Propositions

1. The gut commensal *Akkermansia muciniphila* expands its glycolytic repertoire to human milk oligosaccharides utilization.
   (this thesis)
2. Mucolytic activity is essential for *A. muciniphila* to survive in the complex gut ecosystem.
   (this thesis)
3. Human gut microbiome is considered as "the invisible organ" (Li, et al. 2020), however its contribution to human's health is not acknowledged the same as the visible ones.
4. Science is always playing with your sanity.
5. As a PhD student multi-tasking can be your best friend but also your worst enemy.
6. Humans should use learnings from bacterial collaborations in complex ecosystems to make this planet a better place.

Belonging to the thesis entitled:

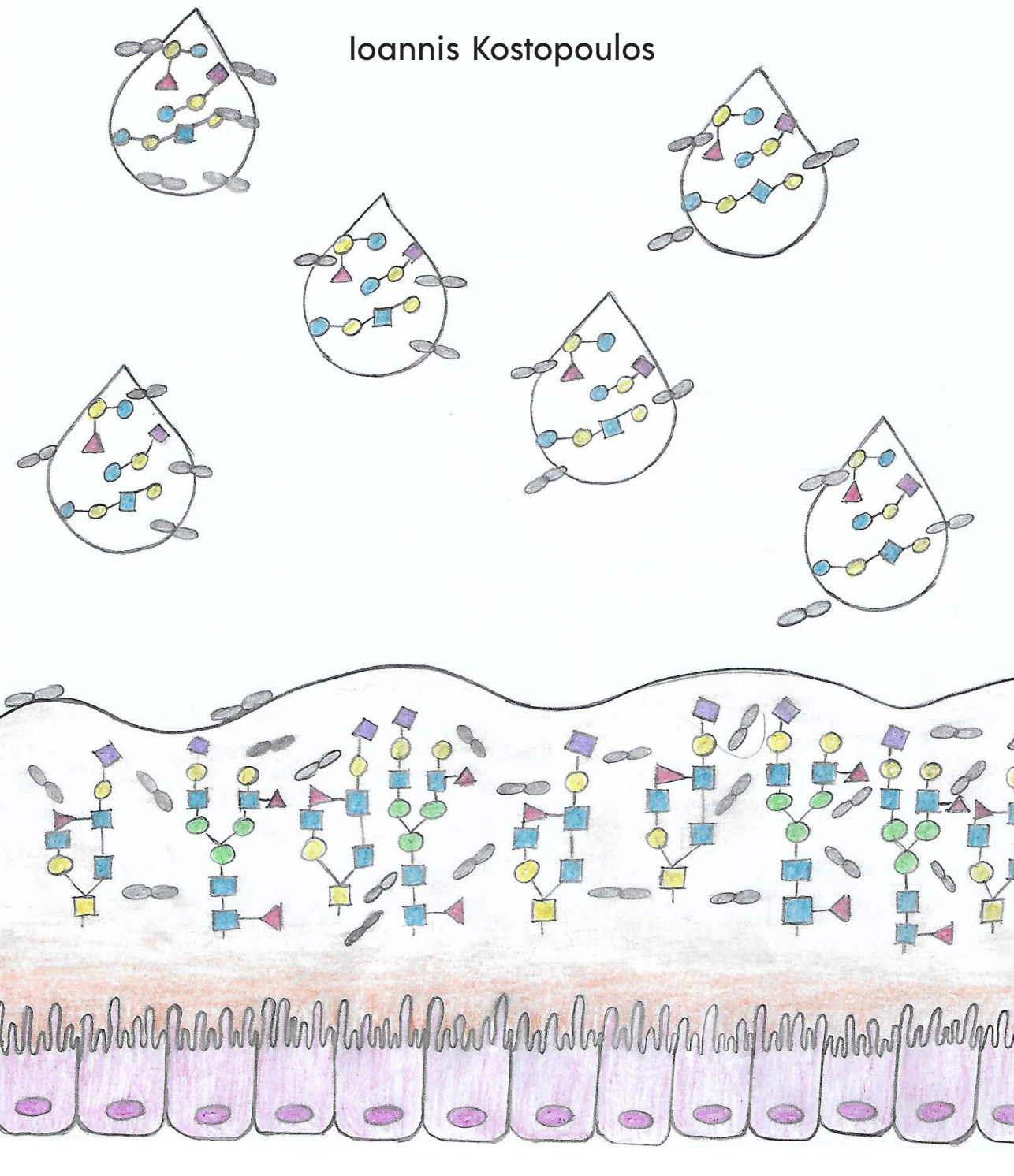
"Mucin and Human milk oligosaccharides utilization: a strategy of *Akkermansia muciniphila* to ensure survival in the human gut"

Ioannis Kostopoulos

Wageningen, January 8th 2020

Mucin and Human Milk Oligosaccharides utilization: a strategy of *Akkermansia muciniphila* to ensure survival in the human gut

Ioannis Kostopoulos



Mucin and Human Milk Oligosaccharides utilization: a strategy of *Akkermansia muciniphila* to ensure survival in the human gut

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Mucin and Human Milk Oligosaccharides utilization: a strategy of *Akkermansia muciniphila* to ensure survival in the human gut

Ioannis Kostopoulos

Thesis

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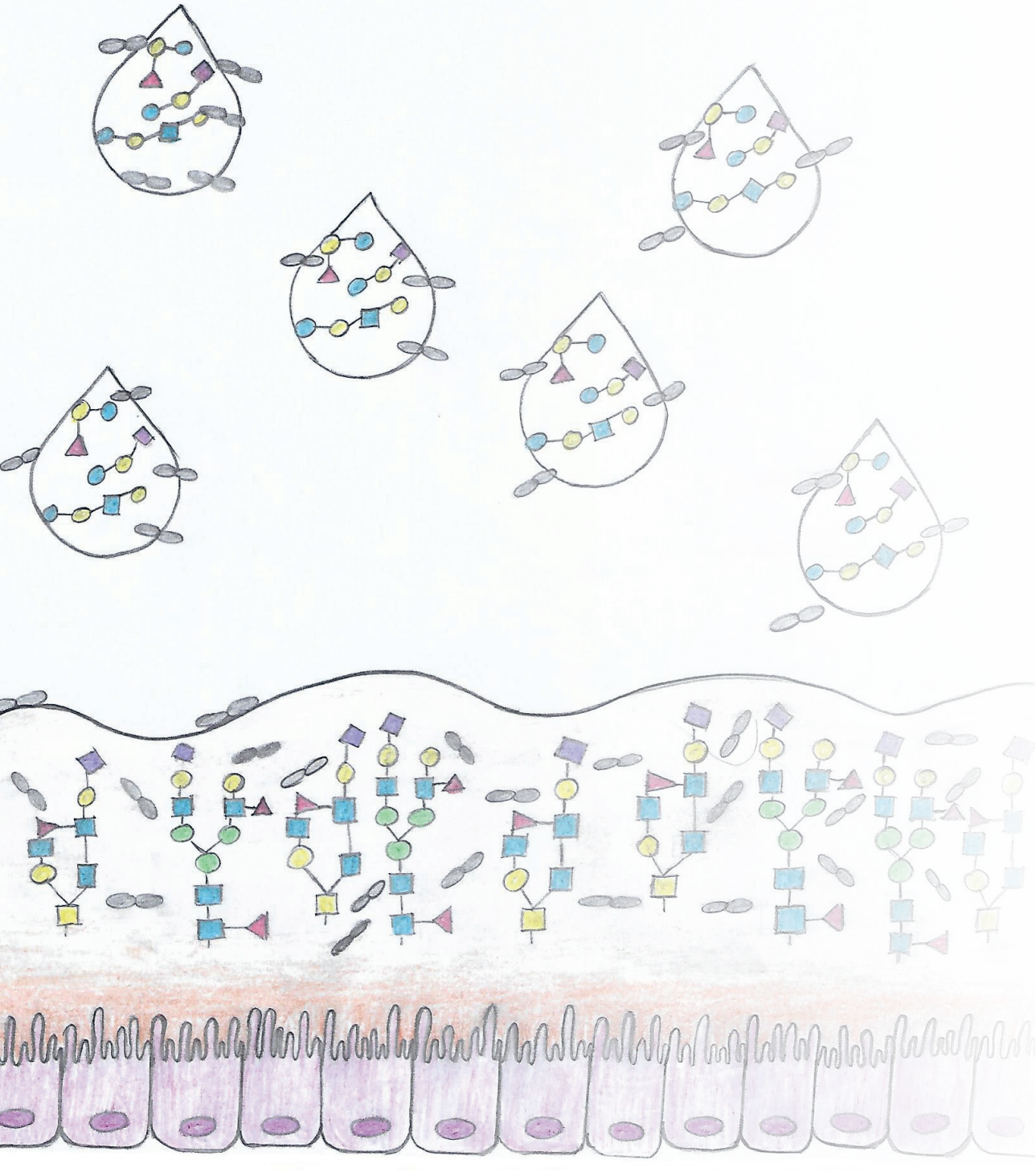
Table of contents

Chapter 1	General introduction and Thesis outline	7
Chapter 2	*Akkermansia muciniphila* in the Human Gastrointestinal Tract: When, Where and How?	31
Chapter 3	*Akkermansia muciniphila* used Human Milk Oligosaccharides to thrive in the early life conditions *in vitro*	63
Chapter 4	A continuous battle for host-derived glycans in the gut between a mucus specialist and a glycan generalist *in vitro* and *in vivo*	101
Chapter 5	Minimalist Approach for Deciphering the Ecophysiology of Human Gut Microbes	131
Chapter 6	The main functions of key species *Akkermansia muciniphila* remain stable across different microbial ecosystems	177
Chapter 7	General discussion	199
	Thesis Summary	213
	Nederlandse Samenvatting	219
	References	225
	Appendices	287
	Co-author affiliations	288
	Acknowledgments	289
	About the author	299
	List of Publications	300
	Overview of completed training activities	301



Chapter 1

General introduction and Thesis outline

General Introduction

This thesis describes the ability of *Akkermansia muciniphila* to survive in competitive ecological environments through its capacity to utilize and degrade host-derived glycans, such as mucins and Human Milk Oligosaccharides (HMOs). Furthermore, this thesis examines *A.* muciniphila's capacity to survive in competitive environments by degrading mucin glycans. In this thesis, the microbe-to-microbe interaction between *A. muciniphila* and other glycan-degrading bacteria is also discussed. This bacterium is the focus of this thesis. *A. muciniphila* is a mucin-degrading colonizer of the gastrointestinal (GI) tract and was first isolated at the Laboratory of Microbiology at Wageningen University (Derrien et al. 2004). *Akkermansia*'s name is named after Dr. Antoon Akkermans, who led the Microbial Ecology group of the Laboratory of Microbiology at Wageningen University at the time that *A. muciniphila* was isolated (Derrien et al. 2004).

The establishment of the gut microbiota

The human gut harbours a complex and diverse bacterial community known as the gut microbiota (Dethlefsen, McFall-Ngai, and Relman 2007), which plays an important role in the balance between health and disease. The intestinal microbiota consists of approximately 500 to 1000 species that belong to only few of the known bacteria phyla. The most abundant phyla in the human gut are Firmicutes and Bacteroidetes, but also include members of the phyla Proteobacteria, Verrucomicrobia, Actinobacteria, Fusobacteria, and Cyanobacteria (Huttenhower et al. 2012; Qin et al. 2010). The establishment of the human gut microbiota starts at birth, when microbes rapidly colonize the infant gut, although the composition and diversity only stabilize after seven years in humans (Bäckhed et al. 2015; Yatsunenko et al. 2012). The microbiota composition is influenced by numerous factors such as delivery mode, administration of antibiotics to the infant or the mother, mode of feeding (breastfeeding and/or formula) and the intake of dietary fibre (Bergström et al. 2014). The development of the microbiota in early life is important, as this period is critical in shaping the host's long-term metabolic, immunological and neurological development (Kundu et al. 2017; Thompson 2012). The mode of delivery has a significant effect on the development of the microbial ecosystem in neonates (Van den Abbeele et al. 2019). Initial microbial exposure during vaginal delivery predominately originates from the maternal

microbiota, while exposure upon caesarean delivery is related to the environment (Lennox-King et al. 1976a, 1976b). The desirable transmission of vaginal and gut microbiota from mother to infant is observed in vaginally delivered infants during birth, but this is not the case in caesarean-delivered infants (Dominguez-Bello et al. 2016). In general, *Escherichia coli* and *Streptococcus* spp. are the first bacterial species to colonize the gastrointestinal tract of neonates. These facultative anaerobes create an optimal environment for the subsequent colonization by obligate anaerobes such as *Bacteroides* spp., *Bifidobacterium* spp., and *Clostridium* spp.

Apart from the delivery mode, another factor that contributes significantly to the development of the gut microflora is the intake of dietary fibre (glycans) into the intestine, mostly from diet and host mucosal secretions (Koropatkin, Cameron, and Martens 2012). The dietary fibre passes undigested to the human intestine because of the lack of hydrolytic enzymes in the human genome (Koropatkin, Cameron, and Martens 2012). Gut bacteria utilise and transform these indigestible glycans into short-chain fatty acids (SCFAs), which serve as nutrients for colonocytes and other gut epithelial cells (Koropatkin, Cameron, and Martens 2012). Human intestinal microbiota, therefore, plays a significant symbiotic role in helping humans access and utilise the indigestible dietary fibre. Glycan-degrading bacteria in the intestine have various glycan preferences. Therefore, selective consumption of these nutrients can influence the composition and balance of the gut microbiota (Koropatkin, Cameron, and Martens 2012).

Switching from HMOs to mucin glycans

The acquisition of a stable intestinal microbial community during the first months of life constitutes a critical developmental window (L. M. Cox et al. 2014). Distinct faecal microbial compositions differ between infants fed with human milk and those fed with formula. The microbiota of breastfed infants, for example, is characterised by high levels of *Actinobacteria* (mainly bifidobacteria) and low microbial diversity (Koropatkin, Cameron, and Martens 2012). Mother's milk is the only source of nutrients and dietary glycans for breastfed infants, and it is considered the best nourishment for the development of the new-born (Neville et al. 2012). The glycans in human milk are known as HMOs, and they have proven to influence infant intestinal microbiota composition (Koropatkin, Cameron, and Martens 2012). Human milk contains 5-15 g/L HMOs, with more than 200 different HMO structures reported, of

which 100 have been successfully described (Ninonuevo et al. 2006; Ruiz-Palacios et al. 2003; Stahl et al. 1994; Urashima et al. 2018). The presence and quantity of these HMO structures vary per individual and are related to the genetic Secretor and Lewis status of the mother (Ayechu-Muruzabal et al. 2018). The major building blocks of monosaccharides present in HMOs are D-glucose (Glc), D-galactose (Gal), *N*-acetyl-glucosamine (GlcNAc), L-fucose (Fuc), and *N*-acetylneuraminic acid (sialic acid, Neu5Ac) (Zivkovic et al. 2011). These sugars form several complex glycans that contain well-defined different glycosidic linkages that result in linear and branched structures (S. Wu et al. 2010). In human milk, 70% of the oligosaccharides are fucosylated and 30% are sialylated (Ninonuevo et al. 2006; Weiss and Hennet 2012). HMOs can function as prebiotic substrates by promoting and stimulating the growth of beneficial bacteria such as bifidobacteria (Bode 2012). Supplementation of infant formulae with HMOs, such as 2'-FL and LNnT, is gaining increasing interest to mimic human milk's composition (Vandenplas et al. 2018).

The colonic epithelial cell surface is covered by an inner mucus layer that firmly adheres to the cells, virtually free of bacteria, and a loose layer that consists predominantly of mucin glycoproteins that provide a nutrient-rich habitat for the microbiota (Johansson, Holmén Larsson, and Hansson 2011; Johansson et al. 2008, 2014). Secreted mucins are the main structural components of the mucus gel (Tailford, Crost, et al. 2015). Along the GI tract, synthesis and secretion of these polymeric glycoproteins occur in the goblet cells of the small intestine and colon, or the surface mucus cells of the stomach (Moncada, Kammanadiminti, and Chadee 2003). MUC2 is the best characterized secreted mucin of the GI tract (H. Nilsson et al. 2014). These endogenous glycans provide consistent sources of nutrients to the intestinal microbiota, despite significant changes in the host diet (Koropatkin, Cameron, and Martens 2012). Host-secreted glycans are presented to the commensal bacteria in the intestinal lumen as *O*-linked glycans attached to secreted or cell-associated mucin glycoproteins or as *N*-linked glycans present in shed epithelial cells. A proportion of the endogenous glycans is found in the protective mucus layer (Koropatkin, Cameron, and Martens 2012). Certain microorganisms penetrate and degrade mucus, which then provides a nutrient source (Tailford, Crost, et al. 2015). During the dysbiosis state in the intestine, the species that can use these host-secreted glycans may exert a disproportionate effect on colonic health (Koropatkin, Cameron, and Martens 2012).

Microbe to microbe interactions within the human gut microbiota and mucosal layer

In a complex environment such as the human gut microbiome, the search for nutrients is crucial for the survival of the gut residents (Bauer et al. 2018). Bacterial communities exist under continuous competition and the collaborative efforts of the bacteria in the gut. Many different types of interactions take place within a complex microbial ecologic network (Hibbing et al. 2010; Case and Gilpin 1974). Two mechanisms of competition exist: the exploitative competition, in which members compete for shared nutrients, and the interference competition, in which a member directly attempts to harm a competitor, often through the production of an antimicrobial molecule (Ghoul and Mitri 2016; Roelofs et al. 2016). Moreover, microbial communities contain symbiotic and cross-feeding relationships (Seth and Taga 2014). For example, when *B. thetaiotaomicron* colonized the distal guts of adult germ-free mice maintained on a diet rich in plant glycans, it increases the gene expression involved in the catabolism of dietary substrates (Sonnenburg 2005). In contrast, in adult mice fed a diet devoid of complex glycans, *B. thetaiotaomicron* alters its response to express genes involved in targeting host glycans (Desai et al. 2016). A recent example of such a model used rats colonized with seven bacterial species from the human gut to investigate how the microbiota composition changes in response to dietary challenges (Becker et al. 2011). It was shown that the bacterial community declined during feeding with a fibre-free diet compared to the fibre-rich standard chow diet. Colonized mice with a defined community of human bacteria were used to investigate microbe-microbe interactions (Mcnulty et al. 2013; Rey et al. 2013) or the interactions between microbiota, dietary fibre, and the colonic mucus barrier (Desai et al. 2016). A recent study with simplified intestinal microbiota and changes in dietary fibre intake demonstrated that these dietary changes affect the Carbohydrate-Active Enzyme (CAZy) genes in the community as well as the host's metabolism (Kovatcheva-Datchary et al. 2019). The simplest method to study gut bacteria is through the monocultivation of pure strains. This technique is often used to assess the influence of environmental gut conditions in single species (Ouwerkerk, van der Ark, et al. 2016; Turroni et al. 2014). Furthermore, co-culture has been used to study interactions such as cross-feeding or competition (Chia et al. 2018) (Egan et al. 2014; Falony et al. 2006). However, recently, the use of defined synthetic communities was developed to study the gut microbiota in a defined but more complex

ecosystem. This was done conducted in bioreactors and mouse models (Desai et al. 2016; Kovatcheva-Datchary et al. 2019; Oliphant et al. 2019).

Microbial metabolism of host-derived glycans and dietary fibers degradation in the gut

Within the gut microbiota community, several species can utilize host-derived glycans and different dietary fibres that pass undigested to the human intestine (Tailford, Crost, et al. 2015). To date, several bacterial species/strains from the Bacteroidetes, Firmicutes, Actinobacteria, and Verrucomicrobia phyla have been studied for their ability to consume host-derived glycans (Tailford, Crost, et al. 2015).

For example, the ability of the *Bacteroides* species to utilise diverse glycans depends on a series of gene clusters known as Polysaccharide Utilisation Loci (PULs) (Bjursell, Martens, and Gordon 2006). PULs encode cell envelope systems that typically include glycolytic enzymes and homologs of two outer membrane proteins (SusC and SusD) that are part of the first described PUL, the starch utilisation system (Sus) locus (Martens et al. 2009). One well-studied glycan-degrading bacterium is *Bacteroides thetaiotaomicron* VPI-5482, which possesses 88 PULs and 221 mucin-degrading enzymes (Martens, Chiang, and Gordon 2008). *B. thetaiotaomicron* is a prominent member of the human gut microbiota capable of growing on many different plant and host glycans (Salyers et al. 1977; Martens, Chiang, and Gordon 2008). *B. thetaiotaomicron* VPI- 5482 can grow on different fractions of glycans purified from pig gastric mucosa, including an *O*-glycan-rich fraction (Martens, Chiang, and Gordon 2008). Transcriptomic analyses highlighted specific polysaccharide-utilization loci (PULs), including genes coding for putative glycoside hydrolases (GHs) such as α-L-fucosidase, endo-β-N-acetylglucosaminidase, endo-β-galactosidase and α-mannosidase, which were upregulated when *B. thetaiotaomicron* was grown on mucin O-glycans or in monoxenic mice as compared to *in vitro* glucose control (Martens, Chiang, and Gordon 2008; Martens et al. 2011). Interestingly, these PULs were not upregulated when *B. thetaiotaomicron* was grown on glycosaminoglycans (GAGs), as compared to glucose (Martens, Chiang, and Gordon 2008; Martens et al. 2011). Colonization competition experiments demonstrated that *B. thetaiotaomicron* mutants for O-glycan PULs were able to colonize germ-free mice in a similar way to the wild-type strain when mice were fed a plant glycan-rich diet, but were outcompeted by the wild-type on a simple-sugar diet (Martens, Chiang, and Gordon 2008). *B.*

thetaiotaomicron induces its mucus-utilisation genes to also consume human milk oligosaccharides (Marcobal et al. 2011). Additionally, *Bacteroides fragilis* was able to grow on mucin as its sole carbon source (Salyers et al. 1977; Robertont and Stanley 1982) and it contains a subset of PULs dedicated to host mucin O-glycan utilization (Marcobal et al. 2011). Furthermore, *Ruminococcus torques* strains (ATCC 35915 and VIII-239) and *Ruminococcus gnavus* ATCC 35913, from the Firmicutes phylum, have been shown to degrade mucin (Hoskins et al. 1985). It has been also confirmed that both *R. gnavus* and *R. torques* species were able to degrade and utilize human MUC2 as a sole carbon source, which provides further evidence of their adaptation to the human colonic mucosal environment (Png et al. 2010). Several enzymatic activities were detected in the spent media of these mucin-grown strains that could explain their ability to degrade mucin (Hoskins et al. 1985; Corfield et al. 1992; Crost et al. 2013). Recently, it was shown that *B. fragilis* encodes a specific sialidase that allows potential pathogens to degrade sialomucin in a dedicated way (Tailford, Owen, et al. 2015). Also, *Bifidobacterium bifidum* ATCC 35914 and *Bifidobacterium longum subsp. infantis* VIII-240, from the Actinobacteria phylum, were capable of growing on host-derived glycans (Hoskins et al. 1985; Png et al. 2010; Turroni et al. 2010).

In the Verrucomicrobia phylum, *Akkermansia muciniphila*, a strictly anaerobic Gram-negative bacterial species, was identified as a key mucin degrader. *A. muciniphila* can utilize mucins' sole carbon and nitrogen source (Derrien et al. 2004). *A. muciniphila* has since been shown to be a common member of the human gut with a high prevalence and variable abundance, present both in faeces and at the mucosal surface (Eckburg et al. 2005; Collado et al. 2007; Derrien et al. 2008). The capacity of *A. muciniphila* to utilize host-derived glycans is discussed later in this chapter.

Microbial glycan degrading enzymes and their functions

The saccharolytic bacteria express glycan-utilizing bacteria, which are known as carbohydrate-active enzymes (CAZymes). The CAZymes include glycoside hydrolases (GHs) and polysaccharide lyases (PL), which are required to fully degrade mucins (McKeen et al. 2019). Genomic and metagenomic sequencing has greatly expanded the number of predicted microbial enzymes that degrade polysaccharides (Cantarel et al. 2009). These GHs hydrolyse the glycosidic linkages between carbohydrates to breakdown glycans into fermentative monosaccharides (Koropatkin, Cameron, and Martens 2012). The GHs are classified into families according to their hydrolytic

potential (Naumoff 2011). To date, 168 glycoside hydrolases families (GH1 – GH168) are known (Lombard et al. 2014). The mucin-degrading GHs include exo-α-sialidases (GH33), α-fucosidases (GH29 and GH95), exo- and endo-β-N-acetylglucosaminidases (GH84 and GH85), β-galactosidases (GH2 and GH42), β-hexosaminidases (GH20), α-N-acetylglucosaminidases (GH89) and α-N-acetylgalactosaminidases (GH101, GH129) (Lombard et al. 2014) (Figure 1). These enzymes are typically exo-acting enzymes, meaning that they release monosaccharides from the non-reducing terminus of the glycan. They often secrete cell-surface associated GHs likely to facilitate the liberation of terminal monosaccharides from the massive mucin molecules (Marcobal et al. 2013). GH33 sialidases release the α2-3/6 linked sialic acid to galactose that can be found in mucin and HMOs (Juge, Tailford, and Owen 2016; Tailford, Crost, et al. 2015; Tailford, Owen, et al. 2015). Furthermore, in host-derived glycans, fucose units can be found in *O*-glycans linked to galactose by α1-2 linkages or to GlcNAc by α1-3/4 linkages. It is also common that fucose is linked by an α1-6 bond to terminal β-GlcNAc of *N*-linked glycans. α-fucosidases from families GH29 and GH95 are employed by the commensal to liberate the terminal fucose from the fucosylated substrates (Tailford, Crost, et al. 2015). The production of bacterial α-N-acetylgalactosaminidases allows the hydrolysis of the linked β1-3/4-N-acetylgalactosamine (GalNAc) (GH101, GH129) and serine/threonine in mucin peptide backbone (Ruas-Madiedo et al. 2008). Furthermore, the presence of N-acetyl-β-hexosaminidases (GH20), β-galactosidases (GH2 and GH42) indicate hydrolyzation of β1-4/6 linked GlcNAc to galactose and GalNAc, and β1-3/4 linked galactose to GalNAc and GlcNAc, respectively.

The characterization of bacterial GHs will help to elucidate the glycan-utilization strategies of gut bacteria and the way that we can use their function to promote the growth of beneficial species in the gut.

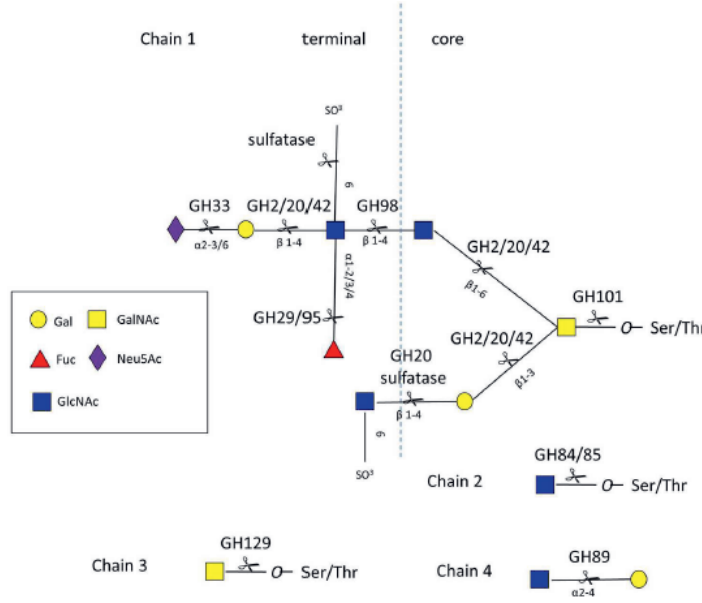


Figure 1:*O*-glycan chains showing sited of action of GHs and sulfatases. Image adapted from Tailford et al. 2015.

Akkermansia muciniphila in the gut

A. muciniphila is a Gram-negative bacterium and the only representative of the phylum Verrucomicrobia that colonizes the mucus layer of the human gastrointestinal (GI) tract (Derrien et al. 2008). While *A. muciniphila* is described as a strictly anaerobic bacterium (Derrien et al. 2004), it has been discovered that it is can tolerate oxygen exposure in the mucosal layer as well low levels of oxygen (Espey 2013; Reunanen et al. 2015). Recently, *A. muciniphila* was cultured in a growth experiment with low oxygen levels, which demonstrates a complex transcriptional response to oxygen (Ouwerkerk, van der Ark, et al. 2016). *A. muciniphila* is one of the most abundant species, and its abundance ranges from 0.5-5% (Cani and de Vos 2017). The genome of *A. muciniphila* is composed of one circular chromosome of 2.7 Mbp, which codes for 2,176 protein-coding genes, of which 65% are assigned a putative function (van Passel et al. 2011). *A. muciniphila* is a common member of the adult and infant microbiota (Derrien et al. 2008). The infant's gut colonisation with *A. muciniphila* has been detected from the first month of life, with a continuously increasing abundance during adulthood (Derrien et al. 2008; Collado et al. 2007). Additionally, *A. muciniphila* was detected in the breast tissue of lactating mothers and in human milk

(Collado et al. 2012; Aakko et al. 2017; Urbaniak et al. 2014). Interestingly, two studies reported lower numbers of *A. muciniphila* in breast-fed infants compared to formula-fed infants (Azad et al. 2013; Bergström et al. 2014). In a more recent study with 98 Swedish infants, though, the abundance and the prevalence of *A. muciniphila* increased between 4 and 12 months old and showed no significant difference related to delivery mode or type of feeding (Bäckhed et al. 2015). In the human gut, *A. muciniphila* has the extraordinary capacity to degrade mucin as the sole energy, carbon and nitrogen source and convert this polymer into mostly acetate and propionate (Derrien et al. 2004).

Glycolytic machinery of *A. muciniphila*

A. muciniphila contains 61 mucin-degrading enzymes that consist of sulfatases, proteases and glycoside hydrolases (GH) for the effective metabolism of mucin glycans, such as β-galactosidases, β-*N*-acetylhexosaminidases, exo-α-sialidases, α-L-fucosidases, and α-*N*-acetylglucosaminidases (Derrien et al. 2010; Ottman, Davids, et al. 2017) (Table 1).

Table 1: Mucin-degrading enzymes encoded in *A. muciniphila*'s genome. Updated from Derrien et al. 2010)

Locus_tag	Protein name	CAZy
Amuc_0010	alpha-L-fucosidase	GH29
Amuc_0060	alpha-N-acetylglucosaminidase	GH89
Amuc_0146	alpha-L-fucosidase	GH29
Amuc_0186	glycoside hydrolase family protein 95	GH95
Amuc_0290	glycoside hydrolase family 2 sugar binding	GH2
Amuc_0369	beta-N-acetylhexosaminidase	GH20
Amuc_0392	coagulation factor 5/8 type domain-containing protein	GH29
Amuc_0397	beta-N-acetylhexosaminidase	GH20
Amuc_0539	glycoside hydrolase family 2 sugar binding	GH2
Amuc_0623	glycosyl-hydrolase BNR repeat-containing protein	GHnc
Amuc_0625	exo-alpha-sialidase	GH33
Amuc_0697	beta-glucanase	GH43
Amuc_0698	beta-glucanase	GH43
Amuc_0771	beta-galactosidase	GH35
Amuc_0824	glycoside hydrolase family 2	GH2
Amuc_0846	coagulation factor 5/8 type domain-containing protein	GH29
Amuc_0863	glycosyl hydrolase family protein 88	GH105
Amuc_0868	beta-N-acetylhexosaminidase	GH20
Amuc_0875	beta-glucanase	GH16
Amuc_1008	alpha-xylosidase	GH31,CBM32
Amuc_1032	beta-N-acetylhexosaminidase	GH20
Amuc_1120	glycoside hydrolase N-terminal domain-containing protein	GH95
Amuc_1187	alpha-galactosidase	GH27
Amuc_1220	alpha-N-acetylglucosaminidase	GH89
Amuc_1438	glycosyl hydrolase family protein 98	CBMnc
Amuc_1666	glycoside hydrolase family 2	GH2
Amuc_1667	glycoside hydrolase family 2 sugar binding	GH2,CBM32
Amuc_1669	beta-N-acetylhexosaminidase	GH20
Amuc_1686	beta-galactosidase	GH35,CBM32
Amuc_1815	beta-N-acetylhexosaminidase	GH20
Amuc_1835	exo-alpha-sialidase	GH33
Amuc_1924	beta-N-acetylhexosaminidase	GH20
Amuc_2018	beta-N-acetylhexosaminidase	GH20
Amuc_2019	beta-N-acetylhexosaminidase	GH20
Amuc_2108	glycoside hydrolase family protein 16	GH16
Amuc_2136	glycoside hydrolase family 20	GH20
Amuc_2148	beta-N-acetylhexosaminidase	GH20
Amuc_2164	glycoside hydrolase family 18	GH18
Amuc_0121	arylsulfatase	-
Amuc_0176	peptidase S1 and S6	-
Amuc_0187	peptidase M28	-
Amuc_0253	M23 family peptidase	-
Amuc_0391	M23 family peptidase	-
Amuc_0451	sulfatase	-
Amuc_0465	M23 family peptidase	-
Amuc_0482	alpha/beta superfamily hydrolase	-
Amuc_0491	sulfatase	-
Amuc_0565	sulfatase	-
Amuc_0670	Trypsin-like protein serine protease	-
Amuc_0953	sulfatase	-
Amuc_1003	alpha/beta hydrolase	-
Amuc_1033	sulfatase	-
Amuc_1074	sulfatase	-
Amuc_1106	peptidase M24	-
Amuc_1118	sulfatase	-
Amuc_1480	Peptidase S11 D-alanyl-D-alanine carboxypeptidase	-
Amuc_1631	tail-specific protease	-
Amuc_1655	sulfatase	-
Amuc_1755	sulfatase	-
Amuc_1791	peptidase S1 and S6	-
Amuc_1801	alpha/beta hydrolase	-
Amuc_1934	hypothetical protein	-
Amuc_2040	M3 family peptidase	-
Amuc_2040	peptidyl-dipeptidase Dcp	-

The functional characterisation of these proteins and their role in mucin glycan degradation had not been reported until recently. Several studies assessed the ability of *A. muciniphila* GHs to cleave different glycosides present in mucin (Kosciow and Deppenmeier 2019, 2020; Meng Wang et al. 2018; B.-S. Guo et al. 2018). For example, Kosciow *et al* evaluated the enzymatic activity of *A. muciniphila* GH35 β-galactosidases (Amuc_1686, Amuc_0771) and GH2 β-galactosidases (Amuc_0824, Amuc_1666) against β-galactosidic bonds and porcine stomach mucin (PSM). It was shown that when these enzymes are incubated with desialylated PSM, only the mono-saccharides galactose, GlcNAc and GalNAc, are released. Furthermore, it was noticed that *A. muciniphila*'s β-galactosidases effectively hydrolyzed the β1-3-linked galactose to GlcNAc (Lacto-N-biose) and GalNAc (Galacto-N-biose) as well as the β1-4-linked galactose to GlcNAc (LacNAc) (Kosciow and Deppenmeier 2019, 2020). The activity of Amuc_0771 has been characterized in a previous study in which it was reported that *A. muciniphila* was able to liberate the β1-6-linked galactose to GalNAc and GlcNAc when tested against synthetic substrates (Bz-GalNAc1,6Gal and *p*NP-GalNAc[1,3-Gal]1,6GlcNAc (B.-S. Guo et al. 2018). *A. muciniphila* encodes in its genome twelve β-hexosaminidases from GH20 family (van Passel et al. 2011). β-N-acetylhexosaminidases are responsible for the degradation of carbohydrate components, such as chitin and complex glycans, by releasing the terminal β-linked GlcNAc and GalNAc (Okada et al. 2007; Sakai et al. 1994). Therefore, β-hexosaminidases are predicted to be employed by *A. muciniphila* for mucin degradation. Wang *et al*. recently described two *A. muciniphila* GH20 β-N-acetylhexosaminidases (Amuc_2018 and Amuc_2136) that were able to cleave the terminal GlcNAc off the *N*- and *O*-glycans, which confirms their exo-activity as glycoside hydrolases (Meng Wang et al. 2018). Additionally, another β-N-acetylhexosaminidase (Amuc_0868) was recently characterized, and it showed high specificity to β-GlcNAc linkages found in mucin structure (W. Xu et al. 2020). Natural mucins are often decorated with terminal fucose, sulfate and sialic acid units, which play an important role in preventing degradation by microbial hydrolases. The removal of these protective groups by sialidases, fucosidases, and sulfatases is crucial for the enzymatic degradation of mucin (Corfield et al. 1992; Tsai et al. 1992; Wright et al. 2000). *A. muciniphila*'s four GH33 family sialidases from (Amuc_0623, Amuc_0625, Amuc_1547, Amuc_1835) annotated in its genome have been characterised and validated their ability to cleave sialic acid from mucin-like substrates (X-gal sialosides)

(Huang et al. 2015). While it is known that *A. muciniphila* employs its fucosidases and sulfatases to remove the terminal fucose and sulfate, the function and activity as single enzymes has not been characterised. Furthermore, the way in which *A. muciniphila* accesses complex glycans is still unknown. However, *A. muciniphila* β1,3/4-glucosidase (Amuc_2108) has been characterised as belonging to the GH16 family, which has helped to elucidate the initiation of mucin breakdown from endo-acting *O*-glycanases (Crouch et al. 2020). Therefore, it is very well established that *A. muciniphila* contains all the necessary tools to access and utilize complex oligosaccharides. It was also predicted via a genome-scale metabolic model (GEM) that *A. muciniphila* can degrade the monosaccharides that are released from mucin degradation, such as galactose, fucose, GalNAc and GlcNAc, as well as glucose (Ottman, Davids, et al. 2017). The prediction that *A. muciniphila* utilize of monosaccharides was also confirmed by growth experiments, where it was shown that *A. muciniphila* can degrade glucose, GlcNAc, GalNAc and fucose (van der Ark et al. 2018). Despite the ability of *A. muciniphila* to utilize different monosaccharides, sialic acid metabolism is not in its repertoire. *A. muciniphila* cannot utilise the liberated sialic acid as it lacks the Nan Cluster (NanA/K/E) that is necessary for sialic acid utilisation in other microorganisms (van Passel et al. 2011).

A. muciniphila depends on mucins as a growth substrate, as also discussed in an *in vitro* gut model (SHIME) (Van Herreweghen et al. 2017). While *A. muciniphila* is a well-known mucin degrader, its ability to degrade other plant-derived glycans and/or host-derived glycans, such as HMOs, has not been demonstrated. HMOs constitute the only source of nutrients and dietary fibre during the first months of life in the infant's gut, apart from the mucin glycans (Koropatkin, Cameron, and Martens 2012). HMOs and mucin glycans have a similar glycosidic structure (Garrido, Dallas, and Mills 2013; Tailford, Crost, et al. 2015; Thurl et al. 2017) (Figure 2a). It has been proposed that *A. muciniphila* uses its mucin-degrading enzymes to metabolise HMOs and other human-milk-derived components to facilitate its passage through the gastrointestinal tract for infant gut colonisation (Korpela et al. 2018; Ottman 2015). In theory, *A. muciniphila* acquires all the necessary enzymes to degrade glycolytic linkages found in HMOs (Figure 2b). Tailford et al. demonstrated that *A. muciniphila*'s sialidases (Amuc_0625 and Amuc_1835) liberated terminal α2-3 and α2-6-linked sialic acid from the sialylated HMOs 3'-SL and 6'-SL, respectively (Tailford, Owen, et al. 2015). The HMOs'

degradation from *A. muciniphila* is crucial to secure healthy microbial colonization of the mucosal layer, since the high abundance of *A. muciniphila* is often associated with a healthy state in adults (Derrien, Belzer, and de Vos 2017; Png et al. 2010).

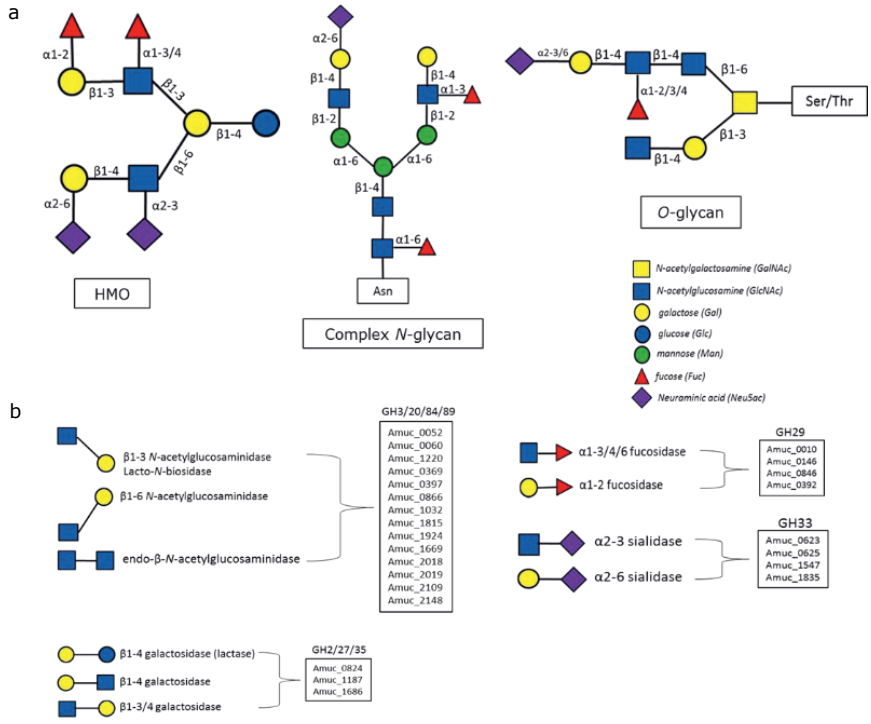


Figure 2: The structure of HMO and mucin show glycosidic similarities. a) Schematic representation of HMO molecule, complex *N*-glycan, and *O*-glycan. b) Glycosidic linkages found in host-derived glycans and the associated predicted *A. muciniphila* GHs involved in their breakdown. The information for the GHs involved in hydrolyzation of host-derived glycans is retrieved from cazy.org

The role of *A. muciniphila* in microbial network formation in the gut

Microbial communities are known to contain symbiotic and cross-feeding relationships (Seth and Taga 2014). The ability of *A. muciniphila* to degrade mucin glycans and release simpler glycan structures is important for cross-feeding in the gut (Derrien, 2004) and the flourishing of other commensal bacteria (Chia et al. 2018; Belzer et al. 2017). For example, *A. muciniphila* releases the sialic acid that is found in *O*-mucin glycans throughout the sialidases that encode to its genome (Juge, Tailford, and Owen 2016). The released sialic acid in the gut promotes the growth of other gut bacteria such

as *Bifidobacterium breve, R. gnavus* and *B. fragilis* (K. Nishiyama et al. 2017, 2018; Crost et al. 2016; Brigham et al. 2009). Additionally, the monosaccharides released by *A. muciniphila* during mucin degradation were used by the butyrate producer *Anaerostipes caccae* for central metabolism (Chia et al. 2018). It has been suggested that a media containing mucus supported the production of butyrate by co-cultures, while neither of the species in pure culture on the same medium did not result in butyrate production (Belzer et al. 2017) (Figure 4). During mucin breakdown, *A. muciniphila* leads to fucose liberation. Fucose is further metabolised by other commensal bacteria, such as *B. thetaiotaomicron,* as an energy source (Sonnenburg 2005). *A. muciniphila* is also able to utilize the released fucose to produce 1,2-propanediol. The liberated 1,2-propanediol could lead to cross-feeding from *Eubacterium halii* and *Lactobacillus reuteri* to produce propionate (Engels et al. 2016; Amin et al. 2013).

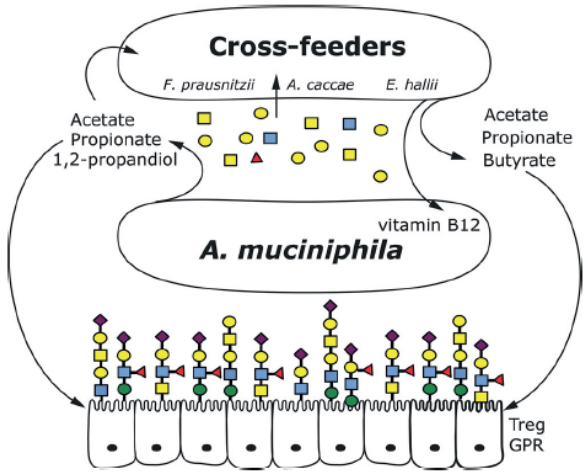


Figure 2: Schematic overview of mucus-dependent cross-feeding network and the role of *A. muciniphila*. Image is adapted from Belzer et al. 2017.

Akkermansia muciniphila in health and disease

A. muciniphila is often associated with a healthy mucosal layer and metabolic state as it has been inversely correlated with obesity (Dao, Everard, Aron-Wisnewsky, et al. 2016; Karlsson et al. 2012) metabolic diseases (Type 2 diabetes) (X. Zhang et al. 2013) as well as intestinal disorders (inflammatory bowel disease (IBD) and appendicitis) (Png et al. 2010; Rajilić-Stojanović et al. 2013; Swidsinski et al. 2011). For example, a higher faecal abundance of *A. muciniphila* was associated with a healthier metabolic status in overweight and obese humans (Dao, Everard, Clément, et al. 2016), while the concentration of *A. muciniphila* was significantly lower in obese/overweight children. It was recently demonstrated that supplementation with *A. muciniphila* in overweight and obese humans reduced blood marker levels that pertain to liver dysfunction and inflammation, while the overall gut microbiome remained unaffected (Depommier et al. 2019). Another study showed a negative correlation of *A. muciniphila*'s relative abundance in faecal samples of children with autism, suggesting impairment of the mucus barrier (L. Wang et al. 2011).

The direct effect of *A. muciniphila* on obesity and metabolic disorders, such as Type 2 diabetes, has been investigated, in which live cells of *A. muciniphila* were administered to mice fed a high-fat diet (HFD). *A.* muciniphila's abundance was decreased in obese and Type 2 diabetic mice. The administration of only live cells of *A. muciniphila* showed improved gut-barrier function and fat-mass storage when compared to the control group. Furthermore, in the same study, *A. muciniphila*'s abundance was normalised during prebiotic intervention, which indicates a correlation with an improved metabolic profile (Everard et al. 2013). A follow-up study illustrated similar, or even stronger, effects of *A. muciniphila* pasteurized cells before administration to HFD-fed mice. The pasteurisation of *A. muciniphila* enhanced its ability to reduce fat-mass gain and insulin resistance. Additionally, a purified outer membrane protein (Amuc_1100) that was administered to the mice improved the gut barrier (Plovier et al. 2017). The function of Amuc_1100 was also monitored *in vitro*. Amuc_1100 was found to be a pili-like gene that could modulate the immune response (Ottman, Reunanen, et al. 2017).

Despite the negative correlation of *A. muciniphila* with metabolic diseases, the opposite has also been observed. For example, a relative abundance of *A. muciniphila* was correlated with obesity and colorectal cancer in humans and allergic diarrhoea in mice

(Collado et al. 2010; Sonoyama et al. 2010; Weir et al. 2013; X. Wang et al. 2017). This increase of *A. muciniphila* in colorectal cancer patients could be due to low caloric intake. A low caloric intake is generally associated with a higher abundance of *A. muciniphila* because of its independence of the host diet. In another recent study, *A. muciniphila* was co-isolated from a blood culture with *Enterococcus faecium* and *Escherichia coli* in a woman with severe diarrhoea (Dubourg et al. 2017). This suggests that *A. muciniphila* can survive in the bloodstream in severe cases of intestinal barrier disruption. These studies, in which the negative and positive correlations are described, indicate that *A. muciniphila* has a profound influence on human health.

Akkermansia muciniphila as the next-generation probiotic

The gut microbiota plays a significant role in human health, and it participates in several functions beneficial to the host (R. Patel and Dupont 2015; Kristensen et al. 2016). For instance, gut microbiota prevents pathogen colonization, shapes the immune system, stimulates the production of gastrointestinal hormones (Saulnier et al. 2013) and regulates brain behaviour (De Palma et al. 2014, 2017). Furthermore, the gut bacteria can ferment the non-digestible carbohydrate in the colon into SCFAs, which elicit health effects (Den Besten et al. 2013). Passive or active processes can manipulate the composition of human gut microbiota. For example, passive factors include hygiene, lifestyle, and diet, while probiotic supplementation can be regarded as an active process in human-gut microbiota manipulation (El Hage, Hernandez-Sanabria, and Van de Wiele 2017; Scott et al. 2015). Probiotics are defined as live microorganisms that, when administered in adequate amounts, confer a health benefit to the host (Gibson et al. 2017). The effect of probiotics on human health has been discussed in various studies. For instance, the administration of probiotics prevented metabolic syndromes such as obesity, type 2 diabetes (Kasińska and Drzewoski 2015) and dyslipidaemia (Asemi et al. 2013). Moreover, *Bifidobacterium spp.* and *Lactobacillus* spp. can reduce body weight gain and adipose tissue in mice that are fed a high-fat diet (Yin et al. 2010; Plaza-Diaz et al. 2014; Reichold et al. 2014; Savcheniuk et al. 2014; Kim et al. 2013; Kobyliak et al. 2016; J. Chen et al. 2012; J. Wang et al. 2015). Probiotics were described as regulating the mucosal immune response (Klaenhammer et al. 2012) and improving the activity of macrophages (Sang et al. 2010). It is been also suggested that probiotics may interact with TLR and downregulate the expression of NF-κB and pro-inflammatory cytokines (Ng et al. 2009; Plaza-Diaz et al. 2014). The conventional

probiotic strains often originate from a narrow taxonomic range of bacteria that mainly consists of *Bifidobacterium* spp. and *Lactobacillus* spp. (O'Toole, Marchesi, and Hill 2017). On the other hand, non-conventional strain candidates for probiotics include *A. muciniphila*, *Faecalibacterium prausnitzii*, *B. fragilis*, and members of Clostridia clusters IV, XIVa, and XVII (El Hage, Hernandez-Sanabria, and Van de Wiele 2017).

A. muciniphila's behaviour and function in the gut makes it the perfect candidate for the title of the next-generation beneficial microbe. Few studies have discussed *A. muciniphila*'s potential to be the next-generation probiotic (Zhai et al. 2019; T. Zhang et al. 2019; Cani and de Vos 2017). *A. muciniphila* is the only cultured representative of the Verrucomicrobia phylum in the human gut (Derrien et al. 2004). It resides in the mucus layer of the large intestine, whereas *A. muciniphila*'s sequences have been detected in human milk, the oral cavity, the pancreas, the biliary system, the small intestine, and the appendix (Geerlings et al. 2018). It has been demonstrated that *A. muciniphila* has the enzymatic capacity to degrade and utilize mucin glycans (Derrien et al. 2004, 2010; Derrien 2007). Additionally, this thesis (**Chapter 3**) describes, for the first time, *A. muciniphila*'s ability to use its mucin-degrading enzymes for HMOs utilization and degradation (Kostopoulos et al. 2020). HMOs are regarded as natural prebiotics in the gut (Bode 2009; Moossavi et al. 2018) and their degradation from *A. muciniphila* releases sugars that can serve as nutrients for other beneficial microbes in the gut, thus contributing to cross-feeding in the human gut (Chia et al. 2018; Belzer et al. 2017; Sonnenburg 2005). The relative abundance of *A. muciniphila* exhibited more than a 100-fold increase after the ingestion of prebiotics (Everard et al. 2014). The ability of *A. muciniphila* to survive also in the early life environment by utilising HMOs from the breast milk and/or infant formulae might provide beneficial effects during initial colonisation (Kostopoulos et al. 2020). *A. muciniphila*'s high relative abundance is often associated with a healthy state and is, inversely, correlated to obesity (Dao, Everard, Aron-Wisnewsky, et al. 2016; Karlsson et al. 2012), Type 2 diabetes (X. Zhang et al. 2013), inflammatory bowel disease (IBD) and appendicitis (Png et al. 2010; Rajilić-Stojanović et al. 2013; Swidsinski et al. 2011). For example, the administration of *A. muciniphila* can improve the metabolic state of obese, diabetic mice, as well as mice with alcoholic liver disease (Everard et al. 2013; Grander et al. 2018). Treatment with live and pasteurized *A. muciniphila* cells showed less body weight gain in mice fed a high-fat diet (Everard et al. 2013; Plovier et al. 2017). Often, non-viable or

inactivated microbial cells that confer benefits to the host, when administered in sufficient amounts, are referred to as postbiotics (Taverniti and Guglielmetti 2011; Aguilar-Toalá et al. 2018). Postbiotic efficacy relies on the microbial metabolites, proteins, lipids, carbohydrates, vitamins, organic acids, cell wall components or other complex molecules that are generated in the matrix that is fermented (Konstantinov, Kuipers, and Peppelenbosch 2013; Aguilar-Toalá et al. 2018). For example, exopolysaccharides (EPS) and extracellular vesicles (EVs) are fermentation products associated with health effects on the host. *A. muciniphila* secreted outer membrane vesicles (OMVs) showed to decrease gut permeability and activate signalling through the intestinal epithelial barrier *in vitro* (Chelakkot et al. 2018). Another recent study showed that *A. muciniphila*'s OMVs reduced body and adipose weight gain and ameliorated HFD-induced intestinal barrier dysfunction in obese mice (Ashrafian et al. 2019). Furthermore, *A. muciniphila* prevented the development of metabolic endotoxemia, an effect associated with the restoration of a normal mucus layer thickness (Everard et al. 2013). These observations indicate the strengthening of the epithelial barrier function, which is often considered a key effect of probiotic therapy (Mennigen and Bruewer 2009). Additionally, the endogenous production of antimicrobial peptides was restored after the administration with *A. muciniphila*. Cani et al. observed that live *A. muciniphila* cells increased the endogenous production of specific bioactive lipids that are known to have anti-inflammatory activities (Cani and Everard 2016). More specifically, they showed that these bioactive lipids regulate the production of gut peptides (glucagon-like peptide-1 and 2) that are involved in glucose regulation and the gut barrier. The positive effect of *A. muciniphila* on mucus layer was also observed in another mouse trial, where administration of *A. muciniphila* increased the number of mucin-producing goblet cells and adipose tissue resident CD4 Foxp3 regulatory T cells (Tregs) (N. R. Shin et al. 2014). *A. muciniphila* was also shown to induce the production of a wide range of cytokines in human-derived peripheral blood mononuclear cells (PBMCs) and to activate TLR2 and TLR4 receptors in reporter cell lines (Ottman, 2015 thesis). A purified *A. muciniphila* protein (Amuc_1100) was found to induce production of IL-6, IL-8 and IL-10, to activate TLR2 and increase transepithelial electrical resistance (TER) (Ottman et al. 2016). In addition, Amuc_1100 showed stability at pasteurization temperature as well as improvement of the gut barrier (Plovier et al. 2017). *A. muciniphila* was shown to adhere to human colonic cell lines but not to colonic mucus (Reunanen et al. 2015). Adherence is

important for probiotic colonization and antagonism against pathogens by competitive exclusion. Taking into consideration all the above, *A. muciniphila* can be seen as a novel therapeutic option for human obesity and associated disorders (Plovier et al. 2017).

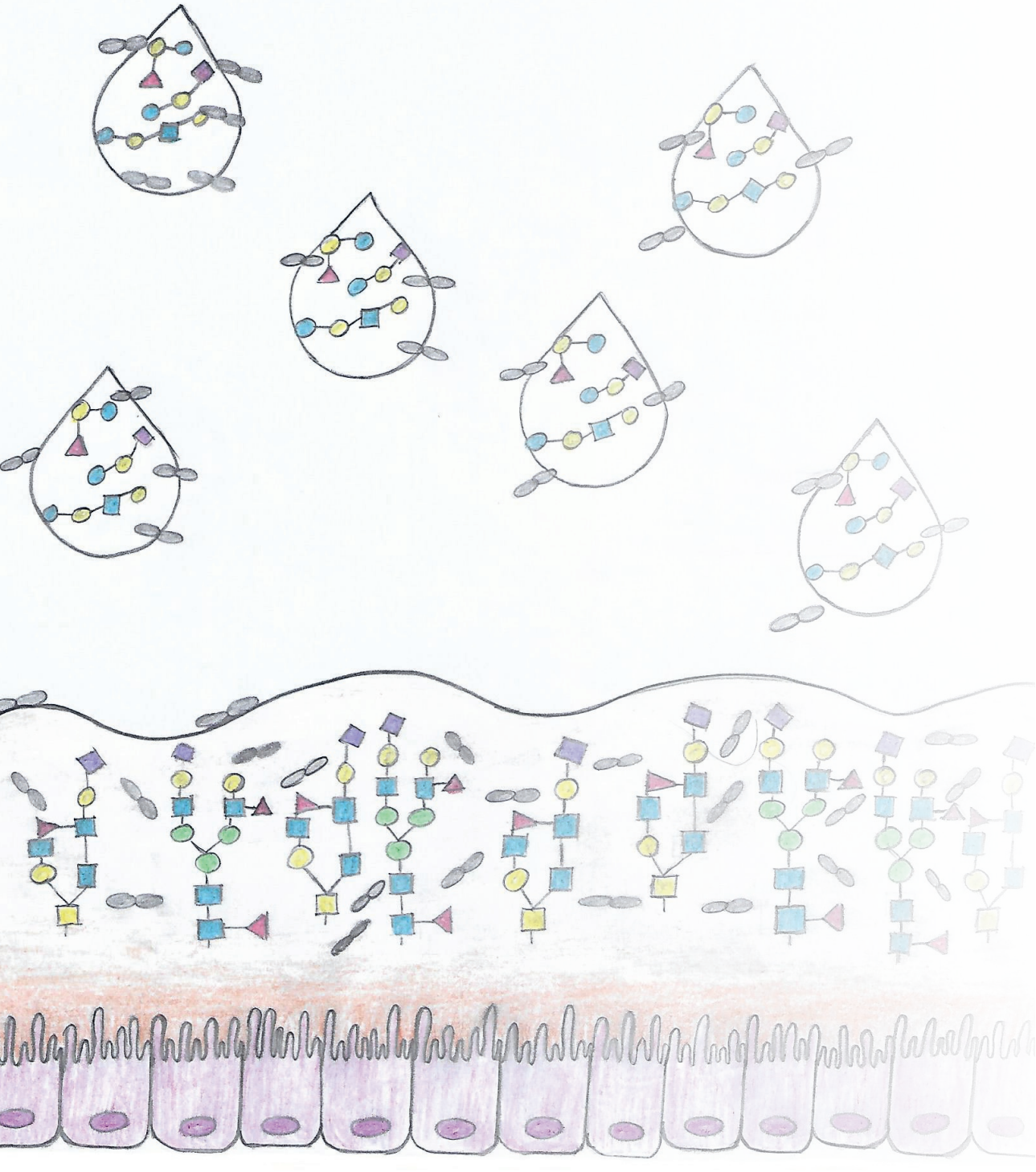
In this thesis, we attempt to elucidate and decipher *A. muciniphila*'s ability to survive and thrive in the gut in the presence of other competitive species by evaluating its capacity to utilise mucin glycans and HMOs. We combined transcriptomics, proteomics, 16S rRNA sequencing and enzymatic assays techniques to gain insight into the carbohydrate metabolism and microbe-to-microbe interaction of this prominent member of the gut bacteria.

Research aim and thesis outline

The research described in this thesis aims to elucidate *A. muciniphila*'s potential to expand its glycolytic repertoire to other carbohydrates other than mucin glycans such as HMOs. Additionally, this research attempts to decipher the mechanisms that *A. muciniphila* uses to survive and thrive in a complex and competitive environment as the human gut, as well as how *A. muciniphila* responds to different environmental changes, such as the introduction of other dietary fibre. Transcriptome, proteome and 16S rRNA sequencing analyses in combination with *in vitro* and *in vivo* growth experiments and enzymatic assays were used.

Chapter 1 introduces the readers to human intestinal microbiota, with a specific focus on the dietary factors that affect the physiology and composition of human intestinal microbiota. The functions and effect of *A. muciniphila*, which is the focus of this thesis, on human health are also introduced. **Chapter 2** provides an overview of *A. muciniphila* functionality in different parts of the gastrointestinal tract. For instance, *Akkermansia muciniphila* sequences have been found in human milk, the oral cavity, the pancreas, the biliary system the small intestine, and the appendix. We proposed as well hypothetical functions of *A. muciniphila* in these regions, highlighting nevertheless the fact that further research is needed to confirm its role among the different regions of the digestive tract. **Chapter 3** highlights the ability of *A. muciniphila* to expand its glycolytic repertoire to another source of glycans present in early life environments, such as HMOs. Using proteome analysis and enzymatic assays, we were able to validate and show the capacity of *A. muciniphila* to be grown on human breast milk and its derivatives to thrive in early life conditions. We discuss the contribution of *A. muciniphila* to cross-feeding interactions in the gut from early life through the liberation of monosaccharides and simpler sugars that serve as nutrients for other beneficial bacteria in the gut. The ability of *A. muciniphila* to survive in the early life environment might provide beneficial effects during the initial early life colonization of *A. muciniphila*. In **Chapter 4**, we assess the microbe-to-microbe interaction of *A. muciniphila* with another mucin-degrading generalist, *B. thetaiotaomicron,* under the continuous influx of mucin glycans *in vitro* and *in vivo* by colonizing germ-free mice fed a high-fibre diet. We employed transcriptomics analysis from both *in vitro* and *in vivo* to monitor the differential gene expression between mono- and co-culture. In **Chapter 5**, we assembled in bioreactors a synthetic

community of 16 different species including *A. muciniphila*. Mucin glycans were constantly supplied to the community, providing also the community members with dietary fibre (pectin, starch, inulin and xylan) three times per day. We use different omics techniques (transcriptomics, 16S rRNA sequencing) to evaluate the microbe-to-microbe interactions as well as the metabolic interactions between key bacteria in the human intestinal microbiome. **Chapter 6** compares the *A. muciniphila* transcriptional response between different experiments described in this thesis (**Chapter 4** and **Chapter 5**). We study the transcriptional landscape of *A. muciniphila* under varying growth conditions, such as complexity of the community, diet, media composition, and experimental design (*in vitro* vs. *in vivo*). We focus on *A. muciniphila*'s key functions, for example, mucin degradation, EPS and pili-associated gene expression. We show that *A. muciniphila* similarly exerts the key functions in all the different conditions included in this study. **Chapter 7** summarizes and discusses the observations of the research data presented in this thesis and provides future perspectives for research.



Chapter 2

Akkermansia muciniphila in the Human Gastrointestinal Tract: When, Where and How?

Sharon Y. Geerlings, Ioannis Kostopoulos, Willem M. de Vos and Clara Belzer

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Abstract

Akkermansia muciniphila is a mucin-degrading bacterium of the phylum Verrucomicrobia. Its abundance in the human intestinal tract is inversely correlated to several disease states. *A. muciniphila* resides in the mucus layer of the large intestine, where it is involved in maintaining intestinal integrity. We explore the presence of *Akkermansia*-like spp. based on its 16S rRNA sequence and metagenomic signatures in the human body as to understand its colonization pattern in time and space. *A. muciniphila* signatures were detected in colonic samples as early as a few weeks after birth and likely could be maintained throughout life. The sites where *Akkermansia*-like sequences were detected apart from the colon included human milk, the oral cavity, the pancreas, the biliary system, the small intestine and the appendix. The function of *Akkermansia*-like *spp.* in these sites may differ from that in the mucosal layer of the colon. *A. muciniphila* present in the appendix or in human milk could play a role in re-colonization of the colon or breast-fed infants, respectively. In conclusion, even though *A. muciniphila* is most abundantly present in the colon, the presence of *Akkermansia*-like spp. along the digestive tract indicates this bacterium might have more functions than is known so far.

Keywords: *Akkermansia muciniphila*, mucin, gut microbiota, ecological niches, digestive tract, human breast milk

Introduction

The microbial community in the human gut plays a role in the balance between health and disease. The gastrointestinal (GI) microbiota has recently emerged as an important factor in human physiology, both under homeostatic and pathological conditions (Blaser 2014). Characterization of the GI microbiota may identify gut-related abnormalities and play an important role in establishing functional linkages to health status (Verdu, Galipeau, and Jabri 2015). Some of the GI tract disorders with associated microbiota imbalance include coeliac disease (Verdu, Galipeau, and Jabri 2015), irritable bowel syndrome (IBS) (Distrutti et al. 2016; Lopez-Siles et al. 2014), inflammatory bowel disease (IBD) (Sheehan, Moran, and Shanahan 2015; Png et al. 2010; Rajilić-Stojanović et al. 2013) and Type 2 diabetes (T2D) (X. Zhang et al. 2013; Schneeberger et al. 2015; Everard et al. 2013).

A. muciniphila type strain Muc^T of the phylum Verrucomicrobia was first described in 2004 (Derrien et al. 2004). This bacterium has been isolated from the fecal sample of a healthy individual using purified mucin as the sole source of carbon, nitrogen and energy for growth. *A. muciniphila* shares only little similarity to other representatives of Verrucomicrobia (van Passel et al. 2011). Interestingly, *Akkermansia* is the only genus of the Verrucomicrobia phylum found in gastrointestinal samples. Large differences were observed between Verrucomicrobial genomes, in terms of the GC content and genome sizes. In contrast, similarities were observed within the Verrucomicrobia phylum as a large proportion of the proteins in Verrucomicrobial proteomes were found to contain signal peptides (26.1% for *A. muciniphila*). In the colon of a healthy human being, *A. muciniphila* is present in high levels with an abundance of approximately 3% (Png et al. 2010; de Vos and Vos 2017) . The core activity of *A. muciniphila* is to degrade mucus using the many mucolytic enzymes encoded in its genome (Png et al. 2010; van Passel et al. 2011) .

The presence of *A. muciniphila* has been associated with healthy intestine and its abundance has been inversely correlated to several disease states (Png et al. 2010; X. Zhang et al. 2013; Rajilić-Stojanović et al. 2013; Swidsinski et al. 2011; Karlsson et al. 2012). For example, in cases of IBD (patients with ulcerative colitis and Crohn's disease), the abundance of *A. muciniphila* was found to be decreased (Dao, Everard, Aron-Wisnewsky, et al. 2016; Png et al. 2010; Rajilić-Stojanović et al. 2013). Also, individuals with acute appendicitis were found to harbour a decreased amount of *A.*

muciniphila (Swidsinski et al. 2011). In this case, the abundance of *A. muciniphila* was inversely related to the severity of the appendicitis. Furthermore, obese children were shown to have a significant reduction in *A. muciniphila*-like bacteria (Karlsson et al. 2012). In a comprehensive study of day care infants, *A. muciniphila*-based sequences were found to be reduced in children that had received multiple antibiotic courses and were at risk for later life obesity (Korpela et al. 2018). In concordance with these studies, another reported the association of *A. muciniphila* with a healthier metabolic status in obese and overweight individuals (Dao, Everard, Aron-Wisnewsky, et al. 2016). Moreover, the genus *Akkermansia* and its metabolic pathways were found to be enriched in athletes with a low body mass index (Clarke et al. 2014; Barton et al. 2018). Lastly, the abundance of Verrucomicrobia was significantly reduced in pre-diabetes and T2D (X. Zhang et al. 2013). It is important to note that these diseases may influence the integrity or thickness of the mucus layer, and thereby effect the abundance of *A. muciniphila*. To confirm this, studies taking both the microbial composition and mucus layer integrity into account should be performed. Altogether, these studies indicate the correlation of *A. muciniphila* to a healthy status and indicate the possible use of *Akkermansia* spp. as biomarker for disease.

Interactions between the host and *A. muciniphila* have been studied in mice (Everard et al. 2013; Derrien et al. 2011). Colonization by *A. muciniphila* resulted in transcriptional changes, leading to an increase in expression of genes associated with immune responses (Derrien et al. 2011). Furthermore, *A. muciniphila* was found to strengthen the gut barrier function in mice (Everard et al. 2013). By doing so *A. muciniphila* played a role in normalizing metabolic endotoxemia and adipose tissue metabolism. These findings have been supported by another study showing that *A. muciniphila* affects genes involved in cellular lipid metabolism (Lukovac et al. 2014). The effect of a fiber-free diet was studied in mice colonized with a synthetic community consisting of 14 species, including *A. muciniphila* (Desai et al. 2016). Feeding these mice, a fiber-free diet was found to damage the mucus barrier. The changes in microbial community included an increased abundance of *A. muciniphila* and a switch in metabolism of gut microbiota species from fiber degradation to mucus glycan degradation (Desai et al. 2016; Makki et al. 2018). An in vitro study using human colonic cell lines (Caco-2 and HT-29) demonstrated adherence of *A. muciniphila* to the intestinal epithelium thereby strengthening the epithelial integrity rather than causing a pro-inflammatory reaction

(Reunanen et al. 2015). Lastly, outer membrane proteins of *A. muciniphila* were found to have a role in modulation of immune responses (Ottman et al. 2016). Recently, one of the outer membrane proteins was identified (Amuc_1100) (Ottman, Reunanen, et al. 2017). This study demonstrated that the outer membrane pili-like protein is involved in immune regulation and enhancement of trans-epithelial resistance.

Several studies have purposely or un-purposely revealed the presence of *Akkermansia*-like *spp.* in other segments of the human body than the colon, where *A. muciniphila* might also have important functions. In this review, we will discuss the presence of Verrucomicrobia and *Akkermansia*-like *spp*. in different anatomic regions of the digestive tract. The physiology and environmental parameters of these anatomic regions will be considered to assess the possibility of *A. muciniphila* to colonize and be active at these niches.

Prevalence of *Akkermansia muciniphila* through geography and age in the human gastrointestinal tract

Akkermansia muciniphila is present in the intestinal tract throughout different stages of life (Collado et al. 2007; Derrien et al. 2008). This was determined using fecal samples from healthy subjects divided in groups based on their age, analysed using fluorescence in situ hybridization (FISH) and quantitative PCR (qPCR). The number of bacteria related to *A. muciniphila* significantly increased from early life to adult subjects (Collado et al. 2007; Derrien et al. 2008). When focusing on the prevalence of bacteria related to *A. muciniphila*, 16% of one-month old infants in this study were found to harbour *Akkermansia* in their intestinal tracts (Collado et al. 2007). At this very young age the concentration of *A. muciniphila* ranged between 2.05-4.36 log cells per gram of feces. Subsequently, at 6 months of age the percentage of infants where *A. muciniphila* could be detected increased to 72%, with a further increase to 90% in 12 months old infants. The concentrations ranged between 2.50-7.30 and 2.80-9.50 log cells per gram of feces in 6 months and 12 months old infants respectively. However, in this study no correlation was made between the *A. muciniphila* abundance and the feeding mode of the infant (breast and/or formula feeding). Other studies, not primarily focusing on the presence of *A. muciniphila* have also shown the presence of *Akkermansia*-like spp. or the Verrucomicrobia phylum in the infant's gut at different geographical locations, such as Finland, Germany and Malawi (Grzeskowiak, Gronlund, et al. 2012; Grzeskowiak, Collado, et al. 2012; de Weerth et al. 2013). The presence of *A. muciniphila* in the

infant's gut could be considered as a marker for gut microbiota development and diversity (Grzeskowiak, Gronlund, et al. 2012). Next to infants, adults (25-35 years old) were found to harbour 5 to 8.8 log cells per gram of feces of *A. muciniphila*, while a significant decrease was noted for elderly (80-82 years old) namely, 95.5% (Collado et al. 2007). These outcomes differed in another study, where young adults harboured significantly less *Akkermansia* spp. than elderly both in terms of prevalence and abundance (Biagi et al. 2010). In addition, centenarians were found to harbour a higher concentration of *Akkermansia* spp. Also, semi-supercentenarians (individuals with an age of 105 or higher) had a higher concentration of *Akkermansia*-like spp. than other (younger) age groups in the study (Biagi et al. 2017). *A. muciniphila* is proposed to have a role in immunological and metabolic health of semi-supercentenarians, rendering it as a biomarker for healthy aging. In contrast to age, gender does not play a role considering the amount of *A. muciniphila* (Derrien et al. 2008).

