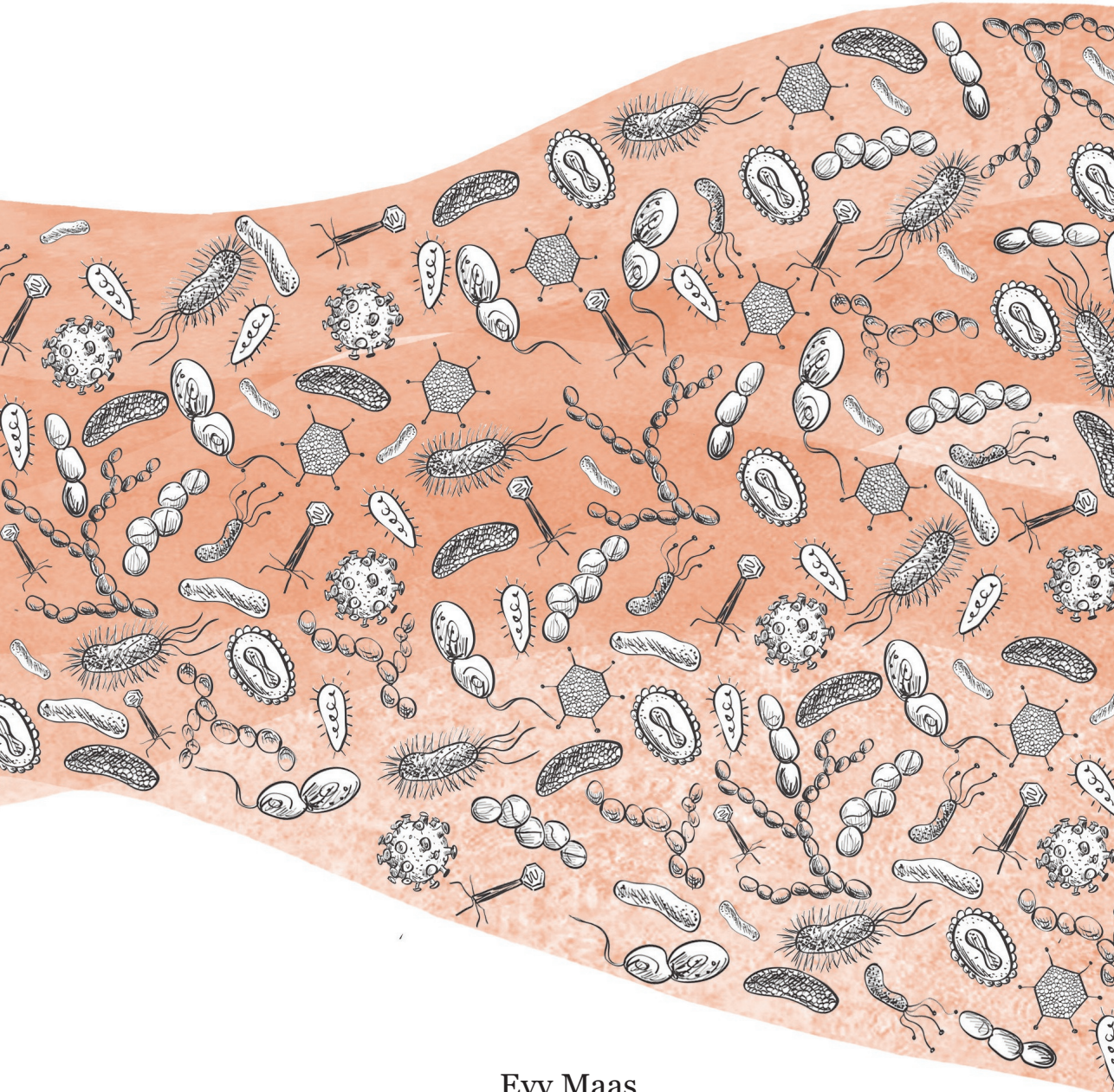# A CLASH OF KINGS:

Tools to study cross-kingdom interactions in the human gut microbiota

Evy Maas

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PROEFSCHRIFT

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Evy Theresia Maria Maas

Promotor

Prof. Dr. K. Venema

Co-promotor

Dr. J. Penders

Beoordelingscommissie
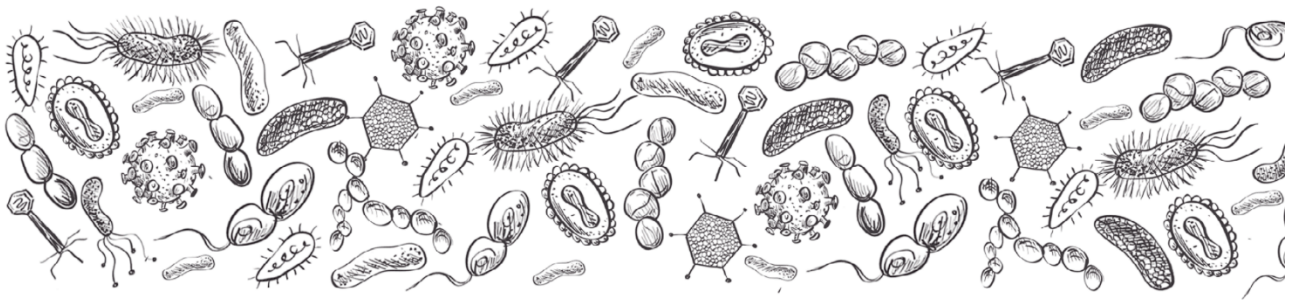
Prof. dr. E.E. Blaak (voorzitter)

Dr. S. Green, Katholieke Universiteit Leuven, België

Prof. dr. P.H.M. Savelkoul

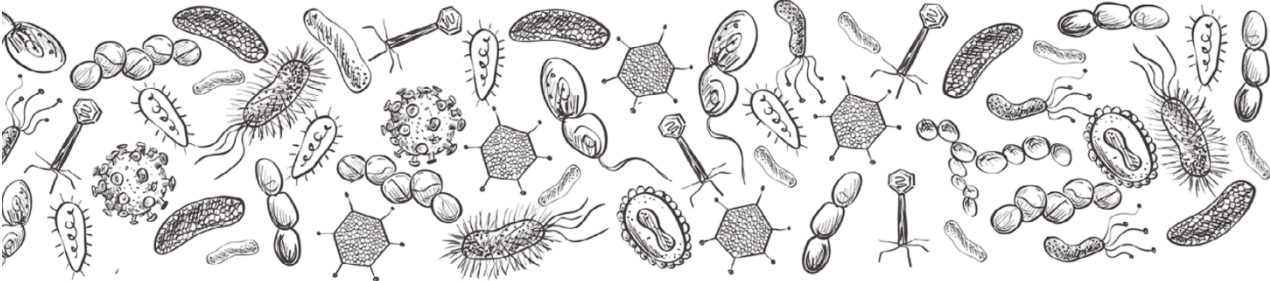Dr. R.M.J.G.J. van den Wijngaard, Amsterdam University Medical Center

Contents

Chapter 1

General Introduction

The human body harbors a large amount of microbial cells, collectively called the microbiota, which in numbers are comparable to the amount of human cells ($\sim10^{14}$) (Sender, Fuchs, & Milo, 2016). The majority of the microbiota is found in the human gastrointestinal tract, where numbers increase across the length of the GI tract, from stomach to colon, with the lowest numbers found in the stomach, followed by the small intestine (duodenum, jejunum, ileum) and the majority of the microbiota is found in the colon (10^{11-12} bacteria per gram) (Dieterich, Schink, & Zopf, 2018). The gut microbiota plays an important role in host health, amongst others through the break-down and absorption of otherwise non-digestible nutrients (Cockburn & Koropatkin, 2016), the proliferation and maturation of intestinal cells (Matsuki et al., 2013; Sommer & Bäckhed, 2013), the development and activity of the immune response (Belkaid & Hand, 2014; Round & Mazmanian, 2009) and the protection against pathogenic microbes (Cameron & Sperandio, 2015). The microbiota found in the gut consists of microbes from different kingdoms: bacteria, which take up the majority of total cell numbers and therefore received most attention in research (Qin et al., 2010; Sender et al., 2016), but also viruses and fungi. The focus of research on the bacterial component of the microbiota has led to a gap of information about the viral and fungal component.

Viruses

Viruses are infectious agents that can infect the cells of all kinds of organisms, including animals, plants and bacteria. The most abundant biological entities present on the planet are viruses, of which the majority are bacteriophages (in short also named phages): viruses that infect bacteria (Edwards & Rohwer, 2005). Also in the gut, high numbers of viruses are found. In stool samples, 10^{9}-10^{10} virus like particles per gram are observed (Hoyles et al., 2014). The majority of viral genomes in the gut are bacteriophages (97.7%), the remainder being eukaryotic and archaeal viruses (Gregory et al., 2020). Due to limited tools available for the study on the phageome, the function and impact of gut bacteriophages remains largely unknown (Shkoporov & Hill, 2019). However, with the recent development of high-throughput metagenomic sequencing technologies, the complexity of the phageome became clear (Breitbart et al., 2008). Furthermore, it was shown that the majority of the

bacteriophages in the gut were not yet identified, as taxonomic classification and connection to a bacterial host was not possible (Manrique et al., 2016; Reyes et al., 2010). The high abundance of these bacteriophages indicate that they can play an important role in maintaining the balance in the complex environment in the gut, mainly by their ability to shape the bacterial community. Different types of life cycles for bacteriophages can be distinguished: lysogenic and lytic cycles (Brady et al., 2021; Hobbs & Abedon, 2016). During a lysogenic life cycle, the phage integrates as a prophage into the genome of the bacterial host. The replication of the bacterial cell is not disturbed, but upon induction of replication, it is transmitted to new cells. In the case of a lytic cycle, phages bind to the bacterial cell via specific receptors. This allows penetration and infection of the bacterial host cell by the phage's nucleic acid and subsequently takeover of its mechanisms for replication and translation to produce a multitude of new virions. The bacterial cell is lysed and these virions are released into the environment where they are able to infect new cells (Barr, 2017). Historically, lytic bacteriophages have been used as therapy to treat bacterial infections. After the first description of bacteriophages by both Frederick Twort and Félix d'Hérelle in 1915 and 1917 respectively (d'Herelle, 1917; Twort, 1915), soon the first use of phages as antibacterial therapy in patients with shigellosis was performed (Sulakvelidze & Kutter, 2004; Summers, 1999). These are the first described examples of phage therapy as treatment of bacterial infections, and it was followed with more usage of phage therapy (d'Herelle, Malone, & Lahiri, 1930; Smith, 1924). Although not all attempts of phage therapy were successful (Eaton & Bayne-Jones, 1934), it was seen as a potential strategy to combat against bacterial infections. The interest in the use of phages as antibacterial therapeutics declined with the discovery and rise of antibiotic use during the 1940s (Aminov, 2017). The lack of availability of antibiotics in Eastern Europe however, has led to a continuation of research and use of bacteriophage therapy in this geographical area (Alisky, Iczkowski, Rapoport, & Troitsky, 1998). The narrow bacterial host range of bacteriophages could possibly explain the fact that phage therapy was not always successful. The bacterial host range of phages can vary from a specific bacterial strain to several closely related species of the same bacteria (de Jonge, Nobrega, Brouns, & Dutilh, 2019). This

characteristic of phages creates difficulties for the widespread use of phage therapy, since the pathogenic strains causing bacterial infections can vary across individuals. Therefore, for a successful phage therapy, it is important not to rely on a single bacteriophage that is active against a single bacterial strain that causes the infection, but create a combination of bacteriophages that target multiple strains within the pathogenic species. A solution for this is the use of bacteriophage cocktails, which contain a range of phages to broaden the bacterial host range (Abedon, Danis-Wlodarczyk, & Wozniak, 2021). Another challenge is that the phage must survive and act in the complex environment of the gut. Several factors such as pH, bile acids, oxygen levels, presence of mucin and nutrient availability have an influence of the population dynamics of bacteria in the gut, and therefore also on the success of phage therapy (Zuppi, Hendrickson, O'Sullivan, & Vatanen, 2021). Although phage research was continued in Eastern Europa, well-described studies in English and available for the Western society on the development of phage therapy are lacking. Several examples of recent studies on phage therapy are a clinical trial on oral phage therapy of children who suffer from acute diarrhea with two different *E. coli* phage cocktails in Bangladesh. Although no severe adverse effects were observed, the phage therapy was not efficacious, possibly due to a too low dosage of the phage (Sarker et al., 2016). A case-report on a 57 year old woman with Crohn's disease suffering from a multisite infection (including the GI tract), describes that the treatment with a phage against *Klebsiella pneumonia* led to the eradication of the original host bacteria (Corbellino et al., 2020). In a study on infant mice, the oral administration of a three-phage cocktail prior to a *Vibrio cholerae* challenge, led to a signification reduction of *V. cholerae* found in the gut (Yen, Cairns, & Camilli, 2017). In addition, studies on fecal microbiota transplantation (FMT) in *Clostridioides* (previously *Clostridium*) *difficile* infection (CDI) show the possible involvement of bacteriophages. The transplantation of sterile fecal filtrate, that still contained phages, to five patients suffering from CDI led to elimination of symptoms in all patients (Ott et al., 2017). These results were supported by the findings that FMT success in CDI was correlated with bacteriophage abundance in the donors (Park et al., 2019; Zuo et al., 2018). As can be seen from these examples, more research is

needed on how bacteriophages can be used to shape the bacterial community, and subsequently influencing host health.

Fungi and yeasts

In addition to viruses, also fungi and yeasts (for sake of simplicity referred to as 'fungi' in the remainder of this chapter) are present in the human gut. When looking to the cell numbers, fungi make up only a limited amount compared to bacteria: approximately 0.1% of the total microbiome in the gut (Chin et al., 2020; Qin et al., 2010), but they still can have a big role on the host health. Comprehensive information on gut fungi is lacking, leading to missing insights on the mycobiome and its interaction with the gut bacterial community and the human host. Historically it was very difficult to study gut fungi, because it was not possible to culture most species in a laboratory situation outside of the human body. Only limited species were culturable and known to colonize the gut. The rise of culture-independent techniques, such as next-generation sequencing, gave new insights on the mycobiome. Nash et al. performed a sequencing study on the mycobiome of samples of the Human Microbiome Project healthy cohort (Nash et al., 2017). This study showed that the gut mycobiome is low in diversity and has a high variability between individuals. With the rise of sequenced-based studies, it became clear that the gut fungal community can influence health and is involved in several diseases. Both in gastrointestinal diseases and in systemic diseases changes in the fungal community were observed. For example, in patients with inflammatory bowel disease (IBD) it was shown that the fungal composition and diversity was altered in IBD patients (Beheshti-Maal et al., 2021; Ott et al., 2008). Also, an increase in several fungal species was correlated with pro-inflammatory cytokines in patients with ulcerative colitis (Qiu et al., 2017). Moreover, specific fungal species were altered in Crohn's disease (CD) patients, where an increase in *C. albicans* and *Malassezia* spp. was observed compared to healthy controls (Limon et al., 2019; Sokol et al., 2017). When looking at oncogenesis, treatment with antifungals was associated with a reduction in tumor progression in pancreatic carcinoma, while a subsequent re-introduction of *Malassezia* increased tumor growth (Aykut et al., 2019). In colorectal cancer, an increased abundance of *Malassezia* was described (Coker et al., 2019).

Fungi are probably also involved in the gut-brain axis, as gut fungal dysbiosis has been described in several neurological disorder such as schizophrenia, autism and Parkinson's disease (De Pablo-Fernandez et al., 2022; Severance et al., 2012; Strati et al., 2017). These different studies show that gut fungi are potentially involved in a range of processes that can have an influence on health. These effects can be either directly by affecting the host, but also indirect via interactions with/modulation of the gut bacterial community. For example, the direct anti-inflammatory effect of *Saccharomyces boulardii* was shown by a decrease in T-helper cell 1 migration and secretion of pro-inflammatory cytokines in the inflamed colon after *S. boulardii* treatment (Dalmasso et al., 2006). Furthermore, the cell wall polysaccharides of *C. albicans*, mannan and β-glucan, interacted with pathogen-associated molecular patterns (PAMPs), thereby increasing the secretion of pro-inflammatory cytokines (van de Veerdonk et al., 2009; Zhang et al., 2016). There are also some examples about fungal-bacteria interactions, although research on this is limited. The outgrowth of fungi in individuals after broad-spectrum antibiotic treatment shows that the commensal bacteria play a role in maintaining a balanced fungal community (Ianiro, Tilg, & Gasbarrini, 2016). Fungal dysbiosis also has an influence on the bacterial community as was shown by Qiu et al., where the disruption of the mycobiome in dextran sulfate sodium (DSS) treated mice by antifungal treatment led to changes in the microbiota (Qiu et al., 2015). Also, specific species of fungi and bacteria can interact with each other. The previously described *S. boulardii* can interact with bacteria by the production of a protease that degrades toxins of *C. difficile* (Castagliuolo, Lamont, Nikulasson, & Pothoulakis, 1996; Castagliuolo, Riegler, Valenick, LaMont, & Pothoulakis, 1999). Most interactions described between bacteria and a fungus are interactions with *Candida albicans*. Several studies describe how bacteria negatively influence *C. albicans* growth. The addition of *Salmonella enterica* serovar Typhimurium led to a reduction of *C. albicans* colonization *in vitro* (Tampakakis, Peleg, & Mylonakis, 2009). A mixture of probiotics (*L. acidophilus*, *L. reuteri*, *L. casei* and *B. animalis*) inhibited *C. albicans* infection in mice (Wagner et al., 1997). The combination of four probiotic strains (*S. boulardii* ATCC MYA-796, *L. acidophilus* ATCC 43121, *L. rhamnosus* ATCC 39595,

and *B. breve* ATCC 15701) limited the formation of a mixed biofilm containing *E. coli, Serratia marcescens, C. albicans and Candida tropicalis* (Hager et al., 2019). Also metabolites produced by bacteria can reduce *C. albicans*: metabolites produced by *Roseburia* spp. and *Bacteroides vulgatus* inhibited *C. albicans* growth (García et al., 2017). A comparative study between Japanese and Indian individuals looked at the fungal-bacterial interactions, and how these correlate with diet (Pareek et al., 2019). Higher levels of *Candida* and *Prevotella* were observed in Indian subjects who have a diet that is mostly plant-based and it was shown that the plant polysaccharide arabinoxylan could act as a growth factor for *Candida* spp. This shows that diet can also shape the gut mycobiome. Moreover, diet is an important source of live fungi and fungal DNA (Fiers, Gao, & Iliev, 2019; Graves & Hesseltine, 1966; Tournas & Niazi, 2018). For example, *Saccharomyces* is found in a variety of food products and levels found in feces could be directly linked to the diet (Auchtung et al., 2018). These different interactions show how complex the gut microbial community is, and that the mycobiome can possibly play an important role in maintaining a healthy microbiota balance.

Antibiotic use

A factor that can cause a dysbiosis in the gut microbiota is the use of broad-spectrum antibiotics. Since their discovery, antibiotics have been used worldwide to treat bacterial infections, which has drastically declined infectious disease mortality (Adedeji, 2016). Next to this positive effect, there are also downsides to the widespread use of antibiotics: e.g., the extensive use as therapeutics in both human and veterinary health care and the prophylactic (over)use in livestock has led to a global rise of antibiotic resistance (Llor & Bjerrum, 2014; Vidovic & Vidovic, 2020). The last years it has become more clear that the commensal microbial community plays an important role in maintaining host health. Antibiotic treatment does not only remove the pathogenic bacteria, but also impacts the commensal microbiota, leading to a decreased diversity and the removal of valuable microbes (Blaser, 2014; Dubourg et al., 2014). Blaser and Falkow described the theory of 'missing microbes', which states that the disappearance of important microbes can lead to conditions such as obesity and diabetes (Blaser & Falkow, 2009). This link is factually supported

by several studies (Candon et al., 2015; Del Fiol, Balcão, Barberato-Fillho, Lopes, & Bergamaschi, 2018). Another consequence of the use of antibiotics is the increase in the pool of antibiotic resistance genes (ARGs). It was shown that the human body is an important reservoir for ARGs (Bailey, Pinyon, Anantham, & Hall, 2010; Clemente et al., 2015; Kamenshchikova et al., 2021; Sommer, Church, & Dantas, 2010), and most ARGs can be found in the gut (Carlet, 2012). The rise of ARGs worldwide is a dangerous development, because it endangers the treatment of infections due the loss of efficacy of antibiotics . The increase in antimicrobial resistance is a global crisis, which leads to increased length of hospital admission, morbidity and mortality and consequently increased healthcare costs (Murray et al., 2022). To combat this global crisis, alternatives to antibiotic treatments are explored, and this has led to a renewal of interest in phage therapy. There are some case-reports that describe the successful removal of antibiotic resistant bacteria by phage therapy (Cao et al., 2015; Dedrick et al., 2019; Schooley et al., 2017). Phage therapy has as an additional advantage that they have a small bacterial host range compared to antibiotics, therefore minimizing the disruption of the commensal microbiota (Cieplak, Soffer, Sulakvelidze, & Nielsen, 2018). These results are promising for the use of phage therapy in the combat against antibiotic resistant bacteria, but more well-designed studies are needed to gain more evidence. The use of antibiotics also leads to the fungal community being disrupted, as eluded to above. The outgrowth of fungi following antibiotic treatment is a serious problem (Dollive et al., 2013; Esaiassen, Fjalstad, Juvet, van den Anker, & Klingenberg, 2017). This could lead to dangerous fungal infections which are difficult to treat and which have a high mortality (Ben-Ami et al., 2012; Cornely et al., 2008; Uzun, Ascioglu, Anaissie, & Rex, 2001). The combination of the risks posed by antibiotic use, show that research is needed into alternatives for antibiotic treatment such as bacteriophage therapy. Also, more information is needed on cross-kingdom (fungal, bacterial and viral) relations in the gut which are important to maintain a healthy state.

In vitro gut fermentation models

Most current research on the gut microbial community is done with the use of high-throughput sequencing, and although this technique has given very valuable

information about the composition of the microbiota found in the gut, information about the functionality of these microbes is lacking. To gain more insight in the functionality of microbes, and the interactions that take place in the complex gut environment, mechanistic studies are necessary. A strategy for this kind of research is to make use of *in vitro* gut fermentation models, which allow for the research on both microbial composition and functionality. In general, these gut fermentation models consist of one or more chemostats with physiological conditions, which are inoculated with a fecal microbiota (Macfarlane, Macfarlane, & Gibson, 1998; Minekus et al., 1999; Molly, Vande Woestyne, & Verstraete, 1993). The complexity of these *in vitro* model varies from simple batch models to more sophisticated continuous models, and can model a single region of the GI tract or combine several regions (Payne, Zihler, Chassard, & Lacroix, 2012). The most innovative models, do not only include continuous fermentation, but also include host digestive functions thereby simulating the physiological functions that can have an influence on the microbiota. They can include the different regions of the GI tract: stomach, small intestine and colon. The **T**NO *i*n vitro **m**odels of the gastro-intestinal tract (TIM) are an example of such artificial digestive systems (Minekus, 2015; Venema, 2015).

TNO gastro-intestinal model of the upper GI tract (TIM-1)
TIM-1 is a multi-compartment model of the upper GI tract, which has four compartments: stomach, duodenum, jejunum and ileum, that are made of glass containers with a flexible membrane on the inside. The different compartments are connected with valves, which can open and close to simulate transit of the chyme, similar to what happens *in vivo*. In the compartments, the chyme is mixed through peristaltic movements. Throughout the model, temperature and pH are controlled, and gastric enzymes, pancreatin and bile are secreted and mixed with the chyme. Water soluble products are filtered out via a dialysis system, and samples can be taken at various spots for analysis. Also from the ileum outlet, which simulates the fraction that is transported to the colon, samples can be taken.

TNO in vitro model of the colon (TIM-2)
On the basis of the TIM-1 model, a model of the colon was developed: TIM-2. This model also consists of glass compartments, a flexible membrane on the inside and

water in between. This water is temperature controlled at physiological levels (37°C for humans). By applying pressure on the water, peristaltic movements can be mimicked which allow for optimal mixing. The pH is constantly measured and kept at a level of 5.8 or higher, by addition of sodium hydroxide (2M NaOH) to neutralize acids that are produced by the microbiota. The TIM-2 also has a dialysis system, which allows keeping microbial metabolites at physiological concentrations. The model is continuously flushed with nitrogen to create an anaerobic environment. For experiments, the model is inoculated with a pooled microbiota after which an adaptation period is started that allows microbiota to adapt to its new environment. This is followed by a test period that usually takes 72 hours, but experiments up to 3 weeks with a live microbiota have been reported (Minekus et al., 1999; Venema, Nuenen, Smeets-Peeters, Minekus, & Havenaar, 2000). During experiments, a feeding medium, which simulates the fraction of the diet that reaches the colon, is continuously fed in the compartments. This allows different interventions to be carried out, to investigate the effect of nutritional of pharmaceutical products. Samples can be taken directly from the lumen and from the dialysis solution to observe the direct effect of the feeding on the microbiota composition and activity.

Until now, research with the TIM systems have focused on the bacterial component of the microbiota, but as described before the gut microbiota is much more complex. The use of TIM in research on bacteriophage therapy could give valuable insights on how bacteriophages behave in the complex environment of the GI tract, studying both survival under the harsh conditions (low pH, bile, digestive enzymes) of the upper GI tract, and in the microbial community of the colon. This information could add to the success of bacteriophage therapy, since *in vitro* studies on the functionality of phages are lacking. In addition to the bacterial microbiota, an attempt should be made to analyze the fungal microbiota, since this community can also play an important role in health and disease, and should not be neglected in studies on the microbiome. The use of an *in vitro* model of the GI tract can give more insights on the composition and functionality of gut fungi.

Scope of this thesis

As outlined in the previous paragraphs, the gut microbiota is important in health and disease, and this is true for both the bacterial and fungal component. The interaction between these, as well as the broadening of the use of phage therapy to modulate these microbial components, require the development of a set of tools to study this.

Therefore, the aim of this thesis was to:

- Develop tools to study mechanistically the survival and efficacy of bacteriophages in the human gut and thereby optimizing oral phage therapy.
- Optimize tools to study the fungal community *in vitro* and the fungal-bacterial interactions that occur in the human gut.

Chapter 1 (this chapter) summarizes the importance of the study of the different kingdoms found in the gut, and discusses the need for the development of tools to study bacteriophages as well as fungi in the gut. **Chapter 2** describes the importance of identification of bacteriophages before using them in therapy, as well as testing the survival of bacteriophages in the upper GI tract with the use of the *in vitro* model TIM-1. In **chapter 3**, the survival and efficacy of bacteriophages in the colon and the effect on the bacterial community in the colon is studied with the use of the *in vitro* model TIM-2. In **chapter 4**, the use of TIM-2 as a tool for the study of the gut fungal community is investigated. In addition, dietary interventions are carried out to see if this modulates the mycobiota. The gut fungal community is further investigated in **chapter 5**, where the mycobiota of healthy individuals is analyzed and fungal-bacterial interactions are examined. The interactions between the fungal and bacterial community in the gut are further analyzed in **chapter 6** with the use of TIM-2, where interventions with antibiotics and fungicide are carried out to investigate whether the microbial community and the cross-kingdom interaction is disrupted. Finally, all major findings, identified limitations and important future directions are discussed in **chapter 7**.

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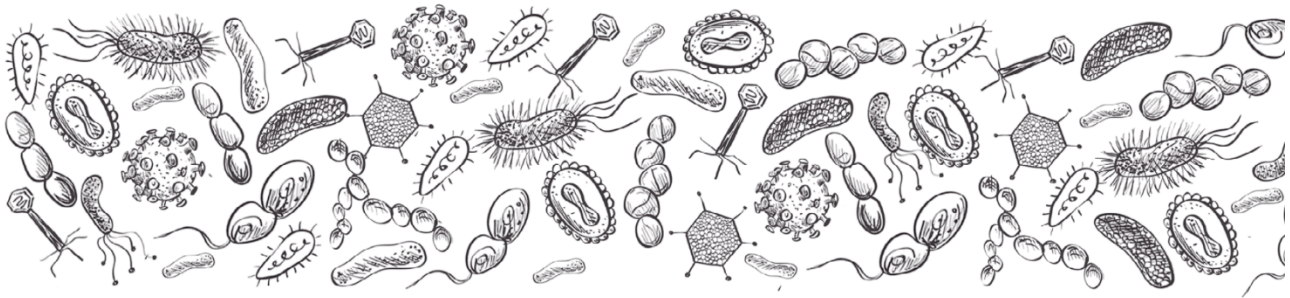
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Chapter 2

Survival of two phages as free particles during transit through a computer-controlled dynamic system simulating the upper gastrointestinal tract (TIM-1)
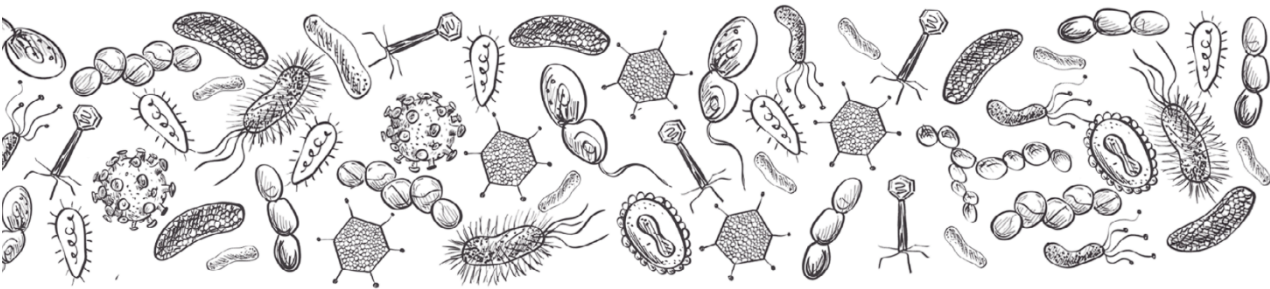
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Abstract

Excessive use of antibiotics in the last few decades has led to a global rise of antibiotic resistance. Alternative therapeutic options for bacterial infections should therefore be explored. Bacteriophages are known to eliminate bacteria and have a narrow bacterial host range, therefore they do not disrupt the gut microbial community as is the case for antibiotics. Studies on effective phage therapy are limited. More insight in the survival of bacteriophages in the gastrointestinal (GI) tract could help in the development of successful phage therapy. When taken orally, bacteriophages have to survive the conditions in the upper GI tract, such as low pH, bile and gastric and pancreatic enzymes. In this study, the survival of two bacteriophages (a novel isolated *E. coli* phage and phage J1 against *L.casei*) was investigated with the dynamic computer-controlled TNO gastro-intestinal model of the stomach and the small intestine (TIM-1). Gastric experiments were performed as well as experiments using the complete model. The cumulative survival through the stomach compartment was 7.38% for the *E. coli* phage and 19.15% for phage J1. The cumulative survival for transit through the stomach and small intestine compartments was 13.31% for *E. coli* phage and 6.81% for phage J1. These experiments show that bacteriophages are affected by passage through the upper GI tract, and also that the survival rate can differ between different phages. When phages are ingested in a fed-state, roughly 5-10% reaches the lower GI tract. In conclusion, the use of TIM-1 in the design of phage therapy could help with increasing the efficacy of the therapy by optimizing the dosage or exploring the use of survival strategies, such as encapsulation, to enhance survival.

Introduction

The last decades, a global rise in antibiotic resistance has been observed (Aslam et al., 2018). The World Health Organization acknowledges this continuing global rise in antibiotic resistance to be a serious threat for global health ("WHO's first global report on antibiotic resistance reveals serious, worldwide threat to public health," 2014). The (excessive) use of antibiotics to prevent and treat bacterial infections in both human and veterinary medicine has led to a dramatic increase in antibiotic resistance among pathogenic as well as commensal bacteria. Multi-drug-resistant bacteria, which have become unsusceptible to various classes of antibiotics, are most difficult to treat and therefore lead to increased morbidity and mortality. To avoid a further rise in antibiotic resistance, alternative therapeutic options should be explored.

Already discovered 100 years ago, bacteriophages are known to eliminate bacteria (d'Hérelle, 2007; Twort, 1961). The potential of (bacterio)phage therapy was neglected during the rise of the antibiotic era, but with the problems caused by antibiotics, phage therapy should be investigated as alternative (Kakasis & Panitsa, 2019). There are some advantages of bacteriophages compared to antibiotics. Antibiotics usually act on a large range of bacteria, thus will also affect beneficial bacteria, which can lead to dysbiosis (Duan et al., 2020). Dysbiosis in the gut is characterized by a decreased diversity and allows potential harmful bacteria, such as *Clostridioides* (formerly *Clostridium*) *difficile*, to bloom. This can lead to side effects ranging from self-limiting antibiotic-associated diarrhea to life-threatening inflammatory conditions such as psuedomembranous colitis. In addition, the widespread use of antibiotics is believed to have led to the extinction of certain beneficial microbes in the gut (Blaser, 2014). Bacteriophages on the other hand have a narrow host-range, they can act only on one or several related bacterial species, or even on a specific strain (Hyman & Abedon, 2010). There are several examples of (successful) use of phage therapy in bacterial infections, but more research is needed to make phage therapy more effective, to be able to use it as a more common option for treatment (S. Aslam et al., 2020; Gindin, Febvre, Rao, Wallace, & Weir, 2018; Gupta, Singh, Shukla, Nath, & Bhartiya, 2019; Ujmajuridze et al., 2018).

In order for orally administered phage therapy to be effective, the bacteriophage should survive the gastric and small intestinal passage. In literature, several experiments describe the survival rate after exposure to low pH, bile and gastric enzymes (Jakobsen et al., 2021; Lorenzo-Rebenaque, Malik, Catalá-Gregori, Marin, & Sevilla-Navarro, 2021; Śliwka et al., 2019). In addition, several mechanisms to increase survival are investigated, for example encapsulation (Malik et al., 2017). When the bacteriophage has successfully survived the harsh conditions in the upper gastrointestinal tract, it encounters a next challenge: the complex microbial community in the large intestine. This consists of bacteria, but also bacteriophages are present in the gut at high numbers. For the phage therapy to be effective, the phage has to survive transit through the (upper) gastrointestinal (GI) tract. Next, it should act on its host bacteria, where successful lysis can lead to removal of these (harmful) bacteria. To get more knowledge about the efficacy of bacteriophages in the gut, but in terms of survival during passage as well as targeting its host, more research is needed.

A method to mechanistically study the survival in the stomach and small intestine is by making use of an *in vitro* model. A validated dynamic computer-controlled intestinal model of the stomach and small intestine is TIM-1 (Minekus, 2015), which is validated for studying the digestion of different compounds, as well as survival of probiotic strains (Marteau et al., 1995) encompassing several different genera (Surono, Verhoeven, Verbruggen, & Venema, 2018; Venema, Verhoeven, Beckman, & Keller, 2020; Venema, Verhoeven, Verbruggen, Espinosa, & Courau, 2019). This complex model allows the prediction of the survival of bacteriophages, due to accurate mimicking of the complex and dynamically changing conditions that occur in the stomach and small intestine.

E. coli is present in the gut as a symbiont, but there are also many virulent strains that can cause a variety of infections. The prevalence of multi-drug resistant *E. coli*, which are ranked as global priority pathogens by the WHO has risen dramatically over the past decades (Asokan, Ramadhan, Ahmed, & Sanad, 2019).

There are several described candidate coli phages to treat *E. coli* infections (Costa, Pereira, Gomes, & Almeida, 2019; Dalmasso et al., 2016; Sarker et al., 2016). Given the clinical relevance of this bacterium and the well-described phages, it is a good candidate to proof that TIM-1 can be an effective tool to study bacteriophage survival. To obtain a relevant phage from fecal samples, a new isolate was obtained from wastewater, as isolation from a fecal sample itself proved to be difficult due to the fecal consistency (not shown). To extend the versatility of the model, an additional phage-host pair was used. For this *Lacticaseibacillus* (formerly *Lactobacillus*) *casei* and phage J1 were chosen. When these experiments are successful for these bacteriophages, it can also be used for clinically relevant (multi-drug-resistant) bacteria and novel phages, which are more challenging to work with in the lab. Therefore, the aim of the study was to study survival of a newly isolated phage against *E.coli* and phage J1 against *L.casei* during passage through the upper GI tract in the well-established TIM-1 model.

Material and methods

Growth of bacterial strains, isolation, and multiplication of phages

Bacteriophages against *E.coli* were isolated and purified from wastewater received from a sewage treatment plant (RWZI Aarle-Rixtel, The Netherlands). The wastewater (40 mL) was incubated with 5 mL 5x concentrated LB medium and 5 mL overnight culture of *E. coli* K12, as the host to let the coli phage multiply. The mixture was incubated for 24 hours at 37°C in a water bath. After the incubation, the wastewater – bacteria – bacteriophage mixture was centrifuged at 2000×g for 5 minutes. The supernatant was filtered through a 0.45μm filter and subsequently through a 0.20μm filter. After this, the phage-containing solution was tested (double layer agar method; see below) against an *E. coli* DH5α containing the plasmid pMG36e, which confers resistance against erythromycin (van de Guchte, Van der Vossen, Kok, & Venema, 1989).

For the TIM-1 experiment a high titer bacteriophage stock was needed (PFU/ml ≥ 10^8). Therefore, *E. coli* (pMG36e) from a -80°C freezer glycerol stock, were grown in rich medium to create high bacterial growth. The medium used was Terrific Broth (TB) (24 g/l Yeast Extract, 20 g/l Tryptone, 4 ml/l Glycerol, 2.2 g/l KH_2PO_4 and 9.4

g/l K_2HPO_4), and Tryptone Yeast (TY) medium (26.8 g/l Tryptone, 21.4 g/l Yeast Extract, 10 ml/l Glycerol, 8.5 g/l NaCl, 1.2 g/l MgSO4, 5.4 g/l KH_2PO_4 and 1.6 g/l K_2HPO_4) (Romano, Molla, Pollegioni, & Marinelli, 2009).

Bacteriophages were propagated via liquid lysate as described before (Bonilla et al., 2016). In sterile 50 mL Falcon tubes, TB and TY were supplemented with 0.001 M $CaCl_2$ and $MgCl_2$ to promote adhesion of bacteriophages. Next, 0.1 volume of overnight culture of *E. coli* (pMG36e) was added and incubated for 1 hour at 37°C with shaking. After this 1 mL of bacteriophage lysate was added and incubated overnight at 37°C until medium was cleared. Lysate was cleaned by centrifugation for 20 min at 4000×g, supernatant was collected and filtered through a 0.22 μm filter to remove bacterial debris. For further clean-up, 0.1 volume of chloroform was added to the supernatant, mixed by vortexing and incubated for 10 min at room temperature without shaking. After this, the supernatant was centrifuged for 5 min at 4000 × g, transferred to sterile 100 mL bottles, and stored at 4°C until further use.

For titer determination, *E. coli* (pMG36e) was grown overnight in rich medium (TB and TY). The high titer bacteriophage stock was 10-fold serially diluted to 10^{-10} dilution in SM buffer, which contained 5.8 g/l NaCl, 2 g/l $MgSO_4\cdot7$ H_2O and 50 ml Tris-HCl (1M, pH 7.5), to obtain individual plaques. LB agar plates (10 g/l tryptone, 10 g/l NaCl, 5 g/l Yeast Extract, and 15 g/l agar) were prepared with erythromycin (50μg/μL final concentration). LB top agar (10 g/l tryptone, 10 g/l NaCl, 5 g/l Yeast Extract, 7.5 g/l agar) was prepared and 3 ml was added to 15 ml tubes, which were kept liquid in a water bath at 56°C. To the top agar, 1 ml of bacterial culture and 1 ml of phage dilution was added, mixed, and quickly poured onto pre-warmed LB plates. The top agar was evenly spread and plates were left until the top agar was set. To check for contamination in the media and cultures, always a control with only top agar 9for contamination with bacteria) and top agar + bacterial culture (for contamination with (other) phages) was included. Preparation of plates was done in a laminar flow cabinet. Plates were incubated overnight at 37°C, after which phages were enumerated by counting the plaques and multiplying with the dilution-factor.

A single plaque was taken, the phage eluted from the top-agar and re-seeded in multiple dilution again to obtain a pure phage-culture.

In addition, bacteriophage J1 (ATCC 27139-B1) active against *L. casei* was acquired commercially (ATCC, Manassa, VA, USA). For this, similar propagation and enumeration steps were followed as described above. The medium used to grow the *L. casei* BL23 was BD Difco™ Lactobacilli MRS broth or agar (Difco, Franklin Lakes, NJ, USA). To increase the visibility of plaques, glycine (100mM final concentration) and $CaCl_2$ (10mM final concentration) were added to the agar. Plates were incubated under anaerobic conditions overnight at 37°C.

