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Investigating Moringa Oleifera Lam As A Dietary Supplement To Promote Immune Health: A Mouse Model

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Investigating *Moringa oleifera* Lam as a Dietary Supplement to Promote Immune Health: A Mouse Model

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Biographical Sketch

Donja S. Smith was born May 1, 1988, in Alexandria, Virginia. At a very young age Donja S. Smith, had a passion for animals and science. While in middle school, a teacher gave an assignment instructing the class to write a report on what they wanted to be and how they planned to achieve this goal. Donja quickly researched careers that involved animals and science and she narrowed in on becoming a veterinarian. From that point on her sole career path has always been to become a veterinarian.

In 2006, her senior year at West Potomac High School, in Alexandria, Virginia, she applied to several schools with programs in Animal Science. The anticipation was unbearable, waiting for letters to return. Her heart was set on going to North Carolina Agricultural and Technical State University and her prayers had been answered the day she was accepted for the spring semester of 2007.

She graduated May of 2011 with a bachelor's degree in Animal Science at North Carolina Agricultural and Technical State University. Seeking more academic challenge she applied to the graduate program at North Carolina Agriculture and Technical State University. She was accepted for the fall of 2011 into the Food Science program with a concentration in animal health and has gained respect for the scientists that strive to improve this world little by little.

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Key to Symbols/Abbreviations

APC	Antigen-Presenting Cell
B	Bursa
BCRs	B-Cell Receptors
CD	Cluster of Differentiation
CCL_4	Carbon Tetrachloride
CON A	Concanavalin A
DSHEA	US Dietary Supplement Health and Education Act
EDTA	Ethylenediaminetetracetate Acid
EPA	Environmental Protection Agency
ERK	Extracellular signal-regulated kinase
FACS	Fluorescence-Activated Cell Sorting
FDA	Food and Drug Administration
g	Gram
Ig	Immunoglobulin
IM	Intramuscular
JNK	c-Jun N-terminal kinase
LARU	Laboratory Animal Resource Unit
LLNA	Local Lymph Node Assay
MAPKs	Mitogen Activated Protein Kinases
ME	Methanolic Extract
MEST	Mouse Ear Swelling Test
ml	Milliliter

MOL	Moringa oleifera Lam
MT	Moringa Tea
p38	Stress-activated kinase of 38kDa
SBM	Soybean Meal
SI	Stimulation Index
T	Thymus
VDR	Vitamin D Receptor

Abstract

Moringa oleifera Lam (MOL) is a tree, native to the tropics and sub-tropics of Asia and Africa. All parts are edible and because it is rich in vitamins, minerals, and antioxidants it has many nutritional benefits. For example, many of the vitamins and minerals are known to modulate immunity, suggesting that MOL could be a good dietary supplement to boost immunity. Our lab is interested in identifying natural products that can be used to enhance immunity and health among animals. Therefore, the objective of this study was to investigate immune modulation by Moringa using a mouse model. Two trials were conducted to evaluate whether and how consumption of Moringa tea (MT) affects T and B-cell number and proliferation. In each trial, mice were divided into two groups: water (control group) or MT (experimental group). Each group was given fresh water or MT every day for 14 days (trial 1) or 21 days (trial 2). On the final days of the trials (14 and 21), mice were sacrificed and the total number of lymphocytes (T and B-cells) within the spleen and lymph nodes were measured by flow cytometry. In addition, proliferation in response to mitogen-induced activation was compared. Results show that mice consumed MT and water at similar rates during both trials and there were no significant differences in weight gain. There were no differences in the percentages of B-cells or T-cells ($CD4^+$ or $CD8^+$) in the spleen or lymph nodes. However lymphocytes isolated from the spleen of mice that consumed MT had decreased proliferation after stimulation with anti-$CD3^+$ and anti-$CD28^+$ (a specific T-cell activator) but not LPS (a specific B cell activator) as compared to mice that drank water. These data suggest that the tea prepared from the leaves of Moringa may have a modulating effect on the function of T-cells but not B-cells.

CHAPTER 1

Introduction

The public has grown increasingly concerned about how farmers are raising their livestock, specifically food animals. In particular, there is concern over the use of antibiotic growth promoters. Traditional farmers depend on antibiotics not only for treating sick animals but also in a sub-therapeutic capacity, for disease prevention and growth promotion. Sixty to eighty percent of antibiotics used in in the U.S. livestock production are used as sub-therapeutic growth promoters and are incorporated into animal feed (Sapkota et al, 2007). Sub-therapeutic use of antibiotics is thought to be associated with the increase in antibiotic-resistant bacteria and these bacteria can be passed along to people from contaminated food and through occupational exposure (Ahmad et al, 2011).

According to the World Health Organization, bacterial strains are becoming resistant to antibiotics faster than scientists can develop new drugs to eliminate them (Holtcamp, 2011). The problem and concern over antibiotic resistance is the reason many consumers are demanding antibiotic free and organic meats. As of 2012, there are 177 organic meat operations in the U.S., including beef, poultry, lamb, swine, fish and shellfish (U.S. Department of Agriculture, 2013). Consumer retail sales of organic meats have grown from less than $100 million in 2003 to over $600 million in 2008 (Dimitri and Oberholtzer, 2009). Organic livestock production satisfies consumers because it eliminates the health concern with regards to antibiotic residues (Eijck and Kijlstra, 2006). Research to discover natural alternatives to sub-therapeutic antibiotics that can improve health and immunity and help with growth of livestock animals is ongoing.

Plant and plant based products used for medicinal purposes in humans are also being used by farmers and traditional healers to improve the performance and health of livestock (Githiori et

al, 2006). Ethno veterinary medicine is a form of veterinary medicine that uses plants with extraordinary properties as treatment (Benitez et al, 2011). Certain plants in Afghanistan and Pakistan have medicinal properties in animals. For example, *Gambila* is a plant used to treat gastrointestinal worms and *Korai* is a plant used to treat trauma and coughing (Davis et al, 1995). Ritter et al (2012) surveyed the residents in Brazil to categorized and study the uses of veterinary phytopharmacology. The survey revealed a total of 56 naïve plants used for various medicinal purposes on domestic animals such as dogs, cats and livestock. Alawa et al (2001) surveyed the herdsmen of northern Nigeria of their indigenous practices using plants and plant products on livestock. The survey revealed 18 of the most common plants used to treat 21 of the most common animal diseases. The long-term goal of the lab is to find natural solutions for current and emerging health of animals and disease issues by identifying plant based products that promote health by enhancing immunity.

Moringa oleifera Lam (MOL) is known by many different names, such as horseradish, drumstick tree, rawag (Arabic), la ken (Chinese) and morungue (French) (Roloff et al, 2009). This tree's native territory is very broad; it includes South Asia, tropical Africa, Central America and the Caribbean. Fuglie (1999), showed the use of MOL leaf powder as a nutritional lifesaving substance in relation to starvation in developing countries. The multipurpose uses of MOL are not uncommon to the native people in these countries. MOL possess a wide variety of medicinal functions (Table 1), such as, anti-microbial, anti-tumor, anti-diabetic, anti-ulcer, anti-hypertensive, hepatoprotective and anti-inflammatory activities (Devaraj, 2011). MOL is rich source of vitamins, minerals and antioxidants that promote health and have modulating immune responses. In this study a mouse model was used to evaluate the immune modulating effect of

Moringa, specifically focusing on its effect on lymphocytes. The knowledge gained from this experiment will be used to design studies in livestock animals.

CHAPTER 2

Literature Review

2.1 Antibiotic Resistance

The use of antibiotics on food animals has been practiced for years. Antibiotics are used typically when an animal shows signs of an infection and under these circumstances using antibiotics are not an issue. However, traditional farmers depend on antibiotics not only for treating sick animals but also for disease prevention and growth promotion. In this instance when an animal is sick, the entire herd/flock is treated to minimize a potential spread of the illness. Society has been increasingly concerned with how farmers and food animal production companies care for their animals prior to being sent to the meat processing plants, and there is greater consumer demand for organic meats. Recent articles state that antibiotics used on food-producing animal could be the cause of antibiotic resistant food-borne bacteria (Sapkota, 2011).

Antibiotic residues can remain in the muscle of the animal. When the animal goes to slaughter, those residues can be present in the meat. When consumers ingest these meats with antibiotic residue it can increase the risk of generating antibiotic resistant bacteria in the intestines of the person who consumed the meat. This contributes to the growing problem of antibiotic resistant bacteria and ultimately resulting in antibiotics not working properly making it harder on medical doctors and veterinarians to treat sick humans and animals. Sub-therapeutic use of antibiotics has increased antibiotic-resistant bacteria. According to the World Health Organization, bacterial strains are becoming more resistance to antibiotics, faster than scientists can develop new drugs to eliminate them (Holtcamp, 2011).

Githiari et al (2006), reports that various plants and plant products have been used to treat parasitism in developing countries by farmers and herders. To treat animals for gastrointestinal

parasitism, seeds or foliage of garlic, onion, mint, walnuts, dill or parsley are used. Bharati and Kumar (2013) report the findings of multiple plants being used for medicinal purposes with livestock in the local villages of Uttar Pradesh, India. Some of the plants were: Kakai, used to treat blood in urine via oral administration; Pyaj, used to treat mastitis and anorexia via oral administration; Tulsi, used to treat conjunctivitis via eye drop. The use of plants and plant products for nutritional and medicinal purpose are a common practice in developing countries (Table 1). *Moringa oleifera* Lam is a tree that is well-known for its multiple health benefits, which could be an ideal feed additive to promote health in animals.

