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ABSTRACT

The microbiome is the organisms and their gene content that occupies a particular space, perhaps most saliently the gut microbiome which has diverse and important implications in organism functioning. The previous decade has had a veritable explosion of microbiome literature owing to the development of 16S gene sequencing, which can taxonomically identify the microbes in a microbiome, and refinement of analytical and bioinformatic techniques for more effectively extracting information from this data. Despite this, the chemistry of the microbiome, a key facet in health and medicine, remains under-explored. We present here three case studies which utilize 16S sequencing and subsequent analysis to explore the microbiome through a chemical lens, making strides in understanding the physiology of diverse subjects of neointimal hyperplasia, Alzheimer's disease, and γ-aminobutyric acid supplementation. This is followed by a review of microbiome precision medicine to contextualize these findings and point towards future research in the field.

CHAPTER 1

INTRODUCTION

1.1 The microbiome and its characteristics

Disease is often what comes to mind when considering the presence of microbes inside the human body. This is not surprising, given the tight coupling of the discovery of microbes with germ theory by Robert Koch and Louis Pasteur and the ensuing focus on the pathogenicity of microbes in both the scientific and popular literature. Infectious diseases remain a huge global health burden [1] and a salient aspect of day-to-day life. Often overlooked are the commensal and even mutualistic interactions of microbes and humans. However, these interactions are not just important to human health; they are, in fact, fundamental to what it means to be human. This is because our bodies harbor approximately as many microbial cells as human cells, mainly in the gut [2].

Collections of microbial taxa (mainly bacterial) associated with an environment are known as the microbiota, and these organisms and their gene content, are collectively known as the microbiome. Here we are chiefly concerned with those microbiomes which inhabit animals and humans, much of which supplements physiology. Note that while microbiome sometimes strictly refers to gene content, in analogy to genome, the previous looser definition is more common in the literature [3]. Speculations on the existence of a microbiome first began when Antonie van Leeuwenhoek observed oral and fecal microbes in the 1700s [4]. However, advances in culturing and sequencing technology in the last 20 years have begun to reveal the true scope of the microbiome [5]. The gut microbiome is ultimately a presence so important that many have taken to considering it an organ of the body [6].

The microbiome comprises of a highly interconnected network of microorganisms, primarily located in the environmentally suitable descending colon, though with complex biogeography [7]. In addition to bacteria (the primary focus of this chapter), archaea, protists, fungi, and vast numbers of viruses and bacteriophages inhabit the gut and contribute to micro-

biome function. The bacteria are dominated by the phyla Firmicutes and Bacteroides, with major genera in many healthy individuals being *Bacteroides*, *Bifidobacterium*, *Clostridium*, *Eubacterium*, *Fusobacterium*, *Peptococcus*, *Peptostreptococcus*, and *Ruminococcus* [8]. At the species level, however, the notion of a core microbiome is weak, with very high variability between individuals, despite its significance to human physiology. Despite the variance in taxonomic composition, there remains a relatively stable functional potential between people, suggesting that most microbial metabolisms are conserved [9]. The lack of a compositional core microbiome may be related to a lack of genetic control over gut microbial content. A recent study placed the average genetic heritability of gut microbial taxa at 1.9% [10], while environmental variability described 20% of the variance in microbiome composition. Furthermore, the microbiome is malleable, rapidly changing in response to dietary changes [11] and differing across age and geography [12]. This is not to say the microbiome is completely unstable; one study found an average of 60% of strains were conserved across the first 5-years of life [13]. Certain early gut colonizers and certain taxa, especially those belonging to the phyla Bacteroidetes and Actinobacteria, may be particularly persistent across time and within a human population. However, the variability of the stability of each microbe is poorly understood, despite being extremely important to assessing clinical relevance of each strain.

The mutable characteristics of the microbiome are a positive benefit for medical approaches, as they allow for the possibility of targeted and lasting alterations to the microbiome, but they have also has led to health problems as our lifestyle behaviors and environment have diverged from our ancestors, which may carry potential negative consequences for host-microbe relationships. The microbiome co-evolved with humans who had a vastly different diet than humans living in the developed world, with far more non-digestible carbohydrates and fibers [14]. In particular, the "western diet," characterized by high fat and high simple sugar consumption, is known to have deleterious effects on the microbiome and thus, overall health [15]. Other environmental risk factors include pollution [16] and C-section de-

livery [17]. Sterility in the built environment, often allowing for establishment of pathogens [18], can prevent the natural acquisition of and exposure to microbes [19], which may also contribute to poor outcomes in host health, especially if experienced during early phases of development (i.e. in utero and early life). The concept of an interruption in the interaction between our developing immune system and potentially beneficial microbes, is often known as the "hygiene hypothesis" [20]. Thus, the challenges and conditions of modern life may well require microbial supplementation and alteration for ideal health. It is important to appreciate that selection pressures on the microbiome imposed by environment, behavior, disease and pharmaceutical use, are so crucial to human health because of how closely coupled the microbiome is with its host, to the point of the combination of human and microbiome being termed a "superorganism" [21]. This tight relationship causes astonishingly profound physiological effects both within and far beyond the gut. We will now turn the discussion to some of the primary functions of the microbiome.

First, the microbiome supplements and improves human metabolism and nutrient absorption by degrading of indigestible compounds such complex polysaccharides, especially those from plants [22]. The result is a mutually beneficial partnership: energy that would otherwise be wasted is harvested by the microbiota, and the host is able to absorb some of the metabolic byproducts. Another major function of the microbiome is its network of interactions with the intestinal epithelium, the barrier between the gut luminal contents and the underlying immune cells. The complex homeostatic processes between host and microbiome crucially involves the mucosal layer and innate immune system to regulate the commensal microbiota while preventing invasion or overgrowth of pathogens [23], indicating a delicate interplay between the host and the microbiota. Importantly, gut microbes can modulate intestinal permeability and junctions between epithelial cells, which, in a disease state, can break down, leading to unwanted translocation of substances across the gut barrier, termed "leaky gut" [24]. In addition, gut microbiota can moderate the immune system even as it acts upon them [25]. This is the interface where pathogens are often recognized and re-

jected, or conversely, take hold, so it is key that these systems work correctly. A healthy microbiome moderates immune responses and gut physiology to exclude pathogens without over or under-stimulating the immune system or damaging the gut. This is especially important developmentally, as the effects of early life dysregulation may be persistent and likely increase the risk of disease later in life [26].

Perhaps one of the most surprising major functions of the microbiome is its interactions with the nervous and neuroendocrine systems, a set of pathways known as the gut-brain axis. The gut-brain axis encompasses communications acting locally via the gut walls (and connected blood vessels) and the enteric nervous system (including the vagus nerve) to ultimately affect even central nervous system functioning [27]. Furthermore, this signaling is strongly suggested to be bi-directional; for example, stress-associated gut alterations can increase the proliferation and virulence of pathogens [28]. This link is critical developmentally, with anxiety and motor control neural circuits being especially implicated [29].

The microbiome is additionally crucial in pathogen resistance. This is most obvious in infections contracted following antibiotic use; the microbiome is significantly disrupted allowing for pathogens to take hold. These pathogens are either externally acquired or are members of the normal microbiome that become pathogenic (pathobionts). There are several mechanisms by which pathogens flourish. Immune dysregulation can occur; for example, communication of T lymphocytes with the microbiota is impaired. Direct microbial colonization resistance is hampered as well with lower cell densities, less competition for resources, and less production of pathogen-inhibiting chemicals allowing for growth [30]. While they are more difficult to study and determine, disease states, especially inflammatory bowel disease (IBD) [31], and general dysbiosis [32], may allow for pathobiont expansion through similar mechanisms. Interestingly, IBD symptoms are reduced if bacterial load in the intestine is decreased through antibiotics, supporting the aforementioned hypothesis on the role of pathobionts [33]. Clearly, given their profoundly important roles in human health, microbiome states have been shown to cause or correlate with a number of diseases, which include

autism, cancer, chronic heart disease, diabetes, IBD, liver disease, and obesity [34]. Thus, there is a great health need to develop treatments for these diseases, and microbiome-based interventions present a promising opportunity.

1.2 Microbiome techniques and technologies

The technologies for growing and querying bacteria in both the natural environment and the human body are necessarily strongly tied to the discovery and development of microbiome science. The earliest techniques for culturing the human microbiota were crude, especially with respect to survival of oxygen intolerant anaerobes, which make up the vast majority of the microbiome. It wasn't until the 1970s when what are considered to be the first proper explorations of the human gut microbiome took place, following significant advancements in anaerobic techniques as well as identification methods [35]. Not only were studies done detailing the composition of the microbiome [36] but intriguing work on diet [37] and even a tentative gut-brain connection [38] proceeded shortly after. However, a significant problem remained: the vast majority of gut microorganisms were still unculturable, despite many more of them being observable under a microscope. This has been termed the "great plate anomaly" [39] and remains a significant problem even today. However, in the 1990s, a revolution began in our understanding of the human microbiome: the development of culture independent techniques able to describe microbiome compositions as they are in vivo [40]. These techniques mainly focus on the 16S rRNA gene present in all bacteria (and archaea, chloroplasts and mitochondria). This gene contains both highly conserved and highly variable regions, allowing for extremely general probes that can nonetheless differentiate microbes to a species or even strain level. Due to the slow evolution of this gene and developed computational analysis techniques, it is therefore possible to construct phylogenies and characterize bacterial sequences from both cultured and uncultured bacteria by preferentially amplifying and querying this gene [41].

In one of the first large scale 16S rRNA studies, a huge amount of uncultured bacte-

rioplankton were found in the Sargasso Sea, and a novel group was observed, expanding ecological understanding of that habitat [42]. Since then, innovations such as next generation sequencing (NGS) techniques have lowered the price of sequencing to the point that a veritable explosion in microbiome sequencing studies took place [40]. These include numerous detailed studies of the gut microbiome, which form the basis of our understanding of that ecology. However, simply knowing what microbes are present hardly explains the microbiome. A better understanding is often framed as three questions that can be answered to varying degrees by existing technologies: who's there, what can they do, and what are they doing [43]. 16S sequencing does much to answer who's there, though other technologies such as qPCR and FISH can supplement it with more specific microbe targeting [44].

The capability of members of the microbiome of influence their host (what can they do), also called functional potential, is explored through genome sequencing to construct metabolic and other pathways. This can be done on cultured bacteria relatively easily, though these bacteria are a small fraction of the human microbiome. Furthermore, culture-independent single-cell isolation techniques where the cells aren't required to propagate have been developed, such as microfluidics and flow cytometry [45]. Notably, functional potential makes up a large part of explicating the chemistry of the microbiome, since it at minimum requires only 16S sequences for actionable insights to be generated [46]. However towards this end, whole genomes can also be characterized and genes identified rather than inferred [47]. In the case of complex communities, this is known as metagenomic analysis, whereby DNA from a sample is amplified in an unbiased fashion and genomes are reconstructed bioinformatically from the resulting data [48]. Metagenomics is currently a promising though expensive and computationally intensive process.

Techniques for uncovering expressed function (what are they doing) of whole microbiomes and of specific microbiota within those communities include metabolomics, metatranscriptomics, and metaproteomics. These omic techniques are named after what they interrogate: metabolites, RNA transcripts, and proteins. While metatranscriptomics [49] and especially

metaproteomics [50] are theoretically powerful and likely necessary techniques for a full understanding of in situ function, they are in their infancies with regards to the microbiome, requiring further methodological and technological improvements to be feasible and economical. Metabolomics is quickly becoming a widely adopted technique with significant successes, though it is still somewhat more of a specialized technique than sequencing approaches [51].

In this thesis,16S was the modality by which microbiomes were assayed; therefore we focus on the analysis that can be done with this technology. When performing a microbiome 16S survey, first the DNA is liberated from the sample through chemical and physical means dependent on the sample type (stool, soil, etc.) [52], a region (V4 in our protocol [53]) is carefully amplified via PCR [54] to reach a usable concentration, and the sequences are barcoded by sample for identification. Then, this is sequenced, reading out the physical basepairs onto a computer, in our case via Illumina Miseq technology using V3 chemistry following the Earth Microbiome Project protocol [55]. For an overview on the chemistry of high throughput sequencing see [56] and for a discussion of the biases and error profiles of this specific platform see [57]. What results is simply a list of every sequence in the sample and how many times it occurs.

This then requires substantial computational work to get into a form which is human readable and where conclusions can be drawn about the composition and function of the microbiome. A great number of bioinformatic pipelines and various software packages exist which can be utilized towards this end. While a comprehensive review is beyond the scope of this document, the packages and techniques utilized in this thesis work are summarized briefly in the methods section.

1.3 The microbiome through a chemical lens

Ultimately a fruitful and often under-appreciated way to frame the above insights and concepts is to regard the microbiome as a chemical-ecological system. In traditional ecology, communication between organisms and between the environment and organism occur primar-

ily at the macroscopic scale. But as the microbiome is a complex system of microorganisms interacting with an environment often at the microscopic scale, direct chemical interactions are paramount. Chemicals rather than sensory organs determine how microbes perceive the environment and likewise rather than emitting sounds, visual cues, etc. microbes communicate through direct chemical means [58]. This includes interactions such as antimicrobials produced to compete with other microbes, cross-feeding, and alterations in availability of metabolites in the extracellular space [59]; notably these can as a side-effect affect a host organism and in some cases these natural products isolated [58]. However, this is not to say chemical signaling are absent coming from higher organisms, for example coral reefs produce defense chemicals against consumers [60], and of course the host of the microbiome in human and animal systems abundantly utilizes chemical interactions, including with the microbiota. Thus, the environment and microbial chemistries define the overall ecology of the system in complex ways. An example of such an interaction is early life colonization with *Bifidobacterium longum* enables the processing of some complex carbohydrates altering the immune homeostasis as reflected in the large changes in prostoglandin E2 and bile acids [61]. In addition to interacting with each other and the environment though chemical means, the microbiota can also interact with external perturbations. One major source of ecological impact is endogenous chemicals, known as xenobiotics, which the microbiome can modify directly or change their action. This has significant impacts on pharmacochemistry as the action of a drug can not always be predicted solely by the host metabolism [62]. One class of xenobiotics of particular interest is prebiotics, compounds which act on the microbiome primarily (rather than the host) to promote the growth of specific members, shifting overall ecology [63]. One chief goal of chemical understanding of the microbiome is to predict these changes in xenobiotics (including computationally) so as to understand what the host ultimately receives [64].

Furthermore, live ingested microorganisms themselves can be considered complex drugs [65]. Etymologically (and in many ways functionally) contrasting antibiotics, these are re-

ferred to as probiotics [66]. However, as they are live organisms, probiotics pose unique challenges and novel possibilities compared to traditional drugs. The mechanisms by which probiotics achieve their therapeutic properties are varied but can generally be divided into two factors: "adaptation" and "probiotic". The microbes survive transit through the gastrointestinal tract, adhere in the intestinal mucosal layer, are adapted metabolically to thrive on available nutrients, and survive among other microbiota. Often these adaptations are beneficial to the host, such as in the case of lactic acid bacteria where produced lactic acid reduces competition allowing for establishment in the gut by inhibiting other microbes, in particular some pathogens, leading to overall increased host health [67]. Additionally cell surface proteins presented when adhering to a host can often have immunomodulatory effects, which can be therapeutic in nature [68]. The most prominent directly "probiotic" reaction is the production of short chain fatty acids (SCFAs), which have profound systemic effects, including roles in appetite, gut integrity, glucose homeostasis, lipid metabolism, and immune function [69]. Positive effects have even been reported on neural functioning and plasticity [70]. This is supplemented by gut microbiota's ability to produce and transform numerous neurologically active metabolites, including gamma-aminobutyric acid, histamine, serotonin, and tryptophan [71]. In addition, gut microbiota synthesize a number of vitamins that can be utilized by the host, transform bile acids, and ultimately produce metabolites in ways still being discovered and explored [51]. Lastly, the microbiome may also mediate gaseous compounds within the host, affecting host physiology [72].

1.4 Understanding the chemistry of the microbiome

Currently in microbiome research, we unfortunately know not enough about the chemistry driven functional roles of microbes within certain ecologies, the chemical mechanisms of microbiota interactions with itself and the host, and the ecological and chemical effects of microbiome compositional change. Therefore, the chemistry of the microbiome is a subject of interest and necessary to understanding the microbiome as a whole. A powerful tool for

expanding our knowledge in this space is case studies. In this thesis is presented three such studies.

Firstly, a rat model of hyperplasia is explored with respect to microbiome remodeling following cohousing. In this experiment, rats of a strain susceptible to arterial inflammation and hardening following surgery were allowed to live in cages with rats more resistant to this injury. We found that simply by this cohousing procedure, the microbiome was altered in the susceptible strain such that hyperplasia was significantly reduced. This was then correlated with microbiome changes to explore the taxa and resultant chemical changes that may be driving this process.

Secondly, an aggressive Alzheimer's disease mouse model was found to have significantly better outcomes when dosed with antibiotics. This process was furthermore seen to be gender dependent, and it is known that Alzheimer's progression in humans is likewise influenced by gender. Excitingly, the palliative effect of antibiotics could be reduced by transplantation of non-treated stool into the antibiotic treated mice. I explored, via functional gene profiling, ways in which these changes may have been effected, leading to actionably hypotheses which can be tested in future experiments.

Lastly, antibiotic treated rats were orally gavaged with a γ-aminobutyric acid supplement in an attempt to reduce anxiety-like behaviors, a controversial but possibly observed effect in humans. These were not observed in the current experiment, possibly due to noisy measurements. However, subtle but possibly important shifts in the microbiome were seen leading to a hypothesized change in chemical production by the microbiome (namely SCFA). This opens the door to exploring a drug which may directly affect the microbiome in an unexpected non-antibiotic manner.

Thus we observed how environmental changes, classically microbiome targeted drugs (antibiotics), and microbiome affecting xenobiotics can change the microbiome and consequently the possible chemistry of the microbiome ultimately leading to changes in disease state and maybe even behavior. This then represents a solid foundation for additional chemical stud-

ies of the microbiome, an aspect which should be considered vital for its functioning and host-microbiome interactions. Following these chapters, a review of microbiome precision medicine is presented, providing a context for which to understand the current experiments and the field as a whole.

In the future, we would like to validate these findings and gain a deeper understanding of the chemistry of the microbiome through additional omic techniques, especially measuring metabolites, to directly assay chemical changes we expect to see and through deeper theoretical models to better predict the action and dynamics of the microbiome. Ultimately, when combined with animal models and human studies, these types of studies and techniques can advance the discovery and development of both microbiome science and the chemistry of the microbiome, ultimately with the goal of translating these discoveries to the commercial or (personalized) therapeutic spaces.

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CHAPTER 2

METHODS

2.1 Bioinformatic methods for 16S sequencing

Raw reads were processed in Quantitative Insights Into Microbial Ecology 2 (QIIME2) [1], an end to end pipeline for analyzing microbiome data. It was chosen for it's ubiquity of use, well tested and maintained open-source code, standardization across projects, backwards compatibility with other formats and pipelines, and portability between research labs. Furthermore, QIIME2 data is wrapped in "artifacts" which are zip files containing the data and the provenance metadata indicating all previous bioinformatic steps taken, making reproducibility and documentation easier.

First, the reads are paired and demultiplexed. This involves matching forward and reverse sequences then mapping these barcoded, paired sequences to the sample from which they came through a sample metadata file. Paired sequences are used because generally speaking, sequence quality drops off toward the end of a read, especially true for the reverse read. The higher sequence quality at the start of both forward and reverse reads can be used to correct poor-quality bases toward the ends of the reads, generating a combined high-quality sequence. Barcodes and other non-biological portions (such as primers) of the sequences are then removed or masked.

Next quality control is performed. In QIIME2 this is done as part of the Divisive Amplicon Denoising Algorithm 2 (DADA2) denoising package [2]. First, poor quality base calls are dropped by the user by trimming the start and end of the sequences where it seems appropriate. For paired end sequences, this is less necessary and here no trimming was generally considered to be required. PhiX sequences, which are added to the samples to improve sequencing quality, are then removed [3]. Chimeric sequences, those which are composed of two organisms, are identified if they can be exactly reconstructed by combining a left-segment and a right-segment from two more abundant parent sequences and then removed.

DADA2 was chosen for denoising as it tended to find more ESVs than the other popular pipelines (including QIIME2 supported deblur) when analyzing both a real soil dataset and two other host-associated datasets, which suggests that it could be better at finding rare organisms, but at the expense of possible false positives [4]. Denoising models the errors introduced during sequencing, and uses that error model to infer the true sample composition. This replaces the traditional "OTU-picking" step in sequencing workflows [5], whereby sequences are clustered into operational taxonomic units (OTUs) based on sequence similarity, producing instead higher-resolution tables of exact sequence variants (ESVs) which are then identified taxonomically via subsequent OTU clustering or Basic Local Alignment Search Tool (BLAST) [6]. Using ESVs allows for better data sharing between studies by using exactly identifiable sequences, more accurate measurements of diversity by not losing diversity artificially through clustering methods, and less loss of data by clustering without keeping original sequences attached compared to OTUs [7].

Once the sequences are thus in their final quality controlled form, biologically relevant inferences can be made. Comparisons of diversity between samples is often the first step, namely alpha and beta diversity. Alpha diversity measures the diversity at a local scale. This variously interpreted as measurements such as the richness [8], evenness [9], or entropy [10], but intuitively it represents the effective number of species (or sub-species in the case of ESVs) in a sample. Beta diversity measures the ratio between regional and local diversity and can be referred to as the differentiation among habitats. It is thus useful for comparing sample composition in a way that reflects how similar the ecologies of samples from different environments are. The most commonly used diversity metric is unifrac which calculates the distance between pairs of samples by placing all taxa on a phylogenetic tree and then marking which branches leading to these taxa are shared between samples [11]. This can be weighted by abundance to limit the influence of possibly outlier low abundance microbes which additionally are often phylogenically more dissimilar than shared higher abundance microbes. Differences in beta diversity can then be visualized by principal coordinates anal-

ysis and statistical significance determined by Monte Carlo simulations (both implemented in QIIME2).

