* engraftment in the gut (García-Bayona and Comstock 2019; Whitaker, Shepherd, and Sonnenburg 2017; Shepherd et al. 2018). An interesting aspect of *Bacteroides* species is their ability to transfer gene clusters horizontally in the human gut (**Manuscript II**). When considering the potential of *Bacteroides* as a NGP, this ability may become an

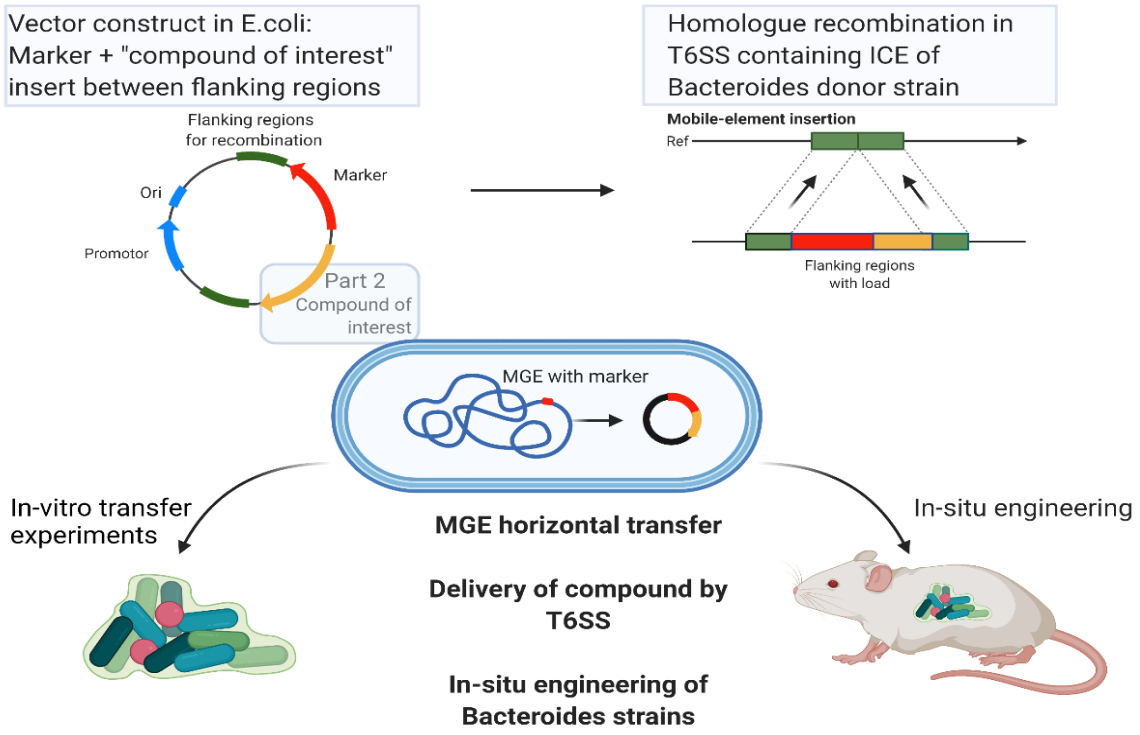


Figure 3.2. Genetic engineering in mobile genetic elements (MGE) of Bacteroides species. A vector can be constructed with a marker element and a "gene of interest" that might encode a therapeutic compound. By a homologue recombination event the construct can be inserted in a mobile element of a Bacteroides species. The marker and "compound of interest gene" can now be transferred horizontally in the environment, e.g. in the mouse gut enabling in situ engineering. Modified from **Manuscript II**. Created with Biorender.com

advantage or a disadvantage. A disadvantage could be that it is more difficult to obtain containment of the engineering strain if genetically modified components are readily being transferred among strains in the gut environment (García-Bayona, Coyne, and Comstock 2021). On the other hand, horizontal transfer of genes could also confer interesting applications so as engineered *Bacteroides* species used for microbial modulation *in situ* (Figure 3.2).

There is much to be uncovered about the different mechanisms of horizontal transfer among *Bacteroides* species and elucidation of some of these could be the first step in future research. The T6SS shared within the gut community is an interesting system and we have taken the first steps to better understand them (**Manuscript II**). For example, we identified a mechanism of exclusion that might be controlled by a novel integrase-defense regulator. Understanding the molecular function of these systems will probably require a great effort but will bring the applications of *Bacteroides* for *in situ* engineering closer. Further, understanding the mechanism of antagonism better may aid in the comprehension of the gut ecosystem as a whole.

Conclusion

We were able to increase the abundance of *Bacteroidaceae* and specifically *Phocaeicola vulgatus* in a conventional mouse model by use of HMOs and specifically 3FL. The increase was both acutely inducible and reversible. We further reported an effect on the microbial composition and showed a concurrent decrease of the butyrate-producing family of *Lachnospiraceae* and decreased levels of fecal butyrate.

We genome-sequences four *Bacteroides* isolates obtained from a healthy human donor. We reported the horizontal transfer of a large mobile element containing a T6SS between the four different *Bacteroides* species. We tagged a genome-sequenced *Bacteroides* strain with tetracycline resistance in a mobile genetic element to enable further studies of the mechanism of transfer. We identified the T6SS as a possible target for *in situ* engineering.

Future directions

A first step to further elucidate the effects of HMO-supplementation in humans could be a clinical study of healthy adults to assess the amounts of SCFA and specifically butyrate in fecal waters before, during and after an HMO-supplementation period and to correlate the findings with general microbiota analysis such as microbial compositions and strain isolation (**Manuscript I**). Further, the identification of general "gut health" parameters related to the microbial composition and function, might be of value. This could be formulated as a general guideline of reporting in clinical trials with pre- and probiotics. Clinical trials are under strict regulations but the effects on microbiota and microbiota-related health parameters can be more difficult to assess and are sometimes lacking (Palsson et al. 2020; Elison et al. 2016; Fonvig et al. 2021). A guideline as suggested might become even more important with the introduction of "live therapeutic products" that are now emerging in clinical trials.

The genome sequenced strains of *Bacteroides* provides a good starting point for future research in the new avenue of NGP research (**Manuscript II**). The experimental setup with an HMO-induced *Bacteroides* increase can be applied for other research questions, for example for induction of a genetically modified strain of *Bacteroides.* One interesting approach for strain engineering could be a design with tagging of a mobile genetic element to enable *in situ* modifications of the microbiota (Figure 3.2). The clinical use of genetically engineered NGP might have great potential but many fundamental questions about these gut-relevant strains are still unanswered (Mimee, Citorik, and Lu 2016; Riglar and Silver 2018). In example, the finding of a putative integrase-defense system regulating the horizontal transfer of genes within the *Bacteroides* species warrens further investigation, and might be of great importance for the sharing of genes in the gut ecosystem (**Manuscript II**).

Precision medicine is emerging as a concept to improve the treatment of human diseases (Veiga et al. 2020; OToole, Marchesi, and Hill 2017). The microbial composition is one important factor that can differentiate individuals and influence disease parameters. In the future, prebiotics, probiotics and even "live biotherapeutics" might be applied as modulatory agents or therapeutic compounds.

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Chapter IV: Manuscript I

Human milk oligosaccharides induce acute yet reversible compositional changes in the gut microbiota of conventional mice linked to a reduction of butyrate levels.

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Keywords: HMO, Bacteroides, Prebiotic, Microbiota, Butyrate, Microbiome

One-sentence summary: The study investigates effects of human milk oligosaccharides on the intestinal bacterial community in a rodent model and highlights changes affecting colonic butyrate concentrations.

Abstract

Human Milk Oligosaccharides (HMOs) are glycans with prebiotic properties known to drive microbial selection in the infant gut, which in turn influences immune development and future health. Bifidobacteria are specialized in HMO degradation and frequently dominate the gut microbiota of breastfed infants. However, some species of *Bacteroidaceae* also degrade HMOs, which may prompt selection also of these species in the gut microbiota. To investigate to what extent specific HMOs affect the abundance of naturally occurring *Bacteroidaceae* species in a complex mammalian gut environment, we conducted a study in 40 female NMRI mice administered three structurally different HMOs, namely 6'sialyllactose (6'SL, n=8), 3-fucosyllactose (3FL, n=16) and Lacto-N-Tetraose (LNT, n=8), through drinking water (5 %). Compared to a control group receiving unsupplemented drinking water (n=8), supplementation with each of the HMOs significantly increased both the absolute and relative abundance of *Bacteroidaceae* species in faecal samples and affected the overall microbial composition analysed by 16s rRNA amplicon sequencing. The compositional differences were mainly attributed to an increase in the relative abundance of the genus *Phocaeicola* (formerly *Bacteroides*) and a concomitant decrease of the genus *Lacrimispora* (formerly *Clostridium* XIVa cluster). During a one-week wash-out period performed specifically for the 3FL group, this effect was reversed. Short-chain fatty acid analysis of faecal water revealed a decrease in acetate,butyrate and isobutyrate levels in animals supplemented with 3FL, which may reflect the observed decrease in the *Lacrimispora* genus. This study highlights HMO-driven *Bacteroidaceae* selection in the gut environment, which may cause a reduction of butyrate-producing clostridia.

Introduction

The newborn gut microbiota is typically characterized by a low bacterial diversity and dominated by facultative anaerobic bacteria, reflecting the oxygenated state of the gut at birth (Bäckhed *et al.* 2015; Ferretti *et al.* 2018). The microbiota then undergoes large, but mostly conserved structural changes on a successional path towards a well-established microbiota achieved at about three years of age (Stewart *et al.* 2018; Laursen, Bahl and Licht 2021). Several different external factors influence these structural changes including early life exposure to antibiotics (Nobel *et al.* 2015), while the most important natural influencer is human breast milk (Stewart *et al.* 2018). Breast milk contains structurally diverse Human Milk Oligosaccharides (HMOs, 5-25 g/L) with about 200 different natural forms categorized into fucosylated, sialylated and non-fucosylated neutral structures (Bode 2012; Chen 2015). The HMOs have no direct nutritional value for the infant, as humans lack the enzymatic capabilities to hydrolyse these compounds (Brand-Miller *et al.* 1998; Gnoth *et al.* 2000). However, low levels of HMOs have been detected in the blood of the breastfed infants suggesting they may confer systemic health benefits (Goehring *et al.* 2014). However, the majority of HMOs reach the colon where they selectively promote growth of certain bacteria in the developing gut microbiota and thus have a prebiotic capacity (Bode 2015). Specifically, HMOs have been found to be highly important in establishing and maintaining a community rich in infant-associated bifidobacteria such as *Bifidobacterium longum* ssp. *infantis* with relatively low overall bacterial diversity (Laursen *et al.* 2021). In accordance with this, a recent study in 12 infants sampled densely during the first two years (n=1048), points to the cessation of breastfeeding as the key to the switch from an infant-like bifidobacteria-dominated community towards an adult-like community dominated by Clostridiales and Bacteroidales (Tsukuda *et al.* 2021). Notably, the study also demonstrated that

the increase in Clostridiales is associated with a concurrent increase in faecal butyrate, which is typically almost absent in very early life. Some bacterial species within the *Bacteroidaceae* family are also able to utilize HMOs as a carbon source (Yu, Chen and Newburg 2013; Salli *et al.* 2021). Specifically, the species *Phocaeicola vulgatus*, formerly *Bacteroides vulgatus* (García-López *et al.* 2019), has in several independent studies been shown to obtain high *in vitro* growth on the major fucosylated and sialylated HMOs namely 3-fucosyllactose (3FL), 2′-fucosyllactose (2′FL), lactodifucosyllactose (LDFL), 3′-sialyllactose (3′SL) and 6’-sialyllactose (6′SL) (Yu, Chen and Newburg 2013; Salli *et al.* 2021).

The potential competition for HMOs between bifidobacteria and Bacteroidales has been investigated both *in vitro* and *in vivo* and is proposed to be based on differences in specificity for uptake of the HMO (Marcobal *et al.* 2011). *Bacteroides* species are generalists and use a mucin-glycan degradation pathway for HMO utilization, while infant-type bifidobacteria are specialists and grow efficiently on HMOs but not on mucin glycans. The specificity of bifidobacteria might give them the selective advantage observed when inoculated in a 1:1 competition with *Bacteroides* species using a HMO supplemented germ-free mouse model (Marcobal *et al.* 2011). Nonetheless, in experiments with animals colonized with a complex adult microbiota, *Bacteroidaceae* respond positively to HMO supplementation (Pruss *et al.* 2020). However, in this study animals were consuming a microbiota-accessible carbohydrate deficient diet, and it is unknown if similar *Bacteroidaceae* enriching effects of HMO supplementation would apply if animals are consuming a complex fibre rich diet.

It is possible that expansion of Bacteroides species observed during the complementary feeding period (the transition from exclusive breastfeeding to family foods) could be driven by the combined presence of HMOs and introduction of more complex carbohydrates and fibres

reflecting their metabolic capacities (Laursen *et al.* 2017; Stewart *et al.* 2018). In addition, in the adult setting, with limited abundance of HMO-degrading *Bifidobacterium* species, *Bacteroidaceae* may enrich during prebiotic HMO supplementation. Indeed, potential benefits of HMOs as a prebiotic supplement besides infant nutrition have been considered (Elison *et al.* 2016; Fonvig *et al.* 2021; Iribarren *et al.* 2021). A recent trial in healthy adults showed that oral supplementation with 2'-O-fucosyllactose (2'FL), lacto-N-neotetraose (LNnT) or a mix of the two up to 20 g/day for 2 weeks (n=10/group) was well tolerated and linked to an increase in the relative abundance of Actinobacteria and specifically *Bifidobacterium* coupled to a reduction in Firmicutes and Proteobacteria (Elison *et al.* 2016). The relevance of bifidobacteria as a marker for a healthy intestinal community in adults is however not well established (Schnorr *et al.* 2014), so other HMO induced microbiota changes with potential health effects should also be considered. In the present study, we sought to investigate modulatory effects of oral HMO supplementation, including 3-fucosyllactose (3FL), Lacto-*N*-Tetraose (LNT) and 6'sialyllactose (6'SL) on bacterial community composition, short-chain fatty acids concentrations and colonic gene expression in a NMRI mouse model lacking infant-type bifidobacteria. The chosen compounds represent abundant fucosylated, basic neutral and sialylated HMOs found in human breast milk.

Results

HMO supplementation increased the absolute abundance of *Bacteroidaceae* family bacteria enumerated by culturing.

The conventional NMRI mice used in this study tolerated supplementation of 5% HMO in their drinking water well and no differences in weight between groups were observed (Fig. S1A). From experimental Day 0 to Day 15 *Bacteroidaceae* colony forming units (CFU) in fecal

samples were enumerated (Figure 1A). No differences were found between the groups on Day 0 before HMO supplementation commenced (Fig. 1C, p=0.28, Kruskal-Wallis test). Supplementation with HMOs resulted in significant changes in *Bacteroidaceae* levels (Fig. 1C, p=0.0006, mixed-effects analysis). After two days of supplementation of HMOs in drinking water (5% 6'SL, LNT or 3FL), higher counts of *Bacteriodaceae* were observed in the LNT and 3FL groups compared to the control group (CTR) while all three treatment groups were significantly higher on Day 5 (Fig. 1C, p<0.05, Dunnett's multiple comparisons test). On Day 8 most of the animals were terminated, yet a wash-out group was maintained in the 3FL group (3FL-WO, n=8) and followed for an additional seven days without HMO supplementation, which resulted in a significant decrease in *Bacteroidaceae* (Fig. 1C, p<0.0001, one-way ANOVA) compared to Day 8. Already one day after removing 3FL from the drinking water (Day 9) a significant decrease in *Bacteroidaceae* was observed as compared to Day 8 (p=0.0004, Dunnett's multiple comparisons test). Counts of *Bacteroidaceae* remained decreased in 3FL-WO mice compared to Day 8 during the remainder of the wash-out period (Fig. 1C).

Bacteroidaceae CFUs were enumerated in the cecum and colon content after euthanization on Day 8 (n=8 per group) and Day 15 from the 3FL-WO (n=8). We found ignificant differences between both compartments (p=0.0014), with overall higher counts in colon and between treatment groups (p<0.0001) by mixed-effects analysis. In both cecum and colon, the 6'SL group had significantly higher levels of *Bacteroidaceae* compared to the CTR group, while all three HMO treatment groups resulted in higher *Bacteroidaceae* counts in the colon. (Fig. 1D, p<0.05, Dunnett's multiple comparison test). After the washout period, levels of *Bacteroidaceae* were not significantly different from the CTR group in any of the compartments (Fig. 1D).

Cultured *Bacteroidaceae* belonged to several genera and matched the Amplicon Sequence Variants identified by 16S rRNA gene sequencing.

A total of 21 bacterial isolates from Day 8 faecal and colon samples from several different animals were picked randomly from different animals, excluding the CTR group. Identification by partial 16s rRNA gene Sanger sequencing confirmed that colonies mostly belonged to the *Bacteroidaceae* family with strains representing several different *Bacteroides* and *Phocaeicola* species (Table S1). Four of the 21 isolates did not belong to the *Bacteroidaceae* family. Alignment of the V3 regions of the 16S rRNA gene sequences obtained from isolated colonies with the most prevalent *Bacteroidaceae* ASVs identified by 16S rRNA gene profiling (Table S2) showed an overall good match between the two methods (Fig. 2). The generated phylogenetic tree including also type strains revealed distinct clades for the genus *Bacteroides* and the genus *Phocaeicola*, underlining the taxonomic differences between these recently separated genera. The only exception was the species *Bacteroides fragilis*, which seemed more closely related to *Phocaeicola faecalis* based on this dataset. The genus *Phocaeicola* itself also appeared to be further divided into two distinct clades represented by *Phocaeicola vulgatus* and *Phocaeicola fragilis* respectively, with phylogenetic distances as large as those found between *Bacteroides* and *Phocaeicola* genera, indicating substantial phylogenetic variation between species within the *Phocaeicola* genus (Fig. 2).

HMO supplementation affected microbial composition dependent on the specific compound

Microbial profiling by 16S rRNA gene sequencing before and after HMO supplementation showed a marked reduction of both richness (observed number of ASVs) and Shannon diversity in animals that had received 6'SL (Fig. 3A,B, $p<0.05$, paired *t*-test), while no differences in

alpha diversity were observed in the LNT and 3FL groups. The relative abundance of bacterial classes in faecal samples for individual animals at Day 0, Day 8 and Day 15 showed no clear indications of specific changes for HMO supplemented animals at this level, which could be explained by the Bacteroidia class predominantly consisting of *Muribaculaceae* (approximately 70% on average) while the *Bacteroidaceae* only constituted approximately 6% of this class (Fig. 3C). Principal coordinate analysis based on Bray-Curtis dissimilarity matrices clearly indicated differences in faecal microbial composition between treatment groups and CTR after HMO supplementation (Day 8), which CTR samples clustering separately from the other samples (high PC3-value). No differences were observed before supplementation (Day 0), indicating a specific effect of the HMOs on the microbiota (Fig. 3D,E). Analysis of similarities (ANOSIM) revealed that the faecal microbiota in animals receiving 6'SL and 3FL were significantly different from that in the CTR group on Day 8, while the LNT group did not differ from the CTR group (Fig 3D,E). Interestingly, the 3FL-WO samples obtained on Day 15 after the washout period showed a reversion from the 3FL sample cluster towards the CTR sample cluster, with no significant difference found by ANOSIM analyses between CTR Day 8 and 3FL-WO groups (Fig. 3E, p=0.184, ANOSIM test). We did not observe notable cage-effects in the study (Fig 3C and Fig S1F).

The genus *Phocaeicola* increased in relative abundance during HMO supplementation while the genus *Lacrimispora* decreased.