2.2 *Moringa oleifera*

Moringa oleifera Lam (MOL) is a tree that is indigenous to the tropics and sub-tropics of Asia and Africa. This tree is known by over 200 names, the most common names are miracle tree, horseradish tree and drumstick tree. This tree has been used in developing countries for centuries for nutritious and medicinal purposes. Every part of the tree is edible and varies in properties as described in Table 2 and its nutritional content is plentiful as described in Table 3. Manaheji et al (2011) evaluated the analgesic properties of methanolic extracts MOL leaves on arthritis rats and concluded that there was pain reduction.

Table 1

Herbal Uses in Small Ruminants, Cattle, Swine and Poultry

Plant	Purported Uses
Crushed Garlic (in food) or Coconut Water (in drinking water)	Enhances Appetite
Lime/Lemon/Sour Orange Juice (in drinking water)	Respiratory/Heat Stress
Aloe Vera (gel or leafs in drinking water)	Enhance Livability
Kojo Root, Cedar, Black Sage	(In bedding) Repels External Parasites
Goldenseal Root, Black Cohosh Rhizome	Uterine Infections

Table 1

Cont.

Motherwort Leaves	Increases Milk Production
Flaxseed	Aids in Conception and Pregnancy Support
Wild Raspberry Leaf	Pregnancy Support
Ginger Root Extract	Car Sickness
Chamomile	Reduces Anxiety
Garlic + Mullein	Ear infections
Calendula	Abrasions
Goldenseal Tea	Eye infections

(Lans and Brown, 1998) (Lans et al, 2009)

Table 2

List of Some of the Nutritional and Medicinal Properties of MOL

Medicinal Uses of Moringa	**Part of Tree**
Urinary Tract Infection	Leaves
Common Cold	Fruit, Root, Bark
HIV-AIDS	Leaves
Parasites (Helminthes)	Leaves, Fruit, Pods
Bronchitis	Leaves
Fever	Leaves, Root, Gum, Seeds
Asthma	Root, Gum
Anti-tumor	Leaves, Fruit, Seeds, Bark
Diarrhea	Leaves, Root
Lupus	Oil (from seeds)
Headache	Leaves, Roots, Bark, Gum
Energy	Leaves, Seeds, Oil (from seeds)
Protein	Leaves, Seeds
Birth Control	Bark
Gout	Root, Oil (from seeds)

(Fahey, 2005)

Moyo et al (2012) investigated antioxidant potential of MOL leaves in different *in vitro* systems using standard phytochemical methods by using MOL as a dietary supplement for goats. The results suggested that MOL could be a potential source of compounds with strong

antioxidant potential as all parts of MOL were rich in potassium, magnesium and calcium; low levels of selenium were only detected in whole seeds.

Table 3

Nutritional Values of Moringa Dry Leaves in Comparison to Common Foods

	Common Foods Nutritional Value	**Moringa Dry Leaves Nutritional Value***
Calcium (Milk-Whole)	0.276/g	2.003/g
Potassium (Bananas)	0.0008/g	28.2/g
Vitamin C (Oranges)	0.422/g	1324/g
Iron (Spinach)	0.0511/g	0.0173/g
Vitamin A (Carrots)	3×10^{-6}/g	0.0189/g
Protein (Yogurt)	11/g	27.1/g

Shah, Shakeel Ahmad. "Moringa – The Miracle Tree." Healthy Manners. N.p., n.d. Web. 9 Oct. 2013. http://healthymanners.com/moringa-the-miracle-tree/ *Comparison of 100g of dry leaves

Hamza (2010) evaluated the effect of MOL seed extract on liver fibrosis on rats via oral administration. Results concluded that MOL seed extract can act against carbon tetrachloride (CCl_4)-induced liver injury and fibrosis in rats; suggesting its antioxidant and anti-inflammatory effect on reducing the effect on liver stellate cell activation.

Mendieta-Aracia (2011) investigated the effect of MOL leaf meal as a source of protein in milk yield and milk composition in dairy cows in tropical areas. Results showed MOL leaf meal to be a successful replacement of commercial constituents in concentrate for dairy cows. Aregheore (2002) investigated the intake and digestibility of MOL and batiki grass mixtures of growing goats. The results stated that the mixture at 20% and 50% levels of total daily forage allowance could be used as an inexpensive protein supplement in combination with batiki grass for goat based diets.

These studies show that Moringa has the potential to be used to enhance animal health. However, limited research has focused on whether and how Moringa modulates immune responses. Therefore, for the purposes of this study, we will focus on the high content of vitamins and minerals and its potential to modulate immune responses, specifically those of lymphocytes. Immunity and lymphocyte function, in correspondence with the effect of vitamins and minerals that are found in high quantity in Moringa that have been shown to affect lymphocyte function will be presented.

2.3 Immunity

The word immunity is derived from the Latin word *immunis*, meaning exempt. According to Coico (2009), "immunity refers to all the mechanisms used by the body as protection against environmental agents that are foreign to the body". Thus the role of the immune system is to protect the body from foreign organisms that invade the body to cause harm or damage to the body. The immune system works on a self/non-self-recognition mechanism; therefore if any foreign organism were to enter the body, the body would recognize this organism as non-self and immediately try to eliminate it (Coico, 2009). Non-self would include everything from environmental to physical factors that could affect the immune system and its responses.

Immunity can be classified as innate or adaptive/acquired. Innate immunity is an antigen non-specific, response that occurs minutes to hours after exposure and has no memory. The cells associated with the innate immune system are mast cells, phagocytes, macrophages, neutrophils, dendritic cells, basophils, eosinophils, natural killer cells and gamma delta (γδ) T-cells. Innate immunity also consists of natural barriers such as skin and mucous membranes. In contrast to the innate system, the adaptive/acquired immune system is found only in vertebrates, it is antigen specific, slow in response that occurs days to weeks after exposure and has memory. This type of

immunity is composed of lymphocytes, secreted molecules and antigen recognition molecules such as T and B-cells.

B-cells

B-cells were given their name after early experiments with birds, when an organ called the Bursa of Fabricus that synthesizes antibodies was discovered. B-cells in mammal are produced from the primary lymphoid organ, the bone marrow, and travel through the blood and are stored in the secondary lymphoid organs such as the spleen and lymph nodes. After B-cells have matured in the bone marrow and they remain in peripheral tissues until an antigen invades the body. B-cells main function is to produce membrane bound immunoglobulin, and recognize pathogens through B-cell receptors (BCRs) (Coico, 2009).

Antigen activation in B-cells requires two signals. The first activation signal occurs when an antigen binds to B-cell receptors (BCRs) and the antigen is engulfed by receptor-mediated endocytosis, digested and major histocompatibility complex (MHC) II molecules, such as dendritic cells and macrophages, on the B-cell surface. The second activation signal occurs via either a thymus-dependent or a thymus-independent mechanism. As results of activation, B-cells proliferate and differentiate into memory B-cells or plasma cells that secrete antibodies. These antibodies then seek to bind to the pathogen to assist in neutralization and clearance by phagocytes, such as macrophages and dendritic cells (Parham, 2009).

Lipopolysaccharide (LPS) is a strong activator of the immune system and inflammatory responses. It has been found to be an effective B-cell mitogen causing non-specific polyclonal B-cell activation (Bucala, 1992; Kim, 2006; Sabarinto, 2013). When murine B-cells are incubated with LPS they undergo proliferation and secrete of polyclonal antibodies (Bucala, 1992).

B-cell function can be affected by nutrition. If the nutritional value of an animal's diet is in some way less than efficient or lacks quality the immune system will not function properly (Munoz, 1995). It's extremely important to have functional B-cells because otherwise antibody production would be inefficient and the animal would correspondingly become ill quite frequently until its immune defenses were completely defeated. For example, B-cell function is essential for certain diseases. Mucosal Disease is an infectious disease that affects calves. Clinical signs involve bloody diarrhea, erosions of the mucosal membranes and if the disease isn't treated early on calves could die within 1-2 weeks. From an immunological aspect, this disease modifies the lymphatic tissues and immunohistochemical evidences shows that this disease disturbs the quantity and production of B-cells in the Peyer's patches.

T-Cells

Thymus cells are called T-cells because they fully mature in the thymus rather than the bone marrow, like B-cells. The thymus is also a primary lymphoid organ that is especially important to the development of T-cells. The thymus is a butterfly-shaped organ that resides directly above the heart. The role of T-cells is to respond quickly and effectively to pathogens such as, viruses, bacteria and parasites that may be harmful to the organism that is exposed (Coico, 2009).

Cluster of differentiation (CD) are subsets of cellular surface receptors (epitopes) that are expressed on cells as can be used to identify cell type, their stage of differentiation. There are many different clusters that are expressed on the surface of T and B-cells. Some are specific to the certain cell types for example all T-cells express CD3, helper T-cell CD4, and T-cell CD8 and B- cells CD19.

$CD3^+$ and $CD4^+$, also known as helper T-cells are cells that secrete proteins that aid in defending the organism against pathogens. Helper T-cells only recognize antigen in the Class II MHC markers. An antigen-presenting cell (APC), such as a macrophage or a dendritic cell breaks down the antigen it devours, and then it places small pieces (peptides) on its surface along with a Class II MHC marker. APCs then enable specific receptors on helper T-cells to bind the antigen and confirm that an invasion has occurred. After binding, an inactive helper T-cell quickly becomes an activated helper T-cell it proliferates and secretes cytokines. These cytokines send signals to other immune cells. For example cytokine signals to B-cells help to increase the number of specific antibody to better eliminate the antigen.

