Food for microbes. The interplay between indigestible carbohydrates, gut microbiota, and cardiometabolic disease

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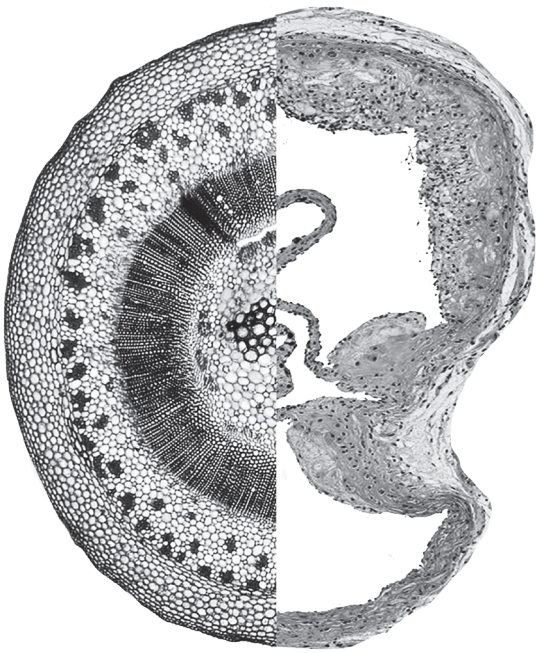

GENERAL DISCUSSION

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In this thesis, we aimed to understand the interplay between the indigestible carbohydrates inulin and mannan oligosaccharides (MOS), gut microbiota composition and function, and the development of cardiometabolic disease in mouse models. In this chapter, methods to map gut microbiota composition and function, factors that determine gut microbiota function, the role of the gut microbiota in the development of atherosclerosis, the translatability of mouse models in gut microbiota research, and implications for prebiotics will be discussed.

16S RIBOSOMAL RNA GENE V-REGION SEQUENCING OR WHOLE METAGENOME SHOTGUN SEQUENCING TO MAP GUT MICROBIOTA COMPOSITION?

The development of next generation sequencing has made it feasible to determine the bacterial composition at virtually any physical location, varying from ocean waters to human body surfaces and cavities. Currently, the 'golden standard' methods used to map gut microbiota composition are 16S rRNA gene amplicon sequencing and whole metagenome shotgun sequencing.

16S RIBOSOMAL RNA GENE SEQUENCING

The 16s rRNA gene is an approximately 1.5KBp gene that is unique to prokaryotes. The 16S rRNA gene is composed of highly conserved regions interspaced with nine variable regions named V1 to V9. A 16S rRNA V region can be amplified by PCR with primers that recognise the conserved flanking regions. The 16S rRNA variable regions are unique for specific genera [1], yielding clusters of similarity termed Operational Taxonomic Units (OTUs)[2,3]. A nearly complete 16S rRNA gene sequence is relatively easy to obtain for a novel bacterial isolate. This provides sufficient phylogenetic information to identify the isolate at least down to the genus level, thanks to the huge database of 16S rRNA gene sequence information that is publicly available and easily searchable [4]. 16S data lends itself to computational analytical techniques including diversity measures within (alpha) and between (beta) samples, which can be defined quantitatively (based on abundance) or qualitatively (based on presence/absence)[2]. Functional

annotation of microbiota communities after 16S sequencing is based on OTU assignment and linking this to databases with reference genomes. The presence of gene families in the microbiota communities are thus inferred based on the OTU assignment. Software tools for such "predictive metagenomics approaches" have been developed [5].

The fact that the 16S rRNA gene is unique to prokaryotes is an advantage in studying the host microbiome as this immediately eliminates host-derived and viral DNA contamination during the amplification and sequencing stage. In addition, important reasons to use 16S sequencing for the characterisation of gut microbiota composition, are its relatively low cost, fast turnaround time, and relative ease and availability of computational tools to analyse the data. A limitation of 16S rRNA sequencing is that a specific V-region often not uniquely identifies the species. Thus, taxonomic assignment will be of variable depth. Furthermore, additional functional analyses is not based on direct sequencing, but predicted based on the OTUs. Another disadvantage of 16S sequencing is the possibility that 16S rRNA genes are derived from horizontal gene transfer which may distort relationships between taxa in phylogenetic trees [6].

