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THE BENEFICIAL EFFECTS OF WHITE BUTTON MUSHROOMS ON THE GUT HEALTH

A Thesis in

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by

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ABSTRACT

White button (WB) mushrooms have been reported to have beneficial effects in the gut. The purpose of this study was to determine the effect of WB mushrooms on 1) the composition of the gastrointestinal microflora, 2) the host resistance to *Citrobacter rodentium* infection and 3) healing effect on the gut. Wild type mice were maintained on either WB or the Control diets. As early as two weeks after introducing WB mushrooms into the diet, several changes in the composition of the gastrointestinal microflora were detected by denaturing gel gradient electrophoresis. The 16s rDNA obtained from feces of both groups of mice was then analyzed through sequencing technique to obtain the species differences due to different diet treatments. The most striking results from the metagenomic sequencing were the decrease in the *Clostridia* species of bacteria and increase in the *Bacteroidetes* species in the WB fed mice. The *Clostridia* species belongs to the phylum *Firmicutes*. This phylum contains various pathogenic groups of bacteria including *Clostridium spp*., *Bacillus* spp. and *Erisypelotrix* spp. The decrease in the Phylum *Firmicutes* in mice maintained on WB diet compared to the controls indicates that the WB helps in decreasing the pathogenic microflora in the gut. Though, the role of *Bacteroidetes* as a beneficial bacterium in the gut is still unclear. Both the groups of mice were experimentally challenged with *Citrobacter rodentium*, which induces gut inflammation that is very similar to lesions that appear in patients suffering from Ulcerative colitis and Inflammatory Bowel disease. The bacterial counts of the *C. rodentium,* from feces of each group of mice, were measured during its peak days i.e., day 10^{th} and day 14^{th} post infection. It was observed that, there was no significant change

in the bacterial loads of *C. rodentium* in the WB fed mice compared to controls. Cytokine profile analysis of the inflamed gut tissue indicated that the WB fed mice showed higher expression of pro-inflammatory cytokines such as, IFN-γ and IL-17 in the colon. Also, it was observed that WB fed mice showed increased resolution and healing of the gastrointestinal mucosa following *C. rodentium* infection. The reasonable hypothesis can be that the pro- inflammatory cytokines work locally on the gut to induce a faster protective gut immune response in the WB fed mice. Thus, adding modest amounts of WB mushrooms to the diet changed the composition of the normal flora and these changes resulted in better control of inflammation and healing in the colon following infection with *C. rodentium*.

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Chapter 1

INTRODUCTION

1.1. Introduction

Medicinal mushrooms have been used either whole or as various extracts in traditional Oriental therapies. Numerous bioactive components have been identified, and mushroom extracts are sold world-wide as dietary supplements and make up an over 5 million dollar industry in the US. Understanding the mechanisms by which mushrooms affect health is complicated by the numerous different varieties of mushrooms and the differing purities and compositions of extracts being tested. There is evidence that edible mushrooms and/or mushroom extracts regulate the immune system (Chang, 1996; Wu et al., 2007).

In a model of gastrointestinal colitis, edible mushrooms improved healing following colonic injury with dextran sodium sulfate (DSS) (Yu et al., 2009). DSS treatment of mice induces inflammation in the gastrointestinal tract, and the inflammation is most notable in the colon (Westbrook et al.). The colitis induced in the DSS model is characterized by the local overproduction of cytokines, primarily by macrophages, and the disease occurs in T and B cell–deficient mice (Axelsson et al., 1996; Dieleman et al., 1994). Feeding mice diets that contained 1-2% white button (WB) mushrooms resulted in protection from DSS colitis (Yu et al., 2009).

The composition of the gastrointestinal microflora is a critical determinant of the severity of experimental colitis in many models including DSS colitis (Sellon et al., 1998). Experiments using germfree animals and animals treated with probiotics highlight the importance of the composition and existence of the gastrointestinal bacterial flora for maintaining health and in some cases causing disease. The bacteria in the gut are important for normal immunity and changes in the microflora affect diseases of the gut.

One of the ways the commensal flora protects the gastrointestinal tract is to prevent the infection by competition. *Citrobacter rodentium* is an extracellular enteric pathogen, which includes the enteropathogenic *Escherichia coli* that cause human infections. In addition, *C. rodentium* infection has been used as a model of inflammatory bowel disease (IBD) where the infection causes inflammation in the gut and the inflammatory mediators including iNOS, TNF-α, and IL-12 are all important for regulating the extent of pathology in the gut (Mundy et al., 2005). Infection and resolution of infection with *C. rodentium* in mice is affected by host immunity and shifts in the bacterial communities found in the gut (Hoffmann et al., 2009).

Feeding mice diets that contained 1% freeze dried *Agaricus bisporous* (white button, WB) mushrooms resulted in changes to the composition of the gastrointestinal microflora. Changes in mircoflora were evident at 2 weeks and stabilized after 4 weeks of WB feeding. The diversity of the microflora was increased in the WB fed mice, and several classes of bacteria associated with disease (*Clostridia, Erysipelotrichi*) were decreased in the WB fed mice. Interestingly, there was a significant increase in the commensals such as all classes of *Proteobacteria*, *Bacteroides* spp. WB fed mice were then infected with *C. rodentium*. There were no differences in bacterial clearance between WB and control fed mice, but there was significantly more IFN-γ and IL-17 and less inflammation in the gut of the WB fed mice. WB feeding resulted in changes to the composition of the gastrointestinal microflora and improved healing of the gastrointestinal tract following an enteric infection.

To date, there are no reports on the beneficial interaction of mushrooms and the gut microflora. It is anticipated that the findings of this study would help us understand

the microbial changes occurring in the gut due to mushroom feeding in mice that help in healing the gut faster during colitis by inducing the immune response against the injury.

Chapter 2
LITERATURE REVIEW

2.1. The mice gut microflora

The gut of mice harbors a diverse microbial population, the combined genetic content of which even exceeds the whole human genome (Shanahan, 2002). Microbial colonization usually occurs in the distal part of the small intestine and large intestine owing to their fairly less acidic environment compared to that of the stomach and proximal intestine (Eckburg et al., 2005). Bacteria form a predominant population in the gut of mice and their colonization begins soon after birth and *Enterobacteria* and *Bifidobacteria* are known to be the early colonizers (Mountzouris et al., 2002).

The significance of gut microbes of mice, bacteria in particular, has extensively been studied and a recent study has suggested that bacteria in the gut exert protective, structural and metabolic effects on the epithelial lining of the gut (Savino et al., 2005). Shanahan et al., 2002, demonstrated the influence of microflora on the intestinal physiology and concluded that germ-free animals were more susceptible to infection. Furthermore, it was noticed that these animals had reduced vascularity, digestive enzyme activity, muscle wall thickness and suppressed immune profile. Reconstitution of germ-free mice with intestinal microflora was found to be sufficient to restore the mucosal immune system (Umesaki et al., 1995). Further, it is reported that commensals markedly influence the development of humoral immunity in the gut mucosal level (Weinstein and Cebra, 1991) and also modulate T-cell repertoires and cytokine profiles (Shanahan, 2002; Weinstein and Cebra, 1991).

