PROBIOTIC EFFECTS DURING AN ENTERIC INFECTION

THE EFFECT OF *LACTOBACILLUS REUTERI* ON HOST IMMUNE AND CELL ALTERATIONS DURING AN ENTERIC PARASITIC INFECTION

By

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ABSTRACT

Parasite infections around the world are a huge economic burden and decrease the quality of life for many people. Probiotic bacteria are being investigated as a possible treatment for many enteric issues due to their beneficial effects by altering the immune system. Goblet cells are the main source of mucins in the gut, and play an important role in host defense. Alterations in goblet cells and mucin have been implicated in a number of gastrointestinal (GI) diseases and infections. The aim of this study is to develop a probiotic based strategy to modulate goblet cell function in relation to host defense in enteric infection. Utilizing a murine model of parasite infection, *Trichuris muris*, we examined the effect of daily administration with probiotic *Lactobacillus reuteri* in different strains of mice and investigation of goblet cell alterations, immune and inflammatory responses in gut, and host defense mechanisms.

Treatment with live *L. reuteri* significantly enhanced worm expulsion in resistant C57BL/6 mice and this was associated with significant increase in goblet cells numbers and an increase in IL-10. This lead to investigation of the probiotic effects in IL-10 knock out (KO) and Muc2 KO mice during the infection. There was no difference of worm burden or goblet cell amounts in infected IL-10 KO mice infected treated with probiotic or medium. In infected Muc2 KO mice treated with *L. reuteri*, there was an earlier increase of goblet cells, and a corresponding decrease in worm numbers. Finally, assessment of this probiotic in susceptible ARK mice revealed no alterations in worm burden, but the treatment prevented the increase in IFN-γ and IL-1β and significantly increased goblet cell numbers.

These data demonstrate that altering the flora with probiotic *L. reuteri* treatment can modulate intestinal goblet cell biology and immune responses in gut, and promote worm expulsion, possibly through an IL-10 mediated mechanism. The increases in goblet cell numbers may also play a role in the early expulsion of the parasite. In addition to enhancing our understanding on the beneficial effect of probiotics in host defense in enteric infection, this research provides new information on gut function in the context of goblet cells and mucins.

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LIST OF ABBREVIATIONS

5-HT	5-hydroxytryptophan, serotonin
CD	Crohn's Disease
DC	Dendritic cell
dH_2O	Distilled water
DSS	Dextran sulfate sodium
EC	Enterochromaffin
FAO	Food and Agriculture Organization
PAS+	Periodic acid-Schiff Staining
PBS	Phosphate buffered saline
pi	Post infection
GI	Gastrointestinal
IBD	Intestinal Bowel Disease
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
KO	Knock out
LR	*Lactobacillus reuteri*
mAbs	Monoclonal antibodies
MLN	Mesenteric lymph nodes
MPO	Myeloperoxidase
Muc	Mucin gene
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK	Natural killer
TGF	Transforming growth factor
TLR	Toll like receptor
TNBS	Trinitrobenzene sulfuric acid
TNF	Tumor necrosis factor
TSLP	Thymic stromal lymphopoietin
UC	Ulcerative Colitis
WHO	World Health Organization

1.0 INTRODUCTION

1.1 Enteric Parasitic Infections

Enteric infections are all infections of the gastrointestinal (GI) tract, and have a significant impact on health status and the economy. Enteric infections of interest in this thesis are parasitic infections, specifically nematode infections. In human populations, nematode infections have a high prevalence, infecting 1/6 - 1/4 of the world's population (Grencis, 1993). These infections are of high interest as they have widespread prevalence and have the ability to cause human disease, both acute and chronic (Jamison *et al.* 2006). In humans, parasitic infections are responsible for extensive diseases that cause high morbidity rates, anemia, vitamin and nutrient deficiency, stunted growth, decreased learning abilities, and possibly surgery (WHO, 2002, 2011). Some of these may last into adulthood, even after deworming (WHO, 2002, 2011). Parasitic infections are responsible for the decreased quality of life for billions of people, a huge economic burden, as well as economical losses in domestic animals which are also affected by parasitic infection (Artis, 2006). Many anti-parasitic medications are commercially available, although there is a rising in the resistance rates, which will lead to a huge increase in infection numbers as well as financial need for the assistance of infected individuals, and new research developments for new therapeutic strategies. New therapies that are being researched in several different enteric infections, including parasite infections, are probiotic therapies.

1.2 Probiotic Bacteria

1.2.1 Overview of Probiotics

There are thousands of different bacterial species that inhabit the GI tract, and there have been many advances in a class of bacteria termed, probiotics. In 1989, Fuller redefined the term probiotics as, 'live microbial feed supplement which beneficially affects the host by improving its intestinal microbial balance' (Fuller, 1989). The World Health Organization (WHO) and Food and Agriculture Organization of the United Nations (FAO) further defined these bacteria as, 'live microorganisms that when being administered in appropriate dose, they confer a benefit of health to the receiver' (WHO, 2002; Galdeano *et al.* 2007).

The area of the human body in which probiotics are believed to exert the most beneficial effects is the GI tract, but studies have been done on the respiratory tract, skin, bladder and genitals (Marco *et al.* 2006; Reid, 2005). The most common types of probiotics used in scientific studies as well as the food industry are *Lactobacillus*, *Bifidobacterium*, *Streptococcus*, and *Lactococcus* (Naidu *et al.* 1999). This is due to their safe and beneficial effects on the host (Galdeano *et al.* 2007; Sheil *et al.* 2007).

1.2.2 Qualities Needed in Probiotics

To maximize their advantageous effects on the host, probiotics must exhibit several qualities. Firstly, they must not be a virulent strain of bacteria, and be safe to administer, to ensure the host is not negatively affected by the bacteria (Reid, 2005). Other important traits probiotics must have are that they are capable of tolerating the low pH levels that are present in the stomach and intestine, as well as the bile in the

GI tract (Collins *et al.* 1998; Galdeano *et al.* 2007; Marco *et al.* 2006). Viable probiotics adhere to the intestinal epithelial cells, and are able to persist for a short amount of time by avoiding the peristalsis effect (Galdeano *et al.* 2007; Sheil *et al.* 2007). Although probiotics can avoid peristalsis for a short time, they do not colonize the GI tract, and in turn they do require continual administration (Corthesy *et al.* 2007; Marco *et al.* 2006). While residing in the host gastrointestinal tract, probiotic bacteria are metabolically active, although they divide slowly in the intestine (Marco *et al.* 2006). It has been shown that probiotics must inhabit the gut for a minimum of 48 to 72 hours in order to induce gut immunostimulation (Galdeano and Perdigon, 2004). Most probiotics need to be viable when ingested for their beneficial effects, although there is some research is being done on non-viable bacteria as well (Collins *et al.* 1998; Galdeano *et al.* 2007; Marco *et al.* 2006).

1.2.3 Effects of Probiotics

Probiotics affect many aspects of the GI tract., including: gut physiology, homeostasis, gut morphology, stabilization of cytoskeletal functions, stabilization of the tight junctions holding enterocytes together, mucosal immunity, disease and virus prevention, and disease treatment (Galdeano *et al.* 2007; Limdi *et al.* 2006; Ma *et al.* 2004; Sheil *et al.* 2007).

1.2.3.1 Physical Changes Due to Probiotics

The physiological changes that arise from the presence of probiotic populations are mainly in relation to the gut mucosal barrier. This barrier becomes

more stabilized which enhances it in addition to strengthening it (Salminen *et al.* 1996). Due to this effect, external pathogen growth is inhibited (Galdeano *et al.* 2007; Marco *et al.* 1996). Probiotics can modulate tight junction proteins, regulate the phosphorylation of proteins, and alter the overall cytoskeletal dynamic (Ng *et al.* 2009). Tight junctions control transcellular intestinal permeability, keep harmful bacteria and pathogens out of the systemic system, and hold epithelial cells together (Limdi *et al.* 2006; Ma *et al.* 2004). Some enteric pathogens are able to induce alterations to the permeability of tight junctions to benefit their virulence (Sherman *et al.* 2005). Certain strains of probiotics are able to prevent the pathogen induced changes to the tight junctions (Corthesy *et al.* 2007; Sherman *et al.* 2009). Some research on secreted products of probiotic bacteria has also unveiled that the soluble proteins may be helping in the permeability changes seen when probiotics are administered (Madsen *et al.* 2001).

The administration of probiotic bacteria can also alter the protective layer over the epithelium, which also enhances the barrier function. The mucin layer, and the genes associated with mucus are influenced by the bacterial populations (Mack *et al.* 2003). When probiotics are present in the GI tract, they can send signals that in turn strengthen the overall mucus barrier which aides in protection against pathogens (Mack *et al.* 1999). When some probiotics, such as *Lactobacillus*, adhere to the epithelial layer, there is an upregulation of mucin genes that are found in the GI goblet cells, which further enhances the mucus barrier (Mack *et al.* 1999, 2003). The tightening of tight junctions, decreased permeability, and the increased mucus barrier,

can benefit the host by the prevention of pathogenic bacterial binding, and reduction of translocated bacteria (Ng *et al.* 2009).

Probiotics also improve homeostatic conditions, and help with disease and infection prevention. The correct probiotic treatment can improve the overall normal flora to stabilize and balance homeostasis. Probiotics can help restore altered flora to a more normalized state during a pathogenic situation (Alander *et al.* 1999). Short term probiotic colonization has the ability to act as antagonists against pathogens by colonization resistance (Sherman *et al.* 2009). There are only a limited number of possible binding sites in the intestine and the administration of probiotics proposes a competition for these binding sites and nutrients. This in turn protects the host by preventing adhesion of infectious bacteria, inhibit growth, or decrease the duration of some infections, which will in turn, prevent epithelial injury, and improve overall host health (Bernet-Camard *et al.* 1997; Coconnier *et al.*1998; Reid *et al.* 1987). Probiotics also have the ability to alter the pH in the lumen of the GI tract where they reside which will deter the growth and adherence of pathogenic bacteria (Ng *et al.* 2009).

1.2.3.2 Interaction between Host and Probiotics

Probiotic bacteria residing in the intestine have the ability to modulate the normal intestinal microflora, which in turn alters the typical immune system's response (Johnson-Henry *et al.* 2004; Sheil *et al.* 2007). Probiotics help maintain a stable mucosal immune system by modulating the normal flora to a more beneficial balanced environment (Galdeano *et al.* 2007; Tancrede, 1992). A healthy mucosal

immune system allows for the interaction of commensal or safe bacteria with the immune system without initiating a signal cascade to induce an inflammatory response, promoting overall homeostasis (Galdeano *et al.* 2007).

There are many different types of interactions between the host and bacteria in the GI tract and these interactions may drive different host responses. One common interaction between bacteria and host is the sampling of bacteria by immune cells such as dendritic cells (DCs) or macrophages in the lamina propria (Galdeano *et al.* 2007; Rescigno *et al.* 2001). Commensal bacteria and safe bacteria such as probiotics also interact and activate toll-like receptors (TLRs) that are present on membranes of epithelial and immune cells (Rakoff-Nahoum *et al.* 2004). Probiotics that are present in the GI tract interact with and send signals through immune cells or can be internalized or sampled, and possibly induce activation of different immune mediators and cells (Galdeano *et al.* 2007). When bacteria is sampled through M cells of the Peyer's Patches, they can either be cleared from the area, or sent to the mesenteric lymph nodes (MLNs), to interact with T and B cells residing in the MLNs (Mowat, 2003). The interaction between the epithelial cells and probiotic components has a role in triggering underlying immune cells in the lamina propria (Corthesy *et al.* 2007). The recognition of the bacteria by the host will help determine the alterations, if any, the host will make in response to the probiotic's presence in the GI tract.

Different probiotics have different immune results, activations and signalling pathways. It has been shown that probiotics have the ability to induce immune cell maturation, recruitment, antibody production, as well as overall cytokine expression, production and release (Haller *et al.* 2000; Hart *et al.* 2004; Malin *et al.* 1996).

1.2.3.3 Probiotic Influence on Immune Cells

The inferaction between probiotic bacteria and the host immune system can alter the cytokine milieu, and this change occurs through the bacterial and immune cell communication (Corthesy *et al.* 2007). These bacteria are known to work on many immune cells, such as T cells, B cells, and DCs, in different tissue areas, or lymph nodes (Ng *et al.* 2009).

DCs are usually the first cells to interact with administered bacteria, as they are able to sample the contents of the GI tract lumen (Rescigno *et al.* 2001). Both probiotics and secreted factors from probiotics have been shown to differentially induce the maturation, activation and survival of DCs, and altering their cytokine expression (Marco *et al.* 2006). One suggested pathways of DC activation is the binding of probiotics to DC-SIGN, on the DC, which then primes the cells to further induce regulatory T cells (T regs), and drive these regulatory immune cells to produce regulatory cytokines such as IL-10 (Hoarau *et al.* 2006; Smits *et al.* 2005).

T and B cells interact with probiotic bacteria in the Peyer's Patches and MLNs. Some probiotics help balance the overall T cell responses, although not all species influence T cell populations (Walker, 2008). As mentioned above, probiotics influence DCs to induce T reg cells, and some probiotics of the *Lactobacillus* origin, influence and increase in CD25+ T cells in the lamina propria, as well as an increase in Foxp3 transcription factor (Feleszko *et al.* 2007; Karimi *et al.* 2008). B cells are also stimulated in response to certain probiotic species to increase the IgA production and secretion (Macpherson *et al.* 2000; Malin *et al.* 1996). B cells are influenced by probiotics to increase immunoglobulins, but it is important to note that probiotic

signals are also able to interact with the gut epithelial cells to signal an increase in the number of immunoglobulin A (IgA) producing cells (Galdeano *et al.* 2007). An increase in IgA levels in the gut environment helps to promote a more effective gut immunological barrier (Sheil *et al.* 2007). The overall modification of secretory IgA production in the gut is important for mucosal immunity by aiding in blocking some pathogens and viruses from inhabiting the intestine (Limdi *et al.* 2006).

1.2.3.4 Alteration of Cytokine Expression Due to Probiotics

Probiotics can signal through TLR pathways, or interact directly with epithelial and immune cells to alter cytokine production and release (Galdeano *et al.* 2007). It has been shown, that when in the presence of the probiotic *Lactobacillus*, colonic and intestinal epithelial cells secrete a decreased amount of TNF-α, a pro-inflammatory cytokine (Marco *et al.* 2006). Lymphocytes and splenocytes are also affected by probiotic treatment. When in the presence of probiotics these immune cells are down regulated, and therefore synthesize and secrete less pro-inflammatory IFN-γ and TNF-α in vivo (Ma *et al.* 2004). Furthermore, probiotics act to lessen inflammation by inducing the release and production of regulatory cytokines such as IL-10 (Galdeano *et al.* 2007; Ma *et al.* 2004). Not only do the resulting cytokine alterations due to probiotic bacteria reduce inflammatory conditions, it also prevents cytokine induced apoptosis of epithelial cells, as well as promoting overall homeostasis of the GI tract and immune system (Otte and Podolsky, 2004; Yan *et al.* 2007).

1.2.4 Probiotics and GI Disease

As discussed above, probiotic treatment has the ability to create a beneficial GI environment, and promote overall host health. Probiotics may have the ability to reverse or even prevent some pathological conditions of the GI tract through stimulation or modification of the immune system (Limdi *et al.* 2006). The exact mechanisms of how probiotics benefit the host health are not fully understood, and further research is required in order to comprehend the complete process of probiotic and host interaction.

Many studies have used probiotics to reduce the effects of GI diseases, specifically inflammatory bowel disease (IBD). With the current treatments for IBD, several issues arise such as chronic relapses, side effects, and low drug compliance (Chen *et al.* 2009; Seksik *et al.* 2008). Ulcerative colits (UC) is the inflammation of the lamina propria of the colon, disruption of the mucosal barrier, and a decrease in mucosal layer (Herias *et al.* 2005; Seksik *et al.* 2008). On the other hand, Crohn's disease (CD) consists of inflammation of the entire wall of affected area, with possible lesions, anywhere in the GI tract (Herias *et al.* 2005; Seksik *et al.* 2008). Since bacteria play a role in many GI conditions, including UC and CD, probiotics are being considered for a new role in treatment and maintenance of these diseases (Seksik *et al.* 2008). Different GI diseases affect different areas of the GI tract, so it is important to understand not only the disease, but the area the probiotic colonizes and adheres when choosing a species or strain of bacteria (Chen *et al.* 2009; Osman *et al.* 2004).

Although successful clinical trials showing the efficacy of probiotics are rare, there are a few trials with UC patients (Seksik *et al.* 2008). One of these trials treated patients with a probiotic, *Bifidobacterium longum*, and the normal medication. This treatment strategy showed a decrease in inflammation and pro-inflammatory cytokines, as well as a reduction of lesions (Furrie *et al.* 2005). Another study of similar nature was done and showed that the probiotics normalized the flora, as well as prevented recurrence exacerbations of the condition (Ishikawa *et al.* 2003). In the case of UC, the administration of probiotics to the patient alleviates the symptoms by modulating the cytokine expression and stabilizes the mucosal barrier. This resolves some of the symptoms of the disease (Galdeano *et al.* 2007; Sheil *et al.* 2007). Unfortunately trials with CD are less common due to the variability of the location of the inflammation, and some of the underlying factors that contribute to CD (Seksik *et al.* 2008).

All clinical trials have evolved from animal models of colitis and GI inflammation to better understand the diseases, pathologies and mechanisms underlying the condition. Dextran sulfate sodium (DSS) is a commonly used model of UC, which is a chemical toxic to the epithelial layer causing bloody diarrhea, weight loss, shortening of the colon, immune cell infiltrate, altered mucosal layer, and mucosal ulcerations (Okayasu *et al.* 1990).

In laboratories around the world, researchers are using the DSS model and treating these mice with probiotics to determine which probiotics will be beneficial in treatment or suppression of UC in humans. So far many different probiotic mixtures and strains have been suggestive as a therapeutic role. Studies that have given

probiotics before DSS, such as VSL#3, *Lactobacillus fermentum*, *Lactobacillus casei*, or a mixture of *Lactobacilli* species, have shown that the bacteria were able to prevent weight loss, prevent an increase in the inflammatory marker myeloperoxidase (MPO), decrease the disease severity, and prevent an overgrowth of certain species of harmful bacteria (Chen *et al.* 2009; Geier *et al.* 2007; Herias *et al.* 2005; Rachmilewitz *et al.* 2004). A mixture of probiotics have been shown to decrease the disease activity index, while also preventing bacterial translocation, which has been linked to a more severe colitis and inflammation (Osman *et al.* 2004). Probiotics are able to alter the cytokine environment in this experimental colitis by increasing anti-inflammatory cytokines while decreasing the pro-inflammatory cytokines to reduce inflammation and cell infiltrate (Chen *et al.* 2009). One hypothesis on the mechanism of this prevention is through comminucation with the TLR9 mechanism, as this phenomenon was not seen in TLR9 knock out (KO) mice (Rachmilewitz *et al.* 2004).

Other models of colitis are less studied using probiotics, but are still common models of GI inflammation. Trinitrobenzene sulfuric acid (TNBS) is a model of IBD which causes GI inflammation and damage, thickening and shortening of the colon, ulceration of the mucosa, neutrophil infiltration, and crypt loss (Amit-Romach *et al.* 2008). A *Lactobacillus GG* was given during TNBS, and these mice have improved crypt architecture, less neutrophil infiltrate, improved histological scores, and improved recovery time after TNBS is stopped (Amit-Romach *et al.* 2008; Peran *et al.* 2007).

The IL-10 KO mouse is another model of colitis as these mice spontaneously develops colitis if colonized with bacteria (Sellon *et al.* 1998). Once these mice have

developed colitis, they have an abolished population of protective *Lactobacillus* species. Treatment with *Lactobacillus reuteri* is able to increase the total number of protective bacteria, and in turn normalize the number of adherent and translocated bacteria, and limit some of the invasive aerobic species (Madsen *et al.* 1999). Probiotics also improve the barrier function in IL-10 KO mice that have developed colitis, which is a possible mechanism of preventing translocation of bacteria (Madsen *et al.* 1999, 2001).

Many of these experimental models have administered probiotics as a preventive measure, and more studies need to be done to rule out mechanisms, and if the bacteria can help patients who have the underlying condition and need a treatment and a way to prevent relapses. The microbiota in the GI tract is important in IBD pathogenesis, and modulation of the microenvironment may be therapeutic by preventing the overgrowth of certain species by the competition for similar niches (Amit-Romach *et al.* 2008). So far these researchers have proven that probiotic treatment may help prolong remission of patients with GI disorders, possibly by restoring protective bacteria and altering the immune environment (Amit-Romach *et al.* 2008; Madsen *et al.* 1999; Osman *et al.* 2004).

1.2.5 Probiotics and Enteric Infections

With regards to infectious diseases, parasites and viruses, probiotic treatment may improve viral associated diarrhea, produce antimicrobial substances, act as antagonists and protect host from infections (Galdeano *et al.* 2007; Marco *et al.* 2006; Sheil *et al.* 2007). Probiotics have been shown to not only to limit binding sites and

exclude the infection, but also inducing mucin mRNA levels and increasing the mucus layer to trap the bacteria to be flushed out of the GI tract (Mack *et al.* 2003; Sherman *et al.* 2005). Probiotic treatment has also been proven to protect, or if infected, decrease the duration of infection, and prevent some of the increased permeability that accompanies a rotavirus infection (Isolauri *et al.* 1993). Probiotic treatment reduces the increased permeability that some viral infections cause, as well as decreasing the length of time a virus sheds in the intestinal tract (Limdi *et al.* 2006). If a specific *Lactobacillus* species is administered to mice before a *Clostridium rodentium* infection there is a reduction in overall colonization, minimal epithelial thickening, decreased influx of immune cell infiltrate to the lamina propria, and less overall damage and morphological changes. This reduction of the severity of the disease is thought to be from the alteration of immune response from a T_H1 response, to more of a T_H1/T reg response and the overall exclusion of the pathogenic bacteria and prevention of growth (Johnson-Henry *et al.* 2005). Probiotics have been studied a great deal in respect to *Helicobacter pylori* infections. Co-incubation of a *Lactobacillus* mixture with *H. pylori* decreases colonization of the pathogenic bacteria, decreases overall inflammation of the infection, and decrease the growing capabilities of the bacteria (Johnson-Henry *et al.* 2004). Some *Lactobacillus* species have the ability to directly inhibit the growth of *H. pylori* infections, because they have bactericidal and antimicrobial properties (Chatterjee *et al.* 2003; Johnson-Henry *et al.* 2004). In a more clinical sense, administration of *Lactobacillus* species to *H. pylori* infected patients helped to eradicate the infection and to normalize and restore homeostasis while on a standard therapy (Sheu *et al.* 2002).

