Evaluating Immunotoxicity of Quaternary Ammonium Compounds

Valerie A. McDonald

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Terry C. Hrubec, Co-Chair

Jill Sible, Co-Chair

Caroline N. Jones, Member

Xin M. Luo, Member

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Abstract

Alkyl dimethyl benzyl ammonium chloride (ADBAC) and didecyl dimethyl ammonium chloride (DDAC) are common quaternary ammonium compounds used as disinfectants in households, medical, and restaurant settings. They cause occupational skin and respiratory hazards in humans, and developmental and reproductive toxicity in mice. They also cause increased secretions of proinflammatory cytokines in cell lines and vaginal inflammation in porcine models; but have not been evaluated for developmental immunotoxicity. We assessed immunotoxicity *in-vitro* with J774A.1 murine macrophage cell line by analyzing cytokine production and phagocytosis; and evaluated developmental immunotoxicity in CD-1 mice by analyzing antibody production. Additionally, because of the associations between gut microbiome dysbiosis and immune disease, we monitored changes in the microbiome as a result of ADBAC+DDAC exposure. Production of cytokines TNF-alpha and IL-6 increased at low ADBAC+DDAC concentrations, and IL-10 decreased in the murine macrophages with ADBAC+DDAC exposure. The phagocytic function of macrophages was also severely decreased. ADBAC+DDAC altered the mouse microbiome by decreasing the relative abundance of *Bacteroides* and increases in *Clostridia* in F0 and F1 generations. IgG primary and secondary responses were altered in F1 male mice; and IgA and IgM production were decreased in secondary response in F2 male mice. Since ADBAC+DDAC show signs of immunotoxicity in mice, further studies are needed to reassess risk for human exposure as ADBAC+DDAC may be contributing to immune disease.

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General Audience Abstract

Disinfectants are used every day in households, hospitals, and restaurants. Two common ingredients in disinfectants are alkyl dimethyl benzyl ammonium chloride (ADBAC) and didecyl dimethyl ammonium chloride (DDAC). These chemicals can cause asthma and allergic dermatitis in humans. In animals, they cause reduced fertility, altered development, and tissue inflammation. Disinfectant exposure could potentially alter bacterial populations in the gut. Altered microbial populations are associated with many inflammatory diseases. This study evaluated ADBAC and DDAC for their ability to alter immune function and change bacterial populations in the gut. Exposure to ADBAC and DDAC caused inflammation and altered antibody production for two generations. ADBAC and DDAC exposure also significantly altered bacterial communities in the gut. Both changes in the immune function and changes in the gut bacteria could contribute to inflammatory disease. Humans are exposed frequently to ADBAC and DDAC. If these chemicals alter immune function in humans, they could be contributing significantly to human disease.

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Abbreviations

Alkyl dimethyl benzyl ammonium chloride (ADBAC), Analysis of variance (ANOVA), Antigen Presenting Cell (APC), Benzalkonium chloride (BAC), Bisphenol A (BPA), Bovine serum Albumin (BSA), Delayed type hypersensitivity (DTH), Developmental immunotoxicity (DIT), Didecyl dimethyl ammonium chloride (DDAC), Dioctyltin dichloride (DOTC), Di(2-ethylhexyl) phthlate (DEHP), Enzyme linked Immunosorbent assay (ELISA), Extended One Generation Reproductive Toxicity Study (EOGRTS), Gut microbiome (GM), Immunoglobulin M (IgM), Immunoglobulin G (IgG), Immunoglobulin A (IgA), Interferon (INF), Interleukin-1-Beta (IL-1β), Interleukin-8 (IL-8), Interluekin-18 (IL-18), Interluekin-1 (IL-1), Interleukin-12 (IL-12), Interleukin-6 (IL-6), Interleukin-12 (IL-12), Interleukin-10 (IL-10), Keyhole limpet hemocyanin (KLH), Lipid Polysaccharide (LPS), Lowest observed adverse effect level (LOAEL), Nitric oxide (NO), Neural tube defects (NTDs), Non steroidal anti-inflammatory drug (NSAID), No observed adverse effect level (NOAEL), Phosphate buffered saline (PBS), Plaque-forming cell assay (PFC), Polyvinyl Chloride (PVC), Principal component analysis (PCA), Sheep red blood cells (SRBC), T-cell dependent antibody response (TDAR), The Organization for Economic Cooperation and Development (OECD), Tumor necrosis factor (TNF-α), Quaternary Ammonium Compound (QAC)

I. Literature Review

Introduction

The purpose of this review is to provide an overview of the general immune system, move into toxicity type and testing methods, and how the immune system is tied into the microbiome and give background on quaternary ammonium compounds.

Immune system

A Brief Overview of the Immune System

The immune system is typically divided into two functional groups, innate and adaptive. These two arms of the immune system communicate frequently. A key example of this, are macrophages and other leukocytes that phagocytose cellular material and degrade it for presentation of the antigens to T-cells which then induce B cells to produce antibodies (Chaplin, 2003; LeBrec et al., 2014). Macrophages are also integral to the immune system for regulatory purposes . They release messenger proteins called cytokines to enact functional changes within the same cell and throughout the immune system (Chaplin, 2003).

Macrophages typically act through one of three pathways, proinflammatory (classically activated), anti-inflammatory (alternatively activated), and regulatory. Classically activated macrophages release proinflammatory cytokines such as tumor necrosis factor (TNF-alpha), interleukin-6 (IL-6), and interleukin-12 (IL-12). These macrophages activate immune cells to increase inflammation and microbicidal activity. Macrophages activated through the alternative pathway release interleukin-10 (IL-10) which dampens the

inflammatory response and promotes tissue repair and wound healing (Mosser et al., 2008). Macrophages and other phagocytes produce nitric oxide (NO) in response to cytokines which aid in the destruction of viruses, bacteria, fungi, protozoa, and tumor cells (MacMicking et al., 1997). Similar to macrophages, neutrophils and other granulocytes are in charge of phagocytosing invaders. Generally, the immune system is weaker in the young but matures as development progresses (Luebke et al., 2008; DeWitt et al., 2011). Many granlucytes are found in smaller populations in newborns compared to adults because the neonatal bone marrow produces fewer cells (Wilson, 1986). Complement proteins work with these phagocytes to opsonize bacteria so that phagocytes are attracted to foreign invaders. Natural killer cells are responsible for limiting the spread of viral infection and tumor cells. Eosinophils and mast cells are cells that are associated with the allergic response (Chaplin, 2003).

T-cells and B-cells are essential to the immune system. There are several types of T-cells including Th1, Th2, Th17, Treg and T-follicular helper cells. Th1 are aid in defense from intracellular pathogens, Th2 aid in fighting parasites, Th17 aid in defense from extracelluar bacteria, and T-follicular helper cells work with B-cells to produce antibodies (Hirahara et al., 2016). T-cells work with antigen presenting cells (APCs), i.e., macrophages and dendritic cells, to communicate with B-cells which produce antibodies. Antibodies help the immune system tag infectious and toxic materials to be attacked by complement or other white blood cells. There are five main classes of antibodies: IgM, IgG, IgE, IgA, and IgD. IgM is produced quickly after antigen uptake, usually about 5 days after antigen exposure, and circulates the blood. IgG is produced a little later than IgM (10 days after exposure)

and is secreted into the blood. IgE is associated with the allergic response and is essential for attaching helminths in the body. IgA is associated with mucosal immunity and is predominantly secreted by mucous membranes and along the digestive path. IgD is associated with basophils and mast cells which are associated with the allergic response (Chaplin, 2003; Schroeder et al., 2010).

Antibodies can be formed via T-cell dependent or T-cell independent mechanisms. T-cell independent antigens are multivalent with repeating structure, for example, bacterial lipopolysaccharide. B cells bind directly to the antigen, without needing to first be processed by the T-cell. This allows the B cell to quickly make IgM antibodies. T-cell dependent antigens have protein epitopes that need to be presented by phagocytic cells to a T-cell. Once activated by a T-cell B-cells can differentiate into antibody producing cells, called plasma cells (Chaplin, 2003; Schroeder et al., 2010; LeBrec et al., 2014).

Overview of Critical Windows of Immune Development

Critical windows of immune development are specific unique time windows during development that are clustered heavily during gestation and early life. Toxicant exposure during these events can result in life long changes to the structure of function of the immune system. To properly evaluate immunotoxicity, one must study critical windows of immune development. There are generally five critical windows in humans and rodents: initiation of hematopoiesis, stem cell migration and cell expansion, colonization of the bone marrow and thymus, maturation of immunocompetence, and lastly immune memory (DeWitt et al., 2011). Xenobiotic exposure during one stage of development may alter

immune responses for the rest of the lifetime of the individual, while the same exposure at another stage may have no adverse effect (Dietert, 2011). Different sensitivities may be found at different exposure timelines because they could affect the immune system at a different critical point. Exposures within critical windows can have a large variety of effects including delayed immune maturation, failure of negative and positive selection in the thymus, and higher rates of autoimmunity or immunosuppression (West, 2002).

Developmental Immunotoxicity

Toxicology: A general introduction to type and testing.

Toxicology is the study of exposures that cause adverse effects on living systems. Toxicity testing was not standardized until several chemicals caused loss of human life. This prompted the federal government to pass laws to ensure the safe use of food chemicals and environmental exposures. One of the first laws regulating toxic exposures was The Food and Drug Act which was passed in 1906, and required labeling of food additives (Weideman, 1993). Even after the Food and Drug Act was passed, many products still lacked proper warning labels and used dangerous combinations of chemicals. In 1937, over 100 people died after drinking an "Elixir of Sulfanilamide" sold by the Massengill Company (Weideman, 1993; Krewski et al., 2010). An elixir means that the solvent in a solution is alcohol. Massengill's elixir used the toxic solvent, diethylene glycol. This mixture was considered mislabeled which allowed the product to be removed from the market (Weideman, 1993; Krewski et al., 2010). In response, the Food, Drug, and

Cosmetics Act, which made toxicity testing in animals mandatory before products could put into the market, was passed in 1938 (Weideman, 1993; Krewski et al., 2010).

A similar trend was enacted for environmental chemicals. The 1947 Federal Insecticide, Fungicide, and Rodenticide Act required registration with the Department of Agriculture before chemicals could be sold internationally or interstate nationwide (Krewski et al, 2010). Jurisdiction was moved in 1970 to the EPA as it was created. Amendments were created in 1972 that required testing to show that there were no adverse effects of each pesticide (Kreski et al., 2010). Additional amendments were passed to ensure the safety of food chemicals, additives, and environmental chemicals, including establishing maximum tolerated doses in different populations.

Today, a variety of toxicity testing is mandated depending on function and potential exposures of the chemical or drug. Before pesticides can be registered for use in the U.S., certain toxicity tests are required. The Code of Federal Regulations, Title 40 Chapter 1, part 158, lists the toxicology testing requirements for pesticides. Immunotoxicity testing is required if evidence from the previous testing suggests that it is necessary, but there is not a required test guideline for developmental immunotoxicity. Test guidelines include acute, chronic, sub chronic, mutagenic, carcinogenic, teratogenic, reproductive, and immunological toxicity testing. Testing often focuses on skin irritation, acute and chronic toxicity, carcinogens, and mutagens. These focus may miss key elements of noncommunicable diseases (Dietert, 2014). Many noncommunicable diseases, such as asthma, allergy, cardiovascular disease, diabetes, inflammatory bowel disease, and other

autoimmune diseases, are inflammatory in nature. Inflammation is regulated by the immune system, so it is vital to seek out exposures that can affect and perhaps cause these diseases.

Immunotoxicology emerged in the 1970's to study agents that caused immunosuppression (Dietert, 2014). The field expanded, driven by the rise and emergence of diseases in immunocompromised patients. While it was known from animal studies that challenging the immune system early in life resulted in an altered immune function, it took until the early 2000's for developmental immunotoxicity (DIT) to become a research focus (Dietert, 2014).

Developmental immunotoxicity results when a biological or chemical exposure in early life periods functionally alters the immune system (DeWitt et al., 2011*).* The EPA does have an immunotoxicity test guideline, but it focuses on adult animals, not on the developing animal, where the risk to the immune system is highest (LeBrec et al., 2014; Leubke et al., 2006). DIT overlaps with other toxicology fields. DIT is most closely related to immunotoxicity, which focuses on agents that directly affect immune system function, and developmental toxicity, which focuses on exposures, that alter normal development. The difference between developmental toxicity and DIT is the focus on the development of immune system function. DIT testing is not currently included in regulatory testing, but developmental and reproductive toxicity are tested. The Organization for Economic Co-operation and Development (OECD) offers a test guideline for an Extended One Generation Reproductive Toxicity test (EOGRTS), which includes a cohort of rodents for

DIT. This has led to more labs testing for potential immunotoxicants, but more screening is needed to protect the population from adult onset disease. Older screening for immunotoxicity used relatively high doses and structural endpoints. (Tonk et al., 2015). This is problematic because the developing immune system is highly sensitive (Leubke et al., 2006). Altered immune function can be observed before structural changes are discernable and the functional changes occur at lower doses than those required to cause structural changes (Luebke et al. 2006; Tonk et al. 2005).

Developmental Immunotoxicity (DIT)

In humans, immune system maturation and development takes many years starting *in-utero* and continuing through puberty. Inflammatory disease, autoimmune and allergic disease, as well as cancer, are becoming increasingly common. Immune cells are found throughout the body and are involved in many homeostatic processes. When an exposure affects the immune system, it may have a large effect on underlying causes of common non-communicable diseases (Dietert, 2011; DeWitt et al., 2011*).* The overall risk for immunotoxicity is higher during pre-natal and early development. Exposures that may not cause ill effects in adults may adversely harm exposed fetuses and increase the risk of immune disease both as in infant and as an adult (Dietert et al., 2005; Dietert et al., 2008). Thus, more stringent DIT testing should be conducted, especially on chemicals that have widespread exposure to both adults of reproductive age and children alike.

DIT from Drug Exposure

A number of drugs when tested show DIT. For example, diethylstilbestrol and diazepam are both drugs that show DIT in humans and rodents (reviewed by Luebke et al., 2006). Diethylstilbestrol, previously prescribed to prevent miscarriages and premature delivery, has immunotoxic effects both in humans and rodents (Leubke et al., 2006). Babies of exposed mothers have increased incidence of asthma and some autoimmune disease. In rodents, female rodent offspring from exposed dams, have decreased T-cell independent IgM production and suppressed delayed-type hypersensitivity (DTH) responses (Luebke et al., 2006). Diazepam, prescribed for anxiety disorders, has been associated with increased risk of pneumonia, indicating possible immunotoxicity (Luebke et al., 2006). Rodent offspring of exposed dams demonstrated immunosuppression with decreased T-lymphocyte proliferative response and decreased IgG production. General immunosuppression of the offspring persisted into adulthood, with increased parasitic larvae populations after immune challenge and decreased IgG production (Leubke et al., 2006).

Dexamethasone is prescribed for autoimmune disease, cancer treatment, inducing labor in adults, and treating respiratory distress in newborns (Dietert et al., 2003). In rodents, dexamethasone exposure during gestation, caused altered immune function. The offspring had decreased juvenile DTH responses, and adherent splenocytes showed decreased production of IL-2 and IL-4 (Dietert et al., 2003). Adult offspring had persistent effects from gestational exposure including alterations in antibody production with increased IgG and decreased IgE production (Dietert et al., 2003).

Even common non-steroidal anti-inflammatories (NSAID), such as indomethacin, acetyl salicylic acid, and diclofenac sodium salt, have been tested as immunotoxicants in rodents (Kushima et al., 2007). Juvenile offspring from NSAID exposed dams had alterations in immune cell populations and dose-dependent decreases in secondary antibody response. This indicates that young offspring may be more susceptible to disease (Kushima et al., 2007). Most of these effects were transient and did not persist into adulthood (Kushima et al., 2007).

Cyclosporin-A is another drug used to suppress the immune system in order to treat autoimmune disease and prevent rejection of organ transplants (Hussain et al., 2005). *In-utero* exposure in rodents caused decreased DTH, increased IgG production, and IL-4 production (Hussain et al., 2005). Increased IgG production persisted into adulthood. This indicates that early exposure to the developing immune system can have lifelong effects (Hussain et al., 2005).

DIT from Environmental Exposures

A variety of environmental contaminants exhibit DIT. Di(2-ethylhexyl) phthalate (DEHP) is a high production volume chemical used in polyvinyl chloride (PVC) products, and can leak out from PVC products and into the environment (Tonk et al, 2012). Phthalates are known reproductive toxicants in rodents (Tonk et al., 2012). Phthalates are associated with immune diseases such as allergy, asthma, and eczema in humans (Tonk et al., 2012). DEHP exposed juveniles and exposed adults had increased IgG production, decreased DTH, and

increased IL-2, IL-4, IL-6, IL-10, IL-13, interferon gamma, and TNF alpha. Generally, adults needed a higher dose before these immune modulations were observed. This indicates that the juvenile immune system is more sensitive than that of adults (Tonk et al., 2012). Exposed juveniles also had decreased natural killer cells, which are essential for preventing cancer and viral infections (Tonk et al., 2012). Di-n-octyltin dichloride (DOTC), another chemical that is used in PVC production, causes DIT in rodents. Juvenile and young adult rodents exhibited dose-dependent decrease in the secondary antibody response for IgG, as well as alterations in NO and TNF-alpha production (Tonk et al., 2011). Developmental toxicity was seen at higher doses while DIT was observed at lower doses (Tonk et al., 2011).

As reviewed by Luebke et al., (2006) lead exposure can result in several different types of toxicity including developmental, reproductive, immunotoxicity and DIT. Lead is a heavy metal naturally found in the environment and frequently used in industry. Lead use is common in some countries, but it is restricted in the United States. In humans, early exposure can cause increased IgE antibody production (Leubke et al., 2006). DIT has been observed in rodents exposed in early and late gestion to lead. Exposure to lead during gestation increased thymus weights, decreased DTH, and decreases NO production. Sex-specific effects of DIT were observed. Males had increased IL-12 and decrease IL-10, while females had increased IL-10 (Bunn et al., 2001). This indicates that there may be sex-specific effects from early exposure to developmental immunotoxicants.

DIT from Food Additives

Ethanol and some food additives such as 4-methyl anisole have shown both immunotoxicity and DIT. Ethanol is well known as a developmental toxicant causing fetal alcohol syndrome in humans. When evaluated for DIT in rats, alterations in NO production, TNF-alpha, IL-10, DTH and increased IgM and IgG antibody production were found (Tonk et al., 2013). Immune changes were observed at doses lower than needed to cause developmental defects. Again this indicates that the immune system is more sensitive to functional changes than structural changes (Tonk et al., 2013). Another food additive, A4-methyl anisole, is used in fragrance oils, as a food flavoring agent, as well as in cleaners, and other biocidal products. Exposure to this chemical increased IL-13, TNF-α production, reduced IgG antibody production and reduced eosinophil cell numbers in rodents (Tonk et al., 2015).

DIT Testing: Extended One-Generation Reproductive Toxicity Study and T-cell Dependent Antibody Response

The OECD produces the EOGRTS test guide that covers toxicants not covered by other regulated toxicity testing which affect early life stages (OECD, 2012). Researchers also use this to determine the No Observed Adverse Effect Level and the Lowest Observed Adverse Effect Level (NOAEL and LOAEL). EOGRTS is an effective protocol for testing because it assesses several types of toxicity in both the parental and F1 generations. A drawback of this testing regime is the long exposure time and the number of animals. The test guide recommends four cohorts to determine reproductive, developmental, DIT, and neural toxicity. Each cohort has different endpoints to screen for its respective toxicity. Body

weights, as well as food and water intake, are monitored in all cohorts. Cohort 1 measures reproductive and developmental toxicity. Cohort 2 is for the assessment of neurotoxicity and studies the behavior and motor function. Cohort 3 assesses DIT. Tests may include lymphoid organ weights and immune cell counts, but the main endpoint is IgM serum titers through a T-cell dependent antibody response (TDAR).

The TDAR assesses the primary and secondary response of the immune system to T-cell dependent antigens. These are antigens that require T-cell interaction with B-cells in order to produce antigen-specific antibodies. Since this is a multi-step process, a TDAR reflects many functional aspects of the immune system including: the processing of antigens through APCs, the ability of those APCs to meet and differentiate or activate CD4+ T-cells, and activation and differentiation of B-cells in the lymphoid organs. The ability of B-cells to undergo somatic hypermutation, antibody secretion, and isotype switching is also reflected in the TDAR (LeBrec et al., 2013).

The TDAR is stimulated by injection of a protein antigen. Animals are bled 4-6 days post injection of the antigen when IgM titers have peaked and are measured either by ELISA or a plaque-forming cell assay (PFC) (OECD, 2012; LeBrec et al., 2014). Different antigens and assessment methods can be used. The two most common antigens are keyhole limpet hemocyanin (KLH), and sheep red blood cells (SRBC). While EOGRTS allows for SRBC or KLH to be used, the EPA mandated immunotoxicity test guide, specifically calls for the use of SRBC. There are drawbacks to SRBC. Sources for the antigen can be difficult to identify, there is variability between lots necessitating the use of the same lot even in large

scale experiments, and lastly, the SRBC has a short storage time (LeBrec et al., 2014). In contrast, KLH is commercially available, very immunogenic and has low variability between batches making it the protein of choice for TDAR. Less commonly used are tetanus toxoid and hepatitis B antigen (LeBrec et al., 2014).

The PFC assay measures IgM titers, approximately 4 days after immunization. In this test, splenocytes are isolated and mixed with complement proteins that have been previously immunized with SRBCs. This mixture is then added to a sterilized agar solution and incubated at 37°C for 3 hours (LeBrec et al., 2014). Plaques (areas of clearing) occur from anti-SRBC antibodies produced by B-cells (LeBrec et al., 2014). Antibodies tag complement through the classical pathway and the red blood cells are lysed so that the agar becomes clear. While the PFC has been used for over 35 years and is a historically trusted method, it is limiting because it assesses the immune function of one organ and because the spleen must be assayed the same day as harvest (LeBrec et al., 2014). ELISA, on the other hand, can be performed from stored serum, reflects systemic antibody production, and can evaluate a variety of different antibodies (LeBrec et al., 2014). Additionally, ELISA is faster and less time intensive than the PFC assay and therefore is becoming more popular than PCF.

The Microbiome, Disease, and the Immune System

The microbiome interacts with its host in many ways, including contributions to metabolic interactions, digestion, maturation of immune tolerance, and prevention of pathogenic

bacteria colonization (reviewed by Lozupone et al., 2012; Shreiner et al., 2016). The biodiversity of the microbiome is high with over 1000 species present. Most of the species are from phyla *Bacteroidetes* and *Firmicutes*, but also include *Proteobacteria* and *Actinobacteria* (Shreiner et al., 2015; Shi et al., 2017). As reviewed by Lloyd-Price et al.. (2016), variation in taxa within a healthy adult human microbiome is large. Generally, the most abundant genera are *Bacteroides* followed by *Clostridium, Bifidobacterium, Eubacterium, Lactobacillus, and Escherichia* (Lloyd-Price et al., 2016). The relative abundances of different species vary with environment, diet, age, and disease.

Dysbiosis is the shift in microbial taxa populations that are associated with disease. Dysbiosis has been seen with cardiovascular disease, irritable bowel disease, inflammatory bowel disease, Crohn's disease, obesity, type-1-diabetes and multiple sclerosis (Lloyde-Price et al., 2016; Shreiner et al., 2016). For example, in a study of healthy vs. obese people, it was discovered that *Bacteroidetes* abundance was decreased in obese individuals, but returned to higher levels with diet therapy and weight loss (Ley et al., 2006). Microbial communities can follow patterns or trends in certain phenotypes, for example the lowered *Bacteroidetes* in an obese phenotype compared to an individual of healthy weight. The microbiome is highly variable and within an individual of a certain phenotype and their microbiome will always be more similar to their previous microbiome another individuals. For example if an obsese individual, who then loses weight and changes their diet to become a healthy, the similarity of the individuals microbiome would better match the general phenotype of a healthy microbiome, but still be even more similar to their previous microbiome (Ley et al., 2006;).

As reviewed by Bendtsen et al., (2015), the gut microbiome (GM) is formed during and immediately after birth. Evidence indicates that the microbiome may even start developing during gestation (Bendtsen et al., 2015; Koleva et al., 2015). The early GM is affected by the type of delivery, whether a baby is formula or breast fed, and also the mother's microflora when she is breastfeeding (Bendtsen et al., 2015). Ingestion of breast milk also selects for types of bacteria like *Bifidobacterium longum,* which can utilize breast milk as an energy source (Zivkovic et al., 2011). In the first few years of human life, the GM is labile and can rapidly change due to diet and other environmental stimuli, but later in life becomes more 'set', although still capable of change (Bendtsen et al., 2015). Babies born by C-section have higher opportunistic pathogen populations, such as *Staphylococcus, Haemophilus,* and *Enterobacter* (Lloyd-Price et al., 2016; Bendtsen et al., 2015).

The microbiome has an immediate and long-lasting effect on the host immune system. Mice born of transiently infected dams have greater production of antibacterial molecules and reduced inflammatory responses which indicates that the mother's microbiome and the pre-natal microbiome can influence health responses even after birth (Gomez de Aguero et al., 2016). In Swiss-Webster germ-free mice, invariant natural killer T cells accumulated in the intestinal lamina propria and lungs resulting in inflammatory bowel diseases and asthma in susceptible mouse models (Olszak et al., 20212). When these neonatal germ-free mice were colonized with microbes, natural killer T-cells did not accumulate and disease symptoms were alleviated (Olszak et al., 2012). This effect was age-sensitive indicating that the microbiota is tightly intertwined with healthy host development. In another study

mice lacking a gut microbiota had fewer intraepithelial lymphocytes, fewer IgA producing cells, reduced regulatory T-cell populations, and smaller germinal centers (Shi et al., 2017).

The effect of the microbiome on health may relate to its role in digestion, and vitamin and metabolite production. Gut microbes can utilize dietary fiber, which is indigestible to the host but can be utilized by microbes to produce short chain fatty acids, which then interact with the host in many ways. Short chain fatty acids can regulate regulatory T (Treg) cell populations, inhibit pathogens, and aid in suppression of colon cancer (Lloyd-Price et al., 2016). In humans, a high fat, high sugar diet changed the microbial populations quickly, increasing *Alistipes, Bilophila,* and *Bacteroides*, but decreasing the amount of *Firmicutes*. This caused alterations to the short chain fatty acids, which can alter the immune system through mucosal immunity (David et al., 2014; Shi et al., 2017). Other metabolites such as acetate produced from *Bifidobacteria* species induce protection against enteric pathogens (Fukuda et al., 2011).

Butyrate is a short chain fatty acid produced by gut microbes that regulate macrophages and other immune cells. Butyrate exposed macrophages down regulate proinflammatory cytokines like IL-6 and IL-12 and produce less NO. This makes macrophages in the gut less likely to react to commensal populations through down regulation of proinflammatory cytokines (Change et al., 2014). Butyrate also increases the amount of Treg cells in the gut, which also down regulates hosts’ reaction to commensal gut bacteria (Belkaid et al., 2014).

