CALCIUM MONTMORILLONITE FOR THE MITIGATION OF AFLATOXICOSIS AND GASTROINTESTINAL INFLAMMATION

A Dissertation

by

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Submitted to the Office of Graduate and Professional Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

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August 2014

Major Subject: Toxicology

ABSTRACT

Clays have been used for centuries as 'ancient medicine' for their therapeutic benefits. One particular clay, calcium montmorillonite, has historically been used as an anti-caking agent in animal feeds, but has also demonstrated the ability to bind toxins and alleviate infectious diarrhea. The full breadth of therapeutic applications and molecular mechanisms of montmorillonite is still unknown. Therefore, the purpose of this research was to explore novel therapeutic applications for NovaSil (NS), a calcium montmorillonite clay to reduce the risk of aflatoxicosis in farm-raised fish and alleviate gastrointestinal inflammation and dysbiosis in a mouse model of Crohn's disease (CD).

Aflatoxin B_1 (AFB_1) is a fungal mycotoxin that commonly contaminates corn and peanut crops. It is produced by the fungi *Aspergillus flavus* and *A. parasiticus* during times of drought or due to improper post-harvest storage. Aflatoxin B_1 is known to cause hepatocellular carcinoma, immunosuppression and growth stunting in several species. Recently, incorporation of plant-based alternatives into feed for farm-raised fish has become a trend, thereby increasing the risk for mycotoxin contamination. Inexpensive strategies to reduce AFB_1 exposure are needed. Calcium montmorillonite clay, which is both inexpensive and abundant, has a dioctahedral structure that is known to sequester AFB_1 in its negatively-charged interlayer, thereby reducing systemic bioavailability. There is also some evidence to suggest that calcium montmorillonite clays may possess gastrointestinal anti-inflammatory properties.

NovaSil was used as a strategy to reduce the effects of AFB_1 in Nile tilapia (*Oreochromis niloticus*) and red drum (*Scieanops ocellatus*). Juvenile tilapia and red

drum were dosed with AFB_1 and NS over the course of 10 and 7 weeks, respectively. Additionally, proinflammatory cytokine-clay binding was characterized using isothermal analysis, X-ray diffraction (XRD) and transmission electron microscopy (TEM). Furthermore, a TNBS (2,4,6-Trinitrobenzenesulfonic acid)-colitis gastrointestinal mouse model was employed to study the anti-inflammatory properties of NS and its ability to protect the gut microbiome.

Results suggest that NS can prevent aflatoxicosis in red drum at a 2% inclusion level over the course of 7 weeks. NovaSil also prevented some toxicity in Nile tilapia; however, these results were not significant. *In vitro* results also indicate that NS sorbs proinflammatory cytokines such as TNFα and IL-1β in its interlayers. Additionally, NS was found to reduce serum pro-inflammatory cytokine levels in TNBS-induced mice and reduce gut dysbiosis. These results could positively impact both human and animal populations with AFB_1 exposure and/or chronic gastrointestinal inflammation.

DEDICATION

I would like to dedicate this dissertation to my mother, Sylvia Zychowski.

ACKNOWLEDGEMENTS

I would like to thank my committee chair, Dr. Timothy D. Philips for his guidance and support throughout these years. I could not have asked for a better advisor and mentor for this journey. Thank you to my committee members Dr. Michael Criscitiello, Dr. Delbert M. Gatlin III. and Dr. Roger Harvey.

To the Phillips' laboratory members both past and present: Sarah Elmore, Natalie Johnson, Alicia Marroquin-Cardona, Nicole Mitchell, Abraham Robinson, Amelia Romoser, Kristal Rychlik and John Taylor. Thank you for your help and support both in and outside of the lab for all of these years.

A special thanks to my parents William and Sylvia Zychowski, my brother Gregory Zychowski, my sister-in-law Lena Timmons and my fiancé, Daniel Bufford.

TABLE OF CONTENTS

Page

LIST OF FIGURES

Page

LIST OF TABLES

Page

1. INTRODUCTION

1.1 Aflatoxin

1.1.1 Origins of aflatoxin contamination

Aflatoxins are toxic fungal metabolites produced by the fungi *Aspergillus flavus* and *A. parasiticus.* These fungi typically grow in tropical and subtropical climates on multiple agricultural products [1]. While some agricultural commodities are contaminated in the field, the majority of crops are contaminated during the post-harvest storage [2]. Aflatoxins can frequently contaminate feedstuffs such as peanuts, corn, soybean, maize, rice, dried fish and fishmeals. These feed components are used for both commercial animal feeds and also for human consumption. Aflatoxin B_1 (AFB_1), one of the aflatoxin metabolites, is a complete carcinogen causing initiation and promotion [3, 4]. Often, AFB_1 occurs in feed concurrently with other mycotoxins such as fumonisin B_1 (FB_1), ochratoxin (OTA), and zearalenone (ZEA) [5, 6].

A. Flavus is the most abundant soil-borne mold and can be found in various environments. This fungus is saprophytic, a term used to describe its ability to grow on wood and organic matter and can thrive in temperatures ranging from 12 to 48°C. *A. Flavus* is exceedingly heat tolerant, which contributes to its resilience on agricultural commodities in both humans and animals. *A. flavus* has the ability to infect maize, cotton, peanuts and tree nuts. *A. flavus* can also attack monocot and dicot seeds above and below the surface. In corn, it typically causes “ear rot” especially when plants are stressed at high ambient temperatures. Natural sources of stress such as insects, hail and disease can predispose plants to AFB_1 contamination [7].

The fungal cell wall of *A. flavus* is the key characteristic that influences the pathogenicity and the fungal hyphae is responsible for penetrating the host's cell wall. Aflatoxin contamination is not evenly distributed throughout crops and "hot spots" are a notable issue, especially in maize. While *A. flavus* is typically responsible for producing AFB_1, *A. parasiticus* is also responsible for producing aflatoxins such as AFG_1, AFG_2 and AFM_1.

While temperature is the most influential factor in pre-harvest aflatoxin contamination, moisture is a secondary influential impetus. Water stress occurring from 40 to 75 days before harvest causes aflatoxin contamination in mature kernels. Drought for 20 days with normal soil temperatures (28-30.5°C) resulted in *A. flavus* contamination in 19.5% of peanut crops, and 75% of damaged peanuts with up to 784 ppb AFB_1 contamination [8]. While aflatoxin is a world-wide issue, extensive economic losses have occurred in the United States during times of drought in both peanut and corn crops as well as livestock [9, 10].

1.1.2 Aflatoxin metabolites

Aflatoxins and their metabolites vary considerably in prevalence and toxicity. Aflatoxin B_1 is one of the most toxic congeners because of its carcinogenic, immunosuppressive and growth stunting capabilities. Aflatoxin B_1, B_2, G_1 and G_2 commonly contaminate grains and agricultural crops. Aflatoxin M_1 and M_2 are prevalent in milk and dairy products. Action limits for AFB_1 in dairy feed and AFM_1 in milk and dairy products are country specific, however, in the United States, the limit for dairy feed is 20 ppb aflatoxin B_1 and 0.5 ppb aflatoxin M_1 in milk When animal feed or milk is

above these levels, it is rejected for animal and human consumption , which results in nearly a billion USD loss per year [7].

1.1.3 Aflatoxin B_1 metabolism

Aflatoxin B_1 is metabolized in three different stages: bioactivation, conjugation and deconjugation. In the bioactivation phase, AFB_1 becomes hydroxylated forming AFM_1, aflatoxicol, and the AFB_1 8,9 epoxide. Other metabolites, such as AFQ_1 and AFB_2, are produced from the reaction but are not as toxic as the other metabolites. Because the 8, 9 epoxide is very unstable, often it will bind to DNA and other nucleophilic compounds (Fig. 1). This causes the formation of multiple DNA adducts including AFB_1-N7-guanine, -pyrimidyl, and -diol adducts [11]. Carbon 8 on AFB_1 reacts with the N^7 position of guanine to form the 8,9-dihydro-8-(N^7-guanyl)-9-hydroxy-AFB_1 adduct. Multiple CYP450 isoenzymes are responsible for AFB_1 epoxidation, such as CYP2C11. CYP1A2 is also capable of catalyzing the oxidation of AFB_1 to AFM_1 in the rat liver [12]. Mammalian cytochromes and piscine cytochromes differ in nomenclature according to the Cytochrome P450 Nomenclature Committee. Some of the Phase I metabolizing enzymes that are responsible for AFB_1 biotransformation in fish include CYPLMC1- CYPLMC5 [13].

There are five known cytochromes that are responsible for transforming AFB_1 into the epoxidated form in humans. These include CYPs1A2, 2A6, 2B7, 3A3, and 3A4, with 1A2 and 3A4 enzymes which cause the majority of epoxidation. Inhibition of liver microsomes with CYP450 inhibitors such as furafylline and troleandomycin have shown

to be very effective in inhibiting CYP1A2 and CYP3A4, respectively, thereby preventing formation of the AFB_1 epoxide [14].

Following bioactivation (phase I metabolism), conjugation (phase II metabolism) is facilitated via glutathione-S transferase (GST), β-glucoronidase and/or sulfate transferase enzymes to produce products such as AFB_1-glutathione, the major metabolite, and other lesser metabolites including AFB_1-glucoronide and AFB_1-sulfate. The amount of GST activity in rodents is inversely related to species susceptibility to AFB_1-induced hepatocarcinogenesis.

Intestinal bacteria are also responsible for deconjugation and can recirculate AFB_1 metabolites which ultimately reach the liver [15]. Aflatoxin B_1, a direct mutagen, forms an epoxide through cytochrome P450-mediated oxidation [16] (Fig. 1). In addition, proto-oncogenes of the ras family are activated with the formation of the AFB_1 adduct and can cause a direct transversion of guanine to thiamine in p53 tumor suppressor genes in the liver [17]. RNA damage and interaction with intercellular proteins are also consequences of AFB_1 exposure. Proteins can bind to the epoxide in the same way that the epoxide binds to DNA, and albumin is the most common protein that is known to bind to AFB_1, creating the AFB_1-albumin adduct that can be detected in serum [18]. In addition to CYP-450 mediated oxidation as the dominant pathway in AFB_1 epoxidation, other pathways have also been identified that contribute to AFB_1 activation. Prostaglandin H synthase (PHS) can also cause epoxidation; however, levels

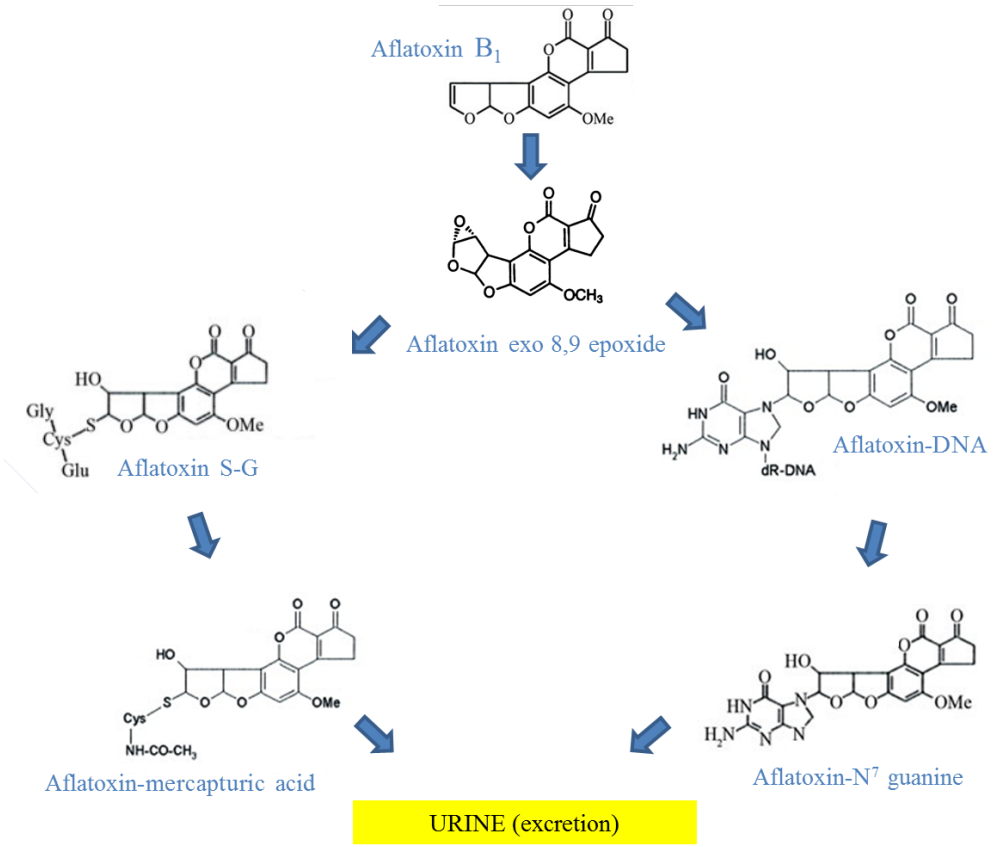


Figure 1. Aflatoxin metabolism. Aflatoxin metabolism results in urinary excretion of metabolites. (adapted from [16].)

are largely tissue-dependent [19]. Lipooxygenases in the liver and kidney of mammals can also activate AFB_1-DNA binding.

Further oxidation of AFB_1 at the 8,9 carbon may also form aflatoxin $B_{2\alpha}$. This particular metabolite can contribute to the formation of Schiff bases with amines, but is still less toxic than AFB_1. Epoxide hydrolase can convert the AFB_1 epoxide to the AFB_1-dihydrodiol. Therefore, AFB_1-DNA binding can be reduced with the presence of epoxide hydrolase, even in the absence of GST [20].

1.1.4 Aflatoxin toxicity in multiple species

One of the first recorded instances of AFB_1 carcinogenicity was demonstrated in farm-raised rainbow trout. In the 1960s, a trout farm had a series of aflatoxin outbreaks and ultimately, the source was traced back to a batch of heavily contaminated cottonseed meal. To this day, trout are used as a model for hepatocellular carcinoma (HCC), due to their relatively high level of phase I metabolizing enzymes and relatively low level of phase II metabolizing enzymes [21]. Aflatoxin-induced hepatocellular carcinoma is often dictated by the proportion of phase I and phase II metabolizing enzymes in the liver. Within a few years after the trout aflatoxicosis outbreak, “Turkey X” Disease was discovered and was ultimately attributed to AFB_1 [22]. The disease was characterized by odd behavior followed by coma and death. Upon necropsy, multiple necrotic liver lesions were discovered. Scientists eventually traced the incident to moldy peanut meal originating from South America [23]. Other fowl that can be affected by Turkey X disease include ducklings and pheasants. Younger poults ranging from 2 to 8 weeks are also susceptible [24].

Since those historic events, other animals have been assessed for AFB_1 susceptibility and have exhibited varying responses. The LD_{50} values determined from this work are a good predictor of toxicity, and also indicate the likelihood of cancer progression in each species. Initially, while carcinogenicity was the primary concern with AFB_1, studies have also focused on delineating its association with immunosuppression, teratogenesis and other physiological effects. Because of the high levels of phase I metabolizing enzymes, species such as rabbits, pheasants, ducks, turkeys and trout and other species are highly sensitive to AFB_1. On the other hand, chickens, which have lower levels of phase I metabolizing enzymes are highly resistant to AFB_1, and show minimal signs of aflatoxicosis up to 2.5 ppm [25]. In general, species that possess high levels of glutathione-s-transferase (GST) are more resistant to aflatoxicosis. Human liver microsomes are only one-quarter efficient at activating AFB_1 when compared to rat microsomes at high concentrations. Mouse microsomes can efficiently create the AFB_1 epoxide, but it is rapidly detoxified due to the efficient conjugation of the epoxide with GSH. Therefore, mice are less prone to hepatocarcinogenesis than other animals where this mechanism is not as efficient [26].

1.1.5 Aflatoxin immunosuppression

Aflatoxin B_1 can significantly alter the immune system. Controlled laboratory studies in animals have demonstrated that AFB_1 immunosuppression greatly increases the risk of secondary infection [27]. Young pigs exposed to 800 ppb AFB_1 showed signs of immunosuppression after 25 days. These pigs had depressed blood lymphocyte proliferation and oxidative radical burst was inhibited. Additionally, *in utero* piglets

whose mothers were exposed to AFB_1 had a decrease in cortical thymus lymphocytes and a decrease in thymus weight after exposure [28, 29]. Weanling rats and adult rats, alike, have shown immune alterations after exposure toAFB_1 [30]. Raisuddin et al. 1994 described this toxicity. In the study, 5-6 week-old rats were fed 60 to 600 ppb AFB_1 every other day for 4 weeks. This had a substantial impact on cellular immunity, including LPS-induced lymphocyte proliferation, but did not affect humoral immunity. Aflatoxin B_1 administered to rats intravenously over 2 wks negatively affected thymus and bone marrow cellularity, LPS-lymphocyte proliferation, phagocytic function of peritoneal macrophages and delayed hypersensitivity responses in mice exposed to AFB_1, helper T cells as well as IL-2 levels decreased at 0.75 mg/kg [31]. Aflatoxin B_1 negatively impacts the immune system by decreasing production of lymphokines and interfering with antigen processing by macrophages [32, 33].

In vitro studies have also demonstrated immune dysfunction in murine peritoneal macrophages following AFB_1 exposure. A decrease in nitric oxide (NO) production and reactive oxygen intermediates (ROI) was demonstrated when *in vitro* cultures were treated with LPS. Proinflammatory cytokines produced from macrophages were also significantly reduced, including TNFα, IL-1 and IL-6 [34]. Low doses of AFB_1 (0.05-0.1 pg/mL) decreased phagocytosis and bacteriocidal activity against *Candida albicans* in human monocytes [35], indicating increased disease susceptibility with AFB_1 exposure. Direct exposure to 20 pg AFB_1 in chicken macrophages resulted in a significant reduction in substrate adherence ($P=0.0007$) [24]. In the same study, lower doses (5-10 pg) caused a less dramatic decrease in substrate adherence ($P=0.05$). Five to

twenty pg/mL doses of AFB_1 resulted in morphological alterations, cytoplasmic blebbing and nuclear disintegration, characteristic of cell damage. Aflatoxin B_1 is also a notable pro-oxidant [36]. When bovine neutrophils were evaluated for ROS production after AFB_1 exposure, *in vitro*, O_2^- production and MPO (myeloperoxidase) activity significantly decreased. When challenged with *E.coli* and *S.aureus*, phagocytic activity significantly decreased in AFB_1-exposed neutrophils, compared to the controls.

Broiler hens fed between 0.2 and 5 ppm AFB_1 for 3 weeks demonstrated cell-mediated immunity, humoral immunity and phagocytic cell compromise in their offspring [37]. Turkeys, chickens and other livestock exposed to aflatoxin frequently result in economic losses for producers because of their reduced immunity and increased susceptibility to disease. Low-level exposure to AFB_1 over a long-period of time is known to enhance susceptibility to infection and tumorigenesis in many vertebrates [38]. Sub-chronic exposure to AFB_1 in chickens over an extended period of time has been known to contribute to increased disease susceptibility [39]. In one study, chicks were fed chicks from 7-days old to 7 wks with 1 ppm AFB_1, and the chicks were subsequently challenged with FAV-4 (fowl adenovirus serotype 4). There was a marked depletion of lymphocytes in all immune organs including the bursa, thymus, spleen and tonsils in AFB_1-exposed chicks, compared to the control group. Furthermore, AFB_1-exposed chicks were more adversely affected from the FAV-4 virus than their control counterparts [40].

Metabolic studies in salmonid fishes including trout, coho salmon and others such as zebrafish and channel catfish have revealed the mechanism behind AFB_1-

induced toxicity. The aflatoxin B_1-8,9 epoxide (AFBO) is the toxic intermediate that is responsible for causing DNA adduct formation in salmonid fish liver. Within the family of ray-finned fish, rainbow trout exhibit high sensitivity to AFB_1, while coho salmon are fairly resistant [41]. Sensitivity towards AFB_1 in fish is due to the balance between phase I and phase II metabolizing enzymes. Salmon liver contains nearly 5 times less CYPLM2 (CYP2K1) when compared to trout liver [42]. Glucuronidation (phase II metabolism) and glutathione conjugates in trout are often negligible, as compared to other salmonids and mammals [43, 44]. This means that because phase I metabolism is extremely efficient, AFBO is readily formed. However, because of the lack of phase II metabolizing enzymes, limited detoxification occurs and the epoxide forms the DNA adduct more easily than in other species.

1.1.6 Aflatoxin decontamination

Decontamination of aflatoxins by chemical means or by processing has been explored in years past. There are a number of factors that are considered regarding aflatoxin decontamination: 1) removing, destroying or inactivating the mycotoxin, 2) retaining the nutritive value of the feedstuff 3) limiting or not leaving any toxic residue 4) not altering the properties of the product and 5) destroying the fungal spores that may produce additional mycotoxins during the storage process In a study by Cole et al. (1989), it was demonstrated that removing mold damaged kernels can result in a 40-80% reduction in AFB_1 [45]. Additionally, Lopez-Garcia et al. (1998) found that flotation and density segregation were somewhat effective for contaminated corn and

peanuts however AFB_1 residues still remained The food industry has investigated this method to decontaminate peanuts that are used for peanut butter [46].

Aflatoxins are relatively heat stable. Processes such as boiling and autoclaving are not entirely effective in eliminating AFB_1 from feed components. Fermentation results in a 70-80% reduction in AFB_1 contaminants in different grains [47]. Gamma-irradiation has been attempted and did not degrade AFB_1 in peanut meal [48]. However, e-beam irradiation resulted in a 75.49% decrease in AFB_1 contamination in coconut agar [49].

Nixtamalization is the process of using an alkaline solution to prepare maize or grains, such as the production of tortillas. Aflatoxin B_1 is susceptible to alkaline pH and can be degraded at the lactone ring using this process [51, 52]. The resulting reaction at equilibrium produces two less toxic metabolites, ; however, the reaction is reversible at intermediate stages and can result in reformation of parent AFB_1 under acidic conditions found in the stomach.

Human and animal intervention strategies have also been explored. However, several of these post-harvest strategies, such as Oltipraz, chlorophyllin, isothiocyanates and triterpenoids are not realistic for large-scale implementation in livestock and commercial farms due to their high cost. Intervention strategies that are inexpensive, effective, and safe are needed [50].

1.2 Aquaculture

1.2.1 Aquaculture industry

Aflatoxins can affect several livestock species including turkeys, chickens, pigs and fish. There are currently over 123 finfish species that are cultured worldwide. Aquaculture is a $50 billion industry and the largest growing food sector due to decreases in wildstock and overfishing [51]. Due to the growing global population and associated strain on the food supply, aquaculture is expected to become increasingly important over the next century. Fishery products are some of the most traded food commodities globally. Subsequently, demand is expected to continue rising as aquaculture production expands (Fig. 2). In the next decade, production of aquaculture products is expected to exceed total production of beef, pork or chicken [52]. Recently, the United Nations Conference on Sustainable Development, also known as Rio+20, facilitated discussion of world hunger issues and methods to improve food security, while using fewer natural resources. The focus on improving small-scale aquaculture operation sustainability to promote poverty eradication is pertinent to both the domestic and international communities, especially in countries throughout sub-Saharan Africa, where food shortages are common. Promoting sustainable fish farming can promote economic stewardship and environmental responsibility [53].