In terms of intestinal parasitic infections, there have been fewer studies regarding probiotic therapy. Studies have focused on *Giardiasis intestinalis*, a protozoa causing diarrhea and chronic intestinal symptoms around the world (Shukla *et al.* 2008). Treatment of *Giardia* with different strains of probiotics has shown promising outcomes. Using *L. casei*, researchers have prevented histopathological changes in the small intestine, decreased the number of fecal cysts, and reduced the duration of the infection. This is possibly due to the inhibition of initial adhesion of the protozoa (Shukla *et al.* 2008). Similar results have been shown using *L. johnsonii* La1 probiotic against *Giardia.* This probiotic decreased overall infection rate, and those mice which were infected had less morphological changes due to the protozoa (Humen *et al.* 2005). The findings show a possible antagonism effect of the bacteria, as well as a greater splenocyte response in the probiotic treated mice infected with the parasite (Humen *et al.* 2005). Another group showed therapeutic potential with the probiotic *Enterococcus faecium* SF68 against *Giardia intestinalis* (Benyacoub *et al.* 2005). Treatment with this probiotic increased expulsion of the intestinal trophozoites and increased recovery. This correlated with an increase in CD4+ T cells and IgA, both of which have been shown to be important in recovery from this parasite (Benyacoub *et al.* 2005).

Many probiotic species and mixtures are currently being used in treatment therapies as well as prevention of infections in the clinical field. As promising as this is, there are still many mechanisms to be defined and more research to improve the overall effects of probiotics. Further research will help in administration of

probiotics, and what dose, species and strain of bacteria will work best in each situation.

1.2.6 Safety of Probiotic Therapy

There are about 20 billion doses of probiotics used every year and as supplementation is on the rise, safety is a concern (Reid, 2002). Many probiotics, specifically *Lactobacillus* species have been shown to be safe to use in many different situations, with both adults and children, but there are several incidences when there have been adverse effects due to a *Lactobacillus* species (Mackay *et al.* 1999; Rautio *et al.* 1999). The most common reported adverse effects due to probiotics are endocarditis, liver abscesses, and sepsis due to bacteremia (Land *et al.* 2005; Mackay *et al.* 1999; Rautio *et al.* 1999).

In one case study, an elderly lady taking a dairy product containing *L. rhamnosus* to relieve abdominal discomfort was admitted to the hospital with increasing abdominal pain, was found to have liver abscesses (Rautio *et al.* 1999). The patient had previous medical issues such as diabetes and hypertension, and liver biopsies were positive for gram positive *L. rhamnosus* (Rautio *et al.* 1999). Another case study reported a male patient, who was admitted to the hospital complaining of a dry cough, shortness of breath, and weight loss. Blood cultures were positive for gram positive bacteria, later identified as *L. rhamnosus* (Mackay *et al.* 1999). With further history, it was relevant that the patient recently had dental work done, and was chewing probiotic supplement pills containing *L. rhamnosus*, instead of swallowing them (Mackay *et al.* 1999). There have also been case studies on infants and children

that have had a complication possibly related to probiotic supplements given to treat antibiotic associated diarrhea. The first case was with a 6 week old infant that had heart surgery and was given antibiotics, but then developed severe diarrhea. The diarrhea was treated with *L. rhamnosus GG*, and the diarrhea improved, but shortly after, the infant developed a fever, respiratory distress and metabolic acidosis. Blood cultures came back positive for *Lactobacillus*, probiotic treatment was stopped, and the baby improved after 3-4 days (Land *et al.* 2005). The second case was with a 6 year old child with cerebral palsy and many other medical conditions, who was being treated for a urinary tract infection with antibiotics. This child also developed diarrhea, and was treated with *L. rhamnosus GG*, but then developed a fever. Blood cultures showed *Lactobacillus* growth, suggesting sepsis caused by the probiotic. The child was taken off the probiotics and given antibiotics and symptoms improved (Land *et al.* 2005).

Many of the reported cases have not been fatal, and sepsis and complications due to probiotics is rare, and in all reported cases, there had been an underlying condition, immune suppression, or the patient has been in a debilitated state (Land *et al.* 2005; Mackay *et al.* 1999; Rautio *et al.* 1999). Clinicians need to be cautious when administering probiotics to patients who are immunocompromsed or have immune deficiencies, neonates, or those who have serious underlying medical conditions, as there may be increased risks (Boyle *et al.* 2006). Overall, the risks are very minimal when taking probiotics, although these risks should not be forgotten when being prescribed.

1.2.7 Probiotic Conclusion

Currently there are many different species and strains of probiotics that are on the market and being studied in laboratories around the world. Different probiotics colonize in different areas of the GI tract, and choosing the right probiotic for each condition is critical to see beneficial effects. It is important to keep in mind that not all probiotic species have the same effects on the immune system, and not all of these alterations will occur with every bacterial species used (Limdi *et al.* 2006). Probiotics have the potential to reverse or even prevent some pathological conditions of the GI tract through stimulation or modification of the immune system (Limdi *et al.* 2006). The exact and complex mechanisms of how probiotics benefit the host health are not fully understood, and further research is required in order to comprehend the complete process of probiotic and host interaction.

1.3 *Trichuris muris (T. muris)*

1.3.1 Overview of *T. muris*

The influence probiotic therapies are having on enteric infections opens many doors for research on their effects during parasitic infections. Animal models of parasitic infections are extremely useful in exploring mechanisms of host defense and immune responses. There are several parasitic animal models available, one being the *Trichuris muris* model, which is extensively used for many reasons.

T. muris is a natural parasitic helminth nematode that dwells in the caecum and large intestine of mice (Fahmy, 1954). This parasite is very similar to the human parasite *Trichuris trichura* which affects over 1 billion people worldwide. Susceptible

individuals develop a chronic infection which consists of bloody diarrhea, histological changes of the large intestine, anemia, and a possibility of a rectal prolapse (Mestecky *et al.* 2005). This is a preventable soil transmitted helminth which is among the most common infections in the world, making it an economic burden on those developing, mainly tropical countries which the parasite is an endemic (MacDonald *et al.* 2002; Mestecky *et al.* 2005). *T. trichuria* rarely causes death, but does produce high levels of morbidity in infected individuals (Cliffe *et al.* 2007). *T. muris* is used for a model of *T. trichuria*, as well as other intestinal helminth infection responses, and other local immune responses during enteric infections (Else and deSchoolmeester, 2003).

1.3.2 Life Cycle of *T. muris*

T. muris has an oral-fecal life cycle, and does not replicate within the host. The soil phase of the cycle allows the ova to embryonate, resulting in infective eggs (Mestecky *et al.* 2005). Embryonated eggs are orally ingested, reaching the caecum in about 2.5 hours. The pH range of 7.0 in the caecum, and 6.0 in the colon, as well as a temperature of 37°C are cues for the larval emergence from the egg (Hayes *et al.* 2010). Bacterial contact is also a necessary cue for larval hatching. Hayes *et al.* cultured *T. muris* eggs in media with *Escherichia coli*, a common commensal, and in media in which the bacteria has been removed. Eggs hatched only when bacteria were present. This shows that direct contact between the egg and a structural component of the bacteria is necessary for the hatching of *T. muris,* and not a secreted product (Hayes *et al.* 2010). The larvae hatch in the caecum and proximal colon and embed in

the mucosal epithelium by 24 hours post infection (Cliffe *et al.* 2007; Fahmy, 1954; Grencis, 1993). The worms stay in their intraepithelial niche, and mature in about one month after penetration of the mucosa (Else and deSchoolmeester, 2003; Fahmy, 1954). The parasite causes alteration in homeostasis of the epithelium, inflammatory cell infiltrate as well as cell hyperplasia in caecal and colonic crypts (Artis *et al.* 1999; Cliffe *et al.* 2007). This model is ideal for studying direct immune responses, as this parasite does not penetrate farther than the epithelium and there are no confounding systemic responses, which makes it a well defined parasite model that is beneficial to investigate the host's responses (Fahmy, 1954).

1.3.3 Mouse Responses to *T. muris*

A unique feature of the *T. muris* parasite is that different strains of mice respond to the infection differently, and in turn have different outcomes (Cliffe and Grencis, 2004). CD4+ T cells have a major role in determining the immune response and susceptibility of different mouse strains (Grencis, 1993). T cells not only determine the cytokine milieu in the intestine during infection, but they also affect physical aspects of the gut. T cells regulate or influence mucosal alterations such as epithelial cell turnover and hyperplasia, cell populations, goblet cell hyperplasia, fluid and mucin secretions, smooth muscle control, enterochromaffin (EC) cells hyperplasia, serotonin (5-hydroxytryptophan, 5-HT) secretion and overall architecture, which are all important in the clearance of a *T. muris* infection (Khan and Collins, 2004; Mahida, 2003; Wakelin, 1978). Certain aspects of the immune system are beneficial in fighting a parasite response, but are not necessary for the

clearance of worm. The outcome is independent of mast cells, eosinophils, natural killer (NK) cells, and antibodies, even though these aspects of the immune system are all activated and enhanced during a parasite infection (Betts and Else, 1999; Else *et al.* 1996, 2003). See Figure 1 for a summary table of both classic and KO models of mice that are either susceptible or resistant to a *T. muris* infection

1.3.3.1 Resistant Mice Strains

Certain strains of mice, such as C57BL/6 and BALB/c, mount a T_H2 immune response, producing mainly IL-4 and IL-13 during the infection (deSchoolmeester and Else, 2002; Dixon *et al.* 2006). These mice are resistant to *T. muris*, and can shed the parasite in 21 to 35 days (deSchoolmeester and Else, 2002). Protection from this parasite in these strains of mice is dependent on many factors related to the T_H2 immune response (deSchoolmeester *et al.* 2003; Else and Grencis, 1991). Once a resistant mouse is infected with *T. muris*, T cells in the MLNs are activated to become T_H2 T cells and produce T_H2 cytokines (Else and Grencis, 1991). This occurs without the production of IFN-γ or IL-12, as these cytokines are blocked by the increase in the T_H2 response (Artis *et al.* 1999; Else and Grencis, 1991). The Stat6 pathway is then activated by IL-4 and IL-13 and this pathway is essential in the overall T_H2 development, which increases the worm expulsion (Khan *et al.* 2001).

Mice lacking Stat6 have impairment in the production of T_H2 cytokines and have a reduction in EC cells and 5-HT amounts. These together cause this KO mouse to become chronically infected with *T. muris* because it is unable to expel the worm (Khan, 2008). Mice deficient in the two main T_H2 cytokines, IL-4 or IL-13, have

significant delays in worm expulsion as well (Bancroft *et al.* 2000). In these KO mice, there is a decrease in important T_H2 cytokines, as well as IL-9, and IL-9 is associated with muscle hypercontractility in the colon of *T. muris* infected mice (Artis *et al.* 1999; Khan *et al.* 2003). Other non classical T_H2 cytokines and proteins are also important in the expulsion of *T. muris* from the caecum and colon of resistant mice. The regulatory cytokine, IL-10, is important during a *T. muris* infection, playing a regulatory role as well as an anti-inflammatory role by suppressing T_H1 cytokines (Schopf *et al.* 2002). A *T. muris* infection in IL-10 KO mice causes a high morbidity early on in the infection leading to death, due to an increase in T_H1 cytokines, weight loss, decreased mucus amounts, and an overgrowth of opportunistic bacteria (Schopf *et al.* 2002).

There are other physical and biochemical changes that occur in resistant mice during a *T. muris* infection that are directly or indirectly related to the enhanced worm expulsion compared to other strains of mice. There is an increase of smooth muscle contractility in resistant mice which increases the propulsive movement of the large intestine (Shea-Donohue and Urban, 2004). This increased peristaltic movement may be a mechanism which allows for the worm expulsion in resistant mice (Collins, 1996; Farmer, 1981). It has also been noted that there is an increase in epithelial shedding in the caecum and large intestine in resistant mice infected with *T. muris* (Cliffe *et al.* 2005). The increase in cell turnover rate is IL-13 dependent, because IL-13 KO mice have a turnover rate similar to the rate seen in susceptible mice (Cliffe *et al.* 2005). T_H2 cells have an increased expression of amphiregulin during a *T. muris* infection which may be a link between the T_H2 response and the increased

proliferation and cell turnover rate of the epithelial cells of the gut, which helps in parasite expulsion (Zaiss *et al.* 2006). Also linked with an increased T_H2 response is goblet cell hyperplasia, and the increase in Muc2 as well as Muc5ac (Hasnain *et al.* 2010, 2011). Muc2 has been shown to be important in host defense, although not necessary, but Muc5ac is critical for the expulsion of *T. muris* from the colon and caecum of mice (Hasnain *et al.* 2010, 2011). Thymic stromal lymphopoietin (TSLP) is a protein produced mainly by epithelial cells, and this protein activates DCs to produce T_H2 attracting chemokines, and promote the overall T_H2 response from T cells (Liu *et al.* 2007). TSLP has been shown to be increased during a *T. muris* infection, and this protein also prevents IL-12 upregulation and suppresses the T_H1 response (Massacand *et al.* 2009). Serotonin is an enteric mucosal signalling molecule produced by EC cells, which is critical for intestinal homeostasis (Wang *et al.* 2007). During a *T. muris* infection the number of EC cells in the large intestine, as well as the levels of mucosal 5-HT are increased during the enteric infection. This upregulation is induced by CD4+ T cells and T_H2 cytokines (Wang *et al.* 2007).

These alterations work together to promote the helminth expulsion from the caecum of resistant strains of mice. The overall T_H2 environment and the physical changes that occur in response to the *T. muris* infection allow these mice to expel the parasite and return to homeostasis quickly after infection.

1.3.3.2 Susceptible Mice Strains

Other strains of mice, such as AKR and B10.BR, are susceptible to a *T. muris* infection, and are unable to expel the parasite and develop a chronic infection (Else

and Grencis, 1991). In these strains of mice, there is a strong T_H1 response, an overall increase in pro-inflammatory cytokines, mainly IFN-γ, TNF- α and IL-12, and an absence of T_H2 cytokines (deSchoolmeester and Else, 2002; Else and Grencis, 1991; Grencis *et al.* 1993). It has been noted that the increase of TNF-α promotes IFN-γ, which in turn recruits inflammatory cells to the gut mucosa (Cliffe *et al.* 2007).

Several knockout (KO) mice are also susceptible to a chronic *T. muris* infection, such as IL-10 KO and NF-κB KO (Artis *et al.* 2002; Schopf *et al.* 2002)**.** IL-10 KO mice highly susceptible to *T. muris* and susceptibility is dependent on IL-12, noted when IL-10/IL-12 double KO mice we infected with *T. muris* and were resistant to the infection (Mestecky *et al.* 2005; Schopf *et al.* 2002). A *T. muris* infection causes IL-10 KO mice to develop severe inflammation, loss of Paneth cells, and they do not have an increase in mucus amounts in the caecum (Schopf *et al.* 2002). It has also been shown by Schopf *et al.* that treatment with broad spectrum antibiotics reduces the morbidity and mortality of these mice, suggesting a role of luminal bacteria in the severity of the *T. muris* infection (Schopf *et al.* 2002). In another study done with antibiotics given to susceptible AKR mice before and throughout a *T. muris* infection, these mice had a decreased worm burden and had a stronger T_H2 response, but similar IFN-γ levels (Hayes *et al.* 2010). These data show that there is also a role for commensal bacterial composition in determining the immune response to a *T. muris* infection, and in the overall outcome (Hayes *et al.* 2010). AKR mice and some susceptible KO mice have a decrease in goblet cell numbers during infection compared to wild type mice (Artis *et al.* 2002). This suggests that there is a role for goblet cells in a *T. muris* infection, and the lack of

goblet cells in some susceptible mice may attribute to the severity of infection. Susceptible strains of mice also undergo some crypt hyperplasia and an increased rate of cell turn over starting around day 21 of infection (Cliffe *et al.* 2005). This late increased cell turnover causes crypt hyperplasia and because *T. muris* worms have quadrupled in size from day 14 to day 21, the worms are too large for the increased cell turnover rate to help flush the worms from the intestine (Cliffe *et al.* 2005). The chemokine CXCL10 has been shown to reduce the rate of epithelial cell turnover in mouse colitis models (Sasaki *et al.* 2002). CXCL10 expression was detected on day 21 in susceptible AKR mice, when IFN-γ levels were high (Cliffe *et al.* 2005). These studies suggest that the late cell turnover rate is another mediator in delaying worm expulsion and allowing a chronic *T. muris* infection to occur.

The overall T_H1 immune response is not appropriate to induce worm expulsion from the GI tract of susceptible mice (Else and deSchoolmeester, 2003). Not only are cytokines a key player in determining resistance of susceptibility, but other factors as well. Mucin and goblet cell hyperplasia, cell turnover, and other immune pathways all play a role (Artis *et al.* 2002; Else and deSchoolmeester, 2003; Hasnain *et al.* 2010).

Mice	Resistant	Other Resistant	Susceptible	Other Susceptible
Strains	C57BL/6	Muc2 KO	AKR	IL-10 KO
Immune Response	T_H2	T_H2	T_H1	Mixed
Cytokine Response	IL-13, IL-4	IL-13, IL-4	IFN-γ	Mixed
Goblet Cell Alterations	Increase	Increase-Muc5ac	No increase	No increase
5-HT+ EC Cell Alterations	Increase	Unknown	No increase	Unknown
Expulsion Time	21-30 days	25-30 days – delayed onset	Chronic infection	Mortality after ~25 days
References	Motomura *et al.*, 2008	Hasnain, *et al.*, 2010	Motomura *et al.*, 2008	Schopf *et al.*, 2002

Figure 1: Summary of the immune and cell responses in different strains of mice used in this thesis, outlining specifically the cytokine responses, goblet and EC cell alterations, and the worm burden. Chart was adapted from several papers.

1.4 Goblet Cells

T. muris infections induce many host alterations, including cellular responses such as changes in lymphocytes, mast cells, eosinophils and goblet cells (Mahida, 2003). Among the cell changes that occur, much attention has been paid on the role of goblet cells in the expulsion of the parasite from the GI tract (Hasnain *et al.* 2010).

1.4.1 Overview of Goblet Cells and Mucins

Goblet cells are exocrine cells found throughout the colon and small intestine in both human and mice, with a turnover rate of about 48 hours (Specian and Oliver, 1991). Goblet cells synthesize and release mucins, which are highly glycosylated proteins. Mucins are either secreted or membrane bound, and form a gel layer which acts as a protective barrier over the epithelial cells to protect from the harsh lumen environment (Corfield *et al.* 2000; Specian and Oliver, 1991). Mucins are highly glycosylated proteins that are either membrane bound or secreted, and form a gel layer (Karlsson *et al.* 2000; Theodoropoulos *et al.* 2001).

There are many genes that code for mucins, in particular, MUC2 and MUC3 are of interest in the GI tract, as these are the two types of mucins found in high levels in the intestine (Corfield *et al.* 2000). There are two layers of mucin over the epithelial layer. The outermost layer is a loose layer which bacteria can penetrate and get trapped in. The inner layer is sterile, because it is a more adherent layer that bacteria cannot penetrate (Johansson *et al.* 2008). MUC2 is the main secreted form of mucin in the large intestine, which forms the viscous gel layer over the epithelial layer (Ho *et al.* 1993). MUC3 is the main membrane bound mucin, and found in less

amounts in the large intestine compared to the small intestine (Ho *et al.* 1993). Another mucin of interest in the GI tract is MUC5ac, which is normally found in significant amounts in the respiratory tract, but has been recently been shown to be critical in parasite expulsion (Hasnain *et al.* 2011). There are mouse models developed to study in depth the role of goblet cells in different situations. The model of interest in the GI tract is the Muc2 KO mouse model. The background strain of these KO mice determines the overall outcome and well being of these mice (van der Sluis *et al.* 2008).

1.4.2 Goblet Cells during Parasitic Infections

During an enteric parasitic infection, goblet cell numbers have been shown to increase significantly to help the expulsion of the worms. Mucus is able to trap the parasite and protecting the host (Miller, 1987). The changes and upregulation of goblet cells are under the regulation and control of the host's immune system, specifically T_H2 cells (Ishikawa *et al.* 1997). T_H2 lymphocytes produce factors that induce stem cells to differentiate into goblet cells, and these are increased in a parasitic infection (Ishikawa *et al.* 1997). The fact that CD4+ lymphocytes are important in goblet cell hyperplasia was shown by Khan *et al.* in 1995, when anti-CD4-antibodies were used during a *Nippostrongylus brasiliensis* infection, and there was no spontaneous expulsion of the parasite, possibly due to a decreased amount of mucus production (Khan *et al.* 1995). During a *T. muris* infection, it has been shown that goblet cell hyperplasia occurs in resistant mice, and the mucins help trap and remove the intestinal parasite (Hasnain *et al.* 2010; Khan *et al.* 2003; Miller, 1987).

In IL-13 KO mice and Stat6 KO mice, there was decreased goblet cell hyperplasia during an enteric parasitic infection. Both of these strains of mice were deficient in a cytokine or pathway important in the T_H2 response, which is important in the determination of susceptibility or resistance. This reinforces the previous work that goblet cell hyperplasia is T_H2 dependent (Ishikawa *et al.* 1997; Khan *et al.* 2001). Another way T_H2 cells and their subsequent cytokines have been associated with the increase in goblet cell numbers is at the stem cell level. During another type of nematode infection, *Trichinella spiralis*, after T_H2 cells are stimulated, it has been suggested that factors are produced to induce stem cells to differentiate into the goblet cell lineage opposed to other endocrine cells (Ishikawa *et al.* 1997). Muc5ac increases during not only *T. muris* infections, but also *T. spiralis* and *N. brasiliensis* and is needed for expulsion from the host (Hasnain *et al.* 2011). The increase in Muc5ac is linked with IL-13 increases, and Muc5ac is able to decrease the energy of *T. muris* and decrease the viability of the worm, and the overall infection (Hasnain *et al.* 2011).

Not only are there quantitative changes in goblet cell numbers during a parasitic infection of the GI tract, it has also been shown that there are qualitative changes of the mucin components (Ishikawa, 1993). Under normal, healthy conditions, mucins of the gut are of neutral charge but during a parasitic infection, these mucins are sulphated, and become slightly acidic (Koninkx *et al.* 1988). The exact reason for these changes in the mucin is still unknown, although the changes occur at the time of the parasite expulsion (Ishikawa *et al.* 1993, 1994; Karlsson *et al.* 2000). After the parasite is successfully expelled, the mucins return to their normal

neutral state, suggesting that the changes are not long term, and are only needed for the clearance of the infection and perhaps in the host's recovery (Karlsson *et al.* 2000).

The alterations of the goblet cell mucins may be triggered by a substance secreted by the parasite, or a product found on the surface of the worm, and more studies need to be done to determine the exact mechanism (Karlsson *et al.* 2000). The overall changes in the mucus production in response to parasitic or bacterial infections may be due to the alterations in the goblet cell population. This enhances the barrier protection to help protect the epithelium and increase the expulsion of the infection.

