DISSERTATION / DOCTORAL THESIS

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“Bacterial degradation of polysaccharides in the gut mucosal eco-system”

verfasst von / submitted by

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Science is a wonderful thing if one does not have to earn one's living at it. *Albert Einstein*

Summary

A healthy diet is one of the main pillars of healthy living, where fibers play an important role in host health. Not only do fibers reduce chances of disease onset, fibers are also vital for a healthy digestive tract which includes the gut microbiome. In return the bacteria in the intestines provide the host with additional nutrients such as vitamins and short chain fatty acids the host is unable to produce itself. In a healthy system a mucus layer covers the intestine in order to prevent bacteria from coming into direct contact with host cells and thus causing an inflammatory response. However, with many diseases this mucus layer is diminished.

The main focus of this thesis was to investigate bacterial interactions with the mucus layer. This was done in a holistic *in* vivo approach using mice models fed a fiber deficient diet investigating the entire mucosal eco-system as well as *in vitro* where we selected two specific host compounds of the mucosal environment and a investigated a specific selection of bacterial species and their degradation abilities. The *in vivo* experiments showed, contrary to popular belief, mucus reduction is not a result of an increasing number of mucus degrading bacteria but rather a host-led response. Additionally, our results verified the loss of bacterial diversity upon the absence of fiber and showed the importance of using absolute quantification methods in such instances as relative abundances can lead to false conclusions. Lastly, we showed that not only mucus and microbes were affected by the lack of fibers but that there were additional physiological changes in the intestines including a shortened length.

Host glycosaminoglycans such as chondroitin sulfate and hyaluronan play an important role in shaping the gut mucosal environment. Additionally, the different taxa within the gut microbiome are vital to host health as seen with the fiber deficiency. We thus selected closely related *Bacteroides* species in order to investigate the mechanisms behind degradation of these specific components as well as the potential co-operation between species via cross-feeding. Here we found that closely related species have different degradation profiles yet do share degradation products in order to keep a balanced eco-system. The results in this thesis shed a new light on the complexity of the gut mucosal eco-system and the important role of (balanced) nutrition therein.

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Chapter 1

General introduction to human nutrition, mucus, and the gut microbiome

Life as we know it

Long before dinosaurs, let alone *homo sapiens*, inhabited the earth, there were microbes (Madigan, 2012). Microbes can be found anywhere; from the air we breathe (Madigan, 2012), to the soil we stand on (Trojan, 2021); from Antarctic permafrost (Pelikan, 2019) to hot water springs such as those in Yellow Stone, USA (Havig, 2021). Microbes are versatile and everchanging in order to survive and thrive. Our life literally would not be possible without microbes; without them the earth's atmosphere would never have become oxygenated (Madigan, 2012). Even today we need microbes for a multitude of things; to clean wastewater (Wagner, 2002), stimulate plant growth (Semchenko, 2022), or to train our immune system (Gensollen, 2016). They are also vital for our food; fermentation of cheese, yoghurt, beer, or bread are not possible without microbes (Madigan, 2012). Even the flavor of strawberries is enhanced by microbes (Verginer, 2010). Have you ever noticed the delightful smell lingering after a rain shower? The smell is known as Geosmin, and is once more, produced by microbes (Gerber, 1965). However not all microbes have beneficial effects, *Brevibacterium* for example, results in smelly feet (Hulley, 2019). Others, known as pathogens, can make us sick. These include *Salmonella*, commonly found in raw meat, which can result in vomiting and/or diarrhea; *Mycobacterium tuberculosis* causing tuberculosis or *Rhinoviruses* responsible for the common cold (Madigan, 2012). Scientific research is required for us to understand more about microbes, both the beneficial and harmful so we can prevent and/or treat disease and optimize the benefits we can obtain from these tiny organisms invisible to the naked eye.

The importance of a healthy diet

According to Webster's dictionary the definition of food is "something that nourishes, sustains or supplies" (Merriam-Webster). In this context 'food' can range from sunlight for plants, any host cell for viruses, grass for cows or anything you find in the supermarket for humans. In other words, food is the fuel of life; without it we cannot survive. However, that is where the simplicity ends. The wrong (balance) of foods can negatively impact human health; too many saturated fats can increase cholesterol, clog arteries and lead to heart failure (Stanfield, 2011; Korver, 2006). Too little fresh fruits containing vitamin C can lead to scurvy (Maxfield, 2022). Eating more calories than one uses on a daily basis will cause weight gain and can potentially result in obesity (Stanfield, 2011), to name a few. What is then considered to be a healthy diet? Although a precise definition varies depending on culture and geolocation, in general it entails a foundation with variety of fruits, vegetables, nuts and legumes accompanied by a moderate amount of fish, lean-meat, low-fat dairy and whole grain carbohydrate sources such as brown rice or whole wheat bread, with little to no consumption of refined sugars, sodium and saturated fats (US department agriculture 2020). A healthy, balanced diet can elongate life expectancy and reduce morbidity (Stanfield, 2011). Nutrition has even been shown to reduce disease symptoms or improve treatment effectiveness. Broccoli for example, contains sulforaphane, which has been suggested to inhibit cancer development by increasing antioxidants and induce apoptosis of cancerous cells (Priya, 2011; Meeran, 2010). Another example

where nutrition has been shown to affect disease symptoms is Attention Deficit Hyper-Activity Disorder (ADHD). Individuals with ADHD have a hard time concentrating, combined with hyper-activeness which interferes with normal functioning (NIMH, webpage). Currently there is no cure, and treatment often involves medication with a multitude of side-effects (Stevens, 2013). However, recently, it has been shown that a so called "Few-foods Diet" can strongly reduce ADHD symptoms without the need of medication (Hontelez, 2021). Nutrition of course is not a cure-all, but the above examples do highlight why it is vital to focus significant research efforts investigating the role of nutrition to take care of our bodies and pay more attention to what we eat.

The most common suggestion for healthy food choices are fibers. Fibers are found in a multitude of food options such as whole grains, vegetables, or fruits (US department agriculture 2020). Fiber-rich foods are low in calories but increase satiety (Anderson, 2009) and it has been suggested that increasing fiber intake reduces the risk of obesity (Tucker, 2009). Type 2 diabetes is a metabolic disease often linked to obesity; dietary intervention, in particular an increase in fiber intake, can reduce the onset of the disease by 62% (Lindstrom, 2009). On a more general note, dietary fibers increase water retention and improve stool consistency, as well as delaying transit time and thus increasing the opportunity for nutrient uptake (Anderson, 2009). Fibers seem like magic; they can greatly improve our health status, they can even reduce risk for disease onset, yet 85% of adults in the Western world do not meet the daily recommended intake of 14-gram fiber / 1000 kcal (US department agriculture 2020)!

The mucus layer and it's slimy business

The gastro-intestinal tract (GIT) is technically a hollow tube which starts at the mouth and ends at the anus, consisting of the esophagus, stomach, small intestine, and colon (Figure 1). The inside 'tube' is a part of the outside world, yet cleverly enfolded into the body. It has been believed the surface area of the GIT was comparable to a tennis court (300 m^2), recent studies however, estimate it to be the size of half a badminton court (32 m^2), which is still a considerably larger surface area than that of the human skin (2 m^2) (Helander, 2014). Anything we ingest passes through the GIT, after which it is broken down into molecules either taken up by the body or excreted via de urine or feces. To protect the GIT from the dangers of the outside world, it is lined with a mucosal layer (Johansson, 2011). This layer varies in thickness throughout the GIT, being thinnest in the small intestine to enhance nutrient uptake, and thickest in the colon (Ermund, 2013). In the colon it consists of two layers; one tightly adherent layer which prevents direct contact of bacteria or other large molecules with the epithelial cells and a second, looser layer on top (Figure 2) (Ermund, 2013). Not only does this layer form a physicall barrier to protect against pathogens, it also contains antimicrobial and immune-related proteins (Johansson, 2011).

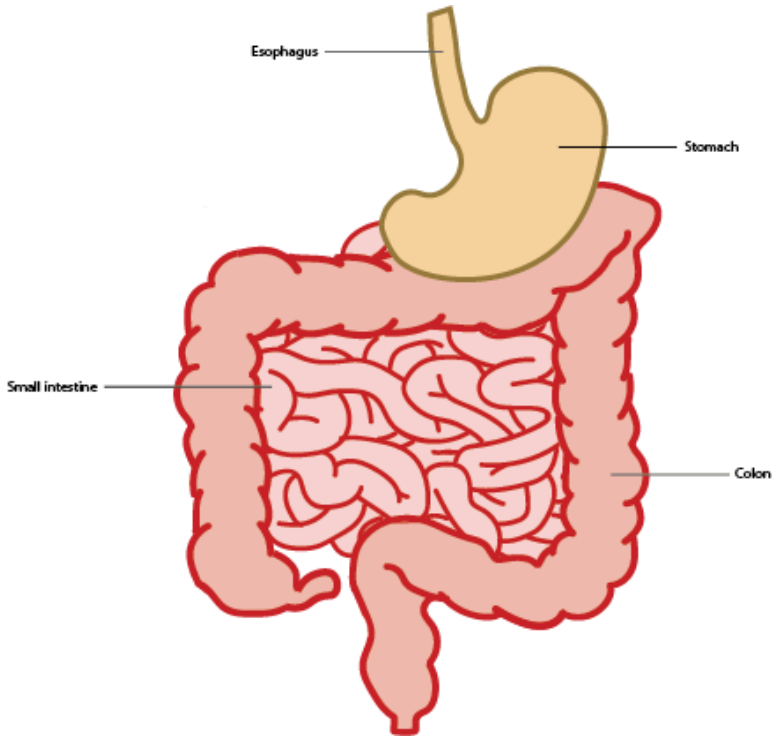

Figure 1: Diagram of the gastro-intestinal tract
After food is ingested, it passes through the esophagus, stomach, small and then large intestine before excretion.

Glycoproteins called mucins make up the majority of mucus throughout the body (Ermund, 2013; Larsson 2013). Mucins can be either gel-forming (MUC2, MUC5AC, MUC6, MUC5B) or transmembrane (MUC1, MUC3, MUC4, MUC12, MUC13, MUC17) (Pelaseyed, 2014). The two main gel-forming mucins in the GIT are MUC5AC, found mainly in the stomach, and MUC2 found mainly in the intestines (Ermund, 2013; Rodríguez-Piñeiro 2013). Gel-forming mucins are produced by goblet cells found throughout the intestinal epithelium but increase in number from the small intestine to the distal colon (Ermund, 2013). As soon as mucins are released from the goblet cells, they start binding water and greatly increase in volume, giving them their gel-like structure (Johansson, 2011). Despite the structure of the mucus differing between the small and the large intestine they both consist of MUC2 glycoproteins. Interestingly, proteomics did not indicate a different protein composition when comparing the small with the large intestinal mucus, despite the completely different structure (Rodríguez-Piñeiro 2013). Glycosylation can be either N-linked or O-linked depending on where and how the sugar is attached to the protein. If the sugar is attached at the asparagine, it is N-linked and takes place in the ER and Golgi apparatus whereas O-linked glycosylation takes place in the Golgi only and is attached to a serine or threonine (Larsson, 2013; Moran 2011). Mucins are O-linked and although the protein-backbone is the same, entailing repeats of threonine, serine, and proline, the glycans covering the backbone are highly diverse and consist of various sugars such as N-acetyl-D-galactosamine (GalNAc), N-acetyl-D-glucosamine (GlcNAc), D-galactose (Gal) and D-glucuronic Acid (GlcA) in different compositions (Moran, 2011). Interestingly, glycans in the small intestine have less complexity in comparison to those found in the colon (Ermund, 2013; Larsson, 2013). Furthermore, glycosylation of the mucins is highly diverse and dynamic and can be influenced by other factors such as

inflammation (Larsson, 2013). The biological role and relevance of the various glycosylation patterns remain to be unraveled.

Membrane-bound mucins, such as MUC17, expressed by gut epithelial cells, play an important role in the protective layer of the cells, known as the glycocalyx (Pelaseyed, 2014). Aside from membrane-bound mucins, the glycocalyx also contains a variety of glycosaminoglycans (GAGs) including chondroitin sulfate (CS) and hyaluronan (formerly known as hyaluronic acid, HA). Both CS and HA consist of repeating disaccharides; HA has GlcA and GlcNAc, whereas CS has GlcA and GalNAc, and, as the name suggests, can be sulfated (Bartlett & Park, 2010). These two compounds are found in varying concentrations throughout the human body and have numerous functions. CS for example, is the main GAG in blood and is a potent antioxidant (Campo, 2006). HA is found in virtually every tissue of the (human) body and plays an important role in tissue structuring (Hill, 2013). Both CS and HA can also be found in human milk, where HA has been shown to play an essential role in the innate immune defense of newborns, where it stimulates the production of antimicrobial peptides (Hill, 2013).

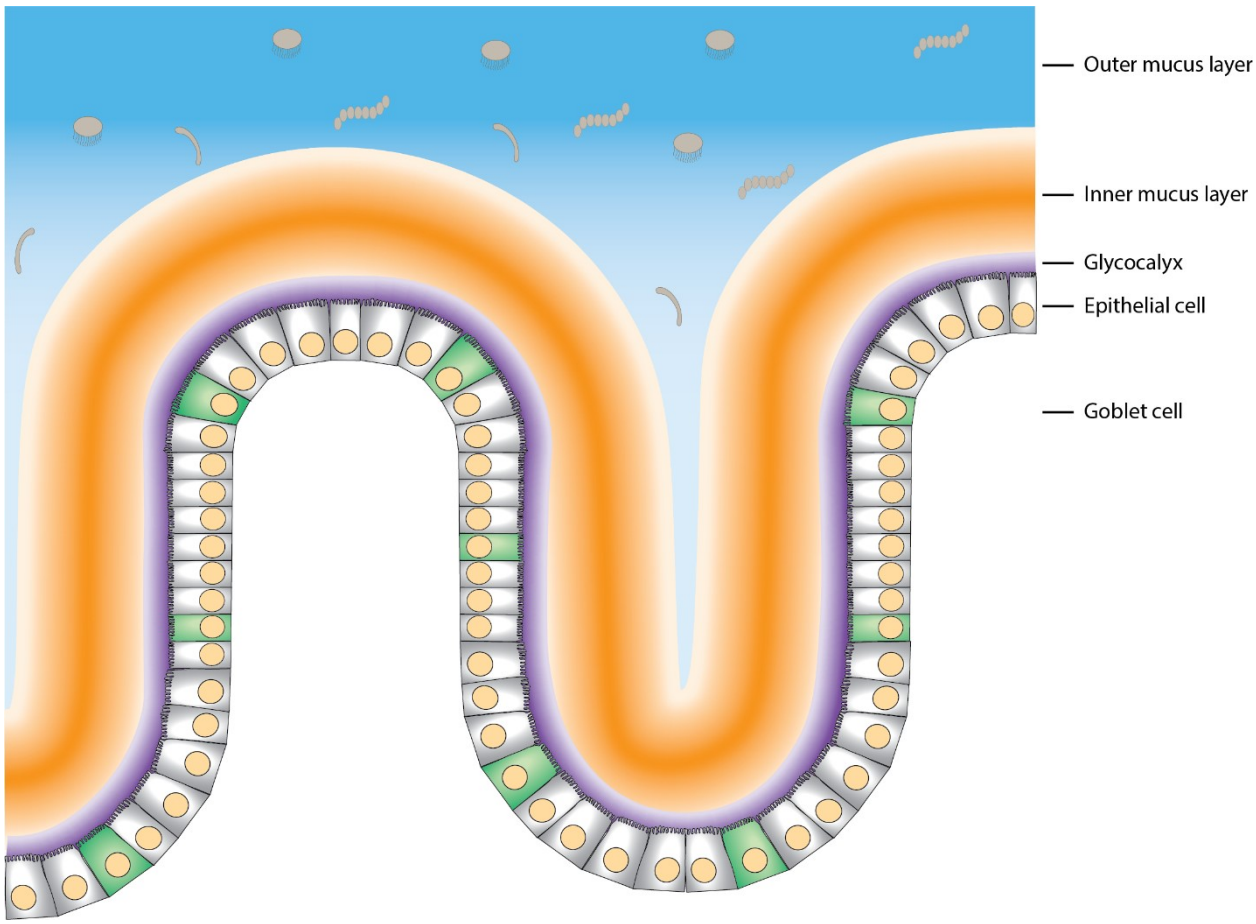

Figure 2: Graphical depiction of the mucosal environment in the colon

Inflammation is a key modulator of the mucus layer, including the glycocalyx. Inflammation affects mucosal glycosylation (Larsson, 2011) as well as increasing the amount of GAGs present in both blood (Campo,

2006) and intestine (Kessler, 2008). HA in particular is interesting in this situation as under normal circumstances it exists as a high molecular weight (HMW) form, however during inflammation it becomes fragmented and obtains a low molecular weight (LMW). HMW-HA has been linked to afore-mentioned antioxidant properties whereas the LMW-HA has been proven to stimulate an inflammatory response including leukocyte recruitment (de la Motte, 2011; Hill 2013; Kessler, 2008). During inflammation, including chronic inflammatory bowel diseases (IBD), both CS and HA levels are greatly enhanced in the mucosa observed in combination with structural loss of the glycocalyx (Belmiro, 2005; de la Motte, 2011).

The benefits of the gut microbiome

The gut microbiome consists of a variety of bacteria, viruses, and fungi. Here, however, we will focus on bacteria. In the entire human body, there are many more bacterial cells than human cells. In the GIT, the colon has the highest amount of bacteria with most belonging to either the phylum *Bacteroidota* (previously known as *Bacteroidetes*), or *Bacillota* (previously known as *Firmicutes*) (Madigan, 2012; Eckburg, 2005). Most bacteria are commensals and form a harmless symbiosis providing their host with a multitude of benefits. They can provide us with essential amino acids we cannot produce (Madigan, 2012), or digest fibers we cannot digest ourselves, again supplying the host with valuable nutrients (Zafar, 2021). Additionally, they inhabit niches to prevent the colonization of pathogens and are able to condition, develop and stimulate the immune system when needed (Zafar, 2021; Madigan, 2012). In the intestines most of the bacterial niches are found in the lumen just above the villus, or in the colon in the loose outer mucus layer. In healthy hosts bacteria will rarely be in between villi or in direct contact with the epithelial cells as these are protected by a tight mucus layer which is impermeable to bacteria (Figure 2) (Ermund, 2013).

A sub-group of bacteria is capable of Short Chain Fatty Acid (SCFA) production from fiber fermentation or mucin degradation. The most prominent SCFA in the intestines are propionate, acetate, and butyrate (Martin-Gallausiaux, 2021). Butyrate is currently one of the most studied SCFAs as it has many beneficial functions and is an energy source for colon epithelial cells, regulates mucus secretion as well as anti-inflammatory processes (Martin-Gallausiaux, 2021; Flint, 2007; Louis 2017). Acetate is another beneficial SCFA, not only can it be used to form butyrate by butyrate producing bacteria, it can also stimulate intestinal IgA production to preserve homeostasis of the intestinal immune system (Morrison, 2016; Flint, 2007). Thus, the excretion of SCFA by bacteria can directly influence the gut eco system and affect the immune system and thus host health.

Sharing is caring

As previously mentioned, the two main phyla in the gut are the *Bacteroidota* and the *Bacillota*. The ratio between these two phyla is an indicator for host health as dysbiosis has been linked to obesity, autism or IBD (Turnbaugh, 2006; Hills, 2019). It is thus not surprising that they have been the focus of research for

decades. *Bacteroidota* in particular have been extensively studied, for example in carbohydrate break-down as they can degrade both dietary as well as host-derived glycans (Salyers, 1977). Most bacteria use carbohydrate-active enzymes, better known as CAZymes, to break down polysaccharides extra-cellularly and then import them as simple sugars into the cell. *Bacteroidota* however, have groups of clusters of co-regulated genes required for the breakdown of a specific polysaccharide, so called Polysaccharide Utilization Loci (PULs) (Terrapon, 2018). These PULs entail proteins needed for binding the polysaccharide to the cell-surface after which it gets imported into the periplasm before further breakdown (Terrapon, 2018).