Many studies have shown protective effects from specific bacterial species in the gut microbiome. *Lactobacillus kefiri* and *Bacterioides fragilis* are two common examples (reviewed by Shi et al., 2017). *Lactobacillus kefiri* reduced inflammation after LPS exposure. *Bacteroides fragilis* produces polysaccharide A, which increases the Treg cells in the gut and reduces production of proinflammatory IL-17 (Shi et al., 2017).

By altering microbial communities and metabolite interactions in the gut the microbiome can affect the immune system by stimulating populations of Treg cells other immune system response. This process starts in early life by forcing the immune system to remain tolerant to commensal bacteria and protecting the body from harmful bacteria. As different metabolites are produced by different bacteria, alterations in microbial communities can change the metabolites that are being produced in the gut. Changes that move the GM from a healthy GM can risk the development of inflammatory and autoimmune diseases. For example, the microbiome can increase the risk of cardiovascular disease by having microbial communities that metabolize phosphatidylcholine from the host's diet into trimethylamine-N-oxide which is associated with atherosclerosis. The level of this metabolite is decreased both in vegans and patients recently treated with antibiotics (Shreiner et al., 2015).

Quaternary Ammonium Compounds

Quaternary ammonium compounds (QAC) are cationic detergents that are common in the household, medical, and commercial products. Structurally, QACs contain a central

nitrogen with four alkyl groups and an associated negative anion, such as chlorine or bromine (Gerba et al., 2014). QACs are popular choices in cleansers and antimicrobials because they are versatile and highly effective disinfectants. They are biocidal for bacteria, algae, and both enveloped and enveloped viruses (Gerba et al., 2012). The likely mechanism for biocidal action is a reaction between QACs' positive cation and negatively charged cell walls or membranes, where the long alkyl chains disrupt the cell membrane and causing cell leakage (Gerba et al., 2014). While QACs are widely viewed as safe, new research is demonstrating that they cause adverse effects *in-vitro* and *in-vivo*.

Alkyl dimethyl benzyl ammonium chloride (ADBAC) and didecyl dimethyl ammonium chloride (DDAC) are commonly used in a variety of settings summarized by the EPA (2006) (Tables 1 and 2).

Summary of Uses:

Use Category	Use Site
Industrial processes and water systems	Industrial re-circulating water systems, pulp and paper facilities, cooling water towers, disposal water, oil field operations, and oilfield water flood or saltwater disposal.
Swimming Pools	Swimming Pools, Outside Spas, Whirlpools, and hot tubs
Aquatic Areas	Golf courses, recreational parks, amusement parks, universities, cemeteries, and greenhouse/nurseries
Wood Treatment	Pressure Treatment, Double vacuum, and dip/spray surface treatment
Agricultural Premise and Equipment	Hatcheries, swine/poultry/turkey farms, animal housing facilities, farrowing barns, dressing plants, mushroom farms, citrus farm, florist/flower shops, and greenhouses/nurseries
Residential and Public Access Premises	Homes, mobile homes, cars, boats, playgrounds, boats, public facilities, campgrounds, trailers, campers, trailers, and trucks
Medical Premises and Equipment	Hospitals, health care facilities, medical/ dental offices, nursing homes, autopsy rooms, funeral homes, mortuaries, medical research facilities, acute care institutions, alternative care institutions, newborn nurseries, day-care facilities, and sick rooms
Commercial, Institutional, and Industrial Premise and Equipment	Athletic/recreational facilities, exercise facilities, health clubs, dressing/locker rooms, schools, colleges, universities, transportation terminals, libraries, motels, hotels, barber and beauty salons, convenience stores, offices, commercial/ institutional laundry mats, emergency vehicles, factories, commercial florist, and correctional facilities
Food Handling/Storage Establishments Premises and Equipment	Restaurants, food service establishments, food processing/storage/handling plants and facilities, beverage processing plants, supermarkets, breweries, bars, cafeterias, fishery/citrus/wine/ice cream/ potato processing plants, egg processing plants, dairies, institutional kitchens, fast food operations, rendering plants, school lunchrooms, and packing plants

Table 1 Summary of uses for ADBAC (EPA, 2006).

Summary of Uses:

Use Category	Use Sites
Industrial Processes and Water Systems	Industrial recirculating water systems, cooling water, disposal water, oil field operations, oil field water flood or salt water disposal
Swimming Pools	Swimming pools, outside spas, whirlpools, and hot tubs
Aquatic Areas	Greenhouses/nurseries, golf courses, recreational parks, amusement parks, universities, and cemeteries
Wood Treatment	Pressure treatment, double vacuum, and dip/spray surface treatment
Agricultural Premise and Equipment	Hatcheries, swine/poultry/turkey farms, dressing plants, farrowing barns, mushroom farm, citrus farm, animal housing facilities, florists/flower shops, greenhouses, and nurseries
Residential and Public Access Premise	Homes, mobile homes, cars, trucks, campgrounds, playgrounds, trailers, campers, boats, and public facilities
Medical Premise and Equipment	Hospitals, health care facilities, medical/dental offices, nursing homes, medical research facilities, autopsy rooms, newborn nurseries, acute care institutions, alternate care institutions, funeral homes, mortuaries, day-care facilities, sick rooms
Commercial Institutional and Industrial Premise and Equipment	Athletic/recreational facilities, exercise facilities, schools, colleges, dressing/locker rooms, transportation terminals, libraries, motel, hotels, barber/beauty salons, health clubs, emergency vehicles, correctional facilities, factories, commercial florists, conveniences stores, offices, commercial and institutional laundry mats,
Food Handling/Storage Establishments Premises and Equipment	Restaurants, food service establishments, food storage, handling, processing plants/facilities, beverage processing plants, bars, cafeterias, supermarkets, dairies, egg processing plants, institutional kitchens, breweries, fast food operations, rendering plants, school lunchrooms, packing plants

Table 2 Summary of uses for DDAC (EPA, 2006).

Benzalkonium chloride (BAC), the parent chemical of ADBAC, causes several different toxicities in both *in-vitro* and *in-vivo* models. In pigs, exposure to 2% BAC caused increased in IL-1β, IL-8, IL-18 (D'cruz et al., 2005). These cytokines are associated with observed histological changes that indicate inflammation (D'cruz et al., 2005). BAC is commonly used in eye drops as preservatives at 0.004% to 0.02% (Cha et al., 2004). When human conjunctival and corneal cell lines were exposed to BAC, it increased biomarkers indicating inflammation, including TNF-alpha, IL-1, IL-12, and IL-10 (Epstien et al.,

2009). Occupational exposure to BAC caused asthma in humans (Purohit et al., 2000; Gonzalez et al., 2013). Airway inflammation was observed in rodent models following BAC exposure (Larsen et al., 2011). *In-vitro* QAC exposure has caused mammalian cell toxicity and decreased mitochondrial function (Inacio et al., 2013).

Often, ADBAC+DDAC are combined in disinfectants for increased efficiency and few toxicity studies have examined if there is a synergy in their toxic effects. Combinations of ADBAC and DDAC caused significant developmental and reproductive toxicity in mice. Neural tube defects (NTDs) are severe birth defects of the brain and spinal cord. In ADBAC+DDAC exposed litters the incidence rate of NTDs increased from 0% to 17% with a dose dependent response (Hrubec et al., 2017). ADBAC+DDAC disinfectant use in the building and room is sufficient to cause NTDs, even if mice have no direct exposure through ingestion (Hrubec et al., 2017). This exposure is referred to as the ambient exposure, as the disinfectant is used in the environment the mice are housed in.

Reproductive toxicity was also found in association with combined ADBAC+DDAC exposure. Chronic exposure in mice decreased fertility with fewer pups per litter, fewer pregnancies, and longer pregnancy intervals (Melin et al., 2014). Exposed female mice had decreased numbers of corpora lutea and shortened estrus times, while male mice had decreased sperm concentrations and decreased mobility (Melin et al., 2016)

Additionally, there is evidence that QACs alter anti-biotic resistance genes in the gut microbiome and that microbial resistance to QACs is growing (Buelow, 2015). BAC

resistance is found in the human microbiome as active efflux genes (Buelow, 2015). These genes could pass resistance through horizontal gene transfer to other disinfectants and antibiotics (Buelow, 2015). QACs that enter the body are excreted unmetabolized in the feces (Thorsteinsson et al., 2003). This indicates that active disinfectant could alter the GM as it passes through the digestive tract Wide spread use of QACs could be contributing in the increase in antibiotic resistance and may be altering immune function by interacting with the GM.

Summary

Previous studies have identified toxicity from QACs, particularly ADBAC and DDAC, at the subcellular, cellular, and organismal levels. Taken together, these data indicate potential significant toxicity from QAC exposure and demonstrate the need for further study into the safety of ADBAC and DDAC. Particularly, DIT needs to be studied due to the unique sensitivity of the developing immune system. QAC's also come into contact with the gut microbiome, which interacts with the host immune system. Thus QAC alterations on the gut microbiome could affect the developing immune system.

II. Decreased Macrophage Function Following *in-vitro* ADBAC+DDAC Exposure

Valerie A. McDonald[1], Hoang Nguyen[2], Terry C. Hrubec[2,3]

1. Department of Biological Sciences, VA Tech, Blacksburg, VA 24060
2. E. Via College of Osteopathic Medicine VA Campus, Blacksburg, VA 24060
3. VA-MD Regional College of Veterinary Medicine, Blacksburg, VA 24060

Abstract

Alkyl dimethyl benzyl ammonium chloride (ADBAC) and didecyl dimethyl ammonium chloride (DDAC) are common quaternary ammonium compounds used as disinfectants in households, medical, and restaurant settings. They cause occupational asthma and allergic dermatitis in humans. They also cause increased secretions of proinflammatory cytokines in corneal cells and inflammation in the vaginal tract in pigs and animal models. ADBAC and DDAC have not been evaluated for immunotoxicity directly in immune cells. Production of TNF-alpha, IL-6, and IL-10 cytokines and phagocytic function were assessed in J774A.1 mouse macrophages. ADBAC+DDAC exposure caused significant increases in TNF-alpha and IL-6 and decreases in IL-10 indicating significant proinflammatory effect. ADBAC+DDAC also drastically decreased phagocytosis. These data suggest that ADBAC+DDAC directly alter immune function.

Introduction

Quaternary ammonium compounds (QACs) are a class of cationic surfactants and disinfectants. They are popular chemical in commercial products with a wide variety of functions. Due to their biocidal properties, they are used in outdoor environments for wood treatments and landscape treatments and in swimming pools. In indoor environments, they are used as cleaners in medical, restaurant, and household products (EPA, 2006). Alkyl dimethyl benzyl ammonium chloride (ADBAC) and didecyl dimethyl ammonium chloride (DDAC) are commonly used as disinfectants. They are popular for their wide array of functions and over 1 million pounds each of ADBAC and DDAC are produced yearly in the US alone (PubChem Database). The potential for human exposure to QACs is high as they are prevalent in everyday environments. Exposure occurs through inhalation, and from oral and transdermal routes.

QACs are generally considered relatively safe; however, both animal and *in-vitro* studies, have indicated that may not be the case. In mice, ADBAC+DDAC exposure caused developmental and reproductive toxicity (Melin et al., 2014; Melin et al., 2016; Hrubec et al., 2017). Other studies also indicate that ADBAC and DDAC may alter immune function. Combinations of ADBAC+DDAC in cleaning agents caused severe airborne contact dermatitis in humans as well as occupational asthma (Mauleon et al., 2006; Purohit et al., 2000; Gonzalez et al., 2014). In pigs, exposure to 2% benzalkonium chloride (BAC) the parent compound of ADBAC, caused increased in IL-1 Beta, IL-8, IL-18 in a cervicovaginal lavage, which are all cytokines associated with inflammation (D'cruz et al., 2005). Airway inflammation has also been observed in rodent models after BAC exposure

(Larsen et al., 2011). When human conjunctival and corneal cell lines were exposed to BAC, biomarkers of inflammation, including TNF-alpha, IL-1, IL-12, and IL-10 were increased (Epstien et al., 2009). *In-vitro* QAC exposure has caused decreased mitochondrial function (Inacio et al., 2013). Additionally, low concentrations of QACs are cytotoxic to corneal cells and epithelial cells in the respiratory tracts (Deutschle et al., 2006; Pauloin et al., 2008; Levin et al., 1997).

All tissue systems have resident macrophages, which are important cells in both innate and adaptive immune function. Macrophages phagocytize potential pathogens and particulate matter, which is then degraded in phagolysosomes. The degraded contents are processed and expressed on the cell surface for presentation to adaptive immune cells. Macrophages also are major secretors of regulatory cytokines including. TNF-α, IL-10, and IL-6, and. TNF-α is generally a pro-inflammatory cytokine that also has functions associated with cell proliferation, differentiation, and survival. IL-10 is an anti-inflammatory cytokine with a negative feedback loop acting on the macrophages to down regulate cell activation. Altered macrophage function is associated with immunosuppression. For example, in diseases such as obstructive pulmonary disease and type 2 diabetes, resident tissue macrophages are less effective; and have reduced ability to clear infections (Hiemstra et al., 2013; Hodgson et al., 2011).

The results from previous studies tie QAC exposure to altered immune function, yet their specific effect on immune cell function has not been tested. If QACs are immunotoxic, their widespread use could be contributing to dysregulated immune function and immune

disease. We will examine potential immunotoxicity from combined ADBAC+DDAC exposure by assessing phagocytosis and cytokine production in J774 mouse macrophages *in-vitro*. We hypothesize that ADBAC+DDAC will cause cytotoxicity and alter macrophage function.

Methods

General Culture Conditions

Mouse macrophage J774A.1 (ATCC, Manassas, VA) cells were cultured in a base media of Eagle Minimum Essential Media (Corning, Corning, NY) supplemented with sodium bicarbonate at 1.5g/L and 1% penicillin streptomycin solutions (Corning, Corning, NY) and 10% Fetal Bovine Serum (CellGro, Herndon, VA). Cells were grown at 37 ˚C with 5% $C0_2$ in a humidified HeraCell Vios incubator (ThermoScientific, Waltham, MA).

Viability and Phagocytosis

Cells were seeded into 12-well tissue culture plates (Corning, Corning, NY) at 1×10^5 cells. Cells were exposed for 24 to ADBAC+DDAC (6.76% ADBAC (60% C-14, 25% C-12, 15% C-16) and 10.1% DDAC), (ADBAC (Sigma Chemical, St Louis, MO) and DDAC (AK Scientific Inc., Union City, CA) at 5×10^{-3} %, 1×10^{-3} %, 5×10^{-4} %, 1×10^{-4} %, 5×10^{-6} %, and 0% in duplicate. Viability was calculated using the live/dead stain trypan blue (ThermoScientific, Waltham, MA) at 0.2%. Cells were incubated with trypan blue for one minute at room temperature and examined by hemocytometer. Each well was counted in duplicate and averaged.

Phagocytic ability was determined by flow cytometry at concentrations where the viability was not affected, 0%, $1x10^{-5}$ %, $5x10^{-5}$ %, $1x10^{-4}$ % ADBAC+DDAC. Cells were plated in triplicate at 1 X 10^5 cells/well on a 12 well tissue culture treated plate (Corning, Corning, NY). Cells were exposed to ADBAC+DDAC for 24 hours, after which, $1x10^7$ fluorescent carboxylated-modified microspheres (FluoSpheres, Molecular Probes, Eugene, OR) were added and incubated for an additional 24 hours. Cells were lifted by scrapping and pooled for flow cytometry. Cells were analyzed with an Image Stream flow cytometer (Amnis, Seattle, WA), using IDEAs software to measure median fluorescence and the number phagocytic cells. Percent phagocytosis was calculated using the number of observed cells divided by the number of cells that up took beads.

Cytokine Production

Production of IL-10, IL-6, and TNF-alpha cytokines was tested in cell supernatants using sandwich ELISA (murine Quantikine kits R&D Systems, Minneapolis, MN). Cells were plated in duplicate at 1 X 10^4 cells/well on a 12 well tissue culture treated plate (Corning, Corning, New York) and exposed to the $6x10^{-5}$ % ADBAC+DDAC. Cells were primed and stimulated for either classical or alternative pathways. For the analysis of the classical pathway, macrophages were primed for 24 hours with 150U INF-gamma (R&D Systems, Minneapolis, MN) and then stimulated with 10ng LPS (LPS (Escherichia coli 0111:B4) Sigma-Aldrich, St. Louis, MO) for 24 hours (Mosser, 2015). The supernatant was collected and concentrations of secreted TNF-alpha and IL-6 were determined. To test alternatively activated cytokine secretion, macrophages were stimulated with 150U prostaglandin, PGE_2, (Sigma Aldrich, St. Louis, MO) and 10ng LPS. The supernatant was collected and

concentrations of secreted IL-10 were determined. Cytokine production was analyzed using a standard curve and the standard error of the mean was calculated. Duplicate wells were averaged for each treatment

Statistics

Statistical analysis was conducted using a two sample t-test to compare between dosed and control samples using Statistix 8 (Tallahassee, FL). The p-value was set to 0.05 for statistical significance.

Results

Effects of ADBAC+DDAC on J774 Viability

Over all, ADBAC+DDAC had little effect on the viability at $5x10^{-5}$ %, but severely decreased viability at concentrations higher than $5x10^{-4}$ % (Fig. 1) This data was used to determine conditions for the phagocytosis assay.

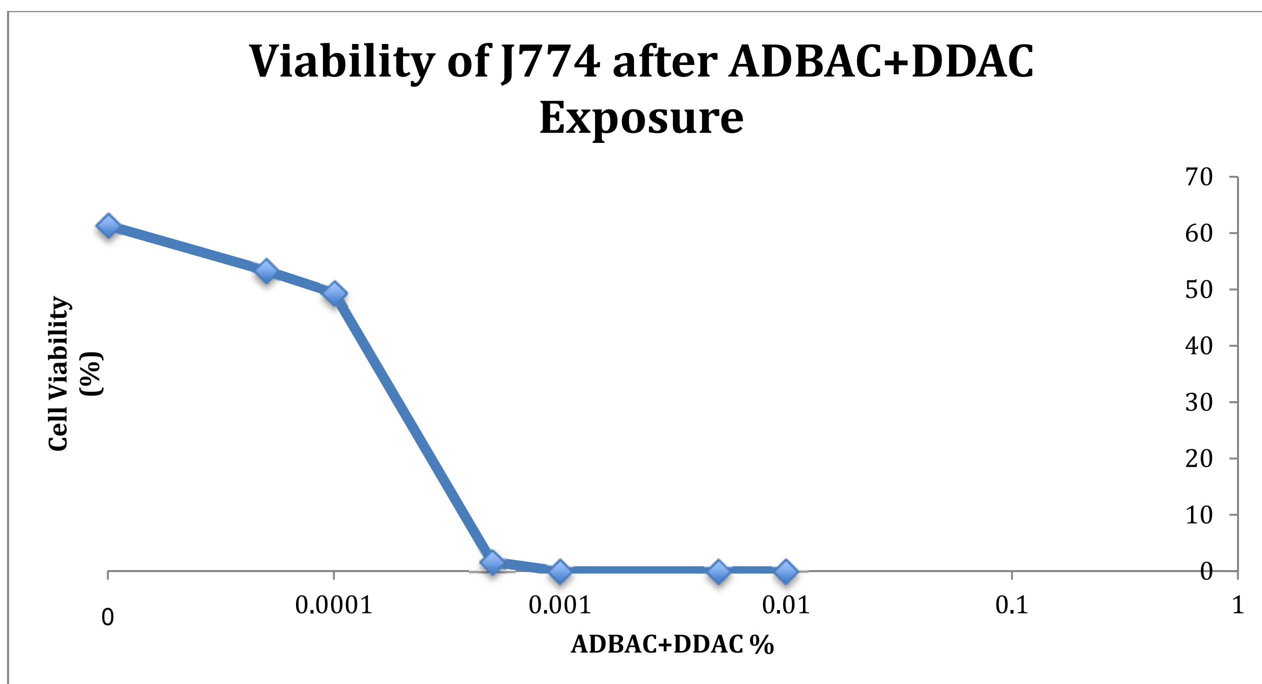

Figure 1: Murine macrophages (J774) were exposed to 5x10^{-3} %, 1x10^{-3} %, 5x10^{-4} %, 1x10^{-4} %, 5x10^{-6} %, and 0% concentrations of ADBAC+DDAC for 24 hours and viability was assessed using trypan blue staining. Cells demonstrated a dose dependent decreases in viability after 24h ADBAC+DDAC exposure.

ADBAC+DDAC %	**# Cells**	**Beads+Cells**	**% Phagocytosis**	**Median Fluorescent Intensity**
Cells, No Beads	4,276	4	0.09	650,530
0.0%	1,940	1,919	98.9	3,474,403
1x10^{-5}%	1,731	1,583	91.5	2,790,478
5x10^{-5}%	884	848	95.9	2,437,151
1x10^{-4}%	1,275	115	9.02	744,797

Table 1: The % of phagocytic cells and the medium fluorescent intensity from murine J774 macrophages. Cells were exposed to ADBAC+DDAC for 24 hours, and then exposed to fluorescent microspheres for an additional 24 hours. Samples were pooled for by flow cytometry to analyses phagocytosis, N= 884 to 4,276.

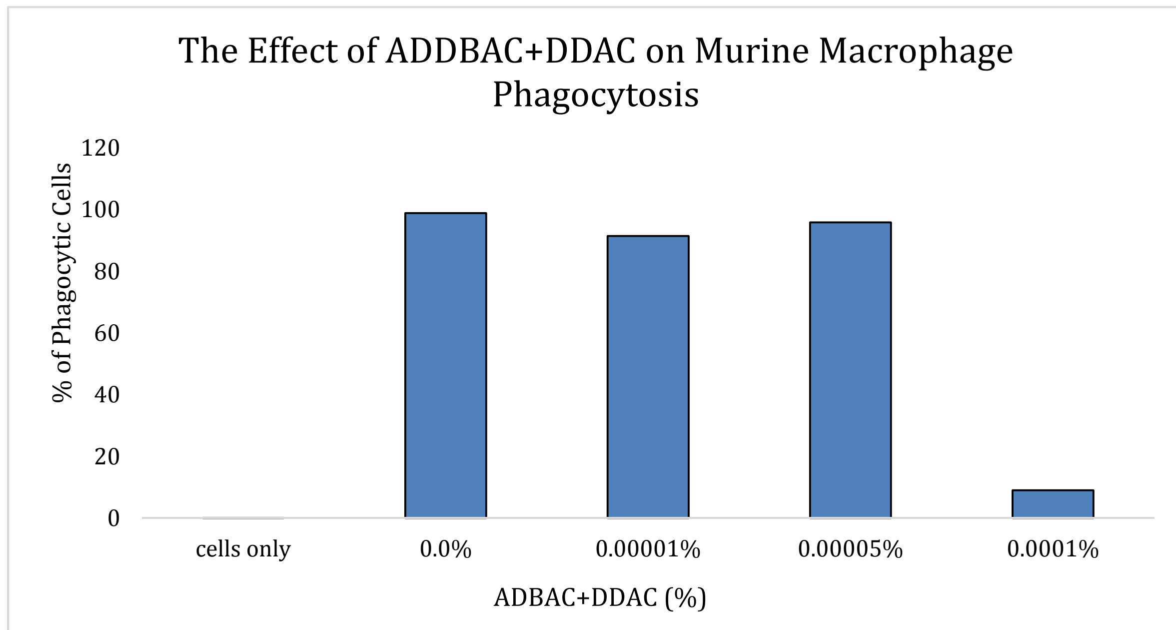

Figure 2: J774A murine macrophages exposure to ADBAC+DDAC and fluorescent beads demonstrated decreased uptake of the % of phagocytic J774 macrophages following ADBAC+DDAC exposure. Cells were exposed to ADBAC+DDAC for 24 hours, and then exposed to fluorescent microspheres for an additional 24 hours. Samples were pooled for by flow cytometry to analyses phagocytosis, N= 884 to 4,276

The viability of the cells was not deceased at $1x10^{-4}$%; (Fig. 1), however phagocytosis decreased from almost 100% to 9% at $1x10^{-4}$% (Fig. 2). This indicates that ADBAC+DDAC dramatically decreases J774 macrophage's ability to phagocytosis the beads at concentrations where the cells were still alive.

Significant alterations to cytokine production were observed for TNF-alpha, IL-6 and IL-10. Production of IL-6 and TNF-alpha increased after LPS and INF stimulation. At the same time production of IL-10 decreased after LPS and PGE_2 stimulation.

	IL-6	TNF-alpha	IL-10
Cells alone	0 ± 0	0 ± 0	0.01 ± 0.005
Stimulated Cells	8.36 ± 0.16	7.05 ± 0.58	0.32 ± 0.04
Stimulated + QAC	13.9 ± 0.23*	19.4 ± 0.58*	0.15 ± 0.02*

Table 2: Summary of cytokine production. Cells were cultured alone or with $6x10^{-5}$ % ADBAC+DDAC for 24 hours then stimulated. Cytokines in duplicate cultures were determined by ELISA (R&D Systems). Units are $1x10^{-2}$ pg/cell. = Significantly different, T test $p < 0.05$. The standard devotion is indicated by $\pm$.

Discussion

ADBAC and DDAC are found in many common household products such as cleaners, disinfectants, and preservatives. Human exposure to QACs is high but unquantified. The EPA lists the maximum rate of use for many types of products. The maximum rate for ADBAC in air fresheners, food utensil disinfectant, cleaning eggs from hatcheries, and spray disinfectants in everything from day cares to medical offices is 0.2% (EPA, 2006). In eye drops, ADBAC is used as a preservative from 0.004% to 0.01%. Our results show that murine macrophages are affected at lower concentrations than what are typically contained in household products. At $5x10^{-4}$ %, ADBAC+DDAC viability was decreased to almost zero. This is similar to other studies which found necrosis, apoptosis, and decreased cell size following BAC exposure in ocular cells (Debbasch et al., 2000). Since ADBAC is used frequently in spray products, it is possible that small amounts could be inhaled and affect resident alveolar or mucosal macrophages. In mice, inhalation exposure to BAC decreased respiratory ability at $0.23mg/m^3$; significantly increased the number of polymorphonuclear

leukocytes. There was a trend towards increase in number macrophages (Larsen et al., 2012).

In our study, phagocytic function sharply decreased at $1x10^{-4}$% ADBAC+DDAC. This is below the concentration of ADBAC found in many products. At this concentration of ADBAC+DDAC cell viability was minimally effected and similar cell numbers were observed in the flow cytometry results. These data indicate that our decreased phagocytosis is a treatment effect and not just the result of reduced cell number from ADBAC+DDAC induced cell death. QACS are associated with respiratory disease in both rodents and humans, so these reduced phagocytic function could potentially increase symptoms of allergic and inflammatory diseases.

ADBAC+DDAC caused increases in pro-inflammatory cytokines TNF-alpha and IL-6 and decreased IL-10 at $6x10^{-5}$ % ADBAC+DDAC. This means that proinflammatory cytokines are increased and anti-inflammatory cytokines are decreased, pushing the system to a pro-inflammatory state. Diseases such as Crohn's and major depressive disorder are correlated with increased TNF-alpha and IL-6. (Liu et al., 2012; Reinecker et al., 1993). In type II diabetes, nitric oxide production and TNF-alpha concentrations are increased (Doganay et al., 2002). A heightened level of IL-6 is particularly associated with autoimmune diseases such as arthritis (juvenile and rheumatoid) and psoriasis (Ishihara et al., 2002).