The genome of *A. muciniphila* (ATCC BAA-835) was sequenced and annotated (van Passel et al. 2011). These sequences were used to mine 37 reported GIT metagenomes derived from adults belonging to 6 nationalities to evaluate the presence and genetic diversity of *Akkermansia spp.* in the human gut. The prevalence of *Akkermansia* spp. in these metagenomes was found to be 30%, using a cut-off of >95% identity to 16S rRNA. When queried with the *Akkermansia* genome (identity >90%) 62% of the metagenomic libraries showed to be *Akkermansia* carriers, which is comparable to earlier findings of a Finnish cohort (Collado et al. 2007). The relative abundances of *Akkermansia* spp. DNA in these libraries varied between <0.01% to almost 4% (van Passel et al. 2011). However, higher abundances of Verrucomicrobia in the GIT have also been reported, for example in biopsy samples (up to ~15% depending on the method and individuals) (Momozawa et al. 2011). The analysis of fecal samples from the Metagenomics of the Human Intestinal Tract (MetaHit) project derived from Danish and Chinese individuals, revealed country-specific differences in gut microbiota (J. Li, Jia, Cai, Zhong, Feng, Sunagawa, Arumugam, Kultima, Prifti, Nielsen, et al. 2014). In terms of *Akkermansia*-like spp. the mean relative abundance of Danish individuals (0.0137) was higher than that of Chinese individuals (0.0015). Moreover, a recent study showed that Verrucomicrobia sequences were found to be enriched in the guts of industrialized regions than in those of traditional populations ($P < 2 \times 10^{-16}$) such as the traditional Hadza hunter-gatherers (Smits et al. 2017). However, as with many

comparative microbiota analyses, confounding factors related to sample processing, DNA extraction, and subsequent processing cannot be ruled out (Costea et al. 2017). A study performed in China showed that the frequency of *A. muciniphila* is lower in Southern China than in European populations (X. Guo et al. 2016). The frequency in Southern China was found to be 51.71%, which is significantly lower than ~75% found in European populations (Collado et al. 2007; X. Guo et al. 2016). Interestingly, in southern China the highest frequencies were detected among elderly, while in European populations a significant decrease was noted for elderly. This observed difference may be due to external factors affecting the microbiota composition, including geographic location, diet and age.

The human intestine may be colonized by different *Akkermansia*-like spp. (van Passel et al. 2011). The 16S rRNA sequences detected in metagenomic datasets suggested colonization by at least 8 different *Akkermansia*-like spp. But as these sequences derived from short Illumina reads, misassembly and other technical biases may have affected this conclusion. Moreover, it is also possible that simultaneous colonization by different species occurs. Recently, 39 *A. muciniphila* strains were isolated from human and mouse feces and subsequently analysed for their 16S rRNA sequences and draft genomes (X. Guo et al. 2016, 2017). All 16S rRNA sequences from these strains shared over 97% sequence identity with that of the type strain *A. muciniphila* Muc^T isolated from Europe, suggesting they represent isolates from the same species. Using these isolates, three phylogroups (AmI, AmII and AmIII) were identified based on core genome single nucleotide polymorphisms (SNPs). We constructed maximum likelihood tree based on the available 16S sequences of the Chinese *A. muciniphila* strains in the NCBI sequence database and all other 16S rRNA *Akkermansia*-like sequences derived from colonic and ileal biopsy samples derived from the SILVA SSU Ref 132 dataset (Figure 2a and Supplementary Figure S1). In this tree, the newly isolated Chinese *A. muciniphila* strains fall into two out of three distinct clades (clade one and two) that however have only minimal differences. In comparison with the type strain, the first clade has 99-100% identity, the second clade 98-99% and the third clade 98%. Interestingly, the Chinese *A. muciniphila* strains have reportedly distinct metabolic and functional features (X. Guo et al. 2017). However, unlike *A. muciniphila* type strain Muc^T, complete closed genomes of these Chinese *A. muciniphila* strains have not been reported. Furthermore, functional analysis of the Chinese *A. muciniphila*

strains has not been performed, but the strains are able to grow on mucus (X. Guo et al. 2017). Next to *A. muciniphila* only one other *Akkermansia*-like sp. has been validly described, namely *Akkermansia glycaniphila* strain Pyt^T isolated from reticulated python feces (included in the phylogenetic tree shown in Figure 1a) (Ouwerkerk, Aalvink, et al. 2016; Ouwerkerk et al. 2017). The closest relative of *A. glycaniphila* is *A. muciniphila* Muc^T sharing 94.4% 16S rRNA sequence similarity (Ouwerkerk, Aalvink, et al. 2016). The average nucleotide identity between the *A. glycaniphila* Pyt^T genome and the genome of *A. muciniphila* Muc^T was found to be 79.7%. Comparatively to *A. muciniphila*, *A. glycaniphila* is also able to use mucin as sole carbon, energy and nitrogen source. However, the relatedness between these two species, determined by DNA-DNA hybridization, is low namely 28.3%. To be able to compare functional differences between Chinese *A. muciniphila* strains, the *Akkermansia*-like sequences found in biopsies of the ileum and large intestine (included in the tree) and *A. muciniphila* Muc^T, enclosed genomes and functional analysis are needed.

Altogether, *A. muciniphila*'s presence varies among individuals. Its abundance varies not only from person to person but also from age group to age group. On top of that, other factors such as geographical location may also play an important role in the presence and richness of *Akkermansia*-like spp. in the human GIT.

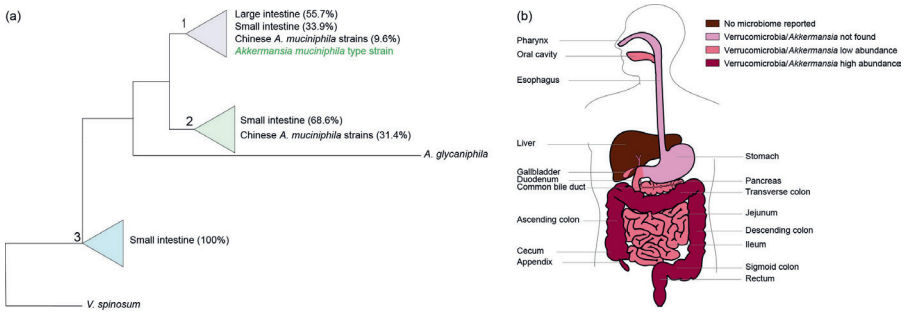


Figure 1: (a) *Akkermansia* is not only present in the large intestine, but also in other anatomic regions of the digestive tract. A schematic overview of the positioning of 16S rRNA clones in the small and large intestine and the available sequences of the Chinese *A. muciniphila* strains towards the *A. muciniphila* type strain Muc^T and *A. glycaniphila* Pyt^T. The percentages indicate the compositions of the clades. The tree was generated using the maximum likelihood (RAxML) method in ARB using a 40% positional conservatory filter (Ludwig 2004). The original detailed maximum likelihood tree is shown in Supplementary Figure S1. Similar groups were observed using the neighbor joining method (Supplementary Figure S2). (b) Overview of Verrucomicrobia/*Akkermansia* sequences in the human digestive tract.

Physiologic adaptation of *Akkermansia muciniphila* to the human GIT

A. muciniphila is an oval shaped, anaerobic Gram-negative bacterium that was first described by Derrien *et al*. (Derrien et al. 2004). Transmission electron microscopy revealed the presence of filamentous structures on Muc^T cells when grown on mucin medium. On top of this, strain Muc^T is able to exclude Indian ink, suggesting that the filamentous structures are capsular polymers. More recently, outer membrane proteins of *A. muciniphila* were analyzed, which resulted in the identification of pili proteins (Ottman et al. 2016; Ottman, Reunanen, et al. 2017).

When focusing on the growth parameters of *A. muciniphila*, it is known that growth was observed between temperatures of 20 and 40°C and pH values of 5.5-8.0 (Figure 2) (Derrien et al. 2004). However, the optimum growth temperature and pH are 37°C and 6.5, respectively. *A. muciniphila* is an obligate chemoorganotroph, utilizing mucus as sole carbon, nitrogen and energy source (Derrien et al. 2004). Consequently, the short chain fatty acids acetate, propionate and to smaller extent 1,2-propanediol and succinate are produced (Derrien et al. 2004; Ottman, Davids, et al. 2017). Another factor that influences the growth of *A. muciniphila* is the presence of oxygen. *A. muciniphila* was found to be able to tolerate and even benefit from nanomolar concentrations of oxygen in liquid medium (Ouwerkerk, van der Ark, et al. 2016). Upon the presence of oxygen, there is a change of acetate to propionate production by *A. muciniphila*. This results in an increased production of ATP and NADH, which enhances growth of *A. muciniphila*.

To compose a minimal medium for *A. muciniphila*, a genome-scale metabolic model was constructed (Ottman, Davids, et al. 2017). This model predicts the degradation of mucin-derived monosaccharides. The model showed *A. muciniphila* is able to synthesize all essential amino acids, except for L-threonine of which the pathway was missing. Furthermore, growth experiments revealed that *A. muciniphila* can degrade a variety of sugars such as glucose, N-Acetylglucosamine (GlcNAc), N-Acetylgalactosamine (GalNAc) and fucose. However, to obtain growth large amounts of casein tryptone, mucin or a rich medium was required. Hereafter, another study showed that *A. muciniphila* does not code for GlmS, which mediates the conversion of fructose-6-phosphate to glucosamine-6-phosphate (van der Ark et al. 2018). This reaction is essential for peptidoglycan formation. Therefore, the degradation of glucose does not lead to biomass production. *A. muciniphila* does code for NagB which

catalyses the reverse reaction indicating that the addition of GlcNAc is essential for growth of *A. muciniphila*. Altogether, this information has led to the development of a defined minimal medium for *A. muciniphila,* in which L-threonine and GlcNAc or GalNAc are essential components for growth (van der Ark et al. 2018).

Recently, the growth of *A. muciniphila* in the presence of bile has been studied (van der Ark et al. 2017). Interestingly, the *A. muciniphila* abundance was positively correlated to circulating primary bile acids in mice (Pierre et al. 2016). The addition of 0.1%, 0.5% and 1% porcine bile extract resulted in increased growth of *A. muciniphila* in comparison to the medium that did not contain bile (van der Ark et al. 2017). In contrast, purified bile salts addition of 0.5% or higher resulted in inhibited growth of *A. muciniphila*, whereas the addition of 0.1% purified bile salts did not inhibit growth (Van der Ark 2018). Moreover, survival of *A. muciniphila* in gastric juice was found to be very low .

Several studies have described the antibiotic resistance of *A. muciniphila* (Dubourg et al. 2013, 2017). The type strain of *A. muciniphila* (Muc^T) was found to be susceptible to imipenem, piperacillin/tazobactam and doxycycline, while resistance was noted for vancomycin, metronidazole and penicillin G (Dubourg et al. 2013). Another *A. muciniphila* strain was resistant against vancomycin and ofloxacin, but susceptible to penicillin, amoxicillin, ceftriaxone and imipenem (Dubourg et al. 2017). *A. muciniphila* MucT has potential beta-lactamase genes and may code for a 5-nitroimidazole antibiotic resistance protein (van Passel et al. 2011). The *in silico* prediction of a strain directly sequenced from stool (*A. muciniphila* strain Urmite) predicted the presence of antibiotic resistance genes for the classes beta-lactamases, glycopeptides, MLS, phenicol, sulphonamide, tetracycline and trimethoprim (Caputo et al. 2015). Guo *et al*. described the presence of 3 antibiotic resistance genes in *A. muciniphila* strain GP36, which originated from plasmid pRSF1010 (8684 bp) of *Salmonella enterica* (X. Guo et al. 2017). This indicates that *A. muciniphila* might acquire antibiotic resistance genes through lateral gene transfer.

The growth parameters of *A. muciniphila* described above coincide with the environmental parameters found in the large intestine. The oxygen concentration in the intestine follows a steep gradient from the intestinal submucosa to the lumen, where the oxygen concentrations decrease to near anoxia (Espey 2013). *A. muciniphila* may take advantage of this oxygen concentration in the mucus layer, enhancing its growth.

The mucosal layer of the large intestines serves as carbon, nitrogen and energy sources for the use of *A. muciniphila*. The mucin backbone is rich in threonine (among other amino acids) and contains many sugar groups, including GlcNAc and GalNAc (Johansson et al. 2011). As mentioned earlier these are among the minimal growth requirements of *A. muciniphila*. Although, the mucus layer in the large intestine is thought to be the optimal niche for *A. muciniphila*, mucus is also present at other locations of the GIT. Several conditions may vary in the GIT such as type of mucin that is secreted, pH, oxygen concentration and concentration of bile acids. The ability of *A. muciniphila* to cope with these conditions will be discussed below.

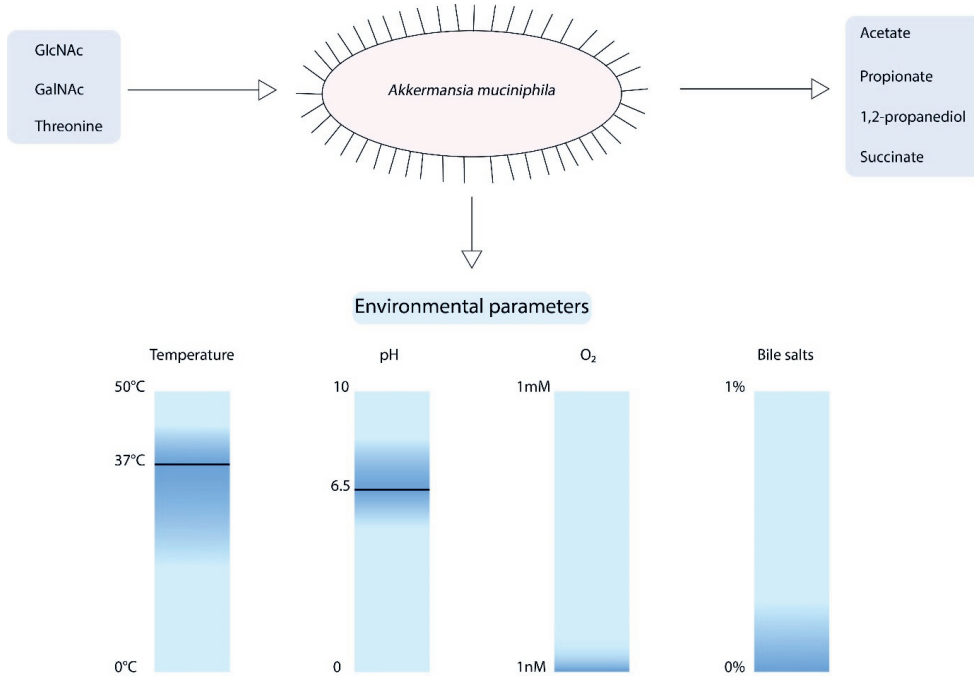


Figure 2: Schematic overview of the growth parameters of *A. muciniphila*. Optimum growth temperature and pH are 37°C and 6.5, respectively. In addition, *A. muciniphila* is able to tolerate nM concentrations of oxygen and is able to grow in the presence of 0.1% purified bile salts.

Akkermansia muciniphila along the human gastrointestinal tract

Akkermansia-like spp. were found to be present in different anatomical regions of the digestive tract, including the oral cavity, breast milk, pancreas, the biliary system, the small and large intestine and the appendix (Figure 2b). Next to the aforementioned

regions, *Akkermansia*-like sequences were also detected in human blood (Dubourg et al. 2017; Santiago et al. 2016; Traykova et al. 2017). However, the presence of *Akkermansia*-like sequences was only detected in subjects with diseases as septicemia and cirrhosis. In a mice study, *A. muciniphila* was detected in the oral cavity, stomach, small intestine and large intestine upon administration of breast milk and formula milk (Gómez-Gallego et al. 2014). However, in this review we will mainly focus on the presence of *Akkermansia*-like spp. in the human body.

Oral cavity

The oral cavity is a moist environment with a relatively constant temperature between 34 and 36°C (Marcotte and Lavoie 1998). There are several ecological niches in the oral cavity that mostly have a neutral pH. The mean pH of the mucosal sites was found to be 6.78+/-0.04 (Aframian, Davidowitz, and Benoliel 2006). Examples of different sites in the oral cavity are the tongue, soft and hard palate, tooth surfaces and tonsils (Marcotte and Lavoie 1998). In terms of temperature and pH, the oral cavity can support the growth of a wide variety of microorganisms. Therefore, the microbial community of the oral cavity is site specific and highly diverse (Aas et al. 2005).

Several studies found a high abundance of the phyla Firmicutes, Proteobacteria, Bacteroidetes and Actinobacteria with *Streptococcus* (belonging to the phylum Firmicutes) as most abundant genus (Shaw et al. 2017; Bik et al. 2010). The presence of Verrucomicrobia in the oral cavity is often not described (Shaw et al. 2017; Bik et al. 2010; Nasidze et al. 2009; Sarkar, Stoneking, and Nandineni 2017). However, the presence of *Akkermansia*-like sequences were found in the oral cavity of a choledocholithiasis patient (Ye et al. 2016) . The salivary samples were taken by gargling with 20 mL of sterile saline water. Thus, the microorganisms in these samples originate from multiple niches in the oral cavity.

The relative abundance of *Akkermansia*-like spp. in this sample was low, namely 0.02% (Ye et al. 2016). In addition, these sequences were only found in one out of six patients included in this study. In terms of pH and temperature, *Akkermansia* spp. could be able to survive in the oral cavity (Derrien et al. 2004). Furthermore, Gram-negative and obligate anaerobes with proteolytic lifestyles are present in healthy gingival crevice biofilms (Marsh, Head, and Devine 2015). As discussed before, *A. muciniphila* has a mucin-degrading lifestyle (Derrien et al. 2004). The mucins found in the oral cavity are

MUC5B, MUC7, MUC19, MUC1 and MUC4 (Linden et al. 2008). Of these mucin structures, MUC5B is the most abundant gel-forming mucin in the oral cavity (Nielsen et al. 1997; D. J. Thornton et al. 1999). As *A. muciniphila* has mucin-degrading enzymes similar to those found in oral *Streptococcus* spp., *A. muciniphila* might be able to use the mucin structures in the oral cavity as a substrate for growth (Derrien et al. 2010; van der Hoeven, van den Kieboom, and Camp 1990) . However, further research is needed to confirm this hypothesis.

Mucin oligosaccharides are able to bind microbes and in some cases exert functions in antimicrobial activity or carry antimicrobial proteins (Linden et al. 2008). For example, MUC7 and MUC5B both bind statherin and histatin-1. By binding these molecules, mucins exert a protective function in the oral cavity. The potential role of *Akkermansia* spp. in the oral cavity is unknown. However, one could hypothesize that its capability to stimulate mucus production of the epithelial cells enhances the protection e.g. against pathogens. Another possibility could be that *Akkermansia* spp. produces compounds in mucin degradation that could be useful for other bacteria in the oral microbial community. Lastly, *Akkermansia* spp. might be involved in modulation of the host response. Altogether, more studies should be conducted to confirm the presence of *Akkermansia* spp. and its function in the oral cavity.

Pancreas

The pancreas is a complex organ comprised of both exocrine glands (secreting digestive enzymes into the intestinal lumen) and endocrine glands, called the islets of Langerhans, which secrete hormones directly into the blood stream (Tan 2014). The pancreas plays a central role in human metabolism, allowing ingested food to be converted and used as fuel by cells throughout the body. The pancreas may be affected by devastating diseases, such as pancreatitis, pancreatic adenocarcinoma (PAC), and diabetes mellitus (DM), which generally results in a wide metabolic imbalance (Leal-Lopes et al. 2015). Nutrient metabolism in pancreatic cells is not only essential for providing energy for the cell, but also serves as a mechanism to sense and react to circulating levels of macronutrients. This gives the pancreas a central role in metabolism regulating the whole-body energy homeostasis. Efficient energy metabolism in pancreatic endocrine cells of the islet is required to permit secretion of many hormones, mainly insulin and glucagon, that regulate glucose and lipid utilization throughout the body. The pancreas is thought to be devoid of bacteria. However,

microbial translocation as a result of disease states has led to measurements of microbes also in pancreatic tissue. Because it is dangerous and impossible to take biopsies of pancreatic tissue, due to the risk of leakage of pancreatic fluid, pancreatic samples can only be obtained via surgery. As such, samples of healthy individuals are not yet reported. Healthy controls are usually healthy tissue surrounding a pathologic site. Recent research has shown that disruption of pancreatic metabolism is often a consequence of disruptions in the gut microbiome (Jouvet and Estall 2017). Another study has shown that the pH in the pancreas drops significantly (p-value< 0.05) in patients with painful chronic pancreatitis (7.02 ± 0.06) compared to healthy individuals (7.25 ± 0.04) (A. Patel et al. 1995). That pH change in patients with chronic pancreatitis might be one of the reasons why the bacterial barrier in the pancreas is ruptured and the abundance of bacterial phyla and species is elevated (Memba et al. 2017).

A. muciniphila plays an important role on the maintenance of the gut barrier function (Dao, Everard, Aron-Wisnewsky, et al. 2016). A few studies have shown the association of *A. muciniphila* with the pancreas and its health. A recent study with patients undergoing pancreatic fluid pancreaticoduodenectomy (PD) revealed that the mean relative abundance of the Verrucomicrobia phylum and *Akkermansia* genus respectively was 0.0005 and 0.0004 in the pancreas of these patients (Rogers et al. 2017). The study continues highlighting that in the pancreas of the patients other commensal bacteria were found to be present. The Proteobacteria phylum and *Klebsiella genus* were the most abundant with mean relative abundances of 0.5410 and 0.2011, respectively. *Faecalibacterium*, *Bacteroides* and *Prevotella* were also detected in the pancreas of the PD patients. The presence of other gut microbes in the pancreas apart from *Akkermansia* may indicate trophic interactions between them.

Interestingly, it was found that mice treated with pancreatic enzyme replacement therapy (PERT), had a significant increase of 58-fold of *A. muciniphila* sequences compared to the control samples (mice treated with tap water) (H. Nishiyama et al. 2018). Furthermore, it is stated that pancrealipase diminishes PEI-associated symptoms by inducing colonization of *A. muciniphila* followed by normalization of the intestinal barrier. Therefore, it is hard to speculate on the role of *Akkermansia* species and/or *A. muciniphila* in the pancreas.

The *Akkermansia* genus is detected in the pancreas, mainly in conditions of pathology. The significant change of pH of the pancreatic fluid in patients with chronic pancreatitis

(7.02 ± 0.06) might be a reason that *Akkermansia* was detected in pathological conditions. There is not any evidence so far, showing that the pancreas is colonized by bacteria in healthy individuals or that it is a bacterial-free organ. All the studies that have been described so far; the bacterial colonization of the pancreas is inextricably linked to the cause of pancreatic disease. The higher abundance of bacteria in patients with pancreatic diseases could be associated with an overall higher abundance of microbiota members in these states of disease due to bacterial overgrowth and translocation. Decreased gut barrier function in both acute and chronic pancreatitis, increases bacterial translocation. This bacterial translocation could have significant impact on the nutrient absorption and therefore on the availability of nutrients for intestinal microorganisms, and microbial composition of the gut.

Bile ducts and gallblader

Bile is a complex aqueous solution secreted by the liver (Boyer 1986). Both gallbladder bile and common bile duct bile of patients undergoing cholecystectomy were found to have an alkaline pH ranging between 6.8-7.65 and 7.5-8.05, respectively (Sutor and Wilkie 1976). In most animal/mammal species bile contains less than 5% of solid contents (Boyer 1986). The most abundant organic substances in bile are bile salts (Boyer 2013). The primary bile salts in the mammalian liver are cholic acid and chenodeoxycholic acid (CDCA). They are produced from cholesterol in the liver and are then excreted into the duodenum (Islam et al. 2011). Bile salts exert several functions. For example, they were found to have a role in antimicrobial defence, promoting of lipid absorption and protein digestion and assimilation (Inagaki et al. 2006; Gass et al. 2007). Conjugation of these bile salts occurs at the side chain, where either taurine or glycine is added and leads to the formation of stronger acids (Boyer 2013). Intestinal bacteria can convert the stronger acids producing secondary bile acids, by deconjugating them. Large amounts of bile salts are secreted into the intestine, however only limited amounts are excreted from the human body (Hofmann 1976). More than 95% of the bile salts are reabsorbed in the ileum and redirected to the liver for recirculation. According to this enterohepatic circulation mechanism, each bile salt is recirculated approximately 20 times.

Interestingly, the bile duct was first considered to be generally sterile (Csendes, Fernandez, and Uribe 1975). However, more recently several studies have focused on the microbial community present bile (Ye et al. 2016; Rogers et al. 2017; Shen et al.

2015; P. Pereira et al. 2017; Scheithauer et al. 2009; T. Wu et al. 2013). The phyla Firmicutes, Fusobacteria, Proteobacteria, Actinobacteria and Bacteroidetes (among others), have been identified in bile samples (Rogers et al. 2017; P. Pereira et al. 2017; Ye et al. 2016; T. Wu et al. 2013). These phyla are all commonly found in the human GI tract (Stearns et al. 2011). Subsequently, the bacteria found in the biliary tract are likely to originate from the human duodenum (Ye et al. 2016). The most common genera in bile samples were found to be *Prevotella*, *Streptococcus*, *Veillonella*, *Fusobacterium* and *Haemophilus* (P. Pereira et al. 2017).

To study the function of the biliary microbiota, predictive functional profiles were constructed using Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) (Ye et al. 2016). This method revealed that biliary bacteria have significantly enriched pathways, in comparison to the upper digestive tract microbiota, related to environmental information processing, cell motility, carbohydrate metabolism, amino acid metabolism and lipid metabolism. Furthermore, several studies have focused on the role of the biliary microbiota in the development diseases such as, gallstone disease and biliary neoplasia in primary sclerosing cholangitis (PSC) (T. Wu et al. 2013) (P. Pereira et al. 2017; Belzer et al. 2006; Saltykova et al. 2016). However, to explore the exact role of the microbiota in gallstone formation, more research needs to be conducted. In addition, PSC was not associated with changes in the microbial community of the biliary system (P. Pereira et al. 2017). However, *Streptococcus* species were found to be positively correlated to disease progression and might therefore have a pathogenic role in progression of PSC.

Although the presence of Verrucomicrobia and/or *Akkermansia*-like sequences. has not been described in all studies involving the biliary microbiota, they have been found to be present in a proportion of studies including bile samples (Ye et al. 2016; T. Wu et al. 2013; Rogers et al. 2017; P. Pereira et al. 2017). In a study describing the bacterial community in bile and gallstone samples, *Akkermansia*-like sequences were detected (T. Wu et al. 2013). The relative abundance of *Akkermansia*-like sequences in 12 out of 26 bile samples, ranged between 0.03-0.4%. In addition, 10 out of 29 gallstone samples contained *Akkermansia* spp. with a relative abundance ranging between 0.02 and 0.3%. *Akkermansia*-like sequences were also detected by another study in the bile sample of one out of the six gallstone patients included using V3-4 Illumina sequencing (Ye et al. 2016). The relative abundance of *Akkermansia*-like sequences in this sample

was low; 0.153%. In addition, the mean relative abundance of Verrucomicrobia and *Akkermansia* spp. was determined to be 0.05% and 0.04% respectively, in bile samples from 50 patients undergoing pancreaticoduodenectomy (Rogers et al. 2017). Furthermore, bile samples of subjects with opisthorchiasis (bile duct infection by *Opisthorchis felineus* (Fedorova et al. 2018)) contained higher amounts of Verrucomicrobia (among other phyla) than subjects with gallstone disease without infection of *O. felineus* (Saltykova et al. 2016). The presence of Verrucomicrobia was not specified to the *Akkermansia* genus and the exact relative abundance of Verrucomicrobia is not provided. Even though five phyla including Verrucomicrobia were found to be more abundant in infected subjects than non-infected subjects, there were no functional differences between these groups based on analysis using PICRUSt. The studies described in this section were properly controlled for contaminants.

The pH values of gallbladder bile and common bile duct bile are both within the growth range of *A. muciniphila*. However, for *Akkermansia* spp. to be able to remain in bile, it is expected to harbour a mechanism for protection against bile. A putative bile acid transporter gene (Amuc_0139) is annotated in the genome of *A. muciniphila* that might function to export bile acids from the cell (Van der Ark 2018). This process could reduce the effects of bile acids inside the cell of *A. muciniphila*. Another possible mechanism, as identified in *Bifidobacterium spp.* is the protection of the cell wall against bile acids by the production of exopolysaccharides (Fanning et al. 2012; Ruas-Madiedo et al. 2009). However, this mechanism has not been identified for *Akkermansia muciniphila.*

Several mucins have been identified in the biliary tract, namely in the gallbladder. The mucins that are expressed in the gallbladder are MUC3, MUC5B, MUC5AC and MUC6 (Gum Jr. et al. 1997; Keates et al. 1997; Pigny et al. 1996; Yoo et al. 2016). Even though colonic mucin consists mainly of MUC2, *A. muciniphila* might be able to use the mucins in the gallbladder as a substrate. Next to *Akkermansia* spp. other bacteria with mucin degrading capacities in the GI tract have been identified in biliary system. As such, *Streptococcus anginosus* (Saltykova et al. 2016) and bacteria with OTU ID's with 99% identity to *Bacteroides vulgatus* (T. Wu et al. 2013) were found to be present in bile samples. Although the function of *Akkermansia* spp. in the biliary system is unknown, it might have a role in strengthening of the mucosal barrier. In this way, the mucosal layer may provide increased protection against pathogens. This could explain

the increase of Verrucomicrobia during infection of *Opisthorchis felineus,* since it might function to strengthen the mucosal barrier and thereby provide protection during infection.

Small intestine

The GIT supplies the human body with energy and essential nutrients (Zoetendal et al. 2012). This is done by the conversion and absorption of food components reaching the small intestine. The small intestine can be divided into three segments: duodenum, jejunum and ileum (Savage 1977). The transit time of the small intestine was found to be between 30 minutes and 4.5 hours (Hung, Tsai, and Lin 2006). Once the gastric content enters the duodenum, it is neutralized by bicarbonate derived from the pancreas, liver and duodenal mucosa causing pH fluctuations (Ovesen et al. 1986). More recent investigations of pH profiles revealed that pH values increased from 5.9-6.3 in the proximal part of the small intestine (duodenum) (Koziolek et al. 2015). In the distal parts, pH values were found to increase to pH 7.4-7.8.

The epithelial cells within the small intestine are covered with mucus. In contrast to the mucosal layer in the stomach and the colon, the mucosal layer in the small intestine is thinner and less dense (Atuma et al. 2001). In addition, this layer is not firmly attached to the epithelial surface and forms a soluble mucus gel (Atuma et al. 2001; Johansson et al. 2008). The mucus gel layers observed in the duodenum and jejunum are similar in thickness, although no loose/sloppy mucus was found in the jejunum (Atuma et al. 2001). In contrast to the duodenum and jejunum, the mucus layer in the ileum is significantly thicker. The accumulation rates were similar throughout the different segments of the small intestine.

The fast transit time in comparison to the large intestine, contributes to limited microbial growth in the small intestine . In addition, the secretion of digestive enzymes and bile into the small intestine creates a harsh environment in terms of microbial growth (Zoetendal et al. 2012). The bacterial concentration in the duodenum and jejunum is only 10^3-10^4 bacteria/mL content (Sender, Fuchs, and Milo 2016). This concentration increases in the ileum where the bacterial concentration is 10^8 bacteria/mL content. Due to the challenging conditions for microbial growth in the small intestines (acidity and higher oxygen levels than the colon), the microbial community is dominated by bacteria that are facultative anaerobic, able to grow fast

and able to tolerate bile acids and antimicrobials (Donaldson, Lee, and Mazmanian 2015). At the same time, these bacteria are also competing with the host and other microorganisms for simple sugars in the small intestine. Interestingly, phagocytes in the small intestine are thought to play a role in immune surveillance of the small intestinal mucosa (Morikawa et al. 2016). This means that phagocytes are able to selectively take up bacteria, which might be linked to maintaining immune homeostasis.

The location of the small intestine in the human body causes difficulties in sampling, in comparison to for example the oral and fecal microbiota. Therefore, fewer studies have been performed describing the microbiota in the small intestine (Zoetendal et al. 2012; Sundin et al. 2017). In the duodenum, the phyla Firmicutes and Actinobacteria were found to be predominant in the duodenal fluid of both the obese and healthy groups (n=5 for each group) (Angelakis et al. 2015). Other (less abundant) phyla detected in the duodenum are Proteobacteria, Fusobacteria, TM7, Bacteroidetes and Tenericutes. However, an inter-individual variability in the taxonomic composition between these samples was observed. Even though the duodenum was found to have fewer OTUs than mouth, colon and stool samples, it does harbour most phyla observed in the other sites (Stearns et al. 2011). The mucosa-associated microbiota of the duodenum was found to be dominated by the phylum Firmicutes and genus *Streptococcus* (Shanahan et al. 2016). The genera *Prevotella, Veillonella* and *Neisseria* were also found to be present in the mucosal layer. Interestingly, the duodenal mucosa-associated microbiota found in this study overlaps in broader levels of classification with that of the oral cavity and saliva. Further down the small intestine, the most dominant phyla in jejunal fluid were found to be Firmicutes, Proteobacteria and Bacteroidetes (Sundin et al. 2017). Less abundant phyla (5-10%) were Actinobacteria and Fusobacteria. In comparison to the findings of microbiota composition in the duodenum, the abundance of Proteobacteria and Bacteroidetes were found to be dominant over Actinobacteria in the jejunum (Sundin et al. 2017). When comparing the microbiota composition found in samples obtained after the washing procedure and mucosal biopsies, these compositions were highly similar. The last part of the small intestine (ileum) is dominated by the phyla Bacteroidetes, *Clostridium* cluster XIVa, and Proteobacteria (Zoetendal et al. 2012; E. Li et al. 2012).

Several studies have focused on the small intestinal microbiota in disease states, such as IBS, Crohn's disease and liver cirrhosis (Hartman et al. 2009; Haberman et al. 2014; Dlugosz et al. 2015; Y. Chen et al. 2016; Assa et al. 2016). A study focused on IBS showed that the small intestinal microbiota of IBS patients and healthy individuals did not differ in terms of major phyla or genera (Dlugosz et al. 2015). In contrast, the duodenal mucosal microbiota of cirrhotic patients were surprisingly different to that of healthy controls (Y. Chen et al. 2016). The dysbiosis observed in duodenal samples of cirrhotic patients might be associated with an altered oral microbiota or an altered environment of de duodenum.

Even though the phylum Verrucomicrobia is not included in the dominant microbial compositions described above, Verrucomicrobia and *Akkermansia*-like spp. have been detected in all segments of the small intestinal tract. In duodenal fluid, Verrucomicrobia and *Akkermansia*-like spp. were found in three out of six subjects (with relative abundances of 0.17%, 0.012% and 0.013%) using V3-4 illumina sequencing (Ye et al. 2016). In addition, Verrucomicrobia (not specified to *Akkermansia*) were detected in duodenal biopsies (0.0688%) and mucus (0.0387%) using 454/Roche GS FLX sequencing (G. Li et al. 2015). Jejunal contents also showed to harbour *Akkermansia*-like spp. with a mean relative abundance of 0.01% (n=17) (Rogers et al. 2017). Analysis of swabs from jejunal contents were performed using Illumina MiSeq. Another study detected *Akkermansia*-like spp. in 4 out of 20 subjects with concentrations ranging from 3 to 90 number of hits equalling to 0 to 0.029 percent of total hits, also using Illumina MiSeq (Rogers et al. 2017). Furthermore, Verrucomicrobia were found in the distal ileum using direct cloning and sequencing, making up 5% of the detected microbial community (Mei Wang et al. 2005). Lastly, *Akkermansia*-like sequences were detected in the ileocecal biopsies of patients with PSC and ulcerative colitis, as well as non-inflammatory controls (relative abundances of 0.49±0.52%, 0.37±0.37% and 0.36±0.31% respectively) (Rossen et al. 2015). In the schematic tree in Figure 2a, the *A. muciniphila* sequences derived from ileum biopsies cluster together. This occurs in particular in the third clade, which is made up entirely of *Akkermansia*-like sequences derived from the ileum, with a 16S rRNA sequence identity of 98% in comparison to *A. muciniphila* Muc^T. Isolation of *Akkermansia*-like species in the ileum is needed to study possible differences between the strains found in the small intestine and the strains in the large intestine. Considering the pH in the small intestine, the

Akkermansia-like spp. found in the ileum and other parts of the small intestine could have a different optimum pH for growth.

The function of the small intestinal microbiota has also been studied using comparative metagenomics and RNAseq (Zoetendal et al. 2012). This study, in which *A. muciniphila* was not detected, revealed that the metabolic focus small intestinal microbiota lies within carbohydrate uptake and metabolism. In more detail, simple carbohydrate transport phosphotransferase systems, fermentation, central metabolism, metabolism of amino acids and production of cofactors were enriched. A more recent study emphasizes that the ileum mucosal microbiota might have a role in plant cell wall polysaccharide (PCW) degradation (Patrascu et al. 2017). A large portion of the glycans that reach the small intestine are PCW polysaccharides. These polysaccharides cannot be degraded by humans, whereas the ileal microbiota associated with the mucus layer was found to have enzymatic potential to break down PCW polysaccharides. The exact role of *A. muciniphila* in the small intestine remains unknown, but *Akkermansia*-like spp. could have a role in immune signaling in this part of the GIT. In mice, the addition of *A. muciniphila* in comparison with germ free mice resulted in more significantly modulation in the ileum of PPARα-RXRα activation, tryptophan metabolism, serotonin receptor signaling and dopamine receptor signaling among others (Derrien et al. 2011). The number of differentially expressed genes in the ileum of *A. muciniphila* mono-associated mice was 253 (144 upregulated and 99 downregulated genes). Administration of *A. muciniphila* resulted in an increase of Reg3g expression under control diet and a decrease of Lyz1 expression in the ileum . Another study also showed a decrease in Cnr1 expression and increase of Cldn3 expression in the ileum upon administration of *A. muciniphila* in mice (Plovier et al. 2017). As discussed before, the mucus layer in the small intestine is thinner and less dense than that of the colon (Atuma et al. 2001). This allows closer contact between the microbial community and host cells, promoting immune signaling in this region of the gastrointestinal tract. The presence of *Akkermansia*-like spp. in the ileum might contribute to immune health.

Large intestine

The large intestine is specialized in digestion and consists of several different segments, namely cecum, ascending colon, transverse colon, descending colon, rectum and anus (Macfarlane and Macfarlane 2007). The colonic transit time of healthy individuals is longer than the transit time of the small intestine, ranging between 9-46 hours (mean

28 hours) (Madsen 1992). In healthy individuals, a decrease in luminal pH is observed in the cecum in almost all subjects (pH ranging from 5.5-7.5) (Nugent et al. 2001). This drop in pH is due to the fermentation of carbohydrates by colonic bacteria, leading to the production of short chain fatty acids. Then, the pH increases along the large intestines to pH values ranging between 6.1-7.5. The mucosal pH of the large intestine parallels the luminal pH (McDougall et al. 1993). However, the mucosal pH is less acidic than the luminal pH in all anatomic regions of the large intestine.

The epithelial cells along the large intestine are covered by the mucosal layer (Johansson, Holmén Larsson, and Hansson 2011). As such, the mucosal layer protects the epithelial cells from direct contact with microorganisms. On top of this, the mucosal layer also contains antimicrobial proteins such as IgA. The mucosal layer can be divided into two parts: the inner and outer mucus layer. The inner mucus layer is firmly attached to the epithelial cells and devoid of bacteria, whereas microbes are capable of colonizing the outer layer due to higher permeability (Johansson et al. 2008). Both layers are mainly composed of gel-forming mucin MUC2, consisting of large polymers that are formed by N-terminal trimerization and C-terminal dimerization (Ambort et al. 2012). Expansion of the mucus layer occurs due to an increased pH and decreased calcium (Ca^{2+}) levels. N-terminal interactions are weakened by the decreased calcium concentrations. Therefore, water is able to bind to the mucin domain glycans, leading to the formation of flat mucin sheets. Furthermore, the less dense outer layer is the result of endogenous proteases, promoting the possibility of microbes to colonize the mucus layer (Johansson et al. 2008). The degradation of these mucins by mucin-degrading bacteria of the colon microbiota affects the host cells e.g. by producing SCFA's (Johansson and Hansson 2016).

The gut microbiota is mainly studied using fecal samples, since these samples can be obtained without colonoscopies. However, microbial communities detected in fecal samples mainly reflect the luminal microbiota in the distal large intestine. Therefore, the microbial communities in biopsies can differ distinctly from that in fecal samples (Stearns et al. 2011). Several studies have confirmed that the microbial communities in biopsy samples of different anatomic regions of the colon show similarities focusing on the major phylogenetic groups (Stearns et al. 2011; Mei Wang et al. 2005; Z. Zhang et al. 2014). Along the intestinal tract, Firmicutes and Bacteroidetes were predominant with lower proportions of Proteobacteria and Fusobacteria observed in biopsy samples

(Stearns et al. 2011; Z. Zhang et al. 2014). Sequencing of fecal samples of 22 individuals from four different European countries revealed the presence of three robust clusters, called enterotypes (Arumugam et al. 2011). These enterotypes are either enriched in 1) *Bacteroides,* 2) *Prevotella* and co-occurring *Desulvibrio* or 3) *Ruminococcus* and co-occurring *Akkermansia*. Recently, a method of restricting enterotyping space was proposed to increase the ability to detect samples outside of these enterotyping spaces (Costea et al. 2018). Overall, the dominant phyla in fecal samples derived from healthy individuals are Firmicutes, Bacteroidetes and Actinobacteria. Less abundant phyla are Proteobacteria and Verrucomicrobia (Arumugam et al. 2011).

In addition to studies about the role of the gut microbiota in health, the gut microbiota has also been studied in diseases such as IBD, IBS, obesity and type-2 diabetes. A shift in microbiota composition was observed in IBD patients and may have a role in the onset, maintenance and severity of the disease, although this shift could also partly be due to the disturbed gut environment (Walker et al. 2011). In IBS patients a decrease in bacterial diversity was observed (Codling et al. 2010; Carroll et al. 2012). However, a consistent gut microbiota pattern in IBS patients is lacking (Tap et al. 2017). In obesity inconsistent findings have been reported. For example, one study reported an increase of Firmicutes and decrease of Bacteroidetes while another reported the opposite (Ley et al. 2005; Schwiertz et al. 2010). Lastly, a decrease in canonical butyrate-producing bacteria was found in patients with T2D (Qin et al. 2012). A decrease in butyrate-producing bacteria, was associated with an increase in opportunistic pathogens, mainly Proteobacteria. Taken together, the studies on the gut microbiota in individual diseases are not all uniform highlighting the difficulties in appointing markers for disease.

The gut microbiota plays an important role in metabolism of host nutrients and health maintenance of the host. Carbohydrates (mainly polysaccharides) that have not been hydrolysed in the small intestine become available for the microbial community in the colon (Cummings and Macfarlane 1991). The main substrates entering the colon are resistant starch and polysaccharides derived from plant cell walls. The major end products produced by the gut microbiota are SCFAs (e.g. acetate, propionate and butyrate) and gases (e.g. H_2 and CO_2) using the available substrates. Of these, butyrate is used as energy source by the colonic epithelial cells (Clausen and Mortensen 1995). Furthermore, propionate is able to signal to the host through the GPR41 and GPR43

receptors (Le Poul et al. 2003). Interestingly, short chain fatty acids activate free fatty acid (FFA) receptor 2 and FFA3 in the gut (Le Poul et al. 2003; Brown et al. 2003; N. E. Nilsson et al. 2003). These receptors control peptides (peptide YY and glucagon-like peptide 1) involved in appetite regulation (Flint et al. 2012). Therefore, short chain fatty acids production in the gut may be associated with food intake. Next to dietary carbohydrates, the colonic microbiota also has a role in the degradation of host derived glycans (mucin), xenobiotics and drugs (Cummings and Macfarlane 1991; Possemiers et al. 2011). The gut microbiota is also able to stimulate host immunity in order to protect the host against pathogens (Kamada et al. 2013). In this way, the gut microbiota enhances the innate immune response and has a role in increasing gut barrier function. One of the microbial species in the gut involved in immune regulation and increasing gut barrier function is *A. muciniphila* (Everard et al. 2013; Ottman, Reunanen, et al. 2017).

A recent study revealed the presence of Verrucomicrobiae in all anatomical regions of the large intestine by sequencing the V2 region (Momozawa et al. 2011). The concentrations of Verrucomicrobiae (in two individuals) ranged between 0.3% and 15.8%. Interestingly, one individual harboured only low concentrations ranging between 0.3 and 1.4%, while concentrations in another individual were ranging between 9.8% and 15.8% throughout the anatomic regions. Notably, the individual with higher Verrucomicrobiae concentrations was a Crohn's disease patient. Differences were not only observed between individuals, but also between the experimental designs. The use of another DNA extraction method resulted in lower amounts of Verrucomicrobiae with concentrations ranging between 0.3-7.3% including both individuals. Next to this study, there are more studies identifying the Verrucomicrobia phylum and/or *Akkermansia-like* spp. focusing on several anatomic regions of the large intestine (Stearns et al. 2011; McHardy et al. 2013; Lyra et al. 2012; Hong et al. 2011; Sanapareddy et al. 2012; Mei Wang et al. 2005).Verrucomicrobia were identified in the cecum by another study, although quantities were not shown (McHardy et al. 2013). Even though the pH is lower in the cecum (pH 5.5-7.5), it is still within the growth range of *A. muciniphila*. In the ascending colon, Verrucomicrobia were identified with a concentration of 6% in the large intestine of a healthy volunteer (Mei Wang et al. 2005). Using a qPCR approach within the same region, a concentration of 4.17 +/- 0.6 log 10 genomes per gram of sample of *Akkermansia*-like spp. is described (Lyra et al.

2012). Furthermore, the transverse colon of two out of four included subjects showed the presence of Verrucomicrobia with 563 and 7771 sequence counts of this phylum in each sample (Stearns et al. 2011). In the sigmoid colon compared to the transverse colon, the same study reported a decrease in one of the subjects (from 563 to 64 sequence counts), whereas an increase was noted in another subject (from 7771 to 11941 sequence counts). The qPCR approach resulted in a similar concentration to that found in the ascending colon, namely 4.16+/-0.56 log 10 genomes per gram of sample (Lyra et al. 2012). Several studies have also described the presence of *Akkermansia*-like sequences in the rectum (Stearns et al. 2011; Sanapareddy et al. 2012; Mei Wang et al. 2005). Where one study reported a higher concentration in the rectum (9%) than in the ascending colon, another reported a rapid decrease in sequence counts from 64 and 11941 in the sigmoid colon, to sequence counts of 1 and 2 respectively (Stearns et al. 2011; Mei Wang et al. 2005). In conclusion, based on these studies the presence and abundance of *A. muciniphila* in the large intestine is subject-specific.

To compare the 16S rRNA *Akkermansia*-like sequences in biopsies to fecal samples, a maximum likelihood tree was constructed (Figure S3). Interestingly, the majority of the *Akkermansia*-like sequences derived from biopsies cluster together. This cluster contains a low amount of *Akkermansia*-like sequences derived from fecal samples. Therefore, one could hypothesize that sub-populations of *Akkermansia* spp. exist within the large intestine. However, it should be noted that complete *Akkermansia* spp. genomes derived from biopsies and lumen are needed to support this hypothesis.

In contrast to the presence of *A. muciniphila* in other regions of the GIT, its function has been more explored in its ecological niche. A. *muciniphila* was found to be correlated to health and inversely correlated to several disease states (as explained in the introduction). But next to the presence in health and disease, *A. muciniphila* was also found to be involved in syntrophic interactions (Belzer et al. 2017). For example, co-cultivations of *A. muciniphila* with butyrate producing bacteria (*Anaerostipes caccae, Eubacterium hallii* and *Faecalibacterium prausnitzii*) resulted in butyrate production. Therefore, it is suggested that the mucus-degrading capacity of *A. muciniphila* stimulates intestinal metabolite pool and specifically butyrate levels, which is beneficial for the host. Another example is the release of sulfate during mucin degradation. This sulfate might be used by sulfate reducing bacteria in the colon producing hydrogen sulfide (Derrien 2007; Willis et al. 1996) . In turn *A. muciniphila*

predictively harbours genes involved in L-cysteine biosynthesis using hydrogen sulfide, suggesting *A. muciniphila* might have a role in the detoxification of hydrogen sulfide in the intestines (Ottman, Geerlings, et al. 2017).

As mentioned earlier, colonization of *A. muciniphila* in mice led to an increased expression of genes associated with immune responses and strengthening of the gut barrier function (Everard et al. 2013; Derrien et al. 2011). In addition, the outer membrane protein (Amuc_1100) was found to be involved in immune regulation and enhancement of trans-epithelial resistance (Ottman, Reunanen, et al. 2017). Altogether, these studies suggest an important role of *A. muciniphila* in the microbial community of the large intestine as well as its role in host interactions, promoting the use of this bacterium as a therapeutic agent for intestinal disorders.

Appendix

The human appendix extends from the cecum and is 5-10 cm long and 0.5-1 cm wide (Randal Bollinger et al. 2007). The function of the appendix has been up for debate for quite some time. Charles Darwin described the lack of function of the appendix and noted that the appendix is a remainder from primate ancestors that ingested leaves, in which the appendix functioned to ferment plant material (Darwin 1871; Smith et al. 2013). The appendix is covered in gut-associated lymphoid tissue, suggesting its involvement in immune function (R. J. Berry 1900). An apparent function for the human appendix was described, suggesting that the appendix functions as a "safe house" for beneficial bacteria (Randal Bollinger et al. 2007). This same study revealed a higher abundance of microbial biofilms in the appendix than other areas of the human colon. To our knowledge, a description of the pH in the human appendix is lacking. However, the rabbit appendix was found to have a pH ranging between 6.2-6.7 (Merchant et al. 2011). Secretions of the rabbit appendix are rich in bicarbonate and occur spontaneously and at a relatively rapid rate (1-12 mL/h). Therefore, it has been suggested that in rabbits, the appendix may have a major role in regulation of pH in the cecum. However, similar data is not available for the human appendix.

Considering the difficulty of obtaining samples of the human appendix, there are few studies describing the microbiota of the appendix. These studies mainly focus on the microbiota in appendicitis in comparison to healthy controls (Jackson et al. 2014; Salo et al. 2017; Zhong et al. 2014). In healthy controls, the taxa *Fusibacter*, *Selenomonas,*

and *Peptostreptococcus* were increased in comparison to the rectal microbiota. This finding indicates that the human appendix harbours a distinct microbiota. A wide variation of abundances in phylum, genus and species level was detected within groups divided by health and severity of inflammation (Salo et al. 2017). In healthy controls, Firmicutes and Bacteroidetes were found to be the most abundant phyla. Other abundant phyla detected in these samples were Fusobacteria, Actinobacteria and Proteobacteria. Phyla with lesser abundance (<2%) were Spirochaetes, Cyanobacteria, Synergistetes, Tenericutes and Verrucomicrobia.

While some studies found significant differences between the microbiota of appendicitis patients and healthy controls (Jackson et al. 2014; Zhong et al. 2014) or between severity of inflammation (Guinane et al. 2013), another did not (Salo et al. 2017). One of the genera linked to appendicitis is *Fusobacterium* (Zhong et al. 2014; Guinane et al. 2013). Increased abundances of this genus were observed in appendicitis patients in comparison to healthy controls (Zhong et al. 2014) and the presence of *Fusobacterium* could be linked to the severity of inflammation (Guinane et al. 2013). In contrast, the presence of *A. muciniphila* was found to be inversely correlated to the severity of appendicitis (Swidsinski et al. 2011). Using fluorescence in situ hybridization (FISH), the mean proportion of single bacterial groups for *A. muciniphila* was 4.0±4.6, 1.0±2.1 and 0.2±-0.6 for No appendicitis, catarrhal appendicitis and suppurative appendicitis, respectively.

The mucus layer of the appendix was found to contain a more concentrated biofilm than other parts of the large bowel (Randal Bollinger et al. 2007). Therefore, the appendix might be a favourable niche for mucin-degrading bacteria, including *A. muciniphila*. Although the role of *A. muciniphila* in the appendix is not specified, one could hypothesize that as being part of the appendiceal microbiota, *A. muciniphila* might have a role in re-colonizing the colon after an infection or colonic dysfunction. Thereby, *A. muciniphila* could function in the maintenance of a healthy gut microbiota by restoring mucus barrier function subsequent to infection/inflammation.

Human breast milk and early life intestine

In a breast-fed infant the main source of glycans are human milk oligosaccharides (HMOs) (Bode 2012). Human milk consists of a mixture of nutrients for infants conveying immunologic and other health benefits (Flint et al. 2012). Human milk

contains 5-15 g/l HMOs, and over 200 different HMO structures exist. The major monosaccharides present in HMOs are D-glucose, D-galactose, N-acetyl-glucosamine, L-fucose, and N-acetylneuraminic acid (sialic acid) (Zivkovic et al. 2011). HMOs in the infant gut act as substrates for specific bacteria in the gastrointestinal tract, functioning as natural prebiotics by stimulating the growth of beneficial intestinal bacteria such as bifidobacteria and lactobacilli (Zivkovic et al. 2011; Bidart et al. 2014). It is remarkable to mention that milk oligosaccharides and glycoconjugates are able to prevent the development of pathogens and toxins inhibiting their binding on the surface of the epithelial cells (Jost et al. 2015). The structure of HMOs has chemical similarities to mucus glycans (D S Newburg 2000).

A. muciniphila has been identified in human milk samples after delivery (colostrum), and at 1 and 6 months (Collado et al. 2012). *A. muciniphila* cell counts in breast milk were measured after conducting qPCR revealing *A. muciniphila* was higher in abundance in overweight than normal weight mothers with mean concentrations of 1.25, 1.09 and 1.20 log number of gene copies/ml in colostrum samples and breast milk samples. Furthermore, *A. muciniphila* was observed to be present in colostrum samples that were collected from eleven women after elective caesarean with median counts number of 0.9 (interquartile range from 0.0 to 1.5) analysed by qPCR (Aakko et al. 2017). In turn, in samples from human breast tissue from 43 women (aged 18 to 90) the presence of *Akkermansia*-like species was found using 16S rRNA sequencing (Urbaniak et al. 2014).

As mentioned earlier, *A. muciniphila* is also present in the infant's intestine from the first months of life (Collado et al. 2007; Grzeskowiak, Gronlund, et al. 2012; Grzeskowiak, Collado, et al. 2012). The structures in HMOs can also be found in mucus glycans (Koropatkin, Cameron, and Martens 2012; Tailford, Crost, et al. 2015). *A. muciniphila* was able to break down structures of HMOS into simpler sugars releasing SCFAs (acetate and propionate) in the media. *A. muciniphila* was expressing enzymes that were involved in carbohydrate and glycan degradation such as α-L-fucosidases, exo-α-sialidases, β-galactosidases and β-hexosaminidases (Ottman 2015). This indicates that *A. muciniphila* might be able to use HMOs using human milk as sole energy, carbon and nitrogen source, which could also explain its presence in breast milk and breast tissue of lactating woman.

To confirm this, further research should be conducted to gain more insight in the mucolytic activities and the function of *A. muciniphila* in human milk. The presence of *Akkermansia* spp. and *A. muciniphila* specific in human milk may benefit the maturation of the infant's microbiota establishment and immune maturation, as its outer protein was found to be involved in immune regulation (Ottman, Reunanen, et al. 2017). Last but not least, *A. muciniphila* glycan degradation ability might be proved to play an important role on the initial colonization on infant's gut having in that way a major impact on human's later life.

Akkermansia muciniphila in in vitro gut models

In contrast to invasive sampling of the human body, in vitro models have also been introduced to study the spatial organization of the human gut microbiota. Multiple in vitro models are available for this purpose, such as Gastro-Intestinal Model (TIM-1 and TIM-2) and the Simulator of Human Intestinal Microbial Ecosystem (SHIME). Small intestinal model TIM-1 consists of four compartments representing the stomach, duodenum, jejunum and ileum (Mateo Anson et al. 2009), while TIM-2 simulates the large intestine (Rajilic-Stojanovic et al. 2010). The SHIME was developed in 1993 (Molly, Vande Woestyne, and Verstraete 1993). This model simulates five compartments of the digestive tract, namely the stomach, small intestine and the ascending, transverse and descending colon. In addition, a variation on SHIME was developed, named the mucosal SHIME (M-SHIME) (Van den Abbeele et al. 2012). The M-SHIME has a mucosal compartment, developed to study microbial colonization of the mucus layer.

Most of the studies including *A. muciniphila* used the SHIME model. The presence of *A. muciniphila* in different compartments of in vitro models has also been evaluated (Van Herreweghen et al. 2017; Van den Abbeele et al. 2010). In SHIME, *Akkermansia* spp. are more abundantly present in the transverse and descending colon compartments than proximal compartments (ascending colon) of this model (Van Herreweghen et al. 2017; Kemperman et al. 2013; Garcia-Villalba et al. 2017; Van den Abbeele et al. 2010). This is not in concordance with findings using biopsy samples, where no clear depletion of Verrucomicrobiae was observed in the ascending colon (Momozawa et al. 2011). Another SHIME experiment also described the distal location of *Akkermansia* spp. (Kemperman et al. 2013). In addition, in this model the growth of *Akkermansia*-like spp. was stimulated by black tea and red wine grape extract. Interestingly in M-

SHIME, *A. muciniphila* did not reach high densities as was observed in distal compartments in the SHIME setup (Van Den Abbeele et al. 2013). This might be due to the setup of the M-SHIME model, which is lacking distal colon compartments, where *Akkermansia*-like spp. reached highest densities (Van den Abbeele et al. 2010; Kemperman et al. 2013; Garcia-Villalba et al. 2017). Recently, a study using the SHIME model demonstrated that *A. muciniphila* is pH and mucin dependent (Van Herreweghen et al. 2017). An increase of *A. muciniphila* was observed upon addition of mucin. When the pH in the distal colon was lowered, a decrease in *A. muciniphila* was observed in comparison to the same compartment with a higher pH. Altogether, these studies suggest that these models can be used to study the effect of environmental parameters and diet on the human gut microbiota in health and disease states.

Conclusion

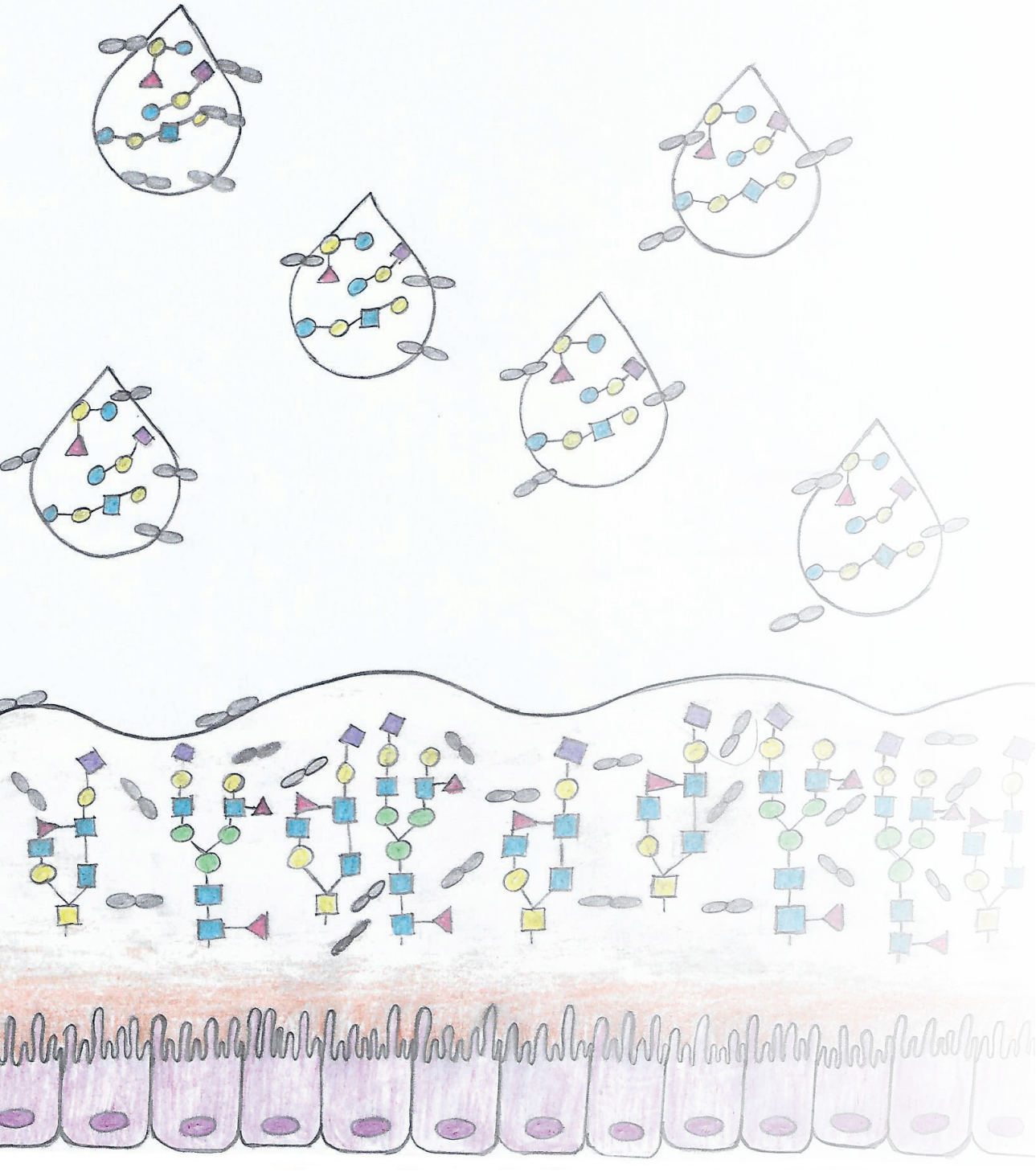
Up until now, the presence of *A. muciniphila* has mainly been associated with the mucus layer of the colon. However, in this review we have collected results from other studies and showed that *Akkermansia*-like sequences were also found to be present in other anatomical regions of the digestive tract and human breast milk. In short, these regions are the oral cavity, pancreas, bile ducts and gallbladder, the small intestine, large intestine and the appendix (Figure 2b). The environmental parameters (e.g. pH, oxygen and nutrient availability) differ among anatomic regions of the human body, affecting the growth of *A. muciniphila*. As the aforementioned organs have different functions, the function of *A. muciniphila* might also differ in different regions of the digestive tract. In this review, we proposed hypothetical functions of *A. muciniphila* in these regions, but research is needed to confirm its role among different regions of the digestive tract. Altogether, the presence of *Akkermansia*-like spp. along the digestive tract indicates this bacterium might have more functions than is known so far. However, as can be concluded from abundance of *Akkermansia*-like spp. the optimal ecological niche remains to be the mucus layer in the colon.

Acknowledgments

We would like to thank Asimenia Gavriilidou and Daan van Vliet who provided insight and expertise in the phylogenetic analysis. The work was supported by the Netherlands Organization for Scientific Research (SIAM Gravity Grant 024.002.002) and the EU Joint Programming Initiative – A Healthy Diet for a Healthy Life (JPI HDHL, http://www.healthydietforhealthylife.eu).

Supplementary Materials

The following are available online at www.mdpi.com/link, Figure S1: Detailed maximum likelihood phylogenetic tree (RaxML) including *Akkermansia*-like sequences derived from large intestine and ileum biopsies and Chinese *A. muciniphila* strains. Figure S2: Detailed Neighbor Joining phylogenetic tree including *Akkermansia*-like sequences derived from large intestine and ileum biopsies and Chinese *A. muciniphila* strains. Figure S3: Detailed maximum likelihood phylogenetic tree (RaxML) including *Akkermansia*-like sequences derived from fecal samples, large intestine, ileum biopsies and Chinese *A. muciniphila* strains.



Chapter 3

Akkermansia muciniphila uses human milk oligosaccharides to thrive in the early life conditions *in vitro*

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Abstract

Akkermansia muciniphila is a well-studied anaerobic bacterium specialized in mucus degradation and associated with human health. Because of the structural resemblance of mucus glycans and free human milk oligosaccharides (HMOs), we studied the ability of *A. muciniphila* to utilize human milk oligosaccharides. We found that *A. muciniphila* was able to grow on human milk and degrade HMOs. Analyses of the proteome of *A. muciniphila* indicated that key-glycan degrading enzymes were expressed when the bacterium was grown on human milk. Our results display the functionality of the key-glycan degrading enzymes (α-L-fucosidases, β-galactosidases, exo-α-sialidases and β-acetylhexosaminidases) to degrade the HMO-structures 2'-FL, LNT, lactose, and LNT2. The hydrolysation of the host-derived glycan structures allows *A. muciniphila* to promote syntrophy with other beneficial bacteria, contributing in that way to a microbial ecological network in the gut. Thus, the capacity of *A. muciniphila* to utilize human milk will enable its survival in the early life intestine and colonization of the mucosal layer in early life, warranting later life mucosal and metabolic health.

Keywords: *Akkermansia muciniphila,* microbiota, Human Milk Oligosaccharides (HMOs), early life, Fucosyllactose (FL), Sialyllactose (SL)

Introduction

Akkermansia muciniphila is a Gram-negative anaerobe, belonging to the phylum Verrucomicrobia (Derrien et al. 2010), that colonizes the mucus layer of the human gastrointestinal (GI) tract (Derrien et al. 2004). *A. muciniphila* is associated with a healthy mucosal layer and metabolic state as it has been inversely correlated with obesity (Dao, Everard, Aron-Wisnewsky, et al. 2016; Karlsson et al. 2012), metabolic diseases (Type 2 diabetes) (X. Zhang et al. 2013) as well as intestinal disorders (inflammatory bowel disease (IBD) and appendicitis) (Png et al. 2010; Rajilić-Stojanović et al. 2013; Swidsinski et al. 2011). This intestinal bacterium has an extraordinary capacity to degrade mucin as the sole energy, carbon and nitrogen source and convert this polymer into mostly acetate and propionate (Derrien et al. 2004).

A. muciniphila is a common member of the adult and infant microbiota (Derrien et al. 2008). The infant's gut colonisation with *A. muciniphila* has been detected from the first month of life, with a continuously increasing abundance during adulthood (Derrien et al. 2008; Collado et al. 2007). Additionally, *A. muciniphila* was detected in the breast tissue of lactating mothers as well as in human milk (Collado et al. 2012; Aakko et al. 2017; Urbaniak et al. 2014). Interestingly, two studies have reported that *A. muciniphila* was found to be lower in abundance in the breast-fed infants compared to formula-fed infants (Azad et al. 2013; Bergström et al. 2014). In a more recent study with 98 Swedish infants though, the abundance and the prevalence of *A. muciniphila* increased between 4 and 12 months old, and showed no significant change depending on delivery mode or type of feeding (Bäckhed et al. 2015). Early in life, mother's milk is often the only source of nutrients and dietary glycans for infants, and it is considered the best nourishment for the development of the new-born (Neville et al. 2012). The glycans in human milk are named human milk oligosaccharides (HMOs), and they have proven to have an impact on infant intestinal microbiota composition (Koropatkin, Cameron, and Martens 2012). Human milk contains 5-15 g/l HMOs, with more than 200 different HMO structures reported of which 100 have been successfully elucidated (Ninonuevo et al. 2006; Ruiz-Palacios et al. 2003; Stahl et al. 1994; Urashima et al. 2018). The presence and the quantity of these HMOs structures vary per individual and are related to the genetic Secretor and Lewis status of the mother (Ayechu-Muruzabal et al. 2018). The major building blocks of monosaccharides present in HMOs are D-glucose (Glc), D-galactose (Gal), *N*-acetyl-glucosamine (GlcNAc), L-fucose (Fuc), and *N*-

acetylneuraminic acid (sialic acid, Neu5Ac) (Zivkovic et al. 2011). These sugars form a number of complex glycans containing well-defined different glycosidic linkages resulting in both linear and branched structures (S. Wu et al. 2010). In human milk 70% of the oligosaccharides are fucosylated and 30% are sialylated (Ninonuevo et al. 2006; Weiss and Hennet 2012). HMOs can function as prebiotic substrates by promoting and stimulating the growth of beneficial bacteria such as bifidobacteria (Bode 2012). Nowadays, supplementation of infant formulae with HMOs, such as 2'-FL and LNnT, is gaining more and more interest to bring infant formula composition even closer to human milk (Vandenplas et al. 2018). In addition, some of these oligosaccharide molecules inhibit the colonisation of pathogenic bacteria by acting as receptor analogues and binding to the bacterial surface (Zivkovic et al. 2011).

The resemblance of glycosidic structure between HMOs and mucin glycans might explain why some bacteria are capable of utilising both human milk glycans and host mucosal glycans (mucins) (Garrido, Dallas, and Mills 2013; Tailford, Crost, et al. 2015). Mucins are the main structural components of the mucus layer that covers the gut epithelium surface. Mucins' protein core consists of 80% carbohydrates, mainly *N*-acetylgalactosamine, *N*-acetylglucosamine, fucose, galactose and sialic acid (Bansil and Turner 2006; Abodinar et al. 2016). The mucus layer in the human gut is divided into an outer layer, which provides a nutrient-rich habitat for the microbiota, and an inner layer, which is firmly attached to the surface of the epithelium and virtually free of bacteria (Johansson et al. 2008). In the human gut, *A. muciniphila* has the extraordinary capacity to degrade mucins by employing a large arsenal of sulfatases and glycoside hydrolases (GH) for effective metabolism of mucin glycans such as α-fucosidases, α-sialidases, β-galactosidases, β-acetylhexosaminidases, and α-acetylglucosaminidases (Derrien et al. 2010; Ottman, Davids, et al. 2017).

We hypothesize that the presence of *A. muciniphila* in the early life intestine is the result of its ability to use is mucin degrading machinery to breakdown HMOs. Its presence in the early life intestine will enhance microbial ecologic network formation and healthy microbial colonisation of the mucosal layer warranting later life health. To assess this, we tested the ability of *A. muciniphila* to grow on human milk and different HMOs. Subsequently, we identified the HMOs structures that *A. muciniphila* was able to break down and the enzymes responsible for the degradation.