1.5 *Lactobacillus reuteri (L .reuteri,* LR*)*

Lactobacillus species are common bacteria used as probiotics, and *Lactobacillus reuteri* has been shown in many studies to be beneficial bacteria when administered during certain circumstances (Forsythe *et al.* 2007; Naidu *et al.* 1999). *L. reuteri* is a gram positive, rod shaped bacteria that produces lactic acid and is naturally found in human breast milk and the GI tract (Martín *et al.* 2005). This bacteria is being studied in the GI tract, respiratory tract, in vivo, and in patients of all ages, with many potential therapeutic strategies and outcomes. Bringing this probiotic into an enteric GI infection will allow for the investigation of the possible beneficial effects on the host in a *T. muris* infection.

1.5.1 *Lactobacillus reuteri* in the Respiratory Tract

With regards to the respiratory tract, *L. reuteri* has been sown to be beneficial in the treatment of allergic airway inflammation. Ovalbumin sensitized mice develop an allergic airway response similar to allergic asthma in humans (Forsythe *et al.* 2007). These mice have been treated with *L. reuteri* during sensitization by gavage, and there was an attenuation of the classical influx of eosinophils to the lung tissue, and a decrease in overall cytokine response (Forsythe *et al.* 2007). An attenuation of the increase of pro-inflammatory cytokines was noted, specifically a decrease in MCP-1, TNF-α, IL-5, and IL-13, possibly through activation of the TLR9 pathway, as these attenuations were not seen in TLR9 KO mice (Forsythe *et al.* 2007). T regs are also an interest in mice receiving oral treatment of live *L. reuteri*. After wild type mice received *L. reuteri*, there was an increase in Foxp3+ CD4+ T cells and IL-10 production in the splenocytes of treated mice (Karimi *et al.* 2008). It has been suggested previously that *L. reuteri* has the ability to prime DCs, possibly by binding to DC-SIGN to drive the development of T reg cells (Smits *et al.* 2005). If isolated T reg cells from *L. reuteri* treated mice are transferred into ovalbumin sensitized mice, these asthmatic mice have a decrease in bronchoalveolar fluid, a decrease in overall airway response, and an attenuation of similar cytokines seen when mice received the probiotic directly (Karimi *et al.* 2008). These studies focused on asthmatic mice show that *L. reuteri* has beneficial effects in the respiratory tract, through a regulatory T cell response, and through TLR9 pathway activation (Forsythe *et al.* 2007; Karimi *et al.* 2008).

1.5.2 *Lactobacillus reuteri* in the GI Tract

L. reuteri has also been studied in the GI tract, focusing on colitis, and less so on pain and floral alterations. As mentioned previously, probiotics are being widely studied for the treatment and prevention of many inflammatory intestinal diseases. There are many types of experimental mouse models of colitis where *L. reuteri* treatment has been promising. When this specific probiotic was administered to mice before receiving DSS colitis there was an overall improvement of clinical inflammation, a decrease in TNF-α and MCP-1, and a decrease infiltration of neutrophils to the lamina propria (van der Kleij *et al.* 2008). In a TNBS model of colitis, *L. reuteri* treatment improved weight gain at the end of the study, decreased MPO and TNF-α production, and modified microflora (Peran *et al.* 2007). Microflora alteration with *L. reuteri* treatment is also of importance in the IL-10 KO model of colitis, as bacteria are necessary for the development of colitis in IL-10 deficient mice (Madsen *et al.* 1999; Sellon *et al.* 1998). These mice have a decrease in protective *Lactobacillus* species, and supplementing with *L. reuteri* helps to normalize the mucosal bacteria and improve the amount of translocated and invasive bacterial species. This alteration in microflora attenuates some of the colonic injury and inflammation (Madsen *et al.* 1999). In a clinical study, *L. reuteri* supplemented to breastfed infants with colic quickly decreased the total crying time in a day, as well as the number of times a day the baby cried (Savino *et al.* 2007). The probiotic supplement possibly alters the intestinal flora, which may be important in the development of colic (Savino *et al.* 2007).

The promising research being conducted on this strain of *Lactobacilli* makes this probiotic a good choice for looking into the alterations it may have on mice infected with *T. muris.* The possibility of the probiotic treatment altering flora, T regs, cytokines, and cell infiltration during the infection is not only interesting, but also may turn out to be a beneficial treatment or preventative method to help fight this common parasite.

1.6 Aims and Hypothesis

The main goal of this thesis is to examine and explore the effect of *L. reuteri* administration in host response to different strains of mice with a *T. muris* infection. Probiotics are a huge area of research currently and very little is known about mechanisms of action. My hypothesis is that treatment with *L. reuteri* will promote host resistance in *T.muris* infection by increasing worm expulsion from the GI tract. Investigation into the probiotic's role as a beneficial supplement to help in parasite eradication may open new treatment and prevention methods to help millions of people worldwide. Mechanisms such as alterations in levels of cytokines, changes in intestinal cellular response, and looking at overall host responses and expulsion rates to a *T. muris* parasite infection will likely open many new doors of probiotic research and treatments of parasitic infections.

This thesis has three main objectives:

1. To elucidate whether treatment with probiotic *L. reuteri* can alter host response and promote *T. muris* expulsion in resistant mice.

2. To determine the potential effector mechanism by which the probiotic *L. reuteri* promotes host resistance in *T. muris* infection.
3. To characterize the role of probiotic treatment in susceptible mice with a *T. muris* infection, and if the treatment benefits the host's overall response, and reduces susceptibility.

In summary, as probiotic treatment are on the rise, investigating the role and mechanism of the treatment in different strains of mice will allow for the better understanding on how these bacteria work on the host in different situations. Furthering the research on probiotic treatments will allow for better strategies and a better understanding on what bacteria is the best choice, and what effects they might have on the host in certain situations.

2.0 MATERIALS AND METHODS

2.1 Mice

All mice were housed in the Central Animal Facility at McMaster University. All protocols used were in accordance with McMaster University Care Committee and the guidelines set by Canadian Council of the Use of Laboratory Animals. Experiments were approved by McMaster animal ethics committee and Canadian Guidelines for Animal Research. All mice were kept in sterilized, filter topped cages under specific pathogen free conditions. Mice were on a 12h:12h light dark cycle, and allowed water and autoclaved chow *ad libitum.* Mice were between 6-8 weeks old at beginning of experiment and never reached past 12 weeks of age during experiments. Mice were caged according to group, and never with more than 5 mice per cage. All mice ordered in from other facilities were allowed a 7 day acclimatization period before the beginning of the experiment.

2.1.1 C57BL/6

Male C57BL/6 mice were ordered from Taconic Farms Suppliers Inc. (Germantown, NY, USA). This specific strain of mice are resistant to a *T. muris* infection and expel the parasite within 25-30 days (deSchoolmeester and Else, 2002).

2.1.2 IL-10 KO

Six week old IL-10 KO mice from Jackson Laboratory (Bay Harbor, ME, USA) on a C57BL/6 background (B6.129P2-Il10 ^{tm1Cgn}/J) were used for the IL-10 study. These mice have a targeted gene deletion in the IL-10 allele though a specific

mutation (Kuhn *et al.* 1993). These mice spontaneously develop colitis, with timing and severity depending on bacterial colonization of the GI tract. Mice were closely monitored daily to ensure there was no development of colitis throughout the experiments.

2.1.3 Muc2 KO

A breeding colony of Muc2 KO mice were housed at McMaster University Animal Center, and were raised to at least 6 weeks of age before starting the experiments. These mice occasionally develop rectal prolapses, but do not develop spontaneous inflammation or colorectal cancer in the facility. If a mouse during the experiment developed a prolapsed rectum, the mouse was removed from the experiment, as it reached its endpoint. Muc2 KO mice were originally made by replacing a fragment of the genome with phosphoglycerate kinase-meomycin, and were developed on a C57BL/6 background (Albert Einstein Medical College, Bronx, NY, USA) (Velcich *et al.* 2002).

2.1.4 AKR

Five week old male AKR mice were ordered from Jackson Laboratories (Bay Harbor, ME, USA). These mice are susceptible to a chronic *T .muris* infection and develop a mild colitis with this infection (Else and Grencis, 1991). Mice were monitored throughout infection course to ensure that severe inflammation and weight loss was not occurring.

2.2 Probiotics and Medium

2.2.1 Live *Lactobacillus reuteri*

This strain of probiotics was purchased by Dr. John Bienenstock originally from America Tissue Type Culture Collection (ATCC#23272)(Manassas, VA, USA). The probiotics were given to the Khan laboratory as a kind gift from the laboratories of Dr. John Bienenstock and Dr. Paul Forsythe (McMaster University, St. Joseph's Hospital, Hamilton, ON, Can.). Briefly, these bacteria were grown at 37°C under anaerobic conditions for 48 hours, collected, centrifuged for at room temperature at 2000 rpm, and washed twice in sterile PBS. Bacteria were measured in Vitek colorimeter (bioMeraux, Hazelwood, MO, USA), then diluted to give a final concentration of of $5x10^9$CFU/mL. Bacterial suspension was then centrifuged again, supernatants discarded, and bacteria were resuspended in 1mL of MRS to give the optimal concentration of bacteria/mL. This particular strain of *L. reuteri* is of human intestinal origin. Aliquots of bacteria at a concentration of $5x10^9$CFU/mL were frozen, and used at time of experiment. Mice were fed 200µL daily ($1x10^9$ CFU) by gavage for 15 or 35 days total, depending on experiment. This is an optimal dose previously optimized by Dr. Bienenstock and Dr. Forsythe.

2.2.2. γ-irradiated *Lactobacillus reuteri*

The same viable *L. reuteri* was used and prepared in the same manner as described above. The bacteria were then killed using gamma irradiation with Cobalt 60 for 20 hours at 8.05 Gy/min. Viability was determined by plating killed bacteria on MRS agar plates under anaerobic conditions for 72 hours at 37°C. No bacterial

growth was detected in irradiated *L. reuteri* preparations. The same dose as the live probiotic was used for the γ-irradiated probiotic.

2.2.3 MRS Medium: deMan-Rogosa-Sharpe Medium (MRS)

Lactobacilli MRS broth was the medium used throughout all experiments as a control (BD-Difco Laboratories; Ref#288130, Lot#0049647, Sparks, MD, USA). This medium is used in the isolation, enumeration and cultivation of *Lactobacillus* species. The formulation of this medium was shown previously to support the growth of all *Lactobacillus* species. This broth contains peptone and dextrose, and other ingredients to supply nitrogen, carbon, and other elements necessary for optimal growth of bacteria, and hinders growth of gram negative bacteria (deMan *et al.* 1960).

2.3 *Trichuris muris* Parasite Techniques

2.3.1 Maintenance of the Parasite

T. muris eggs were originally a kind gift from Dr. Richard Grencis. The colony is now maintained at McMaster University in Dr. Khan's laboratory through serial infections of immunodeficient mice, age 6-8 weeks, infected with ~400 eggs of *T. muris*. These mice are susceptible to a *T. muris* infection and in 2 months, the worms mature and reproduce. The caecum of the mouse was removed after 8 weeks and rinsed in PBS with 5% PenStrep antibiotics. The caecum was then placed in RPMI 1640 medium with 5% PenStrep, and worms were gently removed. Worms were then transferred into a 6 well plate with 4mL RPMI+5% PenStrep medium and incubated overnight at 37°C in a humid box. Twenty four hours later the supernatant

containing the released eggs was collected. Eggs were then resuspended in sterile distilled H_2O and filtered through a 100μm filter. Eggs were washed 2-3 times with sterile dH_2O and centrifuged. Eggs were resuspended and transferred into a non-filter top tissue culture flask and filled half way with sterile dH_2O. The flask was covered with tin foil and stored in a dark area for 8 weeks for the eggs to grow and embryonate. Flasks are checked monthly to change dH_2O and to check for contamination and other fungal growth.

2.3.2 Oral Infection of *T. muris*

Eggs were checked before infection to ensure they were viable, and not contaminated. Amounts of eggs were also checked to calculate the appropriate dose to give each mouse to ensure successful infection. Mice were gavaged with approximately 400 eggs on day 0 of each experiment.

2.3.3 Worm Burden Analysis

After each time point during the experiment, the caecum was removed from each infected mouse for post mortem analysis. The caecums were frozen at -20°C for at least 24 hours. When counted, caecums were thawed, slit open, and the mucosa was scraped in dH_2O to remove any embedded worms. Examination of the fecal content as well as the mucosa under a dissecting microscope was performed to determine the number of worms in the caecum at each time point throughout the experiment.

2.4 Bromodeoxyuridine (BrdU)

BrdU (Sigma, St. Louis, MO, USA) incorporates into newly synthesized DNA in proliferating cells (Vega and Peterson, 2005). This allows for the visualization and analysis of the amount of cells undergoing proliferation and division at that specific time point. BrdU is diluted in sterile PBS to create a 20mg/mL solution. A dose of 10mg/mL was injected interperitoneally at a volume of 500µL, 6-8 hours before sacrificing the mouse.

2.5 Immunohistochemistry

After caecal and colonic tissues were retrieved, they were immediately submerged in 10% buffered formalin to be fixed, and sent to the Department of Pathology (McMaster University, Hamilton, ON, Can.) for paraffin embedding, and sectioning at 3-4 µm. When staining was complete, cells were counted blindly, recording the number of positive cells per 10 crypts, for a total of 50 crypts at a magnification of 200x.

2.5.1 Serotonin (5-HT) Staining

Slides were deparaffinized at 60°C for 30 minutes then submerged in CitriSolv (Fisher Scientific, Nepean, ON, Can.) and rehydrated through a series of ethanol gradients. Endogenous peroxidase was blocked by addition of peroxidase blocking agent (Dako North America, Carpinteria, CA, USA) for 15-30 minutes then washed in phosphate buffered saline (PBS). Antigen retrieval was done using citrate buffer (10nµ, pH 6.0, heated in microwave) then washed in PBS before blocking

unwanted non-specific binding sites with 1% bovine serum albumin (Sigma, St. Louis, MO, USA) diluted in PBS. Slides were then incubated with rabbit anti-5-HT antibody (ImmunoStar Inc., Hudson, Wisconsin, USA) at 1:5000 dilution in PBS for one hour. After the slides were rinsed with PBS, sections were incubated with Envision, a horseradish peroxidase-coupled anti-rabbit secondary reagent, (Dako North America, Carpinteria, CA, USA) for 30 minutes. Sections were then developed with 3, 3' diaminobenzidine (Sigma, St. Louis, MO, USA). The chromogen from the tablets targets the peroxidase on the Envision bound areas. Finally, slides were counter stained using Meyer's haematoxylin, mounted and cover slipped.

2.5.2 Periodic acid-Schiff (PAS+) Staining

Periodic acid-Schiff stain is used to detect glycogen containing cells, such as goblet cells. Slides were stained in the Pathology Department (McMaster University, Hamilton, ON, Can.). Briefly, slides were deparaffinized and treated with 1% Periodic acid at room temperature for 5-10 minutes. After they were rinsed in dH_2O, they were treated with Schiff's reagent at room temperature for 10 minutes then rinsed in warm dH_2O for 10 minutes. Sections are counterstained with Mayer's hematoxylin for 1 minute and rinsed well in tap water before being mounted.

2.5.3 BrdU Staining

Slides were deparaffinized in an oven at 60°C for 30 minutes, submerged in CitriSolv (Fisher Scientific, Nepean, ON, Can.) then rehydrated through a series of ethanol gradients. Antigen retrieval was done using citrate buffer (10nμ, pH 6.0,

heated in microwave) then washed in PBS before blocking unwanted non-specific binding sites with 1% bovine serum albumin (Sigma, St. Louis, MO, USA) diluted in PBS. Slides were then incubated for one hour with primary antibody, rat anti-Brdu (AbD Serotec, Langford, UK) at 1:100 dilution in PBS and 1% BSA. After the slides were rinsed with PBS, sections were incubated with secondary antibody at a 1:200 dilution in PBS (donkey anti-rat; AlexaFluor 488; Invitrogen) for 30 minutes at room temperature, in the dark. Finally slides were washed in PBS and mounted. Slides were examined using florescent microscope and analyzed using NIS-Elements Basic Research (Version 3.10, 2009, Nikon) using object count settings taking into consideration minimal and maximum area as well as mean intensity. Settings were applied to each slide, and one full crypt was analyzed. A minimum of 7 crypts were analyzed per slide, at a magnification of 400x.

2.6 Spleen Cell Cultures

Spleens from each mouse were taken aseptically and placed in Hank's solution to be crushed and filtered through a 5µm filter. This allows lymphocytes and red blood cells to pass, but fat and debris to be removed. Cells were then centrifuged for 10 minutes at 2000 rpm, and red blood cells were lysed using Tris-NH_4Cl mixture (pH 7.2) and washed with Hank's solution. Lymphocytes were diluted to one million cells per one mL of culture media made from RPMI containing 10% Fetal Calf Serum (Invitrogen, Burlington, ON, Can.), 1% PenStrep antibiotics (Invitrogen, Burlington, ON, Can.), and 50µM/L 2-mercapto-ethanol (Sigma, St. Louis, MO, USA). One million cells (1mL) were cultured with either 5µL of Concavalin A (concentration of

1µg/µL) (Sigma, St. Louis, MO, USA), control media, or *T. muris* antigen purified from worm culture at a concentration of ~500µg/mL for 24 hours. The supernatants were collected for analysis of cytokine release from stimulated lymphocytes.

2.7 Cytokine Analysis

Segments of proximal colon were snap frozen upon retrieval and homogenized in 1mL of lysis buffer containing Tris-HCl, NaCl and Triton X-100 and 50µL protease inhibitor cocktail (Sigma, St. Louis, MO, USA) per 10mL lysis buffer. The protease inhibitor prevents endogenous enzymes from degrading proteins. The homogenized samples were then centrifuged at 6000rpm for 5 minutes at 4°C, and supernatants were collected and stored at -80°C until used in the cytokine ELISA kit. All cytokine Quantikine ELISA kits (IL-1β, IL-4, IL-10, IL-12, IL-13, IL-17, IFN-γ, and TGF-β) were purchased from R&D Systems (Minneapolis, MN, USA) and are commercially available. Assays were run according to manufacturer's protocols. Kits were used for both tissue and cell cultures supernatants. Amounts of tissue cytokines are expressed as pg/mg of protein, determined with the D_c Protein Assay Kit (Bio Rad Laboratories, Hercules, CA, USA). Plates were read using and Absorbance Microplate Reader (BioTek, Winooski, VT, USA) and Gen5.1 software (BioTek, Winooski, VT, USA).

2.8 Serotonin Analysis in Colonic Tissue Samples

Segments of proximal colon were snap frozen upon retrieval and stored in -80°C until they were homogenized in 0.5mL of 0.2 perchloric acid ($HClO_4$) then

centrifuged at 10000g for 5 minutes at 4°C. Supernatants were collected and neutralized to a pH of 7-8 with 0.5mL of 1.0mol borate buffer (pH 9.25) then centrifuged for 1 minute at 10000g at 4°C. Supernatant was collected and 5-HT content was analyzed using a commercially available ELISA kit, as per the directions from the manufacturer's protocol (Beckman Coulter, CA, USA). The total 5-HT content in the colon tissue was expressed as a function of wet weight (ng/g).

2.9 Myeloperoxidase (MPO) Assay from Colonic Tissue Samples

The MPO assay measures the amount of MPO in the tissue, and is a common marker of neutrophil degranulation and overall amount of infiltrating neutrophils. The amount of MPO in the tissue is used to quantify acute intestinal inflammation (Krawisz *et al.* 1984). Briefly, colon tissue samples were homogenized in 50mM potassium phosphate buffer (pH 6.0) containing 0.5% hexadecyltrimethylammonium bromide buffer. Supernatants were collected then O-dianisidine dyhydrochloride (Sigma, St. Louis, MO, USA) and hydrogen peroxide (EMD Chemicals, Gibbstown, NJ, USA) were added. A spectrophotometer measured absorbance of the colorimetric reaction. Colonic MPO amounts are expressed in units per milligram of wet tissue, and one unit is the amount of enzyme able to convert 1µmol of hydrogen peroxide to H_2O in one minute at room temperature (Krawisz *et al.* 1984).

2.10 Flow Cytometry of Mesenteric Lymph Nodes (MLNs) of AKR Mice

On day 35, MLNs were removed from ARK mice to form a single cell suspension in sterile PBS and kept on ice. Suspension was then transferred to St.

Joseph's hospital to run flow cytometry by Jessie Chau. Briefly, cells were washed and suspended in FACS buffer (PBS containing 0.1% Azide and 2% BSA) and stained with fluorochrome labelled antibodies including CD4-FITC (1:300), CD3-APC Cy7 (1:300), CD25-APC (1:200), and Foxp3-PerCP-Cy5.5 (1:200) (all from eBioscience, San Diego, CA, USA). For intracellular staining of Foxp3, cells were permeabilized beforehand. Stained cells were acquired using the LSR II using FACSDiva software (BD Biosciences, Mississawga, ON, Canada) and analyzed with Flowjo software (TreeStar Inc., Ashland, OR, USA).

2.11 Overall Experimental Design

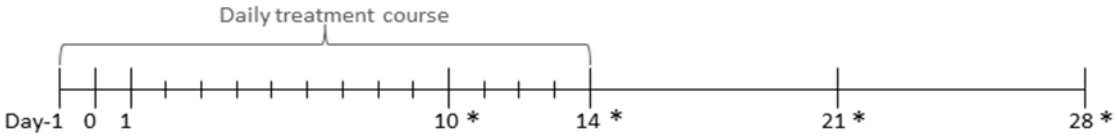
L. reuteri treatment by gavage was started on day -1 and continued until day 14 for all experiments except for AKR mice, where the probiotic treatment was carried out until day 35. MRS media was the control for all experiments and was given in the same volume, daily, for as long as the probiotic treatment was continued. Mice were infected with ~400 *T. muris* eggs on day 0 of experiment. Mice were sacrificed by cervical dislocation on day 10, 14, 21 for each experiment except for IL-10 KO mice, which were only taken out to day 14, and AKR mice, which were only sacrificed on day 35 (Figure 2). There were also some C57BL/6 mice taken out to day 28 to check if worms were expelled at this time, and restoration of homeostasis was achieved. The n numbers varied depending on the groups and experiment. N=5 for most infected mice treated with probiotics or media, except for AKR mice, which n=6. Control numbers were either n=2 for no infection and no treatment, or n=4 for no infection but did receive treatment of either media or the probiotic. The weight of

each mouse was monitored 2-3 times per week to continually monitor for signs of inflammation and illness. If mice ever reached an end point or developed a prolapsed rectum, they were removed from the experiment.

