Impact of antibiotic use, alternative therapies and inflammation on the intestinal microbiota

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Impact of antibiotic use, alternative therapies and inflammation on the intestinal microbiota

by

Anne-Marie Caroline Overstreet

A dissertation submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Major: Microbiology

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Iowa State University
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TABLE OF CONTENTS

Acknowledgements  iv

Abstract  vii

CHAPTER 1. The Role of the Microbiota in Gastrointestinal Health and Disease 1
   An Introduction to the Microbiota 2
      Initial Colonization of the Gastrointestinal Tract 3
      Impact of the Method of Delivery on Colonization 3
      Impact of Feeding Method on Colonization 5
      Microbial Changes that Occur After Weaning 6
      The Microbiota and Gastrointestinal Homeostasis 8
      Effect of Bacterial Colonization on Immune Development 11
      Effect of Bacterial Colonization on Other Aspects of the Body 15
      Bacterial Production of Short Chain Fatty Acids 17
   Introduction to Inflammatory Bowel Disease 20
      Genetic Factors Affecting IBD 22
         Nod2 23
         ATG16L1 24
      The Immune Response and IBD 26
      Environmental Factors Affecting IBD 30
         Antibiotic Usage 31
         MAP 33
         Adherent-invasive Escherichia coli 34
      The Microbiota and IBD 36
   Canine IBD 40
      Clinical and diagnostic features 42
      Therapeutic approach 44
      Future directions in canine IBD 46
   Treatment for Human IBD 47
      Antibiotics 47
      Corticosteroids 49
      Immunosuppressive Therapy 50
      5-Aminosalicyclic Acid (5-ASA) 50
      Anti-TNF monoclonal antibodies 51
      Complementary and alternative therapies 54
      Probiotics 55
      Prebiotics 60
   Mouse Models of IBD 62
      Chemically-induced models 62
         Dextran sodium sulfate (DSS) 62
         TNBS (2,4,6-trinitrobenzene sulfonic acid) 64
         Oxazolone 65
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10 Things I Learned in Graduate School a.k.a. Amanda-isms

1. There is always someone smarter, faster, and prettier than you. On the flip side there is always someone dumber, slower, and uglier too.

2. Life isn’t fair.

3. You have to look out for yourself.

4. Take initiative.


6. Sometimes you just have to be a jerk.

7. If you never ask, the answer is always no.

8. The bumble bee story.

9. Don’t interrupt people.

10. Most things happen as they should.
Abstract

The gut microbiota is a complex community that consists of $10^{11}$-10$^{12}$ organisms per gram of colonic contents and over 150-fold more genes than the human genome. This community has evolved with its eukaryotic host to form a mutualistic relationship. It has been known that the microbiota is involved in the production of vitamins, the breakdown of dietary substances and the maturation of the immune system. Recent work has expanded the role of the microbiota in human health. The microbiota has been implicated in the regulation of fat storage and diseases such as ulcers, diabetes, obesity, cardiovascular disease, and inflammatory bowel disease (IBD).

The work in this dissertation focuses on the role of the microbiota in IBD, a disease of unknown etiology. Several factors contribute to the development of disease, including environmental factors, host genetics, the immune response, and the gut microbiota. An environmental factor that has become increasingly recognized as a critical component in shaping the microbiota is the use of antibiotics and the potential link to IBD. The research summarized in chapter 2 reveals the effects that antibiotics have on a limited microbiota, the altered Schaedler flora (ASF). Use of a limited microbiota affords the ability to monitor effects of antibiotics on the entire community that would be missed when using a conventional microbiota. Following treatment with ampicillin, mice harboring the ASF developed more severe disease in response to a colitic insult than did mice bearing a conventional microbiota, indicating a central role for the microbiota in reducing disease severity. Those conventional mice that received ampicillin prior to colitic insult revealed that the ASF
in a more complex community was less affected by the administration of antibiotics. However, conventionally-reared mice harboring a complex microbiota did exhibit low grade inflammation following colitic insult and eventually developed a physiological profile consistent with the onset of metabolic syndrome.

The research summarized in chapter 3, evaluated the immunomodulatory and anti-bacterial properties of ethanolic extracts of *Prunella vulgaris* and *Hypericum gentianoides*, which have the potential to be used as complementary and alternative treatments for gastrointestinal diseases including IBD. There is no cure for ulcerative colitis or Crohn’s disease, and many of the treatments have severe side effects. This has caused some patients to seek alternative therapies to manage their disease. In chapter 3, herbal therapies were administered prophylactically to mice harboring the ASF to assess their ability to prevent or attenuate DSS-induced colitis. The results revealed that one of the herbal therapies, *P. vulgaris*, was actually pro-inflammatory in the ASF mice as evidenced by the increased production of pro-inflammatory cytokines/chemokines. The other botanical extract, *H. gentianoides*, seemed to exert both anti-inflammatory and anti-bacterial effects in this murine model of colitis indicating a possible role for *H. gentianoides* as a therapy for IBD.

One of the consistent observations noted in IBD patients is that their microbiota is abnormal (i.e., dysbiotic) compared to healthy controls. Whether this change in the microbiota occurs post-inflammation, or if dysbiosis initiates the inflammation, is still up for debate. Summarized in chapter 4 are the results of a temporal study that chronologically evaluated changes in the microbiota and the host’s inflammatory response over the course of disease using a spontaneous
model of murine colitis. The results revealed that in this model, the mucosal inflammation occurs early, before the changes in the microbiota. This indicates that the inflammation is what drives the microbial changes seen in the diseased mice. In summary, studies described herein indicate that the microbiota plays a large role in the inflammatory processes of murine models of colitis.
CHAPTER 1
The Role of the Microbiota in Gastrointestinal Health and Disease

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1. An Introduction to the Microbiota

Antony van Leeuwenhoek’s observation of “animalcules” in the tarter of teeth in 1683 marked the beginning of humans trying to understand their relationship with microbes. Now commonly referred to as “bacteria” or the “microbiota” we share our inner and outer space with these single-celled organisms. They are present all around us—in the oceans, soil, leaves, and even air [1]. One of the microbial niches that has recently garnered much attention is the human body. In this niche, we share the table with $10^{14}$ microbial cells, which outnumber our eukaryotic cells ten to one [1, 2]. These organisms colonize most body sites, including the skin, oral cavity, gastrointestinal, urogenital and respiratory tracts. Additionally, there is 150 times more genetic material associated with our resident bacteria when compared to our own DNA and comprises what is known as our body’s “second genome,” a concept recently reviewed by Zhu et al. [3]. In an effort to understand the role that these organisms have in human development, health and disease, the National Institutes of Health launched the “Human Microbiome Project” in 2007 in an effort to elucidate this host-microbe interaction [4-6].

The role of bacteria is most obvious in the gastrointestinal tract (GIT). Bacteria in this niche have the ability to break down substances that otherwise could not be digested by GIT cells; they also produce metabolites needed by the body such as vitamin K (menaquinones), folate, B12, and riboflavin [7-12]. The GIT has a surface area the size of a tennis court and is home to $10^{11}$-$10^{12}$ organisms per gram of colonic content [1, 13]. The predominant bacterial phyla present in the GIT include the Firmicutes and Bacteroidetes with low levels of Proteobacteria, Actinobacteria,
Fusobacteria, and Verrucomicrobia [14-16]. It has been noted that members of the predominant factions (the Firmicutes and Bacteroidetes) belong to only three groups, *Bacteroides, Clostridium coccoides* (cluster XIVa) and *Clostridium leptum* (cluster IV) [15, 17, 18]. There are also Archaea present in the GIT and have been found to belong to one phylotype, *Methanobrevibacter smithii* [14, 19].

1. a. Initial Colonization of the Gastrointestinal Tract

Infants are born sterile and establishment of the GI microbiota begins shortly thereafter. Studies in mice have revealed that the first organisms to colonize include lactobacilli, flavobacteria and Group N streptococci [20]. These three groups were found in the stomach, and small and large intestine. When the mice were 12 days of age, the flavobacteria disappeared from all three sites. Concurrently, there was a rapid increase in the presence of enterococci and slow lactose fermenting coliform bacilli reaching $10^9$ bacteria per gram of tissue in the large intestine. This spike was transient however, and as the mice aged, a shift toward a more strict anaerobic population occurred in the large intestine. This ordered colonization occurs because aerotolerant organisms are able to colonize the GIT early in the presence of oxygen. Through their metabolism, they reduce the redox potential and allow for the later colonization by and replication of strict anaerobes [21].

1. b. Impact of the Method of Delivery on Colonization

In humans, the method of delivery impacts the initial microbiota of the infant [22-24]. In a study of Venezuelan women, vaginal delivery was associated with initial
colonization by *Lactobacillus*, *Prevotella*, *Atopobium*, and *Sneathia* spp.; in contrast, Cesarean delivery was associated with colonization of *Staphylococcus*, *Propionibacterium*, and *Corynebacterium* spp. (organisms typically found on skin). It was also noted that the initial bacteria present in infants born vaginally were vertically transferred from the mother. This was not the case for those born via Cesarean. A Finnish study analyzed infant fecal samples collected between three days of age and six months of age to evaluate temporal colonization [23]. The results revealed that at three days of age, all but one infant, regardless of the delivery method, was colonized with aerobic bacteria. However, a higher percentage of vaginally delivered infants were colonized with *Bifidobacterium*-like (BLB) and *Lactobacillus*-like (LBB) bacteria as compared to Cesarean delivered infants. These differential colonization patterns were corrected by day ten for LBB and by day 30 for BLB. Additional characterization of the fecal microbiota revealed that at one month of age, infants born via Cesarean had a significant increase in *Clostridium perfringens* (25 % to 56 %); an increase not seen in vaginally delivered infants. This is an important observation, as *C. perfringens* has been thought to play a role in sudden infant death syndrome (SIDS) [25]. Also of note, significant differences in the numbers of *Bacteroides fragilis* were observed between the two methods of delivery [23]. Specifically, this bacterium was present at statistically higher numbers in vaginally delivered infants as compared to Cesarean delivered infants at all time points throughout the six months of the study.

These differences in initial colonizers have been associated with adverse effects, as infants born via Cesarean are more susceptible to skin infections with
methicillin resistant *Staphylococcus aureus* (MRSA). A 2004 report indicated that 82% of infant MRSA cases in Chicago and 64% in Los Angeles were delivered via Cesarean, all of which involved healthy, full-term infants [26]. An additional prospective cohort study indicated a link between Cesarean delivery and *Clostridium difficile* infection, which was further associated with the development of wheeze, asthma, eczema, and food allergies throughout the first seven years of life [27]. Consistent with these findings, a Norwegian study documented an association between asthma and Cesarean delivered children [28]. Although the epidemiological connections among Cesarean delivery, the composition of the microbiota, and disease are still being studied, strong evidence exists that early colonization of the intestines with aerobic skin microbes is associated with health risks.

1. c. Impact of Feeding Method on Colonization

Another factor affecting early colonization of the human gut is determined by feeding method [29]. In one study of vaginally delivered infants, the fecal microbiota of breast-fed compared to formula-fed infants within the first month of life was analyzed using fluorescence *in situ* hybridization (FISH) to evaluate 11 separate groups of organisms [29]. *Bifidobacterium* spp. were the most prominent organisms found to colonize infants fed breast milk, comprising 69% of the bacterial population, with the next most prevalent group being the *Bacteroides* and *Prevotella* spp. making up 12%. With respect to the fecal microbiota of formula-fed infants, the microbiota was actually more diverse than that found in the breast-fed babies. Formula-fed infants had *Bifidobacterium* spp. as the most predominant group;
however, they only comprised 32% of the bacterial population while *Bacteroides* and *Prevotella* spp. comprised 29% of the composition. Additionally, *Atopobium* spp. were found to account for 8% of the microbiota in formula-fed infants, but comprised only 1% of the community in the breast-fed infants.

### 1. d. Microbial Changes that Occur After Weaning

The next major shift in the microbial population occurs during weaning. In a study consisting of infants from five European countries, the composition of the fecal microbiota at four weeks post-weaning was analyzed via FISH using 10 different probes [30]. When considering the results from all of the infants, it was noted that the microbial communities consisted primarily of *Bifidobacterium* spp. (37%), *C. coccoides* (14%) and *Bacteroides* spp. (14%). When samples were obtained from infants that were separated by geographic location, differences among the groups were noticeable. Infants from Spain had significantly greater populations of *Lactobacillus* spp. and fewer *Bifidobacterium* spp. as compared to infants from the UK, Sweden, Germany, and Italy. The Spanish fecal samples also had significantly greater presence of *Bacteroides* spp. and *C. leptum* compared to those from the UK, Sweden, or Italy. The numbers of *C. leptum* was highest in samples obtained from German infants. The microbiota of infants from Spain also had significantly greater numbers of enterobacteria when compared to those from the UK, Sweden, or Germany, and higher numbers of *Streptococcus* spp. compared to those from Germany. The infants with the highest numbers of *Bifidobacterium* spp. were from Sweden and the UK. Further separation of the data revealed that breast-fed infants
had an increased percentage of *Bifidobacterium* spp. while *Bacteroides* spp. and *C. coccoides* were reduced post-weaning. Additionally, delivery method also influenced the microbiota composition, with vaginally delivered infants having higher numbers of *Bacteroides* present.

This study also reported results from infants who provided samples before (at six weeks of age) and four weeks after weaning [30]. Switching to solid food during this time caused a significant reduction in the presence of bifidobacteria, enterobacteria and *C. difficile/C. perfringens* proportions. These reductions were accompanied by significant increases in both *C. leptum* and *C. coccoides* clusters. Again, location also played a significant role in the microbial differences at weaning. Spanish infants had the greatest increase in both *C. coccoides* and *C. leptum* post-weaning compared to the other four countries, while enterobacteria numbers were significantly decreased in the Spanish samples compared to those from the other countries. The samples from Spanish infants also had significant increases in *Atopobium* spp. compared to those from Sweden and Germany and *Streptococcus* spp. compared to Germany and Italy. The authors also reported significant decreases post-weaning in the enterobacteria present in the Italian infant samples as compared to samples from Sweden and the UK. As mentioned previously, feeding and delivery method alter the composition of the microbiota at birth; this is also true after weaning. Formula-fed infants had higher proportions of *C. leptum* in their feces compared to breast-fed infants post-weaning. Infants born via Cesarean possessed increased levels of *Bacteroides* spp. after weaning, whereas there was no change in the abundance of this group in vaginally delivered infants. Additionally,
*Atopobium* spp. decreased in vaginally delivered children but increased in Cesarean delivered children post-weaning [30].

Studies describing development or maturation of the human microbiota reveal that as an individual ages, the microbiota shifts from predominantly facultative anaerobes to strict anaerobes, just as previously observed in rodents [20]. Notably, variations in initial colonization associated with different delivery and feeding methods seem to profoundly impact the microbial community post-weaning. Additionally, geographic location plays an important role in the initial colonization of the GIT. In spite of all these variables, several themes for colonization can be described. Initially, *Bifidobacterium* spp. are the primary colonizers of the infant GIT along with other species such as *Bacteroides, Lactobacillus, Prevotella,* and *Atopobium.* As the infant matures, the oxygen presence in the gut is reduced and the infant begins eating solid food, which promotes a transition towards the growth and maintenance of the anaerobes found in the *C. coccoides* and *C. leptum* clusters.

1. e. The Microbiota and Gastrointestinal Homeostasis

The commensal microbiota has a tremendous influence on the development and functional capabilities of the GIT of its host. Numerous studies have documented the effects that the microbiota has on GIT development in mice. Specifically, the morphology of the intestines differs significantly in animals devoid of microbes (i.e., germfree) compared to conventionally-reared (CONVR) mice [31]. The mucus layer is thinner and epithelial cells have a slower rate of turnover compared to those from conventional animals, primarily because of extended time in
the S and G\textsubscript{1} phases of the cell cycle [32]. This can be corrected by bacterial colonization. In Figure 1, panel A shows intestinal tissue from germfree (GF) mice and panel B shows tissue from a mouse colonized with \textit{Brachyspira hyodysenteriae}. Colonization with \textit{B. hyodysenteriae} elicits the production of goblet cells and increases the heights of the mucosa, resembling what is seen in CONVR mice.

![Figure 1. Photomicrographs of cecal tissue from a germfree mouse (Panel A) and a mouse monoassociated with \textit{Brachyspira hyodysenteriae} (Panel B). Note the changes in the cellularity, presence of goblet cells, and height of the mucosa in the ceca from the monoassociated mouse 28 days after colonization with \textit{B. hyodysenteriae}.](image)

In the small intestine, this change in epithelial cell transit time is doubled, going from 53 hours in a CONVR mouse to 115 hours in a GF mouse [33]. The most noticeable gross change in GF mice is the enlargement of the cecum, which can comprise up to 19\% of the mouse’s body weight [34]. The cecum of a GF mouse can assume a more normal size (4.5 \% body weight) following colonization with a mixture of \textit{Lactobacillus} spp., Group N streptococci, \textit{Bacteroides} spp., enterococci and coliform bacilli recovered from CONVR mice [34]. As shown in Figure 2, monoassociation with \textit{B. hyodysenteriae} also returns the cecum to a more normal size.
Of interest, not all bacteria tested were able to return the cecum to a normal size. It was noted that colonizing mice with *Lactobacillus* spp. and Group N streptococci only was not sufficient to change the morphology of the cecum. However, when *Bacteroides* spp. colonized, the mice the morphology assumed a more normal size. Other differences between GF and CONVR mice include the consistency of the cecal contents, with the contents from a GF mouse being more liquid, hypotonic and alkaline than in a CONVR mouse [35]. Additionally, GI transit time is slower in GF mice, which are also prone to vitamin deficiency due to a lack of bacterial metabolism [36-38]. In GF rats, monoassociation with either a rat-derived *Escherichia coli* strain or a sarcina-like micrococcus was able to reverse the vitamin K deficiency. Research identifying the response to *Salmonella Typhimurium* challenge indicated that there was a 50-fold greater translocation of bacteria to the mesenteric lymph node, and organisms were present in the blood of GF mice, something not seen in CONVR mice [37]. This phenomenon occurred due to the
decreased transit time in the small intestine of GF mice, thereby allowing the 
Salmonella to multiply in the host and translocate to other tissue sites.

1. f. Effect of Bacterial Colonization on Immune Development

In addition to its importance in maintaining gut homeostasis, the GI microbiota 
is also critical for normal priming and development of the immune system [39-41]. 
GF mice have reduced numbers of immune cells in the lamina propria and have 
smaller Peyer’s patches compared to CONVR mice. They also have a diminished 
capacity for antibody production, fewer plasma cells, smaller mesenteric lymph 
nodes, and reduced numbers of germinal centers compared to CONVR mice [39]. 
However, this underdeveloped immune system is fully capable of mounting a 
response comparable to that of a CONVR mouse when stimulated with bacterial 
antigen or protein [40, 41]. Macrophages from CONVR mice process antigen faster 
than those from GF mice, likely because the continued exposure of CONVR 
macrophages to bacterial antigen allows them to be “primed” for antigen degradation, 
a phenomenon that does not occur in GF mice [41]. The presence of bacteria in the 
GIT promotes decreased immunoreactivity towards commensal organisms. The 
barrier between the gut microbiota and the underlying gastrointestinal-associated 
lymphoid tissue consists of a single layer of epithelial cells covered by two layers of 
mucus [42, 43]. The inner layer of mucus is approximately 100 µm thick while the 
outer layer is approximately 700 µm in the rat colon [43]. The inner layer is firmly 
attached to the epithelial cells and devoid of bacteria, while the outer layer is “loose” 
and contains commensal organisms [44]. The major constituent of these layers is a
protein known as Muc2 [42]. The importance of Muc2 has been highlighted by the development of Muc2\(-/-\) mice. These mice fail to gain weight, have diarrhea by seven weeks of age, occult blood present in their feces by eight weeks of age, and the majority had gross bleeding and reversible rectal prolapse by nine weeks of age [45]. These mice also developed microscopic evidence of colitis as early as five weeks of age.

Underneath the mucus layers lie the intestinal epithelial cells, which are held together by tight junction proteins such as occludins, claudins, and junctional adhesion molecules [46]. These proteins seal the paracellular junction between the cells and regulate the entry of nutrients, ions and water. They also act as a barrier against bacterial entry. Loss of epithelial barrier function promotes a break in immunological tolerance and facilitates immunoreactivity towards the normal commensal microbiota (Figure 3). This phenomenon has been demonstrated in mouse studies employing 2,4,6 trinitrobenzensulfonic acid (TNBS), a chemical that disrupts the epithelial barrier to allow translocation of bacteria, resulting in the induction of both innate inflammatory responses and antigen-specific immune responses [47]. Subsequent to the loss of epithelial barrier function, these mice develop severe gastrointestinal inflammation, which can be ameliorated by pretreating with antibiotics to reduce the microbial load [47].
The role of the commensal bacteria in regulating the immune response was elegantly demonstrated in a study by Wlodarska et al. [48]. Mice administered metronidazole were more susceptible to subsequent infection with the pathogen, *Citrobacter rodentium*. Metronidazole-treated mice had increased submucosal edema, ulcerations, mucosal hyperplasia and decreased numbers of goblet cells. The authors also noted enhanced expression of *IL-25* and *Reg3γ* mRNA and an increased presence of NK cells and macrophages in the lamina propria of these mice, indicating an increase in microbial stimulation. The inner mucous layer of metronidazole-treated mice was significantly thinner and mRNA expression of genes encoding for the goblet-cell specific proteins Muc2, TFF3, and RelmB was also decreased in these mice [49, 50]. The thinning of the inner mucous layer following
metronidazole treatment allowed for *C. rodentium* to closely associate with the epithelium and promote production of the pro-inflammatory cytokines and chemokines TNF-α, IFN-γ, and MCP-1. This study highlights the contribution of the commensal microbiota in the production of the mucus barrier and subsequently in maintaining mucosal homeostasis [48].

It has also been noted that specific members of the resident microbiota are able to elicit specific immune functions. Colonization with *Bacteroides fragilis* expressing polysaccharide A (PSA) suppressed development of T helper 17 (Th17) CD4+ T cells by promoting the development of Foxp3+ regulatory (Treg) CD4+ T cells and inducing IL-10 expression via TLR2 signaling [51]. Additional studies have demonstrated that purified PSA is sufficient to expand the numbers of Foxp3+ Treg cells [52]. *B. fragilis* inhabits colonic crypts. This niche puts the organism in close contact with the immune system but its production of PSA ensures that no inflammatory response is induced. Both prophylactic and therapeutic treatment of mice with PSA ameliorate TNBS-induced colitis in mice [52]. More recently, work from Atarashi et al., elegantly demonstrated a role for indigenous *Clostridium* species in the induction of colonic Treg cells [53]. Colonization of GF mice with a cocktail of 46 *Clostridium* strains enriched for clusters IV and XIVa promoted TGF-β production from intestinal epithelial cells and increased the number of Foxp3+ Treg cells in the colon. Furthermore, inoculation of young CONVR mice with *Clostridium* species facilitated resistance to both dextran sodium sulfate (DSS)- and oxazolone-induced colitis as well as increased systemic Immunoglobulin E (IgE) production as adults.
The segmented filamentous bacteria (SFB) is another microorganism of interest that has been implicated in the induction of Th17 responses [54]. Ivanov et al. found that C57BL/6 mice purchased from the Jackson Laboratory had significantly less IL-17-producing CD4+ T cells in the small intestine compared to those from Taconic Farms. This discrepancy could be corrected via gavage of the Jackson mice with contents from the small intestines of the Taconic mice. Using a 16S ribosomal RNA PhyloChip analysis, SFB were identified in the microbiota of mice from Taconic Farms but not in those from the Jackson Laboratory. Monoassociation of mice with SFB induced the production of Th17 cells and up-regulated genes encoding antimicrobial peptides and serum amyloid A (SAA). SAA co-cultured with naive CD4+ T cells and lamina propria derived dendritic cells induced Th17 cell differentiation in vitro. The authors concluded that SFB, which tightly adheres to and imbeds itself among the microvilli on the epithelial cell surface, induces the production of SAA by intestinal epithelial cells. In turn, the SAA acts on lamina propria dendritic cells to stimulate the induction of Th17 cells. Colonization with SFB protects against C. rodentium infection. Th17 cytokines such as IL-22 likely stimulate intestinal epithelial cells to secrete antimicrobial peptides to limit the growth of the pathogen and its infiltration into the colonic wall [54].

1. g. Effect of Bacterial Colonization on Other Aspects of the Body

In addition to promoting immune maturation, the commensal microbiota also helps to regulate fat storage. GF mice eat 29% more food than conventional mice yet have 42% less body fat and a decreased metabolic rate [55]. Colonization of GF
mice with cecal contents from conventional mice for 14 days caused a 57 % increase in total body fat with a concomitant 27 % reduction in food intake. These “conventionalized” mice also had increases in leptin, fasting glucose, and insulin levels compared to GF mice; they also developed insulin resistance. Levels of mRNAs specific for transcription factors SREBP-1 and ChREBP were also elevated leading to increased production of lipogenic enzymes. Fat formation is aided by the regulator lipoprotein lipase (LPL) and is inhibited by the fiaf gene product whose expression is suppressed in microbiota-bearing conventional mice. The authors suggest that the bacteria in the gut breakdown dietary polysaccharide into monosaccharaides that are then transported to the liver to activate lipogenic enzymes. This process promotes fat formation in the peripheral tissues due to the suppression of fiaf. In addition to the production of white adipose tissue, the microbiota may also play a role in eye health [56]. A recent study revealed differences in the lipid profiles in the lens and retinas of GF mice as compared to CONVR mice. The CONVR mice had reduced concentrations of multiple phosphatidylcholines and an overall reduced presence of phospholipids in the lens. The authors postulate these changes may be due to the increased exposure of the CONVR mice to more oxidative stress than their GF counterparts [56]. Together, these studies help us realize the effects that the gut microbiota may have on host systems that, at the surface, seem to have limited or no connection to the GIT.
1. h. Bacterial Production of Short Chain Fatty Acids

Another health benefit that the microbiota provides its host revolves around the production of short-chain fatty acids (SCFA) such as acetate, propionate, and butyrate as end products of anaerobic fermentation [57]. These SCFA (predominantly butyrate) can be utilized as energy sources by eukaryotic cells. Members of the *Clostridium* clusters IV (e.g., *Faecalibacterium prausnitzii*) and XIVa (e.g., *Roseburia* spp. and *Eubacterium rectale*) are the primary butyrate producers in the GIT, and they comprise approximately 2-15% of the total gut microbiota [14, 58-61]. Butyrate, a four-carbon fatty acid, is produced by bacteria via one of two metabolic pathways. The first pathway utilizes the enzymes phosphotransbutyrylase and butyrate kinase to form butyrate from butyryl-CoA to yield one ATP per one molecule of butyrate produced [62, 63]. The second pathway, which is utilized by most organisms in the gut, uses butyryl-CoA acetate CoA transferase to form butyrate and acetyl-CoA from butyryl-CoA [64, 65].

Once produced, butyrate has multiple effects on gut health. A primary use is as a preferred energy source for colonocytes, and the mechanisms by which butyrate is utilized in the GIT is summarized in Figure 4 [66-69]. Two biomarkers of energy homeostasis, ATP and NADH/NAD⁺ levels, are both significantly reduced in only the colonic tissues of GF mice as compared to CONVR mice [70]. This observation indicates that GF mice have a reduction in TCA cycle activity, and subsequently less ATP is generated for cellular energy.
Figure 4. Microbial regulation of colonocyte metabolism. Schematic depicting how dietary fiber is fermented by microbes into butyrate in the lumen of the colon, which is then transported into the colonocyte. In the colonocyte, butyrate promotes oxidative metabolism and inhibits autophagy. Based on transcriptome and proteome experiments, enzymes regulated by microbes are shown in boxes. In all cases, boxed enzymes that function in β-oxidation and the TCA cycle are downregulated in GF colonocytes, revealing that microbes positively regulate their expression. Diminished ATP results in phosphorylation of AMPK and p27, which culminates in autophagy. (Cell Metab. 2011 May 4;13(5):517-5) Reprinted with permission from the publisher license number 2977121495018
Moreover, this reduction in ATP was correlated with increased signs of energetic stress in colonocytes, including increased expression of 5’-adenosine monophosphate-activated protein kinase (AMPK). Consistent with previous reports describing a role for AMPK in inducing autophagy [71], GF colonocytes also expressed elevated levels of the autophagosome marker LC3-11. Transmission electron microscopic analysis revealed that significantly more GF colonocytes were undergoing autophagy than colonocytes from CONVR mice. Colonizing GF mice with a conventional microbiota reversed these effects, as did incubation of isolated colonocytes with butyrate. Additional experiments employing the fatty-acid oxidation blocker, etomoxir, demonstrated that colonocytes consume butyrate as an energy source and not as a histone deacetylase (HDAC) inhibitor, another known function of butyrate [72, 73].

Butyrate functions as an HDAC inhibitor by blocking cellular deacetylase activity and allowing histone acetylation [72, 73]. Histone modification causes changes in cellular gene expression patterns; compounds and molecules that can elicit these modifications are being studied as potential anti-cancer therapeutics [74]. A comparative study of gene expression patterns in HT-29 cells (a colon carcinoma-derived cell line) treated with either butyrate or trichostain A (a known HDAC inhibitor) revealed that both substances had similar effects on gene expression [75]. Upregulated genes (21 total) were found to regulate the cell cycle, signal transduction, DNA repair and genome transcription. Only two genes were down-regulated—*lactoferrin δ* and *MAPKAP kinase*. It is also important to note that both butyrate and trichostain A inhibited the growth of HT-29 cells by creating an arrest in
the G₁ phase of the cell cycle [76]. Butyrate may also down-regulate pro-
inflammatory responses via its HDAC activity, as incubation of butyrate with inflamed
biopsy samples or LPS-induced peripheral blood mononuclear cells (PBMCs)
reduced the mRNA expression of IL-6, TNF-α, TNF-β, and IL-1β. Additionally, in
murine studies of TNBS- and DSS-induced colitis, and in ulcerative colitis (UC)
patients, administration of butyrate enemas ameliorates disease activity via NFKB
inhibition [77-79]. Butyrate decreases both COX-2 and PGE₂ expression in HT-29
cells stimulated with TNF-α [80].