WHOLE METAGENOME SEQUENCING

Whole metagenome shotgun sequencing is based on full length DNA from the entire metagenome. Short read sequencing subsequently results in millions of short random DNA fragments that can then be assembled using reference genomes or used individually as markers for specific gut microbial organisms and their metabolic functions [2,7]. This results to the identification of microorganisms with augmented taxonomic resolution [8,9] as the entire genomes of organisms in the community become available for characterisation rather than the more limited single 16S rRNA gene.

While whole genome sequencing provides much more information, including genetically encoded functions of the gut microbiota, the extensive amount of sequence data obtained, however, also leads to a vast amount of challenges with regard to data processing, storage and analysis. For instance, the Illumina HiSeq 2500 platform can yield over 1 Tbp

of raw sequence data, which may even further increase during downstream processing and subsequent analysis. This also comes at much higher costs per sample since it may be necessary to sequence a metagenome with high coverage and thus less samples per run [3,10]. Whole genome sequencing also results in DNA sequences from other microorganisms such as viruses in a community. This may be an advantage or disadvantage based on the premise of the study. Nevertheless, these data need to be dealt with. However, it is expected that the high costs and the necessity of extensive advanced computational skills continue to decrease every year due to optimisation of short read next generation sequencing methods. Moreover, novel "long read" next generation sequencing techniques are in development that hold great promise for metagenomics analysis (reviewed in [11–13]).

The choice for 16S sequencing or whole metagenome sequencing approaches for microbiome analyses is usually dictated by the nature of the studies being conducted. Both of these sequencing methods have their advantages and disadvantages. In our experiments in **chapter 5** and **chapter 7**, we aimed to confirm that indigestible carbohydrates modulate gut microbiota composition. Our research questions required a method that provided enough detail to indicate major gut microbiota changes. We also aimed to perform the bioinformatics analyses in house at relatively low experimental costs. Therefore, in our studies 16S sequencing for the analysis of gut microbiota composition seemed sufficient. However, as shown in **chapter 5**, where 16S sequencing allowed us to identify specific genera that expressed properties that could explain some of our observations, whole genome sequencing will undoubtedly almost always provide additional detail and insight.

1H-NMR OR GC/LC-MS FOR THE IDENTIFICATION OF GUT MICROBIOTA FUNCTION?

DNA sequence analysis provides valuable information on the microbiota composition, from which the presence of potential gene families and biochemical pathways can be inferred. A more direct measure to determine the biochemical activities of the species that are present in the microbiota is analysis of the input metabolic substrates and output of bacterial metabolic products that are present in the feces and in the blood. Fecal/cecal metabolomics provides a complementary functional readout of microbial metabolism as well as its interaction with host and environmental factors [14]. The most commonly used methods for the large scale identification of metabolites (metabolomics) are either based on 1H-nuclear magnetic resonance (NMR) or on gas or liquid chromatography mass spectrometry (GC or LC-MS).

1H-NMR

1H-NMR is based on the principle that every proton behaves as a small magnet due to the fact that it has a spinning electrical charge. In a strong external magnetic field, these tiny magnets align and depending on their particular environment in the molecule require a certain amount of energy to be misaligned. This energy is transferred by a radio wave and can be measured. 1H-NMR can measure multiple metabolites in samples such as blood, serum/plasma, or cecal and fecal material [15]. NMR-based metabolite profiling is a well-established technique producing rapid, robust, and reproducible profiles without the need for extensive sample preparation. Identification of individual compounds is based on deconvolution of the measured spectra as well as 2D-NMR techniques. However, NMR is not an extremely sensitive method and the number of metabolites that can be detected in a given sample is around 70-80 depending on the matrix as well as the employed extraction techniques [16–18].

GC/LC-MS

GC/LC-MS analyses are based on molecular separation of a specific fraction of a sample by GC or LC followed by detection of the molecules employing MS. Sample preparation can be extensive depending on the type of compounds that is measured. Particularly absolute quantification using MS detection can be very cumbersome and usually involves spiking with stable isotope labelled internal standards. Although quantification can be cumbersome and is not as accurate as by 1H-NMR,vast numbers of metabolites (>1000) can be monitored and assessed by a single analysis. For targeted analysis of a specific class of compounds, such as fatty acids (FA) in blood and feces method choices usually depend on the expected concentrations and specific coverage of the available techniques.

