Microbiome-mediated colonization resistance: defense against enteropathogens and multi-drug resistant organisms

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Chapter 10

General discussion and future perspectives

Enteric colonization with multi-drug resistant organisms (MDROs) or enteropathogenic bacteria such as *Clostridioides difficile* can precede development of an infection and is considered an important public health concern. It has long been suspected that the gut microbiome can provide protection against these potentially pathogenic bacteria and research into this field increased considerably in the last 10-15 years. The gut microbiome is able to confer resistance against colonization and infection by endogenous and exogenous microorganisms through a myriad of mechanisms, including nutrient competition, secretion of antimicrobial compounds such as short-chain fatty acids and bacteriocins, maintaining gut barrier integrity and its interaction with the host immune system. It is hypothesized that in the case of enteric colonization or infection, there is a lack of microbiome-mediated colonization resistance against the potentially pathogenic microorganism. Therefore, appropriate restoration of colonization resistance may prevent colonization or contribute to the eradication, before an infection can develop. The latter strategy may be especially valuable for vulnerable patient populations such as nursing home residents, stem cell transplant patients and patients admitted to the intensive care unit. The work in this thesis largely aimed at identifying gut bacteria involved in conferring microbiome-mediated colonization resistance against enteropathogens and MDROs. In addition, this thesis contributes to several technical challenges that the microbiome research field is currently facing, namely standardization of wet-lab and dry-lab procedures for clinical microbiome studies and development of novel computational tools for functional microbiome profiling. The work in this thesis is primarily computational and over the years we progressed from using 16S rRNA gene amplicon sequencing for microbiota profiling to studies using metagenomic sequencing and metabolomics, which present more challenges in data analysis. By building on the experience and knowledge gained in the first 2.5 years of this PhD, we designed and performed a very stringent cross-sectional study and combined multi-omics with machine learning approaches.

Technical challenges in the era of multi-omics

Research in the fields of (bio)medicine and biology is rapidly changing and is becoming increasingly quantitative of nature, with large and complex high-dimensional data being commonly used[1, 2]. This can especially be attributed to the arrival of the many -omics techniques which allow for deep resolution at the molecular level (DNA, RNA, proteins and metabolites) and thereby make a systems biology approach feasible[3]. These technical advancements are accompanied by decreasing costs for such measurements, which makes obtaining these large data sets easier, cheaper and more common[4]. However, the scientific community, including journals, funding bodies, education and software/ tool development do not always develop at the same pace, while this is imperative

for optimal usage of obtained data. In the sections below I will discuss the current challenges and opportunities of these technical issues.

The solution of the reproducibility crisis: findable, accessible, interoperable and reusable (FAIR) data

One of the main challenges in recent scientific research is the so-called "reproducibility crisis", which means that many studies cannot be reproduced, including studies in the gut microbiome research field[5-7]. One relatively simple approach that can help to resolve this crisis is that data reported in manuscripts should be FAIR[8]. One of the current main issues with regard to FAIR data is that not all study-related data are made available for the scientific community by researchers[9]. While this is understandable, as a lot of effort, time and money can be involved with collecting data from a large cohort, not sharing all data used for analysis can impede scientific advancement and hinders reproducibility of results[10]. Not all journals

are implementing stricter rules and guidelines for sharing microbiome data and it remains frequently stated that 'raw sequence data is available on request' or that the data cannot be shared due to potential privacy issues. The underlying explanation is that human reads are present in fecal metagenomes, which can in theory lead to identification of an individual[11]. The latter issue can be easily tackled by filtering out human reads prior to uploading the data to a central archive such as the European Nucleotide Archive (ENA). In this way, privacy would no longer be an argument for not sharing sequence data. However, the 'privacy issue' may actually be representative of an underlying aversion to data sharing. This could be one possible explanation for the fact that of manuscripts published in Nature and Science in which is stated that data is available upon request, only in less than half of the cases the data can actually be obtained[9]. This study by Tedersoo and colleagues evaluated data availability in 875 articles published between 2000 and 2019 and they contacted authors of 310 papers to investigate if data could be obtained[9]. An encouraging finding of this paper was that a yearly decay rate of 5.9% was found with respect to the 'data available upon request' statement, which implicates that data sharing is becoming more common. Unfortunately, sharing of metagenomic data in the microbiology field was found to be an exception to this trend, as its public availability has decreased over the past years[9]. The reason for keeping data within a research group may be to ensure a consistent stream of (high-impact) publications. The aforementioned issue of data being 'available upon request' is in general a way of complying with journal policies while not always having the intention of actually sharing raw data[9]. It should be stressed that this does not apply to all researchers with such a statement in their paper, as many of them are willing to share data without any specific requirement.

For the research conducted in this thesis, we have always made raw sequence data and associated metadata publicly available (**Chapter 6 – Chapter 9**). For several

manuscripts, we have also made all the applied statistical code and other necessary data files available (**Chapter 7** and **Chapter 8**), which further improves the reproducibility and transparency of our work. In view of this, it is a good development that journals are also changing their policies and require sharing and publishing of raw data and associated statistical code upon publication[12]. For example, a journal can instruct authors to make references to both the location of raw sequence data and to the location of all applied code, ensuring reproducibility and findability. Such a journal policy was also encountered in one of the studies in this thesis (**Chapter 8**). However, it is important that journals, similar to the funding bodies as discussed below, also control whether data is actually uploaded to public repositories in a FAIR manner. In any case, I hope that more journals will follow the encouraging trend of data and code sharing, which will allow the microbiome field to advance more quickly.