$CD3^+$ and $CD8^+$, also known as cytotoxic T-cells, kill foreign cells and cells that are infected with viruses. Killer T-cells only recognize antigen in the Class I MHC markers. An inactive cytotoxic T-cell recognizes virus fragments, which are displayed by a macrophage in combination with a Class I MHC marker. A receptor on an inactive cytotoxic T-cell ($CD8^+$) recognizes the antigen-protein complex and binds to it. Since the surfaces of other infected cells have the same virus fragments in combination with Class I MHC markers, once activated cytotoxic T-cells proliferate, attack, and destroy the virus infected cells (Coico, 2009).

2.4 Vitamins, Health, Disease and Lymphocyte Function

Vitamins A, C, E are antioxidant molecules that assist in the protection against free radicals that enter the body and could damage cells. Antioxidants, vitamin A, C, E, are plentiful in the leaves and oil from MOL. A study evaluated the content of antioxidant properties in MOL and concluded that the aqueous and acetone extracts of MOL have antioxidant properties in the presence of polyphenolic compounds; Meaning that a diet that contains MOL could protect an

organism against disease induced by oxidative stress (Moyo, 2012). Another study of the same purpose concluded that 70% ethanol extracts of MOL produced the highest content of antioxidant activity. These vitamins also have been shown to affect the function of T and B-cells. Proper nutrition is essential to being healthy and an improper nutritious diet can cause dysfunction of the immune system and being more vulnerable to infectious diseases. Vitamins such as A, C, E and D are all molecules that hold great importance to nutrition and immune function.

2.4.1 Vitamins A. Vitamin A is essential for vision, reproduction, maintenance of epithelial surfaces, immunity and cell differentiation and development (Debier, 2005; Hanekorn, 1996).Vitamin A-derived medicines have been used to treat acne, Psoriasis and skin cancer prevention (Chapman, 2012).Vitamin A increased mitogen-induced lymphocyte proliferation and cell-mediated cytotoxicity was enhanced in mice that received a vitamin A supplemented diet (Chew, 1996). Retinoic acid, also known as, vitamin A is a lipid soluble molecule that aids in the function of sight and a regulator of macrophage function and cytokine production. Vitamin A deficiency can cause various effects to the immune system as well as other functions of the body systems. Apagar (1995) stated that vitamin A deficiency during pregnancy is parallel to improper development of fetal immune function. Yang et al (2009) sought to find the effects of vitamin A deficiency on mucosal immunity in rats to an intestinal infection. Results concluded that vitamin A deficiency affects the mucosal immune response against infection. Vitamin A stimulates the growth of organs, however if there is a vitamin A deficiency the growth of organs, including lymphoid organs are depressed, the production of B-cells are increased and T-cell production is suppressed (Liao et al, 1996). Adaptive immune responses to vitamin A deficiency can decrease the lymphocyte affinity, IgA and helper T-cell responses (Duriancik and Hoag, 2010).

Sailstad et al (1995) conducted a study focused on the effect of vitamin A diet supplementation to increase the sensitivity on two assays, local lymph node assay (LLNA) and mouse ear swelling test (MEST). These two assays are important to evaluate the skin contact sensitivity to repeated chemical exposure to products. These products would include pesticides, textile dyes, cosmetics, skin care products and cleaning supplies. MEST measures the elicitation phase of hypersensitivity to contact and LLNA uses T-cell proliferation in the draining lymph node caused by contact sensitizers to predicate the contact sensitivity of chemicals. Previous studies have indicated that a vitamin A diet increases the quantity and efficiency of APCs in the skin and lymph node, thus an increase in T-cell proliferation. Therefore, the scientists sought to investigate the effect of a vitamin A diet on a mouse model on the LLNA to improve sensitivity. This study concluded that vitamin A supplementation increased T-cell proliferation, therefore improving the sensitivity of the LLNA.

2.4.2 Vitamin C. Vitamin C (ascorbic acid) is essential for the forming of critical proteins to make skin, tendons and ligaments. Vitamin C is also an antioxidant and aids in fighting against free radicals that cause harm to the body. Its properties include promoted proliferation and inhibiting apoptosis of T-cells (Jeong, 2010). Vitamin C supplementation may improve responses to antibody production and cell-mediated immunity. A study showed reduced bursal lymphocytes and improved antibody response to infectious bursal disease in chickens that was related to vitamin C supplementation in diet (Wu et al, 2008). Vitamin C alters immune responses toward Th1 helper T-cells (Jeong, 2011). Deficiency of vitamin C can induce the onset of a disease called scurvy that is common only among humans, guinea pigs and monkeys. The signs of scurvy are improper formation of bones, lethargy, anemia and lesions in the oral cavity

and skin. The deficiency of vitamin C depresses the T-lymphocyte response to respiratory disease in cattle and other species (Hemila and Douglas, 1999).

Maeng (2011) suggests that vitamin C increases T-cell proliferation when injected intramuscular in higher than recommended daily intake doses. However, the study showed that high doses of vitamin C lowered cell viability and cytokine secretion. Although, Noh (2005) also shared similar ideas of vitamin C and its ability to increase proliferation of T-cells and inhibit cell death. It was concluded that high doses of vitamin C actually only affect activated T-cells because they seem to be more vulnerable to change and cause a shifts in the immune system in mice toward Th1 T-cells.

2.4.3 Vitamin D. Vitamin D is a lipid soluble molecule that assists in calcium homeostasis and bone formation. Calcitriol receptor, also known as, Vitamin D receptor (VDR) is an immune system regulator, suggesting that vitamin D plays a role in the development and function of T-cells by inhibiting the development of Th1 cells *in vivo* and the inhibiting of the Th1 mediated response helps to protect against disease pathology (Cantorna, 2006). VDR is present on almost all immune cells, such as, T-cells, B-cells, neutrophils and APCs. Pludowski (2013) states that the amount of VDR expressed are in relation to the degree of T-cell activation and modulate the response of helper 17 T-cells. Vitamin D deficiency can lead to certain illness and diseases such as osteoporosis, muscle weakness, increase risk to auto immune disease and infection.

DeLuca (2011) hypothesized that vitamin D deficiency could be the direct cause of the onset of various diseases, such as, multiple sclerosis, type 1 diabetes, rheumatoid arthritis and lupus. They sought to prove the theory that vitamin D deficiency increased the symptoms,

severity and onset of experimental autoimmune encephalomyelitis in mice. Results showed that supplementation of vitamin D suppressed the symptoms of experimental autoimmune encephalomyelitis and delayed the onset in mice. Van der Stede (2001) conducted a study and sought to evaluate the immunomodulatory activity on antibody responses in pigs when intramuscular (IM) immunized with the active metabolite of vitamin D, 1,25-dihyroxyvitamin D_3. They were interested in the mucosal protection (saliva, feces and nasal) via the secretion of IgA antibodies. IgA antibodies fight against harmful microorganisms that come in contact with the mucosae. The results concluded the IM immunization of 1,25-hydroxyvitamin D_3 enhanced antibody production of IgA and IgM in the mucosae of the mouth, nasal and intestines.

2.4.4 Vitamin E. Vitamin E is an antioxidant in the blood that reduces lipid free radicals and protects tissues from free radicals. Vitamin E regulates humoral immunity via production of IL-2 cytokines (Chew, 1996). Vitamin E has many biological functions, the antioxidant function, enzymatic activities, gene expression, and neurological function and cell signaling. Supplementation of vitamin E has been reported to enhance growth, reproduction, prevention of diseases, and protectant against oxidative stress in newborn ruminants and enhances antibody production in response to immunizations (McDowell, 1996; Debier 2005; MacGlafin, 2011). Maternal colostrum in ruminants has high vitamin E concentration and is essential for newborn ruminants that usually are born with low levels of vitamin E (McDowell, 1996; Debier 2005). The main issue in animals that are vitamin E deficient is tissue degeneration, also known as, white muscle disease. White muscle disease's clinical signs are stiffness, weakness, rapid breathing, infertility, severe increase appetite and polyarthritis.

Anugu et al (2013), reports that the period of transition is the most vulnerable time during pregnancy in sheep and cattle. During this time antibody response and cytokine production is

reduced. This study was conducted to evaluate the immune response with intramuscular (IM) injections of vitamin E on pregnant and lactating sheep. The study proved that supplemental vitamin E increased serum IgG concentration and a potential treatment for immune suppression for sheep during the transition period. MacGlafin et al (2011) sought to resolve the gastrointestinal nematode infection in sheep in the United States, since the current anthelmintic control has become resistant to majority of the parasite populations. Based on previous studies on mice models, they believed that vitamin E supplementation enhanced antibody production in response to immunization. Parasite populations was observed in sheep after administering vitamin E injections of either 5ml or 10ml for 2 weeks, however there was no effect on parasite populations. Similar studies have been conducted in relation to vitamin E supplementation to evaluate immunological effects. Albers (2005) analyzed the immunomodulatory effect when a mouse was given vitamin E via injections, Giadins (2000) investigated vitamin E effect on antibody production of sheep vaccinations against Chlamydia infection, and Hossain et al (1998) evaluated the effects of vitamin E supplementation on performance and immune response in poultry. Unfortunately, all studies had the same results of little to no effect. Beharka et al (1996) reported that aging is linked to the decline in T-cell-mediated immunity, including the decrease of interleukin IL-2 production and mitogen-induced T-cell proliferation. It was hypothesized that increased production of prostaglandin E2 would decrease T-cell function with aging and that decrease in prostaglandin E2 production by dietary antioxidants would enhance T-cell-mediated function. They concluded that vitamin E improves T-cell responsiveness in old mice.

