Faecal matters

How transplanting gut bacteria and their viruses impacts cardiometabolic disease

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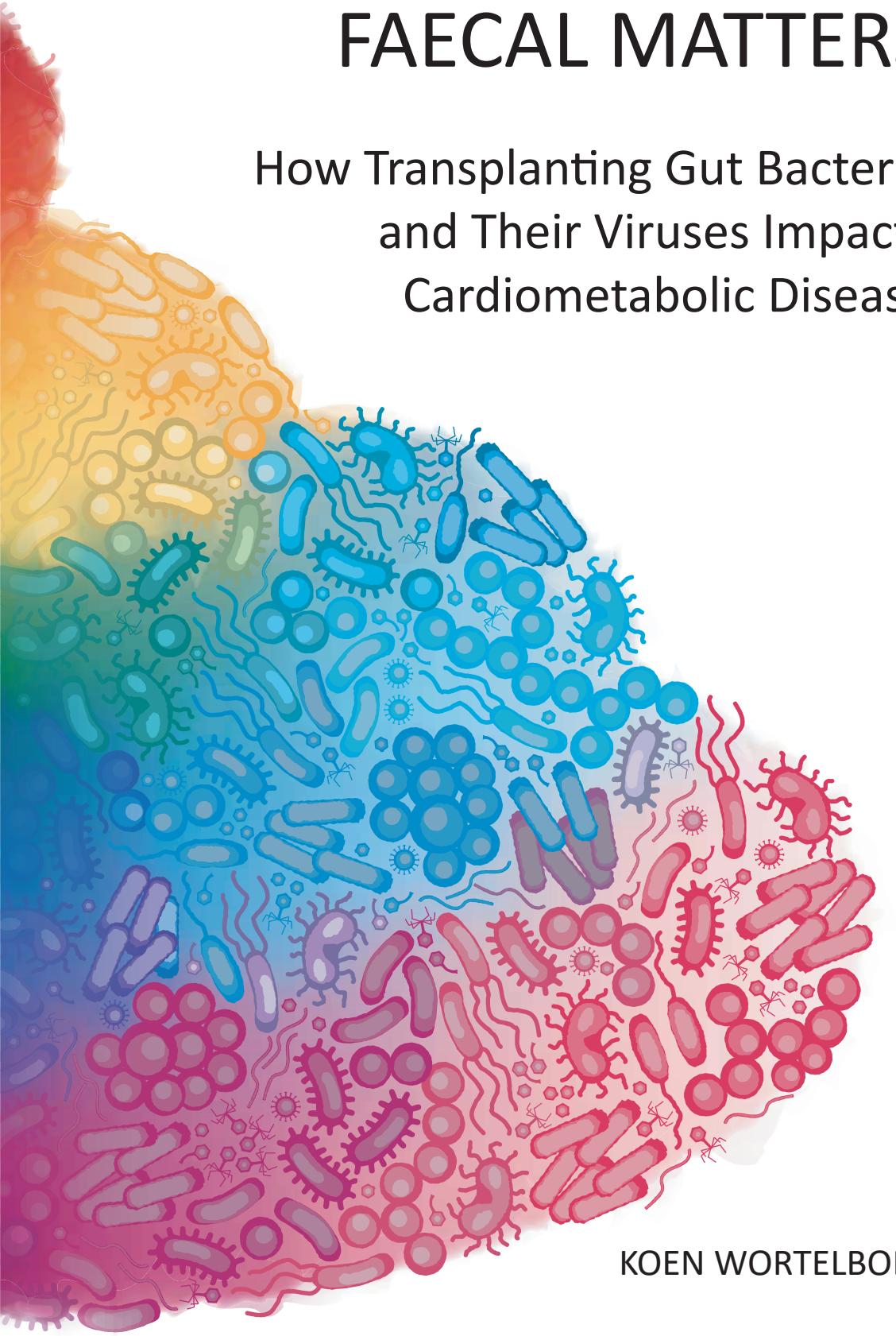

KOEN WORTELBOER

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Faecal matters: how transplanting gut bacteria and their viruses impacts cardiometabolic disease

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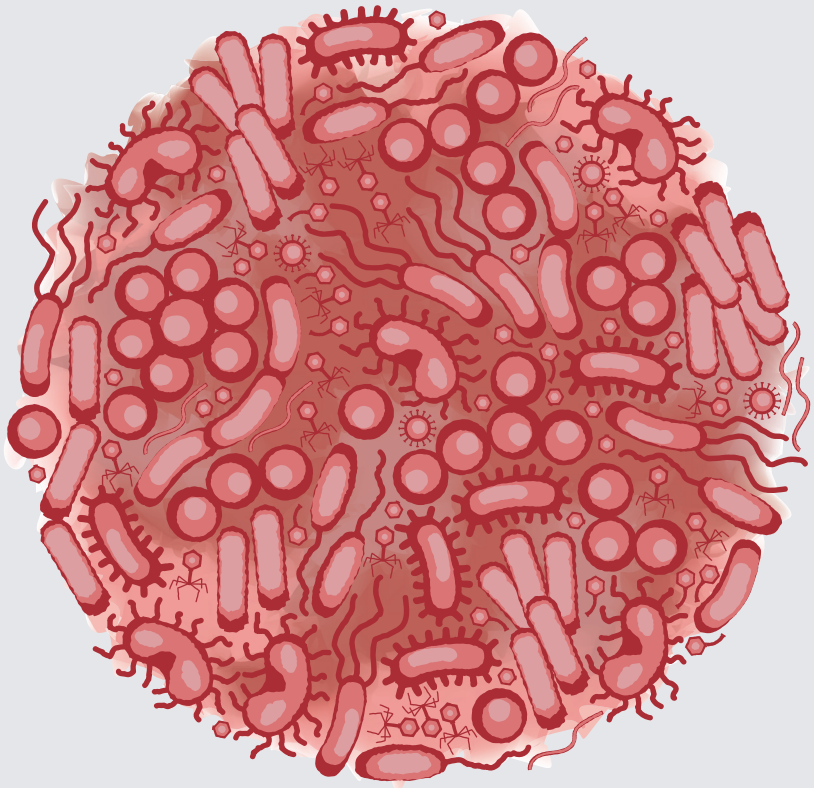
CONTENTS

CHAPTER 1

General Introduction & Thesis Outline



GENERAL INTRODUCTION

The pandemic of cardiometabolic diseases

The global prevalence of cardiometabolic diseases (CMD) is increasing worldwide, especially in low- and middle-income countries[1]. CMD encompass a range of interconnected cardiovascular and metabolic health conditions, such as obesity, type 2 diabetes (T2D), and cardiovascular diseases. Overweight and obesity, key components of the metabolic syndrome (MetSyn), are particularly important risk factors for other CMD and have an increasing socio-economic impact[2]. Since 1975, the obesity prevalence has nearly tripled[3], affecting globally 1.0 billion people (14% of the world population) in 2020, which is expected to rise to 1.9 billion (24%) by 2035[4]. Along with the rapid increase in the number of overweight and obese individuals, the prevalence of other CMD such as MetSyn has increased, while the number of individuals with diabetes has quadrupled worldwide between 1980 and 2014[5,6]. Importantly, CMD are a leading cause of morbidity and mortality, with cardiovascular diseases accounting for 31% of global deaths[1]. This highlights the importance of reducing the incidence of CMD and associated morbidity and mortality.

Cardiometabolic diseases have a multifactorial pathophysiology

The pathophysiology of CMD is complex and multifactorial. Many different factors may be involved, including lifestyle, diet, environment, genetic, and epigenetic factors[7]. For example, overeating and lack of physical activity have been identified as major contributors to the development of MetSyn. Overweight and obesity are characterized by visceral adiposity, which in turn can lead to insulin resistance, a chronic state of low-grade inflammation, and neurohormonal activation[8]. These mechanisms subsequently can lead to an increased blood glucose[9], abnormal lipid and cholesterol levels[10], and hypertension[11], which in turn increase the risk of progressing towards T2D and cardiovascular diseases[12]. While some symptoms of CMD can be treated with medication, the main approach to treat them is via lifestyle changes designed to lose weight, for example by increasing physical activity and improving the diet[13]. Strikingly, loss of 15% or more of bodyweight can have a disease-modifying effect in T2D, improving not only glycaemic control, but also risk factors for cardiovascular diseases and quality of life[14,15].

The gut microbiota is associated with disease

Beside the above-mentioned factors that contribute to development of CMD, a growing body of evidence suggests a role for the gut microbiota in the development of CMD[19–21]. The gut microbiota refers to the vast and diverse community of microorganisms residing in the gastrointestinal tract, including bacteria, viruses, fungi, and archaea. Every person harbours trillions of gut microbes, which are estimated to be equal to the number of human cells and weigh up to 200 gram[22]. This complex ecosystem is involved in various

physiological processes, including digestion, metabolism, and immune regulation, which influence our well-being. In line, research has linked alterations in the gut microbiota to a wide range of diseases, including cardiometabolic disorders such as obesity, diabetes, and non-alcoholic fatty liver disease[23,24]. However, association does not necessarily mean a causal contribution of the gut microbiota to a specific disease. Before moving towards causality of the gut microbiota in CMD and methods to study this, it is important to first have a better understanding of the gut microbiota and its functions.

The gut microbiota – who are there?

The gut microbiota constitutes a complex ecosystem comprising various microorganisms, including bacteria, archaea, protists, and fungi, as well as viruses. While their numbers equal the number of human cells, their combined genetic material, or the gut microbiome, encodes a hundred-fold more genes than the human genome[25]. These genes enable many microbial activities, which produce a plethora of metabolites that contribute to human health. Bacteria account for more than 90% of the genetic material in the gut microbiome, which is why the majority of research efforts have focused on bacteria in the past decades[26]. In addition, with the development of high-throughput sequencing techniques to study bacteria, such as the 16S rRNA gene amplicon sequencing, affordable technology to study bacteria became widely available[27].

BOX 1: prevalence of obesity and diabetes worldwide and in the Netherlands

Globally, 2.6 billion (38%) adults, adolescents, and children were estimated to be overweight or obese (body mass index (BMI) ≥25 kg/m2) in 2020, and this number is expected to rise to 4.0 billion (51%) by 2035[4]. During this period, the number of individuals with obesity (BMI ≥30 kg/m2) is anticipated to rise from 1.0 billion (14%) to 1.9 billion (24%)[4]. In the Netherlands, these numbers are similar, with 44% of the population being overweight or obese in 2020 and 12% having obesity[16]. Notably, the prevalence increases with age, with 51% of adults being overweight or obese in the Netherlands, of which 14% is obese[16].

In line with the obesity prevalence, it is estimated that 537 million (10%) adults were suffering from diabetes worldwide in 2021, which is expected to increase with 46% to 783 million (12%) adults by 2045[6]. The vast majority of these people (over 90%) suffer from type 2 diabetes (T2D), which is characterized by hyperglycaemia resulting from insulin resistance[6]. Compared to type 1 diabetes, which is characterised by an autoimmune destruction of the insulin-producing beta cells, the symptoms of T2D are in general less severe and the condition can even be symptomless. As a result, it is estimated that 240 million people are living with yet undiagnosed diabetes worldwide[6]. In addition, 860 million (17%) adults are estimated to have an impaired glucose tolerance or an impaired fasting glucose[6]. This state, also called prediabetes, signifies an increased risk for progression towards T2D[17] and an increased risk for cardiovascular disease (CVD)[18]. In the Netherlands, the diabetes prevalence is slightly lower compared to the world, yet it is still alarmingly high: 1.2 million people (7%) have diabetes and 1.1 million (6%) have prediabetes.

Bacteria

The bacteria within the gut microbiota are dominated by several bacterial phyla, including the *Bacillota* (previously *Firmicutes)*, *Bacteroidota* (previously *Bacteroidetes)*, *Actinomycetota* (previously *Actinobacteria)*, *Pseudomonodota* (previously *Proteobacteria)*, *Verrucomicrobiota* (previously *Verrucomicrobia)*, and *Fusobacteriota* (previously *Fusobacteria)*, of which the *Bacillota* and *Bacteroidota* represent about 90% of gut bacteria[28]. The *Bacillota* phylum comprises more than 200 different genera, including *Clostridium*, *Ruminococcus*, *Lactobacillus*, *Bacillus*, and *Enterococcus*. The *Bacteroidota* phylum is predominated by the *Bacteroides* and *Prevotella* genera, while the well-known *Bifidobacterium* genus represents the *Actinomycetota* phylum. More than 2500 bacterial species have been identified in the human gut so far[26], while individuals harbour on average 160 bacterial species in their intestine[29]. Moreover, bacterial species and functions vary substantially, both throughout the GI tract and between individuals, which can be explained by several intrinsic and extrinsic factors (see below). To better understand the origins and implications of this bacterial variation, different clusters of bacterial populations that tend to co-occur in individuals have been identified, so called enterotypes[30]. There are three enterotypes, which are characterized by different dominant clusters, namely the Bacteroides, Prevotella, and Ruminococcus enterotype. These distinctive clusters of bacteria are thought to form trophic networks, which have functional characteristics and define distinctive ways of generating energy from fermentable substrates available in the colon[31].

Viruses

While often overlooked in the past, the emergence of affordable shotgun metagenomics has increased research efforts into the virome. The gut virome is the collection of pro- and eukaryotic viruses present within the gut microbiota. Although the viruses only account for approximately 6% of total DNA within the microbiome due to their much smaller genomes, they are estimated to be as abundant as the gut bacteria [32,33]. Bacteriophages (phages), viruses that exclusively infect bacteria, dominate the viral population, accounting for 98% of the viruses present[34]. Phages infect bacteria and most often incorporate their genetic material into bacterial genomes as a prophage (lysogeny), or kill the bacteria (lysis)[35]. Thus, phages modulate bacterial communities in every environment, including the intestine[36–38].

Like bacteria, the viral composition within the gut microbiota can be classified into various viral families, each displaying distinct characteristics and interactions with the bacterial counterparts. Historically, phages were classified as other viruses, based on the type of nucleic acid (i.e., double-stranded (ds) or single-stranded (ss) DNA or RNA), particle morphology (e.g., presence or absence of a tail) and presence of an envelope. Interestingly, most phages are non-enveloped, dsDNA *Caudoviricetes*, ssDNA *Microviridae*, or ssDNA

Inoviridae[39]. However, the traditional differentiation within the *Caudoviricetes* order based on tail types into the *Siphoviridae*, *Myoviridae*, and *Podoviridae* families is not completely coherent with their phylogeny. Moreover, until recently, 85-99% of phages in each human sample were novel, uncultured, or not taxonomically classified[40,41]. Assembly of uncultured phage genomes from metagenomic data has led to the continuous discovery and classification of new phages[42,43]. Examples of these are the recently described order of *Crassvirales* (crAss-like phages), which comprises several clades of dsDNA phages with a short non-contractile tail[44,45], the Lak megaphages, with genome sizes of more than 0.5 Mb[46], and the Gubaphage[47]. As a result, phage classification is moving from morphology-based classification towards genome-based classification, with the traditional *Myoviridae*, *Podoviridae*, and *Siphoviridae* families abolished in 2023[48].

Archaea, fungi, and protists

Archaea, fungi, and protists are less abundant within the microbiota and outside the scope of this thesis. Archaea are unicellular prokaryotes that share many properties with both bacteria and eukaryotes. Methane-forming archaea of the *Methanobacteriales* and *Methanomassiliicoccales* phyla are the most prevalent and abundant within the gut microbiota, with *Methanobrevibacter smithii* being the most common methanogen with a prevalence of around 96%[49,50]. In general, archaea account for 0.8% of total DNA within the microbiome, with numbers ranging between the 10^8 – 10^10 colony-forming units (CFU) per gram of faeces[51]. The fungi that inhabit the gut microbiota, also referred to as the mycobiome, account for 0.1% of the total DNA, with numbers reaching 10^2 – 10^6 CFU/g faeces[52]. Fungal communities are relatively less diverse than the gut bacteria and are mainly dominated by *Saccharomyces*, *Candida,* and *Malassezia* [53,54]. One study identified 701 fungal species encompassing 247 genera in stool samples of 147 healthy individuals, although their individual gut microbiomes contained only 2 – 92 species per individual[55]. Protists are a diverse group of unicellular eukaryotes, estimated to account for 0.2% of the microbiome DNA, although their exact numbers are unknown[30]. Historically, protists (but also helminths) identified in humans were considered harmful parasites. However, recent studies showed that protists such as *Blastocystis spp.* and *Dientamoeba fragilis* are more common than previously thought, which have been associated with healthy and diverse microbiomes, and could beneficially influence the immune system[56–58].

Development of the gut microbiota in early life

Our intestinal microbiota develops within the first 2-3 years of life, starting from the moment of birth. Although there is ongoing debate regarding microbial colonization in utero[59], it is in general believed that the amniotic sac remains free of living microbes[60,61]. However, evidence suggests that maternal microbiota-produced metabolites can cross the placental barrier, influencing the (immune) development of the foetus[62]. Upon birth,

BOX 2: terminology of gut microbiota research

- **Microbiota**: a community of microorganisms, including bacteria, viruses, archaea, protists, and fungi, that reside in a particular site or environment, such as the human gut.
- **Microbiome**: the collective genetic material of all microorganisms, both alive and dead, within a particular community or ecosystem.
- **Bacteria & bacteriome**: single-cell microorganisms that belong to the domain Bacteria. They are among the most abundant and diverse organisms on Earth and can be found in various environments. The bacteriome represents the collective genetic material of bacteria that reside in a particular site or environment.
- **Viruses & virome**: microscopic infectious agents that consist of genetic material (DNA or RNA) enclosed in a protein coat. They are obligate intracellular parasites because they require a host cell to replicate. Viruses infect various organisms, including bacteria, plants, animals, and humans. They play important roles in ecosystems, can cause diseases, and have complex interactions with their host organisms. The virome refers to the collective viral genetic material present in a specific site or environment.
- **Bacteriophages & phageome**: phages, short for bacteriophages, are viruses that specifically infect and replicate within bacterial cells. Phages play a significant role in shaping bacterial populations, influencing bacterial evolution, and impacting ecosystem dynamics. The phageome refers to the collective genetic material of phages within a particular community or ecosystem.
- **Fungi & mycobiome**: eukaryotic organisms, including microorganisms such as yeast and moulds that belong to the Fungi kingdom. The mycobiome represents the collective genetic material of fungi that reside in a particular site or environment.
- **Metagenome & metagenomics**: the collective genetic material obtained from microorganisms present in a specific sample or environmental niche, such as a microbiome. Metagenomics is the study of metagenomes, which provides insights into composition, functional potential, and ecological interactions of the microorganisms in a given habitat.
- **Shotgun sequencing**: a high-throughput laboratory technique for determining the DNA sequences of organisms present in a sample. It involves randomly breaking the DNA into small fragments, sequencing them, and then assembling the resulting sequences to reconstruct the genomes of the organisms present in the sample.
- **16S rRNA gene amplicon sequencing**: a targeted sequencing method that focuses on amplifying and sequencing a specific region of the bacterial 16S rRNA gene. This gene is commonly used as a molecular marker for bacterial identification and classification, thereby enabling studying bacterial composition and diversity.
- **Metabolome & metabolomics**: the complete set of small molecules, known as metabolites, present in a biological sample or environment. Metabolomics is the study of metabolomes, which provides insight into metabolic processes and pathways occurring in a biological system.
- **Transcriptome & transcriptomics**: the complete set of RNA molecules, including messenger RNA, non-coding RNA, and other functional RNA molecules, present in an individual or a population of cells at a specific time. Transcriptomics is the study of transcriptomes, which provides insight into gene expression patterns, regulation, and functional pathways.
- **Proteome & proteomics**: the complete set of proteins expressed by a cell, tissue, or organism at a specific time. Proteomics is the study of proteomes, providing insight into protein expression, structure, function, interactions, and modifications.
- **Symbiosis**: a close and long-term interaction between two or more different species, often involving physical and biochemical associations. It can be mutually beneficial (mutualism), where both species benefit, or have varying degrees of benefit and harm (parasitism and commensalism). Symbiotic relationships are widespread in nature and can occur between microorganisms and their hosts.
- **Dysbiosis**: an imbalance or disruption in the composition or function of the microbiota, typically characterized by a decrease in beneficial microorganisms, an increase in potentially harmful or pathogenic microorganisms, and/or reduced microbial diversity. Dysbiosis can result from various factors such as antibiotic use, diet, lifestyle, or disease conditions, and is associated with adverse health effects.
- **Probiotics**: live microorganisms, primarily bacteria or yeasts, that when consumed in adequate amounts, confer health benefits to the host. They are commonly found in certain fermented foods or dietary supplements and are believed to improve or restore the microbial balance in the gut, enhance digestion, support immune function, or promote overall well-being.
- **Prebiotic**: non-digestible dietary fibres or compounds that selectively promote the growth and activity of beneficial microorganisms in the gut, primarily bacteria. They serve as a substrate for the growth of specific beneficial bacteria, providing them with a competitive advantage.
- **Synbiotic**: a combination of probiotics and prebiotics, where the prebiotic compound is specifically selected to enhance the survival and activity of the beneficial microorganisms in the probiotic mixture. Synbiotics aim to maximize the health benefits of both probiotics and prebiotics by providing a supportive environment for the growth and activity of the beneficial microorganisms.

newborns leave the protective environment of the amniotic sac and are exposed to a variety of microbes, which immediately begin colonizing all body surfaces, including skin, lung, and GI tract. In the first weeks of life, the newborns gut microbiota is primarily dominated by bacterial families such as *Enterococcacae*, *Clostridiaceae*, *Lactobacillaceae*, *Bifidobacteriaceae*, and *Streptococcaceae*[63]. In the first months, especially the *Bifidobacteriaceae* thrive, which feed on the oligosaccharides that are highly abundant in maternal milk. When solid foods are slowly introduced around 4-6 months, also known as the weaning phase, the abundance of the *Lachnospiracea*, *Clostridiaceae*, and *Oscillospiraceae* increases, while the *Bifidobacteriaceae* decrease[63]. By the age of 2-3 years, the infants microbiota starts to resemble that of adults, characterized by a high abundance of *Bacteroidaceae*, *Lachnospiraceae*, and *Oscillospiraceae*, which remain relatively stable into adulthood[63]. This establishment of commensal microbes is important for the maturation of the infant's immune system and lifelong health[64].

The development of the early life microbiota is influenced by various factors, which in turn can affect the development of the neonatal immune system[64]. The mode of delivery is one such factor, as it determines the initial colonizers of the body surfaces. Vaginally born infants are first exposed to their mothers' vaginal microbiota, which includes microbes inhabiting the maternal gut lumen[65]. On the other hand, infants born via caesarean section are initially exposed to skin-colonizing microbes, such as *Staphylococcus aureus*, or microbes circulating in hospital environments, such as *Enterococcus faecalis*, *Enterobacter cloacae*, and *Klebsiella pneumoniae*[66]. Although the microbiota of these infants usually recover within the first year of life[67], the initially altered composition may interfere with a healthy development of the immune system, increasing the risk of childhood asthma and other chronic inflammatory diseases[68,69]. Another factor that influences microbiota development in the first months of life is whether the neonate is fed breast milk or formula milk[70]. Breastmilk contains a variety of biologically active molecules, ranging from oligosaccharides, antibodies and immune cells, to growth factors, cytokines and exosomes carrying miRNAs[64]. These components can either directly influence the gut microbiota, such as prebiotic oligosaccharides or antibodies targeting pathogens[71,72], or indirectly promote tolerance of commensal microbes by influencing the neonatal immune system[73]. Other factors known to influence the infant's microbiota are the use of antibiotics[74,75] and the introduction of solid foods[76]. Interestingly, while the host's genetic background contributes to the composition of the intestinal microbiota, its estimated impact is relatively modest, accounting for only 8.8% of the variation observed[77].

Intrinsic factors that shape the microbiota

The composition and function of the gut microbiota are influenced by a wide range of intrinsic and extrinsic factors, highlighting the dynamic nature of this microbial community.

Throughout the anatomical regions of the GI tract, a large variety in bacterial communities is observed, also referred to as biogeography[78]. While the oral and saliva contain millions of microbes that are daily ingested with our food, their survival in the gut is impeded by several factors. These include the acidity of the stomach, the secretion of bile acids in the duodenum, and the secretion of digestive enzymes, antimicrobial peptides and antibodies throughout the GI tract[23,78]. Consequently, the microbial abundance in the duodenum is more than a thousand-fold lower compared to oral samples[79]. Other factors that affect microbial colonization throughout the GI tract are the pH, oxygen concentration and redox potential, as well as the gut anatomy, mucus layer, peristalsis and transit time[23,78]. Conversely, these intrinsic factors are influenced by host genome, age, sex, BMI, external factors, including diet and lifestyle, and interaction with the microbes themselves[80–82].

Increasing transit times and pH lead to an increasing microbial density along the GI tract, with abundances in the small intestine increasing from 10^3 to 10^8 cells per gram, culminating in the colon with up to 10^{11} cells per gram[83]. While the small intestine are primarily dominated by partly oxygen-tolerant *Firmicutes* and *Proteobacteria*, the composition shifts towards more obligate anaerobic bacteria as oxygen concentrations decrease towards the lower intestine[84]. The colon harbours a more diverse microbiota consisting of the major phyla of *Firmicutes* (predominantly *Ruminococcaceae* and *Lachnospiraceae*), *Bacteroidetes* (including *Bacteroidaceae*, *Prevotellaceae* and *Rikenellaceae*), *Actinobacteria*, *Proteobacteria* and *Verrucomicrobia* [23,78]. Besides these vertical variations in gut microbiota throughout the GI tract, also a horizontal gradient exists from the mucosal surface towards the lumen due to decreasing oxygen concentrations, redox potential, and mucus thickness. For example, in the colon, the outer mucus layer is colonized by microaerophilic mucin-degrading bacteria, including *Bacteroides fragilis*, *Bifidobacteriaceae* and *Akkermansia muciniphila*[85]. Moving towards the anoxic lumen, primarily strict anaerobic microbes are found, with a high abundance of *Ruminococcaceae* and *Lachnospiraceae* in the inner-folds of the lumen, and *Bacteroidaceae*, *Prevotellaceae,* and *Rikenellaceae* in the central lumen compartment through which the digesta flows[86,87]. While there is increasing evidence of how the other members of the microbiota are structured along the GI tract, e.g. the viral biogeography[88], data are limited and mainly derived from animal models.

Extrinsic factors that influence the microbiota

In addition to age and the intrinsic factors described above, external factors exert significant influence on the gut microbiota, with diet being one of the most important modulators. Different diets, based on geography or culture (e.g. hunter-gatherer), or personal choices (e.g. vegetarian) have been associated with a different gut microbiota[89,90]. Several studies have shown that dietary interventions can induce acute shifts in gut microbiota composition and functionality within one day[91,92]. However, despite these rapid changes, long-term

dietary habits are required to induce major changes in the gut microbiota, e.g. in the enterotype[92,93]. In addition, alterations in microbiota composition in response to dietary changes vary largely between individuals[94,95]. Notably, nondigestible carbohydrates, including starches and fibre, serve as food source for many colonic microbes, which ferment these nutrients into short-chain fatty acids (SCFA)[96]. These complex carbohydrates also function as prebiotics that can induce a selective pressure on the gut microbiota, favouring specific microbes[96].

Besides diet, other lifestyle choices or cultural habits influence the gut microbiota, including exercise and sleep. Exercise has been shown to enrich microbial diversity and increase the number of beneficial microbes, which have been implicated in improving metabolic and inflammatory markers[97,98]. But not everybody responds the same to exercise: in some cases exercise results in a lack of or even adverse response on metabolic health, an effect partly determined by an individual's gut microbiota and it's fermentative capabilities[99]. Like exercise, sleep quality and duration have been associated with the gut microbiota[100]. In line, the light-induced circadian rhythm has been found to modulate microbial composition, while microbial circadian oscillations in turn can affect host metabolism and immunity[101].

Furthermore, geographical location, household dynamics, and social interactions affect the composition of the gut microbiota[102,103]. Recent research highlighted the impact of cohabitation, demonstrating the highest bacterial strain sharing between mothers and their offspring, followed by individuals within the same household, and those residing in the same village[103]. Even the presence of a pet within the household influences the gut microbiota composition[104]. In addition, certain medication and dietary supplements impact the gut microbiota. While the effects of antibiotics on the gut microbiota are well-established[105,106], other medication such as antidiabetic drugs like metformin and proton-pump inhibitors that reduce gastric acid production have been found to alter the gut microbiota composition[107,108]. Moreover, antibiotic treatment in early life, which hampers the natural development of the gut microbiota, has been implicated in weight gain and the development of immunological diseases later in life[109,110].

Why do we need the gut microbiota?

Far from being passive bystanders, the intestinal microbes affect their host's health through a myriad of functions. Even though microbial genomes are much smaller than that of the human host, their joint genomes have a greater metabolic capability. Their metabolic activities range from catabolism and bioconversion of complex molecules to synthesis of compounds that affect both the microbiota and the host[111]. Through these activities, the microbiota modulate the dietary nutrient availability to the host, thereby increasing the energy harvest of the diet[112]. In addition, it has been known for over 40 years that the gut

microbiota can synthesize certain vitamins, particularly vitamin K and several B group vitamins[113]. Below, several other microbial metabolites of interest are discussed.

Short-chain fatty acids

A very important activity of gut microbes is the fermentation of non-digestible substrates like dietary fibres and endogenous intestinal mucus, producing a variety of compounds, most notably SCFAs. The most commonly produced SCFAs are acetate, propionate, and butyrate. Butyrate is the primary energy source for colonocytes and contributes to the anaerobic condition in the colon by activating β-oxidation in the mitochondria of colonocytes, thereby increasing oxygen consumption[114]. Besides maintaining the integrity of the intestinal barrier, butyrate has anti-inflammatory properties and can activate intestinal gluconeogenesis[115]. Propionate, itself a substrate of intestinal gluconeogenesis, can regulate gluconeogenesis and satiety through interaction with the free fatty acid receptor (FFAR) 3[115]. Acetate is the most abundant SCFA and an essential metabolite for the growth of other bacteria. Upon absorption, it is used for the synthesis of cholesterol and lipids in peripheral tissues, and has been implicated in central appetite regulation[116]. By binding FFAR2 and FFAR3 receptors on neuroendocrine L cells, butyrate and propionate regulate gut hormone secretion, including peptide YY (PYY) and glucagon-like peptide-1 (GLP-1), which in turn stimulate insulin release and regulate satiety[117–119]. In addition, FFAR2 activation can stimulate browning of white adipose tissue and reduce fat accumulation in adipocytes[120]. Finally, SCFAs such as butyrate are inhibitors of histone deacetylases, leading to histone hyperacetylation and thus inhibiting gene expression, which contributes to their anti-inflammatory properties[121].

Bile acids

An example of host-microbe co-metabolism is the bile acid metabolism. Primary bile acids are synthesized from cholesterol in the liver and are, after conjugation with glycine or taurine, secreted in the intestine to aid in the absorption of dietary lipids and fat-soluble vitamins. Although the vast majority of bile acids are reabsorbed in the distal small intestine, a small portion reaches the colon where the gut microbiota converts them into unconjugated secondary bile acids[122]. These hydrophobic molecules are easily absorbed into the circulation and act as signalling molecules through a broad range of receptors, including the Takeda G-protein-coupled receptor 5 (TGR5) and the farnesoid X receptor (FXR). Activation of the bile acid membrane receptor TGR5 has been shown to induce GLP-1 secretion, increase energy expenditure and reduce inflammation[123,124]. Moreover, both activation and inhibition of the nuclear FXR receptor by bile acids has been shown to regulate bile acid synthesis and secretion, as well as glucose and lipid metabolism[124–126].

Other microbial metabolites

There are several other examples of microbial metabolites which are less beneficial and have been implicated in disease, including trimethylamine, phenylacetylglutamine, and imidazole propionate. Trimethylamine is produced from dietary phosphatidylcholine and carnitine (dairy and meat) by gut microbes, which is oxidised in the liver to trimethylamine N-oxide and increases the risk of atherosclerosis and other cardiovascular diseases[127,128]. Another microbial metabolite that has been implicated in cardiovascular disease is phenylacetylglutamine. Derived from the amino acid phenylalanine, phenylacetylglutamine can influence adrenergic receptor signalling an is associated with heart failure and thrombosis[129,130]. Moreover, histidine can be converted by gut microbes to imidazole propionate, which has been shown to directly impair hepatic insulin signalling and contribute to insulin resistance in humans[131,132].

In contrast, 4-cresol, a microbial metabolite derived from the fermentation of the amino acids phenylalanine and tyrosine, has been found to enhance insulin secretion and pancreatic β-cell function[133]. Likewise, indole-3-propionic acid, which is a microbe-derived metabolite from tryptophan, has been shown to have antibiotic, anti-inflammatory and antioxidative properties, and has been associated with a lower risk of developing T2D[134,135]. Other indole metabolites derived from tryptophan, a nitrogen source for gut microbes, have been found to bind the transcription factor aryl hydrocarbon receptor (AhR). The AhR regulates several processes in the GI tract, including gut motility, epithelial barrier integrity, and stimulation of intestinal immune cells[136–138]. It's important to realize that production of the metabolites described above is highly dependent on the presence of microbes that express the enzymes necessary for these conversions and the availability of the substrates.

Intestinal colonization resistance, immune system and barrier integrity

In addition to their metabolic functions, the gut microbiota protects the host from colonization by and disease from potentially pathogenic microbes. This colonization resistance is achieved through a combination of direct competition for nutrients, metabolic activities, and immunologic effects on the host[139]. Furthermore, the gut microbiota actively participates in the development and maturation of the host's immune system, contributing to immune regulation and homeostasis[140]. However, the interactions between the gut microbiota and intestinal immune system that allow them to coexist in a mutually tolerant state are complex. For example, the SCFA butyrate can stimulate regulatory T cells, which leads to a reduced inflammation[141]. On the other hand, certain microbes can activate T helper 17 (Th17) cells, which can increase intestinal inflammation upon activation and thereby protect against intestinal pathogens[142]. However, while their activation can improve the immune response and healing in some settings, pathogenic Th17 activation has been linked to inflammatory and autoimmune disease[143]. Finally, the gut microbiota

prevents systemic immune activation and induction of a proinflammatory state by maintaining the gut barrier integrity[144]. By regulating the mucus production and tight junction strength, primarily through butyrate production, gut microbes prevent the translocation of opportunistic pathogens and bacterial products such as lipopolysaccharide to the portal vein and beyond[145,146].

Are gut microbiota involved in cardiometabolic diseases?

Given the gut microbiota's dynamic nature and its impact on various metabolic and immunological processes, disruptions in its composition or function could potentially contribute to disease, including CMD. Indeed, observational studies suggest that the gut microbiota contributes to our metabolic health and, when perturbed, to the pathogenesis of CMD like obesity, MetSyn, and T2D[19,147]. For instance, obese individuals often have a reduced microbial diversity and an altered abundance of certain bacterial taxa, suggesting a potential link between the gut microbiota and energy metabolism[148,149]. Similarly, alterations in the gut microbiota have been observed in individuals with T2D, implying a potential role of microbial "dysbiosis" in glucose metabolism and insulin resistance[150,151]. Although these associations provide valuable insights, establishing causality of the gut microbiota in CMD is crucial to understand their true impact on disease pathogenesis.

Mechanistic evidence from mice studies

To elucidate the mechanisms underlying the association between the gut microbiota and CMD, animal studies have provided valuable insights. Pioneering work with germ-free mice has shown that the gut microbiota affects energy harvest and storage from the diet[152], while a lack of gut microbiota protects these mice from obesity induced by a high-fat, sugar-rich diet[153]. Furthermore, mice harbouring an 'obese microbiota' have an increased capacity to harvest energy from their diet, a trait which can be transferred to germ-free mice through a faecal microbiota transplantation[154,155]. Interestingly, perturbation of the gut microbiota of young mice via subtherapeutic antibiotic therapy has been shown to induce adiposity through metabolic alterations[75,156]. While these mechanistic studies highlight a potential role of the gut microbiota in CMD, it is important to exercise caution when extrapolating findings from mice to humans. Gut microbiome composition and functionality are simpler in mice than humans, and the two species differ in anatomy and genetic background, which strongly drives disease phenotypes[157]. Finally, laboratory mice live in a controlled, sanitized environment. These pitfalls should be considered when interpreting the results of microbiota research in mice.

Intervention studies in humans

To prove gut microbiota cause CMD development in humans, they should satisfy the first and third of Koch's postulates, meaning that removal and addition of the microbiota

alleviates or worsens the metabolic/disease phenotype[158]. This is tested with interventions that impact the gut microbiota composition or function, including dietary modifications, the use of probiotics, prebiotics, bacteriophages (phages), and faecal microbiota transplantation (FMT). FMT studies in human individuals with MetSyn, for example, have shown that a faecal transplant from a lean healthy donor alters the gut microbiota of the recipient and improves peripheral insulin sensitivity[159,160]. This improvement correlated with increased levels of butyrate-producing bacteria, including an increased abundance of *Anaerobutyricum spp*. in the small intestine, which could regulate insulin sensitivity through the production of the short-chain fatty acid (SCFA) butyrate[159]. Another example can be found in **Chapter 5** of this thesis, in which we used FMT-induced microbiota alterations to study the effect on intestinal microRNA (miRNA) secretion[161].

Finally, to confirm a causal contribution, the microbe(s) of interest should affect the metabolic/disease phenotype in a prospective intervention study, e.g. when administered to individuals with MetSyn[158]. An example is *Anaerobutyricum soehngenii*, previously named *Eubacterium hallii*[162], which was identified as potential beneficial microbe[159]. After confirming *that A. soehngenii* was safe and improved insulin sensitivity dose-dependently in mice, it was administered to human individuals with MetSyn[163,164]. The administration of *A. soehngenii* was well tolerated and faecal levels correlated positively with peripheral insulin sensitivity [163]. The second study observed an increased postprandial excursion of glucagon-like peptide 1 (GLP-1), which was accompanied by a reduced glucose variability[164]. The development of this specific beneficial microbe is described in more detail in **Chapter 6** of this thesis[165].

Another example of such a beneficial microbe is *Akkermansia muciniphila*, a mucin-degrading bacterium, which could be safely administered for 3 months and of which the pasteurized form significantly improved insulin sensitivity, reduced insulin levels, and reduced cholesterol[166]. In contrast to supplementing a beneficial microbe, deletion of detrimental microbes can also confirm causality, e.g., by antibiotic treatment or administration of selective phages. Recently, it has been shown that non-alcoholic fatty liver disease could be alleviated by administration of bacteriophages targeting a high alcohol-producing *Klebsiella pneumoniae*[167]. The above human intervention studies have provided evidence of specific gut microbes that can affect CMD, although for many microbes it still remains the question whether they are causative of disease or merely disease modifiers[168]. Moreover, it is plausible that, rather than a single microbe, a consortium of microbes orchestrates the altered physiological functions contributing to disease[169].

Targeting the gut microbiota

To study causality of the gut microbiota in humans, relevant interventions that target the microbes or their activity are required. In addition, these interventions can serve as treatment modalities for those diseases in which the gut microbiota is shown to contribute to the disease pathophysiology. Several interventions have already been mentioned previously and a selection of the most frequently used and promising interventions can be found below, each with its unique mechanism, potential benefits, and disadvantages.

Dietary interventions

Dietary modifications, such as adopting a high-fibre or low-fat diet, are known to induce shifts in microbiota composition and function. However, as described above, long-term dietary interventions are necessary to induce major and lasting changes to the gut microbiota[170]. In addition, the overall adherence to a diet is low[171,172], while the interindividual responses are high[173]. Interestingly, the clinical response to a diet has been shown to correlate with the baseline microbiota composition[173]. Moreover, based on the existing gut microbiota, the glycaemic control of T2D patients could be improved with a personalized diet compared to a traditional Mediterranean diet, both short-term and long-term[174]. These studies demonstrate the reciprocal relationship between diet and the gut microbiota, where both modulate the effect of each other on the human host.

Pro-, pre-, syn- and postbiotics

Besides diet, specific nutrients or prebiotics, usually nondigestible carbohydrates, can be used to selectively promote the growth and activity of certain (beneficial) microbes[175]. Examples of prebiotics are resistant starches and oligosaccharides, including fructo-oligosaccharides, inulins, and galacto-oligosaccharides, which have been found to influence the gut microbiota and promote the growth and activity of beneficial bacteria[176,177]. Alternatively, beneficial or missing microbes can also be directly administered in the form of a probiotic supplement or via fermented food. Traditional probiotics are predominantly from the *Lactobacillus* and *Bifidobacterium* genera, which represent only a small fraction of the adult gut microbiota, and clinical studies have yielded conflicting results[178,179]. Next-generation probiotics, or live biotherapeutic products, are (endogenous) microbes without a long history of safe and beneficial use, that are more likely to colonize the GI tract or be metabolically active, and thus confer a health benefit[180]. Examples of these have been mentioned above, such as *A. muciniphila* and *A. soehngenii*, of which the latter's development is described in **Chapter 6**. Combinations of pro- and prebiotics, also called synbiotics, aim to enhance their synergistic effects on the gut microbiota and host health. While synbiotics are increasingly used in clinical studies, the optimal combination or a personalized formulation warrants further research[181,182]. Finally, postbiotics, defined as non-viable bacterial or metabolic products, confer beneficial metabolites or proteins of the

gut microbiota directly to the host. Examples of postbiotics are the administration of butyrate or the use of the Amuc_1100 protein instead of the live *A. muciniphila* cells from which it was derived[183,184].

Antibiotics & bacteriophages

Altering the gut microbiota by suppressing (bacteriostatic) or killing (bactericidal) specific members or groups of the microbiota can be achieved with the help of antibiotics and other antimicrobial products. An obvious disadvantage of this approach is that there usually is collateral damage, especially with broad-spectrum antibiotics. Furthermore, we cannot precisely predict how a course of antibiotics will affect a specific microbial community or its functional capacity. While more narrow-spectrum antibiotics have been developed, such as fidaxomicin for *Clostridioides difficile* infections (CDI), broad-range antibiotics can be used to temporarily disrupt the human gut microbiota to confirm a microbial contribution to a disease phenotype. To illustrate this, a recent study used a course of antibiotics to prove that gut bacteria, more specifically *Lactobacillaceae*, produced endogenous ethanol in individuals with non-alcoholic fatty liver disease[185].

An elegant method to target specific (harmful) bacterial members of a community is the use of bacteriophage therapy. As phages infect and eliminate specific bacteria, this prevents off-target effects[186]. Host specificity varies among phages and is determined by the specific receptors they can bind on the bacterial cell surface, with some phages being strain specific, while others can infect a range of bacterial strains and even genera[187]. Phage therapy has been extensively used in the former Soviet Union and Eastern Europe as alternative for antibiotics for almost a century, and the increasing worldwide incidence of infections with antibiotic-resistant bacteria has sparked a renewed interest in phage therapy[188]. However, clinical research with phages is still in its infancy in Western societies[189].

Faecal microbiota transplantation

In cases where significant alterations in the gut microbiota are needed, FMT has emerged as a popular intervention. FMT involves transferring faecal material from a healthy donor into the gastrointestinal tract of a diseased individual with an altered or dysbiotic gut microbiota[190]. This procedure aims to restore a more balanced microbial ecosystem and has shown remarkable success in treating recurrent *Clostridioides difficile* infection (rCDI). Having shown high cure rates compared to antibiotic therapy, with diarrhoea resolution rates up to 90%, FMT has become a routine treatment for rCDI[191]. FMT is currently being explored as a potential therapeutic approach for a wide range of conditions, including inflammatory bowel disease, metabolic disorders, and even neuropsychiatric diseases[192]. These endeavours are described in more detail in **Chapter 2**.

History of faecal microbiota transplantation

The administration of faecal suspensions has a rich history dating back thousands of years. While many believe the first documentation was by the traditional Chinese doctor Ge Hong in the 4th century, records on the use of oral faecal matter have been found in older texts of Chinese medicine dating back to the 8th century BCE[193]. These texts described details of the preparation of a faecal slurry called “yellow soup” or “golden juice”, which was used as a treatment for food poisoning and severe diarrhoea[194]. Subsequent Chinese medicine handbooks added supplemented and new versions of faecal preparations, with formulae containing faeces of human, animal, and even insect origin[193]. These include a range of faecal preparations described by the Chinese doctor named Li Shizhen, which were used as treatment for a variety of GI-diseases, including constipation, fever, vomiting, and pain[195]. A widely cited story suggests that North African Bedouins advised German soldiers to consume fresh camel faeces containing *Bacillus subtilis* as a treatment for bacterial dysentery during world war II[196]. However, the accuracy of this claim has recently been questioned, as no direct evidence could be identified and camel faeces was found to contain only low amounts of *Bacillus subtilis* spores[197]. Although the concept of microbial benefits to health had been proposed by Metchnikoff in 1907, it was not until 1958 that faecal enemas were first described in scientific literature as a treatment for pseudomembranous enterocolitis by Dr. Ben Eiseman, an American surgeon[198]. This pivotal discovery has paved the way for further exploration of FMT's potential applications and therapeutic benefits, first as treatment for rCDI and later for many other conditions[192].

How does it work?

FMT aims to restore a more balanced microbial ecosystem in diseased individual with an altered or dysbiotic gut microbiota[199]. However, microbial dysbiosis is a controversial term, which refers to an imbalance or disruption of the composition and function of the microbiota, usually characterized by a reduced microbial diversity, altered abundances, and disrupted microbial interactions and functions. The controversy surrounding this term stems from the lack of consensus regarding the definition of a "normal" healthy microbiota and, consequently, the characterization of a dysbiotic state[200]. In addition, the complexity and dynamic nature of microbial communities make it challenging to establish clear cause-and-effect relationships between dysbiosis and specific diseases.

The precise mechanisms of FMT efficacy are incompletely understood and are likely different for the wide range of disease for which FMT is currently tested. For rCDI it was shown that FMT responders had an increased bacterial diversity and that their microbiota shifted towards an remained similar to that of their donors[201]. Specific bacteria, phages, archaea and fungi have been described which associated with FMT success, although results heterogenous and the precise mechanisms require further research[201]. Examples of these

are a decreased *Enterobacteriaceae* abundance post FMT[202], a higher donor *Caudoviricetes* richness[203], or increased *Methanobrevibacter smithii* post FMT[204]. While incompletely understood, the downstream effects of the altered microbes, including metabolic products such as SCFAs and secondary bile acids, immune cross-talk, and colonization resistance, likely mediate the efficacy of the FMT[205–207].

Screening healthy donors

Screening of stool donors is a crucial step in FMT to ensure the safety of the procedure. Potential donors undergo a rigorous screening process that includes a thorough questionnaire on medical history and high-risk behaviours, and examination of blood and stool samples[208]. Based on the questionnaire, people that have gastrointestinal comorbidities, have a high risk of infectious agents, or have factors that can perturb the intestinal microbiota (e.g., antibiotic use), are excluded. In addition, the blood and stool tests screen for the presence of pathogenic bacteria, viruses and parasites, examine liver and kidney function, and look for an impaired immunity[209]. This stringent screening is essential to minimize the risk of transmitting infections or other adverse effects from the donor to the recipient. However, many potential donors fail this extensive screening, with success rates estimated between 0.8–31%[210–215]. In **Chapter 3** of this thesis, our experiences with the donor screening for FMT are described.

Historically, spouse and close relatives were considered ideal stool donors, as they would have a more similar microbiota due to the shared environment, which was thought to be better tolerated by the recipient's intestinal immune system[216]. However, no clinical evidence has proven this association between donor relatedness and FMT outcome[217,218], and unrelated donors were hypothesized to be favourable in cases where genetics may contribute to disease pathophysiology, such as in inflammatory bowel disease[219]. In addition, several studies have suggested that FMT success depends on microbiota diversity and composition of the donor, which lead to the concept of "super-donors"[220]. However, this concept is highly disputed and a recent meta-analysis has shown that FMT outcome was independent of donor strain colonization or displacement of recipient species[221]. Moreover, recipient strain diversity and complementarity of donor and recipients microbiomes promoted donor strain colonization[221]. Therefore, besides screening for donor health, it might be worthwhile to match donors to recipients based on microbiome complementarity.

Processing of the faeces

After collection of the donor faeces, it has to be processed before administration. The optimal preparation of the faecal suspension remains to be determined and practices vary significantly between researchers, FMT centres and stool banks[222–224]. Fresh faeces are

usually processed within six hours of collection. The amount of faeces used differs, but is usually 50 grams or more. The faeces are mixed with sterile saline (0.9% sodium chloride solution) in a 1:3 ratio, which can be done manually or in a blender. Next, the faecal slurry is filtered through a sterile gauze to remove particulate matter that would otherwise clog the endoscope channel or nasoduodenal tube. The faecal slurry can alternatively be centrifuged at low speed to pellet any large particles. The resulting faecal suspension is used to fill 60 ml syringes for administration via colonoscopy or nasoduodenal tube, or poured in an enema. This faecal suspension can subsequently be directly administered to the patient.

Alternatively, the faecal suspension can be stored in a freezer[225]. However, to preserve microbial viability during freezing and thawing a cryoprotectant is necessary. Most often glycerol in concentrations of 10% to 20% is used, which is either added to the sterile saline during homogenization, after filtration, or further concentration of the faecal suspension. Several studies have compared fresh and frozen FMTs and found a similar efficacy as treatment of rCDI[226,227]. Furthermore, in recent years, capsules with frozen faecal suspensions or freeze-dried faecal material have been developed[228,229]. These faecal microbiota capsules or “crapsules” usually have acid-resistant properties, preventing the capsules from opening in the stomach, or have delayed-release properties. While promising for FMT therapy, production of capsules is more complex than a relatively simple faecal suspension and research is ongoing to reveal the most optimal formulation, production method, and treatment regimen.

Administering the faeces

Prior to administration of the FMT, patients need to be prepared and instructed. Administration of any antibiotics should cease 12-48 hours prior to FMT[208]. In addition, patients receive a standard bowel preparation or laxative to remove any faecal material prior to infusion of the donor faeces[208]. Some clinicians have suggested the use of loperamide, an antidiarrhoeic agent, prior to FMT via colonoscopy or enema to ensure the administered donor faeces remain in the intestine for at least 4 hours[230,231]. In addition, in some studies proton-pump inhibitors are used prior to ingestion of faecal microbiota capsules to reduce stomach acid and preserve microbial viability[232].

There are different routes of administering the FMT as previously mentioned, each with their own advantages and disadvantages[233]. The upper GI route via a nasoduodenal tube is less inconvenient and expensive as an endoscopy and exposes the entire GI tract to the donor faeces. On the downside, tube placement causes discomfort and there is a small risk of an aspiration pneumonia[233]. The advantage of a colonoscopy or endoscopy is the ability to evaluate the intestinal mucosa and collect tissue samples. However these methods are more risky, expensive, and require sedation of the patient[233]. Both FMT via the upper and

lower GI route has been found to be safe and equally effective for treating rCDI[234]. Enemas are minimally invasive, inexpensive, and relatively easy to administer, but the donor faeces can only be delivered to the distal colon. In addition, FMT delivered via enema has been shown to be initially slightly less effective than administration via nasoduodenal tube or colonoscopy, although administration for multiple days is an option[235]. Finally, faecal microbiota capsules are a less invasive, more patient-friendly alternative compared to the traditional administration routes via nasoduodenal tube, colonoscopy, or enema. FMT via capsules has been shown to be equally effective for rCDI as FMT via traditional routes of administration and is increasingly used[236–238].

Faecal microbiota transplantation in research

FMT not only serves as a therapeutic intervention but also as a valuable research tool to study the relation between the gut microbiota and various diseases[239]. By transplanting a complete microbial community, FMT allows researchers to investigate whether specific microbial configurations can induce or alleviate disease phenotypes in animal models or humans. This approach has helped uncover important mechanistic insights into the role of the gut microbiota in health and disease and has paved the way for further studies exploring causal relationships and potential targeted interventions. Examples of these are described in **Chapters 5** and **6**.

What about the viruses?

Over the past decade, there has been a growing focus on the viral component of the gut microbiota, particularly phages. It has been shown that a "healthy" gut microbiota is dominated by integrated prophages[240,241], while diseases such as inflammatory bowel disease have been associated with higher levels of extracellular phages[242,243]. In addition, gut virome alterations have been linked to several other diseases, including malnutrition[244] and colorectal cancer[245]. Also in the context of CMD, alterations in the gut virome have been observed in individuals with T2D[246], hypertension[247], and MetSyn[248]. The latter association between the virome and MetSyn is described in more detail in **Chapter 7** of this thesis. These alterations are thought to influence the gut microbial composition and metabolic functions, potentially contributing to disease development and progression. However, the specific mechanisms and causal relationships between phages and CMD require further research.

Phage dynamics in the human gut

Phage dynamics in the gut are complex and comprehensively described in several reviews[33,39,249,250]. Briefly, intestinal phage communities are individual-specific, temporally stable, and strongly correlated with bacterial populations and their replication[251,252]. The balance between the lysogenic and lytic life cycle of phages, which is influenced by

environmental signals or stressors, can impact homeostasis of the gut microbiota and has been implicated in human health[253,254]. In the lumen of the gut, many phages are thought to reproduce via a lysogenic lifecycle, integrating in successful bacterial strains and thus ensuring their own survival and dissemination ("piggyback-the-winner" model)[255]. This is illustrated by the many bacteria in the gut that carry prophage genes[256]. Moreover, prophage integration facilitates horizonal gene transfer, potentially improving fitness, metabolic capacity and resistance to infection by related phages[257–259]. Towards the end of the colon, stressors such as nutrient starvation and oxidative stress can lead to more prophage induction, increasing the number of phage virions[33].

The mucus layer is colonized by less bacteria compared to the lumen, which is thought to induce a density-dependent switch to the lytic cycle in temperate phages ("kill-the-winner" model)[255]. These phage virions can adhere to the mucus and thereby reduce bacterial colonization of the mucus layer ("bacteriophage-adherence-to-mucus" (BAM) immunity)[260]. Moreover, recent studies suggest that phages can translocate from the mucus layer into the lamina propria and directly interact with the mammalian immune system, modulating both innate and adaptive immunity[261–263]. For example, phages have been found to be taken up by intestinal macrophages and dendritic cells, directly promoting or dampening immune responses through stimulation of toll-like receptor (TLR) 9 and TLR3, respectively[264]. Understanding the intricate dynamics of phages in the human gut will provide valuable insights into their interplay with the gut microbiota and their potential impact on human health.

Studying phages in a human setting

To further study phages as modulators of (immune)metabolism, assess their contribution to CMD, and treatment potential, more randomized controlled studies are needed. Phage therapy, in which lytic phages specifically eliminate pathogenic bacteria, is an increasingly used approach to systematically study the effect of phages in a human setting[265]. However, while such phages or phage cocktails can be very effective to treat monoclonal infections or target a specific bacterial strain, they are in general insufficient to alter the entire gut microbiota[266,267]. In contrast, FMT has shown to induce long-term changes in the gut microbiome and virome of the recipient[268,269], and phages have been identified to contribute to treatment outcome[203]. This has sparked the interest for faecal virome transplantations (FVT), in which (phage) virions are isolated (and concentrated) from the faecal suspension prior to administration. In mice FVTs have shown comparable effects as standard FMTs[270,271]. Moreover, an FVT in diet-induced obese mice was able to reduce weight gain and improve glucose tolerance[272]. In addition, a faecal filtrate depleted of bacteria, also known as a faecal filtrate transplantation (FFT), was successful at curing five human individuals with rCDI[273]. These findings have led to the first FFT study in individuals

with MetSyn to assess the effect of healthy donor phages on glucose metabolism, which is described in **Chapter 8**.

Summary and conclusion

In summary, the prevalence of CMD is rising worldwide and the gut microbiota have been implicated in the complex pathophysiology. Comprising a diverse community of bacteria, viruses, archaea, fungi, and protists, the gut microbiota plays a vital role in digestion, metabolism, and immune regulation. Alterations in the gut microbiota been implicated in CMD and mechanistically linked to various disease phenotypes. However, well-controlled clinical studies are needed to establish a causal contribution of the microbiota to CMD. Various interventions, including FMT, offer potential for modulating the gut microbiota and investigating its impact on disease. Although FMT has shown promise in treating multiple conditions, it is currently only indicated for rCDI. Furthermore, FMT serves as a valuable research tool for studying causality of the gut microbiota in disease and identifying novel beneficial microbes for future next-generation probiotics. Finally, growing interest in the human gut's bacteriophages highlights the importance of studying their interactions with bacteria and the immune system, providing insights into their contributions to health and disease. Overall, studying the gut microbiota, including its bacterial and viral components, presents a promising avenue for exploring new therapeutic strategies and advancing our understanding of cardiometabolic diseases.

AIMS AND OUTLINE OF THE THESIS

In this thesis, we explore the potential of the gut microbiota in cardiometabolic disease. **Part I** focuses on faecal microbiota transplantation and how (faecal) microbes can be used to study and treat cardiometabolic diseases. In **Chapter 2** we searched the literature for the potential future indications for FMT and summarized the evidence for FMT beyond rCDI. The availability of suitable stool donors is one of the challenges of FMT and in **Chapter 3**, we reported our experience with the recruitment and screening of stool donors and the associated costs. **Chapter 4** is a short commentary we wrote on the use of FMT within Europe. Besides being an interesting treatment modality, FMT also offers the opportunity to study the interaction between the gut microbiota and human host, which is what we did in **Chapter 5**. In this study, we studied the interaction between the microbiota and intestinal micro-RNAs after FMT in human individuals with MetSyn. Finally, in **Chapter 6**, we described the development of a next-generation beneficial microbe, *Anaerobutyricum soehngenii*, which was identified during a previous FMT study in MetSyn subjects.

Part II of this thesis focuses on another component of the gut microbiota, namely the bacteriophages, and explores their role in cardiometabolic diseases, specifically MetSyn.

Since alterations of the gut bacteria have been implicated in MetSyn, we hypothesized that the composition of the phages which infect these bacteria will be altered as well. In **Chapter 7**, we therefore compared the gut viromes of individuals with MetSyn and healthy controls and identified a previously undescribed phage family, dubbed the *Candidatus Heliusviridae*, Finally, we hypothesized that the transfer of faecal phages could induce similar effects as an FMT and thus improve the glucose metabolism in human individuals with MetSyn. Thus, we performed a double-blind, randomized, placebo-controlled pilot study, in which we treated 24 MetSyn subjects with either a faecal filtrate from a healthy lean donor or a placebo, which we described in **Chapter 8**.

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PART I

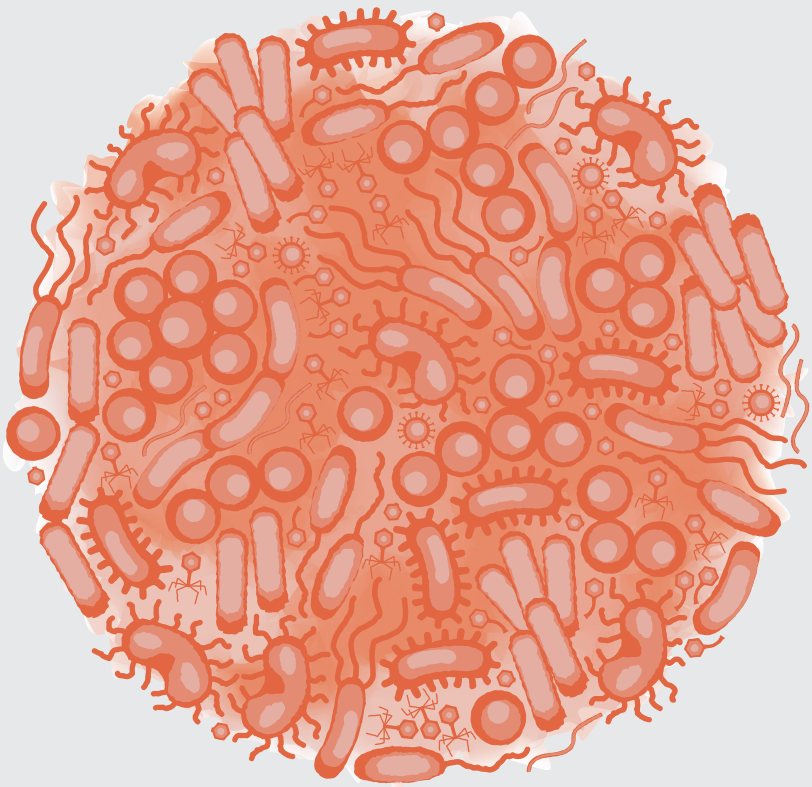
Faecal Microbiota Transplantation to Study Gut Bacteria in Cardiometabolic Disease

CHAPTER 2

Faecal Microbiota Transplantation Beyond *Clostridioides difficile* Infections

Koen Wortelboer, Max Nieuwdorp, and Hilde Herrema

EBioMedicine (2019)



ABSTRACT

The importance of the commensal microbiota to human health and well-being has become increasingly evident over the past decades. From a therapeutic perspective, the popularity of faecal microbiota transplantation (FMT) to restore a disrupted microbiota and amend imbalances has increased. To date, most clinical experience with FMT originates from the treatment of recurrent or refractory *Clostridioides difficile* infections (rCDI), with resolution rates up to 90%. In addition to CDI, a role for the intestinal microbiome has been implicated in several disorders. FMT has been tested in several randomized controlled trials for the treatment of inflammatory bowel disease, irritable bowel disease and constipation with mixed results. FMT has also been explored for extra-gastrointestinal disorders such as metabolic syndrome, hepatic encephalopathy and graft-versus-host disease. With the exception of recurrent CDI, FMT is currently used in experimental settings only and should not yet be offered as standard care. In addition, it is critical to further standardize and optimize procedures for FMT preparation. This includes determination of active components of FMT to develop (personalized) approaches to treat disease.

INTRODUCTION

The human body harbours on average 10–100 trillion microbes, which is more than ten times the estimated number of human cells[1]. The majority of these microbial cells reside in the gastrointestinal (GI) tract and this complex community of microorganisms in the GI tract is termed the intestinal microbiota. The historical view that the gut microbiota is largely pathogenic has undergone a paradigm shift. Over the past few decades, the importance of the commensal microbiota to human health and well-being has become increasingly evident, as the impact of a healthy and diverse intestinal microbiota on metabolic activities, the immune system and homeostasis of the intestine has become more clear[2]. Furthermore, it has been shown that the gut microbiota influences the gut-brain axis, affecting brain function and development, and to confer colonization resistance against pathogenic bacteria[3,4].

Dysbiosis of the intestinal microbiota is defined as decreased bacterial diversity or a shift in bacterial species compared to a healthy control, e.g. a decrease in butyrate producers[5]. Many studies have shown that this dysbiosis is implicated in the development of a wide range of diseases (Figure 1)[5,6]. However, for most diseases it is currently unknown whether the changes in microbiota are causally related to the pathophysiology or merely a consequence of the disease. For *Clostridioides difficile* infection (CDI), there is a clear causal relationship with disease phenotype. For other diseases such as obesity and metabolic disease, a causal relationship still needs to be clarified[7]. In both scenarios, however, modulation of the intestinal microbiota to restore a balanced and diverse microbiota might hold merit to treat or prevent microbiome-related disease.

Faecal microbiota transplantation (FMT), also called “faeces transplantation”, “human intestinal microbiota transfer” and “faecal bacteriotherapy”, is the transfer of the faecal microbiota from a healthy, screened donor to a recipient[8]. FMT aims to restore a disrupted microbiota and amend imbalances through establishment of a stable, complex microbiota. The earliest documented administration of a faecal suspension was by the traditional Chinese doctor Ge Hong in the 4th century[9]. He used so-called ‘yellow soup’ as a treatment for food poisoning and severe diarrhoea. However, it wasn't until the 16th century that another Chinese doctor named Li Shizhen recorded a range of faecal preparations for effective treatment of GI-diseases, such as constipation, fever, vomiting and pain. Subsequently during World War II, African Bedouins advised German soldiers stationed in Africa to consume fresh camel faeces as a treatment for bacterial dysentery[10]. Although the potential health benefits of microbes were already mentioned by Metchnikoff in 1907, it wasn't until 1958 that faecal enemas were first described for the treatment of pseudomembranous enterocolitis by Dr. Ben Eiseman, an American surgeon[11]. Thereafter,

a plethora of articles on the potential of FMT to treat recurrent CDI (rCDI) have been written. In this review, the potential of FMT beyond treatment of CDI and the current evidence in support of FMT as a therapeutic approach will be discussed.

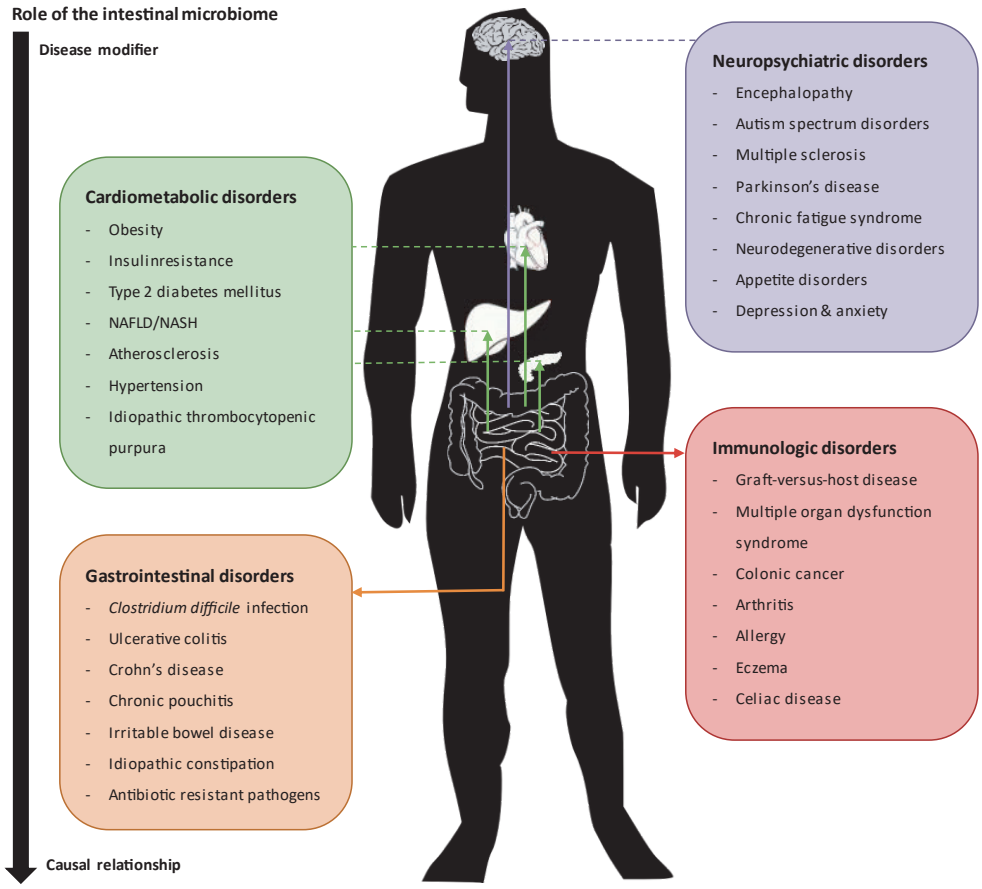


Figure 1: Associations between the intestinal microbiome and disease. At this moment, for most diseases it is not known whether the microbiota is causally related or merely a result of the pathophysiology. Abbreviations: NAFLD = non-alcoholic fatty liver disease, NASH = non-alcoholic steatohepatitis[6,8,102].

