EXAMINATION OF PARASITES AS A TREATMENT FOR INFLAMMATORY BOWEL DISEASE USING MURINE MODELS.

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ABSTRACT

EXAMINATION OF PARASITES AS A TREATMENT FOR INFLAMMATORY BOWEL DISEASE USING MURINE MODELS

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Inflammatory Bowel Disease (IBD) is a serious, life-threatening chronic inflammatory disease of the gastro-intestinal tract that has been reported to affect 1.1 million adults in the US. Patients with IBD often become refractory to traditional treatments which include antibiotics, immune modulators and corticosteroids. IBD can result in a life-threatening condition and lead to the necessitation of surgical interventions such as for bowel resections. In 2003 Dr. Joel Weinstock proposed using *Trichuris suis* as a treatment for inflammatory bowel disease in patients who were otherwise refractory to standard treatments and subsequently published the results of two small-scale clinical trials. Here, he reported that *T. suis* resulted in amelioration of clinical signs of disease in many patients and no adverse side effects. Despite these positive results, the mechanism(s) by which *T. suis* improved patient outcomes remains unknown. In this work we set forth to address possible mechanisms, and to investigate the possibility of adverse side effects due to *T. muris* infections in a murine model. We addressed the hypothesis that infections with *T. muris* alter the colonic microbiota in mice, which may serve as one mechanism to explain the improvement observed in the human patients treated with this worm. We found that C57 BL/6 IL-10$^{-/-}$ mice infected with *T. muris* had sex and dose dependent morbidity and mortality. Additionally, these infections resulted in severe peritonitis with or without gastrointestinal perforations in some mice. In a subsequent experiment C57 BL/6 IL-10$^{-/-}$ mice infected with *T. muris* were concurrently treated with prednisolone or metronidazole, neither of

which resulted in a significant decrease in mortality when compared to the untreated infected mice. Finally we infected C3H/HeJ and C3Bir IL-10$^{-/-}$ mice, which are predisposed to developing a spontaneous colitis, with *T. muris* to test our hypothesis that infections with *T. muris* would change the colonic microbiota. Using Terminal Restriction Fragment Length Polymorphisms (TRFLPs) we determined that mice infected with *T. muris* had significantly different proximal colon microbial microbiota when compared to uninfected mice. To further assess the effects of colitis without *T. muris* infections on proximal colon microbiota we obtained samples from C3Bir IL-10$^{-/-}$ mice within our breeding colony who developed spontaneous colitis and compared them to C3Bir IL-10$^{-/-}$ mice without colitis. From this study we found that overall there were no differences, as assessed using TRFLP, between the two groups. From the entirety of this work we conclude that *T. muris* has the possibility of causing adverse side effects in some individuals, which could include gastrointestinal perforations and peritonitis, particularly those who are deficient in IL-10. Additionally, we concluded that *T. muris* does result in changes in the structure of the proximal colon microbiota, which may be one mechanism through which it worked as a treatment for IBD in human patients.

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CHAPTER 1

Introduction

Trichuris infection as a double-edged sword

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TRICHURIS OVERVIEW

Trichuris spp. are nematodes which have been traditionally considered a health threat in both veterinary and human medicine. The *Trichuris* life cycle begins with the host ingesting embryonated eggs in the first larval stage (L1); the eggs hatch in the host's cecum and/or colon and undergo four molts to become sexually mature adults. Larval and adult stages burrow into the host's colonic epithelium forming syncytial tunnels and produce well characterized excretory-secretory products (ESP) with antimicrobial activity (Abner, Parthasarathy et al. 2001). This review will cover *Trichuris* host interactions that result in disease described as *trichuriasis*, as well as interactions that result in enhancements to health after *Trichuris* eggs are used as a therapeutic intervention. This discussion is meant to introduce a wide span of *Trichuris*-host interactions rather than to cover in depth the immunological interactions this helminth induces in the host which have been well reviewed previously by Artis (Artis 2006), Wang *et al* (Wang, Cao et al. 2008), Bradly and Jackson (Bradley and Jackson 2004) and Grencis *et al* (Hayes, Bancroft et al. 2004). The ultimate goal is to stimulate further research into the potential benefits and adverse effects of *Trichuris* in the host.

Trichuris host interactions resulting in disease

Disease in humans

In humans, *Trichuriasis* has long been considered an important disease throughout the world and is associated with malnutrition, anemia and rectal prolapse (Cooper and Bundy 1988). Individuals with high adult *Trichuris* worm burdens can go on to develop *Trichuris* Dysentery Syndrome (TDS) (Krishnamurthym, Samanta et al.). This syndrome has multiple clinical presentations in patients but most often results in chronic dysentery, clubbing of fingers, anemia

and developmental, growth and cognitive deficiencies. In 1994 Callender *et al* (Callender, Grantham-McGregor et al. 1994) reported their findings of a study assessing the cognitive function of children with TDS compared to those without TDS, and also followed up with the same children a year post-anthelminthic treatment. They found that children with TDS, prior to anthelminthic treatment, had significant developmental deficits, as measured using the Griffiths mental developmental scale, when compared to uninfected children. By a year post treatment, they were showing improvement in locomotive development and were rapidly catching up to their peers in terms of body condition/wasting, height-for-age and hemoglobin levels. Furthermore, four years later Callender *et al* (Callender, Walker et al. 1998) reported a follow up study with these same children. Although children with TDS given anthelminthic treatment did catch up in terms of height, their school achievement and cognitive function scores were still significantly lower than the uninfected control children, even 4 years post anthelminthic treatments. Thus, *Trichuris* infections in children—especially those producing dysentery—significantly impair health, growth, development and mentation in a manner similar to other helminth infections (Nokes and Bundy 1994).

These systemic effects resulting from trichuriasis are mediated by specific sequelae of infection. Malnutrition is a common consequence of *Trichuris* infection with effects on multiple organ systems (Stephenson and Holland 1987); micro nutrients such as vitamins and iron and macronutrients such as protein can be deficient. In a study in Zanzibari children, decreases in iron status were associated with several parasitic infections including *T. trichuria*, *Ascaris lumbricoides*, hookworms, and malaria, although the association was greatest with hookworms (Stoltzfus, Chwaya et al. 1997). Blood loss or red blood cell hemolysis without adequate scavenging may occur concurrently. Iron deficiency anemia appeared when the dietary intake of

iron is insufficient for the proper synthesis of hemoglobin. Clinically apparent anemia most often resulted in fatigue and weakness, but if it occurred during early childhood impaired neurological development (Lozoff, Beard et al. 2006). In this case there can be a permanent decrease in dopamine receptors and serotonin levels, reduced myelination of the spinal cord and changes in myelin composition (Lozoff, Beard et al. 2006). These mechanisms lead to decreased ability to learn and altered motor function. Iron deficiency anemia can decrease growth because growth hormone levels are related to serum transferrin levels that drop during helminth infections.

Disease in animals.

Trichuris spp. are a recognized cause of diarrheal disease in veterinary medicine as well, particularly in dogs and weaning age pigs. Mucohemorrhagic diarrhea is frequently reported as a cause of clinical signs and economic losses in weaned swine (Tubbs 1987; Company 1991). In 1975 Rutter and Beer hypothesized that *Trichuris* synergized with the host's gastrointestinal (GI) microbiota to produce clinical signs of disease, primarily dysentery. They tested this hypotheses using conventionally raised, specific pathogen free and gnotobiotic pigs which were infected with *T. suis* and followed throughout the course of infection. From this work they showed that the conventionally raised pigs developed severe mucohemorrhagic enteritis as opposed to the specific pathogen free and gnotobiotic pigs which developed few and mild signs of enteritis. From this work they concluded that *T. suis* was synergizing with the microbiota or certain members of the microbiota in the conventionally raised pigs leading to the severe clinical signs of disease. In more recent work, *T. suis* was linked to diarrhea in weaning age pigs where interactions with enteric bacteria, such as *Campylobacter jejuni*, caused mucohemorrhagic colitis (Mansfield and Urban 1996; Mansfield, Gauthier et al. 2003) . In the 2003 study, germ free pigs

were infected with *T. suis*, *C. jejuni* or dually infected with both *T. suis* and *C. jejuni* and followed throughout the course of disease. Pigs infected with *T. suis* and *C. jejuni* developed clinical signs of disease, primarily diarrheal disease, and at necropsy had more severe colon lesions than those pigs receiving either *T. suis* or *C. jejuni* which experienced minimal to no disease and pathology and mild disease and pathology respectively.

Trichuris vulpis is a recognized and common large bowel parasite in canids. Many dogs can harbor this parasite with no or few clinical signs of disease, but large numbers of *T. vulpis* have been associated with varying degrees of disease states (Malik, Hunt et al. 1990). Typical disease presentations include ill-thrift, weight loss, and abdominal discomfort. Diarrhea in infected dogs is typical of other diarrheas of large bowel origin which includes small frequent amounts, with mucus and sometimes frank or undigested blood and may occur in "bouts" which are interrupted by seemingly normal periods of elimination. In a limited number of cases dogs have presented with life threatening disease that was associated with *Trichuris*; these dogs had severe dehydration that was associated with acid base and electrolyte imbalances (Smith 1954; DiBartola 1985; Malik, Hunt et al. 1990)

Additionally, *Trichuris trichiura* has also been found associated with enteric pathogens including *Campylobacter coli*, *C. jejuni*, *Shigella flexneri*, *Yersinia enterocolitica*, adenovirus, and *Strongyloides fulleborni* in captive rhesus monkeys with chronic enterocolitis (Sestak, Merritt et al. 2003). This work builds upon the previous studies performed using experimental infections with *T. suis* and *C. jejuni* and shows that *Trichuris* can be found in association with enteric pathogens in a non-experimentally induced setting.

Trichuris host interactions resulting in beneficial effects

It has been recognized for some time that experimental challenge with *Trichuris* spp. produces adaptive immune responses that favor an anti-inflammatory milieu in the GI tract. Based on these observations, *Trichuris* has been proposed as a treatment for inflammatory conditions such as Inflammatory Bowel Disease (IBD) and most recently allergies (Summers, Elliott et al. 2003; Rodrigues, Newcombe et al. 2008). Weinstock and colleagues reported success using deliberate infection with the swine helminth, *T. suis*, to treat IBD patients whose disease had been refractory to standard treatments (Summers, Elliott et al. 2003). Their hypothesis, that *Trichuris* embryonated ova challenge is a successful treatment for patients with inflammatory bowel disease, was based on the "hygiene hypothesis." In this form, the hygiene hypothesis was proposed as an explanation for the relatively low incidence of autoimmune disorders in less industrialized nations. The hypothesis is founded on the idea that exposure to common infectious agents at a young age is necessary to program the immune system for proper future responses (Koloski, Bret et al. 2008). Early exposure to microbes is proposed to establish a necessary immunological balance between pro-inflammatory Th1 responses and tolerant regulatory T (Treg) cells. Later, this "educational exposure" could prevent hyperactive responses to microbes and other stimuli. With this hypothesis in mind, Weinstock and colleagues postulated that parasitic worms play an important role in educating the human immune system for appropriate responses to foreign microbes and resident microflora and that this effect can be impaired in "clean" environments (Summers, Elliott et al. 2005). They conducted and reported the results of a preliminary open label study (Summers, Elliott et al. 2003) and a randomized controlled trial (Summers, Elliott et al. 2005) to assess the effects of *Trichuris suis* infections on

Crohn's Disease (CD) and Ulcerative Colitis (UC). Their 2003 open label study involved seven patients (four with CD and three with UC) which had been refractory to other treatments for IBD. Patients were assessed based on several indices including the Crohn's Disease Activity Index (CDAI), Simple Clinical Colitis Activity Index (SCCAI) and the Inflammatory Bowel Disease Quality of Life Index (IBDQ). Based on their biweekly IBDQ scores 6 of the 7 patients achieved remission of disease by 8.3 weeks into the treatment and all of the patients reported a decrease in clinical signs of disease. They did note that in some patients the decrease in clinical signs of disease was temporary, although in four patients multiple doses were given and these patients remained in remission. In their 2005 report of the randomized control study involving 30 patients receiving *T. suis* ova (TSO) and 24 patients receiving a placebo, 44.8% of the patients were classified as having experienced a positive response, which signified a decrease in the CDAI of ≥4. The authors also reported that they did not observe any adverse side effects during the treatment period of these IBD patients. They concluded that based on several clinical indices, patients with CD and UC experienced significant improvement with this therapy without any adverse events. Although deliberate *Trichuris* infections served as a safe and effective treatment for IBD in clinical trials on a small scale, the mechanism(s) underlying clinical improvements in *Trichuris* treated IBD patients remained unknown (Summers, Elliott et al. 2003).

Epidemiological studies have found that children infected with *Trichuris* at a young age had protective antigens that prevented the development of atopy later in life (Cooper, Chico et al. 2003). Cooper *et al* reported the results of an epidemiological cross-sectional study in school aged children. Here, they found that individuals defined as being chronically infected with helminths, including *T. trichuria*, were less likely to react to skin allergen testing. Additionally,

Rodrigues *et al* (Rodrigues, Newcombe et al. 2008) performed a cohort study with school-aged children in Brazil and found that children heavily infected with *T. trichuria* were significantly less likely to respond to skin allergy testing. In light of the epidemiology studies that revealed a negative correlation between *Trichuris* infections and reactivity on skin allergen testing, Bager *et al* conducted a double-blinded placebo control study looking at the effects of TSO as a treatment for allergic rhinitis in humans (Bager, Arnved et al. 2010). Individuals were chosen for the study by the following criteria: 1) if they had symptoms of grass-pollen induced allergic rhinitis during the last two pollen seasons, 2) skin allergen testing with grass allergen that produced a wheal diameter of ≥3mm, 3) specific IgE levels against grass allergen of ≥0.7 kilo Units antigen per liter (kUA/L), and 4) a spirometric FEV_1 ≥70% or predicted and no significant asthma. These individuals were then given 10 to 8 treatments with TSO 21 days apart. Measurements of clinical signs and effect of TSO on patient allergies and evidence of infection were based on a self-scoring system, need of rescue medication, skin prick allergen testing, total histamine blood levels, serum antibody titers for grass-specific IgE and *T. suis* specific IgE, IgG, IgG_4 and IgA, exhaled nitric oxide, hemoglobin levels and differential counts of leukocytes and erythrocytes. Using these clinical and laboratory measurements to assess both the effects of *T. suis* infection and the clinical signs and symptoms of allergic rhinitis, Bager *et al* concluded that although there was immunological and clinical evidence of *T. suis* infection, there was no supporting evidence that repeated treatment with *T. suis* resulted in a substantial therapeutic effect on allergic rhinitis. In a 2010 correspondence, Summers *et al* (Summers, Elliott et al. 2010) argued that several flaws existed in the experimental design that may have caused the potential benefit of TSO as a treatment for Allergic Rhinitis to be missed. Namely, they claim that the timing of the TSO

administration was not ideal, because the majority of the subjects began treatment too late for therapeutic levels to be optimal prior to the peak allergen season.

Mechanisms underlying *Trichuris* host interactions

Effects of excretory secretory products (ESP)

Trichuris ESP have been demonstrated to aid the worm in both burrowing into the host's colonic epithelium and in feeding (Abner, Parthasarathy et al. 2001; Abner, Hill et al. 2002). Additionally, a handful of helminth-derived immune-modulating products have been identified in other parasites that may also be found in *Trichuris* (Harnett and Harnett 2010). These products were found in a number of different parasites including *Schistosoma mansoni*, *Fasciola hepatica*, *Onchocerca volvulus*, *Heligmosomoides polygyrus*, *Ascaris lumbricoides* and *Acanthocheilonema viteae*; their activities cover a broad array of immune-modulating properties.

Trichuris spp. appears to have multi-functional excretory secretory products (ESP) a number of which have been identified with *in vitro* and *in vivo* studies. In a 1997 report, Hill and Sakanari reported on a thiol protease excreted by *T. suis* (Hill and Sakanari 1997). Although the exact function still remains unknown, it was found to be localized in the gut tissues, which suggests that it may play a role in feeding and digestion behaviors of *T. suis*. The authors hypothesized that the thiol protease may have a roll in heme digestion. In support of this hypothesis it was later shown that *T. suis* is not capable of heme synthesis even though they incorporate heme into their own hemoproteins (Rao, Carta et al. 2005). This suggested that the parasite incorporates the host's heme into its hemoproteins. In 2000 a secreted protease inhibitor was also identified by Rhoads *et al* (Rhoads, Fetterer et al. 2000). This protease inhibitor was hypothesized to act as a *Trichuris*-defense mechanism aimed at the host's attempts to expel the

parasite by inactivating serine proteases of the host's mast cells and neutrophils. A second serine protease with anti-chymotrypsin and elastase activities was identified and characterized (Rhoads, Fetterer et al. 2000). Furthermore, another secreted product was identified in *Trichuris trichuria* and *T. muris* that has also been shown to have a similar component in their excretory secretory product with *in vitro* ion conducting pore forming abilities in phospholipid membranes (Drake, Korchev et al. 1994).

Excretory Secretory Products from *Trichuris suis* has been shown to have anti-microbial activity termed trichuricin (Abner, Parthasarathy et al. 2001). Specifically, these *in vitro* experiments showed that the *T. suis* ESP had broad spectrum antimicrobial activity against gram negative bacteria, including *Campylobacter jejuni*, *C. coli* and *Escherichia coli* and gram positive bacteria, including *Staphylococcus aureus*.

Immune Response Elicited by Trichuris

Trichuris spp are known to modulate host immunity. Within 24 h post infection (PI) Balb/c and AKR mice both elicit colonic epithelial innate response to infection with *T. muris* which includes increased expression of INF and TNF mRNA levels; these expression levels remain increased for the next 7 days post infection (DeSchoolmeester, Manku et al. 2006). These results were confirmed *in vitro* using *T. muriss* ESP and a colonic epithelial cell line, CMT-93.

The classical immediate-type hypersensitivity response is the general immune response in mammals towards gastrointestinal nematodes (Urban Jr 1998; Mansfield 2000). It is partially characterized by mucosal mastocytosis and eosinophilia, the production of IgE and IgG_1 antibodies, and the alteration of the composition and quantity of goblet cell mucins (Urban Jr, Madden et al. 1992; Urban Jr 1998; Mansfield 2000). Investigators have studied the components

of the immediate-type hypersensitivity response as it relates to *Trichuris* infection using a mouse model. From this work, much has been discovered about the roles of mucosal mast cells, eosinophils, B cells, $CD4^+$ T cells and cytokines in trichuriasis. For example, the classical immediate-type hypersensitivity response was not effective in providing resistance against *T. muris* infections. While mastocytosis and eosinophilia occur in response to *Trichuris* infections, they were neither necessary for resistance to *T. muris* nor were they involved in expulsion of the worm (Betts and Else 1999; Koyama and Ito 2000; Onah and Nawa 2000). These conclusions are based on *T. muris* challenge studies in which eosinophilia or mastocytosis were ablated within mice, or by using mice that were genetically mast cell-deficient, the WBB6F1 mice (Betts and Else 1999; Koyama and Ito 2000). Ablation of eosinophilia was obtained by injecting neutralizing anti-IL-5 antibodies into the mice, and ablation of mastocytosis was obtained by injecting neutralizing anti-c-*kit* antibodies that block stem cell factor (Betts and Else 1999). Results showed that activated B cells were not required for expulsion of *T muris* in infected mice; however, they were required for resistance in that they lead to the development of a Th2-type cytokine response (Blackwell and Else 2001). *Trichuris muris*-specific IgE and IgG_1 were also not involved in worm expulsion after primary infection, but were shown to play roles in resistance to subsequent challenge infections of *Trichuris* (Blackwell and Else 2001). Furthermore, antibody-dependent cell-mediated cytotoxicity (ADCC) was also shown not to play a significant role in *T. muris* resistance or expulsion. $Fc\gamma R^{-/-}$ mice (C57BL/6 background) that were deficient in effector function requiring high affinity IgE or IgG binding, behaved similarly to the C57BL/6 controls, expelling the worms 21 to 24 days after infection and exhibiting cytokine profiles and antibody production consistent with resistant strains of mice (Betts and Else 1999).

$CD4^+$ T cells are very important in resistance to *Trichuris muris* (Koyama, Tamauchi et al. 1995; Onah and Nawa 2000) . Studies have shown that athymic BALB/c mice and mice where the $CD4^+$ T cell population has been ablated using neutralizing antibodies are susceptible to whipworm infections (Blackwell and Else 2001). Furthermore, Severe Combined Immune Deficiency (SCID) mice (which lack B and T cells) reconstituted with purified $CD4^+$ T cells from infected BALB/c mice were resistant to whipworm infection (Blackwell and Else 2001). The ability of the different inbred mouse strains to expel the whipworm is based on genetic background, especially MHC-linked genes (Else and Wakelin 1988). The majority of existing inbred mouse strains are resistant to infection and expel the worms before patency is reached, which is approximately 32 days post-infection. Resistance and worm expulsion are associated with the $CD4^+$ Th2 cytokine response, in which interleukins (IL)-4, IL-5, IL-6, IL-9, IL-10, and IL-13 are secreted by $CD4^+$ cells (Else and Grencis 1991; Else, Hültner et al. 1992; Else, Finkelman et al. 1994; Urban, Fayer et al. 1996; Bancroft, McKenzie et al. 1998; Faulkner, Renauld et al. 1998; Bancroft, Artis et al. 2000; Koyama and Ito 2000; Mansfield 2000; Onah and Nawa 2000; Richard, Grencis et al. 2000; Grencis 2001; MacDonald, Araujo et al. 2002). Th2-type cytokines promote differentiation of precursor Th cells into Th2 cells, class switching of B cells to IgE and IgG_1, eosinophilia, and mucosal mastocytosis (Koyama, Tamauchi et al. 1995; Urban, Fayer et al. 1996; Faulkner, Renauld et al. 1998; Urban Jr 1998; Betts and Else 1999; Koyama and Ito 2000; Mansfield 2000; Richard, Grencis et al. 2000; Grencis 2001) . Using IL-10- and IL-4/IL-10-knockout mice (C57BL/6 background) Schopf and colleagues discovered that IL-10 is important in resistance to *T. muris* and in the survival of mice subject to the inflammatory process brought about by secondary bacterial infections (Schopf, Hoffmann et

al. 2002). More recently, tumor necrosis factor (TNF)-α has been demonstrated to play a role in Th2 cytokine-mediated *T. muris* immunity (Artis, Humphreys et al. 1999). Artis et al. demonstrated that the expulsion is hindered by blocking TNF-α *in vivo* using an anti-TNF-α antibody (Artis, Humphreys et al. 1999). Additionally, intraperitoneal injections of recombinant TNF-α in *T. muris*-infected, BALB/c male IL-4 knockout (KO) mice, which cannot expel worms by day 35 post-infection, enhanced the ability of these mice to clear worms by day 35 post-infection. It is important to note that the TNF-α treatments did not change levels of IL-5, IL-9 or IL-13 production (Artis, Humphreys et al. 1999).

Mice that are susceptible to *T. muris* infection mount a Th1-type response characterized by IL-18, IL-12 and interferon (IFN)-γ, and harbor adult worms even after patency (Else and Grencis 1991; Else, Finkelman et al. 1994; Bancroft, Else et al. 1997; Betts and Else 1999; Koyama and Ito 2000; Grencis 2001; Helmby, Takeda et al. 2001; Swain 2001) . The role of IL-18 in susceptibility is considered novel because it acts to downregulate Th2 cytokines such as IL-4 and IL-13 instead of acting as an inducer of IFN-γ (Grencis 2001; Helmby, Takeda et al. 2001; Swain 2001) .

The cytokine profile in *T. muris*-infected mice is not always distinctly polarized to either the Th1 or Th2 response, and discrepancies among cytokine responses to experimental whipworm infections have been reported. Mice challenged repeatedly with low doses of *T. muris* embryonated eggs mount mixed cytokine responses and eventually expel the worms (Bancroft, Else et al. 2001). Also, mixed cytokine responses have been demonstrated in swine and human trichuriasis. Both IL-10 (Th2) and IL-12 (Th1) were shown to be elevated in swine exposed to *T. suis* on dirt lots. However, experiments have shown that only IL-10 is significantly elevated in *T.*

suis-infected pigs kept in confinement, while IL-12 is not detectable (Mansfield, Urban et al. 1998). A small percentage of mixed white blood cells from whole blood cultures from *T. trichiura*-infected humans secreted IL-4 (7%), IL-9 (5%), and IL-13 (17%) and a larger percentage secreted IL-10 (97%), TNF-α (93%) and IFN-γ (32%) when stimulated with *T. trichiura* antigen *in vitro* (Faulkner, Turner et al. 2002). Turner and colleagues have also shown using whole blood culture from *T. trichiura*-infected individuals that *Trichuris* ESP elicits a different cytokine response (IL-10 and TNF-α) than *Trichuris* somatic antigens (IL-4, IL-13 and proliferative responses of white blood cells). IFN-γ was not induced (Turner, Faulkner et al. 2002).

Thoughts for the future of Trichuris as a friend and foe

Our knowledge of the many effects that helminths have on mammalian systems is rapidly increasing. Many of these advancements in our knowledge may also apply to *Trichuris* spp. as well, and may help to guide future investigative efforts as the driving forces for new hypotheses behind *Trichuris* as both a disease causing agent and a treatment for various disease processes. As an example of recent advancements in terms of investigations of the effects of parasites on the gastro-intestinal environment, Walk *et al* (Walk, Blum et al. 2010) reported that the parasitic helminth *Heligsmosomoides polygyrus* results in changes in the murine gastrointestinal microbiota, including a significant increase in the abundance of the bacterial family *Lactobacillaceae* in the ileum of these mice. If this holds true for other parasites, specifically *Trichuris*, it may result in a greater understanding of its successful use as a treatment for inflammatory bowel disease.

INFLAMMATORY BOWEL DISEASE (IBD)

Who, What, Where, When and Why

Inflammatory Bowel Disease (IBD) is a serious chronic relapsing inflammatory disorder that primarily affects the gastrointestinal (GI) tract, but more recently has been considered a systemic disease due to extra-intestinal manifestations (Danese, Semeraro et al. 2005). IBD is reported to affect 1.1 million adults in the US and the number of individuals that it affects is on the rise (Bonen and Cho 2003). To put this in perspective, Morrison et al (2009) reports that an individual is more likely to have or to develop IBD than epilepsy or to be involved in a traffic accident and that the probability is similar to developing type 1 diabetes or schizophrenia. The two most commonly recognized forms of IBD are ulcerative colitis (UC), where inflammation involves the rectum and may extend continuously as far as the cecum and Crohn's disease (CD), which may involve any region of the GI tract, but most commonly affects the ileum and colon (Cho 2008). Although there are two different classifications of IBD, they can occur within the same family suggesting that individuals with different forms share genetic susceptibilities (Lennard-Jones 1989). In a report from ACCESS, individuals with IBD reported that they have experienced and noted a number of impacts that IBD has on their life. These include development of functional GI symptoms, anemia, sexual dysfunction, and interference with employment, education, quality of life, obtaining proper nutrition, and development (Access Economics, 2009). In many cases, patients reported the need for psychological help besides the need for recurring medical care.

IBD is thought to be caused by a multitude of factors which may include host genetics, host adaptive cellular immune responses, exposure to pathogens, and abnormal interactions of

microbiota with the host's GI tract (Hugot 2004). CD and UC are not considered single gene disorders, as over 30 susceptibility and IBD associated genes have been identified (Cooney and Jewell 2009). Twin studies showed that while there is a genetic basis for IBD, it is not inherited in a simple Mendelian fashion (Farmer, Michener et al. 1980; Monsen, Broström et al. 1987; Halme, Paavola-Sakki et al. 2006). Genetic linkage analysis studies have identified nine disease loci, five of which meet the most stringent linkage analysis criteria, the remaining of which were at least suggestive (Cooney and Jewell 2009). From these genetic link analyses specific susceptibility genes were also identified. *CARD15/NOD2* was the first susceptibility gene that was identified (Hugot 2004). CARD15 encodes a protein that is expressed in multiple immune system components and part of the gut barrier as well. Some of these locations include: Paneth cells, monocytes and tissue macrophages and intestinal epithelial cells (Ogura, Bonen et al. 2001; Hisamatsu, Suzuki et al. 2003; Lala, Ogura et al. 2003; Ogura, Lala et al. 2003; Tanabe, Chamaillard et al. 2004; Begue, Dumant et al. 2006). Additionally, two autophagy genes, ATG16L1 and IRGM— both of which have roles in the processing of microbial antigens as part of the innate immune system—were identified as susceptibility genes. Recently, IBD has been linked to IL-10 deficiencies in humans (Glocker, Kotlarz et al. 2009; Noguchi, Homma et al. 2009). In the Glocker *et al* 2009 study, investigators found through genetic linkage analysis and candidate gene sequencing from samples of two unrelated consanguineous families with children who had developed early onset IBD, mutations in genes encoding the *IL10R* subunit proteins (Glocker, Kotlarz et al. 2009). Specifically, they were able to show that mutations in either *IL10RA* or *IL10RB* are associated with severe early onset enterocolitis in children. In 2009, Noguchi *et al* reported finding NOD2 mutations in patients with IBD. The functional importance

of this mutation was linked to inhibition of IL-10 in human monocytes (Noguchi, Homma et al. 2009).

In addition to the genetic susceptibilities that have been identified that appear to predispose an individual to developing IBD, some environmental factors have also been identified that have been epidemiologically linked to an increased incidence of IBD (Molodecky and Kaplan 2010). One environmental association hypothesis is referred to as the "hygiene hypothesis", which states that the frequency of immunological disorders has increased due to a decrease in childhood exposure to various enteric pathogens (Bernstein and Shanahan 2008; Shanahan and Bernstein 2009). Epidemiological studies have linked *H. pylori* infections, breast feeding and large family sizes as having a negative correlation with the development of IBD (Lashner and Loftus Jr 2006; Luther, Dave et al. 2010). Additionally the use of birth control, stress, some enteric pathogens such as *Salmonella* and *Campylobacter* have all been associated with an increase in the incidence of IBD (Gradel, Nielsen et al. 2009).

IBD has been reported to have extra-intestinal manifestations (Bernstein, Blanchard et al. 2001). These manifestations affect many functions, organs and organ systems including: bones and joints (rheumatic arthritis), metabolic system, skin, eyes, liver, blood, urinary tract, the pulmonary system and the pancreas. The most common extra-intestinal complications are rheumatic (peripheral and axial), dermatological, ophthalmologic and hematologic. Rheumatic complications are of the most commonly noted in IBD patients and are reported to affect up to 10-35% of patients with IBD (Kethu 2006; Juillerat, Mottet et al. 2008). In some cases the extra-intestinal manifestations are immune related and thought to be due to activation of the individual's immune system against antigens that are cross reactive with colonic mucosa, also known as antigen mimicry (Das 1999). These antigens are also present on other organs, leading

to the observed extra-intestinal manifestations. An example of this occurs in patients with primary sclerosing cholangitis where the anti-colonic mucosa auto-antibodies cross react with the patient's biliary epithelium (Chapman, Selby et al. 1986). Additionally, extra-intestinal manifestations have been reported to have familial tendencies, with the HLA system being one of the main genetic markers of interest in these cases (Satsangi, Parkes et al. 1996).

Despite the wide range of traditional pharmaceuticals, surgery is necessary in approximately 75% of IBD cases (Isaacs and Sartor 2004). Unlike patients with Crohn's disease where surgery is only palliative, in patients with Ulcerative Colitis surgical removal of the colon and rectum can be curative and can also eliminate the possibility of subsequent malignancies in this area (Grucela and Steinhagen 2009). Unfortunately, surgical corrections do not come without their own consequences and site-specific considerations. Bowel resections are most common in individuals with disease of the terminal ileum, with or without proximal colon involvement. Unfortunately, even with surgery, their IBD rarely goes into lifelong remission (Loftus Jr and Sandborn 2002), and as inflammation returns surgeons are faced with challenges of what else can be removed without putting the patient at risk for short bowel syndrome (Hyman 2007). When colonic involvement necessitates surgery, subtotal colectomy with end ileostomy is usually the safest technique (Tekkis, Purkayastha et al. 2006) and like small intestinal surgery, re-occurrences post-surgery are common.

INTESTINAL MICROBIOTA: A ROLE IN HEALTH AND DISEASE

Normal Microbiota Gone Bad

Host microbiota have been shown not only to play a crucial role in maintaining normal GI physiology but also in the pathogenesis of enteric diseases and have been implicated as having a key role in the development of IBD (Cho 2008). Individuals with IBD are believed to have a reduced tolerance to the commensal microbiota of their GI tracts. This hypothesis has been supported through the study of murine models in germ-free environments. Several studies demonstrated that a germ-free status resulted in amelioration of clinical signs in several murine models of IBD (Cho 2008). Although it was originally postulated that IBD is associated with pathogens, current studies link IBD to commensal enteric bacteria (Packey and Sartor 2008). These IBD-associated commensal bacteria may carry virulence factors and/or evoke elevated immune responses. One potential pathogen associated exception, is *Mycobacterium avium* subspecies *paratuberculosis* (MAP), which causes Johne's disease in ruminants. In ruminants this obligate intracellular pathogen causes a spontaneous granulomatous enterocolitis resulting in diarrhea and wasting. Numerous studies have attempted to tie MAP to CD and thus far the hypothesis has been neither supported nor disproven (Packey and Sartor 2010).

Additionally, it has been reported that there is evidence of reduced microbial diversity in IBD patients (Frank, St Amand et al. 2007; Scanlan and Marchesi 2008) including a decrease in methanogenic community members (Scanlan and Marchesi 2008), another study has shown an increase in fungal members (Kuhbacher, Ott et al. 2006; Ott, Kühbacher et al. 2008). Specific bacteria and/or virulence factors have not yet been identified, although various candidates have been suggested. Changes in resident *Escherichia coli* have been recorded in CD patients (Frank, St Amand et al. 2007). Specifically, Arnott et al (2004) noted an increase in antibody titers *to E.*

coli outer membrane protein C (OmpC) (Arnott, Landers et al. 2004). Additionally, in one study, the presence of adherent invasive *E. coli* (AIEC) was found in 65% of ileal resections in chronic inflammation (Darfeuille-Michaud, Boudeau et al. 2004). Using fecal samples, an increase in the presence of *Bacteroides* spp. was noted in human patients with IBD (Prindiville, Sheikh et al. 2000); similar results were reported in IL-$10^{-/-}$ murine models (Sellon, Tonkonogy et al. 1998). Patients with IBD have also been noted to have a decreased level of protective commensal bacteria such as *Clostridium* Groups XIVa and IV. In addition to alterations in individual microbial communities, the overall diversity of bacterial species appears to be less in IBD patients than in normal individuals (Frank, St Amand et al. 2007) and the composition and diversity of the microbial community members tends to fluctuate in CD patients (Scanlan, Shanahan et al. 2006). The increased incidence of microbial fluctuation may contribute to the increased immune response to what would otherwise be considered normal GI microbial members (Reiff and Kelly 2009).

You are what you eat

The environment of the host's GI tract is thought to be influenced by metabolic products of resident microbial species (Roediger, Duncan et al. 1993). One of these products, butyrate, is a source of energy for colonic epithelial cells which improves the GI epithelial barrier integrity and the host's immune response (Roediger, Duncan et al. 1993). Increases in sulphate-reducing bacteria have been recorded in IBD patients; sulphate-reducing bacterial species produce hydrogen sulfide and their presence has been linked to blockage of butyrate use by colonocytes.

Intestinal microbiota and mucosal immune system interaction

Experimental data support the hypothesis that IBD can be triggered by an abnormal immune response to GI commensal microbiota in a genetically susceptible individual (Cho 2008). In contrast, in normal individuals the immune system elicits appropriate anti-microbial responses against pathogens while tolerating the host's normal microbiota. The underlying basis for this is cross-communication between microbiota and host receptors and regulators. It has been suggested that an abnormal composition of microbiota may lead to changes in innate signaling and have downstream effects on the immune system (Pettersson 2008).

Four potential defective microbiota-immune system interactions have been identified that are expected to lead to IBD (Sartor 2008). In the first and second, an increase in pathogenic bacteria or virulence of the normal microbiota may cause increased stimulation of both adaptive and innate immune responses. Thirdly, an altered, although non-pathogenic, composition of the gut microbiota can adversely affect GI physiology making mucosal surfaces more susceptible to damage and invasion. In the fourth situation, even when commensal bacteria remain unaltered, if the host's ability to prevent bacteria from crossing the mucosal barrier is diminished, activation of inflammatory responses can occur. Early work suggests that there are multiple factors that control the integrity of the gut epithelial barrier.

RATIONALE FOR THIS STUDY

Although published reports of human clinical trials using *T. suis* as a therapy for inflammatory bowel disease reported that it is a safe and effective treatment for inflammatory bowel disease, the mechanism(s) by which it works remains unknown (Summers, Elliott et al. 2003; Summers, Elliott et al. 2005) . Additionally, very little, if any published information is available regarding adverse side effects or the potential for adverse side effects. Given that the two published studies were performed on a relatively small sample size it is possible, particularly given the multi-factorial nature of IBD, that when applied to a larger population some individuals will experience adverse side effects due to this therapy. The overarching goal of this thesis was to develop murine models to explore *Trichuris* as a therapy for IBD.

This study was multi-pronged in its aims. First, we sought to identify axenic and accurate methods by which we could deliver *Trichuris* to mice, so that our later studies would not be complicated by the possibility of inoculating the mice with extraneous pathogens and contaminants at the time of *Trichuris* inoculations. Our second objective was to investigate the possibility of adverse side effects associated with the administration of *Trichuris* therapies in a mouse model. A previous study by Schopf et al (Schopf, Hoffmann et al. 2002) reported 100% mortality in C57BL/6 IL-$10^{-/-}$ mice when administered *Trichuris muris*, but there was little in the depth of characterization of the pathology associated with their morbidity and mortality. Additionally, recent studies have linked IL-10 deficiencies with IBD in some individuals (Leach, Davidson et al. 1999; Farmer, Sundberg et al. 2001) making this model of more relevance to *T. suis* therapy in individuals with IBD, particularly if it were applied to an increased number of individuals. Finally, we aimed to investigate the effect that *Trichuris* might have on an individual's colonic microbiota as a possible mechanism by which it ameliorates clinical signs

and symptoms associated with IBD. In doing so, we also sought to further enhance the microbial community profiling technique, Terminal Restriction Fragment Length Polymorphisms (TRFLPs) and our understanding of the effects of siblingship and caging cohorts on the murine colonic microbiota. In conduct of this work we posed the following hypotheses and specific aims.

Specific Aim 1: Identification of accurate axenic methods for delivery of *Trichuris muris* ova.

Hypothesis: Further investigations of currently employed *Trichuris* dose preparations and delivery will identify better methods for sterilization and delivery of *T. muris* eggs.

Specific Aim 2: Determine the effect of *Trichuris muris* in a C57 BL/6 IL-$10^{-/-}$ (a Th1 polarized) murine model of adverse effects.

Hypothesis 1: Infection with *Trichuris muris* is associated with significant gastrointestinal (GI) pathological changes.

Hypothesis 2: Antibiotic or steroid treatment given concurrently with *Trichuris muris* infection protects infected C57BL/6 IL-$10^{-/-}$ mice from previously observed mortality associated with *T. muris* infections.

Specific Aim 3: Determine changes in colonic microbial composition in C3Bir IL-$10^{-/-}$ *Trichuris muris* infected mice.

Hypothesis 1a: Murine fecal Terminal Restriction Fragment Length Polymorphism (TRFLP) Operational Taxonomic Units (OTUs) are more similar to other fecal TRFLP OTUs than full-thickness colon TRFLP OTUs from the same mouse.

Hypothesis 1b: A mouse's TRFLP OTUs from full thickness colon samples will be most similar to other full thickness colon samples, regardless of fecal contents, from the same mouse.

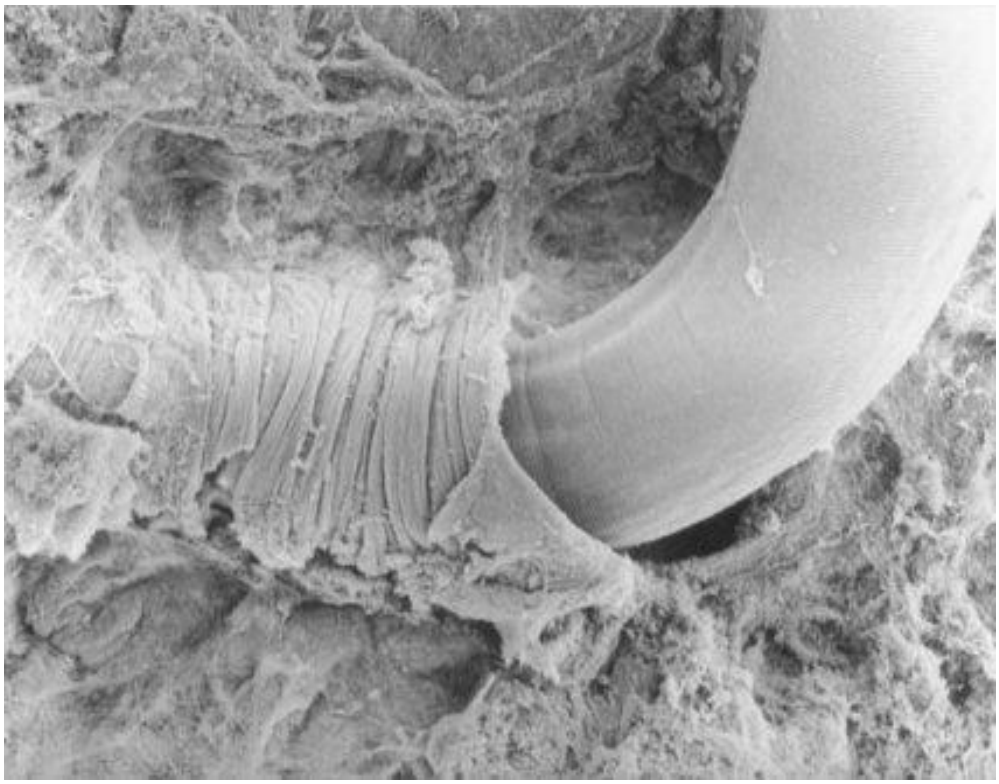
Hypothesis 1c: Use of different restriction enzymes, specifically *Hae*III, *Msp*I and *Bsl*I, in TRFLP analyses will not significantly change the resolution of the technique.

Hypothesis 2: Mice will retain GI microbiota that is more similar to their littermates than other mice from the same colony regardless whether they are housed in groups or individually

Hypothesis 3: Mice infected with *Trichuris muris* will have a significantly different GI microbial composition than uninfected mice.

The findings for specific aim 1 are reported in chapter 2 and the findings for specific aim 2 are reported in chapter 3. Finally, the findings for specific aim 3 are reported in chapter 4 with hypotheses 1a-c addressed in part 1, hypothesis 2 addressed in part 2 and hypothesis 3 addressed in part 3. Chapter 5 serves to summarize the findings of chapters 2-4 and suggests future directions for work in this field.

Figure 1.1. Electron micrograph showing the close association of *Trichuris suis* adult worms with the epithelium of the colon. Worms lie within syncytial tunnels formed from epithelial cell debris.



CHAPTER 2

Development of improved methods for delivery of *Trichuris muris* to the laboratory mouse

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ABSTRACT

Murine immunological responses to experimental infection with *Trichuris muris* and the effects of the resident microbiota on these responses are of increasing interest. For these studies, accurate dose delivery and improved sterilization of inocula are essential to prevent co-infection with unknown contaminants. We found that washing *T. muris* eggs with antibiotics may not be sufficient for sterilization of inocula. However, washing eggs in 6.25% hypochlorite/bleach eliminated bacteria and fungi, as determined by culture and PCR, did not harm viable *T. muris* eggs and reduced the number of non-viable eggs in inocula. A hatching assay and propidium iodide staining method were developed and found to increase accuracy for assessing *T. muris* egg viability prior to infection for rapid dose evaluation. Additionally, metal gavage feeding needles increased the accuracy and precision of the dose delivered to mice compared to flexible rubber tubes. These methods will improve experimental *Trichuris* studies by decreasing the variability in outcome due to unintended carryover of adherent microorganisms and unrecognized variation in inocula.

INTRODUCTION

Trichuris spp. are parasitic nematodes demonstrated, through a large body of literature, to be a health threat in both veterinary and human medicine. The *Trichuris* life-cycle begins when the host ingests embryonated eggs in the first larval stage (L1), which hatch in the host's distal small intestine, invade the cecum and/or colon and undergo four molts to become sexually mature adults. Larval and adult stages burrow into the colonic epithelium, forming syncytial tunnels (Tilney, Connelly et al. 2005) and produce excretory-secretory products (ESP) which have antimicrobial activity (Abner, Parthasarathy et al. 2001; Abner, Hill et al. 2002). Natural infection of humans with *Trichuris trichuria* can lead to *Trichuris* dysentery syndrome (TDS) (Stephenson, Holland et al. 2000). TDS results in chronic dysentery, rectal prolapse, anemia, poor growth, and clubbing of the fingers. *Trichuris suis* can cause disease under some circumstances and has been linked to diarrhea in weaning age pigs where interactions with enteric bacteria such as *Campylobacter jejuni* exacerbate mucohemmorhagic colitis (Mansfield and Urban 1996; Mansfield, Gauthier et al. 2003). *Trichuris muris* infections of mice can also cause typhlocolitis (Schopf, Hoffmann et al. 2002). However, *Trichuris* spp. can also exert protective effects. *Trichuris* has been applied in human clinical trials, where it appeared to be a safe and effective treatment for inflammatory bowel disease (IBD) (Summers, Elliott et al. 2003).

The rationale for this study is that more information is needed on how *Trichuris* spp. induces disease, interacts with the colon and the colon microbial community, induces anti-inflammatory responses and ameliorates pre-existing gastrointestinal (GI) inflammation. *Trichuris muris* experimental infections in mice have been used extensively to show that a primary infection induces polarized T helper (Th)-2 responses that are protective and can further modulate the host's immune responses (Williams, Montenegro et al. 1994; Else and deSchoolmeester 2003;

Zaph, Rook et al. 2006). Mice also show a chronic inflammatory response to this helminth that can lead to consistent production of anti-inflammatory responses with Treg cells that secrete TGF-β and IL-10 (Schopf, Hoffmann et al. 2002; Else and deSchoolmeester 2003; Veldhoen, Uyttenhove et al. 2008). It is clear from this and other work that pathogen products encountered during antigen presentation will determine the character and ultimate polarization of Th cell responses in the host. Thus, because parasite-bacterial interactions can participate in the pathogenesis of trichuriasis (Mansfield and Urban 1996; Mansfield, Gauthier et al. 2003), exacting immunological studies focusing on *Trichuris* spp. should be conducted using live ova that are free of contaminants. More importantly, improved dosing methods to insure axenic and accurate treatment doses are essential to prevent disease due to contaminating microorganisms for both clinical and experimental applications.

Ensuring that eggs are not contaminated with bacteria and fungi is essential for accurate studies of the host's immune response to challenge infection with *T. muris*, biochemical analysis of constituent antigens, and *in vitro* studies. Without assurance of contaminant-free inoculum it is difficult to attribute experimental results to *Trichuris* alone versus the potential contaminants. Furthermore, in human clinical trials, the inoculated ova used were reported to be tested and declared free from pathogens; therefore, to accurately simulate these conditions in animal models, a standardized and effective protocol for delivering axenic doses of *Trichuris* is of increasing importance. These tasks are difficult because collection of *Trichuris* ova inherently involves carryover of bacterial and fungal contaminants from the digesta in which the adult female worms reside. *Trichuris* ova are reported to have three outer layers, the outermost of which has a similar appearance to the vitellin layer noted in other nematode eggs (Wharton and Jenkins 1978). Electron microscopic images have demonstrated the adherence of particulate

material to the outer surface of *T. muris* egg shells. Another consideration is that carryover of bacterial and/or fungal contaminants must be eliminated without impairing the development or infectivity of the *Trichuris* larva.