The information presented in this section clearly defines a central role for the
gut microbiota in many physiological processes; the microbiota has a tremendous
impact on the host’s health status. Causal links likely exist between the methods by
which infants are delivered, their gut microbial colonization patterns and subsequent
health concerns, including asthma, eczema and food allergies. Associations
between gut bacterial communities and obesity, diabetes and cardiovascular
disease have also been documented [81-83]. A significant body of literature also
links changes in the composition of the gut microbiota with inflammatory bowel
diseases (IBD), which is discussed in detail in the following section [84-89].

2. Introduction to Inflammatory Bowel Disease

In 1932, three physicians, Burrill Crohn, Leon Ginzburg, and Gordon
Oppenheimer described a disease of unknown etiology in the terminal ileum of
young adults [90]. The disease was characterized as being similar to UC (fever,
diarrhea and weight loss) and having ulcerations of the mucosa that would
eventually lead to stenosis of the lumen and formation of multiple fistulas. Eventually, surgical intervention was used to resect the affected portion of the intestinal tract and patients recovered with little signs of the disease persisting. In their 1932 report, Crohn and colleagues discuss observations from other physicians regarding observations of granulomas in the small and large intestine of unknown etiology being classified under the umbrella term “benign granulomas.” Still today Crohn’s disease (CD) and UC are both included under the umbrella term “inflammatory bowel disease.” Crohn and colleagues described the disease as beginning at the ileocecal junction with lesions separated by normal mucosa [90]. They described inflammation of the submucosa and the muscularis resulting in a markedly thickened bowel wall. The presence of “giant cells” was also noted, which they attributed to vegetable matter becoming entrapped in the ulcers and becoming encapsulated during healing. The authors concluded that the presence of these giant cells in the granulomatous lesion led others to believe that the inflammation was due to an unusual form of tuberculosis. However, the authors could not find any evidence of tuberculosis in their 14 patients.

Clinically, the patients with IBD were characterized as young adults with fever, diarrhea, dull abdominal pain, and vomiting; they were also weak, anemic and had poor appetite. Upon physical examination, the author noted five commonalties: 1) a mass in the right iliac region, 2) evidence of fistula formation, 3) emaciation and anemia, 4) approximately half of the subjects had undergone an appendectomy, and 5) evidence of intestinal obstruction. Treatment was supportive and surgical resection of the affected portion of the intestinal tract was recommended; 13 of the
14 patients had no symptoms post-operatively. For the one patient that developed recurrent symptoms, it was later determined that not all of the affected intestinal tissue had been resected during the first surgery.

Eighty years after the first published article of what would become known as “Crohn’s disease,” there is still no cure for this disease nor do we fully understand its etiology. Unfortunately, the number of people being diagnosed with IBD is increasing yearly [91]. There is not one specific, causative agent of IBD; instead the etiology is thought to be multifactorial in nature, with host genetics, environmental factors, the induction of aberrant immune responses, and the gastrointestinal microbiota contributing to disease pathogenesis.

2. a. Genetic Factors Affecting IBD

As of 2011, genome-wide meta-analyses had identified 71 susceptibility loci associated with CD, 47 with UC and 28 associated with both, some of which are shown in Figure 5 [92-95]. Here, we focus on genetic variants within specific genes involved in mediating host responses to microbial components
2. a. I. Nod2

One of the first candidate genes linked to IBD susceptibility was Nod2.

Located on chromosome 16, the defective allele affects the activation of the transcription factor NF-κB. NOD2 is expressed in antigen-presenting cells, macrophages, lymphocytes, ileal Paneth cells, and intestinal epithelial cells and functions as an intracellular microbial recognition molecule [96-100]. NOD2 senses muramyl dipeptide (MDP), a minimally bioactive motif of peptidoglycan from both Gram-negative and Gram-positive bacteria. Once activated by its ligand, NOD2 undergoes a conformational change to expose its CARD domains and recruits the kinase RIP2 to the complex. RIP2 associates with NOD2 via homophilic CARD-
CARD interactions [97]. This process leads to the activation of IKK and the subsequent degradation of IκB and ultimately results in the translocation of NF-κB to the nucleus [96, 101].

Petnicki-Ocwieja et al. demonstrated that MDP stimulation of ileal crypts induced bactericidal secretions capable of killing *E. coli* [102]. Moreover, the same authors showed that NOD2 was required for the bactericidal activity of crypt secretions of the terminal ileum. The composition of the ileal microbiota of the NOD2−/− mice was significantly altered in comparison to their WT counterparts. Specifically, NOD2−/− mice had increased numbers of Firmicutes, specifically *Bacillus* species and *Bacteroides* species. Wild-type mice were able to clear an infection of *Helicobacter hepaticus*, an opportunistic mouse pathogen, within seven days while NOD2−/− mice remained colonized with *H. hepaticus* for at least 14 days post-infection [102]. Also of note, the presence of a gut microbiota was required for the expression of NOD2. GF mice possessed decreased levels of NOD2—a phenotype that could be reversed following monoassociation with either Gram-negative or Gram-positive organisms. Together, these results outline a reciprocal regulatory relationship between NOD2 expression and the gut microbiota.

2. a. II. ATG16L1

Another gene implicated in the pathogenesis of IBD that regulates cellular autophagy and is known as *ATG16L1* [95, 103]. Autophagy is a process utilized by cells to recycle cellular components. This process may also be utilized by cells as a non-apoptotic method of programmed cell death [104]. *ATG16L1* is expressed by
CD4+ and CD8+ T cells, CD19+ B cells, epithelial cells, and macrophages [105, 106]. Studies by Caldwell et al. utilizing mice engineered to express low or hypomorphic levels of ATG16L1 revealed that this gene is indeed an autophagy-related protein. Elevated levels of cytosolic proteins normally degraded by a rapamycin-induced degradation pathway were found in the cells of the ATG16L1 hypomorphic (ATG16L1\textsuperscript{HM}) mice [95]. Additionally, cells from ATG16L1\textsuperscript{HM} mice possessed fewer autophagosomes following rapamycin treatment or nutrient deprivation. The small intestines and colons of ATG16L1\textsuperscript{HM} mice had no morphological defects in crypt height or villus length, but did possess abnormalities in their Paneth cells. Specifically, lysozyme within Paneth cells was either depleted/absent or diffuse, which is in contrast to the normal and orderly packaging of lysozyme within granules. Decreased levels of lysozyme were also observed in the ileal mucus layer of ATG16L1\textsuperscript{HM} mice. However, intact granules were observed in the crypt lumen, indicating a potential role of ATG16L1 in the maintenance of the Paneth cell granule exocytosis pathway. The authors observed no differences between WT and ATG16L1\textsuperscript{HM} mice in terms of resistance to a challenge with Listeria monocytogenes. However, others have reported a reduction in intracellular bacteria targeted to autophagic vacuoles following in vitro infections of HeLa or Caco-2 cells with S. typhimurium in conjunction with siRNA to reduce the expression of ATG16L1 [105, 107]. Additional work by Caldwell et al. revealed that 100 % of ileocolic resection specimens obtained from humans with the ATG16L1 risk allele had abnormal Paneth cells that looked similar to those found in the ATG16L1\textsuperscript{HM} mice [95].
Using siRNA to reduce ATG16L1 expression in THP-1 macrophages revealed an increase in intracellular adherent and invasive E. coli (AIEC) as compared to normal THP-1 infected macrophages [106]. AIEC promote enhanced production of TNF-α and IL-6 from macrophages. Silencing of ATG16L1 expression resulted in significant increases in both cytokines. The authors postulate that bacterial clearance is less efficient in individuals with the risk alleles for ATG16L1. When AIEC are present, this reduction in bacterial clearance likely leads to increased production of pro-inflammatory cytokines, which could tip the immune balance to an inflammatory state. Thus, it appears that the presence of bacterial provocateurs (e.g., AIEC) in the microbiota increases the risk of developing IBD in individuals possessing polymorphisms in genes such as ATG16L or NOD2.

2. b. The Immune Response and IBD

The mucosal immune system is charged with the monumental task of balancing responsiveness and tolerance to a tremendous number of environmental antigens, including those from food and bacteria. Although the exact cause of IBD remains elusive, significant evidence supports the hypothesis that GIT inflammation is initiated and perpetuated by a dysregulated immune response directed against the gut microbiota resulting in deleterious responses in genetically susceptible individuals following an environmental trigger. The host with a genetic predisposition for IBD may possess defects in epithelial permeability and/or altered regulation (i.e., NOD2 deficiency) of commensal bacteria. Potential environmental triggers may include smoking, certain medications, or a gastrointestinal illness that induces a
break in homeostasis. Regardless of the specific genetic or environmental trigger, what ensues is an exaggerated, inappropriate mucosal immune response characterized by chronic activation of T cells and the production of cytokines and other inflammatory mediators.

The success of cytokine-targeted immunotherapies for a subset of IBD patients supports the idea that these chemical messengers of the immune system play an important role in disease pathogenesis. However, the nature of the immune phenotypes observed in CD and UC patients do differ [108]. The immune response of CD is typically associated with a T helper 1 (Th1) phenotype while UC is characterized by a T helper 2 (Th2) phenotype [108]. Isolated lamina propria (LP) CD4+ T cells from CD patients produce IFN-γ when stimulated via the CD2/CD28 pathway; in contrast, LP CD4+ T cells from UC patients secreted mostly IL-5 [109]. Another cytokine with a strong link to CD is IL-12 [110]. Messenger RNA for IL-12p40 in LP mononuclear cells (LPMC) was found in 85 % of CD patients, a percentage significantly higher than that found in healthy and UC GIT tissue samples. Similarly, IL-12p35 mRNA was detectable in the LPMC of 92 % of CD patients; expression was again significantly higher than for healthy and UC patients. Elevated levels of IL-12 were also detectable in the serum of CD patients [110], and this potent Th1-promoting cytokine is able to induce IFN-γ production from LP lymphocytes (LPL) isolated from CD patients [111]. Additionally, studies employing a murine model of TNBS-induced colitis have revealed that administration of anti-IL-12 antibodies ameliorates disease severity, presumably by decreasing IFN-γ secretion from LP CD4+ T cells[112].
In UC, production of the cytokines IL-5 and IL-13 appears to mediate the disease process. Using LPMC from IBD patients, those with UC produced increased amounts of IL-13 and IL-5 upon \textit{in vitro} restimulation as compared to LPMCs recovered from CD patients or healthy individuals [113]. Levels of IFN-\(\gamma\) produced by LPMC from UC patients were similar to those produced by cells from healthy controls. The authors specifically identified CD4\(^+\) CD161\(^+\) NK T cells as the primary source of IL-13 in UC patients, as these cells produced 30-fold more IL-13 than NK T cells from CD patients following \textit{in vitro} restimulation.

More recent work has implicated the T helper 17 (Th17) lineage of CD4\(^+\) T cells in the chronic inflammation observed in IBD [114, 115]. The development of Th17 T cells is dependent upon the presence of both IL-6 and TGF-\(\beta\) [115]. Colonic biopsies from UC and CD patients expressed higher levels of IL-17A mRNA as compared to those from healthy controls [115-117]. Immunohistochemical analysis of these tissues identified increased numbers of IL-17A\(^+\) cells in both the LP and the epithelium of UC and CD patients as compared to controls [116]. Additional work also noted the presence of IL-17\(^+\) cells in patients with active IBD [114]. However, the IL-17\(^+\) cells were found predominantly in the LP of UC patients but in the submucosa and muscularis propria of CD patients [114]. These different locations of IL-17\(^+\) cells complements prior observations that CD lesions often present as transmural while those in UC are superficial. Also of note, a comparison of biopsies from patients with active versus inactive disease revealed that IL-17\(^+\) cells were only increased in numbers during active disease. The authors also determined that both T cells and monocytes/macrophages are a source of IL-17, and that IBD patients
have elevated levels of IL-17 in their sera as compared to undetectable levels of this cytokine in the sera of healthy individuals.

The maintenance of Th17 T cells requires the presence of IL-23 [115]. Of interest, a gene variant significantly associated with CD encodes for the IL-23R, which, along with IL-12Rβ1, comprises the IL-23 receptor complex [118, 119]. This receptor complex interacts with IL-23 (a heterodimer of IL-12p40 and IL-23p19) to direct the production of a Th17 immune response [120-122]. Anti-IL-23 antibodies have been shown to both prevent and ameliorate established disease in a T-cell transfer model of murine colitis [123]. Studies of human tissues demonstrate that IL-23R expression is upregulated not only on IL-17 producing CD4+ T cells, but also on IFN-γ+ T cells in both UC and CD patients [115]. Stimulation of LP CD4+ T cells from UC patients with IL-23 significantly increased IL-17 production; in contrast, IL-23 stimulation of LP CD4+ T cells from CD patients resulted in enhanced IFN-γ secretion [115]. In addition to promoting the production of Th17 cells, IL-23 also inhibits the production of Foxp3+ Treg cells [124] and suppresses production of IL-10, a key regulatory cytokine [125].

Another hallmark of the immune response observed in IBD is the production of IgG antibodies against the normal commensal microbiota [126]. Increased intestinal permeability, be it through genetic predisposition or environmental trigger, promotes enhanced immunoreactivity to bacterial antigens. The mucosal immunoglobulin profile of healthy adults consists predominantly of IgA with only a small amount of IgG. In contrast, patients with active IBD presented with significantly higher amounts of IgG in their mucosal secretions, but with no difference in amounts
of IgA, as compared to controls. IgG antibodies present in IBD patients were found
to bind to non-pathogenic bacterial commensals, including *E. coli*, *B. fragilis*, *C.
perfringens*, *Klebsiella aerogenes*, and *Enterobacter faecalis*. Patients with CD had
significantly higher titers than UC patients, while antibody titers to these commensal
bacteria were at or below the limit of detection in serum samples from healthy
controls. Notable differences were also observed in the isotypes of IgG present in
UC versus CD patients. Specifically, UC patients produced predominantly IgG\(_1\) and
IgG\(_3\) antibodies to bacterial antigens, while IgG\(_1\), IgG\(_2\), IgG\(_3\) were the predominant
isotypes reacting with the bacterial antigens in serum samples from CD patients
[126]. Conversely, other reports have described an increase in IgG\(_1\) antibodies in UC
patients and an increased in IgG\(_2\) antibodies in CD patients [127-130]. Additional
analyses have determined that IgG antibodies in CD patients are primarily directed
toward bacterial cytoplasmic proteins rather than membrane associated proteins
[126]. Despite the presence of many types of antibodies in both the mucosal
secretions and serum of IBD patients, no direct evidence for their involvement in IBD
immunopathogenesis has been reported, indicating that they may simply be a
marker for immune responsiveness and/or dysregulation [131].

2. c. Environmental Factors Affecting IBD

Although there are allelic differences in many people with IBD, genetics alone
cannot completely account for the development of IBD nor for the increase in the
incidence of IBD worldwide. Studies of monozygotic twins best highlight this concept,
as there are disease concordance rates of only 50 % for CD and only 20 % for UC
Of interest, a British study assessing discordant twins with CD found an association with mumps infection, smoking, and oral contraceptive usage with the development of CD [134]. Additionally, the twin(s) with CD had suffered both a medical illness, more episodes of gastroenteritis, and spent more time with animals. Smoking is one confounding environmental factor that is of special interest, because it appears to have a protective effect against UC, but increases the risk of CD [132, 135-137]. Another factor that appears to protect against UC is an appendectomy; its effect on CD is not as evident [138-140]. The equivalent procedure performed in mice ameliorates colitis in both chemically-induced and genetically-engineered murine colitis models [141, 142]. Modest associations between oral contraceptive use and IBD have been documented, while others have found breast-feeding to be protective against both UC and CD [143, 144]. A meta-analysis performed by investigators in New Zealand identified other potential environmental factors, including being an only child, using antibiotics prior to and during adolescence (four or more courses in year), and having a pet in the house during childhood [145]. IBD also tends to occur in extended families as first and second degree relatives of IBD patients reported the occurrence of disease with a significantly higher frequency than the general population [146]. These findings strengthen the interconnection between genetics, environmental factors, and the incidence of IBD.

2. c. I. Antibiotic Usage

Antibiotics are used in the treatment of IBD to reduce microbial load and dampen inflammatory immune responses. However, their use prior to disease
diagnosis is now being identified as a risk factor for developing IBD. A Danish study of IBD patients revealed that antibiotic users were 1.84 times more likely to develop IBD, which correlated with a 12% increase in disease risk for each course of antibiotics taken [147]. Further analysis revealed that antibiotic users were 3.41 times more likely to develop CD than UC, which correlated to an increased risk of 18% per course of antibiotics used. Specifically, use of penicillin V and extended spectrum penicillins were associated with the greatest disease risks. A Swedish study focused on the use of antibiotics from birth to age 5, a time when the microbiota and immune response are still developing and/or maturing, as a risk factor for IBD. They reported an association between a diagnosis of pneumonia, subsequent antibiotic treatment and the onset of both pediatric and adult CD [148]. Another study by Card et al. revealed a statistically significant association between antibiotic usage 2 to 5 years prior to diagnosis of CD in patients from the United Kingdom [149]. This finding was confirmed in a Canadian study, which reported that the more antibiotics taken within 2 to 5 years of diagnosis, the greater the risk of developing IBD [150]. That same study found that disease risk was weakly associated with penicillin use and greatly associated with metronidazole use, which was prescribed primarily for “non-infectious gastroenteritis.” This association with antibiotic usage may be explained by the failure of the gut microbiota to reestablish its normal community structure and function following a course of antibiotics [151]. This dysbiosis or alteration in the microbiota may be a predisposing factor to the onset of IBD in a susceptible individual.
2. c. II. MAP

Another potential environmental risk factor for the development of IBD is a chronic infection in the GIT. To date, no one particular organism has been found to be the causative agent of IBD. Although many microbial pathogens have been implicated as causative, only two have been significantly investigated. The first organism hypothesized to be associated with IBD was *Mycobacterium avium* subsp. *paratuberculosis*, which causes Johne’s disease in cattle [152, 153]. The granulomatous inflammation observed in CD patients led many early researchers to investigate various types of *Mycobacterium* spp. in CD pathogenesis but confirmation was never achieved (reviewed in [154]). The first report of *M. paratuberculosis*-like organisms isolated from patients with CD in was published 1984. Now known as *M. avium* subsp. *paratuberculosis* (MAP), there is still no consensus as to whether or not it plays a role in the onset of IBD in a subset of genetically susceptible patients [155]. Using PCR to amplify the DNA insertion element, IS900, (which is specific to MAP) in biopsy sections, Sanderson et al., reported that 65 % of adult CD patients examined were PCR positive [156]. Dell’Isola et al. reported that 72 % of pediatric CD patients examined were PCR positive for MAP [156, 157]. While many studies have reported MAP DNA in CD patients, just as many have found MAP DNA in samples from healthy individuals and UC patients [156-162]. Moreover, multiple studies have failed to detect MAP-specific DNA in any CD patient sample examined [163-169]. The ability to detect MAP in some tissue samples and not others may be due, in part, to the organism’s fastidiousness, slow growth, and/or the presence of PCR inhibitors in fecal/tissue
samples that inhibit the detection of IS900. Serological evidence also fails to provide definitive answers for the involvement of MAP in the pathogenesis of IBD. Some studies assessing the presence of serum antibody titers against MAP-derived antigens have found an association with IBD [170-176] while others have not [177-182]. More recent work has identified a possible link between variants in the Nod2 gene and the presence of MAP [183]. In this study, 68 % of CD patients were positive for MAP DNA based on PCR analysis as compared to 21 % of the healthy individuals. Fifty-one percent of the CD patients were carriers of NOD2 polymorphisms as compared to 21 % of healthy individuals, and 74 % of the CD patients possessing one of the mutated NOD2 alleles were positive for MAP DNA. Even with all of the data collected, the debate continues as to whether or not MAP plays an important role in the pathogenesis of CD [184, 185].

2. c. III. Adherent-invasive Escherichia coli

Another organism implicated in IBD pathogenesis is adherent-invasive Escherichia coli (AIEC). First described in 1998, AIEC isolates were recovered from the ileal mucosa of CD patients [186]. These organisms are found in higher numbers in CD patients (22 %) as compared to healthy controls (6 %) [187]. They adhere to Caco-2 cells and do not possess any of the virulence genes associated with known E. coli pathotypes (e.g., ETEC, EHEC) [186]. A characterization of expressed adhesins in AIEC strains revealed the Pap and Sfa adhesin as colonization factors, both of which are found in uropathogenic E. coli; however, many of the AIEC strains did not possess any of the known adhesins associated with pathogenic E. coli (e.g., intimin).
Some of the cytotoxic strains expressed the *hly* operon, and it was subsequently demonstrated that these organisms produce a type 1 pilus similar in sequence to that of an *E. coli* strain associated with avian colisepticemia and meningitis [188]. Type 1 pili are involved in both the adherence and internalization of *E. coli* into host cells. They specifically bind to the CEACAM6 receptor on ileal enterocytes, which is expressed at significantly higher levels in the inflamed small intestinal epithelium of CD patients as compared to healthy controls [189].

Further research involving the prototypic AIEC strain, LF82, has shown that these pathogenic *E. coli* strains invade epithelial cells via engagement of actin microtubules and microfilaments and replicate intracellularly even without possessing any of the known invasive determinants found in other invasive *E. coli* strains [190]. AIEC strains are able to survive and replicate within macrophages without causing apoptosis and induce production of the pro-inflammatory cytokine TNF-α [191]. LF82 is also able to induce *in vitro* aggregation of peripheral blood mononuclear cells (PBMCs), an observation reminiscent of the granulomas observed in the colonic tissue of CD patients [192].

In addition to being detected with increased frequency in human IBD patients, AIEC strains have also been isolated from Boxer dogs with granulomatous colitis. These *E. coli* isolates were found to have the same adherent-invasive phenotype as human AIEC strains. The Boxer dog isolates were also of the same phylotype (B2 and D) and possessed similar virulence gene profiles as LF82. In contrast to the human AIEC strains, however, the canine AIEC strains were only isolated from diseased dogs and not healthy controls. Simpson and colleagues also demonstrated
that remission of colitis in Boxer dogs could be achieved by treatment with enrofloxacin, the use of which resulted in the eradication of AIEC *E. coli* [193].

2. d. The Microbiota and IBD

Although no single organism has been implicated in the induction of IBD, a preponderance of studies indicate that the GI microbial community of IBD patients is different from that of healthy individuals (dysbiotic). Characteristics of a dysbiotic community in IBD include a reduction in numbers of organisms in the *C. leptum* and *C. coccoides* clusters (members of the Firmicutes phyla and major butyrate producers) and an increase in numbers of *Enterobacteriaceae* and Bacteroidetes, thereby leading to a reduction in the microbial diversity of the gut. Another notable difference is the greater concentration of mucosa-associated bacteria in IBD patients as compared to healthy individuals [194-197]. Using FISH, patients with CD were found to have predominantly *Enterobacteriaceae*, γ−*Proteobacteria*, or *Bacteroides/Prevotella* adherent to their mucosa and present in their submucosa [195]. Another FISH-based analysis revealed that *Bacteroides* spp. were the dominant gut bacteria, representing up to 80 % of the total mucosa-adherent bacterial population in some samples. Of interest was the authors’ additional finding that treatment of IBD patients with mesalamine (an anti-inflammatory therapeutic drug) significantly reduced the numbers of mucosa-adherent bacteria [198].

Sample origin is an important factor to consider when interpreting data for microbial analyses. Stool samples are easy to obtain, but may not accurately reflect the microbial community in the cecum and proximal colon of patients [14]. In a
comparative study of the microbial populations detected in cecal versus fecal
samples, more anaerobes and *Bifidobacterium* spp. were detected in fecal samples
using culture-based methods [199]. Molecular probe hybridization revealed that
facultative anaerobes represented by *Lactobacillus*, *Enterococcus*, and *E. coli* were
higher in numbers in cecal contents, yet the number of strict anaerobes represented
by the *Bacteroides*, *C. leptum*, and *C. coccoides* groups were significantly lower in
the cecal contents. Differences in microbial composition have also been observed
when comparing colonic biopsies versus feces [200]. Analysis of feces from
Japanese IBD patients showed that *Faecalibacterium* spp. were significantly
decreased in CD patients, while *Bacteroides* spp. were significantly increased only in
patients with active IBD [201]. Based on the Shannon diversity index, this study
demonstrated that microbial diversity was significantly reduced in CD patients both
during active disease and remission as compared to healthy individuals.

Several studies have noted a specific decrease in the numbers of *C. leptum*
and *C. coccoides* clusters present in colonic contents or feces in IBD patients [18,
197, 202-206]. A reduction in the *C. coccoides* group in UC patients and *C. leptum* in
CD patients has been shown using FISH probes on patient feces [202]. Another
study employing a combination of PCR and FISH analysis of fecal samples found a
reduced presence of both *C. leptum* and *C. coccoides* clusters in IBD patients [197].
Additional analyses revealed significant decreases in the concentrations of the
SCFAs (e.g., butyric and propionic acid) in the feces of IBD patients [197]. In other
work, high throughput sequencing demonstrated reduced numbers of
*Faecalibacterium*, *Ruminococcaceae*, *Alistipes*, *Collinsella*, and *Roseburia* and
increased numbers of *Enterobacteriaceae* in a twin with ileal CD as compared to the healthy twin [204]. This latter study again emphasizes the involvement of the microbiota in the development of IBD in a genetically susceptible host, as only one of the twins developed disease.

One specific member of the *C. leptum* cluster, *Faecalibacterium prausnitzii*, has recently been the subject of many published studies. This organism is a major butyrate producer that also possesses some anti-inflammatory properties [59, 207]. A study by Sokol et al. described a reduction of both *C. leptum* and *C. coccoides* clusters in the stool of patients with active IBD along with a skewed Firmicutes/Bacteroidetes ratio [203]. The authors specifically identified a reduction in *F. prausnitzii* in IBD patients, confirming results obtained using biopsy samples from twins analyzed via qPCR [205]. In other work, denaturing gradient gel electrophoresis (DGGE) analysis of biopsy samples also demonstrated a significant decrease in *Faecalibacterium* spp. along with increased levels of *E. coli* and *Clostridium* spp. in CD patients as compared to healthy individuals [208]. Using fecal cylinders and 11 different FISH probes to analyze sections GIT tissue specimens, Swidsinski and colleagues found the presence of *F. prausnitzii* to be significantly reduced in CD patients [209]. Of special note, analysis of samples from IBD patients given high-dose cortisol or infliximab to reduce inflammation revealed a dramatic increase (>14 x10⁹) in the levels of *F. prausnitzii* within days of initiating treatment. This increase was short-lived, however, as levels of *F. prausnitzii* decreased when the cortisol dose was reduced or the time between infliximab infusions was
increased. Together, these data indicate that inflammation plays a major role in shaping the intestinal microbiota.

Work in murine models of colitis also demonstrates that inflammation in the gut, be it chemically or bacterially induced, causes an increase in the numbers of *Enterobacteriaceae* [210]. Colonization of mice with *C. rodentium* resulted in a reduction of the total number of bacteria in the colon at 7 and 14 days post-infection; this decrease coincided with the highest levels of both *C. rodentium* and intestinal inflammation [211]. Further analysis specifically determined that members of the Cytophaga-Flavobacter-Bacteroides group were decreased. Infection with *C. rodentium* infection is self-limiting, and the total bacterial levels returned to normal following pathogen clearance. These changes in the bacterial load were not simply associated with colonization of the microbial community by a new organism, as colonization with *Campylobacter jejuni*, an organism that does not cause intestinal inflammation in immunocompetent CONVR mice [212], induced no detectable changes in microbial load.

The induction of GIT inflammation in mice via administration of dextran sulfate sodium (DSS) in the drinking water for seven days was associated with increased numbers of aerobic bacteria, specifically *Enterococcus faecalis* [210]. Bacteroidetes were eliminated from the community and the total number of bacteria was also decreased. A dramatic increase in the numbers of a non-pathogenic *E. coli* following DSS treatment was also observed. The authors subsequently colonized IL-10−/− mice, which spontaneously develop colitis [213], with this non-pathogenic *E. coli*. Upon the development of GIT inflammation, the *E. coli* proliferated to the same level observed
in DSS treated mice, but displaced the Firmicutes phylum instead of the Bacteroidetes. These results indicate that regardless of the source, GIT inflammation creates a niche that favors the colonization and population expansion of Enterobacteriaceae. Collectively, these data suggest that the induction of inflammation following colonization of the GIT by a bacterial provocateur along with perturbations of the microbiota together have a deleterious impact on the microbiota and mucosal homeostasis.

2. e. Canine IBD

In addition to affecting humans, IBD can also occur in dogs as well [214]. As in human IBD, the interactions among genetics, the mucosal immune system, inflammation, and environmental factors (i.e., diet and imbalances in the intestinal microbiome) all contribute to the pathogenesis of canine IBD (Figure 6) [215-217].