Improved sequence and metadata sharing will allow researchers to conduct higher-quality meta-analyses, something that has proven to be crucial for establishing robust disease-specific microbial signatures. For example, a large gut microbiome meta-analysis has led to identification of *Fusobacterium nucleatum* as more prevalent and abundant in colorectal cancer patients than in healthy controls, which was largely independent of geography or technical variation[13]. Also in this thesis (**Chapter 5**) we have been able to profit from publicly available data, as we re-analyzed metagenomic data from eight cross-sectional studies comparing gut microbiomes from colorectal cancer patients with controls. Also for **Chapter 6,** several studies have been published in the meantime that would allow for verification of our results and potentially a meta-analysis, but these were unfortunately not published at the time of publication[14, 15]. When consistent results are obtained from several cohort studies, this increases the degree of confidence of findings, which can serve as an incentive, foundation and guide for conducting follow-up experiments.

To tackle the aforementioned issues with regard to data sharing, there may be an important role for both funding agencies and journals/editors to enforce stricter rules, as outlined in editorials of the journals Nature in 2017 and Microbiome in 2018[11, 16]. For example, funding agencies may include a requirement to make all generated data publicly available immediately after publication. It is encouraging in this respect to see that one of largest Dutch funders in medical research, ZonMw, has adopted the FAIR guidelines for their funding calls and they strongly support open science[17]. They have even released a call in 2019 termed ‘Tackling antibiotic resistance by reusing data and increasing FAIRness’, where the goal was to get a better grip on tackling antibiotic resistance by reusing existing data resources[18]. Importantly, funding agencies will also have to dedicate resources to ensure compliance with data sharing and to provide technical support, as a mere data sharing requirement has shown to be insufficient due

to limited compliance[19]. A similar role is reserved for journal editors with regard to ensuring that authors comply with data sharing instructions, even though this may be a time-consuming effort. While time-consuming, it has been found that studies with publicly shared data get more citations on their work, an important metric for journals[20, 21].

The 'modern' biologist

The omnipresent availability of large data sets obtained from -omics technologies can pose researchers without a quantitative or computational background with a lot of challenges. In light of these developments, extensive collaboration between wet-lab and dry-lab researchers is becoming more important to obtain a detailed understanding of the generated data, which is also increasingly becoming clear in the microbiome field[22, 23]. It remains of crucial importance that researchers in biomedical research fields have some basic understanding of computational data processing for properly interpreting and judging -omics data described in scientific papers. This is not a one-way street though, as computational scientists also need to have at least a basic understanding of applied experimental methods for making optimal use of the data[24]. However, as biomedical research programs (BSc/MSc) at this point mostly offer traditional biomedical courses like physiology, genetics and biochemistry, during which experimental methods and wet-lab experiments are often part of the curriculum, it is necessary to incorporate more in-depth data science and statistics courses that focus on analyses often performed in -omics studies, such as principal component analysis[25-27].

Functional characterization of the microbiome

Microbiome researchers have, thus far, mostly investigated taxonomic profiles of microbial communities, e.g. through 16S rRNA gene amplicon sequencing and shotgun metagenomics. However, this has a large disadvantage of not obtaining information about the functional capacity of the microbial community, which is likely much more relevant than taxonomy when investigating the microbiome's relation with health and disease[28, 29]. For example, several studies have shown that the functional repertoire encoded in metagenomes displays much higher sensitivity to perturbations than the taxonomic profile[30-32]. One of several reasons for the lack of functional analyses in the gut microbiome field is the current scarcity of good and easy-to-use tools for this purpose[29]. With the decreasing costs of sequencing techniques[4], it becomes particularly important to have reliable and informative computational tools to determine the functional capacity of the microbiome. To the best of my knowledge, the first (and so far only) manual curation of functional information for the gut microbiome was performed on Kyoto Encyclopedia of Genes and Genomes (KEGG) annotations by the Raes group, which were grouped into so-called gut metabolic modules[33]. These curations mainly focused

on microbial metabolism of carbohydrates, amino acids and lipids and are useful for obtaining better understanding of the utilization of these three macronutrients by the gut microbiome[33]. However, for these gut metabolic modules to be more readily applicable for (gut) microbiome research, modules with other metabolic pathways should be added, e.g. pathways involved in bile acid and short-chain fatty acid metabolism.

In this thesis we systematically curated known carbohydrate-active enzymes (CAZymes) involved in glycan breakdown and grouped these into several functional categories (**Chapter 5**). Such an annotation is not only much more intuitive and easier to interpret than a list of CAZyme families, but it can also reduce the number of features when analyzed at a different level. As the number of features (CAZymes in this case) is collapsed into approximately 100x fewer functional categories, this lowers the chance for finding false negatives due to multi-error correction. While multi-error correction may not be a major problem for CAZymes yet, since approximately ~700 CAZyme (sub) families are currently known, functional annotation using KEGG families can easily provide researchers with thousands of features and lists of genes from metagenomes can even result into millions of features per sample. Importantly, obtaining substrate information through CAZyme annotation allows for analyses that are not common in the microbiome field, but have proven their value in the transcriptomics field by detecting up or downregulated pathways in e.g. different types of leukemia, amongst many other discoveries[34-36]. An example of such an analysis technique is gene set enrichment analysis (GSEA), which can also be applied to the microbiome field (**Chapter 5**)[34]. GSEA mainly derives its strength from grouping genes into informative functional pathways (or in the case of CAZymes, into substrates). This has several major advantages over testing single features. For example, when testing individual genes there may be no significant differentially abundant genes, but all genes may show a trend towards a specific effect. When grouping these genes into functional pathways, a very significant effect at the pathway level may become apparent, which is in any case much more informative than gene-by-gene testing[34, 37]. The opposite may also happen, where a researcher observes many significantly differentially expressed genes, challenging the identification of commonalities across all the genes and only experts in the field may be capable of identifying and interpreting these correctly at the functional level. While GSEA(-like) tools and manually curated annotations are almost non-existent in the microbiome field, I am convinced that this is the future of functional metagenomics and will allow for a much more detailed understanding of the functional capacity of microbial communities and its relation to health and disease.