The percent viability of *T. muris* embryonated ova prior to inoculation of animals is another factor that could have important effects on outcomes of experimental infections and must be assessed accurately. Few published papers on *Trichuris* spp. address this factor. The ultimate test of viability of a pathogen is its ability to induce infection in a susceptible host. One technique for determining percent viability of *T. muris* egg stocks involves infecting severe combined immune deficient (SCID) mice with the intended inoculum and later counting the number of larva in the GI tract at necropsy (Schopf, Hoffmann et al. 2002). Although this method allows for accurate determination of the infective dose at the time of inoculation, the 32 day lag period between inoculation and worm counting from mice may allow for further die-off of eggs in the original stock. Another technique uses morphology and physical development of the embryo within the egg as a means of assessing the developmental state of the larva within the egg and the potential for viability (Black, Scarpino et al. 1982). Black and colleagues assessed morphological changes in *T. suis* and *Trichuris vulpis* eggs associated with larval death, including cytolysis of egg cells after cytokinesis had begun, formation of large refractile granules within the cell, vacuolation or hyalinization in the cytoplasm, shrinkage of the egg, disintegration of the membrane surrounding the egg cell, and collapse of the egg shell. However, functional analysis such as hatching assays revealed that some eggs assessed for viability by this means contain embryonated larvae that had died and were no longer infective. Practical experience indicates that *Trichuris* spp. inocula vary in their viability. Thus, lack of viability testing prior to inoculation means that the number viable versus the number dead is unknown. In our lab,

preliminary studies have shown that dead eggs are capable of provoking host responses (Kopper, unpublished).

Propidium iodide (PI) staining has been used widely to assess both prokaryotic and eukaryotic cell viability (Fernandez and Sanchez 2002; Panka and Mier 2003). This stain can cross damaged cell membranes produced by programmed cell death or necrosis and bind to the cell's DNA. PI has been used to evaluate the viability of *Sarcocystis neurona* sporocysts, but its utility in assessing viability of helminth eggs has not been determined (Elsheikha and Mansfield 2004).

Thus, for experimental *Trichuris* spp. studies, improved methods are needed for 1) determining accurately the percent viability of egg inocula, 2) delivering accurate doses of clean, viable eggs, and 3) eliminating carryover of contaminants in inocula. The existing literature gives little information on preparation of inocula for experimental animal model studies. To our knowledge, detailed methods for and evidence of successful sterilization for *Trichuris* inocula have not been previously published. Our specific hypotheses were that (1) use of hypochlorite sterilizes *Trichuris* spp. egg inoculum without detrimental effects on egg viability, (2) propidium iodide (PI) staining allows for determination of percent viability of *T. muris* eggs, and (3) metal gavage needles deliver more accurate doses to experimental mice than flexible plastic tubing such as 3.5 Fr rubber catheters. Our specific aims were to develop methods to surface-sterilize embryonated *T. muris* ova, to assess the viability of those eggs, and to deliver highly accurate doses so that controlled experimental studies could be designed to examine the role of the microbial community in *T. muris* pathogenesis in mice.

MATERIALS AND METHODS

Mouse Breeding and Handling

All animal protocols were approved by the Michigan State University (MSU) Institutional Animal Care and Use Committee (IACUC) (07/06-044-00) and complied with National Institutes of Health guidelines. For maintenance of the *T. muris* strain, CBySmn.CB17-Prkdc^{Scid}/J mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and were housed in a containment facility in a laminar flow hood in sterile filter top cages, and fed irradiated feed (mouse breeder diet 7904; Harlan Teklad, Indianapolis, IN) and sterile water *ad libitum*. For experimental studies, Non-Obese Diabetic (NOD), C57BL/6 IL-10$^{-/-}$, and C3Bir IL-10$^{-/-}$ breeding mice and their progeny were produced, maintained and monitored in a specific-pathogen-free colony at MSU as previously described (Mansfield, Bell et al. 2007). Experimental mice were housed in a containment facility in sterile filter top cages, and fed irradiated feed and sterile water *ad libitum*. Personnel wore sterile coveralls, cap, mask, boots and gloves for work with all mice. Transfer of mice to clean caging was performed with sterile forceps. Prior to all *Trichuris* inoculations, fecal samples were collected from one mouse in each cage and were shown to be free of colitogenic bacteria including *Campylobacter* spp., *Helicobacter* spp., and *C. rodentium* by subjecting DNA isolated from the fecal pellets to a specific PCR assay as described previously (Mansfield, Bell et al. 2007).

Parasites

T. muris eggs were obtained from Dr. Joseph Urban and passaged in CBySmn.CB17-Prkdc^{Scid}/J mice. *Trichuris muris* strain was confirmed to be of the J-isolate via western blot analysis. Mice were orally infected with 100 embryonated ova of *T. muris*. At 40 days post infection mice were humanely euthanatized by inhalation of an overdose of CO_2. At necropsy, the GI tract was removed and placed in wash medium; Phosphate Buffered Saline (pH 7.4) (PBS) with 1X 100 U/ml Penicillin/100 µg/ml Streptomycin and 2.5 mg/ml Amphotericin B (PSF). The cecum and colon were slit and teased apart, and *T. muris* adults were individually removed and placed in a Petri dish with 15 ml Dulbecco's Modified Eagle's Medium 5% Glucose (DMEM) supplemented with PSF. Worms were washed with 5 changes of DMEM medium by transferring them into fresh sterile medium using sterile instruments which were re-sterilized for each wash. After the fifth wash, *T. muris* worms were placed in a Petri dish at a concentration of roughly 4 worms/ml of medium and incubated at 37^{o}C with 5% CO_2. Every three days for a total of four collections the medium was changed and eggs were collected by low speed centrifugation for embryonation. Cultures with bacterial and/or fungal contamination observed by microscopy and Gram staining were discarded.

After *T. muris* eggs were collected, they were stored in 30 ml filter top tissue culture flasks with 15 ml of PSF wash medium in the dark in an incubator set at 22^{o}C. The flasks were monitored weekly for the presence of fungal and/or bacterial contamination by microscopy and Gram staining.

Assessing optimal methods for embryonation of *Trichuris muris* ova

In the first trial, optimal conditions to maximize embryonation of *T. muris* eggs was determined by storage under three conditions: room temperature (approximately 22^{o}C, RT), 37^{o}C, and 37^{o}C on a slowly rotating shaker. Percent embryonation of ova was judged using morphological methods (Black, Scarpino et al. 1982); eggs were photographed using a Nikon Eclipse E600 microscope with a SPOT camera with Windows version 4.09 software (RT-Slider Diagnostic Instruments, Inc., Sterling Heights, MI). In a second trial, *Trichuris muris* eggs were stored at room temperature (RT) or 4^{o}C and assessed for embryonation each week for a total of ~16 weeks. Once again, the percent embryonation of eggs was evaluated using morphological methods and documented as described.

Methods to remove contaminants from *Trichuris muris* ova and to assess microbial carryover and percentage of inocula developing to adult stage

Equal quantities of embryonated *T. muris* eggs were used to compare the efficacy of antibiotic treatment (100 U/ml Penicillin/100 µg/ml Streptomycin and 2.5 mg/ml Amphotericin B (PSF)(Group 1), 6.25% hypochlorite (Group 2) for surface sterilization of *T. muris* eggs or sterile media-treated alone (Group 3). The *T. muris* egg inoculum in Group 1 was incubated for approximately two hours in PBS with PSF and rinsed three times in wash medium by centrifugation. Group 2 eggs were incubated in 6.25% hypochlorite (bleach) in 12 increments of 5 minutes each (5 min – 60 min) followed by 3 washes in PBS. *Trichuris muris* eggs from both groups were subsequently assessed for sterility and viability by microscopy, bacterial culture,

and inoculation into specific pathogen free mice of three genotypes NOD, C57BL/6 IL-$10^{-/-}$ and C3BirIL-$10^{-/-}$.

Light microscopy was done using a Nikon Eclipse E600 microscope with a SPOT camera with Windows version 4.09 software to determine potential damage to the shells and/or larvae using criteria described by Black et al. (Black, Scarpino et al. 1982). Signs of damage included degraded or broken shell walls and larvae found exterior to the shell. Bacteriological culture was performed as follows. After washing, both groups were centrifuged at 3220 x g for 5 min to pellet the *T. muris* eggs, and a 100 µl volume of supernatant was spread on each of the following solid media plates: Luria Broth (LB) agar, MacConkey (MAC) agar, KF Streptococcus agar (KF) and Potato Dextrose (PD) agar to determine presence of contaminants. Plates were incubated at 37° with 5% CO_2 and assessed at 24, 48, and 72 hours for bacterial growth. To increase the sensitivity of detection of bacterial contaminants that would not be expected to grow on the four media in the described conditions, a 10 µl volume of supernatant from each treatment group was assessed by PCR using universal 16S primers for detection of the 16S rRNA gene present in all bacteria. Primer sequences, reaction mixture, and thermocycler conditions were based on methods of Nagashima and colleagues (Nagashima, Hisada et al. 2003). PCR products were visualized on a 1.2% agarose gel in 40 mM Tris-Acetate and 2 mM Na_2EDTA TAE, stained using ethidium bromide, visualized using UV illumination and photographed (Alpha Innotech Gel Documentation System, Alpha Innotech Corporation, San Leandro, CA).

Mouse bioassays were used to determine percentage of *T. muris* inocula developing to the adult stage. Three separate experiments were conducted using mice of the following genotypes (NOD, C57BL/6 IL-$10^{-/-}$ and C3BirIL-$10^{-/-}$) to assess viability of *T. muris* inocula in a natural

host bioassay by observing the percentage of the inocula developing to the adult stage. There were two groups of 5 mice in each experiment; one receiving *T. muris* eggs sterilized with 6.25% hypochlorite and the other an uninfected group receiving a sham inoculation. Thereafter, mice were monitored daily for adverse clinical signs for 40 days using our standardized score sheet (Mansfield, Bell et al. 2007). At 38 to 40 days post infection fecal pellets were collected and saturated sugar ova flotation performed with McMaster counting to determine *T. muris* egg shedding (Mines 1977). If mice experienced adverse signs that met our preset criteria at any time during the 40 day observation period they were humanely euthanized with an overdose of $C0_2$ (Mansfield, Bell et al. 2007). At 40 day, the remaining mice were euthanized similarly and necropsied to remove the colon and tissues fixed for histopathology.

In vitro hatching assay

After hypochlorite treatment, eggs were evaluated *in vitro* for hatching and motility. One ml Dulbecco's Modified Eagle's Medium (DMEM) with 0.05% agarose was poured into the wells of an 8-well tissue culture plate. Prior to the agar solidifying, 7 wells were inoculated with embryonated *T. muris* eggs that were hypochlorite-treated as described above. One ml of DMEM with 0.05% agarose was poured, without eggs, into the eighth well as a control for bacterial contamination. Plates were incubated at 37^{o}C with 5% CO_2 for increasing times. Wells were assessed for *T. muris* larva hatching and motility using an inverted microscope (Olympus CK2, Olympus America Inc.) at 0, 1, 2, 3, 4, 5, 6, and 12 h and then once per day for 21 days after inoculation. Using criterion similar to those reported by Kotze and colleagues (Kotze, Coleman et al. 2005), hatching of *T. muris* larva was determined using a dissecting microscope (Wesco,

Western Scientific Co., Valencia CA) by observation of motile larvae free from egg shells. Eggs with unhatched larvae were also counted to establish the percentage hatched.

Determining larval viability using Propidium Iodide (PI) staining.

A PI staining method developed for use with parasitic protozoa was modified for evaluating viability of *T. muris* ova (Elsheikha and Mansfield 2004). *T. muris* eggs were divided into four groups: (1) no treatment, (2) hypochlorite treatment, (3) propidium iodide treatment, and (4) hypochlorite treatment followed by propidium iodide exposure. *Trichuris muris* eggs either pre-treated with 6.25% hypochlorite for 5 minutes and subsequently washed with PBS as described above or not hypochlorite treated were stained with propidium iodide by the method above (Elsheikha and Mansfield 2004). Red eggs were assessed for both (1) presence or absence of propidium iodide stain (stained vs. unstained appearance) and (2) morphological characteristics of embryonated eggs (presence or absence of a larva) using fluorescence microscopy with a Nikon Eclipse E600 microscope and photography with a SPOT camera with Windows version 4.09.

Effect of metal vs. rubber instruments for delivery of *Trichuris muris* ova to mice

Multiple factors including the delivery method (metal gavage feeding needle vs. 3.5 French rubber catheter) and the length of time the inoculum spent in the gavage needle were tested to evaluate their effect on the accuracy and precision of the dose delivered to the mice. To assess the delivery methods, the inoculum was prepared using embryonated *T. muris* eggs treated with 6.25% hypochlorite for 5 minutes and washed as described above. The inoculum was then

drawn up in either (1) a sterile 22 gauge metal gavage feeding needle or (2) 3.5 French rubber feeding tube and 200 µl were delivered into McMaster counting chambers. All the eggs present within the 200 µl were counted. Most eggs sedimented and were counted on the bottom of the chamber. However, because the eggs were not suspended in a traditional flotation media that would bring the eggs to the surface, multiple planes of focus were assessed and counted for the presence of *T. muris* eggs. The eggs were counted and classified as viable or non-viable using both morphological and PI staining methods (Black, Scarpino et al. 1982; Elsheikha and Mansfield 2004). The intended dose was 100 embryonated *T. muris* eggs. Four replicates of each treatment were performed and delivery results compared as described in statistical methods.

Statistical Methods

One-way ANOVA (SigmaStat 4, SPSS Inc, Chicago, IL) was used to analyze the dose and variation in doses of *T. muris* eggs delivered using a metal feeding needle vs. 3.5 Fr Rubber catheter.

RESULTS

Optimal conditions for *Trichuris muris* egg embryonation

Based on the morphological methods of Black et al. (Black, Scarpino et al. 1982) in trial 1, *T. muris* eggs in all treatment groups (room temperature, 37^{o}C, 37^{o}C on a shaker) began embryonating at approximately the same time about two weeks after shedding from the host (Figure 2.1A). In a second trial, *T. muris* eggs stored at room temperature or 4^{o}C and assessed for embryonation each week for 16 weeks showed that both conditions allowed embryonation, but more eggs embryonated at room temperature than at 4^{o}C (Figure 2.1B). Embryonated ova kept at room temperature or 4^{o}C persisted in a viable state for much longer periods of time than those incubated at 37^{o}C with or without shaking.

6.25% hypochlorite was sufficient and Penicillin-Streptomycin-Fungizone (PSF) antibiotic treatment was insufficient for surface sterilization of *Trichuris muris* eggs

After antibiotic treatment of *T. muris* eggs, bacterial and fungal colonies grew on Luria broth (LB), Maconkey agar (MAC), (KF) and potato-dextrose (PD) agar plates (Table 2.1). Treatment of *T. muris* eggs with 6.25% hypochlorite eliminated bacterial and fungal growth on these media, but increased time of exposure to the hypochlorite resulted in increased levels of *T. muris* shell degradation (Table 2.1; Figure 2.2). Five and 10 minute exposures of eggs to 6.25% hypochlorite had limited detrimental effects on the integrity of viable egg shells but eliminated bacteria and fungi. 16S Universal PCR of egg-free media was also negative but the untreated

controls were positive for the expected approximately 1 kb sized band (Table 2.1). Eggs that were treated with hypochlorite for 5 minutes and subsequently subjected to *in vitro* hatching assays resulted in hatching of eggs and motile larvae. In the *in vivo* bioassays, NOD, C57BL/6 IL-10$^{-/-}$, C3 Bir IL-10$^{-/-}$ mice gavaged with 6.25% hypochlorite treated *T. muris* ova developed patent infections with consistent numbers of adult worms in those mice that did not develop severe disease (Table 2.2). All *T. muris* infected mice that survived until 40 days post infection had *T. muris* ova in feces based on fecal flotation (Table 2.2).

In vitro hatching assays improved the determination of the percentage of viable larvae

In the *in vitro* hatching assay, *T. muris* eggs hatched and were observed to be viable for up to 21 days, at which time the larvae died. In the hatching assay, eggs settled in multiple planes of focus in the agar layer and hatched over a period of approximately 12 hours. Hatched larvae became highly motile and traveled significant distances from the original egg shells. An unexpected finding was that hatched larvae aggregated distant from their shells, making correlation of an empty egg shell to a specific viable larva difficult. In subsequent trials it was found that inoculation of a smaller number of eggs into the media increased the ability to track egg shells and larvae, but a significant decrease in hatching was observed. When larger inocula were used, larvae were most often found aggregated around the un-hatched eggs.

Propidium Iodide (PI) staining of hypochlorite-treated eggs differentiated viable and non-viable *T. muris* larvae

PI staining was found to be unreliable when used on non-hypochlorite treated eggs (those washed by other methods) as a method to determine viability (Figure 2.3). In this experiment,

when eggs were evaluated after exposure to PI, some eggs took up the stain whereas others did not. Staining was not correlated with viability; a large proportion of eggs lacking an identifiable embryo were either red or unstained. In the experimental group using hypochlorite treated eggs, the eggs judged non-viable by morphological criteria stained red (Figure 2.4). Additionally, a proportion of eggs judged morphologically viable stained red (Figure 2.4). When inoculated into the previously described *in vitro* hatching assay, red-stained eggs regardless of their morphological features failed to hatch, and eggs that were clear (unstained) hatched and produced viable larvae. These results suggest that PI may serve as a more accurate tool than previous morphological assessment methods.

Metal gavage feeding needles delivered a more accurate and higher amount of the dose than a 3.5 Fr rubber feeding tube

Well mixed stock *T. muris* inocula in PBS pH7.4 that were calibrated using the PI method described above, 200 µl were drawn into multiple metal gavage feeding needles or 3.5 Fr Rubber catheters and each dispensed into a McMasters counting slide to evaluate the best dose delivery method. Results for this experiment are presented in Figure 5. It was found that use of a metal gavage needle resulted in more precise and accurate delivery of *Trichuris* eggs when compared to inocula delivered via a 3.5 French rubber catheter (Figure 5A). The number of times a metal feeding needle was used did affect the accuracy of delivery of the *T. muris* embryonated egg dose (Figure 2.5B). Most accurate delivery occurred on the first expulsion from the needle (Figure 2.5B). Most repeatable delivery occurred from the second to fifth expulsion from the needle although the dose is significantly less than the 100 eggs intended (Figure 2.5B). We expect that the first delivery wets the inside of the metal gavage needle evenly and after five

doses delivered with the same needle ova begin to build up in the needle causing increasing numbers of ova to be delivered. There was no difference in embryonation rates based on amount of time *T. muris* eggs spent in the gavage needle (Figure 5C).

DISCUSSION

The aim of these experiments was to develop improved methods for (1) delivery of axenic *T. muris* inocula to mice, (2) accurate and precise assessment of *T. muris* egg viability immediately prior to inoculation and (3) assessment of the effects of inoculum preparation and delivery on the accuracy and precision of the dose delivered. We demonstrated that carryover of fecal microorganisms is likely to occur if preparation methods lack a step to sterilize the surface of *T. muris* eggs. Furthermore, the standard dose of PSF commonly employed for this purpose was not adequate to remove bacterial and fungal contaminants from *T. muris* ova, even with a larger number of washing steps. Axenic inocula can be prepared using 6.25% hypochlorite for sterilization. Our data showed that *T. muris* eggs can withstand treatment and remain infective and that hypochlorite treatment had the added benefit of eliminating many of the non-viable eggs by dissolving them.

When studying responses to experimental infections, delivering axenic inocula is important for interpretation of experimental results. Because *Trichuris* is a GI-dwelling nematode, obtaining, collecting and storing its eggs inherently involves potential contamination with bacteria and fungi. Many labs report using techniques for collecting eggs from adult female worms removed from GI tracts and thereafter cultured in medium without gross evidence of bacterial and/or fungal growth. In our studies, we found evidence of bacterial and/or fungal contaminants via culture independent mechanisms even when antibiotic-treated egg cultures lack visual or cultural evidence of contamination. In batches of *T. muris* eggs intended for inoculation, pre-treatment with 6.25% hypochlorite eliminated bacteria and/or fungi as demonstrated by the culture dependent and independent testing methods used here and did not decrease larval viability. The mouse infection studies shown here demonstrate that hypochlorite

treated ova retain infectivity and result in patent *T. muris* infections that appear to be less variable within groups than in some published studies (Schopf, Hoffmann et al. 2002). Furthermore, after hypochlorite treatment the number of visible nonviable, unembryonated eggs decreased. This observation may be due to the action of the hypochlorite causing the inner lipid layer, which is usually impermeable to most chemicals, to break down (Wharton and Jenkins 1978). A method such as this that removes some nonviable eggs could be an advantage in experimental studies because some animals develop immune responses to *T. muris* when inoculated with non-viable *T. muris* eggs (Kopper, unpublished).

The use of hypochlorite for sterilization prior to use of PI had the added benefit of enhancing the accuracy of the PI method as an indicator of viability when compared to microscopic examination of egg morphological features. When preparing inocula, we expect that some proportion of the *T. muris* eggs with visible embryonated larvae have already died, but have yet to decompose within the shell. It was this nonviable population of eggs that we wished to identify in the PI staining assay. Using the *in vitro* hatching assay here, we were able to confirm that eggs which had stained red failed to hatch. Identification of this population of eggs will help improve delivery of accurate and precise doses of all *Trichuris* spp. to experimental animals.

Some laboratories employ a biological assay that involves inoculating immune compromised SCID mice with *T. muris* and subsequently counting the number of established larvae approximately 14 days after infection (Schopf, Hoffmann et al. 2002). While this method may provide an accurate number of larvae for infectivity in those animals, the larvae are difficult to see at this stage of development and might not provide good repeatability between experiments. One reason for differences between viability of the dose and the number of worms maturing in the experimental animals is the amount of time that elapses between the inoculation of mice in

the biological assay and the inoculation of experimental mice during which the percentage of embryonated *T. muris* eggs could change significantly. Based on our experience, egg viability varies over time, and sometimes sudden and significant drops in viability can occur which could result in significantly different numbers of viable eggs delivered to mice. Using an egg stock that has been stored for many days may lead to delivering a different number of viable eggs compared to the inoculation time point of mice used for the viability assay. Calibration of the experimental inocula on the day of delivery to the experimental mice improves the accuracy and precision of the dose delivered and the subsequent experimental results. Another consideration is that SCID mice require special facilities and considerable expense.

Finally, we recognize that the concentration of *Trichuris* spp. eggs suspended in inocula is often vastly different from the number of eggs delivered to an experimental animal. Accuracy and precision both play critical roles in maximizing the quality of *T. muris* infection studies; reporting accurate doses results in more uniform cross-study comparisons and use of higher levels of precision results in robust and repeatable study designs. It was found that inoculating mice using a 3.5 Fr rubber feeding tube delivered far fewer eggs than use of a metal gavage needle. We suspect that this is phenomenon is due to (a) increased adherence of eggs to rubber surfaces and (b) the larger surface area the inocula passes through and is exposed to in the rubber feeding tube. Because many delivery devices have plastic or rubber components, operators should consider that metal gavage needles will produce more accurate results. These results also show that delivery is more accurate when a separate metal needle is used for each mouse.

Acknowledgements

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TABLES

Table 2.1. Results of 16S Universal PCR (Nagashima, Hisada et al. 2003) to detect carryover of bacterial contaminants in *Trichuris muris* inocula washed in either antibiotic-containing media or 6.25% hypochlorite. Group 2 hypochlorite treated *Trichuris muris* ova inocula supernatants were negative by 16S Universal PCR, but Group 1 (Antibiotic medium-treated ova) and Group 3 (Sterile medium alone-treated ova) and the positive control for the PCR assay were positive for the expected approximately 1 Kb sized band.

Table 2.1 Continued

Group	Treatment Group	16S Universal PCR Result	Bacteriologic Culture Results
1	Antibiotic medium-treated *Trichuris muris* ova	Positive	Positive
2	6.25% hypochlorite-treated *Trichuris muris* ova	Negative	Negative
3	Sterile medium alone-treated *Trichuris muris* ova	Positive	Positive
Assay Controls	16S Universal PCR positive control or positive control bacterial strains (*Campylobacter jejuni*)	Positive	Positive
Assay Controls	16S Universal PCR negative control (no DNA) or sterile medium	Negative	Negative

Table 2.2. Murine bioassays. All mice were given 100 *Trichuris muris* embryonated ova treated with 6.25% hypochlorite for 5 minutes followed by three washes in sterile medium without antibiotics. Mice were monitored twice daily for clinical signs of disease. If clinical signs of disease were present they started as early as approximately 10 days post infection, but most frequently at 21 days post-infection and included rough hair coat, hunched posture, anorexia, dehydration and in some severe cases labored breathing. If mice exhibited clinical scores exceeding preset limits, as outlined in the materials and methods, they were immediately humanely euthanized. Several *Trichuris muris* infected C57BL/6 IL-10$^{-/-}$ mice in Group 2 were euthanized when clinical signs approached humane limits.

Table 2.2 continued. Murine bioassays

Group	Treatment Group	Clinical Signs of Disease	Number of adult *Trichuris muris**	Mean of *Trichuris muris egg shedding*[1]
1	*Trichuris muris* infected NOD mice	None	90.2 ± 14.02	275 ± 35.3
2	*Trichuris muris* infected C57BL/6 IL-10$^{-/-}$ mice	Severe	ND[2]	399.6 ± 38.3
3	*Trichuris muris* infected C3Bir IL-10$^{-/-}$ mice	Mild	ND[2]	331.6 ± 23.7
4	Sham inoculated NOD mice	None	0	0

[1]Values represent the mean plus or minus the standard deviation.

[2] ND, "not done." Tissue was used for histopathology assessments rather than adult worm counting. In both groups 2 and 3, all infected mice had many adult worm cross sections within colon tissue.

FIGURES

Figure 2.1. Assessment of optimal conditions for *Trichuris muris* embryonation. These data shows the optimal conditions for *Trichuris muris* ova embryonation. Percent embryonation was judged using morphological methods (Black, Scarpino et al. 1982). (A) Embryonation rate of *Trichuris muris* eggs post collection (B) Long term assessment of storage conditions for maintenance of optimal *Trichuris muris* egg embryonation. One year post storage all eggs stored at room temperature spontaneously hatched and died, while those at 4^{o}C remained viable (data not shown).

Figure 2.1continued

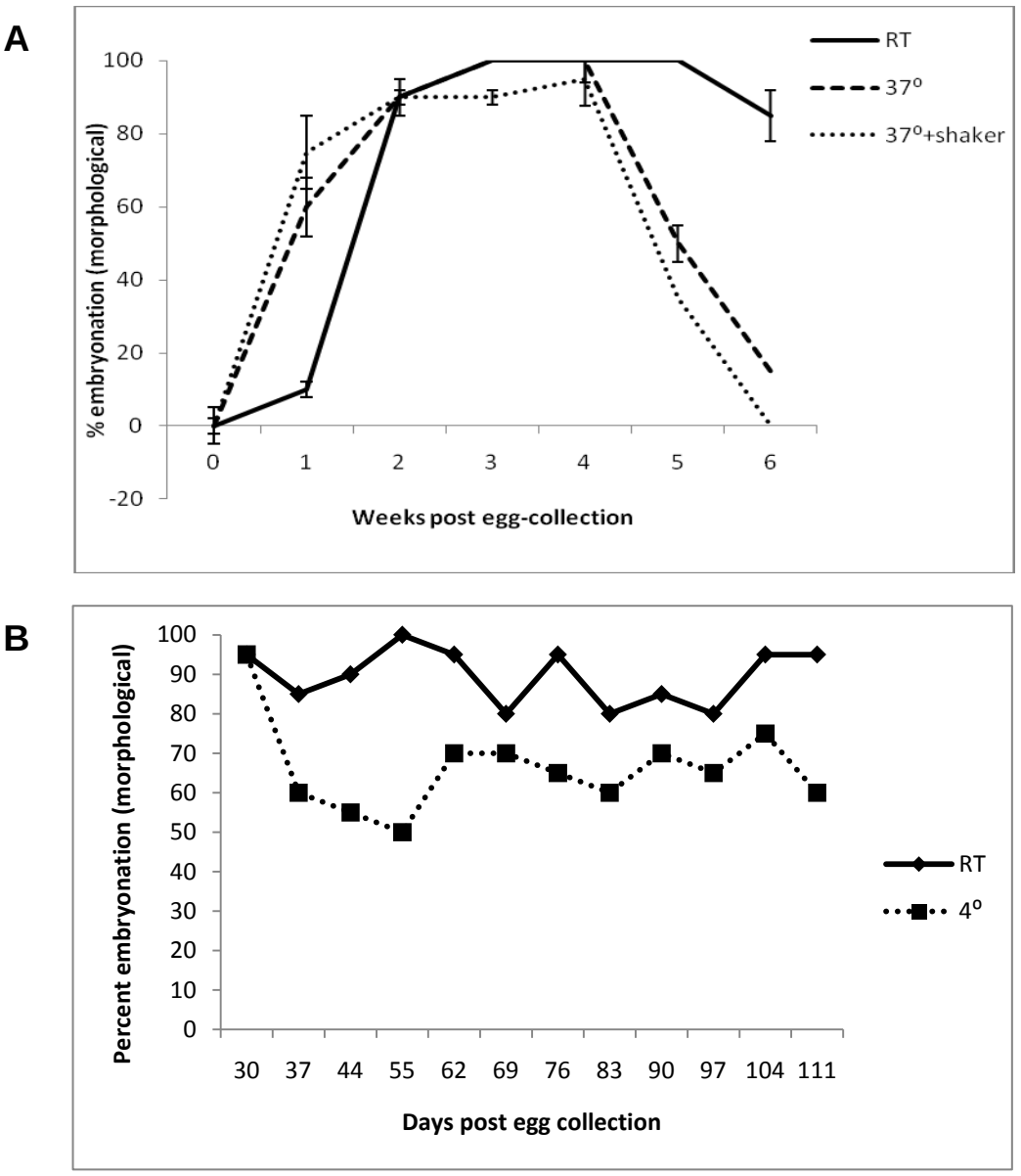


Figure 2.2. Light microscopic images of *Trichuris muris* eggs post treatment with 6.25% hypochlorite in increasing time increments of 5 minutes. (A) No treatment, (B) 5 minutes, (C) 10 minutes, (D) 15 minutes, (E) 20 minutes, (F) 25 minutes, (G) 30 minutes, (H) 35 minutes, (I) 40 minutes, and (J) 45 minutes. 15-20 minute treatments were for hypochlorite treatment where no damage to the eggs was observed. In practice 5 minutes of hypochlorite treatment was adequate to prevent carryover of bacteria and was employed in later studies. Note barrel shape and brownish coloration of ova in panel A versus F–J where eggs are clear and distorted in shape, appearing almost round. Larvae within the eggs were also damaged at the longer incubations in hypochlorite (see panels I–J). For interpretation of the references to color in this and all other figures the reader is referred to the electronic version of this dissertation.

Figure 2.2 Continued

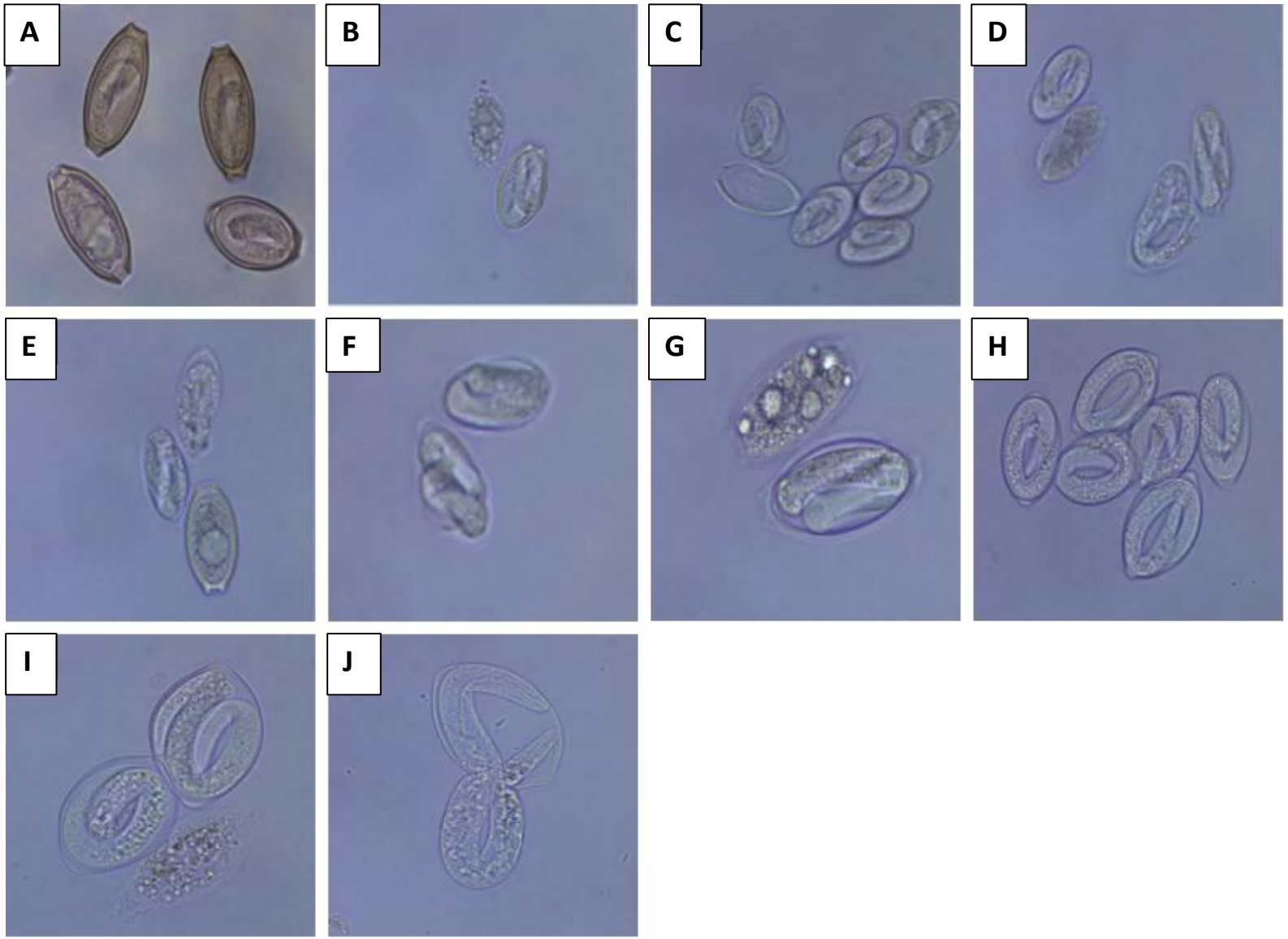


Figure 2.3. Propidium iodide as a tool for determination of viability of *Trichuris muris* eggs without prior exposure of ova to hypochlorite treatment. All pictures were taken at 40X magnification. Panels A, C and E were viewed under bright field microscopy and panels B, D and F were viewed using ultraviolet fluorescence microscopy. (A and B) morphologically non-viable egg (indicated by arrow) did not take up propidium iodide stain whereas eggs judged as viable by morphological criteria took up stain in the shell but not within the egg. (C and D) morphologically determined non-viable egg took up the stain within shell. (E and F) morphologically viable and non-viable eggs (indicated by arrow) stained similarly with only the shell taking up the propidium iodide stain. These results show that propidium iodide staining when used as the only technique for assessing viability on a population of *Trichuris muris* ova with many eggs in various stages of decay and degradation will sometimes fail to identify nonviable eggs.

Figure 2.3 Continued

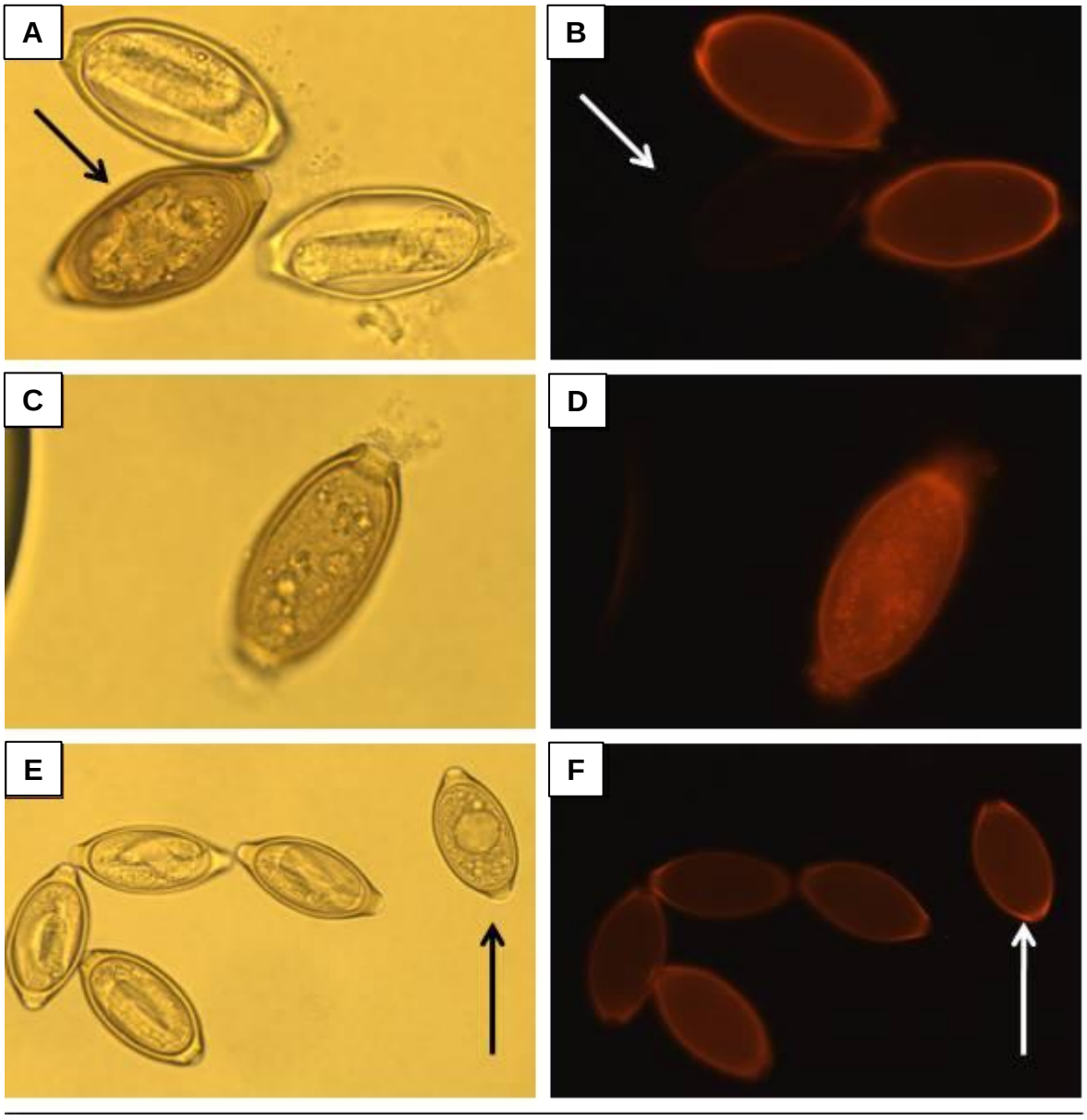


Figure 2.4. Use of hypochlorite and PI to detect embryonated *Trichuris muris* eggs. *Trichuris muris* eggs treated for 5 minutes with 6.35% hypochlorite followed by staining with propidium iodide shown in either bright field microscopy (panels A and C) or ultraviolet fluorescence microscopy (panels B and D) both at 40x magnification. (A) Shows a dead larva next to a degrading egg. (B) Shows the same image as panel A demonstrating that after bleach treatment both dead eggs and larvae took up propidium iodide and fluoresce orange. (C) Shows a dead egg (lower left corner) and a viable egg (upper right corner) and (D) shows the same image under fluorescence. The egg that is viable based on morphological criteria did not take up the propidium iodide stain, while the nonviable egg stains bright orange.

Figure 2.4 Continued

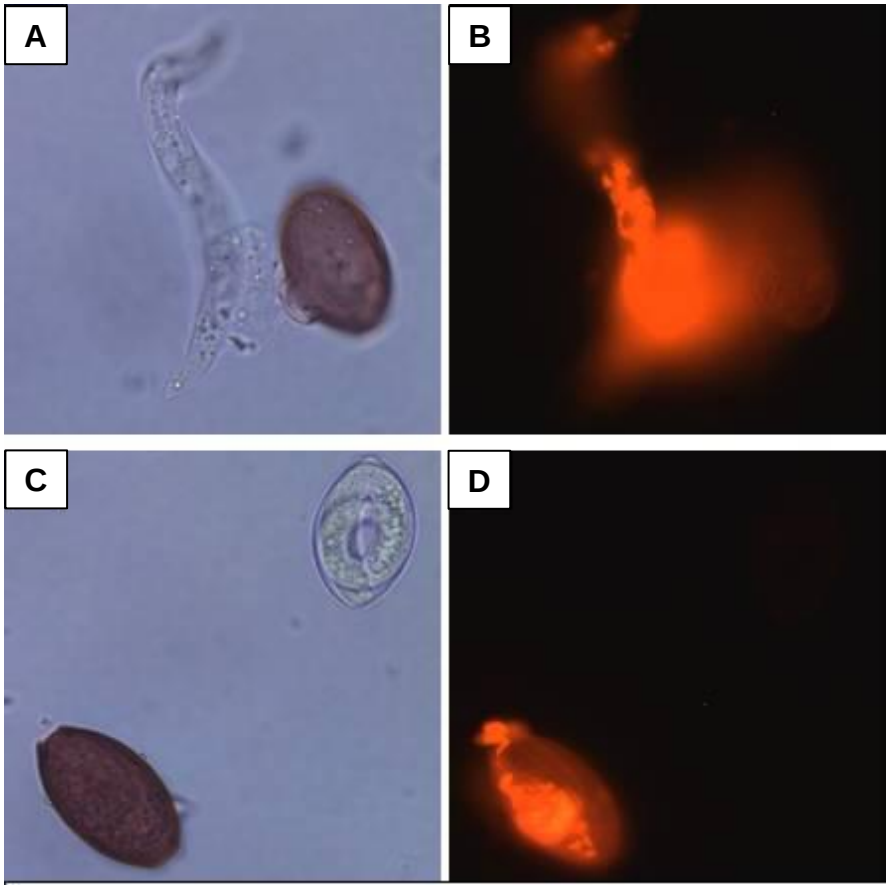
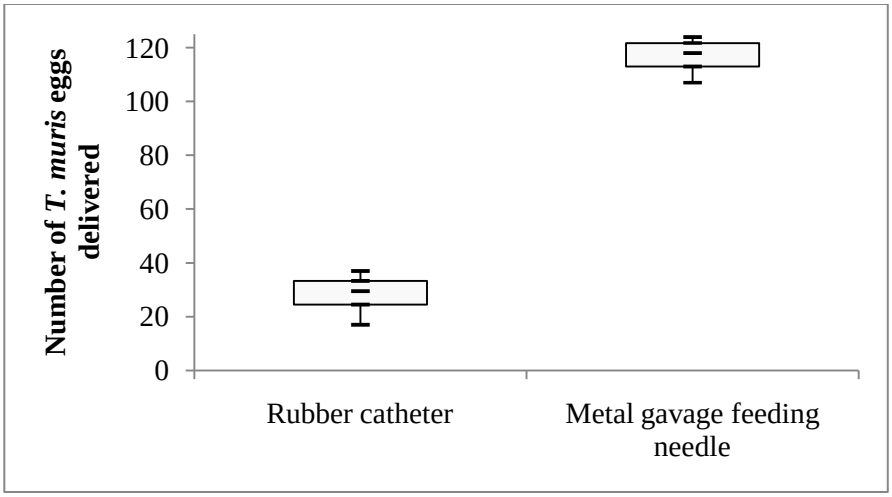


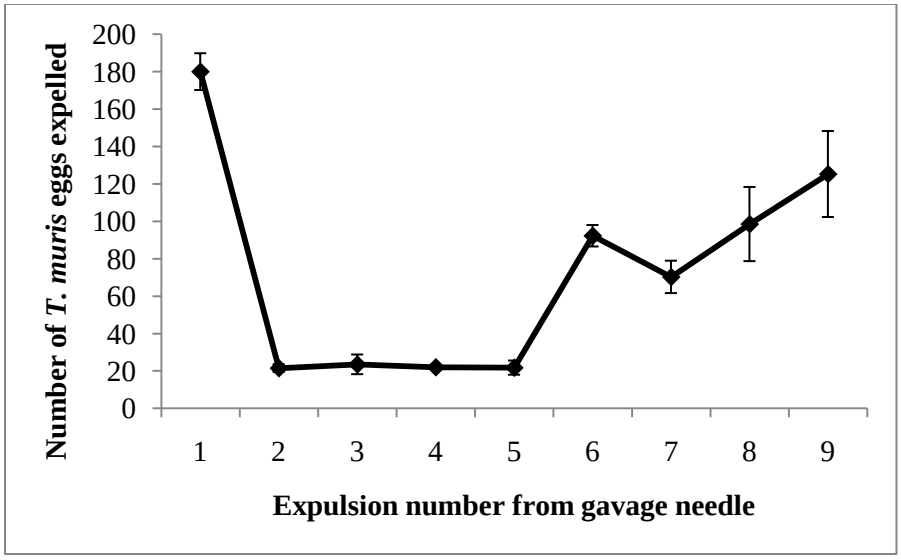
Figure 2.5. Number of *Trichuris muris* embryonated ova delivered to McMaster slide chambers and percentage embryonation. In all cases the intended dose was 100 embryonated *Trichuris muris* eggs. (A) Number of eggs delivered after passing the dose through either a 3.5 gauge French rubber catheter or a metal gavage feeding needle. The difference between the two groups is significant at $p<0.05$ using a one-way ANOVA showing the superiority of the metal needle. (B) Shows the number of *Trichuris muris* embryonated eggs expelled after repeated use of a metal gavage needle. The most accurate delivery occurred on the first expulsion from the needle. The most repeatable delivery occurred from the second to fifth expulsion from the needle although the dose delivered was significantly less than the 100 eggs intended. (C) Percent embryonation of *Trichuris muris* eggs after being left in a metal gavage needle showing that there was no effect of time in needle to percent embryonation. Standard errors are noted by error bars. There was no difference in embryonation rates based on amount of time *T. muris* eggs spent in the gavage needle.

Figure 2.5 Continued

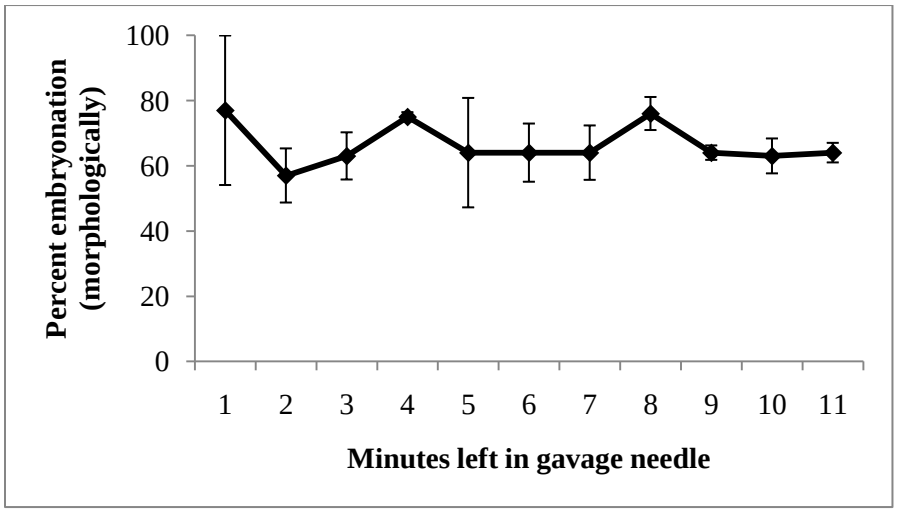
A



B



C



CHAPTER 3

Further characterization of pathology associated with *T. muris* infection of C57BL/6 IL-10$^{-/-}$ mice.