2.12 Statistical Analysis

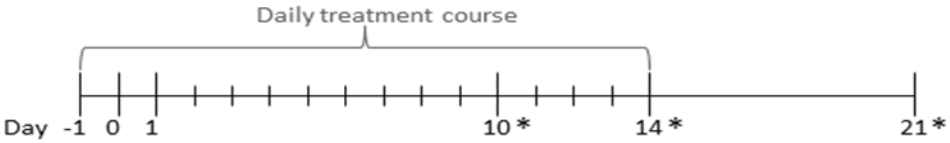
Statistical significance was determined using GraphPad Prism software (Version 4, 2004, San Diego, CA, USA) using either a one way ANOVA to compare more than two treatment groups, or the unpaired Student t-test for two groups with normally distributed data. Significance was reported if values were $p<0.05$ with a 95% confidence level. Outliers were determined using GraphPad QuickCalcs Outlier Calculator using the Extreme Studentized Deviate method, also known as Grubb's tests. This test determined values that are significant outliers, where 0.05 was the significance level, meaning that there is less than a 5% chance this value would be seen by chance in the population. All data are presented as mean ± stand error of mean.

a) *L. reuteri* (live or γ-irradiated) treatment in C57BL/6 with *T. muris* infection.



b) *L. reuteri* treatment in IL-10 KO mice with *T. muris* infection.

c) *L. reuteri* treatment in Muc2 KO with *T. muris* infection.



d) *L. reuteri* treatment in AKR mice with *T. muris* infection.

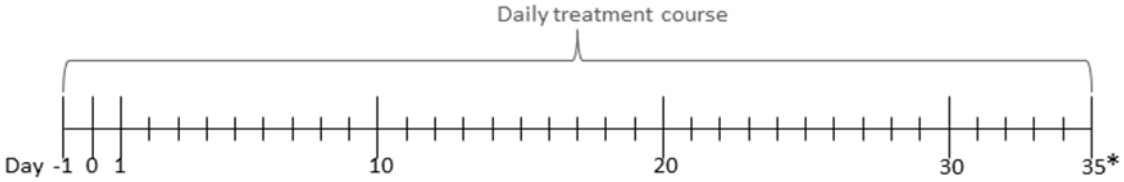


Figure 2: Experimental design of each strain of mice. Daily treatment of *L. reuteri*, live or γ-irradiated, or MRS media starts on day -1 and continues for appropriate number of days. Volume gavaged daily of media was 200μL, or 200μL of media containing $1x10^9$ CFU of the probiotic. Gavaged infection of each mouse with ~400 viable eggs of *T. muris* was done on day 0. * represents sacrifice points throughout experiment where the caecum, spleen and 4 sections of proximal colon were collected.

3.0 RESULTS

3.1 Resistant C57BL/6 mice and live *L. reuteri*

In the first experiments using the probiotic to treat a *T. muris* infection we used the resistant C57BL/6 mice, which expel *T. muris* naturally in 25-35 days. This allowed for the understanding of the probiotic treatment in a T_H2 immune environment. Mice were treated with *L. reuteri* until day 14, but checked on day 10, 14, 21 and 28 for all parameters.

Worm burden was quantified at each time point to determine if the probiotics had any effect on worm expulsion. On days 10 and 21 the mice infected with *T. muris* but getting treatment with *L. reuteri* had a reduced amount of worms in the caecum, suggesting that the probiotic treatment increased worm expulsion in this strain of mice (Figure 3). Worm burden on day 14 did not reach significance, but possibly with an increased number of mice there would be a significant difference. Mice treated with *L. reuteri* probiotic expelled the worms at a more rapid rate than the medium treated controls, which would allow these mice to return to their normal immune status earlier and have a homeostatic GI environment again. All groups expelled most of the worms by day 28, which is expected in mice with a T_H2 immune response to the infection.

The cytokine milieu of the GI mucosal tissue, as well as the cytokines secreted by T lymphocytes is important in the overall infection model. The overall immune response of the mice determines the susceptibility of the mouse, and T_H2 cytokines are important in the resistant strains. Both T_H2 and T_H1 cytokines, as well

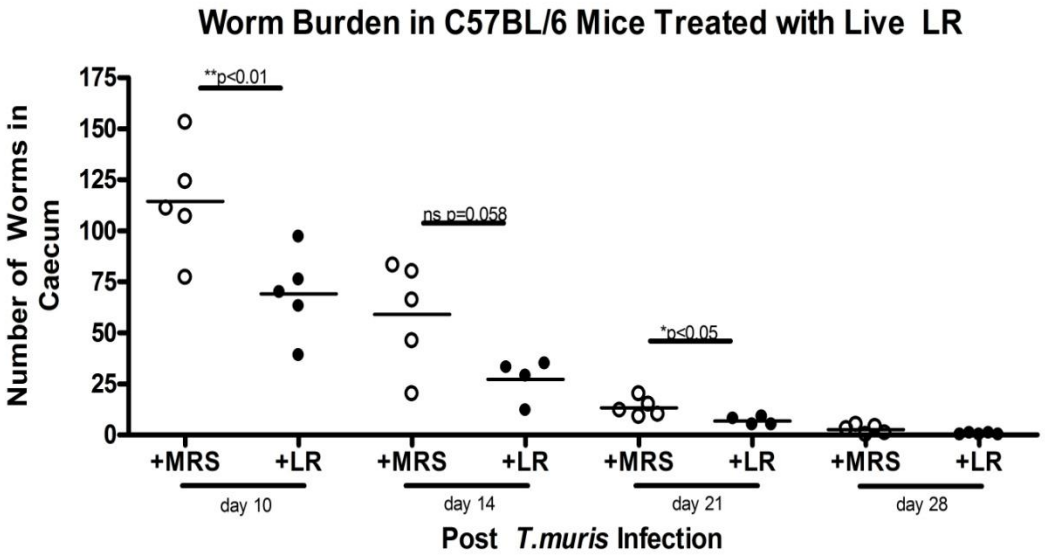


Figure 3: Worm burden in resistant C57BL/6 mice treated with live *L. reuteri* or MRS. Mice received treatment for 14 days post *T. muris* infection. Worm burden was determined by the number of worms in the caecum of each mouse. There was a significant decrease of number of worms on day 10 and 21 when mice received *L. reuteri* treatment. *p<0.05, **p<0.01, with n=5.

as a few T reg associated cytokines were investigated in the tissue of the colon of infected mice at each time point, as well as in spleen cell T lymphocyte culture supernatant. In the tissue, there were no alterations in T_H2 cytokines, IL-4, IL-13, or TGF-β, in the probiotic treated infected groups compared to the medium treated infected controls (Figure 4). With respect to other cytokines that might have been affected, there was no change between groups in IL-12, MCP-1, IL-17 or IFN-γ (Figure 4 continued). Interestingly, IL-10 amounts in the tissue of probiotic treated infected mice were significantly increased on day 10 of infection. (Figure 4). The cytokine responses from splenic T lymphocytes were measured in vitro, with stimulation with Con A and *T. muris* antigen. There were no significant differences in any of the T_H2 or T_H1 cytokines checked, or IL-10 amounts.

Alterations in muscle function and cellular responses in the GI tract are also correlated with *T. muris* infections, and are different in resistant and susceptible mice (Else and Grencis, 1991; Khan *et al.* 2003). Cells in the lamina propria such as mast cells and eosinophils are altered during parasitic infection, as well as cells of the intestinal epithelium (Else and Grencis, 1994; Richard *et al.* 2000). Epithelial located cells that are of interest during a *T. muris* infection are both the goblet cells and serotonin secreting enterochromaffin (EC) cells. In these studies, the main focus is on goblet cells responses, but EC cell alterations were also investigated. These cells were checked in the colon of infected mice treated with medium or *L. reuteri* at each time point. Goblet cells are known to increase in numbers in many different parasite infections of the GI tract, to increase the amount of mucin, and possibly help in flushing the parasite out of the colon (Ishikawa *et al.* 1997). When mice were

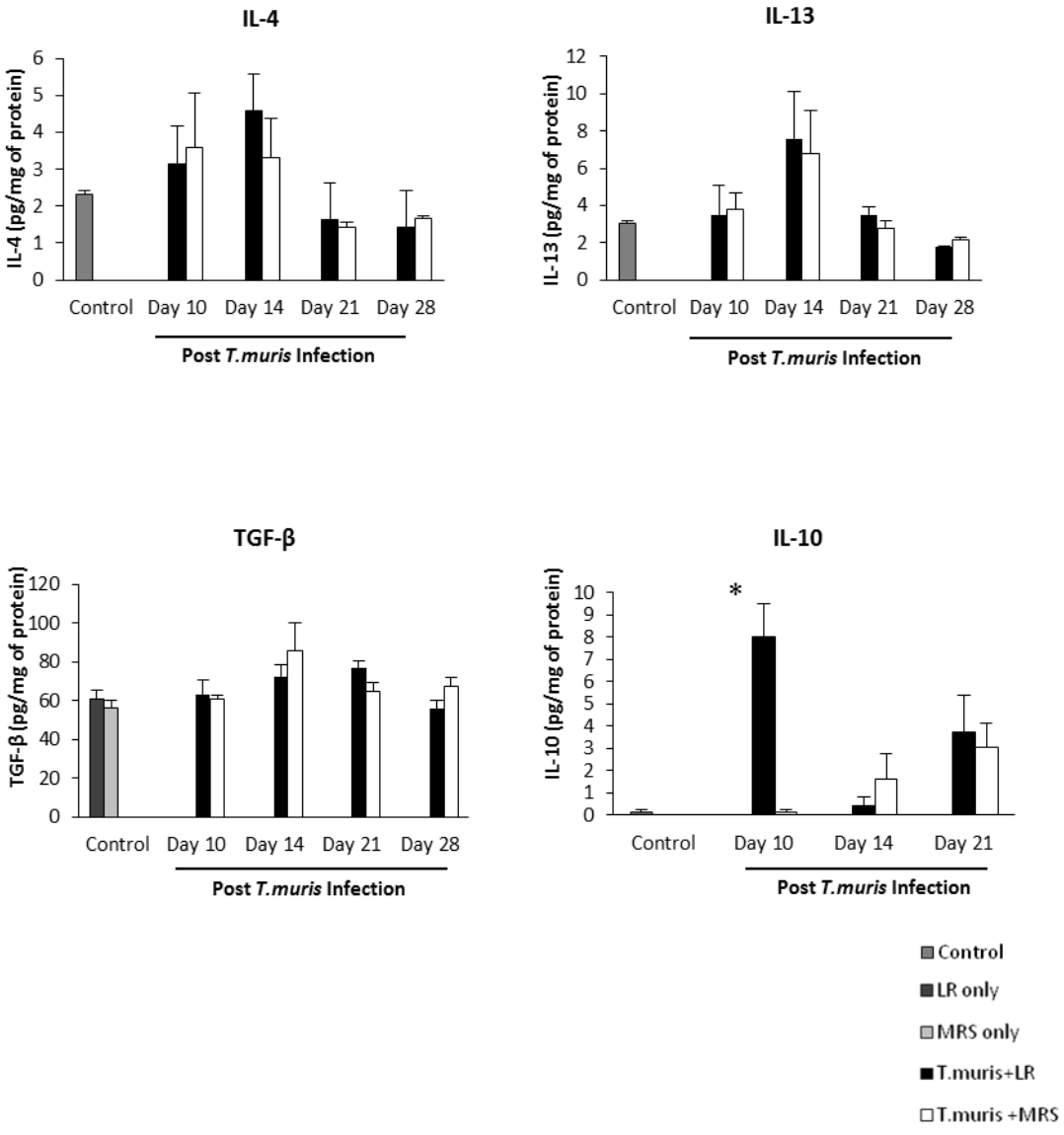


Figure 4: Cytokine responses from colon tissue of various cytokines from C57BL/6 mice. All values were normalized for protein content and expressed as pg/mg of protein. n=5 for all groups except controls, where n=4 or 2. *L. reuteri* values are compared to MRS treated groups, and expressed as mean +/- SEM. *p<0.05.

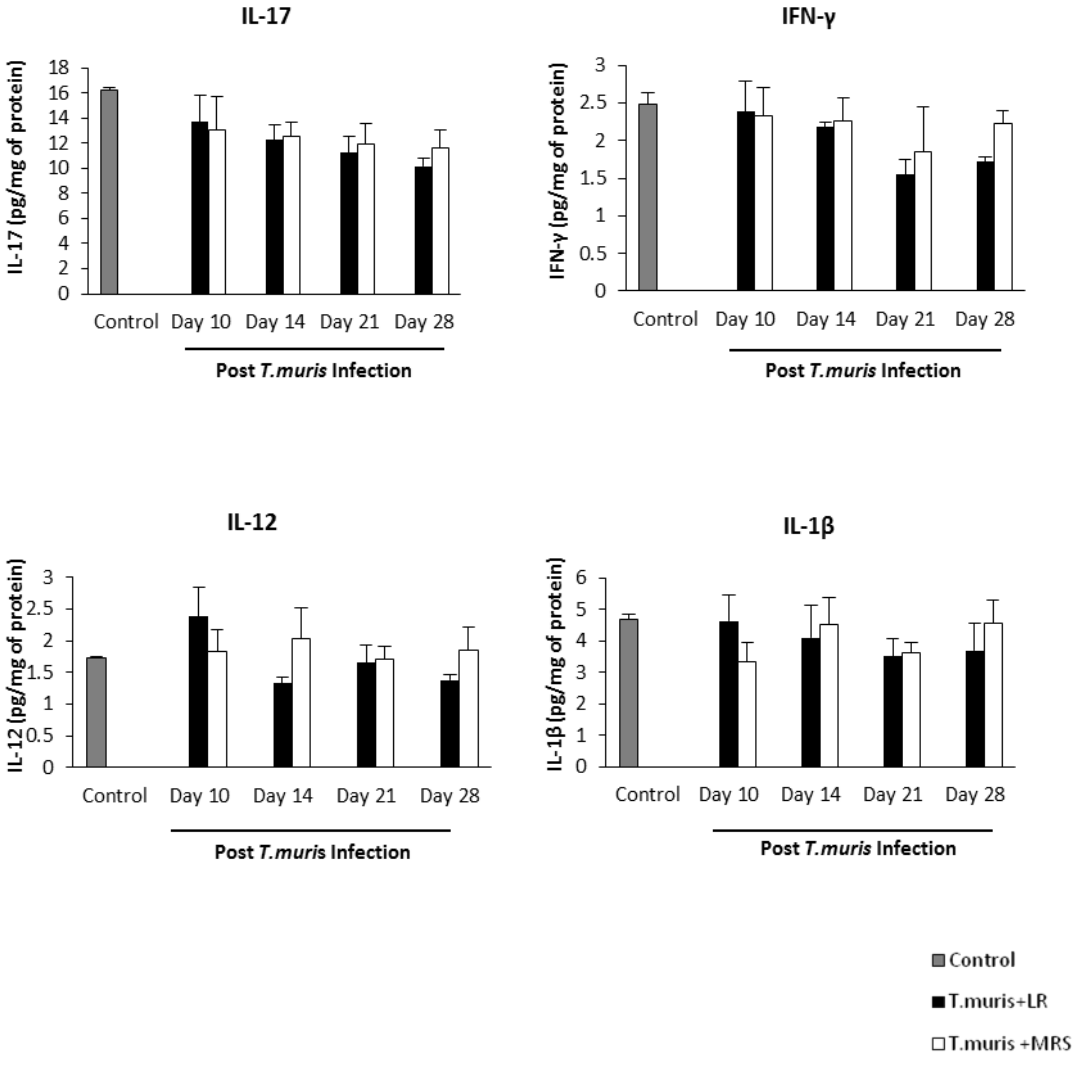


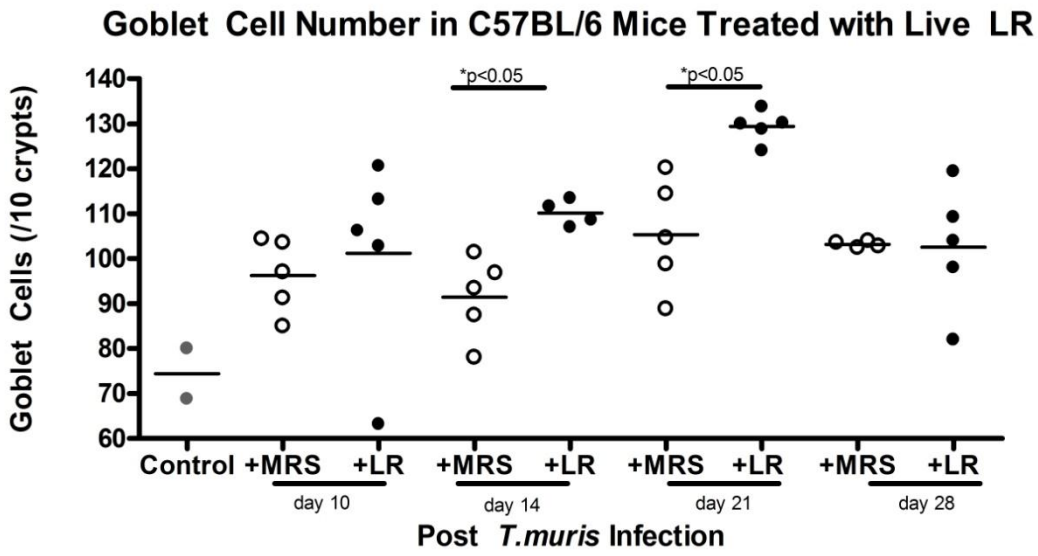
Figure 4 continued: Colon tissue concentrations of various cytokines from C57BL/6 mice. All values were normalized for protein content and expressed as pg/mg of protein. n=5 for all groups except controls, where n=4 or 2. *L. reuteri* values are compared to MRS treated groups, and expressed as mean +/- SEM. *p<0.05.

infected with *T. muris* there was an overall increase of goblet cells in the colon. This increase in hyperplasia of goblet cells was significantly higher on days 14 and 21 in mice that were treated with the probiotic (Figure 5). When the EC cells of the GI tract in infected C57BL/6 mice were stained and quantified, it was noted that the probiotic treatment did not alter the number of EC cells in the colon or caecum, when compared to the EC cell number of infected mice treated with media only (Figure 6).

Other mediators were investigated during the infection such as tissue 5-HT amounts, MPO amounts as well as the weights of the mice. The mice were weighed to ensure they were getting all of the nutrients needed to thrive throughout the experiment. All of the mice gained weight steadily and gradually as they would without a parasitic infection. Serotonin content of the mice was also measured at each time point. There were no differences in 5-HT amounts between probiotic or medium treated mice, correlating with unaltered EC cell amounts between treatment groups. MPO levels were also measured in the tissue of mice as a marker of inflammation and neutrophil infiltration. MPO amounts did not differ between *L. reuteri* or MRS treatment. In subsequent studies, the focus remains on cytokine and goblet cell alterations, more so than EC cell responses and 5-HT amounts, as these remained unaltered with the probiotic treatment.

The probiotic treatment given to infected mice increased IL-10 amounts, as well as goblet cell amounts. These increases may have contributed to the more rapid worm expulsion of these mice. The next experiments not only address these factors, but also if the probiotic bacteria need to be viable for the benefits that were seen.

a)



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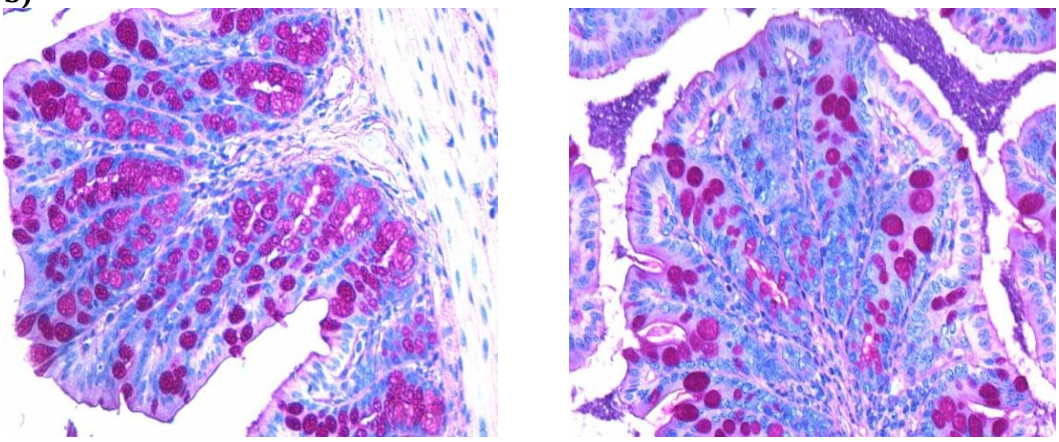
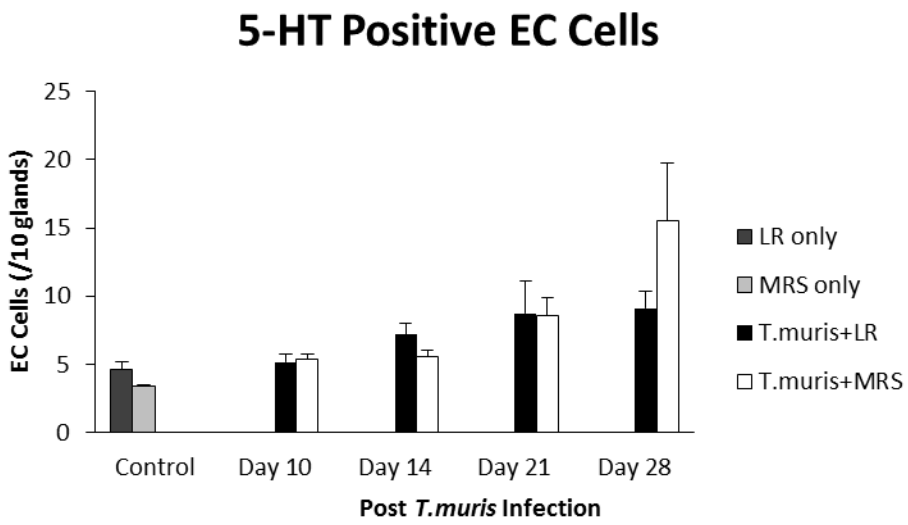


Day 21 *T. muris*+LR

Day 21 *T. muris*+MRS

Figure 5: a) Goblet cell counts from the colon of resistant C57BL/6 mice. Uninfected controls, and *T. muris* infected mice treated with either *L. reuteri* or MRS. There is a significant increase in the total goblet cell numbers in the infected mice treated with *L. reuteri* on days 14 and 21 post infection. A minimum of 50 crypts were counted, expressed as goblet cells/10 crypts. *$p<0.05$ where n=5 in each group. b) Corresponding histological sections of the colon stained with PAS show the increase of PAS+ cells, representing goblet cells. Magnification of 200x.

a)



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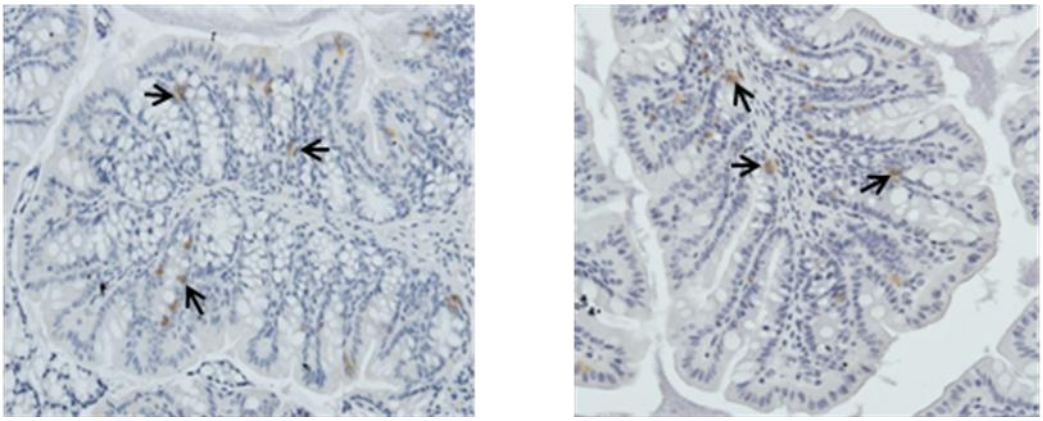


Day 21 *T. muris*+LR Day 21 *T. muris*+MRS

Figure 6: a) Cell count for enterochromaffin (EC) cells stained for 5-HT in the colon of resistant C57BL/6 mice infected with *T. muris* and treated with either *L. reuteri* or MRS. There were no differences in the number of 5-HT positive EC cells in mice treated with *L. reuteri* compared to the medium treated mice. A minimum of 50 crypts were counted, expressed as goblet cells/10 crypts. b) Corresponding histological sections of the colon stained for 5-HT (orange stained cells), representing 5-HT positive EC cells. Magnification of 200x.