2.5 Minerals on Lymphocyte Function, Health and Disease

Minerals are essential molecules for the maintaining of a healthy balanced immune system. Macrominerals are minerals that the body needs in large amounts such as calcium,

phosphorus, magnesium, sodium, potassium, chloride and sulfur. Microminerals are minerals that are only needed in small amounts such as iron, manganese, copper, iodine, zinc, cobalt, fluoride and selenium.

Zinc is thought to be one of the leading elements that assist the correct functioning of tissues, organs, systems and immune response (Mocchegiani, 2000). Zinc is present in the following foods oysters, dark chocolate, lamb and crabs. Zinc is essential for the proper function of T-cell mediated immunity and the balancing between Th1 and Th2 cells (Siberry, 2002). Zinc is a micromineral that has antioxidant properties, that is involved with the regulation of the endocrine system and is essential for basic cellular function on all stages of the cell cycle (Caine, 2009). Zinc is also known for its immune boosting properties that aid in wound healing, prevention of disease in dairy cattle, reduces the risk of mastitis, abortion, lameness and mortality, in sheep zinc improves growth performance and feed efficiency and in weaned pigs zinc improves growth performance and reduces post-weaned diarrhea (Caine et al, 2009; Kendall, 2012; Anderson et al, 2012). Mandal et al (2007) states that zinc is needed to regulate activity of lymphocytes and supplements of zinc into diets increase antibody production.

Iron has various essential functions that are needed to maintain homeostasis, such as, the ability to make hemoglobin and myoglobin for red blood cells and muscles. Iron is present in the following foods red meat, egg yolks, collards, turkey and beans. Iron also has the role of carrying and storing oxygen in the body. Lack of the proper amount of iron in the body will lead to immunological dysfunctions of T-cell lymphocyte and cytokine production (Bergman et al, 2004).

2.6 Preliminary Data

Moringa possesses high amounts of vitamins and minerals, as illustrated in Table 3 that are known to effect lymphocyte function. Therefore, we investigated the immune modulatory effect that a diet supplemented with MOL would have on lymphocytes, specifically T-cells and B-cells. A preliminary study on the immune modulating effects of Moringa, conducted by a rotating student in the lab, Micheal Locklear, was aimed at optimizing a mouse model. The objectives were to optimize the preparation and delivery of Moringa Tea (MT), determine if mice would drink it, and assess white blood cell levels in the blood. Mice consumed MT at a rate similar to water and gained weight at a similar rate (figure 1 and Figure 2). We also compared the number of leukocytes in the blood of the mice (figure 3). In figure 3, blood analysis suggested that there was an increase in the total white blood cell count (figure 3a) and the number of lymphocytes in mice that consumed MT (figure 3b).

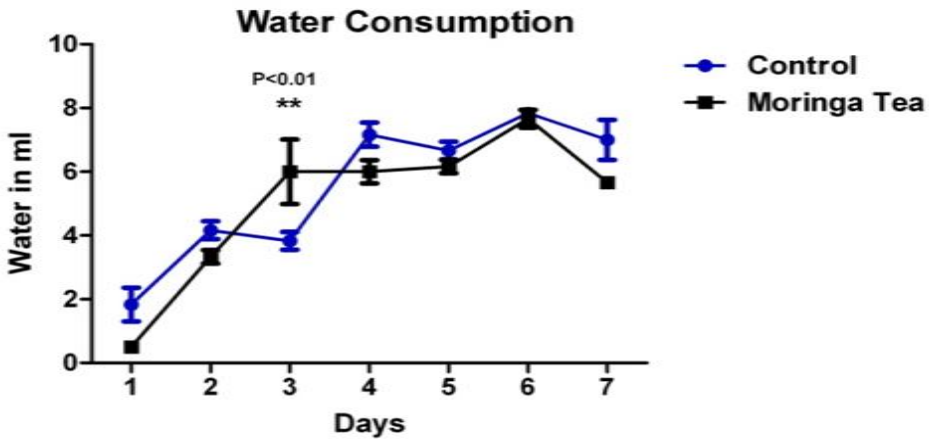


Figure 1. Assessment of Water and Moringa Tea Consumption.

In figure 1, 12 female wild-type Balb/c mice between 5-8 weeks of age were offered MT for a total of 7 days. Total average consumption was calculated to find average daily

consumption per mouse by dividing the amount consumed by the number of mice per cage. Data are an average of 6 mice per group.

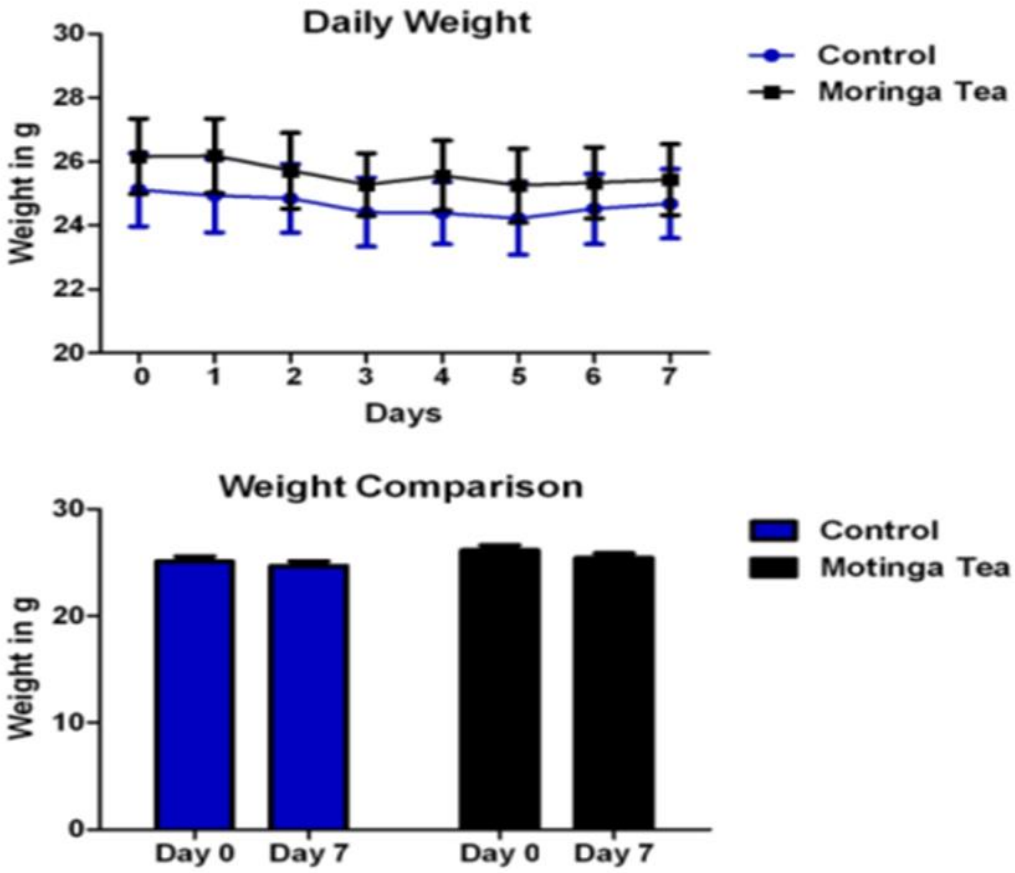
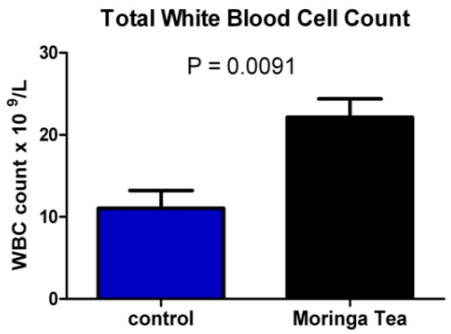


Figure 2. Comparison of Weight Gain.

In figure 2, all mice were weighed daily on an Arbor 1605 electronic balance. Data are an average of 6 mice per group. Two-way ANOVA analyses were used to determine significance.

A.



B.

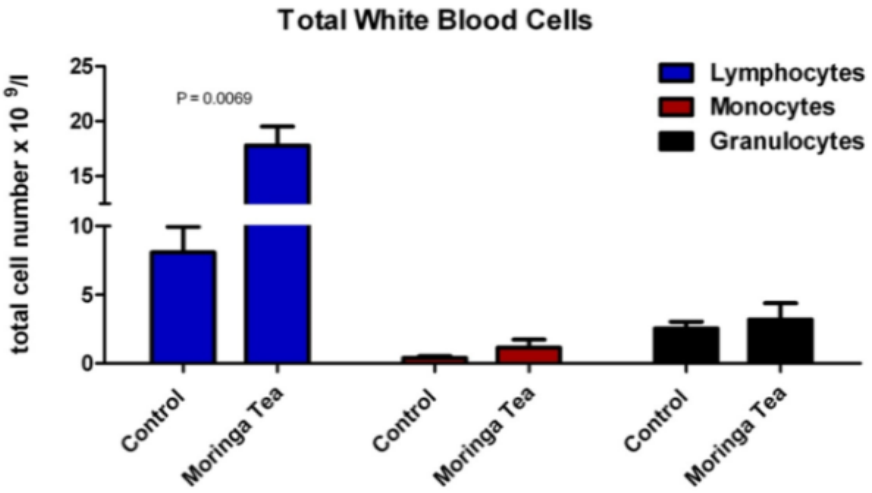


Figure 3. Comparison of Total White Blood Cell Count.