CLOSTRIDIOIDES DIFFICILE INFECTION

Currently, most clinical experience with FMT is derived from the treatment of CDI, in particular recurrent or refractory infections[12]. Over the past decades, the incidence of CDI has risen, while the success rate of prolonged anti-microbial therapy is low (20–30% resolution rate)[13]. FMT has emerged as an important treatment option for rCDI with high resolution rates (up to 90%)[13-15]. Over 100 case reports and clinical trials on the treatment of rCDI with FMT have been published to date; most report high resolution rates of *C. difficile* associated diarrhoea. The first randomized controlled clinical trial (RCT) for FMT in

CDI was performed in the Netherlands by *Van Nood et al*. In this study, authors observed a primary and cumulative resolution of 81% and 94% after one and two FMTs, respectively, compared to 31% after a vancomycin regimen[16].

Subsequently, the number of RCTs addressing the use of healthy donor (allogenic) FMT to treat rCDI has increased. In several publications, FMT via colonoscopy has been shown to be superior to fidaxomicin, vancomycin and autologous FMT[17-19]. The cumulative resolution rate after FMT via colonoscopy was over 90% compared to 42% for fidaxomicin, <30% for vancomycin and 63% for autologous FMT[17-19]. Comparison of nasogastric and colonoscopic administration of a freeze-thawed faecal suspension could not demonstrate a significant difference in resolution rate (both 90%), although the patient groups in this RCT were fairly small[20]. One RCT showed that freeze-thawed faeces was as effective as fresh faeces, both administrated via enema, with resolution rates of 75% and 70% respectively[21]. In contrast, another RCT reported higher resolution rates with fresh FMT via colonoscopy compared to freeze-thawed and lyophilized FMT via colonoscopy, with resolution rates of 100%, 83% and 78%, respectively[22]. However, two RCTs found oral FMT capsules with either frozen or lyophilized faecal microbiota to be as effective as FMT via enema or colonoscopy, with resolution rates around 90%[23,24]. Recently, two small pilot RCTs evaluated the efficacy of FMT for primary CDI instead of rCDI with mixed results[25,26]. One study observed a higher primary resolution with FMT compared to metronidazole, while vancomycin performed better than FMT in the second study[25,26].

Several meta-analyses have confirmed the superiority of FMT over standard antibiotic treatment and indicated that FMT is a safe treatment for patients with rCDI[15,27,28]. In addition, colonoscopic delivery of FMT was associated with higher resolution rates, while duodenal infusion, enema and faecal amount < 50 g were associated with lower resolution rates[29]. Cost-effectiveness analyses have shown that FMT by colonoscopy (or enema, if colonoscopy is unavailable) is cost-effective, as the FMT procedure is relatively cheap and has a high efficacy[30,31]. The above findings have established FMT as an evidence-based treatment option for rCDI, which has been adopted by the European Society for Microbiology and Infectious disease (ESCMID) and the American College of Gastroenterology (ACG) (see Table 1)[32,33].

GASTROINTESTINAL DISORDERS

Inflammatory bowel disease

Inflammatory bowel disease (IBD) comprises ulcerative colitis (UC) and Crohn's disease (CD). Both are characterized by recurring inflammation of the intestine. While UC is restricted to the large intestine, CD can affect the entire gastrointestinal tract. Both pathologies have

been linked to dysbiosis of the gut microbiota, with a decreased diversity and decreases in *Bacteroidetes* and *Firmicutes*[34]. However, it remains unclear whether these shifts are a cause or a consequence of IBD. Some animal studies support a role for the gut microbiota in the pathogenesis of IBD, demonstrating that intestinal exposure to colitogenic microbiota induced spontaneous colitis[35]. Based on these findings, restoration of the gut microbiota through FMT has been explored as a treatment for IBD in several clinical trials.

Table 1: Current and potential indications for faecal microbiota transplantation.

Current indication		Studies	Quality of Evidence	Outcome
Recurrent *Clostridioides difficile* infection		>10 RCTs[16,17,26,18–25] Meta-analyses[15,27–29]	High	Highly effective, with resolution rates around 90%
Potential future indication				
Gastrointestinal disorders				
-	Ulcerative colitis	4 RCTs[34,37-39] Meta-analyses[36,40]	Moderate	Clinical remission around 36-37%
-	Crohn's disease	Cohort studies[42-44] Meta-analyses[36]	Low	Clinical remission around 50-57%; decrease over time
-	Irritable bowel syndrome	2 RCTs[47,48]	Low	Mixed results
-	Slow-transit constipation	1 RCT[49]	Low	Clinical remission around 37%; decrease over time
-	Antibiotic resistant bacteria	Cohort studies[53-57] Open-label RCT[58]	Low	Promising results on decolonization of ESBL-producers, VREs and CREs
	Metabolic disorders			
-	Metabolic syndrome	2 RCTs[61,62]	Low	Increased insulin sensitivity, but no effect on clinical endpoints
-	Cardiovascular disease	1 RCT[68]	Low	Enrichment of SCFA-producers, but no clinical effect
	Neuropsychiatric disorders			
-	Hepatic encephalopathy	1 RCT[70]	Low	No new episodes of HE and fewer SAE's
-	Autism spectrum disorder	Cohort study[74]	Low	Decrease in gastrointestinal and neurologic symptoms
	Immunologic disorders			
-	Graft-versus-host disease	Cohort study[86]	Low	Overall survival and progression-free survival of 85%

The above disorders were only listed if there was at least a cohort study published. Abbreviations: RCT= randomized controlled trial, ESBL= extended-spectrum beta-lactamase, VRE= vancomycin-resistant *Enterococcus*, CRE= carbapenem-resistant *Enterobacteriaceae*, SCFA= short-chain fatty acid, HE= hepatic encephalopathy, SAE= serious adverse event[103].

Ulcerative colitis

Promising case reports and uncontrolled observational cohort studies have been published on the treatment of IBD with FMT, although the response rate to FMT is lower compared to rCDI. Of these studies, 53 were summarized in a systematic review, demonstrating clinical remission in 201/555 (36%) of UC, 42/83 (51%) of CD and 5/23 (22%) of pouchitis patients after FMT[36]. To date, four RCTs on the treatment of UC using FMT have been published, all showing promising results[34,37-39]. These results were pooled in a recent Cochrane meta-analysis, which demonstrated a significantly higher clinical remission at eight weeks in the FMT arm compared to the control arm, with 52/140 (37%) and 24/137 (18%) patients achieving remission, respectively[40]. Serious adverse events and adverse events did not significantly differ between groups. Importantly, methodology, FMT strategies and primary endpoints varied considerably among the RCTs. This exemplifies the necessity of additional dedicated studies.

Some studies indicated that the efficacy of a faecal transplant differed between transplant donors. These observations gave rise to the concept of super donors and highlight the importance of careful donor selection[37,38]. Furthermore, an enrichment in *Eubacterium hallii* and *Roseburia inulivorans* with increased levels of short-chain fatty acids and secondary bile acids was consistently found in patients in remission after FMT[41]. In addition, in patients that did not achieve remission, an enrichment in *Fusobacterium gonidiaformans*, *Sutterella wadsworthensis*, and *Escherichia* species was observed, characterized by increased levels of heme and lipopolysaccharide biosynthesis.

Crohn's disease

Evidence for FMT to treat CD is sparse and to date no RCTs have been published. In a meta-analysis of 11 uncontrolled observational cohort studies and case series in CD, 42/83 (51%) patients achieved clinical remission[36]. A prospective study observed clinical remission in 13/25 (52%) CD patients three months after FMT, which decreased to 5/22 (23%) 18 months after FMT[42]. Another study demonstrated that a second FMT, administered within 4 months after the first FMT, maintained the clinical benefits of the first FMT[43]. The largest prospective cohort study published to this day found a clinical remission in 79/139 (57%) patients one month after FMT and observed mild adverse events in 14% of patients one month after FMT[44]. However, the potential of FMT to treat CD is still uncertain and well-designed controlled studies addressing this question are needed. Compared to UC, CD is a more heterogeneous disease and it might be necessary to focus on specific disease phenotypes, instead of a general CD population.

Functional bowel disease

The efficacy of FMT is actively being explored in functional GI disorders, in particular irritable bowel syndrome (IBS) and chronic constipation. IBS is a chronic noninflammatory GI disorder, characterized by abdominal pain with diarrhea and/or constipation. Although the pathophysiology of IBS is not completely understood, several associations with the intestinal microbiota have been found. In line, this suggests a potential role for FMT to treat IBS[45,46]. Two RCTs were recently published showing conflicting results. In the first study, 36/55 (65%) patients in the FMT group had relief of IBS symptoms (>75 points reduction on IBS severity scoring system (IBS-SSS)) three months after a single FMT via colonoscopy compared to 12/28 (43%) patients in the placebo group[47]. The second study reported a larger reduction in IBS-SSS (−125·71) in the placebo group (n = 23) after three months compared to the FMT group (n = 22) that received FMT-capsules for 12 days (−52·45)[48]. Although diversity of the microbiota increased in patients receiving FMT capsules, clinical improvement of IBS symptoms was not achieved. Discrepancies in study outcomes might originate from the different FMT administration strategies or the included IBS-subtypes. Results of several ongoing RCTs (see Figure 2) will further disentangle the therapeutic potential of FMT to treat IBS.

The gut microbiota has also been implicated in the etiology of constipation. In one RCT, 60 adults with slow transit constipation (STC) received standard of care treatment (education, behavioral strategies, and oral laxatives) either with or without an additional 6 days FMT by nasoduodenal infusion[49]. After 12 weeks, clinical improvement was observed in 16/30 (53%) versus 6/30 (20%) patients and clinical cure (≥ three complete spontaneous bowel movements per week) in 11/30 (37%) versus 4/30 (13%) patients in the FMT and control group, respectively. Two prospective studies from the same group showed a decrease in clinical cure rate over time and observed higher efficacy of FMT in combination with the polysaccharide pectin in patients with STC[50,51]. A third prospective trial reported alleviation of bloating and pain symptoms in patients with chronic intestinal pseudo-obstruction (CIPO), a serious life-threatening motility disorder[52]. Although these results are promising, the FMT treatments were quite intense and invasive, with patients receiving up to 18 nasoduodenal FMTs over three months[51]. These limitations of dosing frequency and nasal tube placement could be addressed by using FMT capsules. Furthermore, the efficacy of the faecal microbiota in the FMT can be debated as the glycerol, used to protect the microbiota from freezing, could have a laxative effect.

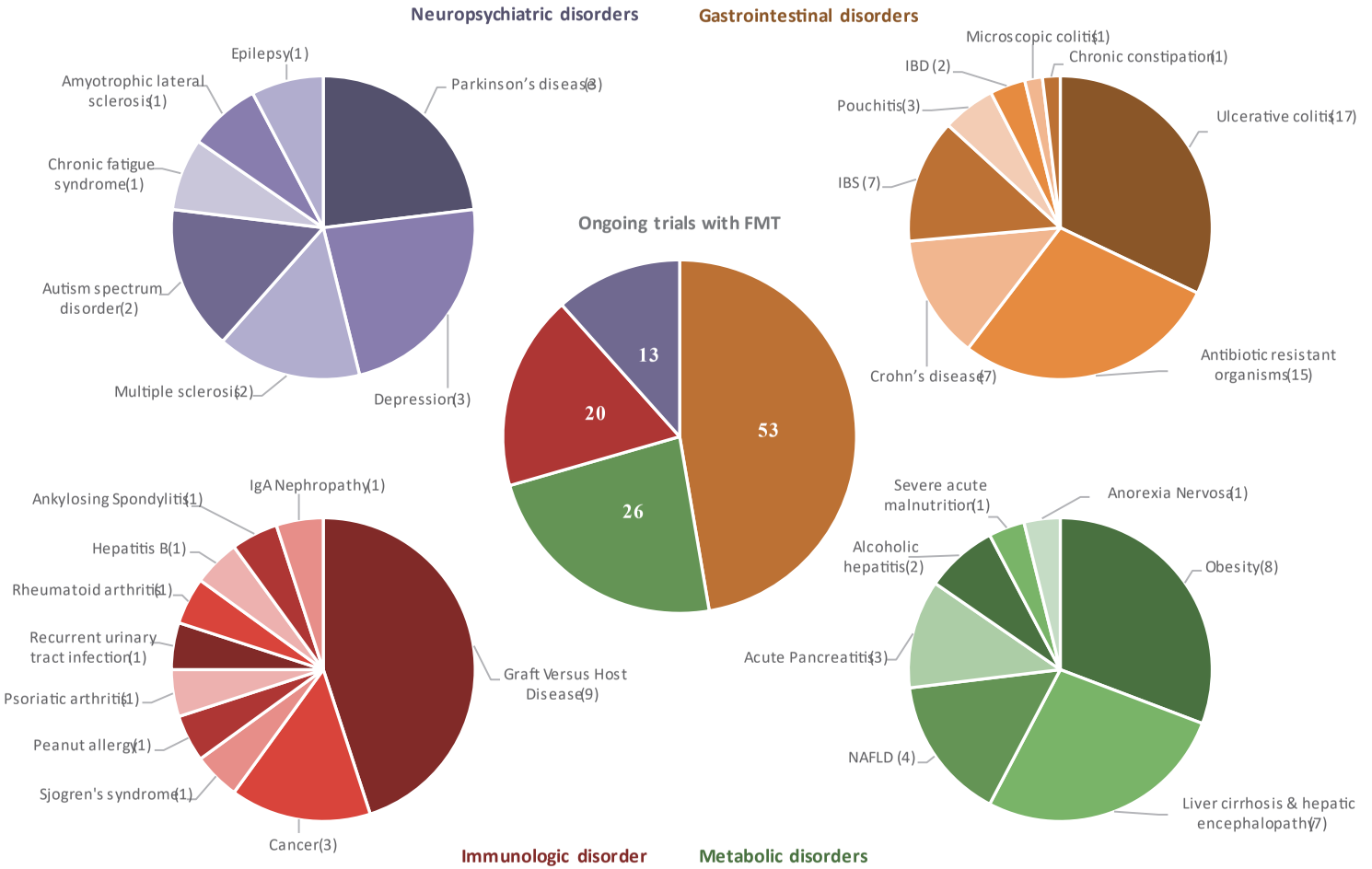


Figure 2: Overview of ongoing trials in which the efficacy of FMT is tested for a range of diseases. Only intervention studies were included that were registered as 'active', 'recruiting', 'enrolling via invitation' or 'not yet recruiting'. In addition, studies examining the effect of FMT in treating CDI were excluded, since this is out of the scope of this review. Table 2 gives a more detailed overview of the ongoing studies. Abbreviations: FMT = faecal microbiota transplantation, IBD = Inflammatory Bowel Disease, IBS = Irritable Bowel Syndrome, NAFLD = Non-Alcoholic Fatty Liver Disease.

Antibiotic-resistant bacteria

An increasing healthcare threat is intestinal colonization with multidrug-resistant organisms, which may cause life-threatening infections. Through direct ecological competition, FMT may potentially stimulate decolonization of antibiotic-resistant bacteria (ARBs) and increase resistance to colonization by these pathogens. This was first described in a case report where FMT was used for the successful eradication of an extended-spectrum beta-lactamase-producing (ESBL) *Escherichia coli*[53]. Thereafter, many case reports and small prospective cohort studies have been published, showing efficacy of FMT in decolonization of ESBL-producers, vancomycin-resistant *Enterococcus (VRE)*, carbapenem-resistant *Enterobacteriaceae* (CRE) and other ARBs[54-57]. One open-label RCT has been published, which demonstrated decolonization of CRE or ESBL producers in 9/22 (41%) patients who received a five-day course of oral antibiotics followed by FMT compared to 5/17 (29%) patients who didn't receive an intervention[58]. Interestingly, two prospective studies found a higher decolonization effect of FMT in the absence of periprocedural use of antibiotics, reporting decolonization in 6/7 (79%) and 7/8 (88%) patients after FMT[56,57]. Although the number of treated patients is small, these studies show that FMT might be an effective therapy for decolonization of antibiotic-resistant organisms from the GI-tract and more trials are currently under way to assess its safety and efficacy (see Figure 2).

METABOLIC DISORDERS

Metabolic syndrome

There has been an increasing interest in the role of the gut microbiota in metabolic diseases, as microbes play a crucial role in digestion and absorption of nutrients from the diet. Furthermore, gut bacteria produce metabolites with critical properties for host metabolism including -but not limited to- short-chain fatty acids (SCFA) and bile acids. Dysbiosis of the intestinal microbiota has been linked to an impaired mucosal barrier function, also known as a “leaky gut”, a proinflammatory state and a disturbed production of signalling molecules, such as SCFAs and bile acids[59]. Animal studies suggest a causal link between the intestinal microbiota and obesity. For example, mice colonized with obesogenic microbiota were shown to have increased body fat and insulin resistance compared to mice colonized with lean donor microbiota[60]. Currently, two placebo-controlled RCTs have been published, which determined the effect of nasoduodenal FMT in obese Caucasian males with metabolic syndrome[61,62]. Six weeks after nasoduodenal infusion of lean donor faeces, insulin sensitivity was significantly increased. This coincided with an increase in butyrate-producing intestinal microbiota. Importantly, the effect on insulin sensitivity disappeared after 18 weeks and no long-term clinical effects were found. In addition, metabolic response to FMT was found to be associated with a low microbial diversity at baseline[62]. Although the mechanisms underlying the favourable effects on insulin sensitivity are yet to be

determined, these studies highlight a role for the intestinal microbiota in metabolic diseases.

NAFLD and NASH

Non-alcoholic fatty liver disease (NAFLD) is characterized by accumulation of fat in the liver, which may lead to inflammation and liver damage, commonly known as non-alcoholic steatohepatitis (NASH). NASH is a major cause of liver cirrhosis and liver cancer; both primary indications for liver transplantation. Differences in microbiota composition have been observed in patients with NAFLD or NASH compared to subjects with healthy liver[59]. In addition, increased intestinal permeability and a proinflammatory environment in the gut are frequently observed in NAFLD/NASH patients[63]. Although human studies are yet to be performed, studies in high-fat diet-fed mice found that FMT reduced weight gain and non-alcoholic fatty liver score[64]. To further investigate the potential of FMT in NASH there are several RCTs underway (summarized in Figure 2), determining the efficacy of FMT compared to standard therapy in NASH related cirrhosis.

Cardiovascular disease

Accumulating evidence has implicated a role of the intestinal microbiota and microbial metabolites in the development of cardiovascular disease such as atherosclerosis and hypertension[65]. In an animal model, the introduction of a proinflammatory microbiota low in SCFA-producers enhanced systemic inflammation and accelerated atherogenesis[66]. In another study, mice with myocarditis were subjected to FMT. This resulted in reshaping of the microbiota composition and restoration of the *Bacteroidetes* population, which was accompanied by attenuation of myocarditis through reduced inflammatory infiltration of immune cells[67]. Currently, one small RCT in humans has addressed the effect of a single lean vegan-donor FMT on vascular inflammation and trimethylamine-N-Oxide (TMAO) production. TMAO is a microbial metabolite which increases atherosclerotic burden and stimulates a prothrombotic phenotype[68]. Although SCFA-producers were significantly enriched in the allogenic FMT group, no differences were detected in TMAO production or vascular inflammation at two weeks.

NEUROPSYCHIATRIC DISORDERS

Hepatic encephalopathy

Hepatic encephalopathy (HE) comprises a spectrum of neuropsychiatric abnormalities as a result of end-stage liver cirrhosis. HE has been associated with differences in the microbiota and an increased relative abundance of ammonia-producing bacteria[69]. Subsequent hyperammonaemia is associated with impaired neuronal function[69]. Current treatment strategies for HE consist of lactulose supplementation and treatment with the

nonabsorbable antibiotic rifaximin, which both influence the intestinal microbiota. Currently, one RCT has been published in which patients with HE were treated with a single FMT via enema in addition to standard of care treatment[70]. Fewer serious adverse events (2 versus 8) and new episodes of HE (0 versus 6) were observed in the FMT arm (n = 10) compared to the control group (n = 10) receiving solely standard of care. Furthermore, cognition and dysbiosis improved after FMT. In a small pilot study, eight patients with steroid-ineligible severe alcoholic hepatitis were treated with a nasoduodenal FMT for seven days[71]. Liver disease severity reduced and coincided with resolution of ascites and HE. Moreover, survival rate improved compared to historically matched controls. These studies show promising results and multiple clinical studies addressing the use of FMT in HE are underway (see Figure 2).

Autism spectrum disorder

Autism spectrum disorders (ASD) are not only characterized by impairments in social interaction and communication, but often coincide with GI symptoms such as constipation or diarrhea[72]. Experiments in ASD mouse models have mechanistically linked the gut microbiota to abnormal metabolites and behavior[73]. To this day, one open-label study has explored the effect of FMT on GI and ASD symptoms in children aged 7–16 years[74]. 18 children were placed on a two-week antibiotic regimen, a bowel lavage and either an initial rectal or oral high FMT dose, followed by a daily lower oral maintenance dose for 7–8 weeks. After FMT, both GI and ASD symptoms significantly improved, which persisted for 8 weeks after treatment. These are promising results, although the potential causal contributions of the gut microbiota to ASD remain speculative.

Multiple sclerosis

A number of studies have shown intestinal microbiota dysbiosis in patients with multiple sclerosis (MS), a chronic inflammatory disease of the central nervous system[75]. Additionally, animal models have shown that faeces from patients with MS could precipitate an MS-like autoimmune disease in mice, which suggests microbiota involvement in the pathogenesis of MS[76]. Some case reports describe improvement of neurological symptoms and disease stability after FMT, although more research is needed to determine the benefit and safety of FMT in MS[77,78].

Parkinson's disease

Also for Parkinson's disease (PD) intestinal dysbiosis has been reported, with decreases in *Prevotella* and butyrate-producing bacteria[79]. An observational study found a decrease in total count of gut microbiota during PD progression and changes in gut microbiota could be correlated with a rapid or slow disease progression[79]. In a mouse model, gut microbiota transplantation from donor mice with PD reduced striatal neurotransmitter release with

subsequent motor impairment in healthy recipient mice[80]. Furthermore, healthy mouse donor FMT had neuroprotective effects in PD mice through suppression of neuroinflammation and reduction of TLR4/TNF-α signaling[80]. FMT could have a potential benefit in PD, but studies in humans have not been performed yet.

IMMUNOLOGIC DISORDERS

Graft-versus-host disease

Acute intestinal graft-versus-host disease (GVHD) is a major cause of mortality in patients that receive an allogenic hematopoietic stem cell transplantation (HCT). Standard treatment consists of immunosuppressive steroids, although some patients develop steroid-refractory GVHD for which no well-established treatment is available. Growing evidence suggests that the risk of GVHD is influenced by host-microbiota interactions and one study observed an increased mortality in recipients with a lower phylogenetic diversity[81]. In a mouse model, alterations in intestinal microbiota following HCT resulted in a decreased butyrate production, potentially contributing to a proinflammatory state of the intestine[82]. Results from several case series on FMT to treat acute steroid-refractory GVHD have shown some promise, demonstrating resolution of clinical symptoms, restoration of microbiota composition and a higher progression-free survival[83-85]. In a recent prospective open-label study, 13 patients were treated with FMT capsules to restore their intestinal microbiome diversity at a median of 27 days after HCT[86]. During a median follow-up period of 15 months after FMT, two patients developed acute GI GVHD which resulted in one death. The 12-month overall survival and progression-free survival were both 85%. Although the results of FMT in GVHD are promising, larger controlled studies are needed.

Cancer

Microbial dysbiosis has been extensively observed in human malignancies[87]. Several bacterial species are linked to colorectal cancer (CRC), including *Enterococcus faecalis*, *Escherichia coli* and *Bacteroides fragilis*[88]. This finding was supported by animal studies, in which the infusion of faeces from patients with CRC could promote tumorigenesis in germ-free mice[89]. Although FMT has not been tested as a treatment for CRC, it has been used to increase the efficacy of immune checkpoint inhibitors (ICI). Only a minority of patients with CRC responds to ICIs, which has been linked to an abnormal gut microbiome composition[90]. Moreover, it has been shown that antibiotics inhibit the clinical benefit of ICIs[90]. It has been hypothesized that restoration of the microbiome reinforces the intestinal barrier integrity and reduces systemic inflammation[90]. When the faeces from cancer patients who responded to ICIs was transplanted into germ-free mice, the antitumor effects of ICIs were ameliorated, whereas faeces from non-responders failed to do so[90]. In one case-series, FMT was used to successfully treat refractory ICI-associated colitis, reconstitute the

GI microbiome and increase the proportion of regulatory T-cells in the colonic mucosa[91]. These results indicate the important role of the microbiota in ICI-related toxicity and efficacy and point to a potential role for microbiota-modifying therapies, such as FMT. A recently published review by Wardill et al. extensively describes the use of FMT in supportive oncology more into depth[92].

DISCUSSION & CONCLUSION

Interest in FMT to treat disease has risen over the last few years and its therapeutic benefit is currently being explored for a variety of diseases. Table 1 provides an overview of the current and potential indications for FMT. Besides the above described disorders, the use of FMT has been described in case reports as treatment for multiple organ dysfunction syndrome, chronic pouchitis and celiac disease[93-95]. However, for most diseases it is not fully known whether the changes in microbiota are causally related to the pathophysiology, or merely a result of the disorder. If the intestinal microbiota plays a causal role in disease pathophysiology, altering the microbiota may influence its course. In most cases, however, a single microorganism is not likely to be a causal pathogen or missing beneficial microbe. Therefore, an advantage of FMT over prebiotics and probiotics is the introduction of a complete healthy gut microbiota. FMT can be used as a tool to dissect association from causality in human intervention studies by assessing the effect of the microbiota on a disease. Figure 2 and Table S2 give an overview of currently ongoing clinical trials that study the potential of FMT as a treatment for a variety of disorders.

Currently, FMT is a non-standardized treatment which should be optimized and standardized for specific indications. This is supported by the finding of super donors, which suggests a specific bacterial composition can be more effective to treat a certain disease[37,38]. In addition, treatment strategy and route can impact the microbiota composition and colonization, which can influence the therapeutic effect. With the development of FMT capsules, the therapy became less invasive, more standardized, and less expensive[96]. However, some microbes (or metabolites) critical for the efficacy of an FMT might not survive the processing required for capsulation. Therefore, it is important to determine the differences between fresh and processed faecal microbiota and the efficacy in particular diseases. Furthermore, optimal location of delivery and 'dose' of FMT to treat microbiota-mediated diseases are largely unknown. A small cohort study showed that capsules releasing faecal microbiota in the colon achieved a slightly higher cure rate (81%) compared to gastric release (75%) in treating patients with rCDI[97]. Delivery of microbiota to a specific area of the intestine via targeted opening of a capsule might be an interesting future approach to further investigate.

Given the variable composition of faeces, FMT will probably be replaced by other microbiome-targeting therapeutics. While the knowledge of the microbiota and host-commensal interactions in dysbiotic environments increases, it is to be expected that dietary manipulation and specific alteration of key microbes will be emerging in the future. Furthermore, it appears FMT is not a one-size-fits-all therapy and needs a more personalized approach for several disorders. For instance, donors with a specific microbiota profile are more likely to provide a beneficial effect for patients with IBD[37,38]. Other studies have shown that the microbiota profile of the recipient is predictive for the outcome of the FMT[62]. Future studies should therefore focus more on donor-recipient compatibility and suitability prior to FMT.

With the rapid increase in novel and more affordable techniques to analyse the gut microbiota, implications for a role of this 'endocrine organ' in disease development has risen exponentially. It is important to emphasize however, that besides bacteria, the microbiota consists of archaea, viruses (especially bacteriophages) and fungi. Bacteriophages, viruses that specifically infect and eliminate bacteria, were found to be 20 times more abundant than bacteria in mucosal samples[98]. Given the high number of bacteriophages in an FMT (1–10 times the number of bacteria), these viruses might be important drivers of FMT efficacy. In a small prospective study, the effect of a sterile (bacteria-free) FMT was tested in rCDI patients[99]. Although only five patients were included, all patients had resolution of their CDI-associated diarrhoea. Interestingly, shifts in viral and bacterial composition towards the donor's microbiota profile were observed. Another prospective study observed highly individualized virus colonization patterns depending on specific donor-recipient pairings[100]. The intestinal microbiome is a complex ecosystem with many yet to be identified components likely to affect human metabolism.

In addition, there is a significant knowledge gap in the link between the (small) intestinal microbiota and disease development and progression in humans. This is in large part because the accessible, faecal microbiome is usually used to analyse the microbiome composition and associate with the disease of interest (faecal bias). The small intestinal microbiome differs significantly form the faecal microbiome[101]. Together with the fact that the small intestine plays a major role in human metabolism and disease development, it is critical to develop strategies to sample small intestinal microbiome. In line, nasoduodenal administration of FMT exposes the upper GI to a lower GI/faecal microbiome. This might be a potential drawback for diseases where the upper GI microbiota or the mucosal microbiota is the main culprit. Future research needs to investigate whether and to what extent FMT is capable of modifying the upper and mucosal microbiota.

In conclusion, FMT is a promising treatment strategy for many microbiota-related indications. However, with exception of rCDI, FMT is still experimental and should not be offered as treatment option outside of a research setting. More controlled trials are needed to assess the potential benefit of FMT compared to or in addition to standard therapy.

OUTSTANDING QUESTIONS

FMT remains an unstandardized procedure and the optimal location of delivery and 'dose' of FMT to treat specific microbiota-mediated diseases remain largely unknown. Some studies show a specific microbiota profile is more effective in treating disease, while others show the microbiota profile of the recipient is predictive for the outcome of the FMT. For treating rCDI, the above appears less relevant, as high effectivity rates are observed regardless of the route, dose, processing or donor. However, FMT as a treatment for other disease comes with a smaller effect size along with a larger group of non-responders. Further optimization and personalization of the FMT strategy might improve the outcome in diseases beyond CDI.

The gut microbiota remains a complex ecosystem with lot of unknowns and therefore, future research should focus more on other key players beside bacteria. In addition, there is a significant difference in microbiota composition throughout the GI tract and the role of each section in human metabolism and disease development is hardly understood. Therefore, it is critical to develop strategies to sample the microbiome throughout the GI tract. In line, future research needs to investigate whether and to what extent FMT is capable of modifying the upper and mucosal microbiota.

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Declaration of interests

M. Nieuwdorp is on the scientific advisory board of Caelus Health, the Netherlands. The remaining authors have nothing to disclose.

Author contributions

K. Wortelboer performed the literature search, wrote and corrected the article and made the figures. H. Herrema and M. Nieuwdorp critically reviewed and corrected the manuscript.

Search strategy and selection criteria

Embase, Web of Science and Pubmed were searched in December 2018 for articles using the search term “Faecal Microbiota Transplantation”, which resulted in 2027, 1581 and 1414 articles respectively. Duplicates were merged. Articles on clinical trials, systematic reviews and meta-analyses, written in English and published before 1 January 2019 were included. To identify additional relevant studies, reference lists were manually searched. In addition, we searched ClinicalTrials.gov for new and ongoing trials with FMT for indications other than CDI using the search term “[disease]” in combination with “FMT” OR “faecal microbiota transplantation”.

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Table S2: Ongoing trials with faecal microbiota transplantation for indications other than CDI.

Indication	NCT number	Intervention	Study design	Primary outcome	Patients (n)
IBD	NCT03399188	I: FMT via colonoscopy	Single group, open label trial	- Clinical remission (PUCAI / Mayo score / PCDAI / CDAI) [6 months]	100
IBD	NCT02575040	I: FMT	Single group, open label trial	- number of patients with worsened disease [1 year] - Safety: (serious) adverse events [1 year]	60
Ulcerative colitis	NCT01790061	I: FMT via DJ C: standard of care treatment	Controlled, open label trial	- Clinical remission (Montreal score S0) [1 year]	500
Ulcerative colitis	NCT03843385	I: faecal microbiota filtrate capsules II: FMT capsules C: placebo capsules	3- arm, randomized, placebo-controlled, quadruple blinded trial	- Clinical remission (Mayo score) [12 weeks]	174
Ulcerative colitis	NCT03483246	I: FMT via colonoscopy C: sham via colonoscopy	Randomized, placebo-controlled, single blinded trial	- Steroid-free clinical and endoscopic remission [12 weeks]	150
Ulcerative colitis	NCT03804931	I: FMT C: Saline	Randomized, placebo-controlled, single blinded trial	- Clinical remission (Mayo score) [12 weeks] - Clinical improvement (Mayo score) [12 weeks]	120
Ulcerative colitis	NCT03110289	I: super donor FMT C: autologous FMT	Randomized, controlled, quadruple blinded trial	- Clinical remission (Mayo score) [8 weeks] - Endoscopic remission [8 weeks]	108
Ulcerative colitis	NCT02291523	I: allogenic FMT via colonoscopy C: autologous FMT via colonoscopy	Randomized, controlled, quadruple blinded trial	- Clinical remission (PUCAI) [12 months]	101
Ulcerative colitis	NCT03582969*	I: FMT capsules C: placebo capsules	Randomized, placebo-controlled, triple blinded trial	- Clinical remission (SCCAI) [12 weeks]	100
Ulcerative colitis	NCT03561532	I: allogenic FMT via colonoscopy C: autologous FMT via colonoscopy	Randomized, controlled, quadruple blinded trial	- Clinical and endoscopic remission [52 weeks]	80
Ulcerative colitis	NCT02606032	I: FMT + metronidazole + doxycycline + terbinafine C: FMT	Randomized, controlled, quadruple blinded trial	- Clinical and endoscopic remission [9 weeks]	80
Ulcerative colitis	NCT03016780	I: FMT C: Saline	Randomized, placebo-controlled, open label trial	- Safety: (serious) adverse events [3 months]	60
Ulcerative colitis	NCT03104036	I: FMT via enema C: mesalazine enema	Randomized, controlled, open label trial	- Clinical remission (Mayo score) [12 weeks]	60

Table S2 continued.

Indication	NCT number	Intervention	Study design	Primary outcome	Patients (n)
Ulcerative colitis	NCT03006809	I: antibiotics + FMT via colonoscopy + FMT capsules II: FMT via colonoscopy + FMT capsules III: antibiotics + FMT via colonoscopy + FMT via enema IV: FMT via colonoscopy + FMT via enema	4-arm, randomized, controlled, open label trial	- Safety: (serious) adverse events [1 year] - Clinical and endoscopic remission [8 weeks]	40
Ulcerative colitis	NCT02734589	I: FMT via colonoscopy II: diet for UC + FMT III: diet for UC	3-arm, randomized, controlled, single blinded trial	- Clinical remission (SCCAI) [56 days]	34
Ulcerative colitis	NCT03948919	I: low sulfur FMT capsules C: placebo capsules	Randomized, placebo-controlled, quadruple blinded trial	- Engraftment of sulfate reducing microbiota [12 weeks]	20
Ulcerative colitis	NCT02390726	I: FMT C: placebo FMT	Randomized, placebo-controlled, double blinded trial	- Endoscopic stage [2 years] - Biologic inflammatory markers [2 years] - Symptomatology and quality of life [2 years]	20
Ulcerative colitis	NCT03949257	I: FMT via DJ	Single group, open label trial	- Gut microbiome [7 days]	20
Ulcerative colitis	NCT03716388	I: FMT + mesalazine granules II: FMT + placebo granules III: mesalazine granules + placebo FMT	3-arm randomized, controlled, double blinded trial	- Clinical remission (Mayo score) [14 weeks]	15
Crohn's disease	NCT01793831	I: FMT via DJ	Single group, open label trial	- Clinical remission (HBI score) [1 year]	200
Crohn's disease	NCT03078803	I: FMT via colonoscopy and capsules C: water via colonoscopy and placebo capsules	Randomized, placebo-controlled, quadruple blinded trial	- Clinical and endoscopic remission [8 weeks]	126
Crohn's disease	NCT03378167	I: FMT via colonoscopy + FMT capsules C: Placebo FMT	Randomized, placebo-controlled, triple blinded, crossover trial	- Monthly Recruitment Rate [30 weeks] - Dropout Rate Post Enrolment [30 weeks] - Rate of Patient Protocol Adherence [30 weeks] - Safety: (serious) adverse events [30 weeks]	45
Crohn's disease	NCT02417974	I: FMT via colonoscopy C: no intervention	Randomized, controlled, single blinded trial	- Endoscopic recurrence after ileo-cecal resection (Rutgeert's score) [6 months]	44

Table S2 continued.

Indication	NCT number	Intervention	Study design	Primary outcome	Patients (n)
Crohn's disease	NCT03267238	I: FMT	Single group, open label trial	- Treatment-related adverse events (CTCAE v4.0) [5 years]	40
Crohn's disease	NCT03747718	I: FMT via enema C: placebo via enema	Randomized, placebo-controlled, triple blinded trial	- Safety: (serious) adverse events [6 months]	30
Crohn's disease	NCT03194529	I: FMT via DJ	Single group, open label trial	- Safety: (serious) adverse events [24 weeks]	10
Pouchitis	NCT03524352	I: FMT C: sterile saline	Randomized, placebo-controlled, double blinded trial	- Clinical and endoscopic relapse [PDAI) [106 weeks]	42
Pouchitis	NCT03545386	I: FMT via enema C: saline via enema	Randomized, placebo-controlled, triple blinded trial	- Clinical remission (PDAI) [7 weeks]	34
Pouchitis	NCT03378921	I: allogenic FMT via colonoscopy C: autologous FMT via colonoscopy	Randomized, controlled, triple blinded trial	- Clinical remission (PDAI) [52 weeks]	26
Microscopic colitis	NCT03275467	I: FMT via colonoscopy + FMT via enema	Single group, open label trial	- Remission (<3 stools/day) [6 weeks]	10
IBS	NCT03613545	I: FMT via colonoscopy C: sham via colonoscopy	Randomized, placebo-controlled, single blinded trial	- IBS severity (IBS-SSS) [6 months]	120
IBS	NCT03125564	I: FMT C: placebo FMT	Randomized, placebo-controlled, double blinded trial	- Proportion of responder [12 weeks]	90
IBS	NCT02847481	I: FMT C: placebo FMT	Randomized, placebo-controlled, double blinded trial	- Engraftment of donor microbiota [10 weeks]	80
IBS	NCT02857257	I: Anaerobic cultured human intestinal microbiota	Single group, open label trial	- Symptom relief (IBS-SSS) [4 weeks]	50
IBS	NCT03074227	I: allogenic FMT via DJ C: autologous FMT via DJ	Randomized, controlled, quadruple blinded trial	- >50% reduction in severity and frequency of abdominal pain [12 weeks]	30
IBS	NCT02092402	I: allogenic FMT C: autologous FMT	Randomized, controlled, quadruple blinded trial	- Symptoms of IBS patients (GSRS-IBS) [6 months]	17
IBS	NCT02651740	I: Rifaximin 400 mg + FMT via DJ	Single group, open label trial	- Relief of IBS condition [6 months]	10
Chronic constipation	NCT03018613	I: FMT C: Saline	Randomized, placebo-controlled, open label trial	- Safety: (serious) adverse events [3 months]	60
Abx resistant organisms	NCT03061097	I: autologous FMT via enema C: placebo via enema	Randomized, placebo-controlled, triple blinded trial	- Safety: (serious) adverse events [7 days]	180

Table S2 continued.

Indication	NCT number	Intervention	Study design	Primary outcome	Patients (n)
Abx resistant organisms	NCT03643887	I: FMT capsules C: placebo capsules	Randomized, placebo-controlled, triple blinded trial	- VRE/CRE decolonization [6 months]	90
Abx resistant organisms	NCT03391674	I: FMT capsules + omeprazole C: no intervention	Randomized, controlled, open label trial	- CRE Decolonization (3 consecutive negative stool samples for CRE) [3 months]	60
Abx resistant organisms	NCT03167398	I: FMT capsules + omeprazole	Single group, open label trial	- CRE Decolonization (3 consecutive negative stool samples for CRE) [1 month]	60
Abx resistant organisms	NCT03834051	I: FMT via enema	Single group, open label trial	- Clearance of antimicrobial resistant organism [2 years]	50
Abx resistant organisms	NCT03029078	I: FMT via DJ	Single group, open label trial	- Intestinal colonization with CRE/VRE [6 months]	50
Abx resistant organisms	NCT03802461	I: FMT via enema C: standard of care treatment	Randomized, controlled, open label trial	- Intestinal colonization with CRE [3 months] - Feasibility: randomization rate [12 months] - Feasibility: retention of 90% of patients [6 months]	40
Abx resistant organisms	NCT03479710	I: FMT via DJ C: no intervention	Controlled, open label trial	- Intestinal colonization with CRE/VRE [12 months]	40
Abx resistant organisms	NCT02472600	I: colistin + neomycin, followed by FMT capsules or ND infusion C: no intervention	Randomized, controlled, open label trial	- Intestinal colonization with ESBL-E / CRE [48 days]	39
Abx resistant organisms	NCT02922816	I: FMT via enema C: no intervention	Randomized, controlled, open label trial	- Safety and feasibility (CTCAE v4.0) [30 weeks]	20
Abx resistant organisms	NCT03527056	I: FMT capsules C: no intervention	Controlled, open label trial	- CRE decolonization [10 days] - Safety: (serious) adverse events [10 days]	20
Abx resistant organisms	NCT02543866	I: FMT via DJ	Single group, open label trial	- Safety and Tolerability of FMT [1-5 years]	20
Abx resistant organisms	NCT02312986	I: FMT via enema	Single group, open label trial	- Safety: (serious) adverse events [12 months]	20
Abx resistant organisms	NCT02816437	I: FMT via enema	Single group, open label trial	- Safety: (serious) adverse events [10 months]	20
Abx resistant organisms	NCT03063437	I: FMT capsules C: placebo capsules	Randomized, placebo-controlled, quadruple blinded trial	- VRE decolonization [10 days] - Safety: (serious) adverse events [10 days]	9

Table S2 continued.

Indication	NCT number	Intervention	Study design	Primary outcome	Patients (n)
Obesity	NCT03727321*	I: FMT + fiber supplement II: FMT + placebo supplement III: fiber supplement C: placebo supplement	4-arm, randomized, controlled, quadruple blinded trial	- Insulin resistance (HOMA-IR) [12 weeks]	68
Obesity	NCT03127696	I: FMT + lifestyle modification program II: FMT III: lifestyle modification program	3 –arm randomized, placebo-controlled, double blinded trial	- 5% reduction in weight [24 weeks]	60
Obesity	NCT03273855	I: allogenic FMT via enema C: autologous FMT via enema	Randomized, controlled, quadruple blinded trial	- Changes in body weight [12 months]	60
Obesity	NCT02970877	I: allogenic FMT C: autologous FMT	Randomized, controlled, quadruple blinded trial	- Insulin Resistance (HOMA-IR) [3 months]	48
Obesity	NCT03391817	I: allogenic FMT C: autologous FMT	Randomized, controlled, triple blinded trial	- Reduction of weight [1,5 years]	40
Obesity	NCT02530385	I: FMT capsules C: placebo capsules	Randomized, placebo-controlled, double blinded trial	- Insulin resistance (HOMA-IR) [6 weeks]	24
Obesity	NCT02741518	I: FMT capsules C: placebo capsules	Randomized, placebo-controlled, triple blinded trial	- Safety: (serious) adverse events [52 weeks]	22
Obesity	NCT03789461	I: FMT	Single group, open label trial	- 10% reduction in weight [6 weeks]	20
NAFLD	NCT02868164	I: FMT via DJ C: standard of care treatment	Randomized, controlled, open label trial	- Adverse events complication rate in NASH [1 year]	120
NAFLD	NCT02721264	I: FMT via DJ C: standard of care treatment	Randomized, controlled, open label trial	- Hepatic Venous Pressure Gradient [1 year]	112
NAFLD	NCT03803540	I: FMT via DJ	Single group, open label trial	- Histological resolution of NASH [72 weeks]	15
NAFLD	NCT02469272	I: FMT via DJ	Single group, open label trial	- Hepatic steatosis (by MRI) [12 weeks]	5
Alcoholic hepatitis	NCT03091010	I: FMT via DJ C: prednisolone 40 mg	Randomized, controlled, open label trial	- Overall Survival [3 months]	130
Alcoholic hepatitis	NCT03827772	I: FMT via DJ C: standard of care treatment	Controlled, open label trial	- Overall Survival [3 months]	40

Table S2 continued.

Indication	NCT number	Intervention	Study design	Primary outcome	Patients (n)
Liver cirrhosis & hepatic encephalopathy	NCT03796598	I: FMT capsules + FMT enema II: FMT capsules + placebo enema III: placebo capsules + FMT enema C: placebo capsules + placebo enema	4-arm, randomized, placebo-controlled, triple blinded trial	- Serious adverse events related to FMT [6 months]	100
Liver cirrhosis & HE	NCT03363022	I: FMT via enema C: placebo via enema	Randomized, placebo-controlled, double blinded trial	- Survival [21 days]	40
Liver cirrhosis & HE	NCT02862249	I: FMT via DJ C: placebo via DJ	Randomized, placebo-controlled, single blinded trial	- Feasibility of FMT [18 months] - Safety: (serious) adverse events [18 months]	32
Liver cirrhosis & HE	NCT03439982	I: FMT via colonoscopy + FMT via enema	Single group, open label trial	- Time to hepatic encephalopathy breakthrough [9 weeks]	30
Liver cirrhosis & HE	NCT03420482	I: FMT capsules C: placebo capsules	Randomized, placebo-controlled, quadruple blinded trial	- Psychometric Hepatic Encephalopathy Score [28 days]	30
Liver cirrhosis & HE	NCT03416751	I: FMT via enema C: saline via enema	Randomized, placebo-controlled, double blinded trial	- Safety: (serious) adverse events [15 days] - Related transmissible infectious disease [15 days]	20
Liver cirrhosis & HE	NCT02255617	I: FMT via colonoscopy and enema	Single group, open label trial	- Time to hepatic encephalopathy breakthrough [6 months]	10
Acute Pancreatitis	NCT03015467	I: FMT C: Saline	Randomized, placebo-controlled, open label trial	- Mortality [3 months]	80
Acute Pancreatitis	NCT02318134	I: FMT via enema + traditional treatment C: traditional treatment	Randomized, controlled, single blinded trial	- Gastrointestinal Failure score [7-14 days]	60
Acute Pancreatitis	NCT02318147	I: FMT via enema + traditional treatment C: traditional treatment	Randomized, controlled, single blinded trial	- Control of infectious complications [from admission to discharge]	60
Severe acute malnutrition	NCT03087097	I: FMT via enema C: placebo via enema	Randomized, placebo-controlled, quadruple blinded trial	- Safety: serious adverse events [56 days]	20
Anorexia Nervosa	NCT03928808	I: FMT via DJ	Single group, open label trial	- Safety: (serious) adverse events [30 days] - Feasibility: participants recruited [3 years] - Tolerability: participants able to complete 4 FMT administrations [3 years] - Tolerability: GI distress Post FMT [3 years]	10

Table S2 continued.

Indication	NCT number	Intervention	Study design	Primary outcome	Patients (n)
Graft Versus Host Disease	NCT03862079	I: total gut decontamination + FMT via enema II: FMT via enema C: standard of care treatment	3-arm, randomized, controlled, open label trial	- Development of acute GVHD [100 days] - Relapse-free survival [6 months]	120
Graft Versus Host Disease	NCT03678493	I: FMT capsules C: placebo capsules	Randomized, placebo-controlled, open label trial	- Incidence of infections [4 months]	120
Graft Versus Host Disease	NCT03492502	I: autologous FMT via DJ	Single group, open label trial	- Safety: (serious) adverse events [7 days]	70
Graft Versus Host Disease	NCT03720392	I: FMT capsules C: placebo capsules	Randomized, placebo-controlled, double blinded trial	- Gut microbiome diversity [1 month]	48
Graft Versus Host Disease	NCT03359980	I: FMT	Single group, open label trial	- GI and overall GVHD response [28 days]	32
Graft Versus Host Disease	NCT03812705	I: FMT via colonoscopy of ND tube	Single group, open label trial	- Response rate [12 weeks]	30
Graft Versus Host Disease	NCT03549676	I: FMT via DJ	Single group, open label trial	- Efficacy in the treatment of refractory GVHD on day 7 and 28.	15
Graft Versus Host Disease	NCT03819803	I: FMT via colonoscopy	Single group, open label trial	- GI-GVHD remission [90 days]	15
Graft Versus Host Disease	NCT03214289	I: FMT capsules	Single group, open label trial	- Safety: (serious) adverse events [28 days]	4
Cancer	NCT03819296	I: FMT	Single group, open label trial	- Safety: (serious) adverse events [4 months] - Incidence of toxicities [1 year]	100
Cancer	NCT03353402	I: FMT via colonoscopy + FMT capsules with faeces of responders to immunotherapy	Single group, open label trial	- Safety: (serious) adverse events [4 years] - Engraftment of faecal microbiota [4 years]	40
Cancer	NCT03341143	I: FMT via colonoscopy + pembrolizumab	Single group, open label trial	- Objective Response Rate [3 years]	20
Psoriatic arthritis	NCT03058900	I: FMT via DJ + methotrexate C: saline via DJ + methotrexate	Randomized, placebo-controlled, triple blinded trial	- Treatment failure [6 months]	80
Rheumatoid arthritis	NCT03944096	I: allogenic FMT via DJ + methotrexate C: autologous FMT + methotrexate	Randomized, controlled, triple blinded trial	- Clinical response (ACR20) [16 weeks]	30

Table S2 continued.

Indication	NCT number	Intervention	Study design	Primary outcome	Patients (n)
Ankylosing Spondylitis	NCT03726645	I: allogenic FMT C: autologous FMT	Randomized, controlled, triple blinded trial	- Clinical activity of ankylosing spondylitis [12 months]	20
Hepatitis B	NCT03429439	I: FMT + antiviral therapy C: antiviral therapy	Randomized, controlled, open label trial	- Serum hepatitis B virus e antigen [6 months]	60
IgA Nephropathy	NCT03633864	I: FMT via enema	Single group, open label trial	- Change of Urinary protein [8 weeks]	30
Recurrent urinary tract infection	NCT03050515	I: FMT via enema	Single group, open label trial	- Frequency of culture proven urinary tract infections [6 months]	12
Peanut allergy	NCT02960074	I: FMT capsules	Single group, open label trial	- Safety: (serious) adverse events [12 months]	10
Sjogren's syndrome	NCT03926286	I: FMT via enema	Single group, open label trial	- Safety: (serious) adverse events [7 months] - Stable microbiome engraftment [3 months]	10
Parkinson's disease	NCT03876327	I: FMT C: no intervention	Controlled, open label trial	- Motor symptoms (UPDRS III) [6 months] - Constipation level [6 months]	100
Parkinson's disease	NCT03808389	I: allogenic FMT via DJ C: autologous FMT via DJ	Randomized, controlled, quadruple blinded trial	- Changes in clinical symptoms (MDS-UPDRS) [12 months]	40
Parkinson's disease	NCT03671785	I: FMT capsules C: placebo capsules	Randomized, placebo-controlled, single blinded trial	- Microbiome diversity in faecal Samples [9 months] - Microbiome Richness in Faecal Samples [9 months] - Safety: (serious) adverse events [9 months]	12
Multiple sclerosis	NCT03183869	I: FMT C: no intervention	Randomized, controlled, open label, crossover trial	- Cytokines levels in peripheral blood [6 months]	40
Multiple sclerosis	NCT03594487	I: FMT via colonoscopy C: no intervention	Controlled, open label trial	- Subjects who complete the study protocol [1 year] - Change in faecal microbiota [12 weeks] - Safety: (serious) adverse events [12 weeks]	30
Amyotrophic lateral sclerosis	NCT03766321	I: FMT via DJ C: sham FMT via DJ	Randomized, placebo-controlled, quadruple blinded trial	- Tregs number [6 months]	42
Depression	NCT03279224	I: allogenic FMT via colonoscopy C: autologous FMT via colonoscopy	Randomized, controlled, quadruple blinded trial	- Depressive symptoms (MADRS) [24 weeks]	60

Table S2 continued.

Indication	NCT number	Intervention	Study design	Primary outcome	Patients (n)
Depression	NCT03281044	I: FMT capsules C: placebo capsules	Randomized, placebo-controlled, triple blinded trial	- Depressive symptoms (HRSD) [8 months]	40
Depression	NCT03233100	I: FMT	Single group, open label trial	- Complete spontaneous bowl movements [12 weeks] - Anxiety symptoms (HAMA) [12 weeks] - Depressive symptoms (HAMD) [12 weeks]	40
Autism spectrum disorder	NCT03408886	I: FMT capsules C: placebo capsules	Randomized, controlled, quadruple blinded trial	- Autism symptoms CARS) [10 weeks]	84
Autism spectrum disorder	NCT03426826	I: FMT via DJ C: placebo via DJ	Randomized, placebo-controlled, quadruple blinded trial	- Safety and tolerability [24 weeks] - Symptom changes (RBS-R) [24 weeks]	10
Chronic fatigue syndrome	NCT03691987	I: allogenic FMT via enema C: autologous FMT via enema	Randomized, controlled, quadruple blinded trial	- Clinical response (FSS) [3 months]	80
Epilepsy	NCT02889627	I: FMT via DJ	Single group, open label trial	- Frequency of the seizures [3 months]	50

The above table summarizes ongoing trials studying the effect of FMT for a variety of diseases. Studies are grouped in 'gastrointestinal', 'metabolic', immunologic' and 'neuropsychiatric' disorders, sorted by disease and number of included patients. These studies were derived from Clinicaltrial.gov in May 2019. Ongoing trials were included when they were registered as 'Active', 'recruiting', 'enrolling via invitation' or 'not yet recruiting'. Only intervention studies were included. In addition, studies examining the effect of FMT in treating CDI were excluded, since this is out of the scope of this review. There were several methods for upper administration of FMT (i.e. gastroscopy, nasoduodenal tube, transendoscopic enteral tubing, etc.), which were pooled for simplicity as duodenal/jejunal infusion (DJ). Within brackets, the time frame of a primary outcome can be found. Abbreviations: I= intervention, C= control, FMT= faecal microbiota transplantation, DJ= duodenal/jejunal infusion, IBD= Inflammatory Bowel Disease, UC= Ulcerative Colitis, IBS= Irritable Bowel Syndrome, CRE= Carbapenem-Resistant *Enterobacteriaceae*, VRE= Vancomycin-Resistant *Enterococci*, ESBL-E= Extended Spectrum Beta-Lactamase *Enterobacteriaceae*, NAFLD= Non-Alcoholic Fatty Liver Disease, NASH= Non-Alcoholic Steatohepatitis, GI= Gastrointestinal, GVHD= Graft Versus Host Disease.
*Entry NCT03582969 was the same as NCT03273465, which were therefore merged. The same goes for entry NCT03477916, which was the same as NCT03727321

CHAPTER 3

Challenges and Costs of Donor Screening for Faecal Microbiota Transplantations

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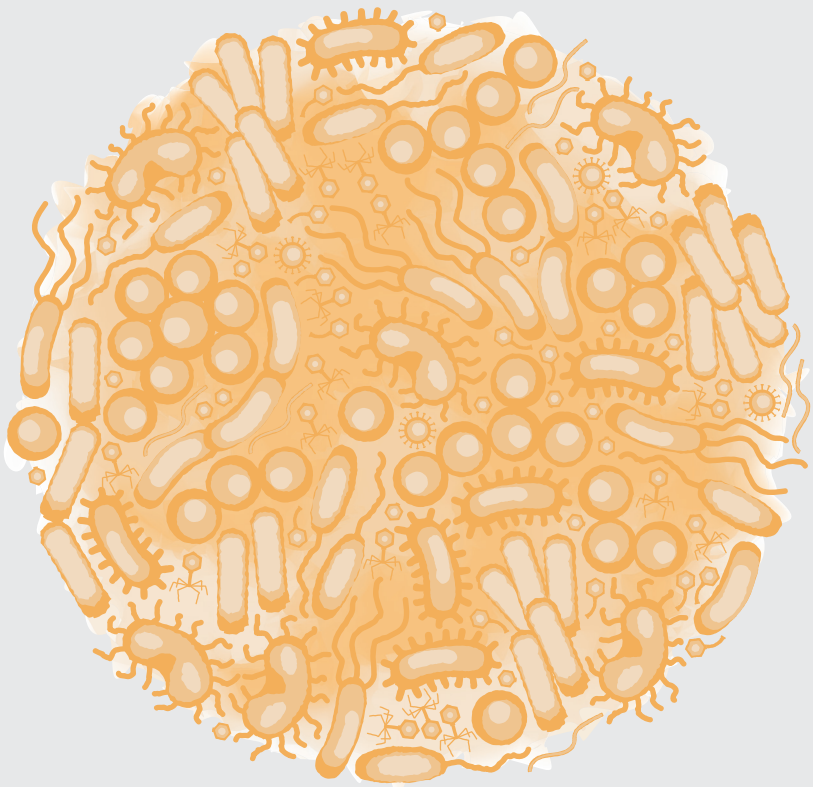

ABSTRACT

Background

The increasing interest to perform and investigate the efficacy of faecal microbiota transplantation (FMT) has generated an urge for feasible donor screening. We report our experience with stool donor recruitment, screening, follow-up, and associated costs in the context of clinical FMT trials.

Methods

Potential stool donors, aged between 18−65 years, underwent a stepwise screening process starting with an extensive questionnaire followed by faeces and blood investigations. When eligible, donors were rescreened for MDROs and SARS-CoV-2 every 60-days, and full rescreening every 4−6 months. The costs to find and retain a stool donor were calculated.

Results

From January 2018 to August 2021, 393 potential donors underwent pre-screening, of which 202 (51.4%) did not proceed primarily due to loss to follow-up, medication use, or logistic reasons (e.g. COVID-19 measures). 191 potential donors filled in the questionnaire, of which 43 (22.5%) were excluded. The remaining 148 candidates underwent parasitology screening: 91 (61.5%) were excluded, mostly due to *Dientamoeba* fragilis and/or high amounts of *Blastocystis* spp. After additional faeces investigations 18/57 (31.6%) potential donors were excluded (mainly for presence of Helicobacter Pylori and ESBL-producing organisms). One donor failed serum testing. Overall, 38 out of 393 (10%) potential donors were enrolled. The median participation time of active stool donors was 13 months. To recruit 38 stool donors, €64.112 was spent.

Conclusion

Recruitment of stool donors for FMT is challenging. In our Dutch cohort, failed eligibility of potential donors was often caused by the presence of the protozoa *Dientamoeba* Fragilis and *Blastocystis* spp.. The exclusion of potential donors that carry these protozoa, especially *Blastocystis* spp., is questionable and deserves reconsideration. High-quality donor screening is associated with substantial costs.

INTRODUCTION

Faecal microbiota transplantation (FMT) is defined as the infusion of faeces from healthy individuals into diseased recipients. FMT is thought to be effective because it has the potential to restore a recipient's distorted microbiota, by introducing a new and diverse microbiome associated with a healthy state to normalize microbiota composition and function. In daily practice, FMT is a widely accepted and highly effective treatment for recurrent *Clostridioides difficile* infection (CDI)[1,2]. Over the past couple of years, evidence is growing for the application of FMT as a treatment for other diseases, such as inflammatory bowel disease (IBD)[3], irritable bowel syndrome (IBS)[4], obesity and related metabolic diseases[5], acute graft-versus-host disease[6], and autism spectrum disorder[7]. The interest in FMT increased tremendously recently, with more than 357 registered ongoing clinical trials worldwide at the time of writing[8-10].

This increasing interest in FMT has generated an urge for feasible donor screening programs to secure an ongoing supply of healthy stool donors. Enrolled donors need to fulfil strict safety criteria, which are continuously adjusted to new insights[11]. For example, due to the current COVID-19 pandemic, additional screening procedures to assess COVID-19 symptoms before donation and regular testing for SARS-CoV-2 RNA are needed[12,13]. In addition, measures to reduce the risk of transmitting multi-drug resistant organisms (MDROs) via FMT were advised earlier by the United States Food and Drug Administration (FDA) after two immunocompromised adults developed invasive infections with extended-spectrum beta-lactamase (ESBL)-producing *Escherichia coli*[11]. Although international recommendations on donor screenings exist[14], stool donor selection processes in practice are highly heterogeneous, and standardized procedures are lacking[9]. Experience from clinical practice indicates that finding a safe, eligible stool donor is complicated. Previous studies performed in the USA, Canada, Hong Kong, and Denmark have shown variable donor acceptance rates ranging between 0.8 - 31%[15-21]. Challenges in donor screening comprise initial donor recruitment and prolonged donor eligibility. A major disadvantage of the extensive screening procedures is the high associated costs[15], leading to an economic burden for patient care and research departments. Therefore, more insights into donor screening programs and accompanying costs are warranted to optimize and further standardize donor screening procedures.

At present, limited data is published on FMT donor screening and associated costs within the context of clinical trials. In recent years, one of the largest University hospitals in the Netherlands −the Amsterdam UMC− has conducted four randomized controlled clinical FMT trials: the FAIS[22], IMITHOT and PIMMS trials have evaluated the efficacy of multiple donor FMTs using fresh faecal material in respectively IBS (in adolescents), subclinical

autoimmune hypothyroidism and metabolic syndrome, whereas the TURN2-trial is evaluating the efficacy of frozen faecal suspensions in active ulcerative colitis. To perform these trials, a pool of healthy stool donors who were able to provide regular stool donations was established. The donor screening was performed according to a predefined standardized screening protocol. With the current study, we aim to describe the process of recruiting and screening stool donors, evaluate the follow-up of eligible donors, and report the associated costs in the context of clinical FMT trials in a Dutch tertiary University hospital.

METHODS

Donor recruitment

In this retrospective observational cohort – study, potential healthy faecal donors were recruited through advertisements via posters, announcements in the hospital magazine and intranet network (employee website), and word-of-mouth advertising among staff at the Amsterdam UMC (location AMC). The Amsterdam UMC, location AMC, is a University hospital with over 7000 employees and 2300 healthcare student placements. Potential donors were invited to participate in the FAIS, IMITHOT, PIMMS, and/or TURN2-trial and oral and written information about the study aims, donation process, and screening requirements were provided. Clinical trials registration numbers are NCT03074227, NL7931, NL8289, and NL7770, respectively. All trials were approved by the Medical Ethics Research Committee of the Amsterdam UMC, the Netherlands. Written, signed and dated informed consent forms were obtained separately for each study as participation in multiple trials was optional. Financial compensation was offered, with reimbursements ranging between €10 – 50 per donation plus additional travel expenses, depending on the trial.

Population and screening procedure

The study population consisted of non-smoking adults, aged 18 − 65 years (except for the TURN2 trial, in which the age ranged between 18 – 54 years), and with a body mass index between 18 − 25 kg/m.^{2} No specific diet restrictions were required. After informed consent was signed, potential donors were thoroughly screened based on the screenings protocol of the Netherlands Donor Faeces Bank (NDFB)[23], a Dutch stool bank that supplies FMT for the treatment of CDI in the Netherlands since 2016. Before accepting a donor, a rigorous screening was performed as shown in Table 1. The screening started with an extensive questionnaire regarding risk factors for infectious diseases and factors potentially perturbing the intestinal microbiota. When potential donors passed the screening questionnaire, they subsequently underwent elaborate faecal and blood laboratory testing in a stepwise approach (Table 2). First, stool samples -collected in a plastic stool container- were screened for parasites presence by a combination of PCR and direct microscopy (Dual

Faeces Test). Next, faeces samples were tested for pathogenic bacteria and viruses, multi-drug resistant organisms and calprotectin. Subsequently, routine biochemical analysis of blood was performed, followed by serological testing for pathogenic viruses, bacteria, and parasites. Once qualified as faecal donors, rescreening of active faecal donors was performed regularly to reduce the risk of transmission of infectious diseases as much as possible. In line with FDA recommendations[11], screening for MDROs (faecal culture) and molecular stool testing on SARS-CoV-2 was performed every 60 days. Frozen FMT material (TURN2 trial) remained quarantined until successful complete rescreening, performed every four months. Complete rescreening was executed every six months when fresh FMT was used (other trials). During the trials, the study staff were in regular contact with the active stool donors, especially before each donation. If there were any concerns about symptoms or risk factor exposure of the faecal donor, donation was suspended and an additional rescreening was performed. In addition, since the outbreak of coronavirus pandemic in 2019 (COVID-19) questions to assess the risk on SARS-CoV-2 infection were asked, including the presence of fever, cough, sore throat, dyspnoea, anosmia or ageusia, or close contact to subjects with suspected or proven infection. Independent of SARS-CoV-2 vaccination status, in case of any suspicion on COVID-19 infection, nasopharyngeal swab and reverse transcription polymerase chain reaction (RT-PCR) were performed and the potential donor was temporarily excluded. During the screening and rescreening process, all positive laboratory tests were discussed with the (potential) donor and counselling was provided accordingly. Qualified faecal donors were matched to patients based on gender (with exception of the TURN2-trial) and cytomegalovirus (CMV)/ Epstein–Barr virus (EBV) status. Donors of the TURN2-trial were additionally selected on a putatively favourable microbiota profile based on results from a previous TURN1 trial, including high alpha-diversity and high predicted butyrate production[24,25].