After measuring diversity, which is done on ESVs to best preserve observed diversity, ESVs are clustered into OTUs so that they can be identified and statistics and techniques can be done at the OTU level if desired. Clustered OTUs are small sets of ESVs with closely related sequences that are named by similarity to a database, such as GreenGenes [12]. This annotation gives a species or higher (when not available) level name to the ESV and subsequent cluster so that researches know what taxonomy is represented by the DNA sequence. Members of these OTUs should in theory have similar behaviors so downstream steps are often done on both ESVs and OTUs (especially since at the OTU level power is increased since less comparisons are being made that must be multiple comparison corrected). This is performed in QIIME2 via a specialized machine learning package [13] or in uclust [14] when closed reference, where reads that are not hits against a reference database are discarded, OTUs are needed.

In addition to diversity, differences in distributions of individual ESVs or clustered OTUs are of great interest to the investigator. One of the most powerful techniques for determining differentially abundant microbes which can then be focused on for analysis (though culturing or known characteristics through the literature) is Analysis of Composition of Microbiomes (ANCOM) [15]. This statistical framework importantly accounts for compositional constraints in microbiome data (namely that sequencing depth is not the same for either sample, often leading to observed differences in abundance where there are none) by modeling the underlying structure in the data through log-ratios rather than raw numbers of sequences before comparing the microbiome compositions. The compositionality of microbiome datasets is a long running issue with analysis, and ANCOM represents a strong step towards addressing this issue, performing better than many normalization techniques which are known to introduce certain biases when trying to correct for sequencing depth differences [16]. An alternate, older approach to handling data is to rarefy, or randomly subsample the

data, so that equal sequencing depth is simulated. This is an easy way to approach the problem at the expense of throwing out data. Nonetheless, it remains a valuable approach for its simplicity and the ability to use standard, better understood statistical tests on the resulting data, such as Kruskal-Wallis (KW) analysis [17]. We have found this is useful for generating hypotheses in situations where ANCOM does not have sufficient power to resolve any differentially abundant microbes and where normalization techniques should be avoided due to possible high false positive rates and bias.

From here, a multitude of differing bioinformatic approaches can be utilized to further explore the data, such as network theory approaches [18] and longitudinal data analysis [19]. Here we primarily focused on functional gene approaches. These infer the gene content of the microbiome so that this can be compared between samples and inferences made about what the microbiome is capable of doing metabolically. Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) takes closed reference OTUs and uses ancestral state reconstruction to make predictions about the genomes of unsequenced microbes from closely related ones (as opposed to traditionally ancestral organisms from living organisms). This step is pre-calculated allowing for quick resolution of predicted metagenomic content from 16S sequences in addition to the ability to backtrack and output which microbes contributed to which gene hits [20]. Piphillin uses ESVs to perform functional inference without the aid of phylogenetic trees. This has the advantage of working with sequences independent of closed reference database. allowing for more diverse sample types and constantly updated genomic information to be used. However, from laboratory animal samples, no performance advantage was observed for Piphillin over PICRUSt, so choice was made based on what best suited the individual experiment [21].

Essentially, these bioinformatic steps take raw sequences from a sample and result in a picture of how ecologically distant each microbiome is so that meaningful sample groupings can be made, what microbes are differentially represented in samples so that these can be further interrogated, and what genes might be present in differing microbiomes to ultimately

produce different metabolites and effects on the host. This represents a pipeline from raw data to insights on the chemistry of the microbiome in varying environments and experimental conditions, which then inform thinking about measured phenotypes and behavior as well as follow up experiments.

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CHAPTER 3

MICROBIOTA COMPOSITION MODULATES INFLAMMATION AND NEOINTIMAL HYPERPLASIA AFTER ARTERIAL ANGIOPLASTY

3.1 Introduction

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Atherosclerosis, hardening and narrowing of the arteries, is a leading cause of morbidity and mortality globally, with cardiovascular disease (CVD), primarily coronary and cerebrovascular atherosclerosis, causing over 800,000 deaths in the US in 2014 [2]. Currently, many interventions are done to treat atherosclerosis, such as balloon angioplasty, but these interventions often fail with surgery related narrowing of the arteries. This is due to pathological vascular remodeling because of proliferation and migration of vascular smooth muscle cells in the tunica intima layer. This results in vascular wall thickening and the gradual loss of luminal openness, restenosis, which may lead to the return of vascular symptoms fostered initially by atherosclerosis. Neointimal hyperplasia, the mid-term phase of restenosis, is unfortunately common, affecting 20-50% of patients in the first year after revascularization treatment [3]. This is in contrast to atherosclerosis which takes many years to manifest.

In experimental rat and mouse models, differences in neointimal hyperplasia after arterial injury have been ascribed to genetic background, which determine extracellular matrix formation, vascular fibroelastic content, wall fragility, and other biological processes [4]. Chemically, not much is known about the etiology of neointimal hyperplasia. Nitric oxide likely plays a role; it was shown to be a potent stimulator of guanylate cyclase to form cyclic guanylate monophosphate and cause relaxation of vascular smooth muscle cells. Otherwise, there are not many candidates for chemical effectors of neointimal hyperplasia.

However, the Ho lab at Northwestern University, who we are collaborating with here, is

advancing the study of metabolites with respect to neointimal hyperplasia and have found targets of interest. Some of these metabolites were indole and phenyl derivatives, such as kynurenine and tryptophan [5]. Futhermore, they previously advanced the novel concept that restenosis is inversely associated with the synthesis by intestinal microbiota of the short chain fatty acid butyrate, which has anti-proliferative and anti-migratory effects on vascular smooth muscle cells [6]. Short chain fatty acids produced by gut microbiota are known to have wide-ranging interactions with host physiology, including modulation of the peripheral vasculature by inducing vessel relaxation and lowering blood pressure [7]. This represents a strong step in the direction of exploring the chemistry of neointimal hyperplasia, especially microbially produced compounds.

We thus hypothesize that the arterial remodeling response can be modulated more broadly by commensal microbes. The gut microbiome represents a dynamic ecosystem that is shaped by host diet composition and complexity, maturation of the immune system, host genetics, lifestyle, social interactions, environmental exposures, and antibiotic therapy [8]. The microbiome may produce or consume metabolites affecting neotiminal hyperplasia. It is, for example, heavily involved in the regulation of tryptophan metabolism [9]. Importantly, the microbiome helps regulate and affects immune function [10], which can then propagate to advance or hinder neointimal hyperplasia, with an anti-inflammatory milieu benefiting the patient [11].

To elucidate the complexity of microbiome-microbiome interactions in the context of a viable mammalian host, I chose (through collaboration with Dr. Ho's laboratory) to examine whether the exchange of microbes between rats with differing phenotypes of neointimal hyperplasia phenotypes and differing intestinal microbiota influenced their neointimal hyperplasia phenotype. Thus, the purpose of this study is to investigate whether we could alter neointimal hyperplasia by cohousing and mixing bedding of rats with known robust neointimal hyperplasia responses and rats with reduced arterial remodeling responses. Changes in the remodeling phenotype could then be correlated with shifts in the microbiome.

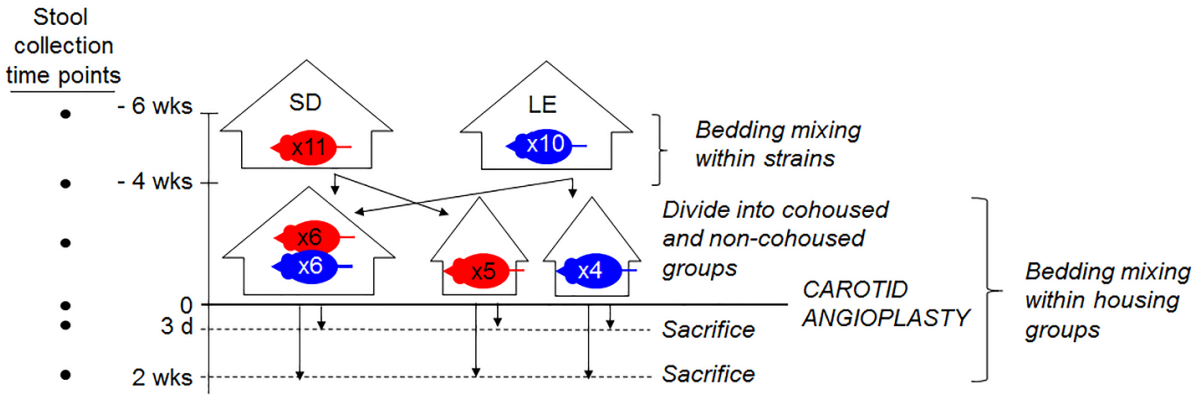

Figure 3.1: Experimental schema. Bedding mixing among cages of similar rat strains (Lewis [LE] and Sprague-Dawley [SD]) was carried out for 2 weeks. Thereafter, rats were divided into cohoused and non-cohoused groups for 4 weeks, followed by carotid angioplasty and then sacrifice at either 3 days or 14 days post-angioplasty. Stool samples were collected at time points designated on the left.

3.2 Results

3.2.1 Strain differences in hyperplasia and related metrics

In order to equilibrate initial animal housing conditions, bedding (including stool pellets) from all cages within one strain were intermixed 3 times per week for 2 weeks. A cohort of LE and SD rats were then cohoused (CH) (separated and then re-caged with the opposing strain), while a separate cohort of LE and SD rats (non-cohoused [NCH]) remained in cages segregated by rat strain (see experimental schema in figure 3.1). Bedding intermixing continued thereafter among cages in the same housing groups. After four weeks, all animals underwent left carotid artery balloon angioplasty; the right carotid artery served as the uninjured control artery. Animals were sacrificed for tissue and blood collection either 3 or 14 days after carotid angioplasty. Stool samples were collected upon arrival to the animal facility, prior to the cohousing period, 2 weeks after the start of the cohousing period, on the day of carotid angioplasty, and on the day of sacrifice. Freshly collected stool pellets were immediately frozen and stored at -80C until use in 16S sequencing pipeline.

All animals underwent left carotid artery balloon angioplasty using a 2-French Fogarty

catheter and at the appropriate time point, rats were euthanized and preserved with carotid arteries fixed and cryoprotected. Whole blood was collected by cardiac puncture prior to perfusion fixation at the time of sacrifice. Carotid arteries were examined for neointimal hyperplasia after hematoxylin-eosin staining of artery sections at evenly-spaced 350-μM intervals.

As anticipated from prior work by others demonstrating differences in neointimal hyperplasia development due to strain differences [4], arterial remodeling 14 days after carotid angioplasty differed significantly between NCH LE and SD rats, as assessed by intima area, media area, intima+media (I+M) area, and intima area/intima+media area. Cohousing modulated these differences (Figure 3.2 a and b). CH LE rats had significantly more neointima area compared to NCH LE counterparts. In contrast, CH SD rats had significantly less neointima area compared to their NCH SD counterparts. When we compared I+M area as a measure of neointimal hyperplasia, there was a slight difference between LE rats by housing group that did not reach statistical significance, but CH SD rats again had less I+M area compared to NCH SD rats. Representative photomicrographs of hematoxylin-eosin-stained post-angioplasty carotid arteries from each rat strain and both housing groups are shown in figure 3.2 c (top). Of note, there was no qualitative histological difference between the uninjured right-sided carotid arteries of LE and SD rats (Figure 3.2 c, bottom).

To determine if the observed changes in neointimal hyperplasia were due to altered cellular proliferation in the arterial wall, Ki67 immunoreactivity in the carotid arteries of NCH and CH LE and SD rats was compared 3 days after carotid angioplasty. There was more Ki67 staining in the medial and adventitial layers of NCH SD rats compared to LE rats. There was no significant difference in Ki67 staining in the intimal or medial layers between the NCH and CH groups of either rat strain. However, there was significantly more adventitial Ki67 staining in the CH compared to the NCH LE rats and significantly more adventitial Ki67 staining in the NCH SD rats compared to the CH rats, corresponding to the pattern of neointimal hyperplasia at the 14-day time point.

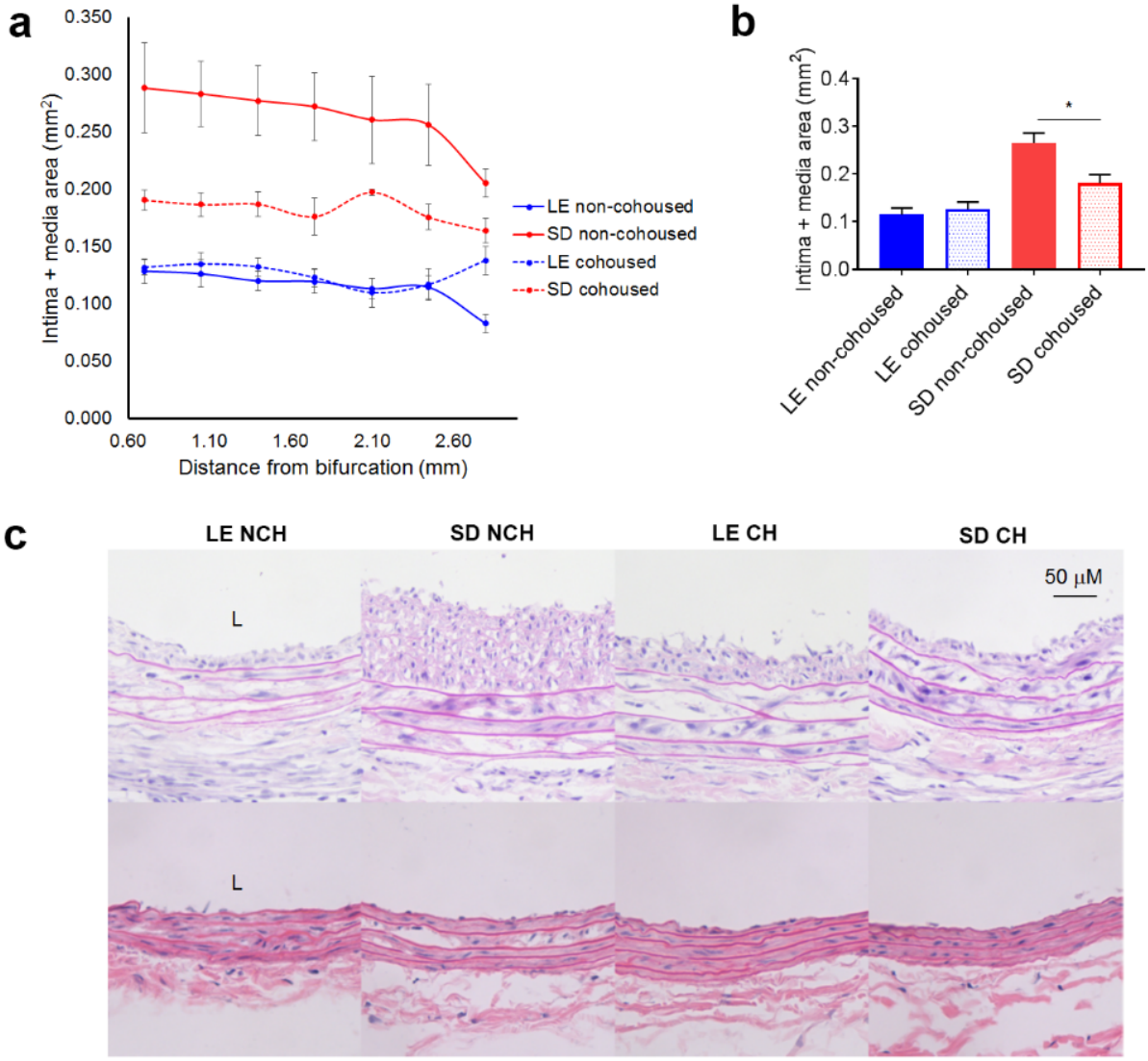

Figure 3.2: Neointimal hyperplasia 14 days after carotid angioplasty in NCH and CH LE and SD rats. a Differences in mean intima + media area (mm2 ± SEM) along the entire length of the angioplastied carotid artery in NCH LE and SD rats (solid lines) were attenuated upon cohousing (dotted lines). b Mean intima and media area (mm2 ± SEM) of NCH and CH LE and SD rats. *, P<.001; Mann-Whitney U test. c Representative cross-sectional photomicrographs of NCH and CH LE and SD rat carotid arteries after carotid angioplasty (top). Un-injured right carotid artery sections are grossly similar (bottom). Original magnification 40x. Scale bar, 50 μm. N=6-8 rats per group.

With the exception of IFN-γand IL-17A, circulating cytokines, chemokines, and growth factors were not detected. There were no significant differences between NCH LE and SD rats at either the 3-day and 14-day time points, and IFN-γdid not discriminate between housing group within each strain at either time point. IL-17A was significantly higher in the NCH SD group than the NCH LE group at 3 days but not at 14 days. NCH and CH LE rats had similar IL-17A serum concentrations at both time points. However, NCH SD rats had higher IL-17A concentrations at 3 days compared to CH SD rats, a difference that was no longer seen at 14 days, suggesting that early increased IL-17A in SD rats is associated with greater neointimal hyperplasia, a response that is attenuated upon cohousing with LE rats.

3.2.2 Composition of rat fecal microbiome

NCH SD and LE rats had microbiomes significantly differentiated by both weighted (P=.012) and unweighted (P<.001) unifrac beta diversity distances, though without significant differences in alpha diversity (P=.43). This is indicative of similarly rich microbiota that are nonetheless distinct in composition between rat strains. The baseline between-strain microbiome composition differences are summarized in figure 3.3. Note that the differentially abundant OTUs between these strains are not entirely consistent across experiments and investigators due to external variables such as handling and vendor [12], so what we observed is likely not generalizable to all SD and LE rats.

3.2.3 Reshaping the fecal microbiome by cohousing and bedding mixing

Bedding mixing and cohousing of rats in the same treatment group reduces fecal microbial variation between individuals via coprophagy and direct animal-to-animal contact. This results in an increased similarity in microbial structure and composition and is an accepted method of normalizing microbiota communities in mice [13, 14]. To probe the role of commensal microbes in regulating neointimal hyperplasia, we cohoused LE and SD rats for 4

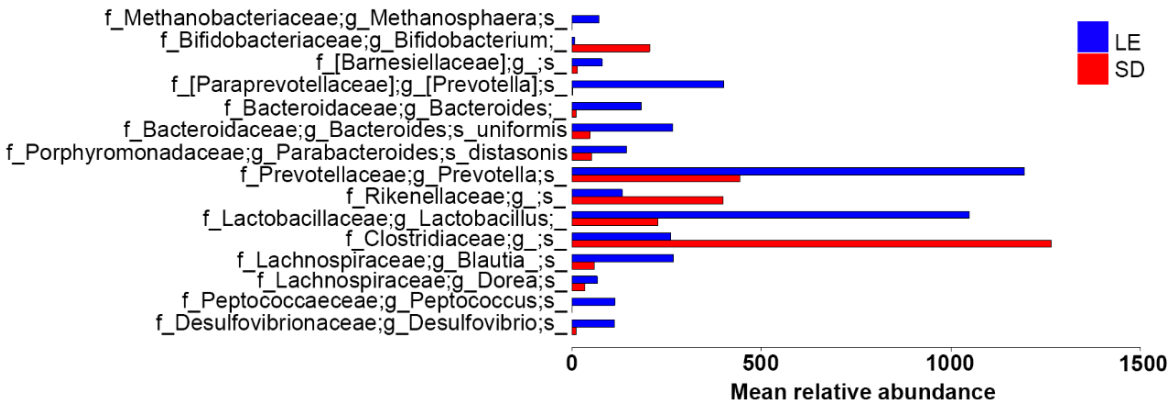

Figure 3.3: Relative abundances of differentially abundant OTUs at baseline (prior to cohousing). Relative abundances were defined by the Benjamini-Hochberg multiple comparison corrected Kruskal-Wallis test with FDR P<.05 using samples collected prior to any manipulations.

weeks to modulate their microbiome composition, and mixed bedding within experimental groups to reduce cage effects, before performing carotid angioplasty.

Over the course of the experiment, the microbial community composition and structure was shifted, associated with cohousing status. Although alpha diversity and weighted unifrac beta diversity were not significantly correlated with cohousing, unweighted beta diversity was significantly different between all groups (PERMANOVA P<.05). This is indicative of changes in the presence of relatively rare microbes after cohousing, rather than changes in the relative abundance of numerically dominant taxa. Significant differences between strains at baseline were observed in both weighted and unweighted beta diversity, which was consistent across the experiment in non-cohoused groups, indicating a distinct microbial makeup of the 2 strains (P<.05). The difference in unweighted beta diversity between the fecal microbiota of SD and LE rats was significantly reduced by cohousing (see principal component analysis in figure 3.4 a), showing a convergence in the microbiota along the first principle axis (13% of variance explained). Strikingly, while an SD rat cohoused with LE rats became significantly more similar to LE rats, an LE rat cohoused with SD rats stayed more similar to LE rats than SD (Figure 3.4 a). This asymmetry might be attributed to a more

stable LE microbiome, where new taxa cannot as easily take residence, and/or behavioral differences (primarily coprophagia). To test this hypothesis, OTU co-occurrence networks were constructed via SPIEC-EASI for the 2 rat strains at baseline. While the 2 networks were similarly dense, measured by degree distribution, (Figure 3.4 b), progressively removing nodes revealed a lower natural connectivity in SD rats (Figure 3.4 c). The connectivity is defined by the eigenvalue of the adjacency matrix of the shrinking graph and accesses the stability of the network. This is indicative of an interaction network of microbes that is relatively similar but less robust to change; a result that corroborates the observation that the SD rat microbiome shifts more dramatically with cohousing.

A total of 17 OTUs were significantly different between the four animal groups (cohoused or non-cohoused LE and SD; Figure 3.5), and as expected from beta diversity analysis, the majority were low abundance taxa. Of these, Lactobacillus increased the most in cohoused LE rats and Methanosphaera increased the most in cohoused SD rats.