The bacterial colonization depends on various factors like infant diet, hygiene levels, and medication (Gronlund et al., 1999). Mountzouris et al., 2002, evaluated the

effect of feed on the microbial populations in the gut of infants and found that there is an obvious difference in microbial populations between breast-fed and formula-fed infants. Zoetendal (2001) demonstrated varying degrees of genetic relatedness, and thereby the microbial diversity, by taking into consideration the influence of the host genotype over the environmental factors. Considering the importance of diversity of gut microflora it becomes mandatory to identify and classify individual species that can aid in mapping their population shift due to environmental pressures. Additionally, it will help in the better comprehension of host-bacterial interaction in gut as well. However, most of the bacterial species, except some members of *Eubacterium*, *Fusobacterium* and *Clostridium* genera (Dewhirst et al., 1999), cannot be cultured. Keeping this drawback in view, DNA based techniques have recently been devised for taxonomic identification of bacteria through sequencing of 16S ribosomal RNA in gut microflora (Hopkins et al., 2001). With the advent of this technique, many novel bacterial species of gut microflora of mice have been discovered and a 16s rRNA library of diverse bacterial species of the gut of mice has been developed (Salzman et al., 2002). Owing to the extensive work being carried out to study the microbial diversity and host-bacteria interaction in the gut of mice, it can be used as a model study to investigate and understand the host-microbial relationship in the gut of humans.

2.2. Mushrooms and their properties

Mushrooms are a type of spore forming fungi. The most commonly grown mushroom is the white button/ *Agaricus bisporus spp*. It is a type of edible mushroom and it would be interesting to study its beneficial role.

Mushroom extracts are sold as dietary supplements with a world market of around 5–6 billion US dollars per year (Wasser, 2002). From amongst the thousands of different mushroom species available across the world, about 700 are reported to have significant pharmacological properties (Chang, 1996; Wasser SP, 1999). The so called 'Medicinal mushrooms' have long been used in traditional Oriental therapies such as the use of water-soluble fractions of some mushrooms as medicine in the Far East (Wasser, 2002). This traditional use of mushroom extracts has generated interest in the exploration of biologically active components in mushrooms. Active components of mushrooms, mostly are large polysaccharides/ β-(1→6)-branched β-(1→3)-linked glucans have been shown to have beneficial effects on experimental cancer (Lull et al., 2005; Yin et al., 2007; Yuminamochi et al., 2007) and the latter have been reported to inhibit tumor growth (Fujimiya et al., 1998). A study recently concluded that components like selenium, B vitamins and polysaccharides present in mushrooms have a significant role in immunoregulatory responses in vitro and the amalgam of these components elicited different responses in comparison to the response they elicited when used individually (Yu et al., 2009). In the same study, it was further reported that the use of white button mushroom suppressed IL-10 production and enhanced the production of IL-1β, and TNF-α in macrophages. These activated macrophages preferentially stimulated T cell production of TNF-α and IFN-γ while diminishing IL-10. The inhibition of IL-10 and activation of other cytokines may stimulate production of T cells and further cytokines that may help in clearance of tumorogenic cells or killing of infectious organisms. This immune modulation by mushrooms may in part be due to the bacteria, yeasts and molds associated with them. It is reported that, normal healthy mushrooms are associated with

bacterial populations ranging from 6.3 to 7.2 log CFU/g of fresh mushrooms (Chikthimmah N, 2006). The bacteria associated with mushrooms are mainly the *Pseudomonads spp.* (Godfrey et al., 2001). This organism results in activation of the innate immune response by interactions with the Toll like receptors (TLR), especially the TLR-5 (Ramphal et al., 2005; Zhang et al., 2005). Thus, it means that probably the microbes ingested with the mushrooms are responsible for activating the mucosal innate immune response through the TLRs.

2.3. Gut microbiota and immunity

The gut microflora is a very diverse and dynamic system. It gets established during the birth, depends on various factors like antibiotics, food host genetics and environmental factors (Gronlund et al., 1999). Turnbaugh et al. (2009), suggested a significant influence of diet on gut microflora, which in turn affects the normal immune response of the gut. One of the studies demonstrated that the decreased intake of fiber in the diet resulted in a drastic change of gut microflora and a significant decrease in immunomodulatory products like short-chain fatty acids (SCFA) (Reimer et al., 2012). The SCFA consists of three main acids which are acetate, butyrate and propionate. They are the end products of the unabsorbed starch and fiber by the action of gut bacteria. They play a very crucial role in maintaining the gut morphology and health by proliferating the enterocytes and in certain cases preventing the colitis (Scheppach, 1994).

The commensals of the gut play a protective role and evoke Pattern Recognition Receptors (PRRs), which is the integral parts of the innate immune system that recognize conserved regions of bacteria and viruses and activate pro-inflammatory pathways that

activate the host's immune system during an infection (Philpott and Girardin, 2004). There are two classes of PRRs viz. Toll-Like Receptors (TLRs), associated with enterocyte's cell membrane, and Nucleotide-Binding Oligomerization domain (Nod) molecules, present in the cytoplasm of epithelial and immune cells (Inohara and Nunez, 2003; Takeda and Akira, 2004). Both, the PRRs activate transcription factor NF-κB that leads to the activation of the pro-inflammatory gene expression (Inohara and Nunez, 2003; Takeda and Akira, 2004), which in turn signals the gut's mucosal immunity to release certain cytokines such as IFN-γ, IL-12 and IL-17 to combat the inflammation. The TLRs 1-9 and Nod 1 and 2 are all expressed by the gut epithelium (Otte et al., 2004). Nod 1 and 2 have an impact on intracellular infection by recognizing different muropeptide motifs from bacterial peptidoglycans (Viala et al., 2004). On the other hand, TLRs recognize various bacterial and viral components, some of the archetypes being the recognition of cell wall polysaccharides of gram negative bacteria by TLR4 and identification of bacterial flagellin by TLR5 (Philpott and Girardin, 2004). MyD88, the adapter molecule, plays a crucial role in signaling TLR and also expresses cytoprotective proteins viz. hsp25 and hsp72 in colonic epithelial cells (Rakoff-Nahoum et al., 2004). Neish et al., (2000) noticed that MyD88 mutation leads to high susceptibility of inflammatory bowel disease (IBD) in mice. The above studies suggest that the commensals in the gut help in maintaining a healthy gut environment by activating innate immune responses against a toxic insult.

Besides the above beneficial effects of the commensals, they also evoke the mucosal immunity that plays a critical role in the protection of gut epithelium from deleterious effects of food and gut pathogens (Brandtzaeg and Pabst, 2004). The mucosal

immunity mainly consists of CD4+ T cells (Monteleone et al., 2005), macrophages, dendritic cells, and eosinophils, along with abundant intraepithelial lymphocytes (IEL) which contains CD8+ T cells (Hayday et al., 2001). Thus, it can be suggested that the mucosal and innate immunity act synergistically with the commensals in a tightly regulated manner to fight against any invading infection in the gut.

2.4. *Citrobacter rodentium* infection in mice

Citrobacter rodentium is an opportunistic Gram-negative extracellular bacterium present in soil and water and causes enteric disorder in mice (Barthold et al., 1976; Schauer and Falkow, 1993; Schauer et al., 1995). *C. rodentium* is classified under the family of bacterial pathogens that cause intestinal attaching and effacing (A/E) lesions, characterized by adherence of the bacteria to the host enterocytes and disruption of microvilli. Although, *C. rodentium* primarily causes the disease in mice, it has also been found to be infectious in other rodents (de la Puente-Redondo et al., 1999). *Citrobacter* species have been reported to cause extra-intestinal infections in animals and humans also under certain circumstances (Luperchio et al., 2000). Discovered in 1960 (Brennan et al., 1965), the outbreaks due to *Citrobacter rodentium* in mice and hamster colonies was earlier considered either an atypical mouse-pathogenic *E. coli* (MPEC) (Itoh et al., 1978; Itoh et al., 1988; Muto et al., 1969) or atypical *Citrobacter freundii*, which was later, reclassified as *Citrobacter genome species 9* (Barthold et al., 1976; Brennan et al., 1965; Brenner et al., 1993; Ediger et al., 1974). Being highly infectious, *Citrobacter rodentium* is easily transmissible resulting in frequent outbreaks in mice, hence, sometimes also known as Murine Colonic Hyperplasia (TMCH) (Barthold et al., 1977).