To investigate which bacterial genera contributed to the observed changes in microbial composition during HMO supplementation, a statistical analysis of the compositional changes between Day 0 and Day 8 (ANCOM test) was performed at the genus level for all four treatment groups separately. Using this stringent test we found one genus, *Phocaeicola* (formerly

Bacteroides) significantly increase (3FL group) and one genus, *Lacrimispora* significantly decrease (6SLgroup) from day 0 to day 8, although same trends were observed for both 3FL and 6'SL treatment groups (Fig. S2). Focusing specifically on the genera found to be differently abundant by the ANCOM analysis, paired *t*-tests were applied to compare the relative abundances of *Phocaeicola* and *Lacrimispora*, respectively, within each group between Day 0 and Day 8. This showed that all three HMOs caused an expansion of *Phocaeicola* and a concomitant decrease in *Lacrimispora*, particularly pronounced in the 3FL group (Fig. 4A, B). In accordance with culturing data, the wash-out period resulted in a relative abundance of *Phocaeicola* on Day 15 that was significantly reduced compared to Day 8 (Fig. 4A). Additionally, *Lacrimispora* was significantly increased on Day 15 compared to Day 8 (Fig. 4B). No HMO-induced changes in levels of *Bacteroides* (not including *Phocaeicola*) nor *Bifidobacterium* between Day 0 and Day 8 were found in any of the treatment groups (Fig. 4C,D). Strong positive and negative correlations were found between the calculated PC3 coordinates (Fig. 3E) and the relative abundance of *Lacrimispora* (Rho=0.60, $p<0.0001$, Spearman's rank correlation), and *Phocaeicola* (Rho=-0.69, $p<0.0001$, Spearman's rank correlation) respectively indicating that these genera were driving the observed differences in the PCoA plot. Another interesting finding from the ANCOM analysis was that the abundance of the genus *Faecalibacterium* was significantly lower on Day 15 in the 3FL-WO group compared to both Day 0 and Day 8, with the genera being below detection level in all samples on Day 15 (Fig. S1E and Fig S2E,F).

Supplementation with 3FL reduced faecal concentrations of acetate, butyrate and isobutyrate.

The concentrations of short-chain fatty acids measured in faecal samples of all animals on Day 8 revealed significant differences in concentrations of acetate, butyrate and isobutyrate for HMO treated animals compared to the CTR group (Fig. 5, $p<0.05$, Kruskal-Wallis tests). Specifically lower concentrations of acetate ($p=0.0027$, Dunn's multiple comparisons test), butyrate ($p=0.025$, Dunn's multiple comparisons test) and isobutyrate ($p=0.048$, Dunn's multiple comparisons test) were found in the 3FL group compared to CTR. No other HMO-induced differences in SCFA levels were found.

Colonic occludin expression lowered during and after HMO supplementation

We found no effects of HMO supplementation on colonic gene expression levels of Tjp1, TNFα and Muc2 compared to the CTR group on Day 8 (Fig. 6A-D). However, a significantly lower gene expression level of occludin was observed in the 3FL-WO group on Day 15 compared to the CTR group on Day 8 ($p=0.0005$, Dunn's multiple comparisons test). A comparison between the CTR group and all three treatment groups aggregated on Day 8 also revealed slightly, but significantly lower levels of occludin gene expression compared to the CTR group (Fig 6A, $p=0.012$, Mann-Whitney test).

HMO-induced expansion of *Phocaeicola* and reductions in *Lacrimispora* is associated with reduced faecal acetate and butyrate levels.

A highly significant negative correlation was found between the relative abundance of *Phocaeicola* and *Lacrimispora* (Fig. 7A, Rho=-0.614, $p<0.001$, Spearman's rank correlation). Focusing on the the negatively correlated *Phocaeicola* to *Lacrimispora* we found that the ratio

between these two genera correlated negatively with the number of observed species (Fig. 7B, Rho=-0.247, p=0.023, Spearman's rank correlation), indicating general effects on the community composition. We further found negative correlations between the *Phocaeicola* to *Lacrimispora* ratio and levels of both faecal acetate (Fig. 7C, rho=-0.359, p=0.025, Spearman's rank correlation) and butyrate (Fig. 7D, rho=-0.489, p=0.002, Spearman's rank correlation) but not isobutyrate (rho=-0.11, p=0.51). Clustering of samples according to specific HMO treatment group (indicated by different colours) were notable in the dot-plots (Fig. 7A-D).

Discussion:

The investigated HMOs were well tolerated in mice at the given dosage, which is consistent with previous studies in humans (Elison *et al.* 2016) at comparable dosages (Nair and Jacob 2016). To investigate the effects of HMO supplementation specifically on the culturable members of the *Bacteroidaceae* family we used Brucella lacked blood agar supplemented with kanamycin and vancomycin as previously described (Sheppard, Cammarata and Martin 1990). This growth media proved to be a reliable method for tracking acute changes in the *Bacteroidaceae* abundance during HMO supplementation (Fig. 1C) and strain isolation (Fig. 2 and Table S1). Interestingly, the positive correlation between *Bacteroidaceae* CFU/g and *Phocaeicola* relative abundance on Day 8 (Fig. S1D) was not present on Day 0 (Fig. S1C), indicating that the strains enumerated on Day 0 were not predominantly *Phocaeicola* strains but probably a broader collection of *Bacteroidaceae* species, while the positive correlation observed on Day 8 indicated that mainly *Phocaeicola* strains were cultured at this time point and thus selected for by HMOs. Enumeration of CFUs from cecum and colon samples likewise showed a general increase in *Bacteroidaceae* in HMO-supplemented animals compared to control animals which were more pronounced in colon samples than in the cecum samples (Fig 1D), which could reflect a higher

selective effect in the colon, possibly enhanced by mucus glycan metabolism (Donaldson, Lee and Mazmanian 2016; Patnode *et al.* 2019). A positive selection for *Bacteroidaceae* species during administration of selective carbohydrates has been reported in several previous studies, although *Bacteroidaceae* species may be outcompeted by specialist HMO degrading *Bifidobacterium* species when co-inoculated in a germ-free mouse model during HMO supplementation (Marcobal *et al.* 2011). Also, the mucin-derived O-glycans versus HMO availability may affect growth competition between *Bifidobacterium* and *Bacteroidaceae* (Pruss *et al.* 2020). As very low abundances of bifidobacteria were detected in the mice applied in the present study, and these most probably were not adapted to HMO degradation, the *Bacteroidaceae* strains were likely not challenged by direct competition with bifidobacterial species (Fig.4D). This study underlined the general ability of *Bacteroidaceae* species to readily respond to changes in carbohydrate availability and exploit a new nutritional niche especially in the absence of specialist HMO degrading bifidobacteria.

In the present study, HMOs were found to affect the faecal microbial composition after eight days supplementation period (Fig. 3) with effects observed already on Day 2 (Fig. 1C). The negatively correlated genera *Phocaeicola* and *Lacrimispora* were found to be the main drivers of the observed difference of the HMO-supplemented animals in all three treatment groups. The observed expansion of *Phocaeicola* is consistent with previous *in vitro* studies (Yu, Chen and Newburg 2013; Salli *et al.* 2021). The phylum Bacteroidetes has recently undergone taxonomic reclassification based on a large genome-scale survey (García-López *et al.* 2019). Here it was proposed to reclassify some former *Bacteroides* species into the genus *Phocaeicola* including *B. dorei* and *B. vulgatus* while most other prevalent species remain in the *Bacteroides* genus

including *B. fragilis* and *B. thetaiotaomicron*. Interestingly, this study showed that HMO supplementation did not affect the now constricted *Bacteroides* genera (Fig 4C).

Analysis of SCFA levels revealed a significant decrease of butyrate and acetate in the 3FL supplemented group (Fig. 5A,D) compared to the CTR group, which could be linked to the observed reduction in *Lacrimispora* spp. The family of *Lachnospiraceae* (formerly designated as part of *Clostridium* cluster XIVa) are among the main producers of butyrate in the gut (Van Den Abbeele *et al.* 2013; Vacca *et al.* 2020). The *Clostridium sphenoides* group has recently been reclassified as the genus *Lacrimispora* under the *Lachnospiraceae* family and most species from this genus are confirmed to have the genetic capacity to synthesize butyrate through the acetyl-coenzyme A (CoA) pathway (Vital, Howe and Tiedje 2014; Haas and Blanchard 2019). In line with this, the relative abundance of *Lacrimispora* was significantly and positively correlated with faecal levels of butyrate (Fig. S3D). *Lacrimispora* also correlated positively with faecal acetate levels collectively indicating a role of *Lacrimispora* in faecal SCFA levels (Fig. S3B), while no correlation with isobutyrate was found. Whether the observed HMO induced decrease in *Lacrimispora* was caused by a change in the gut environment (e.g. pH) or by competition with *Phocaeicola* remains unresolved. *In vitro* studies have shown that lowering pH levels by one unit in anaerobic continuous cultures curtails the dominating bacterial population of *Bacteroidaceae* species relative to that of *Clostridia* species such as the family of *Lachnospiraceae* and that the shift is correlated to a metabolic response resulting in a large increase in butyrate production (Walker *et al.* 2005). The butyrate producing *Faecalibacterium* was present in most faecal samples on Day 0 and Day 8 but was, surprisingly, absent (below level of detection), in samples on Day 15 (Fig. S1E, Fig. S2E,F). We speculate that *Faecalibacterium* may also be negatively affected by 3FL-induced changes in the community composition possibly due to the significantly

decreased levels of acetate (substrate for butyrate production) observed on Day 8 (Duncan *et al.* 2002; Wrzosek *et al.* 2013), but further studies are needed.

The observed reduction of occludin gene expression (Fig. 6A) linked to barrier function through tight junction stability (Cummins 2012; Panwar, Sharma and Tripathi 2021; Pérez-Reytor *et al.* 2021) was most pronounced in the 3FL wash-out group (Day 15) compared to CTR (Day 8) but also significant when all HMO groups were combined (Day 8). The cause of this reduction is difficult to determine based on available data, but could be speculated to be linked to the observed reduction in faecal butyrate levels (Fig. 5D), although no significant correlation was found.

The HMOs 3-fucosyllactose (3FL), Lacto-*N*-Tetraose (LNT) and 6'sialyllactose (6'SL) are considered safe for human consumption as a novel food supplementation (Turck *et al.* 2019, 2020, 2021) and several clinical trials have reported no adverse effects upon HMO-supplementation of adults and children 6-12 years of age (Elison *et al.* 2016; Palsson *et al.* 2020; Fonvig *et al.* 2021; Iribarren *et al.* 2021). None of the clinical studies conducted in either adults or children have analysed changes in microbial short-chain fatty acids as a marker of overall microbial activity and gut health (Pérez-Reytor *et al.* 2021). However, our findings suggest that this may be relevant. A limitation of the study was that combinations of HMOs were not addressed in the experimental design.

In conclusion, this study demonstrates an acute yet reversible HMO-induced increase in the human-relevant *Phocaeicola* (formerly *Bacteroides*) concurrent with a reduction in butyrate-producing *Lacrimispora* in the context of a complex, mature microbiota of conventional mice. This was linked to a decrease in faecal butyrate levels especially following supplementation with the fucosylated HMO 3FL. The reported results additionally emphasize the importance of

including other effects than bifidogenicity when evaluating the effects of HMOs in a complex adult-like microbiota.

Materials and methods

HMO drinking water solutions

3-fucosyllactose (3FL), Lacto-*N*-Tetraose (LNT) and 6'sialyllactose (6'SL) in powder form was obtained from Glycom A/S with a purity above 92% (3FL = 92.4%; LNT =95.5%; 6'SL =98.8%). Human milk oligosaccharides were separately dissolved in autoclaved water, sterile filtered (0.45 µm) and adjusted to a concentration of 50 g/L (5% w/v) in a total volume of 600 mL. From these stock solutions water was transferred to individual drinking water bottles. Animals in the CTR group received un-supplemented autoclaved water. Drinking bottles were re-filled when necessary. In a previous pilot trial the drinking water intake of NMRI mice (same age) was found to be approximately 5 mL per day with no difference observed between pure (autoclaved) and 3FL (5%) supplemented drinking water. The daily intake of HMOs was thus calculated to be approximately 0.25 g/animal/day.

Design of the animal study

40 conventional NMRI, 6 weeks old, female mice were obtained from Taconic and kept in a Scantainer under a 12 h light:dark cycle at a temperature of 22± 1° and relative humidity of 55± 5%. The mice were fed ad libitum Altromin 1314 chow (Brogaarden ApS, Lynge, Denmark) and autoclaved water in drinking bottles. Four days after arrival, on experimental Day -5 the mice were pseudo-randomized according to weight in four experimental groups; three groups of eight mice and one group of 16 mice and housed in cages of two (Fig. 1A). On experimental Day 0,

before starting the treatment, the mice were weighed and faecal samples collected. The drinking bottles from each cage were exchanged with bottles containing clean autoclaved water, which for the treatment groups were supplemented with 50 g/L Lacto-*N*-Tetraose (LNT), 50 g/L 6'-sialyllactose (6'SL), or 50 g/L 3-fucosyllactose (3FL), respectively. Animals in the control group (CTR) were provided non-supplemented autoclaved water. On experimental Days 2, 5, and 8, the mice were weighed and faecal samples were collected directly from the animals. On Day 8, all mice of the CTR, 6'SL and LNT groups (n=8/group) and half of the mice in the 3FL group (n=8) were anesthetized (hypnorm/midazolam) for collecting portal vein blood before being euthanization by cervical dislocation. The remaining half of the 3FL group mice (n=8) continued into the washout period where the drinking bottles were exchanged with clean bottles containing autoclaved water without the supplementation. The 3FL group was selected based on data from a pilot study. On experimental Days 9, 12 and 15, the remaining mice were weighed and faecal samples collected. On Day 15, the eight remaining mice of the washout group were euthanized as described. All faecal samples were kept at room temperature in a 2mL tubes until processing immediately after the sampling. The mouse experiment was approved by the Danish Animal Experiments Inspectorate (license no. 2020-15-0201-00484 C nr.: C-1) and was overseen by the National Food Institute's in-house Animal Welfare Committee for animal care and use.

Dissection

The intestines were dissected to obtain the tissue samples from mid-colon stored in RNA*later* (Invitrogen™) for gene expression analysis and pellets from distal-colon for culturing and SCFA analysis. Colon samples were kept on ice until processing immediately after the dissection.

Plating, enumeration and isolation of *Bacteroidaceae* strains

Selective plating of *Bacteroidaceae* was performed on Brucella laked blood agar prepared from Brucella Agar with Hemin and Vitamin K1 (B2926 Sigma-Aldrich) supplemented with 50 mL/L filter-sterilized defibrinated sheep blood (No.8545090 E&O Laboratories), 50 mg/L kanamycin and 10 mg/L vancomycin (BrLa+Kan+Van agar). On the day of use, the plates were pre-warmed at room temperature for approximately 4 hours before plating appropriate dilutions of intestinal content. Faecal samples obtained from individual animals or intestinal content obtained upon dissection were weighed and then homogenized in 500 µL phosphate-buffered saline (PBS) by the pipet tip followed by 10 s vortexing on maximum speed. The resulting faecal/intestinal solution was 10-fold serially diluted in 96-well plates using a multichannel pipet with automatic mixing steps between rows. From each dilution, 5 µl was spot plated onto BrLa+Kan+Van agar plates and incubated for 2 days under anaerobic conditions at 37°C before enumeration. Enumeration of *Bacteroidaceae* was performed as Colony Forming Units (CFU) counts of spots with 5-20 visible isolates, summed between triplicates of each dilution and multiplied by the dilution factor. Additionally, colonies from 12 randomly selected plates from dissections on Day 8 (HMO-supplemented animals) were further used for strain isolation and taxonomic identification. Two colonies from each of the 12 plates were re-streaked on fresh BrLa+Kan+Van agar and grown for additional two days as stated above. Purified colonies were aseptically inoculated into a 1 mL cryo-vial containing Luria-Bertani (LB) broth and 15% glycerol, vortexed and stored at -80°C until further use.

Isolate identification

The putative *Bacteroidaceae* isolates obtained from colon and faecal samples and intestinal content on the dissection day were plated from glycerol stocks on fresh BrLa+Kan+Van agar and

grown for 2 days under anaerobic conditions at 37°C. Genomic DNA from the 22 isolates that re-grew was extracted by use of the DNeasy UltraClean Microbial DNA Isolation kit (No. 12224-50, Qiagen) according to the manufacturer's protocol. Mechanical lysis of bacterial cells was performed at 30 cycles/sec for 10 min on a bead beater MM300 (Retsch VWR). DNA concentrations were measured by the Qubit dsDNA HF kit (Q33266, Invitrogen) and samples diluted with nuclease-free water (W4502 Sigma-Aldrich) to a concentration of 5ng/µl. The 16S rRNA gene sequences were amplified in a 50 µL PCR reactions containing 10µL 5X Phusion™ HF-Buffer, 1µL dNTPs (10 mM of each oligo), 1µM universal forward primer 27F (AGA-GTT-TGA-TCM-TGG-CTC-AG), 1µM universal reverse primer 1492R (TAC-GGY-TAC-CTT-GTT-ACG-ACT-T), 1µL template DNA (5ng/µL) and 0.5 µL Phusion™ High-Fidelity DNA polymerase (F-530 Thermo Scientific). Reaction conditions were as follows: Initial denature 98°C for 30sec, 35 cycles of 98°C for 15s, 61°C for 15s and 72°C for 60s and lastly 72°C for 5 min before cooling to 4°C. The PCR products were purification by use of the MinElute PCR purification kit (No. 28004 Qiagen) and diluted to 20-80ng/µl with nuclease-free water. Each purified PCR product (5 µl) was mixed with 5 µl forward primer 27F (5pmol/µl) and shipped to Eurofins facility (Eurofins Genomic Sequencing GMBH 51105 Köln, Germany) for Sanger-sequencing.

Handling of fecal samples for gut microbiota and SCFA analyses

After the initial dilution of faecal samples described in the above section, the samples were centrifuged at 16,000 x G for 10 min at 4°C and 500µL supernatant saved in a 1.5 mL tubes at -20°C for later SCFA analysis. The pellet was stored at -20°C until bacterial DNA extractions. Bacterial DNA extraction was conducted by use of the DNeasy PowerLyzer PowerSoil Kit (No.

12855-50 Qiagen) essentially according to manufactures recommendations. Mechanical lysis of bacterial cells was performed at 30 cycles/sec for 10 min on a bead beater MM300 (Retsch VWR). DNA concentrations were measured by the Qubit dsDNA HF kit (Q33266, Invitrogen) and adjusted to 5 ng/µl in nuclease-free water (W4502 Sigma-Aldrich).

Gut microbiota analysis

Microbiota profiling was performed essentially as previously described (Laursen *et al.* 2021). Briefly, the V3 region of the 16s rRNA gene in extracted community DNA was PCR amplified by using a universal forward primer with a unique 10-12 basepair barcode for each sample (PBU 5´-A-adapter-TCAG-barcode-CCTACGGGAGGCAGCAG-3´) and a universal reverse primer (PBR 5´-trP1-adapter-ATTACCGCGGCTGCTGG-3´) in 20 µl reactions containing 4µL 5X Phusion™ HF-Buffer, 0.4 µL dNTPs (10 mM of each oligo), 1µM forward primer, 1µM reverse primer, 1µL template DNA (5ng/µL) and 0.2 µL Phusion™ High-Fidelity DNA polymerase (F-530 Thermo Scientific). Reaction conditions were as follows: Initial denature 98 °C for 30s, 24 cycles of 98 °C for 15s and 72 °C for 30s and lastly 72°C for 5 min before cooling to 4°C. The PCR products were purified by the HighPrep ™ PCR clean-up system (AC-60500 Magbio) according to the manufacturer's protocol. The resulting DNA concentrations were determined by Qubit HS assay and libraries constructed with mixing equimolar amounts of each PCR product. Partial 16S rRNA gene sequencing was performed on an Ion S5™ System (ThermoFisher Scientific) using OneTouch 2 Ion5: 520/530 kit - OT2 400bp and an Ion 520 Chip.

Sequence data handling

Raw sequence data was initially quality checked and sequencing depth deemed satisfactory. The sequences were imported into CLC genomic workbench (v8.5, CLCbio, Qiagen) as FASTQ files, de-multiplexed and trimmed using defaults settings. Reads below 125 bp and above 180 bp were

discarded. The trimmed sequences were exported to Rstudio (version 4.0.5 (Team 2015)) and the Divisive Amplicon Denoising Algorithm 2 (DADA2) pipeline (Callahan *et al.* 2016) was used (standard settings, except pool=true and adjustments recommended for Ion Torrent reads, namely HOMOPOLYMER_GAP_PENALTY = -1, BAND_SIZE = 32, were implemented in the dada() function) to generate Amplicon Sequence Variants (ASVs), which were taxonomically classified using the Ribosomal Database Project database (rdp_train_set_18) (Wang *et al.* 2007). The ASV taxonomic classification table and the ASV sequences and counts per sample were imported into Quantitative Insights Into Microbial Ecology 2 software (QIIME 2 Core - 2020.11 (Bolyen *et al.* 2019)) and data sorted to contain only taxa of bacterial origin with very rare reads sorted out by setting a minimum frequency of 100 across all samples. Alpha and beta diversity metrics were calculated by the function "Diversity Core-metrics-phylogenetic" based on a rooted phylogenetic tree. When performing diversity analysis each sample was rarefied to 11,000 reads to obtain even sampling depths. For beta diversity, when applicable, the data were sorted according to either experimental day (when performing ANOSIM or ANCOM analysis) or to taxonomic level when performing beta diversity analysis on a subset of the data. Relative abundance calculations were based on non-rarefied reads.