Results

A. muciniphila grows on human milk via HMOs utilisation

Incubation of *A. muciniphila* on human milk resulted in growth (Figure 1a). The fermentation profile of the cultures showed production of acetate, propionate, and succinate (Figure 1b,c) as well as release of glucose and galactose due to the utilisation of the lactose that is already present in the breast milk and in the HMOs (Figure 2a).

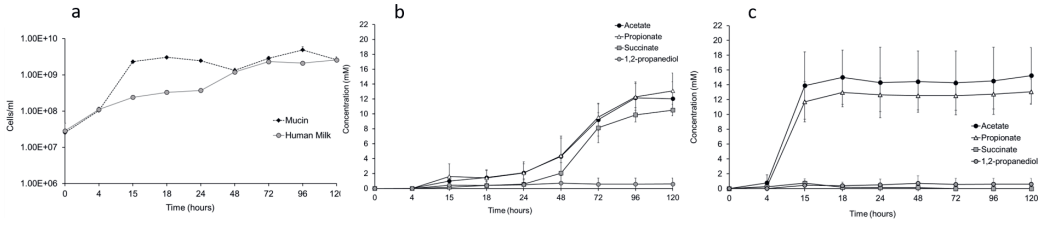

Figure 1: *A. muciniphila* growth in human milk. a) *A. muciniphila* growth in human milk or porcine mucin as the sole carbon and nitrogen source. Error bars indicate the standard error of qPCR for three biological replicates. *A. muciniphila* SCFA production b) in human milk and c) in mucin. Error bars indicate the standard deviation of three biological replicates.

The amounts of produced short-chain fatty acids (SCFAs) and utilised sugars are shown in the Supplementary Table 2. We next sought to investigate which HMOs structures were utilised by *A. muciniphila* during the growth on human milk. The HMOs profile showed utilisation of neutral trioses (2'-fucosyllactose [2'-FL] and 3-fucosyllactose [3-FL]), tetraoses (difucosyllactose [DFL], lacto-N-tetraose [LNT], lacto-N-neotetraose [LNnT]), pentaoses (lacto-fucopentaose I [LNFP I], lacto-fucopentaose II [LNFP II], lacto-fucopentaose III [LNFP III], lacto-fucopentaose V [LNFP V]), and acidic trioses (3'-siallylactose [3'-SL], 6'-sialyllactose [6'-SL]) (Figure 3). All measured HMOs were reduced at least 2-fold (Figure 3). The HMO profile analysis revealed that *A. muciniphila* utilises almost completely 2'-FL and 3'-SL present in human milk (99.65% and 97.49% respectively) (Figure 3).

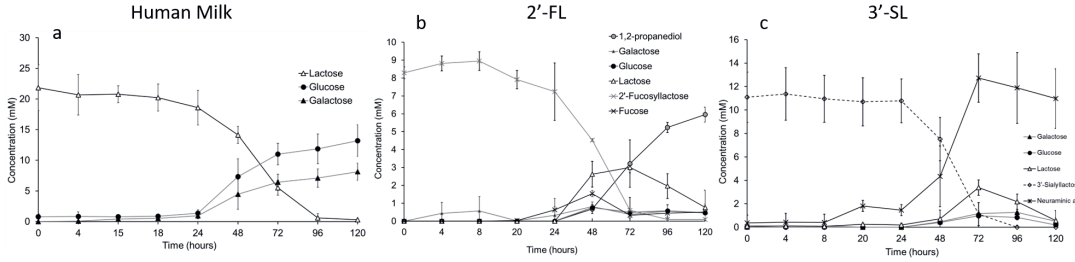

Figure 2: *A. muciniphila* HMO degradation. a) Human Milk, b) 2'-fucosyllactose (2'-FL), and c) 3'-sialyllactose (3'-SL). Error bars represent the standard deviation of three biological replicates.

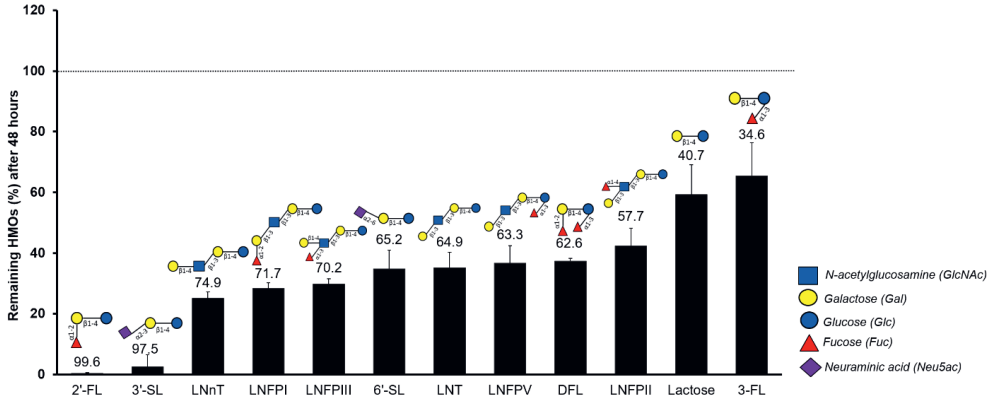

Figure 3: Utilisation of HMOS structures by *A. muciniphila* incubated in 10% human milk. The numbers above each bar indicate the degradation percentage of each HMO. Error bars represent the error propagation of three biological replicates.

Next, it was tested whether these nutritional components by themselves could promote the growth of *A. muciniphila*. Incubation of *A. muciniphila* with pure 2'-FL or 3'-SL (10 mM) resulted in growth (Supplementary Figure 1) and demonstrated the release of lactose from both 2'-FL or 3'-SL (Figure 2b,c). The resulting lactose was further broken down to its monosaccharides (glucose and galactose). Growth on 2'-FL resulted in the production of 1,2-propanediol (5.95 ± 0.42 mM), which is an indication of the fucose metabolism by *A. muciniphila*. The liberated neuraminic acid from 3'-SL was not further catabolised by the bacterium. The overall fermentation efficiency was determined by calculating the carbon balance; the recovery of carbon atoms at 48 h of 2'-FL and 3'-SL was 86.58 and 82.58% respectively (Supplementary Table 3a). Furthermore, the amounts of produced short-chain fatty acids (SCFAs) and utilised

sugars of *A. muciniphila* grown in 2'-FL and 3'-SL are shown in the Supplementary Table 3b.

A. muciniphila exhibits glycoside hydrolase expression during growth on human milk

We performed proteomic analysis on the *A. muciniphila* cultures grown on human milk to identify the active enzymes that could contribute to the degradation of human milk and its components. A total of 832 bacterial proteins were detected after growth on human milk. We mined the proteome data for proteins predicted to participate in carbohydrate metabolism and this returned 109 proteins and over half of them (62 proteins) are primarily involved in this specific metabolic pathway (Table 1). *A. muciniphila* possesses 58 proteins that encode for glycoside hydrolases (GHs). In our results, we detected 43 GHs and 19 of these proteins belong to the six GH families (GH2, GH20, GH29, GH33, GH35 and GH95) that target the most common linkages found within HMOs. We identified four β-galactosidases that belong to GH2 family, two GH35 β-galactosidases, seven β-hexosaminidases from GH family 20, two GH29 α-fucosidases, two GH95 α-fucosidases and two exo-alpha sialidases from GH33 (Table 1). Therefore, we proposed *A. muciniphila* employed the fucosidases to release the terminal α1-2/3/4 linked fucose from the fucosylated HMOs; 2'-FL (Fucα1-2Galβ1-4Glc), 3-FL (Galβ1-4Fucα1-3Glc), DFL (Fucα1-2Galβ1-4 Fucα1-3Glc), LNFPI (Fucα1-2Galβ1-3GlcNAcβ1-3Galβ1-4Glc), LNFP II (Galβ1-3Fucα1-4GlcNAcβ1-3Galβ1-4Glc), LNFP III (Galβ1-4Fucα1-3GlcNAcβ1-3Galβ1-4Glc) and LNFP V (Galβ1-3GlcNAcβ1-3Galβ1-4Fucα1-3Glc). Additionally, *A. muciniphila* expressed the 2 sialidases that can liberate the α2-3 and α2-6 linked sialic acid from the sialylated glycans 3'-SL (Neu5Acα2-3Galβ1-4Glc) and 6'-SL (Neu5Acα2-6Galβ1-4Glc) present in the human milk. Furthermore, the hydrolysis of the terminal β1-3 and β1-4 galactose residues found in the HMOs structures; LNT (Galβ1-3GlcNAcβ1-3Galβ1-4Glc), LNnT (Galβ1-4GlcNAcβ1-3Galβ1-4Glc), lactose (Galβ1-4Glc), as well as LNFP II, LNFP III and LNFP V can be the result of β-galactosidases' action expressed by *A. muciniphila* during growth on human milk. *A. muciniphila* expresses also 7 β-hexosaminidases which are responsible for releasing the terminal β1-3 and β1-4 *N*-acetylglucosamine (GlcNAc) bound to glycan structures. However, GlcNAc is usually found as terminal sugar in HMO glycans but rather is often decorated by other monosaccharides (galactose, fucose, sialic acid). Thus, *A. muciniphila* might use its hexosaminidases to

degrade simpler HMOs such as Lacto-*N*-biose (Galβ1-3GlcNAc), LNT2 (GlcNAcβ1-3Galβ1-4Glc). In addition, *A. muciniphila* expressed some of the necessary enzymes to metabolise the liberated monosaccharides from the HMOs degradation. *A. muciniphila* employs, for example, Amuc_1833 (L-fucose transporter-fucP), Amuc_1832 (L-fucose isomerase-fucI), Amuc_1830 (L-fuculokinase-fucK), and Amuc_1829 (class II aldolase-fucA) for the utilisation of the liberated fucose to 1,2-propanediol. Furthermore, Amuc_0969 (Galactokinase) participates in the first steps of the galactose metabolism by converting the free galactose into α-D-galactose-1-phosphate, while Amuc_0097 (ROK family protein) can be used by *A. muciniphila* to convert glucose to α-D-glucose-6-phosphate during the first step of the glycolysis pathway (Supplementary Table 4). Nevertheless, *A. muciniphila* lacks all the necessary enzymes for the sialic acid utilisation, since no expression of these enzymes was observed in the proteome data. These data demonstrate that *A. muciniphila* has the enzymatic capacity to utilise a broad range of HMOs as well as their constituents.

Table 1: Abundance of *A. muciniphila* enzymes involved in carbohydrate metabolism with their corresponding KEGG identifier (KO ID). The average of Log10 transformed LFQ values is shown. Colouring is based on abundance from the most abundant (red) to medium abundant (orange) to least abundant (green).

CAZy	Locus tag	Protein	LFQ	KO ID	Pathway
GH20	Amuc_2136	Glycoside hydrolase, family 20	8,64	ko00511 ko00520	Other glycan degradation Amino sugar and nucleotide sugar metabolism
GH2	Amuc_0824	Glycoside hydrolase family 2 TIM barrel	8,55	ko00052 ko00511	Galactose metabolism Other glycan degradation
GH2	Amuc_0290	Glycoside hydrolase family 2 sugar binding	8,43	ko00052 ko00511	Galactose metabolism Other glycan degradation
GT35	Amuc_0235	Alpha-glucan phosphorylase	8,42	ko00500	Starch and sucrose metabolism
GH29	Amuc_0392	Coagulation factor 5/8 type domain protein	8,23	ko00511	Other glycan degradation
CE11	Amuc_1918	Beta-hydroxyacyl-(Acyl-carrier-protein) dehydratase FabZ	8,21	ko00061 ko00540	
	Amuc_0155	Phosphoglucomutase/phosphomannomutase alpha/beta/alpha domain I	8,19	ko00051 ko00520	Fructose and mannose metabolism Amino sugar and nucleotide sugar metabolism
GH31	Amuc_1008	Glycoside hydrolase family 31	8,16		
GH109	Amuc_0017	Glycosyl hydrolase family 109 protein 1	8,14		
GH84	Amuc_0052	Hyaluronon glucosaminidase	8,09	ko00531	Glycosaminoglycan degradation
CE9	Amuc_0948	N-acetylglucosamine-6-phosphate deacetylase	7,84	ko00520	Amino sugar and nucleotide sugar metabolism
GT4	Amuc_1869	Glycosyl transferase group 1	7,83		
GH2	Amuc_0539	Glycoside hydrolase family 2 sugar binding	7,76		
	Amuc_1436	Malate dehydrogenase	7,74	ko00020 ko00620 ko00630 ko00680	Citrate Cycle (TCA cycle) Pyruvate metabolism Glycoxylate and dicarboxylate metabolism Methane metabolism
GH89	Amuc_1220	Alpha-N-acetylglucosaminidase	7,73		
GH95	Amuc_1120	Putative uncharacterized protein	7,72	ko00511	Other glycan degradation
GH36	Amuc_0216	Putative uncharacterized protein	7,66		
GH13	Amuc_1751	Glycoside hydrolase family 13 domain protein	7,65	ko00500	Starch and sucrose metabolism
	Amuc_1543	Formate acetyltransferase	7,54	ko00620 ko00640 ko00650	Pyruvate metabolism Propanoate metabolism Butanoate metabolism
GH57	Amuc_1868	Glycoside hydrolase family 57	7,42	ko00500	Starch and sucrose metabolism
GH35 CBM32	Amuc_1686	Beta-galactosidase	7,37	ko00052	Galactose metabolism
GH110 CBM51	Amuc_0480	Alpha-1,3-galactosidase B	6,90		
GH33	Amuc_0625	Exo-alpha-sialidase	6,89	ko00511	Other glycan degradation
GH98	Amuc_1438	Glycosyl hydrolase family 98 putative carbohydrate binding module	6,86		
GH35	Amuc_0771	Beta-galactosidase	6,79	ko00052 ko00511	Galactose metabolism Other glycan degradation
GH16	Amuc_0724	Glucan endo-1,3-beta-D-glucosidase	6,73		
GH33	Amuc_1835	Exo-alpha-sialidase	6,70	ko00511	Other glycan degradation
GH123 CBM32	Amuc_0803	Coagulation factor 5/8 type domain protein	6,66		
CBM50	Amuc_0821	Peptidoglycan-binding LysM	6,30		
GH20	Amuc_1032	Beta-N-acetylhexosaminidase	6,29		
CE4	Amuc_1500	Polysaccharide deacetylase	6,27		
GH95	Amuc_0186	Glycoside hydrolase family 95	6,24		
GH89	Amuc_0060	Alpha-N-acetylglucosaminidase	6,18		
GT28	Amuc_0659	UDP-N-acetylglucosamine-N-acetylmuramyl-(pentapeptide) pyrophosphoryl-undecaprenol N-acetylglucosamine transferase	6,18	ko00550	Peptidoglycan biosynthesis
GH77	Amuc_1621	4-alpha-glucanotransferase	6,14	ko00500	Starch and sucrose metabolism
GT51	Amuc_2122	Glycosyl transferase family 51	6,09		
	Amuc_0756	ADP-L-glycero-D-manno-heptose-6-epimerase	6,01	ko00540	Lipopolysaccharide biosynthesis
	Amuc_1880	Isocitrate dehydrogenase [NADP]	5,97	ko00020 ko00480	Citrate Cycle (TCA cycle) Glutathione metabolism
	Amuc_0523	KpsF/GutQ family protein	5,91	ko00540 ko01100	Lipopolysaccharide biosynthesis Metabolic pathways

GH16	Amuc_2108	Glycoside hydrolase family 16	5,86		
GH88	Amuc_0863	Glycosyl hydrolase family 88	5,86		
GH16	Amuc_0875	Glycoside hydrolase family 16	5,71		
GH43	Amuc_0698	Beta-glucanase	5,65		
GH20	Amuc_1669	Beta-N-acetylhexosaminidase	5,52	ko00511 ko00520	Other glycan degradation Amino sugar and nucleotide sugar metabolism
GH27	Amuc_1187	Alpha-galactosidase	5,47	ko00052 ko00561	Galactose metabolism Glycerolipid metabolism
	Amuc_1572	2-methylcitrate synthase/citrate synthase II	5,43	ko00020 ko00630	Citrate Cycle (TCA cycle) Glycoxylate and dicarboxylate metabolism
	Amuc_1756	Phosphoenolpyruvate-protein phosphotransferase	5,42	ko02060	Phosphotransferase system (PTS)
GH20	Amuc_0369	Beta-N-acetylhexosaminidase	5,41	ko00511 ko00520	Other glycan degradation Amino sugar and nucleotide sugar metabolism
GH20	Amuc_1924	Beta-N-acetylhexosaminidase	5,41		
	Amuc_1242	Inositol-phosphate phosphatase	5,24	ko00521 ko00562	Streptomycin biosynthesis Inositol phosphate metabolism
GH29	Amuc_0010	Alpha-L-fucosidase	5,00	ko00511	Other glycan degradation
GH36	Amuc_0517	Raffinose synthase	5,00		
GH43	Amuc_0697	Beta-glucanase	5,00		
GT2	Amuc_0757	Glycosyl transferase family 2	5,00		
GH109	Amuc_0920	Glycosyl hydrolase family 109 protein 2	5,00		
GH97	Amuc_1420	Putative uncharacterized protein	5,00		
GT2	Amuc_1582	Glycosyl transferase family 2	5,00		
	Amuc_1616	Polysaccharide deacetylase	5,00		
GH13	Amuc_1637	Alpha amylase catalytic region	5,00		
GH2 BM32	Amuc_1667	Glycoside hydrolase family 2 sugar binding	5,00		
GH20	Amuc_1815	Beta-N-acetylhexosaminidase	5,00		
GH20	Amuc_2018	Beta-N-acetylhexosaminidase	5,00	ko00511 ko00520 ko00531	Other glycan degradation Amino sugar and nucleotide sugar metabolism

Characterization of *A. muciniphila* HMO degrading enzymes

The next step of the study was concerned with the characterisation of *A. muciniphila* enzymes that are effective in HMO-degradation. Therefore, we assessed five glycan-degrading enzymes identified by proteomics that are also predicted to hydrolyse the glycosidic bonds of lactose and the HMOs; 2'-FL, LNT and LNT2 (Table 1); one α-fucosidase (Amuc_0010), two β-galactosidases (Amuc_0771 and Amuc_1686) and two β-acetylhexosaminidases (Amuc_0369 and Amuc_2136). The enzymatic activity of the recombinant proteins was first assessed using synthetic substrates (Table 2). Amuc_0771 and Amuc_2136 showed a pH optimum of 5.0, while Amuc_0010 and Amuc_0369 displayed optimal pH at 5.6. For Amuc_1686 the rate of pNP release (μM min^{-1}) was similar for all the different pH values that were tested (Supplementary Figure 2). pH 5.0 was selected as the optimal pH for this enzyme. Each enzyme's optimal pH and temperature of 37ºC were used to assess the kinetic parameters of these proteins against synthetic substrates pNP-α-L-Fuc, pNPG and GlcNAc-β-pNP (Table 2 and Supplementary Figure 3). The α-L-fucosidase (Amuc_0010) showed a K_M 839 ±

46.72 μM against pNP-Fucose. Both β-acetylhexosaminidases (Amuc_0369 and Amuc_2136) exhibited hexosaminidase activity by cleaving GlcNAc from pNP-GlcNAc. A lower K_M for Amuc_0369 (322.37 ± 43.64 μM) was observed than Amuc_2136 (714.55 ± 47.96 μM). Cleavage of galactose from pNPG was detected with β-galactosidases (Amuc_0771 and Amuc_1686). Amuc_0771 showed higher K_M (2599 ± 565.27 μM) than Amuc_1686 (319.40 ± 259.30 μM). The ability of the α-L-fucosidase (Amuc_0100) for cleaving α1-2 linked fucose from 2'-FL (Fucα1-2Galβ1-4Glc) was assessed (Table 2 and Supplementary Figure 4). The enzyme exhibited a high K_M and lower activity (152.08 min^{-1} k_{cat}) against 2'-FL than pNP-Fuc (2.27 x 10^4 min^{-1} k_{cat}). The activity of β-acetylhexosaminidases (Amuc_0369 and Amuc_2136) on the HMOs, LNT (Galβ1-3GlcNAcβ1-3Galβ1-4Glc) and LNT2 (GlcNAcβ1-3Galβ1-4Glc) was assessed as well. The substrate and product specificity were monitored by HPAEC-PAD. For both enzymes no liberation of GlcNAc was observed in the presence of LNT, while both *A. muciniphila* β-acetylhexosaminidases were able to cleave the terminal GlcNAc off lactose resulting in LNT2. Amuc_0369 exhibited a k_{cat} 1.42 x 10^4 min^{-1} and a K_M 3980 ± 210.30 μM against LNT2. Amuc_2136 showed higher substrate affinity than Amuc_0369 (2435.82 ± 289.51 μM K_M) but no significant difference in the catalytic activity (1.45 x 10^4 min^{-1} k_{cat}). The β-galactosidases (Amuc_0771 and Amuc_1686) were tested for their capability to break down galactosidic linkages from the HMO, LNT (Galβ1-3GlcNAcβ1-3Galβ1-4Glc) and lactose (Galβ1-4Glc). Amuc_0771 showed a k_{cat} 4.42 x 10^3 min^{-1} and a K_M 1223 ± 171.7 μM against LNT (β1-3) (Table 2). In addition, Amuc_0771 was able to release 711 μM of glucose and 936 μM of galactose when was incubated in 2000 μM of lactose overnight (Supplementary Figure 5). However, the reaction in lactose was slow and therefore we were not able to assess the kinetics of Amuc_0771. The enzyme displayed cleaving capacity for both β1-3 and β1-4 glycosidic linkages, but with higher substrate specificity towards β1-3 linkages. The other β-galactosidase (Amuc_1686) showed no enzymatic activity against LNT or lactose. *E. coli* BL21 Rosetta strain harbouring the pCDF1-b empty vector induced with IPTG was used as negative control and it was incubated overnight in 2 mM LNT, 2'-FL, lactose and 5 mM LNT2 showing no activity at all, against these substrates (Supplementary Figure 7).

Table 2: Kinetic parameters of α-L-fucosidase (Amuc_0010), β-hexosaminidases (Amuc_0369 and Amuc_2136) and β-galactosidases (Amuc_0771 and Amuc_1686) with synthetic substrates and HMOS.

α-L-fucosidase	**Amuc_0010**				
	Substrate	**Vmax (μM min^{-1})**	**k_{cat} (min^{-1})**	**K_M (μM)**	**k_{cat}/K_M (min^{-1} μM^{-1})**
	PNP-Fucose	19.76 ± 11.30	2,27E+04	841.23 ± 46.72	27,07
	2'-fucosyllactose	25.13 ± 6.80	152,08	$1.03 \times 10^4 \pm 6.98 \times 10^3$	0,02
	Amuc_0369				
β-hexosaminidases	**Substrate**	**Vmax (μM min^{-1})**	**k_{cat} (min^{-1})**	**K_M (μM)**	**k_{cat}/K_M (min^{-1} μM^{-1})**
	PNP-GlcNAc	55.64 ± 2.02	4.02×10^4	323.38 ± 43.64	124,50
	Lacto-N-triose II	982.61 ± 37.68	1.42×10^4	3980 ± 210.30	3,56
	Amuc_2136				
	Substrate	**Vmax (μM min^{-1})**	**k_{cat} (min^{-1})**	**K_M (μM)**	**k_{cat}/K_M (min^{-1} μM^{-1})**
	PNP-GlcNAc	109.46 ± 4.22	8.93×10^4	714.55 ± 47.96	125,10
	Lacto-N-triose II	443.75 ± 47.20	1.45×10^4	2435.82 ± 289.51	5,95
	Amuc_0771				
β-galactosidases	**Substrate**	**Vmax (μM min^{-1})**	**k_{cat} (min^{-1})**	**K_M (μM)**	**k_{cat}/K_M (min^{-1} μM^{-1})**
	PNP-Galactose	60.08 ± 7.02	825,63	2599 ± 565.27	0,34
	Lacto-N-tetraose	302.08 ± 53.71	4.42×10^3	1223 ± 171.70	3,61
	Amuc_1686				
	Substrate	**Vmax (μM min^{-1})**	**k_{cat} (min^{-1})**	**K_M (μM)**	**k_{cat}/K_M (min^{-1} μM^{-1})**
	PNP-Galactose	29.34 ± 3.80	1.63×10^3	319.40 ± 259.30	4,21

A. muciniphila expresses mucus-utilisation enzymes to consume human milk oligosaccharides

A. muciniphila is adept at mucus glycans degradation and we showed in this study that *A. muciniphila* was able also to utilise human milk glycans. Thus, we investigated whether the enzymatic capacity is adapted to the different environmental conditions. Consequently, we compared the protein expression profile between *A. muciniphila* grown on human milk and grown on mucin.

First, we tested the activity of *A. muciniphila* cell lysates by measuring α-fucosidase, β-galactosidase and sialidase activities from cultures grown in either human milk or mucin. The lysates from mucin- and human milk cultures were incubated with synthetic substrates and both demonstrated fucosidase, β-galactosidase and sialidase activity. Human milk cell lysates showed significantly higher β-galactosidase and sialidase activity than in mucin lysates (Figure 4 a,b). Fucosidase activity was similar for both human milk and mucin (Figure 4c). The cell lysates were also incubated with lactose or 2'-FL. Samples were taken at 0, 20 h and analysed by HPAEC-PAD. The lysates from *A. muciniphila* grown both on human milk and mucin showed partial degradation of lactose into its constituent monosaccharides (glucose and galactose) and partial degradation of 2'-FL into lactose, glucose, galactose and fucose (Supplementary Figure 6 a,b,c,d).

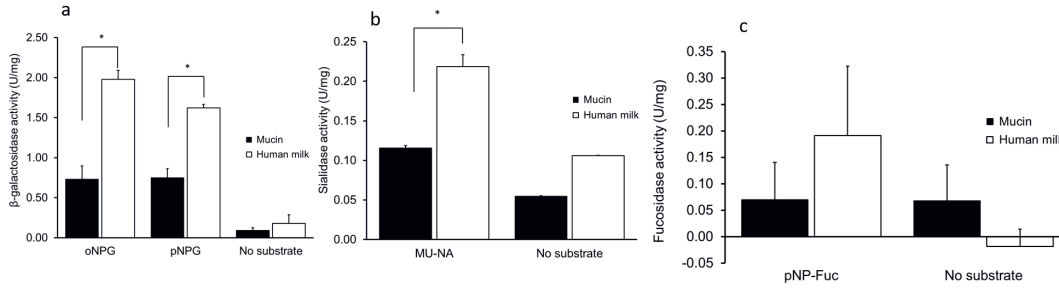

Figure 4: Enzymatic activity of cells lysates of *A. muciniphila.* The activity of a) β-galactosidases, b) sialidases and c) α-fucosidases in cell lysates was tested with 2.5 mM oNPG/pNPG, 2.5 mM MU-NA and 0.5 mM pNP-Fuc, respectively. One unit (U) is the amount of enzyme that converts 1 µmole substrate per minute.

Second, a comparative proteome study identified the enzymes of *A. muciniphila* that are expressed in either human milk or mucus conditions. A total of 832 proteins were detected in both human milk and mucin. Forty-six proteins were significantly more abundant in human milk (p-value < 0.05), while 219 proteins more abundant in mucin condition (Supplementary Figure 7). The rest of the proteins were expressed in similar amounts between the two conditions. We identified 108 proteins that were involved in carbohydrate metabolic process, of which the majority (67%) was expressed in similar amounts in human milk and mucin (Table 3), while 36 (33%) were significantly influenced by the environmental conditions (Supplementary Table 5 and 6). When focusing on mucus glycan degradation enzymes, 42 out of the 61 annotated enzymes in the *A. muciniphila* proteome were detected in both milk and mucin conditions (Table 3). The majority (64%) was expressed in similar amounts in both conditions. Three proteins were significantly more abundant in human milk (Amuc_1755 and Amuc_1033 encoding for sulfatase activity, Amuc_0670 encoding for trypsin-like protein serine protease), and 12 proteins were more abundant in mucin condition (Amuc_1631 - Carboxyl terminal protease, Amuc_1220 - α-N-acetylglucosaminidase, Amuc_0451- Sulfatase, Amuc_0824 - Glycoside Hydrolase Family 2, Amuc_1008 - Glycoside Hydrolase Family 31, Amuc_1835 - Exo-α-sialidase, Amuc_1182 - Sulfatase, Amuc_0010 - α-L-fucosidase, Amuc_0369 - β-N-acetylhexosaminidase, Amuc_1187 - α-galactosidase, Amuc_1924 - β-N-acetylhexosaminidase, and Amuc_1815 - β-N-acetylhexosaminidase). Most of the glycan-degrading enzymes were found to be carbohydrate-active enzymes belonging to the glycoside hydrolases (GH) family (Table 3). Finally, human milk conditions showed a higher expression of the

pili-associated protein (Amuc_1100), which has been characterised as an outer membrane protein (Ottman et al. 2016). All proteins of the pili gene cluster (Amuc_1098 – Amuc_1102), were expressed in (Amuc_1099, Amuc_1100, Amuc_1101) in higher levels in human milk, while Amuc_1098 was more abundant on mucin (p-value < 0.05). The results indicate that *A. muciniphila* has a highly adapted lifestyle to thrive on complex host-derived glycan structures such as the ones in human milk and the mucus layer, where similar enzymes are used by the organism to degrade either substrate.

Table 3: *A. muciniphila*'s saccharolytic enzymes. Locus tags in grey indicate the enzymes that are predicted to be in mucin degradation with their corresponding CAZy Family group. Positive values (Log10 Fold change) indicate higher abundance in human milk than in mucin condition. P-values less than 0.05 are shown in with light blue colour.

Locus tag	Protein	Log10 Fold change	p-value	CAZy Family
Amuc_1815	Beta-N-acetylhexosaminidase	-1,74	0,00	GH20
Amuc_1924	Beta-N-acetylhexosaminidase	-1,72	0,01	GH20
Amuc_1500	Polysaccharide deacetylase	-1,53	0,08	CE4
Amuc_1187	Alpha-galactosidase	-1,48	0,02	GH27
Amuc_0369	Beta-N-acetylhexosaminidase	-1,22	0,03	GH20
Amuc_0863	Glycosyl hydrolase family 88	-1,19	0,06	GH105
Amuc_1616	Polysaccharide deacetylase	-1,14	0,02	CE4
Amuc_0010	Alpha-L-fucosidase	-1,13	0,03	GH29
Amuc_1686	Beta-galactosidase	-1,01	0,25	GH35, CBM32
Amuc_1667	Glycoside hydrolase family 2 sugar binding	-0,90	0,13	GH2
Amuc_1032	Beta-N-acetylhexosaminidase	-0,81	0,32	GH20
Amuc_1669	Beta-N-acetylhexosaminidase	-0,66	0,35	GH20
Amuc_1835	Exo-alpha-sialidase	-0,57	0,01	GH33
Amuc_0060	Alpha-N-acetylglucosaminidase	-0,57	0,21	GH89
Amuc_1438	Glycosyl hydrolase family 98 putative carbohydrate binding module	-0,54	0,43	CBM51
Amuc_0186	Glycoside hydrolase family 95	-0,51	0,27	GH95
Amuc_0771	Beta-galactosidase	-0,42	0,52	GH35
Amuc_1637	Alpha amylase catalytic region	-0,38	0,36	GH13
Amuc_0697	Beta-glucanase	-0,37	0,36	GH43
Amuc_2018	Beta-N-acetylhexosaminidase	-0,35	0,36	GH20
Amuc_1008	Glycoside hydrolase family 31	-0,34	0,01	GH31
Amuc_0824	Glycoside hydrolase family 2 TIM barrel	-0,32	0,01	GH2
Amuc_1220	Alpha-N-acetylglucosaminidase	-0,29	0,05	GH89
Amuc_0724	Glucan endo-1,3-beta-D-glucosidase	-0,18	0,77	GH16
Amuc_0290	Glycoside hydrolase family 2 sugar binding	-0,14	0,12	GH2
Amuc_0625	Exo-alpha-sialidase	-0,13	0,85	GH33
Amuc_2136	Glycoside hydrolase, family 20, catalytic core	-0,07	0,23	GH20
Amuc_0539	Glycoside hydrolase family 2 sugar binding	-0,03	0,79	GH2
Amuc_1868	Glycoside hydrolase family 57	0,00	0,97	GH57
Amuc_0698	Beta-glucanase	0,01	0,98	GH43
Amuc_0392	Coagulation factor 5/8 type domain protein	0,03	0,54	GH29

Amuc_0235	Alpha-glucan phosphorylase	0,09	0,46	GT35
Amuc_0875	Glycoside hydrolase family 16	0,19	0,73	GH16
Amuc_2108	Glycoside hydrolase family 16	0,86	0,13	GH16
Amuc_1880	Isocitrate dehydrogenase [NADP]	-1,52	0,03	-
Amuc_1480	Peptidase S11 D-alanyl-D-alanine carboxypeptidase 1	-1,25	0,13	-
Amuc_1756	Phosphoenolpyruvate-protein phosphotransferase	-1,18	0,03	-
Amuc_0756	ADP-L-glycero-D-manno-heptose-6-epimerase	-1,02	0,13	-
Amuc_0391	Peptidase M23	-0,93	0,16	-
Amuc_1182	Sulfatase	-0,90	0,03	-
Amuc_1543	Formate acetyltransferase	-0,83	0,00	-
Amuc_0523	KpsF/GutQ family protein	-0,63	0,27	-
Amuc_1436	Malate dehydrogenase	-0,58	0,03	-
Amuc_0155	Phosphoglucomutase/phosphomannomutase alpha/beta/alpha domain I	-0,46	0,04	-
Amuc_0451	Sulfatase	-0,29	0,04	-
Amuc_1631	Carboxyl-terminal protease	-0,26	0,00	-
Amuc_0491	Sulfatase	-0,24	0,36	-
Amuc_0953	Sulfatase	-0,19	0,09	-
Amuc_2040	Oligopeptidase A	-0,18	0,36	-
Amuc_1106	Peptidase M24	-0,15	0,50	-
Amuc_1074	Sulfatase	-0,11	0,39	-
Amuc_1572	2-methylcitrate synthase/citrate synthase II	-0,07	0,92	-
Amuc_0121	Sulfatase	0,17	0,20	-
Amuc_1033	Sulfatase	0,24	0,02	-
Amuc_1242	Inositol-phosphate phosphatase	0,24	0,17	-
Amuc_0670	Trypsin-like protein serine protease typically periplasmic contain C-terminal PDZ domain-like protein	0,29	0,01	-
Amuc_0465	Peptidase M23	0,73	0,10	-
Amuc_1755	Sulfatase	1,02	0,01	-

Discussion

This study demonstrates that *A. muciniphila* can grow on human milk thanks to the expression of a set of HMO degrading enzymes. Human milk fermentation by *A. muciniphila* resulted in the degradation of fucosylated and sialylated HMOs (Figure 3). We demonstrated that *A. muciniphila* GH29 α-fucosidase (Amuc_0010) could cleave the α1-2-linked fucose to galactose. However, the high K_M value obtained during incubation with 2'-FL indicates that this substrate might not be one of the preferred substrates for Amuc_0010. In mucus, fucose is mostly found linked through α1-6 to the reducing, terminal GlcNAc (Tailford, Crost, et al. 2015) in *O*-glycans, indicating that this enzyme from *A. muciniphila* might prefer mucus glycans structures over glycosidic linkages that are present in HMOs. HMO content differs per person based on the expression of certain glycosyltransferases. For instance, the presence of terminal α1-2 fucosylated HMOs requires a functional α1-2 fucosyltransferase (FUT2), encoded by the secretor gene in humans (Moran, Gupta, and Joshi 2011; Kumazaki and Yoshida 1984). Interestingly, it was reported that *A. muciniphila* is more abundant in the caesarean-born infants of FUT2 secretor mothers than in the non-secretor mothers (Korpela et al. 2018). In this study, we showed that *A. muciniphila* can use fucose from human milk and basal media supplemented with 2'-FL as an energy source resulting in the production of 1,2- propanediol. This agrees with the ability of *A. muciniphila* to use fucose released from mucin. The liberation of 1,2-propanediol could lead to cross-feeding in the gut as previous studies have shown that 1,2-propanediol was utilised by other bacteria in the gastrointestinal tract (Engels et al. 2016; Amin et al. 2013; Staib and Fuchs 2015; Faber et al. 2017). For instance, *Eubacterium hallii* and *Lactobacillus reuteri* were able to utilise 1,2-propanediol derived from the fermentation of fucose and rhamnose to produce propionate (Amin et al. 2013; Engels et al. 2016). In this way, the milk and mucus degrading ability of *A. muciniphila* would support microbial network formation in the early life intestine and the early life mucosal environment in the gut.

A. muciniphila encodes sialidases that could cleave the 3'-sialyllactose into lactose and neuraminic acid. The activity of *A. muciniphila* against sialylated mucus-glycans has been assessed before (Huang et al. 2015). We found that the two exo-α-sialidases (Amuc_0625 and Amuc_1835) were present in our proteomics dataset. The release of α2-3 and α2-6-linked sialic acid from sialylated HMOs 3'-SL and 6'-SL (Tailford, Owen, et al. 2015) are therefore considered to be the result of their activity. Sialic acid

resulting from the degradation of 3'-SL could not be further utilised by *A. muciniphil*a, which could be due to the fact that it lacks the Nan cluster (NanA/K/E) that is described to be necessary for the sialic acid utilisation in other microorganisms (van Passel et al. 2011). Both human milk and mucin are rich in terminal sialyl-groups. The sialidase activity of *A. muciniphila* enables the release of sialic acid, however it seems most likely that *A. muciniphila* uses its sialidase activity to reach the sugars that are difficult to attain. At the same time, the released sialic acid could serve as substrate for community members, rather than for *A. muciniphila* itself. This implies a key role for *A. muciniphila* in the formation of a microbial network in the infant gut stimulating growth of bacteria that can use sialyl-groups and thus increasing diversity. *Bifidobacterium breve's* growth, for example, is promoted by the sialic acid that is liberated from the degradation of sialyl-oligosaccharides in the gut by *Bifidobacterium bifidum* (K. Nishiyama et al. 2017, 2018). Additionally, *Ruminococcus gnavus* ATCC 29149 that is present in the digestive tract of humans encodes a complete Nan cluster for the sialic acid consumption (Crost et al. 2016). It has been also reported that *Bacteroides fragilis* was able to metabolise the released sialic acid in the gut via an alternative pathway (*nanLET*) (Brigham et al. 2009). To further support the cross-feeding properties of *A. muciniphila*, it has been observed before that *A. muciniphila* is stimulated by the presence of the butyrate producer *Anaerostipes caccae* when grown on mucin. Indeed, *A. muciniphila* when co-cultured with *A. caccae* upregulates mucin-degrading genes that can play a role in the degradation of oligosaccharide chains consisting of monomeric sugars such as GalNAc, GlcNAc, mannose, galactose, fucose and sialic acid (Chia et al. 2018).

In the proteome data, we further identified two β-galactosidases (Amuc_0771, Amuc_1686) of *A. muciniphila*, that showed similar expression between human milk and mucin cultures. The recombinantly-expressed protein Amuc_0771 displayed activity on lactose by releasing galactose and glucose. However, the rate of hydrolysis of the reaction was slow and we were unable to determine the kinetics of this enzyme on lactose. This finding is consistent with that of Kosciow *et al*. who observed that Amuc_0771 exhibited low relative hydrolytic activity against β1-4 linked galactose to glucose (lactose) and β1-4 linked galactose to N-acetyl-D-glucosamine (LacNAc) (Kosciow and Deppenmeier 2020). Guo *et al*., recently characterised the enzymatic activity of an *A. muciniphila* GH35 β-galactosidase, Am0874. In this study, a different

tool was used for annotation *A. muciniphila's* genes that is not in any other common genome or proteome database (ncbi.nlm.nih.gov, genome.jp/keg, uniport.org,). When we aligned the protein sequences from both β-galactosidases NCBI we found a 100% identity between Amuc_0771 and Am0874. Based on their data we can confirm that this enzyme can hydrolyse glycosidic bonds in synthetic substrates and N-glycans. They pointed out that the enzyme showed higher efficiency of cleaving β1-3 and β1-6 than β1-4-linked galactose. Interestingly, they found that Am0874 had lower cleaving capacity when galactose was linked to GlcNAc in β1-4-configuration compared to galactose linked in β1-3-configuration GalNAc (B.-S. Guo et al. 2018). In our results this β-galactosidase (Amuc_0771) exhibited higher hydrolysis against β1-3-linked galactose (LNT) than β1-4 configuration (lactose). The other β-galactosidase (Amuc_1686) that we studied, showed no hydrolysing capacity against LNT or lactose. The activity of Amuc_1686 has been characterised in another study where it is reported that the enzyme exhibits no activity against lactose either, but it showed preference over Galacto-N-biose (Galβ1- 3GalNAc) (Kosciow and Deppenmeier 2019). In the same study, it is mentioned that Amuc_1686 can cleave only the β1-3-linked galactose to GalNAc and not the β1-3-linked galactose to GlcNAc. Their findings might explain the reason why Amuc_1686 was not active against the β1-3-linked galactose to GlcNAc (LNT) in our experiments. *A. muciniphila*'s β-galactosidases are responsible for the liberation of the terminal galactose linked to milk's glycan structures. The free galactose is consumed further by the action of a galactokinase (Amuc_0969). Surprisingly, closer analysis of the proteome data highlighted that *A. muciniphila* produced Amuc_0969 in higher amounts than Amuc_0097, indicating that galactose continued being utilised by *A. muciniphila* compared to glucose (Supplementary Table 4).

In the absence of glycoproteins from mucus *A. muciniphila* has an essential need for GlcNAc (van der Ark et al. 2018). In case of growth on human milk GlcNAc could be released by β-acetylhexosaminidases. Wang *et al*., recently described two *A. muciniphila* GH20 β-acetylhexosaminidases (Am2301 and Am2446) that were able to cleave the terminal GlcNAc off the N- and O-glycans, confirming its exo-activity as glycoside hydrolases (Meng Wang et al. 2018). Our biochemical experiment on the purified β-acetylhexosaminidases (Amuc_0369 and Amuc_2136) revealed that both enzymes were able to hydrolyse only the terminal β1-3 linked GlcNAc to lactose

(LNT2) that results to the liberation of lactose molecule and not the GlcNAc that is located inside the HMO structure (LNT).

A. muciniphila grown on human milk led to the production of a significant amount of succinate (~10 mM). *A. muciniphila* uses the succinate pathway to produce propionate in the gut, as has been described before (Reichardt et al. 2014). Recently, it has been demonstrated that *A. muciniphila* is dependent on the presence of vitamin B12 as a cofactor of Methylmalonyl-CoA synthase in order to convert succinate to propionate (Chia et al. 2018; Belzer et al. 2017) *A. muciniphila* grown on human milk expresses all the necessary enzymes to successfully convert succinate to propionate (Methylmalonyl-CoA mutase; Amuc_1984, Amuc_1983 and Methylmalonyl-CoA epimerase; Amuc_0200). It is described that in human milk vitamin B12 can be tightly bound to haptocorrin, and the concentration varies depending on the diet of the mother, especially on the intake of animal products (Allen 2012). An explanation why succinate is not converted to propionate in human milk in our experiment could be that *A. muciniphila* is not efficient in using the B12 present in human milk.

A. muciniphila grown on human milk shows significant higher abundance of the pili-protein (Amuc_1100) (p-value = 0.02, 1.29-fold change) (Ottman, Reunanen, et al. 2017). This outer membrane pili-like protein plays an important role in immune regulation and enhancement of trans-epithelial resistance. Recently, it has been demonstrated that Amuc_1100 is able to improve gut barrier and restrain the high-fat-diet-induced obesity in mice (Plovier et al. 2017). Therefore, expression of this protein in the infant gut might also contribute to immune maturation and gut health in early life.

Interestingly, the proteome analysis showed high abundance of seven sulfatases functioning as sulfate ester hydrolases. Four of these expressed sulfatases (Amuc_0451, Amuc_1033, Amuc_1182, Amuc_1755) were significantly higher expressed in human milk. It has not been reported so far that HMOs could have sulfate residues attached to their glycan structure like in *O*-glycans of mucin. However, it has been described that human milk can contain more than 100 μmol/L of sulfate esters (McPhee, Atkinson, and Cole 1990). These sulfate esters are part of the glycosaminoglycans (GAGs). GAGs are present in human milk (416 mg/L) and are highly sulfated linear polysaccharides constituted by disaccharidic units where the sugar unit is made up of an *N*-acteylhexosamine (GalNAc or GlcNAc) (Coppa et al. 2011). The most abundant GAGs

found in human milk are chondroitin sulfate (CS) (231 mg/L) and heparin (Hep) (173 mg/L). Therefore, it is possible that *A. muciniphila* deploys its sulfatases for the hydrolysis of the sulfuric esters that are present in human milk glycosaminoglycans.

In this study, it is shown that *A. muciniphila* can grow on human milk and is able to degrade HMOs by using its glycan-degrading enzymes. These glycan-degrading enzymes hydrolyse the HMOs extracellularly into mono- and disaccharides and then the liberated sugars are imported in the cell by transporters (Ottman, Davids, et al. 2017). These findings allow us to propose a model for the utilisation of 2'-fucosyllactose, 3'-siallylactose, lacto-N-tetraose, lacto-N-triose II and lactose by *A. muciniphila* (Figure 5).

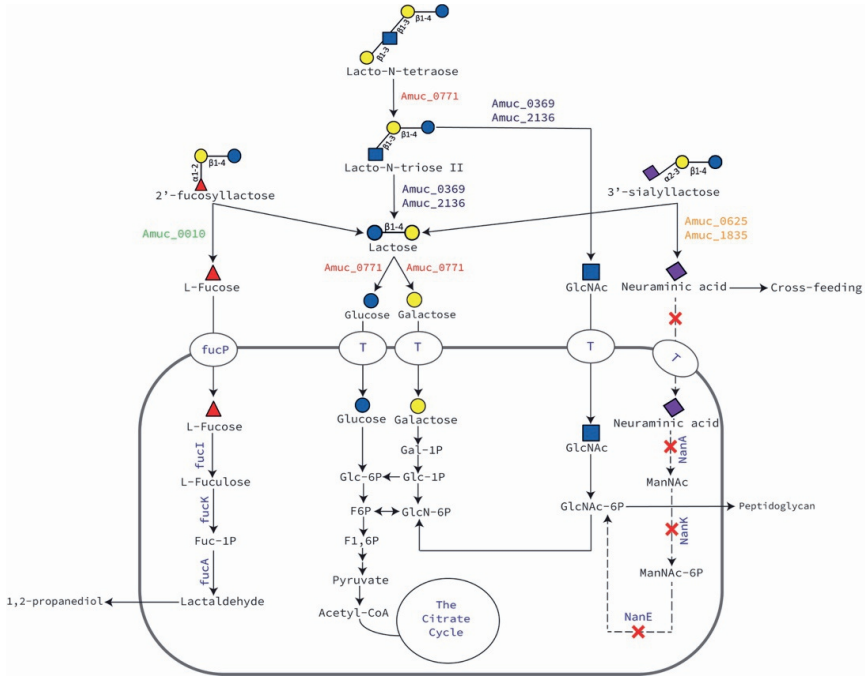

Figure 5: Schematic representation of the proposed pathway for the metabolism of pure 2'-fucosyllactose, 3'-sialyllactose, Lacto-N-tetraose, Lacto-N-triose II and lactose by *A. muciniphila*. The proteins in green colour represent α-fucosidases, in orange represent sialidases, in red represent β-galactosidases and in blue represent β-N-acetylhexosaminidases. fucP: L-fucose transporter, T: putative substrate transporter.

The results of the study suggest that *A. muciniphila* can survive in early life environment by consuming oligosaccharides coming either from the breast milk or infant formulae. This might provide beneficial effects during the initial early life colonisation of *A. muciniphila* before it reaches its natural niche, the outer mucosal

layer. Furthermore, the insufficient degradation of HMOs by *A. muciniphila* liberates simpler glycan structures and metabolites, which then become accessible to other beneficial bacteria. The milk environment also activated the expression of *A. muciniphila* outer membrane protein Amuc_1100 which could benefit the infant immune system (Tseng, Tyler, and Setubal 2009; Galdiero et al. 2012). Human milk carries its microbiota to the infant's intestine facilitating early life colonisation. Development of a healthy gut microbiota from the beginning of life with glycan-degrading microbes could be associated with health in later life by guiding the development of the immune system and protecting against pathogens (Brugman et al. 2015; Duerr and Hornef 2012). As such, *A. muciniphila* might be one of the key members of the early life guiding healthy microbiota and immune development.

Acknowledgments

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Material & Methods

Bacterial growth conditions

A. muciniphila Muc^T (ATCC BAA-835) was grown in basal medium as described previously (Derrien et al. 2004). The medium was supplemented with either hog gastric mucin (0.5% w/v Type III, Sigma-Aldrich, St. Louis, MO, USA), which was purified by ethanol precipitation as described previously (Hoskins et al. 1985), human milk (10% v/v), 2'-fucosyllactose (2'-FL, 10 mM, purity > 90%) or 3'-sialyllactose (3'-SL, 10 mM, purity > 90%). The growth conditions containing HMOs (i.e. 2'-FL and 3'SL) were supplemented with 25 mM *N*-acetyl-glucosamine (GlcNAc) as nitrogen source for *A. muciniphila* (Sigma-Aldrich). The bottles containing human milk and HMOs were further supplemented with L-Proline and L-Threonine (Sigma-Aldrich) (6 g/L). All the anaerobic bottles were supplemented with 1 % v/v of $CaCl_2$ and vitamin mixture as described previously (Stams et al. 1993). Human breast milk was collected under sterile conditions from a healthy donor (approximately 3-6 months post-partum) and stored at -20^{o}C until use. Incubations were completed in serum bottles sealed with butyl rubber stoppers at 37^{o}C under anaerobic conditions provided by a gas phase of 182 kPa (1.5 atm) N_2/CO_2T. The bacterial growth on mucin by measuring the optical density at 600 nm and quantitative Polymerase Chain Reaction (qPCR). The optical density of the cultures of *A. muciniphila* grown in human milk could not be determined due to the high turbidity of the milk. Therefore, bacterial numbers were solely measured by qPCR.

Quantitative real-time PCR (qPCR)

The abundance of *A. muciniphila* in human milk and mucin was determined by qPCR as described previously (Collado et al. 2007). Cells (1mL) were harvested at 21,000 x *g* for 15 min. DNA extractions were performed using the MasterPure^{TM} Gram Positive DNA Purification Kit (Epicentre, Lucigen, USA). DNA concentrations were measured fluorometrically (Qubit dsDNA BR assay, Invitrogen) and adjusted to 1 ng/µL prior to use as the template in qPCR. Primers targeting the 16S rRNA gene of *A. muciniphila* (AM1 5'-CAGCACGTGAAGGTGGGGAC-3' and AM2 5'-CCTTGCGGTTGGCTTCAGAT-3'; 327 bp [10]) were used for quantification. A standard curve was prepared with nine standard concentrations from 10^0 to 10^8 gene copies/µL. qPCR was performed in triplicate with iQ SYBR green supermix (Bio-Rad, USA) in a total volume of 10 µL prepared with primers at 500 nM in 384-wells plates

with the wells sealed with optical sealing tape. Amplification was performed with an iCycler (Bio-rad): one cycle of 95 ºC for 10 min; 40 cycles of 95 ºC for 15 s, 60º C for 20 s, and 72 ºC for 30 s each; one cycle of 95 ºC for 1 min; and a stepwise increase of temperature from 60ºC to 95ºC (at 0.5 ºC per 5 s) to obtain melt curve data. Data were analysed using Bio-Rad CFX Manager 3.0. The copy number was corrected for the DNA concentration and for the number of 16S rRNA genes encoded in *A. muciniphila*'s genome.

Human Milk Oligosaccharide extraction

HMOs were recovered from 1 mL aliquots of *A. muciniphila* grown in 10% v/v human milk cultures as described before (Mank et al. 2019). An internal standard of 1,5-α-L-arabinopentaose (Megazyme, Ireland) was added, at the volume of 10 µL per sample to minimize pipetting error, to reach a final concentration of 0.01 mmol/l. The solution was diluted 1:1 with ultrapure water and centrifuged at 4,000 x *g* for 15 min at 4ºC. The supernatant was filtered through 0.2 µm syringe filter followed by subsequent centrifugation with a pre-washed ultra-filter (Amicon Ultra 0.5 Ultracel Membrane 3 kDa device, Merck Milipore, USA) at 14,000 x *g* for 1h at room temperature. Finally, the filtrate was vortexed and stored at -20ºC.

Electrospray ionisation liquid chromatography mass spectrometry (LC-ESI-MS^2) analysis

HMOs were identified and quantified with LC-ESI-MS^2 (Mank et al. 2019). This method allows the study of distinct HMO structures more in particular their monosaccharide sequence, glycosidic linkage and the molecular conformation. Thereby the HMOs isobaric isomers such as Lacto-N-fucopentaose (LNFP) I, II, III and V could be distinguished as described by Mank et al (Mank et al. 2019). We used the latter approach with adaptations. Micro LC-ESI-MS^2 analysis was performed on a 1200/1260 series HPLC stack (Agilent, Waldbronn, Germany) consisting of solvent tray, degasser, binary pump, autosampler and DAD detector coupled to a 3200 Qtrap mass spectrometer (ABSciex, USA). After extraction of HMOs, 2.5 µL of this extract was injected into the LC-MS system. Oligosaccharides were separated by means of a 2.1x30 mm Hypercab porous graphitized carbon (PGC) column with a 2.1x10 mm PGC pre-column (Thermo Scientific, USA) using a water-methanol gradient for 30 min, and 2 min equilibration. Solvent A consisted of 0.3% ammonium hydroxide solution (28-

30%, Sigma-Aldrich, St. Louis, Missouri United States) in water and solvent B of 0.3% ammonium hydroxide solution in 95% methanol (all v/v). Pre-equilibration was performed using 97.5% solvent A. The gradient started with 97.5% solvent A for 0.5 min, decreased to 60% in 12.5 minutes and decreased to 40% in 3 min, where it was kept for 4 min. In a next segment, solvent A decreased in 0.5 min to 2.5% where it was kept for 3 min. In 0.5 min, solvent A increased to 97.5% for re-equilibration of 6 min. Eluent flow was 400 µL/min and the columns were kept at 45ºC. The LC-effluent was infused online into the mass spectrometer and individual HMO structures were analysed semi-quantitatively by multiple reaction monitoring (MRM) in negative ion mode. Specific MRM transitions for neutral HMOs up to pentaoses and acidic HMOs up to trioses were included. The spray voltage was -4500V, the declustering potential and collision energy were optimized to individual compounds measured. A segmented method was used to obtain higher sensitivity. Each MRM-transition was measured for 70 ms. The instrument was calibrated with polypropylene glycol (PPG) according the instruction of the manufacturer.

Protein extraction from *A. muciniphila* cultures

A. muciniphila was grown in basal medium supplemented with 0.5% purified mucin or 10% human milk (4 biological replicates). After 15 and 48 h incubation at 37ºC, cells (2mL) were pelleted at 4,816 x *g* for 30 min at 4 ºC, re-suspended in 1 mL PBS, washed twice (21,130 x *g* at 4 °C), and finally re-suspended in 500 µL lysis buffer (100 mM Tris HCl, pH 8.0, 4% (w/v) SDS, 7.7 mg/mL Dithiotreitol [DTT]). Cells were lysed by sonication (four pulses of 20 s with 30 s rest on ice) with an amplitude of 20-30% on ice using an MS-72 probe, followed by centrifugation at 21,130 x *g* for 30 min at 4ºC. Qubit® Protein Assay Kit (Life technologies, Oregon, USA) was used according to the manufacturer's instructions to determine the protein content of cell extracts. Protein samples (15 h for mucin and 48 h for human milk samples) (40 µg) were loaded on a Bolt 4-12% Bis-Tris Plus separation gel (Invitrogen, Life Technologies, USA) using the XCell Surelock Mini-Cell (Novex, Life Technologies, USA). The electrophoresis procedure was according to the manufacturer's instructions. Gels were stained overnight using QC Colloidal Coomassie Blue G250 stain (Bio-rad Laboratories, USA).

In-gel digestion identification and relative quantification of proteins from *A. muciniphila* cell extracts

Each of the used gel lanes was cut into three slices to increase the number of identified proteins. Slices were further processed to pieces of about 1 mm^2 and put in 1.5 mL low binding tubes (Eppendorf) prior to their reduction, alkylation, and trypsin digestion, as described previously (Rupakula et al. 2013). The supernatant obtained was used for LC-MS/MS analysis. Samples were measured by nLC–MS/MS with a Proxeon EASY nLC and a LTQ-Orbitrap XL mass spectrometer as previously described (J. Lu et al. 2011; Wendrich et al. 2017). LC – MS data analysis was performed as described previously (Rupakula et al. 2013; Hubner et al. 2010; Smaczniak et al. 2012) with false discovery rates (FDRs) set to 0.01 on peptide and protein level, and additional result filtering (minimally 2 peptides necessary for protein identification of which at least one is unique and at least one is unmodified). Any remaining hits against the reversed database as well as all human proteins were removed. To analyse the abundance of proteins in the fractions, their label-free quantification (LFQ) intensities were compared (J. Cox et al. 2014). Non-existing LFQ intensity values due to not enough quantified peptides were substituted with a value slightly lower than the lowest LFQ intensity value measured. The normal logarithm was taken from protein LFQ MS1 intensities as obtained from MaxQuant. Relative protein quantitation of sample to control was done with Perseus (Tyanova et al. 2016) by applying a two sample T-test using the "LFQ intensity" columns obtained with FDR set to 0.01 and S0 set to 1. nLC-MSMS system quality was checked with PTXQC (Bielow, Mastrobuoni, and Kempa 2016) using the MaxQuant result files. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (Vizcaíno et al. 2016) partner repository with the dataset identifier PXD011357. All the *A. muciniphila* proteins differentially expressed between milk and mucin conditions are listed in Supplementary Table 5 and 6.

Cloning, expression and purification of selected *A. muciniphila* glycoside hydrolases

The selected proteins coded by the genes with locus tags Amuc_0010 (57.51 kDa), Amuc_0369 (72.02 kDa), Amuc_0771 (70.53 kDa), Amuc_1686 (85.71 kDa), and Amuc_2136 (81.59 kDa) without their signal peptides were cloned into the pCDF-1b vector, introducing a 6xHis-tag at the C-terminus. The genes of interest and the plasmid

backbone were amplified by PCR with gene-specific primers, using Q5 DNA polymerase (New England BioLabs, USA) (Supplementary Table 1). The amplified genes and vector were being assembled with NEBuilder HiFi DNA Assembly Master Mix (NEB BioLabs, USA) according to manufacturer's instructions. *E. coli* DH5-aplha competent cells were transformed using 5 µL of assembly reaction and sequences were verified by DNA sequencing by Eurofins (Ebersberg, Germany). *E. coli* BL21 Rosetta competent cells were transformed with the recombinant plasmid harbouring the genes of interest. The recombinant cells were grown to an OD_{600} between 0.5 and 0.6 in 250 mL Lysogeny-Broth (LB) supplemented with 50 µg/mL spectinomycin and 25 µg/mL chloramphenicol. Then, they were induced with 1mM isopropyl β-D-1-thiogalactopyranoside (IPTG) at 22ºC for 16 h. The cells were harvested by centrifugation at 6,000 x *g* for 20 min and re-suspended in 15 mL of lysis buffer. Cells were lysed by sonication on ice (1 s pulse with 2 s rest for 10 min, amplitude of 20-25%) using a MS-72 microtip (Bandelin, Germany). The lysate was cleared by centrifugation at 120,000 x *g* for 1 h at 4 °C. The proteins were His-tag purified using Ni^{2+}-nitrilotriacetate (Ni-NTA) agarose affinity columns (500 µL bed volume, Qiagen) equilibrated with binding buffer. Samples were loaded, followed by 2 mL of binding buffer and 2x 500 µL plus 1 mL of elution buffer. Purity of the enzymes was checked by SDS-Page (4 -10% polyacrylamide gel) (Supplementary Figure 2) and pure samples were concentrated using 50-kDa MWCO Amicon Ultra-15 Centrifugal Filter Units (Merck, Germany) with storage buffer. Protein content was measured using the Qubit Protein Assay (manufacturer's protocol) and fluorometer (DeNovix, USA). pCDF-1b empty vector was cloned, expressed and purified as it is described above. The empty vector was used as negative control in all the enzymatic assays.

Activity assays and kinetics

The purified enzymes were incubated with different substrates in McIlvaine buffer solution adjusted to the optimum pH of each enzyme at 37ºC. To quantify α-fucosidase, β-galactosidase and β-acetylhexosaminidase activity on synthetic substrates, 4-nitrophenyl-a-L-fucopyranoside (pNP-α-L-Fuc), 4-nitrophenyl-b-D-galactopyranoside (pNPG) and 4-nitrophenyl 2-acetamido-2-deoxy-b-D-glucopyranoside (GlcNAc-β-pNP) were used respectively. The enzymes were incubated with substrate concentrations ranging from 0 to 5 mM in McIlvaine buffer. At intervals of 2.5 min, samples were taken and added to 2.5x volume of glycine buffer (pH 9.6) to stop the

reaction. Absorbance was monitored at 405 nm and quantified using a standard curve of 4-Nitrophenol (pNP). To determine the optimal pH of each enzyme, the assay was carried out in McIlvaine buffer with pH values ranging from 5.0 to 8.2 and a substrate concentration of ~3 times the K_M as determined at the initial pH. Next, the aforementioned assays with 0-5 mM substrate were repeated at the optimal pH. For incubations of β-galactosidases and hexosaminidases with lactose and LNT, the assays were performed in a similar way, but the products were monitored by HPAEC-PAD. The sugars were separated by HPAEC with 10 mM NaOH at 0.5 mL/min on a CarboPac PA20 protected with a guard column and detected using PAD on a Dionex ICS5000 system (Thermo Scientific). The column was cleaned for 10 min with 200 mM NaOH and re-equilibrated with 10mM NaOH. Standards of galactose and glucose in different concentrations were used to quantify the results. For incubations of the α-fucosidase with 2'-FL, reactions were performed in a similar way, but the fucose release was measured directly (without adding glycine buffer first) by α-L-fucose kit (Megazyme, Ireland) based on a fucose dehydrogenase-coupled method (Morris 1982). Reactions without enzyme and with only the purified plasmid incubated in both synthetic and human milk derived sugars were served as negative controls. Kinetic data of the enzymes were obtained from triplicate experiments, and the kinetic parameters were calculated by fitting the initial raw data to the Michaelis-Menten equation using linear regression analysis, and the error bars (SD).

Cell lysate activity

Enzymatic activity of *A. muciniphila* cell lysates was tested with colorimetric substrates as described previously (Rosendale et al. 2012). The following substrate/enzyme combinations were used: 4-nitrophenyl-α-L-fucopyranoside/fucosidase (pNP-Fuc), 4-nitrophenyl-β-D-galactopyranoside (pNPG) and 2-nitrophenyl-β-D-galactopyranoside (ONPG)/β-galactosidase, and 2'-(4-methylumbelliferyl)-α-D-N-acetylneuraminic acid (MU-NA)/sialidase. Reactions were performed in a final volume 20 µL, using 0.5-2.5 mM substrate and ~1 µg/mL enzyme in 0.05 M citrate buffer pH 6.0 (fucosidase, sialidase) or 0.25 M phosphate buffer, pH 7.0 (β-galactosidase). After 1 h of incubation at 37 °C, reactions were stopped by adding 50 µL 0.5 glycine buffer, pH 9.6. Absorbance was read at 405 nm (pNPG and pNP-Fuc) or 420 nm (oNPG) and fluorescence intensity was measured at λ_{ex} 390 nm, λ_{em} 460 nm on a 96-well plate reader (Biotek, USA). Activity is expressed in units (U), in which one unit is the amount of

enzyme that converts 1 µmole substrate per minute. To test fucosidase and galactosidase activity of cell lysates in 2'-FL and lactose, ~10 µg/mL of each enzyme was incubated with 1 mM lactose and 1 mM of 2'-FL in 0.05 mM citrate buffer, pH 6.0 in a final volume of 1 mL, shaking at 300 rpm. Reactions of aliquots at different time points were stopped by adding 2.5 x the volume of 0.5 M glycine buffer, pH 9.6. The reaction was monitored by HPAEC-PAD as it was described previously.

High-performance liquid chromatography

For fermentation product analysis, samples were obtained at different time points of the incubation period. Crotonate was used as the internal standard and the external standards were lactate, formate, acetate, propionate, butyrate, succinate, 1,2-propanediol, lactose, *N*-acetyl-glucosamine (GlcNAc), *N*-acetyl-galactosamine (GalNAc), 2'-fucosyllactose (2'-FL), 3'-sialyllactose (3'-SL), glucose, galactose, fucose and sialic acid. Substrate conversion and product formation were measured with Thermo Scientific Spectrasystem high-performance liquid chromatography (HPLC) system equipped with a Varina Metacarb 67H 300 x 6.5 mm column kept at 45ºC and running 0.0005 mM sulfuric acid as eluent. The eluent had a flow of 0.5 mL/min and metabolites were detected by determining the refractive index (RI-150) and identified by using standards of pure compounds as described previously (van Gelder et al. 2012).

Statistical analysis

Statistics were performed using **s**tudent's t-test and corrected for multiple testing using False Discovery Rate (FDR). Data are presented as mean ± standard deviation (SD), unless stated otherwise. *P*-values below 0.05 were considered significant.

Ethical consideration

The usage and analysis of human milk samples described in this study were performed with written consent by the donor.

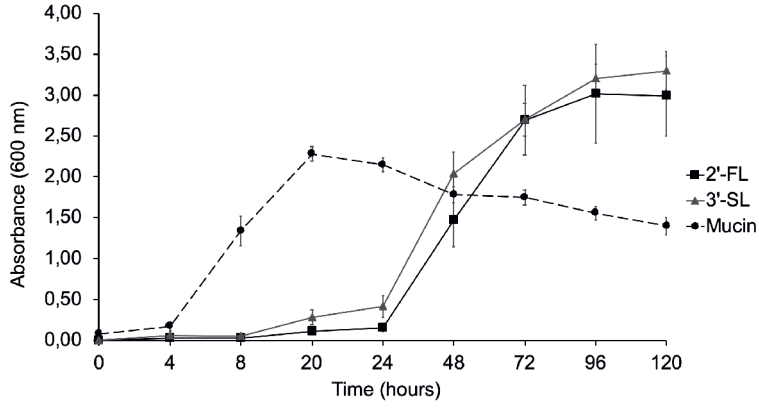
Supplementary Data

Supplementary Table 1: The primers used for transformation of Amuc_0369, Amuc_2136, Amuc_0010, Amuc_0771, Amuc_1686 and vector into *E. coli* BL21 Rosetta, including 6xHis-Tag on C-terminus. His-tags are given in bold.

Protein name	Protein	Forward primer (5'-3')	Reverse primer (5'-3')
β-hexosaminidase	Amuc_0369	GGATCCCAATTGGGAGCTCATGGAACAAATCATCCCGAAACC	TTA**GTGGTGATGGTG**ATGATGTGGCGTTTCAAAATACACTGTC
β-hexosaminidase	Amuc_2136	GGATCCCAATTGGGAGCTCATGAAGCAGATTGCGGATTCC	TTA**GTGGTGATGGTG**ATGATGCAACGGCTGTACGTTCAT
α-fucosidase	Amuc_0010	GGATCCCAATTGGGAGCTCATGCAGTCCGCCACTAAAATC	CTA**GTGGTGATGGTG**ATGATGTTTGCTCAGTTTGATGACGG
β-galactosidase	Amuc_0771	GGATCCCAATTGGGAGCTCATGGCATTGGCATGGGC	TTA**GTGGTGATGGTG**ATGATGCCACTGGTTGTCCAGGTTTTT
β-galactosidase	Amuc_1686	GGATCCCAATTGGGAGCTCATGCCCATGCCTTTGC	TTA**GTGGTGATGGTG**ATGATGCTTGGCAGGCTTGAACG
Backbone (pcdf-1b)	-	**CATCATCACCATCACCAC**TAGCTCGAGTCTGGTAAAGAAACCG	GAGCTCCCAATTGGGATCC
	-	**CATCATCACCATCACCAC**TAACTCGAGTCTGGTAAAGAAACCG	
	-	**CATCATCACCATCACCAC**TGACTCGAGTCTGGTAAAGAAACCG	

Supplementary Table 2: Fermentation products and sugar utilisation of *A. muciniphila* grown on human milk and mucin.

Mucin							
Time (hours)	Acetate (mM)	Propionate (mM)	1,2-propanediol (mM)	Succinate (mM)	Lactose (mM)	Glucose (mM)	Galactose (mM)
0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.03 ± 0.06	0.04 ± 0.10	0.04 ± 0.10
4	0.94 ± 1.06	0.27 ± 0.30	0.00 ± 0.00	0.24 ± 0.12	0.00 ± 0.00	0.13 ± 0.20	0.13 ± 0.20
15	16.53 ± 4.55	11.72 ± 2.70	0.45 ± 0.60	0.74 ± 0.58	0.00 ± 0.00	0.08 ± 0.13	0.08 ± 0.13
18	17.17 ± 3.65	12.98 ± 1.93	0.40 ± 0.48	0.18 ± 0.42	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
24	17.21 ± 4.76	12.66 ± 2.26	0.50 ± 0.80	0.15 ± 0.38	0.02 ± 0.06	0.14 ± 0.22	0.14 ± 0.22
48	17.05 ± 4.14	12.55 ± 1.91	0.72 ± 1.00	0.14 ± 0.34	0.00 ± 0.00	0.09 ± 0.15	0.09 ± 0.15
72	16.82 ± 4.32	12.56 ± 1.99	0.57 ± 0.81	0.00 ± 0.00	0.00 ± 0.00	0.13 ± 0.21	0.13 ± 0.21
96	17.33 ± 4.53	12.75 ± 2.01	0.58 ± 0.80	0.00 ± 0.00	0.00 ± 0.00	0.11 ± 0.18	0.11 ± 0.18
120	17.55 ± 3.78	13.08 ± 1.72	0.59 ± 0.79	0.00 ± 0.00	0.00 ± 0.00	0.07 ± 0.11	0.07 ± 0.11
Human Milk							
Time (hours)	Acetate (mM)	Propionate (mM)	1,2-propanediol (mM)	Succinate (mM)	Lactose (mM)	Glucose (mM)	Galactose (mM)
0	0.00 ± 0.00	0.00 ± 0.00	0.90 ± 1.43	0.00 ± 0.00	21.85 ± 3.75	0.78 ± 0.48	0.01 ± 0.03
4	0.01 ± 0.03	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	20.69 ± 3.31	0.83 ± 0.76	0.07 ± 0.16
15	1.00 ± 0.68	1.61 ± 1.71	0.00 ± 0.00	0.16 ± 0.40	20.78 ± 1.37	0.79 ± 0.31	0.40 ± 0.13
18	1.46 ± 1.02	1.42 ± 1.00	0.00 ± 0.00	0.39 ± 0.61	20.23 ± 2.21	0.88 ± 0.25	0.54 ± 0.18
24	2.09 ± 1.49	2.08 ± 1.23	0.00 ± 0.00	0.56 ± 0.60	18.58 ± 2.82	1.34 ± 0.58	0.94 ± 0.48
48	4.29 ± 2.44	4.39 ± 2.65	0.00 ± 0.00	2.05 ± 1.41	14.13 ± 1.40	7.31 ± 2.93	4.44 ± 2.44
72	9.23 ± 2.19	9.55 ± 1.79	0.00 ± 0.00	8.14 ± 2.00	5.52 ± 1.22	11.00 ± 1.76	6.41 ± 1.30
96	12.15 ± 2.15	12.28 ± 1.79	0.03 ± 0.06	9.87 ± 0.96	0.59 ± 0.69	11.87 ± 2.43	7.10 ± 1.50
120	12.05 ± 2.28	13.10 ± 2.40	0.04 ± 0.11	10.51 ± 0.77	0.30 ± 0.25	13.19 ± 2.59	8.12 ± 1.40



Supplementary Figure 1: Growth curve of *A. muciniphila* grown in mucin (black dotted line), 2'-FL (black line), 3'-SL (grey line) measured by optical density at 600 nm.

