ROLES OF THE MICROBIOTA IN INFLAMMATORY BOWEL DISEASES

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Abstract

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Studying the roles of the microbial community structure as it relates to disease can give important insight into development of inflammation. This study employs concepts present in ecology to understand how altering bacterial communities in mammalian intestines affect inflammation at different stages of disease. The overall goal is to understand the role of the resident microbiota in initiation and progression of inflammatory bowel disease (IBD). Changes in the community can possibly act as a biomarker to diseases allowing preventative action to be taken or development of novel therapies.

Work was done on two different models of IBD, dextran sodium sulfate (DSS)-induced murine colitis and *Helicobacter hepaticus* triggered inflammation in IL-10$^{-/-}$ mice (model of Crohn's disease). Through culture independent techniques, changes in the microbiota were investigated, together with alterations in host mediators.

In the first model, whether DSS-induced colitis was associated with changes in the microbiota was investigated. DSS administration was associated with microbial changes in intestinal communities in mice and these changes were

present at onset of inflammation and persisted through later stages of disease. In the second model, whether changes in microbial community structure affected disease severity was investigated. The *H. hepaticus* triggered disease model was used to explore this question, and it was found that *H. hepaticus* infection of mice with different microbial community structures resulted in similar disease outcomes. This indicated that disease severity was independent from antibiotic-induced changes in the microbial community in this model. We also show that the microbiota is essential in disease induction in mice in our colonies, as *H. hepaticus* monoassociated mice do not develop disease.

These experiments demonstrate that the microbiota has important roles at several stages of disease, and these roles can be affected by other factors that influence IBD. In investigating stages at which the microbiota plays a role in IBD, we can better understand stages along disease progression when prognosis or treatment can be most effective.

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Chapter 1

The mammalian intestinal microbiota

Introduction

The human intestinal microbiota is comprised of seven of 70 known bacterial phyla: Firmicutes, Bacteriodetes, Actinobacteria, Proteobacteria, Verrucomicrobia, Fusobacteria and Cyanobacteria; and one of 13 archeal divisions: Euryarchaeota (1, 2). Of these, Bacteriodetes and Firmicutes are most dominant. This may suggest that communities are not very diverse (low richness and evenness), especially when compared to those in soils or oceans that have over 12 major phyla represented (3, 4), but this is far from the truth when genus or species-levels are investigated. There are more than 400 species identified in the mammalian intestines, which were found to contribute to host development (5, 6).

The participation of this microbial community is integral to our well-being and we are sometimes referred to as a "super-organism," as both the microbes and host work in unison. This mutualism, however, is only maintained when the microbial community possesses a particular structure, composition, richness and evenness i.e. components of diversity. If this balance is perturbed, health can be compromised (7-9). Fortunately, studies on the diversity of the intestinal community demonstrate its resistance and resilience to external pressures (7, 10).

This review attempts to summarize roles of the mammalian gut microbiota in health and disease. As previously mentioned, a stable microbial community maintains health; therefore, how various factors change this structure will be discussed, as well as techniques used to measure these changes. Also, inflammatory bowel disease (IBD), a gastrointestinal condition, will be used as an example of a disease linked to changes in resident microbial communities. But first, I will address direct contributions of the microbiota to hosts in maintaining health and promoting proper host development.

Effects of the microbiota on the host

The microbiota is very active in molding its host. Gut microbes function to activate host genes in order to facilitate their own survival, as in the case of *Bacteroides thetaiotaomicron* activating production of defensins to kill potential pathogens and prevent competition for available space (11); or to benefit hosts via metabolism of previously unavailable nutrients such as butyrate (12). Microbial communities can also contribute to the host's physiological and immunological development.

Microbiota on host gut physiology

There is a wealth of information on the benefits of intestinal microbiota on host development. A review by Wostmann summarizes a multitude of studies done on mapping differences between germfree animals and conventionally raised ones

(13). Some of these differences include increased ceca size, decreased intestinal weight and decreased colon surface area in germfree mice compared to their conventionally raised counterparts (14, 15). This might be due to the absence of butyrate-producing bacteria since this short chain fatty acid is the sole energy source for colonocytes, and lack of butyrate would lead to poor cellular development in germfree animals. Germfree mice also had less fat deposition seen post-mortem, indicating bacteria were important in lipid metabolism (16).

Microbes contribute greatly to development and maintenance of healthy gut tissue. Intestinal architecture of germfree animals shows poor development of colonocyctes, as noted earlier. This is because these cells need the short-chain fatty acid, butyrate, as an energy source to proliferate, which is produced by the microbiota (17). A study also reported shorter crypt lengths and increased number of goblet cells that secreted mucus associated with the presence of a microbial community (18). Mucin is also very important in the barrier function of intestinal epithelium. Presence of a resident microbiota can stimulate production of polysaccharides and enhance the protective ability of the gut by providing a thicker layer between potentially harmful bacteria, or toxic components, and the host itself. Germfree mice also possess leaky tight junctions when compared to mice colonized with bacteria. Tight junctions are composed of a protein mesh that seals intercellular spaces in the epithelial layer. One study in particular shows that colonization with the probiotic *Escherichia coli* Nissle 1917 caused increased expression of the protein zonula occludens 1, involved in the formation of tight junctions (19). This leads to improved barrier function and protection against invasion by pathogens or antigens, and underscores the contribution of “beneficial bacteria” to the host.

Microbiota and the immune system

The intestinal tract is an interface between hosts and their environments. As such, it is a location where cues are presented to the immune system from the external environment. Thus it is necessary that host’s defenses respond to appropriate signals; otherwise we would be constantly inflamed. It is seen, through work with germfree animals, that the intestinal microbiota is key in educating the immune system.

Germfree mice were found to have fewer Peyer’s patches, thinner lamina propria, fewer plasma cells in germinal centers and fewer lymphoid follicles as reviewed by Round et al. (20). These mice also contained fewer T cells, paneth cells with reduced gene expression and B cells that produced less IgA compared to conventionally raised mice. All of these show importance of the resident microbiota in immune system development. Paneth cells are located in crypts and secrete defensins, small antimicrobial peptides. These cells also express RegIIIγ, which is involved in killing gram-positive bacteria. Use of antibiotics was shown to lower expression of this gene (21). This indicates that expression of this C-lectin may depend on the gut microbiota and emphasizes the degree of cross-talk between host and microbes in maintaining homoeostasis.

There are several mechanisms known for immunological responses elicited by pathogenic bacteria, such as effector systems on pathogenic islands like the Type III secretion system possessed by many pathogens such as *Yersinia pestis,* or flagella of *Salmonella* (22, 23). However, limited information is present for why resident bacteria do not trigger the host's immune system and thereby can co-exist with the host. Studies have cited various mechanisms that particular bacteria have evolved to suppress immunological responses. Many bacteria act on inhibiting NF-κB activation. *Bacteroides thetaiotaomicron,* for instance, is known to act down stream of TLR receptor signaling and NF-κB activation (24). Intestinal epithelial cells were incubated with *Salmonella* alone or *Salmonella* and resident microbe, *B. thetaiotaomicron*. It was found that with *Salmonella* alone, there was much less Rel A, the transcriptionally active subunit of NF-κB, in nuclei of host cells. This indicates that *B. thetaiotaomicron* is involved in export of this subunit and thus limits activity of NF-κB. Other studies show different mechanisms for tolerance of the indigenous bacteria (25, 26). Some of these are described by Cario et al. who reviewed tolerance of intestinal immunity through toll-like receptors (TLRs) (27). Consequently, the host is not simply blind to the presence of indigenous bacteria, but employs a two-way communication that results in tolerance.

Microbiota on other locations besides the gut

As discussed above, gut microbiota is key in proper development of adaptive and innate responses against pathogens and irritants. Therefore, it is not surprising that the microbiota would also have far reaching effects in host organisms, and not just on the intestines in which they are located.

It is known that signals from the brain have effects on the microbiota, but the converse is also true, the microbiota has an effect on the brain's function as well. Vagus nerves, for instance, can trigger vomiting during food poisoning (28). Studies with germfree mice showed that these animals had an increase in levels of adrenocorticotrophic hormone (ACTH) and corticosterone, during a particular stress experiments, compared to specific pathogen free controls. This study indicates that the gut microbiota may have a role in suppression of ACTH and corticosterone production (29). A study was also conducted investigating whether there was a connection between the microbiota and pain. The authors reasoned that the perception of pain was necessary for organisms to adapt to their environment and external stresses. They conducted experiments using germfree mice and found that a microbiota was indeed necessary for proper development of the response to inflammatory pain (30).

The gut microbiota was also shown to play a role in liver functioning, as well as with deconjugation of bile acids (by some *Lactobacilli*), and insulin resistance (31). Dumas et al. conducted a study on BALB/c mice to study the metabolic profile associated with insulin resistance and found that metabolites indicative of this condition was related to host diet (31). They saw that the metabolic phenotype associated with disease could be correlated to amounts of fat in the diet. By extension, since the microbiota plays a large role in lipid available to the host, the gut's microbial community plays a role in altering host metabalomics and occurrence of certain disease (32).

To underscore the far reaching effects of the gut microbiota, one recent paper also reported effects of this community on lens of the eyes (33). Lipid content of lens and retina of eyes, from mice with a gut microbiota, was measured together with those from germfree animals. They found that conventionally raised mice had less phosphatidylcholines in their lens indicating exposure to oxidative stress. It is thus clear that the intestinal microbial community affects more than digestion.

Analysis of microbial communities

Earlier, the importance of community diversity and stability on the maintenance of health was briefly mentioned. Before discussing the nature and impact of specific changes in the microbial community, I will first summarize techniques used for measuring these changes.

Tools and techniques

For a long time microbiologists studied organisms by growing them in culture. However, more than 80% of intestinal bacteria cannot be cultivated using current techniques, as described by "the great plate count anomaly" which addresses differences between the greater number of microbes seen with a microscope than those actually grown on culture plates (34).

When Carl Woese introduced the 16S rRNA-encoding gene as a target to study microbial phylogeny, new doors opened for investigating microbial diversity (35).

Ashelford et al. have described features of the 16S rRNA gene that makes it appropriate and useful for phylogenetic classification (36). This gene is highly conserved given its function in protein synthesis; however, nine variable regions interrupt this conserved gene. The first characteristic allows us to design broad-range primers that bind conserved regions so that amplicons can be retrieved that include many groups of microbes. The second feature allows these amplicons to be distinguished from each other since aligned sequence of these variable regions will differ from gene to gene, and thus bacterium to bacterium. Many molecular techniques used for studying diversity within the microbial community depend on these features of the 16S rRNA-encoding gene. Some of these will be described below.

Methods used to study the microbiota include denaturing gradient gel electrophoresis (DGGE)/ temperature gradient gel electrophoresis (TGGE), terminal restriction fragment length polymorphism (T-RFLP), fluorescent in-situ hybridization (FISH), clone libraries, and more recently, high throughput pyrosequencing.

In DGGE, the 16S rRNA genes from a sample are amplified through PCR and 16S rRNA gene amplicons are run across a polyacrylamide gel that has a vertical gradient of denaturing substance. As the double stranded amplicons travel down the gel, they pass through changing concentrations of this denaturing substance and the double stranded helices dissociate at various points. Since each helix is composed of DNA that spans a hypervariable region, these strands would have different base pairs and hence separate at different points along the gradient. The resulting gel would have a ladder-like appearance. If amplicons from two different communities, of differing community structure, were run side-by-side, it would be expected that patterns of “rungs” of the ladders would be different. Further analyses are normally done to identify microbes according to patterns on the gel. For example, hybridization analysis with species-specific probes can be used to identify specific microbes (37). Bands from the gel can also be excised and sequenced in order to ascertain microbial identities. An alternative approach involves using a temperature gradient (TGGE) instead of a denaturing gradient, to allow double stranded DNA helices to denature along the gel (38).

T-RFLP is another fingerprinting technique. Here PCR-amplified 16S rRNA genes of a bacterial community are digested with restriction enzymes and cleaned product is visualized using spectroscopy. This visualization is possible because terminal fragments are fluorescent as one of the primers, either forward or reverse, has an attached fluorophore (e.g. 8-carboxy-fluorescein label) (39). Using T-RFLP, relative abundance of different operational taxonomic units (OTUs) can be seen, as intensity of light emissions is proportional to abundance of each OTU, and different OTUs are designated by peaks generated at cut sites (39). This makes the use of traditional macro-ecological indices (e.g. Shannon or Simpson Indices), employed to measure richness and evenness, easy to apply.

As mentioned briefly above, sequence-specific probes can be used to investigate microbial identities using hybridization techniques. FISH is commonly used for a quantitative measure of particular bacteria in an environmental community, such as the gut, in situ (40, 41). In this technique, a fluorescently labeled oligonucleotide is used to bind to genes of interest (frequently the 16S rRNA gene) of microbes in a community. This oligonucleotide sequence would have a certain degree of specificity according to its design, that is, compliment a variable region for species-level detection or a more conserved region for higher phylogenetic resolution such as phylum-level detection (40-43). The labeled microbes can then be visualized by microscopy or flow cytometry.

Another method used to study the microbial communities is construction of clone libraries. Clone libraries are typically constructed from full-length 16S rRNA gene amplicons using primers that bind to conserved regions (10). The amplicons are then ligated into vectors and transformed into a host cell, typically *Escherichia coli*. These cells, carrying individual 16S rRNA gene fragments are cultured to retrieve sufficient copies of the gene that are then sequenced. These sequences are then aligned, and assigned identities by comparing to known bacterial sequences that are already archived. This technique allows researchers to study communities by comparing who is there and in what relative abundance. Although this is a very powerful technique for analyzing a community, it is limited in that there is relatively little sampling effort exerted (44, 45). Here, pyrosequencing has bridged the gap with deep sampling, with hundreds of thousands of sequence reads per run compared to only about a hundred sequences with clone libraries (46, 47).

In pyrosequencing, a hypervariable section, about 300 base pairs, of the 16S rRNA gene is amplified from a community. Adaptors are ligated on either end of PCR fragments, which aid in community separation in future analyses, and facilitate binding to beads. Beads are captured in droplets that house PCR reactions so that there are millions of DNA strands associated with each bead. Droplets are then broken and the beads placed into a microtitre plate, along with enzymes. Sequencing occurs by measuring emissions generated when deoxynucleotides wash over the plate (47, 48). This techniques generate massive amounts of data and allows investigators the opportunity to observed the “rare biosphere” (49).

These methods described above have all been invaluable in studying gut communities. DGGE was first reported in a 1993 publication where it was used to investigate microbial communities from various locations from microbial mats and waste water samples (37). These authors used PCR to amplify the third variable (V3) region of the 16S rRNA gene and used this amplicon to conduct the DGGE. Zoetendal et al. also used this technique to study the microbiota of the gut by comparing community composition of feces and tissues from different locations along the colon (50). Likewise, through T-RFLP, a broad overview of the community was observed when Keuhl et al. investigated whether there were changes in community richness and evenness after infection with *Helicobacter hepaticus* (39). The authors noted that presence of *H. hepaticus* had minimal effects on other members of the community by comparing changes in resulting traces. Although these techniques present information on general differences between communities, they do not provide identities of members in the community. Supporting techniques are used for this purpose, such as sequence-specific probe hybridization in DGGE that would bind to DNA of certain members of the microbiota, or in silico digests of known bacteria to generate predictive traces to compare with T-RFLP data.

FISH has the advantage of showing identities of certain groups in a particular location. Franks et al. studied changes in abundance of certain bacteria in fecal microbiota of nine participants over eight months (40). This hybridization technique provided rapid results that were quantitative and allowed investigators to track certain species of interest, *Bacteriodes fragilis* and *Bacteriodes distasonis* among others. One constraint however, is since bacteria are measured using emissions from fluorophores attached to probes, there is a limit to the number of probes that can be used. This issue can be overcome using sequencing methods. These methods allow generation of data that have components of relative abundance, richness and identity of community members. With pyrosequencing, communities can be sampled to such an extent that previously rare members are observed. Dethlefsen et al. used this method to study changes in the human microbiota induced by antibiotic treatment (49). They identified more than 5000 taxa associated with the human microbiota, and were then able to further analyzed communities by focusing on specific groups, including the Bacteriodes, Clostridiales and Lachnospiraceae. At the moment, this method is more expensive than others previously mentioned, however, the volume of data generated makes this technology very appealing.

Parameters in measuring microbial diversity

To begin to understand microbial communities, data need to be sorted and interpreted. There are two important parameters that are measured in studying diversity of intestinal microbial communities: richness and evenness. Richness involves enumerating different bacteria that are present and is represented by numbers of operational taxonomic units (OTUs) in communities; therefore, defining what makes each OTU different is necessary. In T-RFLP, an OTU is defined by each peak in the trace generated; or in clone libraries, the designation can be applied after 16S rRNA gene sequences are aligned and compared. Sequences that are 97% similar to each other are generally considered to be of the same species (6). This is still subjective since some authors prefer to use more stringent criteria for defining “species” such as 99% similarity (5). Once cut-off levels are defined though, analysis of communities is quite powerful and represents an accurate and easy way of measuring diversity. Evenness refers to relative abundance of groups within communities. This can be measured using quantitative methods such as clone libraries, pyrosequencing, real-time PCR, FISH, and microscopy. Fingerprinting methods, such as T-RFLP, also gives data on relative abundance by noting relative fluorescent units associated with each peak. This method is relatively cheap, and useful in retrieving a broad overview of changes in a community. It is becoming clear that relative abundances of certain groups, and not merely their presence or absence, is key in onset of disease (10, 51). Two previous parameters, richness and evenness, in part, describe structure of the community. Another feature of microbial communities is that of function, and depends on community composition. Composition refers to identities of the various bacteria present and can be measured using sequencing methods. Denoting membership allows investigators to potentially assign function and thereby begin to understand mechanisms of community dynamics.

Resisting and rebounding from change: Stability of the microbiota

We have described how to measure community structure and composition. This structure is subject to shifts that may be due to a number of factors. Ecologists have been considering changes in terrestrial and aquatic communities for decades and have generated concepts to explain and predict changes in these communities. Microbiologists have now applied these concepts in studying the microbial world, be it in soil communities or gut microbiota (3, 10). Intestinal microbial communities experience many perturbations, including infection by pathogens or treatment with antibiotics. In studying communities' responses to these disruptive forces, one must consider resistance and resilience, two key ecologic concepts developed for studying communities of macroorganisms. Resistance is the ability of a community to maintain its structure under stress, while resilience is its ability to return to its pre-stress state after brief change in structure (52). These two terms are associated with stability of a community, a condition thought to be necessary for health since alterations in diversity of gut microbial communities have been believed to play a role in some diseases such as inflammatory bowel disease (IBD) and antibiotic associated diarrhea (10, 53).

The question of whether diversity begets stability has been ongoing for at least the past 50 yrs (54). Ecologists first believed that more diverse communities are more stable. This was supported by work on terrestrial communities from Odum (55), Elton (56) and Tilman (57) who thought that simple communities were susceptible to perturbations that left these communities open to invasion. MacArthur had similar views after studying predator-prey interactions (58). More recently, however, this view was challenged. Robert May used mathematical models to show that diversity does not lead to stability in a community (59). In fact, he showed that increased diversity destabilized the community. This issue was further complicated when looking at food web relationships where strength of associations also played a major role in the stability of community structure. In these cases, it was seen that observations noted by Odum, Elton and MacArthur did not apply since there was more than one trophic level involved (55, 56, 58, 59).

Microcosm experiments show that there is a positive correlation between diversity and stability. This is seen at community-level (different groups of bacteria) and not population-level (within a particular group, be it at phylum or genus-level). At the population-level, it was seen that increasing diversity did not affect community stability. Findings from these experiments led scientist to note that two explanations were possible in explaining community stability. First, increasing diversity increased the chances of variable species response to a perturbation and hence some species can help maintain the normal functioning of the ecosystem. Secondly, increasing diversity increased chances of functional redundancy so if certain species are lost during a disturbance, communities still function as all the tasks are still occurring (60, 61). These two explanations give rise to the “insurance hypothesis.” It is also noteworthy that diversity is not the “driver” of stability, but merely shows a positive correlation with it. Mechanisms by which this occur are still under investigation. With this hypothesis, it is thought that in the event of a perturbation, a community either shows resistance, in that it generally does not show great change, or it is resilient, and bounces back after an initial change because hardier members of the community compensate for decrease of susceptible ones until they recover (61). The complexity of this issue is emphasized by contradictions observed as one study showed that a less diverse community was more resistant and resilient than highly diverse one (62). Authors explained that relationships between diversity and stability in communities depend on pre-stress states of those communities and mechanisms that drive them.

In considering the gut microbial community, the same ideas apply. One would expect that diversity correlates with stability at the community level. As seen with some of the antibiotic work, communities can be resilient (10, 46). And as suggested by the insurance hypothesis, this resilience may be due to functional redundancy in communities. Functional redundancy is known to be present in our gut microbiota, for instance several members are able to produce butyrate (12, 63).

Influences on the microbiota

There are many factors that sculpt microbial community structure in the intestine and, on occasion, cause disruption. From birth, when colonization occurs, the microbiota is shaped according to its anatomical location as conditions (pH, oxygen concentration) vary, diet changes, pathogens infect and antimicrobials are administered. It is not surprising then that there is great inter-individual variation. However, there are also great similarities due to evolutionary commonalities among vertebrates that give rise to the idea of a “core” microbiome (6, 64).

Microbiota changes with age

The fetal environment is thought to be sterile and colonization starts during birthing. Colonization, thus, depends on both the method of birthing (caesarian section versus natural child birth) (65), and whether or not the child is breast-fed (66, 67). This early microbiota then evolves into a mature community that begins to stabilize soon after weaning. This community is then influenced by diet, antibiotics and other environmental factors that contribute to inter-individual variation, and persists into old-age.

Palmer et al. investigated development of the intestinal microbiota by following infants through their first year of life (68). They assessed colonization through stool sampling and comparisons with stool samples from other members of the family. Mothers’ milk and vaginal swabs were also investigated in an attempt to map route of colonization. The authors found that the gut microbiota in the first days to months of an infant’s life is variable and is related to environmental exposure, i.e. vaginal route, breast-feeding. The microbiota of these 12 infants, however, converged toward the end of their first year. This community resembled that of an adult, consisting of an abundance of Firmicutes, Bacteriodetes, with some Verrucomicrobiae and Proteobacteria. Contrary to many publications (69, 70) though, there was a low prevalence of *Bifidobacteria*. The authors suggest that previous papers may have exaggerated the importance of this group due to its probiotic effects, and lost focus on other more prevalent groups (68).

Once the community reaches this stabilized adult structure, fluctuations from life-style, e.g., diet and disease, are its main effectors. This was demonstrated in another recent investigation involving more than 35 000 adults (71). It was found that bacterial communities, in stool samples, were generally the same over age and gender. A few individual groups such as Enterococci, increased with age while Bacteriodes decreased. *Bifodobacteria* and *Lactobacillus* appeared to be stable. These data were retrieved by culture so do not represent species that may be difficult to grow via methods used. Other limitations included the lack of knowledge of antibiotics taken or diet of participants. Diet is known to play a role in the structure of gut microbiota and these authors suggest that change in bacterial communities with age could be due to changing diets and not the actual process of aging (72, 73).

Microbiota changes with location

Studies often use fecal samples in analyses (68, 73). Although, stool is convenient and non-invasive, it may not be representative of the gut microbiota since microbial communities are not homogenous along the gastrointestinal tract, or across the lumen. Gut microbiota is divided into two groups, autochthonous (commonly referred to resident communities that are closely associated to the host) and then allochthonous (transient bacteria that do not get permanently associated with host and may include pathogens) (74). In studies investigating resident microbiota, the autochthonous community can be investigated using biopsy samples, which capture mucosal microbes that interact intimately with a host. It was also found that certain resident bacteria associate with hosts at particular locations along the intestine, at varying abundances (75).

The alimentary canal, although continuous, harbors various environments from mouth to anus (51, 75). Focus will be placed on the intestinal section since much emphasis has been placed on this community for its role in host development and maintenance of health (1, 12, 76). Differences in anatomy between intestinal sections are obvious with the cecum being a blind end, while the colon is tubular. Other differences are detailed in a review by Snipes (77). There are also differences in the immune elements among different regions of the intestines (78), such as Paneth cells being present in small intestines and not colon. These characteristics may contribute to the variation seen along different sections of the intestinal tract. Wang et al. (75), through molecular techniques, saw that human jejunum had the lowest microbial richness compared to distal ileum, ascending colon and rectum. By comparing sequences against those of known bacteria in the database of the National Center for Biotechnology Information (NCBI), they also found that the main difference was that the jejunum had the least Bacteriodetes but the most *Streptococcus* (68%) and Proteobacteria compared to other three regions. Another group suggests that acid-tolerance by *Streptocococcus* may select for this bacterium in the upper intestine since this environment has a low pH (79). They also showed a complete lack of detection of Clostridium XIVa group in the jejunum while other parts had between 20 and 33% prevalence. The ileum and rectum both had a high prevalence of Bacteriodetes (49 and 44%, respectively). Previous work by this group showed a gradient of increasing load and richness from terminal ileum to colon in a single individual (80). However, Zoetendal et al. (50), using DGGE, noted that bacteria along colon mucosa of 10 individuals were evenly distributed. Wang et al. (75) also showed similar distribution except in the case of Bacteriodetes. Since Wang et al. (75) studied a single subject while Zoetendal et al. (50) had 10 participants, this difference could be due to variation noted among individuals, as well as the sampling methods employed.

Certain microbes may also be associated with different regions along the cross-section of the lumen since different microenvironments are present; for instance, anoxic conditions exist at the center of the lumen particularly within stool (81), whereas microaerophilic conditions are present near the vasularized epithelium (82).

Communities retrieved also depend on samples and sampling techniques since there are differences in dominant bacteria in feces and those attached to mucosa as observed through TGGE, for instance (8). However, fecal and biopsy communities look more similar to each other in one individual than two fecal samples from two different individuals due to inter human variability (5, 83). We can even go further in describing variation among mammals where it was seen that grouping not only depends on genetics but also diet (64).

Microbiota changes with diet

Diet has been known to influence gut bacteria since it selects for certain groups that can carry out certain functions, such as Bacteroides to break down protein or Clostridia to act on complex carbohydrates in fiber degradation. Therefore, one would expect a predominance of Clostridia in an individual consuming a vegetarian diet. This was supported in a study done by Hayashi et al. (72) where gut microbiota of a female vegetarian was investigated and compared to those of three healthy men. It was found that the vegetarian had a high proportion of Clostridium XIVa and Clostridium IV, as well as *Clostridium ramosum* from cluster XVIII. This latter bacterium may be important in individuals on a vegetarian diet since Finegold et al. also reported that this microbe was present in 52% of vegetarians sampled in their study (84). Actual function, however, is still under investigation. Researchers have also found that Bacteriodes were higher in individuals with a greater meat intake. In addition, Bacteriodes convert bile into products that are known carcinogens and thus a high proportion of this group has been linked to colon cancer (85). This has been the subject of debate as a vegetarian diet is proposed to reduce chances of developing colon cancer, emphasizing a link between diet, microbiota and disease. Thus, diet is not only associated with shaping gut communities, but also selecting microbes that are associated with development of disease.

Microbiota changes with a pathogen

Pathogens are organisms that cause disease. Many, such as *Clostridium difficile* and *Salmonella enterica*, are linked to intestinal disease in humans, and are associated with shifts in resident microbial communities (7, 51). Studies on these organisms show that infection normally occurs after shifts in community structure, due to antibiotic treatment, and implies deterioration in colonization resistance.