Classically activated macrophages are associated with increased phagocytosis and proinflammatory cytokines, while alternatively activated macrophages are associated with

wound repair and anti-inflammatory states. However, most macrophages exist in a state where they are either proinflammatory or anti-inflammatory, but phagocytosis and cytokine production are regulated in difference ways, so it possible both states can exist at the same time (Martinez et al., 2014). Our results demonstrating that ADBAC+DDAC alter macrophage cytokine production and decrease phagocytic ability indicate overall alteration to macrophage function. This is a concern because if ADBAC+DDAC can cause similar effects in humans, then humans could be at risk for decreased response to infection and inflammatory diseases. Overall, we conclude that ADBAC+DDAC are an immunotoxic concern, but more studies in-vivo and in eventually in humans must be done to accurately assess human risk.

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III. The Effect of ADBAC+DDAC on the Microbiome and Development of the Immune Response

Valerie A. McDonald [1], Kristin Blount [2], Xin M. Luo [2], Terry C. Hrubec [2,3]

1. Department of Biological Sciences, VA Tech, Blacksburg, VA 24060
2. Department of Biomedical Sciences and Pathobiology, VA-MD College of Veterinary Medicine, Blacksburg, VA, 24060
3. E. Via College of Osteopathic Medicine VA Campus, Blacksburg, VA 24060

Abstract

Alkyl dimethyl benzyl ammonium chloride (ADBAC) and didecyl dimethyl ammonium chloride (DDAC) are common quaternary ammonium compounds used as disinfectants in households, medical, and restaurant settings. They have caused occupational skin and respiratory hazards in humans and developmental and reproductive toxicity in mice. They also have caused increased secretions of proinflammatory cytokines in cell lines and in animal models, but have not been evaluated for developmental immunotoxicity. The developing immune system is most sensitive to toxicants because it encompasses many unique developmental events. Toxicant exposure during development can have lifelong effects on the immune system. The microbiome is formed during and after birth and affects the development of immune tolerance. Alterations to the gut microbiome have been associated with immune disease. QAC exposure in hospital patients has been shown to alter resistance genes of the gut microbiome. We tested a combination of ADBAC+DDAC for developmental immunotoxicity through an extended one generation study in CD-1 mice. In addition to evaluating immune function, the effect of ADBAC+DDAC on the fecal microbiome and developing immune system were evaluated. ADBAC+DDAC exposure significantly affected the gut microbiome in F1 mice and these changes persisted into the F2 females. ADBAC+DDAC also significantly affected antibody production in male F2 in mice. It appears that alterations in the microbiome are not directly responsible for ADBAC+DDAC immunotoxicity since microbial changes to the gut microbial community mostly recovered after the dose was removed, while altered antibody production persisted in F2 males.

Introduction

Quaternary ammonium compounds (QAC) are a class of cationic surfactants and disinfectants. They are popular chemicals in commercial products with a wide variety of functions. Due to their biocidal properties, they are used in outdoor environments such as wood treatments and swimming pools, as well as indoor environments as cleansers in medical, restaurant, and household products (EPA, 2006). QACs are considered relatively safe; however, both animal and *in vitro* studies have demonstrated acute and chronic toxicity form QAC exposure.

QACs are cytotoxic to human epithelial cells of the eyes, respiratory, and vaginal tract (Deutschle et al., 2006; Pauloin et al., 2008; Levin et al., 1997). Alkyl dimethyl benzyl ammonium chloride (ADBAC) and didecyl dimethyl ammonium chloride (DDAC), two common QACs, cause developmental and reproductive toxicity in mice. Mice exposed to ADBAC+DDAC had decreased measure of fertility in in both male and female mice; decreased pregnancy rates and fewer pups per litter (Melin et al., 2014; Melin et al., 2016). Additionally, *in utero* exposure to ADBAC+DDAC resulted in developmental toxicity and caused neural-tube birth defects in a dose-dependent manner (Hrubec et al., 2017). Ambient exposure from the use of QACs in the mouse room was sufficient to cause both reproductive and developmental toxicities (Melin et al., 2014; Melin et al., 2016; Hrubec et al., 2017)

QACs appear to alter immune function. Combinations of ADBAC+DDAC in cleaning agents caused severe airborne contact dermatitis in humans as well as occupational asthma

(Mauleon et al., 2006; Purohit et al., 2000; Gonzalez et al., 2014). In pigs, exposure to 2% benzalkonium chloride (BAC), the parent compound of ADBAC, caused increased in IL-1 beta, IL-8 and IL-18, which are all cytokines associated with inflammation (D'cruz et al., 2005). Airway inflammation has also been observed in rodent models after BAC exposure (Larsen et al., 2011). When human conjunctival and corneal cell lines were exposed to BAC, biomarkers of inflammation including TNF-alpha, IL-1, IL-12, and IL-10 were increased (Epstien et al., 2009).

Many non-communicable diseases have immune-based mechanisms, and these immune-mediated diseases are on the rise suggesting an environmental component (Dietert et al., 2011; Dietert, 2014). The immune system has a long developmental period starting during gestation and lasting until puberty, where it is particularly sensitive to toxicants (Dietert et al., 2003; Leubke et al., 2006; Tonk et al., 2015; West et al., 2002). Thus, when screening for possible immunotoxicity, it is essential to encompass the temporal development of the immune system. There is a trend for chemicals to show developmental and reproductive toxicity at higher doses than immunotoxicity or developmental immunotoxicity (DIT) (Kushima et al., 2007; Tonk et al., 2012; Tonk et al., 2015) If QACs are immunotoxic, their widespread use could contribute to the increase in amount of immune diseases.

Inflammatory diseases are due to dysregulation of the immune system. A number of inflammatory diseases have been tied to altered patterns in the gut microbiome (GM) including allergic respiratory disease, atopic dermatitis, inflammatory bowel disease, and type 1 diabetes (Bendtsen et al., 2015). The GM begins to develop during gestation but is

mainly colonized during delivery and shortly after birth (Bendtsen et al., 2015; Koleva et al., 2015). Different microbial community compositions have been associated with regulatory T-cell numbers and activity (Bendtsen et al., 2015; Koleva et al., 2015). It is likely that stimulation of regulatory T-cells and initiation of tolerance are how the GM influences overall health. Antibiotic treatment increases the risk of disease development later in life (Bendstsen et al., 2015).

Any ADBAC or DDAC that enters the body is excreted unmetabolized in the feces (Thorsteinsson et al., 2003). This active disinfectant could alter the GM as it passes through the digestive tract. There is evidence that QACs interact with the GM to alter bacterial resistance genes (Buelow, 2015). ADBAC+DDAC alterations to the microbiome could be a mechanism by which these chemicals alter immune function.

ADBAC and DDAC had not been tested for DIT. We screened for DIT *in-vivo* by measuring IgM, IgG, and IgA dependent antibody responses and changes in the fecal microbiome following multigenerational ADBAC+DDAC exposure. We hypothesized that ADBAC+DDAC will alter microbial communities which in turn affect the timing and magnitude of antibody production

Methods

Animal Husbandry and dosing

Animal care and generation of the ADBAC+DDAC free mice were described in detail in Melin et al. (2016). Briefly, CD-1 mice were procured from Charles River Laboratories

(Raleigh, NC) and housed in 12-h light/dark cycle at 20 to 25 ˚C with 30–60% relative humidity. Mice were fed Harlan 7013 rodent chow (Harlan Teklad, Madison, WI) and distilled water was provided *ad libitum*. Mice for the dosed group were bred in a ADBAC+DDAC free vivarium and transferred to a facility using ADBAC+DDAC disinfectants prior to dosing to prevent contamination of QAC-free controls (Melin et al., 2016). Experimental mice were fed Nutra-gel (purified dry mix formula, Bio-Serv, Frenchtown, NJ), which was prepared following the manufacturer's instructions. The mice were dosed with 60 mg/kg/day ADBAC + DDAC [6.76% ADBAC (60% C-14, 25% C-12, 15% C-16; Sigma Chemical, St Louis, MO) and 10.1% DDAC (AK Scientific Inc., Union City, CA)]. Body weight and food intake were monitored daily when dosing was in progress to ensure accurate dosing. No overt toxicity was observed. Mice were acclimated to the gel food for one week before dosing, and then were dosed for 3 weeks. Mice were then combined into breeding pairs, and dosing continued throughout gestation and lactation. Male mice were euthanized before dams gave birth to prevent additional pregnancies. Pups received indirect dosing through lactation and direct dosing as they transition to solid diet (OECD, 2012). Litters were standardized to 5 males and 5 females at postnatal day 3 to reduce variation from differences in litter size. On day 15 litter sizes were further reduced to 4 male and 4 female pups. Dietary dose was stopped at weaning (day 21).

DIT Test

The DIT study was modified from the extended one-generation reproductive toxicity test guide 443 from the OECD and from the testing procedure described by Tonk et al., (2011). Keyhole limpet hemocyanin (KLH) (BioVision, Milpitas, CA) was administered intraperitoneally to induce a T-dependent antibody response, as summarized in Figure 1. To assess primary response, mice were immunized on postnatal day 21 with 0.3 mg of KLH. On day 26, one mouse per sex per litter was bled. Sera from this blood was used to assess the primary response and if the litter had reached immunocompetence. An additional mouse per sex per litter was bled on day 35 to assess class switching from IgM to IgG. To assess the secondary response, one mouse per sex per litter was given a second injection of 0.3 mg of KLH on day 35 and were bled on day 40. Mice were anesthetized before bleeding with isoflurane and bled by cardia puncture with a 26g needle. Blood was collected in pediatric serum separator tubes and clotted for one hour before centrifugation. Serum was stored frozen at -40 ˚C until use.

One male and female from each litter were not injected with KLH and were breed to unrelated mice in the same treatment to produce the F2 generation. These F1 parents were moved to the ADBAC+DDAC free vivarium and were provided un-dosed Nutra gel diet. F1 parents were paired and bred in the same manner as the F0. Thus, F2 mice were only exposed to QACs as gamete cells in the F1 mice. Injections and bleeding of F2 pups were completed as described for the F1.

ELISA

IgM, IgG, and IgA serum responses to KHL were determined. ELISA plates (Nunc 96 well Maxisorp plates; Nunc, Roskilde, Denmark) were coated with 1 μg/well of KLH in 50 mM carbonate buffer (pH 9.6) overnight at 4 ˚C. Plates were washed with phosphate buffered saline (PBS) and 0.05% tween-20 (Sigma-Aldrich, St. Louis, MO) four times and then blocked for at least two hours in 3% bovine serum albumin (BSA; Sigma-Aldrich, St. Louis, MO) in PBS at room temperature for IgM and IgG and in 5% BSA in PBS for IgA. Sera were incubated at 1:100 dilution in 3% BSA+PBS+0.05% tween-20 for one hour at room temperature for IgM and IgG and incubated at 1:100 dilution in 3% BSA+PBS overnight at 4 ˚C for IgA. Plates were washed four times. The peroxidase-conjugated secondary antibodies against IgG and IgM (AffiniPure goat anti-mouse IgG and IgM; Jackson Immuno-Research Laboratories, Inc., West Grove, PA) and peroxidase-conjugated goat anti-mouse IgA (NOVUS, Littleton, CO) were diluted in 3%BSA+PBS+0.05% tween-20 to 1:40,000 for IgG and 1:3,000 for IgM and IgA and 100 ul were added to each well. Plates were washed 4 times and 100 ul Single-Component TMB Peroxidase EIA Substrate Kit (Bio-Rad, Hercules, CA) was added to each well. The reaction was allowed to proceed for a minute at room temperature for IgG and IgM and 10 minutes at 37 ˚C for IgA. The reaction was stopped with 2M sulfuric acid and the absorbance were read at 450 nm on a SpectraMax spectrophotometer (Molecular Devices, Sunnyvale, CA). Sera for the positive controls were derived by injecting mice twice 10 days apart with 0.6 mg of KLH, and bleeding 5 days after the second injection. Antibody concentrations were determined as a percent of the plate positive for each sample.

Analysis of fecal microbiome

Fecal samples were collected from all 3 generations. Fecal samples were collected into sterile microcentrifuge tubes and frozen at -20 ˚C. F0 mice were sampled one week after the gel diet started to establish a baseline of normal microbiota. A second sample was collected one week after dosing started. F1 samples were collected on day 23, right after the dosing ceased and again on day 40, to compare dosed to un-dosed. F1 samples were pooled by litter. The above collection pattern was repeated for the F2 study. Fecal DNA extractions were conducted using the phenol-chloroform method and PCR of the 16S-V4 region were performed with the same methods described in a previous report (Zhang et al., 2014). Purified amplicons were sequenced bi-directionally (paired-end 150 bp) on an Illumina MiSeq at Argonne National Laboratory. Sequence read merge, quality filtering, de-replication, chimera removal, and OTU clustering were performed with the UPARSE pipeline implemented in the USEARCH program version 8.1/1831 (Edgar et al., 2013). Bacterial taxonomy was assigned by using the 'UclustConsensusTaxonAssigner' implemented in QIIME (Caporaso et al., 2010) against the Greengene reference database (McDonald et al., 2012), and summarized at all taxonomic levels. Alpha- and beta-diversity metrics were computed with QIIME. Alpha-diversity included Shannon diversity index and observed OTUs, and beta-diversity included unweighted UniFrac distance metrics.

Statistics

Statistical analysis for ELISA results were calculated using a Wilcoxin Rank Sum test, a nonparametric t-test using Statistix 8 (Tallahassee, FL) with significance set at $p \leq 0.05$. The microbiome data were analyzed with the Adonis test for principle component analysis, a student t-test for alpha diversity, a one-way ANOVA for relative abundadnce with significance set at $p \leq 0.05$. For the F0 microbiome analysis, the N ranged from 9-10 males and females. For the F1 antibody production and the microbiome analysis, the N was between 6 and 8 litters for each treatment group. For the F2, the N ranged from 3 to 6 for the microbiome and the N for the antibody production was ranged from 3 to 5 for each treatment group.

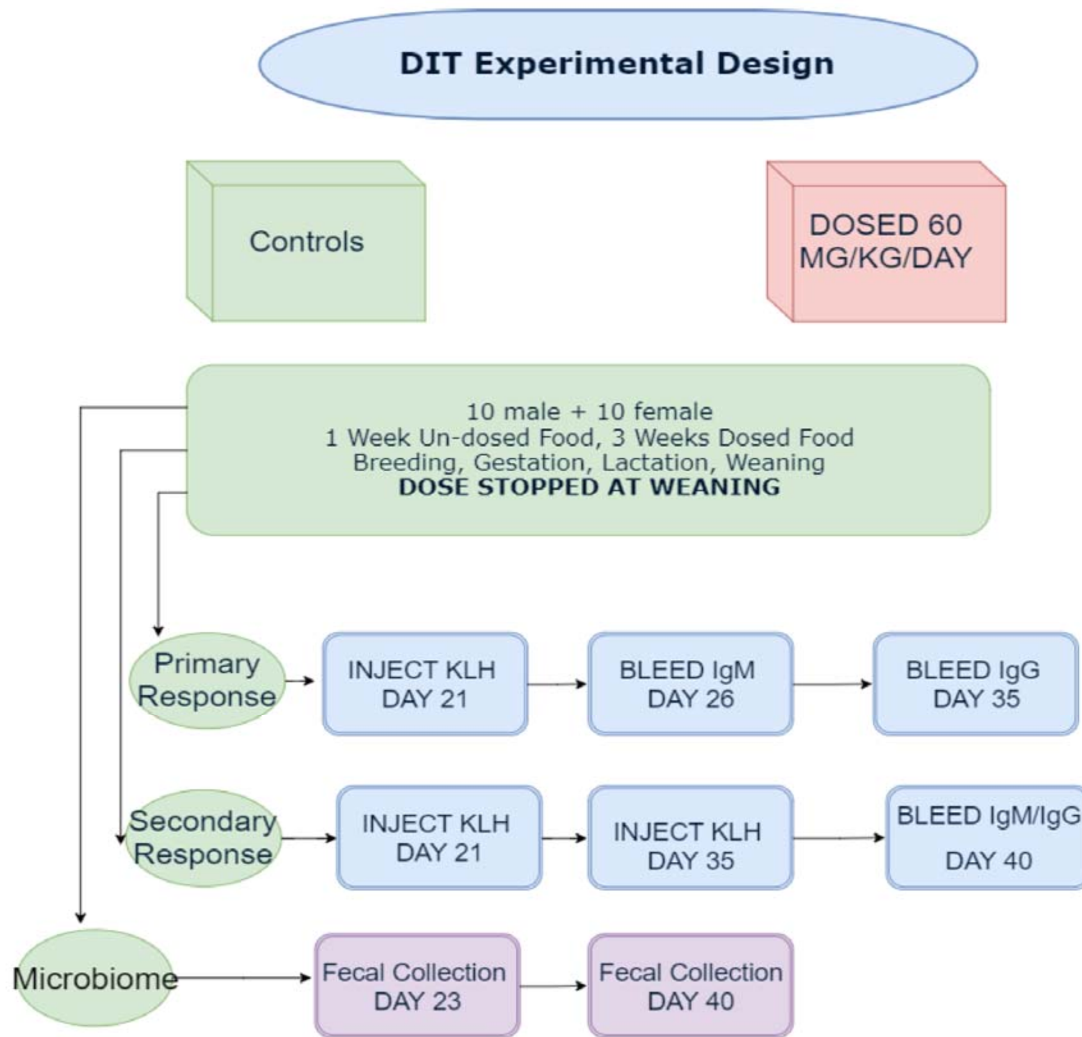

Figure 1: Experimental design - summary of key experimental time points. Dosing started in the F0 and continued until F1 postnatal day 21.

Results

Daily Weights and Food Checks

Mouse weights and cumulative food intake were monitored throughout dosing. Food intake did not vary between the dosed and un-dosed groups (Fig. 2). Mice in both groups continued to grow and gain weight after dosing began. Weight did decrease slightly after mice were combined for breeding but the decline was observed in both groups so it is unlikely a treatment effect (Fig. 2).

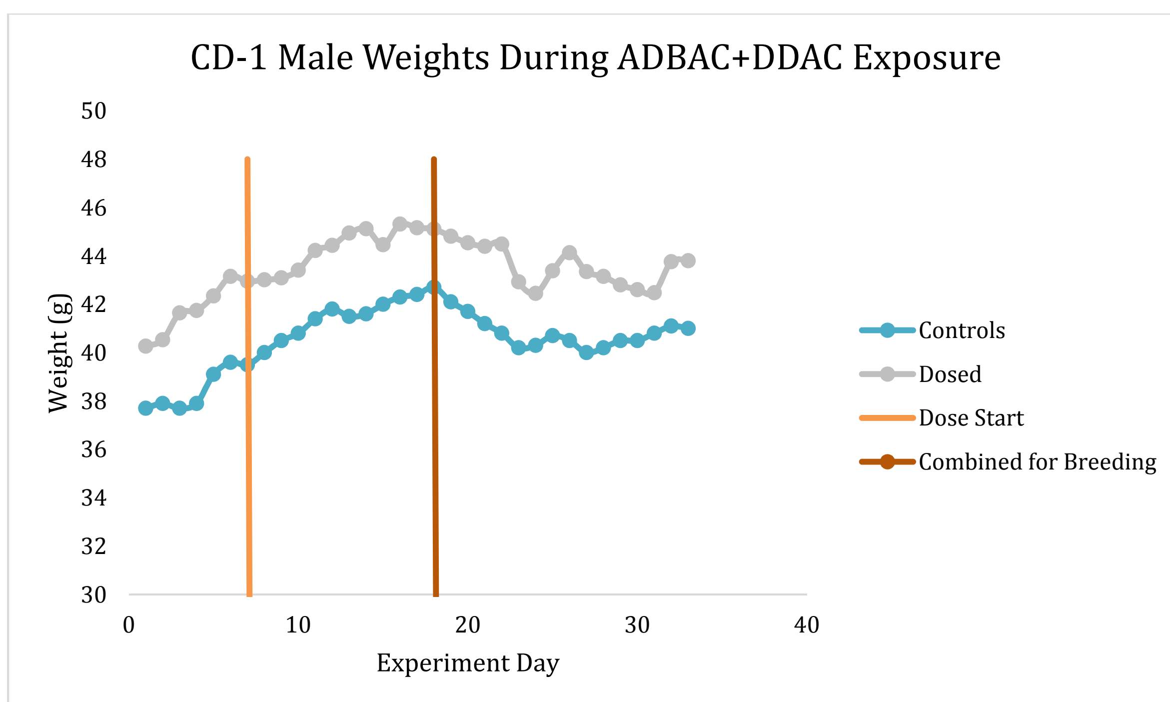

Figure 2: Mouse weights were assessed daily. Both treatment groups showed similar gains and plateaus in weights. Only male weights are shown because female weights varied with number of pups. N=10 per treatment group. Average standard deviation is 4.5 for the controls and 3.7 for the dosed.

ADBAC+DDAC was administered through diet and therefore the food intake could affect the amount of dose actually received. Food intake and actual dose were tracked daily; food intake varied slightly between controls and dosed, but the ADBAC+DDAC dose remained around 60mg/kg/day (Fig. 3). Control mice weights were always lower than dosed mice weights, but these starting weight differences existed before treatment started and both groups follow the same pattern of weight changes.

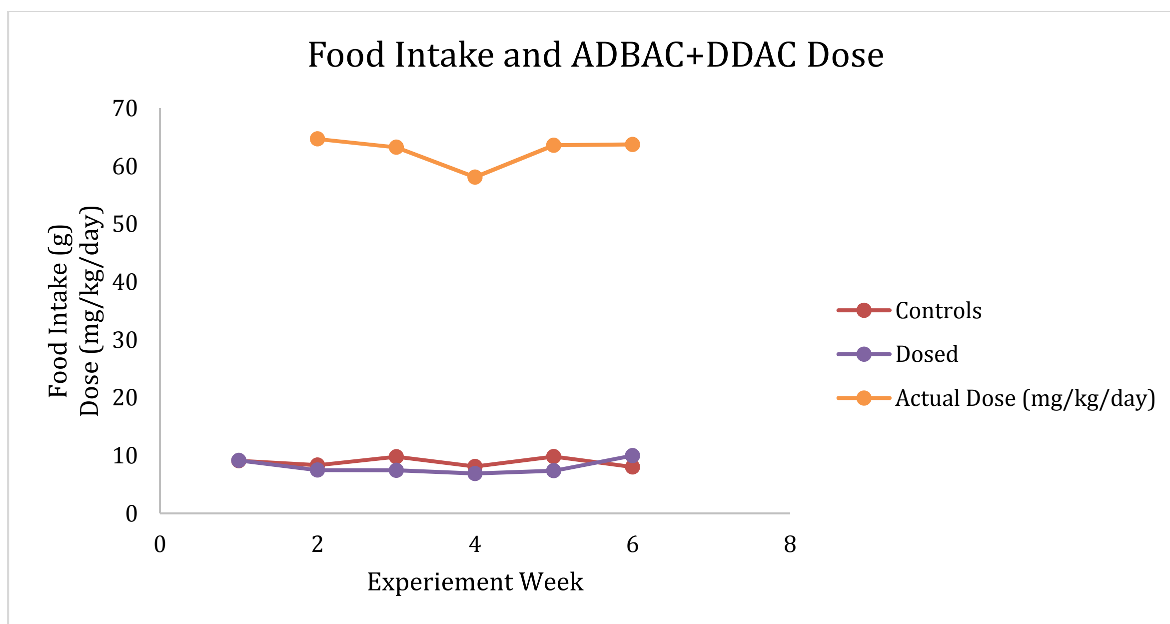

Figure 3: Food intake was assessed daily and average each week over six week dosing period is shown above. Food intake did not vary significantly between treatment groups and the dose concentration was steady around 60mg/kg/day.

ELISA

IgM Antibody Production

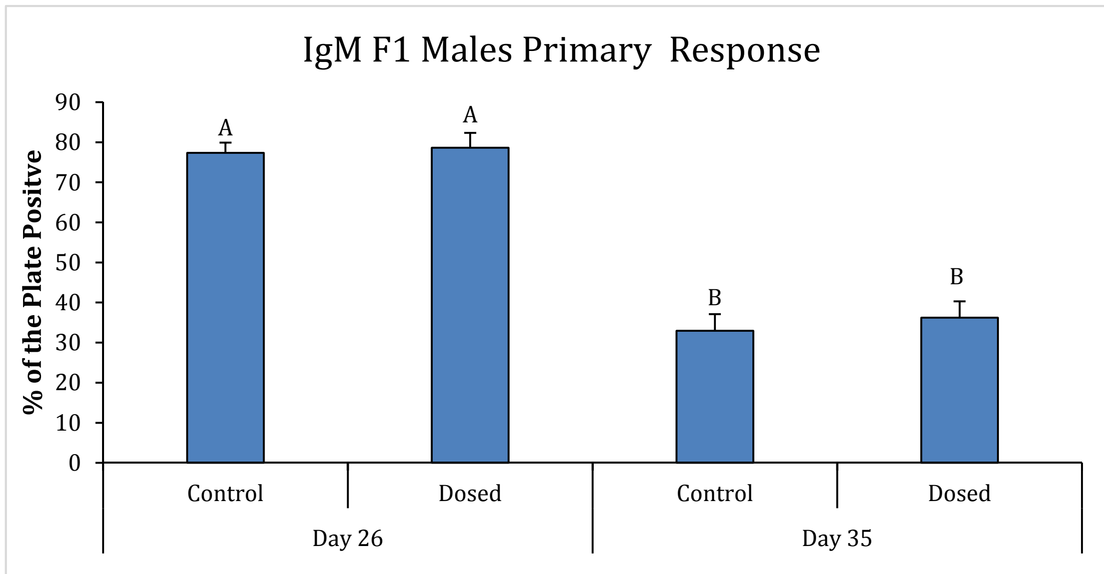

Figure 4: F1 male primary response of IgM was assessed by bleeding mice both 5 and 10 day post injection of .3mg KLH on post-natal days 26 and 35. No significant differences were observed between treatment groups. N= 7-8, p≤0.05 on Wilcoxin Rank Sum test.

