GASTROINTESTINAL MICROBIOTA COMMUNITY COMPOSITION HAS SIGNIFICANT EFFECTS ON SYSTEMIC IMMUNE RESPONSES

BY

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DISSERTATION

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ABSTRACT

This study explored the utility of an oral microbial inoculum as a therapeutic tool to affect systemic immune responses. Colonization of the gastrointestinal (GI) tract is initiated during birth and continually seeded from the individual's environment. Gastrointestinal microorganisms for a mutualistic relationship with the host, playing a central role in developing and modulating host immune responses. Animal studies have demonstrated the impact of GI microbiota on the development of GI and systemic immune systems; however, the full spectrum of action of early gastrointestinal tract stimulation and subsequent modulation of systemic immune responses is poorly understood. Human trials have shown the successful use of probiotics and fecal transplantations to treat GI disorders. In addition, patients receiving fecal transplants have also reported improvements in systemic disorders such as multiple sclerosis. These results, in addition to increased incidence of allergic and autoimmune diseases associated with reduced GI microbial diversity has increased interest in the effect of early life GI colonization on the development of the systemic immune system. In order to address this issue, we sought to determine the effects of early life colonization on microbiome composition and systemic immune responses. One group of newly weaned pigs was inoculated with an oral microbial inoculum (modulated), while another group (control) was not. Sequencing results show a successful modulation of the GI microbiome through oral inoculation. The effects of GI microbial modulation on systemic immune responses were evaluated by experimentally infecting with the respiratory pathogen *Mycoplasma hyopneumoniae* (*M. hyopneumoniae*). The *M. hyopneumoniae* infection study showed beneficial effects of the oral inoculum on systemic immune responses including antibody production, severity of infection and cytokine levels. These results suggest an oral microbial inoculum can be used to modulate microbial communities, as well as have a beneficial effect on systemic immune responses as demonstrated with *M. hyopneumoniae* infection.

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CHAPTER ONE

INTRODUCTION: HYPOTHESIS AND SPECIFIC AIMS

1.1 INTRODUCTION

The mammalian gastrointestinal (GI) tract is home to a complex microbial community with a population over 10 times greater than the total number of somatic cells present in the host [1]. Colonization of the GI tract starts at birth with exposure to bacteria from the mother and the surrounding environment, and this initial colonization is thought to have significant effects on microbial community structure later in life [2].

Germ-free animal studies have shown that GI microbiota and their hosts do not simply co-exist, but rather form a mutualistic relationship [1]. The human gastrointestinal tract is estimated to contain over 1,000 microbial species who's collective genome size is two orders of magnitude larger than the human genome [3], and provides important biological and metabolic functions for its host [4]. Some benefits accounted for by this relationship include sharing of nutrients and organic substrates, pathogen colonization resistance, regulation of fat storage and maturation and modulation of gastrointestinal immunity [1], [5]. The composition of an individual's GI microbiota is dependent on a number of factors, including early environmental exposures, hygiene and diet [6–8]. Studies have shown the human gastrointestinal tract to be dominated by anaerobic bacteria belonging to the Bacteroidetes, Firmicutes and Actinobacteria phyla. Furthermore, increases in the relative abundance of Firmicutes and decreases in Bacteroidetes phyla have been correlated to obesity [9–11], and colonization of germ-free mice with microbiota from obese animals results in increased body fat and insulin resistance [12]. The association between obesity and the abundance of these bacterial phyla has also been confirmed in human studies [2].

The central role of GI microorganisms in developing and modulating host intestinal immune responses has been a subject of investigation over the last few decades [13]. Germ-free and conventional animal studies have shown that microbial exposure early in life is

associated with protection from immune-mediated diseases, such as inflammatory bowel disease (IBD) and asthma [14]. Animal studies using pigs raised in indoor or outdoor environments have demonstrated differences in mucosa-adherent microbial diversity as well as increased gastrointestinal immune gene expression levels in indoor-housed pigs [15], while another study has shown that the time and length of exposure to microbes early in life may be crucial in establishing the porcine GI microbiota [16]. There is also increasing evidence of strong associations between particular GI microbial populations and the incidence of enteric and/or metabolic disorders, such as obesity and diabetes [11], [17], as well as differential intestinal immune responses [15]. In addition, recent studies have shown the successful use of GI microbial modulation as a therapy to combat chronic *Clostridium difficile* infections and other GI conditions in humans [18–20].

The GI microbiota are in constant contact with the epithelial surfaces of the intestinal mucosa, where they interact with dendritic cells (DC) in Peyer's patches [21]. The microbe-associated molecular patterns present in the gut microbiota are recognized by various DC pattern recognition receptors, such as toll-like receptors (TLRs), which migrate into mesenteric lymph nodes, where the antigens are bound to MHC class II receptors and presented to T cells, causing activation and differentiation [21]. This process serves as a bridge between GI microbiota and the systemic immune system, and helps to explain how GI microbial diversity is involved in the development and regulation of immune responses outside of the GI tract. This interaction, as well as the hygiene hypothesis, which proposes that infections in early childhood and unhygienic contact with older siblings and the environment mitigate allergic diseases [22], [23], has led to the testable hypotheses that GI microbiota could modify the hosts immune responses outside the GI tract [24], [25]. However, the full spectrum of early GI tract stimulation and the subsequent modulation of systemic immune responses are far from understood, and even more uncertain is how the GI microbiota may be modulated and subsequently serve as a therapeutic tool.

1.2 Hypothesis and Specific Aims

The hypothesis of this thesis proposal is an oral microbial inoculation is sufficient for successful alteration of the gastrointestinal microbiome composition, and that this alteration in gastrointestinal microbiome composition will result in the modulation of systemic immune responses. In order to test this hypothesis, the two specific aims for this project are as follows:

1.2.1 Evaluate the effects of gastrointestinal modulation on gastrointestinal and respiratory microbiomes

In order to validate the objectives of this project, an effective protocol for modulating the gastrointestinal microbiome must first be created. The protocol should allow quick and permanent modulation of the gastrointestinal microbiome without directly altering other microbiome communities or the health status of the host. We hypothesized that an oral inoculation could be used to effectively modulate the gastrointestinal microbiome, and that the difference in GI micrbiome composition between this group and uninoculated subjects would be sufficient to elicit different systemic immune responses to a pathogenic challenge. Chapter 3 describes the development and validation of this gastrointestinal microbiome modulation protocol.

1.2.2 Evaluate the host response after experimental infection with *Mycoplasma hyopneumoniae* in pigs with altered gastrointestinal microbial diversity

We hypothesized that the gastrointestinal microbiome modulation would be sufficient to produce statistically significant differences in immune responses to a pathogenic challenge. To appropriately support this hypothesis, the pathogenic challenge cannot have any direct contact with the gastrointestinal tract. Therefore the swine respiratory pathogen *Mycoplasma hyopneumoniae* was chosen for the pathogenic challenge. Chapter 4 describes in detail the process of evaluating host systemic immune responses to a pathogenic challenge following successful modulation of the gastrointestinal microbiome.

CHAPTER TWO

LITERATURE REVIEW

2.1 INTRODUCTION

Humans are host to vast communities of microorganisms that have evolved with us over time to form symbiotic relationships [26]. While microbial communities are present on every surface of the human body, the GI tract boasts the greatest bacterial load, with the number of bacteria in the large intestines estimated to reach levels of 10^{12} cells per gram of fecal material [27].

The process of GI colonization begins at birth with the vertical transfer of microbes from the mother to the fetus (Figure 2.1). Once colonization has been established, the composition of the GI microbiome evolves in response to environmental factors such as diet and the genetic background of the host [26]. Studies have shown newborns are exposed to vaginal and intestinal microbiota from the mother's birth canal and feces during birth, while caesarean derived infants are colonized by environmental microbes more generally associated with the skin [4], [28]. Furthermore, caesarean derived infants have been shown to have lower colonization rates than naturally born infants as late as 6 months after birth [29]. The delayed colonization of the GI tract in caesarean derived infants has been associated with increased risk of developing allergies and asthma [30], [31].

Humans and the microbial communities they harbor form mutualistic relationships that include the sharing of nutrients and growth factors, pathogen colonization resistance, promotion of differentiation of mucosal structures and functions, regulation of fat storage, and maturation of the gastrointestinal immune system. Studies have shown that germ-free mice have less total body fat than conventionally raised mice, even when fed a higher calorie diet [12]. The composition of the gastrointestinal microbiome has been shown to be important as well, with increases in the Firmicutes and decreases in Bacteroidetes phyla shown to be associated with obesity [10].

Decreases in bacterial diversity have also been associated with increased incidence of allergies and asthma, an aspect of the hygiene hypothesis, which states that early childhood exposure to microbes mitigates these diseases [22], [23]. GI microbes are especially important in this regard, as they are in constant contact with the gut-associated lymphoid tissue, the largest portion of the host immune system [32]. Decreases in microbial diversity have been associated with gastrointestinal diseases, including *Clostridium difficile* infections and inflammatory bowel disease [33], [34]. Treatments using probiotics and fecal transplants have shown to be effective in combating many of these diseases [35–37], [38], [39]. In addition to gastrointestinal diseases, recent evidence has shown correlations between GI microbiome composition and systemic allergic and autoimmune diseases [13]. However, further studies are required in order to determine if GI microbiome composition has an effect on disease progression in these cases.

2.2 The Hygiene Hypothesis

Allergic diseases are the result of inappropriate immunological responses to otherwise harmless antigens, which are recognized by immunoglobulin E (IgE) and cause the production of histamines and the symptoms of allergic reactions. Studies have shown increases in allergic responses associated with the absence of microbial exposure while the immune system is still under development [40], [41]. This theory has come to be known as the hygiene hypothesis, and has since been expanded to include the increased risk of developing autoimmune diseases.

2.2.1 History of the Hygiene Hypothesis

The hygiene hypothesis was first proposed by David Strachan in a 1989 article which suggested that allergic diseases were prevented by infections in early childhood transmitted by unhygienic contact with older siblings [22]. He further concluded that the increased incidence of asthma, childhood eczema and hay fever were associated with declining family size, improvements in household amenities, and higher hygiene standards which reduce cross infection opportunities among family members [22]. Since then the hygiene hypothesis has been altered to include the importance of contact with symbiotic microorganisms and parasites in reducing the susceptibility to allergic disease by initiating the natural development of the

immune system. Studies in third world countries have demonstrated an increase in immunological disorders as a country develops and becomes cleaner [42], while antibiotic use during the first year of life has been linked to asthma and other allergic diseases [43]. These phenomena are thought to be due to a lack of stimulation and subsequent development of the immune system, and these interactions are known to affect other areas of health as well.

2.2.2 Evolution of the Hygiene Hypothesis

The first mechanism by which the hygiene hypothesis was proposed to work suggested insufficient stimulation of the T helper 1 branch of the immune system early in life leads to overcompensation by the T helper 2 branch, causing allergic diseases [44]. T helper cells are a subset of T cells that drive immune responses by releasing cytokines that promote B cell antibody class switching, activation of cytotoxic T cells and bactericidal activity of macrophages. T helper lymphocytes can be divided into two subtypes, Th1 and Th2. The Th1 subset drives cell-mediated responses, while Th2 cells drive antibody-mediated responses. Together these two cell types produce a balanced immune response against invading pathogens, while imbalances in this Th1/Th2 subset have been shown to be associated with certain diseases. A shift towards Th2-mediated responses is associated with allergic disease [45], while a shift towards Th1-mediated responses has been associated with autoimmune disorders [46].

While this mechanism can be used to explain increases in allergic diseases, it cannot explain the increased incidence of Th1-mediated diseases in developed countries, such as inflammatory bowel disease, multiple sclerosis and type I diabetes. Since then, a new theory has been proposed to include both Th1 and Th2 responses, stating that early life contact with pathogens, symbiotic microorganism and parasites is essential for the proper development of regulatory T cells. Regulatory T cells are a type of lymphocyte that modulates the immune system by repressing immune responses and maintaining self-tolerance. Regulatory T cells have been shown to inhibit interactions between dendritic cells and CD4+ T cells, a subset of lymphocytes that includes both Th1 and Th2 cells [47]. Individuals with underdeveloped regulatory T cells have been shown to be unable to properly regulate Th1 and Th2 immune responses, making them more susceptible to autoimmune and allergic diseases [48]. Regulatory

T cells that have the ability to suppress immune responses have been identified in humans and mice [49], [50], while studies in mouse IBD models have shown regulatory T cells have significant control over gut inflammation [51]. This mechanism provides a means by which GI microbiota can alter both Th1 and Th2 responses, and provides an avenue by which exposure to GI microbes early in life could have an impact on autoimmune and allergic diseases later in life.

2.3 The Role of Gastrointestinal Microbiota in Intestinal Immunity

2.3.1 Gut-Associated Lymphoid Tissue

The GALT is the largest portion of the host immune system, coming into contact with more antigens than any other part of the body [32]. The GALT is where the GI microbiota come into contact with the host immune system (Figure 2.2). The only barrier separating the contents of the GI lumen from the intestinal immune system is the gut epithelium, which has a surface area of over 400 m^2 [52]. In addition to processing antigens, the intestinal immune system must also discriminate between invasive pathogens and harmless antigens from commensal bacteria and food proteins. The ability of the host to induce both local and systemic immunological tolerance to these harmless antigens is known as oral tolerance [53]. The regulation of oral tolerance is thought to be due to unique features of the GALT, including specialized cells involved in the uptake of antigens, unique subsets of antigen-presenting cells, and conditions which favour the generation of T regulatory cells [32]. An inability to properly regulate the intestinal immune response to harmless antigens can result in hypersensitivity responses, which can lead to such inflammatory disorders as celiac and crohn's disease.

The GALT is comprised of two main areas, effector sites and organized tissues. The effector sites are areas in which lymphocytes are scattered throughout the epithelium and lamina propria of the intestines, while the organized tissues consist of peyer's patches and mesenteric lymph nodes [32]. Peyer's patches are macroscopic lymphoid aggregates consisting of B-cell follicles and intervening T-cell areas found along the intestines. The lymphocytes are separated from the intestinal lumen by the follicle-associated epithelium (FAE), a single layer of epithelial cells. Within the FAE are specialized enterocytes called microfold (M) cells. M cells lack microvilli and bind invasive pathogens as well as particulate antigens and pass them to

antigen-presenting cells such as dendritic cells in the subepithelial area. In addition, dendritic cells can squeeze processes between the tight junctions of the epithelial cells to sample the GI luminal contents. Dendritic cells process and present the antigen bound to MHCII receptors to naive T and B lymphocytes. When B cells recognize a particular antigen, they undergo immunoglobulin class switching in response to signals presented by dendritic cells and T cells to express IgA [54]. These activated lymphocytes then travel to the mesenteric lymph nodes, where they interact with immune cells and eventually migrate to the lamina propria.

The mesenteric lymph nodes are the largest lymph nodes in the body, and it is believed that they may serve as an intersection between peripheral and mucosal pathways, providing a link between systemic and intestinal immunity. Mesenteric lymph nodes are considered crucial in the induction of mucosal immunity and tolerance, as studies have shown antigen recognition occurs in mesenteric lymph nodes after oral inoculation [55–57]. In addition induction of oral tolerance has not been possible in mice lacking mesenteric lymph nodes [58], and IgA antibody responses are also absent in these mice [59], [60].

Once the primed lymphocytes leave the mesenteric lymph nodes, they travel through the circulatory system and make their way to the lamina propria, where B cells mature into IgA-producing plasma cells. These plasma cells are extremely important in the intestinal immune response, being responsible for the secretion of 3 to 5 grams of IgA into the GI lumen every day [52]. In addition to antibody producing plasma cells, CD4+ and CD8+ T cells are found throughout the lamina propria. CD8+ T cells, in addition to their cytotoxic activity, are believed to be effector cells important for the production of IgA by B cells [61]. Studies have also shown CD4+ T cells in the lumen may in fact be regulatory T cells responsible for maintaining local tolerance to environmental antigens [62].

There is evidence to suggest that alternative pathways may also be important in the recognition and presentation of GI antigens to lymphocytes. Recent studies suggest dendritic cells originating from the peyer's patches present naive T cells with antigens of intestinal origin mainly in the mesenteric lymph nodes [63]. This route of activation allows T cells to travel throughout the circulatory system and enact their immunological responses on other areas of

the body. Enterocytes may also be crucial in regulating intestinal immune responses by producing pro-inflammatory chemokines and cytokines in response to the recognition of pathogen-associated molecular patterns by pattern recognition receptors such as toll-like receptors (TLRs) and nucleotide-binding oligomerization domain (Nod) molecules [52]. However the signals responsible for this activation have been shown to be inhibited by interactions between epithelial cells and commensal bacteria [64], [65]. In addition, enterocytes express MHCII in most species and have been shown to process antigens and present them on their basal surface to T cells *in vitro* [66]. Because enterocytes normally lack co-stimulatory molecules required for T cell activation [67], this mechanism of antigen presentation has been submitted as a means by which enterocytes can act as tolerogenic antigen-presenting cells.

2.3.2 GI Microbiota in Health and Disease

In order for the intestinal immune system to function properly, interactions between the host and GI microbiome are necessary. Mice kept in germ-free environments have been shown to have underdeveloped Peyer's patches lacking germinal centers, as well as few IgA plasma cells and CD4+ T cells in the lamina propria [68], [69]. In addition, the inactive intestinal immune system has been restored in these mice by the addition of a GI microbiome. While this symbiotic relationship is extremely important, it can also be dangerous, as alterations in the immune or epithelial homeostasis can lead to diseases such as inflammatory bowel disease (IBD). Symptoms associated with IBD include chronic diarrhea, weight loss, abdominal pain, fever and fatigue. Two of the more common versions of IBD are Crohn's disease and ulcerative colitis (UC). These hypersensitivity responses are an exaggeration of the Th1 response [70], [71]. Immunosuppressive drugs designed to enhance mucosal T cell death have been effective in down-regulating intestinal inflammation associated with these diseases [72–74], however the identification of antigens that drive the Th1 inflammatory response has not been successful.

Despite the inability of researchers to determine specific antigens associated with IBD, studies suggest that these antigens are derived from GI microbes. In mouse models of IBD, raising animals under germ-free conditions leads to a significant reduction of the disease [75]. In addition, studies have shown CD4+ T cells reactive to GI microbial antigens cause colitis [76].

Although no single component of the GI microbiota has been identified as being significant for the development of IBD, specific bacteria have been shown to be associated with the disease. *Bacteroides vulgates* and *Escherichia coli* induce IBD in animal models, but these results are strain specific [76], showing the importance of an individual's immune response to the GI microbiota in IBD.

The overall evidence suggests that altering the GI microbiome can have significant effects on intestinal immune responses and disease state. Recent studies administering probiotics to infants with high risk of developing allergies have resulted in decreases in the incidence of atopic dermatitis later in life [77]. The concept of using probiotics to improve health is not a new concept. The use of GI microbiota to improve health dates back to a paper published by Elie Metchnikoff in 1907 [78]. He correlated the large amounts of fermented milk consumed by specific Eastern European populations with increased longevity, going as far as to start consuming fermented milk himself. Dr. Metchnikoff noted an improvement in his health after regularly consuming the fermented milk, and concluded that microbes associated with increased health could be consumed to replace potentially harmful ones. Probiotics have been shown to improve the symptoms of diseases such as IBD, atopic dermatitis and asthma [35–37]. However the strain-specific nature of these results suggests further work is needed to determine which probiotics are effective at treating specific diseases. In addition, probiotics have been shown to persist in the GI microbiome for up to 14 days after usage has stopped, showing continual usage is necessary for long term benefits to be realized [79].

2.3.3 Treatment of GI Disorders via Fecal Transplantation

In addition to probiotics, the use of fecal transplantations to treat gastrointestinal diseases has become of interest recently. The process of fecal transplantation takes probiotics one step futher, by transferring gut microbiota from a healthy donor to the patient in order to establish a stable microbial community in the GI tract (Table 2.1) [80]. The use of fecal transplantations to treat gastrointestinal diseases dates back to the 1950s, when surgeons from Colorado used fecal retention enemas to treat four patients with severe pseudomembranous colitis [81]. The result was a quick recovery for all patients within a few days of treatment.

Recent studies have shown the advantages of fecal transplantation over probiotic use, with reports of the persistence of the donor microbiome up to 24 weeks after transplantation [82].

The most common disease that is currently being treated using fecal transplantation is chronic diarrheal disease caused by *Clostridium difficile* infections [18–20]. This disease is most commonly acquired as the result of major disruptions in the GI microbiome, caused by the use of oral antibiotics [20]. *Clostridium difficile* infections are associated with reduced species diversity, with decreases in Bacteroidetes and increases in Proteobacteria and Verrucomicrobia [34]. In extreme cases the success rate of fecal transplantation treatment of *C. difficile* infections is around 90% after a single treatment (Table 2.2) [83], much higher than with traditional antibiotic treatments, which cause 20-25% of patients to relapse after discontinuation [84], [85]. Moreover, patients treated by fecal transplantation have been shown to remain asymptomatic, without the need of any therapies, for years after treatment.

Although no adverse effects have been reported with the use of fecal transplants to date [80], the increased use of this procedure has led to the establishment of basic guidelines for donor screening, including testing for pathogens as well as communicable diseases (Table 2.3). In addition, the recent success of treating *C. difficile* infections has prompted many researchers to look towards fecal transplants as a means to treat a wide variety of intestinal disorders. For example, reduced microbial diversity is associated with other gastrointestinal disorders, such as IBD [3]. IBD, Irritable bowel syndrome (IBS) and ulcerative colitis have been treated using fecal transplants, with patients remaining asymptomatic for up to 13 years without medication [38], [39]. However multiple transplants have been required to treat most patients with ulcerative colitis, showing a need for a better understanding of the mechanism by which fecal transplantation treats these disorders.

2.4. The Role of Gastrointestinal Microbiota in Systemic Immunity

In addition to the role of the GI microbiome on intestinal function and immunity, there is growing evidence to suggest that GI microbes are important in the development of the systemic immune system [13]. As with diseases such as IBS, alterations in GI microbiome composition have been observed in the development of non-gastrointestinal disorders.

However the role of individual bacterial species still needs to be determined, as well as whether the altered community compositions observed have a causative role in these diseases.

Studies examining the development of the systemic immune system in germ-free mice have noted fewer and smaller germinal centers in the spleen, decreased CD4+ T cell numbers and a Th2 cytokine profile [86], [87]. However the mechanisms by which the GI microbiome stimulates development of the systemic immune system are not fully understood. Some individual effects, such as T cell deficiencies and Th1/Th2 imbalances in germ-free mice, have been shown to be corrected by monocolonization with *B. fragilis* due to a capsular polysaccharide on its surface [87]. In addition, colonization of germ-free mice with *Clostridia* species resulted in reduced serum IgE responses to ovalbumin [88].

2.4.1 GI Microbiome and Allergic Diseases

Decreased contact with microbes early in life has been associated with increases in the incidence of allergies and asthma for decades. However, finding a causative link between altered GI microbiome composition and allergic diseases has been difficult, and few experiments support the role of GI microbes in the development of systemic immune-mediated disorders. Decreases in the abundance of *Lactobacillus* and *Bifidobacterium* species have been associated with increased allergies, and probiotics are being used to try and correct these levels in high risk infants [89]. Children derived by caesarean section have reduced incidence of eczema and IgE related food allergies when administered prebiotics (galacto-oligosaccharides) and probiotics (*Lactobacillus rhamnosus* GG, *Lactobascillus rhamnosus* LC705, *Bifidobacterium breve* Bb99 and *Propionibacterium freudenreichii* spp. Shermanii JS) during the first 6 months of life compared to caesarean derived children who received a placebo [90]. Because the benefits of this treatment were not seen in vaginally derived infants, one can conclude that this treatment increases the abundance of GI microbiota to a level more similar to that of vaginally derived infants.

A recent study has shown increased allergic airway disease associated with mice orally administered the antibiotic cefoperazone [91]. Other studies have shown TLR4 activation is necessary to prevent anaphylaxis in response to the peanut allergen Arah 1, and that oral

administration of antibiotics (kanamycin, gentamicin, colistin, metronidazole and vancomycin) increases Th2 cytokine responses compared to control animals [92]. In addition, stimulation of TLR9 by oral administration of CpG oligodeoxynucleotides also reduced allergic symptoms and corrected the Th1/Th2 imbalance, showing further evidence for the importance of GI microbiome signalling in allergic disorders [92].

2.4.2 GI Microbiome and Autoimmunity

In addition to the involvement of the GI microbiome in allergic disorders, recent studies have focused on the connection between the GI microbiome and development of systemic autoimmunity. Altered GI microbiome compositions have been reported in patients with rheumatoid arthritis when compared to control groups [93]. However, while these studies show a connection between altered microbiome composition and disease state, they cannot link altered microbiome composition with development of the disease. Many autoimmune studies have been done comparing the development and severity of disease in germ-free and control mice, with conflicting results. While some studies have shown no differences in autoimmune disease progression between germ-free and control mice [94–96], other studies have shown significant connections between the GI microbiome and autoimmune disease development. For example, a mouse strain that spontaneously develops the autoimmune disease ankylosing enthesopathy does not develop the disease under germ-free conditions [97]. The disease also develops in mice colonized by culturable anaerobes, but not mice colonized by *Lactobacillus* or *Staphylococcus* species, suggesting specific components of the GI microbiome are important in disease progression.

Mouse studies focused on autoimmune arthritis and encephalomyelitis have found increased disease prevalence associated with the presence of GI microbiota, specifically segmented filamentous bacteria [98], [99]. In these models Th17 cell responses are induced by the GI microbiota and lead to the induction of disease state. However, studies have also shown a protective role for GI microbiota in collagen-induced arthritis in mice, showing that these results are disease/strain specific and require further experimentation to determine the mechanisms by which GI microbiota affect systemic immunity [100], [101].

Type 1 diabetes is an autoimmune disease caused by self-reactive T-cell destruction of insulin-producing β-cells in the pancreas. Researchers studying type 1 diabetes in mice have shown that mice treated with oral antibiotics develop type 1 diabetes at a higher rate than untreated mice, showing a connection between GI microbiota at the development of the disease [101]. A similar increase in the incidence of type 1 diabetes was also seen by this group in mice under germ-free conditions compared to mice under specific pathogen free conditions. However, studies using diet to reduce the number of bacteria in the cecum where shown to help prevent type 1 diabetes in both rats and mice [102], [103], with higher numbers of Bacteroidetes associated with the development of diabetes. Despite the contradiction in the results, these studies suggest a link between GI microbiota and the development of autoimmune diseases like type 1 diabetes.

2.4.3 GI Microbiome and Infection

The effects of the GI microbiome on immune responses to viral infections has also become of interest as of late. Studies in mice have shown the importance of the GI microbiome for effective immune responses to the respiratory influenza virus [104]. Mice orally administered antibiotics (ampicillin, vancomycin, neomycin sulphate and metronidazole) had reduced virus-specific antibody levels and CD4+ T cell responses compared to control mice. In addition, antibody levels and T cell responses were restored in antibiotic treated mice inoculated with various TLR ligands intrarectally, suggesting that signals from the distal GI tract affect immune priming in the lungs. The distribution of dendritic cells in the lungs, and their migration to the mediastinal lymph nodes was also reduced in antibiotic treated mice. Once again, the introduction of TLR ligands into the rectum restored DC distribution and migration in the lungs.

2.4.4 Treatment of Systemic Disorders via Fecal Transplantation

In addition to the promising results of current studies treating various gastrointestinal disorders using fecal transplantation, improvements in non-intestinal disorders of patients treated for constipation and ulcerative colitis have been reported. These include significant improvements in patients with severe multiple sclerosis, an effect that was observed for over

15 years, as well as normalization of platelet counts in patients with idiopathic thrombocytopenic purpura [20].

Obesity is a major health issue in the developed world, and correlations between GI microbiota and obesity have been detected. Differences in gastrointestinal microbiome composition and the metabolites they produce have been shown in both human and animal studies comparing obese and lean individuals [9], [11], [105]. In addition recent studies have shown reduced fasting triglyceride levels, as well as improved peripheral and hepatic insulin sensitivity in obese patients who received fecal transplants from lean donors [106].

2.5 Conclusions and Future Directions

The composition of the GI microbiome has a significant impact on the host, including energy storage, nutrient sharing, pathogen colonization resistance and the development of intestinal and systemic immune responses. Over the past few decades researchers have determined the GI microbiome is important in the development of both intestinal and systemic immune responses. However, more research is needed in order to determine the mechanisms responsible for these host/microbial relationships and how they can be manipulated to benefit human health. This project sought to show a correlation between GI microbiome composition and systemic immune responses to a pathogenic challenge, further strengthening the argument for a connection between microbiome composition and development of the systemic immune system.