As figure 3 shows, (A) Blood was collected from all 12 mice via cardiac puncture into tubes containing EDTA to prevent coagulation. Each blood sample was run in a Vet Scan HMII to determine the total white blood cell count and percentage of lymphocytes, monocytes and granulocytes. (B) Differential cell counts on leukocytes were performed on stained blood smeared slides. Hundred total cells were counted under a light microscope under oil immersion. Cells were differentiated based on color and morphology. Data is an average of an n=6 for control and n= 4 for Moringa tea. An unpaired two tailed student t-test was used to determine significance.

The MT in this study was prepared in a large batch and was not replaced on a daily basis. As a result bacteria accumulated in the bottle during the 7 days trial period. Therefore, we could not determine whether the Moringa was causing the increase or if the increase in T-cells was in response to the bacteria. Furthermore, our analysis did not distinguish the type of lymphocytes (B-cells vs. T-cells). Therefore, for the current study, we sought to further investigate whether MT can be used as a supplement to improve the health of food animals and specifically expanded our investigation of its effect on lymphocytes (B-cells and T-cells). Using flow cytometry, allowing for specific cell detection, and refreshing MT daily. We hypothesized that the consumption of water infused with MOL would increase the lymphocyte production and function. Our specific aims are:

1. Determine the influence of Moringa Tea on lymphocyte numbers *in vivo* using flow cytometry.
2. Measure the effect of Moringa Tea on lymphocyte proliferation *ex vivo*.

CHAPTER 3

Materials and Methods

3.1 Animals

The mice used in this study were housed in the Laboratory Animal Resource Unit in Webb Hall on the campus of North Carolina Agricultural & Technical State University. This study was approved by the Institutional Animal Care and Use Committee. The mice used were given a standard commercial rodent chow (Purina 5001) throughout the study. For this study, a total of 12 6-8 week old female Balb/c mice (Harlan Labs, Bethesda, MD) were used for the two trials. The mice were divided into two groups. The control group received only water and the experimental group received only Moringa Tea.

3.2 Preparation of Moringa Tea

Dried Moringa leaves were harvested from North Carolina Agricultural and Technical State University Farm at the mature stage of the growth (at least 24 inches in height) and used to prepare Moringa Tea. Forty grams of dried Moringa leaves were boiled in 2L of distilled deionized water for 30 minutes. After the 30 minutes, MT was filtered through cheese cloth to remove large particles of MOL and then again through a funnel lined with filter paper into a 2L flask to remove the smaller particles. Based upon the preliminary study and additional filtration was added. To ensure all particles were removed, the potential of bacterial growth was minimized and MT was sterilized, a final filtration was conducted using 0.22 micron 250-mL vacuum sterile filtered bottles. MT was poured from the flask into sterile 50ml tubes under a tissue culture hood and then stored in the freezer at -20◦C until used.

3.3 Monitoring of Weight and Liquid Consumption

The mice for the two trials were randomly subdivided into two groups, control and experimental. Mice were given 100-mL of fresh water or 100-mL of fresh MT for 14 (Trial 1) and 21 (Trial 2). Daily weights and liquid consumption were recorded. An Arbor 1605 electronic balance was used to record all weights and feed consumption. Weights were recorded daily for 14 and 21 day studies. Water and MT consumption were measured using graduated cylinders and recorded daily for all studies on MT or water remaining in the bottle at the time of change. Graph Pad Prism software was used to generate graphs of the data.

3.4 Analysis of Moringa Tea

A 250-mL sample of MT from trial 2 was sent to the Center for Excellence in Post-Harvest Technologies in Kannapolis, NC for its quantitative nutritional values. The analysis of MT involved evaluation of caloric, mineral, vitamin, fat and sugar values. All methods or procedures were conducted using Association of Analytical Communities (AOAC) methods or modified (MOD) methods.

3.5 Necropsy

On day 14 and 21 the mice were asphyxiated by CO_2 inhalation. A ventral midsagittal incision was made and the superficial dermal layer was peeled back and pinned down. The lymph nodes from the inguinal, brachial, axillar and superficial cervical regions of the body were harvested. Another incision was made on the visceral dermal layer and the spleen and mesenteric lymph nodes were harvested.

3.6 Preparation of Cell Suspensions

Single cell suspensions from the lymph nodes and spleens were prepared via mechanical dissociated using the plunger of a syringe. The cell suspension was passed through a 70μm cell

strainer with complete Roswell Park Memorial Institute (RPMI) media, centrifuged. Complete RPMI is a media that contains vitamins, glucose, salts, amino acids, glutathione, a pH indicator and Fetal Bovine Serum. However, this media contains no protein or growth promoting agents, which is essential for cell and tissue culture. The cell pellet was re-suspended and centrifuged again. Cells were re-suspended in RPMI media and a viable cell count determined using a hemocytometer via trypan blue exclusion. The cells were suspended in complete RPMI at a concentration of [$1x10^7$/ ml].

3.7 Flow Cytometry

Cells were stained with FACS antibodies [CD3-FITC, CD4-PE, CD8-APC, CD19-PerCP cy5.5 and CD49b-PE-cy7 from eBiosciences (San Diego, CA, USA) and collected and analyzed on an Acurri C6 Flow cytometer to determine the amount and type of lymphocytes that are present in the spleen and lymph nodes. Using a 96-well rounded bottom plate, 50µl of cell suspension was deposited into each well. The plate was centrifuged for 2 minutes at 2,000 rpm, and then the supernatant was discarded. Twenty microliters of the blocking reagent, Fc-Block, was added to prevent non-specific binding of FACS antibodies to the lymphocytes. After 20 minutes of incubation on ice, each well was added 50µl of an antibody cocktail incubation for 30 minutes in darkness on ice. As illustrated in Table 3 (Appendix D), each antibody cocktail consisted of three or more antibodies and FACS buffer (PBS, 5% Fetal Bovine Serum, 0.1% Sodium Azide). Two hundred microliters of FACS buffer was added to each well and plate was centrifuged twice. Fifty microliters of 2% paraformaldehyde was added to each well and incubated for 10 minutes. FACS buffer of 200µl was added to each well and plate was centrifuged as described twice for the final wash. Data was then acquired on the flow cytometer collecting 10, 000 events per sample.

3.8 Proliferation Assay

The effect of Moringa Tea on proliferation of lymphocytes was determined using the CELLTITER 96 AQueous ONE KIT from Promega (Madison, WI, USA) per manufacturer's protocol. Briefly, lymphocytes isolated from the lymph nodes and spleen were stimulated in the presence and absence of T-cell stimulators anti-$CD3^+$ and anti-$CD28^+$, CONA and B cell stimulator, lipopolysaccharide (LPS) at a concentration of 1 µg/ml and incubated at 37°C in a humidified, 5% CO_2 atmosphere. At the 66-72 hour time point the 10µl of solution reagent from the CELLTITER 96 AQueous ONE KIT was added to each well and incubated at 37°C for 4 hours in a humidified CO_2 incubator. The plate was read in a Versamax 96-well plate reader at an absorbance of 490 nm using a reference wavelength in the range of 650nm. The level of proliferation was determined by calculating the stimulation index (SI) using the following equation. SI = OD value of stimulated cells / OD value of unstimulated cells. Graph Pad Prism software was used to generate graphs of the data and perform statistical analysis.

3.9 Statistical Analysis

Graph Pad Prism, Inc. software (Version 5) was used to generate graphs of the data and a two-way ANOVA Bonferroni post-test was performed on all samples.

CHAPTER 4

Results

4.1 Analysis of Moringa Tea

As described in Table 3, the nutritional values of Moringa dry leaves are to contain more vitamin A than carrots, more calcium than milk, more iron than spinach, more vitamin C than oranges, more protein than eggs and more potassium than bananas (Farley, 2005). MT was evaluated used varies of association of analytical communities (AOAC) methods to assess its nutritional values in minerals, vitamins and antioxidants. These assays were performed at the Center for Excellence in Post-Harvest Technologies in Kannapolis, NC. The results revealed that the calcium, potassium, iron, vitamin A, vitamin C and protein nutritional contents were significantly lower than what was projected from previous studies, which claimed to be high in vitamins, minerals and antioxidants.

Table 4

Nutritional Analysis of Moringa Tea

Analysis	Result	Units	Method
Fat (Total Triglycerides)	1.44	g/100g	AOAC 966.06 MOD
Saturated Fat	0.62	g/100g	AOAC 966.06 MOD
Trans Fats	<0.10	g/100g	AOAC 966.06 MOD
Polyunsaturated Fat-cis,-cis	0.12	g/100g	AOAC 966.06 MOD
Monounsaturated Fat –cis	0.09	g/100g	AOAC 966.06 MOD
Cholesterol	<1.10	mg/100g	AOAC 966.10 MOD
Sodium	45.8	mg/100g	AOAC 990.08C/SW846 6010B
Potassium	2000	mg/100g	AOAC 990.08C/SW846 6010B
Carbohydrate, Total	40	g/100g	Calculation
Dietary Fiber, Total	29	g/100g	AOAC 991.43
Sugars Total	0.6	g/100g	AOAC 982.14 MOD
Protein	0.2	g/100g	AOAC 992.15 MOD

Table 4

Cont.