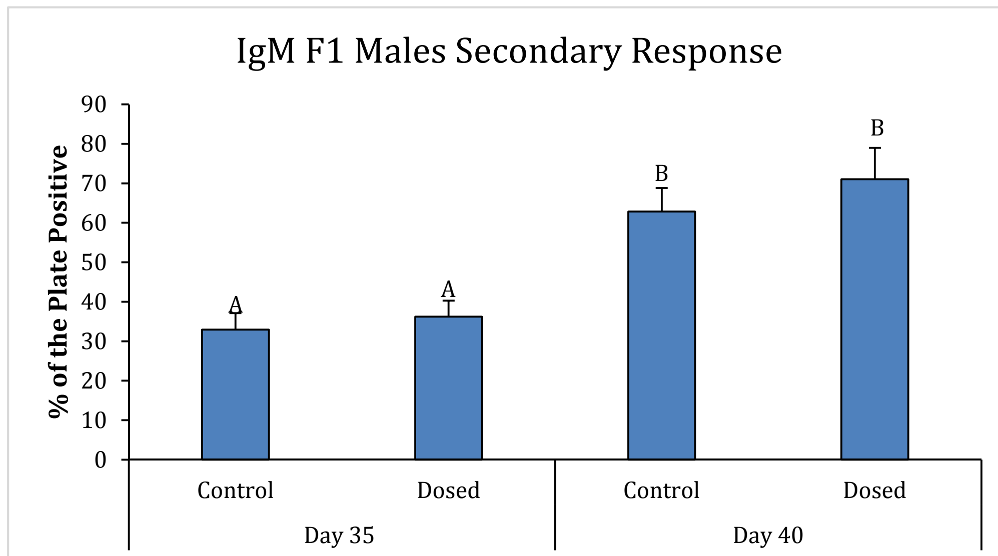

Figure 5: F1 male secondary response of IgM was assessed by bleeding mice 5 days after the secondary injection of 0.5mg KLH on post-natal day 40. No significant differences were observed between treatment groups. N= 87-8 p≤0.05 on Wilcoxin Rank Sum test.

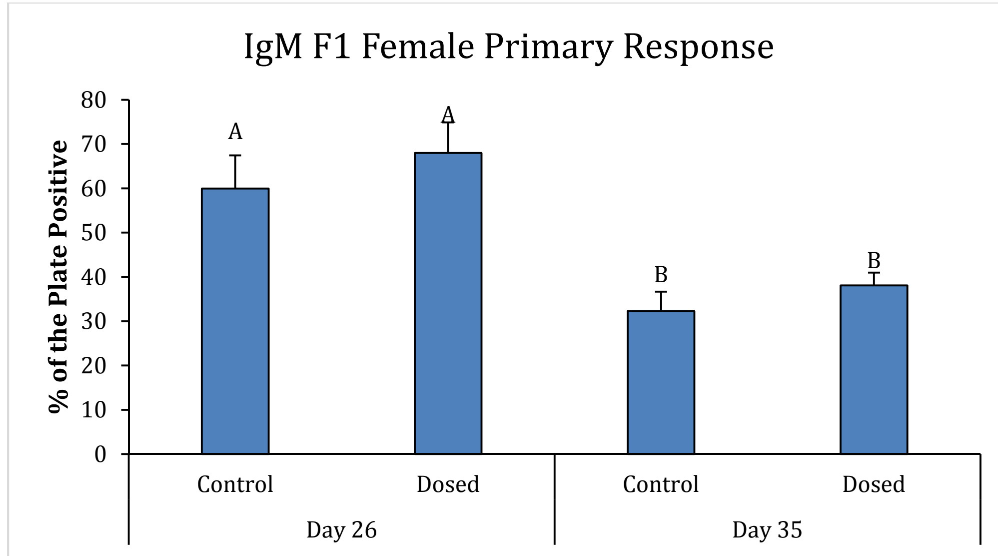

Figure 6: F1 female primary response of IgM was assessed by bleeding mice both 5 and 10 days post injection of 0.3mg KLH on post-natal days 26 and 35. No significant differences were observed between treatment groups. N= 8, $p \leq 0.05$ on Wilcoxin Rank Sum test.

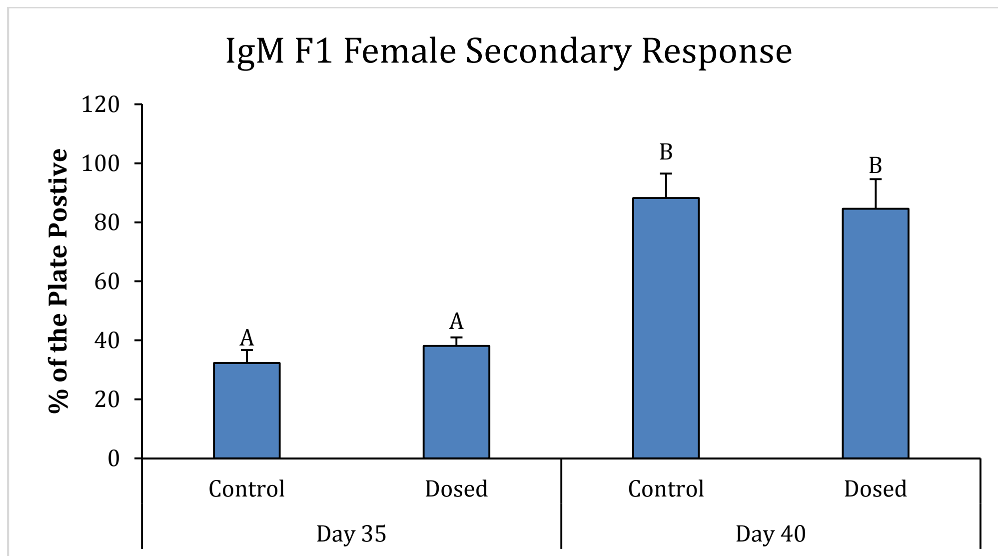

Figure 7: F1 female secondary response of IgM was assessed by bleeding mice 5 days after the secondary injection of 0.3mg KLH on post-natal day 40. No significant differences were observed between treatment groups. N= 8, $p \leq 0.05$ on Wilcoxin Rank Sum test.

In both F1 males and females, the primary IgM response at day 26 was higher than that at day 35, which was expected as IgM is known to peak 5 days after injection (Fig. 4, Fig. 6). The IgM secondary response was higher than the primary response, which was also expected, as the immune system has memory and increase production on subsequent antigen exposure (Fig. 5, Fig. 7). No significant differences between dosed and control groups were observed in the F1 IgM antibody response at any time point.

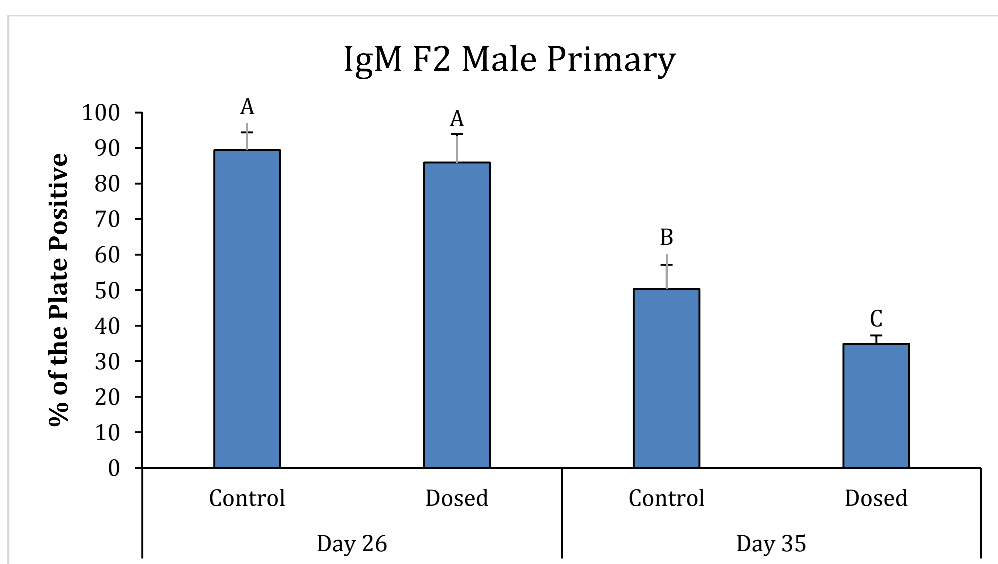

Figure 8: F2 male primary response of IgM was assessed by bleeding mice 5 and 10 days post injection of 0.3mg KLH on post-natal days 26 and 35. Significant decreases production was observed in the day 35 response. N=4-5 p≤0.05 on Wilcoxin Rank Sum test.

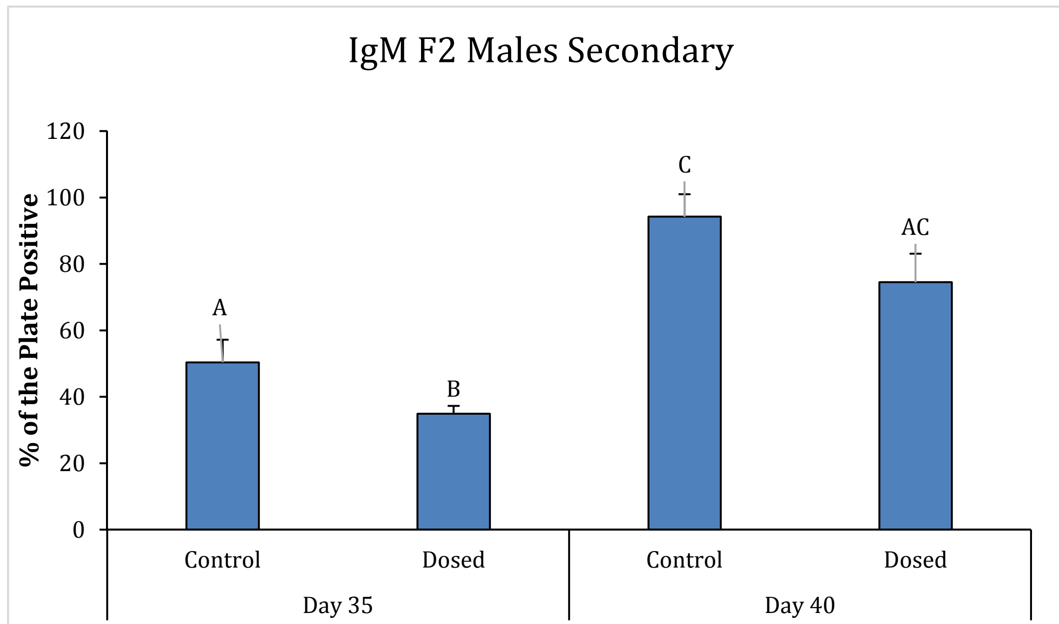

Figure 9: F2 male secondary response of IgM was assessed by bleeding mice 5 days after the secondary injection of 0.3mg KLH on post-natal day 40. The dosed secondary response is not different than the primary response of the control on day 35, indicating a decrease in antibody production. . N= 4-5, p≤0.05 on Wilcoxin Rank Sum test.

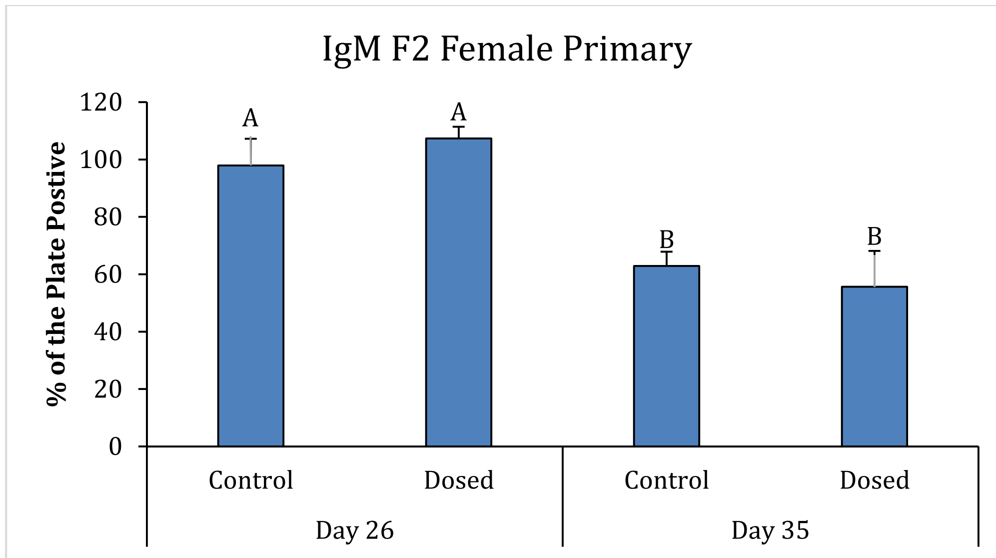

Figure 10: F2 female primary response of IgM was assessed by bleeding mice 5 days post injection of 0.3mg KLH on post-natal Days 26 and 35. No significant differences were observed. N= 3-5, $p \leq 0.05$ on Wilcoxin Rank Sum test.

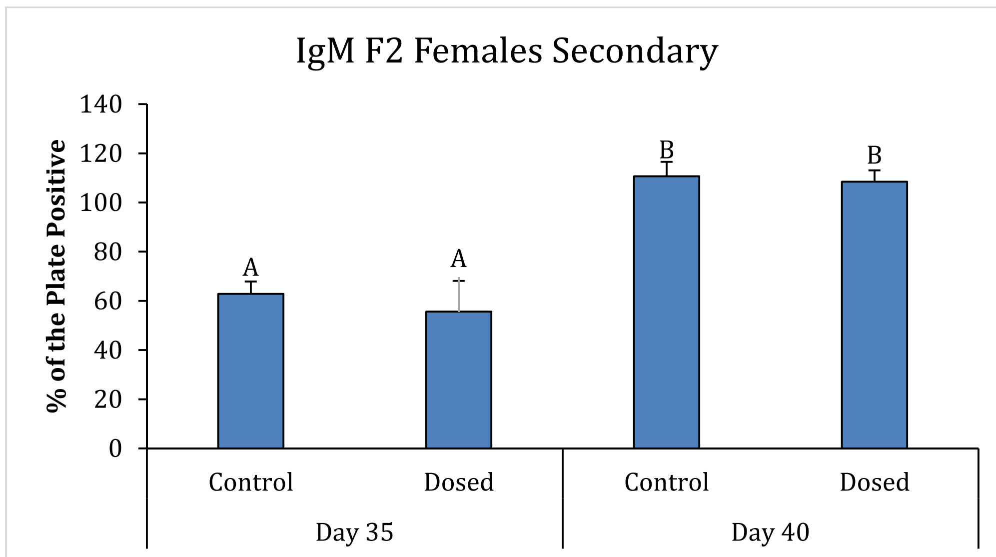

Figure 11: F2 female primary response of IgM was assessed by bleeding mice 5 days after the secondary injection of 0.3mg KLH on post-natal day 40. No significant differences were observed. N= 3-5, $p \leq 0.05$ on Wilcoxin Rank Sum test.

The F2 female IgM primary and secondary responses followed expected temporal patterns with no significant treatment effects observed at any time point (Fig. 10, Fig. 11). The IgM response in dosed F2 males was significantly decreased in the primary response on day 35 (Fig. 8). The F2 male IgM secondary response in the dosed males was not different from the day 35 primary response (Fig 9). This indicates that there is a decrease in antibody production despite the day 40 not being from the controls because the secondary response should have been greater than the primary.

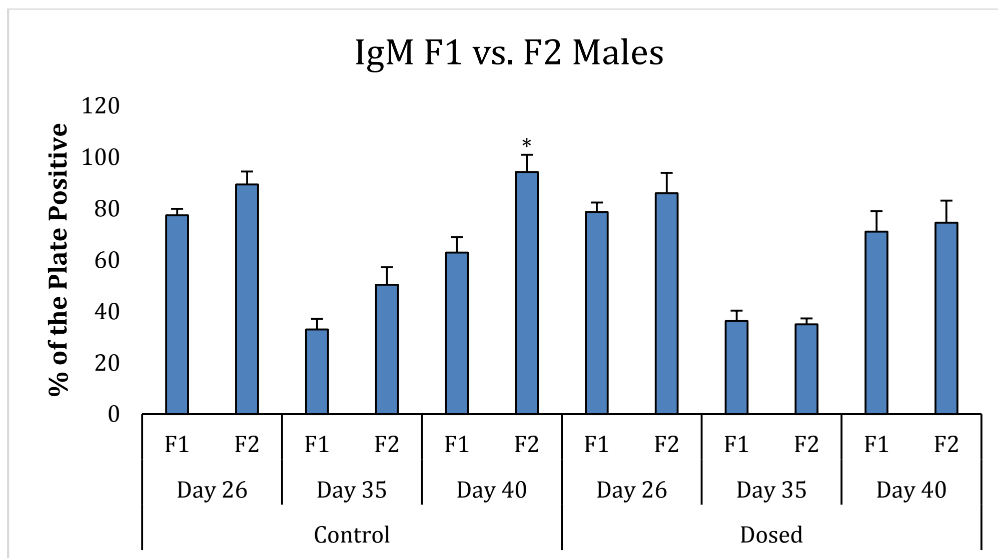

Figure 12: F1 IgM primary and secondary responses were compared to F2 IgM responses. A pattern of F2 increased responses were observed in the control but not in the dosed groups. N=4-8, p≤0.05 on Wilcoxin Rank Sum test.

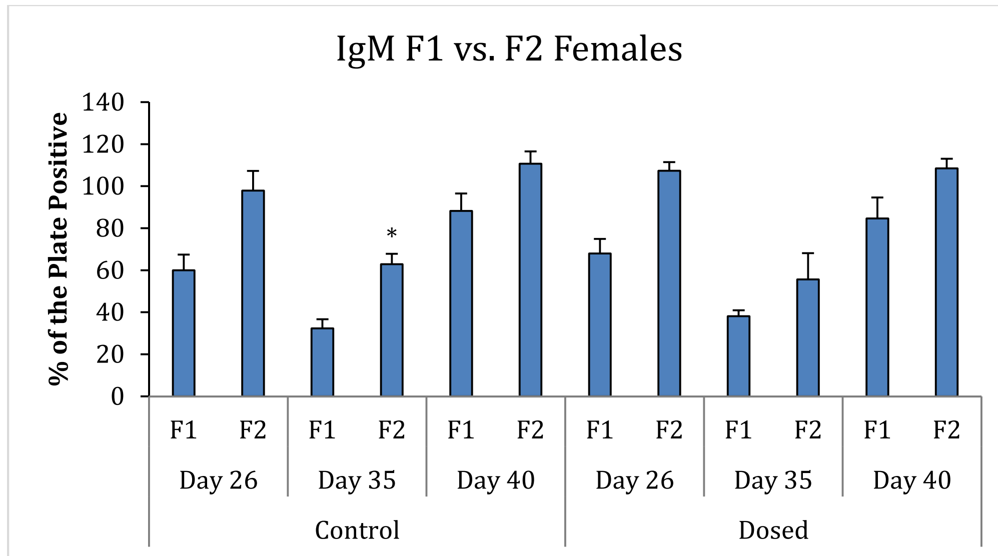

Figure 13: F1 IgM primary and secondary responses were compared to F2 IgM responses. A pattern of F2 increased responses were observed in the control. N=3-8, p≤0.05 on Wilcoxin Rank Sum test.

When the F1 and F2 generations were compared, there was a trend toward a higher F2 response in both males and females (Fig. 12, Fig. 13). This effect was significant in F2 males on day 40 and F2 females on day 35. This trend was absent for dosed males on days 35 and 40 supporting the finding of decreased response in the dosed F2 males.

IgA Antibody Response

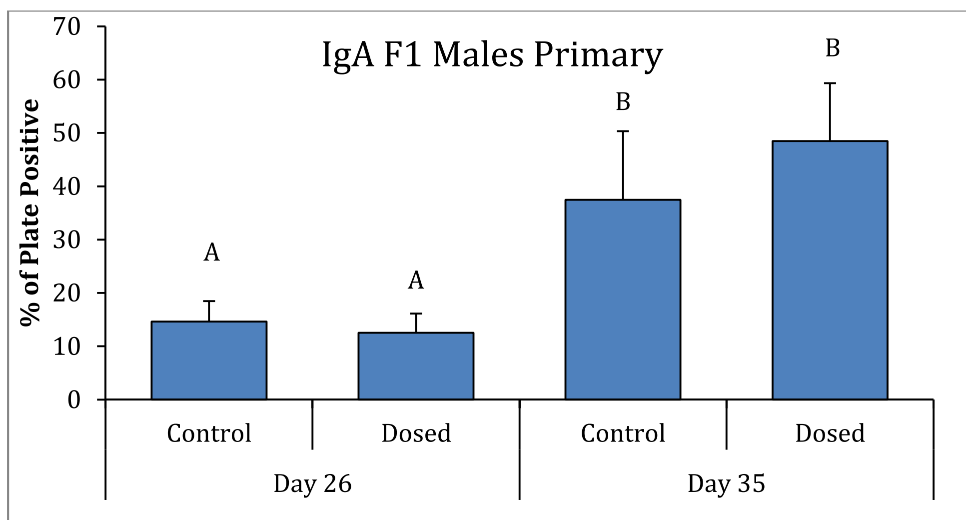

Figure 14: F1 male primary response of IgA was assessed by bleeding mice 5 and 10 days post-injection of 0.3mg KLH on post-natal days 26 and 35. No significant differences were observed. . N=7-8, $p \leq 0.05$ on Wilcoxin Rank Sum test.

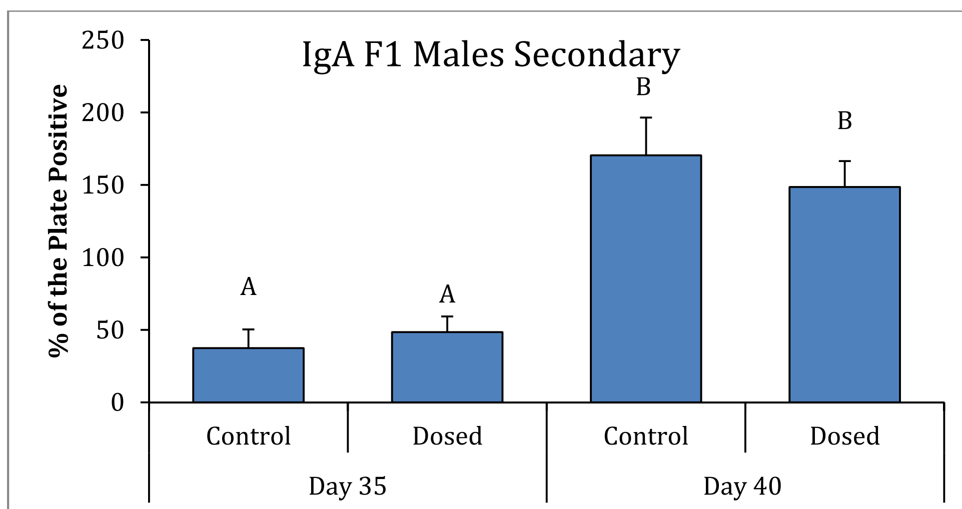

Figure 15: F1 male secondary response of IgA was assessed by bleeding mice 5 days after the secondary of injection of 0.3mg KLH on post-natal day 40. No significant differences were observed. . N=7-8, $p \leq 0.05$ on Wilcoxin Rank Sum test.

IgA production in most groups followed a typical pattern with lower primary response on day 26, and peaks 10 day after antigen exposure on day 35. The secondary response on day 40 was greater than the primary response. IgA production in F1 males showed no significant differences with ADBAC+DDAC treatment (Fig. 14, Fig. 15, Fig. 16, Fig. 17).

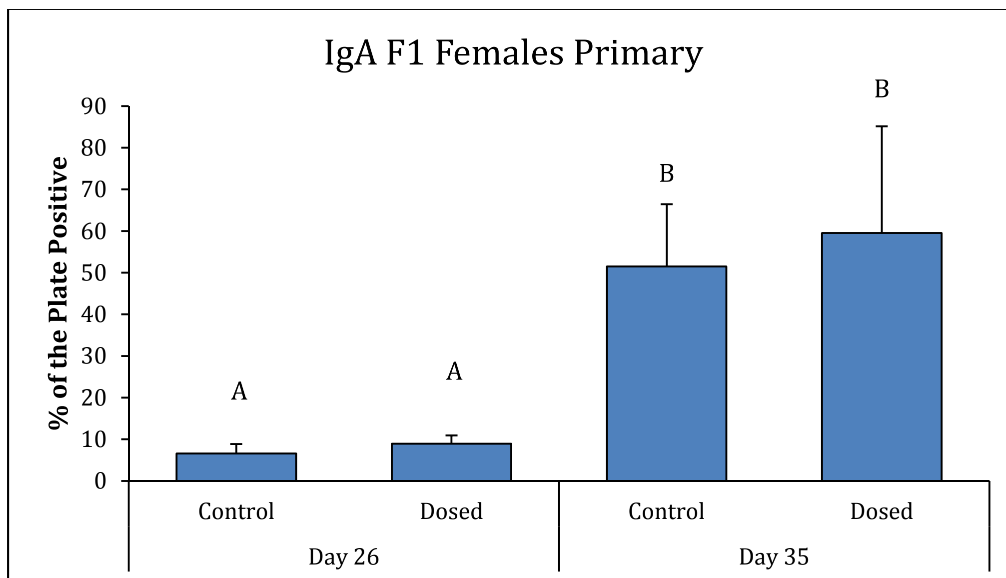

Figure 16: F1 female primary response was assessed by bleeding mice 5 and 10 days post injection of 0.3mg KLH on post-natal days 26 and 35. No significant differences were observed. N=8, $p \leq 0.05$ on Wilcoxin Rank Sum test.

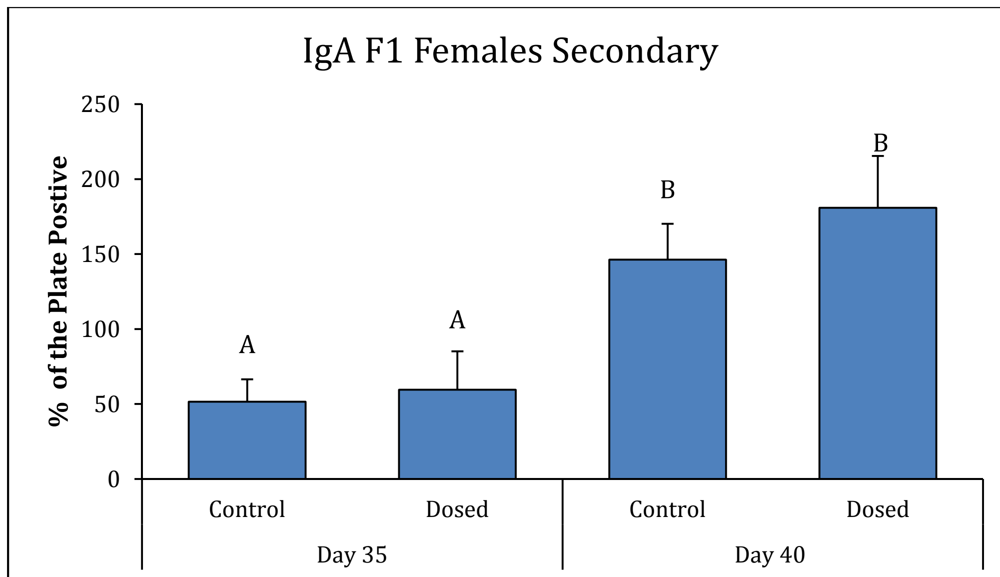

Figure 17: F1 female secondary response was assessed by bleeding mice 5 days after the secondary injection of 0.3mg KLH on post-natal day 40. No significant differences were observed. . N=8, p≤0.05 on Wilcoxin Rank Sum test.