2.6 FIGURES AND TABLES

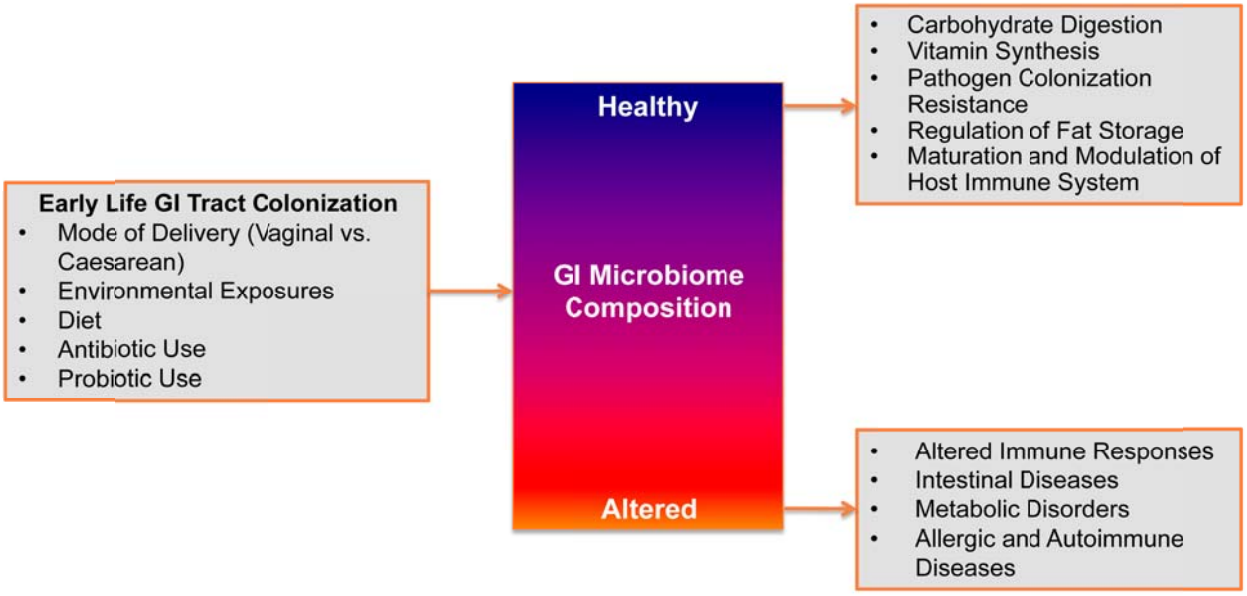

Figure 2.1: Importance of early life exposure in GI microbiome composition and health. Early life exposures are important in determining GI microbiome composition. Colonization of the GI tract starts at birth, and the mode of delivery is very important in determining the microbiome composition early in life. In addition, bacteria we come into contact with through environmental exposures can colonize the gut. Our diet, as well as the use of antibiotics or probiotics plays an important role in shaping the microbiome composition. These early life exposures are important in establishing a healthy GI microbiome that can provide benefits to the host, including carbohydrate digestion, vitamin synthesis, pathogen colonization resistance, regulation of fat storage and maturation of the host's immune system. Alterations in the microbiome composition caused by delivery via caesarean section, poor diet and high antibiotic use early in life can lead to altered immune responses, which can result in increased risk of developing intestinal, metabolic, allergic and autoimmune disorders.

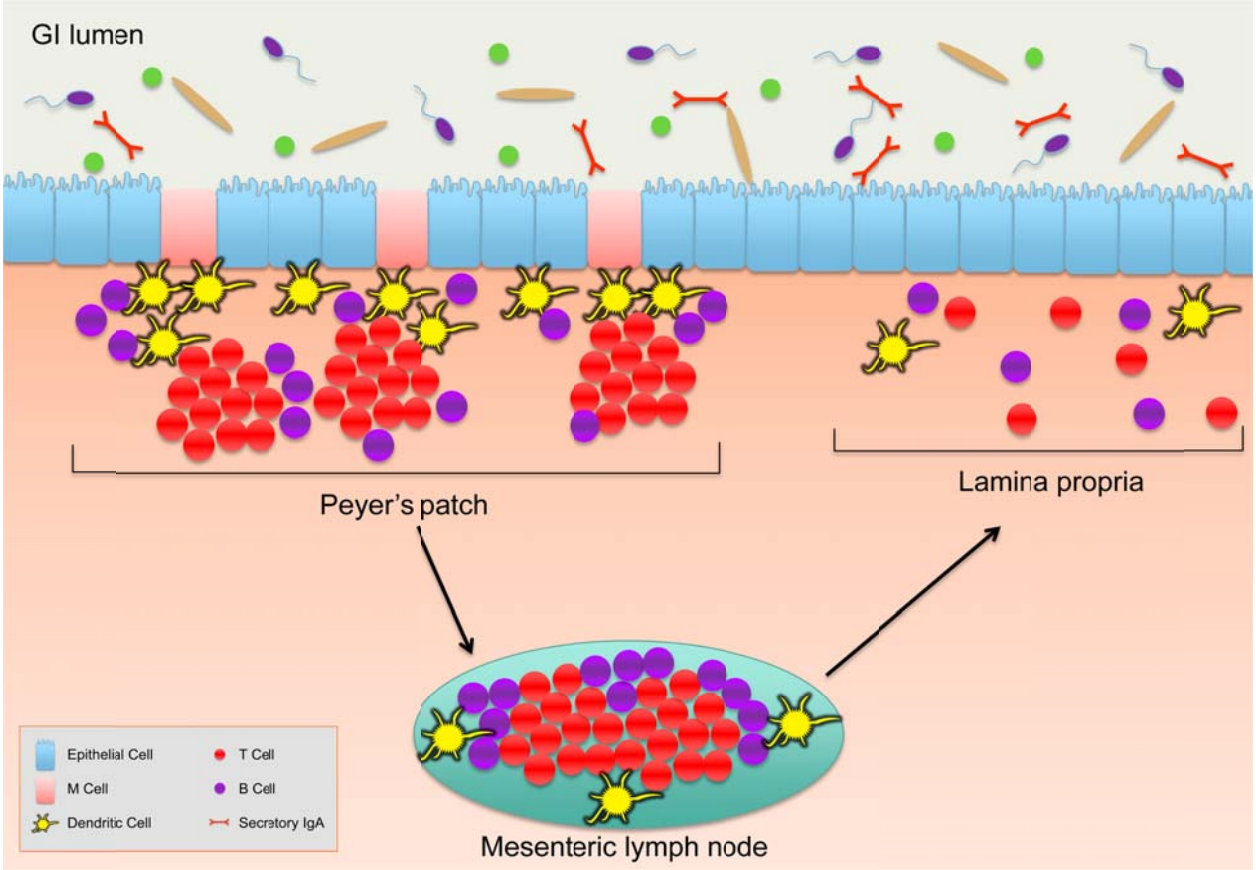

Figure 2.2: Interactions between GI microbiota and the gut associated lymphoid tissue. The GALT consists of networks of peyer's patches and mesenteric lymph nodes. Bacterial antigens in the GI lumen are taken up by M cells in the peyer's patches. The M cells pass the antigens to dendritic cells, which process the antigens and present them to T and B cells bound to MHC class II receptors in the peyer's patches and mesenteric lymph nodes. B cells capable of recognizing the antigen are activated and differentiate into a variety of cell types including memory cells and plasma cells. The immune cells exit the mesenteric lymph nodes through the circulatory system, where they make their way to the lamina propria of the GALT. Once in the lamina propria, plasma cells produce secretory IgA that is transferred across the epithelial surface into the GI lumen to prevent bacteria from breaching the epithelial cell barrier. This process is important for maturation of the host immune system, as well as keeping the GI microbiome in check.

Timeline	Donor	Recipient
Less than 2 weeks before fecal transplantation	Donor Screening	
1-3 days before fecal transplantation		Terminate antibiotic use
Evening before fecal transplantation	Mild laxative	Polyethylene glycol electrolyte lavage of GI tract
Less than 6 hours before fecal transplantation	Stool sample collection and processing Suspend stool sample in sterile saline solution and filter	
During fecal transplantation		Moderate sedation may be used Conduct standard colonoscopy to terminal ileum Deliver suspended stool sample through colonoscope working channel
After fecal transplantation		Administer antidiarrheal medication Avoid bowel movements for up to 4 hours Bedrest for remainder of the day Standard post-procedure dietary instructions

Table 2.1: Process of fecal transplantation via colonoscope.

Disease	# Patients	Mode of Delivery	Success Rate	Citation
Recurrent *C. difficile* colitis	18	Nasogastric tube	83.30%	Aas et al. (2003)
Chronic *C. difficile* infection	24	Colonoscope	83.30%	Borody et al (2003)
Recurrent *C. difficile* associated diarrhea	5	Fecal enema	100%	Jorup-Ronstrom et al. (2006)
Recurrent *C. difficile* infection	16	Colonoscope	93.80%	Wettstein et al. (2007)
Recurrent *C. difficile* infection	45	Rectal catheter	95.60%	Louie et al. (2008)
Fulminant *C. difficile* infection	1	Fecal enema	100%	You et al. (2008)
Recurrent *C. difficile* infection	7	Jejunal infusion	100%	Niewdorp et al. (2008)
C. difficile associated diarrhea	1	Colonoscope	100%	Hellemans et al. (2009)
Recurrent *C. difficile* associated diarrhea	15	Nasogastric tube	86.70%	MacConnachie et al. (2009)
Recurrent *C. difficile* infection	37	Colonoscope	92%	Arkkila et al. (2010)
Recurrent *C. difficile* associated diarrhea	1	Colonoscope	100%	Khoruts et al. (2010)
Recurrent *C. difficile* associated diarrhea	12	Colonoscope	100%	Yoon et al. (2010)
Recurrent *C. difficile* infection	19	Colonoscope	94.70%	Rohlke et al. (2010)
Recurrent *C. difficile* infection	7	fecal enema	100%	Silverman et al. (2010)
Recurrent *C. difficile* associated diarrhea	40	Duodenal instillation	82.50%	Garborg et al. (2010)
Recurrent *C. difficile* infection	1	Nasogastric tube	100%	Russell et al. (2010)
Recurrent *C. difficile* infection	12	Colonoscope	100%	Kelly et al. (2010)
Recurrent *C. difficile* infection	13	Colonoscope	92.30%	Mellow et al. (2010)
C. difficile associated diarrhea	14	Fecal enema	100%	Kassam et al. (2010)
Relapsing *C. difficile* infection	26	Colonoscope	92.30%	Kelly et al. (2011)
Recurrent *C. difficile* infection	70	Colonoscope	94.20%	Mattila et al. (2012)

Table 2.2: Success rate of fecal transplantations for the treatment of *C. difficile* infections.

	Exclude individuals who:
Donor Screening	Received antibiotics in the past 3 months
	Participate in high-risk sexual behaviors
	Use illicite drugs
	Have had a tattoo or piercing within 6 months
	Have recently been incarcerated
	Have traveled to countries where endemic diarrhea is prevalent
	Have a history of GI disorders
Donor stool screening	**Exclude individuals who's samples test positive for:**
	Ova and parasites
	Bacterial cultures including, but not limited to:
	Salmonella, Shigella, Escherichia coli, O157:H7, *Yersinia enterocolitica, Campylobacter*
	Clostridium difficile toxin A and B
Donor serum screening	**Exclude individuals who's serum tests positive for:**
	HIV-1 and HIV-2
	Hepatitis A, B, and C

Table 2.3: Basic guidelines for fecal transplant donor screening.

CHAPTER THREE

EFFECTIVE USE OF MICROBIAL ORAL INOCULATION TO MODULATE THE GASTROINTESTINAL AND RESPIRATORY MICROBIOMES

3.1 INTRODUCTION

The mammalian gastrointestinal tract is estimated to be home to over 1,000 microbial species with a collective genome that provides important biological and metabolic functions [4]. The composition of an individual's GI microbiome is dependent on early environmental exposure, hygiene, diet and many other factors [2], [6–8]. Colonization starts at birth with exposure to bacteria from the mother's vaginal and GI tracts. The GI microbiome is an important aspect of our body, with alterations in its composition being associated with many health issues, including allergic and autoimmune diseases. Studies have shown the route of delivery to be important in the establishment of the GI microbiome, and that caesarean derived infants have increased risk of developing allergies and asthma [30], [31].

Humans and their microbial communities form mutualistic relationships that include the sharing of nutrients, pathogen colonization resistance, regulation of fat storage and maturation of the host immune system. Studies in mice have shown differences in disease development and immune status associated with germ-free mice, as well as varying GI microbiome compositions, including decreased bacterial diversity associated with gastrointestinal and systemic diseases [77], [98]. In addition, many of these studies have shown the beneficial effects of introducing microbes to germ-free animals on immune responses and disease progression [70], [71].

Similar results have been seen in humans using probiotics and fecal transplants. Human trials using probiotics have shown reductions in intestinal disease symptoms such as IBD [87], while fecal transplants have successfully treated *Clostridium difficile* infections, with patients remaining disease free for years after a single treatment [18–20]. In addition, patients who received fecal transplants for intestinal disorders have reported improvements in non-intestinal

diseases, including multiple sclerosis and idiopathic thrombocytopenic purpura [20]. These results have led to the testable hypothesis that the GI microbiome can be modulated by fecal transplantation, and that this modulation can have profound effects on both gastrointestinal and systemic immune responses.

This study demonstrates the use of GI microbial modulation as a therapeutic tool to improve systemic immune responses by altering the gastrointestinal and respiratory microbiome composition. Briefly, a litter of pigs was removed from their mother immediately following birth and raised in controlled research units until weaning (28 days old), after which the pigs were randomly assigned to 2 groups. One group was inoculated (modulated) with the GI microbiota from a healthy adult boar for seven consecutive days, while the other was not (control). Following inoculation, the effects on GI and respiratory microbial community composition were determined (Figure 3.1).

3.2: RESULTS

3.2.1 MICROBIAL DIVERSITY

The dataset consisted of fecal samples, nasal swabs, bronchial swabs and lung lavages from all the pigs, in addition to the oral inoculum samples and a vaginal swab from the gilt. The dataset contained a total of 319,026 sequences represented by 9,954 operational taxonomic units (OTUs) after removal of low quality reads (Table 3.1). Taxonomic analysis revealed a total of 17 phyla, 27 classes, 47 orders, 111 families and 364 genera contained in the dataset (Table 3.1). The total number of sequences and OTUs in the GI and respiratory microbiome samples was also determined (Table 3.1). The dominant phyla in the dataset were Firmicutes (45.31%) and Bacteroidetes (39.10%; Table 3.2), while the dominant genera were *prevotellaceae* (12.30%), *oscillibacter* (7.70%) and *barnesiella* (6.72%; Table 3.3).

Shannon's diversity index and Chao1 estimates were used to calculate diversity and richness of the microbial community samples, respectively (Table 3.4). Before the oral inoculation was performed the average Shannon index was 2.14 and 2.32 for the GI microbiome (feces) of the modulated and control group, respectively. After the oral inoculation, Chao1 estimates suggested a total increase in richness of 656 OTUs in the

modulated GI samples and Shannon's index also revealed an increase in diversity (3.14 and 2.19, p=0.012; Table 3.4) compared to the control group, respectively. No significant difference was seen in the respiratory samples for the modulated and control group following oral inoculation, with Chao1 estimates suggesting no increased richness in the modulated group and Shannon's indices also revealing no increase in diversity compared to the control group for both upper (p=0.7735) and lower respiratory samples (p=0.4555; Table 3.4). The oral inoculum samples had an average Shannon's diversity index of 3.56.

3.2.2 OTU Analysis of the Gastrointestinal Microbiome Samples

Due to the immediate removal of the piglets from the gilt at birth, as well as the use of antibiotics during the first 4 weeks of life, only 0.70% of the OTUs in the modulated, and 0.55% of the OTUs in the control group GI samples at 27 days of age were found to be shared with the sow vaginal swab sample. 48.24% of OTUs were shared between the GI microbiomes of the modulated and control groups for all time points before GI modulation, and 42.43% for all time points after GI modulation. Successful modulation of the GI microbiome is evidenced by a significant increase (p=0.023) in the number of OTUs present in the modulated group (445.83%) one day after oral inoculation (40 days of age) compared to the control group (20.98%). The modulated GI samples were found to share significantly more OTUs with the oral inoculum samples (13.06%) compared to the control GI samples (7.99%) after modulation (p=0.0003). No difference in the number of OTUs shared with the oral inoculum samples was seen between the modulated (1.69%) and control group (1.36%) before modulation (p=0.159). ANOSIM results and MDS plots of the GI microbiome samples revealed no significant difference in GI microbial composition between modulated and control groups before oral inoculation (ANOSIM R=0.056, p=0.26; Figure 3.2a). After 7 consecutive days of exposure to the oral inoculum, a statistically significant difference in the composition of the GI microbiomes was observed between the two groups (ANOSIM R=0.82, p=0.002; Figure 3.2b). This difference in GI microbiome composition was observed for the remainder of the study (Figure 3.2c & 3.2d). Analysis of the similarity between GI microbial samples within groups revealed no difference in similarity or variation before oral inoculation, and significantly less similarity and variation in the modulated group compared to the control group one day after oral inoculation (p=0.0063 & p=0.0038,

respectively; Figure 3.3a & 3.3b). Significant differences in the within group similarity and variation in similarity were observed for all time points following oral inoculation (p=0.0024 & p<0.0001, respectively; Figure 3.3c).

3.2.3 Taxonomic Analysis of the Gastrointestinal Microbiome Samples

The relative taxonomic abundance of the GI microbiome samples at the phylum and genus level were determined (Table 3.2 and Table 3.3, respectively). Results from the taxonomic analysis show no significant difference in the phylogenetic distribution of the two groups 1 day before oral inoculation (32 days of age), with Bacteroidetes (49.3% and 45%) and Firmicutes (37.2% and 45%) representing the dominant phyla in the modulated and control group, respectively (Figure 3.4a). One day after the completion of the oral inoculation (40 days of age), statistically significant differences in the relative abundance of both Bacteroidetes (44% and 58.5%, p=0.0043) and Firmicutes (47.3% and 34.8%, p=0.0036) phyla were visible between the modulated and control groups, respectively (Figure 3.4b). No significant differences were seen for any other phyla. A significant difference in the relative abundance of the Firmicutes phylum (47.9% and 55.9%, p=0.0489) was still seen 1 day before euthanasia (103 days of age), as well as a significant difference in the relative abundance of Synergistetes (0.25% and 1.12%, p=0.021) between the modulated and control groups, respectively (Figure 3.4c). A significant difference in the relative abundance of the Bacteroidetes phylum was no longer apparent at euthanasia, with a relative abundance of 36.6% and 35.6% in the modulated and control group, respectively.

Analysis at the genus level shows a significant difference in the relative abundance of only one genus (parasegetibacter, p=0.026) 1 day before oral inoculation (32 days of age), with a relative abundance of 0.06% and 1.2% in the modulated and control group, respectively (Figure 3.4d). One day after oral inoculation (40 days of age) Barnesiella (2.17% and 38%, p=0.0006), Prevotella (16.18% and 4.41%, p=0.0035), Oscillibacter (4.45% and 2.79%, p=0.0364), Robinsoniella (3.96% and 0.35%, p=0.0026), Coprococcus (3.75% and 0.86%, p=0.007), Anaerotruncus (2.8% and 1.2%, p=0.0438), Bacteroides (2.36% and 0.99%, p=0.0252), Anaerostipes (1.22% and 0.04%, p=0.0064), Roseburia (1.03% and 0.42%, p=0.0301) and Parasegetibacter (0.12% and 1.42%, p=0.0091) were all found to have significantly different

relative abundances in the modulated and control group, respectively (Figure 3.4e). Significant differences in the relative abundance of Barnesiella (6.7%% and 1.28%%, *p*=0.02) and Roseburia (1.45% and 0.49%, *p*=0.0043) were still seen 1 day before euthanasia (103 days of age), as well as significant difference in the relative abundance of Thermovirga (6.40% and 1.09%, *p*=0.0123), Blautia (2.54% and 5.22%, *p*=0.0225) and Dorea (1.15% and 4.75%, *p*=0.0234) between the modulated and control groups, respectively (Figure 3.4f).

3.2.4 OTU Analysis of the Respiratory Microbiome Samples

0.70% of the OTUs in the modulated, and 0.56% of the OTUs in the control group upper respiratory samples taken throughout the study were found to be shared with the sow vaginal swab sample. 47.47% of OTUs were shared between the upper respiratory microbiomes of the modulated and control groups for all time points. 14.92% of OTUs were shared between the modulated upper respiratory and the oral inoculum samples, and 15.06% were shared between the control upper respiratory and oral inoculum samples, revealing no significant difference between the two groups (*p*=0.8885). ANOSIM results and MDS plots of the upper respiratory microbiome samples revealed a statistically significant difference in the composition of the upper respiratory microbiomes between the two groups for all time points after oral inoculation (Figure 3.5). Cluster analysis of all the upper respiratory samples taken after oral inoculation showed clustering of the samples by group (Figure 3.6). No respiratory microbiome samples were available for analysis prior to the oral inoculation. Lower respiratory tract samples showed no significant difference in microbial composition between the two groups (ANOSIM R=-0.029, *p*=0.724; Figure 3.7). Analysis of the similarity between respiratory microbial samples within groups revealed the modulated group tended to be more similar (*p*=0.063) and significantly less variable (*p*=0.0006) compared to the control group for all time points following oral inoculation (Figure 3.3d).

3.2.5 Taxonomic Analysis of the Respiratory Microbiome Samples

The relative taxonomic abundance of the respiratory microbiome samples at the phylum and genus level were determined (Table 3.5 and Table 3.6, respectively). Results from the taxonomic analysis show Bacteroidetes and Firmicutes as the dominant phyla in both the

modulated and control group's upper respiratory microbiome samples for all available time points (69 to 91 days of age), with Bacteroidetes representing an average of 46.4% and 38.3% (p=0.001) and Firmicutes representing an average of 44.9% and 51.8% (p=0.002) in the modulated and control group after oral inoculation, respectively. No respiratory samples were available before oral inoculation. Significant differences in the relative abundance of 4 phyla and 19 genera were seen between the upper respiratory samples of the two groups for all time points (Table 3.7). No significant differences in the relative abundance of any phyla or genera were detected between the two groups in the lower respiratory microbiome samples (data not shown).

3.3: DISCUSSION

Results from this study show successful modulation of the GI microbiome via oral inoculation. These results are evident by the increased diversity and richness in the modulated group after oral inoculation, as well as clustering of the microbiome samples by group. Previous studies have shown delivery via caesarian results in infants being colonized by bacteria similar to those found on the skin surface rather than natural GI tract colonizers [28]. Based on these results piglets in this study were born naturally as opposed to caesarean derived to ensure the piglet's GI tract was colonized by microbes that would naturally colonize the neonatal gut, as opposed to skin derived microbes. Piglets were caught at birth using nitrile gloves and removed from the mother immediately to prevent contact with maternal feces and limit the vertical transfer of microbes. Analysis of the piglet microbiome samples and the gilt vaginal swab sample revealed that a minimal number of OTUs represented in the piglet microbiome samples originated from the sow. These results are not unexpected and likely due to the use of a medicated milk replacer, as well as antibiotic treatment during the first 4 weeks of the experiment. It is important to note that none of the piglets received maternal antibodies or immune molecules, as they were not allowed to suckle prior to being removed from their mother.

Taxonomic abundances of the modulated and control group 1 day before oral inoculation (32 days of age) were comparable to normal pigs, with Firmicutes and Bacteroidetes

representing the dominant phyla. Only the relative abundance of the low abundance *parasegetibacter* genus was significantly different between the two groups before oral inoculation. Significant differences in the relative abundance of 2 phyla and 10 genera in the GI microbiome samples of the two groups were observed one day after oral inoculation. Chao1 estimates, Shannon's diversity index and the number of OTUs present in the GI microbiome samples were all increased in the modulated group compared to the control group after oral inoculation. MDS and ANOSIM analysis shows the GI microbial communities are more similar within groups than between following oral inoculation. These results, in addition to the increase in the number of OTUs shared between the modulated group GI microbiome and the oral inoculum samples compared to the control group confirm the successful modulation of the GI microbial community.

It was thought that because the same oral inoculum was administered to all the pigs in the modulated group, their within group similarity would be greater. Surprisingly, within group similarity was significantly lower in the modulated group than the control group following oral inoculation. Despite the lowered similarity, the variation in the similarity within the modulated group was significantly reduced following oral inoculation. This significantly lower variation in within group similarity was also seen in the respiratory samples of the modulated group.

Despite no difference in richness or diversity between the upper respiratory samples of the two groups, significant differences in the relative abundance of 4 phyla and 19 genera were observed for all time points following oral inoculation, with Bacteroidetes and Firmicutes representing the dominant phyla in both groups. MDS and ANOSIM analysis shows the upper respiratory microbial communities are more similar within groups than between following oral inoculation. No samples were available for analysis prior to oral inoculation. Similar numbers of shared OTUs between the oral inoculum and upper respiratory microbiome samples of both groups (p=0.8885) show that the GI modulation did not directly cause modulation in the respiratory tract, and these differences were therefore an indirect result of the altered GI microbial communities. Overall, this study shows the successful modulation of the GI microbiome and the subsequent effects on the respiratory microbiome.

3.4: Materials and Methods

3.4.1 Ethics Statement

This study was approved by the Institutional Animal Care and Use Committee (IACUC) and the Institutional Biosafety Committee of the University of Illinois at Urbana-Champaign (protocol # 09141 and 09146). All animals were cared for following the guidelines of the IACUC and the Institutional Biosafety Committee of the University of Illinois at Urbana-Champaign, and all efforts were made to minimize suffering throughout the study.

3.4.2 Experimental Design

A litter of pigs (12) was removed from their mother immediately following birth in order to prevent exposure to the maternal GI microbiota. The pigs were raised in controlled research units and fed medicated milk replacer until weaning (28 days old) when the pigs were randomly assigned to 2 groups based on weight and gender. At 33 days of age one of the groups was inoculated (modulated) with the GI microbiota from a healthy adult boar for seven consecutive days, while the other was not (control). Nasal swabs and fecal samples were collected throughout the study and sequenced to determine the effects of the oral inoculation on GI and respiratory microbial communities.

3.4.3 Source of Animals and Housing

A pregnant gilt obtained from a high health herd was housed under commercial conditions and then transferred to a research facility 3 weeks before farrowing. The gilt was induced with 3 doses of 10 mg dinoprost tromethamine (Lutalyse®, Pfizer Inc., New York, NY, USA) intramuscularly at 12 hour intervals starting at day 113 of gestation. Plastic was placed under the gilt and piglets were caught at birth using nitrile gloves to prevent contact with fecal matter. The piglets were removed immediately following birth and raised in controlled research units in order to prevent exposure to GI colonizing microbiota. Research suites were equipped with HEPA filters and the ventilation system was individualized for each room. Biosecurity measures were followed at all times to avoid cross-contamination between experimental groups.

3.4.4 Feeding Protocol

To avoid vertical transfer of porcine immune molecules, piglets were removed from gilts before suckling and syringe fed 20-25 mL of previously frozen bovine colostrum, obtained from the University of Illinois Dairy Farm, every 2 hours for the first 48 hours of life. The colostrum tested negative for *M.* hyopneumoniae antibodies. Piglets were then switched to Advance Liqui-Wean Medicated Pig Milk Replacer® (Oxytetracycline and Neo-Terramycin) (MSC, Carpentersville, IL, USA), which was pumped into bowls every 60 min at a rate of 360 mL/kg/day. Both antibiotic and colostrum were used in order to ensure the piglets health, as previous attempts by our group to artificially raise piglets without colostrum or antibiotic treament resulted in severe *E. coli* infections and gastrointestinal clinical signs. In order to prevent respiratory infection and gastrointestinal clinical signs, Baytril (enrofloxacin) was injected subcutaneously into the ear in 100mg/30lbs body weight doses 24 hours after birth. To prevent gastrointestinal infection, neomycin sulfate was administered orally at a rate of 10 mg/lb body weight every day for the first 2 weeks of life. At 10 days of age the piglets were introduced to phase I dry feed and were eventually weaned off the milk replacer over a 2-day period once they reached an average weaning weight of 6 kg (28 days of age). Piglets were kept on the dry feed *ad libitum* for the remainder of the study.

3.4.5 Collection, Preparation and Delivery of Oral Inoculum

Fresh feces was collected daily from a single boar from a high health herd (*M. hyopneumoniae,* PRRSv, *Pasteurella multocida* and *Bordetella bronchiseptica* free) for use as an oral inoculum. The farm has clinical and historical data backing up their high health status. Depopulation and repopulation had occurred just months before the samples were taken. The boar was showing no clinical signs of infection at the time of collection, and flotation tests done by the University of Illinois Veterinary Diagnostic lab were negative for GI parasites. Samples were immediately mixed 1:1 with sterile phosphate buffered saline (PBS) and fed by syringe to the 33 day old piglets (modulated) at a rate of 2 mL/kg, as previously described [107]. This process was repeated for 7 consecutive days in order to ensure GI colonization.