Due to the high density of the microbial population in the gut, there is competition between species for nutrients and niches. Being able to degrade a wide array of polysaccharides and being able to selectively keep break-down products due to the PULs is a huge advantage (Rakoff-Nahoum, 2014). In some cases, bacteria may be selfish and keep any break-down products for themselves, there are however also many instances where bacteria "share" with others, thus providing what is known as public goods. This may seem counter-productive; however, this 'sharing' can be used to promote growth of certain species beneficial to the public-goods supplier, which in turn can enhance its own growth (Rakoff-Nahoum, 2016).

The symbiosis of microbes, mucus & nutrition

We have established the entire GIT is lined with a mucosal layer and that bacteria have benefits to the host and rely on host-nutrition to thrive. Yet what happens when this balance is disturbed? With the absence of dietary fibers, it has been shown that the mucosal layer thins, and bacteria are able to come in close contact with epithelial cells, damaging them and causing an inflammatory response (Earle, 2015). In germ-free mouse models it has been shown that mucosal gene expression is altered, and the mucosal layer is negatively affected without the presence of bacteria (Johansson, 2008; Comelli 2008). Currently however, there are no specifics known how the mucus layer is affected, or what is cause or effect. It is thus of the utmost importance to enhance our knowledge on the delicate interaction between the host and our gut microbiome to not only understand more about how they interact, but also how we can (positively or negatively) influence this symbiosis with our dietary habits.

Current studies investigating the intricate relations between fiber (deficiency) and the gut microbiome are mostly observational with causal relations at best or preformed in simplified bacterial communities with many open niches which could result in incorrect conclusions (Desai, 2016; Earle, 2015). In this thesis the aim is to understand more about the causing factors and mechanisms of mucosal turn-over. For **chapter 2** the objective was to determine if the mucosal thinning observed with fiber deficiency was a host or bacterial initiated response. Whereas in **chapter 3** two specific mucosal compounds, CS and HA, were investigated in respect to the degradation of CS and HA in closely related species in order to understand more about community dynamics in the gut mucosal environment.

Traditional methods for analyzing mucus secretion commonly use histological stains. However, fixatives and dehydration steps required for staining may alter both the properties and structure of the mucus giving an incomplete picture (Hasegawa, 2017). By using stable isotope probing, as done in **chapter 2**, it was possible to evaluate mucus secretion in a quantitative way and for the entire intestine rather than representative samples. By combining this with *muc2* gene expression mucus secretion could be quantitatively assessed. Furthermore, flow cytometry was used to calculate absolute abundances of microorganisms instead of the simpler and more commonly used, relative abundances, to get enhanced accuracy when analyzing changes within the *in vivo* bacterial community as response to fiber deprivation.

For **chapter 3** the initial aim was to study the cooperation of numerous *Bacteroides* species *in vitro* whilst degrading host glycosaminoglycans. However, when it became apparent there was a division between these closely related species in the ability to degrade CS but not HA, the objective changed to know why this was happening. Here transcriptomic analysis was used in one model species (*B. theta*) to determine if there was a separate pathway between CS and HA. This high-lighted genes of interest which were then compared to the other species in an attempt to hypothesize a potential mechanism specific for HA degradation.

This thesis gives valuable insights into processes of mucus degradation previously unknown. Not only is it shown that the mucosal thinning during fiber deprivation is initiated by the host, it also demonstrates the importance of using quantitative measures which led to the conclusion that the existing model of a mucus degrader bloom should be revised. Additionally, it was shown that even in a competitive environment bacteria can co-operate, and many more bacteria can benefit from mucus degradation than the primary degraders.

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Chapter 2

Impaired mucosal homeostasis in short-term fiber deprivation is due to reduced mucus production rather than overgrowth of mucus-degrading bacteria

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Abstract

Introduction: The gut mucosal environment is key in host health; protecting against pathogens and providing a niche for beneficial bacteria, thereby facilitating a mutualistic balance between host and microbiome. Lack of dietary fibers results in erosion of the mucosal layer, suggested to be a result of increased mucus-degrading gut bacteria. This study aimed to use quantitative analyses to investigate the diet-induced imbalance of mucosal homeostasis.

Results: Seven days of fiber-deficiency affected intestinal anatomy and physiology, seen by reduced intestinal length and loss of the colonic crypt-structure. Moreover, the mucus layer was diminished, muc2 expression decreased, and impaired mucus secretion was detected by stable isotope probing. Quantitative microbiome profiling of the gut microbiota showed a diet-induced reduction in bacterial load and decreased diversity across the intestinal tract, including taxa with fiber-degrading and butyrate-producing capabilities. Most importantly, there was little change in the absolute abundances of known mucus-degrading bacteria, although, due to the general loss of taxa, relative abundances would erroneously indicate an increase in mucus degraders.

Conclusion: These findings underscore the importance of using quantitative methods in microbiome research, suggesting erosion of the mucus layer during fiber deprivation is due to diminished mucus production rather than overgrowth of mucus degraders.

Introduction

Nutrition can play a major role in health and disease. A healthy diet is considered to incorporate a variety of different nutritional sources such as nuts, fruits, legumes, and vegetables, whilst moderate in, fish, poultry, and dairy products [1]. Many studies have shown that a variant to this, known as the Mediterranean diet, can extend lifespan, thus emphasizing the importance of our nutritional intake [2]. Optimal nutrition is somewhat individualized, as genetic predisposition can affect response to nutrition, and diet can in turn influence gene expression as well as the gut microbiome [3–5]. The gut microbiome itself can also influence health and response to dietary components, for example by microbial fermentation of fibers, as well as modifying fat accumulation and leptin sensitivity [6].

Fiber is an important component of a balanced diet, giving stool its consistency, delaying transit time, increasing water retention, and acting as an energy source for gut bacteria [7,8]. Additionally, fiber intake has been linked to lowered cholesterol levels as well as increased number of colonic goblet cells [9,10]. The recommended daily fiber intake is 14 grams / 1000 kcal, yet 85% of adults do not meet these recommendations [11]. This is problematic as fiber deficiency is related to cardiovascular diseases, colon cancer and obesity [12–16].

The gastro-intestinal tract is lined with a mucosal layer consisting of secreted mucin glycoproteins produced by goblet cells, of which MUC2 is the main component in the intestine [10,17,18]. The mucosal layer protects colonocytes from pathogen invasion, microbial and dietary toxins as well as antigens. Additionally, it represents a vital niche for microbes, which in return produce short-chain fatty acids (SCFAs) important for host health [18,19]. The main SCFAs produced in the gut are acetate, propionate, and butyrate. The latter for example, has numerous beneficial functions, including being an energy source for epithelial cells, regulating mucus secretion and having anti-inflammatory effects [19–21]. Propionate enhances satiety and reduces cholesterol, whereas acetate can be rerouted into butyrate and in itself can maintain intestinal immune homeostasis by stimulating intestinal IgA production [22–25]. A fiber-rich diet is imperative to support the synergistic relationship between the host mucosal environment and the gut microbiome [26,27].

A lack of fibers has been shown to cause erosion of the mucosal layer in gnotobiotic mouse models with low complexity gut microbiota [28,29]. Current literature proposes that a bloom of mucosal degraders during fiber deprivation drives this erosion [28]. However, as diet has been associated with altered goblet cell function, it remains unclear whether the host or the mucus-degrading microbiota is driving the imbalance in mucosal homeostasis. In this study we performed quantitative microbiome profiling, analysis of host mucosal anatomy and physiology, and stable isotope probing to determine compartment-specific changes in the host and intestinal microbiota. Our results indicate that the reduction of the mucosal layer is driven by impaired mucus production rather than by overgrowth of mucus degrading bacteria.

Materials and Methods

Animal experiments

A total of 38 C57BL/6J mice (18 males, 20 females) were bred at the Centre for Biomedical Research, Medical University of Vienna, under specific pathogen-free (SPF) conditions and transferred to the Max Perutz Laboratories, University of Vienna, between 4-6 weeks of age. After an initial week of acclimatization on a standard chow control diet (CD, Ssniff, Soest, Germany), half the mice were switched to a fiber-free diet (FFD: without polysaccharides and cellulose with glucose as the only carbohydrate source; Ssniff, Soest, Germany) for another 7 days (Supplementary Figure 1). The control diet contained numerous sources of polysaccharides such as wheat, soy, and barley. The remaining mice continued to receive the CD. Mice were co-housed yet split from the start by gender and diet in a controlled environment (14h/10h day/night cycle) with unlimited access to food and water. Two biological replicates were performed, yet not all methods were used for both experiments. Fecal pellets were collected on the morning of the diet switch, 2 days, and 7 days after switching the diet. Labeling was performed with stable isotope-labeled L-threonine (98 atom % ^{13}C, 98 atom % ^{15}N, Sigma-Aldrich) in order to quantify mucus secretion. In 4 mice unlabeled L-threonine (Merck, Germany) was used as a ^{12}C control. Compounds were dissolved in a sterile 0.9% NaCl solution and mice were injected with 50 µl via tail vein injection on day seven as previously described [30]. Mice were sacrificed 6 hours post-injection by cervical dislocation after which contents from cecum, small intestine and colon were collected. Intestinal contents were split in 2 for both quantitative microbiome profiling and elemental and isotope analyses. A section of the colon was prepared for histological analyses. In the second replicate, the wet weight of the small intestine, cecum and colon were measured with and without contents, to determine luminal content weight. In addition, the length of the small intestine and colon was measured.

Quantitative microbiome profiling

Flow cytometry was used to quantify the absolute number of bacterial cells per sample for small intestine, cecum and fecal pellets using FACS Melody and the FACS_Chorus software (BD, Germany). Samples were dissolved in 500 µl PBS and then strained through a 40 µm cell strainer (Corning Inc., Corning, New York, USA). After filtering, 5 µl SYBR green was added which was later used for gating samples. Absolute counting beads (CountBright, Invitrogen, ThermoFisher Scientific, Austria) were used for cell counts according to the manufacturer's instructions. Each sample was counted in triplicate and the mean total cell counts g^{-1} were calculated. DNA from intestinal contents was extracted using the FastStool DNA extraction kit automated on a QIACube Connect, following the manufacturer's instructions. Thereafter, amplicons were generated using primers targeting the V4 region (515F Parada [5'-GTG YCA GCM GCC GCG GTA A-3'] and 806R Apprill [5'-GGA CTA CNV GGG TWT CTA AT-3']) [31] of bacterial and archaeal 16S rRNA genes, barcoded in a unique dual setup, and sequenced on the Illumina MiSeq system as further described by Pjevac

et al. [31]. Sequencing was performed at the Joint Microbiome Facility of the Medical University of Vienna and the University of Vienna under the project IDs JMF-1904-2 and JMF-2009-3. After sequencing, amplicon sequence variants (ASVs) were inferred [32] and classified following the analysis workflow detailed by Pjevac et al. [31]. Absolute taxon abundances, expressed as total counts per compartment, were derived from the flow cytometry cells counts in combination with the genus read table after copy number correction using *rrn*DB (5.7 pantaxa) based on Van de Putte et al [33,34]. Sample load was calculated by summing the absolute abundances corrected by copy numbers. Load per compartment was calculated by multiplying the copy number corrected abundance by the total weight inside the intestinal compartment. To calculate the log2-fold change (log2FC) per diet, the top 50 genera where selected based on mean abundance. A pseudocount was added before taking the ratio of the FFD / CD. T-tests were used to evaluate significant statistical diet-induced abundance changes.

Elemental Analysis – Isotope-Ratio Mass Spectrometry

For Elemental Analysis – Isotope-Ratio Mass Spectrometry (EA-IRMS), 5 mg intestinal biomass was washed to eliminate free L-threonine. After washing, the pellet was dried overnight in a speedvac (Eppendorf Concentrator 5301) and another 24 h at 60 °C. These samples were used for wet and dry-weight analysis. Next, 0.05-0.3 mg (dry weight) was transferred into a tin capsule. Samples for elemental analysis and ^{13}C and ^{15}N quantification were analyzed with an elemental analyzer (EA 1110, CE Instruments) coupled via a ConFlo III device to the IRMS (DeltaPLUS, Thermo Fisher) [30].

Mucosal gene expression analysis

Epithelial cells were isolated according to the protocol by Gracz et al. [35]. After isolation, RNA was extracted using the RNAqueous-Micro Total RNA Isolation Kit (Thermo Fisher) following the manufacturer's instructions. Subsequently cDNA synthesis was performed using the High-Capacity cDNA Reverse Transcription Kit (Thermos Fischer). Real-time quantitative PCR was performed in a 20 µl reaction volume containing 1 µl template (cDNA diluted 1:5), 1x SYBR green Master Mix (Bio-Rad), 7.4 µl H_2O, and 0.8 µl of primers targeting either *muc2* (Forward: 5'-ACTGCATGTGCGCGGCTCTT-3', Reverse :5'-TGAGCTTGGGCAAGCGTGCA-3') or the housekeeping gene 36B4 (Forward: 5'-GCTTCATTGTGGGAGCAGACA-3', Reverse: 5'-CATGGTGTTCTTGCCCATCAG-3'). Amplification and detection were performed using a CFX96™ Real-Time PCR Detection System (Bio-Rad) using the following cycling conditions: 95 °C for 5 min, followed by 40 cycles of 95 °C for 15 s, 56 °C for 20 s, and 72 °C for 30 s. The concentration of cDNA of *muc2* was normalized to the concentration of the housekeeping gene 36B4. The ratio of normalized target concentrations ($2^{-\Delta\Delta C}$T method) was used to determine the fold change in gene expression [36].

Histological analysis

Sections of the distal colon were fixed in 10% formalin overnight, after which samples were embedded in paraffin. Using a microtome, 4 µm thick sections were cut. Before staining with Alcian Blue, slides were heated at 60 °C for 30 min followed by two 5-minute baths in xylene. Next, slides were submerged into 100% ethanol twice for one min, after which they were rehydrated in 96%, 80% and 70% ethanol and water, each for 1 min. Slides were then incubated with Alcian Blue for 30 min at room temperature and washed with water to remove excess stain. After air-drying, Vectashield Hardset™ Antifade Mounting Medium (Vector Laboratories) was applied together with a cover slip and hardened at 4 °C overnight. Slides were scanned with a 40x objective (137.766 nm/pixel, SLIDEVIEW VS200, Olympus). ImageJ [37] software was used for crypt length measurements and staining intensity quantification.

Statistical analysis

Statistical analyses and data visualization were performed in R (version 4.1.1) [38], using the R packages data.table (version 1.14.0) [39], rstatix (version 0.7.0) [40], and ggplot2 (version 3.3.5) [41]. The differences between diet and day were tested using a 2-way analysis of variance (ANOVA) for weight change, nutritional intake & alpha diversity, and with a 2-way ANOVA between diet and compartment for all other analyses, followed by Students T-test with a Bonferroni adjustment for significant factors. Prior to ANOVA, data was tested for normality with Shapiro-Wilk and Levene's test was used to test homoscedasticity. A square root transformation was applied when data was not normally distributed. For the sequencing data, samples were subsetted for a minimum of 1000 reads and rarified to a richness of 1763 after which alpha (observed OTUs, Shannon diversity and Simpson index) and beta diversities (PCoA with Bray-Curtis dissimilarity) were calculated using the R package vegan (version 2.5-7) [42] and visualized using ampvis2 (version 2.7.9) [43]. Vegan was used both for permutational multivariate analysis of variance (PERMANOVA) calculations and for the Kendall coefficient of concordance [42]. Silhouette scores were determined to deduce the optimal number of clusters for identification of concordant clusters of bacterial taxa, by Ward clustering based on Spearman correlations [44].

Results

FFD alters intestinal anatomy and decreases colonic mucus production

In order to investigate the role of acute dietary fiber deprivation on mucus secretion and utilization by the gut microbiota, mice were fed either normal chow as a control diet (CD, composition in Supplementary Table 1) or a fiber-free diet (FFD, composition in Supplementary Table 1) lacking cellulose and polysaccharides (two biological replicates; Supplementary Figure 1). Fecal samples were taken immediately before, 2, and 7 days after diet switch, at which point mice were sacrificed and intestinal compartments were sampled. Food intake was not significantly different between groups for either diet or day of the dietary

intervention (ANOVA, p = 0.170 & p = 0.224), and although FFD-fed mice had, on average, roughly 9000 joules per day higher energy intake (ANOVA, R^2 = 0.37, p = 0.043), the overall gain in body weight was not affected by diet (ANOVA, p = 0.527; Supplementary Figure 2). Notably, however, FFD induced a significant shortening of both the small intestine (CD: 36.1 ± 1.0, FFD: 34.1 ± 1.1 cm; T-test, p = 0.007) and the colon (CD: 8.9 ± 0.5 cm, FFD: 7.3 ± 0.6 cm; T-test, p < 0.001; Figure 1A). Consistent with this shortening, there was a marked reduction in the weight of the small intestine, cecum, and colon tissue (emptied of luminal contents) in FFD mice (T-test, p = 0.01, p = 0.01 & p < 0.001 ; Figure 1B).

To further explore FFD-induced changes in mucosal physiology, tissue structure and mucus production were evaluated in colon samples at day 7 (representative images of colon sections are shown in Figures 1C and 1D). There was a trend towards loss of large-scale tissue structure in FFD mice, with a slight, though not statistically significant, reduction in the number of intestinal longitudinal folds per colon section (T-test, p = 0.115; Figure 1E). Crypt height, as measured from the base to the top of the crypt, was significantly reduced in FFD mice (T-test, p < 0.001; Figure 1F). Although the number of mucus-containing goblet cells, as determined by Alcian Blue staining, was not affected by diet (T-test, p = 0.371; Figure 1G), the expression of the *muc2* gene, which encodes the most abundant secreted mucin in the colon, was greatly reduced in FFD mice (T-test, p = 0.002; Figure 1H). These results indicate that acute dietary fiber deprivation greatly impacts intestinal anatomy and colonic mucus production.

FFD reduces luminal contents and mucus secretion

We next evaluated how FFD affects the luminal environment. Water content, as expected, decreased from proximal to distal compartments regardless of diet (ANOVA, R^2 = 0.48, p < 0.001), but also water content in the colon was significantly lower in FFD-fed mice (T-test, p = 0.038; Figure 2A). The wet weight of luminal contents was reduced in FFD mice (ANOVA, R^2 = 0.36, p < 0.001; Figure 2B), though the dry weight was not affected by diet (ANOVA, p = 0.477; Figure 2C). These differences likely reflect the water-retaining capacity of fibers and suggest a decreased nutrient absorption capacity which may result from the shortened intestines in FFD mice and is consistent with the unaffected body weight gain despite the higher energy intake by FFD-fed mice. Next, the percentage of total carbon and nitrogen in the luminal contents was used to establish a carbon to nitrogen ratio (C:N). The C:N ratio is an important measure of nutrient limitation in ecosystems, as N levels in the gut can affect bacterial load [45]. FFD-fed mice had a lower C:N ratio in each intestinal compartment (ANOVA, R^2 = 0.18, p < 0.001; Figure 2D), which could largely be attributed to a reduction in the relative carbon content of the luminal biomass (%C; ANOVA, R^2 = 0.49, p < 0.001; Figure 2E). This may be due to the lack of recalcitrant carbon-rich fiber as well as an alteration in the ratio of assimilable carbon and nitrogen-containing nutrients. In support of the concept of reduced assimilable nutrient levels, quantification of total bacterial load using flow cytometry indicated that there were fewer bacteria per intestinal compartment in FFD-fed mice (ANOVA, R^2 = 0.15, p = 0.007; Figure 2F).

Overall, these data suggest that fiber deficiency results in a marked reduction in overall luminal wet weight contents and microbial load.