The choice for either NMR or GC/LC-MS based analytical methods to measure metabolites associated with gut microbial activity and function is dependent upon the specific research question that needs to be addressed. For a rapid screen to determine whether overall activity differs between microbial communities, 1H-NMR based analyses likely suffices. However, when the question is whether a specific class of compounds is affected in blood, cecum, or feces, GC/LC-MS is likely the preferred method. In our research group, high fat/high cholesterol diets are used to induce obesity, insulin resistance and atherosclerosis. Therefore it is of particular interest to determine the spectrum of FA in the blood. Similarly, fermentation of the indigestible carbohydrates MOS and inulin by bacteria in the gut results in the production of short chain fatty acids (SCFA) in the large intestine. In order to have the ability to determine FA in blood, cecum, and feces with high specificity and sensitivity, we have setup a method using GC-MS for the characterisation of FA including SCFAs in blood, fecal and cecal samples (**chapter 2** and **chapter 3**).

SHORT-CHAIN FATTY ACIDS AS MARKERS FOR GUT MICROBIAL FUNCTION

Generally, and also in this thesis, the SCFAs acetate, propionate, and butyrate are measured as indication for gut microbiota function, since they are the main bacterial breakdown products of indigestible carbohydrates [19–21]. However, gut microbiota do more than the production of SCFAs. The gut microbiota contribute to the production and/or metabolism of a large spectrum of metabolites, including BAs, choline metabolites, vitamins, and lipids (reviewed in [20]), that differently may affect the gut microbiota itself, but also the host. Despite our global understanding of metabolite production by the gut microbiota that is used by the host, for many reasons, including the difficulty in culturing anaerobic bacteria, our knowledge of which bacterial species synthesise which metabolites is currently limited. Therefore, gut-bacteria-derived metabolites other than SCFAs need extensive research in the future in order to determine their relative contribution to health and disease. However, understanding the response of gut microbial communities to diet and other factors in order to predict gut microbiota function, presents a distinct set of challenges. Continued innovation in analysis tools to monitor microbial metabolic shifts and host interactions is needed, and especially to track these events under *in vivo* conditions. Although next-generation sequencing methods can provide an assembly of DNA sequences and insight into the competence of organisms to perform metabolic functions, these analysis' are not suitable for the provision of an overview on the functionality of particular gut bacteria under complex and dynamic environmental conditions. Metabolic profiling of biofluids (e.g. cecum content, plasma, or urine) that uses high-resolution spectroscopy offers additional information to some extent. In **chapter 5** and **chapter 7** we used both 16S rRNA gene sequencing and GC-MS metabolomics to identify the effect of inulin and MOS on gut microbiota composition and SCFA production. Although these two separate platforms provided useful compositional and functional information, the challenge for future research lies in optimising the computational capacity for co-analysis of these two (and other) analytical platforms in order to link gut microbiota composition to the produced metabolites.

WHAT DETERMINES GUT MICROBIOTA FUNCTION?

Microbial function is not only dependent on the individual separate microbial genera present in the microbiota but also on the interaction of the bacteria with each other, the host and the diet. Therefore, prebiotic feeding in one particular host or setting might lead to other results than when given to one another. Some important examples of the interaction of gut bacteria with their environment and how they determine microbial function will be discussed.

CROSS-FEEDING BETWEEN GUT BACTERIA

Co-culturing studies of different bacterial species have demonstrated that metabolites produced after fermentation of indigestible carbohydrates by one particular bacterium, may aid in the provision of substrates to support growth of other bacterial species, termed cross-feeding [22]. Cross-feeding can induce metabolic consequences that would not have been predicted simply by the substrate preferences of isolated bacteria [23]. For example, in a recent study only 8 of 55 bifidobacterial strains had the ability to degrade long-chain inulin. This leads to the suggestion that the observed blooming of *Bifidobacteria* by inulin *in vivo* is mainly due to cross-feeding of end-products released by other inulin-degrading gut bacteria [24]. Another example of cross-feeding was inferred from the increased production of butyrate by *Roseburia sp.* strain A2-183 when co-cultured with *B. adolescentis* L2-32. In plain culture medium, *Roseburia sp.* strain A2-183 is incapable of utilising lactate or to grow on fructo-oligosaccharides (FOS), while in co-culture with *B. adolescentis* L2-32 these bacteria are able to produce butyrate. Butyrate production observed in these *in vitro* co-culturing experiments is most likely due to cross-feeding on products released by partial hydrolysis of FOS from *B. Adolescnetis* [25]. In this thesis, we found increased abundance of *Allobaculum* and *Coprococcus* after feeding mice a high cholesterol diet supplemented with inulin (**chapter 5**). It remains possible that these genera did not thrive on inulin themselves but were increased in abundance due to cross-feeding.