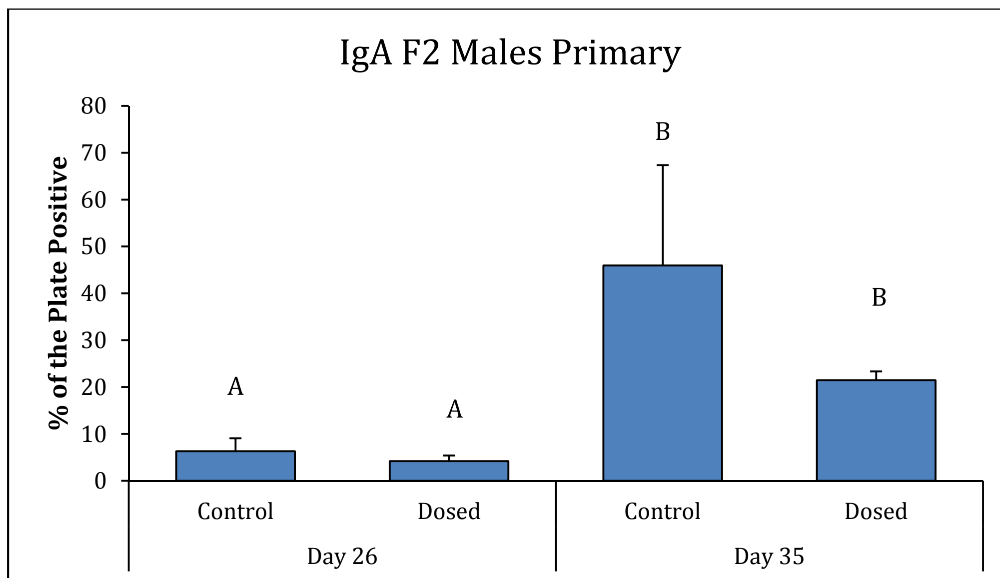

Figure 18: F2 males primary response of IgA was assessed by bleeding 5 and 10 days post injection of 0.3mg KLH on post-natal Days 26 and 35. A large decrease was observed in the day 35 response. No significant differences were observed. N=8, p≤0.05 on Wilcoxin Rank Sum test.

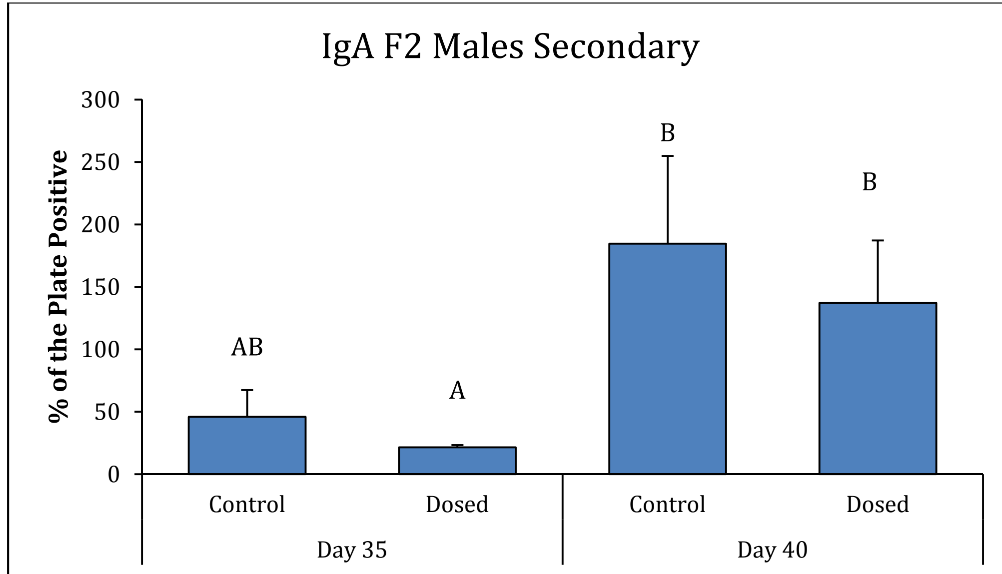

Figure 19: F2 male secondary response of IgA was assessed by bleeding mice 5 days after the secondary injection of 0.3mg KLH on post-natal day 40. The secondary response was not significantly different from the primary response on the control day 35 indicating a possible decrease in the secondary response due to treatment. N=4-5, $p \leq 0.05$ on Wilcoxin Rank Sum test.

IgA production in the F2 males did not follow this general pattern. Although no significant decrease was found in response to treatment on day 35 and day 40 in the F2 males, there was a trend toward decreased antibody response in both these days particularly on day 35 (Fig. 18, Fig. 19).

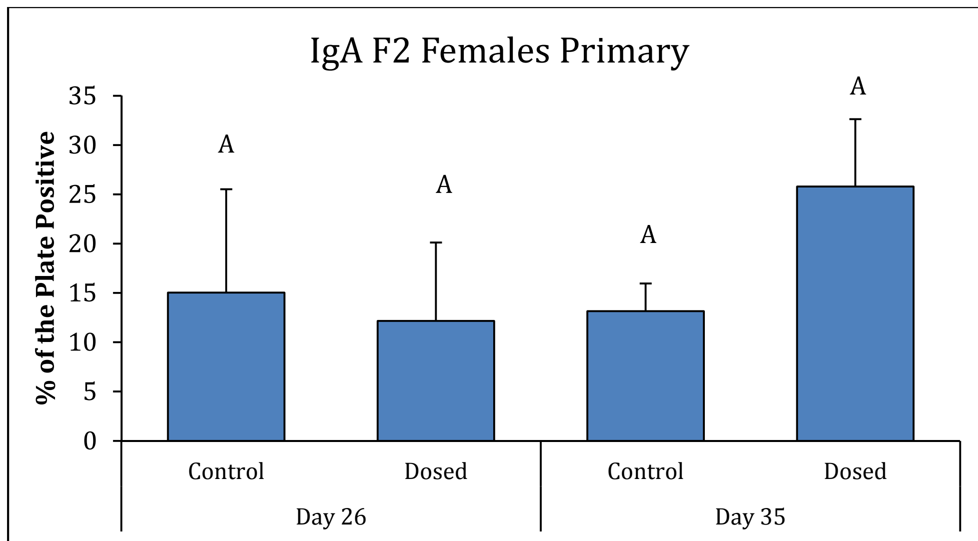

Figure 20: F2 female primary response of IgA was assessed by bleeding 5 and 10 days post injection of 0.3mg KLH on post-natal days 26 and 35. No significant differences were observed. N=3-5, $p \leq 0.05$ on Wilcoxin Rank Sum test.

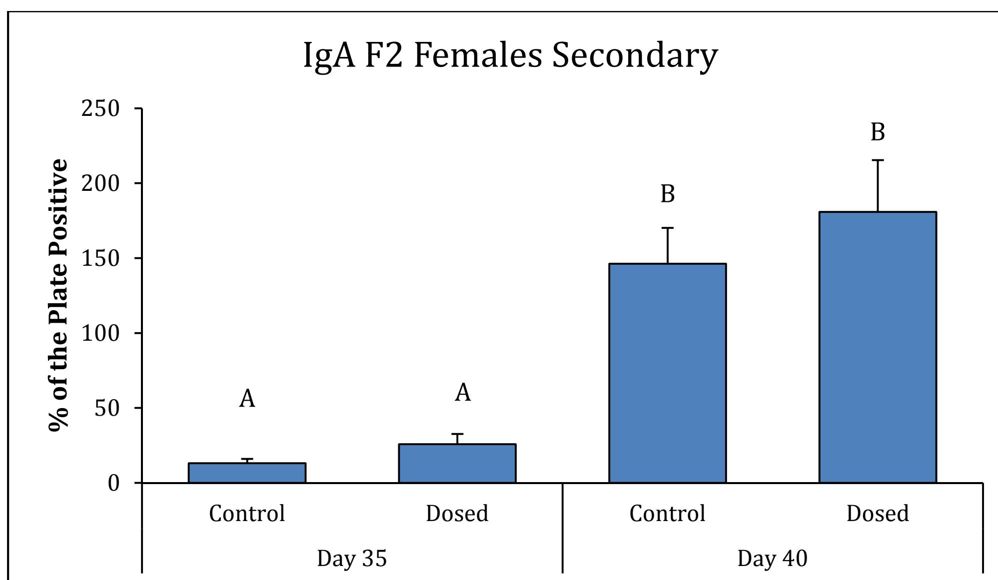

Figure 21: F2 female secondary response of IgA was assessed by bleeding mice 5 days after the secondary injection of 0.3mg KLH on post-natal day 40. No significant differences were observed. N=3-5, $p \leq 0.05$ on Wilcoxin Rank Sum test.

In F2 females, IgA production on day 35 was decreased and not significantly different than the day 26 response (Fig. 20). It is unlikely that this was an effect of ADBAC+DDAC treatment because IgA was decreased in day 35 controls as well. The secondary response for the F2 female IgA production followed the standard pattern and showed no treatment effect (Fig. 21).

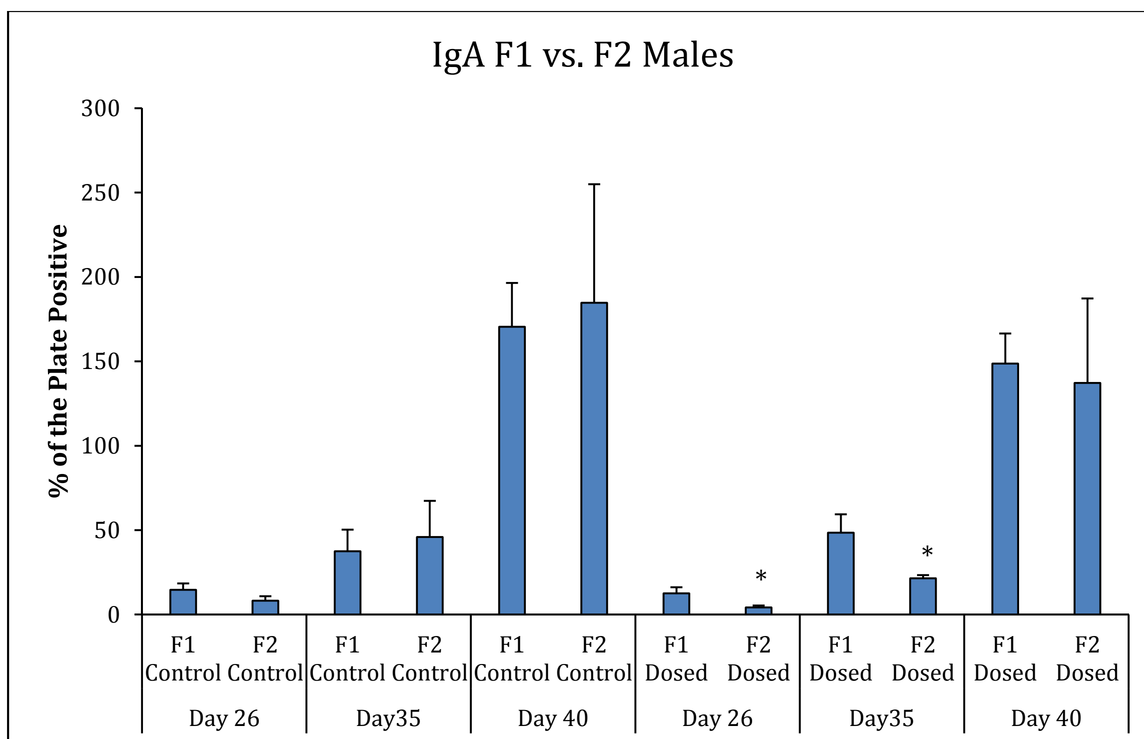

Figure 22: IgA F1 and F2 responses were compared. Significant decreases were found in the dosed group on days 26 and 35. N=4-8, $p \leq 0.05$ on Wilcoxin Rank Sum test.

When IgA production between the F1 and F2 generations was compared, a significant decrease was observed in F2 dosed males on day 26 and 35 (Fig. 22). This implies that the decrease in the F2 males on day 35 compared to controls (Fig. 18) may be real.

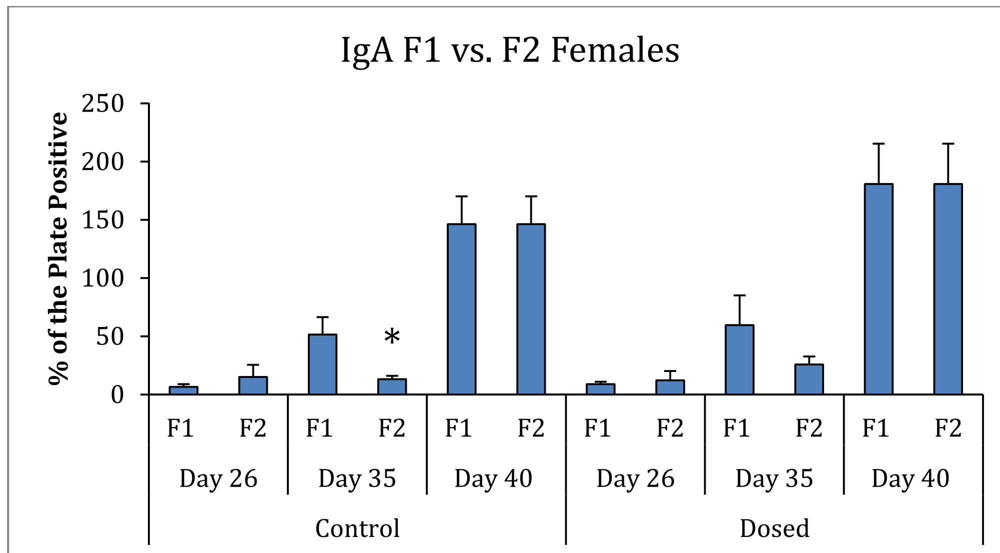

Figure 23: IgA F1 and F2 responses were compared. A significant decrease was found in the F2 controls on day 35. N=4-8, $p \leq 0.05$ on Wilcoxin Rank Sum test.

The F2 female controls, when compared to the F1 had a significant decrease in day 35 (Fig. 23). This could explain why the IgA primary response was not different on days 26 and 35 (Fig. 20).

IgG Antibody Response

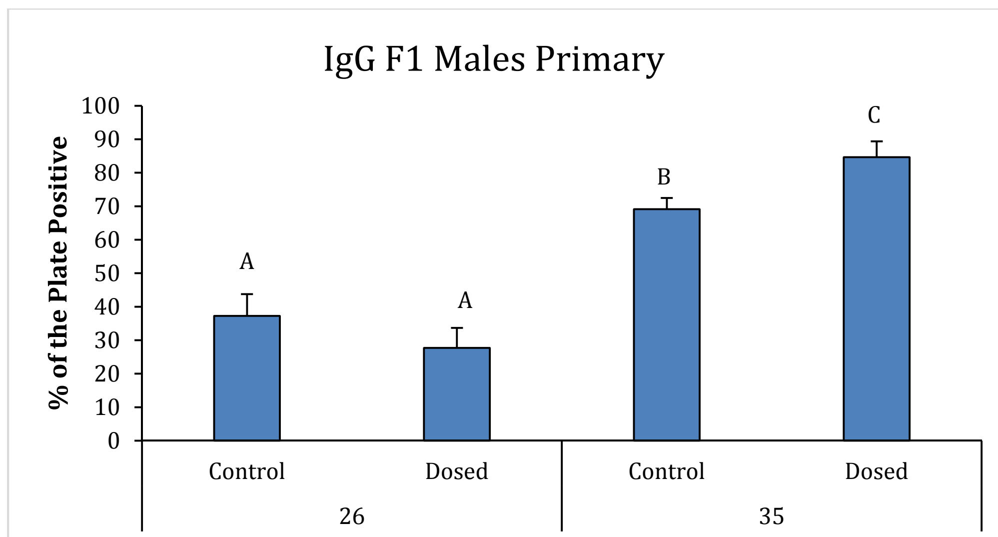

Figure 24: F1 male primary response of IgG was assessed by bleeding mice 5 and 10 days post injection of 0.3mg KLH on post-natal days 26 and 35. A significant increase was observed on the day 35 primary response. N=7-8, p≤0.05 on Wilcoxin Rank Sum test.

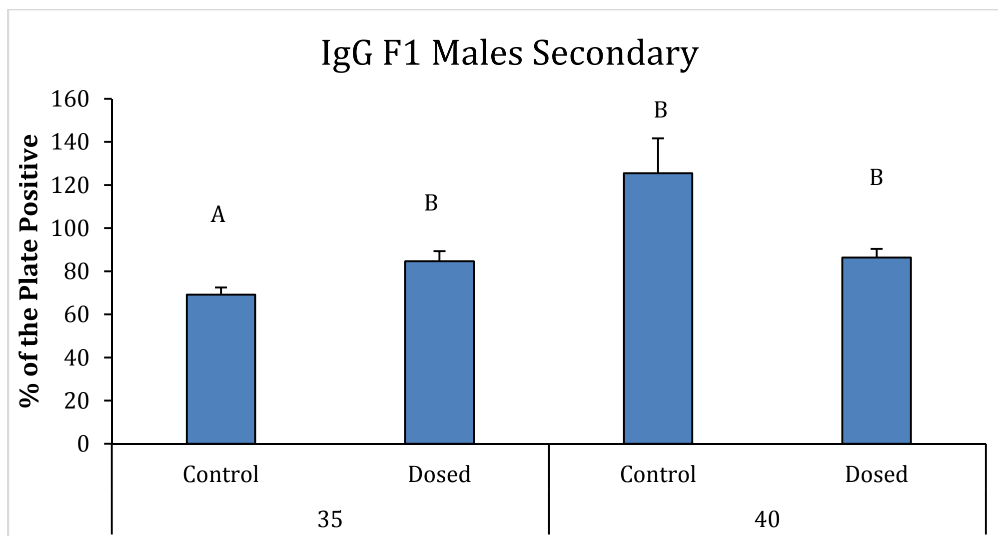

Figure 25: F1 male secondary response of IgG was assessed by bleeding mice 5 days after the secondary injection of 0.3mg KLH on post-natal day 40. The secondary response was depressed in both the control and dosed mice. N=7-8, p≤0.05 on Wilcoxin Rank Sum test.

IgG followed a typical pattern similar to IgA with higher primary responses on day 35 than day 26, and higher secondary responses on day 40. In F1 there was a significant increase in IgG production in dosed F1 males on day 35 (Fig. 24). The secondary response in the dosed F1 males was not significantly different from the primary response (Fig. 25). This suggests that secondary response on day 40 dosed may be depressed, despite not being significantly different from the control on day 40.

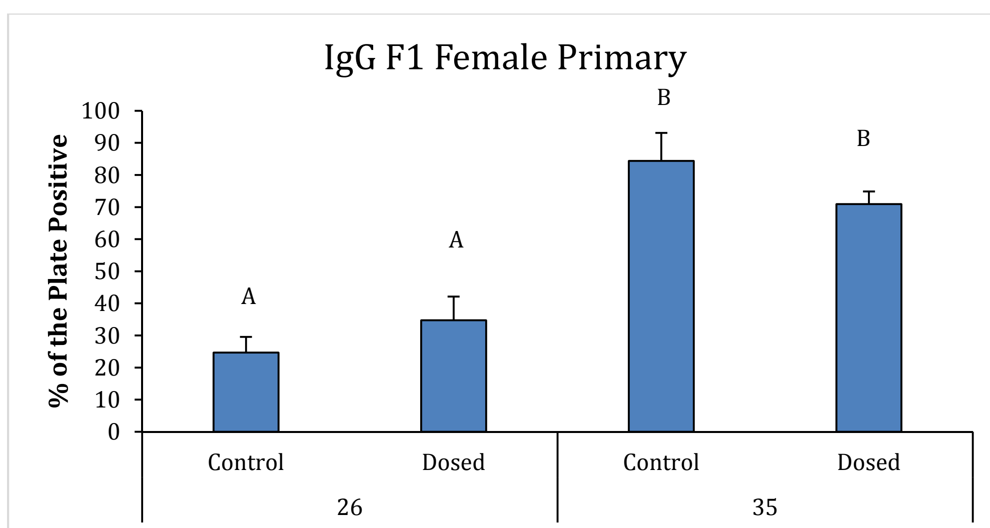

Figure 26: F1 female primary response of IgG was assessed by bleeding mice 5 and 10 days post injection of 0.3mg KLH on post-natal days 26 and 35. No significant differences were observed N=8, p≤0.05 on Wilcoxin Rank Sum test.

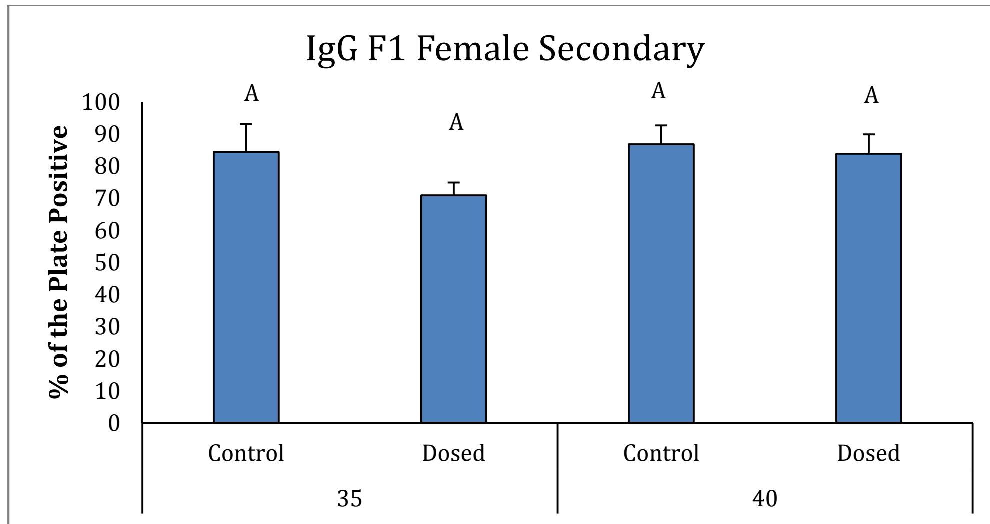

Figure 27: F1 female secondary response of IgG was assessed by bleeding mice 5 days after the secondary injection on post-natal day 40. No significant differences were observed N=8, $p \leq 0.05$ on Wilcoxin Rank Sum test.

The F1 female IgG response followed the expected pattern in the primary response, but the secondary response was not significantly different from the primary response on day 35 (Fig. 26, Fig. 27).

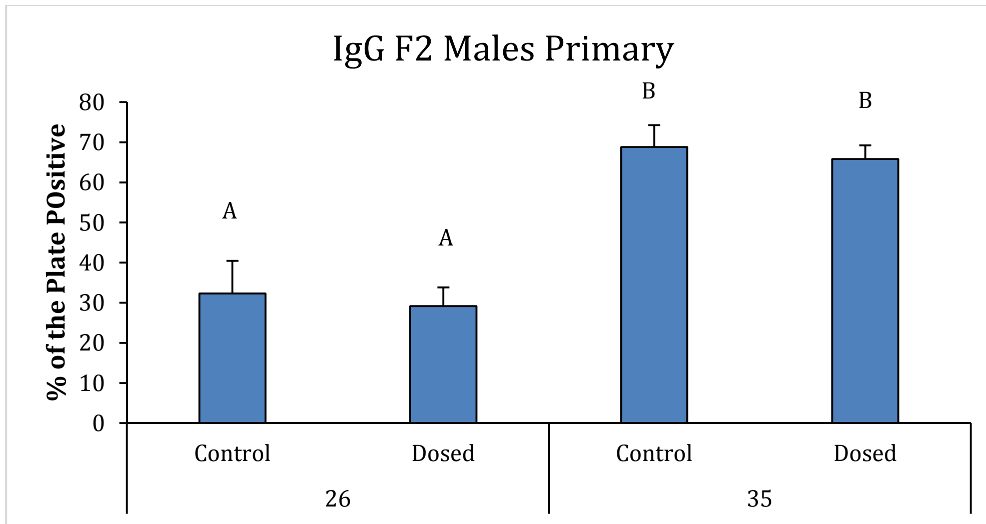

Figure 28: F2 male primary response of IgG was assessed by bleeding mice 5 and 10 days post injection of 0.3mg of KLH on post-natal day 26 and 35. No significant differences were observed N=4-5, p≤0.05 on Wilcoxin Rank Sum test.

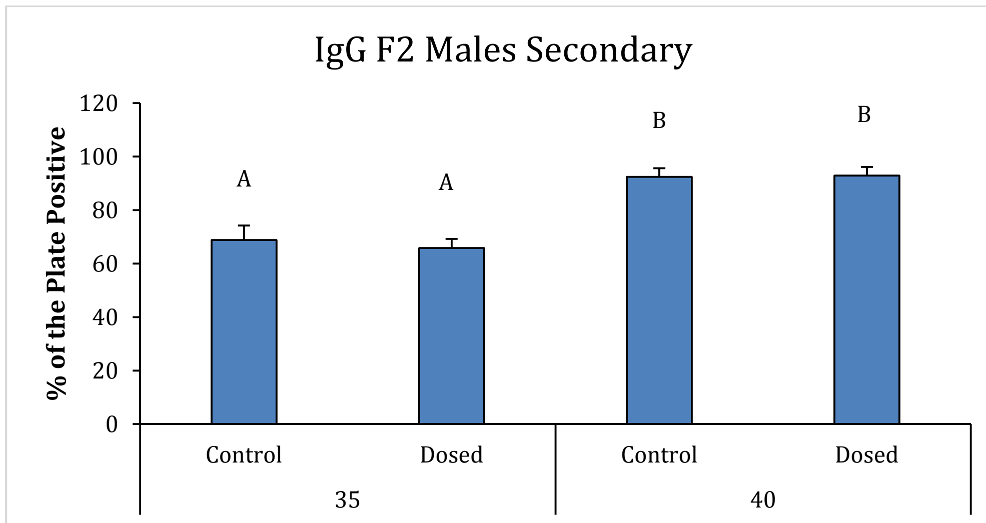

Figure 29: F2 male secondary response was assessed by bleeding mice 5 days after the secondary injection of 0.3mg KLH on post-natal day 40. No significant differences were observed N=4-5, p≤0.05 on Wilcoxin Rank Sum test.

The F2 male IgG antibody response shows no significant differences by treatment in either the primary response or the secondary response (Fig. 28, Fig. 29).

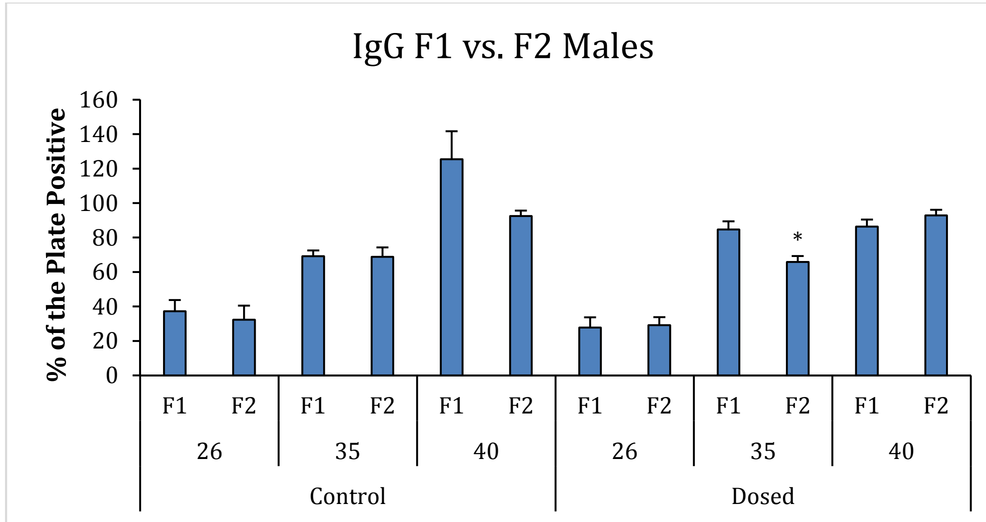

Figure 30: IgG responses were compared between the F1 and the F2. A significant decrease was observed in the F2 dosed day 35 response. N=4-8, p≤0.05 on Wilcoxin Rank Sum test.