Alignment and generation of a phylogenetic tree for 16S rRNA genes from isolates and microbial profiling.

The 16s rRNA gene sequences obtained by Sanger sequencing of each isolate were quality assessed by CLC Main Workbench and trimmed to obtain only high-quality nucleotide reads. The sequences were searched against the National Center for Biotechnology Information (NCBI) Nucleotide database by the NCBI BlastN tools and the top match was applied as the putative taxonomic classification (Altschul *et al.* 1990). From the Ribosomal Database Project

(rdp.cme.msu.edu) the 16S rRNA gene sequence from a total of 11 type strains representing both the *Bacteroides* genus and the *Phocaeicola* genus were downloaded and exported into CLC Main Workbench. Furthermore, sequences of ASVs taxonomically classified as either *Bacteroides* or *Phocaeicola* were sorted and the relative abundance of each ASV was summed across all samples. ASVs with a relative abundance sum above 1% were also imported into CLC Main Workbench using the ASV number and genus name as the identifier of the sequence. All sequences were trimmed to the same length (V3 region) before performing a multiple alignment and creating a phylogenetic tree with the algorithm "Neighbour-Joining", the distance measure of "Jukes-Cantor" and the bootstrap setting of 100 replicates.

Short-Chain Fatty Acids analysis of faecal waters

Faecal water samples obtained on the dissection day were prepared by thawing faecal supernatants at room temperature followed by centrifugation at 16,000xG at 4C for 5 min. The supernatants were then filtered through Costar SpinX centrifuge filters 0.22 µm (CLS8160 Sigma-Aldrich) at 15,000xG for 5 minutes until clear. The filters were removed from the columns and the solutions were immediately couriered to MS-Omics (Vedbæk, DK-2950, Denmark) where they were stored at -80°C until analysis as follows. Samples were acidified using hydrochloride acid, and deuterium-labelled internal standards were added. All samples were analysed in a randomized order. Analysis was performed using a high polarity column (Zebron™ ZB-FFAP, GC Cap. Column 30 m x 0.25 mm x 0.25 µm) installed in a GC (7890B, Agilent) coupled with a quadrupole detector (5977B, Agilent). The system was controlled by ChemStation (Agilent). Raw data were converted to netCDF format using Chemstation (Agilent) before the data was imported and processed in Matlab R2014b (Mathworks, Inc.) using the PARADISe software described by Johnsen et al (Johnsen *et al.* 2017).

RNA isolation from colonic tissue

Tissue samples from the colon were collected at dissection and stored in RNAlater (Sigma-Aldrich) at -80° C until further analysis. Approximately 30 mg of the tissue sample was used to purify total RNA. Samples were homogenized using a tissue-lyzer (QIAGEN Tissue lyser II) followed by total RNA purification (No. 74106, Qiagen), using Qiagen RNeasy with on-column DNase digestion using Qiagen RNase free DNase kit (No. 79254, Qiagen). cDNA synthesis was performed using omniscript c-DNA synthesis kit (No. 205113, Qiagen), Random primer mix (No. S1330S, Bio Nordica) and Anti RNAse (No. AM2694, Invitrogen) according to manufacturer's protocol.

RT- qPCR, Gene expression analysis

The real-time quantitative PCR was carried out using Roche light cycler Real-Time PCR System (Roche) and threshold cycle values were calculated by light cycler software (Roche). Reactions were performed in triplicates in 384-well PCR plates (Thermo Scientific). The total volume in each well was 10 µL, containing 3 µL diluted cDNA (1:24), 5 µL Taqman Fast Advanced Master Mix (No. 4444963, ThermoFisher Scientific) and 0.5 µl Taqman Gene expression Assay primer/probe mix (ThermoFisher Scientific). Gene assays used were Occludin (Mm00500912_m1), Tight junction protein 1 (Tjp 1 Mm00493699_m1), Tumor necrosis factor-a (*TNF*-α Mm00443258_m1) and Mucin 2 (Muc2 Mm01276696_m1) using Glyceraldehyde 3-phosphate dehydrogenase (GAPDH Mm99999915_g1) and Beta-Actin (*β-actin* Mm00607939_s1) as reference genes. Thermal cycling conditions for the reaction were as follows: 1 cycle at 50 ^{o}C for 2 min, 1 cycle at 95 ^{o}C for 20 sec, 45 cycles at 95 ^{o}C for 3 sec and 60 ^{o}C for 30sec. The relative gene expression of the target genes was calculated using the $2^{-\Delta\Delta Ct}$ method and normalized with the housekeeping genes *GAPDH and β-actin*.

Statistics

GraphPad Prism Software was used for statistical analysis unless otherwise stated. *t*-tests were applied for testing differences in means between two groups when data were normally distributed while a Mann-Whitney test was applied when data were not normally distributed. One- or two-way ANOVA tests or mixed-effects analysis were used when appropriate with multiple comparisons performed by Dunnett's post-hoc test. Alternatively, Kruskal-Wallis non-parametric tests with Dunn's multiple comparisons test were used. Correlation analysis was performed by Spearman rank's analysis. Microbial profiling data obtained by 16S rRNA gene sequencing were analysed with QIIME2, employing ANOSIM for testing differences in the community between groups (beta diversity) and the ANCOM test for assessing differently abundant taxa between groups and sampling times using default settings (Clarke 1993; Mandal *et al.* 2015).

Data availability

The 16S rRNA gene sequence data are deposited in the NCBI Sequence Read Archive with the accession number PRJNA787049.

Author contribution

AQH, TRL, MFL and MIB conceived and designed the study. AQH and HJ performed the experimental work. AQH, HJ and MIB analysed and interpreted the data. AQH drafted the manuscript. All authors made substantial intellectual contributions, revised the manuscript and approved the final version of the manuscript.

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Figure legends

Figure 1: Experimental design and *Bacteroidaceae* culturing data in NMRI mice. (A) The experimental study design of the animal trial is shown indicating period of HMO supplementation and sampling times. (B) chemical structure of the three different HMOs, 6'-sialyllactose, 3-fucosullactose and Lacto-N-Tetraose included as study substrates. (C) Enumeration of CFUs from faecal samples obtained before, during and after the HMO supplementation period is shown as mean values with error bars indicating standard deviations. The period of HMO supplementation in drinking water is highlighted as a shaded box. (D) Box-plots showing CFU counts of samples obtained from the cecum and colon at the end of the HMO supplementation period and after the 1-week washout period for the 3FL-WO group. Individual values are shown with whiskers highlighting minimum and maximum values. *P* values were obtained by mixed-effects analysis followed by Dunnett's multiple comparisons tests between CTR and all HMO supplementation groups individually with $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$ or repeated-measures ANOVA followed by Dunnett's multiple comparisons tests comparing wash-out period time points to Day 8 CFU counts in the 3FL group with $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$.

Figure 2: Phylogenetic tree based on cultured strains, ASVs identified by 16S rRNA gene sequencing and reference type strains. The tree was generated by the “Neighbour-Joining” method using the “Jukes-Cantor” distance measures with bootstrap values shown (100 replicates). The bar shows phylogenetic distance.

Figure 3: Effects of HMO supplementation on bacterial diversity and community composition.(A) Boxplots of total number of observed ASVs and (B) Shannon index based on

16S rRNA amplicon sequencing. Statistical significance between time-points was evaluated by paired *t*-tests within groups with *p < 0.05. (C) Profiling of bacterial composition in faecal samples obtained from individual animals indicates relative abundance at the class level. Grey lines indicate co-caging of animals. (D) Principal coordinate analysis based on Bray-Curtis dissimilarity on Day 0 and (E) Day 8 / Day 15 coloured by group. The table shows results of pairwise comparisons performed by ANOSIM tests indicating R and P values with significant differences highlighted in bold (p<0.05).

Figure 4: Effects of HMO supplementation on relative abundance of specific bacterial genera. (A) Boxplots showing the relative abundance of *Phocaeicola*, (B) *Lacrimispora*, (C) *Bacteroides* and (D) *Bifidobacterium* based on 16S rRNA amplicon sequencing. Statistical significance between time-points was evaluated by paired *t*-tests within groups. *p < 0.05, **p < 0.01, ****p < 0.0001.

Figure 5: Effects of HMO supplementation on faecal short-chain fatty acid concentrations. (A) Boxplots showing the concentration of acetate, (B) formate, (C) propionate, (D) butyrate, (E) valerate and (F) isobutyrate. Statistical significance between groups was evaluated by Kruskal-Wallis tests followed by Dunn's multiple comparisons tests comparing to the CTR group. *p < 0.05, **p < 0.01.

Figure 6: Effects of HMO supplementation on colonic tissue gene expression. (A) Boxplots showing the relative gene expression of occludin, (B) Tjp1, (C) TNFα and (D) Muc2. Statistical significance between all groups was evaluated by Kruskal-Wallis tests followed by Dunn's multiple comparisons tests comparing to the CTR group or Mann-Whitney test between the 6'SL, LNT and 3FL groups aggregated versus the CTR group. *p < 0.05, **p < 0.01.

Figure 7: Correlations between affected bacterial genera, alpha diversity and short-chain fatty acids in faecal samples. (A) Scatter dot plots of *Lacrimispora* vs. *Phocaeicola* relative abundances, (B) *Phocaeicola*/*Lacrimispora* ($\log_2$) ratio vs. number of observed ASVs, (C) *Phocaeicola*/*Lacrimispora* ($\log_2$) ratio vs. concentration of acetate and (D) *Phocaeicola*/*Lacrimispora* ($\log_2$) ratio vs. concentration of butyrate. Associations were assessed by Spearman's rank correlation analysis with rho and p-values indicates.

Supplementary figure legends

Figure S1: Effects of HMO supplementation on animal weight and microbial communities. (A) Average body-weight of animals in the four treatment group with error bars indicating standard deviations. (B) Principal coordinate analysis based on Bray-Curtis dissimilarity of ASVs classified within the Bacteroidetes phylum coloured according to HMO treatment status. (C) Correlation between cultured Bacteroidaceae (CFUs) and relative abundance of Phocaeicola on Day 0 and (D) Day 8 coloured according to treatment group. Associations were assessed by Spearman's rank correlation analysis with rho and p-values indicates. (E) Relative abundance of *Faecalibacterium* spp. in individual animals in the 3FL group. (F) Principal coordinate analysis based on Bray-Curtis dissimilarity of ASVs classified within the Bacteroidetes phylum coloured according to cage for the Day 8 FL3 sub-samples.

Figure S2: Results of ANCOM analysis. The *ctr*-value (effect size difference) and *W*-statistic (number of genera that a single genus is tested to be significantly different against) values of ANCOM analysis comparing Day 0 and Day 8 relative abundances at the genus level for (A) the CTR group, (B) the 6'SL group, (C) the LNT group and (D) the 3FL group as well as between (E) Day 8 and Day 15 and (F) Day 0 and Day 15 for the 3FL/3FL-WO group. Significant genera are indicated with asterisks.

Figure S3: Correlations between bacterial genera and SCFA and alpha diversity in faecal samples. (A) Scatter dot plots of *Phocaeicola* relative abundances vs. acetate, (B) *Lacrimispora* relative abundances vs. acetate, (C) *Phocaeicola* relative abundances vs. butyrate, (D) *Phocaeicola* relative abundances vs. Shannon index and (F) *Lacrimispora* relative abundances

vs. Shannon index. Associations were assessed by Spearman's rank correlation analysis with rho and p-values indicates.

Figure 1

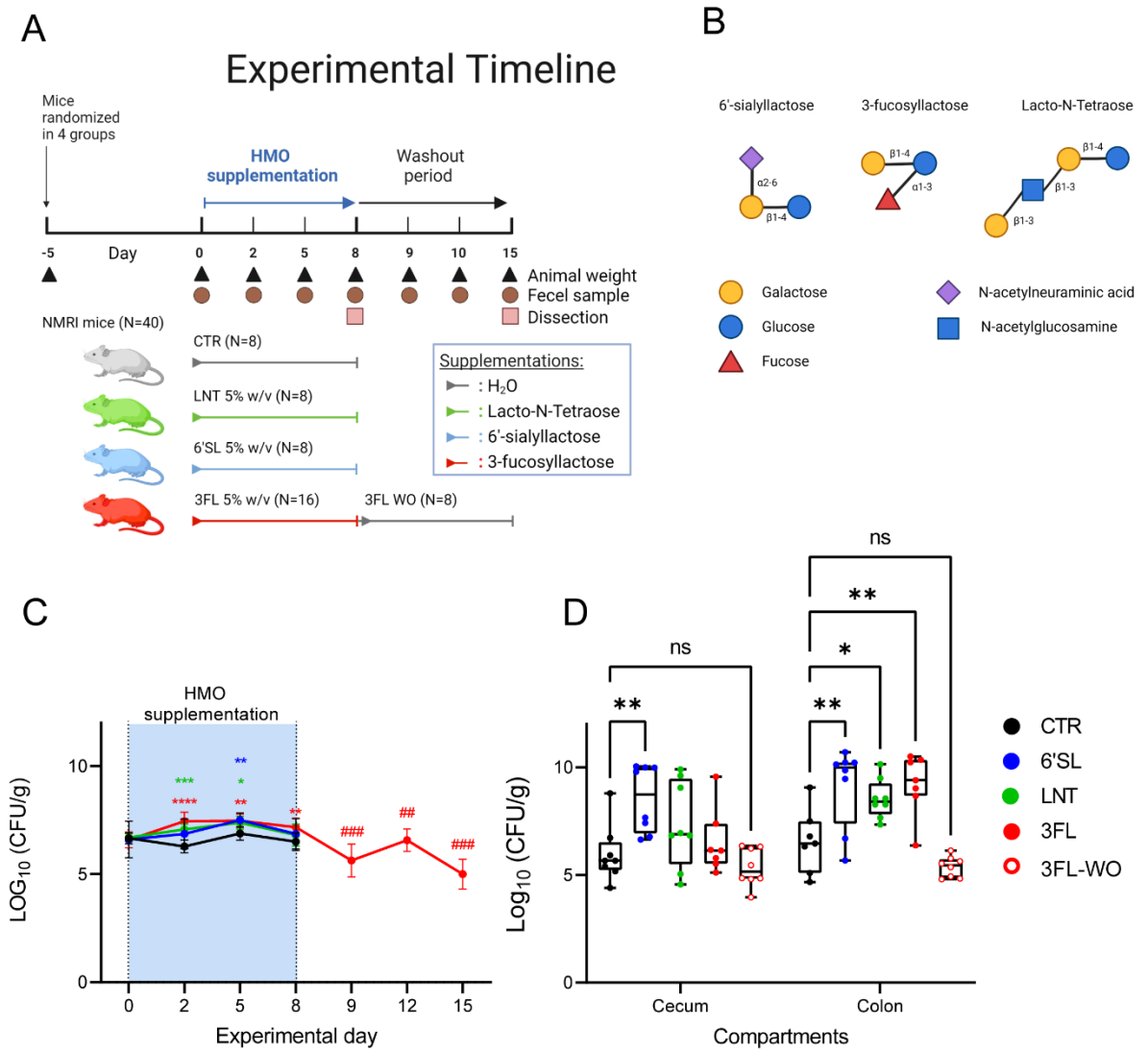


Figure 1

Figure 2

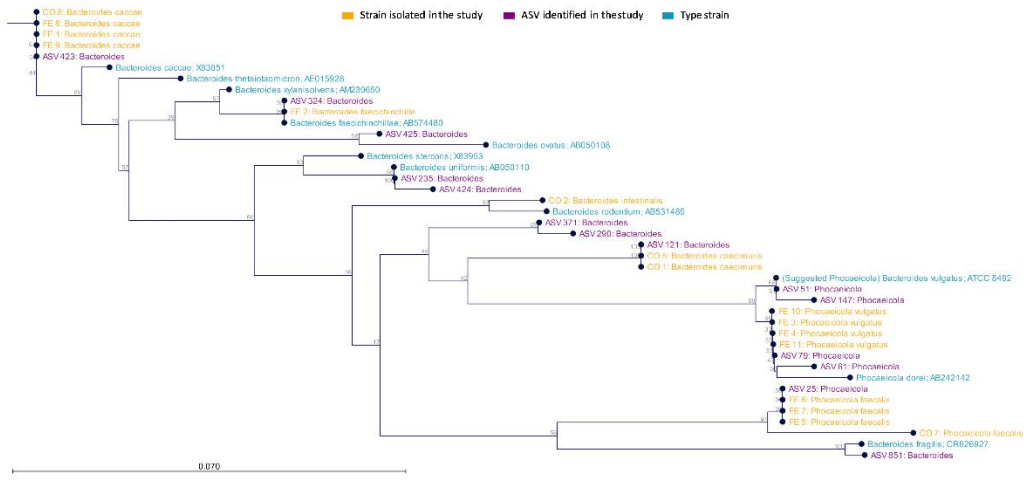
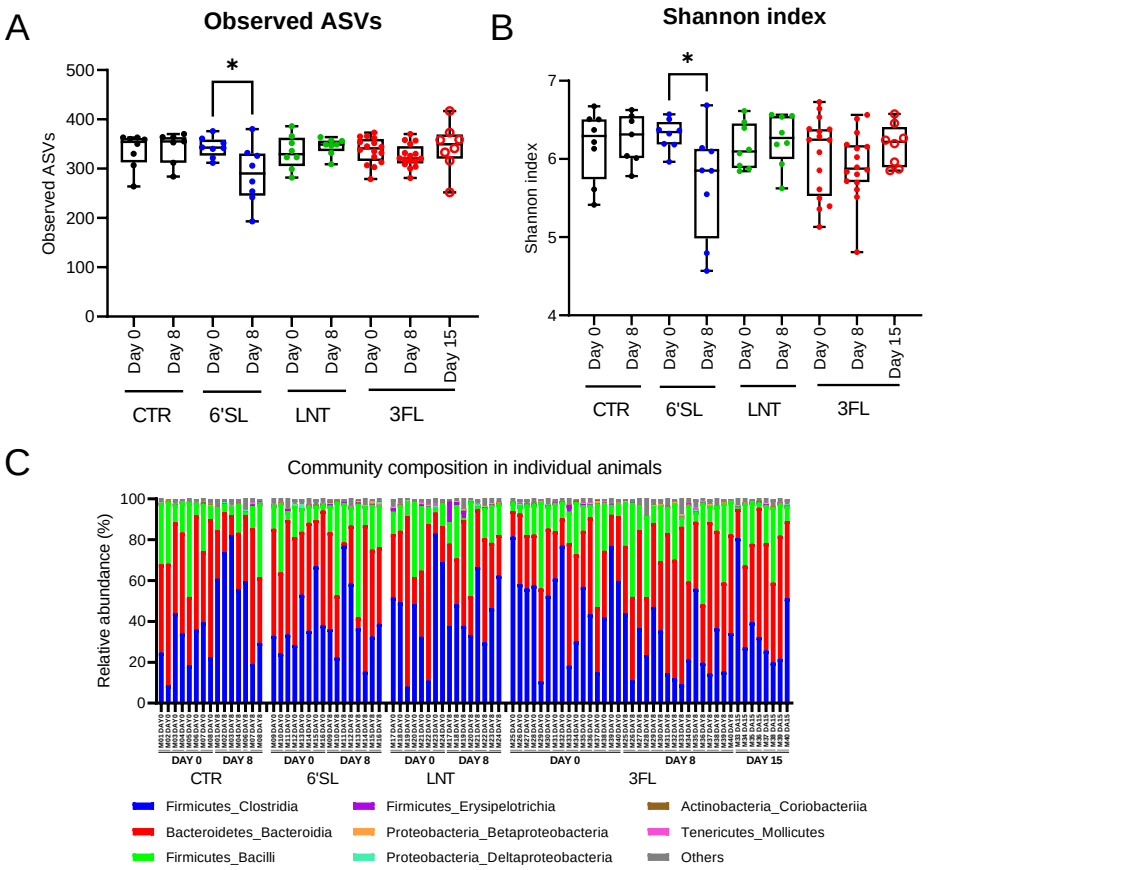
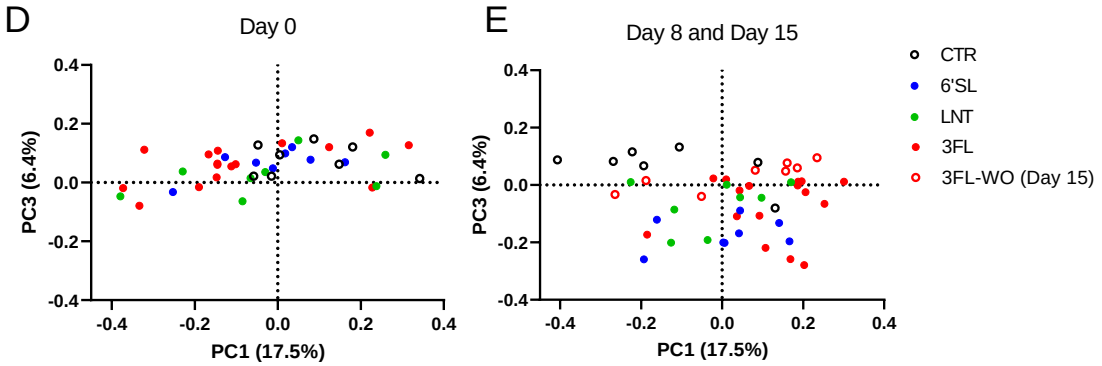