Vitamin A, Total	10400	IU/100g	AOAC 2001.13 MOD, JAOAC 67:1, 69:5
Vitamin A (Retinol)	<40.0	IU/100g	AOAC 2001.13 MOD, JAOAC 67:1, 69:5
Vitamin A (B-carotene)	10400	IU/100g	AOAC 2001.13 MOD, JAOAC 67:1, 69:5
Vitamin C	<1.50	mg/100g	AOAC 967.22, JAOAC 75:5
Vitamin D2 (Ergocalciferol)	480	IU/100g	AOAC 2002.05 MOD
Vitamin D3 (Choleccalciferol)	<1.0	IU/100g	AOAC 2002.05 MOD
Vitamin E	0.9	IU/100g	AOAC 967.18MOD 974.29 MOD
Vitamin K1 (Phylloquinone)	5210	mcg/100g	AOAC 999.15
Thiamine (B1)	0.6	mg/100g	AOAC 942.23, 970.65, 981.15
Riboflavin (B2)	1.04	mg/100g	AOAC 942.23, 970.65, 981.15
Niacin (B3)	0.8	mg/100g	AOAC 944.13 960.46
Vitamin B6	0.5	mg/100g	AOAC 961.15, 985.32, 960.46
Vitamin B12	0.9	mcg/100g	AOAC 952.20, 986.23
Folate/Folic Acid	1059	mcg/100g	
Biotin	61.3	mcg/100g	JAOAC 49:882 MOD; AFC 23 (6) PSEBM 56:95
Copper	0.72	mg/100g	AOAC 990.08C/SW846 6010B
Magnesium	335	mg/100g	AOAC 990.08C/SW846 6010B
Phosphorus	380	mg/100g	AOAC 990.08C/985.35D/SW846 6010B
Zinc	2.73	mg/100g	AOAC 990.08C/SW846 6010B
Protein Factor	3.74		AOAC 992.15 MOD
Calcium	1798	mg/100g	AOAC 990.08C/SW846 6010B
Iron	65.1	mg/100g	AOAC 990.08C/SW846 6010B
Moisture	47.23	%	AOAC 925.09
Ash	2.3	%	AOAC 923.03

4.2 Body Weight of Mice

The recording of the weights were essential for the evaluation of weight gain for Trial 1 and Trial 2. All mice were weighed daily using an Arbor 1605 electronic balance. Each mouse was weighed and the weight was recorded in research log in the LARU. In figure 4, there was no

significant difference in weight in either trial; and the average daily weight gain was the same rate of 0.5g/day.

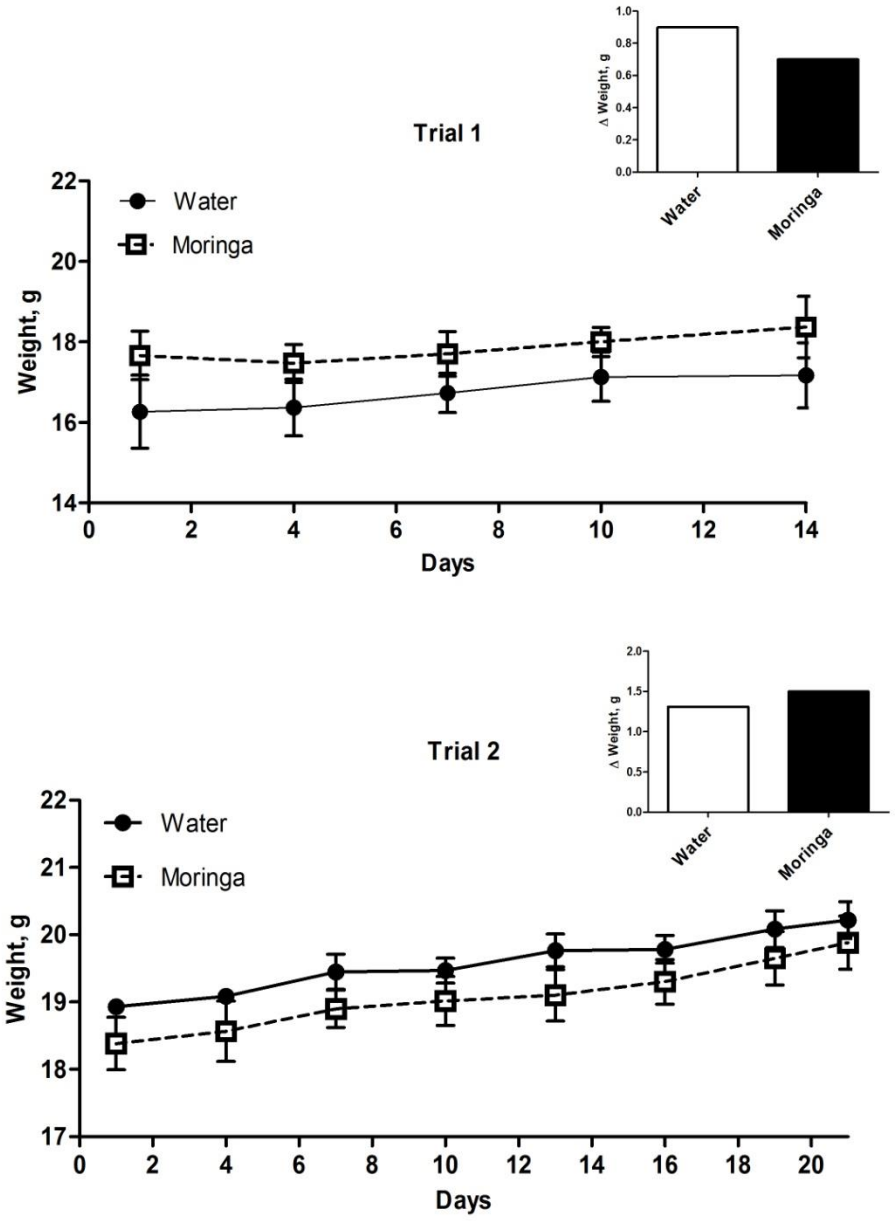


Figure 4. Change of Weights Over Time. Data is an average of n=3 at each point.

As figure 4 shows, Graph Pad Prism, Inc. software was used to generate graphs of the data. Insets are the average weights of the water and Moringa groups for each trial. No significant differences were observed.

4.3 Liquid Consumption of Moringa Tea and Water

The mice were given 100ml of fresh water or MT daily. The MT was made with a 2% concentration of dried Moringa leaves. Fresh Moringa leaves grown on the North Carolina Agricultural and Technical State University farm has a spicy taste when eaten in abundance. It was suspected that the MT may have a spicy taste, which may make the mice reluctant to drink the tea. However, based upon a previous study it was proven that the mice willingly drank MT. In this study, trial 1 and 2, mice in the MT group drank more than the mice in the control group (figure 5).

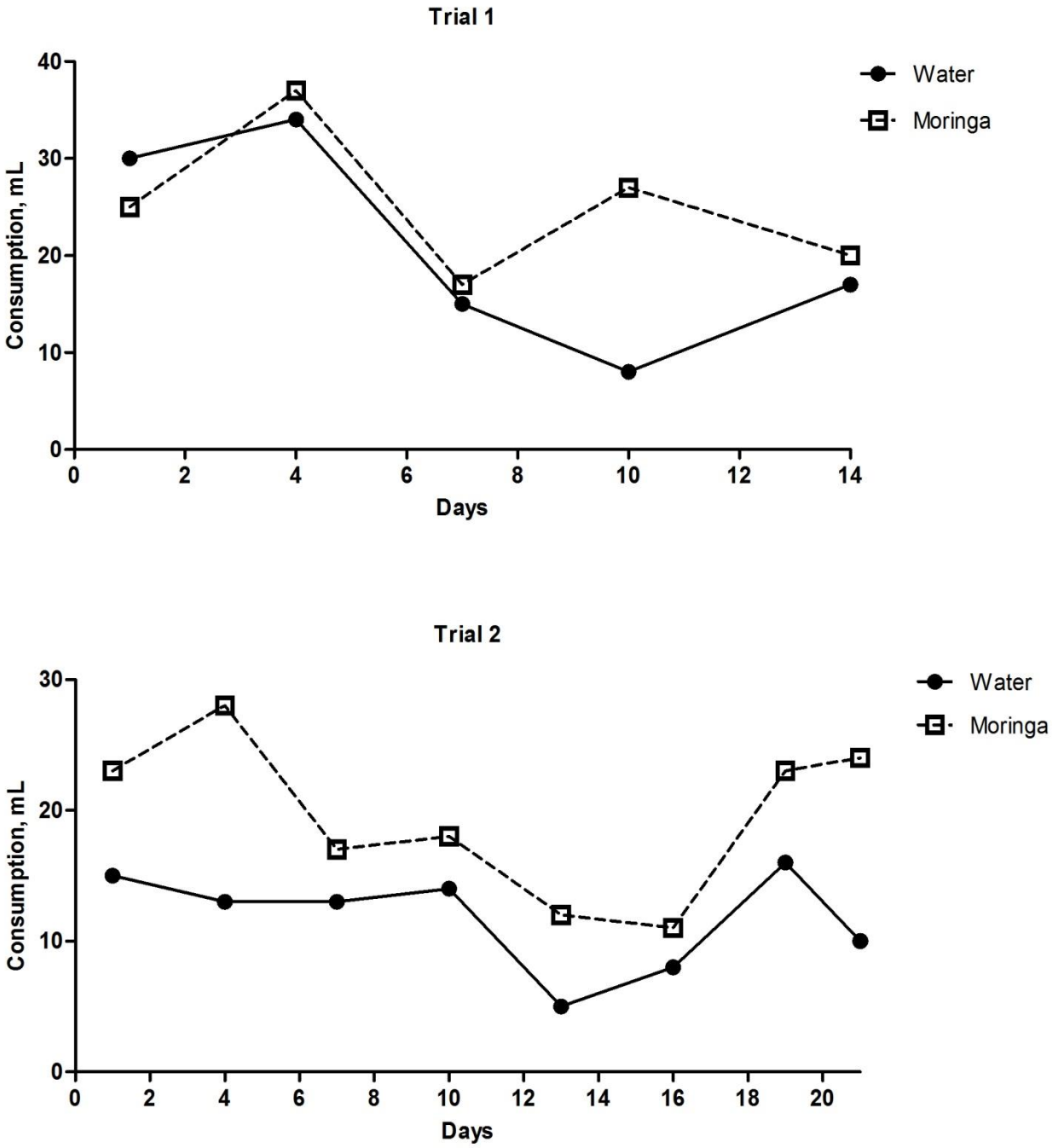


Figure 5. Comparison of Liquid Consumption. Data is an average of n=3 at each point. Graph Pad Prism software was used to generate graphs of the data.