The bacteria adhere to the host epithelial cells by the formation of pedestal like extensions by the host actin with the adherent bacteria (Wales et al., 2005). The A/E bacteria contain a conserved region called the locus of enterocyte effacement (LEE), which causes, gastrointestinal colonization and formation of A/E lesions (Deng et al., 2001; Wales et al., 2005). As the other known A/E pathogens, such as *E. coli* (EPEC) and enterohaemorrhagic *E. coli* (EHEC) can not cause pathogenesis in mice, *C. rodentium* is a powerful model organism for studying infection, colonization, virulence factors and mechanism of pathogenesis of such clinically significant human A/E pathogens (Luperchio and Schauer, 2001; Mundy et al., 2005; Wales et al., 2005). The 5′ of the LEE gene contains transcriptional regulators and structural components of a type III secretion system (TTSS) ((Luperchio and Schauer, 2001; Mundy et al., 2005; Wales et al., 2005). The central part of LEE has genes that code for outer membrane adhesion protein, intimin and its translocated intimin receptor (Tir) (Frankel et al., 2001), and the 3′ end expressing additional TTSS structural, translocator and effector proteins. Both intimin and Tir are conserved regions in *C. rodentium* and *E. coli* EPEC. Intimin was the first protein discovered which causes A/E lesions in humans and has various types such as α, β, γ, δ, and ε. The most common one found in *C. rodentium* infection is intimin β. The intimin protein plays a critical role in causing infection in mice. It is responsible for the attaching/effacing (A/E) lesion. It activates T cells by binding to the β1 integrins present on T cells' surface (Frankel et al., 1996), but the interaction function is yet to be studied in detail. Similar immune responses are observed with intimin α of *E. coli* EPEC/EHEC, which suggests that, developing anti intimin drugs could be the future therapy for entero-pathogenic infections in humans (Gansheroff et al., 1999).

Role of Tir is to form the intimate attachment with the enterocytes. Any mutation in the Tir can result into avirulence of the bacteria (Deng et al., 2003). TTSS is a common complex system, used by various Gram negative bacteria such as, *E. coli* and *C. rodentium*, to invade the gut cells and cause pathogenicity. The system's function is, to secrete infectious proteins into the host cells. This happens by forming an injectosome complex that helps to inject the proteins into the host cell membrane (Blocker et al., 2001). The injectosome complex consists of structural/ basal proteins, which form the base of the needle, effector or infectious proteins and chaperone proteins, which protect and transfer the infectious proteins towards the needle complex (Galan and Wolf-Watz, 2006). The TTSS effector protein present in the *C. rodentium* is the espB. It interferes with the host's mitochondrial function, and disturbs the intestinal barrier functions and induces bacterial adhesion. This ultimately results in cell death (Newman et al., 1999). A/E bacteria such as *E. coli* EPEC and EHEC present similar destructive functions (Mundy et al., 2004; Nagai et al., 2005). Therefore, it suggests that LEE genes play a crucial role to cause A/E lesions in the gut by the bacteria.

C. rodentium can easily be introduced experimentally by oral gavage at a concentration of 10^8- 10^9 cfu/ml and takes 3 days for the bacteria to appear in the intestine. However, in natural infection, the bacteria colonize in the lymphoid follicles in the caecum around the second day and localize in the intestines at day tenth post infection (Wiles et al., 2004). Naylor et al., 2003 reported that the infection generally peaks on day 10th- 14th.

The clinical symptoms of Murine Colonic Hyperplasia include, thickening of colon, diarrhea, loose feces, rectal prolapse, and destruction of the brush border

microvilli. This epithelial thickening and crypt hyperplasia in the gut shows the presence of a vast population of CD3 infiltrate, mainly the CD4 cells (Higgins et al., 1999). Two mechanisms studied lately, define the immune response of the host against *Citrobacter rodentium* and *E. coli* EPEC infection; one is via M-cells present in the follicle-associated epithelium and identified by the immune system and other is the mucosal immune responses to the infection (Celli et al., 2001; Klapproth et al., 2000). Subsequent studies have shown that iNF Kappaβ and MAP kinases get activated which causes production of pro-inflammatory cytokines and chemokines (Mundy et al., 2005; Savkovic et al., 1997). *C. rodentium* infection has been observed to elicit an increased Th1 response entailing IL-12, IL-17 and IFN γ cytokines (Higgins et al., 1999). Spahn et al. (2008) demonstrated the protective role of CD4+ cells in healthy mice challenged with *C. rodentium*. IL-23 also plays a protective role in *C. rodentium* infection. IL- 23, which is an IL-12 cytokine family member, induces production of IL-17 cytokine, from the Th17 cells (Mangan et al., 2006). Mangan et al. (2006) also observed high mortality in IL-23 gene knockout mice infected with *C. rodentium* even in the presence of IFN-gamma and IL-17. In addition, IL-22 cytokine is suggested to mediate an early host defense against A/E bacteria (Zheng et al., 2008). IL-22 is produced by Th 17 cells and comes under IL-10 family of cytokines (Pestka et al., 2004; Zheng et al., 2007). IL-22 receptors are present on the gut epithelial cells and are thought to be inducing epithelial innate immunity (Gurney, 2004; Wolk et al., 2004). IL-22 production is controlled by the presence of IL-23 in CD4 T cells and monocytes and absence of the IL-23 disables the mice to control C. rodentium infection (Mangan et al., 2006; Zheng et al., 2007).

The humoral response also plays an important role in gut protection during *C. rodentium* infection by decreasing the bacterial burden on account of enhanced clearance of the bacterium from the gut of mice (Simmons et al., 2003). Recent studies suggest that, LEE encoded proteins such as, EspA, Esp B, Tir and intimin, induce a humoral response consisting of antibodies especially, IgA and IgG, that mediates gut protection, during re-infection and provides resistance to the same (Simmons et al., 2003). The favorable hypothesis for the same is that, the LEE encoded antigens are exposed to B cells present in the gut associated lymphoid tissue and/or in lamina propria of humans infected with EPEC/ EHEC, or mice infected with *C. rodentium*. In contradiction to the above stated studies, a separate study has demonstrated that the bacterial clearance is independent of secretory antibodies such as IgA or IgM (Maaser et al., 2004) which could be possible considering the passive leakage of either Ig A or Ig M across the epithelium that can alone provide sufficient protection. On the other hand, role of Ig G antibodies in host defense against *C. rodentium* especially during sub-cutaneous immunization with an intimin derivative has been established (Ghaem-Maghami et al., 2001).

Current studies suggest that, the infectious effector proteins can also be found outside the LEE locus mostly, on prophages of the bacteria (Garmendia et al., 2005). Espl was one such protein identified in the *C. rodentium*, and its function is yet to be explored (Garmendia et al., 2005). A gene called colonization factor *Citrobacter* (cfc) type IV has been identified in *C. rodentium*, that is thought to help in colonization, transmission and infection in the hosts (Mundy et al., 2003). Similarly, a gene called LifA identified in *E.coli* is a lymphostatin that deactivates hosts' lymphocytic activity in vitro (Klapproth et al., 2000).