In recent years, other -omics techniques such as metabolomics and, to a lesser extent, metatranscriptomics and metaproteomics have been used to get information on the actual activity of the microbiome (**Chapter 1**)[28, 38, 39]. We had the opportunity to perform

metabolomics in our study investigating the associations between enteric colonization of ESBL-producing *Escherichia coli* and the microbiome and metabolome (**Chapter 9**). While in this study the metabolomics data confirmed the results of the metagenomics (no differences between the groups), we previously experienced in a collaborative project on the effects of the herbicide glyphosate on the gut microbiome, that metabolomics can be very powerful to detect functional differences between groups at the level of metabolic pathways and may be more sensitive than metagenomics[40]. As we did not detect any differences between individuals colonized with ESBL-producing *E. coli* and non-colonized individuals with either metagenomics or metabolomics in **Chapter 9**, we did not take the next step of integrating both data sets, which could have provided more insight in case differences would have been found[41]. The importance of metabolomics for studying functionality of the microbiome is also reflected by the fact that multiple research groups have built tools that allow for prediction of metabolite concentrations based on metagenomic sequence data[42, 43]. With regard to metatranscriptomics and metaproteomics, we unfortunately did not apply these techniques during the research described in this thesis. These techniques have already been successfully used to increase understanding of microbial alterations in the gut in Type I Diabetes Mellitus (T1DM) patients on taxonomic and functional level[44] and to reveal differences in transcriptional activity in microbes over time in IBD patients[45]. Nevertheless, significant challenges remain for both techniques to be overcome before they can be more widely implemented in microbiome research in the coming years[38].

Microbiome-mediated colonization resistance

Microbiome-mediated colonization resistance is a relevant topic from both a therapeutic and evolutionary point of view, and its importance for health and disease is already recognized for many decades[46-49]. From a therapeutic perspective, obtaining a detailed understanding of microbiome-mediated colonization resistance against a microorganism can pave the way for targeted restoration of colonization resistance to eradicate or prevent colonization by this microorganism. With regard to the more evolutionary aspects, it is imperative for our understanding of microbial community dynamics in general to define what is necessary for a community to outcompete a specific microorganism. In this thesis we provided an extensive overview of the mechanisms through which the microbiome can confer colonization resistance (**Chapter 2**). A very recent, breakthrough finding that builds upon the concept of colonization resistance is that the resistance conferred by the gut microbiome to an infection can be strengthened by having contracted a prior infection[50]. It was shown that an initial infection of *Yersinia pseudotuberculosis* (a food-borne pathogen) could protect against subsequent colonization and infection by other potential pathogens, like *Klebsiella pneumoniae*. The initial infection rewired the

microbiome such that taurine, an intermediate in bile acid metabolism, was produced in higher amounts. Taurine was subsequently converted into antimicrobial sulfide, which in turn inhibited *K. pneumoniae* respiration by limiting access to oxygen through inhibition of enzymes involved in aerobic electron transport chains[50]. This effect could, importantly, be replicated in a mouse model infected with enteropathogenic *Citrobacter rodentium*, providing further support for the hypothesis that taurine can enhance colonization resistance by restricting pathogen respiration. It should however be noted that the gut microbiome-mediated effect could not be fully uncoupled from the host immune response[50].

In this thesis we aimed to identify bacteria that could play a role in providing colonization resistance against enteropathogens or MDROs (**Chapter 6 – Chapter 9**) (Figure 1 Step 1). We identified several bacterial taxa (e.g. *Fusicatenibacter* and *Eubacterium hallii*) associated with protection against *C. difficile* colonization (**Chapter 6**) and several bacterial taxa (*Dorea*, *Atopobiaceae* and *Lachnospiraceae ND3007 group*) associated with protection against MDRO colonization. However, these findings were not followed-up with more targeted experiments (Figure 1, Step 2 and Step 3), which leaves the question whether these bacteria actually play a causative role unanswered. In sharp contrast, we did not find associations pointing towards a role for the microbiome in mediating protection against infection caused by *N. americanus* (**Chapter 7**) or against asymptomatic colonization of ESBL-producing *E. coli* (**Chapter 9**).

With the successful implementation of fecal microbiota transplantation (FMT) for treating recurrent *Clostridioides difficile* infection (rCDI) almost a decade ago, it was expected that restoring colonization resistance through defined microbiome-based therapeutics would soon replace FMT as a treatment for rCDI[52]. Despite the extensive research that has been performed since, this has not yet led to widespread microbiome-based therapeutic intervention options (as is the case for the microbiome field in general)[53]. In my opinion this can mainly be attributed to study design and subsequent computational analyses issues and to the lack of follow-up wet-lab experiments and mechanistic research.