Data and statistics

Data were collected from January 2018 to August 2021. To date, donor recruitment is still carried out for the IMITHOT and TURN2-trial. Data were collected in the Electronic Data Capture system Castor EDC. Descriptive statistics were used to summarize variables. Normally distributed continuous data are expressed as mean (SD). Not normally distributed continuous data are presented as median (IQR). Categorical data are displayed as frequencies (percentages). Data were analysed using IBM SPSS Statistics for Windows, Version 26.0 (Armonk, NY: IBM Corp).

Table 1: **Exclusion criteria donor recruitment.**

Risk of infectious agent
Active hepatitis A, B-, C- or E-virus infection or known exposure within recent 12 months
Acute infection with *Cytomegalovirus* (CMV) or *Epstein-Barr virus* (EBV)
An extensive travel behaviour
Higher risk of colonization with multidrug resistant organisms including: • *Health care workers with direct patient contact* • *Persons who have recently been hospitalized or discharged from long term care facilities* • *Persons who regularly attend outpatient medical or surgical clinics* • *Persons who have recently engaged in medical tourism*
History or current use of (IV) drugs
Individual working with animals[a]
Positive blood tests for the presence of: HIV, HTLV, *Treponema pallidum*, *Strongyloides stercoralis*
Positive faecal test for MDROs, pathogenic bacteria, viruses and parasites as listed in Table 2
Previous reception of blood products (<12 months) or recent needle-stick accident (<6 months)[a]
Tattoo or body piercing placement within last 6 months
Unsafe sex practice (assessed with standardized questionnaire)
Gastrointestinal comorbidities
A positive history/clinical evidence (e.g. elevated faecal calprotectin) for inflammatory bowel disease, including Crohn's disease or ulcerative colitis
A positive history/clinical evidence for other gastrointestinal diseases, including chronic diarrhea or chronic constipation
Abnormal bowel motions, abdominal complaints or symptoms indicative of irritable bowel syndrome
Factors affecting intestinal microbiota composition
Antibiotic treatment in the past 12 weeks[b]
History of or present known malignant disease and/or patients who are receiving systemic anti-neoplastic agents
History of cholecystectomy
History of treatment with growth factors
Patients receiving immunosuppressive medications and/or a positive history/clinical evidence for autoimmune disease including: • *Type 1 Diabetes Mellitus* • *Hashimoto's hypothyroidism* • *Graves' hyperthyroidism* • *Rheumatoid arthritis* • *Celiac disease*
Recent (gastrointestinal) infection within last 6 months[c]
Smoking
Use of any medication including PPI, except contraceptives and over the counter medication
Use of pre- and probiotics in the past 12 weeks[a]
Other conditions
Abnormal liver function[d]: ASAT >40 U/L, ALAT >45 U/L, AF >120 U/L, GGT >60 U/L, bilirubin >17µmol/L
Abnormal renal function[d]: creatinine >110 µmol/l, urea >8,2 mmol/l
Alcohol abuse (>3 units/day)
Chronic pain syndromes (e.g., fibromyalgia)[c]

Impaired immunity[d]: CRP >5 mg/L, haemoglobin <8,5 mmol/L, MCV 80-100 fL, leukocytes 4,0-10,5 x10^9/L, thrombocytes 150-400 x10^9/L
Known chronic neurological/neurodegenerative disease (e.g., Parkinson's disease, multiple sclerosis)
Known psychiatric disease (i.e., depression, schizophrenia, autism, Asperger's syndrome)
Known risk of Creutzfeldt Jacob's disease
Major relevant allergies (e.g., food allergy, multiple allergies)
Presence of diabetes mellitus type 1 and 2 or hypertension[d]
Presence of chronic low-grade inflammation or metabolic syndrome (NCEP criteria)[e]

[a] Not included in screening protocol of FAIS and TURN2-trial; [b] For the TURN2-trial the exclusion criteria included antibiotic treatment in the past 4 weeks; [c] Additional exclusion criteria FAIS trial; [d] Not included in screening protocol of TURN2-trial; [e] Additional exclusion criteria PIMMS trial; Abbreviations: AF, alkaline phosphatase; ALAT, alanine aminotransferase; ASAT, aspartate aminotransferase; GGT, gamma-glutamyl transferase; CRP, c-reactive protein; HIV, human immunodeficiency virus; HTLV, human T-lymphotropic virus; MCV, mean corpuscular volume; MDROs, multidrug-resistant organisms, NCEP, National Cholesterol Education Programs; PPI, proton pump inhibitors.

Table 2: Specification of donor screening and associated costs.

Faeces screening		**€**
Calprotectin[a] (ELISA)		*20*
***Bacteria** (PCR or stool antigen detection[b])*		***150***
Clostridium difficile	*Salmonella spp.*	
Helicobacter pylori	Shiga toxin-producing *Escherichia coli* (STEC)	
Pathogenic *Campylobacter spp*.	*Shigella spp*.	
Plesiomonas shigelloides	*Yersinia enterocolitica*	
***Multidrug resistant organisms** (culture)*		***150***
Carbapenem-resistant *Enterobacteriaceae* (CRE)	Multidrug-resistant Gram-negatives (MRGN) 3	
ESBL-producing *Enterobactereacceae*	MRGN 4	
Methicillin-resistant *Staphylococcus aureus* (MRSA)	Vancomycin-resistant *Enterococcus* (VRE)	
***Viruses** (PCR)*		***125***
Adenovirus non-41/41	Norovirus Type I and II	45
Adenovirus type 40/41	Parechovirus	
Astrovirus	Rotavirus	
Enterovirus	Sapovirus	
Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)		45
Hepatitis E virus		35
***Parasites** (PCR and/or microscopic evaluation)*		***212***
Blastocystis spp.[c]	*Entamoeba moshkovskii*[d]	
Cryptosporidium spp.	*Entamoeba polecki*[d]	
Cyclospora	*Giardia lamblia*	
Dientamoeba fragilis	*Iodamoeba bütschlii*[d]	
Endolimax nana[d]	*Isospora spp*.	
Entamoeba coli[d]	Larvae[c]	
Entamoeba dispar[d]	*Microsporidium spp*.	
Entamoeba gingivalis[d]	Parasitic worm eggs[c]	
Entamoeba hartmanni[d]	Protozoan Cysts and Oocysts[c]	
Entamoeba histolytica		

Table 2 continued.

Serum screening			
Hematology[a]		***44***	
Alanine aminotransferase (ALAT)	Complete Blood Count (CBC)		
Alkaline phosphatase (AF)	C-reactive protein (CRP)		
Aspartate aminotransferase (ASAT)	Estimated Glomerular Filtration Rate (EGFR)		
Bilirubine	Kreatinine		
Gamma-glutamyl transferase (GGT)	Ureum		
Bacteria *(ELISA)*		***8***	
Treponema pallidum			
Viruses *(CLIA or PCR)*		***CLIA: 119***	***PCR: 293***
Cytomegalovirus (CMV)		36	35
Epstein-Barr Virus (EBV)		25	
Hepatitis A virus[a]		15	
Hepatitis B virus		10	67
Hepatitis C virus		11	77
Human immunodeficiency viruses (HIV)		11	63
Human T-lymphotropic virus Type I and II (HTLV)		11	
Parasites *(ELISA)*		***18***	
Strongyloides stercalis		18	

[a] Not included in screening protocol of TURN2-trial; [b] All bacteria were detected with the use of PCR, with exception of Helicobacter pylori were ELISA was used; [c] Microscopic evaluation, exclusion of donor only if high amounts *Blastocystis* spp. are seen, defined as 'moderate' or 'many'[38]; [d] Presence of only one non-pathogenic parasite is acceptable; Abbreviations: ELISA, quantitative enzyme-linked immunosorbent assay; CLIA, chemi-luminescence immunoassay.

RESULTS

Initial donor screening

From January 2018 to August 2021, a total of 393 potential donors underwent prescreening. A flowchart of donor screening is presented in Figure 1. The main causes for failing prescreening were lost to follow-up (N=97), logistics problems (N=35, *e.g.,* working from home as a result of national COVID-19 measures), occupation as a health care worker with direct patient contact (N=23), and the use of medication, including pre- and probiotics (N=19). Eventually, only half of the initial respondents signed informed consent and continued the screening procedure (N=195). After consenting, four individuals did not respond to further communication and were lost to follow-up. All other potential donors filled in the online screening questionnaire (N=191). Based on 191 completed questionnaires, 43 individuals (23%) were excluded for various reasons (Figure 1).

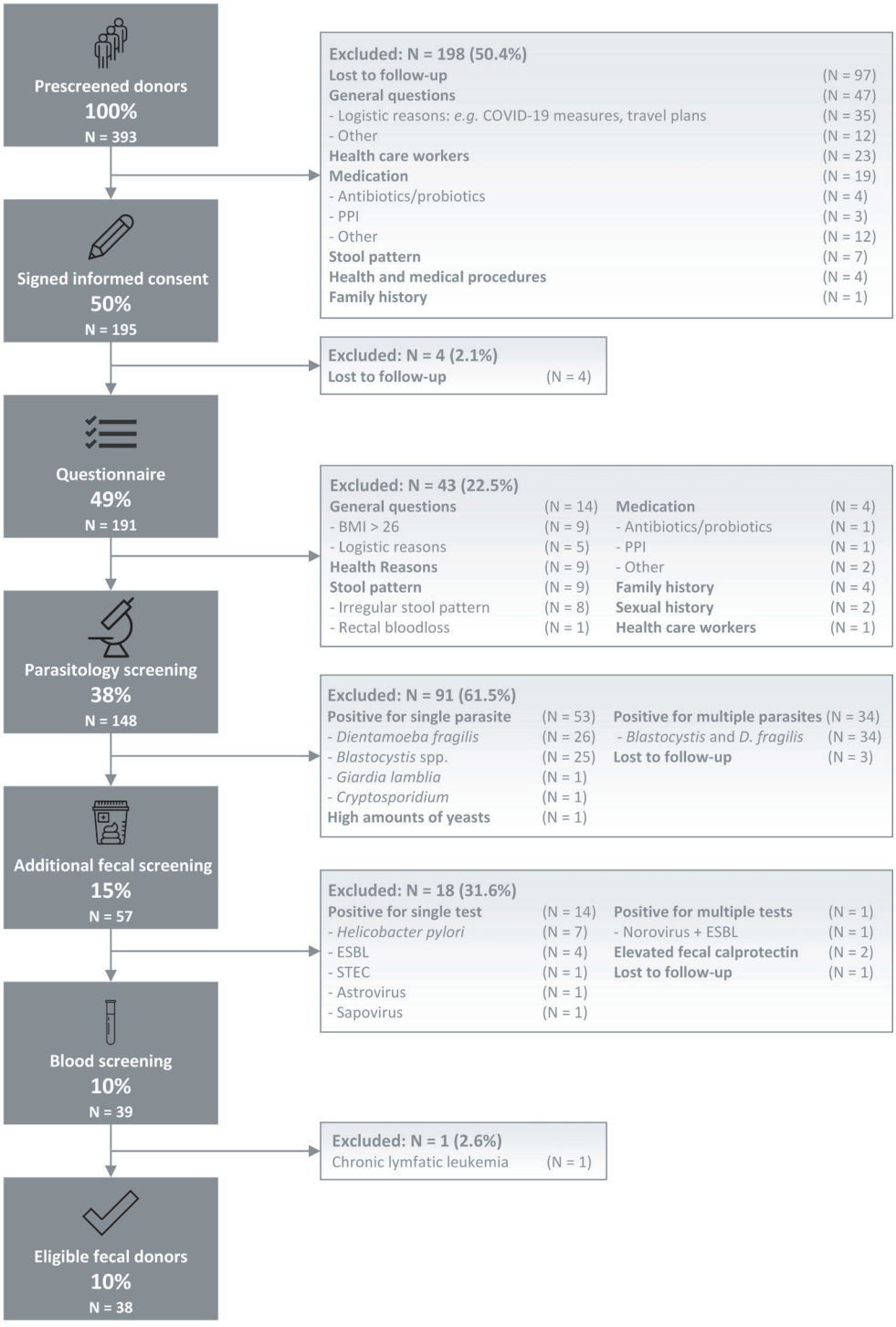

Figure 1: Flow diagram of donor screening outcomes.

Hereafter, 148 potential donors remained and sent in faecal samples for parasitology screening. This screening step resulted in the largest relative loss of potential donors, with positive test results in 91 out of 148 samples (61%). Potential donors tested most frequently positive for *Dientamoeba fragilis* (N=26, 29%), microscopic quantification of 'moderate' or 'many' *Blastocystis spp. (*N=25, 27%), or a combination of both (N=34, 37%). Asymptomatic infestation with *Giardia Lamblia* and *Cryptosporidium* resulted in the exclusion of two additional donors. One donor was dismissed from further screening steps because remarkably high amounts of yeasts were noticed during microscopy evaluation of the stool. Next, 57 potential donors continued screening and delivered stool samples for biochemical, bacterial, and viral analysis. Eighteen out of 57 individuals (32%) failed these stool tests: 7 had Helicobacter pylori, 4 an ESBL-producing strain of E. coli, 1 individual had a Shiga toxin-producing *E.coli* (STEC), 2 potential donors tested positive for a pathogenic virus (astrovirus, sapovirus) and one individual tested positive for multiple tests (norovirus plus an ESBL). Two additional potential donors were excluded due to elevated faecal calprotectin levels (79 and 87 ug/g). The penultimate screening step consisted of blood analysis and resulted in the exclusion of only one individual who had remarkably high levels of lymphocytes and was later diagnosed with chronic lymphatic leukemia. Serum screening for the presence of Hepatitis B and C, HIV, recent infection of CMV and EBV, *Strongyloides,* and *Treponema pallidum* didn't result in any positive tests. In the end, only 38 of the initial 393 individuals (10%) could be enrolled as faecal donors.

Eligible faecal donors

A flowchart of the follow-up of eligible donors is presented in Figure 2. The median age of the 38 eligible faecal donors was 28 years (IQR: 25 – 31.5 years), and 14 donors (36.8%) were male. Eligible donors had a healthy weight with a median BMI of 22.5 kg/m2 (IQR: 20.3 – 24.0 kg/m2). Twenty-four of the 38 eligible faecal donors (63.2%) donated at least one time, further referred to as 'active donors'. The other 14 'non-active' donors could not be matched to a patient due to their microbiota profile (TURN2-trial), gender and/or CMV/EBV status, and therefore did not donate (demographic and referral reasons are listed in Table S1). The number of donations per active donor ranged from 2 to 48 with a median of twelve donations (IQR: 5.3-18.8). Seven donors donated for and participated in multiple studies. The active donors (N=24) had a median participation time of 13 months (IQR: 8 – 16 months). Additional screenings due to symptoms or exposure to risk factors were performed in 11 donors with a total of 34 tests, of which 11 (32.6%) returned positive. Five donors had transient positive tests that didn't lead to definite exclusion, most frequently a transient infection with enterovirus. Reasons for definite exclusion of active donors varied; six donors were excluded due to recurrent positive stool testing of which the majority tested positive for *Dientamoeba fragilis* and/or microscopic quantification of 'moderate' or 'many' *Blastocystis spp*. (N=4). Demographic characteristics of the active donors, details on

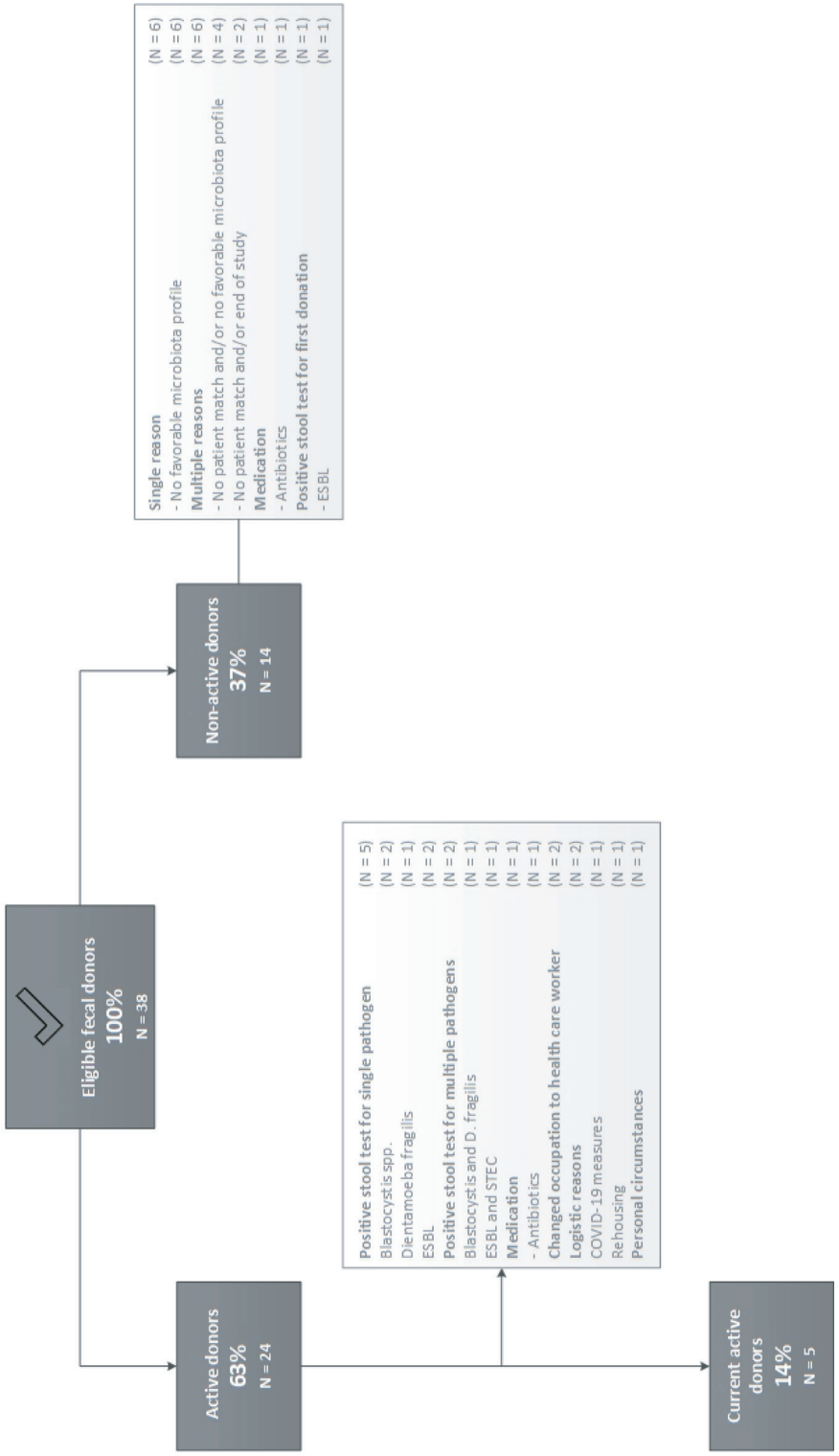

Figure 2: Flow diagram of follow-up of eligible donors.

(re)screenings, and reasons for later exclusion are listed in Table S2. At time of writing (August 2021), only five out of the 38 eligible donors (13%) were still qualified and active donators. The median time of their participation up till August 2021 was 9 months (IQR: 4 – 21.5 months).

Screening costs

An initial safety screening at our centre amounted to €846 for all faecal and blood tests only, not including microbiota profiling (TURN2), costs for location, travel allowance and compensation for donors, and wage of study coordinators (Table 3). The total cost of all performed biochemical tests was €64,112 to find 38 eligible faecal donors. Total screening costs per active donor were estimated at €2,774 a year, including full initial screening, one full rescreening (every six months), four times an additional 60-day screening, and average costs of additional screenings per active donor (€197,-). In the TURN2-trial, in which frozen faeces is used, the total screening costs per year are even higher; €5,388 a year per active donor, including full initial screening, two complete re-screenings (every four months) with PCR assays, three additional 60-day screening, and average costs of additional screenings per active donor (€197,-).

Table 3: Total costs donor screening procedure.

Screening	€
Full screening	**846**
Faeces screening	*657*
Serum screening	*189*
60-day screening	**195**
Multidrug resistant organisms	*150*
SARS-CoV-2	*45*
Full rescreening 4 months (*TURN2*)	**940**
Faeces screening	*612*
Serum screening[a]	*328*
Full rescreening 6 months (*FAIS, IMITHOT, PIMMS)*	**846**
Faeces screening	*657*
Serum screening	*189*
Additional rescreening[b]	**Variable**
Faeces bacteria	*150*
Faeces MDRO	*150*
Faeces viral gastroenteritis	*45*
Faeces SARS-CoV-2	*45*
Faeces parasites	*212*
Serum haematology	*44*

[a] Full rescreening in TURN2-trial included PCR assays of HIV, CMV, HBV and HCV instead of serology; [b] In case of concerns donation was suspended and an additional rescreening was performed depending on symptoms and/or exposed risk factor of the faecal donor.

DISCUSSION

The expanding use of FMT in daily practice and clinical trials is accompanied by a need for more long-term available faecal donors and feasible donor screening programs. In this study, we reported our experience with stool donor recruitment, screening, follow-up, and associated costs in the context of clinical FMT trials. Our study showed that only 10% of potential donors passed all screening steps and could be enrolled as stool donors. Adding to the current literature, we reported the follow-up of eligible donors. In our experience, once qualified, active donors were eligible to donate for about a year before exclusion. Recruiting eligible donors is not only challenging, but also costly; we spent over €64,000 on biochemical tests only to detect 38 suitable faecal donors. This study highlights the obstacles in donor screening and provides practical insights for FMT researchers.

Previous research on donor screening showed variable success rates between 0.8 - 31%[15-21]. Our 10% eligibility rate is similar to smaller studies performed by Craven et al.[15] and Paramsothy et al.[19]. A higher success rate compared to our data was reported in a Danish study, and may be explained by the fact that in this study potential donors were recruited among an existing cohort of eligible blood donors, in which the risk of transmittable infectious diseases by blood transfusion is already assessed[21]. Lower success rates were published by Openbiome, the first public stool bank based in the USA, in which over 15.000 candidates were (pre-)screened and only 3% eventually qualified as faecal donors. The majority of candidates (66%) failed pre-screening mainly due to social history reasons and body-mass index higher than 30 kg/m^2 [27]. In our cohort approximately half of all potential donors failed pre-screening (N=198) of which half was lost to follow-up after initial contact (N=97). Especially during the COVID-19 pandemic, when in periods employees were requested to work from home in accordance to national measures, we experienced high rates of exclusion due to logistics of stool donations. It could be assumed that the COVID-19 pandemic also impacted our high rates of lost to follow-up during pre-screening. More insight into motivation and preferences around stool donation is needed to improve initial donor recruitment and to reduce drop-out rates. Limited data on this subject is available[28]. Based on a multinational questionnaire study, McSweeney and colleagues identified that a male gender and being a blood donor is associated with a high willingness to stool donation, whereas a lack of knowledge on FMT and logistic burdens around screening and stool donation were reported as deterrents[28]. These variables should be taken into consideration. In general, the process of screening and donating should be as easy and convenient for donors as possible.

The global distribution of donor exclusion reasons varies not only as a result of different screening criteria between FMT centres and stool banks[9] but also on diagnostic approach

and geographic location. For example, in the Hong Kong study stool tests were failed by the majority (86%) due to the carriage of ESBL-producing *Enterobacteriaceae*[16]. High prevalence of ESBL in this area is the result of several factors, including a high population density and diet habits. High carrier ship of ESBL-producing organisms seems less of an issue for donor selection in the USA and the Netherlands, where stool bank Openbiome tested only 3 of 571 (0.5%) stool donors positive for MDROs[27], and in our experience, ESBL positive stool tests accounted for the exclusion of five (8.8%) Dutch individuals at initial screening. Moreover, the US FDA has warranted screening for enteropathogenic E. coli (EPEC) by stool nucleic acid amplification testing (NAAT) in addition to Shiga toxin-producing E. coli (STEC)[29]. In our cohort one individual failed stool testing due to the presence of STEC. Currently, EPEC is not included in our screening, because data on pathogenicity of EPEC is inconclusive[30]**.** Including EPEC in our screening protocol could result in even higher rates of donor exclusion.

In our cohort, we found positive parasite testing as the most common exclusion reason during the laboratory screening stage (91 out of 148 stool samples, 61.5%), in specific the presence of *Dientamoeba fragilis* or high amounts of *Blastocystis* spp*.* This is why, at least in certain cohorts, parasitology testing should follow as first step of the laboratory testing phase after (pre-)screening questionnaires. *D. fragilis* and *Blastocystis* spp. were also leading reasons for exclusion in the Canadian study by Craven et al.[15] and the Australian study by Paramsothy et al.[19], but not in others[16,17]. Despite the recommendation of an international guideline to screen and exclude for these protozoa, heterogeneity between screening procedures in practice exists. According to a systematic review evaluating 168 FMT studies, only 15.7% and 14.5% of studies specifically report screening for *D. fragilis* and *Blastocystis* spp*.,* respectively[9]. Moreover, many studies do not state the methods to screen for these organisms, even though the specific diagnostics used has a considerable influence on the detection rate. To illustrate this, the introduction of a *Blastocystis spp.* polymerase chain reaction (PCR) test by the NDFB in 2018 resulted in the discovery that faeces from previously by-microscopy-regarded *Blastocystis* spp*.*-negative donors did actually contain DNA of *Blastocystis subtypes 1* or *3* and that these *Blastocystis* spp*.* were transferred to 31 patients via FMT[31]. Importantly, this did not have a negative effect on the efficacy of treatment for CDI nor resulted in gastrointestinal symptoms. The potential risk of harming recipients by transferring *Blastocystis* spp., might be overestimated. In fact, patients that received *Blastocystis* spp*.*-positive donor stool evaluated their defecation pattern in the long-term as more improved than those receiving *Blastocystis spp.*-negative donor stool[31].

Current consensus recommendations for screening stool donors are based on safety criteria, drawn up by FMT experts in the field, and aim to minimize the risk of inadvertently transmitting a communicable disease to an FMT recipient. Once a potential pathogen is

added to the screening norms it can be difficult to defer it later. However, since the field of FMT research is still relatively new, these criteria are not always supported by solid data and should therefore be adjusted to risk-benefit analysis and progressive insights. For example, whether the exclusion of *D. fragilis*- and *Blastocystis* spp.-positive donors is justified could be questioned, especially for *Blastocystis* spp. of which the pathogenicity is still under debate[32,33]. Both *Blastocystis* spp. and D. *fragilis* appear more commonly in asymptomatic individuals than in patients with gastrointestinal symptoms or disorders, suggesting that these protozoa can have a commensal relationship with human hosts[34-36]. Interestingly, recent literature shows a link between the presence of the above-mentioned single-cell eukaryotes, especially *Blastocystis* spp., and gut microbiota features[37]. For example, stool containing *Blastocystis* spp. has been associated to higher bacterial diversity and distinct microbial profiles (*e.g.* enterotype Bacteroides[38] and co-occurrence with the beneficial bacteria *Akkermansia*[39]), and their presence may reflect a healthier state of the gut microbiota[38-43]. The application of the current consensus screening protocol that suggests the exclusion of *Blastocystis* spp. positive donors[14] could therefore result in the elimination of stool donors that have a favourable bacterial community. This led us to adjust our initial screening protocols where we now accept donors with microscopic quantification of 'rare' or 'few' *Blastocystis* spp. and only exclude individuals with 'moderate' or 'many' *Blastocystis* spp.[26]. Due to the double-blinded nature of the described ongoing clinical studies, it is not yet established if *Blastocystis* spp. positive FMT products have been transferred to our study patients. To prevent unnecessary elimination of valuable stool donors, future research should look into the influence of co-transplantation of common protozoa (and their subtypes) on the microbiota structure and efficacy of FMT.

Since there is limited understanding of what constitutes a successful stool donor for different conditions, most current screening protocols do not comprise potential predictors for FMT efficacy. Nevertheless, it is clear that FMT can improve disease outcome in some recipients (responders), but not in all (non-responders). Hence, the current 'one stool fits all' approach may not be the way to go[44-46]. A more personalized donor-recipient matching strategy where donors are screened for taxa associated with metabolic pathways, or directly for metabolites[47], that are disturbed in a particular disease phenotype, might enhance FMT efficacy. Conversely, the more tailor-made matching strategies will become, the harder the search for suitable donors will be. Evidently, future larger-scale studies in the FMT field are needed to further explore donor-dependent predictors of treatment success.

In the current trials, 14 eligible donors could not be matched to recipients based on gender and/or CMV/EBV status. These mismatches led to expiration of costly screening results and non-activity of valuable stool donors. This waste of screening costs is partly explained by

the fact that the current trials started with establishing a pool of healthy stool donors, whereas at that time no patients were included and stool donation was not yet required. Donor-recipient incompatibilities could be prevented by a more synchronous approach of execution of donor screening programs and patient recruitment. Alternatively, especially in trials using fresh faecal material for FMT, another approach could be applied where patients are first recruited and serologically profiled and subsequently a suitable donor is being sought. The stepwise approach for donor screening could then start with serological testing for pathogenic viruses. Only in case of gender and/or CMV/EBV match, the potential donor could continue full screening program. However, postponing the search of stool donors until a study patient has been screened, might result in an unnecessary delay in the start of study treatment.

Direct costs of an initial safety screening at our centre was €846 (891 USD) per donor. These costs did not include overhead, administration costs and personnel. Limited data is available on associated donor screening costs in other centres. In accordance with our study, Kazerouni et al.[48]. evaluated screening costs for Openbiome to be 885 USD per donor, including clinical assessment, stool and serum screening. The Canadian study by Craven et al.[15] reported that the costs for a full donor screening work-up (including history, examination, blood, stool, and urine screening, and administration) were approximately 440 USD per donor. Differences in costs can be explained by lower costs of biochemical tests in Canada. As discussed previously, minor differences in screening protocols occur since no current consensus on the perfect screening program exists. It should be considered that stricter regulations can lead to increased rates of (temporarily) donor disqualification and even higher associated donor screening costs. Examples of stricter regulations compared to our donor protocol are more regular rescreening of active faecal donors, screening for more enteric pathogens (e.g. EPEC implemented by OpenBiome[49]), broader assessment of conditions (e.g. anti-nuclear antibody test for autoimmune diseases[50]), and mandatory donation of faeces in a supervised bathroom. By reporting the average costs associated with our donor screening program we provide an estimate for clinicians thinking of establishing a pool of healthy stool donors for FMT research. Collaboration with other FMT researchers or national stool banks, in order to share screening costs and eligible donors, will presumably be more cost-effective. Furthermore it lowers the chance of discarding valuable FMT products when a suitable patient match cannot be found within a relatively small study cohort.

Nowadays, FMT is a widely accepted treatment for recurrent *Clostridioides difficile* infection[1,2]. The application of FMT as a treatment for other conditions associated with alterations in the gut microbiome, is limited to the context of clinical trials[8-10]. This barrier has driven some patients to seek for alternative options, including Do-It-Yourself-FMT

procedures with self-administration of (mostly) unscreened donor faeces[51]. The high rates of donor exclusions in seemingly healthy individuals reported by our study and other FMT programs[15-21] illustrates that Do-It-Yourself-FMT procedures can be accompanied by several risks, most importantly the risk of inadvertent transmission of an infectious disease to an FMT recipient. Ekekezie et al. studied factors that influenced willingness to pursue DIY-FMT. Results showed that majority of respondents would have preferred to have FMT performed in a clinical setting[51]. However, lack of access drives these patients to try FMT at home. Regulated stool banks could partially attenuate this problem by enabling compassionate use of FMT in carefully defined clinical cases. A major advantage of regulated (national) stool banks is to ensure safety of FMT products by following strict safety criteria for screening stool donors. Nevertheless, health care professionals must acknowledge the fact that DIY-FMT is an actual phenomenon and therefore clinicians should discuss concerns regarding safety and potential harms with patients considering such a procedure. On the other hand, commercial developers argue that the development of synthetic microbial community products seem to be a safe and sustainable alternative to conventional FMT[52]. However, most colonic bacteria are yet unculturable not and current synthetic microbial products contain limited strains and therefore poorly represent the gut microbiome. Data on clinical efficacy of these products as well as their long-term safety is yet unavailable. Also, data on transmission of uncovered harmful species (i.e. potentially procarcinogenic or pathogenic) can only be derived retrospectively from performed conventional FMT studies[53]. Using synthetic microbial products in FMT trials would rule out the possibilities for these ancillary findings.

This study has several strengths. Firstly, our study included data regarding recruitment and selection procedures of healthy faecal donors from four different clinical FMT trials, creating a large cohort. Secondly, by presenting follow-up data we provided information on the time frame in which donors were qualified to donate faeces after successful screening. Furthermore, this study included an estimation of donor screening costs. By presenting discussed data, this study provides insights in the challenges for creating a sustainable faeces donor pool and is accordingly relevant for researchers setting up clinical FMT trials.

Nonetheless, this study also has some limitations. First, the FMT trials required donors to deliver fresh faecal samples to the hospital for rapid procurement. Therefore, only donors living within a short travel distance were included, comprising mostly urban areas. This potentially influenced the presence of pathogenic microorganisms as mentioned above and limits the generalizability of our results to other regions and countries. Secondly, due to our stepwise screening approach not all faecal and blood laboratory tests were executed on every potential donor. Therefore, presented data on donor deferral reasons per step should be interpreted with caution. Lastly, as discussed, minor differences in the screening

protocols of the four included clinical trials were present. Pre-screening approaches through advertising and short telephonic interviews to discuss in- and exclusion criteria were not standardized. As a consequence, possible exclusions of potential donors and multiple donor deferral reasons could have been missed. Nevertheless, the most relevant in- and exclusion criteria were similar and our approach is in line with current available screenings protocols[14,23]. Therefore, we believe that the effect of the minor (pre-) screening differences is limited.

CONCLUSION

In conclusion, this study shows that a thorough screening protocol for stool donors in the context of clinical FMT trials results in only 10% being eligible donors and is associated with substantial costs. The majority of healthy asymptomatic donors failed stool testing, predominately due to positive parasite testing. The need to exclude donors that carry certain protozoa, especially *Blastocystis* spp., is questionable. The high rates of donor exclusions in seemingly healthy individuals reported by our study illustrates that Do-It-Yourself-FMT procedures can be accompanied by several risks, Further research into the centralization of stool donor screening and procurement of FMT products is warranted.

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SUPPLEMENTARY DATA

Table S1: Demographics and reasons of exclusion of non-active donors.

Donor	Included study/studies	Age	BMI	Sex	Reason of exclusion
1	PIMMS	41	24,7	M	Antibiotic use
2	IMITHOT	24	21,3	F	ESBL-strain *Escherichia coli*
3	FAIS / TURN2	25	23,8	F	No patient match [a], end of study [b] ; no favourable microbiota profile [c]
4	PIMMS	28	24,8	M	No patient match [a], end of study [b]
5	FAIS / TURN2	23	23,4	F	No patient match [a], end of study [b] ; no favourable microbiota profile [c]
6	FAIS	28	22,8	M	No patient match [a], end of study [b]
7	TURN2	29	19,0	F	No favourable microbiota profile [c]
8	TURN2	43	24,9	F	No favourable microbiota profile [c]
9	TURN2	33	23,9	F	No favourable microbiota profile [c]
10	TURN2	31	23,5	F	No favourable microbiota profile [c]
11	FAIS / TURN2	26	20,1	F	No patient match [a], end of study [b] ; no favourable microbiota profile [c]
12	IMITHOT/ TURN2	27	22,4	M	No patient match [a], end of study [b] ; no favourable microbiota profile [c]
13	TURN2	29	23,9	F	No favourable microbiota profile [c]
14	TURN2	30	19,7	F	No favourable microbiota profile [c]

[a] based on gender and/or CMV/EBV status; [b] PIMMS or FAIS study; [c] Donors of the TURN2-trial were additionally selected on a putatively favourable microbiota profile based on results from a previous TURN1 trial. Abbreviations: ESBL, extended spectrum beta-lactamase.

Table S2: Demographics, specifications of screening, and reasons of exclusion of active donors.

Donor	Included study/studies	Age	BMI	Sex	Donations	Full screenings	60-days screenings	Additional screenings	Transient positive tests	Transient pathogens	Reason of exclusion
1	IMITHOT	34	19.6	F	6	2	3	0	0	NA	Antibiotic use
2	FAIS	27	21.8	F	12	5	3	5 (DFTs)	2	*Blastocystis spp.* ('rare' or 'few') [a]	*Blastocystis* spp. ('moderate' or 'many') [a]
3	FAIS / TURN2	27	20.4	F	5	1	0	1 (serum CBC, CRP)	0	NA	*Blastocystis* spp.
4	TURN2	28	20.2	F	21	4	3	0	0	NA	Changed occupation to health care worker
5	TURN2	29	25.0	M	19	3	3	5 (feces viral)	2	Enterovirus	Changed occupation to health care worker
6	IMITHOT / FAIS	25	20.2	M	12	3	5	0	0	NA	COVID-19 measures
7	TURN2	22	24.7	F	18	4	2	6 (DFTs)	0	NA	*Dientamoeba fragilis*
8	FAIS	29	23.9	M	2	2	0	0	0	NA	*Dientamoeba fragilis* + *Blastocystis* spp.
9	PIMMS	29	23.7	M	9	2	2	0	0	NA	End of study [b]
10	PIMMS	33	20.6	M	3	2	1	1 (feces bacterial)	1	*Yersinia enterocolitica*	End of study [b]
11	IMITHOT / FAIS	38	24.4	F	2	2	1	0	0	NA	End of study [b]
12	TURN2	51	22.6	F	7	2	3	3 (MDROs)	0	NA	ESBL-strain *Escherichia coli*
13	PIMMS / TURN2	45	21.1	M	18	4	3	0	0	NA	NA (active donor)
14	IIMTHOT / FAIS	23	23	M	13	3	4	1 (SARS-CoV-2)	0	NA	NA (active donor)
15	TURN2 / IMITHOT / FAIS	25	24.0	F	28	3	5	5 (feces viral)	5	Noro-, Sapo-, Enterovirus	NA (active donor)
16	TURN2	21	24.2	M	16	2	0	0	0	NA	NA (active donor)
17	IMITHOT	23	21.7	F	6	1	1	0	0	NA	NA (active donor)
18	TURN2 / IMITHOT / FAIS	27	19.0	F	28	4	3	1 (SARS-CoV-2)	0	0	No patient match [c]
19	FAIS	31	21.0	F	2	1	0	0	0	NA	No patient match [c]
20	IMITHOT	22	21.6	M	3	1	3	3	0	NA	No patient match [c]
21	TURN2	47	25.4	M	48	5	5	0	0	NA	Personal circumstances
22	IMITHOT / PIMMS FAIS	28	25.3	F	27	1	1	0	0	NA	Rehousing
23	TURN2	26	18.9	F	14	3	4	3 (feces viral, STEC, MDROs)	1	Enterovirus	STEC + ESBL-strain *Escherichia coli*
24	FAIS / PIMMS	25	20.7	F	6	2	1	0	0	NA	Travel

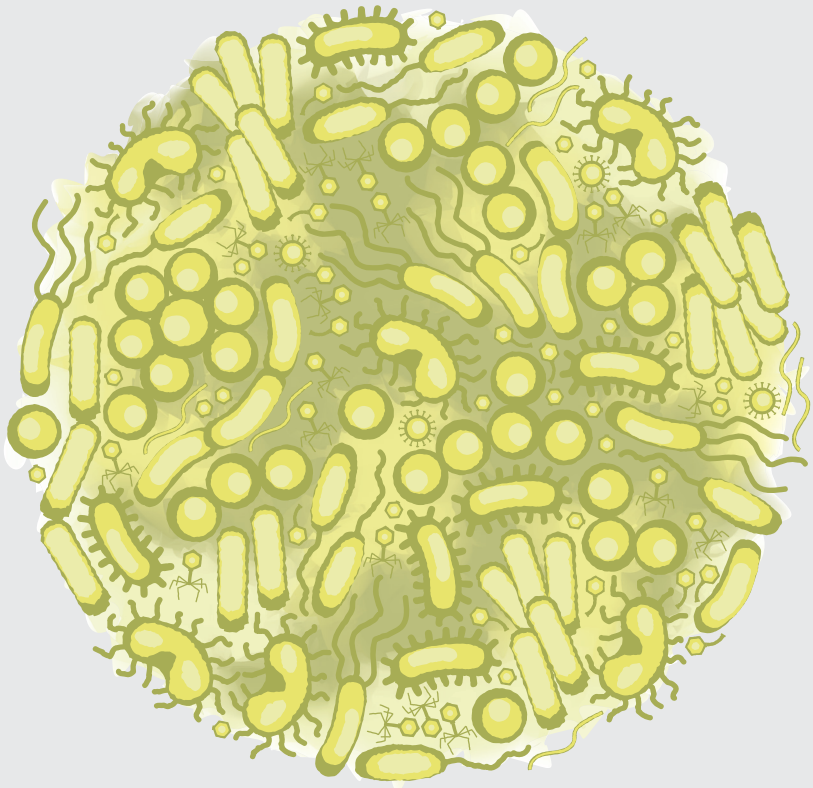
[a] Determined microscopically by an experienced laboratory analyst[26]; [b] PIMMS or FAIS study; [c] based on gender or CMV/EBV status. Abbreviations: CBC, complete blood count; CRP, c-reactive protein; DFT, dual faeces test; ESBL, extended spectrum beta-lactamase; NA, not applicable; STEC, shiga toxin-producing *Escherichia coli*; MDROs, multidrug resistant organisms; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2.

CHAPTER 4

Shedding Light on Dark Matter - Faecal Microbiota Transplantation in Europe

Koen Wortelboer and Hilde Herrema

The Lancet Regional Health - Europe (2021)



COMMENTARY

Faecal microbiota transplantation (FMT) is an emerging treatment modality. FMT entails the transfer of the intestinal microbiota from a healthy donor to a recipient to beneficially alter the intestinal microbiota and change the course of recipients' disease. Having shown high cure rates compared to antibiotic therapy, FMT has become a routine treatment for recurrent *Clostridiodes difficile* infection (rCDI)[1]. In many other microbiota-related diseases including gastrointestinal, metabolic and immunological disorders, the potential of FMT is still in an experimental phase[2]. In addition, FMT allows researchers to study causality of the gut microbiota in human disease[3]. Although the demand for safe FMT is growing, current clinical use, organisation and dissemination of FMT are unknown.

Baunwall and colleagues therefore set out to describe the clinical use and potential for FMT in Europe[4]. A total of 42 hospital-based FMT centres within the European Union were identified. Of these FMT centres, 31 centres from 17 countries replied to a digital survey organised by the United Gastroenterology European (UEG) working group for stool banking and FMT. The survey inquired FMT-related clinical activities, organisation and regulation of approached centres. A total of 1874 FMT procedures were reported, more than half of which (57%) were performed for the treatment of rCDI. Authors state that the reported number of FMTs for rCDI covers only 10% of annual cases of multiple, rCDI in Europe. The significant underuse of FMT in rCDI emphasizes the need to raise clinical awareness for FMT as recommended treatment for rCDI and increase European FMT activity by 10-fold.

The FMT centres in Europe operate with high safety standards and adhere to international consensus guidelines as well as formal or informal regulations from health authorities. Nevertheless, the survey showed a wide variation in donor screening, production and delivery of FMT among the European centres. Safety and accessibility of FMT are relevant concerns for clinical use of FMT. These can largely be overcome by establishing centralised FMT centres or faeces banks as proposed by Baunwall and colleagues. Although cost effectiveness of large FMT centres remains to be determined, these centres can facilitate FMTs via strict standards for donor screening, production, storage and handling. While this infrastructure would benefit FMTs to treat rCDI, it is yet to be determined if such standardised preparations are effective for indications that are more likely to benefit from fresh, (anaerobically) processed FMTs[5]. In such cases, local centres are to be preferred over centralised large centres.

The preferred delivery method for FMT was colonoscopy, followed by rectal enema and nasoduodenal tube. In 2019, 6/31 FMT centres offered FMT in encapsulated form, half as glycerol-based frozen FMT capsules and half as lyophilized FMT capsules. FMT capsules

achieve comparable cure rates for rCDI compared to more traditional means to administer FMT and are quite patient friendly[6,7]. Capsules can be self-administered and, if disease conditions allow, FMT capsule treatment does not necessarily require a hospital visit. Long-term storage can be efficiently realised and capsules provide the opportunity for repeated treatment (*e.g.,* maintenance therapy) and targeted delivery, which might be important for specific indications[8,9]. Production methods for FMT capsules vary and protocols best preserving viability and diversity of donor microbiota still need to be optimised. Nevertheless, encapsulated FMT provides many advantages and opportunities and deserves close attention from initiatives that aim to foster and increase use of FMT for the treatment of rCDI and beyond.

A question which remains to be answered is whether live microbes are necessary for the clinical efficacy of FMT[10]. Other components in FMT such as bacterial remnants, metabolites and bacteriophages could modulate the microbiota as well and might broaden applicability of FMT for patients currently excluded from FMT (*e.g.,* immunocompromised patients). To further standardize the FMT treatment, the active components need to be identified. FMT can be used to identify these promising microbial or metabolic leads, which could replace FMT in time as pre-, pro- or postbiotics. Indeed, ongoing studies are investigating the efficacy of rationally selected bacterial consortia produced under GMP as alternative for FMT to treat rCDI. In time, treatments like these will probably replace the use of FMT.

There are some minor limitations of the study. Authors identified FMT centres via their joint networks and via the trial registry clinicaltrials.gov. In addition, only hospital-based FMT centres were included, leading to an underrepresentation of smaller or peripheral FMT centres. Therefore, the estimated FMT activity in the European union is a conservative measure and will likely be higher. In addition, the results from the survey reflect the situation in 2019 and current practices and FMT activity might have changed. Especially with the recent outbreak of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), many FMT centres were forced to temporarily cease activity and implement additional donor screening measures.

Nevertheless, FMT and similar treatments have an exciting future ahead. By mapping the current FMT landscape in Europe, Baunwall and colleagues provide important guidance for future clinical practice; for decision-makers to regulate FMT and for upscaling FMT and FMT centres in Europe.

Author Contributions

KW and HH contributed equally to writing and revising this commentary.

Declaration of Interests

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CHAPTER 5

Faecal Microbiota Transplantation as Tool to Study the Interrelation between Microbiota Composition and miRNA Expression

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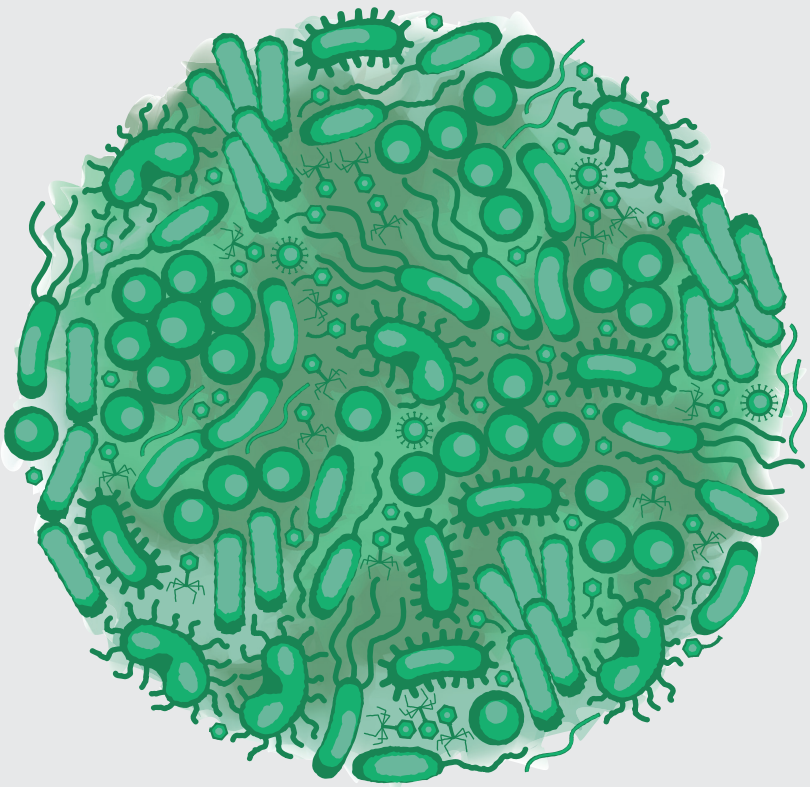
Microbiological Research (2022)



ABSTRACT

The intestinal gut microbiota is important for human metabolism and immunity and can be influenced by many host factors. A recently emerged host factor is secreted microRNA (miRNA). Previously, it has been shown that secreted miRNAs can influence the growth of certain bacteria and conversely, that shifts in the microbiota can alter the composition of secreted miRNAs. Here, we sought to further investigate the interaction between the gut microbiota and secreted miRNAs by the use of faecal microbiota transplantation (FMT). Subjects with the metabolic syndrome received either an autologous (n = 4) or allogenic (n = 14) FMT. Faecal samples were collected at baseline and 6 weeks after FMT, from which the microbiome and miRNA composition were determined *via* 16S rRNA sequencing and miRNA sequencing, respectively. We observed a significant correlation between the faecal miRNA expression and microbiota composition, both before and after FMT. Our results suggest that the FMT-induced shift in microbiota altered the faecal miRNA profile, indicated by correlations between differentially abundant microbes and miRNAs. This idea of a shift in miRNA composition driven by changes in the microbiota was further strengthened by the absence of a direct effect of specific miRNAs on the growth of specific bacterial strains.

HIGHLIGHTS

- Using faecal microbiota transplantation, we observed that changes in the microbiome coincided with changes in faecal miRNA expression.
- We identified twelve strong correlations between differentially abundant intestinal microbes and faecal miRNAs.
- We did not observe a direct effect of specific miRNAs on their associated bacteria in an *in vitro* model.

INTRODUCTION

In past decades, the importance of the intestinal microbiota in human metabolism and immunity has become evident[1]. There are several known factors through which the host can influence the intestinal microbiota, such as secretory IgA, antimicrobial peptides and mucins[2,3]. A more specific host factor that has only recently emerged are secreted microRNAs (miRNA)[4]. MicroRNAs are non-coding RNAs, comprising 18-23 nucleotides, which regulate gene expression at the post-transcriptional level[5]. Studies concerning miRNAs have mainly focused on their role within eukaryotic cells and replication of eukaryotic viruses[6,7]. However, miRNAs are present extracellularly and circulate in body fluids[8], including the intestinal lumen[9]. In line, miRNAs in human faeces have been identified as potential biomarkers for intestinal diseases[10-12].

Recently, studies have found that miRNAs secreted by the hosts intestinal epithelial cells can alter the intestinal bacterial composition[13,14] and that conversely the gut microbiota can influence the expression of miRNAs, mainly through metabolites[15-18]. In addition, plant-derived exosome-like particles containing miRNAs have been shown to influence bacterial growth, localization and production of microbial metabolites[19,20]. While the molecular mechanisms via which eukaryotic miRNAs affect prokaryotes remain to be further elucidated, these studies suggest that miRNAs can play a role in interspecies communication. Conversely, the microbiota can alter the expression and secretion of faecal miRNAs by IECs, thereby promoting proliferation and regulating permeability of IECs[16,21]. In line, it has been shown that bacteria can influence colorectal inflammation and cancer through regulation of miRNAs that enhance the intestinal barrier function[22].

To further study the relation between the intestinal microbiota and intestinal miRNA expression, we used faecal microbiota transplantation (FMT) as a tool. During an FMT, the faecal microbiota from a healthy, thoroughly screened donor is administered to a recipient via an upper or lower gastrointestinal route, thereby inducing a shift in the recipients microbiota to restore a balanced microbiota composition and function[23,24]. Currently, FMT is used as an (experimental) treatment for a plethora of diseases, providing an interesting opportunity to determine the contribution of the intestinal microbiota to disease pathology and thereby leading to important mechanistic insight[25,26]. In the past years, we have performed two randomized controlled clinical trials in which subjects with the metabolic syndrome (MetS) were treated with either an allogenic or autologous FMT[27,28]. Here, we studied whether an FMT altered the faecal miRNA expression in a subpopulation of the most recent clinical trial[28]. Next, we investigated whether observed changes in miRNA composition were correlated with the intestinal microbiota composition. Finally, we tested

whether the associated miRNAs could influence the growth of the specific microbes in an *in vitro* model.

MATERIALS AND METHODS

Participants and faecal samples

Human faecal samples were collected from subjects who underwent an FMT[28]. In short, subjects were Caucasian males, who had obesity (body mass index (BMI) ≥30 kg/m^2), fulfilled at least 3 out of 5 criteria for metabolic syndrome (NCEP criteria[29]: fasting plasma glucose ≥5.6 mmol/l, triglycerides ≥1.7 mmol/l, waist-circumference >102 cm, high-density lipoprotein (HDL) cholesterol <1.03 mmol/l, blood pressure ≥130/85 mmHg) and were treatment naïve. Main exclusion criteria were a history of cardiovascular events, cholecystectomy and use of probiotics or medication.

Subjects were randomized to receive either allogenic (faeces from lean healthy donor) or autologous (control = own faeces) FMT via a nasoduodenal tube. Subjects and faeces donors collected fresh faeces on the morning of FMT and after 6 weeks subjects collected a follow-up faecal sample. Samples were directly stored at -80 °C. Faeces from 18 subjects (n = 14 allogenic, n = 4 autologous) and 5 lean faeces donors was used for 16S rRNA gene amplicon sequencing and miRNA sequencing. Characteristics of these groups are depicted in Table 1. Study procedures were in compliance with the principles of the declaration of Helsinki and approved by the Academic Medical Center ethics committee. All subjects provided written informed consent.

Table 1: characteristics of MetS subjects and lean donors.

	MetSyn				Healthy donor	
	Allogenic (n = 14)	**Autologous** (n = 4)	**p-value**	**Combined** (n = 18)	(n = 5)	**p-value**
Age (years)	54 (6)	53 (8)	*0.865*	**53** (7)	**29** (6)	***0.000***
Male sex (n (%))	14 (100)	4 (100)	*1.000*	**18** (100)	**5** (100)	*1.000*
BMI (kg/m^2)	35.0 (2.9)	33.9 (3.7)	*0.539*	**34.8** (3.0)	**22.3** (1.5)	***0.000***
Waist circ. (cm)	120.0 (7.8)	118.4 (7.7)	*0.722*	119.6 (7.5)		
Syst. BP (mmHg)	139 (14)	147 (17)	*0.357*	141 (15)		
Diasyst. BP (mmHg)	87 (9)	89 (14)	*0.699*	87 (10)		
Glucose (mmol/L)	6.4 (1.2)	6.1 (0.7)	*1.000**	**6.4** (1.1)	**5.2** (0.3)	***0.000****
Triglycerides (mmol/L)	1.73 (1.07)	1.70 (0.71)	*0.798**	**1.72** (0.98)	**0.86** (0.25)	***0.046****
HDL (mmol/L)	1.14 (0.19)	1.20 (0.18)	*0.605*	**1.16** (0.18)	**1.63** (0.36)	***0.040***

Unless otherwise specified, data are reported as mean (SD) and statistical testing is performed by independent t-test. *Data not normally distributed; p-value calculated by independent Mann-Whitney U test. MetS = metabolic syndrome; Waist circ. = waist circumference; syst. BP = systolic blood pressure; diasyst. BP = diasystolic blood pressure; HDL = high-density lipoprotein cholesterol.

16S rRNA sequencing

DNA was extracted from faecal samples using a repeated bead beating protocol and subsequently purified using the Maxwell RSC Whole Blood DNA kit[30]. Next, 16S rRNA gene amplicons spanning the V3-4 region were generated using a single step PCR protocol using universal primers B341F and B806R. Amplicon libraries were purified using Ampure XP beads and pooled equimolarly[31]. An Illumina MiSeq platform using v3 chemistry with 2x251 cycles was used to sequence the library.

Forward and reverse reads, truncated to 240 and 210 bases respectively, were merged using USEARCH[32]. Merged reads were removed if they did not pass the Illumina chastity filter, had an expected error rate higher than 2, or were shorter than 380 bases. Amplicon sequence variants (ASVs) were inferred for each sample individually using UNOISE3 and ASV abundances were determined by mapping unfiltered reads against the joint ASV set[32]. Taxonomy was assigned to ASVs using the RDP classifier[33] and SILVA 16S ribosomal database V132[34].

miRNA profiling

Total RNA was extracted from faecal material using the Qiagen RNeasy PowerMicrobiome kit. RNA concentration and integrity were assessed by Nanodrop and Bioanalyzer, respectively. Thereafter, small RNA fragments were separated from large RNA fragments. To monitor the size distribution and to normalize data between samples, synthetic RNA spike-ins were added as described previously[35]. Small RNA libraries were prepared using the Ion Total RNA-seq kit v2 and barcoded with IonXpress RNA-Seq BC01-BC16 according to the manufacturer's protocols. Library templates where clonally amplified on Ion Sphere particles using the Ion PI Template OT2 200 Kit on an Ion OneTouch 2 Instrument, followed by enrichment of template-positive Ion Sphere Particles using the Ion OneTouch ES. Libraries were sequenced on the Ion Proton system using the Ion PI Sequencing 200 kit and Ion PI Chip v2.

Reads were first mapped to the synthetic spike-in sequences, after which remaining reads were categorized based on length (small <15 nucleotides (nt), medium 15-45 nt and large >45 nt). Next, reads were aligned to human miRNA sequences from miRbase version 21[36–40] and mapped reads were counted. Finally, IsomiR analysis was performed to identify miRNA sequence variants with respect to the reference sequence. Only the miRNAs found within the medium size fragments were used for further analysis, since miRNAs are generally ~18-23 nucleotides in length.

Bioinformatics and statistical analysis

Microbiome and miRNA data were analysed in R Studio 4.0.5. Distance matrices were calculated using the clr transformed count tables and Euclidean distance. Compositional differences were tested using subjected stratified permanova as implemented in vegan, while Procrustes was used to test compositional correlations[41]. All permanova and Procrustes analysis were performed with 999 permutations. Compositional shifts were visualized using inter-individual variance corrected multilevel PCA[42]. Then, for the subjects who received an allogenic FMT, ASV and miRNA deltas were normalized and a univariate Spearman correlation matrix was built for the 250 most abundant ASVs and miRNAs. False discovery rate (FDR) was used to correct for multiple comparisons.

Finally, we investigated whether the identified miRNAs could impact the growth of the associated bacteria *in vitro*, which has been shown in previous studies[13,14,20]. Therefore, we first identified whether there was any overlap between the genome of the identified ASV and the miRNA. In addition, we focused on correlations with a high significance and a negative slope, since growth inhibition by host secreted miRNAs would be biologically more likely. The methods and results of this *in vitro* validation model are described in the supplementary material.

RESULTS

16S rRNA gene amplicon sequencing resulted in a total of 3411 ASVs, of which 554 were present in more than 12 subjects in at least one sample. FMT induced a significant shift in intestinal microbiota composition ($p = 0.005$), which was apparent for both the allogenic and autologous FMT groups (Figure 1A).

Ion Proton sequencing resulted in at least a million reads per sample. Since most of the small RNA molecules were of bacterial origin, merely 7128 reads on average (range: 2049 – 14425 reads) could be assigned as miRNAs. Nevertheless, this resulted in a total of 3753 identified miRNAs, of which 2813 were annotated as mature and 940 as putative mature. Of these identified miRNAs, 1286 were present in more than 12 subjects in at least one of the timepoints. No obvious difference in miRNA composition at baseline was identified between MetS subjects and healthy donors (Figure 1B). However, this could be a power issue due to the low number of healthy donors ($n = 5$). The miRNA profiles were subject specific ($p = 0.001$) and 53% of the variance was inter-individual. Unlike the changes in gut microbiota, shifts in the miRNA composition were not significant ($p = 0.085$; Figure 1C). Using a Procrustes correlation analysis, we did observe a significant correlation between ordinations of the microbiome and miRNA expression on both time points ($p = 0.004$, $p = 0.001$ respectively). This indicates that subjects that share similar microbiota also share

similar miRNA profiles (Figure 1D). Furthermore, Procrustes analysis of the multi-level PCA indicated that changes in microbiome did coincide with changes in miRNA expression (p = 0.007).

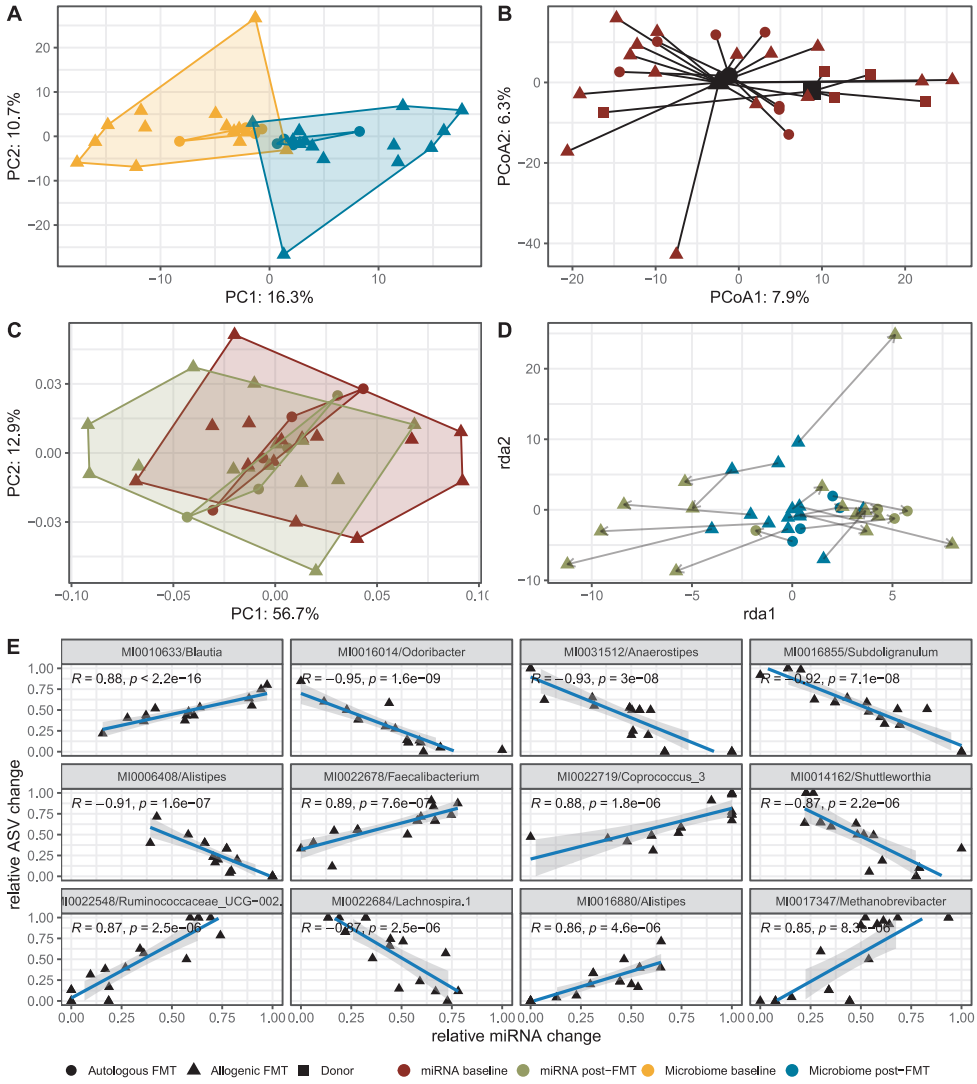


Figure 1: (**A**) Multi-level PCA of the intestinal microbiota composition before and after FMT; (**B**) Ordination of the faecal miRNA composition of MetS subjects at baseline and healthy donors; (**C**) Multi-level PCA of the miRNA composition before and after FMT; (**D**) Procrustes rotation of the post FMT multilevel ordinations; (**E**) Correlation plots between the normalized ASV and miRNA abundance (top 12 based on the strongest Spearman correlation coefficients and highest significance).

Thus, we next investigated whether there were any direct correlations between changes in microbe and miRNA abundance. Figure 1E shows the 12 correlations with the strongest Spearman correlation coefficients between differentially abundant faecal ASVs and miRNAs. Within this selection, *Blautia* and *Faecalibacterium* showed a strong positive correlation with hsa-miR-2114-5p and hsa-miR-6833-5p, respectively, which remained significant after correction for multiple testing. Conversely, *Odoribacter*, *Anaerostipes*, *Subdoligranulum* and *Alistipes* showed a strong negative correlation with hsa-miR-3622b-5p, putative hsa-miR-3648-2-3p, putative hsa-miR-4493-5p and hsa-miR-1272-5p, respectively. Table S1 describes the 12 univariate correlations and summarizes the known literature on the biological role of the identified miRNAs.

Interestingly, we found hsa-miR-3622b-5p aligned with the DNA/mRNA for the DNA polymerase III subunit alpha of *Odoribacter splanchnicus*. To test whether this miRNA could impact the growth of *O. splanchnicus,* we co-cultured them in an *in vitro* experiment (see supplementary material). However, we did not observe a direct effect on the growth of the bacterium, as depicted in figure S1. Similarly, we did not observe an effect of putative hsa-miR-4493-5p on the growth of *Subdoligranulum variabile*. The lack of discernible effect of the miRNAs on growth of these specific bacteria make it more likely that the changes in the microbiome induce the differences in miRNA expression.