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Prepared for the journal Comparative Medicine

ABSTRACT

*Trichuri*s spp, traditionally considered to be a health threat in both veterinary and human medicine, are a frequently studied immunological model of helminth infections. Although a large body of literature exists describing immunological responses to *Trichuris*, little published information describes the pathology associated with *Trichuris* infections. We hypothesized that C57BL/6 IL-$10^{-/-}$ and IL-$10^{+/-}$ mice would develop significant gastro-intestinal pathology when infected with *T. muris* and that with decreasing doses of *T. muris* the IL-$10^{-/-}$ mice would show comparatively fewer clinical signs of disease. In addition we hypothesized that the C57Bl/6 IL-$10^{+/+}$ mice would support patent infections. Here, C57BL/6 IL-$10^{+/+}$, IL-$10^{+/-}$ and IL-$10^{-/-}$ mice were infected with *Trichuris muris* eggs in a dose response study. Additionally, in a separate experiment C57BL/6 IL-$10^{-/-}$ mice infected with *Trichuris muris* were treated with either metronidazole or prednisolone prior to and throughout the duration of their infection. *Trichuris muris* produced dose-dependent disease in IL-$10^{-/-}$ mice. Ninety percent of mice receiving the high dose (75 ova) had severe disease necessitating early euthanasia. The medium dose (50 ova) resulted in 100% early euthanasia of male mice and 75% of female mice, and the low dose (25 ova) resulted in 100% early euthanasia of males and 25% early euthanasia of females. Some IL-$10^{-/-}$ mice in the high dose group experiencing severe disease had extra-intestinal *T. muris* adults, extra-intestinal bacteria and severe peritonitis. Mice receiving metronidazole had a significantly lower mortality rate than those receiving prednisolone, but pathology of similar severity including peritonitis and histopathologic changes that extended from the stomach to colon. We conclude that *Trichuris* produces dose and sex dependent disease in C57BL/6 IL-$10^{-/-}$ mice. In

addition, higher doses of *Trichuris* increased the likelihood of severe disease including the possibility for gut perforation and *T. muris* infected C57BL/6 IL-10$^{-/-}$ mice treated with metronidazole had a significantly higher survival rate, but still experienced severe multi-organ pathology.

INTRODUCTION

Trichuris spp. are nematodes traditionally considered as a health threat in both veterinary and human medicine. The *Trichuris* life cycle begins with the host ingesting embryonated eggs in the first larval stage (L1); the eggs hatch in the host's cecum and/or colon, L1s penetrate the epithelium and undergo four molts to become sexually mature adults. Larval and adult stages burrow into the host's colonic epithelium forming syncytial tunnels and produce well characterized excretory-secretory products (ESP) with antimicrobial activity (Abner, Parthasarathy et al. 2001).

Trichuris suis can cause disease under some circumstances and has been studied in a number of animal models. In pigs, infections with *T. suis* has been linked to diarrhea in weaning age pigs where interactions with enteric bacteria, such as *Campylobacter jejuni*, causes mucohemorrhagic colitis (Mansfield, Hill et al. 1996; Mansfield, Gauthier et al. 2003). Disease states have been associated with *Trichuris trichiura* when found in association with enteric pathogens including *Campylobacter coli*, *C. jejuni*, *Shigella flexneri*, *Yersinia enterocolitica*, adenovirus, and *Strongyloides fulleborni* in captive rhesus monkeys with chronic enterocolitis (Sestak, Merritt et al. 2003). In mice, work by Schopf et al (Schopf, Hoffmann et al. 2002) reported 100% mortality by day 25 post infection (PI) in *Trichuris muris* infected C57BL/6 IL-$10^{-/-}$ mice given high infective doses. In contrast, they observed resistance to infection in congenic IL-$10^{+/+}$ mice given the same high doses of *T. muris*. They concluded that IL-10 is necessary for survival in C57BL/6 mice infected with *T. muris.* Thus, we aimed to use C57BL/6

IL-10$^{-/-}$ as a murine model to better understand the pathology associated with *Trichuris* infections in these immune compromised mice and their congenic IL-10 sufficient counterparts.

We predicted that *T. muris* causes disease in a dose dependent manner and that those IL-10$^{-/-}$ mice previously observed to develop severe clinical signs of disease resulting in morbidity and mortality would be rescued by concurrent administration of prednisolone or metronidazole. We infected C57BL/6 IL-10$^{+/+}$ mice and congenic IL-10$^{-/-}$ and IL-10$^{+/-}$ mice with *T. muris* to model the adverse events following *Trichuris* infection. All infected IL-10$^{-/-}$, IL-10$^{+/-}$ and IL-10$^{+/+}$ mice had evidence of adult worms in the cecum and colon and infected IL-10$^{-/-}$ mice required early euthanasia due to severe clinical signs that developed in a dose- and sex-dependent manner. In IL-10$^{-/-}$ and some IL-10$^{+/-}$ mice, pathological changes in the colon were dominated by mononuclear and neutrophilic infiltrates, and neutrophils predominated in areas of tissue damage surrounding worms. Additionally, some infected IL-10$^{-/-}$ mice became acutely moribund and were found to have severe peritonitis with extra-intestinal *T. muris* in the peritoneal cavitydue to suspected GI perforation. In our second follow-up study we found that mice infected with *T. muris* and treated with metronidazole had a significantly higher survival rate than those treated with prednisolone, but they did not have significantly decreased GI pathology and histopathology. Although not statistically significant, the prednisolone treated *T. muris* infected mice did tend to develop more severe clinical signs of disease more rapidly resulting in mortality than *Trichuris* infected mice that did not receive any additional treatments. Additionally, we found gastric lesions histologically in all *T. muris* infected mice and in prednisolone treated uninfected mice. From this study we concluded that concurrent treatment

with metronidazole does not rescue *T. muris* infected mice from mortality at the *T. muris* infection dose received but did increase the rate of survival and decrease the clinical signs observed. Also, prednisolone did not help to increase the rate of survival and may even tend to increase the mortality rate of these infected mice.

MATERIALS AND METHODS

Mouse Breeding and Handling

All animal protocols were approved by the Michigan State University (MSU) Institutional Animal Care and Use Committee (IACUC) and complied with National Institutes of Health guidelines (04/07-044-00). To limit the exposure of the mice to extraneous microorganisms and to increase the likelihood that the murine GI microbiota would not be unintentionally influenced by outside parameters, the handling, diet/feeding, cage changing and screening for enteric pathogens, etc., were kept constant and clean throughout the experiment to prevent introduction of microorganisms. C57BL/6J (IL-$10^{+/+}$) and B6.129P2-IL-10^{tm1Cgn}/J (referred to below as C57BL/6 IL-$10^{-/-}$) mice were obtained from Jackson Laboratories (Bar Harbor, ME). A breeding colony was established in a *Campylobacter/Helicobacter*-free facility. Mice heterozygous for the IL-10 allele (Il-$10^{+/-}$) were obtained by crossing the IL-$10^{+/+}$ and IL-$10^{-/-}$ mice in our breeding colony. Mice were housed in pathogen-free conditions using a FlexAir ventilated mouse rack (Alternate Design Manufacturing & Supply Inc., Siloam Spring, AR), fed an irradiated mouse diet (mouse breeder diet 7904; Harlan Teklad, Indianapolis, IN), kept on autoclaved bedding and given filter sterilized water (autoclaved water in bottles for weanlings) in an MSU limited-access room. PCR assays obtained from Jackson Laboratories were used to confirm mouse genotypes ((http://jaxmice.jax.org/pub-cgi/protocols/protocols.sh?objtype=protocol &protocol_id=346) both before and after experiments. Experimental mice were screened for colitogenic bacteria including *Campylobacter* spp.(Linton, Owen et al. 1996), *Helicobacter* spp. (Riley, Franklin et al. 1996), *Enterococcus*

faecalis (Dutka-Malen, Evers et al. 1995), and *Citrobacter rodentium* (McKeel, Douris et al. 2002) using DNA isolated from fecal samples both prior to and at the conclusion of the experiment. The screening assays used were 16S rRNA gene PCR assay for *Campylobacter* spp. and *Helicobacter* spp., *espB* gene-specific PCR for *C. rodentium*, and *ddl* gene-specific PCR for *Enterococcus faecalis*.

A dedicated sentinel mouse was used to assess for extraneous infection with bacterial, protozoan and viral agents and was monitored by the MSU Laboratory Animal Resources Facility (ULAR). Additionally, we monitored the mouse colony for the incidence of spontaneous colitis by examining euthanized retired breeding mice and those euthanized for other reasons for enlargement of the proximal colon, cecum, ileocecocolic lymph node and spleen. If mice exhibited signs of colitis feces were screened for the presence of known colitis causing bacteria using the same assays described above.

Mice between the ages of 8 and 12 weeks were transported to the Michigan State University Research Containment Facility in autoclaved polycarbonate filter-topped cages for experiments. All mice were housed individually in similar filter top cages (Ancare, Bellmore, NY) on sterile bedding, fed irradiated diet 7904 (Harlan-Teklad, US) , given autoclaved water and autoclaved cotton nestlets and randomly assigned to cage slot locations on the rack without respect to their genetic background or treatment group.

Parasites

T. muris eggs were obtained from Dr. Joseph Urban (USDA, Beltsville, MD) and used to infect CBySmn.CB17-Prkdc^{Scid}/J mice (SCID) mice to obtain adult *T. muris* worms. At

necropsy, the GI tract was removed and placed in phosphate buffered saline (PBS) with 100 U/ml Penicillin/100 µg/ml Streptomycin and 2.5 mg/ml Amphotericin B. We individually removed and placed the adult *T. muris* worms in a petri-dish with 15 ml Dulbecco's Modified Eagle's Medium 5% glucose (DMEM) supplemented with penicillin/streptomycin and amphotericin B. Worms were washed free of intestinal contents by transferring them through 5 changes of fresh DMEM media without antibiotics. After the fifth wash, *T. muris* worms were stored in a petri dish at a concentration of roughly 4 worms/ml of medium and incubated at 37˚ with 5% CO_2. Every three days the medium was changed and eggs were collected (via centrifugation).

After *T. muris* eggs were collected they were stored in the dark at room temperature in 30 ml tissue culture flasks with 15 ml of PBS media and 100 U/ml Penicillin/100 µg/ml Streptomycin and 2.5 mg/ml Amphotericin B to prevent bacterial and/or fungal growth while the eggs embryonated. The flasks were monitored via microscopy for fungal and/or bacterial infection and discarded if contamination occurred.

Parasite Inoculum Preparation and Infection

T. muris egg inoculum was prepared as previously described (Kopper and Mansfield 2010). Eggs were sterilized using 6.25% hypochlorite (bleach), washed in sterile PBS and infectivity and viability confirmed using Propidium Iodide staining and hatching assays. Mice were orally gavaged with embryonated *T. muris* eggs in 200 µl PBS according to their experimental group assignment using 1 ml tuberculin syringes with 22 gauge, bent, autoclaved feeding needles.

Monitoring for clinical signs

Mice were evaluated one to four times per day by trained individuals with a standardized score sheet. Mice were monitored for clinical signs including, but not limited to rough hair coats, hunched posture, decreased activity and dehydration. Each factor, if present, conferred a numerical score. Any mouse receiving a score of 8 or higher was immediately euthanized and necropsied. This served as a means for standardizing the stage of disease at which mice were sacrificed.

EXPERIMENTAL DESIGNS

Two experiments were conducted: a *T. muris* dose response and an antibiotic/steroid treatment trial. All mice were age matched and randomized according to sex and treatment group.

We conducted a dose response experiment in which C57BL/6 IL-$10^{-/-}$ mice received either 25, 50 or 75 embryonated *T. muris* eggs or PBS sham inoculations. To control for the effects of genotype, C57BL/6 IL-$10^{+/+}$ and IL-$10^{-/-}$ received the high dose of 75 embryonated *T. muris* eggs (Table 3.1). Treatment groups were age-matched and randomized from the available litters. If mice exhibited adverse signs based on the standardized scoring system they were euthanized and asymptomatic IL-$10^{+/+}$ and IL-$10^{+/-}$ mice were chosen at random for euthanasia to provide time-post-infection-matched comparisons (Table 3.1). All remaining mice were euthanized at day 40 post inoculation (PI). This end-point was chosen based on the *T. muris* life cycle and data from a preliminary mouse experiment with the same mouse genotype and *T. muris* strain that showed peak egg-shedding in fecal pellets at this time. After the single oral challenge infection, mice were grouped according to their time of end-point for analysis.

In the antibiotic and steroid treatment trials 30 (15 males and 15 females) C57BL/6 IL-$10^{-/-}$ mice were infected with *T. muris*. One week prior to inoculation with *T. muris* mice were started on their drug treatment protocol (Table 3.2). Mice were randomly assigned to infection and drug treatment groups. Mice received either 0.6 mg of Prednisolone by mouth (PO) once daily (SID), 0.02 mg of Metronidazole PO SID or suspension only PO SID (compounded by

Wedgewood pharmacy, Swedesboro, New Jersey). Mice were given their drug SID by using a micropipette to deliver 10µl of the compounded suspension into their mouths.

Necropsy, Gross Pathology and Histopathology

For both experiments mice were humanely sacrificed by CO_2 overdose according to AVMA guidelines (2001) and weighed. Immediately following euthanasia a blood sample was obtained by cardiac puncture using 3.8% sodium citrate to prevent coagulation. Spleens and livers were removed aseptically, weighed and cut longitudinally. One half was placed on a sponge histological cassette (Histocette II; Simport Plastics, Beloeil, Quebec Canada) and submerged in Carnoy's solution for 24 hours, then the solution was decanted and the spleen placed in 60% ethanol in preparation for histological analysis. The other half was snap frozen using dry ice for subsequent DNA extraction. The GI tract was removed in its entirety and gross pathological changes were noted and photographed. The cecum with approximately 1 cm of both the terminal ileum and proximal colon was placed on a sponge histological cassette (Histocette II), immediately injected with Carnoy's solution in separate containers according to treatment group and submerged in Carnoy's solution for 24 hours. Thereafter, the solution was decanted and the tissue was placed in 60% ethanol in preparation for histological analysis. Formalin-fixed tissues that were previously stored in ethanol were embedded in paraffin, and 5-µm sections were stained with hematoxylin and eosin (H&E) at the Investigative Histopathology Laboratory, Division of Human Pathology, Department of Physiology, Michigan State University. Sections were examined microscopically and lesions were photographed using a Nikon Eclipse E600 microscope with a SPOT camera with Windows™ version 4.09 software (RT-Slider Diagnostic Instruments, Inc., Sterling Heights, MI.

Histopathological scoring

A scoring system was developed to evaluate histopathological changes in the ileocecocolic junction of each mouse (Mansfield, Bell et al. 2007). Specific features were evaluated as follows. The lumen was evaluated for excess mucus and inflammatory exudates. The epithelium was evaluated for surface integrity, number of intraepithelial lymphocytes, goblet cell hypertrophy, goblet cell depletion, crypt hyperplasia, crypt atrophy, crypt adenomatous change and crypt inflammation. The lamina propria and submucosa were evaluated for increases in inflammatory or immune cells and their distribution; the submucosa was evaluated for fibrosis. All histological sections were scored by a single investigator (JJK) who was blinded to the identities and experimental group of the individual mice. Spleen histopathology sections were scored using a standardized criteria (Mansfield, Patterson et al. 2008), which evaluates periarteriolar sheaths size (PALS), degree of extramedullary hematopoiesis, and degree of mononuclear lymphocytes infiltrates by a single individual (JJK). Liver histological slides were scored using a standardized scoring system. This system evaluated for the presence of extra-hepatic inflammation and intra-hepatic inflammatory cells. Additionally, the slides were evaluated for presence of absence of damage to the three zones within the liver lobules. Standardized scores were assigned to each slide based on the type of damage and the extent or severity of the damage.

Slides for stomach histology were assessed for the following key histopathological features: the presence of non-glandular hyperplasia, keratin cysts, ulceration, parietal cell vacuolation, fundic hyperplasia, inflammatory exudates, extra-gastric inflammation, glandular edema and non glandular edema. Each feature observed on histology counted as one point and an

overall histopathology score was given based on the presence or absence of each of these pathological features.

Fluorescent in situ Hybridization

A 16S Universal DNA probe sequence (Steel, Malatos et al. 2005) with 6'FAM fluorescent tags was used to investigate the presence of extra-intestinal bacterial colonization. Tissue sections from the small intestine and ileocecocolic junction on the slide were first digested with Proteinase K (10ug/ml Proteinase K in PBS with 1 mM EDTA) for 10 min. at 37^0C and then placed in a stop solution (0.2% glycine in PBS) for 5 minutes. Slides were subsequently washed in 10 mM PBS pH 7.4 and autoclaved deionized water, and air dried. Then, slides were hybridized to the probe of interest using a hybridization buffer (50% deionized formamide, 5xSSC, 10% dextran sulfate, 5x Denhardt's solution and 100ug/ml of denatured herring sperm DNA) using a parafilm coverslip. Slides were then incubated to allow for hybridization in the dark at 37^0C for 22-24 hours, after which time the parafilm coverslip was removed by soaking slides in 2x SSC. Slides were then washed in 2x SSC, allowed to air dry and coverslipped with ProLong Gold (Invitrogen, Carlsbad, CA). Slides were then evaluated for the presence or absence of positive fluorescence.

Statistical Analysis

Statistical Analysis. Comparisons of ileocecocolic junction (ICC-J) histological scores between groups were conducted using ANOVA. For this analysis, scores of the *T. muris* infected mice sacrificed at both time points (29 or 40 days post infection) were compared to their

respective genetically matched uninfected control group. The null hypothesis was rejected if $P \leq 0.05$.

Comparisons of spleen histological scores between groups were conducted using Fisher's exact test. For this analysis, scores of the *T. muris* infected mice sacrificed at both time points (29 or 40 days post infection) were compared to their respective genetically matched uninfected control group by Fisher's exact test. In infected groups, scores from mice given different doses of *T. muris* were grouped as a single infected group. Thereafter, scores were grouped in the two-way table so that mice having scores falling into grade 0 (scores ≤ 9) formed one class and mice having scores falling into grades 1 and 2 (scores ≥ 10) formed the second class. After two-tailed P values were calculated using Fisher's exact test, the Holm step-down procedure was used to apply the Bonferroni correction for multiple comparisons (Ludbrook 1998). The null hypothesis was rejected if $P \leq 0.05$.

RESULTS

Mice were free of colitogenic bacteria. All fecal samples taken from (1) experimental mice before and after induced infections with *T. muris* and (2) healthy mice from the breeding colony tested negative by PCR for *Campylobacter* spp., *Helicobacter* spp, *C. rodentium*, and *E. faecalis*. All DNA samples extracted from stomach, jejunum, cecum, and colon tissues and feces of the sentinel mice in the containment facility gave negative results in the four PCR assays. No spontaneous colitis was observed in these mice during the course of these experiments.

C57 BL/6 IL-10$^{-/-}$ developed clinical signs that dictated early euthanasia in a sex and dose dependent manner. Figures 3.1 and 3.2 shows the clinical signs and survivorship curves for C57BL/6 IL-10$^{-/-}$ mice infected with *T. muris*. None of the uninfected mice displayed clinical signs of disease throughout the course of infection. Of mice receiving the high dose (75 embryonated *T. muris* eggs) 11/12 male and 9/9 female mice required early euthanasia due to severe clinical signs by day 30 post infection. Infected male mice had a slightly higher mortality rate at day 21 post infection than the female mice. Of the mice receiving the medium dose (50 embryonated *T. muris* eggs), 5/5 male mice and 3/6 female mice required early euthanasia due to severe clinical signs, and of the mice receiving the low dose (25), 5/5 male mice and 1/5 female mice required early euthanasia before the conclusion of the experiment. Additionally, in the low dose group, appearance of clinical signs was delayed, compared to mice receiving high and medium doses of *T. muris* eggs (Figure 3.1C). These survivorship curves showed an increased mortality rate with an increase in dose, and associated with sex (male mice).

In steroid and antibiotic treatment trials, all infected mice developed clinical signs of disease, but those mice treated with prednisolone had a higher and more rapid mortality rate. As shown in Figure 3.1, prednisolone treated, metronidazole treated and untreated groups of *T. muris* infected mice all developed significant clinical signs of disease. These clinical signs included a hunched posture, rough hair coat, and decreased activity. Figure 3.2, depicts a survival curve for the three groups of infected mice. Here, we see that the mice treated with metronidazole survived for a statistically significant longer period of time and in higher numbers than those treated with prednisolone.

C57BL/6 IL-10$^{-/-}$ and some IL-10$^{+/-}$ infected with *T. muris* had significant gross pathologic changes. All infected C57BL/6 IL-10$^{-/-}$ mice in all treatment groups developed significant gross pathologic abnormalities of the ileocecocolic (ICC) junction and proximal colon compared to the uninfected controls. This included a thickened wall, watery contents and enlarged ileocecocolic lymph nodes (Figure 3.3). Some of the infected IL-10$^{+/-}$ mice developed lesions in these sites that were judged to be less severe. None of the IL-10$^{+/+}$ had notable gross lesions at necropsy. Some IL-10$^{-/-}$ mice in the high-dose (75) infection group with severe clinical signs were observed to have white raised, firm plaques of various shapes and sizes on the surface of the liver at necropsy (Figure 3.4). Titers for known pathogenic mouse viruses were negative in these mice (Charles River Laboratories, Wilmington, Massachusetts).

At necropsy all infected mice in the antibiotic and steroid treatment trials had significant gross lesions in their jejunum, duodenum, cecum, and proximal and distal colon (Figure 3.5). Although there were some differences in the lesions observed at necropsy, there were no statistically significant differences in lesions of the gastrointestinal tract among the *T. muris* infected groups. The gross pathology included enlarged, fluid filled and thickened intestinal walls almost uniformly throughout their ileum, cecum and colon. Approximately half of the mice from each infected treatment group had thickening and distension of their jejunum and duodenum. 10-30% of the mice from each group were observed to have digested blood in their upper gastrointestinal tract. Two infected mice that were also treated with metronidazole and survived throughout the course of the experiment were noted to have significant cecal adhesions on the serosal side, which adhered to other segments of gastrointestinal tract tissues.

Histopathological changes of the ileocecocolic junction included neutrophilia, thrombi and chronic-active inflammation. IL-10$^{-/-}$ mice receiving the high (75) dose of *T. muris* ova developed significant histologic lesions of the ileocecocolic junction with extreme crypt hyperplasia, inflammatory cell infiltrates, and sloughing of the mucosal epithelium (Figures 3.6a, 3.7). In some mice, inflammation was chronic yet active; mononuclear cell infiltrates (lymphocytes, plasma cells, macrophages) predominated with evidence of healing in some areas. Additionally, these mice were observed to have thrombi in the submucosa associated with large amounts of edema (Figures 3.7B, 3.6H). Significant neutrophilic infiltrates and exudates were noted around *T. muris* worms (Figure 3.8C). IL-10$^{+/-}$ mice receiving the high dose either had severe histopathology scores similar to IL-10$^{-/-}$ infected mice or minimal to no histological

lesions (similar to those seen in the IL-10$^{+/+}$ mice). All IL-10$^{-/-}$ mice receiving the medium dose (50) had severe crypt hyperplasia, inflammatory cell infiltrates and sloughing of the mucosal epithelium. Additionally, marked neutrophilic infiltrates of the lamina propria were also noted. Finally, the IL-10$^{-/-}$ mice receiving the low (25) egg dose had lesions with the same features as IL-10$^{-/-}$ mice receiving 50 *T. muris* eggs but to a lesser degree of severity. Infected and uninfected IL-10$^{+/+}$ mice had few or no histologic lesions in their GI tracts. Figure 6A-C show representative gastro-intestinal sections from the IL-10$^{+/+}$ mice. Although none of the IL-10$^{+/+}$ mice displayed clinical signs of disease a portion of the high dose (100 embryonated egg) group was euthanized early (prior to day 28 post infection) to serve as matched end-point controls for the IL-10$^{-/-}$ mice experiencing severe clinical signs of disease.

All infected mice in antibiotic and steroid treatment groups had more severe lesions of their ileocecocolic junctions, as judged by histology when compared to their uninfected controls, and prednisolone uninfected mice had more severe lesions of their ileocecocolic junctions than the other uninfected mice. As shown in Figure 3.6B, all infected mice had significantly higher histopathological changes to their ileocecocolic junction. The inflammatory cell changes were primarily neutrophilic, particularly in the exudates that were observed. Additionally, many of the mice had submucosal edema and fibrosis, and extra-intestinal bacteria and inflammation were noted. Although some mice were treated with metronidazole or prednisolone there was not a significant difference in the number of mice from each group which was also noted to have evidence peritonitis on histological examination.

All infected mice in antibiotic and steroid treatment trials groups had significant gastric histopathologic lesions when compared to their respected uninfected control group (Figure 3.6B,C). Although the use of prednisolone or metronidazole was not associated with an overall score difference, there were some differences regarding individual histologic features that were evaluated. Although no significant changes were noted between the infected groups and any of their respective controls, there were changes that were noted in the prednisolone treated uninfected mice. Mice treated with prednisolone had significant vacuolation of parietal cells when compared with the other uninfected mice and metronidazole treated *Trichuris* infected mice. Additionally, 90% of the prednisolone uninfected mice were noted to have fundic hyperplasia, which was not noted in any other group of mice that were *Trichuris* infected or uninfected.

IL-10$^{-/-}$ and IL-10$^{+/-}$ receiving 75 embryonated eggs had significant histologic lesions in splenic tissues. Mice from both genotypes and treatment groups were noted to have marked extramedullary hematopoiesis (EMH) and prominent and irregularly shaped periarteriolar sheaths (Figures 3.9, 3.10). IL-10$^{-/-}$ and IL-10$^{+/-}$ mice receiving the high 75 egg dose had splenic histological scores that were significantly different from IL-10$^{+/+}$ mice but not from each other. IL-10$^{-/-}$ mice given 50 and 25 eggs had splenic changes that were not statistically significant from other groups ($p>0.05$, data not shown)

Mice infected with *T. muris* and given antibiotic or steroid treatments trials had significant splenic lesions when compared to their respective control groups (Figure 3.9B*)*. Mice infected with *T. muris* had similar histopathological changes, including extramedullary hematopoiesis and enlargement of PALS despite being treated with metronidazole or prednisolone. These changes were statistically different from their corresponding uninfected groups.

Adult *Trichuris muris* and *Trichuris* eggs were found in some moribund IL-10$^{-/-}$ mice in extra-intestinal sites. Histopathological evaluation of the ileocecocolic-junction of IL-10$^{-/-}$ mice receiving the high (75) dose revealed that some mice had adult *Trichuris* on the serosal side of the gastrointestinal tissues (Figure 3.11). Additionally, *Trichuris* eggs were observed embedded within extra-intestinal inflammatory exudates on the serosal side of the GI tract, implying that sexually mature egg-producing females were present within the peritoneal cavity (Figure 3.11A).

Extra-intestinal bacteria were found adherent to the serosal membranes in some IL-10$^{-/-}$ mice receiving 75 and 50 embryonated *T. muris* eggs. Further investigation of gross lesions noted at necropsy in the liver and small intestines of IL-10$^{-/-}$ mice receiving the high dose (75 eggs) revealed aggregates of inflammatory cells and bacteria adherent to the capsules of the liver and spleen and the serosal surfaces of the small intestine (Figures 3.12, 3.13). No lesions were noted within the parenchyma of the liver. 16S universal rDNA specific fluorescent *in situ*

hybridization confirmed the presence of bacteria on the serosae of small intestines and liver and within splenic tissue (Figure 3.14).

DISCUSSION

Previous work identified significant morbidity and mortality in C57BL/6 IL-10$^{-/-}$ mice (Schopf, Hoffmann et al. 2002) and a large body of literature exists investigating immunological responses to *Trichuris* in laboratory mice. The overall goal of these experiments was to test our hypotheses that (1) C57BL/6 IL-10$^{-/-}$ and IL-10$^{+/-}$ mice would develop morbidity/ mortality in a dose dependent manner and (2) that concurrent treatment of *T. muris* infected C57BL/6 IL-10$^{-/-}$ mice with antibiotics or glucocorticoid steroids would decrease the rates of morbidity and mortality. C57BL/6 mice with IL-10 had a significantly enhanced rate of the survival, but presence of this cytokine did not strictly control survival, resistance or mortality. While Schopf et al (Schopf, Hoffmann et al. 2002) reported 100% mortality in IL-10$^{-/-}$ mice and 100% resistance in the IL-10$^{+/+}$, we report adult patent infections of the IL-10$^{+/+}$ mice and dose and sex dependent effects on survivorship in IL-10$^{-/-}$ mice infected with *Trichuris muris*. Differences between our results and published studies where *T. muris* precipitated death in C57BL/6 IL-10$^{-/-}$ mice and resistance to patent infections in C57BL/6 IL-10$^{+/+}$ mice may be due to the dose of *T. muris* eggs delivered. We chose to give doses that were significantly reduced in comparison to those previously used in mouse models based on severe outcomes in mice given the higher doses in the dose response studies.

These results show that *Trichuris* is capable of producing severe disease in an IL-10 deficient murine model which includes gut perforation, the presence of adult egg-producing *T. muris* in the peritoneal space, severe peritonitis and intestinal bacteria in extra-intestinal sites. To our knowledge, *Trichuris* has never been found outside of the gastro-intestinal tract, and current

published studies have not reported dose and sex dependent adverse side effects. Although previous literature does report dose-dependent susceptibility in models that were otherwise thought to be resistant to *Trichuris* (Bancroft 1994), to our knowledge dose-dependent increases in severity of clinical signs have not been reported, particularly in an IL-10 deficient model with documented severe morbidity and mortality. Additionally, although worm burdens were not determined quantitatively in mice due to a desire to preserve and examine the histopathology of the ileocecocolic junction of these mice in detail, infective egg doses were carefully determined using previously published methods (Kopper and Mansfield 2010). We showed here that a relatively small change in dose (75, 50 and 25 eggs) produced a significant change in mortality in the IL-10 deficient mice. Blinded histopathology evaluations reveal that even when IL-10$^{-/-}$ mice display few, if any, clinical signs of disease, the level of inflammation does not vary significantly from those mice euthanized early due to severe clinical disease. Furthermore, having some IL-10 present as observed in the heterozygotes does not rescue the *Trichuris* infected mice from the effects of the high dose. These data suggest that mortality is due to factors other than sheer numbers of inflammatory cells in the definitive site of worms in cecum and proximal colon. Additionally, these data make a compelling case for enteric bacteria in the pathogenesis of this disease.

Histopathological analyses of the gastrointestinal tract and other abdominal organs revealed the presence of *T. muris*, and significant inflammation, and bacteria in the peritoneal cavity. At this time the mechanism by which bacteria escape the gastro-intestinal tract and establish in the peritoneum is unknown, although we hypothesize that escape is facilitated when worms breach the mucosal barrier and cause severe inflammation and weakening of the wall of the distal ileum, cecum and proximal colon. Even in mice that were not observed to have *T.*

muris or *T. muris* eggs on the intestinal serosa, many of the mice had extra-intestinal bacteria, as noted on evaluation of H& E stained sections and this observation was confirmed using a 16S Universal rDNA probe which is a classic tool specific for bacteria. It is also unknown if the inflammatory response demonstrated by intense mononuclear and neutrophilic infiltrates is generated towards the extra intestinal bacteria, worms, or both. However, it is reasonable to assume that the generalized peritonitis observed here results from reactivity to both worms and bacteria. Based on results reported by Zaph et al (Zaph, Troy et al. 2007) it is highly likely that these animals succumbed to endotoxic shock with endotoxin absorbed into the blood stream following penetration of intestinal bacteria beyond the mucosal barrier. A bacterial mechanism for the severe disease observed here is supported by the presence of numerous neutrophils in the extra-intestinal sites of inflammation. Also, histopathologic examination showed that in many infected IL-$10^{+/+}$, IL-$10^{+/-}$ and IL-$10^{-/-}$ mice *T. muris* worms at the mucosal surface and within the intestinal wall were surrounded by neutrophilic exudates. Traditionally, neutrophilic responses are considered to be a key component in a host's response to bacterial infection (Williams, Montenegro et al. 1994) whereas characteristically, helminth infections induce strong eosinophilic responses in the host. These results also suggest a bacterial component associated with the worms.

Despite the fact that *T. muris* infected mice treated with metronidazole had an increased rate of survival, they did not have lower histopathology scores of their ileocecocolic junctions (ICC-Js), spleens, livers or stomachs or a decreased incidence of extra-intestinal inflammation when compared to prednisolone or untreated mice infected with *T. muris*. This suggests that the morbidity of the untreated and prednisolone treated infected mice is mediated by something other than sheer inflammation, as a decrease in inflammation was not observed in the

metronidazole treated mice. Additionally, there were few statistically significant differences when individual histopathological features were assessed with our scoring system. These differences were limited to the prednisolone uninfected mice which had a statistically significant number of mice with fundic hyperplasia and vacuolated parietal cells when compared to other infected and uninfected treatment groups. We hypothesize that this is due to the administration of prednisolone long term. Surprisingly, none of the prednisolone treated mice infected with *T. muris* had fundic hyperplasia (compared to 9/10 of the uninfected prednisolone treated mice) and only 5/9 (compared to 9/10 uninfected prednisolone treated mice) had significant decreases in parietal cells. This could be due, to the fact that many of the prednisolone treated mice were euthanized early due to adverse clinical signs, and as a result did not have prednisolone treatment for as long a period of time (maximum of 6 days difference). Alternatively the effects could have been modulated by the presence of the *T. muris* worms, but we cannot determine that from these studies.

Although there was no overall decrease in the histopathology scores of the ileocecocolic junctions of these mice associated with antibiotic treatment, two metronidazole treated infected mice at the end of the experiment had fibrous adhesions on their cecal serosae. This suggests chronic damage, with eventual scarring on the peritoneal surface of these organs. Based on the evidence of bacterial peritonitis, serosal adhesions and *T. muris* cross sections on the serosal side of the gastrointestinal tract, we speculate that a few worms migrated in the peritoneum of these mice.

Gastric lesions were noted in all of the *Trichuris* infected mice in the second study, the antibiotic and steroid treatment trials. This was an unexpected result, as *T. muris* is a proximal colon dwelling nematode and has not been reported as hatching from their eggs until reaching

the distal small intestine, cecum and colon. There were no significant differences in the characteristics of the pathologic lesions, particularly the presence of digested blood or the microscopic lesions among the three *Trichuris* infected treatment groups, and therefore the lesions noted cannot be attributed to the administration of metronidazole or prednisolone. It is possible, that these mice stopped or decreased in eating due to adverse clinical signs, as their infection progressed which would have left them more prone to gastric pathology.

In the natural host, *Trichuris* spp. infections, including *T. suis*, frequently cause disease. Mansfield and colleagues have demonstrated the augmentation of clinical disease and pathologic changes with concurrent infections of *T. suis* and *C. jejuni* in immunologically naïve swine (Mansfield, Gauthier et al. 2003). In this study, germ free neonatal swine were infected with *T. suis, C. jejuni, or C. jejuni* and *T. suis* or left uninfected. Those animals with concurrent infections had more frequent and severe diarrheic episodes, more severe gross lesions and significantly more inflammation demonstrable histologically in the proximal colon. Animals used as controls had no disease and those infected solely with *T. suis* or *C. jejuni* had minimal pathologic changes and only mild clinical signs. In this model of dual infection, disease was most severe in the later stages of the prepatent period when L4 larvae and adults were present, suggesting a role for *Trichuris* excretory secretory products in the pathogenesis. In our mouse model, we see a similar time course with clinical signs and lesions arising at approximately 21 days post infection, coinciding with the development of L3 and L4 larvae. This may be due to burrowing activity or simply the rupture of the epithelial barrier when third stage larvae begin to extend into the colonic lumen.

As previously stated, the difference in results reported here compared to the 2002 study by Schopf et al (Schopf, Hoffmann et al. 2002), may be due to differences in the doses of

infective *T. muris* eggs delivered. Furthermore, the finding of extra-intestinal *T. muris* worms, extra-intestinal colonization of bacteria and severe peritonitis in some IL-10$^{-/-}$ infected mice in our study here demonstrates that *T. muris* has a significant potential to cause adverse side effects in some mice. It is possible that IL-10 deficiency impairs gut barrier function leading to an increased chance for escape of *Trichuris* larvae and resident bacteria. It is clear that more work is needed to understand the mechanism by which *Trichuris* escaped the GI tract in this murine model.

In conclusion, these studies revealed a dose and sex-dependent effect on the morbidity and mortality of IL-10$^{-/-}$ mice infected with *T. muris*. In mice of the C57BL/6 genetic background, histological assessment revealed extra-intestinal migration, bacteria and peritonitis that occurred mainly in those immunocompromised individuals lacking IL-10. Further investigation of dose dependent reactions as well as *Trichuris* strain differences could be of benefit to further understand the host-*Trichuris* relationship and host reaction. We also demonstrated that while antibiotic treatment increases the rate of survival of infected mice, it does not prevent mortality or even peritonitis, in the IL-10$^{-/-}$ mice. The presence of gastric inflammation, despite the absence of worms in this portion of the gastrointestinal tract, suggests that *Trichuris* infection may trigger inflammation in these mice beyond the site(s) of the primary lesions in the proximal colon, cecum and distal ileum.

TABLES

TABLE 3.1A

Experiment	C57BL/6 IL-10$^{-/-}$ (no. uninfected, no. infected)	C57BL/6 IL-10$^{+/-}$ (no. uninfected, no. infected)	C57 BL/6 IL-10$^{+/+}$ (no. uninfected, no. infected)
High Dose (75)	6,20	6,17	6,16
Medium Dose (50)	7,14	None	None
Low Dose (25)	10,10	None	None

TABLE 3.1B

Experiment	Prednisolone	Metronidazole	No treatment
Infected (75)	10	10	10
Uninfected	10	10	10

FIGURES

Figure. 3.1**.** Judged clinical signs of IL-10$^{-/-}$ mice. All other mice remained free of clinical signs throughout the course of the experiment. Dots represent the individual clinical sign scores in a mouse on each day. (A) High dose experiment receiving 75 embryonated eggs (B) Medium dose experiment receiving 50 embryonated eggs (C) Low dose experiment receiving 25 embryonated eggs, (D) Prednisolone treated infected mice receiving 75 embryonated eggs, (E) Metronidazole treated infected mice receiving 75 embryonated eggs and (F) Untreated infected mice receiving 75 embryonated eggs. Closed circles represent female mice and closed squares represent male mice.

Figure 3.1 Continued

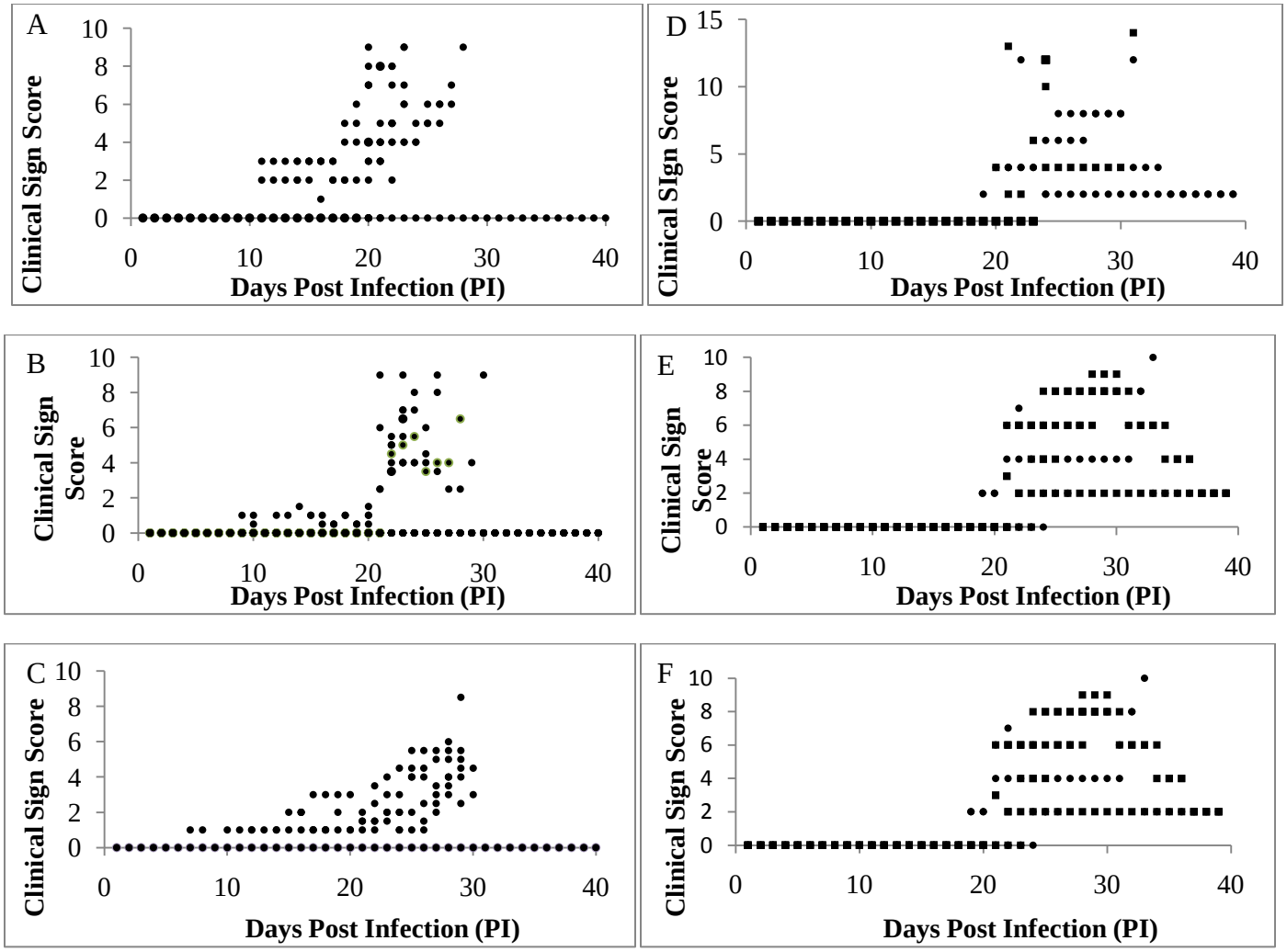

Figure 3.2. Survivorship curves with percentage of IL-10$^{-/-}$ mice surviving. A) High-Dose Experiment (75 embryonated *T. muris* eggs) N= 11(m), 9(f), B) Medium-Dose Experiment (50 embryonated *T. muris* eggs) N= 5 (m), 6 (f) and C) Low dose experiment (20 embryonated *T. muris* eggs) N=4 (m), 4 (f), D) prednisolone, metronidazole and untreated treated infected mice N= 10 per group.

Figure 3.2 Continued

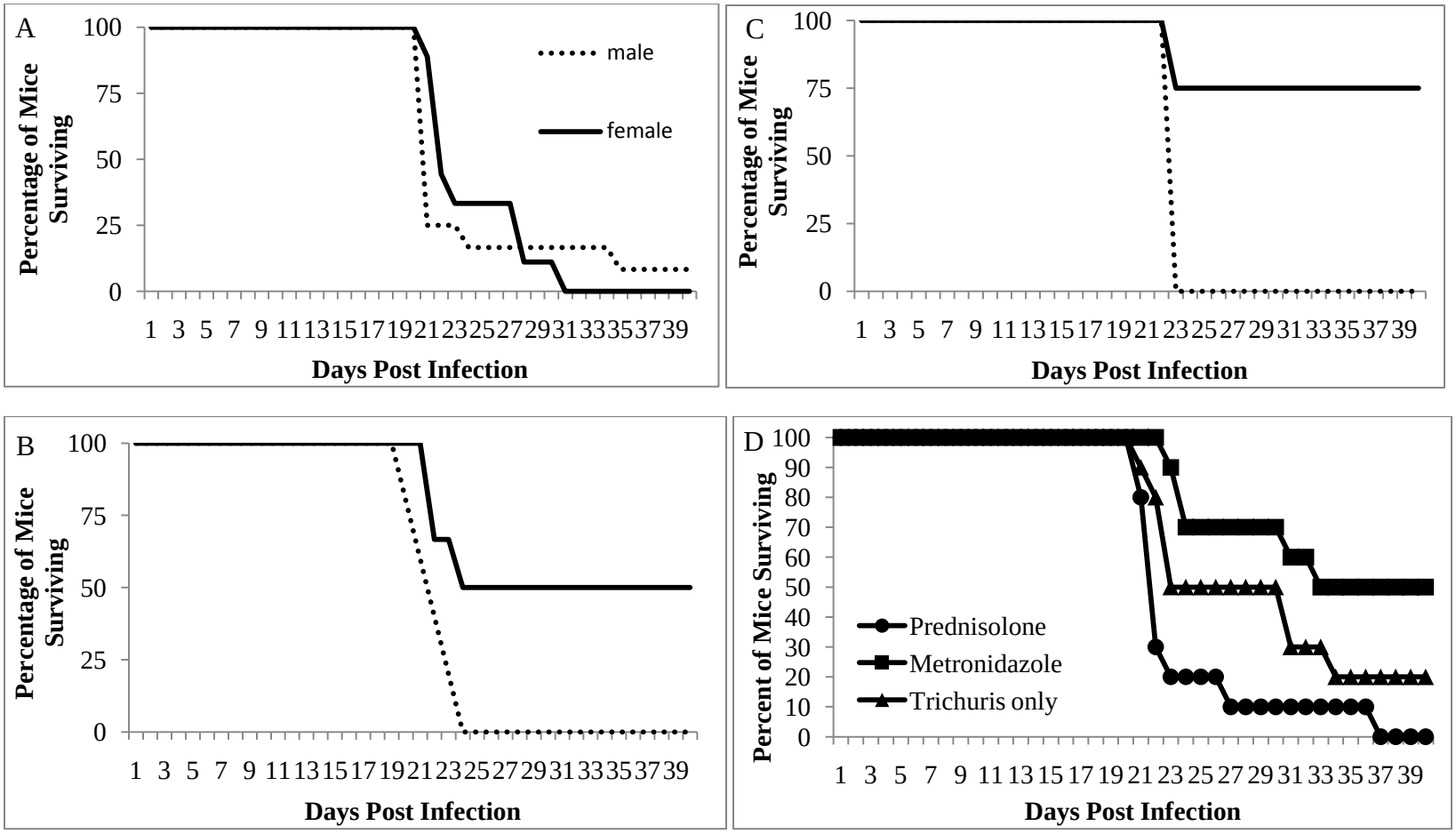

Figure 3.3. Distal ileum, cecum and proximal colon pathology of (A) Uninfected IL-10$^{+/+}$ (B) Uninfected IL-10$^{+/-}$ (C) Uninfected IL-10$^{-/-}$ (D) Infected IL-10$^{+/+}$ (75 embryonated eggs) (E) Infected IL-10$^{+/-}$ (75 embryonated eggs) and (F) Infected IL-10$^{-/-}$ (75 embryonated eggs) all taken at the same magnification. Notice that only IL-10$^{-/-}$ (F) mice severely distended intestine and cecum associated with *T. muris* infections.