DNA isolation and sequencing of the newly isolated E. coli phage
The high titer bacteriophage stock of the newly isolated *E. coli* phage was used as starting material for nucleic acid isolation. First, the lysate was treated to remove bacterial DNA and RNA and to digest the phage capsid as described before (Jakociune & Moodley, 2018). For this, 900 μl of phage lysate, filtered through a 0.22 μm filter, was combined with 100 μl DNase I 10x buffer, 2 μl DNase I (1 U/μl) and 1 μl RNase A (10 mg/ml) (Thermo Fisher Scientific, Walham, MA, USA). This was incubated for 1.5 hour at 37°C. Reaction was inactivated by adding 40 μl of 0.5M EDTA. Next, 2.5 μl of proteinase K (20 mg/μl) was added and this was incubated for 1.5 hour at 56°C. For the isolation of DNA, the phage DNA isolation kit from Norgen Biotek (Norgen Biotek Corporation, Thorold, Canada) was used. In brief, to the pre-treated phage lysate, 500 μl of lysis buffer B was added, this was mixed and incubated for 15 min at 65°C after which 320 μl isopropanol was added. A spin column was assembled and 650 μl lysate was loaded and centrifuged for 1 min at 6000×g, and this was repeated until all lysate passed through the spin column. In the next step, 400 μl of wash solution A was loaded on the column, centrifuged for 1 min at 6000×g, the flow through was discarded and this was repeated two times. The spin column was then centrifuged for 2 min at 14000×g to dry the resin, and placed in an elution tube. 75 μl elution buffer B was loaded and the tube was centrifuged for 1 min at 6000×g. To increase the yield of DNA, the column was placed in a new elution tube, the eluted DNA solution from the previous step was again applied on the column and centrifugation was repeated. The DNA concentration was measured

using the high sensitivity ds DNA assay on the Qubit 2.0 fluorometer (Thermo Fisher Scientific).

For sequencing of the phage DNA, libraries were prepared with the Native barcoding genomic DNA protocol, using the 1D genomic DNA by ligation kit (SQK-LSK109) and the Native Barcoding Expansion (EXP-NBD104) (Oxford Nanopore Technologies Inc., Oxford, UK). The barcoded libraries were loaded on a MinION flow cell (FLO-MIN106) and sequenced on the MinION device (Oxford Nanopore Technologies Inc). After the run, the sequenced reads were base called using Guppy (version v 5.0.2) using default parameters. Base called reads were filtered using NanoFilt from NanoPack (De Coster, D'Hert, Schultz, Cruts, & Van Broeckhoven, 2018), and reads shorter than 1000bp and an average quality less than 10 were removed. Filtered reads were assembled into contigs using the metaFlye de Novo assembler (Kolmogorov et al., 2020). Resulting contigs were subjected to an extra round of polishing with medaka (https://github.com/nanoporetech/medaka) and annotated with Prokka (Seemann, 2014).

Electron microscopy (EM) of bacteriophage
The bacteriophage sample was prepared for cryo-EM. The sample was applied on Lacey Carbon grids and vitrified using a vitrobot (FEI, Eindhoven, The Netherlands) (Ravelli et al., 2019). Next, the grids were clipped into Autogrids. Imaging was done with the Artica 200 kV transmission electron microscope using the Falcon3 detector (Thermo Fisher Scientific).

Survival of the phages in TIM-1
In order to test the survivability of the bacteriophages, the dynamic *in vitro* model of the stomach and small intestine (TIM-1; Figure 1) was used (Minekus, Marteau, Havenaar, & Veld, 1995). The model consists of glass compartments that mimic respectively the stomach (Figure 1A), duodenum (Figure 1C), jejunum (Figure 1E) and ileum (Figure 1G). To create peristaltic movements, the glass compartments contain silicone membranes inside that can mimic the movements, by pumping water in between the silicon membrane and glass jackets. The water temperature is set to 37 °C, and the pressure on the water allows for realistically mixing and transit

of the chyme through the system. In the whole system, the pH is controlled by the secretion of hydrochloric acid (stomach; Figure 1I) or sodium bicarbonate (small intestinal compartments; Figure 1J). The TIM-1 is computer controlled; the protocol sets the pH-curve in and the emptying of the stomach compartment, the transit time in the small intestine and concentrations of electrolytes, enzymes, bile, and pancreatic juice over the course of the experiment. To keep the concentrations of these compounds in physiological amounts, two hollow fiber membranes are used for dialysis of digested and small compounds from the jejunum (Figure 1M-left) and ileum (Figure 1M-right). In the experiments for gastric survival, only the stomach and the duodenum compartment were used, where in the duodenum only neutralization took place, but no bile or pancreatin was secreted. Over the course of the 3-hour experiment, the stomach content was slowly transported into the duodenum according to the loaded protocol, mimicking transit of a meal. In subsequent experiments for gastric and intestinal survival, over the course of 6 hours the content was delivered gradually to the sample bottle (Figure 1H), which represents the large intestine, which was placed after the ileum compartment. The bacteriophages (1ml of ~10^8 PFU/mL) were added to the 'meal' and introduced at the start of the experiment. The 'meal' used in this experiment was not an actual meal, but mimics the buffering capacity of ingested food with the use of a 300 ml sodium citrate buffer solution. The experiments were performed in duplicate with both the *E. coli* and J1 bacteriophage. Each hour samples were taken from the ileal efflux (Figure 1H) for assessing phage survival. At the end of the experiments, the residue in the model was collected as well. Serial dilutions were prepared of the 3 gastric efflux, 6 ileal efflux and the residue samples taken from TIM-1, as well as from the initial phage solution and the meal introduced in the stomach compartment, and these were plated on Difco MRS agar + glycine + $CaCl_2$, for the J1 phage and LB agar for the *E. coli* phage according to the double layer agar method as described above. Subsequently, the plates were incubated at 37 °C for 24 hours under aerobic (*E. coli* phage) and anaerobic (J1 phage) conditions. Cumulative survival was calculated as the sum of the surviving phages in the different efflux samples taken over time from TIM-1.
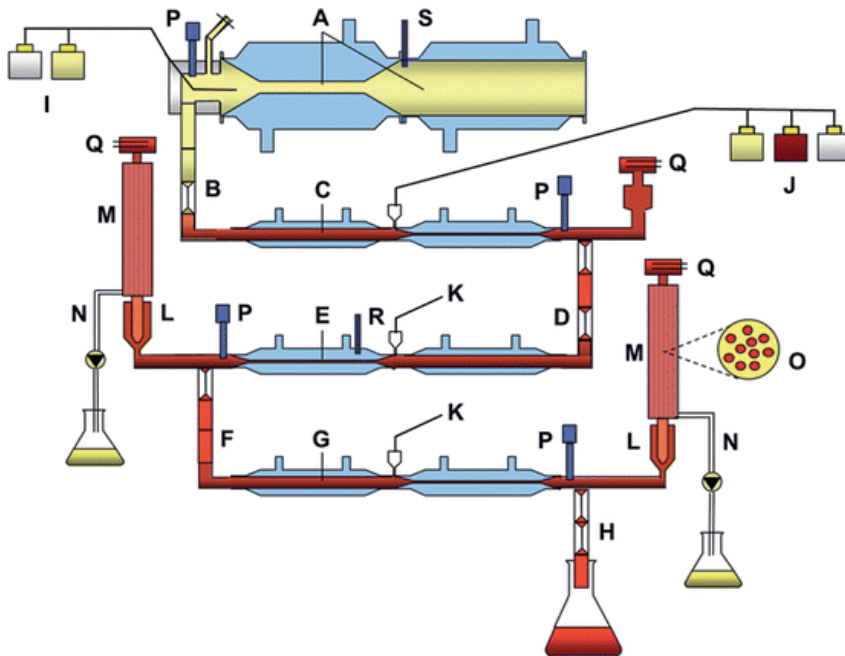
Figure 1. Schematic presentation of TIM-1. **A.** gastric compartment; **B.** pyloric sphincter; **C.** duodenal compartment; **D.** peristaltic valve; **E.** jejunal compartment; **F.** peristaltic valve; G. ileal compartment; **H.** ileal-cecal valve; **I.** gastric secretion; **J.** duodenal secretion; **K.** bicarbonate secretion; **L.** pre-filter; **M.** filtration system; **N.** filtrate with bio-accessible fraction; **O.** hollow fiber system (cross section); **P.** pH electrodes; **Q.** level sensors; **R.** temperature sensors; **S.** pressure sensor (Minekus, 2015).

Results and Discussion

The isolation of a phage from wastewater was successful. Wastewater has shown to be a fruitful source of bacteriophages (Alharbi & Ziadi, 2021). Phages exist at all sites where their hosts are present, such as soil, water reservoirs and the human body. The isolation from wastewater has the advantage that there is less debris, which makes it easier to filter out exclusively the bacteriophages, which proved in our hands difficult in a fecal sample. This shows that wastewater is a source of clinically relevant phages acting on bacterial infections.

After the isolation, the bacteriophage showed activity against *E. coli* (pMG36e). To identify the phage, the MinION with Nanopore sequencing technique was used. This sequencing showed that the contig with the highest coverage (~150) was a novel phage of the family *Siphoviridae*, with a size of ~44 kb, named phage WW_EC

(Figure 2). The closest relatives were phages also active against *E. coli* (Figure 3). Subsequently, cryo-EM images of the bacteriophage against *E. coli* (pMG36e) were acquired (Figure 4). From the images the head-and-tail morphology can be observed, the tail has a long flexible tail, which confirmed that the isolated phage belongs to the family *Siphoviridae*. The identification of bacteriophages prior to the use in phage therapy is important to optimize the therapy as well for regulatory standards needed for the phage therapy to be accepted as clinical therapy (Abedon, 2017). In this experiment, also the well-described bacteriophage J1 active against *L. casei* was used, which was isolated during the production of Yakult (Japan) (Hino & Ikebe, 1965) to show the tools used in this study are useful for different kinds of bacteriophages (Dieterle et al., 2014).
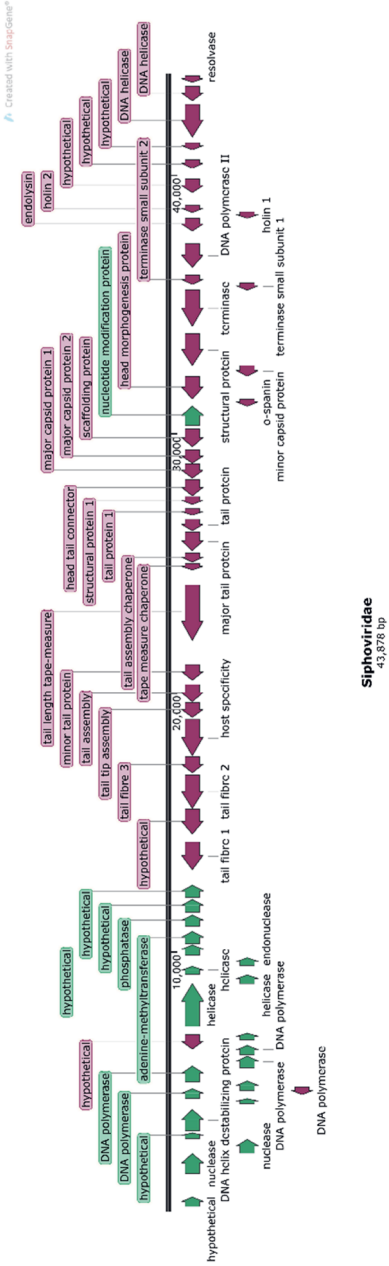
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Figure 2: Genetic map of novel isolated phage WW_EC

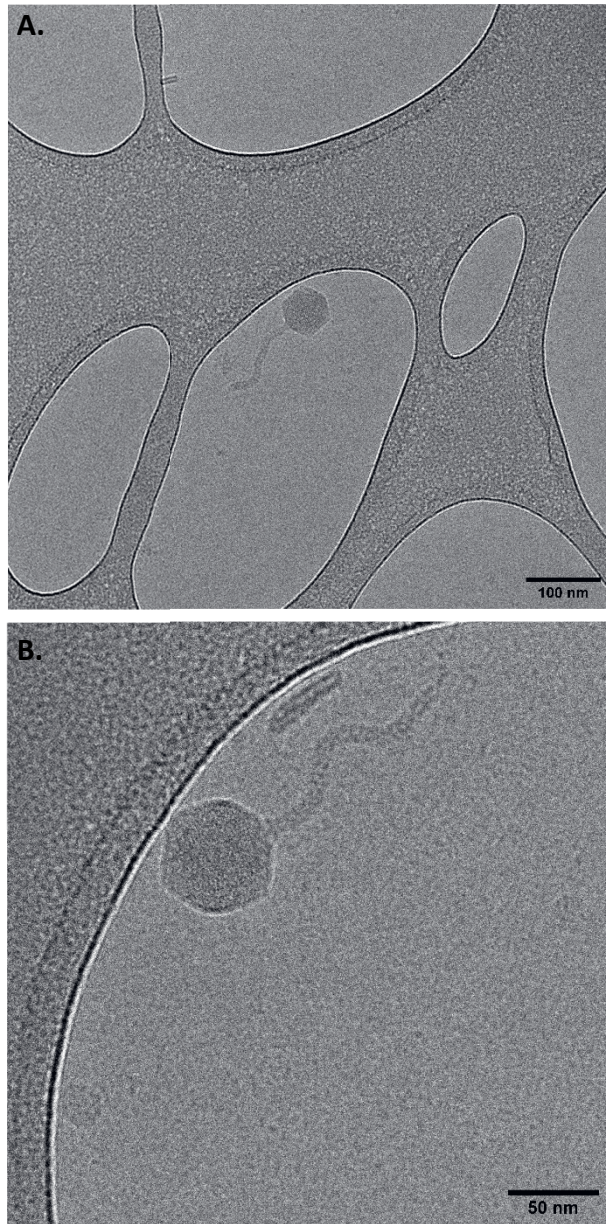**Figure 3:** Phylogenetic tree isolated phage WW_EC

Figure 4. Cryo-EM images of bacteriophage against *E. coli* (pMG36e) at different magnifications; **A.** 55,000x **B.** 110,000x

In this study, the use of the TIM-1 system to evaluate the survival of bacteriophages in the upper GI tract was investigated. In this era where an antibiotic resistance pandemic is threatening us, tools to help the development of bacteriophage therapy are very much needed. The TIM-1 system has been shown before to be a valuable tool to accurately mimic physiological conditions of the upper GI tract (Minekus et al., 1995). The complex system realistically simulates the pH and concentrations of pancreatic enzymes and bile that are dynamically changing over time (Minekus et al., 1995). With the use of the TIM-1 model, the stability during transit of different compounds through the stomach and small intestine can be followed. The model allows for non-invasive and reproducible experiments, that *in vivo* would not be as feasible. The model has been successfully validated (Marteau, Minekus, Havenaar, & Huis, 1997) and used to study the survivability of probiotics (Surono et al., 2018; Venema et al., 2020; Venema et al., 2019).

Gastric experiments were performed with the in-house isolated bacteriophage against *E. coli* (WW_EC) and a commercial acquired bacteriophage against *L. casei* (J1). The starting conditions for the experiments were similar (PFU/mL = 10^8 added to the stomach content) therefore the experiments can be compared. The PFU/mL was determined for the gastric efflux after 60, 120 and 180 minutes and as well in the residues left in the system at the end of the experiment. Also, the PFU/mL was determined immediately after it was added to the meal, and as expected a 2log drop could be observed (Figure 5 and Supplemental Figure 1). The bacteriophage titer dropped after 120 and 180 minutes for both bacteriophages, which can be explained by the drop in pH to ~2 after the buffering capacity of the meal is over. In the residues left in the system, no phages were detected, so all phages were either delivered to the small intestine or killed. From the PFU/mL and total volumes of the efflux, the total phages present in the system were calculated and this was used to determine the cumulative survival (Figure 6 and Supplemental Figure 2). For the *E. coli* bacteriophage, 7.4 % of bacteriophages survived transit through the stomach compartment, while for the bacteriophage J1 this was 19 %. The higher survival rate of the J1 bacteriophage could be due to the lower pH tolerability of the host (*L. casei*), compared to *E. coli*, and phage J1 may have evolved with the host to resist this lower

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pH. The large drop in cumulative survival shows that both bacteriophages are sensitive to low pH and/or the proteolytic activity of pepsin in the gastric compartment. The enzyme may digest the proteins in the tail-tip of the phage that are required to attach to the receptors on the host cells, thereby rendering the phage ineffective (and un-infective). The plaques on the plates of the bacteriophages in the samples were sometimes difficult to see. The addition of glycine and $CaCl_2$ was helpful to increase visibility of these plaques (Ebrecht, Guglielmotti, Reinheimer, & Suarez, 2010).
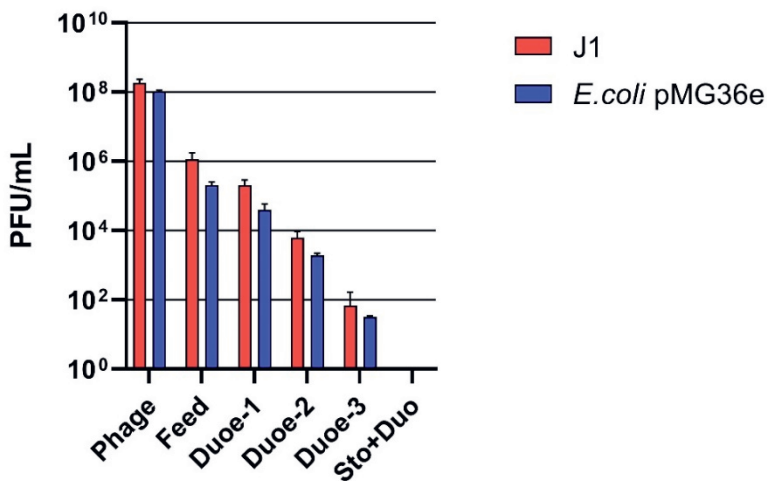

Figure 5. Bacteriophage titers (PFU/mL) for gastric experiments for bacteriophage E. coli and J1. Titer in initial phage solution (Phage); in meal at the start of the experiment (Feed); in duodenal efflux (Duoe) after 1, 2 and 3 hours; the residue in stomach and duodenum compartment at the end of the experiment (Sto+Duo).
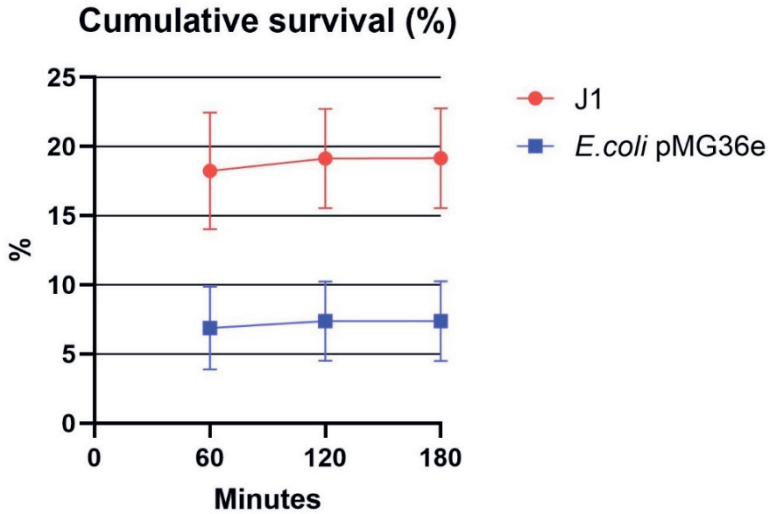
Figure 6. Cumulative survival stomach experiment (as % of intake) as calculated from PFU/mL and total volumes in efflux

Next to the gastric experiments, also experiments with the whole system were performed. In these experiments, the effect of pancreatic enzymes and bile could be observed, next to the influence of the pH and pepsin. From the samples of the ileal efflux also the PFU/mL was determined (Figure 7 and Supplemental Figure 3). A drop in titer was also seen in these samples, which was expected since the titer already was lower after the stomach compartment. Remarkably, the titer stayed more or less stable in the samples after 2 – 6 hours running the system, whereas we expected a reduction in viability due to e.g. degradation of phage protein by pancreatic enzymes. This suggests that most phages are already delivered in the system after the first hour. In the residues also bacteriophages could be detected. For these experiments, also the cumulative survival was calculated (Figure 8 and Supplemental Figure 4). For the *E. coli* bacteriophage this was 13 % and for J1 bacteriophage 6.8 %. For the bacteriophage J1, the survival rate is lower compared to the gastric experiments. This suggests that the bacteriophage is also somewhat sensitive to bile and pancreatic enzymes, although the largest drop was observed after the gastric compartment. The survival of the *E. coli* bacteriophage in the

complete experiment is higher compared to that in the gastric experiments, which was unexpected. Several hypotheses can be entertained for this. First, there could be some variation in the host *E.coli* culture that was used, since the experiments were performed in the course of a few weeks. Secondly, the TIM-1 system is not completely sterile, and a background microbiota is introduced from bile and/or pancreatin. The increase in survival compared to the gastric experiments then suggests that the bacteriophage was able to multiply in the small intestine compartments, due to the presence of a 'background' *E. coli* that could act as a host for this bacteriophage. Already a few *E.coli* cells could affect the titer enormously, due to the burst size of the phage.
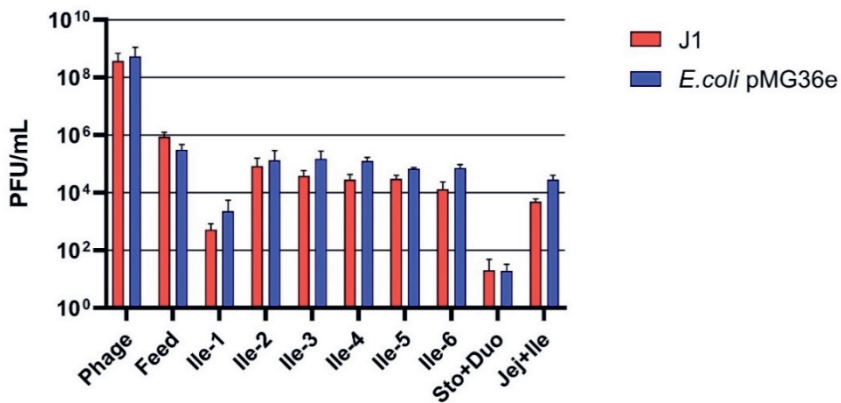

Figure 7. Bacteriophage titers (PFU/mL) for complete experiments for bacteriophage E. coli and J1 (mean + SD; n=2). Titer in initial phage solution (Phage); in meal at the start of the experiment (Feed); in ileal efflux (Ile) after 1-6 hours; residue in stomach+duodenum (Sto+Duo) and Jejunum+ileum (Jej+Ile) compartment at the end of the experiment.
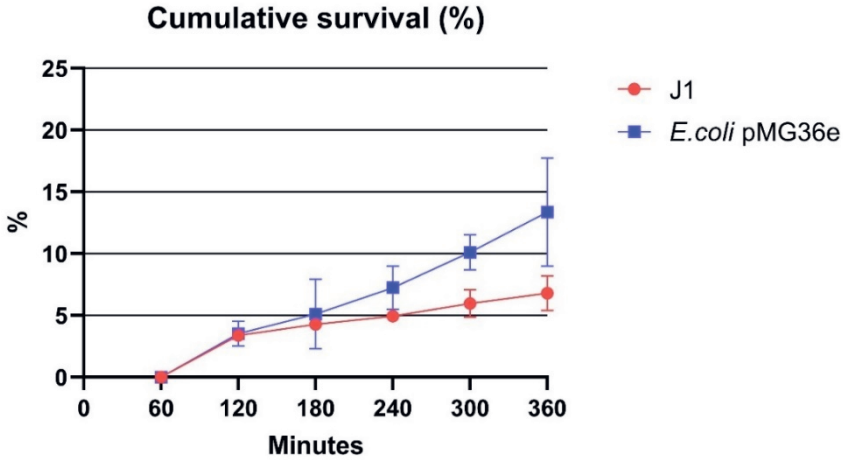
Figure 8. Cumulative survival of complete experiment (%) as calculated from PFU/mL and total volumes in efflux (mean + SD; n=2)

The detection of replication-competent bacteriophages in all efflux samples shows that the TIM-1 model is a suitable tool to follow the survival of bacteriophages during transit trough the upper GI tract. Although a significant drop of titer could be observed, bacteriophages were delivered to the large intestine. The experiments in this study were performed in a fed-state; the phage was ingested together with a meal. The buffer capacity of the meal ensures that the pH changes gradually, therefore enabling the phage to (at least partly) survive the gastric conditions and transportation to the small intestine. Similar findings are described by Jakobsen et al. where they describe the survival of a *Listeria monocytogenes* bacteriophage cocktail under simulated stomach and small intestine conditions and showed that the phages could survive when the pH was 4 or higher (Jakobsen et al., 2021).

Detailed information about clinical trials with bacteriophages are limited. In studies describing the use of an oral T4-like phage cocktail and the commercial available Microgen ColiProteus cocktail (McCallin et al., 2013; Sarker et al., 2012; Sarker et al., 2016), dosages in the range of 10^8-10^9 PFU are described, which is in the similar range as in our study. In the first study, the T4 phage cocktail was used by 15 healthy adults, and with a dosage of 3×10^9 PFU, the phage could be detected in 60% of the stool samples. In the second study, both phage cocktails were used to treat acute

bacterial diarrhea. The treatment was not successful, as intestinal amplification of the phage failed, and the diarrhea was not improved. This outcome could possibly be explained by the loss in titer of the bacteriophage cocktail during passage through the GI tract. The use of the TIM-1 model prior to the design of clinical trials could aid in an improvement of successful oral phage therapy design with the appropriate dosage, and help in the development of strategies to protect the phage against degradation in the upper GI tract. Now that we have established that roughly 5-10% of the orally ingested phage survives passage through the upper GI tract, its efficacy in eradication of its host in the large intestine can be investigated. This is described in **chapter 3** of this thesis.

Acknowledgements

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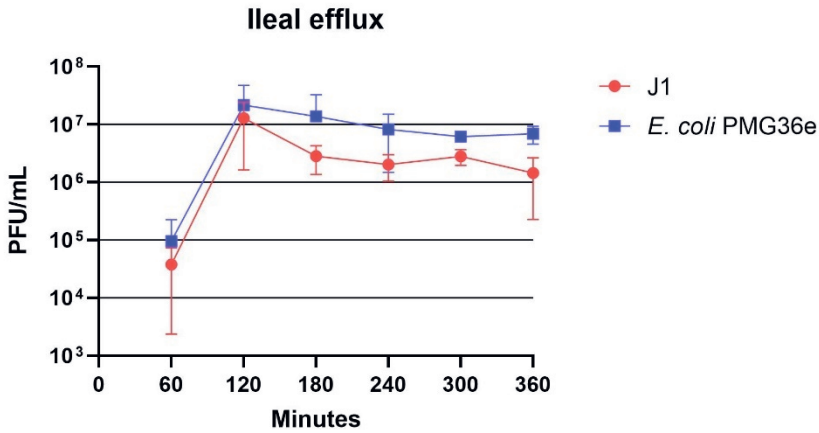
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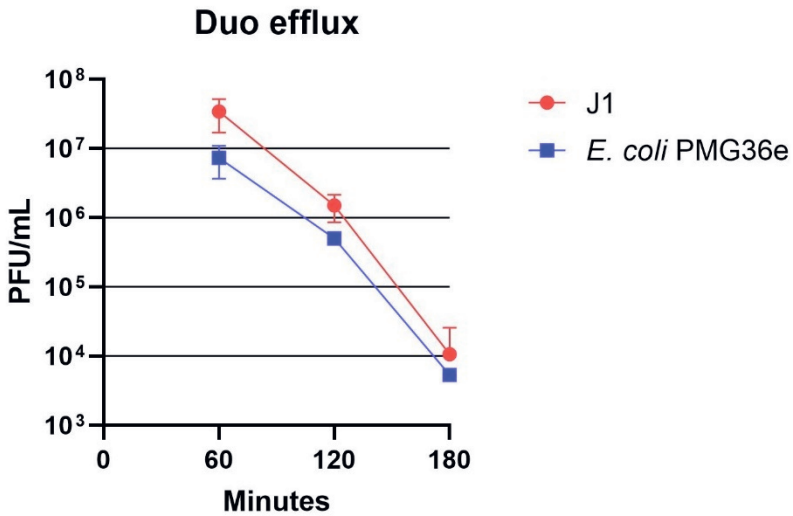
Supplemental Figure 1. Bacteriophage titers (PFU/mL) for complete experiments for bacteriophage *E. coli* and J1 in duodenal efflux samples.
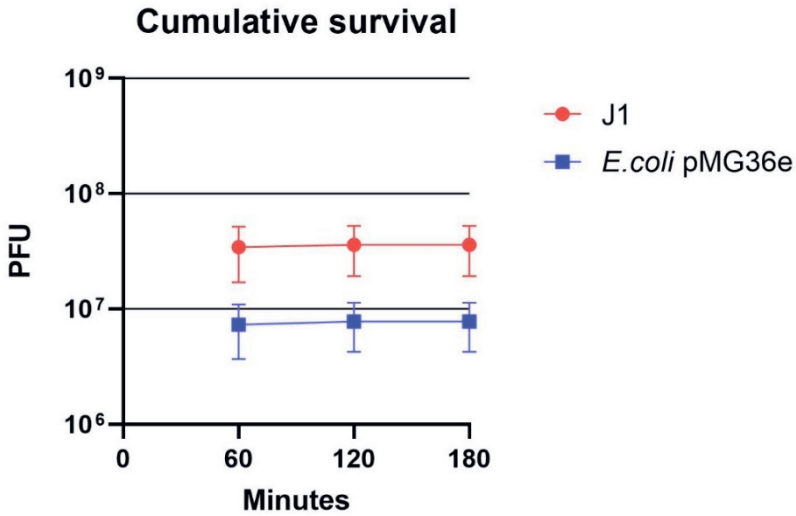
Supplemental Figure 2. Cumulative survival of stomach experiment (PFU) as calculated from PFU/mL and total volumes in efflux
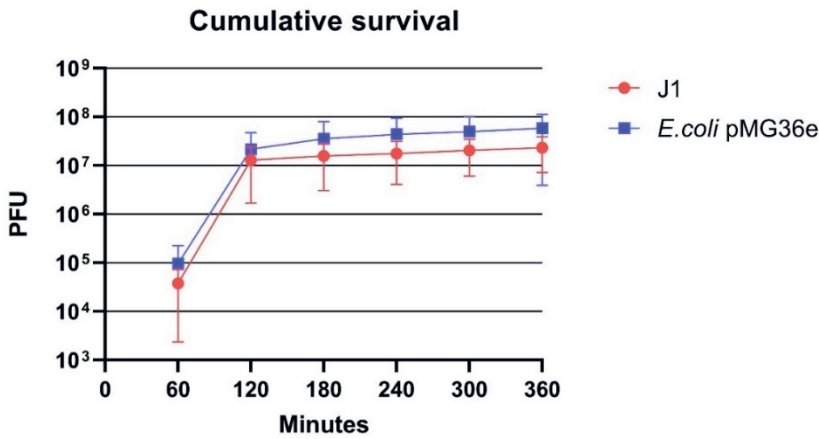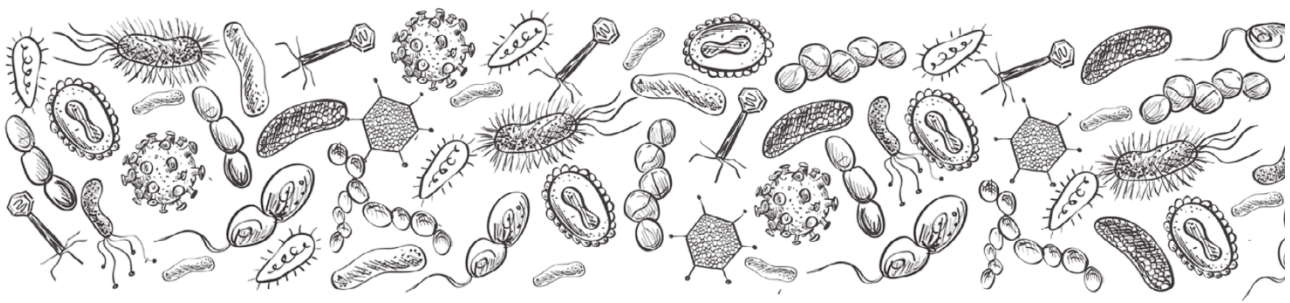
Supplemental Figure 3. Bacteriophage titers (PFU/mL) for complete experiments for bacteriophage *E. coli* and J1 in ileal efflux samples.

Supplemental Figure 4. Cumulative survival of complete experiment (PFU) as calculated from PFU/mL and total volumes in efflux
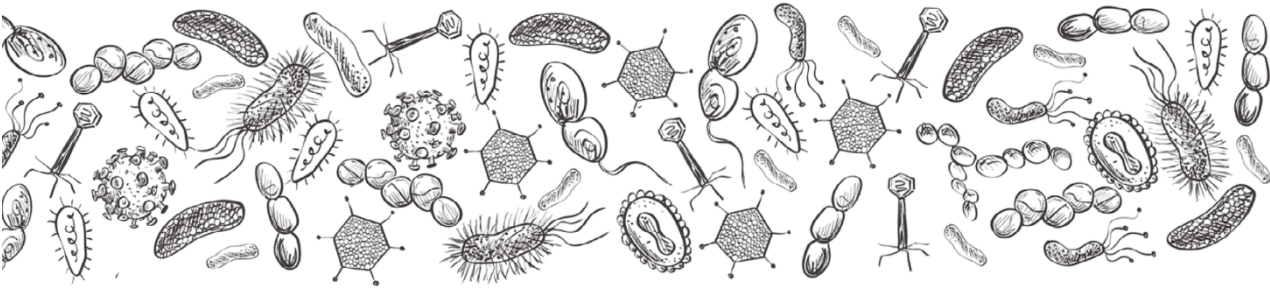
Chapter 3

Investigating the survival and activity of a bacteriophage in the complex colon environment with the use of a dynamic model of the colon (TIM-2)

Evy Maas

John Penders

Koen Venema

submitted

Abstract

The rise of antibiotic resistance poses a global problem and could potentially lead to a high mortality rate due to bacterial infections. To avoid this, alternative therapeutic options should be explored. One of these options is lytic bacteriophage therapy, where orally ingested phages are used to treat bacterial infections. Well-designed and described research on effectivity of oral bacteriophage therapy is lacking, therefore the aim of this study was to study whether the *in vitro* model of the colon (TIM-2) could be used to investigate the survival and efficacy of therapeutic bacteriophages. For this, an antibiotic-resistant (Cm^R) *E. coli* DH5α (pGK11) was used in combination with a corresponding bacteriophage. For the survival study, the TIM-2 model was inoculated with the microbiota of healthy individuals and a standard feeding (SIEM) was fed over the course of the 72 hour experiment. To test the bacteriophage, different interventions were carried out: i) SIEM as a control run, ii) SIEM spiked with high titer phage effective against *E. coli*(pGK11) (1 mL of 10^{10} PFU), iii) SIEM spiked with both *E. coli*(pGK11) (~10^6 CFU/mL TIM-2 content) and its phage, iv) SIEM spiked with a dose of *E. coli*(pGK11) ~10^8 CFU/mL) and multiple shots of the phage. Survival of bacteriophages and bacteria was followed by plating of the lumen samples at different time points 0, 2, 4, 8, 24, 48, and 72 hours. In addition, the stability of the bacterial community was determined with the use of 16S rRNA sequencing of the V3-V4 gene region. Results showed that the phage titers could be decreased by activity from the commensal microbiota. Levels of the phage host (here *E.coli*) were decreased in the interventions with the phage shot. Multiple shots did not seem to be more effective than a single shot. At the same time, the bacterial community was not disturbed and remained stable throughout the experiment, which is in stark contrast to treatment with antibiotics. This high specificity shows that phage therapy could be a promising alternative to antibiotic treatment in treating GI infections, because the microbial community is less affected.

Introduction

The discovery of antibiotics has drastically decreased the mortality due to bacterial infections, but the rise of antimicrobial resistance (AMR) has resulted in an increasing loss of effectivity of antibiotics and concurrent increase in mortality rates. As such, AMR is one of the most important global public health concerns of future generations. For a long time antibiotics were the golden standard for the treatment of these infections. During the 1940-1960s, a lot of new antibiotics were discovered, and they were widely used (Aminov, 2017). The discovery of new antibiotics rapidly waned after this 'Golden Era', meanwhile the widespread use of these antibiotics led to rapid emergence and dissemination of antimicrobial resistance. In particular, the development of multi-drug resistance (MDR) in pathogenic bacteria is worrisome, as standard options to treat infections with such bacteria are becoming more and more limited. Some examples of bacteria that are worrisome according to the WHO when they develop MDR are *Enterococcus faecium, Staphylococcus aureus* as well as members of the Enterobacteriaceae family (e.g., *Acinetobacter baumannii, Pseudomonas aeruginosa and Escherichia coli*) (Tacconelli et al., 2018). It is therefore pivotal to identify alternative strategies to treat infections caused by MDR bacteria and one such alternative is bacteriophage therapy (Kaur, Agarwal, & Sharma, 2021).

Bacteriophage therapy makes specific use of the lytic function of bacteriophages (or in short 'phages'). Lytic phages can release phage virions over short intervals with lethal disruption of bacterial cells, and, unlike antibiotics, they are highly specific and bind specific receptors on the bacterial cell surface, after which they can invade the cell, use the host's enzyme machinery to multiply and subsequently start lysis. With this lysis, numerous new viral particles are released and the process is repeated and more bacterial cells can be lysed (Kakasis & Panitsa, 2019). In the antibiotic era, phage therapy was neglected as treatment option, except from some Eastern European countries where antibiotics were not readily available (Chanishvili, 2016). Although phage therapy is used as treatment in these areas, well-documented studies are scarce. More evidence is needed to develop effective phage therapy. For the acceptance of phage therapy as treatment, double-blind cross-over clinical trials are

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needed, but to optimize the outcome of clinical trials, phage efficacy should be investigated *in vitro* (Abedon, 2017).

One of the advantages of phage therapy, compared to antibiotics with a broad spectrum of activity, is that phages are highly specific (Moebus & Nattkemper, 1981). The consequential advantage is that the microbial community of the gut is less disturbed, but it also poses some challenges, because it is more difficult to use phage therapy widespread on different strains or even different species. A strategy used to overcome this is to make use of a phage cocktail. In such a cocktail, different phages are combined to increase the host-range and therefore the efficacy of the therapy (Abedon, Danis-Wlodarczyk, & Wozniak, 2021). For the analysis of the activity of bacteriophages in the colon, information about the growth and multiplicity of infection is important, which can be obtained through a simple test-tube experiment with a combination of phage and its cognate host. This information can help in the design of the therapy and dosage (Gelman et al., 2021), but to predict the activity of the bacteriophage in a complex environment such as the colon, more complex experiments are needed.

A method to mechanistically study the gut microbiota is by making use of an *in vitro* model. This eliminates host factors such as immune response but is a realistic reproduction of the microbial processes in the gut. A validated dynamic model to study the large intestinal bacterial communities is TNO's *in vitro* large intestinal model (TIM-2) (Aguirre et al., 2016; Venema, 2015). This sophisticated model is computer controlled, allowing for the control of pH and temperature. Also, it can realistically mimic the peristaltic movements in the colon. Unique to TIM-2 compared to other (more simple) *in vitro* models, is the dialysis system that allows for the absorption of metabolites. The use of this complex *in vitro* model can aid in the development of effective phage therapy and provide essential information about the survival and activity of phages in the colon. Therefore, the aim of this study was to use the TIM-2 as a tool to investigate the survival and efficacy of bacteriophages in the colon, as a follow up of experiments that were performed in the upper GI tract to study survival during passage through stomach and small intestine (**chapter 2** of

this thesis). We used *E. coli* and the newly isolated coli phage (**chapter 2**) as a model system for this.

Material and methods

One-step growth experiment

The isolation and characterization of the phage used in these experiments was reported before (**chapter 2** of this thesis). To examine the growth performance of the phage, a one-step growth experiment was performed. *E. coli* DH5α (pGK11) (containing a chloramphenicol resistance gene on the plasmid; generous gift from Dr. Jan Kok, Groningen University) was used for these experiments, because the chloramphenicol resistance (Cm^R) marker allowed for specific determination of this Cm^R *E.coli* strain against the background of other *E.coli* strains in the TIM-2 system (see below). The strain was grown overnight in LB broth containing 50 μg/mL chloramphenicol. Bacterial cells were pelleted by centrifugation at 6000×g and resuspended in 500 μL LB broth. Then, 500 μL phage stock (PFU/mL = 10^8) was added and this was spun down for 30 s at 18000×g, to remove unabsorbed phages. The pellet was resuspended in 50 mL LB broth and incubated at 37°C (time zero (T_0)). After 15 min, 100 μL was removed and centrifuged for 30 s at 18000×g, the supernatant was used for phage enumeration with the double layer agar method as described in **chapter 2**. In short, bacteriophage solution was diluted in SM buffer (5.8 g/L NaCl, 2 g/L $MgSO_4·7H_2O$, 50 mL Tris-HCl (1M, pH 7.5)) to get individual plaques after plating. LB agar plates (10 g/L tryptone, 10 g/L NaCl, 5 g/L yeast extract, 15 g/L agar) were prepared with chloramphenicol (final concentration 50 μg/mL). LB top agar (10 g/L tryptone, 10 g/L NaCl, 5 g/L yeast extract, 7.5 g/L agar) was prepared and 3 mL of this top agar was added to a 15 mL tube and kept in a water bath at 56°C to avoid it to solidify. One mL of bacterial culture and 1 mL of (diluted) phage solution were added to the top agar, mixed and poured on the pre-warmed LB plates. This procedure was done in the laminar flow cabinet to avoid contamination. Plates were incubated at 37°C overnight and enumerated by counting the plaques the next day. This procedure was repeated every 15 min for 2 hours. The moment of maximum phage release from lysed cells was used in the TIM-2 experiments to determine frequency of sampling.