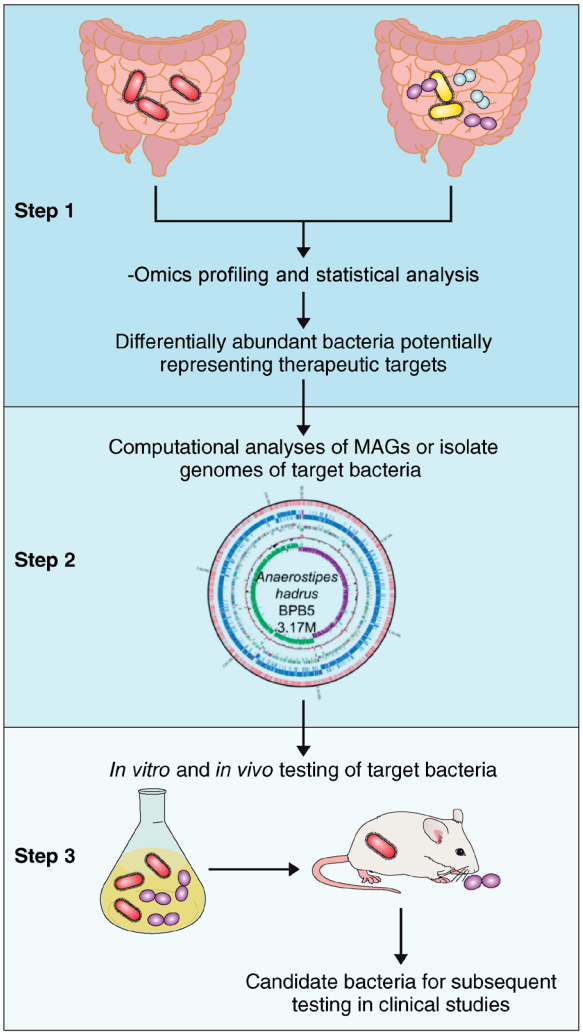

Figure 1: Workflow of studies in this thesis investigating microbiome-mediated colonization resistance and suggestions for follow-up research directions. In this thesis, studies were limited to the first step in the infographic, namely -omics profiling and identifying bacteria that may represent potential therapeutic targets, but no follow-up computational or wet-lab experiments have been performed. Follow-up computational experiments should involve analyses of the genomes of the bacterium of interest and understanding their encoded metabolic capacities (Step 2). After obtaining a detailed picture of such encoded capacities of the bacterium of interest, targeted in vitro and in vivo experiments can shed further light on the antagonistic actions against the potential pathogen. In vitro experiments can include co-culture of the bacterium of interest with the potential pathogen and in vivo experiments can include colonizing an animal with the potential pathogen and subsequently administering the bacterium of interest to investigate whether this leads to eradication of the potential pathogen (Step 3). Red bacteria indicate potential pathogenic bacteria, while yellow, purple and blue bacteria indicate potential bacteria of interest. The *A. hadrus* genome image was obtained from Zhang et al.[51].

The importance of study design and targeted follow-up computational analysis

Currently, there are too many studies which do not have an appropriate study design to make strong conclusions about which bacteria/microorganisms can potentially be protective against, or contribute to, a specific disease or pathogen colonization. It becomes increasingly clear that it is important to control for confounding factors in the study design, as many clinical variables have been shown to significantly affect gut microbiome composition[7, 54, 55]. A classic example illustrating the importance of uncoupling confounders is the original report that individuals with Type 2 Diabetes Mellitus (T2DM) had a very different gut microbiome as compared to healthy individuals[56]. However, a later publication showed that metformin, the first-line treatment for T2DM, usage was a very strong confounder and could explain the majority of the microbiome-modulating effect previously thought to be explained by having T2DM or not. The authors of this paper also strongly recommend future studies to disentangle effects of medication from effects of disease[57]. In this thesis, we could also not always fully dissociate effects of disease or colonization status from confounding factors (**Chapter 6** and **Chapter 8**). While in most epidemiological studies one can correct for confounding factors using (advanced) statistical models, there are no known statistical frameworks which can incorporate confounding variables for microbiome research. Ideally, one would have access to large enough cohorts so that sample selection can be applied to select for the phenotype of interest and to simultaneously exclude individuals positive for variables known to affect the microbiome. In cases of more severe disease, it is often not possible to exclude confounders such as medication use in the investigated population. In these cases it is possible to match cases against controls for more general confounders like age and sex, to at least minimize their effect on the obtained results[7]. Using samples from a large cross-sectional Dutch population cohort ("Pienter cohort"), we had the opportunity to select individuals who were negative for many microbiome confounders while positive for our phenotype of interest (ESBL-producing *E. coli* colonization) and could afterwards match them based on age, sex, travel history and ethnicity to individuals negative for our phenotype of interest (**Chapter 9**). While previous research has not taken confounders into account and showed several bacteria to be associated with MDRO protection (**Chapter 8** and [58, 59]), we demonstrated that there is no difference in the gut microbiome and metabolome between ESBL-producing *E. coli* colonized individuals versus those who are not colonized. This also suggests that microbiome-based therapeutics may not be as effective against ESBL-producing *E. coli* as they potentially are against other antibiotic-resistant bacterial species, such as vancomycin-resistant *Enterococcus* (VRE) (further discussed in the next paragraph).

After identifying bacteria that may be important in providing colonization resistance, follow-up research with more targeted computational and wet-lab analyses is warranted (Figure 1). To illustrate this point I will take the example of our recent finding that

the anaerobic and butyrate-producing bacterium *Anaerostipes hadrus*, amongst other bacterial species, was significantly associated with protection against *C. difficile* colonization (unpublished data, Figure 2).