4.4 T and B-cell Populations of Mice Determined by Flow Cytometry

Flow cytometry is an immunological assay that identifies cell types and quantity using lasers and sensors. In this study, the cells collected from the spleen and lymph nodes of the MT group and water group were used for flow cytometry to identify and count T-cells (helper and cytotoxic) using cell specific markers $CD3^+$, $CD4^+$, $CD8^+$ and B-cells using $CD19^+$. The data show that there was no significant differences in the percentage of T-cells and B-cells within the spleens (figure 6A) and lymph nodes (figure 6B) of MT or water groups. Further there was no difference in the percentages of $CD4^+$ and $CD8^+$ cells within the spleen nodes (figure 6C) and lymph nodes (figure 6D).

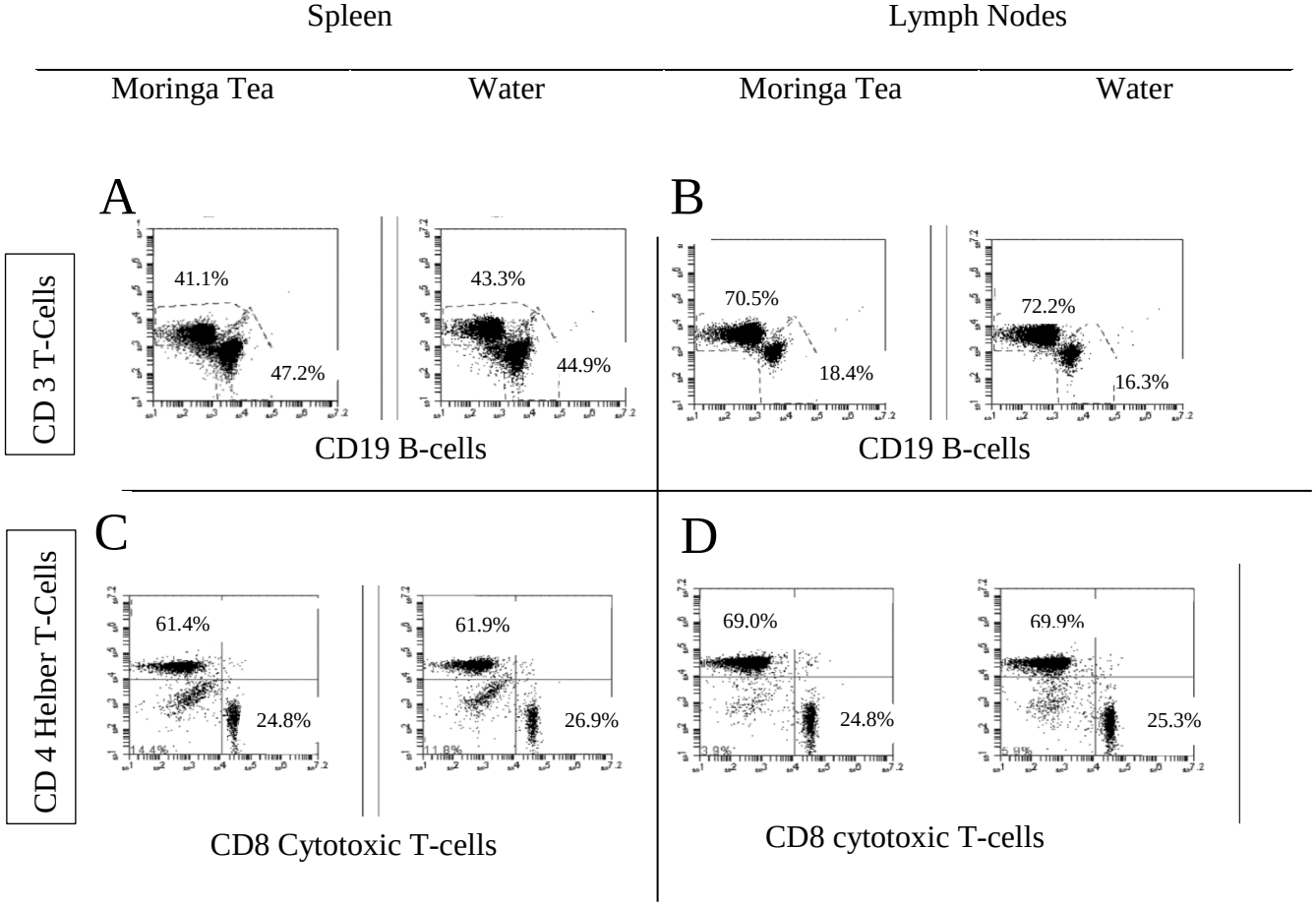


Figure 6. Flow Cytometric analysis of B and T-cell populations for 14 day study. Each panel is a representative dot plot chosen from an n= 3 in each group.

4.5 Proliferation of B and T-cells

Next we sought to determine whether MT had an effect on the function of T and B-cells, focusing specifically on proliferation. To measure the effect of MT on lymphocyte proliferation a we used the (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) or (MTT) proliferation assay. The MTT assay is a colorimetric assay that measures cell growth and viability. Cells that are alive and proliferating will convert the water soluble MTT to insoluble formazan. The concentration of the formazan, which gives a purple color concentration can then determine by optical density. Therefore, the color intensifies as the number of cells increases. This is a sensitive assay with linearity up to approximately 10^6 cells per well (van Meerloo, 2011). Cells isolated from either the spleen or lymph nodes were stimulated in the presence or absence of a stimulator. To compare proliferation of B-cells LPS was used and for the proliferative response of T-cells Concanavalin A (Con A) and anti-$CD3^+$ and anti-$CD28^+$ were used. LPS is a mitogen that is used to stimulate B- cell proliferation. (Con A) is a plant mitogen, originally extracted from the jack-bean, and is known for its ability to stimulate mouse T-cells (Gantner et al 1995). The data show no significant differences in the proliferation of cells isolated from the spleen or lymph nodes, in response to either Con A or LPS stimulation. However, when T-cells were stimulated with plate bound antibodies against the co-receptors in the TCR activation complex $CD3^+$ and $CD28^+$, we observed an inhibitory trend. As demonstrated in figure 7, there was less proliferation by T-cells isolated from the spleen of the MT group compared to that of the water group

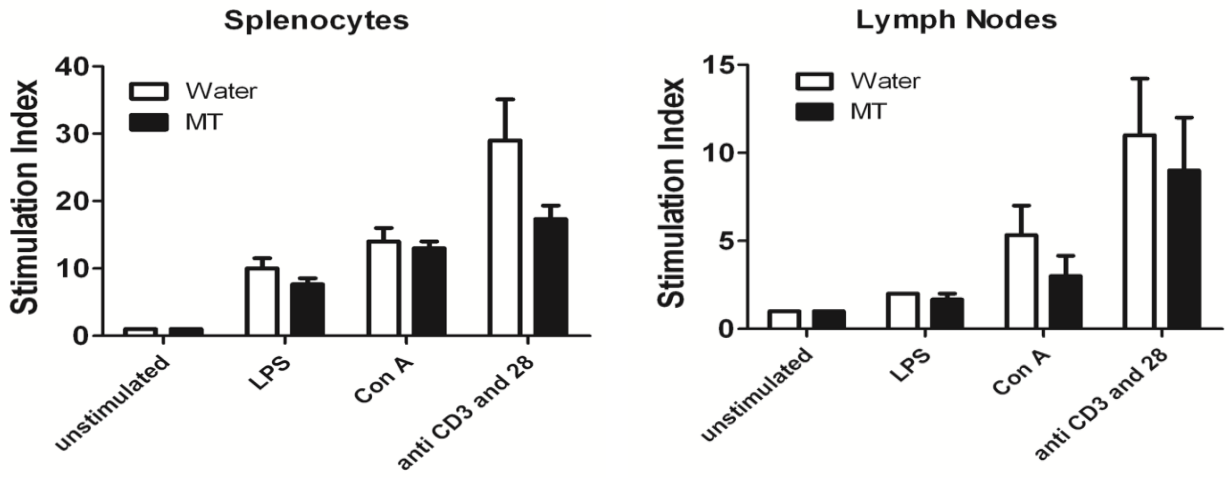


Figure 7. Mitogen-Induced Proliferation of Mice B and T-cells.

As figure 7 shows, cells were stimulated with LPS [1 µg/ml] and CON A [0.5 µg/ml] and anti-CD3 and CD28 [1µg/ml] each. The level of proliferation was determined by calculating the stimulation index (SI) using the following equation. SI = OD value of stimulated cells / OD value of unstimulated cells. Graph Pad Prism software was used to generate graphs of the data and perform statistical analysis. Data is an average of n=3.