Interestingly, the *C. rodentium* bacterial infection gets cleared by three weeks, which makes it distinct from the *E. coli* infection. This clearance is due to the role of T and B cells (Simmons et al., 2003; Vallance et al., 2002b). Lately, it is also shown that, serum antibodies such as Ig A and Ig M, are not necessary for the clearance of bacteria (Maaser et al., 2004; Uren et al., 2005) but serum Ig G does help in lowering the bacterial numbers in the gut (Maaser et al., 2004). The reason could be that Ig G being a small antibody, is able to pass through the enterocytes, block the binding of bacteria to the host epithelial cells, activate the complement cascade and thereby help in bacterial clearance. Lebeis et al., suggested that innate immunity has a significant role in the clearance of *C. rodentium* infection along with the humoral response (Lebeis et al., 2007). The major player in the innate immunity that acts against these A/E pathogens is the TLR response. They studied the signaling cascades initiated by TLRs and its adaptor protein MyD88 which activated various transcription factors and controlled chemokines and cytokines production during an A/E infection (Higgins et al., 1999; Kaisho and Akira, 2004; Savkovic et al., 1997). MyD88 signaling induces an inflammatory response in the gut due to *C. rodentium* infection, and also limits the bacteria to the lumen and helps timely recruitment of neutrophils by CXCL1/ KC and mediates activation of humoral response for the clearance of the bacteria (Lebeis et al., 2007). Also, it would be interesting to study role of macrophages along with the neutrophils in the clearance of *C. rodentium* as macrophages are also found in the gut during the infection. In addition to the same, beta defensins, the cationic peptides expressed by Paneth cells and epithelial cells provided gut protection against the bacteria (Muller et al., 2005). Also, nitric oxide (NO) that has antimicrobial activity showed enhanced expression of inducible NO

synthase (iNOS) in gut epithelial cells during *Citrobacter* infection (Vallance et al., 2002a). Furthermore, the cathelicidin-related antimicrobial peptide (mCRAMP) expressed by colonic surface epithelial cells illustrated antimicrobial activity against *C. rodentium* (Iimura et al., 2005). Therefore, the A/E causing bacteria are controlled by fascinating and highly regulated innate and adaptive responses and further investigation is required to define the interaction more promptly.

Chapter 3

MATERIALS AND METHODS

3.1. Mice

C57BL/6 male and female mice were bred and maintained at the Pennsylvania State University (University Park, PA). Groups of 6-7 wk old mice were used for experiments and all of the mice came from one of two breeding females.

3.2. Diet

Mice were started on purified diets complete for all nutrients as described previously (Cantorna et al., 2000). Commercially available *Agaricus bisporous* or common name white button (WB) mushrooms were obtained from Modern Mushroom Farm, Inc. (Toughkenamon, PA). The whole mushrooms were freeze-dried and ground into a fine power. Half of the mice were fed the basil control (CTRL) diet and the other half of the mice were fed the CTRL diet with 1% WB mushrooms added exactly as described (Yu et al., 2009). Feces, urine and blood were collected at the start of the diet treatment (time 0) and every 2 wks for a total of 6wks.

3.3. Denaturing gradient gel electrophoresis (DGGE).

Total DNA was isolated from fecal samples using a QIamp DNA stool minikit (Qiagen, Valencia, CA). Extracted fecal DNA was amplified with Universal 16s rDNA/UNIBAC primers that target the V3 region of the 16s rDNA which is highly conserved across bacterial species (Muyzer et al., 1993). DGGE was performed with a DCode, Universal Mutation Detection System (BioRad, Hercules, CA). The electrophoresis was performed with 0.75-mm-thick 6% polyacrylamide gels (ratio of acrylamide to bisacrylamide, 37.5:1) submerged in 1× TAE buffer (40 mM Tris, 40 mM

acetic acid, 1 mM EDTA; pH 7.4) at 60°C. 200 ng/µl of PCR product was applied to individual lanes in the gel. The electrophoresis conditions were selected based on the results of perpendicular DGGE and time travel experiments which were set at 18 h at 70V in a linear 30 to 60% denaturant agent gradient (100% denaturant agent was defined as 7 M urea and 40% deionized formamide) (Diez et al., 2001). The gels were stained for 20 min in 1× TAE buffer with ethidium bromide and visualized with UV radiation by using MultiAnalyst imaging software (Bio-Rad). Each of the DGGE band/ fingerprint obtained was defined as a specific bacterial species within each lane. Gels were analyzed using MultiAnalyst imaging software (Bio-Rad). Standard strains used were *Clostridium propionicum* (ATCC strain 25522), *Lactobacillus murinus* (ATCC strain 35020) and *Parabacteroides distasonis* (ATCC strain 8503). These standards were DGGE bands generated from DNA isolated from purified cultures of the three organisms and were used so that gels run on different days and with different samples could be compared to each other based on the migration of the standards. The UNIBAC primers sequence is 341F- GC (5'- CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGCCT ACGGGAGGCAGCAG- 3') and 534R (5'-ATTACCGCGGCTGCTGG-3'). PCR amplification was performed with a PTC-100 Thermocycler as follows: 94^0C for 5 minutes, 95^0C (denaturation) for a minute, 55^0C (annealing) for 45 seconds, 72^0C (elongation) for 10 minutes. 30 cycles were performed.

3.4. Metagenomic analysis.

Four DNA samples from 2 CTRL fed and 2 WB fed mice at 6 wks were sequenced on a 454 Titanium sequencer. The sequences obtained were analyzed using the MOTHUR software (Schloss et al., 2009). All analysis scripts and datasets are available on the Penn State Bioinformatics Consulting Center website (http://www.bcc.bx.psu.edu). The sequencing reads have been filtered to remove reads that had an average read quality of less than 35. This initial filtering removed approximately 30% of the reads leaving 176,122 reads distributed over the 4 samples. Two additional read quality filtering steps were done. First potential chimeric reads were removed (ChimeraSlayer developed by Broad Institute) and a preclustering step was applied that merged reads due to pyrosequencing errors. At the end of the quality filtering steps 130,232 reads were retained and the read counts for each group was from 22,827 to 46,692 reads. To determine operational taxonomic unit (OTU), the filtered data were aligned via MOTHUR and against the SILVA 16S rRNA database containing 14,956 prealigned representative bacterial references. The resulting alignments were then clustered according to the furthest neighbor distance metric. For phylotyping analyses the filtered data was classified using the SILVA taxonomy with the Bayesian classification method implemented in MOTHUR.

3.5 Analysis of *Clostridium* spp. by RT PCR.

The amplified16s rDNA from the mice feces were analyzed for the *Clostridium spp.* by real time using PCR ABI 7500 Fast RT PCR machine (Applied Biosystems,

Carlsbad, CA). The primer sequences used for detection of *Clostridium* spp. were forward CTC AAC TTG GGT GCT GCA TTT and reverse ATT GTA GTA CGT GTG TAG CCC (Rani, 2006). The PCR conditions used were 94^0 C for 5 minutes, 94^0C for 30 seconds, 53^0C for 1 minute, 72^0C for 1 minute and 30 cycles was run with elongation being the final step.

3.6. *Citrobacter rodentium* infection.

The *C. rodentium* strain ICC169 was a kind gift of Gad Frankel (London School of Medicine and Dentistry, London UK). For inoculations, bacteria were grown overnight in Luria bertani (LB) broth containing 50 μg of nalidixic acid (EMD chemicals, Gibstown, NJ per ml. Five $X10^9$ CFU were gavaged orally to each mouse. On day 10 and 14 post-infection feces were collected, weighed and suspended in phosphate-buffered saline. Serial dilutions were plated onto nalidixic acid containing LB agar plates. Bacterial colonies were counted after 24 hours of incubation at 37^0 C.

