EXAMINATION OF THE EFFECT OF REDUCTION OF PROBIOTIC SPECIES *LACTOBACILLUS* DUE TO BROAD SPECTRUM ANTIBIOTIC TREATMENT ON ORAL TOLERANCE

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ABSTRACT

THESIS: Examination of the Effect of Reduction of Probiotic Species *Lactobacillus* due to Broad Spectrum Antibiotic Treatment on Oral Tolerance

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Antibiotic usage is on the rise in industrialized countries and as a result the prevalence of autoimmune and atopic diseases has risen. The use of antibiotics is connected to a depletion of the microflora located within the gastrointestinal tract. The microflora contains a variety of different bacterial species, including some that are probiotic species, Lactobacilli and Bifidobacteria, which have a beneficial effect on the host. Probiotic species of bacteria are important for immune function due to their ability to regulate oral tolerance, a state of unresponsiveness to antigens that have been introduced orally to the host. The goal of this study was to assess the effect of broad spectrum antibiotic treatment on the probiotic species Lactobacilli and the resulting effect on the induction of oral tolerization to the antigen ovalbumin.

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INTRODUCTION

A Brief Overview of the Immune System

There are two branches that comprise the immune system, innate and adaptive [1]. The branches differ in the form of activation, level of specificity, and the cells included; however, the innate and adaptive branches are able to communicate and work together. The innate system is considered primitive because it functions in a less sophisticated manner than the adaptive immune system. The innate system reacts to general sequences of proteins found only in microbes and is the first line of defense for the body. The roles of the innate immune system include surveying, isolating and breaking down foreign particles that have entered the body. The adaptive immune system is able to respond to very specific antigen and decipher if the antigen is self or foreign. After activation by the innate immune system the adaptive immune system is able to regulate and control the intensity and duration of the immune response generated. The adaptive immune system is able to have memory that will allow for a faster immune reaction involving a higher amount of cells upon a second exposure. The immune system is able to function remarkably well in defending the body against harmful antigens due to the specific functions of both the innate and adaptive immune systems and the ability of the two branches to function together.

The Innate Immune System

In order to understand the connection between the innate and adaptive immune systems it is necessary to understand the process of the activation of the innate system [1]. The innate system includes neutrophils, dendritic cells (DCs), macrophages, natural killer cells (NK cells), and a variety of other cells. Neutrophils are granular cells that will typically be the first cells to appear at the site of infection. Neutrophils work as phagocytes to uptake foreign antigen. DCs are unique in appearance having long arms that extend in a nature similar to neurons and survey the body looking for foreign antigen. Once identified the antigen will be engulfed by DCs using one of the three different methods; phagocytosis, pinocytosis, or endocytosis. Macrophages are the largest cells of the immune system and generally found in various locations throughout the body including; gut, lung, connective tissues, liver, kidney, brain, and bone. The main function of macrophages is phagocytosis of foreign antigen. Natural killer cells are granular cells that comprise 5-10% of the cellular component of blood. NK cells are capable of killing other cells marked as infected by releasing cytotoxins directed at the cell. Any foreign particle, or antigen, that enters the body can bind to and activate the cells of the innate system. The job of the innate immune cells is to scan for general patterns of molecules found only on microbes. If one of the patterns are identified the foreign particle (antigen) is taken up by specialized cells called antigen presenting cells (APCs). APCs, including dendritic cells and macrophages, are able to engulf antigen and digest it to form a specific protein sequence called an epitope. The epitope is combined with a specific receptor,

the major histocompatibility complex receptor (MHC receptor), within the APC. After the epitope is joined with the MHC receptor, the complex is displayed on the surface of the APC. The MHC is specific for the individual host and is critical for communication between the innate and adaptive branches of the immune system. The antigen attached to the MHC will activate the cells of the adaptive immune system, B and T cells.

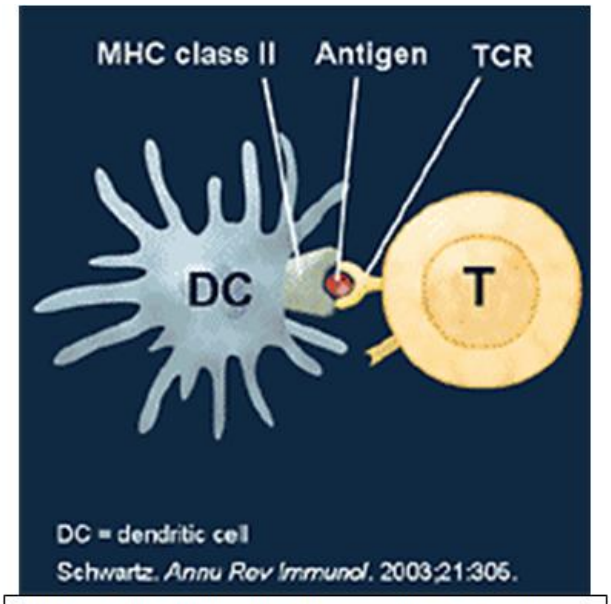

Figure 1 *Dendritic cell acting as antigen presenting cell.* Dendritic cells are able to work as antigen presenting by presenting antigen through the MHC receptor on their surface to the T cell receptor; activating the T cell.

The Adaptive Immune System

In addition to macrophages and DCs working as APCs one of the cells of the adaptive branch of the immune system B cells also functions as an APC. B cells are able to uptake foreign antigen through receptor-mediated endocytosis

[1]. The uptake of antigen allows it to be broken down and presented to T cells. In addition to presenting antigen to T cells, B cells are also able to be influenced by T cell interaction. The interaction occurs through the secretion of cytokines by T cells and will influence B cells to proliferate and secrete proteins capable of binding to an epitope called antibodies [1]. Antibodies can be membrane bound to serve as the antigen specific B cell receptor, or they can be secreted. As soon as a B cell comes into contact with an antigen for which the B cell receptor is specific, the B cell will become activated to become either an effector B cell or memory B cell. Effector B cells have a short life span and function to secrete antibodies into the blood flow. Memory cells will remain within the system for a longer duration of time than effector B cells and will become active upon subsequent exposures to the antigen for which it is specific. Either form of B cells will secrete antibodies at one point in time when activated by contact with antigen; however, the amount of time that the B cell is within the system will vary depending on the form of B cell.

Antibodies, in general, perform the following functions: increasing phagocytosis by binding to antigen, clearing foreign antigen from the body through complement activation or the process of agglutination, and preventing invading microbes from attaching to the cells of the body [1]. All antibodies are similar in structure; they all have a variable and a constant region. The variable region will determine the specific antigen for which an antibody will be specific for. The constant region will determine the antibody class. The five main classes of antibodies secreted vary in function and location due to their differences in

structure. The classes of antibodies (immunoglobulins) are: IgA, IgD, IgE, IgG, and IgM [1]. IgA, comprises 10 to 15% of antibodies present within serum and is found in bodily secretions and mucosal linings of the body. The least amount of information is known about IgD. It was first identified in a myeloma and the function of IgD remains a mystery. IgE is found in low concentrations within serum and is well known due to its role in hypersensitivity or allergic reactions. IgG is found in the highest amount (80%) within serum and is capable of passing across the placenta from mother to fetus. IgM is the antibody that makes up 5 to 10% of the antibodies within the serum and is secreted first after antigen contact. Despite the differences in function and location between the classes of antibodies they all play an important role in the immune system.

In an active immune response, different antibody classes with the same antigen specificity will be generated to ensure that the microbe is properly cleared. The B cell will first generate IgM after encountering antigen. It will be able to be detected within the serum after a period of 4 to 5 days, with a peak being found around 7 to 10 days. In order for the other classes of antibodies to be generated, class switching needs to occur [1]. This is a process where the immunoglobulin heavy chain genes are rearranged during antibody synthesis resulting in the generation of a different class of antibody. The order of antibody class production is IgM, IgD, IgG, IgA, and lastly IgE. The antibody that is generally detected in the highest amounts after class switching has occurred is IgG; with the peak in IgG detected around 14 days after antigen contact [1]. Upon a second exposure with the same antigen IgG is the first class of antibody

produced and the amount generated is 100-fold higher in amount than the amount of IgM or IgG from the first exposure. Class switching is an important mechanism that will allow for the different antibody classes to be generated.

The other main cell of the adaptive immune system is the T cell. It is activated through contact with the combination of MHC and epitope presented on the surface on an APC resulting in a specific and complex reaction [1]. There are different types of T cells. Some T cells with have a CD8 surface protein and will become cytotoxic T cells (Tc). Tc cells have the ability to directly kill cells displaying foreign antigen that the Tc cell specifically recognizes. The other main type of T cells has a CD4 surface protein and will differentiate into different forms of T helper cells (Th). Th cells secrete large amounts of cytokines, and thus are able to recruit and activate several immune cells, adaptive and innate, to aid in an active immune response. The presence of cytokines is an indicator of an active immune response and can identify the type of immune response that is mounted. Some common cytokines released by T cells include interleukin-2 (IL-2), IL-4, and interferon-γ (IFN- γ). IL-2 is able to cause the proliferation of T cells, B cells, and the activation of the innate cell, NK cell. IL-4 plays a role in the activation of the Th subset of T cells, more specifically T helper 2 cells (Th2 cells). IFN-γ also functions in the differentiation, activation and growth of T cells, B cells, macrophages, and NK cells. IFN-γ is the key cytokine in an active immune response. Once additional immune cells are activated through the release of the cytokines the newly activated immune cells will secrete cytokines leading to a chain of activated cells and the consequential release of cytokines.

The end result is an active immune response; a strong immune reaction with a large amount of innate and adaptive immune cells activated.

In contrast to an active immune response, suppressive immune responses can be mounted. They are directed by a different subset of CD4 T cells, Tregs, and function antagonistically to the functions of Tc and Th cells. Tc and Th cells are involved in stimulating active immune responses, while Treg cells induce suppressive immune responses. Identification of Treg cells is difficult, but can be accomplished through the combination of surface proteins and the expression of transcription factors. Treg cells express CD4 and CD25 (generally expressed by T cells following antigen stimulation) surface proteins; however these surface proteins are expressed on other subsets of T cells [2, 3]. Therefore, expression of the transcription factor forkhead box P3 (FoxP3) has also been used as a means to identify Treg cells [2, 4, 5]. Tregs will either develop in the thymus, natural Tregs, or by interaction with antigen within the periphery, adaptive Tregs. Natural Tregs express CD4, CD25 and FoxP3 immediately [4], while adaptive Tregs are stimulated to express FoxP3 in the presence of the suppressive cytokine TGF-β. The TNF cytokine receptor and CTLA-4 are also located on the surface of Treg cells [2, 3]. The main types of natural or adaptive Treg cells that have been identified to date are Tr1, and Th3. Tr1 and Th3 cells are able to be triggered through antigen introduced orally [6]. The unique property of Tr1 cells is that they are also activated when IL-10 or TGF-β is found in high concentrations [2, 5]. TGF- β is thought to be secreted by nearby dendritic cells causing the proliferation of immature T cells into Tr1 cells [7]. Th3 cells need IL-4 to be

present for development [6]. Upon activation Tr1 cells will secrete high amounts of IL-10 and IL-5 and low amounts of IL-2 and IL-4. Tr3 cells will secrete the cytokines IL-4, IL-10, and TGF-β upon activation [6]. Th3 and Tr1 cells are able to function in a suppressive immune response through the release of TGF-β and IL-10. Different forms of Tregs will function in a suppressed immune response. The differences between the types of Tregs are found in either the cytokines they require in order to proliferate or the cytokines they release after activation.

The exact mechanism behind the activation of Treg cells and the suppressed immune reaction are not known, however, several theories exist. The cytokines secreted by Tregs are important in the suppressed immune reactions and include TGF-β and IL-10; which work against the cytokines of an active immune reaction, IL-2, IL-4, and IFN- γ [1]. The presence of TGF-β is vital for activation of Tregs to occur as well as the inhibition of the proliferation of Th1 and Th2 cells [2, 7]. TGF-β, by blocking required transcription factors, is able to block Th1proliferation and thereby prevent an active immune response [7]. The proliferation of Th2 cells is prevented by TGF-β stopping IL-4 activity. Tregs are also thought able to control Th1 or 2 cells by either decreasing the amount of IL-2 or through direct contact with the Th1 or 2 cell [2, 5]. Importantly, some forms of Tregs can also be activated by contact with antigen introduced orally [2, 6]. The induction of antigen orally could lead to the generation of APCs that are specific for the antigen introduced [6]. The activated APCs would then lead to activating T cells to become Tregs and secrete suppressive cytokines including TGF-β, and IL-10. IL-10 is able to increase proliferation of B cells leading to an increase in

the amount of IgA as well as aid in the development of T cells into Treg cells [1, 8]. Dendritic cells found in Peyer's patches are able to secrete large amounts of IL-10, making them an important cells for a suppressed immune reaction to occur [9, 10]. Despite the mysteries that remain regarding the specific details of a suppressed immune reaction it is known that Th cell proliferation is capable of being prevented via secretion of suppressive cytokines by Tregs; which is vital for the immune system to function properly.

An Introduction to Oral Tolerance

Oral tolerance is defined as a state of unresponsiveness (or hypo-responsiveness) that occurs to orally introduced antigen upon a second exposure [11], and it is dependent upon the activity of Treg cells. This state of tolerization was first observed in 1911 by Wells in guinea pigs [11, 12]. After guinea pigs were fed hen egg protein, the reaction observed was a state of protection to the hen egg protein as opposed to a state of activation. Typically guinea pig contact with hen egg protein will result in anaphylaxis; however anaphylaxis was not found in guinea pigs that were fed hen egg protein indicating tolerance.

The state induced during oral tolerance is very important. Oral tolerization is a method of controlling the immune system by decreasing immune responses to common antigens [13]. Examples of common antigens are found in foods or bacteria residing within the gastrointestinal tract [6, 14]. Common antigens are not pathogenic and should not cause an activated immune reaction [14]. Function of the immune system is important for a host, however, for the immune

system to be functioning correctly active immune reactions should only occur with an antigen that is harmful. Tolerance is vital to control the reactions that occur within the immune system, however in some cases reactions are not properly controlled for some reason autoimmune diseases are developed. Oral tolerance can also be used to suppress autoimmune diseases after development [6, 14]. Autoimmune diseases found to be controlled through oral tolerance include arthritis, diabetes, colitis, allergies, and experimental autoimmune myasthenia gravis. The range of diseases that oral tolerance can suppress is vast in nature. Other diseases that have been able to be suppressed with oral tolerance in the experimental setting include nerve injury, stroke, and atherosclerosis. Oral tolerization is able to suppress the previously listed diseases by orally introducing a reactive form of the antigen in order to change the form of immune response that is generated.

The Mechanisms for Inducing and Maintaining Oral Tolerance

The specifics regarding the orally-tolerized state still remain unknown. While the location where the tolerization occurs remains a mystery [15], certain tissues are known to be required for the induction of oral tolerance. The removal of the spleen in rats is connected with an inability to induce the orally-tolerized state [16]. Gut associated lymphoid tissue (GALT), including peyer's patches (PP) and mesenteric lymph nodes (MLN), is also necessary for the induction of oral tolerance [12, 15, 17, 18]. Mice that did not have PP or MLN were not able to be tolerized orally, but could be tolerized to TNP-OVA through intraperitoneal

injections of TNBS [14, 15]. While the exact location where oral tolerization occurs may remain a mystery, it is clear that these tissues are necessary and that may presumably be because these tissues are sites where dendritic cells and Tregs can interact.