Figure 2

Figure 3





Anosim	Day 0					
	CTR vs. 6'SL	CTR vs. LNT	CTR vs. 3FL	6'SL vs. LNT	6'SL vs. 3FL	LNT vs. 3FL
R	0.08	0.02	0.07	0.01	0.09	0.06
P	0.191	0.554	0.186	0.424	0.123	0.226

Anosim	Day 8						Day 8 vs. Day 15	
	CTR vs. 6'SL	CTR vs. LNT	**CTR vs. 3FL**	**6'SL vs. LNT**	6'SL vs. 3FL	**LNT vs. 3FL**	CTR (day 8) vs. 3FL-WO	3FL (day 8) vs. 3FL-WO
R	**0.31**	0.13	**0.37**	**0.27**	0.09	**0.32**	0.1	0.08
P	**0.005**	0.092	**0.006**	**0.01**	0.111	**0.008**	0.184	0.124

Figure 3

Figure 4

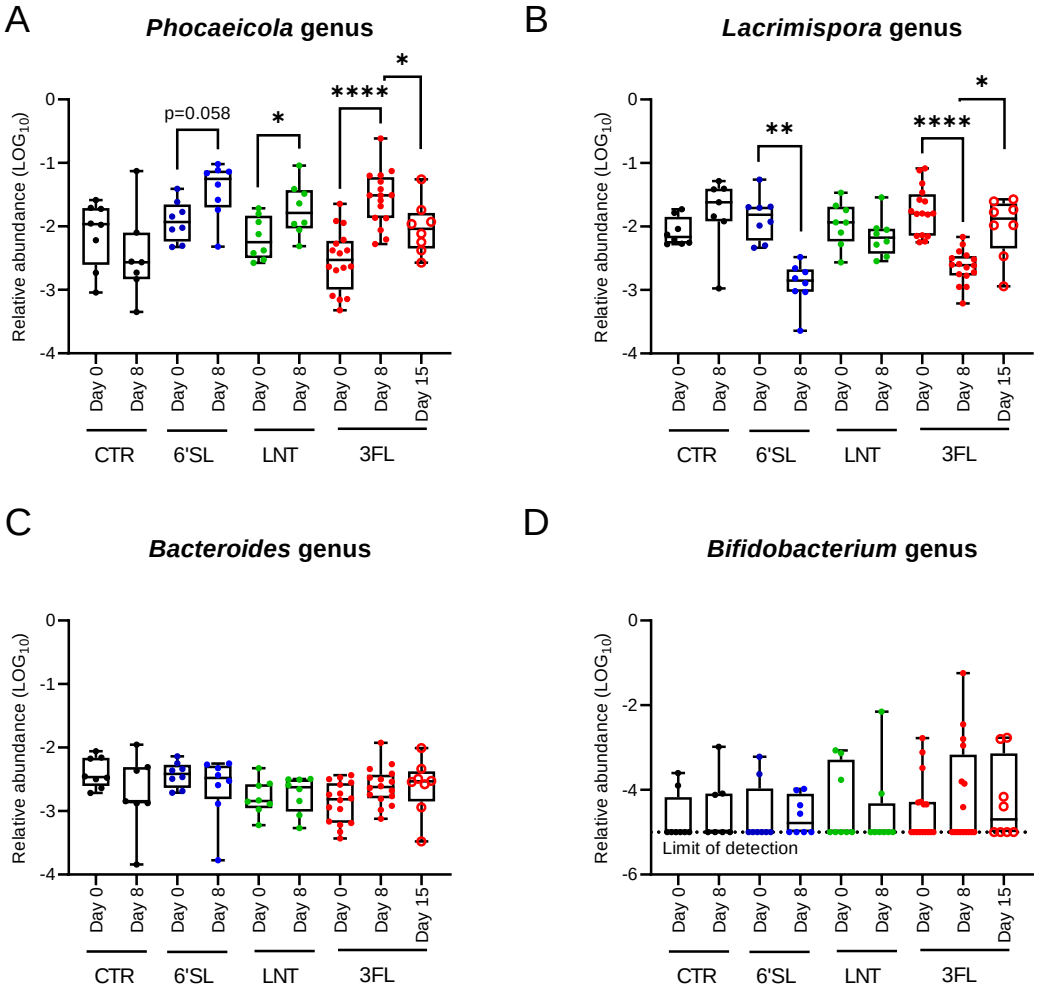


Figure 4

Figure 5

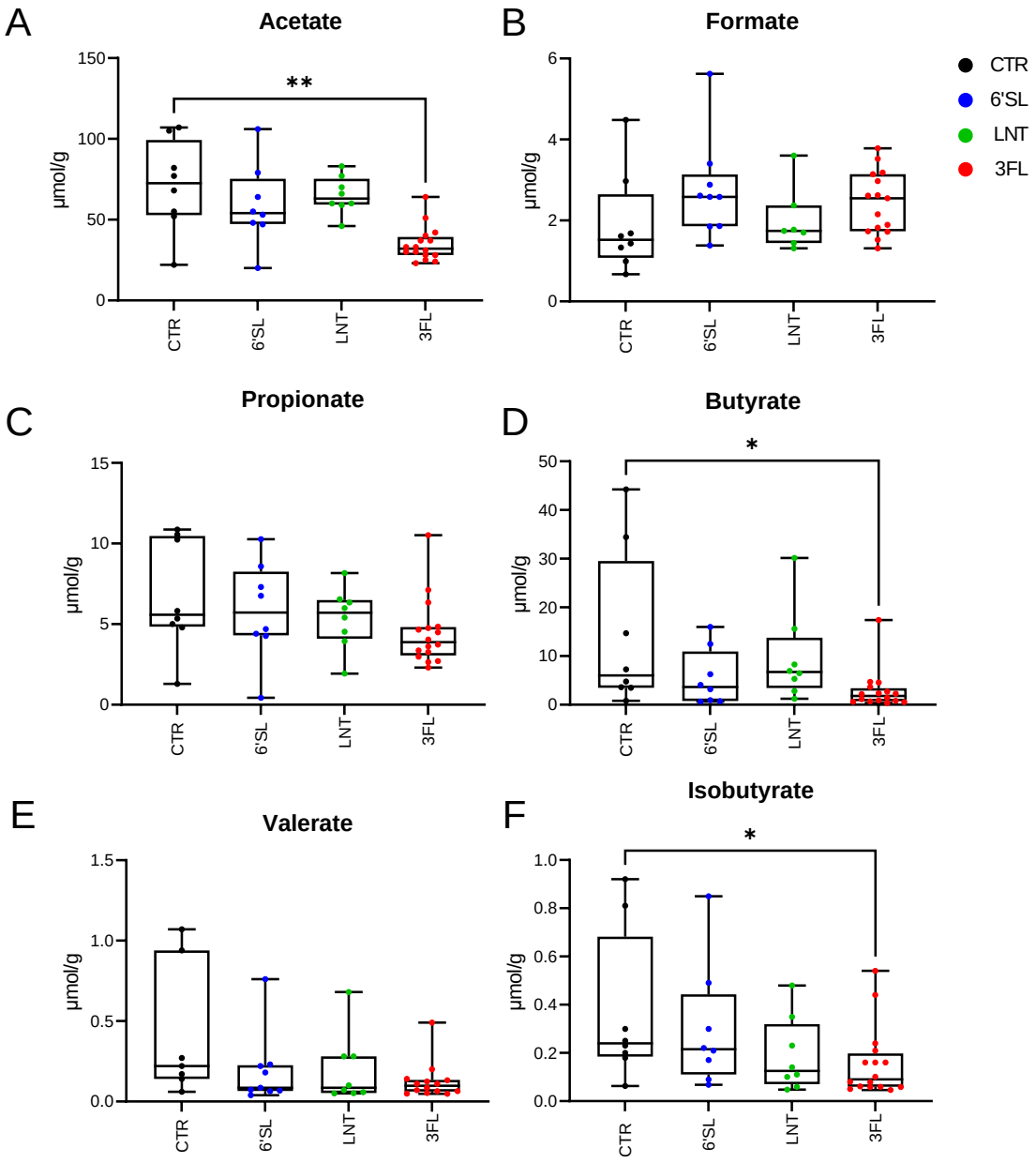


Figure 5

Figure 6

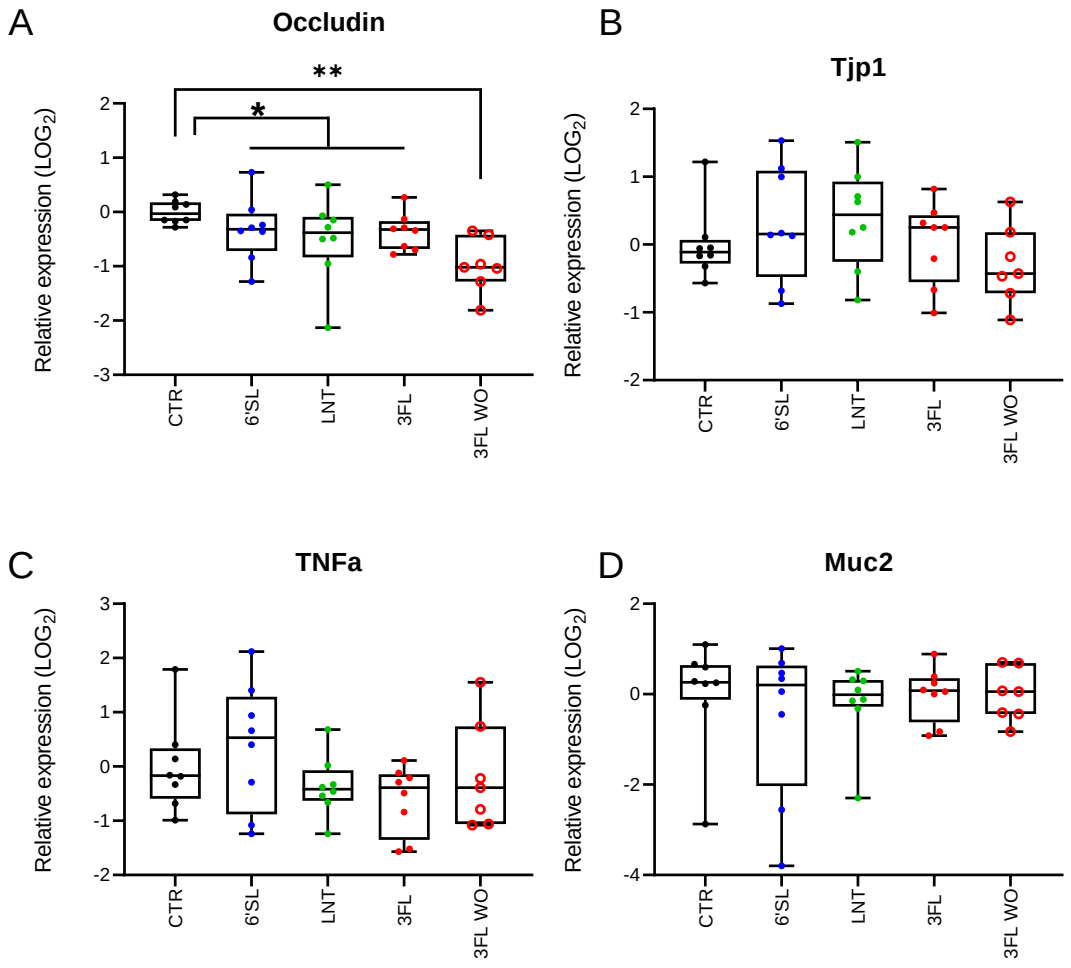


Figure 6

Figure 7

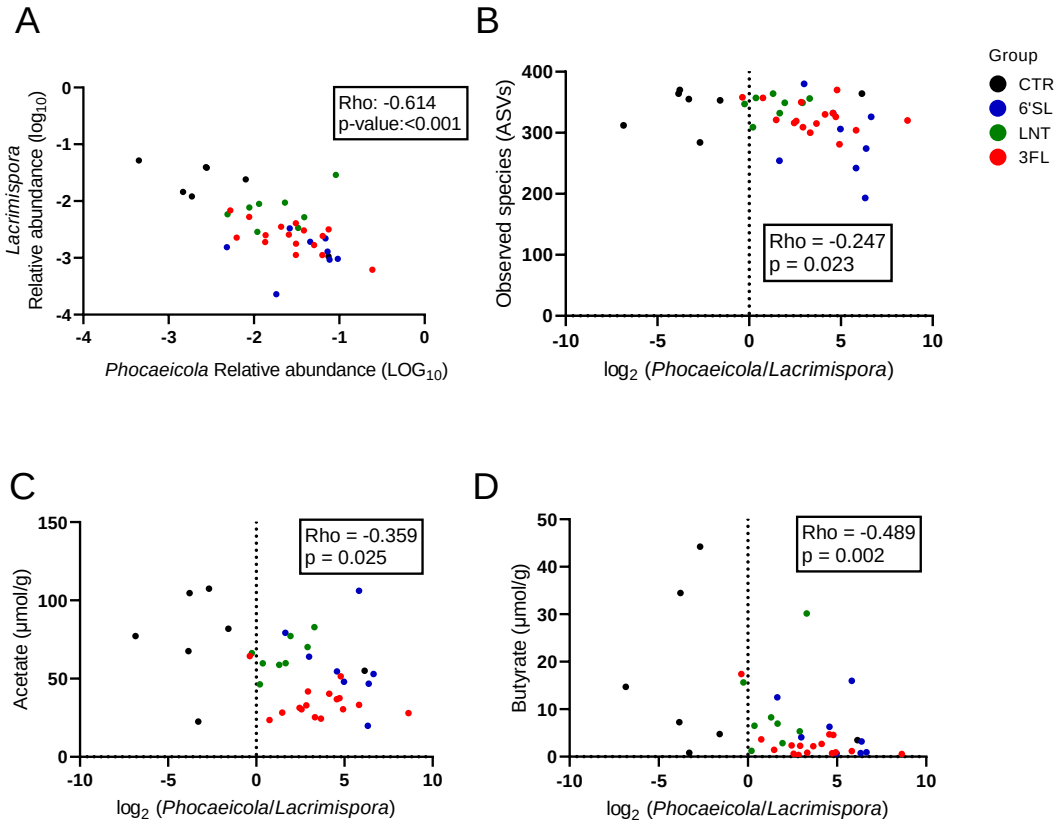


Figure 7

Figure S1

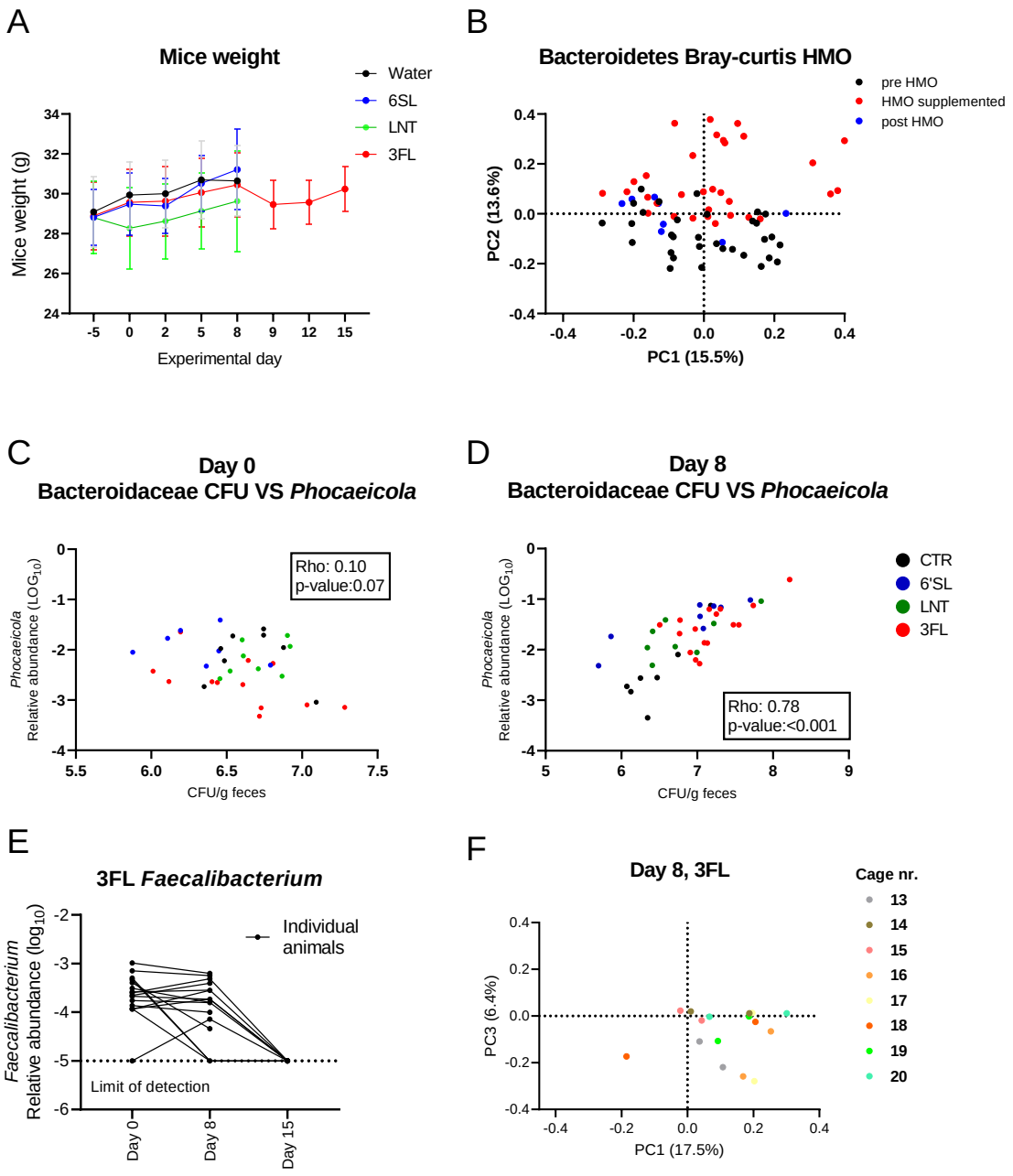


Figure S1

Figure S2

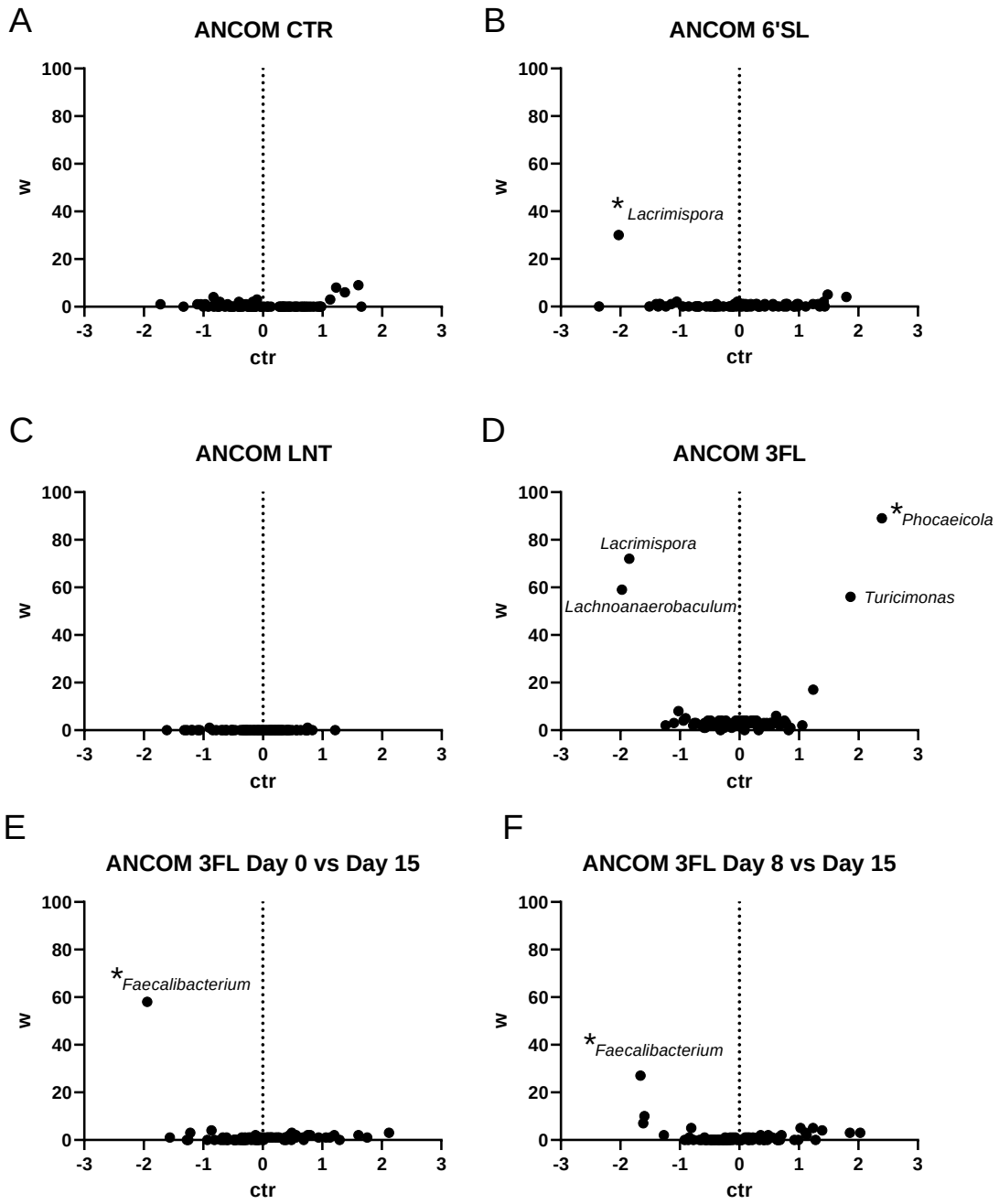


Figure S2

Figure S3

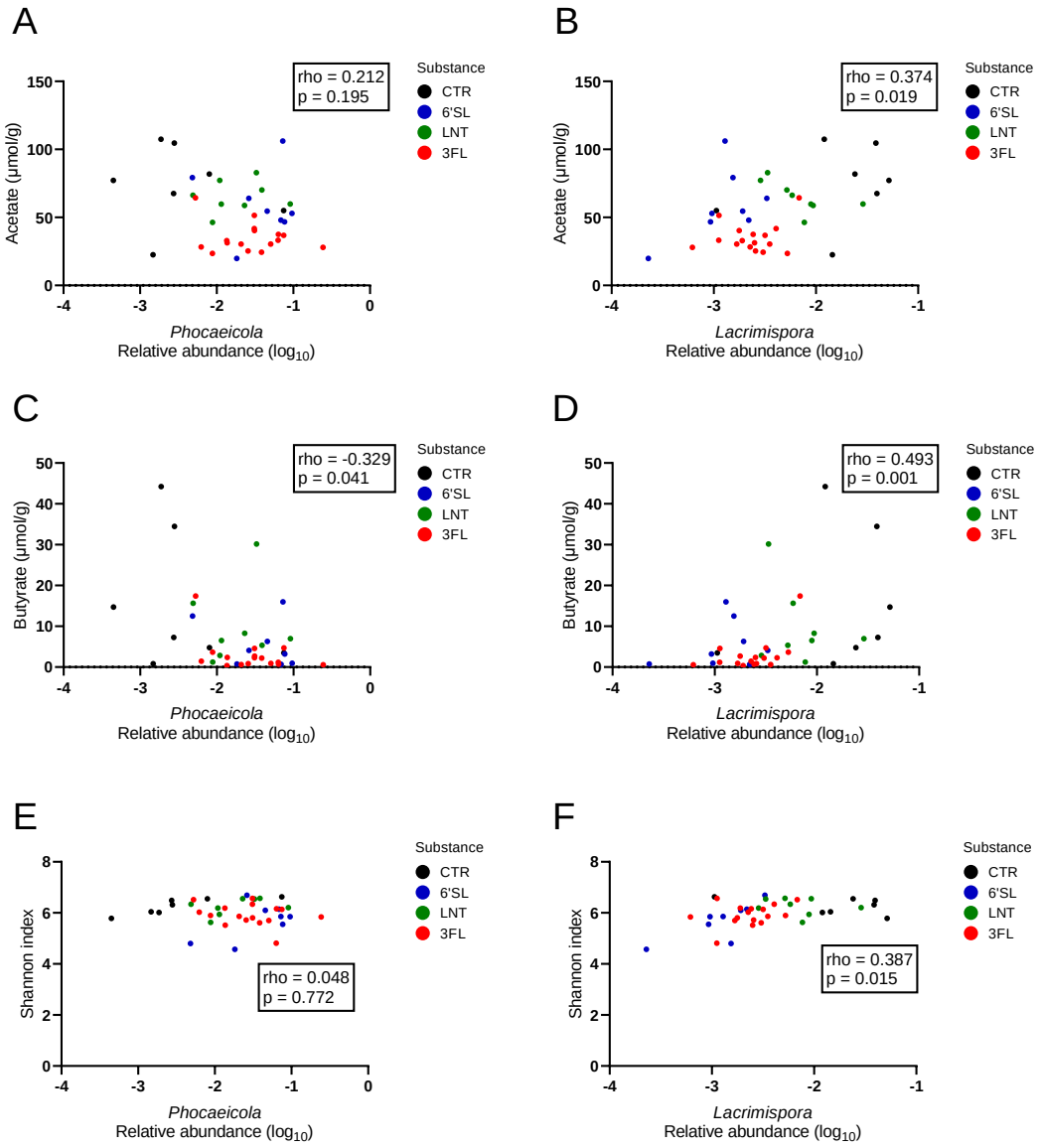


Figure S3

Table S1. Bacterial isolates recovered from intestinal samples from colon and feces.

Isolate ID	Animal No. (group)	Sample material	Sequence length (nt)	Closest BLAST hit (NCBI)	Query Cover	NCBI % Indentity	Accession	Isolate match with *Bacteroidaceae* ASV sequence[1]	Isolate % identity with ASV
FE 1	#12 (6'SL)	Feces Day 8	491	*Bacteroides caccae*	100%	99.83%	AB714287.1	YES	100%
FE 2	#12 (6'SL)	Feces Day 8	496	*Bacteroides faecichinchille*	100%	99.23%	MN854703.1	YES	100%
FE 3	#14 (6'SL)	Feces Day 8	492	*Phocaeicola vulgatus*	100%	99.03%	MN854705.1	YES	100%
FE 4	#14 (6'SL)	Feces Day 8	484	*Phocaeicola vulgatus*	100%	99.61%	MT268990.1	YES	100%
FE 5	#15 (6'SL)	Feces Day 8	495	*Phocaeicola faecalis*	100%	99.40%	MN854703.1	YES	100%
FE 6	#17 (LNT)	Feces Day 8	500	*Phocaeicola faecalis*	100%	99.29%	MN854703.1	YES	100%
FE 7	#17 (LNT)	Feces Day 8	490	*Phocaeicola faecalis*	100%	100.00%	MN854703.1	YES	100%
FE 8	#32 (3FL)	Feces Day 8	500	*Bacteroides caccae*	100%	99.22%	MK743932.1	YES	100%
FE 9	#32 (3FL)	Feces Day 8	476	*Bacteroides caccae*	100%	100.00%	AB714287.1	YES	100%
FE 10	#33 (3FL)	Feces Day 8	490	*Phocaeicola vulgatus*	100%	99.35%	MT268990.1	YES	100%
FE 11	#33 (3FL)	Feces Day 8	502	*Phocaeicola vulgatus*	100%	99.14%	MT268990.1	YES	100%
FE 12	#37 (3FL)	Feces Day 8	690	*Faecalibaculum rodentium*	100%	99.86%	CP011391.1	NO	N/A
FE 13	#37 (3FL)	Feces Day 8	744	*Faecalibaculum rodentium*	99%	99.06%	CP011391.1	NO	N/A
CO 1	#12 (6'SL)	Colon Day 8	725	*Bacteroides caecimuris*	100%	99.80%	CP015401.2	YES	100%
CO 2	#15 (6'SL)	Colon Day 8	740	*Bacteroides intestinalis*	100%	98.73%	NR_041307.1	NO	N/A
CO 3	#19 (LNT)	Colon Day 8	884	*Erysipelatoclostridium ramosum*	100%	99.55%	EU869233.1	NO	N/A
CO 4	#21 (LNT)	Colon Day 8	537	*Bacteroides caecimuris*	100%	99.23%	LC416470.1	YES	100%
CO 5	#21 (LNT)	Colon Day 8	730	*Bacteroides caecimuris*	100%	99.62%	LC416470.1	YES	100%
CO 6	#22 (LNT)	Colon Day 8	859	*Parasutterella excrementihominis*	100%	99.87%	LT558827.1	NO	N/A
CO 7	#31 (3FL)	Colon Day 8	414	*Phocaeicola faecalis*	100%	98.31%	MN854703.1	YES/NO	97.20%
CO 8	#32 (3FL)	Colon Day 8	650	*Bacteroides caccae*	100%	99.08%	AB714287.1	YES	100%

[1]The 16S rRNA gene sequence of the isolate obtained from Sanger sequencing matched an ASV sequence classified as *Bacteroidaceae* as listed in Table S2

ASV No.	Read length	ASV % presence total samples	BLAST (NCBI)	Query Cover	NCBI %ID	NCBI Accession	Isolate obtained[1]	Species type strain
ASV 25	155	96.59%	*Phocaeicola faecalis*	99%	98.78%	MN854705.1	YES	Not available
ASV 51	155	100%	*Phocaeicola vulgatus*	100%	100.00%	MT152628.1	NO	*Bacteroides vulgatus* (RDP : ATCC 8482)
ASV 61	155	96.59%	*Phocaeicola vulgatus*	100%	99.35%	MT515977.1	NO	*Bacteroides vulgatus* (RDP : ATCC 8482)
ASV 79	155	94.32%	*Phocaeicola vulgatus*	100%	100.00%	MT515977.1	YES	*Bacteroides vulgatus* (RDP : ATCC 8482)
ASV 121	155	98.86%	*Bacteroides caecimuris*	100%	100.00%	CP015401.1	YES	*Bacteroides caecimuris* (NCBI: CP015401)
ASV 147	155	76.14%	*Phocaeicola vulgatus*	100%	99.35%	MT152628.1	NO	*Bacteroides vulgatus* (RDP : ATCC 8482)
ASV 235	155	94.32%	*Bacteroides uniformis*	100%	100.00%	LC515590.1	NO	*Bacteroides uniformis* (RDP : AB050110)
ASV 290	155	82.95%	*Bacteroidales* sp.	100%	99.35%	LC333722.1	NO	Not available
ASV 324	154	87.50%	*Bacteroides faecichinchillae*	100%	100.00%	MK929066.1	YES	*Bacteroides faecichinchillae* (RDP : AB574480)
ASV 371	154	70.45%	*Bacteroides* sp.	100%	99.35%	MH682255.1	NO	Not available
ASV 423	155	65.91%	*Bacteroides caccae*	100%	100.00%	MT539041.1	YES	*Bacteroides caccae* (RDP : X83951)
ASV 424	155	77.27%	*Bacteroides uniformis*	100%	99.35%	LC515590.1	NO	*Bacteroides uniformis* (RDP: AB050110)
ASV 425	155	11.36%	*Bacteroides koreensis*	100%	100.00%	MT464342.1	NO	*Bacteroides ovatus* (RDP: AB050108)
ASV 851	155	20.45%	*Bacteroides fragilis*	100%	100.00%	MN629228.1	NO	*Bacteroides fragilis* (RDP: CR626927)

Table S2. Prevalent amplicon sequence varients (ASVs) in 16S rRNA gene sequence data

[1]The obtained isolate had 100% homology in terms of 16S rRNA gene sequence

Chapter V: Manuscript II

Type VI secretion systems contained on almost identical 94.2 kb Integrative and Conjugative Elements indicate recent horizontal transfer events between four Bacteroides spp. co-residing in the gut.

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In preparation.

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Abstract

Species of the order of Bacteroidales express antagonism systems known as Type VI Secretion Systems (T6SS). The T6SS are classified into three different Genetic Architectures (GA1, GA2 and GA3) where GA1s and GA2s are contained on Integrative Conjugative Elements (ICE). These GA1 and GA2 are mobilizable across several Bacteroidales species and recent studies have confirmed the horizontal transfer of a GA1-containing ICE between four species in the gut of a human donor. An interesting aspect of GA1s and GA2s is that they rarely coexist in the same strain, which has been confirmed by a comprehensive data-driven analysis of available Bacteroidales genomes. In the present study, we isolated a diverse collection of Bacteroidales species from six human donors and screened for GA1 and GA2 conserved gene regions by PCR. We identified an interesting pattern of GA2 in isolates of one specific donor and further found evidence of both a GA1 and GA2 T6SS in two of these isolates. Genome sequences of four isolates from this donor showed the horizontal transfer of a 94.2 kb GA2 ICE across the species *Bacteroides thetaiotaomicron*, *Phocaeicola vulgatus*, *Bacteroides ovatus* and *Bacteroides uniformis*. The mutations present on the GA2 ICE indicated that *B. thetaiotaomicron* was the original donor of the ICE to the three other recipient strains. We observed the rare event of a GA2 co-existing with a GA1 in two isolates which, interestingly, both showed unique disruptions of the *mghA* gene. Defects in the *mghA* gene have previously been correlated with the presence of both GA1 and GA2 ICE in strains. It has been suggested that MhgA may be a novel integrase defense system, a prediction supported by this study. By conferring tetracycline resistance (*tetQ*) in the GA1 ICE of a previously genome sequenced *Bacteroides finegoldii* strain we were able to show a horizontal transfer of the GA1 ICE from *B. finegoldii* to two different *Bacteroides* species. This proof-of-concept study allows for further characterization of the horizontal transfer of GA1s and GA2s in the Bacteroidales order.