Figure 6 - The etiology for canine IBD involves complex interactions between host genetics, mucosal immunity, and the enteric microbiota. Therapeutic intervention with diet, antibiotics and immunosuppressive drugs is aimed at reducing inflammation and dysbiosis.
Mutations in innate immune receptors in German shepherd dogs (TLR5, NOD2) have been linked to IBD susceptibility, which in the presence of an inappropriate enteric microbiota may lead to upregulated pro-inflammatory cytokine production (e.g., IL-17, IL-22, TNF-α) and reduced bacterial clearance, thereby promoting chronic intestinal inflammation [218, 219]. Commensal bacterial antigens are likely to be important in disease pathogenesis because it has been observed that boxer dogs with granulomatous colitis (GC) show clinical remission following the eradication of mucosa-associated AIEC that share a novel adherent and invasive pathotype which bears phylogenetic similarity with AIEC strains recovered from patients with ileal CD [193, 220, 221]. Moreover, genome-wide analysis in affected boxer dogs has identified disease-associated single nucleotide polymorphisms (SNPs) in a gene (NCF2) involved with killing intracellular bacteria [222]. Still others have shown that CD11c+ cells are significantly decreased in the intestines of dogs with IBD suggesting that chronic mucosal inflammation may involve an imbalance in the intestinal dendritic cell population leading to aberrant immune activation [223].

Molecular analysis of the intestinal microbiome in different breeds of dogs with IBD have consistently shown that diseased tissues are enriched with members of the families Enterobacteriaceae and Clostridiaceae [224, 225]. These bacteria are believed to contribute to the pathogenesis of GIT inflammatory disease in dogs as in humans [187, 226]. A recent trial using high throughput 16S rDNA sequencing methods (i.e., 454 pyrosequencing) on intestinal biopsies of IBD dogs revealed a dysbiosis in the mucosa-adherent microbiota with an increase in sequences belonging to Proteobacteria and a decrease in Bacteroidetes, Fusobacteria, and the
Clostridiales [227]. Taken together, these studies suggest that chronic intestinal inflammation of canine IBD may be due to overly aggressive adaptive immune responses to enteric bacteria (or fungi) [225] in hosts with genetic defects that fail to properly regulate microbial killing, mucosal barrier function, or immune responses. As in human IBD, environmental factors (i.e., diet and microbiota imbalances) likely govern the onset of inflammation or reactivation and modulate genetic susceptibility to disease.

2. e. i. Clinical and diagnostic features

The clinical manifestations of IBD are diverse and are influenced by the organ(s) involved, presence of active versus inactive disease, and physiologic complications seen with enteric plasma protein loss and/or micronutrient (cobalamin) deficiency [215, 216, 228, 229]. Canine IBD is a disease that predominantly affects middle-aged animals. Vomiting and diarrhea are most commonly observed and are often accompanied by decreased appetite and weight loss. Gastric and duodenal inflammation is associated with vomiting and small bowel diarrhea while colonic involvement causes large bowel diarrhea with blood, mucus, and straining. The clinical course of IBD is generally cyclical and is characterized by spontaneous exacerbations and remissions. Importantly, the clinical signs of IBD are not disease specific and share numerous over-lapping features with other canine disorders. A diagnosis of IBD is one of exclusion and requires careful elimination of IBD mimics [230]. The possible causes for chronic intestinal inflammation may be excluded through the integration of history, physical findings, clinicopathological testing,
diagnostic imaging, and histopathology of intestinal biopsies. A baseline CBC, biochemistry profile, urinalysis, and diagnostic imaging are useful in eliminating the most common systemic and metabolic disorders (e.g., renal disease, hepatopathy, hypoadrenocorticism) causing chronic GI signs in dogs. The measure of clinical disease activity by means of quantifiable indices is well established in human IBD [231-233].

A canine IBD activity index (CIBDAI) used for assessment of inflammatory activity in dogs has been recently designed [234]. Similar to other indices, the magnitude of the numerical score is proportional to the degree of inflammatory activity. This index serves as the principal measure of response to a therapeutic regimen and may be used to tailor medical therapy for an individual patient's needs [235]. Intestinal biopsies are required to confirm histopathological inflammation and to determine the extent of mucosal disease. Diagnostic endoscopy is preferred since this technique allows for direct assessment of mucosal abnormalities and the acquisition of targeted biopsy specimens. The microscopic findings in canine IBD consist of minimal to pronounced inflammatory cell (lymphoplasmacytic) infiltration of the intestinal lamina propria accompanied by varying degrees of mucosal architectural disruption similar to that observed in tissue from human IBD patients (Figure 7).
Unfortunately, biopsy interpretation is notoriously subjective and suffers from extensive intra-observer variability and the technical constraints of procurement/processing artifacts inherent in evaluation of endoscopic specimens [236]. Although several histopathological scoring schemes have been proposed there are no uniform grading criteria that pathologists can universally agree on. One small study has resulted in development of a ‘simplified model system’ for defining intestinal inflammation of IBD that is presently being tested in a separate clinical trial.

2. e. II. Therapeutic approach

Treatment principles for canine IBD are empirical and consist of combination therapy using both dietary and pharmacologic interventions. As compared to clinical trials evaluating the efficacy of therapy for CD and UC, only one randomized, controlled drug trial for canine IBD has been reported [235]. There are, however, abundant evidence-based observations that feeding elimination diets and administering corticosteroids, immunosuppressive drugs, and/or select antibiotics are useful in the clinical management of canine IBD. Some clinicians prefer a sequential approach to nutritional and drug therapy for IBD. The optimal drug or drug
combinations as well as duration of therapy for induction and maintenance of remission of clinical signs have not been determined for most protocols [216, 230]. In general, the administration of corticosteroids (i.e., prednisone, prednisolone or budesonide), antimicrobials (i.e., metronidazole or tylosin), and immunosuppressive drugs (i.e., cyclosporine, chlorambucil, azathioprine) used alone or in some combination are effective in inducing clinical remission in most animals. Some dogs will require intermittent or life-long drug therapy.

The rationale for nutritional therapy of IBD is that restricting exposure to antigens (i.e., dietary proteins) known to evoke sensitivity will reduce exaggerated host responses and attenuate intestinal inflammation. Other indications for specialized nutrition include the presence of decreased appetite, impaired nutrient absorption, or enteric plasma protein loss seen with moderate-to-severe mucosal inflammation. While evidence-based observations indicate that most dogs respond favorably to dietary intervention, the superiority of one novel protein source versus another or the advantage in feeding an intact protein elimination diet versus a hydrolyzed protein elimination diet has not been shown to date. Modifying the dietary n3:n6 fatty-acid ratio may also modulate inflammatory responses by reducing production of pro-inflammatory metabolites [237]. There is relatively sparse clinical data investigating prebiotic or probiotic therapy for canine IBD (see subsequent section on probiotics).
2. e. III. Future directions in canine IBD

Canine IBD represents a common and frustrating GI disorder in veterinary medicine. More research is needed to unravel the mechanisms responsible for disease development and to translate these findings directly to human IBD. The primary features of IBD in humans and animals are remarkably similar (Table 1).

Table 1. Comparative features of IBD in humans and dogs.

<table>
<thead>
<tr>
<th>Feature</th>
<th>Human IBD</th>
<th>Canine IBD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genetic basis</td>
<td>Yes</td>
<td>Likely</td>
</tr>
<tr>
<td>Etiology</td>
<td>Unknown but multifactorial</td>
<td>Unknown but multifactorial</td>
</tr>
<tr>
<td>Involves the microbiota</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Hematochezia</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Diarrhea</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Definitive Diagnosis</td>
<td>GI biopsy</td>
<td>GI biopsy</td>
</tr>
<tr>
<td>Disease activity assessment</td>
<td>Clinical indices, biomarkers (ASCA, pANCA, CRP, calprotectin)</td>
<td>Clinical indices, biomarkers (pANCA, CRP, calprotectin ?)</td>
</tr>
<tr>
<td>Responsive to anti-inflammatory drugs</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Responsive to antibiotics</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Spontaneous GI flares</td>
<td>Yes</td>
<td>Yes</td>
</tr>
</tbody>
</table>

Recent advances in clinical indices, histopathological standards, and the development of species-specific immunologic reagents and innovative molecular tools have made the dog an excellent ‘spontaneous’ animal model to study chronic immunologically-mediated intestinal inflammation. In addition, the dog has higher genomic sequence similarity to that of humans than do mice, a species traditionally used for comparative disease genetics [238]. However, clinical manifestations of
complex disease in the mouse do not compare to the human form as closely as they do in the dog. Furthermore, the lifespan of the dog is much shorter than that of a human; thus, clinical trials aimed at treatment of IBD can be carried out much quicker and yield results that should have relevant application to human trials [230, 235, 239].

2. f. Treatment for Human IBD

Treatments for human IBD patients typically involve the use of anti-inflammatory drugs, antibiotics, and there is a growing trend of probiotics and prebiotics being studied to determine their effects on disease activity. When patients fail to respond to treatment, the last and most drastic treatment option is surgery to resect sections of the inflamed bowel [240]. Most treatment options are primarily focused on reducing the inflammation associated with IBD. Some of these drugs also impact the microbiota and those will be discussed below.

2. f. l. Antibiotics

Given the importance of the microbiota in the pathogenesis of IBD, antibiotic therapy may seem an obvious treatment option. A meta-analysis by Khan et al. in 2011 reviewed randomized controlled trials utilizing antibiotics in the treatment of IBD [241]. Rifamycin derivatives, ciprofloxacin, and clofazamine all induced remission in CD patients. Rifaximin (a rifamycin derivative) is effective against both Gram-negative and Gram-positive anaerobes and aerobes; it is also poorly absorbed after oral administration, resulting in little to no systemic side effects [242,
Analysis of the effect of the drug using an *in vitro* continuous culture method with fecal samples from CD patients revealed increases in beneficial bacteria post-rifaximin administration [244]. Significant increases in *Bifidobacterium* spp., the *Atopobium* cluster, and *F. prausnitzii* were noted, as were increases in levels of lactate, acetate, and propionate as determined by $^1$H-NMR spectroscopy [244].

Ciprofloxacin is a fluoroquinolone with broad-spectrum antibiotic activity [245]. In addition to having anti-bacterial properties, this drug has also been shown to have immunomodulatory properties as well [246]. In a TNBS mouse model of colitis, administration of ciprofloxacin ameliorated disease as compared to mice given ceftazidime (an antibacterial with a similar spectrum of activity compared to ciprofloxacin) [245]. Clinically, mice treated with ciprofloxacin did not lose weight and had reduced histopathological inflammatory scores associated with their colons. Ciprofloxacin treated mice also had reduced expression of IL-1$\beta$, IL-8, and TNF-$\alpha$ as measured from colonic homogenates as well as reduced expression of NF-$\kappa$B. Since ceftazidime was less effective in ameliorating GIT inflammation, there appears to be additional benefits (i.e., anti-inflammatory) to the use of ciprofloxacin in addition to its spectrum of antimicrobial activity.

Clofazamine has also been documented to significantly affect the rate of remission in CD patients [241]. This drug, used to treat leprosy, is similar to ciprofloxacin, in that it has both anti-bacterial and anti-inflammatory properties [247-249]. It is only effective against Gram-positive organisms, and its effectiveness increases in anaerobic environments [250]. Its effects on the immune system include increasing the presence of lysosomal enzymes in macrophages [251] and increasing
phagocytosis by macrophages resulting in enhanced uptake and digestion of immune complexes [252]. Clofazamine has also been shown to inhibit TCR-mediated IL-2 production by T cells, thereby limiting T cell activation, a component of the pathogenesis of IBD [253].

2. f. II. Corticosteroids

The most commonly used corticosteroids used are prednisolone, methylprednisolone, and budesonide [254]. These drugs are very effective at inducing remission, however, they are not without side effects. In a study comparing prednisolone and budesonide, both were found to be effective at inducing remission [255]. Patients on budesonide had reduced evidence of adrenal axis suppression and peripheral leucocyte counts compared to those treated with prednisolone. These results indicate that budesonide is a safer choice yet it is still as effective as prednisolone. Corticosteroids are able to prevent NFκB activation [256] as well as block infiltration of neutrophils, prevent vasodilation and enhanced vascular permeability and downregulate the production of pro-inflammatory cytokines [254]. Although it is well established that these corticosteroids have an anti-inflammatory effect, little research has been conducted on the effects they have on the gut microbiota. A study by Swidsinski et al., mentioned previously, indicated that administration of cortisol increased the population of *F. prausnitzii* in a dose dependent manner [209].
2. f. III. Immunosuppressive Therapy

Drugs such as methotrexate, 6-mercaptopurine (6-MP) and azathioprine (the prodrug of 6-MP) work by inhibiting the proliferation and activation of lymphocytes and decreasing the production of pro-inflammatory cytokines [254]. 6-mercaptopurine (6-MP) and azathioprine are purine antagonists and inhibit cellular metabolism by interfering with DNA replication [257, 258]. Methotrexate is a folic acid analog that inhibits DNA synthesis and, therefore, has an anti-proliferative effect [259]. Antibacterial effects, including growth inhibition of MAP, have also been demonstrated for methotrexate and 6-MP [258].

2. f. IV. 5-Aminosalicyclic Acid (5-ASA)

Sulfasalazine was the first 5-ASA-type drug developed and is a combination of sulfapyridine (an antimicrobial) and salicyclic acid (an anti-inflammatory) to form a pro-drug. Upon entering the colon, this pro-drug is cleaved by colonic bacteria into the two separate molecules [260, 261]. Unfortunately, the frequency of gastrointestinal side effects was quite high due to the sulfapyridine moiety [262, 263]. It was later determined that the active moiety is 5-aminosalicyclic acid (5-ASA, mesalazine) [264]. More recently formulations of this pro-drug have eliminated the sulfapyridine moiety and replaced it with either a second salicyclic acid moiety (disodium azodisalicylate [265]) or an inert carrier (balsalazide [266]) thereby reducing the side effects.

In addition to being an anti-inflammatory agent [267-270], 5-ASA also affects the gut microbiota. As mentioned previously, patients taking mesalamine had a
reduction in mucosa-adherent bacteria [198, 271]. 5-ASA has also been shown to inhibit the growth of MAP [272] and *Bacteroides* spp. [273] and moderately inhibit the growth of *C. difficile* and *C. perfringens* in culture [273]. This drug also effects bacterial gene expression, as *S. Typhimurium* incubated with 5-ASA had no change in growth but differentially expressed 110 genes [274]. Those genes characterized were found to be involved in invasion, metabolism, and antibiotic and stress resistance. *In vitro* assays revealed attenuation in the invasiveness of *S. Typhimurium* towards HeLa cells when pretreated with 5-ASA. These results indicate that in addition to inhibiting host-mediated inflammation, 5-ASA also has the potential to affect the intestinal microbiota.

**2. f. V. Anti-TNF monoclonal antibodies**

The induction of TNF-α is most often a downstream event following the interaction of phlogistic microbial components with toll-like receptors (TLRs) on host cells. The interest in TNF-α as a therapeutic target for IBD treatment began when the expression and secretion of this cytokine was found to be increased in IBD patients [275, 276]. For example, pediatric patients with either active UC or CD present with elevated levels of TNF-α in their stool [275]. Additionally, the incubation of GIT tissue sections in culture medium has demonstrated that significantly elevated levels of TNF-α are spontaneously secreted from inflamed tissue of both UC and CD patients when compared to the amounts produced by non-inflamed tissue and tissue from otherwise healthy individuals [276]. The predominant cell type producing the TNF-α has been shown to be the macrophage [276]. Based on the
central role TNF-α appears to play in the pathogenesis of IBD, there was interest in developing a therapeutic approach to control the harmful effects of this cytokine.

Clinically, the use of monoclonal antibodies to treat IBD patients began with Infliximab, an IgG1 murine-human chimeric monoclonal antibody specific for TNF-α, which was approved for human use in 1998 for CD [277, 278]. This monoclonal antibody consists of human constant regions and murine antigen binding regions [277]. These chimeric antibodies reduce the risk of immunoreactivity that occurs when murine antibodies are used. In addition to being less immunoreactive, this chimeric antibody had improved binding and neutralization characteristics for TNF-α than that of the original murine antibody [277]. Another monoclonal anti-TNF antibody, Adalimumab, is a fully humanized IgG1 antibody that avoids the induction of anti-species IgG that neutralize the effectiveness of the anti-TNF-α reagent [278, 279]. Lastly, Certolizumab is a monoclonal antibody fragment with a polyethylene glycol moiety (PEGylated) [278]. Certolizumab lacks the crystallizable fragment (Fc) portion of the immunoglobulin molecule and is an IgG4 isotype unlike Infliximab and Adalimumab, which are IgG1 antibodies [278, 280, 281]. In addition, the PEGylation increases the half-life of the antibody thereby reducing the frequency of administration.

Anti-TNF-α therapy works via multi-factorial mechanisms. First, it neutralizes TNF-α by blocking its ability to bind to TNF receptors, thus, inhibiting the pro-inflammatory response. Second, Anti-TNF-α binds to cell surface bound TNF-α on CD4+ T cells and macrophages, resulting in both complement- and antibody-
dependent cell-mediated cytotoxicity [282]. All three monoclonal antibodies bind to and neutralize both soluble and membrane forms of TNF-α [281]. Infliximab and Adalimumab both mediate complement- and antibody-dependent cell-mediated cytotoxicity; however, Certolizumab only mediates complement-dependent cellular cytotoxicity as it lacks of an Fc region. Furthermore, Infliximab and Adalimumab induce apoptosis in peripheral blood lymphocytes and monocytes, as well as cause degranulation and loss of membrane integrity of PMNs. These activities were not induced with Certolizumab. Lastly, all three monoclonal antibodies inhibit the production of IL-1β after LPS stimulation in vitro, suggesting that there is a sequential production of pro-inflammatory cytokines induced by microbial components. Infliximab and Adalimumab both inhibit T cell proliferation in mixed lymphocyte reactions in vitro, again suggesting that anti-TNF-α monoclonal antibodies ameliorate the inflammation associated with IBD via more than one mode of action [283]. The impact of these therapies on the microbiota, however, is not well studied. As previously mentioned, treatment with Infliximab resulted in increased levels of *F. prausnitzii* after administration [209]. It is clear that the host inflammatory response often negatively impacts (i.e., shapes) the composition of the GIT microbiota, and that controlling mucosal inflammation benefits the health of the microbiota as well. Otherwise, there have been no specific studies performed to directly evaluate the role of anti-TNF-α therapies on the gut microbiota.
2. f. VI. Complementary and alternative therapies

An estimated 70% of IBD patients have reported using complementary and alternative medicine (CAM) products at some time to treat their symptoms. In a Canadian study of IBD patients, some of the most commonly used CAM treatments included massage therapy, chiropractic visits, probiotics, herbs, and fish oils [284]. A systematic review of the literature on the use of herbal medicines reveals some anti-inflammatory benefits associated with the administration of these herbs to both animals and humans [285]. It should be stressed that prior to utilizing any herbal remedy, which are potentially biologically active, patients need to consult their physician. This is especially important because the number two reason patients using CAM gave as to why they sought these products was that “natural therapy is safe” [286]. Some of the biological properties associated with CAM products include the reduction of pro-inflammatory cytokines, increased antioxidant production, inhibition of leukotriene B4, decreased NF-κB activation, and inhibition of platelet activation[285].

Increasing evidence supports a potential therapeutic role for prebiotic and probiotic therapy in human IBD [287, 288]. If IBD in dogs is indeed driven by loss of tolerance to components of the intestinal microbiota as it is in humans, then prebiotics and probiotics may also prove beneficial as primary or adjunct therapies with diet and drugs. **Probiotics** are living microorganisms that, upon ingestion in sufficient numbers, impart health benefits beyond those of inherent basic nutrition [289]. Lactobacilli and bifidobacteria have been the most commonly used human probiotics, but multi-strain cocktails (e.g., VSL#3), *E. coli* Nissle 1917, and
nonbacterial Saccharomyces boulardii have also been used as probiotics [226]. Probiotic bacteria have measurable host benefits, including the ability to improve epithelial barrier function, modulate the mucosal immune system, and alter the intestinal flora [290]. **Prebiotics** are non-digestible dietary carbohydrates, such as lactosucrose, fructo-oligosaccharides (FOS), psyllium, and bran which beneficially stimulate the growth and metabolism of endogenous enteric bacteria upon consumption [291]. Beneficial effects of prebiotics are also associated with the production of short chain fatty acids due to fermentation by colonic bacteria. **Synbiotics** are combinations of probiotics and prebiotics that are an emerging therapeutic modality. Increasing evidence supports a therapeutic role for probiotics, prebiotics, and synbiotics in treating gastrointestinal diseases of humans, including infectious diarrhea, *H. pylori* infection, irritable bowel syndrome, lactase deficiency, and IBD [84]. A comparison of prebiotic and probiotic preparations is outlined in Table 2.

2. f. VII. Probiotics

VSL#3 is one of the most commonly used probiotic cocktails and contains a very high bacterial concentration per gram of product characterized by greater number of different bacterial species as compared to traditional probiotic preparations [292]. This commercially prepared formulation consists of 450 billion bacteria/g of viable lyophilized bacteria comprised of eight bacterial strains (*Lactobacillus casei*, *L. plantarum*, *L. bulgaricus*, *L. acidophilus*, *Bifidobacterium longum*, *B. breve*, *B. infantis* and *Streptococcus thermophiles*). While the exact
mechanism of action of VSL#3 is unknown, several studies have demonstrated the effects of VSL#3 on epithelial barrier function and down regulation of cytokine secretion from immune cells. For example, Madsen [293] has shown in *in vitro* studies that epithelial barrier function could be enhanced by exposure to a soluble factor secreted by VSL#3 bacteria. Moreover, this same study demonstrated that VSL#3 did not alter the ability of the epithelial cell to activate a mucosal inflammatory response to a bacterial invasion. Studies with VSL#3 formulations have also been conducted in several animal models of colitis, inflammatory liver disease, sepsis, and irritable bowel syndrome (IBS). In models of experimentally-induced colitis, these studies demonstrated that VSL#3 normalized gut permeability and barrier function, and that VSL#3 modulated inflammatory and immune responses [226, 294]. Animal models of sepsis have also demonstrated that VSL#3 administration reduced bacterial translocation and significantly attenuated damage to the liver and intestinal mucosa [295]. The use of VSL#3 as an innovative probiotic preparation, developed specifically to balance the intestinal microbiota, is supported by studies in humans with IBD (ulcerative colitis and pouchitis) [296], and in other patients with diverse gastrointestinal disorders, such as IBS [297, 298].
Table 2. Basic Features of Probiotics and Prebiotics

<table>
<thead>
<tr>
<th></th>
<th>Probiotics</th>
<th>Prebiotics</th>
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</thead>
<tbody>
<tr>
<td><strong>Definition</strong></td>
<td>Live microorganisms which, given in adequate amounts</td>
<td>Non-digestible carbohydrate which stimulate replication of protective</td>
</tr>
<tr>
<td></td>
<td>confer health benefits to the host</td>
<td>enteric bacteria when consumed</td>
</tr>
<tr>
<td><strong>Examples</strong></td>
<td><em>E. coli</em> Nissle 1917</td>
<td>Fructo-oligosaccharide (FOS)</td>
</tr>
<tr>
<td></td>
<td>VSL#3</td>
<td>Galacto-oligosaccharide (GOS)</td>
</tr>
<tr>
<td></td>
<td><em>Lactobacillus</em> species</td>
<td>Inulin</td>
</tr>
<tr>
<td></td>
<td><em>Bifidobacterium</em> species</td>
<td>Lactulose</td>
</tr>
<tr>
<td></td>
<td><em>Saccharomyces boulardii</em></td>
<td>Psyllium</td>
</tr>
<tr>
<td></td>
<td>Prostora Max®</td>
<td>Bran</td>
</tr>
<tr>
<td></td>
<td>Forti Flora®</td>
<td>Beet pulp, pumpkin</td>
</tr>
<tr>
<td></td>
<td>Proviable-DC®</td>
<td>Resistant starch</td>
</tr>
<tr>
<td><strong>Protective</strong></td>
<td>Alters microbiota to suppress pathogens</td>
<td>Stimulates replication of beneficial bacteria</td>
</tr>
<tr>
<td><strong>Mechanisms</strong></td>
<td>Improved intestinal barrier function</td>
<td><em>(Bifidobacterium)</em></td>
</tr>
<tr>
<td></td>
<td>Increased production of antimicrobial peptides</td>
<td>Enhances production of SCFA (butyrate)</td>
</tr>
<tr>
<td></td>
<td>Decreased expression of proinflammatory cytokines</td>
<td>Improved intestinal barrier function</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Decreases proinflammatory cytokines</td>
</tr>
</tbody>
</table>

Studies have shown VSL#3 to induce remission of inflammation in 77 % of adult UC patients with no adverse effects [298] and 56 % of pediatric UC patients [299]. These same pediatric UC patients had a reduction in disease activity index and sigmoidoscopy scores following VSL#3 treatment. They also had reduced levels
of the pro-inflammatory cytokines TNF-α and IFN-γ following therapy [299]. *In vitro* analysis of the effects of VSL#3 on Mode-K epithelial cells revealed that only one of the eight organisms, *L. casei*, inhibited TNF-induced secretion of the pro-inflammatory chemokine IP-10 [300]. The most recent studies with VSL#3 treatment of UC show increased fecal concentrations of beneficial bacterial species, improved clinical, endoscopic and histopathological scores in most patients, and higher rates of remission compared to placebo [298]. VSL#3 is available without prescription and can be ordered via the internet or obtained locally at the pharmacy in the U.S. [301].

Recent studies have also shown that dogs with IBD have distinctly different duodenal microbial communities compared to healthy dogs. Current treatments for IBD include the administration of nonspecific anti-inflammatory drugs which may confer serious side effects and do not address the underlying basis for disease, namely, altered microbial composition. The use of probiotics offers an attractive, physiologic, and non-toxic alternative to shift the balance to protective species and treat canine IBD. The authors (AEJ) have initiated a clinical trial to investigate the clinical, microbiologic, and anti-inflammatory effects of probiotic VSL#3 in the treatment of canine IBD. We hypothesize that VSL#3 used as an adjunct to standard therapy (i.e., elimination diet and prednisone) will induce a beneficial alteration of enteric bacteria leading to induction and maintenance of remission in dogs with IBD. A randomized, controlled clinical trial of eight weeks duration will assess the efficacy of standard therapy in conjunction with VSL#3 versus standard therapy alone in the management of canine IBD. There is a need for additional data to be generated to provide proof of efficacy in probiotic therapy before these agents can be applied to
widespread clinical use. These studies will also provide highly relevant insight into
the anti-inflammatory effects of probiotics for treatment of human and canine IBD.

Another popular probiotic is *E. coli* Nissle 1917 also known as Mutaflor®. This
probiotic has been shown to be just as effective as mesalazine in achieving and
maintaining remission of inflammation in UC patients [302-304]. It has recently been
shown that when *E. coli* Nissle 1917 is genetically modified to produce the quorum-
sensing molecule Al-2, it affected the beneficial probiotic properties of this organism
[305]. Colonization of healthy newborn infants with *E. coli* Nissle 1917 has shown
that the presence of pathogenic bacteria in the gut was significantly reduced
compared to non-colonized infants [306]. *In vitro* studies have shown *E. coli* Nissle
1917 is able to reduce the invasive ability of multiple pathogenic organisms [307],
and also to reduce the adherent and invasive ability of AIEC [308]. *E. coli* Nissle
1917 was also shown to either inhibit the growth (49 %) or overgrowth (30 %) of
79 % of uropathogenic organisms recovered from children with urinary tract
infections [309]. Unlike VSL#3, Mutaflor® cannot be purchased in the U.S. due to its
reclassification by the F.D.A. from a “medical food” (which is what VSL#3 is
classified as) to a “biologic” [301, 310].

There are few reports on the use of probiotic bacteria in dogs and cats.
Recent *in vitro* studies have confirmed the capacity of a lyophilized probiotic cocktail
(e.g., three different *Lactobacillus* spp. strains) to modulate the expression of
regulatory versus pro-inflammatory cytokines in dogs with chronic enteropathies
[311, 312]. One commercially-manufactured probiotic (FortiFlora™ – *Enterococcus
faecium* SF68, Nestle Purina) is reported to potentially control diarrhea and enhance
immune responses in dogs and cats. Several recent trials attest to the short-term efficacy of probiotics in treating acute diarrhea in dogs and cats [313]. The link between the intestinal microbiota and gastrointestinal health in companion animals is now obvious. Future developments in the pharmabiotic field must include performance of randomized clinical trials to determine the role of probiotics and prebiotics in the management of canine chronic enteropathy. One large multicenter trial investigating the efficacy of VSL#3 in reducing inflammatory activity of canine IBD is presently underway.

2. f. VIII. Prebiotics

Prebiotics are substances that can be used to promote specific changes in the microbiota. Administration has been shown to shift the microbiota in healthy adults; for example, individuals who consume either soluble corn fiber or polydextrose had increases in *F. prausnitzii* and those who consumed the soluble corn fiber had increases in *Roseburia* spp. [314]. As mentioned previously, both of these organisms metabolically produce butyrate and *F. prausnitzii* also has other anti-inflammatory properties. In animal models, prebiotics have also shown to be effective. The severity of DSS-induced colitis in rats was attenuated by administering inulin orally as evidenced by a reduction in histopathological scores and myeloperoxidase accumulation in the colons [315]. In addition, the acidity of the colonic contents increased in rats fed inulin as well as the numbers of *Lactobacillus* spp. in the feces. In a multicenter trial feeding UC patients germinated barley foodstuff (GBF), patients consuming GBF had improved clinical activity index scores
GBF has also been shown to be effective in maintaining remission [317] and reducing pro-inflammatory cytokine levels in UC patients [318]. In CD patients, dietary supplementation with fructo-oligosaccharide (FOS) reduced their disease activity index, increased fecal *Bifidobacterium* spp. levels as well as increased production of IL-10 and expression of TLR-2 and TLR-4 from lamina propria dendritic cells [319].