3.1.1 Proliferation in Response to Treatment and Infection

Resistant C57BL/6 were used in the proliferation study, to examine the effects of the probiotics on the proliferation of mucosal epithelial cells during a *T. muris* infection, or if the proliferation rates were unchanged with the treatment. After the BrdU injection and mice were sacrificed, tissue sections of the proximal colon were fluorescently stained using immunohistochemistry techniques, imaging, and quantification. The tissue sections of infected mice on day 21 and treated with *L. reuteri* showed a significant increase in the number of cells per crypt undergoing proliferation in the colon. In accordance with other research, an infection with *T. muris* in C57BL/6 mice increased the number of proliferating cells significantly by day 21. Interestingly, with *L. reuteri* treatment, there is a significant increase of proliferating cells compared to the medium treated mice (Figure 7).

3.2 Resistant C57BL/6 mice and γ-irradiated *L. reuteri*

To determine if *L. reuteri* has beneficial effects on the host only if viable, killed γ-irradiated *L. reuteri* was obtained and used in the same model as the previous experiment. This form of the probiotic is killed using exposure to γ-irradiation, and was used to shed light on if the effects seen previously were from direct interaction, secreted product, or if a factor on the surface of the probiotic was altering host responses. This experiment was taken out to day 21 and the same parameters were checked as in the experiment with the live *L. reuteri*.

The worm burden was quantified to see if there was an increase in expulsion rate when treated with γ-irradiated *L. reuteri*. The worm burden was unchanged in

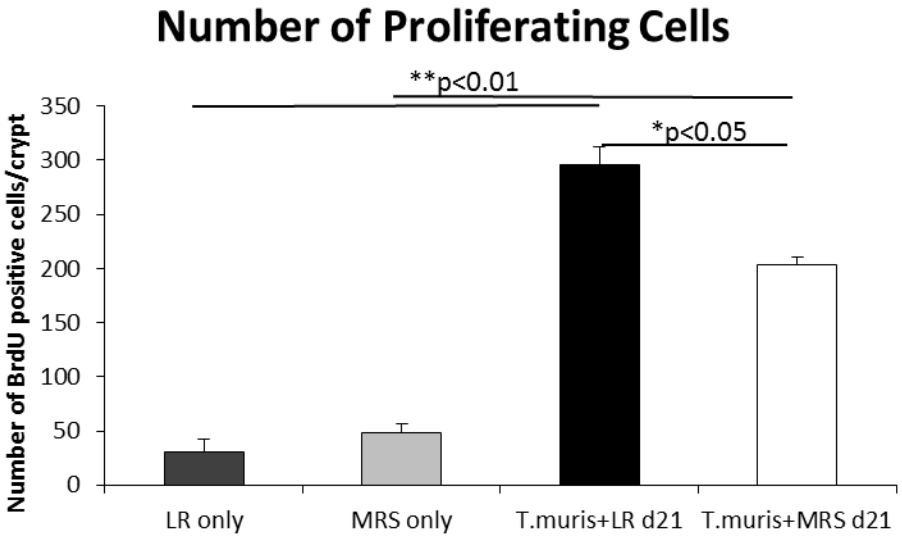


Figure 7: Number of proliferating cells per crypt in C57BL/6 mice. Both groups with *T. muris* infection were significantly higher in the number of proliferating cells when compared to groups that were not infected with the parasite. Infected mice treated with *L. reuteri* had a significant increase in the number of proliferating cells compared with medium treated mice. 7-10 crypts examined in each group, all values expressed as mean+/- SEM. * $p<0.05$, **$p<0.001$.

mice that were treated with γ-irradiated *L. reuteri* compared to medium treated mice. The worm numbers were similar in the dead probiotic group as the medium treated group at each time point that was checked (Figure 8). The killed probiotic did not have the same effects on the host's increased expulsion as the live probiotic administration did.

Cytokines were also investigated, in case the γ-irradiated *L. reuteri* altered cytokines without affecting worm burden. Similar cytokines were checked as in the previous experiment. Of the T_H2 cytokines checked in the colonic tissue of infected mice, there were no alterations in amounts of the cytokines with γ-irradiated *L. reuteri* treatment compared to the medium treated group (Figure 9.). The same was noted for all of the T_H1, T_H1 promoting, and regulatory cytokines (Figure 9). There were also no changes in cytokine levels when comparing the two treatment groups from the lymphocyte supernatants from the spleen. These results show that killed *L. reuteri* treatment to resistant mice infected with *T. muris* has no affect on the cytokine profile immune response.

It was still possible that the γ-irradiated *L. reuteri* treatment could alter the cells of the epithelial layer in similar ways as the live probiotics did. Similar amounts of goblet cells were seen in the γ-irradiated *L. reuteri* and medium treated infected mice (Figure 10). The dead probiotic bacteria treatment had no significant effect on the alterations of goblet cell hyperplasia.

When the inflammatory marker MPO was measured from the colonic tissue of the infected mice with the killed *L. reuteri* or MRS treatments, there were no differences between the amounts of MPO detected. The weights of all the mice were

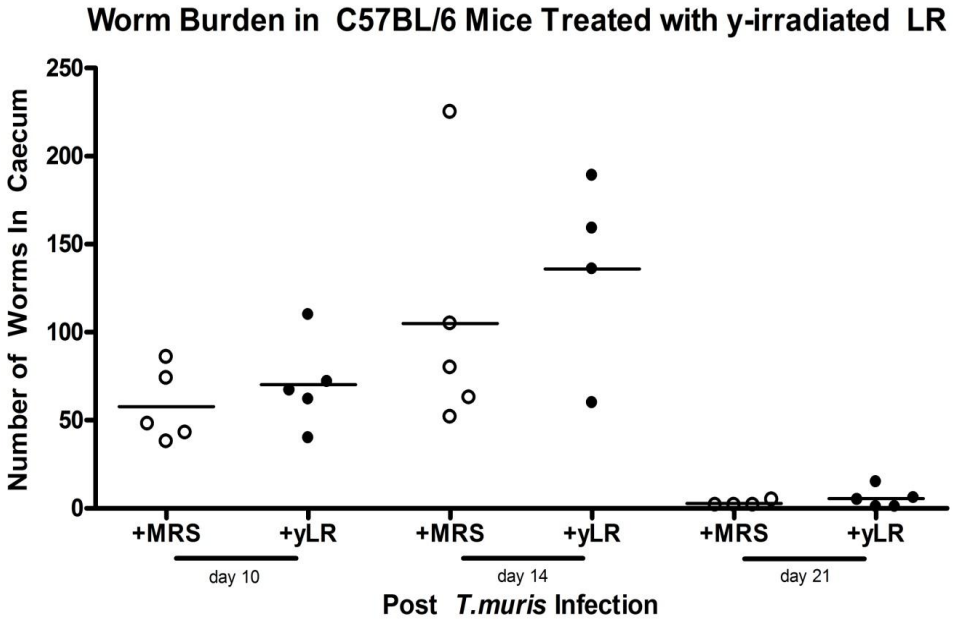


Figure 8: Worm burden in resistant C57BL/6 mice treated with γ-irradiated *L. reuteri* or MRS. Mice received treatment for 14 days post *T. muris* infection. Worm burden was determined by the number of worms in the caecum of each mouse. There was no significant difference of number of worms at any time point when mice received γ-irradiated *L. reuteri* treatment compared with medium treated mice. n=5 in each group, except day 14 when n=4 in the γ-irradiated *L. reuteri* group.

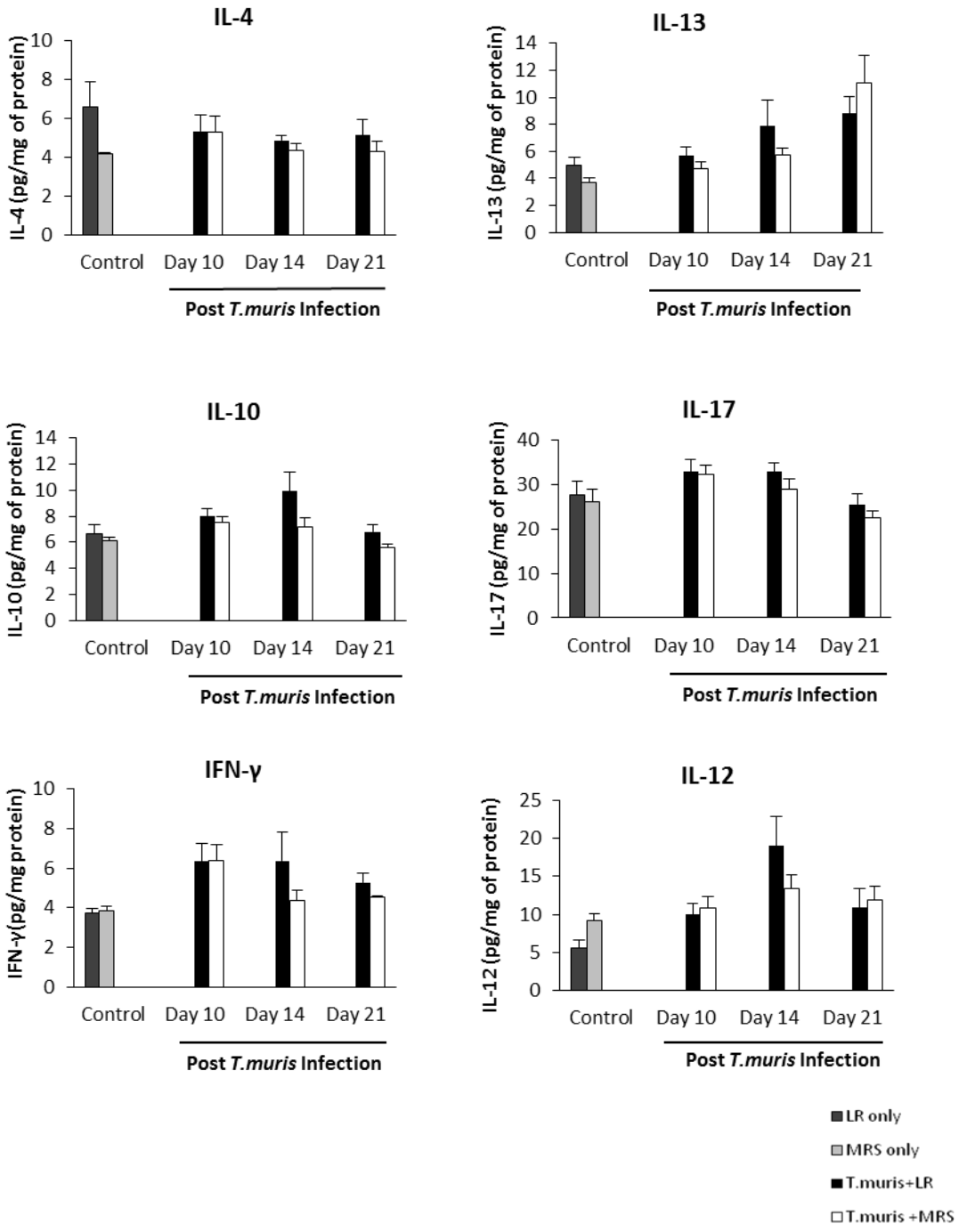
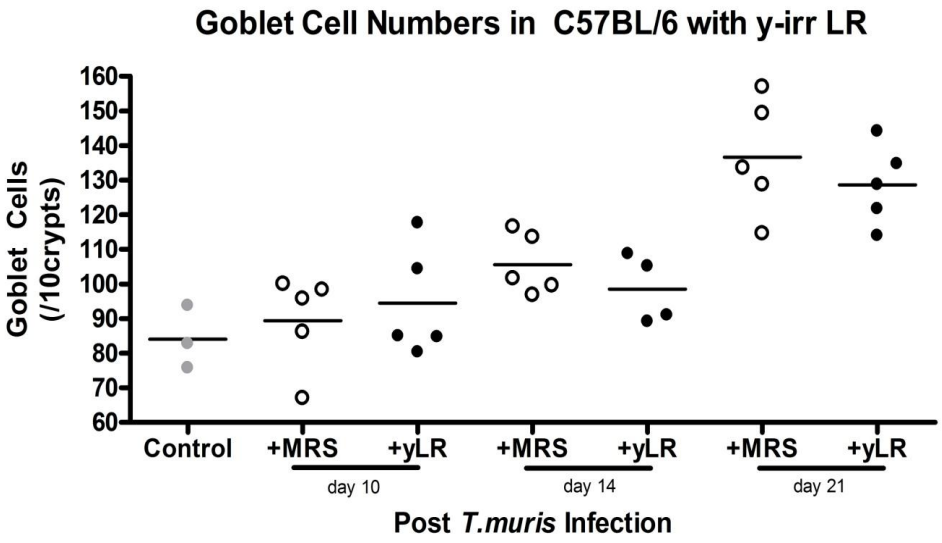
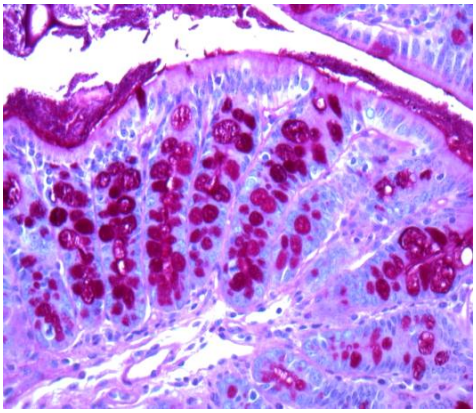
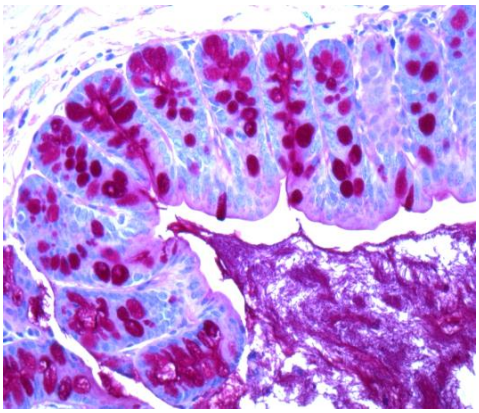


Figure 9: Tissue concentrations of C57BL/6 mice infected with *T. muris* and treated with γ-irradiated *L. reuteri* or MRS. γ-irradiated *L. reuteri* treatment had no effect on cytokine values when compared to medium treated mice. All values were normalized for protein content and expressed as pg/mg of protein. n=5 for all groups except controls, where n=4, values are expressed as mean +/- SEM.





Day 21 *T. muris*+γ-irradiated LR



Day 21 *T. muris*+MRS

Figure 10: a) Goblet cell counts from the colon of resistant C57BL/6 mice infected with *T. muris* and treated with either γ-irradiated *L. reuteri* or MRS. There were no differences in the number of goblet cells in the crypts of the colon in γ-irradiated *L. reuteri* or MRS treated mice. A minimum of 50 crypts were counted, expressed as goblet cells/10 crypts. b) Corresponding histological sections of the colon stained with PAS show the numbers of PAS+ cells are similar in each group. Magnification of 200x.

steadily increasing gradually throughout the entire experiment. These data suggest that for *L. reuteri* to have a beneficial effect on the host's defense in worm expulsion rates and other immunological and cellular changes the bacteria must be alive and viable.

3.3 Role of IL-10 using IL-10 KO mice and live *L. reuteri*

In the initial experiment, it was noted that viable *L. reuteri* treatment in *T. muris* infected C57BL/6 mice, increased IL-10 on day 10. This cytokine is known to have anti-inflammatory properties and contributes to homeostasis of the GI tract (Sanchez-Munoz *et al.* 2008). During parasitic infections, IL-10 plays an important regulatory role, and IL-10 KO mice are susceptible to a *T. muris* infection, and have high mortality rates starting around day 23 (Schopf *et al.* 2002). To determine if IL-10 plays a role in the increased worm expulsion and overall health of *T. muris* infected C57BL/6 mice treated with live *L. reuteri*, the same experiment was preformed, but taken out to only day 14 to ensure the mice did not have severe morbidity and mortality.

The worm amounts were studied at day 10 and day 14 in the experiment to determine if probiotic treatment would benefit the IL-10 KO mice, and possibly reduce the morbidity of this strain of mice. On both day 10 and day 14, the live *L. reuteri* treatment had no significant effect on the number of worms inhabiting the caecum (Figure 11). The probiotic effect on host expulsion is in some way dependent on IL-10 pathways or effects.

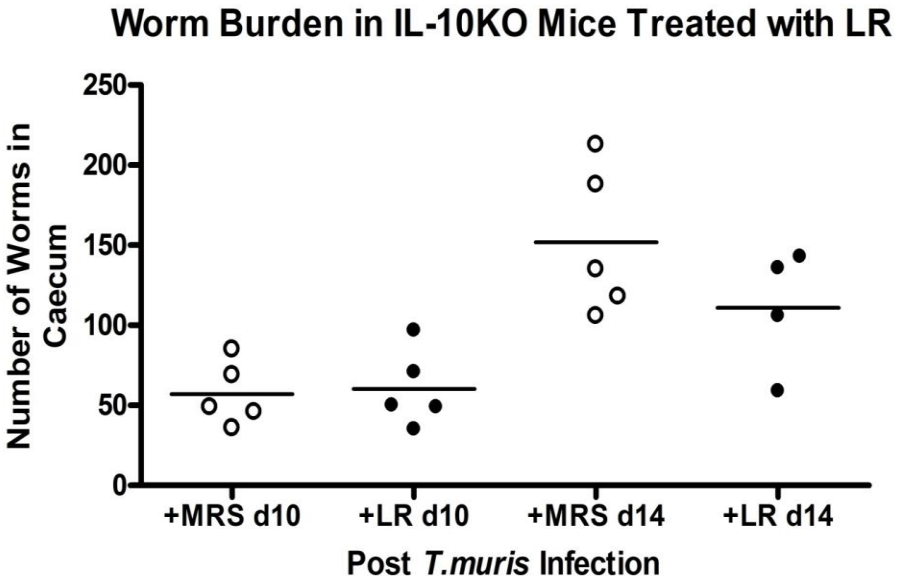


Figure 11: Worm burden in IL-10 KO mice treated with live *L. reuteri* or MRS. Mice received continuous treatment for 14 days post *T. muris* infection. There were no significant changes of the number of worms in the caecum when mice received *L. reuteri* or MRS treatment at either time point.

Cytokine data from the tissue of infected IL-10 KO mice treated with probiotics or medium were also measured to determine if probiotic treatment altered immune responses, or if these benefits seen in previous experiments were solely due to the presence of IL-10. Cytokines were checked in both the tissue and T lymphocyte supernatant from splenocytes. The amounts of IL-13, IL-4 and TGF-β in both of these areas were unchanged with the probiotic treatment compared to the medium treated infected mice (Figure 12). Interestingly enough, the amounts of TGF-β and IL-13 in the tissue of infected mice were not much different than the amounts of uninfected controls. IL-13 has previously been shown to be important in resistance in *T. muris* infections, and the lack of IL-13 may contribute to the susceptibility of IL-10 KO mice in response to the parasitic infection. Other cytokines in the colonic tissue that were checked that showed no difference when comparing the *L. reuteri* treated to the MRS treated mice are IL-17, IL-1β, and IFN-γ amounts. Interestingly enough, there was a significant increase in IL-12 amounts in the colon tissue of *L. reuteri* treated infected mice on day 10 of the experiment (Figure 12).