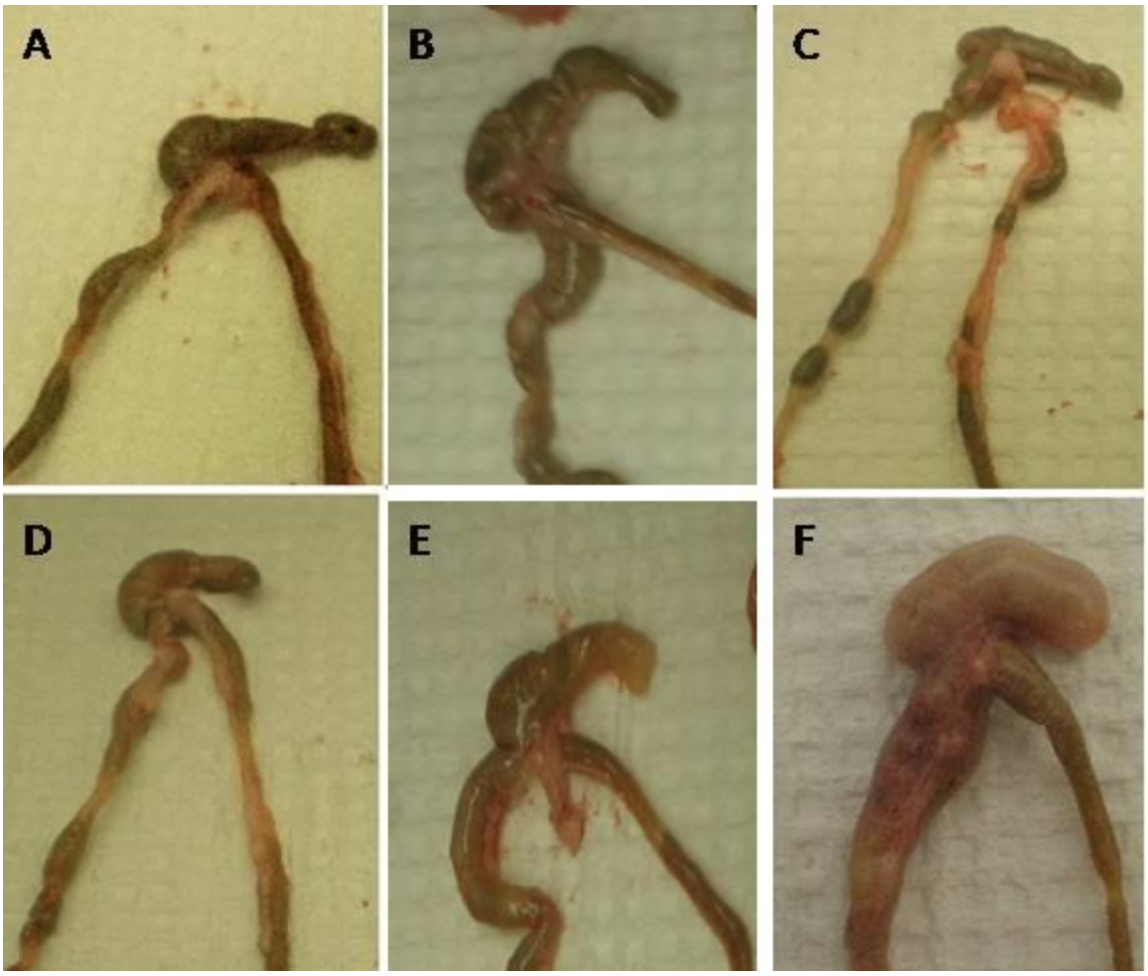

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Figure 3.4. Liver gross pathology of a representative infected IL-10$^{-/-}$ mice. (A) Liver of uninfected IL-10$^{-/-}$ mouse, (B) Liver of infected moribund IL-10$^{-/-}$ mouse receiving the high dose of *T. muris* eggs (75). Note the white areas on the surface of the liver (B).

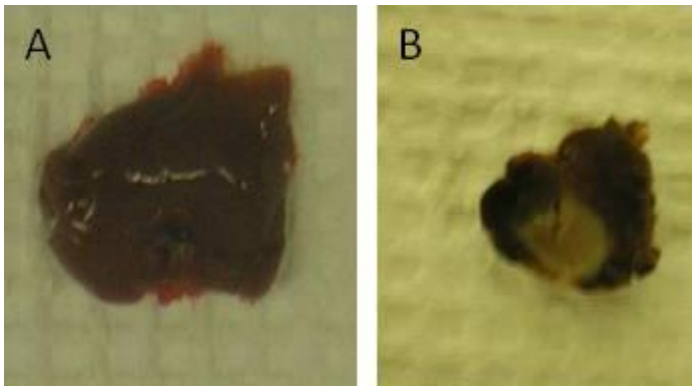


Figure 3.5. Summary of gross lesions seen in C57BL/6 IL-$10^{-/-}$ mice infected with *Trichuris*, and either untreated, treated with metronidazole or treated with prednisolone. Uninfected mice had no gross pathologic changes. (A) Duodenal gross pathology, (B) Jejunal gross pathology, (C) Ileal gross pathology, (D) Cecal gross pathology, (E) Colonic gross pathology and (F) Miscellaneous gross pathology. None of the changes noted were statistically significant ($P \geq 0.05$). Enl demonstrates areas that were enlarged due to thickening, hemorrhage of the tissues and fluid filled gastrointestinal lumens.

Figure 3.5 Continued

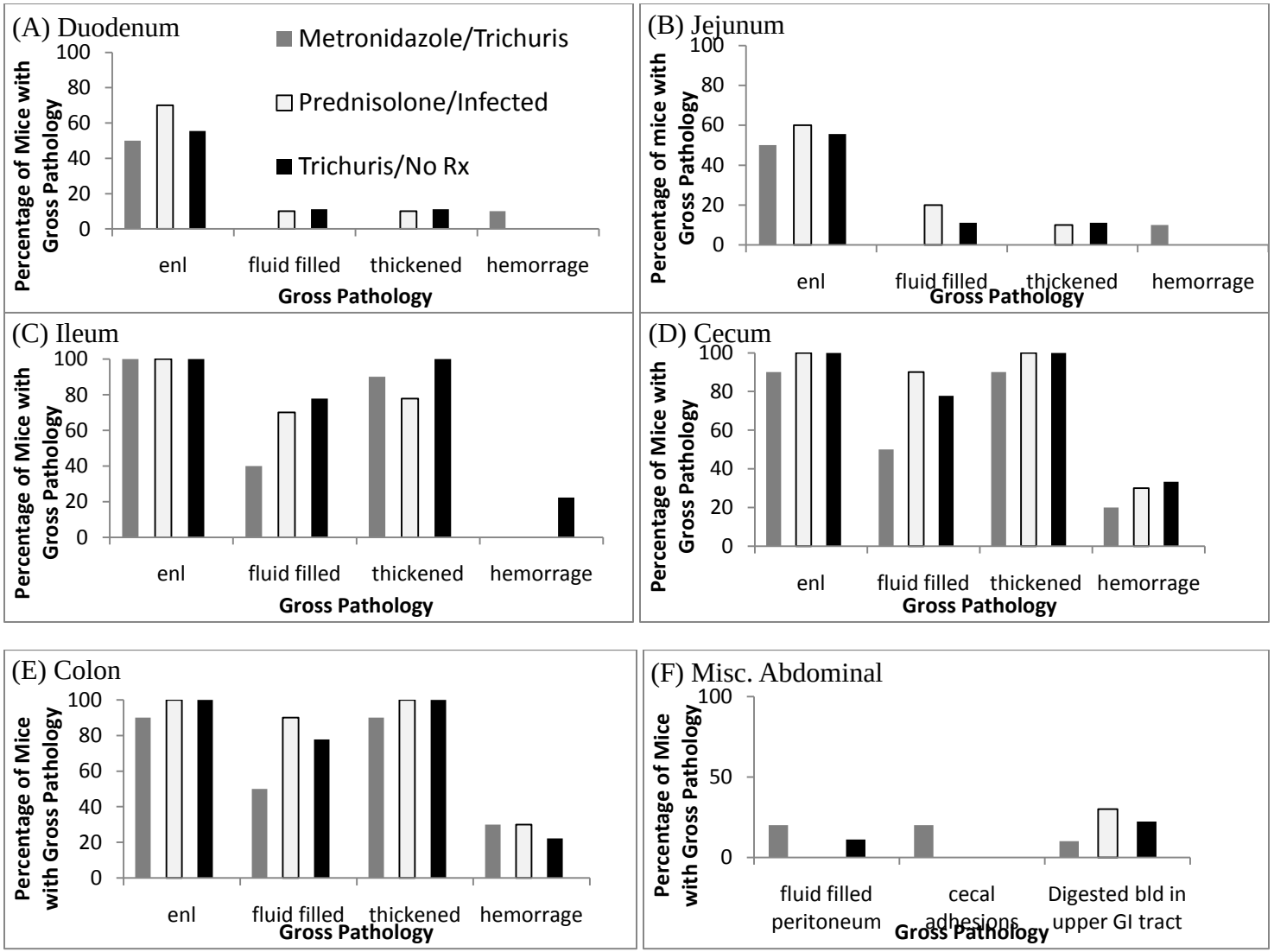
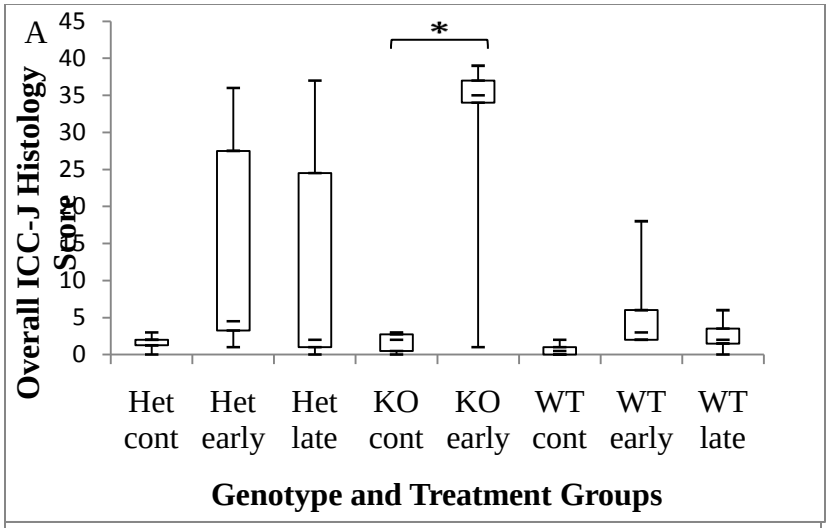
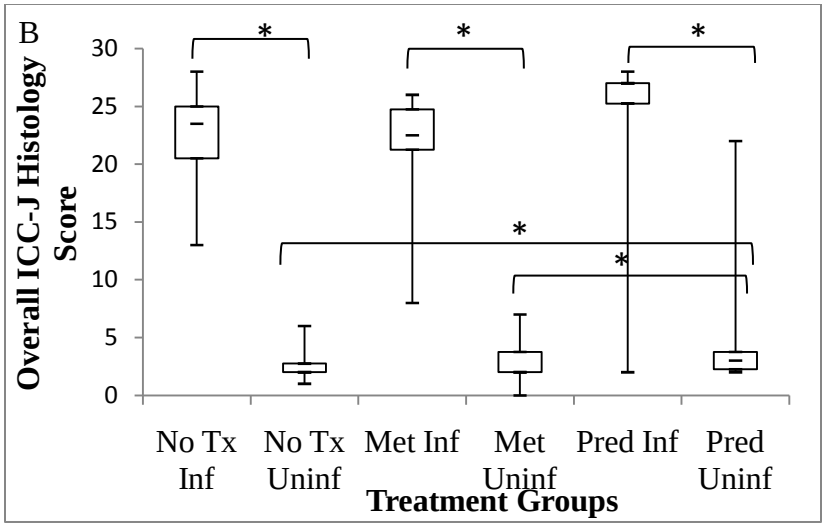


Figure 3.6. Overall histopathology scores of ileocecocolic junctions, stomach and spleen. (A) Histopathology scores of the ileolcecocolic (ICC) junctions of C57BL/6 IL-$10^{+/+}$ (WT), IL-$10^{+/-}$ (Het) and IL-$10^{-/-}$ (KO) mice receiving the high dose (75) of *T. muris* ova. Mice were scored on a standardized scale of 0-40. (B) Histopathology scores of the ileocecocolic (ICC) junctions of C57BL/6 IL-$10^{-/-}$ mice receiving 75 embryonated eggs and those concurrently treated with prednisolone or metronidazole. (C) Histopathology scores of stomach tissues of mice infected with 75 embryonated eggs compared to those treated concurrently with metronidazole or prednisolone and (D) Histopathology scores of splenic tissue of mice infected with 75 embryonated eggs compared to those treated concurrently with metronidazole or prednisolone. Asterisk (*) denotes data are statistically significant, compared to its respective control group with $p<0.05$. Cont = control/uninfected mice, early = refers to mice euthanized early/prior to day 38 and late refers to mice euthanized at days 38-40 post inoculation.

Figure 3.6 Continued





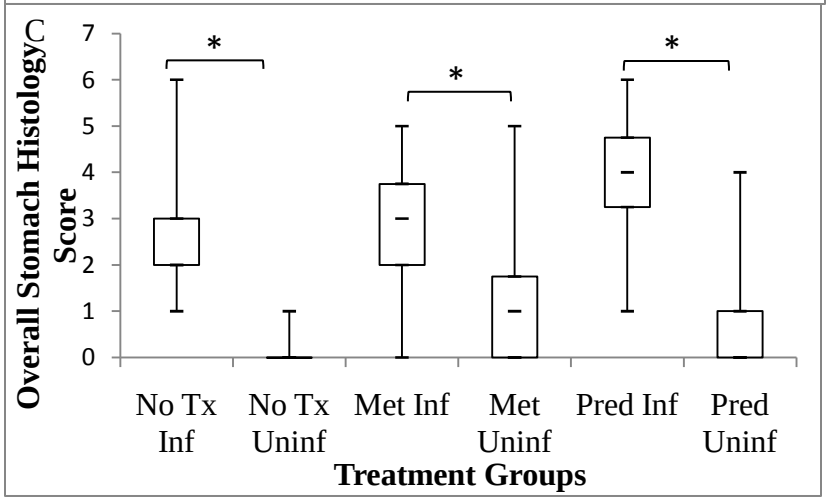


Figure 3.7. Histopathology of ileocecocolic junctions. Sections stained with Hematoxylin and Eosin. (A) IL-10$^{+/+}$ uninfected mouse, (B) IL-10$^{+/+}$ *Trichuris*-infected early end-point mouse receiving 75 embryonated eggs. Note submucosal edema (Ed) present under the epithelium. TE = *T. muris* eggs. (C) IL-10$^{+/+}$ *Trichuris*-infected late end-point mouse. Note mild hyperplasia of the villi. (D) IL-10$^{+/-}$ uninfected mouse. (E) IL-10$^{+/-}$ infected early end point mouse having received 75 embryonated eggs with mild villous hyperplasia (F) IL-10$^{-/-}$ *Trichuris*-infected mouse having received 75 embryonated *T. muris* eggs. Note submucosal edema (Ed) (G) IL-10$^{-/-}$ Uninfected mouse. (H) IL-10$^{-/-}$ early-end point *Trichuris*-infected mouse having received 75 embryonated eggs. Note large area of edema (Ed) and multifocal loss and distortion of epithelial architecture. Due to the significant expansion by inflammation, the mucosal layer extends beyond what could be captured in one photograph given that all micrographs are taken at the same magnification. (I) IL-10$^{-/-}$ infected late end-point mouse having received 75 embryonated eggs with mild villous hyperplasia, mild inflammatory cell infiltration of the mucosa, and goblet cell hyperplasia.

Figure 3.7 Continued

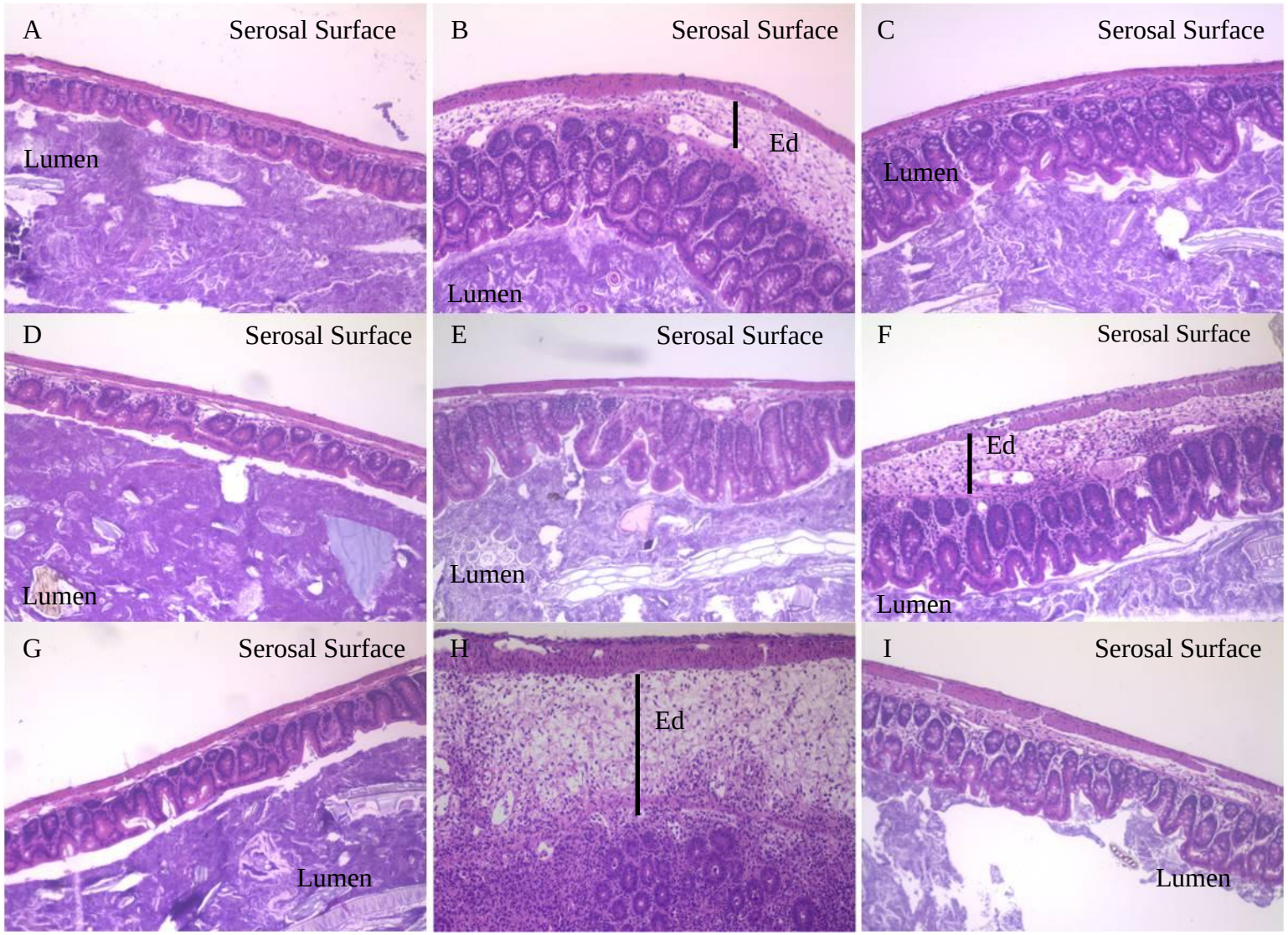


Figure 3.8. Histopathology of IL-10$^{-/-}$ mice infected with 75 embryonated *T. muris* eggs. Sections stained with Hematoxylin and Eosin. A. Neutrophilic exudates in the cecal lumen (40X) B. Thrombi (TH, arrow) in cecal tissue (10X) C. Thrombi (TH, arrow), Edema (ED, arrow) and *T. muris* worm (TM, arrow) in mucosa(10X)

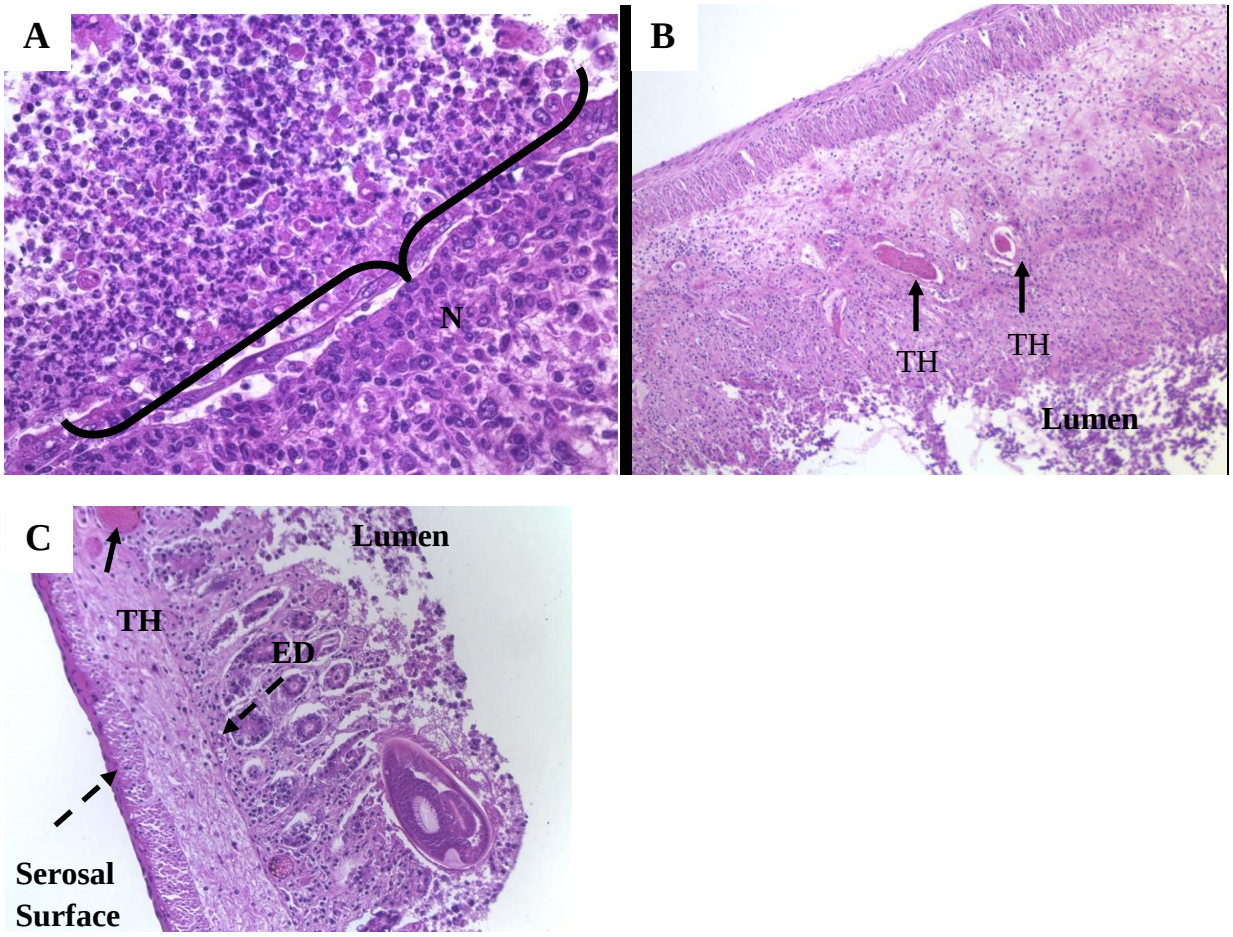
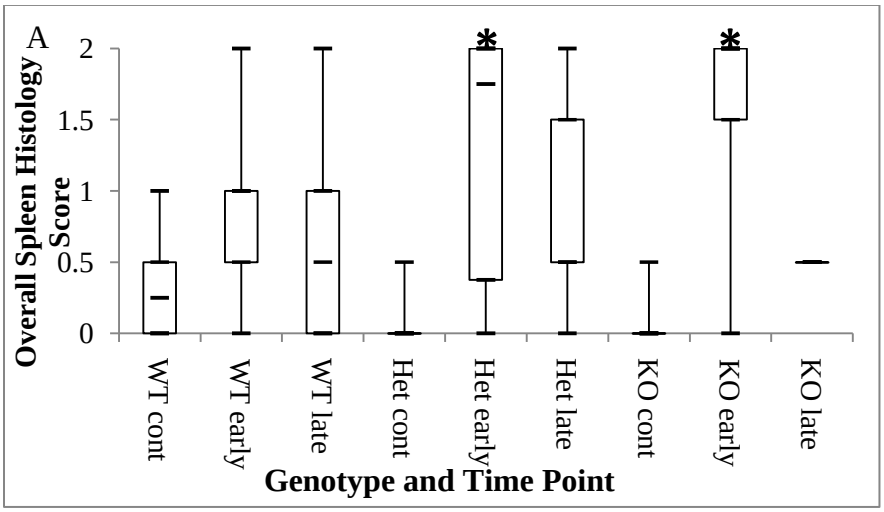


Figure 3.9. Spleen histopathology scores. Mice were given a score of 0-2 based on the presence of marked extramedullary hematopoiesis (EMH) and/or irregular periarteriolar sheaths (PALS). (A) Graph shows mice of all three genotypes (IL-$10^{+/+}$, IL-$10^{+/-}$ and IL-$10^{-/-}$) receiving the high dose (75 ova). Whiskers indicate maximum and minimum values. Boxes enclose data points falling within the second and third quartile. (B) Graph shows mice that were infected with 75 embryonated *T. muris* eggs and those that were concurrently treated with Metronidazole or Prednisolone. Asterisk (*) denotes data that is statistically significant from its respective control group with $p<0.05$



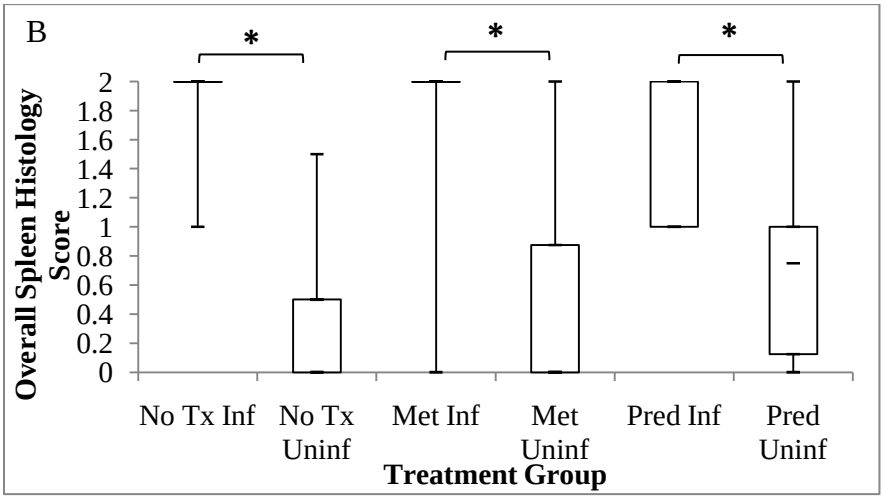


Figure 3.10. Histopathology of spleen tissue in Hematoxylin and Eosin stained sections. Panels A–I show histological changes in spleen according to group, (A) IL-$10^{+/+}$ uninfected mouse, (B) IL-$10^{+/+}$ infected early end-point mouse infected with 75 embryonated *T. muris* eggs. Note irregularly shaped and poorly defined PALS, (C) IL-$10^{+/+}$ infected late end-point mouse showing large irregularly shaped PALS infected with 75 embryonated *T. muris* eggs, (D) IL-$10^{+/-}$ uninfected mouse, (E) IL-$10^{+/-}$ infected early end point mouse infected with 75 embryonated *T. muris* eggs, (F) IL-$10^{-/-}$ mouse infected with 75 embryonated *T. muris*. Note small irregularly shaped PALS due to proliferation of cells in the rest of the spleen, (G) IL-$10^{-/-}$ uninfected mouse, (H) IL-$10^{-/-}$ early-end point infected mouse. Note large irregularly shaped and convalescing PALS, and (I) IL-$10^{-/-}$ infected late end-point mouse. Note large irregularly shaped PALS.

Figure 3.10 Continued

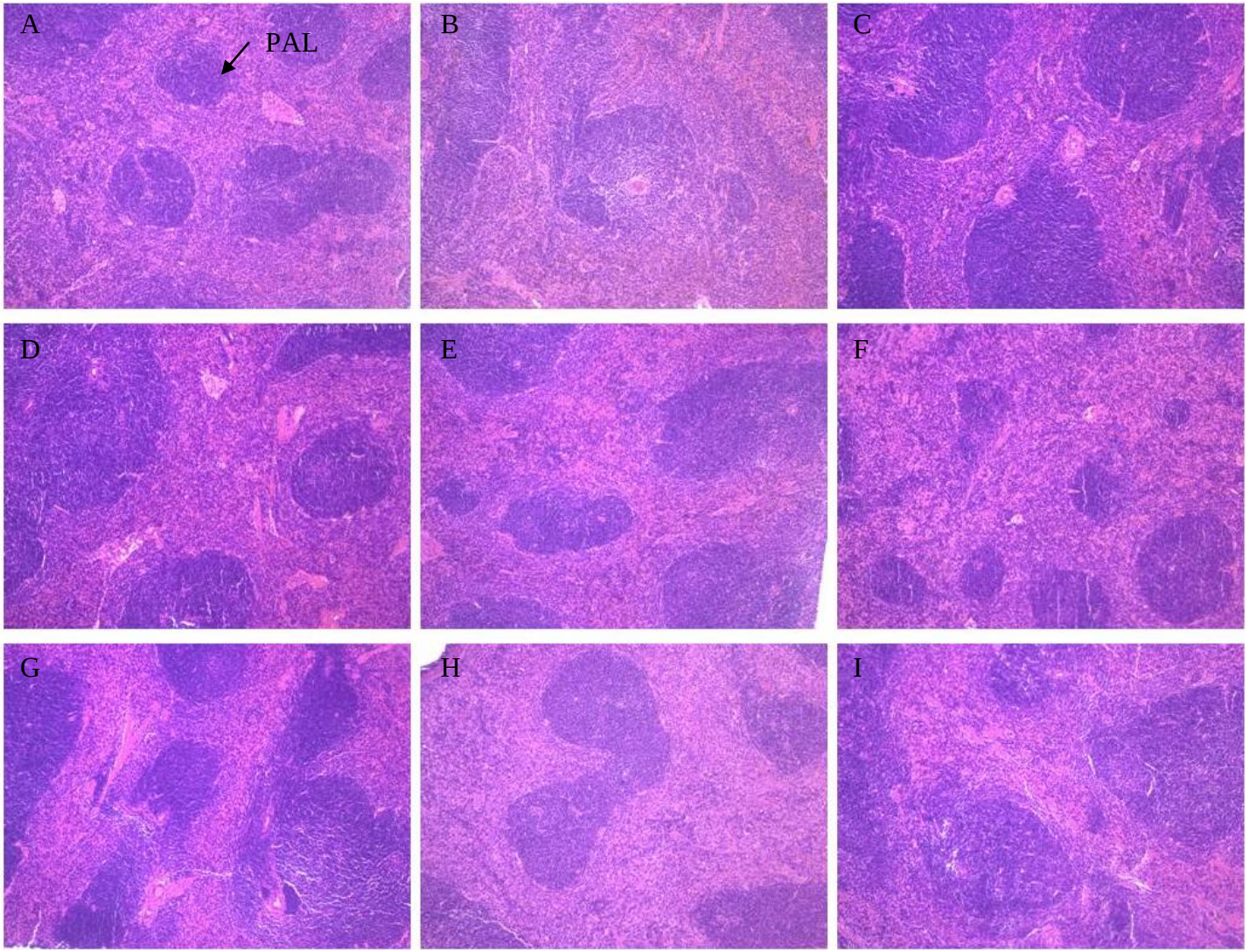


Figure 3.11. Evidence of *T. muris* outside of the GI tract in mice that were infected with 75 embryonated *T. muris* eggs. Sections stained with Hematoxylin and Eosin. (A) *T. muris* (TM, arrow) worm on the serosal side of the cecum in an IL-$10^{-/-}$ mouse (10x) (B) Cross-section of *T. muris* worms (TM, arrows) on serosal side in an IL-$10^{-/-}$ mouse (10x) (C) *T. muris* egg (TE, arrow) on serosal side, *T. muris* worm cross-sections in lumen of an infected IL-$10^{-/-}$ mouse.

Figure 3.11 Continued

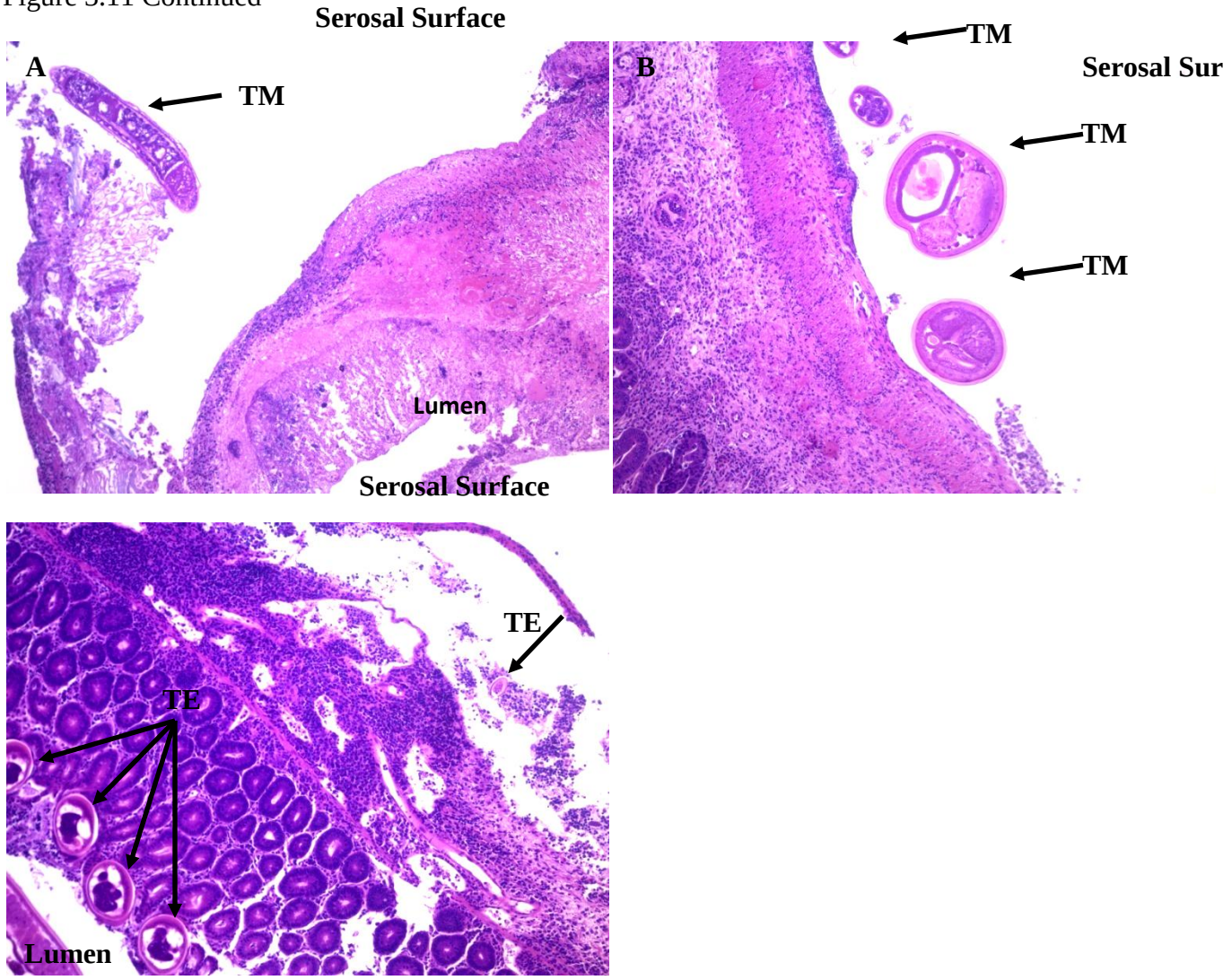


Figure 3.12. Extra-splenic bacteria identified in histopathologic sections from *T. muris* infected IL-10$^{-/-}$ mice receiving 75 embryonated *T. muris* eggs. Sections stained with hematoxylin and eosin (40X). (A) Section of spleen showing the serosal (to the left) from IL-10$^{-/-}$ uninfected mouse (B) marked inflammatory response (inf) within and exudates adherent to spleen capsule in an IL-10$^{-/-}$ mouse. (C) Inflammatory cells (inf) adherent to spleen capsule (D) Bacteria (Bact, arrow) and inflammatory (Inf, arrow) cells on splenic capsule.

Figure 3.12 Continued

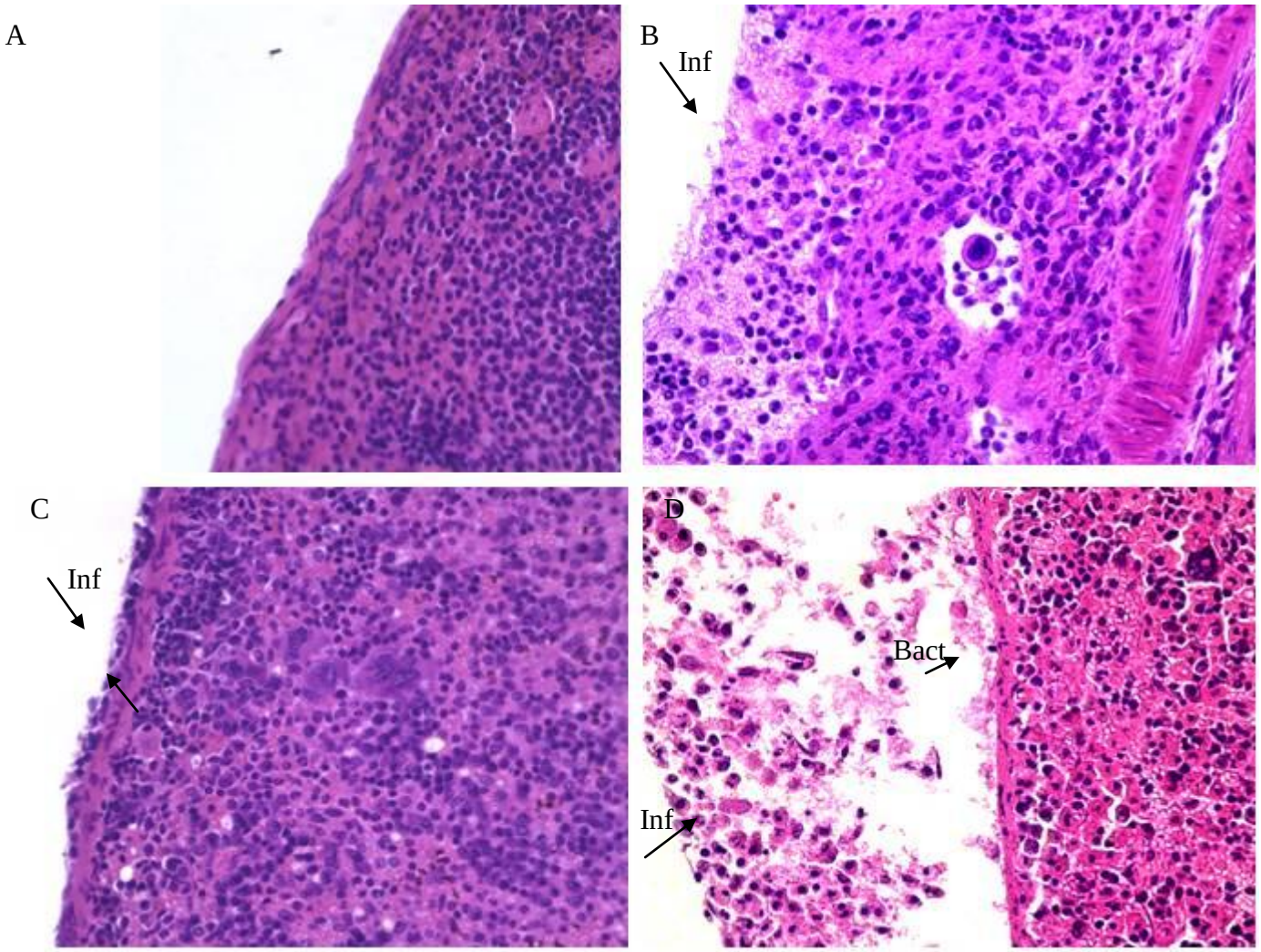


Figure 3.13. Extra-intestinal bacteria and inflammatory response in IL-10$^{-/-}$ mice infected with the high dose (75) of *T. muris*. Sections stained with hematoxylin and eosin. (A) Uninfected mouse small intestine (20X), (B) Cecum cross-section from infected mouse, note the presence of inflammatory cells present on serosal side. (20X), (C) Small intestinal cross-section from infected mouse. Note numerous amounts of bacteria (bact, arrow) present on serosal side (40X). (D) Small intestinal cross-section of infected mouse. Inflammation (Inf, arrow) present on the serosal surface of intestine.

Figure 3.13 Continued

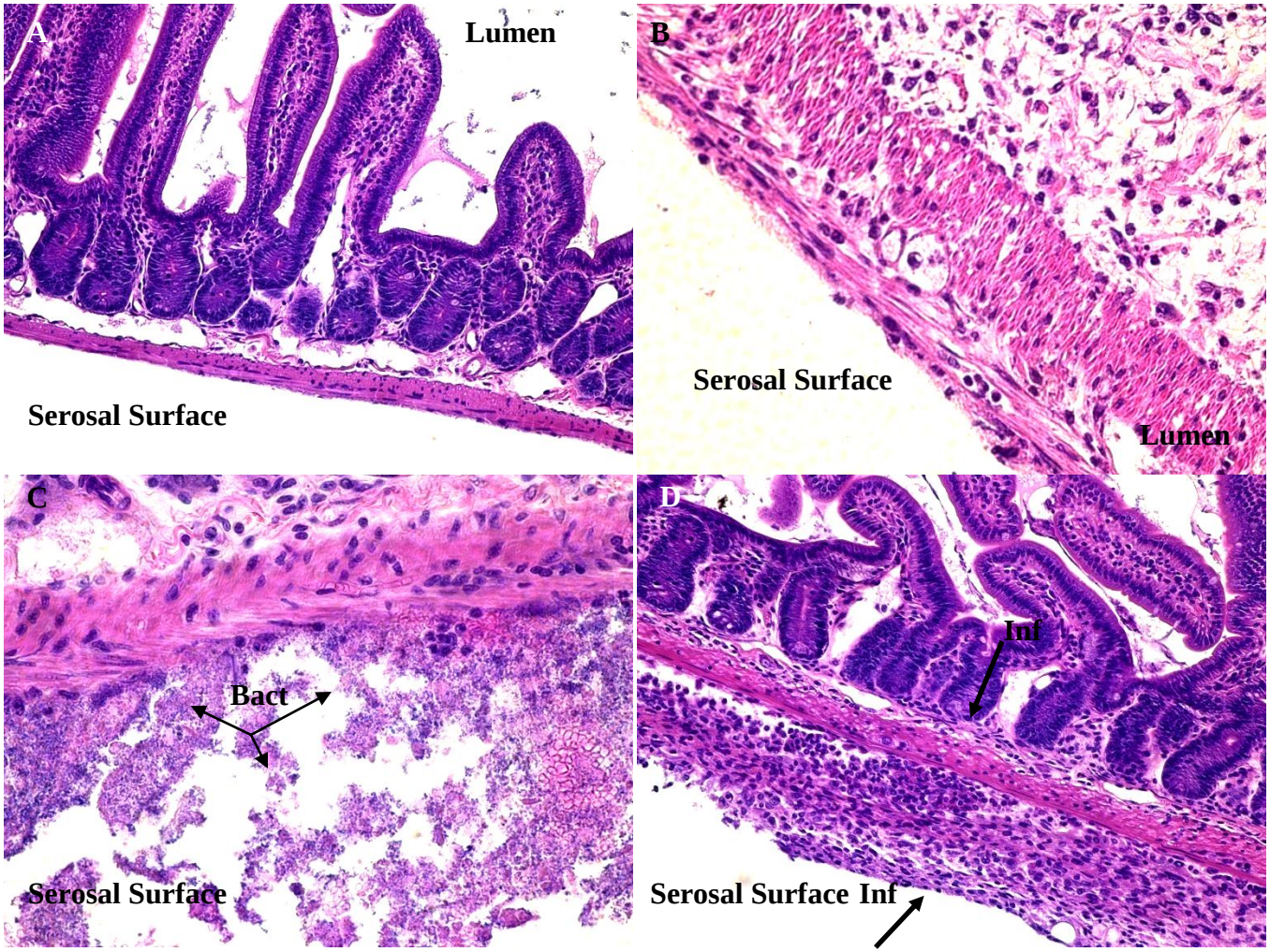
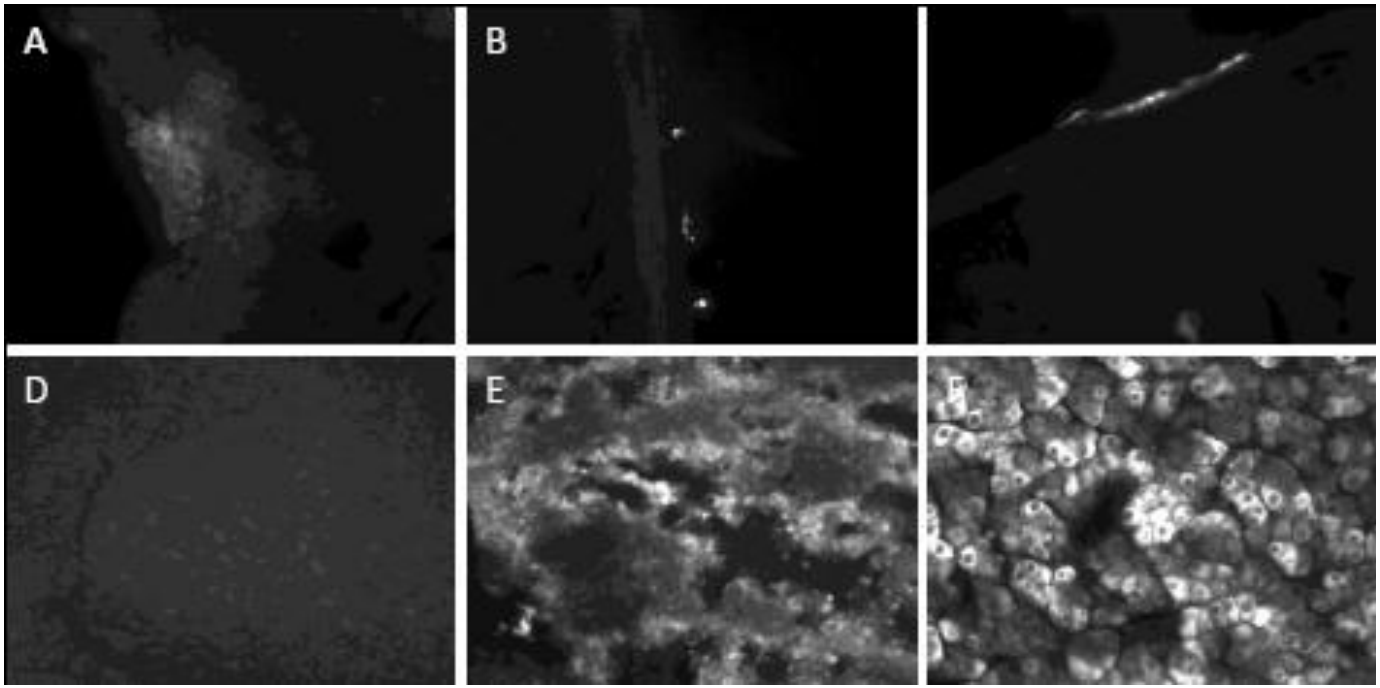


Figure 3.14. Fluorescence from 16S universal *in situ* hybridization confirms the colonization of extra-intestinal bacteria in some IL-$10^{-/-}$ mice infected with 75 embryonated *T. muris*. (A) Diffuse fluorescence intermixed with inflammatory cells on the serosal side of murine small intestinal tissue. (B) and (C) Fluorescence on the serosal side of murine small intestinal cross-sections. Murine tissues are outlined in pale grey fluorescence due to tissue autofluorescence. (D) Uninfected spleen tissue from an IL-$10^{-/-}$ mouse, (E and F) Spleen tissue from two IL-$10^{-/-}$ mice infected with the high dose of *T. muris* showing bacteria within the cells of the spleen.



CHAPTER 4

Assessments of Colon and Fecal Microbiota in a Murine Model

CHAPTER 4 SECTION 1

Further Assessment of Terminal Restriction Fragment Length Polymorphisms in the Study of Murine Gastrointestinal Microbiota.

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Prepared for Microbial Ecology.