Determination multiplicity of infection (MOI)

For the determination of the MOI, *E. coli*(pGK11) was grown to ~10^8 CFU/mL at the exponential growth stage. Subsequently, 1 mL of this bacterial culture was mixed with 1 mL phage solution in different ratios. The ratios tested were (phages:bacteria): 1:1000, 1:100, 1:10, 1:1, 10:1, 100:1, and 1000:1. After mixing, adsorption could take place for 15 min while the mixture was incubated at 37°C. Free phages were removed by centrifugation at 5000×g for 10 minutes. The pellet was resuspended in LB broth and incubated for 4 hours at 37°C after which the phages were enumerated using the double layer agar method as described above.

Survival and activity of bacteriophage in the dynamic in vitro model of the colon (TIM-2)

For the experiments in the TIM-2 system, a pool of fecal samples from healthy individuals was used to inoculate the model as described before (Aguirre, Ramiro-Garcia, Koenen, & Venema, 2014). In brief, feces was collected from 6 individuals (male:female = 3:3) which did not use antibiotics three months prior to collection. Samples were collected in a box and an anaerobic environment was created by placing an anaerobic pouch immediately after defecation. The boxes were transferred to an anaerobic cabinet within 2 hours, and the pooled microbiota was prepared by mixing the samples, with addition of an equal weight dialysate (used in the TIM-2 system; see below) and 12% glycerol (final concentration). The microbiota was divided in 30 mL portions in 50 mL Falcon tubes, snap-frozen in liquid nitrogen and stored at -80°C until further use.

Before the experiment in the TIM-2 system, the 'background' of *E. coli* resistant to chloramphenicol in the pooled inoculum was determined. Therefore, the pooled microbiota was 10-fold serially diluted in PBS and plated on LB agar plates supplemented with chloramphenicol (50µg/mL). In addition, the presence of 'background' phages active against *E. coli*(pGK11) in the fecal samples was verified. The pooled microbiota was centrifuged at 4000×g, the supernatant was subsequently filtered through a 0.45 µm and a 0.22 µm filter to remove bacteria. The supernatant was diluted with SM buffer and the double layer agar method was used to enumerate the phages present in the fecal material.

The model used in this experiment is TNO's intestinal model of the colon (TIM-2), which is a validated, dynamic, computer-controlled model of the colon (Figure 1) (Venema, 2015). It simulates the human colon by control of body temperature, pH and removal of microbial metabolites by dialysis. Peristalsis is mimicked to allow optimal mixing. The model was inoculated with 60 mL of previously prepared microbiota and 60 mL dialysate solution (which contained 2.5 g/L $K_2HPO_4 \cdot 3H_2O$, 4.5 g/L NaCl, 0.005 g/L $FeSO_4 \cdot 7H_2O$, 0.5 g/L $MgSO_4 \cdot 7H_2O$, 0.45 g/L $CaCl_2 \cdot 2H_2O$, 0.05 g/L bile and 0.4 g/L cysteine-HCl, plus 1 mL of a vitamin mixture [containing 1 mg/L menadione, 2 mg/L D-biotin, 0.5 mg/L vitamin B12, 10 mg/L pantothenate, 5 mg/L nicotinamide, 5 mg/L p-aminobenzoic acid and 4mg /L thiamine]). The microbiota was allowed to adapt to its new environment for 16 hours during which it was fed with simulated ileal effluent medium (SIEM) adjusted to pH 5.8 (Figure 2). This medium represents the fractions of undigested fibers and other undigested and/or unabsorbed (dietary) components that can reach the colon (Gibson, Cummings, & Macfarlane, 1988). SIEM contained 9.0 g/L pectin, 9.0 g/L xylan, 9.0 g/L arabinogalactan, 9.0 g/L amylopectin, 43.7 g/L casein, 74.6 g/L starch, 31.5 g/L Tween 80, 43.7 g/L bactopepton, 4.7 g/L $K_2HPO_4 \cdot 3H_2O$, 8.4 g/L NaCl, 0.009 g/L $FeSO_4 \cdot 7H_2O$, 0.7 g/L $MgSO_4 \cdot 7H_2O$, 0.8 g/L $CaCl_2 \cdot 2H_2O$, 0.05 g/L bile, 0.02 g/L haemin, and 0.3 g/L cysteine-HCl, plus 1.5 mL of the previously described vitamin mixture. In this experiment, SIEM was also used during the test period, which took 72 hours. Different interventions were carried out: i) SIEM as a control run, ii) SIEM spiked with high titer phage effective against *E. coli*(pGK11) (1 mL of 10^{10} PFU), iii) SIEM spiked with both *E. coli*(pGK11) (~10^6 CFU/mL TIM-2 content) and its phage, iv) SIEM spiked with a dose of *E. coli*(pGK11) ~10^8 CFU/mL) and multiple shots of the phage. The bacteria and bacteriophages were added as a shot at t0 for variables i), ii) and iii), and for intervention iv) bacteria were added as a shot at t0, with multiple shots of the phage at t0, t24, and t48. Lumen samples were taken after the adaptation period (t0), after 2, 4, 8, 24, 48 and 72 hours of test period (Figure 2). In the samples taken, both *E. coli*(pGK11) and bacteriophage against *E. coli*(pGK11) were enumerated on LB plates supplemented with chloramphenicol (50μg/mL).

Additionally the microbiota composition of the lumen samples was analyzed with Illumina sequencing of the V3-V4 variable regions of the 16S rRNA gene.
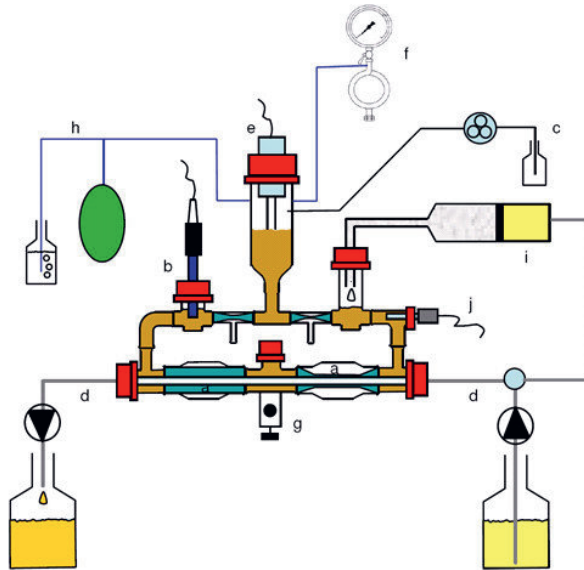

Figure 1. Schematic representation of the TNO TIM-2 in vitro model with **A.** peristaltic compartments containing fecal matter; **B.** pH electrode; **C.** alkali pump; **D.** dialysis liquid circuit with hollow fibre membrane; **E.** level sensor; **F.** N2 gas inlet; **G.** sampling port; **H.** gas outlet; **I.** 'ileal efflux' container containing SIEM; **J.** temperature sensor.
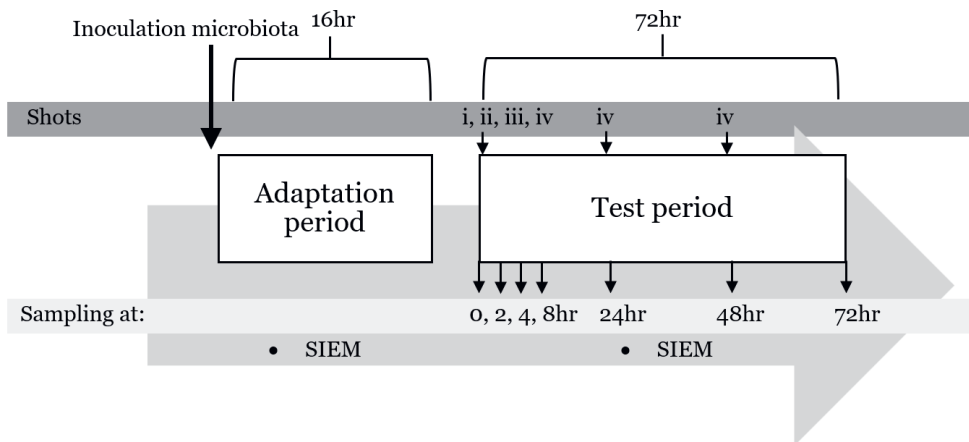

Figure 2. Experimental set-up

Illumina MiSeq sequencing of the V3-V4 region of the 16S rRNA gene

The bacterial community composition was analyzed by sequencing PCR amplicons of the V3-V4 region of the 16S rRNA gene. Isolation of genomic DNA from lumenal samples (1 mL) was done using the QIAamp Fast DNA stool mini kit (Qiagen, Venlo, The Netherlands), with the addition of a bead-beating step at the start of the isolation to increase yield as described before (Knudsen et al., 2016). The library preparation was done by following the Illumina protocols (Nextera XT DNA Library Preparation Kit, Nextera XT Index Kit v2 Set A, Illumina, Eindhoven, The Netherlands). The primers used were 341F (5′-CCTACGGGNGGCWGCAG-3′) and 785R (5′-GACTACHVGGGTATCTAATCC-3′) with Illumina adaptors. Sequencing was performed using the Illumina MiSeq system (MiSeq reagent kit v3, Illumina) and FASTQ files were generated using the Local Run Manager Generate FASTQ Analysis Module v3. Further microbial analysis was carried out using the Quantitative Insights Into Microbial Ecology 2 (QIIME2) software package (version 2019.7) (Caporaso et al., 2010; Estaki et al., 2020). The dada2 plugin was used for demultiplexing, quality filtering and denoising data (Callahan et al., 2016). Classification of sequencing data was done with the q2-feature-classifier using the reference 16S rRNA database SILVA (version 132) (Bokulich et al., 2018).

Results and discussion

Bacteriophages active against *E. coli*(pGK11) were used to test the TIM-2 model as suitable tool to investigate activity and survival of phages in the colon. First, the growth kinetics were determined to set-up the experiment for the TIM-2 model. The growth of the bacteriophage was followed for 120 minutes, samples were taken every 15 minutes (Figure 3a). After 30 minutes, ~10^6 phages were released and after 75 minutes the plateau was reached at the level of 8×10^8 PFU/mL. According to the growth performance of the bacteriophages, the sampling in the TIM-2 experiment was started after two hours, when the burst has taken place, and new bacteriophages are released.

After this, the optimal ratio phages:bacteria was determined for the best performance of the bacteriophage. Bacteria and bacteriophages were incubated together in different ratios (Figure 3b). From this graph it is clear that the ratio

(phages:bacteria) of 10^2 and 10^3 led to the highest numbers of phages (PFU/mL = 10^{11}). In the following TIM-2 experiment, the ratio used was 10^2.

The proper determination of the burst time and size helped in the design of experiments to test the survival and activity of bacteriophages, and eventually in the design of the actual phage therapy *in vivo*. Also, the optimal MOI is important to create favorable conditions for the bacteriophage, which helps determining the efficacy of activity and design of phage therapy.
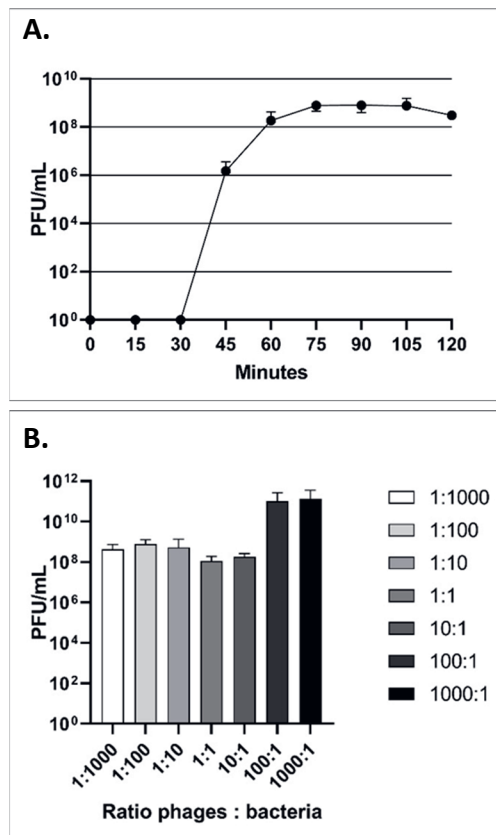

Figure 3: **A.** One-step growth curve for phage active against E. coli(pGK11) and **B.** determination of optimal ratio phages:bacteria.

To specifically measure our target bacterium *E. coli*(pGK11), the background of chloramphenicol resistant bacteria in TIM-2 was tested. None of the chloramphenicol supplemented plates inoculated with dilutions of the pooled fecal sample showed any growth . As such, we could assume that in subsequent experiments all colonies found on the chloramphenicol supplemented plates are resulting from the added *E. coli*(pGK11).

The activity and survivability of the bacteriophage was tested in TIM-2. Samples were taken at 2, 4, 8, 24, 48 and 72 hours after the start of the experiment to monitor the dynamics of both bacteriophages and bacteria over a period of 72 hours (Figure 4a and 4b). Chloramphenicol resistant bacteria were only found in the two interventions where they were added as a shot at t0, as expected from the negative background of Cm[R] bacteria. Similar levels were observed in these two interventions. After the shot, the level of Cm[R] bacteria dropped in the first two hours with about 1 log-factor after which levels stabilized up to 8 hours after the start. The drop in the first two hours could be explained by the fact that the burst of the bacteriophages takes place after approximately 75 minutes. Phages were observed in all samples where they were added as a shot, and the levels found in these interventions were similar. Although contamination during plating was prevented as much as possible by working in a laminar flow cabinet, it could not entirely be ruled out. Since some phages (750 PFU/mL) were observed in the control sample after 8 hours, this could mean that there are already minor proportions of bacteriophages present in the fecal samples that could be active against *E. coli(*pGK11) and that multiplied to above levels of detection in the first 8 hours or is an indication of contamination.
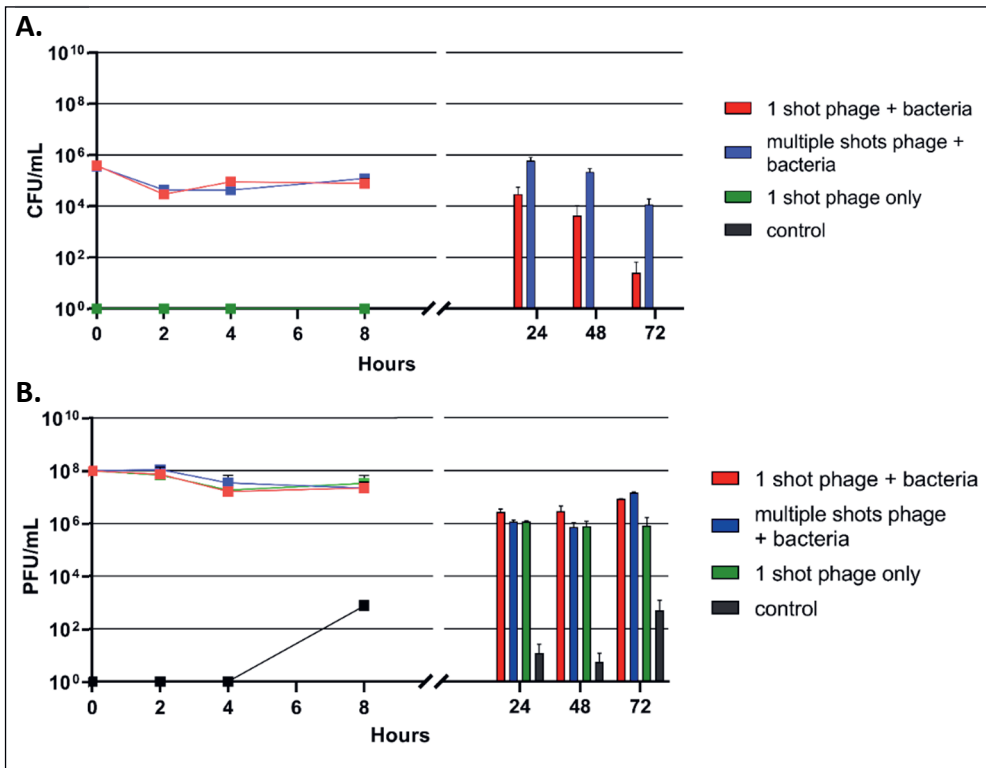
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Figure 4. Bacteria (CFU/mL) **A.** and bacteriophage titers (PFU/mL) **B.** for TIM-2 experiments for 0, 2, 4, 8, 24, 48 and 72 hours

In the experiment with a single shot of phage at t0, the added *E.coli* cells (also at t0) were drastically lowered in the system, in the subsequent 72 hours, despite remaining relatively stable at ~10^5 CFU/mL between time points 2 and 8 hours after the shot. This is a reduction of about 10^3-fold. A similar phenomenon is observed for the experiments with multiple phage shots (every 24 h), but because in this experiment the level of *E.coli* at time point 24 is about 10-fold higher, the strain is not as drastically lowered, despite the multiple phage shot. Regarding the phage titer, this dropped slightly (~1 log factor) at time point 24 compared to time point 8, and then remained relatively constant. From the experiment with only phage added to the system, one might conclude that the phage survival is slightly affected by the endogenous gut microbiota (Bernheim & Sorek, 2020). And even though in the initial experiments with *E.coli* to determine MOI, the phage replicates, it seems that

the titer does not increase in the TIM-2 luminal content over time, perhaps indicating too that the endogenous microbiota keeps the phage at a certain level by affecting its survival. Whether this is by degrading the ligand in the phage tail that attaches to the *E.coli* receptor (making it ineffective) or by another mechanism remains to be seen. A limitation of these experiments is that the Cm[R] *E. coli* alone, without addition of the phage, has not been tested to follow its development in the system. It has previously been observed that members of the microbiota feed on each other in the absence of sufficient growth substrates (Little, Robinson, Peterson, Raffa, & Handelsman, 2008; Zusheng, Clarke, & Beveridge, 1998). So, it might well be that the phage is considered a substrate too and is (partly) degraded by proteolytic enzymes, and perhaps even further by enzymes degradation of its nucleic acid. Why this would not lead to further reduction in phage titer is currently unclear.

To observe the potential changes in composition of the microbial community, the bacterial composition was determined using Illumina sequencing of the V3-V4 region of the 16S rRNA gene. Since we used a pooled microbiota, the start community for all interventions was identical. To observe the effect of the addition of the bacteriophage and *E.coli* to the microbial diversity, the observed richness and Shannon diversity indices were calculated. No significant differences in microbial richness and diversity were observed between the interventions (Figure 5), suggesting that the bacteriophages did not influence the stability of the microbial community, as expected. This is a tremendous advantage for the use of bacteriophages to treat GI infections compared to antibiotics, which drastically disturb the bacterial composition (Andremont, Cervesi, Bandinelli, Vitry, & de Gunzburg, 2021; Patangia, Anthony Ryan, Dempsey, Paul Ross, & Stanton, 2022).
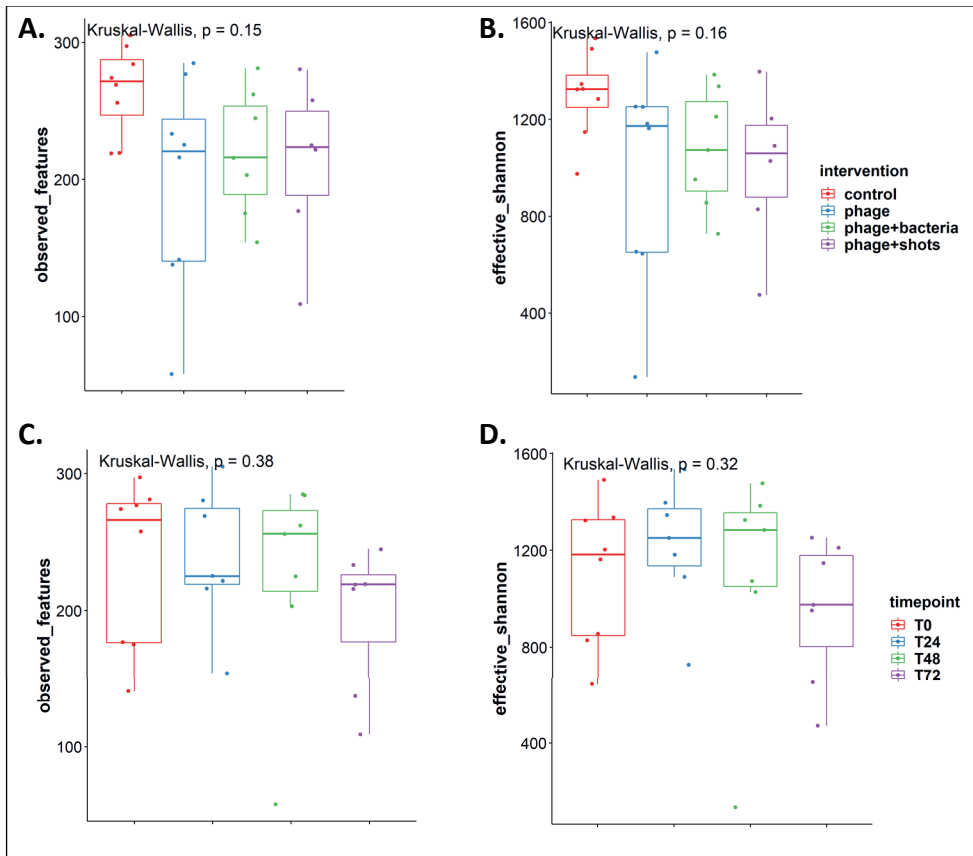
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Figure 5. Alpha-diversity of the lumen samples during the test period. **A.** Observed OTUs different interventions **B.** Shannon diversity index different interventions **C.** Observed OTUs over time **D.** Shannon diversity index over time.

Next to the alpha-diversity, also the beta-diversity was determined to see potential differences in the overall microbial community structure between samples. Samples were compared using the unweighted UniFrac distance. The samples from the control cluster together, but the samples for the other intervention do not cluster separate from each other (Figure 6).
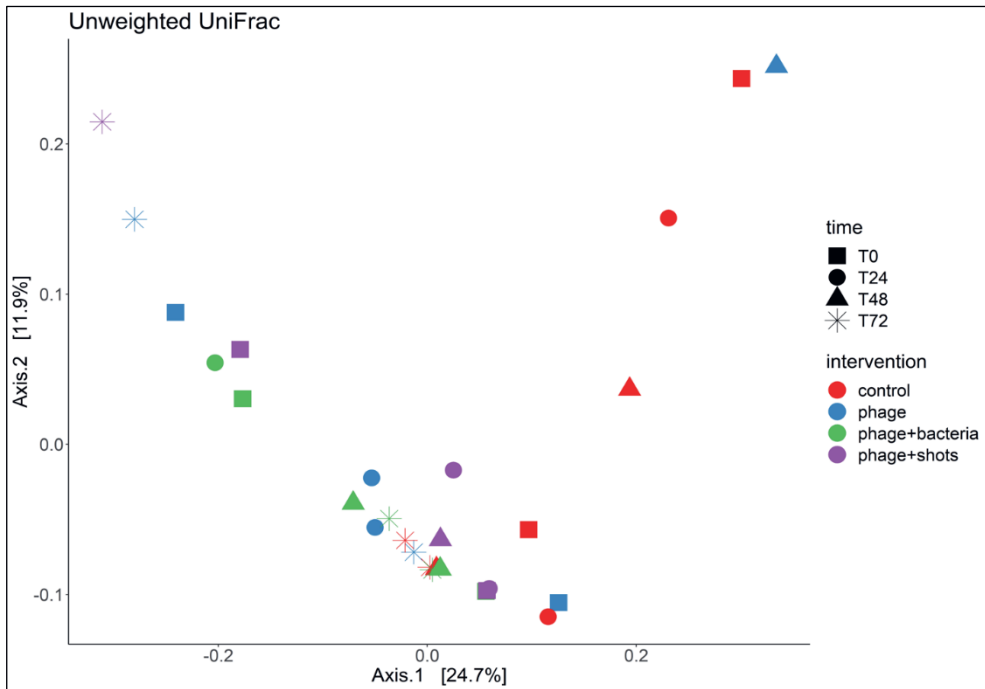
Figure 6. Beta-diversity of the bacterial community during the test period visualized using PCoA plot using Unweighted UniFrac.

To further analyze the bacterial composition, taxonomic analysis were performed. The most abundant phyla and genera were determined. The most abundant phyla were *Bacteroidetes* and *Firmicutes*, which were dominant throughout all samples (Figure 7A). Also, the 20 most abundant genera are shown (Figure 7B). The genera did not change significantly between the different interventions. *Escherichia* is not found to be dominant in the samples, also not in the samples where *E. coli* was spiked in.

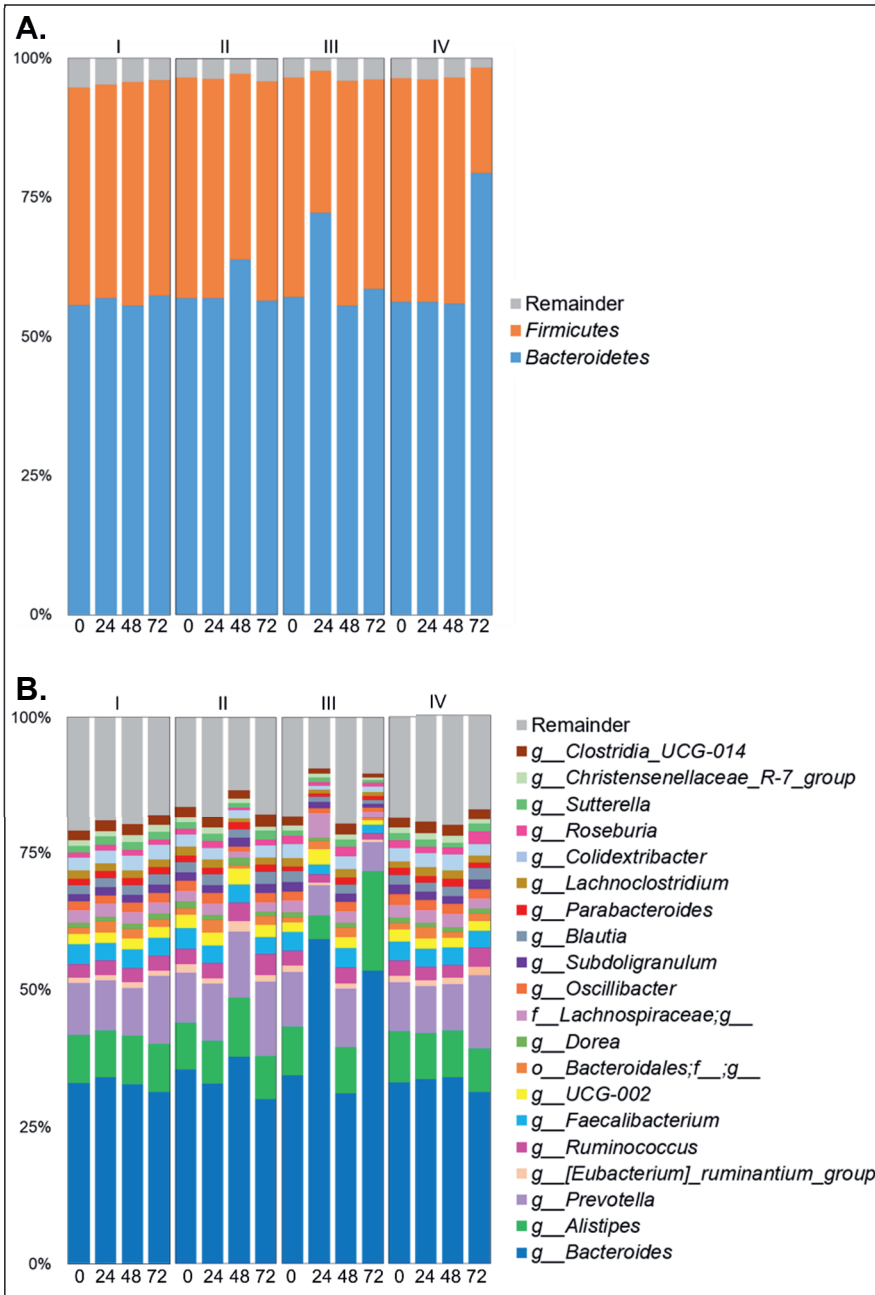**Figure 7.** Relative abundance (%) of major groups of bacteria during the test period after inoculation in TIM-2 for the different interventions; **I** control; **II** phage only; **III** 1 shot phage + bacteria; **IV** multiple shots phage +bacteria **A.** Phyla, **B.** Genera

Conclusions

Phage therapy has great potential for the treatment of GI tract infections in a time where antibiotic resistance is a global problem. To optimize phage therapy efficacy, the survival of orally administered bacteriophages in the GI tract should be investigated. A study on the survival of phages in the upper GI tract showed that approximately 10% of ingested bacteriophage reaches the colon (**chapter 2** of this thesis). In this chapter the survival of bacteriophages in the GI tract was further researched in the colon. It was shown that the phages can be affected by the microbiota. Levels of the target for the phage (here *E.coli*) were decreased in the interventions with the phage shot. Multiple shots did not seem to be more effective than a single shot. At the same time, the bacterial community was not disturbed and remained stable throughout the experiment, which is in stark contrast to treatment with antibiotics. More research is needed to evaluate the stability of orally administered bacteriophages in the complex microbial ecosystem of the colon. In the combat against antibiotic resistant bacteria, also the possibility of small-range antibiotics in the combination with phage therapy should be investigated, to optimize the treatment without major disturbances of the commensal microbiota in the colon.

Acknowledgements

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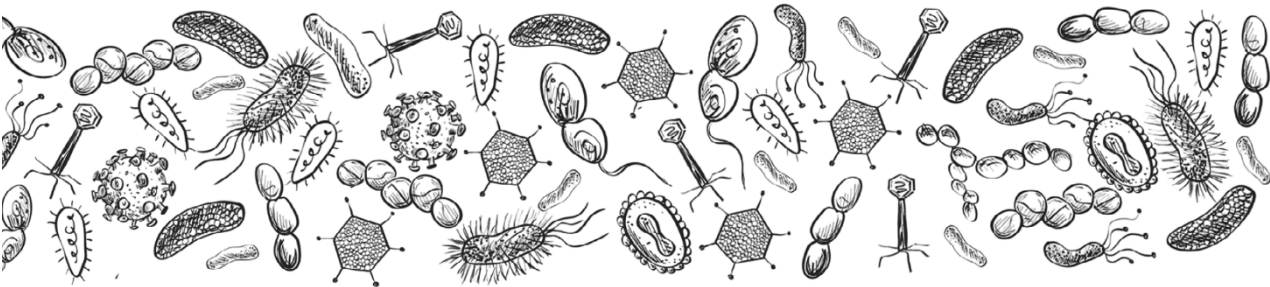
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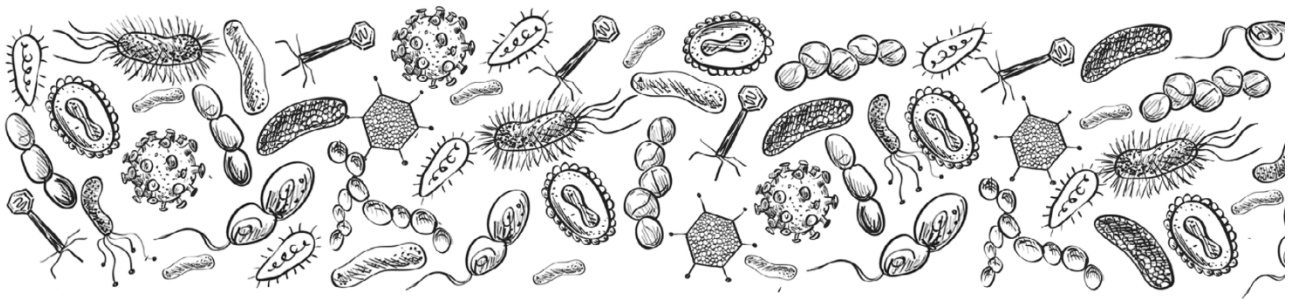
Chapter 4

Modelling the gut fungal community in TIM-2 with a microbiota from healthy individuals

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Abstract

Most research on the human microbiome focuses on the bacterial component, and this has led to a lack of information about the fungal component (mycobiota) and how this can influence human health, e.g. by modulation through the diet. The validated, dynamic computer-controlled model of the colon (TIM-2) is an *in vitro* model to study the microbiome and how this is influenced by interventions such as diet. In this study, it was used to the study the gut fungal community. This was done in combination with next-generation sequencing of the ITS2 region for fungi and 16S rRNA for bacteria. Different dietary interventions (control diet (SIEM), high carbohydrate, high protein, glucose as carbon source) were performed to see if diet could shape the mycobiome. The mycobiome was investigated after the adaptation period, and throughout the intervention period which lasted 72 hours, and samples were taken every 24 hours. The fungal community showed low diversity and a greater variability when compared to bacteria. The mycobiome was affected most in the first hours of the adaptation period. Taxonomic classification showed that at the phylum-level *Ascomycota* and *Basidiomycota* dominated, while *Agaricus*, *Aspergillus, Candida, Penicillum, Malassezia, Saccharomyces, Aureobasidium, Mycosphaerella, Mucor* and *Clavispora* were the most abundant genera. During the intervention period, it was shown that the change of diet could influence the diversity. Clustering of samples for different time points was analyzed using Bray Curtis dissimilarities. Samples of t0 clustered together, and samples of all other time points clustered together. The Bray Curtis dissimilarity analysis also showed that for the different dietary interventions, samples treated with glucose clustered together and were different from the other groups (p<0.05, PERMANOVA). Taxonomic classification showed that the genera *Alternaria, Thanatephorus, Candida* and *Dekkera* differentially changed for the various diet groups (p<0.05, Kruskal-Wallis). These results show that the mycobiota could be modelled in TIM-2, however the low diversity and high variability make studying fungal, as compared to bacterial communities, much more challenging. Future research should focus on optimization of the stability of the fungal community to increase the strength of the results.

Introduction

The human gut harbors a complex microbial community that not only consists of bacteria, but also of fungal and viral communities. Most of the microbiome research focuses on the bacterial component, thereby neglecting the fungal community, or mycobiome. Gut fungi make up about 0.1% of the gut microbiome (Qin et al., 2010), but in size, they take up a considerable volume of the gut ecosystem. Although there are already some studies that show that the fungal community correlates with diseases and disorders (Beheshti-Maal et al., 2021; Nagpal et al., 2020; Salamon et al., 2021), the exact role of fungi is unknown. In addition, ways to modulate the fungal population in the gut have not been explored extensively. More research in this area can help in understanding the role of gut fungi on (intestinal) health.

The balance of the microbial community can be disturbed by antibiotic use, and the elimination of bacterial species can lead to fungal overgrowth (de Oliveira, Atobe, Souza, & de Castro Lima Santos, 2014). A well-known example is the opportunistic pathogen *Candida*, where infections with *Candida* can occur after antibiotic treatment (Kennedy & Volz, 1985; Shirtliff, Peters, & Jabra-Rizk, 2009). Diet has an important influence on the intestinal fungi (Fiers, Leonardi, & Iliev, 2020). Besides, it is a source of fungi that can pass or even colonize the gut. There are several examples that show that diet can have an influence on the gut mycobiome, for example it was shown that mice on a high-fat diet had a different mycobiota compared to mice on a standard diet (Heisel et al., 2017) and *Methanobrevibacter* and *Candida* abundance was correlated with a high-carbohydrate diet (Hoffmann et al., 2013).

The use of a predictive *in* vitro model can provide important insights on the direct impact of diet on the human gut fungal community. The validated, dynamic computer-controlled model of the colon (TIM-2), has been used extensively used to study the bacterial microbiome; for a review see (Venema, 2015). Different interventions can be carried out, such as dietary, pharmaceutical or nutraceutical (e.g., probiotics) (Aguirre et al., 2016; Rehman et al., 2012). Also, the microbial inoculum can be chosen to fit the target group, such as children, elderly, or a specific

patient group (Cuevas-Tena, Alegria, Lagarda, & Venema, 2019; Martina et al., 2019; Míguez, Vila, Venema, Parajó, & Alonso, 2020). The pooled microbiota used in the model is demonstrated to represent a standardized microbiota and allows to perform a series of experiments with the same starting microbiota (Aguirre, Ramiro-Garcia, Koenen, & Venema, 2014). The colon environment is mimicked by controlling the pH, temperature and low oxygen levels. The model also makes use of a dialysis system to avoid accumulation of metabolites, which keeps the metabolite levels in a physiological range. Samples can be taken from the lumen and the dialysate to give insight in the microbiome and metabolites. The model has been validated and extensively used to study the bacterial community, but can also help to study fungi or even the fungal-bacterial interactions.

Historically, fungi were studied with culture dependent methods, which gives a distorted image of the fungi present in the gut. With the rise of culture-independent techniques, such as sequencing, it is now possible to study fungi in a different way, such that species that are non-culturable under standard culture conditions can also be detected (Huseyin, Rubio, O'Sullivan, Cotter, & Scanlan, 2017). Most commonly used is the amplicon-based sequencing of the internal transcribed spacer unit (ITS) (Schoch et al., 2012). To fill the knowledge gap of the fungal community, ITS sequencing combined with *in vitro* modelling can be a useful tool. Therefore, the aim of this study was to develop and optimize the use of the TIM-2 system in combination with ITS2 sequencing to mechanistically study the fungal population in the human gut, and study ways to modulate the fungal community.

Material and methods

Test products
For the adaptation period and as control diet, simulated ileal efflux medium (SIEM), which simulates the undigested components of a diet that reaches the colon, was used as described before (Maathuis, Hoffman, Evans, Sanders, & Venema, 2009). SIEM consists of 100 g CHO-medium (containing 12 g/L pectin, 12 g/L xylan, 12 g/L arabinogalactan, 12 g/L amylopectin, 100 g/L starch), 25 g TBCO 6.25x (containing 270 g/L Tween80, 375 g/L bactopepton, 375 g/L casein, 6.25 g/L ox-bile), 2 g $MgSO_4$

(50 g/L), 2 g cysteine (20 g/L), 0.2 mL vitamin mixture (containing 1 mg/L menadione, 2 mg/L D-biotin, 0.5 mg/L vitamin B12, 10 mg/L pantothenate, 5 mg/L nicotinamide, 5 mg/L p-aminobenzoic acid and 4 mg/L thiamine), 4 mL salts solution (containing 4.7 g/L $K_2HPO_4 \cdot 3H_2O$, 8.4 g/L NaCl, 0.8 $CaCl_2 \cdot 2H_2O$, 0.009 g/L $FeSO_4 \cdot 7H_2O$, 0.02 g/L haemin) and 1 mL antifoam emulsion. For the dietary interventions, modifications of SIEM were prepared (Table 1). To create a high carbohydrate diet (10x CHO), SIEM was modified by diluting ten times the concentration of TBCO, while keeping the rest of the ingredients the same. For the high protein diet (0.1x CHO), SIEM was modified by dilution of CHO ten times, where the rest of the ingredients stayed the same. In the diet with glucose as carbon source, CHO was replaced by 15 g glucose.

Table 1: Dietary interventions TIM-2 experiment

Standard diet (SIEM)	SIEM (Maathuis et al., 2009)
High carbohydrate diet (10x CHO)	SIEM with 10:1 CHO:TBCO (Aguirre et al., 2016)
High protein diet (0.1x CHO)	SIEM with 1:10 CHO:TBCO (Aguirre et al., 2016)
Glucose as carbohydrate source (Glucose)	SIEM-CHO + Glucose

TIM-2 model

To create an anaerobic environment, the TIM-2 model was flushed with N_2 prior to inoculation and throughout the experiment. The pH and temperature were set to mimic a healthy adult at 5.8 (mimicking the proximal colon) and 37°C, respectively. The pH was maintained by computer-controlled addition of 2M NaOH. Accumulation of fermentation products was avoided with the use of a hollow membrane dialysis system. The growth media (Table 1) were added constantly at a rate of 2.5 mL/hour in the adaptation and test period, leading to the introduction of 45mL SIEM in the adaptation period and 180 mL of test medium in the intervention period. The volume in the units was kept constant with the use of a level sensor. The model is described in more detail before (Venema, 2015).

Fecal samples

Fecal samples were collected from healthy adult volunteers (n=6, male:female 50:50) and homogenized under anaerobic conditions to create a standardized pool as described by Aguirre et al. (Aguirre et al., 2014). The fecal pool was snap-frozen in liquid nitrogen and stored in -80°C until use. All experiments were performed with the same fecal pool, and thus the same starting microbiota/mycobiota, which allowed direct comparison between experiments. At the day of the experiment, four tubes were taken out of the freezer and thawed in a water bath for 1 hour at 37°C, after which they were mixed under anaerobic conditions with an equal volume of dialysate (which contained 2.5 g/l $K_2HPO_4 \cdot 3H_2O$, 4.5 g/l NaCl, 0.005 g/l $FeSO_4 \cdot 7H_2O$, 0.5 g/l $MgSO_4 \cdot 7H_2O$, 0.45 g/l $CaCl_2 \cdot 2H_2O$, 0.05 g/l bile and 0.4 g/l cysteine-HCl, plus 1.5 mL of the previously described vitamin mixture). Of this fecal-dialysate mixture, 60 ml were introduced in the model under anaerobic conditions.