Salmonella enterica Serovar Typhymurium infection will induce a typhoid-like illness in mice, i.e. mice develop a disease resembling human typhoid fever, with temperatures as high as 104°F and non-bloody diarrhea. As a human pathogen, this microbe induces enteritis. FvB mice are susceptible to *Salmonella* infection without antibiotic pretreatment. This provided a model to observe alterations of gut microbiota in the presence of a pathogen. Upon infecting mice with *Salmonella*, it was found that the diversity of the resident microbial community changes (86). *Salmonella* colonized cecum and colon better than distal small intestines, as shown by qPCR data. In the colon, numbers actually decreased after 1 week compared to the 3 days post infection. This colonization coincided with changes in load of other bacterial groups. Bacteriodetes decreased in the cecum and colon. *Lactobacillus/Enterococcus* decreased significantly in the distal small intestine after 3 days *Salmonella* infection; and the *Eubacterium rectale/Clostridium coccoides* group decreased in all sites measured, with a corresponding increase in *Clostridium perfinges*. However, these changes could be due to host responses to infection. Actual onset of diarrhea can only be associated with the 1 week post infection group since no diarrhea was seen 3 days post infection. Once *Salmonella* was cleared, the microbiota rebounded to it previous set point. The authors observed only certain groups of bacteria to be indicative of changes in the microbiota. It should be noted that there may be other changes seen in groups that are not represented by the primer sets used. A more wholistic approach, using “universal” primers, would be more informative in probing community changes more thoroughly (10, 46). However, the study shows subsequent shifts in the microbiota due to presence of a pathogen. Although this change in the microbiota may not be directly caused by *Salmonella* invasion, it is definitely associated with onset of diarrhea. Similarly, a study by Chang et al. showed that patients recovering from *C. difficile* infections had gut microbial communities more similar to healthy participants, than to those with active disease (7), indicating a return of a “healthy microbiota” in recovering patients. *C. difficile* infections are normally treated with an antibiotic regime, which does not only target this pathogen, but also members of the resident community. This introduces another agent of perturbation to gut microbial communities.

Microbiota changes with antibiotics

It has been shown that antibiotics can alter intestinal murine microbial communities (51, 87). In so doing, colonization resistance is lowered and infection by a pathogen such as *Salmonella* is very efficient (51). Antibiotics have long been recognized to significantly altering the gut community (88). Van der Waaij et al. touted the use of antibiotics in "decontaminating" the digestive tract since they saw dramatic reduction in aerobic and anaerobic bacteria after treatment with various drugs (88). This survey was done by culture methods thus many bacteria would have been undetectable (34). Never the less, antibiotics can greatly impact gut communities. Antonopoulos et al. (46) showed that degree of perturbation depended on specific treatments used. Researchers, through pyrosequencing technology, showed that cefoperazone induced long-term reduction in community diversity (up to six weeks after cessation of treatment). Another drug combination, amoxicillin/metronidazole/bismuth (AMB), only showed short-term reduction in community diversity with richness being restored after only 2 weeks after antibiotic treatment stopped. This exemplifies elasticity of community diversity under certain conditions. Previous work from this group also showed remarkable resilience of some members of the gut microbiota after a perturbation caused by an antibiotic regime (10). Although the antibiotic administered was for treatment of sinusitis, the gut microbiota of the patient in this study showed shifts among different microbial groups. This change in diversity of the gut community was associated with diarrhea. On completion of treatment, diversity of the microbiota resembled that of the community before antibiotics.

Bowel movements also returned to normal. This suggests that certain microbial diversity is associated with health, and that disturbing it can lead to disease. Alternatively, health may be attributed to overall community structure with a pre-determined balance perpetuating a healthy state.

The microbiota and disease

The microbiota contributes tremendously to health and development of its host, as reviewed above. We have also seen that this community is very resilient. But what happens when the microbiota is altered and cannot compensate for associated changes? In some cases, this community disturbance has been associated with onset of disease.

Inflammatory bowel disease

Inflammatory bowel disease (IBD) includes both Crohn's disease (CD) and ulcerative colitis (UC). The former is characterized by lesions, which may occur anywhere along the gastrointestinal tract. These lesions can penetrate through layers of the GI tract, and may even corrode through the gut wall and create fissures into other organs such as the bladder. The latter is normally confined to the lower intestines and rectum, and lesions are more superficial, affecting the epithelial layer and lamina propria (89).

Since no specific bacteria can be linked to the development of IBD in humans, Koch's postulates cannot be fulfilled, leading to the current view that this disease is a result of dysbiosis (changes in the microbial community structure). Koch's postulates are as follows:

1. The microorganism must be found in abundance in all organisms suffering from the disease, but should not be found in healthy animals.
2. The microorganism must be isolated from a diseased organism and grown in pure culture.
3. The cultured microorganism should cause disease when introduced into a healthy organism.
4. The microorganism must be re-isolated from the inoculated, diseased experimental host and identified as being identical to the original specific causative agent.

Koch's postulates are especially challenging to fulfill in the context of intestinal disease since many microbes are difficult to culture by traditional methods (32), therefore may not be easily isolated. Even if they are isolated, interactions with other microbes or host may be required for growth, preventing pure culture.

However, many studies have been cited that link the microbiota with IBD (90, 91). IL-10$^{-/-}$ mice have been convenient in showing roles of microbes in onset of IBD. Since this anti-inflammatory cytokine is absent in these mice, inflammation perpetuates without the inhibitory force of immune regulation that wild type animals possess. Disease is then mediated from a Th1 type response. It was found that wild type conventionally raised or germfree mice did not develop colitis, neither did germfree IL-10$^{-/-}$ mice (92). However, IL-10$^{-/-}$ conventionally raised mice developed disease leading authors to suggest that a resident intestinal microbiota was necessary for development of colitis in these immune altered mice (92). Also, it was found that disease was only present after mice were gavaged with fecal slurry and not sterile filtrate of fecal slurry or sterile bacterial lysates, indicating that microbes are responsible for immune stimulation (93).

Alternatively, it was postulated that one mechanism by which resident bacteria cause colitis is by compromising the colonic epithelial barrier (94). Ohkusa et al. showed that different indigenous bacteria were capable of inducing varying levels of cytokine production from the host intestinal tissue (94). Activity of these inflammatory molecules suggests that resident microbes could induce and maintain inflammation and perhaps, did not cause disease in vivo since host mediators kept them at bay. The host thus appears to control intestinal bacteria from invading, emphasizing the tug-of-war that occurs in this dynamic system. Nonetheless, resident microbes have an important role in onset of IBD.

Animal models of IBD

IBD is of unknown etiology. There are several animal models available that mimic appropriate immunological and histopathological responses. Although these models do not encompass all the complex parameters involved in human disease, they have been used to elucidate pathophysiology of disease, and have also been useful in determining possible therapies. There are two major types of animal models used to study IBD: chemically induced (dextran sodium sulfate, trinitro-benzene sulphonic acid) or immuno-deficient (T cell adoptive transfers, IL-$10^{-/-}$). In the following, the dextran sodium sulfate (DSS)- induced colitis model, as well as the *Helicobacter hepaticus* infected IL-10$^{-/-}$ mouse model are discussed. These are frequently used models employed by our laboratory in studying IBD related changes in the microbiota (39, 95).

Dextran sodium sulfate (DSS) is commonly used as a means to induce colitis reliably (96). This chemical is heparin-like in nature and too large to transit intestinal walls (97). This chemical can induce two types of disease according to administration, acute or chronic. Acute disease occurs after one time dosage at about 3.5% DSS, while chronic colitis occurs at a lower dosage that is repeated at intervals (98). Mechanisms by which it causes diseases is still under investigation (99).

There has been vast work done on effects of DSS on host tissues and immune response as it relates to ulcerative colitis (UC) (100, 101). Using this model, Medzhitov and colleagues have shown that TLR 4 knockout mice did not develop colitic lesions on treatment with this chemical (102). MyD88 knockout mice also fail to develop characteristic lesions of UC (25). More recently, Ungaro et al. found that TLR 4 antagonist antibody ameliorates inflammation in colitic mice (103). These studies suggest that intestinal bacteria can play a role in development of UC via the MyD88 pathway.

Another model of IBD linked with the microbiota is infection of IL-10$^{-/-}$ mice by *Helicobacter hepaticus* (104). There have been several studies showing development of colitis in IL-10$^{-/-}$ mice infected with this microbe (95, 105, 106). *H. hepaticus* induced colitis is a T cell response (104). Recent evidence has also linked Th 17 that produces IL-23 and INFγ in development and modulation of this infection (107). The mechanism by which this bacterium causes colitis is unknown; however, it has been found that *H. hepaticus* has a pathogenicity island (PAI) that is involved in induction of disease (106). This PAI was involved in significantly increasing disease of the cecum, as seen through histology and proinflammatory mediators, of B6.129-IL10^{tm1Cgn} (IL10$^{-/-}$) mice compared to those infected with an isogenic mutant (106). The PAI region investigated showed immuno-regulatory effects by showing lower IgG2c and IgG1 responses than wild type, but did not affect colonization ability. Another paper from this group also showed that the B component of cytolethal distending toxin (CdtB) was important in intestinal colonization. Again, there were also lower levels of IgG2c and no IgG1 response indicating that CdtB was involved in immuno-regulation (105). Therefore, for this bacterium, PAI and CdtB are important in establishing inflammation and causing colitis.

It is important to note that *H. hepaticus* induced colitis only occurs in mice containing a conventional microbiota. Dieleman et al. has shown that disease only ensues in the presence of a resident microbiota and not in *H. hepaticus* monoassociated IL-10 deficient mice, on a mixed (C57BL/6 x 129/Ola) or inbred (129/SvEv) genetic background (108). It should be noted that germfree animals are not “normal” in that they do not have a developed immune system and would not react as conventional animals. Also, disease does not occur in mice with an intact immune system since several studies with this bacterium in wild type animals have not resulted in inflammation (39). As previously discussed, a pathogenicity island facilitates onset of disease, and CdtB is needed for establishment (95, 105). In this model, disease then seems to depend on several factors: resident community, host and *H. hepaticus* itself.

Probiotics

Non-resident bacteria can be used to enhance or rescue deficient functions of the gut microbiota so that disease is prevented or ameliorated (109, 110). Probiotics is defined as “living microorganisms which when administered in adequate amount confer a health benefit on the host (17).” These benefits can be conferred by several different ways. Probiotics can colonize the gut and occupy niches that would have been available to potential pathogens as reviewed (111). This is competitive exclusion. Probiotics can achieve this by using up nutritional resources, or altering the environment (pH, mucus production) to make conditions undesirable for other microbes (112). Beneficial bacteria can also alter gene expression of potential pathogens so that virulence decreases (113). These microbes work synergistically with the intestinal community to maintain health.

Many of the genera used, such as *Lactobacillus*, are predominant members of the intestinal community, and functionally important. Therefore, these bacteria colonize and persist in the host long enough to convey benefits. The mechanism of action of many probiotics is enhanced abilities to implement functions that are done by the resident microbiota. Probiotics can act through modulation of the host responses. They may induce the production of defensins or produce proteases that cleave and activate this antimicrobial peptide (114). *Bacteroides thetaiotaomicron*, a resident microbe in the human gut, also has this effect (11). Probiotics can also activate expression of immuno-regulatory genes. *Lactobacillus rhamnosus* GG activate NF-κB in macrophages (115). They activate the immune response and allow hosts to clear potential pathogens. Conversely, another species, *Lactobacillus plantarum*, act by blocking signaling to this gene and suppresses inflammation (116). It was found that solution from *L. plantarum* culture inhibited the NF-κB expression of murine intestinal epithelial cells. They found that *L. plantarum* medium (without bacterium) inhibited NF-κB activation from MyD88 dependent and independent pathways via TNF receptor.

Sometimes it is the bacterial metabolites, and not direct immunological effects of the microbes themselves that have health benefits. Martin et al. studied effects of two probiotics, *Lactobacillus paracasei* and *L. rhamnosus* in mice colonized with human baby flora (HBF) (117). Presence of these bacteria altered metabolitic profiles from blood and tissue of mice. *L. paracasei* changed energy metabolism pathways of the host by allowing accumulation of bile acids (bacteria cannot deconjugate this compound). This led to high lipid absorption in the intestine. Efficient metabolism of soybean oil from mouse chow also allowed mice to reduce plasma lipoprotein and liver cholesterol. Bacteria also changed metabolism of amino acids via glucogenesis. Wostmann summarizes studies done on contributions of the microbiota on biosynthetic pathways in the gut (13). Bacteria change lipid composition of fecal content and vitamin and mineral uptake in the gut. Also, complex carbohydrates are broken down into short chain fatty acids with the aid of gut microbes. These functions indicate that it is a product of bacterium and not the microbe itself that has beneficial effects exemplifying various ways microorganisms affects the community.

Conclusion

Mammalian microbota is, without a doubt, a complex and dynamic ecosystem. Not only do microbes interact with each other to exist harmoniously (among resident microbes) or antagonistically (attack by parasites or predators such as *Bdellovibrio*), but also communicate and cooperate with the host.

The gut microbiota is important in many aspects of host health. From discussions above, it can be seen that it is necessary for the microbiota to be resistant and resilient to disruptive forces in order for its function to be maintained. In the event that diversity of the gut community is altered, diseases such as IBD may develop.

To study the relationship between the microbiota, host and disease, I intended to use known models of IBD to induce disease in mice and investigate subsequent changes in the intestinal microbiota. Studies have been done associating changes in the microbiota with disease, but none investigating changes in the DSS model in mice, or whether the relative abundance of *H. hepaticus* in the gut of IL-$10^{-/-}$ mice impact on disease severity. I intended to address these questions through experiments conducted in the upcoming chapters in order to attempt to identify possible roles of the microbiota in influencing disease.

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Chapter 2

Models of IBD

There are many models of inflammatory bowel disease (IBD) geared towards understanding various aspects of this idiopathic disease. They address issues that are difficult to investigate in humans, such as changes in early stages of disease and effects of new therapies. Generally, these models are categorized into three groups: chemical, immune dysregulation and environmental. However these groups often overlap and some models involve more than just one factor, for example in the case of immuno-compromised animals exposed to antigens employ both immunological and environmental triggers (1, 2). These environmental triggers may include luminal stimulation from ingested material as well as from the microbial community present in the gut. The modern view on the role of luminal microbial communities extends from invasion of pathogens, to alterations in resident community structure, also known as dysbiosis. However, changes in the microbiota associated with disease in many of these models are yet to be characterized. Moreover, roles of the microbiota at various stages of disease, initiation and progression, as well as exacerbation or reduction of inflammation, are still to be determined.

Here, we choose two well-known models of IBD, DSS-induced colitis and the *Helicobacter hepaticus* triggered IL-10 deficient murine model, to investigate various roles of the microbiota in IBD. The former causes disease in mice that resembles human colitis, where inflammation is superficial, occurring in the mucosa and submucosa, as well as being confined to the lower intestine. Although there is some evidence that the microbiota plays a role in stimulating immune dysregulation in this model, there has been limited evidence that alterations in community structure occur after DSS administration, and no studies characterizing changes that may occur over the course of disease progression. The latter resembles Crohn's disease in humans due to its Th1 immune response and non-contiguous pattern of mucosal inflammation that occurs in deep layers of the intestine. Importance of the role of the microbiota is inherent in this model since a microbe invades the resident community and triggers disease. However, the role of the microbiota in disease progression is unknown. Before presenting experiments designed to address some of these questions at hand, the following chapter aims to describe models that will be used in upcoming studies.

Value of animal models

Animal models provide a means to test questions that may be difficult or impossible to investigate in humans. An ideal model should be inexpensive, easy to use and reproducible. There are typically several models designed to study a single disease due to many aspects of physiology, immunology, microbiology and genetics that interact to maintain a healthy biological system. A model is chosen since it mimics, or is believed to mimic, a feature of the disease as is it is occurs in humans.

Often times the condition studied in an animal model may not represent human disease entirely, but rather only certain aspects are recapitulated, hence the use of several models to answer questions about one disease. Each model is typically used to answer specific questions and can be investigated at various phase of disease where pathogenic components may be scrutinized.

Some conditions, such as IBD, are very complex and occur by interplay of several mechanisms making prognosis and treatment challenging. As such, several models have been developed to study disease. These systems have proven to be very important in teasing apart various mechanisms by which disease may be caused (3, 4). For example, models utilizing TLR, IL-10 and IL-2 deficient mice have been used to investigate various roles of the immune system and genetic defects in disease (5-7). Furthermore, the role of the microbiota has been studied using germfree and antibiotic treated animal models (5, 6); and chemically induced disease models have been used to study loss of mucosal tolerance and break-down of the epithelial barrier as potential mechanisms of disease development (7, 8).

Two-widely used animal models in IBD

There are several types of animal models available to study various aspects of IBD (3). They are generally divided into four groups: Those that are triggered by dysfunction of innate immunity such as A20 deficient mice (9), by dysfunction of adaptive immunity such as CD45RB^{Hi} transfer models (10), by the environment such as *H. hepaticus* triggered disease in immuno-compromised mice and by chemicals such as DSS and TNBD- induced disease (1, 8, 11). Here we focus on two models investigated in following chapters: DSS-induced colitis and *H. hepaticus* triggered disease in IL-10 deficient mice.

DSS-induced colitis

This murine model has been used since 1990 when Okayasu et al. modified it from the original guinea pig system (8). Disease produced in this model is highly dependent on mouse strain used as well as concentration of DSS administered and duration of treatment (12-14). Variations of this model can also lead to acute or chronic disease, and carcinomas (15).

DSS-induced colitis in mice can progress to dysplasia then carcinoma, similar to the disease sequence in humans. This model of disease also responds to therapies administered to humans such as steroids (16) and antibiotics (16), and has an immunological response and genetic component (12). The model mimics aspects of human disease, and its outcome is reproducible.

Origin of the model

In 1961, the bacteria *Leuconostoc* spp. and *Streptococcus* spp. were found to produce a chemical polysaccharide called dextran from sucrose. Dextran sodium sulfate (DSS) is produced by esterification of dextran with chlorosulfonic acid, which produces a molecule with 17% sulfur. This chemical has a large range in molecular weight, from 5000 to 1.4 million Da; however, usually 40 kDa is used in inducing colitis (13).

Eight years after the discovery of DSS, the carrageenan model of IBD was developed when rodents fed with seaweed developed disease which led to the discovery of this sulfated polysaccharide that induced colitis (17). Later, due to its similar characteristics to carrageenan, DSS was also used to induce disease in hamsters (18), and then Okayasu and colleagues modified the model for mice (8). DSS has been administered to hamsters, rabbits, rats and mice, with larger rodents initially used because clinical signs could be easily monitored. Okayasu et al. found that mice administered 3 to 10% DSS solutions quickly developed inflammation resembling colitis in humans. They also found that changing the treatment regimen by cycling the DSS treatment with periods of water, instead of with continuous DSS treatment only, could induce chronic disease.

Mechanism of the model

The mechanism by which DSS-induced colitis develops is still unclear. However, it is generally believed that inflammation is triggered after the epithelial barrier is compromised by DSS, subsequently facilitating interactions between the host and resident microbes, which then triggers a host immune response that develops into inflammatory bowel disease.

DSS is thought to work by damaging the epithelial layers followed by immunological stimulation by resident microbes (19). Researchers found that DSS treatment led to reduction of zona occludens-1 protein production that is involved in maintenance of tight junctions and these changes preceded inflammation. There was also deposition of DSS in organs such as the liver and uptake by macrophages, adding to evidence of DSS permeability. Others have shown that DSS penetrates and disrupts the normally impervious mucin layer allowing bacteria to penetrate the barrier within 12 hours post-DSS administration, before inflammatory changes are detected (20).

Another proposed mechanism for DSS action is by direct damage to intestinal cells. Investigators found that the chemical caused cytotoxicity that increased with time and concentration of DSS used (21). This group also found that expression of various cell adhesion molecules were affected by DSS, indicating that this alteration, particularly in β7 integrins, may lead to inflammation (21). Araki et al. found that DSS inhibits generation of reactive oxygen species (ROS) in Caco-2 cells, yet DSS administration in this murine model increases ROS, leading authors to suggest that ROS production comes from deeper cells and not the epithelial layer (22).

DSS also increases permeability of the epithelial layer, as seen in human IBD (19), with significant increase in Evans Blue dye from the gut lumen after only 3 days of DSS administration, although no signs of inflammation or changes in architecture were seen at this time point (19). This group also showed that DSS may be responsible for basal crypt cell damage, and together with other cytotoxic effects of DSS, it is clear that this chemical is involved in epithelial cell damage (21, 23, 24), an outcome that may play an important role in induction of disease.

Another line of research suggests that the microbiota plays a major role in DSS-induced disease. Some have shown that germfree mice administered DSS develop more severe disease than their conventionally raised counterparts, suggesting a protective effect of the microbes (25). This indicates that the microbiota's beneficial effects may be due to production of metabolites such as short chain fatty acids (SCFA) that are necessary for colonocyte development and epithelial cell health (26). However, others have shown that administration of anaerobic bacterial antigens reduced severity of disease in mice treated with DSS (27), leading these authors to speculate that ingested antigens increased tolerance to intestinal bacteria. This indicates that altered homeostasis between the microbiota and host immune response may be the driver for immune reaction to DSS (27). These effects may be due to the antigens altering the microbial community by competing for binding sites on epithelial surfaces, altering the immune system that may have subsequently exerted effects of the microbiota.

Antibiotic studies show that certain antimicrobial therapies reduce inflammation (16), indicating both a protective and detrimental effect of the microbiota on colitis. Araki et al. treated Caco-2 cells in vitro with DSS and showed that H_2S (22), thought to be associated with DSS-induced colitis in mice and UC in humans, was not produced by host reaction to the chemical, indicating that it may be a by-product of metabolism by the microbiota (28). Others have tried to explain the negative role of the microbiota by postulating that microbes act on

DSS and metabolize it to a form that causes inflammation. Kitajima et al. incubated DSS with luminal contents for 24 hours before the components were studied though spectrometry (29). They found that microbes did not metabolize DSS, dispelling this potential mechanism of action. However, other evidence still indicates an important role for the microbiota in this model of IBD (14, 20, 30).

Although there are many unknowns surrounding the mechanism of DSS disease induction in mice, certain aspects of the model are known to be important. For example molecular size of DSS and strain of mouse utilized can affect disease severity. The molecular weight of the DSS administered to animals is relevant to disease induction also (13). Investigators found that after testing the effects of 5% DSS solution using three different weights of DSS, mice fed with DSS weights less than 5 kDa or over 500 kDa do not produce severe inflammation while 40 kDa is typically used to trigger colitis (13). Others have also found that the sulfur content was dose dependent on the ability of DSS to induce disease with amounts lower than 16.6 % causing a significant reduction in inducing inflammation (31). Authors speculated that larger molecular weights contained more sulfur that was important in inducing disease when molecules permeate the mucin layer, however DSS over 500 kDa are thought to be too large to penetrate this layer, thus unable to induce inflammation.

This model is also dependent on strain of mice used with C57BL/6 mice being very susceptible to disease and BALB/c being more resistant (12). There are also strain dependent induction differences with BALB/c mice producing disease in the distal colon while Swiss Webster mice show more inflammation in colon and cecum (32, 33). It is suggested that these differences are due to genetically controlled variation in inflammatory response or healing (34). Age and dose dependence was also seen in rats with 4 week old Wistar rats being more sensitive to DSS-induced colitis than those eight weeks old, and 4% DSS producing more severe inflammation that 2% DSS over 7 days administration (35).

Immunology of the model

Different phases of DSS-induced disease, acute and chronic, produce different inflammatory responses. The acute state is normally characterized by an increase in the Th1-Th17 response with up-regulation of TNFα, IL-6, IL-17 and KC (40). Other immunological characteristics of the acute state include up-regulation of IL-12, INFγ and minor roles of Th2 mediators IL-10 and IL-4 (36). A Th2 response is dominant in the chronic phase with a decrease in the aforementioned mediators and increase in IL-4 and IL-10 (37). Egger et al. also found that TNFα, IL-1 and IL-12 expression increased with increasing dose of DSS, but not volume of DSS solution ingested.

Both Th1 and Th2 responses in Swiss Webster mice trigger DSS-induced colitis. Dieleman et al. showed that the acute phase of DSS colitis is initiated by neutrophils and macrophages and can lead to up-regulation of INFγ, IL-5 and IL-4 expression (32), while the acute model requires macrophages predominantly while the chronic disease model requires both macrophages and T cells (23).

Leung et al. showed that gamma/delta T cells interact with LPS and macrophages before onset of inflammation and therefore likely to play an important role in DSS-induced colitis in rats (38). However, findings that SCID mice, which lack B and T cells, also developed disease after DSS treatment, indicate that macrophages are the main drivers (23). More recently, it was reported that the adaptive response is required for both acute and chronic phases of disease (39).

Variation in the model

This model was modified to be used in chronic colitis in hamsters in 1992 by Yamada et al. (40), before being used in mice. DSS causes disease in several strains of rodents with varying degrees of severity, allowing flexibility in choosing an appropriate system for the biologic question at hand (12, 15, 41). Guinea pigs develop severe inflammation within 3 days after start of DSS administration (42). Among mice, C3H/HeJ are highly susceptible to treatment with BALB/c mice being more resistant (12).

Long-term administration of DSS results in colorectal carcinoma, very similar to dysplasia-carcinoma seen in cancer development in human ulcerative colitis (40, 43). Investigators treated hamsters with 1% DSS over 100 days and found that the animals developed colorectal cancer. This was to provide a model of disease seen in patients with long-term ulcerative colitis eventually developing colorectal carcinomas.

Another variation of the DSS model is also used to study dysplasia and carcinoma development by administering 3% of the chemical over seven days followed by water for 14 days, for three cycles (44). Or mice are given a single dose of azoxymethane (AOM) before three cycles of DSS prior to water treatment.

Helicobacter hepaticus **infected IL-10$^{-/-}$ murine model of IBD**

Helicobacter species are a relatively newly identified group of bacteria that has been documented to cause disease in humans and animals since the 1980's (45). Diseases are varied and dependent on infecting bacterial species. For example, *H. pylori* causes peptic ulcers in humans, while *H. rappini* is associated with intestinal disease in human and other mammals (46), and abortions in guinea pigs (47). However, it was in 1992 that a new pathogen was found in mice at the National Cancer Institute-Frederick Cancer Research and Development Center (48), *H. hepaticus*, which was found in hepatic tissue of animals with liver tumors. It is also triggers inflammation in immuno-compromised mice that resembles human Crohn's disease (1).

This model relies on an immuno-altered background since *H. hepaticus* does not cause disease in wild-type mice (5, 49, 50). Disease resembles Crohn's disease in humans with a Th1 response and transmural inflammation, but like many other models, it occurs in the lower intestine while human CD can occur anywhere along the gastrointestinal tract. This model is very desirable as it is highly reproducible, easy and cheap to perform. This model is also conducted in SCID mice and severity of disease is dependent on mouse strains used (51).

Origin of the model

H. hepaticus is a microaerophilic, gram negative, flagellated (bipolar, sheathed), spiral-shaped bacterium (may be curved or have several spirals), 1.5 to 5.0 μm long and 0.2 to 0.3 μm wide. It has urease, oxidase and catalase activity, produces H_2S, and reduces nitrate to nitrite (52). It is associated with the mucosal lining of the lower intestinal tract of mice and commonly occurs in the cecum and colon (53, 54). It is not known as a human pathogen; however, recent reports have indicated its presence in immuno-compromised children.

At one point 88% of mice in laboratory and animal facilities were infected with *H. hepaticus* (55-57). This bacterium can be a normal part of the resident community of these mice and only in an immuno-altered state does it become pathogenic. In wild-type mice this bacterium can represent up to 40% of the microbiota (58). SCID mice and IL-10 deficient mice infected with *H. hepaticus* then became a useful model to study human IBD since it produces a Th1-Th17 type immune response similar to Crohn's disease in humans.

Mechanism of the model

The genome of *H. hepaticus* has been completely characterized and has been shown to possess similarities to other pathogenic bacteria such as *H. pylori* urease genes and the *Campylobacter jejuni* gene cluster that comprises cytolethal distending toxin (59). Although urease is not necessary for intestinal colonization, it plays a role in liver disease and triggers the immune response (60). Additionally, the HHGI1 region, a pathogenicity island, contains 70 coding regions which are mostly hypothetical proteins, three coding regions that are similar to regions that code for the type IV secretion system, that also plays a role in inducing disease. In a study with 12 strains of *H. hepaticus* it was observed that the HHGI1 region was either completely in some strains or partially detected in others which may be responsible for the varying virulence observed (29).

H. hepaticus produces cytolethal distending toxin (Cdt), which not only arrests cell cycle in the G2 phase of cell division, but also has a role in colonization with the *Cdt* mutant being cleared from infected animals within eight months of infection (61). Researchers speculate that this may be due to the *CdtB* gene functioning to suppress host's immune response, as seen by lower colonic inflammation in mice infected with the mutant, thereby allowing the bacterium to avoid detection or clearance (61).