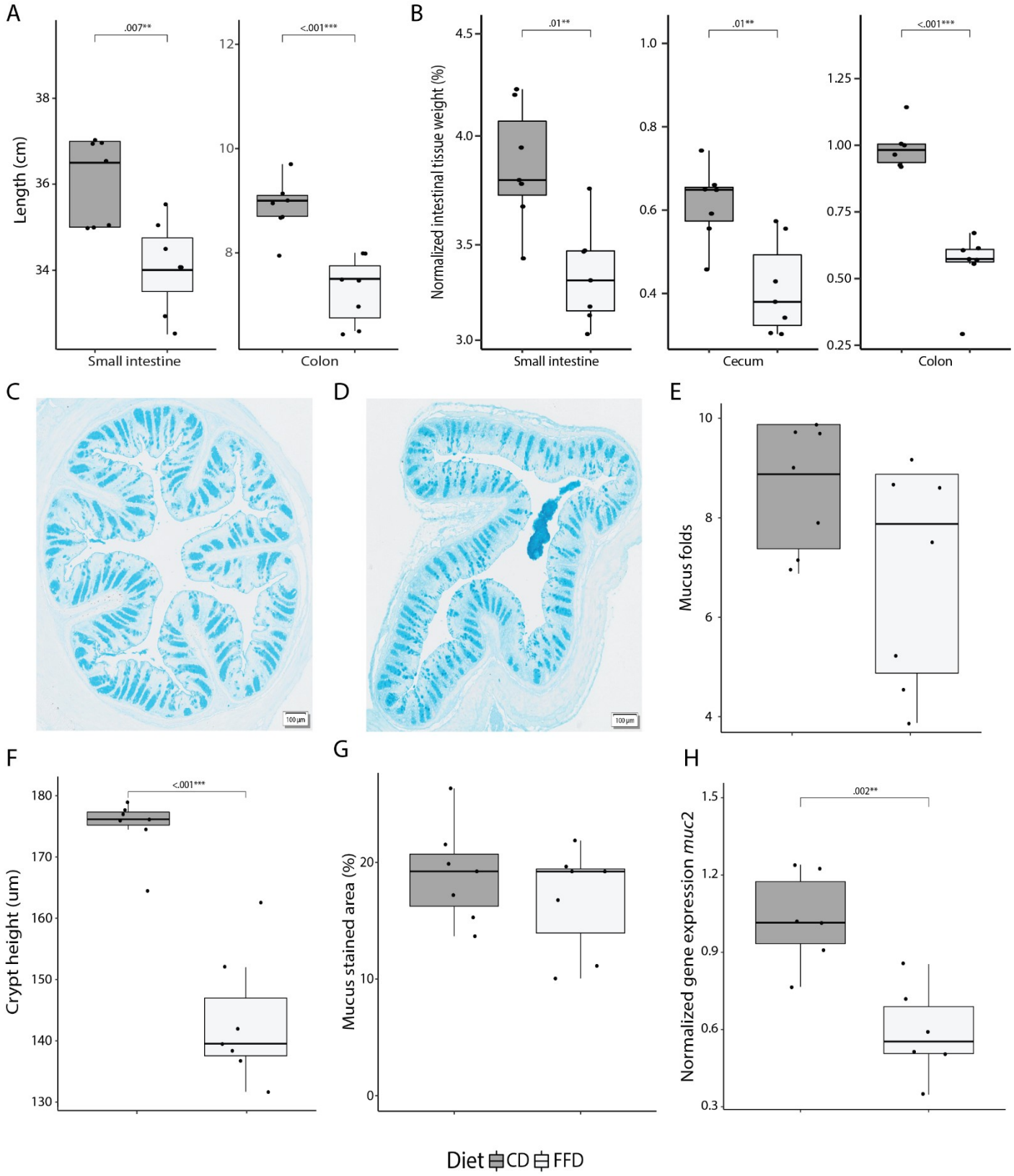

Figure 1. Dietary fiber deficiency affects intestinal anatomy and colonic mucus production

A. Length of the small and large intestine is significantly decreased after 7 days of fiber deficiency (ANOVA, $p < 0.001$) **B.** Fiber deficiency decreases intestinal tissue weight (ANOVA, $p < 0.001$). Values were normalized to the total body weight of the mouse **C.** A representative colon cross-section of a control mouse and **D.** fiber deficient mouse

displaying a loss in tissue structure at 40x magnification **E.** Using a cross-section of the colon, intestinal longitudinal folds were counted. A decreasing trend was observed in fiber-deficient mice **F.** Crypt height, as measured from the bottom to the top of the crypt, significantly decreased with fiber deficiency (T-test, $p < 0.001$). **G.** Quantification of alcian blue, which stains acidic mucus and is an indirect measure for mucus-containing goblet cells, did not show a diet-dependent difference in the colonic cross-sections. **H.** *muc2* expression, as quantified by qPCR, was significantly decreased with a fiber-deficient diet (T-test, $p = 0.002$). Box plots show the group median and interquartile range, with each dot representing a single sample. Dark grey indicates the control diet, light grey the fiber-free diet.

To determine if the FFD-induced anatomical and physiological changes in mucosal tissue affect the amount of mucus secreted into the lumen, secretion was quantified using a previously-established stable isotope probing approach employing an intravenously-administered $^{13}C^{15}N$ threonine tracer [30]. Since MUC2 has a protein domain rich in threonine, proline, and serine, isotopically labeled threonine will be incorporated into the mucin glycoprotein and can thus be used as a read-out for secretion. Mucus secretion was detectable in all compartments, but the overall flux of secreted compounds (calculated by multiplying the amount of ^{13}C with the total amount of carbon and the dry weight of the intestinal contents) per compartment was significantly lower for FFD-fed mice (ANOVA, $R^2 = 0.13$, $p = 0.041$; Figure 2G). This result is in line with the reduced *muc2* gene expression in colonic tissue and indicates impaired mucus secretion due to fiber deficiency.

FFD affects gut microbiota composition

We next evaluated how FFD affects gut microbiota composition using 16S rRNA gene amplicon sequencing combined with flow cytometry to determine absolute abundances of bacterial taxa per intestinal compartment [34]. There was a reduction in bacterial alpha diversity (observed OTU, Shannon diversity and Simpson index) in stool samples of fiber-deficient compared to control mice collected 2 and 7 days after the start of the dietary intervention (ANOVA; $R^2 = 0.3$, $R^2 = 0.34$ & $R^2 = 0.32$, $p < 0.001$ for all measures; Figure 3A). Principal coordinates analysis (PCoA) of Bray-Curtis dissimilarities showed a clustering of day 2 and 7 stool from FFD mice distinct from baseline (Figure 3B), and permutational multivariate analysis of variance supported that diet was a significant driver of stool microbiota composition (PERMANOVA; $R^2 = 0.15$, $p < 0.001$). Notably, there was no statistically-significant difference in microbiota composition between days two and seven (PERMANOVA, $p = 0.73$), indicating that an alternative stable microbiota state had been rapidly induced by FFD. Consistent with the stool diversity, the bacterial alpha diversity in the small intestine, cecum, and colon compartments also significantly decreased at day 7 in FFD-fed mice (ANOVA, $R^2 = 0.37$, $R^2 = 0.61$ & $R^2 = 0.56$, $p < 0.001$ for all measures; Figure 3C). Samples in the PCoA showed clustering by diet and incomplete clustering by intestinal compartment (Figure 3D), although PERMANOVA testing revealed that both diet and compartment were significantly associated with microbiota composition (PERMANOVA; diet: $R^2 = 0.18$, $p = 0.001$, compartment: $R^2 = 0.15$, $p = 0.002$).

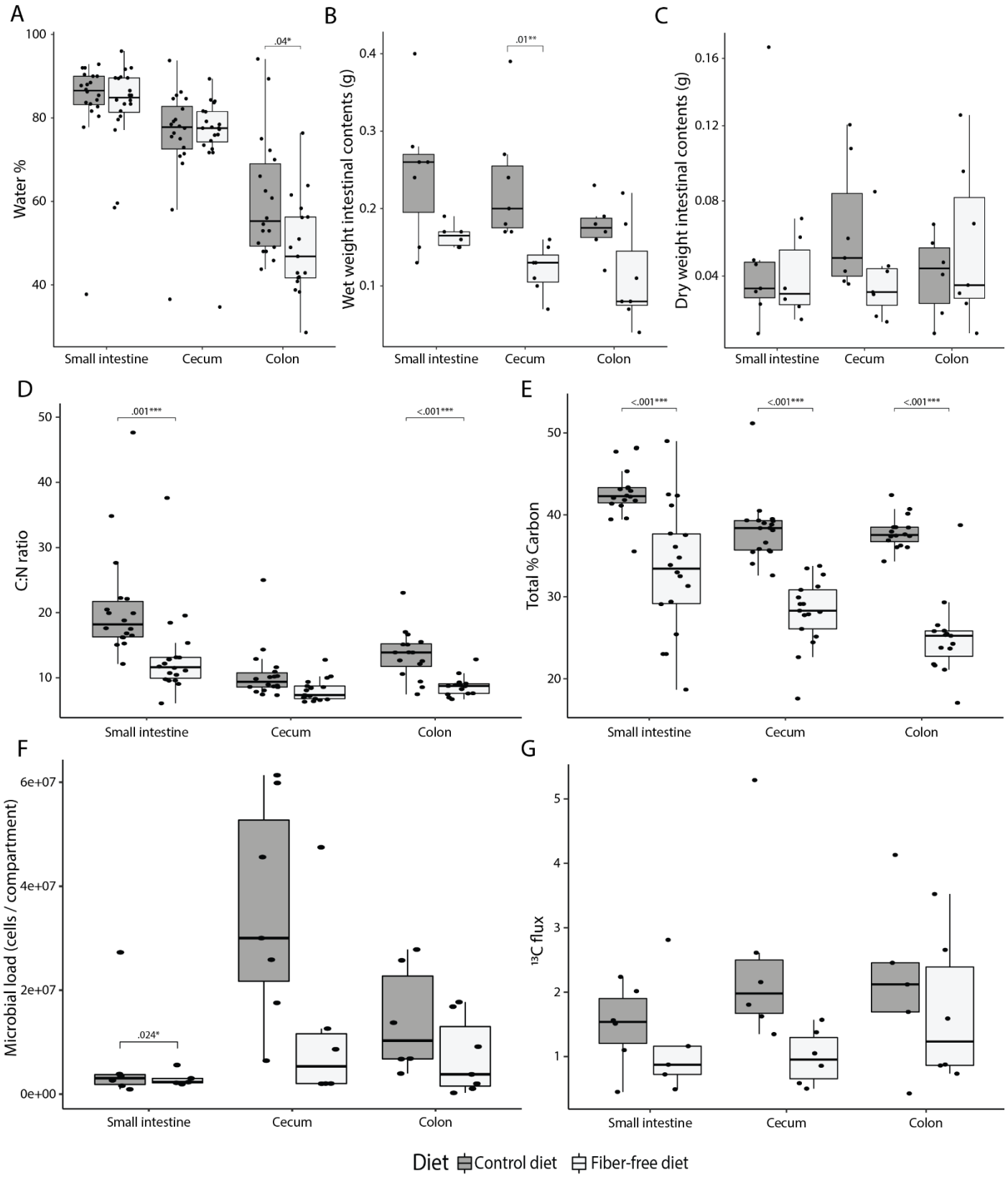

Figure 2: Effect of fiber deficiency on luminal contents and mucus secretion

A. Percentage of water in the intestinal content decreases along the intestinal tract (ANOVA, $p < 0.001$), and is significantly less in the colon of mice fed a fiber-free diet (FFD) compared to a control diet (CD) **B.** Fiber deficiency reduces the wet weight of intestinal contents (ANOVA, $p < 0.001$) **C.** Dry weight of intestinal contents is not affected by diet **D.** Fiber deficiency significantly reduces the carbon to nitrogen ratio (ANOVA, $p < 0.001$) **E.** Total percent of carbon in the lumen is significantly decreased with fiber deficiency (ANOVA, $p < 0.001$) **F.** Fiber deficiency decreases the total amount of bacteria per compartment (ANOVA, $p = 0.007$) **G.** Host-secreted carbon flux - calculated by

multiplying the amount of ^{13}C with the total amount of carbon and the dry weight of the intestinal contents - was significantly lower in FFD-fed mice (ANOVA, p = 0.041). Box plots show the group median and interquartile range, with each dot representing a single sample. Dark grey indicates the control diet, light grey the fiber-free diet.

The taxonomic composition of the intestinal microbiota was typical of previous reports of murine microbiota [4,46], and the most abundant genera included members of the *Bacteroidota* (*Muribaculum*, *Duncaniella*, *Paramuribaculum*, *Alistipes*, and *Odoribacter*), *Bacillota* (*Faecalibaculum*, *Intestinomonas*, and *Lactobacillus*), *Verrucomicrobia* (*Akkermansia*), and *Pseudomonadota* (*Desulfovibrio*) (Figure 3E).

To determine which taxa were altered in their abundance due to FFD, we calculated the mean log2-fold change of the top 50 genera with respect to diet in each intestinal compartment (FFD/CD). This was done for both absolute abundances as well as relative abundances to facilitate comparison to previous studies [28,47,48]. In the small intestine, no genera were significantly altered by diet based on absolute or relative abundances (Figure 4A). In the cecum, 31 taxa were significantly decreased in their absolute abundance such as *Muribaculum, Duncaniella*, *Alistipes* and *Paramuribaculum,* whereas in relative abundance data 37 taxa were significantly altered - both increased and decreased (Figure 4B). Notably, *Akkermansia*, which has previously been reported to bloom in FFD, was in fact not changed in absolute numbers, although it displayed a 1.47 log-2 fold increase in relative abundance. In the colon, 20 taxa were different in their absolute abundance, where 28 were shifted in their relative abundance, again including *Akkermansia* (Figure 4C). As expected, most of the genera affected by diet belonged to the *Bacillota*, including butyrate producers such as *Kineotrix* and *Clostridium,* as well as fiber degrading *Bacteroidota* such as *Muribaculum* [21,49,50]. These results highlight the issue with inferring population dynamics using relative abundance-based methods and indicate that *Akkermansia* does not bloom during dietary deprivation, but rather maintains its population size as other taxa are lost.

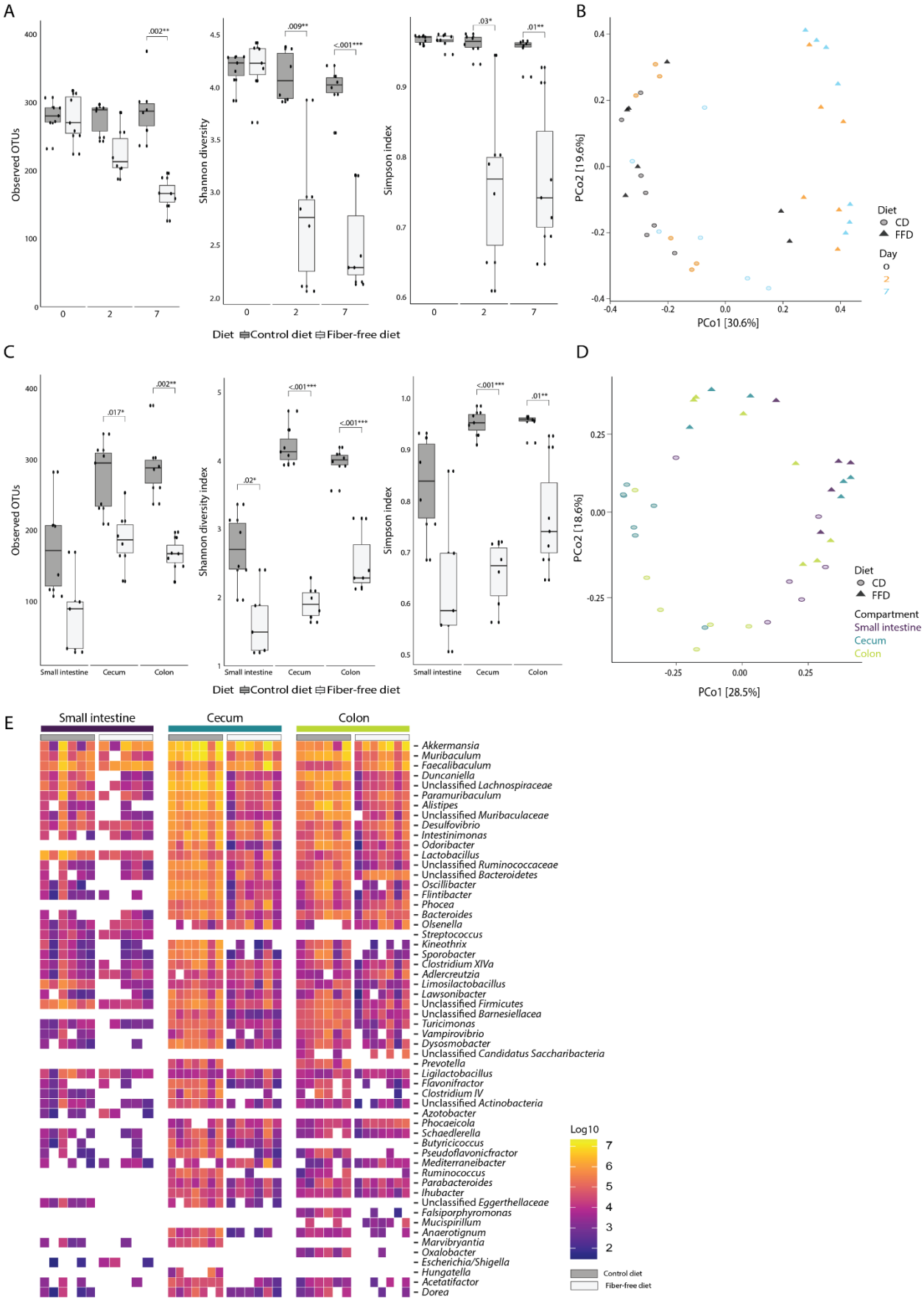

Figure 3. Dietary fiber deficiency decreases microbial alpha-diversity and is a key driver of microbial beta-diversity

Fecal samples were taken 0, 2 and 7 days after dietary intervention. **A.** Fiber deficiency decreases bacterial

richness and alpha diversity (ANOVA, $p < 0.001$ for all measures) in fecal samples taken 0, 2 and 7 days after dietary intervention **B.** Principal Coordinates analysis based on Bray-Curtis dissimilarities shows a significant separation of samples based on diet (PERMANOVA; $p < 0.001$) **C.** After 7 days, fiber deficiency significantly reduces bacterial richness and alpha diversity in all three intestinal compartments (ANOVA, $p < 0.001$ for all measures) **D.** Principal Coordinates analysis based on Bray-Curtis dissimilarities at genus level shows a significant separation by diet (PERMANOVA $p = 0.001$) and incomplete clustering by compartment (PERMANOVA $p = 0.002$). E. Absolute genus abundances, ranked from highest to lowest, separated by diet and intestinal compartment (the 50 most abundant genera are shown). Box plots show the group median and interquartile range, with each dot representing a single sample. Dark grey indicates the control diet, light grey the fiber-free diet.

Taxa that co-vary in abundance across samples may have similar environmental niche preferences, be involved in similar metabolisms, and/or be interacting with one another [51], and can be considered to be an ecological guild [52]. Co-variances in this dataset would be expected to be largely driven by host diet, yet could also arise from other factors such as inter-host variability. Concordance analysis indicated that there was indeed concordance of bacterial genera (Kendall concordance test; $p < 0.001$ for all compartments). To identify clusters of concordant genera, Ward clustering based on pairwise Spearman correlations of absolute abundances was performed. Silhouette scores indicated that genera could be optimally clustered into two groups in each compartment, and that cluster membership varied by compartment (Figure 4 A-C). In the small intestine, diet did not influence the absolute abundance of either cluster which corresponds to no significant differences found in the genera (Figure 4D). In the cecum, however, cluster Ce1, which was the numerically-dominant group of bacteria in CD, was dramatically reduced in FFD (T-test, $p = 0.01$). Cluster Ce2, which included *Akkermansia*, *Faecalibacilum*, unclassified *Bacteroidales*, and *Desulfovibrio*, was not significantly affected by diet (T-test, $p = 0.77$), although as observed for the individual taxon dynamics, relative abundance analysis gave contradictory results (Figure 4E). A similar trend was observed in the colon, with the dominant cluster Col1 decreased in FFD (T-test, $p = 0.048$) and the less abundant cluster containing *Akkermansia* unchanged (T-test, $p = 0.3$, Figure 4E). These results provide evidence that dietary fiber deprivation does not induce a bloom of mucus degraders but instead induces a dramatic loss of non-mucus-degrading bacterial diversity.