3.4.6 Collection of Sow Vaginal Swab

Sow vaginal swab was collected two days before birth by introducing a sterile BD CultureSwab® (Becton Dickinson and companies, Franklin Lakes, NJ, USA) into the vagina of the gilt and rotating it clockwise and counter clockwise, and *stored at -80^{o}C.*

3.4.7 Collection of Fecal Samples

Fecal samples were collected daily from the piglets starting one week after birth and continuing up until euthanasia. Samples were collected in Whirl-pak sample bags (Nasco, Fort Atkinson, WI, USA) individually for each pig and stored at -20 *^{o}C.*

3.4.8 Collection of Nasal and Bronchial Swabs

Nasal swabs were collected at 0, 7, 9, 12, 14, and 21 days post infection (dpi) by introducing approximately 4 mm of a sterile BD CultureSwab® (Becton Dickinson and companies, Franklin Lakes, NJ, USA) into each pig nostril and rotating it clockwise and counter clockwise. Bronchial swabs were collected at euthanasia using the same type of sterile swabs used for nasal swabbing and rotating them in the bronchia instead of the nostrils. All swabs were stored at -80^{o}C.

3.4.9 Collection of BALF

Bronchoalveolar lavage fluids (BALF) were collected at euthanasia. Cytokine levels associated with *M. hyopneumoniae* infection were analyzed in the BALF. Sterile PBS (20 mL) was introduced into the bronchoalveolar space, massaged through the lungs and re-collected in 50 mL conical tubes. BALF was stored at -80^{o}C.

3.4.10 DNA Extraction

DNA from fecal and sow vaginal samples were extracted using the QIAamp DNA Stool Mini Kit (Qiagen, Valencia, CA, USA), and DNA from nasal swabs, bronchial swabs, and BALF were extracted using the DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA, USA).

3.4.11 Sequencing

DNA extracted from nasal swabs, lung lavage, bronchial swabs, vaginal swabs and fecal samples was subject to 454 pyrosequencing of the V1-V3 region of the 16S rRNA gene. PCR

primers flanking the V1-V3 hypervariable region of the bacterial 16S rRNA gene were designed for amplification. The oligonucleotide primers were HPLC-purified and included an A or B sequencing adapter at the 5' end and template specific sequences at the 3' end. Barcodes were located between the A sequencing adapter and the template specific sequence of the forward primer. The primer sequences were: 5' CCATCTCATCCCTGCGTGTCTCCGACTCAG – BARCODE – AGAGTTTGATCCTGGCTCAG 3' (forward) and 5' CCTATCCCCTGTGTGCCTTGGCAGTCTCAG – ATTACCGCGGCTGCTGG 3' (reverse). The PCR amplification mixture contained 1.25 units HotStarTaq Plus DNA Polymerase (Qiagen, Valencia, CA, USA), 2.5 µl 10X PCR Buffer (Qiagen, Valencia, CA, USA), 0.2 mM dNTPs, 0.4 µM forward and reverse primer and 5-20 ng DNA in a reaction volume of 25 µl. The PCR conditions were an initial denaturation at *95* ^{o}C for 15 minutes, followed by 35 cycles of 95 ^{o}C for 15 seconds, 65 ^{o}C for 45 seconds, 72 ^{o}C for 1 minute, and a final 10 minute elongation at 72 ^{o}C. Samples were run on a 1.5% agarose gel to verify product amplification. The PCR products were cleaned up using the Agencourt AMPure XP beads kit (Beckman Coulter, Inc., Brea, CA, USA). The PCR products were pooled into groups of 15 in equal concentration ratios based on the quantification results using the NanoDrop 1000. The pooled PCR amplicons were sequenced using 454 FLX-Titanium technology at the W.M. Keck Center for Comparative and Functional Genomics (University of Illinois, Urbana, IL).

3.4.12 DATA ANALYSIS

Data was evaluated using the Students *t* test or Kruskall-Wallis one-way analysis of variance, were appropriate. Following sequencing, 16S rRNA gene reads were assessed for quality. Sequences shorter than 200 nucleotides, with homopolymers longer than 6 nucleotides, containing ambiguous base calls, or with an average quality score <30 were removed. Sequences were aligned against the silva database [108]. Potentially chimeric sequences were detected using mothur's [109] implementation of UCHIME [110] and removed. The remaining reads were pre-clustered as previously described [111] and then clustered using ModalClust (https://bitbucket.org/msipos/modalclust). OTUs were defined as sharing $\geq$ 97 % sequence complete-linkage identity with the most abundant sequence forming the OTU seed. OTUs detected in less than three samples and fewer than three times were removed as possible artifacts. The relationships among the samples was compared using Bray-Curtis dissimilarity

statistics following normalization of the data to their total read depth (i.e. the proportional representation of each OTU) and transformation of this data by square root to reduce the influence of higher abundant over less abundant OTUs. The total number of bacteria in each sample was not reported, as it is our position that relative abundance is far more informative. This view is supported by previous microbiome studies in which total number of bacteria are not reported [11], [15–17], [112–117]. Shannon's diversity indices were performed in R using the Vegan package [118]. Bias-corrected Chao1 richness estimates were obtained in mothur [109] using methods described previously[119]. Resemblance matrices and non-metric multidimensional scaling (MDS) plots were constructed using this data and visualized in Primer6 [120]. Boxplots were constructed and analysis of the resemblances was done using SAS software, Version 9 of the SAS System for Windows (SAS Institute Inc., Cary, NC, USA). Taxonomic profiles were generated for all reads using the RDPclassifier v2.4 [121] with a cutoff of 0.7. Detection of differentially abundant taxonomic groups was done using Metastats [122].

3.5: CONCLUSIONS

From these results we conclude that a non-pathogenic oral inoculum successfully modulated the GI microbial community and had an indirect regulatory effect on the respiratory microbial community. This hypothesis is supported by the increased diversity and richness in the modulated group after oral inoculation, as well as clustering of the microbiome samples by group. These results, in addition to studies using probiotics and fecal transplants, provide further evidence for the ability of live microbial inoculums to alter GI microbiome composition. Furthermore, similar levels of diversity and richness in the respiratory microbiome samples of the two groups, as well as similar numbers of shared OTUs between the oral inoculum and respiratory samples support the hypothesis that differences in respiratory microbiome composition are an indirect effect of the oral inoculation. This is significant, as it shows the composition of the GI microbiome affects the composition of microbiome communities outside the GI tract. This is proposed to be due to modulation of the systemic immune system by the GI microbiome, however further studies need to be done to determine how the GI microbiota regulates microbial communities outside the gut.

3.6 FIGURES AND TABLES

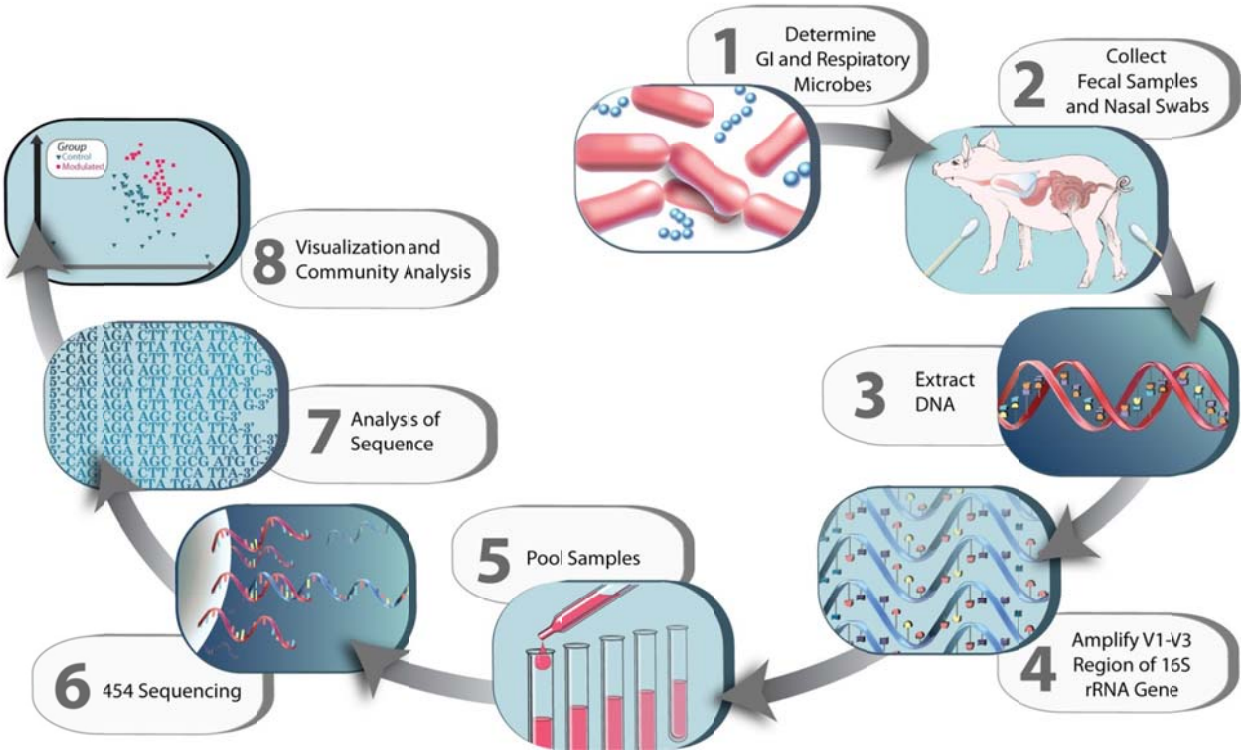

Figure 3.1: Sequencing Design. 1. GI and respiratory microbiomes were determined by sequencing DNA from fecal samples and nasal swabs. 2. Fecal samples and Nasal swabs were collected at multiple time points throughout the experiment and stored at -20^{o}C. 3. DNA was extracted from fecal samples and nasal swabs using the QIAamp DNA Stool Mini Kit (Qiagen, Valencia, CA, USA) and the DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA, USA), respectively. 4. The V1-V3 region of the 16S rRNA gene was amplified using universal primers containg barcodes and pyrosequencing adapters. 5. The PCR products were pooled into groups of 15 in equal concentrations. 6. The pooled PCR amplicons were sequenced using 454 FLX-Titanium technology. 7. Following sequencing, 16S rRNA gene reads were analyzed by removing low quality reads, aligning against the silva database, removing chimeric sequences, clustering using ModalClust, assigned taxonomic profiles using the RDPclassifier, and compared following normalization of the data to their total read depth. 8. The analyzed sequence data was visualized and analyzed for community composition using Primer6, Metastats, SAS and R.

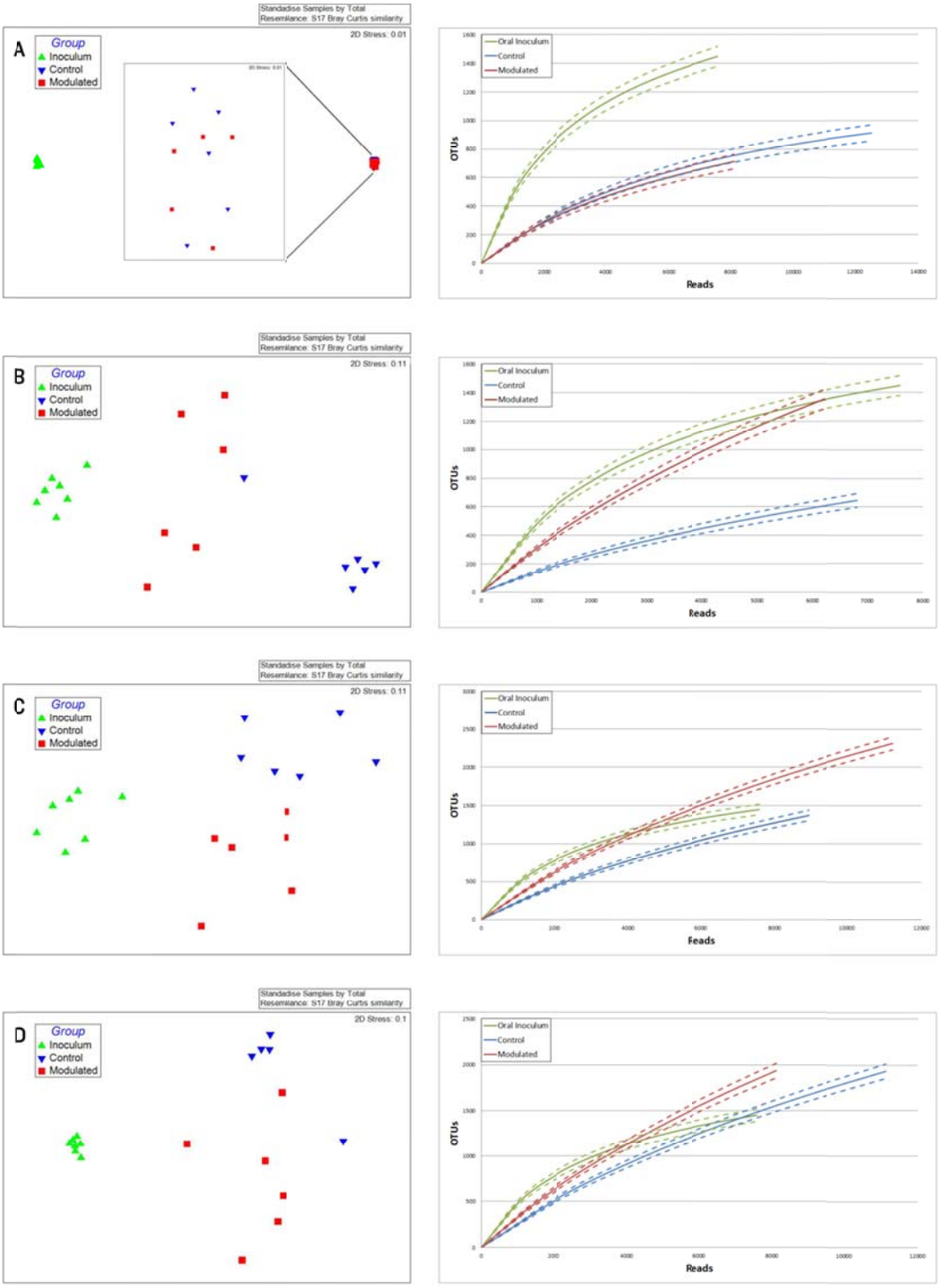

Figure 3.2: MDS plots and rarefaction curves of GI samples. MDS plots and rarefaction curves of GI microbiome samples A) 1 week before the start of oral inoculation (28 days of age; R=0.056, *p*=0.26), B) 1 day after completion of oral inoculation (40 days of age; R=0.82, *p*=0.002), C) at 56 days of age (R=0.502, *p*=0.002) and D) at 70 days of age (R=0.483, *p*=0.015).

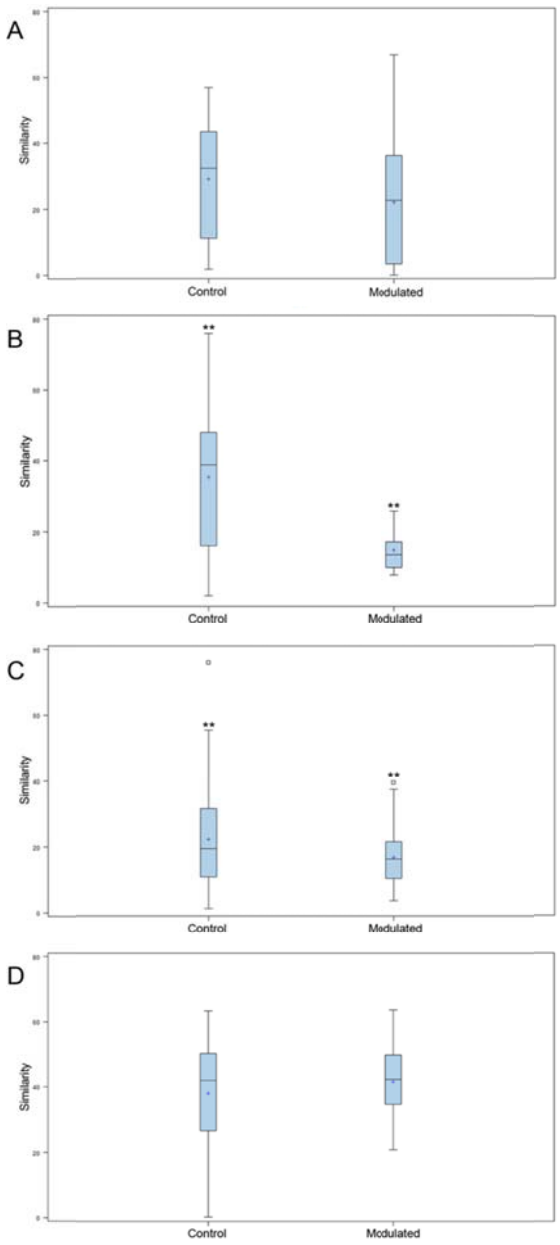

Figure 3.3: Differences in within group similarity for GI and respiratory microbiome samples at multiple time points. Boxplots showing no significant difference for within group similarity of the GI microbial communities A) for multiple time points before oral inoculation, B) significant differences for within group similarity (p=0.0016) and variation (p=0.0038) one day after completion of the oral inoculation (40 days of age) and C) significant differences for within group similarity (p=0.0024) and variation (p<0.0001) for all time points following oral inoculation. D) Boxplots showing differences for within group similarity (p=0.063) and variation (p=0.0006) of the respiratory microbial communities for all time points following oral inoculation. ** denotes statistical significance of p<0.005

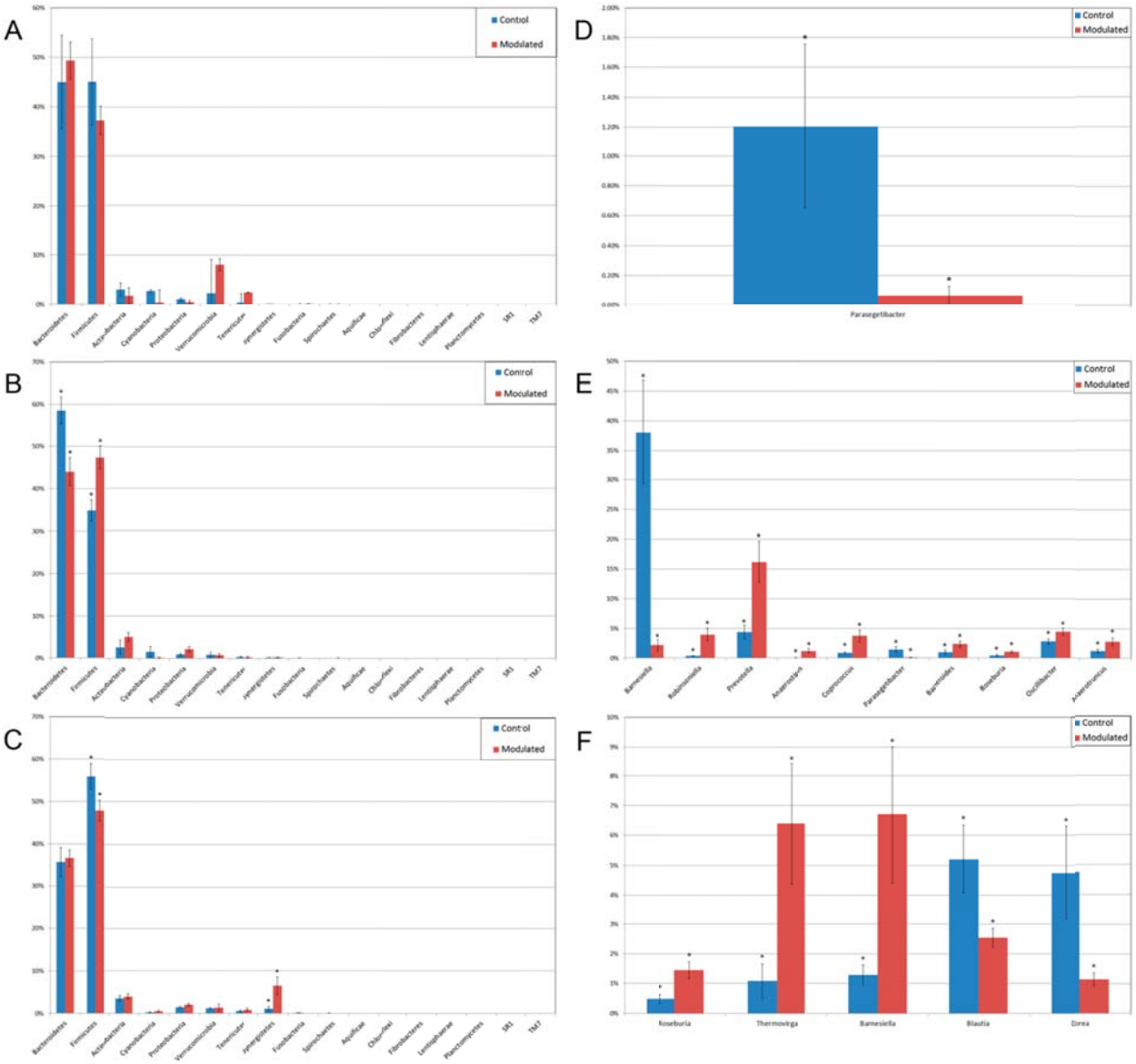

Figure 3.4: Relative taxonomic abundances for GI microbiome samples at the phylum and genus level. The relative abundance of each phylum A) one day before oral inoculation (32 days of age), B) one day after completion of oral inoculation (40 days of age) and C) one day before euthanasia (103 days of age). The relative abundance of each genus showing statistically significant differences between the modulated and control group D) one day before oral inoculation (28 days of age), E) one day after completion of oral inoculation (40 days of age) and F) one day before euthanasia (103 days of age). * denotes statistical significance of $p<0.05$.

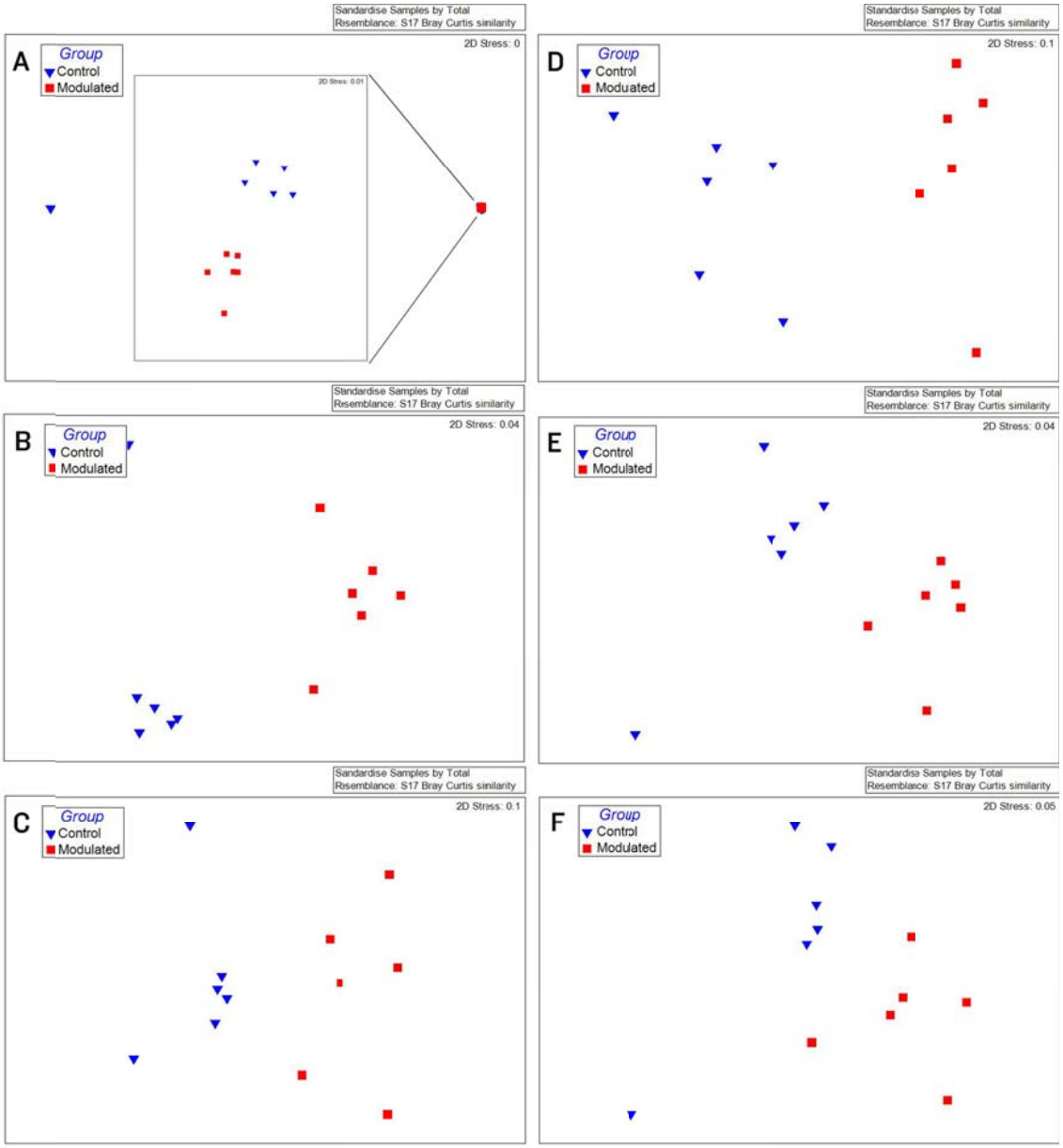

Figure 3.5: MDS plots for upper respiratory microbiome samples. MDS plots of upper respiratory microbiome samples A) the day of *M. hyopneumoniae* infection (69 days of age) (R=0.667, *p*=0.002), B) 7 days after *M. hyopneumoniae* infection (R=0.763, *p*=0.002), C) 9 days after *M. hyopneumoniae* infection (R=0.704, *p*=0.002), D) 12 days after *M. hyopneumoniae* infection (R=0.807, *p*=0.002), E) 14 days after *M. hyopneumoniae* infection (R=0.719, *p*=0.002) and F) 21 days after *M. hyopneumoniae* infection (R=0.576, *p*=0.002).

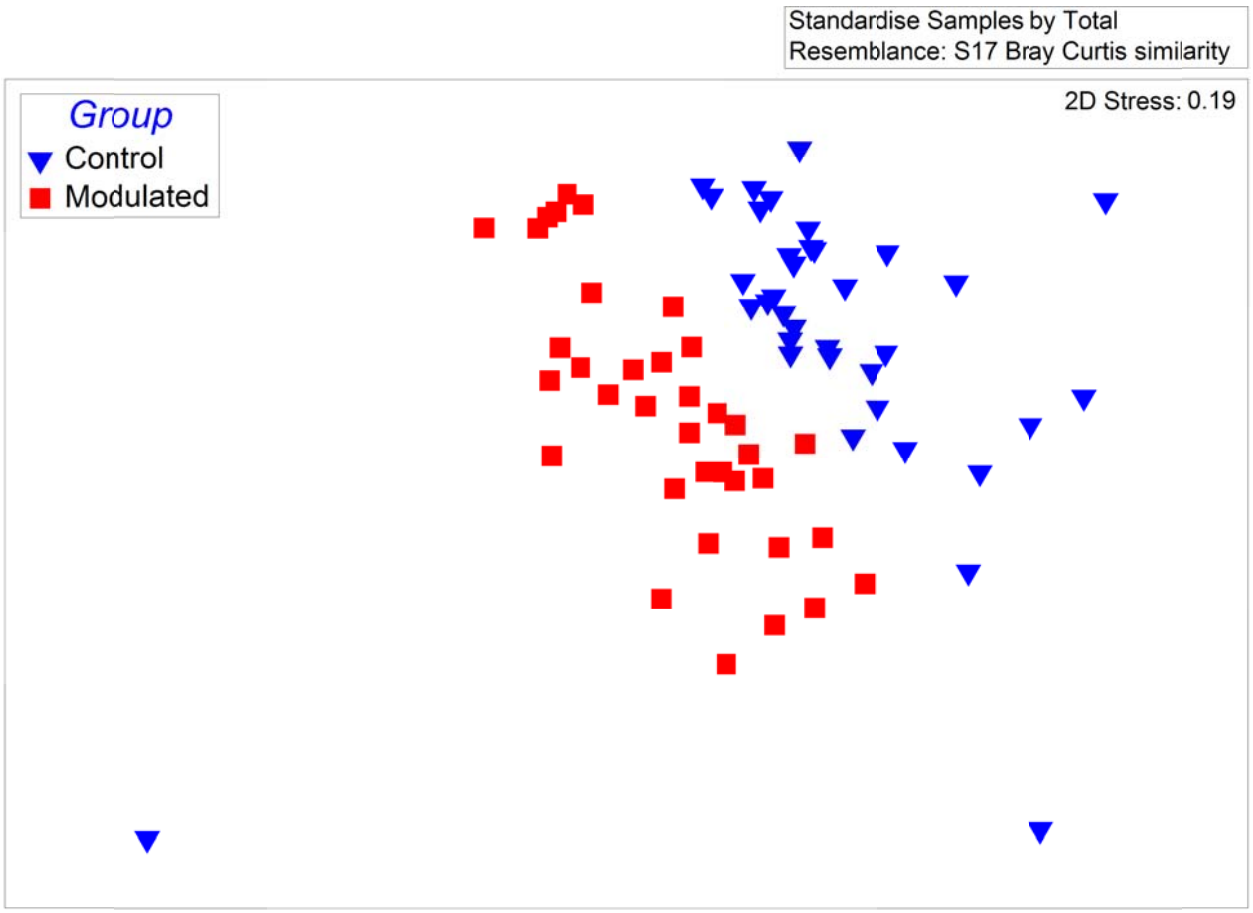

Figure 3.6: MDS plot of all upper respiratory microbiome samples following oral inoculation. MDS plot of all upper respiratory microbiome samples taken at multiple time points throughout the study (R=0.368, p=0.001).