In 2010 alone, aquaculture and capture fisheries produced 148 million tons of fish, 128 million of which were utilized as food for people. Sustained increases in fish production have occurred as a result of improved distribution channels and the farmed

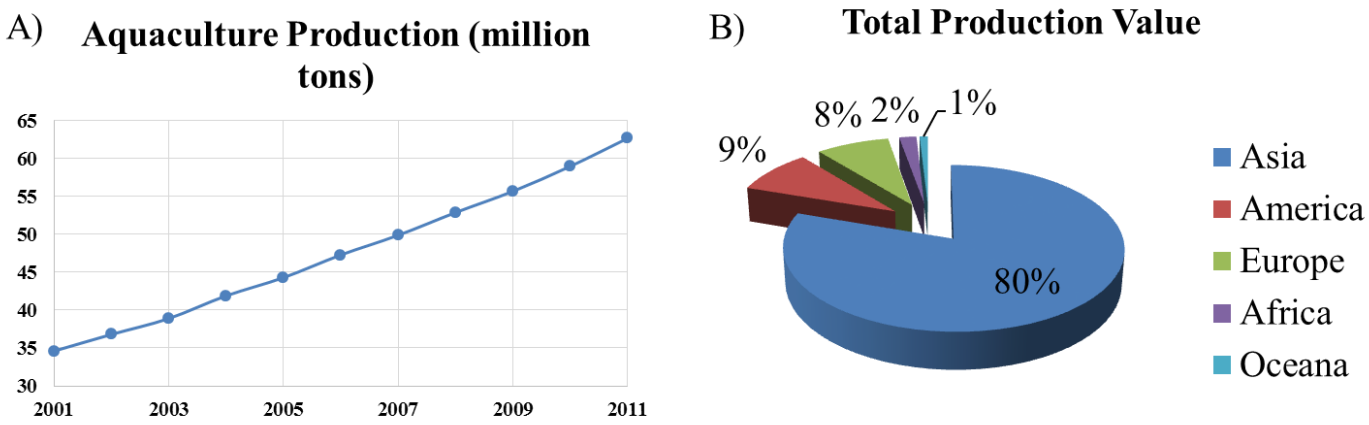


Figure 2. Aquaculture production growth and total production value according to geographical region [54].

fish supply has grown dramatically with a rate of 3.2% per year. Consumption of fish has increased from 9.9 kg in the 1960s to 18.4 kg in 2009 [55].

China's utilization of the farm-raised fish has a substantial effect worldwide and has been responsible for the greatest increase in fish production [56]. China's production grew from about 7 percent in 1961 to 35% in 2010. Likewise, fish consumption in China has risen to approximately 31.9 kg in 2009, and has increased 6% each year for the last 10 years. In 2009, 16.6% of fish accounted for the world's intake of animal protein and 6.5% of all protein consumed globally. Nearly 3 billion people consume fish worldwide and fish account for 20% of total intake of animal protein [57, 58].

Freshwater sources account for 60% of world aquaculture production, despite the fact that only 3% of the planet's water is fresh [54]. Brackish water aquaculture is also growing in popularity, especially in regions such as Africa and Latin America. Because of increasing pressure on freshwater supplies, future aquaculture development is expected to utilize brackish water more frequently. The most widely farmed fish are salmon, carp, tilapia, sea bass, catfish and cod. Red drum is another commercially important species and is farmed in Ecuador, Israel the Gulf Coast of the United States and parts of China [59]. Red drum thrive in brackish water and farming them can alleviate some of the strain on the freshwater aquaculture industry [60].

1.2.2 Fish immunology

The fish immune system is similar to other vertebrates and can be divided into two categories: the innate immune system, and the adaptive immune system. The innate

immune system is non-specific and is considered the “first line of defense” (Fig. 3). It consists of macrophages, neutrophils, mast cells, basophils and natural killer cells. Conversely, the adaptive immune system is specific and is responsible for immunological memory (recognition of a previously encountered pathogen). Major cells of the adaptive immune system include T and B cells. Dendritic cells are classified as part of both the innate and adaptive immune system.

Because fish are free-living organisms during early embryogenesis, they rely more on their innate immune system than mammalian species [61]. Innate immunity has three components: the mucosal barrier, the humoral parameters and the cellular components. An immune response typically involves all three components.

Lysozyme is a bacteriolytic enzyme that is one of several humoral parameters and is present in serum, secretions, and mucous membranes. The kidney and intestine have the highest concentrations of lysozyme [62, 63].

Protease inhibitors such as anti-trypsin are also present in the serum and other fluids [64]. These molecules can promote the degradation of proteolytic enzyme-secreting bacteria [65]. Assessment of anti-trypsin is a good measure of humoral immune function. Other processes in the innate immune system include neutrophil and macrophage phagocytosis and pinocytosis. These cells engulf pathogens by producing reactive oxygen species (ROS) such as O_2^-, H_2O_2, and NO^- [66]. These compounds are produced both intracellularly and extracellularly and kill bacteria by breaking down their cell walls.

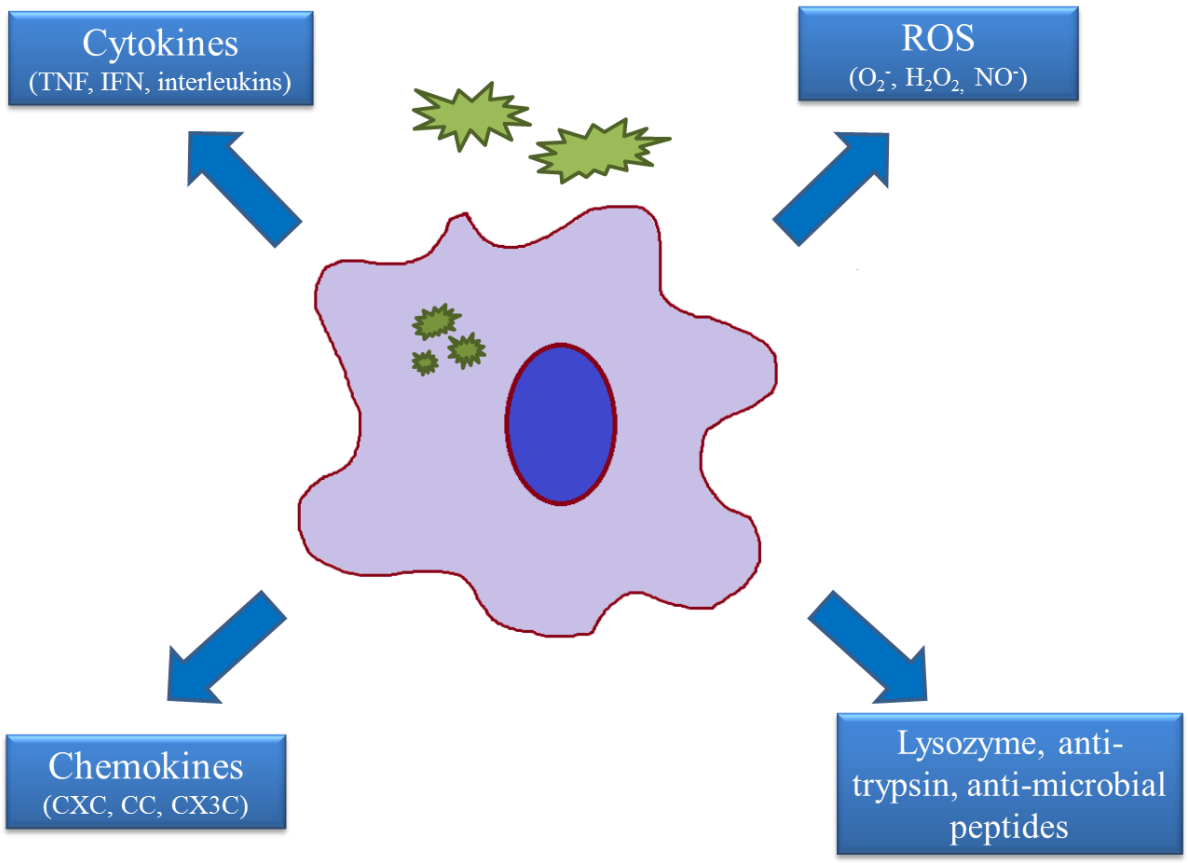


Figure 3. Components of the innate immune system in fish. Includes a series of cytokines, chemokines, ROS, lysozyme and serum components.

1.2.3 Farmed fish feed, fish nutrition, and potential contaminants

Immunity in fish can be affected by nutritional factors in their feed as well as naturally occurring and anthropogenic contaminants. There are two major categories of nutrients for fish: 1) macronutrients, including carbohydrates, lipids and proteins and 2) micronutrients, consisting of vitamins and minerals. Typically, proteins in commercial feed contain amino acids from both animal and plant sources. Protein is also the most expensive component in fish feed, and excess protein in the feed is not economically feasible and also causes excess nitrogen build up in the water. Herbivorous and omnivorous fish consume about 25-30% protein in the diet, however carnivorous fish require more (approximately 40-55% protein). There are ten essential amino acids that are included in commercial fish diets including arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine [67].

Lipids are the next group of essential nutrients in fish feed. Essential fatty acids (EFAs) typically provide fish with long-chain unsaturated fatty acids such as linoleic (18:3 n-3 and linoleic acids 18:2 n-2). Dietary lipids are precursors to steroids, hormones and prostaglandins. Prostaglandins are important for intestinal absorption of fat-soluble vitamins. Lipids and lipid-soluble products, including certain toxins, can bioaccumulate in the muscle and fat.

In order to meet all of the necessary physiological requirements of growing fish, all essential nutrients must be present in the feed for growth and reproduction. While progress has been made in the last half-century in fish nutrition, quantitative

requirements for nutrients and limitations of contaminants in fish feed have not been well-established. Recently, nutritional requirements for well-known commercial species such as rainbow trout, Pacific salmon, channel catfish, tilapia and carp have been established due to their frequent farming in the industry [68].

Some species, such as tilapia can be fed either an herbivorous or omnivorous diet, and are a much sturdier species than rainbow trout, which require highly monitored cultivation. Aquaculture feed is composed of a series of ingredients to meet all of the nutritional requirements of a specific fish species. Red drum, for example, is a carnivorous species and therefore need a higher percentage of protein in the feed than tilapia. Excess intake of certain nutrients can potentially harm the fish, causing nutrient build-up in the liver and fat tissues. For example, fat-soluble vitamins such as vitamin A or trace elements, such as copper can be toxic in excess [69]. Deficiencies in primary essential nutrients and exposure to anti-nutrients might contribute to disease development, survival rate, integrity of the skin, liver, and other organs or the composition of tissues in the body [70].

There are other physiological factors that affect metabolism in fish such as environmental temperature. Fish are poikilothermic, meaning that their metabolic rate and body temperature is determined by the water temperature. Drastic changes in environmental temperature can dramatically affect energy expenditure and food and nutrient intake. Fish are very efficient at utilizing fats and protein over carbohydrates, and can absorb minerals and/or potential contaminants through their skin and gills. Because fish are primitive vertebrates, they also possess anatomical features important in

bioavailability and metabolism that mammalian species do not. They have distinguishing features such as a lack of mandibular teeth and non-differentiated small and large intestine. Fish intestinal cells can also absorb molecules via pinocytosis [71].

Food consumption in farm-raised fish can be affected by daily, annual and lunar rhythms. Because of this, most aquaculture systems provide the fish with diurnal lighting to maintain their natural circadian rhythms. In a farmed-fish setting, fish are fed at their optimal feeding time to promote efficient feeding and prevent waste. In addition to circadian rhythms, other factors affect feed consumption. Due to the piscine gustatory system, fish are able to discriminate between high-quality and low-quality feed that is either rancid or was stored improperly. Behavioral effects such as feed refusal are evident when poor-quality feed is administered [72].

Fish can bioconcentrate or bioaccumulate contaminants either through their food or through their gills and skin. The rate that contaminants are accumulated is based on the age, size, species, and nutrition of the fish [73, 74]. The composition of fish feed includes fish oil and fish meal as well as organochlorine residues that have been frequently detected in these components. Salmon feed can contain up to 30% fish oil and 50% fish meal. Other contaminants enter fish as a result of airborne industrial chemical release [75]. Fatty, carnivorous fish that are high on the food web are more likely to contain high contaminant loads.

With the advent of plant-based feed alternatives in aquaculture feeds such as cottonseed, soybean meal, corn meal, and peanut meal, the unintentional addition of anti-nutritional organic compounds is common. Well-known antinutritional ingredients

include protease inhibitors, phytates, glucosinolates, saponins and tannins, oligosaccharides, mycotoxins, phytoestrogens, and antivitamins. There are four subcategories of antinutrients: 1) factors that affect protein utilization and digestion 2) antivitamins 3) factors that inhibit mineral utilization and 4) other toxic substances including mycotoxins, phytoestrogens, nitrates, alkaloids, and saponins [76].

1.2.4 Aflatoxin in aquaculture

Fishmeal has typically been the most prevalent protein source in feeds; however, due to cost, fish farmers are seeking alternative sources from plants. Increased usage of plant materials in fish feed may result in an increased prevalence of mycotoxin contamination. Mycotoxin exposure is known to cause a vast array of pathological effects in fish and fish cells (Table 1). Evidence continues to mount regarding the threat they pose to farm-raised fish. While aflatoxins have been studied in multiple species such as turkeys, chickens, ducks, swine, rodents and more recently humans, they were first discovered to be extremely toxic in a farm-raised fish species, rainbow trout (*Oncorhynchus mykiss*), due to their development of AFB_1-induced liver tumors [77, 78]. Other researchers have reported that chronic dietary exposure to <1 ppb AFB_1 can cause hepatomas in rainbow trout [79, 80]. Warm-water fish tend to demonstrate more resistance to aflatoxins than cold-water fish like rainbow trout. Channel catfish (*Ictaruslus punctatus*) fed between 2,154 ppb up to 10,000 ppb AFB_1 exhibited decreases in weight gain, increases in liver histopathology, necrotic hepatocytes and infiltrating macrophages in the gastrointestinal tract [81].

Table 1. Mycotoxin-aquaculture studies

Species	Mycotoxin	Effects	Citation
Sea bass (*Dicentrarchus labrax L*)	AFB_1	Five ppb deposited in fillet, increased levels of transaminases and alkaline phosphatase activities, and significantly decreased plasma protein	El-Sayed and Khalil 2009 [82]
Tilapia (*Oreochromis niloticus* × *O. aureus*)	AFB_1	Decreased growth, HSI and lipid content, increased hepatic disorder, ATL, CYP450 1A	Deng et al. 2010 [83]
Tilapia (*Oreochromis niloticus L.*)	AFB_1	Caused cataracts, blindness, weight loss, fin and tail rot, abnormal swimming	Cagauan et al. 2004 [84]
Indian major carp (*Labeo rohita*)	AFB_1	Reduced total protein, globulin, NBT, bactericidal activities, bacterial agglutination titer	Sahoo and Mukherjee 2001 [85]
Catfish *(Ictalurus punctatus)*	AFB_1	Increased immature blood cells, excessive amounts of iron in mucosal epithelium, necrotic glands in the stomach with infiltrating macrophages	Jantrarotai and Lovell 1990 [81]
Rainbow trout *(Salmo gairdneri)*	AFB_1	Loss of B-cell memory	Arkoosh and Kaattari 2006 [86]
Guppies (*Lebistes reticulatus*)	AFB_1	Development of hepatic tumors, hyperplastic nodules, kidney lesions	Sato et al. 1972 [87]
Walleye (*Sander vitreus*)	AFB_1	Aflatoxins up to 20 ppb detected in the musculature, hepatic histopathological lesions	Hussain et al. 1993 [88]
Asian tiger shrimp (*Penaeus monodon*)	AFB_1	Poor growth rate, high susceptibility to shell diseases	Bautista et al. 2006 [89]
Juvenile hybrid sturgeon *(Acipenser ruthenus* ♂ × *A. baeri*♀)	AFB_1	Nuclear hyptertrophy, hyperchromasia, focal hepatocyte necrosis, biliary hyperplasia, inflammation in the liver, 10 ppb AFB1 accumulation in the muscle	Raghavan et al. 2009 [90]
Gibel carp (*Carassius auratus gibeli)*	AFB_1	Decreased specific growth rate and feed efficiency	Han et al. 2010 [91]
RTL W-1 trout cell line	Deoxynevalenol (DON)	Biphasic cellular response, decreased ROS production	Pietsch et. al 2011 [92]
Zebrafish (*Danio rerio*)	Zearalenone (ZEA)	Reduced spawning frequency, increased plasma vitellogenin in male zebrafish	Schwartz et al. 2010 [93]

Table 1. Continued

Species	Mycotoxins	Effects	Citation
Rainbow trout (*Oncorhynchus mykiss*)	Fumonisin B1 (FB_1) and aflatoxin B_1 (AFB_1)	FB_1 promoted AFB_1 liver tumors	Carlson et al. 2001 [94]
Catfish *(Ictalurus punctatus)*	T2 toxin and ochratoxin (OTA)	Significantly higher mortality compared to control when challenged with pathogen, *Edwardsiella ictaluri*	Manning et al. 2004 [95]
Sea bass (*Dicentrarchus labrax L*)	OTA	Fin erosion, behavioral changes, nervous and respiratory problems, hemorrhagic patches, congestion of kidney and liver	El-Sayed et al. 2009 [96]

Another species, *Oreochromis niloticus* (Nile tilapia), has been assessed for AFB_1 susceptibility in multiple studies [83, 97, 98]. In these studies, it was determined that Nile tilapia are susceptible to AFB_1 at concentrations between 1.5 and 1.6 ppm with the development of hepatic lesions, weight loss, immunosuppression and clouding of the eyes. However, while adult tilapia are relatively resistant, young fingerlings are more sensitive and have significantly reduced survival when exposed. The same age-dependent sensitivity is also noted in other species. "Yellow disease," which is characterized by yellowing of the body and skin, fin and tail rot, cataracts, abnormal motility and reduced feed intake is seen in tilapia as a consequence of AFB_1 exposure [84]. Carp fed 2.5 mg AFB_1/kg body weight over a period of 9 mo. had lesions in the liver, severely damaged kidney tissue and pathology in the gastrointestinal tract, heart and brain [77]. Sea bass exposed to 1.8 mg/kg body wt. AFB_1 for 42 d. resulted in 5 ppb AFB_1 accumulation in the muscle [82]. Therefore, one can conclude that AFB_1-

exposure can exert a bioaccumulative effect throughout the food web and humans who consume farm-raised fish are at risk for AFB_1 exposure.

Immunosuppressive effects arising as a result of AFB_1 exposure have also been cited in aquaculture studies. When trout are exposed as embryos to AFB_1, they suffer long-term immunodeficiency [99]. In this study, rainbow trout were exposed to 0.5 mg AFB_1 for 30 min., after which leukocytes were cultured and mitogens were tested after LPS stimulation. Anterior kidney immunoglobulin production was roughly 2/3 of control levels in AFB_1-exposed embryos. Nile tilapia exhibit reduced macrophage phagocytic activity after exposure to 200 ppb AFB_1 [100]. Other species such as the *Labeo rohita* experience suppressed serum bactericidal activity, neutrophil function and globulin levels after AFB_1 exposure. Aflatoxin injection in this species at 1.25 mg/kg body weight resulted in lymphocytolysis [85].

Although aflatoxins are known to adversely affect several types of fish, they have not been thoroughly investigated in all aquaculture species. More information is needed on aflatoxin contamination in feeds and fishmeals, toxicological effects induced in farmed aquatic species, and DNA damage in fish genomes and its impact on carcinogenesis [13]. Aflatoxin contamination has been detected in aquaculture feeds and occurs in several feed components including soybean, peanuts, maize, rice and menhaden meal. A study carried out in Egypt detected aflatoxin levels exceeding 1,000 ppb in fish feed [101]. Others in Portugal determined that out of 35 samples of fish feed, over 40% tested positive for AFB_1 [102]. Another investigation quantified aflatoxin and fumonisin levels from tilapia feed in Nayarit, Mexico and determined that 63% of

samples tested positive for aflatoxins, however all levels were below the European maximum for animal feed [103]. In the Philippines, researchers determined that shrimp feed contained AFB_1 levels from 0 to 120 ppb [89]. One study from Brazil analyzing 60 fish feed samples from Rio de Janeiro State resulted in 35% of the samples testing positive for *A. flavus*, and 55% of samples testing positive for AFB_1 contamination [104]. Animal and aquaculture feeds are often co-contaminated with AFB_1 and other mycotoxins, including fumonisin B_1 (FB_1), ochratoxin and zearalenone [105].

1.3 Therapeutic clays

1.3.1 Clays in ancient medicine: a historical perspective

Geomaterials such as soils and clays can have major beneficial impacts on human health. Clays are aluminosilicates mainly composed of oxygen, silicon and aluminum, with traces of Na, K, Mg and Ca. Geophagy is described as the "deliberate consumption of earth, soil or clay", a practice that has existed worldwide for thousands of years. Geophagy is well-documented in southern parts of the United States, Africa, and Asia and is considered "culturally acceptable" [106]. Pregnant women in developing countries are known to consume earth, especially during famine [107]. Early Greek and Roman medical textbooks document the practice of geophagia, and early reports indicate an association between geophagia and alleviating anemia [108]. "Hippocrates and Aristotle both wrote about medicinal clays and also gave classification to several clays based on their geographic origin such as *Terra Samia, T. Sigillata, T. Lemnia, T. Cimolia, T. Sonoptica, T. Eretia, and T. Negra* [109, 110].

1.3.2 Clay as an aflatoxin intervention

Because of AFB_1-related morbidity and mortality in multiple species, there is a need for intervention strategies to reduce AFB_1 exposure. There are several methods of controlling aflatoxin exposure, including food and feed processing, microbial inactivation, dietary strategies, chemical degradation or chemisorption with clay. Adsorbents including zeolites, silicates and organoclays have been evaluated, however smectite clay has been the most effective *in vitro* and has demonstrated tight binding complexes with the aflatoxins [111]. These complexes are stable from pH 2-10 *in vitro* as well as at temperatures of 25 and 37° C. Less than 10% of parent AFB_1 can be dissociated from the complex by a series of solvents. Ellis et al. (2000) determined that a minimal level of smectite (1%) was capable of removing 44% and 52% of AFM_1 in cattle and goat milk samples, respectively. Two-percent bentonite clay inclusion has been used in rainbow trout feed to reduce toxicity resulting from 20 ppb AFB_1 exposure and significantly decreased liver cancer rates [112]. Dosage of clay-based additives in animal feeds to prevent aflatoxin toxicity has varied substantially. In pigs dosed between 500 and 800 ppb AFB_1, 1 and 2% Ca-bentonite was just as effective as 0.5% in reducing AFB_1 serum levels [113]. Consumption of clay-based minerals has resulted in decreased bioavailability of AFB_1 to target organs such as the liver, muscle and blood [114].

Originally used as an anti-caking agent in animal feeds, NovaSil (NS) is a calcium-montmorillonite clay that has demonstrated AFB_1-binding ability both *in vitro* and *in vivo*. Broiler and leg-horn chickens were rescued with NS from the effects of exposure to 7500 ppb AFB_1 [115]. Importantly, NovaSil has been used safely and

efficaciously in humans in a number of clinical trials to reduce exposure from both AFB_1 and fumonisin (FB_1), both in the United States and in Africa [116, 117]. NovaSil does not significantly alter serum levels of vitamins and minerals including, calcium, vitamin A and iron in rodents or human subjects [118]. NovaSil has also been tested for its ability to reduce transfer of AFM_1 to milk following ingestion of aflatoxins in cows [119]. In that study, cows were fed diets contaminated with 200 μg AFB_1/kg or 0.5% NS+200 μg/kg AFB_1 for 7 days. During the final 7 days, NS was removed from the diet. The presence of 0.5% NS decreased AFM_1 milk levels by an average of 0.44 μg/L (44% reduction).

There are two types of NovaSil currently available for animal and human use. The original parent compound, NovaSil Plus (NSP), has been frequently used in animal feeds. More recently, a more refined version, Uniform Particle Size NovaSil (UPSN) has been developed to minimize the content of particles >100 μm. This was developed to decrease batch to batch differences and increase palatability.