Scientific studies have also investigated the effects of dietary supplementation with prebiotics on the intestinal microbiota of healthy dogs and cats. In one study, FOS supplemented at 0.75 % dry matter produced qualitative and quantitative changes in the fecal flora of healthy cats [320]. Compared with samples from cats fed a basal diet, increased numbers of lactobacilli and *Bacteroides* spp. and decreased numbers of *E. coli* were associated with cats fed the FOS supplemented diet. However, bacteriologic examination of the duodenal juice in these same cats showed wide variation in the composition of the duodenal microbiota, across sampling periods, which was not affected by FOS supplementation [321]. Moreover, healthy Beagle dogs fed a 1 % FOS diet over a three-month trial showed inconsistent fecal excretion of *Lactobacillus* spp. and *Bifidobacterium* spp. [322]. While FOS supplementation has been shown to have health benefits, these studies demonstrate that FOS does not have an adverse affect on the microbiota and suggest that it may have positive physiological benefits as seen in humans. This observation and the lack of significant side effects associated with FOS supplementation provide evidence that FOS should be considered as an attractive alternative or adjunct therapy for IBD in dogs and cats.
3. Mouse Models of IBD

Many different mouse models have been utilized in IBD research to elucidate the roles that bacteria, genetics, the immune response, and environment play in the induction and maintenance of IBD [323]. They fall into two main categories: chemically-induced and genetically-engineered models. The composition of the microbiota in the various mouse models is also discussed. Regardless of the strain of mouse employed in chemically-induced or genetically-engineered models, there are only five options for its microbiota—conventional, specific pathogen free, restricted, gnotobiotic, or germ-free. Note that the word gnotobiotic (gnostos – “known” bios – “life”) indicates that all the organisms present, regardless of the numbers of species, are known and does not apply only to mice that are completely devoid of microbes (i.e., germfree).

3. a. Chemically-induced models

3. a. I. DSS

Dextran sulfate sodium (DSS) is formed by the esterification of dextran with chlorosulphonic acid [324]. Administered *ad libitum* in the drinking water, this compound causes enterocolitis in mice with disease severity being dependent on mouse strain, DSS molecular mass, and sulfur content [325-328]. Within three to seven days after the addition of DSS (1 to 10 % w/v) to the drinking water, mice exhibit loose stools, weight loss, and occult blood. Upon necropsy of mice treated
with DSS, cecal atrophy and colonic shortening are noted, and histopathological examination reveals mucosal ulceration, inflammatory infiltrate, and hyperplastic epithelium in the colonic mucosa. It has also been reported that glandular dropout occurs prior to signs of inflammation. Although the exact mechanism of action for DSS-induced colitis is currently not known, it is thought to manifest epithelial toxicity [329, 330]. Other reports indicate that DSS increases mucosal permeability within three days of administration (before the appearance of inflammatory infiltrate) [331]. Studies have also shown that DSS is taken up by macrophages in the colon and mesenteric lymph node and by Kupffer cells in the liver [326, 330]. When macrophages become laden with DSS, they have a reduced ability to perform normal homeostatic functions such as tissue repair and phagocytosis of bacteria [332]. DSS was demonstrated to be cytotoxic to Caco-2 cells, binding to their nucleus, causing cell cycle arrest and reduced production of reactive oxygen species [333].

Microbial populations of the GIT are altered after DSS administration, with the microbiota of treated mice having increased numbers of Bacteroidaceae and Clostridium spp. [326]. This result indicates that the changes to the microbiota may play a contributory role (e.g., reduction of butyrate production) in the induction of DSS-induced disease. However, DSS induces more severe colitis and increased mortality in GF mice as compared to their conventional counterparts [334, 335]. GF mice given either 5 % or 1 % DSS died at days three or 14, respectively, after the start of DSS administration [335]. Collectively, these studies indicate that the composition of the microbiota may influence the sensitivity of mice to DSS-induced
colitis as well as support the idea that the resident microbiota affords cytoprotective benefits for the host.

3. a. II. TNBS (2,4,6, -trinitrobenzene sulfonic acid)

TNBS is a haptenating agent that causes a disease similar to CD when mixed with ethanol and administered rectally as an enema. Mice treated with TNBS develop a pan-colitis with the peak of clinical signs, such as diarrhea and rectal prolapse, occurring two to four weeks post-administration [112]. Microscopically, transmural inflammation is noted along with neutrophil infiltration, loss of goblet cells, edema, and granulomas. T cells isolated from the lamina propria secrete elevated levels of IFN-γ and IL-2 following stimulation with anti-CD3 and anti-CD28. However, administration of anti-IL-12 antibodies after induction of TNBS-induced colitis reduced the disease severity, and treated mice also showed reduced production of IFN-γ [112]. Further studies have shown that CD4+ T cells recovered from mice with TNBS-induced colitis could induce mild colitis when adoptively transferred into naive control mice; the colitic lesions were characterized by inflammatory cell infiltrate that produced IFN-γ [336]. In that same study, researchers found that feeding mice TNBS-haptenized colonic protein caused the mice to develop oral tolerance. These mice subsequently failed to develop colitis after TNBS administration or the transfer of CD4+ T cells from mice with TNBS-induced colitis. T cells from these tolerant mice secreted elevated amounts of TGF-β, IL-4, and IL-10 [336].
3. a. III. Oxazolone

Oxazolone is a haptenating agent that causes a disease in mice similar to UC when mixed with ethanol and administered rectally [337, 338]. SJL/J mice rapidly develop diarrhea and weight loss that peaks at day two post-administration with a 50 % mortality rate by day four. At day two, the distal half of the colon becomes hemorrhagic and edematous and histologically shows signs of superficial inflammation. There is epithelial cell erosion, goblet cell depletion, edema, and inflammatory infiltrate composed of neutrophils and eosinophils. This is similar to what is observed microscopically in the colonic tissues of human UC patients. These mice also develop elevated levels of IL-4 and IL-5, but no IFN-γ, indicating that oxazolone induces a Th2 response. Elevated levels of TGF-β are also noted and may play a role in the induction of disease in only part of the colon. The model has also been examined for its role in determining efficacy of IBD treatments. BALB/c mice given either 5-ASA or sodium prednisolone phosphate intra-rectally prior to and during induction of oxazolone colitis had decreased severity of disease [337]. The disease is self-limiting, and the mice that survive beyond day four show increased weight gain and are healthy by days 10-12 post-administration.
3. b. Genetically engineered models

3. b. I. IL-10^{−/−}

In 1993, Kuhn et al. discovered that mice deficient in the anti-inflammatory cytokine IL-10 spontaneously develop enterocolitis [213]. This model has been popular in IBD research and this genetic deficiency has been crossed onto many different genetic backgrounds of mouse [339-345]. The availability of different strains has highlighted the role that genetics plays in enterocolitis, as the severity of disease is strain dependent. The order of severity from most severe to least severe is as follows: C3Bir > 129 > BALB/c or NOD/Lt > C57BL/6 or C57BL/10. In addition to strain differences, development of enterocolitis is also dependent on the microbiota, as GF IL-10^{−/−} do not develop enterocolitis and disease is attenuated after administration of antibiotics to IL-10^{−/−} mice harboring a conventional microbiota [346, 347]. The lack of IL-10 does not affect the development of the B or T cells, but its absence does result in a lack of regulatory T cells [213, 348]. Studies to understand the development of disease in these mice have shown that B cells (while present in high numbers in the lamina propria) are not needed for the initiation of disease, but that disease is mediated by CD4^{+} T cells [342, 349]. Transfer studies using RAG2^{−/−} mice as recipients have specifically shown that naive CD4^{+} T cells are capable of inducing colitis, and CD45BR^{low} CD4^{+} T cells from IL-10^{−/−} mice can induce disease in these RAG2^{−/−} mice [348, 349]. This latter observation implicates IL-10 as a central mediator of regulatory T cells (CD25^{+} Foxp3^{+} CD4^{+} T cells). It has also been shown that IL-12 and IFN-γ are needed for initiation but not the continuation of colitis [342,
The increase in the production of IL-12 and IFN-γ along with undetectable levels of IL-4 indicates that the enterocolitis in these mice is mediated by a Th1 immune response, similar to that observed in humans with IBD [342, 346].

3. b. II. Mdr1a−/−

In 1994, mice lacking the gene mdr1a were generated. This gene encodes a P-glycoprotein, which is a drug efflux pump, thus protecting host cells from the build-up of toxic compounds. It was noted later that these mice spontaneously develop a colitis similar to human IBD and that disease can be exacerbated by colonization with Helicobacter bilis [351, 352]. These mdr1a−/− mice have reduced growth rates compared to their WT counterparts and their histological lesions begin in the proximal colon and proceed distally as disease severity progresses. Mdr1a−/− males are more susceptible to the onset of severe disease than females [353, 354]. Mdr1a−/− mice also have increased epithelial permeability and reduced phosphorylation of both occludin and ZO-1, tight junction proteins, compared to WT counterparts [354]. They exhibit increased bacterial translocation with bacteria detected in both the spleen and lymph nodes that correlates with disease severity. Therefore, the induction of disease is associated with a defect in the epithelial barrier function of the GI tract. In addition to the similar disease progression found in IBD patients, studying these mice are of interest because the human MDR1 gene has been mapped on a loci that is associated with susceptibility of IBD, although this relationship is under debate [355-358].
3. b. III. TRUC

TRUC mice are both T-bet\textsuperscript{−/−} and RAG\textsuperscript{−/−} and spontaneously develop colitis by four weeks of age [359, 360]. T-bet (T-box expressed in T cells) is a transcription factor that aids in the development of a Th1 response [359]. These mice have increased permeability of their colonic epithelium that increases with age and increased rate of epithelial cell death. Microscopically, there is inflammatory cell infiltrate, goblet cell dropout, crypt loss, and the presences of ulcers. The only cytokine elevated in these mice is TNF-\(\alpha\) and disease can be ameliorated using an anti-TNF-\(\alpha\) antibody. The microbiota also contributes to disease in this model, as treatment with antibiotics was able to “cure” the mice of their colitis. Additionally, the TRUC colitic microbiota can be horizontally transferred to both WT and RAG\textsuperscript{−/−} mice, and 16S rRNA analysis of feces from TRUC mice revealed that the presence of Klebsiella pneumoniae and Proteus mirabilis correlate with colitis. Interestingly, GF TRUC mice colonized with these two organisms alone do not develop colitis unless a more complete microbiota is present [361].

3. c. CD45RB\textsuperscript{hi} CD4\textsuperscript{+} T-Cell transfer model

C.B.-17 scid mice administered CD45RB\textsuperscript{hi} CD4\textsuperscript{+} T cells develop a wasting disease which is not seen if CD45RB\textsuperscript{lo} CD4\textsuperscript{+} T cells or CD45RB\textsuperscript{hi} CD8\textsuperscript{+} T cells are adoptively transferred [362-364]. Disease occurs three to five weeks post-administration and is limited to the large intestine. The mucosa, submucosa, and muscularis all presented with inflammatory cell infiltrates (macrophages and CD4\textsuperscript{+} T cells predominately), and there was also a loss of goblet cells, epithelial cell
hyperplasia, and deep fissure ulcers. Elevated levels of IFN-γ were present in these mice, and treatment with anti-IFN-γ or anti-TNF (α and β) antibodies ameliorated disease in these mice. However, the protection garnered by using the antibodies was not as great as when CD45RBlo CD4+ T cells were transferred along with CD45RBhi CD4+ T cells [362]. This disease type mimics CD with respect to the type of inflammatory response generated (Th1) and the type of intestinal inflammation present (transmural infiltrate of CD4+ T cells) [365]. There is also a definite microbial component in this model of disease, as restricted microbiota mice have less severe disease as compared to their SPF counterparts [364].

3. d. Bacterial-induced models

3. d. i. Helicobacter spp.

Although not considered a microbial cause of IBD, the presence of Helicobacter spp. does adjuvant or predispose mice to the onset of colitis in some models. In our lab, we utilize a dual hit model of colitis consisting of both Helicobacter bilis colonization and low-dose (1.5 %) DSS to elicit colitis, as shown in Figure 8 [366].
Individually, *H. bilis* colonization or 1.5% DSS alone only induce mild typhlocolitis (Figure 9). However, when DSS is administered to mice colonized with *H. bilis*, severe typhlocolitis is induced [367, 368]. In this model, the colitic mice display diarrhea and both the cecum and colon are involved as evidenced by macroscopic cecal atrophy and colonic shortening. Microscopically, these mice exhibit inflammatory cell infiltrate, ulcerations, and loss of colonic glands (Figure 9).
It has been noted that colonization of mice with *H. bilis* alone can change mucosal gene expression and alter the immune response in C3H/HeN mice bearing the altered Schaedler’s flora (ASF) [366, 369]. Genes involved in T cell receptor signaling, the survival and activation of peripheral B cells, and chemotaxis are a few examples of the host genes that were upregulated by *H. bilis* colonization. Genes that were down regulated were involved in fatty acid metabolism and detoxification. After *H. bilis* colonization, serum antibodies directed at antigens derived from members of the ASF are induced [366, 369]. *H. hepaticus* colonization of A/JCr mice also induced changes in gene expression in the cecum, with female mice being more susceptible to the onset of disease [370, 371].
It appears that an over zealous host response to the introduction of the novel organism (i.e., provocateur) predisposes certain strains of mice for the onset of typholocoltiis following a secondary colitic insult. In a study comparing A/JCr mice (that develop mild inflammation) to C57BL/6 (who do not develop inflammation) mice, cecal gene expression profiles revealed that A/JCr mice had more genes differentially regulated (176) compared to C57BL/6 (80). Differentially expressed genes were predominantly those associated with immune response, chemotaxis, signal transduction, and antigen processing in the A/JCr mice while the genes in the C57BL/6 mice were predominately associated with immunoglobulin production.

In the mdr1a⁻/⁻ mouse, it is not simply the presence of Helicobacter that results in the induction of colitis but the specific species of Helicobacter influences the induction of a differential immune response. In mdr1a⁻/⁻ mice, H. bilis colonization causes colitis as early as three weeks post-infection, whereas H. hepaticus colonization ameliorated the severity of colitis in these mice compared to the uninfected control mdr1a⁻/⁻ mice [352]. The disease phenotypes between spontaneous versus Helicobacter-induced colitis in mdr1a⁻/⁻ mice were different, with ulcer formation not present in the Helicobacter-induced colitis. When mdr1a⁻/⁻ mice were co-colonized with both Helicobacter species, the morbidity and mortality rate was between that of mice colonized by either H. bilis or H. hepaticus and the colitis that developed was characterized by dysplasia. H. bilis was also able to out compete H. hepaticus in vivo as evidenced by recovery of higher numbers of H. bilis, suggesting that these two species may compete for similar niches in the GIT.
Additionally, colonization of IL-10−/− C57BL/6 mice with *Helicobacter* species results in the induction of colitis in otherwise disease-free mice [372]. It was also shown that the onset and severity of colitis was species specific in relation to colonization by *H. bilis* or *H. hepaticus* [373]. With respect to C3Bir.129 IL-10−/− mice, the presence of *Helicobacter* spp. is required for the spontaneous onset of disease [339]. Further studies show that colonization of RAG1−/− mice with *Helicobacter* species fails to elicit clinical signs of disease after nine months of colonization and only very mild inflammation was detected at necropsy [373]. Taken together, these observations indicate that disease induction can be mediated by an aberrant adaptive immune response initiated by bacterial provocateurs entering an otherwise stable host-microbe environment.

3. d. II. *Brachyspira hyodysenteriae*

Similar to the need for the a resident microbiota in the TRUC model of colitis, mice colonized with *Brachyspira hyodysenteriae* also require the presence of a microbiota for the induction of typhlocolitis. *B. hyodysenteriae* is an anaerobic spirochete that is the causative agent of swine dysentery [374]. While the pathogenesis of *B. hyodysenteriae* is associated with the production of a β-hemolysin [375], disease does not develop in the absence of a resident microbiota as was demonstrated by the inoculation of germfree pigs [376-378]. The importance of the microbiota was further demonstrated when the microbiota of C3H/HeSnJ or BALB/c mice was depleted by adding a cocktail of antibiotics (rifampicin, colistin, spectinomycin, spiramycin, and vancomycin) to their drinking water. As can be seen
in Figure 10, the antibiotic cocktail depleted the numbers of Gram-positive, Gram-negative, and strict anaerobes by as much as 5 to 7 log\(_{10}\) (Nibbelink and Wannemuehler, unpublished observations). On day seven, antibiotic treated and sham treated mice were inoculated with 1 \(\times\) 10\(^8\) \textit{B. hyodysenteriae} strain B204 and severity of disease was evaluated at 5, 10, and 15 days post-infection (DPI). As can be seen in Figure 11, the sham-treated mice developed severe typhlocolitis while the antibiotic treated mice had no lesions. At 15 DPI, the antibiotics were withdrawn from the drinking water of the remaining \textit{B. hyodysenteriae}-infected mice to allow the microbiota to recover. On day 30 PI, the mice that had been treated with antibiotics through 15 DPI now presented with severe typhlocolitis. The presence and severity of typhlocolitis in these mice correlated with the presence of TNF-specific mRNA in the cecal tissue of the \textit{B. hyodysenteriae}-infected mice (data not shown). Lastly, \textit{B. hyodysenteriae}-induced typhlocolitis could also be prevented when the host’s inflammatory response was inhibited [379, 380]. Collectively, these observations indicate that certain bacterial provocateurs (\textit{K. pneumoniae}, \textit{P. mirabilis}, and \textit{B. hyodysenteriae}) may fail to induce disease in the absence of an appropriate resident microbiota. As depicted in Figure 12, the etiology of colitis is complex and may require the presence of a microbial provocateur, the resident microbiota, and a host inflammatory response.
Figure 10. To assess the role of the microbiota in *Brachyspira hyodysenteriae*-induced typhlocolitis, C3H/HeSnJ mice were treated with an antibiotic cocktail to deplete the resident microbiota. Six days later, mice were infected with *B. hyodysenteriae*. The data indicate that there was a five to seven log_{10} reduction in the resident microbiota.

Figure 11. Assessment of *Brachyspira hyodysenteriae*-induced typhlocolitis in C3H/HeSnJ mice treated with an antibiotic cocktail. While *B. hyodysenteriae* colonized the antibiotic-treated mice to the same extent as it did the sham-treated mice, there was no evidence of typhlocolitis in the antibiotic treated mice through 15 days post-infection (DPI). However, 15 days after the antibiotics were withdrawn (30 DPI), the mice that had been treated with the antibiotics had developed severe disease once the microbiota recovered.

Figure 12. The pathogenesis of inflammatory bowel disease is complex. Studies from animal models indicate that the etiology of disease involves the presence of a bacterial provokateur, the resident microbiota, and the host response.
3. e. Conventional mice

The majority of commercially available mice harbor a “conventional” microbiota. This simply means that the composition of the community is unknown. There are different types of conventionally-reared mice. For example, Taconic Farms maintain two types of conventional microbiota mice, restricted flora™ (RF) and murine pathogen free™ (PF). RF mice are not colonized by β-hemolytic Streptococcus species, K. pneumoniae, K. oxytoca, Pseudomonas aeruginosa, or Staphylococcus aureus. Mice that are PF are Helicobacter free but can contain organisms not found in RF mice [381]. Additionally, mice on the same background purchased from different vendors can harbor different microbiota, as highlighted in work by Ivanov and colleagues demonstrating the presence of SFB in C57BL/6 mice from Taconic but not Jackson Laboratories [54]. This lack of consistency in microbiota from mice of the same strain has even been identified at different facilities from the same vendor (Overstreet and Wannemuehler unpublished observation). Therefore, comparison of studies evaluating the microbiota of mice are difficult because there are hundreds of unknown bacterial species present in the murine microbiota and there is no standardized microbiota used by investigators. The advent of next-gen sequencing has helped to alleviate some of this challenge as all of the organisms in the gut can be sequenced. However, it still does not resolve the issue related to the use of disparate strains of mice from different suppliers.
3. f. Germfree mice

GF mice are completely devoid of microbial life. As mentioned previously, this does grossly affect the anatomy of these mice, which is most evident by the enlargececa being the most prominent feature [34]. The discovery that many GF mouse strains that carry genetic deficiencies associated with IBD (notably the IL-10−/−) do not develop colitis has led to the popularity of GF models to study the role of bacteria in the pathogenesis of IBD [346]. To determine if an organism is capable of initiating colitis, GF mice are monoassociated with a single bacterial species and then monitored for clinical signs of disease. Some of the bacterial strains used to date to evaluate their ability to induce disease in IL-10−/− mice are shown in Table 3. Although the information gathered from these studies has been useful in analyzing the immune response to specific organisms, trying to relate the resultant disease to that characteristic of IBD is marginal at best because of the differences in the complexities of the microbiota. IBD itself is a multi-factorial disease and it has been fairly well-established that the role of bacteria in IBD occurs via a shift in community dynamics (i.e., dysbiosis) and not the presence/absence of one particular species. A perfect example of this complexity is the fact that *K. pneumonia* and *P. mirabilis* fail to induce colitis in GF TRUC mice (Figure 12) [361].
### Table 3. Bacterial strains used to monoassociate GF mice to examine the ability of the strain to induce colitis

<table>
<thead>
<tr>
<th>Organism</th>
<th>Colitis Severity</th>
<th>Time of Disease Onset</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Enterococcus faecalis</em></td>
<td>Severe</td>
<td>10-12 weeks p.i.</td>
<td>[382, 383]</td>
</tr>
<tr>
<td><em>Escherichia coli</em> (from WT SPF mouse)</td>
<td>Moderate</td>
<td>3 weeks p.i.</td>
<td>[382]</td>
</tr>
<tr>
<td><em>Pseudomonas flourescens</em></td>
<td>No disease</td>
<td>-</td>
<td>[382]</td>
</tr>
<tr>
<td><em>Helicobacter hepaticus</em></td>
<td>No disease</td>
<td>-</td>
<td>[384]</td>
</tr>
<tr>
<td><em>Lactococcus lactis</em></td>
<td>No disease</td>
<td>-</td>
<td>[383]</td>
</tr>
<tr>
<td>Viridans group <em>Streptococcus</em></td>
<td>No disease</td>
<td>-</td>
<td>[385]</td>
</tr>
<tr>
<td><em>Clostridium sordellii</em></td>
<td>No disease</td>
<td>-</td>
<td>[385]</td>
</tr>
<tr>
<td><em>Bacteroides vulgatus</em></td>
<td>No disease</td>
<td>-</td>
<td>[385]</td>
</tr>
<tr>
<td><em>Bifidobacterium animalis</em></td>
<td>Mild</td>
<td>23 weeks p.i.</td>
<td>[386]</td>
</tr>
<tr>
<td><em>Bifidobacterium infantis</em></td>
<td>No disease</td>
<td>-</td>
<td>[386]</td>
</tr>
</tbody>
</table>

### 3. g. Defined microbiota mice

Also referred to as gnotobiotic, defined microbiota (DM) mice have a microbiota in which all members are known and are housed in flexible film isolators to maintain this status [387]. One of the most established DM mouse models harbors the “Altered Schaedler Flora” (ASF). Developed by Dr. Rodger Orcutt and colleagues as a request from the National Cancer Institute, these eight microbial species were originally used to standardized the microbiota of the rodents used as
founders in their breeding colonies [388]. He chose to modify his mentor’s (Dr. Russ Schaedler) cocktail of organisms by eliminating facultative anaerobes (*Escherichia coli* var. *mutabilis* and *Streptococcus fecalis* and the anaerobic *Streptococcus* and *Clostridia* spp. that formed the original “Schaedler Flora”. Dr. Orcutt added four additional species (see Table 4 below). This resulted in an anaerobic community devoid of any cocci or spore-forming blunt ended rods, which comprise the main isolator contaminants, making it easier to monitor for contamination of the gnotobiotic isolator. This new community was dubbed the “Altered Schaedler Flora” and was utilized in the breeding stock by major commercial mouse vendors in the US in that era. The ASF consists of members with different morphologies so that identification using fecal smears for microscopic evaluation was possible. The members, whose full genomes have yet to be sequenced, have had 16S rRNA sequencing performed to assist with the identification of the organisms [389].

Multiple studies have demonstrated the stability of this community both when maintained under gnotobiotic housing conditions or when part of a conventionalized microbiota [390-393]. In our own lab, all eight ASF members have been stably maintained in our breeding colony for over 12 years, indicating the remarkable stability of this model microbial community over time. PCR primers have been developed for each of the eight ASF members as well as group-specific FISH probes [390, 393]. Therefore, the effects of any perturbation of the ASF, such as with antibiotics, inflammation, or CAM treatments can be monitored by bacterial abundance as well as spatial redistribution using qPCR and FISH, respectively. All of the organisms can be cultured and whole cell sonicates produced to measure the
immune response to each organism individually (something that is impossible to do with a conventional microbiota). Additionally, this community, although limited in scope, is able to synthesize all the metabolites needed by the mouse and maintains near normal cecal shape and size, something not possible in GF mice. It is important to note, however, that some of the characteristics of ASF mice are more similar to GF than conventional mice. Both ASF and GF mice have high fecal tryptic activity and possess the ability to degrade mucin and β-aspartylglycine. They also cannot convert bilirubin to urobilinogens or cholesterol to coprostanol [394]. Interestingly, this same research team compared these parameters in CD patients versus healthy subjects and the characteristics of the microbial metabolism associated with the microbiota of CD patients were very similar to those of the ASF[395]. This study suggests that there is benefit to the use of the ASF in mouse models of IBD.

Other defined microbiota mouse studies have been published [396-399]. A study using ten bacterial species specifically chosen for their metabolic function were used to colonize GF mice [396]. Using microbial RNA-seq, the authors were able to build a model relating perturbation of the microbial community to changes in diet. By modeling the functional capacity of a gnotobiotic community under different conditions, this model and similar approaches can be used to unravel the operational dynamics of the gut microbiome with respect to nutrient utilization such that the microbiota might be manipulated to improve human and animal health [396]. Another study colonized mice with *E. rectale* and *B. thetaiotamicron*, and the authors then assessed changes in bacterial gene expression using Affymetrix GeneChips to
show that the cross-talk between these two organisms affected up- and down-regulated genes in response to one another.

**Table 4.** Members of the altered Schaedler flora

<table>
<thead>
<tr>
<th>Strain</th>
<th>Identity</th>
</tr>
</thead>
<tbody>
<tr>
<td>ASF356</td>
<td>Most closely related to <em>Clostridium propionicum</em> (92 % max identity) [400]</td>
</tr>
<tr>
<td></td>
<td>Member of <em>Clostridium</em> cluster XIV</td>
</tr>
<tr>
<td>ASF360</td>
<td><em>Lactobacillus intestinalis</em> (99 % max identity) [401]</td>
</tr>
<tr>
<td>ASF361</td>
<td><em>Lactobacillus murinus</em> [400]</td>
</tr>
<tr>
<td>ASF457</td>
<td><em>Mucispirillum schaedleri</em> [402]</td>
</tr>
<tr>
<td>ASF492</td>
<td><em>Eubacterium plexicaudatum</em> [403]</td>
</tr>
<tr>
<td>ASF500</td>
<td><em>Clostridium</em> sp. with no known related organism in GenBank Database [400]</td>
</tr>
<tr>
<td>ASF502</td>
<td>Most closely related to <em>Ruminococcus gnavus</em> (92 % max identity) [400]</td>
</tr>
<tr>
<td></td>
<td>Member of <em>Clostridium</em> cluster XIV</td>
</tr>
<tr>
<td>ASF519</td>
<td><em>Parabacteroides goldsteinii</em> (99 % max identity) [404, 405]</td>
</tr>
</tbody>
</table>

Three human commensals, *E. coli, B. longum, and L. johnsonii*, have also been used to colonize GF mice to create a gnotobiotic community [397]. This community was used to identify the effects that the introduction of novel organisms has on the community dynamic. When colonized with a second *Lactobacillus* sp., *L. paracasei*, it was noted that both *Lactobacillus* spp. were able to co-habitate reaching similar fecal titers. However, that was not the same when a fifth organism
was added, a second *B. longum* strain. This new strain was only maintained in the mouse at detectable levels for three days. Lastly, a second *E. coli* strain was added to the original community of three. This new *E. coli* reached the highest titer of any organism in the community. This addition caused the reduction of the original *E. coli* to drop to undetectable levels at day two post-addition. Later *L. johnsonii* also reached undetectable levels. *B. longum* decreased initially but then returned to normal titer levels. Lastly, when the original tri-associated mice were exposed to conventional mouse feces, the numbers of the original three organisms decreased.

In this chapter, we have discussed the three most common types of microbiota available for use in IBD research. Because it is clear that IBD results from an imbalance in the microbial community, the use of GF mice (which cannot mimic a "community" dynamic) may be less useful in unraveling the complexities of a multifactorial disease in place of defined microbiota and conventional microbiota mice. Ideally one would want to use a simplified community (such as the ASF) where the actions of all organisms can be assessed. To understand the dynamic interactions that occur between microbes and between the microbes and the host, it will be important to start with what is known in order to begin the unraveling the enigmatic nature of gut health and disease.

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Chapter 2

Impact of antibiotics on the susceptibility to inflammatory insults:
lessons from defined and conventional microbiota mice

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Abstract

The gastrointestinal tract harbors bacteria on which the body depends on for a variety of functions such as vitamin production and energy storage. This community is susceptible to changes from environmental factors such as antibiotics. The effect of the antibiotic on the microbial community lasts longer than the duration of treatment and in some cases the community may never fully re-establish itself. When the community deviates from normal it becomes dysbiotic, which may lead to the development of diseases such as inflammatory bowel disease. We report here the study of two intestinal microbial communities to identify differences in disease severity in response to a DSS-induced model of murine colitis. ASF and CONV mice were given 4 mg/mL of ampicillin for nine days. Mice were then colonized with *Helicobacter bilis* for two weeks. Then either a 1.5 % (ASF) or 2.5 % (CONV) dextran sulfate sodium solution was added to their drinking water for five days followed by a 4 day restitution period. The results of this study revealed major differences in the inflammatory response after the induction of colitis. The administration of ampicillin caused the ASF mice to gain significantly less weight during the study compared to their CONV counterparts. Macropscopic and microscopic lesions within the two communities showed no change in score when comparing ampicillin *H. bilis* DSS (AhbD) and *H. bilis* DSS (Hbd). Ampicillin administration modulated the microbiota of the ASF mice much more drastically compared to the CONV mice. In the ASF mice, 4 of the 8 species present in the community were significantly reduced at the end of the study. ASF mice produced significantly higher levels of pro-inflammatory cytokines compared to the CONV mice.
and also produced significantly more antibody against members of the microbiota. CONV mice had an increased concentration of several diabetes biomarkers, something not seen in the ASF mice.