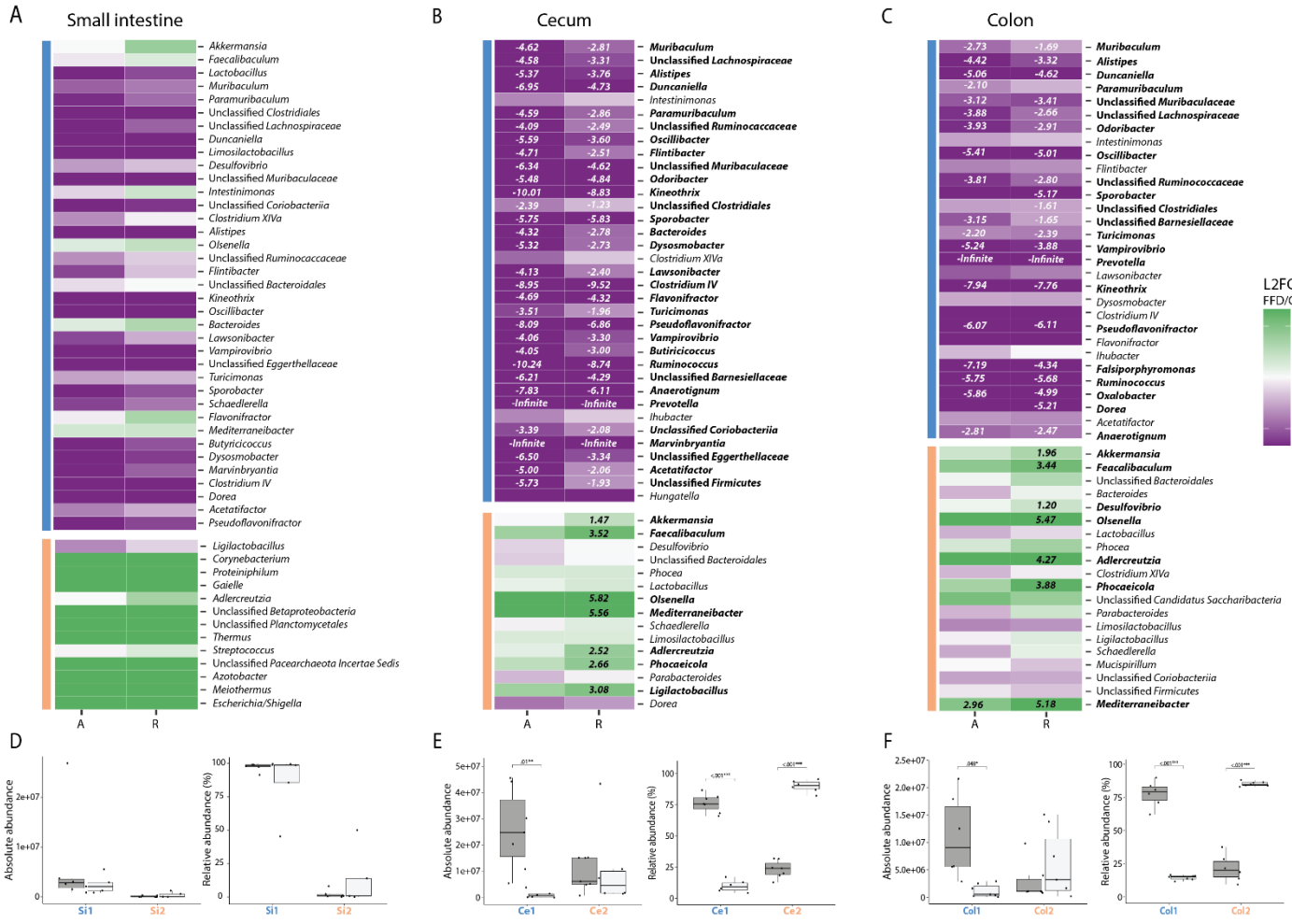

Figure 4. Dietary fiber deprivation induces significant shifts in the composition of the intestinal microbiota
A.-C. Log-2 fold change (L2FC) of each genus with respect to different diets (fiber-deficient/control) in each intestinal compartment, for both absolute (A) and relative (R) abundances. Taxa with significant L2FC values are printed and the L2FC value is given. Taxa are split by concordance group (i.e., cluster) and ranked from highest to lowest mean abundance. The absolute and relative abundances of taxa are grouped by cluster, diet and compartment and displayed in box plots showing the group median and interquartile range, with each dot representing a single sample. Dark grey indicates the control diet, light grey the fiber-free diet. **A + D** for the small intestine**, B+E** for the cecum, **C + F** for the colon.

Discussion

Modern diets are increasingly lacking dietary fibers and studies have shown that numerous diseases have a direct link to a reduced fiber intake [12–16]. Lack of fiber has been linked to a diminished mucosal layer, yet thus far, the processes behind the depletion of the mucus layer remain poorly understood [26,28,29]. By using quantitative microbiome profiling and stable isotope probing, this study reveals that mucus layer depletion upon fiber deprivation is not microbiota-mediated but stems from a direct host response down-regulating mucus production. Besides mucus production, fiber deficiency affected many other host and microbiota endpoints.

Plant materials contain many more components in addition to fiber that can have bioactivity for the microbiome and the host. It should be considered, that with the elimination of fiber from the diet,

polyphenols as well as other plant components are also removed and could contribute to the observed effects. Additionally, that, the replacement of fiber with glucose potentially may influence the results, which is a limitation in our study as well as others [26–28].

Fiber deficiency dramatically affects the intestinal structure, decreasing crypt height and shortening the length of both the small and large intestine (Figure 1). An important function of the intestine is to absorb nutrients, thus when intestines are shortened there is less surface area to do so, and less area for goblet cells and less area to form crypts. The intestinal shortening corroborates previous work by Desai et al. and Hunt et al., which show shortening after long-term fiber deficient diets (40 and 21 + 122 days) as well as 1- and 4-day oscillations in diet [18,20]. Our work shows that this effect already occurs after 7 days. Intestinal shortening due to a fiber deficient diet as well as a high fat and fiber deficient diet has been found to be microbiota-independent, suggesting that this is not due to microbial factors such as lack of butyrate [27,28]. The gut harbors many intestinal hormones, some of which are influenced by diet. GLP-1 secretion is stimulated by fiber [53] and GLP-2 has been shown to play an important role in intestinal size, whereas when acting together, they promote intestinal healing [54,55]. Knockout of GLP-1 and GLP-2 receptors in a mouse model, however, was insufficient to counteract fiber deficiency-induced intestinal shortening, suggesting the presence of another not-yet identified mechanism [47].

Elemental and isotope analysis were used to study changes in the elemental stoichiometry of the gut biomass and mucus secretion *in vivo*. EA-IRMS results showed that there was a lower percentage of total carbon in the gut lumen in FFD-fed mice (Figure 2), most likely from the lack of dietary fibers. The total wet weight of the intestinal lumen content was also decreased in FFD-fed mice (Figure 2). An important function of fiber is to retain water in the colon to increase stool bulk [7], which explains the decreased water content on the FFD seen for the colon yet not for the small intestine or cecum. The water content of mucus is 98% [18], thus less secreted mucus might partially explain the decreased water content. There was a decreased flux of secreted mucus into the gut lumen in FFD-fed mice, as determined using a $^{13}C^{15}N$ threonine tracer as a proxy for intestinal mucus secretion [30], as well as a reduced expression of the *muc2* gene, which encodes the major secreted mucin in the colon (Figure 2). These data indicate that fiber deficiency leads to a decrease in mucus production rather than an increase in microbial mucus degradation activity, as previously implied, supported by unchanged abundances of mucus degraders [28]. In accordance with these results, thinning and increased permeability of the mucus layer as well as reduction in colonic goblet cell numbers due to fiber deficiency have been previously observed in multiple studies [26–28]. This is further supported by the observation that mucus degrading taxa, including *Akkermansia muciniphila*, unclassified *Bacteroidales*, and *Desulfovibrio* did not increase in absolute abundance in fiber-deprived mice. It should be noted that the estimated abundances of rarer taxa are subject to larger measurement error associated with the sequencing depth and are therefore intrinsically of lower accuracy.

In contrast to the mucus-degrading specialists, other taxa quantitatively decreased upon fiber deprivation as reflected by the dramatically reduced alpha diversity of the intestinal microbiota across all compartments, which is in line with previous reports (Figure 3) [26,28,47,56]. Beta diversity showed a clear clustering based on diet (Figure 3). Clustering per compartment was evident for the CD but incomplete for the FFD. Low et al. also found that beta diversity and microbial community per compartment was altered by their high-fat and fiber deficient diet, although they observed clustering to be more apparent on the intervention diet compared to the control diet [48]. Absolute abundance analysis showed that fiber degraders and butyrate producers such as *Lachnospiracea* and *Muribaculum* were lost on the FFD whereas mucus degraders were not influenced by diet (Figure 4). Loss of butyrate-producing bacteria may be detrimental as butyrate is an energy source for colonocytes and plays an important role in gut health [57]. However, butyrate has been found not to affect *muc2* expression unless glucose is restricted [58], and therefore the loss of butyrate producers may not be the reason for the decrease in *muc2* expression observed in this study.

The loss of abundant fiber-associated taxa is likely the reason that an increase in mucus degraders during fiber deprivation has previously been reported [28,48]. Our results, however, showed that such compositional microbiome analysis greatly skews the interpretation of population dynamics and underscores the importance of quantitative microbiome profiling [34].

Conclusions

In conclusion, diet, and in particular fiber, plays a vital role in host health and establishment of the gut microbial community, and thus it is vital to understand its direct and indirect effects on the host as well as the microbiota. Murine studies reversing fiber-deficient diets with normal chow, or transplanting gut microbiota from chow-fed mice to FFD-fed mice, show that the microbial community largely shifts back to a normal composition, whereas the intestines remained shortened, and the thickness of the mucus layer diminished for a prolonged period [27,48]. Thus, microbial shifts are acute yet reversible, whereas physiological changes and damage thereof are more long-lasting. Potential interventions should thus not target the inhibition of mucus degraders, but rather the promotion of fiber-degrading butyrate producers.

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Economics of the Republic of Austria under the license number BMWFW-66.006/0001-WF/V/3b/2016. Dietary intervention was randomized but researchers processing samples and analyzing data were aware which cohort corresponded to which diet of the randomization plan. Animal experiments were performed at the Max Perutz Laboratories of the University of Vienna, Austria.

Data Availability Statement: The 16S rRNA gene amplicon sequencing data has been deposited at the Sequence Read Archive (https://www.ncbi.nlm.nih.gov/sra) under the BioProject accession ID PRJNA800211. FACS data can be found in the flow repository (http://flowrepository.org/) with the ID FR-FCM-Z4UJ.

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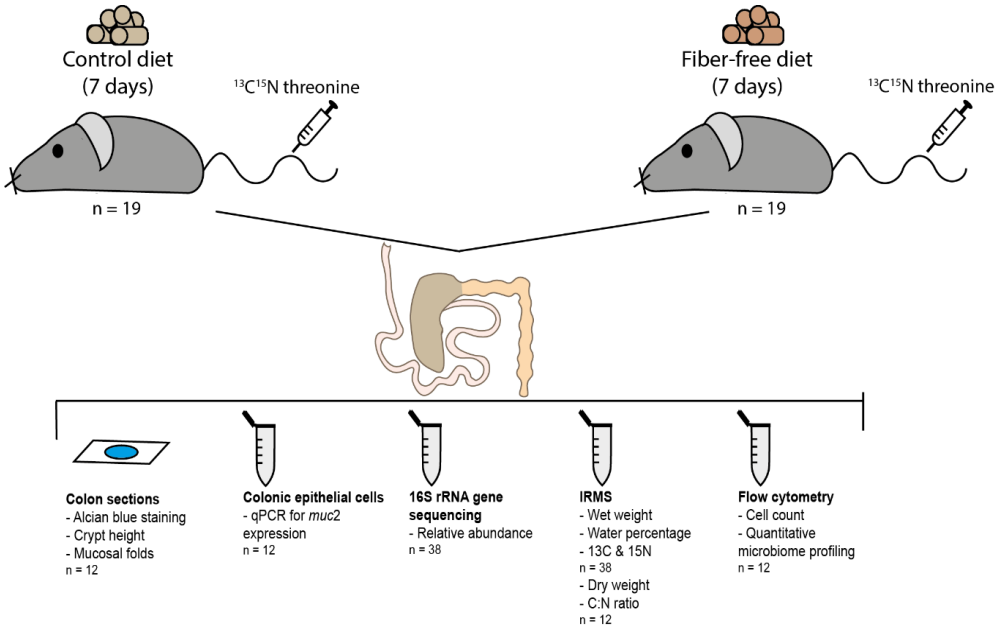
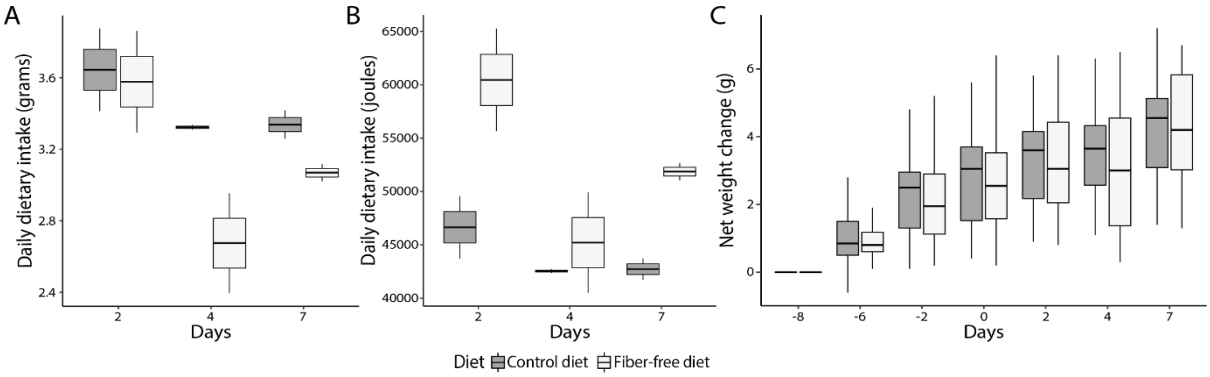
Supplementary materials



Supplementary figure 1: Experimental set-up of harvested samples; after an initial week of acclimatization on a standard chow or control diet, half the mice were switched to a fiber-free diet. All mice were sacrificed after 7 days of dietary intervention.



Supplementary figure 2: Daily dietary intake in grams (**A**) and joules (**B**) per mouse per day. (**C**) The net weight change per mouse during the experiment. Day 0 indicates the day the diets were switched. Box plots show the group median and interquartile range. Dark grey indicates the control diet, light grey the fiber-free diet.

Supplementary Table 1: Nutritional composition of the diets

	Normal chow	**Fiber Free diet**
Crude protein (%)	19	18.5
Crude fat (%)	3.3	7.1
Crude fiber (cellulose) (%)	5 (16.9 = NDF, 7.1 = ADF)	-
Crude ash (%)	6.4	5.3
Starch (%)	35.9	-
Sugar (Dextrose/glucose) (%)	5.4	65.1
Metabolisable Energy (MJ/kg)	**13.5**	**16.9**
Protein, kcal %	33	18.3
Fat, kcal %	9	15.8
Carbohydrates, kcal %	58	65.8

Chapter 3

Nutrient niche specificity for glycosaminoglycans is reflected in polysaccharide utilization locus architecture of gut *Bacteroides* species

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Submitted

Abstract

Introduction: Glycosaminoglycans (GAGs) present in the mucosal layer can be used as nutrients by certain intestinal bacteria, particularly members of the *Bacteroides*. GAG abundances are altered in some diseases such as inflammatory bowel diseases, which may affect microbial composition and activity, and it is therefore important to understand GAG utilization by members of the gut microbiota.

Results: Using growth assays, transcriptomics, and comparative genomics, we found that not all *Bacteroides* species able to degrade chondroitin sulfate (CS) could also degrade hyaluronan (HA), despite having lyases which act on both compounds.

Conclusion: We propose that in the model organism *Bacteroides thetaiotaomicron*, the lyase BT_3328 in combination with surface binding proteins BT_3329 and BT_3330 and potentially BT_4411 are involved in HA breakdown. Furthermore, degradation of both compounds provides public goods for other *Bacteroides*, including non-degraders, suggesting that cooperative degradation as well as cross-feeding may be widespread in the mucosal glycan utilization clade.

Introduction

The human gastrointestinal tract is lined with a layer of secreted mucus that varies in thickness and composition (Pelaseyed et al., 2014). Not only does mucus protect epithelial cells from invasion by pathogens, it also harbors commensal and potentially-beneficial bacteria (Martin-Gallausiaux et al., 2021; Pelaseyed et al., 2014; Sato et al., 2020; Yuan et al., 2020). Beneath the secreted mucus layer there is another defense barrier for the epithelial cells, namely the glycocalyx. This is a thin layer directly covering the epithelial cells consisting of glycoproteins and glycolipids (Moran et al., 2011). Proteoglycans are a subclass of glycoproteins that have glycosaminoglycans (GAGs) attached to a protein backbone. Two predominant intestinal GAGs are chondroitin sulfate (CS) and hyaluronan (formerly known as hyaluronic acid, HA). CS is made up of disaccharides containing glucuronic acid (GlcA) and N-acetyl-galactosamine (GalNAc) and, as the name suggests, can be sulfated on multiple positions (Figure 1A). HA consists of repeating monomers of GlcA and N-acetyl-glucosamine (GlcNAc) (Bartlett & Park, 2010). Similar to the secreted mucus layer, there is a constant turn-over of the glycocalyx, resulting in breakdown products that are released into the mucus, thus supplying bacteria in the gut lumen with additional potential nutrient sources (Moran et al., 2011). GAGs are not only an important microbial nutrient source in the healthy gut, but also in intestinal inflammatory diseases such as ulcerative colitis, where they have been shown to be enriched in the intestinal mucosa (Belmiro et al., 2005). Bacteria capable of degrading GAGs have been shown to proliferate during colitis development (Lee et al., 2009), and it is thus vital to have a better understanding of GAG metabolism by intestinal bacteria. Additionally, hyaluronan supplements have been intensively studied for symptom release in osteoarthritis, inflammation and necrotizing enterocolitis (Asari et al., 2010; Chaaban et al., 2021; Hewlings et al., 2019).

Bacteroidota are one of the main phyla in the intestinal tract (Eckburg et al., 2005) and are capable of degrading both dietary as well as host-derived glycans (Salyers et al., 1977). Enzymes required for glycan degradation include glycoside hydrolases and polysaccharide lyases, which are organized on the genome into polysaccharide utilization loci (PULs) (McKee et al., 2021). *Bacteroides thetaiotaomicron* (*B. theta*) is one of the best characterized gut commensal bacteria and is a widely used model organism to study bacterial physiology and microbe-host interactions. In *B. theta*, degradation of CS and HA is mediated by PUL 57 (Martens et al., 2008; Raghavan et al., 2014). Within this PUL, three periplasmic lyases have been identified for CS-HA degradation (BT_3324, BT_3350 & BT_4410), with two having a higher affinity for CS (BT_3324 and BT_3350) and the third (BT_4410) having a higher affinity for HA (Raghavan et al., 2014). Additionally, a novel surface lyase with a high affinity for CS (BT_3328) has recently been identified (Ndeh et al., 2018, 2020). Although *B. theta* PUL architecture for CS and HA degradation has been well-studied, the ability of other gut *Bacteroides* to use these compounds and the conservation of this PUL among the gut *Bacteroides* remains less well understood.