3.2.4 Correlation of relative abundance with neointimal hyperplasia

Mantel tests of average I+M areas with beta diversity were performed, and concomitant with above, only unweighted beta diversity was significantly differentiated by cohousing ($R=.38$; $P<.001$). Correlation between average I+M area and the relative abundances of OTUs on the day of surgery and day of sacrifice were performed; importantly, no significant difference in microbial composition was found between the day of surgery and the day of sacrifice, indicating recovery of the microbiome from possible perturbation due to surgery. At sacrifice, the relative abundances of 7 OTUs were significantly different between groups and highly significantly correlated with average I+M area per rat (Spearman $r>.55$, $P<.05$; Figure 3.6), including *Parabacteroides distasonis*, *Desulfovibrio*, *Methanosphaera*, *Peptococcus*, and *Prevotella*. Stratifying the analysis by comparing only within rat strains across housing conditions resulted in no significant correlations at the OTU level, likely due to a loss of statistical power and the low relative abundance of the important OTUs. Following OTU

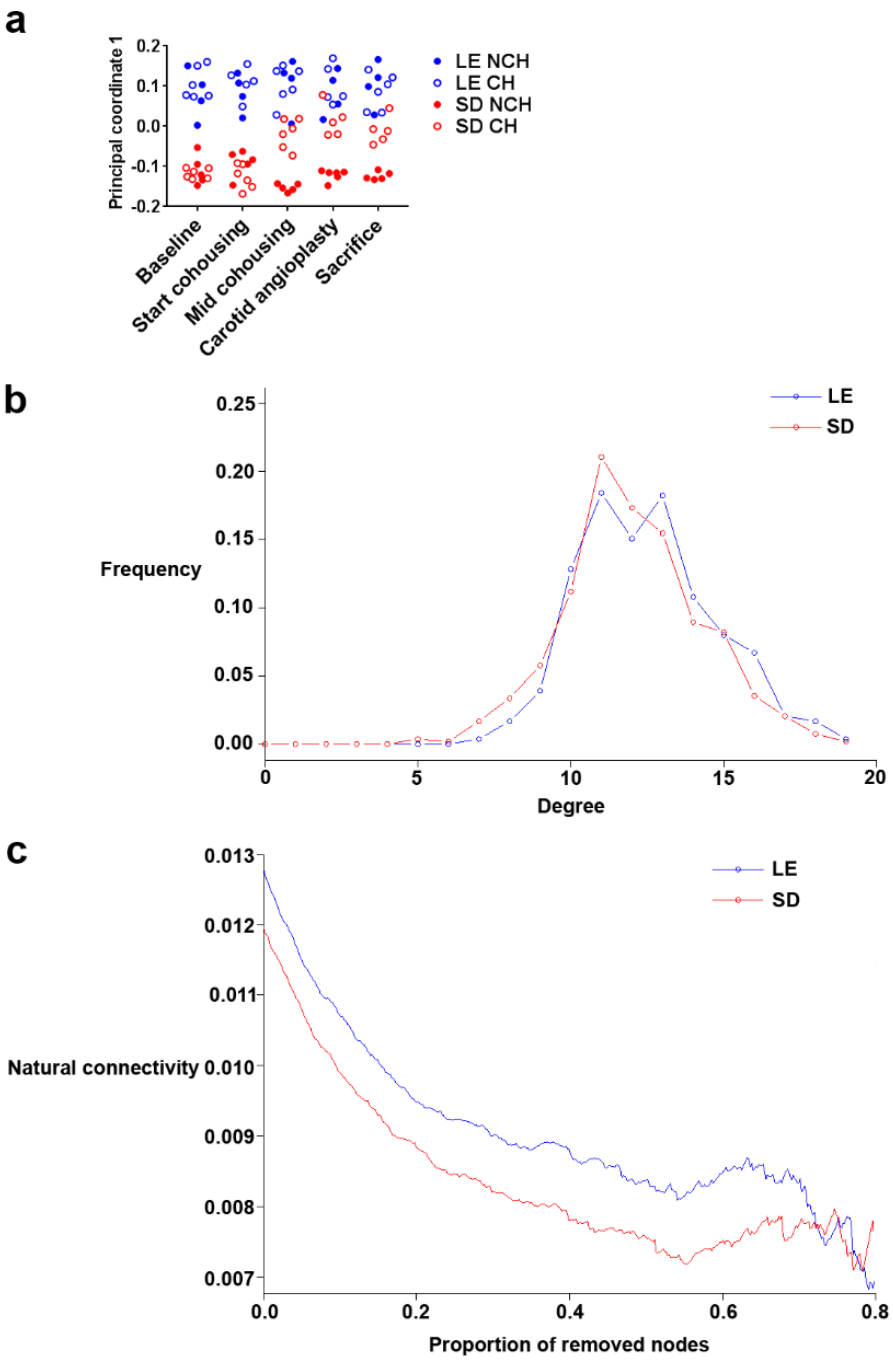

Figure 3.4: Microbial diversity shifts between experimental timepoints and possible basis in baseline microbiome stability. a: Principal coordinate analysis of unweighted unifrac beta diversity of microbiome samples in both strains and housing groups across sampling times. The first component (principal coordinate 1) is shown and explained 13% of the total variance. b: Degree distribution of the OTU co-occurrence graph created by SPIEC-EASI on baseline time point samples. c: Natural connectivity as a function of proportion of removed nodes of OTU co-occurrence network of SD and LE rats at baseline time point.

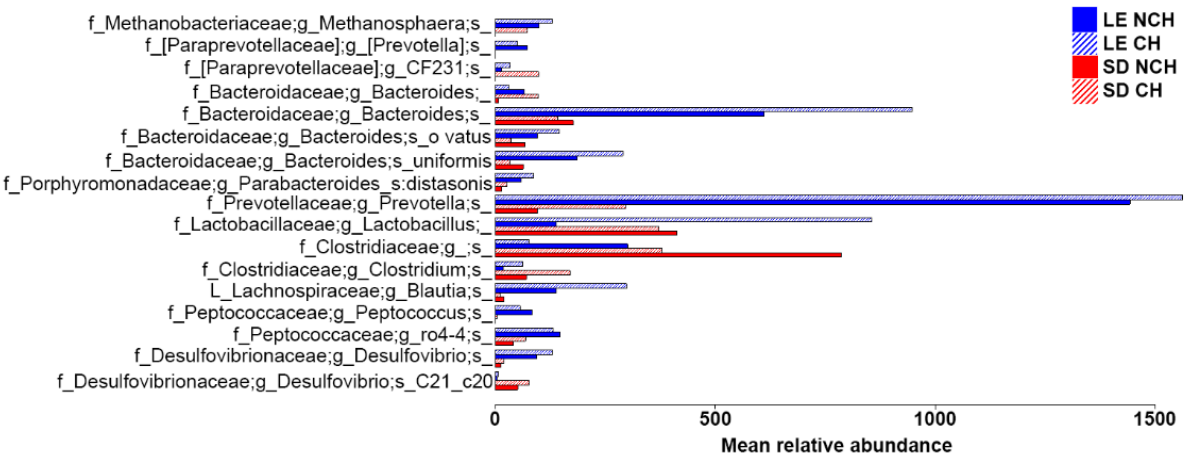

Figure 3.5: Relative abundances of OTUs differentially abundant across experimental groups at the time of sacrifice. Relative abundances were defined by raw 16S read counts and differences between groups found by the Benjamini-Hochberg multiple comparison corrected Kruskal-Wallis test with FDR P<.05.

denoising (DADA2), 5 subOTUs significantly correlated with I+M area and were identifiable at >96% 16S sequence similarity via BLAST (Spearman r>.7; P<.01; Figure 3.6). These subOTUs represent individual 16S sequences instead of multiple sequences clustered into OTUs based on similarity, which increases the specificity of results and in some cases lead to identification of an exact microbe to culture, at the expense of often reduced statistical power. Three of these, *Muribaculum intestinale*, *Peptococcus CF166*, and *Ruminococcus NK4A214* correlated across all groups. Two subOTUs correlated significantly with I+M area in SD rats alone: *Ruminococcus bromii* (Spearman r= .92;P=.001) and *Lactobacillus murinus* (Spearman r=-.85; P=.03). Lastly, the relative abundance of *Sutterella spp.* (Spearman r=-.94; P<.001) and *Ruminococcus flavefaciens* (Spearman r=-.84; P=.002) in SD rats were highly significantly correlated I+M area on the day of surgery, but not on the day of sacrifice. This strong correlation is surprising given that the overall microbial compositions are consistent between these time points as indicated by Kruskal-Wallis testing. This indicates that the overall distribution of these OTUs did not change, but the relative abundances associated with specific rats did in a way that removed the correlation with I+M area. While it is possibly a statistical anomaly, this result warrants additional consideration.

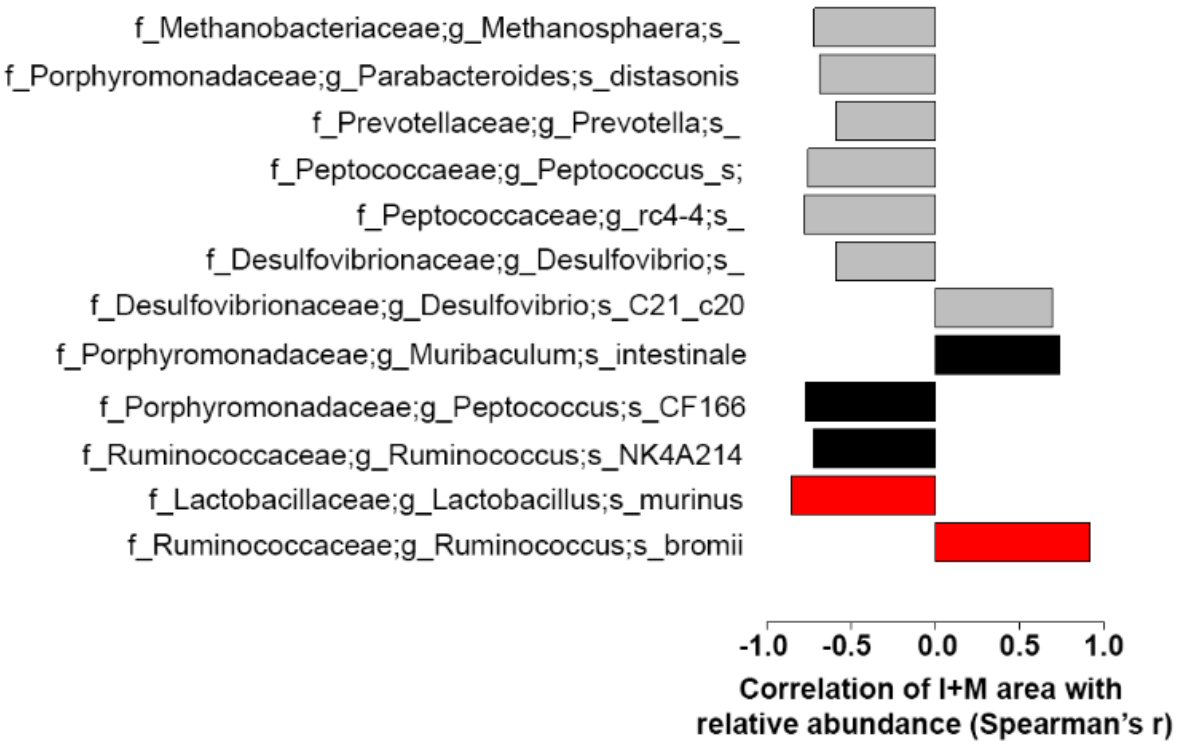

Figure 3.6: Benjamini-Hochberg multiple comparison corrected Spearman's r correlations of specific OTUs and subOTUs with average I+M area with FDR P<.05. Gray bars indicate correlations at the OTU level, black at the subOTU level as defined by DADA2 denoised, BLAST assigned individual sequence variants, and red at the subOTU level only comparing within groups of SD rats. All other correlations are across all rat groups.

3.2.5 Microbe-dependent metabolites and neointimal hyperplasia

Microbe-derived components and metabolic products function as signaling molecules in a variety of inflammatory and immune pathways. For example, short chain fatty acids such as acetate, butyrate, and propionate are produced by gut microbial fermentation of undigested carbohydrates and have wide-ranging effects on host immune system development and function (reviewed by [15]). Furthermore, an untargeted metabolomics study comparing plasma present in conventional and germ-free mice revealed distinct plasma metabolite profiles between the 2-sample set, implying a role for bacterial-dependent metabolism on host-microbial interactions [16]. Some of these metabolites were indole and phenyl derivatives, which we previously have found to be associated with presence of advanced atherosclerosis [5] and which, like neointimal hyperplasia, is driven by chronic systemic inflammation. Thus, we assessed potential associations between indole and phenyl-derived metabolites and neointimal hyperplasia. There were significant differences in mean serum concentration of multiple indole-derived metabolites between the NCH LE and SD rats, including serotonin (2.8±.1 μM LE vs 4.8±.2 SD μM; P=.02), kynurenine (.65±.03 μM LE vs 1.47±.18 μM SD; P=.02), the kynurenine/tryptophan ratio (6.7±.5 LE vs 13.0±1.5 SD; P=.02), and hydroxyanthranilic acid (58.6±.0 nM LE vs 97.8±13.7 nM SD; P=.02). Cohousing and bedding mixing did not significantly alter the serum concentration of any of the assessed metabolites despite the significant changes in gut microbial communities described above (Figure 3.7). However, when we performed a correlation analysis between metabolite concentrations and I+M area in the entire rat cohort, we found significant positive correlations between the kynurenine/tryptophan ratio (Spearman r=.60, P=.007) and 3-hydroxyanthranilic acid (Spearman r=.72; P<.001) (Figure 3.7). Since the SD rats demonstrated decreased mean I+M area upon cohousing, we next examined whether these 3 metabolites had any correlation with I+M area in the subgroups of NCH and CH SD rats. There was no correlation between 3-hydroxyanthranilic acid concentration and I+M area in either the NCH or the CH SD rats. However, in both NCH and CH SD rats, there was a trend towards a correlation between

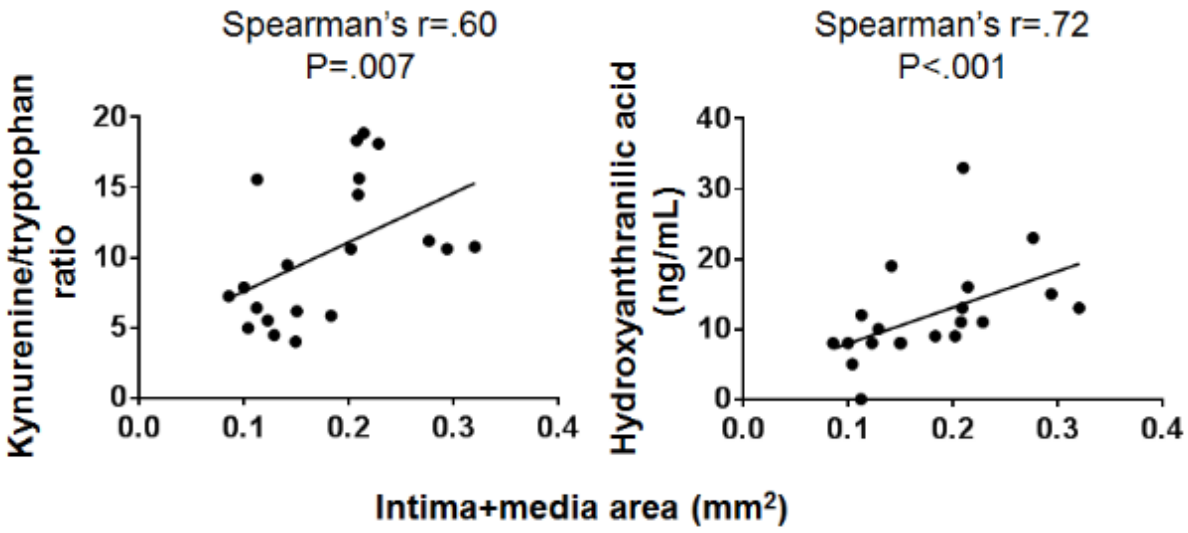

Figure 3.7: Spearman's r correlations of microbe-derived metabolites with neointimal hyperplasia (mean intima+media area) in all rats irrespective of strain or housing group. N=16-20 samples.

kynurenine/tryptophan ratio and I+M area (Spearman r=.81, P=.1 NCH; r=.78,P=.1 CH). Lastly, the possibility of these metabolites being microbially produced was explored through functional gene identification with Piphillin. The TDO2 gene, the first step in the conversion of tryptophan to kynurenine and 3-hydroxyanthranillic acid, was found to be differentially abundant between NCH rat groups, allowing for the possibility that observed differences in metabolites were microbe based, though likely with microbes that were not transferred in cohousing.

3.2.6 Correlation of relative abundances with metabolites

Correlations of relative microbial abundances with kynurenine/tryptophan ratio and 3-hydroxyanthranilic acid were performed. One might expect that microbes that correlate strongly with I+M area will likewise correlate these metabolites regardless of causative connections. Correlations at the species level were difficult to interpret for this reason, but collapsing taxa to the family level led to cleaner results, namely that Prevotellaceae and

Peptococcaceae were significantly negatively correlated with I+M area (Spearman r=-.63, P=.04; r=-.81, P<.001) and kynurenine/tryptophan ratio (Spearman r=-.68, P<.001; r=-.59, P=.05) and 3-hydroxyanthranilic acid (Spearman r=-.63, P=.02; r=-.83, P<.001). Additionally, Bacteroidaceae was found to negatively correlate with kynurenine/tryptophan ratio (Spearman r=-.80; P<.001) and 3-hydroxyanthranilic acid (Spearman r=-.57; P=.01), though not with I+M area.

3.3 Discussion

This is an exploratory and descriptive study of how cohousing and bedding mixing between genetically different rat strains resulted in altered adult host phenotypic differences in neointimal hyperplasia development and in local arterial inflammatory cell infiltration after carotid angioplasty as well as significant shifts in fecal microbiota communities. These results are important because they suggest a direct role for gut microbiota in the arterial remodeling response after injury that occurs through modulation of the acute inflammatory response.

A striking modulation of I+M area after angioplasty was observed in response to cohousing and bedding mixing, especially in SD rats. While NCH LE rats developed less neointimal hyperplasia after balloon angioplasty than NCH SD rats, the relative contributions of genetics and microbiome compositions to this response are unknown. This work demonstrates that the microbiome likely plays a strong role. The finding that CH LE rats exhibit a relatively small change in neointimal hyperplasia as well as a relatively minor shift in gut microbial composition supports this idea.

This difference in resistance of the microbiome to change after cohousing between SD and LE rats may be caused by higher stability of the LE microbiome and/or a stronger selective pressure by the host towards certain microbial consortia. The stability to loss of OTUs was tested through attack on the occurrence networks of the baseline SD and LE microbiomes, revealing that SD microbial networks fell apart more quickly as nodes were removed. This is indicative of a less stable microbiome; however, further experimental

tests (e.g., longitudinal data as the microbiomes are being shifted) are needed to expand on this result. Other investigators have demonstrated that co-housing mice of different strains led bacterial communities to be more similar to each other, but strain-specificity was retained, as we also observed [17]. Furthermore, pretreatment of mice with antibiotics does not necessarily improve, and possibly worsens, the engraftment of exogenous microbiota into non-germ-free animals [12]. The contribution of strain-specific differences in microbiota to strain-specific phenotypes in diseases has also been observed in colitis [18]. Though there have been some experiments on comparisons and manipulations of LE and SD microbiomes, the compositional and functional differences of these strains' microbiota continues to be under-explored given their potentially large effects on study outcomes. This is further confounded by handling and cage effects, which also remain understudied [19]. Nonetheless, by focusing on changes in microbiota from a known starting point and following perturbations such as cohousing longitudinally, these confounders can be partly mitigated.

We observed several notable associations between specific microbial strains, neointimal hyperplasia, and inflammation that can serve as hypothesis-generators for future studies. There was increased M2 macrophage infiltration in the adventitia of CH SD rats compared to NCH SD rats at the 3-day time point (P=.003), which corresponded with increased relative abundance of *Parabacteroides distasonis* in the CH group and with attenuated neointimal hyperplasia. Notably, *P. distasonis* relative abundance in all groups strongly negatively correlated with I+M area (r=-.69). Prior work by others has shown that oral administration of *P. distasonis* has an anti-inflammatory effect in a mouse model of acute colitis, and that its antigens decrease pro-inflammatory cytokine production macrophages in vitro [20]. Thus, this commensal organism may modulate innate immunity mechanisms to attenuate neointimal hyperplasia, a process initiated and potentiated by inflammation. Alternatively, *P. distasonis* may exert its effect via adaptive immunity mechanisms. Treatment of mice with *P. distasonis* increased the number of FoxP3+ regulatory T cells (Tregs), which may represent a change in the balance of Treg-TH17 cells required for commensalism [21]. TH17

cells produce IL-17A, which mediates pro-inflammatory responses and was observed in our study to be significantly higher in NCH SD than in NCH LE SD rats at 3 days (P=.04), corresponding with greater neointimal hyperplasia in the NCH SD rats [22]. Furthermore, IL-17A was significantly lower in CH SD rats compared to NCH SD rats (P=.04), corresponding to attenuated neointimal hyperplasia. IL-17A has also been shown to stimulate vascular smooth muscle cell migration and MMP9 expression in vitro, cellular processes which are relevant to neointimal hyperplasia [23]. Although we did not investigate Treg or TH17 responses in our study and we did not perform *P. distasonis* monoassociation experiments to elucidate the mechanism by which this single strain may affect the inflammatory response to arterial injury, the possibility that *P. distasonis* may employ both innate and adaptive immune mechanisms to effect host phenotype to arterial injury is a fascinating avenue for future investigation.

We observed a negative correlation between *Prevotella* relative abundance and I+M area across all groups (Spearman r=-.59). *Prevotella* is one of the predominant genera in the Bacteroidetes phyla. *Prevotella* relative reduction has been associated with atherosclerotic disease in humans, another chronic inflammatory disease, and with hypertension [24, 25]. Furthermore, patients with new-onset rheumatoid arthritis had an overabundance of *Prevotella spp.*, particularly *P. copri*, which encodes a superoxide reductase and phosphoadenosine phosphosulphate reductase. These 2 genes might favor the development and maintenance of intestinal inflammation by increasing P. copri resistance to host-derived reactive oxygen species [26]. Finally, *Prevotella* abundance has also been associated with increased TH17 immune responses, mucosal inflammation, and systemic dissemination of inflammatory mediators, bacteria, and bacterial products [27]. These conflicting data suggest a likely complex host-*Prevotella* relationship, especially since species-specific roles of *Prevotella* exist.