The results of this study reveal that the severity of the immune response to colitic insults is microbiota specific. Mice with a simplified microbiota had increased antibody responses to the microbiota and increased pro-inflammatory cytokine/chemokine production. CONV mice, which had only a minimal inflammatory response, had an increase in diabetic biomarkers indicating that these mice were entering into a metabolic syndrome-like state.

Introduction

The lower gastrointestinal (GI) tract is home to $10^{11}-10^{12}$ organisms per gram of colonic contents. The microbes that dwell in this intestinal ecosystem harbor 150 times more genes than are present in the human body [1-3]. These bacteria aid their host in nutrient acquisition, vitamin production, metabolism of indigestible foodstuffs and the development of a mature immune system [4, 5]. Therefore, it is obvious that the GI microbiota (i.e., the commensals), with which we share our bodies, provide a great service and help maintain GIT health.

The gut microbiota is now even being considered as a therapeutic target for conditions such as obesity [6]. It has been well established now that the microbiota of lean and obese persons differ [7-9]. Obesity has many associated complications such as type 2 diabetes and cardiovascular disease and also induces a low-grade inflammatory response [10, 11]. A study by Qin et al. revealed that those persons
with type 2 diabetes have an altered microbiota compared to healthy controls [12]. These changes include a decrease in butyrate-producing organisms and an increase in opportunistic pathogens. This and other studies indicate a role for the microbiota in these metabolic diseases [12-14].

This complex microbial community maintains its composition via a delicate balance that can be readily altered by multiple external factors, including infections, dietary changes and drug treatments [4, 15, 16]. Alterations to this balance can have severe consequences for the host, including disruption of normal physiological processes and immune dysregulation [17]. We have long known that antibiotic use has a tremendous impact on the GI microbiota [18]. However, recent advances in next generation sequencing have allowed us to more fully appreciate the significant changes in the composition of GI microbial communities following antibiotic use. In some scenarios, the community never fully returns to its original state even after antibiotic treatment is ceased [19-21]. Antibiotic use significantly alters the composition of the microbiota and also enhances host susceptibility to GI infection and increases the predominance of antibiotic resistance genes and organisms, including Salmonella and adherent and invasive E. coli [21-28]. Disruption of the gut microbiota following antibiotic use is gaining additional attention as a predisposing event in the development of inflammatory bowel disease (IBD) [29-32]. The etiology of IBD is complex; disease is thought to develop in a genetically susceptible host after exposure to an environmental trigger (or triggers) that induces an aberrant immune response directed against and perpetuated by antigens derived from the resident gut microbiota [33-37]. A variety of reports, including those utilizing animal
models, clinical studies and epidemiological data implicate the resident microbiota, including GI infections, in the pathogenesis of IBD. Significant differences in the microbiota composition of IBD patients and healthy controls have been documented [38-40]. The dysbiotic community observed in IBD patients is generally characterized by a contraction of *Firmicutes* and *Bacteroidetes*, expansion of *Proteobacteria*, reduced microbial diversity and increased presence of mucosa-associated organisms [41-47].

Genetically engineered mice do not develop spontaneous colitis unless they are associated with a microbiota [48, 49]. Work by Cadwell et al. detailed the enhanced susceptibility of *Atg16L1* deficient mice infected with a norovirus to a subsequent inflammatory insult administered in the form of DSS [50]. Finally, transient infections with pathogenic enteric organisms, such as *Salmonella* spp., *Campylobacter* spp. and parasites, can serve as triggers to initiate inflammatory responses that are perpetuated by commensal bacterial antigens [51]. Clearly, the composition of the gut microbiota, including the presence of GI pathogens, contributes to the pathogenesis of IBD.

Previous work from our laboratory has shown that colonization of defined microbiota mice with *Helicobacter bilis*, a model mouse GI pathogen, induces host immune responses against antigens derived from the resident microbiota and alters mucosal gene expression profiles in immunocompetent mice [52-54]. We have also demonstrated that prior colonization with *H. bilis* increases host susceptibility to colitis following a subsequent inflammatory insult with a very low dose of the inflammatory trigger, dextran sulfate sodium (DSS) [55]. This dose of DSS alone is
not sufficient to elicit colitis. In this present study, we examine the impact of antibiotic pretreatment on host susceptibility to *H. bilis* infection and subsequent inflammatory insults in C3H mice with either a defined or conventional microbiota.

In contrast to previous reports describing exacerbated disease in mice treated with antibiotics prior to colonization with a GI pathogen [26, 56] pretreatment of defined and conventional microbiota mice with ampicillin prior to *H. bilis* colonization did not enhance host susceptibility to an inflammatory insult administered in the form of low-dose DSS. Despite the apparent lack of differences in disease severity, ampicillin pretreatment enhanced local inflammation as well as the humoral response against the resident microbiota in defined flora mice but not in mice with a conventional microbiota. Ampicillin pretreatment also significantly reduced the density of most of the resident bacterial species in the defined flora mice. Also of note were elevated serum levels of several biomarkers associated with metabolic syndrome following the onset of disease in conventional flora but not defined microbiota mice. Together, these data indicate that antibiotic treatment prior to infection with a GI pathogen may not enhance susceptibility to a subsequent inflammatory insult, but it does change the nature of the host’s immune response and metabolism depending on the complexity of the microbiota.

**Materials and Methods**

**Animals**

Male and female C3H/HeN:Tac mice (6-8 weeks old) defined microbiota (DM) mice colonized with the altered Schaedler flora (ASF) were obtained from Taconic
Farms (Germantown, NY). The ASF includes ASF356, a member of the *Clostridium* cluster XIV; ASF360, *Lactobacillus intestinalis* [57], ASF361, *Lactobacillus murinus* [58], ASF457, *Mucispirillum schaedleri* [59], ASF492, *Eubacterium plexicaudatum* [60]; ASF500, a low-G+C content Gram positive bacteria; AF502, a member of the *Clostridium* cluster XIV; and ASF519, *Parabacteroides goldsteinii* [61, 62]. Mice were bred and maintained in the murine gnotobiotic facility at the College of Veterinary Medicine, Iowa State University. Mice were housed in flexible film isolators to maintain their defined status and allowed *ad libitum* access to an irradiated 2919 diet from Harlan Teklad (Madison, WI) and sterile water. One week prior to the start of the experiment, mice were moved to Innocage® IVC cages supported by an Innovive IVC rack (San Diego CA). Mice were allowed *ad libitum* access to an autoclaved 2019S diet from Harlan Teklad (Madison, WI) and sterile water.

Male and female C3H/HeN (6-8 weeks old) conventional microbiota (CONV) mice were obtained from Harlan Laboratories (Dublin, VA). These mice were confirmed positive for the eight ASF members prior to purchase. Mice were maintained in Innocage® IVC cages supported by an Innovive IVC rack (San Diego, CA). Mice were allowed *ad libitum* access to an autoclaved 2019S diet from Harlan Teklad (Madison, WI) and sterile water. Throughout the study, both ASF and CONV mice were maintained in the same room (12:12 hours dark/light cycle) with a maximum of five mice per cage. All animal procedures were performed in accordance with the experimental protocol approved by the Iowa State University Institutional Animal Care and Use Committee.
Experimental Design

Mice were administered either 4 mg ampicillin sodium salt/mL of sterile water (Sigma-Aldrich, St. Louis MO) or sterile water for nine days. Ampicillin and water were changed daily to ensure freshness. Cages were changed during ampicillin administration to prevent the mice from re-colonizing themselves via coprophagy. On the 9th day, mice were given sterile water and 24 hours later they were colonized 0.3 mL of $10^9$ organisms of Helicobacter bilis. Mice were inoculated on two consecutive days. On the 10th day after colonization (i.e., experimental day 20), fecal samples were collected to confirm H. bilis colonization via PCR analysis as previously described [63]. Fourteen days after colonization, mice were given either 1.5 % (ASF) or 2.5 % (CONV) dextran sodium sulfate (DSS) (MW 36,000-50,000; MP Biomedicals, Solon, OH) in their drinking water for five days followed by four days with sterile water prior to terminating the experiment. The experiment lasted a total of 33 days and the timeline is shown in Figure 1. Mice that were treated with ampicillin, colonized with H. bilis, and treated with DSS will be referred to as AHbD and mice colonized with H. bilis and treated with DSS will be referred to as HbD.
**Figure 1.** Schematic diagram of the experimental plan

**Helicobacter bilis colonization**

*H. bilis* was grown in a biphasic growth medium consisting of a Brucella agar base (BD, Franklin Lakes, NJ) supplemented with 0.1 % activated charcoal (Acros Organics, Geel, Belgium), 20 % heat inactivated newborn calf serum (Hyclone Lab, Logan, UT), 2 % Vitox (Oxoid, UK) and 1.25 % agar (Fisher Scientific, Pittsburgh PA). A 3 ml overlay of brucella agar base supplemented with 20 % heat inactivated newborn calf serum, 1 % IsoVitaleX (BD, Franklin Lakes, NJ), and 1.3 % urea agar base (BD, Franklin Lakes, NJ) was used over the agar base and was the medium inoculated with the organism. Plates were incubated at 37°C in a microaerophilic chamber in which the atmosphere consisted of 85 % N₂, 10 % CO₂, and 5 % O₂.

**Sample Collection and Preparation**

Blood was collected at defined time-points via the saphenous vein. At necropsy, blood was collected via cardiac puncture following euthanasia via CO₂ asphyxiation. Following collection, blood samples were allowed to clot at 4°C.
overnight, and serum was separated by centrifugation and stored at -20 °C until used. The cecum was aseptically excised and contents were snap frozen in liquid nitrogen for DNA extraction and stored at -20°C. Cecal tissues devoid of contents were placed in either 10 % buffered formalin or RNAlater® (Ambion, Grand Island NY) for histopathological evaluation and gene expression studies. During the necropsy, sections of cecal tissue were placed in phosphate buffered saline to maintain hydration. Following the necropsy, the tissue was cut into three 1-cm sections and placed in wash medium (RPMI 1640 supplemented with 50 µg gentamicin sulfate/ml and 20,000 IU/20,000 µg of penicillin/streptomycin) and placed on a rocker for 30 min at 25°C. All cell culture reagents were from Cellgro, Manassas, VA, unless otherwise noted. At the end of the incubation period, the tissues were placed into 96 well plates containing explant medium (RPMI 1640 supplemented with 20,000 IU/20,000 µg of penicillin/streptomycin, 50 µg gentamicin sulfate/ml, 2 mM glutamine, 50 µM 2-mercaptoethanol, and 1 mM sodium pyruvate). Tissues were incubated overnight at 37°C in a humidified atmosphere with 5 % CO₂. The following day, supernatant fluids were aspirated, placed in a new 96-well plate, and stored at -20°C.

**Macroscopic assessment of cecal lesions**

Macroscopic lesions of the cecum were scored, noting the presence of atrophy, enlarged cecal tonsil, diarrheic luminal contents, presence of fresh blood, gross thickening (edema) of tissue. Lesion severity scores were based on the total number of observable macroscopic lesions with a maximum score of 5 indicative of severe disease.
Histopathological assessment

Fixed tissues were embedded in paraffin, sectioned and stained with hematoxylin and eosin (H&E) for light microscopic evaluation. Stained sections of each cecum were scored in a blinded fashion by a Board-certified veterinary pathologist (J. Hostetter) as previously described [52, 53]. The final histological score represented the numerical sum of 5 parameters with each parameter scored from 0-5 (0= no lesion, 5= maximum severity). The maximum cumulative score was 25. Histopathologic parameters evaluated included mucosal ulceration, magnitude of lamina propria infiltration and character of inflammatory cells, mucosal edema, stromal collapse, and crypt hyperplasia.

Cytokine/Chemokine Quantification

Supernatants from the cecal tissue explants were used to measure the local chemokine concentration using a murine multiplexed antibody array according to the manufacturer’s instructions (Millipore, Billerica MA). Analytes measured were: Eotaxin, G-CSF, GM-CSF, IFN-γ, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12(p40), IL-12(p70), IL-13, IL-15, IL-17, IP-10, KC, LIF, LIX, MCP-1, M-CSF, MIG, MIP-1α, MIP-1β, MIP-2, RANTES, TNF-α, and VEGF. The mean fluorescence intensity (MFI) values were converted to analyte concentrations using known standards and commercial software (xPONET®).

Serum Diabetes Analyte Quantification

Measurement of diabetes analyte concentrations in the serum of mice collected at necropsy was performed using a murine multiplexed antibody array according to the manufacturer’s instructions (Bio-Rad, Hercules, CA) and a Luminex
200 (Luminex Corp., Madison WI). The mean fluorescence intensity (MFI) values were converted to analyte concentrations using known standards and commercial software (xPONET®).

**Antigen-specific antibodies**

An ELISA was performed to determine the presence of *H. bilis*- and ASF-specific antibodies (total IgG H&L) in the sera as previously described [52].

**DNA Extraction and qPCR for bacterial quantification**

Cecal DNA was extracted using an UltraClean Fecal DNA Isolation kit according to the manufacturer's instructions with a few modifications (MoBio Laboratories Inc, Carlsbad CA). Those modifications include: adding 20 µL of proteinase K (20 mg/mL) to the sample along with solution S1 and the bead solution and heating the sample to 55°C for 1 hour in the beginning of the procedure, using solution CB3 instead of S3 to bind DNA to the silica column, and rinsing the column twice with 70 % ethanol instead of once. After extraction, DNA was quantified using Quant-it™ PicoGreen® (Invitrogen, Grand Island NY) according to the manufacturer's instructions. All DNA samples were diluted to 0.8678 ng/µL with nuclease free water. One µL was used as template in a PCR using Platinum® SYBR® Green qPCR SuperMix-UDG and with a final MgCl₂ concentration of 4.0 mM (Invitrogen, Grand Island NY). All samples were run in duplicate using the primers in Table 1. The qPCR conditions were 2 min @ 50°C, 10 min @ 95°C, and 40 cycles of 95°C for 15 seconds, 60°C for 60 seconds. The thermocycler was an Applied Biosystems® 5700. The results were compared to qPCR results from plasmid standards of the 16S rRNA genes to obtain final values of bacteria per gram of cecal contents. The
plasmids were kindly supplied by Dr. Martin Polz and the standards created as previously described [64].

### Table 1. ASF Primer Sequences, qPCR parameters and operon numbers

<table>
<thead>
<tr>
<th>Strain</th>
<th>GenBank Number</th>
<th>Species or Group</th>
<th>Primer&lt;sup&gt;A&lt;/sup&gt;</th>
<th>Standard Curve</th>
<th>Operon number&lt;sup&gt;B&lt;/sup&gt;</th>
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</thead>
<tbody>
<tr>
<td>ASF356</td>
<td>AF157052</td>
<td><em>Clostridium sp.</em></td>
<td>F AAGCCCGCGTTGAAAGGAA (800)&lt;br&gt;R ACGYTGGGCTCCCCCTCAGTT (800)</td>
<td>-3.33&lt;br&gt;34.10</td>
<td>5</td>
</tr>
<tr>
<td>ASF360</td>
<td>AF157050</td>
<td><em>Lactobacillus sp.</em></td>
<td>F CGGCCGATGGGTGAGTAAC (800)&lt;br&gt;R GTATTAGCACCCTGGTTCCAAGTGGTA (800)</td>
<td>-3.65&lt;br&gt;36.91</td>
<td>4</td>
</tr>
<tr>
<td>ASF361</td>
<td>AF157049</td>
<td><em>Lactobacillus murinus</em></td>
<td>F GTGCGCAACCGGTGAGTAA (400)&lt;br&gt;R GCACAGCTTCCAAGTGTATCC (400)</td>
<td>-3.26&lt;br&gt;34.09</td>
<td>6</td>
</tr>
<tr>
<td>ASF457</td>
<td>AF157057</td>
<td><em>Mucispirillum schaedleri</em></td>
<td>F TGCCAAGATGAAACTCAAGGAAT (400)&lt;br&gt;R TAAGTTCTTCGCCAGCATCGA (400)</td>
<td>-3.29&lt;br&gt;33.58</td>
<td>14</td>
</tr>
<tr>
<td>ASF492</td>
<td>AF157054</td>
<td><em>Eubacterium plexicaudatum</em></td>
<td>F TGAGAGTGTGTTTTATTCGAGA (400)&lt;br&gt;R TGGCAACCTGCTGGTAGATG (400)</td>
<td>-3.44&lt;br&gt;34.64</td>
<td>4</td>
</tr>
<tr>
<td>ASF500</td>
<td>AF157051</td>
<td>low-G+C content Gram positive bacteria</td>
<td>F TCGCTAGATGCTGTATGACGACGAT (400)&lt;br&gt;R TCACACCTGCTGGTAGATG (400)</td>
<td>-3.27&lt;br&gt;34.17</td>
<td>11.2</td>
</tr>
<tr>
<td>ASF502</td>
<td>AF157053</td>
<td><em>Clostridium sp.</em></td>
<td>F ATGGCCGTTGAAACTGTTGA (400)&lt;br&gt;R CCTCGCAATCGGTTATCG (400)</td>
<td>-3.46&lt;br&gt;34.78</td>
<td>5</td>
</tr>
<tr>
<td>ASF519</td>
<td>AF157056</td>
<td><em>Parabacteroides goldsteinii</em></td>
<td>F ATGGCCGTTGAAACTGTTGA (400)&lt;br&gt;R CCTCGCAATCGGTTATCG (400)</td>
<td>-3.46&lt;br&gt;34.78</td>
<td>5</td>
</tr>
</tbody>
</table>

<sup>A</sup> Primers used were previously published by Alexander et al. [65]

<sup>B</sup> Operon number was previously determined by Sarma-Rupavtarm et al. [64]

Values in parenthesis at the end of the primer sequence indicate the nanomolar concentration of primer used

### RNA Isolation and cDNA synthesis

Total RNA from cecal tissues was extracted using a RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer’s instructions with the addition of an extra RPE wash. RNA integrity was measured using an 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). All RNA used in subsequent steps had a RIN number of 7 or higher. RNA was reverse transcribed to cDNA using RT<sup>2</sup> First Strand Kit (Qiagen, Valencia, CA).
Mouse NF-κB Signaling Pathway

For pathway expression analysis, cDNA from each treatment group was pooled and a RT² Profiler PCR array (Qiagen, Valencia, CA) was used according to the manufacturer’s instructions. The plates were run on an Applied Biosystems® 5700 machine using the cycling conditions provided in the instructions. Determination of fold change differences between treatment groups was performed using the PCR array data analysis web portal found at www.SABiosciences.com/pcraraydataanalysis.php. 84 genes were analyzed and included: Agt, Akt1, Atf1, Atf2, Bcl10, Bcl2a1a, Bcl2l1, Bcl3, Birc3, Card10, Card11, Casp1, Casp8, Ccl2, Ccl5, Cdx2, Cdx40, Cflar, Chuk, Crebbp, Csf1, Csf2, Csf3, Egfr, Egr1, Eif2ak2, Elk1, F2r, Fadd, Fasl, Fos, Hmox1, Icam1, Ifng, Ikbkg, Il10, Il1a, Il1b, Irap1, Irap2, Irf1, Jun, Lta, Ltbr, Map3k1, Map3, Myd88, Nfkbi, Nfkbi2, Nfkbia, Nod1, Raf1, Rel, Rela, Relb, Ripk1, Ripk2, Sdc20a1, Smad3, Stat1, Tbk1, Tlr1, Tlr2, Tlr3, Tlr4, Tlr6, Tlr9, Tnf, Tnfalp3, Tnfrsf10b, Tnfrsf1a, Tnfrsf1b, Tnfsf10, Tnfsf14, Tollip, Tradd, Traf2, Traf3, Traf5, Traf6

Fluorescence Imaging

To evaluate the in vivo inflammatory response, mice were intravenously administered 2 nmol of ProSense® 750 (Perkin Elmer, Walthem, MA) in 100 µL of sterile saline. ProSense® 750 is an activatable fluorescent reagent that is optically visible when the dye is cleaved by degradative enzymes, including cathepsin B, L, S, or plasminogen, that are common at sites of inflammation [66]. After 24 hours, the mice were euthanized by CO₂ asphyxiation and the ex vivo images of excised tissues were captured. Images were captured using 30-second exposures with an
excitation filter of 730 nm and an emission filter of 790 nm. All image analysis was quantified using ImageJ version 1.44 [67]. Raw images were inverted and background subtracted via a rolling ball radius of 150 pixels. The ImageJ lookup table “fire” was applied to the fluorescent exposure. Composite images were constructed using a white light exposure (0.2 seconds) to super-impose fluorescence image for tissue distinction.

**Statistical Analysis**

All values were expressed as mean ± SEM. ELISA, qPCR, and fluorescence intensity data were log transformed prior to statistical analysis to normalize the data. An analysis of variance (ANOVA) model was used for the qPCR, ELISA data, fluorescence intensity data, luminex and feed consumption and diabetes biomarker data with no adjustments. For the temporal ELISA, a repeated measures of analysis was used with treatment group, microbiota and their interaction as fixed effects. Non-parametric data were evaluated using a Wilcoxon Rank Sums test. P-values < 0.05 were considered significant for all tests. SAS software (SAS Institute Inc., Cary NC, USA) was used for all statistical calculations.

**Results**

*Treatment with ampicillin for nine days causes gross cecal changes*

During the experiment, a subset of mice were necropsied immediately after ampicillin administration to determine the effects of the antibiotic on the two microbial communities. As shown in Figure 2, both ASF and CONV mice treated with ampicillin for nine days had an increase in cecal size along with a loss of cecal
architecture resulting in a balloon-like appearance. The cecal contents of the ampicillin-treated mice had a more liquid consistency compared to that in mice given water alone; in addition, softer stools were observed for the mice treated with ampicillin. As a measure of the effect the ampicillin treatment had on the microbiota, cecal contents from both ASF and CONV mice were inoculated onto agar medium to assess the replication of aerobic bacteria; plates were incubated for 24 hours at 37°C and no growth was noted for either group (data not shown).

**Figure 2.** The administration of ampicillin for nine days cases gross changes in the cecal morphology. The changes include cecal enlargement and a loss of architecture. The result is a balloon-like sac which encloses watery cecal contents.
ASF and CONV mice exhibit different patterns in feed consumption and weight gain

Feed consumption in the mice was measured during the course of the nine day ampicillin treatment. As shown in Figure 3 A, there were no differences between ampicillin treated and control mice within the two different microbial communities. However, comparing consumption between the two microbial communities, the CONV mice ate significantly more than their ASF counterparts.

The effect of the antibiotic treatment on weight gain in the mice harboring the two different microbial communities was recorded during the study. Mice were weighed at the beginning of the study, after nine days of ampicillin treatment, and at the termination of the experiment. Figures 3B&C show the plotted weights throughout the study of the CONV mice (Figure 3B) and the ASF mice (Figure 3C). Figure 3D shows the difference in the weights during the nine days of ampicillin administration. There was no difference in the amount of weight gained over the nine days for any group of mice. Figure 3E shows the difference in weights for the duration of the 33-day experiment. The ASF mice treated with ampicillin, colonized with H. bilis and exposed to DSS (AHbD) gained significantly ($p = 0.02$) less weight throughout the experiment compared to CONV mice that received the same treatment. The mice that were not treated with ampicillin had no significant change in weight gain over the course of the experiment regardless of microbial community.
Figure 3. The ASF and CONV mice exhibit different trends in weight gain and feed consumption. (A) CONV mice ate significantly more food during ampicillin administration than their ASF counterpart. There was no difference in feed consumption within mice harboring the same microbiota but who had different treatments. Figure 3B&C show the weights of the mice used in this study potted over time. Figure D shows the difference in the weight gained during the nine days of ampicillin treatment between ASF and CONV mice. Figure E shows the differences in weight gain during the duration of the study. All results are expressed as mean ± SEM. An asterisk indicates p < 0.05.
Macroscopic cecal lesions are more severe in ASF mice compared to CONV mice while there is no difference in microscopic score

Pretreatment with ampicillin did not alter the severity of the macroscopic cecal lesions in either ASF or CONV mice colonized with *H. bilis* and exposed to DSS (Figure 4&5 A). Within treatment groups, the HbD ASF mice had a significantly (*p* = 0.0009) higher gross score compared to the HbD CONV mice (Figure 5 A). The ASF mice had an increased abundance of cecal atrophy, enlarged lymphoid aggregates, watery/mucoid contents and thickening of the cecal tissue compared to the CONV mice. Microscopically, there was no difference in the histopathology scores for the AHbD ASF compared to the AHbD CONV and the HbD ASF compared to the HbD CONV mice (Figure 5 B).
Figure 4. Both ASF and CONV mice given *H. bilis* and DSS exhibit typhlocolitis regardless of ampicillin pretreatment. This is exhibited by cecal atrophy, enlarged cecal tonsil with lymphoid aggregates, watery contents, and thickened cecal tissue.

Figure 5. Both ASF and CONV mice given *H. bilis* and DSS exhibit typhlocolitis. (A) ASF mice colonized with *H. bilis* and treated with DSS exhibited a significantly higher gross cecal score compared to CONV mice receiving the same treatment. There was no significant difference between ASF and CONV mice treated with ampicillin prior to *H. bilis* colonization and DSS treatment. (B) ASF and CONV mice regardless of treatment exhibit similar microscopic cecal scores. The results are expressed as mean ± SEM. An asterisk indicates p < 0.05.
Ex vivo imaging of the ceca shown in Figure 6, reveals a greater amount of cecal inflammation present in the AHbD ASF mice. This was confirmed quantitatively by measuring the mean fluorescence intensity of the cecal inflammation assessed using the Prosense 750 probe (Figure 7). The AHbD ASF mice had significantly more inflammation than their AHbD CONV counterparts (p = 0.01) or HbD ASF mice (p = 0.004). There was no difference in inflammation between the treatments in the CONV mice.

**Figure 6.** Both ASF and CONV mice have increased inflammation when given the colitic insult of *H. bilis* and DSS. Giving ampicillin preceding the inflammatory insult increases inflammation in the ASF mice only. This is revealed by increased fluorescence intensity present only in the cecum of the ASF mice given ampicillin, colonized with *H. bilis* and treated with DSS.
Figure 7. ASF mice pretreated with ampicillin prior to *H. bilis* colonization and DSS administration have significantly more cecal inflammation compared to similarity treated CONV mice or ASF mice only colonized with *H. bilis* and treated with DSS. The results are expressed as mean ± SEM. An asterisk indicates a significant p < 0.05. MFI=mean fluorescence intensity.

**ASF mice develop an antibody response to the microbiota after the administration of DSS**

Serum samples were collected at four time-points throughout the study; before ampicillin administration (experimental day 0), after ampicillin administration (experimental day 9), before DSS (experimental day 23), and at necropsy (experimental day 33). Note that at the first two time-points, none of the mice were colonized with *H. bilis*, but for the last two time-points, all mice were colonized (Figure 8). Comparing ASF-AHbD to ASF-HbD mice, there were no significant
differences in total serum IgG induced to either ASF 356 or 360 at any time point. At necropsy (i.e., day 33), significant ($p < 0.05$) serum antibody specific for seven of the nine bacterial antigen preparations were detected in the ASF-AHbD mice compared to the ASF-HbD mice. The only time that the ASF-HbD mice had higher antibody titers compared to the ASF-AHbD mice was at day 9 and day 23 and only against antigen from ASF519.
**Figure 8.** The antibody response to the microbiota was measured throughout the experiment. The mice given ampicillin prior to *H. bilis* colonization and DSS treatment had an increased antibody response to 7 of the 9 antigens measured. The only antigen that had a significant response in the mice colonized with *H. bilis* and treated with DSS was ASF519. Only O.D. values greater than 0.2 were used in the statistical analysis. The results are expressed as mean ± SEM. An asterisk indicates p < 0.05.

*ASF and CONV mice have different antibody responses after the administration of DSS*

Sera collected at necropsy was used to compare the induction of antibody specific for whole cell lysates of each of the eight ASF members as well as to *H. bilis*. Prior to the initiation of these experiments, it was confirmed that the CONV mice harbored all eight members of the ASF and were not colonized with *H. bilis*. The data depicted in Figure 8 indicated that the ASF-AHbD mice had significantly greater antibody responses to 7 of the 9 whole cell antigen preparations measured compared to ASF-HbD mice. CONV-AHbD mice only developed a significant antibody response to *H. bilis* (Figure 9). The CONV-HbD mice had a significantly higher antibody response to ASF361 (p = 0.002), ASF457 (p = <0.0001), ASF360 (p = 0.005), and ASF502 (p = 0.0005) compared to ASF mice that received the same treatment. The ASF-AHbD mice developed a significantly higher antibody response to only ASF492 (p = 0.02) compared to Conv-AHbD mice. With respect to the antibody response to *H. bilis* and ASF 356, the ASF mice had significantly higher
antibody responses than their CONV counterpart regardless of the treatment regimen.