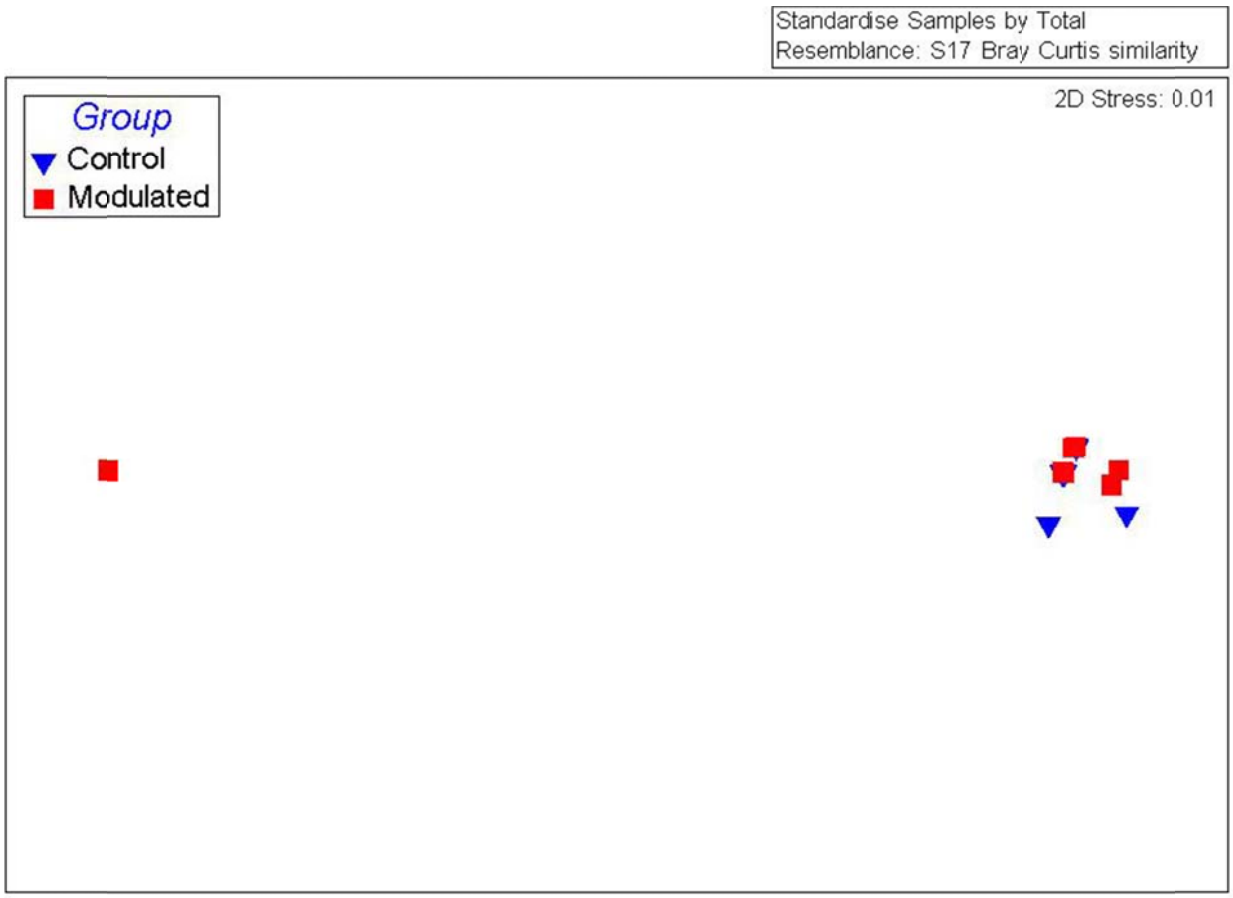

Figure 3.7: MDS plot of lower respiratory microbiome samples. MDS plot of lower respiratory samples (bronchial swabs and lung lavage) collected at euthanasia (103 days of age) show no significant difference in community composition between groups (R=-0.029, *p*=0.724).

	# Sequences	# OTUs	# Phylum	# Class	# Order	# Family	# Genus
Dataset	319026	9954	17	27	47	111	364
Gilt Vaginal Tract	326	72	4	7	7	16	38
Oral Inoculum Samples	7590	1450	11	18	27	59	164
Control GI microbiome before oral inoculation	21235	1384	12	19	32	71	177
Modulated GI microbiome before oral inoculation	10890	893	10	17	27	61	136
Control GI microbiome after oral inoculation	72484	4878	15	25	43	94	271
Modulated GI microbiome after oral inoculation	60874	5059	15	24	43	97	280
Control Upper Respiratory Microbiome	63881	3908	15	25	39	88	248
Modulated Upper Respiratory Microbiome	55409	3862	14	23	37	89	257
Control Lower Respiratory Microbiome	13153	282	10	17	21	38	78
Modulated Lower Respiratory Microbiome	13184	297	9	16	20	39	79

Table 3.1: Sequencing statistics for dataset

		Control		Modulated	
Phylum	Dataset	GI Microbiome Before Oral Inoculation	GI Microbiome After Oral Inoculation	GI Microbiome Before Oral Inoculation	GI Microbiome After Oral Inoculation
Firmicutes	45.31%	38.97%	53.43%	31.89%	47.65%
Bacteroidetes	39.10%	53.60%	37.37%	62.57%	38.59%
Proteobacteria	8.09%	1.33%	1.51%	0.95%	2.62%
Actinobacteria	4.40%	3.85%	3.99%	3.31%	6.08%
Verrucomicrobia	1.08%	1.11%	1.83%	0.86%	1.51%
Synergistetes	0.79%	0.08%	0.73%	0.01%	2.45%
Tenericutes	0.74%	0.34%	0.52%	0.21%	0.56%
Cyanobacteria	0.30%	0.49%	0.40%	0.08%	0.34%
Spirochaetes	0.08%	0.12%	0.06%	0.06%	0.12%
Fusobacteria	0.06%	0.04%	0.13%	0.05%	0.03%
Lentisphaerae	0.02%	0.06%	0.01%	ND	0.02%
SR1	0.01%	ND	< 0.01%	ND	< 0.01%
TM7	0.01%	0.02%	0.01%	ND	0.01%
Planctomycetes	< 0.01%	ND	0.01%	ND	0.01%
Chloroflexi	< 0.01%	ND	ND	ND	< 0.01%
Aquificae	< 0.01%	ND	ND	ND	ND
Fibrobacteres	< 0.01%	ND	< 0.01%	ND	ND

ND – Not Detected

Table 3.2: Relative taxonomic abundance of phyla in the dataset and GI microbiome samples

			Control		Modulated	
Phylum	Genus	Dataset	GI Microbiome Before Oral Inoculation	GI Microbiome After Oral Inoculation	GI Microbiome Before Oral Inoculation	GI Microbiome After Oral Inoculation
Bacteroidetes	*Prevotella*	12.30%	13.75%	10.23%	13.91%	13.49%
Firmicutes	*Oscillibacter*	7.70%	8.74%	11.06%	5.78%	6.52%
Bacteroidetes	*Barnesiella*	6.72%	27.25%	7.62%	34.48%	4.94%
Proteobacteria	*Sandaracinobacter*	6.31%	< 0.01%	0.06%	0.03%	0.17%
Firmicutes	*Blautia*	4.75%	7.04%	5.24%	3.15%	5.26%
Firmicutes	*Coprococcus*	4.70%	1.61%	3.70%	1.07%	4.34%
Firmicutes	*Robinsoniella*	3.35%	2.19%	4.21%	2.41%	4.78%
Bacteroidetes	*Parabacteroides*	3.19%	2.56%	2.74%	2.01%	3.98%
Bacteroidetes	*Tannerella*	3.00%	2.52%	3.71%	2.63%	3.83%
Bacteroidetes	*Bacteroides*	2.50%	1.28%	2.08%	5.70%	3.94%
Bacteroidetes	*Hallella*	2.45%	2.60%	2.03%	1.52%	1.87%
Bacteroidetes	*Rikenella*	2.00%	0.28%	3.29%	0.41%	2.87%
Bacteroidetes	*Pseudosphingobacterium*	1.98%	< 0.01%	0.17%	ND	0.29%
Firmicutes	*Anaerotruncus*	1.79%	1.43%	2.17%	1.31%	2.03%
Actinobacteria	*Olsenella*	1.75%	1.51%	1.22%	1.26%	2.85%
Firmicutes	*Dorea*	1.71%	1.49%	3.58%	0.83%	1.60%
Firmicutes	*Clostridium*	1.64%	1.82%	3.59%	0.60%	1.60%
Firmicutes	*Sharpea*	1.41%	1.05%	1.05%	0.78%	1.93%
Firmicutes	*Faecalibacterium*	1.40%	3.00%	1.60%	0.27%	1.15%
Firmicutes	*Butyricicoccus*	1.38%	1.43%	2.10%	1.50%	1.80%
Firmicutes	*Streptococcus*	1.31%	0.29%	0.78%	0.31%	1.29%
Bacteroidetes	*Paraprevotella*	1.25%	0.60%	0.70%	0.52%	0.66%
Firmicutes	*Roseburia*	1.22%	0.65%	0.94%	0.56%	1.20%
Firmicutes	*Aerococcus*	1.09%	0.59%	0.68%	6.72%	1.69%
Verrucomicrobia	*Akkermansia*	1.07%	1.11%	1.83%	0.86%	1.51%
Firmicutes	*Subdoligranulum*	1.01%	0.54%	1.07%	0.26%	1.02%
Bacteroidetes	*Fluviicola*	0.98%	ND	0.05%	0.01%	0.03%
Firmicutes	*Eubacterium*	0.91%	0.40%	1.46%	0.31%	1.39%
Bacteroidetes	*Alistipes*	0.88%	1.14%	1.37%	0.56%	0.83%
Tenericutes	*Mycoplasma*	0.72%	0.27%	0.48%	0.20%	0.52%
Synergistetes	*Thermovirga*	0.70%	0.08%	0.63%	0.01%	2.34%
Actinobacteria	*Coriobacterium*	0.62%	0.14%	1.14%	0.11%	0.62%
Firmicutes	*Staphylococcus*	0.62%	0.06%	0.72%	ND	0.39%
Firmicutes	*Solobacterium*	0.58%	0.72%	0.35%	0.40%	0.72%
Firmicutes	*Butyrivibrio*	0.55%	0.05%	0.03%	0.02%	0.11%
Firmicutes	*Dialister*	0.52%	0.39%	0.67%	0.20%	0.45%
Firmicutes	*Enterococcus*	0.51%	0.07%	0.22%	0.02%	0.42%
Bacteroidetes	*Xylanibacter*	0.50%	0.34%	0.63%	0.39%	0.66%
Firmicutes	*Mitsuokella*	0.49%	0.64%	0.73%	0.50%	0.42%
Firmicutes	*Lactobacillus*	0.47%	0.21%	0.56%	0.46%	0.92%
Bacteroidetes	*Parasegetibacter*	0.47%	0.49%	1.60%	0.01%	0.29%
Actinobacteria	*Rothia*	0.40%	0.95%	0.29%	0.67%	0.21%
Firmicutes	*Fastidiosipila*	0.35%	ND	0.02%	ND	0.02%
Firmicutes	*Papillibacter*	0.34%	0.48%	0.37%	0.46%	0.36%
Firmicutes	*Syntrophococcus*	0.33%	0.10%	0.45%	0.06%	0.40%
Cyanobacteria	*Streptophyta*	0.29%	0.49%	0.38%	0.08%	0.34%
Firmicutes	*Anaerostipes*	0.28%	0.04%	0.28%	0.11%	0.53%
Proteobacteria	*Succinivibrio*	0.27%	0.02%	0.02%	0.01%	0.38%
Bacteroidetes	*Paludibacter*	0.27%	0.22%	0.50%	0.07%	0.26%
Actinobacteria	*Corynebacterium*	0.27%	0.16%	0.14%	0.51%	0.26%
Firmicutes	*Acetanaerobacterium*	0.25%	0.05%	0.49%	0.05%	0.17%
Actinobacteria	*Collinsella*	0.25%	0.01%	0.10%	0.01%	0.92%
Actinobacteria	*Bifidobacterium*	0.25%	0.18%	0.37%	0.04%	0.20%
Firmicutes	*Sporacetigenium*	0.24%	0.40%	0.32%	0.06%	0.19%
Firmicutes	*Mogibacterium*	0.21%	0.01%	0.34%	0.01%	0.15%
Firmicutes	*Marvinbryantia*	0.21%	0.05%	0.18%	0.15%	0.38%
Actinobacteria	*Eggerthella*	0.21%	0.03%	0.21%	0.01%	0.38%
Firmicutes	*Megasphaera*	0.21%	0.14%	0.46%	0.11%	0.18%
Firmicutes	*Lactonifactor*	0.21%	0.13%	0.22%	0.20%	0.40%
Proteobacteria	*Moraxella*	0.17%	0.07%	0.08%	0.01%	0.10%
Proteobacteria	*Acinetobacter*	0.17%	0.09%	0.10%	0.14%	0.38%
Firmicutes	*Lactovum*	0.16%	< 0.01%	0.32%	ND	0.09%
Firmicutes	*Anaerobacter*	0.16%	0.19%	0.12%	0.22%	0.18%
Proteobacteria	*Escherichia/Shigella*	0.15%	0.05%	0.11%	0.02%	0.21%
Firmicutes	*Pseudobutyrivibrio*	0.15%	0.05%	0.19%	0.06%	0.24%
Firmicutes	*Acidaminococcus*	0.15%	0.03%	0.08%	0.06%	0.19%
Firmicutes	*Turicibacter*	0.15%	0.10%	0.13%	0.11%	0.11%

Table 3.3: Relative taxonomic abundance of genera in the dataset and GI microbiome samples

			Control		Modulated	
Phylum	Genus	Dataset	GI Microbiome Before Oral Inoculation	GI Microbiome After Oral Inoculation	GI Microbiome Before Oral Inoculation	GI Microbiome After Oral Inoculation
Actinobacteria	*Janibacter*	0.14%	ND	0.07%	ND	0.11%
Firmicutes	*Anaerovibrio*	0.14%	0.04%	0.18%	ND	0.11%
Firmicutes	*Ethanoligenens*	0.14%	ND	0.05%	0.01%	0.10%
Firmicutes	*Sarcina*	0.13%	0.02%	0.05%	ND	0.19%
Proteobacteria	*Castellaniella*	0.13%	ND	0.31%	ND	0.26%
Firmicutes	*Lachnobacterium*	0.13%	0.22%	0.08%	0.13%	0.10%
Firmicutes	*Ruminococcus*	0.12%	0.05%	0.17%	0.13%	0.17%
Bacteroidetes	*Butyricimonas*	0.11%	0.08%	0.18%	0.07%	0.19%
Firmicutes	*Pilibacter*	0.11%	0.22%	0.07%	0.34%	0.08%
Firmicutes	*Hydrogenoanaerobacterium*	0.11%	0.19%	0.11%	0.14%	0.11%
Actinobacteria	*Slackia*	0.11%	0.03%	0.03%	0.01%	0.01%
Firmicutes	*Anaerovorax*	0.10%	0.09%	0.22%	0.05%	0.11%
Firmicutes	*Acetitomaculum*	0.10%	0.13%	0.15%	0.06%	0.07%
Firmicutes	*Coprobacillus*	0.09%	0.26%	0.08%	0.62%	0.15%
Firmicutes	*Anaerosporobacter*	0.09%	0.03%	0.10%	0.04%	0.11%
Proteobacteria	*Stenotrophomonas*	0.08%	0.44%	0.18%	0.41%	ND
Firmicutes	*Paralactobacillus*	0.08%	0.03%	0.11%	ND	0.10%
Proteobacteria	*Comamonas*	0.08%	ND	0.09%	ND	0.22%
Firmicutes	*Sporobacter*	0.08%	0.07%	0.11%	0.07%	0.11%
Actinobacteria	*Microbacterium*	0.08%	0.21%	0.10%	0.13%	0.12%
Firmicutes	*Bulleidia*	0.08%	0.03%	0.07%	0.01%	0.03%
Proteobacteria	*Diaphorobacter*	0.07%	ND	ND	ND	ND
Firmicutes	*Lactococcus*	0.07%	0.07%	0.06%	0.07%	0.04%
Spirochaetes	*Treponema*	0.07%	0.11%	0.05%	0.06%	0.12%
Firmicutes	*Allobaculum*	0.06%	0.07%	0.03%	0.01%	0.03%
Synergistetes	*Pyramidobacter*	0.06%	0.01%	0.06%	ND	0.04%
Firmicutes	*Acetivibrio*	0.06%	0.01%	0.02%	0.01%	0.12%
Actinobacteria	*Millisia*	0.05%	0.42%	0.05%	0.39%	< 0.01%
Firmicutes	*Anaerofilum*	0.05%	< 0.01%	0.08%	ND	0.02%
Bacteroidetes	*Chryseobacterium*	0.05%	0.01%	0.08%	ND	0.05%
Firmicutes	*Parasporobacterium*	0.05%	0.02%	0.10%	0.08%	0.03%
Firmicutes	*Catonella*	0.05%	0.04%	0.08%	0.02%	0.06%
Proteobacteria	*Citrobacter*	0.05%	ND	0.02%	ND	0.17%
Bacteroidetes	*Anaerophaga*	0.05%	0.02%	0.01%	0.02%	0.04%
Bacteroidetes	*Empedobacter*	0.05%	< 0.01%	0.05%	ND	0.05%
Bacteroidetes	*Sphingobacterium*	0.05%	0.07%	0.04%	0.15%	0.02%
Firmicutes	*Hespellia*	0.05%	0.10%	0.02%	0.04%	0.10%
Proteobacteria	*Pseudochrobactrum*	0.04%	0.02%	0.03%	0.02%	0.01%
Firmicutes	*Holdemania*	0.04%	0.02%	0.06%	0.07%	0.08%
Firmicutes	*Phascolarctobacterium*	0.04%	ND	0.05%	0.01%	0.08%
Proteobacteria	*Arcobacter*	0.04%	0.01%	0.02%	ND	< 0.01%
Firmicutes	*Sporobacterium*	0.04%	< 0.01%	< 0.01%	0.11%	0.03%
Firmicutes	*Schwartzia*	0.04%	0.04%	0.07%	0.05%	0.03%
Firmicutes	*Bavariicoccus*	0.04%	0.04%	0.14%	0.08%	< 0.01%
Bacteroidetes	*Cloacibacterium*	0.04%	0.04%	0.05%	0.04%	0.02%
Firmicutes	*Vagococcus*	0.04%	0.04%	0.01%	0.01%	0.01%
Firmicutes	*Peptostreptococcus*	0.03%	0.04%	0.05%	ND	0.02%
Firmicutes	*Mahella*	0.03%	0.01%	0.03%	0.04%	0.05%
Firmicutes	*Tepidimicrobium*	0.03%	< 0.01%	0.07%	ND	0.03%
Fusobacteria	*Fusobacterium*	0.03%	0.03%	0.09%	0.03%	0.02%
Bacteroidetes	*Riemerella*	0.03%	ND	0.01%	ND	0.01%
Proteobacteria	*Pseudomonas*	0.03%	0.02%	0.03%	0.04%	0.01%
Firmicutes	*Oxobacter*	0.03%	0.21%	< 0.01%	ND	0.02%
Proteobacteria	*Novosphingobium*	0.03%	0.30%	0.03%	0.08%	< 0.01%
Actinobacteria	*Paraeggerthella*	0.03%	0.01%	0.06%	0.03%	< 0.01%
Firmicutes	*Succinispira*	0.03%	0.01%	0.02%	0.01%	0.08%
Proteobacteria	*Brevundimonas*	0.03%	ND	0.03%	ND	0.06%
Actinobacteria	*Smaragdicoccus*	0.03%	ND	0.01%	ND	0.13%
Actinobacteria	*Leucobacter*	0.03%	ND	0.03%	ND	0.02%
Bacteroidetes	*Flavobacterium*	0.03%	0.19%	0.02%	ND	< 0.01%
Firmicutes	*Anoxynatronum*	0.03%	0.01%	0.05%	ND	0.08%
Bacteroidetes	*Petrimonas*	0.03%	ND	0.05%	ND	0.08%
Bacteroidetes	*Myroides*	0.03%	0.02%	< 0.01%	ND	0.01%
Firmicutes	*Pseudoramibacter*	0.03%	ND	0.01%	ND	0.05%
Firmicutes	*Globicatella*	0.03%	0.02%	0.02%	0.04%	0.04%
Actinobacteria	*Dietzia*	0.03%	ND	0.01%	ND	0.02%
Proteobacteria	*Pasteurella*	0.02%	ND	0.02%	ND	0.02%

Table 3.3: (cont.)

			Control		Modulated	
Phylum	Genus	Dataset	GI Microbiome Before Oral Inoculation	GI Microbiome After Oral Inoculation	GI Microbiome Before Oral Inoculation	GI Microbiome After Oral Inoculation
Actinobacteria	*Propionibacterium*	0.02%	ND	0.04%	ND	0.01%
Firmicutes	*Tepidibacter*	0.02%	ND	0.02%	ND	ND
Firmicutes	*Howardella*	0.02%	< 0.01%	0.02%	ND	0.01%
Proteobacteria	*Paracoccus*	0.02%	< 0.01%	0.01%	ND	0.01%
Actinobacteria	*Kocuria*	0.02%	ND	0.02%	ND	0.02%
Firmicutes	*Anaeroglobus*	0.02%	0.02%	0.03%	ND	0.02%
Bacteroidetes	*Aquiflexum*	0.02%	ND	ND	ND	ND
Fusobacteria	*Cetobacterium*	0.02%	< 0.01%	0.02%	0.02%	0.01%
Firmicutes	*Catellicoccus*	0.02%	0.03%	0.01%	0.01%	0.03%
Firmicutes	*Succiniclasticum*	0.02%	0.17%	0.01%	0.13%	0.01%
Proteobacteria	*Alkanindiges*	0.02%	0.01%	0.04%	ND	0.04%
Proteobacteria	*Enterobacter*	0.02%	< 0.01%	< 0.01%	ND	0.07%
Firmicutes	*Acetobacterium*	0.02%	0.04%	0.02%	0.03%	0.01%
Firmicutes	*Selenomonas*	0.02%	ND	0.03%	ND	0.02%
Proteobacteria	*Wohlfahrtiimonas*	0.02%	0.01%	0.02%	0.01%	ND
Actinobacteria	*Turicella*	0.02%	0.05%	0.03%	ND	0.01%
Actinobacteria	*Actinomyces*	0.02%	0.11%	< 0.01%	0.06%	< 0.01%
Actinobacteria	*Gordonibacter*	0.02%	ND	< 0.01%	ND	0.06%
Proteobacteria	*Enhydrobacter*	0.02%	0.01%	0.02%	0.02%	0.03%
Firmicutes	*Melissococcus*	0.02%	ND	0.01%	ND	0.02%
Synergistetes	*Synergistes*	0.02%	ND	0.03%	ND	0.04%
SR1	*SR1_genera_incertae_sedis*	0.01%	ND	< 0.01%	ND	< 0.01%
Firmicutes	*Oribacterium*	0.01%	< 0.01%	0.01%	ND	0.01%
Firmicutes	*Leuconostoc*	0.01%	ND	0.02%	ND	0.01%
Lentisphaerae	*Victivallis*	0.01%	0.06%	0.01%	ND	< 0.01%
Proteobacteria	*Sphingomonas*	0.01%	ND	0.04%	ND	0.01%
Proteobacteria	*Catellibacterium*	0.01%	0.04%	0.02%	0.03%	0.02%
Firmicutes	*Parvimonas*	0.01%	ND	< 0.01%	ND	0.01%
Firmicutes	*Symbiobacterium*	0.01%	0.01%	< 0.01%	0.17%	< 0.01%
Firmicutes	*Abiotrophia*	0.01%	ND	0.02%	ND	0.03%
Proteobacteria	*Helicobacter*	0.01%	ND	< 0.01%	ND	0.05%
Bacteroidetes	*Phocaeicola*	0.01%	0.03%	< 0.01%	0.01%	0.01%
Firmicutes	*Garciella*	0.01%	ND	ND	ND	0.04%
Firmicutes	*Peptococcus*	0.01%	ND	0.01%	ND	0.01%
Proteobacteria	*Caenispirillum*	0.01%	ND	ND	ND	ND
Firmicutes	*Shuttleworthia*	0.01%	ND	0.02%	ND	0.02%
Firmicutes	*Catenibacterium*	0.01%	< 0.01%	0.03%	ND	0.01%
Tenericutes	*Asteroleplasma*	0.01%	0.04%	0.01%	0.01%	0.01%
Proteobacteria	*Bibersteinia*	0.01%	ND	0.01%	ND	< 0.01%
Bacteroidetes	*Kordia*	0.01%	ND	0.01%	ND	0.03%
Proteobacteria	*Bradyrhizobium*	0.01%	ND	ND	ND	ND
Proteobacteria	*Actinobacillus*	0.01%	ND	< 0.01%	ND	0.05%
Actinobacteria	*Adlercreutzia*	0.01%	ND	0.01%	ND	0.01%
Tenericutes	*Anaeroplasma*	0.01%	0.01%	0.02%	ND	< 0.01%
Proteobacteria	*Tatumella*	0.01%	ND	< 0.01%	ND	0.02%
Proteobacteria	*Klebsiella*	0.01%	ND	< 0.01%	ND	0.04%
Firmicutes	*Eremococcus*	0.01%	ND	0.02%	ND	0.02%
Proteobacteria	*Delftia*	0.01%	ND	ND	ND	0.05%
Proteobacteria	*Pannonibacter*	0.01%	ND	< 0.01%	ND	< 0.01%
Spirochaetes	*Spirochaeta*	0.01%	0.01%	0.01%	ND	< 0.01%
Bacteroidetes	*Lacibacter*	0.01%	0.01%	0.03%	ND	0.01%
Firmicutes	*Bacillus*	0.01%	ND	0.02%	ND	0.02%
Proteobacteria	*Nicoletella*	0.01%	ND	0.01%	ND	0.01%
Firmicutes	*Anaerofustis*	0.01%	ND	0.01%	ND	0.01%
Firmicutes	*Helcococcus*	0.01%	ND	< 0.01%	ND	0.02%
Firmicutes	*Veillonella*	0.01%	0.01%	0.01%	ND	ND
Bacteroidetes	*Odoribacter*	0.01%	ND	0.03%	ND	0.01%
Bacteroidetes	*Dysgonomonas*	0.01%	ND	ND	ND	< 0.01%
Bacteroidetes	*Thermonema*	0.01%	ND	< 0.01%	ND	< 0.01%
Proteobacteria	*Defluvibacter*	0.01%	ND	< 0.01%	ND	< 0.01%
Proteobacteria	*Sutterella*	0.01%	ND	0.01%	ND	0.02%
Firmicutes	*Dethiosulfatibacter*	0.01%	0.01%	< 0.01%	ND	< 0.01%
Actinobacteria	*Devriesea*	0.01%	< 0.01%	< 0.01%	0.01%	< 0.01%
Bacteroidetes	*Pseudozobellia*	0.01%	0.01%	0.01%	ND	0.01%
Proteobacteria	*Sphingopyxis*	0.01%	ND	ND	ND	ND
Synergistetes	*Jonquetella*	0.01%	ND	ND	ND	< 0.01%
Proteobacteria	*Mycoplana*	0.01%	ND	0.01%	ND	ND

Table 3.3: (cont.)