DISCUSSION

Accumulating evidence suggests that the gut microbiota can influence the expression of (circulating) miRNAs, suggesting a new route of communication between microbiota and host[16]. In addition, studies have reported changes in the gut microbiota influenced by secreted host miRNAs[13,14] and plant-derived miRNAs[19,20]. FMT is an interesting approach to assess the interrelation between the gut microbiota and miRNA composition. Previously, our group observed an improvement in insulin resistance in MetS subjects who received FMT from a lean healthy donor[27,28]. This improvement associated with changes in both duodenal and faecal microbiota composition. In the present study, intestinal miRNA profiles correlated with microbiota profiles. Although FMT did not induce a significant global shift in the miRNA profiles, compositional changes within the microbiome could be correlated to changes in miRNA profiles. Furthermore, changes in specific microbe abundance could be correlated to changes in miRNA abundance.

Faecal miRNAs have been characterized in human faeces previously and have been identified as biomarkers for several diseases[10–12]. In addition, shifts in faecal miRNAs as a result of microbiota perturbation have been observed in mouse models[15]. Furthermore, it is known that the intestinal microbiota composition differs between healthy and obese

subjects, which could drive a different miRNA expression[18]. However, in present study we did not observe a significant difference in miRNA composition between healthy and MetS subjects. This is most probably explained by a low statistical power due to the low number of healthy donors.

The introduction and engraftment of new donor-derived microbiota could drive the shift in miRNA excretion by the host, although the specific mechanisms remain poorly understood[4]. One example is the metabolite butyrate, which has been shown to alter the miRNA expression in colorectal cancer cells and thereby reduce the cell proliferation[43–45]. In addition, bacterial endotoxins such as lipopolysaccharide (LPS) and flagella have been shown to influence miRNA expression, thereby maintaining intestinal homeostasis and influencing inflammation[46,47]. Another example is found in colibactin-producing *E. coli*, which can induce the expression of miR-20a-5p, leading to an increased secretion of growth factors and ultimately promoting colon tumour growth[48]. Within our study population, it would be interesting to assess whether microbial metabolism could affect the miRNA expression. However, we felt that using an inferred proxy for microbial metabolism in combination with the limited statistical power of the study would not result in any reliable associations. Therefore, the detailed mechanisms via which the intestinal microbiota influence the miRNA expression warrant further investigation, preferably by directly measuring metabolites of interest.

In two small studies, associations between an altered microbiota of subjects with obesity or type 2 diabetes and circulating miRNAs in plasma were found[49,50]. However, since these are cross-sectional studies, this does not prove any causality. Using our univariate regression model, we identified several correlations between differentially abundant microbes and miRNAs at baseline and 6 weeks after FMT. First of all, we found a strong positive correlation between *Blautia* and hsa-miR-2114-5p. This miRNA was first identified in epithelial ovarian cancer and thereafter shown to be downregulated in pancreatic cancer, while being upregulated in gastric cancer[51–53]. Next, we identified a negative correlation between *Anaerostipes* and putative hsa-miR-3648-2-3p. Previous research reported that expression of this miRNA in macrophages can be induced by LPS and inhibits the NFκB pathway[54]. Moreover, hsa-miR-3648 has been found to downregulate tumour suppressor Adenomatous polyposis coli 2 (APC2), leading to an increased cell proliferation[55,56]. Finally, we found a strong inverse correlation between *Subdoligranulum* and putative hsa-miR-4493-5p. Identified in 2010 from malignant human B cells[57], hsa-miR-4493 has been shown to have a protective function against proliferating glioma cells[58].

To date, no direct relation between the microbiome and the above described miRNAs has been reported in literature. The intestinal epithelial cells (IECs) and some hopx-expressing

cells are the main sources of faecal miRNAs[59]. Previously, it has been observed that miRNA profiles differ between IEC subtypes and that commensal microbes can influence the miRNA expression in IECs, promoting proliferation and regulating permeability of IECs[16,21]. In addition, it has been shown that bacteria can influence colorectal inflammation and cancer through regulation of miRNAs that enhance the intestinal barrier function[22]. In line, the three miRNAs identified here are associated with cell proliferation and could play a role in intestinal barrier function. However, the current study only permits us to speculate about the potential role of the faecal miRNAs and the precise function has to be further investigated.

More recently, studies have found that miRNAs secreted by IECs can directly impact bacterial growth and subsequently alter the microbiota composition[13,14]. Further strengthened by evidence that exogenous diet-derived miRNAs can influence intestinal bacterial growth and metabolites[19,20], these studies suggest that miRNAs mediate in interspecies communication. Within our top 12 correlations we identified one ASV-miRNA pair in which there was overlap between the genome and miRNA sequence, namely *Odoribacter splanchnicus* and hsa-miR-3622b-5p. This miRNA was first identified from a collection of cervical tumours in 2010[60]. Thereafter, hsa-miR-3622b has been found to have antitumor properties in several types of cancer[61–63] and has been implicated as a biomarker for Alzheimer's disease[64]. Binding of this miRNA to the bacterial polymerase transcripts of *Odoribacter splanchnicus* could potentially directly impact DNA synthesis and thereby growth.

Previously, Liu et al. have shown a positive effect of hsa-miR-515-5p and hsa-miR-1226-5p on the growth of *Fusobacterium nucleatum* and *Escherichia coli* respectively[13]. Using a similar approach, we cultured *O. splanchnicus* and *S. variabile* in the presence of their associated miRNA. These miRNAs and bacteria were selected from the univariate analysis because of the negative correlation, meaning the miRNA would impair the growth of the bacterium. We chose this approach since a growth-stimulatory effect of a miRNA on a bacterium was, in our opinion, biologically unlikely. In a highly competitive ecological environment as the intestine, being dependent on specific miRNAs from the host for an optimal growth is detrimental for survival and would be selected against.

Unfortunately, we did not observe any effect of the miRNAs on the growth. The absence of effect could be explained by the fact that there was no overlap between the miRNA and the genome of the tested strain (in the case of *S. variabile*). Since bacterial genes lack introns, alignment of a bacterial gene with a miRNA sequence is predictive for binding capacity of the miRNA to bacterial mRNA[65]. Absence of a target bacterial mRNA which can be inhibited or degraded by the miRNA could explain the absence of growth inhibition. In addition, the

miRNAs were not encapsulated in extracellular vesicles, nor bound to high-density lipoproteins or Argonaute proteins, which probably impaired the uptake by the bacteria and reduced the stability of the oligonucleotides[66]. However, there have been reports of uptake of free miRNA in extracellular vesicles *in vivo*, possibly mediated by electrostatic and hydrophobic interactions between the nucleotides and fatty acids[14,67]. More likely, the interaction could be the other way around, whereby *Subdoligranulum* and *O. splanchnicus* are responsible for the decreased expression of the associated miRNA in the gut. Whether these microbes decrease these faecal miRNAs should be further elucidated in future studies.

Limitations of our study include the small sample size and the fact that solely Caucasian males were included, precluding generalization to females and people of other ethnicities. In current study, univariate correlations were based on the group that received allogenic FMT and we made no comparison between interventions or looked into a specific donor effect. In addition to the small sample size, the unbalanced groups and the fact that FMT effects depend both on the donor and the recipient made it impossible to perform any stratified analyses (*e.g.*, donor stratified correlations). In future studies, larger, more balanced groups should be compared, in which FMT is compared with a real placebo, since an autologous FMT will influence the microbiome as well. In addition, mapping of miRNA sequences to the miRbase database resulted in reads with relatively high E-values, meaning many hits were assigned with low confidence. Unfortunately, the current setup of our study made it impossible to study the effect of donor miRNAs present in the administered FMT. Future studies should further investigate the role of miRNAs transplanted during the FMT and whether these contribute to the effect of the FMT.

Nevertheless, the use of a prospective cohort in which MetS subjects received an allogenic or autologous FMT does show that changes in the microbiome coincide with changes in miRNA expression, and thus provides further evidence for an involvement of the gut microbiota in regulating intestinal miRNA expression.

CONCLUSIONS

We found a correlation between the faecal miRNA profile and microbiome composition in human MetS subjects. Although FMT did not induce a significant global shift in the miRNA profiles, compositional changes in microbiome could be correlated to changes in miRNA profiles. Furthermore, changes in specific microbe abundance could be correlated to changes in miRNA abundance. Finally, we could not to show a direct effect of miRNAs on the growth of specific bacterial strains.

Supplementary material

- Supplementary methods and results of *in vitro* validation model
- Figure S1
- Table S1

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Institutional Review Board Statement

The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the local Institutional Review Board of the Academic Medical Centre (AMC) in Amsterdam, the Netherlands. The study was registered at the Dutch Trial Register (number 2705).

Informed Consent Statement

Informed consent was obtained from all subjects involved in the study.

Data Availability Statement

Processed data and analysis are available at: https://github.com/AMCMC/FL_miRNA

Author statement

Data curation: Koen Wortelboer, Guido Bakker and Maaike Winkelmeijer; Formal analysis: Koen Wortelboer, Guido Bakker, Evgeni Levin and Mark Davids; Supervision: Max Nieuwdorp and Hilde Herrema; Writing – original draft: Koen Wortelboer; Writing – review & editing: Guido Bakker, Maaike Winkelmeijer, Natal van Riel, Evgeni Levin, Max Nieuwdorp, Hilde Herrema and Mark Davids.

Acknowledgments

Not applicable.

Conflicts of Interest

MN is co-founder and member of the Scientific Advisory Board of Caelus Health, the Netherlands. MN is on the Scientific Advisory Board of Kaleido Biosciences, USA. EL is founder and CEO of Horaizon BV. None of these are directly relevant to the current paper.

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SUPPLEMENTARY MATERIAL

Materials and methods

In vitro validation model

To validate our findings from the univariate correlations, we cultured *Subdoligranulum variabile* (DSM 15176) and *Odoribacter splanchnicus* (DSM 20712) in the presence of their associated miRNA, a scrambled variant and a blanc vehicle. *Escherichia coli K12* (DSM 498) was incubated with the mature miRNAs as an additional control. In short, the anaerobic bacteria were cultured in yeast extract, casitone and fatty acid (YCFA) medium supplemented with either 10, 1.0 or 0.1 µM of the respective miRNA. *S. variabile* was grown with putative hsa-miR-4493-5p (sequence: CCAGAGAUGGGAAGGCCUUC) and a scrambled hsa-miR-4493-5p (sequence: AGGCGAGCAUCCGACUGGAU). *O. splanchnicus* was incubated with hsa-miR-3622b-5p (sequence: AGGCAUGGGAGGUCAGGUGA) and scrambled hsa-miR-3622b-5p (sequence: GAGUGGCGAUUACGGAGGAG). Lastly, *E. coli* was grown as control in the presence of the two miRNAs used (putative hsa-miR-4493-5p and hsa-miR-3622b-5p). As negative control, the vehicle of the miRNAs (ultrapure DNase⁄RNase-free water, Invitrogen) was added to the YCFA medium.

All bacteria were grown in duplicate in an anaerobic chamber at 37°C for 3 days. Cultures were sampled in triplicate at baseline, 2, 4, 6, 8, 30, 46 and 52 hours, whereafter growth was assessed as absorbance at 600 nm (OD_{600}), measured by a spectrophotometer (VersaMax™ microplate reader). Data were analysed in GraphPad Prism 8.3.0. Differences between groups were analysed by ANOVA with post hoc testing using Bonferroni correction. A two-sided p-value <0.05 was considered significant. Results are expressed as mean ± standard deviation (SD).

Results

miRNAs do not affect bacterial growth in vitro

We first identified whether there was any overlap between the genome of the identified ASV and the miRNA, which was true for solely one pair: *Odoribacter splanchnicus* and hsa-miR-3622b-5p. This miRNA aligned with the DNA/mRNA for the DNA polymerase III subunit alpha of *O. splanchnicus*. Next, we focused on correlations with a high significance and a negative slope. Since ASVs identified bacteria from different taxonomical levels (family, genus and strain), we looked for commercially available strains that were closely related to the identified family or genus. For *Subdoligranulum*, this resulted in *S. variabile*. Unfortunately, we could not identify a close representative strain for the *Anaerostipes* lineage that we identified.

O. splanchnicus and *S. variabile* were successfully grown together with *E. coli* as negative control in an anaerobic chamber. Addition of hsa-miR-3622b-5p to the growth medium of *O. splanchnicus* or putative hsa-miR-4493-5p to *S. variabile* in increasing concentrations ranging from 0.1 to 10 µM did not affect the growth compared to the water vehicle or the scrambled miRNA variant. Results are shown in Figure S1. Since the identified miRNAs did not negatively influence the growth of the respective strains, this could mean the interaction works in the opposite direction, suggesting that these strains inhibit the expression and secretion of these specific miRNAs in the intestine of the host.

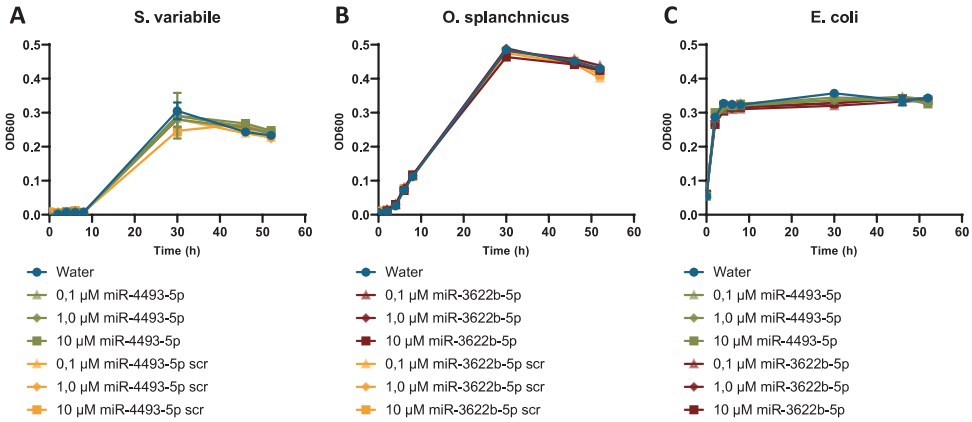

Figure S1: (**A**) Growth of *S. variabile* in the presence of putative hsa-miR-4493-5p; (**B**) Growth of *O. splanchnicus* in the presence of hsa-miR-3622b-5p; (**C**) Growth of *E. coli* in presence of both hsa-miR-3622b-5p and putative hsa-miR-4493-5p. Values are mean ± SD.

Table S1: Overview of the miRNAs that correlated the strongest with microbes in the univariate model.

#	miRNA	mature/putative	correlated ASV	R-value	p-value	padj	biological role miRNA
1	hsa-miR-2114-5p	mature	Blautia	0.88	<1e-16	<1e-16	(1) expressed in epithelial ovarian cancer tissue[1]; (2) upregulated in gastric cancer tissue[2]; (3) downregulated in pancreatic cancer tissue[3]; (4) upregulated in lung adenocarcinoma[4].
2	hsa-miR-3622b-5p	mature	Odoribacter splanchnicus	-0.95	1.6e-09	1.0e-04	(1) expressed in cervical tumors[5]; (2) tumor suppressor in ERBB2-positive breast and gastric cancer cells[6]; (3) regulates Th17 and Treg cell differentiation and plasticity[7]; (4) oncogene in breast cancer[8].
3	hsa-miR-3648-2-3p	putative	Anaerostipes	-0.93	3.0e-08	1.9e-03	(1*) inhibitor of NFκB pathway in macrophages[9]; (2*) downregulation of tumor suppressor APC2[10,11]; (3*) upregulated in non-syndromic cleft lip/palate[12]; (4*) expression inhibited by amyloid precursor protein intracellular domain in neuronal stem cells[13]; (5*) expression increased in microglial cells upon infection with Japanese Encephalitis Virus or West Nile Virus[14]; (6*) upregulated in colon adenomas[15,16].
4	hsa-mir-4493-5p	putative	Subdoligranulum	-0.92	7.1e-08	4.4e-03	(1*) expressed in malignant human B cells[17]; (2*) protective against proliferating glioma cells[18]; (3*) increased in physically active people[19].
5	hsa-miR-1272-5p	mature	Alistipes	-0.91	1.6e-07	1.0e-02	(1) upregulated in pluripotent human embryonic stem cells[20]; (2) upregulated in narcolepsy[21]; (3) inhibition of IL-4 release from Th2 cells[7]; (4) downregulated in lymphoblastoid cell lines after permetrexed treatment[22]; (5) downregulated in progressing upper tract urinary carcinoma[23].
6	hsa-miR-6833-5p	mature	Faecalibacterium	0.89	7.6e-07	4.7e-02	(1) upregulated in colorectal adenomas[24,25].
7	hsa-miR-6872-5p	mature	Coprococcus_3 comes	0.88	1.8e-06	1.2e-01	(1) downregulated in gastric cardia adenocarcinoma tissue[26]; (2) upregulated in alveolar type II cells upon infection with influenza A virus[27].
8	hsa-miR-3139-3p	putative	Shuttleworthia	-0.87	2.2e-06	1.4e-01	(1*) tumor suppressor in triple-negative breast cancer cells[28]; (2*) overexpressed in lung cancer[29]; (3*) downregulated in Huntington's disease prefrontal cortex[30].

Table S1 continued

#	miRNA	mature/putative	correlated ASV	R-value	p-value	padj	biological role miRNA
9	hsa-miR-6715a-5p	putative	Ruminococcaceae _UCG-002	0.87	2.5e-06	1.6e-01	(1) detected in hepatocellular carcinoma tissues[31]; (2) increased expression with age in the inner ear sensory epithelium, targeting Pcdh19[32]; (3*) upregulated in endometrial cancer tissue[33]; (4*) differential expression throughout pregnancy[34]; (5*) downregulation in cholangiocarcinoma tissue associated with poor survival[35]; (6*) overexpression in breast cancer tissue associated with better survival[36].
10	hsa-miR-6838-5p	mature	Lachnospira.1	-0.87	2.5e-06	1.6e-01	(1) tumor suppressor in gastric cancer growth, migration and invasion by targeting USP9X[37]; (2) tumor suppressor in pancreatic cancer through inhibition of HMGA1 expression[38]; (3) upregulation in renal carcinoma cells enhanced tumor proliferation and invasion through inhibition of the DMTF1/ARF-p53 axis[39]; (4) tumor suppressor in gastric cancer cells through inactivation of the Wnt/β-catenin pathway[40]; (5) tumor suppressor in triple-negative breast cancer by targeting WNT3A to inhibit the Wnt pathway[41]; (6) targets the SARS-CoV-2 genome[42,43].
11	hsa-miR-4514-5p	mature	Alistipes	0.86	4.6e-06	2.9e-01	(1) expressed in malignant human B cells[17]; (2) differentially expressed in plasma of colon cancer patients[44]; (3) decreased in blood 72h after intracerebral hemorrhage[45]; (4) downregulated in primary bronchial epithelial cells upon influenza A virus infection[46]; (5) upregulated in exosomes from ultraviolet-irradiated human skin fibroblasts[47]; (6) upregulated in placentas of small for gestational age weight infants[48].
12	hsa-miR-4713-3p	mature	Methanobrevibacter	0.85	8.3e-06	5.2e-01	(1) upregulated in paclitaxel-resistant gastric cancer cells[49]; (2) upregulated in pancreatic ductal adenocarcinoma tissue[50]; (3) differential expression in lung adenocarcinoma tissue[51].

*Since these miRNAs are putative mature miRNAs, these observations have been described for the complementary miRNA strain and might not always be applicable for the putative miRNA. If sampling site or tissue is unspecified, the miRNAs were measured in peripheral blood or plasma. ERBB2 = Erb-B2 Receptor Tyrosine Kinase 2; APC2 = adenomatous polyposis coli regulator of WNT signaling pathway 2; IL-4 = interleukin 4; Pcdh19 = Protocadherin 19; USP9X = ubiquitin specific peptidase 9 X-linked; HMGA1 = high mobility group AT-hook 1; DMTF1 = cyclin D binding myb like transcription factor 1; ARF = alternate reading frame (p14ARF); WNT3A = Wnt Family Member 3A; SARS-CoV-2 = Severe acute respiratory syndrome coronavirus 2.

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CHAPTER 6

From Faecal Microbiota Transplantation Toward Next-Generation Beneficial Microbes: The Case of *Anaerobutyricum soehngenii*

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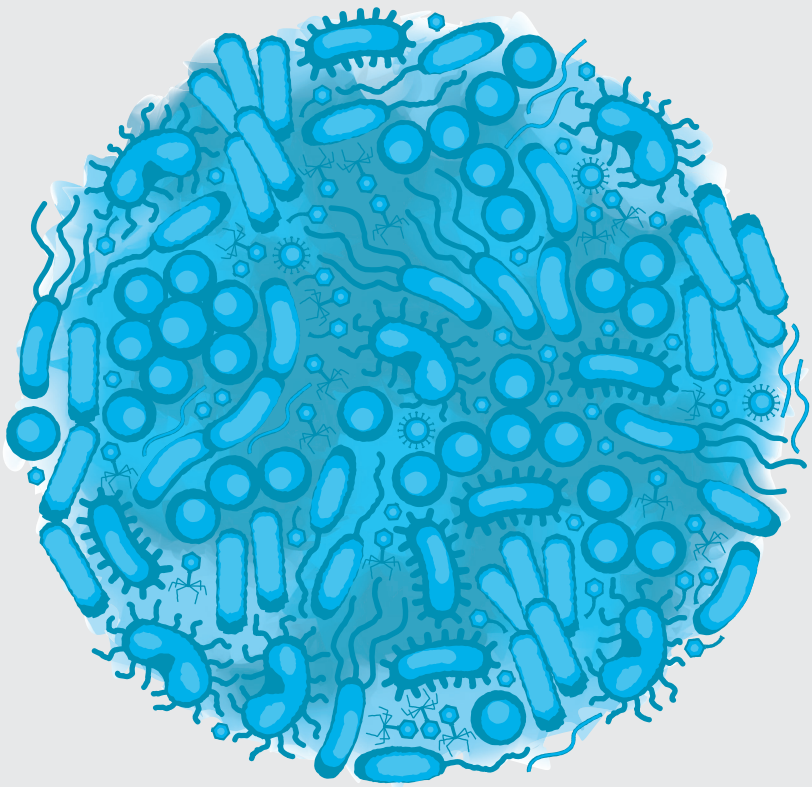

ABSTRACT

The commensal gut microbiota is important for human health and well-being whereas deviations of the gut microbiota have been associated with a multitude of diseases. Restoration of a balanced and diverse microbiota by faecal microbiota transplantation (FMT) has emerged as a potential treatment strategy and promising tool to study causality of the microbiota in disease pathogenesis. However, FMT comes with logistical challenges and potential safety risks, such as the transfer of pathogenic microorganisms, undesired phenotypes or an increased risk of developing disease later in life. Therefore, a more controlled, personalized mixture of cultured beneficial microbes might prove a better alternative. Most of these beneficial microbes will be endogenous commensals to the host without a long history of safe and beneficial use and are therefore commonly referred to as next-generation probiotics (NGP) or live biotherapeutic products (LBP). Following a previous FMT study within our group, the commensal butyrate producer *Anaerobutyricum* spp. (previously named *Eubacterium hallii)* was found to be associated with improved insulin-sensitivity in subjects with the metabolic syndrome. After the preclinical testing with *Anaerobutyricum soehngenii* in mice models was completed, the strain was produced under controlled conditions and several clinical studies evaluating its safety and efficacy in humans were performed. Here, we describe and reflect on the development of *A. soehngenii* for clinical use, providing practical guidance for the development and testing of NGPs and reflecting on the current regulatory framework.

INTRODUCTION

The commensal gut microbiota play an important role in human health and well-being, regulating host metabolism, shaping our immune system and preventing pathogen colonization[1–3]. However, disruption of the intestinal microbiota has been implicated in several diseases, such as gastrointestinal disorders, metabolic disorders and even autoimmune diseases[4,5]. Over the past decades, faecal microbiota transplantation (FMT) has emerged as a potential treatment strategy for such disorders by restoring a balanced and diverse microbiota[6]. In addition, FMT has enabled researchers to study causality of the gut microbiota in disease pathogenesis[7,8]. Even though FMT has shown promising results in several diseases[9], the therapy is currently only indicated for the treatment of recurrent *Clostridioides difficile* infections[10]. Furthermore, FMT faces several logistical challenges such as donor screening and (anaerobic) sample processing and storage[11,12]. In addition, there are potential safety risks with FMT, such as the potential transfer of pathogenic microorganisms missed during donor screening[13]. Other potential risks include the potential transfer of unwanted phenotypes such as obesity or an increased risk of developing disease later in life such a colorectal cancer[14–16].

Due to these limitations and risks of FMT, a more controlled, personalized mixture of beneficial microbes might prove a better alternative. Traditional probiotics are believed to be beneficial for the host health by supporting a balanced microbiota, contributing to the health of the digestive tract and immune system and counteracting pathogenic bacteria through various mechanisms[17–19]. However, even though decades of extensive studies have led to numerous prophylactic and therapeutic health claims[20,21], clinical trials of high methodological quality report conflicting results and debatable conclusions[22]. In addition, the majority of the probiotics currently sold on the market contain microorganisms from the *Lactobacillus* and *Bifidobacterium* genera, while these genera constitute only a minor proportion of the human intestinal microbiota[23,24].

With increasing knowledge of the gut microbiota through affordable genome and metagenome sequencing and the development of better culturing techniques, the list of endogenous microbes with potential health benefits has dramatically increased. Since these microbes are endogenous to the host, they are more likely to engraft and be metabolically active. Even though most of these commensal microbes are still at an early stage of mechanistic investigation, there have been several reports of beneficial microbes restoring the balance of the intestinal ecosystem and improving disease phenotype[25–30]. These microorganisms without a long history of safe and beneficial use are commonly referred to as next-generation probiotics (NGP) or live biotherapeutic products (LBP)[31].

Previously, our group performed a randomized controlled trial studying the effects of lean donor FMT in human obese, insulin resistant subjects[32]. In line with an improved insulin sensitivity, we observed an increased abundance of the commensal *Anaerobutyricum* spp. (previously named *Eubacterium hallii*[33]) in the small intestine upon allogenic FMT compared to autologous FMT. We thus set out to further study and develop this potential beneficial microbe and focused on *A. soehngenii* L2-7 among others since it was best characterized[34–36]. After confirming a dose-dependent improvement of insulin sensitivity and safety of *A. soehngenii* in a mouse model[37], the strain was produced under controlled conditions and tested in a dose-escalating phase I/II clinical trial[38]. Here, we describe the development of *A. soehngenii*, from the identification and production to the first clinicals trial in humans. In addition, we provide a practical roadmap for the development and testing of similar NGPs and reflect on the current regulatory framework.

DEFINITION OF NGP AND LBP

The traditional probiotics are defined as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host"[39]. These microbes have a long history of use and are regarded as safe, having a Generally Regarded as Safe (GRAS) status in the United States or a Qualified Presumption of Safety (QPS) status in the European Union[40]. In contrast, NGPs are microorganisms without a long history of safe and beneficial use, that like traditional probiotics, confer a health benefit on the host when administered in adequate amounts[31]. In 2012 the United States Food and Drug Administration (FDA) introduced the term live biotherapeutic products (LBP), defined as "a biological product that: 1) contains live organisms, such as bacteria; 2) is applicable to the prevention, treatment, or cure of disease or condition of human beings; and 3) is not a vaccine"[41]. This FDA guidance statement was followed up in the European Union in 2019, where LBPs were defined as "medicinal products containing live micro-organisms (bacteria or yeasts) for human use" in the European Pharmacopeia (Ph. Eur.)[42]. However, since LBPs comprise besides the microorganism also the formulation of the final product and are defined as a medicinal product, this term should not be systematically used to replace NGPs. The term NGP is more extensive, including both the microorganisms present in LBPs and those currently being investigated, not formulated in a final product yet[31]. In addition, NGPs could be employed both as a food supplement like traditional probiotics or as a medicinal product in the prevention, treatment, or cure of disease. Finally, genetically modified microorganisms can be viewed a NGPs as well, although the route to market as an LBP is most likely. Figure 1 schematically depicts the various definitions.

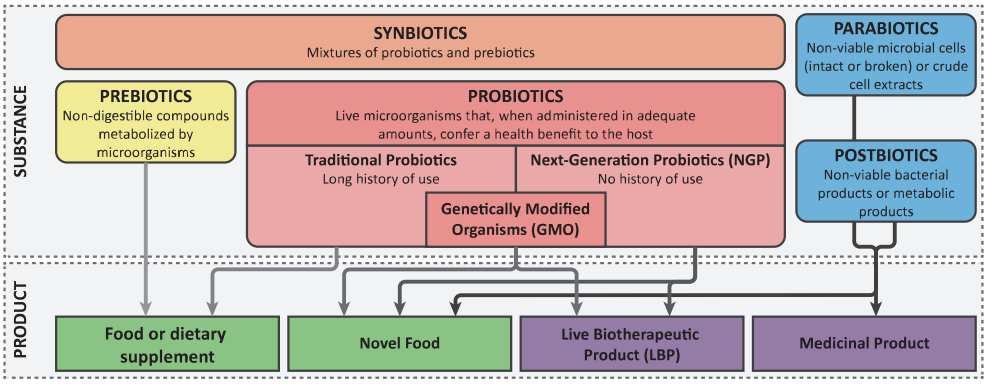

Figure 1: Definitions of probiotics, next-generation probiotics, and live biotherapeutic products. The different "biotics" are colored orange, here denoted as the active substance. The final products are colored green, with the darker green corresponding with products that are considered drugs, while the lighter green falls within the food and food supplements regulation.

DISCOVERY AND ISOLATION OF *ANAEROBUTYRICUM SOEHNGENII*

In line with the worsening global obesity pandemic, the incidence of the metabolic syndrome has dramatically increased, predisposing individuals to developing cardiovascular diseases and type 2 diabetes[43]. Dybiosis of the gut microbiota, defined as a perturbation of the composition and function, has been associated with the emergence of metabolic syndrome[44–46]. To further investigate a causal role of the gut microbiota in metabolic syndrome, we previously infused faecal microbiota from lean healthy donors to male subjects with metabolic syndrome[32]. Six weeks after the infusion of donor microbiota, peripheral insulin sensitivity increased along with levels of butyrate-producing bacteria, as compared to the autologous FMT group. Among these butyrate-producing bacteria, *Anaerobutyricum* spp. were more abundant in the small intestine, pointing towards a potential role in regulating insulin sensitivity through butyrate production. Since insulin resistant metabolic syndrome subjects are characterized by reduced levels of short-chain fatty acid (SCFA)-producing bacteria[47,48] and oral supplementation with butyrate improved insulin resistance and dyslipidaemia in diet-induced obese mice[49,50], we concluded that *A. soehngenii* could be a promising NGP to improve insulin-resistance.

Isolated from the faeces of an infant in 1996[34], *A. soehngenii* strain L2-7, previously designated *Eubacterium hallii*, is a strict anaerobic, Gram-positive, catalase negative bacterium within the family *Lachnospiraceae*[33]. *A. soehngenii* is part of the core microbiota of the human gastrointestinal tract[51,52]. In contrast to other well-known butyrate-producing species such as *Roseburia* and *Faecalibacterium spp*. that produce butyrate from sugars, *A. soehngenii* has the capacity to utilize D- and L-lactate in the presence of acetate instead[53]. In addition, the genome contains bile acid sodium symporter and choloylglycine hydrolase genes, suggesting that *A. soehngenii* can affect host bile acid metabolism[54].

The *A. soehngenii* strain (previously *E. hallii* L2-7^T) was obtained from collaborators in the UK[34,55] and is available from the DSMZ (Deutsche Sammlung van Mikroorganismen und Zellkulturen) as DSM 17630. The strain was cultured routinely under anaerobic conditions using a previously published protocol[33]. Next, we thoroughly characterized the strain. First, the complete genome was sequenced[54], leading to a better understanding of the genetic potential underlying its metabolic capabilities. Next, optimum growth temperature and pH were determined, as well as the tolerability to oxygen. Cell morphology, motility and spore formation were studied using an (electron) microscope and the resistance to heat inactivation and antibiotic susceptibility were determined. Fermentation end products on various carbohydrates were measured and the resistance to bile acids was determined. Finally, the cellular fatty acid contents and the type of peptidoglycan membrane were determined. The results of this thorough characterization led to the reclassification of the previously designated *Eubacterium hallii* type strain L2-7^T to *A. soehngenii* type strain L2-7^T [33].

The metabolic features of *A. soehngenii* were further characterized by proteomic profiling, revealing the complete pathway of butyrate production from sucrose, sorbitol and lactate[56]. This analysis identified a new gene cluster, *lctABCDEF*, which was induced upon growth on D,L-lactate plus acetate. Comparative genomics showed this gene cluster to be highly conserved in only *Anaerobutyricum* and *Anaerostipes* spp., suggesting *A. soehngenii* is adapted to a lifestyle of lactate plus acetate utilization in the human gastrointestinal tract[56]. The capability to convert potentially harmful D- and L-lactate[57,58] to the beneficial SCFA butyrate[59] confirmed that *A. soehngenii* was a promising NGP for further preclinical development.

Learning points and directions

There are two strategies commonly being employed for the development of NGPs. The first method is to associate the presence of a specific strain with a health phenotype and explore whether that strain has a causal effect on the disease phenotype. To date, many NGP candidates have been identified using sequencing technologies to select strains with a depleted abundance in diseased subjects or strains that are associated with successful FMT treatment[60]. The second strategy is to adopt a well-characterized probiotic strain and genetically modify the strain to confer a health benefit, e.g. through production and delivery of bioactive molecules[23]. The latter approach will lead to a genetically modified organism (GMO) that is subject to specific regulations in various parts of the world, such as in the EU[61–63].

Regardless of the strategy used to identify or generate the NGP, before any health benefits can be studied in vivo the candidate strains need to be fully characterized in vitro[64]. Figure

2 summarizes the most important characteristics which have to be assessed besides genotyping and phenotyping the strain. In addition, the strain origin and subsequent manipulation or genetic modifications have to be documented. If there are any antimicrobial resistance genes or virulence genes present, the potential for transmission to other microorganisms of the human microbiota should be assessed, as well as measures taken to mitigate this risk. When the NGP is intended to be used in diseased persons with e.g., epithelial barrier damage of immunosuppression, the risk for bacterial translocation should be determined. A thorough strain characterization is critical for the assessment of the potential safety issues concerning the use of the NGP in healthy or diseased humans.

PRECLINICAL DEVELOPMENT OF *ANAEROBUTYRICUM SOEHNGENII*

After *in vitro* testing of *A. soehngenii*, we moved to an animal model to assess safety and efficacy of the strain on insulin sensitivity. First, we manufactured a preclinical batch of *A. soehngenii* under anaerobic conditions as previously described[33]. In short, cultures were grown under anaerobic conditions to the end of the exponential phase, concentrated by anaerobic centrifugation, washed with phosphate-buffered saline (PBS) and finally diluted in 10% glycerol to concentrations of 10^6, 10^8 and 10^{10} colony-forming units (CFU) in 100 µl. Purity was assessed by 16S rRNA sequencing and microscopic evaluation of cellular morphology. Viability was assessed by most probable number (MPN) analysis and confirmed by microscopic analysis. Samples were directly stored at -80°C and used within 6 months of production, during which time viability was stable. In addition, some of these samples were tested for stability during 2 years to support the product development for the clinical trial.

Next, we performed a dose-finding study in male diabetic (db/db) mice to test the safety and efficacy of orally administered *A. soehngenii* on insulin sensitivity and lipid metabolism[37]. Mice were treated daily with *A. soehngenii* or placebo (10% glycerol) for up to 4 weeks, during which time no adverse events were observed (normal vital signs). A significant improvement on insulin sensitivity was observed during the insulin tolerance test, which was strongest for the 10^8 CFU dose. This was accompanied by a decrease in hepatic fat and a reduced expression of the *Fasn* and *Acc1* genes, both involved in lipogenesis.

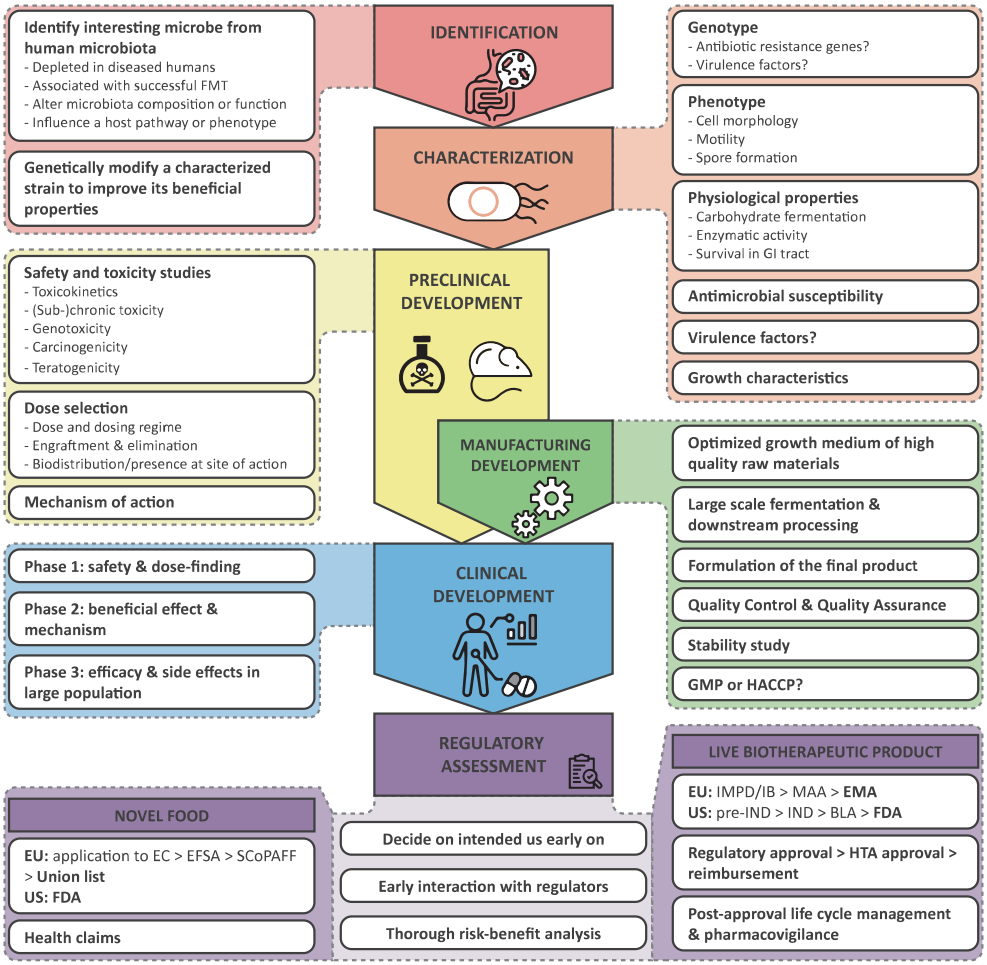

Figure 2: Roadmap for the development of NGP. Important points to consider for the development of NGPs are summarized from the identification to the regulatory assessment. BLA, Biologics License Application; EC, European Commission; EFSA, European Food Safety Authority; EMA, European Medicines Agency; EU, European Union; FDA, Food and Drug Administration; GI, gastrointestinal; GMP, Good Manufacturing Practices; HACCP, Hazard Analysis and Critical Control Points; HTA, Health Technology Assessment; IB, Investigators Brochure; IMPD, Investigational Medicinal Product Dossier; IND, Investigational New Drug; SCoPAFF, Standing Committee on Plants, Animals, Food and Feed, and US, United States.

To confirm these findings and further dissect the therapeutic mechanism of *A. soehngenii*, a second study with db/db mice was performed independently by the lab of prof. Bäckhed (Gothenburg)[37]. Mice were treated with either 10^8 CFU of *A. soehngenii* or heat-inactivated *A. soehngenii* for 4 weeks. An increase in resting energy expenditure was observed after active *A. soehngenii* treatment, while bodyweight remained identical. In addition, active *A. soehngenii* increased faecal butyrate levels and modified bile acid metabolism as compared to the heat-inactivated *A. soehngenii*. These two mouse studies have shown that treatment with *A. soehngenii* is safe and exerts beneficial effects on metabolism, potentially mediated

by butyrate production and changes in bile acid metabolism. These data were used to obtain ethical approval for the clinical studies that we performed in humans.

More recently, a toxicological safety evaluation for *A. soehngenii* CH106, a tetracycline-sensitive derivative from *A. soehngenii* type strain L2-7^T, has been performed to show that the intake at the recommended dosages is safe[65]. As required by the European Food Safety Authority (EFSA) and FDA for safety assessment of new nonabsorbable food ingredients, *A. soehngenii* was assessed for genotoxic potential and subchronic toxicity[66,67]. Both the bacterial reverse mutation and in vitro mammalian cell micronucleus tests showed no genotoxic effects. Furthermore, the 90-day subchronic toxicity in rats did not find any adverse events related to the feeding with *A. soehngenii*, not even at the highest dose (5 x 10^{11} CFU/kg body weight/day) exceeding human recommended daily intake more than 100-fold[65]. These findings support that oral intake of *A. soehngenii* as food supplement is safe.

Learning points and directions

During the preclinical development, adequate information on pharmacological and toxicological properties should be generated to support the proposed clinical trial(s). However, safety and toxicity studies with NGPs are challenging. Since the product generally does not reach the systemic circulation, but its metabolites or its activity could directly or indirectly influence physiological functions in the body, efficacy and toxicity are not necessarily related to the dosage. In addition, other factors such as the human physiology and microbiota composition might influence the safety and efficacy. Furthermore, since most NGPs have coevolved with the human host, the holobiont concept, it is difficult to translate the results from animal studies to the human setting[68–70]. Therefore, it is highly recommended to combine in vitro, ex vivo and in vivo models to establish a global safety profile adapted to the risks within the intended population. It is common to perform the safety and toxicity studies according to the Organization for Economic Co-operation and Development (OECD) principles for Good Laboratory Practice (GLP). However, due to the need for innovative methods and models (e.g. an artificial model of the human gastrointestinal tract) which may not be validated nor at GLP level, this might prove difficult[71].

For food ingredients and dietary supplements, the EFSA advices a tiered approach for toxicological studies[67]. This tiered approach evaluates the toxicokinetics, genotoxicity, subchronic and chronic toxicity, carcinogenicity and teratogenicity of the NGP, balancing data requirements against the risk. This approach was used as well for the toxicological safety evaluation for *A. soehngenii* CH106[65]. If the NGP is intended to be used as medicinal product in a diseased population, it is important that safety for the targeted population is demonstrated. Figure 2 summarizes the most important issues that have to be addressed,

such as the effect of dosage and duration of treatment on toxic response and the teratogenic, carcinogenic and genotoxic potential.

MANUFACTURE OF *A. SOEHNGENII* SUITABLE FOR CLINICAL TESTING

Before we could orally administer *A. soehngenii* to humans, a product suitable for a clinical trial had to be manufactured. At the time of approval by the independent ethics committee (2014), *A. soehngenii* was regarded as a probiotic and had to comply with the Dutch "Warenwet"[72], which was in line with the EU regulations for dietary supplements[73]. This meant the manufacturing had to be performed according to Hazard Analysis and Critical Control Point (HACCP) standards[74]. Therefore, we contracted a third-party manufacturer, which was ISO 9001 accredited and had ample experience with the fermentation of probiotic strains for clinical intervention studies under HACCP standards.

Growth medium

First of all, the growth medium was further optimized for large scale production of a food-grade product. The composition was based on previous experience[33], whereby (1) laboratory chemicals were converted to food-grade sources, (2) only animal-free components were used (no haem or meat peptone), (3) complexity was reduced (removal/reduction of trace minerals, vitamins, carbon sources and organic acids) and (4) the biomass yield was further improved. Raw materials were sourced from audited, reliable suppliers to ensure high quality. Before fermentation, the growth medium was prepared and sterilized inside a large fermenter system, which was made completely anaerobic by nitrogen (N2) flush.

Fermentation

Fermentation was performed in four sequential steps, which are depicted in Figure 3. First, a small volume of food-grade medium was inoculated with a carefully prepared frozen seed stock of *A. soehngenii*. The same strain was used in the animal studies and had therefore been well characterized, was viable, pure and free of any bacterial of viral contaminants. After 24 hours of fermentation at 37 °C, the culture was used to inoculate 1 L of medium, which was again fermented for another 18 hours. Then, this secondary seed culture was used to inoculate 30 L of medium in a small fermenter, which was fermented for 17 hours and which acted as a test run for the large-scale fermentation. Finally, 290 L of medium in the large fermenter was inoculated with 10 L of inoculum of the small fermenter. Both small and large fermenters were controlled for temperature, pH and oxygen level and the optical density (OD) of the culture was used to determine the fermentation time (between 14-18

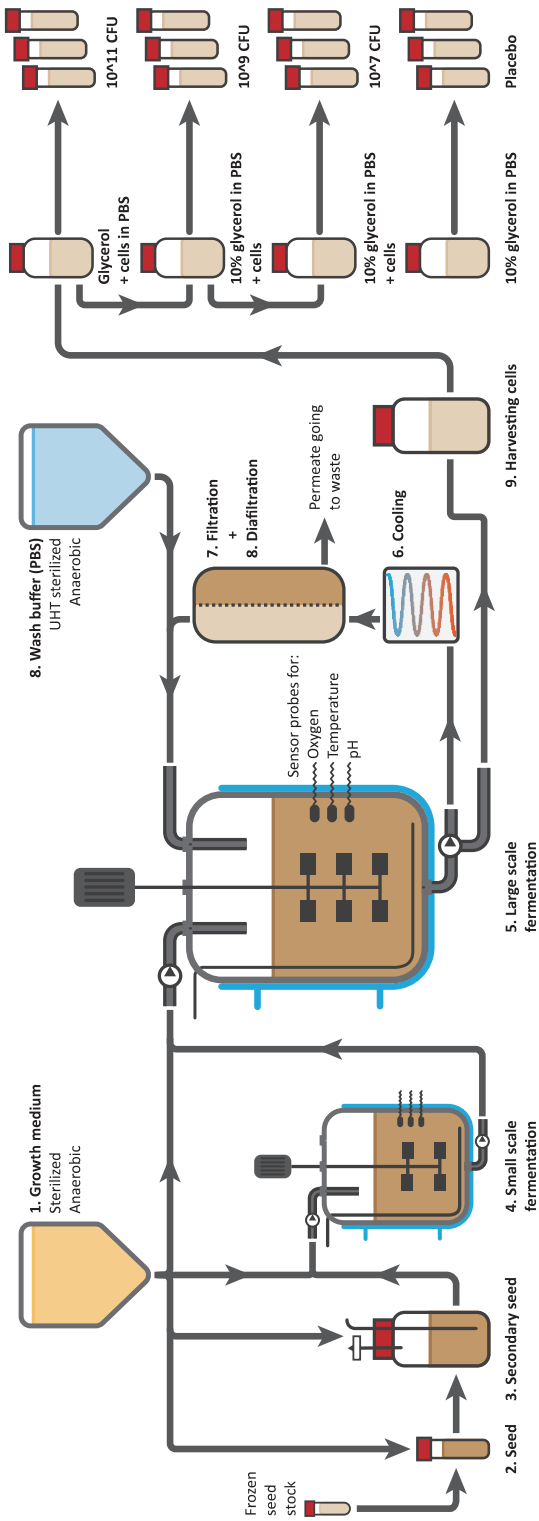

Figure 3: Preparation of Anaerobutyricum soehngenii. Production started with the production of a sterile anoxic growth medium (1), which was used for the seeding (2 and 3) and fermentations (4 and 5) of *A. soehngenii*. The fermentations were well controlled for temperature, presence of oxygen and pH amongst others. After the final fermentation, cells were cooled (6), concentrated (7) and washed by diafiltration (8). Cells were harvested from the fermenter (9), diluted with glycerol (10% final concentration) to 10^{10} CFU/ml and dispensed over 10 ml vials that were labelled and directly frozen. The highest concentration was used to make dilutions for the lower doses.

hours). After 16 hours of fermentation in the large fermenter, *A. soehngenii* grew to an OD of approximately 10.

Concentration and washing

Using hollow fibre membranes (Koch membrane systems; HF3043-25-43-PM500; HF3043-16-106-PM500) and diafiltration with PBS, the cells were concentrated and washed. The fermentate was cooled to 10 °C, pumped through the anaerobic membrane unit and concentrated to 40-50 L within 3 hours. During the second phase diafiltration was performed to reduce the levels of medium components and fermentation products. Wash buffer was sterilized using ultra-high temperature, de-aerated and directly added to the returning cell flow into the fermenter. After 6 hours, the cells were concentrated about 20-fold to 15 L and 99.8% of medium compounds were discarded to waste, leaving solely 2.9% of medium components in the final concentrate. Finally, 9 L of product could be harvested from the system into a sterile, N2-flushed container of 10 L.

Preparation of end-product

Four different batches were produced for the clinical study, consisting of 600 tubes with 10 mL *A. soehngenii* in concentrations of 10^6, 10^8 and 10^{10} CFU/ml in PBS + 10% glycerol and one placebo batch with only 10% glycerol in PBS. For every batch 7 L bottles were prepared with glycerol and PBS for further dilution, which were autoclaved, cooled and flushed with N2. From the 9 L harvested concentrate, the necessary volume was added to these bottles to obtain the correct concentration. Bottles were placed on ice, under continuous stirring and N2 flush. The 10 mL tubes were first filled with N2, followed by 10 mL of product using a dosing-tube-pump. Tubes were immediately closed, labelled and placed in a freezer at -30 °C within 10 minutes of filling. All filling was performed inside a disinfected laminar flow cabinet.

Quality control

During the manufacturing, there was a continuous monitoring of temperature, pH and oxygen level. In addition, the cell count and OD were determined at every step during the process, as well as the absence of any contaminants. Since anaerobes are hard to enumerate quantitatively on agar plates, an MPN analysis was performed under anaerobic conditions to obtain the number of viable cells and cell morphology was assessed microscopically. All above quality controls were performed for the packaged vials, which complied with the standards for human consumption. Table 1 shows the specifications that were defined for the intermediates and final product.

Table 1: specifications for the *A. soehngenii* intermediates and final product

Test	Method	Acceptance Criteria	Intermediate (I), product (P) or stability (S)
Identity	Genome sequencing	confirm strain is *A. soehngenii L2-7*	I*
	Microscopy (visual observation)	Complies with phenotypic characteristics *A. soehngenii L2-7*	I,P
Potency	Culturing/MPN	10^10 CFU/ml	P,S
Purity	Microbial contamination	*Salmonella* spp.: absent *Listeria monocytogenes*: absent *Enterobacteriaceae*: <10 CFU/ml Coagulase-negative *Staphylococci*: <10 CFU/ml *Bacillus cereus*: <10 CFU/ml	I,P,S
Other	pH	6.0 - 7.0	I,P
	Storage	Vial with 10 ml suspension, stored at -20°C	P
	Labelling	According to GMP annex 13	P

*The complete genome of the strain used for seeding has been completely sequenced. CFU = colony-forming unit; GMP = good manufacturing practice.

Subsequently, the stability of the produced vials was tested every 6 months. After production, the vials were given a "best before" date of 6 months, which is required by law for food products in the Netherlands. This gave us the opportunity to extend the expiration date of the vials if the viability and purity criteria were met. Table 2 shows the potency and purity of the vials with the highest dose *A. soehngenii* during a 3-year time period.

Table 2: results of stability testing (potency and purity) of *A. soehngenii*

Storage time (months)		6	12	18	24	30	36
Potency	MPN (CFU/ml)	1.0E+09	1.0E+09	1.0E+09	1.0E+10	1.0E+09	1.0E+09
	Microscopy	Normal	Normal	Normal	Normal	Normal	Normal
Purity	*Salmonella spp.*	Absent	Absent	Absent	Absent	Absent	Absent
	Listeria monocytogenes	Absent	Absent	Absent	Absent	Absent	Absent
	Enterobacteriaceae (CFU/ml)	<10	<10	<10	<10	<10	<10
	Coagulase-negative *Staphylococci* (CFU/ml)	<10	<10	<10	<10	<10	<10
	Bacillus cereus (CFU/ml)	<10	<10	<10	<10	<10	<10

MPN = most probable number; CFU = colony-forming unit

Learning points and directions

Producing a strain at industrial scale sets different requirements for strains and culture media than laboratory scale culturing[75]. Therefore, when a strain qualifies as potential NGP, steps should be taken to see if the strain can be cultured at an industrial scale. The strict conditions necessary for culturing NGPs are one of the technical challenges, such as the need for specific nutrition, the absence of oxygen, a stable temperature and a suitable pH[24]. In addition, longer hold times, sheer stress from pumping, the downstream purification

processes and storage may negatively impact the viability of the bacterial cells. Next, the strains have to be incorporated into a product, such as capsules, a powder or liquid suspension. Since most NGPs are strict anaerobes or facultative anaerobes, the exposure to oxygen should be kept to a minimum. To this end, oxygen permeability into containers should be reduced and antioxidants could be added to reduce the redox potential[76]. Upon ingestion of the product, NGPs have to survive the harsh environment of the gastrointestinal tract. Enteric-coated capsules and microencapsulation are useful strategies to protect the bacteria and deliver them to their site of action[77,78]. Ultimately, manufacturing needs to result in a robust and stable product that will allow for delivery of the NGP in sufficient numbers for an efficacious dose until the expiration date[75].

For medicinal products or LBPs, production according to Good Manufacturing Practices (GMP) is required[41]. For foods and dietary supplements, production in HACCP-certified plants is the standard[74]. Regardless, quality control and quality assurance programs needs to be in place to ensure a consistent quality of ingredients and final product and to secure a reliable production process[75]. The manufacturing process of the strain should be clearly documented, from the raw materials used, the cell bank system, growth and harvesting of the cells, purification and downstream processing to the in-process testing. Likewise, the manufacturing of the final product has to be thoroughly described, including production records and instructions for formulation, filling, labelling and packaging. For both the strain and product manufacturing, the risks for cross-contamination with other products produced in the same rooms or with the same contact equipment has to be assessed. Specifications for the strain and product have to be described, including a description of sampling procedures and the validated test methods. These specifications should describe the identity, potency, purity, contamination, appearance and, if applicable, additional tests for percentage of viable cells, particulate matter, pyrogens, pH and residual moisture. Furthermore, stability data has to be generated, demonstrating the product is stable for the planned duration of use with regards to potency and contamination. For frozen products, the influence of multiple freeze-thaw cycles should be assessed, while for lyophilized products the shelf life after reconstitution should be explored. Finally, the impact of the product on the environment needs to be assessed, especially when the strain is genetically modified, pathogenic, ecologically more fit than the wildtype, or difficult to eradicate.

CLINICAL TRIALS WITH *A. SOEHNGENII*

Safety/dose-finding trial

To validate the murine data in a human setting, we set up a single-blinded, phase I/II dose-escalation trial to determine safety and efficacy of *A. soehngenii* in obese, insulin-resistant subjects[38]. In this study, 27 obese Caucasian males with the metabolic syndrome were

included and assigned to receive *A. soehngenii* in increasing dose of 10^7, 10^9 or 10^{11} cells/day for 28 days. While subjects were blinded for their respective treatment dose, first 9 subjects had to successfully complete the study protocol on the lowest dose before the dose was escalated to a higher concentration. Subjects stored the frozen vials with *A. soehngenii* at -20 °C at home and every day a single 10 mL vial was thawed, mixed with 100 mL of milk and consumed orally. The milk was added to increase the pH in the stomach and thereby protect the living cells during gastrointestinal passage[79]. The primary outcome was safety and in addition the impact on insulin sensitivity and lipolysis was assessed after 4 weeks of treatment.

Treatment with *A. soehngenii* up to 10^{11} cells/day was well tolerated without any serious adverse events[38]. When all treatment groups were combined, the faecal abundance of *A. soehngenii* correlated with an improved peripheral insulin sensitivity, accompanied by beneficial changes in the bile acid profile. Unexpectedly, no increase in faecal butyrate levels was observed, which could be explained by the volatility of SCFAs and the assays' detection limits making butyrate difficult to measure. The increase in (faecal) *A. soehngenii* abundance was transient and mostly gone two weeks after cessation. The viability of the administered strain was negatively affected by stomach acid and oxygen. However, *A. soehngenii* was partially able to survive the gastrointestinal passage as indicated by the highest replication signal in the faeces of subjects that received the highest dose. The viability (and therapeutic efficacy) could be further improved by protecting the strain better from the acidic and oxygenic environment through encapsulation and/or freeze-drying.

Different administration method and mode of action

To further elucidate the mode of action of *A. soehngenii* in humans, a randomized placebo-controlled crossover trial was performed in which the strain was directly administered in the duodenum, thereby circumventing the stomach acid and reducing the exposure to oxygen[80]. Since the small intestine plays a central role in glucosensing, regulation of insulin sensitivity/secretion and glucose homeostasis, it was hypothesized that a direct duodenal infusion of *A. soehngenii* could further enhance the therapeutic effect[81]. Again, obese subjects with the metabolic syndrome (N = 12) were included and randomized to a single nasoduodenal infusion with the highest dose of *A. soehngenii* (10^{11} cells) or placebo (10% glycerol in PBS). After 6 hours, a duodenal biopsy and mixed meal test was performed. In addition, subject monitored their 24-hour glucose and collected several faecal samples. After a 4-week washout period subjects switched to the other treatment arm, which was determined long enough to lose the strain during the first trial.

Again, this study showed that administration of *A. soehngenii* was safe and well-tolerated. Treatment with the strain increased postprandial excursion of insulinotropic hormone

glucagon-like peptide 1 (GLP-1), which was accompanied by a reduced glucose variability[80]. Given that *A. soehngenii* has the capacity to produce butyrate[51,53] and faecal levels of butyrate tended to be higher following *A. soehngenii* treatment[80], the increased GLP-1 secretion could be the result of butyrate activating the G protein-coupled receptor 43 (GPR43) on intestinal L cells[82]. In addition, since *A. soehngenii* expresses a bile acid sodium symporter and bile acid hydrolases[54] and plasma levels of secondary bile acids were elevated[80], the increased GLP-1 expression could also be the consequence of Takeda G protein- coupled receptor 5 (TGR5) activation by secondary bile acids[83]. Moreover, treatment with *A. soehngenii* led to a decreased duodenal expression of the nuclear farnesoid X receptor (FXR) and its target gene *OSTa*, which may also account for an increased GLP-1 availability[84,85]. Finally, the improvement in glucose variability could be explained by the insulin-sensitizing effects of GLP-1 as well as butyrate[49,86].

Furthermore, *A. soehngenii* altered the duodenal transcription of 73 genes, most prominently inducing the expression of *REG1B* along with *REG1A*, which encode for generating islet-derived protein 1A/B[80]. Being strongly expressed within Paneth cells at the base of intestinal crypts, Reg1A and Reg1B are secreted in the lumen and probably act locally, possibly by inducing progenitor or L- cell hyperplasia[80]. Moreover, Induction of *REG1B* was found to correlate with both an increased GLP-1 secretion and a reduced glucose variability 24 hours after administration of *A. soehngenii*[80]. Treatment with a single dose of *A. soehngenii* did not impact the microbiota composition or diversity, as was also seen in the previous studies. In addition, the abundance of faecal *A. soehngenii* was not altered over time, excluding microbiota-mediated carry-over effects at time of crossover[80].

Learning points and directions

The main objective of the first clinical studies is to establish safety and to define the appropriate dosage range and regimen based on the tolerability of the product[64]. This includes the determination of the minimal effective dose or an optimal effective dose range and, if possible, the maximal safe dose. Besides dosing, the focus should be on obtaining safety data to identify common product-associated adverse events. These early clinical studies are commonly performed in healthy volunteers, although inclusion of patients could be more appropriate, for example when the NGP should correct dysbiosis[64]. Risk mitigation measures to ensure the safety of study participants should be taken into account, such as sequential enrolment, dose escalation and monitoring by an independent data monitoring committee. Furthermore, it is expedient to monitor for translocation, inflammation and infection and to establish persistence of NGP and its effects after the final administration.

It is important to account for other confounding factors that influence the function or composition of the microbiota, such as age[87,88], diet[89], lifestyle[90] and environmental

factors[91,92]. In this respect, studies with a placebo-controlled cross-over design are very useful as they can limit the influence of such extrinsic and intrinsic confounding factors, thereby allowing for a smaller sample size. Needless to say, blinding is very important and the washout period should be carefully considered. Increasingly, the baseline microbiota composition is incorporated in the screening criteria as well, looking for example for the presence of specific bacterial groups or clustering within specific enterotypes[93]. This will lead to more comparable study groups and can optimize the efficacy of the intervention when a specific bacterial group is involved in the mechanism of action.

REGULATORY FRAMEWORK NEXT-GENERATION PROBIOTICS

According to the definition of probiotics by the FAO and WHO, probiotics can be classified as both a dietary supplement and a drug, while there is a profound regulatory difference. Similarly, products with NGPs can reach the market as a food, dietary supplement or drug depending on the intended use. In the EU, foods are regulated by the EFSA and drugs by the EMA, while in the US the FDA deals with both categories. When the intended use is related to the prevention, alleviation or cure of disease, the product will be considered a medicinal product or medical device. In contrast, an orally-ingested product with claims relating to enhancement of physiological function or reduction of a disease risk factor could be classified as a functional food or food supplement. Furthermore, topically applied products with a purely cosmetic function could be assessed as a cosmetic. To ensure regulatory compliance, it is important to decide on the indented use and consequent regulatory classification prior to preclinical studies and manufacturing[71].

Functional food or dietary supplement

In the European Union, 'food' is defined as "any substance or product, whether processed, partially processed or unprocessed, intended to be, or reasonably expected to be ingested by humans." Foods and food ingredients are further subdivided into different categories, such as conventional food, food supplements and novel foods, among others. Each of these categories is regulated accordingly, with general requirements and provisions regarding to labelling, presentation and advertising[73,94]. When NGPs are intended for use as food or dietary supplement, they are most likely considered a novel food, since new strains have not been widely consumed within the EU before May 1997[95]. However, if the NGP has been genetically modified, it will be regulated as a genetically modified food[61].

For an NGP to reach the market as a novel food, it needs to be authorized and included in the Union list[95]. One of the most important conditions is that the NGP does not pose a risk to human health, which has to be supported by scientific evidence. This consists of a comprehensive risk assessment, combining biological and toxicological studies in the

context of anticipated human exposure to evaluate the potential risk to human health[96]. In addition, an application should contain detailed descriptions of the NGP, the manufacturing process, the composition of the product, analytical methods used, labelling and conditions for intended use[95].

Many safety-related aspects have been shown to be common at the species level, which has led to the QPS list of the EFSA, expressing a species-based safety evaluation for microbes used as food[40]. If the NGP as a species can be unambiguously identified to a QPS group, the developer does not need to perform detailed tolerance and toxicology studies. However, most NGPs will not belong to a QPS group and must be evaluated by the EFSA to ensure safety[95]. Besides safety, the product must not contribute to the spread of antimicrobial resistance in the food chain or environment, requiring phenotypic and genotypic assessment of antimicrobial resistance.

Any health claims for NGPs have to be submitted to a national competent authority and will be passed on to the EFSA for scientific evaluation[97]. Even the statement "contains probiotics/prebiotics" is considered a health claim in the EU[93]. For a health claim to be accepted, a proper characterization of the NGP is required, as well as a proven beneficial health effect and causal relationship supported by high-quality studies[98].

Live Biotherapeutic Product

Since 2012 and 2019 quality requirements for LBPs have been clarified by the FDA and EDQM[41,42], where LBPs are described as medicinal products containing live microorganisms for human use. Other than these quality requirements, there is currently no specific LBP regulation. However, since LBPs contain live microorganisms, they are considered biological medicinal products and as such have to comply with the legislative and regulatory framework. In absence of a specific LBP subcategory, developers will have to rely on the regulatory concepts available for the other subcategories of biological medicinal products. One of these concepts is a thorough risk-benefit analysis based on quality, safety and efficacy data obtained from preclinical and clinical studies. Cordaillat-Simmons et al. and Rouanet et al. previously elaborated on what a thorough risk-benefit analysis should include[64,71]. Other relevant guidelines for the design of preclinical and clinical studies are the International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use (ICH) guideline on general consideration for clinical trials (ICHE8)[99], the Committee for Medicinal products for Human Use (CHMP) guideline on strategies to identify and mitigate risks for first-in-human and early clinical trials with investigational medicinal products[100], and the CHMP guideline on Human Cell-Based Medicinal Products[101].

For an LBP to reach the market in the EU, marketing authorization has to granted through a centralized or a national route. Under the centralized authorization procedure, EMA's CHMP carries out the scientific assessment, whereafter the European Commission takes a legally binding decision based on EMA's recommendation. To date, no LBPs have reached the EU market, which is partly due to the lack of a defined regulatory framework. Recently, Paquet *et al*. published their experiences with both the EMA and FDA leading up to their first-in-human trial[102]. They described several key considerations for the development and (non-)clinical testing of LBPs based on points raised by the competent authorities. Furthermore, they highlighted the importance of early interaction with the competent authorities to discuss uncertainties and reduce risks in the absence of clear guidelines.

CONCLUDING REMARKS

Above we described our experience with the development of *A. soehngenii* as an NGP and provided several (regulatory) directions. Figure 2 summarizes these points and provides a schematic roadmap for developing NGPs. With the increasing knowledge on our intestinal microbiota, more and more potential NGPs will be discovered and developed, either as novel food/supplement or as LBP. It is important that these new strains are well characterized, of high quality and safe. Though difficult and complex, a thorough safety assessment for NGPs is very important, especially since efficacy and toxicity are not necessarily related to the dosage. Furthermore, since this is a relatively young field and currently no specific LBP regulation, talking to regulators in early stages of development can help to mitigate risks and clarify any uncertainties. This requires a clear view on the route to market (food or drug) early in the development.

We illustrated the development of NGPs with the strict anaerobe *A. soehngenii* as example. Identified as potential beneficial microbe after an FMT intervention, this microbe showed promising results in both preclinical in vitro and in vivo studies as well as in humans. Treatment with *A. soehngenii* was found to be safe and well tolerated. It showed promising effects on improving insulin sensitivity, increased GLP-1 secretion and reduced glucose variability. These effects are potentially mediated through the production of butyrate and secondary bile acids. By protecting the strain better from the acidic and oxygenic environment, e.g., through lyophilization and encapsulation, the viability and thereby therapeutic efficacy could potentially be increased. This NGP is currently being further developed as a food supplement.