Figure 9. The ASF mice had significant antibody responses to 7 of the 9 antigens measured between treatment groups. The CONV mice only had one antigen that exhibited differences between the treatment groups (H. bilis). The results are expressed as mean ± SEM. An asterisk indicates p < 0.05.
ASF and CONV mice have different patterns of microbial dybiosis after ampicillin administration

To identify the effect that ampicillin administration had on the cecal microbial community, qPCR was performed to quantify all eight ASF. Two of the ASF members, 360 and 492, had no significant differences within mouse microbial communities or within treatment groups. As shown in Figure 10, CONV-HbD mice had a significantly higher population of both ASF356 (p = 0.02) and 361 (p = 0.02) than ASF mice that received the same treatment. ASF500 and 502 shared similar patterns in distribution. Those organisms were significantly reduced in ASF-AHbD mice compared to CONV-AHbD mice. Comparing within the ASF mice, those in the AHbD treatment group had significantly reduced levels of ASF 500 and 502 compared to ASF-HbD mice. The results were similar for ASF 457 with the exception that the CONV-AHbD mice also had significantly lower levels compared to CONV-HbD mice. The levels of ASF 519 were significantly reduced in both the ASF-AHbD and CONV-AHbD mice compared to mice in their respective counterparts in the HbD treatment groups. ASF 519 levels were also significantly higher in the ASF-HbD mice compared to the CONV-HbD mice.
**Figure 10.** The administration of ampicillin causes long term changes in the microbial community of the cecum in both ASF and CONV mice. The results are expressed as mean ± SEM. An asterisk indicates a p < 0.05.
**ASF and CONV mice have different cecal cytokine responses after ampicillin administration**

To identify changes in the magnitude of the mucosal inflammatory response induced by DSS subsequent to disrupting the microbiota with ampicillin, secretion of cytokines/chemokines was measured in cecal tissue collected at necropsy (day 33). Among the analytes measured (see Materials and Methods) three patterns emerged. The first pattern was exhibited by only two cytokines/chemokines (Figure 11). Specifically, M-CSF and IL-1β were significantly elevated (p < 0.05) in CONV-AHbD mice compared to CONV-HbD mice; the same was not true between ASF-AHbD and ASF-HbD mice. In the second response pattern (Figure 12) the cytokines/chemokines (IFN-γ, LIX, MCP-1, IP-10, and IL-10) were significantly (p < 0.05) elevated in the ASF-HbD mice compared to ASF-AHbD mice. This suggests that the presence of antigens derived from the resident microbiota were involved in this response. This set of cytokines were also significantly elevated in the ASF-HbD mice compared to the CONV-HbD mice.
**Figure 11.** CONV mice pretreated with ampicillin prior to *H. bilis* colonization and DSS treatment exhibit increased cytokine/chemokine concentrations compared to ASF mice that received the same treatment and CONV mice colonized with *H. bilis* and treated with DSS. The results are expressed as mean ± SEM. An asterisk indicates p < 0.05.

**Figure 12.** ASF mice colonized with *H. bilis* and treated with DSS have elevated cytokines/chemokines concentrations compared to CONV mice given the same treatment or ASF mice pretreated with ampicillin prior to *H. bilis* colonization and DSS treatment. The results are expressed as mean ± SEM. An asterisk indicates p < 0.05.
With respect to the third pattern of cytokine expression (Figure 13) the cytokines/chemokines (Eotaxin, G-CSF, GM-CSF, IL-1α, TNF-α, MIP-1β, MIG, LIF, KC, IL-6, IL-12p40, and IL-12p70) were significantly (p < 0.05) elevated in only the ASF-HbD mice compared to the CONV-HbD mice. Since there was no significant difference in the concentrations of these analytes between the ASF-AHbD and ASF-HbD, this suggests that the microbiota does not play a role in this response.
**Figure 13.** ASF mice treated with HbD have elevated cytokines/chemokines concentrations compared to CONV mice given the same treatment.

The results are expressed as mean ± SEM. An asterisk indicates p < 0.05.

**CONV mice have increased levels of diabetes biomarkers during inflammation compared to ASF mice**

To identify the role that disruption of the microbiota and inflammation may have on the development of metabolic syndrome, eight diabetes biomarkers were measured at the termination of the study. The groups analyzed were HbD, AHbD, control, and ampicillin only (mice treated for nine days and allowed to recover throughout the rest of the study). Of the eight analytes measured, only leptin showed no significant changes between CONV and ASF mice for any of the treatment groups. For the other seven biomarkers only the CONV mice showed increased levels compared to the ASF mice (Figure 14). Inflamed CONV mice (those given HbD or AHbD) had significantly (p < 0.05) increased levels of GLP-1, Glucagon, Resistin, and Ghrelin. The CONV-AHbD mice had significantly increased levels of Insulin and GIP while the CONV-HbD mice had increased levels of these hormones that were trending towards significance compared to their ASF counterparts. Lastly, PAI-1 was found to be significantly (p < 0.05) increased in all CONV mice compared to their ASF counterparts regardless of their treatment status.
**Figure 14.** Inflamed CONV mice have elevated levels of diabetic biomarkers in their sera compared ASF mice.

The results are expressed as mean ± SEM. An asterisk indicates p < 0.05, a # indicates p < 0.1.
Differences in microbiota cause differences in gene expression on the host side

To determine the effects that the two types of inflammation had on the two microbial communities, a NF-κB signaling pathway superarray was used to examine gene expression changes in the cecum (Table 2). See materials and methods for a list of the genes measured.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Gene</th>
<th>Fold Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>ASF HbD compared to CONV HbD</td>
<td>Ccl2</td>
<td>+3.13</td>
</tr>
<tr>
<td>ASF HbD compared to CONV HbD</td>
<td>Csf3</td>
<td>+7.30</td>
</tr>
<tr>
<td>ASF HbD compared to CONV HbD</td>
<td>Il1b</td>
<td>+4.14</td>
</tr>
<tr>
<td>CONV AHbD compared to CONV Hbd</td>
<td>Csf3</td>
<td>+4.57</td>
</tr>
<tr>
<td>ASF AHbD compared to ASF HbD</td>
<td>Tbk1</td>
<td>+3.88</td>
</tr>
</tbody>
</table>

First differences between mice with different intestinal microbiota were examined. Comparing ASF AHbD to CONV AHbD mice revealed no changes in any of the 84 genes measured. ASF HbD compared to CONV HbD mice revealed three genes whose mRNA was up-regulated in the ASF mice. Ccl2 (MCP-1) was up-regulated 3.13 fold which corresponded to what was seen in the luminex data. In the luminex data, MCP-1 was significantly elevated in the ASF HbD mice compared to the CONV HbD mice. It was also noted that G-CSF was up-regulated 7.3 fold in the ASF mice, which corresponded to what was seen in the luminex data. In the luminex data, G-CSF was significantly elevated in the ASF HbD mice compared to the CONV
HbD mice. Lastly, the IL-1β-specific mRNA expression was up-regulated 4.14 fold in the ASF-HbD compared to the CONV-HbD mice.

When comparing the two ASF groups to each other and the two CONV groups to each other, CONV AHbD mice had one gene whose mRNA was up-regulated compared to CONV HbD mice. This gene G-CSF, was increased 4.57 fold in the superarray results. In the ASF mice, there was one gene whose mRNA was elevated in the AHbD mice 3.88 fold compared to the HbD mice. This gene, TANK-binding kinase 1 has been found to activate NF-κB through interactions with TANK and TRAF2 proteins [68].

**Discussion**

The results of this study indicate that the composition of the microbiota plays a role in the host’s response to inflammation. The conventional mice that received ampicillin prior to colitic insult had no change in inflammation compared to those that did not. The only significant differences between those two groups were in the concentrations of M-CSF and IL-1β in cecal explant supernatants, antibody to *H. bilis*, and levels of ASF457 and 519 in cecal contents. Compared to the changes in cytokine levels and the microbiota observed between the two ASF mouse groups (HbD versus AHbD), the corresponding changes in the CONV mice were minor. One potential reason for this could be because the microbiota of the CONV mice was less affected by the administration of ampicillin. The microbiota of the CONV mice is more functionally redundant than the microbiota of the ASF mice. In the CONV mice a loss or decrease of one organism may not result in a loss of the microbiota’s
functional capabilities [69]. The ASF mice had a significant reduction in 50% of their microbiota members. This loss of such a significant portion of their microbiota may also explain the difference in weight gain between the ASF-AHbD mice and the CONV-AHbD mice. The ASF mice treated with ampicillin gained significantly less weight than the ampicillin-treated CONV mice (Figure 2E). A study by Backhed et al. revealed that the microbiota has a role in regulating feed intake and weight gain [70]. In their study, conventionalized C57BL/6J mice had 42% more body fat compared to germfree (GF) controls, even though they ate 29% less feed. Conventionalization of those GF mice for 14 days caused a 57% increase in their body weight even though they consumed 27% less food than GF mice. This increase in weight was attributed to the suppression of *fiaf*, an inhibitor of lipoprotein lipase (LPL), which is responsible for the uptake of fatty acids and triglyceride accumulation. In addition, they found that after the mice were conventionalized, there was a significant increase in enzymes associated with de novo fatty acid biosynthesis [70]. The authors go on to suggest that this increase in fatty acids coupled with LPL leads to the increased weight gain seen in mice with a conventional microbiota.

Further work from Backhed and colleagues demonstrated that CONV mice fed a high fat diet gain significantly more weight than a GF cohort [71]. The GF mice also had increased levels of phosphorylated AMPK, which incites fatty acid oxidation. Additionally Ley et al. [72] has shown that the microbiota of obese and lean persons was significantly different; specifically, obese persons had a higher proportion of Firmicutes relative to Bacteroidetes as compared to lean persons. Further characterization revealed that the microbiota of obese persons was able to extract
more energy from the diet [8]. GF mice colonized with an “obese” microbiota were shown to gain significantly more weight than GF mice colonized with a “lean” microbiota, even when feed consumption was the same over 14 days.

A recent article describes a link between antibiotic use early in life and increased adiposity [73]. In our study, we found that levels of biomarkers associated with metabolic syndrome were significantly increased in the inflamed CONV mice. These markers included GIP, GLP-1, insulin, glucagon, resistin, ghrelin, and PAI-1. These results indicate that CONV mice, compared to the ASF mice, seem to develop metabolic syndrome. These mice gained more weight than the ASF mice, exhibited inflammation, as well as high levels of the majority of the diabetic biomarkers measured. GIP and GLP-1 are incretin hormones and are made by K and L cells, respectively, in the intestine [74]. Both of these hormones are able to stimulate the production of insulin, a response seen in CONV mice that received AHbD or HbD compared to their ASF counterparts. Glucagon has the opposite effect of insulin; glucose promotes insulin release and inhibits the production of glucagon [75, 76]. It has been reported that in diabetes mellitus, there appears to be an abnormal response of glucagon to the presence of glucose [77, 78]. In one study, patients with diabetes mellitus had elevated plasma concentrations of both insulin and glucagon, results that are similar to what is seen in Figure 14 [79]. Resistin has been found to be significantly increased in patients with type 2 diabetes (T2DM) and may have a role in the development of insulin resistance and cardiovascular inflammation [80-84]. It has also been noted that resistin may play an role in the inflammatory process of IBD [85]. PAI-1, GLP-1, glucagon, and insulin, markers
increased in the inflamed CONV mice, have found to be significantly increased in patients with metabolic syndrome and/or T2DM [77-79, 86-88]. High concentrations of PAI-1 have been shown to predict the development of diabetes [89]. Additionally, high PAI-1 is associated with insulin resistance as well as thrombotic vascular disease [90-92]. The last biomarker that was increased in the inflamed CONV mice was ghrelin. The role of this hormone in GI inflammation is still unclear. In some cases, ghrelin is able to ameliorate both DSS and TNBS-induced murine models of colitis [93, 94]. However, levels of ghrelin and resistin have found to be increased in IBD patients [95]. One of the reasons that the CONV mice developed metabolic syndrome and the ASF mice did not may be due to differences in the microbiota composition. It has been postulated that lipopolysaccharide (a component found in Gram negative organisms) may be a factor involved with the onset of metabolic diseases [96]. Of the nine organisms in the mice used in the study only three of them are Gram negative. The CONV mice have a much greater proportion of their microbial community containing LPS that could contribute to the onset of metabolic syndrome.

The results of the studies with the CONV mice reveal that administration of ampicillin prior to colitic insult does not change the severity of the inflammatory response. These mice only had significant production of total IgG H&L antibody to *H. bilis* antigen and no difference in weight gain and no change in mucosal inflammation based on analyses using Prosense 750 that is an inflammatory probe between CONV AHbD and HbD groups. The CONV mice treated with AHbD did have significantly reduced amounts of ASF457 and 519 and significantly elevated
concentrations of M-CSF and IL-1β. Additionally, these CONV mice also showed a pre-disposition toward metabolic syndrome and/or T2DM as indicated by the increased concentrations of diabetes biomarkers in the CONV mice that were inflamed.

The results in the ASF mice given AHbD compared to HbD were strikingly different than those found in CONV mice given the same treatments. The mice treated with ampicillin prior to colitic insult had greater cecal inflammation as indicated by the significantly elevated levels of activation of the inflammatory probe Prosense 750 in the cecum. However, this increase did not translate into enhanced microscopic evidence of disease. The histopathological scores for all of the mice examined in the study ranged between 10-11.8. Compared to control mice whose scores were 0.2 (CONV) and 0.4 (ASF), the microscopic scores of the AHbD and HbD mice indicate that both groups of mice (CONV and ASF) were severely inflamed. There was no significant difference in histopathologic score among any of the treatment groups. However, when comparing inflamed ASF mice to inflamed CONV mice, the ASF mice produced greater amounts of pro-inflammatory cytokines/chemokines compared to the CONV mice. The lack of a demonstrable difference in the histopathological scores indicates that there must be an inflammatory ceiling in regards to the parameters measured.

Measuring the antibody response to the members of the ASF over time revealed that with the exception of ASF519, the only significantly elevated responses were found in the AHbD mice after the onset typhlocolitis. After administration of ampicillin, mice had significantly reduced antibody to ASF519, most
likely due to a significant decrease in the organism. Previous work in our lab has shown that the administration of ampicillin causes a decrease in all the ASF organisms to undetectable levels (data not shown). That reduced antibody response to ASF519 was maintained even after *H. bilis* colonization; however, after the administration of the DSS, the antibody to members of the microbiota in the AHbD mice became significantly increased compared to the HbD mice.

Cytokine/chemokine levels for several of the analytes measured were higher in the ASF mice compared to the CONV mice, indicating that the ASF mice were experiencing more severe inflammation. Additionally, some of the anti-ASF antibody responses were increased in the ASF HbD mice compared to the ASF AHbD mice. This result may simply be due to a lack of antigen to produce the response in the AMP treated mice. The microbiota of the AMP-treated ASF mice had not returned to the pre-AMP levels during the duration of the experiment. At necropsy, there were four ASF members that had a significant reduction in numbers in the AHbD mice compared to the HbD mice: ASF 500, 502, 457, and 519. It has been noted that during an acute GI infection, CD4+ T cells will break tolerance to the commensal bacteria and become reactive to specific antigens derived from resident microbiota. In the AHbD mice, four members of the microbiota were significantly decreased; consequently, there may have not been enough antigen present to stimulate the production of antigen-specific CD4+ T cells. Studies have shown that GF mice have a decreased number of CD25+ CD4+ T cells and those regulatory T cells present have a reduced suppressive function [97, 98]. Therefore it is possible that in the ASF mice, whose microbiota is not as diverse as one found in a CONV mouse, there
would be a decrease in these cells as well. Future studies would be needed to determine the quantity of T\textsubscript{reg} cells in the two groups of mice.

Collectively, the results of this study reveal the importance of the complexity of the microbiota during inflammatory processes. Ampicillin pretreatment did not make ASF or CONV mice more susceptible to an inflammatory insult following colonization with H. bilis. However, ampicillin pretreatment did enhance the local inflammatory response as well as the humoral response against the resident microbiota in defined flora mice but not in mice with a conventional microbiota. Ampicillin pretreatment also significantly reduced the density of most of the resident bacterial species in the defined flora mice. CONV mice, who have a more complex microbiota developed a metabolic syndrome/diabetic condition when inflamed whereas ASF mice did not. Together, these data indicate that antibiotic treatment prior to infection with a GI pathogen may not enhance susceptibility to a subsequent inflammatory insult, but it does change the nature of the host’s immune response and metabolism depending on the complexity of the microbiota.

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CHAPTER 3

The effects of *Prunella vulgaris* and *Hypericum gentianoides* whole extract on DSS induced colitis in C3H/HeN mice

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Abstract

The morbidity and expense of traditional inflammatory bowel disease (IBD) treatments have led patients to explore alternative therapeutic options. Over 50% of IBD patients have used alternative or complementary prebiotic treatments. Botanical supplements represent a subset of alternative therapies of potential value for IBD patients. Additional research is needed to identify the potential biological activity and safety of these products. Ethanolic extracts of the botanicals *Prunella vulgaris* and *Hypericum gentianoides* possess anti-inflammatory and/or anti-microbial activities; however, limited information exists regarding its efficacy as a treatment for IBD. Using mice with a defined microbiota (the altered Schaedler flora or ASF), we have established a colitis model that mimics human IBD and enables us to elucidate the roles of both the host and its microbiota in the progression of disease and the response to nutraceutical treatment. Despite the purported anti-inflammatory effects of *P. vulgaris* and *H. gentianoides*, neither extract was able to significantly lessen disease severity. There was no significant difference in microscopic lesion scores between any of the treatment groups. Use of an inflammatory probe to measure inflammation *in situ* also revealed no significant difference between the groups, but there was a decrease in the fluorescence intensity in mice treated with *H. gentianoides* indicating the potential for an anti-inflammatory effect with that botanical. Increased production of the pro-inflammatory cytokines and chemokines, KC, IL-5, LIX, and RANTES in mice given *P. vulgaris* was not observed in the mice treated with *H. gentianoides*. Mice administered *H. gentianoides* did have a substantial modulation of their gastrointestinal microbial community as *H.
gentianoides treatment resulted in a significant decrease in four of the eight members of the ASF. P. vulgaris had no effect on the bacterial population. In summary, there was no significant amelioration of disease severity in this mouse model of colitis. The presented data do suggest, however, that there may be benefit to the use for H. gentianoides in the modulation of the gastrointestinal microbial community. Further studies are warranted to determine the specific compounds associated with this anti-bacterial activity.

**Key words:** Hypericum gentianoides/Prunella vulgaris/ altered Schaedler flora/Helicobacter bilis

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**Introduction**

Inflammatory bowel disease is a condition that encompasses both Crohn’s disease (CD) and ulcerative colitis (UC). This condition has no specific cause but is known to have genetic, bacterial, environmental, and immunological components [1-3]. Although there is no cure disease, several drug therapies are designed to reduce the inflammation and put the patient into remission [4]. Unfortunately, many of these treatments are not without side effects [5]. One type of therapy that is gaining popularity among patients is the use of complementary and alternative medicines (CAM) [5, 6].

Studies surveying the use of CAM in IBD patients have revealed that up to 74 % of those surveyed did indeed use a CAM at some point to treat their disease [7, 8]. Some of the reasons given for choosing to use CAM include ineffectiveness of conventional therapy (40 % persons surveyed), greater sense of control over the
disease (30 % persons surveyed), safety of therapy (28 % persons surveyed), and perceived adverse effects with conventional therapy (18 % persons surveyed) [7]. In categorizing the types of CAM utilized, Chinese herbal medicine was used by 15 % of those surveyed [7].

Herbal medicines can have multiple mechanisms of action with regard to treatment of IBD [5, 9]. Some of these actions include the reduction of oxidative stress, inhibition of leukotriene B4, antimicrobial effects, and the inhibition of the pro-inflammatory transcription factor NF-κB [9]. A study by Jackson et al. characterized the effects of an herbal remedy used by Chinese physicians to treat IBD in a DSS-induced model of colitis [10]. This remedy consisted of a mixture of seven different herbs: *Forsythia koreana, Corydalis saxicola, Semiaquilegia adoxoides, Taraxacum officinale, Chrysanthemum coronarium, Glycyrrhiza inflate*, and *Lonicera japonica*. The results of this study indicated that extract treated mice had lower disease activity index scores, longer colons, and significantly reduced colonic ulcerations compared to control mice. Additionally, mice treated with the extract also had decreased expression of TNF-α and IL-6 in the colonic mucosa compared to the controls. The results of this study indicate a potential role for botanical extracts in the treatment of IBD [10].

In this present study, two different botanical extracts were used to determine their effects on a DDS-induced model of colitis. *Prunella vulgaris*, also known as “heal-all,” has been shown to have some anti-microbial effects as well as quorum sensing inhibitor activity [11]. *P. vulgaris* has anti-HIV properties attributable to the compound prunellin, a partially sulfated polysaccharide isolated from aqueous
extracts [12]. Another water-soluble polysaccharide isolated from the aqueous extract of *P. vulgaris* has shown to be effective against herpes simplex virus, possibly through inhibition of the virus particles binding to the cells [13]. Two phenolic acids can also be isolated from the organic fraction of *P. vulgaris*—caffeic acid and rosmarinic acid [14]. Caffeic acid has been shown to ameliorate disease in a DSS-induced mouse model of colitis [15]. Mice fed this phenolic acid had longer colons, reduced colonic myeloperoxidase activity, and reduced colonic and cecal microscopic lesion scores. Additionally, mRNA levels of IL-17 and iNOS were reduced in the mice given caffeic acid mice while CYP4B1 (cytochrome p450) mRNA levels were increased [15]. Rosmarinic acid, another phenolic acid found in *P. vulgaris* organic fractions, has been shown to inhibit the expression of LPS-induced COX-2 expression [16].

The other botanical extract used in this study is from *Hypericum gentianoides*. Used by Native Americans for the treatment of multiple disorders, this relative of *H. perforatum* (St. John’s Wart) does not contain the two major biological constituents of St. John’s Wart, hypericin or hyperforin[17, 18]. Using HPLC-PDA it was discovered that the chromatographic profile of *H. gentianoides* was very different compared to that of *H. perforatum* [17]. Extracts of *H. gentianoides* reduce the production of PGE$_2$ in LPS-induced macrophages and are not cytotoxic like extracts of *H. perforatum* [19]. Additional work determined that fractions of *H. gentianoides* extracts were also able to decrease nitric oxide (NO) production Uliginosin A, a specific molecule discovered in one of the fractions, was found to be anti-inflammatory by itself. Pure uliginosin A used at the same concentration found in the
whole extract was able to inhibit NO, TNF-α, and IL-1β production, but not PGE₂ [20]. Anti-bacterial properties for this compound have also been reported [21].

In this present study, we utilized our previously published multiple hit model of typhylcocolitis to examine the effects of these two botanicals on disease severity [22]. Pretreatment of *H. bilis* colonized mice with *P. vulgaris* prior to DSS treatment significantly increased production of the chemokines RANTES, KC, LIX and the cytokines IL-5 and IL-10 as compared to control *H. bilis* colonized mice administered DSS. *P. vulgaris* pretreated mice also had an increased IgG antibody response directed against ASF492, one of the eight members of the Altered Schaedler Flora. Pretreatment of *H. bilis* colonized mice with *H. gentianoides* prior to DSS treatment had a reduced antibody response against ASF519 as well as increased gross cecal scores as compared to mice not receiving the botanical extract. Of interest, ex vivo imaging using a fluorescent dye activated by cathepsins and other enzymes associated with inflammation revealed that *H. gentianoides* pretreatment tended to reduce cecal inflammation despite no macroscopic evidence of disease improvement. There was no definitive amelioration of disease following pretreatment with either botanical, but *H. gentianoides* tended to improve disease severity while *P. vulgaris* increased production of pro-inflammatory chemokines/cytokines.
Materials and Methods

Animals

Male and female C3H/HeN:Tac mice (6-8 weeks old) mice colonized with the altered Schaedler flora (ASF) were obtained from Taconic Farms (Germantown, NY). The ASF included ASF356, a member of the *Clostridium* cluster XIV; ASF360, *Lactobacillus intestinalis* [23], ASF361; *Lactobacillus murinus* [24], ASF457, *Mucispirillum schaedleri* [25], ASF492, *Eubacterium plexicaudatum* [26]; ASF500, a low-G+C content Gram positive bacteria; AF502, a member of the *Clostridium* cluster XIV; and ASF519, *Parabacteroides goldsteinii* [27, 28]. Mice were bred and maintained in the murine gnotobiotic facility at the College of Veterinary Medicine, Iowa State University. Mice were housed in flexible film isolators at Iowa State University to maintain their defined status and allowed *ad libitum* access to an irradiated 2919 diet from Harlan Teklad (Madison, WI) and sterile water. One week prior to the start of the experiment, mice were moved to Innocage® IVC cages supported by an Innovive IVC rack (San Diego CA). Mice were allowed *ad libitum* access to an autoclaved 2019S diet from Harlan Teklad (Madison, WI) and sterile water. Mice were maintained in the same room (12:12 hours dark/light cycle) with a maximum of five mice per cage. All animal procedures were performed in accordance with the experimental protocol approved by the Iowa State University Institutional Animal Care and Use Committee.
**Experimental Design**

Mice were colonized with 0.3 mL of $10^9$ organisms of *Helicobacter bilis* on two consecutive days. At 10 days post-colonization, fecal samples were collected and PCR analysis was performed in order to confirm that the mice were colonized with *H. bilis* as previously described[29]. After 16 days of colonization, mice were orally gavaged with either with *Prunella vulgaris* (2.4 mg/day) or *Hypericum gentianoides* (4.8 mg/day) ethanolic extract for 14 days. On the 7th day of botanical gavage, the mice were given 1.5 % dextran sodium sulfate (DSS, MW 36,000-50,000; MP Biomedicals Solon, OH) in their drinking water for five days followed by four days with sterile water prior to terminating the experiment.

*Helicobacter bilis* colonization

*Helicobacter bilis* was grown in a biphasic growth medium consisting of a Brucella agar base (BD, Franklin Lakes, NJ) supplemented with 0.1 % activated charcoal (Acros Organics, Geel Belgium), 20 % heat inactivated newborn calf serum (Hyclone Lab, Logan, UT), 2 % Vitox (Oxoid, UK) and 1.25 % agar (Fisher Scientific, Pittsburgh PA). A 3 mL overlay of brucella agar base supplemented with 20 % heat inactivated newborn calf serum, 1 % IsoVitaleX (BD, Franklin Lakes, NJ), and 1.3 % urea agar base (BD, Franklin Lakes, NJ) was used over the agar base and was the medium inoculated with the organism. Plates were incubated at 37°C in a microaerophillic chamber in which the atmosphere consisted of 85 % N$_2$, 10 % CO$_2$, and 5 % O$_2$. 
Extract Preparation

The dried plant materials were kindly provided by Dr. Mark Wiederlichner at the USDA-ARS North Central Regional Plant Introduction Station (NCRPIS, Ames IA). Information about the specific provenance of *P. vulgaris* accession Ames 27664 and *H. gentianoides* accession Ames 28015 obtained from the NCRPIS is available on the Germplasm Resources Information Network database at http://www.arsgrin.gov/npgs/acc/acc_queries.html. Arboreal portions of plants from *P. vulgaris* (Ames 27664), harvested in 2008 and *H. gentianoides* (Ames 28015), harvested in 2009 were prepared for storage by drying for 8 days at 38°C in a forced-air dryer with constant humidity. The dried material was ground with a 40-mesh screen and stored at -20°C until extraction. Extractions were made in 95 % ethanol (EtOH) solvent by the Soxhlet method (6 hours) [30]. Upon complete drying of the extract by evaporation, the weight of the extracted material was recorded; the residue was lyophilized and stored at -20°C until solubilized in a final working solution of 5 % EtOH (the lowest concentration of EtOH that would allow solubilization) at a final plant extract concentration of 12 mg/mL (*P. vulgaris*) and 24 mg/mL (*H. gentianoides*). The working extract was divided into 2 mL aliquots and stored at -20°C until use. Extracts from NCRPIS were screened for endotoxin by using the Limulus Amebocyte Lysate Test (BioWhittaker, Inc., Walkersville, MD) according to manufacturers’ specifications for a microplate assay, and there was no detectable endotoxin present in the extract (data not shown).
Sample Collection and Preparation

At necropsy, blood was collected via cardiac puncture following euthanasia via CO₂ asphyxiation. Following collection, blood samples were allowed to clot at 4°C overnight, serum was separated by centrifugation, and stored at -20°C until used. The cecum was aseptically excised and contents were snap frozen in liquid nitrogen for DNA extraction and stored at -20°C. Cecal tissues devoid of contents were placed in either 10% buffered formalin or RNAlater® (Ambion, Grand Island NY) for histopathological evaluation and gene expression studies. During the necropsy, sections of cecal tissue were placed in phosphate buffered saline to keep the tissues hydrated. Following the necropsy, the tissue was cut into three 1-cm sections and placed in wash medium (RPMI 1640 supplemented with 50 µg gentamicin sulfate/ml and 20,000 IU/20,000 µg of penicillin/streptomycin) and placed on a rocker for 30 min at 25 °C. All cell culture reagents were from Cellgro, Manassas VA) unless otherwise noted. At the end of the incubation period, the tissues were placed into 96 well plates containing explant medium (RPMI 1640 supplemented with 20,000 IU/20,000 µg penicillin/streptomycin, 50 µg gentamicin sulfate/ml, 2 mM glutamine, 50 µM 2-mercaptoethanol, and 1 mM sodium pyruvate). Tissues were incubated overnight at 37°C in a humidified atmosphere with 5 % CO₂. The following day, the supernatants were aspirated, placed in a new 96-well plate, and stored at -20°C.
Macroscopic assessment of cecal lesions

Macroscopic lesions of the cecum were scored, noting the presence of atrophy, enlarged cecal tonsil, diarrheic luminal contents, presence of fresh blood, gross thickening (edema) of tissue. Lesion severity scores were based on the total number of observable macroscopic lesions with a maximum score of 5 indicative of severe disease.

Histopathological assessment

Fixed tissues were embedded in paraffin, sectioned and stained with hematoxylin and eosin (H&E) for light microscopic evaluation. Stained sections of each cecum were scored in a blinded fashion by a Board-certified veterinary pathologist (J. Hostetter) as previously described [31, 32]. The final histological score represented the numerical sum of 5 parameters with each parameter scored from 0-5 (0= no lesion, 5= maximum severity). The maximum cumulative score was 25. Histopathologic parameters evaluated included mucosal ulceration, magnitude of lamina propria infiltration and character of inflammatory cells, mucosal edema, stromal collapse, and crypt hyperplasia.