CHAPTER 5

Discussion

The long-term goal of the lab is to find solutions for current and emerging agricultural health and disease issues by identifying immune enhancing nutritional supplements that promote the health of feed and fiber animals. The use of natural medicines to prevent or cure diseases (ethnopharmacology) has been used for centuries in developing and established countries around the world. In countries such as South Africa, more than half of the community utilizes the aid of traditional healers to receive treatments from the plants that are locally grown that have various nutritional and medicinal properties for themselves as well as their livestock. More than 75% of the farmer owners that raise livestock use plant-derived products and remedies to treat and prevent their animals against disease and infection (McGraw, 2008). Even though MOL isn't a novel tree to countries in Asia and Africa, various studies have been conducted to get a better understand of the content and properties that all part of MOL possesses. It was hypothesized that MT would have an immune enhancing or modulating effect on lymphocytes (T-cells and B-cells) in a mouse model.

5.1 The Effect of Moringa Tea on Mice Weight Gain

Moringa leaves added to the feed of dairy cattle that increased daily weight gain 32% increase in daily weight gain (Mendieta-Araica et al, 2011; Sanchez 2006; Foidl 2006). The nutritional value of Moringa leaves contains large amounts of nutrients, much greater than the foods that are typical associated with the same nutrients. Moringa leaves also contain all of the essential amino acids, which are the building block to proteins.

In this study we used a novel administration method of making MT instead of using other methods that have been reported in previous studies that resulted in an increase in daily weight

gain. However, no significant differences in the weight gain over time in the mice that consumed MT or water. The seeping time when preparing MT may have destroyed all nutritional value, potential causing an insignificant change in weight over time for mice that consumed MT. Also the previous studies stated that Moringa leaves were added to the feed of livestock that produced the results that were expected for this study. The leaves of Moringa seem to possess more nutritional value when given in its entirety whether than being infused in water.

5.2 The Effects Moringa Tea on Mice T and B-cell Populations

We were interested in determining the effect of Moringa consumption on the immune system specifically modulation of T-cells. The average normal lymphocyte population in mice range between 65.1% and 93.3%. In previous experiments using differential cell counting with stained cells we determined that consumption of Moringa changed the number of lymphocytes. However, differential cell staining is a subjective method that does not allow the type of lymphocyte (B-cell, vs. T-cell or ($CD4^+$ T-cell vs. $CD8^+$ T-cell) to be distinguished. Flow cytometry, is a more objective technique that allows for cell types to be identified and counted using fluorescently tagged antibodies as markers for cell specific antigens such as $CD4^+$, that are excited by lasers that transmits the data onto a computer. Cells from the spleen and lymph nodes of each mouse were stained with fluorescence activated cell sorting (FACS) antibodies $CD3^+$ (T-cells), $CD4^+$ (Helper T-cells), $CD19^+$ (B-cell), $CD8^+$ (Killer T-cells). For this experiment, results concluded that the percentage of T-cells or B-cells within the spleen and lymph nodes were not affected my consumption of MT. Nor was there a difference in the percentage of $CD4^+$ or $CD8^+$ T-cells in either organ.

5.3 The Effect of Moringa Tea on Mitogen-Induced Proliferation of Mice B and T-cells

Although there was no difference in the numbers of T-cells, we were interested in testing whether Moringa affected function. For this assayed cell isolated from the spleen and lymph nodes and tested lymphocyte proliferation using MTT assay. We found that splenic T-cells (not B-cell) isolated from mice that consumed Moringa tea stimulated with anti-CD3 and CD28 (a specific T-cell activator) had decreased proliferative response as compared to control. The data is suggestive that inhibition of a T-cell activated signaling pathway may have contributed to the decreased proliferative response. The mitogen activated protein kinase proteins (MAPKs) are a group a intracellular signal transduction enzymes found in yeasts, animals and plant cells (Ichimura, et al, 2002). They are triggered in response to extracellular signals and instruct new gene expression, cell survival, growth/proliferation, differentiation or death (Barr, 2001) in response to the stimuli. The MAPKs proteins are; extracellular signal-regulated kinase (ERK), stress-activated kinase of 38kDa (p38) and c-Jun N-terminal (JNK) (MacClorke and Tan, 2005). T-cell receptor (TCR) engagement activates proteins in the MAPKinase pathway in T-cells (Su, 1994) and co-ligation of CD3 and CD28 in mouse T-cells has been shown to lead to the activation of p38 and stimulate T-cell proliferation (Zhang, 1999). Further, an inhibitor of p38 MAP kinase (SB203580) was shown to inhibit CD28-dependent T-cell proliferation (Ward, 1997). Boiled *M. oleifera* pod extracts were found to block inflammatory responses by inhibiting MAPK activation (Muangnoi, 2012) and bioactive compounds from the roots of *Moringa Oleifera* has been shown to inhibit the production of IL-2 a cytokine produced by activated T-cells that promotes proliferation (Sashidhara KV, 2009). Recent studies in our lab have also found decreased activation of MAPK proteins in mice that consumed Moringa tea as compared to controls. This suggests that Moringa may inhibit T-cell proliferation by blocking activation of

the mitogen activated protein kinases (MAPKs) signaling pathway. But further studies are needed to determine this.

CHAPTER 6

Conclusions

In conclusion, these results reported that MT had no significant differences in weight, consumption or in the numbers of lymphocytes present in the spleen or lymph nodes. It has been shown that splenocytes isolated from mice that consumed Moringa tea for 14 days stimulated with anti-CD3 and CD28 (a specific T-cell activator but not LPS (specific B cell activator) had decreased proliferative response as compared to control. The proliferation results would suggest that Moringa may have a modulating effect on the function of T-cells but not B-cell. However, further studies need to conduct to evaluate its potential health benefits as a dietary supplement for livestock in immunity enhancement.

During the course of both trials mice were not immunologically challenged, it therefore might be hard to determine an effect on the cells and function. Furthermore, the longer of the two trial was 21 days, this may not have been enough time for an effect to occur. Therefore, additional studies could be designed where the length of the study is extended and the mice could be challenged immunologically. Amongst the nutritional and medicinal properties that Moringa possesses other areas that could be investigated that were not in this study are antibody production, killer T-cell function, and cytokine production.

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Appendix A

Preparation of Moringa Tea

Materials

3	3000ml Beakers
2	Large Stir Bars
2	Large Funnel
40g	Dried Moringa Leaves
10	24cm Filter Papers
2	2000ml Vacuum Flasks
2ft	Cheese Cloth
Ab libitum	Plastic Tubing
1	Thermometer
1 pair	Thermal Gloves
2	Hot Plates
Ab libitum	Labeling Tape
1	Extra Large Rubber Band
2-6in cuts	Parafilm
Ab libitum	Sterile filtered bottles

Protocol (4-6 hours)

- All glassware and autoclavable materials must be autoclaved at least a day prior to use.
    - Beakers, funnels, stir bars, filter paper, vacuum flasks, cheese cloth (NO PLASTICS)

- Once all materials are sterilized. Fill 2-3000ml Beakers with 2000ml of deionized UV water and add a stir bar to each beaker
    - Place beakers filled with water on hot plates, partially cover top with foil and allow water to come to a boil. Temperature should reach 95°C ± 3°C.
    - Use a clean thermometer to periodically the temperature. DO NOT LEAVE THERMOMETER INSIDE BOILING WATER.
- While waiting for the water to boil, take this time to measure out 40 grams of dried Moringa leaves . Use weigh boats to weight out the Moringa leaves and set aside until ready for use.
- Once water has come to a boil, turn off the heat and pour 20g of Moringa leaves into one beaker and the remainder into the other beaker. Steep for 30 minutes and make sure the stir bar is continuously, but if the stir bar isn't stirring manually stir the tea periodically.
- Before the 30 minutes of steeping is complete, take the cheese cloth and fold it to cover 1-3000ml beaker and secure the cheese cloth with the extra-large rubber to fit around the neck of the beaker.
- Once the 30 minutes are complete, use the thermal gloves and pour the tea through the cheese cloth into the 3000ml beaker.
    - If the beaker gets full from the filtered tea , stop and continue on to the next step, save the cheese cloth and resume once the beaker used to filter the tea has enough space
- Using 2-funnels, line the funnels with filter paper and insert into 2-2000ml vacuum flasks

- Connect the plastic tubing to the vacuum outlet on the flasks, turn on the vacuum and use the parafilm to wrap around the junction between the end of the funnel and the mouth of the flasks to maximize suction prior to secondary filtration

- Slowly pour filtered tea through funnel and allow tea to filter through the filter paper into the flask until the flask is full
- For the final filtration, sterile filter bottles will be used. Plastic tubing will be connected to the outlet of the sterile filter bottles and the vacuum will be turned on prior to final filtration.
  - Pour secondary filtered tea into the filter top of the bottles until full and allow tea to be filtered into the bottle.
  - Once completed, remove filter top and discard, close cap and label all bottles and store in refrigerator or freezer (depending on what dr. minor would want).

Appendix B

Table 5

FACS Antibody Cocktails

	FACS Antibody				
Cocktail	FITC CD 3	PE CD 4	PE-Cy7 CD 49b	PerCp cy 5.5 CD 19	APC CD 8
1	X	X		X	
2	X			X	X
3	X	X			X
4		X		X	X
5	X	X		X	X
6	X		X		