Another important requirement for tolerance for which a mechanism is not completely understood is Treg activation. One of the functions of dendritic cells (DCs) is the ability to induce Tregs. When infection or antigen from vaccines are present DCs will mature and move to areas that have a high number of T cells present [19, 20]. The DCs will work as an APC and present antigen to the Tregs that will active the Tregs to create a suppressed immune response. There are many different forms of DCs, including some populations within the gut, specifically the Peyer's patches that are less mature than other DCs. The immaturity results from a higher percentage of MHC inside of the cell membrane instead of being located on the outside of the DC [20]. DCs, including the less mature form in the gut, have been found to secrete high amounts of IL-10 or TGF-β when in the presence of immature T cells to push them to become Treg cells. It has been found that the presence of DCs is necessary for a suppressed immune response, created by Tregs, to occur [10]. After Treg activation the cytokines secreted by the Tregs, IL-10, will work to block further DC maturation to maintain the suppressed immune reaction exerted by the Tregs.

In addition to tissue location, DC and Treg activation, dose of antigen plays a role in oral tolerization. It is thought that upon the second exposure to antigen, Tregs, activated by DCs, will cause a suppressed response due to their

cytokine secretion (as opposed to an activated response), or possibly Th and Tc cells specific for that antigen will undergo the process of anergy or be deleted through apoptosis [6, 17]. Anergy is a state where T cells specific for a certain antigen become unresponsive to that specific antigen. The difference in T cell responses, suppressed or anergic, is due to the antigen dose and length of exposure to the fed antigen. Repeated low doses are connected with a switch from Th1 and Th2 responses to a Th3 response [15], however, anergy or deletion has been observed when a high amount or prolonged dose is given [15, 21, 22]. A low dose of antigen is any amount under 1mg given repeatedly and a high dose of antigen is equivalent to or greater than 5 mg given repeatedly or a single dose of at least 20 mg [21, 23, 24]. The differences between the T cell reactions are due to the differing levels of cytokines secreted. Low doses of antigen cause the regulatory T cells to secrete TGF-β, IL-4, and IL-10, which act to suppress an immune reaction [17, 23]. A higher amount of TGF- β will be observed after low doses of antigen than found in other immune reactions [21]. A high dose of antigen fed orally is connected to a decreased level of IL-2 secreted by lymph node cells and an absence in T cell proliferation [23]. High doses of oral antigen result in the decrease in activity or even deletion of Th1 and Th2 cells. When the Th1 and Th2 cells are deleted a different set of cells that secrete TGF-β, including Treg cells, have been found to survive which are necessary for suppressed immune reactions [23]. The tolerized state is important for an individual, however, for it to be induced the dose and length of exposure needs to be controlled or else anergy will occur.

An additional factor important in the state of oral tolerization is bacteria that reside within the gastrointestinal tract (GI tract) [18]. At birth the GI tract is sterile; however, as the tract begins to digest food, bacteria start to reside within the GI tract [18, 25]. The process of developing the normal flora takes time and is important for regulation of the immune system [25]. At first the bacteria that begin to colonize the tract come from the flora of the mother, however, through time and different contractual or dietary exposures, the flora will shift in variety [13, 26]. The main species of bacteria that will initially reside within the GI tract are facultative, or anaerobic, in nature. As time passes the bacteria within the GI tract shifts to be predominately lactic acid and coliforms [26]. By adulthood as many as 500 different species could reside and make up 35-50% of the volume within the GI tract [25-27]. The bacteria that become the normal flora are important for preventing the over growth of harmful bacterial species. The process of developing the natural flora of the GI tract is very important for the individual. The process of developing the natural flora within the GI tract takes time and many interactions, however, is very important because of the beneficial effects of probiotic bacteria to the host.

Probiotic Bacterial Species

A portion of bacteria found within the GI tract are probiotic bacterial species. Probiotics have many beneficial effects on the host including creating an immunological barrier within the GI tract, providing signals for proliferation of lymphoid cells, creating a balance between suppressive and activating cytokines,

and importantly helping induce the state of oral tolerance [25, 26]. Probiotics are able to increase resistance to nonspecific microbial antigens that can cause disease [28]. A healthy individual will have a balance between probiotic and pathogenic bacterial species within the GI tract [27, 28]. Treatment with probiotics can be given to help regulate the GI tract in conditions where the balance has been disturbed [26]. This is a way to regulate the immune response to harmful antigen and decrease immune response to self antigens.

The two most common intestinal probiotic bacterial species are Bifidobacteria and Lactobacilli [25, 28]. Both Bifidobacteria and Lactobacilli are gram positive bacteria resistant to both bile and acid allowing them to easily live within the GI tract [29]. Bifidobacteria grow best in anaerobic conditions while Lactobacilli can grow anaerobically, but prefer 2-10% oxygen [30, 31]. The optimum temperature for Lactobacilli growth is between 30 and 40° C and 37 to 41° C for Bifidobacteria growth [31]. Lactobacilli are typically found within the mouth, GI tract, and in the vagina and do not generally cause disease within individuals [30, 31]. Areas where Bifidobacteria are commonly found include the mouth and GI tract of individuals [31]. Similar to Lactobacilli, Bifidobacteria are generally considered non-pathogenic. Bifidobacteria and Lactobacilli have different growth conditions, however, are able to reside together with the GI tract as the most common probiotic bacterial species.

Probiotics and Immune Regulation

Probiotic bacteria have a close connection with the immune system and are able to carry out a variety of actions that aid the immune system, both suppressed and innate immune reactions. Probiotic strains are able to reduce inflammation via Tregs as well as increase the maturation rate for DCs; which affect the suppressed immune reaction. Probiotics are able to function within the innate immune system by increasing immune cell proliferation and antigen presentation. The type of cytokines secreted, as well as, the type of immune response generated are affected by probiotics. There are many different ways that probiotics are able to benefit the immune system which are important in the process of regulating the immune system.

Probiotic bacteria have a great effect on immune activity including both innate, adaptive, and suppressed immune responses. This occurs by increasing the proliferation of macrophages and the amount of antigen presented by APCs [25, 26]. The addition of probiotics, specifically *Lactobacillus casei* and *Lactobacillus bulgaricus*, can cause an increase in proliferation of macrophages [32, 33]. Lactobacilli increase the ability of APCs to take up antigen to be presented [29]. This is accomplished by increasing the amount of MHC located on the surface of dendritic cells or by increasing the amount of cytokine expression [29, 34]. By increasing the proliferation of macrophages and antigen presentation probiotics are able to boost the innate immune system.

Probiotics, including Lactobacilli strains, can affect Tregs and DC cells, which are important for a suppressed immune reaction. *Lactobacilli casei*

reduces the amount of inflammation to the skin as a result of allergies, considered to be regulated by Treg cells [35]. Other Lactobacilli strains are capable of decreasing the amount of time necessary for maturation of DCs. By allowing DCs to mature quicker the ability to activate Tregs to cause suppressed immune reactions can be increased over the amount of standard T cell activation causing an active immune response. Dendritic cells are thought to have a large interaction with probiotic bacterial species within the GI tract [27, 35]. Probiotics are able to control the cytokines secreted by dendritic cells. The interaction that occurs between dendritic cells and probiotics are critical for the activation of suppressed immune reaction led by Treg cells and occurs through toll-like receptors, generally a method of activating the innate immune system [35]. The cytokines released, IL-2, IL-4, and IL-10 by dendritic cells that have come into contact with probiotic bacteria, *Lactobacillus rhamnosus,* activates Treg cells leading to a hyporesponsive state [35, 36]. The activated Treg cells will secrete IL-10 and TGF-β resulting in a suppressed immune state. Probiotics are located within the GI tract; however the effects of probiotic bacterial species on the cells activated can result in a suppressed immune reaction. Once the suppressive immune cells have become activated they are able to travel throughout the body and can reduce active immune responses generated in other locations.

In addition to regulating APCs and Tregs, probiotics influence antibody production. A strain of Lactobacilli, *Lactobacillus casei*, when administered to mice increases the amount of B cells secreting IgA [33, 37]. IgA is found most abundantly within the mucosal linings is necessary for the beginning of oral

tolerance to occur [38]. Antigen will be first introduced within the mouth, which should have IgA present, and typically IgA will be unreactive, allowing the antigen to enter into the GI tract to create an orally tolerized state. In addition to increasing the amount of antibody-secreting B cells, the amount of antigen-specific antibodies is also increased with probiotic treatment. *Bifidobacterium bifidum* when given orally increases the amount of overall antibody generated to ovalbumin (OVA) [25, 33, 39]. Similar results were found when *Bifidobacterium breve* was administered to mice along with the cholera toxin [25, 40]. The end result yielded a higher amount of IgA from the feces and IgA production to the cholera toxin when compared to mice that did not receive the probiotic treatment. It has been demonstrated that different probiotic strains are able to increase either the amount of plasma B cells or antigen-specific antibodies and therefore able to increase the amount of suppressive immune activity that occurs.

Taken together, these findings demonstrate the important role probiotic bacteria play in the induction of oral tolerance.

Antibiotics, Probiotics, and Immune Function

Bacterial growth, including probiotics, within the GI tract is affected by the use of antibiotics [41]. Antibiotics are necessary for the treatment of bacterial infections; however, there are many different side effects that can occur with their usage. Increasing antibiotic treatment is correlated with decreasing bacterial levels within the GI tract as well as decreasing absorption of vitamins and minerals from food intake. The bacteria decreased with antibiotic usage include

the probiotic strains that assist in suppressed immune reactions found in oral tolerance. Overuse of antibiotics has also been connected with the occurrence of atopic and autoimmune diseases including allergy, asthma, and irritable bowel disorders (IBD) such as Crohn's and ulcerative colitis [42, 43]. Antibiotics are necessary at times; the full effect, including that on the immune system, remains unknown.

There are many different classes of antibiotics available. Five classes of antibiotics that are commonly prescribed to treat are aminoglycosides, β-lactams, macrolides, nitromidazoles, and glycopeptides. Common aminoglycosides are used against gram-negative bacteria and include gentamycin, kanamycin, neomycin, and streptomycin [44]. They are able to stop bacterial growth by binding to the small ribosomal subunit and thereby stopping the production of bacterial proteins [30]. β-lactams include both penicillins and cephalosporins (including cephalexin). The current theory for the mechanism of β-lactams is cross-linking the peptidoglycans within the bacterial wall is stopped and the osmotic pressure is affected [30, 44]. The end result is lysis or break down of the bacterial wall. β-lactams are broad spectrum antibiotic and are most effective against gram-positive bacteria. There have been many different generations of cephalosporins and each generation is effective against a different type of bacteria. The generations and targeted bacteria are: 1st generation, primarily gram-positive, 2nd generation, gram-negative and gram-positive, 3rd generation, gram-negative bacteria [30]. Macrolides, including erythromycin, are broad spectrum antibiotics effective against gram-positive and some gram-negative

bacteria [30, 45]. The mechanism employed by macrolides is the binding to the 50s subunit of the ribosome preventing elongation of chain of peptides during the process of protein synthesis. Erythromycin and cephalexin have side effects resulting in the development of colitis or the overgrowth of the bacteria *Clostridium difficile* which are indications of the depletion of probiotic bacteria within the GI tract [46]. The nitroimidazole class of antibiotics includes metronidazole [47]. The target for metronidazole includes anaerobic bacteria, specifically gram negative bacteria. The mechanism for action of nitroimidazoles is to disrupt the nucleotides within the bacterial DNA. The class of glycopeptides is a narrow spectrum antibiotic, which includes vancomycin and targets some gram positive bacteria. Glycopeptides work by stopping the cross-linking of peptidoglycan chains [30, 45]. The result is a poor quality peptidoglycan chain that creates a weak bacterial wall that is easily lysed through osmotic pressure. Additional effects of vancomycin include altered permeability of the bacterial cell membrane and altered RNA synthesis [45]. There are many different forms and classes of antibiotics that have been effective in the treatment of bacterial infections. The mechanisms employed are as vast as the bacteria that the antibiotics are used against.

The specific antibiotics that were included within this study are erythromycin, cephalexin, metronidazole, and vancomycin. Erythromycin and cephalexin both target gram positive bacteria and some gram negative, therefore should be effective against gram positive Lactobacilli and Bifidobacteria [30, 45]. Erythromycin is used to treat upper and lower respiratory infections, skin

infections, diphtheria, and acute pelvic inflammatory disease; when all of the above are suspected to be caused by a bacterial infection [45]. Cephalexin is prescribed for respiratory infections, ear infections, bone infections, and genitourinary tract infections; when all of the above are suspected to be caused by bacterial infections. Thus, treatment of mice with these antibiotics serves as our experimental variable when examining oral tolerance induction. Metronidazole is not known to kill probiotic species and was included in our study as a control for antibiotic treatment that may alter GI bacterial populations not considered to be probiotics [30, 47]. Metronidazole is prescribed to treat abdominal infections, lower respiratory tract infections, skin infections, meningitis, brain abscess, bone infections, endocarditis, and gynecologic infections; all of which are due to a bacterial infection [48]. Vancomycin is prescribed to individuals with bacterial infections, specifically pseudomembranous enterocolitis [45]. It has been reported to kill gram positive organisms, including *Lactobacillus* spp., *Staphyloccus aureus*, and *Clostridium difficile.* Recent work has demonstrated that it targets Lactobacilli species differently [49]. Generally the effect of antibiotics on pathogenic bacteria are known, however, the effects of antibiotics towards non-pathogenic strains of bacteria are not as easily known; only further complicating the issue of susceptibility of Lactobacilli to vancomycin. Research by Cooper et al. and Riebel et al. has found that Lactobacilli generally will be sensitive to vancomycin treatment [49-51]. However, in the research into the rare situation when *Lactobacillus* spp. are responsible for infection Woodford et al. concluded Lactobacilli were resistant [49, 52]. To address some of the

controversy Hamilton-Miller et al. identified 42 different strains of Lactobacilli and treated all of the strains with different antibiotics including vancomycin [49]. It was concluded that vancomycin has an effect on some strains of Lactobacilli, i.e. *Lactobacillus acidophilus* or *Lactobacillus delbreukii*, however, other strains, i.e. *Lactobacillus casei*, are resistant. Additional research has been performed by other groups including Billot-Klein et al. and Handwerger et al. that corresponds with the finding that *Lactobacillus casei* is resistant to vancomycin treatment [49, 53, 54]. Taken together, these reports demonstrate that the effect of antibiotics on probiotic bacterial species could have serious consequences upon breaking down oral tolerance, yet little research has been done to fully investigate this possibility.

Probiotics, Antibiotics, and Oral Tolerance

There have been several studies performed on oral tolerance and the effect of probiotic species on the immune system. One of these studies was performed by Prioult et al. who studied the effect of flora within the GI tract on oral tolerization [55]. The methods used by Prioult et al. included feeding germ-free mice probiotic strains of bacteria including *Lactobacillus paracasei*, *Lactobacillus johnsonii*, and *Bifidobacterium lactis*. Eighteen days after biotic treatment a portion of the mice were orally tolerized with whey protein and then immunized with bovine β-lacto-globulin (BLG). When the mice were euthanized, samples of the GI tract were harvested to assess the bacterial levels of the fed bacteria in addition to harvesting serum to determine BLG- and OVA-specific IgE

and BLG-specific IgG_1 and IgG_2 levels via ELISA. The levels of BLG-specific antibodies were reduced in mice that had been orally tolerized with whey protein, with variations in the amount of immune activity depending on the bacterial strain fed to the mice. They concluded that flora within the GI tract is important for oral tolerization to occur. The strain of probiotic bacteria cultivated within the GI tract also has an effect on oral tolerization, some strains *Lactobacillus paracasei* are better at inducing oral tolerance than other, i.e. *Lactobacillus johnsonii*. An additional conclusion was when the flora was absent or limited to one strain, the amount of tolerance is also reduced. It is important to understand the connection that occurs between probiotic bacteria and oral tolerization before it is altered with the introduction of antibiotics that was explored in our study.