Disease does not occur in IL-10 deficient mice monoassociated with *H. hepaticus* indicating an important role of the resident microbiota in development of disease in this model of IBD (5). Furthermore, *Helicobacter* spp. have been found to cause dysregulation of TLR2 and NOD1 in epithelial cells (62), receptors for bacterial antigens. Investigation of the role of TLR4 and IL-10 in *H. hepaticus* triggered colitis show that TLR/IL-10 deficient mice have elevated INFγ, IL-17, TNFα, IL-1 and IL-6 when infected with this bacterium, emphasizing the role of resident bacteria in induction of disease. Disease was more severe in double knock-out mice than in IL-10 deficient only mice, which may be related to increases the accumulation of apoptotic cells in the lamina propria in double knock-outs.

Immunology of the model

This model elicits a Th1/Th17 type response with the production of TNFα, IL-17 and IL-23 (63). Kullberg et al. found that both IL-23 and IL-12 have p40 subunits, which is an essential subunit in disease induction (63, 64). Previous work pointed to IL-12 as having an important role in *H. hepaticus* triggered disease in IL-10 deficient mice after the use of anti-p40 monoclonal antibodies, however these antibodies also targeted IL-23 (63). After researchers used more targeted approaches to investigate these two cytokines it was determined that IL-23 and not IL-12 is important in this model (63). Furthermore, it was found that in the absence of INFγ, IL-17 cannot produce severe disease. It was also found that disease was inhibited only in IL-23 deficient mice. Authors suggest that when IL-23 is down-regulated by IL-10, lowered IL-23 levels then suppress IL-17 production from TH17 cells.

This model hinges on the role of IL-10 in inhibiting diseases in mice. Wang et al. examined the role of NFκB on IL-10 by testing the role of subunits of NFκB in IL-10 expression with the use of IL-10 immunoglobulin (Ig) fusion proteins in RAG mice (65). They found that the fusion protein prevented disease in *H. hepaticus* infected RAG mice but not in mice deficient in p50. Radiation treatment further revealed that p50/p105 subunit of this transcription factor in homopoietic cells of the innate immune system is important in IL-10 expression and suppression of disease (66). This indicates that there is an important role for the innate immune system in *H. hepaticus* triggered colitis. Authors also found that c-Rel, a subunit of NfκB, is needed for *H. hepaticus* triggered inflammation. This subunit is necessary for proper functioning of IL-12/23 and absence prevented disease in RAG-2 deficient mice and prevented the ability of adoptive transfer of CD4+CD45RB high T cells to ameliorate disease (65).

The model works via the adaptive response with inflammation occurring in SCID mice that had adoptive transfer of CD4+ T cells. Cahill et al. found that mice that were infected with *H. hepaticus* developed severe inflammation if they had CD4+ T cells expressing high levels of CD45RB (1). Disease did not develop in mice raised in germfree conditions indicating a role for resident bacteria, and a dysfunctional immune response, in disease development.

Variations of this model

There are many lines of mice that develop colitis when infected with *H. hepaticus* (51). SCID/NCr mice infected with *H. hepaticus* are used to demonstrate IBD. Natural infection with *H. hepaticus* resulted in lesions in the liver and intestines that progressed with age (49). Disease manifested was less severe than that observed in A/JCr mice (67). This indicates that the T cell response caused reduced disease as also suggested by IgG antibody levels being lower in SCID mice compared to A/JCr mice. It is also possible that different microbiota present in these mice strain play a role in the different disease manifestations observed.

Infection of *H. hepaticus* into BALB-Min and BALB-RagMin mice show that this bacterium is involved with tumors of the colon and not small intestine. These tumors are not dependent on severity of inflammation, but depend on organs on which they occur (68).

Other species of *Helicobacter* have also been used in models of IBD, such as *H. bilis*, *H. cindai* and *H. fennili*. Severity of disease that develops varies with the species used. Co-infections of *H. hepaticus* and *H. bilis* produce colitis that later develops into dysplasia in P-Glycoprotein-deficient mdr1a$^{-/-}$ mice. *H. hepaticus* alone was found to delay colitis in these mice while mono-infection with *H. bilis* accelerated colitis development. The co-infection is thought to progress to dysplasia since the *H. hepaticus* slows progress of disease generated by the *H. bilis* by inducing regulatory T-cells, and allowing abnormal growth as cells are transformed by *H. bilis* activity (69). This system was proposed as a model to study dysplasia, hyperplasia and neoplasia in humans.

Conclusions

By using the DSS-induced model of colitis and the *H. hepaticus* triggered IBD model, roles of the microbiota in development of IBD can be assessed. Upcoming experiments attempt to investigate roles of the microbiota in initiation and progression of disease. With the DSS-induced model of colitis, changes in the microbiota will be investigated at onset and progression of disease since there is no reported data documenting whether dysbiosis is associated with disease over time. Using the *H. hepaticus* triggered IBD model, where the microbiota is essential in onset of disease, the role of changes in the microbiota at initiation and during disease will also be investigated. By assessing how changes in the microbial community during development of IBD affect disease manifestation, advances towards prevention and therapy may be achieved.

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I performed experiments, data retrieval and analyses. Dr. Kao provided custom primers for detection of changes in expression of host mediators, scored histology and provided discussion on observed host changes. I also prepared the manuscript with contribution from two other authors.

Chapter 3

Microbial ecology of the murine gut associated with the development of Dextran Sodium Sulfate-Induced colitis.

Abstract and Key Words:

Background: Dextran sodium sulfate (DSS) is used to induce murine colitis. Although the exact mechanism by which DSS administration causes disease is unknown, evidence suggests that resident bacteria play a role in the development of murine DSS colitis, analogous to their role in human inflammatory bowel diseases.

Methods: C57BL/6 mice received 5% DSS in drinking water, and were euthanized 3 days and 14 days after initiation of DSS treatment. Culture-independent methods were used to follow changes in community structure of the gut's microbiota following DSS treatment. Histologic evidence of disease and changes in host gene expression were assessed.

Results: Histologic colitis was minimal in DSS-treated animals at three days, but severe after 14 days. Analysis of 16S rRNA-encoding gene clone libraries demonstrated that microbial communities in the ceca of DSS-treated mice were distinct from those in control mice. The microbiota in the cecum of DSS-treated animals was characterized by an overall decrease in microbial richness, an increase in members of the phylum Verrucomicrobia, and decrease in Tenericutes. Changes in the host's inflammatory response and microbial communities occurred before histologic appearance of severe disease in the colon, but were seen concurrently in the cecum.

Conclusion: DSS administration is associated with reproducible changes in the gut microbial diversity of mice. Changes in the microbial community and increases in host mediators, such as TNFα and arginase, appeared before development of severe inflammation in the cecum. This indicates that these changes in the microbial community may play role in potentiation of the abnormal inflammatory response seen in DSS-treated animals.

Key words: Microbiota, DSS, 16S rRNA-encoding gene, ecology, colitis

Introduction:

Microbes are essential to the health and well-being of their hosts (1, 2). However, presence of bacteria alone is not enough to impart these benefits, rather composition and relative abundance of specific microbes have an important role in maintaining health (3). Alterations of community structure of the indigenous microbiota have also been implicated in development of disease. One such condition in which resident bacteria are thought to play a critical role in pathogenesis is inflammatory bowel disease (IBD) (3, 4). Dysbiosis, defects in immunoregulation and defects in the barrier function are all thought to contribute to onset of disease (5-7). Evidence of a key role for the microbiota in pathogenesis is provided by studies that demonstrate that antibiotics can reduce or prevent inflammation both in patients and in murine models of disease (8, 9). In IL-$10^{-/-}$ mice, which develop colitis after infection with certain bacteria or in the setting of certain housing conditions, treatment with antibiotics is associated with alterations in the microbial gut community (10), and prevention or amelioration of disease (11).

Of the multiple murine models of IBD, one commonly used system involves administration of dextran sodium sulfate (DSS) which induces disease very similar to human ulcerative colitis (12). Antibiotic administration has been shown to ameliorate DSS-induced colitis (13), and cathelicidin, an antimicrobial peptide, was also found to have protective effects in this model of colitis (14), indicating that the microbiota plays a role in this disease model system.

Multiple hypotheses have been postulated for the mechanism by which DSS-triggers gut mucosal inflammation but the exact pathogenesis remains unclear (14, 15). Given that human IBD is associated with an altered microbial diversity (5, 16) and evidence for a role of the microbiota in DSS-induced colitis (14, 17), we hypothesize that this model is also associated with an altered diversity of the intestinal microbiota. To test whether DSS treatment can alter the microbial community diversity, we employed molecular techniques targeting the 16S rRNA-encoding gene to follow shifts in community structure of gut bacteria in animals receiving DSS. We correlated changes in community structure with development of disease.

Materials and Methods

Animals: C57BL/6 mice from a breeding colony initially established with breeding stock from Jackson Laboratories (Bar Harbor, ME) were used for experiments. Animal studies were conducted at Michigan State University and were approved by the Michigan State University Animal Use Committee. Mice were housed with autoclaved bedding, given sterile food and water *ad libitum,* and exposed to 12:12 h light:dark cycles. Mice, between 12 to 16 wks old, were assigned to cages according to gender. Dextran sodium sulfate (DSS) (36 000-50 000 MW, ICN Biochemicals Inc., CA.) was administered as a 5% solution in drinking water (8).

Three groups of mice were used in this experiment: ten control mice that were maintained on sterile drinking water; ten mice that were placed on 5% DSS in sterile drinking water for 3 days before being euthanized; and eleven mice that were given 5% DSS for 14 days before euthanasia (18).

Necropsy and Histology: Mice were euthanized by CO_2 asphyxiation and the cecal tissue harvested as described previously (19). Tissues were gently washed with 1X phosphate-buffered saline (PBS) to remove fecal contents, cut into sections and snap-frozen in liquid nitrogen. One of these sections was used for terminal restriction fragment length polymorphism (T-RFLP) analyses and clone library analysis, and another for RNA extraction.

The remainder of the cecum was processed for histology as follows: Luminal contents were removed, and the tissue was washed with PBS, placed in tissue cassettes and submerged in 10% formalin for 24 hours. Tissue cassettes were transferred to a 60% ethanol solution and then processed for paraffin embedding and staining with haematoxylin and eosin (H&E).

Scoring was completed using the colitis index histological scoring system used by Berndt *et al.* (20) and adapted from Rachmilewitz *et al.* (21). Briefly, sections assessed on inflammation, transmural infiltration, cell wall thickening and bleeding; and scored on a scale ranging from 0 to 40.

RNA extraction and PCR array analysis: Total RNA from cecal tissue was isolated using TRIzol® (Invitrogen, Carlsbad, CA) as directed by the manufacturer's protocol. The High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) was used to convert the total RNA to cDNA. Changes in host gene expression were measured using an array of gene-specific primers from Superarray® (Frederick, MD), designed for the following targets (22): Jun, Cd80, Cd209a, Il12b, Irak3, Smad3, Arg1, Cd86, Mapk3, Il17a, Mapk8, Tgfb1, Cd274, Creb1, Mapk1, Il1b, Tlr2, Tlr5, Vtcn1, Cx3cr1, Foxp3, Il2, Tlr4, Tlr9, Ccr2, Cxcl1, Tnfrsf18, Il23a, H2-DMb1, Tnf, Ccr7, Cxcl10, Infg, Il4, Nfkb1, Vegfa, Itgax, Cxcl5, Il10, Il6, Mapk14, Gapdh, Cd40, Cxcl2, Il12a, Irak4, Pik3r1, ActB. The neutrophil marker, Ly-6G, was also quantified using the primer set from Sasmono *et al.* (23) (Ly-6G forward primer 5'-TGGACTCTCACAGAAGCAAAG-3' and reverse primer 5'-GCAGAGGTCTTCCTTCCAACA-3') as well as the Gapdh primer set from Cui *et al.* (24) (forward primer 5'-ACCACAGTCCATGCCATCAC-3' and reverse primer 5'-TCCACCACCCTGTTGCTGTA-3). Quantization was performed using LightCycler® 480 SYBR Green I Master and analyzed on a LightCycler® 480 system (Roche Diagnostics, Indianapolis, IN) as directed by manufacture's protocol, with the following cycling conditions: 95°C activation for 10 min; 40 cycles of 95°C denaturation for 15s, and 60°C annealing for 1 min. Resulting threshold values were analyzed by calculating the $2^{-\Delta\Delta Ct}$ values, using GAPDH as reference, to find fold regulation compared to no-DSS controls (25-27).

DNA extraction: Genomic DNA was extracted from tissue using the Qiagen DNeasy® Blood & Tissue kit (Cat. No.69504, Valencia, CA). Briefly, tissue was incubated overnight in lysis buffer and proteinase K at 56 °C. The following day, this enzyme was denatured at 95 °C and DNA was purified using ethanol through filter columns, as directed by the manufacturer's protocol, and eluted in 30µl of elution buffer. This genomic DNA was then used in preparation of clone libraries and T-RFLP.

Clone library construction: For clone library construction, reaction was set up as described previously (28), with illustra PuReTaq™ Ready To Go PCR™ beads (GE Healthcare, Piscataway, NJ). Briefly, amplification by polymerase chain reaction was performed using broad-ranged primers, (8F, 5'-AGAGTTTGATCCTGGCTCAG-3'; 1492R, 5'-GGTTACCTTGTTACGACTT-3'). Amplicon purification was done using a commercial kit (GFX, GE Healthcare, Piscataway, NJ) as directed by the manufacturer. Products were ligated into TOPO 4® vector (Invitrogen K4575-01, Carlsbad, CA) according to manufacturer's specifications, and transformed into *Escherichia coli*. Colonies were picked into Luria Broth (LB) with carbenicillin (50 µg/ml)) and grown overnight at 37 °C. Vector specific primers (M13F, 5'-CAGTCACGACGTTGTAAAACGACGGC-3'; and M13R, 5'-CAGGAAACAGCTATGACCATG-3') were used to screen these colonies for bacterial clones containing appropriate 1.5 KB amplicon insert. Partial 16S sequences were determined by a single sequencing run using the 8F primer, at the Genomic Core at Michigan State University. Raw sequence data were processes through an automated "information pipeline" available through the Ribosomal Database Project (RDP) Web site (http://rdp.cme.msu.edu/). Data were screened using the Chimera Check Program before uploading into the

RDP. Following alignment of sequences via myRDP (29), distance matrices representing each of the libraries were downloaded and then taxonomic assignments designated (80% confidence cut-off) using Classifier through the RDP website. These distance matrices were also input into mothur (30) to be grouped into operational taxonomic units (OTUs). Analyses were performed using a 97% sequence similarity to denote species level. An input table was also generated for the EstimateS program via the RDP Pipeline. EstimateS (31) was used to calculate ecological diversity indices from aligned sequences. Dendrograms based on the Bray-Curtis similarity index were constructed using the Mega3 program (32).

16S small subunit rRNA gene sequences obtained by clone library analysis were subjected to *in silico* terminal restriction fragment length polymorphism (T-RFLP) analysis using the TRF-cut program in the ARB suite of programs (33). The *MspI* enzyme, a four base cutter, was used to calculate predicted terminal restriction fragments (TRFs) from clone sequences. Histograms were then constructed displaying relative abundances of these *in silco* generated TRFs and compared to actual traces retrieved from T-RFLP analysis described below. Fragment sizes obtained by T-RFLP anlaysis were compared to sizes of the TRFs generated *in silico* from cloned 16S sequences. These fragments were identified within two basepairs (± 2 bp) of predicted TRFs (34).

T-RFLP analysis: T-RFLP was performed as described elsewhere (35) using primers 1492R and FAM labeled 8F. In summary, genomic DNA was amplified using the aforementioned primers with PCR conditions: 94 °C denaturation for 2 min; 30 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 45 s, and extension at 72 °C for 90 s; and final extension at 72 °C for 4 min. The 1.5 kb PCR product was verified and purified with GFX columns (GFX, GE Healthcare, Piscataway, NJ), then subjected to digestion with *MspI* restriction enzyme for two hours. Digested DNA was submitted for analysis to the Genome Technology Support Facility (GTSF) at Michigan State University. Traces were visualized using the program Genescan® (Applied Biosystems, Foster City, CA).

The T-RFLP Stats collection of programs (http://styx.ibest.uidaho.edu/ibest/research.html) (36) were used to analyze traces from T-RFLP output data. Briefly, peaks of each trace were binned into operational taxonomic units (OTU) to produce data categorized by both OTUs and abundance. This was then used as input for the EstimateS (http://viceroyeebuconnedu/estimates) (31) program where the Bray-Curtis values were calculated and used to compare the diversity among communities. The Mega3 program (32) was used to construct dendrograms showing the relationship between community structures using the Bray-Curtis values. Significance differences between community structures were calculated using the parsimony test from the mothur suite of programs.

qPCR analysis of microbial communities: The quantity of 16S rRNA operons in samples relative to a single-copy host gene was measured using a primer/probe set that targets a broad range of rRNA-encoding gene sequences (5'-TCCTACGGGAGGCAGCAGT-3'), reverse primer (5'-GGACTACCAGGGTATCTAATCCTGTT-3'), and probe (5'-[6-FAM]-203CGTATTACCGCGGCTGCTGGCAC-[TAMRA]-3') (37). A primer/probe set targeting a 264-bp portion of the TNF alpha gene was used as a reference using 200 nanomoles of forward (TNFα_mu_se; 5'-GGCTTTCCGAATTCACTGGAG-3') and reverse primers (TNFα_mu_as; 5'- CCCCGGCCTTCCAAATAAA-3'), and 100 nanomoles of probe (TNFα_mu_probe; 5'-[Cy5]-ATGTCCATTCCTGAGTTCTGCAAAGGGA-[Iowa Black RQ™]-3') 213 (38). The reaction mix consists of LightCycler® 480 Probes Master reaction mix (Roche) at 1X concentration, and appropriate primer/probe pair. Amplification of each gene was done under separate run conditions: cycling conditions for the 16S target involved an activation step of 50 ^{o}C for 2 min followed by 95 ^{o}C for 10 min. Forty-five cycles were done at 95 ^{o}C for 15 s and 60 ^{o}C for 1 min before holding. For the TNF reference gene cycling conditions included an activation step of 50 ^{o}C for 2 min followed by 95 ^{o}C for 10 min and forty-fives cycles of 95 ^{o}C for 20 s and 64 ^{o}C for 30 s. Calculations of $2^{-\Delta\Delta Ct}$ were made to compare changes in the amount of 16S from samples between treatment groups (21,22).

Statistical Analysis: The non-parametric Kruskal-Wallis test was used to analyze histological scores. Differences in abundances of specific operational taxonomic units among treatment groups were analyzed by ANOVA. Statistical differences in dendrograms comparing distances between microbial communities were calculated using the parsimony test function in mothur (30). Probability values less than 0.05 were considered significantly different.

Results

DSS treatment leads to inflammation of the murine intestinal tract.

Wildtype mice were treated with 5% DSS in drinking water and gastrointestinal tissue examined histologically after 3 and 14 days of treatment. All animals survived DSS treatment and no significant clinical signs were noted. A mild inflammatory infiltrate developed in the cecum 3 days after DSS administration (figure 3.1 b), with notable progression of disease after 14 days characterized development of a massive inflammatory cell infiltrate and severe edema (figure 3.1 c). These changes were significantly different from the control state ($p<0.05$), as well as between 3 day and 14 day treatment outcomes (figure 3.1 g).

Similarly in the colon, a few animals developed morphological and inflammatory changes after 3 days of DSS treatment, but as a group, these changes were not significant when compared to controls. However, following 14 days of DSS administration colonic tissue showed evidence of severe inflammation and edema which was significantly greater than controls of animals after 3 days of treatment (figure 3.1 h).

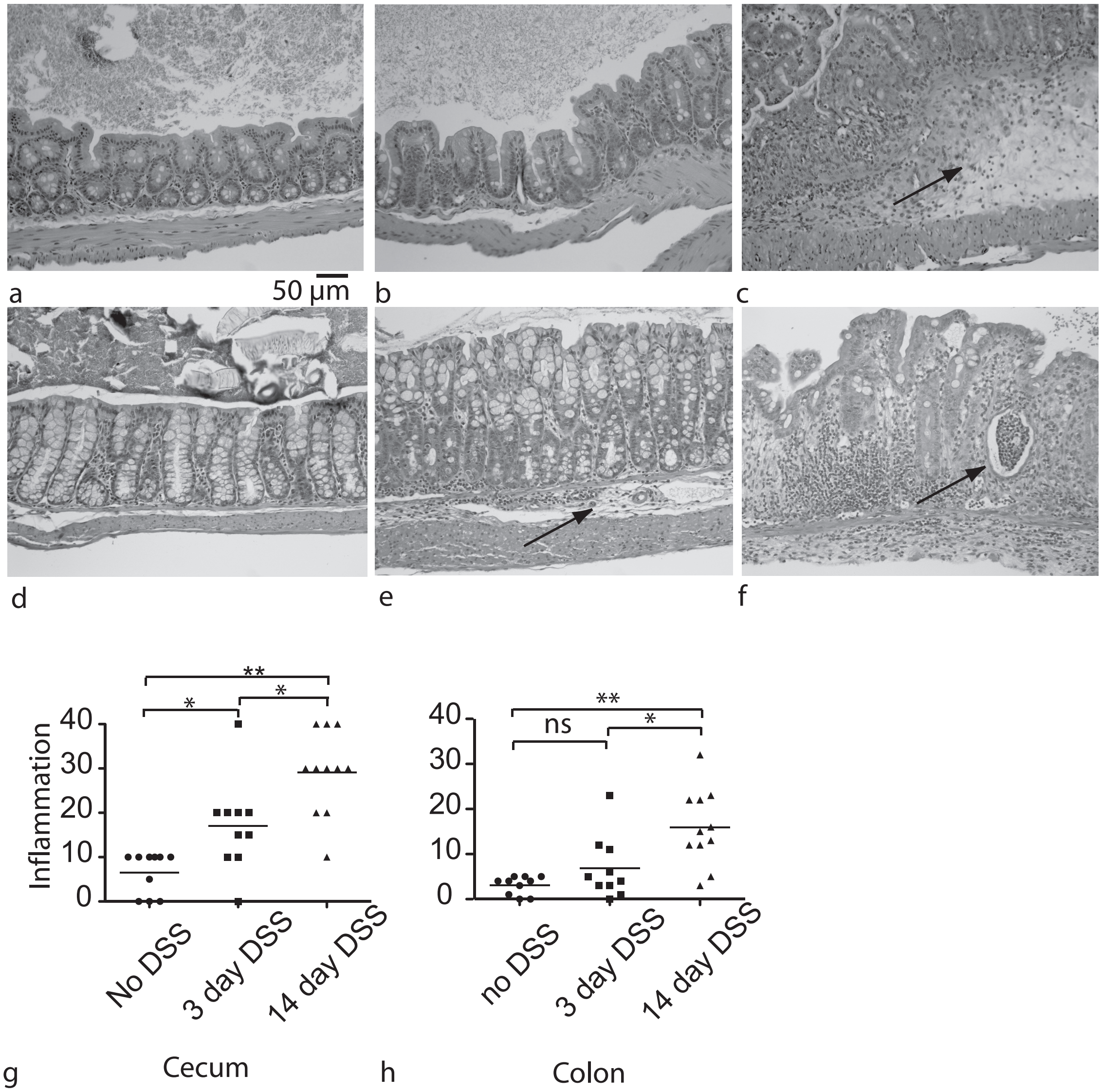


Figure 3.1. Histopathology in DSS-treated mice. Hemotoxylin and eosin (H&E) stained sections were prepared from cecum sections of (a) untreated control mice (b) mice after 3 days of DSS treatment and (c) after 14 days of DSS. Arrow indicates submucosl edema. H&E sections were also prepared from colon samples of (d) untreated controls (e) mice after 3 days of DSS treatment (arrow points to inflammation) and (f) animals after 14 days of DSS (arrow shows abscess). Histopathologic scores were calculated for sections from all 31 animals for (g) cecum and (h) colon sections. Statistical analysis done was Kruskal-Wallis test. *p< 0.05 **p<0.001. Initial magnification 40X.

Changes in host gene expression following DSS treatment

Using a quantitative PCR array, we found significant changes in expression of ten host genes in cecal tissue following DSS treatment (figure 3.2, supplemental figure 1). Expression of arginase 1 began to increase after 3 days and was significantly up-regulated ~seven fold ($p<0.05$) after 14 days of DSS administration. A significant increase in tumor necrosis factor-alpha (TNFα) was also seen after 3 days of DSS treatment, with a 12.9 fold up-regulation ($p<0.05$), and remained significantly up-regulated at the 14-day time point.

Eight other genes were down-regulated, compared to untreated controls. They were TLR 5, IL17, Ccr2, Cx3cr1, Cxcl2, Cd40, Cd80 and Cd209a. TLR 5 had significant down-regulation after 14-days of DSS exposure, as did Cd40 and Cxcl2. Cd80 showed significant change after 3 days of DSS only marginal change after 14 days. Ccr2, Cx3cr1, Cd209a and IL17a showed significant down-regulation both at the 3 day and 14 day time points.

Presence of neutrophils, as indicated by measuring the Ly-6G marker, increased significantly with DSS treatment with a 7.7±1.2 fold increase after 3 days of DSS administration, and 14.0±1.8 fold increase after 14 days of DSS (supplemental table 3.1).

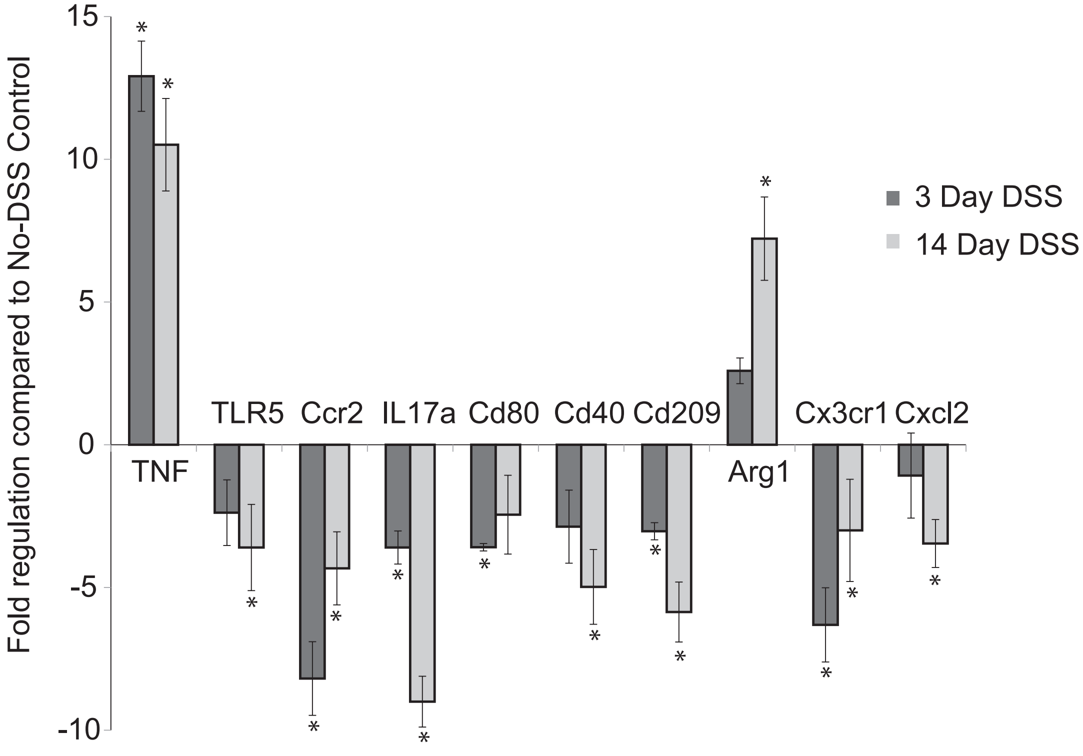


Figure 3.2. Changes in host gene expression after 3-days (dark bars) or 14-days (grey bars) ± standard deviation of DSS administration. Expression levels were compared to expression in tissue from animals not exposed to DSS, after normalization with GAPDH. Statistical analysis done was Student's t-test. Statistically significant differences ($p<0.05$) are denoted by asterisks (*).