Test design

Inoculation with the fecal pool was done at -20 hours (20 hours prior to the start of the interventions), the feeding was started with SIEM and the adaptation period was started for 18 hours (Figure 1). Samples were taken from the lumen at -20, -18 and -16 and all samples were stored at -80°C until analysis. After the adaptation period, the feeding was stopped for two hours (=starvation period) to allow fermentation of the remaining carbohydrates in SIEM by the microbiota (Figure 1). Next, the intervention period started, which lasted 72 hours (Figure 1). Samples were taken at 0, 24, 48 and 72 hours from the lumen and the dialysate. Feeding was started at 0 hours and the different interventions were carried out: SIEM, 10xCHO, 0.1xCHO, Glucose (Table 1). All interventions were done in quadruplicate.
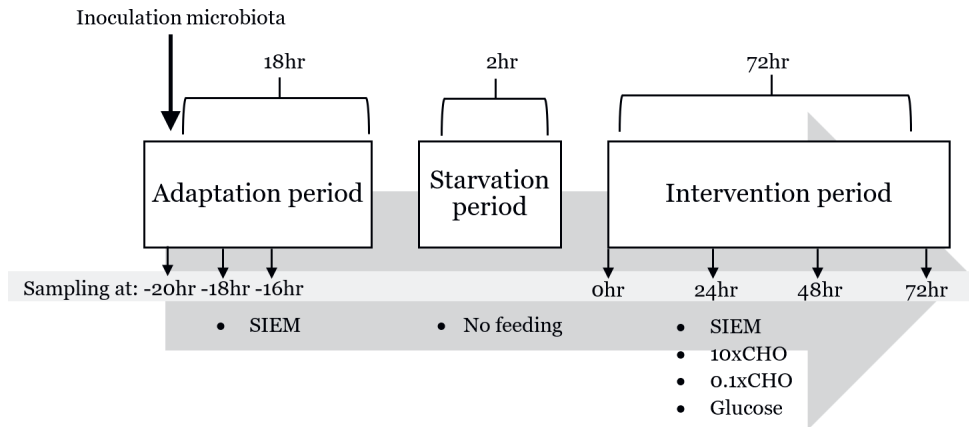
Figure 1: Experimental set-up

Gut mycobiota composition

To determine the fungal composition in the lumen samples, DNA was isolated using the QIAamp Fast DNA stool mini kit from Qiagen (Venlo, The Netherlands), with a bead-beating step according to the protocol described before (Knudsen et al., 2016), which is suitable for optimal isolation of fungal DNA. Starting material was 400µL lumen sample and the final elution was done in 50µL elution buffer to ensure high yield. DNA concentration was measured using the Qubit dsDNA HS Assay and a Qubit 3.0 Fluorometer (Invitrogen, Waltham, MA, USA).

The isolated DNA was used for sequencing the internal transcribed spacer unit 2 (ITS2) for determination of the fungal community composition. For the library preparation of the ITS2 sequencing, the Fungal Metagenomic Sequencing Demonstrated protocol of Illumina was used with some modifications (Nextera XT DNA Library Preparation Kit , Nextera XT Index Kit v2 Set A, Illumina, Eindhoven, The Netherlands). Input DNA in PCRI was increased to 50 ng/µL and the PCRI program was adjusted to 30 cycles. Primers used were ITS F (5'-GCATCGATGAAGAACGCAGC-3') and ITS R (5'-TCCTCCGCTTATTGATATGC-3') (Op De Beeck et al., 2014). After PCRII, all samples were checked with the

Bioanalyzer using the DNA1000 kit (Agilent, Santa Clara, CA, USA) to check the fragment sizes, which are variable for ITS2 sequences. The libraries were quantified, normalized and pooled equimolar before loading on the Illumina Miseq system (Miseq reagent kit v3, Illumina). The Local Run Manager Generate FastQ Analysis Module v3 was used to generate fastq files and further analysis was done with the Quantitative Insights Into Microbial Ecology 2 (QIIME2) software package (version 2019.7) (Caporaso et al., 2010; Estaki et al., 2020). The QIIME2 plugin Q2-ITSxpress was used to trim ITS sequences (Rivers, Weber, Gardner, Liu, & Armstrong, 2018). After this the dada2 plugin was used for demultiplexing, quality filtering and denoising (Callahan et al., 2016). Classification of the ITS2 sequencing data was done with the use of the reference database UNITE (version 02-02-19).

Gut microbiota composition

DNA isolated as above was also used to determine the gut bacterial composition, by sequencing the V3-V4 region of the 16S rRNA gene. The library preparation was done according the Illumina protocol (Nextera XT DNA Library Preparation Kit , Nextera XT Index Kit v2 Set A, Illumina). The primer set used was 341F (5′-CCTACGGGNGGCWGCAG-3′) and 785R (5′-GACTACHVGGGTATCTAATCC-3′). Sequencing was done on the Miseq system from Illumina using the Miseq reagent kit v3. With the use of the Local Run Manager Generate FastQ module v3, fastq files were created. Bioinformatic analyses were done with QIIME2 (version 2019.7). Taxonomic classification was done with the SILVA reference database (version 132).

Statistical analysis

Alpha diversity indexes analyses were calculated in QIIME2. Further statistical analyses were performed in R (version 4.0.4) using RStudio (version 1.4.1106). Data were expressed as median, with range, and significance tested with Wilcoxon-rank sum test. Kruskal-Wallis analyses were performed to find correlations between individual taxa and categorical parameters, Dunn's test was performed as post-hoc analysis. A p-value of < 0.05 was considered significant. Data was visualized using ggplot2 (package version 3.3.5). Beta diversity was expressed as Bray Curtis dissimilarities and Jaccard similarities. Differences between groups were tested using PERMANOVA.

Results and discussion

Adaptation period

The aim of this research was to use the sophisticated *in vitro* model (TIM-2) to mechanistically study the mycobiome. The *in vitro* model was inoculated with a fecal pool of healthy individuals, and after introduction in the model, 18 hours were used as adaptation period where the mycobiota could adapt to the new environment. The mycobiota was analyzed on diversity and this was compared with the bacterial component of the microbiota in the same period to assess the stability. The fungal composition of the lumen samples was determined in the first four hours of the adaptation period (t-20, t-18 and t-16) and at the start of the intervention period (t0). To examine the abundance and the evenness of the fungal microbiota, the alpha-diversity was determined over time. In Table 2, the observed features at ASV level and effective Shannon diversity index are shown for fungi and bacteria. From this table it can be seen that the observed ASVs for fungi are low, with a median of 22.5. The median effective Shannon diversity index was 22.0. This low fungal diversity compared to bacterial diversity is found more in studies on the mycobiome (Nash et al., 2017). In comparison, the diversity for bacteria is much higher, with a median of 488 observed ASVs and an effective Shannon diversity index of 5973.

Table 2: Alpha-Diversity of fungal and bacterial communities in the adaptation period

	Fungi		Bacteria	
	Observed features	*Effective Shannon diversity*	*Observed features*	*Effective Shannon diversity*
Median	22.5	22.0	510	5973
Minimum	9	4.34	217	1654
Maximum	43	47.5	791	9311

Figures 2A and 2B shows pairwise comparisons of the observed ASVs and the effective Shannon diversity index between the different time points in the adaptation period. The observed fungal ASVs changed significantly in the first two hours (p=0.009) and between t-20 and t-16 (p=0.0032), other comparisons were not significantly different (p>0.05). The effective Shannon diversity index of fungi did not change significantly. In addition, the alpha-diversity for bacteria was compared

between the different time points by pairwise comparisons (Figures 2C and 2D). Here, the changes were significant between t-20 and t0 (p=0.00031), t-18 and 0 (p=0.00016), and for t-16 and 0 (p=0.00062) for the observed ASVs and for the effective Shannon diversity index between t-20 and t0 (p=0.00016), t-18 and t0 (p=0.00016) and t-16 and t0 (p=0.00016). This is different compared to fungi, where most changes took place in the first hours of the adaptation period.
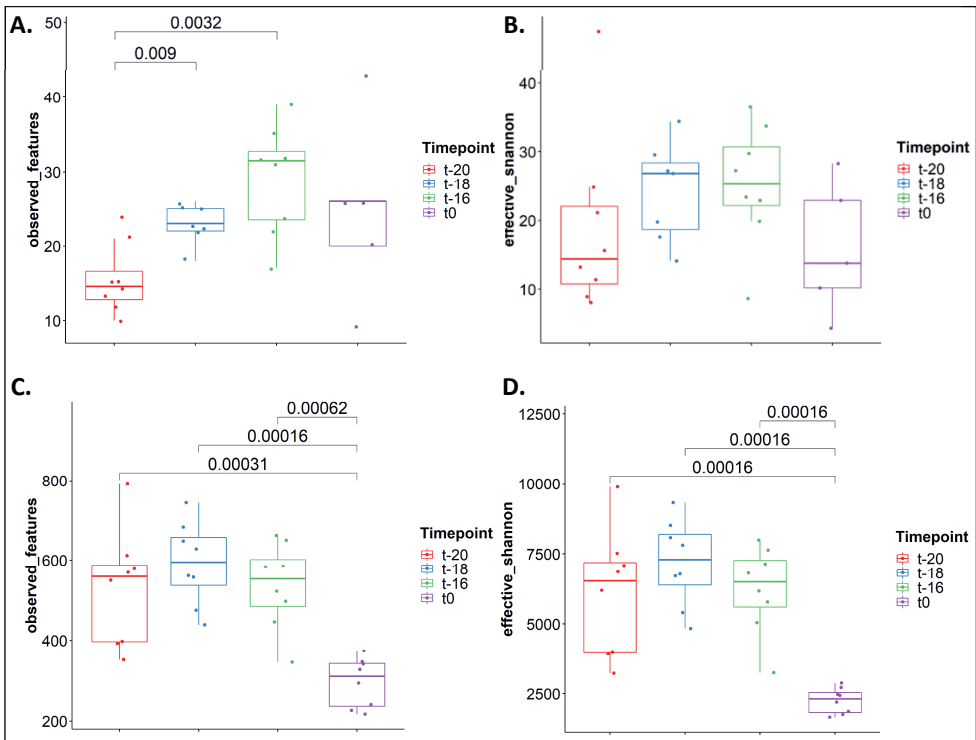

Figure 2: Alpha-diversity of the lumen samples during the adaptation period. **A.** Observed features fungi, **B.** Shannon diversity Index fungi, **C.** Observed features bacteria, **D.** Shannon diversity index bacteria. Significant pairwise comparisons are shown (Wilcoxon signed rank test).

The beta-diversity was determined to measure the variation between the different time points in the adaptation period. Figure 3A shows the beta-diversity (Bray Curtis dissimilarity) for the different time points. The stability of the fungi in the model is shown to be affected mainly in the first two hours of the adaptation period, after which the beta-diversity remained stable, but a high variance between samples was seen at t0. For bacteria, also the Bray Curtis dissimilarity is shown (Figure 3B). Here the time points t-20, t-18 and t-16 cluster together, and t0 samples cluster together. The points at t0 show less variance compared to the fungal samples. The greater variability of the mycobiome between samples could be explained by the low diversity as observed in the alpha-diversity plots, because a change in one of the dominant taxa in the samples drives great differences in the beta-diversity. However, the beta-diversity expressed as Jaccard similarity (presence/absence of taxa) also shows differences in clustering of these time-points (Supplemental Figure 1), which could mean that some taxa are also present in some samples, while not in others.
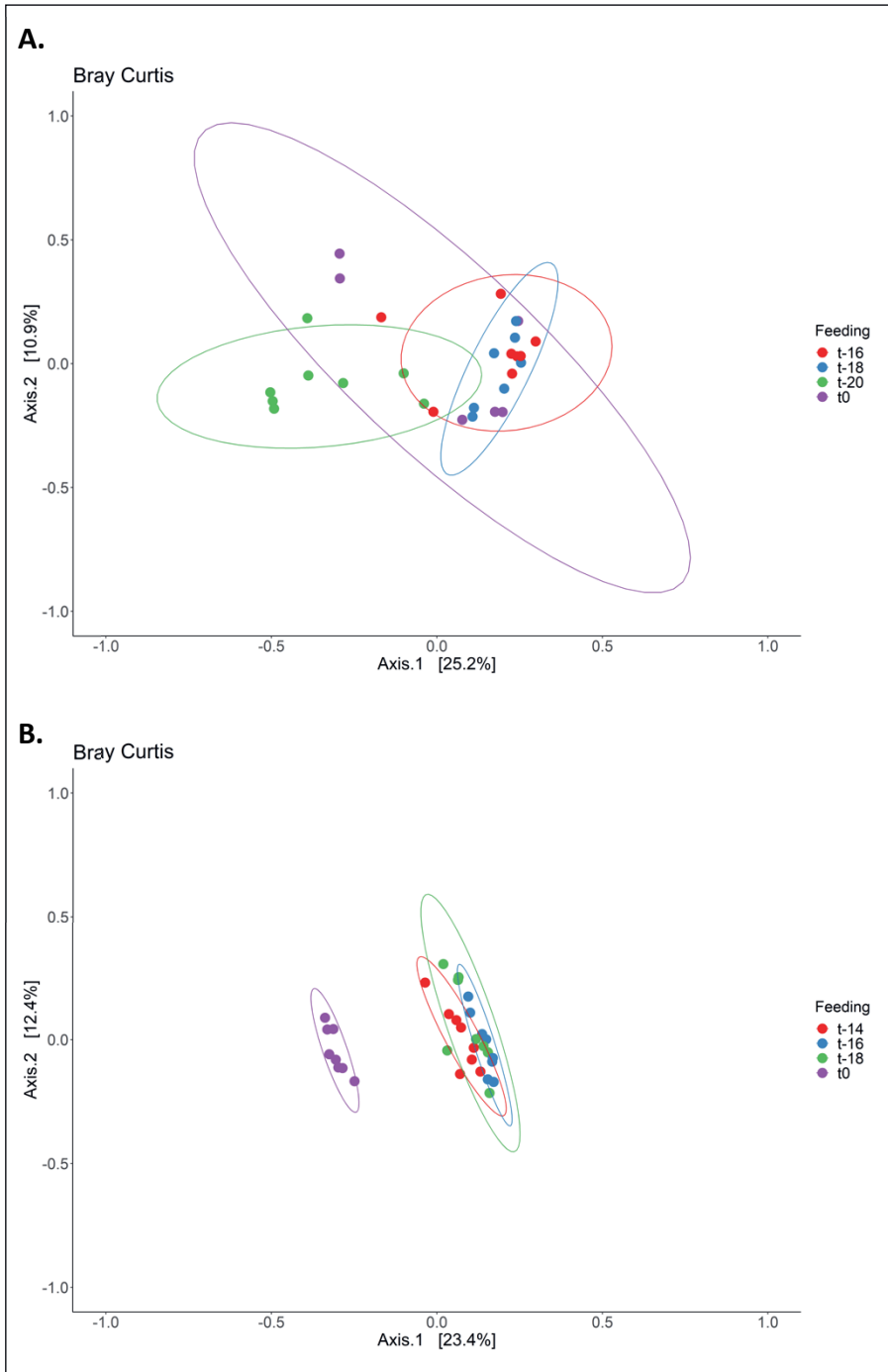
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Figure 3: Beta-diversity during the adaption period visualized using PCoA plot with Bray Curtis dissimilarity distances. **A.** Fungi, **B.** Bacteria. N=8

The fungal composition was further assessed by analysis of the taxonomic composition. The composition was evaluated at the phylum and genus level taxonomical rankings. The phyla *Ascomycota* and *Basidiomycota* were dominant across all samples (Figure 4A). The dominance of these phyla in the human gut has also been previously described (García-Gamboa et al., 2021; Hallen-Adams & Suhr, 2017). The ten most abundant fungal genera across samples are *Agaricus*, *Aspergillus*, *Candida*, *Penicillum*, *Malassezia*, *Saccharomyces*, *Aureobasidium*, *Mycosphaerella*, *Mucor* and *Clavispora* (Figure 4B). These dominant genera make up most of the fungal composition in the different samples. Some genera show a significant change during the adaptation period: *Aureobasidium*, *Pichia*, *Kazachstania*, *Candida*, *Agaricus* and *Mucor* (all $p < 0.05$; Kruskal-Wallis test; Figure 5). Dunn's test was done as post-hoc analysis (Table 3). The genus *Candida* was highly abundant at the time point t-20, but during the course of the adaptation period it became less abundant in most samples. The genus *Agaricus* became more dominant throughout the adaptation period. In a study on the gut mycobiome of the Human Microbiome Project healthy cohort the most abundant genera found in feces with ITS2 and 18S sequencing were *Saccharomyces, Mallasezia, Candida, Cyberlindnera, Penicillum, Cladosporium, Aspergillus, Debarymyces, Pichia, Clavispora* and *Galactomyces* (Nash et al., 2017). The genera *Agaricus* has also been described before to be present in the gut of vegetarians (Suhr, Banjara, & Hallen-Adams, 2016). In these studies on the mycobiome high inter-individual differences were observed in fecal samples. In the *in vitro* model used in this research, a fecal pool was used as inoculum for the experiment. The use of a fecal pool instead of individual samples should lead to less differences between samples (Aguirre et al., 2014). However, some differences can be observed already at the start of the adaptation period (t-20), and these differences increase over the course of the experiment, leading to a greater variability at t0.

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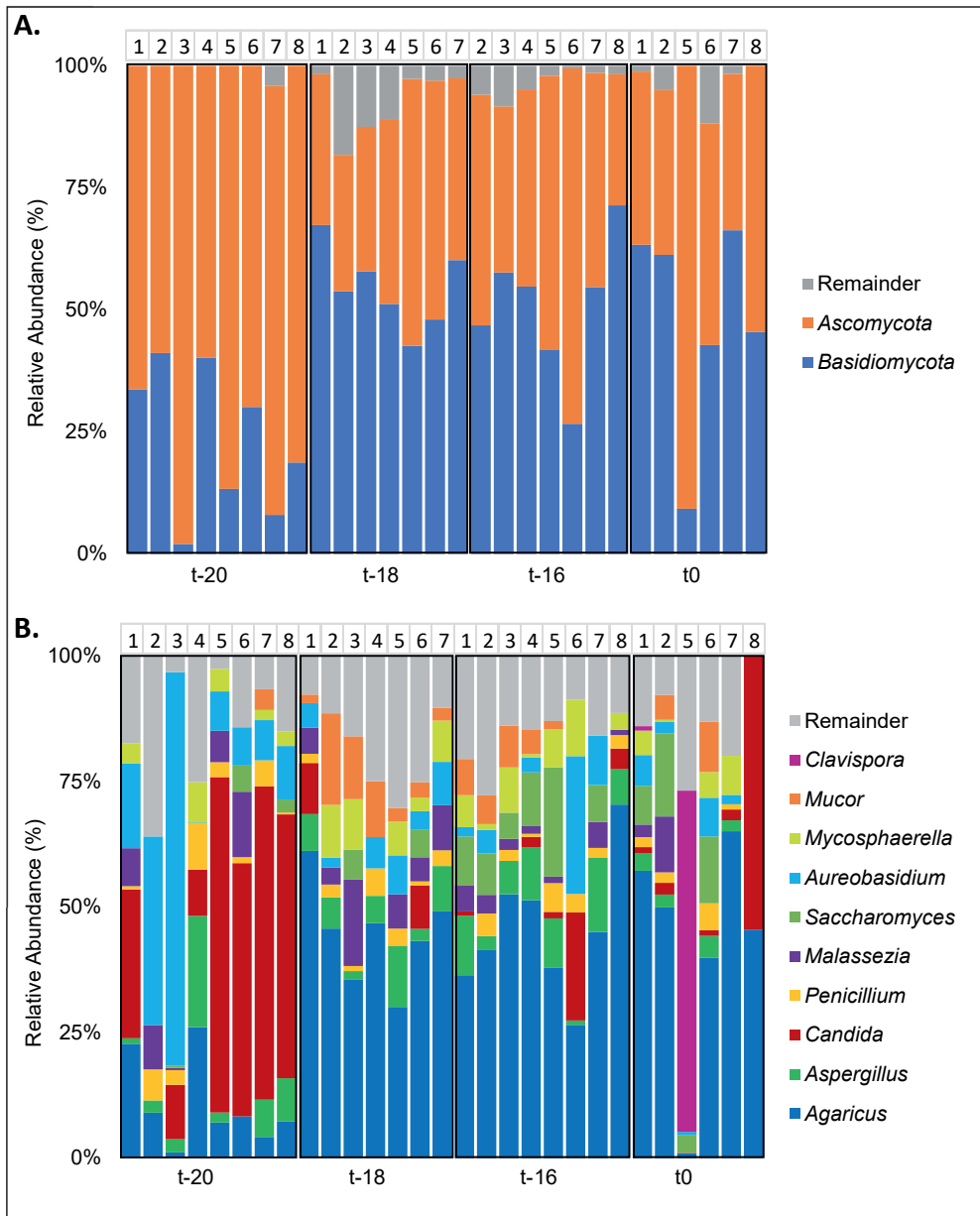
Figure 4. Relative abundance (%) of major groups of fungi during the adaptation period after inoculation in TIM-2 **A.** Phyla, **B.** Genera. N=8 for t-20, t-18 and t-16, n = 6 for T0, due to two samples not passing the quality filtering.

Figure 5. Box-plot of genera *Aureobasidium*, *Pichia*, *Kazachstania*, *Candida*, *Agaricus*, and *Mucor* that were significantly different (Kruskal-Wallis test) at different time points during the adaptation period ($p<0.05$). Dunn's post hoc test was performed to indicate the groups that were significantly different.

Table 3: Genera that are significantly different over time during the adaptation period

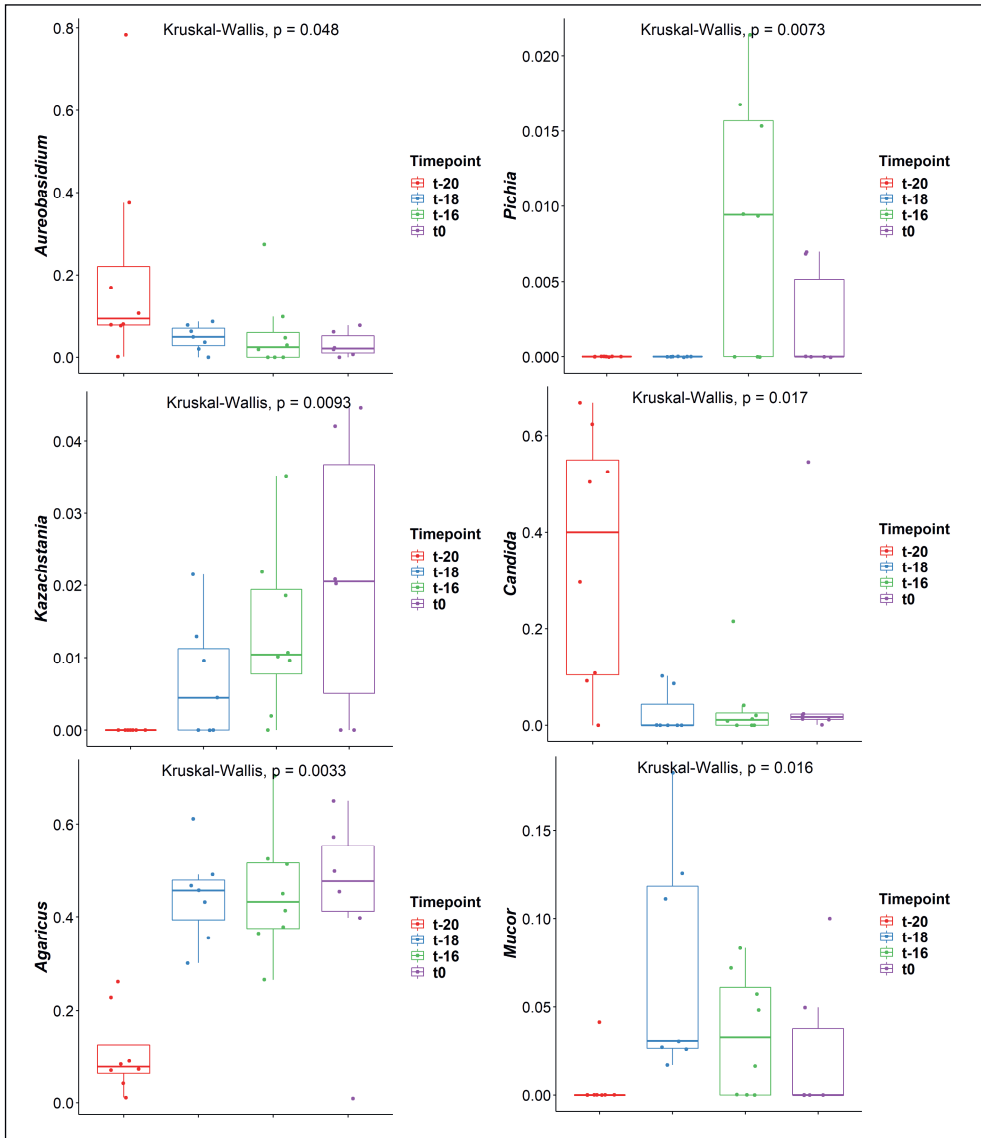| Comparison			Aureobasidium	Pichia	Kazachstania	Candida	Agaricus	Mucor
t-16	-	t-18	0.561	0.003	0.246	0.607	0.990	0.171
t-16	-	t-20	0.019	0.002	0.003	0.013	0.002	0.071
t-18	-	t-20	0.090	1.000	0.092	0.003	0.003	0.002
t-16	-	to	0.792	0.100	0.904	0.377	0.993	0.445
t-18	-	to	0.426	0.261	0.232	0.182	0.984	0.044
t-20	-	to	0.015	0.247	0.004	0.155	0.004	0.364

* p-values Dunn's test for different genera; significant p-values underlined

Intervention period

For the intervention period, the alpha-diversity was determined for fungi and bacteria as well. Similar as in the adaptation period, the observed ASVs were much lower for fungi compared to bacteria (Table 4). In addition, the effective Shannon diversity index was lower for fungi compared to bacteria.

Table 4: Alpha-Diversity of fungal and bacterial communities in the intervention period

	Fungi		Bacteria	
	Observed features	*Effective Shannon diversity*	*Observed features*	*Effective Shannon diversity*
Median	13	10.8	260	1021
Minimum	1	1.0	135	453.6
Maximum	34	54.8	540	3236

To determine whether the dietary interventions carried out in the intervention period had an influence on the diversity, pairwise comparisons were done between interventions; samples were included for the time points t24, t48 and t72. For fungi, the observed ASVs were significantly different between SIEM and 0.1xCHO (p=0.014) and 0.1xCHO and glucose (p=0.0014) (Figure 6A and 6B). The effective Shannon diversity index was significantly different between 0.1xCHO and glucose (p=0.038). The same pairwise comparisons were done for the bacterial community. Here the observed OTUs were significantly different between SIEM and glucose (p=0.0028) and between 0.1xCHO and 10xCHO (p=0.041), the effective Shannon

diversity index was different between SIEM and 0.1xCHO (p=0.013) (Figure 6C and 6D).
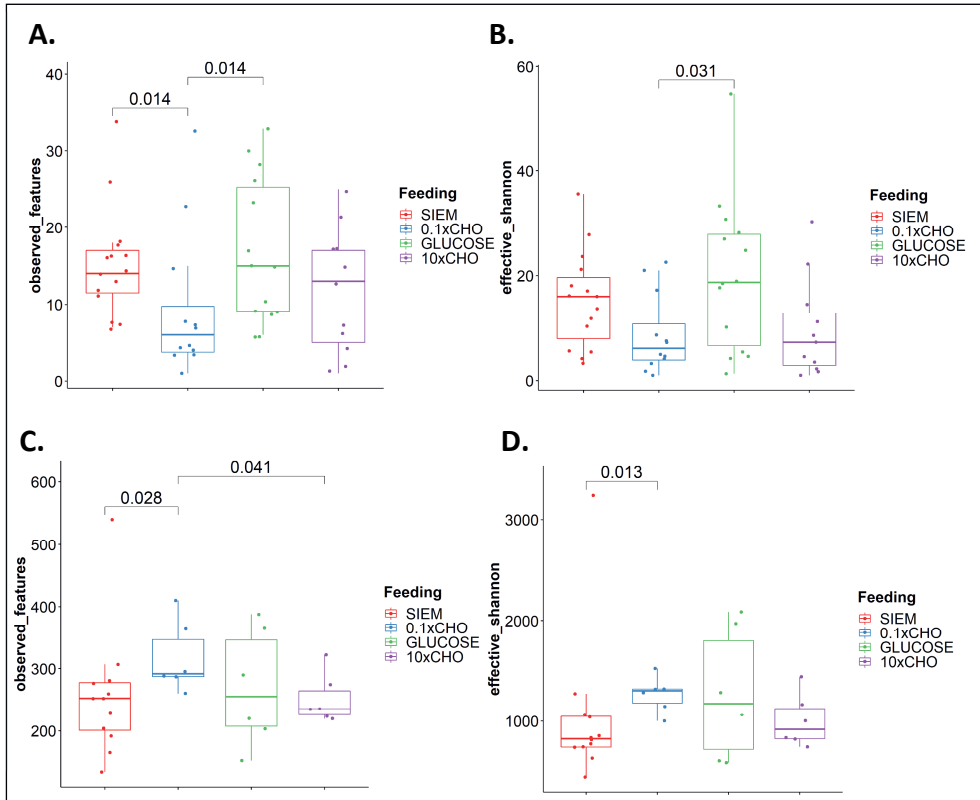

Figure 6. Alpha-diversity of the lumen samples during the intervention period. **A.** Observed features fungi, **B.** Shannon diversity index fungi, **C.** Observed features bacteria, **D.** Shannon diversity index

To check if the samples for the different time points and feeding strategies would cluster together, beta-diversity was determined with the use of Bray Curtis dissimilarities (Figure 7) and the Jaccard index (Supplemental Figure 2) as for the adaptation period. First, the samples were compared between the different time points (Figure 7A). Samples of t0 cluster together and are significantly different compared to t24, t48 and t72 ($p<0.05$). It should be noted though that the first two axes of the PCoA plot only explain 22.5% of the variance in the data. Additionally, the samples were compared between the different feeding strategies (Figure 7B). For this comparison, the samples of t0 were excluded, as the microbiota had not been exposed to the different intervention at that time point. The standard feeding SIEM

clusters together with the 0.1xCHO and the 10xCHO. The samples where glucose was used as carbon source also cluster together and are significantly different from the other interventions ($p < 0.05$) (PERMANOVA).
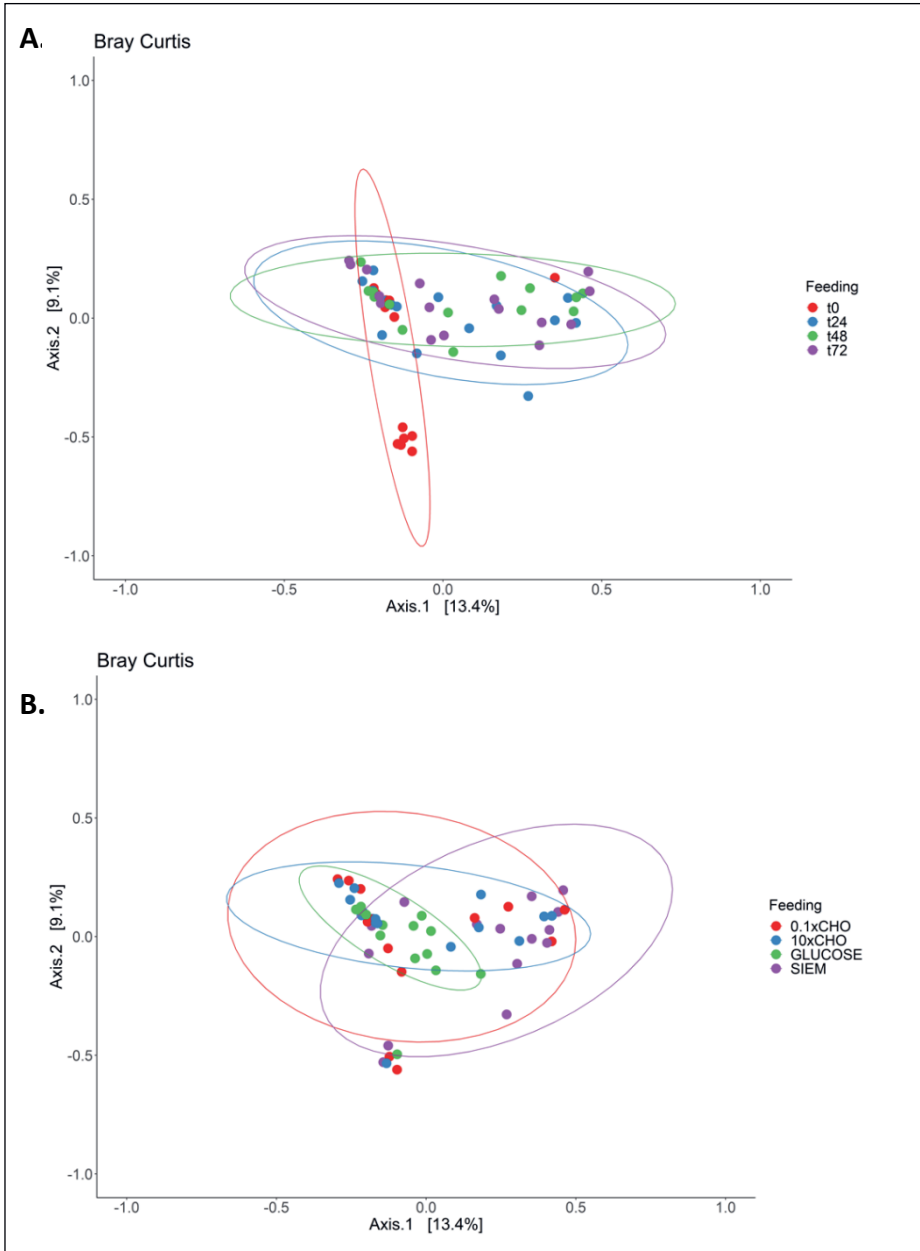

Figure 7. Beta-diversity of the fungal community during the intervention period visualized using PCoA plot with Bray Curtis dissimilarity distances. **A.** Between time points for all feeding interventions, **B.** Between feeding interventions for the time points t24, t48 and t72.

In addition to the diversity analyses, the fungal composition in the samples of the intervention period was also analyzed. In Figure 8 the taxonomic classification at phylum and genus level for the different interventions over the course of the experiment are shown. The phyla *Ascomycota* and *Basidiomycota* are still dominant across all samples (Figure 8A). The ten most abundant genera are shown in Figure 8B; these are *Alternaria, Thanatephorus, Mycosphaerella, Aureobasidium, Penicillium, Aspergillus, Agaricus, Dekkera, Malassezia* and *Candida*. To test whether the genera were significantly different between dietary interventions, the Kruskal-Wallis test was done. The genera *Alternaria, Thanatephorus, Candida* and *Dekkera* were different between interventions ($p<0.05$) (Figure 9). Dunn's test was performed as post-hoc analysis (Table 5). In other studies on the mycobiome, *Candida* was positively correlated with carbohydrate consumption, mainly simple sugars (García-Gamboa et al., 2021; Hoffmann et al., 2013). This is different compared to what was observed in our research, where *Candida* was low in the intervention with glucose. Instead, the genera *Dekkera* and *Alternaria* were dominant in the samples with glucose and probably prevented the increase in relative abundance of *Candida*. This shows that it is difficult to predict how diet can drive the mycobiome composition. Not all fungi found in the gut are stable gut colonizers. Some fungi that are present in the food are transient species and will not settle in the complex microbial environment. In studies on the mycobiome, often samples are taken at one time point, which makes it difficult to discriminate between these transient species and gut colonizers. In the *in vitro* model, the mycobiota is followed for 72 hours, and the feeding is standardized, allowing to control or eliminate fungi introduced by the diet. In addition, there are some challenges in sequencing the ITS compared to 16S for bacteria, including variable length of the ITS, and lower levels of the fungal population in the total microbiota, which makes it more difficult to study fungi in this way. Lastly, it is unknown whether the nucleic acids that were detected at the start of the experiment may also come from non-viable fungi that are present in stool, but will be amplified after DNA isolation.
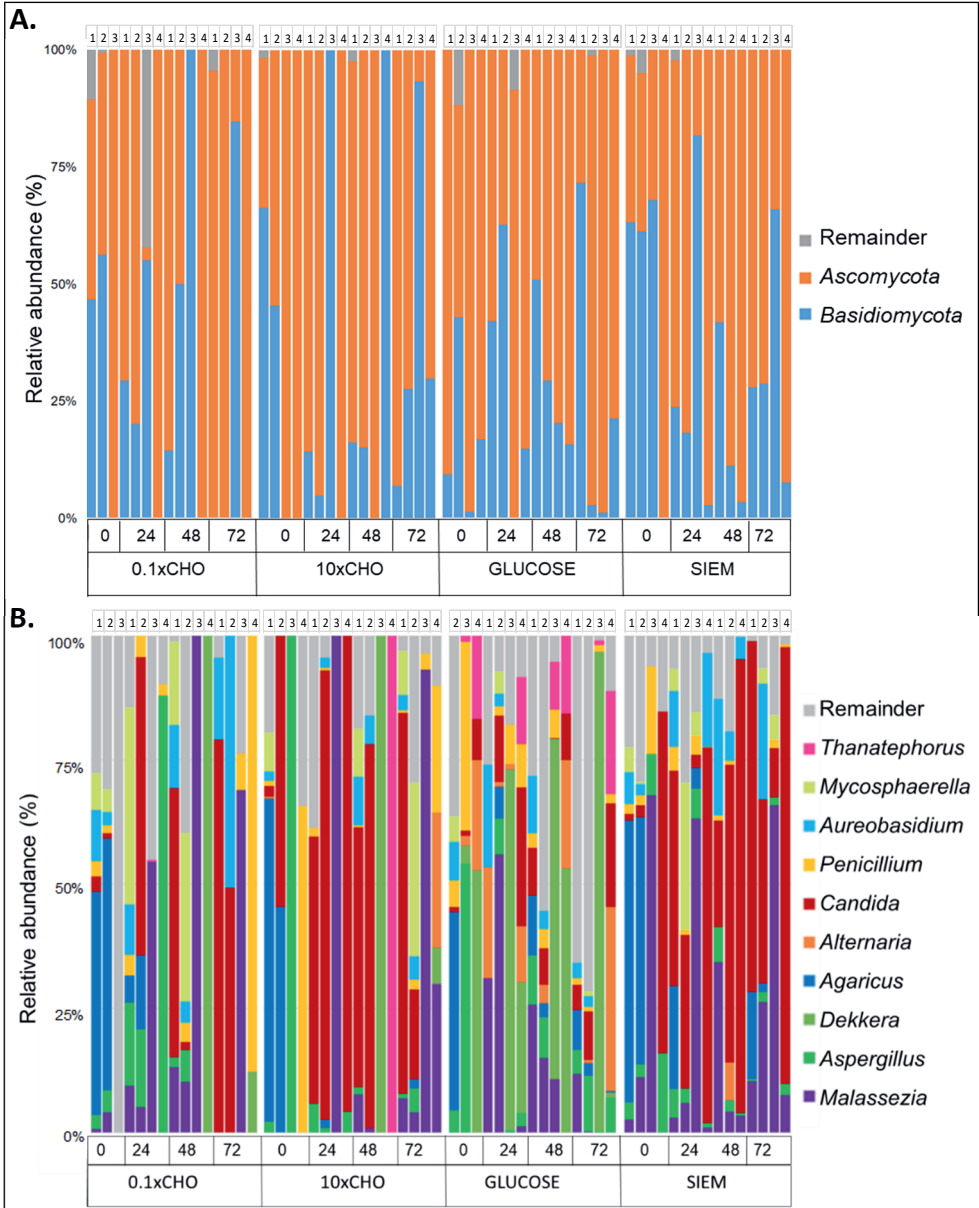
Figure 8. Relative abundance (%) of major groups of fungi during the intervention period after inoculation in TIM-2 **A.** Phyla, **B.** Genera
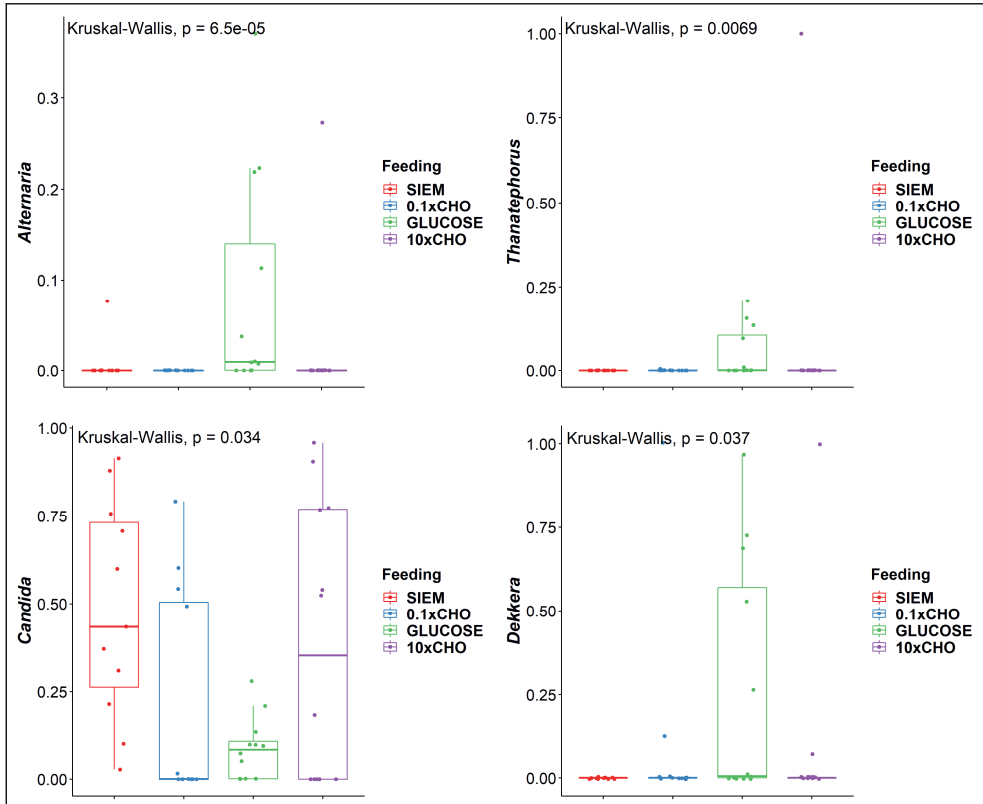
Figure 9. Box-plot of genera *Alternaria*, *Thanatephorus*, *Candida*, and *Dekkera* that were significantly different (Kruskal-Wallis test) for different dietary interventions (p<0.05). Dunn's post hoc test was done to indicate the groups that were significantly different.