While the importance of intestinal microflora in the induction of oral tolerance has been demonstrated, there has been little research performed on the effect of antibiotic treatment on the process of oral tolerization. This is important to explore due the increase in the usage of antibiotics that is occurring within industrialized countries as well as the increase in the prevalence of atopic and autoimmune diseases. One study has been performed that is similar to the proposed work. Pecquet et al analyzed a mixture of gentamycin and vancomycin antibiotics, as well as ibuprofen on the process of oral tolerization [56]. Balb/c mice were treated with either ibuprofen (50 mg/kg of body weight) or the antibiotic mixture (124 mg/kg body weight for gentamycin and 500 mg/kg body weight for vancomycin) for 7 days and then 5 days after the beginning of treatment mice were fed with whey proteins that contained β-lactoglobulin (BLG)

to induce the state of oral tolerance. Four days after the feeding of BLG the mice were immunized intraperitoneally with BLG (80 mg) and five days later the mice were sacrificed. Their findings were that antibiotic and ibuprofen treatments altered the process of oral tolerization. The disruption of oral tolerization was analyzed through analysis of IgG specific for the antigen β-lactoglobulin via ELISA. It was concluded that antibiotic treatment does alter the state of tolerization that can be induced orally. The work by Prioult et al. helped to establish methods and rationale to continue to evaluate the effect of a range of antibiotics on oral tolerance.

Previous Work Done in Our Lab

Published literature has demonstrated that probiotics are beneficial and connected to the induction of the suppressed immune response that occurs in a state of oral tolerization. Prior tolerization studies testing the interaction with probiotic bacteria used germ-free mice, but it is also important to study tolerization in mice kept in standard environments that come into contact with antigens. It is also very important to study different antibiotics because of the differences in the bacterial targets between the different classes. It was found that vancomycin/gentamycin can alter the effect of oral tolerization by Prioult et al., however there are many other antibiotics that are commonly taken by individuals. The effects of antibiotics with other bacterial targets on oral tolerance should also be studied. It is for these reasons that our lab is investigating the effects of several different antibiotics on oral tolerance induction. In order to test

the effect of antibiotics on oral tolerization and probiotic levels, an *in vivo* study similar to Prioult et al. was performed by our lab with some differences to note. The antigen used for tolerization was OVA and the antibiotic used was erythromycin. In order to assess the effect of antibiotic on oral tolerance *in vivo* Balb/c mice were selected and fecal samples were obtained. The samples were diluted and then plated to confirm the presence of Lactobacilli colonies. Once the presence of Lactobacilli was confirmed the mice were fed erythromycin (250 µg/mL) for 7 days using a syringe. After the 7 days of antibiotic treatment ovalbumin (OVA) (10 mg/mL) was fed to the mice for an additional 14 days using a syringe. Post-OVA feedings all of the mice were immunized intraperitoneally and then sacrificed 7 days later. Serum was collected to run for analysis of antibody production via enzyme linked immunosorbent assay (ELISA). The data was analyzed by one-way ANOVA and SEM analysis. The differences between the orally-tolerized mice that had been treated with erythromycin and the other groups of mice in terms of the immune response generated, determined by levels of OVA-specific IgG, was statistically significant. The introduction of erythromycin during the period of oral tolerization resulted in a significant increase in the amount of OVA-specific IgG in comparison to orally tolerized mice that did not received erythromycin treatment or non-tolerized mice that did or did not receive erythromycin. This is evidence that the introduction of antibiotics during the period when oral tolerization occurs is capable of interfering with the tolerization and the immune response is greater than mice that never were tolerized.

The goal of our specific research in this proposal was to expand the study on antibiotics and the process of oral tolerization. This occurred through testing a variety of other broad spectrum antibiotics; metronidazole, vancomycin, and cephalexin on the process of oral tolerization. Both vancomycin and cephalexin are targeted towards gram-positive bacteria, which include some strains of *Lactobacillus* spp. and *Bifidobacterium* spp. It was expected that vancomycin and cephalexin will be able to knock out the probiotic species within the GI tract and alter the process of oral tolerization as was observed with erythromycin treatment. Metronidazole is targeted towards gram-negative anaerobic bacteria and therefore should not impede the process of oral tolerization to the severity that was seen with erythromycin. By testing a variety of broad spectrum antibiotics a larger picture was gained on the interaction of probiotic bacteria during oral tolerization and the effect of antibiotic treatment on that process.

Hypothesis

Antibiotic usage decreases intestinal microbiotic populations, including the probiotic species Lactobacilli, impeding oral tolerance and heightening the response of the immune system in Balb/c mice.

MATERIALS AND METHODS

Mice

We used Balb/c mice (Jackson Laboratories, Bar Harbor, ME) to examine the effect of antibiotic treatment on the induction and maintenance of oral tolerance. Mice were housed individually in non-pathogen free conditions in CL 277 and were maintained by Kelly N. Rider (KNR) under the supervision of Dr. Heather Bruns (HAB). Prior to experimentation approval from BSU ACUC was obtained for the overall project, and modifications specific for this proposed research were submitted for review and approval.

Overview of an Individual Study

There was a minimum of three mice used per group; in order to run statistical analysis. Each mouse receiving treatment (control or antibiotic) was fed a total volume of 100 µL using feeding needles. The groups of mice receiving treatment were given the following solutions: Ovalbumin (OVA) at a concentration of 10 mg/mL (or in some experiments a high dose of 200 mg/mL was given), and one of the following antibiotics; cephalexin (150 mg/mL), metronidazole (45 mg/mL), or vancomycin (1 mg/mL).

Control group 1- was not fed antibiotic or OVA; negative control for oral tolerization

Control group 2- was not fed antibiotic, but was fed OVA; positive control for oral tolerization

Treatment group 1- was fed antibiotic, but not OVA

Treatment group 2- was fed both antibiotic and OVA

Unless otherwise designated in the results, all of the mice were fed 100 µL for 10 consecutive days using ball-tipped feeding needles (South Pointe Surgical, Coral Springs, FL, #FN-7921). The ball on the tip of the feeding needle prevented the needle from entering into the trachea fitting only into the esophagus. The gauge of the feeding needle was determined by measuring the distance from the nose to the last rib of the mouse and using a tube of equal length. To feed the mice, the ball-tipped feeding needle was used as a lever to open the mouse's head upward and slightly to the back to create a straight line into the esophagus. The tube was inserted into the mouth of the mouse and into the esophagus timed with the mouse's swallowing reflex. The total amount of 100 µL was injected into the stomach and then the feeding needle was removed following the swallowing reflex. Any signs of distress to the mouse resulted in immediate removal of the feeding needle. This procedure was done at the recommendation of a veterinarian and did not require anesthesia.

Unless otherwise designated in the results, all of the mice were fed daily for 5 consecutive days with either distilled (DI) water (control) or the designated antibiotic followed by an additional 5 days with OVA added to the treatment solutions for the tolerized mice. After 10 days of treatment, fecal samples were

obtained for plating, and all mice were immunized with OVA. The immunization consisted of 1 µL OVA (100 mg/mL solution) in 99 µL saline, and equal part alum, for a total injection volume of 200 µL. The 100 mg/mL OVA solution was prepared fresh and consisted of 0.5 g OVA into 5 mL of saline. The combination was vortexed, filter sterilized, and stored at 4° C until needed. The alum (Pierce, Rockford, IL, #77161) was added to the OVA/saline solution slowly, vortexed thoroughly, and then rocked for a minimum of 30 minutes at room temperature. After the solution had been rocked, 200 µL of the OVA/alum solution was drawn up into the syringe and all air bubbles were removed. The injection was administered intraperitoneally.

Preparation of Solutions

All solutions were made at the beginning of each round of experimentation. The solutions were made according to the following directions.

Cephalexin

One gram of cephalexin hydrate (Sigma, St. Louis, MO, #C4895) was weighed out and added to 1 mL of 1M NaOH (LabChem, Pittsburgh, PA, #LC24500-1). The combination was vortexed to ensure complete homogenization. Into the 1 g/mL solution, 4 mL of DI water was added and pH adjusted within the range of 6-8; safe for mice to ingest. After the pH was adjusted, the total volume was brought to 6.6 mL with DI water making the final concentration of 150 mg/mL. The solution was vortexed before use each day. A total of 6 mL was needed for the duration of one round of the experiment (3 mL

for 5 days of cephalexin treatment only for all 6 mice, 1.5 mL for 5 days of cephalexin only treatment for 3 mice, and 1.5 mL for the 5 days of cephalexin/OVA for the other three mice).

Metronidazole

Five hundred mg of metronidazole (Sigma, #M1547) was added to 1 mL of glacial acetic acid (Sigma, #695084). The combination was ground together using a mortar and pestle. Once the solution was ground it was placed into a 15 mL centrifuge tube and pH adjusted within the range of 6-8. After the pH adjusted, the volume was brought to 11 mL for a final concentration of 45 mg/mL. The solution was vortexed before use each day. This provided more than enough metronidazole for 10 consecutive days of treatment.

Vancomycin

Six mg of vancomycin hydrate (Sigma, #V1130) was added to 6 mL of DI water for a final concentration of 1 mg/mL. The solution was vortexed before use each day. A total of 6 mL was needed for the duration of one round of the experiment (3 mL for 5 days of vancomycin treatment only for all 6 mice, 1.5 mL for 5 days of vancomycin only treatment for 3 mice, and 1.5 mL for the 5 days of vancomycin/OVA for the other three mice).

Ovalbumin (OVA)

Fifteen mg of OVA (Sigma, #A5378) was added to 1.5mL of either DI water or antibiotic for a final concentration of 10 mg/mL OVA solution. Each mouse was fed a total of 100 µL resulting in 1 mg of OVA fed to each mouse

daily. The solution was vortexed before use each day. A total of 1.5 mL of solution was needed to feed 3 mice 100 µL for a total of 5 days.

For experiments testing the dosage of OVA a high dose solution was made. Two hundred mg of OVA was added to 1 mL of either DI water or antibiotic for a final concentration of 200 mg/mL. Each mouse was fed a total of 100 µL resulting in each mouse receiving a one time dose of 20 mg of OVA. The solution was vortexed before use and was given once to mice 2 days prior to immunization.

Acquisition of Fecal Matter

Fecal samples were obtained from the mice both before and after treatment. The samples were obtained by handling the mouse by the tail and lifting the mouse up and down 2 to 3 times. To allow the mouse to relax, it was placed on a flat surface with the rectum near the edge of a sterile petri dish. The back of the mouse was gently stroked to allow for the acquisition of fecal matter. Immediately after obtaining the fecal samples they were weighed. The same amount (approximately 0.10 g) was obtained from each mouse. Immediately after the weighing the sample, it was placed in sterile tryptic soy broth (TSB) (Sigma, #22092) to prevent desiccation. The proportion of fecal sample to TSB was 0.10 g/100 µL.

Plating and Culturing of Fecal Matter

The fresh fecal samples were homogenized within TSB using sterile pipette tips and then vortexed to guarantee complete mixing. The samples were centrifuged at room temperature and 3000 rpm for 5 minutes. The resulting supernatant was used as a starting solution to make a 1:100 serial dilution. To create the 1:100 serial dilution 5 µL of the starting solution was placed into 495 µL of TSB. The solution was vortexed thoroughly and 50 µL was spread plated on either de Man Rogosa Sharpe agar (MRS) (Sigma, #69964) or tryptic soy agar (TSA) (Sigma, #T4536). The MRS agar was selective for the growth of *Lactobacillus* spp. when grown in aerobic conditions. All of the dilutions and plating were performed within a sterile BL 2 hood. The MRS plates were plated in duplicate and one of the plates was placed within an anaerobic chamber (GasPak EZ anaerobic chamber, BD, Franklin Lakes, NJ, #260672) along with GasPak Anaerobic Sachets (BD, #260678). All of the inoculated plates and anaerobic growth chambers were placed in an incubator at 37° C and 10% CO_2 for a total of 48 hours. After incubation, the plates were removed and the number of resulting colonies counted. After the number of colonies per plate was determined, the bacteria per mL were determined using the following equation:

bacteria/mL = [(dilution factor) X (# of colonies)] / amount plated

Determination of Antibiotic Effectiveness In Vitro

Prior to *in vivo* experimentation, an *in vitro* experiment was performed to determine the efficacy of the antibiotics used. One gram of fecal sample was

obtained from mice and placed into 1000 µL of sterile TSB (refer to *Acquisition of Fecal Matter* or *Plating and Culturing of Fecal Matter*). After centrifugation, 20 µL of the solution was used to inoculate TSA and MRS plates. After the inoculation, 6 mm sterile paper disks were placed in previously prepared antibiotic solutions (refer to *Preparation of Solutions*) and placed on top of the inoculated plates. The plates were made in duplicate and the duplicates were placed inverted within anaerobic chambers during the incubation period. The plates and anaerobic growth chambers were placed into an incubator set at 37° C and 10% CO_2 for a period of 48 hours. After incubation, the diameter of zone of inhibition (zone where growth did not occur due to the antibiotic on the disk) was measured to determine the efficacy of the antibiotics used.

Serum Acquisition

Three weeks post-immunization, mice were sacrificed by CO_2 asphyxiation followed by the opening of the chest cavity. Immediately after opening the chest cavity, blood was collected through cardiac puncture. After obtaining the blood samples, they were placed in a 1.5 mL microcentrifuge tube and left at room temperature for 30 minutes (no longer than 1 hour) to allow clotting to occur. The congealed blood samples were spun down in a centrifuge for 5 minutes at 5000 rpm at room temperature. The supernatant was drawn off and placed into a clean microcentrifuge tube and centrifuged a second time for 2 minutes at 10,000 rpm at room temperature. The supernatant was removed and

placed into another microcentrifuge tube and stored at -20^{o} C until needed for ELISA analysis.

Enzyme-linked Immunosorbent Assay (ELISA)

To determine levels of OVA-specific IgG from serum, an immunoglobulin enzyme-linked immunosorbent assay (ELISA) was performed. The first step of an ELISA was to coat a 96-well plate with the antigen specific for the antibody that we wanted to detect; OVA. The plate was then washed and blocked with a postcoat solution. This was necessary to prevent non-specific antibody binding. After blocking the plate was washed and treated with the serum to be tested. If there were antibodies present within the serum that were specific for the antigen the plate was initially coated with (OVA-specific IgG or IgM) the antibodies would bind. After treating with serum the plate was washed and then treated with a detection antibody. The detection antibody (anti-mouse IgG or IgM) was able to bind to any OVA-specific IgG or OVA-specific IgM antibody that was present within the serum. The detection antibody was conjugated with an enzyme, for the particular substrates the enzyme was either alkaline phosphatase (AP) for IgG or horseradish peroxidase (HRP) for IgM. After treating the plate with detection antibody it was washed and either the p-nitrophenylphosphate (pNpp) for IgG or 3,3’,5,5’-tetramethylbenzidine (TMB) for IgM substrates were added. The substrate reacted with the enzyme that the detection antibody was conjugated to, resulting in a visible color change that indicated the amount of the specific antibody (OVA-specific IgG or IgM) that the ELISA was performed for.