			Control		Modulated	
Phylum	Genus	Dataset	GI Microbiome Before Oral Inoculation	GI Microbiome After Oral Inoculation	GI Microbiome Before Oral Inoculation	GI Microbiome After Oral Inoculation
TM7	*TM7_genera_incertae_sedis*	0.01%	0.02%	0.01%	ND	0.01%
Synergistetes	*Cloacibacillus*	0.01%	ND	0.01%	ND	0.02%
Firmicutes	*Finegoldia*	0.01%	ND	ND	ND	0.02%
Proteobacteria	*Anaeromyxobacter*	0.01%	ND	0.03%	ND	< 0.01%
Tenericutes	*Haloplasma*	0.01%	0.01%	< 0.01%	ND	0.02%
Actinobacteria	*Tessaracoccus*	0.01%	ND	0.01%	ND	ND
Proteobacteria	*Ralstonia*	0.01%	0.03%	ND	0.01%	< 0.01%
Bacteroidetes	*Wautersiella*	0.01%	0.01%	< 0.01%	0.01%	0.02%
Proteobacteria	*Alicycliphilus*	0.01%	0.03%	< 0.01%	ND	< 0.01%
Firmicutes	*Gracilibacter*	0.01%	0.04%	< 0.01%	0.01%	0.01%
Proteobacteria	*Craurococcus*	0.01%	0.05%	0.01%	0.02%	0.01%
Proteobacteria	*Sulfuricurvum*	0.01%	ND	ND	ND	ND
Firmicutes	*Dolosigranulum*	0.01%	0.02%	ND	0.04%	ND
Proteobacteria	*Proteus*	0.01%	ND	ND	ND	0.03%
Firmicutes	*Jeotgalicoccus*	0.01%	ND	0.01%	ND	0.02%
Firmicutes	*Dendrosporobacter*	0.01%	ND	ND	ND	0.01%
Bacteroidetes	*Prolixibacter*	0.01%	ND	ND	ND	ND
Firmicutes	*Desulfonispora*	0.01%	0.08%	ND	0.02%	< 0.01%
Actinobacteria	*Oryzihumus*	0.01%	ND	0.01%	ND	0.01%
Firmicutes	*Weissella*	0.01%	0.05%	< 0.01%	0.06%	ND
Actinobacteria	*Serinibacter*	0.01%	0.02%	ND	ND	0.02%
Actinobacteria	*Myceligenerans*	0.01%	ND	ND	ND	0.03%
Actinobacteria	*Enterorhabdus*	0.01%	ND	0.01%	ND	0.01%
Bacteroidetes	*Olivibacter*	0.01%	ND	< 0.01%	ND	0.01%
Bacteroidetes	*Actibacter*	0.01%	< 0.01%	< 0.01%	ND	0.02%
Bacteroidetes	*Owenweeksia*	0.01%	ND	0.02%	ND	< 0.01%
Proteobacteria	*Oxalobacter*	0.01%	ND	ND	ND	< 0.01%
Actinobacteria	*Asaccharobacter*	0.01%	ND	0.01%	ND	0.01%
Bacteroidetes	*Joostella*	< 0.01%	ND	ND	ND	ND
Firmicutes	*Acidaminobacter*	< 0.01%	ND	ND	ND	ND
Firmicutes	*Anaerococcus*	< 0.01%	ND	ND	ND	ND
Bacteroidetes	*Dyadobacter*	< 0.01%	ND	ND	ND	ND
Proteobacteria	*Roseicyclus*	< 0.01%	ND	ND	ND	ND
Actinobacteria	*Brachybacterium*	< 0.01%	ND	ND	ND	ND
Proteobacteria	*Magnetospirillum*	< 0.01%	ND	ND	ND	ND
Firmicutes	*Desemzia*	< 0.01%	ND	ND	ND	ND
Firmicutes	*Macrococcus*	< 0.01%	ND	ND	ND	ND
Proteobacteria	*Luteimonas*	< 0.01%	ND	0.02%	ND	ND
Proteobacteria	*Ochrobactrum*	< 0.01%	ND	0.02%	ND	ND
Firmicutes	*Filibacter*	< 0.01%	ND	0.01%	ND	ND
Fusobacteria	*Leptotrichia*	< 0.01%	ND	0.01%	ND	ND
Bacteroidetes	*Porphyromonas*	< 0.01%	< 0.01%	0.01%	ND	ND
Firmicutes	*Fructobacillus*	< 0.01%	ND	0.01%	ND	ND
Proteobacteria	*Rhodoferax*	< 0.01%	ND	0.01%	ND	ND
Firmicutes	*Anaerovirgula*	< 0.01%	ND	0.01%	ND	ND
Firmicutes	*Nosocomiicoccus*	< 0.01%	0.01%	0.01%	ND	ND
Bacteroidetes	*Proteiniphilum*	< 0.01%	ND	0.01%	ND	ND
Firmicutes	*Cerasibacillus*	< 0.01%	ND	0.01%	ND	ND
Proteobacteria	*Asticcacaulis*	< 0.01%	ND	0.01%	ND	ND
Proteobacteria	*Zhangella*	< 0.01%	ND	< 0.01%	ND	ND
Proteobacteria	*Massilia*	< 0.01%	ND	< 0.01%	ND	ND
Bacteroidetes	*Chitinophaga*	< 0.01%	< 0.01%	< 0.01%	ND	ND
Firmicutes	*Pullulanibacillus*	< 0.01%	0.02%	< 0.01%	ND	ND
Proteobacteria	*Arsenophonus*	< 0.01%	< 0.01%	ND	0.06%	ND
Actinobacteria	*Varibaculum*	< 0.01%	ND	ND	0.05%	ND
Firmicutes	*Tissierella*	< 0.01%	0.03%	ND	0.04%	ND
Actinobacteria	*Actinobaculum*	< 0.01%	< 0.01%	ND	0.04%	ND
Bacteroidetes	*Perexilibacter*	< 0.01%	< 0.01%	ND	0.02%	ND
Proteobacteria	*Rhizobium*	< 0.01%	0.01%	ND	0.01%	ND
Proteobacteria	*Lebetimonas*	< 0.01%	0.01%	ND	0.01%	ND
Bacteroidetes	*Limibacter*	< 0.01%	ND	ND	ND	0.02%
Firmicutes	*Gallicola*	< 0.01%	ND	ND	ND	0.02%
Proteobacteria	*Mesorhizobium*	< 0.01%	ND	ND	ND	0.01%
Proteobacteria	*Ignatzschineria*	< 0.01%	ND	ND	ND	0.01%
Firmicutes	*Erysipelothrix*	< 0.01%	ND	ND	ND	0.01%
Planctomycetes	*Singulisphaera*	< 0.01%	ND	0.01%	ND	0.01%
Actinobacteria	*Zimmermannella*	< 0.01%	ND	ND	ND	0.01%

Table 3.3: (cont.)

			Control		Modulated	
Phylum	Genus	Dataset	GI Microbiome Before Oral Inoculation	GI Microbiome After Oral Inoculation	GI Microbiome Before Oral Inoculation	GI Microbiome After Oral Inoculation
Firmicutes	***Alloiococcus***	< 0.01%	ND	ND	ND	ND
Actinobacteria	***Phycicola***	< 0.01%	ND	ND	ND	ND
Proteobacteria	***Desulfovibrio***	< 0.01%	ND	< 0.01%	ND	0.01%
Proteobacteria	***Aeromonas***	< 0.01%	ND	ND	ND	< 0.01%
Proteobacteria	***Psychrobacter***	< 0.01%	ND	< 0.01%	ND	ND
Bacteroidetes	***Terrimonas***	< 0.01%	0.02%	< 0.01%	0.01%	ND
Lentisphaerae	***Lentisphaera***	< 0.01%	ND	< 0.01%	ND	0.01%
Proteobacteria	***Wolinella***	< 0.01%	ND	< 0.01%	ND	0.01%
Cyanobacteria	***Bacillariophyta***	< 0.01%	ND	< 0.01%	ND	< 0.01%
Proteobacteria	***Haemophilus***	< 0.01%	0.02%	< 0.01%	0.02%	< 0.01%
Bacteroidetes	***Zunongwangia***	< 0.01%	ND	ND	ND	< 0.01%
Proteobacteria	***Altererythrobacter***	< 0.01%	ND	0.01%	ND	< 0.01%
Proteobacteria	***Lonepinella***	< 0.01%	ND	0.01%	ND	< 0.01%
Cyanobacteria	***Chlorarachniophyceae***	< 0.01%	ND	0.01%	ND	< 0.01%
Proteobacteria	***Alysiella***	< 0.01%	ND	0.01%	ND	< 0.01%
Actinobacteria	***Intrasporangium***	< 0.01%	ND	ND	ND	ND
Proteobacteria	***Ahrensia***	< 0.01%	ND	ND	ND	ND
Firmicutes	***Thalassobacillus***	< 0.01%	ND	ND	ND	ND
Bacteroidetes	***Elizabethkingia***	< 0.01%	ND	ND	ND	< 0.01%
Bacteroidetes	***Nubsella***	< 0.01%	ND	0.01%	ND	< 0.01%
Bacteroidetes	***Sporocytophaga***	< 0.01%	ND	0.01%	ND	< 0.01%
Actinobacteria	***Actinotalea***	< 0.01%	ND	ND	ND	ND
Proteobacteria	***Phocoenobacter***	< 0.01%	ND	ND	ND	ND
Actinobacteria	***Brevibacterium***	< 0.01%	ND	ND	ND	< 0.01%
Proteobacteria	***Daeguia***	< 0.01%	ND	ND	ND	0.01%
Firmicutes	***Atopobacter***	< 0.01%	ND	0.01%	ND	0.01%
Actinobacteria	***Zhihengliuella***	< 0.01%	ND	< 0.01%	ND	< 0.01%
Actinobacteria	***Xylanimicrobium***	< 0.01%	ND	ND	ND	ND
Firmicutes	***Centipeda***	< 0.01%	ND	ND	ND	ND

ND – Not Detected

Table 3.3: (cont.)

	Control GI	Modulated GI	Control Upper Respiratory	Modulated Upper Respiratory	Control Lower Respiratory	Modulated Lower Respiratory
	Shannon's Diversity Index		**Shannon's Diversity Index**		**Shannon's Diversity Index**	
Before Inoculation	2.32	2.14	NA	NA	NA	NA
After Inoculation	2.91	3.14*	3.13	3.15	1.47	1.49
	Chao1 Estimate		**Chao1 Estimate**		**Chao1 Estimate**	
Before Inoculation	1545	1389.6	NA	NA	NA	NA
After Inoculation	1635.6	2291.7	2541.3	2309.6	394.4	339.7

*Denotes statistical significance compared to control $p<0.05$

Table 3.4: Shannon's diversity index and chao1 estimates for GI and respiratory microbiome samples.

Phylum	Control Upper Respiratory	Modulated Upper Respiratory	Control Lower Respiratory	Modulated Lower Respiratory
Firmicutes	50.24%	45.57%	14.81%	14.97%
Bacteroidetes	39.83%	45.45%	6.36%	8.75%
Actinobacteria	5.00%	3.97%	1.63%	2.05%
Proteobacteria	2.31%	2.52%	76.48%	73.32%
Tenericutes	1.19%	1.08%	0.35%	0.54%
Verrucomicrobia	0.82%	0.45%	0.04%	0.05%
Cyanobacteria	0.26%	0.15%	0.27%	0.26%
Synergistetes	0.16%	0.61%	0.01%	ND
Spirochaetes	0.08%	0.06%	0.04%	0.03%
Fusobacteria	0.06%	0.05%	ND	ND
SR1	0.03%	0.04%	0.02%	0.04%
Lentisphaerae	0.02%	0.02%	ND	ND
TM7	0.01%	0.01%	ND	ND
Aquificae	0.01%	ND	ND	ND
Fibrobacteres	< 0.01%	ND	ND	ND
Planctomycetes	ND	ND	ND	ND
Chloroflexi	ND	0.02%	ND	ND

Table 3.5: Relative taxonomic abundance of phyla in the respiratory microbiome samples

Phylum	Genus	Control Respiratory	Modulated Respiratory	Control Lower Respiratory	Modulated Lower Respiratory
Bacteroidetes	***Prevotella***	13.37%	15.16%	2.87%	3.87%
Firmicutes	***Coprococcus***	7.63%	6.60%	1.89%	1.60%
Firmicutes	***Oscillibacter***	7.40%	7.29%	2.72%	2.87%
Firmicutes	***Blautia***	5.27%	4.32%	0.68%	0.93%
Bacteroidetes	***Pseudosphingobacterium***	4.42%	5.68%	0.14%	0.11%
Bacteroidetes	***Parabacteroides***	3.97%	3.44%	0.58%	1.40%
Bacteroidetes	***Hallella***	3.60%	3.46%	0.15%	0.25%
Bacteroidetes	***Barnesiella***	3.09%	1.85%	1.08%	0.87%
Firmicutes	***Robinsoniella***	2.82%	3.29%	0.26%	0.30%
Firmicutes	***Streptococcus***	2.80%	1.50%	0.17%	0.37%
Bacteroidetes	***Bacteroides***	2.25%	2.51%	0.27%	0.40%
Bacteroidetes	***Fluviicola***	2.21%	3.00%	0.03%	0.02%
Firmicutes	***Anaerotruncus***	2.17%	1.17%	0.97%	0.96%
Bacteroidetes	***Tannerella***	2.09%	3.24%	0.11%	0.12%
Firmicutes	***Roseburia***	2.00%	1.57%	0.30%	0.29%
Bacteroidetes	***Paraprevotella***	1.65%	2.76%	0.54%	1.01%
Firmicutes	***Sharpea***	1.57%	1.92%	0.23%	0.30%
Actinobacteria	***Olsenella***	1.43%	1.99%	1.13%	1.45%
Firmicutes	***Faecalibacterium***	1.42%	1.65%	0.02%	0.08%
Firmicutes	***Butyrivibrio***	1.31%	1.47%	ND	0.01%
Firmicutes	***Dorea***	1.27%	0.91%	0.19%	0.20%
Firmicutes	***Fastidiosipila***	1.20%	0.57%	0.02%	0.01%
Tenericutes	***Mycoplasma***	1.16%	1.06%	0.35%	0.54%
Firmicutes	***Subdoligranulum***	1.04%	0.92%	1.44%	1.28%
Bacteroidetes	***Rikenella***	0.98%	2.49%	0.11%	0.11%
Firmicutes	***Solobacterium***	0.94%	0.41%	0.30%	0.46%
Firmicutes	***Butyricicoccus***	0.91%	1.05%	0.17%	0.19%
Firmicutes	***Staphylococcus***	0.82%	0.90%	0.12%	0.12%
Bacteroidetes	***Alistipes***	0.82%	0.63%	0.15%	0.17%
Verrucomicrobia	***Akkermansia***	0.82%	0.43%	0.04%	0.05%
Firmicutes	***Clostridium***	0.80%	0.82%	0.84%	0.63%
Proteobacteria	***Sandaracinobacter***	0.79%	0.31%	74.93%	71.72%
Actinobacteria	***Coriobacterium***	0.71%	0.38%	ND	0.02%
Actinobacteria	***Rothia***	0.66%	0.14%	0.30%	0.40%
Firmicutes	***Enterococcus***	0.63%	1.40%	ND	0.01%
Firmicutes	***Eubacterium***	0.59%	0.69%	0.13%	0.14%
Firmicutes	***Dialister***	0.58%	0.46%	0.21%	0.27%
Actinobacteria	***Corynebacterium***	0.51%	0.30%	0.01%	0.01%
Firmicutes	***Mitsuokella***	0.46%	0.49%	ND	ND
Firmicutes	***Aerococcus***	0.45%	1.35%	0.02%	0.02%
Bacteroidetes	***Xylanibacter***	0.43%	0.49%	0.04%	0.11%
Firmicutes	***Syntrophococcus***	0.41%	0.18%	ND	ND
Firmicutes	***Lactobacillus***	0.41%	0.16%	0.11%	0.08%
Firmicutes	***Mogibacterium***	0.40%	0.12%	0.01%	0.04%
Actinobacteria	***Slackia***	0.38%	0.09%	0.01%	ND
Firmicutes	***Sporacetigenium***	0.36%	0.13%	0.02%	0.02%
Actinobacteria	***Janibacter***	0.35%	0.09%	ND	0.02%
Firmicutes	***Ethanoligenens***	0.31%	0.22%	0.06%	ND
Proteobacteria	***Succinivibrio***	0.31%	0.72%	0.01%	ND
Firmicutes	***Acetanaerobacterium***	0.26%	0.10%	0.30%	0.30%
Firmicutes	***Anaerobacter***	0.26%	0.11%	ND	ND
Cyanobacteria	***Streptophyta***	0.26%	0.14%	0.27%	0.26%
Actinobacteria	***Bifidobacterium***	0.25%	0.32%	ND	ND
Firmicutes	***Acidaminococcus***	0.24%	0.10%	0.27%	0.23%
Bacteroidetes	***Paludibacter***	0.23%	0.23%	ND	ND
Proteobacteria	***Escherichia/Shigella***	0.22%	0.19%	0.02%	0.03%
Firmicutes	***Anaerovibrio***	0.19%	0.16%	0.01%	ND
Firmicutes	***Lachnobacterium***	0.19%	0.12%	0.01%	ND
Firmicutes	***Papillibacter***	0.19%	0.40%	0.28%	0.21%
Firmicutes	***Anaerostipes***	0.18%	0.19%	0.20%	0.36%
Firmicutes	***Bulleidia***	0.18%	0.11%	ND	ND
Actinobacteria	***Eggerthella***	0.17%	0.21%	0.13%	0.11%
Firmicutes	***Lactonifactor***	0.17%	0.14%	ND	ND
Proteobacteria	***Diaphorobacter***	0.17%	0.23%	ND	ND
Firmicutes	***Acetitomaculum***	0.15%	0.08%	ND	ND
Firmicutes	***Marvinbryantia***	0.13%	0.11%	0.36%	0.35%
Firmicutes	***Turicibacter***	0.13%	0.17%	0.31%	0.36%

Table 3.6: Relative taxonomic abundance of genera in the respiratory microbiome samples

Phylum	Genus	Control Respiratory	Modulated Respiratory	Control Lower Respiratory	Modulated Lower Respiratory
Actinobacteria	***Collinsella***	0.12%	0.14%	0.05%	0.02%
Proteobacteria	***Pseudochrobactrum***	0.11%	0.04%	ND	ND
Firmicutes	***Lactovum***	0.11%	0.05%	0.47%	0.40%
Firmicutes	***Paralactobacillus***	0.10%	0.04%	ND	ND
Firmicutes	***Vagococcus***	0.10%	0.05%	ND	ND
Firmicutes	***Anaerovorax***	0.10%	0.02%	ND	ND
Firmicutes	***Pseudobutyrivibrio***	0.09%	0.11%	ND	ND
Synergistetes	***Pyramidobacter***	0.09%	0.11%	ND	ND
Bacteroidetes	***Aquiflexum***	0.09%	0.02%	ND	ND
Firmicutes	***Sporobacter***	0.09%	0.02%	ND	ND
Firmicutes	***Allobaculum***	0.08%	0.16%	ND	ND
Firmicutes	***Tepidibacter***	0.08%	0.01%	ND	ND
Proteobacteria	***Acinetobacter***	0.08%	0.19%	0.05%	0.13%
Firmicutes	***Ruminococcus***	0.08%	0.06%	ND	ND
Firmicutes	***Anaerofilum***	0.08%	0.05%	ND	ND
Bacteroidetes	***Parasegetibacter***	0.08%	0.02%	ND	ND
Bacteroidetes	***Sphingobacterium***	0.08%	0.04%	ND	ND
Spirochaetes	***Treponema***	0.07%	0.05%	0.04%	0.03%
Bacteroidetes	***Empedobacter***	0.07%	0.06%	ND	ND
Proteobacteria	***Paracoccus***	0.07%	0.02%	ND	ND
Synergistetes	***Thermovirga***	0.07%	0.50%	0.01%	ND
Firmicutes	***Lactococcus***	0.07%	0.06%	0.22%	0.20%
Actinobacteria	***Dietzia***	0.07%	0.04%	ND	ND
Actinobacteria	***Leucobacter***	0.06%	0.02%	ND	ND
Firmicutes	***Howardella***	0.06%	0.02%	ND	ND
Bacteroidetes	***Butyricimonas***	0.06%	0.05%	ND	ND
Bacteroidetes	***Myroides***	0.06%	0.04%	0.01%	0.02%
Firmicutes	***Parasporobacterium***	0.05%	0.01%	ND	ND
Firmicutes	***Sarcina***	0.05%	0.36%	ND	ND
Firmicutes	***Megasphaera***	0.05%	0.06%	0.38%	0.27%
Firmicutes	***Pilibacter***	0.05%	0.10%	0.15%	0.10%
Bacteroidetes	***Riemerella***	0.05%	0.10%	ND	ND
Proteobacteria	***Pasteurella***	0.05%	0.02%	ND	ND
Actinobacteria	***Kocuria***	0.05%	0.02%	ND	ND
Bacteroidetes	***Anaerophaga***	0.05%	0.03%	0.23%	0.27%
Proteobacteria	***Arcobacter***	0.05%	0.03%	0.31%	0.20%
Bacteroidetes	***Flavobacterium***	0.05%	ND	ND	ND
Actinobacteria	***Paraeggerthella***	0.05%	0.03%	ND	ND
Actinobacteria	***Propionibacterium***	0.05%	0.02%	ND	0.01%
Proteobacteria	***Moraxella***	0.04%	0.13%	1.14%	1.16%
Fusobacteria	***Cetobacterium***	0.04%	0.02%	ND	ND
Firmicutes	***Anaerosporobacter***	0.04%	0.08%	0.02%	0.05%
Firmicutes	***Anaeroglobus***	0.04%	0.01%	ND	ND
Proteobacteria	***Bibersteinia***	0.04%	< 0.01%	ND	ND
Proteobacteria	***Bradyrhizobium***	0.04%	0.01%	ND	ND
Firmicutes	***Melissococcus***	0.04%	< 0.01%	ND	ND
Firmicutes	***Acetivibrio***	0.03%	0.10%	ND	ND
Actinobacteria	***Microbacterium***	0.03%	0.04%	ND	ND
Firmicutes	***Pseudoramibacter***	0.03%	0.03%	ND	ND
Firmicutes	***Hespellia***	0.03%	0.03%	ND	ND
Firmicutes	***Peptococcus***	0.03%	0.01%	ND	ND
Firmicutes	***Catonella***	0.03%	0.05%	ND	ND
Proteobacteria	***Pseudomonas***	0.03%	0.08%	ND	ND
Firmicutes	***Globicatella***	0.03%	0.02%	ND	ND
Firmicutes	***Acetobacterium***	0.03%	0.01%	ND	ND
SR1	***SR1_genera_incertae_sedis***	0.03%	0.04%	0.02%	0.04%
Proteobacteria	***Citrobacter***	0.02%	0.03%	ND	ND
Firmicutes	***Phascolarctobacterium***	0.02%	0.02%	ND	ND
Proteobacteria	***Wohlfahrtiimonas***	0.02%	0.04%	ND	ND
Proteobacteria	***Defluvibacter***	0.02%	0.01%	ND	ND
Bacteroidetes	***Cloacibacterium***	0.02%	0.05%	ND	ND
Firmicutes	***Tepidimicrobium***	0.02%	0.04%	ND	ND
Proteobacteria	***Ralstonia***	0.02%	< 0.01%	ND	ND
Bacteroidetes	***Chryseobacterium***	0.02%	0.01%	ND	ND
Firmicutes	***Sporobacterium***	0.02%	0.02%	0.26%	0.25%
Firmicutes	***Leuconostoc***	0.02%	0.02%	ND	ND
Proteobacteria	***Tatumella***	0.02%	0.01%	ND	ND

Table 3.6: (cont.)

Phylum	Genus	Control Respiratory	Modulated Respiratory	Control Lower Respiratory	Modulated Lower Respiratory
Proteobacteria	*Castellaniella*	0.02%	0.01%	ND	ND
Proteobacteria	*Comamonas*	0.02%	0.06%	0.02%	0.02%
Firmicutes	*Peptostreptococcus*	0.02%	0.08%	ND	ND
Lentisphaerae	*Victivallis*	0.02%	0.02%	ND	ND
Firmicutes	*Parvimonas*	0.02%	0.05%	ND	ND
Fusobacteria	*Fusobacterium*	0.02%	0.01%	ND	ND
Actinobacteria	*Turicella*	0.02%	0.01%	ND	ND
Bacteroidetes	*Thermonema*	0.02%	0.02%	ND	ND
Proteobacteria	*Mycoplana*	0.02%	0.01%	ND	ND
Firmicutes	*Hydrogenoanaerobacterium*	0.02%	0.04%	0.40%	0.33%
Firmicutes	*Mahella*	0.02%	0.07%	ND	ND
Firmicutes	*Selenomonas*	0.02%	0.01%	ND	ND
Bacteroidetes	*Dysgonomonas*	0.02%	0.03%	ND	ND
Firmicutes	*Dethiosulfatibacter*	0.02%	0.01%	ND	ND
Actinobacteria	*Tessaracoccus*	0.02%	0.01%	ND	ND
Firmicutes	*Catellicoccus*	0.01%	0.04%	ND	ND
Proteobacteria	*Pannonibacter*	0.01%	0.03%	ND	ND
Firmicutes	*Schwartzia*	0.01%	0.01%	0.07%	0.08%
Proteobacteria	*Brevundimonas*	0.01%	0.04%	ND	0.04%
Firmicutes	*Symbiobacterium*	0.01%	0.02%	ND	ND
Spirochaetes	*Spirochaeta*	0.01%	0.02%	ND	ND
Firmicutes	*Helcococcus*	0.01%	0.01%	ND	ND
Firmicutes	*Finegoldia*	0.01%	< 0.01%	ND	ND
Firmicutes	*Allisonella*	0.01%	ND	ND	ND
Firmicutes	*Holdemania*	0.01%	0.03%	ND	ND
Tenericutes	*Anaeroplasma*	0.01%	0.01%	ND	ND
Proteobacteria	*Nicoletella*	0.01%	0.02%	ND	ND
Bacteroidetes	*Pseudozobellia*	0.01%	< 0.01%	ND	ND
Firmicutes	*Abiotrophia*	0.01%	< 0.01%	ND	ND
Proteobacteria	*Klebsiella*	0.01%	< 0.01%	ND	ND
Proteobacteria	*Sutterella*	0.01%	0.01%	ND	ND
Actinobacteria	*Oryzihumus*	0.01%	< 0.01%	ND	ND
Actinobacteria	*Sanguibacter*	0.01%	< 0.01%	ND	ND
Firmicutes	*Bavariicoccus*	0.01%	ND	ND	ND
Actinobacteria	*Actinomyces*	0.01%	0.02%	ND	ND
Proteobacteria	*Sphingomonas*	0.01%	0.01%	ND	ND
Tenericutes	*Asteroleplasma*	0.01%	0.01%	ND	ND
Actinobacteria	*Adlercreutzia*	0.01%	0.02%	ND	ND
TM7	*TM7_genera_incertae_sedis*	0.01%	0.01%	ND	ND
Tenericutes	*Haloplasma*	0.01%	ND	ND	ND
Firmicutes	*Dolosigranulum*	0.01%	0.02%	ND	ND
Bacteroidetes	*Prolixibacter*	0.01%	0.01%	ND	ND
Actinobacteria	*Micrococcus*	0.01%	ND	ND	ND
Bacteroidetes	*Sediminicola*	0.01%	ND	ND	ND
Firmicutes	*Geosporobacter*	0.01%	ND	ND	ND
Bacteroidetes	*Filimonas*	0.01%	0.01%	ND	ND
Proteobacteria	*Samsonia*	0.01%	0.01%	ND	ND
Proteobacteria	*Telmatospirillum*	0.01%	< 0.01%	ND	ND
Actinobacteria	*Intrasporangium*	0.01%	< 0.01%	ND	ND
Proteobacteria	*Alkanindiges*	0.01%	< 0.01%	ND	ND
Firmicutes	*Veillonella*	0.01%	ND	0.08%	0.06%
Synergistetes	*Cloacibacillus*	0.01%	0.01%	ND	ND
Firmicutes	*Dendrosporobacter*	0.01%	0.01%	ND	ND
Proteobacteria	*Hylemonella*	0.01%	ND	ND	ND
Actinobacteria	*Ornithinicoccus*	0.01%	ND	ND	ND
Aquificae	*Hydrogenobaculum*	0.01%	ND	ND	ND
Firmicutes	*Isobaculum*	0.01%	ND	ND	ND
Firmicutes	*Anaeroarcus*	0.01%	0.01%	ND	ND
Proteobacteria	*Acidovorax*	0.01%	0.01%	ND	ND
Proteobacteria	*Ahrensia*	0.01%	< 0.01%	ND	ND
Firmicutes	*Coprobacillus*	< 0.01%	0.03%	0.01%	0.04%
Firmicutes	*Oxobacter*	< 0.01%	ND	0.14%	0.16%
Firmicutes	*Succinispira*	< 0.01%	0.02%	ND	ND
Actinobacteria	*Smaragdicoccus*	< 0.01%	< 0.01%	ND	ND
Bacteroidetes	*Petrimonas*	< 0.01%	< 0.01%	ND	ND
Firmicutes	*Succiniclasticum*	< 0.01%	< 0.01%	ND	ND
Firmicutes	*Oribacterium*	< 0.01%	0.05%	ND	ND

Table 3.6: (cont.)