Shifts in gut microbial diversity following DSS treatment.

To obtain an overview of the status of the microbial community present in the gastro-intestinal tract of each of the animals in this study, 16S terminal restriction fragment length polymorphism (T-RFLP) analysis was performed on DNA extracted from cecal tissue. Analysis of microbial communities in the cecum of all 31 animals in the study revealed that the structure of the microbial communities from DSS treated mice were significantly different from the no-DSS controls ($p<0.001$) (supplemental figures 3.2).

Since T-RFLP provides a broad overview of community structure without information regarding changes in specific organisms, 16S clone libraries were constructed to further investigate the microbial community in four representative mice from each treatment group. A total of 328 partial 16S rRNA-encoding gene sequences were retrieved from control animals (71, 84, 89 and 84 per animal), 364 sequences from animals 3 days after initiation of DSS treatment (88, 92, 93 and 91) and 370 from animals treated with 14 days of DSS (92, 94, 89 and 95). Clone library analysis confirmed T-RFLP findings, indicating that community compositions of the microbiota in DSS treated animals were more similar to each other than they were to those of control mice (figure 3.3, supplemental figure 3.4). T-RFLP traces were compared to in silico TRFs of clone libraries to verify that OTUs matched the traces (supplemental figures 3.5- 3.8). Communities in all DSS-treated animals were significantly different from those in no-DSS controls ($p<0.001$ by the parsimony test implemented in the analysis suite mothur (30)). Rarefaction analysis of clone libraries revealed that DSS treatment resulted in a decrease in phylotype richness (supplemental 3.4). This finding was also supported by a decrease in the Simpson diversity index (1/D) following DSS treatment from 43.0±12.1 to 21.3±8.3 (3 days) and 19.7±7.0 (14 days) ($p<0.05$). However, quantitative PCR of the 16S gene from each treatment group showed that there were no significant changes in overall bacterial biomass relative to no-DSS controls (fold change for 3 day DSS= 0.68±0.92 SD, 14 day DSS= 0.97±0.82 SD).

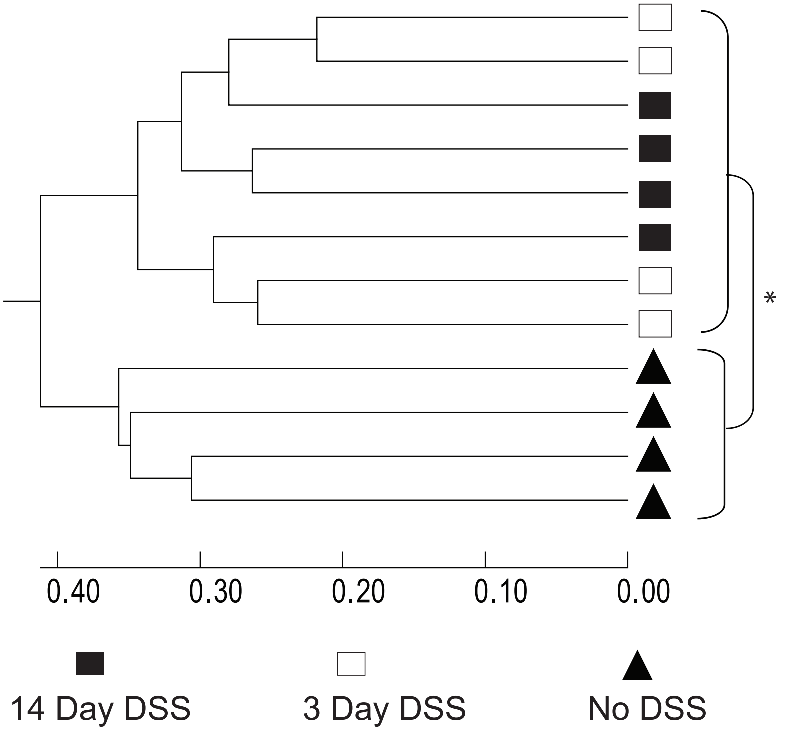
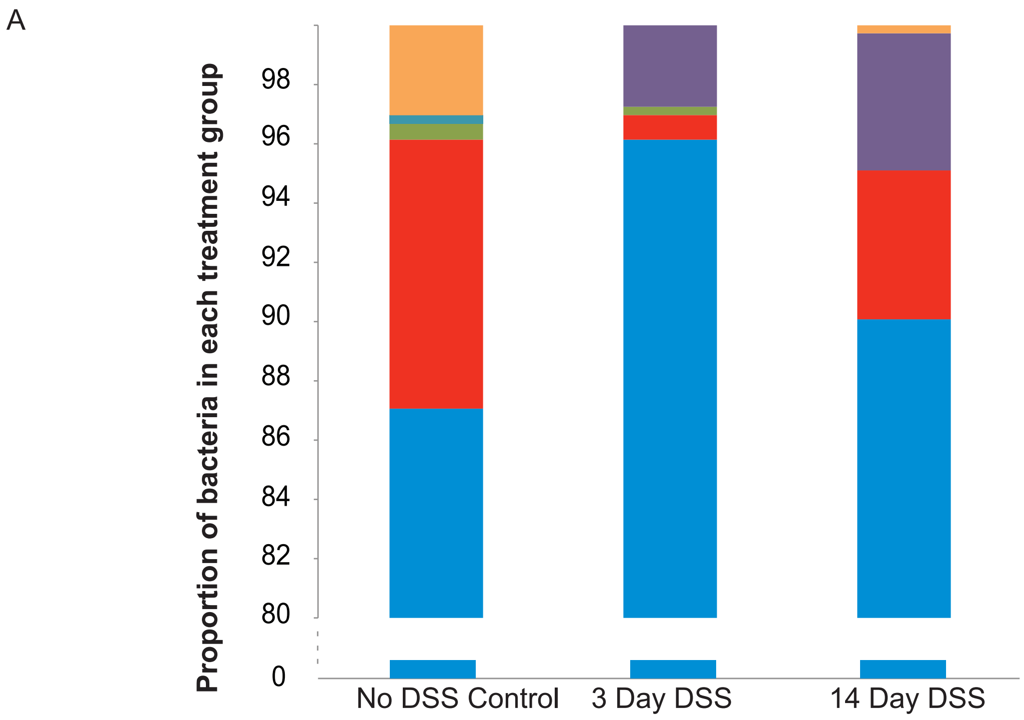


Figure 3.3. Comparison of cecal communities in control animals (black triangles) and in animals following 3 days of DSS treatment (white squares) and 14 days of treatment (black squares). Using an OTU definition of 97% similarity, the Bray-Curtis similarity metric was calculated for each pair-wise comparison and then results displayed in denrogram format. Analysis by parsimony tests indicate that there is a statistically significant (*p<0.001) difference between the communities in control animals and in both groups of DSS-treated animals.

We analyzed changes in the relative abundance of specific phylotypes by classification of the 16S rRNA-encoding gene sequences. DSS treatment resulted in a decrease in members of the phyla Bacteriodetes and Tenericutes. Alterations in the microbial community were also apparent at lower taxonomic levels. For example, there was an increase in unclassified genera under the family Lachnospiraceae (figure 3.4, supplemental 3.3). The majority of classified phylotypes in animals from all three experimental groups were members of the phylum Firmicutes, which include the classes Clostridia and Bacilli. Bacteriodetes was most abundant in control animals and represented to a lesser extent in DSS treated groups (figure 3.4 A, supplemental 3.3). A striking observation was that members of the phylum Verrucomicrobiae were detected only in DSS treated mice and not in control mice (figure 3.4 A).

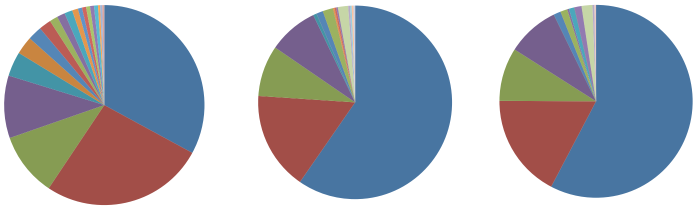
Among members of the phylum Bacteriodetes richness of the population did not change, indicating that changes were mainly due to shift in abundances of different bacteria within this phylum. Among the Firmicutes, unclassified genera among families Ruminoccocaceae and Lachnospiraceae were most prevalent. These groups showed treatment related shifts as seen in pie charts of figure 3.4 B. When analysis was done using 97% sequence similarity, there was high richness among Lachnospiraceae, compared to other major groups within the Firmicutes (table 3.1), indicating increased diversity within this category ($p<0.05$). These groups also showed treatment related shifts (table 3.1).



Phylum Level Assignment	**mean proportion of bacteria per treatment group**		
🟧 Tenericutes*	3.14 % ±1.82	0.00 % ±0.00	0.27 % ±0.54
■ Crenarceota	0.30 % ±0.60	0.00 % ±0.00	0.00 % ±0.00
🟪 Verrucomicrobiae*	0.00 % ±0.00	2.75 % ±1.10	4.64 % ±3.42
🟩 Proteobacteria	0.55 % ±0.67	0.28 % ±0.56	0.00 % ±0.00
🟥 Bacteriodetes	9.39 % ±5.00	0.83 % ±0.55	5.04 % ±4.17
🟦 Firmicutes	86.90 % ±7.02	96.15 % ±1.49	90.05 % ±3.31

Figure 3.4 (A). Changes in the cecal gut microbial community following DSS administration at the phylum level. Analysis was done by ANOVA and statistical significance (p<0.05) is denoted by asterisks (*). **For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this dissertation**.

B



No DSS Control   3 Day DSS   14 Day DSS

Assignments within Firmicutes	mean proportion of bacteria per treatment group		
🟦 Lachnospiraceae (N/A) *	32.93 % ±9.22	59.63 % ±6.27	57.69 % ±11.64
🟥 Ruminococcaceae (N/A)	26.43 % ±9.58	16.43 % ±7.13	17.39 % ± 5.74
🟩 Clostridiales (N/A)	10.30 % ±7.49	8.20 % ±5.62	8.95 % ± 4.03
🟪 Lachnospiraceae Incertae Sedis	10.10 % ±1.53	8.32 % ±7.17	8.75 % ± 3.46

* denotes statistical significance (p<0.05)

Figure 3.4. (B) Changes in the cecal gut microbial community following DSS administration within the phylum Firmicutes. Analysis was done by ANOVA and statistical significance ($p<0.05$) is denoted by asterisks (*). **For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this dissertation**.

	Clostridiales N=17 (%)	Ruminococcus N=36 (%)	Lachnospiraceae N=65 (%)
Shared OTUs among all groups	6 (35)	16 (44)	15 (23)
OTUs in control group only	4 (24)	8 (22)	13 (20)
OTUs in 3 day DSS group only	2 (12)	4 (11)	8 (12)
OTUs in 14 day DSS group only	2 (12)	5 (14)	12 (18)
Shared OTU in DSS treated groups	3 (18)	3 (8)	17 (26)

Table 3.1 Showing distribution of OTU's across treatments from major groups within the Firmicutes.

Discussion:

Indigenous gut microbiota are felt to play a key role in pathogenesis of inflammatory bowel disease. Much of the evidence for the involvement of intestinal bacteria in IBD comes from studies with murine models of disease (39, 40). Many of these models have specific alterations in host defenses ranging from altered epithelial cell function to altered innate or adaptive immunity (41-44). These alterations in host defense result in abnormal interactions between the host and indigenous microbiota that lead to disease (45). Evidence for this comes from the fact that antibiotic administration reduces severity of disease in these models and rederivation of these mice to the germ-free state prevents initiation of disease (13, 46).

Administration of DSS consistently triggers intestinal inflammation in rodents. Similar to other models, antibiotic administration has a beneficial effect in the system again pointing to a role for the indigenous microbiota in disease development (47). Effects of DSS on germfree mice have been variable. In some strains of mice more severe colitis is encountered while early death, without development of significant inflammation, is observed in others (48, 49). Germ-free IQI/Jic mice have a high mortality rate, compared to conventional mice, when given a concentration of 5% DSS in drinking water (45). These mice died secondary to hemorrhage within three days after DSS treatment, indicating a protective function of the microbiota against toxic effects of DSS on the gut epithelium. These results are consistent with the finding that antibiotic administration or genetic defects in TLR signaling also increased mortality in DSS-treated conventional mice (50, 51). Differences in these two studies employing germ-free mice likely reflects differential effects of two doses of DSS on the epithelium, but may also reflects differences in composition of the microbiota of conventional control animals. Since specific subsets of the "normal" microbiota have differing ability to initiate or sustain experimental colitis (47, 52) these results emphasize the importance of characterizing changes in the gut community associated with DSS-induced colitis across time.

Although it was originally suggested that IBD resulted from infection with an unknown bacterial pathogen (53), the more contemporary view is that entire shifts in the microbial community structure may be the trigger for disease (5). An altered gut microbial community has been associated with disease as shown in human studies and animal models (54, 55), suggesting that there is a change in abundance of bacteria that are necessary for maintaining homeostasis (54, 55). After administering DSS to mice, we show that treatment results in changes in microbial community structure in the gut, also noted by others (51). Additionally, we found that these changes occur early (within 3 days) and are characterized by reduced overall diversity while bacterial load remains unchanged. Some community members are below limits of detection after DSS treatment, while others increase in abundance demonstrating a selective effect of DSS treatment on specific members of the community. It has been shown that certain bacteria can de-polymerization DSS and thus can grow in a DSS-rich environment better than others (56). This may explain the bloom of Verrucomicrobia that we observed in DSS treated mice. This group of microbes, which has been found in mammalian intestine at very low abundances (57), metabolize sulfur and degrade mucin (58). Therefore, perturbations of the community can cause previously underrepresented members of the community to become dominant (59), resulting in shifts in community structure, as seen in the phylum Bacteriodetes and families Ruminococcaceae and Lachnospiraceae.

Another study has profiled effects of DSS treatment on the microbial community (51). Similar to our study, it was noted that shifts in relative abundances of specific phylotypes were observed following DSS treatment, although specific changes were different from what we observed. One obvious reason for these differences could be explained by the fact that different anatomic locations were sampled (cecal mucosa used here versus distal colonic contents in study by Heimesaat et al. (51)), as well as length and concentrations of DSS administration. Additionally, it is likely that the baseline microbial communities were quite different between the animals in that study and the animals we used. Recent reports have demonstrated that the baseline microbiota can differ in genetically identical animals obtained from different sources and these differences can have a profound effect on host immune responses (60, 61).

In our study, disease severity in the cecum increased after 3 days and 14 days post DSS administration, concurrent with changes observed in the microbial community, indicating a link between the changes in diversity of the microbiota and disease. Since shifts in the community structure can reduce beneficial members of the indigenous microbiota that act to maintain epithelial health, such as production of short-chain fatty acids and stimulation of mucin production (55, 62-65), it is likely that such changes can affect host inflammatory responses (45, 66).

Others have suggested that DSS-induced colitis occurs due to breaches in the epithelial barrier with exposure to antigens produced by luminal microbes (5, 67-73). However, there is evidence that detection of an *altered* microbial community is an important factor in host response (74, 75). Our data indicate that DSS induces changes in the microbiota, and together with a compromised barrier, appears to facilitate disease initiation and progression. It has been shown that the host responds to microbial-associated molecular patterns (MAMPS) via toll-like receptors (TLRs) in development of colitis (17, 40, 76). We found significant a change in expression of TLR 5, expression of which was decreased as colitis progressed. It has been previously demonstrated that TLR 5 is down-regulated in patients with severe ulcerative colitis (77), in concurrence with our data. However, the role of flagella in DSS colitis is not dependent on TLR5 (40, 78), suggesting that down-regulation is perhaps a response from over-stimulation from microbes through a deteriorating epithelial layer.

It is noteworthy that we saw an increase in arginase 1 after 14 days of DSS treatment. This could be due to an increase of neutrophils after DSS administration, as indicated by increased expression of the neutrophil marker Ly6-G. Arginase 1 also has a protective role in the *Citrobacter rodentium* model of colitis where this enzyme inhibition aggravated disease (79). It has been seen that arginase expression increases with TNFα/lipopolysaccharides (LPS) stimulation in human intestinal microvascular endothelial cells (80). With breaches in the epithelial layer that allow increased stimulation by LPS, together with an increase in TNFα as seen here and in other studies with DSS (70, 81), it follows that arginase expression is likely to be up-regulated. Interestingly, although TNFα appeared to be at lower levels at 14 days compared to 3 days, arginase was up-regulated at 14 days compared to 3 days, consistent with the presumed role for this gene product in the epithelial repair response. This is not unexpected since DSS administration disrupts the epithelial barrier (15). To our knowledge, this study is the first to document the significant increase of this enzyme in DSS-induced colitis.

Although others have noted changes in the gut microbiota following DSS treatment (51) after development of disease, this is the first study to monitor DSS-induced changes in the intestinal microbiota over time. In this study, we not only corroborate that changes in the gut microbial community are associated with DSS-induced disease, but we also show that diversity of the microbiota changed as early as after 3 days and continued to 14 days of DSS administration. Early shifts in the microbial community were characterized with increases in abundances of microbes previously undetectable by methods used. These altered communities are present at onset of inflammation, and maintained as inflammation severity increased, reinforcing the idea that disease occurs, and persists, in the presence of an altered intestinal community. These early changes in the microbiota support the view that disturbance of the resident bacterial community structure may have a very important role in onset of diseases, such as IBD.

Acknowledgment

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SUPPLEMENTAL INFORMATION

Table 3.2. Changes in host gene expression following DSS treatment.

TARGET	**FUNCTION**	**FOLD-REGULATION**	
		3-Day DSS	**14-Day DSS**
Th1 response			
Interleukin 12a (Il12a)	differentiation of naïve T cells	2.35 ± 0.81	1.57 ± 1.66
Interleukin 12b (Il12b)	differentiation of naïve T cells	1.42 ± 0.33	-2.53 ± 0.40
Interleukin 2 (Il2)	leukocytotrophic hormone	1.79 ± 0.00	-2.45 ± 0.10
Tumor Necrosis Factor alpha (Tnf)	proinflammatory cytokine	12.91* ± 1.23	10.51* ± 1.62
Interferon gamma (Infg)	proinflammatory cytokine	2.26 ± 0.20	-2.05 ± 0.12
Toll-like Receptor 2 (Tlr2)	pathogen recogition	-1.22 ± 0.91	-2.51 ± 0.38
Toll-like Receptor 4 (Tlr4)	pathogen recognition	1.21 ± 1.28	1.74 ± 1.31
Toll-like Receptor 5 (Tlr5)	pathogen recognition	-2.38 ± 1.15	-3.60* ± 1.51
Toll-like Receptor 9 (Tlr9)	pathogen recognition	1.05 ± 1.41	-1.58 ± 1.53
Chemokine (C-X-C motif) ligand 10 (Cxcl10)	chemoattractant	2.76 ± 2.90	2.64 ± 1.31
Th2 response			
Interleukin 4 (Il4)	differentiation of naïve T cells	1.82 ± 0.00	-2.26 ± 0.26
chemokine (C-C motif) receptor 2 (Ccr2)	chemoattractant	-8.19* ± 1.29	-4.33* ± 1.28
Th17 response			
Interleukin 17a (Il17a)	induction and mediation of proinflammatory responses (allergic)	-3.60* ± 0.58	-9.00* ± 0.89
Interleukin 23a (Il23a)	differentiation of naïve T cells	2.48 ± 0.80	-2.02 ± 0.13

Table 3.2 (cont'd)

CD4+ T cell marker			
Interleukin 6 (Il6)	pro-inflammatory and anti-inflammatory	-1.41 ± 0.26	-2.94 ± 0.97
Mitogen-activated protein kinase 8 (Mapk8)	"regulation of cellular activities (gene expression, mitosis, differentiation, proliferation, cell survival/apoptosis)"	-1.83 ± 0.35	-1.75 ± 1.03
Cluster of Differentiation 80 (Cd80)	receptor	-3.59* ± 0.13	-2.45 ± 1.38
Cluster of Differentiation 86 (Cd86)	receptor	1.41 ± 2.10	2.23 ± 1.73
chemokine (C-C motif) receptor 7 (Ccr7)	receptor	-2.71 ± 0.43	-2.42 ± 1.97
V-set domain-containing T-cell activation inhibitor 1 (Vtcn1)	ligand on antigen presenting cells	1.61 ± 0.00	-2.8 ± 0.00
Tumor necrosis factor receptor superfamily member 18 (Tnfrsf18)	receptor	1.21 ± 0.66	1.42 ± 1.69
"Integrin, alpha X (Itgax/CD11c)"	receptor	1.47 ± 0.44	1.26 ± 2.05
Tregs			
Transforming growth factor beta (Tgfb1)	differentiation of naïve T cells/anti-inflammatory cytokine	-1.4 ± 0.53	1.01 ± 1.24
Interleukin 10 (Il10)	anti-inflammatory cytokine	-2.85 ± 0.00	-2.78 ± 1.06
forkhead box P3 (Foxp3)	differentiation of naïve T cells/anti-inflammatory cytokine	-1.6 ± 1.24	-2.68 ± 1.02
Transcription factors			
nuclear factor kappa-light-chain-enhancer of activated B cells (Nfkb1)	regulation of the immune response to infection	-1.19 ± 0.56	-1.1 ± 1.92
Jun	early response factor to AP-1	1.43 ± 1.47	1.62 ± 0.91
Smad3		-1.33 ± 0.90	-1.1 ± 1.91
Interleukin-1 receptor-associated kinase 3 (Irak3)	signalling innate response	-1.24 ± 0.90	-1.17 ± 1.46
Interleukin-1 receptor-associated kinase 4 (Irak4)	signalling innate response	1.65 ± 1.84	1.94 ± 1.36

Table 3.2 (cont'd)

Mitogen-activated protein kinase 14 (Mapk14)	"regulation of cellular activities (gene expression, mitosis, differentiation, proliferation, cell survival/apoptosis)"	-1.29 ± 0.38	-1.35 ± 1.84
Mitogen-activated protein kinase 1 (Mapk1)	"regulation of cellular activities (gene expression, mitosis, differentiation, proliferation, cell survival/apoptosis)"	-1.27 ± 0.31	-1.03 ± 2.32
Mitogen-activated protein kinase 3 (Mapk3)	"regulation of cellular activities (gene expression, mitosis, differentiation, proliferation, cell survival/apoptosis)"	-1.49 ± 1.61	-1.57 ± 1.85
CAMP responsive element binding protein 1 (Creb1)	binds DNA to regulate transcription	-1.45 ± 0.25	-1.32 ± 1.76
B cell			
Phosphatidylinositol 3-kinase regulatory subunit alpha (Pik3r1)	insulin receptor	-1.38 ± 0.64	-1.14 ± 1.16
Cluster of differentiation 40 (Cd40)	receptor	-2.87 ± 1.28	-4.98* ± 1.31
Other			
Cluster of differentiation 209a (Cd209a)	receptor	-3.03* ± 0.30	-5.86* ± 1.05
Arginase 1 (Arg1)	wound healing	2.59 ± 0.45	7.22* ± 1.46
Cluster of differentiation 274 (Cd274)	receptor	2.58 ± 1.13	1.23 ± 1.11
Cx3cr1	receptor	-6.31* ± 1.30	-3.00* ± 1.79
Chemokine (C-X-C motif) ligand 1 (Cxcl1)	chemoattractant	2.23 ± 0.00	-1.93 ± 0.13
MHC class II beta chain (H2-DMb1)	antigen processing and presentation	2.23 ± 1.60	2.94 ± 2.13
Vascular endothelial growth factor A (Vegfa)	cell differentiation, maturation, proliferation, anti-apoptosis	-2.4 ± 0.18	-2.65 ± 1.93
Chemokine (C-X-C motif) ligand 5 (Cxcl5)	chemoattractant	1.71 ± 0.20	-2.42 ± 0.29
Chemokine (C-X-C motif) ligand 2 (Cxcl2)	chemoattractant	-1.08 ± 1.49	-3.46* ± 0.84
Lymphocyte antigen 6 complex (Ly-6G)	Neutrophil marker	7.7*± 1.20	14.0*± 1.80

Table 3.3. Taxonomic distribution of rRNA genes clones from RDP classifier analysis of treatment group

PHYLUM	**CLASS**	**FAMILY**	**GENUS**	**No DSS**	**3 Day DSS**	**14 Day DSS**
Total Bacteroidetes				**33**	**3**	**19**
Bacteroidetes	Bacteroidetes	Porphyromonadaceae	*Tannerella*	13	2	10
			Parabacteroides	1	0	0
		N/A		2	1	2
				17	0	7
Crenarchaeota	N/A			1	0	0
Total Firmicutes				**282**	**351**	**334**
Firmicutes	Clostridia	Ruminococcaceae	*Anaerotruncus*	7	4	4
			Papillibacter	2	0	1
			Ruminococcaceae Incertae Sedis	4	6	4
			Ruminococcus	9	0	0
			Fastidiosipila	0	1	0
			N/A	78	58	58
			Sporobacter	1	0	0
		Lachnospiraceae	*Lachnospiraceae Incertae Sedis*	30	29	29
			Roseburia	2	0	0
			Lachnospira	0	1	0
			Catonella	1	0	0
			Butyrivibrio	0	6	6
			N/A	95	209	193
		Peptococcaceae	*Peptococcus*	1	0	0
			N/A	2	1	4
		Peptostreptococca	*Peptostreptococcaceae Incertae Sedis*	2	0	0
		N/A		26	30	30
				3	1	0

Table 3.3 (cont'd)

	Bacilli	Lactobacillaceae	*Lactobacillus*	2	1	0
		N/A		2	0	0
	Erysipelotrichi	Erysipelotrichaceae	*Allobaculum*	12	2	0
			Turicibacter	4	0	3
			Erysipelotrichaceae Incertae Sedis	0	1	1
			Holdemania	0	1	0
			Erysipelotrichaceae Incertae Sedis	0	0	1
	N/A			6	0	0
Total Proteobacteria				**2**	**1**	**0**
Proteobacteria	Betaproteobacteria	Oxalobacteraceae	*Herbaspirillium*	1	0	0
		N/A		1	0	0
	N/A			0	1	0
Tenericutes	**Mollicutes**	**Anaeroplasmataceae**	***Anaeroplasma***	**11**	**0**	**1**
Verrucomicrobia	**Verrucomicrobiae**	**Verrcomicrobiaceae**	***Akkermansia***	**0**	**10**	**17**
N/A				1	0	0
TOTAL				**328**	**365**	**371**

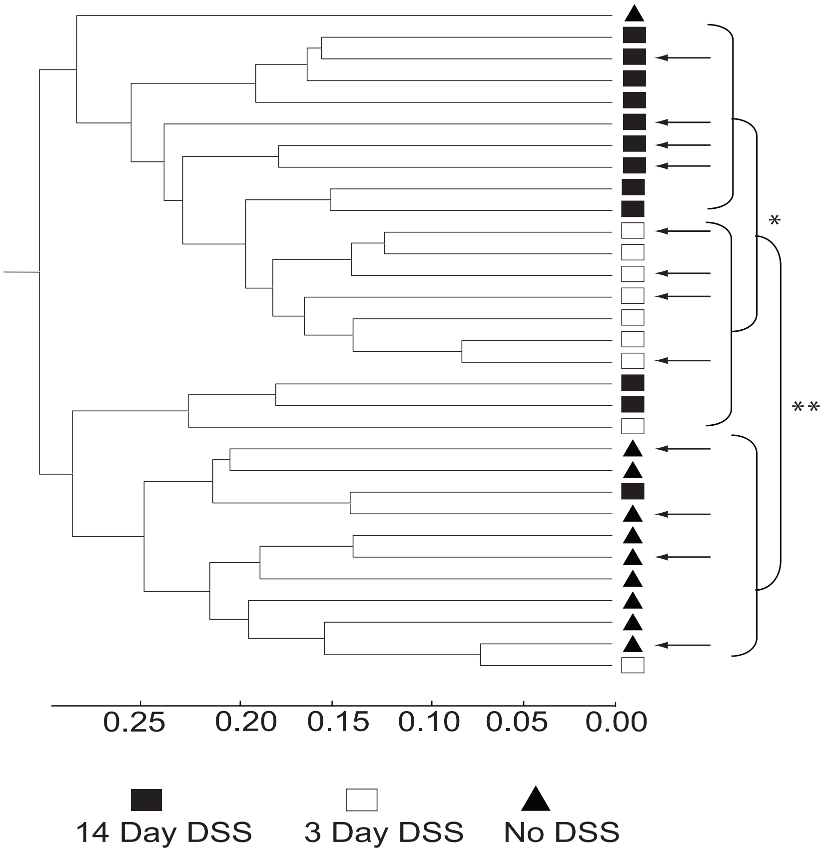

Figure 3.5. Comparison of the cecal community in control animals (black triangles), and animals following 3 days of DSS treatment (white squares) and 14 days of treatment (black squares). Using T-RFLP, the Bray-Curtis similarity metric was calculated for each pair-wise comparison and then the results displayed in dendrogram format. Communities that were further analyzed, by construction of clone libraries, are indicated by arrows. Analysis by the parsimony test indicates that there is a statistically significant difference (*p<0.05 and **p<0.001).