Table 5: Genera that are significantly different over time during the intervention period

Comparison			*Alternaria*	*Thanatephorus*	*Candida*	*Dekkera*
0.1xCHO	-	10xCHO	0.581	0.891	0.208	0.983
0.1xCHO	-	Glucose	0.00003	0.007	0.686	0.060
10xCHO	-	Glucose	0.0003	0.010	0.393	0.057
0.1xCHO	-	SIEM	0.615	0.641	0.006	0.324
10xCHO	-	SIEM	0.971	0.548	0.133	0.334
Glucose	-	SIEM	0.0004	0.002	0.019	0.005

* p-values Dunn's test for different genera; significant p-values underlined

We set out to use the dynamic computer-controlled *in vitro* model of the colon (TIM-2) as a tool to study the fungal communities in the gut microbiota. This model has been extensively used to study the bacterial community, and has been validated for

changes in bacterial composition and activity (Venema, 2015). Validation for the fungal community is more difficult, due to the very high inter-individual variation in fungal community between individuals (Nash et al., 2017). For the bacterial community our group has shown before that pooling a microbiota can at least lead to the same starting microbiota (Aguirre et al., 2014), allowing better comparison between experiments and applied interventions. As such, the model can be applied to mechanistically study what happens to the fungal community, e.g. upon antibiotic or fungicide treatment, and the interaction (e.g., co-occurrence) between fungi and bacteria (**chapter 6** of this thesis). Using more sophisticated tools, such as substrates labelled with the stable isotope ^{13}C, even metabolic cross feeding between fungi and bacteria or vice versa can be studied. We anticipate that those mechanistic studies help in contributing the closure of the gap in knowledge on the role of fungi in the gut microbiota and health and disease.

Conclusions

Although *in vitro* gut models have the limitation that they do not include host factors, they have shown to be useful in the study of the microbiome. These studies on the microbiome usually are restricted to the bacterial component of the microbiome. However, in this study it was shown that also fungi can be modelled in a complex *in vitro* model of the colon (TIM-2). The modelling of the fungi has the additional challenge that the variation between samples is big and the cell-numbers are small, which likely leads to a less stable community as compared to bacteria. Future research should focus on optimizing the stability of the fungi in the *in vitro* model to increase the strength of results found. To see if the mycobiome could be modulated, dietary interventions were carried out. Only in the diet with glucose significant differences could be observed, and only for four genera. To further investigate the fungal population and how it is modulated, bacterial-fungal interactions are studied in **chapters 5** and **6** of this thesis, which focuses on how changes driven by the diet, but also by fungicide and antibiotics use can influence the stability and interaction of these two kingdoms.

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Supplemental Figures
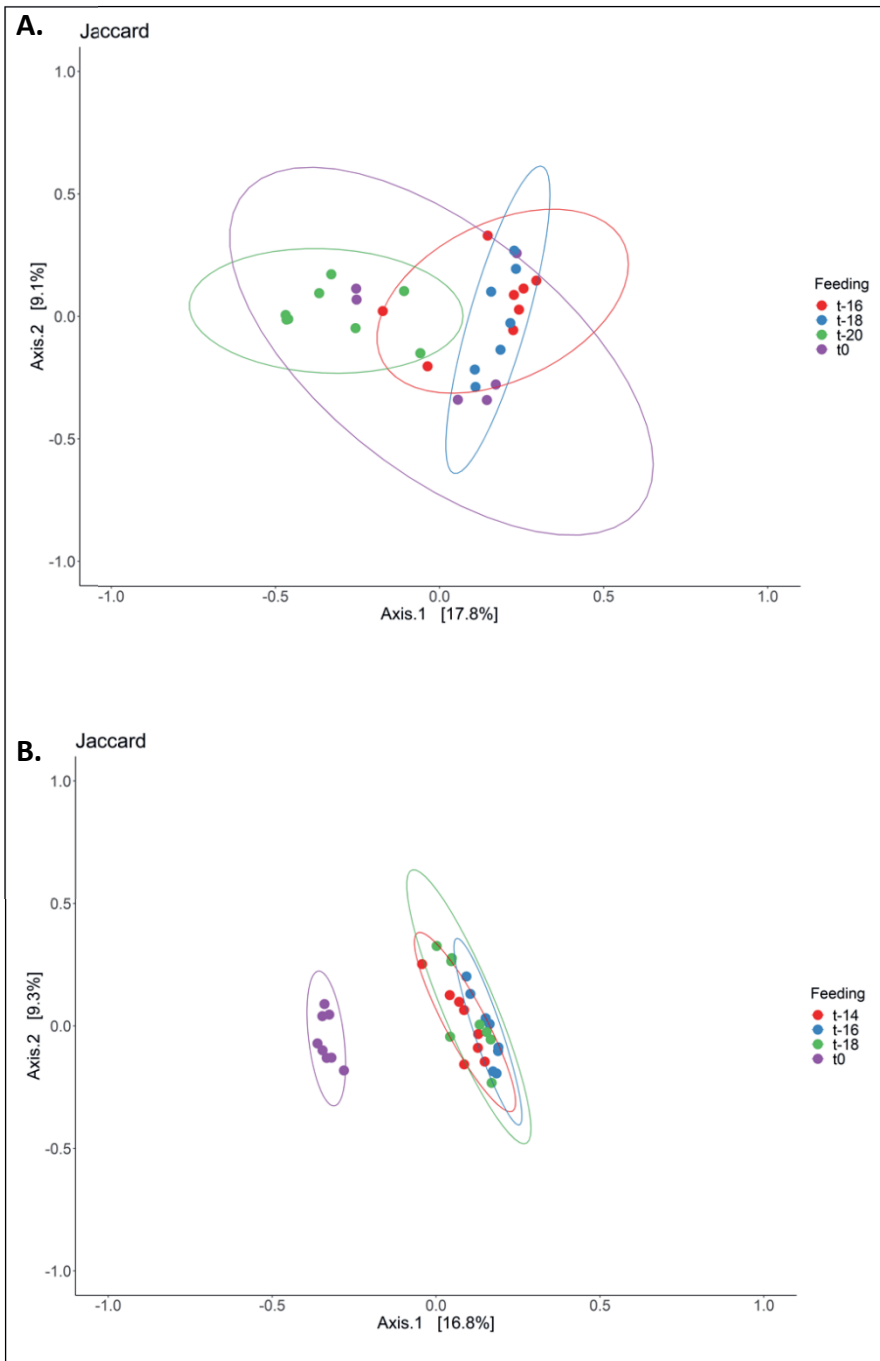
Supplemental Figure 1: Beta-diversity during the adaption period visualized using PCoA plot with Jaccard.similarities **A.** Fungi, **B.** Bacteria. N=8
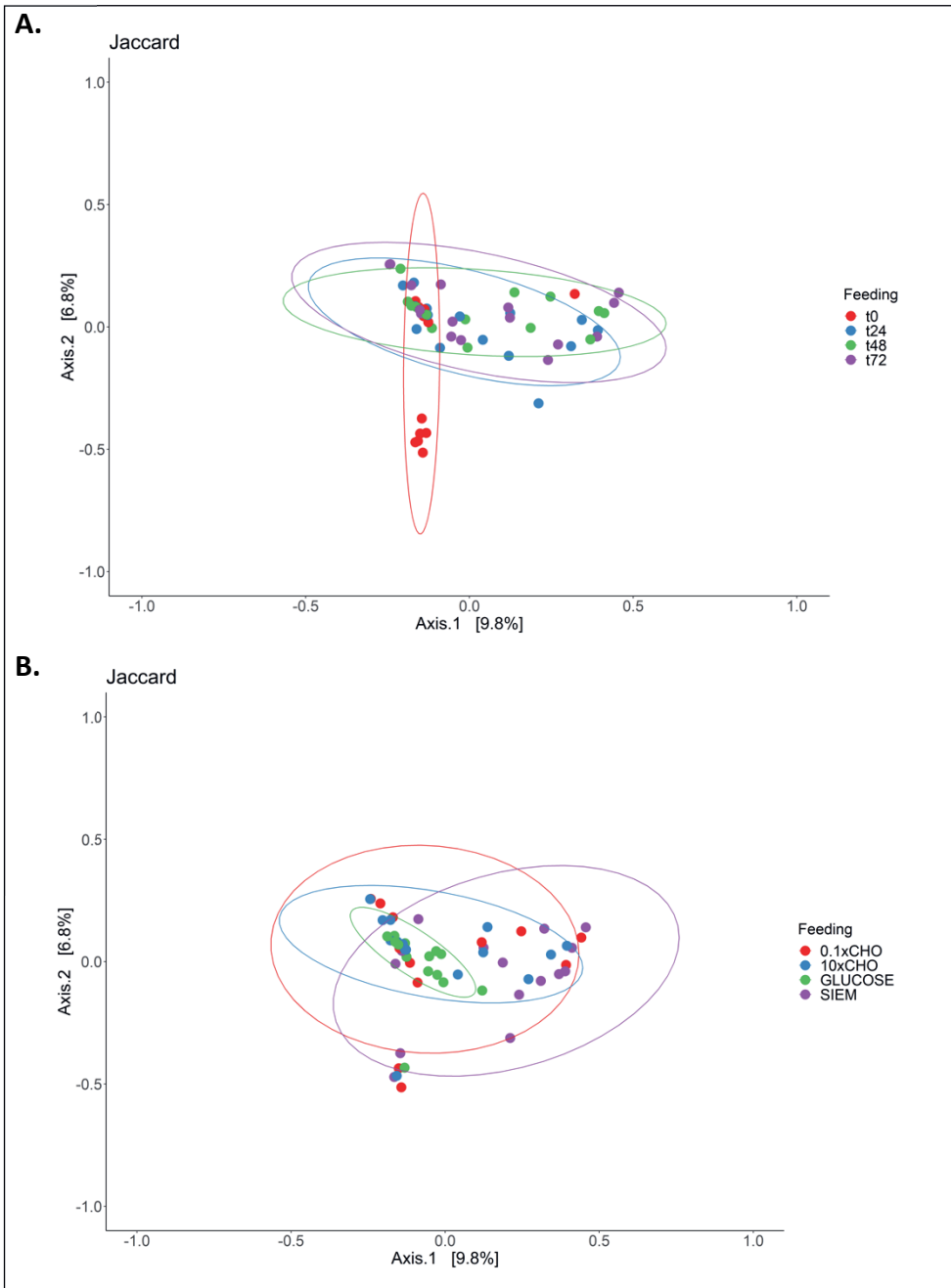
Supplemental Figure 2: Beta-diversity of the fungal community during the intervention period visualized using PCoA plot with Jaccard similarities. **A.** Between time points for all feeding interventions, **B.** Between feeding interventions for the time points t24, t48 and t72.
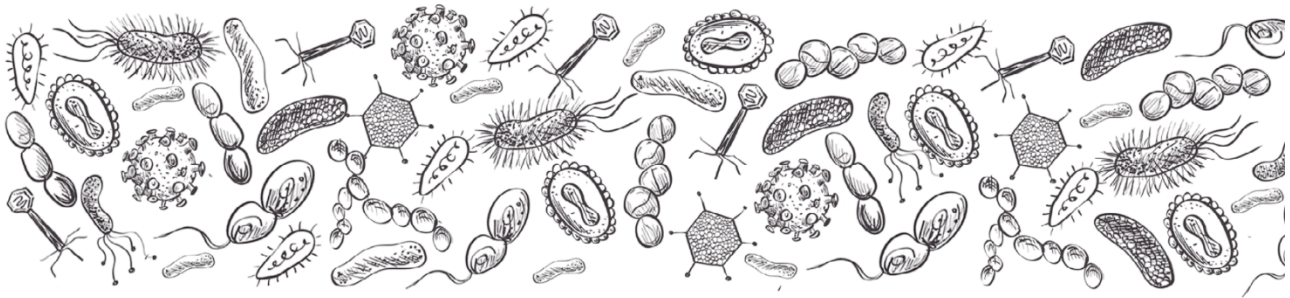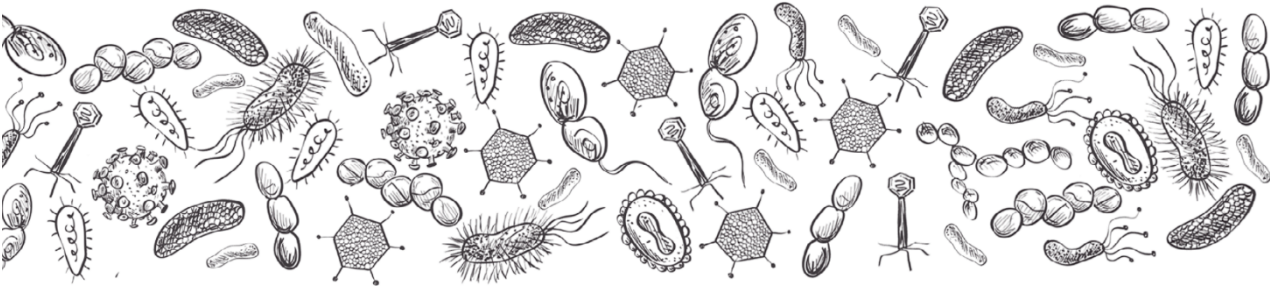
Chapter 5

Fungal-bacterial interactions in the human gut of healthy individuals

Evy Maas

John Penders

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Abstract

Most studies concerning the microbiota in the human gut focus on the bacterial part, but increasing information shows that also intestinal fungi are important for maintaining health. This can either be by directly influencing the host, or by indirectly influencing the gut bacteria that link to host-health. Studies about fungal communities in large cohorts are scarce, therefore this study aims to get more insight in the mycobiome of healthy individuals and how this mycobiome interacts with the bacterial component of the microbiome. For this, on fecal samples of 163 individuals that were available from two separate studies, ITS2 and 16S rRNA gene amplicon sequencing was performed to analyze the fungal and bacterial microbiome, respectively, as well as their cross-kingdom interactions. The results showed a much lower fungal as compared to bacterial diversity. *Ascomycota* and *Basidiomycota* were dominant fungal phyla across all samples, but levels varied enormously between individuals. The ten most abundant fungal genera were *Saccharomyces, Candida, Dipodascus, Aureobasidium, Penicillium, Hanseniaspora, Agaricus, Debaryomyces, Aspergillus* and *Pichia,* and here also extensive inter-individual variation was observed. Correlations were made between bacteria and fungi, and only positive correlations were observed. One of the correlations was between *Malassezia restricta* and the genus *Bacteroides*, which both are described before to be alleviated in IBD. Most other correlations found were with fungi that are not known as gut colonizers, but originate from food and environment. To further investigate the importance of the observed correlations found, more research is needed to discriminate between gut colonizers and transient species.

Introduction

The human gut is a complex ecosystem that consists of numerous microorganisms, not only bacteria but also archaea, viruses and fungi (Breitbart et al., 2003; Hamad, Raoult, & Bittar, 2016; Hoffmann et al., 2013). Studies concerning the human gut microbiota usually focus on the bacterial component of the microbiota, but awareness about the importance of the other components is rising, and especially the fungal component of the microbiota, the so-called mycobiota, is getting more attention. Amongst others, the low density of fungi in the gut compared to bacteria has led to the previous lower attention. Fungi that are cultivable are present in the range of 10^2 to 10^6 CFU/g (Simon & Gorbach, 1984), compared to 10^{11}-10^{12} CFU/g for bacteria (Macfarlane & Macfarlane, 2004). The fungal genes take up around 0.1% of the total microbial metagenome (Qin et al., 2010). In addition, the more complex molecular and phylogenetic characterization of fungi has also contributed to the neglecting of this part of the microbiome community. Historically, fungi were difficult to study with the use of culture-dependent techniques as many fungi are difficult to culture outside the body, leading to an incomplete picture about the gut fungal composition (Finegold, Attebery, & Sutter, 1974). With the rise of culture-independent techniques, also increasing information was gathered on the mycobiome. In bacteria, the use of 16S rRNA gene amplicon sequencing is standard practice, but different regions of ribosomal RNA have been used for studying the mycobiome. Although 18S rRNA sequencing is often used, it is known that 18S primers amplify non-fungal species, e.g. contamination from host cells or food (Hamad, Sokhna, Raoult, & Bittar, 2012; Scanlan & Marchesi, 2008). In addition, the identification is limited to the family or genus level with the use of 18S rRNA sequencing. Therefore, in the last years the regions that are preferred for the sequencing of fungi are the ITS regions. The ITS units are the spacer DNA regions between the small-subunit (SSU or 18S) rRNA and the large-subunit (LSU or 28S) rRNA genes, where ITS1 is positioned between the 18S and 5.8S rRNA genes and ITS2 between the 5.8S and 28S genes. When using the ITS regions for the identification of fungi, the whole ITS region can be used, but also the use of the ITS1 and ITS2 alone is described (Heisel et al., 2015; Hoffmann et al., 2013; Scanlan & Marchesi, 2008).

These new insights by next-generation sequencing of the mycobiome, show that fungi are important in several gut-associated and metabolic diseases (Beheshti-Maal et al., 2021; García-Gamboa et al., 2021; Salamon et al., 2021). The gut mycobiome could also be a reservoir for opportunistic pathogens, which can grow out and cause infections when the gut ecosystem is disturbed (de Oliveira, Atobe, Souza, & de Castro Lima Santos, 2014; Polvi, Li, O'Meara, Leach, & Cowen, 2015). In addition to linking fungi to disease states, it is also essential to define the mycobiome composition in a healthy state, and to determine how the mycobiome is intertwined with the bacterial component of the microbiome. These fungal-bacterial cross-kingdom interactions could give more insights about the complex ecosystem that exists in the gut.

Studies about the gut mycobiome and microbiome in healthy individuals in a larger cohort are very limited. Therefore, this study aimed to analyze the gut fungal composition and to discover fungal-bacterial interactions in 163 healthy individuals.

Methods

Study subjects
Individuals included in this study were healthy volunteers that participated in two prior studies, one study in which the microbiota was analyzed to determine what could be the core microbiota in healthy individuals, and the other where the difference in the gut microbiota of healthy individuals and participants with acne were studied (both studies unpublished). These previous studies did not require medical ethical approval for collection of fecal samples. In total 163 persons were asked to collect their fecal sample. From these persons information on age, sex, weight, length and medication use three months prior to collection was collected. Subjects gave written consent for the determination of the microbial composition.

Sample collection
Volunteers were provided with a fecal collection kit for the collection of fecal samples. The kit consisted of a fecal collection tube with a scoop containing 9 mL DNA shield (Zymo Research, Irvine, CA, USA) and a fecal collection paper (Fe-Col®). Volunteers were asked to donate one fecal sample according to the instruction

provided with the kit. The DNA shield buffer stabilized the nucleic acids, which allowed for transporting and storing samples at ambient temperatures for a longer time (Kazantseva, Malv, Kaleda, Kallastu, & Meikas, 2021). The collection kit was sent to the laboratory facility, where upon arrival they were kept at 4°C until further processing.

DNA isolation

To determine the fungal and bacterial composition of the fecal samples, DNA was isolated using the QIAamp Fast DNA Stool Mini kit (Qiagen, Venlo, The Netherlands). Fungi have a stronger cell wall containing chitin; therefore, a bead-beating step was introduced in addition to the protocol from the kit as adapted from (Knudsen et al., 2016). In short, 500 μL of fecal sample was transferred to a Precellys tube (Bertin Corp, Rockville, MD, USA) which contained 0.5 mm glass beads, after which 1 mL InhibitEX buffer (Qiagen) was added and samples were treated in the Precellys 24 homogenizer (Bertin Corp) at 6000RPM for 3 x 30s. Samples were cooled on ice in between sessions. Next, samples were heated to 95°C for 7 min, subsequently mixed by vortexing and the sample was pelleted by centrifugation for 1 min at 14000×g. Thereafter, 30 μL proteinase K was added and steps were followed as described in the QIAamp Fast DNA Stool Mini kit handbook. The final elution step was done in smaller volume of elution buffer (50 μL) to increase DNA yield. After elution, the DNA concentration was determined using the Qubit 1X dsDNA high sensitivity (HS) assay and a Qubit 3.0 Fluorometer (Invitrogen, Waltham, MA, USA).

Bacterial composition

Isolated DNA was used for the determination of the bacterial composition. Theretofore, the V3-V4 region of the 16S rRNA gene was sequenced. The Illumina protocols for 16S rRNA metagenomic sequencing were used for the library preparation (Nextera XT DNA Library Preparation Kit, Illumina, Eindhoven, the Netherlands). For the first PCR, the following primers were used: 341F (5′-CCTACGGGNGGCWGCAG-3′) and 785R (5′-GACTACHVGGGTATCTAATCC-3′) (Klindworth et al., 2013). In the second PCR, Illumina indexes and adapters were added to the sequences (Illumina Nextera XT Index Kit v2 Set A). After the library preparation, the DNA was quantified using the Qubit 1X dsDNA HS assay and a

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Qubit 3.0 Fluorometer. The length and quality of the sequences were checked on the Bioanalyzer (Agilent, Santa Clara, CA, USA) using the DNA 1000 kit. Subsequently, samples were pooled in an equimolar manner, loaded on the reagent cartridge and sequencing was performed on the Miseq system (Illumina Miseq reagent kit v3). Fastq files were generated with the use of the Local Run Manager generate FastQ module v3.

Fungal composition

In addition to the bacterial composition, the isolated DNA was also used for the determination of the fungal composition. For this purpose, the internal transcribed spacer unit 2 (ITS2), as the phylogenetic marker for the identification of fungi (Schoch et al., 2012), was sequenced. ITS2 was chosen because it is more conserved than ITS1 (Schultz, Maisel, Gerlach, Muller, & Wolf, 2005). The Illumina "Fungal Metagenomic Sequencing Demonstrated Protocol" was used for the library preparation (Nextera XT DNA Library Preparation Kit, Illumina). In the first PCR the primer pair ITS F (5'-GCATCGATGAAGAACGCAGC-3') and ITS R (5'-TCCTCCGCTTATTGATATGC-3') was used (Op De Beeck et al., 2014). Illumina indexes and adapters were added in the second PCR (Nextera XT Index Kit v2 Set A, Illumina). After the library preparation DNA was quantified (Qubit 1X dsDNA HS assay), quality and length of the sequences were analyzed using the Bioanalyzer in combination with the DNA 1000 kit (Agilent). Because of the variable lengths of the sequences from the ITS2 regions, all fragment sizes were checked and the average length was determined to calculate the appropriate concentration for equimolar pooling in the subsequent steps. The library was sequenced on the Miseq system using the Illumina reagent kit v3. Fastq files were generated as described above.

Bioinformatics analysis

Fastq files for both bacteria and fungi generated with the Miseq system were analyzed with the Quantitative Insights Into Microbial Ecology 2 (QIIME2) software package (version 2019.7) (Caporaso et al., 2010; Estaki et al., 2020). To optimize quality of the ITS2 fastq files, the QIIME2 plugin Q2-ITSxpress plugin was used to trim ITS sequences (Rivers, Weber, Gardner, Liu, & Armstrong, 2018). The dada2 plugin was used for both bacteria and fungi libraries for demultiplexing, quality

filtering and denoising (Callahan et al., 2016). Taxonomic analysis was done with the q2-feature-classifier (Bokulich et al., 2018), using the SILVA database (version 132) for bacterial identification and the UNITE database (version 02-02-19) for fungal identification.

Statistics

Alpha-diversity indexes (observed features and effective Shannon) were determined using QIIME2. Data was visualized using GraphPad Prism 9.3.0. Differences between groups were tested with Kruskal-Wallis, with Dunn's test as post-hoc analysis when significant differences (p<0.05) were found. These analyses were done in Rstudio using the packages ggpubr and FSA. Further analysis was done in Rstudio using the packages qiime2R, phyloseq, ggplot2 and ComplexHeatmap. All analyses were performed using R version 4.04.

Results and discussion

To study the gut mycobiota and microbiota in healthy individuals, ITS2 and 16S rRNA amplicon based sequencing was done on fecal samples of 163 individuals. The study population consisted of 38 (23.3%) male and 125 (76.7%) female subjects, with an average age of 35.5 years and an average BMI of 22.5 (Table 1).

Table 1: Characteristics of study subjects (N = 163)

Sex	38 male (23.3%); 125 female (76.7%)
Age [a]	35.5 ± 12.1 years
Weight [a]	67.9 ± 11.3 kg
Length [a]	173.5 ± 8.7 cm
BMI [a]	22.5 ± 2.7
Overweight [b]	23

[a] =mean ± SD; [b] BMI > 25

In the 163 samples, the "observed OTUs" diversity index was determined on ASV level. The observed ASVs were lower for fungi compared to bacteria (Figure 1A). In the fecal samples on average 16.9 (range 2- 45) fungal and 298.4 (range 117-452)

bacterial ASVs were observed. To further assess the alpha-diversity, the effective Shannon diversity index was determined (Figure 1B), this was also lower for fungi (17.29; range 1.07 – 115.67) compared to bacteria (1475; range 503.03 – 3487.31). These results show that the gut fungal population is far less diverse than the bacterial population.

The lower diversity for fungi compared to bacteria here is comparable to what has been observed in previous studies. In the study on the mycobiome of the Human Microbiome Project healthy cohort, the metric "observed OTUs" ranged from 9 to 92 with a mean of 14 OTUs (Nash et al., 2017), in close agreement with the value observed in the current study. Also Shuai et al. observed lower alpha-diversity values for fungi compared to bacteria (Shuai et al., 2022).
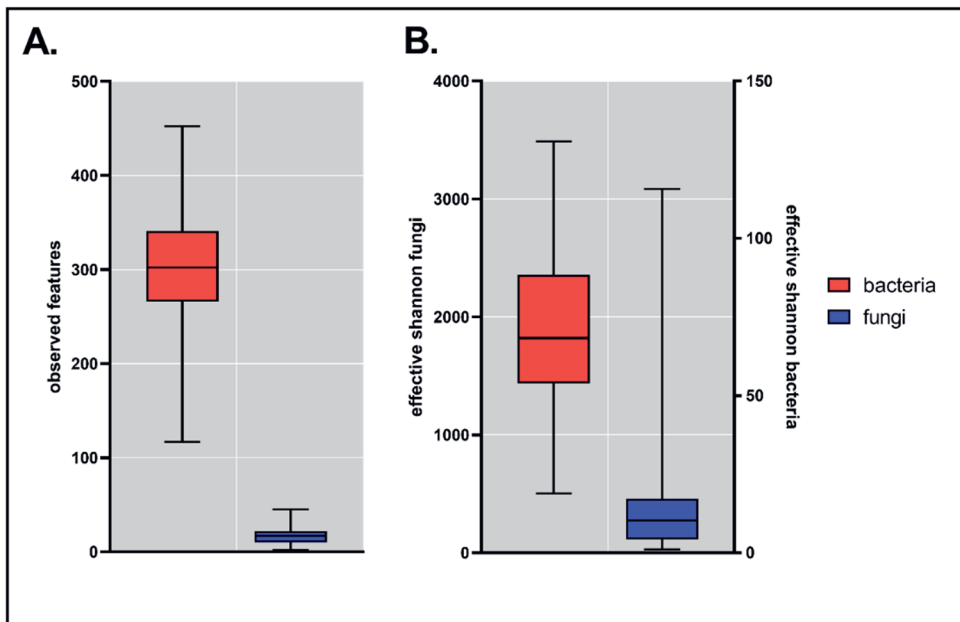
Figure 1: Box-plots of alpha-diversity indexes of the fecal samples (N=163). **A.** Observed features *fungi* (blue) and bacteria (red). **B.** Effective Shannon fungi (blue) and bacteria (red). Whisker: min to max; horizontal line = median; box = 25th to 75th percentile.

We did not observe an association between a higher BMI (>25) and the alpha diversity indexes for bacteria nor for fungi (Figure 2). This is in contrast to what has been described in earlier studies, where a decreased fungal diversity was observed in

obese as compared to lean subjects (Mar Rodríguez et al., 2015). This discrepancy might be explained by that the subjects in this study were not selected to create two evenly groups with obese and non-obese persons as was the case in the study of Mar Rodríguez et al. In our study N=23 for overweight individuals (Table 1) and N=2 for obese individuals, probably does not lead to the proper statistical power.
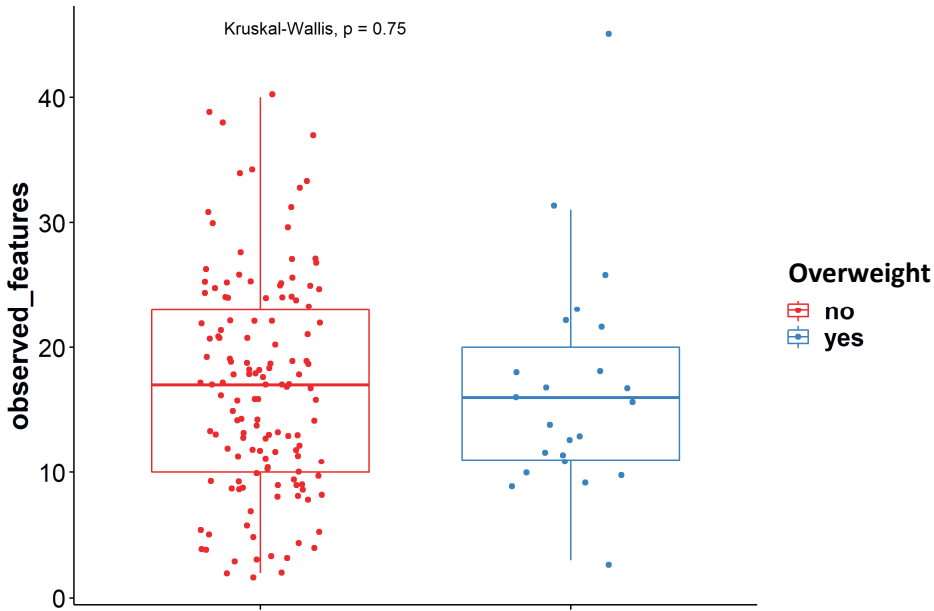
Figure 2: Observed features at ASV levels for obese (n=23) and non-obese (n=140) subjects (Kruskal-Wallis test)

In addition to the diversity analyses, the fungal samples were also studied for their taxonomic composition. At phylum level, *Ascomycota* and *Basidiomycota* were the dominant phyla across all samples, where the average relative abundance for *Ascomycota* was 89.9% ± 14.4% (mean ± SD) and for *Basidiomycota* 9.30% ± 14.4% (mean ± SD). However, the inter-individual variation in the relative abundance of these dominant phyla was extremely large (Figure 3A). The dominance of *Ascomycota* and *Basidiomycota* corresponds with earlier findings in other mycobiome studies (Nash et al., 2017; Shuai et al., 2022). Mar Rodríguez et al.

described the presence of *Ascomycota, Basidiomycota* and *Zygomycota* in fecal samples (Mar Rodríguez et al., 2015).

The mycobiota composition was also analyzed at the genus level. The ten most abundant genera were: *Saccharomyces, Candida, Dipodascus, Aureobasidium, Penicillium, Hanseniaspora, Agaricus, Debaryomyces, Aspergillus* and *Pichia* (Figure 3B).
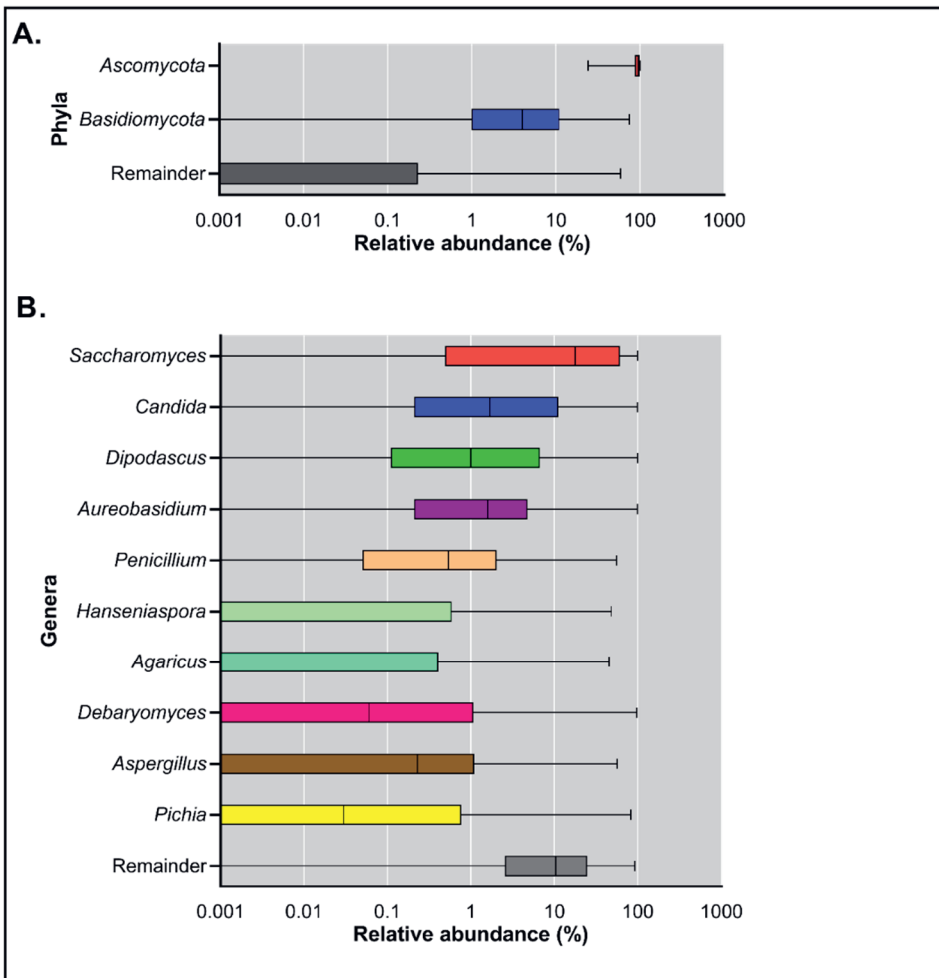

Figure 3: Relative abundance fungi across all subjects; displayed at log scale; **A.** phylum level **B.** genus level.

Many of the genera that dominated in our study (Figure 3B) also belonged to the dominant genera in previous studies on the mycobiome (Table 2). This suggests that some fungi persistently colonize our gut, and are not just transient passengers of the gastrointestinal tract when ingested with the diet.

Table 2: Most abundant genera in mycobiome studies. In bold overlap with genera found in this study.

Most abundant genera	Reference
Penicillum, ***Candida***, ***Saccharomyces***, *Mucor*, ***Aspergillus***	(Mar Rodríguez et al., 2015)
Saccharomyces, Candida, ***Aspergillus***, *Malassezia*	(Shuai et al., 2022)
Saccharomyces, *Malassezia*, ***Candida***, *Cyberlindnera*, ***Penicillium***, *Cladosporium*, ***Aspergillus***, ***Agaricus***, *Fusarium*, ***Pichia***, *Debaryomyces, Galactomyces, Alternaria, Clavispora*	(Nash et al., 2017)

However, from our data and from literature it can be observed that there is a high inter-individual variability in the mycobiome, and when compared to bacteria, fungi are less stable over time (Santus, Devlin, & Behnsen, 2021). Some of this high variability can still be explained by those fungi that are transiently introduced in the human GI tract via the diet or the environment, despite that most genera seem to have been observed in other studies before and therefore are likely gut colonizers. It has been described that fungi and fungal DNA can be found in the diet of humans (Fiers, Gao, & Iliev, 2019; Graves & Hesseltine, 1966; Raimondi et al., 2019). Some of these fungal genera are not able to grow under the conditions found in the gut (temperature, pH and low oxygen levels) (Suhr & Hallen-Adams, 2015). An attempt to discriminate between fungi found in the diet and in fecal samples was done using mouse models, where fungi found in chow were compared with fungi in feces. Some genera were found both in the chow and feces, and others were only found in feces and not in the chow (Iliev et al., 2012; Scupham et al., 2006). The recent study performed by Shuai et al. described the possibility of a core mycobiota that is more stable, because from their data there were some core fungal taxa that were founds in samples of individuals on two time points 3 years apart (Shuai et al., 2022). Since in

our study we only had one time point at which fecal samples were collected, it is difficult to discriminate between the transient species that originate from the diet, and true colonizers that can are consistently found in the gut. We looked into this more in **chapters 4** and **6** of this thesis.

To give more insight on how the mycobiome is shaped in healthy individuals, interactions with the bacterial component of the microbiome were investigated. This was done by correlation of fungal ASVs with bacterial ASVs (by Spearman correlation with FDR correction). In the supplemental material, correlations at genus level can be found. Figure 4 shows correlations that are present in minimal 10% of the study subjects. The picture shows that only positive correlations were found. There are several examples of positive correlations found in literature. For example, the secretion of amino acids by the yeast *Saccharomyces cerevisiae* promotes the bacteria *Lactiplantibacillus plantarum* and *Lactobacillus delbrueckii* subsp. *lactis* which in turn produce lactose that can be used by *S. cerevisiae* (Ponomarova et al., 2017). In addition, it was found that *Candida* spp. could aid in the growth of *Clostridioides difficile* when grown anaerobically (van Leeuwen et al., 2016). Bacteria from the family *Enterobacteriaceae* showed to promote the colonization of *Saccharomyces boulardii* or *Candida albicans* in the mouse gut (Sovran et al., 2018). There are some suggestions on how these positive relationships work. E.g., several studies show that fungi and bacteria are involved in combined biofilms, which creates a favorable environment for both the bacteria and the fungi (Hoarau et al., 2016; Lambooij, Hoogenkamp, Brandt, Janus, & Krom, 2017). Another mechanisms is quorum sensing, where molecules produced by members of one kingdom can promote growth of members of the other kingdom (Kong, Tsui, Kucharíková, Van Dijck, & Jabra-Rizk, 2017). Furthermore, fungi can create an strict anaerobic environment by the consumption of oxygen via the mitochondria, which can be beneficial for certain strict anaerobic bacteria (Lambooij et al., 2017) In our study, however, fungal-bacterial correlations are found that have not been described in literature before (Figure 4). Although the number of fungal and bacterial species found in the gut are high, the information on fungal-bacterial interactions is scarce. For a part of these sort of experiments, the fungi and bacteria that are investigated

are researched *in vitro*, outside of the complex environment in which they normally live, and because of the available research, which is mostly limited to *in vitro* studies, may have missed interesting relationships (Kruger, Vielreicher, Kapitan, Jacobsen, & Niemiec, 2019).