We also found a significant negative association between *Methanosphaera* (Spearman r=-.72) and conflicting associations between *Desulfovibrio* (Spearman r=.70 and =-.59) and I+M area across all groups. *Desulfovibrio* use lactate, pyruvate, ethanol, malate, and formate

to reduce sulfate to produce hydrogen sulfide [28], while *Methanosphaera* (single species stadtmanae) utilize hydrogen to reduce methanol to methane [29]. Hydrogen sulfide has been shown to have a protective effect on restenosis in atherosclerotic rabbits [30] and in balloon-injured rats [31], but given the complex relationship of hydrogen balance to host homeostasis [32], it is not surprising that different *Desulfovibrio* OTUs correlate with I+M area in different directions and magnitude. These taxa may also be indirectly related to acetate levels, since the main pathways of hydrogen consumption are methane, hydrogen sulfide, and acetate production.

Ruminococcus bromii, which had a strong positive correlation with I+M area in the SD rats, is a specialized "keystone" organism that can degrade certain forms of resistant starch to short chain fatty acids [33]. We previously demonstrated an inverse relationship between serum butyrate concentration in antibiotic-treated LE rats and neointimal hyperplasia after carotid angioplasty and a potential direct anti-migratory and anti-proliferative effect on vascular smooth muscle cells [6]. Others have observed attenuated LPS-induced neuroinflammation in rats given supplementary dietary acetate [34]. In contrast, acetate is a pro-inflammatory mediator in hepatitis [35] and in cultured gastric adenocarcinoma cells [36]. These data suggest a tissue-specific effect for acetate whose role in inflammatory processes needs further clarification.

Finally, *Muribaculum intestinale*, which also had a strong positive correlation with I+M area (Spearman r=.8), is a relatively newly isolated member of mouse gut bacteria whose functional effect on the host is not yet known [37]. Similarly, the functional significance of the genus *Peptococcus*, gram-positive anaerobes that negatively correlated across all groups with I+M area, is largely unknown. However, glycosulfatase-like enzymes have been found in *Peptococcus niger*, pointing towards another possible sulfur-driven role in neointimal hyperplasia reduction. *L. murinus*, which also had a negative correlation with I+M area, has been observed to be pro-inflammatory in dendritic cells [38] and modulate Th17 cells in salt-sensitive hypertension [39]. In contrast, *L. murinus* induced production of anti-inflammatory

cytokines in murine peritoneal macrophages [40]. Lastly, the genus *Sutterella* maintains taxa that are widely prevalent and potentially are pro-inflammatory given their ability to adhere to intestinal epithelial cells [41], but little is known beyond their frequent association with diseases such as inflammatory bowel disease and metabolic syndrome [42, 43].

We observed strong correlations between neointimal hyperplasia and microbe-derived metabolites kynurenine/tryptophan ratio and 3-hydroxyanthranilic acid. However, given the diverse effects of gut microbes on host physiology, it is not surprising that there may be multiple mechanistic pathways which are both overlapping and distinct. The kynurenine pathway accounts for the catabolism on 99% of ingested tryptophan not used for protein synthesis [44]. Upon entering the kynurenine pathway, tryptophan is converted to N formyl-l kynurenine by indole-2,3-deoxygenase (IDO) or the similar TDO2 gene and then to kynurenine. IDO1 is known to be upregulated by interferon-gamma, IL-2, and IL-10 activity. Whether or not the effector of observed metabolomic differences was due to murine IDO, bacterial TDO2 or another process is difficult to determine and warrants further investigation. This might be sussed out by the use of selective IDO1 inhibitors which do not inhibit TDO2 [45]. The kynurenine/tryptophan ratio, which is an index of IDO1 activity, has been found to be elevated in states of immune stimulation, such as infection, malignancy, and endotoxin administration [46, 47]. Downstream kynurenine catabolites including 3-hydroxyanthranilic acid reduce Th1 and Th17 responses and affect T cell apoptosis [48]. Thus, as neointimal hyperplasia an inflammatory process, it is conceivable that there is a strong positive correlation with kynurenine/tryptophan ratio and 3-hydroxyanthranilic acid as we observed. However, we observed a negative correlation between Bacteroidaceae with kynurenine and 3-hydroxyanthranilic acid without an accompanying correlation in I+M area. Also, TDO2 differences were tied to rat species, so the lack of effect of cohousing makes genetic and microbiome contributions impossible to disentangle. This means that future directed studies will be necessary to elucidate correlations of these metabolites with microbes. Which microbes have the TDO2 gene also remains to be clarified as Piphillin does not provide this informa-

tion. It might be possible to deduce this if in future germ free or microbiome transplant experiments the TDO2 functional gene is seen to be transferred. Alternately, metagenomic studies may lead to assembled genomes with the TDO2 gene annotated.

This study's strengths lie in its experimental design, which demonstrates that exchange of microbes in adult rats is sufficient to incur changes in acute arterial inflammation and downstream neointimal hyperplasia. Similarly, other investigators have found that microbial exchange and/or fecal microbial transfer in adult mice was sufficient to induce differences in anti-tumor immunity in melanoma [49] and skin transplant rejection [50]. Our findings also add to a growing body of literature linking gut microbiota to the peripheral vasculature, specifically atherosclerosis, arterial stiffness, blood pressure, and endothelial function. While we did not obtain direct mechanistic data linking specific microbial strains with macrophage polarization or neointimal hyperplasia development, we identified several bacterial strains that correlated highly with neointimal hyperplasia severity that can be the subjects of future focused interventions (e.g., monoassociation of germ-free rats, prebiotics, and probiotics). Furthermore, certain biomarkers and microbe-derived metabolites such as hydrogen sulfide compounds might also be targeted based on our data. For example, metagenomic studies that could differentiate and categorize the *Desulfovibrio* strains and correlate marker genes with metabolites are in the immediate future plan. Further metabolomic studies of the kynurenine pathway might lead to a fuller understanding of the role of these metabolites in hyperplasia. Finally, germ-free animal models will be important for dissecting out the specific impact of gut microbiota on the neointimal hyperplasia phenotype regardless of host genetic background.

Limitations of this study include its descriptive nature, which precludes any inference of causality by gut microbiota and a lack of information of how and if genes present are being expressed and if observed metabolite difference are microbe-derived. Additionally, other microbiomes were not explored. Given the possibility of involvement of nitric oxide in neointimal hyperplasia, the oral microbiome, where this metabolite is chiefly regulated [51],

would be of particular interest.

Ultimately, the data presented in this exploratory study argue for in-depth mechanistic studies into a novel mechanism for how microbiome manipulations affect arterial remodeling after injury and for disentanglement of the genetic versus microbial mechanisms for neointimal hyperplasia and, more generally, for immune function. Furthermore, we demonstrate that cohousing and bedding mixing, which are relatively easily undertaken experimental methods, are a valuable starting point for focused interventions.

3.4 Methods

Experimental rats: Eight-week-old male Lewis Inbred (LE) and Sprague-Dawley (SD) male rats obtained from Envigo (Indianapolis, IN) were housed in a barrier facility at Northwestern University under a 12-hour light cycle. Standard irradiated rat chow and autoclaved drinking water were provided ad libitum.

Genomic DNA extraction from fecal samples: Stool pellets were collected and stored at -80C until processing. 100 mg of stool pellets were used for microbial DNA extraction with the DNeasy Powersoil HTP 96 kit (Qiagen, Germantown, MD). The following modifications were made to the manufacturer's protocol: after samples, the bead solution, and the C1 solution were added to the bead plate, the plate was partially submerged in a water bath for 20 minutes at 60C, followed by a 20-minute shaking step on a MM 400 plate shaker (Retsch, Haan, Germany). This was done to further ensure lysis of the cells. The manufacturer's protocol was then followed to obtain clean DNA.

16S rRNA gene amplification and sequencing: The V4 region of the bacterial 16S rRNA gene was amplified using the Earth Microbiome Project primer set (515f-806r). Each 25 µl PCR reaction contained 12.5 µl of AccuStart II PCR ToughMix (Quantabio, Beverly, MA), 1 µl of 5 µM forward primer, 1 µl of 5 µM reverse primer, 9.5 µl of water, and 1 µl of DNA extraction. The PCR program was 94C for 3 minutes to denature the DNA, with 35 cycles at 94C for 45 seconds, 50C for 60 seconds, 72C for 90 seconds, and then a final 72C

step. Amplification was quantified using Picogreen (Invitrogen, Carlsbad, CA), and each sample was pooled at 70 ng per sample. Pools were cleaned using Agencourt AMPure XP beads (Beckman Coulter, Indianapolis, IN), and the clean pools combined. This final pool was quantified and sent to the Environmental Sample Preparation and Sequencing Facility (ESPSF) at Argonne National Laboratory for sequencing on an Illumina Miseq using V3 chemistry, following the Earth Microbiome Project protocol [52].

Sequence data handling and analysis: Raw 16S reads were demultiplexed, chimera and phiX filtered through the QIIME2 pipeline [53] and denoised with DADA2 [54]. The resulting representative sequences were aligned and masked with MAFFT [55]. A phylogenetic tree was constructed via FastTree2 [56]. Samples were subsequently clustered and taxonomically classified into operational taxonomic units (OTUs) with QIIME2 utilizing the greengenes 99% database [57]. Samples were filtered by globally removing OTUs with minimum count fractions of .001. Differences in relative abundances were calculated via bootstrapped Kruskal-Wallis tests (999 permutations) for multiple group comparisons. Correlations of metadata with OTU abundances were calculated as Spearman's rank correlations with raw P values determined via bootstrapping (1000 permutations). All reported P values are Benjamini-Hochberg false discovery rate (FDR) corrected for multiple comparisons. Diversity metrics were calculated after rarefying reads at a depth of 20,000 sequences. Beta diversity was calculated as unweighted [58] and weighted [59] unifrac distances. Faith's Phylogenetic Diversity was used to measure alpha diversity [60]. Beta diversity correlations with metadata were calculated by Mantel test (999 permutations). OTU co-occurrence networks were created with SPEIC-EASI [61]. SubOTUs were taxonomically assigned via the NCBI BLAST web interface [62]. Functional genes were identified with Piphillin [63] and differential abundance determined by DESeq2 [64].

Detection and quantification of serum metabolites by high performance liquid chromatography (HPLC)-tandem mass spectrometry: P-cresyl sulfate (PCS), hippuric acid, indole-3-propionic acid (I3P), tryptophan (trp), kynurenine (kyn), indoxyl sulfate (IS), and serotonin.

Fifty microliters of serum and internal standards (n-methyl serotonin and p-toluene sulfonic acid) were prepared as previously described and analyzed using tandem mass spectrometry (MS/MS) using an Agilent HPLC-MS/MS system with a 6400-series triple quadrupole (QQQ) mass spectrometer described in detail previously. Indole, indole-3-aldehyde (I3A), and 3-hydroxyanthranilic acid. Samples were prepared from 50 μL of serum by solid-phase extraction and analyzed by an API 3000 HPLC-MS/MS system (Applied Biosystems, Foster City, CA) equipped with an Agilent 1100 series HPLC system (Agilent Technologies) operating in positive ion mode as described in detail previously [5].

3.5 Author contributions

Cori Cason: rat work, histology, immunohistochemistry, wrote relevant manuscript sections. Thomas Kuntz: assessed microbiome profiles, performed bioinformatics analysis, co-designed experiment. Ed Chen: multiplex assays, wrote relevant sections. Kelly: histology, immunohistochemistry. Michael Nooromid: metabolomics, LC-MS MS. Liqun Xiong: additional rat work, tissue processing. Neil Gottel: supervised and processed microbial DNA extraction and 16S sequencing process. Katie Harris: metabolomics, LC-MS MS. Tim Morton: metabolomics, LC-MS MS. Mike Avram: metabolomics, LC-MS MS. Gene Chang: planning and troubleshooting. Jack Gilbert: reviewed and critiqued the chapter. Karen Ho: reviewed and critiqued the manuscript.

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CHAPTER 4

GENDER-SPECIFIC PERTURBATIONS IN CEREBRAL AMYLOIDOSIS AND MICROGLIA PHENOTYPES IN ANTIBIOTIC-TREATED APPPS1-21 TRANSGENIC MICE

4.1 Introduction

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Alzheimer's disease (AD) was first described in 1906 by the psychiatrist widely regarded as having founded the field of neuropathology, Alois Alzheimer [2]. While age-related dementia had been described as far back as the 7th century [3], Alzheimer's observation of microscopic structures, senile plaques and neurofibrillary tangles (NFT), was the first step towards a deeper understanding of the disease. Despite subsequent significant research into and large strides in understanding AD, treatment remains elusive. In 2018 an estimated 5.7 million Americans were living with AD with this prevalence set to increase due to an aging population [4]. Therefore it is imperative that AD is approached at new angles; the microbiome is one such promising avenue of AD treatment.

Chemically, AD is characterized primarily by amyloid-β (Aβ) peptides, senile plaques, and accumulation of hyperphosphorylated tau proteins, neurofibrillary tangles. Aβ is an abnormal proteolytic byproduct of transmembrane protein amyloid precursor protein (APP) that at sufficient concentration forms fibrils by conformational change into a beta sheet rich structure. Both the fibril form and a possible intermediate misfolded form of Aβ may contribute to AD, the former through disruption of the cell's calcium ion homeostasis and the latter through physical piercing of the cell membrane. Due to this dual nature of the problem, therapies targeting both agglomeration of Aβ and earlier APP processing are being developed. Other, more complex chemical processes regarding Aβ have been explicated through structural imaging, redox potential investigation, spectroscopic, and other means to further

explicate AD pathology [5]. Tau protein driven AD pathology is possibly caused by the loss of functioning tau protein, a microtubule stabilizing agent, which leads to degradation of the cytoskeleton and aggregation inside neurites, blocking nerve synapses. Much of the work on tau protein involves its interactions with other molecules and chemical modifications, such as glycosylation [6]. Interestingly, gender specific tau gene expression is implicated in the differing progression of AD [7]. Other (related) disease mechanisms include the cholinergic, oxidative stress, metal ion hypothesis, insulin signaling, and neuroinflammation hypotheses [8]. Of these, neuroinflammation is of particular interest for microbiome researchers as the microbiome has both immunomodulatory potential and access to brain signaling through the gut-brain axis. It is now well known that neuroinflammation, rather than being simply secondary to senile plaques and NFT, is a primary driver of AD and that the microbiome can drive neuroinflammation [9].

Gut microbes have been known to be involved in inflammatory processes for some time, but it is now becoming increasingly clear that they can modulate various neurological conditions with inflammatory aspects, such as Parkinson's disease, where germ free mice showed a reduced pathophysiology [10]. Part of what is termed the "gut-brain axis", the bidirectional signaling pathways between the microbiome and the brain, includes the modulation of this and similar diseases [11]. This axis includes the central nervous system, the autonomic nervous system, the enteric nervous system and the hypothalamic pituitary adrenal axis (HPA) with multifarious means of communication between the microbiome and the host. Neuroinflammation can be caused through various aspects of this pathway possibly including modulation of HPA mediated stress response and of microglia by short chain fatty acids (SFCAs). Furthermore, inflammation at the gut, for example reaction to lipopolysaccharide (LPS), can propagate to the nervous system [12].

However, these ways in which the microbiome can alter neurological diseases including AD, especially mechanistically, require further investigation. Previous efforts by the Sisodia group demonstrated that antibiotic (ABX) cocktail mediated perturbations of the gut micro-

biome reduces Aβ plaque pathology and plaque-localized gliosis in a transgenic (Tg) mouse model of Aβ amyloidosis, termed $APP_{SWE}/PS1_{dE9}$. To extend and validate these findings, the highly aggressive APPPS1-21 mouse model, which exhibits pathology at 6 weeks rather than 6 months, was studied here. Using an established ABX treatment protocol consisting of post-natal oral gavage (p14-p21) followed by 1:50 diluted ABX-administration until the age of 7 weeks, we evaluated fecal microbiome, peripheral cytokines, brain Aβ amyloidosis and microglial morphology.

The earlier findings of reduced Aβ amyloidosis and morphological alterations in microglia were confirmed. On the other hand, ABX-treated female mice exhibited no significant differences in Aβ amyloidosis and plaque-localized microglial characteristics. Transcriptomic and immune profiling of showed that ABX-treated males exhibited profound changes compared female mice. The microbiomes showed large differences in diversity between ABX treated and vehicle treated mice in both male and females. This resulted in functional gene differences and compelling taxa differences between groups which may help explain differences seen in pathology. Furthermore, transplants of fecal microbiota from 7-week-old APPPS1-21 male mice into ABX-treated male mice partially restored Aβ amyloidosis and microglial phenotypes that are observed in vehicle-treated APPPS1-21 mice. The FMT resulted in only small changes in diversity and differentially abundant taxa from Tg animals, indicating a largely successful transplant.

Collectively, the previous work and the analysis I present here reveals that ABX-mediated microbiome perturbations influences brain Aβ amyloidosis and microglial homeostasis in a gender-specific manner and that pathology can be partially restored via FMT in this ABX model. My work here details important taxonomic differences created by ABX and FMT treatment and differentiated by gender, from 16S marker gene sequence analysis. Furthermore, functional gene content is inferred from 16S sequences and possible connections to pathology are pulled from that data based on chemical rational. Lastly, I suggest future experimentation including ways in which products of differential gene content can be utilized

to test a novel hypothesis on the role of the microbiome in AD.

4.2 Results

4.2.1 Postnatal antibiotic treatment resulted in similar microbiome changes in male and female APPPS1-21 mice at p22

Male and female APPPS1-21 mice were orally gavaged with either vehicle or high dose antibiotic (ABX) cocktail daily between postnatal days p14-p21. The microbiome profile in fecal pellets was evaluated using Illumina MiSeq amplicon sequencing of the V4-V5 variable region of the 16S rRNA gene. Beta diversity differences between bacterial communities found in vehicle/ABX-treated groups were calculated with the weighted UniFrac metric. Microbial beta diversity was significantly different between vehicle- and ABX-treated groups (PERMANOVA; weighted: pseudo-F=32, $p<0.001$; unweighted: pseudo-F=51, $p<0.001$). There was no significant different in beta diversity between male and female samples. Both alpha diversity (species richness) and evenness were significantly lower in the ABX treatment group compared to control (Faith's PD, Two-way ANOVA: $F(1,34)=17.31$, $P=0.0002$; Pielou's evenness, Two-way ANOVA: $F(1,34)=76.19$, $P<0.0001$). Gender or an interaction between gender and ABX-treatment showed no significant difference in either richness or evenness ($P>0.05$).

ANCOM analyses of 16S rRNA sequencing data at the DADA2 inferred ESV (exact sequence variant) level (taxonomy assigned via GreenGenes) and operational taxonomic unit (OTU) clustered species level were performed to determine the taxa which described the most variance between groups. Vehicle-treated male and female mice showed no differences at p22. Likewise, ABX-treated mice did not differ significantly in ESVs or species by gender. However, as expected ABX treatment showed significant changes in microbial composition in both genders (Table: 4.1) compared to vehicle control.

Predicted metagenome analysis (PICRUSt) showed significant differences via Krustkal-

Wallis (KW) test in pathways between ABX-treated male and ABX-treated female mice compared to their vehicle-treated counterparts but no significant differences between male and female mice irrespective of the vehicle/ABX treatments (Table: 4.2). In both male and female mice, LPS biosynthesis and glycosaminoglycan degradation ($P<0.001$) was increased in vehicle treatment groups. Based on inspection of KEGG orthologs determined to be differential between groups by DESeq2, glycosaminoglycans degraded include Heparan sulfate, Keratan sulfate, Chondroitin sulfate, and Hyaluronan, with male and female mice sharing the same genes other than the addition of IDS in female mice (Figure 4.1). Looking at the PICRUSt databases, Heparan and Keratan genes mainly comes from *Akkermansia* OTUs, Hyaluronan from *Bacteroides uniformis*, and Chondroitin from several *Bacteroides* species including *B. ovatus*. Female mice are distinguished by additionally having greater IDS gene in vehicle, which may influence the degree to which some GAGs are broken down. The top two taxa identified as contributing to this were *Parabacteroides* and *Akkermansia*.

4.2.2 Long-term antibiotic treatment results in gender-specific microbiome changes in APPPS1-21 mice at time of cull

16S amplicon sequencing was then performed to analyze the microbiota in mice at necropsy that were subject to high dose ABX treatment between p14-p21 followed by diluted ABX in the drinking water till the age of 7 weeks (p49). As before, microbial beta diversity at 7-weeks was significantly different between ABX-treated mice compared to their vehicle-treated counterparts (PERMANOVA; weighted: pseudo-$F=79$, $p<0.001$; unweighted: pseudo-$F=44$, $p<0.001$), but no significant difference was observed for male versus female groups. Similarly, richness and evenness were only significantly different by ABX treatment (Faith's PD, Two-way ANOVA: $F(1,35)=367.6$, $P<0.0001$; Pielou's, Two-way ANOVA: $F(1,35)=14.73$, $P=0.0005$), with no significant impact of gender.