Cytokine/Chemokine Quantification

Supernatants from the cecal tissue explants were used to measure the local chemokine concentration using a murine multiplexed antibody array according to the manufacturer’s instructions (Millipore, Billerica MA). Analytes measured were: Eotaxin, G-CSG, GM-CSF, IFN-γ, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12(p40), IL-12(p70), IL-13, IL-15, IL-17, IP-10, KC, LIF, LIX, MCP-1, M-CSF, MIG, MIP-1α, MIP-1β, MIP-2, RANTES, TNF-α, and VEGF. The mean fluorescence
intensity (MFI) values were converted to analyte concentrations using known standards and commercial software (xPONENT®).

**Antigen-specific antibodies**

An ELISA was performed to determine the presence of *H. bilis-* and ASF-specific antibodies (total IgG H&L) in the sera as previously described [32].

**DNA Extraction and qPCR for bacterial quantification**

Cecal DNA was extracted using an UltraClean Fecal DNA Isolation kit according to the manufacturer’s instructions with a few modifications (MoBio Laboratories Inc, Carlsbad CA). Those modifications include: adding 20 µL of proteinase K (20 mg/mL) to the sample along with solution S1 and the bead solution and heating the sample to 55°C for 1 hour in the beginning of the procedure, using solution CB3 instead of S3 to bind DNA to the silica column, and rinsing the column twice with 70% ethanol instead of once. After extraction, DNA was quantified using Quant-it™ PicoGreen® (Invitrogen, Grand Island NY) according to the manufacturer’s instructions. All DNA samples were diluted to 0.8678 ng/µL with nuclease free water. One µL was used as template in a PCR using Platinum® SYBR® Green qPCR SuperMix-UDG and with a final MgCl₂ concentration of 4.0 mM (Invitrogen, Grand Island NY). All samples were run in duplicate using the primers in Table 1. The qPCR conditions were 2 min @ 50°C, 10 min @ 95°C, and 40 cycles of 95°C for 15 seconds, 60°C for 60 seconds. The thermocycler was an Applied Biosystems® 5700. The results were compared to qPCR results from plasmid standards of the 16S rRNA genes to obtain final values of bacteria per gram of cecal contents. The
plasmids were kindly supplied by Dr. Martin Polz and the standards created as previously described [33].

**RNA Isolation and cDNA synthesis**

Total RNA from cecal tissues was extracted using an RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer’s instructions with the addition of an extra RPE wash. RNA integrity was measured using a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). All RNA used in subsequent steps had a RIN number of 7 or higher. RNA was reverse transcribed to cDNA using RT² First Strand Kit (Qiagen, Valencia, CA).

**Mouse NFκB Signaling Pathway**

For pathway expression analysis, cDNA from each treatment group was pooled and a RT² Profiler PCR array (Qiagen, Valencia, CA) was used according to the manufacturer’s instructions. The plates were run on an Applied Biosystems® 5700 machine using the cycling conditions provided in the instructions. Determination of fold change differences between treatment groups was performed using the PCR array data analysis web portal found at www.SABiosciences.com/pcraraydataanalysis.php. 84 genes were analyzed and included: Agt, Akt1, Atf1, Atf2, Bcl10, Bcl2a1a, Bcl2l1, Bcl3, Birc3, Card10, Card11, Casp1, Casp8, Ccl2, Ccl5, Cd27, Cd40, Cflar, Chuk, Crebbp, Csft1, Csft2, Csft3, Egfr, Egr1, Eif2ak2, Elk1, F2r, Fadd, Fasl, Fos, Hmox1, Icam1, Ifng, Ikbkg, Il10, Il1a, Il1b, Irak1, Irak2, Irf1, Jun, Lta, Ltbr, Map3k1, Map3, Myd88, Nfkb1, Nfkb2, Nfkbia, Nod1, Raf1, Rel, Rela, Relb, Ripk1, Ripk2, S1c20a1, Smad3, Stat1, Tbk1, Tlr1, Tlr2, Tlr3,
Fluorescence imaging

To evaluate the in vivo inflammatory response, mice were intravenously administered 2 nmol of ProSense® 750 (Perkin Elmer, Walthem, MA) in 100 µL of sterile saline. ProSense® 750 is an activatable fluorescent reagent that is optically visible when the dye is cleaved by degradative enzymes, including cathepsin B, L, S, or plasminogen, that are common at sites of inflammation [34]. After 24 hours, the mice were euthanized by CO₂ asphyxiation and the ex vivo images of excised tissues were captured. Images were captured using 30-second exposures with an excitation filter of 730 nm and an emission filter of 790 nm. All image analysis was quantified using ImageJ version 1.44[35]. Raw images were inverted and background subtracted via a rolling ball radius of 150 pixels. The ImageJ lookup table “fire” was applied to the fluorescent exposure. Composite images were constructed using a white light exposure (0.2 seconds) to super-impose fluorescence image for tissue distinction.

Statistical Analysis

All values were expressed as mean ± SEM. ELISA, qPCR, and fluorescence intensity data were log transformed prior to statistical analysis to normalize the data. An analysis of variance (ANOVA) model was used for the qPCR, ELISA data, fluorescence intensity data and luminex with a Tukey adjustment. Non-parametric data were evaluated using a Wilcoxon Rank Sums test. P-values < 0.05 were
considered significant for all tests. SAS software (SAS Institute Inc., Cary NC, USA) was used for all statistical calculations.

Results

*Neither* *P. vulgaris* nor *H. gentianoides* pretreatment *significantly reduced cecal inflammation*

Subsequent to *H. bilis* colonization, mice were treated with either *P. vulgaris* or *H. gentianoides* extract via intragastric gavage for seven days prior to DSS administration. Pre-treatment with either botanical extract had only minimal effects on gross cecal pathology (Figures 1 and 2A). *H. bilis* colonized mice pretreated with *H. gentianoides* extract prior to receiving DSS had significantly higher gross scores than the *H. bilis* DSS mice (*p* = 0.032). These mice presented with more watery/mucoid cecal contents and cecal thickening as compared to the mice receiving the vehicle control. There was no difference in the macroscopic scores between the botanical groups. All mice treated with DSS were inflamed compared to naïve mice whose average score was 0.25 (data not shown). Also, there were no significant differences in the histopathological cecal lesion scores among any of the treatment groups (Figure 2B).
Figure 1. Photographs of gross macroscopic lesions from each treatment group.

Treatment with *H. bilis* and DSS or *P. vulgaris H. bilis* and DSS had no effect grossly. The mice given *H. gentianoides H. bilis* and DSS did have significant gross changes due to the presence of water/mucoid contents and cecal thickening.
Figure 2. A) Mice given *H. gentianoides* *H. bilis* and DSS have significantly increased gross cecal scores compared to *H. bilis* and DSS only. B) There was no difference in microscopic lesion scores for any of the treatment groups. Data are expressed as mean ± SEM. Dissimilar letters indicate $p < 0.05$, while similar letter indicate no significant difference.

Independent of extract treatment, the ceca from all *H. bilis*-colonized and DSS-treated mice were highly inflamed compared to naive mice (average score of 1.1; data not shown). There were also no significant differences among the three treatment groups with regards to the mean fluorescence intensity from the inflammatory probe (Figure 3).
Figure 3. Mice treated with botanical have no difference in cecal inflammation compared to *H. bilis* DSS mice. A) Fold increase of mean fluorescence intensity of treatment compared to naïve control who received no treatment. B) *Ex vivo* images of representative ceca from the three treatment groups analyzed during the study. Data are expressed as mean + SEM. Dissimilar letters indicate p < 0.05, while similar letter indicate no significant difference.
**H. bilis colonized mice pretreated with P. vulgaris prior to DSS administration** have increased production of chemokines and cytokines in their cecal explant supernatants

Of the 32 cytokine/chemokines measured, only five were significantly changed in concentration among the treatment groups (Figure 4). *H. bilis* colonized mice pretreated with *P. vulgaris* prior to DSS administration had a significantly increased level \((p = 0.048)\) of the chemokine LIX compared to vehicle control mice. Explant supernatants from the same mice also had increased production of IL-10 \((p = 0.094)\), IL-5 \((p = 0.086)\), KC \((p = 0.071)\), and RANTES \((p = 0.062)\) whose \(p\)-values were trending towards significance. There were no significant differences in cytokine/chemokines secretion when comparing vehicle treated and *H. gentianoides* pretreated mice. A comparison of the two extract treated groups to one another revealed that mice pretreated with *P. vulgaris* secreted significantly more IL-5 \((p = 0.034)\) and tended to produce more KC \((p = 0.062)\) and IL-10 \((p = 0.067)\).
Figure 4. Cytokine and chemokine concentrations from cecal explant supernatants. Treatment with P. vulgaris increased levels of pro-inflammatory cytokines compared to the other treatment groups. Data are expressed as mean ± SEM. ** indicates p < 0.05, ## indicates p < 0.1

Pretreatment with botanical extract alters the antibody response against specific members of the resident microbiota

Serum antibody responses to the eight members of the resident microbiota and H. bilis were measured via ELISA (Figure 5). H. bilis colonized mice pretreated with P. vulgaris prior to DSS administration had a significantly elevated antibody
response against ASF492 compared to vehicle treated *H. bilis* colonized mice given DSS (*p* = 0.012), and an increased anti-ASF492 antibody response compared to *H. gentianoides* pretreated mice (*p* = 0.083) that was trending towards significance. *H. bilis* colonized mice pretreated with *H. gentianoides* and DSS produced significantly less anti-ASF519 antibody than the vehicle treated (*p* = 0.003) and the *P. vulgaris* pretreated mice (*p* = 0.008). *P. vulgaris* pretreatment did not significantly alter the anti-ASF519 response as compared to vehicle pretreatment. Botanical extract treatment did not significantly alter the antibody response to any other members of the gut microbiota, including *H. bilis* (data not shown).

**Figure 5.** Significant total IgG H&L antibody responses to specific members of the microbiota. *P. vulgaris* *H. bilis* DSS mice had elevated responses to antigen from ASF492 compared to the other treatment groups. *H. gentianoides* *H. bilis* DSS mice had significantly reduced responses to antigen from ASF519 compared to the other treatment groups. Data are expressed as mean ± SEM. Dissimilar letters indicate *p* < 0.05, while similar letter indicate no significant difference. Dissimilar letters with ** at the end indicate *p* < 0.1.
**Pretreatment with *P. vulgaris* and *H. gentianoides* extracts elicits unique host gene expression patterns**

To determine the effects of the two botanical extracts on host pro-inflammatory gene expression patterns, a NF-κB signaling pathway superarray was used to examine changes in gene expression within cecal tissues (Table 1).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Gene</th>
<th>Fold Change</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. vulgaris</em> compared to <em>H. gentianoides</em></td>
<td>Ccl2</td>
<td>-3.48</td>
</tr>
<tr>
<td><em>P. vulgaris</em> compared to <em>H. gentianoides</em></td>
<td>Il1b</td>
<td>-3.20</td>
</tr>
</tbody>
</table>

When comparing the *H. bilis* colonized mice pretreated with *H. gentianoides* prior to DSS administration to the vehicle controls, none of the 84 genes measured revealed any changes in mRNA expression. No changes were observed when comparing the *P. vulgaris* pretreated mice to the vehicle controls. Significant changes in host mRNA expression were only seen when comparing the two botanical treatments to one another. Pretreatment with *P. vulgaris* significantly decreased Ccl2 (MCP-1) mRNA expression 3.48 fold and decreased Il1b 3.20 fold as compared to *H. gentianoides* pretreatment.

**H. gentianoides is antibacterial to members of the ASF**

The members of the microbiota were analyzed for changes in quantity using qPCR. Of the eight bacteria analyzed, four showed significant changes in quantity between treatment groups (Figure 6). *H. bilis* colonized mice pretreated with *H. gentianoides* prior to DSS treatment had significantly reduced levels of ASF356, 519,
360, and 361 compared to *P. vulgaris* pretreatment. *H. gentianoides* pretreatment significantly reduced levels of ASF356 and 519 as compared to the vehicle controls. There was no difference between *P. vulgaris* pretreatment and the vehicle controls for any of the bacteria measured.

**Figure 6.** ASF members whose populations changed significantly during the study. Mice treated with *H. gentianoides* *H. bilis* and DSS had significantly reduced levels of ASF356,519,360 and 361. ** indicates p < 0.05.
Discussion

Many IBD patients are turning to the use of CAM to help treat their symptoms; however, studies describing the efficacy of these therapies is lacking [30, 36]. Of the studies that exist, most have used rodent models of colitis to assess the efficacy of certain plant-derived compounds [37-39]. Berberine, an isoquinoline alkaloid, reduces the levels of TNF, IFN-\(\gamma\), IL-27 and KC in the colon and decrease colonic inflammatory scores in DSS-treated C57BL/6 mice [37]. Dried bilberries, rich in anthocyanins (AC), reduced intestinal inflammation and alleviate colitis in BALB/c mice administered DSS [38]. Mice feed bilberries had lower levels of IFN-\(\gamma\) and TNF during acute colitis, while feeding pure AC resulted in reduced levels of IFN-\(\gamma\), TNF, and IL-6 in both chronic and acute colitis. In another study, polyphenols from apples have also been shown ameliorate DSS-induced colitis in C57BL/6 mice [39]. Mice treated with the polyphenols did not lose as much weight and had less severe microscopic inflammation scores as compared to the vehicle treated mice. Additionally, the colonic cytokine profile of the polyphenol treated mice was more similar to that of a naïve mouse than a vehicle treated mouse prior to DSS administration. Collectively, the results of these studies indicate that many plant-derived compounds have anti-inflammatory potential [5].

In this study, neither botanical investigated was able to completely ameliorate DSS-induced colitis in our multiple-hit model. Microscopically, the there was no difference in the mice pretreated with either botanical compared to the vehicle control mice. All the mice had a substantial increase in inflammation, (scores ranged from 10.2-11.6) compared to the naïve mice, whose average score was 1.1.
Macroscopically, the *H. bilis* colonized mice pretreated with *H. gentianoides* prior to DSS administration mice had significantly greater scores compared to the vehicle treated mice. The difference between the two was that in those mice pretreated with *H. gentianoides*, a greater proportion developed watery/mucoid contents and cecal thickening compared to the vehicle treated mice. However, the watery/mucoid contents may be a result of the antibacterial effect that *H. gentianoides* had on the ASF population. Similar results were seen when mice were treated with ampicillin to deplete the microbiota (Chapter 2). Although it was not significant, the *H. bilis* colonized mice pretreated with *H. gentianoides* prior to receiving DSS did have a reduced fluorescence intensity based on the *in situ* analysis inflammation compared to the other two treatment groups (Figure 3B).

The effect of *P. vulgaris* administration to these mice was actually pro-inflammatory in nature. Three chemokines were increased in the *H. bilis* colonized mice pretreated with *P. vulgaris* prior to receiving DSS compared to vehicle treated mice. Chemokines have been found to be elevated in IBD patients and are even being considered as a therapeutic target for treatment of the disease [40, 41]. KC (the mouse equivalent of IL-8) recruits neutrophils to the intestine and is elevated in mouse models of DSS-induced colitis and in IBD patients [42-44]. LIX, similar to ENA-78 found in humans, is also increased in IBD patients [45, 46]. LIX is increased in DSS-induced murine colitis, which can be ameliorated though the use of an antisense oligonucleotide [47]. Use of the antisense oligonucleotide reduced neutrophil recruitment and attenuated the gross macroscopic score in BALB/c mice given 5% DSS. Lastly, RANTES was also significantly increased in the *P. vulgaris*
pretreated mice. This chemokine is also known to elevated in IBD patients[48].
RANTES is a chemoattractant for many difference types of cells, but of interest in this study is its importance to eosinophils [49-51]. IL-5 levels were significantly elevated in the *P. vulgaris* pretreated mice; this cytokine is necessary for the development of eosinophils [52]. Recent studies have shown an increase in the number of eosinophils present in some IBD patients [53-55].

While *P. vulgaris* pretreatment was able to elicit the production of pro-inflammatory cytokines and chemokines, *H. gentianoides* pretreatment was antibacterial to half of the ASF population. The *H. bilis* colonized mice pretreated with *H. gentianoides* prior to DSS administration had significantly reduced levels of ASF356, 519, 360, and 361 compared to *P. vulgaris* pretreated mice. The levels of ASF356 and 519 were also significantly different compared to vehicle treated mice. The bacteria affected by *H. gentianoides* were a mixture of Gram-positive and Gram-negative, indicating that *H. gentianoides* may have a broad spectrum of activity.

Although there have not been any studies published regarding the antibacterial activities of *H. gentianoides* specifically, there have been reports of the antibacterial effects of *H. perforatum* as well as of the compound uliginosin, which is found in *H. gentianoides* [21, 56].

In summary, this study revealed that neither botanical was able to ameliorate the inflammation caused by the combination of *H. bilis* colonization and subsequent DSS administration. *P. vuglaris*, shown in many studies to have anti-inflammatory properties, actually induced the production of pro-inflammatory chemokines and cytokines in our murine model of colitis. Mice pretreated with *H. gentianoides* had
less cecal inflammation as measured in situ by the use of the inflammatory probe, Prosense 750. This treatment also had strong antibacterial properties as well, since 50% of the microbial population was significantly reduced compared to those mice receiving P. vuglaris. With its potential anti-inflammatory properties and substantial anti-bacterial properties, further studies using H. gentianoides are warranted to identify the specific compounds responsible for these actions.

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CHAPTER 4

Temporal dynamics of typhlocolitis in IL-10⁻/⁻ mice

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Abstract

The intestinal microbiota is centrally associated with mucosal inflammation and inflammatory bowel disease. However, the role played by enteric bacteria in initiation, progression, and/or maintenance of the gastrointestinal inflammation process is unclear. In the present study, pyrosequencing was used to identify temporal changes in the cecal microbiota associated with the onset and severity of colitis in IL-10−/− mice. This analysis revealed that the microbiota of the IL-10−/− mice contained significantly more (p ≤ 0.05) Gram-negative organisms compared to age-matched WT mice. In the IL-10−/− mice, beneficial bacteria such as Roseburia, Lactococcus, and Blautia spp. significantly (p ≤ 0.05) decreased between weeks four and seven. Beginning at four weeks of age, the host’s inflammatory response was evaluated to ascertain whether changes in the microbiota correlated with the onset of inflammation. At four weeks of age, GM-CSF, IL-2, IFN-γ, IL-12p40, RANTES, IL-1β, II-13 and IP-10 were elevated in the serum of IL-10−/− mice. At 19 weeks of age, the IL-10−/− mice began to lose weight and this was accompanied by increased G-CSF and IL-17 in the serum. Concomitantly, the Enterobacteriaceae family increased in the IL-10−/− mice comprising 9.5 % of the total cecal bacteria compared to 0.5 % in WT mice. These data suggest that the reduction of Gram-positive bacterial species along with a concomitant increase in Gram-negative organisms occurred as a result of chronic intestinal inflammation in C3Bir IL-10−/− mice.

Key words: IBD/IL-10 deficient mice /microbial composition /Enterobacteriaceae /pyrosequencing
Introduction

The inflammatory bowel diseases (IBD), which include Crohn’s disease (CD) and ulcerative colitis (UC) are chronic immunologically-mediated disorders affecting the gastrointestinal tract. The current hypothesis for the pathogenesis of IBD involves a complex interplay between mucosal immunity and environmental factors (e.g. the enteric microbiota) in a genetically susceptible host. This allows for bacteria and/or their products to directly activate pathogenic CD4+ T cells that may cause chronic intestinal inflammation [1-7].

Accumulating evidence from rodent models and human IBD patients suggests that the pathogenesis of IBD is influenced by the composition of the enteric microbiota [5, 8-10]. Compared to healthy individuals, members of the Enterobacteriaceae are increased in IBD patients with ileal CD [11-14]. Other investigators have shown a decreased concentration in members of the Clostridium clusters IV and XIVa in humans with active IBD [15-17]. It is noteworthy that members of these Clostridium clusters comprise a large portion of the gut microbiota in healthy individuals as well as produce short chain fatty acids, such as butyrate, which serve as an important energy source for colonocytes. [18-21]. Butyrate also has an anti-inflammatory effect including the ability to reduce the expression of IL-6, TNF-α, and IL-1β as well as inhibit the activation of NFκB [22-24]. It is well established that changes in the microbial composition occur in human IBD patients compared to healthy controls. However, there is a gap in our knowledge related to when during the disease progression a shift in the composition of the microbial
community occurs and whether this change precedes or follows the onset of intestinal inflammation [25, 26].

Mice deficient in IL-10 are a popular model used to study the pathogenesis of IBD [27]. These mice lack the anti-inflammatory cytokine, interleukin-10, which regulates gastrointestinal immune responses including inflammation [28]. IL-10−/− mice develop spontaneous enterocolitis of varying severity depending on the composition of the intestinal microbiota and the genetic background of the mice. Compared to conventionally housed IL-10−/− mice, IL-10−/− mice housed under specific-pathogen free (SPF) conditions have attenuated disease and mice housed under germ-free conditions do not develop disease [29-31]. These studies were performed using IL-10−/− C3H/HeJ Bir mice that are predisposed to the development of severe IBD when housed under SPF conditions [32-34].

In this study, using high throughput sequencing, histological observations, and serum cytokine concentrations, changes in the microbial composition and in host immunological responses were assessed in mice 4 - 19 weeks of age. The data indicated that an initial inflammatory response detected at four weeks of age in IL-10−/− mice preceded changes in microbial composition. This was characterized by a significant reduction in Gram-positive spp. and subsequent increase in Enterobacteriaceae in the IL-10−/−, but not WT mice. Over the 19 weeks of this study, the data indicated that there was an increase in the diversity index and the number of observed species a in the WT mice, but not in the IL-10−/− mice. The results from this study indicate that in this mouse model, the intestinal inflammation precedes the microbial changes. Those mice that were colitogenic had more Gram-negative
organisms in their microbial community which was less diverse than that of the WT controls.

Materials and Methods

Animals

Female homozygous IL-10<sup>-/-</sup> mice generated on a C3H/HeJ Bir background (C3Bir.129P2(B6)-Il10<sup>tm1Cgn</sup>/Lt) and C3H/HeJ controls were obtained from Jackson Laboratory (Bar Harbor, ME). At Iowa State University, animals were housed under SPF conditions in Innocage® IVC cages supported by an Innovive IVC rack (San Diego CA). All mice were maintained in the same room (12:12 hours dark/light cycle) with a maximum of five mice per cage. Mice were allowed ad libitum access to 2019S diet from Harlan Teklad (Madison, WI) and acidified (pH 2.8-3.2) water. All provisions were autoclaved prior to administration. Upon arrival, IL-10<sup>-/-</sup> mice were confirmed colonized with <i>Helicobacter</i> spp. using the polymerase chain reaction (PCR) performed on fecal DNA as previously described [35]. Body weights were measured three times per week during the trial. Clinical signs of disease (e.g., weight loss, diarrhea, rectal bleeding) were recorded. All animal procedures were performed in accordance with the experimental protocol approved by the Iowa State University Institutional Animal Care and Use Committee.

Sample Collection and Preparation

Randomly selected groups (n= 5-13) of female IL-10<sup>-/-</sup> mice and their WT age-matched cohorts were necropsied at 4, 7, 10, 12, and 19 weeks of age. Blood was collected using cardiac puncture following euthanasia via CO<sub>2</sub> asphyxiation. The
cecum was aseptically excised and tissue sections with contents were snap frozen in liquid nitrogen for DNA extraction and stored at -20°C. Cecal tissues devoid of contents were fixed in 10% buffered formalin and processed for histopathologic evaluation.

**Macroscopic assessment of cecal lesions**

Macroscopic lesions of the cecum were scored on the basis of atrophy, enlarged cecal tonsil, diarrheic luminal contents, presence of fresh blood, and gross thickening (edema) of tissue. Lesion severity scores were based on the number of gross lesions with a maximum score of 5, indicative of severe disease.

**Histopathological assessment**

Fixed tissues were embedded in paraffin, sectioned and stained with hematoxylin and eosin (H&E) for light microscope evaluation. Stained sections of cecum were scored in a blinded fashion by a Board-certified veterinary pathologist, (J. Hostetter) as previously described [36, 37]. The final histological score represented the numerical sum of 5 parameters with each parameter scored from 0-5 (0 = no lesion, 5 = maximum severity). The maximum cumulative score was 25. Histopathologic parameters evaluated included: mucosal ulceration, magnitude of lamina propria infiltration and character of inflammatory cells, mucosal edema, stromal collapse, 5) crypt hyperplasia.

**Serum Cytokine/Chemokine Quantification**

Blood collected by cardiac puncture was allowed to clot for 24 hours at 4°C. Serum was collected by centrifuging the blood for 10 minutes at 10,000 x G then stored at -20°C until analyzed. Measurement of cytokine and chemokine
concentrations was performed using a murine multiplexed antibody array according to the manufacturer’s instructions (Millipore Milliplex, MA). The mean fluorescent intensity (MFI) values were converted to analyte concentrations using known standards and commercial software (xPONENT®).

**Serum Amyloid A (SAA) Quantification**

The concentration of the acute phase protein, SAA, in the serum was measured by a commercial ELISA kit (Tridelta Development, Ireland) according to the manufacturer’s instructions. After completion of the reaction, the absorbance was measured at 450 nm (Spectra Max 190, Molecular Devices). The concentration of SAA in the serum was calculated based on known concentrations of SAA standards, which were evaluated on the same plate as the samples. All reactions were performed in duplicate.

**DNA Extraction from Tissues and Contents**

Total genomic DNA was extracted from cecal tissues and their contents using a repeated bead beating and column extraction method [38]. Samples were subjected to homogenization using zirconia beads (1:3 ratio of 0.5 mm and 0.1 mm beads) with subsequent heating to 70°C for 15 min. This step was repeated twice. The DNA was purified using a QIAamp column (Qiagen, Valencia CA) and concentrations quantified using a Nanodrop spectrophotometer (Thermo Scientific, Wilmington DE).

**16S rRNA gene bacterial tag-encoded FLX amplicon pyrosequencing (bTEFAP) Sequencing PCR**
The bTEFAP bacterial sequencing method was performed by the Research and Testing Laboratory (Lubbock, TX) as previously described with primers forward 28F: GAGTTTGATCNTGGCTCAG and reverse 519R: GTNTTACNGCGGCKGCTG [39]. Raw sequence data were screened, trimmed, filtered, denoised, and chimera depleted with default settings using the QIIME pipeline version 1.5.0 (http://qiime.sourceforge.net) [40] and UCHIME (http://www.drive5.com/uchime/). To determine the taxonomic assignment of bacterial sequences, the sequences were sorted such that the FASTA formatted file contained reads from longest to shortest. These sequences were then clustered into operational taxonomic unit (OTU) clusters with 96.5 % identity (3.5 % divergence) using USEARCH [41]. For each cluster the seed sequence was placed in a FASTA formatted sequence file. This file was then queried against a database of high quality sequences derived from NCBI using a distributed .NET algorithm that utilizes BLASTN+ (KrakenBLAST; www.krakenblast.com). The BLASTn+ outputs were compiled using a .NET and C# analysis pipeline. The data reduction analysis was performed as previously described [39].

To account for unequal sequencing depth across samples subsequent analysis was performed on a randomly selected subset of 1,900 sequences per sample. This number was chosen to avoid exclusion of samples with lower number of sequence reads from further analysis. Alpha diversity (i.e., rarefaction) and beta diversity measures were calculated and plotted using QIIME. Differences in microbial communities between animal groups and time points were investigated using the phylogeny-based unweighted Unifrac distance metric. This analysis
measures the phylogenetic distance among bacterial communities in a phylogenetic
tree, and thereby provides a measure of similarity among microbial communities
present in different biological samples. To determine if any groups of samples
contained significantly different bacterial communities, the analysis of similarities
(ANOSIM) function in the statistical software package PRIMER 6 (PRIMER-E Ltd.,
Lutton, UK) was used on the unweighted UniFrac distance matrix [42].

Statistical Analysis

All values were expressed as mean ± SEM. Serum cytokine/chemokine data
and SAA data were log transformed to normalize the data. A repeated measures
analysis of variance (ANOVA) model was used, with treatment group, time and their
interaction as fixed effects. The 454 pyrosequencing data were analyzed using a
generalized linear mixed model, with the same fixed effects structure. T-tests were
used to assess the differences between WT and IL-10−/− at each time point. To test
each treatment temporally, an effect comparison of time by group was used. Tukey’s
t-test was used for multiple pairwise comparisons among time points. Non-
parametric data were evaluated using a Wilcoxon Rank Sums test. P-values ≤ 0.05
were considered significant for all tests. SAS software (SAS Institute Inc., Cary NC,
USA) was used for all statistical calculations.
Results

*IL-10<sup>−/−</sup> mice gain weight at a similar rate as WT mice up to week 19.*

Although *IL-10<sup>−/−</sup>* mice weighed slightly less than the WT mice at four weeks of age, both groups gained a similar amount of weight (12.0 g and 12.2 g, respectively) over the course of the study. However, at week 19, the *IL-10<sup>−/−</sup>* mice abruptly lost greater than 10% of their body weight and were taken off study (Figure 1). WT mice showed no weight loss during this same interval (week 19).
Figure 1. Whole body weights of wild type (WT) and IL-10\(^{-/-}\) (KO) mice during the 19 week trial. Data is presented as mean \(\pm\) SEM. Both groups of mice gained a similar amount of weight between weeks 4 and 18 of age (IL-10\(^{-/-}\) = 12.0 g, WT = 12.2 g). At each time point, there were equal numbers of WT and IL-10\(^{-/-}\) mice included in the analysis. The number of mice used for each strain at each time point varied as follows: at 4 weeks of age, \(n = 5\); at 5 weeks of age, \(n = 17\); at 6 weeks of age, \(n = 37\); at 7, 8, and 9 weeks of age, \(n = 27\); at 10 & 11 weeks of age, \(n = 18\); at 12 weeks of age, \(n = 12\); at 13 to 19 weeks of age, \(n = 5\).