Phylum	Genus	Control Respiratory	Modulated Respiratory	Control Lower Respiratory	Modulated Lower Respiratory
Proteobacteria	*Catellibacterium*	< 0.01%	0.01%	ND	ND
Proteobacteria	*Helicobacter*	< 0.01%	0.01%	ND	ND
Firmicutes	*Shuttleworthia*	< 0.01%	0.01%	ND	ND
Firmicutes	*Catenibacterium*	< 0.01%	< 0.01%	ND	ND
Bacteroidetes	*Kordia*	< 0.01%	0.02%	ND	ND
Firmicutes	*Anaerofustis*	< 0.01%	0.01%	ND	ND
Bacteroidetes	*Wautersiella*	< 0.01%	ND	ND	ND
Proteobacteria	*Alicycliphilus*	< 0.01%	ND	ND	ND
Firmicutes	*Gracilibacter*	< 0.01%	0.01%	ND	ND
Proteobacteria	*Sulfuricurvum*	< 0.01%	0.04%	ND	ND
Proteobacteria	*Proteus*	< 0.01%	0.01%	ND	ND
Firmicutes	*Jeotgalicoccus*	< 0.01%	0.01%	ND	ND
Actinobacteria	*Enterorhabdus*	< 0.01%	0.01%	ND	ND
Bacteroidetes	*Olivibacter*	< 0.01%	ND	0.06%	0.02%
Firmicutes	*Paucisalibacillus*	< 0.01%	ND	ND	ND
Verrucomicrobia	*Spartobacteria_genera_incertae_sedis*	< 0.01%	ND	ND	ND
Firmicutes	*Lutispora*	< 0.01%	ND	ND	ND
Firmicutes	*Solibacillus*	< 0.01%	ND	ND	ND
Fusobacteria	*Psychrilyobacter*	< 0.01%	ND	ND	ND
Proteobacteria	*Gulbenkiania*	< 0.01%	ND	ND	ND
Bacteroidetes	*Epilithonimonas*	< 0.01%	ND	ND	ND
Actinobacteria	*Arthrobacter*	< 0.01%	ND	ND	ND
Fibrobacteres	*Fibrobacter*	< 0.01%	ND	ND	ND
Firmicutes	*Oxalophagus*	< 0.01%	ND	ND	ND
Actinobacteria	*Skermania*	< 0.01%	ND	ND	ND
Proteobacteria	*Desulfocurvus*	< 0.01%	0.01%	ND	ND
Proteobacteria	*Neisseria*	< 0.01%	0.01%	ND	ND
Proteobacteria	*Parasutterella*	< 0.01%	0.01%	ND	ND
Firmicutes	*Pelospora*	< 0.01%	0.01%	ND	ND
Proteobacteria	*Pleomorphomonas*	< 0.01%	0.01%	ND	ND
Bacteroidetes	*Haliscomenobacter*	< 0.01%	0.01%	ND	ND
Actinobacteria	*Terracoccus*	< 0.01%	0.01%	ND	ND
Actinobacteria	*Brooklawnia*	< 0.01%	< 0.01%	ND	ND
Firmicutes	*Alloiococcus*	< 0.01%	< 0.01%	ND	ND
Actinobacteria	*Phycicola*	< 0.01%	< 0.01%	ND	ND
Proteobacteria	*Desulfovibrio*	< 0.01%	< 0.01%	ND	ND
Proteobacteria	*Aeromonas*	< 0.01%	< 0.01%	ND	ND
Firmicutes	*Thalassobacillus*	< 0.01%	< 0.01%	ND	ND
Bacteroidetes	*Elizabethkingia*	< 0.01%	< 0.01%	ND	ND
Bacteroidetes	*Nubsella*	< 0.01%	< 0.01%	ND	ND
Bacteroidetes	*Sporocytophaga*	< 0.01%	< 0.01%	ND	ND
Actinobacteria	*Actinotalea*	< 0.01%	< 0.01%	ND	ND
Proteobacteria	*Phocoenobacter*	< 0.01%	< 0.01%	ND	ND
Actinobacteria	*Brevibacterium*	< 0.01%	< 0.01%	ND	ND
Proteobacteria	*Daeguia*	< 0.01%	< 0.01%	ND	ND
Firmicutes	*Atopobacter*	< 0.01%	< 0.01%	ND	ND
Actinobacteria	*Zhihengliuella*	< 0.01%	< 0.01%	ND	ND
Firmicutes	*Anoxynatronum*	ND	< 0.01%	ND	ND
Proteobacteria	*Enterobacter*	ND	0.01%	ND	ND
Actinobacteria	*Gordonibacter*	ND	0.02%	ND	ND
Proteobacteria	*Enhydrobacter*	ND	ND	ND	0.04%
Synergistetes	*Synergistes*	ND	< 0.01%	ND	ND
Firmicutes	*Garciella*	ND	0.02%	ND	ND
Proteobacteria	*Caenispirillum*	ND	0.06%	ND	ND
Firmicutes	*Eremococcus*	ND	< 0.01%	ND	ND
Bacteroidetes	*Lacibacter*	ND	< 0.01%	ND	ND
Actinobacteria	*Devriesea*	ND	< 0.01%	ND	ND
Proteobacteria	*Craurococcus*	ND	< 0.01%	ND	ND
Proteobacteria	*Oxalobacter*	ND	0.02%	ND	ND
Actinobacteria	*Asaccharobacter*	ND	< 0.01%	ND	ND
Verrucomicrobia	*Luteolibacter*	ND	0.02%	ND	ND
Firmicutes	*Thermotalea*	ND	0.02%	ND	ND
Actinobacteria	*Aestuariimicrobium*	ND	0.02%	ND	ND
Chloroflexi	*Caldilinea*	ND	0.02%	ND	ND
Fusobacteria	*Sebaldella*	ND	0.01%	ND	ND
Proteobacteria	*Duganella*	ND	0.01%	ND	ND
Firmicutes	*Sporotalea*	ND	0.01%	ND	ND

Table 3.6: (cont.)

Phylum	Genus	Control Respiratory	Modulated Respiratory	Control Lower Respiratory	Modulated Lower Respiratory
Proteobacteria	***Chelonobacter***	ND	0.01%	ND	ND
Bacteroidetes	***Pedobacter***	ND	0.01%	ND	ND
Firmicutes	***Caminicella***	ND	0.01%	ND	ND
Proteobacteria	***Azorhizophilus***	ND	< 0.01%	ND	ND
Firmicutes	***Moryella***	ND	< 0.01%	ND	ND
Proteobacteria	***Hoeflea***	ND	< 0.01%	ND	ND
Proteobacteria	***Psychrobacter***	ND	< 0.01%	ND	ND
Bacteroidetes	***Terrimonas***	ND	< 0.01%	ND	ND
Lentisphaerae	***Lentisphaera***	ND	< 0.01%	ND	ND
Proteobacteria	***Wolinella***	ND	< 0.01%	ND	ND
Cyanobacteria	***Bacillariophyta***	ND	< 0.01%	ND	ND
Proteobacteria	***Haemophilus***	ND	< 0.01%	ND	ND
Bacteroidetes	***Zunongwangia***	ND	< 0.01%	ND	ND
Proteobacteria	***Altererythrobacter***	ND	< 0.01%	ND	ND
Proteobacteria	***Lonepinella***	ND	< 0.01%	ND	ND
Cyanobacteria	***Chlorarachniophyceae***	ND	< 0.01%	ND	ND
Proteobacteria	***Alysiella***	ND	< 0.01%	ND	ND
Actinobacteria	***Xylanimicrobium***	ND	ND	0.02%	0.02%
Firmicutes	***Centipeda***	ND	ND	0.02%	0.05%

Table 3.6: (cont.)

Phylum	Control Upper Respiratory	Modulated Upper Respiratory	p-value
Actinobacteria	5.17%	3.91%	0.003
Bacteroidetes	38.30%	46.45%	0.001
Firmicutes	51.80%	44.88%	0.002
Synergistetes	0.14%	0.62%	0.0015
Genus	**Control Upper Respiratory**	**Modulated Upper Respiratory**	**p-value**
Janibacter	0.46%	0.11%	0.0071
Rothia	0.68%	0.14%	0.0005
Slackia	0.36%	0.08%	0.0211
Barnesiella	2.93%	1.83%	0.001
Tannerella	2.03%	3.49%	0.001
Paraprevotella	1.57%	2.74%	0.028
Rikenella	0.96%	2.74%	0.001
Pseudosphingobacterium	4.35%	6.35%	0.043
Aerococcus	0.50%	1.35%	0.001
Lactobacillus	0.41%	0.13%	0.0411
Streptococcus	2.65%	1.33%	0.001
Sarcina	0.05%	0.34%	0.0129
Mogibacterium	0.40%	0.11%	0.0307
Anaerotruncus	2.03%	1.15%	0.001
Sharpea	1.56%	2.11%	0.007
Solobacterium	0.90%	0.37%	0.0064
Sandaracinobacter	0.61%	0.27%	0.0496
Succinivibrio	0.29%	0.70%	0.0284
Thermovirga	0.06%	0.51%	0.0004

Table 3.7: Statistically significant differences in relative taxonomic abundance between the modulated and control group upper respiratory microbiome samples taken at multiple time points following oral inoculation.

CHAPTER FOUR

MODULATION OF SYSTEMIC IMMUNE RESPONSES THROUGH COMMENSAL GASTROINTESTINAL MICROBIOTA

4.1 INTRODUCTION

Chapter 3 shows oral inoculation as an effective tool for modulating GI microbiome composition. Because of the connection that has been made over the past few decades between GI microbiota and host health and immune responses [20], [35], [89], [98], [99], [123], [124], it is logical to think that modulation of the GI microbiome could be used as a therapeutic tool. Studies have already shown the technique of fecal transplantation to be effective in treating intestinal disorders such as *C. difficile* infection, IBD and IBS [18–20]. In addition patients treated for such disorders have also reported improvements in systemic disorders, such as multiple sclerosis and idiopathic thrombocytopenic purpura [20]. These results have led to the testable hypothesis that modulation of the GI microbiome could be an effective tool to improve overall health and treat systemic disorders.

The largest lymphoid tissue in the human body is the gut-associated lymphoid tissue (GALT) [125]. The GALT consists of a network of peyer's patches and mesenteric lymph nodes. Bacterial antigens from the intestinal lumen are captured by M cells and dendritic cells in peyer's patches, where they are processed, bound to MHCII receptors and presented to lymphocytes in the peyer's patches and mesenteric lymph nodes. T and B cells capable of recognizing the specific antigen become activated and differentiate into a variety of cell types. B cells differentiate into memory B cells and antibody secreting plasma cells, which travel to the mesenteric lymph nodes. From the mesenteric lymph nodes these cells travel through the circulatory system, eventually migrate back to the lamina propria of the gut and other mucosal-associated lymphoid tissues (MALT) throughout the body, where they secrete IgA. This process not only regulates the intestinal immune system and helps keep GI microbes from invading the

body, but it also shows a connection between the gastrointestinal tract and potential regulation of the systemic immune system.

The swine respiratory pathogen *Mycoplasma hyopneumoniae* (*M. hyopneumoniae)* was chosen as the pathogenic challenge for this study. *M. hyopneumoniae* is a small gram positive bacterium that lacks a cell wall. It is the main cause of porcine enzootic pneumonia, a chronic, porcine specific respiratory disease characterized by high morbidity and low mortality that affects a majority of pigs around the world [126]. *M. hyopneumoniae* infection is tissue specific and results in a chronic respiratory disease characterized by coughing, lung lesions, and decreases in daily gain, as well as predisposes animals to other respiratory diseases of bacterial and viral origin [127], [128]. The microscopic hallmark of swine mycoplasmosis is a strong immune response, evident by perivascular and peribronchial lymphoproliferation [128] that ultimately accounts for lung consolidation, resulting in pneumonia.

Proinflammatory cytokines play a role in *M. hyopneumoniae* infections by coordinating the adaptive immune response [129]. However, if cytokine levels become too high, additional damage to the host's lungs can occur, showing the importance of proper regulation of host immune responses to *M. hyopneumoniae* disease progression. Increases in the levels of IL-1α, IL-1β, IL-6, IL-8 and TNF-α have all been reported in the lungs of pigs with *M. hyopneumoniae* infection [130]. Studies have also recognized *M. hyopneumoniae* as a ligand for TLR2 and TLR6, showing TNF-α production by porcine alveolar macrophages stimulated with *M. hyopneumoniae* in vitro, and a lack of TNF-α production when incubated with porcine TLR2 and TLR6 antibodies [131].

This study demonstrates the use of GI microbial modulation as a therapeutic tool to alter porcine systemic immune responses to a pathogenic challenge by *M. hyopneumoniae*. Briefly, a litter of pigs was removed from their mother immediately following birth and raised in controlled research units until weaning (28 days old), after which the pigs were randomly assigned to 2 groups. One group was inoculated (modulated) with the GI microbiota from a healthy adult boar for seven consecutive days, while the other was not (control). The effects of the oral inoculation on GI and respiratory microbial communities, porcine systemic immune responses and severity of infection following experimental infection with *M. hyopneumoniae*

were determined (Figure 4.1). In order to determine the effectiveness of the GI microbial modulation on porcine systemic immune responses, allergic and delayed type hypersensitivity responses (type I and IV, respectively) were measured in both groups via *A. suum* worm extract skin testing at 54 days of age, and pigs were experimentally infected with *M. hyopneumoniae* at 69 days of age and observed for 5 weeks.

4.2: RESULTS

4.2.1 TYPE I AND IV HYPERSENSITIVITY

Allergic hypersensitivities are IgE mediated responses that typically occur within 20 to 30 minutes after re-exposure to a specific innocuous antigen. Type IV delayed type hypersensitivity (DTH) responses are cell-mediated reactions, which occur 2-3 days after antigen exposure. In this study a difference between the modulated and control group was observed for DTH responses to *Ascaris suum* (*A. suum*) skin testing following oral microbiota inoculation (54 days of age); however, no type I allergic responses were observed in the young pigs. The modulated group had a stronger DTH response than the control group for all allergen concentrations tested (Figure 4.2). The largest DTH response difference between the two groups was observed at 100 µg *A. suum* extract, with the control group having an average increase in skin thickness of 0.643 mm, and the modulated group having an average increase of 1.19 mm (p=0.07; Figure 4.2; Table 4.1).

4.2.2 ANTIBODY PRODUCTION

M. hyopneumoniae antibody levels in blood serum were monitored at 0, 2, 5, 7, 9, 12, 14 and 21 days post-infection (dpi). The results demonstrated seroconversion to *M. hyopneumoniae* in the modulated group prior to the control group. *M. hyopneumoniae* antibodies were detected in modulated animals as early as 9 dpi. At 12 dpi, five of the modulated pigs had seroconverted, compared to only two of the control animals. All pigs in both groups seroconverted by 14 dpi (Table 4.2).

4.2.3 DNA Methylation Patterns

Methylation patterns of monocytes and neutrophils at a predicted CpG island at the 5' end of TLR1 (NM_001031775.1) were determined 1 day before oral inoculation (32 days of age) and 1 day before euthanasia (103 days of age). The results show no significant difference in percent methylation between the two groups at either time point (Figure 4.3). In addition, no significant differences in percent methylation within groups were observed over the length of the study.

4.2.4 Respiratory TLR2 & TLR6 Transcription

Transcription levels of respiratory TLR2 (NM_213761.1) and TLR6 (NM_213760.1) were determined using frozen lung samples collected at euthanasia. Age-matched pigs (6) from a *M. hyopneumoniae* negative farm were used as a *M. hyopneumoniae* free baseline for TLR2 and TLR6 transcription levels. No significant difference was observed in respiratory TLR2 (p=0.32) and TLR6 (p=0.20) gene transcription levels between the modulated and control groups (Figure 4.4).

4.2.5 Inflammatory Cytokine and C-reactive Protein Levels

Inflammatory cytokines IL-1β, IL-6, IL-8, TNF-α and C-reactive protein levels were monitored in the blood serum and bronchoalveolar lavage fluid (BALF). C-reactive protein serum levels were monitored throughout the study, and although an increase in C-reactive protein level was demonstrated following *M. hyopneumoniae* infection, our results showed that there was no difference seen in these levels between the modulated and control groups (Figure 4.5). No statistical difference between the two groups was observed for cytokine levels in blood serum or BALF (Figure 4.6). Despite no difference in the average TNF-α level of the two groups, a statistically significant difference (p = 0.0153) was observed for TNF-α variance in the BALF (Figure 4.7; Table 4.1). The modulated group demonstrated significantly less variation (0.18) than the control group (5.8) for BALF TNF-α levels.

4.2.6 Bacterial Load

M. hyopneumoniae levels (CFU/mL) were determined using nasal swabs taken throughout the study, as well as bronchial swabs and BALF. CFU levels were not significantly

different between the two groups, and the microscopic lung lesions suggestive of *M. hyopneumoniae* infection were apparent in each animal examined (p=0.346; Figure 4.8).

4.2.7 DAILY WEIGHT GAIN

Weights were recorded at 0, 15 and 22 days post infection (dpi) using a calibrated commercial animal scale. No significant differences in daily weight gain were observed between the modulated and control animals (Figure 4.9).

4.2.8 COUGHING SCORES

Coughing levels for each group were recorded at the same time of day throughout the study. Coughing for the control group began as early as 7 dpi, whereas modulated animals did not begin coughing until 12 dpi, despite no difference in bacterial load between the groups. The number of dry coughs suggestive of *M. hyopneumoniae* infection was lower (p<0.005) in the modulated group than in the control group for the duration of the study (Figure 4.10). The average number of coughs/30 min was 9.57 in the control group and 4.39 in the modulated group (Table 4.1).

4.2.9 LUNG LESIONS

Lung lesion evaluations were done at euthanasia (35 dpi). Pigs in the modulated group tended to have lower (p=0.07) lung lesion scores, with an average of 28% of their lungs covered in lesions, compared to 42% in the control group (Figure 4.11; Table 4.1). Microscopic observations of lung lesions confirmed the presence of lesions caused by *M. hyopneumoniae* infection in all pigs. Perivascular and peribronchiolar lymphocyte infiltration, as well as increased numbers of mononuclear and polymorphonuclear cells in alveoli and lymphoid nodules were observed in all the pigs, each indicative of *M. hyopneumoniae* infection, as previously described [132]. No differences in microscopic lung lesions were observed between the two groups.

4.3: DISCUSSION

This study supports the hypothesis that alterations in the composition of the GI microbiome can significantly affect systemic immune responses [133]. Results from this study

clearly show a link between GI microbiota and systemic immune responses. This is evidenced by a stronger DTH response and less variation in respiratory TNF-α levels in the modulated group compared to the control group. The results from the coughing levels and lung lesion scores show a lower severity of infection in the modulated group than the control group as well, despite the lack of a difference in respiratory *M. hyopneumoniae* levels. All procedures performed in this study were applied to both the modulated and control group, with the exception of the oral inoculation. Because the oral inoculation was the only independent variable in the study, the results validate our hypothesis that modulation of GI microbiota has significant effects on systemic immune responses.

Increased incidence of allergies in developed countries is thought to be due to limited microbial exposure early in life [134]. In order to show the importance of early life microbial exposure on systemic immune responses, a litter of newborn piglets was used as the experimental model, and modulation of the GI microbiota was performed at 33 days of age. Results from a previous study show delivery via caesarian results in infants being colonized by bacteria similar to those found on the skin surface [28]. Based on these results piglets in this study were born naturally as opposed to caesarean derived to ensure the piglet's GI tract was colonized by microbes that would naturally colonize the neonatal gut, as opposed to skin derived microbes. Piglets were caught at birth using nitrile gloves and removed from the mother immediately to prevent contact with maternal feces and limit the vertical transfer of microbes. It is important to note that none of the piglets received maternal antibodies or immune molecules, as they were not allowed to suckle prior to being removed from their mother. The use of a single litter was designed to reduce genetic diversity (both individual and maternal) which could be responsible for differences seen in systemic immune responses between individuals. This is a potentially important aspect of the study, as both individual genetic and maternal factors can have an effect on immune function. Adding a second litter with a different genetic background (both individual and maternal) introduces another variable into the experiment that could have significant effects on immune responses.

The GI microbiota are constantly sampled by the host and participate in stimulation of the immune system [135]. This stimulation is believed to be important in establishing baseline

immune responses to pathogenic infection. Previous studies have shown that a lack of GI microbial diversity is associated with increases in allergic disease, as well as reduced immune responses [77], [136], [137]. Both allergic and delayed type hypersensitivity tests were conducted to test for differences in allergic sensitivity and baseline systemic immune responses. No allergic responses were observed for any of the pigs in this study at the time of testing (54 days of age). The lack of an allergic response to the *A.* suum antigen is proposed to be due to the young age of the pigs at the time of exposure. It is possible that the pigs were not given sufficient time to develop an allergy to the *A. suum* antigen, and that testing at a later date during the experiment may be ideal for future studies. This theory is derived from the fact that this antigen has been used in the past with older pigs, yielding positive allergic responses [138]. Despite a lack of IgE mediated type I allergic response to the *A. suum* skin testing, the DTH results established stronger systemic immune responses for the modulated group caused by the modulation of the GI microbiota.

The disease progression and severity of *M. hyopneumoniae* infection can rely on a number of factors, from the immune status of the pig, to the *M. hyopneumoniae* bacterial load, to co-infection with other respiratory pathogens [132]. This study was conducted in a controlled research facility and pigs were tested for porcine respiratory and reproductive syndrome virus (PRRSv) and swine influenza virus (SIV) antibodies in order to show a lack of co-infection. To show the differences seen in the severity of infection were due to differences in systemic immune responses caused by GI microbial modulation, and not differences in disease progression, bacterial load was determined from nasal swabs taken throughout the study, as well as bronchial swabs and BALF. These results showed no difference in bacterial load between the two groups for any time point throughout the study.

Results from the coughing observations show a significant decrease in the number of coughs/30 minutes, and lung lesions were found to cover a smaller percentage of the lungs in the modulated group compared to the control group. Although the source of an individual cough could not be assigned to an individual pig, the location of the coughs within the pen suggests that all of the pigs contributed to the total coughing score. Furthermore, the

subsequent lung lesion data is consistent with a distribution among the group rather than a single pig being responsible for the increased group average.

An important aspect of this study is the idea that the GI microbiota are involved in the modulation of the systemic immune system, having a direct effect on systemic immune responses, and that responses are not due to any interaction between GI microbiota and the pathogens used as systemic immune response triggers. In order to show this effect it was important to keep the GI microbiota and pathogenic challenges contained from one another. The lack of direct contact between GI microbiota and the pathogenic challenges requires there to be GI microbial regulation of the systemic immune system to explain the differences in systemic immune responses. *M. hyopneumoniae's* restriction to the respiratory tract [139] fulfils these requirements. This intestinal-free stimulant has no direct contact with the GI microbiota, and therefore shows that systemic immune responses were altered by GI microbiota in the modulated group.

There is no evidence that we are aware of to suggest that differences in the composition of the respiratory microbiome have effects on *M. hyopneumoniae* infections. The pigs were tested for and found to be free of other prominent respiratory infections (SIV and PRRSv), showing that co-infection was not a factor in this study. The lack of a difference in *M. hyopneumoniae* bacterial load throughout the study also indicates that there was no modulation of the *M. hyopneumoniae* infection (the disease progression was the same, but immune responses were different). Furthermore, the differences in DTH response, antibody production, and TNFα variance observed in this study are all examples of altered systemic immune responses due to microbial modulation.

The acute phase response is an early non-specific immune response to infection and involves the induction of serum proteins known as acute phase proteins [140]. C-reactive protein is an acute phase protein that binds to dying cells, as well as bacteria to activate the complement system [141]. It has been shown that C-reactive protein levels increase in pigs infected with *M. hyopneumoniae* [142]. In addition to C-reactive protein, proinflammatory cytokines are known to play important roles in porcine immune response to infection. IL-1β is known to induce IL-6 production, and IL-8 has been reported to be a chemotactic for T cells and

neutrophils [129]. TNF-α is also an important factor in porcine disease, being responsible for the accumulation of lymphocytes in *M. hyopneumoniae* infections [131]. Increases in all four of these cytokine levels have been reported in pigs infected with *M. hyopneumoniae* [129], [143]. Because of their importance in systemic immune responses, as well as their association with *M. hyopneumoniae* infection, these immune response elements were monitored throughout the study. Although increased levels were observed as the study went on, no significant differences between the two groups were seen, with the exception of the level of variation seen in the TNF-α levels of the BALF. The modulated group had significantly less variation in their TNF-α levels compared to the control group, suggesting a tighter regulation of TNF-α levels in the lungs due to GI modulation.

M. hyopneumoniae has been revealed as a ligand for porcine TLR2 and TLR6, and the stimulation of porcine alveolar macrophages with *M. hyopneumoniae* has been shown to induce TNF-α production in vitro [131]. Furthermore, these studies showed that TNF-α production could be blocked using antiporcine TLR2 and TLR6 antibodies. Because of the connection between TLR2, TLR6 and TNF-α production it was our theory that the variation in TNF-α levels seen in the BALF could be accounted for by differences in TLR expression levels caused by the oral inoculation. In order to test this theory, respiratory transcription levels of TLR2 and TLR6 were analysed for the control and modulated groups of infected pigs as well as a third group (age matched pigs which had not been infected with *M. hyopneumoniae*). The results of these studies showed no significant difference in the expression levels or variances of TLR2 and TLR6 in the lungs of any group studied.

In addition, bisulfite sequencing of a predicted CpG Island at the 5’ end of TLR1 in monocytes and neutrophils collected from blood showed no differences between the two groups at any time point. TLR1 forms a heterodimer with TLR2 and was chosen for analysis because *M. hyopneumoniae* is not a ligand for TLR1, and any observed differences would be due to differences in GI microbiome composition, and not due to stimulation by *M. hyopneumoniae*. Despite the lack of significant differences in transcription levels and methylation patterns of the TLRs, the much tighter regulation of TNF-α production associated with the modulated group suggests that tighter regulation of immune response genes in the

lungs is associated with GI microbial diversity, and further studies are required to determine what mechanisms are responsible for this regulation.

4.4: Materials and Methods

4.4.1 Ethics Statement

This study was approved by the Institutional Animal Care and Use Committee (IACUC) and the Institutional Biosafety Committee of the University of Illinois at Urbana-Champaign (protocol # 09141 and 09146). All animals were cared for following the guidelines of the IACUC and the Institutional Biosafety Committee of the University of Illinois at Urbana-Champaign, and all efforts were made to minimize suffering throughout the study.

4.4.2 Experimental Design

A litter of pigs (12) was removed from their mother immediately following birth in order to prevent exposure to the maternal GI microbiota. The pigs were raised in controlled research units and fed medicated milk replacer until weaning (28 days old). At 33 days of age the pigs were randomly assigned to 2 groups based on weight and gender, one of which was inoculated (modulated) with the GI microbiota from a healthy adult boar for seven consecutive days, while the other was not (control). Nasal swabs and fecal samples were collected throughout the study and sequenced to determine the effects of the oral inoculation on GI and respiratory microbial communities. In order to determine the effectiveness of the GI microbial modulation on porcine systemic immune responses, allergic and delayed type hypersensitivity responses (type I and IV, respectively) were measured in both groups via *A. suum* worm extract skin testing at 54 days of age, and pigs were experimentally infected with *M. hyopneumoniae* at 69 days of age and observed for 5 weeks. Various systemic immune responses and severities of infection were analyzed throughout the study including: *M. hyopneumoniae* antibody production, respiratory TLR2 & TLR6 transcription levels, and cytokine and C-reactive protein levels.

4.4.3 Source of Animals and Housing

A pregnant gilt obtained from a high health herd was housed under commercial conditions and then transferred to a research facility 3 weeks before farrowing. The gilt was

induced with 3 doses of 10 mg dinoprost tromethamine (Lutalyse®, Pfizer Inc., New York, NY, USA) intramuscularly at 12 hour intervals starting at day 113 of gestation. Plastic was placed under the gilt and piglets were caught at birth using nitrile gloves to prevent contact with fecal matter. The piglets were removed immediately following birth and raised in controlled research units in order to prevent exposure to GI colonizing microbiota. Research suites were equipped with HEPA filters and the ventilation system was individualized for each room. Biosecurity measures were followed at all times to avoid cross-contamination between experimental groups.

4.4.4 Feeding Protocol

To avoid vertical transfer of porcine immune molecules, piglets were removed from gilts before suckling and syringe fed 20-25 mL of previously frozen bovine colostrum, obtained from the University of Illinois Dairy Farm, every 2 hours for the first 48 hours of life. The colostrum tested negative for *M. hyopneumoniae* antibodies. Piglets were then switched to Advance Liqui-Wean Medicated Pig Milk Replacer® (Oxytetracycline and Neo-Terramycin) (MSC, Carpentersville, IL, USA), which was pumped into bowls every 60 min at a rate of 360 mL/kg/day. Both antibiotic and colostrum were used in order to ensure the piglets health, as previous attempts by our group to artificially raise piglets without colostrum or antibiotic treament resulted in severe *E. coli* infections and gastrointestinal clinical signs. In order to prevent respiratory infection and gastrointestinal clinical signs, Baytril (enrofloxacin) was injected subcutaneously into the ear in 100mg/30lbs body weight doses 24 hours after birth. To prevent gastrointestinal infection, neomycin sulfate was administered orally at a rate of 10 mg/lb body weight every day for the first 2 weeks of life. At 10 days of age the piglets were introduced to phase I dry feed and were eventually weaned off the milk replacer over a 2-day period once they reached an average weaning weight of 6 kg (28 days of age). Piglets were kept on the dry feed *ad libitum* for the remainder of the study.

4.4.5 Collection, Preparation and Delivery of Oral Inoculum

Fresh feces was collected daily from a single boar from a high health herd (*M. hyopneumoniae,* PRRSv, *Pasteurella multocida* and *Bordetella bronchiseptica* free) for use as an

oral inoculum. The farm has clinical and historical data backing up their high health status. Depopulation and repopulation had occurred just months before the samples were taken. The boar was showing no clinical signs of infection at the time of collection, and flotation tests done by the University of Illinois Veterinary Diagnostic lab were negative for GI parasites. Samples were immediately mixed 1:1 with sterile phosphate buffered saline (PBS) and fed by syringe to the 33 day old piglets (modulated) at a rate of 2 mL/kg, as previously described [107]. This process was repeated for 7 consecutive days in order to ensure GI colonization.