Supplementary Table 3: a) Carbon balance of *Akkermansia muciniphila* on HMOs and GlcNAc after 48 hours of fermentation, b) Production of sugars and metabolites of *A. muciniphila* grown in 2'-FL (top) and 3'-SL (bottom).

a)

		Substrates		Products											Carbon recovery (%)	
HMO	Time point (h)	2'-fucosyllactose	GlcNAc	2'-fucosyllactose	GlcNac	GalNac	Fucose	Lactose	1,2-propanediol	Glucose	Galactose	Acetate	Propionate	Lactate	Average	SD
2'-fucosyllactose	48	81.58	89.26	81.58	89.26	2.79	9.24	31.42	2.05	4.55	4.88	31.75	28.33	0.00	86.58	3.45
HMO	Time point (h)	3'-sialyllactose	GlcNAc	3'-sialyllactose	GlcNac	GalNac	Fucose	Lactose	Neuraminic Acid	Glucose	Galactose	Acetate	Propionate	Lactate	Average	SD
3'-sialyllactose	48	172.66	60.82	172.66	60.82	2.90	0.20	8.59	47.76	2.48	2.81	42.82	35.02	0.00	82.79	9.10

b)

2'-FL											
Time (hours)	Acetate (mM)	Propionate (mM)	1,2-propanediol (mM)	GalNAc (mM)	GlcNAc (mM)	Succinate (mM)	Galactose (mM)	Glucose (mM)	Lactose (mM)	2'-Fucosyllactose (mM)	Fucose (mM)
0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.29 ± 0.04	22.27 ± 1.76	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	8.29 ± 0.33	0.00 ± 0.00
4	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.63 ± 0.14	23.30 ± 1.86	0.00 ± 0.00	0.43 ± 0.61	0.00 ± 0.00	0.00 ± 0.00	8.81 ± 0.42	0.00 ± 0.00
8	0.22 ± 0.29	0.00 ± 0.00	0.00 ± 0.00	0.76 ± 0.14	23.67 ± 2.20	0.00 ± 0.00	0.57 ± 0.81	0.00 ± 0.00	0.00 ± 0.00	8.95 ± 0.52	0.00 ± 0.00
20	2.06 ± 0.08	3.19 ± 0.21	0.00 ± 0.00	0.46 ± 0.32	20.73 ± 1.75	0.00 ± 0.00	0.02 ± 0.03	0.00 ± 0.00	0.00 ± 0.00	7.92 ± 0.51	0.04 ± 0.05
24	4.50 ± 3.32	3.20 ± 1.05	0.00 ± 0.00	0.47 ± 0.22	18.79 ± 3.95	0.00 ± 0.00	0.33 ± 0.47	0.00 ± 0.00	0.00 ± 0.00	7.24 ± 1.61	0.64 ± 0.63
48	15.88 ± 3.52	9.44 ± 2.15	0.68 ± 0.26	0.35 ± 0.10	11.16 ± 0.36	0.00 ± 0.00	0.81 ± 0.26	0.76 ± 0.16	2.62 ± 0.72	4.53 ± 0.06	1.54 ± 0.18
72	26.27 ± 6.89	18.01 ± 5.04	3.22 ± 1.33	0.26 ± 0.10	1.00 ± 0.21	0.00 ± 0.00	0.38 ± 0.18	0.49 ± 0.05	3.00 ± 0.30	0.61 ± 0.72	0.33 ± 0.17
96	33.46 ± 1.38	24.78 ± 0.59	5.24 ± 0.28	0.00 ± 0.00	0.07 ± 0.10	0.00 ± 0.00	0.55 ± 0.38	0.58 ± 0.32	1.96 ± 0.69	0.09 ± 0.09	0.45 ± 0.48
120	35.82 ± 0.34	27.52 ± 0.63	5.96 ± 0.42	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.46 ± 0.49	0.46 ± 0.40	0.77 ± 0.97	0.10 ± 0.06	0.51 ± 0.35

3'-SL										
Time (hours)	Acetate (mM)	Propionate (mM)	GalNAc (mM)	GlcNAc (mM)	Succinate (mM)	Galactose (mM)	Glucose (mM)	Lactose (mM)	3'-Sialyllactose (mM)	Neuraminic acid (mM)
0	0.00 ± 0.00	0.00 ± 0.00	0.38 ± 0.40	24.31 ± 2.16	0.00 ± 0.00	0.02 ± 0.03	0.00 ± 0.00	0.11 ± 0.04	11.09 ± 2.14	0.38 ± 0.66
4	0.00 ± 0.00	0.00 ± 0.00	0.31 ± 0.00	24.66 ± 0.04	0.00 ± 1.71	0.12 ± 0.00	0.00 ± 0.19	0.10 ± 0.00	11.37 ± 2.24	0.44 ± 0.76
8	0.70 ± 0.07	0.00 ± 0.07	0.32 ± 0.00	23.77 ± 0.04	0.00 ± 1.99	0.10 ± 0.00	0.00 ± 0.18	0.07 ± 0.00	10.96 ± 2.00	0.41 ± 0.70
20	3.77 ± 0.95	2.58 ± 0.95	0.32 ± 0.00	21.33 ± 0.02	0.00 ± 1.26	0.00 ± 0.00	0.00 ± 0.00	0.26 ± 0.00	10.70 ± 2.05	1.82 ± 0.48
24	4.68 ± 1.39	2.65 ± 1.39	0.10 ± 0.00	21.10 ± 0.18	0.00 ± 1.63	0.00 ± 0.00	0.00 ± 0.00	0.20 ± 0.00	10.79 ± 1.87	1.46 ± 0.47
48	21.41 ± 3.62	11.67 ± 3.62	0.36 ± 0.00	7.60 ± 0.03	0.00 ± 1.71	0.47 ± 0.00	0.41 ± 0.16	0.72 ± 0.06	7.51 ± 1.85	4.34 ± 2.60
72	31.44 ± 2.20	19.35 ± 2.20	0.52 ± 0.00	2.10 ± 0.14	0.00 ± 0.45	1.17 ± 0.00	0.97 ± 0.22	3.39 ± 0.13	1.11 ± 0.98	12.73 ± 2.06
96	33.96 ± 3.41	22.79 ± 3.41	0.49 ± 0.00	0.54 ± 0.52	0.00 ± 0.54	1.26 ± 0.00	0.84 ± 0.29	2.18 ± 0.23	0.00 ± 0.00	11.89 ± 3.03
120	36.55 ± 2.82	25.67 ± 2.82	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.51 ± 0.00	0.20 ± 0.30	0.57 ± 0.34	0.00 ± 0.00	10.97 ± 2.54

Supplementary Table 4: Abundance of *A. muciniphila* enzymes involved in carbohydrate metabolism with their corresponding KEGG identifier (KO ID). The average of Log10 transformed LFQ values is shown. Colouring is based on abundance from the most abundant (red) to medium abundant (orange) to least abundant (green).

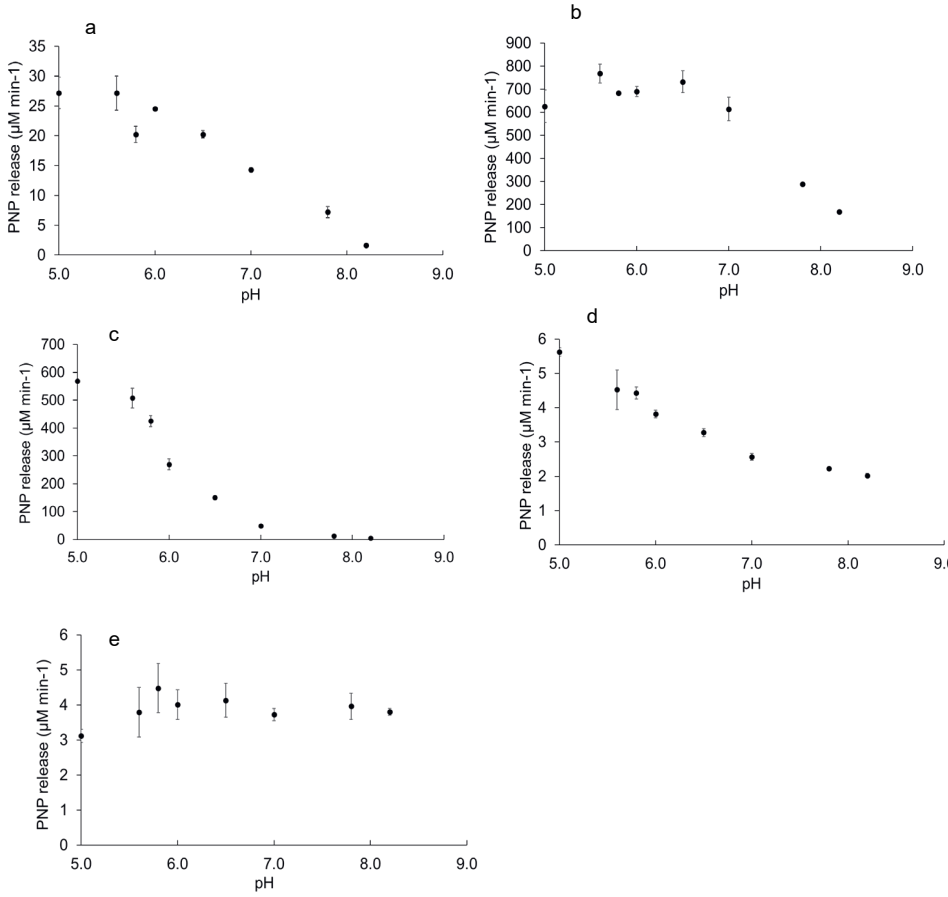
Locus tag	Protein	Average_LFQ_Human Milk	KO ID	Pathway
Amuc_1210	Phosphoenolpyruvate carboxykinase [GTP]	9.03	ko00010,ko00020,ko00620	Glycolysis/Gluconeogenesis; Citrate Cycle (TCA cycle); Pyruvate metabolism
Amuc_1417	Glyceraldehyde-3-phosphate dehydrogenase, type I	8.95	ko00010	Glycolysis/Gluconeogenesis
Amuc_1632	L-fucose isomerase	8.61	ko00051	Fructose and mannose metabolism
Amuc_1630	Serine hydroxymethyltransferase	8.47		
Amuc_0309	2,3-bisphosphoglycerate-independent phosphoglycerate mutase	8.09	ko00010,ko00680	Glycolysis/Gluconeogenesis;Methane metabolism
Amuc_1418	Phosphoglycerate kinase	8.07	ko00010	Glycolysis/Gluconeogenesis
Amuc_1975	Glucose-6-phosphate isomerase	7.95	ko00010,ko00030,ko00500,ko00520	Glycolysis/Gluconeogenesis; Pentose phosphate pathway; Starch and sucrose metabolism; Amino sugar and nucleotide sugar metabolism
Amuc_0721	Fructose-bisphosphate aldolase, class II	7.85	ko00010,ko00030,ko00051,ko00680	Glycolysis/Gluconeogenesis; Pentose phosphate pathway; Fructose and mannose metabolism; Methane metabolism
Amuc_1413	Capsular exopolysaccharide family	7.76		
Amuc_1184	Enolase 2	7.71	ko00010,ko00680,ko03018	Glycolysis/Gluconeogenesis;Methane metabolism;RNA degradation
Amuc_1125	UDP-glucose 4-epimerase	7.56	ko00052,ko00520	Galactose metabolism; Amino sugar and nucleotide sugar metabolism
Amuc_0844	Enolase 1	7.30	ko00010,ko00680,ko03018	Glycolysis/Gluconeogenesis;Methane metabolism;RNA degradation
Amuc_1481	6-phosphofructokinase	7.15	ko00010,ko00030,ko00051,ko00052,ko00680	Glycolysis/Gluconeogenesis; Pentose phosphate pathway; Fructose and mannose metabolism; Amino sugar and nucleotide sugar metabolism; Methane metabolism
Amuc_0690	UDP-N-acetylmuramate/alanine ligase	7.08		
Amuc_1471	Ribose-phosphate pyrophosphokinase	7.01	ko00030,ko00230	Pentose phosphate pathway; Purine metabolism
Amuc_0653	UDP-N-acetylmuramyl-tripeptide synthetase	7.00	ko00300,ko00550	Lysine biosynthesis; Peptidoglycan biosynthesis
Amuc_0654	UDP-N-acetylmuramoyl-tripeptide--D-alanyl-D-alanine ligase	6.95	ko00300,ko00550	Lysine biosynthesis; Peptidoglycan biosynthesis
Amuc_1386	4-hydroxy-3-methylbut-2-en-1-yl diphosphate synthase	6.89	ko00900	Terpenoid backbone biosynthesis
Amuc_1822	Glucosamine-6-phosphate isomerase	6.79	ko00520	Amino sugar and nucleotide sugar metabolism
Amuc_1741	UTP--glucose-1-phosphate uridylyltransferase	6.72	ko00040,ko00052,ko00500,ko00520	Pentose and glucuronate interconversions; Galactose metabolism; Starch and sucrose metabolism; Amino sugar and nucleotide sugar metabolism
Amuc_0431	Pyruvate kinase	6.71	ko00010,ko00230,ko00620	Glycolysis/Gluconeogenesis; Purine metabolism; Pyruvate metabolism
Amuc_0562	Triosephosphate isomerase	6.62	ko00010,ko00051,ko00562	Glycolysis/Gluconeogenesis; Fructose and mannose metabolism; Inositol phosphate metabolism
Amuc_0772	Phosphoglycerate mutase 1 family	6.60		
Amuc_1246	GDP-mannose 4,6-dehydratase	6.59	ko00051,ko00520	Fructose and mannose metabolism; Amino sugar and nucleotide sugar metabolism
Amuc_0969	Galactokinase	6.54	ko00052,ko00520	Galactose metabolism; Amino sugar and nucleotide sugar metabolism
Amuc_0210	6-phosphofructokinase 1	6.09	ko00010,ko00030,ko00051,ko00052,ko00680	Glycolysis/Gluconeogenesis; Pentose phosphate pathway; Fructose and mannose metabolism; Amino sugar and nucleotide sugar metabolism; Methane metabolism
Amuc_1309	Aldose 1-epimerase	6.05	ko00010	Glycolysis/Gluconeogenesis
Amuc_0661	D-alanine--D-alanine ligase	6.04	ko00473,ko00550	D-Alanine metabolism; Peptidoglycan biosynthesis
Amuc_0676	2-dehydro-3-deoxyphosphooctonate aldolase	5.98	ko00540	Lipopolysaccharide biosynthesis
Amuc_1248	NAD-dependent epimerase/dehydratase	5.94	ko00051,ko00520	Fructose and mannose metabolism; Amino sugar and nucleotide sugar metabolism
Amuc_1671	Phosphofructokinase	5.92	ko00010,ko00030,ko00051,ko00052,ko00680	Glycolysis/Gluconeogenesis; Pentose phosphate pathway; Fructose and mannose metabolism; Amino sugar and nucleotide sugar metabolism; Methane metabolism
Amuc_0664	Cell wall hydrolase/autolysin	5.89		
Amuc_1192	Mannose-1-phosphate guanylyltransferase (GDP)	5.71	ko00051,ko00520	Fructose and mannose metabolism; Amino sugar and nucleotide sugar metabolism
Amuc_1435	Nucleotide sugar dehydrogenase	5.63	ko00040,ko00053,ko00500,ko00520	Pentose and glucuronate interconversions; Galactose metabolism; Starch and sucrose metabolism; Amino sugar and nucleotide sugar metabolism
Amuc_0097	ROK family protein	5.58	ko00010,ko00052,ko00500,ko00520,ko00521	Glycolysis/Gluconeogenesis; Galactose metabolism; Starch and sucrose metabolism; Amino sugar and nucleotide sugar metabolism; Streptomycin biosynthesis
Amuc_0550	3D domain protein	5.52		
Amuc_1895	UDP-N-acetylglucosamine 1-carboxyvinyltransferase	5.50	ko00520,ko00550	Amino sugar and nucleotide sugar metabolism; Peptidoglycan biosynthesis
Amuc_1830	L-fuculokinase	5.47	ko00051	Fructose and mannose metabolism
Amuc_0169	3-deoxy-manno-octulosonate cytidylyltransferase	5.00	ko00540	Lipopolysaccharide biosynthesis
Amuc_0208	Glucose-1-phosphate thymidylyltransferase	5.00	ko00521,ko00523	Streptomycin biosynthesis; Polyketide sugar unit biosynthesis
Amuc_0414	Phosphoheptose isomerase	5.00	ko00540	Lipopolysaccharide biosynthesis
Amuc_0656	UDP-N-acetylmuramoylalanine--D-glutamate ligase	5.00	ko00471,ko00550	D-Glutamine and D-glutamate metabolism; Peptidoglycan biosynthesis
Amuc_0681	UDP-N-acetylmuramate	5.00		
Amuc_0795	Glycerol-3-phosphate dehydrogenase [NAD(P)+]	5.00	ko00564	Glycerophospholipid metabolism
Amuc_1211	N-acetylmuramoyl-L-alanine amidase family 2	5.00		
Amuc_1541	Lipid A biosynthesis acyltransferase	5.00	ko00540	Lipopolysaccharide biosynthesis
Amuc_1646	4-hydroxy-3-methylbut-2-enyl diphosphate reductase	5.00	ko00900	Terpenoid backbone biosynthesis

Supplementary Table 5: All the differentially expressed *A. muciniphila* proteins found in Human milk compared to mucin cultures.

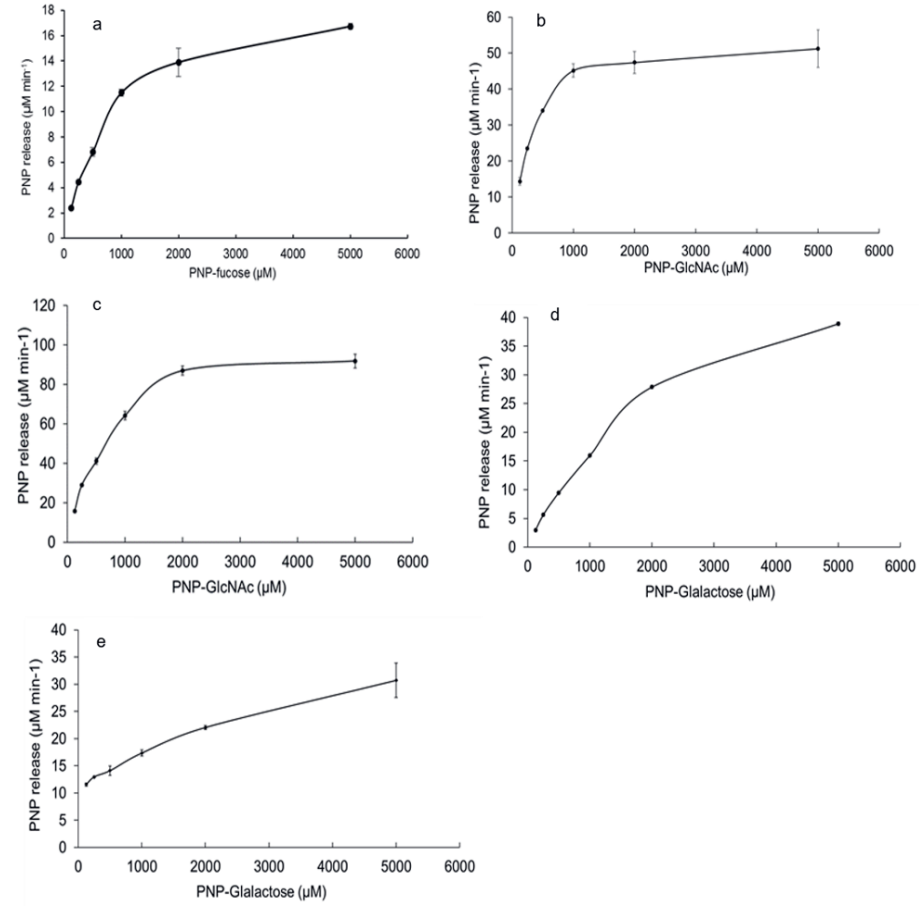
Gene names	Protein names	Log10 fold_change	*p*-value
Amuc_0037	Amino acid permease-associated region	0.65	0.00
Amuc_0172	Putative uncharacterized protein	0.22	0.00
Amuc_0201	Carboxyl transferase	0.27	0.00
Amuc_0335	ABC transporter related	0.51	0.01
Amuc_0372	Glutamate decarboxylase	0.67	0.00
Amuc_0419	General substrate transporter	0.45	0.00
Amuc_0468	Transcriptional regulator, DeoR family	0.42	0.03
Amuc_0541	Ribonuclease, Rne/Rng family	0.28	0.00
Amuc_0543	Putative uncharacterized protein	0.24	0.00
Amuc_0544	Tetratricopeptide TPR_2 repeat protein	0.14	0.01
Amuc_0627	Putative uncharacterized protein	0.41	0.01
Amuc_0670	Trypsin-like protein serine protease typically periplasmic contain C-terminal PDZ domain-like protein	0.29	0.01
Amuc_0687	Outer membrane autotransporter barrel domain protein	0.89	0.00
Amuc_0705	D-isomer specific 2-hydroxyacid dehydrogenase NAD-binding	0.23	0.02
Amuc_0714	UPF0365 protein	0.36	0.04
Amuc_0738	GMP synthase, large subunit	0.27	0.01
Amuc_0836	ATP-dependent chaperone ClpB	0.24	0.00
Amuc_0841	Argininosuccinate lyase	0.25	0.02
Amuc_0876	Heavy metal translocating P-type ATPase	0.18	0.04
Amuc_0955	Ribosome-binding factor A	0.47	0.02
Amuc_0970	SSS sodium solute transporter superfamily	0.23	0.01
Amuc_1033	Sulfatase	0.24	0.02
Amuc_1091	Cytochrome-c peroxidase	0.28	0.01
Amuc_1099	Putative uncharacterized protein	0.46	0.03
Amuc_1100	Putative uncharacterized protein	0.37	0.02
Amuc_1101	Cell division protein FtsA	0.43	0.00
Amuc_1184	Enolase 2	0.62	0.00
Amuc_1225	Inorganic diphosphatase	0.66	0.00
Amuc_1310	17 kDa surface antigen	0.62	0.00
Amuc_1408	60 kDa chaperonin	0.28	0.00
Amuc_1443	Putative uncharacterized protein	0.39	0.00
Amuc_1525	Putative uncharacterized protein	0.68	0.00
Amuc_1584	Type II secretion system protein	0.64	0.00
Amuc_1586	Type II secretion system protein E	0.59	0.00
Amuc_1656	Putative uncharacterized protein	0.52	0.00
Amuc_1689	Dihydrolipoyl dehydrogenase	0.44	0.03
Amuc_1695	Cytochrome bd ubiquinol oxidase subunit I	0.76	0.00
Amuc_1751	Glycoside hydrolase family 13 domain protein/ 1,4 -alpha-glucan branching enzyme	0.61	0.04
Amuc_1755	Sulfatase	1.02	0.01
Amuc_1776	TPR repeat-containing protein	0.93	0.00
Amuc_1782	Glutamyl-tRNA(Gln) amidotransferase subunit A	0.15	0.01
Amuc_1869	Glycosyl transferase group 1	0.74	0.00
Amuc_2001	Putative lipoprotein	0.36	0.02
Amuc_2034	Methylmalonyl-CoA carboxytransferase	0.12	0.02
Amuc_2043	RND efflux system, outer membrane lipoprotein, NodT family	0.45	0.01
Amuc_2127	Carbohydrate-selective porin OprB	0.53	0.00

Supplementary Table 6: Top 10 abundant *A. muciniphila* proteins found in human milk compared to 10 most significantly abundant in mucin cultures.

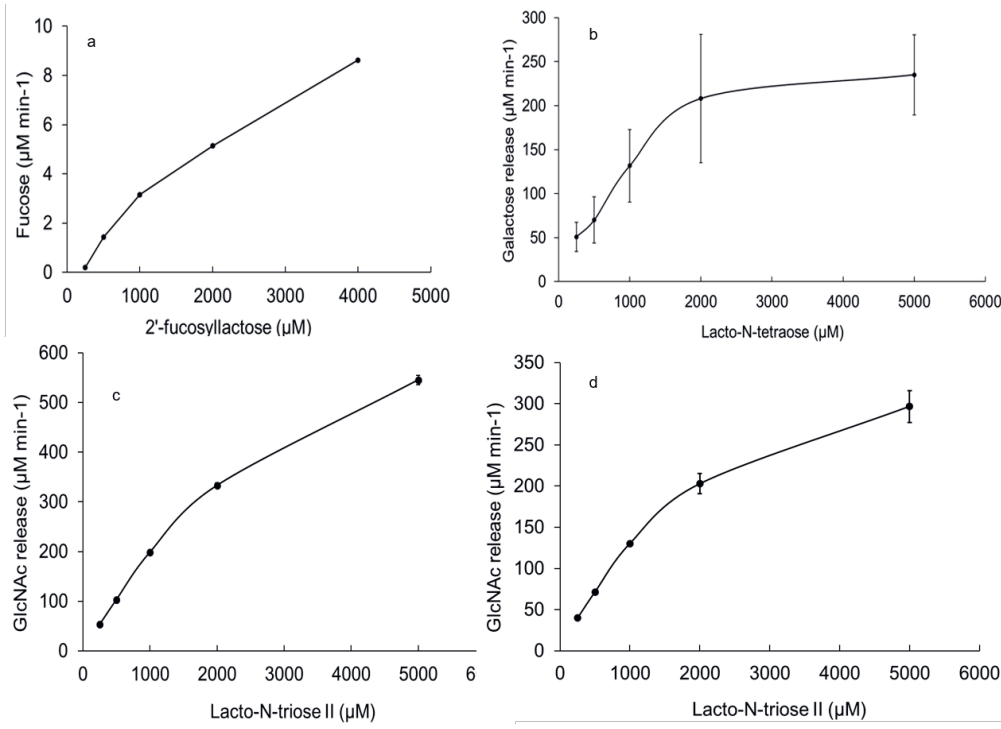
Environmental condition	Protein name	KO ID	Proteins	Kegg Brite Functional Category	Log10 Fold change	p-value
Human Milk	Amuc_1755	x	Sulfatase	x	1.015	0.01
	Amuc_1776	x	TPR repeat-containing protein	x	0.934	0.00
	Amuc_0687	x	Outer membrane autotransporter barrel domain protein	x	0.892	0.00
	Amuc_1695	K00425	Cytochrome bd ubiquinol oxidase subunit I	Energy metabolism	0.758	0.00
	Amuc_1869	x	Glycosyl transferase group 1	x	0.737	0.00
	Amuc_1525	x	Putative uncharacterised protein	x	0.681	0.00
	Amuc_0372	K01580	Glutamate decarboxylase	Carbohydrate metabolism, amino acid metabolism	0.668	0.00
	Amuc_1225	K15986	Inorganic diphosphatase	Energy metabolism	0.655	0.00
	Amuc_0037	K20265	Amino acid permease	Membrane transport	0.648	0.00
	Amuc_1584	K02653	Type II secretion system protein	Membrane transport	0.645	0.00
Mucin	Amuc_0557	K02954	30S ribosomal protein S14	Translation	-2.886	0.00
	Amuc_0243	x	Putative uncharacterized protein	x	-2.441	0.00
	Amuc_1476	K02990	30S ribosomal protein S6	Translation	-2.389	0.00
	Amuc_2106	K00029	Malate dehydrogenase (Oxaloacetate-decarboxylating) (NADP(+))	Carbohydrate metabolism	-2.254	0.00
	Amuc_1430	K02909	50S ribosomal protein L31	Translation	-2.183	0.03
	Amuc_1068	K00789	S-adenosylmethionine synthase	Amino acid metabolism	-2.182	0.00
	Amuc_1059	K07239	Acriflavin resistance protein	Cellular process and signalling	-2.174	0.00
	Amuc_1254	K03217	Membrane protein oxaA	Folding, sorting and degradation, membrane transport, translation	-2.072	0.02
	Amuc_0070	K01867	Tryptophanyl-tRNA synthetase	Translation, amino acid metabolism	-2.045	0.00
	Amuc_2099	x	Putative uncharacterized protein	x	-2.044	0.00



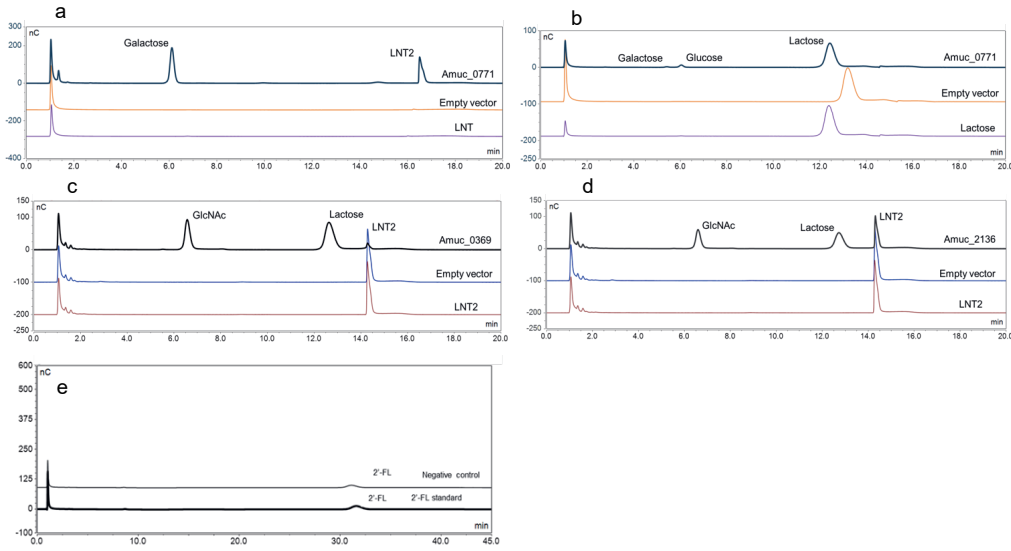
Supplementary Figure 2: pH dependent of *A. muciniphila* GHs using pNP substrates. a) Amuc_0010 and PNP-fucose, b) Amuc_0369 and GlcNAc-PNP, c) Amuc_2136 and GlcNAc-PNP, d) Amuc_0771 and PNPG, e) Amuc_1686 and PNPG



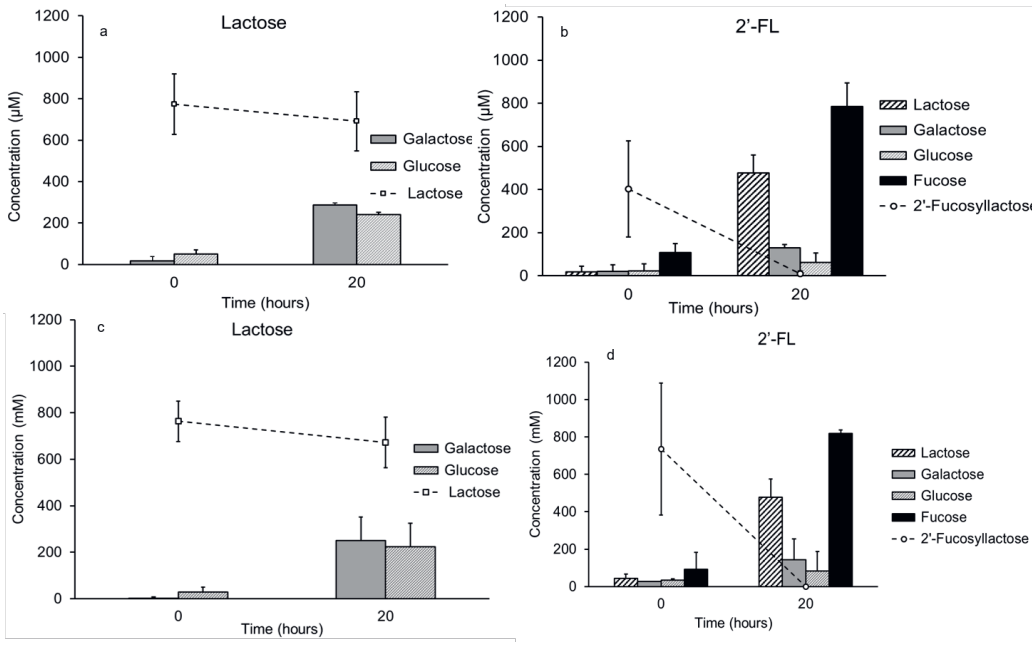
Supplementary Figure 3: Kinetic analysis of *A. muciniphila* GHs against PNP substrates. a) Amuc_0010 and PNP-fucose, b) Amuc_0369 and GlcNAc-PNP, c) Amuc_2136 and GlcNAc-PNP, d) Amuc_0771 and PNPG, e) Amuc_1686 and PNPG



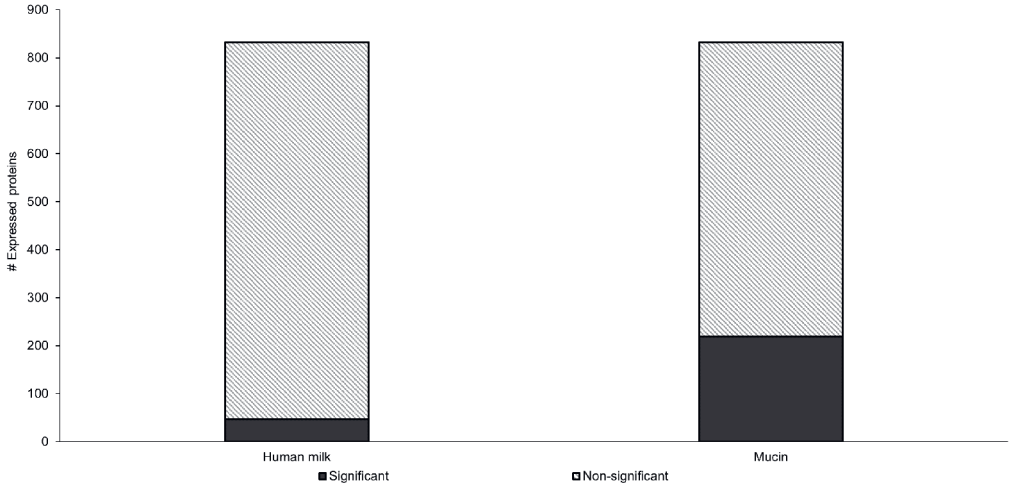
Supplementary Figure 4: Kinetic analysis of *A. muciniphila* GHs against HMOs. a) Amuc_0010 and 2'-fucosyllactose, b) Amuc_0771 and Lacto-N-tetraose, c) Amuc_0369 and Lacto-N-triose II, d) Amuc_2136 and Lacto-N-triose II.



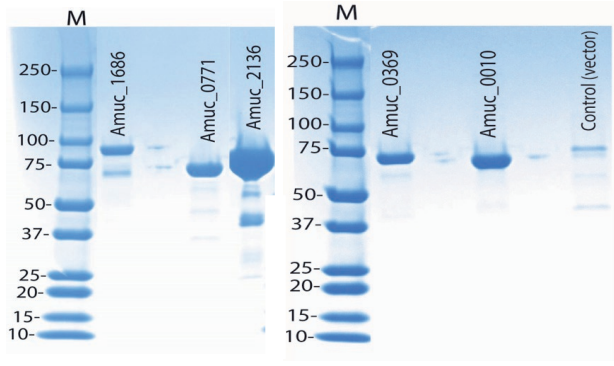
Supplementary Figure 5: Purified *E. coli* BL21 Rosetta strain (Empty vector) specificity against HMOs and lactose. a) Lacto-N-tetraose, b) lactose, c) and d) Lacto-N-triose II, e) 2'-fucosyllactose



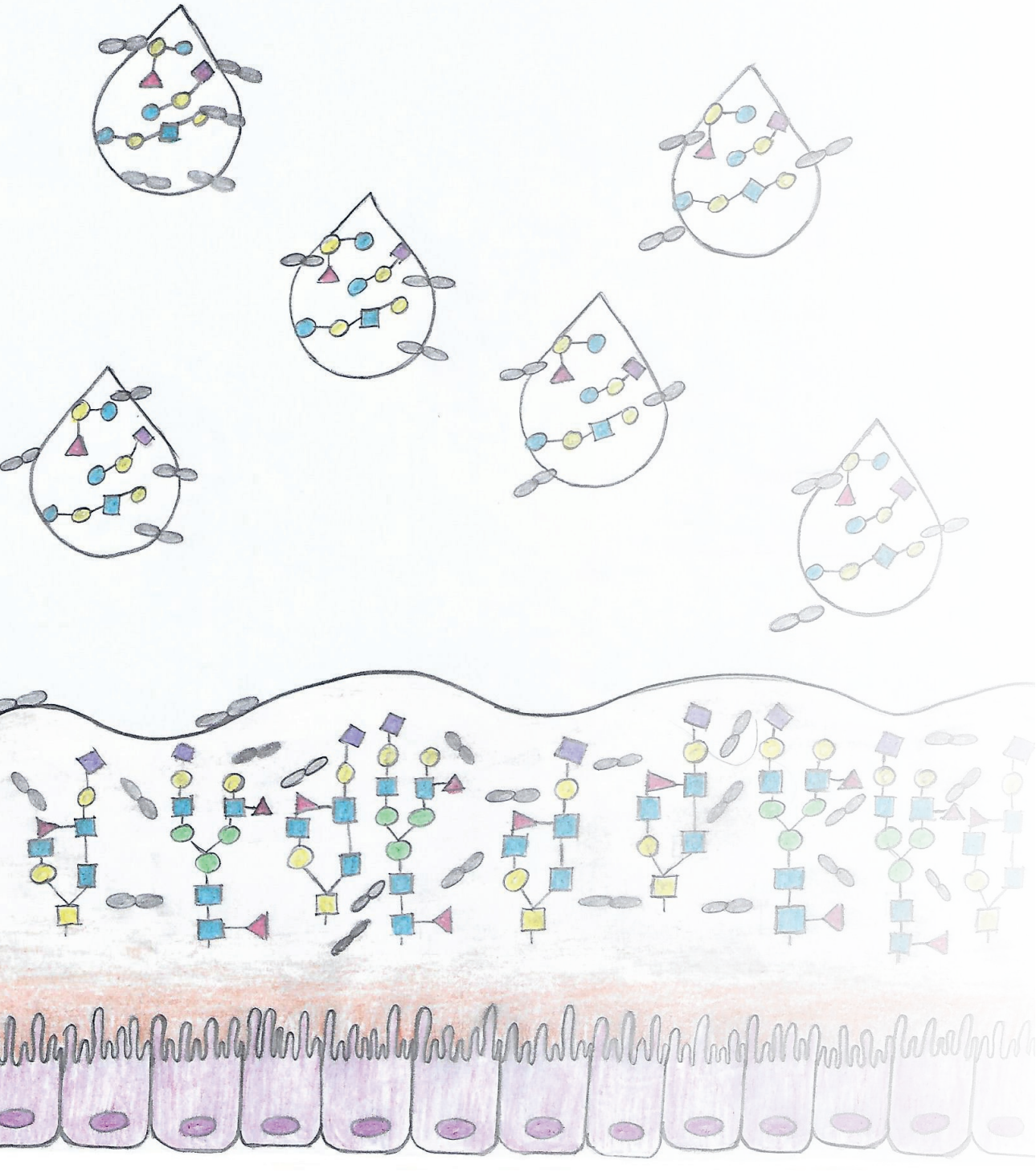
Supplementary Figure 6: Enzymatic activity of cell lysates of *A. muciniphila* against lactose and 2'-FL. a,b) human milk cell lysate and c,d) mucin cell lysate.



Supplementary Figure 7: Indication of the amount of significant and non-significant proteins expressed by *A. muciniphila* either in human milk or mucin condition.



Supplementary Figure 8: SDS-PAGE analysis of the purified recombinant *A. muciniphila* GHs. M- protein marker.



Chapter 4

A continuous battle for host-derived glycans in the gut between a mucus specialist and a glycan generalist *in vitro* and *in vivo*

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Abstract

The human gastrointestinal tract is colonized by a diverse microbial community, which plays a crucial role in human health. In the gut, a protective mucus layer that consists of glycan structures separates the bacteria from the host epithelial cells. These host-derived glycans are utilized by bacteria that have adapted to this specific compound in the gastrointestinal tract. Our study investigated the close interaction between two distinct gut microbiota members known to use mucus glycans, the generalist *Bacteroides thetaiotaomicron* and the specialist *Akkermansia muciniphila in vitro* and *in vivo.* The *in vitro* study, in which mucin was the only nutrient source, indicated that *B. thetaiotaomicron* significantly upregulated genes coding for Glycoside Hydrolases (GHs) and mucin degradation activity when cultured in the presence of *A. muciniphila*. Furthermore, *B. thetaiotaomicron* significantly upregulated the expression of a gene encoding for membrane attack complex/perforin (MACPF) domain in co-culture. The transcriptome analysis also indicated that *A. muciniphila* was less affected by the environmental changes and was able to sustain its abundance in the presence of *B. thetaiotaomicron* while increasing the expression of LPS core biosynthesis activity encoding genes (*O*-antigen ligase, Lipid A and Glycosyl transferases) as well as ABC transporters. Using germ-free mice colonized with *B. thetaiotaomicron* and/or *A. muciniphila*, we observed a more general glycan degrading profile in *B. thetaiotaomicron* while the expression profile of *A. muciniphila* was not significantly affected when colonizing together, indicating that two different nutritional niches were established in mice gut. Thus, our results indicate that a mucin degrading generalist adapt to its changing environment, depending on available carbohydrates while a mucin degrading specialist adapts by coping with competing microorganism through upregulation of defence related genes.

Keywords: ***Akkermansia muciniphila, Bacteroides thetaiotaomicron,*** **mucin glycans, Glycosyl Hydrolases (GHs), antimicrobial proteins, germ free mice**

Introduction

The gut mucosal layer is the first line of defence against damage and infection (Corfield 2015; K. Bergstrom et al. 2020). At the same time, mucin glycans serve as a consistent nutrient foundation for members of the gut microbiota (Martens, Chiang, and Gordon 2008). Mucins consist predominantly of carbohydrates, mainly *N*-acetylgalactosamine (GalNAc), *N*-acetylglucosamine (GlcNAc), fucose (fuc), galactose (gal) and sialic acid (Neu5ac) (Bansil and Turner 2006; Abodinar et al. 2016). In mucus, core-glycans are covalently linked to serine or threonine residues to form complex *O*-linked glycan structures (K. S. B. Bergstrom and Xia 2013). The type of *O*-glycans produced in the human intestinal tract depends on many factors encoded within the human genome, including the expression of genes responsible for mucin synthesis (MUC) (Robbe et al. 2004). The glycan diversity is provided by the peripheral epitopes (Tailford, Crost, et al. 2015). For example, the α1,2-fucosylation of core glycan structures is found in populations that carry the secretor gene (Mollicone et al. 1985). The fucosyltransferase 2 (FUT2) gene is responsible for the synthesis of type 1 H antigens, whose secretion determines the expression of the ABH and Lewis histo-blood antigens in the intestinal mucosa (Wacklin et al. 2014). Moreover, the secretion of FUT2 gene contributes to the microbiota composition (Wacklin et al. 2011; Garrido et al. 2015; Kashyap et al. 2013). Bacteria that can degrade mucin glycans can colonize the mucosal surface and become core species in the gastrointestinal (GI) tract (Tailford, Crost, et al. 2015; K. S. B. Bergstrom and Xia 2013).

Several gut bacteria species are capable of degrading these host substrates (Salyers et al. 1977). For example, the ability of *Bacteroides* species to utilise diverse glycans depends on a series of gene clusters, which are termed the Polysaccharide Utilization Loci (PULs) (Bjursell, Martens, and Gordon 2006). PULs encode cell envelope systems that typically include glycolytic enzymes and the homologs of two outer membrane proteins (SusC and SusD) that are part of the first described PUL, the starch utilization system (Sus) locus (Martens et al. 2009). One well-studied glycan-degrading bacterium is *Bacteroides thetaiotaomicron* VPI-5482, which has 88 PULs and 221 mucin-degrading enzymes (Martens, Chiang, and Gordon 2008). *B. thetaiotaomicron* is a prominent member of the human gut microbiota capable of growing on many different plant and host glycans (Salyers et al. 1977; Martens, Chiang, and Gordon 2008). When *B. thetaiotaomicron* colonizes the distal guts of adult germ-free mice maintained on a

diet rich in plant glycans, it increases the expression of genes involved in the catabolism of dietary substrates (Sonnenburg 2005). In contrast, in adult mice fed a diet devoid of complex glycans, *B. thetaiotaomicron* alters its response to express genes involved in targeting host glycans (Bjursell, Martens, and Gordon 2006). Transcriptomic analyses highlighted specific polysaccharide-utilization loci (PULs) including genes coding for putative glycoside hydrolases (GHs), such as α-L-fucosidase, endo-β-N-acetylglucosaminidase, endo-β-galactosidase and α-mannosidase, which were upregulated when *B. thetaiotaomicron* was grown on mucin O-glycans or in mono-associated mice – when compared to *in vitro* glucose control (Martens, Chiang, and Gordon 2008; Martens et al. 2011).

Another well-studied mucin degrader, *Akkermansia muciniphila* ATCC BAA-835, belongs to Verrucomicrobia phylum and is also a prominent member of the human microbiota. *A. muciniphila* is associated with a healthy mucosal layer, stimulates mucus thickness and barrier function (Van Der Lugt et al. 2019; Reunanen et al. 2015) and metabolic state as it has been inversely correlated with obesity (Dao, Everard, Aron-Wisnewsky, et al. 2016; Karlsson et al. 2012), metabolic diseases (Type 2 diabetes) (X. Zhang et al. 2013) as well as intestinal disorders (inflammatory bowel disease (IBD) and appendicitis) (Png et al. 2010; Rajilić-Stojanović et al. 2013; Swidsinski et al. 2011). Depommier et al. illustrated that supplementation with *A. muciniphila* in overweight and obese humans reduced the levels of blood markers related to liver dysfunction and inflammation, while the overall gut microbiome remained unaffected (Depommier et al. 2019). *A. muciniphila* is a mucin degrading specialist that possesses 61 (~3% of its relatively small genome) mucin degrading enzymes to utilize mucus glycans as the sole carbon, energy and nitrogen source (Collado et al. 2007).

Both *B. thetaiotaomicron* and *A. muciniphila* belong to dominant phyla in the adult human gut (Eckburg et al. 2005). In a complex environment, such as the human gut microbiome, the search for nutrients is vital for the survival of the gut residents (Bauer et al. 2018). Bacterial communities exist under continuous competition and collaborative efforts of the bacteria in them. Many different types of interactions occur within a complex microbial ecological network. In general, two mechanisms of competition exist: (i) exploitative competition, where members compete for shared nutrients and resources and (ii) interference competition, in which a member directly

harms a competitor, often through the production of antimicrobial molecules (Ghoul and Mitri 2016).

To investigate the microbe-to-microbe interaction under the continuous influx of host-derived glycans (mucin), we developed an *in vitro* bioreactor model with a mucin-glycan specialist (*A. muciniphila)* and a glycan generalist (*B. thetaiotaomicron)*. Mono-associated bioreactors were inoculated and used as control. Host-microbe interactions were evaluated through a gnotobiotic mouse model colonized with the same set of microbiomes.

Results

A stable community *in vitro* leads to adaptation of glycan degradative pathways

We established monocultures and a co-culture of *A. muciniphila* and *B. thetaiotaomicron* in bioreactors with a continuous feed of mucin glycans. The bioreactors were sampled for measurements of metabolite production and total mRNA. The co-culture was characterized by higher growth and metabolic profile compared to the mono-associated bioreactors (Figure 1a,b,c). *A. muciniphila* and *B. thetaiotaomicron* co-culture showed a significantly higher production (q-value < 0. 05) of acetate and propionate compared to their monocultures. Moreover, 1,2-propanediol and succinate were also observed during the fermentation period, indicating continued fermentation of the monosaccharides released from the mucus glycans degradation. The amounts of SCFAs in the different conditions are shown in Supplementary Table 1.

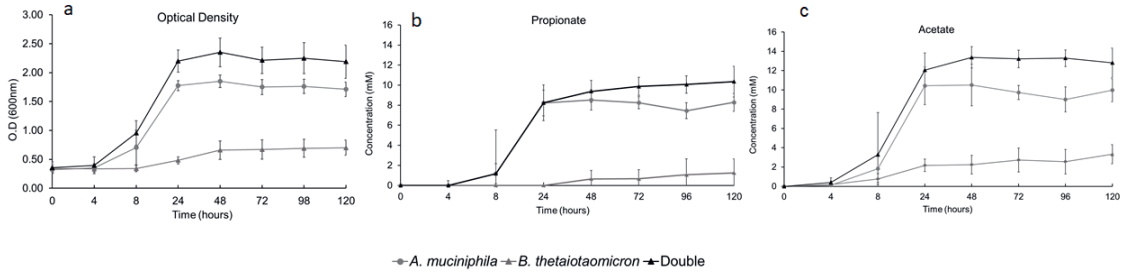


Figure 1: Growth and SCFAs production during *in vitro* fermentation. a) Optical Density (O.D.) measurements at 600 nm, b) Acetate production, c) Propionate production

We first focused on the adaptation of *A. muciniphila* and *B. thetaiotaomicron* to use their glycan degradation machinery. We identified the genes from *B. thetaiotaomicron* and *A. muciniphila* in the community involved in carbohydrate metabolism and glycan biosynthesis and metabolism. The transcriptome data indicated that 306 genes from *B. thetaiotaomicron* were significantly different in the mono- and co-culture conditions. A total of 4% of these genes were significantly upregulated in the co-culture (q-value <0.05). *B. thetaiotaomicron* expressed 218 of the 220-known mucin-degrading genes in both conditions. In the co-culture, *B. thetaiotaomicron* overexpressed (above 7.0-fold change) two genes encoding for D-glycero-D-manno-heptose 1-phosphate kinase (BT_0474) and phosphoheptose isomerase (BT_0475) activity, respectively. Both genes were predicted to be involved in lipopolysaccharide biosynthesis. The capacity

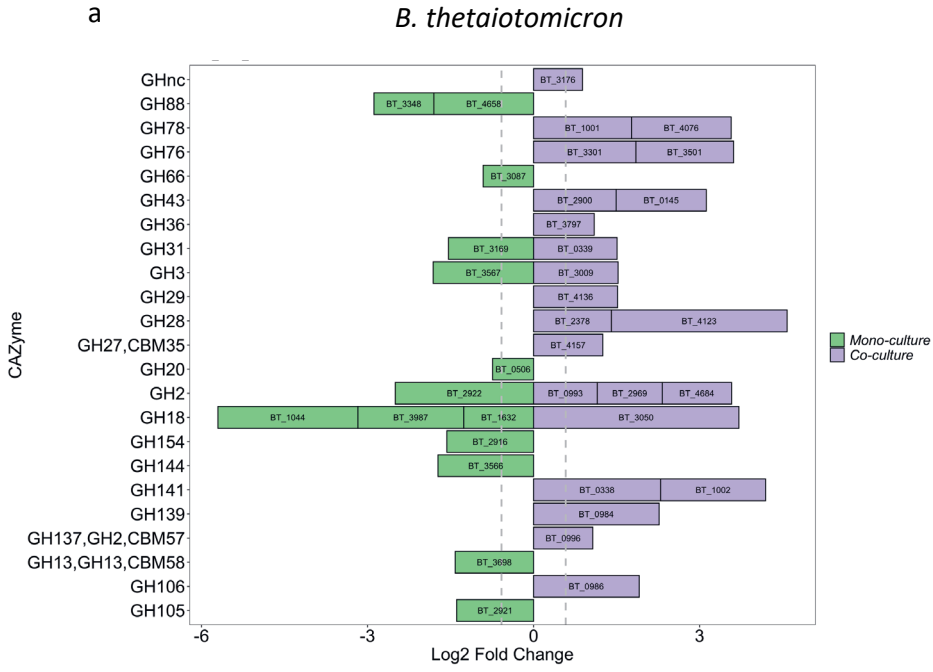
of *B. thetaiotaomicron* to utilize different complex dietary oligosaccharides relies on 88 polysaccharide utilization loci (PULs), which encompasses 18% of its genome (Martens, Chiang, and Gordon 2008). Therefore, we analysed the changes in the PUL expression and compared the mRNA levels between mono- and co-culture conditions. In the co-culture, *B. thetaiotaomicron* significantly upregulated 93 genes belonging to 40 different PULs, while 77 genes associated with 22 PULs were downregulated (Figure 2b). In the co-culture, there was significant upregulation of 40 different PULs that are described as being induced by host *O*-glycans. Three enriched PULs in the co-culture, encompassed within BT_0317-18, BT_2170-73, and BT_4038-40, had more than a 2.0-fold change. Two downregulated PULs, encompassed within BT_4652-62 (heparin sulfate) and BT_3324-49 (chondroitin sulfate) were significantly expressed in the monoculture (Supplementary Table 2).

In contrast, *A. muciniphila*, showed 143 genes, differentially expressed between mono- and co-culture, involved in carbohydrate metabolism and glycan biosynthesis and metabolism, and 5% of these genes were significantly upregulated and 2% were downregulated in the co-culture (Supplementary Table 2). In the co-culture, *A. muciniphila* significantly upregulated two lipopolysaccharide biosynthesis genes encoding for *O*-antigen ligase domain-containing protein (Amuc_0088) and lipid-A biosynthesis acyltransferase protein (Amuc_1541) (>2.0 fold-change, q-value < 0.05). From the 53 mucin-degrading genes found in our *A. muciniphila* transcriptome data, seven were significantly upregulated in the co-culture (>2.0 fold-change) (Supplementary Table 3).

A stable community *in vitro* leads to altered overall expression of Carbohydrate-Active Enzyme Genes

The genes encoding for carbohydrate-active enzymes (CAZymes) in the co-culture compared to monoculture were analysed in detail. *B. thetaiotaomicron* altered most of its hydrolytic machinery between the two conditions. A significant increase in gene expression was detected for β-glucosidases (GH3, GH30), a-glucosidase (GH31), β-galactosidases (GH2, GH137), α-galactosidase (GH27, GH36), α-L-arabinosidase (GH43), α-L-fucosidases (GH29, GH139, GH141), α-mannanases (GH76), and α-L-rhamnosidases (GH78) in the co-culture (Figure 2a). We also observed that, in the co-culture, *B. thetaiotaomicron* expressed genes that target different glycosidic linkages present in mucin. For example, four α-L fucosidases exhibited more than a 2.8-fold

difference in the co-culture compared to the monoculture. Namely, BT_0096 and BT_4136 would cleave α1-2 and a1-3/1-4-linked fucose to *N*-acetylglucosamine in mucin, respectively. BT_0038 and BT_1002 genes encoded for general α-L-fucosidase activity. Moreover, *B. thetaiotaomicron* showed an increase in the overall expression of GHs families in the co-culture (Supplementary Figure 1). Also, polysaccharides lyases (PL1, P10, PL27), encoding for pectate lyase, were enriched in the co-culture. Furthermore, in the co-culture, *B. thetaiotaomicron* significantly expressed more glycosyl transferases (GTs) from different families (> 1.8-fold change, q-value <0.05), with GT2 (8 genes) and GT4 (3 genes) being the most representative GT families in the co-culture (Figure 2b). Interestingly, BT_1709 (GT2) and BT_2864 (GT4) displayed a 29.0 and 39.0-fold change, respectively.



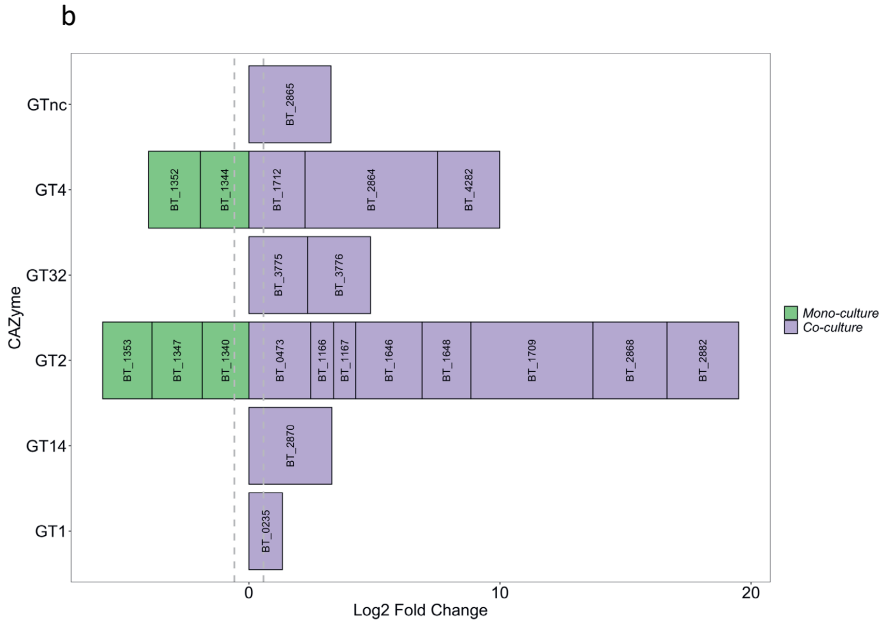


Figure 2: Differential expression of GHs and GTs between mono and co-culture during *in vitro* fermentation. a) *B. thetaiotaomicron* GHs, b) *B. thetaiotaomicron* GTs. Only the CAZymes with adjusted p-value < 0.05 are shown as averages. The grey dotted line indicates Log2 Fold Change threshold (Log2 Fold Change = 0.58)

In the co-culture, *A. muciniphila* showed a significant increase of three GH families that are predicted to be involved in the core glycan degradation pathway. Namely, β-galactosidases (GH2), β-N-acetylhexosaminidases (GH20), and α-L-fucosidases (GH29) were significantly enriched in the co-culture. Furthermore, two families of GTs (GT2 and GT4) showed the highest increase of their overall expression in the co-culture conditions. Specifically, *A. muciniphila* significantly upregulated four genes encoding GT2 (Amuc_2094) and GT4 (Amuc_2088, Amuc_2089 and Amuc_2090) (1.9-fold change, p-value <0.05) (Supplementary Figure 2a,b). The list of *A. muciniphila* and *B. thetaiotaomicron* CAZymes found in the co-culture is summarized in Supplementary Table 5. The overall expression of the CAZymes indicates that both bacteria are expanding their carbohydrate metabolic gene response in the co-culture.

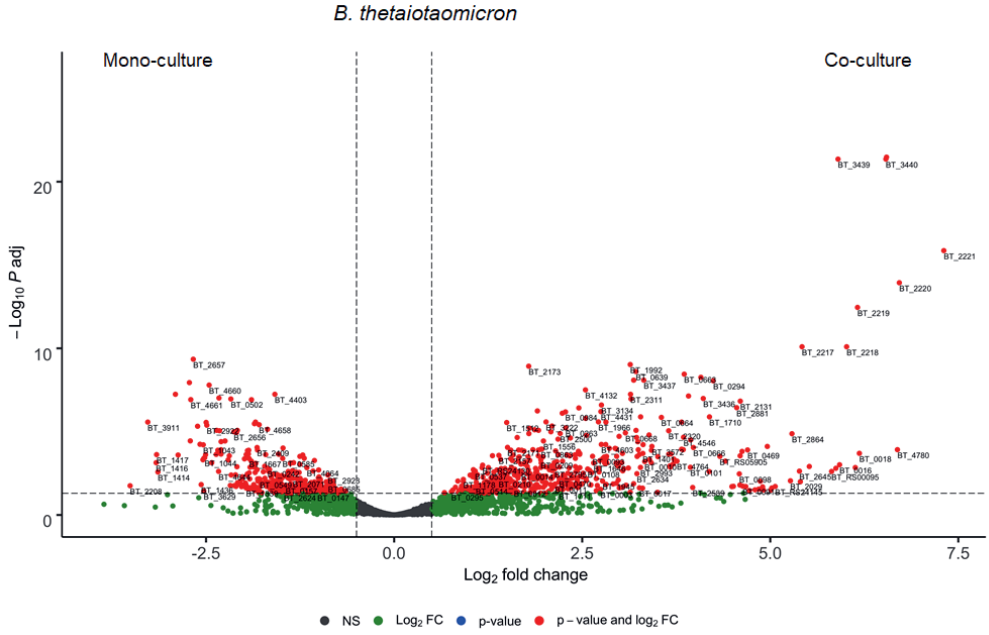


Figure 3: Differential expressed genes of *B. thetaiotaomicron* between mono- and co-culture during *in vitro* fermentation. Volcano plot of the differential expressed *B. thetaiotaomicron* genes of mono- versus co-culture. Positive Log2 fold change indicates upregulation in co-culture. The horizontal dashed black line adjusted p-value threshold (q-value = 0.05). The vertical dashed black lines indicate Log2 fold change threshold (Log2 fold change = 0.58).

In vitro microbe to microbe interaction between *B. thetaiotaomicron* and *A. muciniphila*

Thereafter, we analysed genes involved in the interaction between *B. thetaiotaomicron* and *A. muciniphila*. We identified in *B. thetaiotaomicron*'s transcriptome, 1012 differentially expressed genes, of which 597 genes (59%) were upregulated in the co-culture and 415 genes (41%) were downregulated (Figure 3). In the co-culture, *B. thetaiotaomicron* significantly upregulated two different operons (BT_2217 – 2221 and BT_3439 – 3441) of hypothetical proteins that exhibit the highest fold change compared to the monoculture. BT_2217-2221 exhibited between 30 and 120-fold change (p-value < 0.05). The other operon (BT_3439 – 3441) exhibited between 60 and 96-fold change (p-value < 0.05). The latter cluster contains a BT_3439 gene that encodes for the Membrane Attack Complex/Perforin (MACPF) domain-containing protein. Furthermore, the metabolism of terpenoids and polyketides (K00919, K00991) and the signal transduction (K03100, K07783, K07713) were the most variable metabolic processes in the co-culture (Figure 4a).

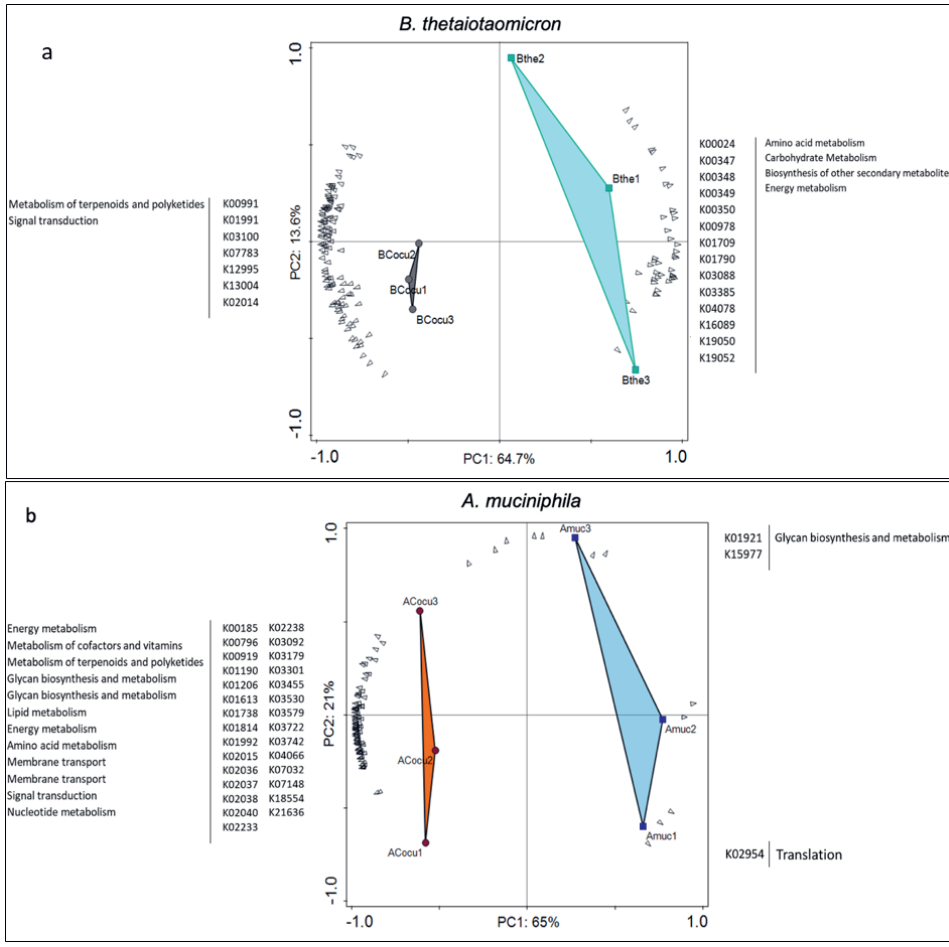


Figure 3: Differential expressed genes and KEGG Orthologies (KOs) of *B. thetaiotaomicron* and *A. muciniphila* between mono- and co-culture during *in vitro* fermentation. a) Principal Component analysis (PCA) showing the most variable KOs between *B. thetaiotaomicron* mono- and co-culture. b) Principal Component analysis (PCA) showing the most variable KOs between *A. muciniphila* mono- and co-culture

A. muciniphila differentially regulated 454 genes, in which 347 genes (76%) were upregulated and the rest 107 genes (24%) were downregulated in the co-culture (Figure 5). Interestingly, 7% of the significantly upregulated *A. muciniphila* genes encoded for transporter activity participate in membrane transport, signal transduction and/or signalling and cellular processes pathways. In the co-culture experiment, *A. muciniphila* demonstrated the highest fold difference (>4.0-fold change, q-value < 0.05) of a gene cluster that consists of two ABC transporter permeases (Amuc_1304 and Amuc_1305), two phosphate-binding proteins (Amuc_1304 and Amuc_1306) and

is found to be part of the same gene operon (Amuc_1295 – 1306). In addition, three genes that belong to the same operon and encode for transporter permease (Amuc_1379 and Amuc_1380) and ATP-binding protein (Amuc_1381) activity, exhibited more than a 2.0-fold difference in the co-culture. Additionally, three membrane transport genes from the same operon (Amuc_0143-45) were predicted to be involved in the oligopeptide transport system. These genes that encode for oligopeptide substrate-binding (Amuc_0145) and permease (Amuc_0143, Amuc_0144) protein activity exhibited above a 1.5-fold difference in the co-culture conditions (p-value < 0.05). Furthermore, the transcriptome analysis revealed a significant upregulation (>1.5-fold change, p-value < 0.05) of genes that were involved in a two-component system pathway (Amuc_0021, Amuc_0828 and Amuc_0831, Amuc_0889, and Amuc_1151 – 1152). The 50 most variable and the most significantly upregulated genes from both members of the community are illustrated in Supplementary Table 4 and Supplementary Table 5.

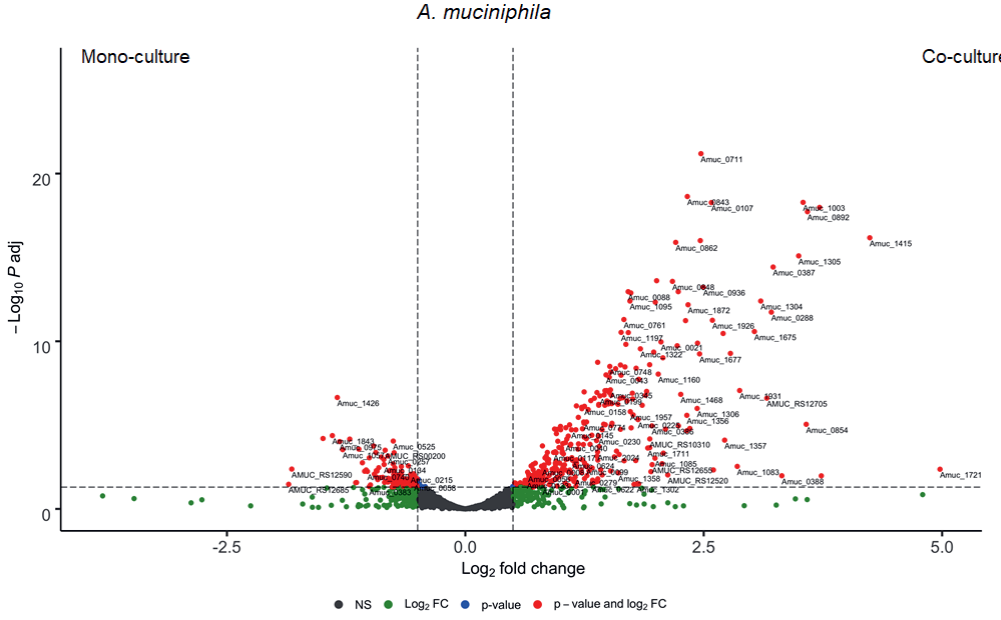


Figure 5: Differential expressed genes of *A. muciniphila* between mono- and co-culture during *in vitro* fermentation. Volcano plot of the differential expressed *A. muciniphila* genes of mono- versus co-culture. Positive Log2 fold change indicates upregulation in co-culture. The horizontal dashed black line adjusted p-value threshold (q-value = 0.05). The vertical dashed black lines indicate Log2 fold change threshold (Log2 fold change = 0.58).

***In vivo* co-colonisation of *A. muciniphila* and *B. thetaiotaomicron* in germ-free mice**

Thereafter, we investigated the mono- and co-culture conditions in a gnotobiotic mouse model. Samples from the cecum and distal colon were taken for total mRNA and transcriptome data were generated. The transcriptome analysis of *B. thetaiotaomicron* in the *in vivo* conditions showed no significant differences in gene expression in the regulation of host glycan-degrading genes or the PULs.