ANCOM comparison of ESVs and clustered species at 7-weeks again showed a large number taxa whose proportions were significantly different between vehicle and ABX-treated

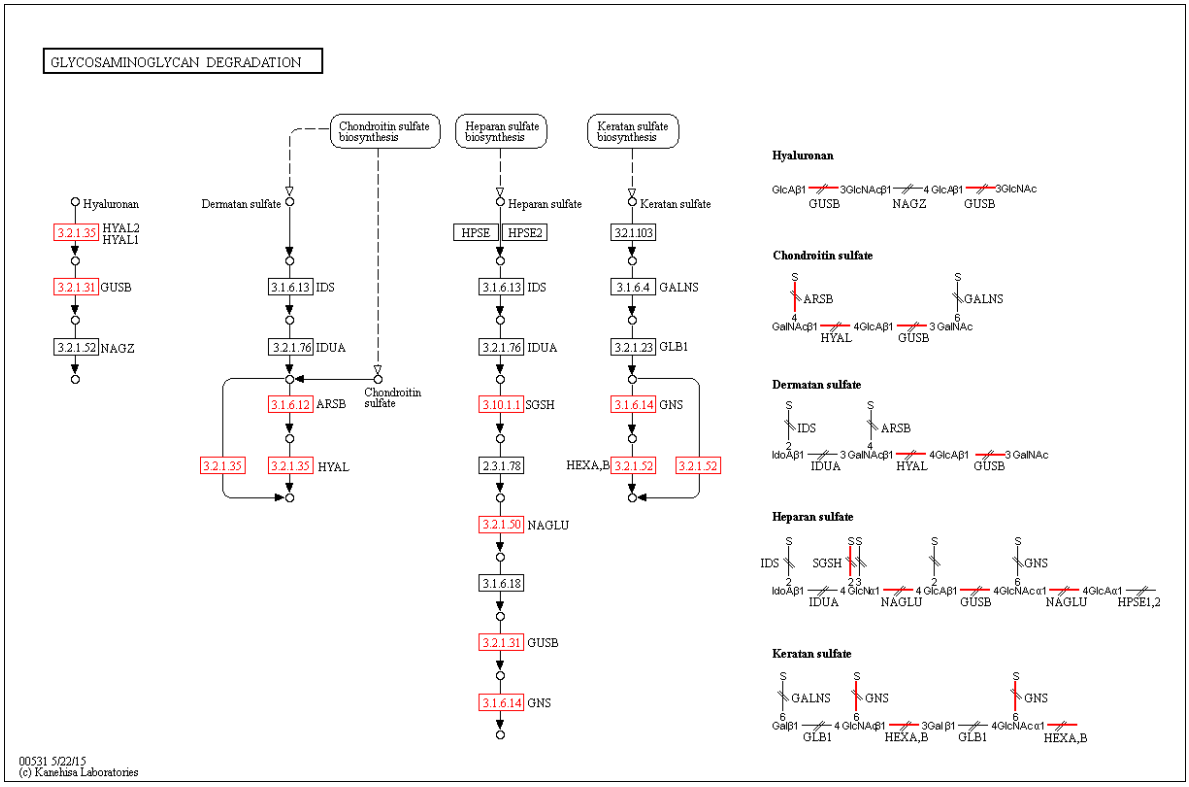

Figure 4.1: Glycosaminoglycan degradation KEGG pathways and gene orthologs in male vehicle treated mice at p22. Differentially abundant PICRUSt predicted genes determined by DESeq2 highlighted in red.

groups in both males and females (Table: 4.3), with some overlapping features. Among ABX-treated mice, an *Akkermansia muciniphilia* ESV and an *Allobaculum spp.* cluster were significantly enriched in females compared to males. In vehicle-treated mice, a Mollicutes RF39 ESV was significantly enriched in males.

PICRUSt showed significant differences between ABX-treated male compared to their vehicle-treated controls, and also between ABX-male and ABX-female mice, but not between Vehicle-male and Vehicle-female mice (Table: 4.4). The pro-inflammatory pathway LPS biosynthesis (FDR-P=0.007) was significantly enriched in ABX-treated female mice compared to ABX-treated male mice. In male mice, vehicle had significantly enriched flagellar assembly (FDR-P=0.002), another possible pro-inflammatory pathway, though less directly.

4.2.3 Fecal microbiome transplantation (FMT) from APPPS1-21 donor into ABX-treated male mice restores fecal microbiome profile and Aβ plaque burden

The microbiome was then profiled at 7-weeks to confirm the effectiveness of FMT protocol. Beta diversity using unweighted UniFrac was significantly different between Tg-donor and ABX-vehicle mice (PERMANOVA; weighted: pseudo-F=8, p=0.003; unweighted: pseudo-F=12, p<0.001). FMT resulted in clear separation between ABX-FMT mice compared to ABX-vehicle mice while Tg-donor and FMT-gavaged ABX mice were clustered very closely, thus suggesting that FMT-treated ABX mice showed similar microbiome profiles as the Tg-donor mice (PERMANOVA; weighted: pseudo-F=3, p=0.084; unweighted: pseudo-F=5, p=0.010). Unweighted unifrac beta diversity was significantly different though with a low effect size, while weighted unifrac was not significant, indicating that lower diversity taxa had a larger effect on differentiating the microbial profiles. Alpha diversity indices showed significantly different Faith's phylogenetic diversity (One-way ANOVA: $F(2,16)=32.9$, $P<0.0001$)

and Pielou's evenness (One-way ANOVA: $F(2,16)=18.7$, $P<0.0001$) with richness and evenness lower in ABX-vehicle mice compared with the Tg-donor, while ABX-FMT mice showed no significant differences in diversity indices compared with the Tg-donor.

ANCOM comparison at the ESV and species levels showed significant differences between both Tg-donor and ABX-FMT groups and between ABX-FMT and ABX-vehicle groups, but with far more differences observed in the latter pairing, as expected (Table: 4.5). Tg-donor groups only differed from ABX-FMT mice in two Bacteroidales S24-7 family member ESVs and a *Bacteroides* species. At the ESV level, ABX-FMT group compared to ABX-vehicle had significantly higher abundances of several members of the Bacteroidales order including *Bacteroides*, *Prevotella*, and S24-7 while ABX-vehicle showed a higher abundance of a single S24-7 ESV. At the L7 level, a number of varying taxa were found, but only those higher in abundance in the ABX-FMT group were significant (Table: 4.5).

Surprisingly, no significant differences were observed in PICRUSt pathways between ABX-FMT and ABX-vehicle groups.

4.2.4 Long-term antibiotic treatment results in gender specific and FMT related AD markers

Via fluorescent antibody analysis of 3D6+ plaque burden exclusively in the cerebral cortex, ABX-treated male APPPS1-21 animals exhibited significantly reduced Aβ burden compared with vehicle-treated male mice. ABX-treatment of female mice did not result in a reduction in Aβ burden compared to vehicle-treated female. Furthermore, the ABX-FMT group showed significantly higher Aβ burden in the cortex compared with the ABX-vehicle group.

Raybiotech cytokine array membranes were used to determine levels of cytokines/chemokines. ABX-treated male mice had relatively lower expression of pro-inflammatory cytokines/chemokines, including CD30, CD40, CCL11 (Eotaxin1), IL1β, IL2, IL3, IL17A, LIX (CXC5), RANTES (CCL5), SCF and higher anti-inflammatory cytokines such as IL10. In addition, ABX-treated female mice showed relatively higher level of pro-inflammatory cytokines (CCL11,

IL1β, IL2, IL3, IL5, IL9, IL17, LIX, SCF) compared with ABX-treated male mice.

Immunofluorescence staining and Imaris 3D reconstructions of plaque localized microglia were then examined. Overall these data suggest that ABX-induced perturbations in the microbiome is associated with less phagocytic plaque-localized microglia, as defined by smaller cell bodies, longer dendritic branch lengths and increased dendritic branch points in male mice only, while female mice exhibited a phagocytic microglial phenotype as defined by larger cell bodies, shorter branch lengths and fewer branch points that are independent of vehicle or ABX treatment. FMT resulted in restored pathological microglia morphology.

Further results by Nanostring analysis suggest that treatment of male APPPS1-21 mice with ABX restores microglial homeostasis likely via induction of TGFβ signaling and several key transcriptional regulators of M0 microglia including Mef2a, Fos and other homeostatic and anti-inflammatory modulators such as Il10ra and Gas6. Microglia switch from M0 homeostatic to a neurodegenerative phenotype (MGnD) during the course of aging and disease. It was observed that that ABX-treated male mice exhibit increases in the homeostatic microglial transcriptomic signature and decreased expression of MGnD genes.

4.3 Discussion

Dysregulation in microbiome-gut-brain axis is now emerging for a variety of neurological diseases, including Parkinson's disease [13] and AD [14], as well as developmental and psychiatric conditions, such as depression [15]. We have explored the impact of the microbiome in AD using a second transgenic mouse model of Aβ amyloidosis. We demonstrate that long-term ABX treatment of male APPPS1-21 results in microbiome changes that are associated with distinct alterations in peripheral cytokines/chemokines, changes in the composition of fecal microbiomes, and a significant reduction in Aβ deposits. In contrast, ABX-treated female mice exhibited greater abundance of bacteria and predicted metabolic pathways that are considered pro-inflammatory, higher levels of plasma pro-inflammatory cytokines/chemokines, and no apparent effects on the levels of Aβ deposits compared with

vehicle-treated animals.

We show that ABX-treated male animals exhibit transcriptional signatures that are consistent with activation of the TGFβ signaling pathway that is a hallmark of MO (homeostatic) microglia [16]. In sharp contrast, the microglia in ABX-treated female mice exhibit conditions that closely mimic that observed in MGnD (disease-associated) microglia. Finally, transplants of feces from age-matched APPPS1-21 male donor mice into long-term ABX-treated male mice resulted in restored microbiome profiles, elevated MGnD-type microglial morphologies and partial restoration in Aβ deposition, thus confirming a causal relationship between the gut microbiome and AD-associated inflammation and Aβ pathology in this model.

Microbiome composition can greatly affect immune functioning which can ultimately propagate to the brain [17]. The evidence that a large number of metabolites in the blood originate from the gut suggests that microbiome-derived metabolites can influence peripheral cytokine production [18]. Several studies show that microbiota can influence the relative populations and function of various subsets of immune cells [19] modulating innate [20] and adaptive [21] immune responses at mucosal surface and finally affecting systemic immunity [22]. It is possible that observed altered peripheral inflammation due to microbiome perturbations in our model could impact upon central inflammation that ultimately affects microglial morphological and transcriptional profiles. Furthermore, other microbial metabolites could impact the progression of AD. While there were many genes found via PICRUSt to be differentially represented between vehicle and ABX treatment, most of these differences remain difficult to interpret and may not be of consequence; further research into the etiology of AD may be required to target more microbial metabolites for analysis. However, it was observed at p22 that vehicle mice had increased degradation of glycosaminoglycans (GAGs). These molecules have a complicated relation with AD and Aβ pathology. It is theorized that high molecular weight GAGs engender Aβ fibrils by promoting aggregation, but that low molecular weight GAGs inhibit this same process by occupying the same bind-

ing site without tangling [23]. The extend to which early time-point (these genes are not differentially present at 7 weeks) GAG degradation affected AD progression is difficult to establish without a cull experiment at p22. Furthermore, the degree to which GAGs are degraded cannot be told from functional gene analysis, and thus vehicle mice may be totally breaking down GAGs, possibly increasing pathology, or they may be creating low molecular weight GAGs from larger ones, ultimately decreasing pathology. However, it may be possible to suss out the effects of this metabolic potential by treating mice with low weight GAGs; some success has already been seen in treating AD this way [24], so if there was a differential effect of GAG administration between ABX and vehicle mice, one may be able to attribute microbiome functioning to this. This could be supplemented by administration of GAG analogues which might not be broken down by the microbiome [25].

Taxonomic differences between vehicle and ABX were large as expected when applying a harsh antibiotic regime, so it is difficult to say which taxa might be contributing to AD pathology, especially since many of these taxa do not have much known about them. However, we will here focus on a few compelling possibilities in male mice (since female mice results are even more difficult to interpret in the absence of ABX pathology effects). At p22, an OTU in the well known probiotic genus *Lactobacillus* was found to be of higher relative abundance in ABX and thus correlated with a reduction in pathology. Some but not all *Lactobacillus* strains are anti-inflammatory inhibiting the release of inflammatory mediators such as TNF-α, IL-6, IL-1β, and IL-10 [26]. In vehicle, *Bacteroides ovatus* and *Prevotella* were found to be of higher relative abundance. *Bacteroides ovatus* was found to be the primary OTU associated with chondroitin sulfate degradation. This GAG not only may have effects on Aβ pathology but also perineuronal nets formed by chondroitin sulfate proteoglycans may have a neuroprotective action against oxidative stress potentially involved in neurodegeneration [27]. Therefore, the breakdown of this compound could result in loss of its beneficial effects. *Prevotella* is a genus often associated with inflammatory conditions, but this linkage is largely correlative and there are indications that *Prevotella* may also have

beneficial health effects [28].

At week 7, *Bacteroides ovatus* and *Parabacteroides distasonis* were found to be higher in ABX. The relative abundance of *Bacteroides ovatus* represents a reversal of the trend at p22, which is unexpected. It is worth noting that *Bacteroides* are (like most bacteria) known to do different things in different milieus. They can be both pro-inflammatory and immunoregulatory depending on the strain and context (ecological and environmental) that they are in [29]. *Parabacteroides distasonis* is sometimes considered a so called next-generation probiotic as they are can have beneficial health outcomes but are not members of the two major probiotic genera, *Lactobacillus* and *Bifidobacterium*. They have been shown to suppress the secretion of pro-inflammatory cytokines IL-17, IL-6, and IFN-γ in mouse models of acute and chronic colitis [30]. If this suppression is directly responsible for differences in cytokines observed in this study is hard to tell; a probiotic model may be indicated in order to determine the effect of *P. distasonis* in this model. *Helicobacter*, *Bifidobacterium*, *Lactobacillus*, and *Allobaculum* were found to be higher in vehicle. *Helicobacter*, strikingly, may predispose one to Alzheimer's disease in a study of a *H. pylori* peptide which modulated inflammatory genes and caused dysregulations in AD pathways. However, the bacterium identified in this study was not necessarily gastric H. pylori, thus this link while interesting may be spurious. *Bifidobacterium* and *Lactobacillus* are both probiotic bacteria so it may seem odd that they were of higher relative abundance in the higher pathology vehicle group. However, this study was not designed to cause AD or even negative health outcomes, it was simply an ABX treatment study, so it is not surprising that not every difference between groups is unhealthy. It is worth noting that the *Lactobacillus* here is a different OTU than the one observed at p22, so it may have different properties. However, *Allobaculum* has been negatively correlated with anti-inflammatory genes FOXP3, IL10 and intestinal barrier ZO-1 and occludin [31], making it a candidate for (partially) causing the negative outcomes in this study.

Comparing sexes, our microbiome data reveals no significant taxa differences in vehicle and ABX treatments at p22, but the microbiome profiles at the time of cull (7-weeks-of-age)

represented a significant effect of gender in ABX-treated mice. Importantly, we observed ABX-treated female mice showed a greater abundance of *Allobaculum* and *Akkermansia* which was associated with higher peripheral pro-inflammatory cytokines, phagocytic microglia and no changes in amyloidosis. Certain *Allobaculum* and *Akkermansia* species are known mucin degraders, a greater proportion of which could lead to a decrease the mucin-layer thickness, which may not only result in greater inflammatory activation, but also could increase the likelihood of translocation of bacteria or bacterial metabolites from the gut to circulation [32]. Increased microbial or metabolite translocation can increase endotoxin levels in the systemic circulation, affecting peripheral and central inflammation as reported [33]. I am not able to prove this with the current data, but it remains a compelling hypothesis to test. One way to do this would be through a dietary fiber deprived model in male mice, which has been shown to degrade the colonic mucus barrier [34]. *Akkermansia* has also previously been associated with various neurodegenerative disorders [35, 36]. *Allobaculum* again has been negatively correlated with anti-inflammatory genes. Thus the differential effect of ABX treatment on male versus female mice may occur due to increased reactivity and gut permeability to inflammatory molecules, especially LPS, which are present in the non-ABX treated mice.

In the FMT study, a large number of organisms of order Bacteroidales were found to be higher in ABX-FMT, possibly driving a restoration in pathology through LPS presentation or some other immune modulating molecules [37]. Significant changes in *Akkermansia* and *Allobaculum* were not observed via ANCOM in this cohort, nor were GAG degradation or LPS gene differences observed, pointing towards multiple mechanisms by which the microbiome influences AD. The lack of PICRUSt differences in LPS genes does not necessarily mean that LPS was not significantly different between groups, since it is not a direct measurement of functional genes and far from all LPS genes are known and accounted for; however, it is odd that this was not observed like it was in the previous cohorts. Further investigation into the role of LPS and other Bacteroidales associated immune interactions in

FMT should be carried out to determine its importance. Other taxa which were increased in ABX-FMT also may have contributed to pathology, in particular *Mucispirillum schaedleri*, a possible pathobiont [38], was higher abundance in the FMT treatment group. *M. schaedleri* has several systems for scavenging oxygen and reactive oxygen species, making it well adapted to the microoxic conditions at the mucosa and in the elevated-redox environment in the gut during inflammation. Furthermore, it has several putative effector proteins with eukaryote-like domains that may play a role during inflammation [39], and it may utilize T6SS, a transport system associated with killing competing bacteria, to carve a niche in the microbiome [40]. Lastly, significant shifts in alpha and beta diversity indicated significant overall differences in microbiome compositions. This may indicate additional differences in microbiome structure and ecology involving taxa which fell under the limit of significance but are nonetheless important.

It thus remains unclear if changes in diversity as quantified by alpha and beta diversity, changes in overall bacterial communities and ecology, or changes in mentioned individual taxa result in observed sex-specific and FMT outcomes in our model. Future transplantation studies involving selected bacteria or full FMT in germ-free mice models will be help resolve these issues. While it has been reported that the microbiome can modulate disease phenotypes in a sex-specific manner [41], our current studies are the first to report sex-specific alterations in AD-related phenotypes by the gut microbiome using a transgenic mouse model of Aβ amyloidosis. We have further identified a number of gene pathways and microbes which may be involved in these processes and warrant further study.

Table 4.1: ANCOM significantly different ESVs and species OTUs at p22

M_Vehicle vs F_Vehicle	
	N.S.
M_ABX vs F_ABX	
	N.S.
M_Vehicle vs M_ABX	
	Higher in M_ABX

Table 4.1 Continued:

ESV	p_Proteobacteria;c_Gammaproteobacteria;f_Moraxellaceae;g_Acinetobacter
	p_Proteobacteria;c_Alphaproteobacteria;f_mitochondria
	p_Proteobacteria;c_Betaproteobacteria;f_Comamonadaceae
	p_Proteobacteria;c_Gammaproteobacteria;o_Pseudomonadales;f_Pseudomonadaceae
L7	p_Bacteroidetes;c_Flavobacteriia;f_[Weeksellaceae];g_Cloacibacterium;s_
	p_Firmicutes;c_Bacilli;f_Lactobacillaceae;g_Lactobacillus;_
	p_Firmicutes;c_Clostridia;f_Lachnospiraceae;g_;s_
	p_Firmicutes;c_Clostridia;f_Ruminococcaceae;g_Oscillospira;s_
	p_Proteobacteria;c_Alphaproteobacteria;f_mitochondria;_;_
	p_Proteobacteria;c_Betaproteobacteria;f_Comamonadaceae;_;_
	p_Proteobacteria;c_Gammaproteobacteria;f_Moraxellaceae;g_Acinetobacter;_
	p_Proteobacteria;c_Gammaproteobacteria;f_Pseudomonadaceae;_;_
	p_Proteobacteria;c_Gammaproteobacteria;f_Pseudomonadaceae;g_Pseudomonas;_
	Lower in M_ABX
ESV	p_Bacteroidetes;c_Bacteroidia;f_S24-7;g_;s_ (x2)
	p_Bacteroidetes;c_Bacteroidia;f_[Paraprevotellaceae];g_[Prevotella];s_
L7	p_Bacteroidetes;c_Bacteroidia;f_Bacteroidaceae;g_Bacteroides;s_ovatus
	p_Bacteroidetes;c_Bacteroidia;f_Prevotellaceae;g_Prevotella;s_
	p_Bacteroidetes;c_Bacteroidia;f_Rikenellaceae;g_AF12;s_
	p_Bacteroidetes;c_Bacteroidia;f_[Paraprevotellaceae];g_[Prevotella];s_
	F_Vehicle vs F_ABX
	Higher in F_ABX
ESV	p_Firmicutes;c_Bacilli;o_Lactobacillales;f_Lactobacillaceae;g_Lactobacillus
	p_Proteobacteria;c_Alphaproteobacteria;o_Rickettsiales;f_mitochondria
	p_Bacteroidetes;c_Flavobacteriia;o_Flavobacteriales;f_[Weeksellaceae];g_Cloacibacterium;s_
L7	p_Bacteroidetes;c_Flavobacteriia;f_[Weeksellaceae];g_Cloacibacterium;s_
	p_Cyanobacteria;c_Chloroplast;o_Streptophyta;f_;g_;s_
	p_Proteobacteria;c_Alphaproteobacteria;f_mitochondria;_;_
	p_Proteobacteria;c_Gammaproteobacteria;f_Moraxellaceae;g_Acinetobacter;_
	Lower in F_ABX
ESV	p_Bacteroidetes;c_Bacteroidia;f_S24-7;g_;s_ (x2)
	p_Bacteroidetes;c_Bacteroidia;f_Prevotellaceae;
	p_Bacteroidetes;c_Bacteroidia;f_[Paraprevotellaceae];g_[Prevotella];s_
	p_Bacteroidetes;c_Bacteroidia;f_Porphyromonadaceae;g_Parabacteroides;s_
	p_Bacteroidetes;c_Bacteroidia;f_[Odoribacteraceae];g_Odoribacter;s_
L7	p_Bacteroidetes;c_Bacteroidia;f_Bacteroidaceae;g_Bacteroides;s_ovatus
	p_Bacteroidetes;c_Bacteroidia;f_Prevotellaceae;g_Prevotella;s_
	p_Bacteroidetes;c_Bacteroidia;f_Rikenellaceae;g_;s_
	p_Bacteroidetes;c_Bacteroidia;f_Rikenellaceae;g_AF12;s_

L7

Table 4.1 Continued:

	p_Bacteroidetes;c_Bacteroidia;f_S24-7;g_;s_
	p_Bacteroidetes;c_Bacteroidia;f_[Odoribacteraceae];g_Odoribacter;s_
	p_Bacteroidetes;c_Bacteroidia;f_[Paraprevotellaceae];g_[Prevotella];s_
	p_Firmicutes;c_Bacilli;f_Lactobacillaceae;g_Lactobacillus;_
	p_Firmicutes;c_Bacilli;f_Lactobacillaceae;g_Lactobacillus;s_
	p_Firmicutes;c_Clostridia;o_Clostridiales;_;_;_
	p_Firmicutes;c_Clostridia;o_Clostridiales;f_;g_;s_
	p_Firmicutes;c_Clostridiaf_Clostridiaceae;g_Candidatus Arthromitus;s_
	p_Firmicutes;c_Clostridia;f_Lachnospiraceae;_;_
	p_Firmicutes;c_Clostridia;f_Ruminococcaceae;g_Oscillospira;s_
	p_Firmicutes;c_Clostridia;f_Ruminococcaceae;g_Ruminococcus;s_
	p_Proteobacteria;c_Epsilonproteobacteria;f_Helicobacteraceae;g_Helicobacter;s_

Table 4.2: PICRUSt KW significantly different pathways at p22

M_Vehicle vs M_ABX			
Pathway	Abundance Mean, M_Vehicle	Abundance Mean, M_ABX	FDR-P
Lipopolysaccharide biosynthesis	49150.4	13221.2	0.000866
Glycosaminoglycan degradation	23565	2132.5	0.000809
F_Vehicle vs F_ABX			
Pathway	Abundance Mean, F_Vehicle	Abundance Mean, F_ABX	FDR-P
Lipopolysaccharide biosynthesis	58487.2	8127.1	0.000365
Glycosaminoglycan degradation	24250	1312.5	0.000365
M_Vehicle vs F_Vehicle			
Pathway	Abundance Mean, M_Vehicle	Abundance Mean, F_Vehicle	FDR-P
N.S.			
M_ABX vs F_ABX			
Pathway	Abundance Mean, M_ABX	Abundance Mean, F_ABX	FDR-P
N.S.			