Prior to ELISA analysis a template was prepared showing the location and dilution factor of each serum sample that was to be run. All of the reagents were ordered from Bethyl Laboratories, Inc (Montgomery, TX) unless otherwise noted. To begin the process of coating a 96-well plate was coated with OVA (4 mg/mL) (Sigma, #A5378). The OVA solution was prepared by adding 100 mg of OVA into 1 mL of DI water. The solution was thoroughly vortexed and then 400 µL was placed into 10 mL of coating buffer (#E107). The coating buffer was prepared according to manufacturer's directions. The OVA/coating buffer solution was mixed and a total of 100 µL was placed into each of the wells where the samples were to be added. The final concentration of the OVA within coating solution was 4 mg/mL.

A small portion of the ELISA plate was coated with bovine serum albumin (BSA) (Amresco, Solon, OH, #0332). BSA is a related to the ovalbumin protein; but, the OVA-specific IgG should specific enough for ovalbumin to not bind to the BSA. This was to measure the amount of non-specific binding that was occurring within the ELISA. Similar to the OVA 4 mg/mL was used to coat the plate. A 100 mg/mL BSA solution was made (100 mg BSA added to 1 mL of DI water), mixed thoroughly and then 200 µL of the BSA solution was added to 5 mL of coating buffer. After the BSA/coating buffer and OVA/coating buffer solutions had been added to the ELISA plate it was covered and allowed to incubate for 1 hour at room temperature. After coating, the ELISA plate was washed three times with ELISA wash buffer (#E106) and blotted dry. The wash solution was prepared according to manufacturer's directions. The wash solution was gently added to

the side of the well to prevent disturbing the coating. After the last wash the plate was blotted dry and prepared for blocking using postcoat solution (#E104).

The postcoat solution contains Tris Buffered Saline (TBS) and 1% BSA. The postcoat solution was purchased and prepared according to manufacturer's directions. Each well of the ELISA plate was filled with 200 µL of postcoat solution. The plate was covered and allowed to incubate for 30 minutes at room temperature. After blocking, the ELISA plate was washed three times with ELISA wash buffer and blotted dry.

After blocking and washing, the samples were added. Before adding the samples, the serum was removed from the -20° C freezer where they have been stored and slowly thawed on ice. For the ELISA 1:10, 1:50, 1:250, and 1:1250 serial dilutions were performed. Into the row for the 1:10 serial dilution a total of 108 µL of sample diluent was added. Into the rows for the other dilutions 100 µL of sample diluent was added. Sample/standard diluent contains TBS, BSA and Tween 20; and prepared according to manufacturer's directions. Once the serum was completely thawed a total of 12 µL was added into the row for the 1:10 dilution. The combination was gently, but thoroughly mixed and then 20 µL was transferred into the 1:50 dilution row. The combination was mixed in a similar manner and the process was repeated until all four dilutions had been made.

After all of the sample dilutions had been prepared, the plate was covered and allowed to incubate at room temperature for 1 hour. After the 1 hour incubation, the plate was washed three times with ELISA wash buffer and blotted dry in preparation for the addition of the detection antibody.

To prepare the detection solution, 10 µL of the detection antibody was added to 10 mL of sample diluent. The antibodies where either Goat anti-mouse IgG-AP (Southern Biotech, Birmingham, AL, #1030-04) or Goat anti-mouse IgM-HRP (Bethyl, #A90-101P). The final dilution of the detection antibody was 1:1000. The antibody/diluent solution was gently mixed and 100 µL was added to each well of the ELISA plate. After the addition was made, the plate was covered and allowed to incubate at room temperature for 1 hour. After the incubation period, the ELISA plate was washed three times in ELISA wash buffer and blotted dry to prepare for the addition of the substrate.

To prepare the ELISA substrate for the IgG detection antibody, 40 mg of p-Nitrophenyl Phosphate, Disodium Salt (pNPP) (Amresco, #0364) was added to 10 mL of prepared ELISA substrate solution, an alkaline phosphatase solution, and mixed well. The TMB substrate (Bethyl, #E102) for the IgM antibody was prepared by adding 5 mL of solution A to 5 mL of solution B. A total of 100 µL of substrate was added to each well of the ELISA plate. Any bubbles in the wells of the ELISA plate were removed by blowing ethanol vapor onto the bubbles using a transfer pipette. As soon as a color change (from clear to yellow) was noticeable in the majority of the wells, the absorbance values were read using the Bio-Rad 680 plate reader (Bio-Rad, Hercules, CA #168-1000) at 415 nm wavelength.

Statistical Analysis of Data

The Bio-Rad plate reader provided absorbance readings for each of the serum samples that were evaluated. Each of the serum samples was run in duplicate; the average for each mouse was calculated. The average of the absorbencies for each mouse was entered into an Excel spreadsheet and then a linear regression graph was produced showing the absorbance values at each dilution for each mouse. The amount of antibody present within each mouse was variable; therefore the graph was used to find the dilution where all of the absorbencies fell within the range of detection. This dilution was the most representative dilution (one where all or the majority of absorbance values were declining as expected by the dilution). Taking the absorbance values for each mouse at that dilution statistics were run using SigmaStat. The one-way ANOVA was calculated to determine the significance between the different treatment groups and the SEM was used to determine the amount of variation within each treatment group. When necessary an additional statistical analysis, Student Neuman-Kheuls, was used in conjunction with one-way ANOVA. Student Neuman-Kheuls was used when statistical significance was found and comparisons were necessary to be made between the means of the different treatment groups in the analysis. Statistical significance was determined by a *p*-value less than 0.05. By using this analysis a more accurate picture can be made to access the activity of the immune system during the breakdown of oral tolerance through antibiotic usage.

RESULTS

Cephalexin significantly reduced levels of Lactobacilli from mouse fecal matter in vitro.

In order to test the efficacy and effect of the antibiotics on the targeted bacteria, Lactobacilli, antibiotic disk testing was performed. The following antibiotics were prepared at the listed concentrations: cephalexin (150 mg/mL), vancomycin (1 mg/mL), and metronidazole (45 mg/mL); refer to *Preparation of Solutions*. Cephalexin and vancomycin were chosen because they are known to target gram positive bacteria, and therefore should have shown a zone of inhibition of growth of Lactobacilli present in the fecal matter. Metronidazole was chosen as a comparison to cephalexin and vancomycin because it targets gram negative bacteria and therefore should not have killed Lactobacilli. Fecal matter was plated because bacterial species found in fecal matter are representative of the bacteria present within the gastrointestinal tract. MRS media was used because it is specific for the growth of Lactobacilli and TSA media was used to show non-specific bacterial growth from fecal matter.

At the end of the *in vitro* testing the zones of inhibitions were measured in mm (**Figure 2**). Cephalexin had the largest average zone of inhibition for each of the plates inoculated and was able to significantly reduce the amount of Lactobacilli (**Figure 3**). Vancomycin had the second largest zone of inhibition for

each of the plates inoculated, and metronidazole exhibited the smallest zone of inhibition. The data obtained from the *in vitro* study demonstrated that cephalexin significantly reduced Lactobacilli growth from fecal matter to the greatest degree. Metronidazole, used to target gram negative bacterial species, was least effective against gram positive Lactobacilli. Vancomycin was used as an experimental antibiotic because it only targets some strains of Lactobacilli [49] and it was not known if the strains it is effective on were actually present within the fecal samples obtained. Based on the *in vitro* disk testing it appeared that vancomycin was effective on some of the strains of Lactobacilli that were present in fecal matter from Balb/c mice.

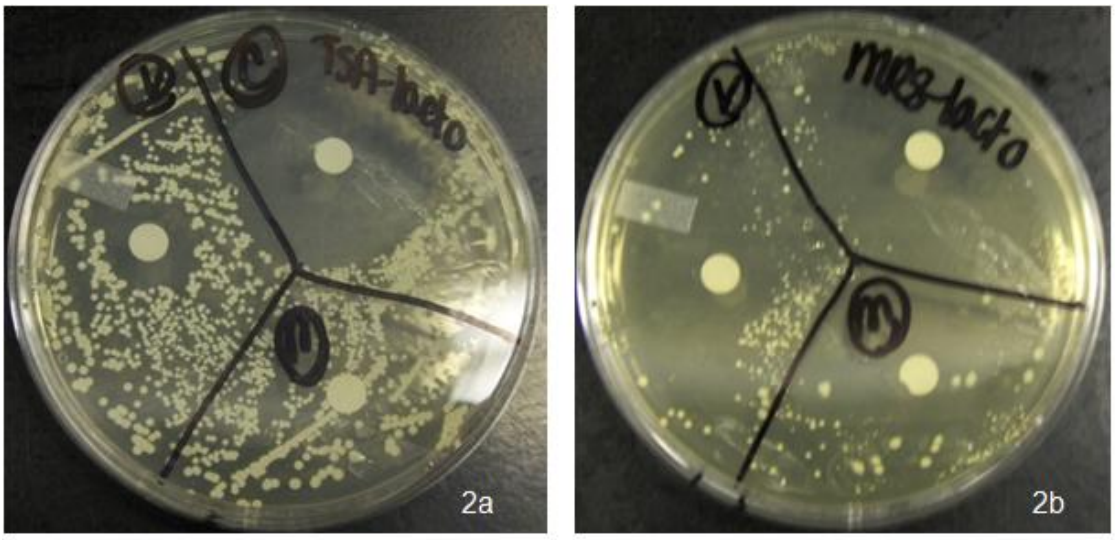

Figure 2 *Cephalexin is most effective at killing aerobic bacteria present in fecal matter.* The *in vitro* antibiotic disk test was performed on non-selective TSA (2a) or Lactobacilli-selective MRS (2b) media inoculated with fecal matter from Balb/c mice. After inoculation, sterile antibiotic-treated disks were placed on the plates. The disks are in the following order going clockwise beginning in the upper right corner: cephalexin (150 mg/mL), metronidazole (45 mg/mL), and vancomycin (1 mg/mL). Incubation was performed at 37°C and10% CO_2 for 48 hours.

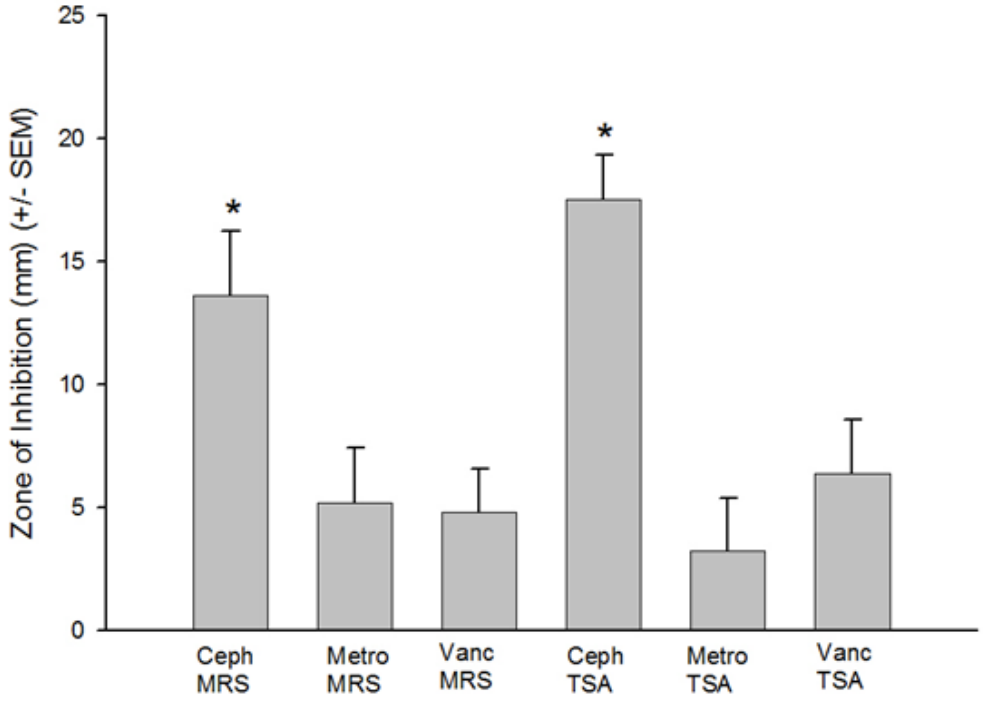

Figure 3 *Cephalexin significantly reduced amounts of aerobic bacteria from mouse fecal matter.* Either non-selective TSA or Lactobacilli-selective MRS media was inoculated using fecal matter obtained from Balb/c mice. After inoculation sterile disks were placed into one of three different antibiotic solutions; cephalexin (150 mg/mL), metronidazole (45 mg/mL), or vancomycin (1 mg/mL) and placed on the media. After 48 hours the zone of inhibition was measured in mm. The values recorded are averages from 5 different plates. Significance was determine using one way ANOVA ($p < 0.05$*).

Cephalexin and vancomycin are effective in vivo; however, oral tolerization is not successful

After confirmation of the efficacy of the antibiotics in vitro, the *in vivo* aspect of the study was initiated. The antibiotics included within the study were cephalexin and vancomycin. Cephalexin was chosen because it targets gram positive bacteria, including Lactobacilli, and works in a similar way as erythromycin, the antibiotic used in previous research in our lab, does. Vancomycin was chosen because it targeted bacteria differently than cephalexin and more importantly it was not known if vancomycin would be effective on the particular Lactobacilli strains that were present in Balb/c mice.

Some random variations were found in the ability of antibiotics to reduce Lactobacilli populations in fecal matter of Balb/c mice (**Figure 4**). This is a challenge of working with animal models and the reason why multiple mice were used for each treatment group. Cephalexin was able to reduce the amount of bacteria per mL in three out the four mice to a greater extent than those treated with vancomycin demonstrating that cephalexin is more effective at reducing numbers of Lactobacilli than vancomycin. These results coincide with the *in vitro* disk testing. While cephalexin is more effective, vancomycin was still able to reduce the amount of Lactobacilli. The amount of bacteria remaining after antibiotic treatment was slightly higher than the amount after treatment with cephalexin. Vancomycin is effective only on some strains of Lactobacilli that would account for the slightly higher amounts that were found; indicating some

strains of Lactobacilli are resistant to vancomycin while other strains are sensitive.

To assess the immune response ELISAs were performed to determine the amount and differences between OVA-specific IgG and IgM after oral tolerization. IgG demonstrates that an active immune response has occurred, evidenced by class switching that has to have occurred for IgG to be generated. IgM is the first antibody produce after an active immune response, however, high amounts of non-specific IgM can be found in the serum regardless of initiation of an active immune response. The ELISA dilutions that demonstrated amount of antibody that fell within the limit of detection was 1:5 for IgG (**Figure 5**) and 1:10 for IgM (**Figure 6**). There were no differences in the absorbance values between the non-tolerized (NT) and orally-tolerized (OT) control mice for either IgG (**Figure 7**) or IgM (**Figure 8**), therefore it was concluded that tolerization did not occur. Because oral tolerization was not successful statistical analysis was not performed and no conclusion can be made regarding the effect of antibiotic treatment on oral tolerization on Balb/c mice.

It was expected to have seen a high absorbance value in the NT control mice and a much lower absorbance value in the OT control mice for IgG. That would have shown the NT control mice had an active immune response, underwent class switching and OVA-specific IgG was generated. The OT control mice should have been tolerized therefore an active immune response should not have been generated, only a suppressed immune response, and the result would have been very low levels of OVA-specific IgG detected. IgM could also

have demonstrated that tolerance did occur. However, due to the higher amount of non-specific IgM present within the serum the differences between NT and OT control group may not have been as pronounced as for IgG.