Introduction

The human gut microbiota is a dense ecosystem with highly competitive bacterial species inhabiting the available niche (Martínez, Muller, and Walter 2013). Bacteroidales is the dominating order of gram-negative bacteria in the human gut and encompasses the prevalent genera of *Bacteroides, Phocaeicola* and *Parabacteroides* (Zitomersky, Coyne, and Comstock 2011). These genera are well adapted to the gut ecosystem and form stable, temporal colonization indicating a symbiotic relationship with the host (Lloyd-Price et al. 2017). Bacteroidales species contain many antagonistic systems such as secretion of bacteroidetocins and expression of Type VI Secretion Systems (T6SS) that target closely related species. While bacteroidetocins are toxins secreted into the environment, the T6SS are, on the other hand, an antagonistic nanomachine firing at cells in close proximity (Coyne et al. 2019; Smith, Vettiger, et al. 2020). T6SS are composed of a core needle-like structure in a complex machinery. The needle and surrounding sheaths are assembled in the cell cytoplasm and anchored to the inner membrane by a baseplate. At the tip of the needle sits the spike where toxins are loaded. Upon attacking, the extended needle will by contraction of the sheaths, protrude through the attacking cell's periplasm and outer membrane and penetrate the target cell. The spike of the needle is then decoupled and the toxins are released in the target. The core structures of the needle are recycled in the cell cytoplasm and a new cycle of T6SS assemble can take place (Cianfanelli, Monlezun, and Coulthurst 2016). The genes for each of the T6SSs are clustered with structural genes conserved between T6SS loci of a particular genetic architecture and genes encoding effector and immunity proteins divergent within these loci. The toxin-immunity pairs contained by the different species of Bacteroidales in a community is important for competition, while their impact in a broader ecological perspective is not well understood (Smith, Brodmann, et al. 2020; Coyne and Comstock 2019)

In the order of Bacteroidales, the T6SS is classified into three different genetic architectures (GA) based on how the core genetic elements are structured (Coyne, Roelofs, and Comstock 2016). The GA3 has so far been found only in species of *Bacteroides fragilis* and is not contained on mobile elements. The GA1

and GA2 are contained on Integrative and Conjugative Elements (ICE) and are widespread in gut isolates of the genera *Bacteroides*, *Phocaeicola*, and *Parabacteroides*. In the recent study by Garcia-Bayona *et al*., it was shown that GA2 obtain a species-level distribution bias with high prevalence in only certain species while the GA1 obtain a seemingly more even distribution among the genera of *Bacteroides*, *Phocaeicola* and *Parabacteroides*. Further, five different GA2 subtypes were identified (classified from GA2a to GA2e) and these subtypes demonstrate geographical clustering (García-Bayona, Coyne, and Comstock 2021). The GA1 and GA2 are transferred by the ICE and studies have confirmed that a community can experience a GA sweep across several species (Coyne et al. 2014; Coyne, Roelofs, and Comstock 2016; García-Bayona, Coyne, and Comstock 2021). The GA1 and GA2 are rarely present in the same strain and analysis of available Bacteroidales genomes revealed that in most cases, when coexistence occurs, either the GA1 or the GA2 are disrupted. In the few cases where an intact GA1 and GA2 coincided, a previously unknown gene designated *mhgA*, present on the GA1 ICE is disrupted. Analysis of the protein sequence of MhgA revealed helicase and methylase domains, leading to the hypothesis that MhgA might represent a novel defense system (García-Bayona, Coyne, and Comstock 2021).

In the present study, we obtained a diverse collection of Bacteroidales isolates from six healthy human donors and performed a PCR screening to detect strains with GA1 and GA2 T6SS loci. We found an interesting pattern of GAs in one specific donor and whole-genome sequenced strains of four species with evidence of GA2 or both GA1 and GA2. Genome sequencing confirmed that a nearly identical 94.2kb GA2 ICE was shared between all four species. In addition, two strains demonstrated the rare event of having both a GA2 and a GA1. In both these isolates, the *mhgA* gene of the GA1 ICE was disrupted but by dissimilar integrase events. Lastly, as a tool to better study the molecular basis of ICE exclusion, we tagged a GA1 ICE with *tetQ* conferring tetracycline resistance so that transfer frequencies to diverse Bacteroidales species can be calculated.

Results

A diverse collection of human gut Bacteroidaceae bacteria obtained by selective plating

Initial screening of all colonies from donor 1 and donor 2 by MALDI-TOF MS indicated that the BrLA+kan+van media was selective for species from the *Bacteroidaceae* and *Tannerellaceae* families, with most isolates (63/65) confirmed to belong to the *Bacteroidacea* family. We, therefore, decided to select 6-14 colonies per donor, chosen based on differences in plate morphology, originating from each of the six human donors for further analysis. Following the taxonomic classification of these strains by partial 16S rRNA Sanger sequencing, we obtained a collection of 52 Bacteroidales isolates belonging to 13 different species from three different families (*Bacteroidaceae, Tannerellaceae* and *Odoribacteraceae*) and constituting between six and 14 strains per human donor (Table 1). The 52 isolated strains all showed very high similarity to reference strains (>99%) except for two isolates (>97.6%) in the NCBI database and represented a diverse collection of bacteria within the Bacteroidales order. Alignment of the partial 16s rRNA gene sequences and generation of a phylogenetic tree revealed an expected clustering of species independent of donors and close clustering of isolated strains of specific species within each donor (Fig. S1). All 52 isolates were assessed for their sensitivity towards erythromycin (10µg/ml) and tetracycline (5µg/ml) revealing only a few resistant strains, which were found in only three of the six donors (Table 1).