SUBSTRATE COMPETITION BETWEEN GUT BACTERIA

Although we know little about the substrate preferences of the majority of the gut bacteria, it is not a surprise that prebiotics can affect non-target populations within the gut. For example, several studies have shown that inclusion of inulin as a dietary prebiotic increase proportions of *Bifidobacteria* in feces, while other studies also showed that inulin stimulate groups of bacteria other than *Bifidobacteria* in animal models [26,27]. Additionally, in *in vitro* gut simulations, two groups of *Clostridium*-related bacteria, and an added strain of *Roseburia inulinivorans*, were shown to be stimulated by inulin in a mixed fecal community [28]. We did not detect *Bifidobacteria* in mice that were fed with inulin in our studies (**chapter 5**), which makes it possible that the type of inulin in combination with cholesterol induced non-target populations within the gut.

BASELINE GUT MICROBIOTA COMPOSITION

Baseline gut microbiota composition may be another determinant for gut microbiota function. For instance, inulin is well-established to exert bifidogenic effects [29,30] and previous research illustrated that increased abundance of *Bifidobacteria* in the gut microbiota was associated with beneficial health parameters [31]. In **chapter 5,** we performed 16S rRNA gene sequencing analysis on cecal samples of the mice and we found that inulin had a profound effect on the microbiota composition [32]. However, we found that inulin mostly drove the growth of the genera *Allobaculum* and *Coprococcus*, while the growth of *Bifidobacteria* was not induced by inulin. In fact, quantification of the gut microbiota composition prior to inulin feeding revealed that *Bifidobacteria* could not be detected in ceca of these mice. It is therefore likely that we were not able to induce blooming of *Bifidobacteria* as they were not present in these mice. This proof-of-principle hypothesis is substantiated by a recent study in which blooming of another species, *Akkermansia Muciniphila*, depended on its initial baseline abundance [33], even though this study used probiotics and not prebiotics. It also remains possible that inulin in our hands differently affected bacterial substrate competition or induced cross-feeding leading to the outgrowth of other gut bacteria.

HOST-GUT-MICROBIOTA INTERACTIONS

Studies comparing the gut microbiome across inbred mouse lines have yielded evidence that host genetics can affect the gut microbiome. The relation between the composition of gut microbiota and the host genetic profile has been evidently demonstrated in murine models [34].

Maternal environment is one of the earliest factors that can have a profound effect on gut microbiota composition. Several studies have shown that genetically identical mice from the same mother and litter have a more comparable microbiome than mice from different litters, even though they may be housed in separate cages [34,35]. Furthermore, there is evidence that genetic polymorphisms help shape the gut microbiota [36]. This implicates that even minor differences in the genetic profile of mammals can play a tremendous role in shaping gut microbiota composition and therefore might affect gut microbiota function.

Another important driving force of shaping the microbiome is the immune system. The innate immune system plays an important part in shaping the community and environment of gut commensal microorganisms in order to be tolerated by the host and to be beneficial for metabolic activities [37]. For instance, gut microbiota dysbiosis has been reported in different mouse models of innate immune deficiency [38], such as in mice that lack the genes *Nod2* [39], *Nlrp6* [40], or *Tlr5* [41]. The other way around, in order for evolution of the mammalian immune system, a homeostatic relationships with the microbiota needs to be maintained (reviewed in [42]). The innate immune system might therefore promote the growth of beneficial gut bacteria and contribute to the preservation of a stable community of microorganisms while affecting their function.