1.3.3 Smectite clays

Smectite clays are a group of silicates with an expandable interlayer and several structural features that affect surface charge. Montmorillonite is one sub category of smectite clays and is characterized by a 2:1 dioctahedral structure (Fig. 4). The chemical formula for montmorillonite is $(Na,Ca)_{0.33}(Al,Mg)_2(Si_4O_{10})(OH)_2*nH_2O$. In geological features over time, isomorphic substitution can cause many elements, including those with a less positive charge, to substitute into these sites, thereby causing an overall negative charge in the interlayer. For example, Mg^{2+} can substitute for Al^{3+} in octahedral

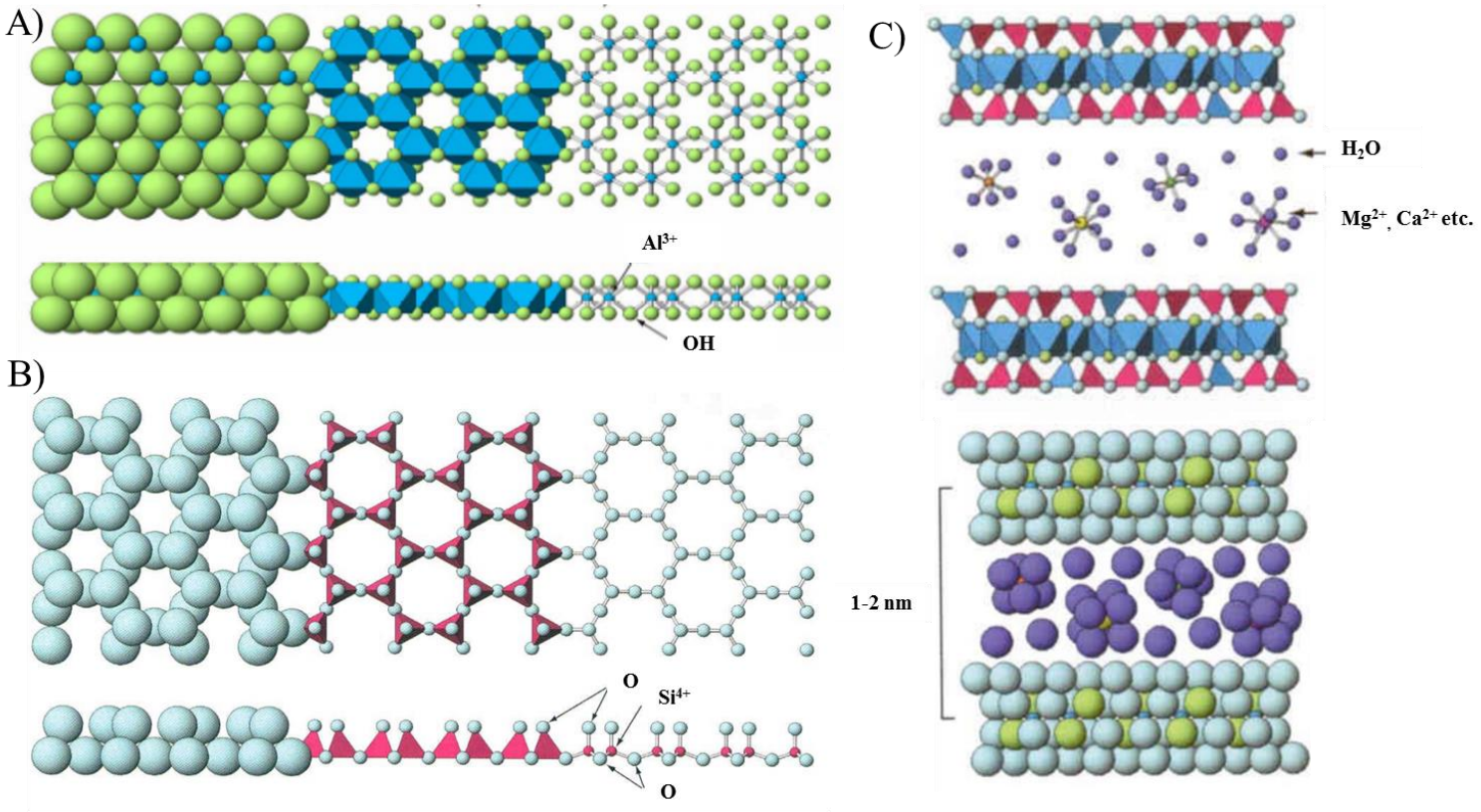


Figure 4. Molecular schematic of smectite. A) Dioctahedral sheet B) tetrahedral sheet C) structural schematic of smectite clay [120].

sheets and Al^{3+} can substitute for Si^{4+} in tetrahedral sheets. This exchange (isomorphic substitution) is responsible for the interlayer charge. Soil smectite usually contains Ca^{2+} and Mg^{2+} that can be replaced by Al^{3+}. The montmorillonite crystal lattice consists of layers that are approximately 1-2 nm thick. There is one octahedral sheet sandwiched between two tetrahedral sheets, similar to talc or mica. Each tetrahedron consists of four oxygen molecules and either an Al or Si. Each octahedron contains Al, Fe or Mg surrounded by four oxygen molecules and two –OH groups. These layers create stacks and the opening between each layer is referred to as the interlayer [120].

Scanning electron microscopy (SEM) is frequently used to characterize smectite clays without damaging the structure. Many montmorillonites display a honeycombed polygonal pattern, though some exhibit a thin film and flake-like structure, such as granordiorite [121]. The cation exchange capacity (CEC) is approximately 110 $cmol_c$ kg^{-1} for soil smectite. Some high-charge smectites possess higher potassium levels and are therefore similar to vermiculite. The edges of smectite can also contribute to the CEC. Approximately 5 $cmol_c$ kg^{-1} of the total CEC is attributed to the edge charge. Isomorphic substitution causing a permanent charge from OH bonds and broken O bonds is called "pH-dependent charge." Typically, swelling and shrinking have also been characteristics of clays and smectite. In particular, crystalline swelling occurs when H_2O molecules are sequestered between the interlayers. Water molecules interstratifying several layers or in different packing arrangements can influence the swelling range of the crystalline structure [122]. Forces of attraction and repulsion can influence

crystalline swelling and coulombic attraction between the negative and positive surface charge sites causing electrostatic attraction to molecules.

X-ray diffraction (XRD) is a way to characterize crystalline materials. In XRD, an x-ray beam hits the material and based on the incident angle, characteristics such as the distance between planes (the d-spacing), can be determined. Heat and humidity can both affect smectite and these factors must be controlled when analyzing smectite with XRD. X-ray diffraction will result in a 1 nm peak for smectite that can collapse slightly at 110 degrees C [123].

1.3.4 Clays in intestinal illness

One potential application for smectite clays is to treat intestinal illness. The first known book that documented the use of "stones" for medicinal benefits was called *De Materia Medica* and mentioned pharmaceutical uses of clays especially for stomach and intestinal illness. Clays that are used frequently for pharmaceutical use include smectites, palygorskite, kaolinite and talc (Table 2). Clay minerals that have been used specifically for gastrointestinal complaints include palygorskite and kaolinite. Clays have been cited to coat the gastric wall and absorb toxins, bacteria and viruses [124, 125]. Binding studies performed with *Salmonella enteriditis* indicated that a cetylpyridinium-exchanged montmorillonite clay was able to reduce bacterial counts by 98%. *In vitro*, montmorillonite clay sorbed rotavirus and coronavirus, two viruses that cause intestinal symptoms [126]. Additionally, they have been known to alter tight junctions between cells, thereby improving cellular adhesion resulting in enhanced pancreas and intestinal enzyme activities and mucosal integrity [127].

Table 2. Clay-based supplementation in aquaculture feed

Species	**Clay**	**Effects**	**Citation**
Eastern oyster (*Crassostrea virginica*)	kaolinite	kaolinite adsorbed organic matter from feed	Urban et al. 1992 [128]
Oyster (*Crussostrea gigus)*	kaolinite, illite, montmorillonite	kaolinite and montmorillonite particles (3-4um) increased ingestion	Sornin et al. 1988 [129]
European seabass (*Dicentrarchus labrax*)	Zeolite	up to 20% inclusion did not affect protein digestibility or growth performance	Dias et al. 1998 [130]
Nile tilapia (*Oreochromis niloticus*)	Cu2+ exchanged montmorillonite	antibacterial activity, protected intestinal mucosa from pathogens, improved growth performance	Hu et al. 2007 [131]
Nile tilapia (*Oreochromis niloticus*)	montmorillonite	montmorillonite reduced Pb accumulation in kidney, intestine, bone, stomach, gill, liver, spleen	Dai et al. 2010 [132]
Mozambique tilapia (*Oreochromis mossambicus*)	Zeolite	reduced Cd levels in fish	Sampath 1999 [133]
Multiple farmed fish species	Bentonite	reduced AFB1 levels in fish feed	Winfree and Alred 1992 [134]
Nile tilapia (*Oreochromis niloticus*)	montmorillonite	reduced sterigomatocystin-related genotoxicity	Abdel-Wahhab et al. 2005 [135]

Diarrhea, which can be caused by a variety of viruses and bacteria, is defined as the passage of three or more loose stools per day. Diarrhea can also be caused by chronic health conditions, defective intestinal absorption, allergies and food poisoning. Diarrhea can be life threatening especially for the very young, the malnourished, or those with impaired immune systems. The WHO has estimated that more than 1 in 10 children die from diarrhea each year, which is approximately 800,000 children [136]. Only 44% of these children with diarrhea are able to receive medical treatment [137].

Most anti-diarrheal agents involve the absorption of excess water from the large intestine, thereby resulting in more compact feces. Calcium smectites have been used to treat diarrhea and are excreted completely in the feces rather than being absorbed. These clays can either be administered directly into the food or administered via tablet or capsule to treat diarrhea. Additionally, Ca^{2+} in the interlayer of the clay can exchange for other divalent ions.

Clinical trials and animal studies alike have extensively tested the efficacy, safety, tolerability and palatability of clays for health supplementation and found little to no adverse side effects [114] (Table 3). One study in Italy with 804 children examined the efficacy of a dioctahedral smectite (DS, or diosmectite) clay for treatment of acute diarrhea [138]. Smectite supplementation was found to reduce the duration and frequency of diarrhea, in addition to improving stool consistency. Another similar study in India examined DS in 117 children with watery diarrhea [139]. There were similar findings in terms of reduced duration of diarrhea, and no adverse side effects from

Table 3. Gastroenterology studies with clay

Condition	**Treatment**	**Results**	**Citation**
Gastroenteritis	Smectite	Reduced duration of diarrhea and prevented a prolonged course	Guarino et al. 2001 [138]
Rotavirus and coronavirus (*in vitro*)	Smectite	Clay sorbed between 77-99% of viral load	Clark et al. 1998 [126]
Infectious diarrhea	Smectite	Shortened diarrhea duration and frequency of liquid stools	Madkour et al. 1993 [140]
Infectious diarrhea	Smectite	Successfully used with rehydration therapy to treat diarrhea	Szajewska et al. 2006 [141]
Acute gastroenteritis	Smectite	Duration of diarrhea reduced and no effect on electrolyte concentrations	Narkeviciute et al. 2002 [142]
Irritable bowel syndrome (Diarrhea)	Diosmectite	Diosmectite was well-tolerated and alleviated pain-related symptoms	Chang et al. 2007 [143]
Chronic functional diarrhea	Smectite	Decreased frequency of daily bowel movements and	Yao-Zon et al. 2004 [144]
Acute diarrhea	Smectite	Shortened diarrhea duration and improved dehydration	Yen et al. 2006 [145]
Acute watery diarrhea	Diosmectite	Reduced duration of diarrhea and prevented a prolonged course, thereby possibly reducing costs	Mujawar et al. 2012 [139]
***Clostridium difficile* toxins (*in vitro*)**	Ditrioctahedral smectite	Neutralized toxins A and B from *Clostridium difficile*	Weese et al. 2002 [146]
Post-operative diarrhea in horses	Ditrioctahedral smectite	Reduced occurrence of diarrhea immediately following surgery	Hassel et al. 2009 [147]
Acute diarrhea	Diosmectite	Diosmectite with rehydration shortened the course of illness	Lexomboon et al. 1994 [148]
Enteric diarrhea in weanling pigs	Palygorskite	Protective effect against diarrheal infection and improved intestinal wall barrier	Zhang et al. 2013 [149]
Diarrhea in weanling pigs	Kaolinite	Alleviated symptoms	Rivera et al. 1978 [150]

administration of the clay, despite the relatively high dose administered (6g/day). Another 2006 study evaluated the efficacy of DS clay to alleviate diarrhea- predominant irritable bowel syndrome [143]. One-hundred and four patients between the ages of 20 and 80 who had reported diarrheal episodes prior to the study were subject to DS treatment. This treatment consisted of a dose equal to one g 3 times a day for 8 weeks. Another study involving cases of constipation-predominant IBS reported that beidellite (a montmorillonite clay) was able to adsorb gas and led to modification of luminal chemical changes [151]. Patients experienced a decrease in IBS symptoms, compared to the placebo. Additionally, pain and discomfort was significantly improved. Diosmectites have been used in studies to treat functional bowel disorders, in which they alleviated abdominal swelling and bloating and provided an alternative to dietary restriction [152].

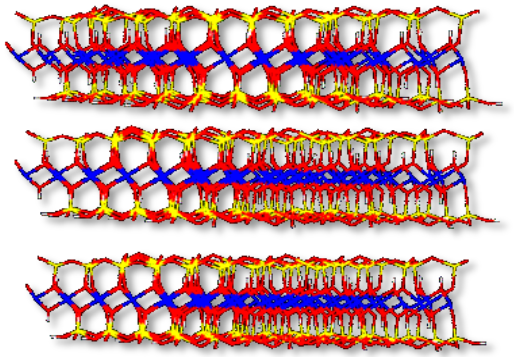
A few studies have been published regarding the use of diosmectites as therapy for IBD [153]. In a short-term investigation, the anti-inflammatory effects of smectite were observed in a rat colitis model. Expression of MUC2, a gene that encodes for a mucin-secreting protein, was upregulated in colitis-induced mice administered smectite, suggesting that mucin production increased. This study determined that colonic IL-1β was reduced, the severity of diarrhea decreased, and histopathological findings diminished with clay supplementation. There was also a marked decrease in diarrhea and reduction of nitric oxide synthase, a proinflammatory mediator. Although long-term clay ingestion has not been evaluated thus far, clinical study results indicate that a low-dose clay regimen is safe and tolerable [116, 154].

1.3.5 Clay-bacterial interactions

Some studies have documented clay-bacterial interactions. In soil environments, clays and bacteria can form "hutches" or aggregates that house bacteria. Lucnsdorf et al. (2000) determined *Pseudomonas putida* bacteria can colonize the surface of montmorillonite clay structures, as indicated through scanning electron microscopy [155]. However, 88.7% were readily desorbed, suggesting that weak, non-electrostatic forces such as hydrogen bonding were involved in the sorption process. Montmorillonite (Fig. 5) itself does not possess anti-bacterial properties, but some investigators have attempted to exchange ions in the clay for other ions that possess anti-bacterial properties. Hu et al. (2005) exchanged copper onto montmorillonite and examined its effects on *E. coli 88*. Bacterial suspensions were prepared and harvested. Antibacterial activity was assayed by using Ca-MMT, Na-MMT, Cu-Ca-MMT and Cu-Na-MMT and the percent reduction was further calculated. The authors reported that the antibacterial activity of Cu-MMT was not affected by pH. Furthermore, copper was found to be exchanged in the interlayer when the pH was above 4.5 by entering the ditrigonal hole in the octrahedral sheets [131].

Another study examined the effect of montmorillonite on growth performance and fecal microflora in weaning pigs subject to zearalenone exposure [156]. In this work, *Lactobacillus* counts increased while *Escherichia coli* counts decreased, regardless of the inclusion rate of montmorillonite. *Lactobacillus* is typically seen as "beneficial" bacteria and *E. coli* more pathogenic, however, the entire microbiome needs to be considered when assessing relative proportions of bacteria. Montmorillonites have

A)



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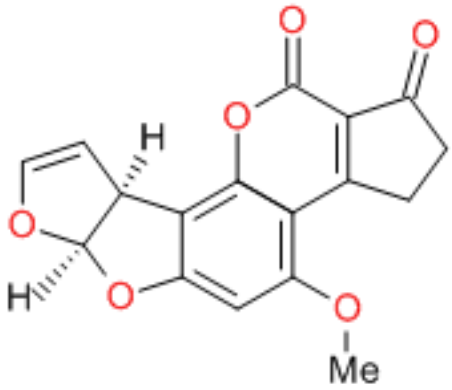


Figure 5. Structure of NovaSil (NS) and AFB_1

NovaSil, calcium montmorillonite clay demonstrating oxygens (red), silica (yellow) and aluminum (blue) composing the 2:1 dioctrahedral structure as well as the interlayer space between each layer (Fig 2A). Structure of aflatoxin B_1 (AFB_1) (Fig 2B).

demonstrated antibacterial ability by altering bacterial cell membrane permeability [157]. Montmorillonite supplementation also significantly increased intestinal villus height and villus height:crypt depth ratio in the jejunum of young pigs [158]. Xia et al. 2005 challenged weanling pigs with *E. coli* and examined the effect on the intestinal barrier and bacterial translocation from the intestinal lumen. When intestinal barrier function is reduced, enteric pathogens migrate more readily from the lumen to the lymph nodes. This study found that *E.coli* increased bacterial translocation, and smectite supplementation reduced translocation [159]. *In vitro* research has shown that smectite clays have the capability of binding bacteria and bacterial toxins, such as *Clostridium perfringens* [160]. To date, there have been no molecular-phylogenetic studies to characterize the intestinal microbiome in animals consuming dietary clay.

While microbiology was once entirely culture-dependent, the field has been revolutionized by next generation sequencing. Next generation sequencing (NGS) is the latest method of phylogenetic characterization for bacterial groups. One of the first high-throughput sequencing methods (pyrosequencing-454) was introduced in 2005 and was then replaced with Illumina Sequencing in 2007. With NGS, bases of fragmented DNA are identified using emitted signals as DNA is resynthesized from the template strand. These reactions take place in a forward and reverse fashion and result in readings that are thousands of sequences deep. The sequences are then compared to a microbial reference database to determine phylogenetic analysis using QIIME software. Other

methods of microbial sequences are available, but Illumina (Solex) sequences is currently the most widespread [161].

There are two predominant measures of microbial diversity, or the number of operational taxinomical units (OTUs) in each sample: 1) alpha diversity and 2) beta diversity. Alpha diversity is defined as the species richness (number) and distribution (evenness) of taxa in a single population. Alpha diversity is visualized using rarefaction curves in which increasing numbers of sequenced taxa demonstrate increased precise estimates of the total bacterial population in the samples. Beta diversity is defined as the number of shared taxa between populations.

1.4 Crohn's disease

1.4.1 Crohn's disease overview

Next generation sequencing has been used to study microbial profiles in Crohn's disease (CD) animal models and in CD patients. Crohn's disease, classified as an inflammatory bowel disease (IBD), is characterized by inflammation and ulceration anywhere along the gastrointestinal tract. Crohn's disease is distinguishable from ulcerative colitis, the other major form of IBD which affects the large intestine only. Crohn's disease was discovered in 1932 by an American gastroenterologist, Dr. Burrill Bernard Crohn. In his publication titled "Terminal Ileitis: A new clinical entity," he describes the symptoms that are commonly experienced [162]. These symptoms described include diarrhea, abdominal pain, rectal bleeding, growth stunting (in children), fatigue, and weight loss.

Formation of fistulae can develop at any time throughout the course of the disease and in women, colonic-vaginal fistulae or colonic-anal fistulae can develop and cause infections in the lower intestine, vagina or urinary tract. Other patients who do not develop fistulae, may develop intestinal scar tissue that causes blockages [163]. Many patients that develop Crohn's will require surgery to remove the diseased section(s) of the GI tract. The disease affects both men and women equally, unlike many autoimmune diseases which disproportionately affect women [164]. While the exact etiology of Crohn's disease is unknown, several factors have been identified that are thought to be primary contributors of the disease.

1.4.2 Causes of Crohn's disease

Currently, there are three theories regarding the etiology of CD: 1) the genetic autoimmune theory, 2) the mycobacterium infection theory, and 3) the immunodeficiency theory [165]. The first theory (genetic autoimmune) suggests that there is a genetic predisposition to dysregulation of gastrointestinal immunity and that inflammation from CD is an inappropriate immune response to endogenous luminal antigens in a genetically susceptible host [166]. Nucleotide-binding oligomerization domain 2 (NOD2) is a pattern recognition receptor and is only expressed in specific cells in the body including Paneth cells and intestinal epithelial cells. This intracellular sensor for small peptides is derived from peptidoglycan, a component of bacterial cell walls. The current thought in Crohn's research is that NOD2 is responsible for several innate immune responses such as type I interferon and autophagy [167]. Additionally, toll-like receptors are known to participate in cross-talk with NOD2. NOD2 is specifically

responsible for activation of NF-kB, an inflammatory mediator that controls the transcription of DNA in the nucleus of the cell. NF-kB is an important component of the immune response and can promote other pro-inflammatory chemokines and cytokines that are downstream, such as TNFα and IL-1β [168]. This can ultimately cause a "cytokine storm," causing excess inflammation along the GI tract. In addition to NOD2, other genetic factors such as CARD15 that exhibit polymorphisms in their LRR (leucine rich repeats) as well as polymorphisms in pattern ligand recognition domain of NOD2 also contribute to the etiology of Crohn's [169, 170]. Mutations in NOD2, an abnormal balance of intestinal flora, environmental factors such as smoking and stress, and a deficiency in IL-10 or other anti-inflammatory cytokines all contribute to Crohn's disease [171].

Mycobacterium avium subspecies *paratuberculosis* (MAP) is one of the first successful human pathogens and infects at least 2 billion people worldwide [172]. MAP can shield itself from a host of immune responses, because of its tough outer cell wall and also its ability to prevent phagosome-macrophage fusion. PCR, serum antibodies, cultures from intestinal tissue, breast milk, and blood cultures all indicate that CD patients have a higher risk for contracting *M. tuberculosis* than controls without CD [173]. For example, one study indicated that 50% of Crohn's patients (14/28) tested positive for *M. Tuberculosis* antibody vs. 0% of controls [174]. The theory that MAP causes CD is one reason why antibiotics are often indicated for clinical treatment, because antibiotics are used to treat bacterial infections. Additionally, it is well documented that mycobacterial infections are difficult to treat in immunosuppressed

patients [175]. The mycobacterial theory is therefore tied to the "immune deficiency" theory suggesting that patients with immune suppression are more susceptibility to mycobacterial-related CD.

The third well-known theory suggests that CD is caused by an immune deficiency. Defects in the innate immune system can be responsible for excessive inflammatory response. This theory suggests that because of these disturbances with the innate immune system, treatment should be focused on augmenting innate immunity, rather than suppressing it [176]. This theory also explains how a pathogen (such as *M. tuberculosis*) might be more likely to invade an already immunosuppressed host. The dysfunction of neutrophils might play a role in the pathogenesis of CD. Post-mortem examinations of Crohn's patients may reveal granulomata, which are present in a number of other diseases with known etiologies such as tuberculosis and berylliosis. In these diseases, it is the failure of the immune system, more specifically, a defect in antigenic clearance from the bowel walls, that causes the granulomata [177].

1.4.3 Environmental factors in Crohn's

Smoking, stress, oral contraceptive use, antibiotic exposure, and diet are all factors that can influence the development, progression, and relapse of CD. Dietary factors can also contribute to CD progression [178, 179].