Figure 4 shows that in our study *Malassezia restricta* is positively correlated with the genus *Bacteroides*. *M. restricta* has been described as a member of the skin microbiota, but it is also seen in other studies on the gut mycobiome, so the presence of this fungus in the fecal samples is probably through introduction via skin contact (Hallen-Adams & Suhr, 2017; Prohic, Jovovic Sadikovic, Krupalija-Fazlic, & Kuskunovic-Vlahovljak, 2016). In patients with Crohn's disease, the presence of *M. restricta* in the colonic mucosa is observed and in mice *M. restricta* can induce colitis (Limon et al., 2019). Also *Bacteriodes* spp. have shown to play a role in inflammatory bowel disease (IBD) (Bloom et al., 2011; Wang et al., 2021). These examples show that both fungi and bacteria are disturbed in disease, and that interactions possibly have a role in this. *Candida sake* is a yeast that has been isolated from sake, and is found as a spoilage organism in a range of food products, such as grape juice, sauerkraut and frozen salmon (Hallen-Adams, Kachman, Kim, Legge, & Martínez, 2015). *C. sake*, which is correlated to *Ruminococcus bromii*, does not grow at 37°C, so it should be a transient species. *R. bromii* is a species described to be important in starch degradation (Ze, Duncan, Louis, & Flint, 2012). Cross-feeding of bacteria with glucose formed from starch that has been degraded by *R. bromii* was described before (Kovatcheva-Datchary et al., 2009; Rangarajan et al., 2022). It is possible that such interactions also take place between fungi and bacteria. *Mycosphaerella tassiana* has been described as a fungus present in the airway mycobiota and possibly could have entered the gut via this route (Petrie & Vanterpool, 1978; Rick et al., 2020). The link with *Gemmiger formicilis* (Figure 4) is not described in literature. *Aspergillus* spp. are commonly found in soil, air or plants, but they can survive at 37° C. The presence in the gut is most likely from environmental origin (Mortensen et al., 2010; O'Gorman & Fuller, 2008). *Aspergillus* has been described to be involved in lung diseases where it can cause dangerous clinical infections (Richardson, Bowyer, & Sabino, 2019). Aspergillosis can also affect the gut

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(Kulkarni, Aruni, Rastogi, Rana, & Gupta, 2020). In our study, *Aspergillus* is positively correlated with *Ruminococcaceae,* which is different than has been described in the piglet gut, where a negative correlation was found (Arfken, Frey, Ramsay, & Summers, 2019). *Dipodascus* showed to be one of the most abundant genera associated with people living in a rural area (Kabwe, Vikram, Mulaudzi, Jansson, & Makhalanyane, 2020). Also *Ruminococcus* spp. are found in the gut of inhabitants of a rural area, as they play a role as important plant-degraders (Schnorr et al., 2014). The other fungi found in this study, *Cystofilobasidium macerans, Baeospora myosura and Debaryomyces prosopidis,* that showed to have interactions with bacteria in this study have not been well described in relation to the human body. They originate from the environment and were probably transferred to the gut via the airway or food, and most likely they are transient species and not true gut colonizers (Libkind, Gadanho, van Broock, & Sampaio,
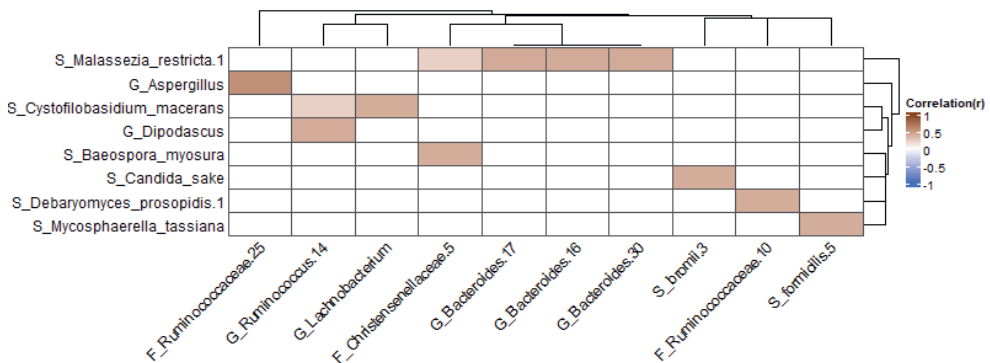
Figure 4: Bacterial - fungal correlations using Spearman correlation present in ≥10% of samples; corrected p-value after FDR cut-off 0.1

2009; Phaff, Vaughan-Martini, & Starmer, 1998; Singer, 1938). The analysis of a single fecal sample for each individual, does not allow to discriminate between species that are passing through the GI tract and true gut colonizers. To further study these interactions to see if they can be relevant for the health in the gut, more research is needed, for example with longitudinal data from subjects with more information on dietary intake. As alternative, also an *in vitro* model of the colon such as TIM-2 could be used to gain more insight in the relations between bacteria and fungi (**chapters 4** and 6 of this thesis).

Conclusions

The aim of this study was to describe the mycobiota of a large cohort of healthy individuals, and to see if fungal-bacterial cross-kingdom correlations could be observed. Comparable to other studies, the mycobiome in this research shows to have a low diversity and a large variation between individuals. Diversity indexes were compared for different BMI: overweight vs. not overweight, but no significant differences were observed. This contrasts with other studies, but could possibly be explained by the fact that this study population was not selected to equally represent obese and non-obese subjects. Taxonomic classification showed dominance of the phyla *Ascomycota* and *Basidiomycota*, but the ratio varies greatly between individuals. This dominance is similar as described in other studies, which is also the case for the majority of the most-abundant genera that were found, such as *Candida, Saccharomyces, Penicillium* and *Aspergillus*. From the taxonomic classification also large inter-individual differences could be observed, possibly explained by the fact that a lot of fungi are introduced via the diet or environment and do not stably colonize the gut. However, a few genera were observed by us and in other studies, possibly representing a core-mycobiota. Due to the single sample point in time, the nature of this study did not allow to discriminate between transient species and gut colonizers. This was also found when looking at fungal-bacterial interactions, where most interactions found were with fungi that were hypothesized to be derived from the environment. The interactions found were all positive correlations. These kinds of correlations have been described before in literature, but the relations observed in this study were new. More research is needed to investigate the relevance of these interactions. The use of an *in vitro* model of colon such as TIM-2 could help to gain more insight in the cross-kingdom correlations and interrelations of bacteria and fungi in the gut and how they could influence the balance of the microbial community, with a link to health and diseases. In the next chapter, TIM-2 is used to further investigate fungal-bacterial interactions, and what happens if one or the other component is disturbed with the use of antibiotic or antifungal drugs.

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Supplemental Figure
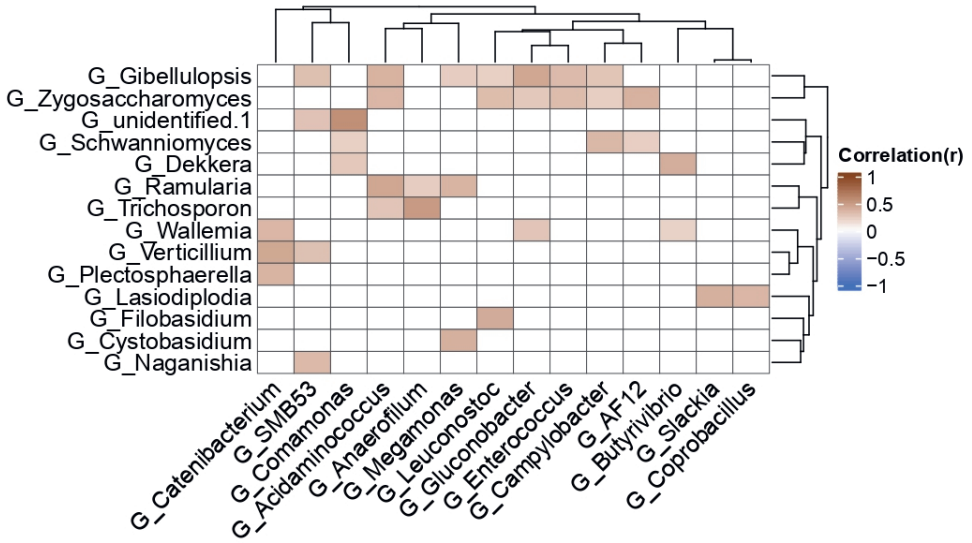
Supplemental Figure 1. Correlations between bacterial and fungal genera present in ≥2% samples; corrected p-value after FDR cut-off 0.2.
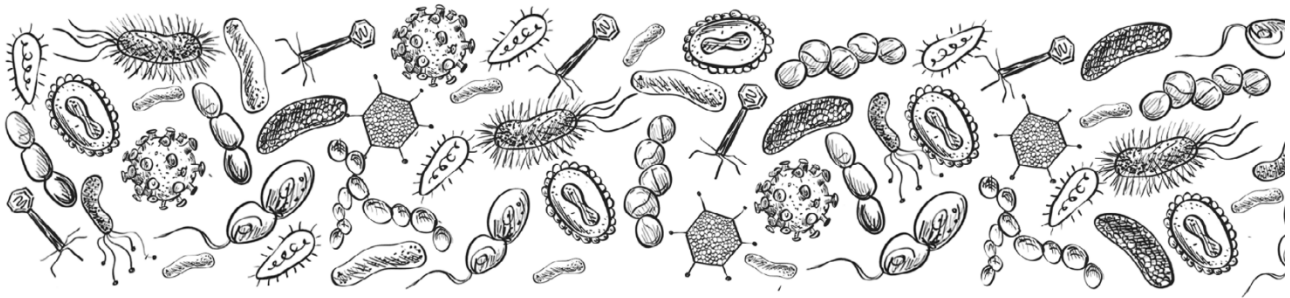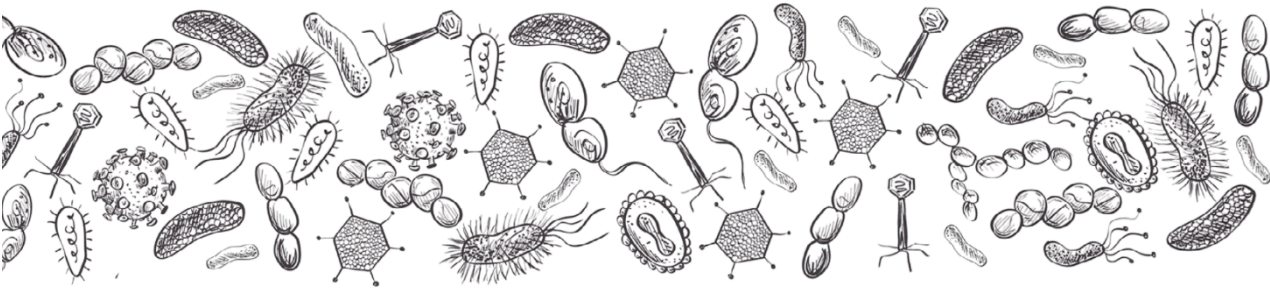
Chapter 6

Studying fungal-bacterial relationships in the human gut using an in vitro model (TIM-2)

Evy Maas

John Penders

Koen Venema

Abstract

The complex microbial community found in the human gut consist of members of multiple kingdoms, among which bacteria and fungi. Microbiome research mainly focuses on the bacterial part of the microbiota, thereby neglecting interactions that can take place between bacteria and fungi. With the rise of sequencing techniques, the possibilities to study cross-kingdom relationships has expanded. In this study, fungal-bacterial relationships were investigated using the complex, dynamic computer-controlled *in vitro* model of the colon (TIM-2). Interactions were investigated by disruption of either the bacterial or fungal community by the addition of antibiotics or antifungals to TIM-2 respectively, compared to a control without antimicrobials. The microbial community was analyzed with the use of next generation sequencing of the ITS2 region and the 16S rRNA. Also, production of SCFAs was followed during the interventions. Correlations between fungi and bacteria were calculated to investigate possible cross-kingdom interactions. The experiments showed that no significant differences in alpha-diversity were observed between the treatments with antibiotics and fungicide. For beta-diversity, it could be observed that samples treated with antibiotics clustered together, whereas the samples from the other treatments were more different. Taxonomic classification was done for both bacteria and fungi, but no big shifts were observed after treatments. At the level of individual genera, bacterial genus *Akkermansia* was shown to be increased after fungicide treatment. SCFAs levels were lowered in samples treated with antifungals. Spearman correlations suggested that cross-kingdom interactions are present in the human gut, and that fungi and bacteria can influence each other. Further research is required to gain more insights in these interactions, and their molecular nature, and to determine the clinical relevance.

Introduction

In the past decades, the development of techniques for the analysis on the microbial community in the human gut has led to new insights in this ecosystem. The use of next-generation sequencing allowed the study of microorganisms that are culture-independent and showed the complexity of the gut microbiota (Zoetendal, Rajilić-Stojanović, & de Vos, 2008). The majority of research on the human gut microbiota is focused on the bacterial component, but the use of sequencing techniques has also shown that fungi are an important component of the microbiota (Nash et al., 2017). Several studies have described the gut fungal community, also named mycobiome, and how these can be linked to several diseases, not only gastrointestinal (GI), but also metabolic diseases (Beheshti-Maal et al., 2021; Nagpal et al., 2020; Richard & Sokol, 2019; Salamon et al., 2021). Both bacteria and fungi can be influenced by external factors, such as diet or antibiotic use (Aguirre et al., 2016; Hoffmann et al., 2013; Seelbinder et al., 2020). Since these two communities share the same environment, the influence on one kingdom is hypothesized to also lead to a change in the other. These fungal-bacterial cross-kingdom interactions are largely unknown, and with that also the influence of these interactions on host health.

An example of a disturbance of these fungal-bacterial interactions, is the disruption of the bacterial community by antibiotic use that can led to dysbiosis, which can subsequently lead to the overgrowth of opportunistic pathogenic fungi, such as *Candida* (Donskey, 2004). The development of antibiotic-induced dysbiosis has been described before (Andremont, Cervesi, Bandinelli, Vitry, & de Gunzburg, 2021). The removal of not only pathogenic microbes, but also commensals by antibiotic use, has led to a disruption of the complex microbial community, which can lead to risks for the human health, such as *Clostridioides difficile* infection after antibiotic use (Blaser, 2014). The widespread use of antibiotics has also led to a rise in fungal infections that occur after this treatment. These fungal infections are often difficult to treat, and have a high mortality rate (Brown et al., 2012). Similarly, treatment with anti-fungal treatment leads to disruptions in bacterial community (Qiu et al., 2015). More information on how these communities interact, and how this is influenced when one or the other community is disrupted, can possibly aid in the prevention of

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some fungal infections that are a problem, particularly in e.g. hospitalized or immunocompromised patients.

The use of sequencing techniques has helped in the study of the microbial community in the gut, but there are still challenges. The diet is an important source of fungi, and it is not always clear if the fungi found through sequencing of fecal samples, are fungal species that can colonize the gut, or are transient species from the diet that only pass through the GI tract (Auchtung et al., 2018). This makes it difficult to interpret results and to show interactions between bacteria and fungi. A tool that can give additional information on these two kingdoms within the gut microbiota and how these are modulated, is an *in vitro* model (Venema & Van den Abbeele, 2013). There are several examples of *in vitro* models of the GI tract (stomach, small intestine, large intestine). The majority of the microbial community in the gut is found in the large intestine, and therefore the complex computer controlled *in vitro* model of the colon (TIM-2) was used in this study to investigate the bacterial and fungal communities and how these interact (Venema, 2015). The TIM-2 system has been used previously for the study of the bacterial microbiota in the human colon (Cuevas-Tena, Alegria, Lagarda, & Venema, 2019; Míguez, Vila, Venema, Parajó, & Alonso, 2020). The model allows for the control of environmental factors, such as temperature, pH and oxygen levels, and it has a complex filtration system, which prevents accumulation of metabolites, thereby maintaining physiological levels of these metabolites. TIM-2 is inoculated with a pooled microbiota to allow all experiments to start with the same starting microbiota (Aguirre, Ramiro-Garcia, Koenen, & Venema, 2014). After an adaptation period where the microbiota can get used to the new environment, an intervention can be performed, usually for a test period of 72 hours. This allows for the study of the mycobiome and microbiome over a longer period of time than just a single time-point of a fecal sample, which could give more insights in the interactions between these kingdoms. The aim of this study therefore was to use TIM-2 to study fungal-bacterial interactions, and to investigate what happens if one or the other of these communities is disrupted.

Materials and methods

TNO's in vitro model of the colon (TIM-2)

To mimic the realistic conditions of the colon, a sophisticated computer-controlled *in vitro* model (TIM-2) was used. The model is explained in detail before (Venema, 2015). In short, in the model the pH and temperature are controlled to resemble the circumstances in the colon of a healthy adult, at 5.8 and 37°C respectively. The pH is controlled by the addition of 2M NaOH. An anaerobic environment is created by a constant flush of N_2 through the model and produced metabolites are removed with the use of a dialysis system. At the start of the experiment, the model is inoculated with a fecal pool mixed with a dialysis solution (explained below), which is the start of the adaptation period, to allow the microbiota to adapt to its new environment. The standard growth medium (described below) is added automatically at a constant rate. After the adaptation period, the test period is started where the different treatments are introduced. The model has been validated and extensively used for studying the bacterial component of the microbiota.

Fecal pool

From healthy adult volunteers (n=6, 50% female), fecal samples were collected and homogenized under anaerobic conditions. The samples were mixed, and after snap-freezing in liquid nitrogen, they were stored at -80°C until the start of the experiment. All experiments were performed with the same fecal pool. Preparation of the fecal pool is described in more detail before (Aguirre et al., 2014). Before the start of the experiment, the tubes containing the fecal pool were thawed at constant temperature (37°C) in a water bath. Next, they were mixed 1:1 with dialysis solution, which contained 2.5 g/L $K_2HPO_4 \cdot 3H_2O$, 4.5 g/L NaCl, 0.005 g/L $FeSO_4 \cdot 7H_2O$, 0.5 g/L $MgSO_4 \cdot 7H_2O$, 0.45 g/L $CaCl_2 \cdot 2H_2O$, 0.05 g/L bile and 0.4 g/L cysteine-HCl, plus 1.5 mL vitamin mixture (containing 1 mg/L menadione, 2 mg/L D-biotin, 0.5 mg/L vitamin B12, 10 mg/L pantothenate, 5 mg/L nicotinamide, 5 mg/L p-aminobenzoic acid and 4 mg/L thiamine). This fecal-dialysate mixture was introduced anaerobically in the TIM-2.

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Test products

During the adaptation period, the standard growth medium was used as feeding. This is the simulated ileal efflux medium (SIEM), which was created to resemble the undigested dietary components that reach the colon (Maathuis, Hoffman, Evans, Sanders, & Venema, 2009), and contains 100 g CHO-medium (containing 12 g/L pectin, 12 g/L xylan, 12 g/L arabinogalactan, 12 g/L amylopectin, 100 g/L starch), 25 g TBCO 6.25x (containing 270 g/L Tween 80, 375 g/L bactopepton, 375 g/L casein, 6.25 g/L ox-bile), 2 g $MgSO_4$ (50 g/L), 2 g cysteine (20 g/L), 0.2 mL vitamin mixture as described above, 4 mL salts solution (containing 4.7 g/L $K_2HPO_4 \cdot 3H_2O$, 8.4 g/L NaCl, 0.8 $CaCl_2 \cdot 2H_2O$, 0.009 g/L $FeSO_4 \cdot 7H_2O$, 0.02 g/L haemin) and 1 mL antifoam emulsion. During the test period, SIEM was also used as growth medium, with the addition of fungicide or antibiotics. The fungicide used was cycloheximide, which was added as a shot (1.5 mg) at the start of the test period, and added to the SIEM (0.75 mg/day) and the dialysate (10 mg/L). As antibiotics, an equal mixture of ampicillin, oxytetracyclin and kanamycin was added at the same level as the fungicide, with in the shot a total of 1.5 mg antibiotics. The experiment with SIEM without any addition was run as a control.

Test design

The experiment started with the inoculation of the fecal pool in the model, which marked the start of the adaptation period for 16 hours. After the adaptation period, the test period was started, which lasted 72 hours. Samples were taken from the lumen and the dialysate at the start of the experiment and every 24 hours. The interventions of SIEM, SIEM+fungicide and SIEM+antibiotics were done in duplicate.

Gut mycobiota and microbiota composition

For the determination of both the fungal and bacterial composition, DNA was isolated from TIM-2 samples. The DNA isolation was started with a bead-beating step as described before (Knudsen et al., 2016), in combination with the QIAamp Fast DNA stool mini kit from Qiagen (Venlo, The Netherlands). To measure the DNA concentration, the Qubit HS Assay and a Qubit 3.0 Fluorometer (Invitrogen, Waltham, MA, USA) were used.

For the mycobiota composition, the internal transcribed spacer unit 2 (ITS2) was sequenced with barcoding according to the Fungal Metagenomic Sequencing Demonstrated protocol of Illumina with some changes (Nextera XT DNA Library Preparation Kit , Nextera XT Index Kit v2 Set A, Illumina, Eindhoven, The Netherlands). Input DNA was 50 ng/μL and the PCRI program was set at 30 cycles. The primer set used was ITS F (5'-GCATCGATGAAGAACGCAGC-3') and ITS R (5'-TCCTCCGCTTATTGATATGC-3') (Op De Beeck et al., 2014). Fragment sizes were analyzed using the Bioanalyzer with the DNA1000 kit (Agilent, CA, USA), and quantification, normalization and equimolar pooling was done before loading the library on the Illumina Miseq system (Miseq reagent kit v3, Illumina).

For the microbiota composition, the V3-V4 region of the 16S rRNA gene was sequenced according to the Illumina protocol with barcoding (Nextera XT DNA Library Preparation Kit , Nextera XT Index Kit v2 Set A, Illumina). The primer set used was 341F (5'-CCTACGGGNGGCWGCAG-3') and 785R (5'-GACTACHVGGGTATCTAATCC-3'). The library was loaded on the Illumina Miseq system according to the manufacturer's protocol (Miseq reagent kit v3, Illumina).

Both libraries were sequenced on the Miseq system with the use of the Local Run Manager Generate FastQ Analysis Module v3 to generate fastq files. Further analysis of fastq files was performed with the Quantitative Insights Into Microbial Ecology 2 (QIIME2) software package (version 2019.7) (Caporaso et al., 2010; Estaki et al., 2020). For the ITS2 sequences, the QIIME2 plugin Q2-ITSxpress was used to trim ITS sequences (Rivers, Weber, Gardner, Liu, & Armstrong, 2018). Demultiplexing, quality filtering and denoising was performed with the dada2 plugin (Callahan et al., 2016). The UNITE database (version 02-02-19) was used as reference database for the classification of ITS2 sequencing data. The SILVA database (version 132) was used as reference database for the classification of 16S rRNA sequencing data.

Short-chain fatty acid (SCFA) analysis
SCFAs (acetate, propionate and butyrate) in the lumen and dialysate samples were analyzed with gas chromatography – mass spectrometry (GC-MS). Samples were prepared for GC-MS as described before (van Nuenen, Diederick Meyer, & Venema,

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2009). In short, the samples were centrifuged and formic acid, 2-ethyl butyric acid (internal standard) and methanol were added to the supernatant. The analysis was carried out on a GC-MS (8890 GC System; Agilent Technologies, Amstelveen, the Netherlands) equipped with a PAL3 RSI 85 autosampler (Agilent) by injecting 1 μL sample on a DB-FATWAX Ultra Inert column (30 m, 0.25 mm, 0.25 μm, Agilent). The temperature settings of the injector port, oven, flame-ionization detector and mass spectrometer detector were 250, 200, 275 and 225 °C, respectively. The flow rate over the column was 1.2 ml|/min. With the use of calibration curves of known quantities of standards, quantities of SCFAs in the samples were determined.

Statistical analysis
QIIME2 was used to obtain alpha diversity indexes (observed features and effective Shannon diversity). Further analysis was done using Rstudio (R version 4.0.4) using the packages qiime2R, phyloseq, ggplot2, ggpubr, FSA and complexHeatmap. Groups were compared using the Kruskal-Wallis test, with Dunn's test as post-hoc analysis. Beta diversity was visualized as Bray Curtis dissimilarity and Jaccard similarity and visualized as principal coordinate (PCoA) plots Differences between groups were analyzed using PERMANOVA. GraphPad Prism version 9.3.0 was used for the visualization of SCFA data.

Results and discussion
To investigate the fungal-bacterial interactions in TIM-2, the fungal and bacterial populations were analyzed on diversity, and perturbations were investigated when one of the populations was disrupted with the antimicrobials. In Figure 1, the alpha-diversity for the microbiota and mycobiota were compared for the different time points to see how time influences these diversity indexes and if this was different for bacteria and fungi. In Figure 1A and 1B, the observed features are shown at ASV level. As described before in other studies, the observed features for bacteria are much higher when compared to fungi, with a median of 242 and 14 respectively. In the fungal population, for observed features a small decrease can be observed over time. Different time points were compared with the Kruskal-Wallis test and for fungi this change was significant (p<0.05). Post-hoc Dunn's test was performed and showed that t0 vs t72 and t24 vs t72 were significantly different. The effective Shannon index

was also determined for both bacteria and fungi (Figure 1C and 1D), with a median of 1470 and 11 respectively, and this was also higher for bacteria compared to fungi. The effective Shannon did not change significantly between the different time points for bacteria and fungi.
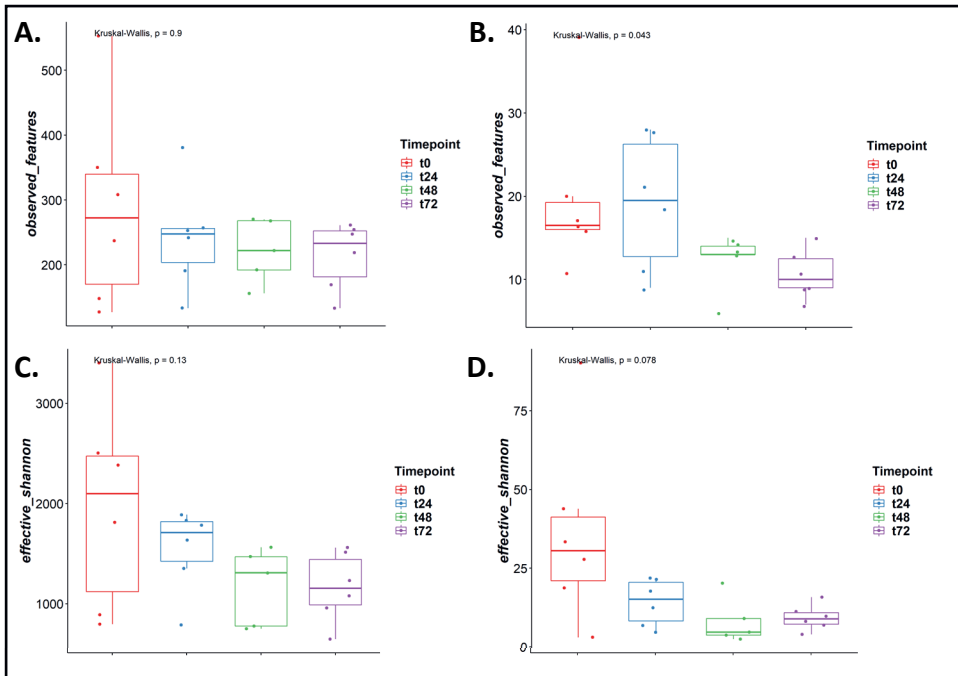

Figure 1: Alpha diversity measures different time points 'observed features' and 'effective Shannon'; **A+C** = bacteria, **B+D** = fungi

Next to the comparison between time points, the alpha diversity was also compared between the different treatments. As can be seen in Figure 2, both the observed features and the effective Shannon did not change significantly between the different treatments.
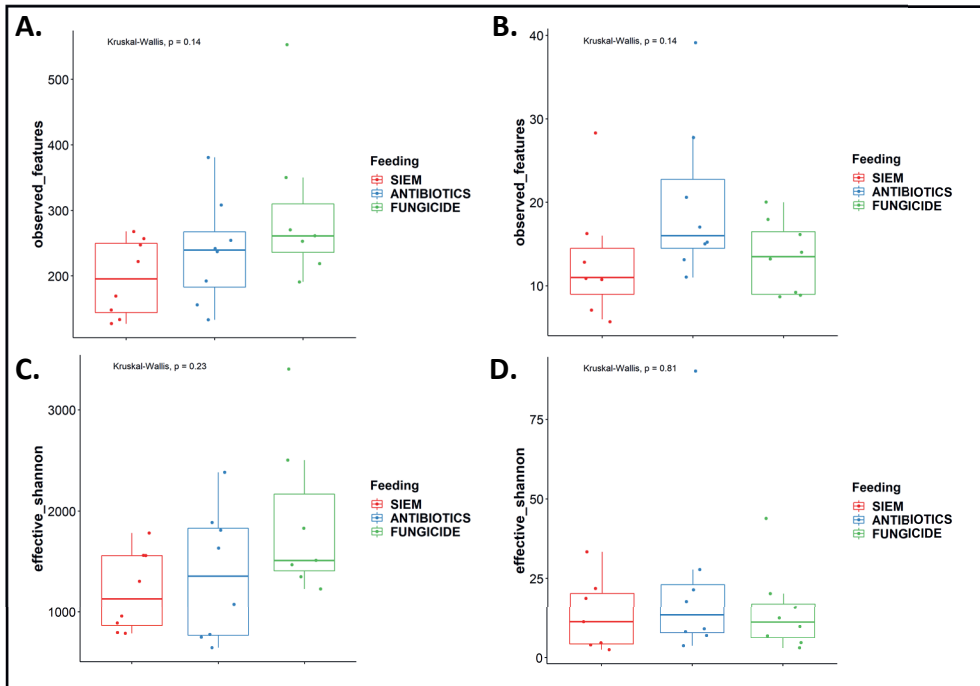
Figure 2: Alpha diversity measures different treatments 'observed features' and 'effective Shannon'; **A+C**= bacteria, **B+D** = fungi

To see if the samples would cluster together for the different time points or the treatments, the beta diversity was analyzed for both bacterial and fungal communities. The Bray Curtis dissimilarity and Jaccard similarity scores for bacteria are shown as a PcoA plot in Figure 3a and 3b. It can be observed that the samples that were treated with antibiotics cluster together, whereas the samples with SIEM and fungicide as intervention were more spread out. The Bray Curtis dissimilarity scores for the different inventions were compared using PERMANOVA, and samples treated with antibiotics were significantly different compared to both other inventions (p<0.05). For the Jaccard index, all pairwise comparisons were significant (p<0.05). The t0 samples treated with antibiotics and fungicide are more close together, after which they move away from each other when the experiment continues. From the Bray Curtis and Jaccard indexes for fungi (Figure 4a and 4b) clustering for the different treatments was less clear. The Bray Curtis dissimilarity scores were not significantly different when compared (PERMANOVA), but the

pairwise comparison of the Jaccard index between the fungicide and the antibiotic treatment was significant ($p<0.05$). For the different time points, there is variation observed. This greater variability in the mycobiome is similar to what is found before, and also seen by us in **chapter 4**.

Antibiotic treatment has been linked to the disruption of the commensal bacterial community found in the gut, which could lead to a reduction in beneficial species and an increase in antibiotic-resistant and/or pathogenic bacteria (Macfarlane, 2014). This is especially the case when broad-spectrum antibiotics are used that can affect a wide range of Gram negative and Gram positive bacteria. Antibiotic treatment has also shown to affect the fungal community found in the gut. Treatment with antibiotics can be followed by an increase in fungal species, which can lead to fungal infections (Samonis et al., 1993; Seelig, 1966). These data suggest that bacteria can have an inhibitory effect on the growth of fungi. This could be because they compete for nutrients, but also metabolites produced by bacteria could have an effect on the mycobiota (Fan et al., 2015; García et al., 2017; Nguyen, Lopes, Cordero, & Nosanchuk, 2011). These findings are confirmed in a mouse model, where normal mice were more resistant to pathogenic fungi than mice treated with antibiotics (Van der Waaij, Berghuis-de Vries, & Lekkerkerk-Van der Wees, 1971). It was also shown that mice treated with antibiotics showed significant changes in their gut microbiota, and it takes a considerable amount of time for the microbiota to return to the state before the treatment (Erb Downward, Falkowski, Mason, Muraglia, & Huffnagle, 2013; Mason et al., 2012). The effect of anti-fungal treatment on gut bacterial and fungal species is less investigated. Human data is scarce, but several studies in mice show that anti-fungal treatment could have an effect on the bacterial component of the gut microbiota. In a mouse model where mice were treated with anti-fungal treatment, the fungal diversity was decreased while bacterial diversity was increased compared to controls (Qiu et al., 2015). Another study in mice showed that mice treated with anti-fungal drugs showed changes in the bacterial composition (Wheeler et al., 2016). The introduction of five species of fungi in gnotobiotic mice induced alterations in the gut bacteria (van Tilburg Bernardes et al., 2020).

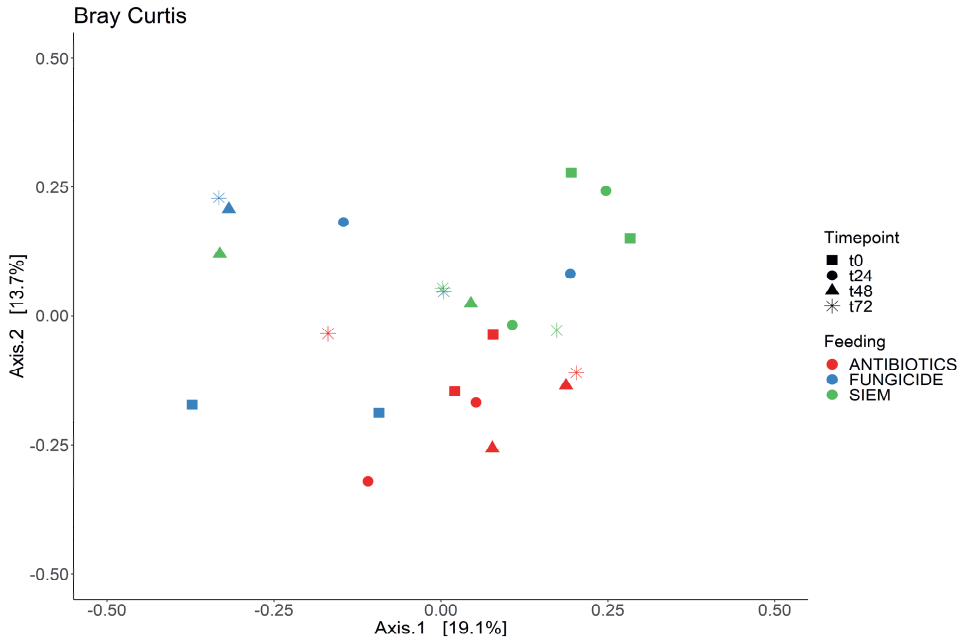**6**

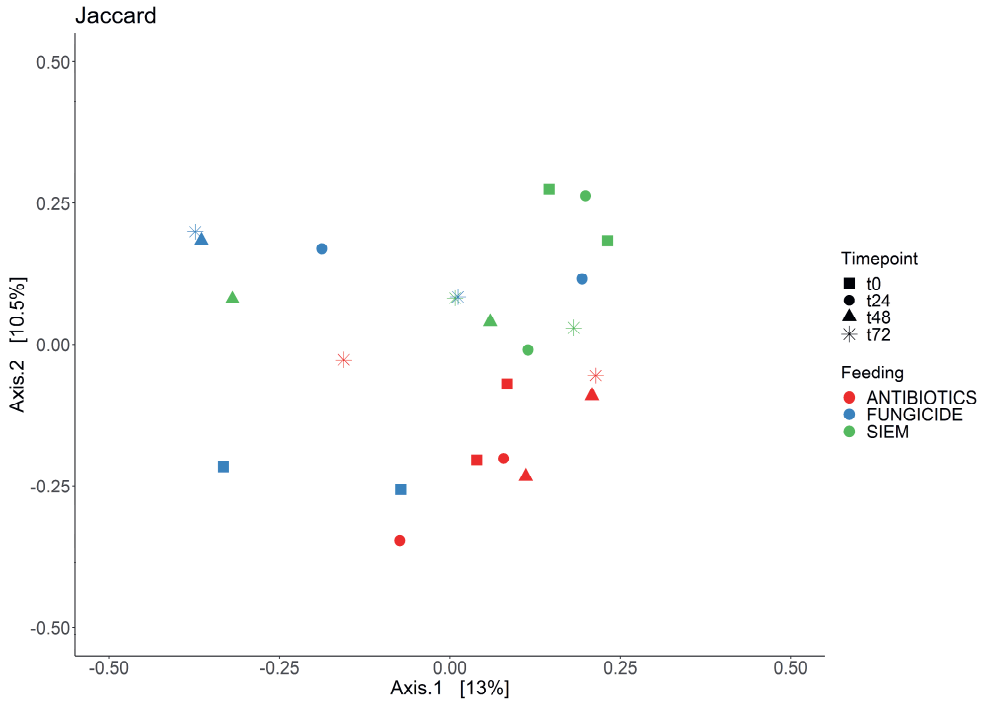**Figure 3a:** PCoA for Bray Curtis dissimilarity for bacteria

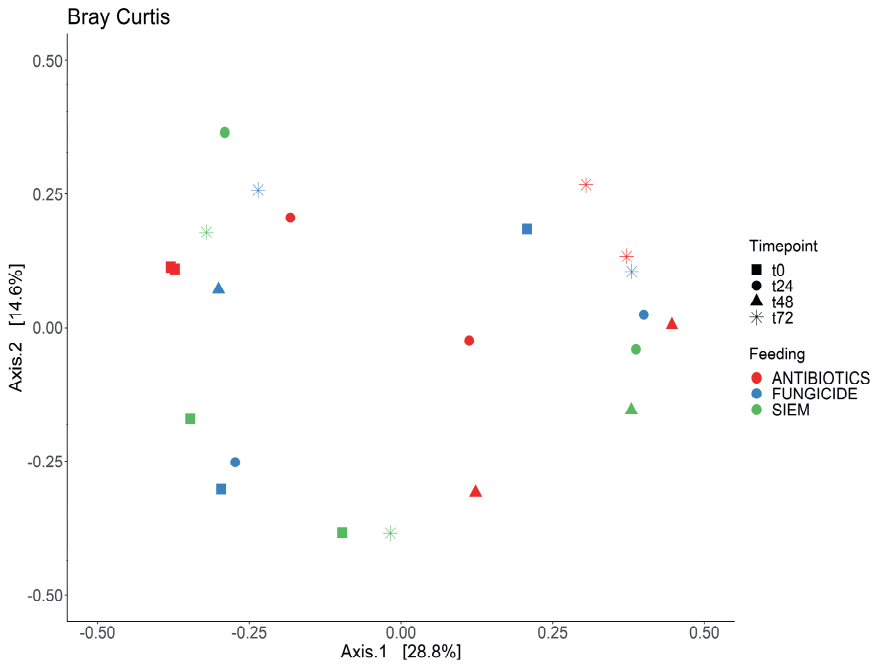**Figure 3b:** PCoA for Jaccard similarity for bacteria

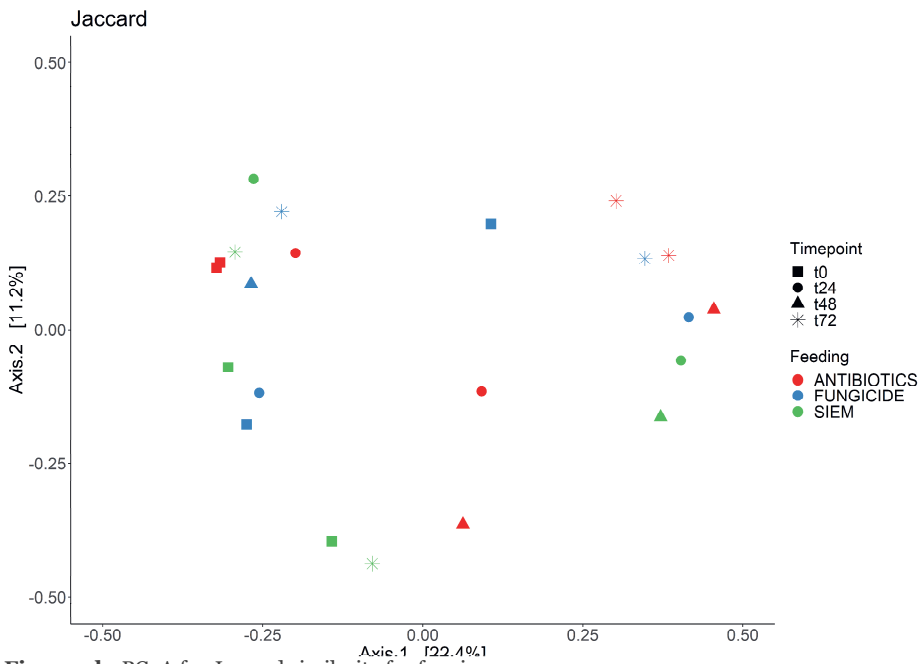**Figure 4a:** PCoA for Bray Curtis dissimilarity for fungi

Figure 4b: PCoA for Jaccard similarity for fungi

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In addition to the diversity analyses, taxonomic classification of samples was also done. In Figure 5, the bacterial relative abundance for the different samples is shown at phylum and genus level. The most abundant phyla found in all samples were *Bacteroidetes* and *Firmicutes* (Figure 5a). No big shifts could be seen for the different treatments. The twenty most abundant genera are also shown, in these genera also no significant differences between the treatments were observed (Figure 5b). Other studies on the effect of fungicides on the microbiota do describe an effect. In a study on the effect of oral exposure to the fungicide carbendazim, disturbances were observed in mice treated with this fungicide. A reduction of the relative abundance of *Bacteroidetes* and an increase of *Firmicutes*, *Actinobacteria* and *Proteobacteria* were found (Jin, Zeng, Wu, Zhang, & Fu, 2015). In another study in mice, on the effect of exposure to propamocarb on gut microbiota, changes were observed. The relative abundances of the genera *Oscillospira, Parabacteroides, Desulfovibrio, Ruminococcus, Bacteroides, Dehalobacterium, Butyricimonas, Prevotella,* and *Dorea* were different after exposure to the fungicide (Wu, Jin, Wang, Fu, & Jin, 2018). Here cycloheximide was used, which may have led to different results.