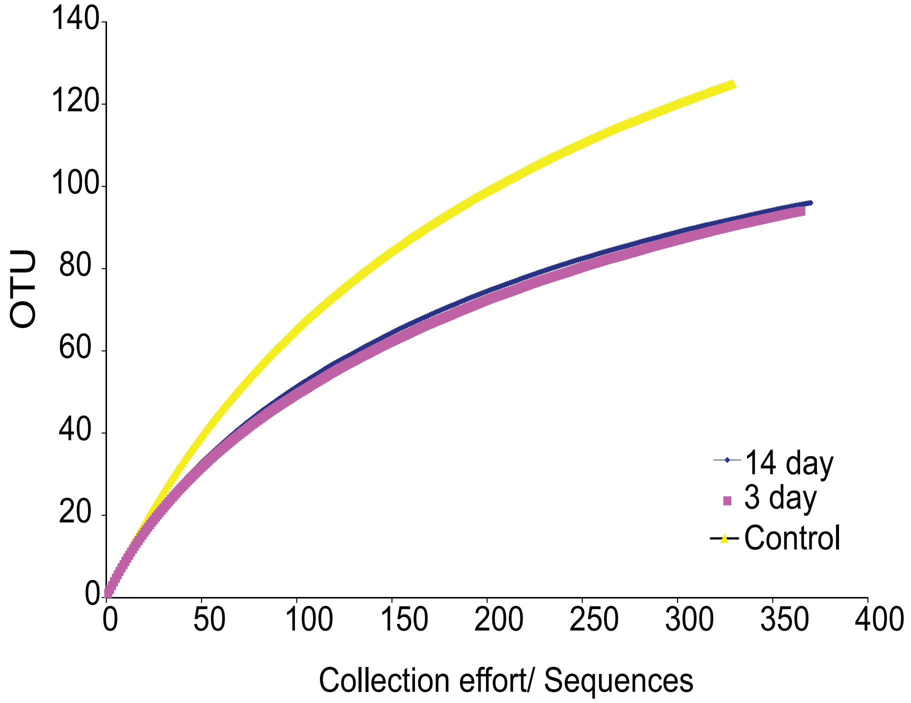

Figure 3.6. The rarefaction curves at 97% sequence similarity comparing the microbial communities in mice after 3-days of DSS treatment (pink), 14-days of DSS treatment (blue) and the controls (yellow). **For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this dissertation**.

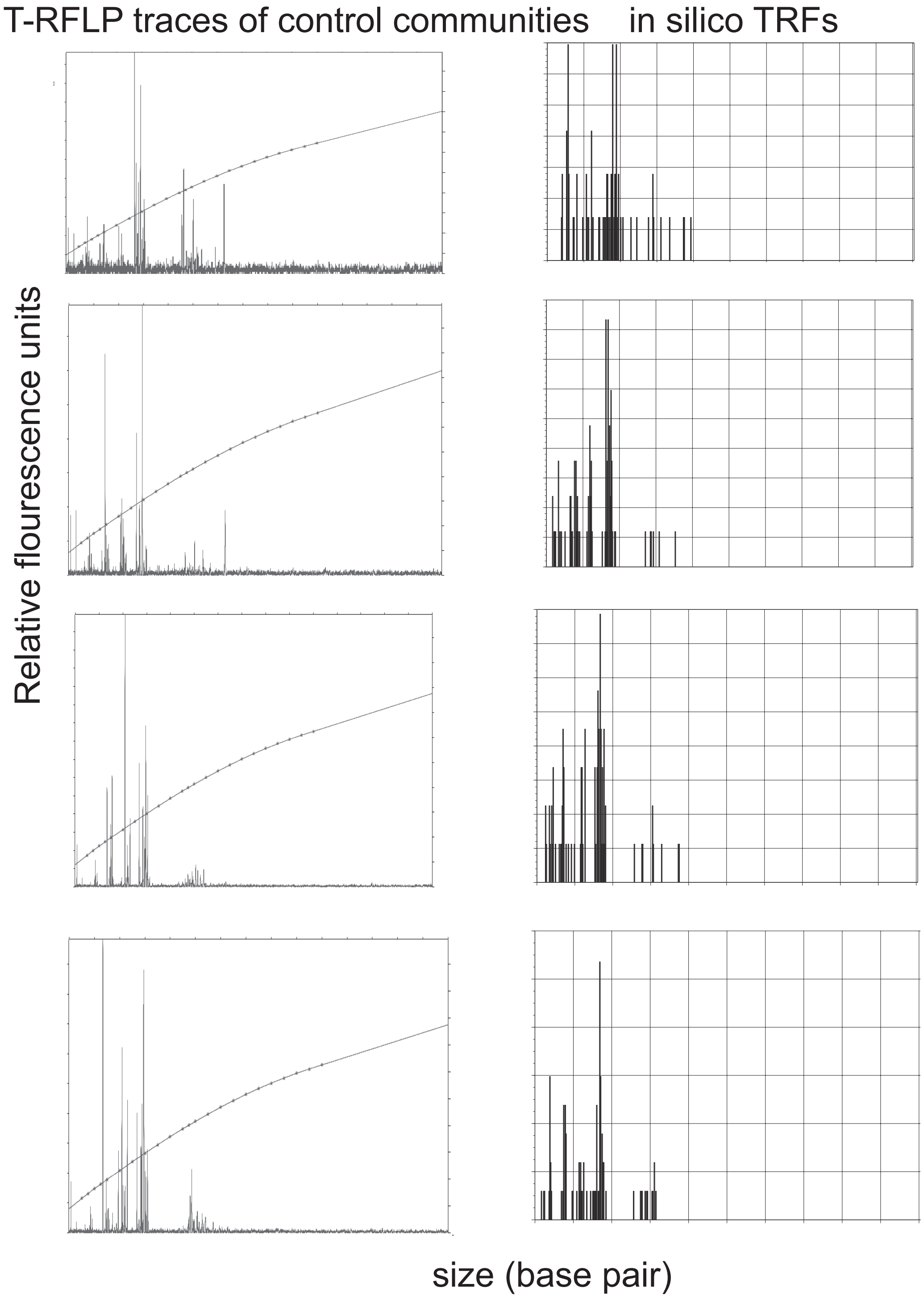

Figure 3.7. Comparisons between T-RFLP traces and histograms of the terminal restriction fragments (TRFs) of in silico digest of the clone library constructed from the control communities.

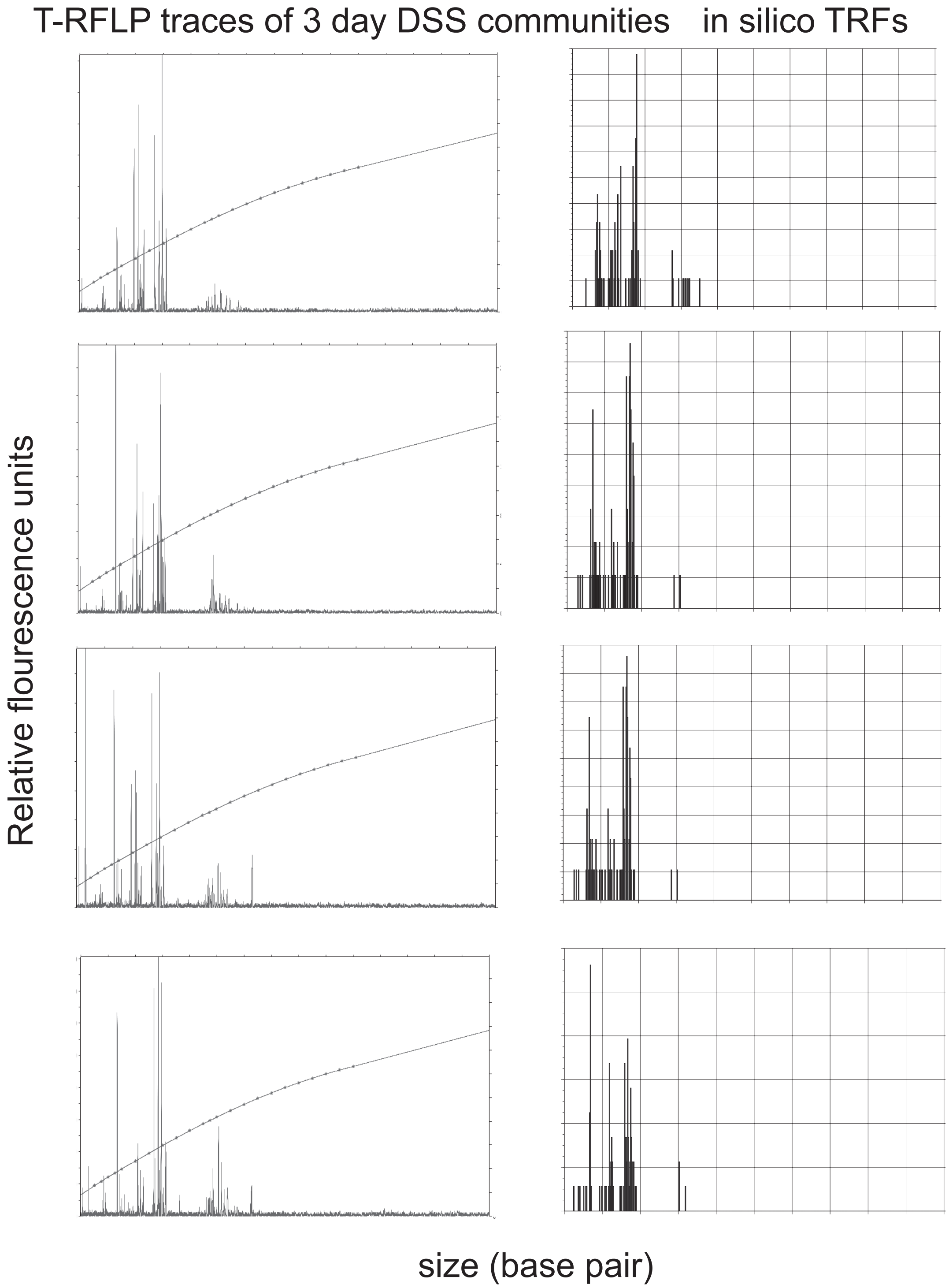

Figure 3.8. Comparisons between T-RFLP traces and histograms of the terminal restriction fragments (TRFs) of in silico digest of the clone library constructed from the 3-day DSS administered communities.

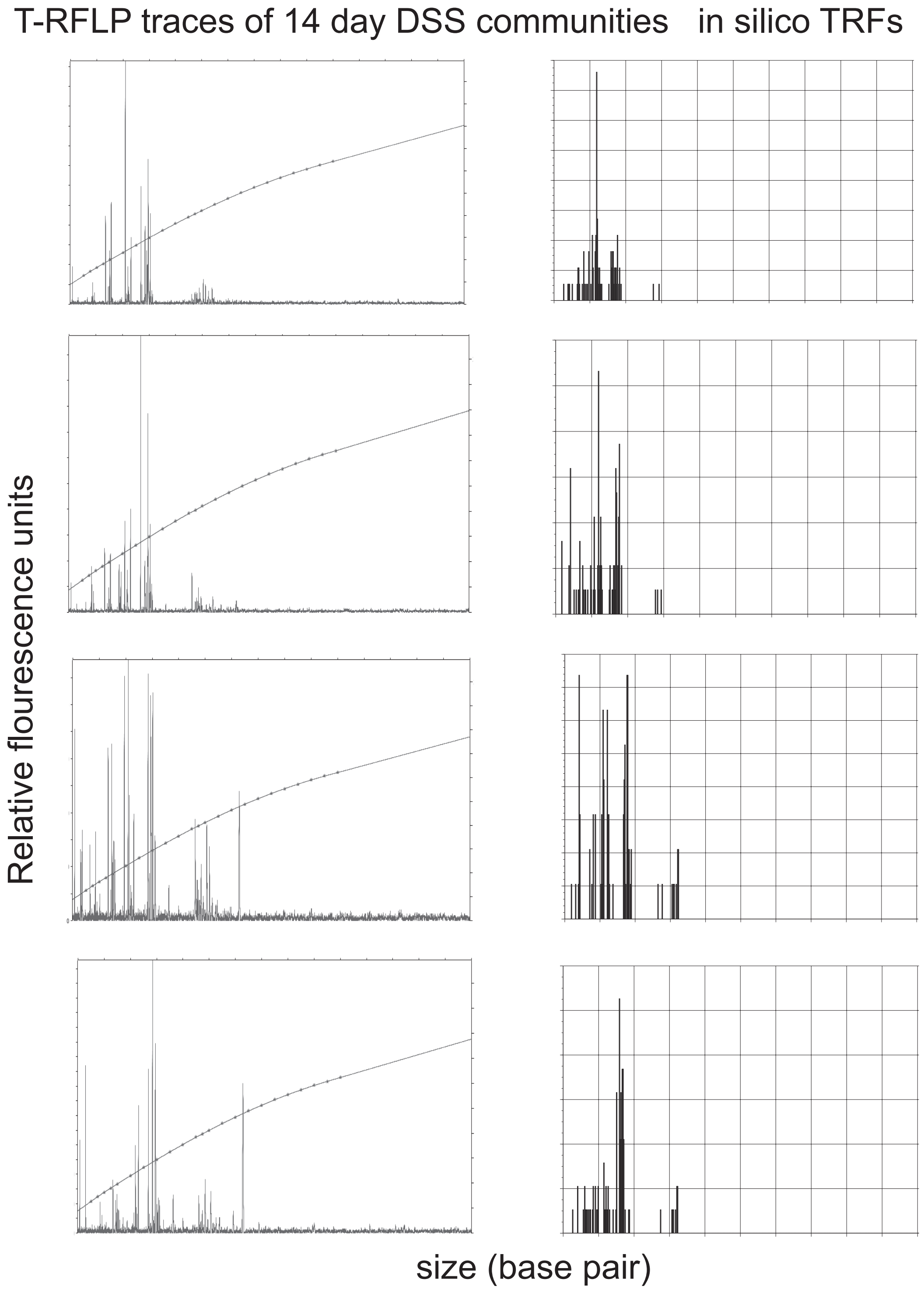

Figure 3.9. Comparisons between T-RFLP traces and histograms of the terminal restriction fragments (TRFs) of in silico digest of the clone library constructed from the 14-day DSS administered communities.

TREATMENT	PHYLUM	GENUS	TRF from clone (size/ base pairs)	TRF from T-RFLP trace (size/ base pairs)
Control	Bacteriodetes	*Tannerella (1)*	83	83
	Firmicutes	*Coprococcus (1)*	261	260
	Firmicutes	*Roseburia*	76	78
	Firmicutes	*Syntrophococcus*	115	116
3 Day DSS	Firmicutes	*Coprococcus (2)*	197	197
	Firmicutes	*Coprococcus (3)*	236	235
	Firmicutes	*Oscillibacter (1)*	259	260
	Firmicutes	*Oscillibacter (2)*	252	253
14 Day DSS	Bacteriodetes	*Tannerella (2)*	58	56
	Firmicutes	*Coprococcus (3)*	176	174
	Firmicutes	*Coprococcus (6)*	142	142
	Firmicutes	*Dorea*	175	174
	Firmicutes	*Peptococcus*	258	258
	Verrucomicrobia	*Akkermansia*	223	223

Table 3.4. Comparison of common terminal restriction fragment lengths generated by *in silico* digests of clones and the terminal restriction fragment lengths from T-RFLP via *Msp1* digests.

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I performed experiments and processed samples for the cefoperazone study, and analyzed sequence data for both cefoperazone and vancomycin studies. Dr. Robinson conducted the vancomycin study and also processed these samples. I prepared the manuscript with contribution from two other authors.

Chapter 4

The effects of intestinal microbial community structure on disease manifestation in IL-10$^{-/-}$ mice infected with *Helicobacter hepaticus*.

Abstract and Key Words:

Backgroud: Inflammatory bowel disease (IBD) is an idiopathic disease where inflammation is associated with several factors, including changes in the intestinal microbiota. Here, we investigated the role of the microbiota in *Helicobacter hepaticus* infected IL-10$^{-/-}$ mice, a commonly used IBD model, in disease manifestation. We hypothesized that alterations in the microbiota affect disease severity during disease progression.

Method: We altered the microbiota in mice by infecting animals with *H. hepaticus* and treating with vancomycin. We also infected animals with *H. hepaticus* after inducing alteration in the microbiota with cefoperazone. Through surveys of the 16S rRNA encoding-gene, analyses of histology and changes in host mediator expression, we measured changes in the microbiota and host response,

Results: We found that severity of disease is independent of antibiotic-induced changes in the microbial community structure. However, we also documented differences in expression of host inflammatory mediators associated with shifts in the microbiota, though these differences led to the same pathology.

Conclusion: In conclusion, we demonstrated that disease is initiated and progresses in the presence of several different microbial communities. Also, *H. hepaticus* is the main driver of inflammation in this model, with changes in the microbiota playing a minor role in disease manifestation once inflammation is triggered. Finally, cefoperazone-induced changes in the microbiota may have long-term effects on the expression of host mediators.

Key words: microbiota, IBD, *Helicobacter hepaticus*, IL-10$^{-/-}$ mice, 16S rRNA

Introduction:

Inflammatory bowel disease (IBD) is most often represented by Crohn's disease (CD) and ulcerative colitis (UC), but is a collective term referring to an idiopathic condition that results in inflammation of the intestinal mucosa. Although incidence of this disease is increasing (1, 2), its cause is yet to be determined. However, advances in the field point to genetic factors, dysregulated immune responses and altered intestinal microbial communities as playing key roles in the onset of disease (3).

Dysbiosis, or alteration of microbial community structure, has been associated with intestinal diseases, both in patients and animal models of IBD (4-8). For example, fecal and biopsy samples from IBD patients have different microbial communities than those from healthy controls (9). Furthermore, patients with CD contain communities that are distinct from those in UC patients (9). Animal models have been key in elucidating the importance of not only the interaction between the immune system and the microbiota, but also the structure of the microbiota in IBD. Mice that are deficient in both *Tbet*, a transcriptional factor, and *Rag2*, recombination activating gene, contain colitogenic communities that can be transferred into healthy recipients and subsequently result in disease (10). Additionally, studies utilizing animals raised in germfree or gnotobiotic conditions provide further evidence for the necessity of the resident community and the significance of community structure in enteric diseases (11-13). The results of these studies suggest that changes in the balance among members of the indigenous microbial community can trigger an inflammatory response.

To further explore the role of the microbiota in IBD, several animal models are available used to monitor changes in these communities and their effects on disease, in a controlled environment (14, 15). A common model of IBD employs the infection of *Helicobacter hepaticus* in interleukin 10 (IL-10) deficient mice. In this model, infection elicits a Th1/Th17 response that most resembles Crohn's disease in humans. Infection with *H. hepaticus* does not cause disease in wild-type mice and only triggers inflammation in immune altered hosts (16, 17). Onset of disease is MyD88 dependent (18). Additionally, *H. hepaticus* only causes disease in the presence of the resident microbiota with no disease observed in germfree mice (19), indicating the importance of the microbiota in the onset of IBD in this model. However, apart from these findings there is limited knowledge of the role that the resident microbiota plays in this model. Therefore, the aim of this study is to investigate the role of the microbiota in this model of IBD by altering community structure and observing disease manifestation.

Previously we showed that treatment with cefoperazone significantly alters the microbial community of the murine gut (20), as does vancomycin (21). We used these antibiotics to modify the gut community structure in IL10$^{-/-}$ C57BL/6 mice before and during infection with *H. hepaticus*, and then monitored disease. We hypothesized that the structure of the microbiota is related to disease severity in IL-10 deficient mice infected with *H. hepaticus*.

Materials and methods:

Animals: C57BL/6 IL-$10^{-/-}$ mice from a breeding colony initially established with breeding stock from Jackson Laboratories (Bar Harbor, ME) were use for all experiments. Mice were housed with autoclaved bedding, given sterile food and water *ad libitum,* and exposed to 12:12 h light:dark cycles. Germfree mice were housed in sterile microisolators. All animal studies were conducted at the University of Michigan and were approved by the University Committee on Use and Care of Animals (UCUCA).

Murine infection with *H. hepaticus*. The wild-type *H. hepaticus* strain 3B1 (type strain, ATCC 51488) was incubated at 37 °C under microaerobic conditions for 48 h, on Tryptic Soy Agar plates (TSA), supplemented with 5% sheep's blood, and then re-suspended in 5 ml of tryptic soy broth (TSB). Mice were inoculated with a suspension of bacteria with an optical density of 1.0 at 600 nm (~10^8 CFU) in a volume of 0.2 to 0.3 ml. Bacteria were introduced directly into the stomach with a 24-gauge ball-tipped gavage needle. Control mice were inoculated with sterile TSB.

Three experimental studies were conducted to investigate roles of the microbiota in disease development: 1) To investigate whether disease can develop in *H. hepaticus* monoassociated IL-10 deficient C57BL/6 mice in our colonies, two groups of germfree mice were used: five mice were gavaged with TSB only and five were gavaged with *H. hepaticus*. Tissues were collected four weeks post-infection. 2) To investigate roles of an altered microbiota in disease development in this model, we changed microbial communities using two different antibiotic treatments. Cefoperazone was administered, *ad libitum*, as a 0.5 mg/ml solution in the drinking water of 10 weeks old, conventionally raised C57Bl/6 IL-10 deficient mice. Mice were assigned to six groups, as shown in figure 4.1A: one group was infected with *H. hepaticus* after 1 day recovery from cefoperazone, another group infected after 6 weeks recovery from cefoperazone, and a third group infected without prior cefoperazone treatment. Three other groups were used as the uninfected controls for respective cefoperazone treatments 3) The second antibiotic treatment involved *ad libitum* administration of 0.1 mg/ml vancomycin in the drinking water of 6-8 week old, conventionally-raised C57Bl/6 IL-10 deficient mice (21). Mice were assigned to six experimental groups as depicted in figure 4.1B: One group of mice was given vancomycin then infected with *H. hepaticus* as antibiotic administration continued. A second group had similar treatment but vancomycin treatment was ceased 10 days post infection as mice recovered fro 11 days from antibiotic treatment. A third group of mice was used as vencomycin untreated controls. Three *H. hepaticus* uninfected control groups were used for respective antibiotic treatments. Mice were grouped into cages according to gender and treatment.

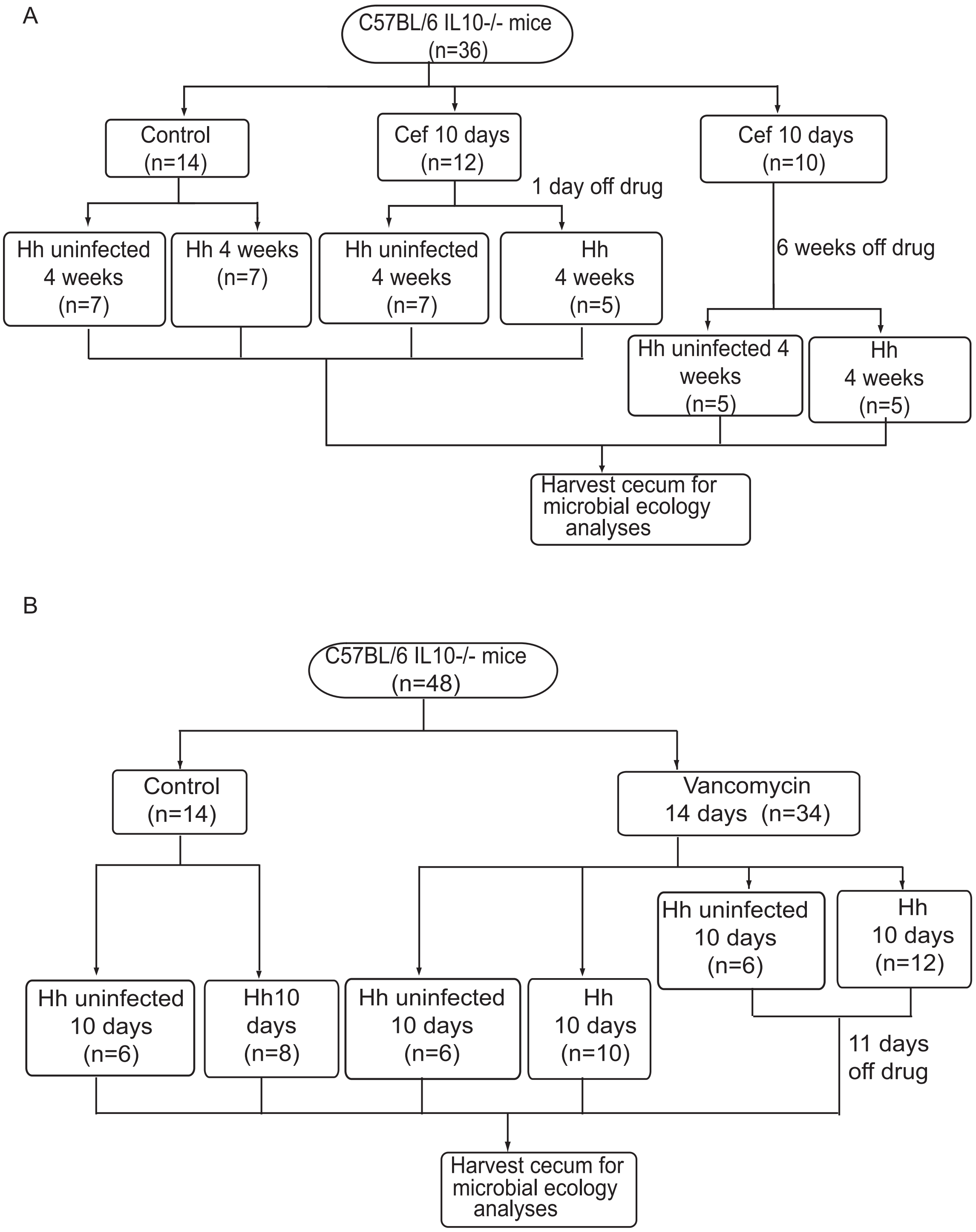

Figure 4.1 Schematic of experimental designs showing (A) cefoperazone (Cef) and (B) vancomycin administration to IL10$^{-/-}$ C57BL/6 mice prior to or during infection with *H. hepaticus* (Hh).

Necropsy and Histology: Mice were euthanized by CO_2 asphyxiation and tissues harvested (22). The cecum sections were gently washed with 1X phosphate-buffered saline (PBS) to remove luminal contents, cut into sections and snap-frozen in liquid nitrogen and stored at -80°C until future use.

The remaining portion of the cecum was processed for histology by removing the luminal contents before washing with PBS, and placing the tissue in tissue cassettes. Cassettes were then submerged in 10% formalin for 24 hours and transferred to a 60% ethanol solution prior to processing for paraffin embedding and staining with hematoxylin and eosin (H&E).

Histologic sections were scored by a veterinary pathologist who was blind to the sample source. The following scoring system was used: 0, no significant inflammation; 1, small multifocal lamina proprial or transepithelial leukocyte aggregates; 2, coalescing lamina proprial leukocyte aggregates, may have submucosal involvement; 3, frequent coalescing leukocyte aggregates with submucosal involvement, may have follicle formation; 3.5, strong submucosal component, infrequent extension to muscularis externa or mesocolon; 4, diffuse or regionally extensive transmural involvement.

DNA extraction: Genomic DNA was extracted from tissue using the MagNa Pur® system (Roche, Indianapolis, IN). Briefly, the tissue was incubated in lysis buffer and proteinase K at 56 °C, before being placed in the MagNa Pur® Compact machine for purification of DNA. The resulting DNA was then used in preparation of the clone libraries and quantitative PCR (qPCR).