ABSTRACT

As our understanding of the importance of gastro-intestinal (GI) microbiota and their impacts on various normal functions and disease processes of the human body increases, our need for further sophistication in culture-independent techniques also increases. The overarching goal of this thesis was to develop murine models to explore *Trichuris* as a therapy for Inflammatory Bowel Disease (IBD). Methods were needed to compare the microbial community richness and evenness in mice with and without *Trichuris muris* treatment. Here, we evaluate the use of Terminal Restriction Fragment Length Polymorphisms (TRFLP) to assess murine GI microbiota and show that fecal TRFLP operational taxonomic units (OTUs) are significantly different from full thickness proximal colon TRFLP OTUs taken from the same mouse at the same time-point. Additionally, we show that TRFLP OTUs from different locations within the murine proximal colon are more like TRFLP OTUs from other proximal colon samples within the same mouse regardless of the presence or absence of significant fecal material and where it was obtained from within the proximal colon. Finally, we assess the use of three different restriction enzymes, *Bsl*1, *Hae*III and *Msp*1, in TRFLP analyses and show that all three restriction enzymes yield similar snapshots of murine GI microbiota.

INTRODUCTION

Current research has noted that the composition and structure of an individual's gastrointestinal (GI) microbiota is affected by numerous disease or normal processes including energy/carbohydrate metabolism (Venema 2010), dietary fat (Hildebrandt, Hoffmann et al. 2009), obesity (Turnbaugh, Ley et al. 2006; Turnbaugh, Hamady et al. 2008; Turnbaugh and Gordon 2009), irritable bowel syndrome (Collins, Denou et al. 2009), Inflammatory Bowel Disease (Macfarlane, Blackett et al. 2009), specifically Crohn's disease (Baker, Love et al. 2009), and infectious disease processes (Stecher and Hardt 2008). Additionally, there is an increasing interest in the study and effect of the use of pro- and pre-biotics (Cox, Huang et al. 2010; Quigley 2010; Rijkers, Bengmark et al. 2010) on an individual's GI microbiota and in the prevention and amelioration of GI diseases.

An increasing interest in and demand to understand the dynamics of the GI microbiota has required community analysis methods that were improved beyond those that rely on traditional culture based techniques. Alternatives to culturing were needed due to both a large number of unculturable organisms (Head, Saunders et al. 1998) and the bias that culture-based techniques impose on community composition analyses (Tan, Reinke et al. 1996). In response to this need, several culture-independent techniques have been developed for analysis of microbial profiles, one of which is Terminal Restriction Fragment Length Polymorphisms (T-RFLP).

T-RFLP is considered to be a rapid, high-throughput, sensitive, culture-independent PCR-based technique (Marsh 1999; Nocker, Burr et al. 2007), that has been used frequently to study the GI microbiota (Li, Hullar et al. 2007) in humans and animals especially mouse models of human disease (Zhang, Zhang et al. 2009). The use of murine models especially inbred mice affords better control of experimental group sizes and environmental conditions and allows for

more invasive sampling techniques than are typically possible in human studies. Additionally, the use of murine models allows for increased ability to choose the sample technique and specimen that they would prefer to use, instead of relying on human samples taken for clinical procedures. Human samples, particularly those involving full thickness gastro-intestinal tissue verses fecal samples, are not readily available due to the invasive nature of sample collection techniques. Recently, it was noted that human fecal microbiota are significantly different than colonic mucosal associated bacteria (Eckburg, Bik et al. 2005). Here, samples were analyzed using biopsies from six different sites within the colon of healthy tissue of three human volunteers and fecal samples taken approximately one month after the tissue samples. They found that while their microbial analyses of the colon biopsies using 13, 355 prokaryotic ribosomal gene sequences within a patient did not reveal any major changes in the microbial communities, there were significant differences between the colon and fecal samples of an individual.

Restriction enzyme analysis provides the discrimination for TRFLP and the use of multiple restriction enzymes can increase the resolving power of the technique. With multiple restriction enzymes available, the question of which restriction enzyme will provide the highest level of discrimination for the bacterial population of interest or if this choice is even of major significance must be evaluated by the user (Schütte, Abdo et al. 2008). While *in silico* analysis tools are available to aid in the choice of a restriction enzyme (Marsh, Saxman et al. 2000), this outcome may vary based on their use in different communities, for example soil versus human GI samples.

In this study we compared TRFLP OTUs from murine fecal and full-thickness colon samples and determined that murine fecal TRFLP patterns are significantly different from full-

thickness colon samples taken from the same mouse at the same time-point. Additionally, within a mouse's proximal colon microbiota we determined that a colon sample from a mouse is more like another colon sample from the same mouse regardless of the presence or absence of significant fecal matter within that portion of the GI tract or the position within the mouse's proximal colon. Finally, we compared the TRFLP results from three restriction enzyme digests: *Bsl*1, *Hae*III and *Msp*I, and found that all three restriction enzyme digests produced comparable characterizations of murine GI microbial community composition.

MATERIALS and METHODS

Mouse Breeding and Handling

All animal protocols were approved by the Michigan State University (MSU) Institutional Animal Care and Use Committee (IACUC) and complied with National Institutes of Health guidelines (04/07-044-00). To limit the exposure of the mice to extraneous microorganisms and to increase the likelihood that the murine GI microbiota would not be unintentionally influenced by outside parameters, the handling, diet/feeding, cage changing and screening for enteric pathogens were standardized. Inbred C57BL/6J mice (C57BL/6) were obtained from Jackson Laboratories (Bar Harbor, ME), and a breeding colony was established in a *Campylobacter/Helicobacter*-free facility. Mice were housed in pathogen-free conditions using a FlexAir ventilated mouse rack (Alternate Design Manufacturing & Supply Inc., Siloam Spring, AR), fed an irradiated mouse diet (mouse breeder diet 7904; Harlan Teklad, Indianapolis, IN), kept on autoclaved bedding and given filter sterilized water (autoclaved water in bottles for weanlings) in an MSU limited-access room. PCR assays obtained from Jackson Laboratories were used to confirm mouse genotypes (http://jaxmice.jax.org/pub-cgi/protocols/protocols.sh?objtype=protocol &protocol_id=346) both before and after experiments. Experimental mice were screened for *Campylobacter* spp (Linton, Owen et al. 1996)., *Helicobacter* spp. (Riley, Franklin et al. 1996), *Citrobacter rodentium* (McKeel, Douris et al. 2002) and *Enterococcus faecalis* (Dutka-Malen, Evers et al. 1995) by 16S rRNA gene PCR assay for *Campylobacter* spp. and *Helicobacter* spp., *espB* gene-specific PCR for *C. rodentium*, and *ddl* gene-specific PCR for *Enterococcus faecalis* using DNA isolated from fecal samples both prior to and at the conclusion of the experiment.

A dedicated sentinel mouse was used to monitor for bacterial, protozoan and viral agents through the MSU Laboratory Animal Resources Facility (ULAR). Additionally, we monitored the mouse colony for the incidence of spontaneous colitis by examining euthanized retired breeding mice and those euthanized for other reasons for enlargement of the proximal colon, cecum, ileocecocolic lymph node and spleen.

Necropsy and Sample Collection

Mice were humanely sacrificed by CO_2 overdose according to the American Veterinary Medical Association (AVMA) guidelines (2001) and weighed. The GI tract was removed in its entirety and gross pathological changes were noted. The distal most fecal pellet was removed, suspended in Tryptic Soy Broth (TSB) with 15% glycerol and frozen at -80^{o}C. The proximal colon was removed and four segments, approximately one cm in length, were removed and snap frozen on dry ice. Sections were taken at the same anatomic sites two of which contained significant fecal contents and two lacked any significant fecal contents.

DNA Extraction and Terminal Restriction Fragment Length Polymorphisms (TRFLP).

DNA was extracted from frozen proximal colon tissue and frozen fecal samples using a DNeasy tissue kit (QIAgen, Valencia, CA) in accordance with the manufacturer's instructions. Each DNA sample was used as the template for the 16S rRNA gene universal primers 5'-fluorescent (6-FAM)'-TGCCAGCAGCCGCGGTA-3' (516f) and 5'-GGTTACCTTGTTACGACTT-3' (1510r) using the reaction mixture described by Nagashima *et al* (Nagashima, Hisada et al. 2003) with the following modifications: (1) The total amount of

sample DNA was increased to 400 ng per 50 µl reaction, (2) 25 pm of primers were used per 50 µl reaction, (3) the annealing time was increased to 60 seconds and (4) the elongation time was increased to 120 seconds. Two 50 µl reactions were combined for each mouse. The combined PCR product was then digested with *Bsl*1 for mouse fecal and colon studies. For the restriction enzyme comparison study the combined product was then digested with either *Bsl*I, *Msp*I or *Hae*III per the manufacturer's instructions (New England Biolabs). All digested samples were purified using QIAQuick PCR Purification Columns (QIAgen, Valencia, CA). Samples were submitted to the MSU Center for Microbial Ecology for TRFLP analysis using an ABI GeneScan Analysis System.

Statistical Analysis

TRFLP peaks with a height of 50 fluorescent units or greater were included in the analyses and reported as fragment size Operational Taxonomic Units (OTUs). For restriction enzyme combination analyses the OTUs from each independent restriction enzyme digest were analyzed together. PAST software was utilized for multivariate ANOSIM analysis and cluster analyses for visual representation (Hammer, Harper et al. 2001). Samples were analyzed comparing both presence versus absence and the relative abundance of OTUs using Jaccard and Bray-Curtis similarity indices, respectively. N was equal to 10,000 permutations for all analyses. In ANOSIM, the statistical null hypothesis that samples were derived from the same community was rejected if $P \leq 0.05$.

RESULTS

Microbial communities were significantly different in fecal samples than in colon samples when compared using Terminal Restriction Fragment Length Polymorphisms (TRFLPs). Fecal and colonic microbial communities were analyzed using TRFLP OTUs and both Bray-Curtis and Jaccard beta diversity indices. ANOSIM cluster analyses are shown in Figure 4.1. Both of these analyses showed that colon TRFLP OTUs were more similar to other colon TRFLP OTUs than to fecal TRFLP OTUs from the same mouse that were obtained at the same sampling time.

Intra-individual colonic microbial communities were similar, regardless of location or the presence or absence of fecal contents within the colon. Four colon samples from each mouse were taken from three mice at necropsy, two containing fecal contents and two without any significant fecal material. The sampled microbial communities were compared based on mouse ID, fecal contents versus no contents, and location in the proximal colon where the sample was taken. The only consistent comparison that was similar based on the statistical significance ($p \leq 0.05$) was that comparing samples based on the mouse that they were obtained from with no regard to contents or lack of contents) or location within the colon (Figure 4.2). Using Jaccard analyses of *Msp*I restriction enzyme digests, significance was found between the samples with contents verses those without contents.

Use of different restriction enzyme digests and combinations of fragments from these digests did not significantly increase the sensitivity of the TRFLP analysis. TRFLP OTUs

were compared from three different restriction enzyme digests, using both Bray-Curtis and Jaccard analyses (Table 4.1). Additionally, each combination of the TRFLP OTUs (*Hae*III-*Bsl*I, *Hae*III-*Msp*I, *Msp*I-*Bsl*I and *Hae*III-*Bs*lI-*Msp*I) were evaluated to determine if the additional fragments from other restriction digests would increase the sensitivity of the analysis under question. Using Bray-Curtis analyses, there were some differences in the P (same) and R values between the different analyses of individual restriction enzymes and their combinations. These differences were minor and all of the restriction enzyme conditions tested still showed significant differences between the colon samples from different mice regardless of their fecal content or the fact that the mice were inbred and considered to be congenic. However, using Jaccard analyses, some differences were apparent among the enzyme combinations. Specifically, *Hae*III and the *Hae*III-*Bsl*I results were significantly less sensitive and *Msp*I results were significantly more sensitive when compared to the other restriction enzymes and combinations of restriction enzymes used.

DISCUSSION

In this study we showed that murine fecal TRFLP OTUs are more like other murine fecal TRFLP OTUs than colon TRFLP OTUs that were obtained from the same mouse at the same collection time. In human studies, fecal samples are often used in the place of gastrointestinal samples due to sampling difficulties with human tissues. This is not necessary in end-point analyses used in murine studies, and based on these data should not be considered an equivalent substitute. One explanation for the differences between fecal and colon bacterial communities seen here could be that mice may have a unique population of mucosal-associated bacterial populations that would not be present in significant quantities in fecal samples. In 2005, Eckberg et al (Eckburg, Bik et al. 2005) reported that they noted significant differences between tissue biopsies that were taken from three patients during colonoscopies and fecal samples taken one month later. More recently, microbial community analyses from samples of seven individual's rectal mucosa and feces were compared. Here, they also noted that the microbiota of an individual's fecal sample was significantly different from rectal samples obtained from the same individual (Durbán, Abellán et al. 2010). They acknowledge some of the potential challenges that are encountered when obtaining human samples for the goal of comparing them to fecal samples. One main issue is the requirement for endoscopy to obtain colon samples. This procedure potentially creates sampling problems due to the necessary pre-bowel cleansing which could significantly disturb an individual's GI microbiota. These sampling issues are not encountered when collecting (end-point) murine samples and thus do not play into our analyses. Given that DNA extractions, PCR assays, restriction enzyme digestions, post-digestion cleanups and submissions for genescan TRFLP analyses were done simultaneously for all of the samples it is unlikely that the differences noted were due to technical differences within the methodology.

Furthermore, we were able to show that colon samples from the same mouse, regardless of the presence or absence of significant fecal matter, were more like other samples from that same mouse than samples from other mice. This further supports the observation that a significant amount of a unique population of bacteria is associated with the colonic tissue itself, which is documented by comparison to the microbiota of fecal material from the same mouse. This is important to investigators studying the microbial composition of the murine gastro-intestinal tract because the presence-or absence of significant fecal material in the colon at the time of necropsy cannot be controlled. In fact, most disease processes affecting this region cause changes in the volume, consistency and presence/absence of feces in the distal colon. This work demonstrates that samples taken anywhere within the proximal colon harbor similar microbial communities when compared to any other samples taken within the proximal colon of the same mouse. It should be noted that there were no signs of gross-pathology or inflammation in these mice, the presence or absence of which could impact the microbial communities in different samples.

In these studies, the robustness of TRFLP results arising from several different restriction enzyme (RE) digestions was compared. Here, either a single RE digest or a combination of REs were used to determine the community OTUs. These comparisons showed that there were not many significant effects of choice of enzyme(s) on the microbial community diversity analyses. These results do appear to show that when using a more quantitative analysis such as Jaccard that *Hae*III produced results that were less sensitive and that *Msp*I is potentially more sensitive in detecting differences in microbial communities. However, no significant differences in community composition based on TRFLP were noted using a qualitative analysis such as Bray-Curtis. This knowledge of the effects of RE choice allows for more flexibility of use with the

technique and also helps to better inform the user of their potential enzyme choices. The repeatability between different enzymes and combinations of enzymes also provides further support for the robustness and repeatability of this technique for use in analyzing murine GI microbial communities.

TABLES

Table 4.1

Two-way ANOSIM results Bray Curtis, Factor B (Mouse ID)

	*Msp*I	*Bsl*I	*Hae*III	*Msp*I-*Bsl*I	*Msp*I-*Bsl*I-*Hae*III	*Msp*I-*Hae*III	*Hae*III-*Bsl*I
R	0.63889	0.6542	0.611	0.6944	0.58333	0.63889	0.52778
P(same)	0.013	0.0186	0.0072	0.0128	0.0098	0.0134	0.0135

Two-Way ANOSIM results Bray Curtis, Factor C (Fecal contents/No fecal contents)

	*Msp*I	*Bsl*I	*Hae*III	*Msp*I-*Bsl*I	*Msp*I-*Bsl*I-*Hae*III	*Msp*I-*Hae*III	*Hae*III-*Bsl*I
R	0.083333	0.25	0.5	0.333333	0.16667	0.16667	0.1667
P(same)	0.3384	0.1134	0.0754	0.0715	0.2243	0.2196	0.2236

Two-way ANOSIM results Jaccard, Factor B (mouse ID)

	*Msp*I	*Bsl*I	***Hae*III**	*Msp*I-*Bsl*I	*Msp*I-*Bsl*I-*Hae*III	*Msp*I-*Hae*III	***Hae*III-*Bsl*I**
R	0.611	0.375	**0.34722**	0.6944	0.555	0.6111	**0.2778**
P(same)	0.0176	0.0083	**0.1043**	0.0123	0.0088	0.0086	**0.1395**

Two-way ANOSIM results Jaccard, Factor C (fecal contents/no fecal contents)

	***Msp*I**	*Bsl*I	*Hae*III	*Msp*I-*Bsl*I	*Msp*I-*Bsl*I-*Hae*III	*Msp*I-*Hae*III	*Hae*III-*Bsl*I
R	**0.41667**	0.333	0.1667	0.333	0.333	0.5	0.1667
P(same)	**0.0351**	0.2176	0.332	0.0763	0.772	0.0731	0.2512

FIGURES

Figure 4.1 A Jaccard cluster analyses of TRFLP OTUs from Fecal and Colon samples obtained from four mice. Both analyses show distinct differences between the colon and fecal microbial populations. ANOSIM R = 0.599 p(same) = 0.0285

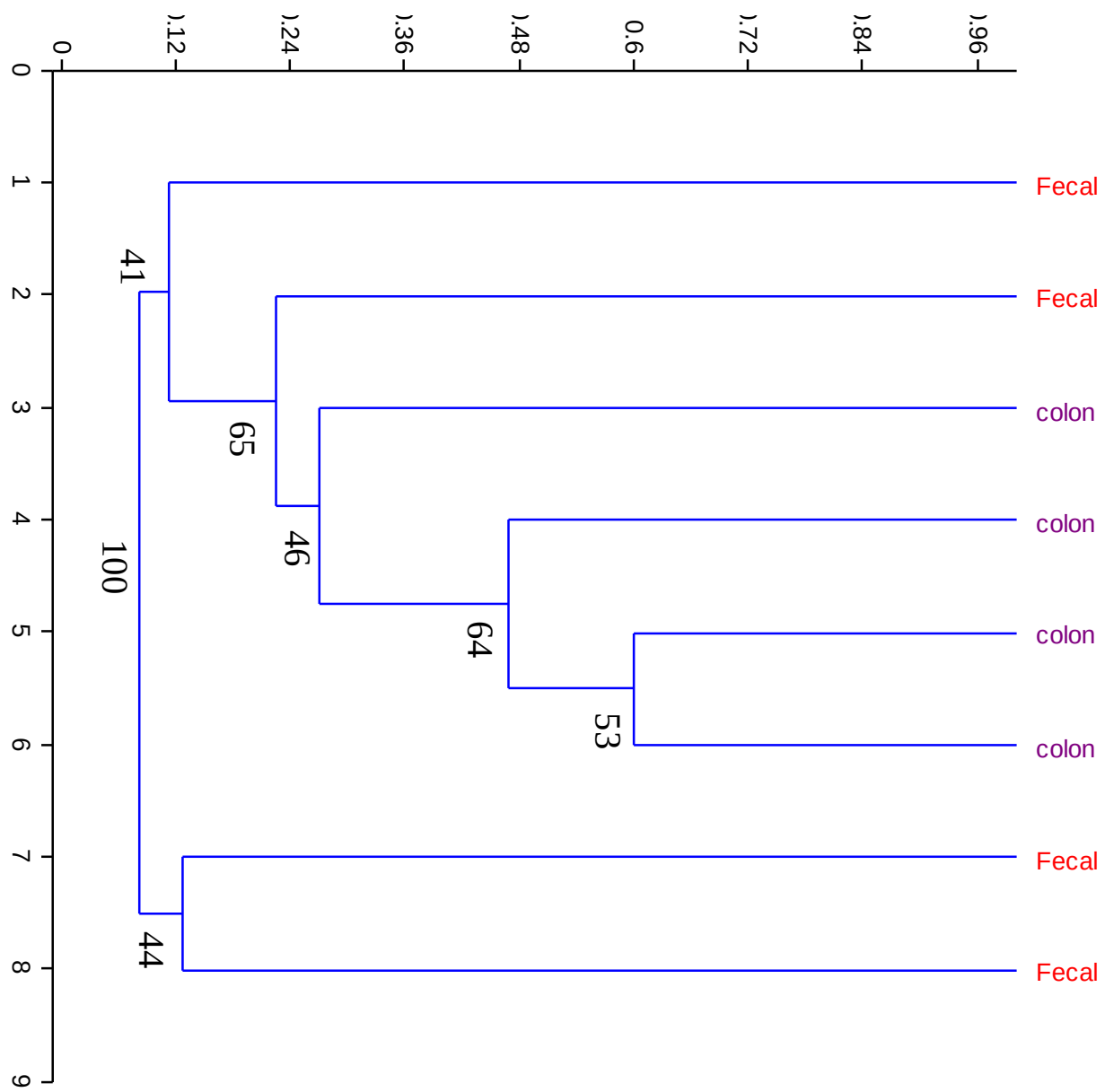


Figure 4.1 B Continued Bray Curtis cluster analyses of TRFLP OTUs from Fecal and Colon samples obtained from four mice. Both analyses show distinct differences between the colon and fecal microbial populations. ANOSIM R=0.5885 p(same)=0.029

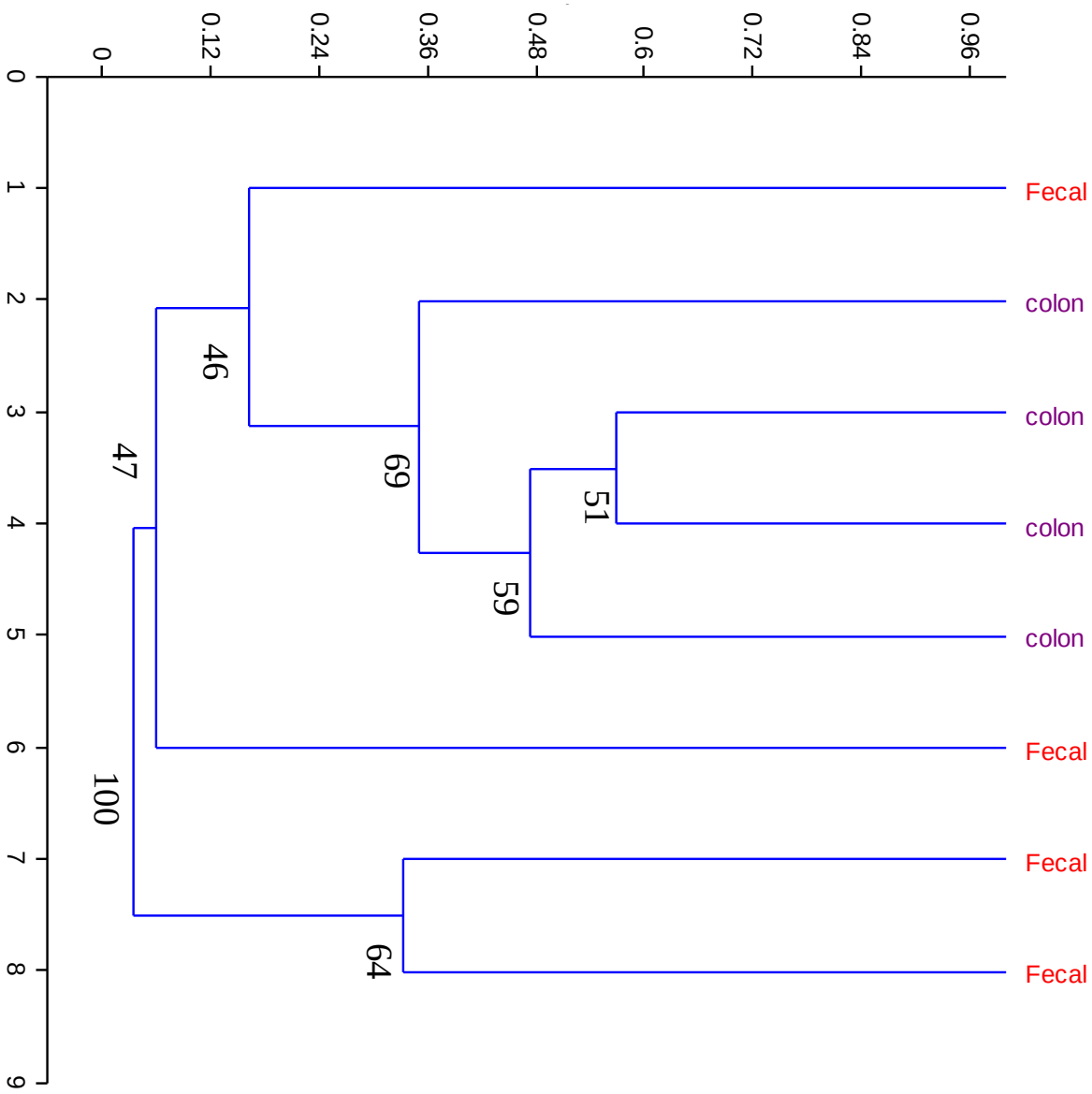


Figure 4.2 A Jaccard and Bray Curtis cluster analyses of TRFLP OTUs from proximal colon samples obtained from three mice (Mouse 1, Mouse2 and Mouse3). Samples either had significant fecal contents (C) or lacked significant fecal samples (N). Two samples were taken with and without significant fecal contents (designated by the number preceding the decimal (.) point. Example Mouse 2 sample 1 with fecal contents = Mouse2.1C, mouse 2 sample 2 with fecal contents = Mouse2.2C. ANOSIM Comparing Mice: R=0.375, P(same)=0.01. ANOSIM Comparing Contents vs. No contents: R=0.33, P(same)=0.2263

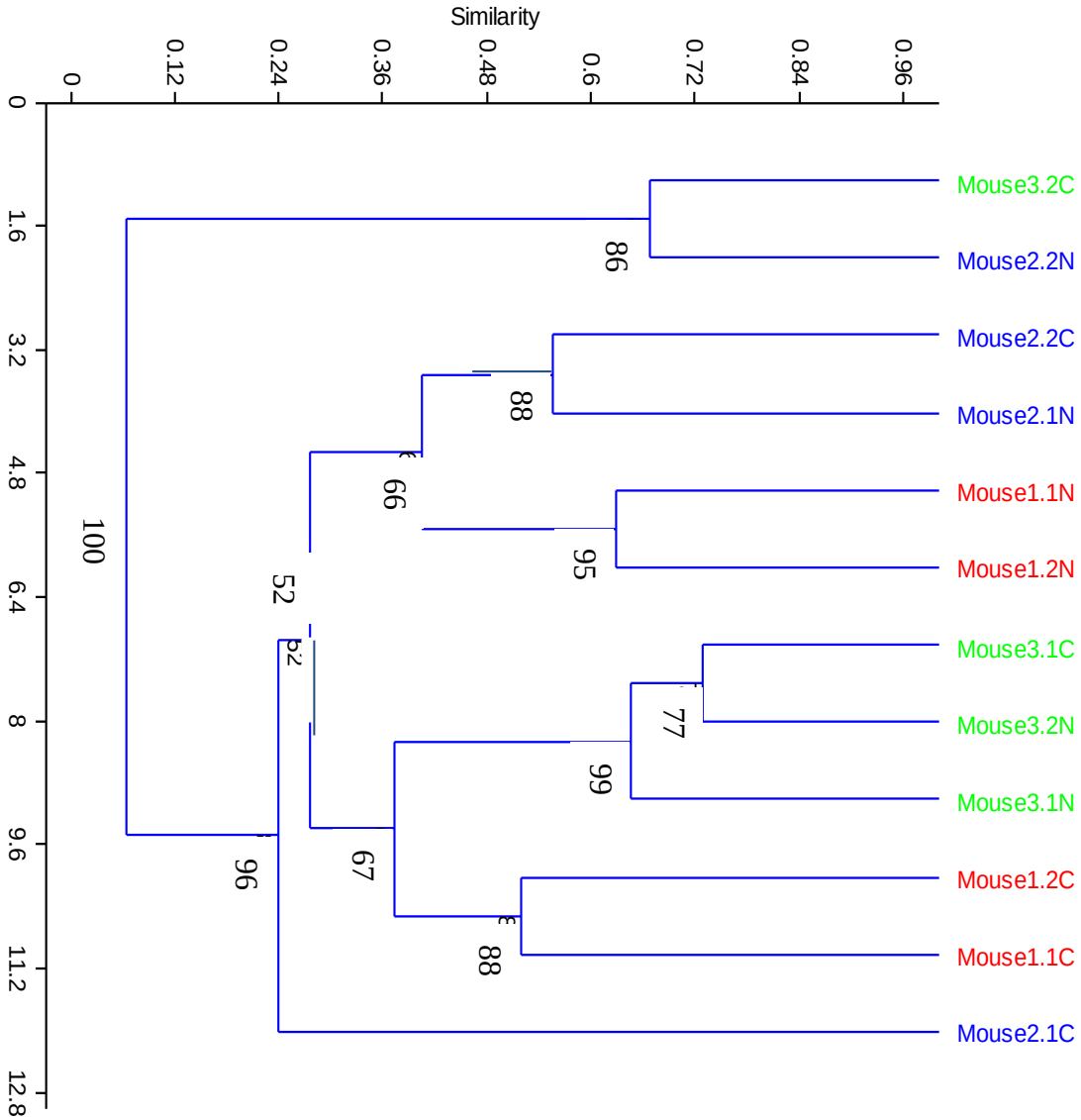
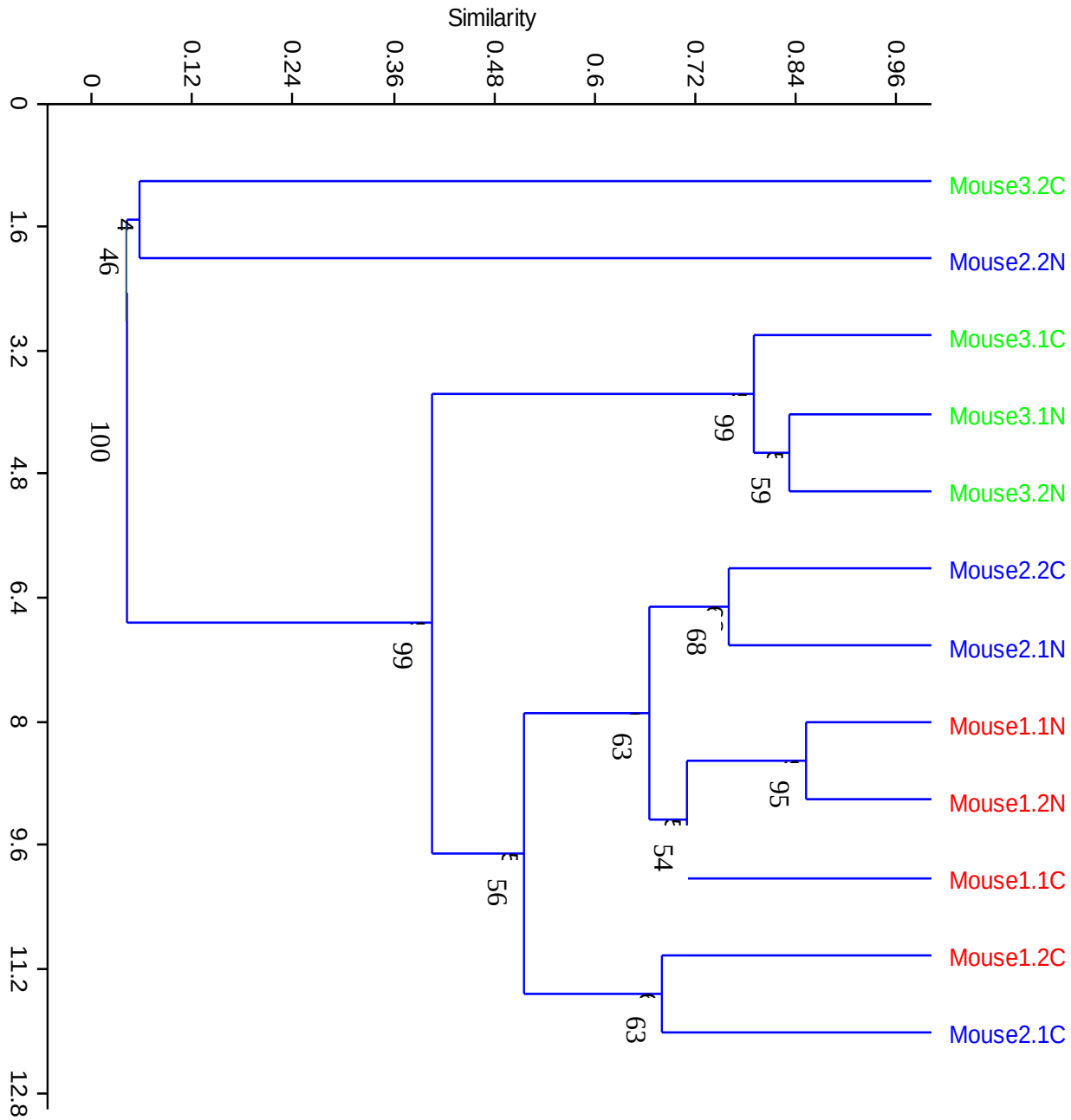


Figure 4.2 B Continued



CHAPTER 4 SECTION 2

The effects of parents and individual caging on fecal microbiota in a mouse breeding colony

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Prepared for Microbial Ecology

ABSTRACT

The impact of composition and function of the gastrointestinal (GI) microbiota is of increasing interest with the acknowledgement of its importance in gastrointestinal disease, digestive functions and nutrient absorption, immune function and obesity. However, the variation in GI microbiota observed between individuals has obscured differences between those in different treatment groups when studying the GI microbiota of humans To examine this in a model system, we hypothesized that mice have microbiota more similar to their siblings than to microbiota of other mice. In this study we have looked at the fecal microbiota of five (5) litters of inbred congenic mice from two breeding pairs over a period of approximately five (5) weeks. To examine the effect of environment, some of the litters were divided into individual cages, while others were left in group housing with their littermates. Based on fecal pellet sampling over time, we found that a mouse's GI microbial composition most closely resembled other mice born to the same parents throughout the course of the experiment. This remained true regardless of which litter they originated from and whether or not they remained with other littermates or were housed individually. Nevertheless, we found that when litters were split and mice were distributed into individual cages their fecal microbiota changed by the 7 week end of the experiment so that it no longer closely resembled that of their littermates. These results shed light into the effect of family versus environment on an individual's gastrointestinal microbiota.

INTRODUCTION

In recent years, there has been an increase in interest in the role that the human gastro-intestinal (GI) microbiota plays in multiple normal and disease associated processes including those that involve organs outside the GI tract. Some examples of these include: nutrient metabolism (Venema 2010), obesity (Turnbaugh, Ley et al. 2006; Turnbaugh, Hamady et al. 2008; Turnbaugh and Gordon 2009), amount of dietary fat (Hildebrandt, Hoffmann et al. 2009), immune function (Stecher and Hardt 2008), Inflammatory Bowel Disease (Macfarlane, Blackett et al. 2009), Irritable Bowel Syndrome (Collins, Denou et al. 2009) and antibiotic-associated diarrhea (Young and Schmidt 2004).

As our understanding of the importance and significance of an individual's GI microbiota increases, an interest in pre- and pro-biotics has grown as well as understanding of the effects of an individual's diet on the stability of the microbial community. Many studies have analyzed or attempted to assess changes that occur in an individual's GI microbiota after using pre- or pro-biotics (Gibson et al 1994, Antoine 2010). Additionally, studies are beginning to look at the effects of specific food stuffs consumed on an individual's GI microbiota, including items such as coffee (Jaquet, Rochat et al. 2009) and whole-grains (Costabile, Klinder et al. 2007). Unfortunately, when measuring richness and evenness of the GI microbiota investigators have found significant variation between results from individual humans which makes detecting changes or shifts in GI microbiota due to specific treatments more difficult (Eckburg, Bik et al. 2005; Ley, Turnbaugh et al. 2006; Turnbaugh and Gordon 2009). These preliminary observations serve, in part, to demonstrate how many environmental factors can influence the composition of an individual's microbiota, but make studying the GI microbiota of individuals and groups difficult, due to wide variability between people. This random individual variability

is thought to mask and make it difficult to detect potential changes due to treatment, in the GI microbiota of humans in studies.

Here, we model this situation and used terminal restriction fragment length polymorphism (T-RFLPs) analysis to assess the fecal microbiota of C57BL/6 IL-$10^{-/-}$ mice over time with the goal of evaluating the effects of parents, siblingship, littermates, and individual housing on the GI microbiota richness and evenness outcomes of the mice under study. We found that overall the variable that had the greatest effect on producing similar GI microbiota between mice was being from the same breeding pair (having the same parents) regardless of which litter from which they originated. We also found that when individual litters were split into individual cages their once similar microbiota tended to drift and become more dissimilar, despite the fact that their diets, bedding, water and other environmental influences remained similar. Despite the drifting of microbiota between litters that were split into individual cages, overall they were still more similar to mice from other litters born to the same parents than to mice originating from different parents after 7 weeks of study.

MATERIALS and METHODS

Mouse Breeding and Handling

All animal protocols were approved by the Michigan State University (MSU) Institutional Animal Care and Use Committee (IACUC) and complied with National Institutes of Health guidelines (04/07-044-00). To limit the exposure of the mice to extraneous microorganisms and to increase the likelihood that the murine GI microbiota would not be unintentionally influenced by outside parameters, the handling, diet/feeding, cage changing and screening for enteric pathogens etc. were standardized as described previously (Mansfield, Bell et al. 2007). Briefly, B6.129P2-IL-10^{tm1Cgn}/J (referred to below as C57BL/6 IL-10$^{-/-}$) mice were obtained from Jackson Laboratories (Bar Harbor, ME) and a breeding colony was established in a colitogenic bacteria free facility. Mice were housed in pathogen-free conditions using a FlexAir ventilated mouse rack (Alternate Design Manufacturing & Supply Inc., Siloam Spring, AR), fed an irradiated mouse diet with 11% fat (mouse breeder diet 7904; Harlan Teklad, Indianapolis, IN), kept on autoclaved bedding and given filter sterilized water (autoclaved water in bottles for weanlings) in an MSU limited-access room. PCR assays obtained from Jackson Laboratories were used to confirm mouse genotypes ((http://jaxmice.jax.org/pub-cgi/protocols/protocols.sh?objtype=protocol &protocol_id=346) both before and after experiments. To prevent typhlocolitis from other causes, experimental mice were screened for for *Campylobacter* spp., (Linton, Owen et al. 1996) *Helicobacter* spp., (Riley, Franklin et al. 1996) *Enterococcus faecalis*, (Dutka-Malen, Evers et al. 1995) and *Citrobacter rodentium* (McKeel, Douris et al. 2002) by 16S rRNA gene PCR assay for *Campylobacter* spp. and *Helicobacter* spp., *espB* gene-specific PCR for *C. rodentium*, and *ddl* gene-specific PCR for

Enterococcus faecalis using DNA isolated from fecal samples both prior to and at the conclusion of the experiment.

Dedicated C57BL/6 IL-10$^{-/-}$ sentinel mice were used to monitor for bacterial, protozoan and viral agents through the MSU Laboratory Animal Resources Facility (ULAR). Additionally, we monitored euthanized retired breeding mice and those euthanized for other reasons in the mouse colony for the incidence of spontaneous colitis by necropsy and examination for other reasons for enlargement of the proximal colon, cecum, ileocecocolic lymph node and spleen. Feces from mice that exhibited signs of colitis were screened for the presence of known colitis causing bacteria *Campylobacter* spp., *Helicobacter* spp., *Enterococcus faecalis*, and *Citrobacter rodentium.* 16S rRNA gene PCR assay was used to detect *Campylobacter* spp. and *Helicobacter* spp., *espB* gene-specific PCR to detect *C. rodentium,* and *ddl* gene-specific PCR to detect *Enterococcus faecalis* all using DNA isolated from proximal colon samples as described below (Mansfield, Bell et al. 2007).

Mouseek software was used to track the relationships of all mice in the breeding colony and on study. Mice were identified by their breeding pair and litter number, and prior to the beginning of the experiment groups of mice were randomly assigned to be split into individual cages, or to be kept housed in groups (Table 4.2). Throughout the experiment individual mice were identified by their cage cards and corresponding ear punches.

Experimental Design

As shown in Table 4.2, mice were randomly assigned prior to the beginning of the experiment to be housed with their original same-sex liter mates, or to be split into individual cages. Two one pellet fecal samples were collected one week prior to the designated date for splitting of their cages. Unfortunately, some mice were lost due to unexpected death, and this is noted in Table 4.2 as well. Fecal samples were collected bi weekly for a total of 9 samples

Fecal Sample Collection

One-pellet fecal samples were collected at a total of nine time points throughout the course of the experiment. Samples were immediately homogenized in Tryptic Soy Broth (TSB) with 10% glycerol and frozen at -80^{o}C for DNA extraction at a later time.

DNA Extraction and Terminal Restriction Fragment Length Polymorphisms (T-RFLP) analysis.

DNA was extracted from frozen proximal colon tissue samples using the fecal DNA extraction protocol with the DNeasy tissue kit (QIAgen, Valencia, CA). Each DNA sample was used as the template for the 16s rRNA gene universal primers 5'-fluorescent (6-FAM)'-TGCCAGCAGCCGCGGTA-3' and 5'-GGTTACCTTGTTACGACTT-3' using the reaction mixture described by Nagashima *et al* (Nagashima, Hisada et al. 2003) with the following modifications: (1) The total amount of sample DNA was increased to 400 ng per 50 µl reaction, (2) 25 pm of primers were used per 50 µl reaction, (3) the annealing time was increased to 60 seconds and (4) the elongation time was increased to 120 seconds. Two 50 µl reactions were combined for each mouse. The combined product was then digested with *Bsl*1 per the

manufacturer's instructions (New England Biolabs) and purified using QIAQuick PCR Purification Columns (QIAgen, Valencia, CA). Samples were submitted to the MSU Center for Microbial Composition for T-RFLP analysis.

Statistical Analysis

T-RFLP analysis. T-RFLP peaks with a height of or greater than 50 fluorescent units were included in the analyses and reported as fragment size Operational Taxonomic Units (OTUs) (Kitts 2001). PAST software was utilized for Multivariate Cluster ANOSIM analysis for Bray-Curtis and Jaccard Beta indices of diversity (Hammer, Harper et al. 2001). Mouse fecal DNA were analyzed both comparing presence verses absence and relative abundance of OTUs using Euclidean and Bray-Curtis analyses, respectively (Hammer, Harper et al. 2001). N was equal to 10,000 permutations for all analyses. The null hypothesis was rejected if $P \leq 0.05$.

RESULTS

Fecal microbiota remained more like mice from other litters of the same breeding pair than mice from another breeding pair throughout the course of the experiment. As shown in Figure 4.3, mice from the three litters from breeding pair 544 (B544-1,-2,-3) had fecal microbiota that was more similar to other mice that resulted from the same breeding pair than from mice from the two litters belonging to breeding pair 549 (B549-1,-2). This remained true through all of the assessed fecal collections regardless of whether the liter mates remained together or if they were split into individual cages.

Fecal microbiota of B44 litters had increased differences in microbial community composition after splitting litters two weeks after splitting them into individual cages. Prior to individualizing the mice in litter B44-1 and half of the mice from B44-3, these two groups (B44-1 and B44-3) had statistically significant differences in the composition of their fecal microbiota as reported using T-RFLP (Table 4.3). Unfortunately, two out of the four mice in litter B44-2 died unexpectedly after the second fecal collection, which resulted in limited data to follow-up with and assess this group. But, at the conclusion of the experiment, the mice in B544-1 and B544-3 were no longer more similar to their litter-mates than other mice from the same breeding pair. This can be noted both by comparing B544-1 to other litters of the same breeding pair (Figure 4.4) and also by comparing them to mice from litters of both breeding pairs (Figure 1). Similar effects can also be noted when following the mice in B549-1 which were also split and housed similarly in individualized cages. At the first collection time, prior to individualizing the mice, they were all found to fall within the same grouping based on assessing their GI

microbial community relative abundance and presence and absence of specific community members (Figure 3). At collection five, the mice had split among two major clades (data not shown), which remained similar at the time of collection seven (Figure 4.5). This suggests that individualizing the mice allowed their fecal microbiota to drift or shift in composition from one another. Despite the lack of statistical significance, it is interesting to note that the three mice from the B544-3 litter that were individualized (1-3) remained in the same Bray Curtis and Jaccard cluster.

DISCUSSION

The major finding from this study was that mice from the same breeding pair had microbiota that was similar to mice within their litter, but also to other mice that were born to the same breeding pair in a later litter; their microbiotas within generations of the same family were more similar to these mice than to mice originating from another breeding pair. This occurred regardless of the age of the mice at sampling. However, the microbiota of mice in the same family changed and shifted from other mice within their same litter after these mice were placed into separate cages and maintained individually. Nevertheless, their microbiota still remained more similar to other mice from the same breeding pair than to mice from another family originating from a different breeding pair over the seven week period of the experiment. In this experiment, the male fathers remained in the cage with the female breeder and the offspring, until they were weaned to allow for re-breeding. Because of this, it is not possible to say if their "signature" microbiota is mostly contributed by the female or the male breeder. Mice are also coprophagic, meaning that they eat their own feces and feces of other mice. This, in effect, allows them to inoculate each other with their feces and any bacteria that may be present. When living in a group housing situation, verses individualized housing, this may help prevent the microbiota of any one individual mouse from changing significantly from their cage-mates. Thus mice may differ from humans in this regard although it may be expected that human families share microbiota through fecal-oral exchange as well through their close associations.

When litters of mice were assessed based on whether they were left together in the same cage or split into individual cages, it was noted that the mice that separated into individualized cages began with microbiota that was similar to other mice within their litter, and then underwent diversification throughout the course of the experiment, until, for the most part, they were no

longer more similar to their litter mates than to other mice from the same breeding pair. This observation was made using three different collections spanning 33 days. Unfortunately we have limited data from mice that were left within the same cage throughout the course of the experiment because the mice died unexpectedly from these groups and were lost to observation. This loss of sample size for the experiment-long data collection complicated the statistical analyses of these groups due to decreasing the group sizes making it difficult to make statistically significant observations. But, it does appear that they tended to stay more like each other than mice that were individualized. In support of the conclusion that mouse microbiota drifted due to separation into individual cages and not just with the age of the mice as their time away from their breeding pair increased, is that at the beginning of the experiment (collection 1) mice had rather distinct microbiota based on litter. Furthermore, all of the samples were taken from all of the experimental mice at the same time, despite the fact that the litters were all different ages and each separated by approximately 21 days. To accomplish this, sample collection began on the same day, regardless of the age of the liters, which varied by up to 16 weeks.

Human studies examining the microbiota composition have been conducted using sets of identical twins, and similar results were found. In these studies, they also found that twins were more likely to have similar microbiota than other unrelated individuals in the same environment. The advantage to using murine litters to further investigate the effects of "individual" on the composition of the GI microbiota, is that there are many more environmental factors that we can hold constant in a murine breeding colony, which allowed us to look more specifically at the effects of parents, siblingship and group housing on this outcome. Further studies to examine the effects of various environmental factors such as diet, bedding, water source, and co-housing with

schemes (i.e. littermates and non-littermates) on microbiota richness and evenness could provide interesting insights to suggest how these same features might affect humans as well.

TABLES

Table 4.2 Experimental design of breeding colony mice for the assessment of the effect of family on gastrointestinal microbiota

Parents	**Litter #**	**# of mice**	**Individualized or group housed**	**Fecal Collection 1**	**Fecal Collection 5**	**Fecal Collection 7**
B544	1	4	individualized	Day 1	Day 23	Day 33
	2	4 (2)**	Group	Day 1	Day 23	Day 33
	3	5	individualized	Day 1	Day 23	Day 33
B549	1	4	Group	Day 1	Day 23	Day 33
	2	6	3 individualized, 3 Group	Day 1	Day 23	Day 33

** Two of the four mice died unexpectedly due to unknown causes on day 6 of the experiment

Group sizes are uneven due to the number of mice born to each litter and due to mice dying unexpectedly for unknown causes.

Table 4.3 P and R values for Bray-Curtis ANOSIM comparing liters at collection time 1, 5 and 7.