DIET

Diet is one of the most important factors shaping gut microbial diversity. Gut bacteria do not only respond to indigestible carbohydrates that are ingested, but also to other dietary components such as lipids. Several independent studies revealed that one particular family of the *Firmicutes*, the *Erysipelotrichaceae*, alters in abundance in response to changes in the amount of dietary fat. For instance, after inducing obesity in mice by feeding them a 'Western-type' high fat diet

(HFD)(high in saturated and unsaturated fats), blooming occurred for specific members of the *Erysipelotrichaceae* family [43–45]. The relative abundance for these members declined when the HFD was changed again to the usual chow diet [43]. *Allobaculum* is a specific member of the *Erysipelotrichaceae* family. In **chapter 5** we fed *E3L.CETP* mice with a high cholesterol diet to induce hypercholesterolemia and we found an increase in the genus *Allobaculum* in inulin supplemented mice compared to controls. Apparently, *Allobaculum* does not only bloom on a HFD but also in the presence of hypercholesterolemic conditions. This is supported by evidence from a study of Martínez *et al.,* where they found that *Allobaculum* was mostly abundant in hypercholesterolemic hamsters [46]. It is therefore likely that the type and amount of dietary lipids are important mediators in shaping gut microbial composition, but also might play a role in gut microbiota function. For example, in **chapter 5** we fed *E3L.CETP* mice either a high cholesterol diet with 0.1% cholesterol or with 0.5% cholesterol supplemented without or with 10% inulin. The only difference between these two groups was the percentage of dietary cholesterol. In both studies, inulin did not beneficially affect hypercholesterolemia or atherosclerosis, but in the group that received 0.5% cholesterol, inulin led to early manifestations in liver inflammation which was not observed in the group that received 0.1% cholesterol with inulin. Therefore, it is important to keep in mind that diet is a major regulator in shaping the gut microbiota composition and unquestionably affect gut microbiota function.

METABOLIC ADAPTATION OF GUT BACTERIA

Many bacteria are well suited to metabolically adapt and grow on a variety of different substrates and produce a variety of different metabolites. For instance, *Roseburia inulinivorans* is predominantly a butyrate producer, however during its growth on fucose, *Roseburia inulinivorans* can completely change its gene expression pattern, switching on genes that are capable of using fucose as an energy substrate, and producing propionate and propanol instead [47]. Similarly, *Ruminococcus obeum* produces acetate, lactate, and formate when grown on glucose, but additionally produces propionate while grown of fucose [48]. This indicates that gut bacteria can metabolically adapt depending on substrate availability and consequently

might also produce different metabolites. As we found increased production of SCFAs in both the inulin (**chapter 5**) and the MOS (**chapter 7**) studies, it remains rather difficult to determine whether the outgrowth of microbial genera in both studies are also the genera that are responsible for the produced SCFAs.

Thus, gut microbiota function can be determined by several factors other than gut microbiota composition alone, e.g. cross-feeding, substrate competition, baseline gut microbiota composition, host-gut-microbiota interactions, diet, and metabolic adaptation to various substrates. It is therefore expected that responses to prebiotics will vary in the context of these environmental factors. Although the interaction of gut microbiota between each other, the host and the diet will be difficult to study, it is a critical area in microbial research that needs further investigation.

IS THERE A ROLE FOR THE GUT MICROBIOTA IN ATHEROSCLEROSIS?

In the last two decades, the gut microbiota have been increasingly linked to metabolic and cardiovascular-related disorders such as atherosclerosis. There are several ways by which microbiota might be linked to and affect atherogenesis as will be discussed below.

THE INTERPLAY BETWEEN THE GUT MICROBIOTA, INFLAMMATION, AND ATHEROSCLEROSIS

Local or distant infections might cause a harmful inflammatory response that can aggravate plaque development or trigger plaque rupture. Previous studies supported this mechanism by findings of bacterial DNA in atherosclerotic plaques [49,50]. Furthermore, regardless of infection, local or systemic inflammation has been shown to trigger the immune system thereby activating inflammatory pathways leading to the production and release of pro-inflammatory cytokines and chemokines [51], which can aggravate the progression of atherosclerosis. In **chapter 5** and **chapter 7**, we have no indications of either changes in infection or local/systemic inflammation as mice were housed under specific-pathogen-free (SPF) conditions and markers of local plaque and systemic inflammation were unaffected. Whether inulin or MOS

altered the presence of systemic or local microbial components (e.g. LPS) remains unknown. Although the presence of bacterial DNA in atherosclerotic plaques is established and there is convincing data linking inflammatory signalling to atherosclerosis, evidence in humans remains scarce. Further clinical studies should therefore focus on whether treatment with antibiotics or fecal gut microbiota transplantation have beneficial effects. Importantly, obtaining such evidence might not be feasible owing to the long experimental duration, the risk of spreading antibiotic resistance, and might even further exacerbate the development of obesity and insulin resistance due to antibiotic side-effects when given prolonged or in early life [52].