There are approximately 800-1000 species of bacteria in the human colon [180]. Dethlefsen et al. (2008) indicated that antibiotic usage had a greater longer-lasting impact on intestinal commensal flora than originally perceived [181]. A different study followed several subjects who were administered a 7-day treatment with

clindamycin [182]. Following this treatment, fecal microbiota samples were collected at nine different time points over the course of two years. Compared to the control group, a substantial disturbance in the fecal bacterial community was detected, especially in *Bacteroides* isolates. Additionally, *Bacteroides* levels never returned to normal during the 2-yr. time frame. While it has not been determined if prior antibiotic use is causal for the establishment of CD, intestinal flora dysbiosis has been a key finding in CD patients [183]. In another study, the influence of smoking and oral contraceptives on relapse of CD patients was examined. While women using oral contraceptives and smokers had an increased risk of CD relapse, ex-smokers did not [178]. Epidemiological evidence has suggested that excess stress can contribute to the course of both ulcerative colitis and CD [184]. Identification of CD risk factors is one step towards management and future prevention of flare-ups.

1.4.4 Incidence of Crohn's disease

People of European Jewish descent are four to five times more likely to develop CD than others [185]. Crohn's disease commonly begins between the ages of 15 and 35, and is characterized by recurring "flares" throughout a patient's life. Individuals with a family member who has CD are 10-30 times more likely to develop CD in his or her lifetime when compared to those who do not [186]. Prevalence of CD in North America can range from 26 to 198 cases per 100,000, which totals 400,000 to 600,000 patients [187]. Prevalence in African Americans has increased in recent years, compared to Caucasians. Additionally, rates are substantially lower among Hispanic and Asian

populations [188]. Between 30-60% of patients with CD that are in remission will relapse within one year [189].

Additionally, geographic variations that correlate with CD incidence are documented, especially at northern latitudes. For example, CD has more of a prevalence in the northern United States than the southern United States and the Canadian province of Manitoba has the highest incidence of CD in the world [190]. In fact, some studies have suggested that exposure to enteric organisms during childhood can program the gastrointestinal immune system and prevent IBD later in life [191, 192, 193].

1.4.5 Cytokines involved in Crohn's disease

Crohn's disease inflammation is mainly driven by a Th1/Th17 (T helper) response that causes an upregulation of proinflammatory cytokines. Proinflammatory cytokines are secreted by T-cells and other immune cells such as macrophages that cause an inflammatory cascade. Multiple cytokines are also involved in the development and progression of CD, however tumor necrosis factor α (TNFα) is the major inflammatory player in CD [194]. Tumor necrosis factor α is involved in multiple inflammatory diseases including CD, ulcerative colitis, psoriasis, and Wegener's granulomatosis. This cytokine is responsible for regulating the innate immune response and is produced by several immune cells including monocytes, dendritic cells, B cells, CD4+ cells, neutrophils, mast cells, eosinophils, fibroblasts, epithelial cells, and smooth muscle cells [195]. Tumor necrosis factor alpha can be produced in membrane-bound or secreted forms that are 26 and 17 kD, respectively. When TNFα binds to the TNFα receptor, this receptor-ligand interaction causes an intracellular cascade of signaling that

leads to phosphorylation of IκBα. Subsequently, transcription factor NF-κB is activated, which increases the production of other proinflammatory cytokines, such as IL-1β, IL-6, IL-8 and TNFα [196].

Tumor necrosis factor α is a key mediator in mucosal inflammation. Dionne et al. (1997) determined that TNFα protein and mRNA levels are upregulated in CD patient biopsies, which have also been found to correlate with serum and stool levels [197]. High levels of TNFα have been implicated in growth stunting of children with chronic IBD. In a study with 31 children, the growth velocity was significantly lower in children with high TNFα levels as a consequence of an IBD [198].

Interleukin 1β is another key inflammatory mediator in CD. Polymorphisms in IL-1β can also contribute to the course of IBD. Patients with polymorphisms in allele 1, position +3953 and allele 2 at position -511 are more likely to suffer from CD [199]. If both allelic pairs are present, then the patient is more likely to need surgical intervention. One study demonstrated that in patients with high concentrations of TNFα, the median CD relapse time was 79 days [200]. When lower concentrations of TNFα were present, the median relapse time was 200 days. Interleukin 1β secretion followed a similar trend and association with risk of relapse. Low concentrations of IL-1β (<75pg/mL) resulted in remission of an average of 180 days, while a high concentration of IL-1β had a relapse rate within 1 year. Stable remission was also dictated by the concentration of IL-1β.

1.4.6 Animal models in IBD

In order to study IBD and its potential treatments, animal models are needed to simulate the disease. There are three predominant rodent models used in IBD research:

1) IL-10 knock-out mice, 2) the Dextran sodium sulfate (DSS) model, and 3) the 2,4,6-trinitrobenzenesulfonic acid (TNBS) model.

TNBS colitis produces a Th1-Th17 response with an upregulation of IL-12 and IL-17 as the disease becomes increasingly chronic. Because of its characteristic cytokine profile, the TNBS colitis model is used as a CD model for acute and chronic studies [201]. Once a week, TNBS is administered via intrarectal injection and symptoms peak approximately three days afterward. SJL/J mice are the most susceptible to TNBS induction because they have a genetically determined high IL-12 response. However, the literature suggests that BALB/c strains are less susceptible to TNBS than SJL/J mice, resulting in several researchers using BALB/c mice over SJL/J mice to reduce mortality rates [202]. Males develop significantly more chronic disease than females in the TNBS model. Additionally, the optimal age range of mice induction is between 4 to 8 wks. Mice under 4 wks suffer high mortality and mice older than 8 wks demonstrate diminished sensitivity [201]. Mechanistically, ethanol (the delivery vehicle) breaks the mucosal barrier and TNBS haptenizes the colonic proteins, which causes an inflammatory cascade. CD4 T cells are responsible for the cell-dependent mucosal immune response in chronic TNBS colitis studies [203].

1.4.7 Microbial ecology in Crohn's disease

Studies in murine models have also suggested that "spontaneous" colitis can develop simply due to the presence of particular strains of bacteria in the lumen [204]. Evidence suggests that the interaction between commensal flora and the immune defensive response can initiate or contribute to the pathogenesis of IBD [193]. Not

surprisingly, fecal microbiota can differ substantially between CD patients with the active disease and CD patients in remission. For example, proportions of *Enterobacteria* are significantly increased [205] and lower diversity of *Firmicutes* exists in patients with active CD [206]. However, because knowledge of microbial interactions is limited, an understanding of microbial impact in the context of IBD is also limited. However, a diagnosis or prediction of CD cannot be made with microbial profiling results alone.

Results from a previous study indicated that twins have highly similar intestinal bacteria composition [207]. This study examined twins with CD and also compared them with sets of healthy control twins. T Terminal-restriction fragment length polymorphism (T-RFLP) and percent guanine to cytosine (% G to C) profiling of the total intestinal microbiome was used to assess the differences. In this study, patients with illeal CD had an overabundance of *E. coli* and decreased abundance of *Faecalibacterium prausnitzii*, compared to healthy controls and patients with CD presenting in the colon only.

The epithelium of the intestinal mucosa is constantly communicating with luminal flora, in addition to a host of adaptive immune cells [208]. It has been suggested that commensal bacteria can lessen intestinal inflammation by inhibition of IKBα degradation, protein ubiquination and PPARγ-mediated nuclear export of RelA, a molecule that is responsible for signal transduction [209]. Goblet cells are highly responsible for epithelial and mucosal repair. Goblet cell specific protein RELMβ is induced on bacterial colonization [210]. The mucosal immune system can detect bacterial antigens on the surface and instigate the appropriate response, depending on symbiotic and pathogenic bacteria [211]. M cells and IgA complexes are responsible for

monitoring luminal microbes and antigens that are detected by antigen-presenting cells (APCs) [212]. Myeloid-derived dendritic cells can then sample bacteria or bacterial antigens by the CX3CR1 mechanism [213]. After contact with microbial antigens, dendritic cells can produce IL-23, which causes intestinal inflammation in murine colitis [145]. Specific classes of receptors recognize microbial patterns and engagement of toll-like receptors by microbes can trigger activation of signal transduction. The leucine-rich repeat domain of NOD2 recognizes muramyl dipeptide (MDP), a component of bacterial peptidoglycans and can activate NF-kB through the receptor serine-threonine kinase-2 [214]. Production of defensins, which are cationic proteins protect the body against bacteria and fungi, are also diminished in NOD2 dependent CD [215]. NOD2 can improve antibacterial immunity and bacterial survival due to lack of defensins and other factors might enhance bacterial survival [216]. The aforementioned study found that knock-out mice ($NOD2^{-/-}$) don't normally develop spontaneous intestinal inflammation, unless introduced to a pathogenic bacteria such as *Listeria*.

1.4.8 Current treatments for Crohn's disease

The effectiveness of CD treatments varies substantially from patient to patient. There are four predominant classes of drugs currently prescribed for CD: 1) TNFα antagonists such as infliximab (Remicade) and adalimumab (Humira), 2) corticosteroids such as prednisolone and budesonide, and 3) antibiotics including metronidazole and ciprofloxacin and 4) immunosuppresants such as cyclosporine, azathioprine and 6MP. Infliximab and similar monoclonal TNFα antibodies, such as adalimumab, have been the

most widely recommended in recent years for refractory CD [217]. In one study, anti-TNFα (Infliximab) was administered to patients for 6 months [218]. At the end of the treatment, red blood cell folate, plasma homocysteine, vitamin B_{12}, methylmalonic acid, vitamin D, β-carotene, serum iron, and total iron binding capacity were tested. Additionally, the prognostic inflammatory and nutritional index (PINI), an index of overall nutrition status and inflammation was assessed through serum measurement of alpha1-acid glycoprotein, C-reactive protein, albumin and prealbumin. Infliximab helped maintain consistent levels of serum vitamins and improved PINI. One study that analyzed 11 clinical trials determined that between 6 and 24% and 21 and 24% of patients on adalimumab and infliximab treatment, respectively, achieved remission when compared to the placebo [219]. In the same study, fistulating CD outcomes were analyzed and the patient remission rate with TNFα antibodies was between 23 to over 50% when compared to a placebo.

Maintenance of CD is a challenge, and can be very costly for the patient. In 2011, one study determined that the median cost of infliximab was $10,275.33 and the median cost of adalimumab was $9328.75 per patient, with no significant cost difference between the two treatments [220]. Infliximab is prepared and administered to the patient as an intravenous injection, as is adalimumab. Most patients undergo a series of injections over the course of several weeks under the supervision of a physician. Side effects of infliximab, adalimumab and other anti-TNFα monoclonal antibodies include respiratory and urinary tract infections, nausea and vomiting, headache, weakness, fever, abdominal pain, rash, flu-like symptoms and high blood pressure. There is an increased

risk of tuberculosis infection as well as an increased risk of certain types of cancers such as T-cell lymphoma and skin cancer. Other side effects include an increased risk of hepatitis B infection, allergic reaction and heart failure [217]. Patients receiving TNFα antagonists should not receive vaccines during therapy because of the potential for an adverse immune response [221].

Corticosteroids are a main staple of Crohn's treatment and are usually prescribed first to reduce inflammation before TNFα antagonists are administered. Corticosteroids such as prednisolone and budesonide are administered either intravenously or in tablet form. Side effects from corticosteroids are different from TNFα antagonists and include facial swelling, excessive hair growth, hyperactivity, high blood pressure, diabetes, glaucoma, cataracts due to increased eye pressure and osteoporosis [222]. A new corticosteroid, budesonide has been shown to produce fewer adverse steroid reactions [223]. Dosage and administration of steroids are closely monitored in children because steroids can cause growth stunting [224]. One of the major problems with corticosteroid therapy is the eventual loss of efficacy with successive treatments, which typically leads to a change in therapy to antibiotics and/or anti-TNFα antibodies [225].

Antibiotics are a third class of drugs that are used in treating CD. Several reviews have compared antibiotics for short-term and long-term use in CD. For patients with active CD, long-term use of either nitroimidazoles or clofazimine is effective [226]. Another study indicated that rifaximin was the most efficacious in inducing remission [227]. Disadvantages of antibiotics include long-standing alterations in

intestinal flora, an increase in antibiotic resistant pathogens, and digestive discomfort [228].

Cyclosporine, an immunosuppressant, is occasionally administered for CD, but has yielded mixed results in clinical trials [229]. It is traditionally used in organ-transplant patients to prevent rejection. Cyclosporine inhibits IL-2, which is responsible for T-cell proliferation [230]. IL-2 inhibition has been somewhat successful in CD therapy and is typically used when other treatments, such as steroids and antibiotics fail. However, cyclosporine has been more successful in achieving remission of ulcerative colitis due to the fact that upregulation of IL-2 is more prominent in ulcerative colitis than in CD [231]. Other immunosuppressive agents including 6-mercaptopurine (6MP) and azathioprine have been used to induce remission of CD [232]. These two drugs are cytotoxic to lymphoid cells which makes them an effective treatment in CD [233].

1.4.9 Alternative/homeopathic treatments

Alternative and homeopathic treatments are also used usually as adjunct therapies. Probiotics have been under investigation for several years as a potential treatment for CD and other diseases and disorders of the bowel. The results from clinical trials have been somewhat mixed as to whether or not probiotics are an effective treatment for CD [234]. Probiotics are defined as living microorganisms that are believed to benefit gastrointestinal health by growing in the intestine and altering the composition of existing bacterial colonies. Several strains such as *Lactobacilli* and *Bifidobacteria* are currently used to treat IBDs [235]. These "good bacteria" allegedly alter the flora and the metabolites produced, ultimately resulting in anti-inflammatory

effects. However, probiotics have disadvantages in certain patients. For example, administration of *Lactobacillus GG* as a therapeutic treatment for antibiotic-induced diarrhea in two pediatric patients resulted in *Lactobacillus* sepsis (systemic inflammation caused by infection) [236]. Another study found that probiotics were ineffective in preventing CD after surgery [237].

Vitamin supplementation is a common additional treatment for CD, because the activity of CD directly correlates to the malnutrition severity. Because of the loss of bone density, it is important to supplement CD patients with vitamin D. One study in the American Journal of Gastroenterology showed that antioxidant vitamin supplementation in Crohn's disease reduced oxidative stress [238]. In this study, 70 patients followed at several gastroenterology clinics were administered a combination of vitamin C (1000 mg) and E (800 IU) or a placebo. Plasma vitamin C, tocopherols, alpha and beta carotene, lycopene, retinol, B-cryptoxanthin and lutein+ zexanthin was measured. Vitamin C and alpha-tocopherol, the type of vitamin E that is absorbed in humans, increased significantly after 4 wks. These vitamins were found to decrease lipid peroxidation and oxidative stress. Vitamin C can regenerate vitamin E during the antioxidant defense mechanism.

One final alternative therapy for CD involves fecal transplantation. In one case study, severe enterocolonic CD was treated with Mycophenolate Mofetil capsules (1.25 g) daily for 3 wks. There was a marked improvement in CD related symptoms as well as a reduction in an intraperitoneal inflammatory mass [239]. Fecal transplants are

commonly used to treat *C. difficile* infection, but less is known regarding their efficacy to treat IBDs [240].

1.5 Objectives

1) To assess the ability of NS to prevent aflatoxicosis in farm-raised tilapia over the course of 10 wk.

2) To evaluate toxicity in red drum exposed to 0-5 ppm AFB_1 and determine efficacy of NS in preventing aflatoxicosis in fish exposed to 5 ppm AFB_1 for 7 wk.

3) To determine the proper dosage of NS supplementation that mitigates TNBS-induced colitis in 5-wk-old BALB/c mice (one-week pilot study).

4) To quantify cytokine sorption onto NS and assess the ability of NS to reduce CD effects and protect the gastrointestinal microbiome in TNBS-induced mice (4- wk study).

2. THE EFFECT OF NOVASIL DIETARY SUPPLEMENTATION ON THE GROWTH AND HEALTH PERFORMANCE OF NILE TILAPIA (OREOCHROMIS NILOTICUS) FED AFLATOXIN-B_1 CONTAMINATED FEED*

2.1 Introduction

The aquaculture industry is one of the fastest-growing food sectors in the world [241, 242]. One of the major challenges facing the aquaculture industry is eliminating food-borne toxin exposure, such as aflatoxin B_1 (AFB_1). Once called "a plague of mankind" [243], aflatoxins are potent, natural toxins produced by *Aspergillus flavus* and *A. parasiticus* [244]. These fungi can contaminate animal feeds when they are stored improperly at high temperatures and humid conditions [245]. Due to climate change, aflatoxins are becoming more prevalent and persistent as temperatures and droughts increase and previously unaffected regions are compromised [246]. Of the aflatoxin congeners, AFB_1 is the most prevalent and toxic [247]. Chronic exposure to AFB_1 causes weight loss, immunosuppression, mutagenesis, reproductive alterations and carcinogenesis in many species including fish [248, 249, 250, 251]. Several studies have noted an *in vitro* binding interaction between AFB_1 and bacteria including *Lactobacillus* and *Bifidobacterium* strains [252, 253, 254]. Aflatoxin B_1 has also been reported to negatively impact bacteria and microbes in culture by inducing mutations in some bacterial species and promoting alterations in microbiota metabolism [255, 256].

*The chapter reported has been reprinted with permission from "The effect of NovaSil dietary supplementation on the growth and health performance of Nile tilapia *Oreobhromis niloticus* fed aflatoxin-B_1 contraminated feed" by Zychowski KE et al. 2013. Aquaculture 376 (2013): 117-123, Copyright 2012 by Elsevier.

Alterations in gut bacterial flora can have significant effects on inflammatory conditions, disease-resistance and the immune system [257, 258]. Therefore, there is potential for AFB_1 to cause alterations in the gut microflora, leading to poor health and growth performance in cultured Nile tilapia.

Due to the high cost of fishmeal and environmental conservation concerns, the aquaculture industry is continually seeking plant-based alternatives as feed ingredients to replace or supplement it in the diet [259]. However, feeds with high concentrations of plant material, such as peanut, corn, soybean and rice components are more susceptible to mycotoxin contamination. Studies have shown that AFB_1 residues in cultured channel catfish (*Ictalurus punctatus*) fillets can be as high as 400 ppb [260], which is well above the current United States Food and Drug Administration (FDA) action level of 20 ppb [261]. Researchers have found correlations between AFB_1 levels in the diet and tissue. For example, 5 ppb levels were detected in marine sea bass (*Dicentratchus labrax L.)* muscle tissue after a 42-day administration of 180 ppb of AFB_1 [262]. Additionally, it has been determined that AFB_1 tissue levels correlate with increasing concentrations of AFB_1 in the consumed feed [263].

Enterosorption therapy (i.e., calcium montmorillonite clay, NovaSil, NS), is one strategy used to decrease the bioavailability and toxicity of ingested AFB_1, and has resulted in successful reduction of AFB_1 exposure biomarkers in the blood and urine from many species. Dietary supplementation with NS is inexpensive and biochemical serum analyses have suggested the overall safety of NS in several species [264, 265, 266]. NS has a dioctahedral structure and negatively-charged interlayer, which enables

AFB_1 to tightly adsorb onto the interlamellar surface of the clay [267]. The objective of this study was to evaluate the efficacy of NS enterosorption therapy in reducing the toxic effects of AFB_1 in Nile tilapia, *Oreochromis niloticus.*

2.2 Materials and methods

2.2.1 Experimental diets

A basal diet (control) was formulated to contain 320 g of protein kg^{-1}, 35 g of lipid kg^{-1}, and an estimated 15.48 kjoules of digestible energy kg^{-1}. This diet satisfied or exceeded the known nutrient requirements of Nile tilapia [268, 269]. The basal diet was spiked with three different levels of AFB_1 (0, 1.5, and 3.0 ppm of the dry weight) and three different NS concentrations (0, 0.5 and 1.0% of the dry weight) in a factorial arrangement (Table 4). Prior to its addition, AFB_1 (Sigma-Aldrich, Saint Louis, MO) was dissolved in chloroform and further added to Celufil, a non-nutritive bulking agent (USB Corporation, Cleveland, OH). The chloroform was then evaporated in the dark under a fume hood. All dry ingredients with the exception of the AFB_1 were mixed in a V-mixer for 20 minutes and then mixed with oil and 1L of water in a Hobart mixer (Hobart Service, Troy, OH) for 1 hr. Aflatoxin B_1-spiked Celufil was then thoroughly mixed into the respective diets to provide a concentration of 1.5, or 3.0 ppm, immediately before the addition of soybean oil and water. $Aflatoxin_1$-free Celufil was also included in the diets without AFB_1 for comparison. All feeds were cold-pelleted through a 3-mm die on a meat grinder attachment, and air-dried in the dark for 24 hr. The diets were then bagged and stored at -20°C until further use. The nine experimental diets contained the following: (1) 0 ppm AFB_1+ 0% NS, (2) 1.5 ppm AFB_1 + 0% NS, (3)

Table 4. Diet formulation and proximate composition of experimental treatments (g/ 100g of dry weight)

Level of AFB_1 (ppm)	**0.0**	**1.5**	**3.0**	**0.0**	**1.5**	**3.0**	**0.0**	**1.5**	**3.0**
Level of NS (%)	**0.0**	**0.0**	**0.0**	**0.5**	**0.5**	**0.5**	**1.0**	**1.0**	**1.0**
Menhaden Meal[1]	11.0	11.0	11.0	11.0	11.0	11.0	11.0	11.0	11.0
Soybean Meal[2]	49.3	49.3	49.3	49.3	49.3	49.3	49.3	49.3	49.3
Dextrinized Starch[3]	20.3	20.3	20.3	20.3	20.3	20.3	20.3	20.3	20.3
Vitamin Premix[4]	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0
Mineral Premix[3]	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0
Carboxymethyl Cellulose[3]	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0
Soy oil[5]	4.8	4.8	4.8	4.8	4.8	4.8	4.8	4.8	4.8
CaPO4, dibasic[3]	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Glycine[6]	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
DL-Methionine[3]	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15
NS[7]	0.0	0.0	0.0	0.5	0.5	0.5	1.0	1.0	1.0
AFB_1(μg)[8]	0.0	150.0	300.0	0.0	150.0	300.0	0.0	150.0	300.0
Celufil[6]	3.5	3.5	3.5	3.0	3.0	3.0	2.5	2.5	2.5
Proximate Composition (g kg^{-1})									
Protein	34.4	33.8	33.6	33.8	34.7	33.6	33.0	33.8	36.4
Lipid	7.5	6.9	6.1	8.0	6.0	8.2	6.3	8.2	9.7
Dry Matter	90.8	90.9	91.3	91.3	90.6	91.3	90.6	90.4	90.3
Ash	8.9	8.9	8.9	9.4	9.4	9.3	9.7	9.8	9.8

[1]Omega Protein, Houston, TX, [2]Producers Cooperative Association, Bryan, TX, [3]MP Biomedicals LLC, Solon, OH, [4]Contains (as g kg^{-1}): $Ca(C_6H_{10}O_6)\cdot 5H2O$, 348.49; $Ca(H_2PO_4)2\cdot H_2O$, 136.0; $FeSO_4\cdot 7H_2O$, 5.0; $MgSO_4\cdot 7H_2O$, 132.0; K_2HPO_4, 240.0; $NaH_2PO_4\cdot H_2O$, 88.0; NaCl, 45.0;$AlCl_3\cdot 6H_2O$, 0.15; KI, 0.15; $CuSO_4\cdot 5H_2O$, 0.5; $MnSO_4\cdot H_2O$, 0.7; $CoCl_2\cdot 6H_2O$, 1.0;$ZnSO_4\cdot 7H_2O$, 3.0; Na_2SeO_3, 0.011., [5]Crisco, Orrville, OH, [6]USB Corporation, Cleveland, OH, [7]Engelhard Corporation, Jackson, MS , [8]Sigma-Aldrich, St. Louis, MO

3.0 ppm AFB_1 + 0% NS, (4) 0 ppm AFB_1 + 0.5% NS, (5) 1.5 ppm AFB_1 + 0.5% NS, (6) 3.0 ppm AFB_1 + 1.0% NS, (7) 0 ppm AFB_1 + 1.0% NS, (8) 1.5 ppm AFB_1 + 1.0% NS, and (9) 3.0 ppm AFB_1 + 1.0% NS.