Table 4.3: ANCOM significantly different ESVs and species OTUs at week 7

M_Vehicle vs F_Vehicle	
	N.S.
M_ABX vs F_ABX	
	Higher in F_ABX
ESV	p_Verrucomicrobia;c_Verrucomicrobiae;f_Verrucomicrobiaceae;g_Akkermansia;s_muciniphila

Table 4.3 Continued:

L7	p_Firmicutes;c_Erysipelotrichi;f_Erysipelotrichaceae;g_Allobaculum;s_
	Lower in F_ABX
	N.S.
M_Vehicle vs M_ABX	
	Higher in M_ABX
ESV	p_Bacteroidetes;c_Bacteroidia;f_Bacteroidaceae;g_Bacteroides;s_ovatus
	p_Firmicutes;c_Erysipelotrichi;f_Erysipelotrichaceae;g_;s_
L7	p_Bacteroidetes;c_Bacteroidia;f_Bacteroidaceae;g_Bacteroides;s_ovatus
	p_Bacteroidetes;c_Bacteroidia;f_Porphyromonadaceae;g_Parabacteroides;s_distasonis
	p_Firmicutes;c_Erysipelotrichi;f_Erysipelotrichaceae;_;_
	p_Firmicutes;c_Erysipelotrichi;f_Erysipelotrichaceae;g_;s_
	Lower in M_ABX
ESV	p_Bacteroidetes;c_Bacteroidia;f_S24-7;g_;s_ (x8)
	p_Proteobacteria;c_Epsilonproteobacteria;f_Helicobacteraceae;g_Helicobacter;s_
	p_Actinobacteria;c_Actinobacteria;f_Bifidobacteriaceae;g_Bifidobacterium
	p_Bacteroidetes;c_Bacteroidia;f_Porphyromonadaceae;g_Parabacteroides;s_
	p_Firmicutes;c_Bacilli;f_Lactobacillaceae;g_Lactobacillus;s_
L7	p_Actinobacteria;c_Actinobacteria;f_Bifidobacteriaceae;g_Bifidobacterium;_
	p_Firmicutes;c_Bacilli;f_Lactobacillaceae;g_Lactobacillus;s_
	p_Firmicutes;c_Clostridia;o_Clostridiales;_;_;_
	p_Firmicutes;c_Clostridia;o_Clostridiales;f_;g_;s_
	p_Firmicutes;c_Clostridia;f_Ruminococcaceae;g_Ruminococcus;s_
	p_Firmicutes;c_Erysipelotrichi;f_Erysipelotrichaceae;g_Allobaculum;s_
	p_Proteobacteria;c_Deltaproteobacteria;f_Desulfovibrionaceae;g_Desulfovibrio;s_C21_c20
	p_Proteobacteria;c_Epsilonproteobacteria;f_Helicobacteraceae;g_Helicobacter;s_
F_Vehicle vs F_ABX	
	Higher in F_ABX
ESV	p_Bacteroidetes;c_Bacteroidia;;f_Bacteroidaceae;g_Bacteroides;s_uniformis
	p_Verrucomicrobia;c_Verrucomicrobiae;f_Verrucomicrobiaceae;g_Akkermansia;s_muciniphila
	p_Bacteroidetes;c_Bacteroidia;f_S24-7;g_;s_
	p_Proteobacteria;c_Betaproteobacteria;f_Alcaligenaceae;g_Sutterella;s_
	p_Firmicutes;c_Erysipelotrichi;f_Erysipelotrichaceae
L7	p_Bacteroidetes;c_Bacteroidia;f_Bacteroidaceae;g_Bacteroides;s_uniformis
	p_Bacteroidetes;c_Bacteroidia;f_Porphyromonadaceae;g_Parabacteroides;s_distasonis
	p_Firmicutes;c_Erysipelotrichi;f_Erysipelotrichaceae;_;_
	p_Verrucomicrobia;c_Verrucomicrobiae;f_Verrucomicrobiaceae;g_Akkermansia;s_muciniphila
	Higher in F_Vehicle
ESV	p_Bacteroidetes;c_Bacteroidia;;f_S24-7;g_;s_ (x11)
	p_Actinobacteria;c_Actinobacteria;;f_Bifidobacteriaceae;g_Bifidobacterium

Table 4.3 Continued:

	p_Bacteroidetes;c_Bacteroidia;f_Porphyromonadaceae;g_Parabacteroides;s_ p_Firmicutes;c_Bacilli;f_Lactobacillaceae;g_Lactobacillus;s_
L7	p_Actinobacteria;c_Actinobacteria;f_Bifidobacteriaceae;g_Bifidobacterium;_ p_Firmicutes;c_Clostridia;o_Clostridiales;_;_;_ p_Firmicutes;c_Clostridia;f_Ruminococcaceae;g_Ruminococcus;s_ p_Proteobacteria;c_Epsilonproteobacteria;f_Helicobacteraceae;g_Helicobacter;s_

Table 4.4: PICRUSt KW significantly different pathways at week 7

M_Vehicle vs M_ABX			
Pathway	Abundance Mean, M_Vehicle	Abundance Mean, M_ABX	FDR-P
Flagellar assembly	49563.6	4706.6	0.001933
F_Vehicle vs F_ABX			
Pathway	Abundance Mean, F_Vehicle	Abundance Mean, F_ABX	FDR-P
N.S.			
M_Vehicle vs F_Vehicle			
Pathway	Abundance Mean, M_Vehicle	Abundance Mean, F_Vehicle	FDR-P
N.S.			
M_ABX vs F_ABX			
Pathway	Abundance Mean, M_ABX	Abundance Mean, F_ABX	FDR-P
Lipopolysaccharide biosynthesis	50195.1	82159	0.007149

Table 4.5: ANCOM significantly different ESVs and species OTUs in FMT experiment

Tg_donor vs ABX+FMT	
	Higher in ABX+FMT
ESV	p_Bacteroidetes;c_Bacteroidia;f_S24-7;g_ (x2)
L7	p_Bacteroidetes;c_Bacteroidia;f_Bacteroidaceae;g_Bacteroides;s_
	Lower in ABX+FMT
	N.S.
ABX+Vehicle vs ABX+FMT	
	Higher in ABX+FMT
ESV	p_Bacteroidetes;c_Bacteroidia;f_S24-7;g_ (x2) p_Bacteroidetes;c_Bacteroidia;f_Prevotellaceae;g_Prevotella;s_ p_Bacteroidetes;c_Bacteroidia;f_Bacteroidaceae;g_Bacteroides _Bacteroidetes;c_Bacteroidia;f_Bacteroidaceae;g_Bacteroides;s_

Table 4.5 Continued:

L7	p_Bacteroidetes;c_Bacteroidia;f_Bacteroidaceae;g_Bacteroides;s_ p_Bacteroidetes;c_Bacteroidia;f_Bacteroidaceae;g_Bacteroides;s_g_Odoribacter;s_ p_Bacteroidetes;c_Bacteroidia;f_[Paraprevotellaceae];g_Paraprevotella;s_ p_Deferribacteres;c_Deferribacteres;f_Deferribacteraceae;g_Mucispirillum;s_schaedleri p_Firmicutes;c_Clostridia;f_Ruminococcaceae;g_Ruminococcus;s_ p_Proteobacteria;c_Alphaproteobacteria;o_RF32;f_;g_;s_ p_Proteobacteria;c_Deltaproteobacteria;f_Desulfovibrionaceae;g_;s_
	Lower in ABX+FMT
ESV	p_Bacteroidetes;c_Bacteroidia;f_S24-7;g_;s_
L7	N.S.

4.4 Methods

Animal housing and handling: APPSWE/PS1L166P mice on a C57BL6Cj background were received from David Holtzman lab at the Washington University. They were housed in sterile micro-isolator cage and fed ad-libitum on standard chow. All animal experimental procedures were performed in accordance with the approved Animal Care and Use Protocols (ACUPs) by the Institutional Animal Care and Use Committee (IACUC) at the University of Chicago.

Antibiotic treatment: Pups receiving ABX cocktail were gastric gavaged (animal feeding needles, cat 7901, Cadence) with established ABX cocktail50 (4mg/ml Kanamycin: Sigma K4000-5g, 0.35mg/ml Gentamicin: Sigma G1914-250mg, 8500U/ml Colistin: Sigma C4461-1g, 2.15mg/ml Metronidazole: Sigma M1547-25g, 0.45mg/ml Vancomycin: Sigma V2002-1g) in autoclaved water from post-natal day 14 to day 21 followed by an ad-libitum access to freshly prepared 1:50 diluted ABX water until the time of sacrifice. During seven days of post-natal ABX gavage, all mice were transferred to a new sterile cage after each gavage to avoid microbial contamination from accumulated fecal pellets in the cages. Parents from the same cage as pups receiving ABX-treatment were euthanized after the pups weaning and were not used for any breeding or future experiments as ABX-treated pups microbiome

could influence the paternal microbiome due to co-housing. ABX water was changed every 5-6 days for their best efficacy.

Fecal pellet collection: To evaluate the microbiome profile after high dose ABX treatment (p14-p21), fresh fecal pellet samples were collected in sterile 1.5ml centrifuge tubes at p22 day and stored in -80C from each pup. Fresh fecal pellets were also collected at the time of sacrifice to analyze the microbiome. After collecting the pellets, they were immediately frozen and stored at -80C until the time of bacterial DNA extraction.

Genomic DNA extraction from fecal samples: Stool pellets were collected and stored at -80C until processing. Pellets were used for microbial DNA extraction with the DNeasy Powersoil HTP 96 kit (Qiagen, Germantown, MD). The following modifications were made to the manufacturer's protocol: after samples, the bead solution, and the C1 solution were added to the bead plate, the plate was partially submerged in a water bath for 20 minutes at 60C, followed by a 20-minute shaking step on a MM 400 plate shaker (Retsch, Haan, Germany). This was done to further ensure lysis of the cells. The manufacturer's protocol was then followed to obtain clean DNA.

Fecal Microbiome Transplantation (FMT): Control age-matched APPSWE/PS1L166P transgenic mice were used to collect the fresh fecal pellets. Donor mice (n=4) were housed under SPF conditions in a separate cage from experimental groups with ad libitum food and water access. Everyday an individual mouse was used to collect the fresh fecal pellets. After collection, the fecal pellets were immediately mixed in sterile water (200mg/ml). The fecal slurry supernatant was collected after allowing the suspension to settle down using gravity for 5 minutes. Each recipient mouse in ABX+FMT group received 200 µl of freshly prepared fecal slurry by gastric gavage daily starting at the post-natal day 25 (p25) of age until the time of sacrifice simultaneously with long-term ABX protocol (original experimental design). ABX + vehicle group received 200 µl of sterile water in a similar fashion to nullify the everyday gavage effect.

Microbiome analysis: The V4 region of the bacterial 16S rRNA gene was amplified using

the Earth Microbiome Project primer set (515f-806r). Each 25 ml PCR reaction contained 12.5 ml of AccuStart II PCR ToughMix (Quantabio, Beverly, MA), 1 µl of 5 µM forward primer, 1 µll of 5 µlM reverse primer, 9.5 µl of water, and 1 µl of DNA extraction. The PCR program was 94C for 3 minutes to denature the DNA, with 35 cycles at 94C for 45 seconds, 50C for 60 seconds, 72C for 90 seconds, and then a final 72C step. Amplification was quantified using Picogreen (Invitrogen, Carlsbad, CA), and each sample was pooled at 70 ng per sample. Pools were cleaned using Agencourt AMPure XP beads (Beckman Coulter, Indianapolis, IN), and the clean pools combined. This final pool was quantified and sent to the Environmental Sample Preparation and Sequencing Facility (ESPSF) at Argonne National Laboratory for sequencing on an Illumina Miseq using V3 chemistry, following the Earth Microbiome Project protocol.

Bioinformatics: Raw 16S reads were demultiplexed, chimera and phiX filtered through the Qiime2 [42] pipeline and denoised with DADA2 [43]. The resulting representative sequences were aligned and masked with MAFFT [44]. A phylogenetic tree was constructed via FastTree2 [45]. Samples were subsequently clustered and taxonomically classified into operational taxonomic units (OTUs) with Qiime2 utilizing the GreenGenes 99% database [46]. Diversity metrics were calculated after rarefying reads at a depth of 10,000 sequences. Beta diversity was calculated as unweighted and weighted unifrac distances. Faith's Phylogenetic Diversity and Pielou's Evenness was used to measure alpha diversity. ESVs are reported with GreenGenes assigned taxonomy and species level clustered to OTUs by the Qiime2 Bayesian classifier [47]. Differences in relative abundances were calculated via ANCOM and considered significant at the $p<0.05$ level [48]. Correlations of metadata with OTU abundances were calculated as Spearman's rank correlations with raw P values determined via bootstrapping (1000 permutations) in QIIME. All reported P values are FDR corrected for multiple comparisons. Predicted functional analysis was performed via PICRUSt [49]. KEGG Mapper was used to map KEGG orthologs to pathways [50].

4.5 Author contribution

Thomas Kuntz: assessed gut microbiota profile, performed bioinformatics analysis, and assisted in the FMT protocol. Hemraj B. Dodiya: conceived and performed ABX experiments and fecal microbiota transplantation experiments, cytokine assessments, immunocytochemistry experiments, and confocal microscope and iMARIS evaluation. Shabana Shaik, Caroline Baufeld and Jeffrey Leibowitz: nanostring experiment, nanostring data analysis, and interpretation. Xulun Zhang: performed biochemistry experiment. Neil Gottel: supervised and processed microbial DNA extraction and 16S sequencing process. Xiaoqiong Zhang: generated APPPS1-21 Tg mice. Oleg Butovsky: reviewed and critiqued the manuscript. Jack A. Gilbert: reviewed and critiqued the chapter. Sangram S. Sisodia: developed the microbiota related hypothesis, co-designed the experimental study, reviewed and critiqued the manuscript.

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CHAPTER 5

MICROBIOME CHANGES INDUCED BY GABA TREATMENT OF RATS

5.1 Introduction

γ-aminobutyric acid (GABA) is the chief inhibitory neurotransmitter in the human (and generally mammalian) nervous system. It acts to reduce neuronal excitability in the nervous system by binding specific transmembrane receptors, GABA-A and GABA-B. These receptors are present across the nervous system, both in the central nervous system (CNS) and enteric nervous system (ENS), and in other locations such as immune cells. However, GABA's most well known and pharmacologically studied role, the reduction of anxiety and anxiety-like behaviors, depends on it's action on the CNS [1].

While GABA was not believed to cross the blood-brain barrier (BBB) based on initial studies [2], limiting its use as an exogenous supplement for anxiety, more recent studies have begun to question this assumption [3]. The usage of GABA as a drug remains understudied, but it is easily available to the public with many anecdotal testaments to its success. It may work because of propagation of effects from the ENS, limited but biologically substantial crossing of the BBB, mediation of effect through related metabolites, or other unknown pathways. Currently, GABA system modulating drugs instead are clearly BBB crossing compounds that either act as analogues or more commonly enhance the effects of endogenous GABA [4]. However, it is desirable because of side effect profiles and to expand treatment options to explore if GABA itself in some delivery mode is effective.

One pathway currently being explored is that of microbially mediated GABA supplementation [5]. It has been shown that microbes both produce and consume GABA [6] and can even be engineered to produce GABA [7]. Specifically, production of GABA is done by synthesis from glutamate via the enzyme glutamate decarboxylase (GAD). Consumption of GABA is done by GABA transaminase enzyme to convert GABA and 2-oxoglutarate

into succinic semialdehyde and glutamate [8]. Notably, glutamate can cross the BBB and then be converted back to GABA. Furthermore, GABA, by acting in the gut on the ENS and immune system could shift the microbial environment, and thus also foster a different ecology, ultimately changing how the microbiome affects it's host. These are two interesting pathways by which GABA systems can interact with the microbiome, propagating up to the host to possibly even change behavior.

Therefore, GABA supplementation should be studied with the microbiome in mind. Towards this end, I treated Sprague-Dawley (SD) rats with GABA in order to observe the shifts in microbiome, functional genes contained in that microbiome, and behavior of the rats. A one month time-point was used to observe longer-term, likely stable shifts in the microbiome, rather than the immediate effects. Results here would importantly break with the usual pharmacological paradigm of directly, solely host targeted drugs that are short acting and need to be taken regularly for effect.

The rat treatment consisted of a broad spectrum antibiotic regimen followed by gavage of GABA in saline vehicle. While oral gavage is unlikely for translational medicine, it is a useful method to determine if the rat-gut microbiome composition and functional potential was influenced by GABA. Without antibiotics, there is a greater possibility of the microbiome simply not being altered because of a resilience to perturbations. With our protocol, subtle but possibly important differences in the microbiome were seen between GABA treatment and controls. We hope nonetheless to develop better systems for influencing the microbiome with minimal confounding and maximum effect; this problem is one that is persistent across many microbiome studies and is a general goal that the field is attempting to ameliorate [9].

To test for anxiety-like behaviors, open field (OF) and elevated plus maze (EPM) test were undertook. Though it is unclear whether or not GABA treatment will affect these behaviors based on human studies, this represents a starting point for understanding the possible consequences of GABA treatment.

5.2 Results

5.2.1 GABA supplementation correlates with changes in fecal microbiota and functional gene pathways

Rats in 12 pairs of 2 caged rats aged 2 months were brought into the central animal facility at the University of Chicago and 2 weeks were given for them to acclimate to the new environment behaviorally and with respect to the microbial environment. Then for 5 days a cocktail of meropenem and vancomycin was added to drinking water. Two groups of rats were gavaged with either control saline vehicle (GABA-) or GABA plus vehicle (GABA+) for 5 days a week over 2 weeks. Based on observations by both bystanders and those gavaging the rats, the GABA+ rats tolerated the gavage better. Audio measurements of squeaks were taken to attempt to corroborate this. However, these proved difficult to quantitatively measure and did not reach into the ultrasonic range where much of the stressed rat vocalizations might have taken place [10]. We then waited 1 month for the microbiome to fall into a presumably steady state and collected stool samples and processed them to measure 16S marker gene abundances.

GABA+ and GABA- groups (n=12 for each) did not differ significantly in either beta diversity (weighed and unweighted unifrac) or alpha diversity (Faith's Phylogenetic Diversity). Based on ANCOM analysis there were two significantly different exact sequence variants (ESVs). These were identified via BLAST as *Alistipes onderdonkii* (100% identity) and *Flintibacter butyricus* (97% identity). There were no significant differences when ESVs were clustered into operational taxonomic units (OTUs) at any taxonomic level. This indicates very small but still significant differences in microbial communities.

Piphillin identified many significantly different functional genes, with the important butanoate metabolism pathway having the most hits. The specific genes identified as differentially higher in GABA+ indicate a possible increase in butyrate production from multiple sources (Figure 5.1). Running Piphillin only on the ANCOM identified taxa did not produce

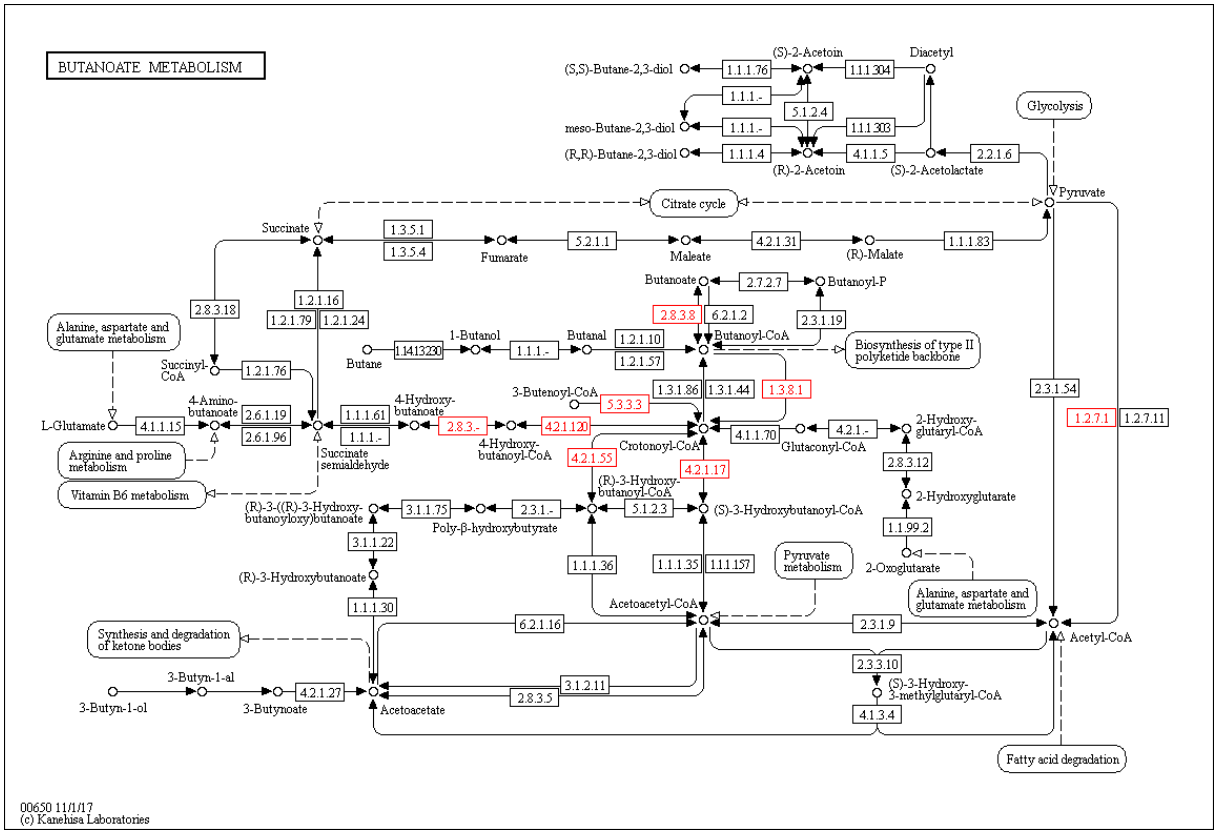

Figure 5.1: Butanoate metabolism KEGG pathways and gene orthologs. Differentially abundant Piphillin predicted genes determined by DESeq2 highlighted in red.

any gene hits (differentially abundant or not), indicating these taxa are not in the database and thus did not contribute to the observed differences between groups.