The transcriptome analysis of *A. muciniphila* in cecum revealed that 185 genes were significantly upregulated, and 116 genes were downregulated in the co-colonized mice (Supplementary Figure 3). *A. muciniphila* in cecum in the co-colonization experiment showed significant differences in the expression profile of genes involved in carbohydrate metabolism and glycan biosynthesis and metabolism. Co-colonization conditions in cecum showed significant upregulation (above 2.0 and up to a 7.0-fold change) of the genes involved in two-component system pathways (Amuc_0827 and Amuc_0831 and Amuc_1151 – 1152) for *A. muciniphila*. Furthermore, 17 genes within the operon Amuc_2077 – Amuc_2097 (Amuc_2078-79, Amuc_2081-94, and Amuc_2096) were differentially expressed, which demonstrated more than a 1.6-fold difference in the co-colonized mice. Interestingly, nine genes of this operon (45%) were found to encode for glycosyl transferase activity (GT2 and GT4). On the other hand, fourteen genes involved in carbohydrate metabolism and four genes participating in glycan biosynthesis and metabolism were found to be significantly downregulated in the co-colonized mice.

Host response towards mono- and co-cultures lead to differential response in immune and gene expression

Host response towards the colonization with mono- and co-cultures of *B. thetaiotaomicron* and *A. muciniphila* was monitored by total RNA-sequencing of the cecum and distal colon. The transcriptional profile showed no significant differences in mouse cecum response between mono-colonization with *B. thetaiotaomicron* and co-colonization. In the distal colon, the comparison of *B. thetaiotaomicron* mono-associated mice with the co-associated revealed 228 significantly upregulated genes in the co-colonized mice. The 10 most significantly impacted genes in the co-colonized mice (Tgm3, Atp12a, Cd74, Lyz2, Slc40a1, B3gnt7, Pag1, Ptprc, Bach2, Lrrk2) exhibited more than a 2.0-fold difference. Next, six genes showed more than a 10-fold

difference in the co-associated mice (St8sia5, Glycam1, Atp12a, Spink5, Nr1h5, Casp14) (Supplementary Table 6). Finally, the co-colonized mice overexpressed three genes encoding for glycosyltransferase activity and involved in glycan biosynthesis (B3gnt7, Chst3, Gal3st2c, St8sia5). Specifically, B3gnt7 (β-1,3-N-acetylglucosaminyltransferase) and Chst3 (chondroitin 6-slfotransferase) participate in the glycosaminoglycan biosynthesis pathway, while St8sia5 (α-N-acetyl-neuraminide a-2,8-sialyltransferase) and Gal3st2c (galactose-3-O-sulfotransferase) are involved in glycosphingolipid biosynthesis and unclassified metabolism pathways, respectively. A trend towards increased expression of genes coding for protein glycosylation activity (B3gat1, Gal3st2, Gal3st2b) was observed in mice colonized with both organisms. In both the cecum and the distal colon, no significant differences were observed in the expression of mucin genes between mono- and co-colonization.

The transcriptional profile showed 23 genes significantly affected by the presence of glycan degraders together in mouse cecum compared to *A. muciniphila* associated mice (Supplementary Table 8). The 10 most significantly abundant genes in the co-colonized mice (Lgals3, Aqp8, Sdc1, Adipor2, Slc2a1, Prdx5, Tsku, Tmem171, Cgref1, Kyat1) exhibited more than a 1.5-fold difference. Five genes (Zzef1, Cckar, Zfp987, Epha6, Gjb4) exhibited more than a 10-fold difference in the co-culture. Zzef1 and Zfp987 genes encoding for zinc finger protein activity were $1.10x10^7$- and 112-fold different in the co-colonization, respectively. Furthermore, we observed that an acetyl-Coenzyme A (Acaa1b) encoding gene was significantly overexpressed in the co-associated mice (2.65-fold change, q-value < 0.05). Additionally, genes encoding for protein glycosylation activity were upregulated non-significantly in the co-colonized mice. No significant differences were noticed in the expression of cecal mucin genes in the mice colonized with the two bacteria. In the distal colon, no significant differences in the mice's responses between mono-colonization with *A. muciniphila* and co-colonization were found. In the distal colon, though, a higher expression of mucin genes (Muc2, Muc3a, Muc4, Muc6, Muc16, Muc20) was detected in the co-colonization study compared to mono-colonization.

Discussion

This study investigated the interaction between a mucus-degrading specialist, *A. muciniphila* and a mucin-degrading generalist *B. thetaiotaomicron* at *in vitro* and *in vivo* conditions. The results demonstrated that these two glycan-degrading bacteria reached a stable state over time *in vitro* where both species co-existent and are metabolically active. Furthermore, the transcriptional response of both bacteria was altered when were cultured together in either a lab bioreactor or animal gut.

We demonstrated that in a mucus environment, *B. thetaiotaomicron* deploys different hydrolytic enzymes to degrade mucin glycans under mono and co-culture conditions both *in vitro* and *in vivo*. In the *in vitro* co-culture, *B. thetaiotaomicron* showed increased expression of its hydrolytic machinery. A possible explanation for this might be that *B. thetaiotaomicron* in the presence of *A. muciniphila* attempts to reach the mucin glycans by deploying as many GHs as possible to gain a competitive advantage. Another possible explanation is that *B. thetaiotaomicron* attempts to reduce *A. muciniphila*'s fitness in the community. The latter is often described as an exploitative competition where a member of the community restricts the competitor's access to nutrients via the secretion of digestive enzymes (Rendueles and Ghigo 2012; Diggle et al. 2007). Moreover, *B. thetaiotaomicron* targeted core mucosal glycosidic linkages by upregulating fucosidases, glucosidases and galactosidases. This result can be explained by the fact that *B. thetaiotaomicron* will alter its transcriptional response when limited nutrients are available. This alteration in the glycan metabolism has been previously described, where *B. thetaiotaomicron* shifted from dietary polysaccharides to mucus glycan metabolism in the absence of fibre (Sonnenburg 2005). Accordingly, *A. muciniphila* in response to *B. thetaiotaomicron* GHs expression, upregulated β-galactosidase (GH2), β-hexosaminidase (GH20), and α-L-fucosidase (GH29), targeting core glycosidic linkages also found in mucin. However, *A. muciniphila* displayed no alterations in its glycan degrading machinery between mono or co-culture conditions, which demonstrates the robustness of the bacterium in a community where the only source of nutrients is mucin glycans. Exploitative competition for nutrients was also observed during the *in vivo* experiment. We observed an overall increase in GHs expression in co-culture conditions from both *A. muciniphila* and *B. thetaiotaomicron*. The chow diet mainly consists of starch (30%), which can be utilized efficiently by *B. thetaiotaomicron* through its starch utilization system (Sus), as described in numerous

studies (Martens, Chiang, and Gordon 2008; Martens et al. 2011, 2009; Marcobal et al. 2011). Since there is no evidence that *A. muciniphila* can utilize starch, we suggest that two different niches for bacterial colonization were established during the mice colonization.

In the *in vitro* co-culture, *B. thetaiotaomicron* significantly upregulated a cluster of genes encoding hypothetical proteins (BT_2217 – 2221). Three genes of this cluster (BT_2217, BT_2219 and BT_2220) were earlier characterized as C10 protease genes that encode for the C10 family of papain-like cysteine proteases, three of which are genetically clustered, and are associated with two staphostatin-like inhibitors (BT_2218, BT_2221) that may play diverse roles in the interaction with the host (R. F. Thornton et al. 2012). Moreover, some studies suggest that cysteine proteases may be located in the outer member vesicles (OMVs), as *B. thetaiotaomicron* preferentially packages lapidated glycosidases and proteases onto OMVs (Hutchings et al. 2009; Schwechheimer and Kuehn 2015; Elhenawy, Debelyy, and Feldman 2014). These OMVs can interact with host epithelial cells and other bacteria, thus aiming to hydrolyse extracellular host and bacterial targets (Schwechheimer and Kuehn 2015). Furthermore, it has also been described that cysteine proteases are used from gut bacteria, such as *Porphyromonas gingivalis,* to cleave the mucus proteins in the gut epithelial barrier (Vergnolle 2016; van der Post et al. 2013). Therefore, in our experiment, the upregulation of cysteine proteases and the associated inhibitors of *B. thetaiotaomicron* in the co-culture *in vitro* could indicate an additional aid to the mucus degradation or a mechanism to prey on or inhibit its competitor – *A. muciniphila,* thus *B. thetaiotaomicron* will share the same nutritional environment.

In vitro, the co-culture conditions significantly upregulated a cluster (BT_3439 – 3441) from *B. thetaiotaomicron,* which encodes a gene for membrane attach complex perforin (MACPF) activity (BT_3439) (Q. Xu et al. 2010; Chatzidaki-Livanis, Coyne, and Comstock 2014). This gene was 60-fold higher expressed (q-value < 0.05) in the co-culture than in the monoculture. *B. thetaiotaomicron* is known to secrete antimicrobial proteins known as Bacteroidales-secreted antimicrobial proteins (BSAPs) (Chatzidaki-Livanis, Coyne, and Comstock 2014; Roelofs et al. 2016; Shumaker et al. 2019). These BSAPs contain a MACPF domain and target a different class of surface molecule. For example, BSAP-1 and BSAP-4 target the β-barrel outer-membrane protein (OMP), and BSAP-2 and BSAP-3 target the lipopolysaccharide (LPS) glycan (core or *O*-antigen)

of the sensitive Bacteroidetes strains that lack the MACPF protein (Roelofs et al. 2016; Shumaker et al. 2019). To date, no study has described the activity of BSAPs against other members of microbial communities, except for Bacteroidetes-sensitive strains. However, in our study, the high fold-change of *B. thetaiotaomicron* MACPF expression in the co-culture may imply that *B. thetaiotaomicron* uses the MACPF to obtain survival advantage in the community. Additionally, the BSAP proteins are always adjacent to the LPS core biosynthesis operon encoding also the replacement glycosyltransferase (GTs) genes of the target strains. The MACPF gene (BT_3439), found in our results, is located close to the lipooligosaccharides (LOS) core biosynthesis operon (BT_3362 – 3385), as it was described before (Jacobson, Choudhury, and Fischbach 2018). Our results demonstrated an increase of *B. thetaiotaomicron* GT2 and GT4 expressed in the co-culture compared to monoculture, showing a more than 20.0-fold change and 30.0-fold upregulation, respectively. The acquisition of the MACPF gene occurs with a new set of glycosyltransferase-encoding genes, resulting in an altered LPS glycan structure (Roelofs et al. 2016; Mceneany et al. 2018). Therefore, these findings indicate that another type of competition has occurred in the co-culture, where *B. thetaiotaomicron* might attempt to attack *A. muciniphila* through the production of an antimicrobial protein (interference competition). On the other hand, one would expect that *A. muciniphila* abundance in the co-culture would be affected by *B. thetaiotaomicron*'s attempt to reduce its fitness, but this is not what we observed. In co-culture conditions, both *in vitro* and *in vivo, A. muciniphila* significantly upregulated ABC transporters. It has been described before that ABC transporters involved in membrane transport provide antimicrobial peptide resistance (Z. Wang et al. 2016). Another study revealed that the ABC transporter YejABEF from *Brucella melitensis* is essential for its survival and resistance against host antimicrobial proteins. *A. muciniphila* may deploy its ABC transporters that are regulated by the adjacent two-component system-encoding genes to efflux the secreted antimicrobial peptides outside the cell that perhaps are released by *B. thetaiotaomicron*. Furthermore, in the co-culture, *A. muciniphila* upregulated in both the bioreactors (3.2-fold change, q-value < 0.05) and the cecum (2.5-fold change, q-value < 0.05), a gene encoding for *O*-antigen ligase activity (Amuc_0088). This *A. muciniphila O*-antigen was found to be a neighbouring gene of the capsular polysaccharide biosynthesis protein (Amuc_2078) (Szklarczyk et al. 2014), which is part of an operon of 20 genes (Amuc_2077 – 2097) predicted to be involved in LPS biosynthesis. The upregulation

of *O*-antigen ligase could be used by *A. muciniphila* to counteract *B. thetaiotaomicron*'s antimicrobial peptides. A previous study observed that an *O*-antigen ligase, a Lipid A and 19 other LPS biosynthesis genes were used by *E. coli* to gain resistance to bacteriocins, such as colicin, and other antimicrobial peptides. In these 21 genes, six GTs were found to be significantly upregulated in the presence of colicins, thus providing fitness benefits to uropathogenic *E. coli* (Sharp et al. 2018). In our results, three *A. muciniphila* glycosyl transferases family 4 (Amuc_2088 – 2090) were above a 2.5-fold change in the co-culture *in vitro*, while, in the cecum, all nine GTs (Amuc_2081 – 2084, Amuc_2088 – 2090, Amuc_2093 – 2094) of this cluster exhibited more than a 1.5-fold difference in the co-culture. Therefore, *A. muciniphila* in a highly competitive environment may be resistant to antimicrobial peptides by using its putative LPS-associated core biosynthesis genes.

In our study, we showed that the presence of *B. thetaiotaomicron* in mice's distal colon and *A. muciniphila* mice's cecum altered the host's response compared to the co-culture. In distal colon co-culture, the host significantly upregulated genes encoding for glycosyltransferase (B3gnt7, St8sia5, Gal3st2c) and sulfotransferase activity (Chst3) involved in protein glycosylation. A recent study observed that more glycosyltransferases, involved in Muc2 O-glycosylation, were detected in the colon than in the small intestine (Kataoka and Huh 2002). It was observed that B3gnt7, which encodes for β1,3-*N*-acetylglucosaminyltransferase activity, is responsible for elongation of glycans by GlcNAc and together with the B4galt enzymes (B4galt1 and B4galt4) synthesize lactosamine elongations (Arike, Holmén-Larsson, and Hansson 2017). Furthermore, B3gnt7 is described to participate in keratan sulfate GAG synthesis (Littlechild et al. 2018). Interestingly, another study highlighted that B3gnt7, together with other transferases, is considered to be responsible for the synthesis of extended sulfated glycans (C. H. Lu et al. 2014). Additionally, Chst3 encodes for chondroitin 6-sulfotransferase and catalyses the transfer of sulfate to position 6 of the *N*-acetylgalactosamine residue of GAG chondroitin sulfate (Uchimura et al. 1998, 2002). Sulfated glycans are abundant in both the small intestine and colon (Arike, Holmén-Larsson, and Hansson 2017) and they are also present in the mucosal layer. Therefore, B3gnt7 and Chst3 are used from the host to synthesize keratan and chondroitin sulfate GAGs, which may constitute a source of nutrients for *B. thetaiotaomicron* in the gut. In the cecum, a non-significant increase of genes that encode from transferase activity

participating in glycan biosynthesis pathway was also observed. The increased abundance of genes that encode for protein glycosylation in the co-colonized mice could be due to increased bacterial diversity in the gut of the mice. In earlier studies, more glycosyltransferases and longer glycans were detected in the mouse intestine as the intestinal colonization was being increased (Arike, Holmén-Larsson, and Hansson 2017). Our data indicate enrichment in the glycosylation process in mice after colonization with glycan-degrading bacteria. This enrichment could constitute a marker for a healthy intestine as abnormal glycosylation was observed in multiple human diseases (Reily et al. 2019; Stanley 2016).

This study demonstrated that two-glycan degrading bacterial species respond to a nutrient deficit by expressing genes encoding for protein activity related to glycan-degrading enzymes as well as ABC transporters and antimicrobial proteins as a result of their interaction. We showed that when mucin glycans were the only source of nutrients in the environment, *B. thetaiotaomicron* expressed genes involved in antimicrobial activity. Furthermore, our study indicates that *A. muciniphila* was able to sustain its abundance in the co-culture by overexpressing genes that are predicted to be involved in LPS core biosynthesis and some ABC transporters. These results suggest how robust and resilient *A. muciniphila* could be in the human gut, provided its access to mucin glycans is not limited. *A. muciniphila* is known for its capacity to utilize and degrade glycans found on the outer mucosal layer (Derrien et al. 2004). Therefore, the ability of *A. muciniphila* to retain its major function (mucin glycans utilization) and its abundance in a competitive environment implies a colonization advantage of *A. muciniphila* in the gut. Overall this study gives more insight in how gut microbes adapt to their environment and respond towards host conditions and other microbiota members.

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Materials and Methods

Bacterial growth conditions

Precultures of *Akkermansia muciniphila* Muc^T (ATTC BAA-835) and *Bacteroides thetaiotaomicron* were grown in basal medium as described previously (Derrien et al. 2004). The medium was supplemented with crude hog gastric mucin (0,5% Type III, Sigma-Aldrich, St. Louis, MO, USA). All the anaerobic bottles were supplemented with 1 % v/v of $CaCl_2$ and vitamins mixture solution as described previously (Stams et al. 1993). Incubations were performed in serum bottles sealed with butyl-stoppers at 37ºC under anaerobic conditions provided by a gas phase of 182 kPa (1.5 atm) N_2/CO_2.

Anaerobic continuous fermentation

In vitro fermentations were conducted in three parallel bioreactors (DasGip, Eppendorf, Germany) filled with 250 ml of basal medium as it has been described before (ref for basal media) at 37ºC, at a controlled pH of 6.5 and at a stirring rate of 150 rpm. The bioreactors and the feed bottle were supplemented with 0.5% of crude mucin, 1% of vitamin solution and at the beginning of the fermentation. Anaerobic conditions were succeeded by sparging the media with N_2/CO_2 continuously (6 sL/h). Experiments were performed with 1% (v/v) supplementation of $CaCl_2$ and vitamin mixture as described previously. The media in both feed and bioreactors were reduced with 0.05% L-Cysteine-HCl in order to achieve anaerobic conditions. The bioreactors were inoculated with a normalized O.D. of 1.0 of both species to achieve same starting cells density in the beginning of the fermentation. The flow rate of the feed was set at 20 ml/h and the recovery rate of media was 12.5 hours. The growth was measured by spectrophotometer as optical density at 600 nm. The experiment was done in three biological replicates.

In vivo experiment

The study was conducted in accordance with institutional guidelines for the care and use of laboratory animals. All animal procedures related to the purpose of the research were approved by the University of Gothenburg Animal Studies Committee under the Ethical license of the national competent authority, securing full compliance the European Directive 2010/63/EU for the use of animals for scientific purposes. Female, 10-12 weeks old Swiss Webster germ free (GF) mice (*Mus musculus*), were used in the current study. All mice were obtained from the in-house breeding facility. The GF status of the mice was tested by culturing fecal bacteria anaerobically and aerobically,

and also by PCR for bacteria 16S rDNA using the primers 27F and 1492R. One week prior the experiment mice were moved from the breeding isolators to the dedicated experimental isolators. For all colonization experiments, mice were maintained the plastic experimental isolators, in open top cages (incl. metal rack and water bottle) with wooden bedding, housed in a climate-controlled room (22°C ± 2°C) and subjected to a strict 12h light/dark cycle (7:00 a.m. – 7:00 p.m.) with free access to autoclaved water and food. Mice were fasted at 9:00 am for 4h prior gavage (intragastrical) with 0.2 ml of active culture (for both mono- and co-colonization). We have estimated the group size based on previously published studies where GF mice were colonized either with *Akkermansia muciniphila* or *Bacteroides thetaiotaomicron* (Derrien et al. 2011; Mahowald et al. 2009) In all these studies at least 3 mice were included in each group. In our study the smallest group was n=5 mice. For *B. thetaiotaomicron* mono-colonization (n=5 mice) and bi-colonization (n=7 mice) 10^8 CFU/ml bacterial culture have been used. For *A. muciniphila* (n=8 mice) mono-colonization 10^9 CFU/ml bacterial culture have been used. For all three colonization, mice were inoculated with a single gavage of the respective culture. The mice received during the 14 day of colonization chow diet (5021 rodent diet, LabDiet; fat 9% wt/wt). At the end of the study mice were euthanized by cervical dislocation. Intestinal segments (cecum and distal colon) were harvested and immediately snap-frozen in liquid nitrogen and stored at -80°C until further processed. Cecum content was collected and stored in RNA Later (Sigma) at -20ºC until further processed.

RNA isolation

Total RNA was isolated for both the bioreactors' samples and mice intestinal segments (cecum and distal colon) by a method combining the Trizol® reagent and the RNeasy Mini kit (QIAGEN, Germany) as described previously (reference). Four microliters of of p-mercaptoenthanol and 0.4 ml of buffer RLT were added to 1 ml of Trizol® reagent containing the bacterial pellet or the intestinal segment. The mixture was transferred to a tube containing 0.8 g of glass beads (diameter 0.1 mm), followed by three times of bead beating for 1 min at 5.5 m/s with ice cooling steps in between. Subsequently, 0.2 ml of ice-cold chloroform was added. The solution was mixed gently followed by centrifugation at 12,000 x *g* for 15 min at 4ºC. The RNA isolation was continued with the RNA clean-up according to the manufacturer's instructions for the RNeasy Mini kit. Genomic DNA was removed by an on-column DNase digestion step during RNA

purification (DNase I recombinant, RNase-free, Roche Diagnostics, Germany). RNA concentration was measured by nanodrop and RNA quality was assessed by Qsep bioanalyzer (BiOptic Inc., Taiwan).

Transcriptome sequencing (RNA-sequencing)

Total RNA samples were further processed by Novogene for RNA-seq.

Transcriptome analysis

Illumina reads have been trimmed for low quality and adapters with fastp (v0.20.0) (21) using default settings. rRNA sequences have been removed with bbduk (v38.79) (https://sourceforge.net/projects/bbmap/) using the following parameters k=31 and ref=riboKmers.fa.gz. Transcripts from the reference strain of *A. muciniphila* (GCF_000020225.1) have been quantified with RSEM (v1.3.1) (B. Li and Dewey 2011) in combination with bowtie2 (v2.3.5.1) (Langmead and Salzberg 2012). Mapping and read quality were inspecting using MultiQC. Tximport (v1.12.3) was used to import read-counts obtained from RSEM into Rstudio (Soneson, Love, and Robinson 2016). These imported reads were used for the differential expression analysis using DESeq2 package. Canoco5 (version 2.8.12) was used for RDA and PCA analysis based on the relative abundance data. All further analysis was done using R version 3.6.3 in Rstudio version 1.2.5019.

High-performance liquid chromatography (HPLC)

For fermentation product analysis, samples were obtained at different time points of the incubation period. Crotonate was used as the internal standard and the external standards were lactate, formate, acetate, propionate, butyrate, isobutyrate, 1,2-propanediol, sialic acid and glucose. Substrate conversion and product formation were measured with Shimadzu LC_2030C equipped with a refractive index detector and a Shodex SH1011 column. The oven temperature was set at 45ºC with a pump flow of 1.00 mL/min using 0.01N H_2SO_4 as eluent. All samples and standards (10µl injection volume) ran for 20 minutes. Standards were prepared with concentrations ranging from 2.5 mM to 25 mM.

Statistical analysis

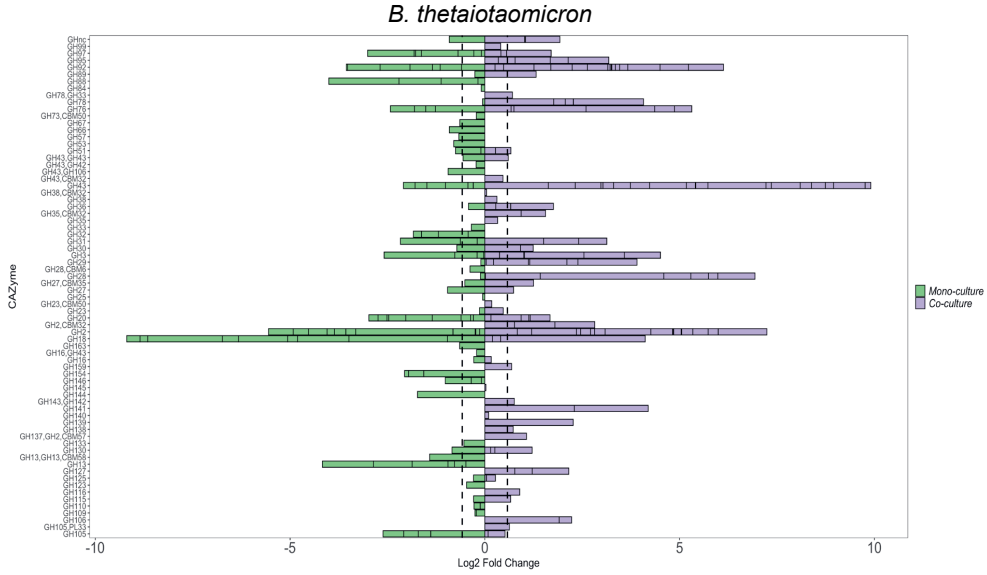
Statistics were performed using student's t-test was used and corrected for multiple testing using False Discovery Rate (FDR) correction for multiple corrections. Data are

presented as mean ± standard deviation (SD), unless stated otherwise. Adjusted p-values of < 0.05 were considered significant.

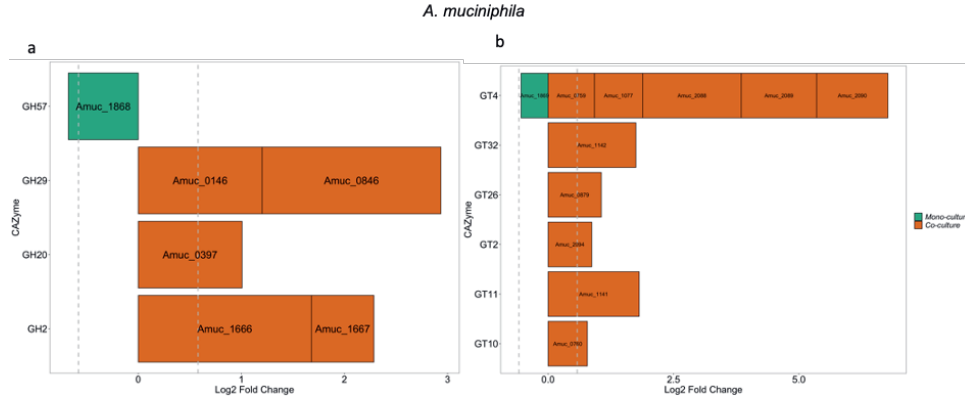
Supplementary Data

Supplementary Table 1: Fermentation products of *A. muciniphila* and *B. thetaiotaomicron* grown in monocultures and co-culture

	Akkermansia muciniphhila				*Bacteroides thetaiotaomicron*				Co-culture			
Time point (hours)	**Acetate (mM)**	**Propionate (mM)**	**1,2-propanendiol (mM)**	**Succinate (mM)**	**Acetate (mM)**	**Propionate (mM)**	**1,2-propanendiol (mM)**	**Succinate (mM)**	**Acetate (mM)**	**Propionate (mM)**	**1,2-propanendiol (mM)**	**Succinate (mM)**
0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.10 ± 0.21	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
4	0.10 ± 0.20	0.00 ± 0.00	0.05 ± 0.11	0.00 ± 0.00	0.19 ± 0.37	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.40 ± 0.48	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
8	1.83 ± 1.42	1.18 ± 1.00	0.07 ± 0.15	0.18 ± 0.21	0.76 ± 0.57	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	3.29 ± 4.35	1.18 ± 2.21	0.14 ± 0.17	0.66 ± 1.25
12	7.97 ± 2.66	5.71 ± 2.34	0.49 ± 0.50	0.47 ± 0.56	1.23 ± 0.54	0.18 ± 0.36	0.00 ± 0.00	0.00 ± 0.00	7.31 ± 5.50	4.47 ± 3.77	0.22 ± 0.18	0.59 ± 0.96
24	10.43 ± 1.95	8.22 ± 1.27	0.69 ± 0.71	0.10 ± 0.19	2.18 ± 0.64	0.00 ± 0.00	0.06 ± 0.12	0.00 ± 0.00	12.05 ± 1.78	8.24 ± 2.32	0.53 ± 0.61	0.32 ± 0.64
48	10.50 ± 2.16	8.53 ± 1.01	0.78 ± 0.72	0.10 ± 0.20	2.25 ± 0.97	0.65 ± 0.85	0.06 ± 0.11	0.00 ± 0.00	13.38 ± 1.10	9.39 ± 1.35	0.54 ± 0.63	0.38 ± 0.60
72	9.73 ± 0.73	8.25 ± 0.61	0.71 ± 0.51	0.10 ± 0.20	2.72 ± 1.23	0.67 ± 0.91	0.06 ± 0.12	0.00 ± 0.00	13.21 ± 0.90	9.87 ± 0.99	0.56 ± 0.55	0.00 ± 0.00
96	9.02 ± 1.30	7.45 ± 0.79	0.54 ± 0.45	0.44 ± 0.65	2.55 ± 1.27	1.07 ± 1.58	0.06 ± 0.13	0.00 ± 0.00	13.28 ± 0.86	10.07 ± 0.70	0.38 ± 0.32	0.00 ± 0.00
120	9.97 ± 1.20	8.29 ± 0.89	0.59 ± 0.23	0.18 ± 0.21	3.33 ± 0.98	1.25 ± 1.41	0.07 ± 0.15	0.00 ± 0.00	12.82 ± 1.53	10.36 ± 0.54	0.31 ± 0.25	0.00 ± 0.00



Supplementary Figure 1: Differential expression of *B. thetaiotaomicron* GHs between mono and co-culture during *in vitro* fermentation. Only the CAZymes with adjusted p-value < 0.05 are shown as averages. The grey dotted line indicates Log2 Fold Change threshold (Log2 Fold Change = 0.58)



Supplementary Figure 2: Differential expression of GHs and GTs between mono and co-culture during *in vitro* fermentation. a) *A. muciniphila* GHs, b) *A. muciniphila* GTs. Only the CAZymes with adjusted p-value < 0.05 are shown as averages. The grey dotted line indicates Log2 Fold Change threshold (Log2 Fold Change = 0.58)

Supplementary Table 2: Upregulated *B. thetaiotaomicron* genes belonging to PULs in the co-culture

GeneName	FoldChange	-Log10_padj	ProteinName	CAZy_family	Description	Inducing_Glycan
BT_0145	3,09	1,39	glycosyl hydrolase 43 family protein	GH43	Glycoside Hydrolase Family 43	unknown
BT_0196	7,68	3,96	MFS transporter		putative hexose phosphate transport protein	unknown
BT_0206	5,42	1,66	TonB-dependent receptor		susC-like	host/residual dietary glycans (unknown type)
BT_0207	4,54	1,97	RagB/SusD family nutrient uptake outer membrane protein		susD-like	host/residual dietary glycans (unknown type)
BT_0208	6,04	2,67	DUF4984 domain-containing protein		hypothetical protein	host/residual dietary glycans (unknown type)
BT_0209	3,85	3,27	DUF5003 domain-containing protein		hypothetical protein	host/residual dietary glycans (unknown type)
BT_0210	2,61	2,11	DUF4458 domain-containing protein		leucine-rich repeat protein, function unknown	host/residual dietary glycans (unknown type)
BT_0211	3,64	1,76	BACON domain-containing protein		hypothetical protein	host/residual dietary glycans (unknown type)
BT_0212	2,68	1,67	S8 family serine peptidase		protease	host/residual dietary glycans (unknown type)
BT_0263	4,82	5,20	six-hairpin glycosidase	PL27	hypothetical protein	unknown
BT_0317	2,30	1,43	TonB-dependent receptor		susC-like	mucin O-glycans (N-acetyllactosamine, adult and suckling mouse)
BT_1024	2,95	3,19	RagB/SusD family nutrient uptake outer membrane protein		susD-like	unknown
BT_1025	2,71	2,43	TonB-dependent receptor		susC-like	unknown
BT_2170	2,71	2,43	hypothetical protein		hypothetical protein	host glycans (unknown type, PMG phases 1 and 2)
BT_2171	2,83	3,98	DUF4974 domain-containing protein		anti-sigma factor	host glycans (unknown type, PMG phases 1 and 2)
BT_2172	3,28	3,74	TonB-dependent receptor		susC-like	host glycans (unknown type, PMG phases 1 and 2)
BT_2173	3,44	8,72	DUF4249 domain-containing protein		susD-like	host glycans (unknown type, PMG phases 1 and 2)
BT_2362	3,41	2,67	TonB-dependent receptor		susC-like	unknown
BT_2363	5,26	2,89	RagB/SusD family nutrient uptake outer membrane protein		susD-like	unknown
BT_2393	2,09	1,31	TonB-dependent receptor		susC-like	probable mucin O-glycans (adult and suckling mouse, N-acetyllactosamine)
BT_2529	7,92	1,61	histidinol-phosphatase		hypothetical protein	unknown
BT_2531	4,23	2,93	TonB-dependent receptor		susC-like	unknown
BT_2532	3,91	3,44	RagB/SusD family nutrient uptake outer membrane protein		susD-like	unknown
BT_2859	3,22	1,75	TonB-dependent receptor		susC-like	unknown
BT_2900	2,81	1,50	family 43 glycosylhydrolase	GH43	Glycoside Hydrolase Family 43	unknown
BT_2903	2,72	2,13	DUF4959 domain-containing protein		hypothetical protein	unknown
BT_2955	4,77	2,28	formate acetyltransferase, formate acetyltransferase, formate acetyltransferase		formate acetyltransferase 2	unknown
BT_2956	5,41	1,88	glycyl-radical enzyme activating protein		putative pyruvate formate-lyase 3 activating enzyme	unknown
BT_2968	1,95	1,64	TonB-dependent receptor		susC-like	unknown
BT_2969	2,26	1,72	beta-galactosidase, beta-galactosidase, beta-galactosidase	GH2	Glycoside Hydrolase Family 2	unknown
BT_3016	2,95	1,34	TonB-dependent receptor		TonB-dependent receptor	host glycans (unknown type, PMG phase 2)
BT_3017	5,02	1,67	acid phosphatase		acid phosphatase	host glycans (unknown type, PMG phase 2)
BT_3024	2,70	2,43	TonB-dependent receptor		susC-like	unknown
BT_3048	3,13	3,47	hypothetical protein		hypothetical protein	host glycans (unknown type, adult and suckling mouse)
BT_3176	1,85	1,36	DUF5006 domain-containing protein	GHnc	hypothetical protein	unknown
BT_3278	4,56	2,88	DUF4974 domain-containing protein		anti-sigma factor	unknown
BT_3301	3,60	1,46	alpha-1%2C6-mannanase	GH76	Glycoside Hydrolase Family 76	unknown
BT_3344	4,72	2,21	DUF4973 domain-containing protein		hypothetical protein	unknown
BT_3345	4,02	1,32	RagB/SusD family nutrient uptake outer membrane protein		susD-like	unknown
BT_3346	3,47	1,34	TonB-dependent receptor		susC-like	unknown
BT_3347	3,04	1,34	hypothetical protein		hypothetical protein	unknown
BT_3489	2,28	1,47	arylsulfatase		arylsulfatase B precursor	host/residual dietary glycans (unknown type)
BT_3498	3,53	1,57	DUF4956 domain-containing protein		hypothetical protein	host/residual dietary glycans (unknown type)
BT_3499	4,41	2,39	DUF2490 domain-containing protein		hypothetical protein	host/residual dietary glycans (unknown type)
BT_3500	2,85	1,60	hypothetical protein		hypothetical protein	host/residual dietary glycans (unknown type)
BT_3501	3,39	2,44	alpha-1%2C6-mannanase	GH76	Glycoside Hydrolase Family 76	host/residual dietary glycans (unknown type)
BT_3570	2,54	1,63	tetratricopeptide repeat protein		TPR-repeat-containing proteins	unknown
BT_3608	2,81	1,87	prolyl oligopeptidase family serine peptidase		hypothetical protein	unknown
BT_3670	3,09	2,44	TonB-dependent receptor		susC-like	host/residual dietary glycans (unknown type)
BT_3671	3,05	1,92	RagB/SusD family nutrient uptake outer membrane protein		susD-like	host/residual dietary glycans (unknown type)
BT_3678	2,06	2,63	response regulator		Hybrid two-component system regulator	unknown
BT_3679	3,57	3,86	hypothetical protein		conserved hypothetical protein, with a conserved domain	unknown
BT_3680	3,86	3,87	TonB-dependent receptor		susC-like	unknown
BT_3775	5,06	4,59	hypothetical protein	GT32	hypothetical protein	a-mannan, host N-glycans
BT_3776	5,66	2,29	hypothetical protein	GT32	hypothetical protein	a-mannan, host N-glycans
BT_3777	4,85	2,17	hypothetical protein		hypothetical protein	a-mannan, host N-glycans
BT_3778	3,31	1,41	hypothetical protein		hypothetical protein	a-mannan, host N-glycans
BT_3989	2,49	3,52	hypothetical protein		hypothetical protein	mucin O-glycans
BT_4038	4,89	1,84	RagB/SusD family nutrient uptake outer membrane protein		susD-like	mucin O-glycans
BT_4039	3,85	1,33	TonB-dependent receptor		susC-like	mucin O-glycans
BT_4040	4,36	1,76	DUF1735 domain-containing protein	CBM32	putative galactose oxidase precursor	mucin O-glycans
BT_4076	3,48	2,48	family 78 glycoside hydrolase catalytic domain	GH78	Glycoside Hydrolase Family 78	host glycans (unknown type, PMG phase 2)
BT_4083	2,09	1,38	DUF4361 domain-containing protein		hypothetical protein	host glycans (unknown type, PMG phase 2)
BT_4119	8,80	6,96	pectate lyase	PL1	Polysaccharide Lyase Family 1	host/residual dietary glycans (unknown type)
BT_4120	3,75	3,87	fibronectin type III domain-containing protein		hypothetical protein	host/residual dietary glycans (unknown type)
BT_4121	3,43	4,77	TonB-dependent receptor		susC-like	host/residual dietary glycans (unknown type)
BT_4122	2,27	1,76	RagB/SusD family nutrient uptake outer membrane protein		susD-like	host/residual dietary glycans (unknown type)
BT_4123	9,03	8,01	glycoside hydrolase family 28 protein	GH28	Glycoside Hydrolase Family 28	host/residual dietary glycans (unknown type)
BT_4124	2,19	1,72	hybrid sensor histidine kinase/response regulator transcription factor		Hybrid two-component system regulator	host/residual dietary glycans (unknown type)
BT_4132	5,80	7,45	chitobiase		putative chitobiase	mucin O-glycans
BT_4133	4,02	2,35	DUF4959 domain-containing protein		hypothetical protein	mucin O-glycans
BT_4134	4,67	6,07	RagB/SusD family nutrient uptake outer membrane protein		susD-like	mucin O-glycans
BT_4135	2,48	2,01	TonB-dependent receptor		susC-like	mucin O-glycans
BT_4136	2,86	2,16	alpha-1%2C3/4-fucosidase	GH29	Glycoside Hydrolase Family 29	mucin O-glycans
BT_4157	2,38	1,34	alpha-galactosidase	GH27,CBM35	Glycoside Hydrolase Family 27	unknown
BT_4266	7,02	3,36	hypothetical protein		hypothetical protein	unknown
BT_4267	5,17	2,24	TonB-dependent receptor		susC-like	unknown
BT_4268	4,51	1,60	RagB/SusD family nutrient uptake outer membrane protein		susD-like	unknown
BT_4269	7,04	1,86	DUF4361 domain-containing protein		hypothetical protein	unknown
BT_4270	4,22	2,20	chitobiase		hypothetical protein	unknown
BT_4708	3,11	3,18	SusD/RagB family nutrient-binding outer membrane lipoprotein		susD-like	unknown
BT_4728	4,49	4,47	MFS transporter		putative hexose phosphate transport protein	unknown

Supplementary Table 3: All *A. muciniphila* mucin-degrading genes expressed in the co-culture

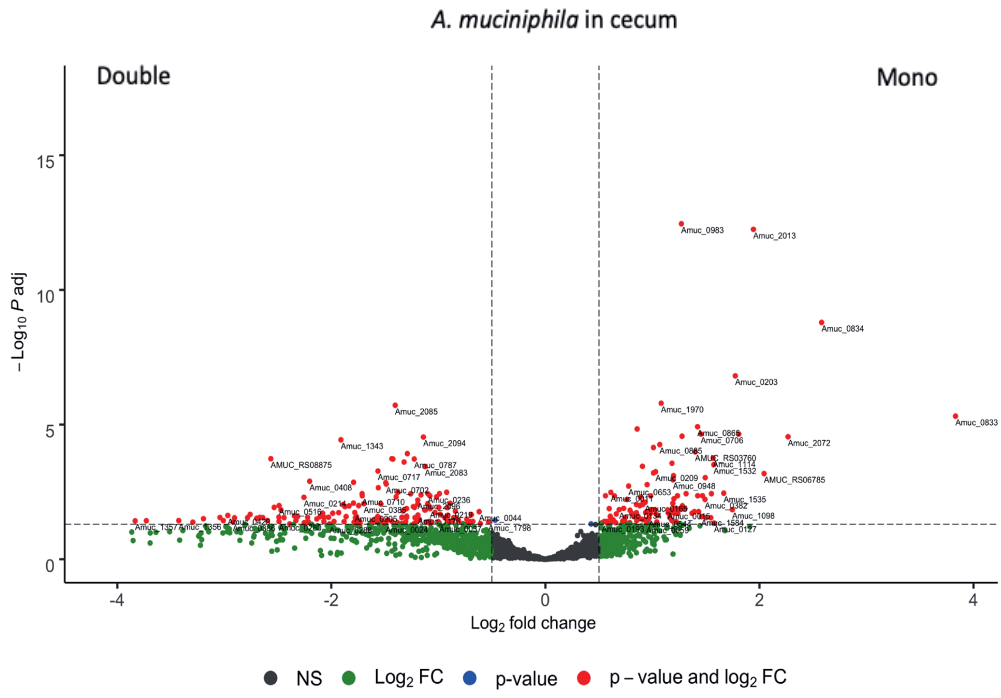
GeneName	baseMean	FoldChange	-Log10_padj	CAZy_family	ProteinName
Amuc_1003	211,05	11,56	17,81		alpha/beta hydrolase
Amuc_1934	102,67	6,46	10,23		hypothetical protein
Amuc_0846	429,93	3,33	6,63	GH29	coagulation factor 5/8 type domain-containing protein
Amuc_1666	282,26	3,20	6,16	GH2	glycoside hydrolase family 2, glycoside hydrolase family 2, glycoside hydrolase family 2
Amuc_2040	518,16	2,46	6,38		M3 family peptidase
Amuc_0146	237,52	2,30	2,19	GH29	alpha-L-fucosidase
Amuc_0397	383,03	2,01	3,73	GH20	beta-N-acetylhexosaminidase
Amuc_1801	213,84	1,60	1,50		alpha/beta hydrolase
Amuc_1667	1045,17	1,52	1,42	GH2	hypothetical protein
Amuc_0697	275,41	1,49	1,25	GH43	beta-glucanase
Amuc_0863	955,34	1,31	0,56	GH105	glycosyl hydrolase family protein
Amuc_0698	302,56	1,28	0,70	GH43	beta-glucanase
Amuc_1815	782,73	1,20	0,25	GH20	beta-N-acetylhexosaminidase
Amuc_0953	3294,08	1,16	0,28		hypothetical protein
Amuc_0565	851,57	1,15	0,25		sulfatase, sulfatase
Amuc_1669	754,53	1,12	0,23	GH20	beta-N-acetylhexosaminidase, beta-N-acetylhexosaminidase, beta-N-acetylhexosaminidase, beta-N-acetylhexosaminidase, beta-N-acetylhexosaminidase, beta-N-acetylhexosaminidase
Amuc_1118	606,58	1,12	0,23		sulfatase
Amuc_0491	718,04	1,12	0,17		sulfatase
Amuc_0060	1266,06	1,11	0,15	GH89	alpha-N-acetylglucosaminidase
Amuc_0539	2573,24	1,11	0,19	GH2	hypothetical protein
Amuc_1187	859,89	1,09	0,12	GH27	hypothetical protein, hypothetical protein, hypothetical protein, hypothetical protein
Amuc_1220	1572,60	1,09	0,16	GH89	alpha-N-acetylglucosaminidase
Amuc_1480	956,72	1,08	0,08		D-alanyl-D-alanine carboxypeptidase
Amuc_0187	724,18	1,06	0,08		peptidase M28
Amuc_1655	635,14	1,04	0,05		sulfatase
Amuc_0875	1069,65	1,02	0,02	GH16	beta-glucanase
Amuc_0369	1307,13	1,01	0,01	GH20	beta-N-acetylhexosaminidase, beta-N-acetylhexosaminidase, beta-N-acetylhexosaminidase, beta-N-acetylhexosaminidase, beta-N-acetylhexosaminidase, beta-N-acetylhexosaminidase
Amuc_0121	3358,09	1,01	0,01		arylsulfatase
Amuc_1924	3243,86	0,99	0,01	GH20	beta-N-acetylhexosaminidase
Amuc_0186	1879,86	0,98	0,02	GH95	glycoside hydrolase family protein
Amuc_0482	245,14	0,98	0,02		alpha/beta superfamily hydrolase
Amuc_0392	4308,91	0,96	0,08	GH29	coagulation factor 5/8 type domain-containing protein
Amuc_1120	6458,88	0,95	0,09	GH95	hypothetical protein
Amuc_1438	3395,89	0,91	0,17	CBM51	glycosyl hydrolase family protein
Amuc_0625	2385,62	0,90	0,16	GH33	exo-alpha-sialidase, exo-alpha-sialidase
Amuc_2164	2480,28	0,89	0,18	GH18	hypothetical protein
Amuc_1074	2324,56	0,88	0,30		sulfatase
Amuc_2108	835,61	1,14	0,25	GH16	glycoside hydrolase family protein
Amuc_0771	2097,93	1,15	0,42	GH35	beta-galactosidase, beta-galactosidase, beta-galactosidase
Amuc_0465	347,67	1,16	0,28		M23 family peptidase
Amuc_0670	2026,14	1,16	0,25		Trypsin-like protein serine protease
Amuc_1008	3368,82	1,16	0,32	GH31	alpha-xylosidase
Amuc_2136	8385,18	1,18	0,35	GH20	glycoside hydrolase family 20, glycoside hydrolase family 20, glycoside hydrolase family 20, glycoside hydrolase family 20, glycoside hydrolase family 20, glycoside hydrolase family 20
Amuc_1106	1692,98	1,19	0,59		peptidase M24
Amuc_0824	8657,71	1,21	0,53	GH2	glycoside hydrolase family 2, glycoside hydrolase family 2, glycoside hydrolase family 2
Amuc_1631	2757,90	1,22	0,66		tail-specific protease
Amuc_1032	2338,78	1,22	0,50	GH20	hypothetical protein
Amuc_0253	581,32	1,25	0,34		M23 family peptidase
Amuc_1686	3086,01	1,31	0,73	GH35,CBM32	beta-galactosidase
Amuc_1755	3031,82	1,35	1,02		sulfatase
Amuc_0176	1116,24	1,36	1,37		hypothetical protein
Amuc_0391	137,88	1,37	0,51		M23 family peptidase

Supplementary Table 4: Top 50 most abundant *B. thetaiotaomicron* genes expressed in the co-culture

GeneName	KO	FoldChange	-Log10_padj	CDS	ProteinName	Metabolic process	Pathway
BT_0016		138,88	5,03	cds15	hypothetical protein		
BT_2221		119,04	15,70	cds2220	hypothetical protein		
BT_2220		101,87	14,60	cds2219	hypothetical protein		
BT_3440		96,05	22,30	cds3438	hypothetical protein		
BT_3441		83,82	19,80	cds3439	hypothetical protein		
BT_1132		78,51	3,53	cds1131	hypothetical protein		
BT_2645		72,16	3,82	cds2644	hypothetical protein		
BT_2641		71,70	3,33	cds2640	hypothetical protein		
BT_2219		70,42	12,60	cds2218	hypothetical protein		
BT_2612		67,67	3,00	cds2611	hypothetical protein		
BT_0020		60,07	2,94	cds19	MACPF domain containing protein		
BT_3439		59,34	21,90	cds3437	hypothetical protein		
BT_1991		59,05	2,67	cds1990	N-acetylmuramoyl-L-alanine amidase		
BT_2312		58,56	2,85	cds2311	hypothetical protein		
BT_4433		51,86	4,42	cds4430	hypothetical protein		
BT_2218		50,27	9,66	cds2217	hypothetical protein		
BT_2960		47,96	3,00	cds2958	hypothetical protein		
BT_2864	K12995	47,02	5,71	cds2862	lipopolysaccharide biosynthesis glycosyltransferase		
BT_1990		46,63	3,02	cds1989	hypothetical protein		
BT_0018		46,05	5,80	cds17	hypothetical protein		
BT_1589		44,87	2,24	cds1588	hypothetical protein		
BT_4023		41,68	2,08	cds4020	transposase		
BT_2996		39,73	2,75	cds2994	hypothetical protein		
BT_4780		39,70	3,12	cds4777	conjugate transposon protein		
BT_4774		37,30	5,14	cds4771	conjugate transposon protein		
BT_4735		37,17	3,49	cds4732	hypothetical protein		
BT_2217		37,07	9,84	cds2216	hypothetical protein		
BT_2652		36,14	1,83	cds2651	hypothetical protein		
BT_2594		31,82	4,30	cds2593	conjugate transposon protein		
BT_1515		30,49	4,43	cds1514	hypothetical protein		
BT_0469		29,78	4,77	cds468	hypothetical protein		
BT_4748		29,57	4,77	cds4745	hypothetical protein		
BT_2131		29,02	8,59	cds2130	hypothetical protein		
BT_0019		26,93	1,31	cds18	hypothetical protein		
BT_1709		25,79	2,44	cds1708	glycosyltransferase		
BT_0538		24,91	1,33	cds537	hypothetical protein		
BT_4435		24,26	4,11	cds4432	hypothetical protein		
BT_4489		23,92	1,72	cds4486	hypothetical protein		
BT_4024		23,25	1,30	cds4021	hypothetical protein		
BT_4491		23,15	1,68	cds4488	hypothetical protein		
BT_1951	K02015	22,67	3,48	cds1950	iron ABC transporter permease		
BT_4490		20,28	1,30	cds4487	hypothetical protein		
BT_2881	K00991	19,20	6,56	cds2879	2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase	Metabolism of terpenoids and polyketides	Terpenoid backbone biosynthesis
BT_0294		18,97	8,55	cds293	hypothetical protein		
BT_3502		17,91	2,35	cds3500	hypothetical protein		
BT_0949		17,62	1,90	cds948	hypothetical protein		
BT_4021		17,34	5,07	cds4018	integrase		
BT_0099		17,08	1,41	cds98	hypothetical protein		
BT_2643		16,22	2,22	cds2642	hypothetical protein		
BT_2696		16,16	1,78	cds2694	hypothetical protein		

Supplementary Table 5: Top 50 most abundant *A. muciniphila* genes expressed in the co-culture

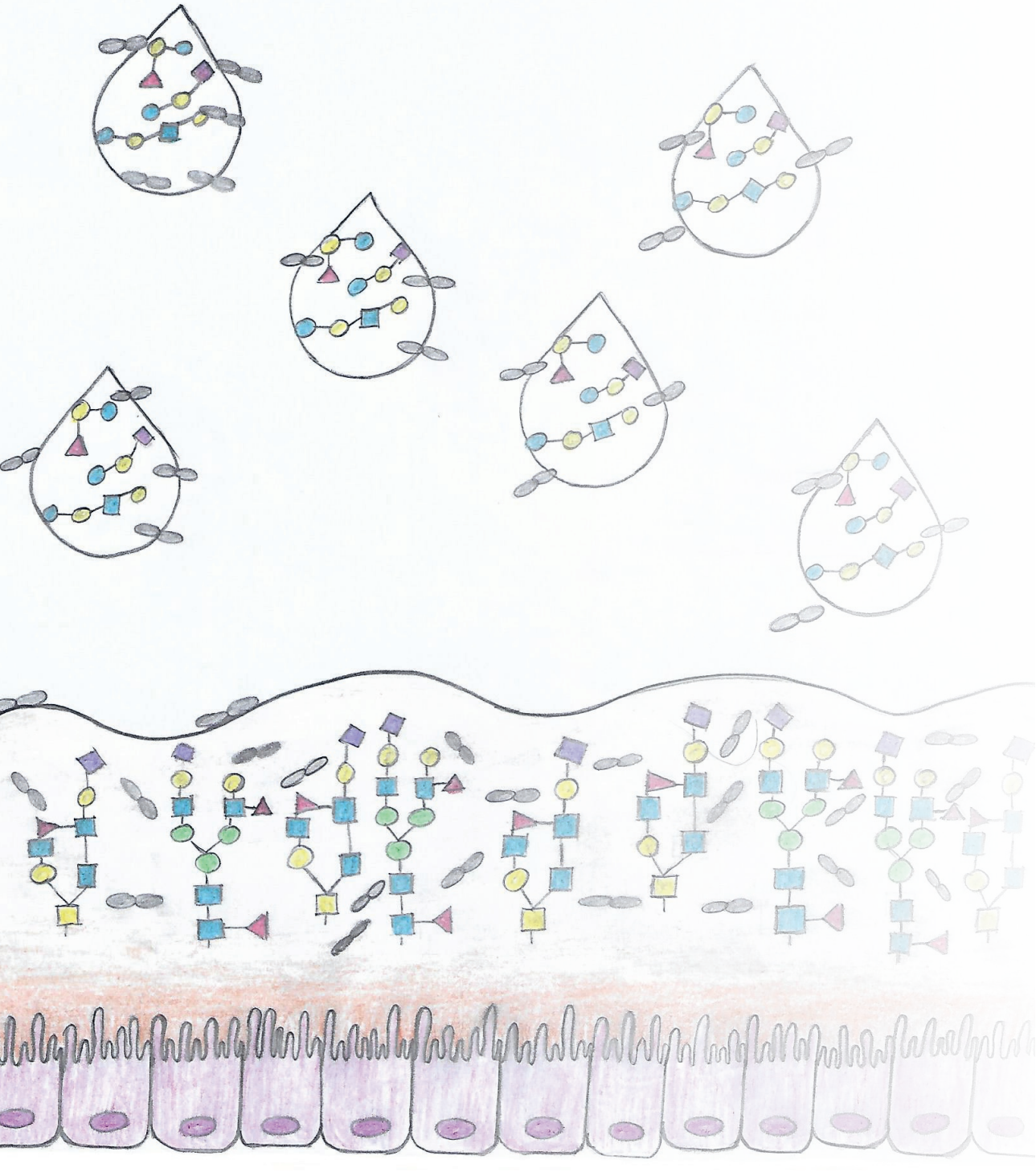
GeneName	KO	FoldChange	-Log10_padj	ProteinName	Metabolic process	Pathway
Amuc_1415		15,43	11,28	hypothetical protein		
Amuc_0892		10,77	12,10	autotransporter domain-containing protein		
Amuc_0388		10,35	1,92	hypothetical protein		
Amuc_1305	K02037	10,03	10,80	ABC transporter permease	Membrane transport	ABC transporters
Amuc_1003	K06889	9,88	13,58	alpha/beta hydrolase		
Amuc_1721		9,49	4,03	hypothetical protein		
Amuc_1303	K02036	9,43	7,48	phosphate ABC transporter ATP-binding protein	Membrane transport	ABC transporters
Amuc_0387		9,22	11,52	hypothetical protein		
AMUC_RS12735		8,25	2,92	hypothetical protein		
Amuc_1304	K02038	8,11	9,75	phosphate ABC transporter%2C permease protein PstA	Membrane transport	ABC transporters
Amuc_0288	K03179	7,92	10,80	hypothetical protein	Metabolism of cofactors and vitamins	Ubiquinone and other terpenoid-quinone biosynthesis
Amuc_1675		7,73	9,38	site-specific integrase		
AMUC_RS12705		7,35	15,93	hypothetical protein		
Amuc_1931	K02015	7,29	8,01	iron ABC transporter permease	signaling and cellular processes	
Amuc_1357		7,11	6,18	hypothetical protein		
Amuc_1083		6,84	4,94	heavy metal translocating P-type ATPase		
Amuc_0854	K03530	6,72	5,57	DNA-binding protein		
AMUC_RS05395		6,48	7,04	alpha/beta hydrolase		
AMUC_RS12525		5,74	1,72	hypothetical protein		
Amuc_1934		5,64	7,16	hypothetical protein		
Amuc_0107		5,64	15,94	hydrophobe/amphiphile efflux-1 family RND transporter		
Amuc_0108		5,41	9,97	efflux RND transporter periplasmic adaptor subunit		
AMUC_RS11805		5,31	4,61	hypothetical protein		
Amuc_1926		5,20	9,90	cytochrome c assembly protein		
Amuc_1387		5,17	13,24	hypothetical protein		
Amuc_1878	K07032	5,16	7,70	lactoylglutathione lyase		
Amuc_1356		5,08	4,25	hypothetical protein		
Amuc_0936		5,06	11,15	hypothetical protein		
AMUC_RS12660		5,01	4,92	glycosyltransferase		
Amuc_1301	K01738	4,98	4,80	cysteine synthase A	Energy metabolism, Amino acid metabolism	Sulfur metabolism, Cysteine and methionine metabolism
Amuc_2091		4,98	9,77	hypothetical protein		
Amuc_1872	K00185	4,95	10,24	polysulfide reductase NrfD	Energy metabolism	Sulfur metabolism
Amuc_1677		4,93	7,43	type II restriction endonuclease		
Amuc_0843		4,84	15,94	hypothetical protein		
AMUC_RS01550		4,75	1,67	hypothetical protein		
Amuc_1930	K02015	4,70	4,24	transporter permease		
Amuc_1160		4,69	9,70	aldo/keto reductase		
Amuc_0386		4,63	5,28	hypothetical protein		
AMUC_RS12600		4,60	3,59	hypothetical protein		
Amuc_1711		4,56	4,22	hypothetical protein		
Amuc_1457	K01814	4,52	10,09	phosphoribosylformimino-5-aminoimidazole carboxamid	Amino acid metabolism	Histidine metabolism
Amuc_1468		4,44	6,35	alpha/beta hydrolase		
AMUC_RS03120		4,43	9,68	ComF family protein		
Amuc_0848	K02238	4,40	12,50	hypothetical protein		
Amuc_1571		4,36	13,58	tRNA (adenosine(37)-N6)-threonylcarbamoyltransferase complex dimerization subunit type 1 TsaB		
AMUC_RS12515		4,35	1,60	hypothetical protein		
Amuc_1351		4,33	2,50	hypothetical protein		
Amuc_1306	K02040	4,30	5,55	phosphate-binding protein	Membrane transport, Signal transduction	ABC transporters , Two-component system
Amuc_0021	K03092	4,26	9,56	RNA polymerase sigma-54 factor	Signal transduction	Two-component system
Amuc_1386	K00796	4,10	10,23	dihydropteroate synthase	Metabolism of cofactors and vitamins	Folate biosynthesis



Supplementary Figure 3: Differential expressed genes of *A. muciniphila* between mono- and co-colonisation in mice cecum. Volcano plot of the differential expressed *A. muciniphila* genes of mono- versus co-culture. Positive Log2 fold change indicates upregulation in co-culture. The horizontal dashed black line adjusted p-value threshold (q-value = 0.05). The vertical dashed black lines indicate Log2 fold change threshold (Log2 fold change = 0.58).

Supplementary Table 6: The most significantly affected host (mouse) genes by the presence of glycan - degraders in cecum. In light blue the 10 most abundant host genes are highlighted.

GeneName	baseMean	log2FoldChange	FoldChange	-Log10-padj	Gene_function
Zzef1-2	58,18	-23,38	10952966,60	7,74	Zinc finger ZZ-type and EF-hand domain-containing protein 1
Cckar	18,00	-8,03	262,04	1,57	cholecystokinin A receptor
Zfp987-2	20,74	-6,81	112,10	1,43	Zinc finger protein 987
Epha6	78,04	-4,82	28,16	1,30	Eph receptor A6
Gjb4	46,46	-3,76	13,56	1,37	gap junction protein%2C beta 4
Cyp4f15	32,87	-2,64	6,25	3,87	cytochrome P450%2C family 4%2C subfamily f%2C polypeptide 15
Insl5	188,08	-2,31	4,94	4,42	insulin-like 5
Mzb1	229,03	-2,07	4,20	6,02	marginal zone B and B1 cell-specific protein 1
Tnfrsf13b	114,56	-1,71	3,28	1,85	tumor necrosis factor receptor superfamily%2C member 13b
Ttll9	47,32	-1,69	3,23	1,57	tubulin tyrosine ligase-like family%2C member 9
Acaa1b	379,23	-1,41	2,65	1,40	acetyl-Coenzyme A acyltransferase 1B
Aqp8	11638,95	-1,39	2,62	4,83	aquaporin 8
Cmtm8	212,97	-1,24	2,37	3,87	CKLF-like MARVEL transmembrane domain containing 8
Tsku	2330,62	-1,24	2,37	1,54	tsukushi%2C small leucine rich proteoglycan
Tmem171	1053,34	-1,12	2,17	1,59	transmembrane protein 171
Pla2g12b	294,90	-1,11	2,16	1,66	phospholipase A2%2C group XIIB
Sdc1	4913,62	-1,04	2,06	2,21	syndecan 1
Kyat1	380,50	-1,00	2,00	1,64	cysteine conjugate-beta lyase 1
Cgref1	575,42	-0,93	1,90	2,41	cell growth regulator with EF hand domain 1
Adipor2	4466,36	-0,70	1,62	1,40	adiponectin receptor 2
Slc2a1	3890,48	-0,63	1,55	1,40	solute carrier family 2 (facilitated glucose transporter)%2C member 1
Prdx5	2341,53	-0,63	1,55	1,82	peroxiredoxin 5
Lgals3	26565,23	-0,62	1,54	1,44	lectin%2C galactose binding%2C soluble 3



Chapter 5

Minimalist Approach for Deciphering the Ecophysiology of Human Gut Microbes

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Abstract

The human intestinal microbiome has an intricate relationship with its host. Microbe-microbe interactions in the gut are largely influenced by host derived glycans and diet. However, investigating and identifying metabolic interactions and trophic roles of key microbes has been limited by the high complexity of the human intestinal microbiome. Synthetic minimal microbiomes provide a pragmatic approach to investigate their ecology including metabolic interactions. Here, we show the assembly of a synthetic microbiome termed **M**ucin and **D**iet **b**ased **M**inimal **M**icrobiome (MDb-MM), in which 16 intestinal microbes stably co-existed for 20 days *in-vitro* in a bioreactor. The MDb-MM exhibited resistance and resilience to temporal perturbations as evidenced by the abundance, metabolic end-products and transcriptional response of its members. We observed microbe-specific temporal dynamics in transcriptional niche overlap and trophic interaction network which explains the observed co-existence. The addition of *Blautia hydrogenotrophica* was used to exemplify vacant niche occupation and established its role as an active homoacetogen in the community. Overall, the present study provides crucial insights into the co-existence, ecological niches and trophic roles of key intestinal microbes in a highly dynamic and competitive ecosystem.

Keywords: Human gut microbiome, Synthetic minimal microbiomes, Niche overlap, Trophic interactions

Introduction

The complexity of interactions within the human gut microbiome contributes to providing health benefits to its host. However, the same complexity presents a major challenge for deciphering metabolic and other ecological interactions between the intestinal microbes. Understanding these complex interactions, at both community and individual taxa level, is crucial for the development of effective microbiome modulation strategies (Shetty et al. 2017; Costello et al. 2012; Gilbert and Lynch 2019). The human intestinal tract includes several hundred species mainly belonging to the phyla Actinobacteria, Bacteroidetes, Firmicutes, Verrucomicrobia, and Proteobacteria and others (Human Microbiome Project 2012; J. Li, Jia, Cai, Zhong, Feng, Sunagawa, Arumugam, Kultima, Prifti, and Nielsen 2014; Forster et al. 2019; Desai et al. 2016). Recently, synthetic microbial communities assembled from host-derived strains have received considerable attention for understanding ecological and metabolic features of the microbiome (Clavel, Lagkouvardos, and Stecher 2017; Shetty, Smidt, and de Vos 2019; D'hoe et al. 2018; Venturelli et al. 2018; Brugiroux et al. 2017; Gutiérrez and Garrido 2019). Synthetic microbial communities of the human intestine can be studied under controlled conditions either in bioreactors, *in-vivo* in animal models, or *in vitro* with or without the host component (Brugiroux et al. 2017; Shah et al. 2016; El Hage et al. 2019; Oliphant et al. 2019). *In vitro* intestinal models allow for stable and controllable conditions as well as frequent sampling of the microbial community that may not be possible with animal models for technical and ethical reasons (Macfarlane and Macfarlane 2007; Venema and Van den Abbeele 2013). Combining *in vitro* intestinal models with defined microbial communities holds potential for understanding community assembly and structure, sub-daily compositional and functional dynamics in time, and plasticity of microbial interactions.

Studies employing *in vitro* intestinal models till date have applied either batch or continuous single or multi-stage fermentation models (El Hage et al. 2019; Silverman et al. 2018; Tanner et al. 2014; Van den Abbeele et al. 2010). However, an important aspect of the host associated microbiome is the dietary intake of the host that often follows circadian rhythms and can give rise to stages of excess carbon and energy source and periodic carbon starvation. Both of these aspects may have a profound influence on the compositional and functional dynamics of the microbial community. In fact, previous studies have revealed that nutrient periodicities can affect microbial

community dynamics and physiological functionality (Carrero-Colón, Nakatsu, and Konopka 2006b, 2006a). Nutrient periodicity is an important factor as it may lead to selection of well adapted taxa, affect microbe-microbe interactions and microbe-environment interactions as well as provide an opportunity for invading species to successfully establish in a community (Carrero-Colón, Nakatsu, and Konopka 2006b, 2006a; Mallon et al. 2015; Symons and Arnott 2014). In the human intestinal tract, two major sources of carbon and energy are dietary and host-derived polysaccharides (mainly secreted mucin) that all have a strong deterministic effect on the microbiome (Cotillard et al. 2013; David et al. 2014; Desai et al. 2016; Salonen and de Vos 2014). The diet can be highly variable on sub-daily time scales posing a major selective pressure on the microbial community. Dietary sources, especially complex fibre-derived polysaccharides that reach the colon in a virtually unmodified way, lead to the creation of diverse niches that can support a higher diversity of microbes (F. C. Pereira and Berry 2017; Chung et al. 2018). On the contrary, mucin is a constant and chemically stable source of carbon and energy within a host and is shown to promote stability of the gut microbiome (K. Duncan, Carey-Ewend, and Vaishnava 2019). Therefore, both diet and mucin play a major role in supporting diverse microbial communities and give rise to complex microbe-microbe interactions. In addition, the periodicity in supply of dietary fibres can give rise to dynamic regimes of niche availability consequently affecting interactions between the diet responsive microbes.

To understand microbe-microbe interactions within a complex community, it is important to create a community that exhibits ecophysiological properties similar to natural ecosystems. Community level ecological properties such as resistance and resilience to perturbations, presence of competitors for nutrients as well as mutualists that support metabolic co-operation can be designed in a synthetic minimal microbiome (Shetty 2019). Here, we sought to investigate microbe-microbe interactions in a synthetic minimal microbiome that represented the human colon. To explore temporal ecophysiological interactions, the community was assembled in a bioreactor with constant supply of mucin and pulse of the main dietary fibers *viz*. pectin, resistant starch, inulin and xylan. The initial community consisted of 15 core intestinal bacterial strains representing the four prominent phyla present in the human colon: Actinobacteria, Bacteroidetes, Firmicutes and Verrucomicrobia. To investigate niche occupation, we introduced a 16th species, *Blautia hydrogenotrophica* (a H_2/formate

utilizing homoacetogen) into the stably co-existing 15-species community. The integrative analysis of temporal measurements of metabolitse, 16S rRNA gene amplicons and metatranscriptomes allowed us to unravel community dynamics and metabolic interactions using a synthetic minimal microbiome.

Results

Experimental setup

We sought to assemble a minimal microbiome that consists of bacterial strains relevant to the human colonic microbiome and mimics key ecological and metabolic properties (Figure 1). Therefore, the selection of strains was rationally guided by ecophysiological aspects such as, high prevalence in human colonic microbiota, ability to degrade mucin or common multiple dietary polysaccharides that reach the colon in a virtual unmodified form (pectin, xylan, starch and inulin) and their breakdown products and/or metabolic end/by products thereof. During selection of the candidate strains, we also considered competition for growth substrates, known metabolic cross feeding on lactate and 1,2- propanediol, and the ability to produce lactate or common short chain fatty acids (SCFAs) such as formate, acetate, propionate and butyrate. The details of the representative strains, their known growth substrates and fermentation end products relevant to the current study are given in Table 1.