2.2.2 Fish stock and culture conditions

Juvenile tilapia were shipped to the Texas A&M Aquacultural Research and Teaching facility from a commercial producer. Fish were conditioned in round tanks and then in the aquaria with a commercial diet (Rangen, Inc. Angelton, TX) for a period of 2 wk, followed by 1 wk on the control diet (0 ppm AFB_1 + 0% NS) prior to the start of the trial. The feeding trial was conducted in a closed, recirculating 110-L aquaria system, where salinity and temperature were maintained at 5 ppt and 37°C. Water flow was set at a rate of 1 L min^{-1} and aeration provided a dissolved oxygen level of at least 80% air saturation. The fish were exposed to a diurnal light:dark cycle of 12:12 hr with fluorescent lighting controlled by timers. Ammonia, nitrate, and nitrite were kept below toxic levels by the use of biofiltration. The nine treatments were randomly assigned to triplicate aquaria. Fifteen fish of similar size (average initial weight 2 ± 0.1 g/fish) were stocked into each of 27 aquaria. The fish were fed twice daily their assigned diet at 8 am and 4 pm over the course of 10 wk. Each diet was fed to the fish at a rate of 7% of initial body weight and adjusted over the course of the trial to 3% in order to minimize overfeeding and maintain a feeding rate close to satiation. Fish in each tank were group-weighed weekly to adjust feeding rates. Mortalities and abnormalities were monitored and recorded on a daily basis.

2.2.3 Fish responses

At the end of the trial, weight gain (% of initial weight), feed efficiency (g weight gain/g dry diet fed), and survival (100 x (final # of fish)/ initial # of fish) measurements were calculated. Additionally, three fish were sampled from each of the 27 aquaria at the end of the feeding trial and each separately homogenized in a blender. Moisture, ash, protein and lipid analysis of whole-body samples were completed according to established procedures [270]. Organ to whole-body weight somatic indices were calculated for heart, spleen, liver (HSI), intraperitoneal fat (IPF), and fillet and averaged based on two fish per tank (six per treatment) using the formula: Somatic Index = (organ weight/body weight)*100. Fish were hand filleted by one individual to maintain consistency. The fillets were skinned and any remaining pin bones were manually removed. The dextral side of each fish was filleted and weighed. The fillet weight was then doubled for each fish and the somatic index was calculated.

2.2.4 Immunological measurements

Extracellular superoxide anion production was measured in head-kidney macrophages using the method described in Secombes et al. [271] and modified by Sealy et al. [272] using three fish per aquaria (9 per treatment). Oxidative radical production in whole blood neutrophils was determined using a nitro blue tetrazolium (NBT) assay [273, 274]. Two fish from each tank (6 fish per treatment) were euthanized and 1 mL of blood was drawn from each fish using 1 mL pre-treated heparinized syringes. Additionally, plasma lysozyme concentration was analyzed from

two fish per tank (6 per treatment) using a turbidimetric method as previously published [274, 275].

2.2.5 Characterization of gut microbiota

Digesta was collected under sterile conditions from the intestines of three fish per aquaria. DNA isolation (QIAamp DNA Mini Kit (Qiagen, Valencia, CA)), PCR and denaturing gradient gel electrophoresis (DGGE) were performed according to a modified method described in Hume et al. [276]. Visualization of bands was accomplished using an imager (Alpha Innotech, San Leandro, CA). Band pattern relatedness (percentage similarity coefficient) and dendrogram construction were determined according to the methods of Hume et al. (2003).

2.2.6 Histology

Upon termination of the trial, livers from six fish per treatment (two per tank) were dissected and stored in Davidson's solution. Within 24 hr, the livers were rinsed with 70% ethanol and transferred to vials containing 10 mL fresh 70% ethanol, until further histological processing. All samples were transported to the Texas A&M Veterinary Integrative Biosciences Histology Laboratory for further processing. Samples were embedded in paraffin and sectioned at 5 μm. The samples were further mounted on glass slides and stained with hematoxylin and eosin. Slides were blind-examined under a light microscope for histopathological changes. Lesions were examined including cellular pleomorphism, nuclear pleomorphism, dysplasia, hydropic degeneration, fatty degeneration, necrosis, leukocyte infiltration and inclusion bodies and were graded on a scale of 0 to 4 for each slide, 0 = no pathological changed

apparent, 1 = mild, 2 = moderate, 3 = moderate to severe, and 4 = severe lesions. All lesions from each slide were then averaged for an overall liver histopathological score per fish.

2.2.7 HPLC analysis of AFB_1 in fish feed

In order to confirm targeted levels and demonstrate the binding capacity of NS, AFB_1 was extracted from each diet with slight modifications from previously established methodology [277, 278]. Briefly, three samples totaling 50 g of diet were pooled according to treatment. A 250 mL solution of 70/30% (v/v) methanol/water was blended with the 50 g diet samples. The sample was then filtered using medium filter paper (VWR, Atlanta, GA), and the filtrate was collected into a clean plastic centrifuge tube, and capped tightly. One gram of NaCl and 20 mL HPLC water (VWR) were added to each sample, followed by thorough vortexing. The solution was then filtered through a microfiber filter (VWR). Filtered extract (6 mL) was pipetted into a plastic syringe barrel reservoir and pushed through a Vicam Aflatest P column (Vicam, Milford, MA) at a rate of 2 drops/sec. Deionized water (10 mL) was added to the syringe barrel and pushed through at a rate of 2 drops/sec. The column was rinsed once more with 10 mL of water. Aflatoxin B_1 was eluted from the column into a glass tube with 1.0 mL of methanol. The solution was filtered through a 0.45-µm syringe filter (VWR). Using a mobile phase of 3:1:1 Water:Acetonitrile:Methanol, the samples were then injected into the HPLC equipped with a Waters Spherisorb® 5 µm 4.6x150 mm column and 2475 Fluorescence Detector (Waters, Milford, MA). Quantification of AFB_1 was achieved using the Breeze Software (Waters Milford, MA). Results from HPLC analyses

indicated that endogenous levels of AFB_1 were negligible (<2.5 ppb AFB_1) in the 0 ppm AFB_1+0% NS, 0 ppm AFB_1+0.5% NS, and 0 ppm AFB_1+1.0% NS diets. Of the extracted AFB_1, NS bound an average of 57.7% of the AFB_1 in the targeted 1.5 ppm AFB_1 dietary treatments and 43% in the targeted 3.0 ppm AFB_1 diets.

2.2.8 Statistical analysis

The Statistical Analysis System (SAS) version 9.2 (SAS Institute Inc., Cary, NC) was used to compute all statistics. All parameters, except for histopathological scores, were first subjected to a two-way ANOVA. Specific differences among treatments and a comparison of means were further analyzed using Duncan's multiple range test. Histopathological scores were analyzed using the nonparametric Aligned Rank Transform as described in [279] and then subject to a two-way ANOVA and Duncan's multiple range test. Treatment effects were considered significant at $P \leq 0.05$.

2.3 Results

2.3.1 Growth performance

Survival was not significantly affected by AFB_1, NS, or the combination of both treatments. However, AFB_1 negatively impacted feed efficiency ($P < 0.001$) and weight gain at 3 ppm ($P = 0.018$), regardless of NS inclusion in the diet (Table 2). The inverse relationship between increasing AFB_1 concentration and decreasing weight gain and feed efficiency occurred in a linear manner, with an $R^2 = 0.94$ and 0.95, respectively (Fig. 6). NS-supplemented fish exposed to 3 ppm AFB_1exhibited higher feed efficiency and weight gain than 3 ppm exposed fish without NS; however, these trends were not found to be statistically significant (Table 5).

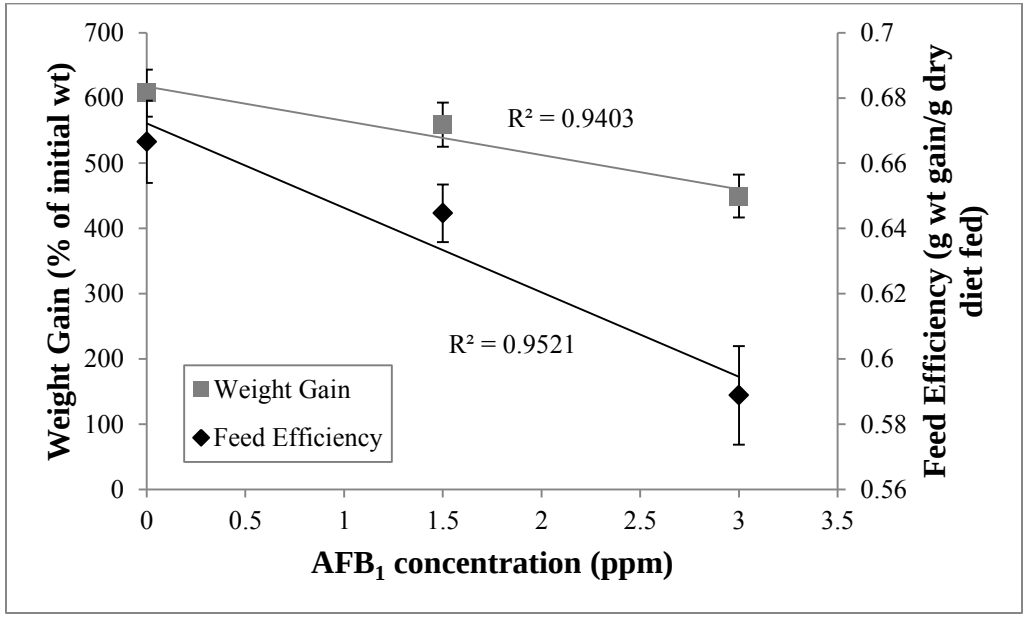


Figure 6. AFB_1 effect on feed efficiency and weight gain. Increasing AFB_1 levels inversely correlated with decreasing feed efficiency (right axis), and weight gain (left axis) in Nile tilapia (*Oreochromis niloticus*), regardless of % NS inclusion (n=9). R^2 values for linear trends were 0.9403 and 0.9521 for weight gain and feed efficiency, respectively.

Table 5. Growth performance of tilapia fed different concentrations of AFB_1 and NovaSil [1,2]

Variable	Level of NS (%)	Weight gain (%)[3]	Survival (%)	Feed Efficiency
Level of AFB_1 (ppm)			**Individual treatment means**	
0	0	636	88.8	0.68
0	0.5	542	97.7	0.64
0	1	643	93.3	0.67
1.5	0	550	93.3	0.64
1.5	0.5	499	97.7	0.63
1.5	1	627	95.5	0.65
3	0	375	95.5	0.54
3	0.5	522	100	0.62
3	1	451	100	0.59
Pooled Std. Error		35.895	2.051	0.012
			Means of main effect	
0		607[a]	98.5	0.66[a]
1.5		559[a]	95.5	0.64[a]
3		449[b]	93.3	0.58[b]
	0	520	98.5	0.62
	0.5	521	96.2	0.63
	1	574	92.5	0.64
			ANOVA: P-values	
AFB_1		0.018*	0.227	<0.001*
NS		0.494	0.147	0.586
AFB1 x NS		0.311	0.948	0.139

[1]Values are means of three replicate groups of fish (n=3).
[2]Values in a column that do not have the same superscript are significantly different according to Duncan's multiple range test (P<0.05).
[3]Initial average weight was 10.9 ± 0.4 g/fish
*Indicates significance (*P*<0.05)

Table 6. Immune parameters of tilapia fed different concentrations of AFB_1 and NovaSil[1]

Variable	Level of NS (%)	Plasma Lysozyme (units/mL)	NBT (mg/mL blood)[2]	Extracellular Superoxide Anion (mmol)[3]
Level of AFB_1 (ppm)			**Individual treatment means**	
0	0	182	2.46	1.60
0	0.5	221	2.40	1.24
0	1	183	2.49	2.25
1.5	0	112	2.64	0.50
1.5	0.5	118	2.89	0.64
1.5	1	220	2.54	0.88
3	0	152	2.31	0.98
3	0.5	127	2.42	1.01
3	1	180	2.57	1.16
Pooled Std. Error		12	0.057	0.119
			Means of main effect	
0		196	2.45	1.7^{a}
1.5		150	2.69	0.67^{c}
3		153	2.43	1.05^{b}
	0	148	2.47	1.03^{x}
	0.5	155	2.57	0.96^{x}
	1	194	2.53	1.43^{y}
			ANOVA: P-values	
AFB_1		0.391	0.243	**<**0.001*
NS		0.687	0.823	0.006*
AFB_1 x NS		0.335	.698	.157

[1] Values in a column that do not have the same superscript are significantly different according to Duncan's multiple range test (P<0.05).

[2] Values are means of determinations on two fish from each of three replicate groups (6 fish/treatment, n=6).

[3] Values are means of three composite samples of kidney cells from three fish in each of the three replicate groups (n=3).

*Indicates significance (P<0.05)

2.3.2 Immune parameters

Plasma lysozyme concentrations were not significantly altered with either the AFB_1 or NS treatments (Table 6). Likewise, there were no significant differences ($P > 0.05$) in the neutrophil respiratory burst (mg/NBT) among treatments. Interestingly, extracellular superoxide anion production was significantly increased with 1% NS dietary supplementation ($P = 0.006$), and decreased in the 1.5 and 3.0 ppm AFB_1-treated fish as compared to controls ($P < 0.001$).

2.3.3 Organosomatic indices and proximate composition of whole-body tissues

There were no statistically significant differences between the somatic indices of heart, spleen or IPF between treatment groups (Table 7). A significantly lower fillet weight was recorded in the 0.5% and 1% NS treatment groups compared to the control ($P = 0.008$). However, this outcome was not observed in the AFB_1 treatment groups. HSI levels were also significantly decreased in groups exposed to AFB_1 at both 1.5 and 3.0 ppm ($P < 0.001$). No significant differences in percent lipid, protein, moisture, or ash were documented in treated fish, indicating that neither AFB_1, NS, nor a combination of the two affected whole-body proximate composition (Table 8).

Table 7. Organ ratios of tilapia fed different concentrations of AFB_1 and NovaSil[1,2]

Variable	Level of NS (%)	Heart	Spleen	Fillet	HSI[3]	IPF[4]
Level of AFB_1 (ppm)			**Individual treatment means**			
0	0	0.092	0.097	26.60	1.81	0.174
0	0.5	0.100	0.089	23.80	1.59	0.204
0	1	0.114	0.162	23.31	1.34	0.158
1.5	0	0.111	0.118	29.60	0.94	0.232
1.5	0.5	0.114	0.117	18.64	1.20	0.257
1.5	1	0.100	0.124	26.58	1.20	0.115
3	0	0.094	0.14	28.09	1.10	0.230
3	0.5	0.097	0.095	26.48	0.81	0.066
3	1	0.088	0.098	24.69	0.95	0.111
Pooled Std. Error		0.002	0.007	0.513	.042	.0300
			Means of main effect			
0		0.102	0.116	24.57	1.58[a]	0.261
1.5		0.109	0.120	24.94	1.12[b]	0.218
3		0.093	0.111	26.42	0.959[b]	0.268
	0	0.099	0.118	28.09[a]	1.28	0.294
	0.5	0.104	0.100	22.97[b]	1.20	0.262
	1	0.101	0.128	24.86[b]	1.16	0.190
			ANOVA: P-values			
AFB_1		0.147	0.905	0.421	<0.001*	0.822
NS		0.819	0.399	0.008*	0.614	0.486
AFB_1 x NS		0.372	0.293	0.055	0.139	0.687

[1] Values in a column that do not have the same superscript are significantly different according to Duncan's multiple range test ($P<0.05$). [2]Values are means of determinations on two fish from each of the three replicates (6 fish/treatment, n=6). [3]Hepatosomatic index[4]Intraperitoneal fat*Indicates significance ($P<0.05$)

Table 8. Whole-body proximate composition of tilapia fed different concentrations of AFB_1 and NovaSil (% of wet weight)[1]

Variable	Level of NS	Lipids	Protein	Moisture	Ash
Level of AFB_1 (ppm)			**Individual treatment means**		
0	0	3.59	15.08	23.78	2.80
0	0.5	3.38	14.94	24.21	4.04
0	1	3.79	13.84	21.01	3.31
1.5	0	3.44	16.76	24.79	3.81
1.5	0.5	4.15	16.23	24.98	4.21
1.5	1	3.32	13.96	20.69	2.72
3	0	2.81	15.99	22.90	3.84
3	0.5	3.92	15.68	22.98	2.82
3	1	3.45	15.73	24.12	3.41
Pooled Std. Error		0.099	0.205	0.242	0.13
			Means of main effect		
0		3.59	14.62	23.00	3.38
1.5		3.64	15.65	23.49	3.58
3		3.39	15.8	23.34	3.35
	0	3.28	15.94	23.82	3.48
	0.5	3.82	15.62	24.06	3.69
	1	3.52	14.51	21.94	3.15
			ANOVA: P-values		
AFB_1		0.829	0.357	0.89	0.902
NS		0.463	0.251	0.105	0.618
AFB_1 x NS		0.644	0.777	0.199	0.379

[1]Values are means of determinations on three fish from each of the three replicates (n=3).

2.3.4 Histology

Hepatocyte necrosis, cellular pleomorphism, nuclear pleomorphism, dysplasia, hydropic degeneration, and fatty degeneration were detected in the liver of fish exposed to AFB_1. Similarly, fish fed AFB_1 had greater liver pathological damage than those without AFB_1 , where the major histopathological lesions observed were related to

cellular changes (Fig. 7), contributing to a higher histopathological score (Fig. 8). Inclusion of NS in the diet did not significantly impact histopathological outcomes.

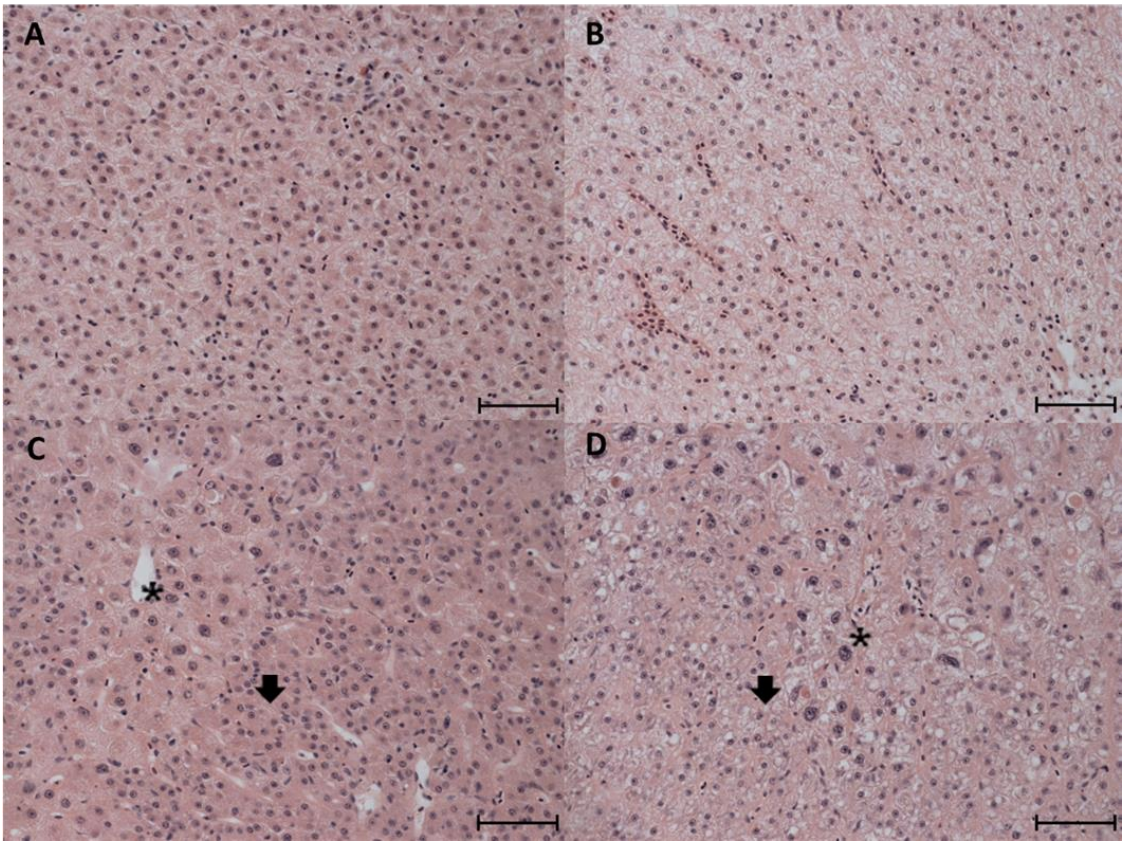


Figure 7. Microphotographs of liver sections Nile tilapia (*Oreochromis niloticus*) were fed combinations of aflatoxin B_1 (AFB_1) and NovaSil (NS) (400× magnification, H/E staining). 0 ppm AFB_1 + 0% NS (A) and 0 ppm AFB_1 + 0.5%NS (B) treatments resulted in normal histological structure, whereas fish fed 1.5 ppm AFB_1 + 0.5% NS (C) and 1.5 ppm AFB_1 (D) showed marked cellular pleomorphism, characterized by zones of enlarged, stellated or spindle-shaped hepatocytes (*) surrounded by small polyhedric-shaped hepatocytes (arrow). Increased fatty degeneration was observed in fish fed 1.5 ppm AFB_1 (D). Scale bar = 50µm. Results were based on two fish from each of the three replicate groups (n=6).

2.3.5 Bacterial microbiotia and DGGE

Denaturing gradient gel electrophoresis analysis indicated that none of the treatments were significantly different from each other. Overall, neither AFB_1, NS, nor a combination of the two significantly altered gut microbiota in tilapia (Fig. 9).

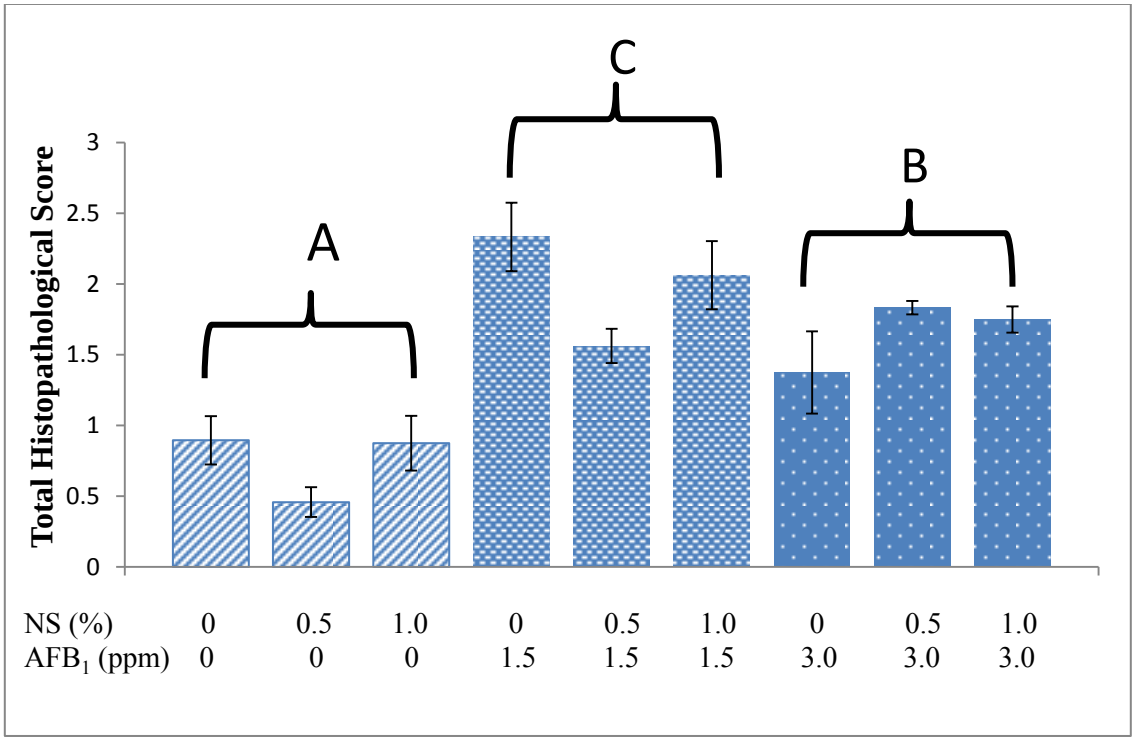


Figure 8. Quantification of histopathological effects. Overall histopathological score per treatment (NS = NovaSil; AFB_1 = aflatoxin B_1) in Nile tilapia (*Oreochromis niloticus*). Bars in the figure that do not have the same letter are significantly different according to Duncan's multiple range test ($P<0.05$). Aflatoxin B_1 had a significant effect on total histopathological score ($P<0.001$). There was no statistical difference between NS levels ($P=0.1347$), or AFB_1xNS ($P=0.1770$). Results were based on two fish from each of the three replicate groups (n=6).