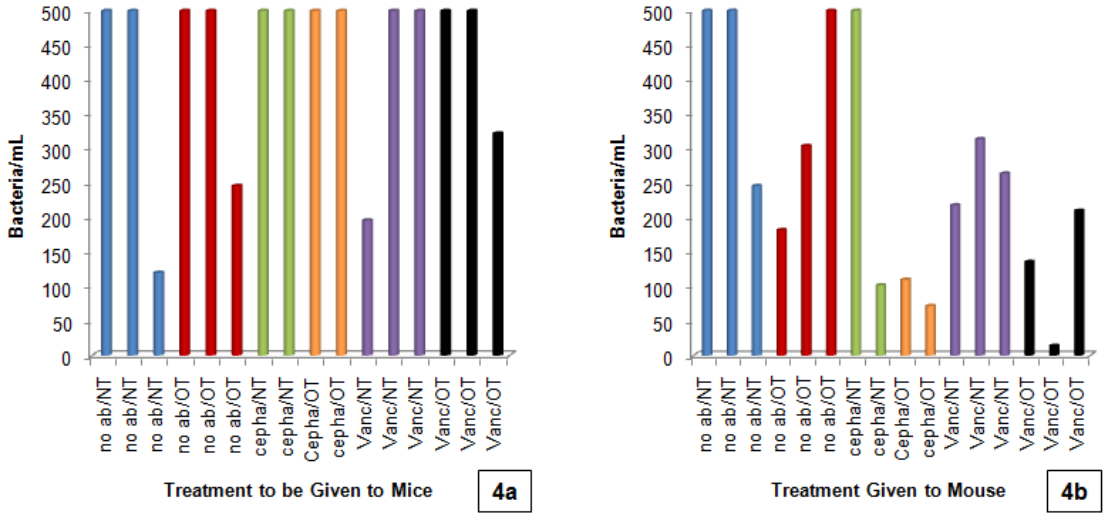

Figure 4 *The amount of Lactobacillus spp. per mL was reduced in the fecal samples after antibiotic treatment.* Fecal matter was diluted and plated on MRS agar. The plates were incubated at 37°C and 10% CO_2 for 48 hours. The resulting colonies were count and bacteria per mL was determined using the following equation: # bacteria/mL = [(dilution factor) X (# of colonies)] / amount plated. Bacterial counts were determined before (**4a**) and after antibiotic treatment (**4b**). There were six different treatment groups within the study; no ab/ NT (no antibiotic, non-tolerized), no ab/OT (no antibiotic, orally-tolerized), ceph/NT (cephalexin, non-tolerized), ceph/OT (cephalexin, orally-tolerized), vanc/NT (vancomycin, non-tolerized), and vanc/OT (vancomycin, orally-tolerized). All of the mice orally tolerized were given 100 µL of 10 mg/mL OVA solution for 5 days. All of the antibiotic treatment was given for 10 and was either cephalexin (150 mg/mL) or vancomycin (1 mg/mL).

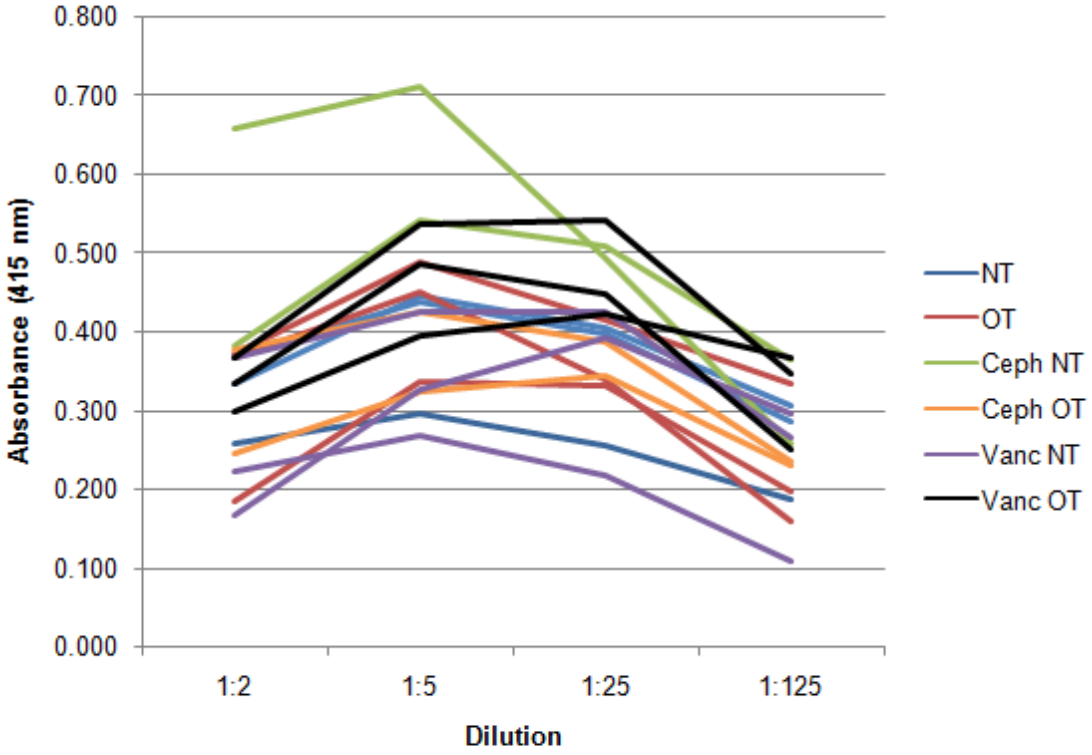

Figure 5 *Absorbance levels from ELISA analysis using serum from antibiotic treated and orally tolerized Balb/c mice to determine amount of OVA-specific IgG.* The mice were sacrificed and serum was harvested to run an ELISA to determine the amount of OVA-IgG present. Mice were treated with cephalexin (ceph) (150 mg/mL) or vancomycin (vanc) (1 mg/mL) for a total of 10 days. The orally-tolerized (OT) mice were given OVA (10 mg/mL) for the last 5 days of antibiotic treatment, whereas the non-tolerized (NT) were given DI water or antibiotic. A total of four different dilutions were performed. The dilution (1:5) where the absorbance values fell within the limit of detection was selected for further analysis.

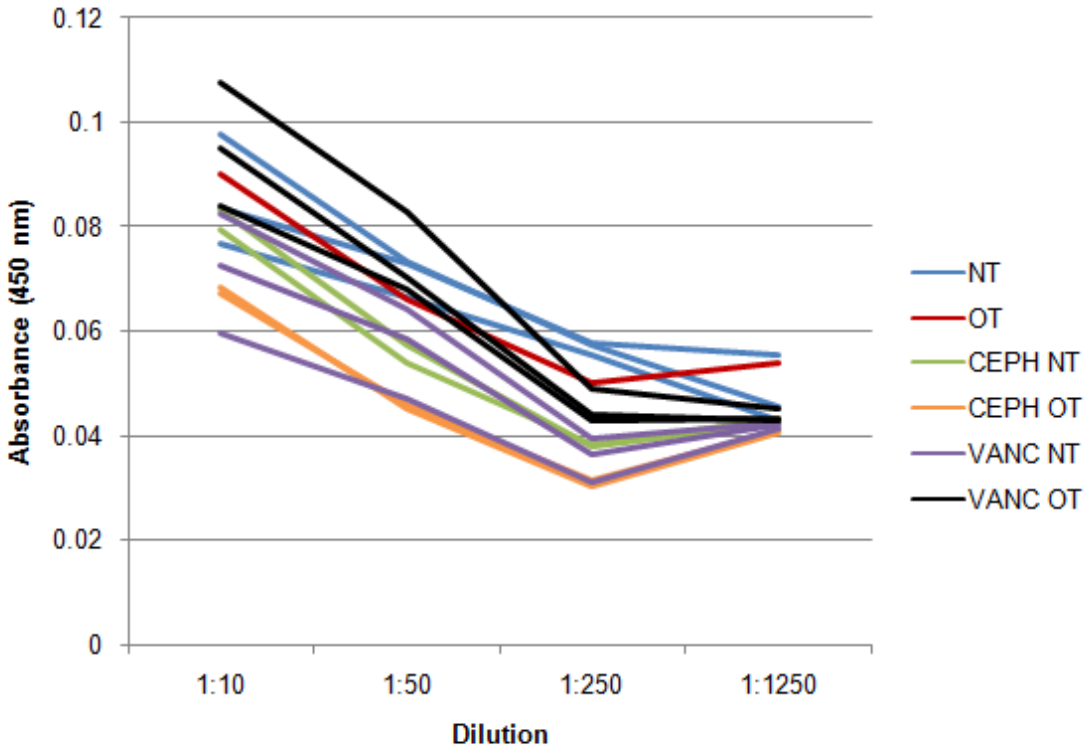

Figure 6 *Absorbance levels from ELISA analysis using serum from antibiotic treated and orally tolerized Balb/c mice to determine amount of OVA-specific IgM.* The mice were sacrificed and serum was harvested to run an ELISA to determine the amount of OVA-IgM present. Mice were treated with cephalexin (ceph) (150 mg/mL) or vancomycin (vanc) (1 mg/mL) for a total of 10 days. The orally-tolerized (OT) mice were given OVA (10 mg/mL) for the last 5 days of antibiotic treatment, whereas the non-tolerized (NT) were given DI water or antibiotic. A total of four different dilutions were performed. The dilution (1:5) where the absorbance values fell within the limit of detection was selected for further analysis.

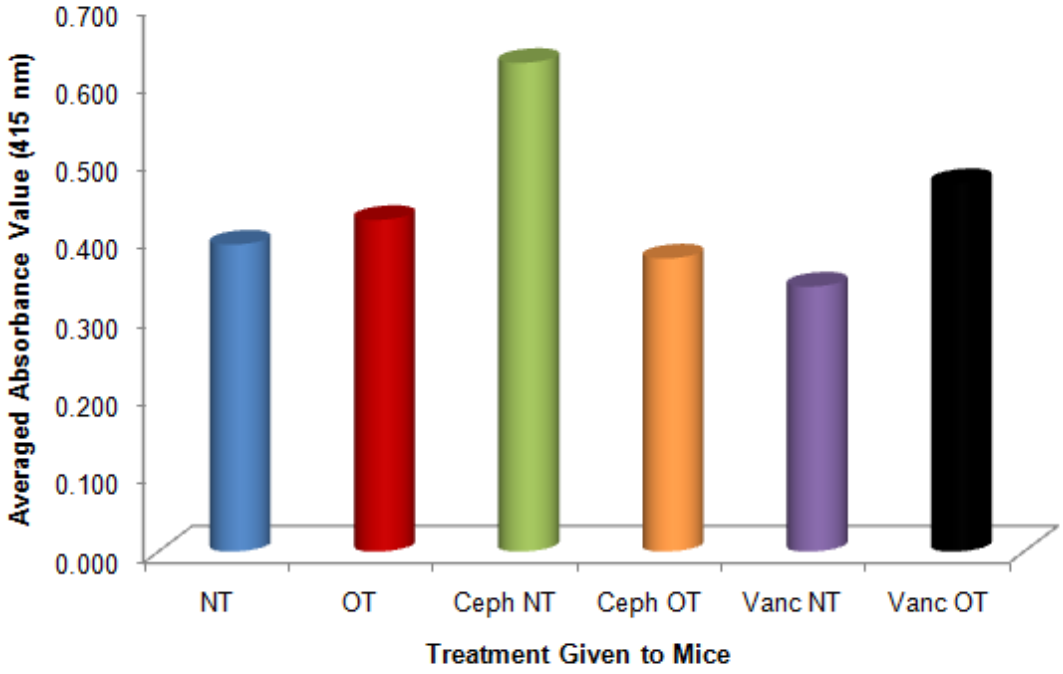

Figure 7 *Oral tolerization was not successful in antibiotic treated Balb/c mice determined by amount of OVA-specific IgG from ELISA analysis*. Graph depicting averaged absorbance levels at the 1:5 dilution from ELISA analysis using serum from antibiotic treated and orally tolerized Balb/c mice. The mice were sacrificed and serum was harvested to run an ELISA to determine the amount of OVA-IgG present. Mice were treated with cephalexin (ceph) (150 mg/mL) or vancomycin (vanc) (1 mg/mL) for a total of 10 days. The orally-tolerized (OT) mice were given OVA (10 mg/mL) for the last 5 days of antibiotic treatment, whereas the non-tolerized (NT) were given DI water or antibiotic. The absorbance values were an average of 3 for each treatment group (except Ceph NT and Ceph OT; n=2).

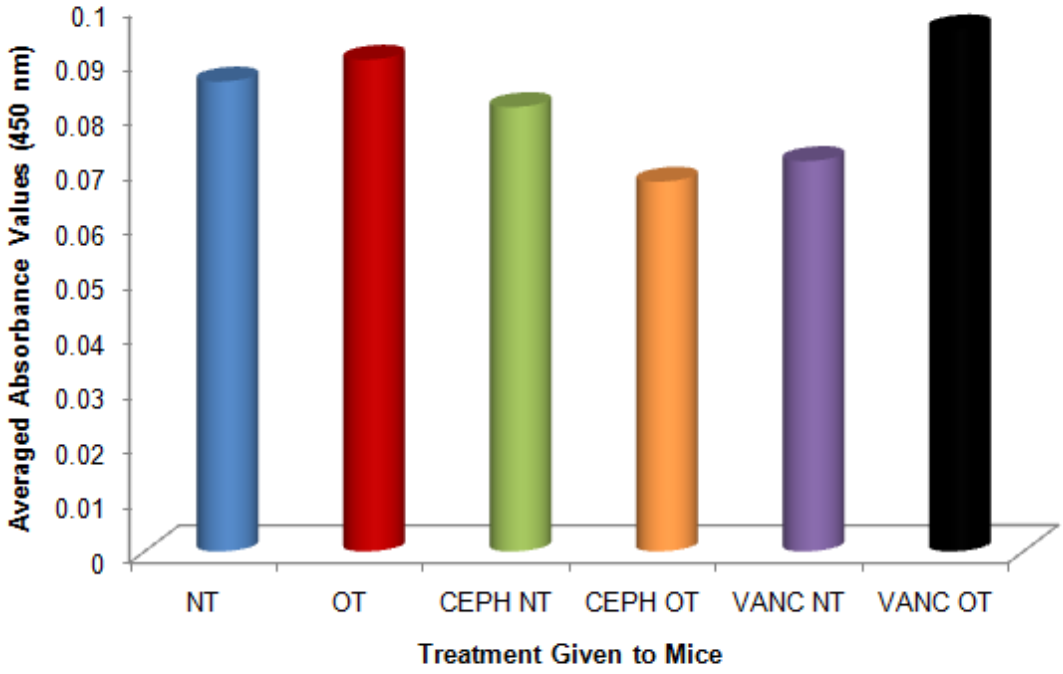

Figure 8 *Oral tolerization was not successful in antibiotic treated Balb/c mice determined by amount of OVA-specific IgM from ELISA analysis*. Graph depicting averaged absorbance levels at the 1:10 dilution from ELISA analysis using serum from antibiotic treated and orally tolerized Balb/c mice. The mice were sacrificed and serum was harvested to run an ELISA to determine the amount of OVA-IgG present. Mice were treated with cephalexin (ceph) (150 mg/mL) or vancomycin (vanc) (1 mg/mL) for a total of 10 days. The orally-tolerized (OT) mice were given OVA (10 mg/mL) for the last 5 days of antibiotic treatment, whereas the non-tolerized (NT) were given DI water or antibiotic. The absorbance values were an average of 3 for each treatment group (except Ceph NT and Ceph OT; n=2).