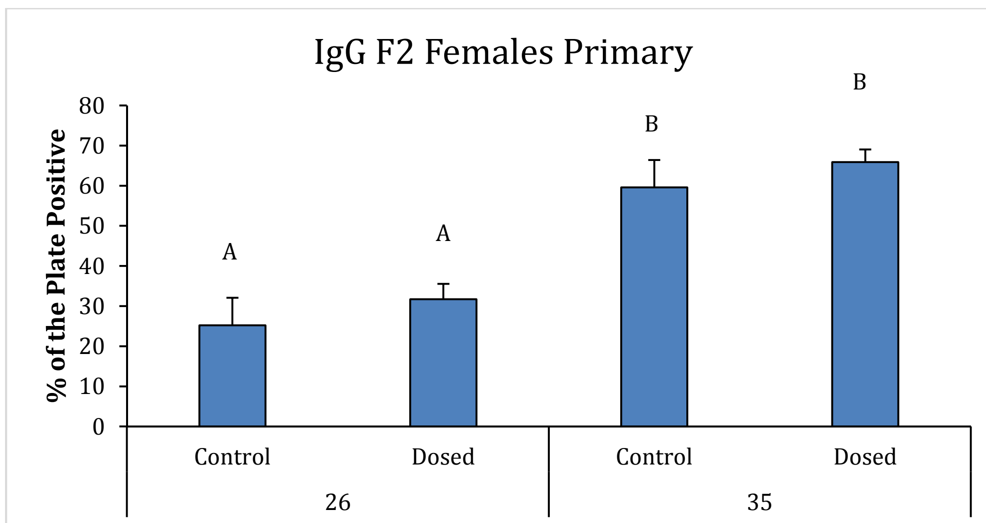

Figure 31: F2 female primary response of IgG was assessed by bleeding mice 5 and 10 days post-injection of 0.3mg KLH on post-natal days 26 and 35. No significant differences were observed. N=3-5, p≤0.05 on Wilcoxin Rank Sum test.

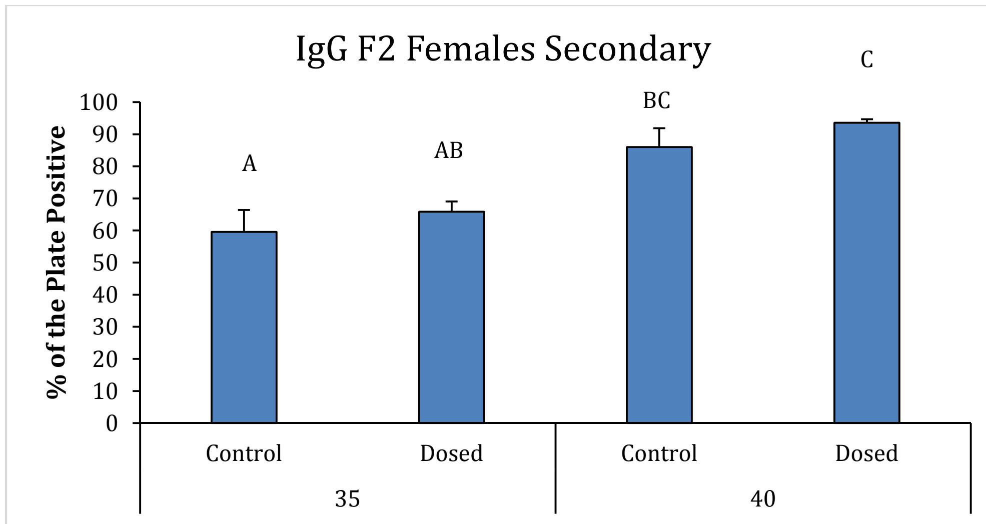

Figure 32: F2 female secondary response of IgG was assessed by bleeding mice 5 days after the secondary injection of 0.3mg KLH on post-natal day 40. A significant increase was observed in the secondary response of dosed mice. N=3-5, p≤0.05 on Wilcoxin Rank Sum test.

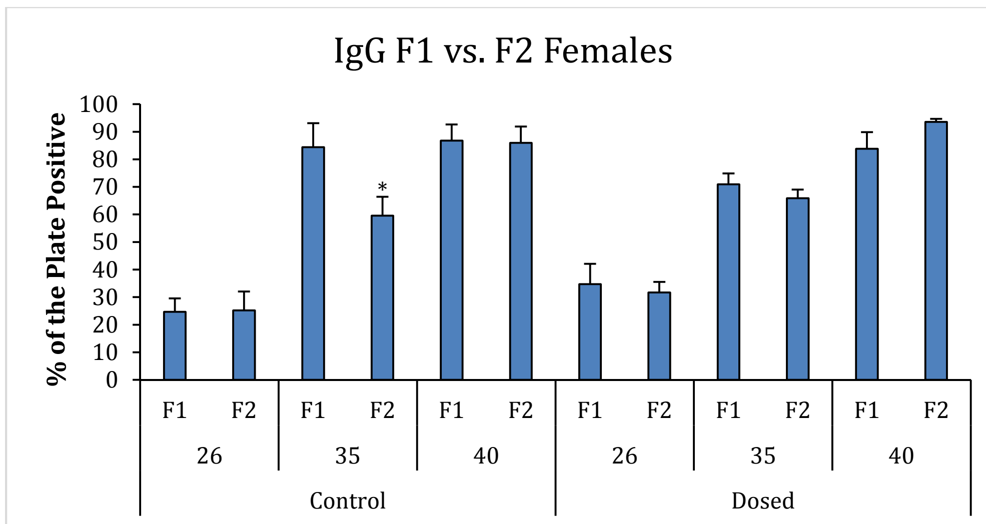

Figure 33: IgG responses were compared between the F1 and the F2. Significant decreases were found in the F2 controls on day 35. N=3-8, p≤0.05 on Wilcoxin Rank Sum test.

When the two generations were compared, dosed F2 males had significantly decreased IgG production on the day 35 primary response (Fig. 32). The F2 females showed a significant decrease in the F2 controls compared to the F1on day 35.

Results

Microbiome

Microbiome data were analyzed using alpha diversity and beta diversity (presented as principal component analysis, or PCA). Alpha diversity measures the number of species in a sample. PCA compares the diversity of samples within a treatment by converting genera and phylogenetic data into a single data point (Tyler et al., 2014). The farther the points are away from each other, the more variance there is between them (Tyler et al., 2014). Both of these measures are used to assess variance in the microbiome and they provide different information about the sample. For example, the same number of species (nonsignificant alpha diversity) can exist in two distinctly different communities (significant PCA).

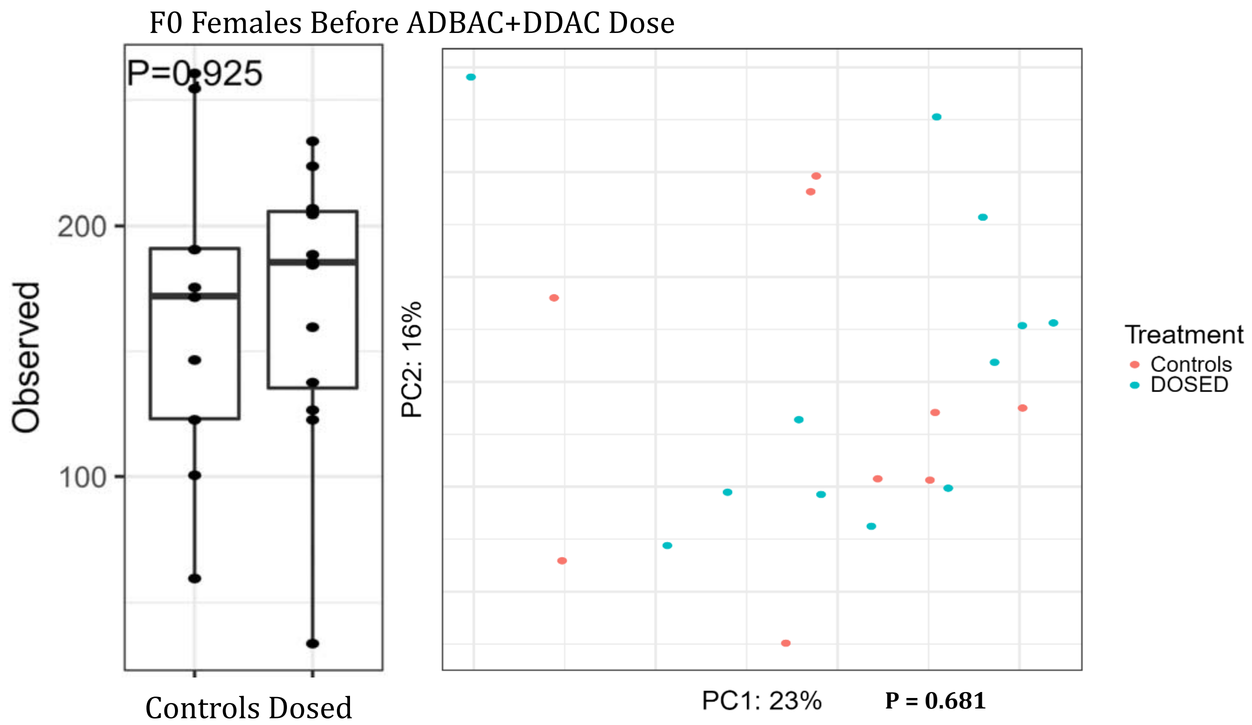

Figure 34: DNA extracted from fecal samples that were collected from F0 females before ADBAC+DDAC exposure was sequenced to assess the gut microbiome. No significant differences were observed. Alpha diversity was analyzed by a student t-test and PCA was analyzed with an Adonis test. N=10 $p \leq 0.05$.

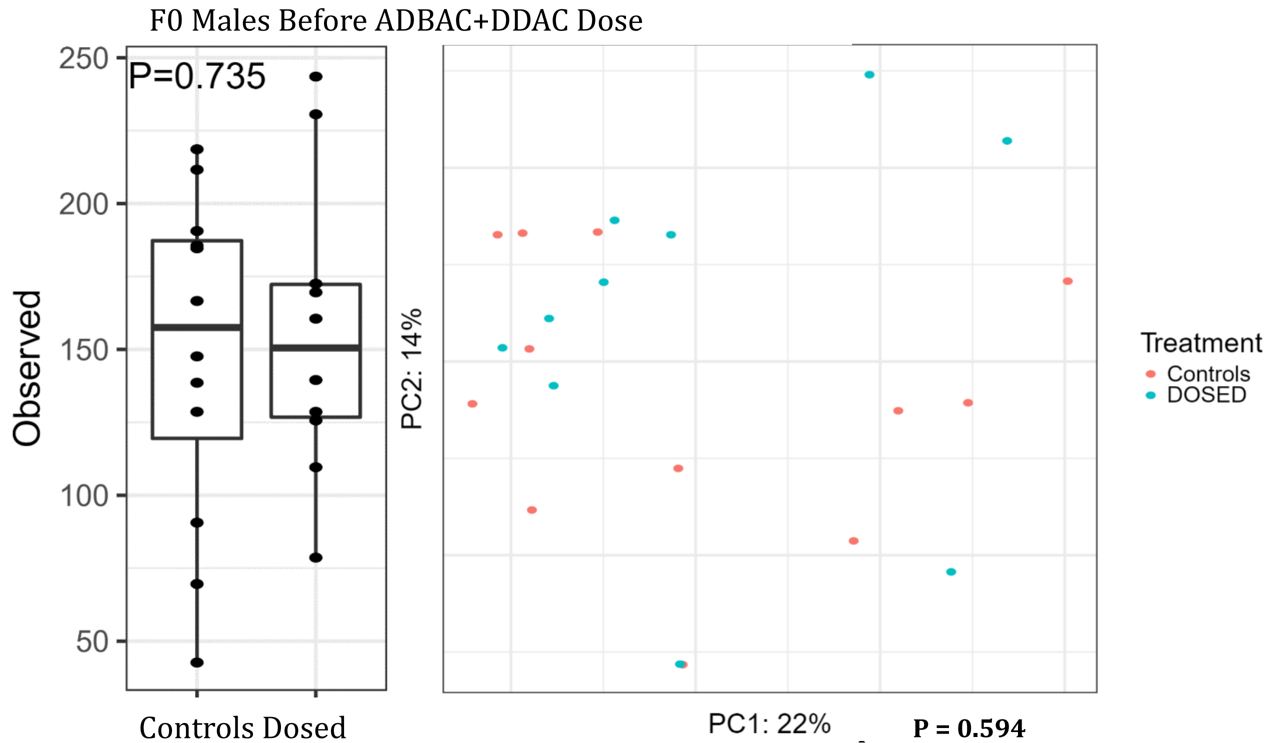

Figure 35: DNA extracted from fecal samples that were collected F0 males before ADBAC+DDAC exposure was sequenced to assess the gut microbiome. No significant differences were observed. Alpha diversity was analyzed by a student t-test and PCA was analyzed with an Adonis test. N=10 $p \leq 0.05$.

Initial fecal samples were first collected from the F0 generation (initial parents) after acclimation to the gel diet. At this time point, there were no differences in microbiome taxa between the control and the dosed groups for both males and females. This was expected as ADBAC+DDAC dosing had not begun.

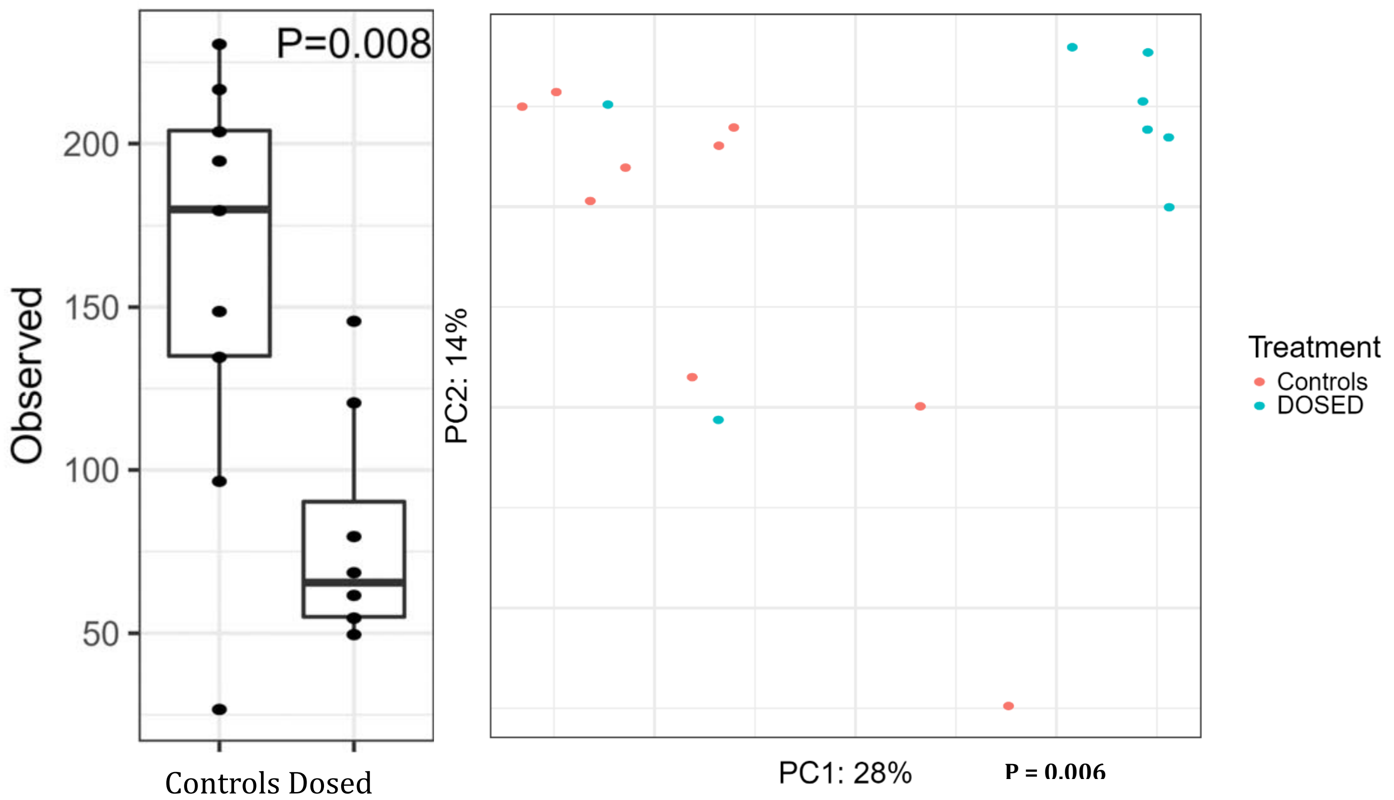

Figure 36: DNA extracted from fecal samples that were collected from F0 females after one week of ADBAC+DDAC dosing was sequenced to assess the gut microbiome. Significant differences were observed in both alpha and beta diversity. Alpha diversity was analyzed by a student t-test and PCA was analyzed with an Adonis test. N=9-10 $p \leq 0.05$.

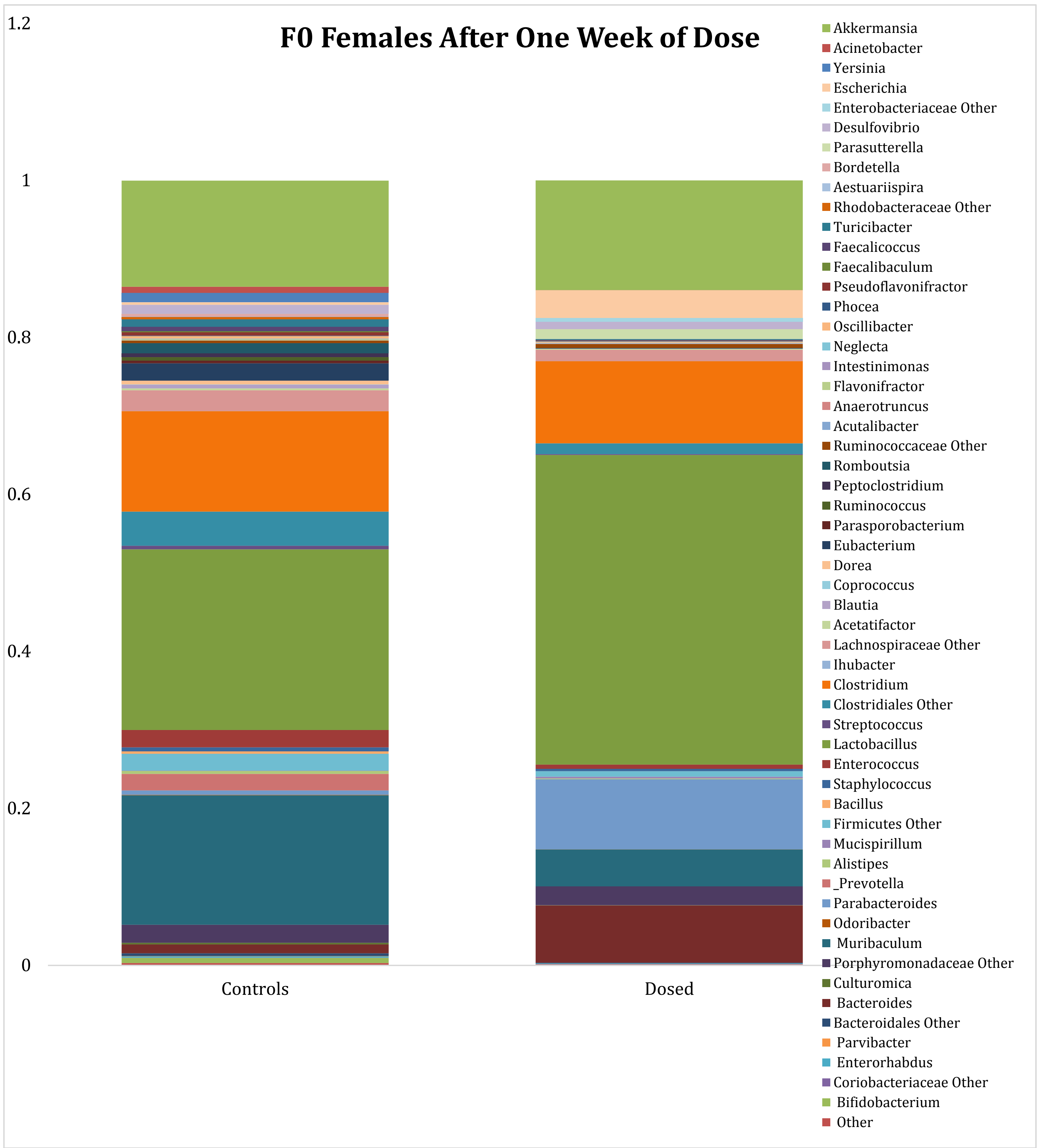

Figure 37: DNA extracted from fecal samples that were collected from F0 females after one week of

ADBAC+DDAC dosing was sequenced to assess the gut microbiome. there were significant decreases in the relative abundances of *Bacteroidales* (genus *Culturomica)* and *Bacteriodales* (genus *Flavonifractor).* N=9-10, p≤0.05.

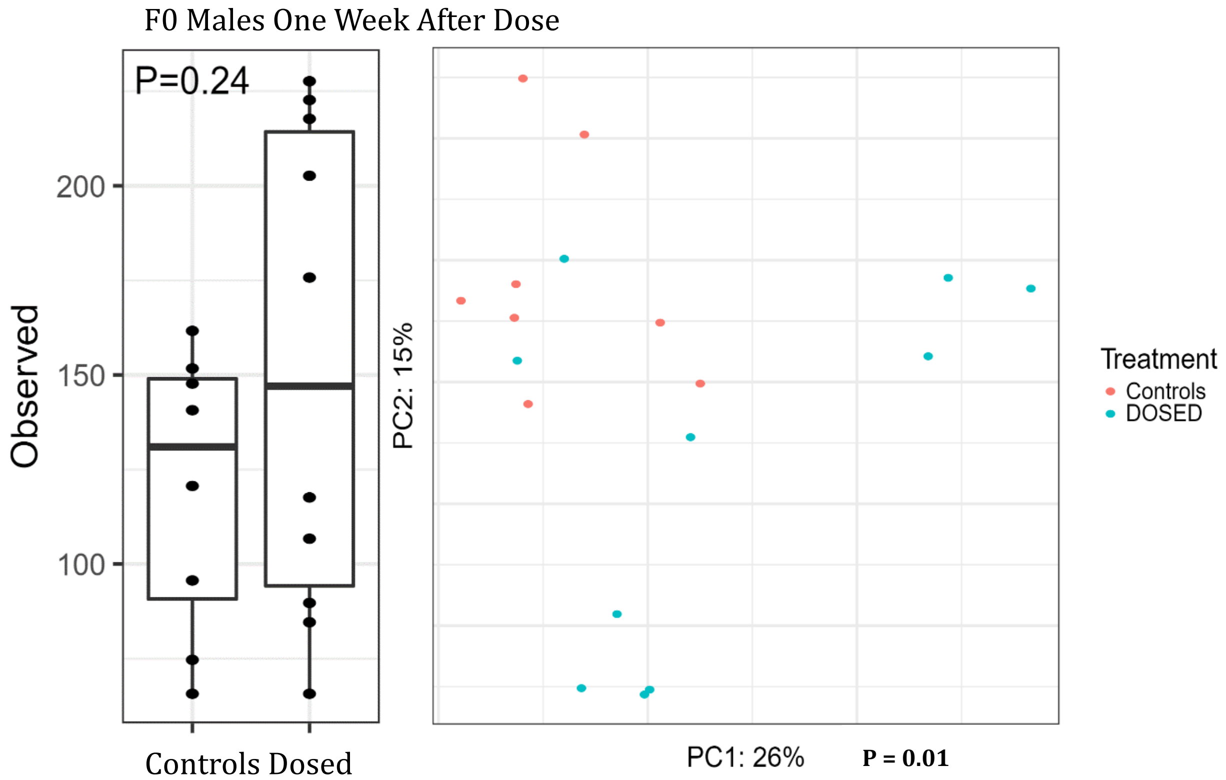

Figure 38: DNA extracted from fecal samples that were collected from F0 males after one week of ADBAC+DDAC dosing was sequenced to assess the gut microbiome. Significant differences were observed in beta diversity. Alpha diversity was analyzed by a student t-test and PCA was analyzed with an Adonis test. N=8-10 p≤0.05.

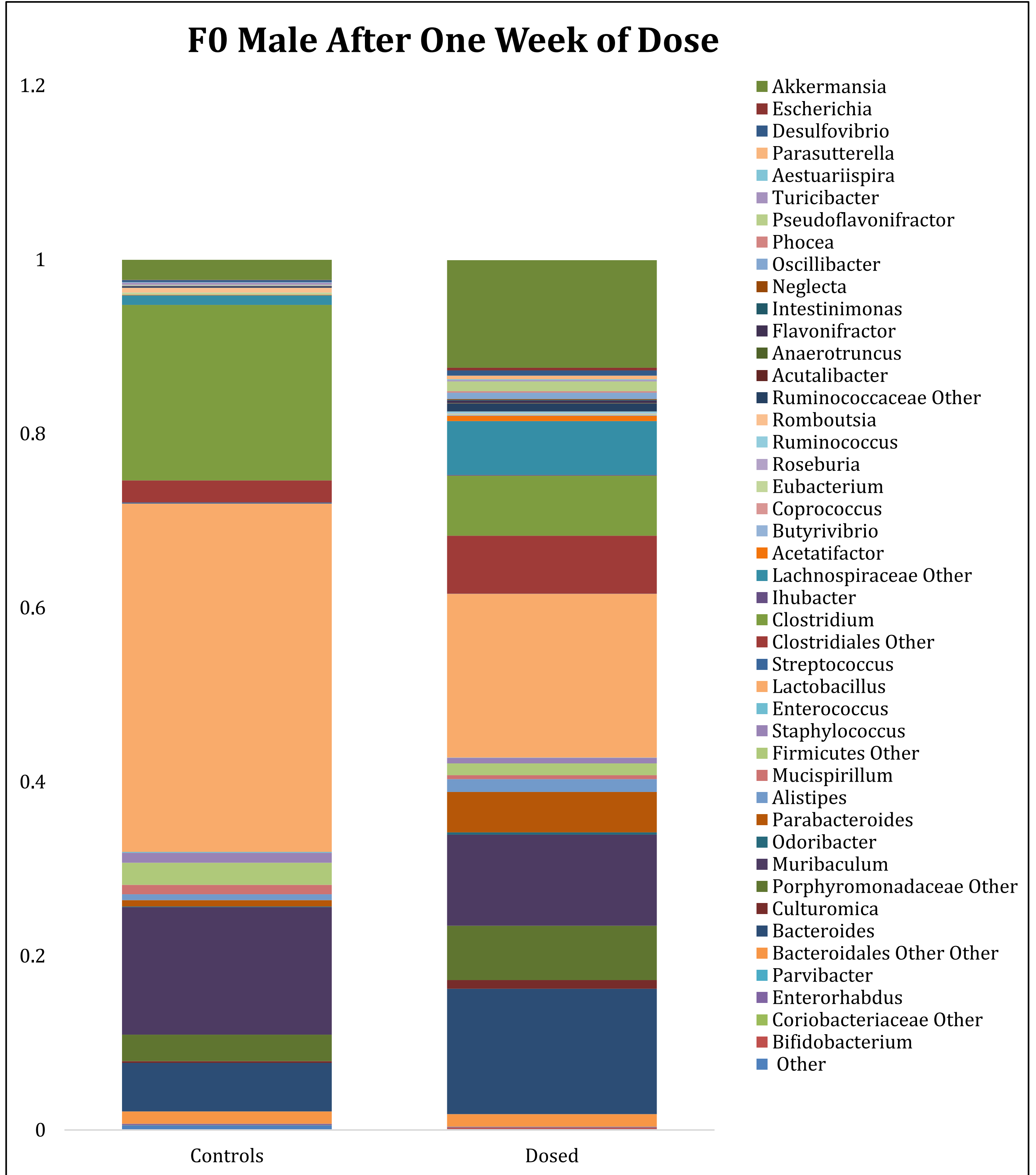

Figure 39: DNA extracted from fecal samples that were collected from F0 males after one week of ADBAC+DDAC dosing was sequenced to assess the gut microbiome. Significant increases were observed in

the relative abundance of family *Ruminococcacea* and decreases in *Streptococcus acidominumus* in the dosed group. Analysis used one way ANOVA, N=8-10 p≤0.05.