Litter Comparison	Collection 1 p value	Collection 1 R value	Collection 5 p value	Collection 5 R value	Collection 7 p value	Collection 7 R value
B544-1 v. B544-2	0.0561	0.6042	0.2075)	0.5	0.4606	0.07143
B544-1 v. B544-3	0.023	0.5438	0.0869	0.2	0.0734	0.3563
B544-1 v. B549-1	0.0304	0.9167	0.048	0.3542	0.0502	0.3021
B544-1 v. B549-2	0.0281	0.3095	0.0295	0.3624	0.0219	0.4643
B544-2 v. B544-3	0.0978	0.2687	0.6286	-0.09091	0.9021	-0.2727
B544-2 v. B549-1	0.0304	0.9792	0.5996	-0.03571	0.7964	-0.3214
B544-2 v. B549-2	0.0575	0.2659	0.0838	0.5591	0.2575	0.1875
B544-3 v. B549-1	0.1651	0.1875	0.2488	0.075	0.4417	0.0125
B544-3 v. B549-2	0.725	-0.07733	0.0098	0.4323	0.0455	0.3227
B549-1 v. B549-2	0.8973	-0.1468	0.0272	0.3651	0.342	0.03968
B544-1,-2,-3 v. B549-1,-2	0.0015	0.2717	0.0063	0.169	0.0008	0.2964

FIGURES

Figure 4.3. One-way ANOSIM microbial community analyses on collection 1 and collection 7 based on parents/breeding pair. A) Bray Curtis analyses of collection 1, R= 0.217 and P(same) = 0.0025. B) Bray Curtis analyses of collection 7, R =0.2964 and P(same) =0.0006. C) Jaccard analyses of collection 1, R = 0.3423 and P(same) = 0.0003. D) Jaccard analyses of Collection 7, R = 0.3495 and P(same) = 0.0002. N = 10,000 permutations for all analyses.

Figure 4.3A Continued

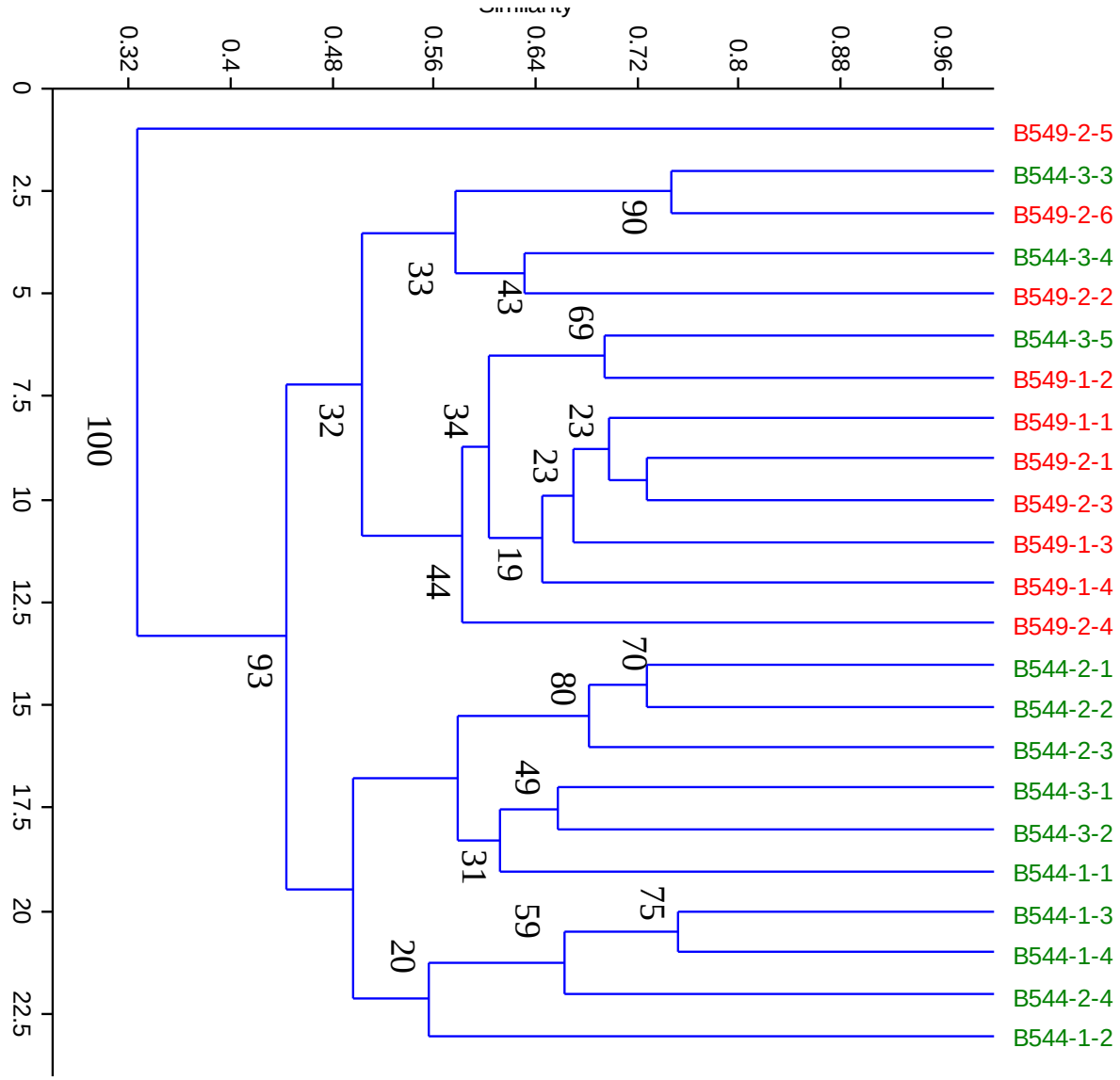


Figure 4.3 B Continued

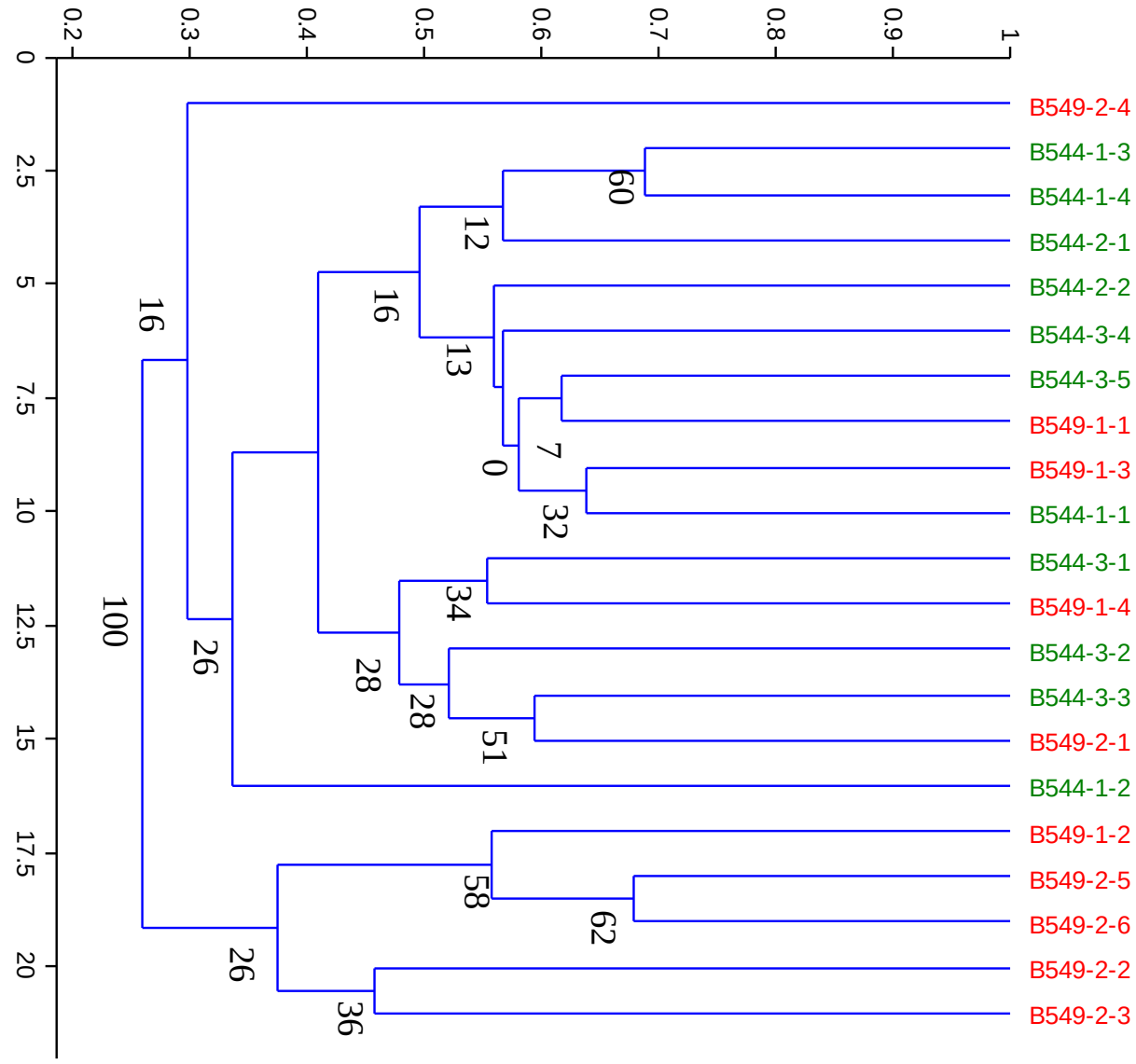


Figure 4.3 C Continued

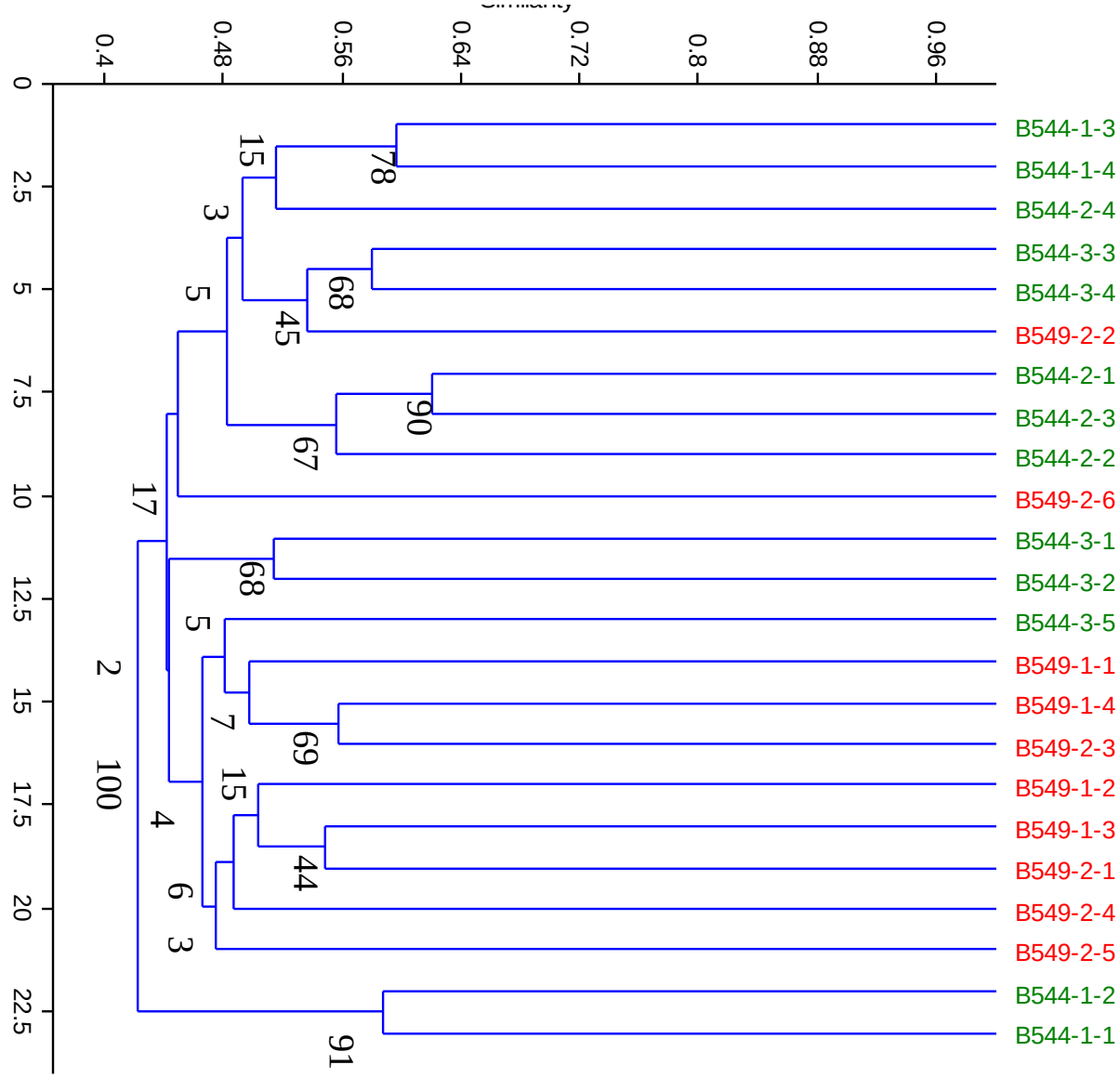


Figure 4.3 D Continued

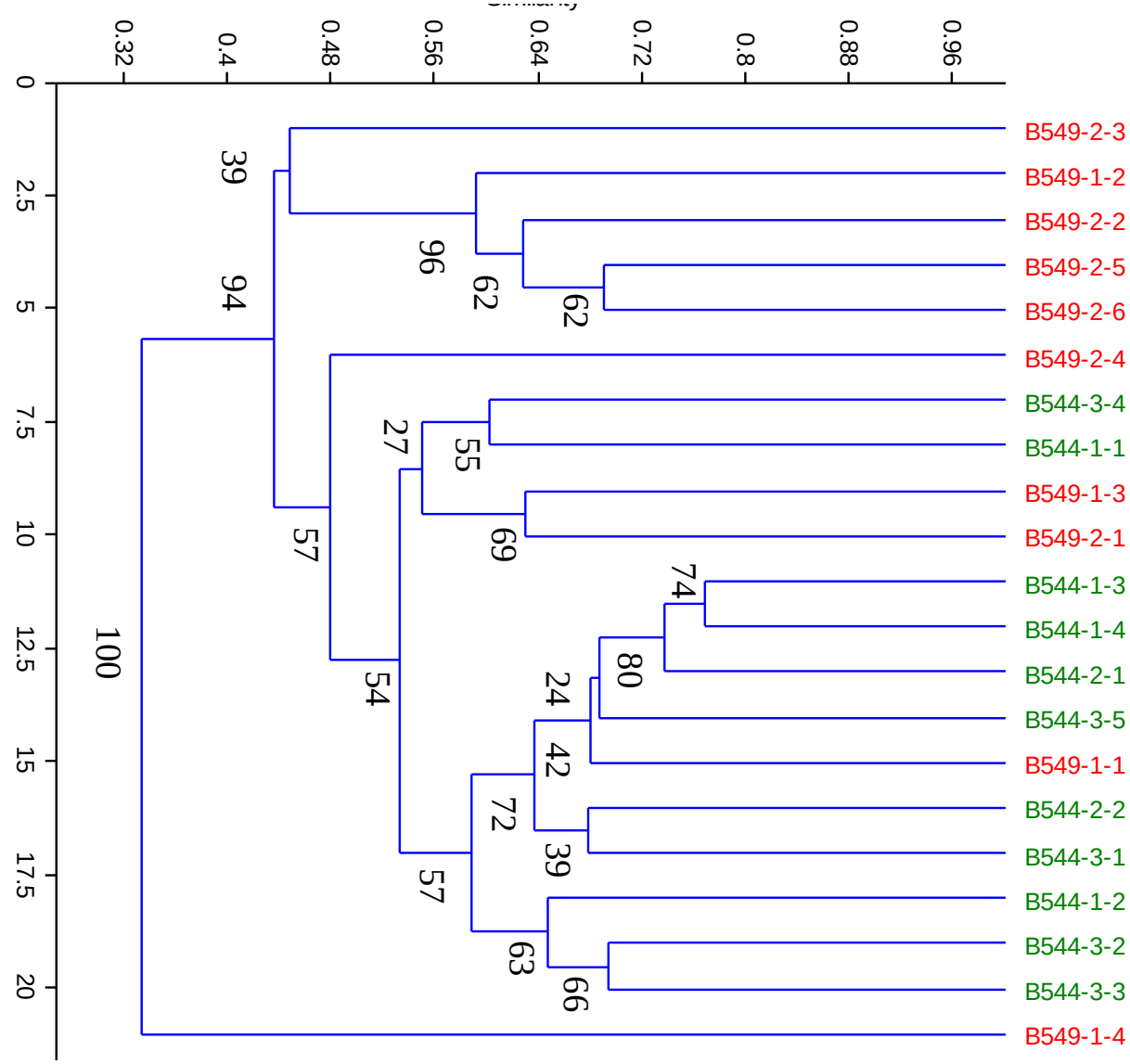


Figure 4.4. One-way ANOSIM analyses of all litters from both breeding pairs on collection 1 and collection 7. A) Bray Curtis analysis of Collection 1 shows statistical significance between B544-1, B544-3, B549-1 and B549-2. B) Bray Curtis analysis of Collection 7 shows significant differences between B544-1 and B549-2 and B544-3 and B549-2 C) Jaccard analysis of Collection 1 shows statistical significance between all groups except B544-2 and B544-3 and B549-1 and B591-2. D) Jaccard analysis of Collection 7 only shows statistical significance between B544-3 and B549-2. For all analyses $p \leq 0.05$ and N=10,000 permutations.

Figure 4.4 A Continued

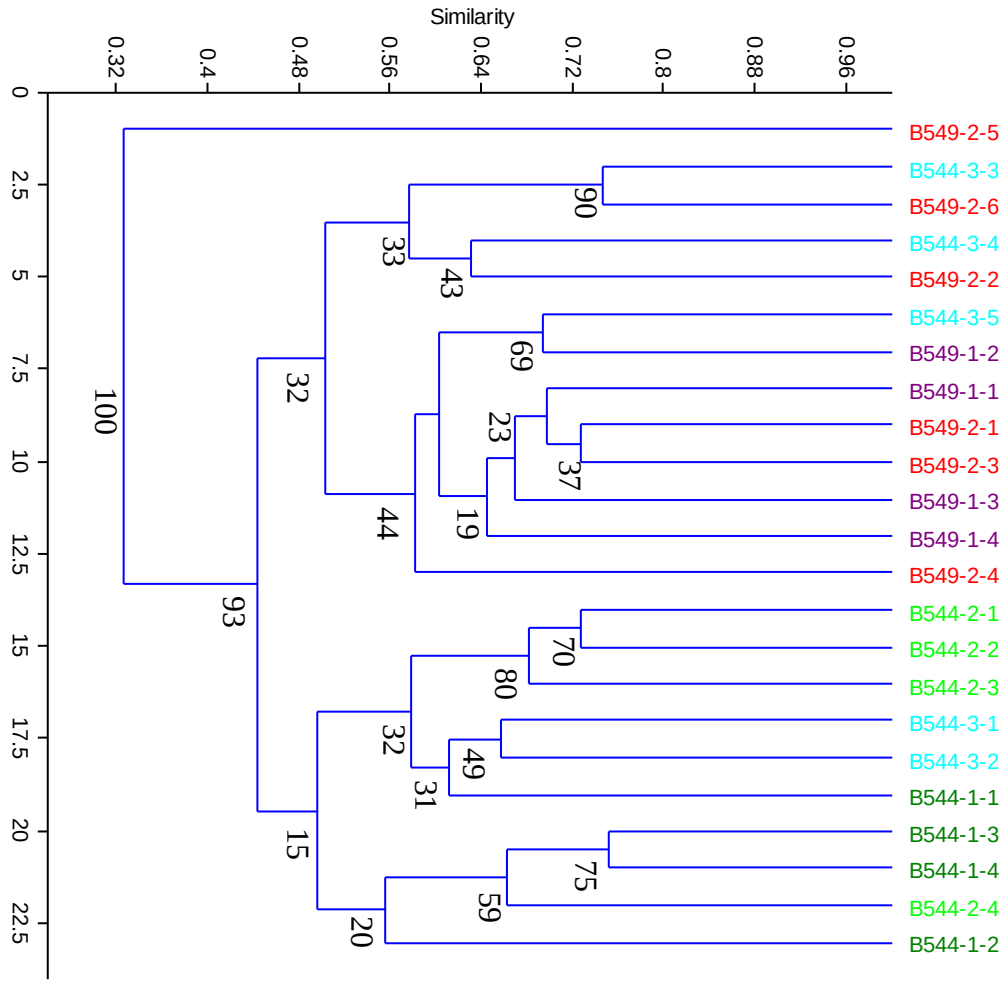
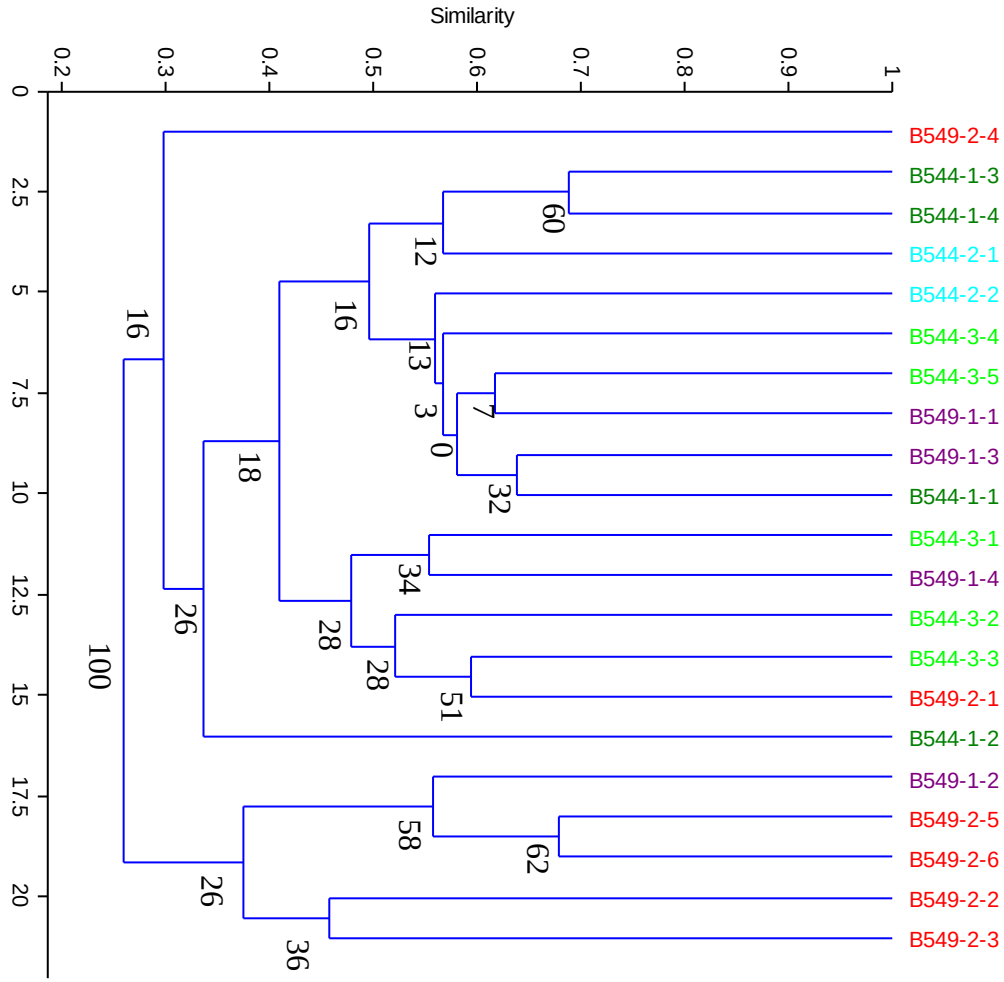


Figure 4.4 B Continued



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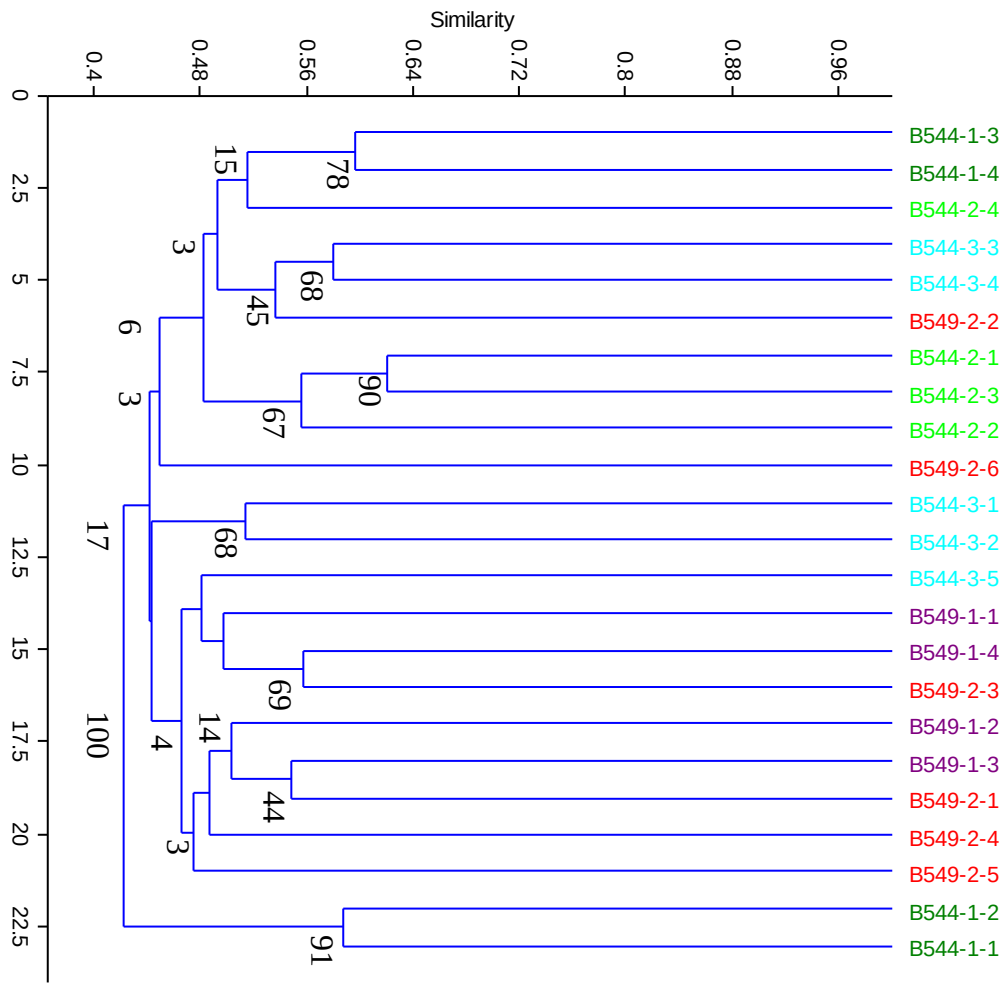


Figure 4.4 D Continued

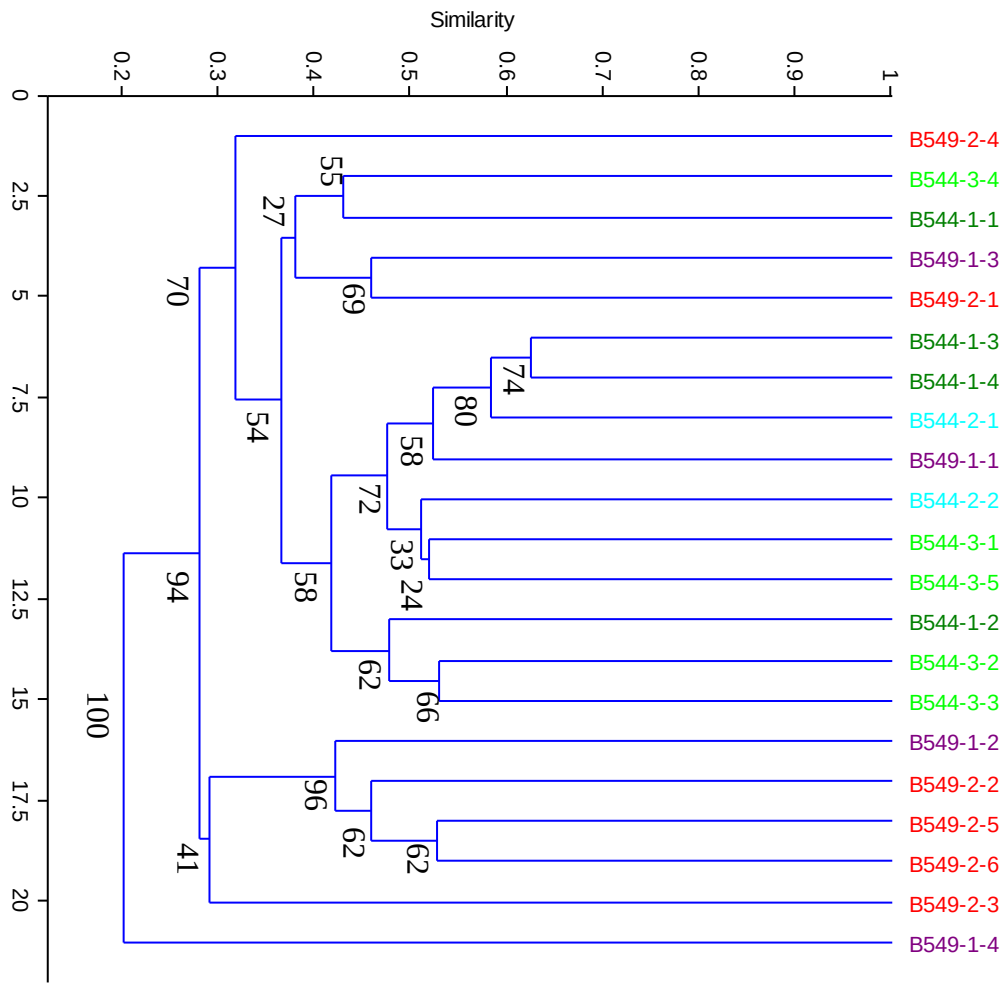


Figure 4.5. One Way ANOSIM analyses of Litters from breeding pair B544 on collection 1 and collection 7. A) Collection 1 Bray Curtis analysis shows significant differences between B544-1 and B544-3 ($P \leq 0.05$) . B) Collection 7 Bray Curtis analysis shows no significant differences between any of the litters. C) Collection 1 Jaccard Analysis shows significant differences between B544-1, B544-2 and B544-3 ($P \leq 0.05$) and D) Collection 7 Jaccard analysis shows no significant difference between any of the litters. N=10,000 permutations for all analyses.

Figure 4.5 A Continued

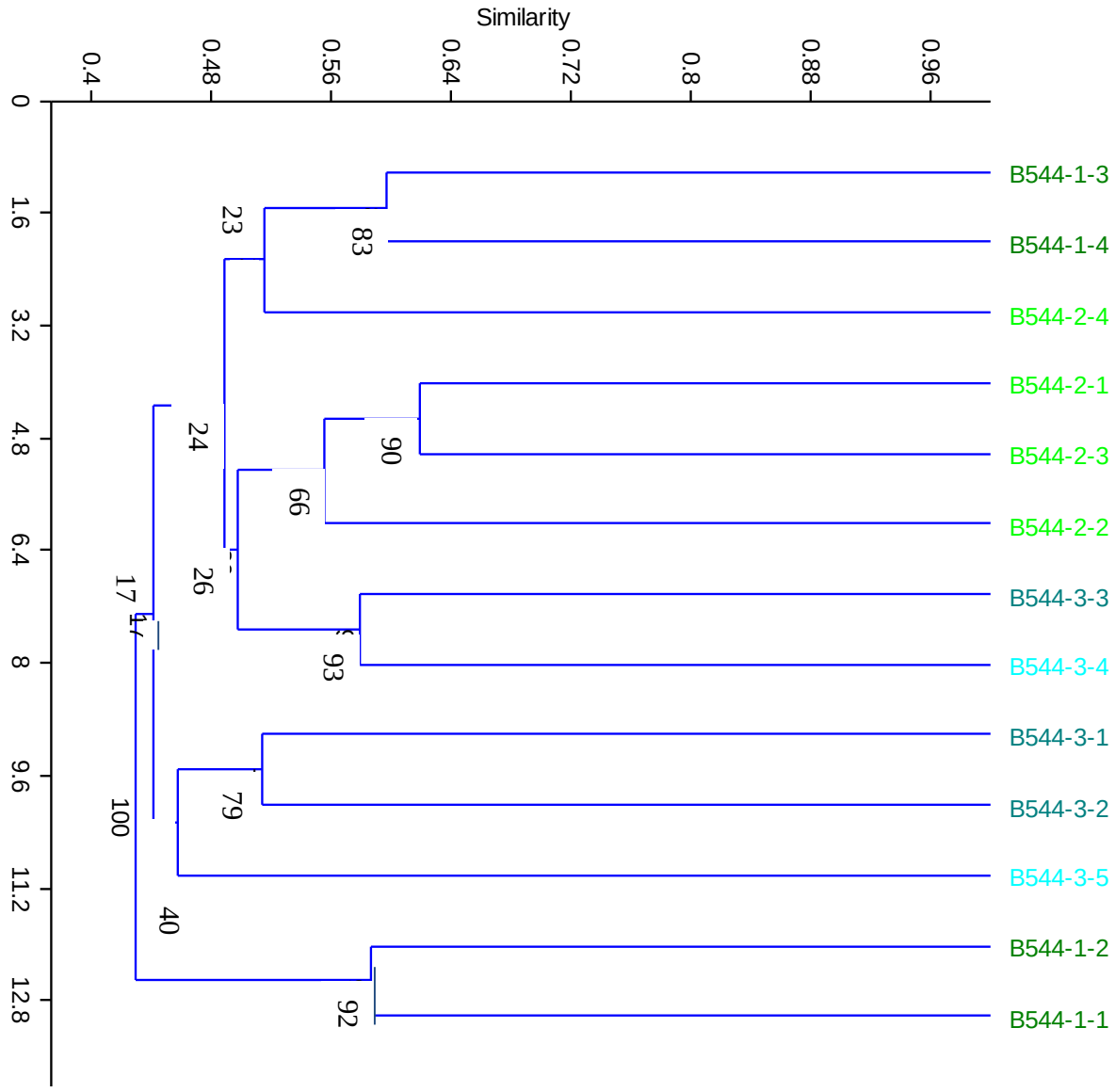


Figure 4.5 B Continued

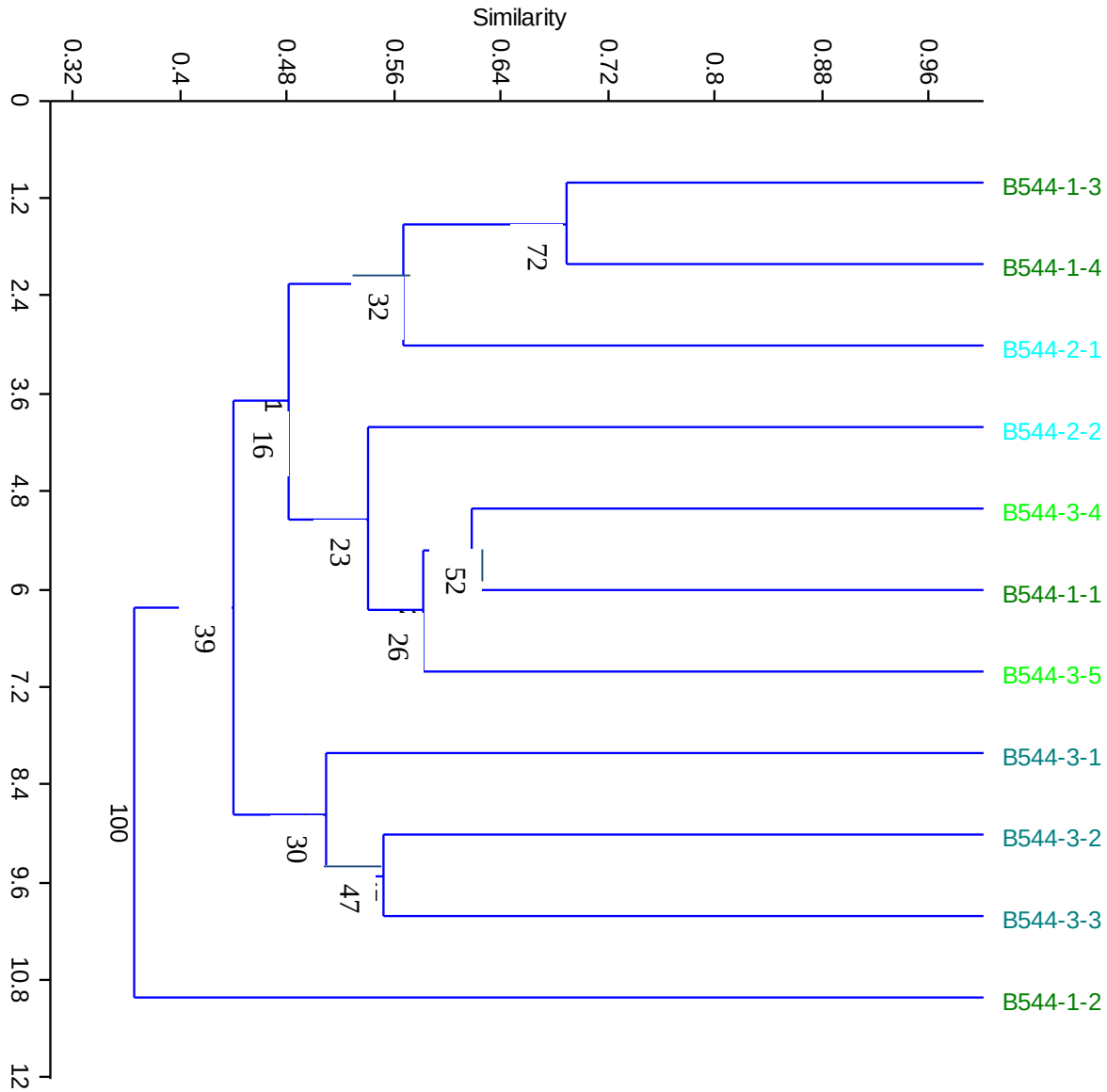


Figure 4.5 C Continued

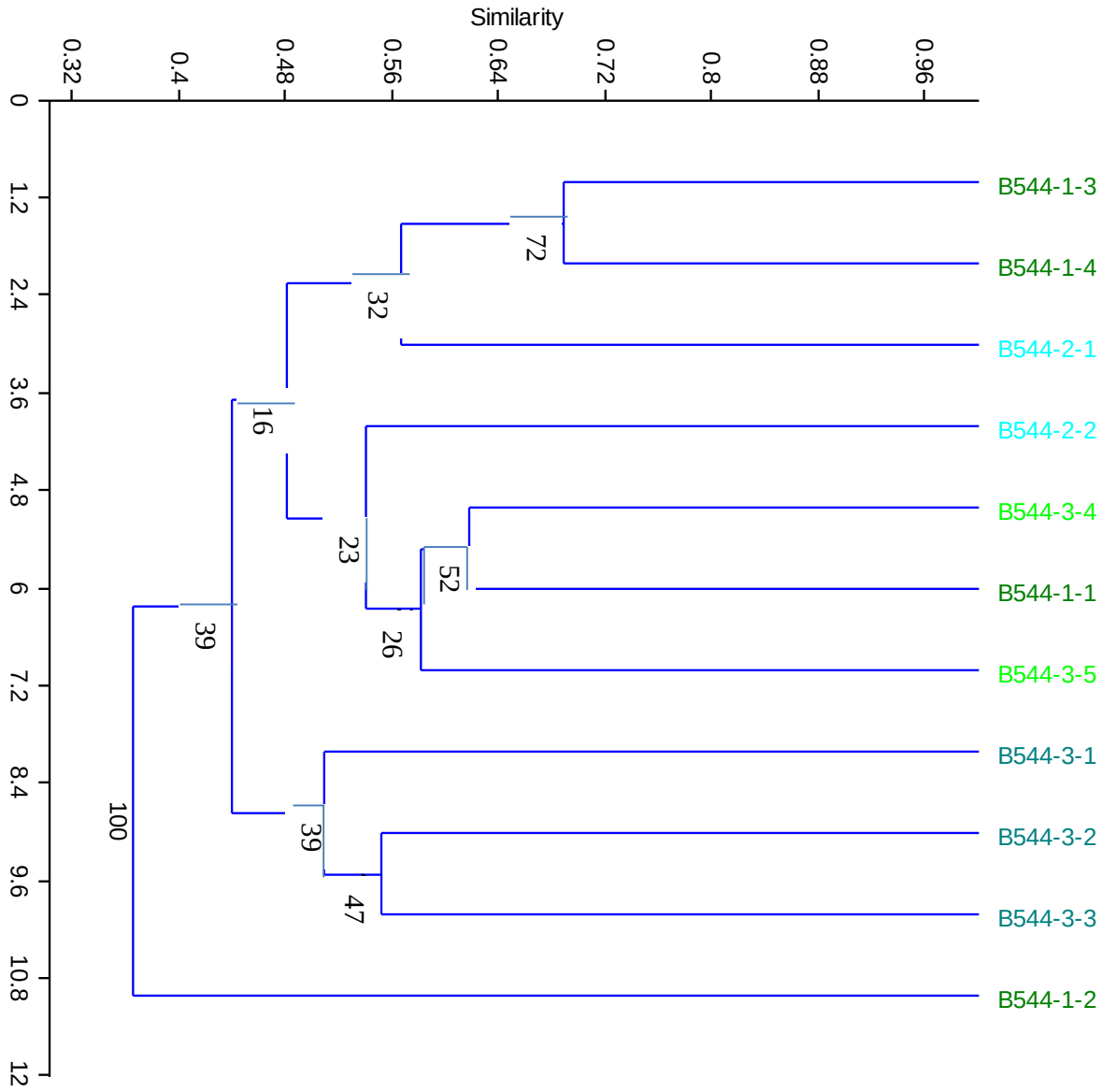
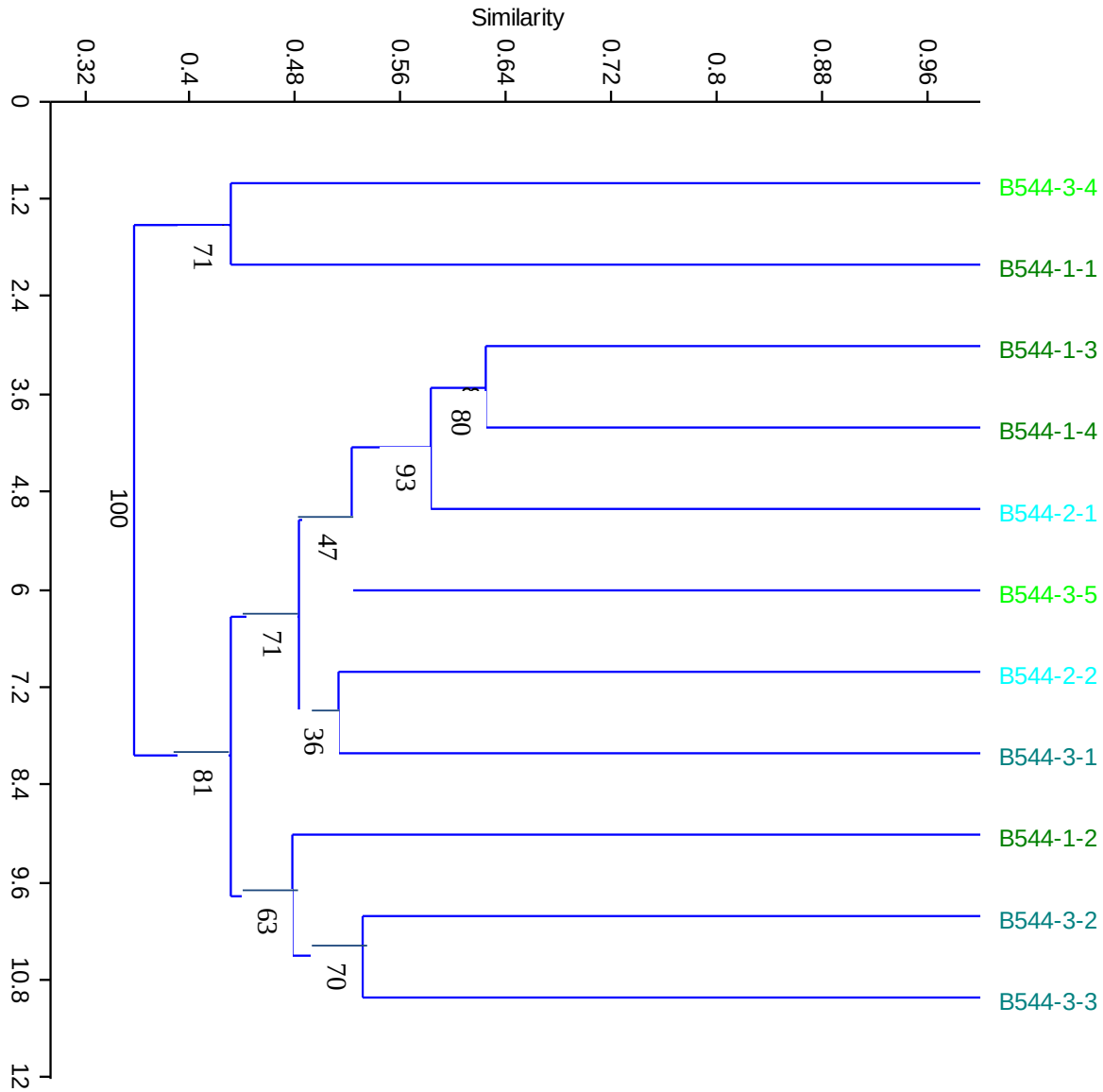


Figure 4.5 D Continued



CHAPTER 4 SECTION 3

Trichuris muris challenge causes a significant shift in the structure of proximal colon microbiota in C3H/HeJ and C3Bir IL-10$^{-/-}$ mice

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ABSTRACT

The nematode *Trichuris* has been shown to interact with particular enteric bacteria, but its effects on the host's microbial community composition are not fully understood. We hypothesized that *Trichuris muris* infected mice have a significantly altered structure of the proximal colon microbiota compared to uninfected mice. Toll-like receptor-4 deficient C3Bir IL-$10^{-/-}$ and congenic IL-10 sufficient mice (C3H/HeJ) orally infected with 100 embryonated *T. muris* eggs were sacrificed after patency at 40 days post infection (PI) or earlier if they demonstrated severe clinical signs of disease; uninfected control mice were sacrificed at 40 days post sham inoculation. Additionally, uninfected C3Bir IL-$10^{-/-}$ mice within our breeding colony that developed spontaneous colitis were assessed and compared to those with no clinical signs of colitis. The composition and structure of proximal colon microbiota were assessed using Terminal Restriction Fragment Length Polymorphism (T-RFLP) analysis targeting the 16S Universal rDNA region. We found that C3Bir IL-$10^{-/-}$ mice infected with *T. muris* had differently structured colon microbial communities than uninfected controls. C3Bir IL-$10^{-/-}$ mice with spontaneous colitis did not have significantly different colon microbiotas than those without colitis. Additionally, we found that although there were few significant differences in the lesions assessed in the gastrointestinal tract using histological scoring, the C3Bir IL-$10^{-/-}$ infected mice had a predominantly neutrophilic response when compared to the other mice. This work supports our hypothesis that mice infected with *T. muris* develop distinct microbial communities in the colon, and that these alterations cannot be solely attributed to the presence of inflammation in the gastrointestinal tract.

INTRODUCTION

Trichuris spp. are gastrointestinal (GI) nematodes that dwell in close association with a complex bacterial community. The *Trichuris* life-cycle begins when the host ingests embryonated eggs containing the first larval stage (L1), which hatch in the host's cecum and/or colon. Larvae penetrate the epithelium and subsequently undergo four molts before becoming sexually mature. Both larval and adult *Trichuris* burrow into the host's colonic epithelium, forming syncytial tunnels (Lee and Wright 1978; Panesar 1981) that enable the worms to maintain their place in the proximal colon. Females produce eggs that pass in feces and embryonate within the egg in the environment.