3.7. Colonic mRNA analyses.

The mice which were challenged with *Citrobacter* infection were sacrificed and their primary third terminal colon was sectioned and weighed. The mRNA was extracted from these colons using RNeasy mini kit (Qiagen, Valencia, CA). cDNA was reverse transcribed from the extracted RNA and real-time PCR was done using a ABI 7500 Fast RT PCR machine (Applied Biosystems, Carlsbad, CA). The PCR cycle was run for 25^0C for 5 minutes, 42^0C for 30 minutes and 85^0C for 5 minutes. Each of the cytokine expression was compared with the GAPDH housekeeping gene expression in the cells in

tne RT PCR and sample were run for 40 cycles in the ABI 7500 Fast RT PCR machine at 50^0C holding temperature, 95^0C denaturing temperature and 63^0C melting temperature.

The analyses included IFNγ, IL-12, IL-17, IL- 22, IL-23 and GAPDH expression. The primer sequences of the same are mentioned in Table 1.

3.8. Histological analysis of colon tissue.

The terminal colon was removed and fixed in 10% formalin. Paraffin-embedded sections were prepared and stained with hematoxylin and eosin at the Pennsylvania State University. For histological grading of colitis six criteria were used – i.e., cryptitis, goblet cell hyperplasia, inflammation, erosion/ulcers and edema. The lesions were scored from 0 to 4, 0= no colitis/epithelial thickening, 1= increased number of leukocytes in the mucosa and/ or slight epithelial cell hyperplasia, 2= multiple loci of inflammation, leukocytic infiltration of mucosa and submucosa and/ or pronounced epithelial cell hyperplasia (2- to 3-fold increase in crypts), 3= extensive leukocytic infiltrate in mucosa, submucosa, ulceration, depletion of mucin-secreting goblet cells and/ or marked epithelial cell hyperplasia (3- to 10- fold increase in crypts) and 4= extensive transmural leukocytic infiltrate, crypt abscesses and/ or marked epithelial cell hyperplasia (crypts more than 10-fold or greater).

3.9. Statistics.

Statistical significance was calculated by one-way ANOVA or 2 sample t-test with Prism software (GraphPad, La Jolla, CA). P values ≤ 0.05 were considered significant.

Cytokines		Primer Sequence	Amplicon Size
IFNr	Forward	5'-TGC ATC TTG GCT TTG CAG CTC TTC -3'	356 bp
	Reverse	5'- GGG TTG TTG ACC TCA AAC TTG GCA -3'	
IL 17 A	Forward	5'- ATG CTG TTG CTG CTG CTG AGC C -3'	220 bp
	Reverse	5'- GGT CTT CAT TGC GGT GGA GAG -3'	
IL 12	Forward	5'- CAC CCT TGC CCT CCT AAA CC -3'	329 bp
	Reverse	5'- CAC GGC AAG GCA CAG GGT CAT CAT C -3'	
IL 22	Forward	5'- AGA AGG CTG AAG GAG ACA GT -3'	83 bp
	Reverse	5'- GAC ATA AAC AGC AGG TCC AGT T -3'	
IL 23	Forward	5'- AGC GGG ACA TAT GAA TCT ACT AAG AGA -3'	245 bp
	Reverse	5'- GTC CTA GTA GGG AGG TGT GAA GTT G -3'	

Table 1. PCR primers used to detect various cytokine responses during gut inflammation induced by *C. rodentium*

Chapter 4

RESULTS

4.1. Changes in diet result in alterations in the DGGE banding patterns.

All of the mice started out on commercial chow diets that were changed at time 0 to either CTRL or WB diets. The DGGE banding patterns of samples at time 0 from mice on chow diet looked to be the most similar (Fig. 1A). Two weeks on the CTRL and WB diet caused a shift in the bacterial species in the feces that is reflected in a change in the DGGE bands detectable (Fig. 1A). In addition, there are differences observed between WB fed and control fed mice (grey boxes) at 2wks (Fig. 1A). Changes in diet result in alterations in the composition of the gut microflora.

The migration distance of the bands in the DGGE profile were measured for each sample and samples that were more similar to one another were clustered based on these measurements (Fig. 1B). The brackets indicated clustering of mice into similar groups. The output shows the degree of similarity between samples. Samples from different diets and at different time points were more similar to each other than to samples from any other time point or diet (Fig. 1B). WB mushrooms altered the composition of the diet at 2 wks and changes continue to occur at 4 wks (Fig. 1B). No further changes were seen in the DGGE banding pattern between the 4 wk and 6 wk time points (data not shown). The diet effects were relatively fixed after 4 wks of feeding.

4.2. Metagenomic analyses.

Two DNA samples from WB and two from CTRL fed mice were sequenced and the sequences analyzed. The coverage, richness, and diversity of the samples at each depth have been assessed. The Chao estimator produces very similar levels of OTU at high levels of sampling. This indicated that all groups have had sufficient coverage and

the comparisons across groups can be made using this data set. The rarefaction curves show support for the use of the 99% level sequence similarity for further downstream analysis. At this similarity measure 47,000 distinct OTUs are detected. The robustness of the samples corresponding to biological replicates is determined using the Jaccard and Bray Curtis similarity measures. The analysis shows that microbial communities from the CTRL fed samples are more similar to each other than the microbial communities found in the WB fed samples. These analyses confirm the less quantitative analyses used on larger numbers of mice and at different time points by DGGE (Fig. 1B). The types of bacteria found in the WB fed groups were more similar to each other than to the CTRL fed groups (Fig. 1B).

For further analyses the two biological replicas are combined. The Berger-Parker index shows the abundance as a percent of the OTU with highest counts. At lower sequence (0.9%) the OTUs in the control group exhibited substantially higher dominance than the WB group. Since the total number of sequences is close to being equal, the result suggests that the control group contains microbial communities that are less diverse than the microbial communities present in the WB fed group.

The sequences were classified against the SILVA taxonomy with 9 levels. The frequencies of the bacteria at the phylum level present in WB and CTRL samples after 6 wks on the diets showed that *Firmicutes* species were decreased in the WB fed group (Fig. 2A). The decrease in *Firmicutes* phyla was associated with an increase in *Bacteroidetes* and the presence of *Verrucomicrobia* that was unique to the WB fed mice. The data is plotted to show significant changes in the number of sequences identified in each phyla, order and class as a result of the diet, and the class level plot is shown (Fig.

2B). The data is scaled logarithmically on the vertical axis to allow high abundance and low abundance changes on the same plots. Only taxa where changes were significantly different ($P<0.001$) are shown and scaled by the appropriate Bonferroni correction to account for multiple testing. At the Class level, bacteria belonging to *Erysipelotrichi*, *Deferribacteres* and *Clostridia* class were decreased in the gut by the WB diets along with an increase in population of the commensals *Mollicutes*, *Epsilon*-, *Delta*- and *Gamma Proteobacteria*, and *Verrucomicrobia*.

4.3. *Clostrida* spp. copy number following WB and CTRL feeding.

The amplified 16s rDNA region from each group of mice feces was run against the *Clostridia* spp. primers by RT PCR. Interestingly, the copy numbers were significantly higher in the WB based 16s rDNA sequences compared to the controls (Fig. 3).

4.4. Protective effects of WB based gut following infection with *C. rodentium*.

Following 6 wk on the CTRL and WB diets mice were infected with the murine gastrointestinal pathogen *C. rodentium*. There was no difference in the weights of CTRL and WB fed mice following *C. rodentium* infection, both groups lost a small but insignificant amount of weight. There were no differences in the numbers of *C. rodentium* in the feces of WB and CTRL fed mice at either d10 or d14 post-infection (Fig. 4A). CTRL mice showed significant induction of inflammation and hyperplasia in the colon at d10 post-infection (Fig. 4B). Severe inflammation including, the identification of ulcers, were seen in CTRL but not the WB fed mice following infection

(Fig. 4B and 4C). There was significantly more inflammation in the d14 than the d10 histopathology sections from both groups (Fig. 4C). In addition, significantly more severe colitis was noted in CTRL colons than WB colons at both d10 and d14 post-infection (Fig. 4C).