With the use of a Kruskall-Wallis test, significant differences for the genera were checked between the different treatments. From this, it was shown that *Akkermansia* was higher in the samples treated with fungicide (Figure 6). The increase of *Akkermansia* after fungicide treatment was also seen in mice (Meng et al., 2022). Here, mice were treated with tebuconazole, after which significant changes in the gut microbiota were observed, with in particular an increase in *Akkermansia*. *Akkermansia* is found in the outer mucus layer of the gut, and plays an important role in maintaining the mucus layer (Tokuhara et al., 2019). In addition, it regulates tight junction proteins, thereby regulating the intestinal barrier function (Li, Lin, Vanhoutte, Woo, & Xu, 2016). A reduction in *Akkermansia* levels could have an effect on the barrier of the intestine and could thereby induce colonic inflammation.

Taxonomic classification was also done for the mycobiota (Figure 7). Here the phyla *Ascomycota* and *Basidiomycota* were most abundant, but some variation in the ratio between the two phyla can be seen (Figure 7a). The twenty most abundant genera are shown in Figure 7b. The dominance of *Candida* can be observed in several samples, but especially in the treatment with antibiotics, this genus becomes dominant over the course of the experiment. The outgrowth of *Candida* after antibiotic treatment is also described in literature. E.g., Gutierrez et al. describe the colonization of *C. albicans* after broad-spectrum use of antibiotics in mice (Gutierrez et al., 2020). Gut bacteria can have an influence on fungal proliferation of *Candida* or other fungi by the production of metabolites, e.g. cell wall components, thereby directly influencing the fungi. The effect could also be indirect, where bacteria influence host responses, which in turn affect fungal growth (Perez, 2021). An example of how bacterial metabolites can influence fungi is the promotion of *C. albicans* hyphal growth after antibiotic treatment in mice, caused by the release of bacteria peptidoglycan in the gut lumen (Tan, Xu, Qiao, & Wang, 2021). In another study, the proliferation of *C. albicans* was inhibited in a continuous-culture bioreactor system, when bacterial metabolites derived from 50 strains from human fecal samples were added (García et al., 2017).
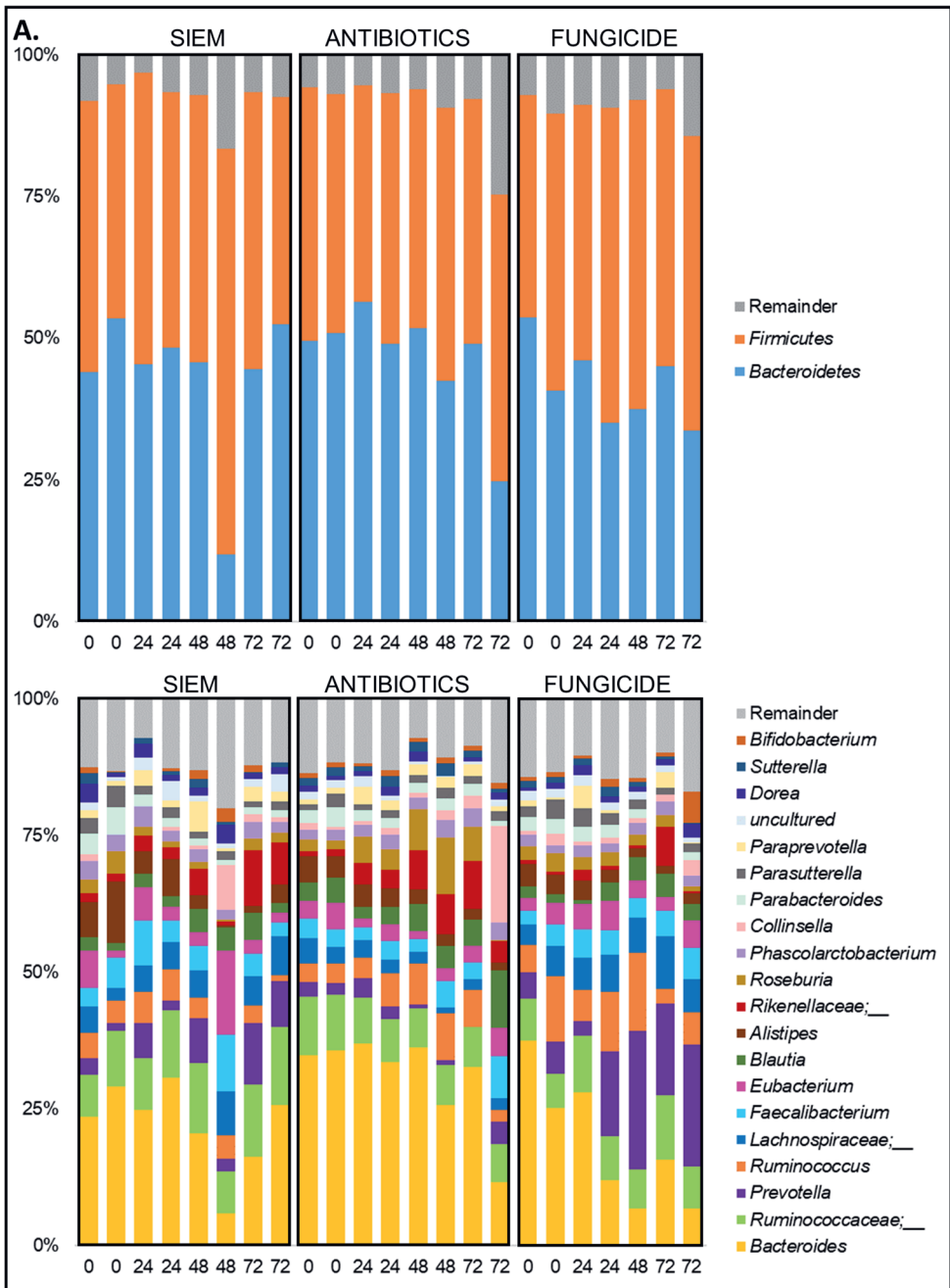
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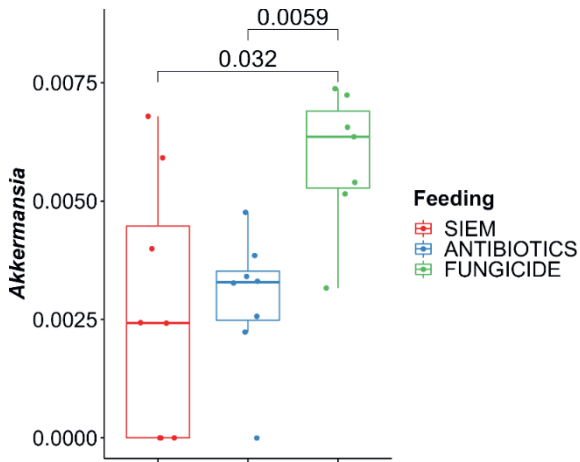**Figure 5:** Taxonomic classification of bacteria at **A.** phylum and **B.** genus level

Figure 6: Kruskal-Wallis with Dunn's post-hoc pairwise comparison for *Akkermansia.*
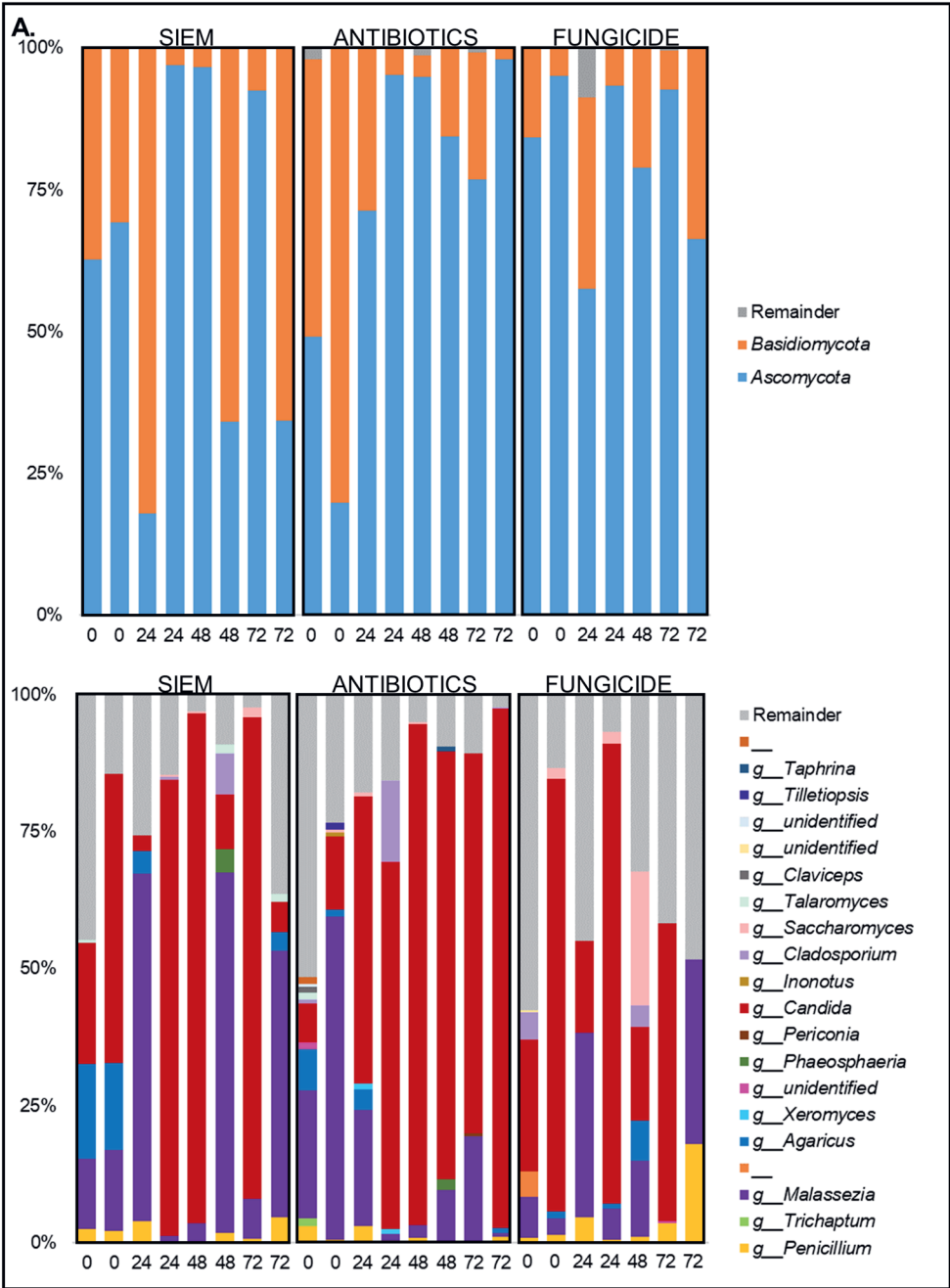
Figure 7: Taxonomic classification of fungi at **A.** phylum and **B**. genus level.

Also short-chain fatty acids (SCFAs) have shown to have an effect on fungal growth, therefore next to the gut microbiota, also the SCFAs (acetate, propionate and butyrate) were determined in the lumen and dial samples of the TIM-2 experiments. These SCFA levels were compared for the different treatments (Figure 8). As has been described in the literature, mainly acetate was found in the samples, and smaller amounts of propionate and butyrate. The disruption of the bacterial community after fungicide treatment can lead to altered SCFA levels in the gut. In line with this, levels found in the treatments SIEM and antibiotics were slightly higher when compared with fungicide treatment. Wu et al. found that after exposure with the fungicide propamocarb, the SCFA propionate and the BCFA isobutyrate were significantly increased in fecal samples of mice (Wu et al., 2018). Research on the effect of fungicides on SCFAs is scarce. The effect of antibiotic use on SCFA production was previously studied using TIM-2, where the SCFA levels were also not effected, similar to what was observed in the current study (Rehman et al., 2012). The interplay between bacteria, fungi and SCFAs should be further investigated to get more insights in these processes. There are some examples where SCFAs show to have an inhibitory effect on fungal growth. For instance, SCFAs have shown to have an inhibitory effect on *C. albicans* growth *in vitro* by influencing germ tube formation and reduction of fungal metabolic activity in biofilms (Guinan, Wang, Hazbun, Yadav, & Thangamani, 2019).

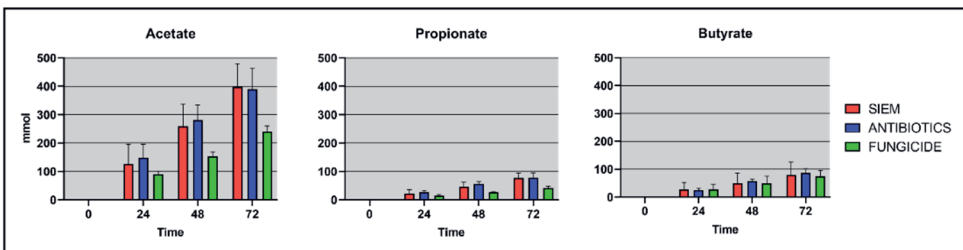**Figure 8:** Cumulative SCFA levels (mmol/L) for the different treatment groups

The results above suggest that interactions between bacteria and fungi are present in the gut. Correlations were studied between bacteria and fungi to see if interactions between specific species were present. The correlations were performed in the groups with the different treatments. The correlations between fungal and bacterial

genera are shown in Figures 9 and 10. In the samples treated with antibiotics, several correlations between genera were found (when these were present in at least 20% of samples; Figure 9). The fungal genera *Agaricus* and *Pichia* were positively correlated with *Ruminococcus* and *Erysipelotrichaceae*. One of the members of the genus *Agaricus* is *A. bisporus*, also known as the white mushroom. Members of this genus possibly have prebiotic properties (Hess, Wang, Gould, & Slavin, 2018). In pigs fed with *Agaricus*, levels of *Ruminococcaceae* were increased, suggesting that *Agaricus* can have a positive effect on the growth of these bacteria (Solano-Aguilar et al., 2018). The family *Erysipelotrichaceae* is commonly described as an inhabitant of the human gut, and in this study, it is positively correlated with the fungal genus *Pichia*. Previous research shows that this bacteria can potentially grow out after treatment with broad-spectrum antibiotics (Kaakoush, 2015). The fungal genus *Aureobasidium* was shown to be negatively correlated with *Lachnospira* in the group treated with antibiotics. *Aureobasidium* is a yeast, which can be found in diverse habitats. They show antimicrobial activity against bacteria and fungi and are therefore used in agriculture and industry (Prasongsuk, Lotrakul, Ali, Bankeeree, & Punnapayak, 2018). The abundance of *Lachnospira* is positively correlated with the consumption of fruits and fiber (De Filippis et al., 2016). They are known fiber and pectin degraders, and with the products released with this degradation, *Lachnospira* can influence the growth of other bacteria via cross-feeding (Biddle, Stewart, Blanchard, & Leschine, 2013; Salyers & Leedle, 1983).

In the group treated with fungicide, the fungal genus *Cladosporium* was positively correlated with several bacteria (Figure 10): *Erysipelothrichaceae*, *Clostridiales* vadin BB60 group*, Eubacterium coprostanoligenes* group and *Desulfovibrio*. The fungus *Malassezia* was positively correlated with the *Ruminococcus gauvreauii* group, *Eubacterium rectale* group and *Turicibacter*, while *Candida* was negatively correlated with these bacteria.

These correlations were found after treatment with antibiotics or fungicide, and not in the samples without treatment. This suggests that these treatments can cause a shift in the microbiota. The correlations that were found in this study should be

explored further to see if they have biological relevance and if they can be replicated *in vivo*. More *in vitro* experiments could help in unraveling mechanisms.

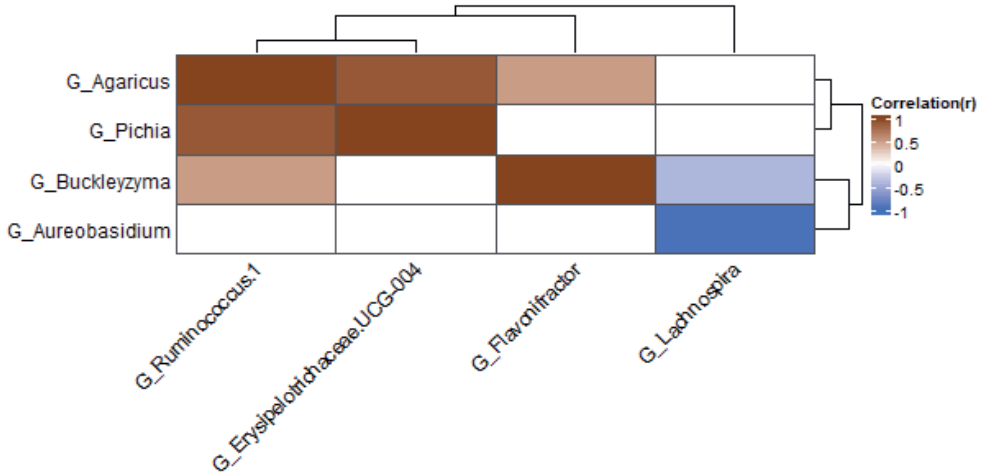**Figure 9:** Heatmap at genus level for antibiotics samples; only genera present in at least 20% of samples were included.
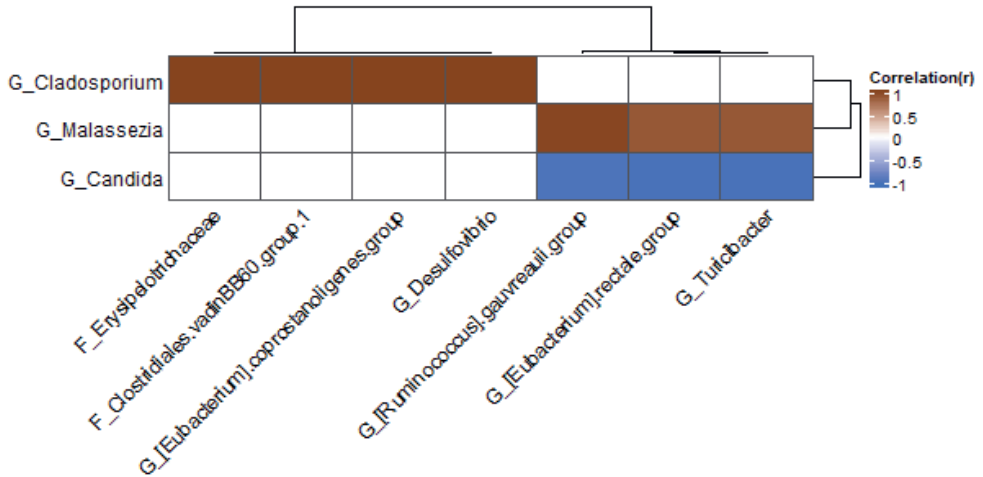
Figure 10: Heatmap at genus level for fungicide samples; only genera present in at least 20% of samples were included.

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Conclusions

In conclusion, in experiment with human fecal samples performed in TIM-2 where the microbiota was treated with antibiotics or fungicide, the bacterial communities were more diverse compared to fungi. No significant differences in alpha diversity were observed between the different treatment groups. From the beta diversity analyses, it could be observed that samples treated with antibiotics cluster together, whereas SIEM and fungicide samples were more spread. Taxonomic classification of both bacteria and fungi were performed, but no big shifts were seen after treatment. The bacteria *Akkermansia* was increased after fungicide treatment. SCFAs levels were determined, and these were slightly lower in samples treated with fungicide. Correlations between fungi and bacteria were made. These correlations suggest that cross-kingdom relations are present and that they can influence each other. Further research is needed to gain more insight in these relationships. For instance, absolute numbers of taxa should be evaluated rather than just relative abundance, to see if the total (viable) count of bacteria and/or fungi changed upon antimicrobial treatment.

Acknowledgements

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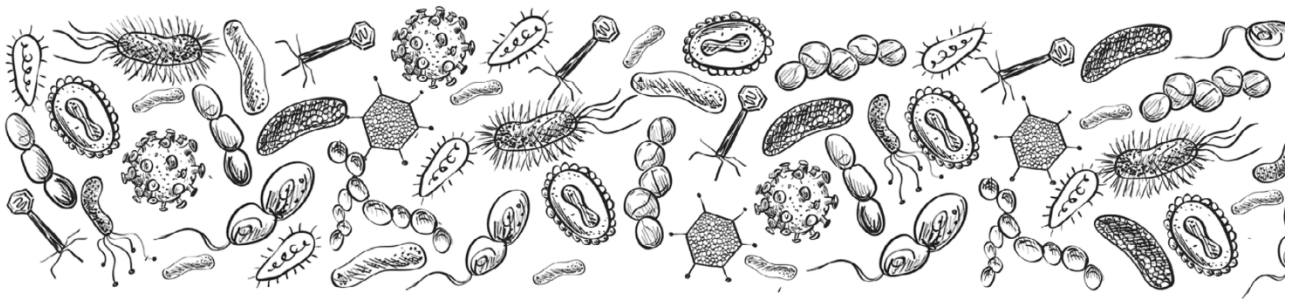
Chapter 7

General discussion
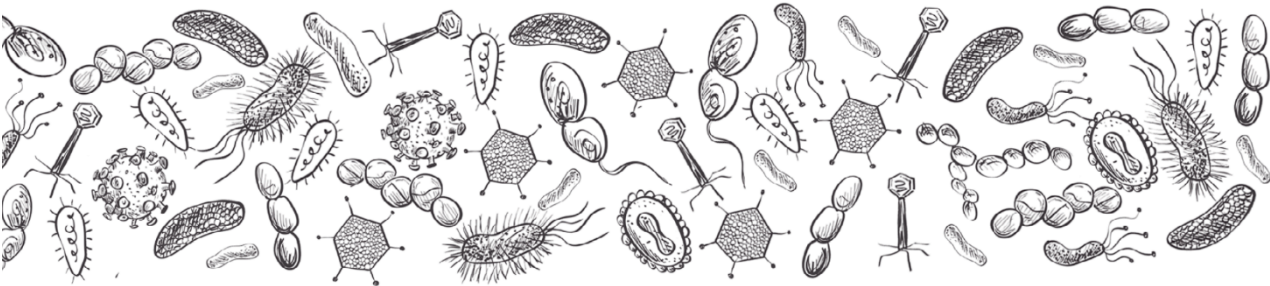
Motivation and aim of this thesis

With the emerging global problem of bacterial resistance to antibiotics, more research into alternative treatment options, such as (bacterio)phage therapy, is warranted. Next to the problem of antimicrobial resistance, the incidence of several diseases characterized by dysbiosis is rising. This dysbiosis can be corrected using food ingredients to stimulate beneficial microbes, while phages can be employed to inhibit opportunistic pathogens or microbes involved in the pathophysiology of non-communicable diseases. Increasing evidence shows a role for bacteriophages in bacterial community- homeostasis as well as host-homeostasis. Moreover, there is increasing evidence that this role in homeostasis is also true for the gut fungal community. However, compared to research on the bacterial component of the gut microbiota, studies into the role of phages and fungi/yeasts in diseases and disorders are still in their infancy. The aim of this thesis was to develop and optimize tools to study the different kingdoms in the gut microbiota and interactions between them.

Therefore, in a study on bacteriophages, the survival in the upper gastrointestinal tract was analyzed with the use of the *in vitro* model TIM-1 **(chapter 2)**. Next to this, the survival and efficacy of phage therapy in the complex environment of the colon was assessed using TIM-2 **(chapter 3)**. To mechanistically study the gut fungal community, TIM-2 was used to study the fungal and bacterial community, and it was investigated whether it was possible to modulate these fungi by diet **(chapter 4)**. The gut fungal community was further analyzed in a large cohort of individuals, and in these individuals, it was investigated whether gut-bacterial interactions could be observed **(chapter 5)**. To further investigate these fungal-bacterial cross-kingdom interactions in the human gut, the impact of antibiotics and fungicides on the bacterial and fungal communities were examined in TIM-2 **(chapter 6)**.

Main findings

The global antimicrobial resistance (AMR) crisis calls for research into all kingdoms found in the human gut microbial community. AMR potentially can lead to a high mortality and likely is one of the biggest health challenges in the coming decades

(Llor & Bjerrum, 2014). Resistant microbes can be ingested through food and once in the gastrointestinal (GI) tract, can transfer their resistance to resident members of the gut microbiota. Moreover, the gut microbiota also contains (opportunistic) pathogens and taxa that have been correlated to various diseases and disorders. Infections were (and still are) treated with antimicrobials (antibiotics and antifungals; see below), but these caused 'collateral damage', by also killing beneficial microbes. Therefore, research is needed into alternative therapeutic options, as well as research on inter-kingdom relationships to get more insight in the complex gut microbial environment and its relations with diseases and disorders.

Apart from the in-effectivity in clearing infections, associated increase in morbidity, mortality and health-care costs, the rise of AMR makes antibiotics not always the best treatment option for bacterial infections when considering the gut microbiota. Especially broad-spectrum antibiotics can do a lot of damage to the gut bacteria, both pathogenic but also commensal/beneficial (Blaser, 2014; Murray et al., 2022). The disruption of the commensal bacteria has shown to have negative effects (Blaser & Falkow, 2009; Candon et al., 2015; Del Fiol, Balcão, Barberato-Fillho, Lopes, & Bergamaschi, 2018). Broad-spectrum antibiotics also have a bigger chance to cause AMR. Narrow range antibiotics, e.g. synthesized chemically, could perhaps be an alternative (Mitcheltree et al., 2021). Another treatment option with a narrow range are lytic bacteriophages (Sulakvelidze & Kutter, 2004). These are viruses that can kill their bacterial host and were already discovered ~100 years ago, but their potential as widespread therapy is never explored with the rise of antibiotic discovery (Aminov, 2017). Only in Eastern European countries research continued, but studies described in English are scarce (Boyd & Portnoy, 1944; Wittig, Raffetto, & Bason, 1966). There are also several examples of studies that did not show a successful phage therapy (Eaton & Bayne-Jones, 1934). These studies are not always well-designed and patient numbers usually are low (Aslam et al., 2019; Gupta, Singh, Shukla, Nath, & Bhartiya, 2019). Considering this, tools are highly needed to improve phage therapy. Several points should be considered when designing phage therapy (Hyman, 2019). The phage should match the bacteria that causes the infection, so proper *in vitro* screening and identification is necessary. Also sources for newly

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isolated bacteriophages and isolation techniques should be explored. In **chapter 2**, it is described how bacteriophages can be isolated from waste water and subsequently can be identified using sequencing and electron microscopy (EM). After unsuccessful attempts of isolation of phages from fecal material, waste water was used as source. The lack of debris allowed for relatively simple isolation of bacteriophages active against *E. coli*. This was followed by identification via sequencing. Using the Nanopore technique, sequencing the whole genome is a relatively simple method for fast identification of bacteriophage genomes. The use of EM can be used for additional identification by assessing the morphology. Proper identification can help in prediction of the success of the bacteriophage therapy. When designing a therapy that should be effective for a bigger patient group, a cocktail of phages should be used that do not only act on a specific strain, but on the species / strains that are found more widespread across the patient population. In this way, a phage therapy can be designed more efficiently instead of individualized therapy and be empirically administered prior to identification of the causative agent (Abedon, Danis-Wlodarczyk, & Wozniak, 2021). Another important point for a successful therapy is dosage of the phage. The most common route for administration of phage therapy is via oral ingestion. For the phage to be effective, it must survive the harsh conditions in the GI tract. In **chapter 2 and 3** it is described how *in vitro* experiments can help to optimize dosage of phage therapy and thereby also increase the efficacy. **Chapter 2** describes the use of a validated *in vitro* model of the upper GI tract (TIM-1) to predict the survival of two bacteriophages in the human body. The use of this model can realistically mimic the conditions found in the human upper GI tract compared to simple batch *in vitro* experiments (Minekus, 2015). When compared to *in vivo* survival experiments, the use of TIM-1 is much faster and easier to gain insights in the survival of bacteriophages. This information can then be used to optimize the dosage used in further experiments, or even to design strategies, such as encapsulation, to protect the phages against upper GI tract conditions. Based on the reported survival in **chapter 2** for the two different phages, which was ~5-10%, it is evident that there is a need to test survival, and to come up with strategies to increase their efficacy. Our experiments also showed that survival

rates differ between phages, whereas one phage was more affected during passage through the stomach, the other demonstrated the greatest drop after passage through the small intestine. This information can aid in designing a suitable protection strategy, e.g. by protection against low pH or bile and/or gastric and pancreatic enzymes. In **chapter 3**, the survival of bacteriophages in the lower GI tract is described. In addition to the decrease in survival due to the low pH and digestive enzymes in the upper GI tract, bacteriophages also need to survive in the complex microbial environment of the colon. The use of the validated *in vitro* model of the colon (TIM-2) has shown to help in the prediction of the survival of bacteriophages and efficacy against their bacterial target host under colonic conditions. This model allows to directly study effects of the therapy on both the target as well as the complete gut microbiota community, to see if eradication of the target bacteria also influences the rest of the community. With the use of plating of both target bacteria and bacteriophages and with 16S rRNA gene sequencing, bacteriophages and bacteria were monitored after addition of bacteriophages. Prior to survival experiments in TIM-2, growth experiments were performed to explore the proper dosage and sample moments for the subsequent experiments. Information about growth characteristics and multiplicity of infection (MOI) is important in the design of phage therapy. In the TIM-2 experiment, it was shown that when phages were provided alone (without target bacteria), the phage titers could be influenced by the commensal microbiota, indicating that the survival of the phages was affected, likely because the microbial community used the phage particles as substrate to incorporate into their own biomass (protein and nucleic acids) (Labrie, Samson, & Moineau, 2010). Levels of the phage host, in this study *E.coli*, were decreased in the interventions with the phage shot. Multiple shots did not seem to be more effective than a single shot. The bacterial community did not show significant disturbances after addition of phages, also not when the target bacteria was reduced several orders of magnitude. The information from these experiments is imperative to optimize the dosage of bacteriophage therapy. Also, the method shows advantage when compared to antibiotic therapy, where the

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commensal microbiota is much more disrupted, usually for several months (Ramirez et al., 2020).

The use of antibiotics in addition has other negative consequences, such as colonization of pathogenic bacteria (Huang, Feng, Huo, & Liu, 2022). Moreover, the widespread use of antibiotics has also led to an increase of fungal infections, as the disruption of the commensal microbiota leads to fungal overgrowth (Esaiassen, Fjalstad, Juvet, van den Anker, & Klingenberg, 2017). The focus of microbiota research has so far been on the bacterial component, thereby neglecting the fungi. This fungal community or mycobiota has gained more attention in the past decade, emphasizing the scientific interest and importance of fungi and yeast in human health. Nevertheless, to date only a few studies using big cohorts gathered some information on fungal communities in the human gut (Nash et al., 2017; Shuai et al., 2022). In **chapter 5**, the microbiota of healthy individuals was analyzed using both 16S rRNA and internal transcribed spacer (ITS) amplicon-based sequencing, for the bacterial and fungal component of the microbiota, respectively. This research indicated the main fungi found in the gut of these subjects to belong to the genera *Saccharomyces, Candida, Dipodascus, Aureobasidium, Penicillium, Hanseniaspora, Agaricus, Debaryomyces, Aspergillus* and *Pichia*. This was in line with several previous studies (Nash et al., 2017; Shuai et al., 2022). Furthermore, large individual differences were detected and when compared with the bacterial community, diversity was much lower. The design of the experiment in **chapter 5** did not allow to discriminate between transient species ingested via the diet and colonizing species. It has been suggested that the fungal community could be driven by diet. To further investigate this, the *in vitro* model of the colon (TIM-2) proved to be a helpful tool. TIM-2 was used to investigate the direct effect of diet on the mycobiota in **chapter 4**. In the experiments, the fungal community was followed for 72 hours to assess its stability on the normal medium used in TIM-2 experiments. Large differences between samples were found and the diversity was far lower for fungi as compared to bacteria. Taxonomic classification showed that similar fungi were found as described in other studies and the human study presented in **chapter 5**. The main genera detected were *Agaricus*, *Aspergillus, Candida, Penicillum,*

Malassezia, Saccharomyces, Aureobasidium, Mycosphaerella, Mucor and *Clavispora*. Subsequently, different interventions were carried out to see if diet could drive changes in the gut fungal community. Some changes could be observed after interventions, especially when glucose was used as sole carbon source. It remains to be explored whether this effect is due to a direct action of glucose on the mycobiota, or whether the glucose was used by the gut bacteria and metabolites of these bacteria affected the mycobiota composition. The interactions between fungi and bacteria were further researched in **chapter 6**, where TIM-2 was used again. Here, the bacterial and fungal community were disrupted with the use of either antibiotic or fungicide treatment and after these interventions, both the fungal and bacterial community were analyzed. No major changes were observed after the treatments, but the samples treated with antibiotics clustered together. Also, SCFA levels were analyzed, and these were slightly lowered in samples treated with fungicide. To see if the bacterial and fungal community have an influence on each other, correlations between the kingdoms were investigated. These correlations suggested that inter-kingdom relations are present and that the two kingdoms can influence each other. Further research is needed to gain more insight in these relationships.

Methodological considerations

When interpreting the results of this thesis, several considerations should be taken into account. In **chapter 2** the Nanopore sequencing technique was used for the identification of a bacteriophage isolated from wastewater. Nanopore sequencing, although getting better in a vast pace, still has some limitations. The method still has a relatively large error rate (Wang, Zhao, Bollas, Wang, & Au, 2021). This makes it difficult to carefully study the interaction of phage receptors with the bacterial target (receptor), due to potential errors in protein predictions from the erroneous DNA sequence, although Nanopore sequencing currently is already used for surveillance of Sars-CoV2 and specific SNPs can be detected (Meredith et al., 2020; Munro et al., 2021). This depends on the coverage if the sequenced genome. This can thus be overcome by increasing the sequencing coverage, which will also increase the accuracy of the bioinformatic base-calling. However, this refutes the benefits of the technology with respect to speed and costs (Jain, Olsen, Paten, & Akeson, 2016).

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Further improvement in the chemistry and nanopores on the flow-cells would be helpful in making this an even better sequencing tool (Rang, Kloosterman, & de Ridder, 2018). Also, nanopore sequencing allows sequencing of larger fragments of DNA, such as the complete 16S rRNA gene, which for determining the bacterial component of the microbiota gives more phylogenetic resolution than sequencing the V3-V4 region as was done in this thesis (Matsuo et al., 2021). Another trend is to do metagenomics (sequencing of the complete genome of the collective community) (Forster et al., 2019). Such sequencing includes more information than 16S rRNA compositional data, and moreover includes information on the presence of fungi and (pro)phages in the sample (Vemuri, Shankar, Chieppa, Eri, & Kavanagh, 2020). However, to get a complete picture of the community deep sequencing is required, and the costs for that is in most studies still prohibitive (Greninger, 2018). Metatranscriptomics (sequencing of the [preferably] mRNA) would be even more advantageous, as it provides information on genes that are activated under certain conditions, e.g. fungicide treatment, or dietary interventions, which may help decipher mechanisms or inter-kingdom cross-talk (Fulci, 2022; Stewart et al., 2019). Also metatranscriptomics has some limitations. An important step in preparation of the samples for sequencing, is the removal of rRNA, which makes up to 90% of RNA, to enrich the mRNA (Shakya, Lo, & Chain, 2019). The absence of polyA signals in bacterial mRNA complicates the purification, and standard methods for this are lacking, which can influence the results (Jiang, Xiong, Danska, & Parkinson, 2016). Another challenge is the lack of well-characterized reference genomes, which results in the need of de novo assembly. Currently, the available de novo assemblers are still in its infancy. The tools that are developed for metatranscriptomics datasets have not been widely tested, so they need to be further developed (Shakya et al., 2019).

In **chapter 2** and **3** of this thesis, the double-agar assay was used to assess survival of the phage-particles in TIM-1 and TIM-2. This is based on the ability of the phage to infect the host (Kropinski, Mazzocco, Waddell, Lingohr, & Johnson, 2009). It cannot provide information on those phage particles that are either degraded completely (and incorporated into bacterial biomass), or where e.g. the base-plate of the phage-particles has been degraded, making them ineffective in infecting the host

(Ács, Gambino, & Brøndsted, 2020). Quantitative analysis of the phage genome (which requires information on the genome to design e.g. specific primers for PCR) would allow to decipher better the fate of these phage particles in the colon (Gutiérrez et al., 2011). A second limitation of the double-agar assays is the inaccuracy in determining the phage titer. This may have caused some variations in the number of phages that were 'fed' to TIM-1 and could be the reason for the observed spread in the data. Alternatively, due to the inaccuracy of the method (estimated to be 0.5 log) there may just have been large differences in the outcome of the determination of PFUs from TIM-1 samples. Also, this would suggest the use of a molecular method to determine the titer of phage particles, although care should be taken then that inactivated phages in TIM-1, which can no longer form a plaque, are likely still detected with this molecular tool based on the whole genome.

This thesis has focused on the use of bacteriophages as therapy. At first, we set out to isolate phages against *E.coli* K12 (as a model microorganism) from a fecal sample. However, we did not find plaques against the strain used when using a fecal sample. So, we next turned to a sample from a waste-water treatment plant and were able to find (a) phage(s) against this strain. Sequencing all phages in a sample might be another manner to find isolates that could be specific against the target bacteria in question (Fitzgerald et al., 2021). Similarly, sequencing the virome in TIM-2 samples will provide information on the presence of all viruses, including bacteriophages in these samples. This provides not only information on phages of potential interest, but information on other phages too. Given the high throughput of sequencing methods, this can speed up development of phage (cocktails) for phage therapy.

In **chapters 2, 3, 4** and **6**, TIM-1 and TIM-2 are used in experiments to mimic the conditions found in the human GI tract. Although the TIM-1 and TIM-2 systems are validated models, they do have limitations (Payne, Zihler, Chassard, & Lacroix, 2012). There is no direct interaction with cells of the host (e.g. epithelial cells, or immune cells). And, although both models have a dialysis system to absorb digestion product and microbial metabolites, they would not allow the phage particles to transverse the epithelial barrier and also be effective against systemic infection. This

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could perhaps be mimicked with cell culture experiments (such as Caco-2 confluent monolayers), but currently this is difficult with TIM-samples, as they contain e.g. bile salts and digestive enzyme, which already affect the Caco-2 monolayer (Deat et al., 2009; Meunier, Bourrie, Berger, & Fabre, 1995).

TIM-2 was previously validated for the research on the bacterial component of the human gut microbiota. In this thesis, also the fungal component was investigated. In **chapter 4** and **6**, the medium that was fed to the microbiota was developed over 3 decades ago for the bacterial component of the gut microbiota (Gibson, Cummings, & Macfarlane, 1988). It may not have been optimal for the mycobiota. Given the observed differences between replicates, it seems that optimization of this medium requires attention. On the other hand, if one wants to study the inter-kingdom interaction the medium also cannot be based solely on growth of fungal species, and probably needs to be a compromise for both kingdoms. Despite this, interesting correlations were found between the presence of fungal and bacterial taxa in TIM-2 that warrant further investigation.

Another aspect of ITS sequencing is that it does not provide any information on the sexual state of the fungi in TIM-2. Both growth as mycelium as well as yeast-like growth can occur, and the molecular method cannot distinguish between these. Metabolically the two states may have a profound effect on the production of fungal metabolites, and it would be good to design methods to determine the sexual state (Almeida et al., 2021; Araújo et al., 2019; Castilho et al., 2012). Plating samples also does not provide sufficient information, as yeast-like cells may grow as mycelium on agar plates and *vice versa*.

Furthermore, in the current setting the dietary interventions applied in TIM-2 did not lead to large changes in the fungal community. On the one hand, this perhaps indicates that this community is rather stable and not greatly influenced by the diet, on the other hand it may indicate that the interventions chosen did not really affect the community. Only glucose led to some changes. It is know that high intake of glucose can lead to fungal overgrowth (Ng, Desa, Sandai, Chong, & Than, 2016; Vargas, Patrick, Ayers, & Hughes, 1993). Moreover, the analysis in this thesis focused

on relative abundance of the different communities. It might well be that in absolute numbers the fungal population was affected. This needs to be investigated further. This can be achieved with the use of the concept of Quantitative Microbiome Profiling (QMP), which is a way to quantify microbial abundances from sequencing data (Galazzo et al., 2020). E.g. with the use of flow-cytometry to determine total microbial load in fecal samples and subsequently normalize 16S rRNA data or in this case, ITS data, taking into account total cell counts (Vandeputte et al., 2017). Another strategy is to use quantitative PCR (qPCR), which is more simple and has lower costs (Jian, Luukkonen, Yki-Järvinen, Salonen, & Korpela, 2020). Lastly, relative few data is available that studies the effect of diet on fungal populations and most of that is in rodent studies (Heisel et al., 2017; Hoffmann et al., 2013; Mims et al., 2021). Therefore, experiments on the effect of different dietary components on the mycobiota warrant further investigations.