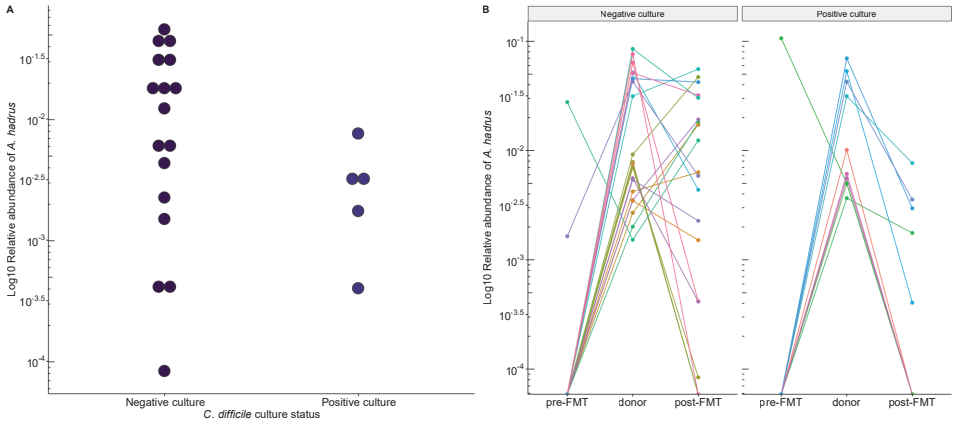

Figure 2: Comparison of *A. hadrus* relative abundance in n = 31 rCDI patients treated with FMT. Relative abundance on a log10 scale is indicated for post-FMT patients based on their status of being *C. difficile* culture positive or negative and only patients with detectable *A. hadrus* levels are shown. Seventeen of 21 samples culture-negative for *C. difficile* had detectable *A. hadrus* and 5/10 samples with a positive culture had detectable *A. hadrus*) (A). We further investigated whether this could be linked to a potential FMT donor effect, but this was not the case, as all patients were treated with donors whose microbiome contained *A. hadrus* (B). Each colored line represents the relative abundance of *A. hadrus* in an individual patient, pre- and post-FMT with the corresponding donor relative abundance of *A. hadrus*.

Ideally, the genomes and/or metagenome-assembled genomes (MAG)s of *A. hadrus* would be downloaded from public repositories and be extensively analyzed for potential antagonistic effects against *C. difficile* (Figure 1 Step 2). This could entail confirming or investigating mechanisms that are known to inhibit *C. difficile*, such as the presence of enzymatic machinery encoded for secondary bile acid conversion. A more generic approach could also be used. For example, one could annotate the *A. hadrus* genomes for biosynthetic gene clusters, which may encode for secondary metabolites that are involved in pathways or in mechanisms known to have an antagonistic function against *C. difficile*[60-62]. While such an approach sounds straightforward, this has long been impossible due to difficulties in culturing many common gut bacteria. In the past two years, however, several public repositories of MAGs have greatly expanded the availability of genomes of difficult-to-culture gut bacteria[63-65]. By leveraging this extensive genomic information, more targeted wet-lab experiments can be performed

to investigate and verify hypothesized mechanisms based on genomic data, as for many common gut bacteria very little is currently known with regard to their functional capabilities[66]. For example, if a putative bacteriocin-producing biosynthetic gene cluster in *A. hadrus* would be identified based on genomic data, one could attempt to purify the bacteriocin and directly test the effect of this bacteriocin on *C. difficile*.

Wet-lab research is necessary to elucidate a potential causative role for a bacterium in providing colonization resistance

After performing extensive computational analysis, mechanistic research in the wet-lab and in animal models is imperative to investigate if identified bacteria are really, and not only predicted to be, involved in providing protection against a given pathogen (Figure 1 Step 3). Such mechanistic research is non-trivial, as one will have to decide on many parameters such as pH, oxygen levels, incorporation of different cells occurring in the human gut (e.g. enterocoytes and goblet cells), growth of a functional mucus layer etc[67-69]. Ideally, conditions should be as representative of the human gut as possible. An important discovery in recent years is the capability of growing organoids from almost any human tissue, including the human gut[70, 71]. These organoids allow for more realistically mimicking the human gut and have been used to study the pathogenesis of various enteropathogenic bacteria, including *Salmonella enterica* and *C. difficile*[72, 73]. In addition, intestinal organoids have been used to identify a specific mutational signature caused by colibactin-producing *E. coli* and this mutational signature was subsequently detected in 10-20% of the investigated colorectal cancer genomes[74]. However, a complication in the organoid field is to incorporate the interactions of a complex human gut microbiome, which is currently one of the most difficult challenges to tackle[71, 75]. It should be mentioned that both the targeted computational analysis of genomes of potentially promising bacteria and the in vitro verification of hypothesized mechanisms involved in microbiome-mediated colonization resistance are unfortunately lacking in this thesis. However, we have started investigating antagonistic actions of *A. hadrus*, *Eubacterium rectale*, *Dorea longicatena* and *Butyricicoccus faecihominis* against *C. difficile* in vitro (unpublished data). Based on known functions of these bacterial species, we hypothesize that these antagonistic actions may be related to SCFA production and / or changes in gut pH and this is currently work in progress.