Significant differences in microbiota between ADBAC+DDAC dosed and control were observed after starting the ADBAC+DDAC diet. Before dosing, neither alpha nor PCA plots were different (Fig. 34, Fig. 35). After the ADBAC+DDAC was added into the diet of the dosed groups, significant differences were observed between treatment groups. Female parents had significant drops in alpha diversity as well as significant differences between the microbial families (Fig. 36). We also show the relative abundance of each genus to show differences in diversity between treatments. With ADBAC+DDAC treatment in females, there were significant decreases in the relative abundances of *Bacteroidales* (genus *Culturomica)* and *Bacteriodales* (genus *Flavonifractor).* In male parents, *Streptococcus acidominumus* was significantly decreased in the dosed group and had statistically significant PCA plots, although the alpha diversity was unaffected (Fig. 38). Additionally, males had increases in a large number of species from the family *Ruminococcacea* (Fig. 39).

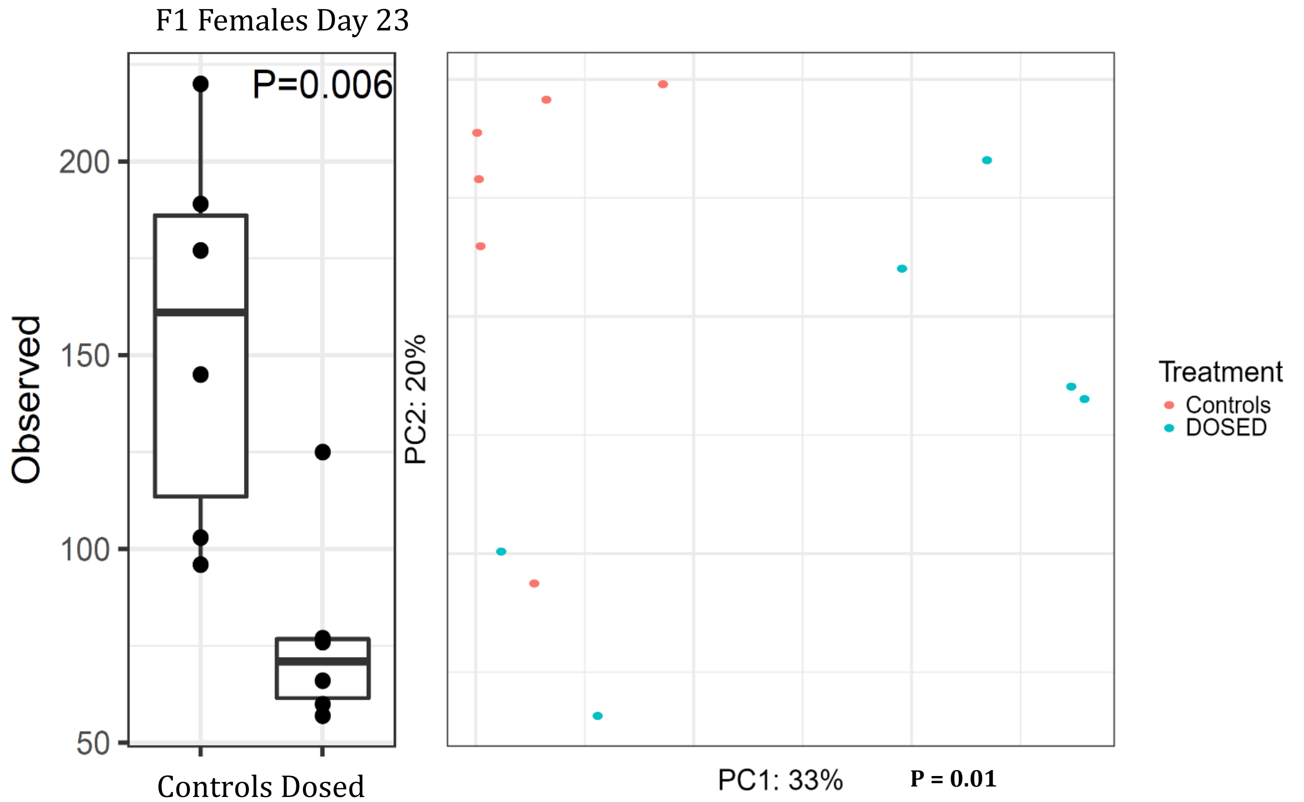

Figure 40: DNA extracted from fecal samples that were collected from F1 females Day 23, shortly after the ADBAC+DDAC dose was stopped, was sequenced to assess the gut microbiome. Significant differences were observed in alpha and beta diversity. Alpha diversity was analyzed by a student t-test and PCA was analyzed with an Adonis test. N=6-7 $p \leq 0.05$.

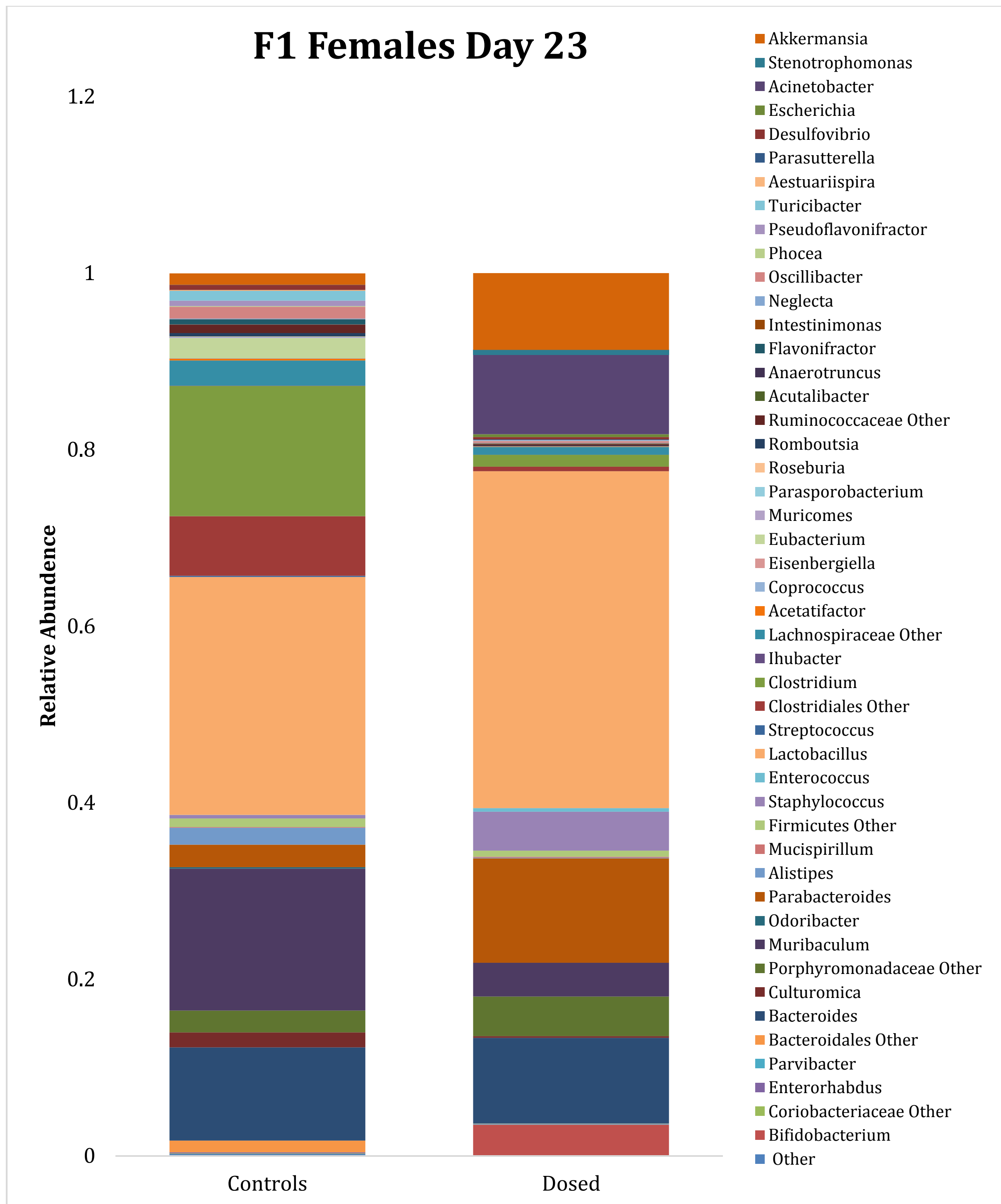

Figure 41: DNA extracted from fecal samples that were collected from F1 females Day 23, shortly after the ADBAC+DDAC dose was stopped, was sequenced to assess the gut microbiome. Significant differences were observed in alpha and beta diversity. Analysis used one-way ANOVA, N=6-7 $p \leq 0.05$

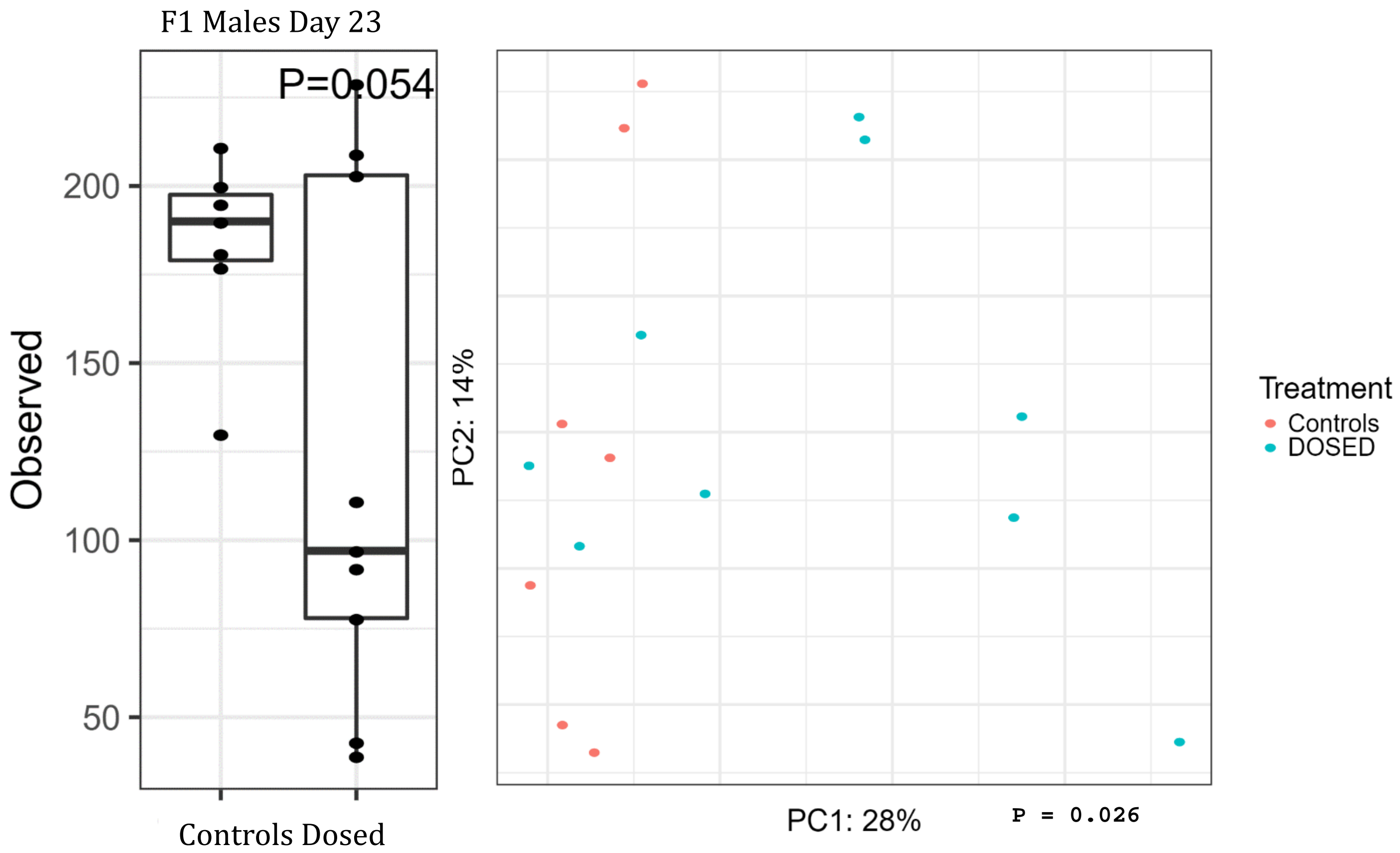

Figure 42: DNA extracted from fecal samples that were collected from F1 Males Day 23, shortly after the ADBAC+DDAC dose was stopped, was sequenced to assess the gut microbiome. Significant differences were observed in alpha and beta diversity. Alpha diversity was analyzed by a student t-test and PCA was analyzed with an Adonis test. N=7-8 $p \leq 0.05$.

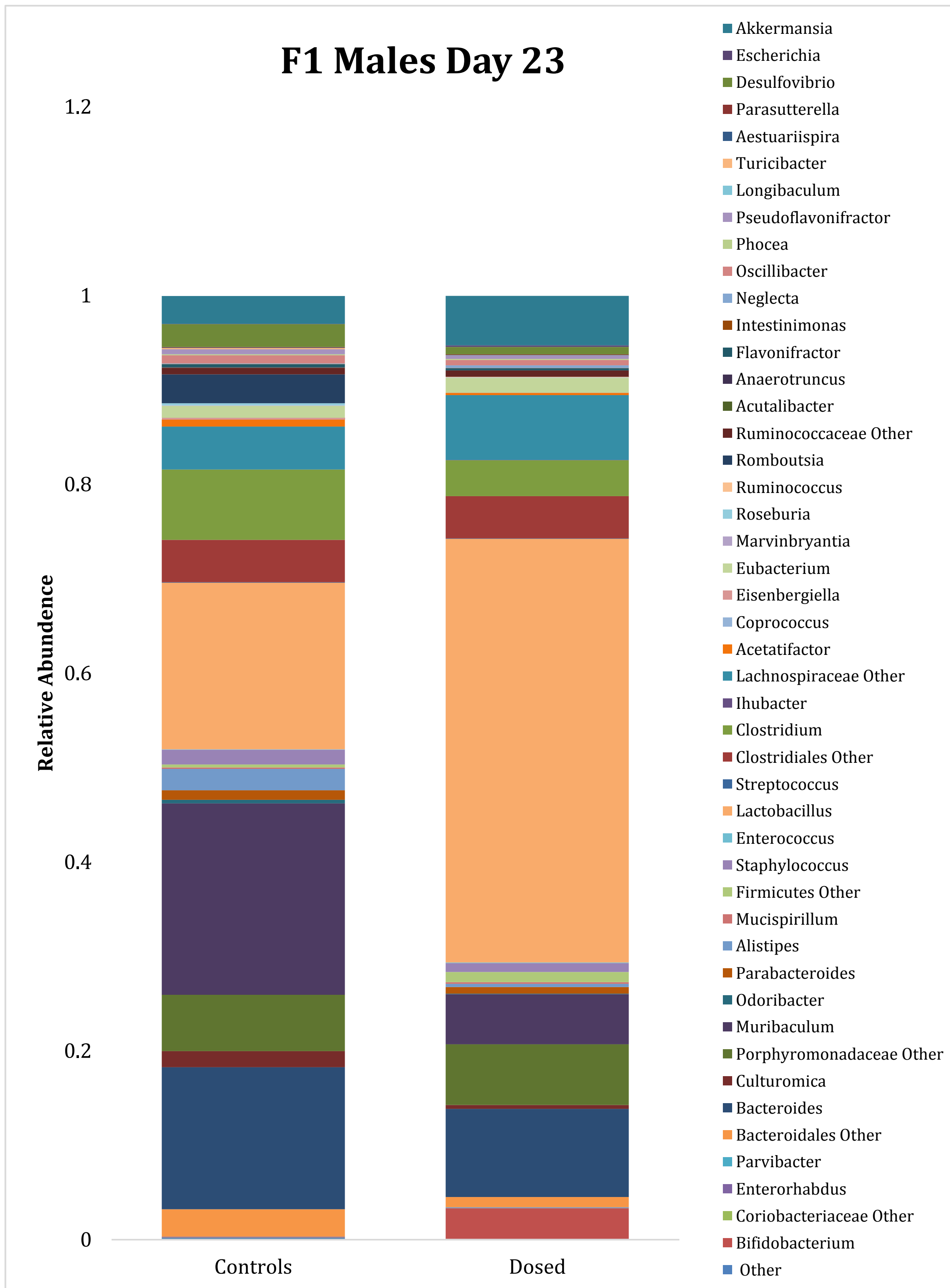

Figure 43: DNA extracted from fecal samples that were collected from F1 males Day 23, shortly after the ADBAC+DDAC dose was stopped, was sequenced to assess the gut microbiome. Significant reductions were

observed in abundances for *Muribaculum intestinale, Alistipes shahii, Lactobacillus frumenti, Clostridium cellobioparum, and Oscillibacter ruminantium* and a significant increase in the *Lactobacillus* genus. Analysis used a one-way ANOVA, N=7-8 $p \leq 0.05$.

Gut microbiota in the F1 were analyzed at day 23. This was two days post weaning and two days after cessation of the ADBAC+DDAC dose. Samples collected on this day represent both indirect exposure during gestation as well as direct exposure through lactation and transitions into solid food. Both alpha diversity and PCA plots were significantly different in both males and females on day 23 (Fig. 40, Fig. 42). This indicates that their GM was significantly altered. In F1 female mice on day 23, the relative abundance of *Clostridia hylemonae* and *Acetatifactor muris were significantly decreased* and *Lactobacillus gasseri* significantly increased. F1 males followed a similar pattern to the F0 males with increased small compositional species changes (Fig. 43). F1 males at day 23 had significant reductions in abundances for *Muribaculum intestinale, Alistipes shahii, Lactobacillus frumenti, Clostridium cellobioparum, and Oscillibacter ruminantium* and a significant increase in the *Lactobacillus* genus with undefined species.

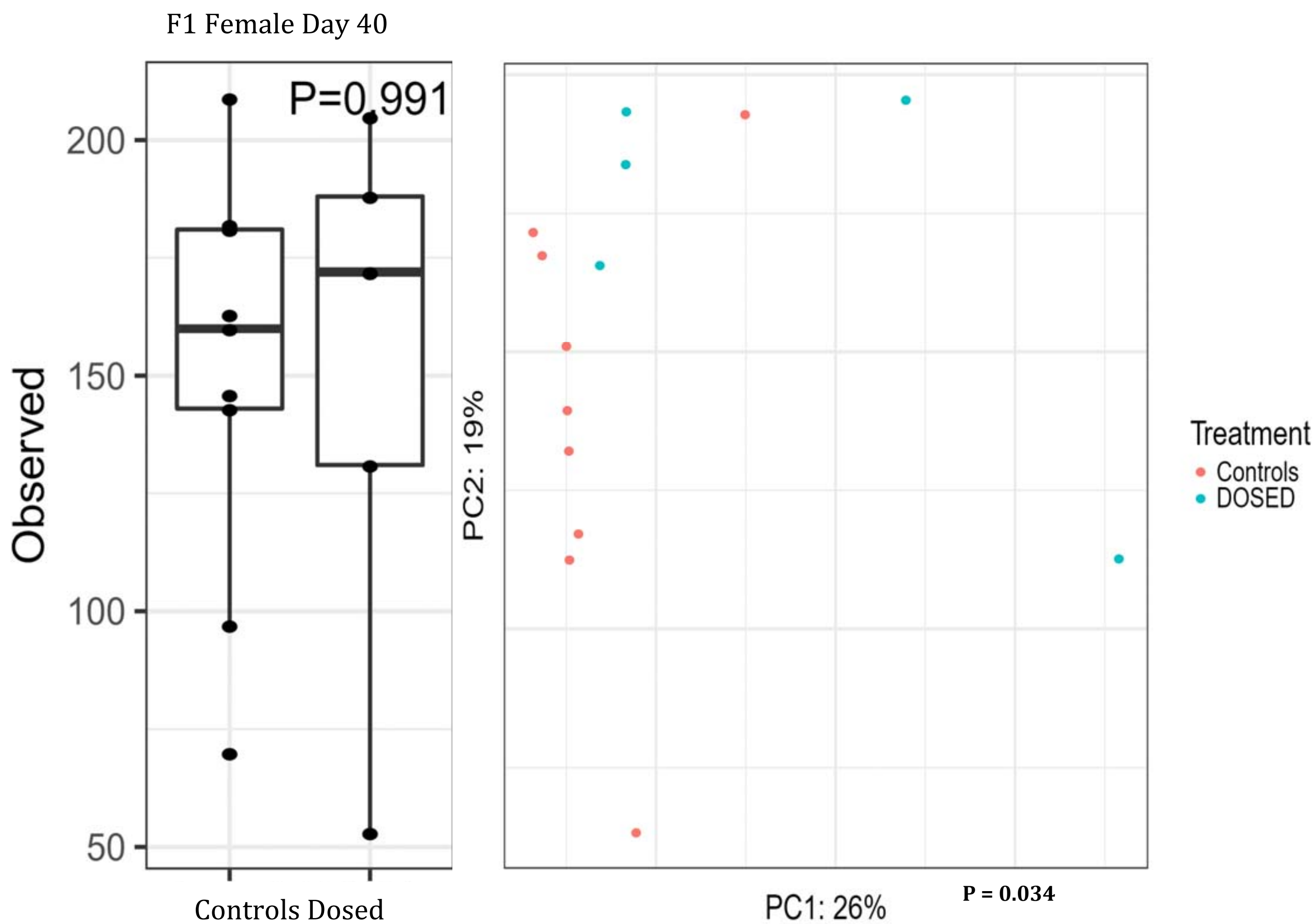

Figure 44: DNA extracted from fecal samples that were collected from F1 females on Day 40, 19 days after dose cessation, was sequenced to assess the gut microbiome. Significant differences were observed in beta diversity, but not alpha diversity. Alpha diversity was analyzed by a student t-test and PCA was analyzed with an Adonis test. N=6-9 p≤0.05.

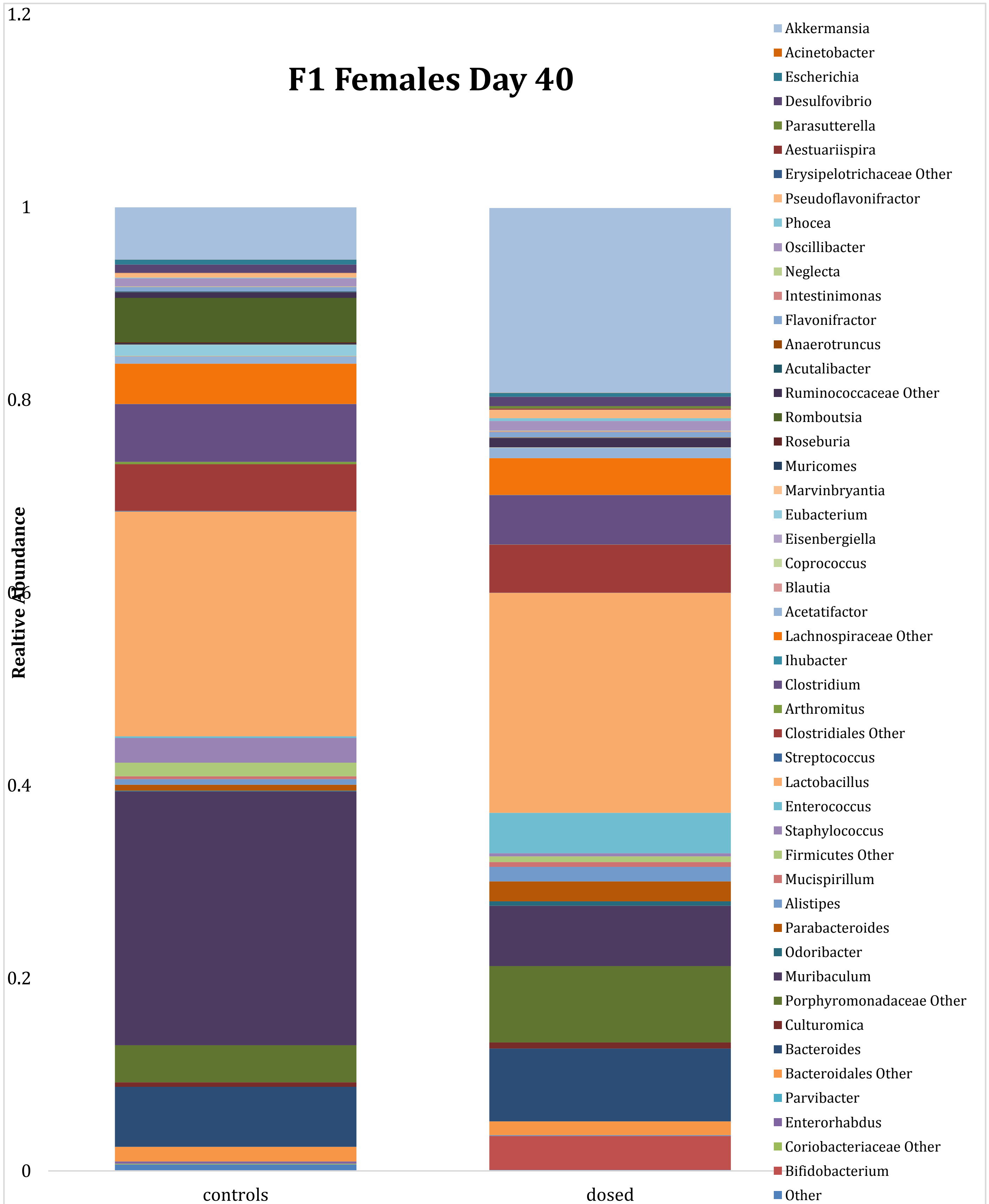

Figure 45: DNA extracted from fecal samples that were collected from F1 females on Day 40, 19 days

after dose cessation, was sequenced to assess the gut microbiome. The relative genera of *Anaerotruncus, Parasotterella, Akkermansia*, and *Clostridium* increased in the exposed mice while *Muribaculum* decreased. Analysis used one-way ANOVA, N=6-9 p≤0.05.