Time is important for oral tolerance to be achieved in Balb/c mice

To investigate the length of time that it takes for tolerance to be established, we chose to induce oral tolerance using a high dose of OVA (20 mg). We only wanted to determine the amount of time that was necessary for class switching to occur resulting in the production of IgG to determine if oral tolerization was successful. The amount of time to orally tolerize mice using a high dose requires one day of feeding as opposed to 5-7 days of feeding OVA (1 mg per day) required for a low dose; which would have made it more difficult to determine how quickly tolerance occurs using a low OVA dose. Oral tolerization should occur using either a high dose or a low dose. The differences between the dosages affect what happens to T cells; high dose leading to anergy or deletion of Th cells, and low dose a switch from Th1 or Th2 cells to the Treg, Th3, cell [15, 21, 23].

To study oral tolerization in mice 2 different control groups were set up. The first group (NT) was not fed OVA, but was given a one-time dose of 100 µL DI water. The second group (OT) was given a one-time high dose of OVA (20 mg). After being fed all of the mice within the study were immunized with OVA. Instead of waiting 5 days after immunization to sacrifice the mice, the time period after immunization was extended for 3 weeks. When the immune system encounters antigen, the first antibody generated is IgM. Class switching from IgM to IgG is necessary to generate more specific and robust immune responses. In order for class switching to IgG to occur a longer period of time is necessary because non-specific IgM is present in moderate quantities throughout

the bodily constantly, IgG is a better indicator of whether tolerization had occurred because it is indicative of an active and strong immune response. Therefore the time frame was extended to see if oral tolerization was successful based on the levels of IgG generated. High levels of IgG indicate that tolerance was not induced. After sacrifice the serum was harvested and used for ELISA analysis to determine the amount of OVA-specific IgG (**Figure 9**) and OVA-specific IgM (**Figure 10**). The dilution that demonstrated the ideal amount of antibody to be detected within the range of detection was selected (1:50). The amount of OVA-specific IgG between the NT and OT mice was significantly different ($p < 0.05$) (**Figure 11**). However, no significance was found between NT and OT in the amount of OVA-specific IgM (**Figure 12**). It was concluded that time period of at least 3 weeks is needed for oral tolerization to be detected based on immunoglobulin levels. IgG is also a better indicator of oral tolerance than IgM.

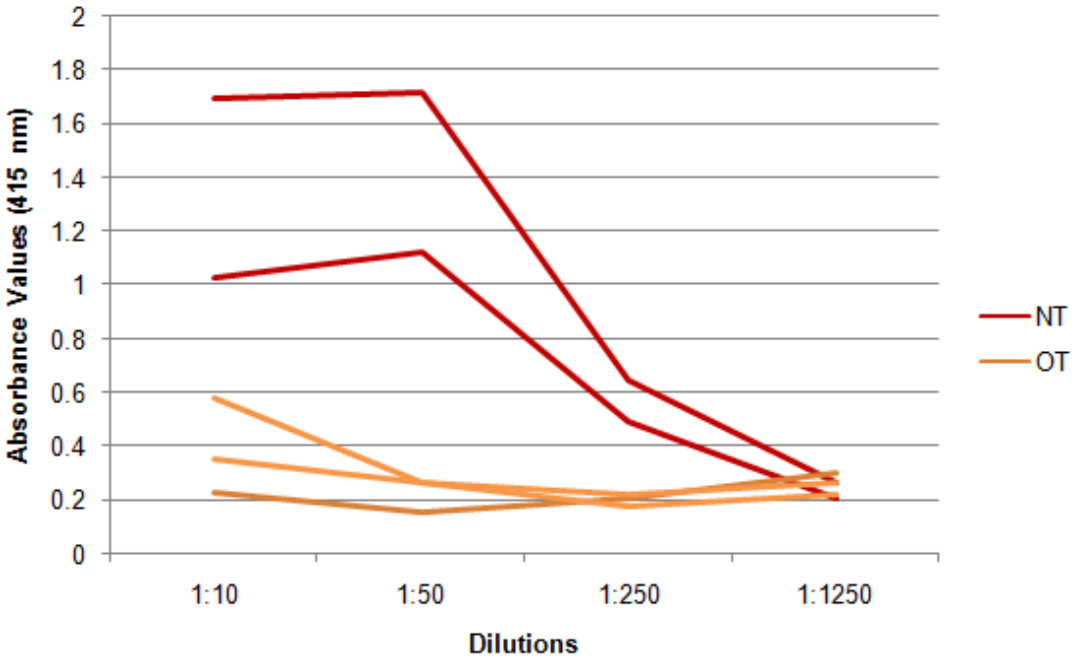

Figure 9 *Balb/c mice were successfully tolerized to OVA based on levels of OVA-specific IgG.* There were two different groups of mice. The non-tolerized mice (NT) were fed a one time dose of distilled water. The orally-tolerized mice (OT) were orally fed a one time dose of OVA (20mg). After being fed all mice were immunized with OVA and 4 weeks later mice were sacrificed and serum was harvested for ELISA analysis to determine levels of OVA-specific IgG.

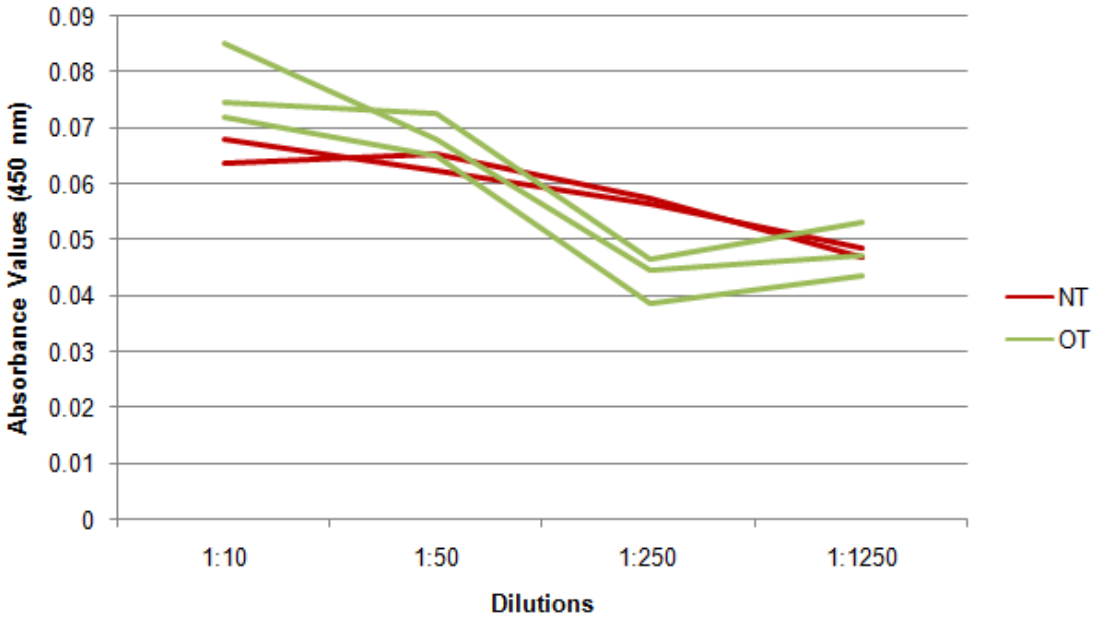

Figure 10 *Balb/c mice did not express levels of OVA-specific IgM indicating oral tolerization was successful*. The non-tolerized mice (NT) were fed a one time dose of distilled water. The orally-tolerized mice (OT) were orally fed a one time dose of OVA (20mg). After being fed all mice were immunized with OVA and 4 weeks later mice were sacrificed and serum was harvested for ELISA analysis to determine levels of OVA-specific IgM. Mice receiving OVA orally did not demonstrate a decrease in OVA-specific IgM indicating oral tolerance was induced.

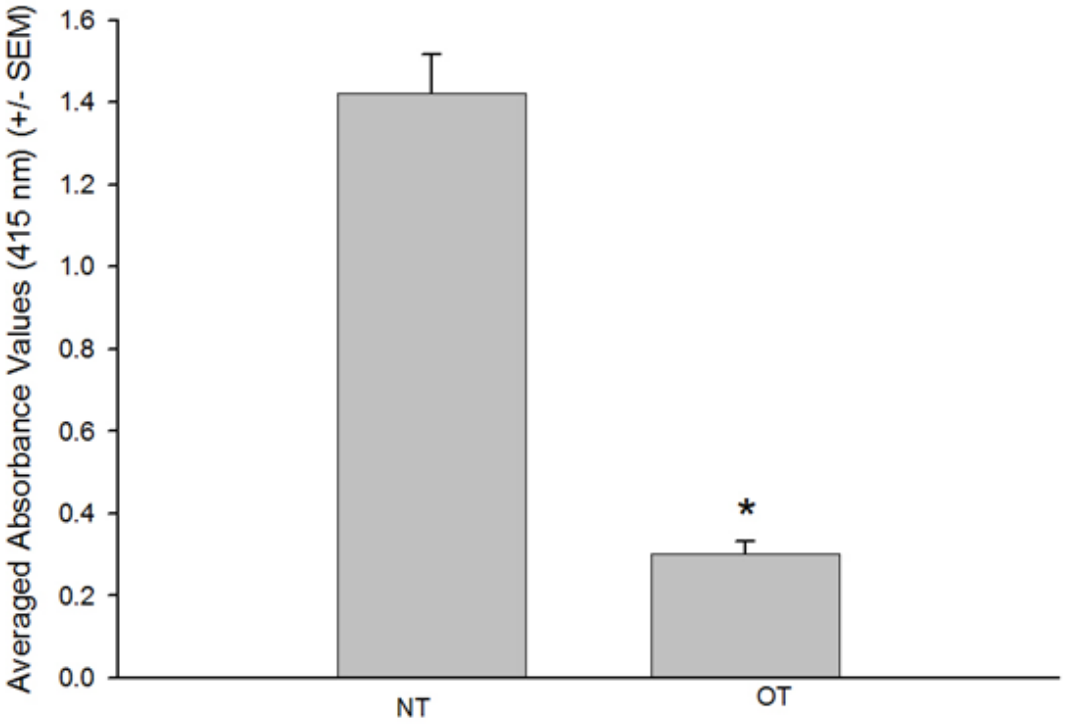

Figure 11 *Balb/c mice were successfully tolerized to OVA based on levels of OVA-specific IgG*. The non-tolerized mice (NT) were fed a one time dose of distilled water. The orally-tolerized mice (OT) were orally fed a one time dose of OVA (20mg). After being fed all mice were immunized with OVA and 4 weeks later mice were sacrificed and serum was harvested for ELISA analysis to determine levels of OVA-specific IgG. The absorbance values for each treatment group were averaged (NT= 2n and OT= 3n). Mice receiving OVA orally showed a statistically significant decrease in OVA-specific IgG indicating tolerance was successful. Significance was determined using one-way ANOVA analysis ($p < 0.05$*).

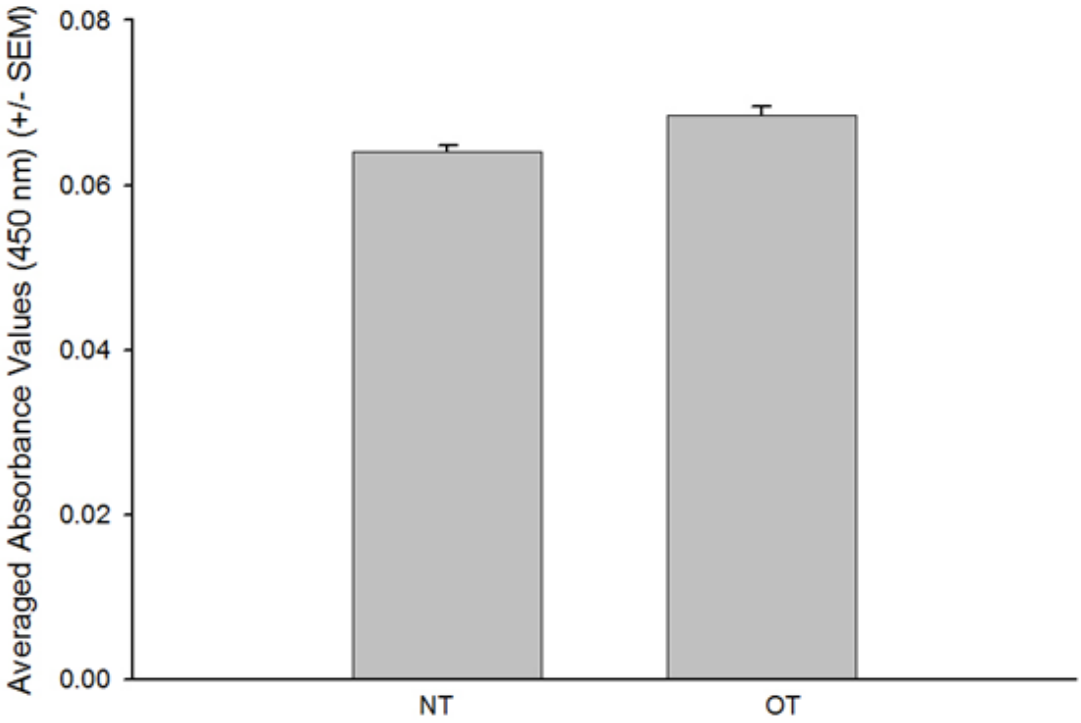

Figure 12 *Balb/c mice did not express levels of OVA-specific IgM indicating oral tolerization was successful*. The non-tolerized mice (NT) were fed a one time dose of distilled water. The orally-tolerized mice (OT) were orally fed a one time dose of OVA (20mg). After being fed all mice were immunized with OVA and 4 weeks later mice were sacrificed and serum was harvested for ELISA analysis to determine levels of of OVA-specific IgM. The absorbance values for each treatment group were averaged (NT= 2n and OT= 3n). Mice receiving OVA orally did not show a statistically significant decrease in OVA-specific IgM indicating tolerance was successful. One-way ANOVA analysis was performed to determine significance.

Studies involving vancomycin and oral tolerization in Balb/c mice are inconclusive

Once time had been determined to be critical for oral tolerization and IgG was identified as the best antibody indicator of tolerance induction (or lack of induction), a new study to test the effect of vancomycin on oral tolerization was initiated. Previous studies performed in our lab demonstrated that erythromycin is able to reduce levels of probiotic bacteria and affect oral tolerance. When mice were orally tolerized following antibiotic treatment, the amount of OVA-specific IgG was elevated above that of non-antibiotic treated, non-tolerized controls, indicating that exposure to fed antigen following antibiotic treatment could result in heightened instead of suppressed immune responses. Erythromycin targets both gram negative and gram positive bacteria and generally is prescribed for infections of the respiratory tract, skin, as well as diphtheria and acute pelvic inflammatory disease [45]. Vancomycin, generally prescribed for bacterial infection including pseudomembranous enterocolitis, was chosen because it is reportedly effective on gram positive bacteria, specifically effective on only some strains of Lactobacilli [45, 49]. Because vancomycin is effective on some strains of Lactobacilli we expected to observe a decrease in Lactobacilli colonies from fecal matter with less alteration to other intestinal species.

Vancomycin partially reduced levels of Lactobacilli from mouse fecal samples, and this was expected because vancomycin is effective against some Lactobacilli strains, but not all (**Figure 13**). Unfortunately, the amount of bacteria among the control mice was also reduced. This was a trend seen in other

experiments and resulted in concluding the study at this point in experimentation in order to avoid waste of reagents. This particular study was continued despite the reduction in bacterial colony levels in control mice to find the effect of this reduction on oral tolerization.