Author Contributions

KW wrote the first draft of the manuscript; All authors contributed to manuscript revision, read and approved the submitted version.

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Conflict of interest

MN and WV are founders and scientific advisors of Caelus Health that is commercializing *A. soehngenii*. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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PART II

Gut Bacteriophages in Cardiometabolic Disease

CHAPTER 7

Gut Virome Profiling Identifies a Widespread Bacteriophage Family Associated with Metabolic Syndrome

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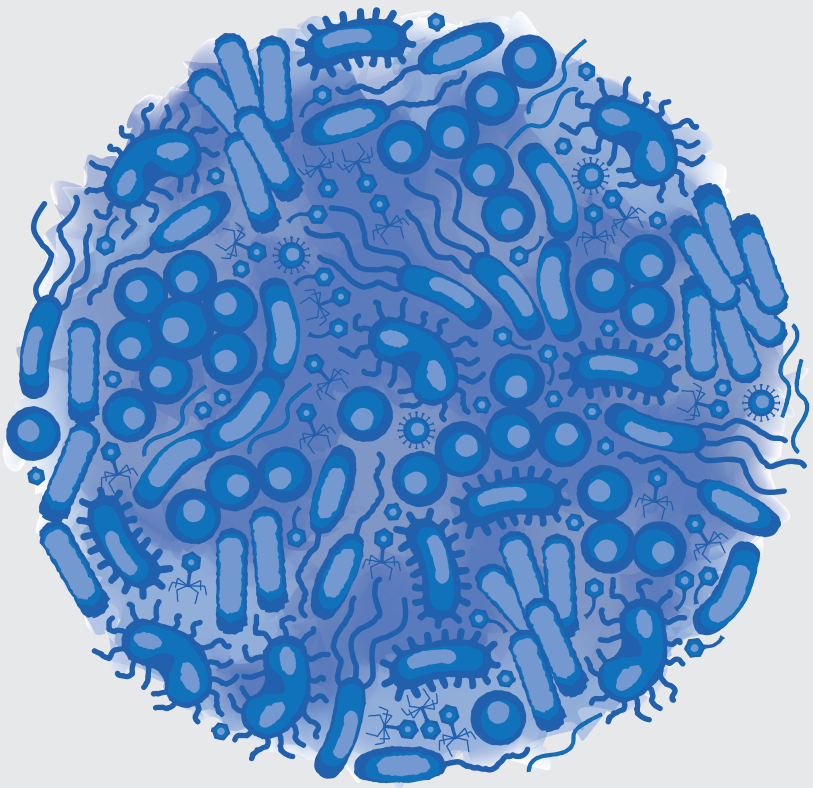


ABSTRACT

There is significant interest in altering the course of cardiometabolic disease development via gut microbiomes. Nevertheless, the highly abundant phage members of the complex gut ecosystem -which impact gut bacteria- remain understudied. Here, we show gut virome changes associated with metabolic syndrome (MetS), a highly prevalent clinical condition preceding cardiometabolic disease, in 196 participants by combined sequencing of bulk whole genome and virus like particle communities. MetS gut viromes exhibit decreased richness and diversity. They are enriched in phages infecting *Streptococcaceae* and *Bacteroidaceae* and depleted in those infecting *Bifidobacteriaceae*. Differential abundance analysis identifies eighteen viral clusters (VCs) as significantly associated with either MetS or healthy viromes. Among these are a MetS-associated *Roseburia* VC that is related to healthy control-associated *Faecalibacterium* and *Oscillibacter* VCs. Further analysis of these VCs revealed the *Candidatus Heliusviridae*, a highly widespread gut phage lineage found in 90+% of participants. The identification of the temperate *Ca. Heliusviridae* provides a starting point to studies of phage effects on gut bacteria and the role that this plays in MetS.

INTRODUCTION

The human gut microbiome influences many (metabolic) processes, including digestion, the immune system[1], and endocrine functions[2]. It is also involved in diseases such as type 2 diabetes[3], fatty liver disease[4] and inflammatory bowel disease[5]. Though studies of these gut microbiome effects on health and disease mostly focus on bacteria, increasing attention is devoted to bacteriophages (or phages).

Phages are viruses that infect bacteria. By infecting bacteria, they can significantly alter gut bacterial communities, mainly by integrating into bacterial genomes as prophages (lysogeny) or killing bacteria (lysis). Such alterations to bacterial communities in turn affect the interactions between bacteria and host, making phages part of an interactive network with bacteria and hosts. For example, an increase in phage lytic action is linked to decreased bacterial diversity in inflammatory bowel disease[6,7], prophage integration into *Bacteroides vulgatus* modifies bacterial bile acid metabolism[8], and dietary fructose intake prompts prophages to lyse their bacterial hosts[9].

Gut virome alterations have been linked to several disease states like inflammatory bowel diseases[6,7], malnutrition[10], and type 2 diabetes[11]. But many such studies have not been able to identify specific viral lineages that are involved in such diseases, mainly due to the lack of viral marker genes[12,13] and high phage diversity due to their rapid evolution[14]. Consequently, human gut phage studies are limited to relatively low taxonomic levels. While recent efforts uncovered viral families that are widespread in human populations, such as the *Crassvirales* phages[15,16], these have not been successfully linked to disease states. In order to develop microbiome-targeted interventions to benefit human health, it is pivotal to study such higher-level phage taxonomies in the gut among relevant cohorts.

Here, we report on gut virome alterations in metabolic syndrome (MetS) among 196 people. MetS is a collection of clinical manifestations that affects about a quarter of the world population, and is a major global health concern because it can progress into cardiometabolic diseases like type 2 diabetes, cardiovascular disease, and non-alcoholic fatty liver disease[17-19]. As gut bacteria are increasingly seen as contributing agents of MetS[20-22], it stands to reason that the phages which infect these bacteria exhibit altered population compositions in MetS. Whereas recent research compared gut viromes in relation to MetS[23], this study was limited to 28 children, in which MetS manifests markedly less well defined than in adults[24]. For our analysis, we focused on dsDNA phages, which form a large majority of gut phages in particular and gut viruses in general[14,25].

Here, we detail differences in the gut virome in MetS versus healthy controls. We find MetS-connected decreases in virome richness and diversity, which are correlated to bacterial population patterns. We further find that MetS viromes are characterized by high levels of *Streptococcaceae* and *Bacteroidaceae* phages, while *Bifidobacteriaceae* phages were less abundant. Finally, among viral clusters (VC) that are differentially abundant in either MetS or controls, we identify four with significant interrelatedness. These phages are part of a previously undescribed family, which we dub the *Candidatus Heliusviridae*, and which is highly widespread in this and several validation cohorts.

RESULTS

Metagenomic sequencing identifies high divergence in MetS viromes

To study gut phage populations, we performed metagenomic sequence analyses on faecal samples of subjects from the Healthy Life in an Urban Setting (HELIUS) cohort[26], a large population study in Amsterdam, the Netherlands. Because gut phages largely exist in two forms: intracellularly (e.g., integrated into bacterial genomes as prophages) and as free-floating particles, we performed sequencing on two types of sample preparations (Supplementary Figure 1). Firstly, for 97 MetS and 99 healthy participants we performed bulk whole genome shotgun (WGS) sequencing, which tends to bias in favour of intracellular phages. Secondly, for a subset of 48 participants (24 each of controls and MetS), we made filtrations of free-floating phage particles and sequenced viral-like particle (VLP) metagenomes. Among the MetS participants, central obesity and high blood pressure were nearly universal, being found in 94/97 participants and 91/97, respectively. For further details on the participants of the present study, see the Methods and Supplementary Table 1. Bulk sequencing yielded an average of 23 ± 3.4 million read pairs per sample (median: 22.6 million read pairs), while VLP sequencing yielded 16.5 ± 2.5 million read pairs (median: 16.3 million). Per sample read assemblies and viral sequence prediction resulted in a database of 45,421 unique phage contigs (non-redundant at 90% average nucleotide identity). We grouped these phage contigs by shared protein content[27] into 6,635 viral clusters (VCs). These comprised 30,161 contigs, while the remainder were singletons that were too distinct to confidently cluster with other phage contigs. Treating such singletons as VCs with one member gave a final dataset of 21,895 VCs.

For further analysis, we mapped quality-controlled reads to viral contigs, and constructed a per-VC RPKM table, which we converted to relative abundances where between-sample comparisons were needed (Supplementary Figure 1). Analysis of relative abundances per VC across the 196 WGS samples (Supplementary Data 1) showed an high inter-individual diversity in bulk gut viromes, as 19,970 VCs (97.4% of the 20,501 VCs present in WGS samples) were either specific to a single individual or present in fewer than 20/196 (i.e.,

<10%) of the participants. Only 59 VCs (0.3%), meanwhile, were putative members of the core human gut virome[28], being present in over 30% of participants (Supplementary Figure 2a). We notably found two VCs that were found in the bulk virome of over 30% of controls and none of the MetS participants, but none vice versa. In both cases, the viral contigs contained in the VCs were genome fragments (i.e., checkv[29] completeness of <25%, Supplementary Data 5). The general prevalence pattern was mirrored among the 48 VLP samples, where 9,147 VCs (93.3% of the 9,800 VCs present in VLP samples) were present in less than 10% of the participants, while 61 (0.6%) were present in over 30% of participants (Supplementary Figure 2b). Interestingly, VCs observed in fewer than 10% of the participants had much higher mean relative abundance among bulk than VLP viromes (WGS: mean 70.1 ± 10.2%, median: 71.8%, VLP: mean 42.1 ± 18.4%, median: 42.6%, Supplementary Figure 2c and d). Much of the interpersonal gut phage diversity is thus contained in the bulk virome.

Gut phage and bacterial populations show altered richness and diversity measures in MetS

To gain a deeper understanding of MetS virome community dynamics, we first examined total read fractions that mapped to VCs. In the bulk phage samples the fraction of reads mapping to VCs was significantly lower in MetS compared to controls (Wilcoxon signed-rank test, $p = 0.023$, Supplementary Figure 3a). This was not caused by differential sequencing depth between the participant groups, as this did not significantly differ between the groups (Wilcoxon signed-rank test, $p = 0.23$). It could instead derive from higher bulk phage micro-diversity causing more fragmented assemblies, thereby decreasing the number of recognized phage sequences. To test this, we constructed cumulative VC ranked-abundance curves of bulk phage samples. These showed that fewer VCs represented the full relative abundance of bulk viromes in MetS than in controls, therefore indicating lower micro-diversity in MetS (Supplementary Figure 3b). Our findings thus imply that MetS is characterized by lower intracellular phage-to-bacteria ratios, for example through decreased lysogeny rates. For VLP phage populations, we observed the opposite: higher fractions of viral reads among MetS (Wilcoxon signed-rank test, $p = 0.011$, Supplementary Figure 3c), while sequencing depth again did not significantly differ (Wilcoxon signed-rank test, $p = 0.65$). But because VLP virome cumulative VC ranked-abundance curves showed the same pattern as those of the bulk viromes, thereby indicating decreased micro-diversity in MetS samples, the increase in viral-mapped read fractions for MetS may reflect less fragmented assemblies of these samples (Supplementary Figure 3d). Thus, while our results suggest decreased lysogeny rates in MetS, we could not definitively determine whether these are paired with increased lytic rates.

For further analysis of phage communities, we examined virome richness and diversity. We determined phage richness by measuring the number of VCs that were present (i.e., had a relative abundance above 0) in each participant, using a horizontal coverage cutoff of 75%[30]. This showed that besides lowered phage-to-bacteria ratios, bulk phage populations in MetS also had lower VC richness than controls, but equal evenness (Wilcoxon signed-rank test, richness $p = 7.1 \times 10^{-7}$, Pielou evenness $p = 0.49$, Figure 1a and b). Nevertheless, due to the strong differences in richness, bulk phage α-diversity was significantly decreased among MetS participants (Shannon H' $p = 0.02$, Figure 1c). This suggested that MetS bulk gut phage populations are distinct from healthy communities. These results were independent of sequencing depth, as significance levels in richness, evenness, and diversity were unchanged upon calculations with the median of 1000 random data sub-samplings. Indeed, the differences between the two participant groups were underscored by our observation of significant separation between controls and MetS when assessed by principal covariate analyses (PCoA) of β-diversity based on Bray-Curtis dissimilarities (Permanova $p = 0.001$, Figure 1d). Similar analyses less notably differed among the VLP phage populations, where richness, evenness, and α-diversity were all non-significantly higher in controls (Wilcoxon signed-rank test, richness $p = 0.11$, evenness $p = 0.26$, and α-diversity $p = 0.089$, Figure 1e-g), though β-diversity still displayed significant separation between the two groups (Permanova $p = 0.038$, Figure 1h). As both richness and α-diversity were highly positively correlated between the VLP and WGS datasets among the subset of 48 participants (richness: Spearman $\rho = 0.68$, $p = 1.1 \times 10^{-7}$, α-diversity: $\rho = 0.5$, $p = 3.6 \times 10^{-4}$), we hypothesize that the lack of significance between controls and MetS VLP datasets was driven by the smaller sample size of the VLP dataset.

Because phages are obligate parasites of bacteria, we also studied bacterial community using 16s rRNA amplicon sequencing data. We opted to analyse 16s rRNA amplicon sequencing data over analysis of the metagenomic samples for its greater taxonomic resolution. Bacterial gut populations are often found to be less diverse in obesity-related illnesses such as MetS[31]. Our data underscored this, and showed that MetS bulk viromes mirror bacterial communities in species richness and α-diversity, but not evenness, which was significantly lowered in MetS bacterial populations (Wilcoxon signed-rank test, Chao1 richness $p = 9.1 \times 10^{-4}$, Shannon H' $p = 1.5 \times 10^{-15}$, Pielou evenness $p = 1.8 \times 10^{-14}$, Supplementary Figure 4a-c). Additionally, bacterial communities separated in PCoA analysis in similar fashion to viromes (Permanova $p = 0.001$, Supplementary Figure 4d). These results were replicable with data derived from taxonomic profiling of the bulk sequences. Population-level bulk virome changes in MetS are thus directly related to a depletion of host bacteria populations, an assertion strengthened by significant direct correlations between bulk phage and bacterial communities in richness (Spearman $\rho = 0.42$, $p = 1.3 \times 10^{-9}$, Figure 2a), evenness (Spearman $\rho = 0.24$, $p = 5.7 \times 10^{-4}$, Figure 2b). Though for the subset of 48

samples with VLP data no such correlations were detected, this could have been due to the smaller sample size.

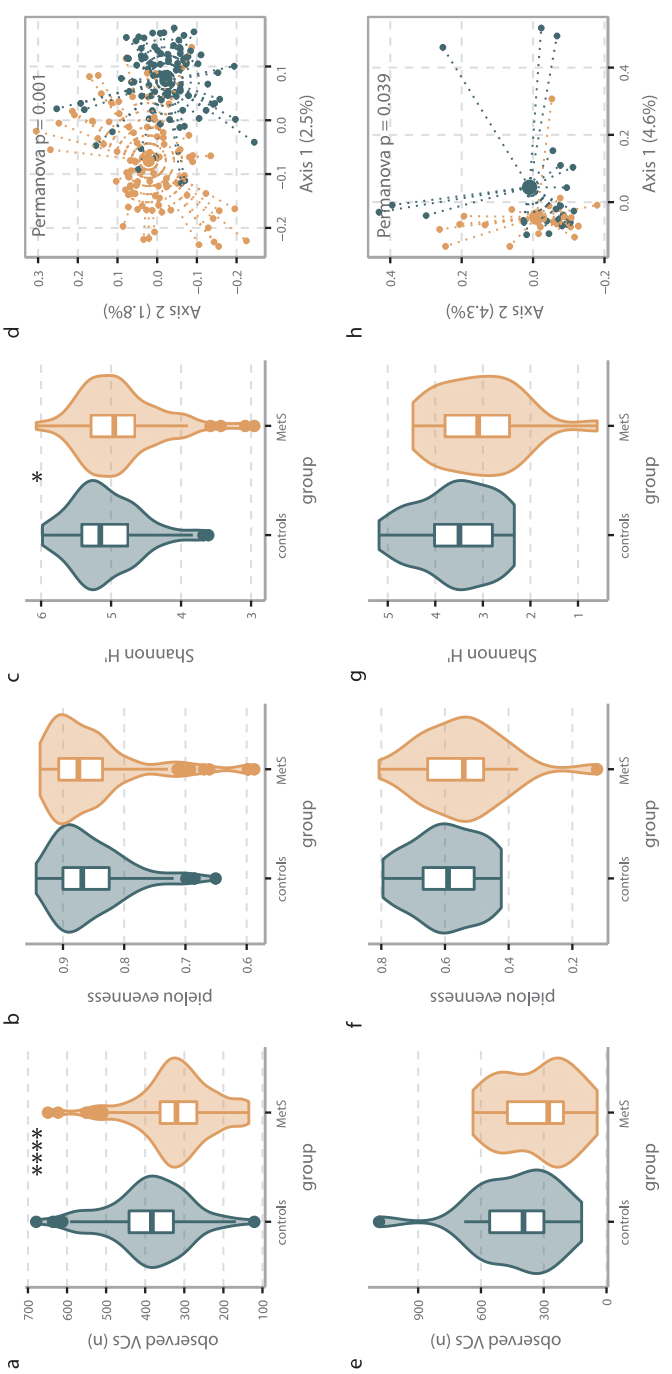


Figure 1: Gut phage populations are altered in MetS.
(a–d) Bulk phage populations measured in WGS samples ($n = 97/n = 99$ biologically independent samples for MetS and controls, respectively), showing: **(a)** MetS-associated decreased species richness is evidenced by the number of unique VCs observed per sample, $p = 7.1 \times 10^{-7}$. **(b)** No change in Pielou evenness measurements, $p = 0.49$. **(c)** Significantly decreased α-diversity measured by Shannon diversity $p = 0.02$. **(d)** Clear separation between populations of MetS (orange) and control (blue) participants as shown by β-diversity depicted in a principal coordinates analysis (PCoA) of Bray–Curtis dissimilarities. **(e–h)** VLP phage populations measured in VLP samples ($n = 24$ biologically independent samples for both MetS and controls), showing no significant difference in **(e)** richness ($p = 0.11$), **(f)** evenness ($p = 0.26$), and **(g)** α-diversity ($p = 0.089$), but **(h)** significantly different populations between MetS (orange) and controls (blue) evidenced by β-diversity. For bulk viromes, Permanova test was adjusted for smoking, age, sex, alcohol use, and metformin use, while analysis of VLP phage populations involved balanced populations that did not need these adjustments. Statistical significance in **(a–c)** and **(e–g)** is according to the two-sided Wilcoxon signed-rank test, where p values are denoted as follows: * ≤ 0.05, ** ≤ 0.01, *** ≤ 0.001, **** ≤ 0.0001. The absence of significance level means p values were above 0.05. Box plots show the median (middle line), 25th, and 75th percentile (box), with the 25th percentile minus and the 75th percentile plus 1.5 times the interquartile range (whiskers), and outliers (single points). Source data are provided as a Source Data file.

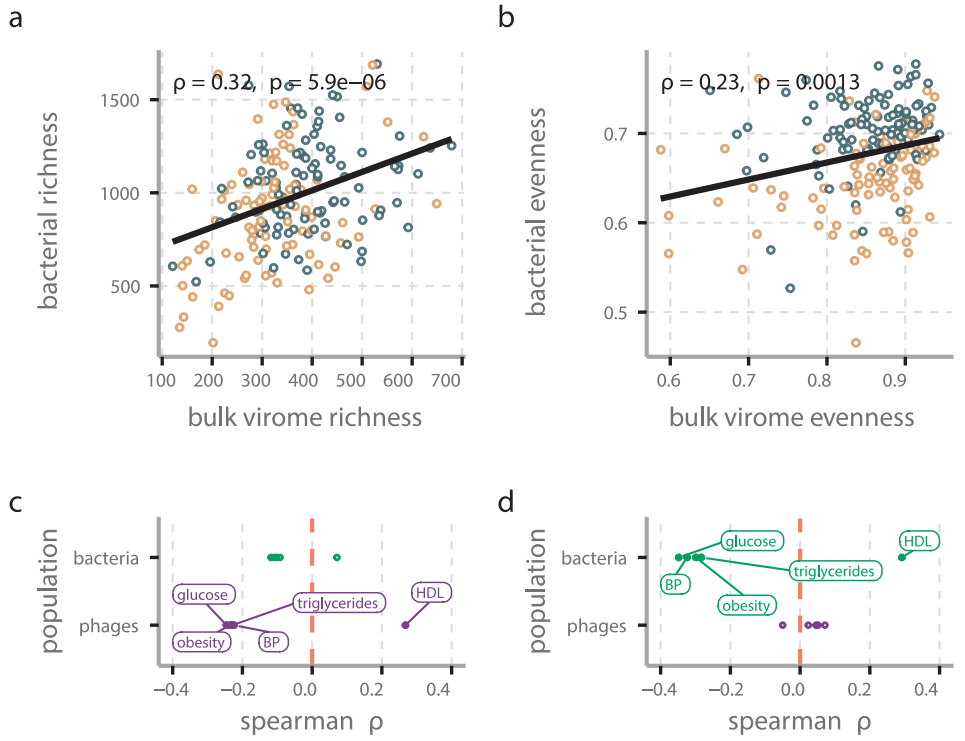


Figure 2: Correlations between phage and bacterial populations as well as between population measures and MetS clinical parameters.
Strong correlations between (**a**) phage richness (observed VCs) and bacterial richness (Chao1 index), as well as between (**b**) phage and bacterial evenness (Pielou's index), both with significant positive two-sided Spearman's rank correlation coefficient. Colours refer to participant groups: MetS (orange) and controls (blue). Both of these measures were correlated to MetS clinical parameters. Plotted are the Spearman's rank correlation coefficients between the five MetS risk factors and (**c**) richness and (**d**) evenness. Points with *q* values below 0.05 are coloured in and labelled. *Q* values were obtained after adjusting *p* values for multiple testing with the Benjamini–Hochberg procedure. Source data are provided as a Source Data file.

Finally, we studied the relationship between both bulk phages and bacteria on the one hand and the five clinical parameters that constitute MetS on the other. As the bacterial and bulk phage populations did not equally decrease in richness and evenness, they also did not equally correlate with MetS clinical parameters. Rather, bulk phage richness was significantly negatively correlated with obesity, blood glucose levels, blood pressure, and triglyceride concentrations but bacterial richness was not ($q < 0.05$, Figure 2c and Supplementary Figure 5). Bacterial evenness, meanwhile, did significantly negatively correlate with these clinical parameters while bulk phage evenness did not ($q < 0.05$, Figure 2d and Supplementary Figure 5). Increasingly severe MetS phenotypes thus result in stronger decreases in bacterial evenness than richness, while bulk phage populations exhibit stronger decreases in richness than evenness. The decreasing bacterial evenness

could be caused by depletion of certain bacterial species in MetS, which results in the bulk phages infecting these depleted bacteria to become undetectable, thereby decreasing richness more than evenness. Otherwise, the success of certain bacterial species could also decrease evenness. In the process this could conceal rare phage species, which could cause the decreased bulk phage richness. Combined with the results showing MetS-associated reduction in total bulk phage abundance and richness, but not those of VLP populations (Supplementary Figure 3), our findings indicate that certain phages are either completely absent from the gut or are too rare to detect in MetS.

Phages infecting select bacterial families are more abundant in MetS viromes

We next studied individual bacterial lineages and the phages that infect them. To do this, we linked viral contigs to bacterial hosts by determining CRISPR protospacer alignments, taxonomies of prophage-containing bacterial sequences, and hosts of previously isolated phages co-clustered in VCs (see methods for details). We found 50,322 host predictions between 7463 VCs (34.1% of all VCs) and 12 bacterial phyla, most commonly *Firmicutes* (5301 VCs) and *Bacteroidetes* (1284 VCs, Supplementary Data 2). We also identified 164 VCs with multi-phyla host range predictions, similar to previous works[32]. To increase statistical accuracy, we selected the predictions between the 12 most commonly occurring host families and 5188 VCs that were present in bulk viromes (23.7% of VCs). We then performed an analysis of compositions of microbiomes with bias correction (ANCOM-BC)[33] on the bulk phage population datasets. This showed higher relative abundances in controls for *Bifidobacteriaceae* ($q = 0.004$), and in MetS for *Bacteroidaceae* ($q = 0.004$), and *Streptococcaceae* ($q = 0.004$, Figure 3a). A complementary analysis of the same 12 families based on 16s rRNA amplicon data showed similar differentially abundance patterns for all three families (Supplementary Figure 6). Notably, the *Ruminococcaceae* and *Clostridiaceae* bacteria were significantly more abundant in controls, while their bulk phages slightly trended toward MetS. This likely indicates that the various species within these families are unevenly predated upon by phages.

We next performed ANCOM-BC on a subset of 2440 VCs that infected within the most abundant host families and for which host predictions were resolved to the species level (Figure 3b). This showed that MetS bulk viromes were dominated by phages infecting *Ruminococcaceae, Clostridiaceae, Bacteroidaceae*, and *Streptococcaceae*. Phages infecting species belonging to the former two families were also differentially abundant among controls, together with those infecting *Bifidobacteriaceae* species. Due to difficulties in taxonomic assignments across metagenomic and 16s rRNA amplicon datasets, we were unable to ascertain whether these specific host species were also differentially abundant in bacteriomes. However, the species found as significantly differentially abundant hosts in

MetS and control bulk viromes largely conformed with previous findings linking these bacteria to either MetS and related diseases or healthy gut microbiomes[34]. Among free-floating viromes, the top 12 most common host families were the same as in the bulk populations, though no host family was differentially abundant in free-floating populations. At the host species level, differential abundance patterns lined up remarkably well to those in the bulk viromes, reflecting how both phage populations mirror each other (Figure 3c).

The findings that *Bacteroidaceae* phages were more abundant in MetS led us to analyze abundance of the widespread *Crassvirales* gut phage order, members of which infect in this family[35,36]. Notably, while *Crassvirales* phage relative abundance did not significantly differ

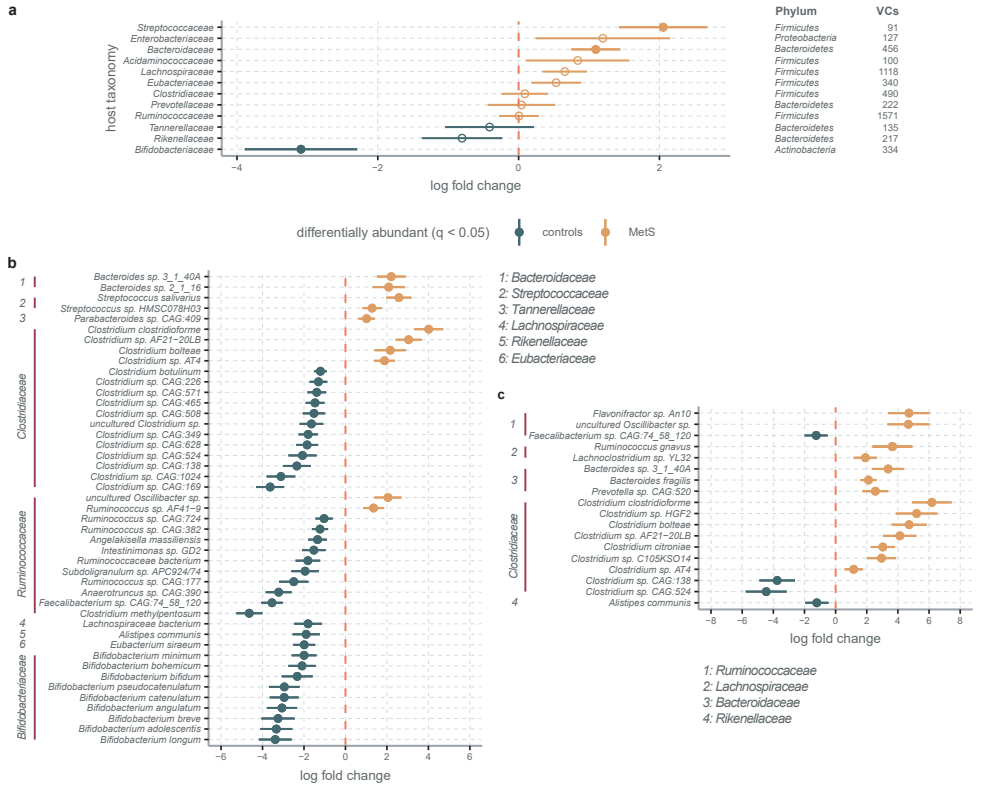


Figure 3: Phages infecting selected bacterial families are differentially abundant in MetS or healthy controls. (**a**) ANCOM-BC[33] analysis of bulk phages that infect the 12 bacterial families to which the most VCs were linked shows significant association between *Bifidobacteriaceae* VCs and controls, as well as between *Streptococcaceae* and *Bacteroidaceae* VCs and MetS. Closed circles denote significance, open circles lack of significance. (**b**) ANCOM-BC of bulk phages infecting the families depicted in (**a**) and with host predictions at the species level. (**c**) Same as (**b**) for VLP phages. For (**a**) and (**b**), $n = 97/n = 99$ biologically independent samples for MetS and controls, respectively. For (**c**), $n = 24$ biologically independent samples for both MetS and controls. Points show the log fold change as given by ANCOM-BC, error bars denote the standard error adjusted by the Benjamini–Hochberg procedure for multiple testing. In (**b**) and (**c**) only, significant species are shown ($q < 0.05$) for brevity. Source data are provided as a Source Data file.

between MetS and controls in either free-floating or bulk phage populations, they were significantly more prevalent in control bulk viromes (prevalence controls: 78/99 participants, MetS: 58/97, Fisher's exact test, $p = 0.005$). This apparent depletion of *Crassvirales* phages in MetS bulk viromes may indicate a decrease in their infectiousness, and is to our knowledge the first link observed between this prominent human gut phage order and a disease state. Alterations to *Crassvirales* phage composition may thus occur at an individual level.

***Bacteroidaceae* VCs are markers of the MetS virome**

The above results all indicate that MetS gut bulk viromes are distinct from those in healthy individuals. In light of this, we surveyed our cohort with ANCOM-BC for individual VCs that were correlated with bulk viromes in either MetS or healthy controls. This uncovered thirty-six VCs that were more abundant in MetS participants, and sixteen more in controls ($q \leq 0.05$, Figure 4a).

In line with the above findings that *Bacteroidaceae* VCs are hallmarks of the MetS bulk virome, six of the seventeen MetS-associated VCs with a positive host prediction infected this family. One of these (VC_1838_0) contained a non-prophage contig (*i.e.*, no detected bacterial contamination) of 34,170 bp with a checkV[29] completion score of 100%. It further co-clustered with a contig that checkV identified as a complete prophage flanked by bacterial genes. Analysis with the contig annotation tool (CAT[37]) identified this contig as *Bacteroides fragilis*. Additionally, the most complete VC_1838_0 contig shared 6/69 (8.7%) ORFs with *Bacteroides uniformis Siphoviridae* phage Bacuni_F1[38] (BLASTp bit score ≥ 50). Besides this, none of the contigs shared marked homology with any isolated phages found in the NCBI nucleotide databases (nr/nt). Some of them did, however, show significant similarity (BLASTn bit score ≥ 50) to phage genomes from an earlier publication by Tisza et al.[39] studying a large phage database in relation to various diseases. Most notably, the largest contig from VC_977_13 (checkv completeness 90.32%) was identical over 99.98% of its genome to a phage that Tisza et al. determined to be significantly associated with fatty liver and atherosclerosis, both diseases related to MetS. We found similar results (with 78% aligned nucleotides from a complete genome) for *Bacteroidaceae* VC_1838_0, of which the most similar Tisza et al. genome was related to atherosclerosis and cirrhosis, as well as for VC_1221_0 (with 62% aligned nucleotides from an 83% complete genome), where relations to atherosclerosis and obesity were found. These disease correlations from independent cohorts support our findings linking these *Bacteroidaceae* VCs to MetS.

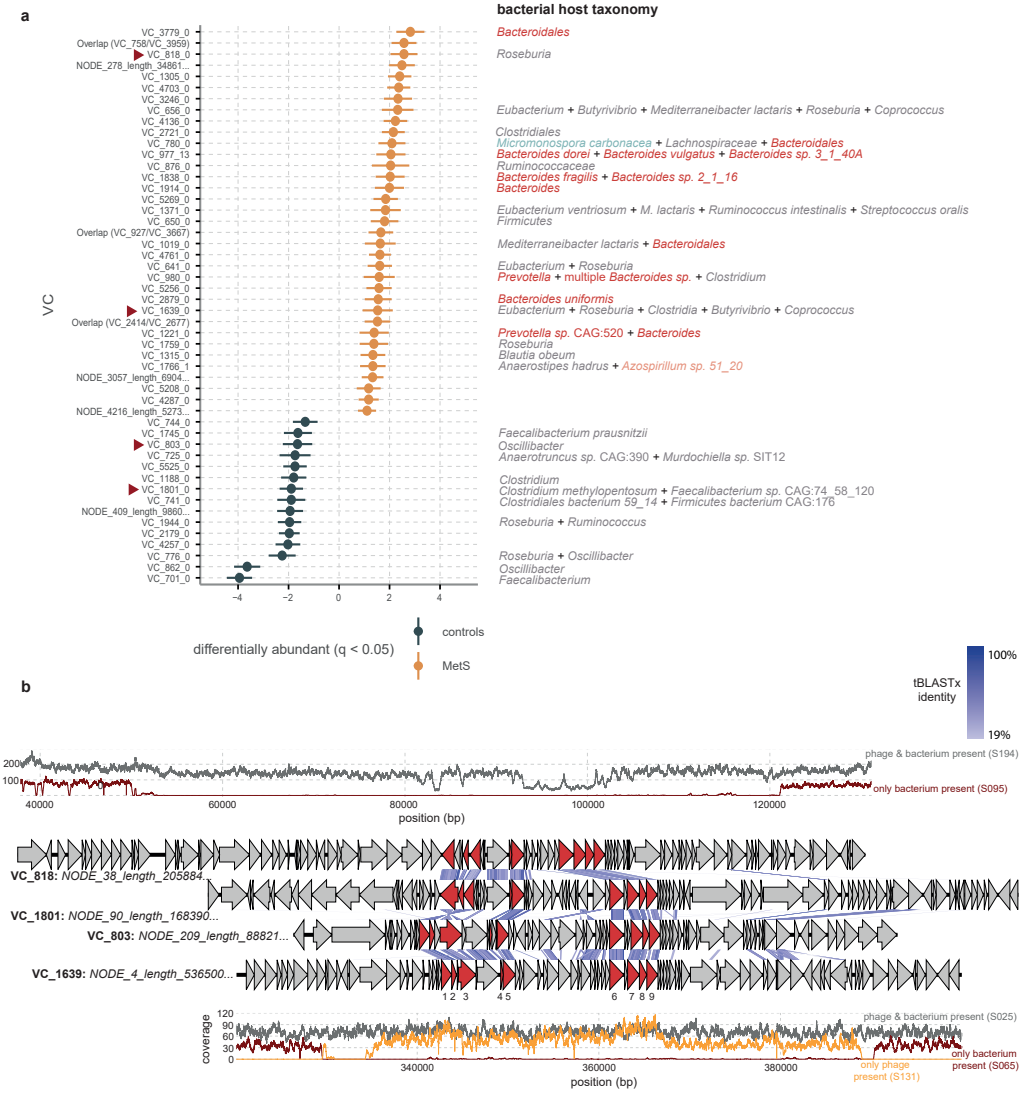


Figure 4: Among significantly differentially abundant VCs some are related.
(**a**) VCs identified by ANCOM-BC as significantly abundant ($q \leq 0.05$ after implementing the Benjamini–Hochberg procedure for multiple testing). Points show the log fold change as given by ANCOM-BC, error bars denote the standard error adjusted by the Benjamini–Hochberg procedure for multiple testing. The analysis was adjusted for smoking, age, sex, alcohol use, and metformin use. Red arrows mark related VCs further depicted in b. Taxonomic names to the right of the plot denote host predictions, which are colored as follows: *Firmicutes;* gray, *Bacteroidetes;* red, *Actinobacteria*; green, *Proteobacteria*; pink. The full taxonomies are listed in Supplementary Data 1 and 3. $n = 97/n = 99$ biologically independent samples for MetS and controls, respectively. (**b**) Whole-genome analysis of four contigs that belong to the VCs marked by red arrows in a. The top and bottom contigs are zoomed in on the prophage region. The read coverage depth of these contigs in samples where they are present/absent is depicted in the graphs at the top and bottom. The nine genes shared by all *Candidatus Heliusviridae* are colored red, and numbered as follows: 1: DUF2800-containing, 2: DUF2815-containing, 3: DNA polymerase I, 4: nuclease (VRR-NUC-containing), 5: SNF2-like helicase, 6: terminase large subunit, 7: portal protein, 8: Clp-protease, 9: major capsid protein. Source data are provided as a Source Data file.

A widespread phage family contains markers for healthy and MetS viromes

Besides the above-mentioned *Bacteroidaceae* VCs, all other differentially abundant VCs with host links, twenty-six MetS- and nine control-associated, infected *Firmicutes*, particularly in the *Clostridiales* order. The sole exceptions to this remarkably had CRISPR protospacer matches to multiple phyla: either *Firmicutes* and *Proteobacteria*, *Fimicutes* and *Bacteroidetes*, or *Firmicutes*, *Bacteroidetes* and *Actinobacteria* (Figure 4a). Though this might result from taxonomically closely related phages that infect taxonomically distant hosts, we also observed one genome fragment in VC_1766_1 that had CRISPR spacer hits from hosts in multiple phyla. This indicated that this may be a phage with an extraordinarily broad host range.

Besides this broad host range VC, our attention was drawn to MetS-associated *Clostridiales* VC_818_0 and VC_1639_0. Both were predicted to infect hosts from Clostridium clusters IV and XIVa[40], which are usually associated with healthy gut microbiomes. Further examination of their largest genomes revealed that they were remarkably similar to each other and to two VCs that were significantly associated with healthy controls: *Faecalibacterium/Clostridium methylopentosum* VC_1801_0 and *Oscillibacter/Ruminococcaceae* VC_803_0 (Figure 4b).

Intrigued by this apparent relatedness of VCs that included markers of MetS and healthy controls among our cohort, we sought to identify additional related sequences among our cohort. For this, we first determined the exact length of a full VC_818_0 genome by analyzing read coverage plots of a prophage flanked by bacterial genes (Figure 4b). By analyzing coverage of the contig in subjects where bacterial genes were highly abundant but viral genes were absent, we extracted a genome of 68,665 bp long. Homology searches of all 74 ORFs encoded by this prophage against all ORFs from all phage contigs in the cohort identified 261 contigs of over 30,000 bp that all shared nine genes (BLASTp bit score ≥ 50, Figure 4b), including thirteen assembled from VLP datasets. Additionally, we identified 61 *Siphoviridae* phage genomes in the National Center for Biotechnology Information (NCBI) nucleotide database that also shared these nine genes. With one exception, these were *Streptococcus* phages, the exception being *Erysipelothrix* phage phi1605.

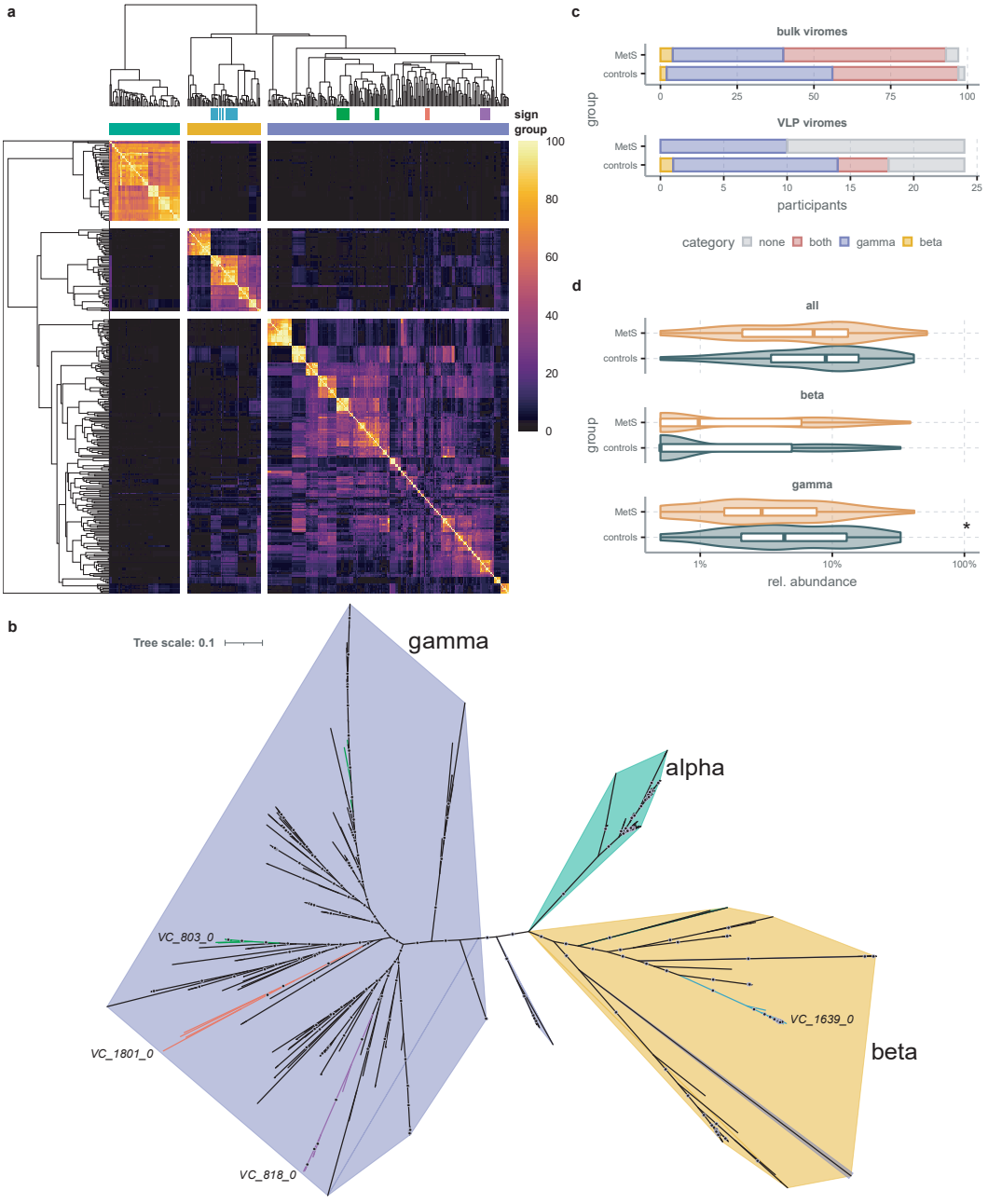
The genes shared by all these phage genomes formed three categories. First are genes encoding structural functions: a major capsid protein, portal protein, CLP-like prohead maturation protease, and terminase. The second group are transcription-related genes encoding a DNA polymerase I, probable helicase, and nuclease. Finally, there are two genes that encode domains of unknown function, but which given their adjacency to the second group are likely transcription-related.

Earlier studies have used a cutoff of 10% gene similarity for phages that are in the same families, 20% for subfamilies, and 40% for genera[41,42], while the international committee for the taxonomy of viruses (ICTV) proposes that phages that form a monophyletic group and share a significant number of genes constitute a family[43]. The nine shared genes form 10-25% of ORFs found on both the characterized phages and non-provirus contigs with checkV 'high-quality' designations. We thus tentatively classify these phages as a family, which we dubbed the *Candidatus Heliusviridae*. Next, we further studied the interrelatedness of *Ca. Heliusviridae* phages by performing pairwise blastp searches for all genes. The resulting bit-score table was then used to form protein clusters[27], from which we calculated the pairwise percentages of shared protein clusters. Hierarchical clustering of the results showed that *Ca. Heliusviridae* phages form three groups (Figure 5a). As the complete genomes in these groups shared less than 70% average nucleotide identity across their genome (median: 28.9%, 48.7%, and 21.8%, Figure 5a), and following proposed guidelines[43], these clusters form subfamilies. We thus designated them the *alphaheliusvirinae, betaheliusvirinae*, and *gammaheliusvirinae*. We confirmed these findings by building a concatenated approximate maximum-likelihood phylogenetic tree from alignments of nine conserved *Ca. Heliusviridae* genes. This also showed three main clades that almost completely aligned with the three groups based on shared protein cluster content (Figure 5b, Supplementary Data 6 and 7).

Members of the *Ca. Heliusviridae* were present in the bulk phage populations of 190/196 participants (96.9%), 97 controls and 93 MetS participants (Figure 5c). Among datasets of VLP phage populations, *Ca. Heliusviridae* phages were found in 25/48 participants (52.1%), 16 controls and 9 MetS, thus precluding the notion that they are defective prophages. It furthermore revealed that this phage family is a part of the core human gut microbiome. To validate our findings, we used three independent cohorts: the phage database constructed

Figure 5: Three VCs that are hallmarks for either MetS or healthy control viromes are part of the widespread *Candidatus Heliusviridae* family of gut phages.
(**a**) heatmap and hierarchical clustering of pairwise shared protein cluster values for 261 contigs from the current study and 61 previously isolated phages that all shared the same nine core *Ca. Heliusviridae* genes (blastp > 50). The dendrogram is cut to form three clusters, which are color coded above the heatmap as *Ca. alpha-* (green), *beta-* (yellow), and *gammaheliusvirinae* (purple). The top row of colors beneath the dendrogram denote the differentially abundant VCs, from left to right: VC_1639_0 (blue), VC_803_0 (green), VC_1801_0 (red), and VC_818_0 (purple). The legend denotes percent of total protein clusters that are shared. As some core genes formed several protein clusters, values can be below 10%. (**b**) An unrooted approximate maximum-likelihood tree built from a concatenated alignment of nine genes shared by all genomes in (**a**), with colors defining subfamily membership according to (**a**), and with the VCs significantly differentially abundant in either MetS or controls denoted. Dots on tree branches signify bootstrap values ≥95. (**c**) the prevalence of the *Candidatus Heliusviridae* groups among bulk and VLP phage populations. (**d**) The relative abundances of the *Candidatus Heliusviridae* and the groups in bulk phage populations. $n = 97/n = 99$ biologically independent samples for MetS and controls, respectively. *Q* values are denoted as follows * ≤ 0.05, ** ≤ 0.01, *** ≤ 0.001, **** ≤ 0.0001. Box plots show the median (middle line), 25th, and 75th percentile (box), with the 25th percentile minus and the 75th percentile plus 1.5 times the interquartile range (whiskers), and outliers (single points). Source data are provided as a Source Data file.

by Tisza et al. mentioned above[39] and one cohort each studying gut virome relations to hypertension[44] and type 2 diabetes[11]. To allow for incomplete assemblies, we searched for contigs in these three cohorts that contain the four conserved *Ca. Heliusviridae* structural genes. A phylogenetic tree containing concatenated alignments of the structural genes revealed two things. First, it clearly showed that contigs from all validation cohorts were



interspersed among both *Ca. beta- and gammaheliusvirinae*. Second, the presence of divergent clades which did not contain any of the genomes in which earlier we identified all nine characteristic *Ca. Heliusviridae* genes hinted at further extensive diversity of the phage family (Supplementary Figure 6). Among the gut viromes from an earlier cohort composed of school-aged children, of which 10 were controls, 10 were obese, and 8 had MetS, we further found Ca. Heliusviridae in 7/10 controls, while among obese and MetS they were present in 4/10 and 4/8, respectively.

Among the two cohorts studying hypertension and type 2 diabetes, *Ca. Heliusviridae* phages were present in 137/196 (69.9%, hypertension) and 98/145 (67.6%, T2D) participants (Supplementary Figure 8). Meanwhile, for the 775 contigs with the four *Ca. Heliusviridae* structural genes, Tisza et al. previously determined the prevalence in the human microbiome project[45]. The data pertaining to this provided by Tisza et al. indicated that three individual *Ca. Heliusviridae* genomes found among their phage database were present in over 50% of human microbiome project participants, of which two had a prevalence of over 80%. Thus, not only are *Ca. Heliusviridae* phages as a family widespread in the human microbiome, several individual phage strains within it may be highly prevalent. In addition to prevalence, Tisza et al. also tested links between phages and various disease states. Among the *Ca. Heliusviridae* phages derived from this database, we found 74 that were previously significantly linked to obesity, and a further 82 related to various other cardiovascular diseases (non-alcoholic fatty liver/steatohepatitis, atherosclerosis, and type 2 diabetes). Our findings relating *Ca. Heliusviridae* phages to MetS are thus in line with findings relating to the Tisza et al. phage database.

***Ca. Heliusviridae* subfamilies have distinct relations to MetS**

The *Ca. alphaheliusvirinae* solely contained previously isolated *Streptococcus* phages, which both in the hierarchical clustering and the phylogenetic tree were distinct from the other genomes. Meanwhile, three of the four VCs that were significantly associated with either MetS (1) or controls (2) where part of the *Ca. gammaheliusvirinae*, by far the largest and most diverse group. Two of these, VC_818_0 and VC_1801_0, formed monophyletic clades in both hierarchical clustering and phylogenetic tree. Meanwhile, VC_803_0 was conversely spread out over multiple clades, indicating it was more heterogenous than the other two.

Of the subfamilies, phages in the *Ca. gammaheliusvirinae* were the most prevalent, being present in the bulk phage populations of 95 controls and 88 MetS participants. These phages were also significantly more abundant in the controls (Wilcoxon signed-rank test, $p = 0.011$, Figure 5d) as a whole, despite the fact that in contains the MetS-associated VC_818_0. Among VLP populations, we also identified them in 15/24 controls and 9/24

MetS participants, though there was no significant difference in abundance. The bacterial hosts of these phages were predicted to be within various families in the *Clostridiales*, as well as the *Veillonellales, Coriobacteriales*, and *Acidaminacoccales*.

While less prevalent than *Ca. gammaheliusvirinae* phages, *Ca. betaheliusvirinae* phages were still identified in the bulk phage populations of 44 controls and 57 MetS participants (Fisher's exact test $p = 0.047$, Figure 5c), though they were not significantly more abundant in the latter (Wilcoxon signed rank test, $p = 0.063$). Remarkably, *Ca. Betaheliusvirinae* phages were completely absent from MetS VLP phage populations whereas they were present in 6/24 controls, making the difference in prevalence significant (Fisher's exact test $p = 0.022$). These results show that Ca. *Heliusviridae* phages are part of both the core human gut bulk and VLP viromes. Counter to *Ca. gammaheliusvirinae*, all host predictions of *Ca. Betaheliusvirinae* phages were within the *Clostridiales*. In summary, *Ca. Gammaheliusvirinae* is the largest and most prevalent subfamily of *Ca. Heliusviridae* phages, which as a whole is more related to the healthy human virome, while *Ca. Betaheliusvirinae* phages are more prevalent in MetS bulk viromes but depleted among VLP populations.

MetS-associated *Ca. gammaheliusvirinae* prophages encode possible metabolic genes

Members of the *Ca. Heliusviridae* are generally linked to bacteria that are associated with healthy human gut microbiomes. It is thus an apparent contradiction that *Ca. Heliusviridae* VC_818_0 (*Ca. gammaheliusvirinae*), which is associated with MetS viromes, contains phages that infect *Roseburia*, which is a short chain fatty acid producer and is often abundant in healthy microbiomes[46]. Due to this contradiction, we explored the phages in this VC further. These included two additional prophages, which where both incomplete (Figure 6a, Supplementary Data 4). Whole-genome alignment showed that all three prophages shared their phage genes, and that the two incomplete ones also shared host-derived genes. Homology searches of the bacterial host ORFs found on these two contigs against the NCBI nr database (BLASTp, bit score ≥50) showed that the most common top hits were *Blautia*, and for the plurality *Blautia wexlerae* (Figure 6a). Thus, VC_818_0 likely contains temperate phages with narrow host ranges that infect bacteria spread out across at least two genera within the *Lachnospiraceae*.

To examine if the hosts infected by VC_818_0 phages were more abundant in MetS participants, we determined mean coverage of bacterial genes found adjacent to the prophages. We thus assured that we analyzed the particular host strains infected by these phages, rather than unrelated strains in the same genera. This showed that both the *Blautia* and the *Roseburia* host genes were more abundant among MetS participants (Wilcoxon signed-rank test, *Blautia* $p = 5.1 \times 10^{-4}$, *Roseburia* $p = 0.042$, Figure 6b and c). The

specific *Lachnospiraceae* strains infected by VC_818_0 phages thus seem to thrive in MetS microbiomes. This could in part be due to functions conferred upon these bacteria by these prophages, as particularly the *Roseburia* prophage which carried several virulence- and metabolism-related genes, including ones encoding a chloramphenicol acetyltransferase 3 (2.3.1.28), Glyoxalase/Bleomycin resistance protein (IPR004360), multi antimicrobial extrusion protein (IPR002528), 2-succinyl-6-hydroxy-2,4-cyclohexadiene-1-carboxylate synthase (4.2.99.20), and NADPH-dependent FMN reductase (PF03358). The latter two in particular are both associated with vitamin K (menaquinone) metabolism, which is part of (an)aerobic respiration in bacteria[47]. We speculate that this opens up the possibility that this *Roseburia* prophage aids its host bacterium, which in turn may contribute to MetS phenotypes.

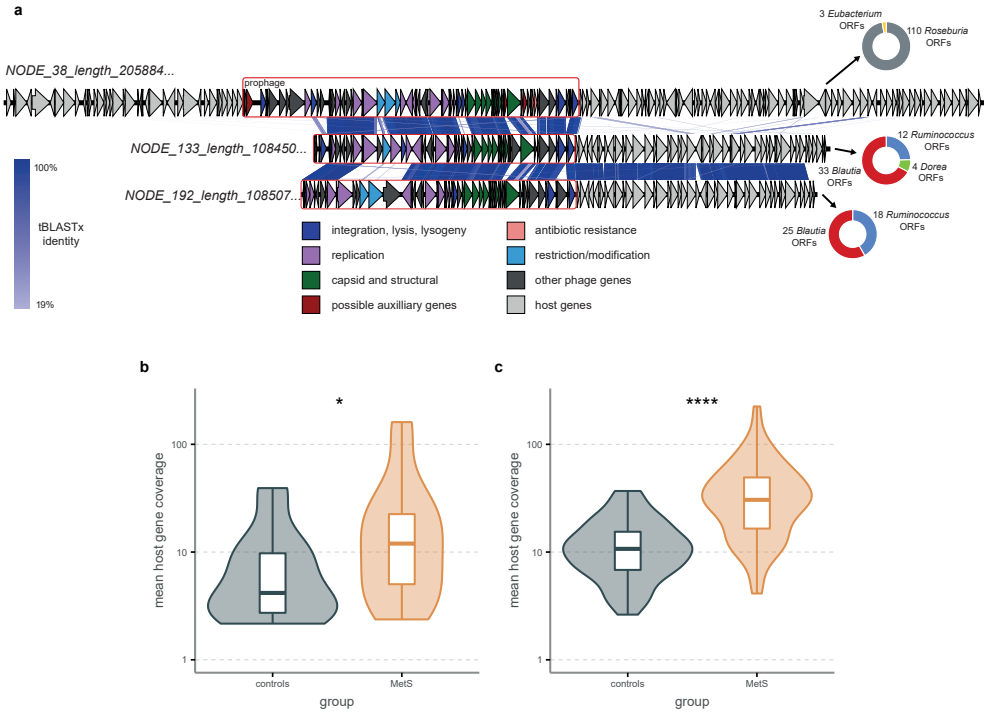


Figure 6: VC_818_0 infects *Roseburia* and *Blautia*, and carries possible auxiliary metabolic genes.
(**a**) Whole-genome alignment of three prophages contained within VC_818_0, with pie charts denoting the top BLASTp hit of all host genes on the contigs. The mean coverage of host-derived regions in NODE_38 ($p = 0.042$) (**b**) and NODE_192 ($p = 5.1 \times 10^{-4}$) (**c**). $n = 97/n = 99$ biologically independent samples for MetS and controls, respectively. Significance according to two-sided Wilcoxon signed-rank test, p-values are denoted as follows * ≤ 0.05, ** ≤0.01, *** ≤ 0.001, **** ≤ 0.0001. Box plots show the median (middle line), 25th, and 75th percentile (box), with the 25th percentile minus and the 75th percentile plus 1.5 times the interquartile range (whiskers), and outliers (single points). Source data are provided as a Source Data file.

DISCUSSION

This is the first study of adult gut viromes in the context of MetS, a widespread global health concern to which the gut bacteria targeted by phages are believed to be a main contributor[18]. We have shown that MetS is associated with decreases in gut bulk virome total relative abundance and richness, but not in evenness. Due to their compositional nature, these virome alterations could be bacterially driven, as phage total relative abundance decreases could be caused by bacterial counts increasing rather than phage counts decreasing. But since we measured decreased bacterial richness and evenness, MetS gut metagenomes would need to have larger numbers of bacterial cells that are distributed among fewer strains that are more unevenly divided than in healthy individuals. Conversely, total phage relative abundances could be lower in MetS due to lower viral loads, which would be in line with decreased phage richness and is in agreement with recently reported direct correlations between gut viral and bacterial populations in healthy individuals[48]. Future confirmation of this would necessitate counts of viable bacterial cells and VLP. In either case, we surmise that the main driver of these effects is diet, which affects bacterial[49-51] as well as viral[52] populations. It is also possible that phage populations as described here may further exacerbate bacterial diversity losses, as low phage abundance may decrease their positive effects on bacterial diversity[53,54]. Our findings of increased richness and diversity in the bulk viromes were in line with a recent study of MetS among 28 school-aged children[23]. Interestingly, their results pertained to VLP datasets, which in our study showed no significant differences in richness and diversity. This could reflect the difference in cohort size, as we analysed double the number of participants, or the previously reported changes in the gut virome with increasing age[14].

We further found strong negative correlations between the risk factors that constitute MetS and bulk phage richness, but not evenness. This likely stems from the nature of bulk viromes, which reflect phages that are actively engaging with their hosts. As phages that target depleted bacteria are more likely to be low in abundance and extracellular, they are not observed among bulk viromes. Thus, the apparent species richness drops because low abundant extracellular phages are below the detection limit of our sequencing approach. This removal of rare phages in turn prohibits significant drops in species evenness in MetS. It could also be that bacteria depleted in MetS reside in phage-inaccessible locales within the gut[55], which perhaps results in removal of the corresponding phages from the gut to below detectable levels. This would explain the stronger correlation between bacterial evenness than richness to MetS risk factors.

As most (gut) phages remain unstudied[14,56], it is often difficult to link phages to host bacteria[57]. Here, we linked roughly one third of all VCs to a bacterial host. The remaining

majority of VCs likely represent phages that infect bacterial lineages lacking CRISPR systems[58], or that integrate into hosts which we could not taxonomically classify. Whichever is the case, our study underscores the great need for methods that link phages to hosts with high accuracy[59,60]. From the phage-host linkages that we obtained, we found that VCs containing phages infecting specific bacterial families tend to be either depleted (*Bifidobacteriaceae)* or enriched (*Streptococcaceae* and *Bacteroidaceae*) in tandem to their hosts. We notably found that several other bacterial families (*Enterobacteriaceae, Lachnospiraceae, Ruminococcaceae, Rikenellaceae*, and *Clostridiaceae*) were either significantly depleted or elevated in MetS microbiomes, but the accompanying phages were not. Though this could reflect an unevenness in predation by phages among the various bacterial families in the gut, it more likely results from the inability to link the majority of VCs to bacterial hosts, as mentioned above.

The identification of *Bifidobacteriaceae* bacteria and their phages as more abundant among healthy controls is in line with established studies that show depletion of these families in MetS[22] and MetS-associated disease states[34]. Phages infecting both the *Bifidobacteriaceae* as a whole and specific *Bifidobacteria* species were strikingly only elevated in abundance among bulk viromes. Their absence among VLP populations may imply a preference of *Bifidobacteriaceae* gut phages toward intracellular lifestyles. This in turn could explain the dearth in isolated virulent *Bifidobacterium* phages when compared to other *Actinobacteria* lineages[61]. For the MetS-associated host families, *Streptococcaceae* are known to be more abundant in obesity-related ilnesses[34]. Within the *Bacteroidaceae*, the *Bacteroides* are often positively associated with high-fat and high-protein diets[62,63]. Simultaneously, however, reports disagree on individual *Bacteroides* species and their associations with MetS-related diseases like obesity, type 2 diabetes, and non-alcoholic fatty liver disease[34]. Such conflicting reports likely reflect the large diversity in metabolic effects at strain level among these bacteria[64]. Based on our results, we drew two conclusions. First, that *Bacteroidaceae*-linked VCs mirror their hosts in MetS-associated relative abundance increase, and second that *Bacteroidaceae*-linked VCs are of significant interest to studies of the MetS microbiome. The latter conclusion is strengthened by findings that *Bacteroides* prophages can alter bacterial metabolism in the gut[8].

While *Bacteroidaceae* VCs at large were thus seemingly associated with MetS phenotypes, we did not find higher abundance of *Crassvirales* phages in MetS. However, we did find higher prevalence of these phages in the bulk viromes of healthy controls. This widespread and often abundant human gut phage family infects *Bacteroidetes*, including members of the *Bacteroidaceae*[65,66]. As these phages are commonly linked to healthy gut microbiomes[42,66,67], it is conceivable that they would be negatively correlated with MetS viromes. But due to the great variety within this family[66], and perhaps also the hypothesized

aptitude of *Crassvirales* phages for host switching through genomic recombination[66], more detailed study is needed to elucidate the exact links of this family to MetS gut viromes despite the apparent elevated abundance of their hosts.

Finally, our study revealed the *Candidatus Heliusviridae*, a highly widespread family of gut phages that largely infect *Clostridiales* hosts. This prospective family is also expansive, and includes at least three distinct groupings. Our uncovering of this human gut phage family underscores the usefulness of database-independent de novo sequence analyses[27,30,68], as well as the need for a wider view on viral taxonomy than has presently been exhibited in the field of gut viromics.

The *Ca. Heliusviridae* are of particular interest to studies of MetS and related illnesses because its member phages include some associated with MetS and others with healthy controls. Most striking is the fact that most of the bacteria infected by MetS-associated *Ca. Heliusviridae* phages are generally producers of short chain fatty acids (SCFA) such as butyrate and commonly depleted in MetS[34]. Such SCFA-producing bacteria are commonly positively associated with healthy microbiomes, as SCFAs that result from microbial digestion of dietary fibres have a role in the regulation of satiation[69,70]. The exception to this is the *Veillonellaceae* that is infected by a phage the *Ca. gammaheliusvirinae*, which displays elevated abundance in non-alcoholic fatty liver disease[34]. While higher abundance of some of the other butyrate-producers infected by *Ca. Heliusviridae* phages is associated with metformin use[71], this is used to treat type 2 diabetes rather than MetS.

Particularly interesting are the *Roseburia/Blautia* phages in VC_818_0, which was the most strongly correlated with MetS out of all VCs. The positive correlation between the relative abundance of these phages and that of their hosts indicates that they have a stable relation with their hosts in the MetS microbiome. This is to be expected, as large-scale prophage induction is generally associated with sudden alterations to the microbiome, such as the addition of a specific food supplement that acts as an inducer of prophages[9]. Such sudden alterations in phage behaviour are unlikely to be captured in large cohorts with single measurements. In fact, as phages are strongly dependent on their host, one might expect the abundance of many gut phages to be positively correlated to that of their particular hosts under the relatively temporally stable conditions of MetS. The strong correlation of VC_818_0 to MetS phenotypes, coupled to the commonly found correlation to healthy microbiomes of VC_818_0 host bacteria, and the presence of potential auxiliary metabolic genes in VC_818_0 phage sequences combined introduce the possibility that prophage formation of these *Ca. Heliusviridae* phages alters the metabolic behaviour of their host bacteria, as is known to happen in marine environments[72,73]. This could make these bacteria

detrimental to health. Proving this hypothesis necessitates future isolation of VC_818_0 phages.

Despite efforts to catalogue the human gut virome[14,32], taxonomically higher structures are still largely absent. This study shows the worth of analysing phages at higher taxonomic levels than genomes or VCs, similarly to what has been shown in recent years regarding the *Crassvirales* phage order[15,16]. Unlike the *Crassvirales*, however, *Ca. Heliusviridae* phages seem to be strongly correlated with human health. We hope that further research will provide a deeper understanding of the effect that these phages have on their bacterial hosts and the role that this plays in MetS, as well as a refinement of their taxonomy.

METHODS

Whole-genome shotgun sequencing

The Healthy Life in an Urban Setting (HELIUS) cohort includes some 25,000 ethnically diverse participants from Amsterdam, the Netherlands. The cohort details were published previously[26]. The HELIUS cohort conformed to all relevant ethical considerations. It complied with the Declaration of Helsinki (6th, 7th revisions), and was approved by the Amsterdam University Medical Centres Medical Ethics Committee. All participants provided written informed consent. For details on stool sample collection from among the participants, their storage, and DNA extraction, see Deschasaux et al.[74]. In summary, participants were asked to deliver stool samples to the research location within 6 h after collection with pre-provided kit consisting of a stool collection tube and safety bag. If not possible, they were instructed to store their sample in a freezer overnight. Samples were stored at the study visit location at −20 °C until daily transportation to a central −80 °C freezer. Total genomic DNA was extracted using a repeated bead beating method described previously[74,75]. Libraries for shotgun metagenomic sequencing were prepared using a PCR-free method at Novogene (Nanjing, China) on a HiSeq instrument (Illumina Inc. San Diego, CA, USA) with 150 bp paired-end reads and 6 Gb data/sample. All bioinformatics software was run using standard settings, unless otherwise stated.