Table 1: General metabolic features of species for which depicted strains were used for MDb-MM. Abbreviations: Acetate (A), Butyrate (B), Propionate (P), Lactate (L), Formate (F), Ethanol (E), 1,2-Propanediol (1,2-PD), Succinate (S)

Species	Strain used/source	Known substrates	SCFA production*
Akkermansia muciniphila	MucT/ATCC BAA-835	Mucin, N-Acetylglucosamine, N-Acetylgalactosamine, Fucose	A, P, L, 1,2-PD
Bacteroides ovatus	HMP strain 3_8_47FAA	Pectin, starch, xylan	A, P, L, 1,2-PD
Bacteroides xylanisolvens	HMP strain 2_1_22	Pectin, starch, xylan	A, P, L
Anaerobutyricum soehngenii	L2-7/DSM 1736	Sugars, DL-Lactate, 1,2-PD	B, P, F, CO_2, H_2
Coprococcus catus	ATCC 27761	Fructose, Mannitol, Glucose, Mannose, lactate	B, P, A, S, H_2
Flavonifractor plautii	HMP strain 7_1_58FAA	Glucose, Maltose, Xylose, Lysine	L, B, P
Eubacterium sireaum	DSM 15702	Starch, Glucose, Maltose	A, E, L, B, S
Agathobacter rectalis	DSM 17629	Starch, Glucose, Lactose, Xylose, Cellobiose, l-Arabinose, Trehalose, Sorbitol, N-acetylglucosamine	B, A, H_2, L
Roseburia intestinalis	DSM 14610	Starch, Glucose, Xylose, Xylan, Arabinose	B, F, L
Faecalibacterium prausnitzii	A2-165	Pectin, Inulin, fructose, glucose	B, A, H_2, L
Subdoligranulum variabile	DSM 15176	N-acetyl-glucosamine, N-acetyl-mannosamine, Cellobiose, Dextrin, Fructose, Fucose, Galactose, Galacturonic acid, α-glucose, α-lactose, Maltose, Maltotriose, Mannose, Melibiose, Rhamnose, Salicin, Sucrose	B, L, A, S
Ruminococcus bromii	ATCC 27255	Starch, Glucose, Fructose, Galactose	A, F, P, L, E
Blautia obeum	DSM 25238	Arabinose, Cellobiose, Lactose, Mannose, Maltose, Raffinose, Xylose, L-fucose	A, 1,2-PD, P
Collinsella aerofaciens	DSM 3979	Starch, Maltose, Glucose, Sucrose	E, H_2, A, L; F
Bifidoabcterium adolescentis	L2-32	Inulin, Starch, Lactose, Glucose, Xylose, Sorbitol, Cellobiose, Maltose	F, A, L

Blautia hydrogenotrophica	DSM 10507	Cellobiose, Lactose, Mannose, Raffinose, Glucose, H_2/CO_2, H_2/Formate	A, L

**SCFA production varies depends on growth substrates*

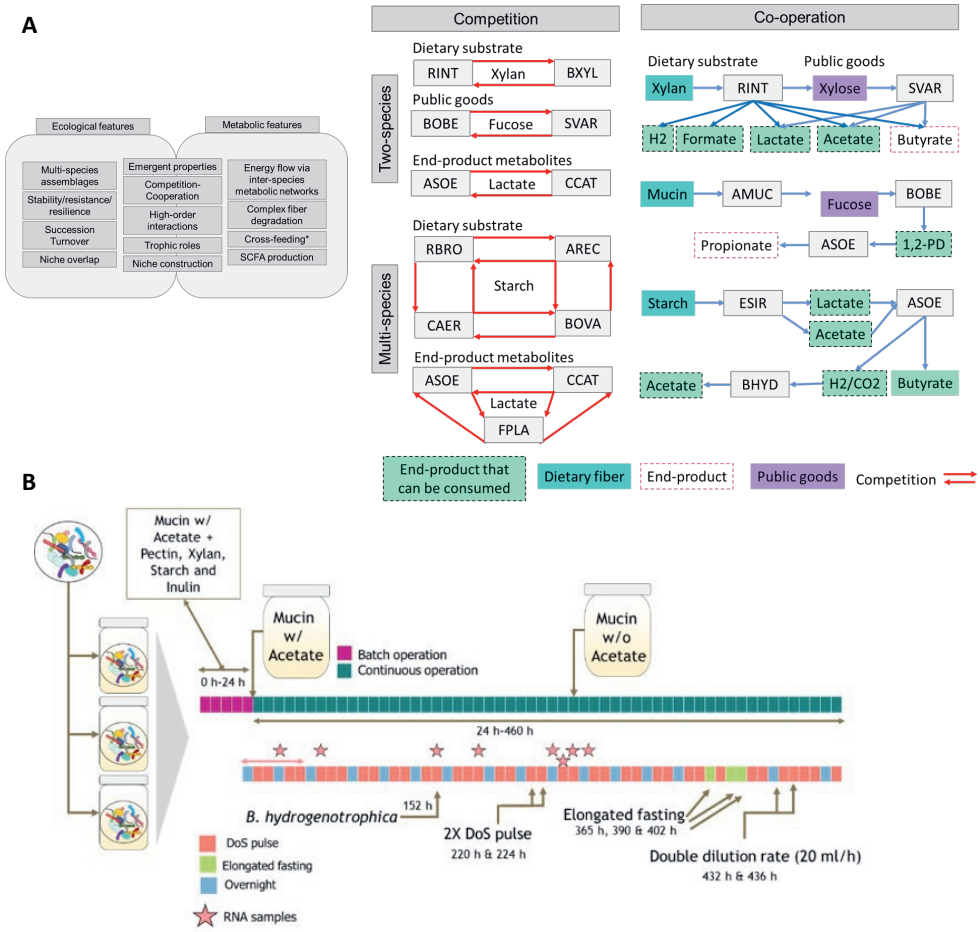

Figure 1: Example of ecophysiological features that were considered during selection of candidates for MDb-MM. A) Potential substrate driven interactions that were considered during selection. While the identity of candidates may vary, the various types of inter-microbial interactions is the focal aspect for design of the MDb-MM. Abbreviations: AMUC, *Akkermanisia muciniphila*; AREC, *Agathobacter rectalis*; ASOE; *Anaerobutyricum soehngenii*; BOVA, *Bacteroides ovatus*; BXYL, *Bacteroides xylanisolvens*; BOBE, *Blautia obeum*; BHYD, *Blautia hydrogenotrophica*; BADO, *Bifidobacterium adolescentis*; CAER, *Collinsella aerofaciens*; CCAT, *Coprococcus catus*; ESIR, *Eubacterium siraeum*; FPRA, *Faecalibacterium prausnitzii*; FPLA, *Flavonifractor plautii*; RBRO, *Ruminococcus bromii*; RINT, *Roseburia intestinalis*; SVAR, *Subdoligranulum variabile*. B) Experimental set-up, bioreactor operation and various events that were executed during the entire duration of the experiment.

First, we assembled a minimal microbiome consisting of 15 strains that were part of the core microbiota (Table 1 & Supplementary Figure S1). The strains were mixed in equal proportions normalized to an optical density of 1 and inoculated at ~1% in three bioreactors where they were initially grown in batch condition with growth media of comprising mucin, pectin, xylan, starch and inulin (Diet origin Substrate, DoS). At 24h, continuous feed was introduced with mucin and acetate (since some of the strains require it for growth), while DoS were introduced as pulsed feeding thrice daily for the majority of the timepoints. During the experiment, various perturbations of varying degree were introduced, such as introduction of a strain, *B. hydrogenotrophica*, doubling concentration of DoS, removal of exogenous acetate (coinciding with replenishing of feed medium), periods of elongated fasting i.e. no addition of DoS for >24 h and increase in dilution rate (Figure 1B). Over a 20-day study, we sampled the three bioreactors at 61 time points each (~3 samples/day) and tested the impact of aforementioned events on the temporal minimal microbiome composition, structure and function.

Assembly, co-existence and ecological properties of MDb-MM

The species abundance in the MDb-MM was tracked by shotgun sequencing of 16S rRNA gene amplicons or specific 16S rRNA gene-based qPCR. The initial 15-species were detected in all the samples from start till the end of the experiment (Figure 2A). The 16th species, *B. hydrogenotrophica* was added at 152 h, however, some low abundance signal was detected prior to addition of this strain. At 264 h, the abundance of *B. hydrogenotrophica* was below the amplicon sequencing detection limit. The DoS pulse events resulted in a significant increase in total biomass (optical density; $O.D_{600}$) while this was not captured with total 16S rRNA gene qPCR (Supplementary Figure S2 A and B). No differences in community evenness and number of species contributing to 90% of the total community abundances were detected after DoS pulse events (Supplementary Figure S2 C and D). A steady increase in butyrate, acetate and propionate concentration was observed until the point of removal of acetate from fresh growth media (Figure 2B).

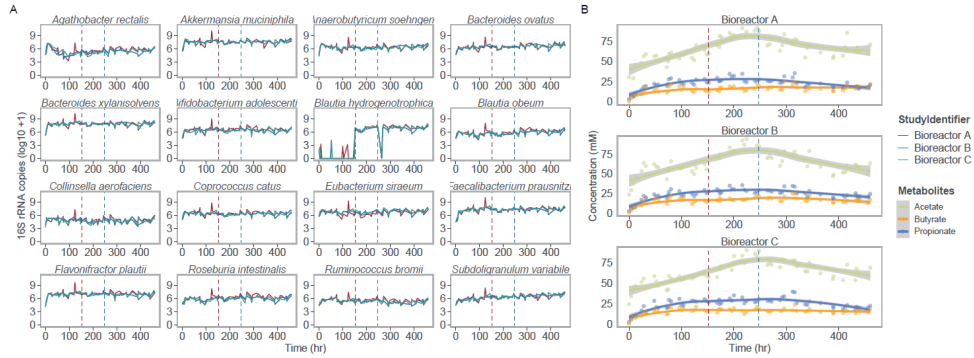

Figure 2: Global response of the MDb-MM. A) Taxon abundances in the MDb-MM. B) Concentration of major SCFAs, acetate, butyrate, propionate produced by MDb-MM in the three bioreactors. The vertical dotted lines, red indicates introduction of *B. hydrogenotrophica* (152 h) and blue indicates removal of acetate/feed change (248h).

In the first 148 h, before the introduction of disturbances, only propionate was produced in significantly higher concentrations in overnight samples (Wilcoxon test, $p<0.001$, Supplementary Figure S3A). Propionate was also significantly higher after addition of *B. hydrogenotrophica* (Wilcoxon test, $p<0.001$, Supplementary Figure S3B). However, after the influx of exogenous acetate was stopped, the concentration of acetate and butyrate were significantly lower in overnight samples (Wilcoxon test, $p<0.0001$) compared to DoS samples, while propionate production was not significantly affected (Supplementary Figure S3B). Thus, successful assembly of the MDb-MM was achieved and stable with presence of all 15 species, production of propionate and butyrate as major fermentation end products for a period of 460 hours. The community was responsive to external forces, primarily, DoS pulse feeding and *B. hydrogenotrophica* was stably engrafted within the community.

Temporal dynamics of MDb-MM community

The MDb-MM community showed changes in community structure over time with similar compositions between triplicate bioreactors (Figure 3A). We evaluated whether the minimal microbiome assembled in the three bioreactors showed time-dependent behaviour and adhered to one of the general scaling laws, i.e. the Taylor's law (Taylor 1961; Taylor and Woiwod 1980). A slope of 0 suggest no species interactions, while competitive interactions can give rise to slope values between 2 to 1 (Kilpatrick and Ives 2003). The MDb-MM in the three bioreactors exhibited a linear relationship

between log variance and log mean abundance with a slope of 1.45, 1.37 and 1.36 for bioreactor A, B and C, respectively (Figure 3B, C and D).

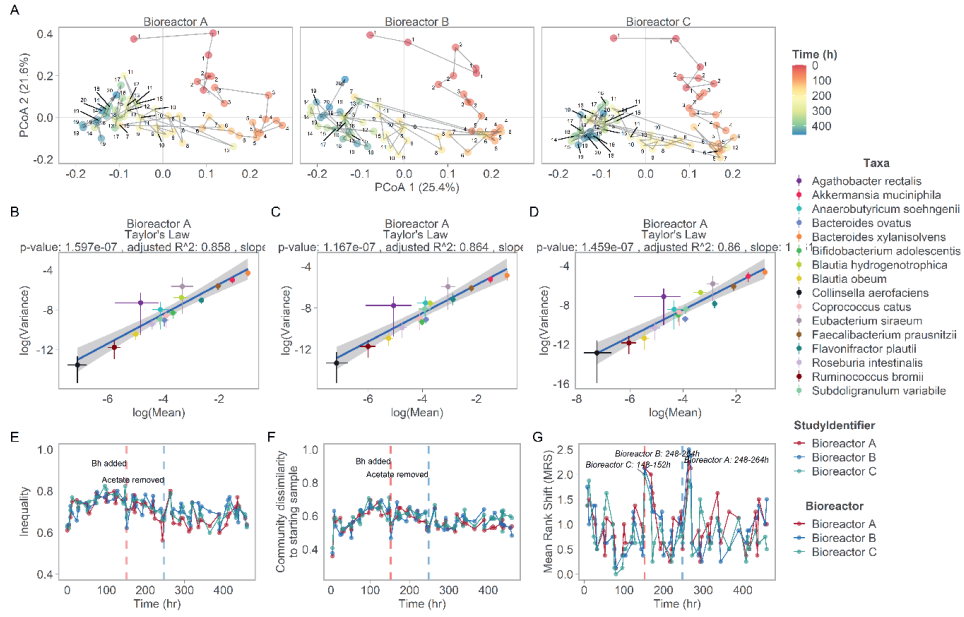

Figure 3: Community level patterns in MDb-MM. A) Principal coordinates analysis plot depicting succession of the MDb-MM community over time, community similarity was calculated using Canberra distance. B, C and D) Power law relationship between variance and mean abundances. The linear regression line is blue and the shaded region represents the confidence interval (geom_smooth function, method=lm). The bars around points represent the lower and upper confidence interval for mean and variance for each of the taxa. E) Temporal inequality (Gini coefficient) in community in the three bioreactors. F) Community divergence based on Canberra distances. G) Mean rank shift of the MDb-MM in the three bioreactors calculated using the codyn R package. In panels E, F and G, the vertical dotted lines, red indicates introduction of *B. hydrogenotrophica* (152 h) and blue indicates removal of acetate/feed change (248h)

Evenness of species abundances can influence functional stability of microbial communities (Wittebolle et al. 2009). We used the Gini coefficient as a measure of evenness, which has values between 0 to 1. Here, 1 indicates a highly uneven community composition (Handcock and Morris 2006). The mean Gini coefficient for the starting minimal microbiome at 0 h was 0.62 (±0.01). At the end of the experiment at 460hr, the Gini coefficient for MDb-MM was 0.6, 0.63, 0.62 for bioreactor A, B and C respectively. The overall mean (± standard deviation) for inequality in MDb-MM was 0.70 ± 0.05, 0.71 ± 0.04, and 0.71 ± 0.05 for bioreactor A, B and C respectively during the entire experiment. The long-term divergence of the MDb-MM in all the three bioreactors follows similar trends over time (Figure 3F). During the first phase of the

experiment, the MDb-MM showed higher deviation from the starting composition, with relatively stable dissimilarities after feed change. Convergence patterns were observed to be similar between the three bioreactors (Supplementary Figure S4A). The correlation between community distances and lagged time intervals further supported directional change which was similar in the three bioreactors (Supplementary Figure S4B). Next, we applied mean rank shift analysis to identify events when drastic changes occurred in the species ranks within the community. During the initial phase, there was a progressive decline in mean rank shift (MRS), but introduction of *B. hydrogenotrophica* resulted in large fluctuations as did the change of feed with removal of acetate in all three bioreactors (Figure 4G). The correlation in relative abundances of species in all the bioreactors was significantly high (p-value < 2.2e-16; A and B, r= 0.92; A and C, r= 0.91; and B and C, r=0.94). These data support highly coherent community level features of the MDb-MM between the three bioreactors.

Temporal stability properties of MDb-MM

The MDb-MM in all three bioreactors showed high resistance to the change of feed that no longer contained acetate (Figure 4A and B). The MDb-MM in all three bioreactors returned to the reference state community (Figure 4A). Among the three bioreactors, MDb-MM in C had highest displacement (DS=0.021) compared to A (DS=0.004) and B (DS=0.005). Notably, MDb-MM in bioreactor C also showed highest resilience (RL=0.282) compared to A (RL=0.194) and B (RL=0.154). Similar patterns were observed when subsequent perturbation events of elongated fasting and increasing dilution rate from 10 ml/h to 20 ml/h were included in the stability analysis (Supplementary Figure S5 A and B). However, the recovery to the reference community state after doubling the dilution rate was borderline at the end of the experiment.

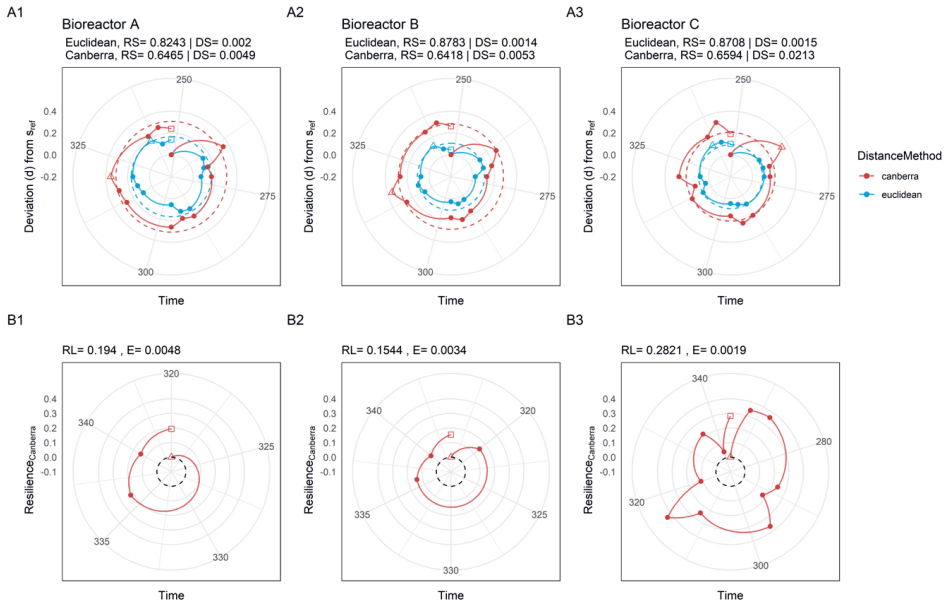

Figure 4: Stability properties of MDb-MM. A) Community changes from reference phase calculated using Canberra and Euclidean distance. The reference boundary was calculated using the method described by Liu et al., 2018 (Z. Liu et al. 2018). The shaded region and brown dashed line depict reference boundary based on Canberra distance, while the blue dashed line depicts reference boundary based on Euclidean distance. The hollow triangles represent timepoints when maximal deviation from reference state was observed. B) Resilience of the MDb-MM after removal of Acetate. The black dashed line depicts reference boundary based on Canberra distance. The stability was calculated with 152 h (introduction of *B. hydrogenotrophica*) as the starting time, removal of acetate/feed change (248h) as the disturbance event and experiment end point was before elongated fasting was initiated in the three systems (344 h).

Community level transcriptional activity

For a subset of the timepoints, we performed metatranscriptome sequencing. We analysed the transcriptional response at two levels, KEGG orthologs (KOs) as well as gut metabolic modules (GMM), the latter of which take into account the combination of KO that are part of specific metabolic modules (Vieira-Silva et al. 2016). The community level functional divergence using relative abundances of and KOs showed similar divergence over time and was linked to changes in the community structure over time (Figure 5A, Supplementary Figure S6). Temporal variation in MDb-MM community composition correlated significantly with transcriptional response at both GMM and KO level (Figure 5A, B and C). Next, to identify community-level transcriptional response to nutrient periodicity, we compared GMM expression at

specific time points (Figure 5B). GMMs linked to carbohydrate degradation were upregulated in the DoS, while mucin and amino acid degradation were upregulated in overnight samples (Figure 5 D, E and F). The butyrate production related module "Acetyl-CoA pathway" was significantly upregulated in the DoS samples, irrespective of presence of *B. hydrogenotrophica* (48 vs 52 h; exogenous acetate in both time points) or removal of acetate (240 vs 248 and 248 vs 264) (Figure 5. D, E and F). In accordance with HPLC data, we observed significantly higher amounts of transcripts encoding enzymes involved in propionate production in overnight samples before addition of *B. hydrogenotrophica* (Figure 5D). Notably, after removal of exogenous acetate, there was a significant upregulation of the GMM for formate conversion and homoacetogenesis (Figure 5F).

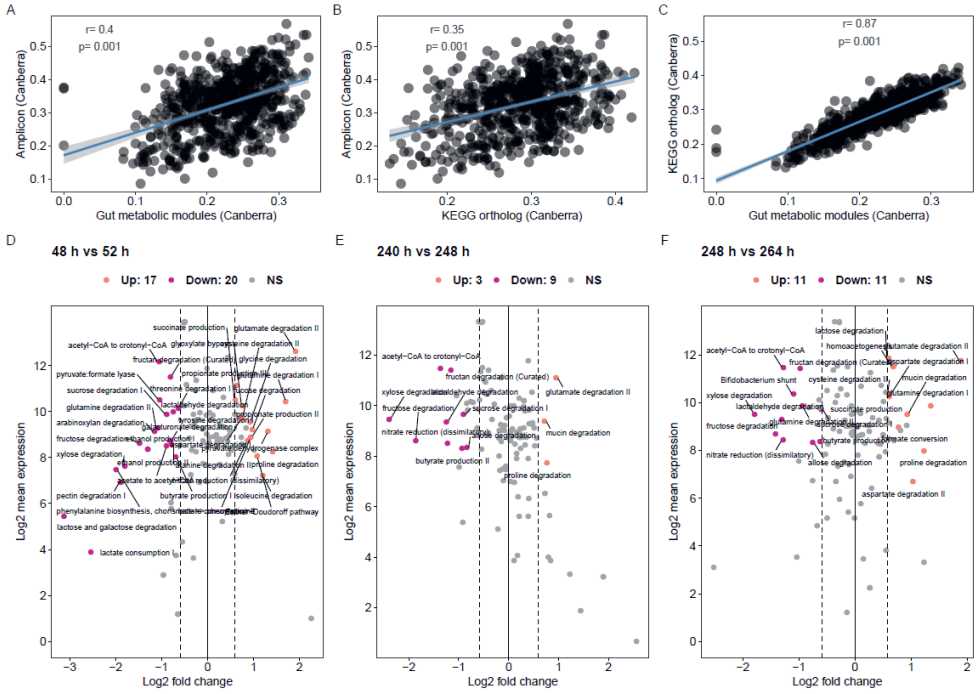

Figure 5: Correlation between compositional and functional succession and transcriptomics response of MDb-MM. Mantel test for correlation between compositional functional community similarity based on Canberra distance. A] Comparison of community similarity based on 16S rRNA gene relative abundance versus gut metabolic module relative abundances. B] Comparison of community similarity based on 16S rRNA gene relative abundance versus KEGG ortholog relative abundances. C] Comparison of gut metabolic module relative abundance versus KEGG ortholog relative abundances. B] Differential expression of GMMs in DoS and overnight samples. Before addition of *B. hydrogenotrophica* with exogenous acetate (48 h vs 52 h). With *B. hydrogenotrophica* and exogenous

acetate (240 h vs 248 h). With *B. hydrogenotrophica* and without exogenous acetate (248 h vs 264 h). Modules with adjusted p value >= 0.01 and with fold change of absolute value >= 1.5 are labelled.

Dynamic niche overlap among MDb-MM species

Thus far, we have demonstrated the dynamic nature of MDb-MM both at compositional and functional level. The 16S rRNA gene analysis showed co-existence of all the 16 species as well as ecological properties of stability at community level. To further understand potential causes of this co-existence and stability, we investigated species traits at transcriptional level using GMMs (Mouillot et al. 2005).

We started by calculating the pairwise niche overlap between each of the species at each of the timepoints for which we had obtained transcriptomes. Metabolic module expression was used as quantitative traits for calculation of niche overlap indices. A lower niche overlap would suggest higher niche segregation and vice-versa. We selected only those GMMs as traits which are involved in either degradation or consumption of substrates and end-product metabolites. A complete list of GMMs and their categorization into trophic guilds is given in Supplementary Table S4.

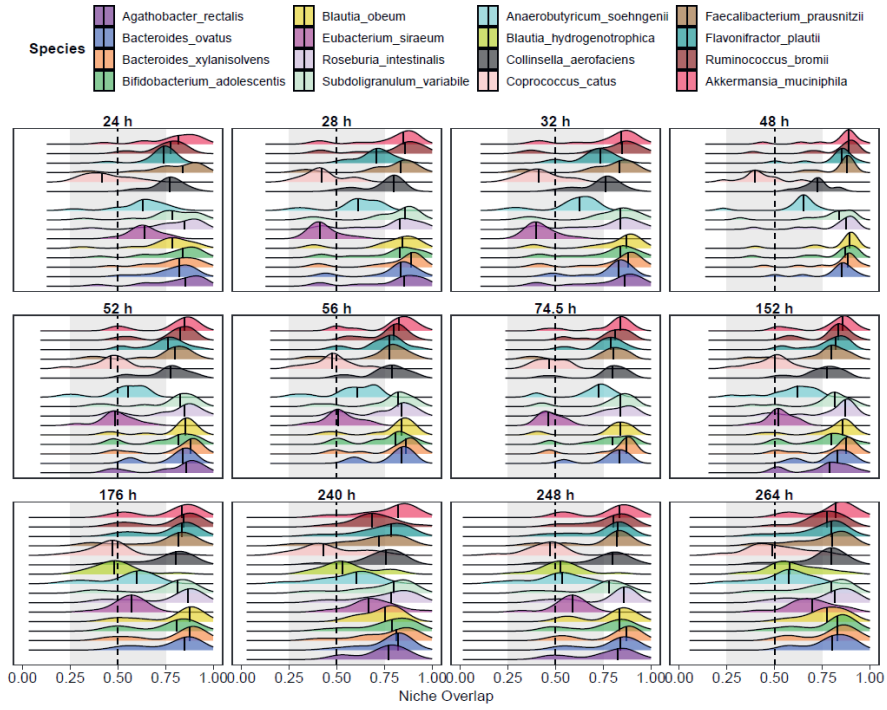

Figure 6: Temporal niche overlap of individual species in MDb-MM. The niche overlap for each species is plotted as density curve to depict variability at each of the time points, facetted boxes are labelled accordingly. The shaded region denotes medium overlap (i.e. >0.25 and <0.75). The vertical line for each taxon in each of the ridge represents quantiles (probability distribution into areas of equal probability. The visualization was done using the R package ggridges.

All species demonstrated temporal variation in niche overlap, highlighting the dynamic nature of inter-species interactions in the MDb-MM (Figure 6). Most fasting timepoints showed larger spread in niche values of bacteria from Firmicutes phylum. Comparison of pairwise distributions of niche overlap values revealed that the complex substrate degraders, *B. xylanisolvens*, *A. muciniphila*, *A. rectalis*, *B. adolescentis*, *S. variabile*, *F. prausnitzii* showed comparatively higher niche overlap (>0.75) with each other (Supplementary Figure S7). *C. catus*, *A. soehngenii* and *E. siraeum* often had the lowest niche overlap with the other strains in the community.

Trophic guilds and realized niches of MDb-MM species

The metabolic flow and biomass distribution within the gut is largely driven by bacteria with specialized molecular machinery capable of degrading complex carbon sources. The action of polysaccharide degraders (primary consumers) results in niche construction that may be dependent on the source substrate as well as their metabolic

pathways. Consequently, this leads to formation of a hierarchal organization within the community into trophic levels (T. Wang et al. 2019). Here, based on metatranscriptomic species-level assignment of transcriptional expression of GMMs, we broadly classified them into four trophic guilds similar to those reported previously from computational simulations (T. Wang et al. 2019) (see methods). Transcriptional contribution of species to each of the trophic guilds revealed the inter-species connectedness of resource utilization.

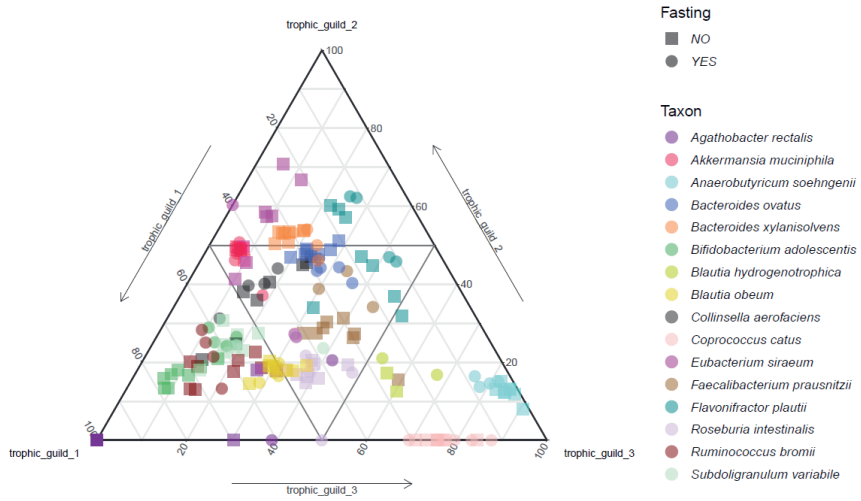

Figure 7: Trophic guilds within the MDb-MM. Ternary plot indicates the trophic status of the minimal microbiome strains. The proximity of the symbols to the apex of the triangle is proportional to the averaged potential contribution of each strain to trophic guilds. Trophic guild 1 is for polysaccharide and mucin degradation, trophic guild 2 consists of mon-di-saccharides and trophic guild 3 consists of consumption of fermentation ends/by-products

The two abundant species in MDb-MM (Figure 1D, 5A), *A. muciniphila* and *B. xylanisolvens* contributed to two trophic guilds: degradation of complex substrates and degradation of simpler carbohydrates (Figure 7). Known starch degraders, *R. bromii*, *B. ovatus*, *C. aerofaciens*, *E. siraeum* and *A. rectalis* showed segregation across trophic guild 1 and 2 axis. Notably, *S. variabile*, *B. adolescentis* and *R. bromii* dominated trophic guild 1 and showed metabolic activity for arabinoxylan, fructan and starch degradation (Supplementary Figure S8).

The action of species occupying trophic guild 1 can give rise to extracellular mono- and di-saccharides that can be utilized by species that lack specialized molecular machinery

for polysaccharide degradation. Breakdown of mucin, pectin, inulin, starch and xylan results in simple mono- and di-saccharides such as fucose, galactose, galacturonate, fructose, maltose, xylose as major simple carbohydrates. Within trophic guild 2, fucose transport and degradation genes were identified to be transcribed in *A. muciniphila* and *B. obeum* (Supplementary Figure S9). In addition, transcription of galactose metabolism genes was predominantly detected in *A. muciniphila*, *B. ovatus* and *B. xylanisolvens* (Supplementary Figure S8). Galacturonate is the main component in pectin and *F. prausnitzii* and to some extent in *B. ovatus* and *B. xylanisolvens* were found to express genes involved in its degradation (Supplementary Figure S9).

Specialist trophic guilds could be assigned to *A. soehngenii*, *B. hydrogenotrophica*, *C. catus* as their transcriptional activity was largely contributing to trophic guild 3 (Figure 7). *F. plautii* showed variation across trophic guild 2 and 3. We classified consumption of fermentation end products such as acetate, lactate, 1,2- propanediol and formate as guild 3. These are mostly major end products of carbohydrate fermentation, while utilization of H_2 and CO_2, inorganic byproducts of acidogenesis, are classified here as the quaternary guild. Acetate was exogenously supplied until 248 h to the MDb-MM and then removed from the feed. Expression of modules for acetate to acetyl Co-A via I and II (acetate kinase pTKA) was observed in *A. soehngenii*, *F. prausnitzii*, *B. obeum*, *B. hydrogenotrophica* and *F. plautii* (Supplementary Figure S9). *A. soehngenii* and *F. prausnitzii* are known to have improved growth in presence of acetate, which would explain the activity for consuming acetate (Shetty 2019). Cross-feeding of lactate resulting from the metabolism of polysaccharide degraders such as *Bifidobacterium* and *Lactobacillus* by butyrate producers in the human gut is well known. Here, we detect very low amounts of lactate in the metabolite analysis. This can be explained by the significant transcriptional activity for lactate consumption primarily *via* the lctABCDE pathway (Supplementary Figure S10). *A. soehngenii* showed high transcriptional activity for utilization of lactate plus acetate which further confirms our previous observation of this being a specialized niche for this organism (Shetty 2019; Shetty et al. 2020). *C. catus* demonstrated activity for lactate consumption but is known only to consume the L-form of lactate, while *A. soehngenii* can use both the D- and L-forms of lactate. Fucose fermentation results in production of 1,2 propanediol (1,2-PD), which is another well-known cross-feeding metabolite (Engels et al. 2016; El Hage et al. 2019; Louis and Flint 2017). While we did not detect any 1,2-PD, there was higher

transcriptional activity for utilization of 1,2-PD in *A. soehngenii* compared to *B. obeum*, *F. plautii* and *C. catus,* of which the latter is not known to utilize 1,2-PD (Supplementary Figure S10) (Reichardt et al. 2014). Transcriptional activity for autotrophic growth on H_2 and CO_2 using formate dehydrogenase and formate-tetrahydrofolate ligase was observed in *B. hydrogenotrophica*. Other than CO_2 and H_2, we observed active processes for dissimilatory nitrate and sulphate metabolism within the quaternary guild. Among the two *Bacteroides* species, *B. xylanisolvens* was the dominant species in the community and notably, had higher contribution to trophic guild 4, which was observed to be linked to higher expression of nitrate reduction module. Respiration *via* nitrate to ammonia may be an advantageous strategy for higher growth rate in competitive ecosystems.

Discussion

Due to technological and practical limitations, deciphering the community dynamics and microbe-microbe interactions is challenging using fecal or other intestinal samples derived from human. Here, we investigated human microbe-microbe and microbe-environment interactions at species and community level within a highly controlled setting, using a defined microbiome that we subjected to detailed community compositional, transcriptional and metabolic analysis. The three most important aspects of this study are (i) assembly of a human minimal microbiome that exhibits ecologically relevant interactions, (ii) the experimental set-up which included nutrient periodicity, and (iii) a set of specific biotic and abiotic perturbations that allowed to address the resilience of the system. All of these aspects are crucial for better understanding the interactions dynamics within human intestinal microbial communities (Shetty, Smidt, and de Vos 2019; De Roy et al. 2014; Großkopf and Soyer 2014). We first demonstrated the applicability of ecological concepts, e.g. Taylor's law, community turnover, divergence, resistance and resilience and then investigated the species level metabolic interactions using metatranscriptomics (Costello et al. 2012; Taylor 1961; Taylor and Woiwod 1980; Kilpatrick and Ives 2003; Collins et al. 2008; Relman 2012). The minimal microbiome exhibited significant correlation with respsect to dynamics of composition, metabolic output, and transcriptional response in replicate bioreactors. Thus, suggesting that a common pool of species shows similar/reproducible assembly and community level dynamics under similar perturbation events. This is equivalent to the classical enrichment experiments where the emergent community assembly can be driven by selecting for specific bacteria or consortia with specific substrates and/or environmental factors such as high salt, pH or temperature (Overmann 2006). Future research is warranted to test whether a different combination of species than the one used here, would result in similar community level behaviours under identical perturbations (Oliphant et al. 2019; Symons and Arnott 2014). Nonetheless, we demonstrated how ecophysiology guided design of synthetic minimal microbiomes combined with metatranscriptomics is a promising avenue for investigating core concepts in ecology and unravelling metabolic interactions at a complexity that bridges the gap between reductionist and holistic approaches.

At individual taxa level, we observed highly variable compositional and functional response. This suggests that stochastic forces are likely higher at species level, while at

community level the behaviour can be rather deterministic as observed with similar divergence, mean rank shift and inequality in triplicate communities when subjected to similar external perturbations (Oliphant et al. 2019; de Cárcer 2019; Vellend 2010; Collins, Micheli, and Hartt 2000; Chase and Myers 2011). However, the system was highly controlled with only one event of immigration (add of *B. hydrogenotrophica*) and stochastic processes such as dispersal limitation were not enforced in our experimental setup (Zhou and Ning 2017). Nevertheless, our observation of deterministic assembly of MDb-MM, has some implications for designing microbiome modulation strategies, where achieving community level stability in both composition and function may be crucial. Examples are resistance to invasion or enhanced butyrate production which can be achieved by targeting ecosystem level properties using appropriate prebiotics (Shetty et al. 2017; Chung et al. 2016; Gibson 1999; Ghosh et al. 2020). These prebiotics may not necessarily target a specific species but a group of species whose fundamental niche allows for "insurance" to absorb impact of daily stochastic, especially destabilizing forces (Relman 2012; Yachi and Loreau 1999).

The investigation of species-specific transcriptional responses revealed that the core gut microbes used in this study have highly evolved metabolic strategies which could explain their co-existence with other seemingly competitive core species. The co-existence is likely due to the ability of these core gut microbes to dynamically regulate the transcriptional response for realized niches among the possible fundamental niches that are vacant (F. C. Pereira and Berry 2017; Plichta et al. 2016). This allows individual species to occupy the niches that become available over time either, due to external (inflow of diet) or changing metabolic behaviour of competitor species. For instance, we observed at transcriptional level, changing patterns of polysaccharide utilization among the species that are part of the first trophic guild where no single species dominates transcriptional contributions for the entire duration of the experiment. These observations provide support for the role of "functional insurance" as result of presence of competitive species in maintaining community composition, structure and functional stability.

Another aspect of host microbial community is the immigration of new species which can have an impact on the overall community (Schmidt et al. 2019; Milani et al. 2019). By introducing *B. hydrogenotrophica* in the established minimal microbiome community, we demonstrated a widely appreciated role of vacant niches in supporting

survival of immigrating species (F. C. Pereira and Berry 2017; Kearney et al. 2018). Despite its fundamental niche being diverse including the ability to utilize several simple carbohydrates that were available, *B. hydrogenotrophica* likely utilized H_2/CO_2 and/or formate as observed with active expression of the formate conversion module (C. Liu et al. 2008). Importantly, when we removed exogenous acetate, *B. hydrogenotrophica* showed high expression of modules linked to homoacetogenesis thus highlighting its contribution to acetate production. This could have aided in stabilizing the community because butyrogenic species such as *A. soehngenii*, *F. prausnitzii* and *R. intestinalis* require acetate for improved growth. These data provide for a realized niche of *B. hydrogenotrophica* that includes inorganic substrates and or formate (D'hoe et al. 2018; Plichta et al. 2016; Bui et al. 2019). Enhancing butyrate production *via* prebiotics can lead to significant amounts of gases and therefore recycling these into acetate by autotrophic acetogens such as *B. hydrogenotrophica* can further support butyrate production (Cummings and Macfarlane 2002; S. H. Duncan, Louis, and Flint 2004). In addition, acetate is a key metabolite for growth of key butyrate producers like *F. prausnitzii*, *R. intestinalis* and *A. soehngenii* (S. H. Duncan, Hold, Harmsen, et al. 2002; Shetty et al. 2018; S. H. Duncan, Hold, Barcenilla, et al. 2002).

The flow of energy in biological ecosystems is widely described via trophic structure where energy flows from one level to another (T. Wang et al. 2019; Rigler 1975). So-called keystone species are usually defined for taxa at higher trophic levels (Trosvik and Muinck 2015; Ze et al. 2012; Cockburn and Koropatkin 2016). Our analysis highlights the difficulties in assigning single and specific trophic roles for individual taxa and supports an acyclic chain of metabolic interactions, especially since the breakdown of complex substrates results in simpler substrates, which the primary degrader can utilize. However, there can be emergent behaviours in bacteria with respect to how the complex substrates are degraded, extracellularly or via membrane bound enzyme complexes (Rakoff-Nahoum, Foster, and Comstock 2016). Nonetheless, in our study, the observations for trophic guild associations and interactions between species can be regarded as emergent behaviours occurring in presence of high order interactions (Grilli et al. 2017; Tilman 2004; Vandermeer 1972; Billick and Case 1994). Notably, we observed certain taxa with a prominent role within specific trophic guilds. For instance, *A. soehngenii* and *C. catus* were predominantly part of the trophic guild

level 3 which involves consuming fermentation end-products, lactate and 1,2 propanediol. On the other hand, *B. hydrogenotrophica* occupied the lowest trophic guild consuming inorganic substrates. The observations for *A. soehngenii* and *C. catus* are in accordance with our previous study of 10-species, where we observed similar functional roles for these two species (Shetty 2019). Thus, MDb-MM could be used to assign realized niches and functional roles of each of the key gut species.

Our experimental system lacks the host-aspect which can have an influence on community composition and dynamics (K. R. Foster et al. 2017). Hence, improvements can be envisaged by incorporating the MDb-MM in an *in vitro* model such as HUMix and organoid cell cultures (Lukovac et al. 2014; Shah et al. 2016), that comprise host features such as aspects of the immune system. One of the major challenges we faced during this study was the difficulty in predicting the metabolic functions based simply on automated annotation and analysis. For instance, the identification of an amylase gene with high expression in *A. muciniphila* suggested its contribution to starch degradation (Supplementary Figure S6). This gene is likely coding for a glycoside hydrolase involved in breaking glycosidic linkages present in crude mucin and is not involved in starch degradation. These observations highlight the need for careful curation and interpretation of -omics based functional analysis of fecal samples where the majority of the species remain uncharacterized. With some manual curation of the published GMMs, we were able to capture >87% of the variation between samples that were identified at KO level annotation. This suggests that it is also valuable to investigate other key functions such as those involved in signaling and processing, virulence, vitamin and co-factor biosynthesis and their role in the species dynamics we observed in this study. We did not include bile salts in our media and several key vitamins and co-factors such as vitamin B_{12} were provided exogenously. Therefore, impact of these key compounds on the community remain unknown. In addition, a bioreactor with similar setup but with constant supply of DoS could help in identifying if the pulse feeding played a role in co-existence of all species till the end of the experiment. Despite these factors, our study was able to unravel complex ecological and metabolic interactions at species level advancing our knowledge of trophic interactions among key gut microbes.

To the best of our knowledge, this study reports the first synthetic human gut minimal microbiome, that incorporates both ecological and metabolic features observed in more

diverse and complex natural ecosystems. We provide experimental evidence for temporal niche segregation as one of the important mechanisms by which species competing for similar resources can co-exists in a dynamic ecosystem. In addition, we demonstrate how metatranscriptomics can be used to assign quantitative traits for identifying niche overlap at transcriptional level. We foresee the use of data generated in this study to serve as a useful resource for ecologists, systems biologists and microbiome experts for developing predictive models (ecological and metabolic) and improving our understanding of the human gut microbiome.

Acknowledgments

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Materials and Methods

Species selection for the composition of the minimal microbiome

Taxonomic composition data from metagenomic studies was obtained from the curatedMetagenomicData data package (v1.18.2) (Pasolli et al. 2017). The data was analysed at species level to identify the taxa that are part of the core microbiota. A total of 64 metagenomic species, which were present in at least 50% of all samples were analysed with a minimum relative abundance of 0.00001. The relative abundance distribution and prevalence in analyzed samples for selected candidate species was visualized using ggridges R package (v0.5.2) (Wilke 2018).

Bacterial strains and pre-culture conditions

The following strains were obtained from the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany) or the American Type Culture Collection (ATCC). *Agathobacter rectalis* (DSM 17629), *Eubacterium siraeum* (DSM 15702), *Roseburia intestinalis* (DSM 14610), *Subdoligranulum variabile* (DSM 15176), *Blautia obeum* (DSM 25238) and *Blautia hydrogenotrophica* (DSM 10507), *Coprococcus catus* (ATCC 27761), *Ruminococcus bromii* (ATCC 27255) and *Collinsella aerofaciens* (DSM 3979/ATCC 25986). *Anaerobutyricum soehngenii* (DSM 1736, L2-7) was kindly provided by Prof. Harry J. Flint's group (University of Aberdeen, UK). The strains from the human microbiome project (HMP) catalogue were *Bacteroides* sp. 3_8_47FAA (*Bacteroides ovatus*), *Bacteroides* sp. 2_1_22 (*Bacteroides xylanisolvens*) and *Flavonifractor plautii* 7_1_58FAA. Furthermore, *Akkermansia muciniphila* (ATCC BAA-835), *Bifidobacterium adolescentis* (L2-32) and *Faecalibacterium prausnitzii* (A2-165) werw taken from the culture collection of the Laboratory of Microbiology, Wageningen University & Research, The Netherlands.

All strains were grown in a medium with the following composition: KH_2PO_4 (0.408 g/L), $Na_2HPO_4.2H_2O$ (0.534 g\L), NH_4Cl (0.3 g/L), NaCl (0.3 g/L), $MgCl_2*6H_2O$ (0.1 g/L), $NaHCO_3$ (4 g/L), yeast extract (2 g/L), beef extract (2 g/L), CH_3COONa (2.46 g/L), casitone (2 g/L), peptone (2 g/L), cysteine-HCl (0.5 g/L), carbohydrates (1.1 g/L), resazurin (0.5 mg/L), 1 mL trace elements in acid (50 mM HCl, 1mM H_3BO_3, 0.5 mM $MnCl_2*4H_2O$, 7.5 mM $FeCl_2*4H_2O$, 0.5 mM $CoCl_2$, 0.1 mM $NiCl_2$, and 0.5 mM $ZnCl_2$, 0.1 mM $CuCl_2*2H_2O$), 1 mL trace elements in alkaline (10 mM NaOH, 0.1 mM

Na_2SeO_3, 0.1 mM Na_2WO_4, and 0.1 mM Na_2MoO_4), 1 mL hemin solution (50 mg hemin, 1 mL 1N NaOH, 99 mL dH_2O), 0.2 mL vitamin K1 solution (0.1 mL vitamin K1, 20 mL 95% EtOH). After autoclaving and before inoculation, 1% of vitamin solution was added (11 g/L $CaCl_2$, 20 mg biotin, 200 mg nicotinamide, 100 mg p-aminobenzoic acid, 200 mg thiamin (vitamin B1), 100 mg panthothenic acid, 500 mg pyridoxamine, 100 mg cyanocobalamin (vitamin B12), and 100 mg riboflavin). For pre-cultures, the bacteria were grown in anaerobic conditions using different combinations of carbon sources (Supplementary table S1). The pre-cultures were incubated non-shaking at 37°C for 24 h.

Anaerobic continuous fermentation

Fermentations were conducted in three parallel bioreactors (DasGip, Eppendorf, Germany) filled with 300 ml of the abovementioned medium at 37°C, at a stirring rate of 100 rpm. The bioreactors were supplemented with 0.5% of crude mucin as well as 1.11 g/L of each of xylan (beechwood, Apollo scientific, U.K.), soluble starch (from potato) (Sigma-Aldrich, USA), inulin (from chicory) (Sigma-Aldrich, USA) and pectin (from apple) (Sigma-Aldrich, USA) at the beginning of the fermentation. The carbon sources, except for mucin, were prepared as 1% stock solution of xylan, soluble starch, inulin and pectin. The pH was controlled at 6.8. The media in both feed and bioreactors were inoculated with 1% of vitamin solution and were reduced because of the cysteine-HCl that was present in the media composition. The bioreactors were inoculated with a normalized O.D. of 1.0 of each one of the abovementioned species in order to have the same cells abundance in the beginning of the fermentation. After allowing the species to grow for 24 hours, the feed was connected containing the growth medium supplemented with 0.5% crude mucin. The flow rate of the feed was set to 10 ml/h. The rate of media refreshing was 30 hours. Upon connecting the feed, the fermentors were spiked three times a day with a 4-hour gap using four carbohydrates (xylan, soluble starch, inulin and pectin) with an end concentration of 0.1% each. During the fermentation period (two weeks) different perturbations than spiking with carbohydrates every four hours took place. These community disturbances were concerned the addition of *Blautia hydrogenotrophica* (152 hours), the increase of the concentration of carbohydrates addition to 2.22 g/L (220 and 224 hours), elongation of the fasting period from 16 to 21 hours (344 and 390 hours), increase of the dilution rate to 20 ml/h (432 and 436 hours). Samples were taken during both the fasting and feeding

period and at every perturbation point (Schematic overview Fig. 1B). Samples for DNA and HPLC were stored in -20°C. Samples for RNA were centrifuged at 4700 rpm for 30 minutes at 4°C. Then, 1 mL of RNAlater was added to the pellet, the pellets were snap-frozen in liquid nitrogen and stored at -80°C.

High performance liquid chromatography (HPLC)

For fermentation product analysis, samples were obtained at different time points of the incubation period. Crotonate was used as the internal standard and the external standards were lactate, formate, acetate, propionate, butyrate, isobutyrate, 1,2-propanediol, sialic acid and glucose. Substrate conversion and product formation were measured with Shimadzu LC_2030C equipped with a refractive index detector and a Shodex SH1011 column. The oven temperature was set at 45°C with a pump flow of 1.00 mL/min using 0.01N H_2SO_4 as eluent. All samples and standards (10µl injection volume) ran for 20 minutes. Standards were prepared with concentrations ranging from 2.5 mM to 25 mM.

DNA isolation and library preparation

Genomic DNA was extracted using the FAST DNA Spin kit (MP Biomedicals, Fisher Scientific, The Netherlands) following the manufacturer's instructions. The concentration of genomic DNA was measured fluorometrically using Qubit dsDNA BR assay (Invitrogen). The hypervariable region V5-V6 (~280 bp) of the 16S rRNA gene was amplified with Phusion Hot Start II DNA polymerase (2 U/µL) for 25 cycles using 0.05 µM of each primer (784F - 1064R) that both contained sample-specific barcodes at their 5'-end. The amplification program for PCR included an initial step of 98°C for 30 seconds, then 25 cycles of at 98°C for 10 seconds, followed by an annealing step at 42°C for 10 seconds and elongation step at 72°C for 10 seconds and a final extension at 72°C for 7 minutes. PCR products were purified using MagBio beads according to the manufacturer's protocol. Purified products were quantified using Qubit dsDNA BR assay kit (Life Technologies, USA) and were pooled in equimolar amounts into one single library. After pooling, the mixed libraries were concentrated using MagBio beads to a concentration needed by the sequencing company. The samples were sequenced on an Illumina NovaSeq platform in 2x150 bp paired-end mode at Novogene (U.K.).

qPCR

The abundance of all species was determined by qPCR as described previously. DNA concentrations were measured fluorometrically (Qubit dsDNA BR assay, Invitrogen) and adjusted to 1 ng/µL by diluting them in DNAse/RNAse free water and prior to use as the template in qPCR. Universal primers targeting the 16S rRNA gene of all the species (1369F 5'-CGG TGA ATA CGT TCY CGG-3' and 1492R 5'- GGWTACCTTGTTACGACTT-3'; 123 bp) were used for quantification. A standard curve targeting the 16S rRNA gene of *B. thetaiotaomicron* was prepared with nine standard concentrations from 10^0 to 10^8 gene copies/µL. qPCR was performed in triplicate with iQ SYBR green supermix (Bio-Rad, USA) in a total volume of 13 µL prepared with primers at 500 nM in 384-wells plates with the wells sealed with optical sealing tape. Amplification was performed with an iCycler (Bio-rad): one cycle of 95 °C for 5 min; 40 cycles of 95 °C for 15 s, 60° C for 20 s, and 72 °C for 30 s each; one cycle of 95 °C for 1 min; and a stepwise increase of temperature from 60°C to 95°C (at 0.5 °C per 5 s) to obtain melt curve data. Data were analysed using Bio-Rad CFX Manager 3.0.

RNA isolation

The cells (10 mL) were centrifuged at 4700 rpm for 15 minutes at 4°C and the supernatant was discarded. Total RNA was isolated by combining enzymatic lysis, the Trizol® reagent and the RNeasy mini kit (QIAGEN, Germany). A mixture of lysozyme (15 mg/mL), mutanolysin (10U/mL) and Proteinase K (100 µg/mL) in 1X TE buffer was added to the pellet normalizing to an OD600 of 2.0 per 100 µL of this mixture. The samples were mixed by vortexing and incubated at room temperature for 10 minutes. After 5 minutes of incubation, the samples were vortexed again. Four microliters of p-mercaptoethanol mixed with 400 µL RLT buffer was added to the sample. Subsequently 1 mL of Trizol® reagent was added to 100 µl of the sample. This mixture was transferred to a sterile tube containing 0.8 grams of glass beads (diameter of 0.1 mm). The tubes were homogenized by bead beating three times for 1 minutes at 5.5 m/s, while cooling the samples on ice in between steps (bead beater, Brand). Then, 200 µL of ice-cold chloroform was added. The tubes were mixed gently and centrifuged at 12.000 x *g* for 15 minutes at 4°C. The RNA isolation was continued following the manufacturer's instructions of the RNeasy mini kit, including an on-column DNase step using DNase I recombinant, RNase-free, (Roche Diagnostics, Germany) incubating at

37°C for 30 minutes. RNA concentration was measured using Qubit and the quality was determined by the Qsep100 bioanalyzer (BiOptic inc, Taiwan). The RNA samples were stored at -80°C until further processing. Further processing such as removal of rRNA, library preparation and sequencing was performed by Novogene using platform Illumina NovaSeq PE150.

Bioinformatics

Amplicon data analysis

The 16S rRNA gene amplicon sequencing data was analysed using the DADA2 R package (Callahan et al. 2016). Raw data (total 4,27,03,796 reads) was filtered to remove low quality reads and reads with more than 2 errors and those matching the PhiX (filterAndTrim function) resulting a total of 4,18,65,602 reads which were then subjected to removal of chimeric sequences (removeBimeraDenovo, consensus method), an average of 225083 ± 102107 reads per samples were obtained. Information of reads for each sample is given in Supplementary table S2. We used a custom database consisting of 16S rRNA gene sequences fetched from the genomes of the 16 bacterial strains used in this study using barnap (available at https://github.com/microsud/MDb-MM) (Seemann 2018). Taxonomic assignment was done using the RDP classifier (Q. Wang et al. 2007). The unique amplicon sequence variants (ASVs) were merged at species level using the *tax_glom()* function in phyloseq (v1.32) (McMurdie and Holmes 2013). The species counts were normalized for the differences in 16S rRNA gene copy number (Supplementary Table S1) and absolute counts were calculated as described previously (Jian et al. 2020). Further analysis of the community composition and structure was done using the microbiome R package (v.1.10.0) (Lahti and Shetty 2018). Data visualization packages, ggplot2 and ggpubr R packages were used for plotting figures (Wickham 2011; Kassambara 2018).

Metatranscriptomics analysis

A total of 816752875 raw paired-end reads totalling to 244.9 giga base pairs were obtained from thirty-six samples (Supplementary Table S3). We followed the approach described in the SAMSA2 pipeline (Westreich et al. 2018). The forward and reverse adaptors were filtered using Trimmomatic (v0.36) (settings: PE -phred33, SLIDINGWINDOW:4:15, MINLEN:70) and then merged using pear (v0.9.10) (Bolger, Lohse, and Usadel 2014; J. Zhang et al. 2013). Merged reads matching the

ribosomal rRNA were removed with SortMeRNA (v2.1) (Kopylova, Noé, and Touzet 2012). A custom database was created from genome sequences of all the bacterial strains used in this study. All the genome sequences in FASTA format were downloaded from the NCBI genome database. For consistency all the genomes were re-annotated using Prokka (v1.12) and the 16S rRNA gene copy numbers for individual strains were identified using the barrnap (v0.9) tool (Seemann 2018, 2014). The amino acid sequences from each strain were then combined to create a database compatible with DIAMOND (v 0.9.22.123) using the *makedb* function (Buchfink, Xie, and Huson 2014). The ribosomal sequences depleted reads were annotated with DIAMOND using blastx. The DIAMOND output files were further analysed in R. The corresponding codes are available at (https://github.com/microsud/Db-MM-10). The amino-acid sequences obtained from genomes were also annotated using the KEGG databases using the GhostKola tool for KEGG ortholog (KO) annotations (Kanehisa and Goto 2000; Kanehisa, Sato, and Morishima 2016).

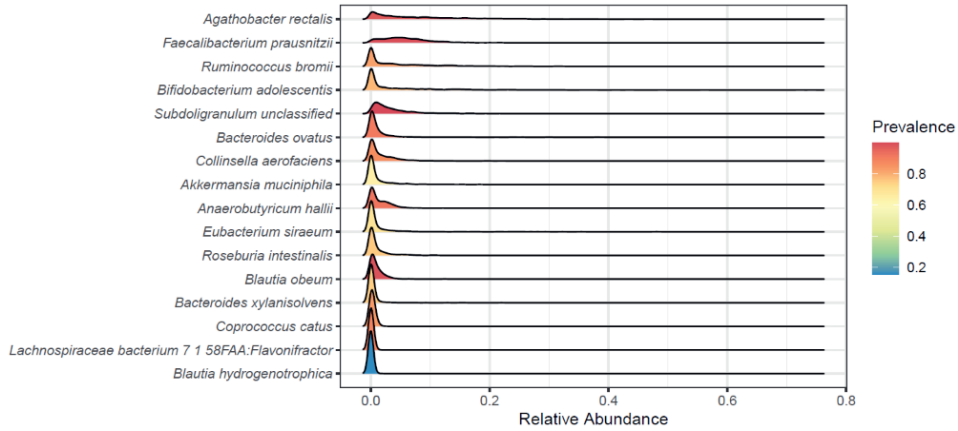
Gut metabolic modules (GMMs)

We did additional curation for the metabolic modules from out previous study to incorporate further refinements for the strains used in this study. The curated GMMs are available at the GitHub repository of this study (https://github.com/microsud/MDb-MM). We used counts per million normalized KO abundances (*cpm* function in edgeR R package v3.24.3) for profiling the metabolic modules using the omixer-rpmR R package (v0.3.1) (Robinson, McCarthy, and Smyth 2010; Darzi et al. 2016). The parameters for the *rpm* function in omixer-rpmR, were as follows, score.estimator= "median", contribute = 0.5, KO=2, distribute=NULL.

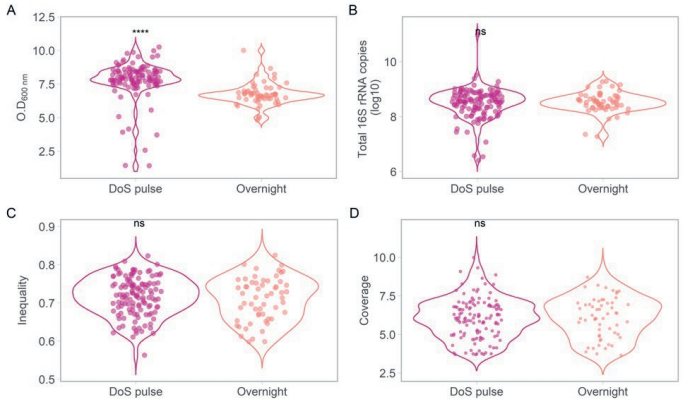
Niche overlap and trophic organisation

The species specific GMM abundances were used a quantitative trait for calculating niche overlap (NO). We used the niche overlap (NO) using the kernel density estimates approached described by Mouillot et al. 2005 (Mouillot et al. 2005). The function to calculate niche overlap was adapted from here https://github.com/umr-marbec/nicheoverlap/blob/master/nicheoverlap.R. We excluded traits (GMMs) for central metabolism such as glycolysis, pentose phosphate pathway, etc. and used only those associated with degradation, consumption or production. A list of GMMs and classification of trophic levels in provided in the Supplementary table S4.

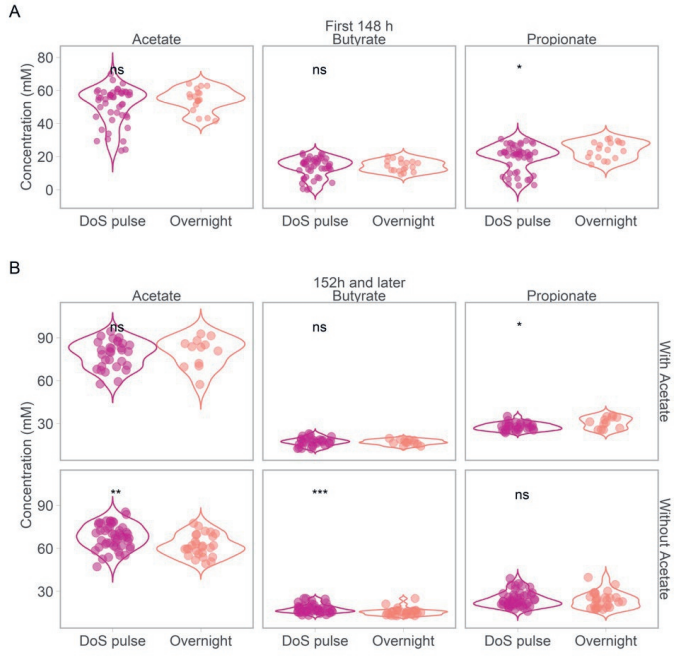
Supplementary Data



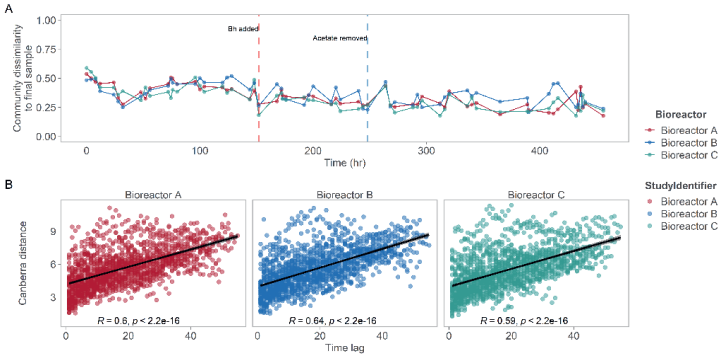
Supplementary figure 1: Relative abundance distribution and prevalence of species. We selected representative strains for each of the species depicted in this figure.



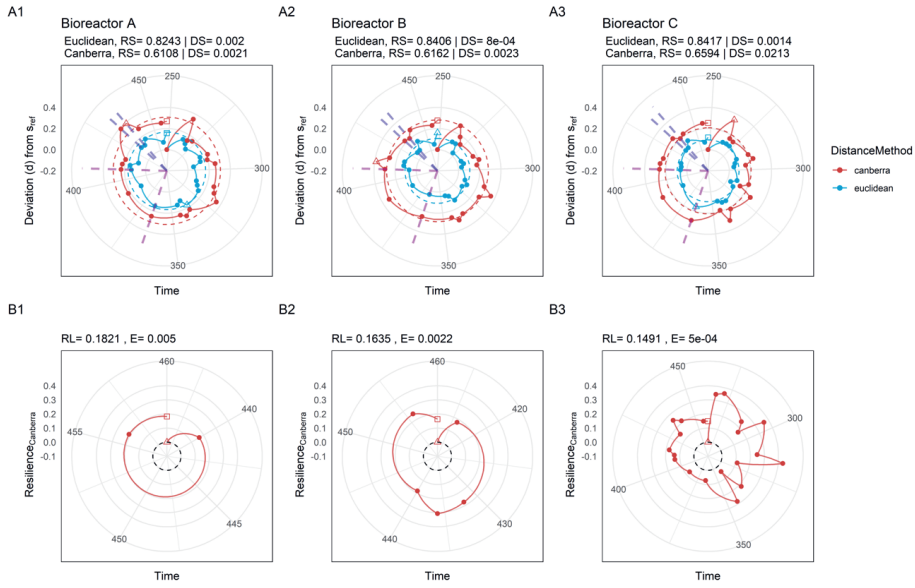
Supplementary figure 2: Comparison of optical density, total 16S rRNA gene copies, inequality and coverage. A) Comparison of optical density (O.D600) after DoS pulse and overnight samples. B) Comparison of total 16S rRNA gene copies after DoS pulse and overnight samples. The counts were transformed to log10. C) Comparison of community evenness between DoS pulsed and overnight samples. D) Comparison of total number of species contributing to 90% of the total abundance after DoS pulse and overnight samples.



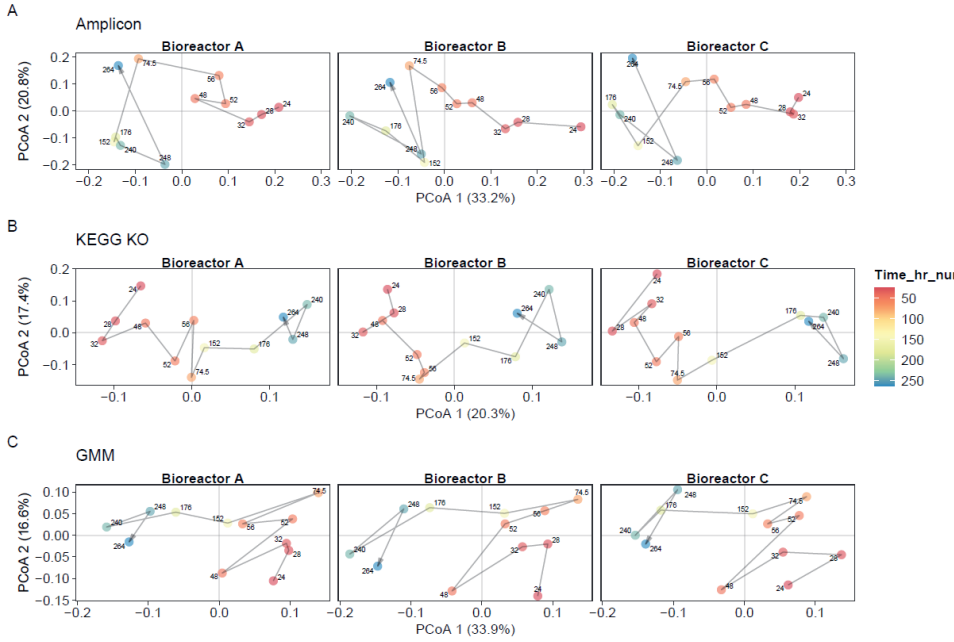
Supplementary Figure S3: Comparison of SCFA production. (A) The first 148 h before introduction of any disturbance event in the system. (B) After introduction of *B. hydrogenotrophica* as well as removal of acetate. The box-and-whisker plots show high, low, and median values, with lower and upper edges of each box denoting first and third quartiles, respectively. * = $p < 0.05$, ** = $p < 0.001$ and *** = $p < 0.0001$, Wilcoxon test.



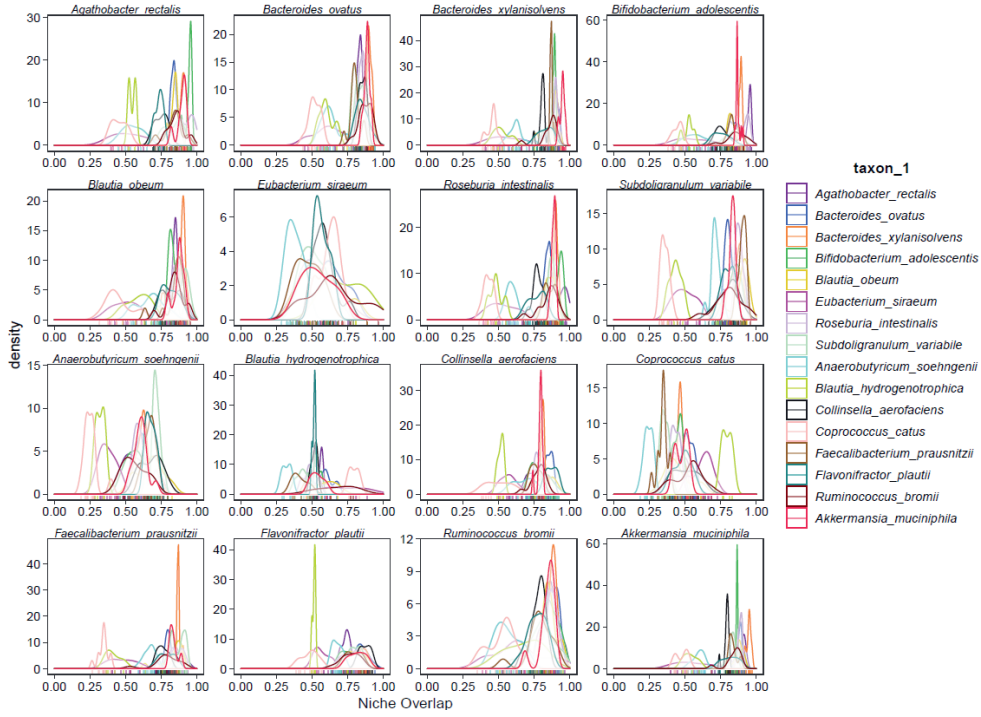
Supplementary Figure S4: Temporal patterns in community succession. A) Temporal convergence patterns of MDb-MM in the three bioreactors (Canberra distance). B) Community divergence based on Canberra distances, with modified codes from the codyn R package which uses Euclidean distances.



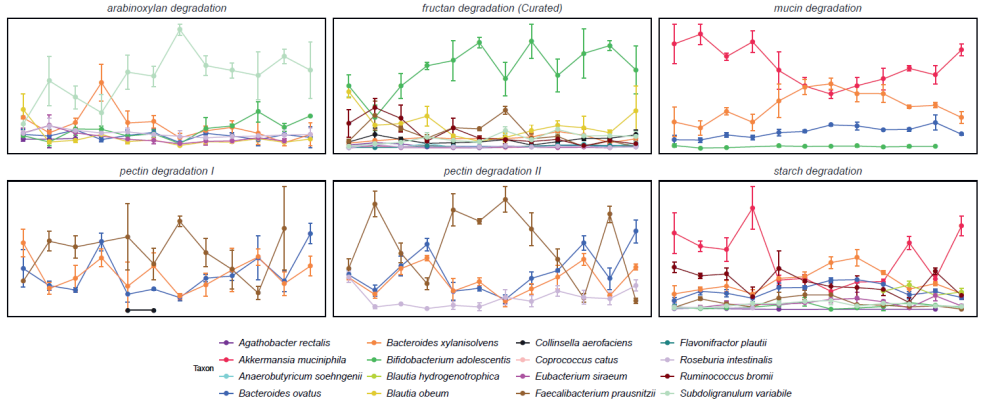
Supplementary Figure S5: Stability properties of MDb-MM in presence of multiple perturbations. A] Community changes from reference phase calculated using Canberra and Euclidean distance. The reference boundary was calculated using the method described by Liu et al., 2018. The shaded region and brown dashed line depict reference boundary based on Canberra distance, while the blue dashed line depicts reference boundary based on Euclidean distance. The hollow triangles represent timepoints when maximal deviation from reference state was observed. The lavender colored dashed line indicates elongated fasting samples and blue colored dashed line indicates the doubling of dilution rate from 10ml/h to 20ml/h B] Resilience of the MDb-MM in presence of multiple perturbations. The black dashed line depicts reference boundary for deviation from disturbance event based on Canberra distance. The stability was calculated with 152 h (introduction of *B. hydrogenotrophica*) as the starting time, removal of acetate/feed change (248h) as the specific disturbance event and experiment end point was 460 h, when the experiment was ended.



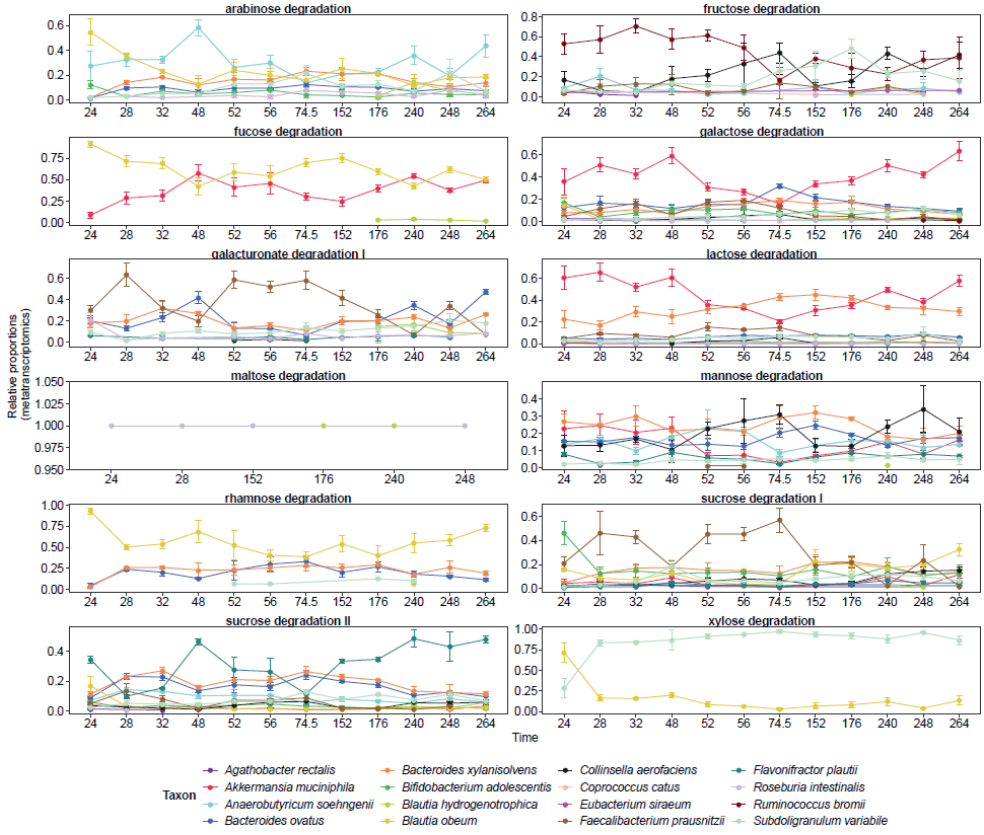
Supplementary Figure S6: Temporal compositional and transcriptional succession of MDb-MM. Comparison of compositional and transcriptional community divergence. Community divergence based on Canberra distances using relative abundances of 16S rRNA gene. Only timepoints with metatranscriptomics data were analysed. Community convergence based on Canberra distances using relative abundances of Kyoto Encyclopedia for Genes and Genomes (KEGG) ortholog. Community convergence based on Canberra distances using relative abundances of Gut metabolic modules (GMMs). Each circle is labelled with corresponding timepoints for clarity.