Changes of the epithelial cell layer were also investigated in this strain of mice in response to not only the *T. muris* infection, but also with the probiotic treatment. The goblet cells in the colon of wild type infected mice increase in numbers to possibly help with parasite expulsion, with an increased amount of goblet cells after *L. reuteri* treatment. In the IL-10 KO mice, there was no overall increase in goblet cell numbers in response to the parasitic infection when compared to uninfected controls. When infected mice were treated with the probiotic throughout

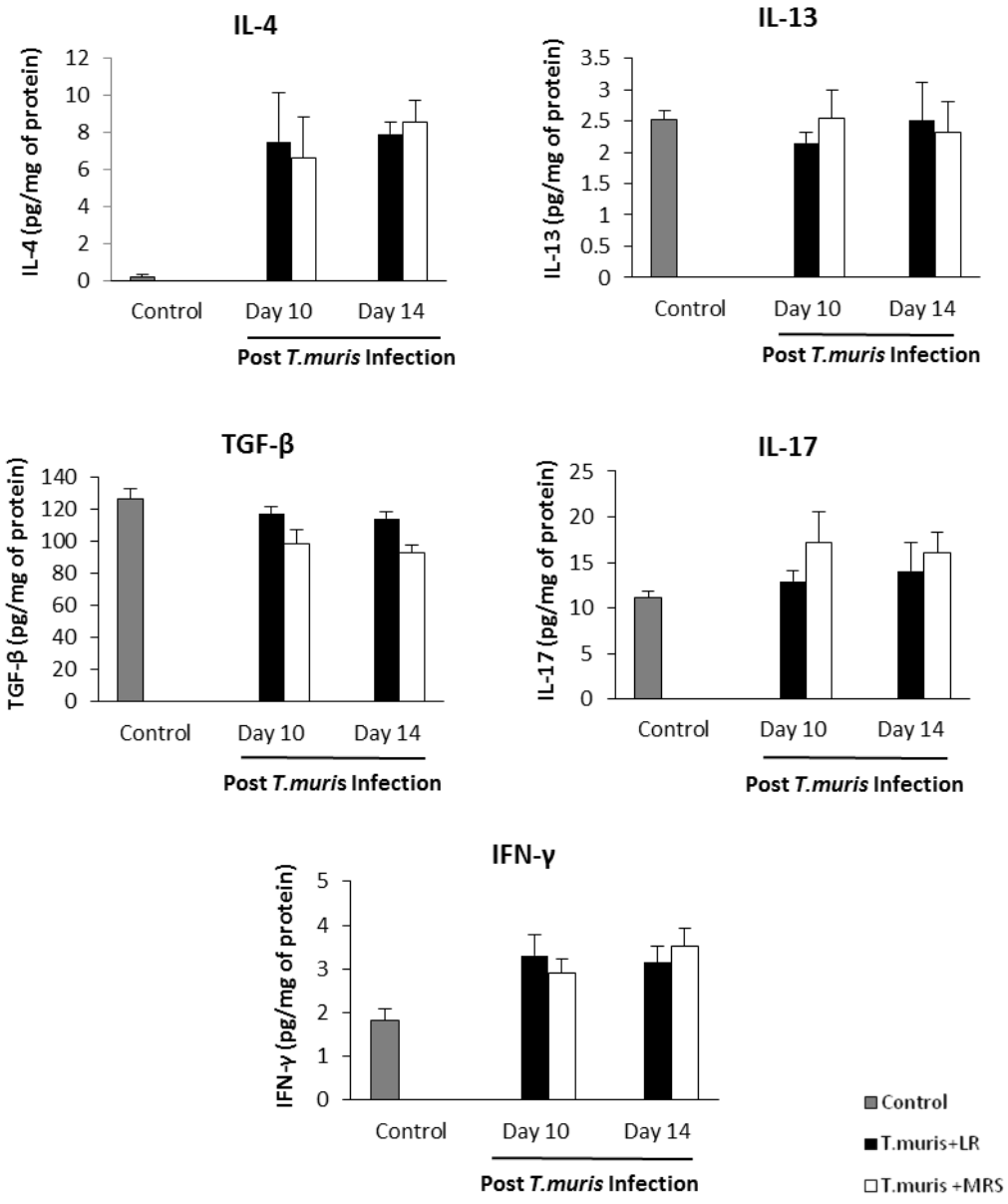


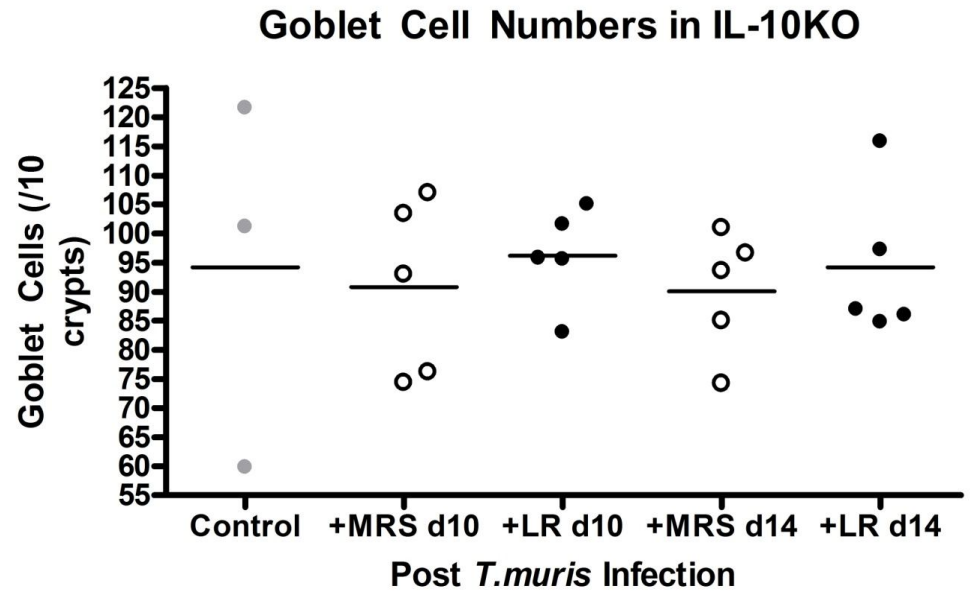
Figure 12: Colon tissue concentrations of various cytokines from IL-10 KO mice treated with either live *L. reuteri* or MRS. Treatment did not have a significant effect on cytokine amounts in the absence of IL-10. All values were normalized for protein content and expressed as pg/mg of protein. n=5 for all groups except controls, where n=2. *L. reuteri* values are compared to MRS treated groups, and expressed as mean +/- SEM.

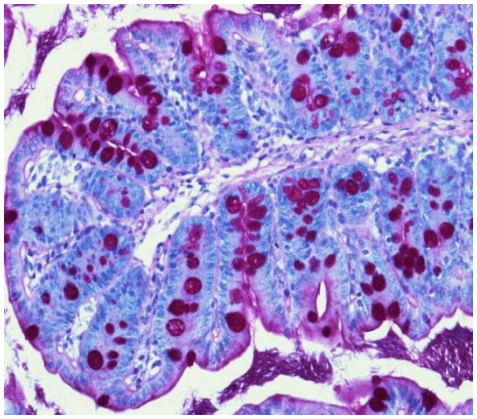
the infection, there was also no increase in goblet cell numbers in the colon compared to medium treated infected mice (Figure 13).

Other parameters were checked in these mice to check for severe inflammation and weight loss. The MPO values in the colonic tissue were checked, and there were no differences in the amount of MPO found in the tissue when comparing the infected mice treated with medium or the probiotic. There was a slight increase in MPO values on day 14 compared to day 10, although this difference was not significant. Weights were also checked to ensure these mice were not sick and beginning to succumb to the infection. The weights of the mice throughout the entire experiment were steady, and there was a minimal, very gradual increase over time. There were no visible signs of colitis throughout the experiment, or seen in histological sections. Although this experiment did not have the same time line as previous work, these findings point to a role of IL-10 in the beneficial effects of the *L. reuteri* treatment.

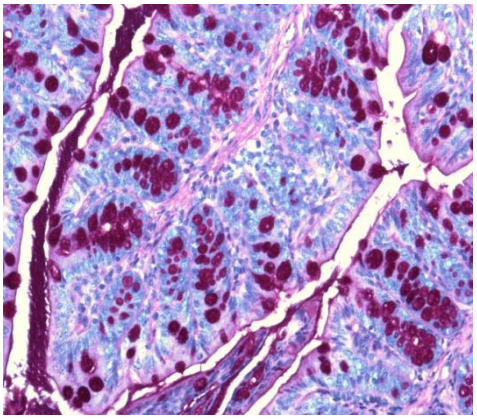
3.4 Role of Goblet Cells using Muc2 KO mice and live *L. reuteri*

Resistant mice infected with *T. muris* have been observed to have an increase in goblet cell hyperplasia, which may help in the expulsion of worms from the caecum and colon. In the previous experiment with C57BL/6 mice, it was noted that there was a significant increase in goblet cells on days 14 and 21 post *T. muris* infection treated with *L. reuteri*. To determine the role of mucins in the beneficial effects of *L. reuteri* during a *T. muris* infection, mice deficient in the main secretory mucin of the gut were used. These Muc2 KO mice are resistant to the *T. muris*





Day 14 *T. muris*+LR



Day 14 *T. muris*+MRS

Figure 13: a) Goblet cell counts from the colon IL-10 KO mice infected with *T. muris* and treated with either *L. reuteri* or MRS. There was no difference on any day, in the total goblet cell numbers in the infected mice treated with *L. reuteri* compared to the mice treated with MRS. A minimum of 50 crypts were counted, expressed as goblet cells/10 crypts. b) Corresponding histological sections of the colon stained with PAS show similar numbers of PAS+ cells, representing goblet cells. Magnification of 200x.

infection due to an increase in Muc5ac, but have an initial delayed worm expulsion (Hasnain *et al.*2010). The possibility of the probiotic treatment increasing the Muc5ac expression earlier on in the infection and promoting expulsion at an earlier time point still needs to be addressed.

When the caecums were removed from these mice for the worm counts to determine if the treatment had a difference in worm expulsion, it was noted that there was a difference in worm burden on day 14 and 21. The mice treated with the probiotic had significantly lower numbers of compared to the mice in the medium treated group (Figure 14).

Cytokines in the infected Muc2 KO mice were investigated to determine if the absence of Muc2 affected the probiotic's effect on the cytokine milieu during infection. It was found that there was an increase in the T_H2 cytokine, IL-4, on day 21 of infection in the tissue of mice that were treated with the probiotic. There was also a decrease in the T_H1 promoting cytokine IL-12 on day 21 of the infection in mice treated with the probiotic. There were no differences in IL-13, IL-10, IL-17, IL-1β or IFN-γ amounts on any of the days, as all amounts were similar in both treatment groups (Figure 15).

These mice are deficient in one of the main mucins found in the colon, and have minimal PAS+ stained goblet cells compared to their wild type counterparts. When these mice are infected with *T. muris*, at the later time points when worm expulsion has become closer to the wild type's expulsion rate, there is an increase of PAS+ stained goblet cells seen in the caecum and colon. When these Muc2 KO mice are treated with the probiotic throughout the experiment, there was a significant

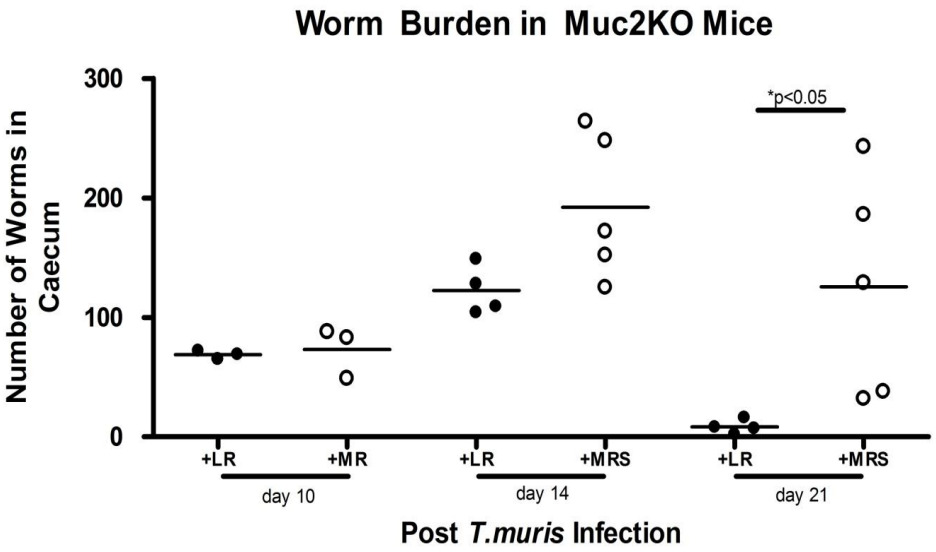


Figure 14: Worm burden in Muc2 KO mice treated with live *L. reuteri* or MRS. Mice received treatment for 14 days post *T. muris* infection. Worm burden was determined by the number of worms in the caecum of each mouse. There was a significant decrease of worm burden in the caecum of mice treated with *L. reuteri* on day 21 post infection compared with medium treated mice. *$p<0.05$, n= 3-5.

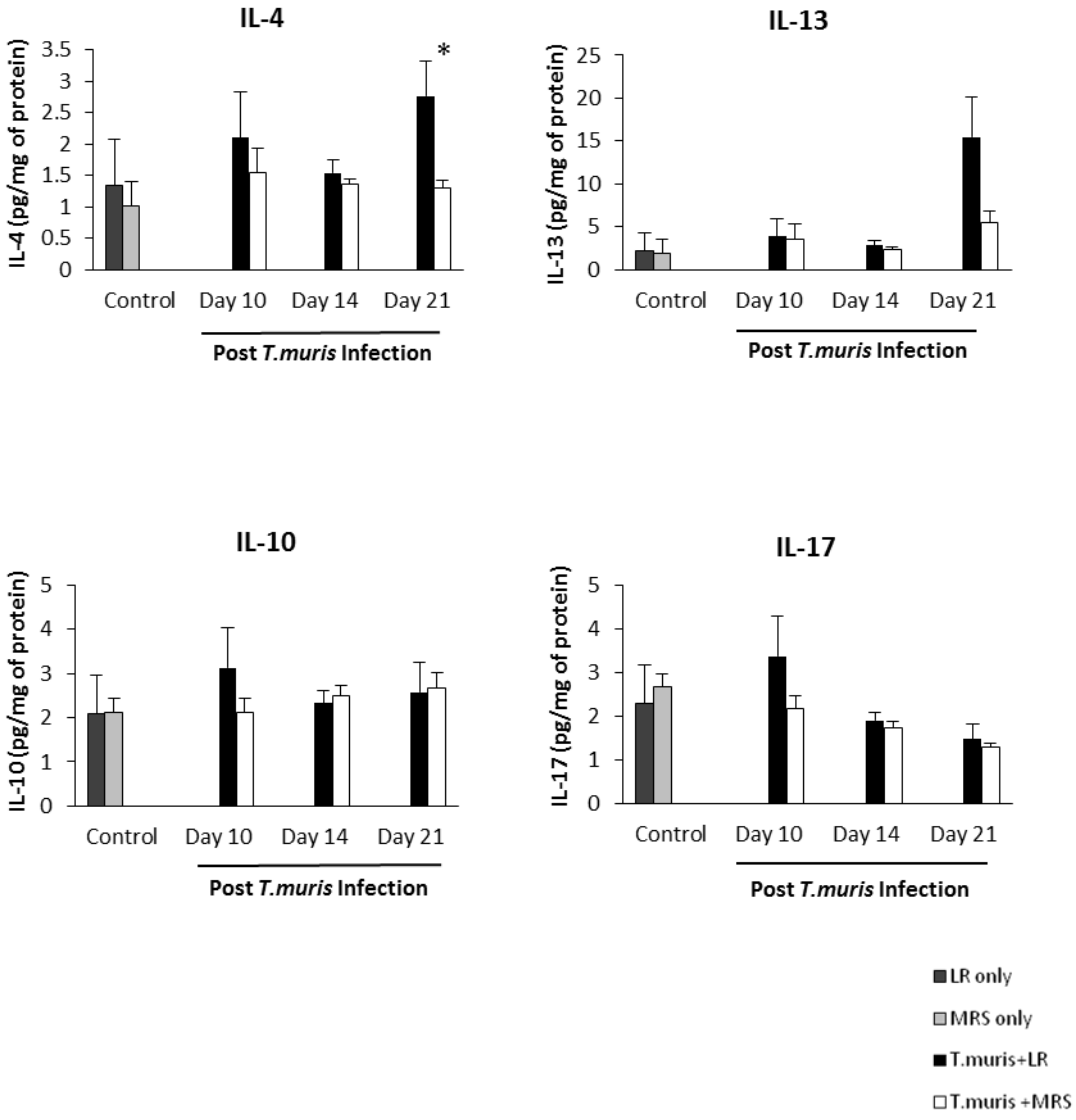


Figure 15: Colon tissue concentrations of various cytokines from Muc2 KO mice. *L. reuteri* treatment increased IL-4 amounts on day 21. All values were normalized for protein content and expressed as pg/mg of protein. *L. reuteri* values are compared to MRS treated groups, and expressed as mean +/- SEM. *$p<0.05$, n=3-5.

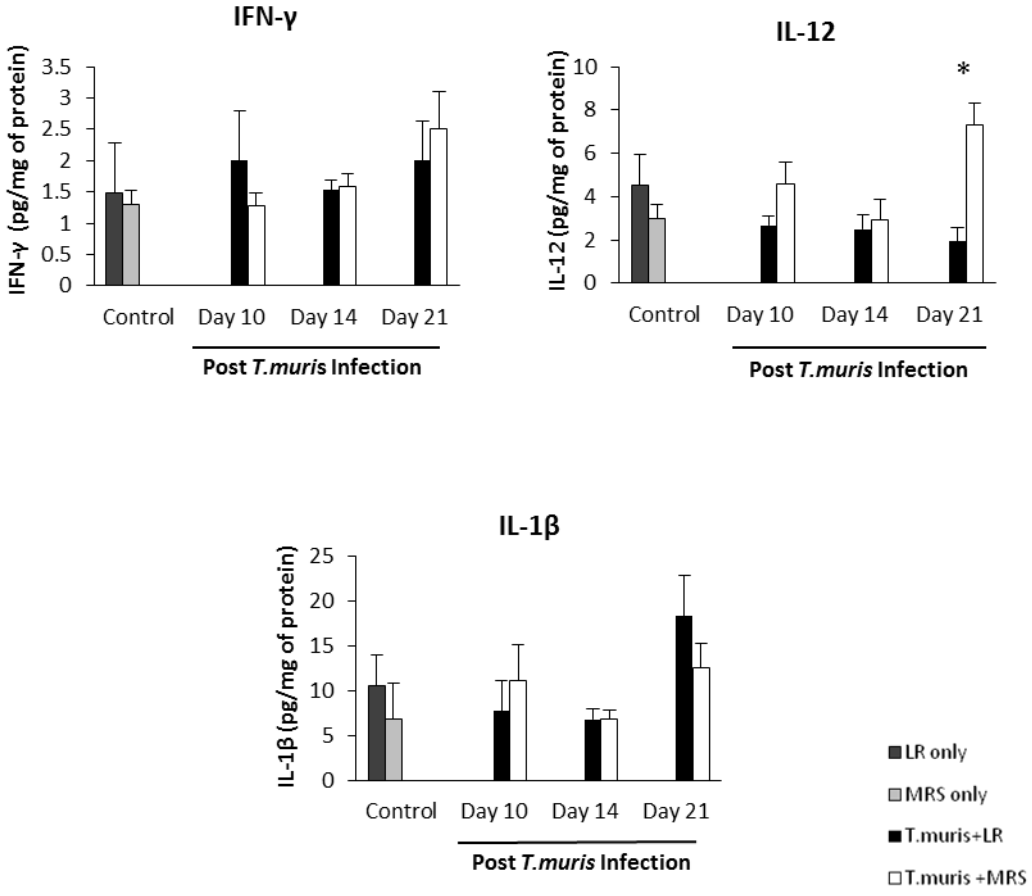


Figure 15 continued: Colon tissue concentrations of various cytokines from Muc2 KO mice. *T. muris* infected Muc2 KO mice treated with *L. reuteri* treatment decreased the amount of IL-12 on day 21 compared with medium treated infected mice. All values were normalized for protein content and expressed as pg/mg of protein. *L. reuteri* values are compared to MRS treated groups, and expressed as mean +/- SEM. *$p<0.05$, n=3-5.

increase of PAS+ goblet cell numbers earlier on in the infection. This increase of PAS+ goblet cells occured on day 21 post *T. muris* infection (Figure 16).

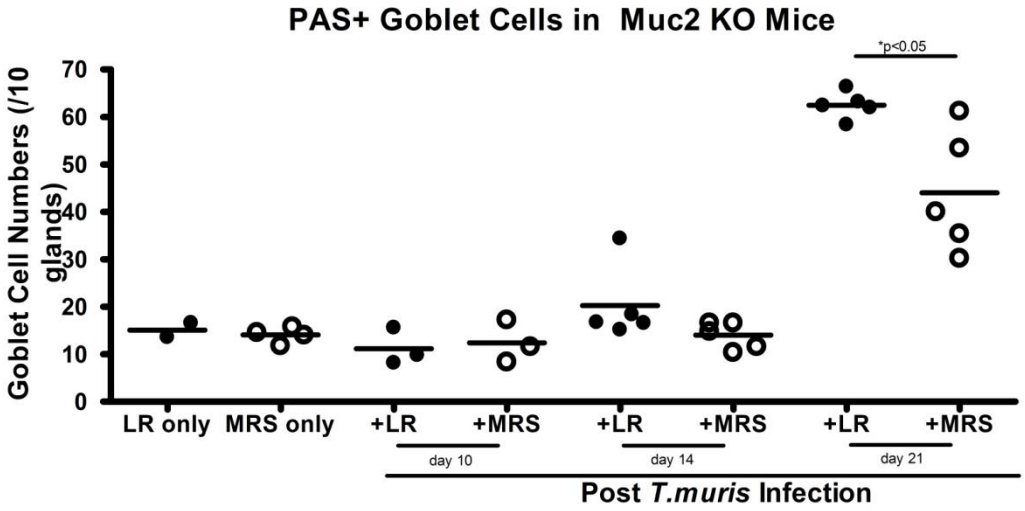
The inflammatory marker MPO was also determined in these mice after they were infected and treated with either probiotics or medium. There were no differences in the amount of MPO in the colonic tissues of infected mice treated with either the medium or the probiotic. The weights of these mice were also monitored, all of the mice, indifferent of the treatment gained weight steadily throughout the experiment.

3.5 Susceptible AKR mice and live *L. reuteri*

The last strain of mice that were studied was the AKR strain of mice, which are susceptible to the *T. muris* parasitic infection, and develop a chronic infection over time which eventually leads to a mild colitis (Else and Grencis, 1991). These mice are known to have a T_H1 immune response when infected with this parasite, which also correlates with non-existent worm expulsion, no goblet cell hyperplasia, as well as a delayed epithelial turnover rate (Cliffe *et al.* 2005; Hasnain *et al.* 2010). The aim of this section was to determine if the probiotic treatment altered the cytokine environment, goblet cell responses, or overall worm burden or expulsion rates, and perhaps helped the outcome of these infected mice.

After mice were sacrificed, the caecums, and the colon were removed for worm burden analysis. Since the worms were larger at day 35, and were present in both the caecum, and colon, worm numbers were quantified using the entire length of colon. There was not a significant difference in the number of worms in the caecum

a)



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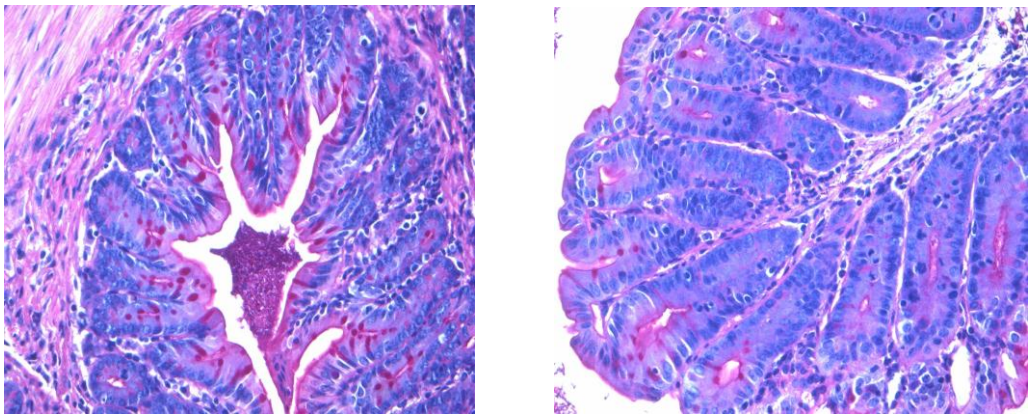


Day 21 *T. muris*+LR

Day 21 *T. muris*+MRS

Figure 16: a) PAS+ stained goblet cell counts from the colon of Muc2 KO mice infected with *T. muris* and treated with either *L. reuteri* or MRS. A significant increase in the total goblet cell numbers in the infected mice treated with *L. reuteri* was seen at day 21 post infection. A minimum of 50 crypts were counted, expressed as goblet cells/10 crypts. *$p<0.05$. b) Corresponding histological sections of the colon stained with PAS show the increase of PAS+ cells, representing goblet cells. Magnification of 200x.

and colon of the mice treated with probiotic compared to medium treated mice (Figure 17). There was however a slight decrease in numbers with the parasitic treatment, which may be significant if the experiment is redone to increase the numbers in each group. There were many difficulties encountered with this experiment, which we have concluded to be due to the strain of mice, as the probiotic and parasite batches were checked, as well as infection administration. It is important that this section be looked at more closely in the laboratory to determine conclusive results.