THE INTERPLAY BETWEEN THE GUT MICROBIOTA, LIPIDS, AND ATHEROSCLEROSIS

There are indications that the gut microbiota affect lipid metabolism and subsequently atherosclerosis development. In subjects from the LifeLines-DEEP population cohort, a large-scale human study, it was found that the gut microbiota contribute to a substantial proportion of the variation found in blood lipids in humans [53]. They found that the family *Clostridiaceae/Lachnospiracease* was specifically associated with low-density lipoproteins (LDL), while the family *Pasteurellaceae*, genus *Coprococcus*, and genus *Collinsella* species *Stercoris* showed strong association with triglyceride (TG) levels. Changes in either plasma LDL or TG levels are major risk factors for the development of atherosclerosis. Although we found an increase in the genus *Coprococcus* after a high cholesterol diet supplemented with inulin (**chapter 5**), we did not find any effect on plasma lipid levels and atherosclerosis development.

In contrast to inulin, in **chapter 7** we found that MOS significantly reduced plasma cholesterol levels and atherosclerosis development. Concomitantly, we identified an increase in the abundance of *Bacteroides Ovatus*. Currently, no other studies have identified an association between *Bacteroides Ovatus* and plasma lipid levels. Nevertheless, it may be possible that *Bacteroides Ovatus* is associated with altering plasma cholesterol levels via interactions with bile acids (BAs). For instance, specific BAs and their signalling pathways play important roles in cholesterol metabolism (reviewed in [54–56]) and atherosclerosis. Primary BAs that are synthesised in the liver end up in the terminal ileum where they can be mostly absorbed from

the terminal ileum. Another part will enter the colon and be modified by the gut microbiota expressing bile salt hydrolases (BSH) to yield so-called secondary BAs [56]. Indeed, *Bacteroides Ovatus* expresses BSH [44, 45] and accordingly is able to deconjugate primary BAs into secondary BAs. Some secondary BAs will be absorbed from the colon and, together with those absorbed from the ileum, transported to the liver for re-secretion into bile. This enterohepatic circulation of BAs contribute to the maintenance of the BA pool. When the BA pool is disturbed and leads to excess BA excretion via the feces, this loss can be compensated by hepatic *de novo* synthesis using cholesterol as a substrate [57,58]. In **chapter 7** we also found increased fecal excretion of BAs which may form an explanation for the reduced plasma cholesterol levels. It seems therefore plausible that interference with the gut microbiota in which BSH-activity and BA metabolism are affected, may eventually alter plasma cholesterol levels and as a result atherosclerosis development.

CAUSALLY LINK GUT MICROBIOTA TO ATHEROSCLEROSIS

When studying the microbiota and its direct role in disease, it is important to keep in mind Koch's postulates, which describe the criteria that are needed in order to determine a causative relationship between microorganisms and disease. As Koch's postulates were established in the late nineteenth century, one should now adapt these postulates and incorporate the substantial amount of knowledge on host-microorganism interactions. Alterations in the entire microbiome should be incorporated rather than only one specific pathogenic species. In order to determine whether the altered microbiota causes, or solely reflects, atherosclerosis in this thesis, a follow-up study is needed in which cecal content of the mice fed either inulin or MOS will be transferred to control mice. This will allow for prove of causality between the gut microbiota and the development of atherosclerosis.

ARE MOUSE STUDIES ON GUT MICROBIOTA TRANSLATIONAL TO HUMANS?

Much of the basic gut microbiota research is performed in mice. However, there are quite some differences between mice and humans. The gastrointestinal tract differs anatomically with for example a relatively short colon, a functional cecum and no appendix in mice compared to in humans [59]. In addition, physiological differences are substantial. For example, even on extreme diets wild type mice are quite resistant to atherosclerosis and overt type 2 diabetes [60,61].