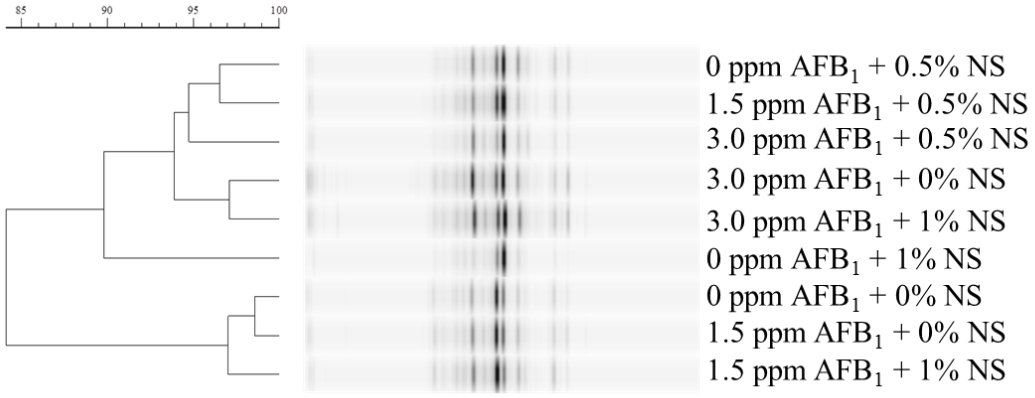


Figure 9. Denaturing gradient gel electrophoresis of bacterial 16S rDNA amplicons from tilapia intestinal contents. The bar above the figure corresponds to the percentage similarity coefficients. Three fish per tank in three separate aquaria were combined per treatment (n=9).

2.4 Discussion

Overall, AFB_1 had a significant impact on tilapia over 10 wk. With decreased weight gain and lower feed efficiency, 3 ppm AFB_1-exposed fish performed poorly compared to 1.5 ppm AFB_1-exposed fish and the controls. Reduction in weight gain and decreases in feed efficiency at the higher dose confirm the anti-nutritional effects of AFB_1 as seen in similar studies [280]. However, this work is the first to report that detrimental effects can occur in tilapia exposed to relatively low AFB_1 concentrations (3 ppm) in 10 wks, a relatively short time-span when contrasted to a full growth cycle. As in other species, the target organ of AFB_1 in tilapia is the liver, where it is known to induce toxicity. AFB_1 binds to DNA, creating the AFB_1-8,9-epoxide which is involved in the development of fatty liver, necrosis and carcinogenesis in fish and other animals [281, 282]. In agreement, we found that HSI decreased significantly in groups

exposed to AFB_1, and damage to the liver was histologically detected at levels as low as 1.5 ppm of AFB_1. Histopathological changes in the liver and lowered HSI suggest a progression towards AFB_1-induced heptocarcinogenesis.

Immunosuppressive consequences have been demonstrated in a broad range of AFB_1-exposed animals including turkeys, chickens, trout, humans and others. Detrimental effects on the animal's immunity are likely due to interference with specific biochemical and physiological pathways [283]. Decreased extracellular superoxide anion production, an indicator of immunosuppression, has been previously reported in murine species as well as fish [284, 285]. One study found a decrease in total superoxide anion production in AFB_1-exposed tilapia [286], but did not report a decrease in extracellular superoxide anion production, as specifically described in this study. Because the production of reactive oxygen species such as superoxide anion is deployed by the immune system to kill invading microorganisms, AFB_1-induced reductions in free radical production may result in a decreased resistance to pathogens including bacteria (Iwama and Nakanishi, 1996). However, further studies examining the effects of AFB_1-exposure in disease-challenged tilapia are needed to determine the extent of this correlation.

Aflatoxin B_1 exposure typically occurs via food consumption and could potentially alter bacterial microbiota in the gut due to its previously established antibacterial activity [288, 289]. Aflatoxin B_1 has also been shown to bind to naturally occurring bacteria in the gut essential for digestion [252, 254, 290]. It is possible that alterations in gut bacterial flora, essential for metabolism and digestion, may negatively

impact animals [291]. However, this hypothesis could not be substantiated given the obtained DGGE data. Therefore, the observed changes in biological parameters caused by AFB_1 could not directly be attributed to alterations in gut bacterial microbiota.

Overall, NS alone at 0.5 and 1.0% inclusion rates did not impact any of the growth parameters and the treatments did not produce results significantly different from the controls, with the exception of a decrease in fillet weight. Interestingly, the 0 ppm AFB_1 + 1% NS treatment produced a sharp increase in extracellular superoxide anion, as compared to the control group ($P < 0.05$), suggesting that NS alone might have a positive influence on the tilapia immune system. In future studies, serum levels of fish supplemented with NS will be monitored and analyzed to detect any changes in mineral and vitamin content that may be responsible for enhanced immunity.

Although not significant, NS had a positive influence on some of the parameters tested. Results were inconclusive regarding its efficacy and varied between 1.5 and 3.0 ppm AFB_1 levels. In published studies using clays to sorb toxins in feeds, the clays were either administered in a dry form added to commercial feeds or in an encapsulated supplement [264, 292, 293]. The efficacy of NS has been cited repeatedly in various species [264, 294], but due to the high degree of processing necessary for the production of fish feeds, there is potential for NS to interact with various components in the feed matrix. Components in the feed may have been sequestered in the negatively-charged interlayer of NS during mixing, thereby rendering it less capable of sorbing 100% of AFB_1 at the administered levels. A previous study determined that 0.5% hydrated sodium aluminosilicate was not effective in preventing liver lesions in rainbow trout

exposed to AFB_1 and may have interfered with some essential feed components [295]. Somewhat similar to this study, the aluminosilicate was added to the feed before the pelletization step and did not prevent the toxic effects of AFB_1 from altering the liver. Conversely, another study suggested that 2% sodium bentonite decreased liver, kidney and feces AFB_1 in 20 ppb AFB_1-exposed trout. However, the diets were prepared by loading the previously dried feed components into hollow gelatin capsules, followed by the addition of 0.1 mL methanol-AFB_1 solution to each gram of sample. The increase in the inclusion level of clay, the difference in feed preparation, or the different experimental endpoints tested may have been responsible for the significant decrease in toxicity [296]. Yet another study found that 0.5% montmorillonite and bentonite both decreased hepatic DNA damage in 1.5 ppm AFB_1-exposed tilapia [297]. Discrepancies in the literature indicate that further research concerning AFB_1 binders in farm-raised fish is needed.

On the whole, AFB_1 had a negative impact on tilapia weight gain and feed efficiency over a relatively short span of 10 wk. AFB_1 also negatively impacted multiple physiological and biological parameters significantly, including HSI and macrophage extracellular superoxide anion production with both 1.5 and 3.0 ppm treatment levels. Although a degree of protection was afforded in some cases with the inclusion of NS in the fish feed, indeed, future studies should strive to achieve mitigation for aflatoxicosis in tilapia by determining an effective dosing method for NS in fish. This method may require supplementation separate from the feed itself, allowing for AFB_1 sorption without food matrix interference. A binder such as NovaSil, if utilized effectively,

could reduce exposure to AFB_1, thereby preventing bioavailability and subsequent effects, such as immunosuppression, liver damage and decreases in growth parameters. Decreasing the incidence of aflatoxicosis in farm-raised fish would ultimately prevent economic loss for the industry.

3. THE EFFECT OF AFLATOXIN-B_1 ON RED DRUM (SCIAENOPS OCELLATUS) AND ASSESSMENT OF DIETARY SUPPLEMENTATION OF NOVASIL FOR THE PREVENTION OF AFLATOXICOSIS

3.1 Introduction

Mycotoxins are toxic metabolites produced by a diverse group of fungi that contaminate agricultural crops prior to harvest or during storage post-harvest [298, 299]. Aflatoxin B_1 (AFB_1), a mycotoxin produced by *Aspergillus flavus* and *A. parasiticus*, is one of the most potent, naturally-occurring carcinogens known to mankind. Aflatoxin B_1 exposure causes decreases in weight gain, growth stunting and immunosuppression in animals, while increasing hepatocellular carcinoma incidence [300]. Different species including humans, poultry, swine, and fish all exhibit varying levels of mortality and morbidity upon exposure to AFB_1 [249, 301, 302]. However, because the damaging AFB_1 effects are largely species and dose-specific, additional studies are necessary to determine AFB_1 susceptibility for at-risk unevaluated species.

As a vital part of the global food industry, aquaculture contributes nearly half of all food of aquatic origin intended for human consumption [303]. Fishmeal, one the most expensive fish feed ingredients, is widely used in the aquaculture industry as the major protein source for farm-raised fish [304]. Menhaden (*Brevoortia sp*) is a clupeid fish species and the most prevalent form of fishmeal used in the North America [305] .

Recent studies have been directed toward the development of plant-based alternative protein sources such as soybean, peanut, corn and cottonseed meal [306, 307, 308, 309]. However, incorporation of plant-based ingredients into feed increases the risk of AFB_1 contamination and subsequent exposure. Aflatoxin B_1 presence in aquaculture feeds and fish feed ingredients has been well-documented, especially in developing countries [310, 311, 312].

One strategy to reduce aflatoxin exposure in humans and animals is the use of enterosorption therapy. NovaSil (NS), a calcium montmorillonite clay, binds AFB_1 in the gastrointestinal tract, thereby reducing overall AFB_1 bioavailability [313]. With a dioctahedral-layered structure and negatively charged interlayer, NS has high affinity and capacity for AFB_1 molecules, which exhibit a partial positive charge [314]. Numerous *in vivo* studies have demonstrated the safety and efficacy of this technology [266, 294, 315], although additional studies are needed to determine the efficacy and proper dosage for farm-raised fish [316].

Red drum, *Sciaenops ocellatus,* is a common recreational and commercial fish native to the Atlantic and Gulf Coast regions of the United States [317]. Red drum is currently farmed in China, Israel, Ecuador and North America [318]. Despite its prevalence and economic importance to the food industry, no studies have evaluated red drum AFB_1 susceptibility. The study presented here was designed to address two objectives: 1) to evaluate red drum susceptibility to AFB_1 using a multi-level AFB_1

challenge incorporated into the feed and 2) to assess the ability of NS to prevent AFB_1 toxicity in red drum.

3.2. Materials and methods

3.2.1 Experimental diets

The control basal diet was composed of 400 g protein kg^{-1} and 110 g lipid kg^{-1}, containing an estimated 3.5 kcal digestible energy kg^{-1} (Table 9) and fulfilling all documented nutrient requirements of red drum [319]. Aflatoxin B_1 (Sigma-Aldrich, Saint Louis, MO) was incorporated into the diet by first dissolving the AFB_1 in chloroform and subsequently adding it to Celufil, a non-nutritive bulking agent (USB Corporation, Cleveland, OH). The chloroform was evaporated to dryness from the mixture in a dark room under a fume hood, leaving the Celufil amended with AFB_1. A V-mixer was used to blend all dry ingredients, with the exception of the AFB_1-spiked Celufil, for 20 min. The dry ingredients were then mixed with the AFB_1-spiked Celufil in a Hobart mixer until homogeneity was achieved. The oil component and 700 mL of H_2O were further added to the dry ingredients and mixed for 1 h. Aflatoxin-free Celufil was incorporated into the basal diet for comparison. The moist feed was cold-pelleted through a 3-mm die on a meat grinder attachment and dried in a dark room for 24 h. Diets were subsequently bagged and stored at -20 ˚C until needed. The ten diets contained the following: 0 ppm AFB_1 (i.e., 0 ppm AFB_1+ 0% NS), 0.1 ppm AFB_1, 0.25 ppm AFB_1, 0.5 ppm AFB_1, 1 ppm AFB_1, 2 ppm AFB_1, 3 ppm AFB_1, 5 ppm AFB_1, 5 ppm AFB_1 + 1% NS and 5 ppm AFB_1+ 2% NS. A NS control group was not included since its safety was previously evaluated in this species (unpublished data).

3.2.2 Fish stock and culture conditions

Fingerling red drum were transported from the Texas Parks and Wildlife hatchery located at Lake Jackson, TX to the Texas A&M Aquacultural Research and Teaching Facility. Fish were stocked and conditioned in round tanks with a commercial diet (Rangen, Inc. Angelton, TX) for 2 weeks, then transferred to aquaria and conditioned for 1 week on the basal diet. A closed, re-circulating system was composed

Table 9. Ingredient and proximate composition of experimental diets (g/ 100 g of dry weight)

Level of AFB_1 (ppm)	**0**	**0.1**	**0.25**	**0.5**	**1**	**2**	**3**	**5**	**5**	**5**
Level of NS (%)	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**1**	**2**
Menhaden Meal[a]	34.9	34.9	34.9	34.9	34.9	34.9	34.9	34.9	34.9	34.9
Soybean Meal[b]	27.3	27.3	27.3	27.3	27.3	27.3	27.3	27.3	27.3	27.3
Dextrinized Starch[c]	16.	16.5	16.5	16.5	16.5	16.5	16.5	16.5	16.5	16.5
Menhaden Oil[a]	5.6	5.6	5.6	5.6	5.6	5.6	5.6	5.6	5.6	5.6
Vitamin Premix[e]	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0
Mineral Premix[c]	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0
CMC[c]	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0
Glycine[f]	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Lysine[f]	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
NS[g]	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.1	2.3
AFB_1-spiked Celufil[h]	0	0.2	0.7	1.6	4.5	0.5	0.8	1.6	1.6	1.6
Celufil[f]	5.5	5.3	4.8	3.9	1.0	5.0	4.7	3.9	2.8	1.7
Proximate Composition (% dry matter)										
Protein	36.2	35.8	35.7	35.2	35.6	35.5	36.1	35.6	35.2	35.5
Lipid	9.5	9.3	10.3	10.4	10.7	10.6	10.6	10.7	10.7	11.1
Dry Matter	94.5	94.7	94.9	93.6	94.3	94.8	95.2	95.4	95.4	95
Ash	11.1	10.9	10.9	11.3	11.1	10.9	10.9	11.1	11.8	12.9

[a] Special Select, Omega Protein, Houston, TX [b] De-hulled, roasted/cooked and solvent extracted, Producers Cooperative Association, Bryan, TX [c] MP Biomedicals LLC, Solon, OH [e] Contains (as g kg^{-1}): $Ca(C_6H_{10}O_6)\cdot 5H_2O$, 348.49; $Ca(H_2PO_4)_2\cdot H_2O$, 136.0; $FeSO_4\cdot 7H_2O$, 5.0; $MgSO_4\cdot 7H_2O$, 132.0; K_2HPO_4, 240.0; $NaH_2PO_4\cdot H_2O$, 88.0; NaCl, 45.0; $AlCl_3\cdot 6H_2O$, 0.15; KI, 0.15; $CuSO_4\cdot 5H_2O$, 0.5; $MnSO_4\cdot H_2O$, 0.7; $CoCl_2\cdot 6H_2O$, 1.0; $ZnSO_4\cdot 7H_2O$, 3.0; Na_2SeO_3, 0.011.[f] USB Corporation, Cleveland, OH [g] Englehard Corporation, Jackson, MS [h] Sigma-Aldrich, St. Louis, MO

of 110-L aquaria with water flowing at 1 L min-1. Biofiltration was used to maintain ammonia, nitrate and nitrite concentrations at non-toxic levels. Salinity was maintained at 7 ppt with artificial salts and water temperature was kept constant at 37 ± 2˚C by controlling air temperature in the wet laboratory. Supplemental aeration provided an adequate dissolved oxygen level of at least 80% air saturation. A 12:12 hour light:dark cycle was maintained throughout the conditioning and trial period and water quality was monitored on a daily basis. Fifteen fish (2.1 ± 0.1g) were stocked in each aquarium. The 10 dietary treatments were randomly assigned to triplicate aquaria, requiring a total of 30 tanks. Fish were fed a morning and afternoon ration over the course of 7 weeks. The diets were fed to fish beginning at a rate of 6% of the initial body weight and tapered to 3% over the span of the trial to prevent overfeeding and to approach apparent satiation. The system was monitored for mortalities and any deceased fish were immediately removed and evaluated for cause of death. With the exception of weight gain and survival which were monitored on a weekly or daily basis, respectively, all other parameters were evaluated at the end of 7 weeks.

3.2.3 Fish growth and health responses

Weight gain (% of initial weight), feed efficiency (g weight gain/ g dry diet fed), and survival rate (% per treatment group) were calculated at the end of the trial. Two fish were sampled from each aquaria and homogenized together using a blender. Whole-body analysis was performed by evaluating moisture, ash, protein and lipid content according to previously established procedures [270]. Somatic indexes including spleen, liver (HSI), intraperitoneal (IPF) fat and muscle (MSI) were averaged based on 2 fish per

aquaria (n=6). Each somatic index was calculated as follows: (organ weight/body weight) *100. Only the dextral side of each fish was filleted, weighed, and then doubled to obtain MSI.

3.2.4 Immunological responses

Immunological parameters were evaluated including plasma lysozyme of white blood cell origin, neutrophil oxidative radical production in whole blood, and % trypsin inhibition. Two fish were randomly selected and bled from each tank, then pooled according to treatment (6 fish per treatment). A total of approximately 1-2 mL of blood was collected per treatment group using heparinized syringes. Plasma lysozyme was analyzed by employing a turbidimetric method [274, 320]. Blood neutrophil oxidative radical production was measured utilizing a nitro blue tetrazolium (NBT) assay [273, 274]. Plasma was also used to determine % trypsin inhibition according to a previously established method [321].

3.2.5 Histological response

Livers were dissected from two fish per tank, or six per treatment. Immediately after dissection, livers were fixed in 10% formalin overnight. Livers were subsequently rinsed with 70% ethanol solution and transferred to vials containing 10 mL fresh 70% ethanol. Samples were processed and paraffin embedded within 48 h for routine histopathology at the Texas A&M Veterinary Pathobiology Histology Laboratory (College Station, TX). Samples were sectioned at a thickness of 5 µm and stained with hematoxylin and eosin (H&E). Lesions were blindly examined and scored according to the criteria listed in Table 10.

3.2.6 Immunohistochemistry

Immunohistochemistry for proliferating cell nuclear antigen (PCNA) was performed on deparaffinized sections of liver mounted on positively charged, silanized slides using an automated staining system for immunohistochemistry (Lab Vision Autostainer 360, Runcom, Cheshire, UK). Briefly, slides were placed in a heated chamber with DIVA decloaking solution (Biocare Medical LLC., Concord, CA) and heated to 121˚C for antigen retrieval. The slides were incubated with a 1:200 dilution of PCNA (Fisher Scientific, Walther, MA) for 20 min followed by a secondary antibody, ImmpRESS (Vector Scientific, Burlingame, CA) for 30 min.

Table 10. Histological evaluation criteria

Score	Evaluation	Description
0	Normal	Intracytoplasmic vacuolation, mostly macrovacuolar with one of the control livers also having micro and macrovesiculation. Nuclei are small and pushed to the periphery with small nucleoli.
1+	Minimal	, Scattered increase in nuclear size and mostly inconspicuous nucleoli.
2+	Mild	Mild hypertrophy and pleomorphism with slightly prominent nuclei and more evident nucleoli. Some loss of intracytoplasmic macrovacuoles, and formation of microvacuoles.
3+	Moderate	Moderate cellular pleomorphism, with anisocytosis, anisokaryosis, megalocytosis and megalokaryosis. Sparse intracytoplasmic vacuoles.
4+	Marked	Diffuse loss of cytoplasmic vacuolation, mostly solid cytoplasm. Marked pleomorphism, anisocytosis, anisokaryosis, megalocytosis and megalokaryosis.

The primary antibody was omitted on negative control tissues. Slides were then stained with DAB Quanto (Vector Scientific) for 5 min, followed by counterstaining with hematoxylin (Biocare Medical LLC., Concord, CA) for 1.5 min. Slides were further dehydrated and mounted. Negative and positive control tissues were stained together with all fish livers. Canine and mouse small intestine, bronchial epithelium and tonsils were used as positive control tissues. All photographs were taken at 400X magnification. Stained nuclei were counted, averaged and evaluated for each treatment using CellProfiler software [322]. The percentage of PCNA positive cells [(positive/total nuclei) x100] was calculated based on 4 fields/ fish x 6 fish/treatment (24 fields/ treatment).

3.2.7 Statistical analysis

All statistics were computed using Statistical Analysis System (SAS) version 9.2 (SAS Institute., Cary, NC). Data from groups exposed to 0-5 ppm AFB_1 were subject to a general linear model regression, while 0 ppm AFB_1, 5 ppm AFB_1, 5 ppm AFB_1+1% NS and 5 ppm AFB_1+2% NS group data were subject to an incomplete factorial ANOVA for all parameters except histopathological scoring. Histopathological scores were first subject to Aligned Rank Transformation [323] and then further analyzed using a general linear model regression or incomplete factorial ANOVA. All differences among treatment means were determined using Duncan's multiple range test. Treatment differences were considered significant at $P< 0.05$.

3.3 Results

3.3.1 Growth parameters

Aflatoxin B_1 treatment effects, including weight gain (%), survival (%), and feed efficiency, did not result in linear trends, with R^2 values of 0.22, 0.01 and 0.1, respectively. Weight gain of individual treatment means were significantly different and varied, with the 0 ppm AFB_1 group experiencing the highest weight gain and the 2, 3, and 5 ppm exposure groups exhibiting the least amount of weight gain (Table 11). Likewise, AFB_1 significantly reduced feed efficiency in a non-linear manner, with the 0 ppm AFB_1 treatment group demonstrating the highest feed efficiency (0.91) and treated groups ranging from 0.49-0.75. Survival also greatly varied across treatments with 0 ppm AFB_1 having the highest survival rate.

Among the NS-supplemented treatment groups, only weight gain and feed efficiency were significantly different compared to AFB_1 controls, with *P*-values of 0.039 and 0.005, respectively. In the case of feed efficiency, 0 ppm AFB_1 and 5 ppm AFB_1 were the most significantly different. NovaSil inclusion at both 1 and 2% positively affected weight gain, feed efficiency, and survival after AFB_1 exposure, although not in a dose-dependent manner.