The group of Eric Pamer has performed various studies in the field of gut microbiome and colonization resistance at the mechanistic level. This research can serve as an example of moving beyond initial observations of differences in gut microbiota towards targeted development of bacterial consortia based on mechanistic understanding. Two of the early publications on VRE and the gut microbiome from this group identified that intestinal domination of VRE (as measured by enterococcal relative abundance by 16S rRNA gene amplicon sequencing) often preceded bloodstream infections

in patients undergoing allogenic hematopoietic stem cell transplantation[76, 77]. These findings were followed up by an elegant mouse study which showed that restoration of the gut microbiome of antibiotic-treated mice could restore colonization resistance against VRE, and that especially the *Barnesiella* genus was associated with clearance and reduction of VRE from the intestinal tract[78]. This was followed up by another mouse study which did actually not show an important role for a *Barnesiella* species, but for for *Blautia producta* and *Clostridium bolteae* instead. Mechanistically, it was further shown that a four-strain bacterial consortium (including *B. producta* and *C. bolteae*) was able to reverse antibiotic-induced susceptibility to VRE infection in mice[79]. An important aspect of the underlying mechanism of this cocktail was the production of a bacteriocin, a lantibiotic, by *B. producta*[80]. In patients at high risk of developing VRE infection, high abundance of this specific lantibiotic gene was associated with reduced *E. faecium* density[80]. Importantly, a patent has recently been filed for a consortium of bacteria including *B. producta* and *C. scindens* (see https://patents.justia.com/patent/20210000887) to reduce the burden of VRE infections in (vulnerable) patient groups. In addition, based on their findings, the Pamer group has performed translational research illustrating the importance of the gut microbiome against infection in stem cell transplant patients. In 2018, they published a study where auto-FMT was administered after successful stem cell engraftment[81]. This procedure was able to restore antibiotic-induced microbiome damage, accelerated restoration of neutrophil, monocyte and lymphocyte counts and was shown to be safe[81, 82]. Considering that the auto-FMT was generally shown to restore the gut microbiome (although this was not the case in all patients), it may therefore play a role in restoring colonization resistance against enteric pathogens and possibly MDROs. However, we are still awaiting results of whether auto-FMT resulted in lower infection rates and decreased graft versus host disease[81]. Noteworthy, none of the patients in the control group (not receiving an auto-FMT) recovered their gut microbiome in the same time period, even though they had a similar microbiota diversity at baseline and a similar drop during antibiotic treatment[81].

While this work is impressive and resulted in the recognition of bacterial consortia that are able to provide colonization resistance, it remains to be demonstrated whether such an intervention results in VRE eradication in immunocompromised patients, like stem cell transplant patients. Secondly, safety is a concern, as it has been previously reported that probiotic capsules containing *Lactobacillus*, which is generally considered a harmless commensal, can lead to a significantly higher chance of developing *Lactobacillus*-caused bacteremia in intensive care unit patients[83]. While safety trials for live biotherapeutic products (LBPs) generally do not report many adverse events, such trials have not yet been conducted in severely immunocompromised patients[84, 85].

Future therapeutic strategies for treating colonization and infection by bacterial enteropathogens and MDROs

Past and current therapeutic strategies for treating infection by bacterial enteric pathogens (e.g. *C. difficile*) and MDROs have largely been based on antibiotics. Asymptomatic colonization of MDROs is generally not treated, since the administration of antibiotics has various disadvantages and is not effective for MDRO eradication[77, 86]. There are some reports which it is described that individuals colonized with *C. difficile* have a higher risk to develop an infection and spread this bacterium to other patients[87-89]. However, it is not advised to treat asymptomatic *C. difficile* colonization with antibiotics, as this might also trigger an environment where *C. difficile* spores persist and are able to germinate and cause infection. However, patients with multiple recurrent CDI have a disturbed gut microbiome and an intervention with only antibiotic treatment fails. Since the landmark study of van Nood et al., FMT has become the best treatment option for rCDI patients, but so far rCDI is the only disease for which FMT is an accepted treatment[52]. All other indications for which FMT is investigated as a treatment are in an experimental or last-resort setting[90]. Also at the LUMC, FMT is applied in experimental and last-resort settings, with pilot studies being underway for IBD, for improving efficacy of checkpoint inhibitors in cancer patients, but also for metabolic disorders such as non-alcoholic fatty liver disease and non-alcoholic steatohepatitis and for neurological diseases such as hepatic encephalopathy and Parkinson's disease (dr. Liz Terveer and prof. dr. Ed Kuijper). One of the difficulties with using FMT as a therapeutic option, is that it is poorly defined as not all components are known and the quality and composition cannot be guaranteed to be consistent (**Chapter 3**)[91]. It is for this reason that in the coming years and decades it is expected that we will move away from administering undefined therapeutics (FMT) towards using live biotherapeutic products (LBPs), which are defined consortia of microorganisms (most often bacteria), or to other, well-defined microbiome therapeutics[91].

Live biotherapeutic products (LBPs)

At the start of my PhD trajectory in January 2018 (and already before that), it was expected that LBPs would quickly replace FMT as a therapeutic intervention against rCDI (and subsequently other diseases)[92]. LBPs have a theoretical advantage over the use of antibiotics for treating bacterial infections, as antibiotic treatment comes with the downside of selecting for antibiotic-resistant pathogens and negatively affecting the gut microbiome[91]. However, no LBPs have been implemented in patient care to date, but the expectations for their therapeutic application remain very high (**Chapter 3**)[91]. This is further illustrated by the fact that there are currently 17 clinical trials registered at clinicaltrials.gov, which investigate the potential of LBPs for highly divergent indications such as different types of cancer, rCDI, asthma, kidney disease and obesity.