4.4.6 *ASCARIS SUUM* ANTIGEN HYPERSENSITIVITY TESTING

Starting at 33 days of age, pigs in both groups were sensitized to *A. suum* antigen by two bi-weekly injections containing 1 mg of *A. suum* worm extract mixed in alum. Solution was made by adding 0.05 μl aluminum potassium sulfate and 24 μl of sodium bicarbonate for every mg of *A. suum* worm extract. The solution was then mixed and allowed to stand at room temperature for 30 min, and then overnight at 4^{o}C. The solution was centrifuged, the supernatant was removed, and the precipitate was resuspended in distilled water at the desired concentration (worm extract and protocol kindly provided by F. Zuckermann, College of Veterinary Medicine, UIUC). Injections (SC) were given in the abdominal wall. One week after the final injection (54 days of age), skin tests for hypersensitivity type I and IV were performed. Pigs were anesthetized in order to obtain accurate measures of antigenic responses. A mixture of 1.5 mg/kg xylazine and 8 mg/kg of a commercial formulation of tiletamine and zolazepam (Telazol®, Fort Dodge Animal Health, Fort Dodge, IA, USA) was used for anesthesia. The skin test consisted of 10 intradermal injections in the abdominal wall performed in duplicate for each pig. The injections consisted of 100 μl of four-fold serially diluted *A. suum* worm extract (4,000; 1,000; 250; 62.5; 15.6; 3.9; 0.97; 0.24; 0.06; 0.015 μg/ml). Saline (100 μl) was also injected as a negative control. Type I hypersensitivity was measured 20 minutes after *A. suum* worm extract injection. Pigs were anesthetized again 24 hours after skin testing to measure for type IV hypersensitivity. Calipers were used to determine skin thickness of injection sites in mm, as previously described [144].

4.4.7 EXPERIMENTAL INFECTION WITH *M. HYOPNEUMONIAE*

69 day old pigs were experimentally inoculated with 10 mL of a $2x10^5$ color changing units (ccu/mL) lung homogenate containing *M. hyopneumoniae* strain 232 (purchased from Iowa State University, Ames, IA, USA) using intra-tracheal intubation to guarantee uniform infection throughout the groups. Due to the fact that growing *M. hyopneumoniae* colonies on agar plates is difficult and requires weeks of incubation, ccu/mL is the standard technique used for determine the concentration of *M. hyopneumoniae* [145]. The ccu/mL technique uses 10-fold serial dilutions of mycoplasma in Friis broth containing a pH sensitive color indicator, usually phynol red, which changes color depending on the acidity of the media. The color changes from red to yellow due to acidification by the cell's metabolism during growth. This change in color is used to determine the concentration of mycoplasma. Endotracheal tubes, syringes and needles employed for inoculation and injection were sterile (individually wrapped) and a different set was used for each animal. The pigs were anesthetized using the same protocol as for the *A. suum* sensitization. An endotracheal tube was placed in the trachea using the lighted guide of a laryngoscope, and the *M. hyopneumoniae* inoculum was administered to animals through the endotracheal tube, as previously described [126].

4.4.8 OBSERVATION OF CLINICAL SIGNS

Starting at 12 dpi the control and experimental groups were observed 30 minutes/day for coughing rates. While remaining out of sight, observations were scored for each group by listening for coughing, as previously described [57]. Coughing scores were recorded by group and not individual pigs in order to minimize stress levels during observation which could lead to inaccurate coughing scores. Observations were performed at the same time each day.

4.4.9 WEIGHT MEASURE

Pigs were individually weighed at the same time of day at 0, 15 and 22 dpi using a calibrated commercial animal scale.

4.4.10 COLLECTION OF BLOOD SERUM

Blood samples were obtained from all pigs at the same time of day at 0, 2, 5, 7, 9, 12, 14, and 21 dpi. Samples were collected in BD Serum Vacutainers® (Becton Dickinson and

Company, Franklin Lakes, NJ, USA) and centrifuged at a rate of 3,000 rpm for 10 minutes. Serum was then removed from the tubes and stored at -80^{o}C in sterile 1.5 mL Eppendorf tubes. ELISA for *M. hyopneumoniae,* PRRSv and SIV antibodies were performed in order to determine the rate of antibody production, as well as rule out the possibility of other respiratory diseases. Observation of C reactive protein, interleukins IL-1β, IL-6, IL-8, and TNF-α blood levels were performed by ELISA, as described in the determination of C-reactive protein, IL-1β, IL-6, IL-8, TNF-α, and specific *M. hyopneumoniae* antibodies section. In addition, blood samples were collected weekly for the isolation of monocytes and neutrophils.

4.4.11 COLLECTION OF NASAL SWABS

Nasal swabs were collected at 0, 7, 9, 12, 14, and 21 dpi by introducing approximately 4 mm of a sterile BD CultureSwab® (Becton Dickinson and companies, Franklin Lakes, NJ, USA) into each pig nostril and rotating it clockwise and counter clockwise. All swabs were stored at -80^{o}C.

4.4.12 COLLECTION OF LUNG TISSUE

Both healthy lung and portions containing lesions were collected at euthanasia. Samples were taken from the same location on the right antero-ventral lobe and placed in sterile 50 mL conical tubes. These tubes were flash frozen in liquid nitrogen and placed at -80^{o}C. Frozen lung samples were used in the gene expression level experiments of porcine TLR2 and TLR6. Samples of both healthy lung and portions containing lesions were stored in 10% fixative as well. Fixed samples were stained with H&E and used in microscopic evaluation of lung lesions.

4.4.13 COLLECTION OF BALF

Bronchoalveolar lavage fluids (BALF) were collected at euthanasia. Cytokine levels associated with *M. hyopneumoniae* infection were analyzed in the BALF. Sterile PBS (20 mL) was introduced into the bronchoalveolar space, massaged through the lungs and re-collected in 50 mL conical tubes. BALF was stored at -80^{o}C.

4.4.14 LUNG LESION EVALUATION (MACROSCOPIC AND MICROSCOPIC)

Macroscopic lung lesion evaluations were done as a single blind study following euthanasia. Lung lesions were scored based on the percentage of lung covered in lesions suggestive of *M. hyopneumoniae* infection, as previously described [127]. Macroscopic lung lesions indicative of *M. hyopneumoniae* infection are purple or grey with a rubbery consolidation, have increased firmness, a failure to collapse and are marked by edema of the lungs [147]. Microscopic lung lesion evaluation was done using the fixed lung tissues collected at euthanasia. Samples were embedded in paraffin, sectioned onto slides for evaluation and observed through an optic microscope [132].

4.4.15 DETERMINATION OF BACTERIAL LOAD IN NASAL SWABS

DNA from nasal swabs and BALF was extracted using the DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA, USA). Extracted DNA was submitted to the Veterinary Diagnostic Laboratory at the University of Minnesota (VDL-UMN) for quantification of the bacterial load by Real Time PCR VetMAX™ (Life Technologies Corporation, Carlsbad, CA, USA) with *M. hyopneumoniae* specific reagents and controls. DNA was also stored at -20 ^{o}C for use in microbiome sequencing analysis.

4.4.16 DETERMINATION OF C-REACTIVE PROTEIN, IL-1B, IL-6, IL-8, TNF-A, AND SPECIFIC *M. HYOPNEUMONIAE* ANTIBODIES

M. hyopneumoniae antibodies, cytokine and C-reactive protein levels were measured in blood serum and BALF. Pig serum samples were submitted to the VDL-UMN for determination of *M. hyopneumoniae* antibodies using the DAKO® ELISA test [148]. Cytokines were measured in the serum and BALF using a porcine specific multiplex ELISA test (Aushon Searchlight, Aushon Biosystems Inc., Billerica, MA, USA). C-reactive protein in serum was measured using the PHASE® (Tridelta Development Ltd, Maynooth, Ireland) ELISA assay kit following the manufacturer's instructions.

4.4.17 RNA ISOLATION, CDNA SYNTHESIS, AND TLR2 AND TLR6 GENE EXPRESSION ANALYSIS

Total RNA was isolated from snap frozen lung tissue of all animals experimentally infected with *M. hyopneumoniae,* as well as 6 age-matched pigs from a *M. hyopneumoniae*

negative farm. The latter group of pigs was used as a control for the gene expression level analysis. The RNeasy Mini Kit (Qiagen, Valencia, CA, USA) was used for RNA extraction from homogenized tissue following the manufacturer's instructions. Total mRNA samples were treated with DNase I (Qiagen, Valencia, CA, USA) in order to remove genomic DNA contamination, and concentrations were determined using an Eppendorf Biophotometer (Eppendorf, Westbury, NY, USA). Two µg of total RNA was used for reverse transcription using the Omniscript RT Kit (Qiagen, Valencia, CA, USA) in a 20µl reaction containing 10 µM Oligo-(dT), 10 U RNase inhibitor, 5 mM of each dNTP, RT buffer and 1 U of Omniscript RT for each sample. The reaction was allowed to occur at 37^{O}C for 90 min. Negative controls, which contained no reverse transcriptase, were processed identically with the samples. Quantitative expression of TLR2, TLR6 and 18S genes was investigated using real-time PCR. The 18S gene was used as an internal control. Gene-specific primers for TLR2 were designed with Primer Express® software (Applied Biosystems, Foster City, CA, USA) and the sequences were: forward primer (5'-GGGCTCTGTGCCACCACTT-3') and reverse primer (5-GGAGCCAGGCCCACAATC-3'). Gene-specific primers (18S) were obtained from previous publications [149]. Quantitative expression of TLR2 and 18S was performed using SYBR® Green PCR Master Mix, while TLR6 expression was determined using a specific TaqMan® Gene Ex Assay (assay ID Ss03392239_s1), both of which were obtained from Applied Biosystems (Foster City, CA, USA). Ten-fold serial dilutions of each gene were prepared from cDNA and a non-template control, and used for the standard curves to determine PCR efficiencies. Real-time PCR was performed in an ABI 7900HT fast real-time PCR system (Applied Biosystems, Foster City, CA, USA). The PCR amplification program steps were 10 min at 95^{O}C, then 40 cycles of: 15 s at 95^{O}C, 1 min at 60^{O}C, 1 min at 72^{O}C, and a final cycle for dissociation analysis of 15 s at 95^{O}C and 15s at 60^{O}C. All samples were run in triplicate, including all dilutions in the standard curve. Automatic cycle threshold (Ct) values obtained from the real-time PCR for TLR2 and TLR6 were normalized using the 18S rRNA transcript. PCR amplification efficiencies and correlation coefficients were analyzed. Final data for relative quantification of gene expression was obtained by applying the comparative Ct method (ΔΔCt) [150].

4.4.18 Monocyte and Neutrophil Isolation

Blood collected in heparinzed tubes was diluted 1:1 with sterile PBS. Up to 35 mL of diluted blood was layered on top of 15 mL of ficoll-Paque PREMIUM (GE Healthcare, Pittsburgh, PA, USA) in a 50 mL tube and centrifuged at 2000 rpm for 30 minutes with the brake off. The top layer (plasma) was then removed from the tube and the PBMC cells (monocytes) at the Ficoll/plasma interface were collected and transferred to a second tube. The original tube was set aside for the isolation of polymorphonuclear cells (neutrophils). The PBMC cells were washed multiple times with PBS and centrifuged at 2000 rpm for 10 minutes between each wash. Any red blood cells present were lysed by resuspending the pellet in 5 mL deionized water for 20 seconds, after which an equal volume of 2x PBS was added to the tube and centrifuged. The PBMC cell pellet was resuspended in 1 ml DMEM and stored at -20^{o}C.

The remainder of the Ficoll/plasma layer was removed from the original tube, leaving a cell pellet containing red blood cells and polymorphonuclear cells (neutrophils). The pellet was then suspended in 20 mL of Hank's Balanced Salt Solution (HBSS, No Ca+, Mg+). In order to separate the red blood cells from polymorphonuclear cells, 20 mL of 3% Dextran T500 (Sigma-Aldrich, ST. Louis, MO, USA) in 0.9% NaCl were added and the solution allowed to sediment for 20 minutes. The supernantant containing polymorphonuclear cells was transferred to a new tube and centrifuged at 1100 rpm for 10 minutes. In order to lyse any remaining red blood cells, the pellet was resuspended in 20 mL ice cold 0.2% NaCl for 30 seconds, after which 20 mL of ice cold 1.6% NaCl were added. The tube was centrifuged at 1100 rpm for 10 minutes and the pellet was resuspended in 2 mL HBSS and stored at -20^{o}C.

4.4.19 DNA Extraction, Bisulfite Sequencing, Cloning and Sanger Sequencing of TLR1 CpG Island

DNA was extracted from monocyte and neutrophil isolates by resuspending the cell pellets in 250 µl DNA Extraction Buffer, 18 µl 20% SDS, 30 µl 0.5 M DDT and 25 µl Proteinase K and incubating overnight at 65^{o}C. The tubes were then place at -20^{o}C until frozen, thawed and 120 µl 5M NaCl added. The tubes were shaken to mix and centrifuged at max speed for 10 minutes. 400 µl of supernantant were added to fresh tubes containing 1 ml of 100% ETOH and mixed by inversion. The DNA was plucked with a pipette tip, resuspended in 500 µl water and

stored at -20^{o}C. Bisulfite conversion was performed using the EpiTect Bisulfite Kit (Qiagen, Valencia, CA, USA) following the manufacturer's instructions. CpG Island prediction for TLR1 was done using the CpG Island Searcher [151], and primers were designed using MethPrimer [152]. A CpG island associated with TLR1 was discovered at base pair positions 557 to 805 of the TLR1 genomic region. The primers designed to quantify this region were HPLC purified and the sequences were: forward primer (5'-TAGTGGTATATGGAGGTTTTTAGGTTAG-3') and reverse primer (5'-TCCTTTAAAAAATTTCAAAAAATATCACT-3'). The PCR amplification mixture contained 2.5 units HotStarTaq Plus DNA Polymerase (Qiagen, Valencia, CA, USA), 5 µl 10X PCR Buffer (Qiagen, Valencia, CA, USA), 0.25 mM dNTPs, 0.25 µM forward and reverse primer and 150 ng DNA in a reaction volume of 50 µl. The PCR conditions were an initial denaturation at 95 ^{o}C for 15 minutes, followed by 35 cycles of 95 ^{o}C for 1 minute, 58 ^{o}C for 3 minutes, 72 ^{o}C for 1 minute, and a final 10 minute elongation at 72 ^{o}C. Samples were run on a 1.5% agarose gel to verify product amplification.

The PCR products were ligated into TOPO vectors and transfected into One Shot Chemically Competent *E. coli* cells (Invitrogen, Grand Island, NY, USA) following the manufacturer's instructions. Transfected cells were spread on agar plates containing X-Gal and incubated overnight at 37^{o}C. A total of 12 white colonies/sample were picked and cultured overnight in LB. Plasmid DNA was purified from the overnight cultures using the QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA, USA) following the manufacturer's instructions. The DNA insert was amplified in a PCR amplification mixture containing 12 µl 12.5% glycerol, 4 µl 5X sequencing buffer, 1 µl ABI BigDye (Invitrogen, Grand Island, NY, USA), 0.5 µM primer (forward or reverse) and 40-60 ng DNA in a 20 µl reaction. The PCR conditions were an initial denaturation at 95^{o}C for 15 minutes, followed by 35 cycles of 95 ^{o}C for 1 minute, 58 ^{o}C for 3 minutes, 72 ^{o}C for 1 minute, and a final 10 minute elongation at 72 ^{o}C. The samples were then sent to the KECK center at the University of Illinois for Sanger sequencing.

4.4.20 DATA ANALYSIS

Data was evaluated using the Students *t* test or Kruskall-Wallis one-way analysis of variance, were appropriate. Equality of variances was measured using the Brown-Forsythe

homogeneity of variance test. DNA methylation patterns were determined by aligning the reads to the TLR1 CpG Island using Sequencher version 5.0 (Gene Codes Corporation, Ann Arbor, MI, USA) and counting the percent methylation at each CpG site with a minimum of 10 reads/site. The proportion of seropositve pigs was compared using a Hypothesis test. The pig was the experimental unit for all comparisons.

4.5: CONCLUSIONS

From these results we conclude that a non-pathogenic oral inoculum successfully modulated the GI microbial community, significantly regulated the systemic immune system of the pig and lowered the severity of infection. This hypothesis is supported by the stronger DTH response, the decreased severity of infection, and the significantly lower amount of variation seen in TNF-α levels in the lungs of the modulated group. This study shows further evidence for the use of microbial inoculums as therapeutic tools to modulate the systemic immune system. Further studies need to be done to determine the mechanisms by which the GI microbiota regulates immune responses outside the gut.

4.6 FIGURES AND TABLES

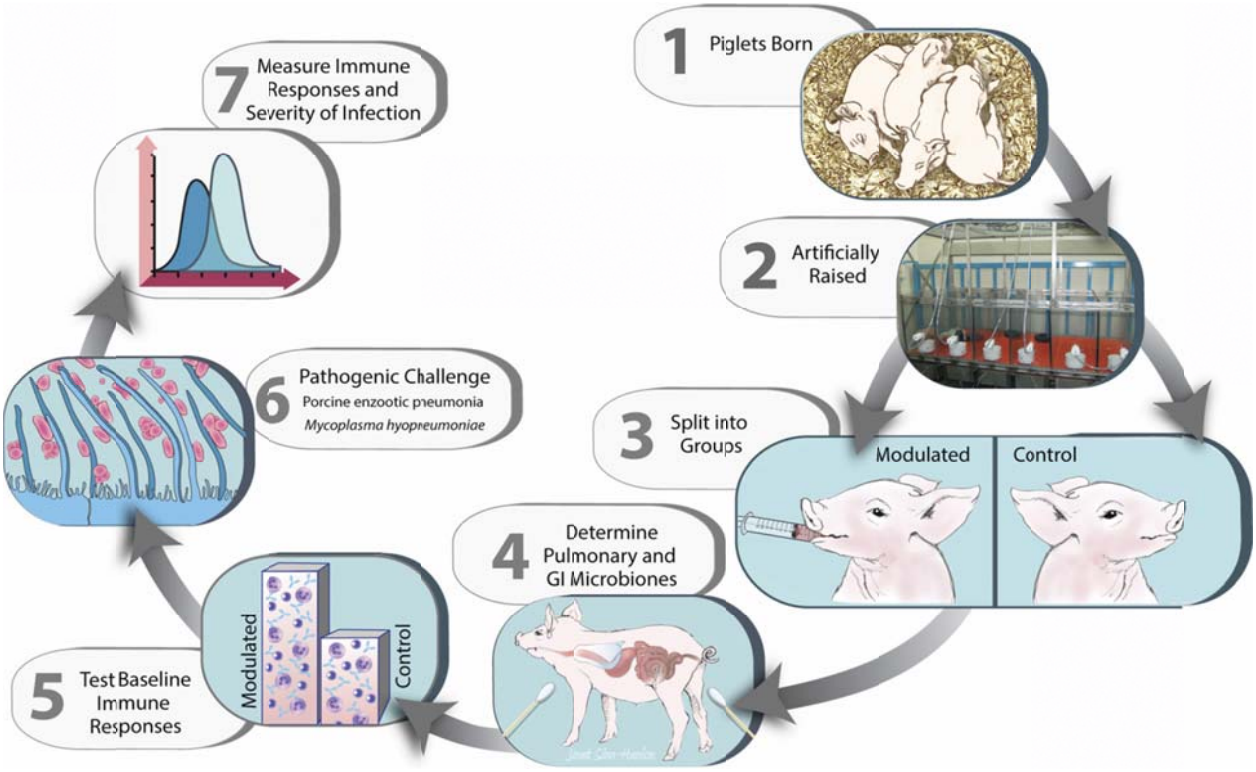

Figure 4.1: Experimental Timeline. 1. A litter of pigs (12) was removed from their mother immediately following birth in order to prevent exposure to the maternal GI microbiota. 2. The pigs were raised in controlled research units and fed medicated milk replacer until weaning (28 days old). 3. At 33 days of age the pigs were randomly assigned to 2 groups based on weight and gender, one of which was inoculated (modulated) with the GI microbiota from a healthy adult boar for seven consecutive days, while the other was not (control). 4. Nasal swabs and fecal samples were collected throughout the study and sequenced to determine the effects of the oral inoculation on GI and respiratory microbial communities. 5. Allergic and delayed type hypersensitivity responses (type I and IV, respectively) were measured in both groups via *A. suum* worm extract skin testing at 54 days of age. 6. Experimental infection with *M. hyopneumoniae* was performed at 69 days of age. 7. Various systemic immune responses and severities of infection were analyzed throughout the study: *M. hyopneumoniae* antibody production, respiratory TLR2 & TLR6 transcription levels, cytokine and C-reactive protein levels, daily weight gain, bacterial load, coughing and lung lesion scores.

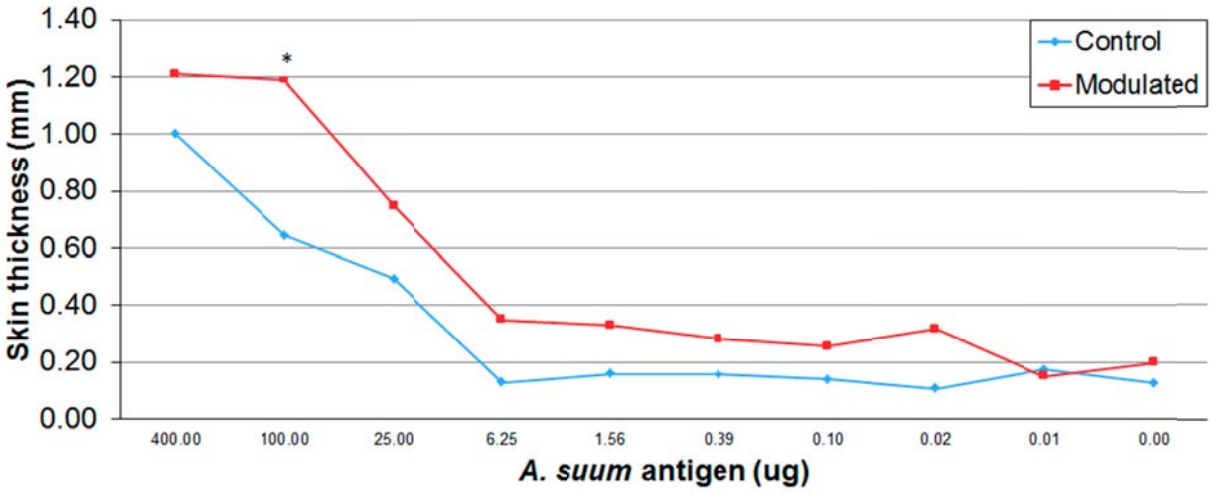

Figure 4.2: Delayed type hypersensitivity response to *Ascaris suum* antigen. DTH responses to *Ascaris suum* antigen injected into the abdomen in four fold serial dilutions. * Denotes $p < 0.05$

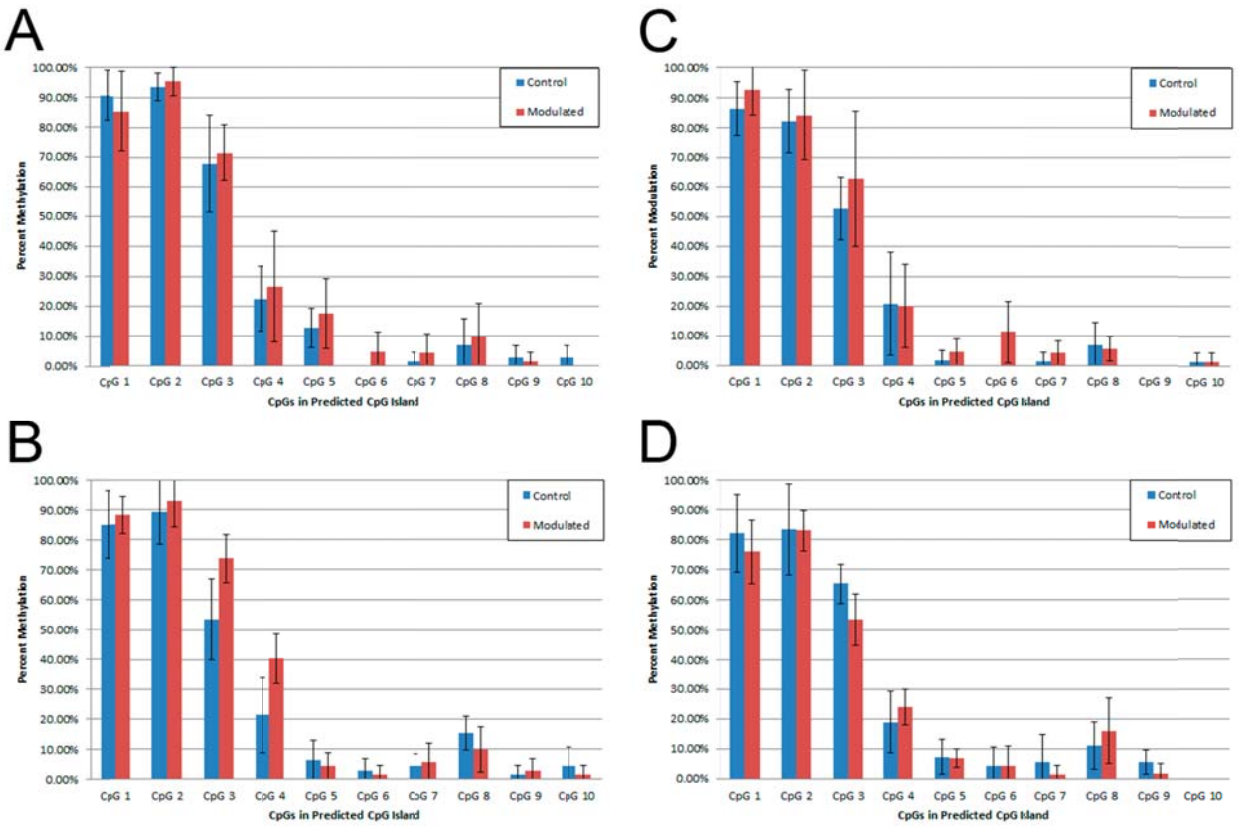

Figure 4.3: DNA methylation patterns of TLR1. Percent methylation at 10 CpG sites within a predicted CpG island located at the 5' region of TLR**1** in A) neutrophils the day before oral inoculation (32 days of age), B) neutrophils the day before euthanasia (103 days of age), C) monocytes the day before oral inoculation (32 days of age), and D) monocytes the day before euthanasia (103 days of age).

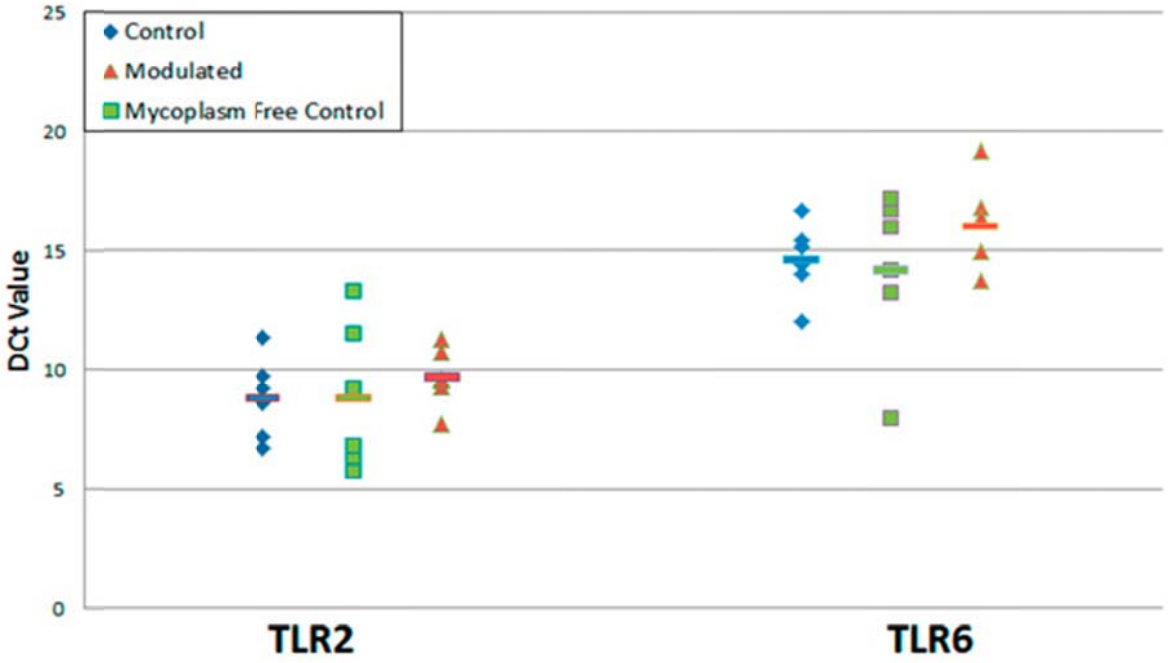

Figure 4.4: TLR2 and TLR6 transcription levels. TLR2 and TLR6 transcription levels in the lungs of the modulated and control group, as well as an age matched *M. hyopneumoniae* free control group.