Bacteroidaceae isolates carry different combinations of Genetic Architectures (GA) of Type IV Secretion Systems

Screening of 52 isolates for the presence of the *vgrG* gene associated with Genetic Architectures 1 (GA1) and Genetic Architectures 2 (GA2) of T6SS revealed some interesting patterns in the strains from the six different donors (Table 1). The GA1 was found in 1-2 different species obtained from donor 1, 2 and 4 while no GA1 was found in species obtained from donor 3, 5 and 6. The GA2 was only present in isolates obtained from donor 2, where it was identified in 11 of the 14 isoates from this particular donor. Based on alignments of the *vgrG* genes identified as belonging to GA1 and GA2 respectively, a phylogenetic tree

was created and showed aligned GA1 regions with 95.8-100% homology between the isolates and 87.1-89.3% homology to the reference gene while aligned GA2 showed 99.3-100% homology between isolates and 81.2-81.6% homology to the reference gene (Fig. 1A). The finding of identical *vgrG* GA2 regions amplified from isolates of four different species from donor 2 led us to focus on this particular community. Screening of GA2 variable gene region of selected isolates followed by partial Sanger sequencing revealed a 100% homology between the identified regions, suggesting these are the same T6SS loci. We found that four unique GA1/GA2-containing strains were present in the community. The four strains all PCR amplified the conserved GA2 region and a PCR that spanned the variable region, and two strains additionally amplified a conserved GA1 region (Table 1). We hypothesized that a horizontal transfer event of the GA2-containing ICE between the four co-residing isolates was a plausible explanation for the finding of identical GA2 elements and were further intrigued by the observation that two of the isolates, D2-S40 and D2-S48, contained both GA1 and GA2. To investigate this further we decided to obtaine genome sequences of the four isolates namely *Bacteroides thetaiotaomicron* D2-S32, *Phocaeicola vulgatus* (D2-S38), *Bacteroides ovatus* (D2-S40) and *Bacteroides uniformis* (D2-S48).

Whole-genome sequencing confirms horizontal transfer of GA2 between four co-residing Bacteroidaceae species

A combination of short read and long read sequencing of *B. thetaiotaomicron* (D2-S32), *P. vulgatus* (D2-S38), *B. ovatus* (D2-S40) and *B. uniformis* (D2-S48) produced contigs of good quality with genomes assemblies of high confidence. As expected, all four strains contained a GA2 ICE and query against concatemers of the different GA2 subtypes classified them as GA2c subtypes (García-Bayona, Coyne, and Comstock 2021). Alignment of the GA2 T6SS loci of the four strains revealed 100% nucleotide identity across the ~29.5kb region and confirmed the presence of the expected core genes of T6SS (Fig. 2A). Alignment of the ICE containing the GA2 T6SS loci of the four strains revealed 100% homology across the ~94.2 kb sequence, with the exception of a few insertion and deletion events, confirming horizontal transfer between these isolates (Fig. 2B). Analysis of the insertion- and deletion events of the

GA2 ICE of the four strains allowed for a prediction on the direction of transfer. The strain *B. thetaiotaomicron* D2-S32 had no unique insertions or point mutations and was suggested to be the donor of the GA2 ICE to the three other strains. *B. uniformis* (S48) had two insertions (of 2562 bp and 2539 bp) relative to the *B. thetaiotaomicron* ICE, *B. ovatus* (S40) had one insertion (of 1839 bp followed by a 22 bp deletion) while *P. vulgatus* (S38) had a single point mutation likely obtained after acquisition from *B. thetaiotaomicron* (Fig. 2B).

Evidence of a disrupted *mhgA* may explain the integration of GA2 in GA1 carrying strains

The two GA1 T6SS loci of *B. ovatus* (D2-S40) and *B. uniformis* (D2-S48) were quite different from each other, demonstrainting only 86.4% DNA identity, and therefore were not obtained by transfer between these strains, as was the GA2 T6SS loci. The *B. ovatus* (D2-S40) GA1 locus lacked two core T6SS genes, *tssB* and *tssE* (Fig. 3A). Alignment of the full GA1 ICE showed 92.3% DNA identity again confirming that the GA1 ICE had not been shared by a recent horizontal transfer event between these strains (Fig. 3B). Notably, the *mghA* gene of both *B. ovatus* (D2-S40) and *B. uniformis* (D2-S48) was disrupted by a gene insertion but the insertions were distinct. Three phage-integrase family genes had disrupted the *mhgA* gene of *B. ovatus* (D2-S40) while *B. uniformis* showed a Group II introns disruption of the *mhgA* (Fig. 3B). MhgA has both helicase and methylase domains, and may represent a novel defense system (García-Bayona, Coyne, and Comstock 2021). These findings are consistent with previous analyses of Bacteroidales geneomes that most strains with coexisting GA1 and GA2 ICE have either defects in one of the two T6SS regions, or have a defective *mghA* gene. We detected both of these disruptions among our two strains with coexisting GA1 and GA2 ICE.

Insert of a tetracycline resistance marker in GA1 of donor enables tracking of transfer in vitro

As a first step to study properties of GA1 and GA2 ICE transfer, we marked a GA1 ICE so that its movement could be tracked. The *tetQ* gene was inserted at a predicted null site of the GA1 ICE of *B. finegoldii* C09T03C10. We performed co-culture experiments to monitor the transfer of this GA1-*tetQ* from *B. finegoldii* to two erythromycin resistant recipients, *Bacteroides ovatus* D2 and *B. fragilis* 638R

ermG. Following 18 hours of co-culture, the bacteria were diluted and plated to erythromycin and tetracycline. As the *ermG* gene is not on a mobile element, transconjugants represent the transfer of the GA1 ICE to the donor strain. Four transconjugants of *B.fragilis* 638R and *B.ovatus* D2 were analyzed by PCR amplifying the vgrG gene of the *B. finegoldii* T6SS regions, which was 100% identical confirming that the *B. finegoldii* GA1 ICE transferred to these recipients (Fig. 4A).

Discussion

We were able to obtain a diverse Bacteroidales isolate collection from six human donors by use of the BrLA+Kan+Van media with isolates of the genera of *Bacteroides* constituting the majority (Sheppard, Cammarata, and Martin 1990). The isolates generally showed a low prevalence of resistance to the antibiotics erythromycin and tetracycline with only a minor fraction of isolates from three donors showing resistance. The present study is not a quantitative assessment of resistance prevalence but similar studies of isolates obtained from American donors have shown that the majority of isolates are resistant to one or more antibiotics (Boyanova, Kolarov, and Mitov 2015; Hastey et al. 2016). When performing genetic work with strains originating from human donors the resistance profiles can pose a challenge for genetic work necessitating alternative strategies (García-Bayona and Comstock 2019). This observation is consistent with the general antibiotic use in the American population, where the Defined Daily Doses of antibiotics per 1,000 inhabitants per day is significantly higher than that of the Danish population (Danmap 2020; US Center for Disease Controls and Prevention 2021). The low antibiotic resistance load in the obtained strain collection will make putative genetic engineering a more straightforward task.

We identified GA1 and GA2 conserved regions in many of the 52 isolates but compared to a more comprehensive data-driven analysis of the global GA prevalence, the present study identified fewer GA elements than expected and only a single donor contained isolates positive for GA2 (García-Bayona, Coyne, and Comstock 2021). Based on the present dataset we could not elude further on the proposed geographical clustering related to GA loci and GA2 subtypes. A finding that was not further investigated was the occurrence of GA1 conserved regions with 100% homology between two isolates of different

species obtained from different donors which might be a coincidence or a putative person-to-person transfer of the GA1.

The genome sequencing of the four selected isolates of donor 2 confirmed our presumption that a horizontal transfer event had conferred a GA2 transfer sweep in this community. Indeed, comparing the GA2 ICE of the four isolates revealed a 100% homology which might indicate the transfer event was relatively recent. We further hypothesized the direction of transfer for the GA2 ICE was with *B. thetaiotaomicron* (S32) as the donor to the recipient strains *P. vulgatus* (D2-S38), *B. ovatus* (D2-S40) and *B. uniformis* (D2-S48) and to our knowledge, this is the first time the route of a GA transfer could be elucidated. Further, the identification of GA2 in the species of *B. thetaiotaomicron* is for unknown reasons rare and have only been identified on one previous occasion (García-Bayona, Coyne, and Comstock 2021)

Studies of isolated *Bacteroides* strains have previously identified GA1 horizontally transferred in the community of a human donor (Coyne et al. 2014) and global screening has also revealed that the transfer of either GA1 and GA2 might not be a rare event but a rather common phenomenon among gut isolates (García-Bayona, Coyne, and Comstock 2021)

Another interesting finding that could be confirmed with the genome sequencing was the co-occurrence of a GA1 and a GA2 in the two strains *B. ovatus* (D2-S40) and *B. uniformis* (D2-S48). Global screening has identified the co-occurrence of a GA1 and a GA2 in the same strain is a rare event why an exclusion mechanism is hypothesized to be in play. In the two strains *B. ovatus* (D2-S40) and *B. uniformis* (D2-S48) it was confirmed that both the GA1 and the GA2 were contained in the expected structure of the ICE element. The GA1 of *B. uniformis* (D2-S48) was missing two of the structural components core genes TssE and TssB indicating that this T6SS might not be functional. An interesting finding of this study was that both *B. ovatus* (D2-S40) and *B. uniformis* (D2-S48) had obtained disruption of the *mghA* gene. This observation is in line with previous findings of strains carrying both a GA1 and a GA2 where it was hypothesized that a novel mechanism of integrase defense might be in play (García-Bayona, Coyne, and

Comstock 2021). This hypothesis was further based on the observation that the structure of the MghA resembled a known restriction-modification system. It is indeed intriguing that the observed exclusion mechanism of GA1 to GA2 could be controlled by a novel integrase entry regulator but more work is needed to elucidate this finding. Further, it should be noted that the targets of the toxins carried on GA1 are generally unknown (García-Bayona, Coyne, and Comstock 2021). The GA1 loci carry toxic effector linked to RHS proteins which typically act on nucleic acid targets in the cytoplasm but the targets of the GA1 toxins are yet to be determined (Koskiniemi et al. 2013).

By tagging a *B. finegoldii* donor with a tetracycline resistance gene of the GA1 ICE in a predicted null locus, we confirmed that it was possible to track horizontal transfer between strains *in vitro*. The donor succeed in transferring the ICE containing the tetracycline resistance to the two recipients species *B. fragilis* 638R and *B. ovatus* D2 and it was confirmed that the donor and the transconjugant carried the same *vgr* gene. Further work is needed to understand the horizontal transfer of GA1 and GA2 and possibly reveal more about the mechanism of exclusion. The strain collection available and the four genome sequenced strains will make a great starting point for in-depth analysis of the interesting and underexplored phenomenon of transfer and sweeps of T6SS in the human gut.

Material and Methods

Bacterial growth conditions and media

Selective Brucella Laked Blood agar (BrLA+Kan+Van) was prepared from Brucella Agar with Hemin and Vitamin K1 (Sigma-Aldrich, MO, USA) supplemented with 50 mL/L filter-sterilized defibrinated sheep blood (E&O Laboratories, UK), 50 mg/L kanamycin and 10 mg/L vancomycin (Sigma-Aldrich, MO, USA) and media used for isolation of *Bacteroidaceae* family bacteria (Sheppard, Cammarata, and Martin 1990). Isolated bacterial strains were routinely grown on Brain Heart Infusion Supplemented media (BHIS) prepared from Brain Heart infusion media (SSI Diagnostica, DK) supplemented with 50 mg/L Hemin and 50 mg/L Vitamin K1 (Sigma-Aldrich, MO, USA) and added 15g/L agar (Sigma-Aldrich, MO, USA) when used for plating. Tetracycline selective plates were prepared by adding 5µg/L tetracycline (Sigma-Aldrich, MO, USA) to BHIS media and erythromycin selective plates prepared by adding 10µg/L erythromycin (Sigma-Aldrich, MO, USA) to BHIS media. Isolates were streaked under atmospheric oxygen tension, immediately transported to anaerobic conditions and hereafter incubated under anaerobic conditions (85% N_2, 10% CO_2 and 5%H_2). Isolates were incubated at 37°C for 2-3 days if not stated otherwise. The *E. coli* strain used for transformation were grown in Luria Bertani (LB) agar or broth (SSI Diagnostica, DK) and incubated at aerobic conditions for 24 hours at 37°C. LB agar or broth was added 100µg/ml ampicillin (Sigma-Aldrich, MO, USA) when appropriate.

Isolation of *Bacteroidaceae* bacteria from human donor samples

Putative *Bacteroidaceae* strains were isolated from human fecal samples by use of BrLA+Kan+Van selective plates. Fresh human feces were sampled by use of the EasySampler Stool Collection Kit (GP Medical Devices, DK) from six healthy anonymous Danish adults and randomly labeled as donor number one to six. Approximately one gram of sample was initially homogenized in 5 mL Phosphate Buffered Saline (Sigma-Aldrich, MO, USA). The suspensions were further serially diluted in PBS and 100 µL from 10^{-4}, 10^{-5} and 10^{-6} dilutions were plated in duplicates on pre-warmed BrLA+Kan+Van plates under atmospheric oxygen tension. Immediately after plating, they were transported to anaerobic conditions and

incubated for 2 days at 37°C. After the initial incubation time, 50 colonies originating from each of the six donors were restreaked onto fresh BrLA+Kan+Van plates under anaerobic conditions bringing the total collection to 300 isolates. After the second incubation of 2 days, single colonies were inoculated into a 1 mL cryo-vial containing LB broth with 15% glycerol, vortexed and stored at -80°C until further use.

MALDI-TOF MS identification of isolates

Single isolates were identified from BHIS media after 1-2 days incubation with a Bruker MALDI-TOF MS Biotyper (Instrument ID 269944.01931) in triplicates using standard settings in server version 4.1.60 (PYTH). Identification in the high-confidence range of 2.00 - 3.00 were assigned to isolates.

Genomic DNA extraction

Based on morphological diversity a total of 52 isolates were applied for genomic DNA extraction by use of the DNeasy UltraClean Microbial DNA Isolation kit (Qiagen, Hilden, DE) according to the manufacturer's recommendation. Mechanical lysis of bacterial cells was performed at 30 cycles/sec for 10 min on a bead beater MM300 (Retsch VWR). DNA concentrations were measured by the Qubit dsDNA HF kit (Invitrogen, MA, USA) and samples diluted with nuclease-free water (Sigma-Aldrich, MO, USA) to a concentration of 5ng/µl. Bacterial DNA was used downstream for 16S rRNA gene analysis and GA1 and GA2 gene region analysis.

Isolate identification

Isolates were submitted for 16S rRNA sequencing. Genomic DNA was extracted as stated above. The 16S rRNA gene sequences were amplified in a 50 µL PCR reactions containing 10µL 5X Phusion™ HF-Buffer, 1µL dNTPs (10 mM of each oligo), 1µM universal forward primer 27F (AGA-GTT-TGA-TCM-TGG-CTC-AG), 1µM universal reverse primer 1492R (TAC-GGY-TAC-CTT-GTT-ACG-ACT-T), 1µL template DNA (5ng/µL) and 0.5 µL Phusion™ High-Fidelity DNA polymerase (Thermo Scientific, MA, USA). Reaction conditions were as follows: Initial denature 98°C for 30sec, 35 cycles of 98°C for 15s, 61°C for 15s and 72°C for 60s and lastly 72°C for 5 min before cooling to 4°C. The PCR products were purified by use of the MinElute PCR purification kit (Qiagen, Hilden, DE) and diluted to 20-80 ng/µl with

nuclease-free water. Each purified PCR product (5 µl) was mixed with 5 µl forward primer 27F (5pmol/µl) or with 5 µl revers primer 1492R (5pmol/µl) and reverse primer 1492R (5pmol/µl) and shipped to Eurofins facility (Eurofins Genomic Sequencing GMBH 51105 Köln, Germany) for Sanger-sequencing (Weisburg et al. 1991).

16S rRNA sequence analysis and alignments for generation of a phylogenetic tree

The 16S rRNA gene sequences obtained by Sanger sequencing of each isolate were quality assessed by CLC Main Workbench and trimmed to obtain only high-quality nucleotide reads. When sequencing was performed with forward and reverse primers, an assembly was created in CLC by use of regions of overlap. The sequences were searched against the National Center for Biotechnology Information (NCBI) Nucleotide database by the NCBI BlastN tools and the top match was applied as the putative taxonomic classification (Altschul et al. 1990). All sequences were trimmed to the same length before performing multiple alignments and creating a phylogenetic tree with the algorithm "Neighbour-Joining", the distance measure of "Jukes-Cantor" and the bootstrap setting of 100 replicates.

Identification of Type VI Secretion System Genetic Architectures (GA) of isolates

Primers to conserved regons of the GA1 and GA2 T6SS loci were previously described (García-Bayona, Coyne, and Comstock 2021 The GA1 conserved region was amplified in a 50 µL PCR reactions containing 10µL 5X Phusion™ HF-Buffer, 1µL dNTPs (10 mM of each oligo), 1µM forward primer oLGB19 (TCTTACCATTCGGTGAACRACCA), 1µM reverse primer oLGB20 (CGGGTATGAATACAAATCCTCTGTTTGT), 1µL template DNA (5ng/µL) and 0.5 µL Phusion™ High-Fidelity DNA polymerase (Thermo Scientific, MA, USA). Reaction conditions were as follows: Initial denature 98°C for 30sec, 35 cycles of 98°C for 10s, 57°C for 20s and 72°C for 20s and lastly 72°C for 5 min before cooling to 4°C. GA2 conserved region was amplified in 50 µL PCR reactions containing 10µL 5X Phusion™ HF-Buffer, 1µL dNTPs (10 mM of each oligo), 1µM forward primer oLGB21 (TGGGAGCAAGTTTTCTGAATTTGG), 1µM reverse primer oLGB22 (TGTTCTCCTGCGCTACATAATCGTATC, 1µL template DNA (5ng/µL) and 0.5 µL Phusion™ High-

Fidelity DNA polymerase (Thermo Scientific, MA, USA). Reaction conditions were as follows: Initial denature 98°C for 3 min, 35 cycles of 98°C for 30s, 54°C for 30s and 68°C for 50s and lastly 68°C for 5 min before cooling to 4°C. GA2 variable region was amplified in 50 µL PCR reactions containing 10µL 5X Phusion™ HF-Buffer, 1µL dNTPs (10 mM of each oligo), 1µM forward primer oLGB27 (CKTGAATTGAAYATCCATTCCAR), 1µM reverse primer oLGB28 (GATCCAGTGGATGCTGGATG), 1µL template DNA (5ng/µL) and 0.5 µL Phusion™ High-Fidelity DNA polymerase (Thermo Scientific, MA, USA). Reaction conditions were as follows: Initial denature 98°C for 30sec, 35 cycles of 98°C for 15s, 59°C for 15s and 72°C for 2min and lastly 72°C for 5 min before cooling to 4°C. The PCR products were purification by use of the MinElute PCR purification kit (Qiagen, Hilden, DE) and diluted to 20-80ng/µl with nuclease-free water. Each purified PCR product (5 µl) was mixed with 5 µl forward primers (5pmol/µl) or with 5 µl reverse primers (5pmol/µl) and shipped to Eurofins facility (Eurofins Genomic Sequencing GMBH 51105 Köln, Germany) for Sanger-sequencing.

Alignment of GA1 and GA2 conserved and variable regions for generation of phylogenetic trees

The Sanger sequences obtained of the *vgrG* of GA1 and GA2 and the variable GA2 gene region spanning between the two conserved TssD regions were quality assessed by CLC Main Workbench. All *vgrG* sequences were trimmed to the same length before performing multiple alignments and creating a phylogenetic tree with the algorithm “Neighbour-Joining”, the distance measure of “Jukes-Cantor” and the bootstrap setting of 100 replicates. Isolate *Bacteroides finegoldii* CL09T03C10 (*B. finegoldii* C10) was used as a reference for the GA1 conserved region while *Bacteroides caccae* CL03T12C61 (*B. caccae* C61) was used as a reference for GA2 conserved region (Coyne et al. 2014; Coyne, Roelofs, and Comstock 2016). GA2 variable gene regions were aligned from sequences obtained from both forward and reverse primers. The homology of the *vgrG* gene of GA1 and GA2 and references were assessed by use of Clustal Omega 2.1 multiple sequence alignment tool (version 1.2.4) and the Percent Identity Matrix applied for tables (Madeira et al. 2019).