Following previously set definitions[76], participants were classified in the MetS group if three of the following five health issues occurred: abdominal obesity measured by waist circumference, insulin resistance measured by elevated fasting blood glucose, hypertriglyceridemia, low serum high-density lipoprotein (HDL), and high blood pressure[76]. All participants of the HELIUS cohort reside in Amsterdam, the Netherlands. Participants were roughly evenly divided by ethnicity, with European Dutch comprising 49 controls and 49 MetS participants, and African Surinamese 50 controls and 49 MetS participants. The MetS group contained 55 women and had a median age of 58 (mean 56.8 ± 8.09), and the

controls 71 and had a median age of 50 (mean 49.1 ± 12). Of the 196 participants, 26 used metformin, of whom 2 were controls who did not concur to the MetS criteria.

VLP isolation and DNA extraction

To gain a full understanding of the dsDNA virome in the current cohort, we performed viral-like particle (VLP) sequencing on faecal matter from a subset of 48 participants. These included 24 controls and 24 MetS participants, with each group being composed of 12 European Dutch and 12 African Surinamese persons. This sub-selection was balanced for age (controls 55.9 ± 8.47, MetS 58.7 ± 7.05, Wilcoxon signed-rank test, $p = 0.27$) and sex (controls 14 women, MetS 14 women).

Studies of the VLP fractions were modelled after Garmaeva et al.[77] and Shkoporov et al.[78]. First, 0.5 g of faeces were resuspended in 5 ml of sterile SM buffer (100 mM NaCl, 8 mM $MgSO_4 \times 7H_2O$, pH 7.5), chilled on ice for 10 min and centrifuged at 27,000 × *g* for 10 min at 4 °C. Supernatant was collected and filtered through a 0.45 µm pore polyethersulfone membrane filter, whereafter the volume of the filtrate was adjusted to 5 ml. Next, free DNA was digested by incubating the VLPs with 5 µl 2.5 U/µl of DNase I (ThermoFisher Cat#R0561) and 555 µl of 10× DNase buffer at 37 °C for 1 h. VLPs were lysed by the addition of 100 µl of 100 mg/ml SDS (Invitrogen Cat#1.5525.017) and 2.5 µl of 20 mg/ml proteinase K (Promega Cat#MC5005) to the samples, which were incubated at 56 °C for 1 h.

Nucleic acids were purified using a two-step phenol/chloroform extraction protocol. First, samples were extracted by mixing with an equal volume (5.7 ml) of phenol/chloroform/isoamyl alcohol 25:24:1 (Sigma Cat#77617) followed by centrifugation at 4000 × *g* for 10 min at room temperature. Subsequently, 5.2 ml of the aqueous upper phase was mixed with an equal volume of chloroform (Merck Cat#102445) and again centrifuged as described above. To precipitate the nucleotides, 4.7 ml of aqueous phase was mixed with 470 µl 3 M sodium acetate (pH 5.2), 4.7 µl glycogen (ThermoFisher Cat#R0561) and 14.2 ml ice-cold absolute ethanol (Merck Cat#100983) and incubated at −20 °C for 1–2 h. Samples were centrifuged at 21,000 × *g* for 15 min at 4 °C, after which the pellet was washed with 500 µl 70% ethanol. After air drying the pellet for ~20 min, the pellet was resuspended in 500 µl ultrapure RNase/DNase-free water (ThermoFisher Cat#10977-035). The resulting solution was subjected to a final round of purification using the DNeasy Blood&Tissue kit (Qiagen Cat#69506) according to the manufacturer's protocol, with a final elution volume of 100 µl.

Metagenomic sequencing of VLP DNA

Next, library preparation was performed using the NEBNext Ultra II FS DNA library prep kit (New England Biolabs Cat#E7805L), complemented with the NEBNext Multiplex Oligos for

Illumina (New England Biolabs Cat#E7600S) dual indexes according to the manufacturer's protocol. Fragmentation with the FS enzyme mix was performed for 5 minutes and the NEB adapters for Illumina were diluted 10 times to prevent dimer formation due to the low input DNA concentrations. After adapter ligation, DNA fragments of 300–500 bp were purified and subsequently amplified with 10 PCR cycles during the PCR enrichment step. After final clean-up, the quality and concentration of the VLP libraries were assessed with the Qubit dsDNA HS kit (ThermoFisher Cat#Q32854) and with the Agilent High Sensitivity D5000 ScreenTape system (Agilent Technologies). Libraries were sequenced using 2 × 150 bp paired-end chemistry on an Illumina NovaSeq 6000 platform with the S4 Reagent Kit v1.5 (300 cycles).

Read trimming and contig assembly

For both WGS and VLP datasets, post-sequencing data analysis was identical. Analysis of sequencing output started with adapter trimming and quality control of sequencing reads using fastp v0.23.1[79], using standard settings. Trimmed reads were mapped to the human genome (GRCh37) using bowtie2 v2.4.0[80], which showed that samples contained 0.13 ± 0.26 % human reads. High-quality reads were then assembled per sample (*i.e.*, 196 WGS and 48 VLP assemblies) into contigs using the metaSPAdes v3.14.1 software[81]. For each sample, we selected contigs of more than 5,000 bp for further analysis. In addition, among contigs between 1,500 and 5,000 bp we identified circular contigs by checking for identical terminal ends using a custom R script that employed the Biostrings R package v3.12[82]. Assemblies yielded a total of 9,108,147 circular contigs and contigs over 5,000 bp. Three VLP samples were subsampled differently due to memory issues encountered in assemblies. These were S038 and S192 (subsampled to 40 million read pairs), and S069 (subsampled to 25 million read pairs).

Phage and bacterial sequence selection

For phage sequences we followed Gregory et al.[83]. We first analysed contigs using VirSorter v1.0.6[84], which analyses both distant protein homologies to viral hallmark genes and genome architecture, and selected those in category 1, 2, 4, and 5. In parallel, contigs were analysed using VirFinder v1.1, which predicts viral sequences with a machine-learning approach, after which we selected those with a score above 0.9 and a p-value below 0.05. We additionally classified contigs as phage if (I) they were both in VirSorter categories 3 or 6 and had VirFinder scores above 0.7 with p-values below 0.05, and (II) annotation with the contig annotation tool (CAT) v5.1.2[37], which classifies contigs using blastp against the NCBI nr protein database, was as "Viruses" or "unclassified" at the superkingdom level. After removing those with CAT classifications as Eukaryotic viruses, this resulted in a database of 45,568 phage contigs. Bacterial sequences were predicted by selecting all contigs that CAT annotated in the "Bacteria" at the superkingdom level, and removing contigs that were also

found in the phage dataset. An exception was made for prophage contigs in VirSorter category 4, 5, and 6, which were left among the bacterial dataset (see "Phage-host linkage prediction"). This resulted in a total of 1,579,361 bacterial contigs. The 1,624,929 bacterial and phage datasets were then concatenated and deduplicated using dedupe from BBTools v38.84 with a minimal identity cutoff of 90% (option minidentity = 90). This identified 759,403 duplicates and resulted in 829,633 non-redundant bacterial sequences and 25,893 non-redundant phage sequences. While the bacterial sequences were used for host prediction (see "Phage-host linkage prediction"), we subsequently predicted open reading frames (ORFs) in phage contigs using Prodigal v2.6.2[85] (option -p meta). These ORFs were then used to group phage sequences in viral clusters (VCs) using vContact2 v0.9.18[27]. For a full accounting of phage contigs, see Supplementary Data 1 and 3. All phage contigs were analysed for completion with CheckV v0.7.0–1[29] (Supplementary Data 5).

To test the robustness of the metagenomic sequencing, we also analysed quality trimmed reads from the bulk sequencing samples with metaphlan v3.0.13 using standard settings. This analysis identified a total of 632 bacterial species across all samples (mean: 88.7 ± 15.7 species/sample, median: 90). Based on the output, richness had a significance of 0.035, Pielou evenness 0.027, and Shannon diversity 0.0015 (according to Wilcoxon signed rank test).

Read mapping and community composition

For bacterial community composition, we used sequencing data targeting the V4 region of the 16s rRNA gene that had been performed previously[74,86]. Details on ASV construction from these samples was described previously in Verhaar et al.[86]. As part of this previous analysis, samples with fewer than 5000 read counts had been removed, and samples had been rarified to 14932 counts per sample.

To determine phage community composition, we mapped reads from each sample to the non-redundant contig dataset using bowtie2 v2.4.0[80]. As previously recommended[30], we removed spurious read mappings at less than 90% identity using coverM filter v0.5.0 (unpublished; https://github.com/wwood/CoverM, option -min-read-percent-identity 90). The number of reads per contig was calculated using samtools idxstats v1.10[87]. As was also recommended[30], contig coverage was calculated with bedtools genomecov v2.29.2[88], and read counts to contigs with a coverage of less than 75% were set to zero. Read counts for each sample were finally summed per VC. For analyses of alpha- and beta-diversity, we adjusted read counts for contig length and library size by calculating reads per kilobase per million mapped reads (RPKM). Where samples were directly comparted, RPKM values were made compositional by dividing them by the total RPKM per sample. On average,

2.71 ± 1.3% of WGS reads mapped to viral sequences (median 2.38%), along with 45.3 ± 20.4% (median 41.8%) of VLP reads.

Ecological measures

In all boxplots, we tested statistical significance using the Wilcoxon rank-sum test as it is implemented in the ggpubr v0.4.0R package (available from: https://cran.r-project.org/web/packages/ggpubr/index.html). Unless stated otherwise, all plots were made using either ggpubr or the ggplot2 v3.3.2R package (available from: https://cran.r-project.org/web/packages/ggplot2/index.html). Alpha diversity measures (observed VCs and Shannon H' for phages and Chao1 and Shannon H' for bacteria) were calculated using read count tables with the plot_richness function in the phyloseq R package v1.33.0[89]. For β-diversity, we converted read counts to relative abundances using the transform function from the microbiome v1.11.2R package. We then used the phyloseq package to calculate pairwise Bray-Curtis dissimilarities and construct a principal coordinates analysis (PCoA). Statistical significance of separation in the PCoA analysis was determined with a permutational multivariate analysis of variance (permanova) using the adonis function from the vegan R package[90]. For this analysis, we adjusted for smoking, sex, age, alcohol use, and metformin use. Direct correlation coefficients between richness and diversity were calculated using the stat_cor function in the ggpubr R package. The resulting P-values were adjusted for multiple testing using the Benjamini–Hochberg procedure.

Phage-host linkage prediction

We predicted VC-bacterium links in three ways: (i) CRISPR protospacers, (ii) prophage similarity, and (iii) characterized phage similarity.

We predicted CRISPR arrays among the bacterial contigs using CRISPRdetect v2.4[91] (option array_quality_score_cutoff 3) and used these to match bacterial contigs and phage contigs. In addition, we used a dataset of 1,473,418 CRISPR spacers that had previously been predicted[60,92] in genomes contained in the Pathosystems Resource Integration Center (PATRIC)[93] database. We matched CRISPR protospacers to viral contigs using BLASTn v2.12.0+[94] with the short option. Spacer hits with less than 2 mismatches were considered valid. This process resulted in 155,173 spacer hits to PATRIC genomes or to bacterial contigs from this study with definite CAT classifications at the phylum level (Supplementary Data 2).

To identify predicted phage contigs with high sequence similarity to prophages, we analysed which viral clusters contained on of the 7691 bacterial contigs with VirSorter prophage predictions in category 4 or 5. CAT was subsequently used to determine the taxonomy of bacterial contigs with prophage regions. In total, we linked 2,391 VCs to prophages with this approach.

Finally, VCs were linked to bacterial hosts by vContact2 clustering with characterized phages from the viral RefSeq V85 database[95] with a known host. To achieve this, we selected all VCs from the vContact2 output that contained both characterized genomes and phage contigs. If all characterized phages infected hosts within the same bacterial family, we took that to mean that the whole VC infects hosts from that family. This approach linked 4457 VCs to hosts.

Differential abundance analysis

To determine which bacteria and VCs were differentially abundant between MetS and control subjects, we employed the analysis of composition of microbiomes with bias correction (ANCOM-BC)[33]. This method, unlike other similar methods like DeSeq2, takes into account the compositional nature of metagenomics sequencing data[96]. To implement this method, we applied the ANCOM-BC v1.0.2 R package to raw read count tables, as ANCOM-BC employs internal corrections for library size and sampling biases[33]. Significance cutoff was set at an adjusted p-value of 0.05, *p* values were adjusted using the Benjamini–Hochberg method, and all entities (bacteria taxa/VCs) that were present in more than 10% of the samples were included (options p_adj_method = "BH", zero_cut = 0.9, lib_cut = 0, struc_zero = T, neg_lb = F, tol = 1e-5, max_iter = 100, alpha = 0.05). For this analysis, we adjusted for smoking, sex, age, alcohol use, and metformin use.

Crassvirales phages

To identify *Crassvirales* phages, we employed a methodology described earlier[42], for which we first made a BLAST database containing all ORFs from all phage contigs (predicted before viral clustering, see "Viral and bacterial sequence selection") using BLAST v2.9.0+[94]. We then performed two BLASTp searches in this database, one with the terminase (YP_009052554.1) and one with the polymerase (YP_009052497.1) of crAssphage (NC_024711.1), with a bit score cutoff of 50. All phage contigs that had (i) a hit against both crAssphage terminase and polymerase and a query alignment of ≥350 bp, and (ii) a contig length of ≥70 kbp were considered *Crassvirales* phages. This resulted in 287 *Crassvirales* phage contigs, which were contained in 88 VCs.

Candidatus *Heliusviridae* analysis

To detect pairwise similarity, whole genome analyses were constructed with Easyfig v2.2.5[97]. The prophage borders in NODE_38_length_205884_cov_102.806990 were determined by determining the read depth along the entire contig from the bam files with read mapping data ("Read mapping and community composition") using bedtools genomecov v2.29.2[88] with option -bg. Resultant output was parsed and plotted in R. Other related phages among the cohort were detected by performing a BLASTp search with all phage ORFs of NODE_38_length_205884_cov_102.806990 against all phage ORFs of the

cohort with Diamond v2.0.4. This identified nine genes that were present in 249 contigs. The ORFs on these contigs were annotated using PROKKA v1.14.6[98] and InterProScan v5.48-83.0[99]. To identify isolated phages that share these nine contigs, we performed a BLASTp against the NCBI nr database using the NCBI webserver[100] on February 26 2021 and collected all genomes with hits against all nine genes (bit score ≥ 50).

The phages sharing all nine genes were clustered by analysing them with vContact2 v0.9.18[27], extracting the protein clustering data and calculating the number of shared clusters between each pair of contigs. Contigs were clustered in R based on Euclidean distances with the average agglomeration method.

To build a taxonomic tree, the nine genes were separately aligned using Clustal Omega v1.2.4[101], positions with more than 90% gaps were removed with trimAl v1.4[102] and alignments were concatenated. From the concatenated alignment, an unrooted phylogenetic tree was built using IQ-Tree v2.0.3[103] using model finder[104] and performing 1000 iterations of both SH-like approximate likelihood ratio test and the ultrafast bootstrap approximation (UFBoot)[105]. Model finder selected LG + F + R8 as the best-fit substitution model. In addition, ten iterations of the tree were separately constructed, as has been recommended[106] (IQ-Tree options -bb 1000, -alrt 1000, and—runs 10).

Validation of *Ca. Heliusviridae* in other cohorts

We used three additional studies to analyse prevalence of the *Ca. Heliusviridae*; one composing of 145 participants used to study the gut virome in type 2 diabetes[11], a second containing 196 participants and used to study the gut virome in hypertension[44], and a final one thousands of phages from various sources[39]. Reads belonging to the former two studies were downloaded from the NCBI sequencing read archive (SRA) and assembled as described above, while for the latter assembled contigs were downloaded. After assembly, ORFs were predicted using Prodigal v2.6.2[85]. *Ca. Heliusviridae* members were identified by blastp using Diamond v2.0.4[107] against ORFs from each study, in which the terminase, portal protein, Clp-protease, and major capsid protein of NODE_38_length_205884_cov_102.806990 were used as queries. This was done instead of all nine signature *Ca. Heliusviridae* genes to better allow for incomplete assemblies. Contigs containing all four genes were selected, and a concatenated alignment was made of the four head genes found in the T2D and hypertension cohorts, plus all *Ca. Heliusviridae* in the tree depicted in Supplementary Fig. 7. These were then used to build a phylogenetic tree. The concatenated alignment and phylogenetic tree were constructed as described above under “Candidatus Heliusviridae analysis”.

We further analysed the data obtained by and earlier study of gut viromes in MetS among 28 school-aged children[23]. We downloaded reads from the NCBI sequencing read archive (sra). As this this project yielded an average 1.3 ± 0.9 M reads, we cross-assembled all 28 samples in one assembly with metaSPAdes with the same settings as described above (Read trimming and contig assembly). This yielded 45,112 contigs of more than 1,500 bp, with an average length of 3,702 bp. No contigs carrying all nine *Candidatus Heliusviridae* were identified, likely because this would require a contig of at least 20,000 bp. We thus performed a blastp using Diamond v2.0.4106 (bit score ≥ 50) against the terminase protein of NODE_38_length_205884_cov_102.806990, which identified 31 potential *Candidatus Heliusviridae* contigs.

Statistics and reproducibility

All statistical analyses were performed in R v4.1.1. Details on the statistical tests that were applied are indicated in the figure captions and the results where necessary. The scripts used to perform statistical analyses are available in Supplementary Data 8. No statistical method was used to predetermine sample size. No data were excluded from the analysis. The experiments were not randomized. Participants were allocated into groups based on clinical measurements of metabolic syndrome-related clinical parameters. Therefore, the investigators were not blinded to allocation during experiments and outcome assessment.

Reporting summary

Further information on research design is available in the *Nature Research Reporting Summary* linked to the online article.

Data availability

The sequencing data generated in this study have been deposited in the European Genome-Phenome Archive database under accession code EGAS00001006260. The sequencing data are available under restricted access for restrictions imposed by the signed consent of participants, access can be obtained by submitting a proposal to the HELIUS Executive Board as outlined at http://www.heliusstudy.nl/en/researchers/collaboration, by email: heliuscoordinator@amsterdamumc.nl. The HELIUS Executive Board will check proposals for compatibility with the general objectives, ethical approvals and informed consent forms of the HELIUS study. There are no other restrictions to obtaining the data and all data requests will be processed in the same manner. The data generated in this study are provided in the Source Data file. The human genome data used in this study is available at the National centre for biotechnology information (NCBI) under accession GRCh37 [https://www.ncbi.nlm.nih.gov/assembly/GCF_000001405.13/]. The CRISPR spacer dataset derived from the PATRIC database is available from Supplementary Table 1 of ref. [92] [https://academic.oup.com/nar/article/48/21/12074/5997439#supplementary-data]. The

reads from the validation cohorts are available from NCBI under the NCBI BioProject accession numbers PRJNA646512, PRJEB13870, PRJNA422434, and PRJNA573942. Source data are provided with this paper.

Code availability

All code describing the statistical analyses performed in this work can be found in Supplementary Data 8. For direct access to the underlying data and participant metadata, see the Data availability statement above.

Author contributions

P.A.d.J. and K.W. conducted data analysis; T.P.M.S., B.J.v.d.B., A.H.Z., F.L.N., B.E.D., and M.N. assisted with experimental design and data interpretation; P.A.d.J. and H.H. designed the study and wrote the manuscript. All authors read and provided input on the manuscript.

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Competing interests

M.N. owns stock in, consults for, and has intellectual property rights in Caelus Health. He consults for Kaleido. None of these are directly relevant to the current paper. The remaining authors declare no competing interests.

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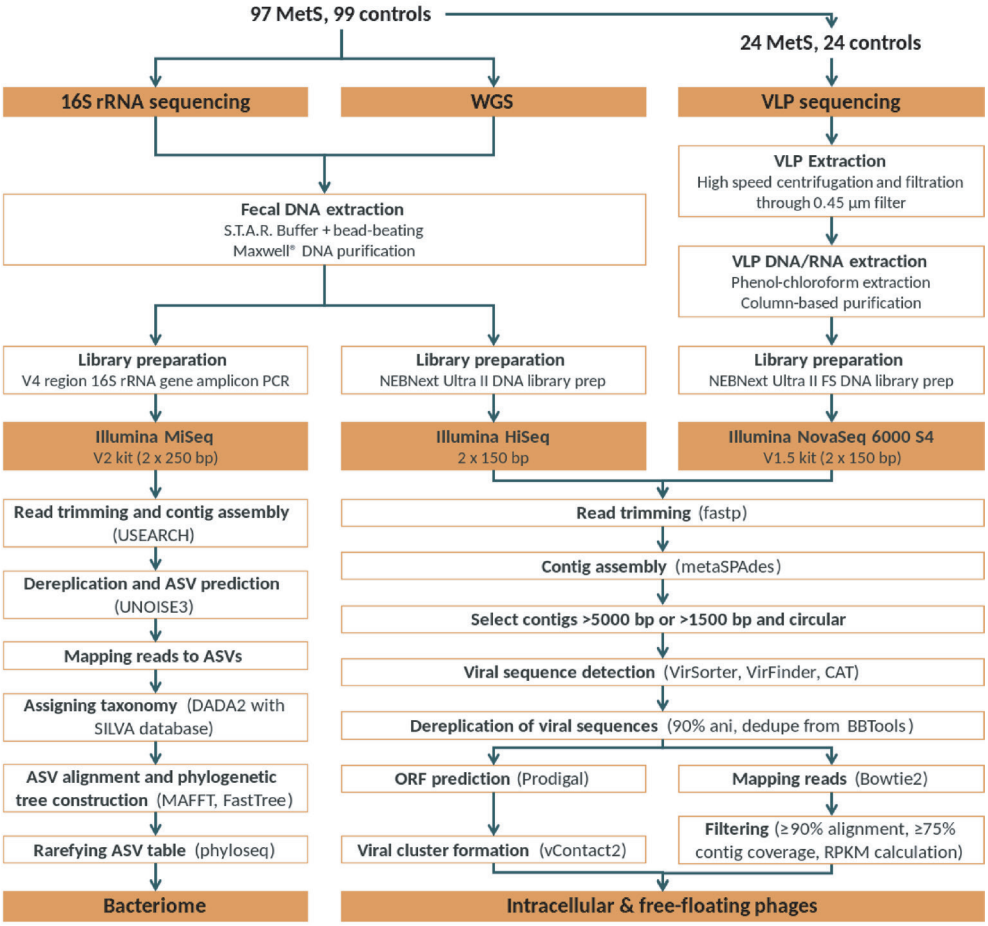
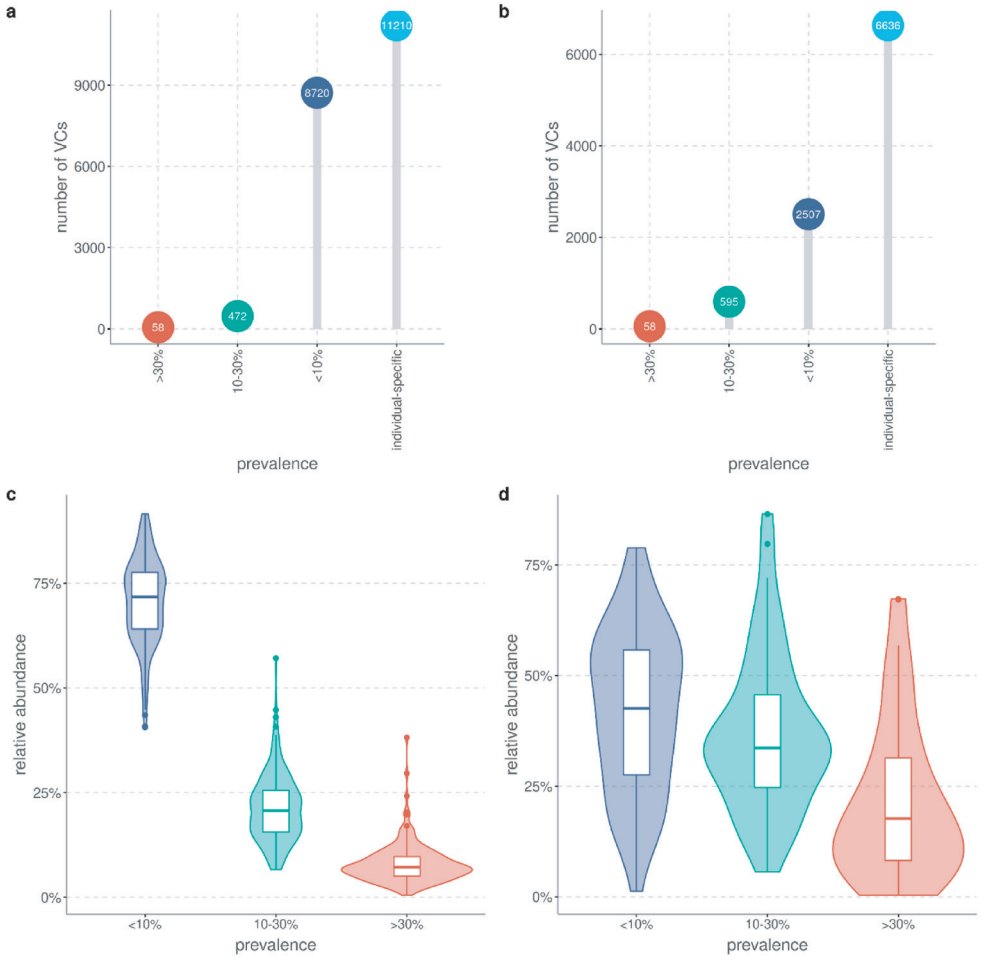
SUPPLEMENTARY MATERIAL

The following supplementary material is available online at:
https://doi.org/10.1038/s41467-022-31390-5.

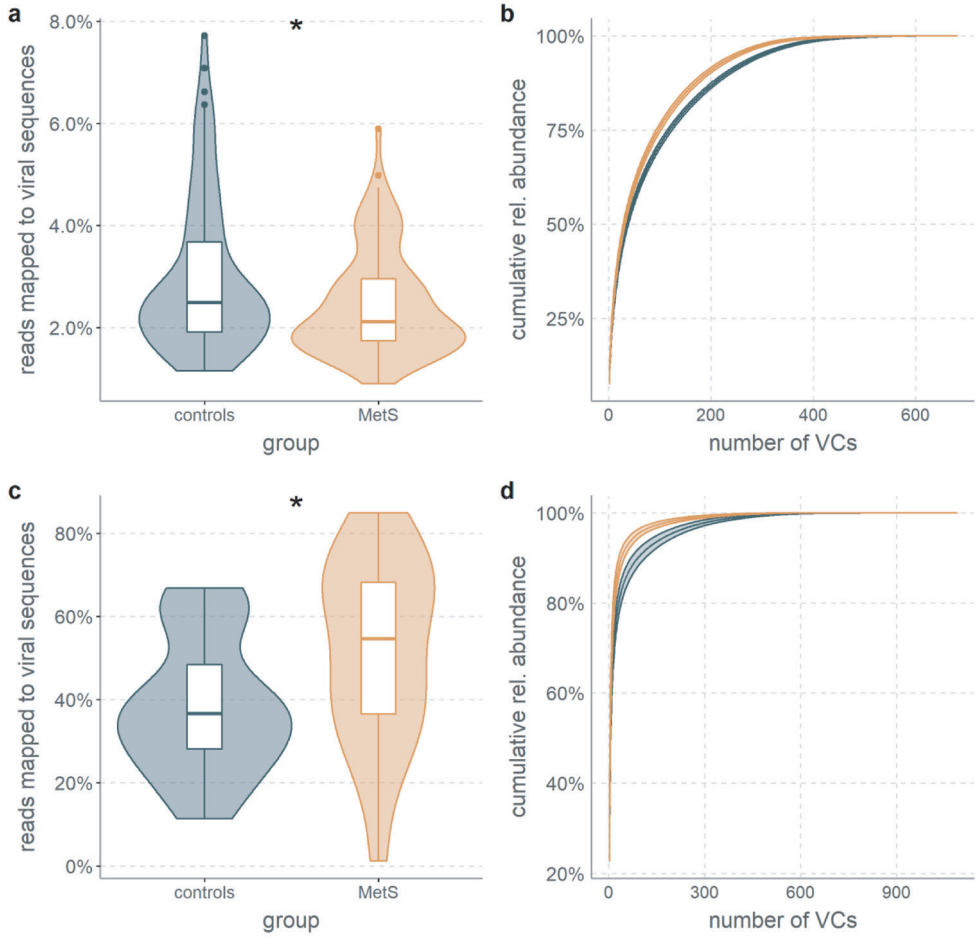
- Source data
- Supplementary Data 1: Relative abundance of all VCs across the WGS samples.
- Supplementary Data 2: VC-host links with taxonomies of the host contigs.
- Supplementary Data 3: Assignments of viral contigs to VCs.
- Supplementary Data 4: Annotation of NODE_38_length_205884_cov_102.806990
- Supplementary Data 5: CheckV results of all viral contigs.
- Supplementary Data 6: Newick file of the phylogenetic tree in Figure 5b.
- Supplementary Data 7: Alignment from which the tree in Figure 5b was constructed.
- Supplementary Data 8: R code of all statistical analysis performed in the study.
- Reporting Summary



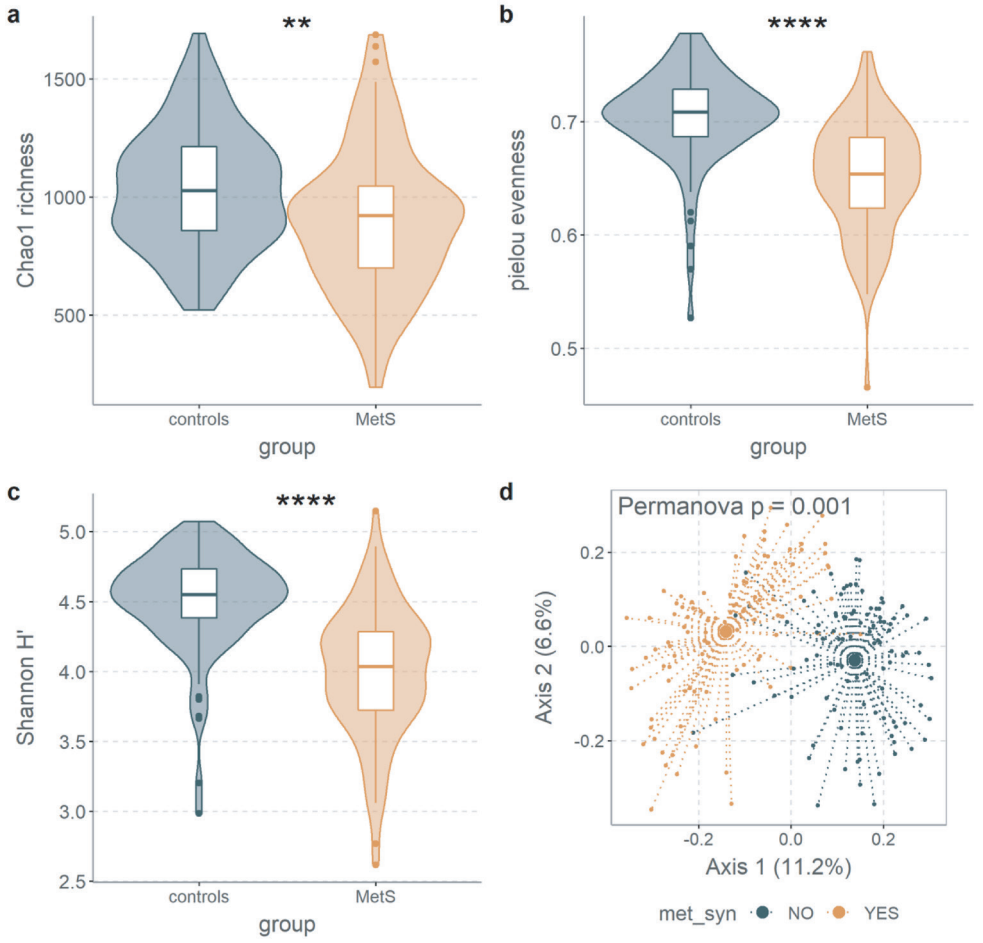
Supplementary Figure 1: Flow chart of the analyses preformed in this study.



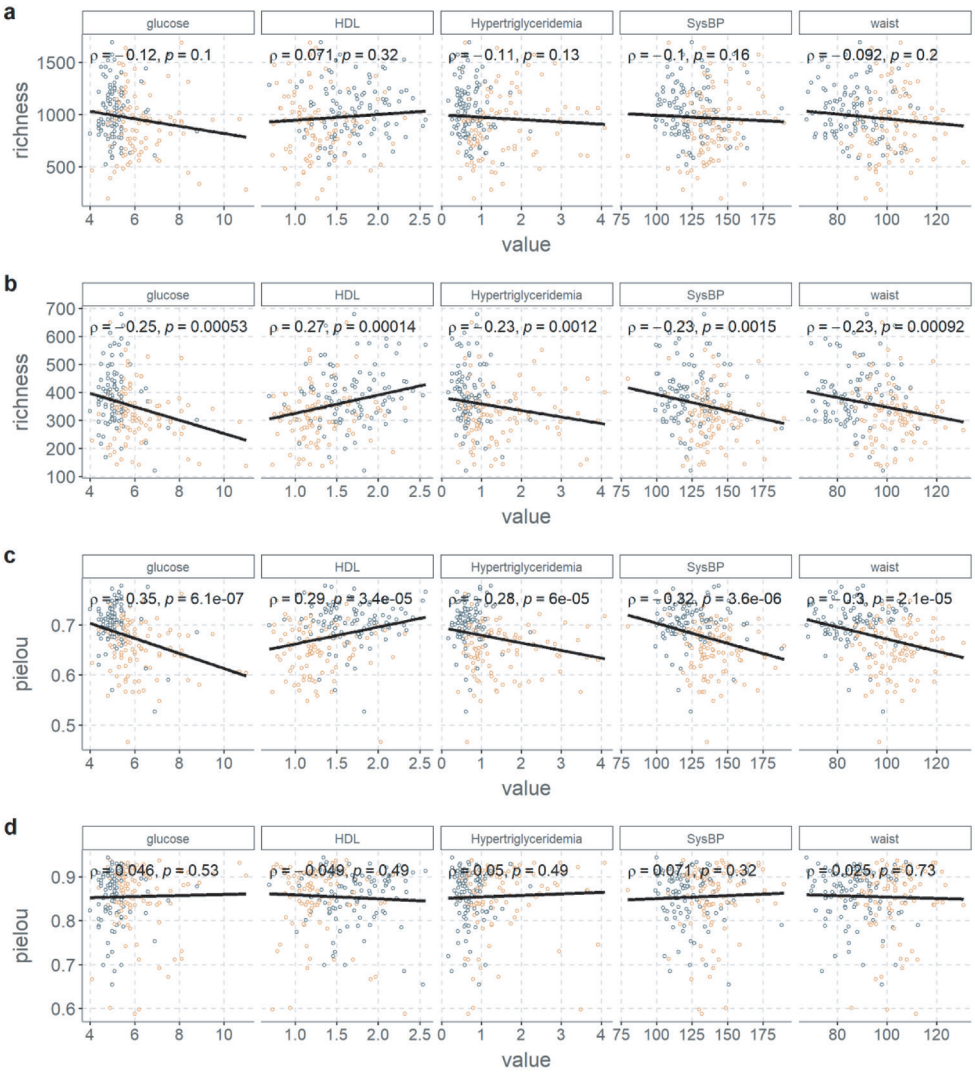
Supplementary Figure 2: Overview of the viromes show high inter-individual variation.
(**a**) Prevalence of VCs among the bulk viromes (**b**) Prevalence of VCs among the VLP viromes. (**c**) Total relative abundance of VCs grouped by their prevalence among the participants among bulk viromes. N=196 biologically independent samples. (**d**) same as (c) for the VLP viromes. N=48 biologically independent samples. Box plots show the median (middle line), 25th, and 75th percentile (box), with the 25th percentile minus and the 75th percentile plus 1.5 times the interquartile range (whiskers), and outliers (single points).



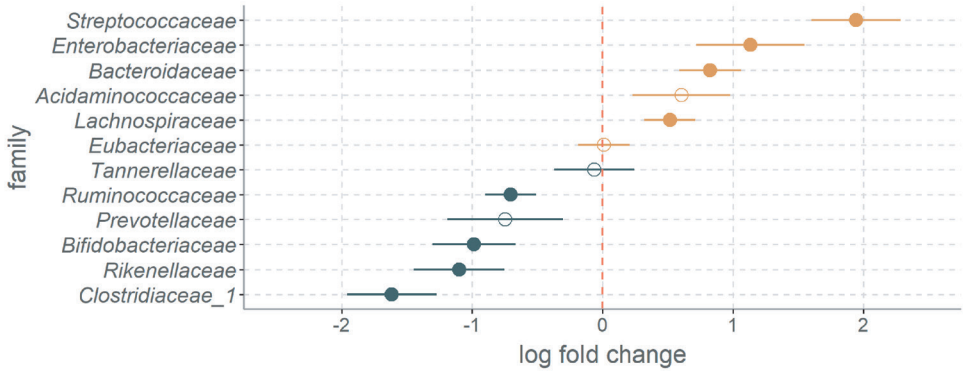
Supplementary Figure 3: Differences in total phage abundance in the two phage populations.
(**a**) total phage abundance in bulk viromes, as shown by the percentage of reads that map to phage sequences. n=97/n=99 biologically independent samples for MetS and controls, respectively (p = 0.023). (**b**) cumulative VC ranked-abundance curves of bulk phage samples. MetS is in orange, controls in blue. (**c**) same as (a) for VLP viromes. n = 24 biologically independent samples for both MetS controls (p = 0.011). (**d**) same as (b) for free floating viromes. Stars denote significance according to the two-sided Wilcoxon signed rank test. * ≤ 0.05, ** ≤ 0.01, *** ≤ 0.001, **** ≤ 0.0001. Box plots show the median (middle line), 25th, and 75th percentile (box), with the 25th percentile minus and the 75th percentile plus 1.5 times the interquartile range (whiskers), and outliers (single points).



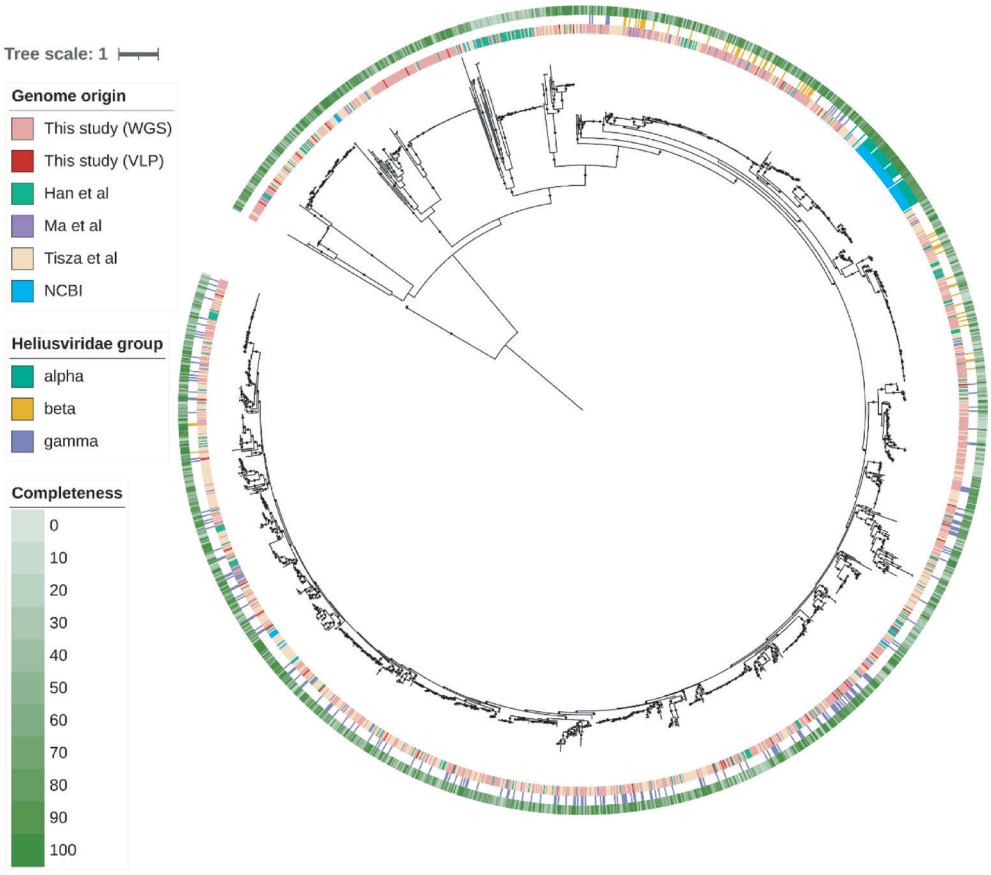
Supplementary Figure 4: Gut bacterium populations are altered in MetS.
(**a**) MetS-associated decreased bacterial species richness is evidenced by the Chao1 index. n=97/n=99 biologically independent samples for MetS and controls, respectively (p = 9.1 x 10-4). (**b**) decreased bacterial pielou evenness measurements (p = 1.8 x 10-14). (**c**) significantly decreased bacterial α-diversity measured by Shannon diversity (p = 1.5 x 10-15). (**d**) clear separation between bacterial populations of MetS (orange) and control (blue) participant as shown by β-diversity depicted in a principal coordinates analysis (PCoA) of Bray-Curtis dissimilarities. Permanova test was adjusted for smoking, age, sex, alcohol use, and metformin use. Statistical significance in A-C is according to the two-sided Wilcoxon signed rank test, where p-values are denoted as follows: ns not significant, * ≤ 0.05, ** ≤ 0.01, *** ≤ 0.001, **** ≤ 0.0001. Box plots show the median (middle line), 25th, and 75th percentile (box), with the 25th percentile minus and the 75th percentile plus 1.5 times the interquartile range (whiskers), and outliers (single points).



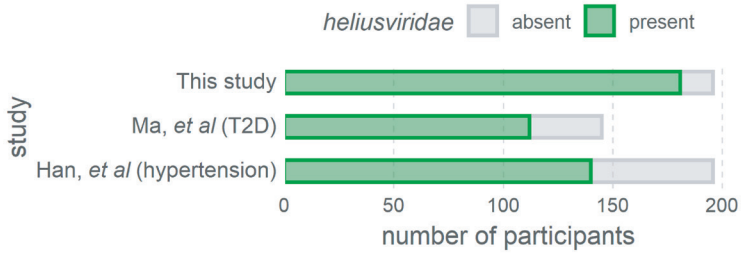
Supplementary Figure 5: Individual correlations between the MetS risk factors and richness of bulk viromes (a) and bacteriomes (b), as well as evenness in viromes (c) and bacteriomes (d).
Plotted are the Spearman's rank correlation coefficients. Point colors denote patient group: MetS is in orange, controls in blue.



Supplementary Figure 6: ANCOM-BC of bacteria in the top twelve most predated upon bacterial families. Closed circles denote significance, open circles lack of significance. n=97/n=99 biologically independent samples for MetS and controls, respectively. Error bars denote the standard error adjusted by the Benjamini-Hochberg procedure for multiple testing. Color shows in which group the family was most abundant: MetS is in orange, controls in blue.



Supplementary Figure 7: A midpoint-rooted approximate maximum likelihood tree made from the concatenated alignments of the four structural *Candidatus Heliusviridae* genes in contigs from this study and multiple cohorts in which the virome was analyzed before.
Dots represent bootstrap values of ≥95. The inner ring of colors denotes the study from which the genome was retrieved. The middle ring shows genomes that were assigned to *Ca. Heliusviridae* groups in Figure 5. The outer rich displays genome completeness according to checkV.



Supplementary Figure 8: Occurrence of *Candidatus Heliusviridae* in this study and two validation cohorts. To circumvent incomplete assemblies, contigs were identified as *Candidatus Heliusviridae* if they 1) contained the terminase, portal protein, major capsid protein, and clp-proteas, and 2) were located in the same clade as *Candidatus Heliusviridae* from this study in the phylogenetic tree depicted in Supplementary Figure 7.

Supplementary Table 1: Characteristics of the Cohort

	MetS	control	p-value (two-sided Wilcoxon)
Participants	97	99	
Participants with MetS risk factors			
0	0	28	
1	0	34	
2	0	37	
3	35	0	
4	32	0	
5	30	0	
Waist Circumference (cm)	102 ± 9.5	86 ± 10.2	**9.2×10^{-07}**
Blood Pressure (mmHg)			
Systolic	139.9 ± 16.6	123.4 ± 16.2	**1.92×10^{-12}**
Diastolic	84.4 ± 10.6	77.6 ± 10.5	**1.72×10^{-06}**
Blood glucose (mmol/l)	6.2 ± 1.2	5.1 ± 0.6	**$< 2 \times 10^{-16}$**
HDL (mmol/l)	1.3 ± 0.3	1.7 ± 0.4	**1.12×10^{-12}**
Triglycerides (mmol/l)	1.5 ± 0.9	0.7 ± 0.3	**1.32×10^{-15}**
Central obesity	94	58	
High Blood Pressure	91	33	
High Blood Glucose	69	9	
Low HDL	69	5	

CHAPTER 8

Sterile Faecal Filtrate Transplantation Alters Phage-Microbe Dynamics in Individuals with Metabolic Syndrome: a Double Blind, Randomised, Placebo-Controlled Clinical Trial Assessing Efficacy and Safety

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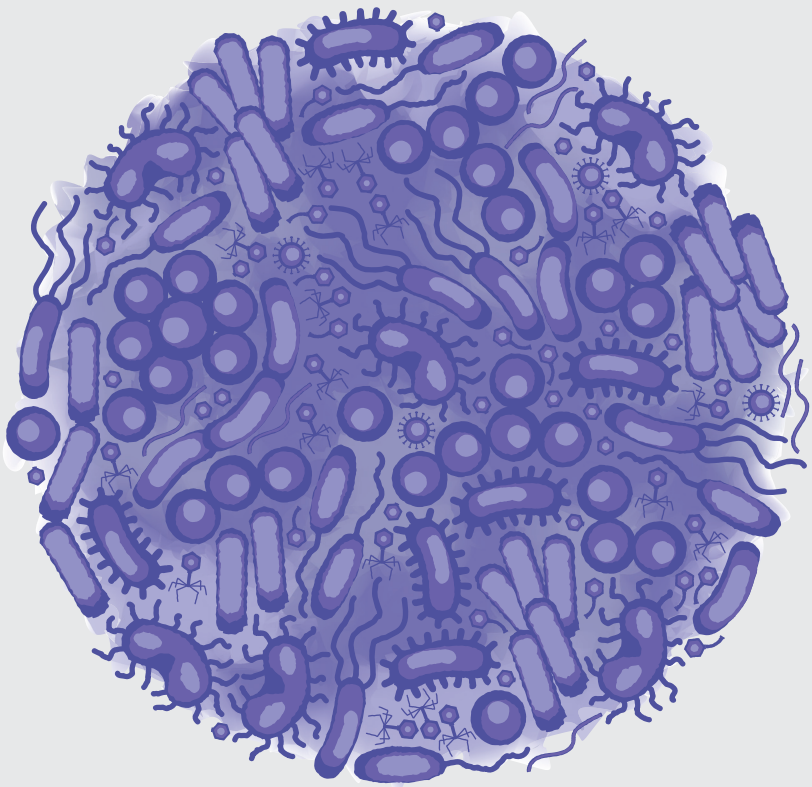

ABSTRACT

Bacteriophages (phages) are bacterial viruses that have been shown to shape microbial communities. Previous studies have shown that faecal virome transplantation can decrease weight gain and normalize blood glucose tolerance in diet-induced obese mice. Therefore, we performed a double-blind, randomised, placebo-controlled pilot study in which 24 individuals with MetSyn were randomised to receive a faecal filtrate transplantation (FFT) from a lean healthy donor or a placebo. From baseline up to 28 days, we assessed safety, effects on glucose metabolism, and longitudinal changes within the intestinal bacteriome and phageome. The FFT was well-tolerated and safe, while the overall changes in glucose metabolism were similar in both groups. The phage virion composition was significantly altered two days after FFT as compared to placebo, which coincided with more virulent phage-microbe interactions. In conclusion, we provide evidence that gut phages can be safely administered to transiently alter the gut microbiota of recipients.

INTRODUCTION

The metabolic syndrome (MetSyn) constitutes a major global health concern[1]. This combination of clinical manifestations that are associated with insulin resistance affects nearly a quarter of the world population and increases the risk for cardiometabolic disease, such as type 2 diabetes (T2D) and cardiovascular disease[2,3]. The intestinal microbiota are increasingly seen as contributors to these diseases, e.g., through production of certain microbial metabolites and induction of low-grade inflammation[4,5].

Previously reported microbial effects on human health have been mainly attributed to the bacterial component of the microbiota[6]. However, the gut microbiome is an ecosystem, which, in addition to bacteria, contains viruses, archaea, fungi, and protists[7]. The viral component predominantly comprises bacteriophages (98%)[8], which are present in similar numbers as bacteria in the gut[9]. Bacteriophages (phages from hereon) are bacterial viruses that exclusively infect bacteria and, by doing so, often either kill bacteria (lysis) or incorporate themselves into the bacterial genome (lysogeny)[10]. Consequently, phages shape microbial communities in many ecosystems[11,12]. Moreover, phages have been implicated in human (gastrointestinal) disease[13–16], including diabetes[17,18]. We recently described decreased richness and diversity of the gut phageome in MetSyn, together with a larger inter-individual variation and altered composition[19].

Considering their ability to modulate gut bacteria and their function[20], phages are of special interest in ongoing endeavours to alter the human gut microbiome to benefit human health. Furthermore, the emergence of multidrug-resistant bacteria has led to an increasing interest in phage therapy, in which host-specific phages target specific pathogenic bacteria without affecting the commensal microbiota[21,22]. Such phage cocktails can be very effective in treating monoclonal bacterial infections, but are in general not sufficient to (beneficially) alter a complete microbiome[23,24]. Therefore, there is growing interest in the transfer of virus-like particles (VLP) isolated from the faecal microbiota, generally called a faecal virome transplantation (FVT). In mice, it has been shown that FVT induced a comparable effect as a faecal microbiota transplantation (FMT), in which the complete faecal microbiota of a healthy donor is transferred[25,26]. Moreover, in a small human pilot study, an FMT depleted of bacteria, also known as a sterile faecal filtrate transplantation (FFT), was successful in curing five individuals from a recurrent *Clostridioides difficile* infection[27]. Compared to FMTs, an FFT or FVT depleted of living microorganisms has a lower risk of transferring unknown pathogenic bacteria, which might improve safety.

Modulation of gut microbiota composition through FMT has been shown to improve peripheral insulin sensitivity in individuals with MetSyn[28,29]. Moreover, an FVT from lean

donor mice was able to decrease weight gain and normalize blood glucose tolerance in diet-induced obese mice[30]. This effect was likely mediated through alterations in the gut microbiota induced by phages, as prior treatment with antibiotics disrupted the bacterial hosts and thereby counteracted the effect of the FVT. This raised the question whether transfer of faecal phages could induce a similar effect as FMT in human individuals with MetSyn.

To study the effect of faecal phages on glucose metabolism, comparing a clean and concentrated faecal virome transplant with a phage-inactivated transplant would be most desirable. Unfortunately, the IRB only allowed us to minimally process the faecal suspension that is usually used for FMT, so we chose an FFT approach. We were hence not able to remove components other than bacteria from the filtrate. However, since phages are self-propagating entities with presumed longer effects on the microbial ecosystem than a single administration of metabolites, peptides or debris, we considered it justified to use the FFT to study phage-bacteria interactions and subsequent effects on glucose metabolism.

In this double-blind, randomised, placebo-controlled pilot study, we provide proof of concept that a faecal filtrate from lean healthy donors containing gut virions can be safely administered to MetSyn recipients. Moreover, gut phages have the potential to improve glycaemic variability and alter phage-microbe dynamics. Although follow up studies with cleaner, better defined, and better matched donor-recipient pairs are needed, this study provides a critical basis to do so and move the field forward.

RESULTS

Inclusion of subjects and donors

To study whether an FFT could induce a similar effect on glucose metabolism as an FMT, we set up a prospective double-blind, randomised, placebo-controlled pilot study (figure 1A). Changes in glucose metabolism between day 0 and 28 were determined by the total area under the curve (AUC) for glucose excursion during an oral glucose tolerance test (OGTT), the primary outcome. Based on previous data from our group[28,29] and our hypothesis that a faecal phage transplant can be equally effective as an FMT[25–27,30], a sample size of 12 patients per group was deemed necessary.

Starting from October 2019, a total of 82 subjects signed the informed consent form and were screened, of whom 24 subjects were included and finished the study before December 2020 (figure S1A). Most subjects were excluded because they did not have MetSyn according to the National Cholesterol Education Program (NCEP) criteria for the metabolic syndrome[31]. For the faeces donors, 24 subjects signed informed consent and were stepwise

screened, resulting in 6 eligible donors (figure S1B). Potential donors failed screening mainly due to carriage of parasites (11/24, 46%), followed by positive stool tests for pathogens (4/24, 17%) and exclusion based on questionnaire (3/24, 13%). Of these 6 eligible donors, only 3 (3/24, 13%) donated faeces for the production of a sterile faecal filtrate. Therefore,

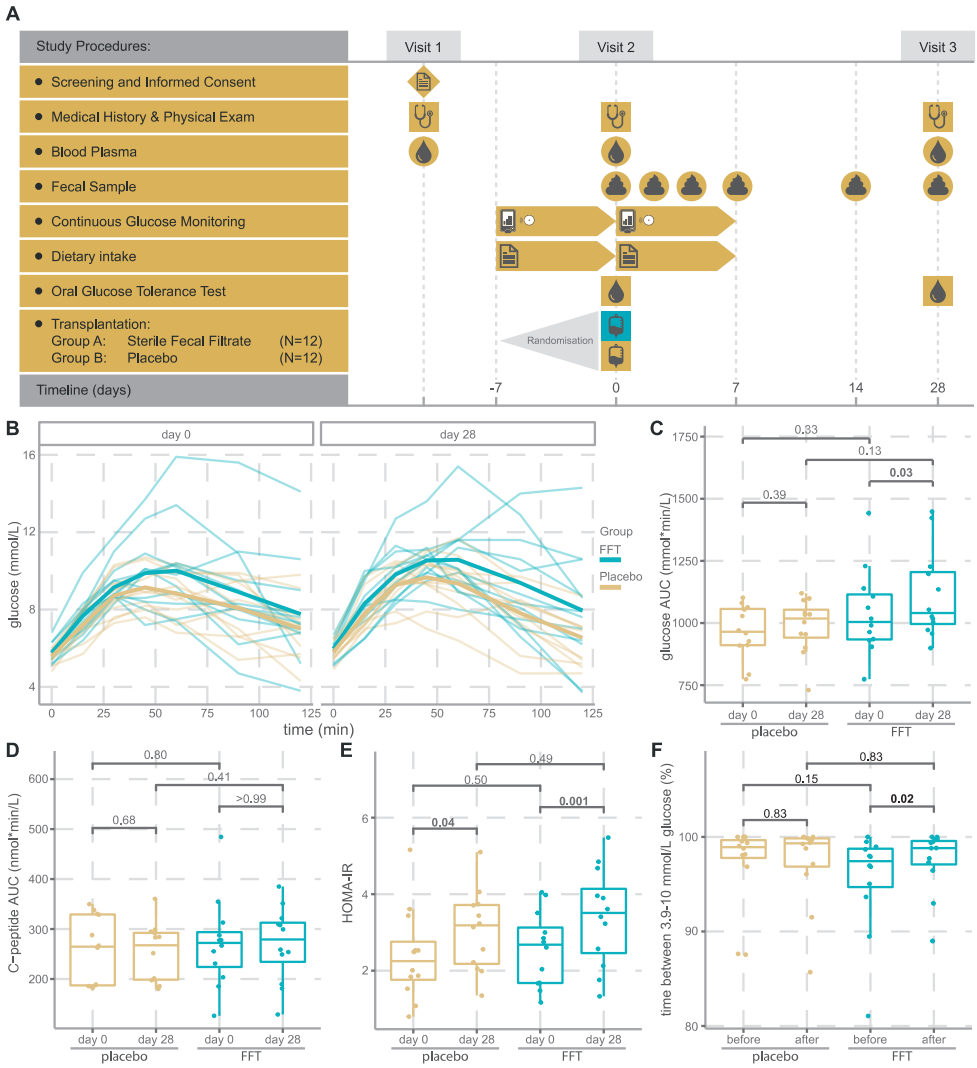

Figure 1: A) Overview of the study. **B)** Glucose excursions during the oral glucose tolerance test. One person who was randomized to the FFT group had progressed to type 2 diabetes, which was not apparent at the time of screening. **C)** Total area under the curve (AUC) for glucose and **D)** for C-peptide did not significantly differ between the groups. Within both groups there was a small increase in glucose AUC between day 0 and 28, which was nominal significant within the FFT group, although this significance disappeared after correction for multiple testing. **E)** Insulin resistance (HOMA-IR) measures did not significantly differ between the groups, but significantly increased from day 0 to day 28 in both groups. **F)** Glucose variability, expressed as time between 3.9-10 mmol/L glucose, improved only within the FFT group between day 0 and day 28, which was nominal significant, but disappeared after correcting for multiple testing.

an additional 2 donors who were already actively donating for other FMT studies were included[32].

The 24 included MetSyn subjects were randomly assigned to receive an FFT (n = 12) or placebo (n = 12). As shown in table 1, both groups were similar in baseline characteristics, such as age, sex, body mass index (BMI), and other MetSyn-associated parameters. Only the systolic blood pressure was significantly higher in the placebo group, although this difference disappeared at baseline and follow-up visits and was therefore probably a case of white coat hypertension during the screening. None of the individuals with MetSyn used concomitant medication and their diets were similar (table S1). Compared to the healthy donors, the MetSyn subjects differed, as expected, in almost every aspect of MetSyn-associated parameters (table 1). All participant completed the follow-up visit at day 28.

FFT is safe and well-tolerated

The FFT was well-tolerated by the participants and there were no serious adverse events. Compared to the placebo group, more subjects in the FFT group reported adverse events (AEs) that were likely or possibly related to the intervention (six vs two subjects), although this difference was not statistically significant (table 2). All adverse events that may have been related to the intervention were mild gastrointestinal complaints, such as diarrhoea, constipation, bloating, and nausea. Besides the transferred faecal phages, these adverse events could theoretically be induced through the transfer of eukaryotic or human viruses. However, as only 0.044 ± 0.3% (median: 0%) of reads mapped to such viruses, we could not ascertain whether these had an effect. To minimize negative effects from eukaryotic viruses, healthy stool donors were thoroughly screened for presence of known pathogenic viruses prior to donation.

Looking at the clinical safety parameters for liver and renal function, haematology, and inflammation, we did not observe any differences between the FFT and placebo groups (table 2). Interestingly, in both groups there was a significant increase in urea levels, which could be explained by the laxative that was used the evening prior to day 0, leading to less degradation of amino acids through the liver at baseline, and therefore less urea.

FFT improved glucose variability

Prior to the intervention and after 28 days at follow-up, subjects underwent an OGTT to assess their glucose metabolism (figure 1A), which was the primary outcome of the study. Glucose and C-peptide excursions during the OGTT at day 28 after the intervention were similar in the FFT and placebo group (figure 1B-D), as were within group alterations (day 0 vs day 28). In addition, we observed similar fasting glucose and insulin levels, insulin resistance (HOMA-IR) (figure 1E), and HbA1c values between the FFT and placebo group at

Table 1: baseline characteristics of MetSyn subjects and lean donors.

	Metabolic Syndrome subjects				Donors	
	Placebo (n = 12)	Fecal Filtrate (n = 12)	*p-value*	Combined (n = 24)	(n = 5)	*p-value*
Age (years)	**49.3** (12.9)	**54.8** (8.9)	*0.20*	**52.0** (11.2)	**32.0** (7.81)	***0.003***
Gender (n (%))						
Male	**7** (58.3%)	**7** (58.3%)	*>0.99*	**14** (58.3%)	**3** (60.0%)	*>0.99*
Female	**5** (41.7%)	**5** (41.7%)		**10** (41.7%)	**2** (40.0%)	
#MetSyn criteria (n (%))						
1	-	-	*0.38*	-	**3** (60.0%)	***<0.001***
2	-	-		-	**2** (40.0%)	
3	**4** (33.3%)	**7** (58.3%)		**11** (45.8%)	-	
4	**5** (41.7%)	**4** (33.3%)		**9** (37.5%)	-	
5	**3** (25.0%)	**1** (8.3%)		**4** (16.7%)	-	
BMI (kg/m²)	**36.1** (4.2)	**35.2** (5.7)	*0.76*	**35.7** (4.9)	**22.2** (1.9)	***<0.001***
WHR	**0.98** (0.09)	**1.00** (0.06)	*0.49*	**0.99** (0.07)	**0.83** (0.07)	***<0.001***
Systolic BP (mmHg)	**147** (17)	**131** (17)	***0.047***	**139** (18)	**124** (5)	***0.039***
Diastolic BP (mmHg)	**93** (10)	**90** (11)	*0.74*	**91** (11)	**76** (5)	***0.004***
Pulse (beats/min)	**66** (12)	**69** (11)	*0.77*	**68** (11)	**60** (11)	*0.17*
Glucose (mmol/L)	**5.6** (0.5)	**5.7** (0.5)	*0.99*	**5.7** (0.5)	**5.0** (0.4)	***0.007***
Insulin (nmol/L)	**85** (24)	**82** (28)	*0.83*	**84** (26)	**38** (12)	***<0.001***
HOMA-IR	**2.91** (0.76)	**2.92** (1.02)	*>0.99*	**2.91** (0.88)	**1.18** (0.37)	***<0.001***
HbA1c (mmol/mol)	**36** (3.2)	**36** (4.3)	*0.75*	**36** (3.7)	**34** (2.1)	*0.19*
Cholesterol (mmol/L)	**5.30** (1.13)	**5.76** (1.53)	*0.63*	**5.53** (1.34)	**4.01** (0.45)	***0.015***
HDL (mmol/L)	**1.15** (0.22)	**1.22** (0.23)	*0.52*	**1.19** (0.22)	**1.52** (0.26)	***0.010***
LDL (mmol/L)	**3.43** (0.98)	**3.71** (1.25)	*0.89*	**3.57** (1.11)	**2.09** (0.58)	***0.003***
Triglycerides (mmol/L)	**1.60** (0.58)	**1.84** (0.75)	*0.59*	**1.72** (0.67)	**0.89** (0.29)	***0.007***
CRP (mg/L)	**4.0** (5.1)	**6.2** (7.3)	*0.25*	**5.1** (6.2)	**1.4** (1.0)	*0.07*

Unless otherwise specified, data are reported as mean (SD). Statistical testing between the placebo and fecal filtrate groups and metabolic syndrome subjects and donors is performed by independent Mann-Whitney U test for continuous variables and by Chi-square test for categorical and binary variables. BMI = Body Mass Index; WHR = waist-hip ratio; BP = blood pressure; HOMA-IR = Homeostatic Model Assessment for Insulin Resistance; HDL = high-density lipoprotein; LDL = low-density lipoprotein; CRP = C-reactive protein.

Table 2: Differences in clinical safety markers after intervention.

		Placebo (n = 12)	Fecal Filtrate (n = 12)	*p-value*
# AEs (n (%))	Total	**13** (44.8%)	**16** (55.2%)	
Relatedness of AEs (n (%))	Likely	**0** (0%)	**2** (12.5%)	0.21
	Possibly	**2** (15.4%)	**6** (37.5%)	
	Unlikely	**4** (30.8%)	**4** (25%)	
	Not	**7** (53.9%)	**4** (25%)	
# Subjects with AE (n (%))	≥1 AE	**2** (16.7%)	**6** (50%)	0.08
possibly or likely related	No AE	**10** (83.3%)	**6** (50%)	
Bilirubin (µmol/L)	Day 0	**12** (6) *	**15** (9)	0.39
	Day 28	**9** (5)	**12** (12)	
AF (U/L)	Day 0	**76** (18)	**69** (16)	0.17
	Day 28	**79** (16)	**68** (16)	
GGT (U/L)	Day 0	**22** (10)	**26** (11)	0.30
	Day 28	**22** (12)	**26** (9)	
ASAT (U/L)	Day 0	**28** (8)	**28** (7)	0.70
	Day 28	**27** (8)	**25** (7)	
ALAT (U/L)	Day 0	**29** (11)	**29** (10)	0.85
	Day 28	**28** (10)	**27** (9)	
Creatinine (µmol/L)	Day 0	**85** (18)	**76** (15)	0.20
	Day 28	**82** (13)	**75** (15)	
eGFR (ml/min/1.73m2)	Day 0	**81** (12)	**86** (6)	0.32
	Day 28	**83** (9)	**85** (7)	
Urea (mmol/L)	Day 0	**4.3** (0.9) *	**4.4** (1.1) *	0.65
	Day 28	**4.8** (0.9)	**5.1** (1.3)	
Haemoglobin (mmol/L)	Day 0	**8.5** (1.0)	**8.8** (0.8)	0.74
	Day 28	**8.6** (0.9)	**8.6** (0.6)	
Haematocrit (L/L)	Day 0	**0.41** (0.04)	**0.42** (0.03)	0.84
	Day 28	**0.42** (0.04)	**0.41** (0.03)	
MCV (fL)	Day 0	**86.0** (4.7)	**88.0** (2.7)	0.25
	Day 28	**86.5** (4.5)	**88.1** (3.0)	
Thrombocytes (x10^9/L)	Day 0	**265** (87)	**259** (45)	0.85
	Day 28	**263** (73)	**259** (48)	
Leukocytes (x10^9/L)	Day 0	**6.2** (1.4)	**6.2** (1.2)	0.56
	Day 28	**5.8** (1.2)	**6.3** (1.4)	

Table 2 continued.