Trichuris suis produces excretory secretory products (ESP) which have antimicrobial properties (Abner, Parthasarathy et al. 2001). *In vitro* experiments showed that the ESP had antimicrobial activity against Gram negative bacteria, including *Campylobacter jejuni*, *C. coli* and *Escherichia coli*, and Gram positive bacteria, including *Staphylococcus aureus*. Additionally, *Trichuris* ESP have been demonstrated to aid the worm both in burrowing into the host's colonic epithelium and in feeding (Abner, Parthasarathy et al. 2001; Abner, Hill et al. 2002).

Trichuris has also been reported to interact with bacteria *in vivo*. For example, early studies demonstrated development of diarrhea in weaning age pigs concurrently harboring *T. suis* and particular bacteria (Rutter and Beer 1975). A mixed inoculum of *T. suis* and cecal scrapings containing *Brachyspira*, *Campylobacter* or *Salmonella* were implicated in this diarrhea by passive transfer to specific pathogen free pigs (Rutter and Beer 1975). Interactions between this helminth and enteric bacteria were also explored by antibiotic treatment of *T. suis* infected pigs (Mansfield and Urban 1996). Results of both passive transfer and antibiotic treatment

experiments showed that *Trichuris* and particular bacteria were necessary to produce the type of diarrhea and colonic lesions seen in weaning aged pigs in production, but did not implicate a single bacterial agent. In 2003, synergism between *T. suis* and *Campylobacter jejuni* was demonstrated to be a cause of mucohemmorhagic colitis when germ free piglets inoculated with these agents in combination developed disease while those infected singly did not (Mansfield, Gauthier et al. 2003). Extensive studies of captive rhesus monkeys with chronic enterocolitis have shown similar interactions. In these studies, severe disease was associated with presence of *Trichuris trichuira* and several enteric pathogens including *Campylobacter coli*, *C. jejuni*, *Shigella flexneri*, *Yersinia enterocolitica*, adenovirus, and *Strongyloides fulleborni* (Sestak, Merritt et al. 2003). Most recently, Hayes *et al* (Hayes, Bancroft et al. 2010) demonstrated that increased levels of colonic microflora favor higher numbers of *T. muris* and chronic infections. These various studies demonstrate that *Trichuris* interacts with—and may even demonstrate synergy in disease production with—the host's colonic microflora.

Trichuris interactions with bacteria have also been studied in murine models. In 2002, Schopf *et al* found 100% morbidity in C57BL/6 IL-$10^{-/-}$ and congenic IL-$10^{-/-}$ IL-$4^{-/-}$ mice following challenge with *T. muris* (Schopf, Hoffmann et al. 2002). They hypothesized that the high morbidity and mortality rates were due to an overgrowth of opportunistic invasive bacteria that use the mechanical damage caused by *T. muris* larva to breach the gastro-intestinal tract. To address this they added neomycin sulfate, a broad spectrum antibiotic that suppresses gastrointestinal bacteria, to the drinking water of IL-$10^{-/-}$ IL-$4^{-/-}$ mice and infected them with *T. muris;* there was a statistically significant increase in the percentage of the C57BL/6 IL-$10^{-/-}$IL-$4^{-/-}$ mice that survived infection with *T. muris* after antibiotic treatment (Schopf, Hoffmann et al.

2002). The authors concluded that a change in the microbiota may have contributed to the previously observed morbidity and mortality.

Thus, *Trichuri*s has been shown to interact with intestinal bacteria in multiple ways. Taken together, these data suggest an important microbial component to the pathogenesis of *Trichuris* infections in a variety of animal models. We suspected that *Trichuris* has community-wide interactions with enteric bacteria. Therefore, we hypothesized that mice infected with *T. muris* have significantly different microbiota in the proximal colon compared to uninfected mice and that these effects contribute to the worm's immunomodulatory properties on its host. To test this hypothesis, we infected C3Bir.129P2(B6)-IL-$10^{-/-}$ (C3Bir IL-$10^{-/-}$) and C3H/HeJ mice, both deficient in TLR-4 and containing the cytokine deficiency colitis susceptibility-1 (*cdcs*-1) allele, with 100 embryonated *Trichuris* eggs and followed the course of infection until patency. C3Bir IL-$10^{-/-}$ mice infected with *T. muris* had significantly different microbial communities in the proximal colon compared to the respective controls. To further explore on the effect of spontaneous colitis on the composition and structure of microbiota in the proximal colon, we assessed murine proximal colon microbiota of C3Bir IL-$10^{-/-}$ mice within our breeding colony that developed spontaneous colitis and compared them to mice of the same genotype that did not develop colitis using T-RFLP. When microbiota Operational Taxonomic Units (OTUs) of all colitic mice were compared to non-colitic mice, we found that there were no statistically significant differences between the groups. This work supports our hypothesis that mice infected with *T. muris* develop distinct microbial communities in the colon, and that these alterations cannot be solely attributed to the presence of inflammation in the gastrointestinal tract.

MATERIALS AND METHODS

Mouse Genetic Background, Breeding and Handling

All animal protocols were approved by the Michigan State University (MSU) Institutional Animal Care and Use Committee (IACUC) and complied with National Institutes of Health guidelines (04/07-044-00). Briefly, C3H/HeJ and C3Bir.129P2(B6)-IL-$10^{-/-}$ (referred to throughout as C3Bir IL-$10^{-/-}$) mice were obtained from barrier facilities at The Jackson Laboratories (Bar Harbor, ME). Previously, Beckwith, Leiter and colleagues documented a role for the Cdcs-1 gene product(s) in these mice in the development of spontaneous colitis in C3Bir IL-$10^{-/-}$, but not C3H/HeJ mice (Farmer, Sundberg et al. 2001; Beckwith, Cong et al. 2005), which we further confirmed in experimental challenge with a colitogenic strain of *C. jejuni* (Mansfield, Patterson et al. 2008). A breeding colony was established in a *Campylobacter/Helicobacter*-free facility. Mice were housed in specific pathogen-free conditions using a FlexAir ventilated mouse rack (Alternate Design Manufacturing & Supply Inc., Siloam Spring, AR), fed an irradiated mouse diet (mouse breeder diet 7904; Harlan Teklad, Indianapolis, IN), kept on autoclaved bedding and given filter-sterilized water (autoclaved sucrose water in bottles for weanlings) in an MSU limited-access room. Mouse genotypes were confirmed using PCR assays obtained from Jackson Laboratories ((http://jaxmice.jax.org/pub-cgi/protocols/protocols.sh?objtype=protocol &protocol_id=346) both before and after experiments. To prevent colitis from other known causes, experimental mice were screened for *Campylobacter* spp. (Linton, Owen et al. 1996), *Helicobacter* spp. (Riley, Franklin et al. 1996), *Enterococcus faecalis* (Dutka-Malen, Evers et al. 1995), and *Citrobacter rodentium* (McKeel, Douris et al. 2002) by 16S rRNA gene PCR assay for *Helicobacter* spp. and *Campylobacter* spp.,

ddl gene-specific PCR for *Enterococcus faecalis* and *espB* gene-specific PCR for *C. rodentium* using DNA isolated from fecal samples both prior to and at the conclusion of the experiment.

A dedicated C3Bir IL-10$^{-/-}$ sentinel mouse was used to monitor for bacterial, protozoan and viral agents through the MSU Laboratory Animal Resources Facility (ULAR). Additionally, we monitored the mouse colony for the incidence of spontaneous colitis by examining euthanized retired breeding mice and those euthanized for other reasons for enlargement of the proximal colon, cecum, ileocecocolic lymph node and spleen. Feces of mice that exhibited signs of colitis were screened for the presence of the known colitis causing bacteria previously listed, all using DNA isolated from proximal colon samples as described below (Mansfield, Bell et al. 2007).

When designated experimental mice reached the ages of 8 to12 weeks they were transported to the Michigan State University Research Containment Facility in autoclaved polycarbonate filter-topped cages in sterile dog crates for experiments. All mice were housed individually in autoclaved polycarbonate filter-topped cages (Ancare, Bellmore, NY) on sterile bedding, fed irradiated 7904 mouse breeder diet (Harlan Teklad, Indianapolis, IN), given autoclaved water and autoclaved cotton nestlets, and randomly assigned to cage locations on the racks without respect to genetic background or treatment group.

C3Bir IL-10$^{-/-}$ mice involved in the second experiment assessing the incidence of spontaneous colitis remained within our barrier breeding colony for the entirety of the experiment.

Parasites

T. muris eggs of the J-isolate were originally obtained from Dr. Joseph Urban (USDA) and used to infect SCID mice to obtain adult *T. muris* worms. Egg stocks were created as previously described (Kopper and Mansfield 2010). After *T. muris* eggs were collected they were stored in the dark at room temperature in 30 ml tissue culture flasks with 15 ml of Phosphate Buffered Saline (PBS) medium, 100 U/ml Penicillin/100 µg/ml Streptomycin and 2.5 mg/ml Amphotericin B to prevent bacterial and/or fungal growth during embryonation. The flasks were monitored by microscopy for the presence of fungal and/or bacterial infection and discarded if contamination was detected.

Parasite Inoculum Preparation and Infection

T. muris egg inoculum was prepared as previously described (Kopper and Mansfield 2010). Briefly, eggs were surface sterilized using 6.25% hypochlorite (bleach) and washed in sterile PBS. Infectivity and viability were confirmed using propidium iodide staining and an *in vitro* hatching assays. Mice were orally gavaged with 100 embryonated *T. muris* eggs in 200 µl PBS according to experimental group assignment using 1 ml tuberculin syringes with sterile 22 gauge curved feeding needles.

Experimental Design

C3H/HeJ and C3Bir IL-10$^{-/-}$ mice were divided into randomized age and sex-matched experimental groups (Table 4.6) and received 100 embryonated *T. muris* eggs or sham inoculations as designated (Table 4.6). Mice displaying clinical signs of disease were euthanized

prior to the scheduled necropsy if necessary in accordance to our clinical signs of disease scoring system (Mansfield, Bell et al. 2007). All remaining mice were euthanized at day 40 post infection (P.I.). For statistical analyses assessing enteric and extra-intestinal lesions based on histological scoring, mice were divided into early (prior to day 29 PI) and late (after day 29 PI) groups. This 40 day experimental end-point was chosen based on data from a previous mouse experiment which showed peak egg-shedding in fecal pellets at this time point. (Kopper et al, in preparation).

In the second experiment, C3Bir IL-$10^{-/-}$ mice that were used to assess the development of spontaneous colitis within the breeding colony were monitored twice daily for clinical signs associated with colitis. Mice with colitis were euthanized if clinical signs progressed to humane endpoints according to an approved score sheet designed to prevent suffering. At this time, if available, a mouse of similar age and sex was euthanized to serve as a control (Table 4.7).

Monitoring of Experimentally Infected Mice for Clinical Signs

Mice involved in the *Trichuris*-infection study were evaluated for clinical signs of disease one to four times per day by a trained individual using a standardized score sheet (Mansfield, Bell et al. 2007). The frequency of monitoring increased with the severity of the clinical score. Briefly, mice were monitored for clinical signs including, but not limited to, rough hair coats, hunched posture, decreased activity, and dehydration. Each factor, if present, conferred a numerical score. For this experiment, the standardized score triggering euthanasia, was modified and lowered. Any mouse receiving a score of 8 or higher was immediately euthanized and necropsied.

Necropsy, Gross Pathology, and Tissue Section Preparation

Mice were humanely sacrificed by CO_2 overdose according to AVMA guidelines (2001) and weighed. A blood sample was obtained by cardiac puncture using 0.1 ml of 3.8% sodium citrate to prevent coagulation. Prior to opening the GI tract, spleens were removed aseptically, weighed and cut on the sagittal plane. One half was infused with fixative and then placed in a histological cassette (Histocette II; Simport Plastics, Beloeil, Quebec Canada) and submerged in Carnoy's solution for 24 hours, then decanted and placed in 60% ethanol in preparation for histological examination. The other half was snap frozen using dry ice for subsequent DNA extraction. The GI tract was removed in its entirety to absorbent paper and gross pathological changes were noted and those from mice involved in the *Trichuris* infection experiments photographed. Care was taken to avoid contaminating the serosal surfaces with either *T. muris* or intestinal contents. A 0.5 cm cross section of colon was sectioned with sterile scissors and snap frozen in a cryovial for T-RFLP and Q-PCR microbial composition studies. The cecum with approximately 1 cm of both the terminal ileum and proximal colon was placed on a sponge in a histological cassette, immediately injected with Carnoy's solution and submerged in Carnoy's solution for 24 hours. Thereafter, the solution was decanted and the cassette placed in 60% ethanol in preparation for histological analysis. Carnoy's-fixed tissues were embedded in paraffin and cut in 5 μm sections; one section was stained with hematoxylin and eosin and another section was used for a Gram stain at the Investigative Histopathology Laboratory, Division of Human Pathology, Department of Physiology, Michigan State University. Sections were observed and photographed using a Nikon Eclipse E600 microscope with a SPOT camera

and Windows™ version 4.09 software (RT-Slider Diagnostic Instruments, Inc., Sterling Heights, MI).

Histopathological Lesion Scoring

A scoring system was developed to evaluate histopathological changes in the ileocecocolic junction of each mouse and each sample assigned a numerical grade according to severity (Mansfield, Bell et al. 2007) . This scoring system involved evaluating the lumen for excess mucus and inflammatory exudates and the epithelium for surface integrity, number of intraepithelial lymphocytes, goblet cell hypertrophy, goblet cell depletion, crypt hyperplasia, crypt atrophy and crypt inflammation. Additionally, the lamina propria and submucosa were evaluated for increases in inflammatory or immune cells and changes in the distribution of those cells and the submucosa for fibrosis, inflammatory cells and edema. All histological sections were scored by a single investigator (JJK) who was blind to the identities and experimental group of the individual mice. Spleens were evaluated to further assess the presence or absence of overall immune response. Spleen sections were scored using separate standardized scoring criterion which graded the size of periarteriolar sheaths (PALS), degree of extra-medullary hematopoiesis, and amount of mononuclear lymphocyte infiltration by a single individual (JJK) (Mansfield, Patterson et al. 2008).

DNA Extraction and Terminal Restriction Fragment Length Polymorphisms (T-RFLP).

DNA was extracted from frozen proximal colon tissue samples using a DNeasy tissue kit (QIAgen, Valencia, CA) according to the manufacturer's instructions. Each DNA sample was used as the template for the 16S rRNA gene universal primers 5'-fluorescent (6-FAM)'-

TGCCAGCAGCCGCGGTA-3' (516f) and 5'-GGTTACCTTGTTACGACTT-3' (1510r) using the reaction mixture described by Nagashima *et al* (Nagashima, Hisada et al. 2003) with the following modifications: (1) the total amount of sample DNA was increased to 400 ng per 50 µl reaction, (2) 25 pm of primers were used per 50 µl reaction, (3) the annealing time was increased to 60 seconds and (4) the elongation time was increased to 120 seconds. Two 50 µl reactions were combined for each mouse. The combined product was then digested with *Bsl*I per the manufacturer's instructions (New England Biolabs, Ipswich MA) and purified using QIAQuick PCR Purification Columns (QIAgen, Valencia, CA). Samples were submitted to the MSU Center for Microbial Ecology for T-RFLP analysis. DNA samples from two C3Bir IL-$10^{-/-}$ infected mice, three C3Bir IL-$10^{-/-}$ uninfected mice and one mouse each from the infected and uninfected C3H/HeJ groups repeatedly had failed 16S rRNA PCRs due to low DNA yields and were not included in T-RFLP analyses.

Quantitative Real-Time PCR.

DNA extracted from the proximal colon (as previously described) was used as the template in species specific Q-PCR assays. *Clostridium* Group I, *Clostridium* Group XIV and *Bacteroides* assays were performed using the primer sequences from Rinttila *et al* (Rinttilä, Kassinen et al. 2004). *Escherichia coli* Q-PCR assays were performed using primer sequences from Khan *et al* (Khan, Gannon et al. 2007). In all assays, 25 µl reactions were performed in triplicate for each sample with SYBR® Green PCR mastermix (Applied Biosystems, Foster City, CA), 12.5 pm of bacterial specific primers and 50 ng of sample DNA. All Q-PCR assays included a 7-point standard curve run in triplicate ($R^2 > 0.90$) and three non-template controls

containing all other reaction components. Q-PCRs were analyzed using a Bio-Rad iQ5 Real-Time PCR Detection System (Bio-Rad, Hercules, USA).

Statistical Analysis

Statistical Analysis of Histological Scoring. Preliminary analyses of histopathological scoring data were conducted using the Kruskal-Wallis nonparametric one-way analysis of variance. If the result indicated that statistically significant differences existed among groups in an analysis, comparisons between groups were conducted using Fisher's exact test (http://www.danielsoper.com/statcalc/calc29.aspx). Thus, overall ileocecocolic junction scores from *T. muris* infected mice were compared to those of uninfected mice using Fisher's exact test. Scores were grouped in the two-way table so that mice that had scores that fell into grade 0 (scores of ≤9) formed one class and mice that had scores that fell into grades 1 and 2 (scores of ≥10) formed the second class. After two-tailed *P* values were calculated using Fisher's exact test, the Holm step-down procedure was used to apply the Bonferroni correction for multiple comparisons (Ludbrook 1998). The null hypothesis was rejected if the *P* value was ≤0.05.

T-RFLP analysis. T-RFLP peaks with a height of 50 fluorescent units or greater were included in analyses and reported as fragment size Operational Taxonomic Units (OTUs) (Kitts 2001). PAST software (version 1.93) was utilized to generate cluster diagrams, and to conduct ANOSIM and SIMPER analyses (Hammer, Harper et al. 2001). The composition and structure of the microbial communities of mice were compared using Dice (Sorenson) and Bray-Curtis

diversity indices, respectively. All analyses included 10,000 permutations, and the statistical null hypotheses were rejected if $P \leq 0.05$.

Quantitative Real-Time PCR analysis. Bio-Rad iQ5 PCR detection system software (Bio-Rad, Hercules, USA) was used to calculate the Ct value for each reaction and the mean Ct value for each set of triplicates. Using the corresponding standard curve, the starting quantity (SQ) of DNA was calculated for each reaction and the mean SQ for the triplicate reactions for each sample. The mice were then grouped according to genotype (IL-$10^{+/+}$ or IL-$10^{-/-}$) and experimental end-point (early[i.e., prior to day 29 PI] or late[40 PI]). The mean SQs of DNA for each mouse within a specific treatment and end-point group were then calculated. The mean SQs of DNA were compared using ANOVA. The null hypothesis was rejected if $P \leq 0.05$.

RESULTS

Mice were free of colitogenic bacteria. All fecal samples taken from (1) experimental mice before and after the experiment of induced infections with *T. muris* (2) healthy mice from the breeding colony and (3) mice which developed spontaneous colitis tested negative by PCR for *Campylobacter* spp., *Helicobacter* spp., *C. rodentium*, and *E. faecalis*. All DNA samples extracted from stomach, jejunum, cecum, and colon tissues and feces of the sentinel mice in the containment facility gave negative results in the four PCR assays.

***Trichuris* infected and uninfected C3Bir IL-10$^{-/-}$ mice developed clinical signs of typhlocolitis with some requiring early euthanasia, while C3H/HeJ mice of both infection groups remained disease free.** C3Bir IL-10$^{-/-}$ mice have been observed to develop spontaneous colitis by multiple laboratories (Sellon, Tonkonogy et al. 1998), including our own. *Trichuris* infected and uninfected C3Bir IL-10$^{-/-}$ mice with severe disease necessitating early euthanasia had clinical signs including hunched posture, decreased activity, rough hair coat, dehydration, and increased frequency and volume of diarrhea. As shown in Figure 4.6, 0/5 (0%) female and 3/6 (60%) male *T. muris* infected C3Bir IL-10$^{-/-}$ mice were euthanized early due to severe clinical signs of disease whereas 1/6 (16.7%) female and 3/5 (60%) male uninfected mice displayed clinical signs requiring early euthanasia. Unlike *T. muris* infected mice, uninfected mice necessitating early euthanasia usually had raw, severely ulcerated perineal tissue due to chronic watery diarrhea.

C3Bir IL-10$^{-/-}$ mice infected with *T. muris* had severe diffuse gastrointestinal inflammation compared to uninfected mice. As shown in Figure 4.7a and Figure 4.7b, C3Bir IL-10$^{-/-}$ mice infected with *T. muris* had inflammation involving a larger portion of the GI tract— including the small intestine (starting as proximal as the anterior ileum), the cecum and the proximal colon—when compared to that of uninfected C3Bir IL-10$^{-/-}$ mice or C3H/HeJ mice. In 3/11 C3Bir IL-10$^{-/-}$ infected mice the distal colon was also involved. The gross pathology observed in these GI tracts included enlarged, thickened wall and/or fluid filled small and large intestines.

Infected C3Bir IL-10$^{-/-}$ mice had significantly higher GI lesion scores than uninfected C3Bir IL-10$^{-/-}$ mice and both infected and uninfected C3H/HeJ mice. Figure 4.8 shows the differences in the overall histopathological score for all genotypes and infection groups. *Trichuris* infected C3H/HeJ mice had significantly higher histopathology scores than those that were uninfected, but C3Bir IL-10$^{-/-}$ infected and uninfected mice did not differ significantly in the severity of their ileocecocolic junction scores, as judged using histological scoring. Although these mice did not differ in their histopathology scores, there were differences in the cell types present in these two groups (Figure 4.9). C3Bir IL-10$^{-/-}$ infected mice had a predominantly neutrophilic response, particularly in the luminal contents, when compared to C3Bir IL-10$^{-/-}$ uninfected mice. Additionally, a significantly higher percentage of the C3Bir IL-10$^{-/-}$ infected

mice had monocytic infiltrates in the epithelial layer of the ileocecocolic junctions where the majority of worms reside.

Assessment of histopathological lesions revealed signs of peritonitis and extraintestinal bacteria in C3Bir IL-10$^{-/-}$ infected mice. Assessment of hematoxylin and eosin stained histological sections of the ileocecocolic junctions showed signs of inflammation and bacteria on the serosal side of the tissues in some *T. muris* infected C3Bir IL-10$^{-/-}$ mice (Figure 4.10). Mice in this group had monocytic inflammatory cells adherent to the serosal side of the GI tissue with or without detectable bacteria in the inflammatory exudates. To further confirm the presence of bacteria in these locations, Gram stains were performed on the tissue sections; Gram positive rods were seen among the inflammatory exudates on the serosal sides of 7/11 (63.6%) ileocecocolic junction mouse sections. Additionally, in one of these mice a cross-section of a mature *Trichuris* worm was observed to have developed under the submucosa (Figure 4.10D). Mature adult *T. muris* worms were observed in close association with neutrophilic inflammatory exudates within the colonic lumen of 9 out of 11 C3Bir IL-10$^{-/-}$ infected mice.

C3H/HeJ and C3Bir IL-10$^{-/-}$ *T. muris* infected mice and uninfected C3Bir IL-10$^{-/-}$ mice that developed spontaneous colitis had enlarged spleens compared to uninfected mice. Spleens were weighed at necropsy and the spleen to body weight ratio calculated. A section of splenic tissue was evaluated histologically for enlargement of the periarteriolar lymphatic sheaths and increased extramedullary hematopoietic tissue. As shown in Figure 4.11, the only statistically

significant differences were noted between the C3Bir IL-10$^{-/-}$ infected and C3H/HeJ infected mice ($P \leq 0.05$). In histological evaluation of sections of splenic tissue, infected C3Bir IL-10$^{-/-}$ mice were not significantly different from uninfected mice, but differences in the overall histological score were noted between C3H/HeJ infected and uninfected mice.

Terminal Restriction Fragment Length Polymorphism analyses revealed significant differences in the structure of microbial communities between *T. muris* infected vs. uninfected C3Bir IL-10$^{-/-}$ mice as shown in Figure 4.12 Microbial communities in the proximal colons of C3Bir IL-10$^{-/-}$ and C3H/HeJ mice differed both qualitatively and quantitatively (Dice: $R = 0.33$, $P < 0.001$; Bray-Curtis: $R = 0.54$, $P < 0.0001$; Figure 4.12). Infection with *T. muris* significantly affected the structure (proportions of OTUs) of colonic microbial communities (Bray-Curtis: $R = 0.15$, $P = 0.019$), but not their composition (Dice: $R = 0.04$, $P = 0.238$). Furthermore, when C3Bir IL-10$^{-/-}$ and C3H/HeJ mice were considered separately, it was apparent that *T. muris* affected the structure of microbial communities of C3Bir IL-10$^{-/-}$ mice ($R = 0.21$, $P = 0.027$), but not those of C3H/HeJ mice ($R = 0.08$, $P = 0.148$). Additionally, among uninfected C3Bir IL-10$^{-/-}$ mice, there were neither qualitative ($R = -0.16$, $P = 0.802$) nor quantitative differences ($R = -0.02$, $P = 0.521$) in the structure of microbial communities of mice that developed colitis and those that did not.

Terminal Restriction Fragment Length Polymorphism analyses did not reveal significant differences in the structure of the microbial communities between mice with and without

colitis. As shown in Figure 4.13A (Dice analyses) and B (Bray-Curtis analyses, there was not a difference in the composition or structure of proximal colon microbial communities among colitic and non-colitic C3Bir IL-$10^{-/-}$ mice (Dice: R = 0.02, P =0.278; Bray Curtis: R = 0.03, P = 0.2687).

Species-specific Quantitative Real-Time PCR (Q-PCR) shows trends in the amounts of specific bacteria in infected vs. uninfected mice. Species specific Q-PCR did not show any differences in *Bacteroides* spp., *E. coli* spp., or *Clostridium* Groups I and XIV spp. between C3H/HeJ and C3Bir IL-$10^{-/-}$ infected mice and uninfected controls (Figure 4.14). Although there were not any significant changes in the specific bacteria detected by our species-specific Q-PCR analyses, two general trends were noted. Based on the *Clostridium* group I and *Bacteriodes* spp. Q-PCR specific analyses, both C3H/HeJ and C3Bir IL-$10^{-/-}$ groups of *Trichuris*-infected mice tended to have lower amounts of these groups in the proximal colon. *Clostridium* Group XIV and *E. coli* assays were also performed, but no trends were evident (data not shown).

DISCUSSION

In these studies, we have demonstrated that infection of C3Bir IL-$10^{-/-}$ mice that carry a spontaneous mutation in TLR4 and the *cdcs-1* colitis susceptibility allele infected with *T. muris* stimulate a significant GI inflammatory response. This leads to severe clinical signs of disease in some mice requiring early euthanasia, but not at a higher rate than those uninfected mice of the same genotype that developed spontaneous colitis. We also assessed the effect of *T. muris* infection on congenic IL-10 sufficient C3H/HeJ mice, which developed a less severe GI inflammatory response to the helminth with subclinical disease and no deaths. In a second experiment, colitis susceptible mice of the same genotype were followed over time to document the development of spontaneous colitis and to compare the composition and structure of their microbial communities with mice of the isogenic strain without colitis. We were able to demonstrate that the microbial community structure differed in the *T. muris* infected C3Bir IL-$10^{-/-}$ mice compared to their congeners that were uninfected, even when their GI lesions appeared to be similar. Furthermore, we have shown that mice experiencing spontaneous colitis did not experience significant changes in their colonic microbiota compared to isogenic mice without lesions. Overall, this work supports our hypothesis that mice infected with *T. muris* develop distinctive microbial communities in the colon, and that these alterations cannot be solely attributed to the presence of inflammation in the gastrointestinal tract.

It is presumed that T-RFLP OTUs typically correspond to fragments from different bacteria and aid in assessing populations that were previously difficult to assess due to large numbers of unculturable bacteria (Liu, Marsh et al. 1997). Compared to whole microbial community genome sequencing, this technique is an inexpensive means for analyzing large

numbers of samples. In this study, using T-RFLP OTUs, we demonstrated that C3Bir IL-10$^{-/-}$ mice infected with *T. muris* have colonic microbial communities that differ quantitatively from the uninfected controls. This indicates that the microbial communities of the infected mice differed significantly from the uninfected mice. However, the specific mechanism and causal relationship between the *Trichuris* infections and the changes in the host's colonic microbiota await further studies. There are a number of likely candidates that may mediate these changes. The excretory secretory products (ESP) of *Trichuris* fourth stage larvae and adults have been previously reported to have antibiotic properties (Abner, Parthasarathy et al. 2001; Abner, Hill et al. 2002), which could provide a rapid mechanism for change in the microbiota. *Trichuris suis* also enhanced *Campylobacter jejuni* infections in gnotobiotic (Mansfield, Gauthier et al. 2003) and specific pathogen free pigs (Mansfield and Urban 1996); in both experiments the presence of the worm in its third larva stage increased mucus production, which is known to enhance growth of this bacterium (Hugdahl, Beery et al. 1988). Additionally, *Trichuris* infections have been reported to induce significant inflammatory responses in some mice (Schopf, Hoffmann et al. 2002) which has been reported to alter the colonic conditions and potentially affect which members of the community predominate. More work is needed to determine specific whipworm/ bacterial interactions in the colon.

With this work we were able to identify an association between a change in microbial communities and experimental infections with *T. muris* in our murine models. Although this association was noted, it cannot be definitively determined whether *Trichuris* adults or larvae directly induced the changes in microbial communities or whether the changes were affected by some other mediator(s). It is possible that the *Trichuris* worms caused inflammatory responses which then led to changes in the microbiota and thus differences in the T-RFLP OTUs. However,

we can ascertain from this work that uninfected C3Bir IL-10$^{-/-}$ mice which developed spontaneous colitis that necessitated early euthanasia did not, as a whole, have T-RFLP OTUs that differed significantly from the other C3Bir IL-10$^{-/-}$ mice which did not develop spontaneous colitis. When ANOSIM analyses were performed on the mice in the experimental study by grouping them based on histopathological lesion scores at the ileocecocolic-junction (data not shown), there was not a significant difference between the mice with inflammation versus those without inflammation regardless of infection group other than those differences seen due to *Trichuris* infection status.

To further assess the affect of colitis on the microbial community composition, uninfected C3 Bir IL-10$^{-/-}$ mice within our specific pathogen free barrier breeding colony were monitored for the development of spontaneous colitis. The proximal colon microbiota of mice that developed spontaneous colitis were then compared to similar mice from the same breeding colony that did not have any signs of colitis. In this study the mice with colitis did not have any significant changes to their proximal colon microbiota as assessed using the obtained T-RFLP OTU. Taken together, the results of these two experiments suggest that the changes observed in the microbial community in *Trichuris* infected mice were specific to the parasitic infection versus simply the affect of acute or chronic inflammation in local tissues.

SIMPER analyses showed that a single OTU alone is responsible for 53% of the 66% average dissimilarity between the *Trichuris* infected and uninfected C3 Bir IL-10$^{-/-}$ mice. Previous work from Nagashima et al (Nagashima, Hisada et al. 2003), suggests that this OTU may correspond with *Clostridium* spp. In an attempt to identify members of the microbial

community that were responsible for the differences noted with T-RFLP, species-specific Q-PCR assays were performed for *Clostridium* Group I, *Clostridium* Group XIV, *Bacteroides* spp. and *E. coli*. Although not statistically significant, it appeared that the *Trichuris* infected mice tended to have smaller amounts of *Clostridium* Group I and *Bacteroides* spp. DNA. At this time, although we know the fragment sizes/OTUs from the T-RFLP analyses that changed between these groups, we do not know what specific bacterium they represent. Further analyses, using either clone libraries, sequencing of 16S PCR products, or microbiome sequencing from the proximal colon of these mice could identify which organisms differed between the two groups. Furthermore, future experiments are needed to identify the functional significance of these changes and their effects on the host.

Finally, we believe the study design used here was adequate to test the hypothesis. We recognize that microbial communities have been shown to vary significantly between individuals even between inbred mice kept in the same colony and fed the same diet (Friswell, Gika et al. 2010). This has necessitated the use of longitudinal study designs where an individual is re-sampled and used as its own control. This type of design allows for smaller sample sizes and analysis using principle component analysis (Wang, Ahrné et al. 2004). Our goals in this work were different. We chose to use large sample sizes of 10 mice per group in a cross sectional design with careful control of housing, pathogen status and diet with the goal of revealing major differences between treatment groups if they were present. Thus, the differences in the microbial community of *Trichuris* infected mice versus other treatment groups demonstrated here is robust and consistent with previous studies showing interactions of this helminth with specific community members.

TABLES

Table 4.4. Experimental design for the study of the effect of *T. muris* on proximal colon microbiota. Mice were given either embryonated *T. muris* eggs or were uninfected and given PBS as a sham inoculation. Groups were age and sex matched.

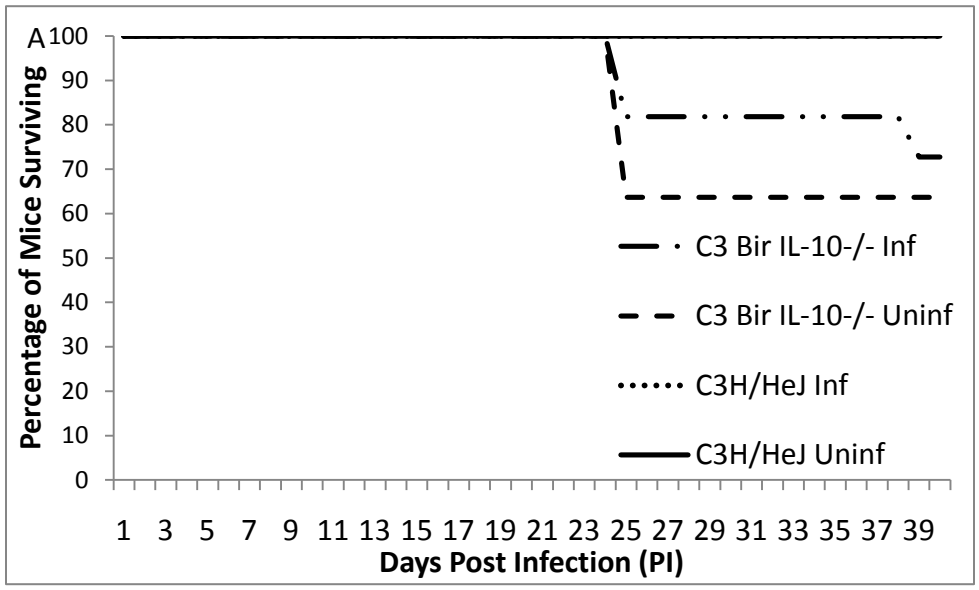
Experimental Group	C3H/HeJ (M,F)	C3Bir IL-10$^{-/-}$ (M,F)
100 Embryonated *T. muris* eggs	10 (5,5)	11 (6,5)
PBS/Sham Inoculation	9 (5,4)	11 (5, 6)

Table 4.5. Experimental design for the study of colonic microbiota in colitic and non-colitic mice. Mice were monitored within the breeding colony for the development of spontaneous colitis. At the time of euthanasia of mice with colitis, if available, a non-colitic mouse that was similar in terms of age and sex was also euthanized.

Experimental Group	Male	Female
Colitic	11	17
Non-Colitic	13	8

FIGURES

Figure 4.6 Survival Curve and Judged Clinical Signs of Disease (A) Survival curve. All mice were infected on day 0 and monitored one to four times a day for clinical signs for the duration of the experiment. Mice were euthanized as dictated by clinical sign scores. Some C3Bir IL-10$^{-/-}$ uninfected mice developed spontaneous colitis and had to be euthanized early. All remaining mice were euthanized at day 40 Post Infection (PI). (B) Graded score based on clinical signs of disease in the C3Bir IL-10$^{-/-}$ infected and uninfected mice. Any mouse with a score over 8 was immediately euthanized.



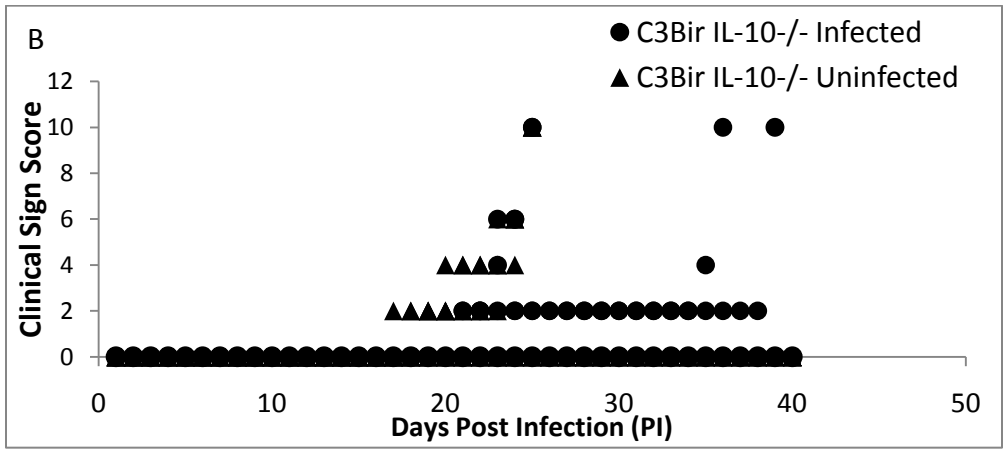
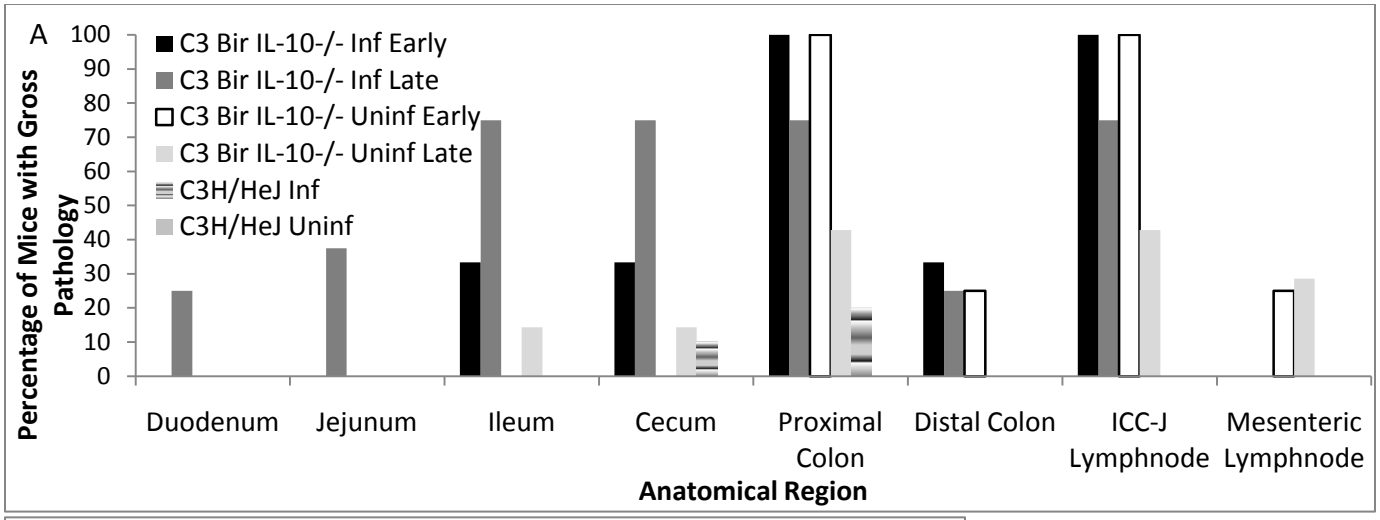


Figure Legend 4.7. Gross pathology noted at time of necropsy. All organs were evaluated for gross pathology at the time of necropsy. Ileocecocolic (ICC) lymph node observations were included as a measure of immune stimulation, particularly from the gastrointestinal tract. The first graph (A) shows the distribution of pathology noted in both genotypes and infection groups and differentiates between those mice that were euthanized early due to clinical signs of disease and those which were euthanized at day 40 PI regardless of clinical signs of disease. The second graph (B) shows the prevalence of pathology between the small and large intestine in infected and uninfected mice.

Figure 4.7 Continued



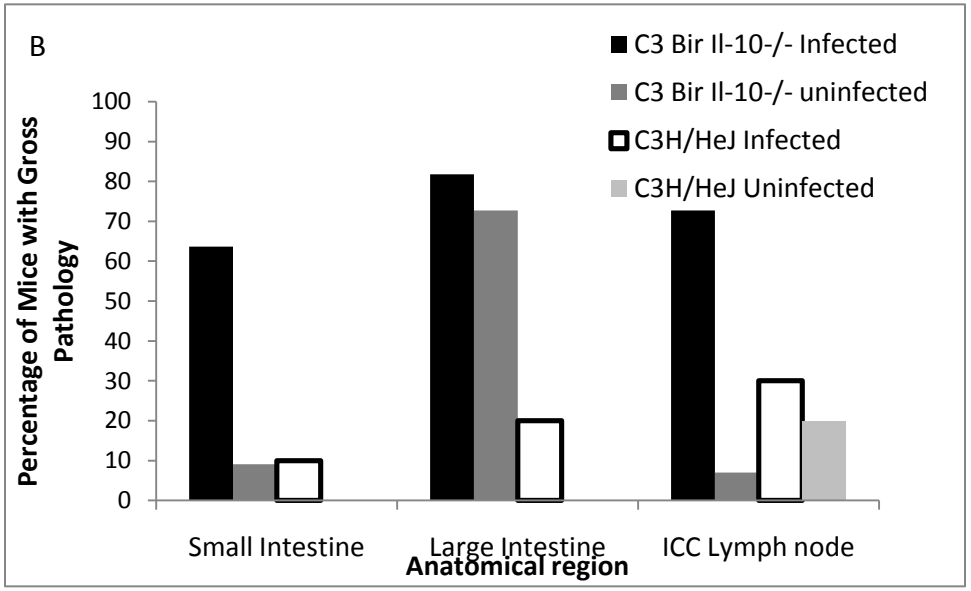


Figure 4.8. Overall histological lesion score from the H and E stained ileocecocolic junction sections.

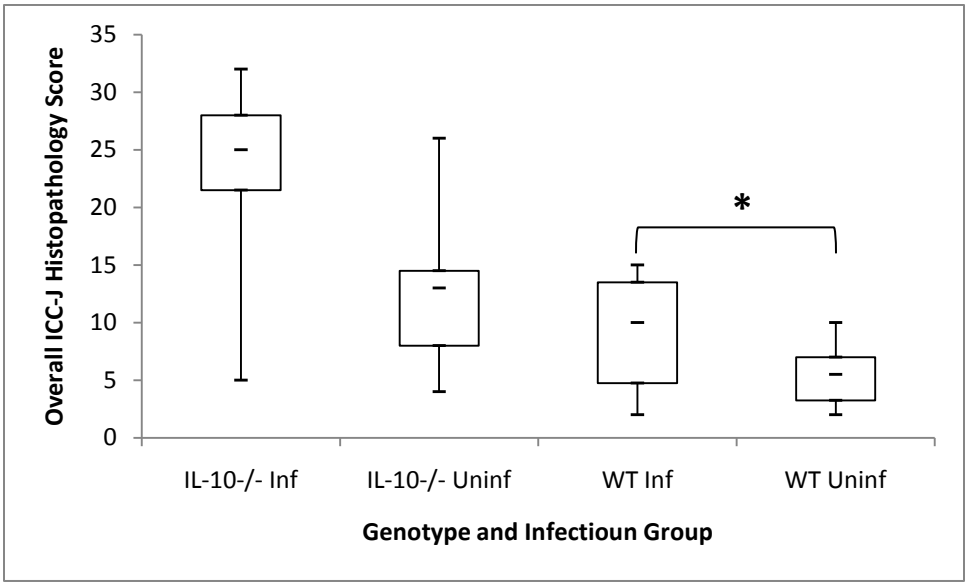


Figure 4.9. Inflammatory cell types present in histological evaluation.

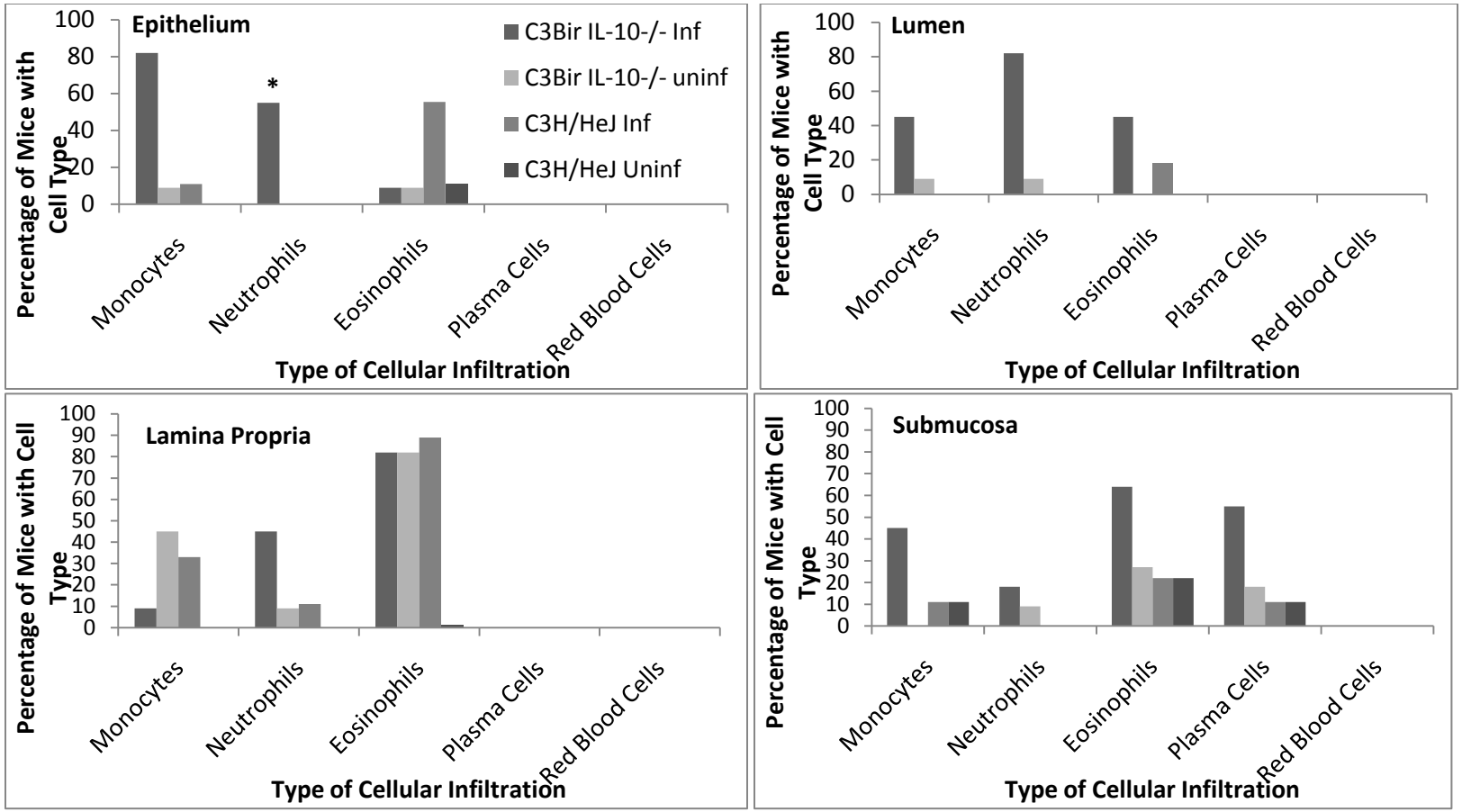


Figure Legend 4.10 Histology of the ileocecocolic junction. All images were obtained at 20x magnification. A. Normal mouse cecum. B. Inflammatory cells present on the serosal side of the cecal tissue. C. Inflammatory cells with bacteria on the serosal side of the tissues. D. *Trichuris* adult worm (W) under the submucosa of the cecum (marked with arrow). E. Neutrophilic exudates surrounding female adult *Trichuris* worm in the lumen of a mouse cecum (outlined with arrows). F. Gram stain of an ileocecocolic junction section of a normal mouse. Note smooth serosal surface free of bacteria and inflammation. G. Gram stain of a section from the ileocecocolic junction of a mouse with rods (arrows) among the inflammatory cells on the serosal side of the cecum.