4.5. Cytokine responses following *C. rodentium* infection.

The colons of mice following *C. rodentium* infection were analyzed for the presence of inflammatory cytokines. IL-12 mRNA was higher at day 10 post-infection than at day 14 post-infection in both the CTRL and WB colons (Fig. 5). IL-22 and IL-23 were not different in CTRL and WB fed colons nor did they change between day 10 and day 14 post-infection (Fig. 5). IFNγ mRNA was higher at day 14 post-infection compared to day 10 post-infection and higher in the WB samples compared to CTRL (Fig. 5). IL-17 did not change between the day 10 and day 14 time points but was significantly higher in the WB colon compared to CTRL (Fig. 5). Ten days post-infection IL-12 was produced that induced a day 14 boost in IFN-γ (Fig. 5). Conversely IL-22 and IL-23 were produced at constant levels between day 10 and day 14 and this result was reflected in the IL-17 levels (Fig. 5). WB colons had more IFN-γ and IL-17 than CTRL colons (Fig. 5).

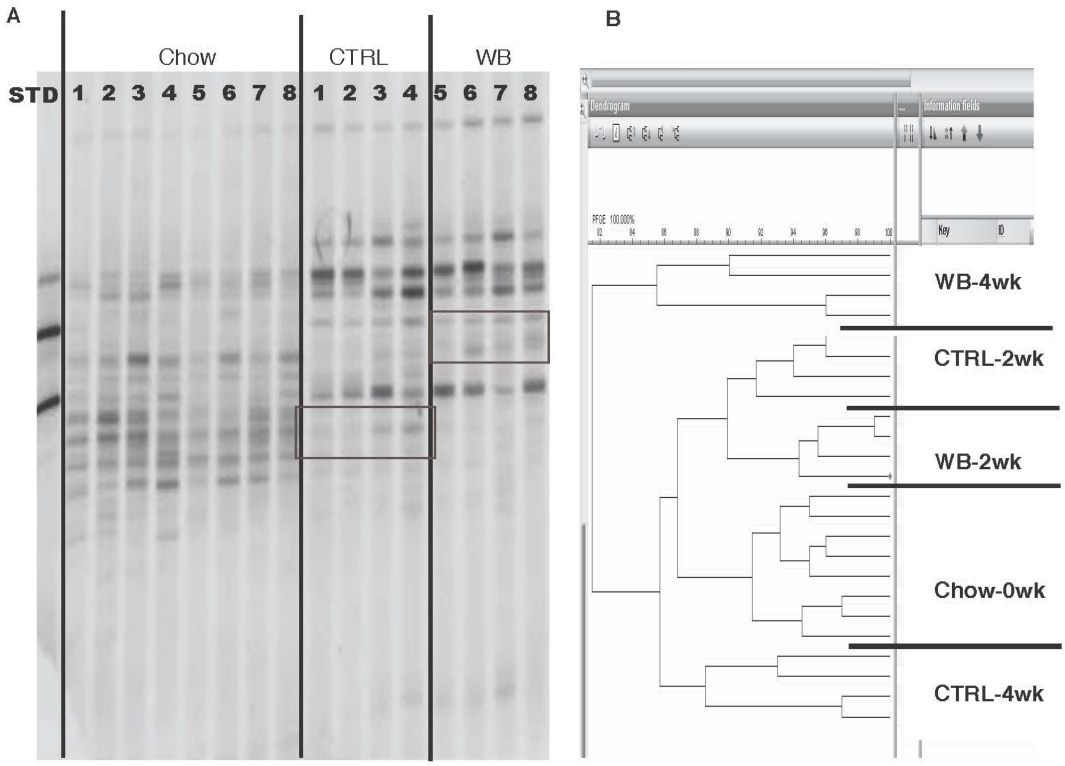

Figure 1. Diet induced changes to the fecal microflora. A) DGGE was run on the PCR amplified fecal bacterial DNA from eight mice (1-8) from two different litters before (Chow) and 2 wks after the change to control (CTRL) and WB diets. Standards (STD) were from pure cultures of (bottom band) *C. propionicum*, (middle band) *L. murinus* and (top band) *P. distasonis*. Selected difference between CTRL and WB groups are highlighted with boxes. B) The migration distance of the DGGE bands from mice before (Chow-0wk) and 2 and 4 wks after CTRL or WB feeding were measured and analyzed by MultiAnalyst imaging software. Clustering analysis show (brackets) the relatedness of the banding profiles between and across group.

A

BACTERIAL GROUP CTRL	(%)	BACTERIAL GROUP WB	(%)
Bacteroidetes	80.3	Bacteroidetes	84.3
Firmicutes	14.7	Firmicutes	8.4
Tenericutes	4.2	Tenericutes	5.5
Proteobacteria	0.5	Proteobacteria	0.8
		Verrucomicrobia	1.0

B

Table and Figure 2. Phylum and Class level changes as a function of WB feeding. The DNA from 2 Ctrl and 2 WB fed mice were sequenced. A) The frequency of the different Phyla sequenced from CTRL and WB fed mice. B) The statistically significant changes in the class of bacteria found in WB fed mice relative to CTRLs. Values that are negative show class level species that are inhibited by WB feeding. Values that are positive show class level species that are more prevalent in WB fed mice.

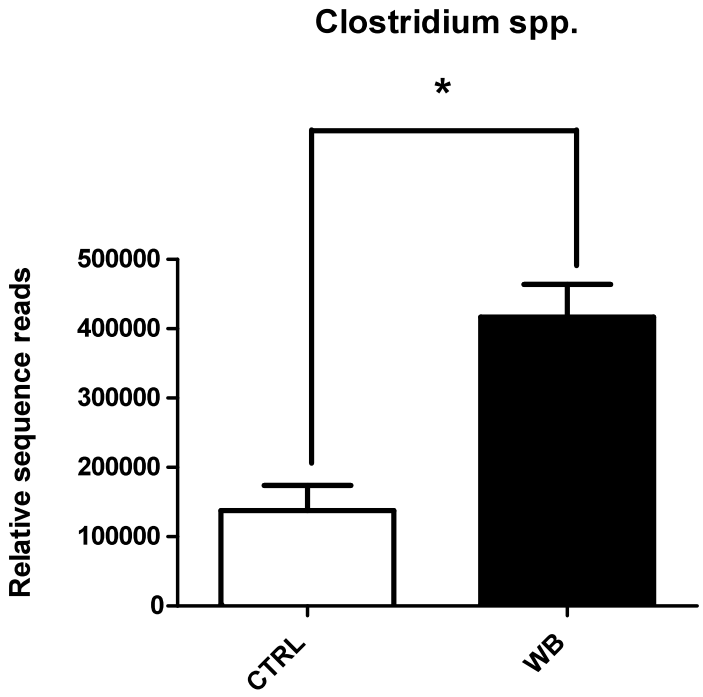

Figure 3. Difference in the *Clostridia* copy number due to WB feeding. Quantitative PCR was used to determine the copy numbers of *Clostridia* in the 16s rDNA regions obtained from the fecal DNA from WB and control group of mice at week 6 after the start of the experiment.

*Indicates significant differences between the WB and CTRL groups connected with the bracket $P<0.05$.