The concept of 'public goods' is an essential aspect of commensal and mutualistic interactions in the gut microbiota. Some bacteria can promote the growth of others by providing them with breakdown products of more complex compounds that they themselves cannot degrade (Banerjee et al., 2018; Rakoff-Nahoum et al., 2014). In marine environments, Bacteroidota have been suggested to be largely selfish utilizers of polysaccharides, meaning that they use cell-surface enzymes for degrading large polysaccharides and directly transport breakdown products into the periplasm (Reintjes et al., 2019). In the gut, it has been shown that some members of the Bacteroidales such as *B. theta* and *Bacteroides ovatus* provide public goods during degradation of the dietary polysaccharides levan and inulin to non-degrading bacteria. Additionally, breakdown products for the same polysaccharide can differ depending on the species responsible for degradation (Rakoff-Nahoum et al., 2014). This potential for public good provisioning has not been studied for host glycans. In this study, we therefore aimed to examine the degradation abilities of CS and HA by a diverse panel of intestinal *Bacteroides* species in order to extend current knowledge on the CS-HA PUL as well as evaluate the extent of potential public good provisioning and utilization due to degradation of these two host-derived glycans among the *Bacteroides*.

Materials & methods

Carbohydrates

Chondroitin sulfate A sodium salt (CS) from bovine trachea, hyaluronic acid sodium salt (HA) from *Streptococcus equi* and D-glucuronic Acid (GlcA) were obtained from Merck (Germany). Glucose, N-acetyl-D-galactosamine (GalNAc) and N-acetyl-D-glucosamine (GlcNAc) were obtained from Carl Roth (Germany).

Culturing of Bacteroides strains

In total, 17 different *Bacteroides* species (Supplementary Table 1) were cultured under anaerobic conditions (atmosphere: 85% N2, 10% CO2, 5% H2, Coy Laboratory Products, USA) at 37 °C using M9 minimal media without glucose (unless stated otherwise, see Supplementary Table 2) (Neidhardt et al., 1974) supplemented with 0.05 g cysteine, 1ml 0.5% hemin, 0.5 ml 0.5% vitamin K1, 0.1 ml 2% FeSO4 and 0.05 ml 0.01% vitamin B12 per liter M9. Either 0.5% CS, HA, or glucose was added as a carbon source. All cultures were pre-grown overnight on M9-glucose before being switched to alternative carbon sources. Cultures were then grown for at least 16 hours in a plate reader (Multiscan Go, Thermo Fischer, Germany) inside the anaerobic chamber. Growth was measured in the plate reader via optical density (OD_{600}) every 30 min with 10 s shaking before measurement. Cultures were grown in triplicate per run and at least two biological replicates were performed. Cultures that did not grow on the positive control (glucose) were excluded as values for the positive control were used for normalization. For each strain, growth rate, generation time, and maximum optical density was calculated in R (R Core Team, 2021) using the Growthcurver package (Sprouffske, 2020). Raw data was blanked by subtracting the starting OD, and all data was trimmed to 19 hours as stationary phase was reached. For growth on conditioned media, values were normalized

to the maximum OD_{600} value obtained on glucose for comparison between conditions and strains. Graphs were visualized using the R package Pheatmap (Kolde, 2019).

Conditioned media

Conditioned media (CM) was prepared by growing bacteria until late log phase, after which cultures were harvested, spun down at 13.4rpm for 10 minutes, and the supernatant was filtered twice using a 0.2 µm poresize. Conditioned media was diluted 1:1 with fresh M9 medium without an additional carbon source, after which cultures were inoculated and introduced into the plate reader. All CM was incubated at 37 °C and measured as negative control to prevent contamination from the donor strain.

Fluorophore-assisted carbohydrate electrophoresis

To determine if any sugars were left in the CM, a fluorophore-assisted carbohydrate electrophoresis (FACE) was performed according to (Midura et al., 2018). Briefly, CM was vacuum-dried and then incubated with 5 µl 2-Amino-9(10H)-acridinone (AMAC) solution at 37 °C for 18 hours. FACE gels were prepared using 25 µl 10% ammonium persulfate and 5 µl TEMED per 5 ml of acrylamide solution. Gels were loaded with 3 µl AMAC-labelled CM and run for 50 min at 300 V and 60 mA in a chamber with chilled TBE.

Transcriptomic analysis of Bacteroides thetaiotaomicron

RNA was extracted from B. theta cell pellets grown on either CS, HA or a mixture containing GalNAc, GlcNAc, and GlcA (harvested from 30 ml late log phase cultures) using the the innuPREP RNA Mini Kit 2.0 (Analytik Jena) following the manufacturer's protocol. Residual DNA in the total RNA extracts was digested using TURBO DNAse (Thermo Fisher) following the manufacturer's instructions. Absence of residual DNA contamination was confirmed by 16S rRNA gene targeted PCR (using primers 515F https://www.ncbi.nlm.nih.gov/pubmed/26271760; and 806R https://doi.org/10.3354/ame01753, following the amplification protocol published in Pjevac et al., 2021). The riboZero Plus Kit (Illumina) was used to deplete ribosomal RNA (rRNA) from the total RNA extracts, following the manufacturer's protocol. Single-index barcoded sequencing libraries were prepared from rRNA-depleted RNA using the NEBNext® Ultra™ II Directional RNA Library Prep Kit for Illumina (NewEngland Biolabs) following the manufacturer's protocol. Sequencing libraries were then pooled and sequenced on a HiSeq3000 (Illumina) in paired-end mode (150 cycles, 2x 75 bp reads) at the Biomedical Sequencing Facility (BSF) of the CeMM Research Center for Molecular Medicine of the Austrian Academy of Sciences/Joint Microbiome Facility (JMF) of the Medical University of Vienna and the University of Vienna (project ID JMF-2012-5).

Reads were quality filtered and trimmed with bbduk (ref=adapters. ktrim=r, k=23, mink=11, hammingdistance=1, qtrim=r, trimq=28, minavgquality=15, minlength=50) and mapped to the *B. theta* reference genome (Xu et al., 2003) using bbmap (minratio=0.96324, pairedonly=t, ambiguous=toss/best/all, Bushnell,

n.d.). Mapped read pairs were counted using featureCounts (-s 2) (Liao et al., 2014). One library failed and was removed from further analysis. Next, DESeq was used to analyze count data using the standard pipeline with alpha = 0.05 (Love et al., 2014). Genes were compared with CS against the mix, HA against mix and CS against HA. Genes with an adjusted p-value < 0.05 and a log-2 fold-change smaller than -2 or greater than 2 were considered to be significantly down or up regulated. Vegan was used for permutational multivariate analysis of variance (PERMANOVA) (Wagner et al., 2020).

Comparative genomics

Orthofinder, using default settings, was used to compare relevant CS-HA degradation PULs from *B. theta* with the 17 other *Bacteroides* species used for CS-HA growth analysis (Emms & Kelly, 2019). Degradation abilities were assigned based on growth results on CS and HA, and those orthogroups only present in CS-only and/or CS-HA degraders were filtered out. Next, 53 *Bacteroides* genomes (translated CDS) were downloaded from NCBI and BlastP was used to blast specific genes from the *B. theta* PULs against these genomes to create a gene presence/absence table (cut-off evalue = $1e^{-40}$). To create a phylogenetic tree, 43 markers were aligned and concatenated using default settings in CheckM for the lineage workflow (Parks et al., 2015) after which a model was estimated with modelFinder (Kalyaanamoorthy et al., 2017). Using Bayesian information criterion scores, the best-fit model was LG+F+R3. Next a tree was calculated using default settings in IQ-TREE 2 (Minh et al., 2020) with the addition of ultrafast bootstraps (Hoang et al., 2018). For visualization, the tree was then uploaded to iTOL (Letunic & Bork, 2021) and combined with the gene presence/absence data.

Results

Not all CS degraders can degrade HA

Until recently, it was believed that all *Bacteroides* capable of degrading CS were also capable of degrading HA (Ndeh et al., 2020; Salyers et al., 1977). To evaluate this, we grew 17 *Bacteroides* species on a minimal media containing either 0.5% CS or HA (Figure 1B). Literature states structures of CS and HA are similar despite the fact that CS is heavily sulfated and they have different monosaccharide compositions as stated previously (Bartlett & Park, 2010). We initially hypothesized that HA degradation capability will be more widespread, and only strains encoding the requisite sulfatases will be able to degrade CS. In total, seven species were able to grow on both CS and HA (*B. acidifaciens, B. caccae, B. clarus, B. finegoldii, B. ovatus, B. thetaiotaomicron,* and *B. xylanisolvens*; Figure 1B). Five strains grew on CS but not on HA (*B. cellulosilyticus, B. eggerthii, B. intestinalis, B. oleiciplenus,* and *B. stercoris*). Additionally, five strains could not degrade either compound (*B. dorei, B. fluxus, B. fragilis, B. uniformis,* and *B. vulgatus*). Strains were also tested for the ability to grow on the monosaccharide subunits of CS (GalNAc) and HA (GlcNAc), and most species were able to grow on both (Figure 1B), with the exception of *B. clarus* and *B. cellulosilyticus*, which

were not able to grow on GalNAc, and *B. oleiciplenus*, which was not able to grow on GlcNAc. For each strain, the growth rate (Figure 1C) was calculated. There were differences between individual strains but no clear distinction between degradation groups.

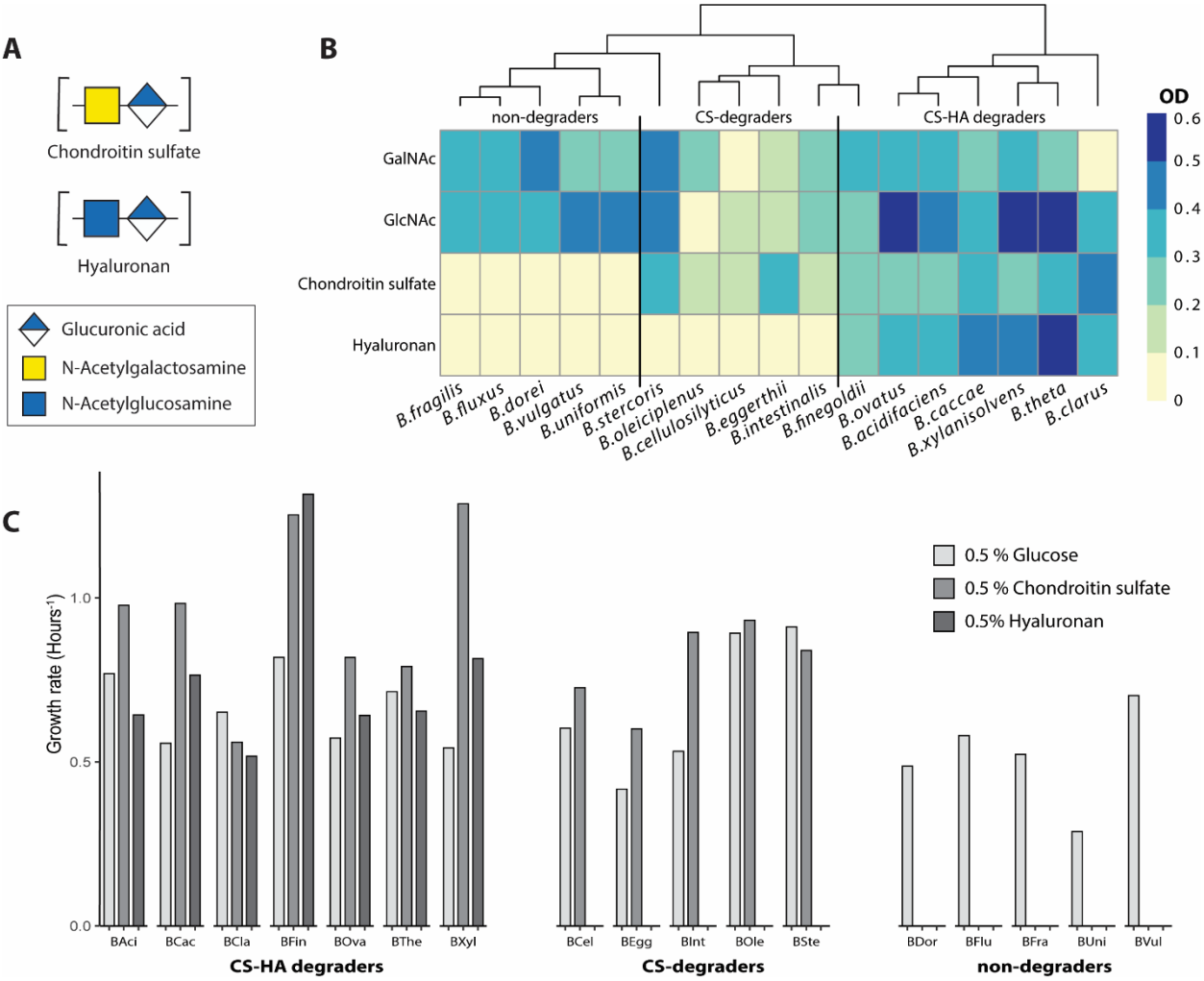

Figure 1: Not all strains capable of degrading CS can also degrade HA

Growth metrics for 17 *Bacteroides* species on 0.5% of glucose, chondroitin sulfate (CS) or hyaluronan (HA). All growth was performed in triplicates with at least two biological replicates. A) Average final optical density (OD_{600}) at 19 hours for 17 *Bacteroides* strains. Almost all species can degrade GalNAc and GlcNAc whereas there are distinct groups for those which can degrade both chondroitin sulfate (CS) and hyaluronan (HA), CS-only or neither. OD < 0.1 was considered to be no growth. B) Growth rate ($hours^{-1}$) measured from the exponential part of the growth curve. BAci = *B. acidifaciens*, BCac = *B. caccae*, BCel = *B.* cellulosilyticus, BCla = *B. clarus*, BDor = *B. dorei,* BEgg = *B. eggerthii,* BFin = *B. finegoldii*, BFlux = *B. fluxus*, BFra = *B. fragilis,* BInt = *B. intestinalis,* Bole = *B. oleiciplenus,* BOva = *B. ovatus*, BSte = *B. stercoris,* BThe = *B. thetaiotaomicron*, BUni = *B. uniformis,* BVul = *B. vulgatus,* BXyl = *B. xylanisolvens*.

Largely overlapping transcriptional regulation of CS and HA utilization by Bacteroides thetaiotaomicron

B. theta is one of the best characterized gut commensal bacteria and is a widely used model organism. To evaluate if *B. theta* has separate pathways for CS and HA degradation, it was grown on either CS or HA and subjected to RNA sequencing. As an additional control, *B. theta* was grown on a mixture of GalNAc,

GlcNAc, and GlcA. On average, 4,663 genes were expressed per library (Supplementary table 3). Nutrient source explained 69% of the total variation in gene expression profiles (PERMANOVA; p = 0.002). When compared to growth on the simple sugar mixture control, there were 802 genes upregulated and 88 downregulated during growth on CS (padj ≤ 0.05 and ≥ 2 log2 fold change; Supplementary table 4), and 399 genes upregulated and 219 downregulated during growth on HA. When directly comparing gene expression profiles during growth on CS or HA, there were 23 genes downregulated on HA and 1 gene upregulated (Supplementary Table 4). For CS degradation there was one PUL with unknown function which showed consistently higher transcription across all genes on CS (PUL 52; Figure 2 & Supplementary table 5). This PUL runs from BT_3235 to BT_3244 and consists of hypothetical proteins and two sus-D homologs. This PUL does not seem to encode known sulfatases, meaning that all sulfatases required for the degradation of CS are found in the characterized PUL for CS and HA degradation, PUL 57. This PUL ranges from BT_3324 to BT_3350 with the addition of BT_4410 being co-regulated by the glycoside hydrolase (BT_3334) in the PUL. Genes previously reported in relation to degradation of CS and HA were manually verified yet had differential expressions below a two log2-fold-change or were found not to have a significant p-value. Our results do not indicate a difference in expression between CS or HA for any of the genes in PUL 57 (Figure 2), thus they do not suggest that there is a separate pathway in *B. theta* for HA degradation. However, the results do show upregulation of PUL 57 when comparing either of the GAGs with simple sugars, validating the transcriptomic approach.

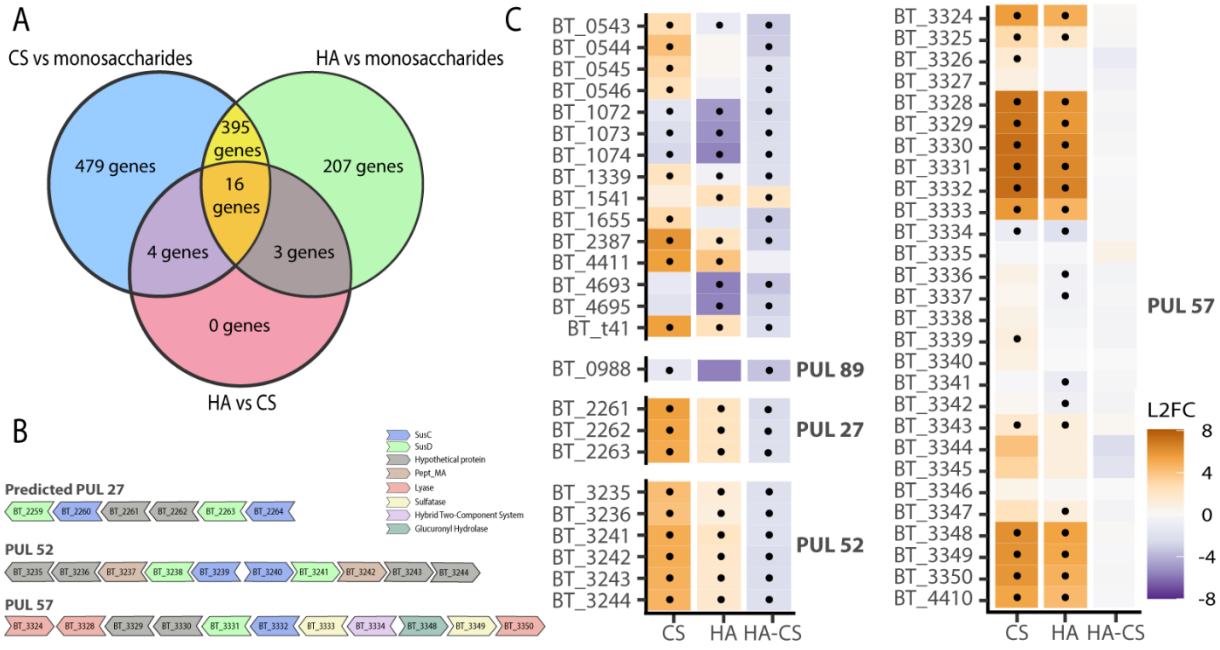

Figure 2: Transcriptomics does not indicate a separate pathway for HA degradation
B. theta was grown on either CS, HA or a control mix containing GalNac, GlcNac or GlcA. A) Venn diagram showing overlapping differentially expressed genes in the different growth conditions. B) Polysaccharide Utilization Loci (PUL) containing differentially expressed genes for CS and/or HA. C) Heatmap showing log 2 fold change (L2FC) for CS – mix, HA-mix or HA compared with CS. Genes are grouped by PULs. PUL57 is the known PUL for CS & HA degradation and does not differ for CS or HA. Dots indicate statistically significant genes (p < 0.05 and L2FC of either ≥2 or ≤ -2).

CS and HA degradation ability is reflected in the gene repertoire of gut Bacteroides

As transcriptomic results did not suggest a separate pathway for HA degradation, we suspected there may be specific genes necessary for HA degradation that were lost in CS-only degraders. First, we compared gene orthologs of *B. theta* to the 17 *Bacteroides* strains used for physiological experiments. In total, 1,193 orthogroups were assigned using 93.2% of the genes. Using the orthogroup gene counts table, we selected only those orthogroups which contained genes for CS and/or CS-HA degraders yet did not contain genes from non-degraders. This resulted in four orthogroups; one for CS-only degraders (OG0003539), one for all degraders (OG0002347) and two for CS-HA degraders (OG0002608 & OG0003140) (Supplementary table 6). The orthogroup for all degraders contained the known CS lyase, BT_3350 (Martens et al., 2008). The two orthogroups containing the other known CS-lyases (BT_3324 in OG0002222 and BT_4410 in OG0002357; Raghavan et al., 2014) contained genes from the non-degrader *B. uniformis*. For the CS-HA degraders the two orthogroups contained the hypothetical protein BT_4411, and the hypothetical protein BT_3328.