Figure 4.10 Continued

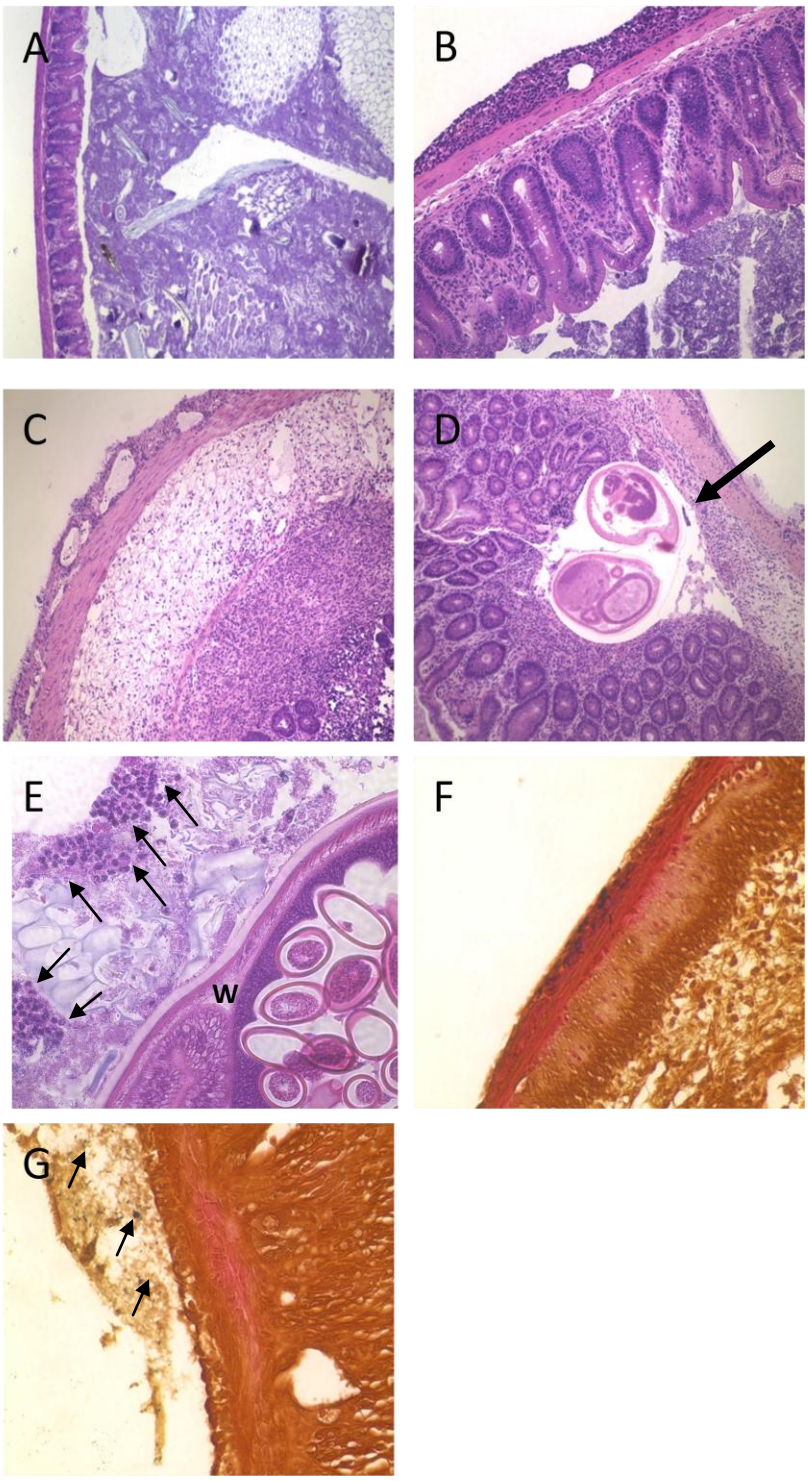
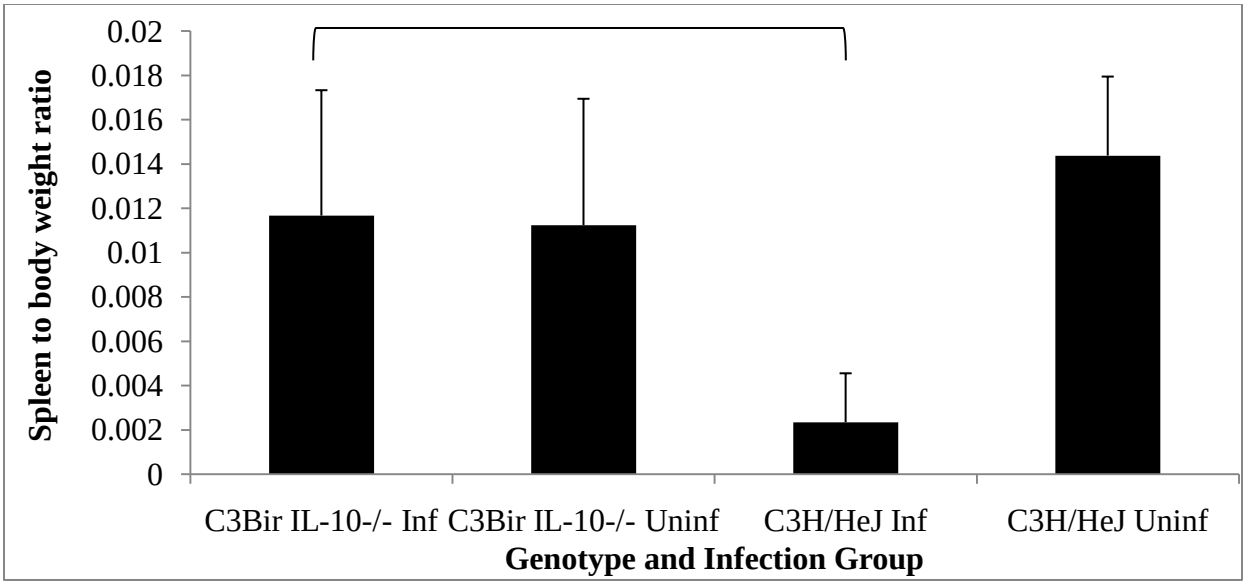


Figure 4.11. Evaluation of spleen weights and histological lesions. A. Weight of spleens at time of necropsy. The only statistically significant differences were noted between the C3Bir IL-$10^{-/-}$ infected and C3H/HeJ infected mice ($p \leq 0.05$). B. Overall histological lesion scores of spleens revealed statistically significant differences ($p \leq 0.05$) between all groups except C3Bir IL-$10^{-/-}$ infected and uninfected mice.

Figure 4.11 Continued



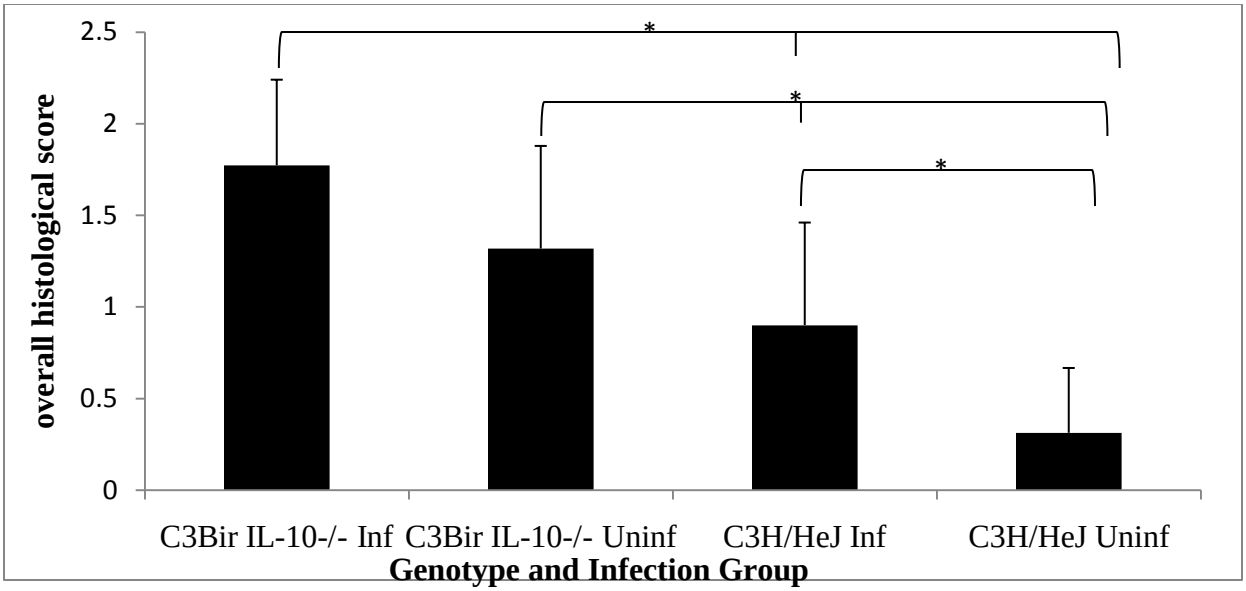


Figure Legend 4.12. non-metric multi-dimensional scaling plots (nMDS) of ANOSIM of T-RFLP patterns for C3H/HeJ (WT) and C3Bir IL-10$^{-/-}$ (IL-10$^{-/-}$) infected and uninfected mice. (A) Bray Curtis analysis of uninfected C3H/HeJ and C3Bir IL-10$^{-/-}$ mice. Bray Curtis ANOSIM P = 0.0003 and R = 0.4821. (B) Bray Curtis analysis of *T. muris* infected and uninfected C3Bir IL-10$^{-/-}$ and C3H/HeJ mice with no regard to genotype. Bray Curtis ANOSIM p = 0.0349 and R = 0.104.

Figure 4.12 A Continued.

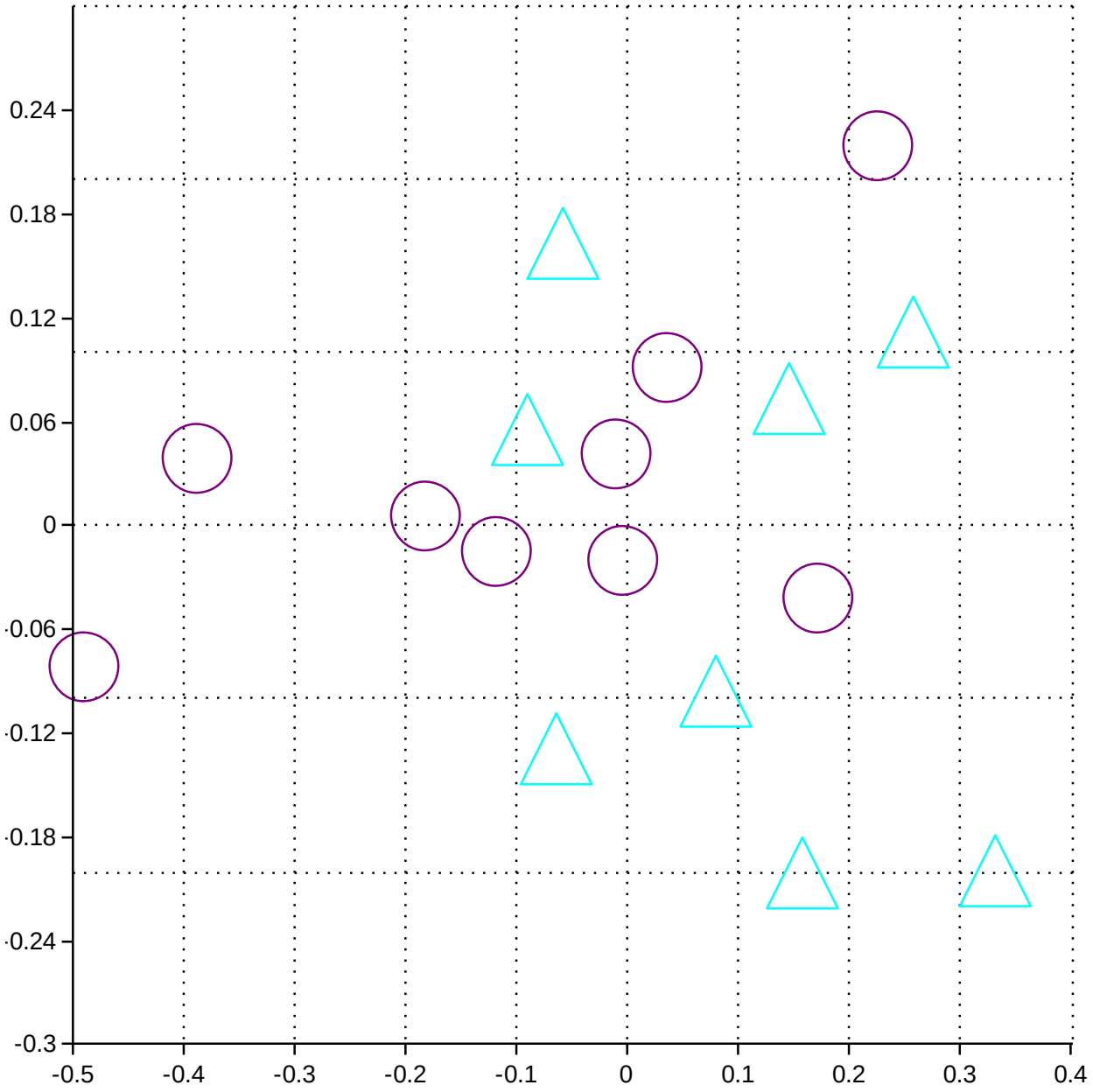


Figure 4.12 B Continued.

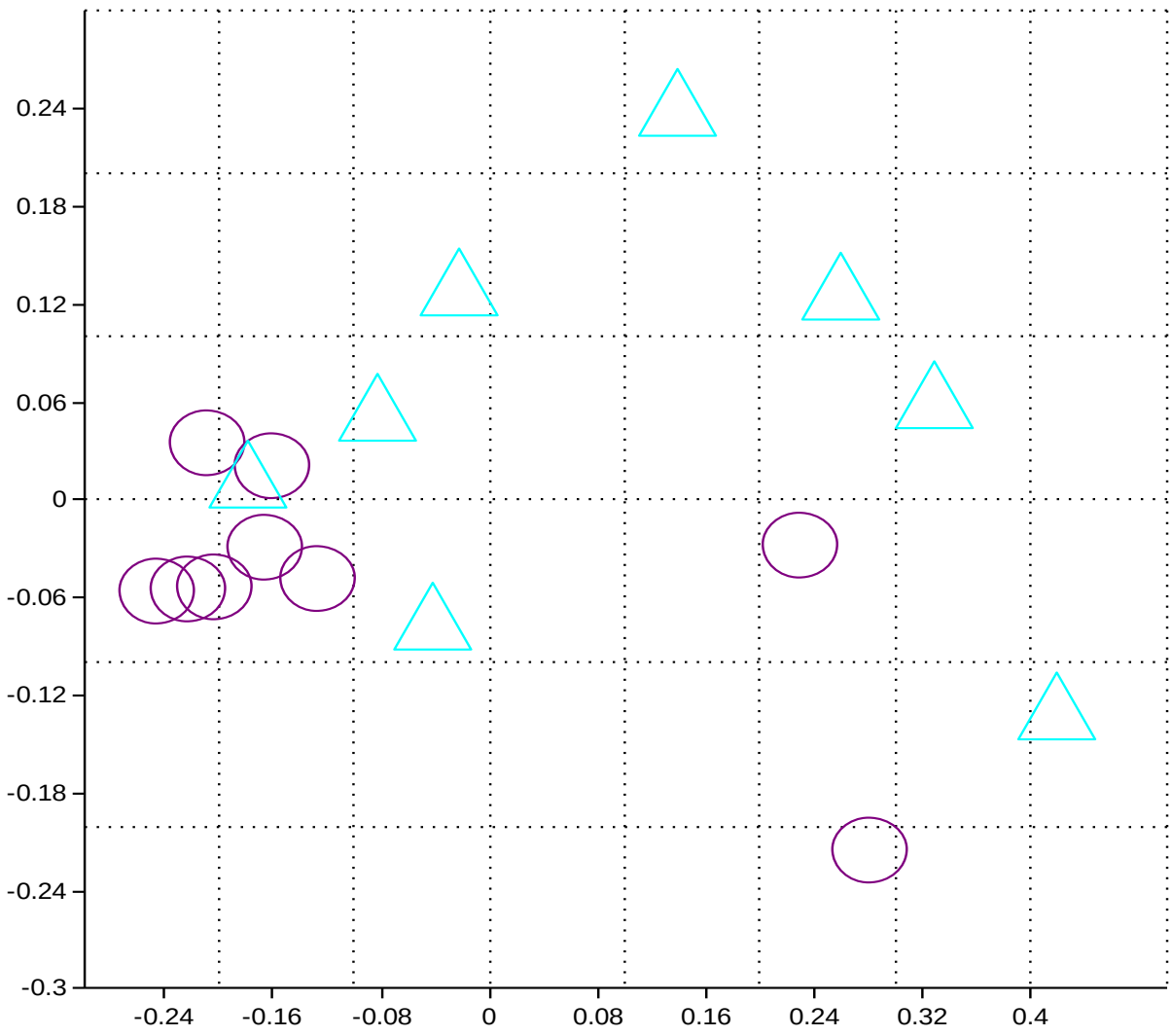


Figure 4.13 nMDS plots comparing C3 Bir IL-10$^{-/-}$ mice with and without spontaneous colitis in a breeding colony. All analyses were performed using Analyses of Similarity (ANOSIM). Mice with spontaneous colitis = Red Cross, mice without spontaneous colitis = purple circles. (A) Dice analyses of mice with and without spontaneous colitis and (B) Bray-Curtis analyses of mice with and without spontaneous colitis. KO = IL-10$^{-/-}$ mice, F = female, M= male, C = colitic, N = non colitic.

Figure 4.13A Continued

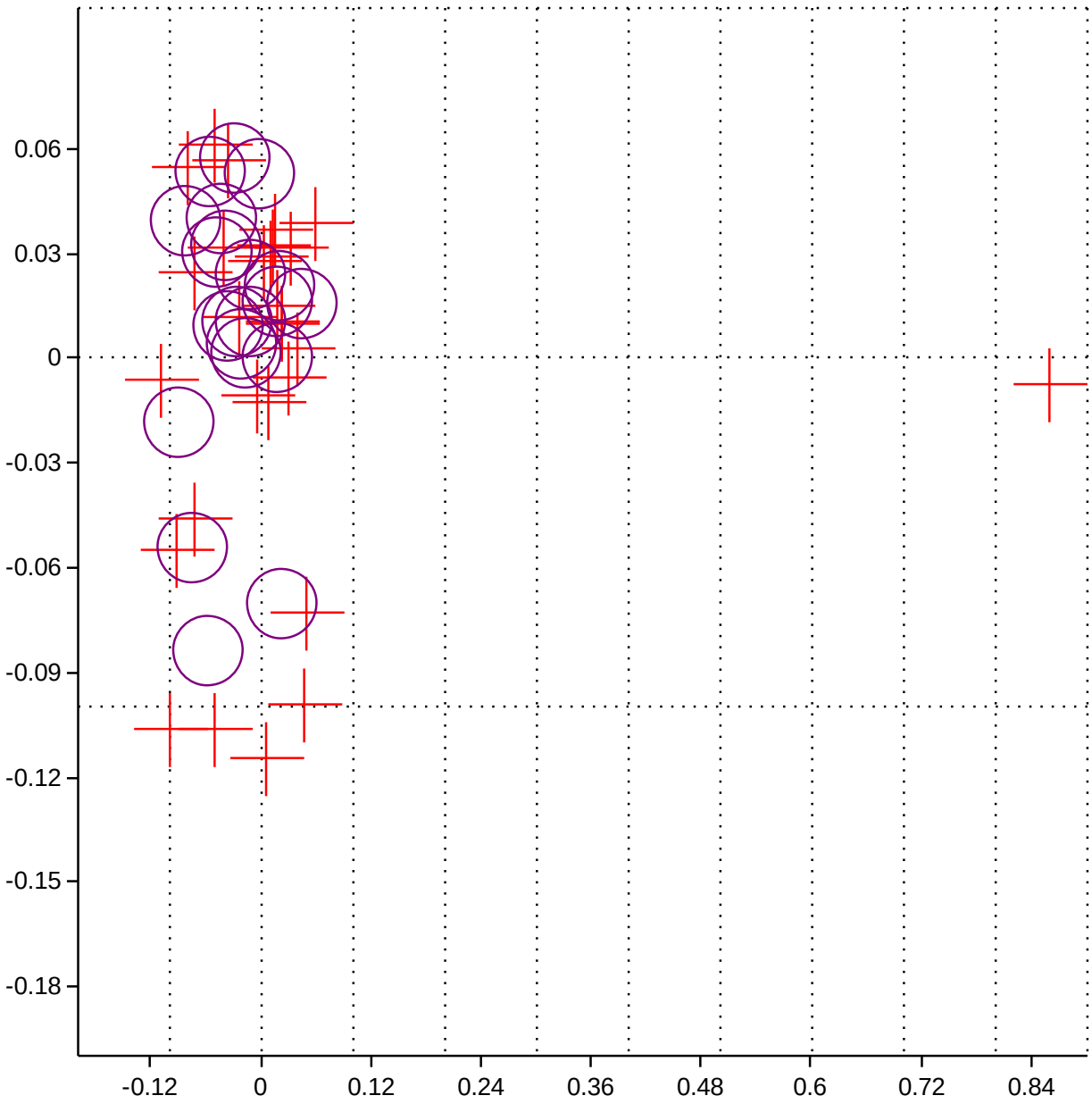


Figure 4.13 B. Continued

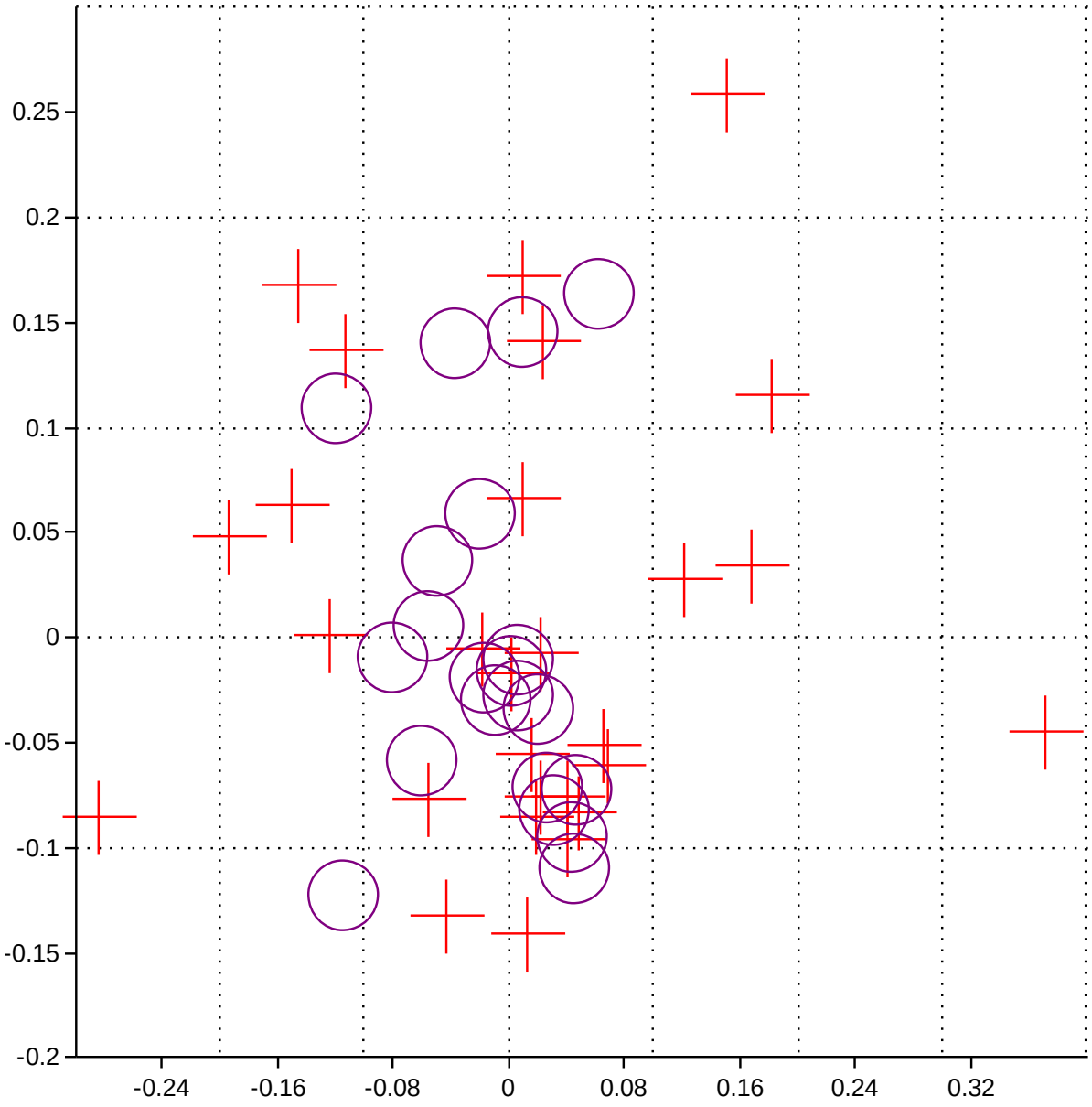
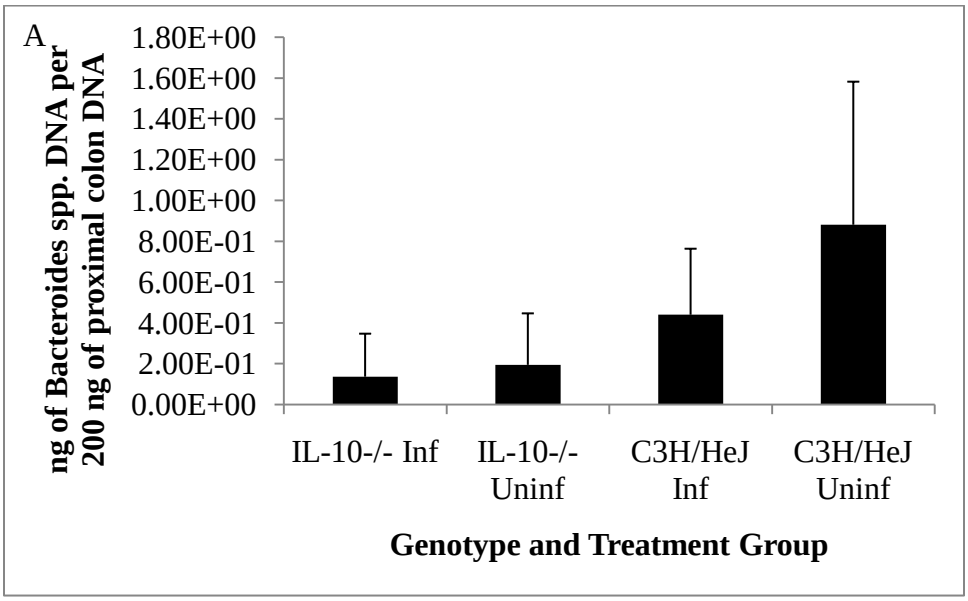
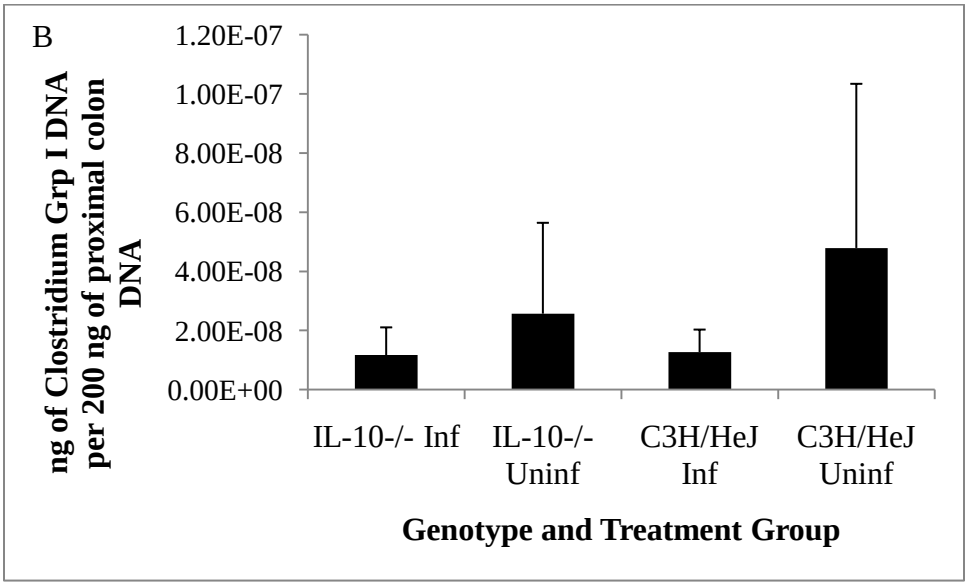


Figure 4.14. Quantitative Real Time PCR (QPCR) for *Bacteriodes-Prevotella-Porphyromonas* (panel A) and *Clostridium* group I (panel B).





CHAPTER 5.

Summary and Future Directions

SUMMARY

Inflammatory Bowel Disease (IBD) is a serious chronic inflammatory condition that not only affects the gastrointestinal tract, but is known to have serious extra-intestinal manifestations (Bernstein, Blanchard et al. 2001; Cho 2008). Approximately 1.1 million people have been diagnosed with IBD, and this number is reported to be on the rise (Bonen and Cho 2003). Despite this prevalence, the exact cause for IBD has not been identified; the task of which is muddled due to the hypothesized multi-factorial nature of the disease (Hugot 2004). Recently, the parasite *Trichuris suis* was proposed as a treatment for IBD in individuals that were otherwise refractory to standard treatments (Summers, Elliott et al. 2003; Summers, Elliott et al. 2005). In these small-scale clinical trials *Trichuris* was reported to be a very effective and safe treatment for IBD with no adverse side effects reported. Despite its apparent success as a treatment for IBD the mechanism by which it works is still unknown. Additionally, the possibility for adverse side effects had not been previously investigated. This thesis was aimed at using mouse models to address relevant hypotheses specific to the use of *Trichuris* as a treatment for IBD.

The body of this thesis work was focused on developing mouse models for studying several important aspects of the use of *Trichuris* as a treatment for IBD. The first experimental work began by investigating the application of axenic and accurate doses of *Trichuris muris* eggs to the laboratory mouse. There were several major flaws in previous experimental challenge studies based on technical problems with handling and delivering the infectious first larval stage *Trichuris* to mouse models. Although *T. suis* challenge doses were reported to be administered "in a sterile manner", a careful search of the older literature on this helminth showed that the

mechanism by which the eggs were sterilized was not published or even described in general terms in later articles. Careful screening of early literature also showed that investigators tended to administer overwhelmingly high doses of *Trichuris* in mouse studies with little focus on dose response effects of this helminth (Else and deschoolmeester 2003). Therefore, one focus in the first chapter was to explore the dose response effects of orally administered axenic *T. muris*. We sought to mimic conditions of the human clinical IBD treatment trials as closely as possible in our studies by using a single oral dose, and also to ensure that our results were not complicated by the potential administration of contaminants or mixed infections. It is well known that infectious stage *Trichuris* eggs are exceedingly tough with a net negative surface charge. The major contaminants expected under standard laboratory conditions were bacteria and fungi. Thus, in this work a means to flash sterilize the surface of eggs immediately prior to challenge infection that was both effective and safe for the mice was devised. We determined that washing the eggs in 6.25% Clorox followed by several washes in a sterile balanced salt medium resulted in sterile doses as judged using both culture and non-culture based techniques. Another major problem with previous methods was that batches of eggs used for challenge studies often varied in viability. This occurred mainly due to the collections methods for eggs from adult *Trichuris* female worms as well as the need to store the doses for long periods before use. Thus, we investigated methods for administering accurate doses – both in terms of preparation of the inocula and also in terms of a delivery system. Here, we found that using a combination of propidium iodide (PI) staining and *in vitro* hatching assays allowed for a more precise estimation of the inocula viability. Furthermore, we determined that metal gavage feeding needles were the device that most accurately delivered the eggs to the mice. The outcome of this work was a publication (Chapter 2) that described in detail the best practices for handling and delivering *T.*

muris to the laboratory mouse as well as the dose response relationships of the commonly used Beltsville strain of *T. muris* in three inbred strains of mice the non-obese diabetic (NOD), C57BL/6 IL-$10^{-/-}$, and C3Bir IL-$10^{-/-}$ mice. This work was published in Parasitology Research in 2010.

The second experimental chapter (Chapter 3) focused on examining the adverse effects of *T. muris* on C57BL/6 IL-$10^{-/-}$ mice observed at even low doses. This work was designed to model a portion of the population with IBD that might be expected to react negatively to *Trichuris* treatments. We hypothesized that, particularly due to the multi-factorial nature of IBD, if applied to a large population of individuals *T. suis* treatments could result in adverse side effects in some people. To further investigate this possibility we infected C57BL/6 IL-$10^{-/-}$ mice with *T. muris*. C57BL/6 IL-$10^{-/-}$ mice were originally chosen due to their previously reported morbidity and mortality when infected with *T. muris* (Schopf, Hoffmann et al. 2002) and due to previously published genome wide association study (GWAS) reports linking IL-10 deficiencies to some forms of IBD (Leach, Davidson et al. 1999; Farmer, Sundberg et al. 2001). In our studies we found that *T. muris* caused significant adverse side effects in a dose and sex dependent manner, which were previously uncharacterized.. Although both sexes were adversely affected at high doses (75 ova) of *T. muris*, at lower doses the female mice were largely unaffected in terms of morbidity and mortality. Adverse side effects, based on gross and histopathological evaluation included severe peritonitis with or without gut perforation by *T. muris*, splenomegaly, and severe gastrointestinal inflammation characterized by a neutrophilic response. In order to mimic the usual treatment scenarios in IBD patients, we infected C57BL/6 IL-$10^{-/-}$ mice with *T. muris* that were concurrently treated with prednisolone or metronidazole.

These treatments were chosen because they are commonly used in IBD treatments, and some patients in the original clinical *Trichuris* treatment trials were concurrently receiving similar interventions. Additionally, we hypothesized that the addition of steroids or antibiotics may counteract some of the previously observed adverse side effects, noted both in our work and that reported in Schopf et al (Schopf, Hoffmann et al. 2002). In this work we found that concurrent treatment with prednisolone or metronidazole did not significantly affect the survival rate of the C57BL/6 IL-$10^{-/-}$ mice when compared to those mice that were infected but not concurrently treated with antibiotics or steroids. Treatment with metronidazole did affect survival rates in *Trichuris* infected mice; a significantly larger number of *Trichuris*-infected mice concurrently treated with metronidazole survived when compared to those that were infected and treated with prednisolone. Additionally, we found that despite the fact that there were differences in the survival rates of these mice, the lesions based on histological evaluation of the stomach, spleen, liver and gastrointestinal tissues were not significantly different among *Trichuris*-infected mice in all treatment groups. We also found that the concurrent treatment of mice with metronidazole or prednisolone did not protect the infected mice from developing severe peritonitis. Finally, we found that infected mice had lesions, as judged using histology, in their gastric tissue, even though this region of the gastrointestinal tract has never been reported as a definitive site for *T. muris* and no evidence for presence of the helminth in the stomach was found in this study. This work has been prepared for publication in the journal Comparative Medicine.

In the last experimental chapters we focused on whether the effects of *Trichuris* on its host were mediated mainly by the effects it exerts on the colon microbial community. Here we use the term microbiota interchangeably with microbial community. We hypothesized that mice infected with *T. muris* have a significantly different microbiota in the proximal colon where the worms

reside. Changes in microbiota in terms of presence/absence and relative abundance of bacterial members along with the expected attendant changes in microbial community functional products and intracellular signaling of the mucosal surface may serve as a mechanism by which *Trichuris* provided amelioration to those individuals with IBD in the reported clinical trials. Before we tested this hypothesis we first further investigated the use of Terminal Restriction Fragment Length Polymorphisms (TRFLPs) in assessing microbiota of the GI tract in mice. At the time of our studies, TRFLP was the most advantageous means for survey of the microbial community particularly for assessing major shifts in community composition over large samples sizes. Therefore, we investigated the use of three different restriction enzymes, *Bsl*I, *Msp*I, *Hae*III and each combination involving two or three of those enzymes, in terms of resolution of quantitative and qualitative differences of the microbial community in the colon of experimental mice. We also assessed the differences between colonic samples with and without fecal contents. This work showed comparable levels of resolution in TRFLP results arising from the three enzymes, and that overall the same results were obtained regardless of which enzyme or enzyme combination was utilized. We also showed that colon samples that included significant fecal material had significantly different TRFLP OTUs when compared to those without significant amounts of fecal material. In fact, we found that samples with fecal material were more like other samples with fecal material from different mice, than the samples without fecal material from the same mouse. Next we assessed the effect of co-housing on proximal colon microbiota in mice over time. We found that the microbial communities in fecal samples of mice from the same parents were more like other mice from those parents regardless of housing together or singularly. Lastly, we tested our hypothesis that *T. muris* infected mice would have a significantly different microbiota of their gastrointestinal tracts. In this study we found that

C3Bir IL-10$^{-/-}$ mice infected with *T. muris* had significantly different microbial communities in their proximal colons when compared to those mice that were uninfected when assessed using T-RFLP. Additionally, we evaluated C3Bir IL-10$^{-/-}$ mice with spontaneous colitis and found that overall there were no differences in the microbial communities in mice that had colitis verses those that did not. Overall, this work supported our hypothesis that mice infected with *T. muris* develop distinct microbial communities in the colon, and that these alterations could not be entirely attributed to the presence of inflammation in the gastrointestinal tract. This answers an important question posed by investigators analyzing the microbial communities in human IBD patients of whether inflammation is solely responsible for changes in the microbial community or whether other specific inciting events such as pathogen infections are ultimately responsible. This work is prepared for submission to The ISME Journal.

FUTURE DIRECTIONS

This thesis was aimed at using mouse models to address relevant hypotheses specific to the use of *Trichuris* as a treatment for IBD. The current studies detailed in this thesis pose several new questions for development into future hypotheses for topics from mouse modeling of IBD treatments to technical challenges to experimental studies posed by the complicated life history of *T. muris*. The paragraphs below highlight proposed next steps from the major findings of my studies.

Testing *Trichuris* treatment modalities in mouse models

Summers and others used *T. suis* ova given by the natural oral route as a treatment in patients with advanced cases of IBD (Summers, Elliott et al. 2003; Summers, Elliott et al. 2005; Summers, Elliott et al. 2005). In this small group of patients positive effects were seen in most and no adverse effects were noted. In the current studies mouse modeling studies, we took the simplest route of observing the effects of *Trichuris* on a host without pre-existing IBD. In future steps it would be interesting to compare and contrast the current results to those of mice with pre-existing IBD given *Trichuris* at various stages of their inflammatory disease process. Therefore, I pose the following questions.

Does *Trichuris* challenge of naïve mice cause the same downstream effects as *Trichuris* treatment of mice with pre-existing IBD?

Previous literature shows us that *Trichuris* has been successfully used as a therapy for inflammatory bowel disease without any reported adverse side effects (Summers, Elliott et al. 2003; Summers, Elliott et al. 2005; Summers, Elliott et al. 2005) and that it can also cause severe

disease in some individuals (Cooper and Bundy 1988; Callender, Walker et al. 1998). Here, in this thesis we have shown that C57BL/6 IL-10$^{-/-}$ mice without pre-existing gastrointestinal inflammation experience high rates of morbidity and mortality when infected with *Trichuris*, and that C3Bir IL-10$^{-/-}$ mice, which are predisposed to developing an IBD-like colitis, do not experience an increased rate of morbidity or mortality when infected with *Trichuris*. But, we have not examined a model which assesses the effects of *Trichuris* on a model with established inflammation comparable to an individual with IBD, which would most similarly model individuals using *Trichuris* as a treatment for inflammatory bowel disease. Even then, the majority of IBD murine models involve using congenic inbred mice, which does not accurately mimic a population of individuals with IBD, which is known to be a multi-factorial disease. From the work reported in this thesis we can ascertain that mice lacking IL-10, but on difference genetic backgrounds (C57BL/6 and C3Bir), have very different outcomes in terms of morbidity and mortality after being challenged with *Trichuris*, and it would be logical to assume that the same would hold true for a human population of patients with Inflammatory Bowel Disease. Despite careful research into mechanisms underlying the effects of whole worm treatments, it may be true that worms will always pose risks to certain individuals in the population with IBD. For that reason I propose the following path for future study.

Can *Trichuris* excretory secretory products substitute for whole worm treatments for IBD?

Patent *Trichuris* infections have been associated with disease in humans (Cooper and Bundy 1988; Callender, Walker et al. 1998) and animals as demonstrated in this thesis and in other work (Rutter and Beer 1975; Schopf, Hoffmann et al. 2002; Mansfield, Gauthier et al. 2003). Additionally, in these studies we noted significant inflammatory responses within

gastrointestinal tissues, including severe peritonitis and perforation of the gastro-intestinal tract associated with infection of some IL-10 deficient mice. One hypothesis is that administration of excretory secretory products (ESP) of *Trichuris* may be sufficient for the induction of a beneficial host response without the mechanical damage caused by the worm forming syncytial tunnels within the gastrointestinal tract and the host's subsequent inflammatory response.

Although we were able to develop a method by which *T. muris* eggs successfully hatched *in vitro* we were not able to sustain them to adulthood. Based on our laboratory investigations the larvae died around day 21 post hatching, while it takes approximately 32 days to reach sexual maturity. Successful maturation of *T. muris* larvae to adulthood, within a controlled laboratory environment could open many avenues to further *Trichuris* research. First, if the adults reached sexual maturity and were able to successfully mate and lay eggs *in vitro*, it would eliminate the need for passage of eggs through an animal/mouse model. This would not only reduce the need for laboratory animals but it would also allow for eggs to be produced in an environment with significantly fewer contaminants.

Secondly, maintenance of adults *in vitro* would allow for the collection of Excretory Secretory Products (ESPs) from the *Trichuris* adults, or larval stages, in an environment without contaminants. Currently, ESPs are collected from adults that first developed in a mammalian host and were then harvested, as adults, from the gastrointestinal tract and kept in an *in vitro* system. ESPs are then purified from contaminants, particularly bacteria which can be troublesome and burdensome.

The creation of an *in vitro* system for growth and development would also allow for observation of and further characterization of different developmental stages. Current methods require mammalian hosts for the development of *Trichuris*, and end-point observations.

Finally, if a component or components of *Trichuris* could be identified as the "active ingredient" for the treatment of IBD, then they could be cultivated *in vitro* which could be collected for administration as a treatment in place of administering the whole live worm to the individuals. One could imagine that this would greatly enhance the applicability and safety of using *Trichuris* as a treatment for IBD in human patients and we hypothesize that the identification and administration of *Trichuris* ESP will result in amelioration of clinical signs and symptoms, similar to published studies using adult *Trichuris* as a treatment for IBD, with a significantly decreased incidence and risk of adverse side effects when compared to treatments involving patent adult *Trichuris* infections.

Can treatment be administered to rescue moribund C57BL/6 IL-10$^{-/-}$ mice infected with *T. muris*?

In this work we found that C57BL/6 IL-10$^{-/-}$ mice infected with *T. muris* resulted in significant morbidities and mortalities. We also found that administration of metronidazole or prednisolone to infected mice did not significantly decrease the rate of mortality or pathological changes to gastrointestinal tissues, when compared to infected but untreated mice. If *Trichuris* is to be used as a treatment for IBD, it would be advantageous to better understand the possibility of adverse side effects and those changes and adversities that occur so that they could be rapidly and appropriately addressed in a clinical setting.

The administration of anthelminthics to moribund mice has not been assessed as a method to rescue these mice from mortality. It is currently unknown if the mice would continue to deteriorate due to damage of their gastrointestinal tissues if the active infection was cured, or if the simple absence of the worms would be sufficient to improve their health status.

Additionally, the administration of fluid support, nutritional support and non-steroidal anti-inflammatories (NSAIDs) has not been evaluated in moribund animal models. The administration of neomycin sulfate in the drinking water of C57BL/6 IL-10$^{-/-}$ mice infected with *T. muris* was evaluated by Schopf et al (Schopf, Hoffmann et al. 2002) and found to be useful in preventing morbidity and mortality in the IL-10 sufficient mice; evaluation of additional antibiotics may prove useful.

Evaluation of changes, based on gross observations and those judged using histology, after implementation of successful support measures may also help determine the cause of death

in these models. Additionally, evaluation of clinical pathological and serum chemistries from blood samples of moribund mice may also provide insight as to the mechanism by which these mice become sick and direct appropriate supportive care or curative treatments. Our work suggests that the cause of death in these mice is due to more than sheer inflammation, as the degree of inflammation observed in moribund mice was not significantly different than those lacking clinical signs associated with disease.

Which bacteria differ in mice infected with *Trichuris muris* when compared to those that are uninfected and are these changes in the microbial community responsible for beneficial or detrimental changes in various strains of inbred mice?

We found that both C3Bir IL-10$^{-/-}$ mice infected with *T. muris* had significantly different microbiota in their proximal colons than those that were uninfected. Additionally, based on our SIMPER analyses of the *Trichuris* infected and uninfected mice one fragment accounted for approximately 35% of the average 66% dissimilarity between these two groups. But, we were unable to determine which bacteria or bacterium changed in these mice. We also do not know what facilitated these changes – whether it was the actual presence of the worm, changes in the colonic tissue in response to the worm which facilitated a different microbial composition or if it was due to excretory-secretory products (ESPs) produced by the worms.

Identifying the bacteria/um that changed may help us better understand what effects this shift in bacteria may have on the host. It may also help us determine if this is a possible mechanism for the amelioration that was reported in human patients (Summers, Elliott et al. 2003; Summers, Elliott et al. 2005). Additionally, if this is a physiologically important affect that the worm has on its host, it can help us assess the affectivity of worm products, including EPSs, as an alternative to active infections.

Identifying the bacteria/um change(s) in *Trichuris* infected mice may prove difficult. Non-culture based techniques will be necessary. Techniques such as 454 shot-gun sequencing may prove fruitful, although the expense may hinder its widespread use. Cloned libraries may also provide useful, potentially in conjunction with TRFLPs. Additionally, once any potential

bacterial changes have been identified, determining the functional significance of these changes may also be difficult, although this would be the ultimate goal.

Finally, once these changes were identified their role in the amelioration of IBD in human patients should be investigated. If these changes were the cause of, or played a significant role in the amelioration of IBD in humans, then perhaps these changes could be pursued using other, non-infective, methods.

Does *Trichuris* have an endosymbiont?

A number of parasites have been noted to have endosymbionts. One of the most notable being Wolbachia, which is an endosymbiont for filarial worms (Sironi, Bandi et al. 1995; Tsai, Huang et al. 2007). Although *Trichuris* has not been reported to have an endosymbiont, the presence of one could provide an explanation for the changes noted in gastrointestinal proximal colon tissues of mice, as we previously demonstrated. Additionally, preliminary data from our laboratory using a 16S Universal rDNA probe on sections of *T. muris* worms *in vivo* suggests that a bacterium may be present in non gastrointestinal areas of *Trichuris*. In support of this hypothesis we have found bacterial 16S Universal PCR product from DNA extracted from *T. muris* eggs, but not from the media where the non-ruptured eggs were suspended. Additionally, a 16S Universal rDNA probes was used on both bleach sterilized eggs which were embedded in parafilm blocks and sectioned and in live adult worms which were also embedded in parafilm and sectioned (Figure 1). But, we have yet to determine the identification of this bacterium or bacteria.

It is possible that, as Wolbachia or another endosymbiont plays a crucial role in the survival and pathogenesis of filarial worms, an endosymbiont could play an important if not necessary role in not only the pathogenesis of but also the reported curative properties of *Trichuris*. Whether this endosymbiont acts to sustain and aide in the survival of *Trichuris* or if actually interacts with the mammalian would also warrant investigation.

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