I still expect that LBPs will ultimately replace FMT for treatment of rCDI with a similar cure rate of ~90%[93], but for other indications expectations should be tempered. While the cause of rCDI is strongly related to a disturbed gut microbiome, diseases like IBD are multifactorial with heavy involvement of the immune system and environmental factors[94]. This is the prime reason why I expect that LBPs for diseases with such a multifactorial etiology will be far less successful. This is currently also shown by the contradictory results for FMT trials against IBD and IBS[95-98], especially since current LBPs are developed based on a 'one-size fits all' approach. This one-size fits all approach is not likely to achieve a very high cure rate for such multifactorial diseases. It is also very likely that specific commensal bacteria will elicit differential immune responses across patients with different diseases[99]. In the oncology field, checkpoint inhibitors have revolutionized cancer treatment and the Nobel Prize of 2018 has been awarded to James Allison and Tasuku Honjo for their discovery[100]. However, 'only' 20% of patients with melanoma treated with checkpoint inhibitors achieves full remission[101]. It should be mentioned that this group of patients would likely not have survived long without checkpoint inhibitors, which is a crucial difference for defining success as compared to IBD or IBS. This is one of the reasons why the recent discovery that the gut microbiome may affect efficacy of immune checkpoint inhibitors was so important[102-104]. Although the exact mechanisms by which the microbiome potentially mediates this effect remain unknown, and different studies report different bacteria to be involved, there may be a role for the microbiome in favorably altering immune cell subsets[104]. Interestingly, in a study where FMT was administered to potentially improve checkpoint inhibitor efficacy in metastatic melanoma patients, donor feces were used from two patients who had previously been treated with checkpoint inhibitors and had achieved clinical remission for more than a year. Subsequent treatment success was only observed using one specific donor, but the underlying reason remained unclear[104]. To conclude, I believe that LBPs will eventually replace FMT for the treatment of rCDI, but we should not expect them to be miracle drugs capable of achieving cure rates of ~90% for other indications.

(Targeted) prebiotics

Prebiotics have been defined as "a substrate that is selectively utilized by host microorganisms conferring a health benefit" by a group of international experts[105]. Following from this definition, suppressing a pathogen may be possible through stimulating potentially antagonistic bacteria by providing the necessary substrates and thereby creating a gut environment unfavorable for enteropathogens, for example by increasing anaerobicity of the gut[106]. This could theoretically be achieved by providing 'good' bacteria with targeted substrates so that they will obtain a competitive advantage and thereby outcompete the pathogen or prevent the pathogen from colonizing in the first place. However, it must be noted that prebiotics (like probiotics) have not met the expectations yet and little to no evidence is available to support their use in

gastrointestinal disease[107, 108]. It is likely that for prebiotics to be more effective, the provided prebiotic should specifically be metabolized by bacteria in the gut that may contribute to reduction of disease burden. In almost all cases, prebiotics are dietary fibers which are not targeted to specific gut microbes. One interesting example where a dietary fiber (acetylated galactoglucomannan) was provided to match specific enzymatic machinery present in *Roseburia* and *Faecalibacterium* species was performed in pigs. This was not related to investigating suppression of a pathogen and rather serves as an example of how to match dietary fiber to bacterial enzymatic machinery[109]. *Faecalibacterium* relative abundance indeed increased post-intervention, but the effects on *Roseburia* relative abundance were less clear. To further investigate this, MAGs were assembled and it was noted that only a small subset of *Roseburia* MAGs contained the necessary enzymatic machinery for metabolizing acetylated galactoglucomannan. These MAGs indeed increased in abundance during the intervention, but this was not the case for *Roseburia* MAGs lacking the necessary enzymatic machinery. From a more general perspective, a 'butterfly' effect was observed whereby widespread community effects occurred through e.g. cross-feeding of products from dietary fiber fermentation[109]. This butterfly effect will likely occur in clinical settings in patients and therefore it remains unknown whether currently available prebiotics are potentially suitable for targeted intervention against pathogens, or can rather be used for inducing broad structural changes in the microbiome (or both). With the current scarcity of data with regard to the effect of targeted modulation of the gut microbiome through prebiotics, it is hard to foresee how this field will develop. Ideally, at least from a theoretical perspective, one would administer an LBP with specific prebiotics that are exclusively metabolized by members of that LBP and thereby create a competitive advantage to facilitate colonization.

Synbiotics

Administration of an LBP together with specific prebiotics, which are ideally metabolized by strains in the LBP, are called synbiotics. The theoretical advantage of synbiotics over LBPs or prebiotics is that the strains will gain a competitive advantage through substrate utilization and this would likely enhance the chance of successful engraftment, which has already been shown in rodent models[110, 111]. For example, Kearny et al. identified a resource, the edible seaweed nori, highly unlikely to be used by bacteria in the lab-mouse gut. They subsequently reasoned that if a microorganism would be introduced into the system during supplementation of seaweed, this microorganism would have a competitive advantage. Indeed, when administering a specific *Bacteroides plebeius* strain capable of porphyran (a polysaccharide present in seaweed) degradation in combination with seaweed supplementation, *B. plebeius* engrafted successfully long-term[111].

While synbiotics seem extremely promising, they are not trivial to produce from a technical point of view, as these strains will need to have very specific metabolic capacities. Preferably, one would like to isolate such strains from humans and not obtain them by genetically modifying bacteria, as this would pose a lot of extra regulatory hurdles[112]. On the other hand, by genetic modification of bacteria there is potential to cure a larger variety of diseases, including diseases that do not have a microbial origin, such as phenylketonuria[113-115]. Phenylketonuria patients are unable to metabolize the amino acid phenylalanine and prolonged consumption of this amino acid can result in severe neurological damage. The idea of using bacteria (next to a protein-restricted diet) to treat this condition is to administer bacteria that specifically metabolize this amino acid and thereby prevent its accumulation. In addition, the large inter-individual variation in gut microbiomes of humans will likely not allow for a 'one-size fits all' approach, as different microbiomes will compete differently with newly introduced strains and have different metabolic capacities. However, if major advances can be made and rare enough substrates can be identified to improve chances of colonization of introduced strains (see the seaweed example in the previous paragraph), there may be potential for development and implementation of synbiotics in the clinical setting.