Clone library construction: PCRs were set-up as described in Young *et al.* (23). Briefly, illustra PuReTaq^{TM} Ready To Go PCR^{TM} beads (GE Healthcare, Piscataway, NJ). and broad-ranged primers, (8F, 5'-AGAGTTTGATCCTGGCTCAG-3'; 1492R, 5'-GGTTACCTTGTTACGACTT-3') were used to amplify 16S rRNA genes. GE illustra^{TM} Microspin^{TM} S-400 HR columns were used to purify amplicons (GFX, GE Healthcare, Piscataway, NJ) as directed by the manufacturer. Purified PCR products were ligated into the TOPO 4$^{®}$ vector (Invitrogen K4575-01, Carlsbad, CA) according to manufacturer's specifications, and transformed into *Escherichia coli* TOP10 cells. Clones were grown overnight at 37 ^{o}C in Luria Broth (LB) amended with carbenicillin (50 μg/ml)). Vector specific primers (M13F, 5'-CAGTCACGACGTTGTAAAACGACGGC-3'; and M13R, 5'-CAGGAAACAGCTATGACCATG-3') were used to screen for clones containing the appropriately sized inserts and to amplify inserts for sequencing. Partial 16S rRNA gene sequences were generated by sequencing with primer 8F, at the Sequencing Core at the University of Michigan. Raw sequence data were processed through an automated "information pipeline" available through the Ribosomal Database Project (RDP) Web site (http://rdp.cme.msu.edu/). Distance matrices containing data from each library were calculated from alignments generated by the RDP aligner (24). The program mothur (25) was used to assign sequences to operational taxonomic units (OTUs) based on values in the distance matrices. Analyses were done using a 95% sequence similarity to denote genus level and at a 80% sequence similarity to approximate phylum level (25). EstimateS (26) and mothur were used to calculate ecological measures from the distance matrices. The Morisita Horn index was then used to measure the similarity among samples taking into account both abundance and richness of the community. Phylogenetic trees were calculated using the Mega3 program and the parsimony test from the mothur suite of programs was used to denote significant differences between treatment groups (25, 27). Additionally, taxonomic assignments were determined using Classifier through the RDP website (80% confidence cut-off).

qPCR analysis of microbial communities: The quantity of 16S rRNA operons in the samples relative to a single-copy host gene was measured using a primer/probe set that targets a broad range of rRNA-encoding gene sequences (forward primer (5'-TCCTACGGGAGGCAGCAGT-3'); reverse primer (5'-GGACTACCAGGGTATCTAATCCTGTT-3'); and probe (5'-[6-FAM]-CGTATTACCGCGGCTGCTGGCAC-[TAMRA]-3')) (28). A primer/probe set targeting a 264-bp portion of the TNF alpha gene was used as a reference using 200 nanomoles of the forward (TNFα_mu_se; 5'-GGCTTTCCGAATTCACTGGAG-3') and reverse primers (TNFα_mu_as; 5'-CCCCGGCCTTCCAAATAAA-3'), and 100 nanomoles of the probe (TNFα_mu_probe; 5'-[Cy5]-ATGTCCATTCCTGAGTTCTGCAAAGGGA-[Iowa Black RQ™]-3') (29). The reaction mix consisted of LightCycler® 480 Probes Master reaction mix (Roche) at 1X concentration, and appropriate primer/probe pair. Amplification of each gene was done under separate run conditions: the 16S rRNA gene target had an activation step of 50 °C for 2 min followed by 95 °C for 10 min. Forty-five cycles were done at 95 °C for 15 s and 60 °C for 1 min before holding at 4 °C. The TNF reference gene was also amplified with an activation step of 50 °C for 2 min followed by 95 °C for 10 min. Forty-fives cycles of 95 °C for 20 s and 64 °C for 30 s were done before holding at 4 °C. Calculations of $2^{-\Delta\Delta Ct}$ were made to compare changes in the amount of 16S from samples between treatment groups (21,22).

RNA extraction and determination for cytokine expression: Total nucleic acids were extracted by MagNaPur® (Roche) as directed by the manufacturer's protocol. Total RNA was then isolated by treating total nucleic acids with DNaseI recombinant, RNase-free (Roche, Indianapolis, IN). The High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) was used to transcribe total RNA to cDNA. Cytokine expression was determined by applying the cDNA to a custom Superarray® (Frederick, MD) PCR plate which contained gene-specific primers designed for the following targets (30): IFNγ, IL-12a, TNFα, IL-4, IL-13, IL-5, TGFβ, IL-10, IL-6, FOXp3, IL-17a, IL-23a, CXCL2, CCL5, NOS2, Arg1, Chi3/4, PTGS2, INDO, GAPDH, βActin, a reverse-transcription control and a genomic DNA control. Quantization was done with LightCycler® 480 SYBR Green I Master and analyzed on a LightCycler® 480 system (Roche Diagnostics, Indianapolis, IN) as directed by manufacture's protocol, with the following program: 95 °C activation for 10 min; 40 cycles of 95 °C denaturation for 15s, and 60 °C annealing for 1 min. Resulting threshold values were analyzed by calculating the $2^{-\Delta\Delta Ct}$ values, using GAPDH as the reference, to calculate fold regulation compared to the antibiotic untreated, *H. hepaticus* uninfected control (31-33).

Statistical Analysis: Statistical significances were determined using Kruskal-Wallis tests for the histological scores, and ANOVA tables for the microbial abundances among the treatment groups using the Metastats, software for metagenomic analysis (34). The differences in the dendrograms were calculated using the parsimony test function in mothur (25). Probability values less than 0.05 were considered significantly different.

Results:

Germfree mice infected with *H. hepaticus* do not get colitis. To investigate whether *H. hepaticus* can colonize germfree mice from our colonies, we infected IL-10 deficient animals and monitored them for 4 weeks. Absence of disease when mice are monoassociated with *H. hepaticus* would indicate that the resident microbiota has an important role in disease manifestation. Detection of *H. hepaticus* was done through qPCR, and revealed that the loads in germfree mice were 2.94 ±1.90 fold less than loads in conventional mice, a level that was not significantly different (p=0.446) (table 4.1). This indicates that not only can *H. hepaticus* colonize germfree mice, as indicated by others (19), but that the bacterium may have a particular niche that is unaffected by the presence of resident microbes.

Treatment		**Fold change ± SD**	
Cefoperazone treatment	***H. hepaticus* infection**	**Total bacterial load**	***H. hepaticus***
No cef	No	calibrator	N/A
1 day recovery from cef	No	-3333.00 ± 0.34	N/A
1 day recovery from cef before infection	No	2.16 ± 1.18	N/A
No cef	Yes	1.25 ± 0.75	calibrator
1 day recovery from cef before infection	Yes	4.48 ± 2.55	-1.12 ± 2.79
6 wk recovery from cef before infection	Yes	-1.35 ± 0.71	3.94 ± 6.30
6 wk recovery from cef before infection	No	1.14 ± 0.04	N/A
Vancomycin treatment			
No vanc	No	1.17 ± 0.68	N/A
Vanc	No	1.95 ± 1.41	N/A
No vanc	Yes	1.90 ± 1.91	1.04 ± 0.30
Vanc	Yes	1.98 ± 1.62	-1.59 ± 0.52
Vanc + 11 days recovery	Yes	1.14 ± 1.16	1.28 ± 0.56
Vanc + 11 days recovery	No	1.41 ± 0.91	N/A
Germfree mice infected with *H. hepaticus*		N/A	-2.94 ± 1.90

Table 4.1. Quantitative PCR measures of fold changes in the total bacterial and in *H. hepaticus* loads from genomic DNA extracted from cecum tissue. The fold change reported ($2^{-\Delta\Delta Ct}$) is normalized to GAPDH and in comparison to the samples indicated as “calibrator” in the respective columns.

Through histological analysis, no inflammation was detected in cecum tissues from germfree mice that were infected with *H. hepaticus* (figure 4.2). This result is in agreement with a previous study (19). However, unlike previous reports (19), IL-10 deficient mice raised in specific pathogen-free conditions did not develop inflammation. This indicates that spontaneous disease is only associated with mice containing specific microbial communities, again emphasizing the important role of the microbiota in disease development.

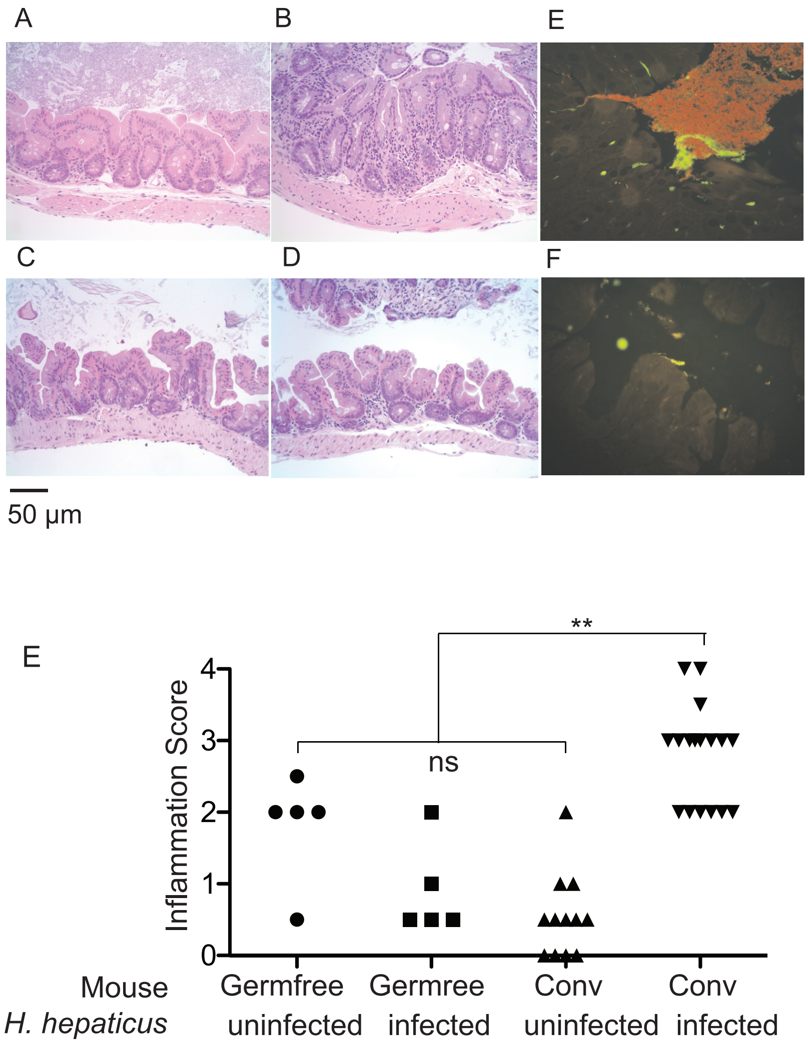

Figure 4.2 *Helicobacter hepaticus* triggered inflammation in conventionally raised IL10$^{-/-}$ C57BL/6 mice compared to germfree counterparts. Representative sections of mouse cecum from experimental groups are depicted. (A) *H. hepaticus* uninfected conventionally raised mice appear normal. (B) *H. hepaticus* infected conventional (Conv) section shows edema, hyperplasia and inflammatory infiltrate. (C) *H. hepaticus* uninfected germfree control, and (D) *H. hepaticus* infected germfree cecum sections are both normal without signs of inflammation. (E) *H. hepaticus* forms close association to the intestinal epithelium, and in the crypts (yellow) compared to other microbes (red) in the lumen. (F) In germfree mice monoassociated with *H. hepaticus*, the bacteria are also found in close association with the epithelial layer (G) Quantification of indicators of pathology showing significant inflammation only in *H. hepaticus* infected conventionally raised mouse sections. Asterisk (*) denotes statistically significant changes ($p<0.05$). **For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this dissertation**.

Vancomycin alters communities in uninfected mice. Vancomycin was administered to mice infected with *H. hepaticus* prior to infection in order to investigate the effects of altering community structure on the development of inflammation. Through clone library construction, we found that vancomycin treated communities were significantly altered and these changes persisted even after administration ceased. Although bacterial load, via qPCR, was not altered by vancomycin treatment (table 4.1), this antibiotic caused a decrease in overall microbial diversity and an increase in relative abundance of members of the Proteobacteria, namely *E.coli/Shigella* (figure 4.3). Also, total community richness was reduced in vancomycin treated mice (Shannon index= 2.10± 0.55) compared to untreated controls (Shannon index= 3.55± 0.15) (supplemental figure 4.3). These results indicate that vancomycin alone causes changes in the microbial community. Communities did not cluster according to gender or housing.

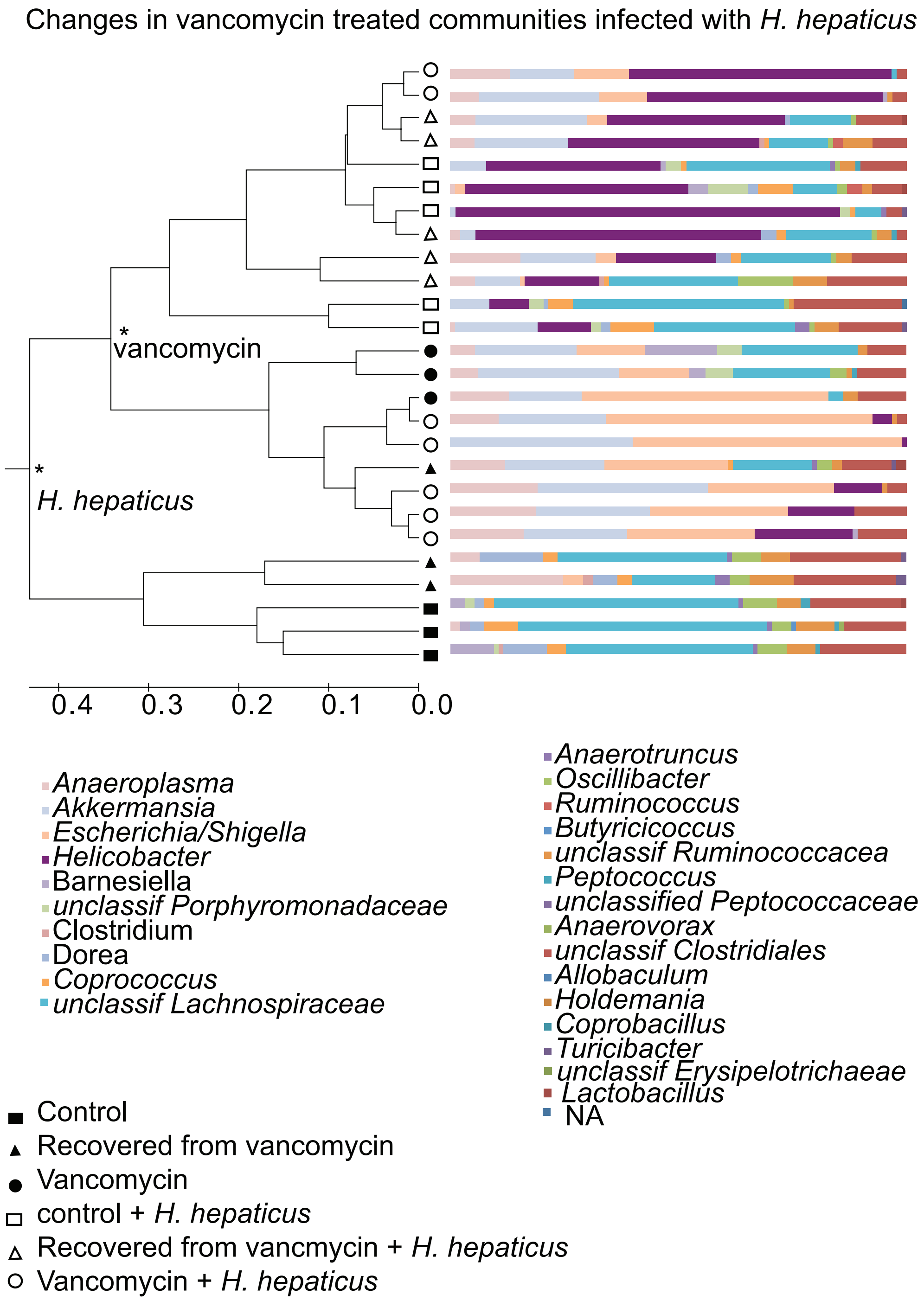

Figure 4.3 *H. hepaticus* infection, as well as vancomycin treatment, significantly alters intestinal community structure. Asterisk (*) denotes statistically significantly different community structures calculated by parsimony test (p<0.05). **For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this dissertation**.

Vancomycin and *H. hepaticus* alter communities in infected mice. Investigations on the effects of *H. hepaticus* infection on the microbial community structure, during vancomycin treatment, showed reduction in community richness (Shannon index= 1.35± 0.35) compared to microbiota in infected, vancomycin-untreated mice (Shannon index= 2.47± 1.01) (supplemental figure 4.3). We also found that mice that were continuously administered vancomycin and infected with *H. hepaticus* (VancHh) contained the same load of *H. hepaticus* as mice that were not administered antibiotics (Hh) (p=0.06). However, VancHh mice contained fewer *H. hepaticus* than mice that had been administered vancomycin previous to *H. hepaticus* infection, but not during infection (VancRecHh) (p=0.009). There was no difference between the loads in Hh and VancRecHh mice (p=0.28).

Structurally, the communities in VancRecHh mice were more like the communities in mice that were only infected with *H. hepaticus* and no antibiotic (Hh) (Morisita-Horn distance= 0.36) than those in VancHh mice (Morisita-Horn distance= 0.43). As compared to the communities in VancRecHh mice, VancHh mice contained communities with decreased population sizes of *Oscillibacter* (p=0.005), Lachnospiraceae (p=0.003), *Coprocococcus* (0.011) and *Dorea* (p=0.038). Additionally, the VancHh communities contained more *E. coli/Shigella* (p=0.001), *Akkermansia* (p=0.001) and *Anaeroplasma* (p<0.001). Shannon diversity index indicates that communities in VancRecHh mice are more diverse than communities in VancHh mice (p= 0.001). Taxonomic analyses indicated that communities in VancRecHh also exhibited a significant increase in

Lachnospiraceae (p=0.001) and decrease in *E. coli/Shigella* (p=0.005) as compared to communities in VancHh mice (figure 4.3).

Inflammation occurs in vancomycin treated mice infected with *H. hepaticus*. To investigate whether changes in the microbiota affect disease manifestation, cecum sections of vancomycin treated mice were scored. All animals infected with *H. hepaticus* developed inflammation, regardless of the shifts in the microbial community structures (figure 4.4).

***H. hepaticus* infection alters communities in infected mice.** Since vancomycin treatment did not result in differences in disease manifestation in *H. hepaticus* infected mice, we administered cefoperazone to animals since it is known to cause significant, broad-spectrum changes to the intestinal microbiota. The community 1 day following cefoperazone treatment has over a 1000 fold lower bacterial load (table 4.1).

After cefoperazone treatment, mice were administered untreated water and allowed to recover for one day*,* or for six weeks before infecting with *H. hepaticus*. Four weeks post infection, all communities had similar total bacterial loads compared to the cefoperazone untreated mice that were *H. hepaticus* uninfected, indicating that the communities had recovered during the four weeks. This was also supported by diversity measures (supplemental figure 4.3). Communities in infected mice contained similar amounts of *H. hepaticus* regardless of cefoperazone treatment or recovery period (table 4.1). It is noteworthy that there was an exception, with one infected mouse having 20-fold less detectable CdtB target, indicating a low *H. hepaticus* load.

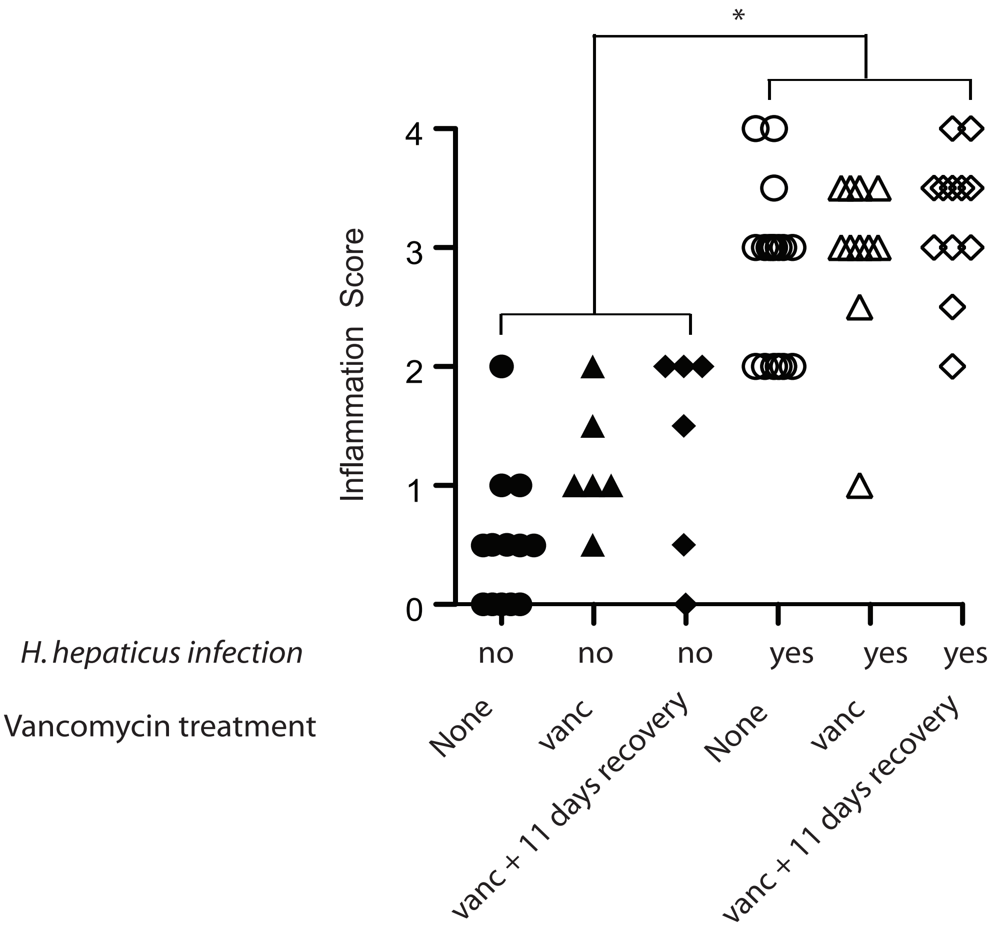

Figure 4.4 Blinded scores showing significant inflammation in all *H. hepaticus* infected sections regardless of vancomycin treatment compared to uninfected controls. Asterisk (*) denotes statistically significant changes ($p<0.05$).

Clone libraries were built from three individual mice from each treatment group (supplemental 4.1). OTU-based analyses show that the communities in *H. hepaticus*-infected mice were significantly different from those in mice that did not harbor *H. hepaticus* ($p<0.001$). In communities of infected mice, the relative abundance of *H. hepaticus* varied between 25% and 70% (figure 4.5). This difference in relative abundance of *H. hepaticus* was independent of cefoperazone treatment.

The mouse that contained low numbers of *H. hepaticus,* as determined via qPCR, contained a community that produced only 2 of 90 clones that affiliated with *Helicobacter.* This supports the finding that *H. hepaticus* did not dominate this community as was typically seen in the other mice in this treatment group.

There were reductions in the genera *Clostridia* ($p=0.032$) and family Lachnospiraceae ($p=0.016$) in communities infected with *H. hepaticus* after only one day off cefoperazone compared to those infected after six-weeks recovery. This indicates differences in recovery dynamics in the two treatment groups. There were no gender or housing effects on communities' relatedness.

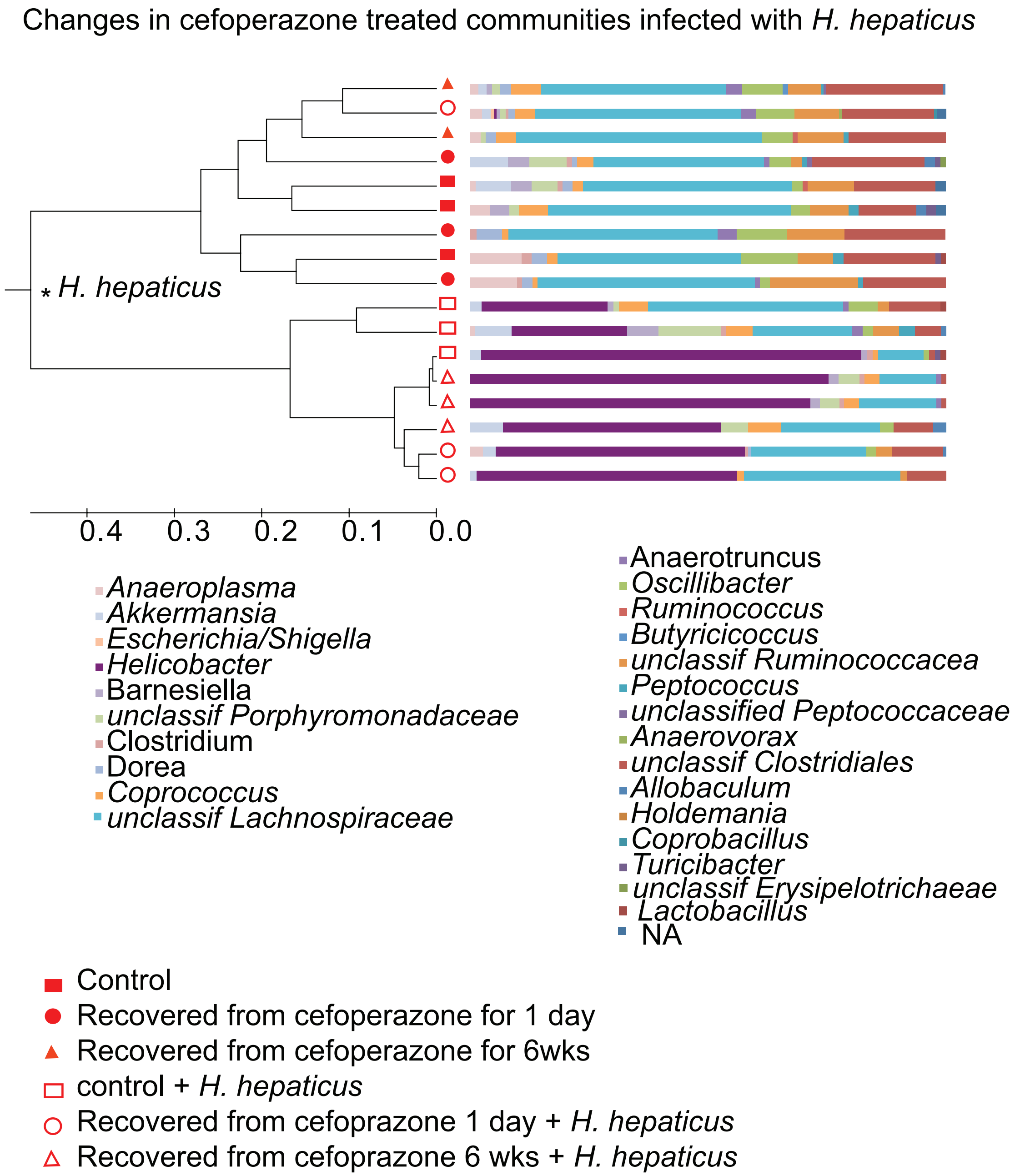
Changes in cefoperazone treated communities infected with *H. hepaticus*

Figure 4.5 *H. hepaticus* infection significantly alters the community structure of mice even after four weeks of termination of cefoperazone treatment. Asterisk (*) denotes statistically significantly different community structures calculated by parsimony test ($p<0.05$). **For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this dissertation.**

Inflammation occurs in cefoperazone treated mice infected with *H. hepaticus*. To investigate whether a major perturbation would affect disease manifestation in *H. hepaticus* infected IL-10 deficient mice, cefoperazone was administered to mice before infecting with the bacterium. Cecum sections were then scored, and it was found that all animals infected with *H. hepaticus* developed inflammation (figure 4.6). There was no correlation between disease scores and relative abundance of *H. hepaticus* (Spearman's ρ = 0.242). Despite changes to the microbiota, cefoperazone did not alter disease severity in *H. hepaticus* infected mice as compared to infected mice that were not administered antibiotics.