Whole-genome sequencing of selected isolates

DNA extraction, library preparation, and sequencing were performed by the Microbiome Metagenomics Facility (MMF), University of Chicago, 900 E 57th Street, KCBD 4100 Chicago, IL 60637. Each genome was sequenced on the Illumina MiSeq platform (2 × 150 paired end reads) and also on the Nanopore platform, and a hybrid assembly was generated using the MMF facility's standard Unicycler-based assembly pipeline (Wick et al. 2017). Gene calling was performed by Prodigal (ver. 2.6.3) and annotation was performed using a custom version of Prokka 1.14.6 (Hyatt et al. 2010; Seemann 2014)
The ICE containing the T6SS system of the four isolates were annotated by an optimized version of the method previously described (Coyne, Roelofs, and Comstock 2016).

Genetic Architectures analysis

GA2 subtype classification was performed using blastn from the Blast suite (version 2.10.0, (Camacho et al. 2009)), comparing the GA2 loci to the subtype concatemers previoudly described (García-Bayona, Coyne, and Comstock 2021)**.** The homology of GA1 T6SS, GA1 ICE and GA2 ICE, respectively were assessed by use of Clustal Omega 2.1 multiple sequence alignment tool (version 1.2.4) and the Percent Identity Matrix applied for tables (Madeira et al. 2019)

Tetracycline resistance marker inserted in nul-loci of Bacteroides donor strain

The tetracycline resistance gene (*tetQ*) was amplified from *Bacteroides caccae* CL03T12C61 (*B. caccae* C61) using forward primer TCAGTCCTTTCTTGCCAGTTGAACCTAC and AAAGAAGTAACCGTATTGCCTTATAGAAATTTC. This tetQ gene was added into the B finegoldii GA1 ICE between genes HMPREF1057_01564 and 01565 by cloning the flanking DNA around the insertion site using primers AGTGGATCCCCCGGGCTGCAGCGAGGAAGTTGTATGGG and AACTGGCAAGAAAGGACTGAAACCGTTC to amplify the left flank and primers GGCAATACGGTTACTTCTTTTCACAAACCTGC and CTTGATATCGAATTCCTGCAGCCGCTATCCATACGGATAAAG to amplify the right flank. These three PCR products were ligated into the PstI site of pLGB13 (García-Bayona and Comstock 2019) using

NEBuilder. The NEBuilder reaction was transformed into E. coli S17 *λ*pir and PCR screened for the correct orientation of the inserted DNA pieces. The resulting plasmid was sequenced to confirm that is was correct and then conjugated from *E. coli* S17 *λ*pir into *B. finegoldii* by a bacterial mating assay as previously described (Salyers et al. 1999; Shoemaker et al. 1986). The next day, the mating spot was plated on gentamycin (200 µg/ml) and tetracycline (6 µg/ml) to select for cointegrates. The correct cointegrate was passaged in non-selective medium and then plated on anhydrotetracycline to select for double cross outs. Double crossouts were screened for the *ermG* gene between genes HMPREF1057_01564 and 01565 of the GA1 ICE of *B. finegoldii* CL09T03C10 (*B. finegoldii* C10-*tetQ*)

GA1 ICE transfer experiments

The transfer of the GA1 ICE from *B. finegoldii* C10-*tetQ* to *Bacteroides* recipient strains was performed as described previously with modifications (Salyers et al. 1999). Recipient strains were human donor isolates *Bacteroides ovatus* D2 and *Bacteroides fragilis* 638R ΔT6SS (Chatzidaki-Livanis 2016). ICE transfer experiments were performed as follows: *B. finegoldii* C10-*tetQ* and recipients *B. ovatus* D2 and *B. fragilis* 638R ΔT6SS were incubated in liquid BHIS under anaerobic conditions to an OD600 of 0.2 for the donor strain and *B. ovatus* D2 ΔT6SS and an OD600 of 0.05 for *B. fragilis* 638R ΔT6SS. The donor strain was mixed with the recipient strain in 1:1 ratio of 4 ml culture each and pelleted by centrifugation at 4000 g for 10 min. The pelleted cells were resuspended in 200 µl phosphate buffered saline (PBS) (Thermo Scientific, MA, USA), spotted in the center of a prewarmed BHIS plate and incubated in anaerobic conditions at 37 °C for 18 hours. Selective plating of the mating culture to select for transconjugants were the recipient strain had received the GA1 ICE was performed by restreaking quarters of mating spots on prewarmed BHIS plates containing erythromycin (10 µg/ml) and tetracycline (5 µg/ml) followed by 24 hours of incubation. Colonies were picked and genomic DNA extracted from transconjugants as stated in the above sections to confirm the recipient strains received the GA1 ICE. Amplification of the conserved *vgrG* gene was performed as stated in above sections and alignments

created using the *vgrG* gene of the donor strain *B. finegoldii* C10-*tetQ* and of the isolates *B. ovatus* (D2-S40) and *B. uniformis* (D2-S48).

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Table 1: Identification of human donor isolates and screening for resistance and conserved and variable GA regions by PCR

Strain ID	Closest BLAST hit (NCBI)	Family[1]	Query Cover	NCBI % Identity	Ery[2]	Tet[3]	Cons GA1[4]	Cons GA2[4]	Var GA2[5]
D1-S3	*Bacteroides thetaiotaomicron*	B	99%	99.40%	+		+		
D1-S12	*Parabacteroides distasonis*	T	100%	100.00%					
D1-S17	*Bacteroides thetaiotaomicron*	B	100%	100.00%			+		
D1-S19	*Bacteroides cellulosilyticus*	B	100%	99.90%					
D1-S21	*Bacteroides uniformis*	B	100%	99.90%		+			
D1-S27	*Bacteroides clarus*	B	100%	99.90%	+	+			
D1-S29	*Phocaeicola vulgatus*	B	100%	99.90%		+			
D1-S37	*Parabacteroides distasonis*	T	100%	100.00%					
D1-S39	*Bacteroides fragilis*	B	100%	100.00%			+		
D2-S2	*Bacteroides uniformis*	B	100%	99.85%			+	+	+
D2-S3	*Phocaeicola vulgatus*	B	100%	99.62%				+	NA
D2-S9	*Phocaeicola vulgatus*	B	100%	99.93%				+	+
D2-S10	*Bacteroides ovatus*	B	100%	99.69%			+	+	NA
D2-S14	*Bacteroides ovatus*	B	100%	99.71%					
D2-S19	*Bacteroides uniformis*	B	100%	100.00%			+	+	NA
D2-S22	*Bacteroides ovatus*	B	100%	99.80%					
D2-S29	*Phocaeicola dorei*	B	100%	100.00%					
D2-S32*	*Bacteroides thetaiotaomicron*	B	100%	99.92%	+			+	+
D2-S36	*Bacteroides uniformis*	B	100%	99.84%			+	+	NA
D2-S38*	*Phocaeicola vulgatus*	B	100%	100.00%				+	+
D2-S40*	*Bacteroides ovatus*	B	100%	99.78%			+	+	+
D2-S48*	*Bacteroides uniformis*	B	100%	100%			+	+	+
D2-S49	*Bacteroides ovatus*	B	99%	99.79%			+	+	+
D3-S1	*Bacteroides ovatus*	B	100%	99.45%					
D3-S3	*Phocaeicola dorei*	B	100%	99.80%		+			
D3-S4	*Parabacteroides* sp.	T	100%	99.40%					
D3-S8	*Bacteroides ovatus*	B	100%	98.63%					
D3-S12	*Odoribacter splanchnicus*	O	100%	99.40%					
D3-S17	*Phocaeicola vulgatus*	B	100%	99.55%		+			
D4-S1	*Bacteroides fragilis*	B	100%	100.00%			+		
D4-S5	*Bacteroides caccae*	B	100%	99.89%					
D4-S9	*Bacteroides fragilis*	B	100%	100.00%			+		
D4-S13	*Bacteroides fragilis*	B	100%	100.00%					
D4-S21	*Bacteroides ovatus*	B	100%	98.95%	NA	NA			
D4-S34	*Bacteroides uniformis*	B	100%	99.76%					
D4-S37	*Bacteroides eggerthii*	B	100%	99.52%					
D5-S1	*Parabacteroides* sp.	T	100%	99.89%					
D5-S5	*Phocaeicola coprocola*	B	100%	99.76%	NA	NA			
D5-S9	*Phocaeicola vulgatus*	B	100%	99.11%					
D5-S16	*Bacteroides uniformis*	B	100%	99.41%					
D5-S25	*Phocaeicola* sp.	B	100%	99.81%					
D5-S29	*Bacteroides uniformis*	B	100%	99.72%					
D5-S32	*Bacteroides uniformis*	B	100%	99.72%					
D5-S33	*Phocaeicola coprocola*	B	100%	99.65%	NA	NA			
D6-S8	*Bacteroides xylanisolvens*	B	100%	99.25%	NA	NA			
D6-S9	*Bacteroides cellulosilyticus*	B	100%	100.00%					
D6-S14	*Bacteroides fragilis*	B	100%	100.00%					
D6-S18	*Bacteroides fragilis*	B	100%	100.00%					
D6-S19	*Parabacteroides distasonis*	B	100%	97.76%					
D6-S35	*Bacteroides xylanisolvens*	B	100%	99.25%					
D6-S44	*Bacteroides xylanisolvens*	B	100%	99.50%					
D6-S47	*Bacteroides uniformis*	B	100%	99.11%					

Table 1: Identification of human donor isolates and screening for resistance and conserved and variable GA regions by PCR

[1]Bacterial family: Bacteroidaceae (B), Tannerellaceae (T) and Odoribacteraceae (O). [2]Growth on Erythromycin plates (10 µg/ml) indicated by +. [3]Growth on tetracycline plates (5 µg/ml) indicated by +. [4]PCR product of expected size for GA1 or GA2 conserved region indicated by +. [5]PCR product of expected size for GA2 variable region indicated by +. *Indicates isolates genome sequenced for this study

Figure 1

A.

Homology GA1 *vgrG*

Strain ID	C10	D1-S39	D2-S40	D2-S19	D4-S1	D1-S3
***B.finegoldii* C10**	100	87.07	89.48	89.09	89.29	89.29
D1-S39 *B.fragilis*	87.07	100	96.16	95.75	97.68	97.68
D2-S40 *B. ovatus*	89.48	96.16	100	98.99	98.59	98.59
D2-S19 *B.uniformis*	89.09	95.75	98.99	100	98.07	98.07
D4-S1 *B. fragilis*	89.29	97.68	98.59	98.07	100	100
D1-S3 *B.thetaiota*	89.29	97.68	98.59	98.07	100	100

Homology GA2 *vgrG*

Strain ID	C61	D2-S32	D2-S38	D2-S40	D2-S48
B.caccae C61	100	81.56	81.56	81.56	81.56
D2-S32 *B.thetaiota*	81.56	100	100	100	100
D2-S38 *P. vulgatus*	81.56	100	100	100	100
D2-S40 *B.ovatus*	81.56	100	100	100	100
D2-S48 *B.uniformis*	81.56	100	100	100	100

Figure 1. GA1 and GA2 obtained from isolates of the human donor collection and references

A. Phylogenetic tree by Neighbor-Joining and the distance measure of Jukes-Cantor showing the separation of the *vgrG* sequence obtained from GA1 and GA2 positive isolates. Homology of *vgrG* of GA1 and GA2, respectively, obtained by Clustal Omega 2.1 multiple sequence alignment of representative isolates indicated in the table. Grey shading indicates 100% homology of *vgrG* obtained from isolates of different species and/or donors.

Figure 2

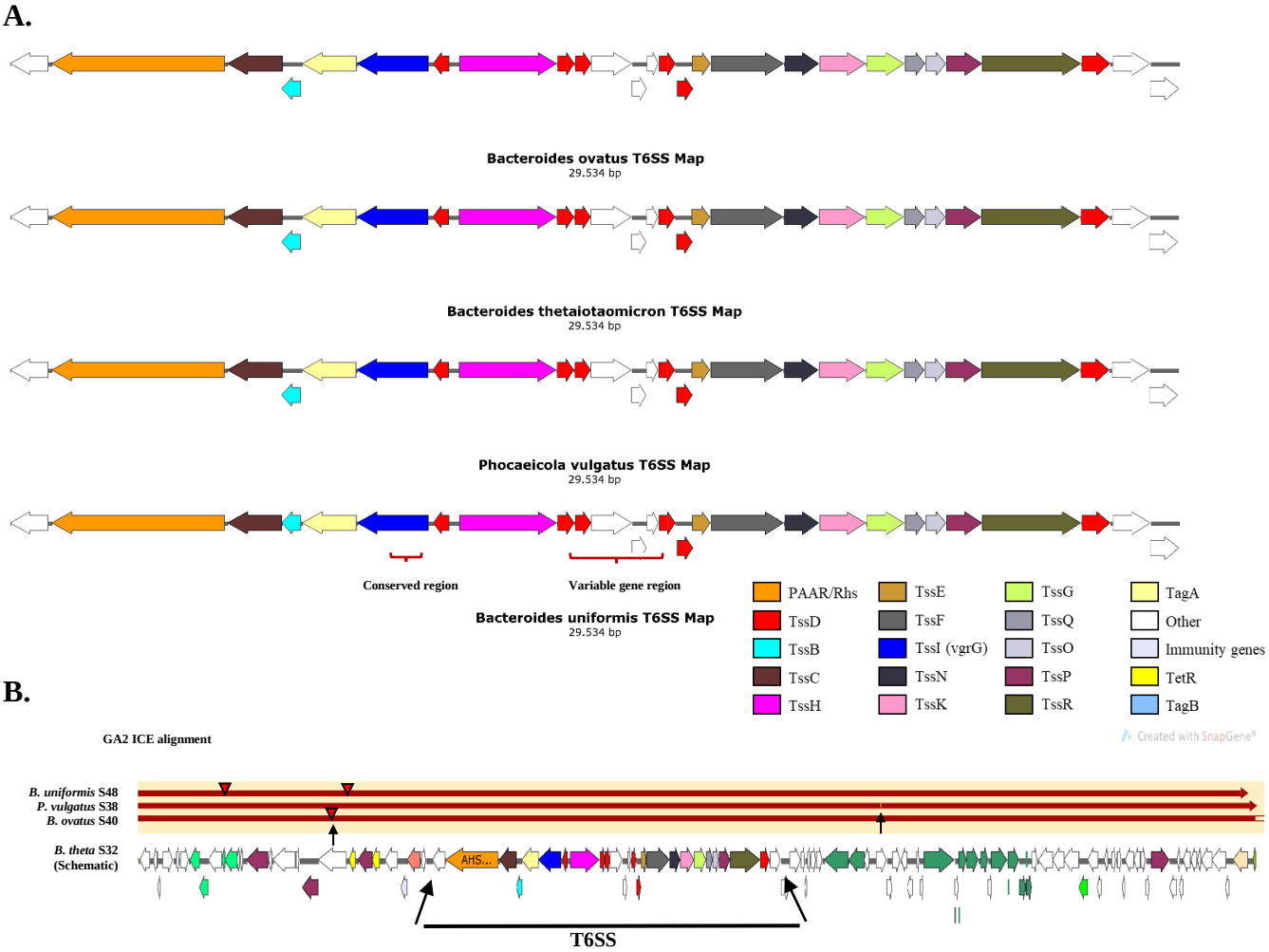


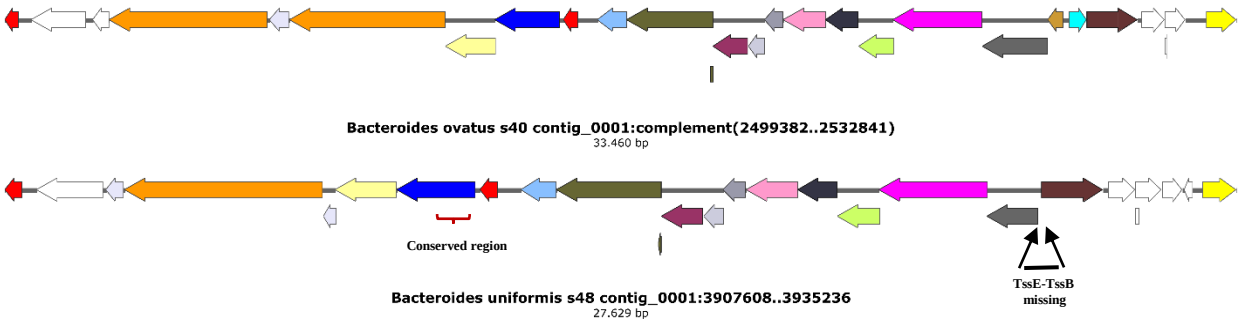
Figure 2. Schematic of GA2 T6SS obtained from the four genomes sequenced isolates and alignments of the ICE containing the GA2.

A. GA2 T6SS of the four isolates obtain the expected structure of core genes. Clustal Omega 2.1 multiple sequence alignment of GA2s shows 100% homology across the ~29.5kb sequence of the four strains. ⊔ Indicates the PCR amplified regions of conserved *vgrG* or variable genes between two TssD. Core T6SS genes are annotated according to legend.

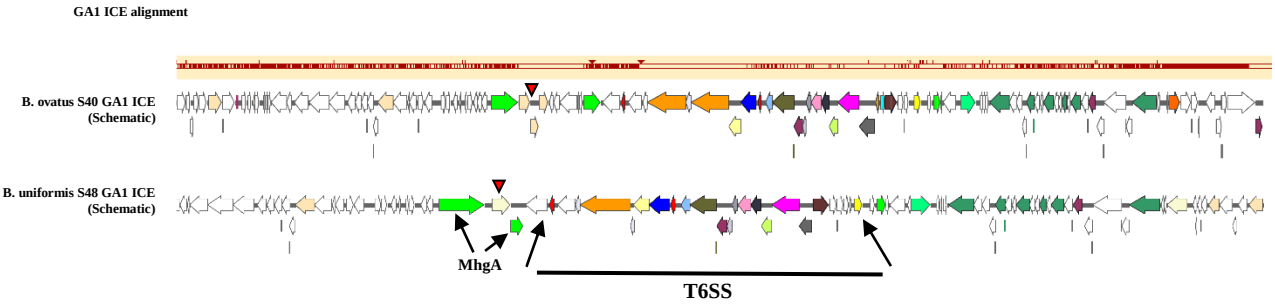
B. The ICE containing the GA2 shows 100% homology across the ~94.2kb sequence by use of Clustal Omega 2.1 multiple sequence alignment. Schematic overview of the alignments shows *B. uniformis* (S48) has obtained two insertions, *B. ovatus* (S40) has obtained one insertion followed by a deletion while *P. vulgatus* (S38) has obtained one point mutation. GA2 T6SS is indicated by angled arrows. ▼ Indicates insertion events. ↑ Indicates point mutations and deletions.

Figure 3

A.



B.



	Homology GA1 T6SS		Homology GA1 ICE	
Strain ID	**S40**	**S48**	**S40**	**S48**
S40 *B. ovatus*	100	86.4	100	92.3
S48 *B. uniformis*	86.4	100	92.3	100

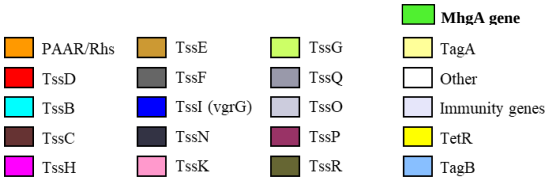


Figure 3. Schematic of GA1 T6SS obtained from the two isolates and alignments of the ICE containing the GA1.

A. GA1 T6SS of the isolates obtain the expected structure of core genes except *B.uniformis* S48 missing the TssB and TssE as indicated by angled arrows. Clustal Omega 2.1 multiple sequence alignment of GA1s shows 86.4% homology across the ~33.5kb sequence. Indicates the PCR amplified regions of conserved *vgrG*. Core T6SS genes are annotated according to legend.

B. Schematic overview of alignment of the ICE containing the GA1 of *B. uniformis* S48 and *B. ovatus* S40. Clustal Omega 2.1 multiple sequence alignment of the ICE shows 92.3% homology across the ~137.5kb sequence. GA1 T6SS is indicated by angled arrows. Indicates phage insertion events of the *MhgA* gene (light green). *B. ovatus* S40 has obtained disruption of the *MhgA* gene by three genes of a phage integrase family while *B. uniformis* S48 has obtained disruption of the *MhgA* gene by an intron.

Figure 4

A.