Next, a phylogenomic tree of 53 gut *Bacteroides* strains was created to determine if there was phylogenetic conservation of CS and HA degradation ability (Figure 3A). We did not observe a phylogenetic separation between degraders and non-degraders, nor between CS and CS-HA degraders. CS degraders were spread over numerous clades. Although most CS-HA degraders cluster together, *B. clarus* prevents CS-HA degraders from being a monophyletic taxon, which could suggest that the CS-only phenotype occurs due to lineage-specific gene loss.

We then wanted to evaluate the conservation of the previously identified *B. theta* genes of interest among the gut *Bacteroides* and thus used BlastP to identify the gene content of other *Bacteroides* species (Figure 3B). An incomplete cluster of *Bacteroides* strains containing the lyase BT_3328 was present, however *B. pyogenes* and *B. clarus* were in separate clades. Predicted PUL 27 and PUL 52, which were upregulated in *B. theta* when grown on CS, were also included in the Blast search (Supplementary table 5). For PUL 27, the genes were only found in 2-3 other *Bacteroides* species. For PUL 52 there was a wider distribution across the different species, although there was no clear distinction between CS and/or HA degraders in comparison to non-degraders. Although there was not a distinguished CS-HA degrader clade, all tested HA-degrading strains had the BT_3328 lyase, whereas this was absent in all non-HA degrading strains.

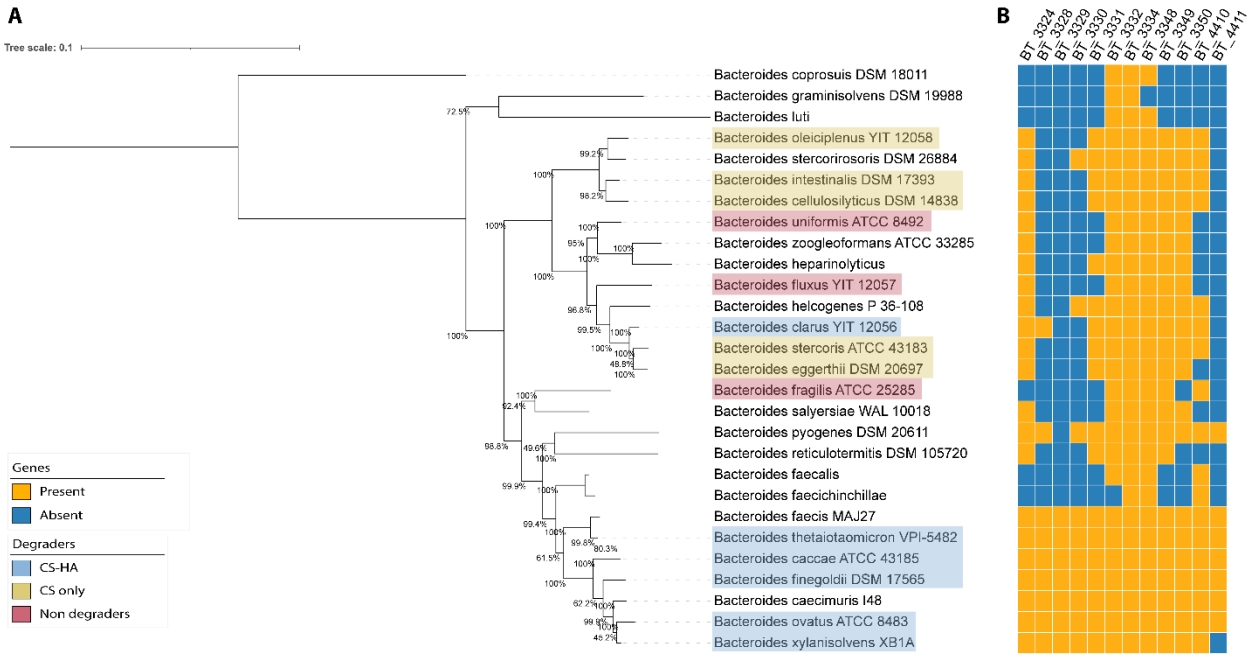

Figure 3: Phylogenomic tree of gut *Bacteroides* and gene repertoire of putative CS and HA utilization genes
Phylogenetic tree of all *Bacteroides* species from NCBI with a translated CDS file. Genomes were aligned with CheckM (Parks, 2015) and the tree was calculated using iqtree2 (Minh, 2020). Clustering is not based on degradation capabilities of CS and/or HA. BlastP (cut-off evalue = $1e^{-40}$) was used to create a presence/absence table of genes from B. theta (potentially) required for chondroitin sulfate (CS) and hyaluronan (HA) degradation based on literature and transcriptomic results. BT_3328 seems to be the crucial gene for HA degradation as bacteria without this gene are incapable of growth on HA.

Breakdown products of CS and HA differ between species

We next wanted to evaluate if the breakdown products from CS and/or HA degradation are potentially available as public goods available for other *Bacteroides*. To do so, fluorophore-assisted carbohydrate electrophoresis (FACE) was used to stain spent supernatant. For all strains tested, CS degradation showed numerous different break-down products, including a strong signal for GalNAc and a variety of di- and oligosaccharides at varying signal strength (Figure 4A, Supplementary Figure 1 for a representative image). For HA, *B. caccae* and *B. ovatus* showed a strong signal for GlcNAc, no signal for GlcA, but a third band of an unknown breakdown product (Supplementary figure 1 & Supplementary Table 7). For *B. ovatus, B. stercoris,* and *B. xylanisolvens* media was harvested at intervals for 24 hours to follow the degradation of CS (Supplementary Figure 2 for a representative image). For all three there was one band observed already after two hours, despite no growth being observable yet via OD. After 24 hours some bands were lost or were less intense, yet degradation products remained in the medium. *B. ovatus* was also grown on HA and showed only two bands during the entire timespan with the first, unknown band occurring after 2 hours and the GlcNAc band appearing after 4 hours. These unknown bands are presumably intermediate break down products of CS and HA in the form of di- and/or oligosaccharides. Based on FACE results, it seems that all strains produce extracellular sugars outside of the periplasm which could potentially be

used by other (non-degrading) bacteria and used as public goods (Figure 4A). To test this, degraders were grown on either CS or HA until late exponential phase, supernatant was spun down and filtered to remove bacteria and is now conditioned media (CM). Next, we diluted the CM with fresh M9 medium without an additional carbon source and inoculated non-degrading bacteria or CS-only degraders and measured OD.

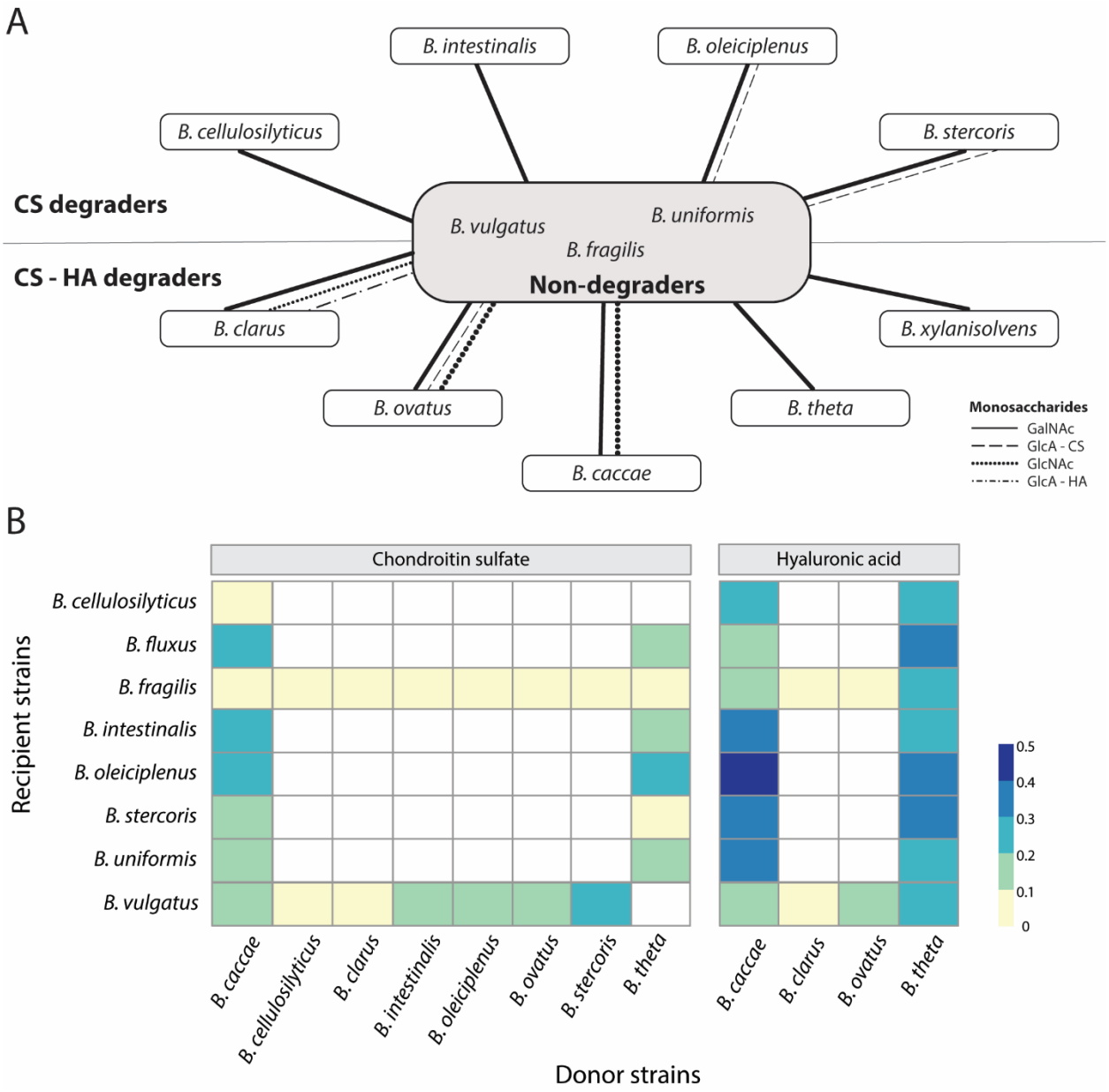

Figure 4: Sharing of public goods depends on carbon source and donor and recipient strains
A) Theoretical public goods provision by CS and CS-HA degraders to non-degraders based on fluorophore-assisted carbohydrate electrophoresis results. CS = chondroitin sulfate, HA = hyaluronan, GalNAc = N-Acetylgalactosamine, GlcNAc = N-Acetylglucosamine, GlcA - CS = glucuronic acid from CS, GlcA – HA = glucuronic acid from HA
B) Not all donor strains (selected CS-HA and CS-only degraders) can supply public goods. Similarly, not all non-degraders are capable of growth on CM. HA donors supply better growth than CS donors. Donor strains were grown until late exponential after which the supernatant was filtered and diluted 1:1 with fresh M9 medium. Recipient strains were then grown on this Conditioned Media (CM) for 20 hours. Optical densities (OD) were normalized to the maximum value grown on glucose. OD at 19 hours is shown. White boxes indicate N/A values.

Interestingly, CM from donors grown on HA provided more non-degrader growth then CM from CS (Figure 4B). Additionally, some non-degraders were better at growing on CM than others. *B. fragilis* for example, was incapable of growing on any of the CMs from CS, regardless of donor whereas *B. oleiciplenus* showed good growth. Interestingly enough, the growth results and CM visualization do not align. FACE-PAGE for *B. theta* only indicated degradation products for CS yet and very limited for HA, yet non-degraders were capable on growing on CM from HA. In contrast, *B. clarus* showed a multitude of degradation products with FACE-PAGE, yet non-degraders were incapable of growth on *B. clarus* CM.

Discussion

It has been thought that bacteria capable of degrading CS were also able to degrade HA, as the characterized lyases worked on both polysaccharides (Ndeh et al., 2020; Salyers et al., 1977). We therefore expected that all tested strains with homologs of these lyases would be able to degrade HA, and those which additionally encoded sulfatases would be able to degrade both CS and HA. Our results do show that there is a separate group of *Bacteroides* only capable of degrading CS (Figure 1A). These results corroborate previous results from Ndeh et al. and show that this holds true for a larger subset of *Bacteroides.* They did not, however, explore the broader significance of these findings for HA metabolism (Ndeh et al., 2020). In this study we set out to investigate the possibility of public goods for host-derived polysaccharides, but upon the finding of the CS-only degrader groups additionally wanted to deduce a potential mechanism for HA degradation.

Using the model organism *B. theta*, we concluded that HA degradation did not require a separate pathway from CS breakdown (Figure 2). This is in line with previous gene expression microarray results from Martens and co-workers (Martens et al., 2008, 2011). Our phylogenetic analysis of *Bacteroides* strains tested suggests the loss of a gene required for HA utilization in CS-only degraders since (with the exception of *B. clarus*) CS-HA degraders cluster together (Figure 3A). Comparative genomic analyses presented in this study indicated that the lyase BT_3328 and hypothetical protein BT_4411 may be together necessary for HA degradation (Figure 3B). BT_4411 has been predicted to be in an operon together with the known HA lyase BT_4410 and is similar to carbohydrate binding domain PF02018, they thus may function together to degrade HA (Liu et al., 2021). Previous knockouts of both BT_4410 and BT_4411 show growth deficits on HA (Liu et al., 2021; Ndeh et al., 2020). However, our results indicate that BT_4411 is absent in two of the CS-HA degraders, we propose this could be a second pathway for HA degradation, similar to the pectin pathway where *B. theta* also has multiple susC-D transporter pairs (Luis et al., 2018).

The CS/HA lyase BT_3328 has been previously identified as the first cell surface lyase for *B. theta* (Ndeh et al., 2018) and interacts together with the predicted surface glycan binding proteins (SGBP) BT_3329 and BT_3330 at the outer membrane. The BT_3328 lyase has been shown to prefer larger molecules with a high degree of polymerization (Ndeh et al., 2018). Since the other lyases capable of HA degradation are

all found within the periplasm, HA may need to be partially broken down outside of the cell by BT_3328 before it can be imported. Previous knockouts for BT_3328 are inconclusive, with one study showing no growth deficits for 1% CS or HA (Ndeh et al., 2018), one study showing a modest growth defect on 0.5% HA and another showing growth on HA to be strongly decreased (Liu et al., 2021). The latter study also shows knockouts for the SGBPs BT_3329 and BT_3330 negatively affect growth on HA (Liu et al., 2021). Interestingly, *B. clarus* does not have BT_3329 nor BT_3330 yet it may have similar SGBPs which can bind HA next to the BT_3328 lyase.

Molecular weights for both CS and HA are highly variable, for CS they range mostly between 50-100 kDA (Kwan et al., 2010), whereas HA is much larger with a range between 1000-8000 kDa (Cowman et al., 2015). A recent paper studying the SusC/D complex for levan degradation have shown there is an upper limit in size for import through this complex (Gray et al., 2021). They suggest 5 kDa to be the upper limit for saccharide import. We thus propose that HA is too large to be transported into the cell with the SusC-SusD transporters of the CS-HA PUL 57. HA would thus require the SGBPs BT_3329 and BT_3330 to bind HA followed by the lyase BT_3328 creating HA oligosaccharides after which it can be imported and further degraded by periplasmic CS-HA lyases (Figure 5). This hypothesis is supported by the lack of BT_3328-BT_3330 in CS-only degraders in our bioinformatic analysis, yet would need wet-lab confirmation by, e.g. double and triple mutants and/or knock-ins of these genes into CS-only degraders.

FACE analysis presented in this study showed that *Bacteroides* strains produce extracellular breakdown products from CS and HA degradation, suggesting the potential for providing public goods to other bacteria (Figure 4A). For CS, this was initially surprising as the lyases are inside the periplasm, however, following the results from Gray et al, CS would be above the maximum threshold size of direct import by SusC/D and would thus also require breakdown by an extra-cellular lyase such as BT_3328 (Gray et al., 2021). Additionally, Koropatkin et al. suggest there is also a minimal size limit to the SusC/D system with monosaccharides being imported via a non-Sus system (Koropatkin et al., 2008). Ndeh et al. reported that the smallest unit of CS and HA degradation by the lyase BT_3328 are disaccharides, indicating there may be an additional cell surface enzyme which can then cleave disaccharides to monosaccharides as is seen for the SusC/D system for pectin (Li et al., 2021). Alternatively, periplasmic lyases encapsulated in outer membrane vesicles could create monosaccharides in the CM (Valguernera et al., 2018; Schwechheimer and Kuehn, 2015). In this study, when looking at CS degradation over time it was observed that bands corresponding to GlcA were present in the media but disappeared after 9 hours, suggesting this was preferred over GalNAc as a carbon source. This is surprising as GalNAc and GlcNAc are cytotoxic for epithelial cells and need to quickly be removed from the lumen (Lee et al., 2009), however both GalNAc and GlcNAc require additional kinases for phosphorylation before they can be metabolized (Ndeh et al., 2020).

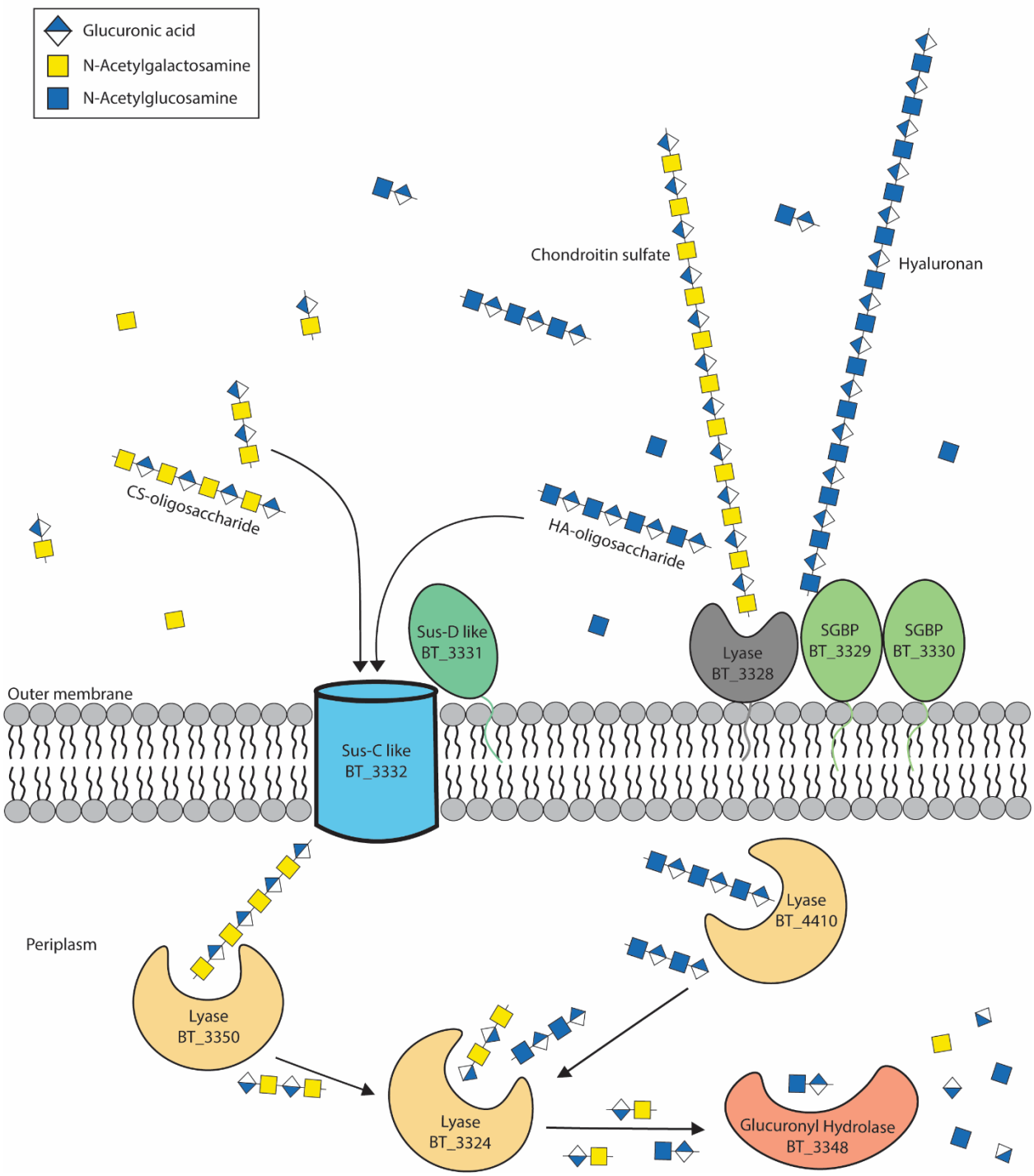

Figure 5: Suggested model for hyaluronan breakdown
Based on our results, we propose that the lyase BT_3328 and surface glycan binding proteins (SGBP) BT_3329 and BT_3330 are required to cleave hyaluronan (HA) before transport and further degradation in the periplasm. Adapted from Ndeh et al. 2020

Lastly, it seems that even when monosaccharides are present in the CM, not all recipient strains are capable of growth (Figure 4B). It should be noted that concentrations of sugars were not quantified or standardized before CM inoculation, resulting in differing concentrations between CM samples, and therefore it is not possible to distinguish between the ability to grow on breakdown products and the availability of

breakdown products in different CMs Nevertheless, plausible explanations for the difference of growth capabilities on CM include the presence of inhibitory compounds like antimicrobials that are packaged in outer membrane vesicles and secreted extracellularly (Coyne et al., 2019). Another possible explanation for selective cross-feeding could be the degree of polymerization of break-down products with not all species being capable of degrading the higher degree components or lacking pathways and deaminases to use monosaccharides (Luis et al., 2018). Thus, our results show *Bacteroides* species not only have differing abilities of GAG degradation and public goods distribution but could also have preference to whom they provide nutrients.