Strengths and limitations

Before coming to the concluding remarks, I would like to take this opportunity to discuss some of the general strengths and limitations of the research described in this thesis.

Two major strengths of this thesis are 1) the versatility of topics and thereby the contributions to different branches of the microbiome field and 2) the progress to much more complex analysis techniques throughout the past ~3.5 years, which allowed me to more successfully extract information about the underlying biology. Most of the chapters in this thesis are devoted to the overarching theme of microbiome-mediated colonization resistance. The chapters range from studies aiming to identify bacteria and metabolites involved in providing colonization resistance, to studies describing the current understanding of the opportunities and challenges necessary for development of LBPs. These varied topics allowed me to obtain in-depth understanding of the many facets of the microbiome field including technical aspects, the implications for biology and medicine and the requirements for developing microbiome-based therapeutics. The fact that I was allowed to work on different potentially pathogenic (micro)organisms (*C. difficile*, the hookworm *N. americanus* and MDROs) enabled me to get a broader overview of infectious diseases than by concentrating on a single (micro)organism and this also facilitated extensive collaboration with different research groups. Furthermore, this thesis includes two technical-oriented chapters describing the effects of technical

variation on obtained microbiota profiles (**Chapter 4**) and the development of a new tool for profiling CAZymes from shotgun metagenomic data (**Chapter 5**). The research described in these chapters required a different approach than the clinical studies, as the underlying research questions were not directly related to biology and medicine, but were rather aimed at method optimization.

Over the course of conducting the research described in this thesis, the applied techniques moved from 16S rRNA gene amplicon analysis to shotgun metagenomics and metabolomics. The first two studies, chronologically speaking, performed during my PhD (**Chapter 4** and **Chapter 6**) merely involved 16S rRNA gene amplicon sequence data analysis and were limited to a single time point. We subsequently moved to longitudinal study designs (**Chapter 7** and **Chapter 8**), metagenomics (**Chapter 5**, **Chapter 8** and **Chapter 9**), metabolomics (**Chapter 9**) and machine learning approaches (**Chapter 5** and **Chapter 9**). The longitudinal studies allowed for investigating the consistency of microbiota patterns over time and to apply longitudinal analysis techniques. By integrating metagenomics and metabolomics with machine learning approaches, identification of potential biomarkers for a given phenotype becomes more likely and reliable than by only performing differential abundance analysis and taxonomic profiling through 16S rRNA gene amplicon analysis. Together, the variety of study designs required me to obtain knowledge of different statistical methods and their strengths and limitations, something which greatly contributed to my development and current knowledge.

There are also several weaknesses and things that I would have approached differently had I possessed the knowledge I currently have at the start of my PhD trajectory. The major weakness of the current thesis is that the mostly associative studies have not been followed up by more targeted genomic analyses of bacteria of interest nor by mechanistic wet-lab research. This holds particularly true for the findings described in **Chapter 6**, where bacterial taxa with the potential to inhibit *C. difficile* were identified. In light of this, we performed another in silico study where shotgun metagenomes of rCDI patients post-FMT were compared and where one group remained colonized by *C. difficile* and patients in the other group fully eradicated *C. difficile* (unpublished data). *A. hadrus*, *E. rectale*, *B. faecihominis* and *D. longicatena* were identified to be more abundant in the non-colonized group. Fortunately, these bacteria are currently being further investigated or their potential antagonistic effect against *C. difficile* at the Experimental Bacteriology group of LUMC. Second, I would have liked to include machine learning approaches in **Chapter 6** to investigate whether *C. difficile* colonization status could reliably be predicted. If the conducted differential abundance analysis and desired feature selection through machine learning shows the same bacterial taxa to be important in protection against *C. difficile* colonization, this would have made our findings more robust. A third

weakness of this thesis is that our studies were exclusively focused on the interactions of enteropathogens or MDROs with the gut microbiome without taking into account any parameters about host immunity function, as this was outside the scope of this thesis. Interactions between host immunity and the gut microbiome, an enteropathogen or an MDRO are complex but necessary for a detailed understanding of infectious diseases processes[50, 116-118]. Fourth, in this thesis we have always relied on fecal samples to investigate the gut microbiome. As was discussed in **Chapter 7**, fecal samples may not be representative for what happens at the mucosal surface of the intestinal tract and this is especially relevant when studying microorganisms which cause infections in other parts of the intestine than the colon. Indeed, the fecal microbiome can differ a lot from the microbiome in other locations of the intestine[119-121]. Lastly, I would like to touch upon my experience of analyzing microbiome data from studies that were not always specifically set up for conducting microbiome analyses (**Chapter 6 – Chapter 8**). For future studies with microbiome analyses, it will be important to involve a microbiome researcher as early as possible at the design of the study. By doing so, appropriate research questions can be formulated a priori, and most importantly, the microbiome researcher can aid in deciding on the appropriate study design to answer these research questions. In addition, advice can be provided on more practical issues like sample collection, storage and subsequent processing steps (**Chapter 4**).

Concluding remarks

The projects described in this thesis are diverse, ranging from methodology optimization to investigating the gut microbiome in clinical cohorts with the goal of finding bacteria associated with providing microbiome-mediated colonization resistance against enteropathogens and MDROs.

While many issues in microbiome research need to be addressed and numerous open biological questions remain, I excitedly look forward to the future of microbiome research and hopefully towards the implementation of the first rationally designed microbiome-based therapeutics into the clinic in the coming years.

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