Why do we still continue to use mice in research aiming for strategies to prevent and modulate human diseases? One of the reasons is that mice and humans share 99% of their genes and differ by 14% in genome size [62]. Furthermore, despite their vastly different overall body size, intestinal anatomy and diet (e.g. mice are coprophagous), the same phyla dominate the distal guts of mice and humans: *Firmicutes*, *Bacteroidetes*, and *Actinobacteria* [35]. However, a main reason to use mice is that mouse models are instrumental in assessing causality of complex gene-environment and host-microbiota interactions in a well-controlled manner. It is very difficult to study gut microbiota-host interactions in humans directly as human bacterial communities are influenced by a plethora of genetic and environmental factors. For example, in a recent study 126 intrinsic and extrinsic factors were found to be associated with inter-individual variation of the gut microbiota [63]. In fact, it is estimated that to adequately assess a relationship between metabolic disease and the intestinal microbiota while correcting for confounding factors, a study should contain at least 1700 subjects [64], which leads to a large variation that is difficult to correct for in human studies. However, even in well-controlled gut microbiota experiments using mouse models inter-study variations can occur due to confounding factors in the experimental setup. These variations include mouse breeding origin and housing, genetic background, maternal effects, and environmental conditions including diet, amount of (day)light, stress, and SPF conditions [59]. When setting up a new experiment, researchers therefore need to specifically take into account these possible study confounders.

To overcome these limitations, recently efforts have been initiated to standardise gut microbiota experiments. An example is the establishment of a standardised microbiota

in isobiotic mice that subsequently can be shared by different institutions performing gut microbiota research [42,65]. Although these efforts are still preliminary, they will increase reproducibility and comparability of experimental results between different studies, which is absolutely essential for progress in the gut microbiota research field.

PROBIOTICS, PREBIOTICS OR SYNBIOTICS?

SUPPLEMENTATION OF PROBIOTICS

Among the first strategies proposed to modulate gut microbiota was the administration of live microbes, probiotics. Some members of the gut bacteria are believed to promote health, whereas others may pose threats to health, particularly if they overgrow. Probiotics must possess specific properties in addition to conveying specific health benefits to the host. They need to stay viable and survive passage through the upper regions of the gastrointestinal tract and persist in the colon. They should be resistant to antagonistic, mutagenic, or pathogenic conditions in the gut. Also, the chosen microorganisms must be amenable to industrial processes and have to remain viable in the final supplemental/food product [66]. Even when probiotics fulfil the above mentioned criteria, there are limitations to the use of probiotics to promote health and/or prevent disease. A major limitation is that bacteria may exert completely different functions depending on environmental factors and the presence of other bacteria. It is therefore difficult to draw general conclusions about universal health effects of probiotics. Indeed, currently, few health claims for probiotics have been approved in Europe or the United States by the responsible regulatory agencies, e.g. European Food Safety Authority (EFSA) and US Food and Drug Administration (FDA). These limitations have not prevented numerous researchers and commercial companies to attribute therapeutic potential to probiotic microorganisms for obesity, insulin resistance syndrome, type 2 diabetes, and non-alcoholic fatty liver disease (NAFLD)(reviewed in [67]).

SUPPLEMENTATION OF PREBIOTICS

Prebiotics are defined as 'selectively fermentable ingredients that allow specific changes in the composition and/or activity of gastrointestinal microbiota that provide benefits to the host'. Multiple studies have reported the occurrence of study participants who respond to prebiotics (responders), whereas in other similar studies, the study participants failed to respond (non-responders) to the same prebiotic treatments [68–71]. This implies significant inter-individual variability in the response to dietary interventions. These responses likely depend on the taxonomic and functional composition of the gut microbiota. However, also other abiotic factors seem to play a role in the response to a given prebiotic. These include the nature of the digestive enzymes provided by the host, stomach and intestinal pH, and transit time, all of which can ultimately affect growth of bacterial members, even if a suitable growth substrate is provided [68]. As a practical strategy and future perspective, the introduction of multiple indigestible carbohydrates simultaneously or the combination of pre- and probiotics (synbiotics) may result in the enrichment and more diverse population of gut microbes.