For the OF test, total distance, time spent in and out of the center of the arena (12 cm radius), and overall velocity and time spent moving were measured by human aided computer image tracking. For the EPM, time spent in the open arm versus closed arm and number of head pokes out of the closed arm were determined from human video analysis. No significant differences were observed between groups in any category in either the OF or EPM. Furthermore, both within and across groups, no ESVs or OTUs correlated with any OF parameters or EPM time spent in the open arm.

5.3 Discussion

The experimental paradigm used was pieced together from several sources since it was a fairly unique experiment compared to typical chemical or probiotic trials. Furthermore, this experiment was being run in tandem with a fecal microbiome transplant (FMT), so methods were used which could suit both purposes, particularly in using a gavage with antibiotics which is more of a requirement for an FMT. However, I believe this treatment represents an interesting and valuable case with antibiotic treatment allowing for a wider change in the microbiome and gavage allowing for a more consistent higher density application of GABA compared to the more standard drinking water treatment, ultimately giving us a greater chance of observing a change compared with less disruptive but more therapeutically relevant methods.

The antibiotics used, vancomycin and meropenem, and their dosages (500 mg/ml) were chosen to wipe as much of the indigenous microbiota as possible while having minimal action on the host, similar to [11] though the near analogue imipenem was used there. I decided on this system of two antibiotics, gram-positive and broad spectrum but weaker against gram-positive respectively, to reduce the complexity of the experiment and possibility of antibiotics acting in unintended ways compared to many other antibiotic regimens. One limitation of this is that meropenem can be difficult to obtain and is often restricted in its use due to the possible induction of drug resistance inherent to carbapenem antibiotics. Determining the therapeutic dosage of GABA is difficult since there is no scientifically accepted amount in rats or humans, but following previous studies which showed host effects, I used about 4 mg/kg (the dosage was not adjusted exactly to individual rat weights) [12, 13].

One month of waiting time was given before investigation of the microbiome and behavioral testing, as almost complete recovery of the microbiome was seen in 1 to 1.5 months in human patients [14, 15]. While about 6 months was required for recovery to be considered full, it was not considered feasible to wait that long, as the rats would have significantly aged over this time. Furthermore, recovery from antibiotics in rats may occur on differing

timescales; further research is required to determine this.

The GABA treated rat microbiome at one month from treatment showed few, but interesting differences from control rats. Taxonomically *Alistipes onderdonkii* and *Flintibacter butyricus* were found to be higher in differential abundance in the GABA+ rats. *Alistipes* species might be butyrate producers based on correlation analysis in a dextran sodium sulfate induced inflammation study [16] and *Flintibacter butyricus* is able to grow on glutamine and glutamate, producing butyrate [17]. Piphillin appeared to validate this by showing functional gene hits along the butanoate metabolism pathway (Figure 5.1) that may indicate butyrate production (many of these pathways are bidirectional). Interestingly however, running Piphillin on *Alistipes onderdonkii* and *Flintibacter butyricus* alone did not produce any functional genes, indicating these ESVs were not in the database or able to have genome reconstructions. Since nonetheless butyrate pathways were seen to be different between control and experimental groups, a more general possible butyrate producing microbiome was correlated with GABA treatment. These changes are likely brought about by small changes in many taxa as unifrac beta diversity was not significantly different between groups, indicating few if any significant changes in microbe abundance (corroborated by the small number of differentially abundant taxa). It will be interesting to see in future GABA related studies if specifically *Alistipes onderdonkii* and *Flintibacter butyricus* continue to play a role, and if this general butyrate pathway change is observed. One also could attempt probiotic or specific colonization in germ free animal experiments with these taxa, since both have been isolated and cultured, unlike many of the species in the microbiome. Of course directly testing for butyrate concentrations through metabolomic investigation is now indicated in GABA treatment, to see if functional gene differences translate to actual observable production of butyrate.

If butyrate is increased in the guts of GABA+ rats, it could have definite effects on physiology as it is a member of an important class of microbially produced compounds, the short chain fatty acids. Butyrate is a key mediator of microbiome-host interactions [18] and

is known to show promising effects in various diseases including obesity and diabetes [19], inflammatory bowel diseases [20], colorectal cancer [21], and neurological disorders, such as Parkinson's [22]. With regards to my research, butyrate has been shown to modulate neointimal hyperplasia severity [23], making GABA an interesting compound to test in this model instead of exogenous butyrate (see Chapter 3). Studies of butyrate's effects on behavior are still in their infancy, but it is possible that it is involved in the impact of the microbiome on social communication [24], a theory which would be interesting to study under specialized but well developed rat behavioral assays [25].

However, since we were expecting behavioral changes in the rats based on GABA's role as a neurotransmitter, we tested for anxiety-like behaviors with EPM and OF [26]. No results, either correlations with microbial taxa or significant differences in behavior, were seen between groups. There are two possible explanations for this. Firstly, that this is a true negative result. For the OF this is especially possible, as there are doubts as to how well it tests the anxiety-like behaviors specifically affected by GABA systems [27]. EPM however would likely show a response as it is the standard for tests of GABA modulating drugs, in fact detecting these differences better than serotoninergic anxiety reductions [28]. If this is a negative result then, we might conclude that at least at the one month time point, GABA supplementation is not having an effect similar to how GABA increases in the CNS would. It is possible (and seems more likely) that GABA supplementation in the short term might have an anxiety-like behavior reducing effect, and these tests should be run, especially since in humans the possible effects of GABA supplementation are considered only as the medication is being used. Secondly, it is possible the test were not run sufficiently well to have the precision needed to see the effects of GABA. There were concerns with the experimental setup, such as having the experimenter in the room, lighting that was brighter than recommended, and planks too thin for the rats to comfortably move on (several fell off the apparatus). It was noted that likely because of these issues the rats did not spend as much time on the open arms as is considered normal and enough for good statistics and

the rats did not tend to come in the open arm that was facing the experimenter at all [29]. Towards this end it would thus be useful to have a control where it is known that the EPM will distinguish between groups if possible, such as benzodiazepine treatment [30], to make sure that we are seeing changes when they do occur. The OF had less issues, though stronger lighting than recommended may have again produced increased anxiety-like behaviors [31]. But as stated, it is less clear that we should see any effect in the OF test. Ultimately, it would behoove us to attempt behavioral tests again with better conditions and suitable positive controls and at differing time points, to make sure that the behavioral component of GABA supplementation, the component most studied with regards to human trials, is not missed.

Nonetheless, I believe this experiment represents a good starting point for the study of GABA supplementation on the microbiome. It is particularly exciting that results were seen in a physiologically relevant and highly studied gene pathway and this alone certainly warrants further study. Even if GABA doesn't produce behavioral effects as was predicted, any drug that affects the microbiome in a non-antibiotic fashion is one that is currently novel [32]. In the future, we predict that microbiome modulating drugs will be an important part of the pharmacological landscape, as the microbiome is an important, mutable aspect of human physiology. That GABA showed effects that were apparent a month after discontinuation of the drug is also an interesting and possibly important phenomenon as the majority of drugs are only active as they are continuously taken, which in many cases is not ideal. Changing the microbiome for the better is a way in which health can be augmented in the long-term yet without unalterable permanent changes, which may be very desirable as a medical paradigm.

5.4 Methods

Experimental rats: Two month old male Sprague-Dawley (SD) male rats obtained from Charles-River were housed in a barrier facility at the University of Chicago under a 12-hour light cycle and were allowed 2 weeks to acclimate to the housing environment and their cagemate before gavage and 1 month before microbiome and behavioral testing. Standard

irradiated rat chow and autoclaved drinking water were provided ad libitum. All animal experimental procedures were performed in accordance with the approved Animal Care and Use Protocols (ACUPs) by the Institutional Animal Care and Use Committee (IACUC) at the University of Chicago.

Antibiotic treatment: Meropenem and vancomycin (Med-Vet, Mettawa, IL) was added to drinking water daily at 500 mg/ml for 5 days. No signs of reduced intake of water or weight loss were detected.

Gavage: 5 days a week for 2 weeks, 1 mL of solution was orally gavaged using a stainless steel bulb tipped gavage needle to deliver approximately 4 mg/kg GABA (Sigma-Aldrich, St. Louis, MO) in PBS. Control rats were gavaged with an equal volume of PBS.

Genomic DNA extraction from fecal samples: Stool pellets were collected and stored at -80C until processing. Pellets were used for microbial DNA extraction with the DNeasy Powersoil HTP 96 kit (Qiagen, Germantown, MD). The following modifications were made to the manufacturer's protocol: after samples, the bead solution, and the C1 solution were added to the bead plate, the plate was partially submerged in a water bath for 20 minutes at 60C, followed by a 20-minute shaking step on a MM 400 plate shaker (Retsch, Haan, Germany). This was done to further ensure lysis of the cells. The manufacturer's protocol was then followed to obtain clean DNA.

16S rRNA gene amplification and sequencing: The V4 region of the bacterial 16S rRNA gene was amplified using the Earth Microbiome Project primer set (515f-806r). Each 25 µl PCR reaction contained 12.5 µl of AccuStart II PCR ToughMix (Quantabio, Beverly, MA), 1 µl of 5 µM forward primer, 1 µl of 5 µM reverse primer, 9.5 µl of water, and 1 µl of DNA extraction. The PCR program was 94C for 3 minutes to denature the DNA, with 35 cycles at 94C for 45 seconds, 50C for 60 seconds, 72C for 90 seconds, and then a final 72C step. Amplification was quantified using Picogreen (Invitrogen, Carlsbad, CA), and each sample was pooled at 70 ng per sample. Pools were cleaned using Agencourt AMPure XP beads (Beckman Coulter, Indianapolis, IN), and the clean pools combined. This final pool

was quantified and sent to the Environmental Sample Preparation and Sequencing Facility (ESPSF) at Argonne National Laboratory for sequencing on an Illumina Miseq using V3 chemistry, following the Earth Microbiome Project protocol [33].

Sequence data handling and analysis: Raw 16S reads were demultiplexed, chimera and phiX filtered through the QIIME2 pipeline [34] and denoised with DADA2 [35]. The resulting representative sequences were aligned and masked with MAFFT [36]. A phylogenetic tree was constructed via FastTree2 [37]. Samples were subsequently clustered and taxonomically classified into operational taxonomic units (OTUs) with QIIME2 utilizing the greengenes 99% database [38]. Diversity metrics were calculated after rarefying reads at a depth of 8,000 sequences. Differences in relative abundances were calculated via ANCOM and considered significant at the $p<0.05$ level [39]. Beta diversity was calculated as unweighted [40] and weighted [41] unifrac distances. Faith's Phylogenetic Diversity was used to measure alpha diversity [42]. SubOTUs were taxonomically assigned via the NCBI BLAST web interface [43]. Functional genes were identified with Piphillin [44] and differential abundance determined by DESeq2 [45]. KEGG Mapper was used to map KEGG orthologs to pathways [46]. Correlations of metadata with OTU abundances were calculated as Spearman's rank correlations with raw P values determined via bootstrapping (1000 permutations). All reported P values are Benjamini-Hochberg false discovery rate (FDR) corrected for multiple comparisons.

Habituation: Animals were habituated to the experimenters (who were kept constant for each cohort of rats) and the testing equipment (OF and EPM) prior to being tested. On day 1, rats were transported to the testing room and left undisturbed in their home cages. On day 2, rats were briefly handled. Starting with the second day of habituation, rats were weighed three times each week for the duration of the experiment; no animal lost weight during the experiment. On days 3-5, rats were marked and handled for 5 min by each experimenter.

Open-field: Above a Plexiglas arena (50 x 50 cm, 60 cm high), a CCD color camera

(KT and C Co, Seoul, Korea) was mounted. The cameras were connected to a video card (Geovision, Irvine, CA) in a dedicated PC.

Elevated plus maze: The black Acrylic plastic maze consisted of two opposing open arms (length: 40 cm, width: 10 cm) and two opposing wall-enclosed arms (wall height: 15 cm) extending off of a center square (9 cm per side) to form a plus shape raised 1 m above the ground. A CCD color camera (KT and C Co, Seoul, Korea) was mounted above the center of the maze and connected to a video card (Geovision, Irvine, CA) in a dedicated PC in the room.

5.5 Author contributions

Thomas Kuntz: designed experiment, assessed microbiome profiles, performed bioinformatics analysis, administered antibiotics, collected stool samples, weighed and handled rats, performed behavioral testing, wrote chapter. Neil Gottel: supervised and processed microbial DNA extraction and 16S sequencing process. Haozhe Shan: collected stool samples, weighed and handled rats, designed EPM apparatus, performed behavioral testing. Yuri Sugano: sacrificed rats. Lindsey Luat and Michael Jordan: gavaged rats. Nora Molasky: video analysis. Peggy Mason: supervised rat work. Jack Gilbert: reviewed and critiqued the chapter.

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CHAPTER 6
INTRODUCING THE MICROBIOME INTO PRECISION MEDICINE

6.1 Abstract

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Understanding how individual people respond to medical therapy is a key facet of improving the odd-ratio that interventions will have a positive impact. Reducing the non-responder rate for an intervention or reducing complications associated with a particular treatment or surgery is the next stage of medical advance. The Precision Medicine Initiative, launched in January 2015, set the stage for enhanced collaboration between researchers and medical professionals to develop next-generation techniques to aid patient treatment and recovery, and increased the opportunities for impactful preemptive care. The microbiome is a key facet of health and disease, it augments drug responses and tolerance, and it can influence endocrinology, physiology and even neurology, altering the outcome of many different disease states. We will review the implications of understanding the microbiome on precision health initiatives and highlight excellent examples, whereby precision microbiome health has been implemented.

6.2 Introduction to precision medicine

The sequencing of the human genome [2] in 2001 fostered advances in both our understanding of the genomic basis of disease and in the DNA sequencing technologies required to bring the results of this understanding to patients. This is often referred to as precision genomic medicine, utilizes a patient?s individual genome to inform treatment and care, based on known genomic markers for disease [3]. The broader, inclusive field of precision medicine couples a person?s treatment with what is known about their population, life style, and

medical history, by matching clinical data and genetic biomarkers. Since the genome is sometimes conceptualized as the core of human individuality, at least in terms of disease, the broader field of precision medicine is often conflated with genomic medicine. Precision medicine, however, includes aspects downstream from the genome, including gene expression and protein expression as well as metabolic markers. Nonetheless, genomic information is the most commonly used and has had great successes [4]. Cancer treatment in particular has been revolutionized by genomic medicine [5], which exemplifies that despite difficulties in implementing precision medicine, it is a deeply important development. In particular, achieving the goals of precision medicine, including diagnosing disease more accurately, and reducing the relative risk of treatments, side effects and non-responses to medications, will revolutionize both treatment courses - ideally at the single patient level [6] - and the structuring of medical care and costs, moving towards cheaper, preventative focused medicine.

6.3 The microbiome as a precision medicine frontier

In this review we focus on a more recent but in many ways analogous development, that of introducing the microbiome into precision medicine. The human microbiome is the the ecological community of commensal, symbiotic, and pathogenic microorganisms that literally share our body space? [7]. These microorganisms, mainly bacteria, fungi, archaea, and viruses in the gastrointestinal tract, are slightly more abundant than the human cells in the body [8], leading some to classify it as an organ. Certainly, the influence of the microbiome on our physiology is significant, affecting immunology [9], neurology [10, 11], endocrinology [12], and, importantly for precision medicine, disease states and clinical outcomes. As it is a nascent and quickly developing field - owing to the faster than exponentially decreasing cost of sequencing [13] - additional important functions of the microbiome are likely to still be discovered. These discoveries are driven by similar sequencing technology as that which has enabled personal genomics and is decreasing rapidly in price, so much so that personal microbiome sequencing is already available to the consumer (e.g. American Gut - ameri-

cangut.org; uBiome - ubiome.com). Furthermore, the well-developed analysis and statistical techniques of genomic medicine have commonalities with microbiome analysis. Since microbiome states are highly individual even between co-raised identical twins [14], but can be rapidly changed [15] (unlike genetics), there is a profound opportunity for individualized treatments. However, the microbiome, like any ecosystem is also profoundly complex, and so the goals of precision microbial medicine require considerably more research before they are appropriately realized [16]. Nonetheless, the microbiome, as we shall exemplify here, is primed and ready for precision medicine, and therefore the clinical application of this new therapeutic area is on the immediate horizon. Various complementary routes of assaying and modifying the microbiome have been proposed and tentatively utilized towards this end; these will be laid out here in the following text as well as diagrammatically (Figure 6.1).

6.4 Review of microbiome analysis techniques

How then could microbiome precision medicine be implemented? Currently two complementary analyses, both beginning with the extraction of microbial genomic DNA, are standard in the field: 16S rRNA sequencing and shotgun metagenomics. The 16S rRNA gene has both highly conserved regions, allowing for the usage of extremely bacterially nonspecific primers, and "hypervariable" regions, where base pair differences can often provide species level identification [19]. Thus, 16S rRNA amplicon sequencing provides a robust tool for identification as well as classification and even discovery of bacteria [20]. A typical 16S rRNA study utilizes the differences in observed communities of bacteria between differing samples to obtain statistically significant correlations between bacterial composition and sample description, for example to identify differences in the gut microbiomes of children born to obese mothers [21]. These studies have led to key insights into the human microbiome. While historically the majority of biomedical research on bacteria has focused on eliminating pathogens, many bacteria as well as communities of bacteria are important in both health and disease [7].

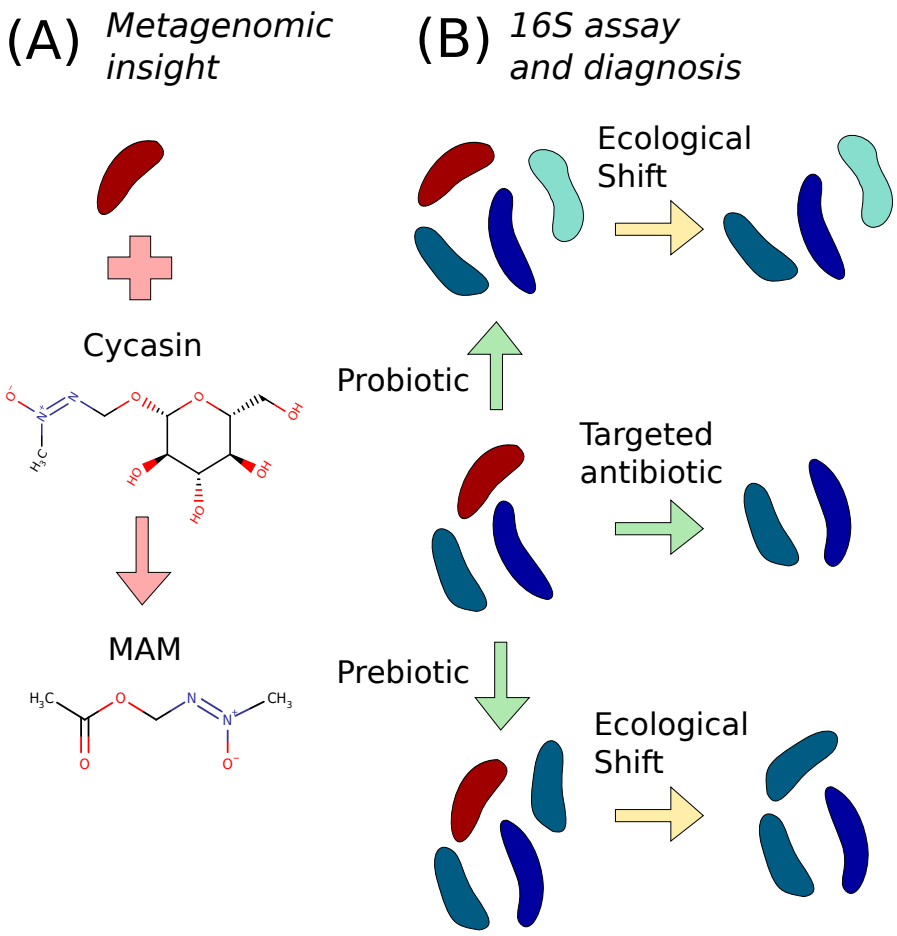

Figure 6.1: A schematic of methods in precision microbiome medicine and their possible interplay: a) As an example, certain microbes, here represented in red, metabolize the compound cycasin to produce a carcinogenic compound methylazoxymethanol (MAM) [17]. This functional potential of the microbe might be discovered through metagenomic sequencing. b) If targeted removal of the red microorganism - identified in a patient via 16S sequencing - was desired, without harming commensal bacteria, represented in shades of blue, three approaches (green arrows) might be utilized. Direct removal of the deleterious microorganism through targeted antibiotics ideally would not affect commensal bacteria. Probiotic treatment introduces new beneficial microorganisms while prebiotic treatment favors the growth of existing beneficial microorganisms. Note that prebiotic and probiotic treatments do not directly remove the targeted microorganism, but in certain cases may shift the gut ecology such that it does not thrive [18]. In all three cases, the specific circumstances may affect which treatment is best employed and what residual outcomes there are on the microbiome.