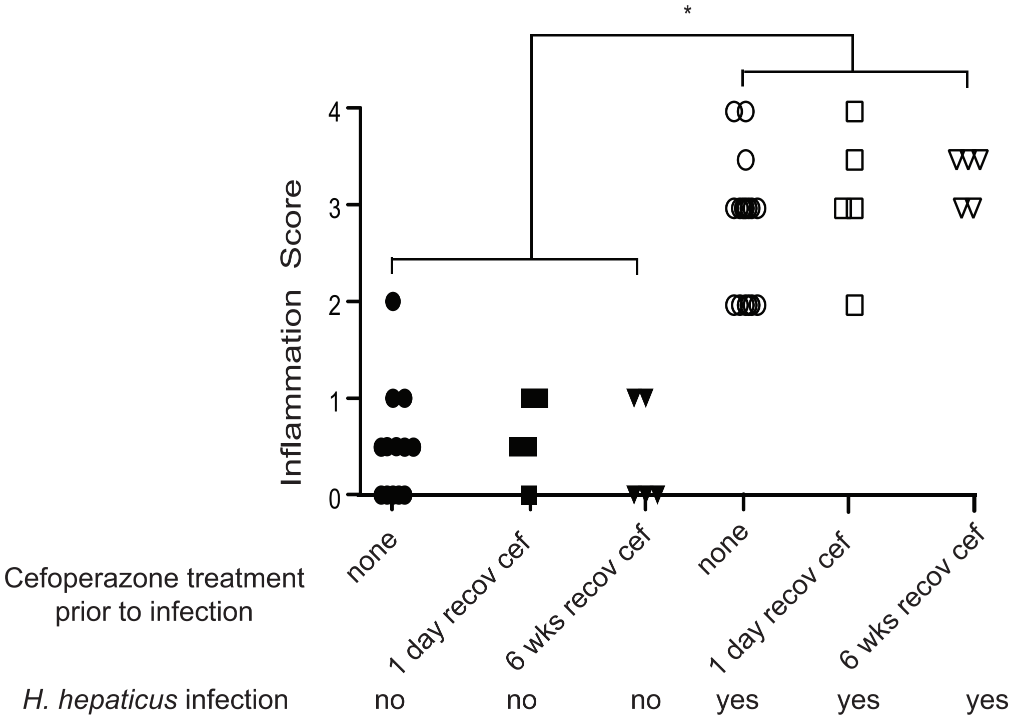

Figure 4. 6 Blinded scores showing significant inflammation in all *H. hepaticus* infected sections regardless of cefoperazone treatment compared to uninfected controls. Asterisk (*) denotes statistically significant changes ($p<0.05$).

Infection with *H. hepaticus* and/or cefoperazone induces host response. Although development of inflammation was not affected by cefoperazone treatment, qPCR showed that antibiotic treated mice that were infected 1 day after treatment ceased, had a lower load of *H. hepaticus* than animals that were allowed to recover from treatment for six weeks prior to infection. To investigate possible host effects due to this difference, we measure changes in expression of host mediators. This analysis indicated that changes were elicited by administration of cefoperazone in communities containing *H. hepaticus*.

After cefoperazone treatment, there was a significant up-regulation of several host mediators including TNFα, CCL5, CCL2, Nos2 and Arg1, or down-regulation in CHI3/4 and IL-23a (figure 4.7, supplemental 4.2). Even when mice were allowed to recover for 6 weeks from cefoperazone treatment, expression of some mediators remained significantly different from the no-cefoperazone, *H. hepaticus* infected control, indicating that treatment with cefoperazone causes an altered immune response in the presence of this bacterium. These changes do not mediate lesions.

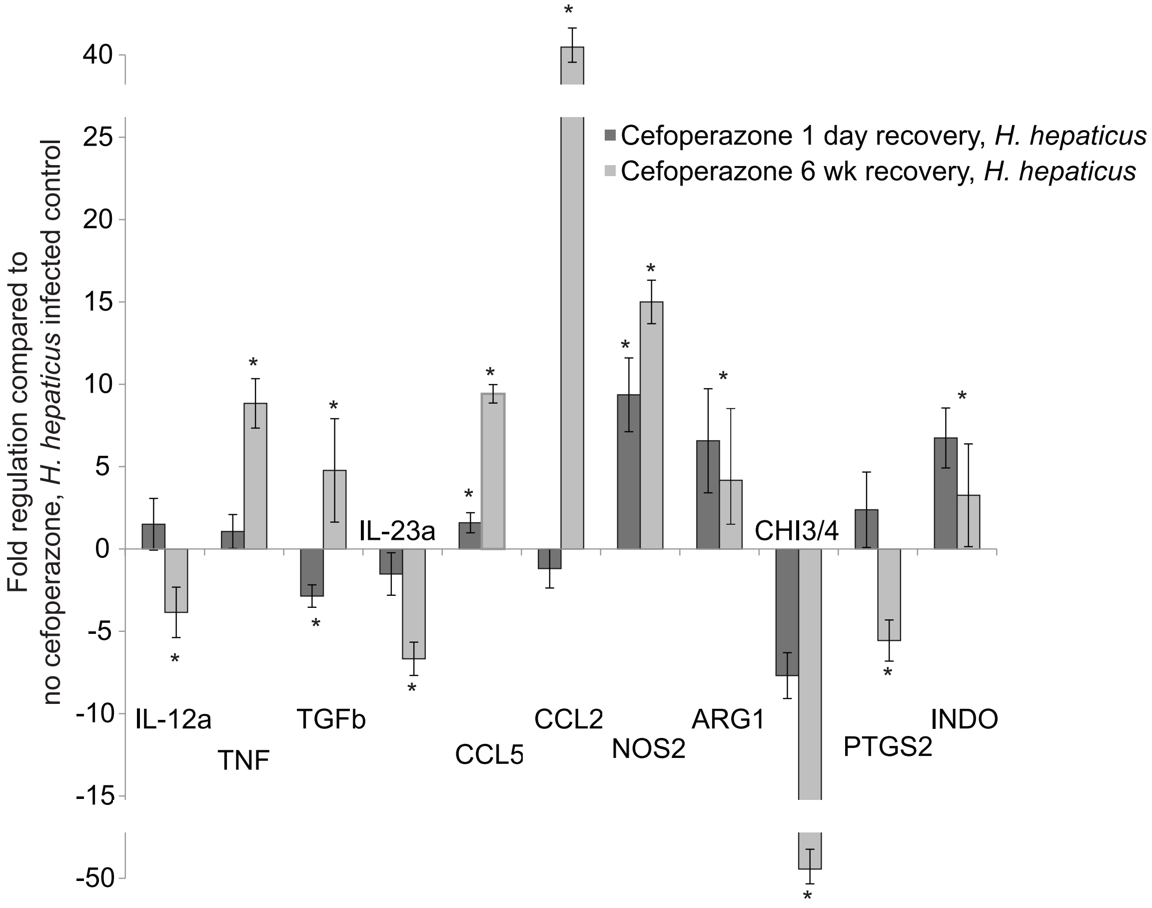

Figure 4.7 Changes in expression of host mediators in cecum tissue of mice administered cefoperazone and infected with *H. hepaticus* (p<0.05).

Discussion:

Intestinal microbes have been credited with many beneficial functions including break-down of complex carbohydrates, protection against pathogens and education of the immune system (35). These are important interactions between the host and its resident microbes as seen by deficiencies in the host development in animals raised in germfree conditions (36-38). Collapse of this delicate balance has been implicated in the onset of diseases, such as inflammatory bowel disease (IBD), a condition triggered by an inappropriate response by the hosts' own immune system. It is also associated with an altered microbiota, a condition commonly referred to as dysbiosis. Patients with IBD often exhibit a reduction in diversity of the microbial community structure compared to healthy participants (39-42). Additionally, some investigators noted a relative increase in particular bacterial groups such as the Bacteriodetes in patients with IBD compared to healthy counterparts (43, 44). There is also evidence that the microbial community is important in the persistence of disease with antibiotic studies showing amelioration of IBD after treatment (45, 46). Apart from this we have little insight into the role of the microbiota in IBD. We therefore used a murine model of IBD, *Helicobacter hepaticus* infected IL-10 deficient C57BL/6 mice, to further investigate how changes in the microbial community associated with disease by testing whether the severity of disease is affected by different microbial community structures.

Helicobacter hepaticus is sometimes found as part of the resident intestinal community of mice (47-49), and triggers inflammation in immuno-compromised mice such as Rag$^{-/-}$, IL-10$^{-/-}$ and SCID (50-52). Therefore, this bacterium is a pathobiont, a member of the resident bacteria with the potential to cause disease (53). Furthermore, *H. hepaticus*-induced inflammation can be ameliorated by the use of antibiotics (54, 55). However, *Helicobacter* causes disease in IL-10 deficient mice only in the presence of the resident microbiota indicating an essential role of the indigenous community towards inducing inflammation in this model (19). Although other groups have shown the development of spontaneous disease in IL-10 deficient mice (11, 19), we report less than 5% of mice in our breeding colony develop disease over a 2 year period (17), suggesting that disease is associated with certain microbial communities. These data collectively suggest that the microbiota is an important driver of inflammation in IBD, particularly in this model, making it an appropriate system in which to study the effects of an altered microbial community structure on severity of inflammation.

To investigate the extent of the role of the microbiota, we infected mice containing various communities with *H. hepaticus* and assessed whether there were differences in disease severity. Two antibiotics, vancomycin and cefoperazone, were used to alter the microbial communities. Each drug causes different changes in the gut community: vancomycin, a drug that targets the gram-positive bacteria, caused shifts in the microbial structure without reducing the bacterial load, and cefoperazone, a broad-spectrum antibiotic, reduced the total bacterial load almost 1000-fold as we have shown here and previously (20, 21). With vancomycin, these shifts were mainly due to the reduction in the Firmicutes, a phylum that typically comprises more than 50% of the gut community (23, 56). After a recovery period communities exposed to vancomycin began to resemble control communities, but also exhibited lasting effects with a reduction of the phylum Firmicutes and an increase in the Proteobacteria. Increases in the Proteobacteria after vancomycin treatment have also been observed by other investigators (57), and are perhaps due to the reduction in gram-positive bacteria, thereby making formerlly occupied niches available to members of the Proteobacteria. Although we did not detect them in control groups in this study, previously we showed that members of the Proteobacteria inhabit the ceca of IL-10$^{-/-}$ mice using 454 pyrosequencing analysis of the microbiota (20). It is likely that they were present in the communities analyzed in the current study, but were below the detection limits of clone library construction. Like other communities (23, 58) after a recovery period, cefoperazone treated communities began to resemble the communities in untreated controls, demonstrating resilience, even after a significant reduction in bacterial loads immediately after treatment.

Perturbation of the microbiota can enhance colonization by pathogens as seen with increased loads of *Salmonella* in mice treated with antibiotics (57, 59-61). In this study, cefoperazone and vancomycin treatments caused different changes in the microbiota, and although the relative proportions of the *H. hepaticus* were different among the various communities, the bacterium's loads were comparable to that in the antibiotic-untreated group. These data suggest that *H. hepaticus* may have a specialized niche as its colonization is unaffected by the alterations in these resident microbiota. One study shows that *Salmonella* exhibits similar colonization abundance when introduced into mice containing two different microbial communities, one perturbed by multi-drug cocktail and another that had recovered (61). The observation suggested to investigators that since the communities were unlikely to be restricting the *Salmonella* colonization, it is plausible that host innate immune system was enforcing the resistance (61). Future ecological studies can contribute to understanding the mechanism by which *H. hepaticus* interacts with the microbiota in induction of disease.

From the histological analyses, *H. hepaticus* infection caused infiltration by inflammatory cells indicative of IBD (62). Disease occurrence was independent of community structure with the only common significant association, between disease and community change, being the presence of *H. hepaticus*. Even then, the relative abundance of this bacterium did not correlate with disease score. This finding also resonates with the outcome that severe disease occurs even at very low colonization loads of *H. hepaticus*, just as it does with relatively high colonization levels. Many factors contribute to the development of IBD (3), however these contributions may not be equal. There may be a skew towards one factor being a predominant contributor to disease development as seen in monozygotic twin studies where environment plays an important part, while genetics' role is to a lesser degree (63); or where fecal transplants or probiotics lead to the amelioration of disease demonstrating an important role of the microbiota over other factors (64, 65). Likewise, this model indicates that the mere presence of *H. hepaticus* is a strong trigger for inflammation and changes in the resident community plays a comparatively minor role, if any.

The host immune response to *H. hepaticus*, such as increase in arginase and Nos2, was consistent with the work of others which revealed increases in iNOS (66) These enzymes are important in wound healing and are associated with increases in neutrophil activity, which was observed here. Alterations in the microbial community by antibiotic treatment have been associated with altered immune responses (67, 68), and since cefoperazone treatment is known to significantly reduce bacterial load in the intestine, it was surprising to observe that final inflammation exhibited was unaffected by antibiotic treatment. We decided to further investigate whether changes in other host response were altered by the treatment regime. We discovered that after recovery from cefoperazone treatment, and *H. hepaticus* infection, TNFα increased significantly while INFγ decreased, which may be a compensatory act for the lack of INFγ response in IL-10 deficient mice (69). Also, the decrease in chitinase (chi3/4) expression in cefoperazone treated mice may be a response triggered by the decrease in microbial load after antibiotic treatment. Chitinase is highly expressed in chemically-induced IBD and has a role in enhanced bacterial adhesion (70). With lower bacterial loads, chitinase expression may also be reduced through lack of microbial stimulation. Likewise, the increases in certain mediators such as TNFα and chemokines CCL5 and CCL2 seen in the cefoperazone treated mice, compared to untreated mice infected with *H. hepaticus* may be due to changes in the microbial stimuli due to shifts in the microbiota after antibiotic treatment (71). These findings not only demonstrate that cefoperazone treatment can have long-term effects on host response, but that different expression of host mediators can result in similar disease manifestation in *H. hepaticus* infected mice.

Perhaps the most significant finding of this work was that disease can be initiated in several different microbial communities. Also, after initiation, disease progressed to exhibit similar manifestations suggesting that microbiota structure may not be a strong factor in exacerbating or reducing disease in this model. The actual mechanism whereby the *Helicobacter* interacts with the microbiota during inflammation is yet to be determined. It is possible that the resident microbes prime the host immune system so that a response can be generated to the *Helicobacter*, or cross-talk with other microbes are needed for the *H. hepaticus* to trigger a host response. The microbiota may contribute to disease in multiple ways, making studies of potential patterns in the community important in unraveling the complexities of IBD perhaps leading to novel therapies or the development of prognostic tools for early detection of this debilitating condition.

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SUPPLEMENTAL INFORMATION

Table 4.2. Clones generated from genomic DNA extracted from cecum samples.

Treatment		
Cefoperazone treatment	***H. hepaticus* infection**	**Clones/sample (N)**
No cef	No	93, 54, 93 (3)
1 day recovery from cef before infection	No	90, 75, 92 (3)
No cef	Yes	83, 92, 86 (3)
1 day recovery from cef before infection	Yes	150, 163, 76 (3)
6 wk recovery from cef before infection	Yes	93, 72, 58 (3)
6 wk recovery from cef before infection	No	
Vancomycin treatment		
No	No	95, 95, 95 (3)
Vanc	No	94, 84, 94 (3)
No	Yes	89, 89, 92, 93, 94 (5)
Vanc	Yes	94, 95, 93, 90, 92, 94 (6)
Vanc + 11 days recovery	Yes	94, 91, 92, 93, 91, 90, 96 (7)
Vanc + 11 days recovery	No	95, 93, 92 (3)

Table 4.3. Fold change in expressions of host mediators from cecum tissue. All values are normalized to GAPDH expression and compared to cefoperazone untreated, *H. hepaticus* uninfected control group. Asterisk (*) denotes statistically significant changes ($p<0.05$).

Host mediator	**1 day recovery from cef, *H. hepaticus***	**6 wk recovery from cef, *H. hepaticus***
IFNγ	1.94 ± 1.12	-2.70 ± 1.09
IL-12a	1.50 ± 1.57 *	-3.85 ± 1.53 *
TNFα	1.06 ± 1.03 *	8.84 ± 1.50 *
IL-4	1.77 ± 1.53	-1.85 ± 0.62
IL-13	-1.37 ± 1.21	-1.03 ± 1.97
IL-5	1.77 ± 1.53	-1.85 ± 0.62
TGFβ	-2.86 ± 0.68 *	4.77 ± 3.14 *
IL-6	1.77 ± 1.53	-1.85 ± 0.62
FOXp	1.77 ± 1.53	-1.85 ± 0.62
IL-17a	1.77 ± 1.53	-1.85 ± 0.62
IL-23a	-1.52 ± 1.29 *	-6.67 ± 1.01 *
CXCL2	1.35 ± 1.85	-2.44 ± 0.94
CCL5	1.59 ± 0.61 *	9.42 ± 0.56 *
CCL2	-1.19 ± 1.18 *	25.00 ± 1.04 *
NOS2	9.36 ± 2.24 *	41.5 ± 1.32 *
ARG1	6.57 ± 3.16 *	4.17 ± 6.28 *
CHI314	-7.69 ± 1.39 *	-50.00 ± 1.05 *
PTGS2	2.38 ± 2.29 *	-5.56 ± 1.25 *
INDO	6.74 ± 1.82 *	3.26 ± 3.12 *

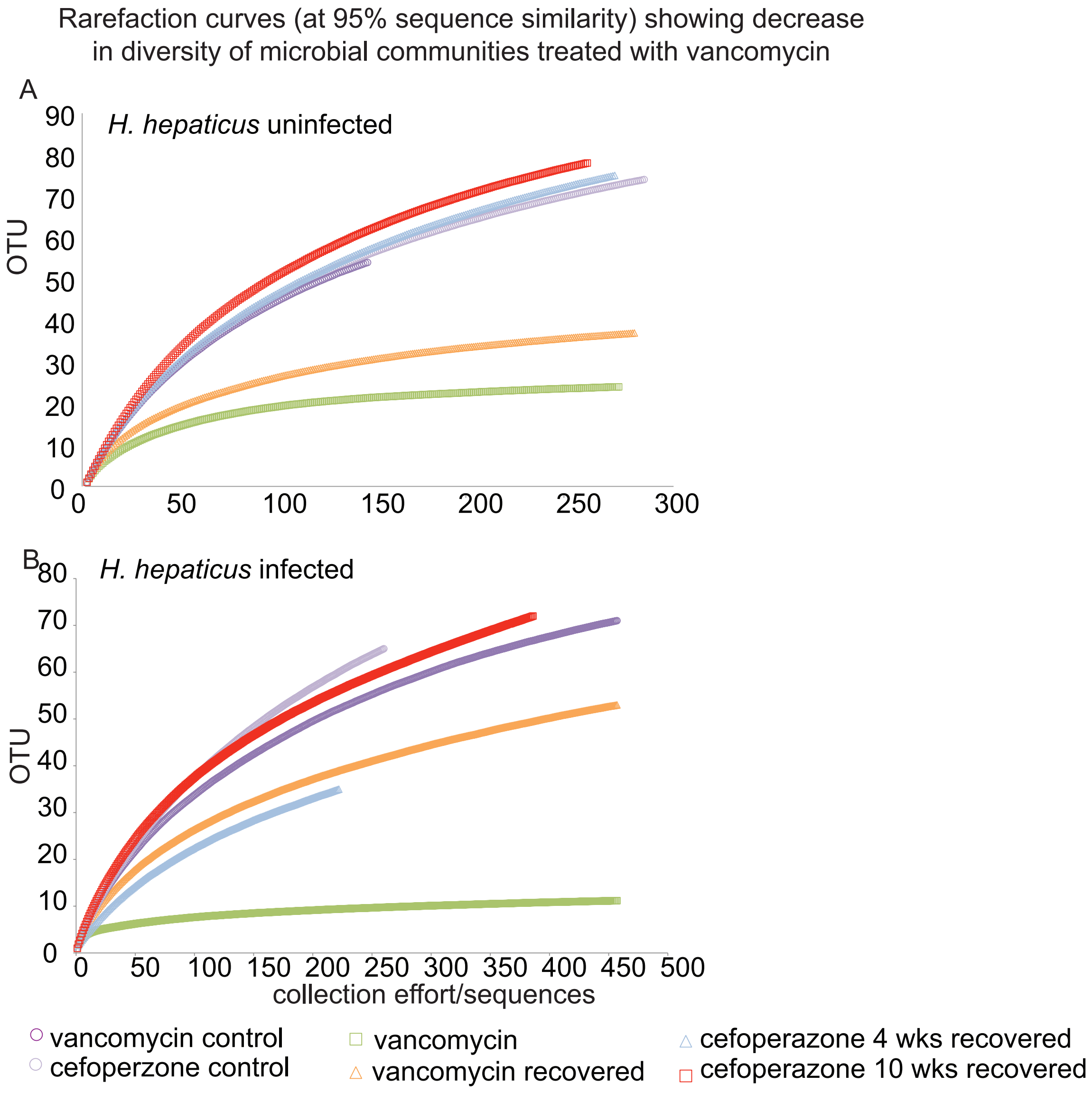

Figure 4. 8 (A) Rarefaction curves show a reduction in richness in communities treated with vancomycin, with or without the presence of *H. hepaticus*. (B) Communities recovering in the presence of *H. hepaticus* have lower community richness than communities in mice infected with *H. hepaticus* after 6 weeks off cefoperazone, or those in mice that were not administered cefoperazone. **For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this dissertation**.

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Chapter 5

Summary, synthesis and future directions

Understanding the changes that occur during onset and progression of disease can also allow for predictive diagnoses from biopsies or fecal samples and help in managing disease. Since it is difficult to investigate changes in IBD in patients due to ethical considerations and inability to predict onset of disease, we must rely on animal models. However, studies on changes in the microbiota associated with IBD in murine models are very sparse. The purpose of the work present here was to investigate the role of the microbiota at various stages of development of IBD through use of mouse models of disease.

In order to investigate the extent to which the microbiota is involved in disease development, two models were used to address two different questions: First, DSS-induced colitis is commonly used to investigate onset of IBD, however, information of microbiota changes that may occur was limited (1, 2). Previously, there was one study, using non-culture based methods, that documented an altered microbiota during DSS-induced colitis (2), however, the role of the microbiota at early stages of disease were unknown. We therefore conducted a temporal study to document changes that occur during early and late stages of disease. The current study demonstrates that community changes are not only associated with later stages of disease, but these changes also appear very early. Alterations in the microbial community occurred as soon as three days post DSS administration, just as host inflammatory responses were also observed. This altered structure persisted to 14 days post DSS administration as inflammatory changes progressed and became more severe. Microbial changes in community structure were associated with changes in host response, linking disease with shifts in microbial community structure at various stages of disease progression. These results indicate that microbiota changes are indeed associated with DSS-induced colitis, and these changes may also be associated with maintenance of inflammation.

In the second model, it is known that *H. hepaticus* alters the microbiota as it becomes a predominant member of the microbial community (3), thus, the model inherently involves changes in the microbiota that triggers disease. However, we did not know whether changes in the resident community would exacerbate or reduce disease once initiated, therefore, the role of the microbiota in this system was further probed by finding by inducing shifts in the resident community by antibiotic treatments. We found that shifts in the resident microbiota, or changes in prevalence of *H. hepaticus* in the community, did not influence severity of disease. We thus demonstrated that the role of the resident microbiota might be minor once disease is initiated in this model. Also, we investigated whether the microbiota was important in disease induction in mice in our colonies through infection of germfree mice with *H. hepaticus*. Results revealed that the cecum sections in mice monoassociated with *H. hepaticus* were similar to uninfected controls indicating that the microbiota was essential in initiating disease. Here, it appeared that the microbiota is essential for initiating disease but not in exacerbating or reducing inflammation during disease progression.

It is thus apparent that there are different roles for the microbiota in different stages of IBD, and these roles may be dependent on other factors associated with IBD such as environmental triggers as in the case of *H. hepaticus* infection. Elucidating these roles of the microbiota associated to IBD will be important in developing methods for prognosis and/or treatment of human disease.

Future Directions

From our work and that of others, we know that the microbiota can be essential in initiation of disease as seen in *H. hepaticus* triggered disease in IL-10 deficient mice, but play a lesser role in exacerbation/reduction of disease as demonstrated here. We also show alterations in the microbiota can play a role at the onset and persistence of disease, as shown by microbiota changes associated with DSS-induced colitis. However, even with these two models, several aspects by which the microbiota is involved in disease development are yet to be determined (table 5.1). The following attempts to present studies that may fill these gaps in knowledge.

Stages of IBD	**DSS-induced colitis**	***H. hepaticus* triggered IBD**
Initiation	Maybe [(4-6)]	Yes [Chapter 4, (7)]
Progression	Maybe [Chapter 3]	Unknown
Exacerbation/ Reduction	Yes [(8)]	No [Chapter 4]

Table 5.1 Roles of the microbiota in at various stages of inflammatory bowel disease.

The role of the microbiota in initiation of IBD

In *H. hepaticus* triggered IBD, there is a clear role for the microbiota in initiation of disease since germfree mice monoassociated with this bacterium do not develop inflammation. However, the role microbes play in DSS-induced colitis is unclear. In some studies, DSS administration resulted in increased inflammation in germfree mice (5), indicating a protective function of the microbiota in onset of disease, while others show more severe disease phenotype in conventional animals compared to germfree counterparts (4), indicating a detrimental role of the microbiota leading to onset of disease. Nonetheless, differences in disease development in germfree and conventional animals demonstrate a role of the microbiota in affecting DSS-induced disease. However, due to conflicting results, more studies are necessary in order to understand the role of the microbiota in this model.

Some of this conflicting results in germfree mice investigations stem from the use of different strains of mice (9), ages and even diet. To investigate the role of the microbiota in disease initiation, several strains of germfree mice, at similar ages, can be given DSS and then monitored for disease development. It will be important to provide elemental diets (minerals, vitamins, amino acids, fats and sugars), which are devoid of potential antigenic material such as bacterial components, plant material and other proteins. If inflammation does not develop in these germfree mice, then they can be conventionalized before, after and during DSS administration to observe effects of introduced microbiota on disease development. If disease manifestations were altered in any way after introduction of the microbiota, this would indicate that microbes play a role in initiating disease. Similar experimental designs have been used in testing the effects of probiotics and antibiotics in IBD (8, 10). Alternatively, inflammation may be worse in germfree mice given DSS suggesting that the microbiota has a protective role against disease induction. Testing whether live bacteria or heat-killed bacteria impart these benefits can then be done.

In conventionally raised mice, DSS-induced disease is known to be highly strain dependent (9, 11, 12). This may be related to the microbiota harbored by various strains since it is known that the microbial community differs according to many factors, including the host (13, 14). Therefore, characterizing the microbiota of various strains that are associated with different disease manifestations may reveal particular community structures linked to disease. If a particular microbial community is associated with severe inflammation, transfer of this microbiota into another strain can be done to test if the community is truly involved in IBD in this model. Others have performed similar experiments in the T-bet Rag$^{-/-}$ ulcerative colitis (TRUC) model where the microbiota of inflamed mice were transmitted to wild type mice that subsequently developed disease (15), indicating that genetic background can alter the microbiota to a community that can induce IBD.

The role of the microbiota in disease progression of IBD

There has been some evidence that the microbiota in DSS-induced colitis is important in sustaining inflammation since antibiotics have been successful in ameliorating disease (8). However, the extent to which microbes are important in disease in *H. hepaticus* triggered infection is unknown.

In *H. hepaticus* triggered disease, we know that *H. hepaticus* is important in maintaining inflammation since eradication of *H. hepaticus* ameliorates disease. Here, we also showed that changes in the community do not affect disease manifestation. However, whether the resident community is at all necessary, after disease is already induced, is unknown. Cefoperazone (0.5 mg/ml) can provide a means to test whether the microbiota is important in potentiating disease since it significantly reduces the intestinal bacteria more than 1000 fold (16). Unfortunately, this antibiotic concentration also eradicates *H. hepaticus* (see appendices 1 and 2). To circumvent this issue, heat-killed *Helicobacter* may be useful.