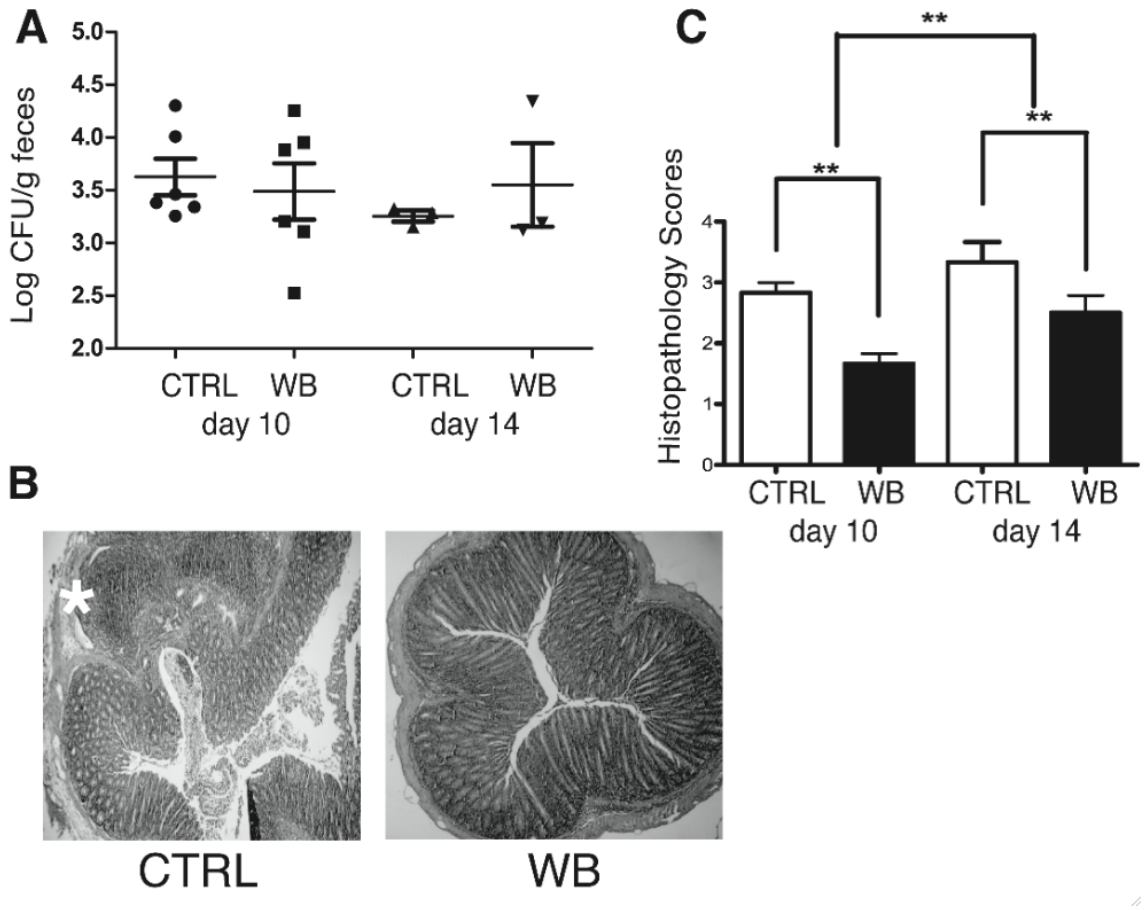

Figure 4. *C. rodentium* infection resolves more rapidly in WB fed mice. A) The numbers of *C. rodentium* isolated from the feces of CTRL and WB fed mice 10 and 14 days post-infection. B) A representative histopathology section of the colons of CTRL (scored 3) and WB (scored 2) fed mice 10 days post-infection. * indicates an ulcer in the section from the CTRL fed mouse. No ulcers were found in WB sections of the colon. C) A summary of the histopathology scores given for CTRL and WB fed mice 10 and 14 days post *C. rodentium* infection. ** Significantly different $P<0.05$ between CTRL and WB fed mice at both day 10 and day 14. There was also a significant difference between day 10 and day 14 scores with the day 14 scores being significantly higher than the day 10 scores.

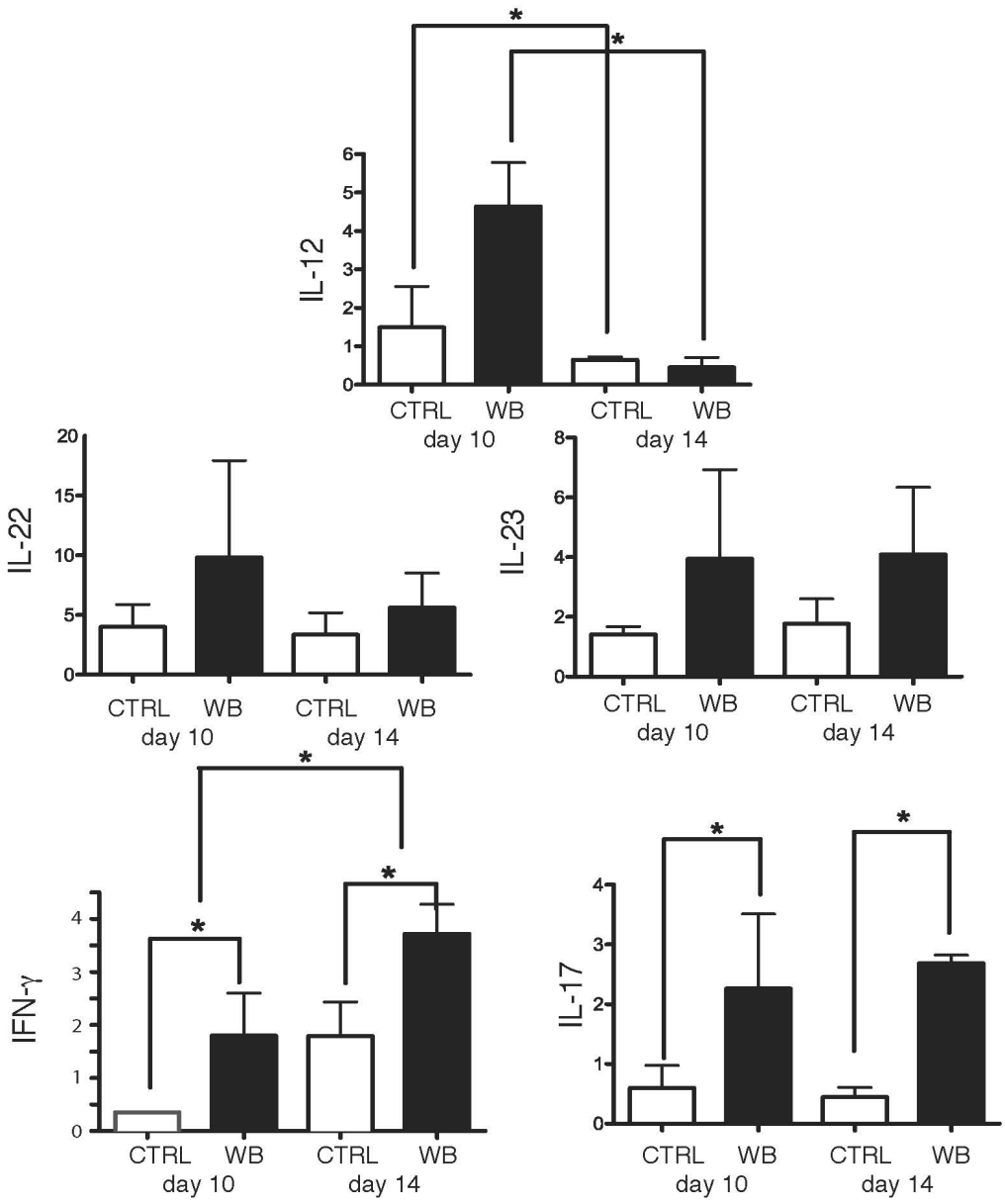

Figure 5. Colonic mRNA expression of inflammatory cytokines. Quantitative PCR was used to determine the level of IL-12, IL-22, IL-23, IFN-γ and IL-17 expressed in the colon of CTRL and WB fed mice at day 10 and 14 post *C. rodentium* infection. *Indicates significantly different than other groups connected by the brackets $P<0.05$.