Serum pro-inflammatory/chemotactic cytokines and SAA are increased in IL-10\(^{-/-}\) mice versus WT mice.

To investigate the progression of the inflammatory response of IL-10\(^{-/-}\) mice over time, concentrations of pro-inflammatory cytokines and chemokines were measured in serum samples. Significant (\(p \leq 0.05\)) concentrations of IL-6, IL-1\(\alpha\), TNF-\(\alpha\) and the neutrophil chemoattractant KC were observed in serum samples from IL-10\(^{-/-}\) mice versus controls (Figure 2).
Figure 2. Elevation of select serum cytokines and chemokines in the serum of IL-10\(^-\)\(^-\) mice between four and 19 weeks of age. Specifically, the amounts of IL-6, IL-1\(\alpha\), TNF-\(\alpha\), and KC were measured in serum samples collected from wild type (white bars) and IL-10\(^-\)\(^-\) (black bars) mice. Data is presented as mean ± SEM. * = p < 0.05 comparing samples between WT and IL-10\(^-\)\(^-\) for each time point. The number of mice used for each strain at each time point varied as follows: at 4 weeks of age, n = 5; at 7 weeks of age, n = 10; at 10 weeks of age, n = 9; at 12 weeks of age, n = 13; at 19 weeks of age, n = 5.
Of interest, two distinct patterns of serum cytokines and chemokines were observed in the sera from these mice. There were increases in eight separate analytes at four weeks of age while two cytokines were increased at week 19. Analytes increased at four weeks of age included GM-CSF, IL-2, IFN-γ, IL-12p40, RANTES, IL-1β, IL-13 and IP-10 (Figure 3), while G-CSF and IL-17 were significantly increased at week 19 (Figure 4). This difference in cytokine expression observed in early versus late course enterocolitis has been previously reported in C57BL/10 IL-10−/− mice. Similar to our results, the study in C57BL/10 mice indicated a prominent role for IFN-γ and IL-12p40 in the induction of early stage enterocolitis in knockout mice [43].
**Figure 3.** Increased expression of select serum cytokines and chemokines in the serum of IL-10$^{-/-}$ mice at four weeks of age. Specifically, the amounts of GM-CSF, IL-2, IFN-\(\gamma\), IL-12(p40), RANTES, IL-1\(\beta\), IL-13 and IP-10 were measured in serum samples collected from the IL-10$^{-/-}$ mice (black bars) and WT mice (white bars). Data is presented as mean ± SEM. * = \(p < 0.05\) comparing WT and IL-10$^{-/-}$ for each individual time point. IL-10$^{-/-}$ mice were also compared to each other across all time points. A different letter or # indicates \(p < 0.05\) for that time point. $ indicates \(p < 0.1\). The numbers of mice used for these analyses are the same as those listed in Figure 2.
Figure 4. Evaluation of G-CSF and IL-17 levels in serum from WT and IL-10−/− mice between four and 19 weeks of age. Data is presented as mean ± SEM for sera from IL-10−/− mice (black bars) compared to WT mice (white bars). For statistical comparisons * = p < 0.05 comparing WT and IL-10−/− mice for each individual time point. When results from IL-10−/− mice were compared across time points, a bracket was used to indicate the specific comparison with # = p < 0.05. The numbers of mice used for these analyses are the same as those listed in Figure 2.

Serum amyloid A has been used as a serological marker of acute inflammation and is a useful biomarker in murine models of intestinal inflammation. It is also widely used as a biomarker in human IBD as it correlates with disease activity and severity of histopathological lesions [32, 44]. In this study, significantly increased SAA concentrations (p < 0.05) were detected in the serum of IL-10−/− compared to WT mice at all time points (Figure 5). These SAA increases were also accompanied by gross and microscopic cecal lesions in the IL-10−/− mice. One important note regarding the SAA sera concentrations is the biphasic response of SAA levels with the inflection point at week 10.
Figure 5. Evaluation of serum amyloid A (SAA) in the serum of WT and IL-10$^{-/-}$ mice between four and 19 weeks of age. Data is presented as mean ± SEM for samples from IL-10$^{-/-}$ mice (black bars) compared to WT mice (white bars). For statistical comparison * = $p < 0.05$ when comparing IL-10$^{-/-}$ to WT mice within each individual time point. The brackets represent statistical comparison at different time points and # = $p < 0.05$ when comparing samples from IL-10$^{-/-}$ mice at different time points. – The numbers of mice used for these analyses are the same as those listed in Figure 2.
**Macroscopic/microscopic cecal lesions provide evidence of early onset of intestinal inflammation**

The cecum of four week old mice revealed no evidence of macroscopic cecal lesions in either group (Figure 6A). However, IL-10⁻/⁺ mice had increased macroscopic lesion scores compared to WT for all other time points beyond four weeks of age. As early as seven weeks of age, the ceca of IL-10⁻/⁺ mice contained intraluminal blood, tissue thickening, and/or watery mucoid contents. Assessment of the colon revealed increased macroscopic lesion scores only at weeks 7, 10, and 12 in the IL-10⁻/⁺ mice compared to WT. There was no significant change in score among the groups of IL-10⁻/⁺ mice evaluated at any time point (data not shown).

Microscopic examination of cecal tissues revealed typhlocolitis of differing severity present in IL-10⁻/⁺ mice at all time points compared to WT mice (Figure 6B and 7). When temporally comparing histopathological inflammatory scores from IL-10⁻/⁺ mice, there was significantly less (p < 0.05) mucosal inflammation at week 10 versus weeks 4 and 7. This reduction in inflammation was characterized by reduced edema and mucosal ulceration in the cecal tissues of the 10 week old IL-10⁻/⁺ mice. Examination of the colon showed increased inflammation at all time points in the IL-10⁻/⁺ mice compared to WT but no change within the IL-10⁻/⁺ mice at any time point (data not shown).
Figure 6. Macroscopic and microscopic evaluation of cecal tissue from WT and IL-10^{-/-} mice. A) Macroscopic cecal scores form IL-10^{-/-} mice (black bars) and WT mice (white bars). Data is presented as mean ± SEM and for statistical comparison * = p ≤ 0.05 when comparing WT and IL-10^{-/-} mice at a given time point. When samples from different time points were compared, brackets were used to identify the comparisons and # = p ≤ 0.05. See Materials and Methods for the criteria used to generate the macro- and microscopic scores. The numbers of mice used for these analyses are the same as those listed in Figure 2.
Figure 7. Photomicrographs of the cecal tissue collected from IL-10−/− and wild type (WT) mice. Tissue samples were fixed in 10 % buffered formalin and routinely processed prior to staining with hematoxylin and eosin. All images were magnified 200X.

454 pyrosequencing shows increased Bacteroidetes and reduced microbial diversity in IL-10−/− mice compared to WT mice.

Culture independent molecular techniques were used to investigate differences in the bacterial community between IL-10−/− and WT mice between 4 and 19 weeks of age. There were significant differences in the microbial composition of WT mice compared to IL-10−/− mice (Figure 8, also see Supplemental Figure S1) PCoA plotted by time. PCoA plots based on the unweighted UniFrac distance metric indicated separatism in the IL-10−/− mice between weeks 4 - 7 (ANOSIM p = 0.001) and 7 – 10 (p = 0.049). In the WT mice, there was clustering of the microbial populations between weeks 4 - 7 (p = 0.001), 7 – 10 (p = 0.023), and 12 – 19 (p = 0.004). Analysis of the cecal samples from the WT mice revealed that there were increases in the number of bacterial species (Figure 9A) as well as the Shannon
diversity index (Figure 9B) as the mice aged compared to the IL-10−/− mice. The IL-10−/− mice had reduced numbers of observed species, which were more tightly clustered over the course of the experiment compared to WT mice (Figure 10A&B).

Figure 8. Principal coordinates Analysis (PCoA) of unweighted UniFrac distances of 16S rRNA genes sequences obtained from cecal tissue and contents. The clustering indicates differences in the microbiota between the WT (blue circles) and IL-10−/− mice (red squares).
**Figure 9.** A) Rarefaction analysis and B) Shannon Diversity Index of 16 S rRNA sequences. Lines represent the average of each group while the error bars represent the standard deviations (blue= WT mice; red IL-10−/− mice)

**Figure 10.** A) Rarefaction analysis and B) Shannon Diversity Index of 16 S rRNA sequences Lines represent the average of each group (red= 4 week, blue= 7 week, orange= 10 week, green= 12 week, purple= 19 week)
Both groups had microbial communities composed predominantly of members from the Proteobacteria, Bacteroidetes, and Firmicutes phyla. The microbiota of the WT mice harbored a minor population of Bacteroidetes (<10 %) throughout the course of the study. The most abundant phylum in the WT mice was the Firmicutes, which comprised approximately 90 % of the microbial population at all time points (Figure 11). The only significant change observed in WT mice occurred in the phylum Proteobacteria. Between weeks 4 and 7 there was an increase (1 % to 3 %, \( p = 0.05 \)) and between weeks 12 and 19 there was a decrease (3 % to 1 %, \( p = 0.03 \)) in abundance of Proteobacteria (data not shown). The microbiota of the IL-10\(^{-/-}\) mice was comprised of a greater abundance of Bacteroidetes but a lower abundance of Firmicutes when compared to the WT controls. At the phylum level, there were no significant changes in microbial composition of IL-10\(^{-/-}\) mice across all time points at the phylum level.

**Phylum**
**Figure 11.** 454 pyrosequencing assessing the cecal microbiota at the phylum level for wild type (WT) and IL-10^{−/−} mice from four to 19 weeks of age. Panel A) The microbiota of the WT mice and panel B) the microbiota of the IL-10^{−/−} mice. The data is presented as the relative abundance of the Firmicutes, Bacteroidetes, Proteobacteria and Other.

![Genus](image.png)

**Figure 12.** 454 pyrosequencing assessing the cecal microbiota at the genus level for wild type (WT) and IL-10^{−/−} mice from four to 19 weeks of age. Panel A) evaluation of the microbiota in cecal samples from WT mice and panel B) evaluation of the microbiota in cecal samples from IL-10^{−/−} mice. The data is presented as the relative abundance of the bacterial genus present at levels > 1% of the total population.
At the genus level, the bacterial community in the ceca of the WT mice showed fluctuations in population percentage over time, most of which were increases in abundance. (Figure 13A). Those changes in microbial composition that occurred between weeks 4 and 7 were all increases (Figure 13C). Of the four genera that increased, *Roseburia* and *Eubacterium* spp. are both associated with the production of butyrate [18]. Between weeks 7 and 10, four additional genera increased. The only one that comprised >1% of the microbial population being *Turicibacter* spp. \( p < 0.0001 \). The only genera to significantly decrease (\( p = 0.01 \)) during this period were *Lactobacillus* spp. Towards the end of the study only three genera changed in abundance; *Prevotella* spp. being the only one to decrease significantly \( p = 0.0006 \).
**Figure 13.** Changes in the genus populations over time. Panels A and B indicate the changes in the microbial population over time in the WT and IL-10^{−/−} mice, respectively. Panels C and D show the direction of the relative change in the microbial abundance (arrows) in cecal samples from WT and IL-10^{−/−} mice, respectively. The p-value is represented parenthetically. Sold arrows indicate either a significant (p < 0.05) increase or decrease in relative abundance. Hashed arrows indicate a statistical trend in the corresponding direction. At 4 weeks n=5; at 7 weeks n=10; at 10 weeks n=9; at 12 weeks n=13; at 19 weeks n=5.

It is well established that the microbial population in CD patients changes over time to a community that is less diverse than seen in a healthy controls [13, 45]. A similar scenario was seen in the IL-10^{−/−} mice when compared to the WT. The most striking difference between mice cohorts was the prominent reduction in bacterial genera between weeks 4 to 7 in the IL-10^{−/−} mice (Figure 13B). During this period, changes in microbial abundance were observed amongst nine different genera with most showing a decrease in abundance (Figure 13D). Genera that were >1 % of the total population prior to reduction were *Lactococcus* spp., *Blautia* spp., and *Roseburia* spp. The reduction of *Roseburia* spp. is especially important as they produce butyrate as a metabolic by-product, which is important for gut health [18].

Between weeks 7 and 10 there were no significant changes in microbial composition in IL-10^{−/−} mice. Between 10 and 12 weeks, the only genera that changed comprised <1 % of the population. At the final time point there was a
significant increase \(p = 0.004\) in two genera in the *Enterobacteriaceae* family, which together comprised 9.5% of the total microbial population.

**Discussion**

It is widely accepted that the commensal microbiota play an important role in the development of experimental colitis. Germfree C57BL/6 IL-10\(^{-/-}\) mice maintained for six months do not develop colitis unlike their SPF counterparts [30]. In addition, antibiotic administration has been shown to reduce the severity of disease in 129 Sv/Ev IL-10\(^{-/-}\) mice harboring a conventional microbiota [46]. Presumably a reduction in bacterial numbers and/or bacterial antigen reduces the phlogistic potential of the microbiota [46, 47]. Intestinal dysbiosis refers to an imbalance in the normal GI microbiota and is routinely seen in IBD patients [48]. These changes consist of a reduction in microbial diversity, a reduction in Firmicutes, an increase in *Enterobacteriaceae*, and an increase in *Bacteroides* spp. [13, 14, 17, 25, 26, 49, 50]. Specifically, increases in adherent and invasive *E. coli* (AIEC) are observed in a subset of CD patients, as well as in Boxer dog colitis [11, 12, 14]. *Faecalibacterium prausnitzii*, a member of the *Clostridium leptum* cluster (cluster IV) and a major butyrate producer, is often reduced in human IBD patients and therefore may have potential benefit as a probiotic strain [20, 51, 52]. Further evidence towards using *F. prausnitzii* as a probiotic is due to its anti-inflammatory properties [52]. A Japanese study noted that in addition to a reduction in *Faecalibacterium* spp. there was also a significant increase in *Bacteroides* spp. in patients with CD [50]. They also noted a
significant decrease in the microbial diversity in CD patients as measured by the Shannon diversity index.

The results presented here indicate that the weight gain of IL-10−/− mice was similar to that of WT mice through 18 weeks of age (Figure 1). Remarkably, this observation occurred in the presence of marked mucosal inflammation, which was noted as early as 4 weeks of age (Figure 7). In addition, increased concentrations of IL-6, IL-1α, TNF-α, and KC were present in sera from IL-10−/− mice compared to WT mice (Figure 2) and indicated active intestinal inflammation that was corroborated by both macroscopic and histological evaluations. Other analytes including GM-CSF, IL-2, IFN-γ, IL-12(p40), RANTES, IL-1β, IL-13, and IP-10 were also increased in the serum of four week old IL-10−/− mice (Figure 3). Increases in these cytokines/chemokines have been found in humans and animals with IBD [53, 54].

The microbiota of the IL-10−/− mice showed the greatest change in composition between weeks 4 and 7 with most of the changes associated with many organisms decreasing in abundance. Roseburia spp. was of particular interest because this genus decreased significantly (p < 0.0001) during this interval. Roseburia spp. are motile, non-spore forming, anaerobic rods which produce SCFA, including butyrate [55]. Roseburia species are commonly isolated from human feces and are potentially important bacteria for maintenance of mucosal homeostasis [56, 57]. Current phylogenetic analysis places these organisms in the Clostridium coccoides cluster (cluster XIVa) group [18, 20].

Recent studies show that the depletion of Roseburia/C. coccoides cluster organisms is a common feature of dysbiosis in human IBD. In a study evaluating IBD
in twins, *Roseburia* spp. was decreased in both the feces and ileal biopsy samples of patients with ileal CD compared to healthy controls [15]. A different study revealed that the microbiota of UC patients contained fewer mucosa-associated *Roseburia* spp. and decreased diversity of the *C. coccoides* cluster in feces compared to samples obtained from healthy controls [58]. In the mice used in this study, the reduction of *Roseburia* spp. is especially important, as they do not have appreciable levels of *F. prausnitzii*. Therefore without the redundancy in butyrate-producing organisms, the significant reduction in *Roseburia* spp. may have exacerbated disease development.

In the present study, most pro-inflammatory cytokines and chemokines were increased in IL-10/− mice at four weeks of age compared to age-matched controls. Many of these cytokines/chemokines had their peak concentrations at four weeks of age and were markedly decreased in concentration through 19 weeks of age. Macroscopic cecal scores were significantly higher for IL-10/− mice at seven weeks of age when compared to IL-10/− mice at four weeks of age; this difference in severity was not seen histologically. With respect to the composition of the microbiota, the only significant change at the phylum level for either WT or IL-10/− mice between 4 and 7 weeks of age was an increase in Proteobacteria in WT mice. However, the IL-10/− mice at week four already had a skewed proportion of Firmicutes/Bacteroidetes, similar to what is reported in IBD patients [49]. At the genus level, the WT mice had increases in organisms such as *Eubacterium* spp., *Ruminococcus* spp., and *Roseburia* spp., which provide a benefit for the host, either through digestion of organic material or production of SCFA. Conversely, the
microbiota of the IL-10−/− mice showed decreased abundance in beneficial bacteria over the same time period. The only genera that increased in the IL-10−/− mice were from the *Enterobacteriaceae*, a family that is often associated with clinical disease in CD patients [13]. The IL-10−/− mice had minimal changes between 7 and 12 weeks of age in their intestinal microbiota. At week 10, the SAA concentration was reduced compared to week seven, which coincided with decreased histopathological scores for the cecal tissue of the IL-10−/− mice.

The IL-10−/− mice abruptly lost >10% of their body weight by 19 weeks of age and had increased levels of IL-17 and G-CSF in their serum. Mucosal IL-17 is increased in humans with IBD and has been associated with intestinal inflammation in several animal models [59, 60]. The increased IL-17 observed in the present study was accompanied by increased abundance of *Enterobacteriaceae* from (i.e. 2.5 % to 9.5 %) at 19 weeks of age. Increased abundance of *Enterobacteriaceae* has been linked to the pathogenesis of mucosal inflammation in diverse species, including humans, rodents, dogs and cats [42, 49, 61-63]. The microbial diversity of IL-10−/− mice was also markedly altered throughout the study. The Shannon index of the WT mice reveals an ordered increase in diversity over time (Figure 10BA). Concomitantly, the number of observed species increased in an ordered fashion as the mice aged indicating maturation of the microbiota (Figure 10AA). In the IL-10−/− mice, there was no change in either the diversity or observed species over time (Figure 10AB and 10). This observation of reduced microbial diversity has been well documented in human IBD patients [49, 61].
This study demonstrated that the critical changes in microbial composition (i.e. dysbiosis) of IL-10\(^{-/-}\) mice occur within the first four to seven weeks of age, after an increase in pro-inflammatory cytokines at week four. Intestinal inflammation eliciting microbial dysbiosis has also recently been reported in a 129/SvEv IL-10\(^{-/-}\) mouse model of colorectal cancer [64]. In our study, changes in the microbiota developed prior to the onset of more severe clinical disease, which was not observed until 19 weeks of age. These data also indicate that attenuation of mucosal inflammation between 7 and 10 weeks of age is associated with modulations of the host inflammatory response as evidenced by the reduction in serum concentrations of the acute phase reactant, SAA. This amelioration of the mucosal inflammation occurred in the absence of regulatory T cells and disease progression ensued in the absence of any medical intervention (e.g., antibiotics).

In this study it was noted that these mice lacked organisms capable of producing butyrate. These observations identify a gap in our understanding that might be addressed in future studies by administering *Roseburia* spp. or *F. prausnitzii* as a probiotic therapy to neonatal mice to ameliorate disease in a genetically-susceptible host. To this end, *F. prausnitzii* has been shown to ameliorate disease in a chemically induced model of IBD [52]. Additionally, the use of prebiotics such as soluble fibers, used alone or in combination with a probiotic preparation, to promote the growth and maintenance of beneficial bacteria (e.g., butyrate producers) already present in the GI tract also necessitates further study.

In conclusion, results of this study indicate that IL-10\(^{-/-}\) mice develop intestinal inflammation as early as 4 weeks of age prior to the change in the microbiota
observed at seven weeks of age. As the WT mice aged, beneficial bacteria such as
*Eubacterium, Ruminococcus, Roseburia, and Lactococcus* increased in abundance.

In addition, there was an increase in the Shannon diversity and observed species present. The opposite occurred in the IL-10\(^{-/-}\) mice, the most noticeable changes being a decrease in numbers of Gram-positive bacteria and an increase in numbers of *Enterobacteriaceae* that were associated with the onset of clinical disease. The decrease in microbial diversity suggests that there would be clinical benefits to treating these mice with a probiotic to create a more normal microbiota or metabolite profile.

**References Cited**


Supplementary Figures

Supplementary Figure 1. Principal coordinates Analysis (PCoA) of unweighted UniFrac distances of 16 S rRNA genes plotted by time (red= 4 week, blue= 7 week, orange= 10 week, green= 12 week, purple= 19 week)
CHAPTER 5
Conclusions and Future Studies

The gastrointestinal (GI) microbiota is an integral part of the body and can readily be considered a "virtual organ" that is vital to human and animal health. Members of this microbial community harbor genes not found in eukaryotic cells, allowing them to produce and break down substances needed by the host. In fact the microbiome is $\geq 150$-times larger than the human genome. Moreover, GI bacterial community is critical for proper development of both GI tract and immune tissues, and for providing nutrients to host cells in the form of short chain fatty acids. The GI microbiota is also responsible for ensuring that the immune system is tolerant towards the resident microbial species as well as preventing GI colonization of potential pathogens.

GI microbial communities are very dynamic, and their composition can be altered significantly by variety of changes (i.e., dietary patterns, antibiotic use, infection, etc.). Unfortunately, these alterations in the composition of function of the GI microbiota can have serious consequences for that host. The microbiota has been implicated in many diseases, including obesity, diabetes, metabolic syndrome, and the disease that was the inspiration of this dissertation—inflammatory bowel disease (IBD). IBD is an umbrella term that encompasses both Crohn’s disease (CD) and ulcerative colitis (UC). First described by Dr. Crohn in 1932, IBD still has no cure and is of an unknown etiology. What is known is that this disease is multifactorial, with environmental, genetic, bacterial, and immunological components.
The main focus of this dissertation was elucidating the role of the microbiota in IBD. One of the most consistent findings regarding the pathogenesis of IBD is that the microbiota of IBD patients is different compared to healthy controls. The microbiota associated with IBD is less diverse, with an increased abundance of Proteobacteria, and a reduction in butyrate-producing organisms. These butyrate-producing organisms are capable of producing short-chain fatty acids, which are utilized as nutrients by the gastrointestinal epithelium.

Environmental factors are also known to contribute to the induction of IBD; one of which is the association of antibiotic usage and subsequent IBD development. Most of the research regarding this association is through recall response surveys of patients indicating past antibiotic usage. In chapter 2, two groups of mice, one with a limited microbiota (i.e., the eight members of the altered Schaedler flora) and one with a conventional (i.e., hundreds of microbial species) microbiota were administered ampicillin to determine the effect of antibiotic usage on the severity of mucosal inflammation subsequent to a colitic insult. Those mice with the limited microbiota had increased inflammation revealed through the increased production of pro-inflammatory cytokines and chemokines and the increased fluorescence intensity measured using the inflammatory probe. The conventional mice, exhibited less inflammation but instead develop metabolic syndrome, which was not seen in the mice with the limited microbiota.

Overall, this study revealed that having a limited microbiota (such as seen with IBD patients after diagnosis) causes the community to be unstable and be more susceptible to perturbations resulting in increased inflammation. This study also
implicated the presence of a normal microbiota in a host with mild inflammation can lead to the induction of metabolic syndrome. Future studies with this model could include further elucidation of the role of the microbiota in the development of metabolic syndrome in the conventional mice. Other parameters, such as serum fatty acid content, glucose levels, and insulin tolerance, should be measured to accurately diagnose the condition. Additional work using a restricted calorie diet as well as modulation of the microbiota through selected depletion of specific members should also be examined to identify if the mice can recover from metabolic syndrome. Other studies could be performed where conventional microbiota mice are fed high-fat versus high-carbohydrate versus high-protein diets to determine the effect diet has on the development of the disease. Because these diabetes biomarkers were measured from the serum, chronological samples could be taken during the study to determine exactly at what point the biomarkers associated with metabolic syndrome appear. Future studies capitalizing on the results from the mice with the limited microbiota could involve manipulation of the microbiota through the use of pro- and prebiotics to overcome the shortcomings that are associated with the reduced microbial diversity.

One of the frustrating aspects for patients with IBD is that there is no cure. There are many treatment options available to help the patient achieve remission, but most are immunosuppressive and not without severe side effects. One facet of medicine that is gaining popularity is the use of complementary and alternative medicine (CAM), including herbal supplements. These can be purchased over the counter and are unfortunately not tightly regulated in regards to purity and content.
Patients, however use these products because they are perceived as safer than conventional therapies because they are natural. However, these compounds can have both anti-inflammatory and/or anti-bacterial properties and should be used with caution. The focus of chapter 3 was to identify the potential therapeutic actions of two botanical extracts on the severity of disease in a DSS-induced model of colitis. The botanicals, *P. vulgaris* and *H. gentianoides*, have been shown to possess anti-bacterial and anti-inflammatory potential *in vitro*. In the study discussed in chapter 3, mice with a limited microbiota were colonized with *H. bilis* and then orally treated with either botanical extract for seven days prior to the administration of DSS. At the end of the study, *H. gentianoides* exhibited anti-bacterial activities *in vivo* and may have also displayed some anti-inflammatory effects as well. *P. vulgaris* treatment correlated with the increased the production of several pro-inflammatory cytokines/chemokines by the host. Those that were increased are associated with the chemotaxis and trafficking of neutrophils and eosinophils; however, no differences in the macroscopic or microscopic disease scores were observed between mice treated with *P. vulgaris* versus the vehicle controls. Treatment with *H. gentianoides* significantly reduced 50% of the microbial community compared to the mice given *P. vulgaris* and also seemed to diminish cecal inflammation as measured by the fluorescence intensity of the inflammatory probe. The effect of the *H. gentianoides* treatment on the microbiota was similar to what was seen in the mice given ampicillin. However, *H. gentianoides* also contained anti-inflammatory properties, which allowed for those mice to not have the increased severity of
disease seen in the limited microbiota mice given ampicillin and then given the colitic insult.

Future studies for this project should include identifying and isolating the specific compounds associated with the anti-bacterial and anti-inflammatory effects of *H. gentianoides*. The extracts used in this study were whole ethanolic extracts. Fractionating the extracts and then repeating the study may lead to the identification and isolation of the compounds responsible for the anti-bacterial and anti-inflammatory effects associated with *H. gentianoides* treatment. Additionally, *H. gentianoides* may be a promising treatment for the metabolic syndrome mouse model described in chapter 2, as its anti-bacterial and anti-inflammatory effects may prevent the onset of the metabolic syndrome in mice with a conventional microbiota.

As previously mentioned, the microbiota of IBD patients is significantly altered compared to healthy controls. One of the main areas of IBD research is trying to decipher whether this microbial change is due to the inflammation associated with the disease or if the change in the microbiota contributes to and accelerates the inflammation. This is the topic of interest in chapter 4 of this dissertation. In chapter 4, IL-10−/− mice, which spontaneously develop colitis, were followed temporally to determine both inflammatory and microbial changes over time concomitantly. Initially, there were no clinical signs of disease, and the IL-10−/− mice gained weight in a similar pattern to the wild type controls up until 19 weeks of age. At that point, the IL-10−/− mice precipitously lost weight and had to be removed from study. During the course of the study, mice were necropsied at predetermined time points, with the first time point at 4 weeks of age. At this early time point, eight pro-inflammatory
cytokines were significantly elevated compared to the later time points (weeks 7, 19, 12, and 19). The microbial community in the IL-10−/− mice at the earliest time point (between weeks 4 and 7) had a significant decrease in many beneficial organisms such as *Roseburia* spp., and *Eubacterium* spp. In the wild type mice, the opposite trend was observed as beneficial organisms increased in number during this time. At week 19, there was also another increase in pro-inflammatory cytokine secretion, predominantly IL-17 and G-CSF. This change was accompanied by a significant increase in members of the Proteobacteria. The results indicate that, in this model of colitis, host inflammation drives the microbial changes, decreasing the abundance of beneficial organisms and creating an environment that promotes the proliferation of Proteobacteria, which have been associated with IBD. Future studies utilizing this model should be aimed at correcting the microbial changes that succeed the inflammation. This could potentially be achieved by colonizing the mice with one of the beneficial bacteria found to be decreased early in the study. *Roseburia* spp. would be a good choice, as it produces butyrate, which may help to offset the inflammatory response of the host. Other studies could include cross fostering IL-10−/− pups to wild type dams and then following disease development. It is possible that IL-10−/− pups are being colonized with a dysbiotic microbiota from their dam, who likely has chronic GI inflammation. By initially colonizing these pups with a more diverse microbiota, the development of disease may be delayed or ameliorated. Lastly, an experiment where exogenous IL-10 is administered to IL-10−/− mice could be performed to determine if the microbiota of these mice change over time in the absence of inflammation.
In summary, the research presented in this dissertation advances our understanding of how the microbiota helps maintain the health of the host. Perturbations that disrupt the community can exacerbate the host response to inflammatory insults, indicating that the bacterial community of the gut exerts a cytoprotective effect on the host in addition to the nutritional benefits. By understanding exactly how these processes interconnect, future research efforts can be aimed at modulating the microbiota in a more favorable direction, thereby dampening the inflammatory process. These modulations might include dietary manipulations as well as feeding probiotics, prebiotics, and even botanical extracts. Using well designed experimental models to elucidate the dynamics of a stable microbial community, as the ones used in the studies outlined in this dissertation, may lead to the development of novel strategies and therapies that can be ultimately translated into treatments to improve GI health.