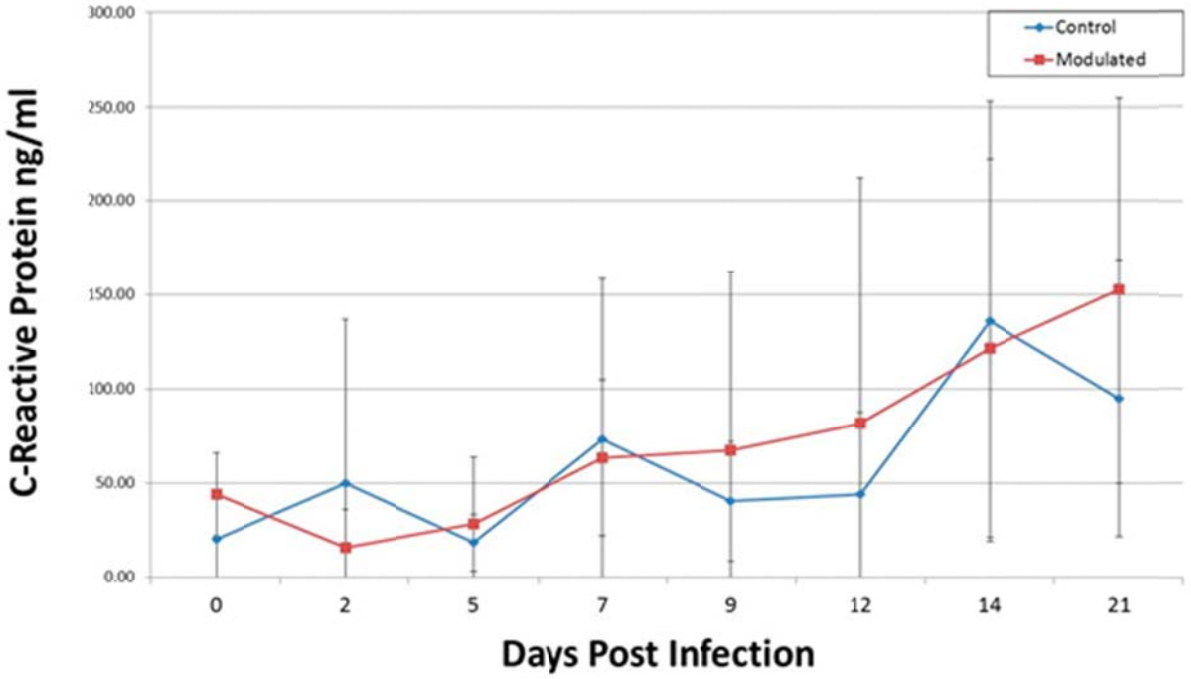

Figure 4.5: C-reactive protein levels in blood serum. C-reactive protein levels in the blood serum at 0, 2, 5, 7, 9, 12, 14 and 21 days post infection with *M. hyopneumoniae*. Results expressed as ng/ml of C-reactive protein.

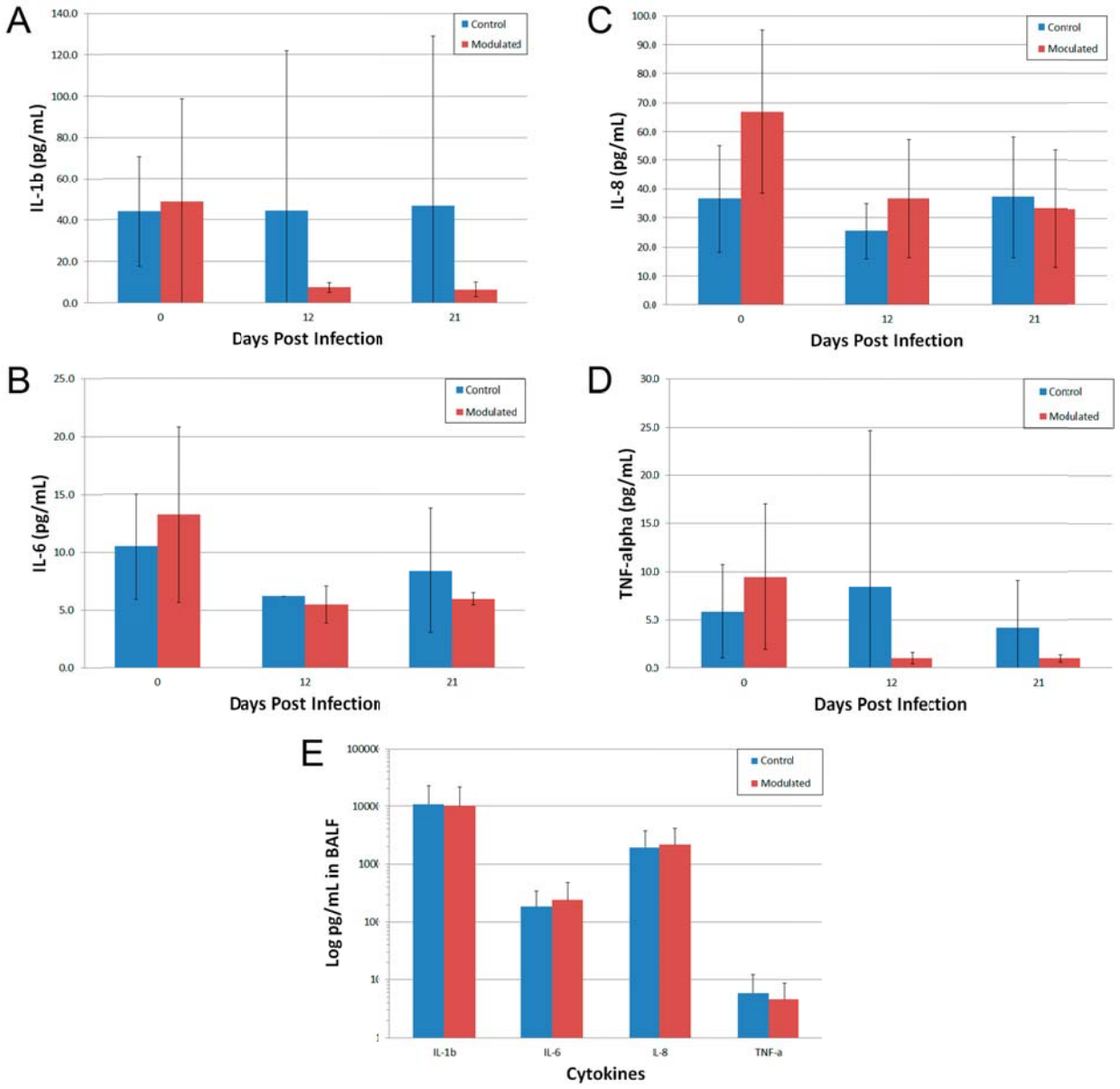

Figure 4.6: Cytokine concentrations in blood serum and BALF. The concentration of A) IL-1β, B) IL-6, C) IL-8 and D) TNF-α in blood serum at multiple time points after experimental infection with *M. hyopneumoniae* expressed as pg/ml. E) The concentration of IL-1β, IL-6, IL-8 and TNF-α in the BALF collected at euthanasia (103 days of age) expressed as log pg/mL.

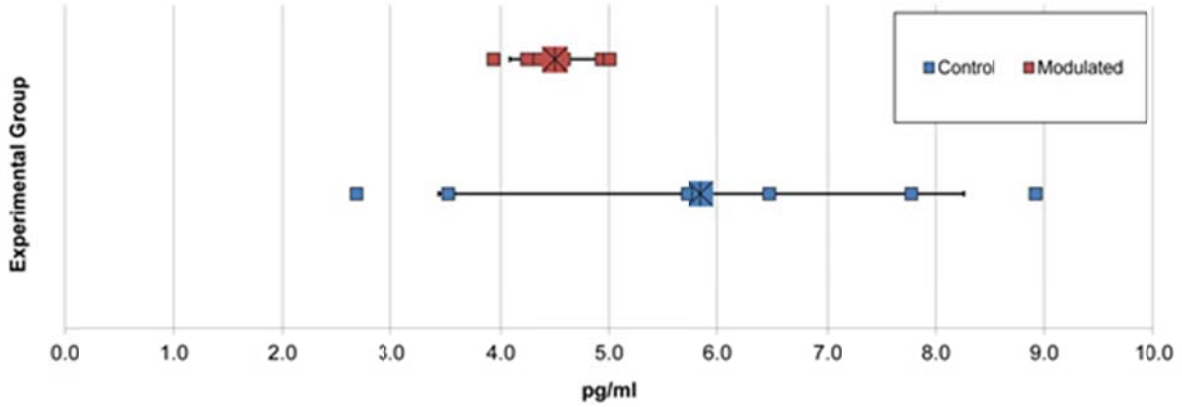

Figure 4.7: BALF TNF-α levels. Results expressed as pg/ml of TNF-α in the BALF. Difference in the variance of the two groups is statistically significant (*p*=0.0153).

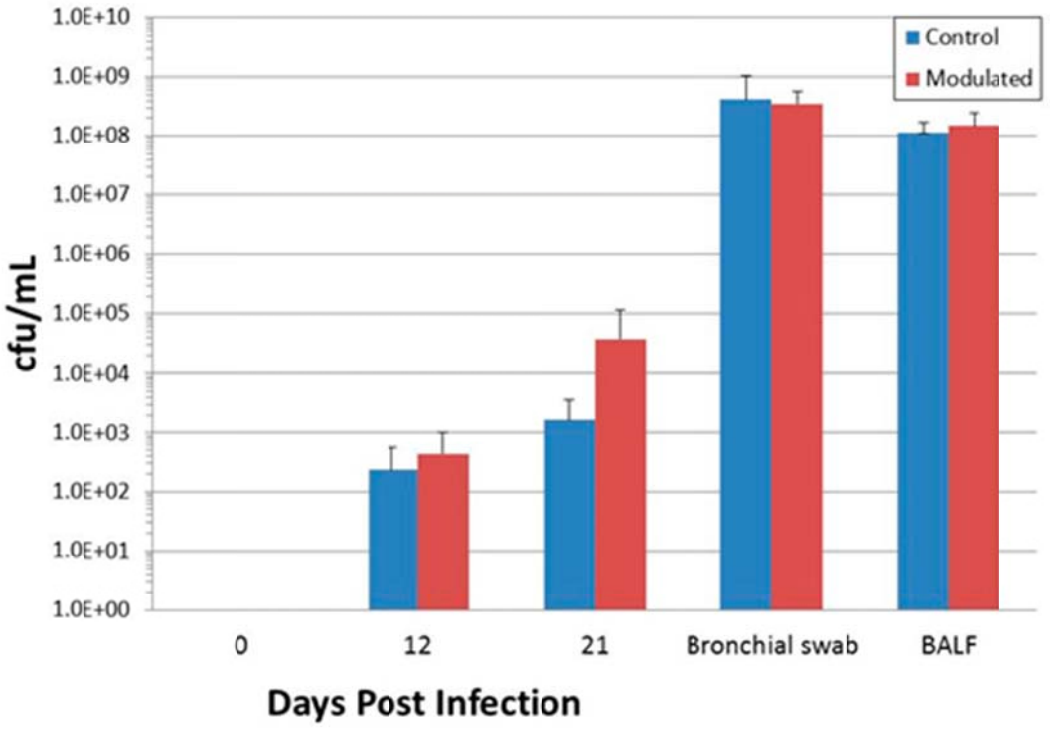

Figure 4.8: *M. hyopneumoniae* bacterial load. *M. hyopneumoniae* bacterial load in the lungs at multiple time points after infection. Expressed as the number of colony forming units (cfu) per mL.

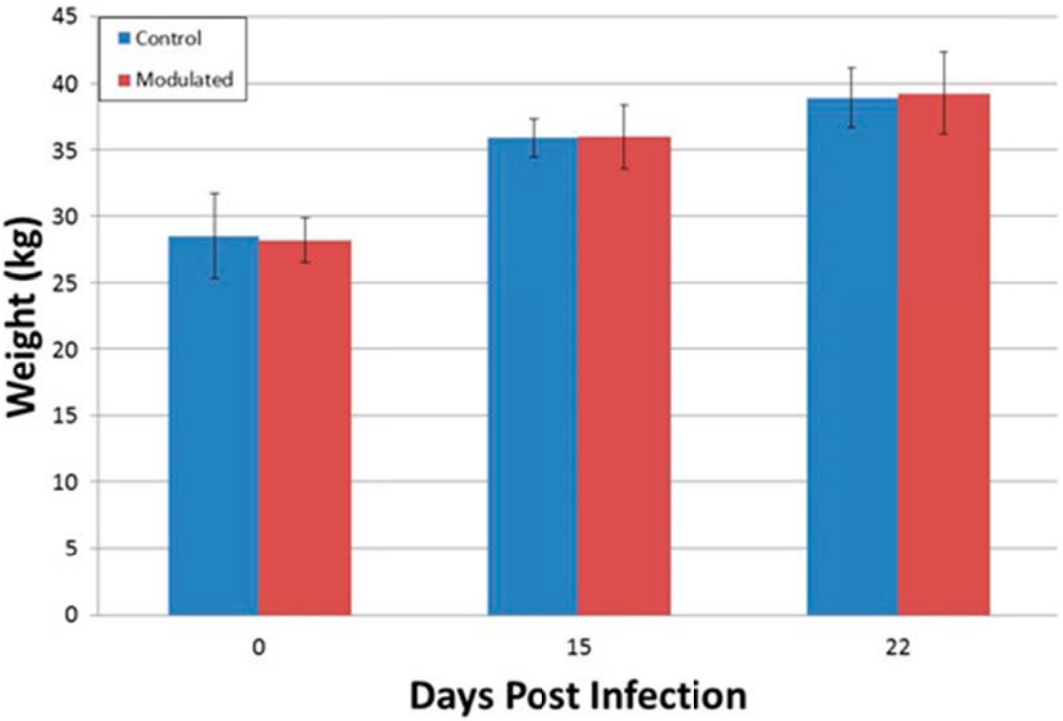

Figure 4.9: Daily weight gain. The weight in kg of pigs in each group at multiple time points after *M. hyopneumoniae* infection.

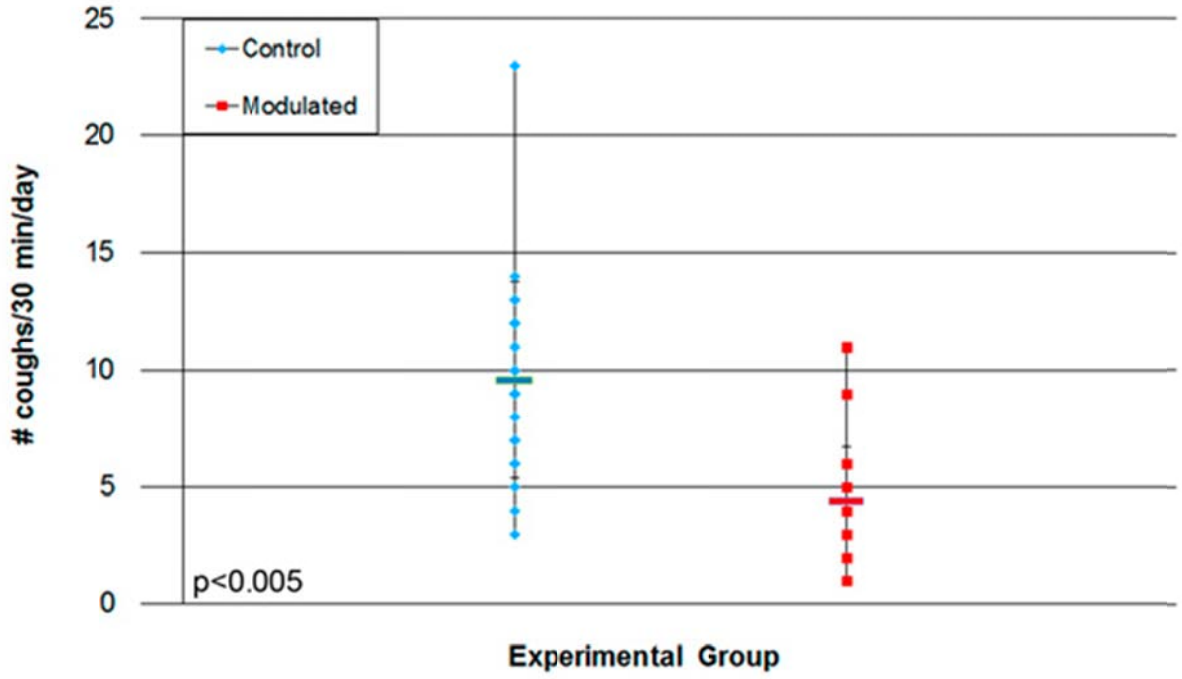

Figure 4.10: Coughing scores of experimentally infected pigs. Scores were obtained by observing pigs for 30 min/d at the same time every day and recording the number of coughs per group. Observations started at 12 dpi and continued for the duration of the study ($p < 0.005$).

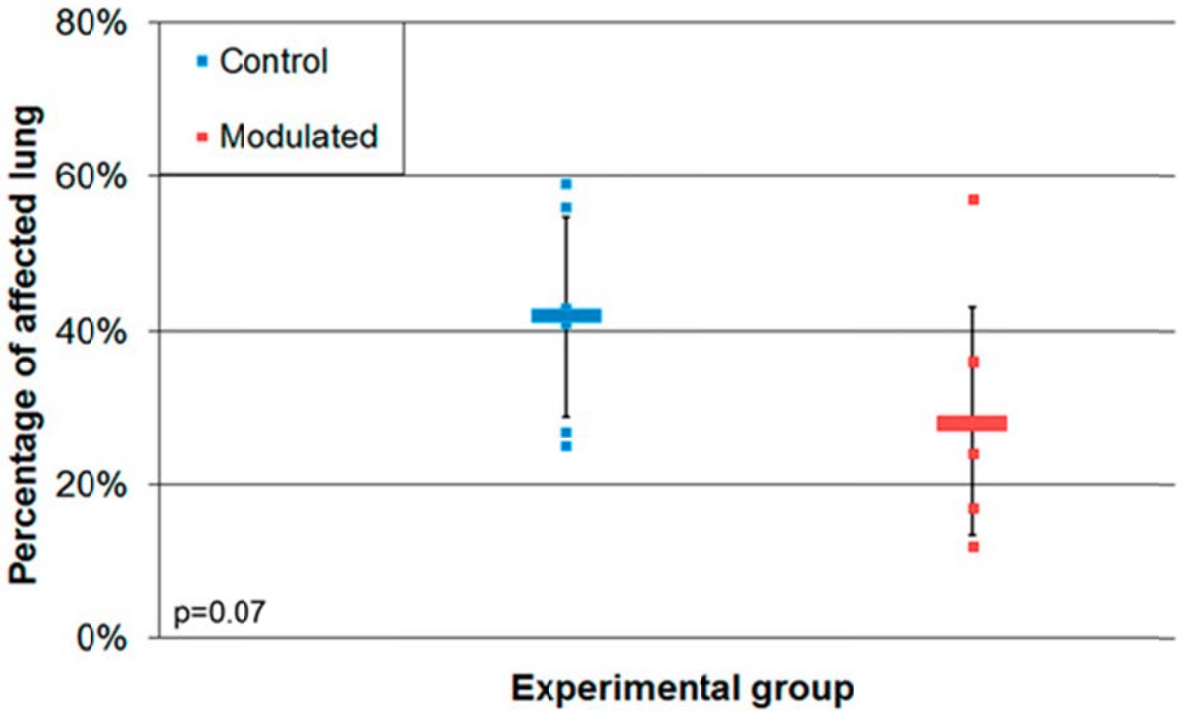

Figure 4.11: Macroscopic lung lesions of pigs infected with *M. hyopneumoniae.* The lungs were removed at euthanasia (35 dpi) and evaluated blindly. Expressed as percentage of lungs with lesions (p = 0.07).

Parameter Measured	Control	Modulated	P-Value
DTH (1,000 Units)	0.643	1.19	0.07
Cough Observations (coughs/30 mins)	9.6	4.4	<0.005
Lung Lesions (lobe area)	42%	28%	0.07
TNF-alpha Variance	5.8	0.18	0.0153

Table 4.1: Summary of host responses.

Days Post Infection	Control	Modulated
0	0/6	0/6
2	0/6	0/6
5	0/6	0/6
7	0/6	0/6
9	0/6	1/6
12	2/6	5/6
14	6/6	6/6
21	6/6	6/6

Table 4.2: Seropositivity to *M. hyopneumoniae.*

CHAPTER FIVE

MODULATION OF SYSTEMIC IMMUNITY: SO NOW WHAT?

5.1 INTRODUCTION

The results from this study show a clear connection between GI microbiome composition and host immune responses. In addition, the work presented here shows the administration of an oral microbial inoculum, similar to fecal transplantation, is sufficient to modulate the GI microbiome early in life. While a connection between microbiome composition and host immune and health status has been well established by studies listed in chapter 2, many questions still remain. Little is known about what components/species present in the microbiome are responsible for the beneficial effects, including reducing the incidence of allergies, asthma and the maturation of both GI and systemic immunity. In addition, germ free mice have been shown to be protected from developing certain autoimmune diseases, suggesting that colonization by certain bacterial species is associated with increased risk of developing such disorders. While the specific mechanisms responsible for these associations are unknown at present, the importance of the GI microbiome in host health and the development of the host immune system has become well established. However, further studies in the field are required in order to deepen our understanding of the symbiotic relationship between the host and its GI microbiome.

5.2 EFFECTS OF MICROBIAL INOCULUMS ON MICROBIOME COMPOSITION OUTSIDE THE GI TRACT

In this study we report the alteration of the respiratory microbiome composition as an indirect result of oral microbial inoculation. To our knowledge this is the first instance that GI microbiome composition has been shown to have an effect on microbiome composition outside of the GI tract. While further studies are required to determine how the GI microbiome is involved in regulating microbiome composition outside the GI tract, we propose this regulation is due to modulation of the host immune system. Lymphocytes activated by GI microbial peptides presented by dendritic cells in peyer's patches travel to the mesenteric lymph nodes

for further differentiation, after which they travel through the circulatory system and home to the lamina propria due to upregulation of $\alpha_4\beta_7$ integrin on their surface [32]. $A_4\beta_7$ integrin recognizes mucosal addressin cell-adhesion molecule 1, which is highly expressed in the vasculature of mucosal surfaces. Once in the lamina propria, the plasma cells secrete sIgA into the GI lumen. Mucosal addressin cell-adhesion molecule 1 has also been shown to be expressed in high endothelial venules of the nasal-associated lymphoid tissue [153], suggesting that lymphocytes originating from the GALT can also home to and act in the mucosal surfaces of the respiratory tract, ultimately altering the respiratory microbiome composition. It is through this process that we propose GI microbiome composition modulates host microbiome compositions at mucosal surfaces outside the GI tract. However, further studies assessing the ability of lymphocytes to migrate from the mesenteric lymph nodes to the nasal-associated lymphoid tissue, as well as the sIgA specificity and production in the nasal-associated lymphoid tissue in animals with varying GI microbiome composition are required in order to support this hypothesis.

5.3 Microbial Inoculums for Improvements in Overall Health

The results of this project support the hypothesis that an oral microbial inoculation is sufficient for successful alteration of the gastrointestinal microbiome composition, and that this alteration will result in the modulation of systemic immune responses. In this study piglets were administered an oral microbial inoculum, resulting in a measurable improvement in host immune responses and reduced severity of infection to the respiratory pathogen *M. hyopneumoniae* compared to healthy controls. In addition, previous studies have shown reduced antibody levels and CD4+ T cell responses to the respiratory influenza virus in mice treated with a combination of antibiotics (vancomycin, neomycin, metronidazole and ampicillin) compared to normal mice [104]. Taken together, these results suggest that in addition to the link between the GI microbiome and allergic and autoimmune disorders, GI microbiome composition is also important for the development of systemic immune responses to infection, suggesting that early life exposure to microbial agents is extremely important for the overall health of an individual.

As evidence for the importance of contact with microbial agents for the improvement of overall health grows stronger, the question becomes how this knowledge can be used to reverse the trend of increased incidence of allergic and autoimmune disorders in developed countries where overall hygiene levels are higher than those of developing countries. While the underdevelopment of regulatory T cell populations has been associated with imbalances in Th1 and Th2 cell responses associated with allergic and autoimmune diseases [48], little is currently known about the mechanisms by which the GI microbiome affects regulatory T cell and general immune system development. Probiotic strains designed to promote the natural balance of the GI microbiome and reduce pathogen levels are currently being used to reduce allergic diseases in infants [36], [40], [90]. However our lack of understanding in how individual microbes effect systemic immune system development and microbiome composition is evidenced by the mixed results of probiotics treatments. Probiotics show reduced effectiveness at producing long term alterations in GI microbiome composition compared to fecal transplants [79], [82] in addition to requiring continuous administration for long term benefits to be achieved. As our understanding of how microbial communities work together and promote host immune system maturation increases, so should our ability to use fecal transplantations and other microbial inoculums to promote long term overall health.

As the rate of caesarean sections continues to rise around the world [154], further research needs to be done in order to determine the effects of non-vaginal delivery on health later in life. Studies have already shown alterations in GI microbiome composition in caesarean derived infants for up to 6 months of age [4], [28], [29], as well as increased risk of allergies and asthma in these individuals [30], [31]. The results presented here suggest that these individuals may also have reduced systemic immune responses due to their altered GI microbiome composition early in life, which could lead to an overall reduction in health over their lifetime. Studies involving germ-free mice inoculated with GI microbes show restoration of the previously underdeveloped GI and systemic immune system [68], [69], [87], [88]. This suggests the administration of fecal transplants shortly after birth could be used to improve immune responses in individuals born by caesarean section, administered antibiotics early in life, or any other individual whose GI microbial diversity is reduced due to a lack of early life exposure to

microbial agents. Future studies assessing the effects of a microbial inoculation on caesarean derived individuals could provide a better understanding of the importance of exposure to the mother's vaginal and fecal microbiomes on the development of host immune responses later in life. These studies could help improve our understanding of the importance of early life exposure to microbial agents and expand the use of fecal transplantations as standard practice for improvements in overall health. The knowledge gained from these studies could lead to the administration of a standardized fecal material designed to promote the healthy maturation of the host immune system when administered to at risk individuals early in life (Figure 5.1).

5.4 CONCLUSIONS

As our understanding of the connection between GI microbiome composition and development of the host immune system increases, we must expand our ability to use microbial inoculums as therapeutic tools. While this use is already well established in a variety of GI infections and disorders, more work is needed to ensure the continued progression of this therapy to the treatment of systemic disorders and the improvement of overall health. The application of such techniques to caesarean derived individuals, as well as others with reduced microbial diversity early in life could help curtail the increased incidence of allergic and autoimmune disorders currently seen in developed countries and improve the overall quality of life for these individuals.

5.5 FIGURES

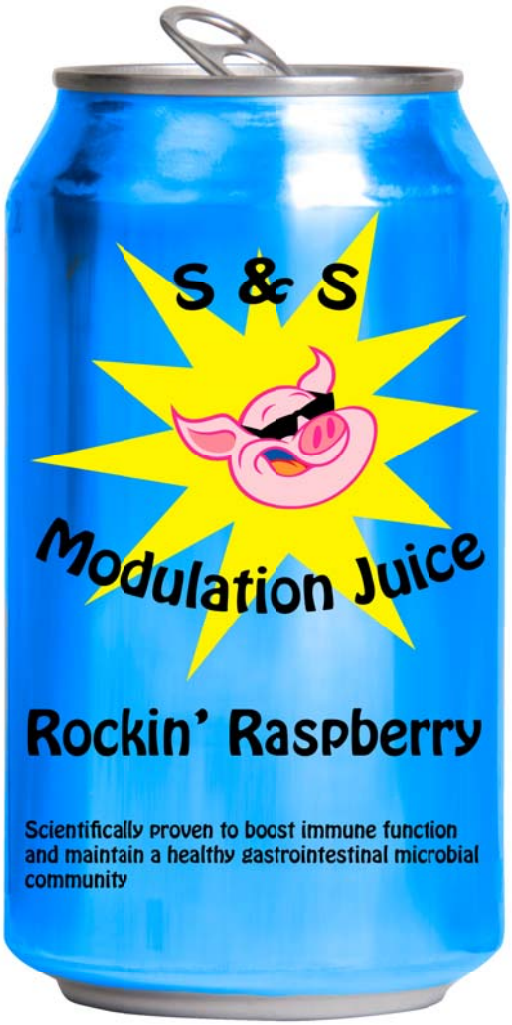

Figure 5.1: Microbial inoculums early in life for improvements in overall health. Research has shown a connection between GI microbiome composition and immune system development, development of allergic and autoimmune disorders, and immune responses to pathogenic infections. Continued research in this field could lead to a better understanding of the mechanisms responsible for this connection, as well as the specific bacteria/communities responsible for proper development of one's immune system. This could lead to the administration of microbial inoculums as standard practice to boost healthy immune system development in high risk individuals such as caesarean derived individuals and individuals exposed to high antibiotic use early in life.

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