3.3.2 Immune response

A summary of immune parameters evaluated for each group is shown in Table 12. The 0.1 ppm AFB_1-exposed fish exhibited the highest plasma lysozyme values (246 units/mL), while the 5 ppm-exposed fish displayed the lowest levels (45 units/mL). Trypsin inhibition (%) results indicated that 1, 2, 3, and 5 ppm AFB_1-exposed groups had

Table 11. Growth performance of red drum fed different concentrations of AFB_1[1] and AFB_1 + NS [2,3,4]

Variable	Weight gain[5] (%)	Survival (%)	Feed efficiency	Variable		Weight gain (%)	Survival (%)	Feed efficiency
AFB_1 (ppm)	**Individual treatment means**			**AFB_1 (ppm)**	**NS (%)**	**Individual treatment means**		
0	332^{a}	80.0^{a}	0.91^{a}	0	0	332.72^{ab}	80	0.91^{a}
0.1	223^{bc}	46.6^{b}	0.62^{bc}	5	0	188.74^{c}	55.5	0.62^{c}
0.25	224^{bc}	55.5^{b}	0.65^{bc}	5	1	339.80^{a}	73.3	0.82^{ab}
0.5	254^{ab}	60.0^{ab}	0.75^{ab}	5	2	218.50^{bc}	57.7	0.71^{bc}
1	212^{bc}	60.0^{ab}	0.73^{abc}	*P*-value		0.039	0.261	0.005
2	136^{c}	60.0^{ab}	0.49^{c}	Pooled Std. Error		7.047	1.801	0.008
3	183^{bc}	62.2^{ab}	0.67^{bc}	**AFB_1 (ppm)**	**NS (%)**	**Means of main effect**		
5	188^{bc}	55.5^{b}	0.62^{bc}	0		332.72	80.0	0.91^{a}
R^2	0.22	0.01	0.1	5		249.04	62.2	0.72^{b}
P-value	0.005	0.132	0.03		0	260.73^{a}	67.7	0.77
Pooled Std. Error	5.189	1.309	0.013		1	339.88^{ab}	73.3	0.82
					2	218.51^{b}	57.7	0.71
						ANOVA: *P*-values		
				AFB_1		0.083	0.138	0.003
				NS		0.043	0.387	0.029

[1] Aflatoxin B_1

[2] NovaSil

[3] Values are means of three replicate groups of fish (n=3)

[4] Values in a column that do not have the same superscript are significantly different according to Duncan's multiple range test ($P < 0.05$)

[5] Initial average weight was 2.1±0.1g/fish

Table 12. Immune parameters of red drum[1]

Variable	Serum lysozyme (units/mL)	NBT (mg/mL blood)[2]	Trypsin inhibition (%)	Variable		Serum Lysozyme (units/mL)	NBT (mg/mL blood)[2]	Trypsin inhibition (%)
AFB_1 (ppm)	**Individual treatment means**			**AFB_1 (ppm)**	**NS (%)**	**Individual treatment means**		
0	165^{ab}	3.52	83.6^{ab}	0	0	165^{ab}	3.52	83.6
0.1	246^{a}	3.35	82.4^{b}	5	0	45^{c}	3.07	81.9
0.25	131^{bcd}	2.54	86.3^{a}	5	1	$76b^{c}$	3.32	81.3
0.5	155^{abc}	3.30	83.2^{b}	5	2	185^{a}	3.21	79.4
1	106^{bcd}	1.78	81.9^{b}	P-value		0.024	0.944	0.577
2	82^{bcd}	3.05	80.5^{b}	Pooled Std. Error		5.550	0.104	0.395
3	63^{cd}	2.21	82.7^{b}	**AFB_1 (ppm)**	**NS (%)**	**Means of main effect**		
5	45^{d}	3.07	81.9^{b}	0		165	3.52	83.6
R^2	0.394	0.015	0.109	5		102	3.20	80.9
P-value	0.004^{c}	0.250	0.038^{c}		0	105	3.30	82.7
Pooled Std. Error	5.705	0.102	0.192		1	76	3.32	81.3
					2	185	3.21	79.4
						ANOVA: P-values		
				AFB_1		0.018	0.622	0.291
				NS		0.021	0.948	0.674

[1] Values in a column that do not have the same superscript letters are significantly different according to Duncan's multiple range test ($P < 0.05$).
[2] Values are means of determinations on two fish from each of three replicate groups (6 fish/ treatment, n=6).

Table 13. Somatic indicies of red drum fed different concentration of AFB_1[1] and AFB_1+NS[2,3,4]

Variable	Spleen	MSI[5]	HSI[6]	IPF[7]	Variable		Spleen	MSI	HSI	IPF
AFB_1 (ppm)	**Individual treatment means**				**AFB_1 (ppm)**	**NS (%)**	**Individual treatment means**			
0	0.044	28.94	1.678^{ab}_{c}	0.329	0	0	0.044	28.9^{a}	1.67	0.329^{a}
0.1	0.041	27.42	1.989^{a}	0.116	5	0	0.043	26.0^{b}	0.88	0.017^{b}
0.25	0.049	26.18	1.796^{ab}	0.200	5	1	0.095	28.3^{ab}	0.82	0.108^{b}
0.5	0.059	27.87	1.201^{ab}_{c}	0.265	5	2	0.201	29.7^{a}	1.56	0.468^{a}
1	0.030	27.79	1.155^{ab}_{c}	0.076	*P*-value		0.528	0.031	0.292	0.003
2	0.188	26.25	0.721^{c}	0.187	Pooled Std. Error		0.015	0.135	0.07	0.012
3	0.054	25.72	0.945^{ab}_{c}	0.067	**AFB_1 (ppm)**	**NS (%)**	**Means of main effect**			
5	0.043	26.06	0.881^{bc}	0.017	0		0.044	28.9	1.67	0.329
R^2	0.004	0.141	0.267	0.152	5		0.113	28	1.09	0.198
P-value	0.503	0.417	0.091	0.466		0	0.044	27.5	1.27	0.173^{a}
Pooled Std. Error	0.015	0.315	0.091	0.03		1	0.095	28.3	0.82	0.108^{a}
						2	0.201	29.7	1.56	0.468^{b}
							ANOVA: *P*-values			
					AFB_1		0.494	0.298	0.205	0.112
					NS		0.429	0.019	0.332	0.002

[1] Aflatoxin B_1
[2] NovaSil
[3] Values in a column that do not have the same superscript letters are significantly different according to Duncan's multiple range test ($P < 0.05$).
[4] Values are means of determinations on two fish from each of three replicate groups (6 fish/ treatment, n=6).
[5] Muscle somatic index
[6] Hepatosomatic index
[7] Intraperitoneal fat

the lowest percent inhibition and 0.25 ppm AFB_1 the highest. Additionally, neither the lysozyme nor the trypsin results suggested linearity with an R^2 of 0.3947 and 0.109, respectively. The NBT test showed no significant differences among any of the AFB_1-exposed groups. NovaSil had a significant impact ($P = 0.021$) on the plasma lysozyme concentration with 5 ppm AFB_1+2% NS outperforming all other treatments. NovaSil did not significantly alter levels of NBT or trypsin inhibition.

3.3.3 Somatic indexes

Somatic indexes for spleen, MSI and IPF did not vary within the AFB_1-treated groups; however, HSI varied slightly between treatments. The highest HSI levels were recorded in the 0.1 AFB_1-treated group, while the 2 ppm and 5 ppm exposure groups exhibited the lowest values (Table 13). A linear trend was not present in any of the groups. In the NS-supplemented groups, muscle and IPF levels recovered to control levels in the treatment group administered 2% NS. Likewise, the means of main effect data indicate that NS inclusion at either 0 and 1% was statistically different than 2%.

3.3.4 Proximate composition

No linear trends were present in the AFB_1-treated groups (Table 14). Percent lipid composition was highest in the 0 ppm AFB_1 group and the lowest at 2 ppm AFB_1, but varied among other treatments. There were some variations in ash values as well; however, these results were not linearly correlated. Inclusion of NS in the diets did not exhibit any statistically significant changes in whole-body proximate composition.

Table 14. Proximate composition of red drum (fresh-weight basis)[1]

Variable	% Lipid	% Protein	% Moisture	% Ash	Variable		% Lipid	% Protein	% Moisture	% Ash
AFB_1 (ppm)	**Individual treatment means**				**AFB_1 (ppm)**	**NS (%)**	**Individual treatment means**			
0	2.70[a]	76.01	78.38	16.38[ab]	0	0	2.21	76.01	78.30	3.54
0.1	2.37[ab]	74.45	79.34	17.56[a]	5	0	1.98	76.52	79.29	3.73
0.25	1.97[bcd]	70.06	79.67	13.64[b]	5	1	2.20	73.92	76.91	4.28
0.5	2.42[ab]	74.33	77.69	16.72[ab]	5	2	2.19	72.93	79.04	4.35
1	2.17[abc]	71.64	78.71	18.04[a]	*P*-value		0.510	0.723	0.173	0.629
2	1.45[d]	74.20	84.55	19.43[a]	Pooled Std. Error		0.022	0.488	0.140	0.098
3	1.77[cd]	71.64	80.60	17.22[a]	**AFB_1 (ppm)**	**NS (%)**	**Means of main effect**			
5	1.98[bcd]	76.52	79.29	18.02[a]	0		2.21	76.01	78.38	3.54
R^2	0.211	0.021	0.024	0.109	5		2.12	74.46	78.41	4.12
P-value	0.002	0.476	0.452	0.038		0	2.10	76.20	78.83	3.64
Pooled Std. Error	0.033	0.441	0.402	1.728		1	2.20	73.90	76.91	4.28
						2	2.19	72.90	79.04	4.35
							ANOVA: *P*-values			
					AFB_1		0.534	0.611	0.964	0.357
					NS		0.394	0.604	0.095	0.663

[1]Values are means of determinations on three fish from each of the three replicates (n=3).

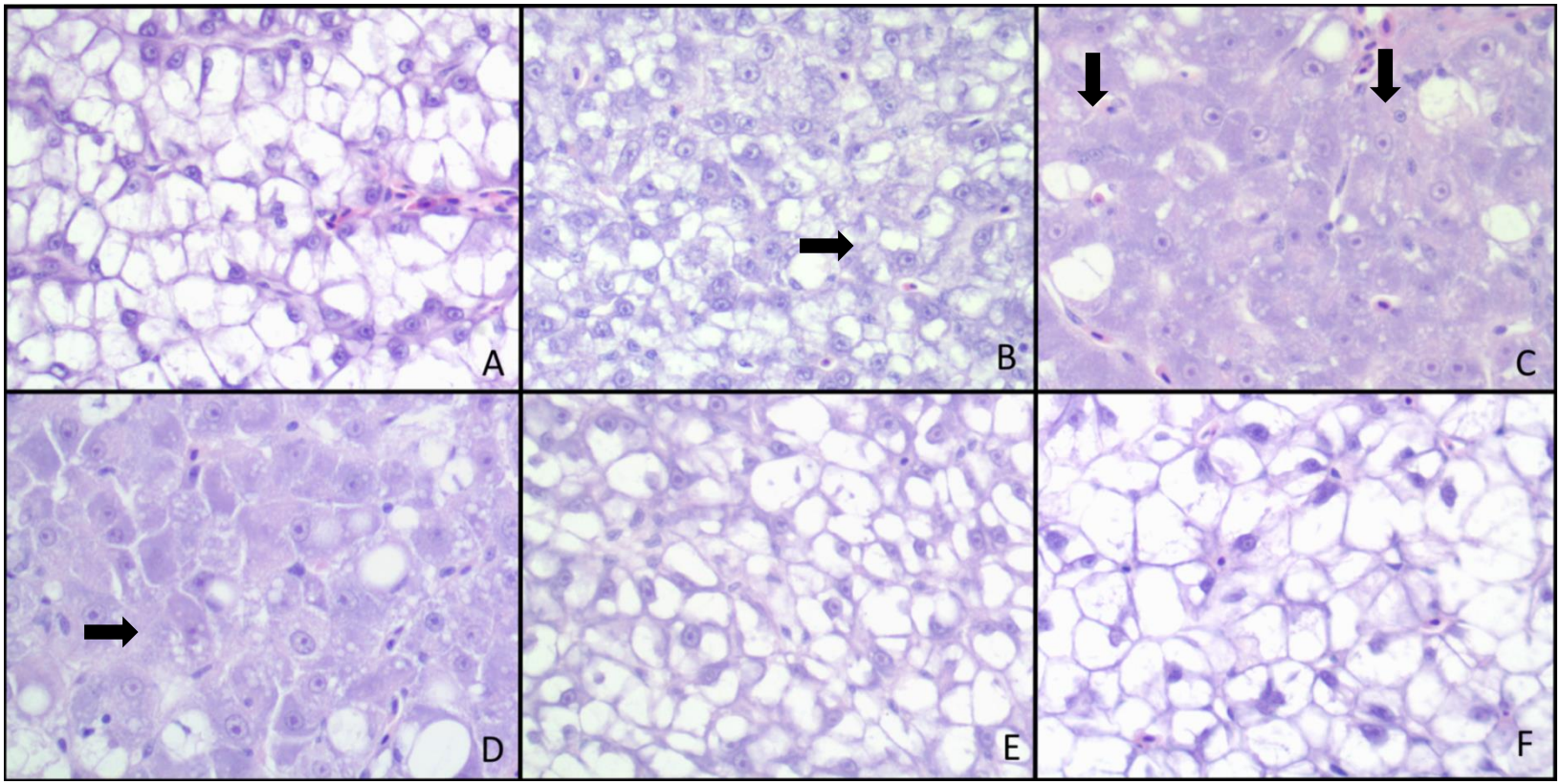


Figure 10. Liver histopathology in AFB_1-exposed red drum. Liver sections were stained with hematoxylin and eosin. Treatments were as follows: A) 0 ppm AFB_1 B) 1 ppm AFB_1 C) 3 ppm D) 5 ppm AFB_1 E)AFB_1+ 1% NS and F) 5 ppm AFB_1 +2% NS. Marked pleomorphism, megalokaryosis with prominent nucleoli (arrows) and loss of hepatocellular cytoplasmic macrovacuolation was observed in the treatment groups that received large amounts of aflatoxin (B,C,D). Although not significant, inclusion of NS resulted in decreased histopathological scores, especially attributed to increased cytoplasmic vacuolation and reduced cellular pleomorphism.

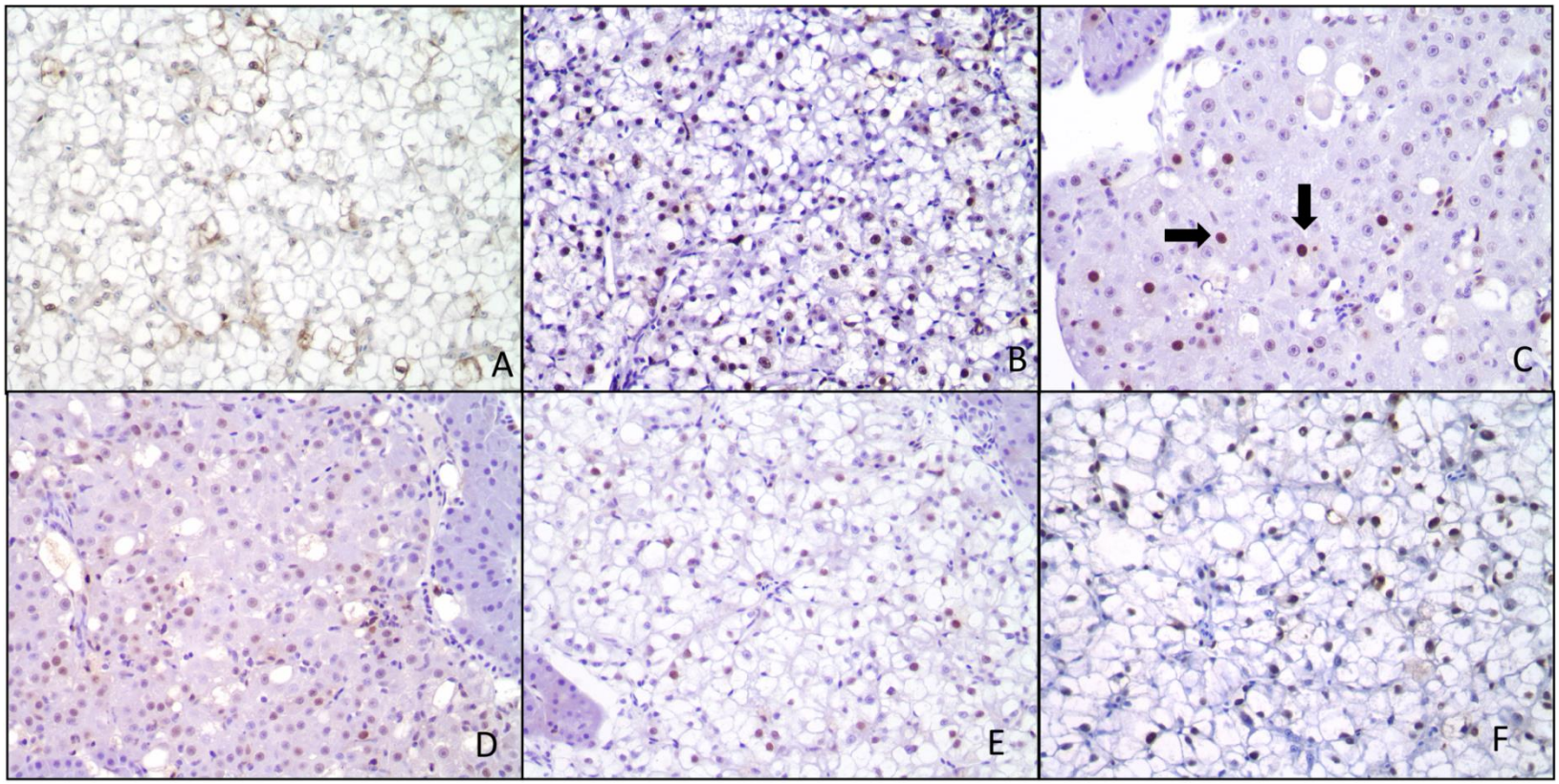


Figure 11. Proliferating Cell Nuclear Antigen (PCNA) positive cells in red drum hepatocytes. Liver sections were stained with PCNA (arrows) and hematoxylin counterstain. Treatments were as follows: A) 0 ppm AFB_1 B) 1 ppm AFB_1 C) 3 ppm AFB_1 D) 5 ppm AFB_1 E) 5 ppm AFB_1+ 1% NS F) 5 ppm AFB_1+ 2% NS. Although not significant, inclusion of NS resulted in a decrease of PCNA-positive hepatocytes. Reduction in cell proliferation suggests that NS afforded some protection from AFB_1 toxicity and cellular proliferation.

3.3.5 Histopathological response and immunohistochemistry

Significant histological changes were observed between treatments (Table 15), with 3 and 5 ppm AFB_1 eliciting the most severe hepatic alterations. Although some samples revealed significant hepatic lesions in groups treated with 5 ppm AFB_1 + 1 or 2% NS, the findings in these fish were considered mild when compared to the 5 ppm AFB_1 without NS. There were no significant differences in PCNA values among all treatments, nor did PCNA staining exhibit a positive linear correlation. Histological changes, characterized by restoration of hepatocellular macrovacuolation and reduced megalocytosis and karyomegaly, were noted with the addition of NS in the diet; however, these results were not statistically significant (Figure 10). A decrease in PCNA staining as compared to the 5 ppm inclusion level was noted, but also did not achieve significant levels with 1 or 2% NS inclusion in the diet (Figure 11).

3.4 Discussion

Aflatoxin B_1 displayed a significant effect across multiple treatment levels. The survival rate for the basal diet group (0 ppm AFB_1) was similar to control survival results reported in other red drum studies [324, 325], although survival was negatively affected by AFB_1 presence. Likewise, the impact on feed efficiency and weight gain found in this study has been similarly documented in other AFB_1-exposure publications, including research analyzing the effects of aflatoxins on several different farmed aquatic species [83, 326, 327, 328, 329]. The majority of AFB_1-sensitive ichthyoids are cold-water species and our findings suggest that red drum may be one of the first identified

Table 15. Histopathology and immunohistochemistry

Variable	Histology Score[1]	PCNA	Variable		Histology Score	PCNA
AFB_1(ppm)	**Individual means**	**treatment**	**AFB_1(ppm)**	**NS (%)**	**Individual treatment means**	
0	5.25[a]	6.27	0	0	13.16[ab]	6.27
0.1	10.67[a]	8.59	5	0	19.00[b]	10.49
0.25	17.33[ab]	9.35	5	1	9.16[a]	9.11
0.5	30.16[c]	11.34	5	2	7.66[a]	9.72
1	25.83[bc]	9.52	P-value		0.0925	0.7542
2	31.83[c]	9.06	Pooled Std. Error		0.838	0.836
3	37.00[c]	10.30	**AFB_1 (ppm)**	**NS (%)**	**Means of main effect**	
5	37.00[c]	10.49	0		13.16	6.27
R^2	0.2353	0.0204	5		11.94	9.78
P-value	0.0001	0.5059		0	16.08	8.38
Pooled Std. Error	1.13	0.8159		1	9.16	9.11
				2	7.66	9.72
					ANOVA: *P*-values	
			AFB_1		0.7248	0.3251
			NS		0.0491	0.9454

[1] Values in a column that do not have the same superscript letters are significantly different according to Duncan's multiple range test ($P < 0.05$).

AFB_1- sensitive warm-water species. However additional studies are necessary to determine the specific metabolic mechanisms responsible for this sensitivity. The published aquaculture literature indicates that incremental increases in AFB_1 exposure do not typically result in dose-dependent, linear responses [90, 91, 97]. Herein, analysis of growth performance factors indicated that some of the most significant AFB_1 effects were present at the lowest level of AFB_1- exposure (0.1 ppm) for feed efficiency, survival, and weight gain. Hormetic responses for growth and immunological parameters have been observed in several species [330]. Hormesis is defined as a biphasic response to an xenobiotic, characterized by a low-dose stimulatory effect and high-dose inhibitory or toxic effect in which a U-shaped or J-shaped model is apparent [331]. Instances of AFB_1-associated hormesis have also been documented in multiple species [332, 333, 334]. Several measured parameters in the current study suggest that AFB_1-exposed red drum exhibited an "inverted U-shaped" immunological hormetic response to AFB_1 as suggested by plasma lysozyme at the 0.1 ppm level and trypsin inhibition at the 0.25 ppm level. Additionally, HSI results indicated a similar increase at the 0.1 ppm level followed by subsequent decreases at higher AFB_1 levels.

Several studies have indicated that PCNA is a suitable marker for cellular proliferation in fish [335, 336] as well as other species [337, 338]. However, our study did not indicate any significant differences in PCNA staining among the treatments. It is possible that the levels of AFB_1 used in this study were not capable of inducing significant cellular proliferation as observed with other species. While there was a slight increase in PCNA with the presence of AFB_1, there was a decrease in HSI. The increase

in PCNA is due to liver damage and mitotic activity from AFB_1-exposure, while the overall decrease in HSI is likely attributed to the loss of vacuolation and fat in the liver.

Histological evaluation indicated liver changes characterized by anisokaryosis, megalocytosis and karyomegaly in AFB_1-exposed red drum, which have been noted in a series of AFB_1 studies with other fish species [42, 339, 340]. Hepatocellular lipid deposition, a well-documented classical sign of aflatoxicosis in fish [341, 342], was present in red drum exposed to AFB_1. However, red drum kept in captivity typically display fatty deposition and hepatocellular macrovacuolation [343], which should be taken into consideration for accurate red drum liver evaluation. The hepatocellular vacuolation seen in control livers was markedly reduced, as anisocytosis and karyomegaly increased, especially in fish exposed to higher levels of aflatoxin. Interestingly, hepatocellular vacuolation and liver fat were restored in fish treated with NS. Ideally, further red drum AFB_1 studies should pair liver histological evaluation with other molecular markers to confirm liver damage, such as inducible nitric oxide synthase ([344] or γ-glutamyl transpeptidase [345, 346]. Additionally, because feed efficiency, IPF and liver fat decreased with AFB_1 exposure, it is possible that there was increased energy expenditure in the fish because less food was utilized. However, more research is needed to determine the exact mechanism of fat loss in AFB_1-exposed red drum.