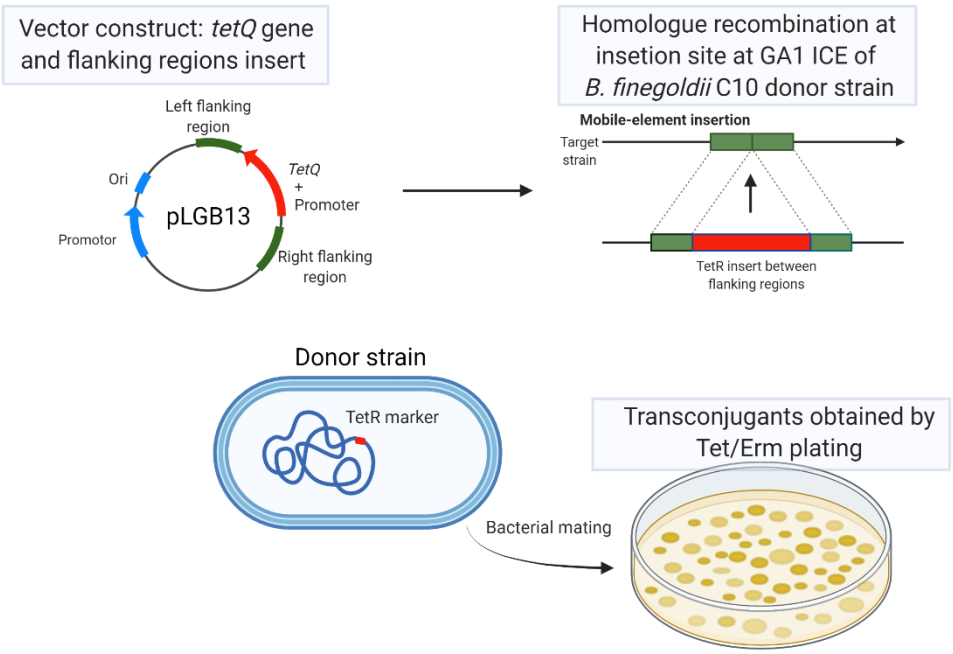


Figure 4. Vector construct for donor *B. finegoldii* C10 GA1 *tetQ* horizontal transfer

A. Tracking of a horizontal transfer event of the GA1 ICE between the donor *B. finegoldii* C10-*tetQ* and the recipients *B. ovatus* D2 ΔT6SS and *B. fragilis* 638R ΔT6SS was enabled by insertion of a tetracycline resistance gene (*tetQ)* in a null locus of the GA1 ICE of *B. finegoldii* C10. The construct is based on the pLGB13 vector and assembled with flanking regions of the donor strain GA1 ICE insertion site to allow homolog recombination of *tetQ* in the genome of *B. finegoldii* C10. The *tetQ* tagged GA1 ICE of *B. finegoldii* C10 was transferred by a bacterial mating assay to recipients and transconjugants obtained on tetracycline and erythromycin selective plates.

Figure S1: Phylogenetic tree of all donors

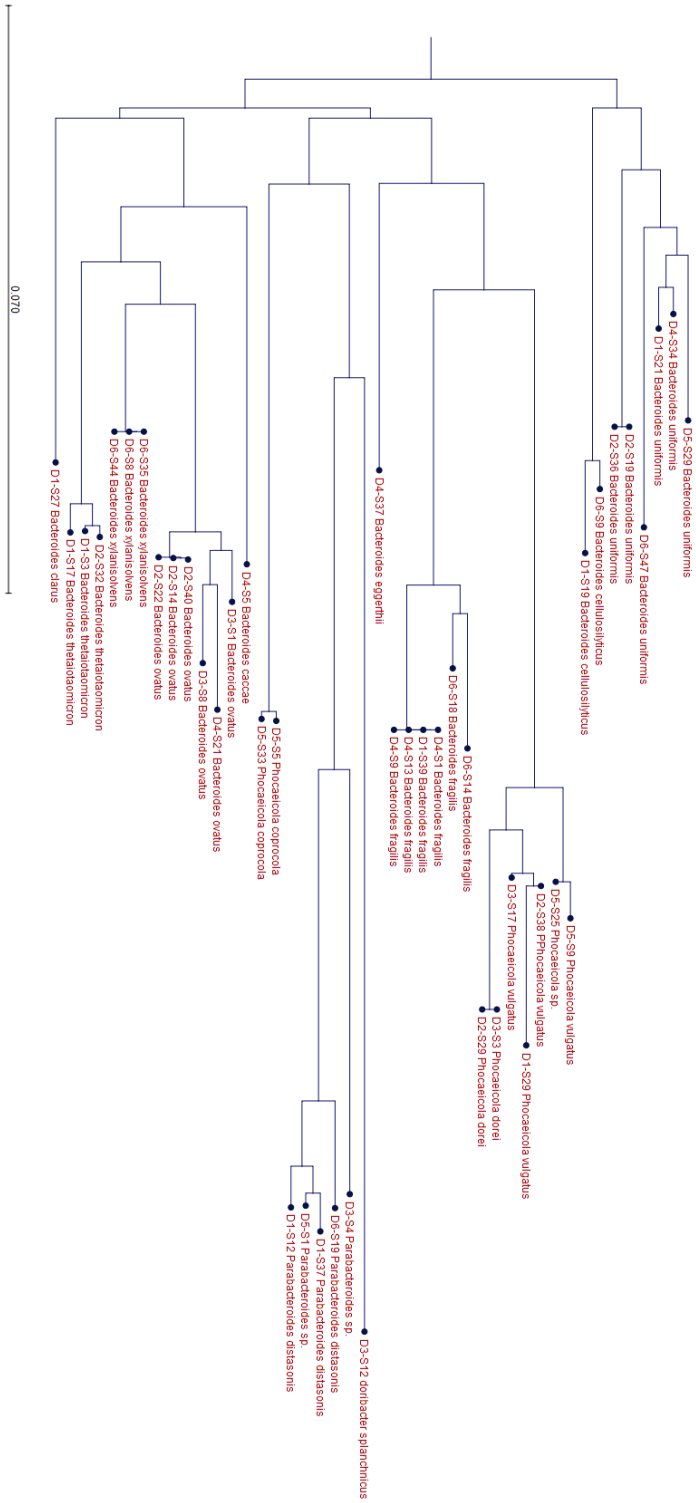


Figure S1
Partial 16s rRNA gene sequences were obtained from 52 human donor isolates. Phylogenetic tree by Neighbor-Joining and the distance measure of Jukes-Cantor show the clustering of strains within each species.

Appendix I: Animal experiments conducted during the PhD

Project name	**Exp. No.**	**Description**	**Measures**	**Data output**	**Findings**
CUP-01	19-05	Melezitose/3-fucosyllactose 5% (w/v) supplementation	Water consumption Fecal/Gut samples	Water consumed PCR of Mel/3FL genes	Melezitose/3FL supplementation (5% w/v) does not affect water consumption in NMRI mice
CUP-02	19-07	Melezitose engraftment (5%) of *E.coli* Me2.4 (+/- strep)	Fecal/Gut samples Weight	CFU counts Relative weight	Streptomycin treatment increase CFU/g of *E.coli* Me2.4 unrelated to the melezitose supplement
CUP-04	19-16	3-fucosyllactose engraftment (5%) of *E.coli* FU2.3ng or *E.coli* MUTng	Water consumption Weight Fecal/Gut samples	Relative weight Water consumed CFU counts 16s rRNA sequence	No difference of FU2.3ng and MUTng engraftment. 3FL supplementation (5% (w/v) increases relative abundance of *Bacteroidacea* and decrease *Lachnospiraceae*
HMO-01	Gut-MicroB 02-20	6'-sialyllactose, Lacto-N-tetraose or 3-fucosyllactose (5%) supplementation	Weight Fecal/Gut samples Fecal waters Colon Sections Blood	Relative weight CFU counts 16s rRNA sequence Strain collection SCFA Gene expression Histology	HMO supplementation (5% w/v) increases relative abundance of *Bacteroidaceae* and decrease butyrate producing genera

Appendix II: Animal experiment pilot studies

Figure S1

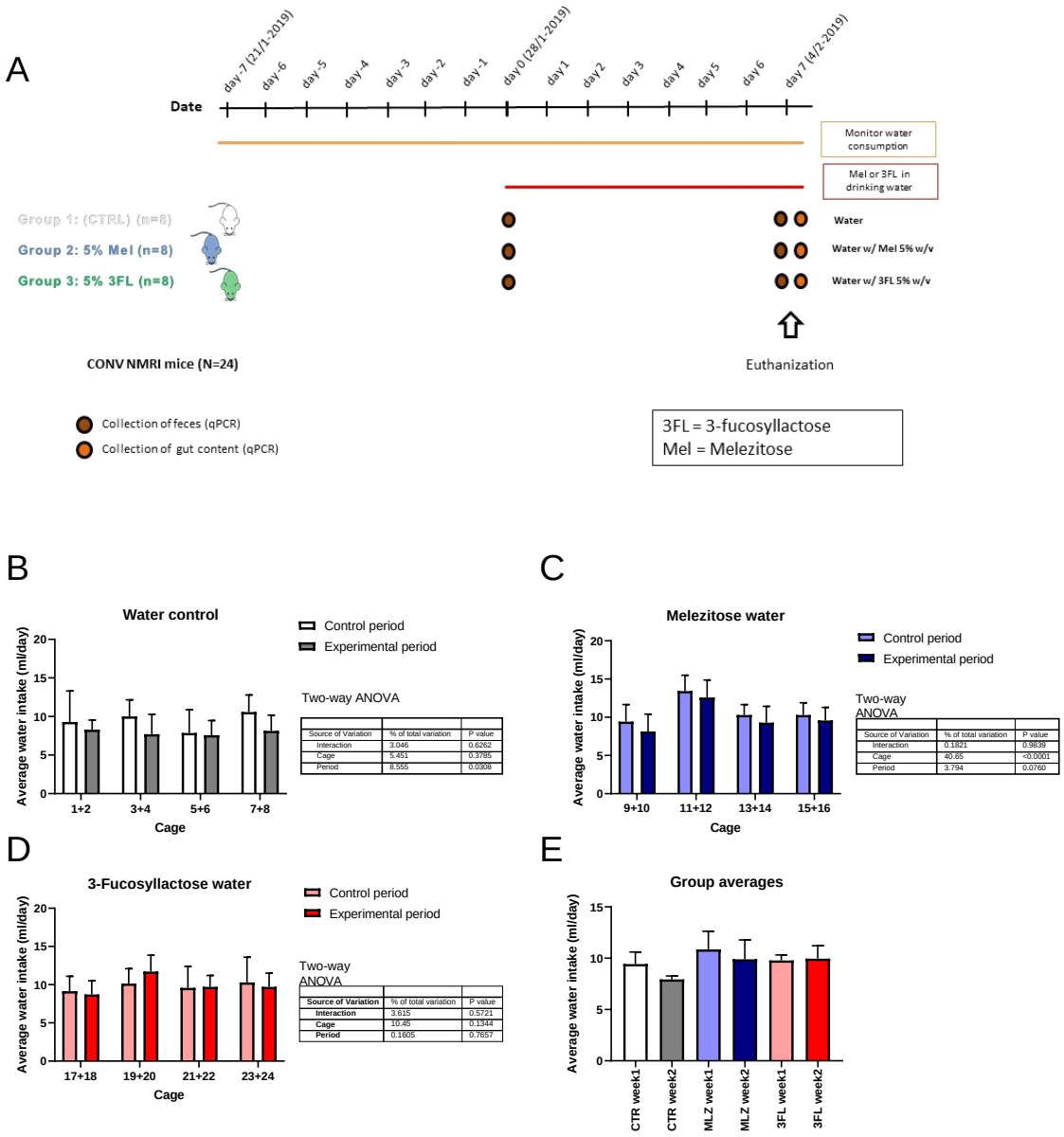


Figure S1. Experimental design of pilot animal study and water intake data for the experimental groups. (A) The water intake of three groups of 8 mice (N=24) were measured by daily weighing of animals drinking bottle per cage. After 7 days animals were supplemented with either 5% (w/v) melezitose or 5% (w/v) 3-fucosyllactose or continued on normal drinking water until euthanizing on day 14. Fecal samples were obtained on day 7 and day 14 and gut content obtained after euthanizing. (B) Water intake data for control cages shows no significant differences between the two periods. (C) Water intake data for cages of animals receiving melezitose shows no significant differences

between the two periods. (D) Water intake data for cages of animals receiving melezitose shows no significant differences between the two periods. (E) Water intake averages for the three groups across the two periods obtained no significant differences. Statistical testing performed with two way ANOVA.

Figure S2

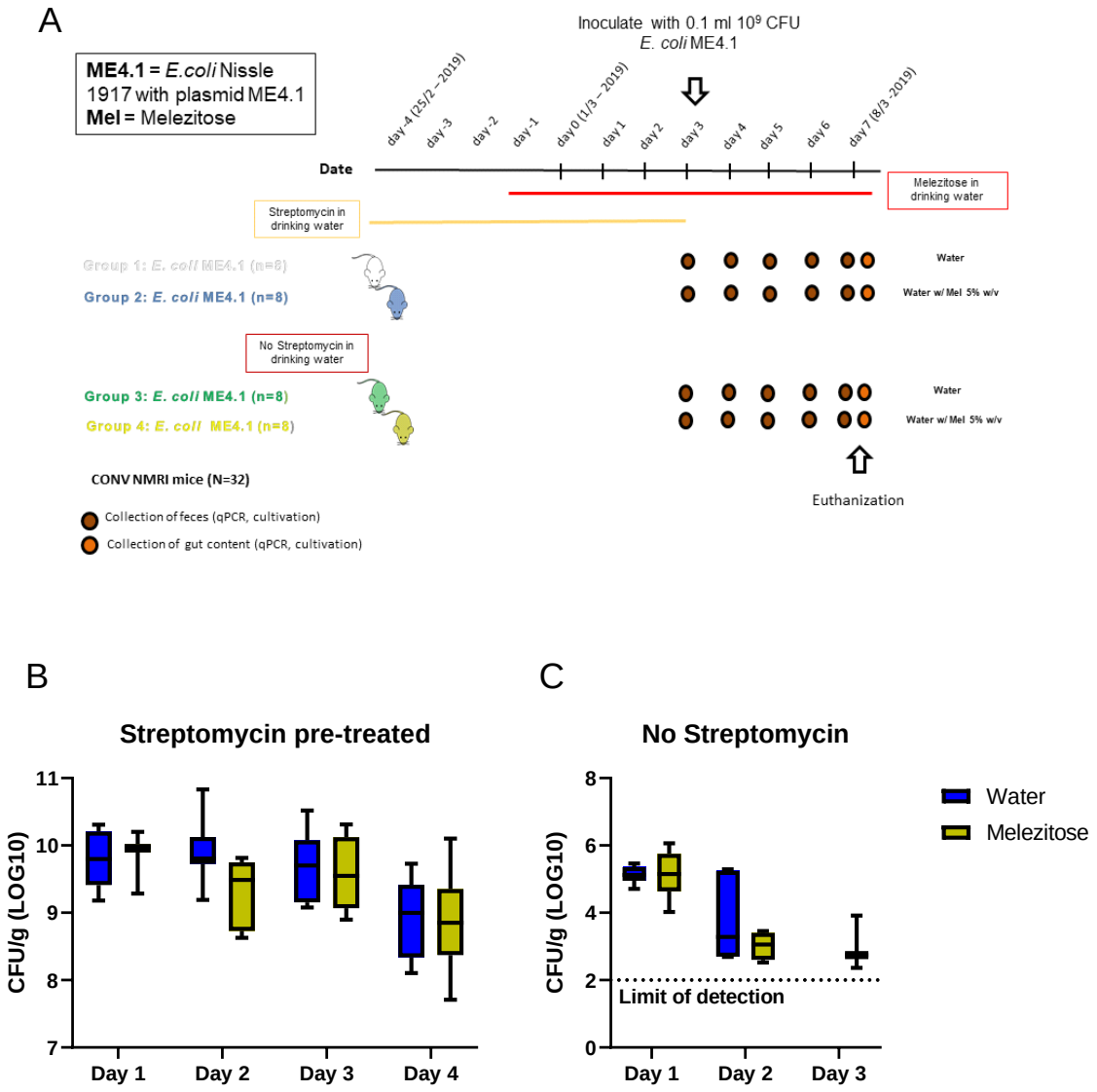


Figure S2. Experimental design of *E.coli* Nissle 1917 ME4.1 melezitose engraftment and CFU/g counts. *E.coli* was enabled to utilize the tri-saccharide melezitose by the incorporation of a gene-cluster from *Bifidobacterium breve*

and the engineered strain named *E.coli* Nissle 1917 ME4.1 (*E. coli* ME4.1) (Erejuwa, Sulaiman, and Wahab 2012). (A) Four groups of 8 mice (N=32) were inoculated with the strains *E.coli* ME4.1 by gavage on day 3 and fecal samples obtained until day 7. From day -1 animals from group 2 and 4 were supplemented with 5% (w/v) melezitose while from day -4 animals from group 1 and group 2 were treated with streptomycin in the drinking water 0.5% (w/v). At euthanization on day 7 gut content was obtained. (B) CFU/g of fecal samples obtained from streptomycin treated animals shows no significant differences between control and melezitose supplemented groups. (C) CFU/g of fecal samples obtained from animals without streptomycin treatment shows no significant differences between control and melezitose supplemented groups on day 1 and day 2 but only animals supplemented with melezitose sustain an *E.coli* ME4.1 population on day 3.

Appendix III. HMO-01 strain collection

Mouse ID	Source	Closest BLAST hit (NCBI)		Strain number	Growth curve
M12_6SL	FECES day 8	*Bacteroides*	*caccae*	12.1	
M12_6SL	FECES day 8	*Bacteroides*	*sp.*	12.2	
M14_6SL	FECES day 8	*Bacteroides*	*sp.*	14.1	
M14_6SL	FECES day 8	*Phocaeicola*	*vulgatus*	14.2	
M15_6SL	FECES day 8	*Bacteroides*	*faecichinchillae*	15.1	
M17_LNT	FECES day 8	*Bacteroides*	*sp.*	17.1	
M17_LNT	FECES day 8	*Phocaeicola*	*vulgatus*	17.2	
M32_3FL	FECES day 8	*Bacteroides*	*caccae*	32.1	
M32_3FL	FECES day 8	*Bacteroides*	*caccae*	32.2	
M33_3FL	FECES day 8	*Phocaeicola*	*vulgatus*	33.1	+
M33_LNT	FECES day 8	*Phocaeicola*	*vulgatus*	33.2	+
M12_LNT	COLON day 8	*Bacteroides*	*caecimuris*	12.1	
M15_6SL	COLON day 8	*Bacteroides*	*intestinalis*	15.2	
M21_6SL	COLON day 8	*Bacteroides*	*caecimuris*	21.1	
M21_6SL	COLON day 8	*Bacteroides*	*caecimuris*	21.2	
M31_3FL	COLON day 8	*Bacteroides*	*sp.*	31.2	
M32_3FL	COLON day 8	*Bacteroides*	*caccae*	32.2	
M25_3FL	FECES day 5	*Phocaeicola*	*vulgatus*	25.3	
M25_3FL	FECES day 5	*Phocaeicola*	*vulgatus*	25.4	+
M31_3FL	FECES day 5	*Phocaeicola*	*vulgatus*	31.4	
M34_3FL	FECES day 5	*Phocaeicola*	*vulgatus*	34.2	+
M34_3FL	FECES day 5	*Phocaeicola*	*vulgatus*	34.3	
M38_3FL	FECES day 5	*Phocaeicola*	*vulgatus*	38.1	
M40_3FL	FECES day 5	*Phocaeicola*	*vulgatus*	40.1	
M40_3FL	FECES day 5	*Phocaeicola*	*vulgatus*	40.2	+
M40_3FL	FECES day 5	*Phocaeicola*	*vulgatus*	40.3	
M40_3FL	FECES day 5	*Phocaeicola*	*vulgatus*	40.4	

Table 1. Strain collection from study HMO-01. A diverse strain collection was obtained from mice fecal and gut samples during the study HMI-01. Isolates were randomly picked after incubation of content from fecal and gut samples from the experimental groups 6SL, LNT and 3FL. Isolates were identified by partially 16s rRNA gene sequencing, searched on the NCBI database and named according to Closest BLAST hit. Strain ID is according to mouse number and number of isolate. Isolates applied for the growth curve experiments is indicated with +.