The cytokine environment of these mice in response to a *T. muris* infection is naturally a T_H1 dominated environment, with an increase of IFN-γ, IL-12 and IL-1β in the colon tissue. Probiotic treatment throughout the entire 35 day experiment had no affect on any of the T_H2 cytokines checked; IL-4 and IL-13, as we all the regulatory cytokine IL-10 (Figure 18). IL-4 and IL-13 were interestingly enough, significantly increased with a *T. muris* infection regardless of treatment, compared to uninfected controls. There was also no difference in the pro-inflammatory cytokine IL-12 in the colon of these infected mice. Interestingly, there was a significant decrease in some of the T_H1 cytokines that were checked. There was an overall decrease in amounts of IFN-γ and IL-1β in the colon of infected AKR mice treated with the probiotic (Figure 18).

Goblet cells were also checked in mice infected and treated with probiotic or medium. Similar to the C57BL/6 strain of mice, goblet cells were altered with *L. reuteri* treatment. When the AKR mice were infected with *T. muris* and treated with probiotics, there was an increase in overall goblet cell amounts in the colon epithelial

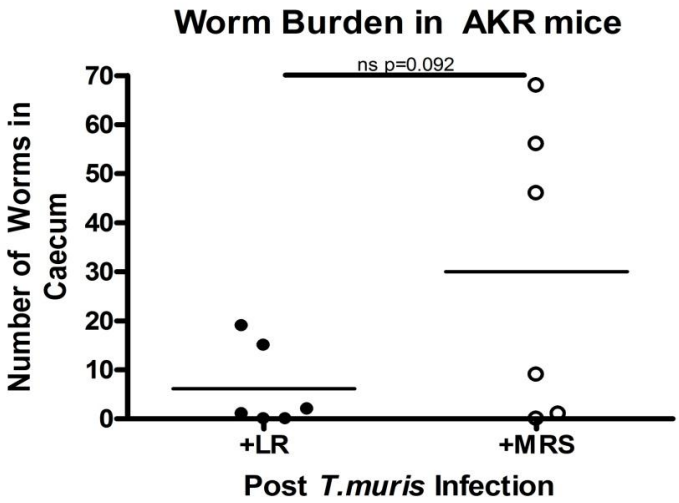


Figure 17: Worm burden in susceptible AKR mice treated with live *L. reuteri* or MRS. Mice received treatment for 35 days post *T. muris* infection. Worm burden was determined by the number of worms in the caecum and colon of each mouse. There was not a significant difference in worm numbers at the end of the treatment when comparing *L. reuteri* and MRS treatment.

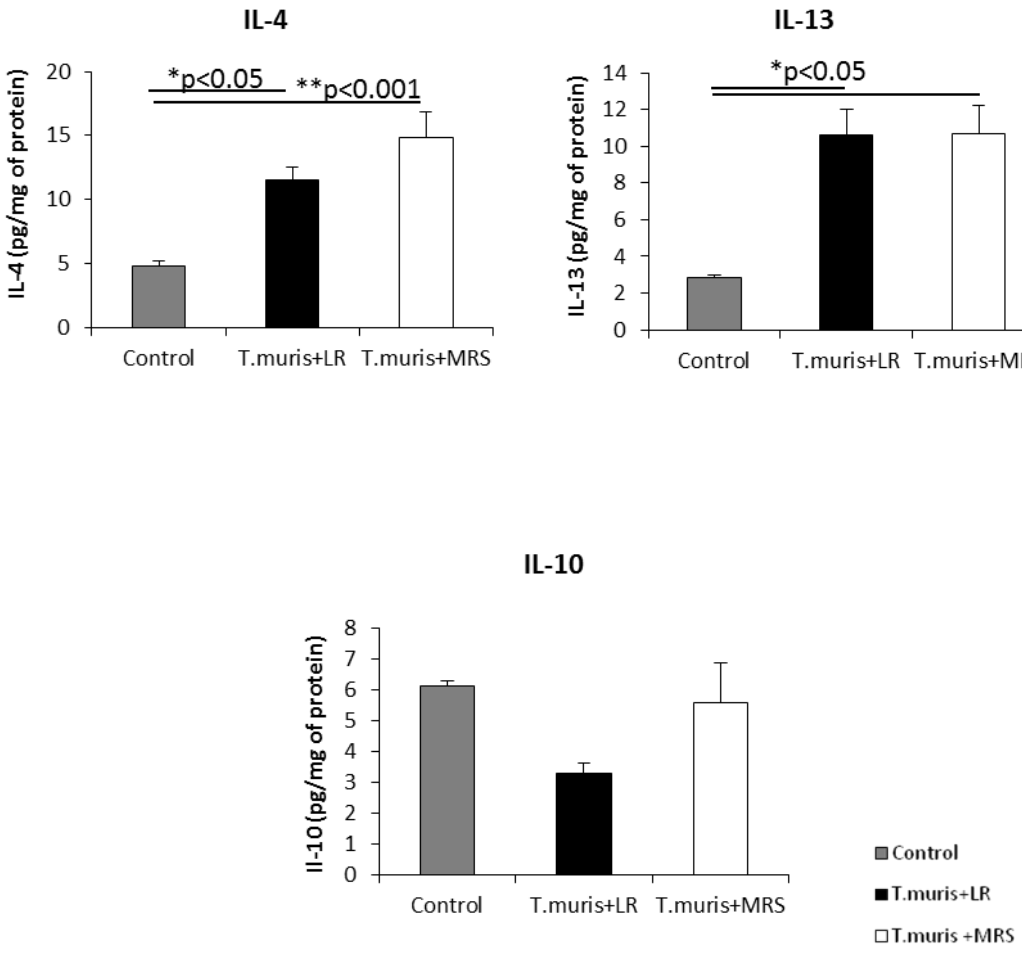


Figure 18: Colon tissue concentrations of various cytokines from AKR mice on day 35. All values were normalized for protein content and expressed as pg/mg of protein. n=6 infected groups, and n=3 for control group. All values are expressed as mean +/- SEM, *p<0.05 and **p<0.001.

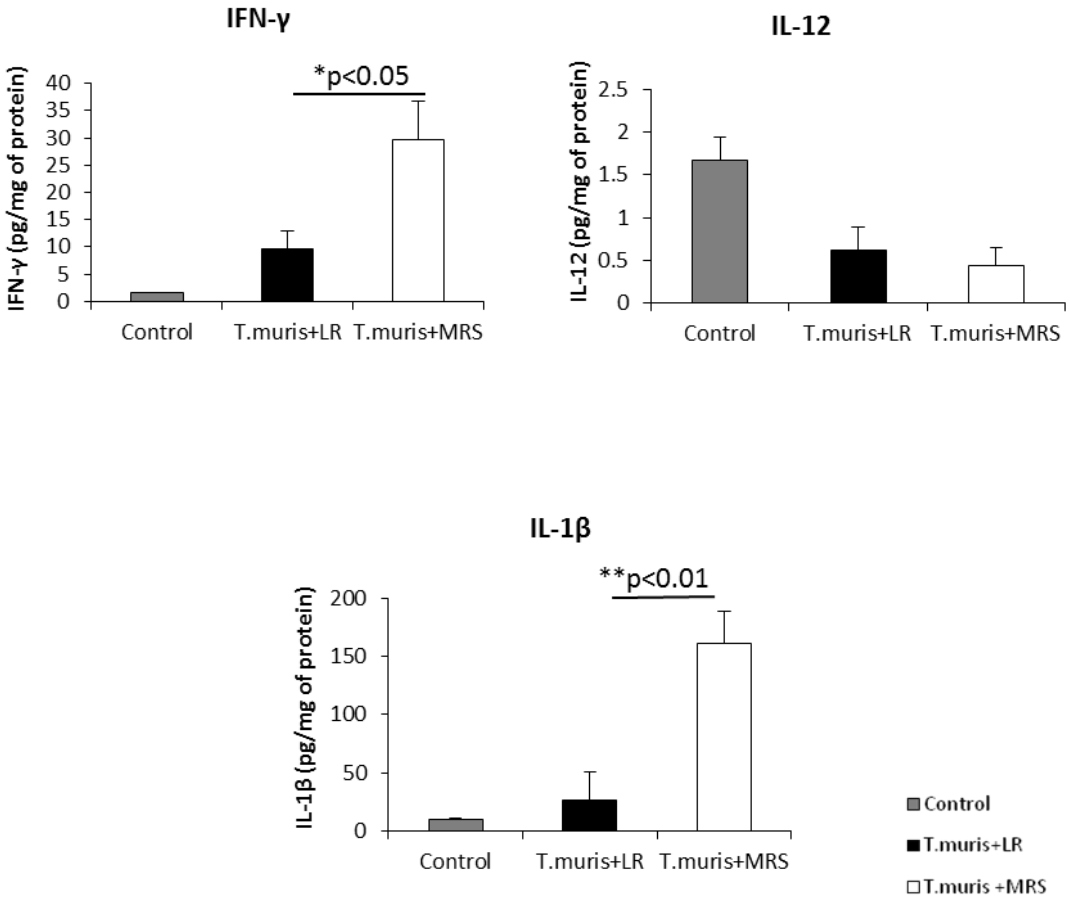
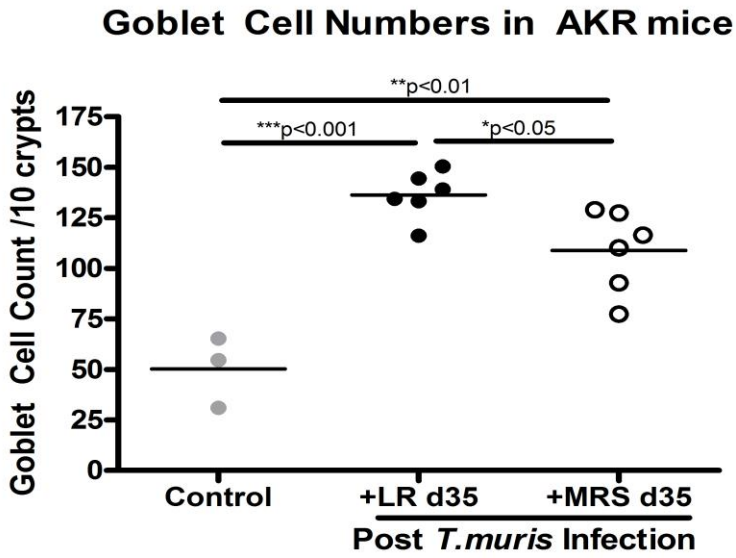
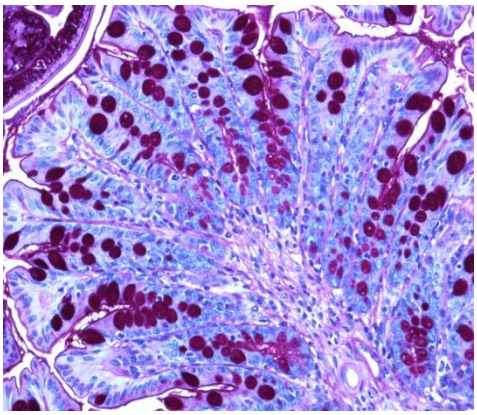


Figure 18 continued: Colon tissue concentrations of various cytokines from AKR mice on day 35. Infected mice treated with live *L. reuteri* had a significant suppression of IFN-γ and IL-1β compared to mice treated with MRS. All values were normalized for protein content and expressed as pg/mg of protein. n=6 infected groups, and n=3 for control group. All values are expressed as mean +/- SEM. *p<0.05 and **p<0.001.

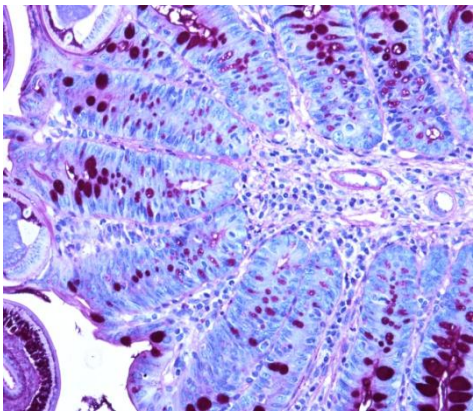
layer (Figure 19). There was also a significant increase in the number of goblet cells in the infected mice compared to the uninfected controls. This is unusual in the AKR strain of mice infected with *T. muris*, and further investigation is needed to rule out the reason for this increase.

Throughout the entire experiment, the mice were gaining weight as they would normally if they were uninfected, and there was no significant weight loss. Inflammation was also checked using MPO amounts in this strain of infected mice. The MPO levels in the colon of the infected mice treated with the probiotics was similar to the level of MPO detected in infected mice treated with medium only. The concentration of 5-HT in the colonic tissue of these mice were at similar levels at day 35 post infection, independent on the treatment the mice received, corresponding to unaltered EC cell numbers. MLNs from these mice were collected, and flow cytometry was performed to quantify the number of T regs. Non infected AKR control mice, and infected mice treated with *L. reuteri* or medium were compared. There were no differences in the amounts of T regs cells in the MLNs of all groups of mice. Of the T cells analyzed AKR controls had about 20% that were CD4+ CD25+ Foxp3+, where infected mice treated with *L. reuteri* had 16.7% and MRS treated mice had 15%. Both the percentage and cell numbers were not significant (Figure 20).





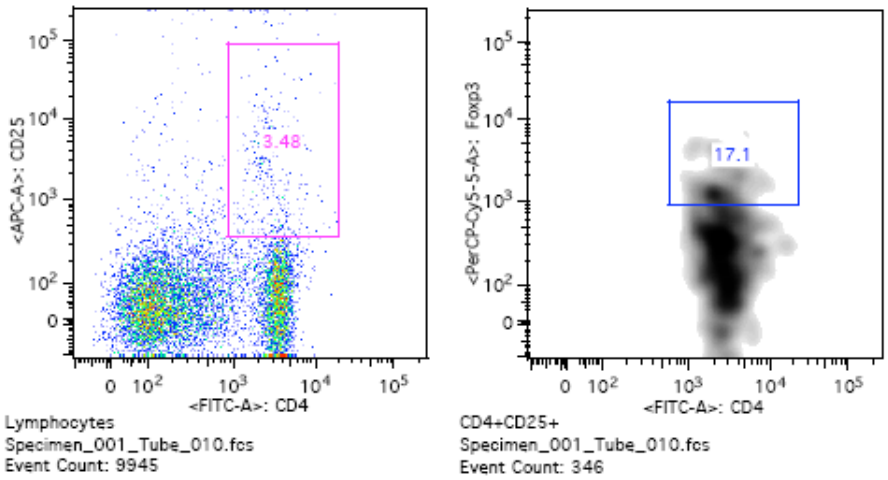
Day 35 *T. muris*+LR



Day 35 *T. muris*+MRS

Figure 19: a) Goblet cell counts from the colon of susceptible ARK mice infected with *T. muris* and treated with either *L. reuteri* or MRS. A significant increase in the total goblet cell numbers in the infected mice treated with *L. reuteri* was seen at day 35 post infection. A minimum of 50 crypts were counted, expressed as goblet cells/10 crypts. *p<0.05, **p<0.01, ***p<0.001, where n=6 in each group, and n=3 for controls. b) Corresponding histological sections of the colon stained with PAS show the increase of PAS+ cells, representing goblet cells. Magnification of 200x.

a)



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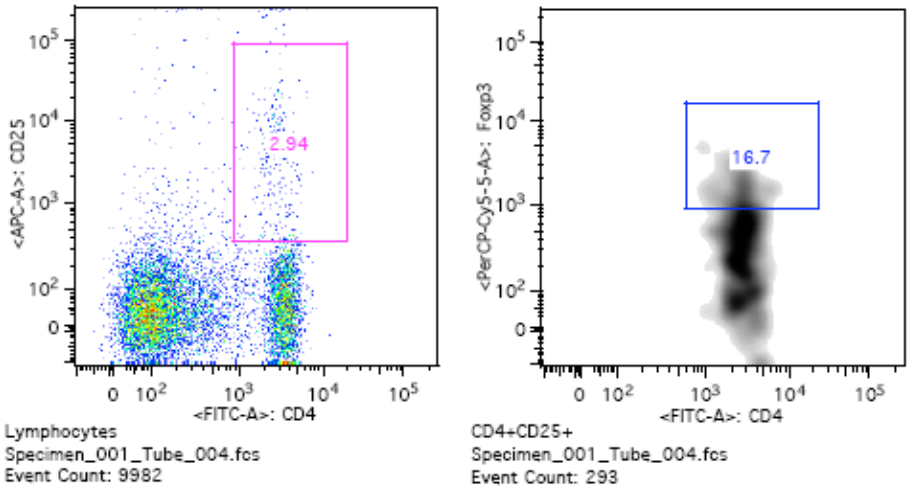

Figure 20: Scatter and density plots representing regulatory T cells found in the MLNs of AKR mice on day 35 infected with *T. muris* and treated with either a) *L. reuteri* or b) MRS. Cells were stained with mAbs for CD4, CD25 and Foxp3. Lymphocytes are gated on CD4+ and CD25+ cells (left panels). Numbers in the gate represent the number of CD4+ and CD25+ cell. These cells are then gated on CD4 and Foxp3 (right panels). The number in the gate represents CD4+ CD25+ lymphocytes which are Foxp3+, representing regulatory T cells.

4.0 DISCUSSION

Probiotic therapy as a treatment or preventative measure has been studied in many different settings. Mechanisms which probiotic bacteria act on to provide benefits are numerous, but also not well understood. Probiotic bacteria have been shown to act on physiology, secretions, immune responses and cell responses to promote homeostatic conditions in the GI tract (Galdeano and Perdigon, 2004). Despite a significant increase on probiotic research in intestinal disorders in recent years, the effects of treatment with probiotic bacteria on host defense in parasitic infections remain unclear. In this comprehensive study, a beneficial role of probiotic treatment has been observed in modulation of immune response and goblet cell response in the context of host defense during a *T. muris* infection.

Immune responses have been shown to be an important factor in determining the resistance or susceptibility of mice strains when challenged with a *T. muris* infection (Grencis, 1993). To investigate the effect of probiotic treatment during a *T. muris* infection, we used different strains of mice to investigate the changes in goblet cell hyperplasia, cytokine responses and worm expulsion. In the resistant strain of mice, C57BL/6, the live probiotic treatment enhanced the host's natural immune response to the parasite which resulted in an increased rate of expulsion. The increase in the regulatory cytokine, IL-10, and an increase in goblet cell numbers in the colon in the probiotic treated mice were key alterations that could help in host resistance. The benefits seen with the live probiotic treatment were not seen in our

experiments with γ-irradiated *L. reuteri.* These data suggest that for *L. reuteri* to have a beneficial effect on the host's defense in worm expulsion and other immunological and cellular changes the bacteria must be alive and viable.

During intestinal parasite infections, IL-10 is important for the regulation of other cytokines such as IFN-γ and IL-12 (Hoffmann *et al.* 2000). IFN-γ and IL-12 are known cytokines upregulated during a *T. muris* infection in susceptible mice, and promote inflammation (Else and Grencis, 1991). It is evident that IL-10 plays a role in host resistance to GI infections, by preventing the over expression of certain cytokines such as IFN-γ and IL-12. The importance of IL-10 during a *T. muris* infection has been previously described by Schopf and colleagues, and the increase of IL-10 with *L. reuteri* treatment may lead to other changes to help expel the parasite and achieve intestinal homeostasis earlier (Sanchez-Munoz *et al.* 2008; Schopf *et al.* 2002). The increases of IL-10 early on in the course of infection with probiotic treatment led to the use of genetically altered mice to investigate the role of IL-10. Using IL-10 KO mice allowed us to determine if IL-10 was a key player in the probiotic treatment benefits in the resistant strain of mice. IL-10 KO mice are susceptible to a *T. muris* infection (Schopf *et al.* 2002) and when these infected mice were treated with *L. reuteri*, the probiotic had no overall beneficial effects on the mice. These data suggest that the increase in host defense with regard to the worm burden reduction with live probiotic treatment is dependent in some way on IL-10 production, secretion, or downstream pathway effects.

Interesting cytokine results were noted in the IL-10 KO strain in both the T_H1 and T_H2 profiles. With regards to T_H2 cytokines, the amounts of IL-13 in the tissue of infected mice with either *L. reuteri* or MRS treatment were not much different than the amounts in uninfected controls. IL-13 has previously been shown to be important in resistance in *T. muris* infections, and the lack of IL-13 increase may contribute not only to the susceptibility of IL-10 KO mice but, but also to the morbidity and mortality of these mice in response to the parasitic infection (deSchoolmeester and Else, 2002). There was a significant increase of IL-12 in the infected mice which were treated with the probiotic. The amounts of IL-1β were slightly higher in the *L. reuteri* treated mice on day 10 post infection (pi), although these differences were not significant with the number of animals in our groups. The increase in IL-12, a T_H1 promoting cytokine, also correlated with a slightly more robust MPO response. This increase in MPO amounts in the colonic tissue of *T. muris* infected mice was not significant, although if taken to a later time point, the amounts may continue to rise. From the results collected, IL-10 seems to have a role in the cytokine balance during a *T. muris* infection, and contributes to the susceptibility or resistance of mice.

Overall, probiotics may promote intestinal expulsion of the parasite by acting on both T_H2 and regulatory cytokine responses in resistant strains of mice. The administration of the probiotic bacteria may not affect the T_H2 environment directly, but the increase in IL-10 may have beneficial effects on the host overall. Without IL-10 the T_H2 response may not be fully developed in response to the *T. muris* infection and allow for a greater T_H1 response and inhibit expulsion. In accordance with the Schopf experiments, IL-10 is an important factor in the polarization of either T_H2 or

T_H1 immune responses in response to a *T. muris* infection (Schopf *et al.* 2002). Considering we saw no beneficial effects of probiotic bacteria, but an increase in IL-12, administration of probiotic bacteria when the host's immune response is not fully functional or skewed, may not be beneficial, but could be slightly harmful (Land *et al.* 2005). The new bacteria being introduced may induce more inflammation in the GI tract and contribute to the susceptibility of the IL-10 KO mice to a *T. muris* infection. Altering the gut flora may have more of a negative effect in these infected mice as they do not have IL-10 to help regulate immune responses. From the data shown and in accordance with other studies, IL-10 is most likely preventing the over expression of pro-inflammatory cytokines and suppressing the overall T_H1 and T_H1 promoting cytokine response during infections of otherwise resistant mice (Hoffmann *et al.* 2000; Schopf *et al.* 2002).