		Placebo (n = 12)	Fecal Filtrate (n = 12)	*p-value*
Eosinophils (x10^9/L)	Day 0	**0.15** (0.07)	**0.12** (0.06)	0.61
	Day 28	**0.16** (0.08)	**0.16** (0.11)	
Basophils (x10^9/L)	Day 0	**0.04** (0.01)	**0.03** (0.02)	0.27
	Day 28	**0.04** (0.02)	**0.04** (0.02)	
Neutrophils (x10^9/L)	Day 0	**3.64** (1.14)	**3.83** (0.97)	0.36
	Day 28	**3.25** (0.99)	**3.82** (1.13)	
Lymphocytes (x10^9/L)	Day 0	**1.83** (0.40)	**1.70** (0.42)	0.61
	Day 28	**1.81** (0.29)	**1.77** (0.48)	
Monocytes (x10^9/L)	Day 0	**0.48** (0.11)	**0.50** (0.10)	0.60
	Day 28	**0.47** (0.14)	**0.50** (0.08)	
Immunoglobulins (x10^9/L)	Day 0	**0.02** (0.01)	**0.02** (0.01)	0.31
	Day 28	**0.02** (0.01)	**0.02** (0.01)	

Unless otherwise specified data are reported as mean (SD). Statistical testing for categorical and binary variables from the adverse events was done by Chi-square tests. Mixed model analyses were used to assess differences between groups and timepoints, whereafter post hoc analyses were performed with Bonferroni correction. Stars indicate statistical significant differences between day 0 and 28 within a treatment group (* = P<0.05). The p-value shows the overall effect of treatment on the variable and only when significant, the adjusted p-values from the post hoc tests are shown. EA = adverse event; AF = alkaline phosphatase; ALAT = alanine aminotransferase; ASAT = aspartate aminotransferase; eGFR = estimated glomerular filtration rate; GGT = gamma-glutamyltransferase; MCV = mean corpuscular volume.

day 28 (table 3). Interestingly, we did observe a significant increase in fasted insulin levels and associated HOMA-IR values between day 0 and 28 within both the FFT and placebo group. However, when comparing these two measures between the screening visit and day 28, they were similar. We can only speculate that this drop in insulin levels and associated HOMA-IR value at the baseline visit resulted from the laxative use the day prior to the intervention. Other baseline characteristics remained stable after intervention and were similar between the FFT and placebo group, such as BMI, blood pressure and cholesterol (table 3).

In addition to the OGGT, subjects wore a continuous glucose monitoring (CGM) device (Freestyle Libre) from one week prior till one week after intervention. Looking at the results from the CGM measurements, the FFT and placebo group showed overall similar glucose levels and glucose variability markers after intervention (table S2). However, within the FFT group we identified a nominal significant improvement from 95.5% to 97.5% in the time between 3.9-10 mmol/L glucose after intervention (p-value = 0.02, Wilcoxon signed rank test, figure 1F). This indicated an improvement in glucose variability within the FFT group in the week after intervention.

Table 3: Changes in physical and metabolic variables after intervention.

		Placebo (n = 12)		Fecal Filtrate (n = 12)		*p-value*
BMI (kg/m²)	Day 0	**35.8** (4.0)		**35.3** (5.6)		*0.75*
	Day 28	**36.1** (3.9)		**35.3** (5.8)		
WHR	Day 0	**0.97** (0.09)		**0.99** (0.07)		*0.58*
	Day 28	**0.97** (0.09)		**0.99** (0.08)		
Systolic BP (mmHg)	Day 0	**134** (15)		**130** (17)		*0.39*
	Day 28	**134** (16)		**126** (17)		
Diastolic BP (mmHg)	Day 0	**88** (11)		**83** (14)		*0.37*
	Day 28	**86** (9)		**84** (14)		
Pulse (beats/min)	Day 0	**66** (9)		**70** (13)		*0.33*
	Day 28	**65** (10)		**70** (11)		
Glucose (mmol/L)	Day 0	**5.5** (0.4)		**5.8** (0.5)		*0.19*
	Day 28	**5.7** (0.5)		**5.9** (0.5)		
Insulin (nmol/L)	Day 0	**71** (34)	*	**72** (26)	**	*0.76*
	Day 28	**87** (30)		**93** (34)		
HOMA-IR	Day 0	**2.41** (1.21)	*	**2.57** (0.97)	**	*0.55*
	Day 28	**3.05** (1.06)		**3.41** (1.28)		
HbA1c (mmol/mol)	Day 0	**36.8** (2.6)		**35.4** (4.7)		*0.53*
	Day 28	**35.5** (2.5)		**35.0** (4.5)		
Cholesterol (mmol/L)	Day 0	**4.87** (0.79)		**5.38** (1.32)		*0.28*
	Day 28	**4.92** (0.78)		**5.33** (1.15)		
HDL (mmol/L)	Day 0	**1.05** (0.20)	**	**1.14** (0.18)		*0.41*
	Day 28	**1.15** (0.30)		**1.21** (0.17)		
LDL (mmol/L)	Day 0	**3.06** (0.77)		**3.43** (1.08)		*0.32*
	Day 28	**3.03** (0.88)		**3.42** (0.99)		
Triglycerides (mmol/L)	Day 0	**1.68** (0.61)		**1.80** (0.61)		*0.96*
	Day 28	**1.66** (0.88)		**1.56** (0.64)		
CRP (mg/L)	Day 0	**3.0** (2.6)		**5.1** (4.6)		*0.17*
	Day 28	**2.9** (2.9)		**4.6** (3.4)		

Unless otherwise specified, data are reported as mean (SD). Mixed model analyses were used to assess differences between groups and timepoints, whereafter post hoc analyses were performed with Bonferroni correction. Stars indicate statistical significant differences between day 0 and 28 within a treatment group (* = $p<0.05$; ** = $p<0.005$). The p-value shows the overall effect of treatment on the variable and when significant, the adjusted p-values from the post hoc tests are shown. BMI = Body Mass Index; WHR = waist-hip ratio; BP = blood pressure; HOMA-IR = Homeostatic Model Assessment for Insulin Resistance; HDL = high-density lipoprotein; LDL = low-density lipoprotein; CRP = C-reactive protein.

Bacterial and viral diversity remain stable after FFT

To assess the effect of the FFT on the bacteriome and phageome, we collected multiple faecal samples from baseline up to day 28, and performed whole genome shotgun (WGS) sequencing (figure 2A)[33]. The phage populations derived from this WGS fraction mainly consist of integrated prophages. To study phage virions, VLPs were isolated from the same faecal samples, lysed, and the purified DNA was shotgun sequenced as previously described[19]. After combining all viral sequences from WGS and VLP fractions, we clustered them at 95% similarity into viral populations (VPs), a level comparable to species in bacteria[34].

Analysis of beta diversity showed that both the VLP and WGS phageomes were indistinguishable between donor and MetSyn participants at baseline (VLP (figure S2A), PERMANOVA $p = 0.725$; WGS (figure S2B), PERMANOVA $p = 0.672$). While this defies our earlier findings[19], this is likely due to the highly individual-specific viromes and the relatively small size of our study. Notably, the VLP phageome was radically different from the WGS phageome (figure S2C, PERMANOVA $p = 0.001$). This is one of the first studies to directly compare the VLP and WGS phageomes within the same patient longitudinally, as previous studies used different cohorts to compare the VLP and WGS phageomes[8].

Next, we looked at the effect of FFT on the bacterial and viral richness (figure 2B) and alpha diversity in MetSyn subjects (figure 2C). These were comparable throughout the study between the FFT and placebo intervention. Interestingly, in both groups the bacterial richness and α-diversity reduced slightly the first days after the intervention, which was resolved by day 14 to 28, though these decreases were non-significant ($p > 0.05$, Wilcoxon signed rank test). A similar non-significant trend was observed for the richness and diversity of the WGS phageome, which consists mainly of prophages that could have been depleted with their bacterial hosts. In contrast, the richness of the VLP phageome increased slightly by day 2 in both groups, while the α-diversity decreased only in the placebo group, albeit non-significant ($p > 0.05$, Wilcoxon signed rank test).

Increase in new phages independent of the intervention

Since we expected transfer of donor phages to the recipients, we looked at the abundance of phages shared between donor and recipient before and after the FFT. Although not significant, after FFT the VPs shared with the donor within the WGS phageome increased up to day 14 ($p = 0.2$, Wilcoxon signed rank test, figure 3A). For the VLP phageome we found an opposite effect, where the VPs shared with the donor decreased non-significantly after the FFT ($p = 0.3$, Wilcoxon signed rank test, figure 3B). The broader effect of the FFT on the phageomes was determined by analysing the abundance of new phages that appeared after the FFT within the WGS phageome (figure 3C) and within the VLP phageome (figure 3D). In

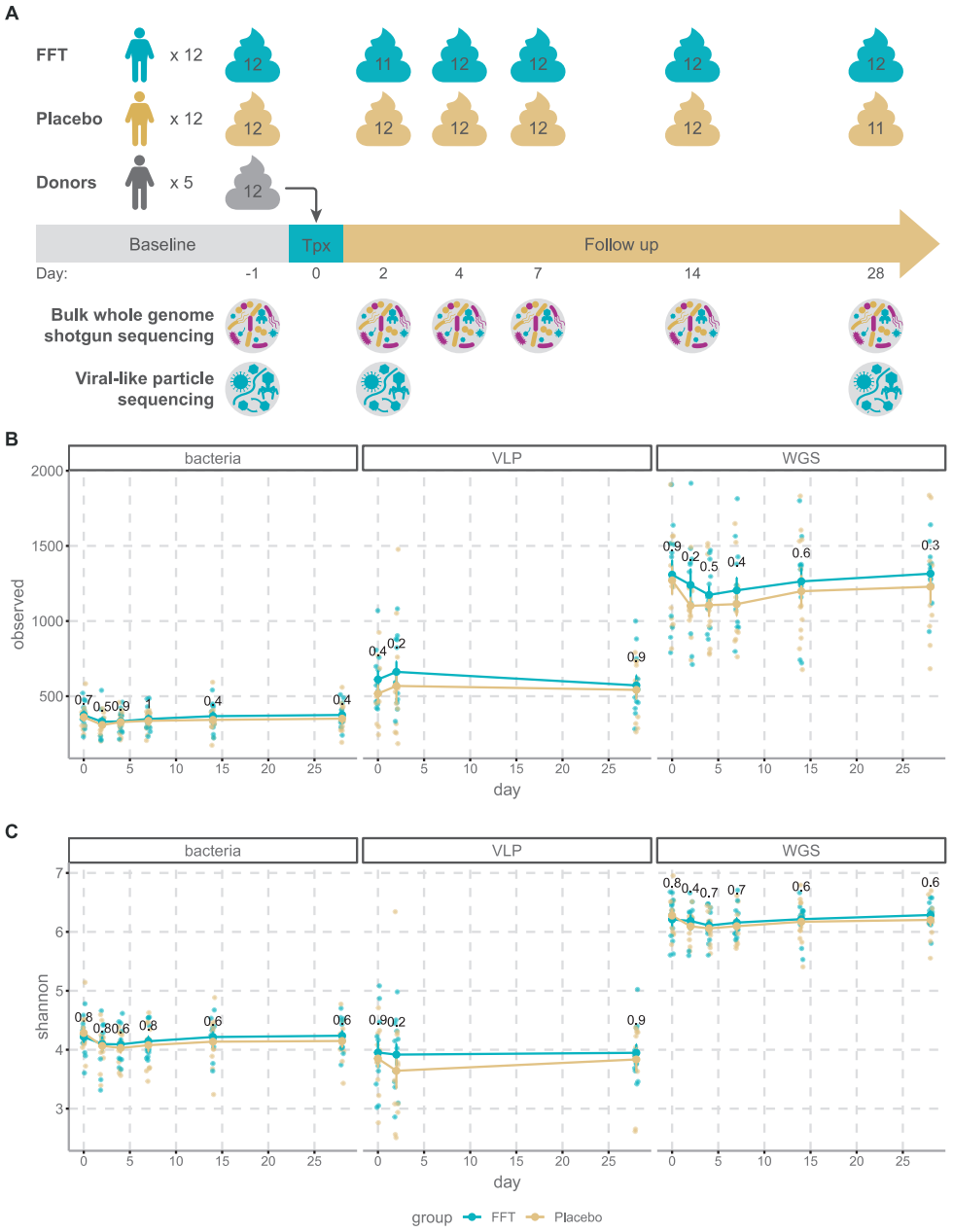

Figure 2: A) Overview of the faecal samples used for the bulk metagenomic sequencing (for bacteriome and phageome) and the metagenomic sequencing of the viral-like particles (VLP). **B)** The richness (number of observed species) in the bacteriome, phage virions (VLP) and bulk-derived phageome (WGS) from baseline until follow-up at day 28. Though there were no significant differences between the placebo and faecal filtrate group, the richness in the bacteriome reduced slightly after both interventions. A similar trend was observed in the phageome (mostly prophages present in bacterial hosts), while the richness in the VLP fraction tended to increase slightly at day 2 for both interventions. **C)** The alpha-diversity (Shannon index) of the bacteriome, phage virions (VLP) and bulk-derived phageome (WGS) from baseline until follow-up at day 28. Again, no significant differences were found between the interventions. Similar to the richness, the diversity of the bacteriome and phageome slightly decreased directly after the interventions. For the free phages, the diversity decreased slightly in the placebo group, but not in the faecal filtrate group.

both groups the abundance of new phages increased over time and although not significant, this increase was slightly higher in the FFT group, especially in the VLP phageome on day 2 (p = 0.2, Mann-Whitney U test). These results seem to indicate that the phageomes were perturbed in both the placebo and FFT groups. It further shows that donor-derived phages, especially the VLPs, were either mostly immediately removed from the gut or their engraftment was balanced with the removal of pre-existing VPs shared with the donors.

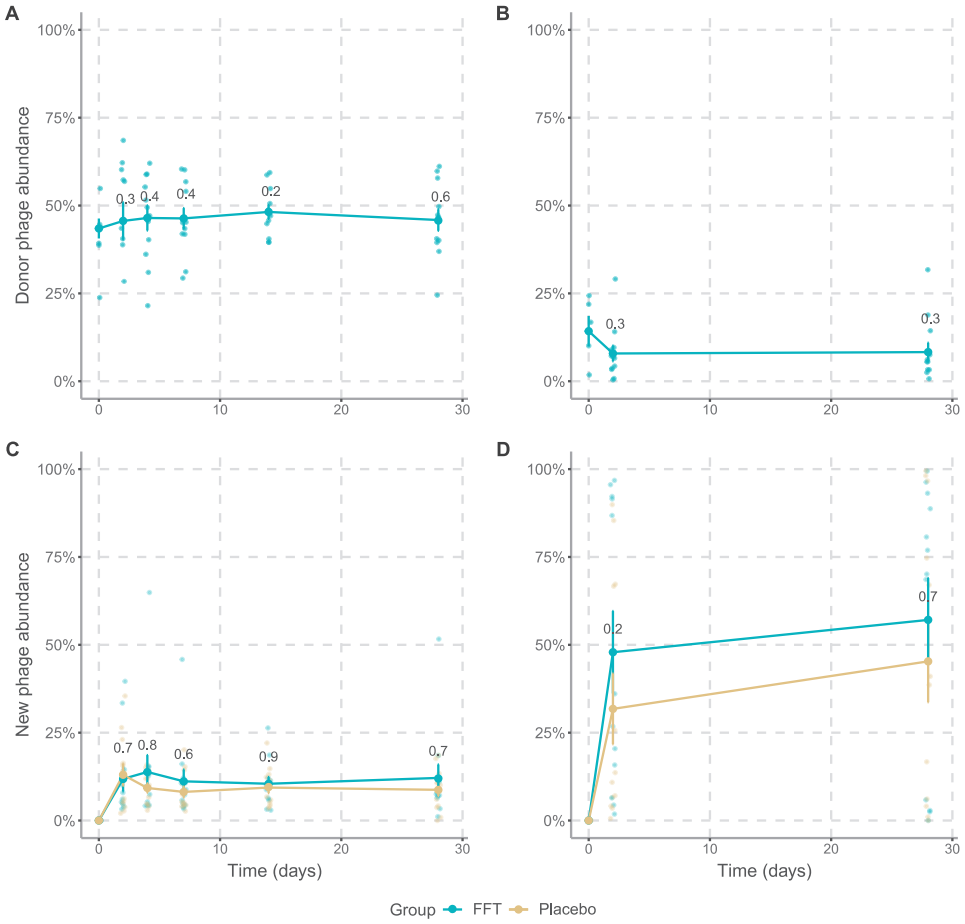

Figure 3: A) The percentage of phages that were shared between the donor and recipient within the phageome and **B)** within the phage virions after the faecal filtrate transplantation. There was a slight, non-significant increase in the relative abundance of (pro)phages shared with the donor after the intervention, while the relative abundance of phage virions that were shared with the donor slightly decreased. **C)** The percentage of new phages that were present after the intervention within the bulk-derived phageome and **D)** within the phage virions. In both, the relative abundance of new phages increased over time and although not significant, this increase was slightly higher in the FFT group.

FFT alters the phage composition of the VLP fraction

Subsequently, we looked at compositional changes within the bacteriome, WGS phageome, and VLP phageome (figure 4A). Principal response curves showed no overall effect of the FFT on any of these communities compared to placebo, except for a significantly different composition of the VLP phageome on day 2 (p=0.02, PERMANOVA). This difference in composition within the VLP phageome on day 2 was also evident from a separate principal component analysis (p=0.028, PERMANOVA, figure 4B). As this pointed toward a short-term effect of the FFT, we looked more specifically into VLP communities on day 2 and found 216 VPs that were differentially abundant between the FFT and placebo groups (figure 4C and table S3).

To get a better understanding of these phages, we looked at the bacterial host species that these differentially abundant VPs can infect. We observed 6 bacterial host species and 5 bacterial host genera of which the phages were significantly enriched among these VPs (figure 4D). The phages infecting some of these host bacteria, like *Roseburia intestinalis* and *Bacteroides* species, were differentially abundant in both FFT and placebo treatment groups. But others, like *Sutterella wadsworthensis* and *Scatocola faecigallinarum,* were notably exclusively differentially abundant in one of the two treatment groups. The only host species enriched among differentially abundant VPs and more prevalent in the placebo group was *S. wadsworthensis*, a betaproteobacterium associated with gastrointestinal infections. Those more prevalent among the FFT group were taxonomically diverse, belonging to the *Bacteroidetes* (*Bacteroides spp.*), *Firmicutes* (*R. intestinalis*, *Faecousia*, *and CAG-882*), and *Proteobacteria* (*S. faecigallinarum* and *CAG-267)*.

FFT induces an antagonistic phage-microbe interaction

Intrigued by the presence of differentially abundant VPs two days after FFT, we determined whether the dynamics between phages and their microbial hosts had changed. For this, we linked VPs to metagenome assembled genomes (MAGs) from our WGS sequencing dataset and calculated the mean abundance change for all VP-MAG pairs belonging to a given species in a given sample. This showed opposing relationships between MAG and VP abundance in the first two days of the intervention (figure 5A): this was negatively correlated for the FFT group ($R = -0.13$, $p = 0.005$) and positively correlated in the placebo group ($R = 0.17$, $p < 0.001$). These results could indicate a difference in the ecological dynamics between the two sample groups, where the FFT group was dominated by lytic phage-bacterium interactions, while they were more likely to be lysogenic or chronic in the placebo group. These effects intriguingly were less pronounced when comparing days 2 and 28 (FFT: $R = -0.043$, $p = 0.24$; placebo: $R = 0.12$, $p = 0.004$; figure 5B), and completely absent when comparing days 0 and 28 (figure 5C). Thus, the overall effect of the FTT on phage-host interactions seemed pronounced but short-lived.

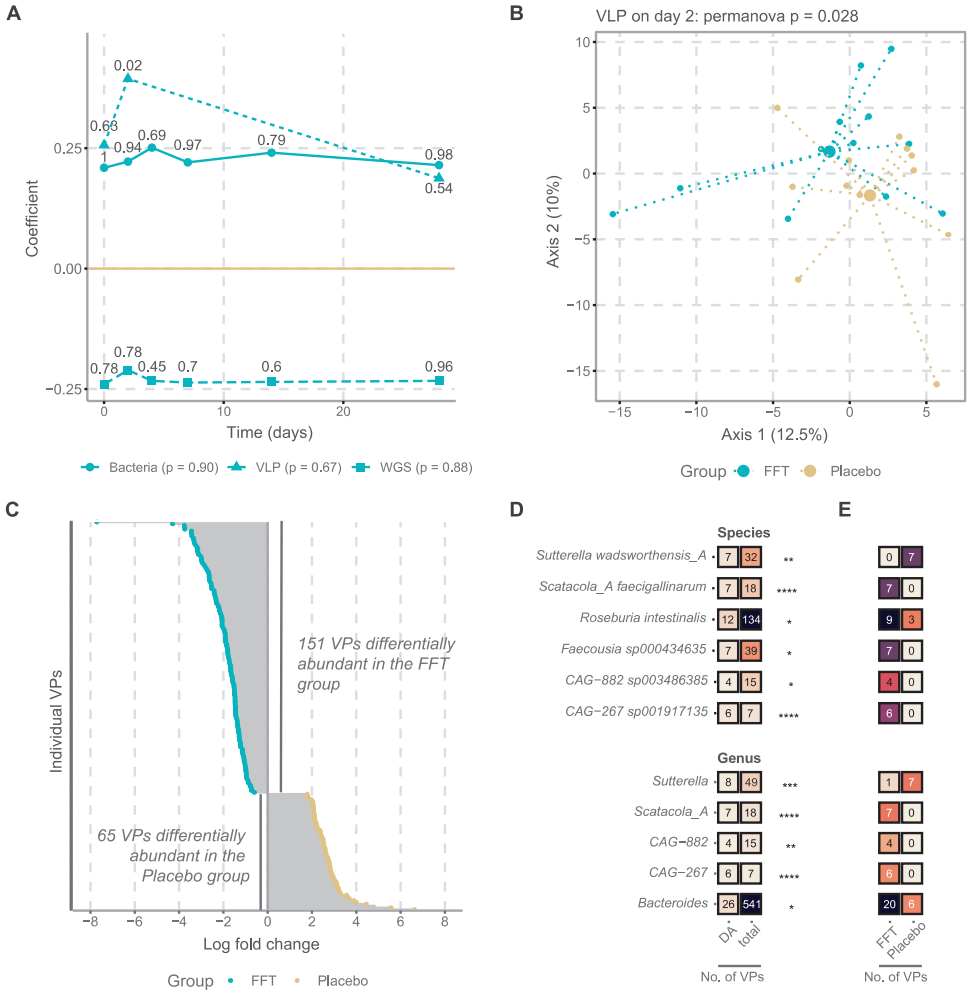

Figure 4: A) Principal response curve showing how the FFT group differs from the placebo (set to zero) in the bacteriome, bulk-derived phageome (WGS), and phage virion (VLP) composition. The coefficient is the canonical coefficient of treatment and significance in dispersion over time and at each separate time-point was calculated with permutation tests, corrected for multiple testing by the Benjamini-Hochberg method. **B)** Principal component analysis of VLP composition after centered log-ratio transformation. Large points show the mean of each group. **C)** Log fold change for all 216 viral populations (VP) indicated as differentially abundant by ANCOM-BC analysis. For legibility, VP names are not shown, these can be found in supplementary table S4. **D)** Bacterial host species of which the phages are enriched among differentially abundant VPs. The first column shows the number of differentially abundant VPs, the second the total number of VPs linked to a given host in the dataset, and stars show the level of significance after testing for enrichment with a hypergeometric test, adjusted for multiple testing by the Benjamini-Hochberg method. **E)** splits up the data on the first column of D by participant group.

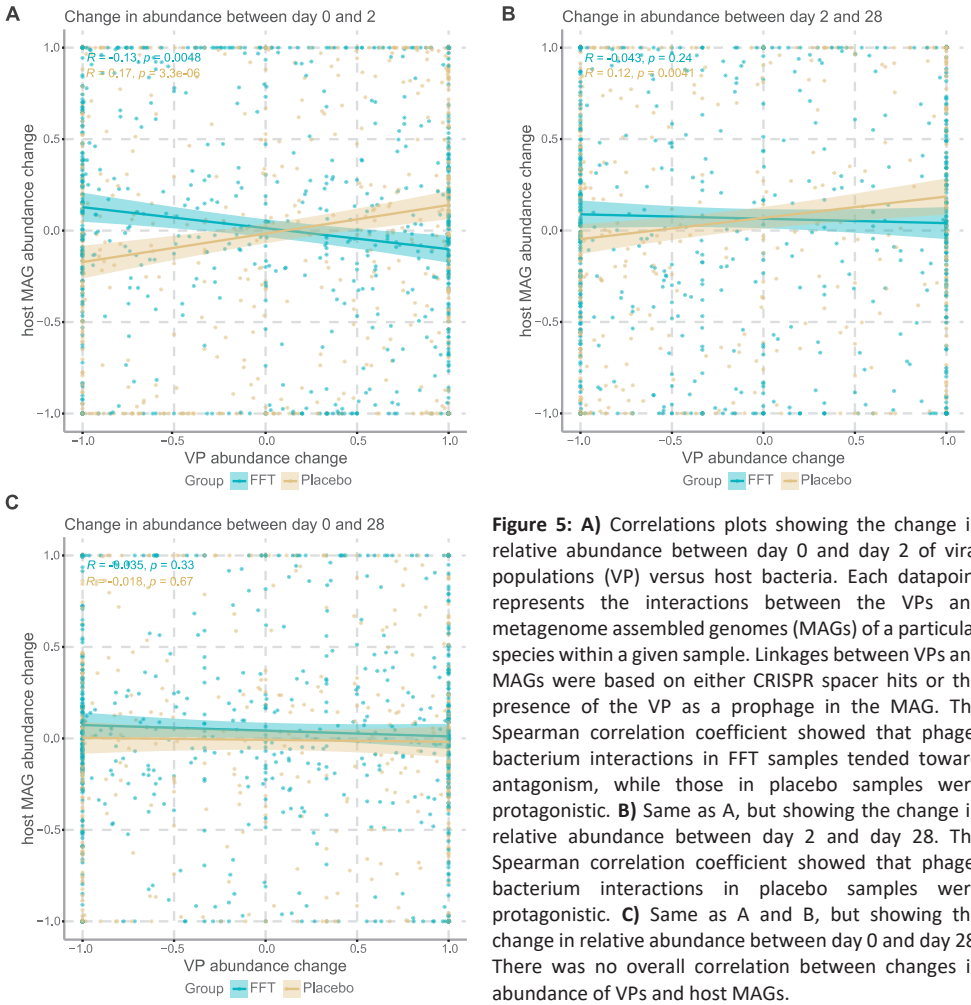

Figure 5: A) Correlations plots showing the change in relative abundance between day 0 and day 2 of viral populations (VP) versus host bacteria. Each datapoint represents the interactions between the VPs and metagenome assembled genomes (MAGs) of a particular species within a given sample. Linkages between VPs and MAGs were based on either CRISPR spacer hits or the presence of the VP as a prophage in the MAG. The Spearman correlation coefficient showed that phage-bacterium interactions in FFT samples tended toward antagonism, while those in placebo samples were protagonistic. **B)** Same as A, but showing the change in relative abundance between day 2 and day 28. The Spearman correlation coefficient showed that phage-bacterium interactions in placebo samples were protagonistic. **C)** Same as A and B, but showing the change in relative abundance between day 0 and day 28. There was no overall correlation between changes in abundance of VPs and host MAGs.

DISCUSSION

This is the first randomized controlled clinical trial in which a sterile faecal filtrate was administered to human individuals. In the present study, the FFT was well-tolerated and safe, with recipients experiencing solely mild gastrointestinal adverse effects. As the study group is small, larger studies with a longer follow-up are warranted to fully assess the safety profile of the FFT. However, compared to FMTs, an FFT depleted of living microorganisms has a lower risk of transferring unknown pathogenic bacteria[27]. Since FMT has a good safety profile[35,36], this most likely holds true for FFTs as well. Compared to FMTs, it is relatively easier to further optimise and standardise FFTs, e.g., through lyophilization and encapsulation of faecal filtrate, as the viability of the many strict anaerobic bacteria does not have to be preserved. Such developments of FFT will ease the administration, reduce

the invasiveness, and provide an option for prolonged or maintenance therapy, even in a home-setting.

While we did find a slight improvement of the glucose variability in the FFT group, expressed as the time between 3.9-10 mmol/L glucose, the FFT and placebo groups showed similar glucose excursions during the OGGT performed at day 28. Previously, an FVT in diet-induced obese mice reduced weight gain and improved blood glucose tolerance[30]. However, FVTs differ slightly from FFTs, with phages being more concentrated and washed to reduce bacterial debris, metabolites and antimicrobial peptides. Moreover, in this previous study, several donor phageomes were combined, resulting in a highly diverse phageome. In addition, compared to humans, microbiomes of mice are more similar due to the same housing and diet[37], thereby increasing the chance of highly specific bacteriophages encountering their host and, subsequently, modulating the microbiota. In humans, improvement of insulin sensitivity after lean healthy donor FMT in individuals with MetSyn has been reported[28,29]. These studies had a comparable study design as present study, with the major difference being the absence of the faecal bacteria in the intervention. Although this is not a direct comparison, we speculate that, in the case of MetSyn, the beneficial bacteria transplanted during an FMT significantly contribute to the improved glucose metabolism observed.

Nevertheless, the FFT was able to alter the phage virions or VLP phageome composition on day 2 compared to the placebo, showing 216 differentially abundant VPs. By day 28 this significant difference disappeared, which indicates the FFT effect was short-lived. Looking at the bacterial hosts of these differentially abundant phages, we found six host species that were significantly enriched, of which five were more prevalent in the FFT group. One of these bacterial hosts is the butyrate producer *Roseburia intestinalis*, which has been found to be depleted in MetSyn[38,39] and contributes to inflammatory signalling inhibition and intestinal barrier repair[40,41]. While the other bacterial species have not been directly linked to MetSyn previously, some of their relatives within the *Oscillospiraceae* (*Faecousia sp000434635*) and *Lachnospiraceae* (*CAG−882 sp003486385*) have been implicated in obesity and MetSyn[42–45]. In line, the genus *Bacteroides* has been associated with obesity and MetSyn, both positively and negatively[46,47].

In addition, we speculate that the FFT induced virulent interactions between phages and their microbe hosts in the first two days after administration, while the phage-microbe interactions appeared more lysogenic/temperate in the placebo group. These virulent interactions in the FFT group could be the result of donor phages infecting and lysing the bacteria from the recipient. On the other hand, the introduction of novel donor phages could have induced the replication of existing prophages[48], thereby leading to more virulent

interactions. As the number of previously unobserved VLP VPs increased on day 2, while donor-shared VLP VPs did not, we hypothesize the latter is more likely. It could be that some non-phage element of the FFT, such as fructose[49] or a phage-derived peptide[50], prompted integrated phages to excise from their bacterial hosts. Otherwise, it could also be that increased infection of bacteria by donor-derived phages caused lower bacterial abundance, resulting in higher phage lysis rates, in line with the piggyback the winner model of phage-host interactions[51]. Following this hypothesis further, growth of (some) bacterial species after the laxative treatment could have caused increased lysogeny among the phageomes in the placebo group.

Interestingly, changes in bacterial and viral diversity over time were similar between both groups. While we did observe a small, non-significant increase in the abundance of VPs shared with the donor in the WGS phageome, this abundance decreased non-significantly within the VLP phageome. This can in part be explained by the large increase in new phages within the VLP phageome (50-60%), which was bigger compared to the increase within the WGS phageome (~15%). This difference may have been caused by either the absence of low-abundance VPs in the WGS sequencing data due to their relatively smaller sizes, or a difference in community dynamics between VLP and WGS phageomes. The increase in new phages indicates that the phageome was perturbed, leading to an accelerated genomic recombination that stimulated phage evolution. However, since this happened in both groups, we hypothesize that this is, in part, an effect of the laxative pre-treatment. This laxative treatment could have removed pre-existing donor-shared VPs, and, by washing away part of the host bacteria, could have reduced the probability of donor phages infecting their host.

This study has several limitations. Although we did not find an overall effect on glucose metabolism, it is not possible to assess whether the FFT intervention was insufficient to alter the glucose metabolism or whether the effect is obfuscated by the small sample size and large heterogeneity within the MetSyn study population. Our sample-size for the current study was based on the assumption that the FFT would be as effective as an FMT in improving glucose metabolism[28,29], which is probably not the case. Unfortunately, based on current results where we observe a small non-significant increase in glucose AUC in both groups, it is not possible to repeat the power calculation. The increased glucose AUC could be seen as natural progression of MetSyn, but we speculate that this was caused by the laxative pre-treatment, which also reduced the fasting insulin levels and associated HOMA-IR values at baseline. The laxative pre-treatment could also have reduced the FFT efficacy, by reducing the number of potential hosts for the transplanted phages. Therefore, for future studies with FFT, we would highly recommend to omit this step. In addition, pooling

of donor phages and matching donors and recipients, thereby increasing the diversity and likelihood of a phage-host match, could further improve the efficacy of the FFT.

Due the ethical reasons, we had to keep the production of the bacteriophage transplant simple and straightforward, which is why we performed an FFT instead of an FVT in this human intervention study. Therefore, we cannot completely rule out any effect of other compounds present in the filtrate besides the phages, such as bacterial debris, metabolites and antimicrobial peptides. In line, we performed tangential flow filtration with sterile, single-use cassettes with a 0.2 µm membrane to reduce the potential risk of cross-contamination between donors. However, not all phages may pass through these pores and a pore size of 0.45 µm will result in higher phage titres, as has been described previously[52]. Our analysis focused on bacteriophages, while we likely also transferred eukaryotic viruses. However, as only 0.044±0.3% (median: 0%) of reads mapped to such viruses, we could not ascertain whether these had an effect. In addition, we focussed on dsDNA phages. Although these phages form the majority of gut phages[7,8], for future studies it would also be interesting to include the ssDNA, dsRNA and ssRNA viruses. The small sample size and large heterogeneity did not allow for any post hoc sex-based analyses. Finally, since we only included Dutch European subjects, the generalizability of our results to other populations is limited.

Besides above-mentioned suggestions for future FFT studies, future research should focus on targeting specific bacteria with phages to get a better mechanistic understanding of how bacterial communities are changed upon phage predation and how these changes could affect disease phenotypes. One example of specific phages targeting pathogenic bacteria is the phage cocktail developed to treat recurrent *Clostridioides difficile* infections[53]. Another interesting target are the *Lactobacillaceae* that are thought to produce ethanol and thereby contribute to non-alcoholic fatty liver disease (NAFLD)[54]. It should be noted that such precision therapy might be very efficient at clearing a specific pathogen, but will unlikely restore any underlying microbial dysbiosis. Therefore, a combination of endogenous phages to modulate a complete microbiome should be further studied, e.g., by matching donors and recipients based on their phageome and bacteriome composition, respectively.

In conclusion, this is a first double-blind, randomized, placebo-controlled trial in which we performed an FFT in human individuals with MetSyn. We provide evidence that gut phages from a healthy donor can be safely administered to transiently alter the gut microbiota of recipients. This study provides a critical basis for follow-up studies, which should better match donors and recipients based on their bacteriome and phageome composition.

METHODS

Study design

We set up a prospective, double-blinded, randomised, placebo-controlled intervention study that was performed in our academic hospital in the Netherlands. After passing screening, 24 subjects with MetSyn were randomised to receive a sterile FFT from a lean healthy donor or a placebo transplant. Prior to the intervention and after 28 days at follow-up, subjects underwent an OGTT to assess their glucose metabolism. In addition, a week prior to one week after intervention, subjects monitored their blood glucose using a flash glucose monitoring device (Freestyle Libre). Fecal samples were collected at multiple timepoints between baseline and follow-up to study dynamic changes in the microbiome. Finally, during every study visit a medical exam was conducted in addition to blood plasma collection to assess the safety of the intervention. Figure 1A provides a schematic overview of the study.

Study subjects

Study participants were all European Dutch, overweight (body mass index (BMI) ≥ 25 kg/m^2) subjects between 18 and 65 years of age and had to meet the National Cholesterol Education Program (NCEP) criteria for the metabolic syndrome[31]. Both male and female participants were included in the study and sex was self-reported. Main exclusion criteria were the use of any medication, illicit drug use, smoking, or alcohol abuse in the past 3 months, as well as a history of cardiovascular, gastrointestinal, or immunological disease. Table S4 summarises all in- and exclusion criteria.

Donor screening

Faeces donors were lean healthy European Dutch subjects who were thoroughly screened according to the guidelines of the European FMT Working Group[55]. Screening of potential donors was performed in a stepwise manner as previously published[32]. Briefly, potential donors first completed an extensive screening questionnaire. If they passed this stage, their faeces were screened for pathogenic parasites. When negative, several faecal samples were screened for presence of pathogenic bacteria, viruses, and multidrug resistant organisms (MDROs), as well as the level of calprotectin. Donors screened after May 2020 were additionally screened for severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)[56]. In addition, blood was collected for serological testing and to screen for an abnormal liver or renal function or an impaired immunity. When donors passed this screening, they were allowed to donate faeces for a period of 6 months. Table S5 lists the specific in- and exclusion criteria for faeces donors. Every two months, active donors underwent a short rescreening, which included, among others, screening for MDROs and SARS-CoV-2. In addition, before every donation, donors had to complete a shortened questionnaire to

confirm their eligibility. We matched donors and recipients based on their sex and whether they have had a prior infection with cytomegalovirus or Epstein–Barr virus.

Sterile faecal filtrate production and administration

Production of the sterile faecal filtrate started the day before administration to the MetSyn subjects. First, 50 g of stool was collected from a screened donor, which was homogenized with 500 ml sterile saline. Large particles were filtered from the faecal suspension using double sterile gauzes. Most of the bacteria were removed in two subsequent centrifugation steps, in which the suspension was spun for 1 hour at 10.000 x g. Finally, the supernatant was filtered through a sterile 0.2 µm membrane using a tangential flow filtration device (Vivaflow 50). Production of the filtrate from donor stool was performed within 6 hours and took, on average, 334 minutes (SD = 27). The filtrate was stored overnight in a fridge until administration. The production is depicted in figure S3A.

The sterile faecal filtrate was administered to the patient via a nasoduodenal tube. The day prior to the administration, subjects were asked to clean their bowel using a laxative (Klean-Prep®, Norgine B.V.), which is a standard pre-treatment for FMT procedures in our hospital. Nasoduodenal tubes were placed with the help of a Cortrak®2 enteral access system (Avanos Medical Inc.), making sure the nasoduodenal tube was correctly positioned. The faecal filtrate was slowly infused with a 60 ml syringe, on average 300 ml during a 15-20 min period. Figure S3B provides a schematic overview of the FFT procedure.

During the optimisation of the tangential flow filtration, we quantified the VLP numbers of the faecal filtrates from four different donors, as previously described[57,58]. Briefly, faecal filtrates were concentrated, from which VLPs were isolated with caesium chloride density gradient centrifugation, stained with SYBR Gold and counted by epifluorescence microscopy. Faecal filtrates contained on average 1.25E+08 VLPs/ml (SD 0.45E+08), which is in line with previous publications[59,60]. We confirmed the absence of bacteria from the faecal filtrate with a qPCR for the bacterial 16S rRNA gene as previously described[61], showing a 10^5-fold decrease in bacterial DNA (figure S3C). We further confirmed this by culturing of the faecal filtrate using Biosart® 100 monitors (Sartorius). 100 ml of faecal filtrate was filtered and the cellulose nitrate membranes were incubated on petri dishes with Columbia agar + 5% sheep blood (bioMérieux) for two days at 37°C under both aerobic and anaerobic conditions. We did not observe any colony-forming units in 100 ml of faecal filtrate (results not shown).

Outcomes

The primary outcome was change in glucose metabolism, as determined by the AUC for glucose excursion during the OGTT. Secondary outcomes related to glucose metabolism

were changes in fasting glucose, insulin, HOMA-IR, and HbA1c between baseline and follow-up after 28 days, as well as changes in glucose variability measured by CGM a week before and after intervention. Other secondary outcomes were the dynamic changes in gut bacteriome and virome populations following FFT or placebo intervention and the comparison of phage composition between lean donors and subjects with MetSyn. Finally, we assessed the safety of the FFT as determined by the occurrence of (serious) adverse events, physical exam, and several blood parameters for renal and liver function and inflammation.

Sample size calculation

Based on previous data from our group in which individuals with MetSyn received an FMT[28,29], and the hypothesis that a faecal phage transplant can be equally effective as a traditional FMT[25–27,30], we assumed a 15% improvement in glucose tolerance upon FFT. With a two-sided 5% significance level and a power of 80%, a sample size of 12 patients per group was necessary, given an anticipated dropout rate of 10%. To recruit 24 individuals with MetSyn, we anticipated a 12-month inclusion period.

Randomisation

Data were captured with electronic case report forms build in Castor EDC[62]. In CASTOR, subjects were randomly assigned to an intervention by block randomization with stratification for age and sex, and block sizes of 4, 6, and 8. The day prior to the intervention, both the faecal filtrate and placebo (sterile saline with brown colour) were prepared and stored overnight. Both the faecal filtrate and placebo looked identical. A randomisation assistant unblinded for the treatment allocation prepared the correct solution for administration and destroyed the other. The investigator administered the allocated treatment in blinded syringes and trough an opaque nasoduodenal tube, making sure both participants and investigator were blinded for the intervention throughout the study.

Oral glucose tolerance test

For the OGTT, overnight fasted subjects ingested a standardized glucose solution (75g). Blood was drawn from an intravenous catheter at baseline and 15, 30, 45, 60, 90, and 120 minutes after ingestion. Both blood serum and plasma were aliquoted and stored at -80°C. From these aliquots we measured glucose and C-peptide, which was performed by the Endocrinology department of the Amsterdam UMC. In addition, additional blood samples collected at baseline and follow-up were used to measure fasted glucose, insulin, HbA1c, and the clinical safety parameters for renal/liver function and inflammation, all measured by the Central Diagnostics Laboratory of the Amsterdam UMC.

Continuous glucose monitoring

To reduce the study burden and prevent daily finger pricks, we used a continuous glucose monitoring device (Freestyle Libre) to monitor blood glucose, which allowed subjects to perform all normal activities while wearing the sensor. Subjects were taught to subcutaneously implant the CGM sensor and were instructed to extract the data from the sensor at least every 8 hours. One week prior to the intervention subjects started to monitor their glucose until one week after the intervention. Compliance among participants was good, with a median 100% (range 76-100%) of data correctly collected, during a median period of 14 (range 11-27) days with a median 1350 (range 1043-2617) sensor readings. During that same period, participants were asked to record their diet using an online food diary (Eetmeter from the Voedingscentrum)[63]. At the follow-up visit, data from the CGM scanner were exported and analysed with a previously published R package for CGM data analysis[64].

Faeces collection

The day before the intervention and 2, 4, 7, 14, and 28 days thereafter, subjects were asked to collect several faecal samples. Faeces were collected by participant in stool collection tubes, which were directly stored in a freezer at home inside a safety bag. In addition, participants registered the time, date, and consistency of the collected faeces according to the Bristol Stool Chart. At the baseline and follow-up visits, these faecal samples were transported to the hospital frozen, where they were directly stored at -80°C until the end of the study.

Bacteriome and virome sequencing

To study the bacteriome and virome, we performed whole genome shotgun (WGS) sequencing. From the stored frozen faeces samples, total genomic DNA was extracted using a repeated bead beating method as described previously[33]. Extracted DNA was stored at -20°C and shipped on dry ice to Novogene (Cambridge, United Kingdom). Libraries for shotgun metagenomic sequencing were prepared using the NEBNext Ultra II Library prep kit (New England Biolabs Cat#E7645L) and sequenced on an Illumina HiSeq instrument with 150 bp paired-end reads and 6 Gb data/sample. Figure S3D summarises the sequencing and bioinformatics pipeline used. For both the WGS and VLP sequencing (see below) negative controls were included to check for contamination during DNA extraction and library prep. These negative controls did not yield any measurable DNA after library prep and were therefore not sequenced. No mock communities were included as positive controls in the current sequencing pipeline.

VLP sequencing

To study phage virions, we isolated the faecal VLP fraction and sequenced dsDNA phages as previously described[19]. Briefly, the VLPs were extracted from 500 mg of faeces using high-speed centrifugation followed by filtration through a 0.45 µm membrane. Any free-DNA debris was digested prior to lysing the VLPs, whereafter the DNA was purified using a two-step phenol/chloroform extraction protocol. Finally, the DNA was purified using the DNeasy Blood&Tissue kit (Qiagen Cat#69506) according to the manufacturer's protocol. Library preparation was done with the NEBNext Ultra II FS DNA library prep kit (New England Biolabs Cat#E7805L) and the NEBNext Multiplex Oligos for Illumina dual indexes (New England Biolabs Cat#E7600S) according to manufacturer's instructions. Quality and concentration of the VLP libraries were assessed with the Qubit dsDNA HS kit (ThermoFisher Cat#Q32854) and with the Agilent High Sensitivity D5000 ScreenTape system (Agilent Technologies). Libraries were sequenced using 2x150 bp paired-end chemistry on an Illumina NovaSeq 6000 platform with the S4 Reagent Kit v1.5, 300 cycles (Illumina Cat#20028312) at the Core Facility Genomics of the Amsterdam UMC.

Sequence assembly

Sequencing resulted in an average of 21.7 ± 3.5 M reads per WGS sample (median: 22.4 M reads), and 23.6 ± 18.3 M per VLP sample (median: 18.1 M reads). Before assembly, reads belonging to the same participant were concatenated. Adapter sequence removal and read trimming were performed with fastp v0.23.2 (option –detect_adapter_for_pe)[65]. As previously recommended[66], reads were then error corrected with tadpole (options mode=correct, ecc=t, prefilter=2), and deduplicated with clumpify (options dedupe=t, optical=t, dupedist=12000), both from bbmap v38.90 (https://jgi.doe.gov/data-and-tools/bbtools). High-quality reads from WGS samples were then cross-assembled per participant using metaSPAdes v3.15.5[67] (option --only-assembler). Due to their great complexity, we were unable to assemble some of the VLP samples. We thus assembled these with MEGAHIT v1.2.9[68], which we did for all VLP samples to keep methodological consistency.

Viral sequence recognition and clustering

To identify viral sequences among the WGS and VLP assemblies, contigs longer than 5000 bp were analysed with virsorter v2.2.3[69] (option --exclude-lt2gene) and checkv v1.0.1[70]. Contigs were taken to be of viral origin if at least one of the following criteria was true: checkv identified at least one viral gene, VirSorter2 gave a score of at least 0.95, VirSorter2 identified at least 2 viral hallmark genes, checkv identified no viral or bacterial genes. In total, we selected 53,204 contigs with at least 1 viral gene, 782 with a virsorter2 score of > 0.95, and 1 with at least 2 viral hallmark genes. The resulting viral sequences were then deduplicated at 100% with bbdupe from bbmap v38.90 (option minidentity=100). This

resulted in a non-redundant database of 50,724 viral contigs, which were subsequently clustered at 90% average nucleotide identity (ANI) into viral populations (VPs) using blastn all-vs-all searches with BLAST v2.12.0+[71]. The longest contigs in each VP were further clustered into viral clusters (VCs) by vContact2 v0.11.3[72]. Since the conclusions of the analyses were identical regardless of whether they were performed with VPs or VCs, only VP-level analyses were reported.

Viral read depth determination

Viral relative abundance was determined by mapping high-quality reads from each sample (i.e., one mapping per participant and time-point) against non-redundant viral sequences with bowtie2 v2.4.2[73]. Following earlier recommendations[74], contigs were considered to be present if at least 75% of their bases were covered by at least 1 read mapped with over 90% ANI. To determine this, reads mapping with less than 90% ANI were removed from alignments with coverm filter v0.6.1 (option --min-read-percent-identity 90, https://github.com/wwood/CoverM), and coverage was determined with bedtools genomecov v2.27.1[75] (option -max 1). Read counts per contigs were then determined with samtools idxstats v1.15.1[76], and those with a horizontal coverage of <75% were set to zero. Read counts and contig lengths were summed per VP, and reads per kilobase per million mapped reads (RPKM) values were calculated to take differential contig lengths.

Bacterial community profiling and binning

Bacterial population compositions of WGS samples were profiled per participant and time point with mOTUs v3.0.3[77]. Binning contigs into metagenome assembled genomes (MAGs) was done per participant. First, high quality reads from each time-point were mapped to cross-assembled contigs of at least 2500 bp with bowtie2 v2.4.2. Read depth tables were then constructed with jgi_summarize_bam_contig_depths v2.15, and contigs were binned with metabat2 v2:2.15[78]. Completion and contamination of putative MAGs were then determined using checkm v1.2.1[79] and, like was previously done[80], MAGs were considered for further analysis if completeness - (5 x contamination) was at least 50. Taxonomy of such MAGs was determined with GTDB-Tk v2.1.1[81] using the R207-v2 database package. This resulted in a database of 3011 MAGs with an assigned taxonomy.

Determining phage-host links

Viral sequences were linked to bacterial MAGs in two ways. Firstly, if a viral contig was contained within a MAG, it was considered to be a prophage. Secondly, viral contigs were linked to MAGs using CRISPR spacer hits. For this, CRISPR spacer arrays were identified among MAGs using CRISPCasFinder v4.2.20[82]. CRISPR spacers between 20 and 30 bp in length were then matched to viral contigs through a blastn search with BLAST v2.12.0+

(options -task blastn-short). Spacer hits were finally filtered for those with 2 or fewer mismatches, minimizing the risk of spurious hits.

Statistical analyses

Richness, α-diversities, principal component analysis (PCA), and principal response curves (PRC) were all calculated with the vegan R package[83]. For richness and α-diversity RPKM values were used, while PCAs and PRCs used centered log ratio (clr)-transformed data so as to account for the compositionality of the data[84]. Before clr-transformation, VPs of low abundance and prevalence were removed by removing those with total RPKM of <100 over all samples, as well as those with RPKM values of >20 in less than 10% of samples. Significance levels of PCAs were calculated with a permutational analysis of variance (PERMANOVA) test, as implemented in the vegan R package v2.6-4 and were controlled for age and sex. For the PRC-analysis, the permutest function was used to calculate significance. Both PERMANOVA and permutest used 1000 permutations. p-values were adjusted for multiple testing using the Benjamini-Hochberg approach where necessary. General linear models were constructed with the glmmPQL function from the MASS R package v7.3-58.1 with the age, sex, day, group, and day:group as fixed effects and participants as random effect.

Differential abundance

Differential abundance of VPs among VLP samples on day 2 was determined with ANCOM-BC v1.2.2[85]. Input of ANCOM-BC consisted of the raw read counts summed per VP in each sample, because this method has its own internal data normalizations to account estimated sample fractions. ANCOM-BC was run on VPs with at least 20 reads reported in at least 10% of samples. To account for the relatively small sample sizes, structural zero discovery was turned on but the usage of the asymptotic lower bound turned off[85]. Differential abundance was corrected for the effects of age and sex. The number of differentially abundant (DA) VPs was then determined per host species. Enrichment of host species among DA VPs was calculated using a hypergeometric test as implemented in the phyper R function, with the number of DA VPs infecting a given species-1 as q, the total number of VPs in the dataset infecting the same species as m, the total number of VPs with host-m as n, the total number of DA VPs as k, and lower.tail set to FALSE.

Phage-host interactions

To determine the dynamics of phage-bacterium interaction across the entire population, the change in relative abundance between days 0 and 2, 2 and 28, and 0 and 28 were determined for all VPs with a host and all MAGs with a known phage. The resulting values were then averaged for both VPs and MAGs at the species level, after which Spearman correlation coefficients were calculated.

Data availability statement

The sequencing data generated in this study have been deposited in the European Nucleotide Archive database under accession code: PRJEB60691. The data are freely available without restriction. Source data are provided with this paper.

Code availability statement

Codes and scripts are publicly available online and from published literature. The following software and codes were used: R v4.2.1, fastp v0.23.2, bbmap v38.90, metaSPAdes v3.15.5, MEGAHIT v1.2.9, virsorter v2.2.3, checkv v1.0.1, BLAST v2.12.0+, vContact2 v0.11.3, bowtie2 v2.4.2, coverm filter v0.6.1, bedtools v2.27.1, samtools v1.15.1, mOTUs v3.0.3, metabat2 v2:2.15, GTDB-Tk v2.1.1, CRISPCasFinder v4.2.20, vegan R package v2.6-4, MASS R package v7.3-58.1, and ANCOM-BC v1.2.2. In the methods section any specific settings are described. No custom software was developed for this project.

Patient and public involvement statement

Patients were involved in the assessment of the grant proposals for this study by the Dutch Diabetes Research Foundation (Diabetes II Breakthrough grant (459001008) and Senior Fellowship (2019.82.004)). Moreover, the patient panel advised on the patient burden of the clinical study. Moreover, patients were involved in the ethical approval of this study (as part of the ethics committee). Once the trial has been published, participants are informed of the results in a letter suitable for a non-specialist audience.

Ethics approval and informed consent statement

This study involves human participants and was approved by the Medical Research Ethics Committee Academic Medical Center Amsterdam. Both participants and faeces donors gave informed consent to participate in the study before taking part. The study was registered at the Dutch National Trial Register (NTR) under NL8289 on the 15th of January 2020, while the first patient was included in October 2019. The delay in registration was due to a miscommunication between investigators. When this mistake came to light during the first monitor visit after the first three patients had been included, the study was directly registered at the NTR. This registry does not exist anymore and all data has been added unaltered to the Dutch Trial Register (LTR) under https://clinicaltrialregister.nl/en/trial/26916. While these data are automatically included in the International Clinical Trial Registry Platform (ICTRP), thereby fulfilling the requirement of prospective registration as required by the International Committee of Medical Journal Editors (ICMJE), it was unfortunately no longer possible to adjust the data.

Supplementary material

Figure S1: Recruitment of individuals with MetSyn and stool donors.

Figure S2: Principal component analyses of viral populations.
Figure S3: Methods of FFT production and sequencing pipeline.
Table S1: Baseline dietary intake of participants.
Table S2: Results from the continuous glucose monitoring devices.
Table S3: Differentially abundant viral populations between the FFT and placebo groups.
Table S4: In- and exclusion criteria for study participants.
Table S5: In- and exclusion criteria for faeces donors.
CONSORT flow diagram
Source data

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Author contributions
HH and MN conceived the research idea and designed the study; KW performed the clinical study; KW and TPMS processed the samples in the laboratory; PAdJ, KW and IA performed data analysis; KW and PAdJ wrote the first draft of the manuscript; All authors contributed to manuscript revision, read and approved the submitted version.

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Competing interests

MN is founder and scientific advisors of Caelus Health, however none of this bears any relevance to the content of the current paper. KW, PAdJ, TPMS, IA, EMK, and HH report no conflict of interest.

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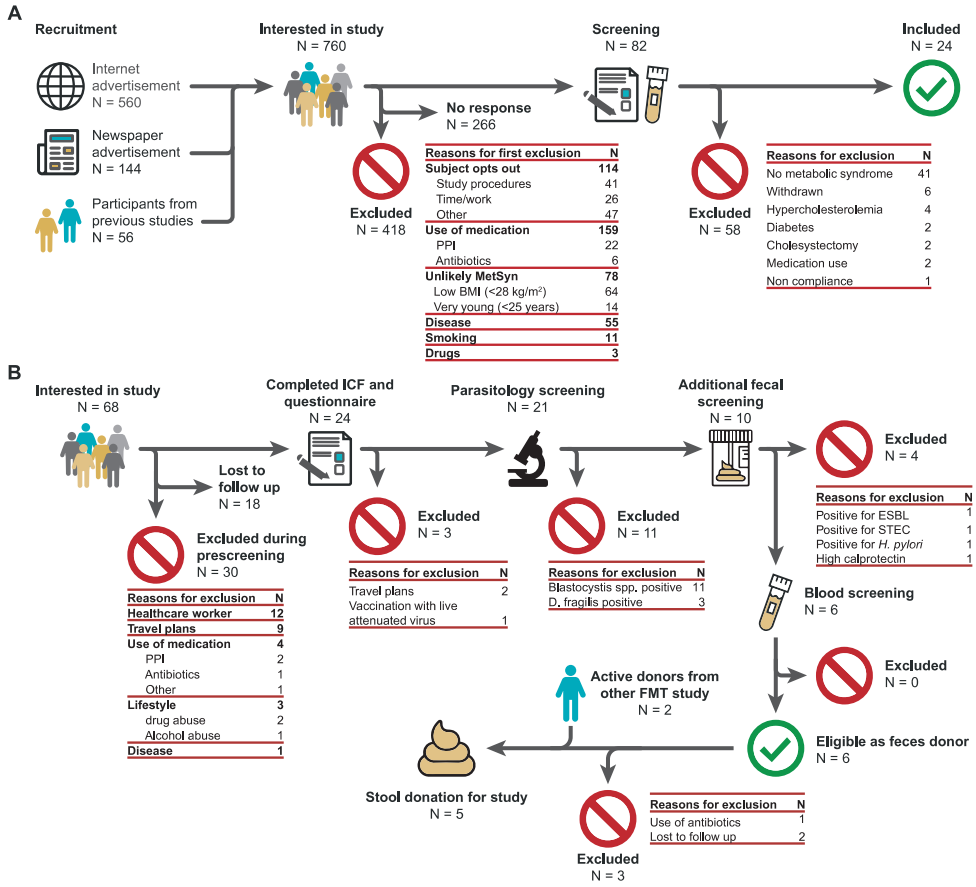
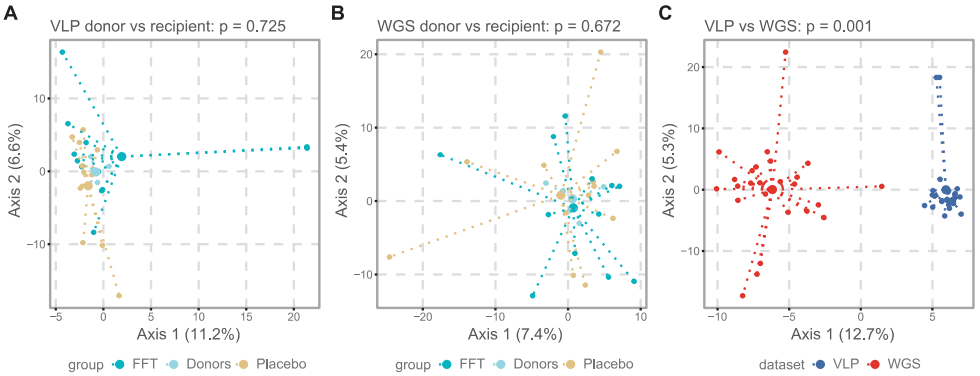
SUPPLEMENTARY MATERIAL



Supplementary figure S1: A) Recruitment and screening of participants with the metabolic syndrome. **B)** Recruitment and screening of healthy donors for the sterile faecal filtrate. BMI = Body Mass Index; D. fragilis = Dientamoeba fragilis; ESBL = extended spectrum beta-lactamase producer FMT = faecal microbiota transplantation; H. pylori = Helicobacter pylori; ICF = informed consent form; PPI = proton pump inhibitor; STEC = shigatoxigenic Escherichia coli.



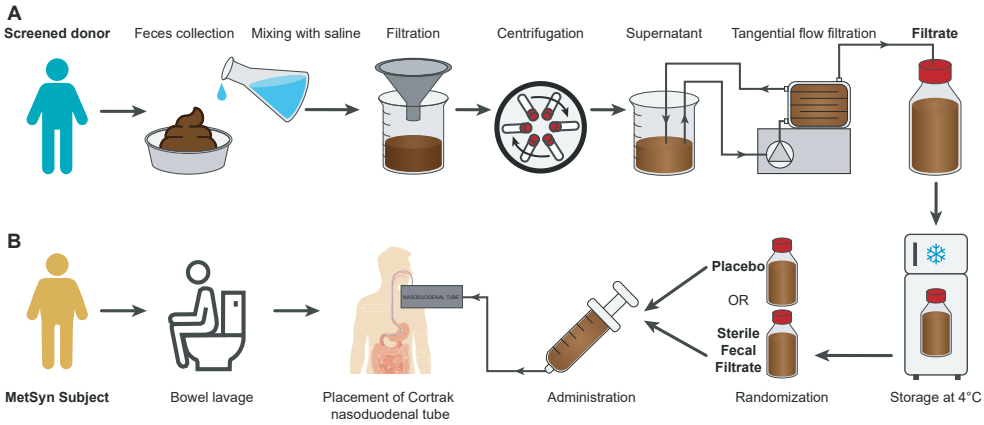
Supplementary figure S2: A) Principal component analysis (PCA) of the viral populations (VP) within the phage virions (VLP) and **B)** bulk-derived phageome (WGS) between the subjects with metabolic syndrome in the faecal filtrate (FFT) and placebo groups and the healthy subjects who donated their stool at baseline. There were no differences in overall composition of the (pro)phages between the groups as determined by permutational analysis of variance (PERMANOVA). **C)** PCA of the VPs showing the difference in overall composition between the free phages (VLP) and bulk-derived phageome (WGS), which was statistically significant as determined by PERMANOVA (p = 0.001).

Supplementary figure S3 (next page)**: A)** Production of the sterile faecal filtrate started the day before the administration to the MetSyn subjects. First, stool was collected from a healthy, thoroughly screened donor, which was homogenized with sterile saline. Large particles were then filtered from the faecal suspension using double sterile gauzes. Most bacteria were pelleted in two subsequent centrifugation steps, in which the suspension was spun for 1 hour at 10.000 RCF. The supernatant was then filtered through a sterile 0.2 µm membrane using a tangential flow filtration device (Vivaflow® 50). The filtrate was stored overnight in a fridge until administration. **B)** The sterile faecal filtrate was administered to the patient via a nasoduodenal tube. The day prior to the administration, subjects were asked to clean their bowel using a laxative, which is a standard pre-treatment for FMT procedures in our hospital. Nasoduodenal tubes were placed with the help of a Cortrak device, making sure the nasoduodenal tube was correctly positioned. The faecal filtrate was slowly infused with a 60 ml syringe, on average 300 ml during a 15-20 min period. **C)** We confirmed with a qPCR for the bacterial 16S rRNA gene the absence of bacteria within the faecal filtrate, showing a 10^5-fold decrease in bacterial DNA. We further confirmed the absence of bacteria by culturing the faecal filtrate, observing no colony-forming units in 100 ml of faecal filtrate (results not shown). **D)** Overview of the used pipeline for the microbiome shotgun sequencing and the VLP shotgun sequencing. *MAGs = Metagenome-assembled genomes; QC = quality control; VLP = viral-like particle; WGS = whole genome shotgun.*

Table S1: baseline dietary intake of participants.

	Placebo (n = 12)	**Fecal Filtrate** (n = 12)	***p-value***
Energy (kcal)	**2199** (271)	**2146** (580)	0.44
Fats (g)	**93.4** (18.9)	**93.3** (29.7)	>0.99
Saturated fats (g)	**33.8** (9.5)	**33.0** (11.8)	>0.99
Carbohydrates (g)	**218.3** (51.8)	**218.4** (70.3)	0.98
Sugars (g)	**82.6** (30.4)	**91.2** (44.5)	0.71
Proteins (g)	**106.0** (26.4)	**89.8** (25.0)	0.16
Fibers (g)	**22.1** (6.7)	**18.7** (6.0)	0.10
Salt (g)	**7.72** (2.93)	**7.50** (2.86)	0.89

Data are reported as mean (SD). Statistical testing between the placebo and fecal filtrate groups is performed by independent Mann-Whitney U test.


A
Screened donor
Feces collection
Mixing with saline
Filtration
Centrifugation
Supernatant
Tangential flow filtration
Filtrate
B
MetSyn Subject
Bowel lavage
Placement of Cortrak nasoduodenal tube
Administration
Placebo
OR
Sterile Fecal Filtrate
Randomization
Storage at 4°C

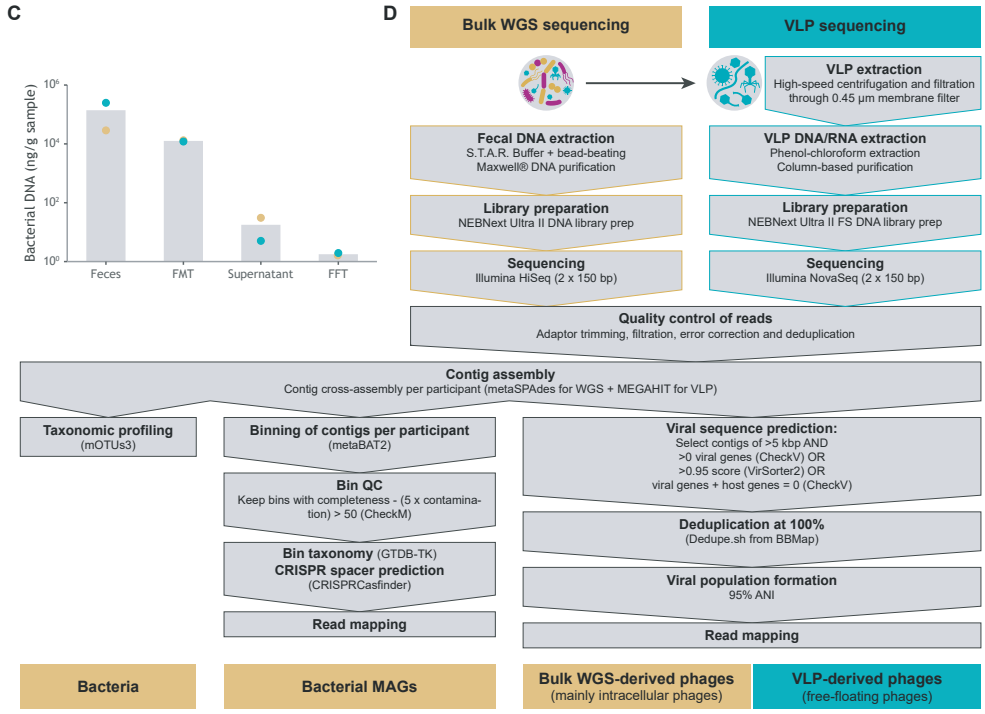

C
Bacterial DNA (ng/g sample)
Feces
FMT
Supernatant
FFT
D
Bulk WGS sequencing
VLP sequencing
VLP extraction
High-speed centrifugation and filtration through 0.45 µm membrane filter
Fecal DNA extraction
S.T.A.R. Buffer + bead-beating Maxwell® DNA purification
VLP DNA/RNA extraction
Phenol-chloroform extraction Column-based purification
Library preparation
NEBNext Ultra II DNA library prep
Library preparation
NEBNext Ultra II FS DNA library prep
Sequencing
Illumina HiSeq (2 x 150 bp)
Sequencing
Illumina NovaSeq (2 x 150 bp)
Quality control of reads
Adaptor trimming, filtration, error correction and deduplication
Contig assembly
Contig cross-assembly per participant (metaSPAdes for WGS + MEGAHIT for VLP)
Taxonomic profiling
(mOTUs3)
Binning of contigs per participant
(metaBAT2)
Bin QC
Keep bins with completeness - (5 x contamination) > 50 (CheckM)
Bin taxonomy (GTDB-TK)
CRISPR spacer prediction
(CRISPRCasfinder)
Read mapping
Viral sequence prediction:
Select contigs of >5 kbp AND
>0 viral genes (CheckV) OR
>0.95 score (VirSorter2) OR
viral genes + host genes = 0 (CheckV)
Deduplication at 100%
(Dedupe.sh from BBMap)
Viral population formation
95% ANI
Read mapping
Bacteria
Bacterial MAGs
Bulk WGS-derived phages
(mainly intracellular phages)
VLP-derived phages
(free-floating phages)

Table S2: Results from the continuous glucose monitoring devices measured one week before and one week after intervention.