In conclusion, although CS and HA degradation have been extensively studied in *B. theta*, not much is known about the degradation in other *Bacteroides* species. We have shown that although multiple *Bacteroides* species encode periplasmic lyases which should be able to break down both CS and HA, some cannot utilize HA. We suggest this is due to the loss of the outer membrane lyase BT_3328 and SGBPs BT_3329 and BT_3330. However, due to cooperative degradation and public good provisioning, loss of these genes may not put these species at a selective disadvantage in the mucosal ecosystem.

Conflict of Interest: The authors declare that they have no competing interests.

Author Contributions: A.O., F.P. and D.B. designed the experiments. A.O. performed all lab work assisted by G.N., with the exception of sequencing which was done by the JMF. A.O., B.H., C.W.H. and D.B. performed bioinformatic analyses. All authors read and approved the manuscript.

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Data Availability Statement: The transcriptomic sequencing data has been deposited at the Sequence Read Archive (https://www.ncbi.nlm.nih.gov/sra) under the BioProject accession ID PRJNA837438.

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Supplements

Supplementary table 1: Strains used in this study

Organism	Strain	Accession
Bacteroides acidifaciens	DSMZ15896 (JCM 10556)	
Bacteroides caccae	Bacteroides caccae 19024, type strain (ATCC 43185)	GCA_000169015.1_ASM16901v1_translated_cds.faa
Bacteroides cellulosilyticus	Bacteroides cellulosilyticus CRE21 DSM No.: 14838, Type strain	GCA_000158035.1_ASM15803v1_translated_cds.faa
Bacteroides clarus	Bacteroides clarus DSM No.: 22519, Type strain (YIT 12056)	GCA_000195615.1_ASM19561v1_translated_cds.faa
Bacteroides dorei	Bacteroides dorei DSM No.: 17855, Type strain	
Bacteroides eggerthi	Bacteroides eggerthii DSM No.: 20697, Type strain	GCA_000155815.1_ASM15581v1_translated_cds.faa
Bacteroides finegoldii	Bacteroides finegoldii DSM No.: 17565, Type strain	GCA_000156195.1_ASM15619v1_translated_cds.faa
Bacteroides fluxus	Bacteroides fluxus DSM No.: 22534, Type strain (YIT 12057)	GCA_000195635.1_ASM19563v1_translated_cds.faa
Bacteroides fragilis	Bacteroides fragilis 2151, type strain (ATCC 25285)	GCA_001997325.1_ASM199732v1_translated_cds.faa
Bacteroides intestinalis	Bacteroides intestinalis DSM No.: 17393, Type strain	GCA_000172175.1_ASM17217v1_translated_cds.faa
Bacteroides oleiciplenus	Bacteroides oleiciplenus DSM NO.: 22535, Type strain (JCM 16102 / YIT 12058)	GCA_000315485.1_Bact_olei_YIT_12058_V1_translated_cds.faa
Bacteroides ovatus	Bacteroides ovatus 1896, type strain	GCA_002959635.1_ASM295963v1_translated_cds.faa
Bacteroides stercoris	Bacteroides stercoris DSM No.: 19555, Type strain	GCA_900106605.1_IMG-taxon_2693429855_annotated_assembly_translated_cds.faa
Bacteorides thetaiotaomicron	Bacteroides thetaiotaomicron 2079, type strain	GCA_000011065.1_ASM1106v1_translated_cds.faa
Bacteroides uniformis	Bacteroides uniformis 6597, type strain (ATCC8492)	GCA_000154205.1_ASM15420v1_translated_cds.faa
Bacteroides vulgatus	Bacteroides vulgatus 1447, type strain (ATCC8482)	
Bacteroides xylanisolvens	Bacteroides xylanisolvens DSM No.:18836, Type strain XB1A	GCA_000210075.1_ASM21007v1_translated_cds.faa

Supplementary table 2: Components of M9 minimal media, adapted from Neidhardt et al., 1974

	1 L
NH_4Cl	1g
Na_2HPO_4	6g
KH_2PO_4	3g
NaCl	0.5g
1M $CaCl_2.2H_2O$	100 µl
1M $MgSO_4.7H_2O$	1 ml
5% Cysteine	10 ml
0.5% Hemin	1 ml
0.5% $VitK_1$	500 µl
2% $FeSO_4$	100 µl
0.01% Vit B_{12}	50 µl

Supplementary table 3: Library results per sample

	Reads	Expressed genes
BT CS 1	180096834	4891
BT CS 2	14230812	3619
BT CS 3	263558	4616
BT CS 4	350	
BT HA 1	25749471	4829
BT HA 2	7121958	4730
BT HA 3	36713662	4856
BT HA 4	2125904	4480
BT mix 1	16542308	4769
BT mix 2	36725544	4812
BT mix 3	36179577	4818
BT mix 4	96901885	4873

Supplementary table 4: Number of statistically significant genes

	padj <= 0.05	L2FC >= 2	L2FC <= -2
CS	1659	802	88
HA	2825	399	219
HA vs CS	42	1	23

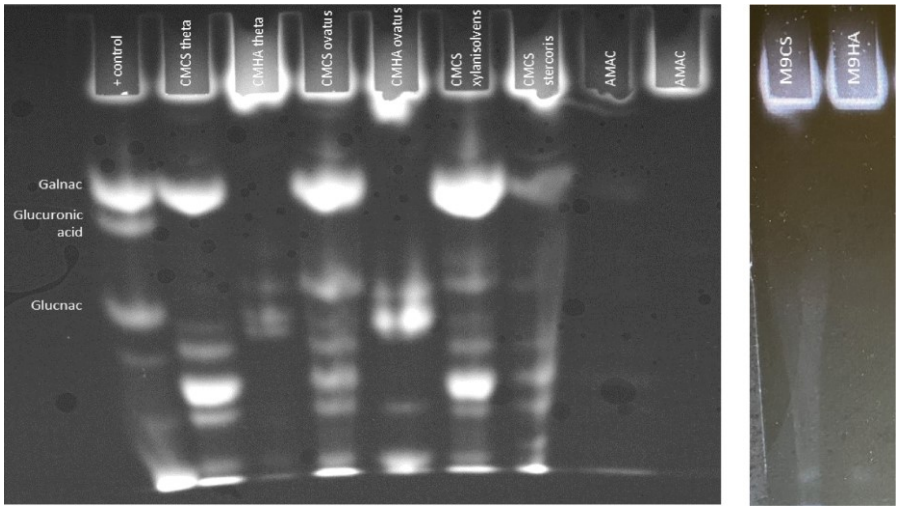
Supplementary table 5: *B. theta* genes upregulated when comparing CS to HA

PUL	Gene	Function	CSL2FC	CS padj	HAL2FC	HA padj	HACSL2FC	padj
CS upregulated								
	BT_0543	Glutamine synthetase	2.8	0.00	-1.2	0.00	-3.2	0.00
	BT_0544	Ammonium transporter	4.1	0.00	0.3	0.46	-3.1	0.00
	BT_0545	Nitrogen regulatory protein P-II	3.1	0.00	0.2	0.63	-2.2	0.02
	BT_0546	Uncharacterized protein	2.5	0.00	-0.6	0.09	-2.3	0.00
PUL 89	BT_0988	Magnesium-transporting ATPase, P-type 1 (EC 7,2,2,14) (Mg(2+) transport ATPase, P-type 1)	-1.5	0.17	-5.7	0.00	-3.5	0.03
	BT_1072	Uncharacterized protein	-1.6	0.00	-4.9	0.00	-2.6	0.00
	BT_1073	DUF4136 domain-containing protein	-2.2	0.00	-5.2	0.00	-2.3	0.00
	BT_1074	OMP_b-brl domain-containing protein	-2.7	0.00	-5.6	0.00	-2.2	0.03
	BT_1339	Undecaprenyl-phosphate alpha-N-acetylglucosaminyl-transferase	2.2	0.00	-0.6	0.00	-2.1	0.03
	BT_1655	Uncharacterized protein	2.9	0.00	-1.0	0.06	-3.2	0.00
Predicted PUL 27	BT_2261	Uncharacterized protein	5.4	0.00	2.2	0.00	-2.5	0.00
	BT_2262	Uncharacterized protein	5.2	0.00	2.2	0.00	-2.2	0.01
	BT_2263	Putative lipoprotein	5.0	0.00	2.2	0.00	-2.1	0.00

	BT_2387	O-acetylhomoserine (Thiol)-lyase	5.8	0.00	2.1	0.00	-3.0	0.00
PUL 52	BT_3235	Uncharacterized protein	4.2	0.00	1.3	0.00	-2.1	0.01
	BT_3236	Uncharacterized protein	4.0	0.00	1.2	0.00	-2.0	0.01
	BT_3241	SusD homolog	4.9	0.00	2.1	0.00	-2.0	0.01
	BT_3242	Uncharacterized protein	4.9	0.00	1.8	0.00	-2.4	0.00
	BT_3243	DUF4987 domain-containing protein	4.7	0.00	1.7	0.00	-2.3	0.01
	BT_3244	BACON domain-containing protein	4.6	0.00	1.7	0.00	-2.1	0.01
	BT_4693	Cation efflux system protein	-1.4	0.18	-5.7	0.00	-3.5	0.01
	BT_4695	Outer membrane efflux protein	-1.9	0.06	-5.6	0.00	-2.9	0.00
	BT_t41	NA	5.4	0.00	2.4	0.00	-2.3	0.04
HA upregulated								
	BT_1541	Putative transmembrane protein	1.0	0.06	2.4	0.00	2.1	0.00

Supplementary table 6: Comparative genomics, relevant orthogroup gene counts ordered by degradation ability

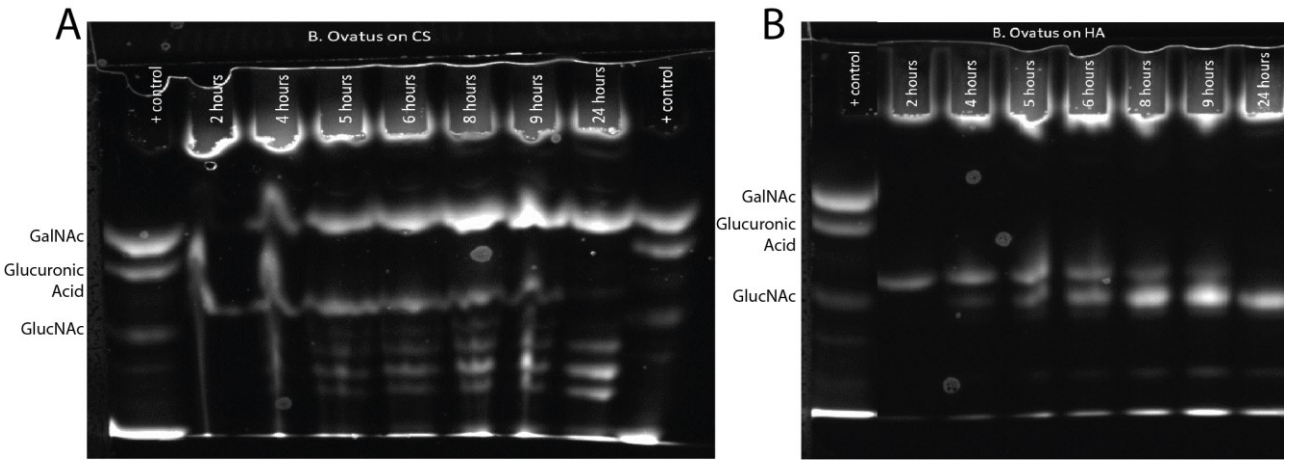
Genome	Organism	Degrader	OG0002222	OG0002347	OG0002357	OG0002608	OG0003140	OG0003539
GCA_000154845.1_ASM15484v1_protein	B. eggerthii	CS	1	1	1	0	0	1
GCA_000155815.1_ASM15581v1_protein	B. cellulosilyticus	CS	1	1	1	0	0	1
GCA_000965785.1_ASM96578v1_protein	B. intestinalis	CS	1	1	1	0	0	1
GCA_001314995.1_ASM131499v1_protein	B. oleiciplenus	CS	1	1	1	0	0	1
GCA_001318345.1_ASM131834v1_protein	B. stercoris	CS	1	1	1	0	0	1
GCA_001404475.1_13414_6_24_protein	B. ovatus	CSHA	1	1	1	1	1	0
GCA_001412315.1_ASM141231v1_protein	B. finegoldii	CSHA	1	1	1	2	1	0
GCA_001578555.1_ASM157855v1_protein	B. plebeius	CSHA	1	1	1	2	1	0
GCA_001578635.1_ASM157863v1_protein	B. clarus	CSHA	1	1	1	2	1	0
GCA_001915515.1_ASM191551v1_protein	B. xylanisolvens	CSHA	1	1	1	1	1	0
GCA_001915535.1_ASM191553v1_protein	B. caccae	CSHA	1	1	1	1	1	0
GCA_002160225.1_ASM216022v1_protein	B. theta	CSHA	1	1	1	1	1	0
GCA_002160605.1_ASM216060v1_protein	B. fragilis	Non	0	0	0	0	0	0
GCA_002161115.1_ASM216111v1_protein	B. vulgatus	Non	0	0	0	0	0	0
GCA_002222615.2_ASM222261v2_protein	B. uniformis	Non	1	0	1	0	0	0
GCA_900106605.1_IMG-taxon_2693429855_annotated_assembly_protein	B. dorei	Non	0	0	0	0	0	0
GCF_014131755.1_ASM1413175v1_protein	B. coprocola	Unknown	0	0	0	0	0	1



Supplementary figure 1: Representative FACE image for CS and HA degradation
Positive control contains GalNAc, GlcNAc and Glucuronic Acid. Samples are filtered media from degraders grown on either CS or HA. 2-Amino-9(10H)-acridinone (AMAC) was used as a negative control.

Supplementary table 7: Degradation of CS and/or HA by selected *Bacteroides* strains as deduced by FACE

		CS			HA		
	Degrader	**GalNAc**	**GlcA**	**Other bands**	**GlcNAc**	**GlcA**	**Other bands**
Cellulosilyticus	CS	Strong	faint	yes			
Intestinalis	CS	strong		yes			
Oleiciplenus	CS	strong	medium	yes			
Stercoris	CS	Strong	medium	yes			
Clarus	CSHA	Strong	faint	yes	medium	medium	yes, multiple
Ovatus	CSHA	Strong	medium	yes	strong		yes, 1
Theta	CSHA	Strong		yes	faint	faint	
Xylanisolvens	CSHA	Strong	faint	yes	n/a	n/a	n/a
Caccae	CS-HA	Strong		yes	strong		yes, 1



Supplementary figure 2: FACE image for *B. ovatus* grown on CS and HA sampled on multiple time-points. A) Growth of *B. ovatus* on CS. After 4 hours, bands are already observed, with strongest GalNAc bands appearing between 8-9 hours. There are many other saccharides observed besides the two monosaccharides. B) Growth of *B. ovatus* on HA. First bands show after 2 hours, GlcNAc bands start showing between 4-5 hours. After 24 hours it seems only GlcNAc is left in the medium. Positive control contains GalNAc, GlcNAc and Glucuronic Acid. AMAC is a negative control.

Chapter 4

General discussion, future outlook and concluding remarks

A gut feeling

This thesis has focused on the mucus degrading abilities of the gut microbiome, giving more insight into mechanisms and potential pathways of mucus degradation as well as highlighting the importance of fibers to maintain the homeostasis between host and (gut) microbes. This relationship is complex and multi-dynamic, as seen in figure 1. Whatever happens in the intestine does not solely have a local effect, it is now known to influence the entire host from top to bottom. Think about that "gut feeling" people have; in recent years it has obtained a scientific foundation in the form of the gut-brain axis (figure 1, arrow 1). The gut microbiome could suddenly be linked to mental health and depression, gut inflammation was potentially causative for Parkinson's, and stress affects microbial composition and vice versa (Almand et al., 2022; Bhattarai et al., 2020; Walace et al., 2017).

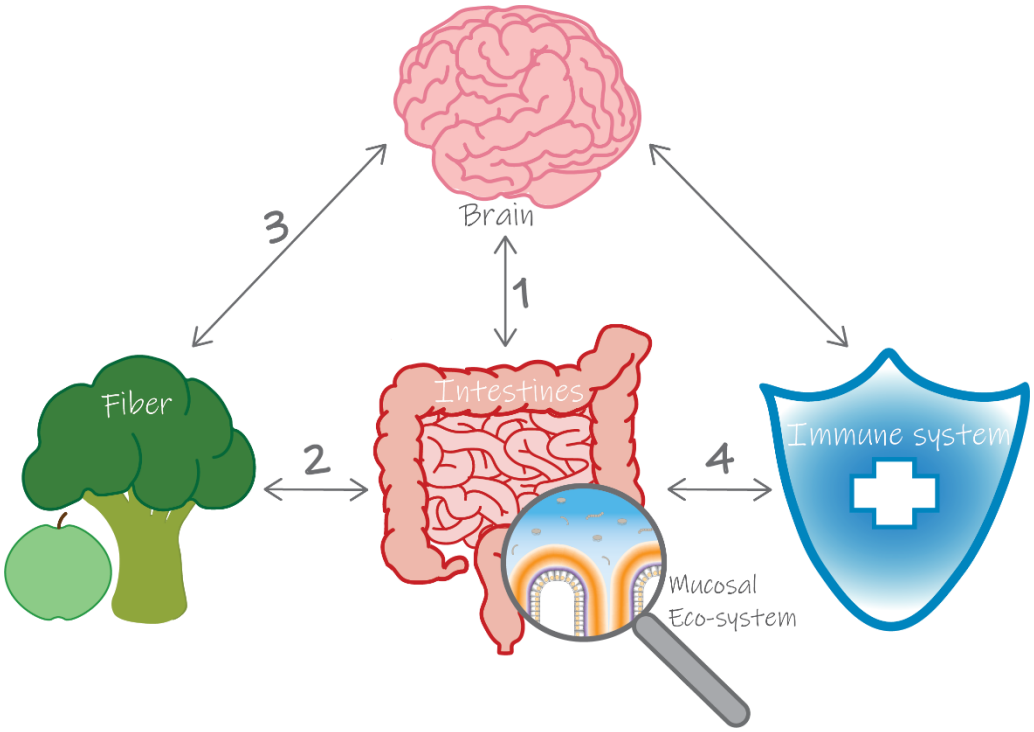


Figure 1: The complex and multi-dynamic relations of the host with its gut microbiome
The gut microbiome influences all aspects of the host, from the gut-brain axis (1) to nutrient intake (2) and health status (4). However, all these aspects, in turn, can also affect microbial composition as well as other host functions. For a larger version of the mucosal eco-system see figure 2 of the introduction.