Stimulation from *Helicobacter* is necessary in this disease model, but whether live bacteria are needed has yet to be determined. By administering heat-killed *Helicobacter* to conventional mice can address this question. Subsequently, if disease is initiated using the heat-killed bacteria, cefoperazone can be administered to mice in conjunction with killed bacteria to investigate effects of significant reduction in microbiota during disease progression. With this experimental design, the resident microbiota can be significantly reduced while maintaining stimulation by *H. hepaticus*. Amelioration of disease by cefoperazone treatment would indicate that the microbiota is essential in reducing or maintaining disease in this model. If, however, disease progresses unaltered during cefoperazone treatment, it may be because the microbiota is not essential for disease perpetuation once it has been induced. Testing whether the microbiota is essential in disease perpetuation would be challenging since removing the microbiota from a conventional animal is impossible. However, by performing gnotobiotic experiments and finding out whether a simple community is sufficient to trigger disease in this model, other experiments may be possible (see upcoming section). For instance, if disease can be initiated in gnotobiotic communities consisting of bacteria that are transient in the intestinal community such as those present in probiotic mixtures (17), we can investigate whether *H. hepaticus* triggered disease perpetuates once the transient microbes have been cleared.

Testing the importance of microbiota complexity in IBD

It is not known whether a complex microbial community is necessary to induce disease, or whether a simple community is sufficient. Murine association with the Altered Schaedler Flora (ASF) can be used as a simple community to investigate changes in the microbiota that may accompany disease in DSS-induced colitis and *H. hepaticus* triggered disease in IL-10 deficient mice. Development of disease in mice harboring simple communities would indicate that complex communities are unnecessary; a single microbe may even be enough for inflammation to occur.

Bacteroides thetaiotaomicron is part of the normal human microbiota (18), and thought to stimulate intestinal development in germfree mice so it resembles that of conventional animals (6). Due to its non-pathogenic nature, as well as its benefits in attenuating some characteristics of germfree animals, including vascularization, architecture and immune modulation (6, 19), this bacterium can be used to infect germfree mice before DSS treatment, or *H. hepaticus* infection, to test whether a simple system of bacterial-host interaction is enough to trigger disease comparable to that seen in conventionally raised mice. If these monoassociated mice develop disease that is similar to that observed in their conventionalized counterparts in each IBD model system, this would imply that simple microbial interaction or even luminal antigenic stimulation from killed bacteria, or bacterial components, might be enough to cause inflammation in the DSS-induced model, as observed in other systems (20). The next step would involve inoculation of mice with heat-killed bacteria and observing whether disease occurs in these models.

DSS-induced colitis: Do alterations in the microbiota precede immune changes?

To attempt to tease apart changes induced by DSS administration, a study can be conducted where frequent samples are collected between day zero and day three of DSS administration. Recent evidence indicates that intimate interactions between the microbiota and host occur within the first 24 hours of DSS administration (21). Bacteria were present in the normally impenetrable mucosa layer indicating DSS increases permeability of the mucin matrix allowing close association with the host within a day, before host inflammatory responses were observed (21). By investigating whether changes in the microbial community structure occurred within this time would be helpful in tracing the sequence of events involving host response and microbiota changes after exposure to DSS.

If changes in host response occur before alteration in the microbiota, it is likely that DSS acts directly on the host in the presence of luminal bacteria and community shifts are not involved in onset of disease. Subsequent changes in the microbial community may simply be a reaction to host’s inflammatory response (22), as it known that components of the immune system can alter the microbiota. This is exemplified by increases in segmented filamentous bacteria in mice with deficient expression of intestinal IgA (23). Subsequent reconstitution of IgA in intestinal tissue resulted in a return to a community resembling that in mice expressing IgA (23).

Alternatively, if changes in the microbial community structure occur before changes in the host immune system are detected, this may be an indication that the altered microbiota plays a role in disease causation. Effects of different microbial structures on development of disease were tested by antibiotic treatment (8), but using gnotobiotic models would also be useful in understanding the role of certain members of the community, as discussed above.

However, due to the almost simultaneous changes in the microbiota and host immune response, teasing apart the sequence of event may be challenging. Germfree and gnotobiotic experiments proposed at the beginning of this section may be more prudent approaches to investigating the role of the microbiota in onset of disease.

Conclusion

Over the past two decades, studies on the microbiota and its influence on disease development has grown exponentially (24). Due to the complexities involved in understanding both the role of the hosts' immune response and changes in diversity and function of the microbiota, studies that lay ahead will be challenging. Nonetheless, development of new tools for investigation of these changes, and continued vigorous research into the field, provide a promising future for patients affected with diseases such as IBD.

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APPENDICES

Appendix A

The effects of cefoperazone on *Helicobacter hepaticus* colonization.

Purpose

Cefoperazone (0.5 mg/ml) was previously used to alter microbial community structure in the intestine (1), and is commonly used in selective media to screen for *H. hepaticus* growth in vitro (2). We therefore initially used this antibiotic to alter the microbial community in mice infected with *H. hepaticus* in order to study effects of changes in the community during disease. Surprisingly however, cefoperazone treatment eradicated *H. hepaticus* in vivo. After reducing the concentration administered to mice by ten fold, there seemed to be negligible effects on total bacterial and *H. hepaticus* loads in vivo. We therefore suspected that bacterial colonization maybe dependent on concentration of cefoperazone administered. We thus tested this hypothesis that changes in the microbiota are associated with cefoperazone concentration administered. Here, effects of various concentrations of cefoperazone on total bacterial load and *H. hepaticus* loads are reported.

Methods and materials

To investigate the effects of cefoperazone treatment on *H. hepaticus* in vivo eleven groups of mice were infected with *H. hepaticus* for ten days before antibiotic was administered. There were three mice in each group. Dosage ranged from 0.5 mg/ml to 0.05 mg/ml. Previously, 0.05 mg/ml was seen to have no effect of either *H. hepaticus* colonization or total bacterial load. Mice were sacrificed seven days after the start of cefoperazone administration as shown in figure A1.1.

Quantitative PCR was performed and clone libraries constructed from mice from each experimental group as described in previous chapters 3 and 4.

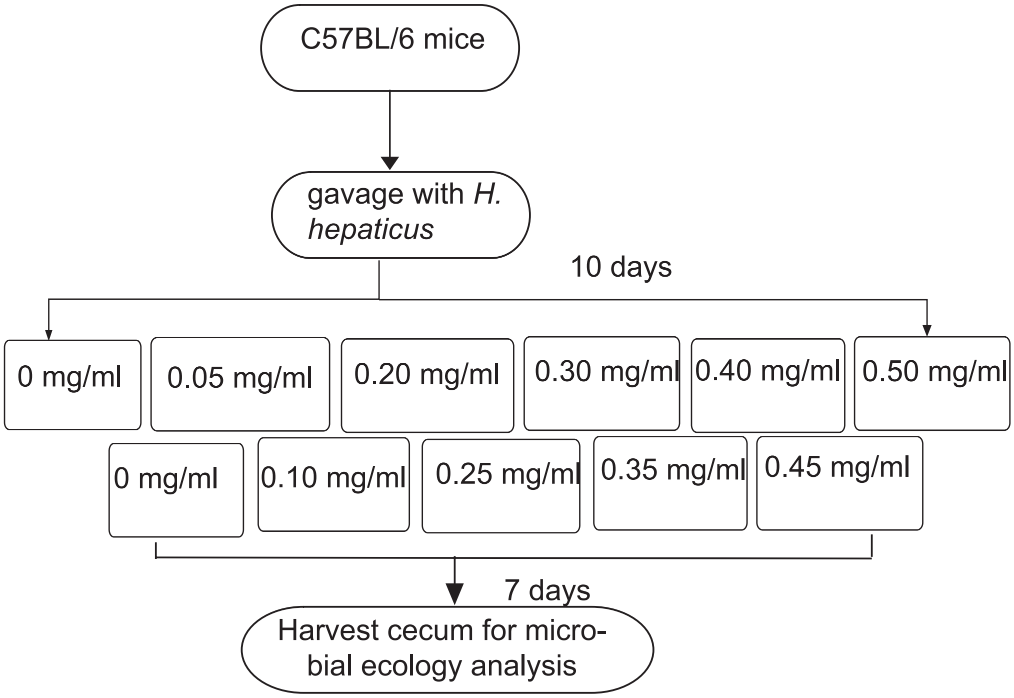

Figure A1.1 Experimental design depicting administration of various cefoperazone concentrations to *H. hepaticus* infected mice.

Results and conclusions

Quantitative data shows that 0.5 mg/ml to 0.30 mg/ml significantly reduced total bacterial loads over 1000 fold and eradicated *H. hepaticus*. At about 0.25 mg/ml, the antibiotic did not affect bacterial load or presence of *H. hepaticus,* indicating a threshold for cefoperazone activity against intestinal bacteria.

Eradication of *H. hepaticus* was confirmed by allowing the intestinal communities to recover after cefoperazone administration. It was found that *H. hepaticus* was not detectable after four weeks cessation of antibiotic administration.

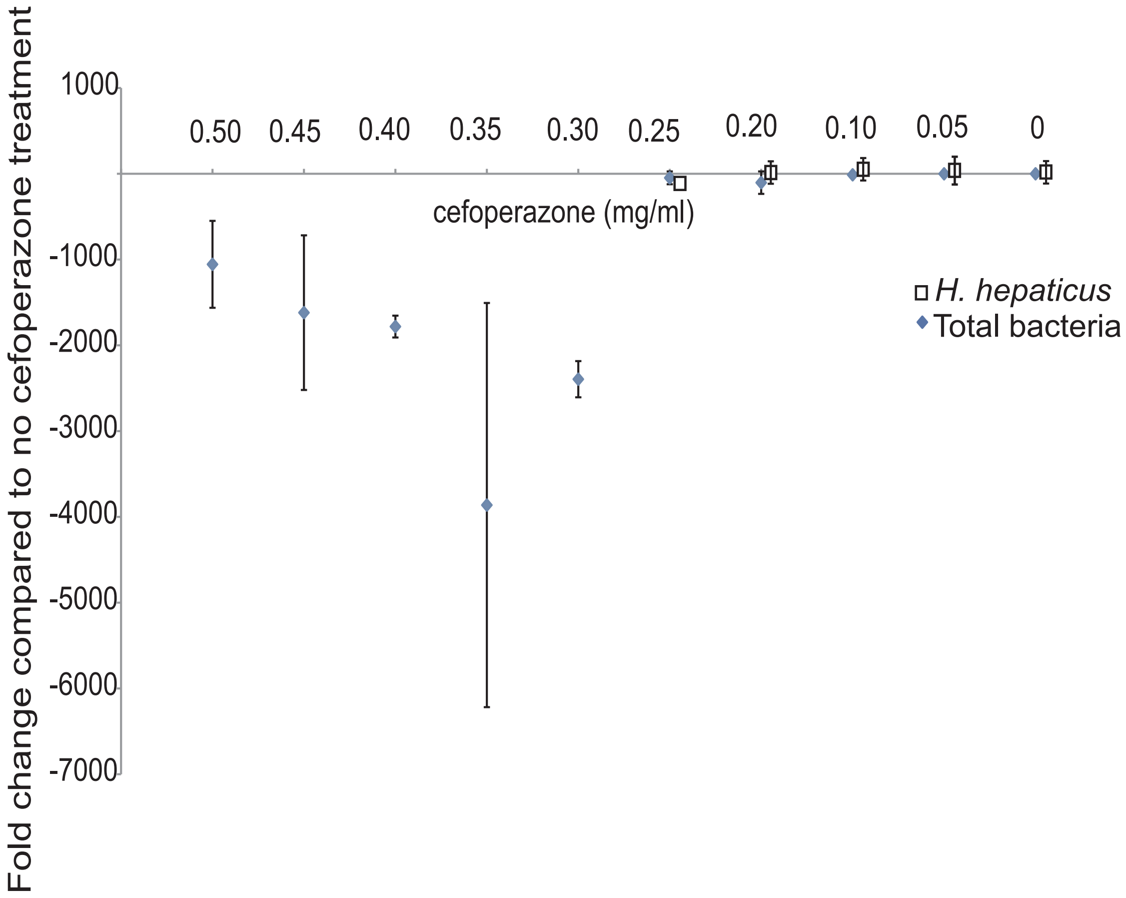

Figure A1.2 Cefoperazone concentrations above 0.25 mg/ml cause significant decreases in total bacterial loads and eradicates *H. hepaticus*.

To investigate these affects more closely, clone libraries were constructed. Clone libraries also show presence of *H. hepaticus* in communities in mice treated with up to 0.25 mg/ml cefoperazone. The community in mice treated with 0.05 mg/ml cefoperazone appeared to be more similar to communities in untreated mice (figure A1.2 A). Furthermore, although bacterial load in mice treated with 0.05 mg/ml was comparable to that in mice treated with 0.10 mg/ml cefoperazone, there was a decrease in richness in the latter community (figure A1.2 B), indicating that this concentration affects colonization of some members of the community more than others.

With 0.30 mg/ml and more, decrease in bacterial loads made it very difficult to construct clone libraries due to poor primary amplification of the bacterial 16S rRNA gene. This indicates that these higher concentrations affect many members of the intestinal community than 0.25 mg/ml cefoperazone. Perhaps the machinery designed to remove cefoperazone from the bacterial cells are overwhelmed at these concentrations causing the bacteria to be susceptible to the antibiotic.

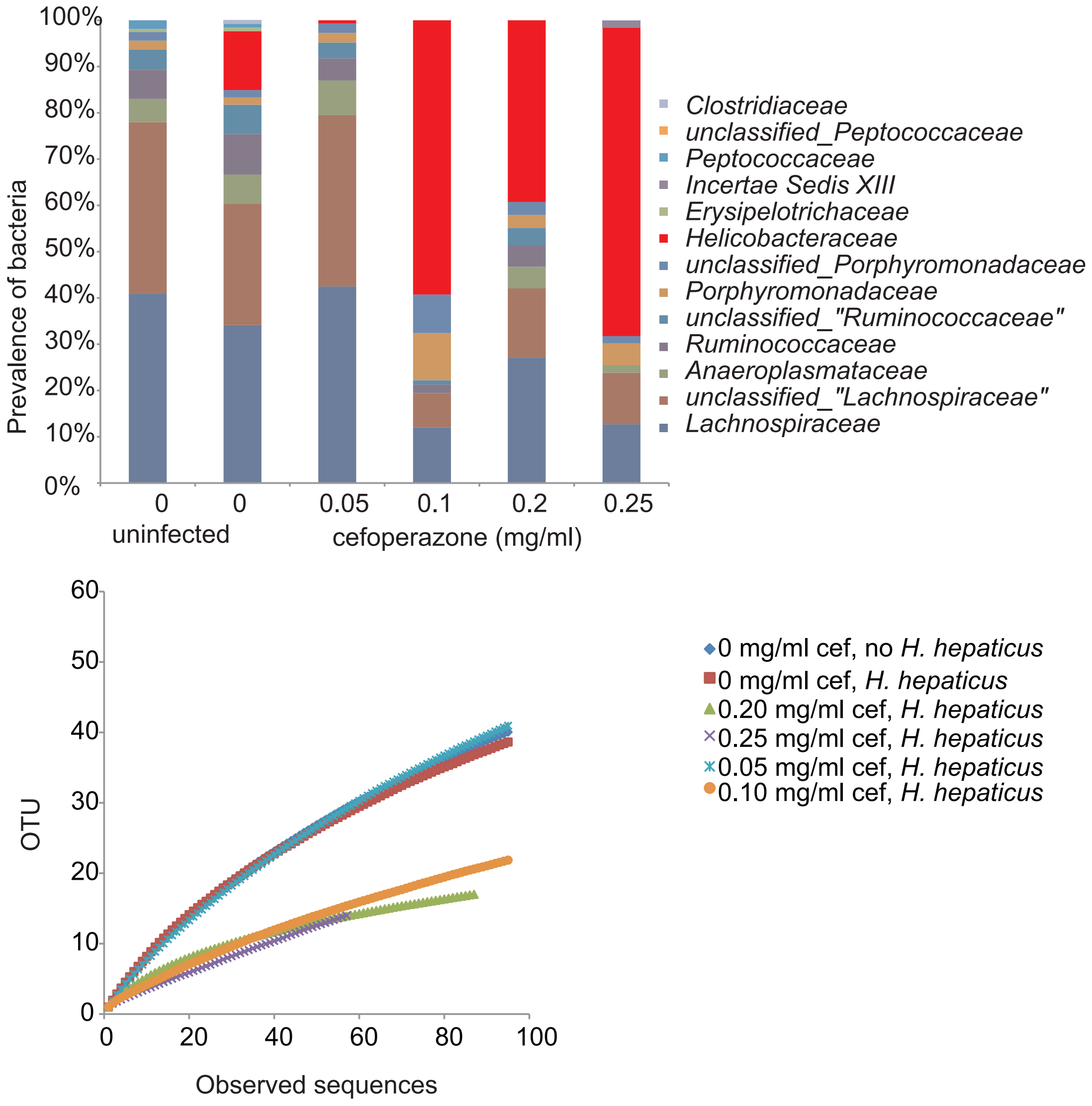

Figure A1.3. (A) *H. hepaticus* were present in the microbiota of communities treated with cefoperazone concentrations below 0.25 mg/ml. Some communities showed significant changes due to *H. hepaticus* infection and cefoperazone treatment. (B) There was also a reduction in intestinal community richness when concentrations of 0.20 mg/ml cefoperazone and above were administered to mice. **For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this dissertation**.

At 0.03 mg/ml cefoperazone, *H. hepaticus* is absent from microbial communities. Below 0.25 mg/ml, however, *H. hepaticus* is present, and together with the finding that total bacterial load is also unaffected at this concentration, it can be postulated that cefoperazone is not active at lower concentrations. This may be due to break down of the compound at low concentrations during in vivo passage since typical signs of cefoperazone treatment, such as dark cecal contents and distended cecum, were absent from these mice (see photographs in appendix B). To test this, cefoperazone levels can be measures from the cecal contents by mass spectrometry.

Another possibility is that at 0.30 mg/ml, *H. hepaticus* is eradicated from the intestinal community suggesting that the bacterium is susceptible to this concentration of cefoperazone. *H. hepaticus* is known to have efflux pumps that functions to remove antibiotics from the bacterial cell (3). It is easy to imagine that by increasing cefoperazone levels, that the pump may not efficiently remove the molecules before they act.

To test the hypothesis that an efflux pump may be responsible for susceptibility of *H. hepaticus* to high concentrations of cefoperazone, mutants with increased pumping activity can be used to test whether they are resistant to higher concentrations of cefoperazone treatment. Alternatively, efflux pump inhibitors may be used, in a dose-dependent manner, to investigate whether there are effects on the ability of *H. hepaticus* to grow at concentrations below 0.25 mg/ml (4). If pumps are indeed important in conferring resistance to cefoperazone in this bacterium, increasing levels of the inhibitor increase susceptibility.

Dosages of 0.10 mg/ml to 0.25 mg/ml were associated with higher relative abundance of *H. hepaticus*. More experiments to investigate how *H. hepaticus* colonizes a community is needed to understand the low relative abundance of the bacterium in communities associated with 0.05 mg/ml cefoperazone administration compared to those administered 0.10, 0.20 and 0.25 mg/ml cefoperazone. It would be expected that *H. hepaticus* would be more prevalent at lower antibiotic concentrations, with communities resembling those of either communities from mice not administered cefoperazone; or more like those associated with 0.10 mg/ml. Perhaps at 0.05 mg/ml cefoperazone treatment, resident microbes shifts so certain members of particular families increase in abundance. This may explain lowered relative abundances of *H. hepaticus* while total loads are unchanged, as well as richness (Shannon index=3.27) of the microbiota similar to that in cefoperazone untreated mice (Shannon index= 3.37), compared to the low richness of antibiotic treated mice (Shannon index= 1.59 with 0.10 mg/ml cefoperazone, 1.52 with 0.20 mg/ml cefoperazone and 1.51 with 0.25 mg/ml cefoperazone) at 3% cutoff.

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Appendix B

The effects of cefoperazone administration in *H. hepaticus* triggered disease in IL-10 defiecient mice.

Purpose

Others have shown effects of antibiotic treatment on *H. hepaticus* infected mice and reported that some are effective in ridding colonies of this bacterium (1). We showed that cefoperazone, an antibiotic normally administered through intravenously in humans, was found to eradicate *H. hepaticus* in wild type mice (appendix A). However, effect of cefoperazone treatment in *H. hepaticus* triggered disease was unknown. We therefore tested the hypothesis that cefoperazone administration was attenuate disease in IL-10 deficient mice infected with *H. hepaticus*.

Methods and materials

Four groups of mice were used in this experiment as indicated in figure A2.1. After euthanasia, the ceca of the mice were removed and used for genomic DNA extraction and histologic preparation (see chapters 3 and 4 of this thesis).

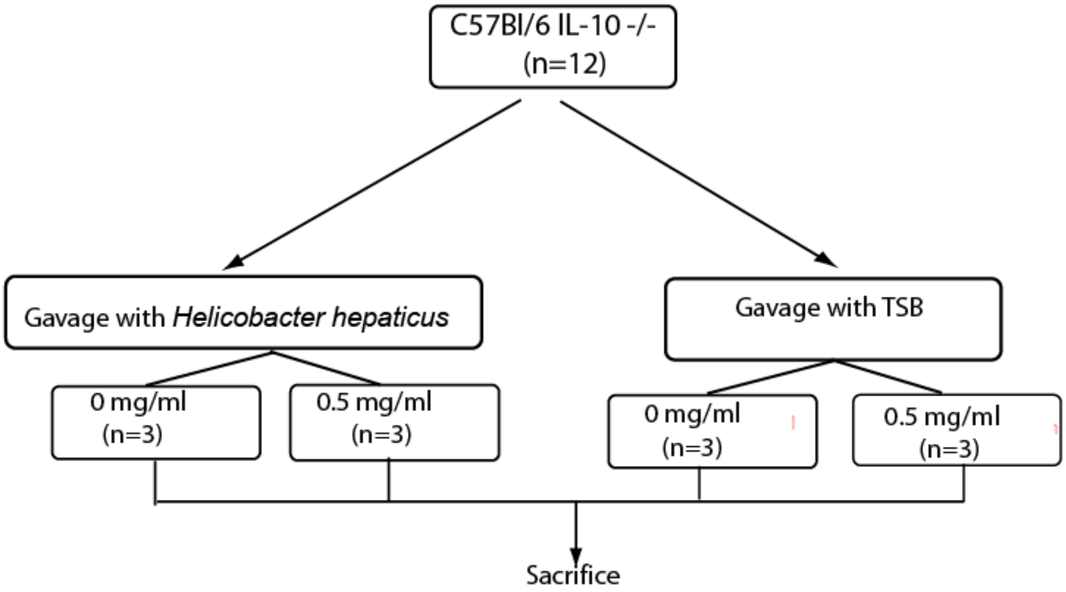

Figure A2.1 Experimental design showing *H. hepaticus* infection prior to cefoperazone treatment.

Results and Discussion

Photographs were taken to document changes that occur with different treatments (figure A2.2). Cefoperazone treatment resulted in distension of the cecum with dark, fluid luminal content, making tissue fragile. This indicates that cefoperazone is associated with alterations in the host, which may be related to changes in the microbiota. Changes in host response mediators were observed after cefoperazone treatment and documented in experiments described in chapter 4 of this thesis. Some of these changes in during immune regulation may affect permeability of the epithelial wall, which may be associated fluid luminal content and distension (3).

H. hepaticus infection was associated with thickening of the cecal tissue making it more rigid, with a reduction in luminal content. This is a common sign of disease as scarring occurs during IBD and was also observed in experiments described in chapter 4. Cefoperazone administration, following *H. hepaticus* infection, appeared similar to tissue from cefoperzone treated, *H. hepaticus* uninfected mice. This may be due to effects of cefoperazone-induced alterations in microbiota. Whether cefoperazone alone can induce host immune changes is yet to be determined. This can be investigated by administering cefoperazone to germfree mice.

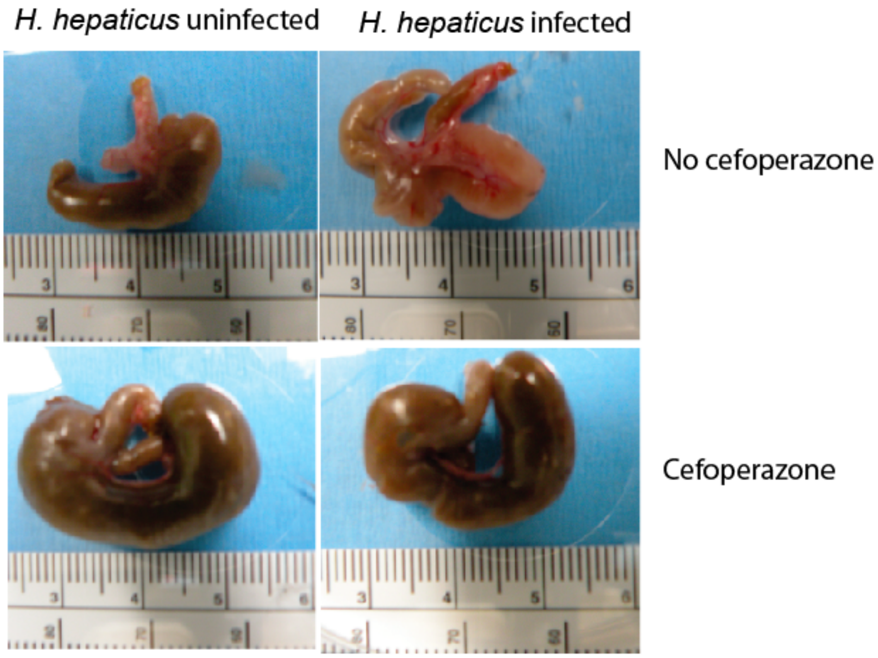

Figure A2.2 Photographs of ceca removed from mice from four treatment groups. (Top left) Cecum is normal with luminal content of paste-like consistency, unlike cecum from mice infected with *H. hepaticus* that have thick tissue (top right). With cefoperazone administration (bottom panels), the cecum become distended and tissue fragile, filled with dark luminal fluid. **For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this dissertation**.

After cefoperazone administration, *H. hepaticus* infected mice appeared to have less inflammation. This corresponded to a reduction in total bacterial load and eradication of *H. hepaticus* (figure A1.3). This finding is supported by reports that eradication of *H. hepaticus* can ameliorate disease in rodents (1). More animals would have to be used in this experiment to attain more statistical power to dram convincing conclusions.

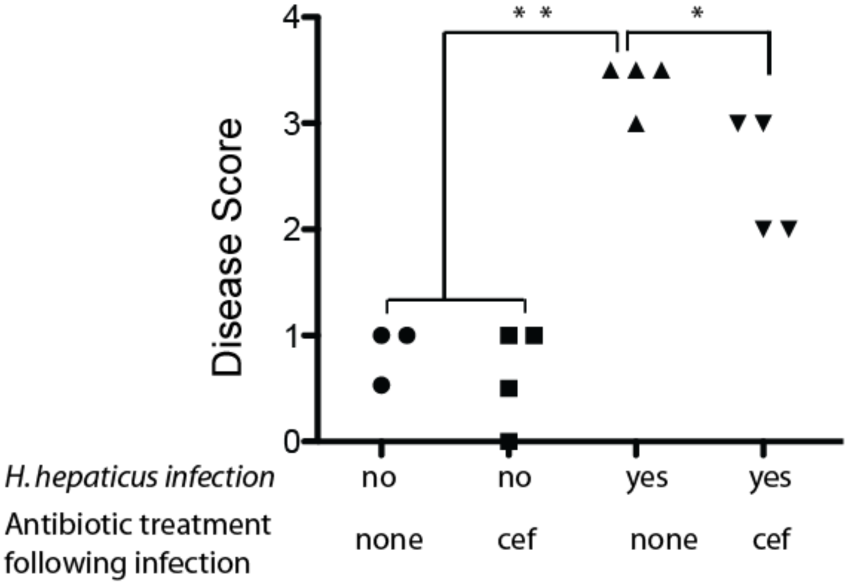

Figure A2.3 show significant inflammation in mice infected with *H. hepaticus*. After administration of cefoperazone, inflammation was attenuated.

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