		Placebo (n = 12)	Fecal Filtrate (n = 12)	*p-value*
Mean glucose (mmol/L)	Before	**5.36** (0.41)	**5.37** (0.70)	*0.75*
	After	**5.34** (0.38)	**5.33** (0.59)	
SD glucose (mmol/L)	Before	**0.89** (0.19)	**0.98** (0.42)	*0.58*
	After	**0.94** (0.26)	**0.94** (0.37)	
CV glucose (%)	Before	**16.7** (3.6)	**17.8** (5.1)	*0.39*
	After	**17.5** (4.7)	**17.3** (4.8)	
Min glucose (mmol/L)	Before	**3.55** (0.47)	**3.43** (0.37)	*0.37*
	After	**3.51** (0.55)	**3.46** (0.48)	
Max glucose (mmol/L)	Before	**8.63** (1.21)	**8.92** (1.74)	*0.33*
	After	**9.18** (1.44)	**8.82** (1.75)	
Time between 3.9-10 (%)	Before	**97.1** (4.5)	**95.5** (5.4) *	*0.19*
	After	**97.3** (4.4)	**97.5** (3.3)	
Est. HbA1c (mmol/mol)	Before	**31.0** (2.8)	**31.1** (4.8)	*0.76*
	After	**31.0** (2.6)	**30.9** (4.1)	
AUC/day	Before	**7621** (991)	**7617** (823)	*0.55*
	After	**7795** (671)	**7841** (919)	
AUC>2SD/day	Before	**33.0** (23.5)	**32.3** (9.5)	*0.53*
	After	**41.4** (15.5)	**40.6** (13.9)	
CONGA 1 score	Before	**0.99** (0.22)	**1.09** (0.42)	*0.28*
	After	**0.99** (0.25)	**1.08** (0.46)	
MODD score	Before	**0.82** (0.17)	**0.87** (0.30)	*0.41*
	After	**0.83** (0.20)	**0.92** (0.39)	
MAGE score	Before	**1.68** (0.87)	**1.81** (1.03)	*0.32*
	After	**1.45** (0.47)	**1.53** (0.72)	

Unless otherwise specified data are reported as mean (SD). Mixed model analyses were used to assess differences between groups and timepoints, whereafter post hoc analyses were performed with Bonferroni correction. Stars indicate statistical significant differences between the week before and after intervention within a treatment group (* = P<0.05). The P-value shows the overall effect of treatment on the variable and only when significant, the adjusted P-values from the post hoc tests are shown. BMI = Body Mass Index; WHR = waist-hip ratio; BP = blood pressure; HOMA-IR = Homeostatic Model Assessment for Insulin Resistance; HDL = high-density lipoprotein; LDL = low-density lipoprotein; CRP = C-reactive protein.

Table S3: Differentially abundant viral populations within the VLP phageome on day 2.

Can be found online with other supplementary data on medRxiv:
https://doi.org/10.1101/2023.03.22.23287570

Table S4: In- and exclusion criteria for study participants.

INCLUSION CRITERIA

- Caucasian male or female
- Age: 18 - 65 years old
- BMI ≥ 25 kg/m^2
- At least 3 of the following criteria:
  o Fasting plasma glucose ≥ 5.6 mmol/L, or HOMA-IR index ≥2.5 (HOMA-IR is measured as (fasting insulin (pmol/L) x fasting glucose (mmol/L)) / 135)
  o Waist-circumference ≥ 102 cm for males, ≥ 89 cm for females
  o HDL-cholesterol ≤ 1.02 mmol/L for males, ≤ 1.29 mmol/L for females
  o Blood pressure ≥ 130/85 mmHg
  o Triglycerides ≥ 1.7 mmol/L
- Subjects should be able to give informed consent

EXCLUSION CRITERIA
- A history of cardiovascular event (e.g., CVA or MI) or pacemaker implantation - Use of any medication including proton pump inhibitors, antibiotics, and pro-/prebiotics in the past three months or during the study period - (Expected) prolonged compromised immunity (due to recent cytotoxic chemotherapy or HIV infection with a CD4 count < 240/mm3) - Presence of overt T1DM or T2D - History of chronic diarrhoea (≥3 stools/day for >4 weeks), chronic obstipation (<2 defecations/week for >3 months), IBS (according to Rome IV criteria), or IBD. - Smoking or illicit drug use in the past three months or use during the study period - Alcohol abuse (>5 units/day on average) in the past three months or use of > 2 units/day of alcohol during the study period - History of cholecystectomy

Abbreviations: CVA = cerebrovascular accident; HDL = high-density lipoprotein; HIV = human immunodeficiency viruses; HOMA-IR = Homeostatic Model Assessment for Insulin Resistance; IBD = inflammatory bowel disease; IBS = irritable bowel syndrome; MI = myocardial infarction; T1DM = type 1 diabetes mellitus; T2DM = type 2 diabetes mellitus.

Table S5: In- and exclusion criteria for faeces donors.

INCLUSION CRITERIA
- Caucasian male or female - Age: 18 – 65 years old - BMI: 18-25 kg/m^2 - Subjects should be able to give informed consent
EXCLUSION CRITERIA
Positive test for infectious agent - Positive Dual Faeces Test for *Giardia Lamblia*, *Dientamoeba fragilis*, *Entamoeba histolytica*, *Microsporidium* spp., *Cryptosporidium* spp., *Cyclospora*, *Isospora,* or *Blastocystis Hominis*. Positive microscopic exam for eggs, cysts, and larvae (e.g. helminth eggs) - Presence of faecal bacterial pathogens *Salmonella* spp., *Shigella* spp., *Campylobacter* spp., *Yersinia* spp., *C. difficile*, *H. pylori*, STEC, *Aeromonas* spp., or *Pleisiomonas shigelloides* in faeces - Presence of ESBL producers, CRE, VRE, or MRSA in faeces - Presence of *Rotavirus*, *Norovirus* I/II, *Enterovirus*, *Parechovirus*, *Astrovirus*, *Sapovirus,* or *Adenovirus* in faeces - Presence of SARS-CoV-2 in faeces - Positive serologic test for HIV 1/2, HAV, HBV, HCV, HEV, active CMV or EBV, *Strongyloides,* or *Treponema pallidum* **Risk of infectious agent** - History of, or known exposure to HIV, HBV, HCV, syphilis, HTLV I and II, malaria, trypanosomiasis, or tuberculosis - Known systemic infection not controlled at the time of donation - Unsafe sex practice - Previous reception of tissue/organ transplant

- Previous (<12 months) reception of blood products
- Recent (<6 months) needle stick accident
- Recent (<6 months) body tattoo, piercing, earring, or acupuncture
- Recent medical treatment in poorly hygienic conditions
- Risk of transmission of diseases caused by prions
- Recent parasitosis or infection from rotavirus, Giardia lamblia, and other microbes with GI involvement
- Recent travel to tropical countries, countries at high risk of communicable diseases, or traveller's diarrhoea
- Recent (<6 months) history of vaccination with a live attenuated virus, if there is a possible risk of transmission
- Healthcare providers having frequent patient contact
- Individual working with animals

Gastrointestinal comorbidities

- History of IBS (according to Rome IV criteria), IBD, functional chronic constipation, or other chronic GI disorders
- History of chronic, systemic autoimmune disorders with GI involvement, such as coeliac disease
- History of, or high risk for, GI cancer, or polyposis
- Recent appearance of diarrhoea (≥3 stools/day), and/or haematochezia
- Elevated faecal calprotectin (> 50 µg/g)

Factors affecting intestinal microbiota composition

- Use of any medication including proton pump inhibitors, antibiotics, and pro-/prebiotics in the past three months or during the study period
- Smoking or illicit drug use in the past three months or during the study period
- History of cholecystectomy

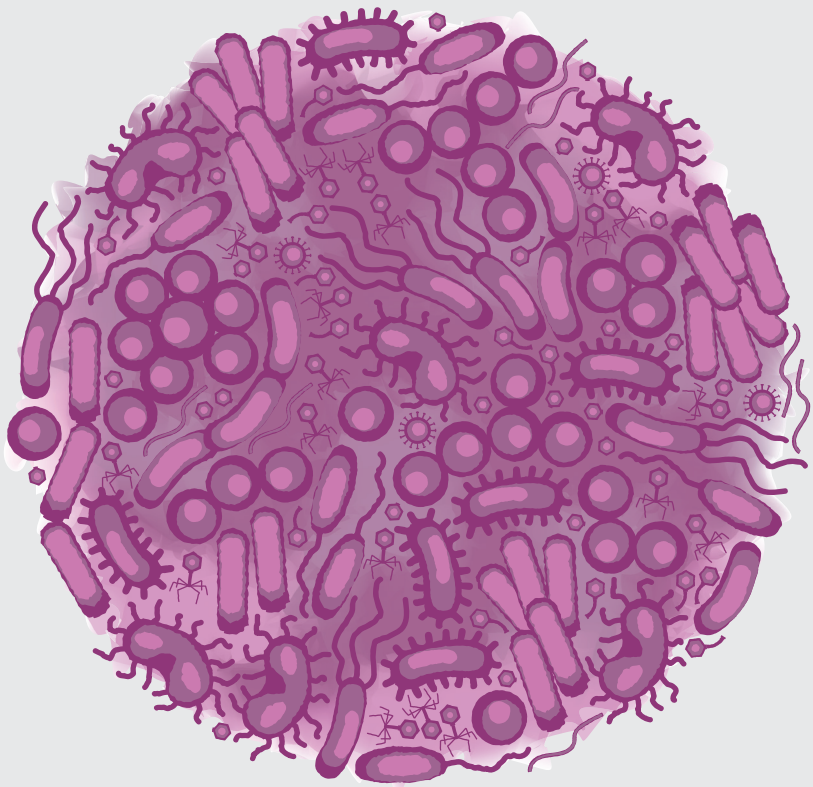
Other conditions

- History of neurological or neurodegenerative disorders
- History of psychiatric conditions
- Presence of chronic low-grade inflammation or metabolic syndrome (NCEP criteria)
- Presence of T1DM, T2DM, or hypertension
- Alcohol abuse (>5 units/day on average) in the past three months or use of > 2 units/day of alcohol during the study period
- Abnormal liver or renal function (creatinine >110 µmol/l, ureum >8,2 mmol/l, ASAT > 40 U/L, ALAT > 45 U/L, AF > 120 U/L, GGT > 60 U/L, bilirubin >17µmol/L), or impaired immunity (CRP > 5 mg/L, haemoglobin < 8,5 mmol/L, MCV: 80-100 fL, leukocytes: 4,0- 10,5 x109/L, thrombocytes: 150-400 x10^9/L).

Abbreviations: AF = alkaline phosphatase; ALAT = alanine aminotransferase; ASAT = aspartate aminotransferase; CMV = cytomegalovirus; CRE = Carbapenem-resistant Enterobacteriaceae; CRP = C-reactive protein; EBV = Epstein–Barr virus; ESBL = extended spectrum beta-lactamase; GGT = gamma-glutamyltransferase; GI = gastrointestinal; HAV = hepatitis A virus; HBV = hepatitis B virus; HCV = hepatitis C virus; HEV = hepatitis E virus; HIV = human immunodeficiency viruses; HTLV = human T-lymphotropic virus; IBD = inflammatory bowel disease; IBS = irritable bowel syndrome; MCV = mean corpuscular volume; MRSA = methicillin-resistant Staphylococcus aureus; NCEP = National Cholesterol Education Program; SARS-CoV-2 = severe acute respiratory syndrome coronavirus 2; STEC = shigatoxigenic Escherichia coli; T1DM = type 1 diabetes mellitus; T2DM = type 2 diabetes mellitus; VRE = vancomycin-resistant Enterococci.

CHAPTER 9

Summary, Discussion and Future Perspectives



SUMMARY

In this thesis, we explored the potential of the gut microbiota in cardiometabolic diseases (CMD). The global prevalence of CMD is increasing, and evidence suggests that the gut microbiota plays a crucial role in their complex pathophysiology. Comprising a diverse community of bacteria, viruses, archaea, fungi, and protists, the gut microbiota are involved in many vital processes, including digestion, metabolism, and immune regulation. Alterations in the gut microbiota have been linked to CMD, but establishing a causal relationship requires well-controlled clinical studies. Therefore, **Part I** of this thesis focused on faecal microbiota transplantation (FMT) as a tool to investigate and treat CMD.

In **Chapter 2**, we focused on potential future applications for FMT beyond recurrent Clostridioides difficile infections (rCDI). We performed an extensive literature search and summarized the evidence for FMT as treatment for rCDI and other gastrointestinal (GI), metabolic, immunologic and neuropsychiatric diseases. FMT has shown high efficacy in resolving rCDI, with success rates of up to 90%. In addition, FMT is superior and cost-effective compared to standard antibiotics for rCDI, which have led to the adoption of FMT as evidence-based treatment for rCDI. At the time of writing (December 2018), FMT had been tested in randomised controlled clinical trials (RCT) as a treatment for inflammatory bowel disease, irritable bowel disease, constipation, metabolic syndrome (MetSyn), hepatic encephalopathy, and vascular inflammation, although with mixed results. We concluded that there was insufficient evidence for FMT to support the use of FMT as standard treatment besides rCDI. The large variation in FMT processing and administration between studies illustrates the need for further standardisation. Furthermore, while FMT appears not a one-size-fits-all therapy, and a more personalized approach might be necessary for diseases other than rCDI.

The interest in FMT generates an increasing urge for suitable stool donors. In **Chapter 3**, we described our experience with recruiting and screening stool donors for FMT, along with the associated costs. In this retrospective study, we combined the data on stool donor screening from four RCTs that were conducted at the Amsterdam UMC, location AMC. These studies used a similar stepwise screening protocol, which consisted of an extensive questionnaire, followed by faeces and blood examination for pathogenic bacteria, viruses, parasites and other abnormalities. Absolutely, most individuals were excluded during the pre-screening (202/393, 51.4%), while relatively, most individuals failed the parasite screening (91/148, 61.5%). Notably, the most commonly found “pathogens” during this parasite screening were the protozoa *Dientamoeba fragilis* and *Blastocystis spp*., which led to exclusion despite their questionable pathogenicity. Ultimately, only 38 out of 393 individuals (10%) were enrolled as stool donor and actively donated for a median duration

of 13 months. Recruitment of these 38 stool donors incurred substantial costs (€64.112). These findings highlight the challenges of finding suitable stool donors and the substantial costs associated with high-quality donor screening. The high rates of exclusion during the screening of seemingly healthy individuals illustrates the risk of do-it-yourself FMTs, most importantly the inadvertent transmission of pathogens.

Chapter 4 consists of a brief commentary we wrote on the clinical use and potential of FMT in Europe. Baunwall and colleagues identified hospital-based FMT centres in Europe and examined their FMT-related clinical activities, organisation and regulation. They noted a significant underuse of FMT in treating rCDI, with only 10% of annual cases receiving FMT. In addition, they found that 43% of all FMTs were performed for experimental indications other than rCDI. By mapping the FMT landscape in Europe, the authors provided useful guidance for clinical practice and for upscaling of FMT. While only 6/31 FMT centres offered FMT in encapsulated form, we argued that capsules provide many advantages and could improve the availability and utilization for rCDI and beyond. Furthermore, the identification of active components of FMT beyond bacteria, such as bacteriophages, metabolites, and bacterial debris, will contribute to further standardization and development of microbiome-targeted interventions.

Besides being an interesting treatment modality, FMT also offers the opportunity to study the interaction between the gut microbiota and human host. In **Chapter 5**, we used this concept to study the interaction between the gut microbiota and microRNAs (miRNAs) in human individuals with MetSyn. Fecal samples were collected at baseline and 6 weeks after FMT, from which the microbiome and miRNA composition were determined. We observed that the microbiota composition and miRNA expression correlated significantly, both before and after FMT. Moreover, the results suggested that the FMT-induced shift in the microbiota correlated with the altered miRNA profile, which was reflected by significant correlations between differentially abundant microbes and miRNAs. Next, we investigated whether the identified miRNAs could directly impact the growth of associated bacteria, but found no direct effect, reinforcing the notion that the observed miRNA alterations were driven by microbiota changes. Further research is needed to elucidate the detailed mechanisms underlying the microbiota's influence on miRNA expression. Nevertheless, this study shows that a prospective FMT cohort provides an interesting opportunity to study the relation between the gut microbiota and its human host.

While FMT shows promise as a treatment strategy, it poses logistical challenges and potential safety risks. Thus, a more controlled and personalized approach utilizing cultured beneficial microbes is likely to replace FMT in the future. Such beneficial microbes are likely to be endogenous commensals to the host, with no prior history of safe and beneficial use,

and are therefore commonly referred to as next-generation probiotics (NGP) or live biotherapeutic products (LBP). In **Chapter 6**, we described the development of one such next-generation beneficial microbe, namely *Anaerobutyricum soehngenii*, which was identified during a previous FMT study in MetSyn subjects. *A. soehngenii* has shown promising effects in preclinical in vitro and in vivo studies, and was found to be safe in humans. It demonstrated beneficial effects on insulin sensitivity, GLP-1 secretion and glucose variability, which could be mediated through the production of butyrate and secondary bile acids. Using the development of *A. soehngenii* as an example, we provided practical guidance for the development and testing of future NGPs, including strain characterization, product quality, and safety assessment.

In **Part II**, our focus shifted to bacteriophages and their role in CMD, specifically MetSyn. Since alterations of the gut bacteria have been implicated in MetSyn, we hypothesized that the composition of the phages that infect these bacteria will be altered as well. In **Chapter 7**, we compared the gut viromes of 196 individuals with MetSyn and healthy controls. We observed a decreased viral richness and diversity in MetSyn viromes and an enrichment of *Streptococcaceae* and *Bacteroidaceae* phages, along with a depletion of phages infecting *Bifidobacteriaceae*. Furthermore, we identified 52 viral clusters (VC) that were significantly differentially abundant in either MetSyn or healthy controls, amongst which were four VCs that showed interrelatedness, notably two MetSyn-associated *Roseburia* VCs and healthy control-associated *Faecalibacterium* and *Oscillibacter* VCs. These phages appeared to be part of a previously undescribed phage family, which we dubbed the *Candidatus Heliusviridae*. We showed that this widespread temperate phage lineage was found in the intestine of 97% of participants, which was confirmed using several validation cohorts. The *Ca. Heliusviridae* clustered into three subfamilies, of which the *gammaheliusvirinae* associated with a healthy gut virome, while the *betaheliusvirinae* were more prevalent in MetSyn viromes. This study underscored the usefulness of de novo assembly-based sequence analyses. Identification of phage families and higher taxonomic levels is crucial to better understand their relation to human health, unveil phage-microbe interactions underlying this association, and to ultimately develop microbiome-targeted interventions to benefit human health.

Since phages can modify bacterial communities and based on previous evidence, we hypothesized that the transfer of faecal phages could induce similar effects as an FMT and thus improve the glucose metabolism in human individuals with MetSyn. In **Chapter 8**, we described a double-blind, randomised, placebo-controlled pilot study we performed to study this hypothesis. We included 24 individuals with MetSyn who were randomly assigned to the faecal filtrate transplantation (FFT) or placebo group, and assessed effects on glucose metabolism, longitudinal changes in bacteriome and phageome, and safety from baseline

up to day 28. While the FFT was safe, the observed changes in glucose metabolism were similar to those in the placebo group. However, we did find a significantly altered phage virion composition two days after FFT compared to placebo. These alterations coincided with an overall negative correlation between differential abundance of phages and their bacterial hosts, which could indicate more lytic phage-microbe interactions. We concluded that phage-containing faecal filtrates can be safely administered to transiently alter the gut microbiota of recipients. However, the FFT effect was small compared to placebo, which could be explained, in part, by the laxative pretreatment, the small sample size and large heterogeneity within the MetSyn study population, or a potential loss of phages during filtration. Furthermore, follow-up studies are needed with cleaner, better-defined phage consortia and well matched donor-recipient pairs, which will increase mechanistic understanding of phage-microbe interactions and how these can benefit human health.

In conclusion, in this thesis we explored the potential of the gut microbiota in CMD. FMT is increasingly being used for diseases beyond rCDI and provides an interesting opportunity to study the contribution of the gut microbiota to CMD. A high-quality feasible donor screening is important however, while further standardisation and use of encapsulated forms could improve the availability and utilization of FMT. Additionally, the role of other components of the gut microbiota, including bacteriophages, in FMT efficacy warrants further attention. Ultimately, we will move towards more personalized, well-characterized (mixtures of) cultured bacteria or phages as alternative for FMT to benefit human health.

DISCUSSION AND FUTURE PERSPECTIVES

With the rapid increase in novel and more affordable techniques to analyse the gut microbiota, a plethora of studies has found associations between the gut microbiome or microbial metabolites and cardiometabolic diseases (CMD)[1–3]. These clinical observations have led to more mechanistic studies, predominantly using mice models, which have broadened our view on how microbes regulate metabolic pathways and interact with the immune system in the mammalian gut[4–9]. However, to study the contribution of gut microbiota to disease pathophysiology, human prospective cohorts and intervention studies are necessary[10]. In this regard, faecal microbiota transplantation (FMT) is a useful research tool and a potential treatment for a broad range of diseases[11,12].

Faecal microbiota transplantation to the rescue?

With the increasing attention for the gut microbiota and hopeful results of FMT trials in diseases without any curative therapies, the demand of patients for an FMT has drastically increased. However, while FMT is very effective in curing recurrent *Clostridioides difficile* infections (rCDI), it is an experimental treatment and should not be routinely offered for other indications[12,13]. This has led to an increased willingness of patients to pursue do-it-yourself FMTs[14]. However, this is not without risk, as pathogenic microbes or parasites can be transferred along with the beneficial microbiota, including antibiotic-resistant bacteria[15–17]. As illustrated by the high rates of exclusion of seemingly healthy individuals during the screening of faeces donors[18], the risk of inadvertent transmission of pathogens is even higher for DIY-FMTs. In addition, a specific gut microbiota might predispose someone for metabolic or disease phenotypes, such as obesity, or increase the risk for future diseases, including auto-immune diseases and colorectal cancer[19–21]. Thus, a proper donor screening is important, as we described in **Chapter 3**.

Which donors should we select?

With the ever-expanding list of screening criteria for stool donors, we are also selecting for a specific microbiota composition which we deem “healthy”. For example, most people that are screened as stool donor in the Netherlands are excluded based on the presence of parasites, predominantly the protozoa *Blastocystis spp*. and *Dientamoeba fragilis*[18]. Interestingly, *Blastocystis spp*. have been found to associate with more diverse microbiomes and distinct microbial profiles, which are in general deemed healthier[22,23]. Moreover, accidental transplantation of *Blastocystis spp*. during FMTs did not negatively impact the efficacy, nor induce any gastrointestinal (GI) side effects[24]. In fact, the defecation patterns of these patients improved more when compared to patients who received faeces from a *Blastocystis*-negative donors[24]. This illustrates that the specific microbiota that is highly

selected for by current screening guidelines might not have the optimal composition or functionality.

Furthermore, the composition of a healthy microbiota is individual specific, established early in life, and shaped by various intrinsic and extrinsic factors, including diet and lifestyle[25]. Based on previous studies, hallmarks of healthy microbiotas are thought to be a high diversity and abundance of beneficial microbes, such as short-chain fatty acid (SCFA) producers like *Faecalibacterium prausnitzii* or *Roseburia intestinalis*[26–28]. However, an increased diversity has also been implicated in disease, including in anorexia nervosa, major depressive disorder and autism[29–31]. Moreover, microbes that are beneficial for one person, might be less beneficial or even harmful for someone else, e.g., *Helicobacter pylori* or *Akkermansia muciniphila*[32,33]. Importantly, the employed method to study the microbiome, including DNA isolation, sequencing technique, read depth, and taxonomic profiling software, greatly affect the outcome and thus the composition and diversity metrics of a microbiome[34,35]. These factors highlight the challenges when trying to improve an individual's "dysbiotic" microbiota towards a "healthy" microbiota.

Notably, someone's existing gut microbiota might be a good predictor for how someone will respond to a microbiota-targeting intervention. Examples of this are personalized diets based on someone's microbiota that outperform traditional diets[36,37], or the finding that baseline microbiota composition is a better predictor for microbiota engraftment and outcome after FMT than the donor's microbiota[38]. This second study also questions the concept of the so-called super donors, which are stool donors with a specific microbiota composition that is supposedly more effective in treating disease[39]. In addition, the authors found that donor-recipient compatibility is an important predictor of strain engraftment besides the recipient's microbiota[38]. Thus, besides screening donors for relevant pathogens, it might be worthwhile to match donors to recipients based on microbiome complementarity.

How should we process the faeces?

Besides the microbiota of donor and recipient, there are several other variables that could influence the effect of an FMT. Processing of the faeces is usually done in a safety cabinet, which negatively affects the strict anaerobic bacteria which are thought to be beneficial for the patient[40–43]. Anaerobic collection, processing in an anaerobic chamber, or the addition of antioxidants could preserve these strict anaerobes. However, to date, clinical studies in rCDI have not shown any difference in efficacy between an aerobic or anaerobically prepared FMT[44,45]. Although there is some evidence that strict anaerobes might be more relevant for other diseases beyond rCDI[46], well-designed randomised controlled clinical trials in humans are needed to establish the added benefit.

How should we administer the faecal microbiota?

Furthermore, the route of administration remains a topic of discussion. While a nasoduodenal or colonoscopic administration of a faecal suspension appears equally effective in treating rCDI[47], this might be different for other indications, e.g., when the upper-GI tract is believed to play a role, such as in type 1 diabetes[48]. However, FMT via nasoduodenal tube, colonoscopy or enema is invasive, time consuming and causes patient discomfort. Encapsulated faecal microbiota might prove a better alternative, as capsules can be easily self-administered and enable treatment of patients for an extended duration, even in a home-setting. Lyophilization of the faecal microbiota provides a more stable product compared to capsules with frozen faecal matter, although any changes in viability and composition have to be thoroughly examined and, if possible, prevented[49]. In addition, the capsules have to protect the microbiota from the gastric acid upon ingestion and have to release the microbiota at the desired location, e.g., in the proximal part of the small intestine or in the colon[50]. Faecal microbiota capsules have been shown to be equally effective compared to a traditional FMT for rCDI, have a higher patient acceptance, and reduced logistical challenges, which can further stimulate the availability and use of faecal microbiota transplantation[51–53].

Stool banks and regulation

The number of FMTs is expected to increase in the coming years, as there is a significant underuse of FMT as treatment for rCDI in Europe in 2019, covering only 10% of annual cases[54]. To facilitate this increasing demand for FMT, for both the clinical and research setting, stool banks or FMT centres are needed that can ensure a safe, accessible and cost-effective FMT therapy[55]. Moreover, while there currently is no regulatory guidance for FMT in Europe, the European Medicine Agency (EMA) and Heads of Medicines Agencies (HMA) published a report in 2022 with several considerations to ensure quality and safety of FMT products[56]. These considerations cover donor selection and screening, traceability and processing of the faecal material, quality control, storage, administration of the FMT product, and long-term monitoring of adverse events. While some countries have classified FMT as a drug, this will ultimately lead to time-consuming and costly registration processes, leading to a steep increase in costs and negatively impacting availability. Faeces should be regarded as a substance of human origin, and as long as only modifications necessary for conservation and administration are made faeces could be regulated under the EU Tissue and Cells Directive (EUTCD; 2004/23/ec)[57].

What are we actually doing?

The goal of FMT is to cure disease by restoring an altered or dysbiotic gut microbiota towards a balanced and stable microbial ecosystem[58]. However, dysbiosis, usually referring to an disrupted microbiota composition and function, is a controversial term due to a lack

of consensus regarding the definition of a healthy microbiome[59,60]. Moreover, the precise mechanisms of FMT are incompletely understood and are likely different for the variety of diseases for which FMT is currently tested. For example, FMT success in rCDI coincided with increased bacterial diversity and donor strain engraftment[61,62], while several bacteria, phages, archaea, and fungi have been described to associated with FMT success[46,63,64]. While these microbes can alter the gut microbiota composition and interact directly with the immune system, it is likely that the downstream effects and functions of these altered microbes mediate the effect of the FMT. These downstream functions include the production of metabolites such as SCFAs and secondary bile acids, crosstalk with immune cells, and provision of colonization resistance[65–67].

It is important to consider that when performing an FMT with a nasoduodenal tube or capsules, we are administering a faecal microbiota in upper GI tract. However, it should be noted that the faecal microbiome differs from the microbiome found in the mucosal or luminal regions of the colon, and particularly from the proximal part of the small intestine[68]. It has been hypothesized that altering the microbiota composition of the small intestine could explain the effect of FMT on autoimmune disorders, since the small intestine is important for training of our immune cells[69,70]. While this hypothesis has to be further explored, a previous study in individuals with type 1 diabetes demonstrated that the beta-cell function was preserved up to 12 months after an autologous FMT via nasoduodenal tube[48]. In addition, in patients with non-alcoholic fatty liver disease, an allogenic FMT into the upper GI tract resulted in reduced small intestinal permeability[71]. These findings illustrate that the mechanism of action of FMT is likely disease-specific and can be influenced various factors, including the route of administration and processing of the stool.

Is there a future for faecal microbiota transplantation?

Although FMT has shown some promising results in treating diseases beyond rCDI, it comes with several logistical challenges, an inconsistent and incompletely defined composition, and potential safety risks[72–74]. Ultimately, FMT will likely be replaced by more controlled and personalized mixtures of cultured beneficial microbes that are lacking in the human host[75]. Nevertheless, FMT has proven a useful research tool to study the contribution of the gut microbiota to human disease pathophysiology. While FMT studies provide interesting correlations between single or groups of microbes and metabolic or disease parameters, it is important to realize that this does not prove causation yet. However, FMT can be used to identify promising beneficial microbes which can be further developed as next-generation probiotics (NGP) or live biotherapeutic products (LBP)[76].

Next-generation beneficial microbes

The majority of the currently available probiotics have little or inconsistent effects on the gut microbiota and metabolic health. Conversely, studies with NGPs or LBPs, which are usually endogenous to the host and thus more likely to engraft or be metabolically active, have shown more promising results. Several examples of these microbes are *A. soehngenii*, which is described in **Chapter 6**, *Akkermansia muciniphila*, and *Faecalibacterium prausnitzii*[77,78]. Importantly, as NGPs or LBPs are without a history of safe and beneficial use, like traditional probiotics, they have to be thoroughly investigated prior to marketing as a food supplement or medicine, respectively. Moreover, while supplementing the intestinal microbiota with a specific bacterial strain appears relatively simple, many factors have to be taken into consideration. These include nutrient requirements of the strain, compatibility with other symbiotic bacteria, and the impact on the host[79]. For example, while A. muciniphila is a promising NGP and has an overall reduced abundance in a variety of metabolic disorders, several studies have reported an increased abundance in patients with Parkinson's disease and multiple sclerosis[80–82]. While the mechanisms underpinning these observations have to be further explored, these observations illustrate the need for careful investigation of new microbial therapies.

Alternatively, microbes could be engineered to produce therapeutic products, perform metabolic functions, or modulate the microbial community. There are several examples of engineered *Escherichia coli* and *Lactococcus lactis* strains that have been used as vehicles for delivery of therapeutic proteins[83–86]. While overall successful, these strains are relatively simplistic, cannot respond to their environment, and generally do not persist in the human gut[87]. With the rapid expansion of gene editing tools, there are many ongoing endeavours to genetically engineer more relevant endogenous obligate anaerobic bacteria[88]. Future challenges encompass the incorporation of environmental sensors (e.g., pH or oxygen concentration) and auxotrophies in these engineered microbes to control their therapeutic activity or colonization, and to provide a biocontainment strategy for when the strain leaves the human body[87].

Microbial consortia

While the above-mentioned beneficial strains appear promising, it is unlikely that a single strain will be able to modulate the entire microbiota and thereby reduce disease incidence or severity. Rather, the development of symbiotic microbial consortia, or synthetic microbial community products, could prove a safe and sustainable alternative to (re-)establish a health-promoting gut microbiota[89]. Using a bottom-up approach that combines insights from computational modelling, microbiota ecology, and disease pathogenesis, will lead to well-characterized consortia of metabolically interdependent strains with a variety of therapeutic functionalities. Examples are the 17-strain consortium GUT-103 and the

refined 11-strain consortium GUT-108, which corrected functional dysbiosis, reduced opportunistic pathogens, and reversed colitis in a humanized mouse colitis model[90]. However, while promising, there are still many challenges to overcome before microbial consortia of designed function and efficacy can be precisely constructed[89]. For example, many bacterial species are still unculturable or underrepresented, such as bacterial species of individuals living non-industrialized lifestyles, and can thus not be included in microbial consortia yet. In addition, predictive models to efficiently design microbial consortia are needed and advanced in-vitro models to test them[91]. Moreover, confirmation of the safety and efficacy of synthetic microbial communities in human intervention studies are much needed.

While these consortia are being developed as a one-size-fits-all therapy, personalization of the microbial consortia tailored to the needs of an individual could even further enhance their efficacy. In this regard, pharmacy compounding could facilitate in formulating and producing individual-specific microbiota products based on someone's initial microbiota composition and clinical parameters or disease phenotype. Such microbiota products would consist of well-defined beneficial microbes that are missing in the patient, complemented with any prebiotic fibres and/or postbiotics to beneficially modulate the existing microbiota. Therapy could be further enhanced by incorporating dietary and lifestyle recommendations based on the gut microbiota composition, which has been previously shown to be effective in improving glycaemic control in individuals with type 2 diabetes[36,37]. As for synthetic microbial community products, many questions and challenges remain before pharmacy compounding of microbiota products will lead to safe and effective personalized therapies. For example, there needs to be a consensus of what a healthy microbiome is, and we need tools to determine someone's optimal microbiota composition. In addition, the added benefit and cost-effectiveness of such personalized microbial consortia compared to the one-size-fits-all products first has to be firmly established in clinical trials.

What about the phages?

Besides supplementing the microbiota with bacteria, there is also the option of altering the abundance of existing species. This may be achieved by harnessing the regulatory mechanisms of bacteriophages (phages), which can be lytic or lysogenic, and can have either negative or positive effects on bacterial growth[92]. Although phages have been found to shape microbial communities in many different ecosystems, phage-host dynamics in the gut are complex and incompletely understood[93–95]. While there are several models that describe phage-microbe interactions in the intestine, such as the "piggyback-the-winner" and "kill-the-winner models", these are predominantly based on observations in different ecosystems, in vitro, and animal models[96–99]. Recently, the viral biogeography of the mammalian gut was studied in a small study with pigs and rhesus macaques[100]. While not

directly representative of humans, studies like these will further our understanding of the virome composition throughout the GI tract, the factors influencing this composition such as pH and transit time, and the interactions between phages and their bacterial hosts.

Before we can use phages as microbiome-targeted intervention, we first have to identify them. However, viruses are underrepresented in reference databases, which is partly due to the lack of an universal phylogenetic marker gene, such as the 16S rRNA gene for bacteria, and high phage diversity as a consequence of their rapid evolution[101]. Consequently, reference-based read mapping approaches, which have been widely used in previous virome studies, are limited by a lack of annotated viral genomes[102]. In addition, previous studies that have linked the gut virome alteration to several diseases, including CMD, have been limited to studying relatively low taxonomic levels[103–105]. However, it may be more useful to study the gut virome at higher taxonomic levels than genomes or viral clusters, which will allow for a more comprehensive understanding of viral diversity, evolution, and ecological roles within the gut ecosystem[106,107]. As phage taxonomy is undergoing a rapid revolution from morphology-based classification towards more genome-based classification, taxonomically higher structures are being implemented that will facilitate the comparison of findings from different phage studies[108].

The discovery of the *Ca. Heliusviridae* phage family in **Chapter 7** emphasizes the usefulness of *de novo* assembly-based sequence analyses. Such database-independent approaches provide a more comprehensive estimation of the complexity of viral communities, including the viral dark matter (uncharacterized viral metagenomic sequences)[109]. However, assembly-based approaches face several challenges that can impact the outcome, such as the read coverage, sequencing technology, and choice of assembly software, which have to be taken into account[110–112].

In line with the high number of uncharacterized gut phages, linking phages to host bacteria is often difficult[113]. In **Chapter 7**, we could not predict a host for roughly two third of the phages, which are likely phages that infect bacteria without a CRISPR system[114], target hosts which could not be taxonomically classified, were not assembled well enough to recognize their host linkages, were virulent and therefore not recognizable as prophages, or a combination of these factors. To better understand the phage-bacteria dynamics in the gut, methods are needed that link phages to hosts with high accuracy[115].

Phages to the rescue?

Phage therapy, in which specific (harmful) bacteria are eliminated by lytic phages, has been extensively used in Eastern Europe as alternative for antibiotics for almost a century[116]. The increasing incidence of infections with multidrug-resistant organisms worldwide has led to

an increasing interest in phage therapy, although in Western societies clinical research with phages is still in its infancy[117]. The advantage of phages as alternative for antibiotics is their specificity, which mostly prevents any off-target effects[118]. An example of this is the phage cocktail that was developed to treat CDI, which could be a future alternative for antibiotics to prevent further disruption of the gut microbiota and increase the risk of a recurrent infection[119]. In addition, phage therapy could be used for specific modulation of the gut microbiota, by repressing specific pathogenic bacteria. As example, the recently described *Lactobacillaceae*, that are thought to produce ethanol and consequently promote development of non-alcoholic fatty liver disease[120], could in theory be selectively inhibited with phages. While promising, it is important to determine the specific bacterial strain that has to be eliminated, identify phages with a narrow host-range that can specifically infect this strain, and assess the consequences of removing that specific strain on the gut microbiota and human host. In addition, any underlying disturbances or missing microbes will probably remain unchanged, and might require additional approaches such as dietary and lifestyle interventions or probiotic supplementation.

As alternative to phage therapy, a consortium of endogenous phages could potentially be used to modulate the entire microbiota. Previous studies have shown promising effects of faecal virome transplantation in animal models[121,122], and two human studies have effectively treated rCDI with a faecal filtrate transplantation (FFT)[123,124]. We performed the first randomised placebo-controlled clinical study with an FFT in individuals with the metabolic syndrome (MetSyn) as described in **Chapter 8**. While we expected that the FFT would have a similar effect as an FMT, we were unable to demonstrate a substantial clinical improvement on glucose metabolism. This could mean that the bacteria from a lean healthy donor microbiota transferred through an FMT are the important driver of the improvement in glucose metabolism[125,126]. However, it could also be that the FFT itself was suboptimal, which could have resulted in the relatively small and transient effect on the gut microbiota. For example, the use of a laxative prior to the FFT could have depleted bacterial hosts for the transplanted phages, the filtration could have resulted in too low phage titres, or the recipients did not harbour the specific bacterial hosts for the donor phages.

In addition, due to the filtration, only phage virions are transplanted, which are usually predominantly lytic phages in a healthy gut microbiota[127], while the temperate phages are mainly integrated as prophages and thus depleted with the removal of the bacteria. Temperate phages have been implicated to facilitate horizonal gene transfer and lysogenic conversion, which has the potential to improve bacterial fitness, metabolic capacity and resistance to infection by related phages[128–130]. With a traditional FMT, these integrated phages are transferred along with the faecal bacteria, and could thus contribute to the FMT efficacy[131]. While the above factors that might have affected FFT efficacy warrant further

investigation, the FFT was well tolerated and safe, and the study provided a basis for follow up studies.

In conclusion, the field of gut microbiota research has witnessed significant advancements and our understanding of the gut microbiota and all its components continues to grow. In this regard, FMT has played a crucial role, although challenges and uncertainties remain. Personalized approaches, improved processing and administration techniques, the development of next-generation beneficial microbes, and the exploration of phages as therapeutic agents hold promise for future microbiota-targeted interventions. Hopefully further research, including well-designed clinical trials, will fully realize the potential of these approaches in improving human health.

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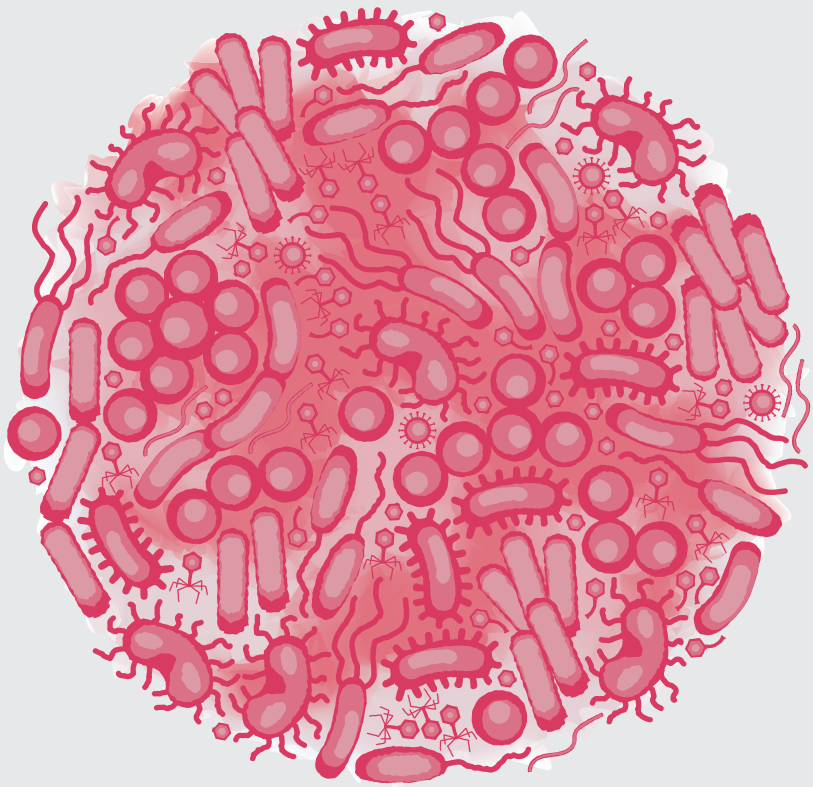
APPENDICES



NEDERLANDSE SAMENVATTING

In dit proefschrift hebben we de betrokkenheid van de darmmicrobiota bij cardiometabole ziekten (CMZ) onderzocht. De wereldwijde prevalentie van CMZ neemt toe en er zijn aanwijzingen dat de darmmicrobiota een cruciale rol speelt in hun complexe pathofysiologie. De darmmicrobiota, bestaande uit een diverse gemeenschap van bacteriën, virussen, archaea, schimmels en protisten, zijn betrokken bij vele essentiële processen, waaronder spijsvertering, metabolisme en regulatie van het immuunsysteem. Veranderingen in de darmmicrobiota zijn gekoppeld aan CMZ, maar om een oorzakelijk verband vast te stellen zijn goed gecontroleerde klinische studies nodig. Daarom richtte **deel I** van dit proefschrift zich op fecestransplantatie, ook wel fecale microbiota transplantatie (FMT) genoemd, als interventie om CMZ te onderzoeken en behandelen.

In **Hoofdstuk 2** hebben we ons gericht op mogelijke toekomstige toepassingen van FMT naast de recidiverende *Clostridioides difficile* infecties (rCDI). We hebben een uitgebreid literatuuronderzoek uitgevoerd en de bewijslast voor FMT als behandeling voor rCDI en andere gastro-intestinale (GI), metabole, immunologische en neuropsychiatrische aandoeningen samengevat. FMT is erg effectief als behandeling van rCDI, met succespercentages tot 90%. Bovendien is FMT superieur en kosteneffectief in vergelijking met de standaard antibiotica voor rCDI, wat ertoe heeft geleid dat FMT als klinische behandeling voor rCDI wordt toegepast. Ten tijde van schrijven (december 2018) was FMT getest in gerandomiseerd gecontroleerd onderzoek (RCT) als behandeling voor inflammatoire darmaandoeningen, prikkelbare darmsyndroom, obstipatie, metabool syndroom (MetSyn), hepatische encefalopathie en atherosclerose, zij het met wisselende resultaten. We concludeerden dat er onvoldoende bewijs was om het gebruik van FMT als standaardbehandeling buiten rCDI om aan te bevelen. De grote variatie in verwerking en toediening van de feces tussen studies illustreert de noodzaak voor verdere standaardisatie. Bovendien lijkt FMT geen 'one-size-fits-all'-therapie te zijn, en een meer gepersonaliseerde aanpak is waarschijnlijk nodig voor andere ziekten dan rCDI.

De interesse in FMT zorgt voor een groeiende behoefte aan geschikte fecesdonoren. In **Hoofdstuk 3** hebben we onze ervaring beschreven met het werven en screenen van fecesdonoren voor FMT, inclusief de bijbehorende kosten. In deze retrospectieve studie hebben we de gegevens over fecesdonorscreening gecombineerd van vier RCT's die zijn uitgevoerd in het Amsterdam UMC, locatie AMC. Deze studies gebruikten een vergelijkbaar stapsgewijs screeningsprotocol, dat bestond uit een uitgebreide vragenlijst, gevolgd door ontlasting- en bloedonderzoek naar pathogene bacteriën, virussen, parasieten en andere afwijkingen. Pre-screening resulteerde absoluut in de meeste exclusies (202/393, 51,4%), terwijl relatief gezien de meeste mensen afvielen bij de screening op parasieten (91/148,

61,5%). De meest gevonden “pathogenen” bij de parasietenscreening waren de protozoa *Dientamoeba fragilis* en *Blastocystis spp*., wat leidde tot exclusie ondanks hun twijfelachtige pathogeniciteit. Uiteindelijk waren slechts 38 van de 393 individuen (10%) geschikte fecesdonoren en hebben zij actief gedoneerd gedurende een mediane 13 maanden. Het werven van deze 38 fecesdonoren ging gepaard met aanzienlijke kosten (€64.112). Deze bevindingen benadrukken de uitdagingen om geschikte fecesdonoren te vinden en de aanzienlijke kosten die gepaard gaan met de uitgebreide screening. Het hoge exclusiepercentage van ogenschijnlijk gezonde individuen bij de screening illustreert de risico’s van doe-het-zelf FMT's, met name de onbedoelde overdracht van ziekteverwekkers.

Hoofdstuk 4 bestaat uit een kort commentaar dat we hebben geschreven over het klinisch gebruik en potentieel van FMT in Europa. Baunwall en collega's hebben FMT-centra binnen Europese ziekenhuizen geïdentificeerd en hun FMT-gerelateerde klinische activiteiten, organisatie en regelgeving onderzocht. Ze constateerden een significant ondergebruik van FMT bij de behandeling van rCDI, waarvoor slechts 10% van de jaarlijkse gevallen met FMT behandeld werd. Bovendien bleek dat 43% van alle FMT's werden uitgevoerd voor experimentele indicaties naast rCDI. Door het in kaart brengen van het FMT-gebruik in Europa hebben de auteurs nuttige inzichten geboden voor de klinische praktijk en voor het opschalen van FMT. Hoewel slechts 6/31 FMT-centra FMT in capsulevorm aanboden, betoogden we dat capsules veel voordelen bieden en de beschikbaarheid en toepassing van FMT voor rCDI en daarbuiten kunnen verbeteren. Bovendien zal het identificeren van de actieve componenten van een FMT, waaronder bacteriën, maar ook bacteriofagen, metabolieten en bacterieresten, bijdragen aan verdere standaardisatie en ontwikkeling van microbiota-modulerende interventies.

Naast een interessante behandelingsmethode, biedt FMT ook de mogelijkheid om de interactie tussen de darmmicrobiota en de menselijke gastheer te bestuderen. In **Hoofdstuk 5** hebben we dit concept gebruikt om de interactie tussen de darmmicrobiota en microRNA's (miRNA's) te bestuderen bij individuen met MetSyn. Ontlastingsmonsters werden verzameld bij aanvang en 6 weken na FMT, waarvan de samenstelling van het microbioom en miRNA werd bepaald. We vonden een significante correlatie tussen de microbioomcompositie en de miRNA-expressie, zowel vóór als na FMT. Bovendien suggereerden de resultaten dat de FMT-geïnduceerde veranderingen in het microbioom samenhingen met het veranderde miRNA-profiel, wat werd weerspiegeld door significante correlaties tussen differentieel abundante microben en miRNA's. Vervolgens hebben we onderzocht of de geïdentificeerde miRNA's direct de groei van gecorreleerde bacteriën konden beïnvloeden, maar we vonden geen direct effect, wat het idee versterkt dat de geobserveerde miRNA-veranderingen worden veroorzaakt door veranderingen in het microbioom. Vervolgonderzoek is nodig om de precieze mechanismen te verduidelijken op

welke manier de darmmicrobiota de miRNA-expressie beïnvloeden. Desalniettemin toont deze studie aan dat een prospectief FMT-cohort een interessante mogelijkheid biedt om de relatie tussen de darmmicrobiota en de menselijke gastheer te bestuderen.

Hoewel FMT veelbelovend is als behandelingsstrategie, brengt het logistieke uitdagingen en mogelijke veiligheidsrisico's met zich mee. Het is daarom waarschijnlijk dat FMT in de toekomst vervangen zal worden door een beter gecontroleerde en gepersonaliseerde aanpak bestaande uit gekweekte nuttige microben. Dergelijke microben zullen waarschijnlijk endogene darmcommensalen zijn, zonder enige voorgeschiedenis van veilig en gezond gebruik, en worden daarom vaak aangeduid als “next-generation” probiotica (NGP) of levende biotherapeutische producten (LBP). In **Hoofdstuk 6** hebben we de ontwikkeling beschreven van een dergelijke NGP, namelijk *Anaerobutyricum soehngenii*, die werd geïdentificeerd tijdens een eerdere FMT-studie met MetSyn-deelnemers. *A. soehngenii* heeft veelbelovende resultaten laten zien in preklinische *in vitro*- en *in vivo*-onderzoek, én is veilig bevonden bij mensen. Het heeft gunstige effecten laten zien op insulinegevoeligheid, GLP-1-secretie en glucosevariabiliteit, welke mogelijk gemedieerd worden via de productie van butyraat en secundaire galzouten. Aan de hand van de ontwikkeling van *A. soehngenii* hebben we praktische adviezen gegeven voor de ontwikkeling en het testen van toekomstige NGP's, waaronder de stamkarakterisering, productkwaliteit en veiligheidsbeoordeling.

In **Deel II** verschoof onze focus naar bacteriofagen en hun rol bij CMZ, specifiek MetSyn. Aangezien veranderingen in de darmbacteriën in verband zijn gebracht met MetSyn, leek het ons aannemelijk dat ook de samenstelling van de fagen die deze bacteriën infecteren veranderd zal zijn. In **Hoofdstuk 7** hebben we het darmviroom van 196 individuen met MetSyn en gezonde controles vergeleken. We vonden een afname in faag-rijkdom en -diversiteit in MetSyn-viromen, evenals een verrijking van *Streptococcaceae*- en *Bacteroidaceae*-fagen en een afname van fagen die *Bifidobacteriaceae* infecteren. Daarnaast hebben we 52 virale clusters (VC's) geïdentificeerd die significant meer aanwezig waren in MetSyn of gezonde controles, waaronder vier VC's die onderling gerelateerd waren, namelijk twee MetSyn-geassocieerde *Roseburia*-VC's en *Faecalibacterium*- en *Oscillibacter*-VC’s die associeerden met gezonde controles. Deze fagen bleken deel uit te maken van een eerder onbeschreven fagenfamilie, die we de *Candidatus Heliusviridae* hebben genoemd. We toonden aan dat deze wijdverspreide fagenfamilie in de darm van 97% van de deelnemers voorkwam, wat werd bevestigd met behulp van verschillende validatiecohorten. De *Ca. Heliusviridae* clusterden in drie subfamilies, waarvan de *gammaheliusvirinae* geassocieerd waren met een gezond darmviroom, terwijl de *betaheliusvirinae* vaker voorkwamen in MetSyn-viromen. Deze studie benadrukt het nut van de-novo-sequencing met genoomassemblage. Identificatie van fagenfamilies en hogere

taxonomische niveaus is cruciaal om de relatie van fagen met de menselijke gezondheid beter te begrijpen, faag-bacterie-interacties die hieraan ten grondslag liggen te onthullen en uiteindelijk microbiota-modulerende interventies te ontwikkelen ter bevordering van de menselijke gezondheid.

Aangezien fagen bacteriële gemeenschappen kunnen beïnvloeden en op basis van eerdere bevindingen, verwachtten we dat toediening van fecale fagen een vergelijkbaar effect als een FMT zou kunnen hebben en daarmee de glucoserespons bij individuen met MetSyn zou kunnen verbeteren. In **Hoofdstuk 8** beschreven we een dubbelblinde, gerandomiseerde, placebogecontroleerde pilotstudie die we hebben uitgevoerd om deze hypothese te onderzoeken. We includeerden 24 individuen met MetSyn die werden gerandomiseerd naar de fecale filtraattransplantatie- (FFT) of placebogroep, en evalueerden de effecten op glucoserespons, longitudinale veranderingen in het bacteriële microbioom en fagoom, en veiligheid van dag 0 tot dag 28. Hoewel de FFT veilig was, waren de veranderingen in glucoserespons vergelijkbaar met die in de placebogroep. We ontdekten wel een significant verandering in de samenstelling van faagvirionen twee dagen na de FFT in vergelijking met de placebogroep. Deze veranderingen werden gekenmerkt door een negatieve correlatie in differentiële abundantie van fagen en hun bacteriële gastheren, wat zou kunnen wijzen op meer lytische faag-bacterie interacties. We concludeerden dat fagen-bevattende fecale filtraten veilig kunnen worden toegediend om tijdelijk de darmmicrobiota van ontvangers te wijzigen. Echter, het effect van de FFT was klein in vergelijking met placebo, wat deels kan worden verklaard door de voorbehandeling met laxeermiddel, de kleine steekproefomvang en de grote heterogeniteit binnen de MetSyn-studiepopulatie, of mogelijk een verlies van fagen tijdens de filtratie. Verder onderzoek is nodig met schonere, beter gedefinieerde fagenconsortia en goed gepaarde donoren en ontvangers, wat zal bijdragen aan een beter begrip van faag-bacterie-interacties en hoe deze kunnen bijdragen aan de menselijke gezondheid.

Concluderend hebben we in dit proefschrift het potentieel van de darmmicrobiota bij CMZ onderzocht. FMT wordt steeds vaker gebruikt voor ziekten buiten rCDI en biedt een interessante mogelijkheid om de bijdrage van de darmmicrobiota aan CMZ te bestuderen. Een hoogwaardige, pragmatische donorselectie is echter belangrijk, terwijl verdere standaardisatie en het gebruik van capsuletoedieningsvormen de beschikbaarheid en het gebruik van FMT kunnen vergroten. Daarnaast verdient de bijdrage van andere FMT-componenten, waaronder bacteriofagen, met betrekking tot de werkzaamheid van FMT meer aandacht. Uiteindelijk zullen we ons bewegen naar meer gepersonaliseerde, goed gekarakteriseerde (mengsels van) gekweekte bacteriën en fagen als alternatief voor FMT ter bevordering van de menselijke gezondheid.

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LIST OF PUBLICATIONS

Included in this thesis

1. **Wortelboer K**, Nieuwdorp M, Herrema H. Fecal microbiota transplantation beyond *Clostridioides difficile* infections. EBioMedicine. 2019;44:716-729.
2. **Wortelboer K**, Herrema H. Shedding light on dark matter - faecal microbiota transplantation in Europe. Lancet Reg Health Eur. 2021;9:100187.
3. **Wortelboer K**, Bakker GJ, Winkelmeijer M, van Riel N, Levin E, et al. Fecal microbiota transplantation as tool to study the interrelation between microbiota composition and miRNA expression. Microbiol Res. 2022;257:126972.
4. de Jonge PA, **Wortelboer K**, Scheithauer TPM, van den Born BH, Zwinderman AH, Nobrega FL, Dutilh BE, Nieuwdorp M, Herrema H. Gut virome profiling identifies a widespread bacteriophage family associated with metabolic syndrome. Nat Commun. 2022;13(1):3594.
5. Bénard MV, de Bruijn CMA, Fenneman AC, **Wortelboer K**, Zeevenhoven J, et al. Challenges and costs of donor screening for fecal microbiota transplantations. PLoS One. 2022;17(10):e0276323.
6. **Wortelboer K**, Koopen AM, Herrema H, de Vos WM, Nieuwdorp M, Kemper EM. From fecal microbiota transplantation toward next-generation beneficial microbes: The case of *Anaerobutyricum soehngenii*. Front Med (Lausanne). 2022;9:1077275.
7. **Wortelboer K**, de Jonge PA, Scheithauer TPM, Attaye I, Kemper EM, Nieuwdorp M, Herrema H. Phage-microbe dynamics after sterile faecal filtrate transplantation in individuals with metabolic syndrome: a double-blind, randomised, placebo-controlled clinical trial assessing efficacy and safety. Nat Commun. 2023;14(1):5600.

Other publications

1. Gilijamse PW, Hartstra AV, Levin E, **Wortelboer K**, Serlie MJ, et al. Treatment with Anaerobutyricum soehngenii: a pilot study of safety and dose-response effects on glucose metabolism in human subjects with metabolic syndrome. NPJ Biofilms Microbiomes. 2020;6(1):16.
2. Koopen A, Witjes J, **Wortelboer K**, Majait S, Prodan A, et al. Duodenal Anaerobutyricum soehngenii infusion stimulates GLP-1 production, ameliorates glycaemic control and beneficially shapes the duodenal transcriptome in metabolic syndrome subjects: a randomised double-blind placebo-controlled cross-over study. Gut. 2022;71(8):1577-1587.
3. Bénard MV, Arretxe I, **Wortelboer K**, Harmsen HJM, Davids M, de Bruijn CMA, et al. Anaerobic Feces Processing for Fecal Microbiota Transplantation Improves Viability of Obligate Anaerobes. Microorganisms. 2023;11:2238.

PORTFOLIO

Name PhD student: Koen Wortelboer
PhD period: 9-2018 t/m 9-2022
Name PhD supervisor: Promotores: prof. dr. Max Nieuwdorp and dr. Hilde Herrema
Co-promotores: dr. Marleen Kemper and dr. Patrick de Jonge

1. PhD training		
Courses	**Year**	**ECTS**
Communication with patients for non-MD's	2018	0.3
The AMC World of Science	2018	0.7
eBROK	2019	1.0
Talents in PhD	2019	0.5
Didactical skills	2019	0.5
Practical Biostatistics	2020	1.0
Mouse Morphology, Function and Genetics	2020	1.5
Collegereeks Human Microbiome in Health and Disease	2021	1.5
AGEM Endocrinology course	2021	2.2
Seminars, workshops and masterclasses	**Year**	**ECTS**
Weekly Department Journal Club	2018-2022	4.0
2-weekly Diabetes Meeting AMC/VUmc	2018-2022	2.0
2-weekly Lab Research Meeting	2018-2022	2.0
2-weekly Microbiota Journal Club	2018-2022	2.0
2-yearly CAMIT consortium meetings	2018-2022	3.0
EPIC training	2018	0.2
Brand en ontruiming training	2018	0.2
FACS theory and training	2018	0.3
Cortrak nasoduodenal tube training	2019	0.3
Venapuncture & peripheral infusion training	2019	0.3
2-yearly ACS symposium Diabetes & Metabolism	2020-2022	0.5
CFG user meeting on Third Generation Sequencing	2020	0.2
AGEM Webinars Series: Owning Obesity & Negating NASH	2020	0.2
Data Visualization	2021	0.2
Personal Microbiome Health kickoff	2021	0.2
ACS PhD Afternoon	2022	0.2
AGEM NAFLD symposium	2022	0.2
VasDia symposium	2022	0.3
Medicijn voor de Maatschappij symposium	2022	0.2

Conferences	Year	ECTS
EMBL/EMBO "The human microbiome", Heidelberg, Germany	2018	1.0
Annual Dutch Diabetes Research Meeting, Oosterbeek	2018	1.0
ACS annual PhD retreat, Soesterberg Pitch: *"PIMMS study"*	2019	1.0
AG&M annual PhD retreat, Garderen Pitch: *"PIMMS study"*	2019	1.0
Annual AMGRO meeting, Lemmer Presentation: *"PIMMS study"*	2019	0.4
Nanopore Day, Utrecht	2019	0.3
U.P.S.V. "Unitas Pharmaceuticorum" Lustrum symposium Presentation: *"The Gut Microbiota in Metabolic Disease - Fecal Microbiota Transplantation a Cure for Diabetes?"*	2019	0.3
Annual Gut Day, Amsterdam	2019	0.3
AGEM annual PhD retreat, online	2020	0.2
Annual DNFB Research Meeting Presentation: *"Role of bacteriophages In gut microbiome composition and glucose metabolism in metabolic syndrome subjects"*	2020	0.3
Word of Microbiome, online	2020	1.0
Annual Dutch Diabetes Research Meeting, online	2020	0.3
ONT London Calling, online	2021	0.3
FMT meeting Denmark, online	2021	0.3
AGEM annual PhD retreat, Garderen Presentation: *"Lyophilized Faecal Microbiota capsules to simplify faecal microbiota transplantation"*	2022	1.0
VJA Voorjaarsdag, Leiden Presentation: *"Improving FMT with faecal microbiota capsules"*	2022	0.3
FIGON/EUFEPS Dutch Medicines Day Presentation: *"Lyophilized Fecal Microbiota capsules to preserve residual beta cell function in T1D"*	2022	1.0
FMT conference, Copenhagen, Denmark Presentation & poster: *"Development of Lyophilized Faecal Microbiota Capsules for Clinical Research"*	2022	1.0

2. Teaching		
Lecturing	**Year**	**ECTS**
Cardiovascular Research & Care, elective course Bachelor Geneeskunde AMC	2019-2022	1.0
Lecture Minor Diabetes, Bachelor Geneeskunde VUmc	2019	0.2
Gastrointestinal and Liver master course, Master Biomedical Sciences	2019	0.2
Supervision	**Year**	**ECTS**
Ana Sousa Gerós; WO master thesis	2018	1.0
Yannick van Schajik; WO bachelor thesis	2019	1.0
Stephanie Handana; WO bachelor thesis	2019	1.0
Ilma Revers; WO master thesis	2019	0.5
Kirby Dick; WO master thesis	2021	0.5
Inaki Arretxe; WO master thesis	2021-2022	1.0
Pleun de Groen; HBO thesis	2021-2022	1.0

3. Parameters of esteem	
Grants	**Year**
Innovatiegrant Amsterdam UMC: *Autologe FecesBank Amsterdam UMC - bundeling van krachten voor een innovatieve, duurzame en kosteneffectieve behandeling van patiënten met autologe feces transplantatie*	2022
Awards and Prizes	**Year**
Best Elevator Pitch award AG&M retreat 2019	2019
Best Presentation award AGEM retreat 2022	2022
Early-stage researcher 2nd prize for flash talk and poster presentation at FMT Conference Denmark	2022

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CURRICULUM VITAE

Koen Wortelboer is op 3 maart 1994 geboren te Hengelo, Overijssel als oudste zoon van ouders Wilma en Rob. Hij groeide op in het groene Twente samen met zijn drie broertjes Thom, Bas en Mick.

Na het behalen van zijn VWO N&T/N&G diploma, besloot Koen zijn passie voor biologie en chemie te combineren in de studie Farmacie te Utrecht. Tijdens zijn studie was Koen actief bij de studievereniging U.P.S.V. “Unitas Pharmaceuticorum” en de overkoepelende K.N.P.S.V., heeft hij het Honoursprogramma Farmacie gevolgd en werkte hij als studentmedewerker bij de Lombok Apotheek Utrecht. Tijdens de bachelor kwam hij in aanraking met de darmmicrobiota en leerde hoe belangrijk deze microben zijn voor onze gezondheid. Daarom heeft Koen voor zijn masterscriptie de productie van gevriesdroogde fecescapsules onderzocht in het AMC bij de Experimentele Vasculaire Geneeskunde onder begeleiding van dr. Hilde Herrema.

Toen Koen na zijn afstuderen de kans kreeg om terug te keren naar het Amsterdam UMC, locatie AMC voor een promotieonderzoek, heeft hij deze kans met beide handen aangegrepen. Hier heeft hij zijn promotietraject bij de Experimentele Vasculaire Geneeskunde doorlopen onder begeleiding van prof. Max Nieuwdorp, dr. Hilde Herrema, dr. Marleen Kemper en dr. Patrick de Jonge. De resultaten van zijn onderzoek zijn beschreven in deze thesis. Naast zijn eigen onderzoek heeft Koen verschillende bijeenkomsten bijgewoond en georganiseerd, studenten begeleid en verschillende onderzoeken gefaciliteerd.

Sinds september 2022 is Koen als postdoc verdergegaan in het Amsterdam UMC, locatie AMC bij de Experimentele Vasculaire Geneeskunde. Na tien jaar met veel plezier in Utrecht gewoond te hebben, is hij verhuisd naar Enschede, terug in het mooie Twente, waar hij samen met zijn man Ruben en katten Ouzo en Momo woont.