In **chapter 5**, *in vivo* samples were analyzed that were not longitudinal, and therefore it is hard to discriminate between fungal taxa that normally colonize the human GI tract and those that were transient passengers, e.g. from food (e.g. *Saccharomyces*) or air (Auchtung et al., 2018; Graves & Hesseltine, 1966). For example, for some of the taxa found in the *in vivo* samples it is known that they cannot grow under the conditions prevailing in the human GI tract and these should therefore be considered transient passengers (Suhr & Hallen-Adams, 2015). For future studies, it would be good to have information on dietary intake, such that assessments can be made about the presence of fungi of food origin. It would be even better to set-up longitudinal studies to see which taxa are stably present and which should be considered transient passengers, here the sampling of air and other environmental samples (e.g., pets) that can contribute to the fungal (but also bacterial) community, could give additional information about the source of gut inhabitants. Due to the low(er) diversity of the mycobiota any 'disturbance from outside' in terms of transient passengers then quickly influence the scientific results and even outcome. Thus, the results of the relative abundance of the mycobiota observed in our clinical samples (**chapter 5**) should be interpreted with care. First of all, the samples were from multiple cohorts that were not selected specifically to

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study the mycobiota. Second, only a single sample was provided by each individual. And, perhaps most importantly as discussed above, no dietary information was available. Moreover, because the amount of fungal cells in the colon is much lower than that of bacteria, the current sequencing protocols also have challenges with respect to obtaining enough sequence reads to get meaningful, interpretable results (Hamad et al., 2017; Richard & Sokol, 2019). On top of that, in this thesis a single DNA isolation method was used for both bacteria and fungi. The structure of the fungal cell-wall is more difficult to lyse (Huseyin, Rubio, O'Sullivan, Cotter, & Scanlan, 2017). Although our protocol made use of a bead-beating step to maximize fungal lysis, using two separate protocols which are specifically optimized for either bacterial or fungal cell lysis would be most optimal.

In the experiments where we tried to disrupt the balance of the gut microbiota by addition of antibiotics or fungicides, relative minor shifts were observed (**chapter 6**). The dose of antimicrobials was based on clinical doses used and pharmacokinetics of the antimicrobials. Despite this, it is difficult to properly mimic a course of antimicrobials, for instance because the TIM-2 system has a dialysis system that removes antimicrobials provided, such that we had to supplement it continuously. In previous work, we have shown that antibiotics led to a shift in microbiota composition and activity (Rehman et al., 2012). In these previous experiments, clindamycin was used, whereas here we used a mixture of ampicillin, oxytetracycline and kanamycin. The idea of the experiments with antimicrobials would be to develop a system with a dysbiotic microbiota (either bacterial or fungal), that could subsequently be treated, e.g. through a dietary approach, bacteriophages or otherwise. Thus, future experiments with antimicrobials would benefit from investigating a dose-response, and/or to check disruption of the microbiota, prior to follow-up interventions.

In these experiments we also looked at inter-kingdom correlations between the bacteria and fungi. Since a standardized microbiota was used, we expected to find some correlations. Due to high inter-individual microbiota composition, especially for the mycobiota, this is not expected to happen quickly in clinical samples. There

were indeed some correlations observed both in the clinical dataset (when filtered for taxa that were present in at least 10% of the samples) and in the TIM-2 experiments, but the clinical relevance of these observations remains to be determined.

Future perspectives

The goal of the experiments performed in this thesis was to develop tools to study inter-kingdom relationship in the human gut. The widespread use of antibiotics as treatment for bacterial infections has led to several problems, such as the global rise of AMR and the disruption of commensal bacteria in the human gut, leading to a dysbiotic state of the gut microbiota. The rise of these problems drives the demand for alternative therapeutic options for the treatment of bacterial and fungal infections. One promising alternative for bacterial dysbiosis and infections is bacteriophage therapy. Phages were discovered a century ago, and although research has been done in their ability to be used as therapy, more information is needed to make it a universal treatment option. Prior to *in vivo* clinical studies of bacteriophage therapy, proper *in vitro* experiments, some of which are described in this thesis, can help in the design of such therapies. With the rise of high-throughout sequencing techniques, easier access to genomic information is available for researchers. The MinION sequencer with the nanopore technique allows for fast and relatively cheap identification of bacteriophages. This kind of information can help in the design for phage therapy. Another important aspect in designing phage therapy is to use the proper efficacious dosage. Realistic survival studies are often lacking in research on bacteriophage therapy. The environment in the human GI tract has an influence on bacteriophages when taken orally. The use of sophisticated *in vitro* models such as TIM-1 and TIM-2 can help in predicting the survival of phages after passage through the GI tract. Results from these experiments can help in setting the right dosage or in designing protective measures, such as encapsulation. The low success rate of phage therapy in clinical studies could be enhanced with good *in vitro* testing leading to better identification of phages and better prediction of the survival rate during passage through the GI tract. In this thesis several tools were used that could be considered for this.

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Research on the human gut microbiota has shown that not only bacteria are involved in human health, but also fungi can play a role. Most studies on the gut microbiota focus on bacteria, so more information on how fungi and fungal-bacterial interactions contribute to health and disease is needed. The rise of culture independent techniques has led to a lot of new information about the human gut mycobiota composition, but information on functionality is scarce. The use of *in vitro* gut models such as TIM-2 can help to mechanistically study the mycobiota. In this thesis, TIM-2 was used for the first time to study fungi and fungal-bacterial interactions *in vitro*. From these experiments, it was shown that there are some additional challenges when studying fungi in TIM-2 compared to bacteria. Fungi have a lower abundance and diversity and a higher variability. Although the model makes used of a pooled microbiota, to ensure that all experiments have the same starting conditions, differences between replicates were observed. This indicates that fungi are less stable in the model compared to bacteria, and additional research on e.g. the medium used could help in increasing the stability. A higher stability will also help in interpretation of the results found after interventions performed, such as different diets. With a more stable mycobiota, TIM-2 can contribute to research on the gut fungal community and how this is influenced by diet, but also how it is affected in different diseases. An advantage of TIM-2 compared to *in vivo* studies is the possibility of studying the direct effect of interventions on the microbiota (both bacterial and fungi), and also interactions between bacteria and fungi can be taken into account. To research these fungal-bacterial interactions, the development of a microbiota in a dysbiotic state should be optimized. In this thesis, this was attempted by giving a cocktail of antibiotics or a fungicide treatment, but this did not lead to major disruptions. Improvement of the administration of antimicrobials can help to reach a dysbiotic microbiota, e.g. by changing the dose or kind of antimicrobial used. Alternatively, donors with bacterial or fungal dysbiosis can be used for the fecal pool for inoculation of the model. Information on fungi, bacteria and interactions between the two kingdoms in this dysbiotic state can give more insights on how the microbiota can influence human health.

In the past decades, next generation sequencing techniques have become available for research. This has led to a lot of microbiome sequencing studies. The majority of these focus on the bacterial component of the microbiota, thereby neglecting the other kingdoms found in the human gut. By ignoring the viral and fungal part of the microbiome in research, important information is lacking. The importance of inter-kingdom interactions in the human gut should be considered when designing microbiome sequencing studies.

In conclusion, this thesis has provided some important tools that can aid in research of interkingdom relations in the human gut, although some optimization might still be required. With the use of TIM-1 and TIM-2, the survival of bacteriophages in the GI tract can be investigated. TIM-2 can also be a helpful tool for the study of the mycobiota and bacterial-fungal interactions. The inclusion of ITS sequencing in addition of 16S rRNA sequencing in microbiome studies gives information on the gut fungal community next to the bacterial community.

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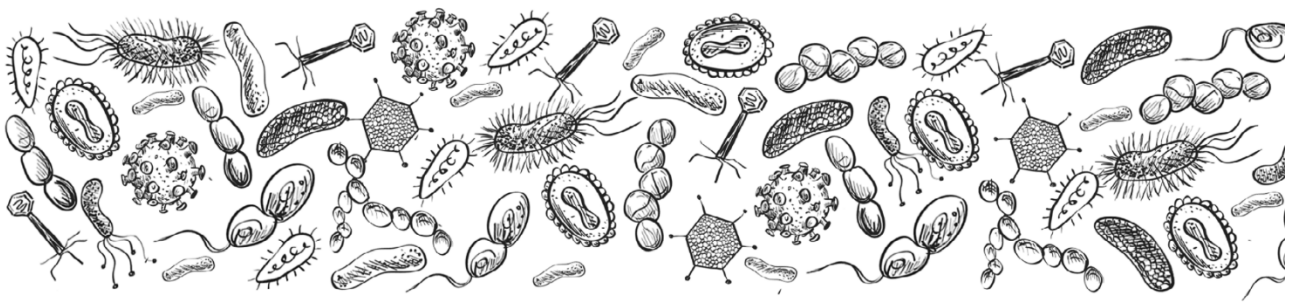
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Summary
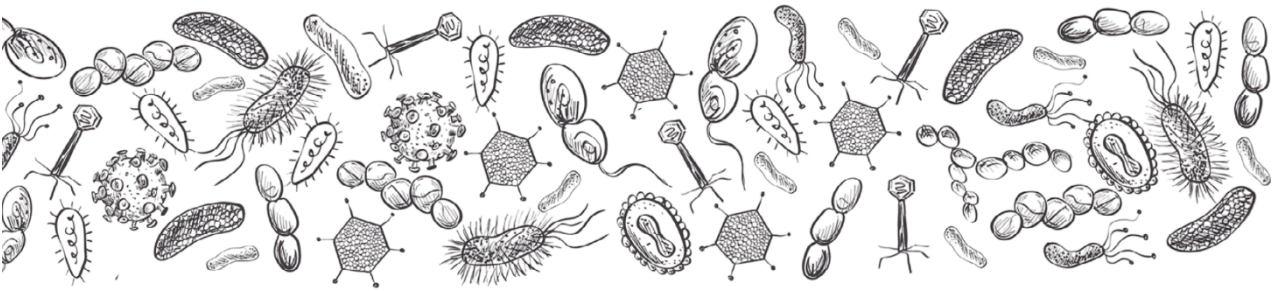
The human body harbors a large amount of microbial cells, called the microbiota. The majority of the microbiota is found in the human gastrointestinal tract, with the highest numbers in the large intestine. The gut microbiota plays an important role in host health, e.g. in absorption of nutrients and protection against pathogenic bacteria. The gut microbiota consists of microbes from different kingdoms: bacteria, viruses and fungi. Research has mainly focused on the bacterial part of the microbiota, thereby neglecting the viral and fungal component. Studies on the gut viruses and fungi show that they can be important in human health and therefore tools to research them are needed. *In vitro* gut fermentation models allow for the research on both microbial composition and functionality and could be helpful in the research on viruses and fungi in the human gut.

Bacteriophages make up the major part of the viruses found in the human gut. Bacteriophages are viruses that can kill bacteria and lytic bacteriophages can be used as therapy to combat bacterial infections. They are very specific, i.e. they have a small host-range, and this has as an advantage that the commensal bacterial community is not disrupted, as is the case with antibiotic therapy. With the rise of antimicrobial resistance, alternatives to antibiotic therapies should be explored. Bacteriophage therapy is a promising treatment strategy for bacterial infections, but more research is needed to design effective therapies. In **chapter 2** the importance of identification of bacteriophages before using them in therapy is described. This identification was done using Nanopore sequencing in combination with electron microscopy. It was also shown how bacteriophages can be isolated from waste water. After the identification, the survival of two bacteriophages (a phage against *E. coli* and phage J1 against *Lacticaseibacillus* [formerly *Lactobacillus*] *casei*) in the upper GI tract (stomach and small intestine) was investigated with the use of the *in vitro* model TIM-1. These experiments showed that bacteriophages are affected by passage through the upper GI tract, and also that the survival rate can differ between different phages. When phages are ingested in a fed-state, roughly 5-10% reaches the lower GI tract. The use of TIM-1 in the design of phage therapy could help with increasing the efficacy of the therapy by optimizing the dosage or exploring the use

of survival strategies, such as encapsulation, to enhance survival. For an effective bacteriophage therapy, after passage through the upper GI tract, bacteriophages need to survive and be effective in the complex microbial environment of the colon. In **chapter 3**, the survival and efficacy of bacteriophages in the colon and the effect on the bacterial community in the colon was studied with the use of the *in vitro* model TIM-2. An antibiotic-resistant *E. coli* was used in combination with a corresponding bacteriophage. The survival of the bacteriophage was tested in TIM-2 in different conditions: addition of only the phage, *E. coli* and the phage, and *E. coli* and multiple shots of the phage. The stability of the bacterial community was followed using 16S rRNA sequencing. It was shown that phage titers could be decreased by activity from the commensal microbiota. Levels of the phage host (here *E.coli*) were decreased in the interventions with the phage shot. Multiple shots did not seem to be more effective than a single shot. At the same time, the bacterial community was not disturbed and remained stable throughout the experiment, which is in stark contrast to treatment with antibiotics. This high specificity shows that phage therapy could be a promising alternative to antibiotic treatment in treating GI infections, because the microbial community is less affected.

In addition to viruses, also fungi and yeasts are present in the human gut. Comprehensive information on gut fungi is lacking, leading to missing insights on the mycobiome and its interaction with the gut bacterial community and the human host. In **chapter 4**, the use of TIM-2 as a tool for the study of the gut fungal community was investigated. In experiments with standard feeding (SIEM), bacteria and fungi were analyzed using 16S rRNA and ITS sequencing. The fungal community showed low diversity and a greater variability when compared to bacteria. Taxonomic classification showed that at the phylum-level *Ascomycota* and *Basidiomycota* dominated, while *Agaricus*, *Aspergillus, Candida, Penicillum, Malassezia, Saccharomyces, Aureobasidium, Mycosphaerella, Mucor* and *Clavispora* were the most abundant genera. In addition, dietary interventions (high carbohydrate, low carbohydrate and glucose as carbohydrate source) were carried out to see if this modulated the gut fungal community and it was shown that the

change of diet could influence the diversity. Overall, the experiments showed that the mycobiota could be modelled in TIM-2, however the low diversity and high variability make studying fungal, as compared to bacterial communities, much more challenging. Future research should focus on optimization of the stability of the fungal community to increase the strength of the results. The gut fungal community was further investigated in **chapter 5**, where the mycobiota of healthy individuals was analyzed. For this, on fecal samples of 163 individuals that were available from two separate studies, ITS2 and 16S rRNA sequencing was performed to analyze the fungal and bacterial microbiome, respectively, as well as their cross-kingdom interactions. The results showed a much lower fungal as compared to bacterial diversity. *Ascomycota* and *Basidiomycota* were dominant fungal phyla across all samples, but levels varied enormously between individuals. The ten most abundant fungal genera were *Saccharomyces, Candida, Dipodascus, Aureobasidium, Penicillium, Hanseniaspora, Agaricus, Debaryomyces, Aspergillus* and *Pichia,* and here also extensive inter-individual variation was observed. Correlations were made between bacteria and fungi, and only positive correlations were observed. To further investigate the importance of the observed correlations found, more research is needed to discriminate between gut colonizers and transient species. The interactions between the fungal and bacterial community in the gut were further analyzed in **chapter 6** with the use of TIM-2, where interventions with antibiotics and fungicide were carried out to investigate whether these two microbial communities were disrupted. The communities were analyzed with the use of next generation sequencing of the ITS2 region and the 16S rRNA gene. Also, production of SCFAs was followed during the interventions. Correlations between fungi and bacteria were calculated to investigate possible cross-kingdom interactions. It was shown that acute treatment with antibiotics or fungicides did not greatly alter the bacterial or fungal communities. SCFAs levels were lowered in samples treated with antifungals. Spearman correlations suggested that cross-kingdom interactions are present in the human gut, and that fungi and bacteria can influence each other. Further research is required to gain more insights in these interactions, and their molecular nature, and to determine the clinical relevance.
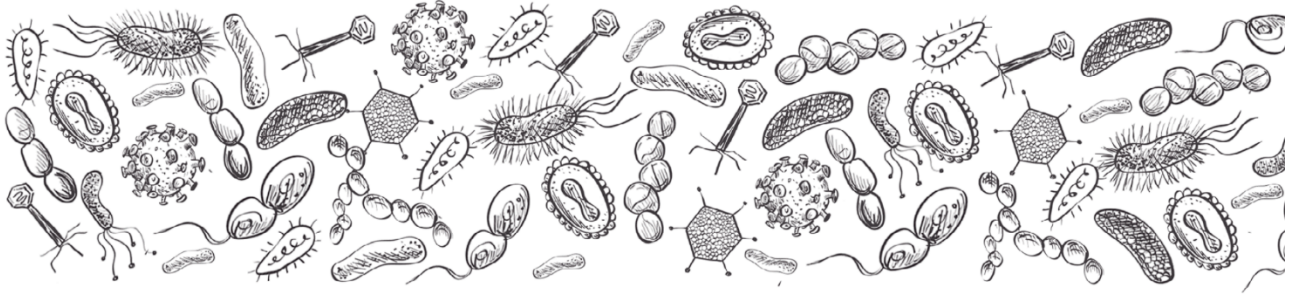
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Samenvatting
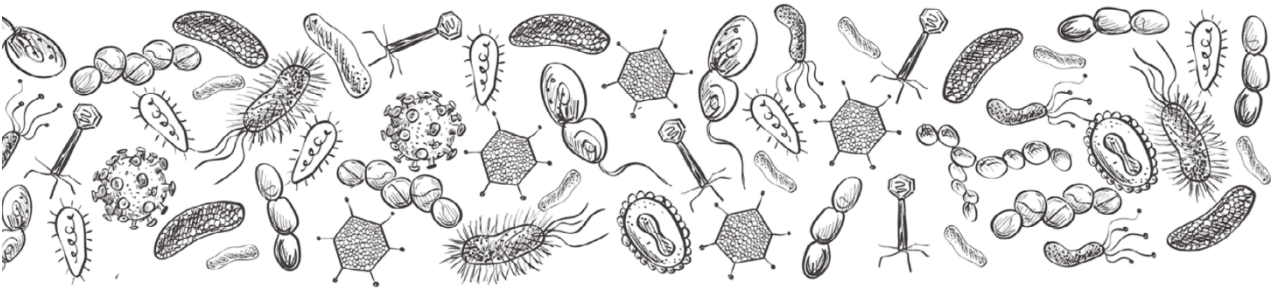
Het menselijk lichaam herbergt een grote hoeveelheid microbiële cellen, de microbiota genaamd. Het grootste deel van de microbiota wordt is aanwezig het maagdarmkanaal, met de hoogste aantallen in de dikke darm. De darm-microbiota speelt een belangrijke rol in de gezondheid van de humane gastheer, b.v. door de opname van voedingsstoffen en bescherming tegen pathogene bacteriën. De darm-microbiota bevat microben uit verschillende koninkrijken: bacteriën, virussen en schimmels. Tot voor kort heeft het onderzoek heeft zich voornamelijk gericht op het bacteriële deel van de microbiota, waarbij de virussen en schimmels werden verwaarloosd. Recente studies naar virussen en schimmels in de darm tonenechter aan dat ze ook belangrijk kunnen zijn voor de menselijke gezondheid, en daarom zijn er 'tools' nodig om dit te onderzoeken. In vitro fermentatiemodellen van de darm maken onderzoek naar zowel de microbiële samenstelling, als de functionaliteit mogelijk en kunnen nuttig zijn bij het onderzoek naar virussen en schimmels in de menselijke darm.

Het grootste deel van de virussen die in de menselijke darm worden aangetroffen zijn bacteriofagen. Bacteriofagen zijn virussen die bacteriën kunnen doden; lytische bacteriofagen kunnen worden gebruikt als therapie om bacteriële infecties te bestrijden. Bacteriofagen hebben een zeer specifieke gastheer, en dit heeft als voordeel dat de commensale bacteriën in de darm niet worden verstoord, zoals bij antibioticatherapie wel het geval is. Met de opkomst van antimicrobiële resistentie moeten alternatieven voor antibiotica worden onderzocht. Bacteriofaag therapie is een veelbelovende behandelingsstrategie voor bacteriële infecties, maar er is meer onderzoek nodig om effectieve therapieën te ontwerpen. In hoofdstuk 2 wordt het belang beschreven van identificatie van bacteriofagen voordat ze in therapie worden gebruikt. Deze identificatie werd gedaan met behulp van Nanopore-sequencing in combinatie met elektron microscopie. Ook werd getoond hoe bacteriofagen uit afvalwater kunnen worden geïsoleerd. Na deze identificatie werd de 'overleving' van twee bacteriofagen (een faag tegen E. coli en faag J1 tegen Lacticaseibacillus [voorheen Lactobacillus] casei) in het bovenste deel van het maagdarmkanaal (maag en dunne darm) onderzocht met behulp van het in vitro model TIM- 1. Deze

experimenten toonden aan dat bacteriofagen worden beïnvloed door passage door het bovenste deel van het maagdarmkanaal, en ook dat de overlevingskans kan verschillen tussen verschillende fagen. Wanneer fagen in een gevoede toestand worden ingenomen, bereikt ongeveer 5-10% de dikke darm. Het gebruik van TIM-1 bij het ontwerp van faagtherapie zou kunnen helpen bij het vergroten van de effectiviteit van de therapie; door de dosering te optimaliseren of het gebruik van overlevingsstrategieën, zoals inkapseling, te onderzoeken om de overleving te verbeteren. Voor een effectieve bacteriofaagtherapie moeten bacteriofagen na passage door het bovenste deel van het maagdarmkanaal, ook in de complexe microbiële omgeving van de dikke darm overleven en effectief zijn. In hoofdstuk 3 werd de overleving en effectiviteit van bacteriofagen in de dikke darm en het effect op de bacteriële gemeenschap in de dikke darm bestudeerd met behulp van het in vitro model TIM-2. Een antibiotica-resistente E. coli werd gebruikt in combinatie met een bijbehorende bacteriofaag. De overleving van de bacteriofaag werd getest in TIM-2 onder verschillende omstandigheden: toevoeging van alleen de faag, E. coli en de faag, en E. coli en meerdere shots van de faag. De stabiliteit van de bacteriële gemeenschap werd gevolgd met behulp van 16S rRNA-sequencing. Er werd aangetoond dat faag titers verlaagd konden worden door activiteit van de commensale microbiota. Niveaus van de faaggastheer (hier E.coli) waren verlaagd in de interventies met het shot van de faag. Meerdere shots leken niet effectiever te zijn dan één shot. Tegelijkertijd werd de bacteriële gemeenschap niet verstoord en bleef deze gedurende het hele experiment stabiel, wat in schril contrast staat met behandeling met antibiotica. Deze hoge specificiteit toont aan dat faagtherapie een veelbelovend alternatief zou kunnen zijn voor behandeling met antibiotica bij de behandeling van maagdarm-infecties, omdat de gezonde leden van de microbiële gemeenschap zo minder wordt getroffen.

Naast virussen zijn er ook schimmels en gisten aanwezig in de menselijke darm. Uitgebreide informatie over darm-schimmels ontbreekt, wat leidt tot ontbrekende inzichten over het mycobioom en de interactie ervan met de bacteriële gemeenschap en de menselijke gastheer. In hoofdstuk 4 werd het gebruik van TIM-2 als

hulpmiddel voor de studie van de darm-schimmels onderzocht. In experimenten met standaardvoeding (SIEM) werden bacteriën en schimmels geanalyseerd met 16S rRNA en ITS-sequencing. De schimmelgemeenschap vertoonde een lage diversiteit en een grotere variabiliteit in vergelijking met bacteriën. Taxonomische classificatie toonde aan dat op phylum-niveau Ascomycota en Basidiomycota domineerden, terwijl Agaricus, Aspergillus, Candida, Penicillum, Malassezia, Saccharomyces, Aureobasidium, Mycosphaerella, Mucor en Clavispora de meest voorkomende genera waren. Daarnaast werden voedingsinterventies (hoog koolhydraat, laag koolhydraat en glucose als koolhydraatbron) uitgevoerd om te zien of dit de darm-schimmels beïnvloedde. Er werd aangetoond dat de verandering van dieet de diversiteit zou kunnen beïnvloeden. Samengevat lieten de experimenten zien dat de mycobiota gemodelleerd kon worden in TIM-2, maar de lage diversiteit en hoge variabiliteit maakt het bestuderen van schimmels, in vergelijking met bacteriële gemeenschappen, veel uitdagender. Toekomstig onderzoek zou zich moeten richten op optimalisatie van de stabiliteit van de schimmels. De darm-schimmels werd verder onderzocht in hoofdstuk 5, waar de mycobiota van gezonde individuen werd geanalyseerd. Hiervoor werd op fecale monsters van 163 personen die beschikbaar waren uit twee afzonderlijke onderzoeken, ITS2- en 16S-rRNA-sequencing uitgevoerd om respectievelijk het schimmel- en bacteriële microbioom te analyseren, evenals hun interacties tussen de koninkrijken. De resultaten toonden een veel lagere diversiteit in schimmels in vergelijking met bacteriële diversiteit. Ascomycota en Basidiomycota waren dominante schimmel phyla in alle monsters, maar de niveaus varieerden enorm tussen individuen. De tien meest voorkomende schimmel genera waren Saccharomyces, Candida, Dipodascus, Aureobasidium, Penicillium, Hanseniaspora, Agaricus, Debaryomyces, Aspergillus en Pichia, en ook hier werd een hoge interindividuele variatie gevonden. Er werden correlaties gemaakt tussen bacteriën en schimmels, en alleen positieve correlaties werden gevonden. Om het belang van de gevonden correlaties verder te onderzoeken, is meer onderzoek nodig om onderscheid te maken tussen schimmels die de darm koloniseren en soorten die uit de omgeving komen. De interacties tussen de schimmel- en bacteriële gemeenschap in de darm werden verder geanalyseerd in hoofdstuk 6 met behulp van

TIM-2, waar interventies met antibiotica en fungicide werden uitgevoerd om te onderzoeken of deze de twee microbiële gemeenschappen verstoorden. De gemeenschappen werden geanalyseerd met behulp van next generation sequencing van het ITS2-gebied en het 16S-rRNA-gen. Ook werd de productie van korte-keten vetzuren gevolgd tijdens de interventies. Correlaties tussen schimmels en bacteriën werden berekend om mogelijke interacties tussen koninkrijken te onderzoeken. Er werd aangetoond dat acute behandeling met antibiotica of fungiciden de bacterie- of schimmelgemeenschappen niet veel veranderde. korte-keten vetzuren-niveaus waren verlaagd in monsters die waren behandeld met fungiciden. Spearman-correlaties suggereerden dat inter-koninkrijkinteracties aanwezig zijn in de menselijke darm en dat schimmels en bacteriën elkaar kunnen beïnvloeden. Verder onderzoek is nodig om meer inzicht te krijgen in deze interacties, en hun moleculaire aard, en om de klinische relevantie te bepalen.
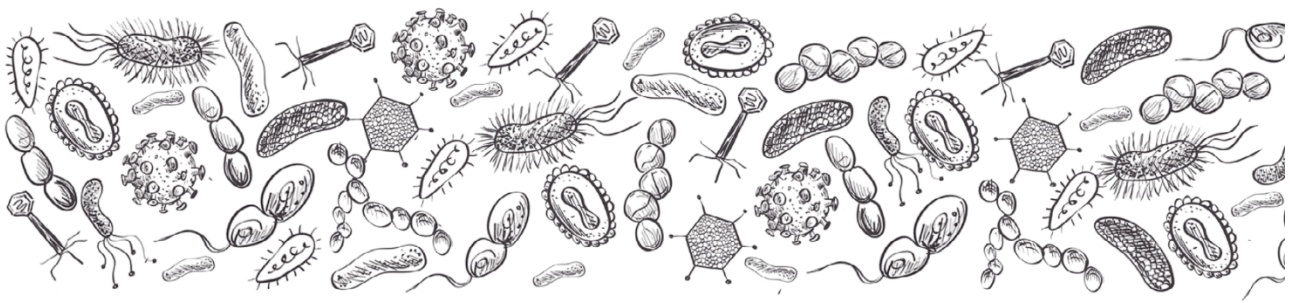
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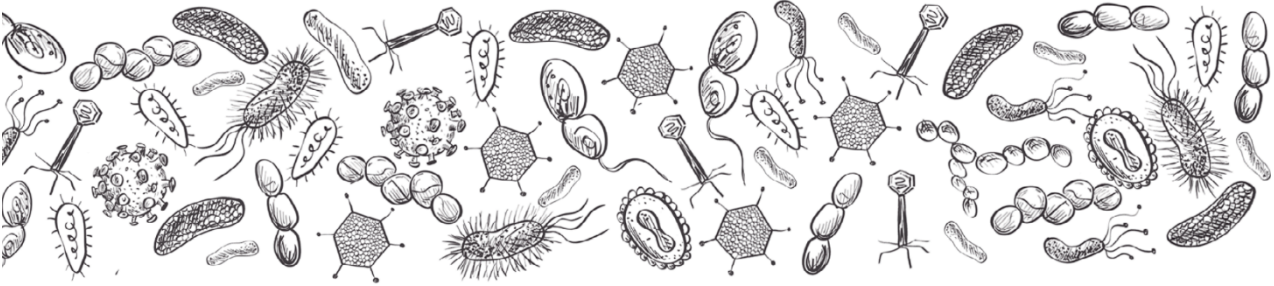
One of the major global health issues in the near future is the rise of antimicrobial resistance (AMR) caused by the widespread use of antibiotics. This 'silent' pandemic has not gained the attention that it should have. It already has a big impact on human health and on healthcare costs and will only do more so in the future. For example, In Europe 390,000 deaths annually are attributable to AMR and worldwide this counts up to 10 million by the year 2050 (O'Neill, 2016). Also economically AMR has a big impact: in Europe the healthcare costs and productivity losses are estimated to cost €1.5 billon each year (ECDC/EMEA, 2009). Furthermore, it is predicted by the World Bank that the economic damage caused by AMR in the next decades could result in a loss of world output between €2-6 trillion (World Bank, 2017).

Antibiotics have been the main treatment option for bacterial infections in humans for decades, and also have been used widely in veterinary medicine. Despite years of successfully treating these infections, it has at the same time also led to an increase of antimicrobial resistance genes (ARGs). Bacteria acquire these ARGs and humans become resistant against antibiotic therapy used. Especially bacteria that are resistant against multiple antibiotics pose a great risk for human health. Patients suffering from infections caused by these multi-drug resistant (MDR) bacteria bring substantial healthcare costs because of their prolonged hospital stays. And more importantly, the difficulty to treat these infections leads to a high mortality in patients suffering from infections with MDR bacteria. To combat these dangerous infections, alternative therapeutic options should be explored to complement antibiotics in the near future.
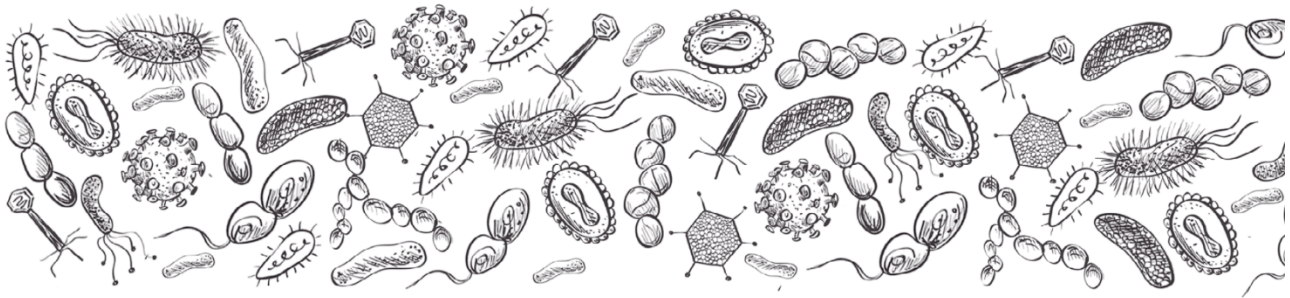
Next to the problems caused by MDR bacteria, antibiotics also have a disruptive effect on the human gut microbial communities. Antibiotics do not only kill or inhibit pathogenic bacteria, but also influence the commensal 'beneficial' microbes found in the gut, leading to dysbiosis. Dysbiosis subsequently affects human health as evidenced by the link between gut microbial dysbiosis and diseases such as inflammatory bowel disease, obesity and diabetes mellitus type II. These chronic conditions pose great health risks to a large part of the human population, and therefore the importance of a balanced gut microbiota should be considered. Not

only bacteria are influenced using antibiotics, but also fungi are affected. Fungi are also an important part of the gut microbial community, and the disruption of the bacterial community by antibiotics has shown to lead to outgrowth of (pathogenic) fungi. Infections caused by these fungi often have a high mortality and are difficult to treat. The increase of fungi after antibiotic use shows the importance of inter-kingdom relations in the human gut. Bacteria as well as fungi form a balanced community, and a disturbance of this community can have great influences on health. Future research should therefore not only focus on bacteria, but also fungi should be taken into account in microbial studies. In this thesis, tools have been explored to study fungi and bacterial-fungal interactions. Further development of these tools can help in understanding and solving problems caused by bacterial and fungal dysbiosis and will contribute to the understanding of their role in (human) health and disease, eventually contributing to prevention and/or therapy. Particularly the cross-kingdom interaction between the two will lead to novel insights and will likely improve medical practices in the future, with an expected reduction in associated healthcare costs.

The problems described above, show the urgent need for replacement of antibiotics as primary treatment options of bacterial infections. One promising alternative option, that is on the rise, is (bacterio)phage therapy, but it has been in the shadow of antibiotics for decades, therefore more research is needed for this to become a more standard clinical practice. In this thesis, tools have been investigated to help in the design of bacteriophage therapy. Good *in vitro* experiments are important to have a solid base for research on phage therapy, because of the specificity of bacteriophages to their bacterial host. Well-designed survival studies can increase the success rate of phage therapy, because it helps in determining the appropriate dosage and helps in exploring the need for protection techniques, such as encapsulation. This increase in success rate of phage therapy will decrease health-care costs due to the 'silent' pandemic caused by infections with AMR bacteria.

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Addendum

Dankwoord
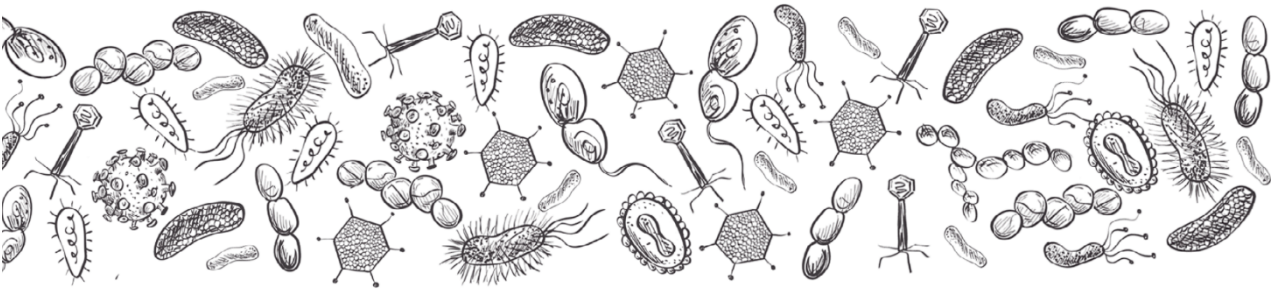
Dit proefschrift luidt het einde in van de PhD-reis die ik afgelopen jaren heb gemaakt. Zonder hulp van collega's, vrienden en familie was dit niet mogelijk geweest en ik wil jullie graag bedanken voor jullie hulp, steun en feedback.

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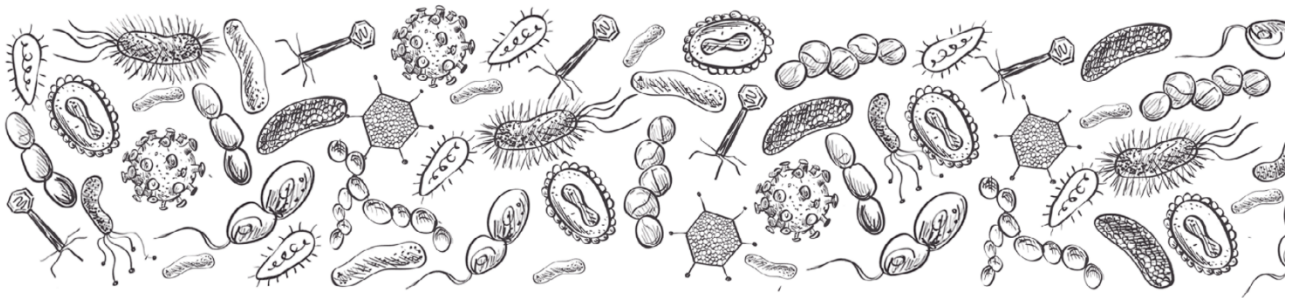
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About the author
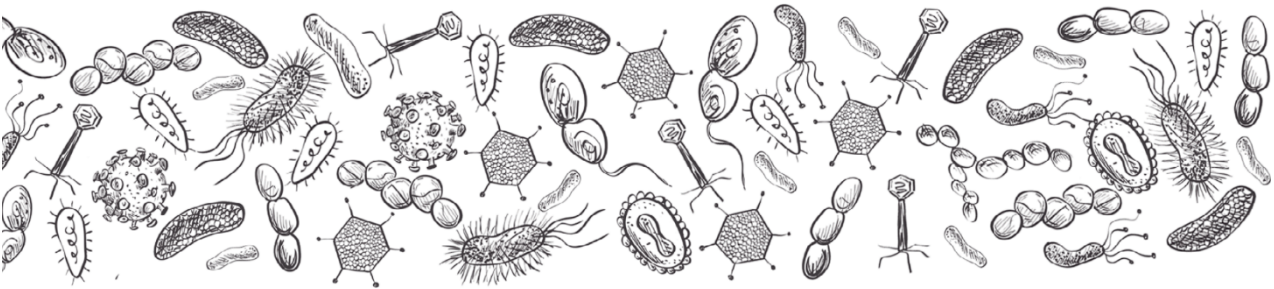
Curriculum vitae

Evy Theresia Maria Maas was born on 31st of July in Veldhoven, The Netherlands. After finishing secondary school at the Rythovius College in Eersel, she started a BSc in Nutrition and Health at Wageningen University in 2010. She wrote her BSc thesis at the department of Human and Animal Physiology about the role of adiponectin on low-level chronic inflammation of white adipose tissue. After taking extra courses in gene technology, enzymology, process engineering and microbiology, she obtained her BSc degree in 2015. Evy continued her studies at Wageningen University in the MSc program of Biotechnology with the specialization Food. During this period the interest for gut microbiology was developed, and for her MSc thesis Evy choose a project at the chair of Microbiology focused on the characterization of a novel homo-acetogenic bacterium isolated from a human fecal sample. After this, she did a 6-month internship at Copenhagen University in Copenhagen on a project on the influence of pectin molecules on the piglet gut microbiota using an in vitro model. Evy graduated from het MSc program in January 2018, after which she immediately started as a PhD candidate at the Gut Microbiology group at the Centre for Healthy Eating and Food Innovation at Maastricht University, Campus Venlo. Her PhD project focused on the development of tools for the investigation of cross-kingdom interactions in the human gut microbiota, and is presented in this thesis.

List of publications

This thesis

Maas, E., Penders, J., Venema, K. (2023). Modelling the Gut Fungal-Community in TIM-2 with a Microbiota from Healthy Individuals. Journal of Fungi, 9(1), 104.

Maas E., Penders J., Venema K. (2023). Fungal-Bacterial Interactions in the Human Gut of Healthy Individuals. Journal of Fungi, 9(2), 139.

Maas, E.; Penders, J.; Venema, K. (2023) Studying Fungal-Bacterial Relationships in the Human Gut Using an In Vitro Model (TIM-2). Journal of Fungi 9(2), 174.

Other

Wiese, M., Hui, Y., Holck, J., Sejberg, J. J., Daures, C., **Maas, E.**, ... & Nielsen, D. S. (2021). High throughput in vitro characterization of pectins for pig(let) nutrition. Animal microbiome, 3(1), 1-15.

Submitted

Maas, E., Umanets A., Penders J., Venema K. Survival of two phages as free particles during transit through a computer-controlled dynamic system simulating the upper gastrointestinal tract (TIM-1).

Maas, E., Penders J., Venema K. Investigating the survival and activity of a bacteriophage in the complex colon environment with the use of a dynamic model of the colon (TIM-2)

Overview of completed training activities

Courses

Safe Microbial Techniques, Maastricht, the Netherlands, 2018

VLAG Summer Course 'Glycosciences', Wageningen, the Netherlands, 2018

Microbiome analysis with QIIME2, Copenhagen, Denmark, 2019

University Teaching Qualification, Maastricht, the Netherlands, 2022

Conferences

<u>Poster presentations</u>

The 7th Beneficial Microbes Conference, Amsterdam, The Netherlands, 2018

Gut Day Symposium, Wageningen, The Netherlands, 2018

Gut Day Symposium, Amsterdam, The Netherlands, 2019

Molecular Mechanisms Linking the Microbiome and Human Health, Miami, USA, 2020

<u>Oral presentations</u>

The 8th Beneficial Microbes Conference, Amsterdam, The Netherlands, [online], 2021

Scientific Spring Meeting Nederlandse Vereniging voor Medische Microbiologie, Arnhem, The Netherlands, 2022