Signals in the gut-brain axis can be sent via hormones, yet there are also certain gut sensory enteroendocrine cells, known as neuropods, which can send direct signals to the brain via nerves found in the intestinal mucosal layer (Bohroquez Walace et al, 2015). These gut sensory epithelial cells can both detect and evaluate the type of nutrient and elicit different responses accordingly (Kaelberer et al., 2021). Interestingly enough, these cells can induce multiple, sometimes even contrasting, signals and, for example, regulate hormones for both satiety and hunger (Glass et al., 2017). Lastly, neuropods can detect bacterial metabolites such as SCFA and are suggested to be a potential mechanism to influence behavior (Kaelberer et al., 2021). The interactions between bacteria and neuropods is intricate as the absence of bacteria

alters enteroendocrine cells (Swartz et al. 2012). Thus, once more stressing the importance of the gut microbial composition in relation to host health.

You are what you eat

A major influencer in bacterial composition is the diet (figure 1, arrow 2); in a high animal fat diet more *Bacteroides* are present whereas *Prevotella* are indicative of a carbohydrate heavy diet. With a diet lacking in fibers, microbial diversity diminishes (**chapter 2,** Riva et al., 2019), something also seen with certain diseases such as depression (figure 1, arrows 1 & 2) (Kelley et al., 2016). The suggestion that a decrease in fiber intake was related to more symptoms of depression underlines the importance of researching this complex topic (figure 1, arrow 3) (Kelley et al., 2016). A recent study By Gao *et al.* investigated the relation between oat fiber and cognitive behavior (including neuroinflammation) in mouse models for atherosclerosis. Fiber is known to reduce cholesterol, so unsurprisingly the fiber fed mice had lower LDL levels and less plaque formation. The fiber fed mice also had less inflammation and enhanced tight junction proteins, most likely due to SCFA produced by fiber-degrading bacteria (figure 1, arrow 4). Furthermore, memory and spatial learning were improved in the fiber fed mice compared to the atherosclerotic controls, which was also suggested to be a result of elevated SCFA levels (Gao et al., 2020). That the gut-microbiota can affect the brain was further corroborated by Fernandez-Real *et al.* who observed that both obesity and the corresponding gut microbial composition can affect the physical structure of the brain, which could consequently be linked to lower cognitive test scores (Fernandez-Real et al., 2015).

Chapter 2 has shown in detail that fiber, or rather the lack thereof, plays a vital role in shaping the gut mucosal eco-system. Not only in bacterial composition, but also in physiological host aspects such as mucosal folds and intestinal length (figure 1, arrow 2). Although reduction in colon length has been previously reported (Desais et al., 2016; Schroeder et al., 2018) it was never observed after such a short time span, in our case a mere 7 days of fiber deprivation. In colitis mouse models, colon shortening is often an indication of inflammation, however then there are also other markers such as thickening and reddening of tissue as well as edema's (Oh et al., 2014). Besides a shorter length, no other inflammatory markers were observed. The reason behind fiber deprivation shortening the intestine is yet to be discovered. From a microbiological point of view the most predictable answer would be microbial shifts and lack of butyrate (producers); however previous studies have shown that colon shortening as a result of diminished fibers is independent of altered butyrate levels (Desais et al., 2016; Schroeder et al., 2018). Others looked at intestinal hormones responsible for intestinal size and intestinal healing such as glucagonlike peptide 1 (GLP-1) and GLP-2; especially interesting is that both GLP-1 and 2 levels decreased during fiber depletion yet were not found to be a causative factor for intestinal shortening (Hunt et al., 2021). Once more it is shown how complex the interplay is between nutrition, host (health) and microbes.

An option could be that neuropod cells and the gut-brain axis play a role in the intestinal shortening by fiber deprivation. In the intestine, GLP-1 is released in vesicles by neuropods. The main function of GLP-1 is to stimulate insulin secretion and reduce glucagon levels after nutritional intake (Holst et al., 2007). Insulin is a hormone needed in order to transport glucose digested from food into the cells so it can be used as fuel. Besides insulin regulation, GLP-1 also plays an important role in the GIT, inhibiting gastric emptying and intestinal motility after nutrient ingestion which has been shown not to be regulated by paracrine systems but instead by inhibiting vagal nerve activity (Wettergren et al., 1997). Recently, it has been shown that this vagal response cannot be elucidated by GLP-1 alone but needs additional ATP which can also be found in the released vesicles, again showing the complexity and delicate interplay of a multitude of factors (Kaelberer et al., 2020). Yet how is this related to microbial composition? Gut bacteria have even been shown to "communicate" with skeletal muscles. In fact, microbial depletion not only reduced muscle endurance, but also affected glucose homeostasis, in part by reducing the Gpr41 receptor which also plays a role in GLP-1 release (Nay et al., 2019). Since the intestine is a muscular tube, could shortening then potentially also be affected by microbial composition?

Another option is that shortening is related to HA degraders, such as those studied in **chapter 3**. A study in 2012 showed that HA is closely linked to intestinal growth in the mucosal area (Riehl et al., 2012). They observed blocking HA receptors resulted in the shortening of both the small intestine and colon, as well as a decreased crypt and villus height (Riehl et al., 2012). Could an altered HA secretion, or perhaps microbial degradation, then be the reason for the observed intestinal shortening on a fiber deficient diet as seen in **chapter 2**? What I can conclude, is that fibers are crucial for health and deserve even more recognition for incorporation into healthy diets than they currently receive.

Deciphering the important taxa of the gut microbiome and their functions

Bacterial composition is of the utmost importance. Each bacterium in a microbiome has a function; certain bacteria can influence the host by degrading mucus and/or producing SCFA, others can influence inflammation (figure 1, arrow 4). *Bacteroides vulgatus* for example, can stimulate certain dendritic cells responsible for producing pro-inflammatory cytokines (Geva-Zatorsky et al., 2017). However, multiple bacteria can have similar functions resulting in unique microbiome compositions. Although this may seem redundant, it prevents functional losses when a specific taxon is wiped out (Geva-Zatorsky et al., 2017). This also creates a challenge as it is difficult to link specific species to individual events. Sometimes, an example observed in the oral microbiome during stress, it is not so much the composition which changes but rather the function that bacterium has at that specific moment, further complicating microbial cause and effect (Almand et al., 2022). It is for this reason, that in **chapter 3** additional transcriptomic analysis was performed to see if *B. theta* had distinct behavior on different GAGs. Additionally, the results in **chapter 3** are

of importance as it sheds new light on how closely related species have diverse degradation abilities and intricate collaborations.

Chapter 2 confirmed previous results showing a diet lacking in fiber reduces microbial diversity in the gut (Desai et al., 2016; Riva et al., 2019). A novel finding in our study was, that contrary to previous beliefs, mucus degraders did not greatly increase in their abundance. This reported increase was rather a side-effect in previous studies using relative abundances to compare composition when so many species are lost. Especially in studies where bacterial load differs greatly between experimental conditions, it is vital to use absolute abundances for comparison as using relative abundance might lead to incorrect conclusions (Barlow et al., 2020; Vandeputte et al., 2017). In **chapter 2** principle co-ordinate analysis was used in order to explore the separation/clustering of samples, both diet and location in the GIT. Although relative abundance data can give similar results, as shown by Barlow *et al.*, relative abundance data indicate different taxa to be responsible for this separation (Barlow et al., 2020). In their studies on a ketogenic diet *Akkermansia* was also indicated to be a key-taxon for separation in respect to relative abundances whereas, similar to our findings, when using absolute abundances this effect was no longer observed. One could thus say that relative abundances show the best surviving / adapting taxa such as *Akkermansia,* which rely solely on mucus for survival and are thus unaffected by dietary changes. This is not to say using relative abundance is wrong, it can answer questions about the distribution of the (remaining) bacteria within a sample, it cannot however indicate if taxa increase or decrease depending on experimental conditions.

Chapter 2 also evaluated microbial composition in the small intestine, cecum, and colon/fecal samples. Often, fecal samples are taken as a representative for the microbiome across the entire GIT. Results in this thesis show that fecal samples can be representative for the cecum, however they do not give an accurate representation for the small intestine, highlighting the importance to take samples from multiple locations or restrict conclusions to the colon. These findings are corroborated by Barlow *et al.*, who urge scientists to use absolute quantifications at different biological locations to obtain the most accurate results, which, too often, is not yet the case (Barlow et al., 2020).

Inflammatory bowel disease and the gut mucosal environment

Inflammatory bowel disease (IBD) is an umbrella term for diseases such as Crohn's disease (CD) and ulcerative colitis (UC). Both consist of inflammation in the GIT, where CD localizes anywhere along the GIT and UC is found only in the colon. For both diseases, symptoms often come in flares with periods of remission in between. One study showed that during disease flares (mucosal) glycan composition differs from remission, when composition is similar to non-diseased controls (Larsson et al., 2011). The controls

had larger and more complex glycans in comparison to active disease glycans which were shorter and thus simpler. This paper suggests that the structural glycan differences are not specific to UC but rather to the occurrence of inflammation during active disease as similar glycosylation alterations were observed in other diseases where inflammation was present. At first, bacterial degradation was thought to play a causative role in the alteration of glycosylation profiles however no differences in hydrolases were observed and the authors concluded glycosyl transferases present in the goblet cells were contributing to glycosylation differences (Larsson et al., 2011).

Using mouse models, two interesting phenomena have been described. The first shows that a deficiency in Muc2 production results in spontaneous colitis (Johansson et al., 2008; Van der Sluis et al., 2006). The other shows colitis doesn't progress in germ-free mice, indicating that the interplay between the mucus layer and the composition of the microbiota are crucial for disease progression (figure 1, arrow 4) (Rath et al., 1996; Rath et al.,2001; Yao et al., 2021). Without Muc2, the protective layer of the mucosal barrier is lost resulting in bacterial infiltration of epithelial cells causing inflammation (Johansson et al., 2008). This would suggest the composition of the bacteria to be irrelevant as any particle interaction would induce inflammatory reactions resulting in colitis. However, over the years numerous studies have shown that germ-free mice do not develop colitis indicating at least the presence of microbes play an important role. These studies suggest that there are specific bacteria responsible for the onset of colitis which would explain the distinct microbial composition when compared to healthy controls (Andoh et al., 2010; Dianda et al., 1997; Frank et al., 2007; Rath et al., 1996; Rath et al., 2001; Veltkamp et al., 2001). Similar to glycosylation pattern, patients in remission clustered closer to healthy controls than active disease patients (Andoh et al., 2010; Larsson et al., 2011). Although diversity decreases, with a specific reduction in *Clostridium* and *Bacteroidetes*, those taxa that are present more strongly induce pro-inflammatory cytokines compared to healthy controls with a loss of anti-inflammatory taxa (Andoh et al., 2010; Frank et al., 2007; Sokol et al., 2008).

As could be expected, diet also plays a role in IBD either alleviating or enhancing disease symptoms. One paper showed most fiber types to significantly reduce the severity of colitis in mice (Llewelyn et al., 2018). Thus, it seems for IBD there are three factors which play a role: glycan composition, bacterial composition, and fiber intake. All of these factors are investigated in this thesis (and depicted in figure 1) and are crucial for a healthy host yet also have complicated and dependent relations to each other. So how then to continue to investigate these intricate relations in order to obtain more insight and hopefully give them greater value such as for treatment with the IBD described above?

In healthy hosts high molecular weight HA (HMW-HA) is the dominant form, during inflammation however, the pro-inflammatory low molecular weight (LWA-HA) is increased (de la Motte et al., 2011; Hill et al., 2013; Kessler et al., 2008). An interesting aspect would be to investigate if CS-only degraders are able to metabolize LMW-HA. Our current hypothesis is that HMW-HA is bound to the cell and cut into smaller pieces by an extra-cellular lyase. With this theory, LMW-HA should be able to be transported into the cell for degradation by CS-only degraders as well. This could perhaps be related to alterations in glycosylation profiles seen with inflammation or potentially in promoting growth of pro-inflammatory species further stimulating an inflammatory response. In respect to colon shortening testing different lengths of HA would also be interesting as it could potentially be that LMW-HA might bind different receptors inducing those related to shortening or not bind to receptors promoting growth. The pro-inflammatory TNF-alpha, for example, has been shown to be induced by medium weight HA but to a lesser extent by LMW-HA and HMW-HA indicating the importance of knowing HA length in order to determine physiological responses (Zheng et al., 2009).

CS and HA levels are known to change during inflammation and/or IBD (Belmiro et al., 2005; de la Motte et al., 2011) thus the role of HA in this story should also be investigated further. HA synthases are upregulated with colitis, suggesting the host needs more HA in order to counter disease progression. It seems, however, that when IBD develops there is not enough HA; when additional exogenous HA is administered to DSS-mice, disease onset and symptoms are greatly reduced (Zheng et al., 2009). To start, it would be interesting to observe levels of HA in germ-free mice models of colitis, if possible, including the different weight forms. This could perhaps shed some light if the altered HA is due to host responses or due to altered microbial composition which effects HA degradation. A follow-up would be to have these germ-free mice then obtain additional fiber-rich diet, fecal transplant from control mice, or both in order to investigate how this alters levels of HA and intestinal shortening. Perhaps there are more HA degraders in the colitis microbiome. This then should not only be investigated using metagenomics to see which taxa are present but also with transcriptomics with a specific interest in GAG degradation. Ideally, results from this can then be translated from mouse models to humans supplementing them with an ideal combination of fiber and/or probiotics which would then hopefully delay onset or reduce symptoms of IBD. Initial studies have already shown that there is a synergistic relationship between lactic-acid bacteria and HA supplementation reducing colitis symptoms, although the mechanisms behind these interactions remain elusive (Lee et al., 2009).

Figure 1 is a simplified summary of a vastly complex system. Fiber intake was already known to be important for host health, reducing risk of developing numerous diseases as well as lowering cholesterol. Especially with recent studies tying fiber intake to the gut-brain axis it is of the utmost importance to

understand more of this intricate relationship (figure 1, arrow 3). In this thesis we have confirmed the importance of fibers for a diverse microbiome and that a diet deficient in fibers has damaging physiological consequences to the mucosal eco-system (figure 1, arrow 2). Results in this thesis have shown that the diminishing mucosal layer is most likely a host-initiated response instead of the previously believed bloom of mucus degrading bacteria. In order to understand more about the interplay between bacteria and degradation of host compounds a variety of *Bacteroides* strains and their ability to degrade CS and HA was studied. Here it was observed that even in closely related species there are vast differences in degradation ability yet also bountiful opportunities for co-operation which are needed for a balanced system. However, it can also be said that figure 1 is missing an essential element, namely HA. Just like the microbial composition can affect a vast range of host aspects, HA in its various forms seems to have many important roles to play. From modulating the enteric nervous system and influencing intestinal motility, to producing natural antibiotics such as β-definsins in the gut mucosal lining, or encapsulation of pathogens to invade the immune system as well as encapsulating probiotics to enhance efficacy against disease flares in patients suffering from IBD (Bosi et al., 2022). Investigating bacterial species capable of (partial) HA degradation and the consequences of enhanced or reduced metabolism seem to be an obvious and important next step. Once more highlighting the complexity of the entire system and all of its interactions and that each answer in science raises many more questions to be investigated.

When starting a PhD, one has ideals that your own research will change the world, or at least have some impact. Very soon one's perseverance is tested, and the question arises if anything will come from all the hard work, will it even matter? Each little bit of result feels insignificant and unimportant. However, when one then puts all these small pieces together and finds a common denominator it all fits together and obtains a purpose. My results will not directly affect IBD treatment, it will not be used to update dietary recommendations, yet they have helped to not only corroborate previous results but have also added new pieces to the puzzle. Science is a group effort, although as a PhD it often feels like an isolated island. Nevertheless, a puzzle cannot be finished with just one single piece. When the right ones are put together, we get a better idea of what the image will be and how to continue. And as soon as the puzzle is done, the next one is already lying-in wait, eager to be begun. The puzzle related to dietary intake and gut microbiome is far from done but the "puzzle piece" from this thesis couldn't be clearer; the situation in the gut is vast and complex with new surprises and unexpected interrelations with various parts of the host. There is far more research to be done to shed more light and create new insights, but in the meantime; eat more fiber!

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Appendix

Deutsche zusammenfassung

Gesunde Ernährung ist ein Grundpfeiler einer gesunden Lebensweise, in der Ballaststoffe eine wichtige Rolle für die Gesundheit des Wirts spielen. Ballaststoffe reduzieren nicht nur das Risiko eines Krankheitsausbruchs, sie sind auch entscheidend für einen gesunden Verdauungstrakt, welcher die Darmflora inkludiert. Im Gegenzug stellen Darmbakterien dem Wirt zusätzliche Nährstoffe, wie etwa Vitamine und kurzkettige Fettsäuren, zur Verfügung, die der Wirt nicht selbst herstellen kann. In einem gesunden System ist der Darm mit einer Schleimhaut ausgekleidet, die verhindert, dass Bakterien in direkten Kontakt mit Wirtszellen kommen und dadurch eine Entzündungsreaktion hervorrufen. Diese Darmschleimhaut ist allerdings bei vielen Krankheiten reduziert.

Das Hauptaugenmerk dieser Doktorarbeit liegt in der Untersuchung von bakteriellen Interaktionen mit der Darmschleimhaut. Dies wurde einerseits durch einen holistischen *in vivo* Ansatz unter der Verwendung von Mausmodellen erforscht, die mit ballaststoffarmer Nahrung gefüttert wurden und eine Untersuchung des gesamten Ökosystems der Darmschleimhaut ermöglichen. Andererseits untersuchten wir in einem *in vitro* Ansatz zwei spezifische Wirtskomponenten der Darmschleimhaut, sowie ausgewählte Bakterienarten und deren Fähigkeit zur Zersetzung der Darmschleimhaut. Die *in vivo* Experimente zeigten wider Erwarten, dass eine Reduktion der Darmschleimhaut nicht auf eine erhöhte Zahl von Schleimhaut abbauenden Bakterien zurückzuführen ist, sondern vielmehr eine Wirtsreaktion darstellt. Unsere Ergebnisse weisen auch einen Verlust an bakterieller Diversität aufgrund von Ballaststoffmangel nach und unterstreichen die Wichtigkeit von Methoden zur absoluten Quantifizierung in diesen Untersuchungen, da relative Häufigkeiten zu falschen Schlussfolgerungen führen können. Zuletzt zeigen wir auf, dass Ballaststoffmangel nicht nur Darmschleimhaut und Mikroben beeinflusst, sondern auch zusätzliche physiologische Veränderungen im Verdauungstrakt, wie etwa eine Verkürzung, hervorruft.

Glykosaminoglykane des Wirts, wie etwa Chondroitinsulfat und Hyaluron, sind ein wichtiger Bestandteil der Darmschleimhaut. Zusätzlich spielen die unterschiedlichen Taxa der Darmflora eine entscheidende Rolle für die Gesundheit des Wirts, wie am Beispiel des Ballatstoffmangels deutlich wird. Wir wählten daher nah verwandte Arten der Gattung *Bacteroides* um die Mechanismen hinter der Zersetzung dieser Wirtskomponenten und mögliche Kooperation zwischen Bakterienarten in der Form von Kreuzfütterung zu untersuchen. Wir zeigen hier, dass nah verwandte Arten zwar unterschiedliche Zersetzungsprofile aufweisen, jedoch die Zersetzungprodukte teilen, um ein ausbalanciertes Ökosystem zu gewährleisten. Die Ergebnisse dieser Doktorarbeit geben Aufschluss über die Komplexität des Ökosystems der Darmschleimhaut und die wichtige Rolle der (ausbalanzierten) Ernährungsweise in diesem Zusammenhang.

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