After the 10 days of antibiotic treatment all of the mice were immunized with OVA to allow for antibodies to be generated for detection by ELISA analysis. The non-tolerized mice should produce high levels of OVA-specific antibodies after immunization, whereas the tolerized mice should produce a lesser amount. Three weeks post-immunization all mice were sacrificed and the serum was used for ELISA analysis. The dilutions performed on the ELISA included 1:10, 1:50, 1:250, and 1:1250 for both OVA-specific IgG (**Figure 14**) and OVA-specific IgM (**Figure 15**). The dilution that had the concentration of antibody that was able to be detected was 1:50 for both IgG and IgM. As demonstrated by the decrease in absorbance values which correlate with a decrease in OVA-specific IgG, a slight amount of tolerization was observed in the OT group compared to the NT group (**Figure 16**). However it was not statistically significant. Importantly, the OT mice showed a decrease in Lactobacilli after 10 days of being fed. Thus, it is possible that the oral tolerization did not occur due to the reduction in probiotic bacteria levels, which have been found to be important for the induction of oral tolerance to occur. While the amount of OVA-specific IgG in the tolerized mice that received vancomycin was elevated compared to the non-tolerized vancomycin treated mice (**Figure 16**), a trend seen in previous work that indicated that antibiotic treatment may enhance immune responses to fed antigens, no

conclusions can be made because tolerance was not able to be induced in this study. OVA-specific IgM ELISA results did not show any amount of tolerization that occurred; in fact the amount of OVA-specific IgM was higher in the tolerized mice than in the non-tolerized mice (**Figure 17**). These results coincide with previous results from the antibiotic/ oral tolerization study (**Figures 8**) and tolerization study (**Figure 12**) which indicates IgM secretion is unaffected by numbers of intestinal bacteria or methods of tolerance induction and this is likely due to the fact that IgM is the first antibody produced upon activation of the immune system and that high levels of IgM is produced non-specifically.

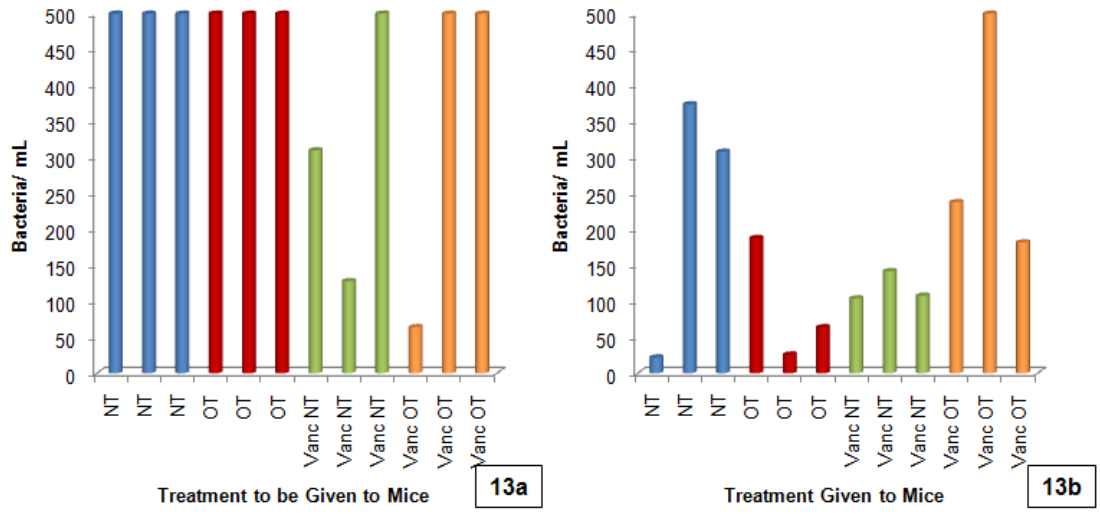

Figure 13 *The amount of Lactobacillus spp. per mL was reduced in antibiotic and non-antibiotic-treated mice.* Fecal matter was diluted and plated on MRS agar. The plates were incubated at 37°C and 10% CO_2 for 48 hours. The resulting colonies were count and bacteria per mL was determined using the following equation: # bacteria/mL = [(dilution factor) X (# of colonies)] / amount plated. Bacteria per mL was determine prior to antibiotic treatment (**13a**) and after treatment (**13b**). There were 4 different treatment groups within the study; NT (no antibiotic, non-tolerized), OT (no antibiotic, orally-tolerized with OVA), Vanc NT (vancomycin, non-tolerized), Vanc OT (vancomycin, orally-tolerized with OVA). The antibiotic treatment , vancomycin 1 mg/mL, was given for 10 days. All of the mice orally tolerized were given 100 µL of 10 mg/mL OVA solution for the last 5 days of treatment.

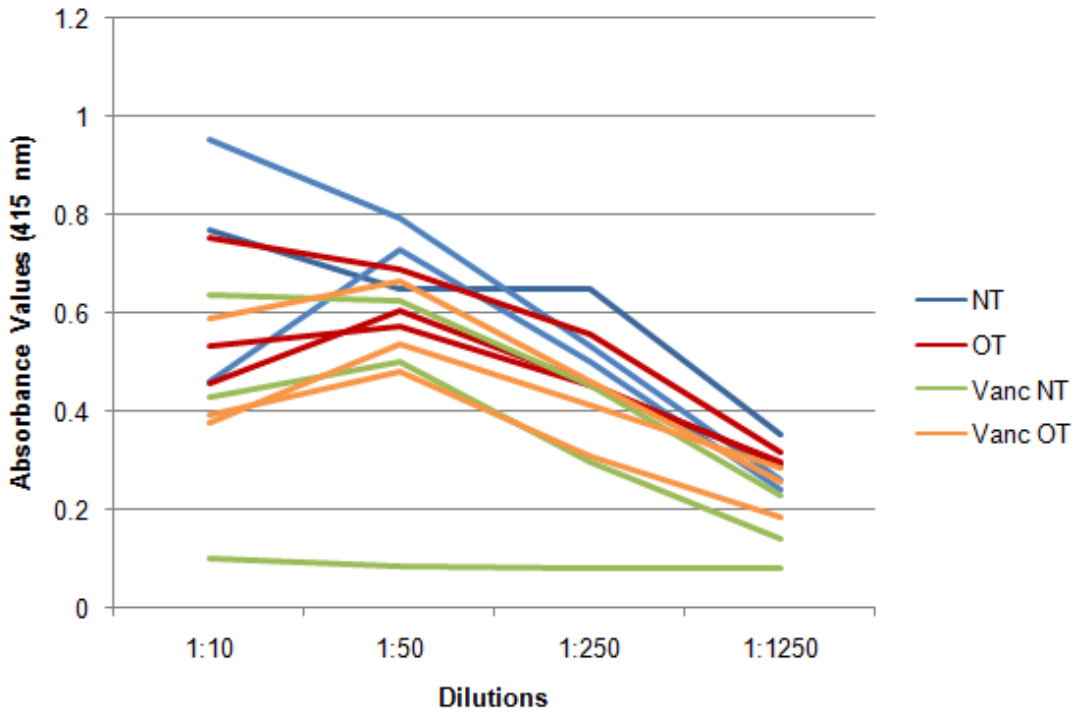

Figure 14 *Absorbance levels from ELISA analysis using serum from vancomycin treated and orally tolerized Balb/c mice to determine amount of OVA-specific IgG.* The mice were sacrificed and serum was harvested to run an ELISA to determine the amount of OVA-IgG present. There were 4 different treatment groups within the study; NT (no antibiotic, non-tolerized), OT (no antibiotic, orally-tolerized with OVA), Vanc NT (vancomycin, non-tolerized), Vanc OT (vancomycin, orally-tolerized with OVA). The antibiotic treatment , vancomycin 1 mg/mL, was given for 10 days. All of the mice orally tolerized were given 100 µL of 10 mg/mL OVA solution for the last 5 days of treatment. A total of four different dilutions were performed. The dilution (1:50) where the absorbance values fell within the limitof detection was selected for further analysis.

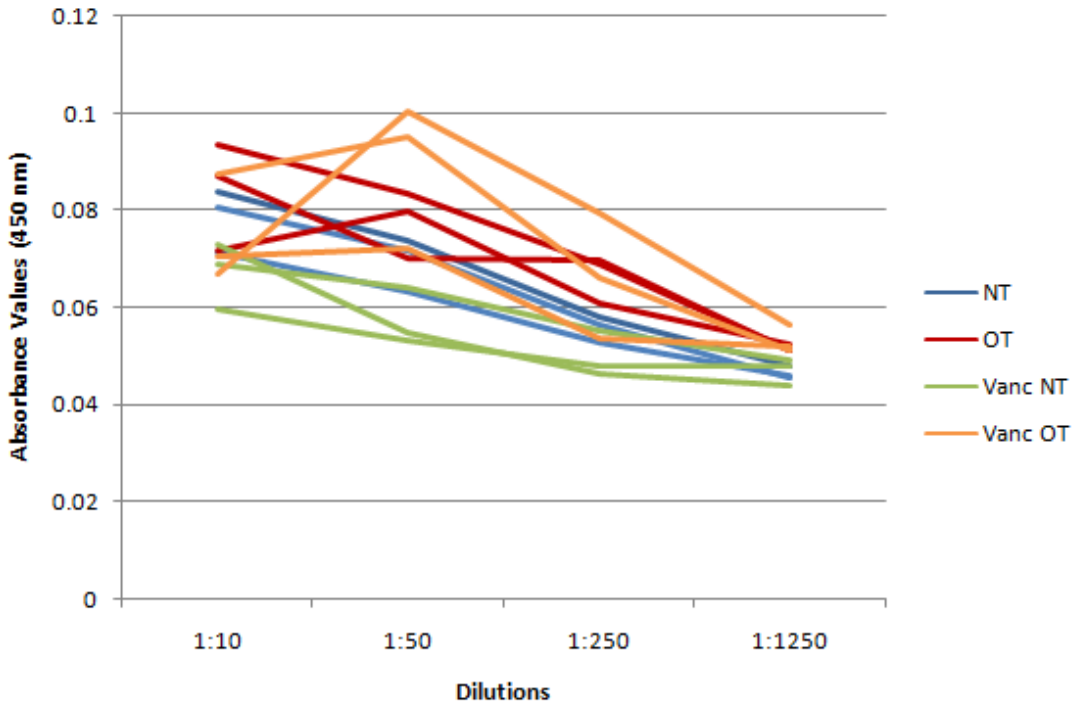

Figure 15 *Absorbance levels from ELISA analysis using serum from vancomycin treated and orally tolerized Balb/c mice to determine amount of OVA-specific IgM.* The mice were sacrificed and serum was harvested to run an ELISA to determine the amount of OVA-IgM present. There were 4 different treatment groups within the study; NT (no antibiotic, non-tolerized), OT (no antibiotic, orally-tolerized with OVA), Vanc NT (vancomycin, non-tolerized), Vanc OT (vancomycin, orally-tolerized with OVA). The antibiotic treatment , vancomycin 1 mg/mL, was given for 10 days. All of the mice orally tolerized were given 100 µL of 10 mg/mL OVA solution for the last 5 days of treatment. A total of four different dilutions were performed. The dilution (1:50) where the absorbance values fell within the limit of detection was selected for further analysis.

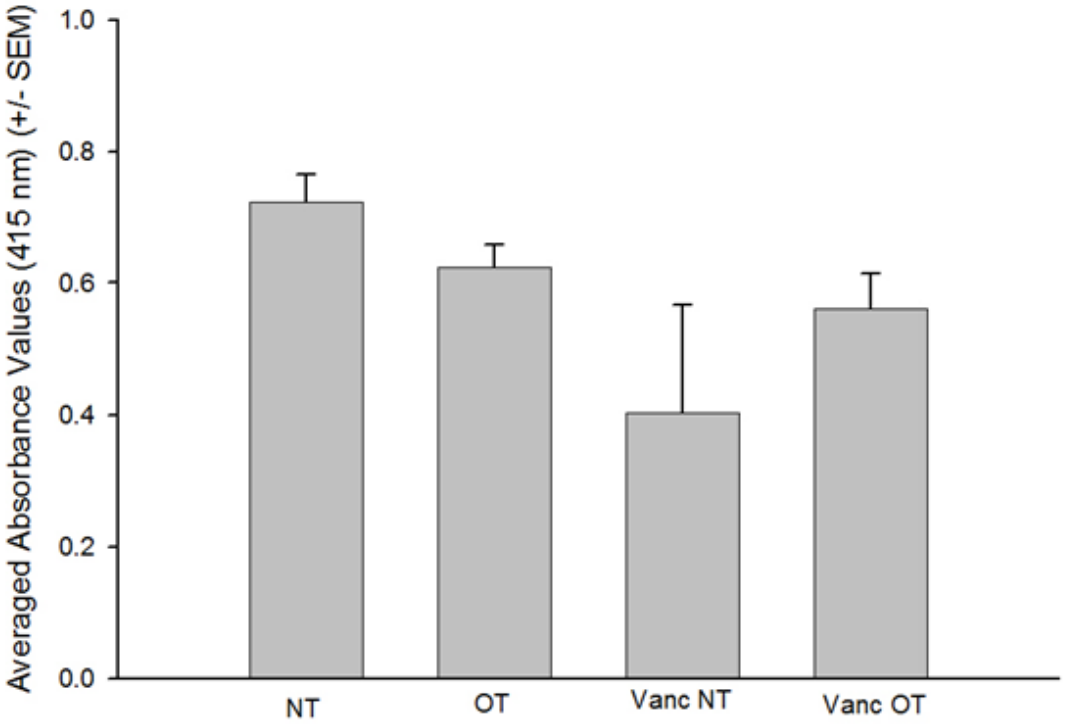

Figure 16 *Vancomycin effect on oral tolerance is inconclusive due to lack of tolerance induction determined by amount of OVA-specific IgG.* Graph depicting averaged absorbance levels at the 1:50 dilution from ELISA analysis using serum from antibiotic treated and orally tolerized Balb/c mice. The mice were sacrificed and serum was harvested to run an ELISA to determine the amount of OVA-IgG present. There were 4 different treatment groups within the study; NT (no antibiotic, non-tolerized), OT (no antibiotic, orally-tolerized with OVA), Vanc NT (vancomycin, non-tolerized), Vanc OT (vancomycin, orally-tolerized with OVA). The antibiotic treatment , vancomycin 1 mg/mL, was given for 10 days. All of the mice orally tolerized were given 100 µL of 10 mg/mL OVA solution for the last 5 days of treatment The absorbance values were an average of 3 for each treatment group. No statistical significance was demonstrated in the amount of OVA-specific IgG.