On the other hand, susceptible strains of mice have a completely different cytokine profile in response to a *T. muris* infection. Unsure if the probiotic would have the same effect in a T_H1 dominant environment; AKR mice were infected and treated with the same probiotic. If the probiotic had similar effects in this strain of mice, it would be an interesting potential therapy to chronic intestinal parasitic infections around the world, as well as GI diseases with T_H1 dominant immune responses. The results showed a notable decrease in the number of worms in the caecum and colon of probiotic treated mice compared to medium treated mice, although this was not statistically significant. It would be important to further the study by increasing the numbers of animals per group.

There were also cytokine differences seen in infected susceptible mice treated with *L. reuteri*. The probiotic treatment had an effect only on T_H1 cytokines, which are the dominant cytokines produced in susceptible mice strains during a *T. muris* infection. The *L. reuteri* treatment prevented the significant increase of both IFN-γ and IL-1β during the infection. This data is interesting and may suggest a beneficial effect of the probiotic in this strain of mice by reducing the T_H1 cytokine environment during a *T. muris* infection. There were also noted changes in some T_H2 cytokines in the colonic tissue of susceptible AKR mice. There was an increase of both IL-4 and IL-13 in infected mice treated with either MRS or LR compared to non infected controls. This increase was unexpected, as T_H2 cytokines are not typically increased in susceptible strains of mice infected with *T. muris* (Else and Grencis, 1991). These results suggest and support the theory that the same species as well as strain of probiotics act differently in different systems, as well as in the same system with different variables. The strain of mice, cytokine environment, infection and many more aspects have an influential role in the effects of the probiotic.

The importance of IL-10 in the parasitic infection has been previously described, although the relationship with IL-10 and regulatory T cells (T reg) during a *T. muris* infection is less understood. There are also previous studies looking at *L. reuteri* and the T reg population alterations in other systems. It has been previously been reported that *L. reuteri* is able to induce FoxP3 CD4+ T cells in wild type mice, 9 days after *L. reuteri* treatment (Karimi *et al.* 2008). This increase also correlated with an increase in the regulatory cytokine IL-10. When these T regs were isolated then transferred into a mouse sensitized with OVA to develop allergic asthma like

symptoms, these mice had a decrease in inflammatory cell influx to the lungs compared with mice given T regs from a mouse not treated with the probiotic (Karimi *et al.* 2008).

Although we did not investigate the effects of the probiotic treatment on the T reg population in *T. muris* infected resistant C57BL/6 mice, the increase of IL-10 early on in the infection and treatment may suggest that there is an alteration in the T reg cell quantities. Looking at this cell population will also help determine what changes the probiotic is having on these cells in the host during a *T. muris* infection. As mentioned previously, we were able to check the regulatory T cell population in the MLNs of control mice and susceptible AKR mice treated with either *L. reuteri* or medium. There were no alterations in the number of T reg cells in the infected mice compared to the controls, and no difference between the numbers of T reg cells in the infected mice treated with the probiotic compared to the mice treated with the medium.

The probiotic treatment in the *T. muris* infected AKR mice had no effect on the T reg population in the infected mice compared to control mice. This does not rule out that the probiotic treatment had no effect on the production of cytokines from T reg cells. The *L. reuteri* treated C5BL/6 mice had an increase of IL-10 which could possibly correlate with a modulation of T reg cell populations by probiotic treatment. Further studies on T reg cells are needed to determine if the probiotic bacteria has an effect on this cell population in the MLNs of *T. muris* infected resistant strains of mice.

Another cytokine that would be interesting to investigate is thymic stromal lymphopoietin (TSLP), which is produced by epithelial cells. This cytokine promotes T_H2 T cell responses during inflammation by activating DCs to increase co-stimulatory molecules on their surface to increase activation of T cells (Artis and Grencis, 2008; Liu *et al.* 2007). TSLP expression in intestinal epithelial cells is upregulated in resistant strains of mice infected with *T. muris* and plays a role by not only promoting T_H2 cell activation, but also suppressing IL-12 secretion from DCs (Artis and Grencis, 2008; Massacand *et al.* 2009). It was shown in the same laboratory that TSLP KO mice on a resistant mouse background have impaired T_H2 responses to a *T. muris* infection and are susceptible to the infection due to an increase T_H1 response (Massacand *et al.* 2008). Blocking IL-12 in these KO mice decreases the susceptibility by decreasing the amount of IFN-γ. Concluding that during a *T. muris* infection, TSLP does not directly act on T_H2 responses, but its benefits come from the limitation of the T_H1 response and inhibition of T_H1 cytokines (Massacand *et al.* 2009).

This role of TSLP during a *T. muris* infection may be an interesting path to look into with the use of probiotics. Perhaps with the probiotics introduced into the gut during infection there may be altered amounts of TSLP which then leads to increased worm expulsion. From the cytokine data that we obtained with the infection and probiotic treatment, we did not see any increase in T_H2 specific cytokines or decrease in T_H1 cytokines in the resistant strain of mice. What we did see however is in the AKR susceptible mice, there was a decrease in certain T_H1 cytokines, IFN-γ and IL-1β. Therefore, the likelihood of an increase of TSLP acting on either the

susceptible or resistant strain of mice with probiotic treatment is low, due to unchanged IL-12 and other cytokines in both models.

A major cell population in the GI tract that plays an important role in homeostasis, normal morphology and overall health and are the goblet cells. These cells are altered by immune and cytokine changes, and are under the control of T_H2 cytokines during enteric parasitic infections (Ishikawa *et al.* 1997; Khan *et al.* 2001). Goblet cells and specifically the mucin secreted have been shown in many papers to be beneficial and important in many types of parasitic infections, including *T. muris* to potentially help to trap and remove parasites from the colon (Miller, 1987).

Interestingly, our data showed an increase of goblet cell numbers after day 14 and 21 pi with probiotic treatment in the infected resistant mice, and on day 35 in susceptible mice. The alteration in goblet cells after probiotic treatment may have benefits to the host's response, as mucins have been shown to help in worm expulsion by enhancing the mucus barrier and trapping worms and preventing further attachment and establishment (Artis *et al.* 2004; Miller, 1987). The increase goblet cell numbers in response to the parasite infection in IL-10 KO mice was not seen, independent of the treatment received. The goblet cell numbers did not alter from control levels, which supports the previous study which noted that *T. muris* infected IL-10 KO mice had unaltered goblet cells and mucus amounts (Schopf *et al.* 2002). The static cell number and mucus amount seen in these KO mice may allow the newly hatched larvae to easily burrow into the caecum mucosa. The growing worms could easily evade peristalsis because they are not coated in as much mucus as they

would be in a wild type resistant mouse. It would be interesting to look at IL-10 and its direct role on goblet cell hyperplasia. The administration recombinant IL-10 (rIL-10) into the lumen or mucosa of the GI tract to naïve wild type mice to investigate whether IL-10 can act directly on stem cells or other proliferative markers and genes. Also, administering rIL-10 to *T. muris* infected C57BL/6 and AKR mice to see if an increase of IL-10 alone without probiotic treatment can influence host responses, and possibly increase the expulsion of the worm. This will give an insight of the role of IL-10 directly on the goblet cell population and host responses of the GI tract of mice in both infected and non-infected controls.

Interestingly, the cell alterations that were seen in the susceptible strain of mice, AKR, were similar to that of resistant strains of mice. There was a significant increase in goblet cell numbers in *T. muris* infected AKR mice treated with the probiotic. The inhibition in the increase of certain pro inflammatory cytokines in the probiotic treated mice may have created an environment which allowed for the increase of proliferation of goblet cells in response to the probiotic treatment and parasite infection. There was also an unexpected increase in goblet cells numbers in infected mice treated with MRS compared to non infected controls. This increase is not normally seen in susceptible AKR mice, and this needs to be further investigated.

The notable increases in goblet cell numbers in response to not only a parasite infection in certain strains of mice, but also the probiotic treatment, led to the investigation of goblet cells as a key player in the benefits of the bacteria. It has been previously shown in our lab in collaboration with Dr. Thornton that Muc2 KO mice expel *T. muris* worms in the same amount of time as the C57BL/6 wild type mice, but

the initial expulsion is delayed. It takes both types of mice 25-30 days to expel the parasite, the wild type mice starting between 13-15 pi and the Muc2 KO mice starting around day 21-24 pi (Hasnain *et al.* 2010). Around the time of expulsion there was a notable increase in Muc5ac expression in the Muc2 KO mice, triggered by the *T. muris* infection (Hasnain *et al.* 2010). Muc5ac was recently shown to be critical to the expulsion of *T. muris* from the GI tract of resistant mice (Hasnain *et al.* 2011). Muc5ac affects the overall energy and ATP level of *T. muris* worms directly, as well as affecting the overall porosity of the mucus layer in the GI tract (Hasnain *et al.* 2011). The next step in the *L. reuteri* experiments was to explore if the benefits of the probiotic were dependent on Muc2 expressed in the gut during a *T. muris* infection. Using Muc2 KO mice, our data show that there was a significant decrease in worm burden on day 21 pi in the Muc2 KO mice treated with *L. reuteri*. This was correlated with a surprising increase in the T_H2 cytokine IL-4, and a decrease in IL-12, a T_H1 promoting cytokine. There was an increase in PAS+ stained goblet cells, on day 21 pi, when treated with the probiotic, correlating with worm expulsion. The alteration in cytokines may have benefited the host by increasing mucins and creating a more resistant environment. The earlier expulsion of the worms from the caecum of Muc2 KO mice with the probiotic treatment suggests that the benefits seen in the wild type mice are independent of Muc2 in the GI tract. Although was cannot rule out all mucins completely as there may have been an earlier increase of Muc5ac in the GI tract with the probiotic treatment (Hasnain *et al.* 2010). Looking more in depth into specific mucins and changes associated with infection and treatment would be beneficial. Checking the gene expression of goblet cell genes such as Muc5ac would

be helpful to get definitive quantitative data to support the immunohistochemistry data. Also, new markers are being looked at within our lab at specific stem cell markers, which could potentially lead to addressing the proliferation question, and if the probiotic treatment is acting on the stem cells of the intestinal epithelium to become part of the goblet cell lineage. If there are specific changes associated with the probiotic treatment, more doors are opened to understand the pathways of the probiotic alterations and how the treatment is benefiting host responses.

The relationship between mucus or goblet cells and worm expulsion and host defense is not completely understood, but from the data shown, the increase of goblet cell numbers correlates with an increased expulsion of the parasite from the GI tract. It is possible that probiotics act on, or have downstream pathways to affect other mucin genes such as Muc5ac, to help with host defense.

Many genes related to goblet cells, mucins, and mucus secretion are also upregulated in response to parasitic infections, including *T. muris* (Artis, 2006). The increased expression of goblet cells, associated genes, and the subsequent hyperplasia, coincides with the maximal expression of T_H2 cytokines and worm expulsion in resistant mice (Artis *et al.* 2004; Hasnain *et al.* 2010). These molecules may play a role in the expulsion of *T. muris* from resistant mice, and the possibility of probiotics acting to further increase the expression of these proteins still needs to be investigated.

Resistin-like molecule β (Relmβ) is a peptide produced and secreted into the lumen from intestinal goblet cells (Artis *et al.* 2004). Relmβ is significantly increased in resistant strains of mice during the expulsion period of *T. muris* infections, when

T_H2 cytokines are at their peak, and goblet cell hyperplasia is occurring (Artis *et al.* 2004, 2008; Hasnain *et al.* 2010). The function of Relmβ is relatively unknown, although it is believed that this peptide is able to bind to *T. muris* directly and interact with its chemotaxic functions, inhibiting attachment and promoting expulsion through peristalsis (Artis *et al.* 2004, 2008). Surprisingly, Relmβ KO mice are able to expel *T. muris* worms, and this molecule is therefore not mandatory in the expulsion in a T_H2 environment (Nair *et al.*2006). Perhaps Relmβ is playing a role in reducing the overall wellbeing of the parasite. The possibility that with probiotic treatment, the increased goblet cell hyperplasia is correlated with a subsequent increase in Relmβ, making worm expulsion happen at a more rapid pace is unknown, and needs to be further investigated.

Other secreted products that are produced by goblet cells and upregulated during a *T. muris* infection in resistant mice are the intelectins (Artis, 2006; Nair *et al.* 2006). Intelectin expression is at its peak when T_H2 cytokines are increased, usually during expulsion of the parasite (Artis, 2006; Nair *et al.* 2006). The role of intelectins during a *T. muris* infection remains unknown, but it has been speculated that these peptides are able to bind certain carbohydrate residues on the helminth, which in turn may impair the parasite's ability to attach to the mucosa (Nair *et al.* 2006). If intelectins do bind residues on the worm directly, this interaction may also inhibit the feeding capabilities of the worm and reduce the parasite's energy and help to promote expulsion (Artis, 2006).

Goblet cells and many things related to goblet cells are of high interest during parasitic infections, but also in mechanisms which probiotic treatment may help the

host. Elucidating the potential mechanisms of probiotic therapy in junction with goblet cell alterations is of importance regarding parasitic infections.

Cell proliferation of not only goblet cells, but also epithelial cells of the mucosa in the crypts of the colon during a *T. muris* infection is also altered (Cliffe *et al.* 2005). Not only the proliferation, but the migratory speed of the cells up the crypts is different during infection depending on the strain of mice (Artis and Grencis, 2008; Cliffe *et al.* 2005). Under natural homeostasis, cells in the crypts of the colon migrate towards the lumen from stem cells at the base, during which cells differentiate into their destined cell lineage, mature, and then undergo programmed apoptosis (Artis, 2006; Cliffe *et al.* 2005). Maintaining a balance of proliferation, migration and death of the cells in the epithelium is needed to maintain a homeostatic morphology (Artis, 2006). In resistant strains of mice, at the beginning of expulsion of the parasite, when T_H2 cytokines are highest, intestinal epithelial cell turnover rates are almost doubled (Artis and Grencis, 2008). The number of cells proliferating quickly returns to baseline soon after the worms are expelled (Cliffe *et al.* 2005). Some of the alterations of the epithelial cell turnover rates and migration are regulated by the immune system during the parasitic infection (Artis, 2006). In susceptible strains of mice, a *T. muris* infection has an effect on the proliferation and migration of epithelial cells of the colon crypts, but slightly different than resistant strains. There is an increase of cell proliferation at the same time point, although the cell migration patterns are delayed until later in the course of the infection (Cliffe *et al.* 2005). The delayed migratory changes cause crypt hyperplasia because of the imbalance of turnover rates and migration (Cliffe *et al.* 2005). The delay in the proliferation and

migration of cells may also prevent the displacement of the smaller worms earlier on in the infection, and thus may allow the larger worms to attach and burrow into the mucosa to create a niche for optimal survival (Cliffe *et al.* 2005). The T_H2 cytokine IL-13 has been shown to promote the rapid epithelial migration up the crypt during worm expulsion, and IFN-γ and its induced CXCL10, reduced the turnover, resulting in accumulation of epithelial cells proliferating in the crypt (Cliffe *et al.* 2005).

Our data with the use of BrdU incorporation into cells and staining showed that in resistant strains of mice infected with *T. muris* and treated with *L. reuteri* there was a significant increase in the number of cells stained with BrdU at day 21 pi. There was an increase of the percentage of cells within each crypt undergoing proliferation as well. Further investigation into the specific cell type and the time course is needed to determine the migratory patterns and rates to draw conclusion on cell types affected by the probiotic bacteria and parasite. If the probiotic treatment has an effect on the rate of proliferation and migration of epithelial cells during infection, it may suggest probiotics act to promote epithelial shedding, leading to an increased expulsion of parasites. Goblet cell hyperplasia was altered with probiotic treatment in many of the mice strains used, and furthering the study with BrdU incorporation and specifically looking into goblet cell progenitor markers would be interesting and beneficial. Perhaps the bacterial treatment is directly working on stem cells to promote the differentiation of these progenitors into goblet cell lineages.

Cell proliferation has been shown to be of importance in parasitic infections and promoting the expulsion of *T. muris* worms from resistant strains of mice. The

immune system acts to regulate this balance and in turn create an environment not suitable for the persistence of the parasite.

Although it has been ruled out that the viable bacteria are needed for beneficial effects, it is still unclear at what pathway, product, or component of the bacteria is specifically needed. As noted, it is of interest to do more studies with the probiotic in the same system, specifically with the conditioned media from a *L. reuteri* culture. Perhaps a secreted product is a key factor in the beneficial effects of this specific treatment for *T. muris* infections. Future studies with conditioned media will be useful to enhance our understanding on the mechanism of the effect of probiotic treatment in host defense in this infection.

A recent study looking at the hatching of *T. muris* eggs and the overall infection with regards to the bacteria present in the caecum has come to the conclusion that specific triggers are needed for hatching to occur (Hayes *et al.* 2010). Incubation of the eggs with *Escherichia coli* (*E. coli*), a common commensal, or *E. coli* conditioned media, showed that there needs to be direct contact of the bacteria, not just a secreted factor, for hatching to occur. This structure was determined to be type 1 fimbriae using modified strains of *E. coli* with mutated fimbriae. Although other hatching cues do exist, as incubation with a gram positive *Staphylococcus aureus* bacteria, which lack type 1 fimbriae, also promoted hatching (Hayes *et al.* 2010). Administration of antibiotics also have the ability to alter hatching cues in the caecum of susceptible AKR mice, further proving that bacteria do have a role in hatching of *T. muris* (Hayes *et al.* 2010). The probiotic treatment that was given to

the mice throughout the experiments may have the potential to not only alter the host responses, but could have also acted on the *T. muris* eggs before even hatching.

The alteration of the microflora populations with administration of *L. reuteri* could have reduced the amount of eggs that were hatching, and possibly preventing some attachment of the larvae to the mucosa. *L. reuteri* treatment that was given ~24 hours before the infection of *T. muris* eggs, and therefore the probiotic may have a role on the hatching of the eggs. Looking at the probiotic and *T. muris* interaction could be a possible next step in this research, to determine if the probiotic alters the gut microflora to prevent some of the hatching of the embryonated eggs by reducing the number of *E. coli* or altering other bacterial species or cues needed for hatching. The probiotic's presence in the caecum of the mice may also prevent the hatched worms from burrowing into the epithelial layer. Investigation of the attachment or brief colonization of the live bacteria may reveal that the probiotic has an effect on the motility of the worm, which the γ-irradiated bacteria do not. There are many possibilities involving the viability and structure of the probiotic and the overall survival and persistence of the *T. muris* worm that remain unknown.

The cytokine IL-9 could also be another potential insight to the action of probiotics. IL-9 is not often a cytokine checked in the gut with working with probiotics, and there have been no insights to whether IL-9 is altered with probiotic treatment during a *T. muris* infection. There are several past studies which may suggest another pathway of the action of probiotics to look into. IL-9 is a cytokine that has been shown to protect the host from gram negative bacteria by altering the

cytokine milieu, and decreasing the amounts of T_H1 cytokines. During a parasitic infection, IL-9 is increased, and has been shown to enhance resistance by increasing amounts of immunoglobulins, mast cells, eosinophils, goblet cells, and by acting on smooth muscle hypercontractility (Khan *et al.* 2003; Richard *et al.* 2000). Blocking IL-9 during a *T. muris* infection in resistant mice increases susceptibility, due to the reduction of muscle hypercontractility and goblet cell hyperplasia, resulting in attenuation of worm expulsion (Khan *et al.* 2003; Richard *et al.* 2000). T_H2 immune responses, specifically IL-13 and IL-4 are needed for muscle hypercontractility, but IL-9 is also involved during the parasite infection (Khan *et al.* 2003). In the study presented here, IL-9 was not checked in either the resistant, susceptible, or the KO mice treated with the probiotic. Had this cytokine been checked and altered, further investigation in the motility of these infected animals would help explain another possible mechanism.

Not only if IL-9 was increased, looking into the contractility and motility of the GI tract during the infection and probiotic treatment would give us an insight to whether the probiotic is altering the smooth muscle of the colon. *T. muris* infection in resistant mice alters the colonic smooth muscle contractility, whereas in a susceptible mouse there is not an increase in contractility, which then leads to gut dysfunction (Motomura *et al.* 2010). Eradication of the worms from the caecum and colon of susceptible mice early on in the infection prevents the dysfunction and hypocontractility (Motomura *et al.* 2010). The low grade T_H1 inflammation during the chronic infection is thought to be a cause of this hypocontractility, which may affect the expulsion of the parasite (Motomura *et al.* 2010).

Not only could the probiotics be altering cytokine responses to alter the muscle movement in the colon of *T. muris* infected mice, but the bacteria could also be working on the microbial composition as well to alter motility. The flora of the GI tract could also be altered during the parasitic infection, which also could be altering contractility. Post infectious dysfunction of gut motility has been shown to be altered by the administration of some types of probiotic bacteria (Verdú *et al.* 2004). Perhaps the administration of *L. reuteri* to the infected mice will alter the flora composition in the gut and help in expulsion by promoting colonic hypercontractility.

If there is a change in motility patterns with probiotic treatment, there may be increased peristalsis to expel the worms or the eggs before hatching. If there are early contractility alterations, it also may be difficult for the worm to initially attach to the mucosa and persist and establish an infection. Studies on motor function of the colon of these mice would help explain the early expulsion of the parasite in the C57BL/6 and Muc2 KO mice. These studies may give us an idea if the probiotics are truly having an effect on the AKR infected mice by altering contractility of the colon.

The ultimate target of this study is not only to help with the millions of parasitic infections around the world, but also to bring these bacteria into chronic inflammatory models, to tests whether *L. reuteri* has similar benefits in other circumstances. Administering *L. reuteri* to mice with other infections inflammation models will give a better understanding of the actions of probiotics in infection and inflammatory conditions of the GI tract.

There are severe consequences of helminth and other parasitic infections not only in humans around the world, but also in the livestock industry (Artis, 2006). The increasing infections with parasites as well as the development of resistance to anti-helminth medications, proposes a huge economical burden on communities and industries that are affected (Artis, 2006). Probiotic therapy could potentially be a cheaper way to prevent or diminish some of these infected areas.

There is also the emerging concern that chronic helminth infections may reduce the effectiveness of certain vaccines. This blunted vaccine response is due to the dampened immune response and inflammation towards to vaccine because of the chronic parasitic infection (Artis, 2006). This would also raise the economical and health concerns of developing new vaccines that are effective. With new probiotic strategies against parasitic infections, not only will the affected individual eliminate the parasite, but the beneficial bacteria will help return to flora and immune homeostasis, but also prevent the dampened immune response, allowing the vaccine to be effective.

The demonstration of upregulation of regulatory cytokine production by probiotic treatment in *T. muris*-induced inflammation has implications in understanding the modulatory role of this probiotic in inflammation which ultimately be helpful in the development of probiotic based effective strategies in combating common GI inflammatory disorders.

A combination of the alterations discussed, and possibly a change in microflora all aided the host to expel the *T. muris* worms effectively and rapidly from the caecum and colon. Probiotic therapy as a treatment or preventative measure to

parasitic infections has the potential to eliminate pandemics of helminth infections, and improve the quality of life of the many individuals affected.

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