Chapter 5
DISCUSSION

The mouse gut microflora majorly consists of bacteria in genera of *Bacteroides, Firmicutes and Tenericutes* (Beaugerie and Petit, 2004; Guarner and Malagelada, 2003; Vedantam and Hecht, 2003) and represents a major metabolic entity in the host. In addition to extracting additional energy from the diet, the gut microbiota is responsible for generating vitamins and amino acids, and can metabolize ingested drugs (Ozturk et al., 2011; Ren et al., 2008; Shin et al., 2010). One of the ways the commensal flora protects the gastrointestinal tract is to prevent infection by competition. *Citrobacter rodentium* is an extracellular enteric pathogen, which includes the enteropathogenic *Escherichia coli* that cause human infections. In addition, *C. rodentium* infection has been used as a model of IBD where the infection causes inflammation in the gut and the inflammatory mediators including iNOS, TNF-α, and IL-12 are all important for regulating the extent of pathology in the gut (Mundy et al., 2005). Infection and resolution of infection with *C. rodentium* in mice is affected by host immunity and shifts in the bacterial communities found in the gut (Hoffmann et al., 2009).

Mushrooms have long known to have active ingredients that have beneficial effects such as antioxidants, antitumors and anti ageing properties (Ozturk et al., 2011).

Feeding mice diets that contained 1% freeze dried *Agaricus bisporous* (white button, WB) mushrooms resulted in changes in the composition of the gastrointestinal microflora as observed by the DGGE analysis (Fig 1 A, B). Changes in mircoflora were evident at 2 weeks and stabilized after 6 weeks of WB feeding. To confirm the visual changes, clustering analysis of the same by Jaccard statistics was performed. The diversity of the microflora was increased in the WB fed mice and, more importantly, several classes of bacteria associated with disease (*Clostridia*) were decreased in the WB

fed mice (Fig. 2 B). There was a clear difference in the species of gut microflora present in CHOW diet and that present in CTRL and WB diet. Additionally, significant differences were also observed between the latter two groups. The phylogenetic analysis also indicated a shift in microbial populations especially the decrease in pathogenic class i.e., Clostridia spp., from 2 weeks to 4 weeks of feeding mushroom as compared to the CTRL group (Fig 2 A, B). This suggests that the length of feeding mushroom diet may be important to obtain the desired effect. It was thus logically concluded that mushroom diet supplement does have a beneficial effect on the gut. Thus, sequencing the 16s rDNA is a good technique as it reveals a varied number of bacteria which can't be cultivated by conventional techniques. Interestingly, the story was different when the copy number of *Clostridia* spp. was compared with real time results. The RT PCR revealed an increase in the *Clostridia* group in WB fed mice as compared to the controls. The logical reasoning behind this would be that there is a possibility that the efficiency to extract the copy numbers/ template of *Clostridia* 16s DNA from each of the samples would be different and hence there is difference in amplification which is not a problem during sequencing. Whether this beneficial effect /change in the microflora is sufficient to provide protection to mice or not was ascertained by challenge experiments carried out by giving *Citrobacter rodentium*, a gram negative bacterial enteric pathogen of the mouse. The bacteria, first attaches to the intestinal epithelial cells and then effaces the cell microvilli, which results into hyperplasia and inflammation in infected mice. The mechanism by which it actually affects the intestine is not clearly identified, but it has been found that *Citrobacter rodentium* affects the intestinal cells using a type III secretion system, where

the bacteria delivers the effector molecules into the host cells causing disturbance in the host cellular process (Mundy et al., 2005).

Also it has been proved that MAP (Mitochondrial associated protein), a multifunctional effector protein that targets host cell mitochondria contributes to infection-induced epithelial barrier dysfunction *in vitro* and Tir, which is the *Citrobacter rodentium* translocated intimin receptor, is another virulence factor that is responsible for colonic hyperplasia in mice. During the infection, Tir gets translocated into mouse enterocytes in the cell by *Citrobacter rodentium*. This then provokes the attaching/effacing (A/E) lesion formation, which is the major mechanism of tissue targeting and infection (Ma et al., 2006).

Recent literature states that Th1 response in the infected enterocytes helps in clearing the *Citrobacter rodentium* infection in the gut (Higgins et al., 1999). Surprisingly, in our study, we did not find differences in bacterial clearance between WB and control fed mice but, there was significantly less inflammation in the gut of the WB fed mice as evidenced by histopathological studies with the mice gut colon (Fig 3 B). On the other hand levels of cytokines such as IFN-γ and IL-17 were higher in WB-fed mice on day-14 as compared to the CTRL group. These cytokines help in communication between cells to trigger the protective defenses of the immune system. They act as a potent mediator in delayed – type reactions by increasing chemokine production in various tissues to recruit monocytes and neutrophils to the site of inflammation (Ren et al., 2008; Shin et al., 2010; Vedantam and Hecht, 2003). This is a paradoxical finding since there were fewer immune cells seen in the histopathology sections from WB fed mice but higher expression of IFN-γ and IL-17. Increased Th1 and Th17 responses are

normally associated with more colitis and inflammation in the colon and faster clearance of *C. rodentium*; which did not occur in the WB fed mice. Previously it was shown that WB feeding protected against colonic injury in DSS colitis and the protection was associated with an increase in TNF-α production (Yu et al., 2009). Therefore it seems likely that the WB feeding is increasing the local expression of inflammatory cytokines in vivo and in addition that this release of inflammatory cytokines might be the signal that results in repair of the injury.

The immune system and the gastrointestinal microflora respond to changes in the diet. WB feeding changed the composition of the gut flora, increased the local inflammatory response and healed the gut faster. The mechanisms that result in the reproducible biological changes that occur with WB ingestion are likely to include direct stimulation of the innate immune system through toll receptors that produce inflammation and affect the composition of the gut flora. It would be dangerous if something in the diet would strongly stimulate or suppress immune function. Instead it seems that WB feeding has a very specific and localized effect on the gut. Small increases in inflammatory cytokines induce changes in the complexity and composition of the microbial flora but do not alter the rate at which an enteric pathogen is cleared. The results raise a number of interesting areas for future investigation including the kinetics and longevity of the WB feeding effects. The more practical message of the work is that eating WB mushrooms could improve gastrointestinal health and responses to injury following an infection.

Chapter 6
CONCLUSION

To the best of my knowledge, this is the first report of studying the influence of mushrooms on the gut microflora. Feeding mice with WB mushroom brought about changes in the gut microflora composition which was evident after two weeks of feeding. The changes occurred till 4 weeks after which the microbial population appeared to get stabilized. . The metagenome sequence analysis suggested that there was a significant decrease in the pathogenic group of bacteria along with increase in the commensals. Moreover, when inflammation in the mice gut was induced by *Citrobacter rodentium*, it was found that the WB maintained mice showed faster healing than the controls as evident by histopathological studies carried out with colonic regions of the gut. These studies indicated the beneficial effects of WB feeding. To understand the mechanism by which such changes are brought about, cytokine profiling was done which revealed that during the course of inflammation, the WB induced a significantly higher levels of IFNr and IL 17 than the controls. It is quite probable that these pro-inflammatory cytokines are inducing the local immune response which does not affect the *Citrobacter rodentium* bacterial counts but instead protects gut from inflammatory lesions. The current study is preliminary in nature and throws open many future research areas with mushrooms, especially in understanding the immunological response, particularly the cytokine interplay during gut inflammation subsequent to WB feeding.

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