SUPPLEMENTATION OF SYNBIOTICS

Synbiotics consist of a probiotic strain and a prebiotic substrate, in which the prebiotic is specifically intended to support the growth of the cognate probiotic [72]. One of the advantages of synbiotics is that such formulations could address the responder/non-responder phenomenon. To become established in the colon, a probiotic must not only secure nutrients and other growth factors but also outcompete the resident microbiota. By providing the probiotic organism with a niche opportunity in the form of a selectively fermentable prebiotic, the strain is given a competitive advantage. The most commonly used synbiotic combinations contain the probiotics *Lactobacilli* and *Bifidobacteria*, together with oligosaccharides, inulin, or fibers as the prebiotic component [73]. As we did not detect *Bifidobacteria* in cecum samples of *E3L.CETP* mice after feeding them with inulin (**chapter 5**), it remains to be investigated whether co-administration of inulin with *Bifidobacteria* would have resulted in different effects on plasma lipids and atherosclerosis development.

SUPPLEMENTATION OF SHORT-CHAIN FATTY ACIDS

Much of the beneficial effects of changes in gut microbiota composition on disease outcome are often attributed to the increased production of SCFAs. It therefore seems tempting and perhaps reasonably to supplement SCFAs directly instead of using probiotics or prebiotics. In fact, oral administration of SCFA has been associated with several beneficial effects. For example, oral administration with butyrate impairs atherogenesis by reducing plaque inflammation [74], protects against non-alcoholic steatohepatitis (NASH)[75], improves insulin sensitivity and increases energy expenditure [76], activates brown adipose tissue and reduces appetite via the gut-brain neural circuit [77]. It is important to consider the site of SCFA production to fully understand the biological effects of SCFA in humans. For instance, oral SCFA are rapidly absorbed in the proximal intestine and oxidised [78] and it is demonstrated that circulating concentrations of SCFAs, except for acetate, are toxic in high concentrations which might even lead to coma [79]. Therefore, is seems very important that either the right concentration of SCFA is administered in order to avoid toxic effects or that SCFAs are being produced in the distal gut after e.g. fermentation of prebiotics.

With prebiotic supplementation one assumes that fermentation of these indigestible carbohydrates takes only place in the distal part of the gut, the colon, where the gut microbiota resides. However, evidence also supports for a role of the gut microbiota in fermentation of e.g. inulin in the upper part of the GI tract [80]. This proximal site of fermentation might play an important role for the actual effect of inulin on host physiology and metabolism. For example, when inulin is fermented by gut bacteria in the colon, inulin is broken down into smaller pieces of fructose units [81]. In mice, the colon does not contain fructose receptors such as Glut5 (Slc2a5) compared to the small intestine [82]. The majority of the fructose formed after fermentation of inulin therefore abide in the colon. On the other hand, when inulin is fermented in the small intestine, fructose can be taken up directly by the fructose receptors, enter the bloodstream, and end up in e.g. the liver. Increased uptake of fructose is implicated in the development of metabolic diseases such as fructose-induced hypertension and NAFLD [83]. In **chapter 5**, we found that inulin both resulted in increased SCFA production in

cecum content, but also affected liver inflammation when combined with higher percentages (0.5%) of dietary cholesterol. Whether fermentation of inulin in our study took place in the small intestine and therefore resulted in increased uptake of fructose in the small intestine is not known.

Future studies should aim for the identification and validation of a role for synbiotics in health and disease. In order to study this, one could introduce an *in vivo* selection method that relies on the selection and isolation of strains whose abundance is significantly enriched in animals or study participants who had consumed a given prebiotic. When recombined as a synbiotic and introduced into a new host, these strains would be expected to colonise at greater levels than in the absence of the prebiotic. Furthermore, the site of fermentation of prebiotics and/or administration of SCFAs should be taken into account. This is a first step in the facilitation of understanding the specific effects of pre-, pro-, and synbiotics, and the processes involved in survival and the crosstalk mechanisms with the human host.

CONCLUSION

The studies described in this thesis increased our knowledge on the potential of the indigestible carbohydrates inulin and MOS in the modulation of the gut microbiota to affect the development of cardiometabolic disease. Specifically MOS induced beneficial effects on gut microbiota composition, atherosclerosis development and minor effects on the immune system. Although inulin did show prebiotic activity by changing gut microbiota composition and increasing the production of SCFAs, inulin adversely affected atherosclerosis development or led to manifestations of liver inflammation. The context in which the prebiotic is administered (e.g. mouse model, dietary background, concentration of the prebiotic) might be important factors that determine the actual effect of the prebiotic on cardiometabolic disease. Therefore, modulating cardiometabolic disease using indigestible carbohydrates suggest a promising strategy to further pursue but also warrants for some caution.

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