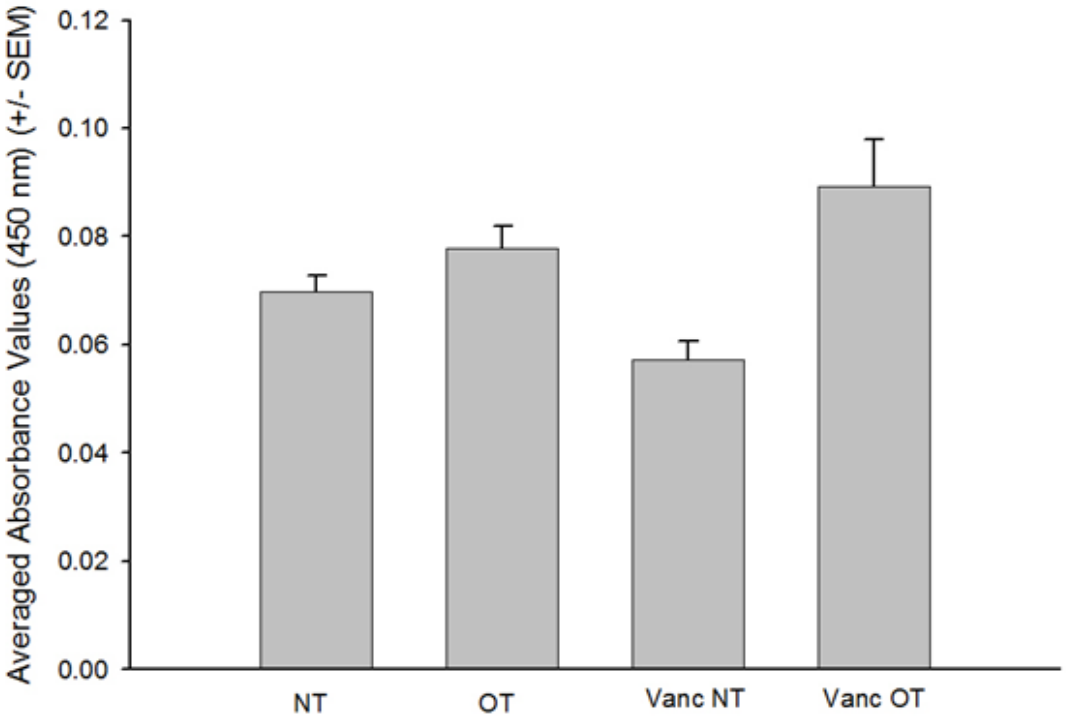

Figure 17 *OVA-specific IgM levels are not significantly altered in response to oral tolerance induction or antibiotic treatment.* Graph depicting averaged absorbance levels at the 1:50 dilution from ELISA analysis using serum from antibiotic treated and orally tolerized Balb/c mice. The mice were sacrificed and serum was harvested to run an ELISA to determine the amount of OVA-IgM present. There were 4 different treatment groups within the study; NT (no antibiotic, non-tolerized), OT (no antibiotic, orally-tolerized with OVA), Vanc NT (vancomycin, non-tolerized), Vanc OT (vancomycin, orally-tolerized with OVA). The antibiotic treatment , vancomycin 1 mg/mL, was given for 10 days. All of the mice orally tolerized were given 100 µL of 10 mg/mL OVA solution for the last 5 days of treatment The absorbance values were an average of 3 for each treatment group. No statistical differences were found in the amount of OVA-specific IgM between treatment groups.

DISCUSSION

This study examined the effect of antibiotic treatment on the process of oral tolerization. Antibiotic use and availability has increased recently within industrialized countries. Antibiotics are frequently and increasingly used and freely given out, at certain pharmacies even given to a patient cost-free [57]. It is well-understood that frequent antibiotic use can lead to drug resistance; however there are other effects taking antibiotics could have on the body. The full extent of antibiotic use on the body, including the immune system, is not fully known. It has been found that along with the increase in antibiotic use the occurrence of many diseases with an immunological basis including allergies, asthma, Crohn's disease, has also risen [42, 43]. Antibiotics come in a variety of classes and are prescribed to treat infections caused by different bacterial species [30, 45]. A consequence with killing the bacteria causing the infection is that other bacterial species, including probiotic strains of Lactobacilli and Bifidobacteria, are also affected. The decrease in probiotic bacterial levels can be devastating to the body due to the many interactions that occur between the probiotic bacteria and the immune system, including both the innate and suppressed immune responses [25, 35]. Suppressed immune responses due to a state of oral tolerization are very important in regulating the immune system from reacting to antigens that are not pathogenic in nature, including antigens found in food or

normal flora within the GI tract [6, 14]. Thus, the goal of these studies was to determine the effect of antibiotic treatment on the probiotic bacteria within the GI tract and the consequential effect on oral tolerization.

Our findings were that antibiotic treatment is able to reduce levels of probiotic bacteria with the GI tract. The antibiotics tested included cephalexin, vancomycin, and metronidazole. Both cephalexin and vancomycin were able to inhibit the growth of Lactobacilli on MRS and TSA agar in *in vitro* antibiotic disk testing (**Figure 2**). Cephalexin is able to significantly reduce populations of Lactobacilli as determined by using MRS agar, which facilitates the growth of Lactobacilli in aerobic conditions (**Figure 3**). Vancomycin was also effective against Lactobacilli from fecal matter *in vitro* as well as *in vivo* (**Figure 3**). Vancomycin was not as effective as cephalexin; however, vancomycin is known to be effective only against some strains of Lactobacilli. This result was also demonstrated by Hamilton-Miller et al [49]. The least effective antibiotic was metronidazole (**Figure 3**). This finding was expected due to difference in the cultured bacteria, gram positive Lactobacilli, and the targeted bacterial species, gram negative bacteria.

Importantly and unfortunately, our experiments have highlighted how differences between individual organisms can affect *in vivo* studies, such that individual mice within a group did not always respond to an antibiotic the same way. Variations of this nature were found in our last study. After 10 days of treatment the amount of Lactobacilli from fecal matter was reduced; this reduction was found in all groups of mice including the control mice (**Figure 13**).

ELISA analysis of the levels of OVA-specific IgG (**Figure 16**) and IgM (**Figure 17**) did not show that tolerization occurred although it was expected. Because oral tolerization was not found in the control mice no conclusions could be made regarding the effect of vancomycin on oral tolerance. One finding from this study was the state of oral tolerance cannot be induced in mice that have altered levels of Lactobacilli within the GI tract. This data furthers coincides with the findings of Prioult et al. that probiotic bacteria species are necessary for the successful induction of oral tolerance [55].

Lastly, our work demonstrated that time is important for the development of antibodies (i.e. IgG) to be detected to determine if oral tolerance was successful. IgG is also a better indicator of an activated immune response than the antibody IgM. IgM is the antibody that is first generated and therefore not necessarily demonstrating an active immune response has occurred instead of a suppressed immune response. IgG will only be generated if class switching has occurred; indicative of an active immune response. In experimentation it was concluded that 5 days post immunization was not enough time for class switching to occur to generate sufficient amounts of OVA-specific IgG to be detected in ELISA analysis showing oral tolerization occurred. Time is necessary for class switching to occur and it takes around 14 days for IgG to be detected within the serum after immunization [1]. The time period of 3 weeks was long enough to allow IgG to be generated to demonstrate oral tolerization has occurred, shown in the tolerization study (**Figure 11**). Oral tolerization found with IgG analysis was

not found using IgM analysis further demonstrating IgM (**Figure 12**) should not be used to demonstrate oral tolerization has occurred.

Our work helps contribute to the controversy that probiotics, while beneficial to the host in other areas, may not be necessary for tolerance induction. Three studies involved in the research over the interaction between probiotic bacterial species and oral tolerization have been performed by Prioult et al., Bashir et al., and Furrie et al [55, 58, 59]. Prioult et al. and Bashir et al. came to the final conclusion that probiotic bacteria are necessary for oral tolerization [55]. The methods of Prioult et al. included feeding germ-free mice probiotic strains of bacteria including both Lactobacilli and Bifidobacteria. Eighteen days after biotic treatment a portion of the mice were orally tolerized with whey protein and then immunized with bovine β-lacto-globulin (BLG). The levels of BLG-specific IgE and BLG-specific IgG_1 and IgG_2 levels were analyzed via ELISA. The levels of BLG-specific antibodies were reduced in mice orally tolerized with whey protein, with variations in the amount of immune activity depending on the bacterial strain fed to the mice. The methods used by Prioult et al. were similar to ours such that the mice were fed a protein, immunized, and then antibody levels were determined via ELISA. Bashir et al. treated a group of mice with an antibiotic cocktail (kanamycin, gentamycin, vancomycin, metronidazole, and colistin) for 1 week prior to introducing antigen, Ara h 1 (a peanut allergen), orally and then after oral introduction antibiotic treatment was continued [59]. A reduction in the bacterial flora after antibiotic treatment was found, however, the amounts of B and T cells in the spleen, lymph nodes, and Peyer's patches were

not affected. Additional experimentation included tolerizing antibiotic-treated mice to *Ara h* 1 along with the cholera toxin. The amount of IgE specific for *Ara h* 1 in the antibiotic-treated orally tolerized mice was significantly elevated compared to the non-antibiotic-treated mice. When the flora depleted with antibiotic treatment was replaced, the amount of *Ara h* 1-specific IgE in the antibiotic-treated mice was reduced. One of the conclusions was that the flora within the GI tract is necessary for oral tolerization; a conclusion that supports the work of Prioult et al. and is in line with the beliefs of our lab. Research by Furrie et al. did not conclude that probiotic bacteria were necessary for oral tolerization [58]. Furrie et al. orally tolerized germ-free mice with OVA (1 mg/g of body weight) harvested the serum 60 minutes after oral induction of OVA and then the serum from orally tolerized mice was injected into a second group of germ-free mice. The second group was later immunized, to test if oral tolerization could be transferred. The second group of mice that were given serum from orally tolerized mice showed a decrease in immune activity (as measured by OVA-specific IgG in an ELISA) when compared to mice that were given serum from non-tolerized mice. Due to oral tolerization occurring in the absence of flora within the GI tract, it was concluded that the flora within the GI tract (including probiotic species) are not necessary for oral tolerization to occur. The methods that were used by Furrie et al. differ greatly from our methods as well as other groups that are doing research in this area. While oral tolerization was still observed by Furrie et al. the means of inducing the oral tolerization were not conventional. Typically, oral tolerization is induced by feeding antigen directly to

a group of mice and not by injecting serum for tolerized mice into a different group of mice. More importantly the time frame after orally tolerizing mice until the time of sacrifice was only 60 minutes. It takes several hours before an immune response can fully be generated [1]. The time frame of 60 minutes is extremely short considering all of the interactions that need to occur for oral tolerization to be induced. There has been a lot of research performed the connection between probiotics and oral tolerance. The methods and conclusions can vary between the different groups that have performed research. Based on the literature available our lab has come to the conclusion that probiotic bacterial species are necessary for oral tolerance to be induced; a conclusion that is in agreement with Prioult et al. and Bashir et al.

Unlike the multitude of experiments performed on the interaction of probiotic bacteria and oral tolerance very few studies have investigated the effect of antibiotics on oral tolerance. One has been performed by Pecquet et al [56]. Their methods included treating mice with either ibuprofen or the combination of gentamycin/ vancomycin for a total of 7 days. After 5 days of treatment whey proteins that contained β-lactoglobulin (BLG) was fed to induce the state of oral tolerance. Four days after the feeding of BLG the mice were immunized intraperitoneally with BLG (80 mg) and five days later the mice were sacrificed. The serum was used for ELISA analysis to find the amount of IgE specific for the antigen BLG present. Their findings were that antibiotic and ibuprofen treatments altered the process of oral tolerization. The amount of BLG-specific IgE detected in tolerized ibuprofen-treated mice was statistically the same as the

amount of BLG-IgE in non-tolerized non-ibuprofen-treated mice. The work by Pecquet et al. has shown that antibiotic usage and ibuprofen treatment is capable of altering oral tolerance. However, there are many other antibiotics that are commonly used and the effects of these other antibiotics on oral tolerance are not known. Pecquet et al. laid the foundation of the implications that antibiotics could have on the immune system. It is because of these findings our lab has decided to expand this study to determine if other antibiotics could have similar effect on oral tolerance.

Given the body of literature on antibiotic, probiotics, and oral tolerance, our hypothesis was antibiotic usage decreases intestinal microbiotic populations, including the probiotic species *Lactobacillus* spp., impeding oral tolerance and heightening the response of the immune system in Balb/c mice. To address our hypothesis we used the study design that was very similar to the methods employed by Pecquet et al [56]. The differences that were found in our study included using the antibiotics cephalexin, vancomycin, and metronidazole and testing for OVA-specific IgG or IgM (as opposed to testing for IgE by Pecquet et al.). For oral tolerization OVA was used in our study instead of BLG and the time frame was also different. Our study design used Balb/c mice treated with antibiotics for a total of 10 days. During the antibiotic treatment the mice were orally tolerized with OVA. After treatment was concluded all of the mice were immunized with OVA and sacrificed either 5 days or 3 weeks after immunization. Serum was harvested and used to analyze OVA-specific IgG and IgM levels. Levels of IgM were detected because it is the first antibody that is generated after

an active immune reaction; however, non-specific IgM can be found in the serum if an active immune reaction has not occurred. IgG levels were detected because it is the antibody most prevalent in serum and is only detected in an active immune response because class switching needs to occur for it to be generated.

The frustrating component of this research that was a major reason why more conclusions could not be drawn was that the antibiotic treatment did not consistently work for every mouse in a group and additionally, mice that were not treated with antibiotic would spontaneously have a drop in intestinal probiotic numbers. There are several reasons that could account for the reduction in Lactobacilli found in the non-antibiotic-treated, OT mice after 10 days of treatment (**Figure 13**). The stress induced from feeding could have affected the bacterial levels within the fecal matter. The act of feeding with needles ensures OVA will reach the stomach, however, is unnatural to the mice undergoing the treatment and therefore could cause a new form of stress. Additional forms of stress could result from separating the mice at the beginning of the study. Initially all of the mice were placed in cages along with liter mates. After the first time obtaining fecal matter all of mice were placed into a new cage individually; creating a change to the living environment and possibly inducing stress. All of the mice are also housed in a non-pathogen free environment; which could allow the mice to come into contact with many different antigens within the environment. This could affect the immune system and create stress to the bodies. There are many variables that exist when using animal models that cannot always be addressed and therefore variations are likely to occur. The

animals could be moved to different cages several days before fecal samples are taken to allow the animals to recover from the stress that could have been inflicted. The animals could also be held daily starting several days before samples are taken to allow the animals to adjust to being handled. Stress will occur when animals are being used, but steps can be taken to reduce or control the amount of stress that the animals have. By experimentation the variations that occur can be connected with differences in results and therefore conclusions revealing new insights to the complexity of the body, and more specifically the workings of the immune system, can be found.

Antibiotic usage is increasing in industrialized countries along with this the prevalence of disease with an immunological basis is also increasing. Antibiotics are commonly prescribed and used to treat infections, however, the range of effects of antibiotics other than killing the pathogenic bacteria are not fully studied or known. Antibiotics kill the bacteria causing the infection along with other bacteria, probiotic species, that are located within the body and the consequences of killing the probiotic species can be devastating in nature. It is possible that antibiotics will alter the important oral tolerized state that is vital for the immune system to be functioning correctly. By studying the effect of antibiotics on the immune system a broader picture of the effect of antibiotics on the entire body can be found. This can also gain some insight into the increase in occurrence of diseases with an immunological basis, including allergies, asthma, Crohn’s disease and many others.

In summary this study explored the effect of antibiotic treatment on probiotic species within the GI tract and the ability to breakdown the orally tolerized state. While it was hoped that we could demonstrate that antibiotic treatment can reduce probiotic bacterial cultures and possibly alter oral tolerance resulting in active immune response where a suppressed immune response should be found, complications prevented this. What these studies did conclude is that the amount of time after immunization before sacrifice is important to detect oral tolerization, IgG is a better indicator of an active immune reaction as opposed to a suppressed immune reaction, and lastly probiotic populations within the GI tract are important for the induction of oral tolerance which will allow further investigation to be done.

Future Directions

The next step in this study would be to determine the reasons why the levels of bacteria within the GI tract are reduced in mice that did not receive antibiotic treatment. Once the reasons behind changes in bacterial levels have been discovered and addressed the effects of antibiotics on oral tolerization can continue to be studied. The experiments that have been performed should be repeated to determine if the results are consistent once reduced bacterial levels in control mice are addressed.

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