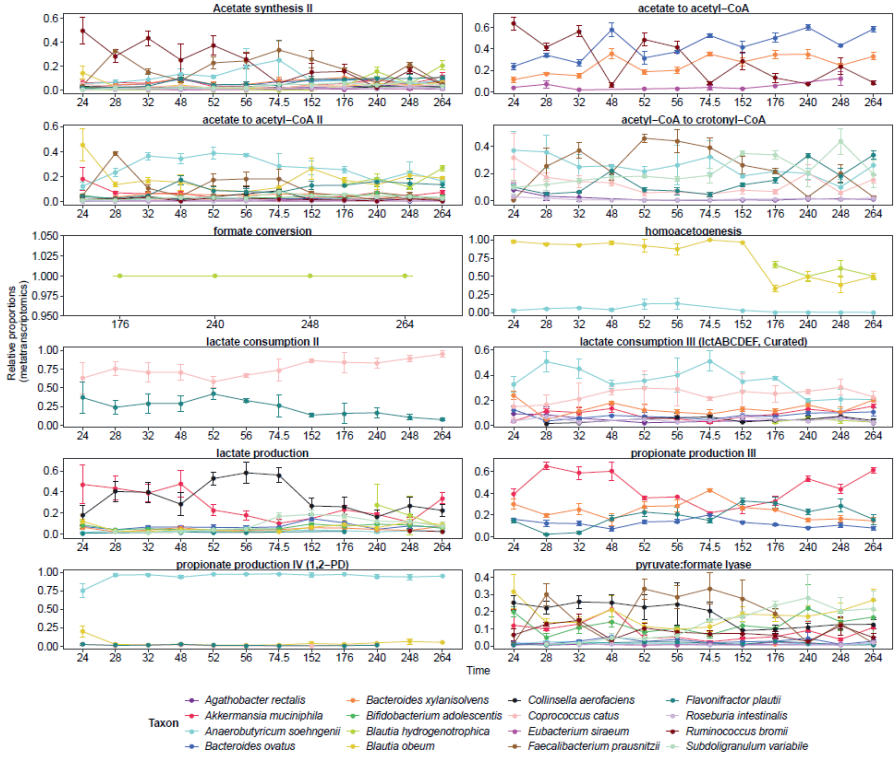
Supplementary Figure S7: Comparison of niche overlap between species. The niche overlap of each of the species was compared with other species in the community across timepoints.
http://www.zo.utexas.edu/courses/bio373/chapters/Chapter13/Chapter13.html



Supplementary Figure S8: Species specific contributions to complex substrate degradation (trophic guild 1). The relative proportions of species specific GMM expression are shown here.



Supplementary Figure S9: Species specific contributions to public goods (trophic guild 2). The relative proportions of species specific GMM expression are shown here.



Supplementary Figure S10: Species specific contributions to SCFA metabolism. The relative proportions of species specific GMM expression are shown here.

Supplementary Table 1: **Candidate strains used in this study.** All strains, except *C. catus* were grown for 24 h. *C. catus* was grown for 48 h due to its flow growth and to achieve sufficient biomass for the experiment.

Species	Strain	Inoculation (%)	16S rRNA gene copies/genome	Substrate pre-culture
A. muciniphila	ATCC BAA-835	1	3	Crude mucin
B. ovatus	HMP strain 3_8_47FAA	1	2	20 mM galactose, 20 mM xylose and 0.2% starch
B. xylanisolvens	HMP strain 2_1_22	1	1	20 mM galactose, 20 mM xylose and 0.2% starch
A. soehngenii	DSM 1736(L2-7)	2	8	60 mM glucose
C. catus	ATCC 27761	1	1	60 mM glucose
Flavonifractor plautii	HMP strain 7_1_58FAA	2	1	20 mM glucose, 20 mM galactose and 20 mM xylose
E. sireaum	DSM 15702	2	1	30 mM glucose, 20 mM maltose and 0.1% starch
A. rectalis	DSM 17629	1	5	20 mM glucose, 20 mM lactose and 20 mM xylose
R. intestinalis	DSM 14610	2	3	30 mM glucose, 20 mM maltose and 0.1% starch
F. prausnitzii	A2-165	1	2	30 mM glucose and 30 mM fructose
S. variabile	DSM 15176	1	4	60 mM glucose
R. bromii	ATCC 27255	2	2	30 mM glucose and 30 mM fructose
B. obeum	DSM 25238	2	5	30 mM glucose and 30 mM fructose
C. aerofaciens	DSM 3979	1	6	30 mM glucose, 20 mM maltose and 0.1% starch
B. adolescentis	L2-32	1	4	20 mM glucose, 20 mM lactose and 20 mM xylose
B. hydrogenotrophica	DSM 10507	2	1	60 mM glucose

Supplementary Table 2: Amplicon sequencing raw data information and total 16S rRNA gene copies in each sample.

Timepoint	SampleID	Raw reads	Filtered reads	Percent surviving	qPCR total 16S rRNA copies
0	F5T0	210840	206942	98.1512	3811346.449
	F6T0	210157	206286	98.15804	3486041.554
	F8T0	267888	262586	98.02081	2576053.019
4	F5T4	289071	282481	97.72028	27489889.77
	F6T4	256351	252155	98.36318	11919416.9
	F8T4	337504	331433	98.20121	87488576.77
8	F5T8	422420	414086	98.02708	268629666.3
	F6T8	384591	377621	98.18769	212352319.4
	F8T8	329572	323392	98.12484	239534469.8
12	F5T12	181667	178044	98.00569	936122605.8
	F6T12	270986	266133	98.20913	443360166.2
	F8T12	263445	257749	97.83788	922600757.1
24	F5T24	288674	281794	97.61669	222347670.3
	F6T24	257533	252976	98.23052	292764928.7
	F8T24	292276	286756	98.11137	604937439.2
28	F5T28	179955	176292	97.96449	171579937.3
	F6T28	283110	278143	98.24556	81282242.74
	F8T28	293628	287326	97.85375	216556050.2
32	F5T32	431612	423642	98.15343	300904450
	F6T32	432546	424692	98.18424	379349453.1
	F8T32	329900	323795	98.14944	278021559.3
48	F5T48	286647	281747	98.29058	415567781.5
	F6T48	286379	280766	98.04001	176832265.7
	F8T48	301374	297383	98.67573	206863233.5
52	F5T52	233339	222394	95.3094	900844321.6
	F6T52	310861	306146	98.48324	553743176.3
	F8T52	272200	266928	98.06319	758584484.5
56	F5T56	162171	159720	98.48863	224283973.6
	F6T56	322208	315806	98.01308	112548896.5
	F8T56	298463	293128	98.21251	57974429.16
72	F5T72	193876	190819	98.42322	709672233.7
	F6T72	420939	412116	97.90397	640409852.9
	F8T72	345906	339524	98.15499	728797534
74	F5T74	89989	88724	98.59427	3340302331
	F6T74	212558	209228	98.43337	347025789.6
	F8T74	245601	241253	98.22965	331978131.1
76	F5T76	230526	227019	98.4787	111713348.3
	F6T76	257017	252626	98.29155	102902522.9
	F8T76	327394	320770	97.97675	98969912.46
80	F5T80	248711	243642	97.96189	539898202.1
	F6T80	382296	375316	98.17419	999117561.6
	F8T80	276439	269318	97.42402	178381303.3
80	F5T80	347580	341800	98.33707	156912853.6

	F6T80	112418	110163	97.99409	212274142
	F8T80	294291	290517	98.7176	422869144.3
96	F5T96	365868	359464	98.24964	19040516.22
	F6T96	388956	382045	98.22319	168486235.1
	F8T96	471210	462344	98.11846	82544366.38
100	F5T100	408868	401376	98.16762	191429422.6
	F6T100	294048	288605	98.14894	476904931.3
	F8T100	338425	332501	98.24954	233403880.2
104	F5T104	137152	134527	98.08607	390467572.3
	F6T104	39021	37355	95.7305	451218769.7
	F8T104	131841	128709	97.62441	601134955.6
120	F5T120	338660	332616	98.21532	196024068.3
	F6T120	228880	224573	98.11823	586485314.9
	F8T120	163372	159914	97.88336	477031944.8
124	F5T124	153385	149463	97.44304	95866766509
	F6T124	183696	180358	98.18287	475763320.9
	F8T124	219202	214663	97.92931	745523354.7
128	F5T128	168687	165799	98.28795	847714466.9
	F6T128	154708	151469	97.90638	595189834.3
	F8T128	183450	179262	97.71709	531853926.2
144	F5T144	32695	31880	97.50726	444916153.3
	F6T144	111683	109416	97.97015	503724206.9
	F8T144	67768	66700	98.42403	1463121363
148	F5T148	377995	371462	98.27167	151166193
	F6T148	388611	381639	98.20592	788775873.3
	F8T148	488616	479462	98.12655	212514528.5
152	F5T152	79979	77608	97.03547	243868402.6
	F6T152	237216	231590	97.62832	347478949.8
	F8T152	214765	210479	98.00433	355885846.5
168	F5T168	112196	109419	97.52487	420659464.2
	F6T168	89610	87863	98.05044	330497181.9
	F8T168	136085	133557	98.14234	615758408.8
172	F5T172	75737	74460	98.3139	266837025
	F6T172	177439	174079	98.10639	556893816.9
	F8T172	162036	159218	98.26088	272839232.3
173	F5T173	161812	153651	94.95649	156027000.5
	F6T173	176820	173056	97.87128	330339381.5
	F8T173	213648	208980	97.8151	225832216.3
176	F5T176	210490	206388	98.05121	80951607.56
	F6T176	167480	164131	98.00036	85175650.97
	F8T176	169790	166588	98.11414	78936721.3
192	F5T192	242578	237194	97.78051	137786044.5
	F6T192	75931	74582	98.22339	207343758.1
	F8T192	85133	83860	98.50469	94219228.23
196	F5T196	421088	413078	98.09778	545519592.3
	F6T196	271859	266700	98.10233	873706311.7
	F8T196	312282	306701	98.21283	860563021.5
200	F5T200	179903	176895	98.32799	342314548.2

	F6T200	173702	170053	97.89928	224106034.3
	F8T200	166959	163260	97.78449	330102734.6
216	F5T216	202287	198344	98.05079	360630081.9
	F6T216	149467	146145	97.77744	348669878
	F8T216	198885	196021	98.55997	322967882.2
220	F5T220	340948	334363	98.06862	171261464.5
	F6T220	429747	422259	98.25758	291049825.1
	F8T220	303549	296468	97.66726	198438613.7
224	F5T224	290979	285129	97.98955	352413111.5
	F6T224	287974	282895	98.2363	193675021.2
	F8T224	238586	232434	97.42147	355810051
240	F5T240	233721	223697	95.71113	215544316.7
	F6T240	285825	281579	98.51448	384018604.1
	F8T240	346143	339661	98.12736	233370625
244	F5T244	106372	104144	97.90546	1092769931
	F6T244	163055	160289	98.30364	655598078
	F8T244	219925	216450	98.41992	590764214.5
248	F5T248	213043	209475	98.32522	534199847.6
	F6T248	78381	75327	96.10365	577591995.7
	F8T248	167425	164199	98.07317	385333536.9
264	F5T264	234603	229664	97.89474	287490600.2
	F6T264	266624	261264	97.98968	258908643.3
	F8T264	141639	137293	96.93164	181813421.3
268	F5T268	165188	162453	98.34431	144846641.8
	F6T268	73166	70252	96.01728	324617721.8
	F8T268	187429	183740	98.03179	364339799
272	F5T272	130408	127139	97.49325	2462501607
	F6T272	369512	361808	97.91509	764086186.9
	F8T272	348184	341568	98.09986	1889205265
288	F5T288	350815	343853	98.01548	389926217.4
	F6T288	426277	418957	98.28281	192033918.3
	F8T288	365267	356972	97.72906	443261372
292	F6T292	244145	239296	98.01389	63050321.24
	F8T292	224190	219609	97.95664	77310401.95
	F5T292	222344	218095	98.089	27582857.1
296	F5T296	74723	73541	98.41816	441374757.3
	F6T296	150069	147349	98.1875	353585909.5
	F8T296	164958	162174	98.3123	341824423.5
312	F5T312	274438	268590	97.8691	1210623309
	F6T312	443578	435392	98.15455	245447356.5
	F8T312	403761	396856	98.28983	375050587.9
316	F5T316	191052	187028	97.89377	818296324.6
	F6T316	70165	68929	98.23844	669411208
	F8T316	98923	97413	98.47356	542521525.7
320	F5T320	337777	331944	98.27312	783894145.6
	F6T320	208340	204445	98.13046	440390676.9
	F8T320	136861	133960	97.88033	715022138.2
336	F5T336	263710	258282	97.94168	216505506.3

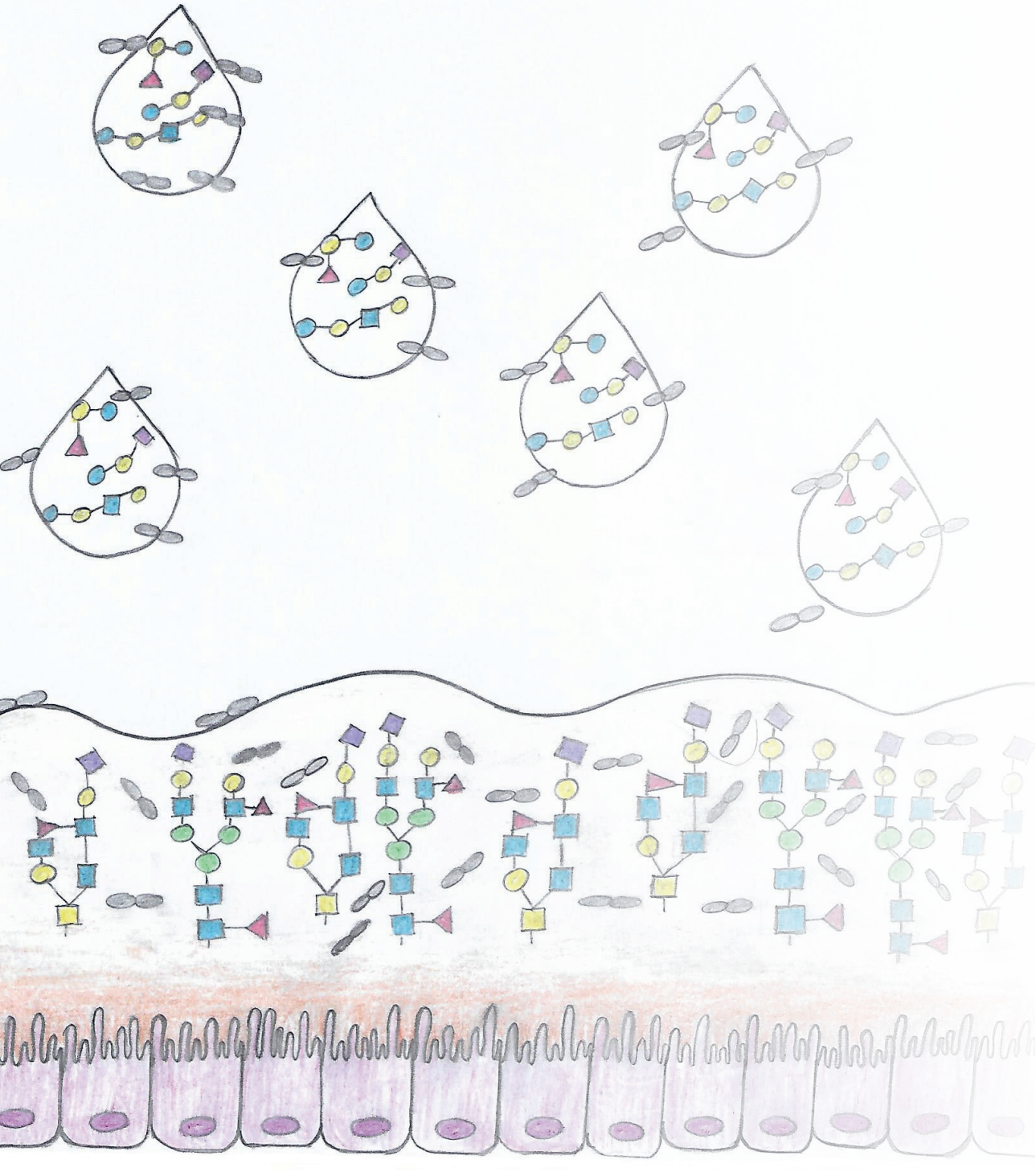
	F6T336	268277	262252	97.75419	583499608.2
	F8T336	261637	257292	98.3393	354471362.9
340	F5T340	51517	50393	97.8182	750163444
	F6T340	161744	158917	98.25218	754064898.6
	F8T340	93422	92077	98.5603	588347287.7
344	F5T344	214682	211447	98.49312	36032437.03
	F6T344	213417	209475	98.15291	591433996.6
	F8T344	303193	296988	97.95345	85602362.58
365	F5T365	249317	245911	98.63387	1219663043
	F6T365	200982	197523	98.27895	422532660.8
	F8T365	249676	245215	98.21328	910852430
389	F5T389	142083	138950	97.79495	132931719.7
	F6T389	197856	193697	97.89797	105111792.2
	F8T389	157593	153641	97.49227	104901279.4
390	F5T390	77908	76143	97.73451	283078844.4
	F6T390	281946	276140	97.94074	22730688.72
	F8T390	339244	332975	98.15207	1295842650
408	F5T408	174450	170261	97.59874	268315303.8
	F6T408	190247	185582	97.54792	270678668.8
	F8T408	163435	160150	97.99003	207804613
412	F5T412	103855	101676	97.90188	929399946.5
	F6T412	176445	173402	98.27538	676731413.4
	F8T412	198037	195009	98.47099	708764184.1
416	F5T416	278198	272688	98.0194	988667622.9
	F6T416	288975	282533	97.77074	1402823509
	F8T416	244715	240297	98.19463	407296698.6
432	F5T432	55443	54430	98.1729	372236302
	F6T432	113149	110776	97.90277	323119950.3
	F8T432	113635	111354	97.9927	256142659.2
434	F5T434	178198	173784	97.52298	474662304.4
	F6T434	235014	230232	97.96523	401200912.7
	F8T434	244691	239931	98.05469	1050501826
436	F5T436	227510	222919	97.98207	959072907.8
	F6T436	164048	160612	97.90549	660149532.3
	F8T436	95561	93271	97.60362	346499010
438	F5T438	113857	110825	97.33701	379745452.1
	F6T438	41071	40268	98.04485	634776777.4
	F8T438	53752	52849	98.32006	417160091.3
440	F5T440	214101	210367	98.25596	129244967.9
	F6T440	236084	231795	98.18327	359900591.5
	F8T440	148357	144614	97.47703	106115248.7
456	F5T456	262064	256565	97.90166	563782664.3
	F6T456	392966	385848	98.18865	1954787393
	F8T456	554165	544757	98.30231	1854261984
460	F5T460	67963	66529	97.89003	1302747839
	F6T460	162087	159239	98.24292	969977502.4
	F8T460	98527	97089	98.5405	615081513.3

Supplementary Table 3: Reads statistics for metatranscriptome sequencing.

Timepoint	Sample ID	Raw Reads	Clean Reads	Error Rate (%)	Q20(%)	Q30(%)	GC Content (%)
24 h	F5T24	22510218	22345873	0.03	97.78	93.59	48.05
	F6T24	22505088	22301265	0.03	97.78	93.62	48.65
	F8T24	23938428	23653126	0.03	97.96	94.03	48.69
28 h	F5T28	23445687	23169642	0.03	97.77	93.53	48.47
	F6T28	20915651	20587416	0.03	97.66	93.36	48.02
	F8T28	31023722	30772505	0.03	97.66	93.34	47.64
32 h	F5T32	23196573	23016159	0.03	97.55	93.03	47.66
	F6T32	21059415	20866971	0.03	97.6	93.16	47.19
	F8T32	23431154	23235304	0.03	97.59	93.15	47.6
48 h	F5T48	24581426	24364344	0.03	97.09	92.07	50.8
	F6T48	27669986	27431356	0.03	97.75	93.59	49.73
	F8T48	19975946	19614457	0.03	97.88	93.89	49.71
52 h	F5T52	24154417	23852997	0.03	97.31	92.42	49.96
	F6T52	24977274	24693836	0.03	97.7	93.43	48.82
	F8T52	21691291	21492803	0.03	97.78	93.56	47.06
56 h	F5T56	24368167	24092192	0.03	97.78	93.59	48.86
	F6T56	21551870	21264878	0.03	97.93	93.98	49.3
	F8T56	20884681	20654025	0.03	97.71	93.45	47.25
74.5h	F5T74T5	24991237	24633215	0.03	97.37	92.79	53.47
	F6T74T5	24787587	24424072	0.03	97.8	93.8	49.12
	F8T74T5	21061858	20831029	0.03	97.83	93.72	47.99
152 h	F5T152	20126710	19789707	0.03	97	91.88	49.77
	F6T152	19982195	19754286	0.03	97.06	92	49.7
	F8T152	23065403	22876366	0.03	96.97	91.8	49.36
176 h	F5T176	25409034	25132415	0.03	97.39	92.63	49.14
	F6T176	23946432	23727878	0.03	96.97	91.73	49.08
	F8T176	20407642	20315381	0.03	97.12	92.12	49.61
240 h	F5T240	23439378	23121953	0.03	97.32	92.55	51.87
	F6T240	21489739	21337826	0.03	97.02	91.9	49.25
	F8T240	21267837	21029334	0.03	97.1	92.06	49.72
248 h	F5T248	21525048	21248891	0.03	96.94	91.72	49.27
	F6T248	24239734	23942665	0.03	97.16	92.14	47.8
	F8T248	24161528	23732221	0.03	97.3	92.52	49.27
264 h	F5T264	23474287	23110742	0.03	97.19	92.23	49.89
	F6T264	20955892	20733904	0.03	96.97	91.84	49.35
	F8T264	19904164	19601841	0.03	96.98	91.76	48.87

Supplementary Table S2: Pearson's product-moment correlation. Correlations were calculated using the cor.test from stats R package for metabolites, composition, gene expression (locus tags expression, KEGG ortholog level, and gut metabolic level expression) between the three bioreactors.

	Correlation	P-value
Short Chain Fatty Acids (Concentrations in mM)		
Bioreactor A vs Bioreactor B	0.98	< 2.2e-16
Bioreactor B vs Bioreactor C	0.97	< 2.2e-16
Bioreactor A vs Bioreactor C	0.98	< 2.2e-16
16S rRNA gene composition (Relative abundance)		
Bioreactor A vs Bioreactor B	0.92	< 2.2e-16
Bioreactor B vs Bioreactor C	0.94	< 2.2e-16
Bioreactor A vs Bioreactor C	0.91	< 2.2e-16
Locus tags (Relative abundance)		
Bioreactor A vs Bioreactor B	0.85	< 2.2e-16
Bioreactor B vs Bioreactor C	0.93	< 2.2e-16
Bioreactor A vs Bioreactor C	0.89	< 2.2e-16
KEGG KO (Relative abundance)		
Bioreactor A vs Bioreactor B	0.97	< 2.2e-16
Bioreactor B vs Bioreactor C	0.97	< 2.2e-16
Bioreactor A vs Bioreactor C	0.96	< 2.2e-16
GMM (Relative abundance)		
Bioreactor A vs Bioreactor B	0.92	< 2.2e-16
Bioreactor B vs Bioreactor C	0.94	< 2.2e-16
Bioreactor A vs Bioreactor C	0.93	< 2.2e-16



Chapter 6

The main functions of key species *Akkermansia muciniphila* remain stable across different microbial ecosystems

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Manuscript in preparation

Abstract

Akkermansia muciniphila resides in the mucosal layer in the gastrointestinal tract. In this niche, *A. muciniphila* was found to be a key species of the gut microbiota. This bacterium has an extraordinary capacity to degrade the mucus glycans in order to thrive in a competitive environment as the gut. We compared existing transcriptional databases to assess the response of *A. muciniphila* in different ecological networks ranging from mono-, to co-cultures to more complex ecosystems of synthetic communities *in vitro* and *in vivo*. Our results indicate that *A. muciniphila*'s overall transcriptional response is altered under different environmental conditions However, the expression of key functions such as mucin glycans degrading ability, pili and EPS production is stable throughout different ecosystems and independent of environmental conditions.

Keywords: *Akkermansia muciniphila,* mucin, glycan degradation, (meta-) transcriptomics, gut microbiota

Introduction

The human gut harbours a complex and diverse microbial community, commonly referred to as the gut microbiota. The gut microbiota is considered to play an important role in host health. Within the gut ecosystem, mucus layer lining the intestinal epithelium is a prominent niche available for bacteria that possess specialized enzymatic machinery for mucin foraging. *Akkermansia muciniphila* is a prominent species residing in the mucus layer with the ability to degrade mucin (Derrien et al. 2004). *A. muciniphila* is currently the only representative of the Verrucomicrobia phylum in the human gut and present in high numbers with an abundance ranging from 0.5-5% (Cani and de Vos 2017). The abundance of *A. muciniphila* is mostly linked to a healthy status in humans, although a few exceptions have been reported likely due to confounding factors (Cani and de Vos 2017). A few examples are the decreased abundance of *A. muciniphila* in humans with diseases, such as type 2 diabetes, obesity and inflammatory bowel disease (IBD) (X. Zhang et al. 2013; Png et al. 2010; Dao, Everard, Aron-Wisnewsky, et al. 2016; Yassour et al. 2016).

A. muciniphila utilizes mucin as the sole carbon, nitrogen and energy source (Derrien et al. 2004). Mucus is continuously produced in the colon by the host epithelial cells. The major component of human colonic mucus layer is the protein MUC2. These MUC2 proteins are rich in proline (P), threonine (T) and serine (S) PTS domains that are densely O-glycosylated (Johansson et al. 2008; Johansson, Holmén Larsson, and Hansson 2011). The genome of *A. muciniphila* is composed of one circular chromosome of 2.7 Mbp coding for 2,176 protein-coding genes, of which 65% was assigned a putative function. Despite its relatively small genome size, this bacterium is, in terms of function, mainly dedicated to mucus degradation (van Passel et al. 2011). A recent study predicted that the genome of *A. muciniphila* contains genes encoding for 61 proteins involved in mucus degradation (Ottman, Davids, et al. 2017). The enzymes involved in this process mainly include proteases, sulfatases, glycosyl hydrolases, and sialidases. Furthermore, it has been shown that N-acetylglucosamine (GlcNAc) and L-threonine are essential for *A. muciniphila*'s growth in minimal medium (van der Ark et al. 2018). Auxotrophy for L-threonine was predicted using the genome scale model for *A. muciniphila* (Ottman, Davids, et al. 2017). Reduced genome sizes with auxotrophies are considered as a hallmark for life in a host associated symbiotic lifestyle for bacteria.

Both GlcNAc and L-threonine are present in mucus. Therefore, the necessity for these components further indicates the adaptation to its mucosal niche.

Considering the important role of *A. muciniphila* in host health, its metabolic properties have been extensively investigated at different levels of complexity, mostly ranging from monoculture to defined consortia (Ouwerkerk, van der Ark, et al. 2016; van der Ark et al. 2018; Van Herreweghen et al. 2017; Chia et al. 2018; Kovatcheva-Datchary et al. 2019). These studies have utilized in vitro, and rodent model systems combined with metabolite and transcriptomic approaches. The availability of transcriptomic data from numerous studies provides an opportunity to unravel the transcriptional landscape of *A. muciniphila* under different environmental conditions. In the present study, we leverage RNAseq data ranging from monoculture to multi-species metatranscriptomes generated recently to investigate the transcriptional landscape of *A. muciniphila* under varying growth conditions. The datasets used in this study consisted of following growth conditions: (i) monoculture of *A. muciniphila* and co-culture of *A. muciniphila* and *Bacteroides thetaiotaomicron in vitro* in bioreactors under a continuous flow of mucin glycans, (ii) mono-colonization of germ-free mice with *A. muciniphila* and co-colonization with *A. muciniphila* and *B. thetaiotaomicron,* and (iii) *A. muciniphila* grown in a 15 species synthetic community under a continuous flow of mucin glycans and periodically addition of carbohydrates (pectin, xylan, starch, inulin). Analysis of the abovementioned transcriptomics and metatranscriptomics networks revealed that *A. muciniphila* has a continuous expression of genes related to mucus degradation, EPS production and pili synthesis, independent of the changing environments. However, genes involved in stress response are regulated in response to the changing environment.

Results

Datasets and RNA sequencing quality control

In this study, we used existing transcriptional databases of *A. muciniphila* in different ecological networks ranging from mono-, to co- cultures to more complex ecosystems of synthetic communities *in vivo* and *in vitro.* The present study includes three transcriptional datasets that were generated from two different experiments. SynCom study is discussed in more details in **Chapter 5**, while the comparison of the *in vitro* mono- and co-culture with the *in vivo* experiment is described in **Chapter 4**.

The RNA sequencing data from several studies ranged from mono-cultivation and co-cultivation to synthetic communities (Table 2). The percentage of reads mapped to the genome of *A. muciniphila* varied within conditions as well as between conditions (Table 1). Notably, the percent of mapped reads varied according to the experimental set-up. Highest mapping of reads was observed in the mono-cultures experiments. In vitro, a higher number of species in the experiment results in a lower number of reads mapped to the genome of *A. muciniphila*. The *in vivo* dataset contains the highest total number of reads. However, three samples of the *in vivo* study were covered by <1% of *A. muciniphila*'s genome.

The variation in the transcriptional response of *A. muciniphila* between different conditions was minimized by using relative abundances of the gene counts. In that way, the abundance of each gene was normalised across all samples of the different studies, allowing us to compare the different ecosystem studies.

Table 2: RNA sequencing coverage and total amount of quality filtered reads of all conditions.

Sample Name	Coverage (%)	Total number of reads
Cecum_mono_1	3.50%	58877899
Cecum_mono_2	16.30%	63665811
Cecum_mono_3	8.80%	59789081
Cecum_mono_4	4.70%	62125268
Distal_mono_1	1.10%	64185933
Distal_mono_2	1.80%	68772752
Distal_mono_3	2.00%	59668290
Cecum_co_1	0.80%	69830646
Cecum_co_2	2.10%	58284293
Cecum_co_3	3.00%	80785928
Cecum_co_4	2.60%	66105956
Distal_co_1	0.50%	69718577
Distal_co_2	0.50%	33520653
Distal_co_3	1.20%	63510452
Monoculture_1	41.10%	8042495
Monoculture_2	52.70%	8290259
Monoculture_3	52.40%	7889334
Co.culture_1	16.10%	9053550
Co.culture_2	17.00%	9277837
Co.culture_3	12.90%	10470948
SynCom_muc_1	9.30%	24051669
SynCom_carb_1	4.40%	24097707
SynCom_muc_2	8.00%	27309425
SynCom_carb_2	3.10%	21306330
SynCom_carb_3	3.60%	20656932
SynCom_muc_3	10.40%	19737563

Transcriptional landscape of *A. muciniphila* under different environmental conditions

We conducted ordination analysis of the overall transcriptional response of *A. muciniphila* to monitor the most variable differences in gene expression under different environmental conditions. Redundancy analysis (RDA) indicated that both the experiment and the location affected gene expression, explaining 29.64% and 19.14% of the variation (Figure 1a & Supplementary Figure 1a). RDA showed that each experimental condition clustered together with distinct separation between the *in vivo* and *in vitro* studies. There was no clear separation between the samples within the *in vivo* experiments. On the other hand, in the *in vitro* experiments, the SynCom study

separated from mono- and co-culture. This indicates an altered gene expression when *A. muciniphila* is in the presence of fifteen other species in the community as well as the different media composition between experiments.

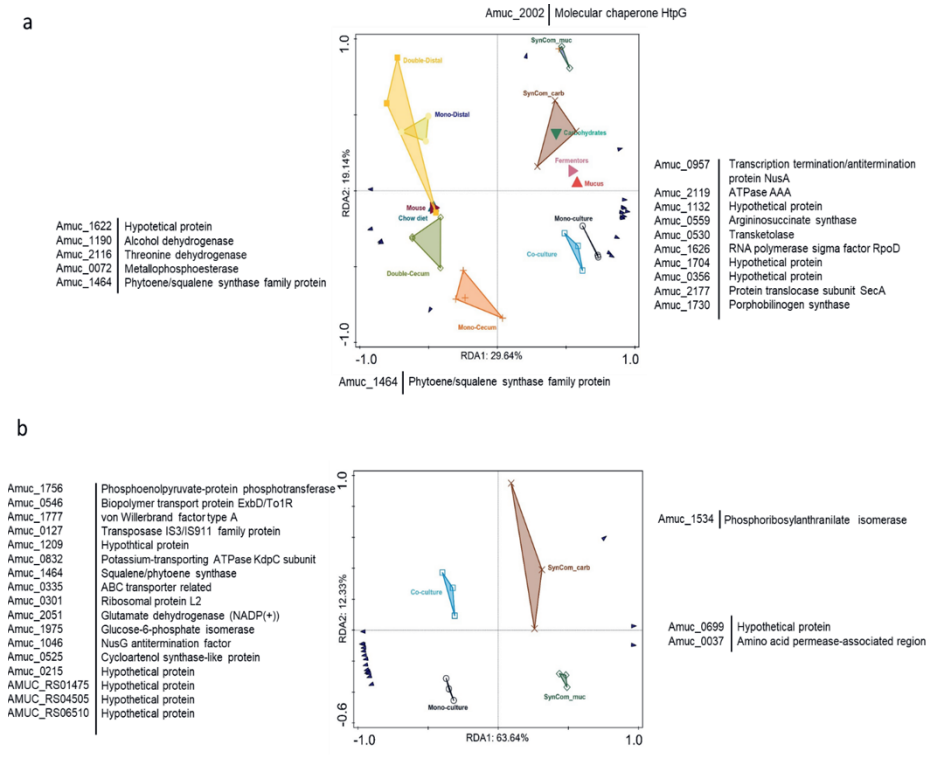

Figure 3: Ordination analysis of the ecosystem studies and the most variable expressed genes. a) RDA analysis showing the 20 most variable genes. b) RDA analysis showing the 20 most variable genes of the *in vitro* studies. The plots were generated by Canoco.

The altered gene expression between *in vitro* experiments was confirmed by a subsequent RDA of the *in vitro* studies. This analysis showed that the gene expression was affected by the microbial population in the culture and/or the different media composition between the two studies, explaining 63.64% and 12.33% of the total variation (Figure 1b & Supplementary Figure 2b). RDA illustrated distinct clustering of monocultures, co-cultures and SynCom_muc. Furthermore, there was a difference observed in *A. muciniphila*'s transcriptional response when carbohydrates were present in the synthetic community.

The analysis of the expression of KEGG pathways in different conditions revealed two pathways (carbohydrate and amino acid metabolism) that were highly abundant across all the environmental conditions in all the datasets (Figure 3). Transcripts belonging to the carbohydrate metabolism pathway exhibited above 4% and up to 6% of relative abundance and those associated with amino acid pathway had above 2% and up to 4% of relative abundance for all the samples included in this study. Moreover, transcripts involved in glycan biosynthesis and metabolism process were highly abundant (>2%). In contrast, transcripts related to genes involved in drug resistance, biosynthesis of other secondary metabolites and metabolism of the terpenoids and polyketides were relatively low abundant.

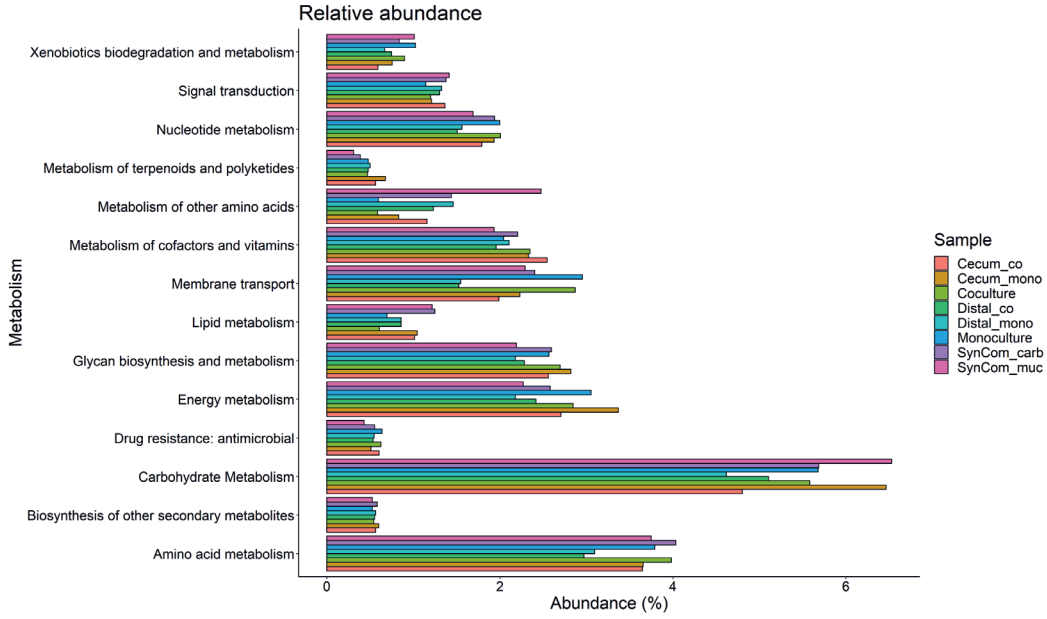

Figure 4: Relative abundances of KEGG pathways expressed by *A. muciniphila* in different environmental conditions.

Top variable genes in response to environmental changes

The comparison of the different studies revealed variability in the gene expression profile of *A. muciniphila* (Figure 4). We assessed the most variable genes detected in the relative abundance RNAseq dataset between *in vivo* and *in vitro* conditions. A high variability across samples was observed of a gene encoding anaerobic ribonucleoside triphosphate reductase activity protein (Amuc_0860). The relative abundance of this gene in *A. muciniphila's* transcriptome was higher in *in vivo* conditions compared to *in*

vitro conditions. In addition, three 23S rRNA genes (AMUC_RS06530, AMUC_RS01455, and AMUC_RS04525) were on average higher in *in vivo* samples compared to in vitro samples. Next to these rRNA genes, the relative abundance of three hypothetical proteins (Amuc_1569, Amuc_0016 and Amuc_0460) and an ATP-dependent chaperone ClpB (Amuc_0836) were on average specifically higher in samples taken from the distal colon of mice mono-colonized with *A. muciniphila*. In contrast, the relative abundance of Amuc_1098 (Ottman, Reunanen, et al. 2017) encoding a type II and II secretion system protein (PiliQ) was highest in the *in vitro* monoculture and co-culture. Furthermore, a subset of genes in *A. muciniphila*'s transcriptome showed higher relative abundances of several genes in the synthetic community with mucus as the main carbon source. These genes encode glutamate decarboxylase (Amuc_0372), autotransporter domain-containing protein (Amuc_0687), thioredoxin (Amuc_0691), molecular chaperone DnaK (Amuc_1406), rubrerythrin (Amuc_2055) and catalase HPII (Amuc_2070).

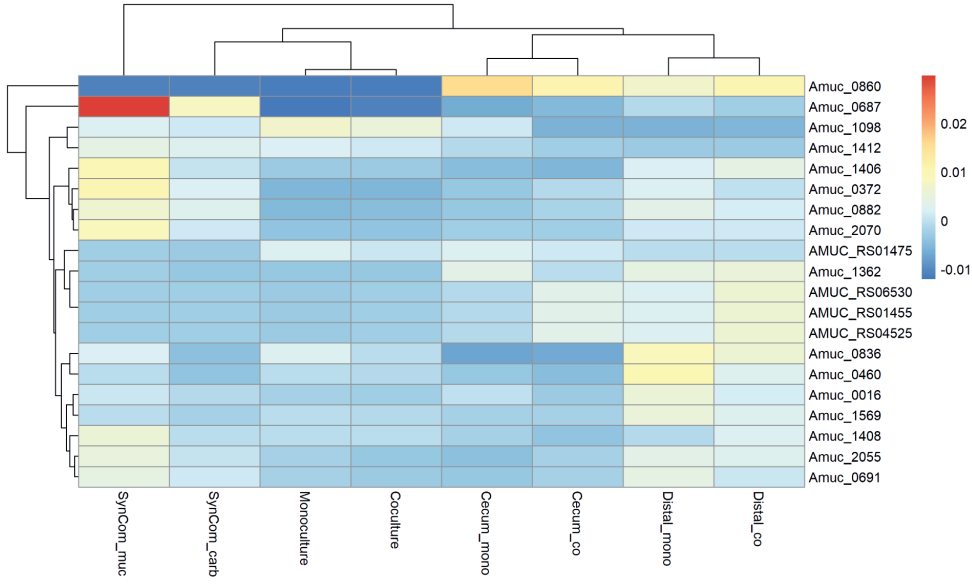

Figure 5: Heatmap of the 20 most variable *A. muciniphila* genes between the different studies

Key functions of *A. muciniphila* across different ecosystems

To assess the key functions of *A. muciniphila* across different ecosystems, we focused on mucus degradation, EPS production and pili production (Figure 5). The data

suggests that all three functions are exerted by *A. muciniphila* in all of the conditions tested and only non-significant differences were found (Supplementary Figure 1).

A. muciniphila is known for being a mucin degrading bacterium, and thereby provides the host with several health benefits. Even though the overall mucus degradation pattern was found to be similar, the data revealed minor differences between *in vitro* and *in vivo* experiments (Figure 5a). The gene with the overall highest relative abundance among mucus degradation genes was Amuc_0824 encoding a glycosyl hydrolase GH family 2 (GH2). However, in synthetic communities three other genes were shown to have higher relative abundances than Amuc_0824. These genes were Amuc_0290 (GH family 2 sugar binding), Amuc_1120 (glycoside hydrolase N-terminal domain-containing protein) and Amuc_2136 (GH20). Even though the order in which the genes are expressed showed differences, all four genes were present in the top 10 genes with the highest relative abundances throughout all the conditions. This indicates that these four genes form the basis of mucus degradation, independent of the environmental condition. When focusing on the genes in the top 10, *in vivo*, *A. muciniphila* seems to adapt to the environment by using an exo-alpha-sialidase (Amuc_0625) and glycoside hydrolase family 18 (Amuc_2164), while *in vitro* this was an arylsulfatase (Amuc_0121).

The mechanism of exopolysaccharides (EPS) produced by *A. muciniphila* has not yet been revealed. Therefore, putative EPS-associated genes in the genome of *A. muciniphila* were used to analyse EPS production between different ecosystems (Figure 5b). Four genes were found to have a stable high abundance across EPS-associated genes among all conditions. These genes include a hypothetical protein that is part of a glycosyl hydrolase cluster (Amuc_1143), two polysaccharide biosynthesis tyrosine autokinases (Amuc_1413 and (Amuc_2078) and a polysaccharide export protein (Amuc_1414).

Another key function of *A. muciniphila* is pili production. In this function the main gene expression pattern was similar. Within all conditions, the key outer membrane protein involved in the production of type 4 pili (Amuc_1098) (Ligthart et al. 2020) was found to have the highest relative abundance among pili associated genes. The second and third most abundant pili-associated genes within all conditions were a type II secretion system protein (Amuc_0394) and pilus assembly protein PilM (Amuc_1101), respectively. Both Amuc_1098 and Amuc_1101 are part of the Amuc_1100 pili cluster.

However, transcription of the Amuc_1100 gene was not detected in any of the conditions included in this study. Furthermore, the overall relative abundances of another cluster Amuc_1583 – 1586 (prepilin-type N-terminal cleavage/methylation domain-containing protein, type II secretion system F family protein, type II/IV secretion system protein and Flp pilus assembly complex ATPase component TadA) were found to be lower than that of the Amuc_1100 cluster in all conditions. Overall, transcripts of pili production genes were present and similar in terms of the expression of different pili production systems in all conditions.

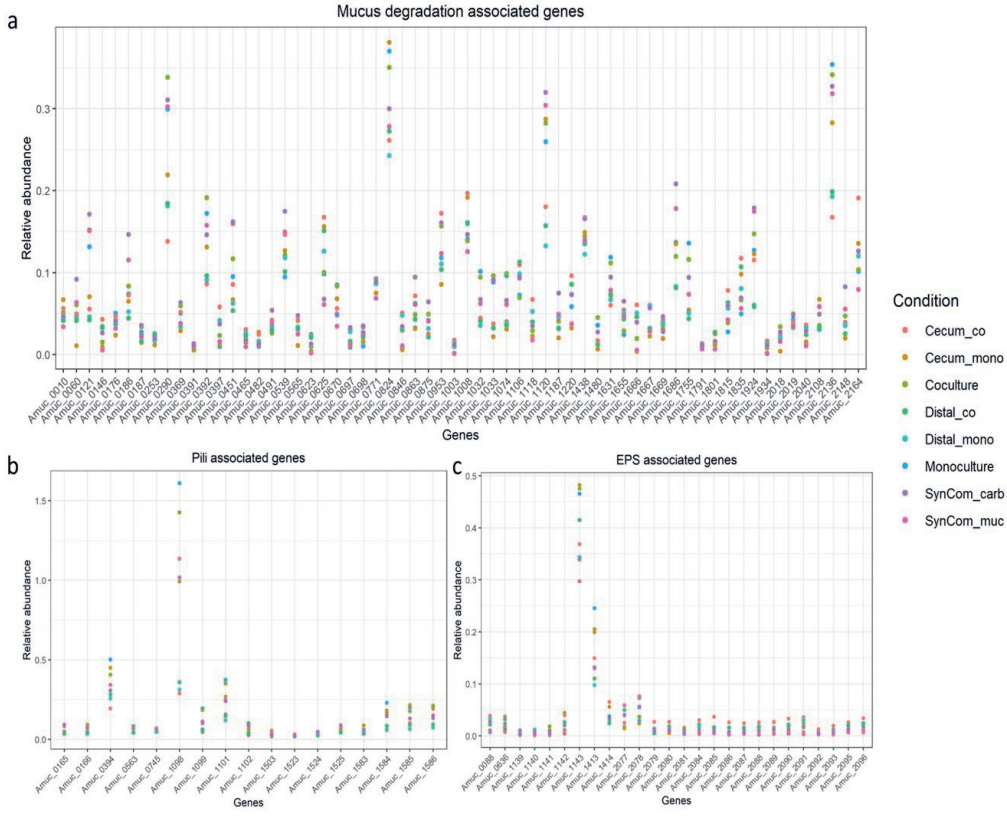

Figure 6: Relative abundance comparison of *A. muciniphila*'s key functions across the different ecosystems. a) mucus degradation associated genes, b) pili associated genes and c) EPS associated genes.

Discussion

This study made use of current transcriptomic databases and mined them for the expression profiles of *A. muciniphila*. This gave new insight into the transcriptional response of *A. muciniphila* in different ecosystems with increasing ecological complexity. Our results indicate that *A. muciniphila*'s overall transcriptional response is altered under different environmental conditions. However, the transcription of genes involved in mucus and glycan degradation, pili production and EPS production by *A. muciniphila* showed no significant differences throughout different ecosystems and are independent of environmental changes.

This study showed that the ecosystem complexity as well as the location and diet supplementation were substantial factors that led to the establishment of four different ecological *A. muciniphila* niches. We demonstrated that carbohydrate metabolism and glycan biosynthesis and metabolism pathways were among the most abundantly expressed pathways across all the samples used in this study irrespective to location (*in vitro* and/or *in vivo*), diet intervention, and complexity of the culture (mono- or co-culture). A possible explanation for this might be that *A. muciniphila*'s ability to access the mucin glycans in any ecosystem will not change its fitness in the community. On the contrary, *A. muciniphila* in a competitive environment would increase its hydrolytic capacity by upregulating Glycoside Hydrolases (GHs) and mucin degrading genes. It is been described before that when *A. muciniphila* shared nutrients with the butyrate producer, *Anaerostipes caccae*, upregulation of mucin-degrading genes was observed (Chia et al. 2018). Additionally, it has also been described that in the presence of *B. thetaiotaomicron* in a solely mucin glycans environment, *A. muciniphila* upregulated GHs that target core glycosidic linkages found in mucin structure (**Chapter 2**). Furthermore, in a mice study with synthetic microbiota, *A. muciniphila* and *Bacteroides caccae* showed increased expression of their *O*-glycan responsive genes in a fiber-free diet compared to a fiber-rich diet (Desai et al. 2016). Therefore, *A. muciniphila*'s glycans degrading activity was not significantly altered under different conditions where mucin glycans can be used as nutrient source. This is also depicted in the comparison of the relative abundance of *A. muciniphila* mucin-degrading genes.

The comparison of the different studies revealed, primary, separation in the transcriptional response of *A. muciniphila* between *in vivo* and *in vitro* studies. Even though the different experimental designs in this study grouped together in the

ordination analysis, no distinct variation was observed between the complexity of the culture. However, the comparison only of the *in vitro* fermentations illustrated variation of *A. muciniphila* transcriptional response between the studies with increasing the microbial population in the bioreactors. A possible explanation for this could be that in the presence of other glycan degrading members in the community, *A. muciniphila* will change its gene response in order to be able to survive in a highly competitive environment. In the co-culture experiment, *A. muciniphila* shared the nutritional environment (mucin glycans) with the glycan generalist *B. thetaiotaomicron*. Bacteroidetes species that are abundant in the human gut (Martens, Chiang, and Gordon 2008; Salyers et al. 1977) have the ability to release antimicrobial proteins, the so-called membrane attack perforin proteins (MACPF) that are used to reduce the antagonism within microbial communities (Chatzidaki-Livanis, Coyne, and Comstock 2014; Roelofs et al. 2016). *B. thetaiotaomicron* significantly upregulated a gene encoding the MACPF protein targeting to reduce *A. muciniphila*'s fitness in the system (Chapter 2). The structure and the activity of this protein has been characterized before (see **Chapter 4**) (Q. Xu et al. 2010). In the same study, it is suggested that *A. muciniphila* was able to counteract *B. thetaiotaomicron*'s attack by upregulating ABC transporters, two-component system encoding genes as well as LPS core biosynthesis genes. It has been described before that LPS core biosynthesis genes and ABC transporters are required for antimicrobial peptide resistance (Z. Wang et al. 2016; Ahmad, Majaz, and Nouroz 2020; Sharp et al. 2018).

In the SynCom study, *A. muciniphila* has a stable abundance throughout the whole period of the experiment. The presence of other glycan-degrading bacteria in the community such as *Bacteroides xylanisolvens, Bacteroides ovatus* and *Bifidobacterium adolescentis* did not affect *A. muciniphila*'s function in the community. Another *in vivo* synthetic microbiota study, showed that *A. muciniphila*'s abundance was not affected by the presence of other glycan-degrading bacteria in the community, but its abundance was increased 2-fold only during the transition from fiber-rich to fiber-free diet [1]. In the present study, the ordination analysis showed that *A. muciniphila*'s transcriptional response differentiated during the periodically addition of carbohydrates. Therefore, a fiber-rich diet could be considered as an influential factor for *A. muciniphila*'s response in a community.

Next, we monitored the most variable genes among the different studies. The analysis revealed the variable expression of multiple genes related to stress responses, including glutamate decarboxylase (Amuc_0372), autotransporter domain-containing protein (Amuc_0687), thioredoxin (Amuc_0691), molecular chaperone DnaK (Amuc_1406), rubrerythrin (Amuc_2055) and catalase HPII (Amuc_2070). These genes were found to be related to acid and oxidative stress responses in bacteria (Storz et al. 1990). Glutamate decarboxylase (GAD) is implicated in acid tolerance in several genera (Feehily and Karatzas 2013). Using this system, the decarboxylation of glutamate results in the uptake of one intracellular proton (H+) and thereby promotes acid tolerance. In *Escherichia coli* the GAD system was found to be the most important acid resistance mechanism (Capitani et al. 2003; J. W. Foster 2004). However, exposure of E. coli O157:H7 to H2O2 indicated the GAD system also promotes resistance against oxidative stress. Furthermore, molecular chaperone DnaK, involved in protein folding, was found to be induced in stresses other than heat shock, including acid and oxidative stresses (Susin et al. 2006). Thioredoxin, rubrerythrin and catalase HPII all play a role in oxidative stress resistance. *Akkermansia* ruberythrin was also detected in another study using mice samples, where it reflected the exposure of *A. muciniphila* to reactive oxygen or nitrogen species during inflammation (D. Berry et al. 2012). The relative abundances of these stress-related genes were highest in SynCom supplemented with mucus as the main carbon source. The oxygen reduction capacity of *A. muciniphila* through aerobic respiration has previously been shown (Ouwerkerk, van der Ark, et al. 2016). In this study it was shown that *A. muciniphila* likely uses the Cytochrome bd complex in combination with other genes involved in the oxidative stress response such as superoxide dismutase, rubrerythrin, catalase HPII and alkyl hydroperoxide reductase. Interestingly, the relative abundance of the cytochrome bd complex was not higher in SynCom_muc compared to the other conditions included in this study. Therefore, it is unlikely that the stress response revealed in SynCom_muc is due to the direct presence of oxygen, but more likely due to the presence of reactive oxygen species. We hypothesize that a lack of additional carbohydrates next to mucus, causes competition between bacterial species takes place leading to the production of antimicrobial products. This hypothesis is also supported by the SynCom OD_{600} measurements. The overnight fasting period, where the community was only supplemented with mucus without other complex carbohydrates, resulted in a drop in OD and qPCR analysis (see **Chapter 5**).

For both EPS production and pili production, the expression pattern of genes was stable in complexity and conditions. In mucus degradation, the genes with the highest and lowest relative abundance within this subset were similar between conditions. However, variation between genes in each condition was observed. This might be due to changes in medium or mucus batches, as well as changes in complexity. For example, the main difference observed between conditions was found in SynCom. In this condition the gene with the highest relative abundance in mucus degradation was Amuc_2136, a beta-N-acetylhexosaminidase belonging to GH20. In contrast, Amuc_0824, a beta-galactosidase belonging to GH2, was shown to have the highest relative abundance among mucus degradation genes in all other conditions. This indicates that in a more complex community, *A. muciniphila* prioritizes to cleave GlcNAc or GalNAc from mucin glycans over beta-D-galactose residues in low complexity experiments. Even though Amuc_0824 is part of the top four mucus degradation genes in SynCom, this shows a consistent difference between conditions. The reason for this difference may be the complexity of the community in SynCom as compared to the other conditions.

Despite small variations in relative abundances of genes involved in the key functions of *A. muciniphila*, this data shows that overall *A. muciniphila* similarly exerts the key functions in all conditions included in this study. This data indicates that the key functions of *A. muciniphila* remain stable throughout different ecosystems and independent of environmental changes as long as mucus is available. This specialistic and competitive niche occupation is indicative for its relative high abundance and occurrence within the mucosal layer.

Acknowledgements

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Material and methods

Studies and samples

We included RNA sequencing data from several studies with environmental and dietary differences (Table X). Each of the conditions included in the study consists of multiple replicates (Figure 6). In the first condition, *A. muciniphila* was cultivated in mono-cultivations and co-cultivations (with *B. thetaiotaomicron*) in continuous fermentors. The second condition is an in vivo mouse study where *A.* mice were colonized with *A. muciniphila* mono-colonization and co-colonization (with *B. thetaiotaomicron*). In the last condition, *A. muciniphila* was cultivated as part of a synthetic community with 14 other species in in continuous fermentors. From this experiment, two different conditions were included: fasting with mucus as the main carbon source and feeding with additional carbohydrates and mucus.

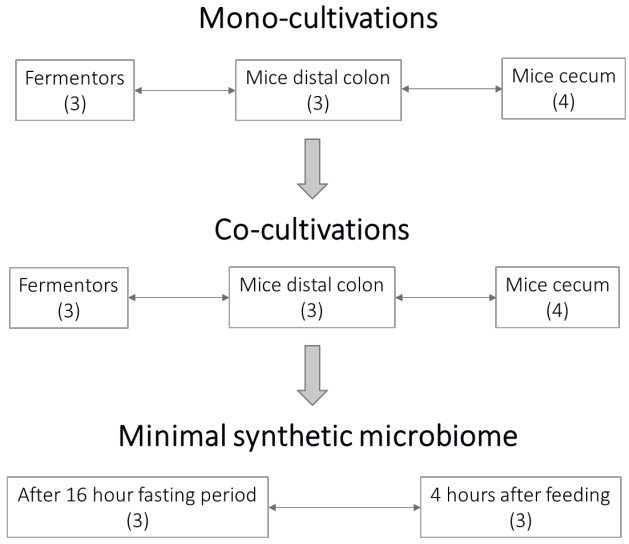


Figure 7: Conditions included in this study.

In vitro fermentation of *A. muciniphila* in mono- and co-culture

In vitro fermentations were conducted in three parallel bioreactors (DasGip, Eppendorf, Germany) filled with 250 ml of basal medium as it has been described before (ref for basal media) at 37ºC, at a controlled pH of 6.5 and at a stirring rate of 150 rpm. The bioreactors and the feed bottle were supplemented with 0.5% of crude mucin, 1% of

vitamin solution and at the beginning of the fermentation. Anaerobic conditions were succeeded by sparging the media with N_2/CO_2 continuously (6 sL/h). Experiments were performed with 1% (v/v) supplementation of $CaCl_2$ and vitamin mixture as described previously. The media in both feed and bioreactors were reduced with 0.05% L-Cysteine-HCl in order to achieve anaerobic conditions. The bioreactors were inoculated with a normalized O.D. of 1.0 of both species to achieve same starting cells density in the beginning of the fermentation. The flow rate of the feed was set at 20 ml/h and the recovery rate of media was 12.5 hours. The growth was measured by spectrophotometer as optical density at 600 nm. Both cultures were normalized to O.D. = 1.0 prior to bioreactors addition. The experiment was done in three biological replicates.

***In vivo* fermentation of *A. muciniphila* in mono- and co-culture**

All the mouse experiments were performed using protocols approved by the University of Gothenburg Animal Studies Committee. Female Swiss Webster germ free mice have been housed in experimental isolators during the colonization period. Mice were fasted 4h prior gavage (intragastrical) with 0.2 ml of active culture (for both mono- and co-colonizations). For *B. thetaiotaomicron* mono- (n=5 mice) and bi-colonization (n=7 mice) have used 10^8 CFU/ml. For *A. muciniphila* (n=8 mice) mono-colonization 10^9 CFU/ml have used. For all three colonization, mice were inoculated with a single gavage of the respective culture. During the experiment, the mice had free to autoclaved water and food. The mice received during the whole period of the colonization chow diet (5021 rodent diet, LabDiet; fat 9% wt/wt) (Supplementary Table X). Intestinal segments (ileum, cecum and distal colon) and faeces were harvested after14-days of colonization and immediately stored in RNA Later (Sigma) at -20ºC until further processed.

Synthetic community

The synthetic communities were cultivated in the following medium: KH_2PO_4 (0.408 g/L), $Na_2HPO_4.2H_2O$ (0.534 g\L), NH_4Cl (0.3 g/L), NaCl (0.3 g/L), $MgCl_2*6H_2O$ (0.1 g/L), $NaHCO_3$ (4 g/L), yeast extract (2 g/L), beef extract (2 g/L), CH_3COONa (2.46 g/L), casitone (2 g/L), peptone (2 g/L), cysteine-HCl (0.5 g/L), carbohydrates (1.1 g/L), resazurin (0.5 mg/L), 1 mL trace elements in acid (50 mM HCl, 1mM H_3BO_3, 0.5 mM $MnCl_2*4H_2O$, 7.5 mM $FeCl_2*4H_2O$, 0.5 mM $CoCl_2$, 0.1 mM $NiCl_2$, and 0.5 mM $ZnCl_2$, 0.1 mM $CuCl_2*2H_2O$), 1 mL trace elements in alkaline (10 mM NaOH, 0.1 mM

Na_2SeO_3, 0.1 mM Na_2WO_4, and 0.1 mM Na_2MoO_4), 1 mL haemin solution (50 mg haemin, 1 mL 1N NaOH, 99 mL dH_2O), 0.2 mL vitamin K1 solution (0.1 mL vitamin K1, 20 mL 95% EtOH). After autoclaving and before inoculation, 1% of vitamin solution was added (11 g/L $CaCl_2$, 20 mg biotin, 200 mg nicotinamide, 100 mg p-aminobenzoic acid, 200 mg thiamin (vitamin B1), 100 mg panthothenic acid, 500 mg pyridoxamine, 100 mg cyanocobalamin (vitamin B12), and 100 mg riboflavin). Initially the medium contained 0.5% crude mucin and 1.11 g/L of each xylan, soluble starch, inulin and pectin. The fermentors were spiked three times a day with a 4-hour gap using four carbohydrates (xylan, soluble starch, inulin and pectin) with an end concentration of 0.1% each. The feed consisted of this medium supplemented with 0.5% crude mucin. Each fermentor, three in total, was inoculated with 15 gut species: *Akkermansia muciniphila* (ATCC BAA-835), *Bacteroides ovatus* (HMP strain 3_8_47FAA), *Bacteroides xylanisolvens* (HMP strain 2_1_22), *Anaerobutyricum soehngenii* (DSM 1736), *Coprococcus catus* (ATCC 27761), *Flavonifactor plautii* (HMP strain 7_1_58FAA), *Eubacterium sireaum* (DSM 15702), *Agathobacter rectalis* (DSM 17629), *Roseburia intestinalis* (DSM 14610), *Faecalibacterium prausnitzii* (A2-165), *Subdoligranulum variabile* (DSM 15176), *Ruminococcus bromii* (ATCC 27255), *Blautia obeum* (DSM 25238), *Collinsella aerofaciens* (DSM 3979/ATCC 25986) and *Bifidobacterium adolescentis* (L2-32).

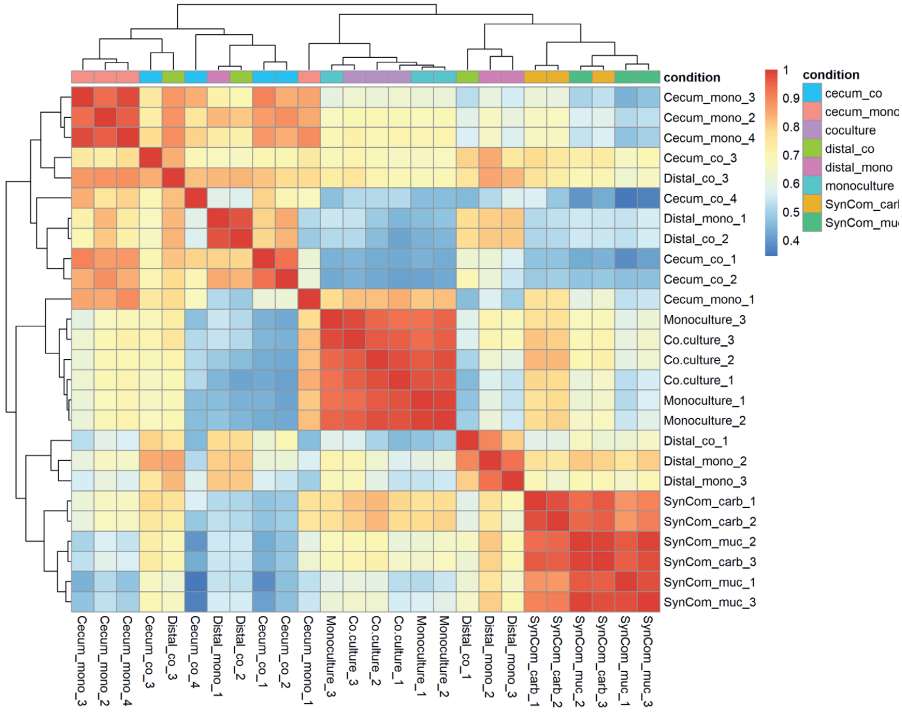
RNAseq analysis

Illumina reads have been trimmed for low quality and adapters with fastp (v0.20.0) (21) using default settings. rRNA sequences have been removed with bbduk (v38.79) (https://sourceforge.net/projects/bbmap/) using the following parameters k=31 and ref=riboKmers.fa.gz. Transcripts from the reference strain of *A. muciniphila* (GCF_000020225.1) have been quantified with RSEM (v1.3.1) (B. Li and Dewey 2011) in combination with bowtie2 (v2.3.5.1) (Langmead and Salzberg 2012). Mapping and read quality were inspecting using MultiQC. Tximport (v1.12.3) was used to import read-counts obtained from RSEM into Rstudio (Soneson, Love, and Robinson 2016). These imported reads were used for the differential expression analysis using DESeq2 package. Canoco5 (version 2.8.12) was used for RDA and PCA analysis based on the relative abundance data. All further analysis was done using R version 3.6.3 in Rstudio version 1.2.5019.

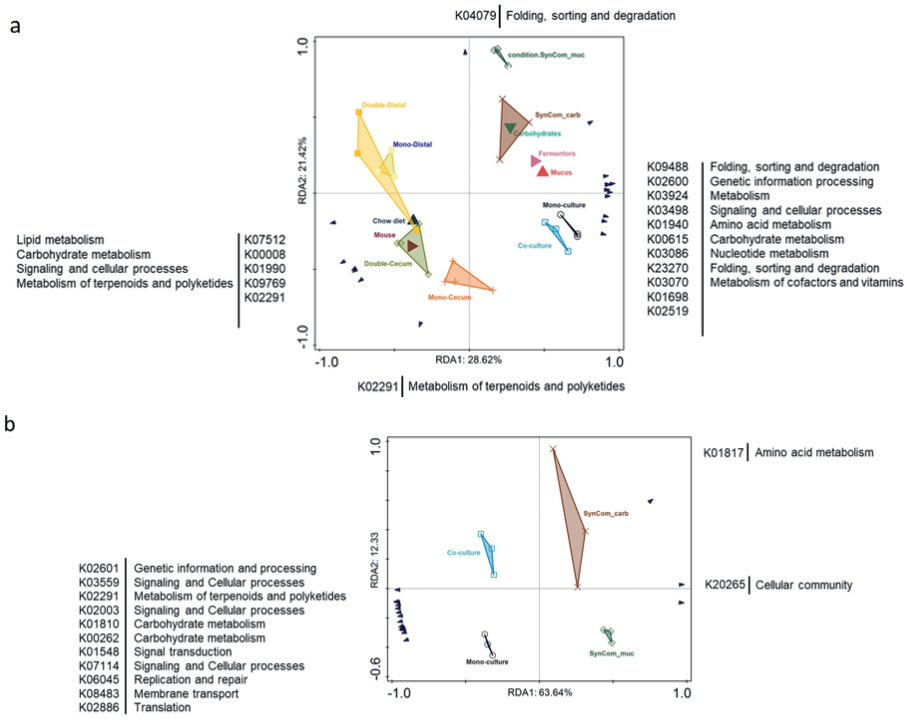
Table 3: Overview of the samples with additional ecosystem information.

Sample	Diet	Location	Condition
Monoculture_1	Mucus	Fermentors	Monoculture
Monoculture_2	Mucus	Fermentors	Monoculture
Monoculture_3	Mucus	Fermentors	Monoculture
Coculture_1	Mucus	Fermentors	Coculture
Coculture_2	Mucus	Fermentors	Coculture
Coculture_3	Mucus	Fermentors	Coculture
Cecum_mono_1	Chow diet	Mice	Cecum_mono
Cecum_mono_2	Chow diet	Mice	Cecum_mono
Cecum_mono_3	Chow diet	Mice	Cecum_mono
Cecum_mono_4	Chow diet	Mice	Cecum_mono
Cecum_co_1	Chow diet	Mice	Cecum_co
Cecum_co_2	Chow diet	Mice	Cecum_co
Cecum_co_3	Chow diet	Mice	Cecum_co
Cecum_co_4	Chow diet	Mice	Cecum_co
Distal_mono_1	Chow diet	Mice	Distal_mono
Distal_mono_2	Chow diet	Mice	Distal_mono
Distal_mono_3	Chow diet	Mice	Distal_mono
Distal_co_1	Chow diet	Mice	Distal_co
Distal_co_2	Chow diet	Mice	Distal_co
Distal_co_3	Chow diet	Mice	Distal_co
SynCom_muc_1	Mucus	Fermentors	SynCom_mucus
SynCom_muc_2	Mucus	Fermentors	SynCom_mucus
SynCom_muc_3	Mucus	Fermentors	SynCom_mucus
SynCom_carb_1	Carbohydrates and mucus	Fermentors	SynCom_carb
SynCom_carb_2	Carbohydrates and mucus	Fermentors	SynCom_carb
SynCom_carb_3	Carbohydrates and mucus	Fermentors	SynCom_carb

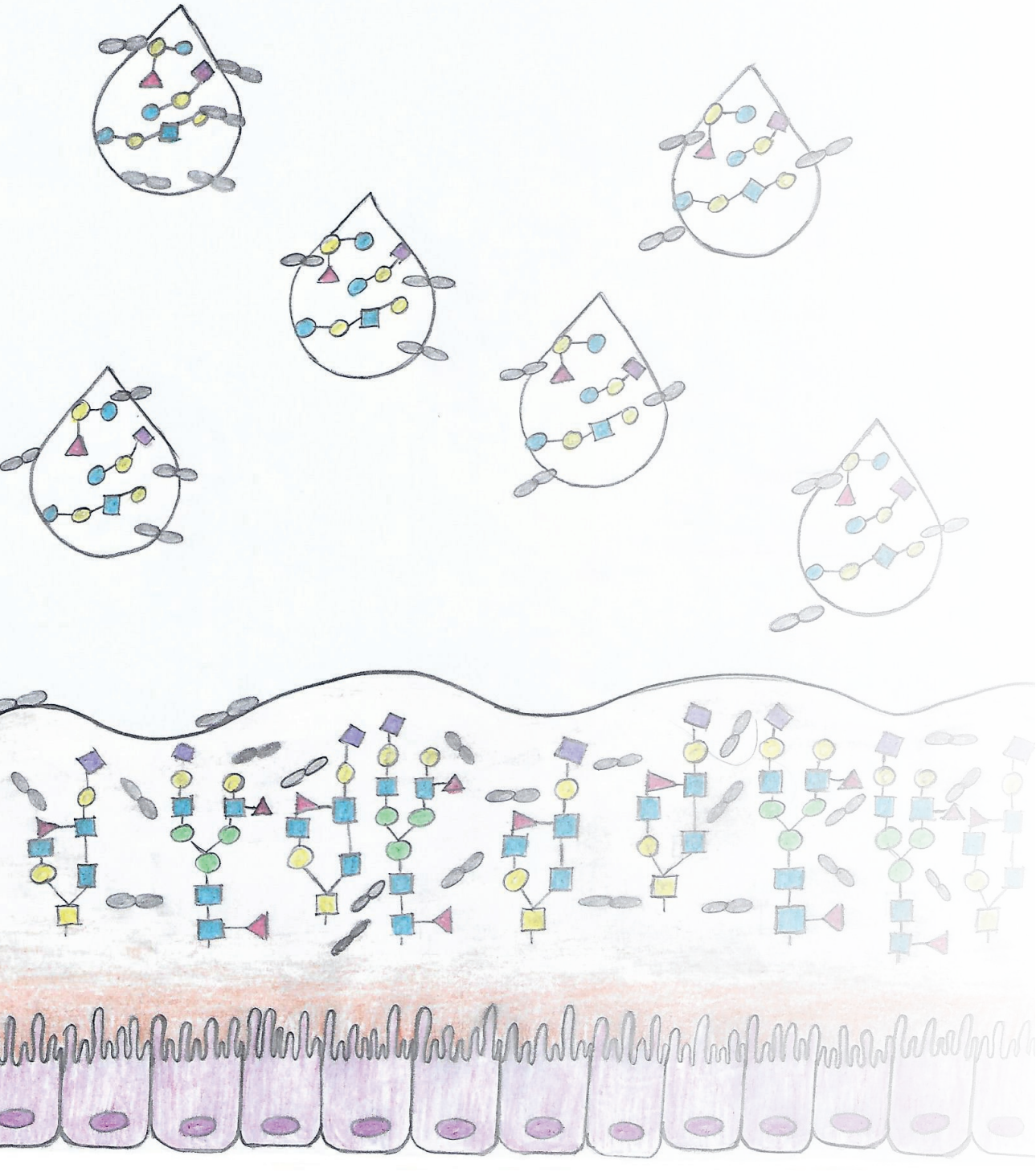
Supplementary data



Supplementary Figure 1: Heatmap of all *A. muciniphila* transcripts across the different ecosystem studies.