Though identifying causative bacteria in disease states will be an important facet of precision medicine, understanding the overall ecology of the microbiome may be equally or even more vital.

The resilience of the microbiome to perturbations is particularly important when a systemic failure of the ecosystem (for example the gut) presages a disease, for example in antibiotic damage mediated *Clostridium difficile* colitis [22]. The dynamics of the microbiome following a perturbation are likely different depending on bacterial community structure, which is has previously been characterized in western populations by stable, long-term diet driven "enterotypes" [23, 24]. However, the initial study proposing enterotypes used a human population of 22 subjects, and defined 3 enterotype groups, which had a predominance of either *Bacteroides* (enterotype 1), *Prevotella* (enterotype 2) or *Ruminococcus* (enterotype 3). Although these clusters are intriguing, further investigation suggests that even within an individual human the microbiome can vary between enterotypes [25], and additional data from larger-scale studies suggest an extended continuum [26], perhaps punctuated by microbial community states, rather than statically maintained in discrete clusters. These ideas have led to diagnostic breakthroughs such as the stratification of irritable bowel syndrome (IBS) patients into microbiome based groups that do not correspond to previously defined IBS subtypes [27] and may have differing therapeutic responses.

To go beyond diagnosis and stratification, it will be essential to understand the functional potential of the microbiome. Shotgun metagenomics enables the researcher to understand this function potential by characterizing the genetic information for the microbial community. Metagenomics is the analysis of the complete genomic repertoire of the community, by sequencing DNA extracted from that community, rather than relying on amplification of a marker gene. Taxonomy can still be determined from signature genes (including 16S rRNA), but it is also possible to assign phylogeny of the functional genes by comparing the DNA sequence against a library of genomes from close relatives [28]. In addition, metagenomics enables the assembly of genomes from organisms in the microbiome that are resistant to cul-

ture, providing a higher resolution exploration of the taxa associated with each person [29] This enables us to determine the metabolic and signaling capacity of each taxon, to determine how it will interact with the immune system and the rest of the body [30] This clearly makes metagenomics of great interest for the development of precision medicine; however, one must be aware of the challenges this technique presents. Metagenomic studies are necessarily more expensive and computationally complex than 16S rRNA based studies. Possible contamination from undesired DNA and biases of analyses towards culturable organisms [31] further complicate matters. Ultimately metagenomics is an extremely useful tool, but there the application of this technology to precision medicine will require a better understanding of the implications of these limitations, especially when scaling up to treatments of large patient populations.

6.5 Avenues towards microbiome based precision therapies

6.5.1 Microbiome-xenobiotic interactions

That gene polymorphisms can drive changes in drug metabolism has been known for some time; it was noted as early as 1957 that atypical forms of serum cholinesterase led to potentially fatal reactions to certain anesthetics [32]. This and other adverse drug reactions are estimated to cost from 30 to 130 billion dollars in the US annually [33, 34] and are a significant source of patient non-compliance and therapy failure [35]. Reducing these adverse reactions is a primary goal of precision medicine. While some interactions are idiosyncratic, a recent survey of adverse drug events observed that about 35% of these events were drug-gene or drug-drug-gene interactions involving cytochrome P450 oxidase (CYP) variants [36]. CYPs are generally considered the body's innate and primary general purpose drug metabolizers; they are involved in about 75% of total human drug modification [37].

However, microbial metabolism in the gut is also a significant factor in biotransformation, especially for low solubility, low permeability compounds [38]. Currently, more than 60

drugs have been identified to have microbiome interactions according to the PharmacoMicrobiomics database [39], and given the vast number of possible unique microbial metabolic transformations [40] and plasticity of the microbiome [41], many more interactions are likely to be discovered compared with the apparently relatively limited number of human genetic interactions. Importantly, the primary forms of xenobiotic metabolism are different between human and bacterial cells: oxidation and conjugation dominate in the former case, reduction and hydrolysis in the latter [38]. Metabolism of drugs is actually a key component of many therapies; so-called "prodrugs" are essentially drugs that will be metabolized into a pharmacologically active drug after consumption. Therefore, production of active drug metabolites from prodrugs is sometimes dependent on the microbiome, with the possibility to either improve or worsen outcomes [42]. This often manifests as a modulation of bioavailability to the human, an important consideration for prediction of appropriate dosing in precision medicine. Efficacy and side effects are also altered directly by microbial metabolism. For example, acetaminophen toxicity shows substantial variability within a given human population [43], and the microbiome has been identified as playing a role in this variability. Members of the genus *Clostridium*, as well as other bacteria can produce p-cresol, which competes as a substrate for SULT1A1 (a human liver enzyme) with acetaminophen [44]. A reduction in the breakdown of acetaminophen by SULT1A1 leads to a build up of NAPQI, which leads to hepatotoxicity. Strikingly, in some cases even strain level differences can lead to altered metabolism, such as inactivation of digoxin by a non-universal *E. lenta* gene. Digoxin has a narrow therapeutic window, and thus a wrong dosage could lead to significant toxicity, highlighting the need for further study of metagenomic diagnostics and insights to adverse outcomes [45].

Furthermore, alternative mechanisms for xenobiotic microbiome interaction including immune [46] and endocrine [12] modulation by bacteria and competition between bacterial metabolites and drugs for human enzyme modification [47] are known to exist, complicating and enlarging the pool of possible drug-microbiome interactions. And lastly, there are

possible reciprocal relations: drugs may both be altered by the microbiome and alter the microbiome. For example, antipsychotic medication has been shown to both alter the microbiome and have microbiome dependent side effects [48]. While this greatly complicates endeavors to understand microbiota-xenobiotic interactions, it also points towards a different microbiome driven approach to precision medicine: directly targeting the microbiome for clinical results.

6.5.2 Targeting the microbiome

It is clear that medication is already utilized to have a direct effect on the microbiome; one needs to look no further than antibiotics. While these drugs are utilized for the eradication of pathogenic bacteria, they have widespread effects on the microbiome, possibly leading to adverse outcomes. Secondary infections caused by antibiotics are well known, most saliently *Clostridium difficile* [49], but it is often less appreciated that antibiotics can have side effects on the human, for instance fluoroquinolone associated cardiotoxic [50] and neuropsychiatric [51] reactions. Importantly, consequences of antibiotic usage, such as reduction of inflammation, are possibly not only human off-target drug effects, but also unintended consequences of microbial community disruption [52]. Studies using mouse models suggest that stress induced increases in circulating cytokines were abrogated by broad-spectrum antibiotic treatment [53]. Furthermore, these types of interactions are not limited to drugs classified as antibiotics; many other drugs have antibiotic and other microbial community structure and function modulating properties that are beginning to be appreciated [54, 55]. While many of these perturbations to the microbiome are associated with poorer outcomes, some drugs may derive some or all of their beneficial qualities from alteration of the microbiome, thus they could be considered a form of discriminatory antibiotic.

A precision medicine therapy that leverages microbial community structural modulation could have beneficial clinical impact. Certainly if pathogen-specific antibiotics were developed the odds ratio could be greatly increased compared to traditional antibiotics. A clear

approach is to design a species-specific enzyme inhibitor or other antimicrobial molecules. For example, a *Streptococcus mutans* targeted drug based on the fusion of a species-specific targeting peptide domain with a wide-spectrum antimicrobial peptide domain has already been developed [56]. However, the bacterial community was also altered when using this peptide, despite its high specificity [57]. It has been suggested that this may shift the microbiome into a healthier state, but of course there is also the potential for negative ecological effects, but these may be less than for traditional antibiotics.

An intriguing approach that may largely avoid the problem of system scale changes in microbial community structure, as well as that of increasing antimicrobial resistance, is to non-lethally target specific enzymes in the bacteria. This has been realized at the multi-species level [58] through targeted inhibition of bacterial tri-methyl amine (TMA) formation by 3,3-Dimethyl-1-butanol (DMB, a structural analog of choline) ultimately attenuating atherosclerosis in a high choline diet mouse model. Surprisingly, slight alterations of bacterial composition were still observed, underscoring the extremely dynamic nature of the microbiome. Nonetheless, this study points towards a microbiome based intervention for a specific (i.e., "Western") diet driven disease. In this case, a single target approach is undesirable, as reduction of global TMA formation is the goal, but given the availability of single isozyme inhibitors [59], precision, non-lethal drugs likely could be developed. These furthermore have the potential to be minimally bioavailable to the human, limiting side effects, and might be exploited not only to target pathogens but also to reduce microbiota-drug interactions through selective elimination of problem microbes.

A final approach for targeted antimicrobials has been successfully employed for approximately 100 years, though not in the western world [60]. Phage therapy was developed predominantly in Russia and was effectively used to control outbreaks of gastrointestinal diseases in Russian armies in World War I and II [61]. The basic premise is that many bacterial species, and maybe even each strain (sub-species), are predated upon by a unique phage [62]. Phage target bacteria cell-membrane protein and sugar complexes that are unique to

each bacterial taxon. Therefore, by identifying the correct phage it should be possible to surgically remove a specific bacterial species from an assemblage. This will enable accurate restructuring of a microbiome so as to precisely augment the functional properties of that consortium. In fact recent evidence from the commercial sector suggests that the same mechanisms employed by phage to target and penetrate bacterial cells, can be programmed into nano-particles that mimic these phage-properties to infect and kill specific cells (Pers. Comm. Jeffrey Miller, UCLA). In this new future, we may have ultimate control over the microbiome.

6.5.3 Prebiotic treatments

Conversely, instead of targeting the microbiome to reduce deleterious bacteria, one could aim to increase the levels of beneficial bacteria or otherwise positively alter the structure or function of the microbiome. Substances applied in this way are often referred to as prebiotics. However, the types of prebiotics currently studied are limited in scope, usually non-digestible fiber compounds that stimulate growth of *Bifidobacterium* and other taxa to produce short chain fatty acids (SCFA) including butyrate and propionate [63]. Though this is promising as a broad treatments for several conditions [64], efforts for precision medicine in this sphere will require the expansion of the scope of prebiotics. Given that metagenomic and metabolomic advances continue to better characterize the metabolic potential of the microbiome, dietary compounds that stimulate alternative beneficial bacteria towards useful metabolic endpoints will be discovered [65].

More audaciously, one might aim at fine-tuning the interactions between microbiota of the gut microbiome. The microbiome is a complex, human co-evolved ecosystem that produces many bioactive compounds, often for intercellular communication [66]. These compounds could be mined to find those which modulate the microbiome in a beneficial way, thus unearthing novel prebiotics [67]. While microbial community disruption is the consequence of both xenobiotic and microbiome targeted drug metabolism, these types of prebiotics might

provide a more gentle perturbation than possible with the former by harnessing already existing biological pathways. This goal certainly seems distant, but as dynamical systems approaches to studying the microbiome continue to develop, we may find that treating certain dysbiotic states require perturbations of varying magnitudes or delicate maintenance of the stability of the microbiome, especially in at-risk populations [68].

6.5.4 Precision probiotics

Perhaps the most direct strategy for altering the microbiome is the usage of probiotics, live microbes administered for health benefits. This idea has been employed since at least 1907 when Elie Metchnikoff hypothesized lactic acid producing bacteria could implant in the gastrointestinal tract to enhance longevity [69]. Today the probiotic landscape is still dominated by lactic acid bacteria, specifically genera *Lactobacillus*, though it is now appreciated that their beneficial properties are not limited to the production of a single metabolite, and that other potential probiotic bacteria could effect various outcomes through multifarious means [70]. This opens the door to precision probiotic development since application of microorganisms is highly specific with regards to both applied agent and effect. Devices now exist for isolating microorganisms based on metabolic output [71], and work is being done to identify probiotic bacteria that produce particular compounds of therapeutic potential [72]. This may include compounds whose efficacies are contingent on route of administration, for example those that are inactive orally. Furthermore, probiotics are being bioengineered to expand their ranges and modes of actions as well as their robustness and incorporation [73]. However, it is important to keep in mind that interactions with diet, established microbiota, and genetics, are known to modulate overall health outcomes if not specific effects and mechanisms of probiotics [70]. Therefore effective patient classification and stratification is required for best results. Success of this program will require detailed insights into metagenomic potential and ecological interactions of presumptive probiotic bacteria, making precision probiotic development a task of considerable difficulty but one that has already

seen demonstrable results [74].

6.6 Regulation and application

Despite the therapeutic promise of the microbiome, its application to precision medicine will require us to overcome considerable hurdles. One may anticipate that failure to successfully apply genomic medicine may lead to delays in the application of the microbiome as a precision therapy. For example, the current legal and R&D model is not well suited for development of genome-informed drugs [75]. Microbiome therapies likewise face difficulties, especially owing to the wide breadth of treatment options, many of which lack analogs in current medical practice. Furthermore, clinicians have been reticent to use the results of genomic information - and thus likely future microbiome data - in treatment due to both uncertainties on its importance and lack of understanding [76]. These problems are highlighted in the case of Plavix (clopidogrel), whereby despite an FDA box warning [77] indicating serious or fatal risk for those carrying certain CYP2C19 variants, this drug is still routinely used on genetically incompatible patients due to poor coverage by insurance and failure to clinically utilize genetic testing [78]. In the case of the microbiome, fecal transplant treatment for *Clostridium difficile* colitis is known to be highly effective especially in recurrent infection [79]; however, this procedure still requires a licensed practitioner to have a protocol approved by their local Institutional Review Board, and therefore each patient needs to be consented prior to therapy. For a therapy with a greater than 90% success rate, this is peculiar. However, it is because we still lack the ability to characterize the microbial community of donor stool appropriately. This means that we do not know the active components of the fecal transplant, and therefore it is very difficult to regulate this using standard legislation under Federal Drug Administration protocols. More importantly, we still don?t fully understand the implications for microbiome therapy on a large scale. While fecal transplants are becoming extremely numerous with few legitimate side effects, it is still hard to predict the outcome across a broad population. The same is true for genomic medicine, whereby the interaction of genes

with the environment is difficult to predict [80]. This requires enormous sample populations for any investigation to be statistically significant [81]. Though the future is bright for genomic medicine, particular issues currently impede efforts towards its development.

Fortunately, some of the difficulties in genomic medicine research and deployment might be lessened in precision microbiome medicine. Environmental-microbiome interactions are potentially more easily studied because there is a more direct interaction between the two, allowing for simpler identification of sample populations and achievement of statistical power. With the correct experimental design, genetic variation can be sufficiently decoupled from microbiome and environmental factors. In fact, studies of this nature already exist, both on humans [82] and especially on mice, where genetics can be well controlled [83]. This bottom up approach can then be extended by genomic studies which better account for confounding factors. Even where genetics is a significant factor, such as in mental health disorders, incorporating the microbiome greatly increases understanding and ultimately treatment of diseases [84]. Of course in disease states where the effects of genetic variation are either entirely or nearly absent, the microbiome is a great candidate for investigation. Conditions such as obesity [85] and inflammatory bowel disease [86] can in subsets of patients be driven by dysbiosis, a chronic, systemic maladaptation of the gut microbiome to the host. Unlike genomic medicine, there are possibilities especially for these conditions to do research in an in vitro environment, most excitingly in artificial gut paradigms [87]. Microbiome precision medicine also has the opportunity to break free of present R&D and legal hurdles to precision medicine. The regulation and marketing of these treatments will at least pose challenges for traditional models [88, 89], as evidenced by the FDA's current stance on probiotics [90], which has led to faster product delivery to the public but also quality control and effectiveness issues [91].

6.7 Notable application: medically underserved communities

Given the above unique assets of the microbiome modality of precision medicine, a promising potential area for its development is in low socio-economic status (SES) and other under-served communities. Low SES is associated with reduced diversity in the gut microbiome [92]. Numerous factors are also present especially in urban communities that reduce immunoregulation, including reduced exposure to microbes in the natural environment [93] and increased stress [53], and increase obesity prevalence and dysbiosis, including increased density of fast-food restaurants [94] and lack of physical activity [95]. This is likely interrelated with microbiome-associated diseases such as asthma [96] and gastrointestinal symptoms [97]. The vast majority of genomic variants discovered are either rare with large effects or common with small effects, unlike in this situation where there is a possibility of appreciable effect size combined with biomarker occurrence. Therefore, these at-risk communities present a potentially illuminating cohort for microbiome.

Of course, great care must be taken to not draw inappropriate or invalid associations between microbiome [98] (or genome [99]) variations and minority status. Lack of cultural understanding and disparities in access to services have driven poor research trends in the past and continue to be a deep issue in the development of precision medicine. Access issues in particular have caused demonstrable problems; statistics on epidermal growth factor receptor testing, for example, show associations of lower educational attainment and income with reduced likelihood of testing [100], and studies suggest health insurance coverage alone does not explain this general effect [101, 102]. For precision medicine to succeed then, under-served populations must be both active participants and beneficiaries of research. Microbiome research in particular could lead to high impact clinical interventions for these communities, hopefully spurring its development. It is both an opportunity and imperative for microbiome precision medicine to address social epidemiological trends, but this is only possible through the combined efforts of researchers, clinicians, the government, and perhaps most importantly the people at large.

6.8 Concluding remarks

Here we have presented a collection of potential avenues towards introducing the microbiome into precision medicine. Though it is difficult to know if and when these techniques will ultimately make it to the clinic, there is substantial evidence that microbiome-based medicine holds great future potential to improve odds-ratios, reduce side effects, stratify patients, and precisely treat previously difficult or untreatable conditions. Ultimately, the microbiome must become an integral part of precision medicine as a whole, since so much of human functioning and metabolism is dependent upon it. If this is to happen in the near future, as it hopefully should, we must better understand the microbiome and its interactions with the human and the environment via a concerted effort and conversation between researchers, clinicians, patients, the government, and most importantly, the broader community.

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CHAPTER 7

CONCLUSION

In this thesis, I've presented three case studies which demonstrate the utility of exploring the microbiome through a chemical lens. This viewpoint is extremely valuable as it offers a window into the fundamental interactions of the microbiome, a chemical ecology, as well as how microbes interact with exogenous chemicals. Practically, these insights can be used in areas such as microbiome targeted precision medicine, a rapidly developing field with profound implications in human health, which was subsequently reviewed here.

In my first case study, the outcomes of neointimal hyperplasia following surgery were seen to be altered by cohousing two rat strains, one which is more susceptible to hyperplasia. Interestingly, this effect was not bidirectional, the susceptible rats had better outcomes after cohousing but the less susceptible rats did not have significant changes. This can possibly be explained by differing stabilities of these strain's microbiomes as determined by graph theoretic methods. Furthermore, several taxa were found to correlate with a measure of hyperplasia. Some of these taxa such as *Peptococcus* have ill-defined roles in the microbiome which hopefully can be elucidated in the future through culturing or other exploratory methods. Others such as *Prevotella* are better characterized and have complex interactions with the immune system and inflammation which may drive development of hyperplasia. Lastly, we observed strong correlations between neointimal hyperplasia and microbe-derived metabolites kynurenine/tryptophan ratio and 3-hydroxyanthranilic acid. These effects might have been produced by either bacterial TDO2 or murine IDO proteins, and disentangling this represents a path forward in the study of microbiome modulated hyperplasia.

In the second study, antibiotics administration was seen to alter Alzheimer's disease (AD) progression in a transgenic mouse model in a gender specific manner. We observed that female antibiotic (ABX) treated mice showed a greater abundance of *Allobaculum* and *Akkermansia*, certain species of which are mucin degraders which could lead to a weakening of the

boundary between the gut and microbiota allowing for greater translocation of metabolites and inflammation. Functional genes corresponding to likely increases in inflammation via lipopolysaccharide synthesis were seen in both genders as were genes indicating glycosaminoglycan (GAG) degradation, which possibly influences creation of Aβ fibrils depending on the molecular weight of the GAGs. Testing this with GAG and non-digestible GAG analogue administration is a high priority. Furthermore, fecal microbiome transplant (FMT) studies demonstrated that pathology could be restored in ABX treated mice by restoration of a similar microbiome to non-treated transgenic mice. *Mucispirillum schaedleri* may be a key player in this process as it was increased in the FMT group and is considered to be a likely pathobiont, playing a role in inflammatory processes.

My third study represented an approach of directly drugging the microbiome and observing the effects this perturbation had at a later time point. γ-aminobutyric acid (GABA) was orally gavaged into broad spectrum antibiotic treated rats and the gut microbiome contents assayed a month later. Small but significant differences were seen in the microbiomes of the GABA treated versus saline control rats, namely higher abundances of *Alistipes onderdonkii* and *Flintibacter butyricus*. These organisms are purchasable and culturable, providing a target for future studies, such as probiotic trials. Behavioral tests, open field and elevated plus maze, did not reveal any differences between experimental groups; however, whether this was a failure to resolve differences or a lack of effect was difficult to resolve as there were issues with the apparatus and testing facility. Either way, GABA treatment was revealed to correlate with increased abundance of predicted microbial genes in the butyrate pathway, likely increasing production of butyrate, a keystone bacterial product with implications on host physiology. Whether this leads to behavioral changes, or if behavioral changes can be induced at different testing time points or with different dosages or delivery methods of GABA remains an interesting open question.

These findings fit well into the context of microbiome precision medicine, whereby personalized therapies are developed based on a subject's individual microbiome profile. One

could for example predict if neointimal hyperplasia is more likely to occur following surgery and ameliorate this with something perhaps as simple as a change in external microbial environment or if necessary a targeted probiotic or antibiotic. Gender differences in AD continue to add complexity to treatment of this disease and exploring how distinctions in the microbiome differentially affects men and women is an important personalized medical principle. Our findings indicate GABA treatment directly affects the microbiome and in the future we would like to see how this effect is changed based on different starting microbiome states in order to best determine when GABA treatment would be indicated as currently in human trials there is conflicting evidence on its efficacy, perhaps owing to differences in the microbiome.

The future is bright for studies of the microbiome which utilize chemical understanding to elucidate key features of microbial interactions with each other and the host. We will certainly see scientists continue to use the intuitions granted by chemical training and techniques to explicate the manners in which the microbiome functions, especially with respect to health and external pressures such as medication and surgery, and I am excited to see where this leads. I hope this work serves as a useful primer for chemists looking to explore the microbiome, for others looking for a chemist's perspective, a document of my research, and a roadmap for where the field is moving.