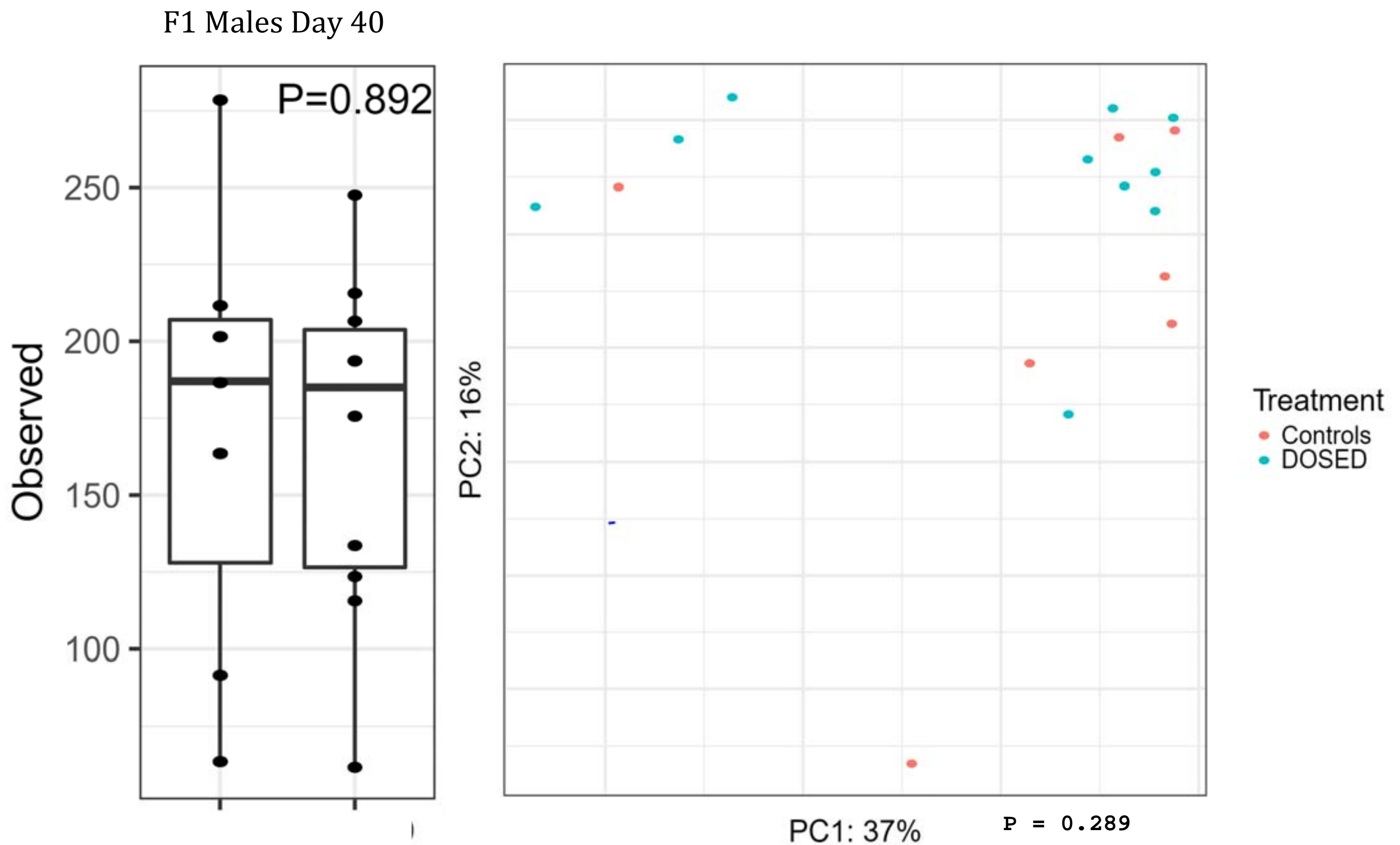

Figure 46: DNA extracted from fecal samples that were collected from F1 males on Day 40,19 days after dose cessation, was sequenced to assess the gut microbiome. No significant differences were observed in either alpha or beta diversity. Analysis used student t-test for alpha diversity and Adonis test for PCA, N=6-9 p≤0.05.

Fecal samples were collected again from F1 mice on Day 40, which was 19 days after cessation of dosing. No significant differences between control and dosed males were observed at this time point (Fig. 46). F1 day 40 exposed females had significantly different microbial composition from controls on the PCA plot, but not in alpha diversity (Fig. 44).

The relative genera of *Anaerotruncus, Parasotterella, Akkermansia*, and *Clostridium* increased in the exposed mice while *Muribaculum* decreased (Fig. 45).

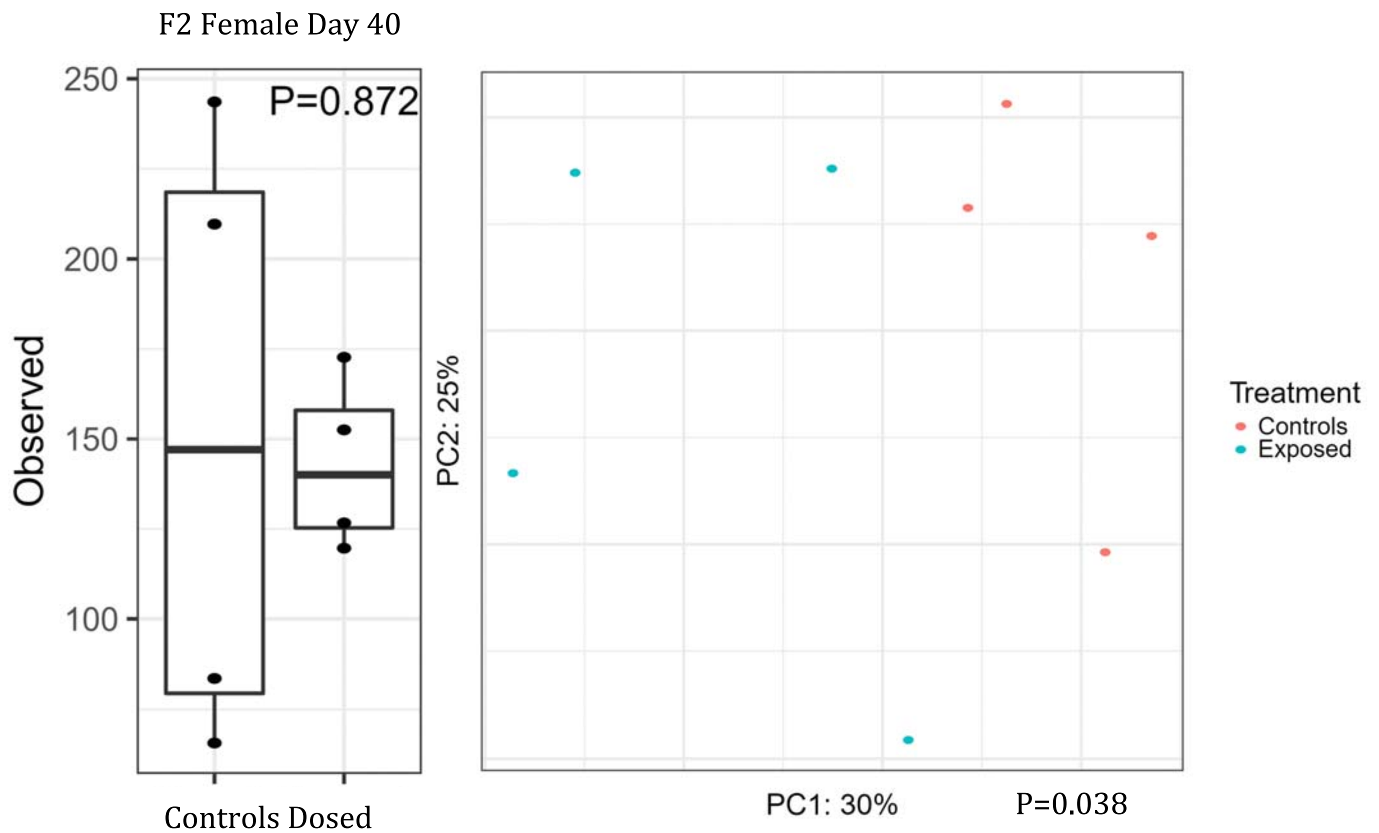

Figure 47: DNA extracted from fecal samples that were collected from F2 females on Day 40 was sequenced to assess the gut microbiome. Significant differences were observed on the PCA plot. Analysis used student t-test for alpha diversity and Adonis test for PCA, N=3-5 $p \leq 0.05$.

Fecal samples were collected from F1 parents at the same collection time points as for the F0 parents. No significant differences in alpha and beta diversity were observed in either sex at either time point. Fecal samples were collected from F2 offspring on days 23 and 40. F2 male mice had no significant differences in alpha or beta diversity on day 23 or day 40. F2 female mice had no significant differences in alpha or beta diversity on day 23, but did have significant differences on day 40 in PCA plot (Fig. 47). Significant decreases in the

relative abundances were observed in *Flavonifractor, Phocea* and an unknown genus within *Bacteriodale*s (Fig. 48).

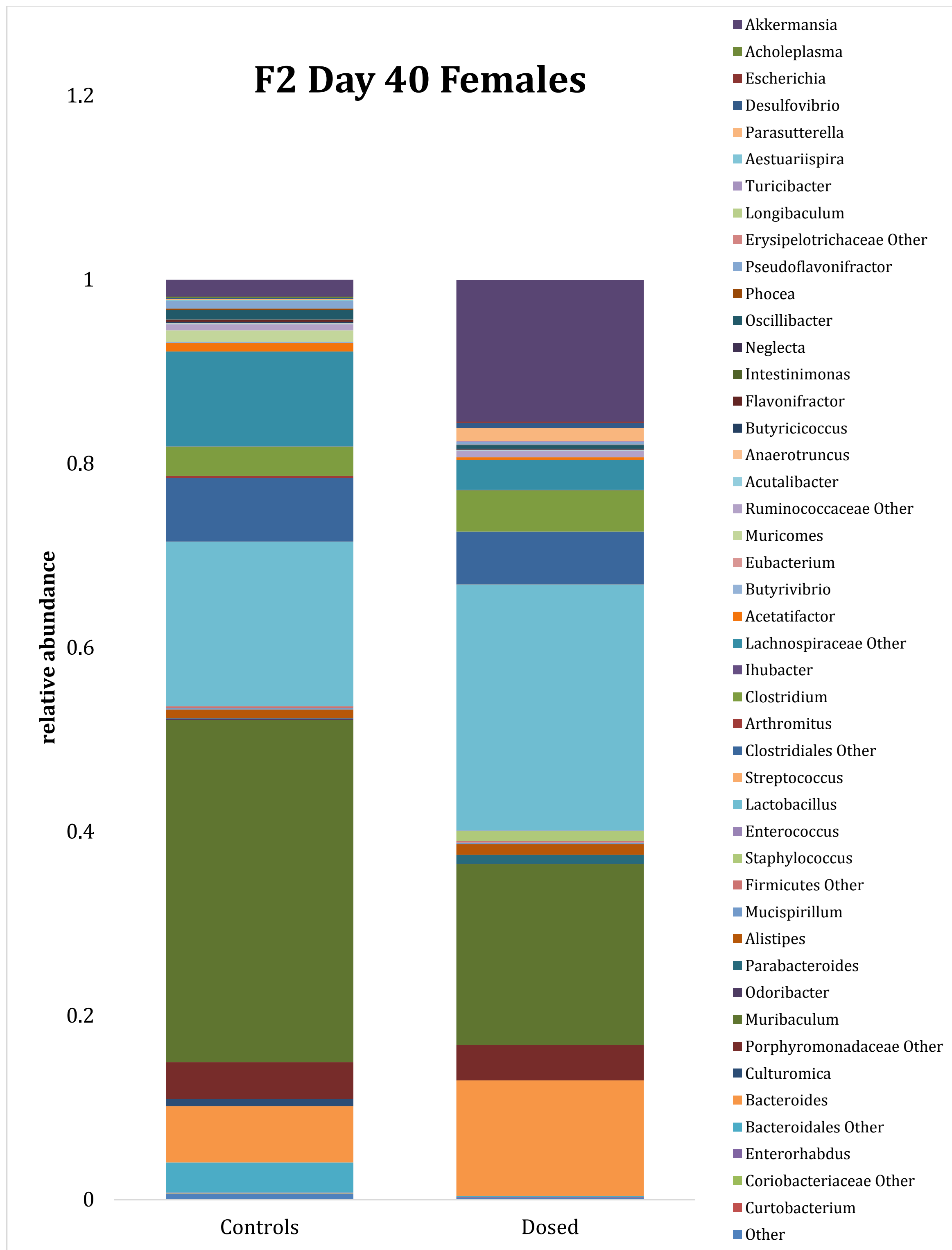

Figure 48: DNA extracted from fecal samples that were collected from F2 females on Day 40 was sequenced to assess the gut microbiome. Significant decreases in the relative abundances were observed in

Flavonifractor, Phocea and an unknown genus within *Bacteriodale*s. Analysis used one-way ANOVA, N=3-5 $p \leq 0.05$.

Discussion

In order to assess the effect of ADBAC+DDAC on the development of the immune system, we assessed immune system function through a TDAR for IgM, IgG, and IgA as well as corresponding changes in the microbiome in a two-generational exposure study.

The TDAR is a measure of antibody production measuring a series of steps: antigen uptake by an antigen presenting cell, such as, a dendritic cell or macrophage, presentation of the antigen to the T-cell, which then activated the B-cell into an antibody producing cell. The TDAR is the gold standard for assessing DIT; and IgM and IgG production are measured frequently (Tonk et al., 2012, Tonk et al., 2015, LeBrec et al., 2014). Our mice were dosed with ADBAC+DDAC from pre-gestation to weaning. This exposes developing mice to several critical immune development windows including initiation of hematopoiesis, stem cell migration and cell expansion, colonization of the bone marrow and thymus, maturation of immunocompetence, and lastly immune memory (DeWitt et al., 2011). Dosing during these windows, when the immune system is most sensitive, allows assessment of DIT at the lowest possible dose (Tonk et al., 2015). When QACs are absorbed into the body, they are excreted unmetabolized in the feces (Thorsteinsson et al., 2003). This places active ADBAC+DDAC disinfectant in contact with the microbiome. Changes to the gut microbiome can affect the developing immune system (Olszak et al., 2012) and increase risk of disease.

Overall ADBAC+DDAC had mild effects on antibody production, mainly in the F2 generation. Significantly altered antibody production was observed mainly in the F2 generation and only in male offspring. Trends toward dosed mice having decreased antibody production was also observed for IgA. These trends, although not significantly different, were supported by similar patterns of response across immunoglobulin type and also by significant decreases in antibody production observed in F2 mice compared to F1.

An exposure to the F0 parents during gestation affects two generations of offspring. The F1 generation is exposed as it develops, and at the same time, germ cells developing within the F1 that will go on to form the F2 are also exposed. Our results showing altered antibody production in the F2 generation suggest that ADBAC+DDAC may have epigenetic effects. DNA expression is regulated by methylation, phosphorylation, and acetylation. Epigenetic changes are heritable changes to DNA or histone modifications that alter gene expression and thus can affect development of the offspring. Our result show altered antibody production in the F2 generation, indicating that ADBAC+DDAC altered the germ cells within the exposed F1. This suggests an epigenetics mechanism for the decreased antibody response. Similar multigenerational effects were observed with ADBAC+DDAC developmental toxicity, where birth defects persisting for two generations following QAC exposure (Hrubec et al., 2017).

Other toxicants have shown multi-generational toxicity as well. It is not uncommon for developmental exposure in utero to have no effect on the F1 generation, but display adverse effects in the F2. Benzo[a]pyrene, a polycyclic aromatic hydrocarbon, has transgenerational

developmental toxicity in zebrafish (Corrales et al., 2013). Zebrafish were only exposed in the F0 generation, and morphological changes were evident through the F2 generation; however craniofacial malformations were only evident in the F2 (Corrales et al., 2013).

Bisphenol A (BPA) is an environmental containment well known for causing reproductive toxicity. In a study examining four generations of reproductive health after BPA exposure in the F0 generation, subsequent generations of mice had delayed vaginal opening and delayed first estrus until the F3 generation (Ziv-Gal et al., 2015). Rats exposed to phenol had decreased litter survival in both the F1 and F2 generations, but the F2 generation was more severely affected than the F1 generation (Ryan et al., 2001). In another study, rats were exposed for 3.5 generations to 4-nonylphenol, a reproductive toxicant. The rats did not show effect in the F0 or F1 generations; however, males in the F2 had decreased sperm health (Chapin et al., 1999). This is another example where toxicity has skipped a generation.

In our study ADBAC+DDAC exposure only altered the antibody production of male mice. Sex differences associated with the immune system are well documented. Typically, in mammals, females have a stronger innate and adaptive immunity which results in better resistance to infections, but higher risks of inflammatory and autoimmune disease; whereas males are more susceptible to some types of cancer (Klein et al., 2016). Additionally, differences in male and female DIT have been studies in lead exposure. Male offspring of exposed rats had increased IL-12 and decreased IL-10, as well as decreased nitric oxide production (Bunn et al., 2001). It is possible that ADBAC+DDAC exposed females were

able to compensate with their stronger innate and adaptive responses to avoid the depressed antibody production.

Our results indicate that ADBAC+DDAC have some adverse effects on the developing immune system. Additional studies are needed to determine the mechanism of the multigenerational responses. Our data supports our hypothesis that ADBAC+DDAC will alter antibody production. The effects were multigenerational and affected males to a greater extent than females.

Altered microbial communities often represent negative health traits and disease. The microbiome is altered in cardiovascular disease, irritable bowel disease, Crohn's disease, immunosuppression, obesity, type-1-diabetes, and multiple sclerosis (Lloyde-Price et al., 2016; Shreiner et al., 2015). At this point, it is not known what comes first, the disease or the altered microbial communities. For example, gut microbial communities that metabolize phosphatidylcholine from the host's diet into trimethylamine-N-oxide can increase the risk of cardiovascular disease as this metabolite is associated with atherosclerosis. The level of trimethylamine-N-oxide was decreased both in vegans and patients recently treated with antibiotics (Shreiner et al., 2015).

The GM is dynamic and can change rapidly. This is particularly true in infants where dietary changes can influence the infants GM even during lactation. Lactation, especially the colostrum, helps seed an infant's microbiome with *Weisella, Leuconostoc, Staphylococcus, Streptococcus,* and *Lactococcus* (Cabrera-Rubio et al., 2012). The

microbial communities found in breast milk can vary as shown in obese mothers with different alpha and beta diversity compared to non-obese mothers (Cabera-Rubio et al., 2012). In our study, the microbiome likely changed as the pups were weaned and transitioning to solid food; however, the mice received the same diets at each time point, so differences in the microbiome should only reflect ADBAC+DDAC effects.

The effects of ADBAC+DDAC on the microbiome were direct and immediate. Within one week of eating dosed food, F0 mice in both sexes showed significant changes to the microbiome. Rapid changes to the microbiome were not unexpected as ADBAC and DDAC are antimicrobials. QACs exposure via injection, inhalation, and ingestion has been tested in animal models for absorption and excretion. QACs do not remain in the blood, lungs, or liver very long and are excreted through the gut (Xue et al., 2004). Furthermore, they are excreted unchanged in the feces (Thorsteinsson et al., 2003). This means they retain their antimicrobial function as they are in the gut allowing them to alter the GM.

Greater differences in microbial communities from ADBAC+DDAC exposure were seen in females. F0 females had decreases in the orders *Bacteriodales* (genus *Culturomica* and an unknown genus) and *Clostridiales* (genus *Flavonifractor*). *Culturomica massiliensis* is a newly discovered bacterium in the *Bacteroidetes* phylum (Ndongo et al., 2016). *Bacteroidetes* tend to be beneficial to the host by helping with host digestion. Decreases in *Bacteroidetes* are associated with Crohn's patients and with obesity (Gevers et al., 2014; Ley et al., 2006; Wexler et al., 2007). Unlike females, F0 males demonstrated an increase

in species from the families *Lachnospiraceae* and *Ruminococcacea*, and a decrease in *Streptococcus acidominum* of the order *Lactobacillales.*

Normally, administrating antimicrobials is associated with a decrease in microbial diversity; however, it is possible the increase in diversity seen in males is the result of an initial decrease followed by re-colonization with additional species. *Lachnospiraceae* and *Ruminococcacea* are both of order *Clostridiales* which have been associated with obesity and high fat diets (Zeng et al., 2016 Lopetusu et al., 2013; Magnusson et al., 2015). *Clostridiales* are found naturally in the microbiome and are introduced by breast milk for humans in the first month of life. *Clostridiales* do have some pathogenic species, but are usually considered commensals and have demonstrated protective effects (Lopetuso, 2013). It is possible that the decrease of *Lactobacillales* in the male mice allowed these new species to colonize. *Lactobacillales* are often used as a probiotic and are considered beneficial or at least harmless (Walter, 2008).

The F1 mice received ADBAC+DDAC exposure indirectly throughout gestation and lactation, but also directly as they transition to solid food prior to weaning. Both males and females F1 mice on day 23 were significantly different in alpha diversity and on the PCA plots with increases in genus *Lactobacillus* and an overall lower diversity. Females had significant decreases in the family *Lachnospiraceae*. Males had significant decreases in *Bacteroidales* genera *Alistipes, Muribaculum, and Clostridium.* The result from the F1 supports the finding that continued ADBAC+DDAC exposure sustains changes into the microbiome. Both male and female F1 mice on day 23 showed an increase in genus

Lactobacillus and an overall lower diversity. The mouse age and recent milk diet could have allowed *Lactobacillus* population to bloom.

In F2 male mice, there was no significant difference in alpha diversity or on the PCA plot indicating that male mice communities recovered after the dose was removed. In F2 females, however, significant differences were seen in the PCA plot, although, alpha diversity was not different. This indicates that the mice dosed in early life had persistent altered microbiomes. The day 40 F2 females had increased populations of Clostridiales, *Verruncomicrobiacea,* and Bacteriodales.

This was not the first time sex differences had been observed in the GM. In germ free non-obese diabetic prone mice, both males and females were equally susceptible to type I diabetes; however, with an intact microbiome, females were more susceptible (Markle et al., 2013). Additionally, transfer of cecal contents from non-obese male mice to female mice could prevented disease onset (Markle et al., 2013). This suggests that the microbiome may interact with the host through sex hormones to affect the immune system in that manner (Markle et al., 2013). Another study in mice suffering from lupus found that *Lactobacillus* increased the amount of Treg cells in the kidney in a sex dependent manner (Mu et al., 2017).

Zaura et al. examined microbiome shifts in adult human feces and saliva after antibiotic exposure at 1, 2, 4 and 12 months. Overall, it was discovered that the salivary microbiome recovered, or stopped being significantly different, in one week to a month. The microbiota

in the feces needed from one to four months, depending on the antibiotic, to recover (Zaura et al., 2015). Our F1 male mice needed at least 19 days to recover from ADBAC+DDAC exposure, this is close to the one month found in Zaura's study. Our mice may have recovered more quickly as they were still growing and the microbiome is more dynamic at younger ages. Overall, it is clear that oral exposure of ADBAC+DDAC to the mouse digestive system results in dysbiosis of the gut microbiome with compositions that favor inflammatory diseases. This dysbiosis persisted though day 40 in the F1 males and females and was intermittent in the F2 females.

Taken together, the changes to the microbiome and TDAR show different patterns of adverse effects from ADBAC+DDAC exposure. ADBAC+DDAC exposure significantly affected the gut microbiome in F1 mice and also significantly affected antibody production in male F2 mice. It appears that alteration in the microbiome are not directly responsible for ADBAC+DDAC DIT since microbial changes to the gut microbe community mostly recovered after the dose was removed, while altered antibody production persisted in F2 males.

ADBAC+DDAC are QACs that are extremely prevalent disinfectants used in households, restaurants, and medical environments. Humans can be exposed orally, transdermally, or by inhalation. If QACs alter the microbiome or immune system in humans, they could be contributing to inflammatory and autoimmune diseases that have been steadily increasing in the past few decades. Further studies should be aimed toxicokinetics to assess the amount of human exposure; and elucidation of the mechanisms of ADBAC+DDAC

toxicity including epigenetic mechanisms due to the multi-generational effects observed in this study.

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IV. Conclusions

Throughout our study, we observed adverse effects of ADBAC+DDAC in both *in vitro and in vivo* models. ADBAC+DDAC exposure resulted in decreased phagocytosis and increased proinflammatory cytokine production in a murine macrophage cell line. This demonstrates the potential for ADBAC+DDAC to directly alter immune cell function and increase inflammation. As immune cells are found in every tissue throughout the body, ADBAC+DDAC exposure may be contributing to a variety of inflammatory diseases in humans.

Results from the developmental immunotoxicity toxicity test in CD-1 mice revealed immunotoxicity in F1 male mice with increased IgG production in the primary response. ADBAC+DDAC exposure did not alter IgA or IgM production in F1 mice but did depress IgM and IgA production in F2 male mice. These data indicate that ADBAC+DDAC exposure effects the ability of the immune system to respond properly. If they induce similar effects in humans, they may result in immunosuppression or other immune disease.

Direct changes in the gut microbiome of ADBAC+DDAC exposed mice were observed in both males and females. Persistent changes in the mouse gut microbiome, however, were rare and only observed in F2 females, indicating that as ADBAC+DDAC are removed from

the environment, the microbiome recovers. The different responses by gender and generation in the microbiome and antibody production indicates that the microbiome may not be the mechanism for immunotoxicity, though it is clear that immune function is altered in CD-1 mice. Dysbiosis is associated with a variety of inflammatory and autoimmune diseases and thus ADBAC+DDAC exposure may contributing to decreased immune health through both direct action on the immune system, and through changes to the microbiome.

The extent of human exposure to ADBAC+DDAC and other QACs is not well quantified. The frequent inclusion of ADBAC+DDAC in commercial products makes human exposure common. The results of this study indicate the serious potential for adverse risk to human health from the frequent use of QACs in products. Further studies should be aimed at determining toxicokinetics to assess the amount of human exposure; and elucidating the mechanisms of ADBAC+DDAC toxicity including epigenetic mechanisms due to the multi-generational effects observed in this study.

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