Supplementary Figure 2: Ordination analysis of the ecosystem studies and the most variable expressed KEGG Orthologies (KOs). a) RDA analysis showing the 20 most variable KOs within all studies involved in this study, b) RDA analysis showing the 20 most variable Kos within the *in vitro* studies. The plots were generated by Canoco.



Chapter 7

General Discussion

General discussion

This thesis describes detailed studies on the ability of *Akkermansia muciniphila* to survive in the complex and competitive environment of the human gut through its extensive capacity to utilize host-derived glycans. The aim of this thesis is to unravel the mechanisms that *A. muciniphila* uses to survive and thrive in this competitive environment using human milk and mucin glycans. The observations and findings of the research described in this thesis are discussed in the following sections.

Establishment of *A. muciniphila* in the gut

A. muciniphila is the only representative of the Verrucomicrobia phylum in the gut (Derrien et al. 2008). While *A. muciniphila* resides in the outer mucosal layer of the colon, sequences of *A. muciniphila* were found in different parts of the gastrointestinal tract. For instance, *A. muciniphila* was found in the oral cavity, the pancreas, the biliary system, the small intestine, and the appendix, where different type of mucin has been identified. **Chapter 2** highlights that the presence of *A. muciniphila* and *Akkermansia*-like spp. along the digestive tract implies that this bacterium might have more functions than those currently known.

A. muciniphila is also detected in human milk (**Chapter 2**) (Geerlings et al. 2018; Borewicz et al. 2019; Collado et al. 2012). It was long believed that human milk was sterile, but it is now recognized that it harbours a microbial community, the composition of which appears to change with maternal characteristics and throughout lactation (Butts et al. 2018). Although human milk and infant microbiome are different, their variations appear to be related, suggesting that milk is an important contributor to early GI tract colonization (Demmelmair et al. 2020). The human milk microbiome plays an important role in seeding the infant gut regardless its low overall biomass. It is been suggested that the process of breastfeeding is a potentially important mechanism for the propagation of breast milk microbes through the retrograde flow via skin contact between the infant's mouth and the areole (Kordy, 2020). A distinct strain of *Bifidobacterium breve* was identified in the maternal rectum, in breast milk and the infant's stool, which suggests a potential translocation of this anaerobic bacteria via the enteromammary pathway in humans, where maternal bacteria translocate across the maternal gut and are transferred to the mammary glands (Kordy et al. 2020). These observations could also indicate how *A. muciniphila* (whose origin is still unknown) is

established in the infant's gut. Therefore, *A. muciniphila* cells in human milk possibly pass to the infant via oral and areolar skin contact, transit the GI tract and reside on the mucosal layer. Furthermore, human milk was found to contain carbohydrate moieties, such as mucins, glycosaminoglycans, glycoproteins and HMOs (David S Newburg and Morelli 2015). Two gel-forming mucins, mucin 1 (MUC1) and mucin 4 (MUC4) have been identified and isolated from human milk (B. Liu et al. 2012; J. A. Peterson et al. 2001). These mucins are transmitted to the infant's gut and play an important role in the inhibition of pathogen adhesion and disease prevention (R. Peterson et al. 2013). The translocation of mucins from human milk to the infant's gut could also promote the initial colonization of mucin-degrading bacteria that are either vertically transmitted from human milk or acquired at the moment of birth from the maternal environment (Ottman et al. 2012). *A. muciniphila* was detected in infant faecal samples during the first month of life while its abundance increased over time through its ability to utilize glycans from the outer mucus layer of the gut epithelium (Collado et al. 2007). Hence, MUC1 and MUC4, which are present in human milk, could initially ensure *A. muciniphila*'s safe transit to the lower parts of the GI tract and consequently facilitate the colonization and growth of *A. muciniphila* in the infant's colon. This is especially the case during the first months of life when the infant's mucosal layer might not be fully developed or the mucus glycans expression is low. Torow et al. stated that the expression of mucins (MUC2, MUC3, and MUC5a) is reduced in the neonate, which results in a thinner mucus layer (Torow et al. 2017). On the other hand, a greater abundance of *A. muciniphila* was found in the gut of formula-fed infants (Bergström et al. 2014; Azad et al. 2013). However, this might be induced by a certain component in infant formula that can trigger *A. muciniphila* growth in the infant gut or the faster maturation of the infant mucosal layer. For example, infant formulas enriched with polyamines influenced microbial colonization patterns with a higher number of *Akkermansia*-like bacteria in neonatal mice (Gómez-Gallego et al. 2012). Additionally, supplementation of infant formulae with polyamines enriched the abundance of *A. muciniphila* in the large and the small intestine of neonatal mice (Gómez-Gallego et al. 2014). Therefore, the effect of infant formulae on *A. muciniphila*'s abundance compared to human milk is still unclear and more studies both *in vitro* and *in vivo* are needed to understand the impact of early nutrition on the colonization of *A. muciniphila* in the human's intestine.

The glycan degrading machinery of *A. muciniphila*'s enables break down of HMOs

Consumption of dietary fibre (i.e. glycans) has been shown to influence the gut microbiota by altering bacterial fermentation and species composition (Sawicki et al. 2017). The influx of glycans into the intestine comes mostly from diet and host secretions, such as mucins and HMOs (Bergström et al. 2014; Koropatkin, Cameron, and Martens 2012). *A. muciniphila* is adapted to mucin glycan utilisation by employing the glycoside hydrolases (GHs) encoded in its genome to cleave the glycosidic linkages present in mucin (Collado et al. 2007). However, the ability of *A. muciniphila* to use the same GHs to degrade other glycan sources, such as HMOs that share structural resemblances, has not been investigated (Figure 1). HMOs play a significant role in the development of the neonatal immune system by promoting healthy microbial diversity, preventing pathogen attachment, stimulating the maturation of the intestinal epithelial surface and modulating immune cells (Walsh et al. 2020). Degradation of HMOs by *A. muciniphila* would contribute to and enhance microbial network formation and a stable microbial colonisation of the mucosal layer supporting (later life) health. It has been speculated that *A. muciniphila* employs its mucin-degrading genes to degrade human HMOs, but experimental evidence was lacking (Ottman 2015; Korpela et al. 2018).

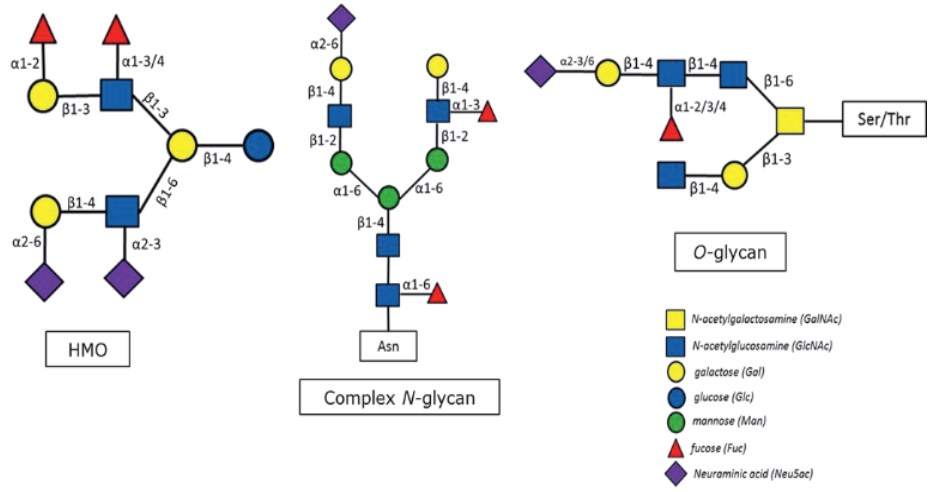


Figure 8: The structure of HMO and mucin show glycosidic similarities

A proof of concept study that addresses this hypothesis is described in **Chapter 3.** We monitored *A. muciniphila*'s growth in human milk and synthetic media that contains purified HMOs, for example, 2'-fucosyllactose (2'-FL) and 3'-siallylactose (3'-SL). *A. muciniphila* showed metabolic activity in human milk and in 2'-FL and 3'-SL. Furthermore, the growth of *A. muciniphila* on human milk revealed the utilization of a wide range of HMOs by *A. muciniphila*, for example, neutral trioses (2'-FL and 3-fucosyllactose [3-FL]), tetraoses (difucosyllactose [DFL], lacto-*N*-tetraose [LNT], lacto-*N*-tetraose [LNnT]), pentaoses (lacto-fucopentaose I [LNFP I], lacto-fucopentaose II [LNFP II], lacto-fucopentaose III [LNFP III], lacto-fucopentaose V [LNFP V]), and acidic trioses (3'-SL, 6'-sialyllactose [6'-SL]). Proteome analysis of *A. muciniphila* grown on human milk demonstrated, indeed, the expression of GHs capable of hydrolysis of glycosidic linkages (**Chapter 3**). For example, *A. muciniphila* expressed α-L-fucosidases (GH29, GH95), α-exo-sialidases (GH33), β-galactosidases (GH2), and β-hexosaminidases (GH20) when grown in human milk. Enzymatic assays showed fucosidase, galactosidase, and hexosaminidase activity of *A. muciniphila* GHs against pure HMOs (2'-FL, LNT) and other human milk constituents (lactose, Lacto-N-triaose [LNT2]). The utilization of HMOs by *A. muciniphila* led to the release of disaccharides and monosaccharides that may contribute to cross-feeding and network formation in the infant gut. Furthermore, the released sialic acid from the degradation of sialylated HMOs (3'-SL, 6'-SL) could serve as a substrate for community members such as *Bifidobacterium breve*, *Ruminococcus gnavus*, and *Bacteroides fragilis*. Lawson et al. recently showed that there is a growth enhancement of non-HMO degraders in the presence of a specific HMO-utilizer (Lawson et al. 2019). This implies a key role for *A. muciniphila* in the formation of a microbial network in the infant gut, which stimulates the growth of bacteria that can use sialyl-groups and, thus, increase microbial diversity in the gut. Furthermore, sialic acid has been characterised as an essential nutrient for brain and cognitive development in humans (B. Wang 2009).

Therefore, the results from **Chapter 3** suggest that *A. muciniphila* uses its mucin-degrading enzymes to utilize and degrade glycosidic linkages of a range of HMOs. In conclusion, *A. muciniphila* can utilise both mucin glycans and HMOs found in the gut, thus facilitating its initial colonization in the gut. Further research is required, however, to determine the role of *A. muciniphila* in the infant's gut colonization through

interactions between *A. muciniphila* and other early life colonizers, in other words, Bifidobacteria spp.

The mucolytic activity of *A. muciniphila* as a survival tool in complex and competitive ecosystems

Host-secreted glycans, such as mucins, are important for barrier integrity, contribute to immune functions, such as antigen sampling and tolerance, and, therefore, to human health (McDole et al. 2012). They are also needed for the establishment of the human gut microbiota (Cornick, Tawiah, and Chadee 2015). Some microbes that colonise the outer mucus layer utilise the mucins as an energy source and, therefore, promote the homeostasis of the intestine (Tailford, Crost, et al. 2015). *A. muciniphila* is a prominent mucus-colonizer that supports the host's health (Derrien et al. 2004; Derrien 2007; Cani and de Vos 2017; Plovier et al. 2017). However, the metabolic interactions within the mucus-residing bacterial community as well as within the overall intestinal microbiota community are difficult to decipher because of the complexity of such communities and the limitations in *in situ* sampling protocols. One way to monitor the metabolic interactions of bacterial communities on the mucosal surface is to assemble, *in vitro*, microbial communities often referred to as minimal microbiomes. Minimal microbiomes are generated to study the microbial interactions and explain the evolution of cooperation and competition between microbial members in the gut microbiome (D'hoe et al. 2018; Venturelli et al. 2018; Desai et al. 2016). One of our primary missions was to evaluate the trophic and metabolic interactions between *A. muciniphila* and other glycan-degrading bacteria and members of the core gut community. For a better understanding of *A. muciniphila*'s role in complex and competitive ecosystems, a co-culture of *A. muciniphila* with the glycan generalist, *B. thetaiotaomicron,* was created *in vitro* under controlled conditions and with limited available nutrients (mucin) (**Chapter 4**). We selected *B. thetaiotaomicron* due to its ability to utilize a wide range of diet- and host-derived glycans (Martens et al. 2011; Martens, Chiang, and Gordon 2008). *B. thetaiotaomicron* can also switch between diet- and host-derived glycans, depending on the availability of these nutrients (Desai et al. 2016; Sonnenburg 2005; Marcobal et al. 2011). The results presented in **chapter 4** suggest that *A. muciniphila* is not affected by environmental changes, such as competition for mucin glycans that were constantly flowing in the co-culture. Transcriptome analysis of *A. muciniphila* revealed that the key function of mucin degradation was expressed at similar levels

between mono- and co-culture with *B. thetaiotaomicron*. Additionally, the transcriptome analysis revealed upregulation of genes associated with LPS biosynthesis (Lipid A, *O*-antigen ligase) and ABC transporters, which might be used by *A. muciniphila* to overcome *B. thetaiotaomicron*'s antimicrobial proteins, such as membrane attack complex-perforin (MACPF). Many components of the bacterial cell envelope, including lipopolysaccharides (LPS), peptidoglycans and lipoteichoic acids, may be involved in triggering immune responses in the gut (Bron, Van Baarlen, and Kleerebezem 2012). Additionally, bacterial products can be involved in these processes, especially as lipopolysaccharides (LPS) and peptidoglycans stimulated mucus secretion and restored mucus properties in GF mice to a similar extent as in conventionally raised animals (Petersson et al. 2011). Hence, we hypothesize that *A. muciniphila* uses its mucin-degrading genes to survive the competition for limited nutrients and that it expresses different genes to sustain its abundance, which counteracts the expression of antimicrobial proteins from its competitors. Future studies should assess the ability of *A. muciniphila* to respond to different antimicrobial proteins and bacteriocins in highly competitive environments, such as the human intestine.

The ability of *A. muciniphila* to survive in complex and competitive ecosystems via mucin-utilization pathways was also confirmed in a mucus- and diet-based minimal microbiome (MDb-MM) where glycan-degrading and core human bacteria from adults with known metabolic features were included (**Chapter 5**). The members of the community were continuously fed with mucin and were given four different carbohydrates (starch, pectin, inulin and xylan) three times per day. *A. muciniphila* was identified as one of the two most abundant species in the community (Figure 2) contributing to two trophic guilds: degradation of complex substrates and degradation of simpler carbohydrates (**Chapter 5** Figure 6). Additionally, transcriptome analysis showed that *A. muciniphila* expressed its glycolytic genes exclusively for mucin degradation. The presence of mucin glycans in complex environments could promote and ensure *A. muciniphila*'s survival *in vitro*. However, more research is needed both *in vitro* and *in vivo* to fully elucidate and understand *A. muciniphila*'s interactions with other members of the human intestinal microbiota.

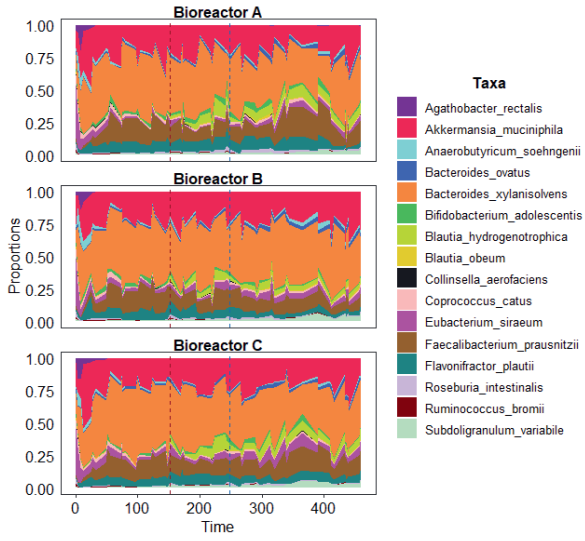


Figure 2: The relative abundances of the different species in MDb-MM (Chapter 5) in all technical replicates (Bioreactors A,B,C). The red dashed line indicates the time point (152 hours) where *Blautia hydrogenotrophica* introduced in the community and the blue dashed line indicates the removal of acetate from the feed (248 h).

In **chapter 4**, we also monitored the host effect of co-colonization of germ-free mice with *A. muciniphila* and *B. thetaiotaomicron*. The presence of dietary fibre (starch, chow diet) in the mouse gut suggests the generation of different ecological niches in the rodent gut, where *B. thetaiotaomicron* uses its glycoside hydrolytic machinery for starch utilization, while *A. muciniphila* focuses only on mucin degradation. Comparison of the transcriptional response of *A. muciniphila* of the abovementioned experiments in **chapters 4** and **5** revealed similar expression of the key functions of *A. muciniphila* such as mucin degradation, LPS/EPS biosynthesis and expression of the pili-like encoding genes (**Chapter 6**). The results in **chapter 6** show that *A. muciniphila* performs the same functions both *in vitro* and *in vivo* independently of the environmental changes and the complexity and competitiveness of the ecosystem. The consistency in expression of *A. muciniphila*'s key functions *in vitro* and *in vivo* indicates how robust and resilient this commensal bacterium is in a mucin-rich environment. On the other hand, our findings suggest that *A. muciniphila* will not dynamically regulate gene expression in different circumstances, for example, the absence of mucin glycans. Therefore, the question that arises is what will *A. muciniphila*'s function and response be to a complex community where there is no or limited access to the nutrients needed for its growth. We expect that our *in vitro*

experimental design used in this thesis will help to investigate such interactions and improve our understanding of *A. muciniphila's* behaviour in the human intestine.

***A. muciniphila*'s contribution to a healthy intestine from early life**

A. muciniphila is known for the health benefits that it offers the host through its degradation of mucin glycans. *A. muciniphila* has been proposed as a candidate for a next-generation probiotic and therapeutic applications that support the host's metabolic health (Van der Ark 2018; Plovier et al. 2017; Cani and de Vos 2017). In this thesis, we demonstrated that *A. muciniphila* uses human milk as an energy source and degrades human milk oligosaccharides. The growth in human milk resulted in the production of metabolites (SCFAs) as well as di- and monosaccharides that could potentially cross-feed other species that are important for health (**Chapter 3**). For instance, *A. muciniphila* released sialic acid from the degradation of sialylated HMOs (3'-SL, 6'-SL) that could serve as a substrate for community members, such as *B. breve*, *R. gnavus*, and *B. fragilis* (H. Nishiyama et al. 2018; Crost et al. 2016; Brigham et al. 2009). Furthermore, it is well-known that SCFAs have key roles in regulating host metabolism (Koh et al. 2016). SCFAs are transported into the systemic circulation and may directly affect host metabolism via binding to G-protein-coupled receptors (GPR) (Canfora, Jocken, and Blaak 2015). Mouse organoids were used to investigate the effect of *A. muciniphila* metabolites (acetate and propionate) and products on host epithelium (Lukovac et al. 2014). *A. muciniphila*'s metabolites modulated the expression of Fiaf, Gpr43, histone deacetylases (HDAC) and Pparγ, which suggests the regulation of host transcriptional response via histone acetylation modifications (Lukovac et al. 2014; Ottman 2015). Furthermore, SCFAs are an important energy source for the intestinal epithelial cells and strengthen the gut barrier function (Makki et al. 2018). Improved gut barrier function reduces the penetration of microbes and microbial molecules into the blood circulatory system, thereby reducing the immune responses associated with metabolic diseases (Knudsen et al. 2018). *A. muciniphila* is associated with the maintenance of GI tract homeostasis and gut-barrier integrity (J. Shin et al. 2019). Moreover, *A. muciniphila* can prevent the age-related decline of the colonic mucus layer (Van Der Lugt et al. 2019). *A. muciniphila* grown on human milk expressed an outer membrane pili-like protein (Amuc_1100) that plays an important role in immune regulation and enhancement of trans-epithelial resistance. It has also been demonstrated that Amuc_0010 can improve gut barrier integrity and reduce high-fat-diet-induced

obesity in mice (Plovier et al. 2017; Ottman et al. 2016; Paone and Cani 2020). *A. muciniphila*'s abundance was inversely correlated with obese and type 2 diabetic subjects (Dao, Everard, Aron-Wisnewsky, et al. 2016; Cani 2018). In a proof-of-concept pilot human intervention in subjects with metabolic disease, *A. muciniphila* improved insulin sensitivity, reduced inflammation, and reduced plasma LPS levels, which suggests a reinforcement of the gut barrier (Depommier et al. 2019). *A. muciniphila*'s ability to thrive and be metabolically active in the intestine from early life creates new opportunities to explore the importance and effect of this bacterium at different stages of life and, consequently, its contribution to human health.

Enrichment of *A. muciniphila* in the gut through a synbiotic diet

Probiotics and prebiotics are crucial for maintaining the balance of human intestinal microbiota, exerting at the same time a positive effect on the host's health (Markowiak and Ślizewska 2017). Probiotic products may contain one or more selected microbial strains. Probiotics that are available for human consumption predominantly include strains from different species found in the intestine of healthy individuals, such as *Lactobacillus*, *Bifidobacterium*, *Lactococcus*, as well as *Streptococcus* and *Enterococcus* (Markowiak and Ślizewska 2017). However, non-conventional strains are qualified for next-generation probiotic candidates, *viz. A. muciniphila*, *Faecalibacterium prausnitzii*, *Bacteroides fragilis*. *A. muciniphila* falls in this category due to the beneficial effects that it exerts on the host (Plovier et al. 2017; Cani and de Vos 2017), which are summarized in **Chapter 1**. In this thesis, we defined, for the first time, the ability of *A. muciniphila* to utilize HMOs (**Chapter 3**) that often function as natural prebiotics, providing selective substrates for gut bacteria (Bode 2009; Moossavi et al. 2018; Marcobal and Sonnenburg 2012). Prebiotics are defined as the substrates that are selectively utilised by microorganisms to confer a health benefit (Gibson et al. 2017). The most promising prebiotic substances that are mostly plant- or dairy derived, include galactans (i.e. galacto-oligosaccharides (GOS) synthesised from lactose) and fructans (i.e. fructo-oligosaccharides (FOS)), isomaltooligosacccharides (IMO), xylooligosaccharides (XOS), transgalactooligosaccharides (TOS) as well as inulin derived from chicory root (Markowiak and Ślizewska 2017; Oozeer et al. 2013; Roberfroid, Van Loo, and Gibson 1998). Often, the combination of probiotics and prebiotics (synbiotics) has been reported to have greater efficacy than either probiotic or prebiotic use alone (Mohanty, 2018). Synbiotics are defined as "a mixture

comprising live microorganisms and substrate(s) selectively utilized by host microorganisms that confers a health benefit on the host" (Swanson et al. 2020). Synbiotics aim to enhance the survival and activity of proven probiotics *in vivo* as well as stimulate indigenous beneficial bacteria (Tuohy, 2003). Various combinations of synbiotics show therapeutic effects against diseases, such as gastrointestinal diseases, respiratory infections, hypercholesterolemia, atopic dermatitis, allergy, diabetes, liver diseases and cancer (Cazzola et al. 2010; Dinleyici et al. 2013; Ogawa et al. 2006; Ooi et al. 2010; Passeron et al. 2006; Rayes et al. 2002). Supplementation with HMOs or prebiotics stimulates and promotes the growth of beneficial bacteria in the infant gut, such as Bifidobacteria. For example, a synbiotic diet (scGOS/lcFOS with *Bifidobacterium breve* M-16 V) in mice demonstrated protection against Western Style Diet (WSD)-induced excessive fat accumulation throughout life, as well as ameliorated adult insulin sensitivity and dislipidaemia (Mischke et al. 2018). The same study also mentioned that synbiotics increased the abundance of *Bifidobacterium* in early life and adulthood. Currently, the supplementation of infant formulae with pure HMOs (2'-FL, LNnT) or prebiotics (GOS and/or FOS) is a well-known practice to mimic, as far as possible, the composition of human milk and infant nutrition (Vandenplas et al. 2018). Hence, administration of *A. muciniphila* in combination with prebiotics such as HMOs (2'-FL) or GOS/FOS may be beneficial to human health from an early stage. For example, children who have autism spectrum disorder (ASD) appeared to have gut microbiota dysbiosis with lower levels of Bifidobacteriales and *Bifidobacterium longum* (Ying Wang et al. 2020), and *A. muciniphila* (L. Wang et al. 2011). Moreover, children with autism who are administered a synbiotic diet with FOS and probiotics demonstrated increased levels of beneficial bacteria and supressed pathogenic bacteria (*Clostridium*), which is accompanied by a significant reduction in the severity of autism and gastrointestinal symptoms. To this end, a synbiotic diet in ASD children with *A. muciniphila* and HMOs or prebiotics could increase the abundance of *A. muciniphila* that confer health benefits to the host and, at the same time, help to restore the Bifidobacterial levels and permit colonization of the mucosal layer. *A. muciniphila*'s administration in overweight/obese insulin resistant individuals showed improvement in several metabolic parameters (Depommier et al. 2019). Moreover, it has been shown that the administration of FOS promoted the growth of *A. muciniphila* in the gut of DIO obese mice and Sprague-Dawley rats (Everard et al. 2013, 2011; Reid et al. 2016). Additionally, the addition of GOS/FOS mixture to mice revealed an increase in the

relative abundance of *A. muciniphila* (Burokas et al. 2017). Therefore, a combination of prebiotic substrates (FOS or GOS/FOS) with *A. muciniphila* could be beneficial to patients with metabolic diseases such as type 2 diabetes and obesity, where *A. muciniphila* was found to be less abundant then in healthy individuals (Karlsson et al. 2012; Collado et al. 2010; N. R. Shin et al. 2014). The delivery of *A. muciniphila* in the gut of individuals associated with metabolic diseases would provide beneficial effects to the host, while the prebiotics would provide enough nutrients to *A. muciniphila* to preserve its abundance in the gut. However, no *in vitro* studies have shown that *A. muciniphila* can utilize substrates such as FOS and/or GOS. In this thesis, we provide data that *A. muciniphila* is no longer specific only to mucin glycans, but it is also present in HMOs. Thus, *A. muciniphila* could also use its mucin-degrading enzymes to degrade those substrates.

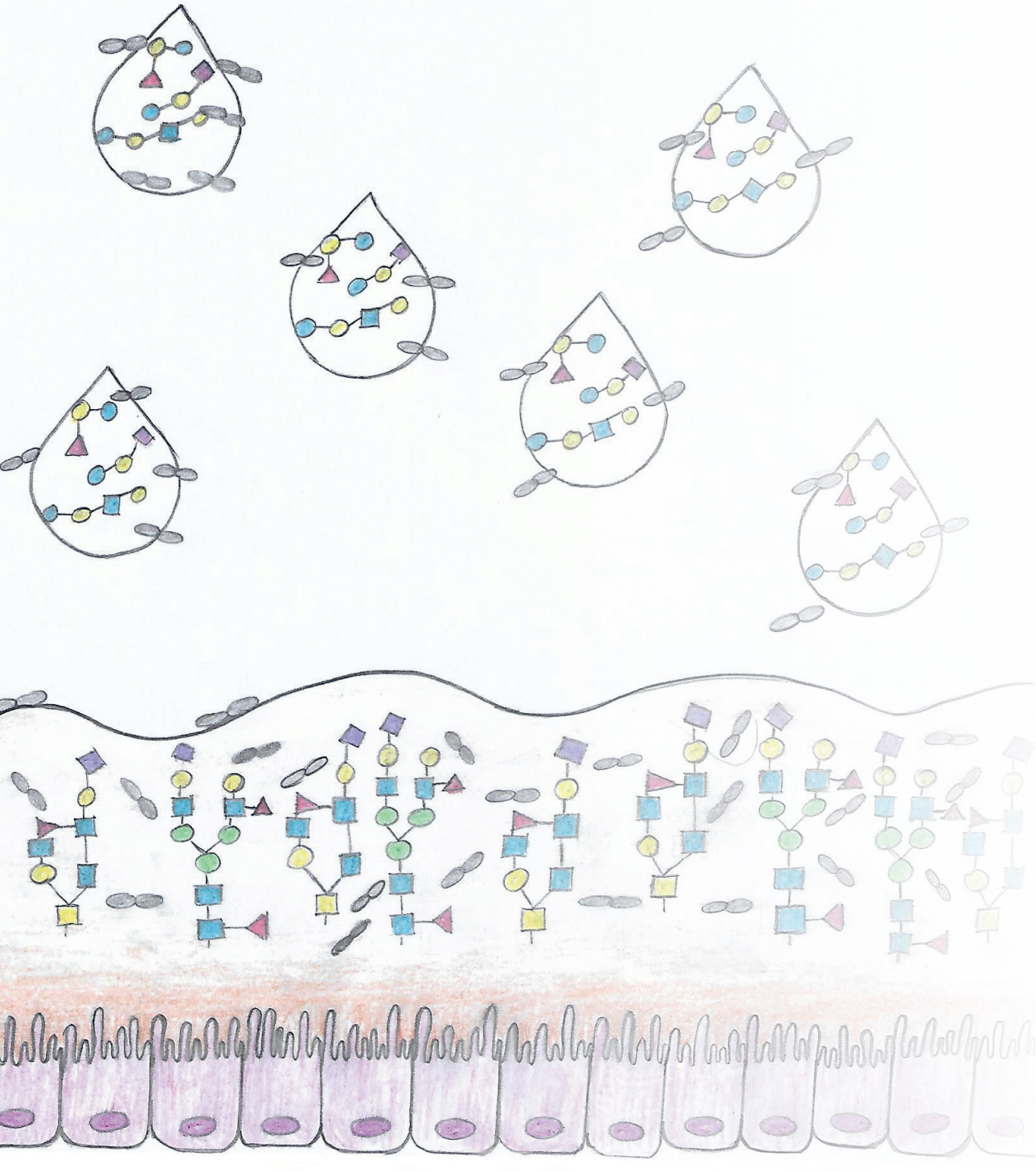
Concluding remarks and future perspectives of *A. muciniphila*

A. muciniphila has been inversely correlated with obesity, metabolic diseases (Type 2 diabetes) as well as intestinal disorders (IBD and appendicitis) (Dao, Everard, Aron-Wisnewsky, et al. 2016; Karlsson et al. 2012; X. Zhang et al. 2013; Png et al. 2010; Rajilić-Stojanović et al. 2013; Swidsinski et al. 2011). *A. muciniphila*'s ability to utilize and degrade host-secreted glycans was comprehensively described after its first isolation (Derrien et al. 2004). In this thesis, the substrate utilization capacities concerning the host-derived glycans, mucin and HMOs as a survival tool in complex and competitive environments was investigated.

A. muciniphila degrades and utilizes mucin glycans as the sole carbon and nitrogen source (Derrien et al. 2004). This mucolytic capacity makes *A. muciniphila* one of the key species in the gut (Ottman, Geerlings, et al. 2017). In this thesis, we demonstrated that physiological responses of *A. muciniphila* are not affected significantly by changes in the environmental conditions, provided mucin glycans are accessible to *A. muciniphila*, exhibiting a similar expression of its key functions in complex microbial communities. Moreover, we suggest that *A. muciniphila* can counteract any effort to reduce its fitness in a co-culture, implying a colonization advantage in the mucus layer of the intestine. This thesis demonstrates that *A. muciniphila* can exploit HMOs, which are the main source of nutrients available in the infant gut. As such, this might provide beneficial effects during the initial early-life colonisation of *A. muciniphila* before it reaches its natural niche, the outer mucosal layer.

Future research should focus on determining the mechanistic role and microbe-microbe interactions of *A. muciniphila* in early life to fully understand its effect on gut health and how the colonization is shaped in early and later life. This can be achieved by assembling a minimal microbiome *in vitro*, where *A. muciniphila* as a member of this community is assessed for its ability to access and utilize HMOs that are provided to the community and how it influences overall ecosystem behaviour. Furthermore, based on the findings of the *in vitro* study, *in vivo* neonate mice and/or human studies would help to elucidate the interaction between *A. muciniphila* and the host and how *A. muciniphila* may stimulate the immune response from early life. Ultimately, the holistic characterization of *A. muciniphila*'s glycolytic machinery will provide more evidence on *A. muciniphila*'s potential in glycan utilisation in the gut, including other dietary fibre besides mucins.

In conclusion, this thesis explores the ability of *A. muciniphila* to exploit host-derived glycan to facilitate its survival in the complex gut ecosystem. The outcomes described in this thesis can be further utilized to interpret future studies to approach and elucidate in detail the cross-talk between *A. muciniphila* and other commensal bacteria that reside close to the mucus layer and the host, and thus gain more knowledge on microbial communities and their contribution to human health.



Thesis Summary

Thesis Summary

The research in this thesis aims to understand the mechanism used by *Akkermansia muciniphila* to survive and thrive in the complex and competitive ecosystems of the human gut. We evaluated the ability of *A. muciniphila* to utilize and degrade HMOs and mucin glycans as a carbohydrate specialist and the advantage of this phenotype in this highly competitive environment. As it has been demonstrated, *A. muciniphila* can have an important role in human (metabolic) health and it is essential to understand the functions and resilience of *A. muciniphila* in such communities. This can further expand our knowledge on the benefits that *A. muciniphila* can bring for human health, but potentially also help to design (nutritional) therapies to support *A. muciniphila's* activity in the gut to even further support human health.

In **Chapter 1**, a brief overview of the human intestinal microbiota is provided, focusing on the host-derived glycans that affect the physiology and composition of the gut microbiota. Furthermore, *A. muciniphila*, a resident of the mucosal layer in the gut, is introduced. The capacity of *A. muciniphila* to use glycoside hydrolases (GHs) to degrade glycosidic linkages found in mucin and human milk oligosaccharides (HMOs) constituted the motivation for the research described in this thesis.

Chapter 2 provides an overview on the presence and functionality of *A. muciniphila* in different parts of the gastrointestinal tract. *Akkermansia muciniphila* sequences have been found in human milk, the oral cavity, the pancreas, the biliary system, the small intestine, and the appendix for example. Hypothetical functions of *A. muciniphila* in these different niches are proposed, highlighting also that further research is needed to fully understand the versatile roles that *A. muciniphila* may have in the digestive tract.

The ability of *A. muciniphila* as a mucus associated micro-organism, to also be able to expand its glycolytic repertoire to human milk oligosaccharides (HMOs) is presented in **Chapter 3**. We hypothesized that the presence of *A. muciniphila* in infant's intestine is the result of its ability to use its glycan degrading enzymes to break down HMOs, whose glycosidic structure resembles mucin's structures. After growth of *A. muciniphila* in human milk, we identified HMOs being utilized by *A. muciniphila* due to the expression of GHs such as α-L-fucosidases, exo-α-sialidases, β-galactosidases, and β-hexosaminidases. We confirmed our hypothesis by testing heterologously expressed and purified GHs against the degradation of pure HMOs (2'-FL and LNT)

and human milk derivatives (lactose, LNT2). Based on the experimental data, we proposed a model for the utilization of 2'-FL, 3'-SL, LNT, LNT2, and lactose by *A. muciniphila*. The ability of *A. muciniphila* to degrade HMOs leads to the release of metabolites, like mono- and disaccharides, which contribute to cross-feeding pathways in the gut, and providing nutrients to the resident bacteria. These findings explain how *A. muciniphila* is able to colonize the gut in early life.

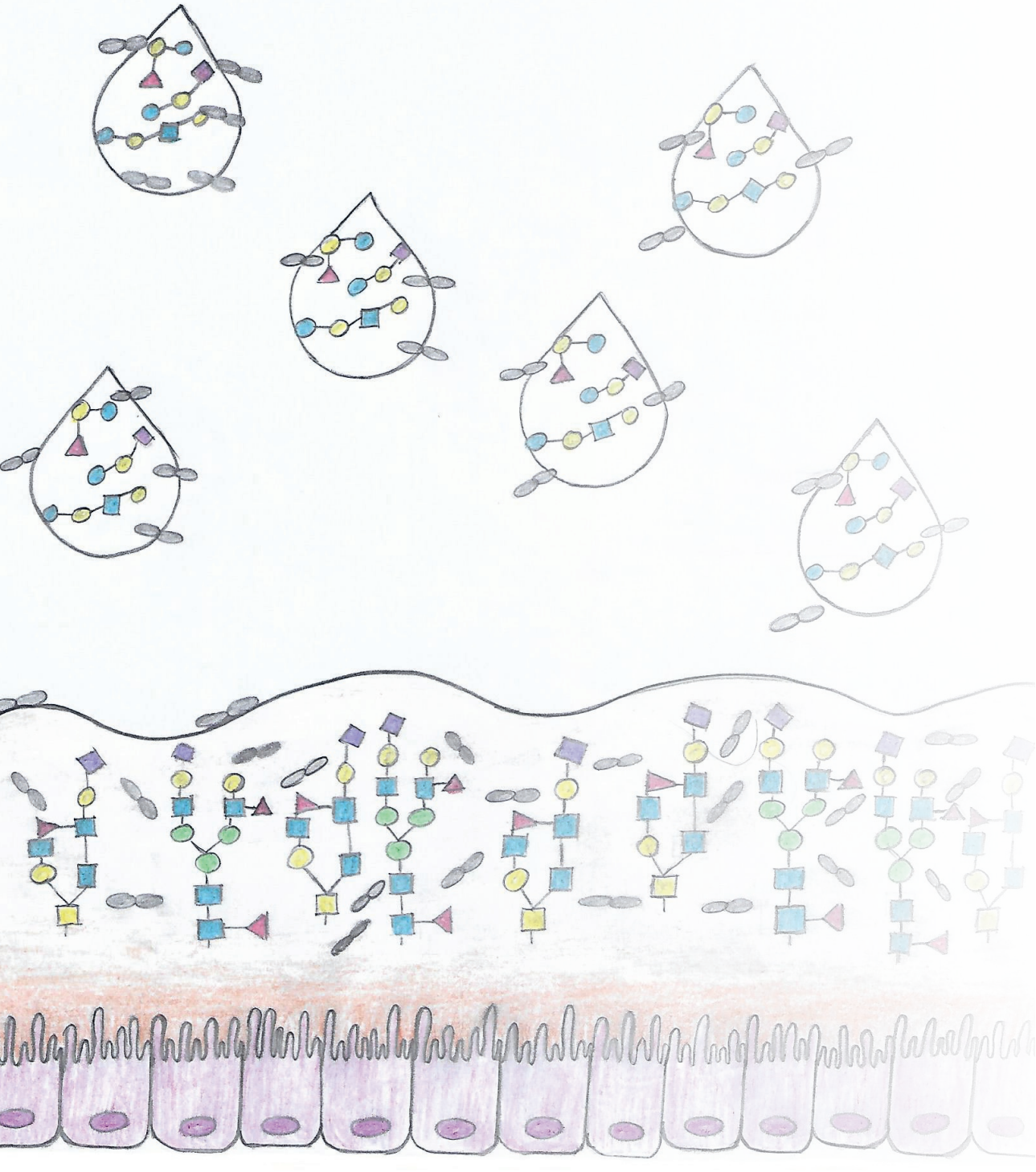
Chapter 4 describes the microbe to microbe interaction of *A. muciniphila* with another mucin-degrading bacterium, *Bacteroides thetaiotaomicron*, a more generalist micro-organism, under continuous influx of mucin glycans *in vitro*. We observed that *A. muciniphila* exhibited little or no alterations in its gene expression profiles for exploitation of mucin glycans, while *B. thetaiotaomicron* showed a trend for increased expression of its GHs coding gene to utilize the available nutrients in the co-culture. We also observed that *B. thetaiotaomicron* tried to gain competitive advantage by expressing genes coding for antimicrobial proteins/peptides. We hypothesize that *A. muciniphila* is able to counteract this by expressing LPS associated biosynthesis genes and ABC transporters that make the organism resistant against antimicrobial proteins. Finally colonizing germ-free mice with the *A. muciniphila* and *B. thetaiotaomicron* showed no significant differences in the gene expression of both species between mono- and co-colonization mice. We explain this by the fact that the high fiber diet used in mice, might sustain two different ecological niches in the rodent gut, and that the micro-organisms do not compete for substrate nor space. Microbe-microbe interactions in complex ecosystems as the human intestinal microbiome are difficult to elucidate, but these simplified *in vivo* models may offer great opportunities for the future. Another approach may also be the use of *in vitro* synthetic communities with a minimal microbiome.

In **Chapter 5**, we describe the assembly of a synthetic community in controlled bioreactors of 16 different species including *A. muciniphila* in order to monitor the trophic and metabolic interactions between mucin degrading species and species that were part of the core microbiota. The members of the minimal microbiome were continuously fed with mucin, while three times per day, dietary fibers (pectin, starch, inulin, and xylan) were fed into the bioreactors. We identified the establishment of four different trophic guilds in the minimal microbiome driven by the available nutrients in the community. For example, *A. muciniphila* and *Bacteroides xylanisolvens* were the

most abundant species in the community, contributing to two trophic guilds; the degradation of complex substrates (mucin) and degradation of simpler carbohydrates.

In **Chapter 6** a comparison of the transcriptional response of *A. muciniphila* between different experiments of chapter 4 and 5 is described. We studied the transcriptional landscape of *A. muciniphila* under varying conditions such as complexity of the community, introduction of carbohydrates, media composition, and experimental design (*in vitro* vs. *in vivo*). Special focus was given to key functions of *A. muciniphila*, e.g. mucin degradation, EPS production and pili associated genes expression. We found that *A. muciniphila* was able to perform these key functions both *in vitro* and *in vivo*, independently of the environmental changes, the complexity of culture and media composition. The consistency in expression of key functions from *A. muciniphila* might indicate how robust and resilient *A. muciniphila* is in an environment rich in mucin glycans.

Finally, in **Chapter 7**, all the research findings both *in vitro* and *in vivo* of this thesis are discussed, in detail highlighting the ability of *A. muciniphila* to thrive in complex environments as the human intestine via degradation of host-secreted glycans. With the research described in this thesis, we have attempted to unravel the metabolic capabilities of *A. muciniphila* and important microbe-microbe interactions of this unique species in complex environments. We described the capability of *A. muciniphila* to utilize and degrade HMOs via glycan-utilizing pathways that are also essential for growth in the mucus environment in the gut. The trait of being able to utilize HMO's also indicates the important role that *A. muciniphila* may play in early life colonization. Also, some suggestions are provided for future research activities concerning the ecophysiology of *A. muciniphila*'s. Finally, potential applications are suggested for the future application of *A. muciniphila* as a probiotic strain either alone or in a combination with HMOs or other prebiotics (synbiotic mixture) to support human health.



Nederlandse Samenvatting

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Dit proefschrift legt de focus op het onderzoeken van de mechanismen die *Akkermansia muciniphila* helpen om te overleven en zelfs te floreren in het complexe en competitieve ecosysteem van de menselijke darm. Wij hebben gekeken naar de mogelijkheid voor *A. muciniphila* om HMO's (Human Milk Oligosaccharides – Oligosachariden die aanwezig zijn in moedermelk) en mucines af te breken en voor zichzelf te gebruiken als specialist hierin en hoe dit een voordeel kan opleveren in een zeer competitieve omgeving. In eerdere studies is al aangetoond dat *A. muciniphila* een belangrijke rol kan hebben in de (metabole) gezondheid van de mens en om erachter te komen waarom dit is, is het van belang om uit te zoeken welke functies een rol spelen in de interactie en de weerbaarheid van *A.muciniphila* in zijn microbiële gemeenschap. Hierdoor weten we straks beter welke voordelen *A.muciniphila* kan hebben op de gezondheid van de mens, maar kan het ons ook helpen met het ontwikkelen van (voedings-) therapieën die de activiteit van *A. muciniphila* bevorderen in de darm om zo de gezondheid van mensen te verbeteren.

Hoofdstuk 1, hier is een kort overzicht te vinden over de darm microbiota, waar voornamelijk de focus ligt op de 'host-derived glycans'(Suikers afgegeven door de gastheer) die een effect hebben op de fysiologie en de samenstelling van de darm microbiota. Daarnaast wordt in dit hoofdstuk *A. muciniphila* geïntroduceerd die hier mogelijk een belangrijke rol in speelt. *A. muciniphila* heeft 'glycoside hydrolases', dit zijn enzymen die de ketens tussen suikers afbreken en die te vinden zijn in mucine en HMO's. Dit aantonen is de belangrijkste drijfveer voor het onderzoek in dit proefschrift.

Hoofdstuk 2 geeft een overzicht van de aanwezigheid en functionaliteit van *A.muciniphila* in verschillende delen van het maagdarmkanaal. Er zijn bijvoorbeeld 16S sequenties van *A. muciniphila* gevonden in de moedermelk, mondholte, alvleesklier, galwegen, dunne darm en de blinde darm. Er zijn al meerdere functionele hypotheses voorgesteld over waarom *A. muciniphila* te vinden is in deze niches van het maagdarmkanaal. Deze studies geven ook aan dat er nog wel meer onderzoek nodig is om volledig te begrijpen welke rol *A. muciniphila* heeft in het maagdarmkanaal.

Het onderwerp over *A.muciniphila* die mogelijk in staat is om zijn glycolytic repertoire te gebruiken op HMOs wordt gepresenteerd in **hoofdstuk 3**. Onze hypothese is dat de

aanwezigheid van *A.muciniphila* in de darmen van kinderen mogelijk gemaakt wordt omdat *A. muciniphila* in staat is om de HMO's met suiker afbrekende enzymen af te breken en te gebruiken. De reden hiervoor is dat de structuur vergelijkbaar is met die van mucine waarvan we al weten dat *A.muciniphila* deze structuur kan afbreken. Na incubatie van *A. muciniphila* op moedermelk vonden we dat *A. muciniphila* in staat was om bepaalde HMO's te gebruiken en dat *A. muciniphila* de volgende 'Glycoside Hydrolases' tot expressie bracht, dit waren α-L-fucosidases, exo-α-sialidases, β-galactosidases en β-hexosaminidases. Deze hypothese hebben we bevestigd door het heterologe tot expressie brengen van GH's (Glycoside Hydrolase) op te zuiveren en te testen of ze in staat waren om ketens te breken in zuivere HMO's (2'-FL en LNT) en moedermelk derivaten (lactose, LNT2). Gebaseerd op experimentele data hebben we een model voorgesteld die het gebruik van 2'-FL,3'SL, LNT, LNT2 en lactose door *A.muciniphila* weergeeft. Het vermogen van *A. muciniphila* om HMO's af te breken kan leiden tot het vrijmaken van andere metabolieten zoals mono- en disachariden. Deze vrijkomende metabolieten kunnen het uitwisselen van voeding stimuleren, waar andere micro-organismen die in de buurt zijn weer baat bij hebben. Deze bevindingen verklaren hoe *A.muciniphila* in staat is om de darmen van jonge kinderen te koloniseren.

Hoofdstuk 4 beschrijft de microbe naar microbe interactie van *A.muciniphila* met een andere mucine afbrekende bacterie, *Bacteroides thetaiotaomicron*, die bekend staat als een algemene suiker afbrekende bacterie, samengebracht in bioreactor met een continue stroom van mucine suikers. Hier zagen we bij *A.muciniphila* weinig verschil in expressie van genen gerelateerd aan het afbreken van mucine, terwijl we bij *B. thetaiotaomicron* een trend in toename van expressie zagen van genen die gerelateerd zijn aan GH's voor het afbreken van de aanwezige voedingstoffen in de co-cultuur. Daarnaast zagen we ook dat *B. thetaiotaomicron* probeerde om de competitie uit te schakelen door het tot expressie brengen van genen voor antimicrobiële eiwitten/peptiden. Onze hypothese is dat *A.muciniphila* deze aanval countert door de expressie van LPS geassocieerde biosynthese genen en ABC transporteiwitten die het organisme resistent maakt tegen antimicrobiële eiwitten. Als laatste vonden we bij de 'germ-free' muizen gekoloniseerd met *A. muciniphila* en *B. thetaiotaomicron* geen significante verschillen in expressie tussen de mono en de co-kolonisatie. De reden hiervoor is waarschijnlijk het vezelrijke dieet van de muizen, wat ervoor zorgt dat de

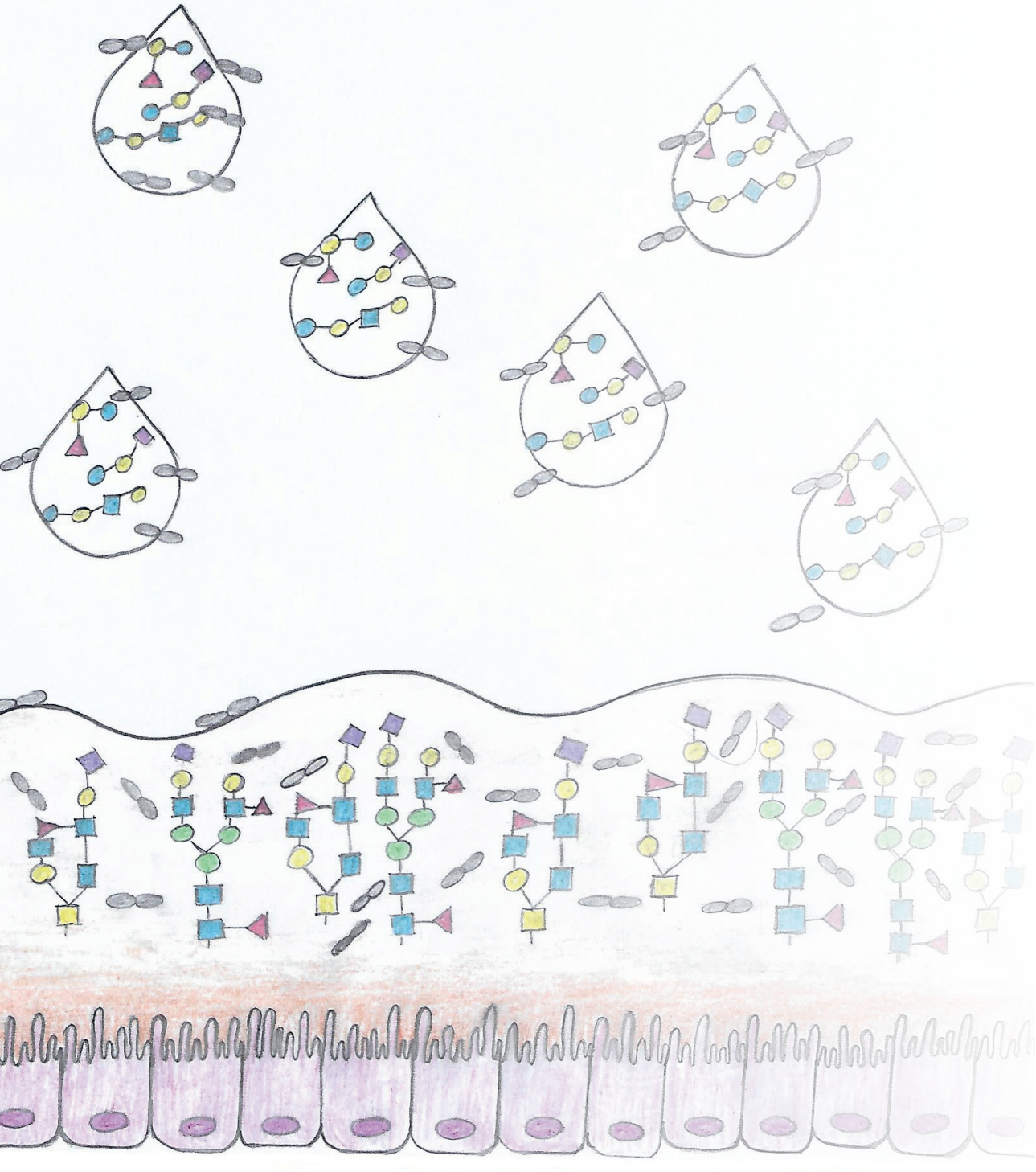
micro-organismen beiden een eigen niche hebben in de muis en ze niet hoeven te concurreren voor voedsel of ruimte. Microbe-microbe interacties zijn moeilijk te onderzoeken in complexe ecosystemen zoals de menselijke darm, maar deze eenvoudige *in vivo* modellen bieden mogelijkheden voor de toekomst. Een alternatief kan het gebruik van synthetische gemeenschap van micro-organismen zijn die vergelijkbaar zijn in samenstelling met een ecosysteem.

In **hoofdstuk 5** beschrijven we een zelf samengestelde synthetische gemeenschap in een continu bioreactor van 16 verschillende soorten, waaronder *A. muciniphila*. Hier onderzoeken we de trofische en metabole interacties van de mucine afbrekende soorten met de soorten die behoren tot de kern van de darm gemeenschap. Deze synthetische gemeenschap wordt continu gevoed met mucine en daarnaast driemaal per dag een toevoeging van voedingsvezels pectine, zetmeel, inuline en xylaan. In deze synthetische samenstelling vonden we dat 4 verschillende trofische gildes zich wisten te vestigen onder deze condities. Een voorbeeld hiervan zijn de 2 gildes die vertegenwoordigd worden door de meest dominant aanwezige stammen *A.muciniphila* en *Bacteroides xylanisolvens* en die individueel bekend zijn om het afbreken en gebruiken van mucine of het gebruiken van minder complexe suikers.

Hoofstuk 6 vergelijkt de gen expressie van *A.muciniphila* tussen de experimenten die worden beschreven in hoofdstuk 4 en 5. Hier wordt gekeken naar de verschillen in complexiteit van de gemeenschap, die toevoeging van suikers, de samenstelling van het medium, en het experimentele ontwerp (*in vivo* and *in vitro*). Hier was in het bijzonder aandacht voor de sleutel functies van *A. muciniphila* bijv. mucine afbraak, EPS (Extracellulaire polysachariden) en genen gerelateerd aan pili. Hier vonden we dat *A.muciniphila* deze sleutel genen tot expressie bracht in beide condities *in vivo* en *in vitro*, onafhankelijk van de complexiteit van de gemeenschap of de medium samenstelling. Het constant tot expressie brengen van deze genen geeft mogelijk aan hoe robuust en weerbaar *A. muciniphila* is in een omgeving rijk aan mucine suikers.

Als laatste wordt in **hoofdstuk 7** alle onderzoeksresultaten van dit proefschrift besproken, hier ligt voornamelijk de nadruk op de mogelijkheid van *A.muciniphila* om te floreren in complexe ecosystemen zoals de darmen door het afbreken van de door de mens afgegeven suikers. Met het onderzoek dat wordt beschreven in dit proefschrift wordt er geprobeerd om de metabole capaciteit van *A. muciniphila* en de belangrijke microbe-microbe interacties van deze unieke soort in complexe ecosystemen te

ontrafelen. Zo hebben we in dit proefschrift beschreven hoe *A.muciniphila* in staat is om HMO's af te breken en te gebruiken met de 'glycan utilizing pathway' die normaal wordt gebruikt voor de groei in een mucine omgeving van de darmen. Deze eigenschap om HMO's af te breken indiceert ook een mogelijk belangrijke rol voor *A. muciniphila* bij het koloniseren van de darmen van jonge kinderen. Daarnaast worden er een paar suggesties gegeven voor toekomstig onderzoek naar de 'ecofysiologie' van *A.muciniphila*. Als allerlaatste worden er een paar applicatie suggesties gegeven voor het toepassen van *A. muciniphila* als probiotica, dan wel alleen of met ander micro-organismen i.c.m. HMO's als prebiotica (Synbiotische mix) om de gezondheid van de mens te stimuleren.



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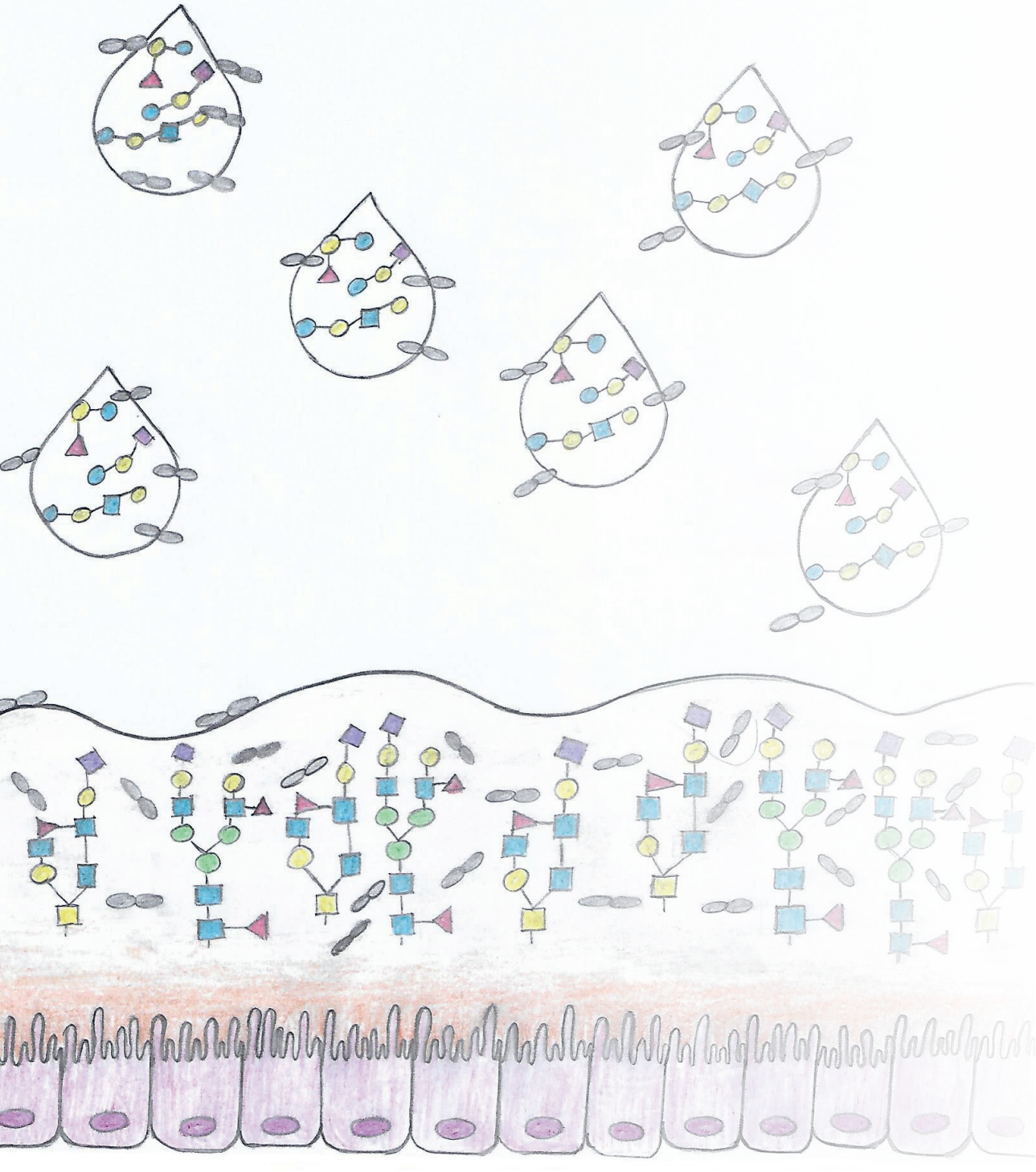

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Κ.Π. Καβάφης, «Ιθάκη»

As you set out for Ithaka hope your voyage is a long one, full of adventure, full of discovery. Laistrygonians, Cyclops, angry Poseidon – don't be afraid of them: you 'll never find things like that on your way as long as you keep your thoughts raised high, as long as a rare excitement stirs your spirit and your body. Laistrygonians, Cyclops, wild Poseidon – you won't encounter them unless you bring them along inside your soul, unless your soul sets them up in front of you…

C.P. Cavafy, "Ithaka"
Translated by Edmund Keeley

My voyage and my quest into the scientific world of the PhD have come to an end after almost 5 years. All these years were not easy, I had to go through a lot of challenges both in personal and professional level. However, I would not have been able to make it so far without the help and support from a lot of people whom I would like to acknowledge here.

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χαρά και είμαι σίγουρος ότι θα έρθουν και άλλες ακόμα καλύτερες στιγμές. Κωστή, σ' ευχαριστώ πολύ για όλα.

Ελευθερία μου, σ' ευχαριστώ πολύ που είσαι δίπλα μου όλα αυτά τα χρόνια να με στηρίζεις, να με υποστηρίζεις, να μου δίνεις κουράγιο και θάρρος να συνεχίσω. Κατά την διάρκεια του διδακτορικού, πέρναγες και εσύ όλα τα άγχη μου, όλες τις ανασφάλειες μου, τις χαρές, τις λύπες, τα γέλια, την ανυπομονησία για αποτελέσματα και χαρούμενα νέα, την απογοήτευση μου για την απόρριψη του άρθρου και όλα όσα συνάντησα αυτά τα τελευταία 4,5 χρόνια. Στην ουσία, έκανες και 'συ διδακτορικό μαζί μου και ας μην το κατάλαβες. Το διδακτορικό αυτό είναι αφιερωμένο σε εσένα. Γιατί πολύ απλά είσαι ο μοναδικός άνθρωπος που ξέρει ακριβώς τις δυσκολίες που αντιμετώπιζα και ανταπεξήλθα για να φτάσουμε στο σήμερα. Και όταν οι δυσκολίες με καταλάμβαναν ήσουν εκεί να μου δώσεις ώθηση να συνεχίσω, να με στηρίζεις. Ήσουν απλά εκεί, δίπλα μου. Πάντα ήσουν εκεί να μου πεις την ειλικρινή γνώμη σου και να με βοηθήσεις να βελτιωθώ. Είσαι η οικογένειά μου και ανυπομονώ για όλα όσα το (άμεσο) μέλλον μας φέρει και θα περάσουμε μαζί. Σε ευχαριστώ πολύ για όλα Ελευθερία μου, από τα βάθη της καρδιάς μου.

Μαμά και **Νάντια**, περάσαμε πολλά για να φτάσουμε ως εδώ, περάσατε πολλά για να φτάσω ως εδώ. Σίγουρα δεν θα φτάναμε σε αυτήν την μέρα, εάν δεν ήσαστε εσείς να με στηρίζετε και να μου δείχνετε πάντα την πίστη σας ότι θα τα καταφέρω. Πάντα μου δείχνατε και δείχνετε πόσο περήφανες είστε για 'μένα. Αλλά, θέλω να ξέρετε ότι εγώ είμαι πιο περήφανος για εσάς και για ό,τι έχετε καταφέρει μέχρι τώρα. **Μαμά**, μακάρι να μπορούσα να εκφράσω όλα αυτά που αισθάνομαι μέσα σε λίγες γραμμές. Η ανιδιοτελής αγάπη σου για εμάς, μας έκανε αυτό που είμαστε σήμερα. Δεν μπορώ να φανταστώ ή μάλλον να χωρέσει ο νους μου όλες τις θυσίες που έχεις κάνει για 'μας, χωρίς να σε δω μια φορά, έστω και μία φορά να το βάζεις κάτω, να λυγίζεις. Δεν το έκανες ποτέ! Μακάρι στη ζωή μου να αντιμετωπίζω τα πάντα όπως εσύ. **Νάντια,** Ναντιούλα μου, δεν ξέρω αν θα μπορούσα να ήμουν πιο τυχερός στη ζωή μου, από το να έχω εσένα για αδερφή μου. Όλα αυτά τα χρόνια έχεις κάνει πολλές θυσίες για να μπορέσω να φτάσω ως εδώ. Είμαι πολύ περήφανος για 'σένα. Έχουμε περάσει πολλές δύσκολες αλλά και αμέτρητες όμορφες στιγμές και είμαι πολύ χαρούμενος που τα περάσαμε όλα αυτά μαζί, σαν μία γροθιά. Το μεγαλύτερο μέρος αυτού του βιβλίου, αν όχι όλο, σας ανήκει. Σας ευχαριστώ πολύ για όλα!!

Μπαμπά…

Πάντα στον νου σου να 'χεις την Ιθάκη. Το φθάσιμον εκεί είν' ο προορισμός σου. Αλλά μη βιάζεις το ταξίδι διόλου. Καλύτερα χρόνια πολλά να διαρκέσει και γέρος πια ν' αράξεις στο νησί, πλούσιος με όσα κέρδισες στον δρόμο, μη προσδοκώντας πλούτη να σε δώσει η Ιθάκη.
Η Ιθάκη σ' έδωσε τ' ωραίο ταξίδι. Χωρίς αυτήν δεν θα 'βγαινες στον δρόμο. Άλλα δεν έχει να σε δώσει πια. Κι αν πτωχική την βρεις, η Ιθάκη δεν σε γέλασε. Έτσι σοφός που έγινες, με τόση πείρα, ήδη θα το κατάλαβες οι Ιθάκες τι σημαίνουν.

Κ.Π. Καβάφης, «Ιθάκη»

Keep Ithaka always in your mind. Arriving there is what you 're destined for. But don't hurry the journey at all. Better if it lasts for years, so you 're old by the time you reach the island, wealthy with all you 've gained on the way, not expecting Ithaka to make you rich.
Ithaka gave you the marvelous journey. Without her you wouldn't have set out. She has nothing left to give you now. And if you find her poor, Ithaka won't have fooled you.
Wise as you will have become, so full of experience, you 'll have understood by then what these Ithakas mean.

C.P. Cavafy, "Ithaka"
Translated by Edmund Keeley

Σε γνώρισα από παιδί μικρό…

About the author

Ioannis Kostopoulos was born on the 29th of August in 1988 in Marousi, Athens, Greece. He studied Food Technology at the Technological Educational Institute of Larissa, Department of Karditsa (Greece) from which he obtained his BSc in 2011. After his BSc studies, Ioannis worked for a year as Quality Management Administrator at ATI S.A., Karditsa (Greece). There, he was responsible for the quality control of the tomato paste products. In 2013, he started his MSc in Food Technology, specializing in Food Biotechnology at Wageningen University and Research from which he obtained his MSc degree in 2016. During his MSc studies, he conducted his Major Thesis project at the Laboratory of Food Microbiology of Wageningen University and Research, under the supervision of Dr. Monica Fernandez Ramirez and Prof. dr. Tjakko Abee, on the characterization of *Lactobacillus* biofilms in mixed cultures. Ioannis concluded his MSc studies with an internship at Danone Nutricia Research in Utrecht (The Netherlands). In April 2016, he started his PhD research project at Molecular Ecology group of the Laboratory of Microbiology of Wageningen University and Research. This research project that is described in this thesis was co-supervised by Dr. Clara Belzer (Associate Professor) and Prof. Jan Knol. Currently, Ioannis has a Postdoc position in Danone Nutricia Research, where he explores the effect of the prebiotics on early life gut microbiota composition.

List of Publications

Fernández, Ramírez, M.D., **Kostopoulos, I**., Smid, E.J., Nierop Groot MN, & Abee, T. (2017). Quantitative assessment of viable cells of Lactobacillus plantarum strains in single, dual and multi-strain biofilms. Int J Food Microbiol. Mar 6;244:43-51. doi: 10.1016/j.ijfoodmicro.2016.12.014. Epub 2016 Dec 29. PMID: 28068587.

Geerlings, S. Y., **Kostopoulos, I**., de Vos, W. M., & Belzer, C. (2018). *Akkermansia muciniphila* in the Human Gastrointestinal Tract: When, Where, and How?. *Microorganisms*, *6*(3), 75. https://doi.org/10.3390/microorganisms6030075

Kostopoulos, I., Elzinga, J., Ottman, N., Klievink, J. T., Blijenberg, B., Aalvink, S., Boeren, S., Mank, M., Knol, J., de Vos, W. M., & Belzer, C. (2020). Akkermansia muciniphila uses human milk oligosaccharides to thrive in the early life conditions in vitro. *Scientific reports*, *10*(1), 14330. https://doi.org/10.1038/s41598-020-71113-8

Kostopoulos, I., Aalvink, S., Kovatcheva-Dutchary, P., Nijsse, B., Bäckhed, F., Knol, J., de Vos, W.M., Belzer, C. (2020). A continuous battle for host-derived glycans in the gut between a mucus specialist and a glycan generalist *in vitro* and *in vivo.* **Submitted**

Shetty, S.A.[†], **Kostopoulos, I.**[†], Geerlings, S.[†], Smidt, H.[*], de Vos, W.M.[*], Belzer, C.[*], Minimalist Approach for Deciphering the Ecophysiology of Human Gut Microbes. **Manuscript in preparation**

Kostopoulos, I.[†], Geerlings, S.[†], Shetty, S.A., Nijsse, B., de Vos, W.M., Belzer, C. The main functions of key species *Akkermansia muciniphila* remain stable across different microbial ecosystems. **Manuscript in preparation**

[†],[*] Equal contribution

Overview of completed training activities

Name of the course/meeting	Organizing Institute(s) and Country	Year
Discipline specific activities		
GutDay	GutDay, The Netherlands	2016
KNVM Fall meeting	KNVM, The Netherlands	2016
KNVM Spring meeting	KNVM, The Netherlands	2017
KNVM Spring meeting	KNVM, The Netherlands	2018
Microbiology Centennial Symposium	MIB, The Netherlands	2017
Advanced Proteomics	WGS, The Netherlands	2017
The Intestinal Microbiome and Diet in Human and Animal Health	VLAG, The Netherlands	2017
GutDay	GutDay, The Netherlands	2018
KNVM Spring meeting	KNVM, The Netherlands	2019
ASM microbe	ASM, USA	2019
Immunity through gut: Biotics in early life	Nutricia Research, The Netherlands	2020
General courses		
VLAG PhD week	VLAG, The Netherlands	2016
Chemometrics	WGS, The Netherlands	2016
Scientific Writing	WGS, The Netherlands	2019
Presenting with impact	WGS, The Netherlands	2020
Optional		
Preparation of research proposal	MIB, The Netherlands	2016
PhD study tour to North Europe	MIB/SSB	2017
Weekly group meetings	MIB, The Netherlands	2016
Programming in Python	WUR/MSc course, The Netherlands	2016
JPI group meeting	JPI, The Netherlands	2017 & 2019
JPI group meeting	JPI, Italy	2018
PhD study tour to East-side USA	MIB/SSB, USA	2019

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Colophon

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