In this study, NS supplementation in the diets of AFB_1-exposed fish resulted in a protective effect, which was evident by the significant improvement in many of the tested parameters. Other studies have reported that a 2% inclusion level of bentonite, a common clay containing montmorillonite, in trout feed reduced toxic AFB_1

effects [112]. Yet other studies suggest that a 0.5% inclusion level was sufficient to protect tilapia from 1.5 ppm AFB_1 [340]. Bentonites have been added into fish feed at concentrations up to 10% with no alteration in whole-body proximate composition [347]. Discrepancies in the aquaculture literature concerning the proper inclusion level of clay-based binders indicate a need to establish a clay dosing regimen for fish at risk for AFB_1 exposure.

3.5 Conclusions

These findings indicate that red drum are susceptible to AFB_1 in levels as low as 0.1 ppm. Other unevaluated species should be tested for AFB_1 susceptibility, especially warm-water species raised in tropical and subtropical environments where the mycotoxin contamination risk is high. NovaSil supplementation at levels between 1-2% may be used in fish feed safely to effectively reduce AFB_1 toxicity. Therefore, this technology could be used by the aquaculture industry as a strategy to reduce aflatoxin-related morbidity and mortality in fish.

4. NOVASIL AS A THERAPY FOR TNBS-COLITIS: A ONE-WEEK PILOT STUDY

4.1 Introduction

Inflammatory bowel disease (IBD) is currently the leading gastroenterological condition that is increasing healthcare expenses in the United States. Direct medical costs related to CD total an average of $18,000 per patient per year [348]. Current therapies for CD are contributing to these excessive costs and often leave patients with side effects such as increased suceptibility to infectious disease and increased risk for certain types of cancer and nervous system disorders [217]. Further investigation of alternative therapies to supplement or replace conventional treatments is warranted. While NovaSil (NS) has historically been used as an anti-caking agent and more recently, a mycotoxin binder [116, 118], little is known about its anti-inflammatory effects or capability to reduce histopathology in the gastrointestinal tract of an IBD patient. Studies suggest that similar clays have been successfully used to treat infectious diarrhea [349] and can alleviate certain IBD effects in rodent models [153]. However, factors such as proper dose, method of administration, and efficacy in mitigating active disease and/or maintaining a state of remission have yet to be determined.

The TNBS colitis murine model is one of the most commonly used models for CD research. It is characterized by a Th1/Th17 cytokine profile and transmural (patchy) inflammation along the GI tract. In this model, rodents under sedation are intrarectally injected with a mixture of TNBS/EtOH or PBS/EtOH. After injection, TNBS haptenates colonic proteins with trinitorphenyl which causes an inflammatory immune response.

Advantages of the TNBS colitis model are that it is highly reproducible and it produces similar CD-like immune and histopathological effects.

The purpose of this study was to provide preliminary data to determine the therapeutic benefit of NS at a 2% inclusion rate in the TNBS colitis model. Feed rejection, colitis symptoms and inflammatory endpoints were assessed.

4.2 Materials and methods

4.2.1 TNBS colitis model

Five-wk old female BALB/c mice were ordered from The Jackson Laboratory (Bar Harbor, Maine) and acclimated for 1 week at the Comparative Medicine Program (CMP) building according to the animal use protocol approved by Texas A&M University (TAMU). Four mice were randomly assigned to each cage. Additional bedding was added into the cages and shallow feeding bowls were implemented to increase comfort and to better maintain hydration level. Feed was moistened with a small amount of H_2O to prevent dehydration during episodic diarrhea. A constant temperature (23°C) and a 12 hr. light:dark cycle were implemented in the room where animals were housed. Mice were randomly assigned to one of three treatment groups: Control, TNBS or TNBS+NS.

Before induction, mice were lightly anesthetized with isofluorane gas in a small chamber. A 4-cm oral gavage tip was used for intrarectal dosing and surgical lubricant was applied to the tip. Once the mice were sedated, they were quickly removed from the chamber and intrarectally injected with 150 uL of TNBS/EtOH or PBS/EtOH. Mice were secured vertically in a “recovery” chamber to ensure thorough distribution and

retention ofTNBS/EtOH orPBS/EtOH in the colon. Mice were induced on day 1 of the study and euthanized on day 7.

4.2.2 Weight gain and somatic indexes

Mice were collectively weighed according to each cage. Weights were recorded at three different timepoints: immediately before intrarectal injection, at 3 days, and at the end of the week (prior to euthanasia). Somatic indexes were calculated using the following equation: (organ weight/ body weight)*100.

Table 16. Histopathological criteria

Feature	Description	Scores
Inflammation extent	None	0
	mucosa	1
	mucosa+submucosa	2
	transmural	3
Damage in crypt architecture	None	0
	hyperplasia/regeneration	1
	destruction	2
Hyperemia/edema	without	0
	With	1
Infiltration with inflammatory cells	without	0
	Mild	1
	moderate	2
	Severe	3
Ulceration	None	0
	focal/mild	1
	multifocal/severe	2

4.2.3 Histopathology

Immediately following euthanasia, the intestines were collected from each mouse and flushed with PBS. Transverse cross sections were cut in 1-cm sections, stored in

10% formalin, and allowed to fix overnight. Intestines were embedded within 24 h. for histopathology at the Texas A&M Veterinary Pathobiology laboratory. Samples were sectioned at 5 μm and stained with hemotoxylin and eosin. Histopathology scoring was performed by Dr. Aline Rodrigues Hoffmann according to the criteria listed in Table 16.

4.2.4 C-reactive protein

Upon CO_2 euthanasia, mice were exsanguinated via cardiac puncture. Blood was allowed to separate for 3 h, and subsequently centrifuged. Serum and collected and stored at -20˚C. C-reactive protein (CRP) was detected in the serum using ELISA (SABiosciences) using serum samples run from each individual mouse.

4.3.5 Statistical methods

JMP software (SAS Institute) was used to analyze all statistics for the study. All data were subject to ANOVA and data were considered significant at $P<0.05$. A post-hoc Student's t-test was performed for all parametric data and a Wilcoxson test was employed for non-parametric data.

4.3 Results

4.3.1 Weight gain, somatic indexes and inflammation

There were no significant changes in weight loss or somatic indexes during the one week study (Figure 12). However, the TNBS group experienced the greatest weight loss followed by TNBS+NS. It was also noted that there was a slight weight decrease in the Control group, possibly due to the temporary lack of appetite following intrarectal injection and sedation. Colon length slightly decreased in the TNBS group, compared to

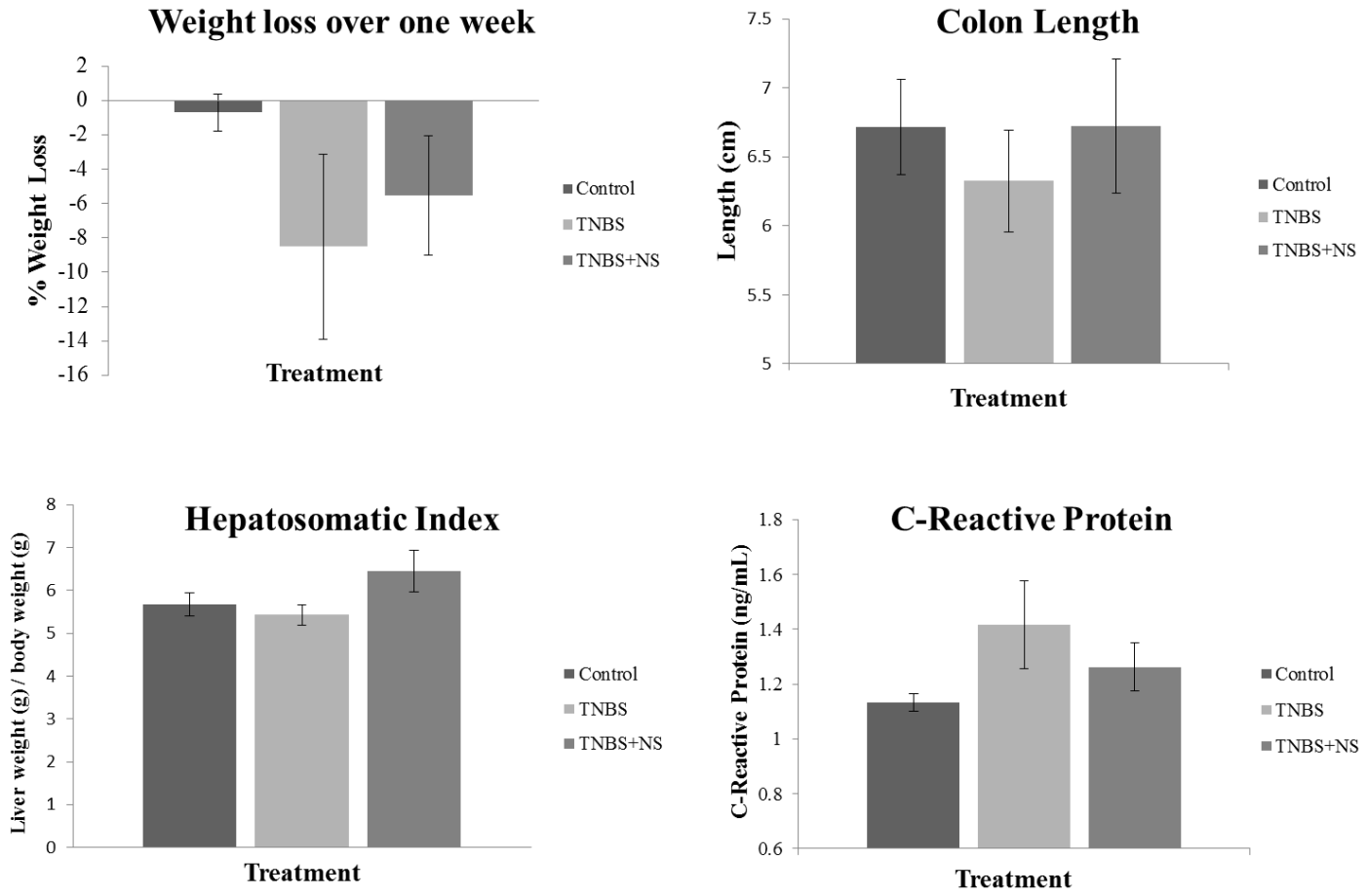


Figure 12. Physiological parameters. Weight loss, colon length, hepatosomatic index and C-reactive protein were examined at the end of 1 week. While results were not significant, colon length decreased slightly with the presence of TNBS. Additionally, weight loss decreased in the TNBS group, but was less severe in the TNBS+NS group.

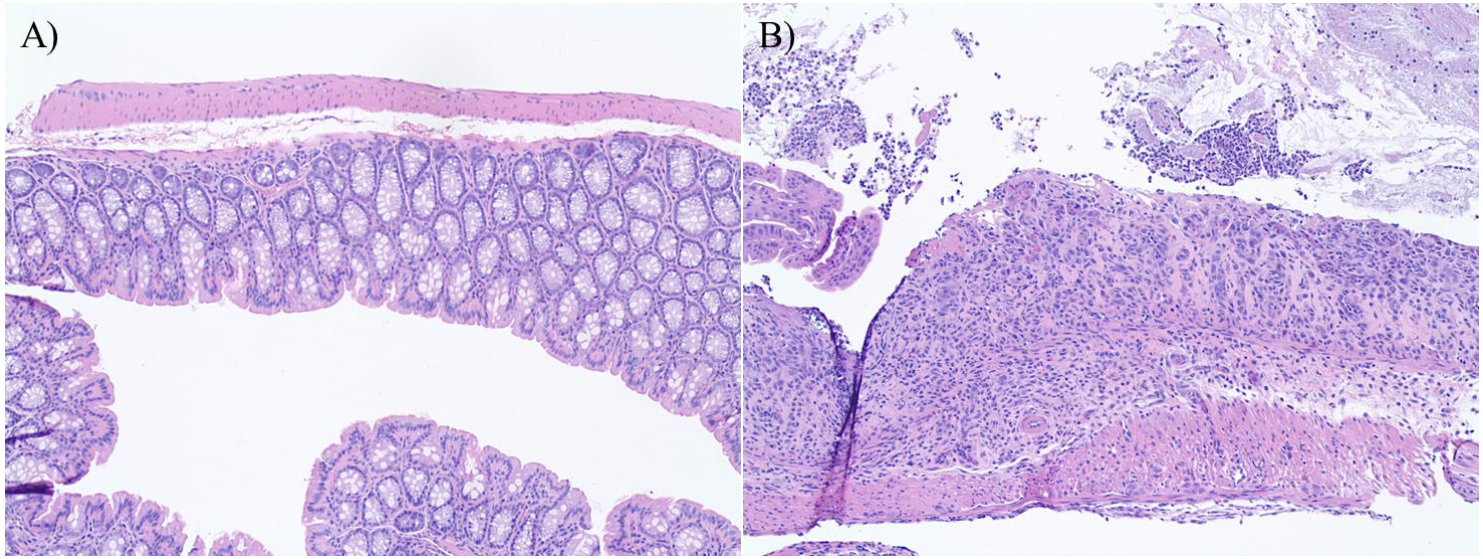


Figure 13. Control and colitis intestinal histopathology. A) Control intestinal tissue demonstrating uniform crypt structure and healthy lamina propria B) Coltis tissue demonstrating mucosal inflammation and distortion of crypt architecture

the Control and TNBS+NS groups, however this trend did not exist for hepatosomatic index values. C-reactive protein levels non-significantly increased in the TNBS group, but TNBS+NS group samples exhibited levels that more closely resembled those of the control group.

4.3.2 Histopathology

Results indicate that there were no significant differences between treatments based on histopathological scoring (P=0.43) (Figure 13). Colitis was successfully induced in mice exposed to TNBS, however the severity of colitis varied substantially between individual mice. Mice in the TNBS or TNBS+NS groups showed signs of inflammation and edema in the intestine. Damage to crypt architecture was the most prominent finding from TNBS group, while mice in the TNBS+NS group demonstrated less severe crypt damage. Control tissues demonstrated mild histopathology, most likely due to the intrarectal injection procedure. NovaSil did not appear to have any significant effect on histopathological scores.

4.4 Discussion

The purpose of this pilot study was to explore the anti-inflammatory effects of NS *in vivo*. Trends resulting in improvement in weight gain, decrease in CRP, and increased colon length with NS supplementation in the feed indicated that NS may provide protection. Other researchers determined that feed efficiency and weight gain were improved solely with clay supplementation in chickens and pigs [350]. Additionally, clay-based supplementation improved weight gain and post-weaning diarrhea in young pigs [351]. Furthermore, children suffering from infectious diarrhea

benefit from oral clay therapy. In a 2001 Italian study with over 800 children, smectite demonstrated a significant decrease in the duration of diarrhea, improved stool consistency and decreased frequency of bowel movements. [138]. Several other rodent study investigators have administered clay therapy using oral gavage. Although oral gavage is an acceptable method to administer therapeutics, mice did not demonstrate feed rejection with 2% NS inclusion in the diet. Therefore, NS administration via feed inclusion appears to be an acceptable method of dosing. Avoiding gavage in a mouse model involving intrarectal injection is advantageous, considering that consecutive intrarectal injections and gavages would likely cause excess physiological and psychological stress.

Based on the results of this study, some minor improvements are warranted for future *in vivo* colitis experiments. Histopathology did not coincide with the other results obtained including weight gain, CRP and colon length. This may be attributed to the manner in which the colon tissue was collected. Sections were sampled transversely and only revealed a small subsection of the entire length of the colon. Due to transmural ulceration in CD, it is possible that the transverse cross sections did not reflect the amount of ulceration that was present throughout the entire length of the colon. Future studies may either need to alter collection of colon samples or utilize indirect methods to assess intestinal damage and inflammation, rather than rely on histopathology.

One-hundred and fifty μL is the standard fluid volume of TNBS administered to mice, based on the previously published TNBS colitis protocol [201]. In this study, the 5-wk-old BALB/c mice induced with 150 μL exhibited symptoms of excess induction.

Subsequently, three out of the eight mice died (38%) in the first 4 days in both the TNBS and TNBS+NS groups. Some studies have reported up to 60% mortality after 1 week [352, 353]. Additionally, the volume was most likely too large and the mice tended to release a portion of the fluid, leading to some inconsistency in dosing. Future studies will incorporate a smaller volume for more accurate dosing and to avoid loss of the TNBS. Additionally, since therapeutic treatment did not yield significant results, prophylactic administration of NS will be administered in future studies at a higher dosage and the length. A longer study is warranted to determine if more time would provide more beneficial therapeutic effects.

4.5 Conclusions

This study suggests that NovaSil may be a promising therapy for TNBS-induced colitis. Though not significant, NS improved weight gain, CRP levels, and colon length. This study also provided valuable information that will improve future studies. Finally, an *in vitro* investigation that will evaluate the mechanism behind NS-based IBD therapy is needed.

5. MITIGATION OF TNBS-INDUCED COLITIS WITH NOVASIL THERAPY

5.1 Introduction

Crohn's disease (CD) is characterized by painful ulceration that can occur along the gastrointestinal tract, from the mouth to the anus, as opposed to ulcerative colitis (UC) which is restricted to the colon [354]. The majority of cases occur in the developed world, similar to other autoimmune diseases. Although the etiology of CD is complex, genetic polymorphisms, alterations in intestinal microbiota, and a modulated immune response have all been attributed to possible causes of the disease. Additionally, cigarette smoking, excess stress and environmental factors are known to play a role in the progression of the disease [355, 356, 357]. Treatments for CD can be expensive and often cause undesirable side effects [358, 359]. Common pharmaceutical treatments include aminosalicylates, antibiotics, corticosteroids, and biologics (anti-TNFα agents) [360]. Immunosuppressive treatments often involve increased risk of infection and certain types of cancer, such as lymphoma [361]. Due to various risks associated with these medications, there is a need to develop alternative therapies. Diarrhea caused by various gastrointestinal (GI) conditions can be mitigated by dietary clays. Historically, DS clays have been utilized effectively for the treatment of diarrhea caused by infectious diseases [138, 139, 362]. From these studies, it can be most easily speculated that clay treatments are capable of pathogen or toxin sorption. However, DS has also been shown to increase mucosal barrier integrity against pepsin and TNFα exposures *in vivo* [363, 364], and to provide protection from immune system disturbances induced in guinea pigs sensitized to cow's milk [365]. Moreover,

inflammation occurring as a result of acute hapten exposures decreased following treatment with dietary clays [153].

Although the specific mechanisms of action have yet to be reported in the case of chronic diarrhea, several theories have been proposed that may support the therapeutic nature of smectite clays, including the possibility of intestinal mucus barrier reinforcement (reducing penetration of luminal antigens) and modulation of pro-inflammatory cytokine production and effects. Of particular importance is the intestinal barrier, which is affected by the presence of luminal inflammatory cytokines, such as IL-6, IL-1β, and TNFα [366]. In the absence of bacterial or viral infection, malfunction of the intestinal barrier is pivotal in diseases causing chronic diarrhea, such as IBD [367]. Additionally, DS clays have demonstrated the potential to shift the population of intestinal flora from a pathological to a balanced state [158, 368, 369], which is known to be important in the etiology and management of IBD [192, 370]. Furthermore, *in vitro* and *in vivo* studies have indicated that smectite clays can form aggregates with *E. coli* [371, 372]. Copper-bearing montmorillonite decreased *E. coli* and *Clostridium* counts in the intestine of male broilers and also improved intestinal mucosal morphology [159]; however, no studies have profiled the intestinal microbiome in smectite-supplemented animals

NovaSil (NS), a calcium montmorillonite clay with a dioctahedral structure and negatively charged interlayer, has been administered as a supplement both in animal feeds and in clinical intervention trials throughout the world to reduce dietary mycotoxin bioavailability [373]. Currently, little information is available concerning the potential

NS anti-inflammatory properties or its impact on gut microbiota. Similar clays have been reported to possess anti-inflammatory properties, but the mechanism remains unclear [153, 374, 375]. For this reason, we investigated the ability of NS to interact with proinflammatory cytokines, protect the intestinal microbiota, and mitigate colitis.

The pH of the normal colon ranges between 6.5 and7.6. Comparatively, the colonic pH in an individual with CD is approximately 5.3 [376], but can drop to a pH of 0.6 in a patient with severe disease. [377]. Based on the fact that the isoelectric point of TNFα is 6.4±0.3 [378], it is expected that TNFα is protonated in individuals living with CD. Likewise, the isoelectric point of proinflammatory IL-1β lies between 6.1 and 6.9 [379], and is increasingly protonated as pH becomes more acidic. This suggests that negatively charged NovaSil could sorb protonated proinflammatory cytokines at a low pH. NS has a long-standing record of safety and efficacy and does not interfere with vitamin or nutrient levels in the serum. If NS demonstrates anti-inflammatory effects in a TNBS model, then it may prove an effective model for CD.

The purpose of this study was two-fold: 1) to characterize the NS-cytokine interaction *in vitro*, and 2) to determine the ability of NS to reduce colitis-related effects and counteract dysbiosis in a TNBS-mouse model.

5.2 Materials and methods

5.2.1 ELISA-based assessment of *in vitro* binding affinity

Deionized H_2O was adjusted to pH 5 to simulate the intestinal pH that would likely be present in diseased intestine. Recombinant TNFα and IL-1β protein (Sigma-Aldrich, St. Louis, MO) were added to the pH-adjusted H_2O in borosilicate glass vials,

resulting in a protein concentration of 210 pg/mL in each vial. Additionally, NS ranging from 0 to 400 μg/mL was added to the vials. To determine if protein was primarily bound to internal or external clay surfaces, interaction with both intact and heat-collapsed NS was investigated. Heat-collapsed NS was synthesized according to a previously published method [380]. The interlayer contributes to the majority of negative charge of the NS structure [381]. Collapsing the structure of NS results in elimination of H_2O from the interlayer as well as dehydroxylation of the clay. Briefly, NS was heated in a furnace for 30 min. at 200° C and then at 800°C for 1 h. The intact and heat-collapsed NS were then separately added to individual vials. The protein was maintained at a constant concentration (210 pg TNFα or IL-1β) and total volume adjusted to 1 mL per vial with H_2O. Controls included one vial for H_2O only, diluted protein only and H_2O+each concentration of NS. The vials were incubated at room temperature on an orbital shaker operating at 100 rpm for one hr. The vials were then removed from the shaker and centrifuged at 2000 rpm for 20 minutes. The supernatant was pipetted and separated from the NS pellet at the bottom of the tube and measured via ELISA for TNFα and IL-1β. The NS pellet was resuspended in H_2O, incubated, centrifuged as described above, and subjected to cytokine detection by ELISA. This procedure was used to determine the amount of unbound protein in the supernatant. Protein concentrations were calculated based on a standard calibration curve.

5.2.2 Transmission electron microscopy (TEM) and powder x-ray diffraction (XRD)

Samples containing 1 μg TNFα/100 μg NS or 1 μg IL-1β/100 μg NS were prepared for TEM in order to further characterize the protein-NS binding interaction. Samples were dehydrated using an ethanol series followed by propylene oxide. Samples were further embedded in epoxy-resin, sealed with epoxy, and cured at room temperature for 24 h, as described by Kolman et al. [382]. Samples were sectioned into 60-100-nm slices. All images were imaged with a Morgagni (FEI) Transmission Electron Microscope at 80 kV (FEI Company, Hillboro, OR). Diffractograms were recorded for NS, heat-collapsed NS, NS+IL-1β and NS+TNFα. A 1-cm-diameter o-ring was coated with a thin layer of petroleum jelly and suctioned onto a custom-made zero background holder. Samples (1000 μg: 1 μg protein/ 100 μg NS) were drop-casted onto the holder. A Bragg-Brentano powder short-arm diffractometer (Bruker Coorperation, Billerica, MA) was used for all diffraction patterns (λ=0.1540 nm) within the 2θ range of 2° to 20°, with a 0.014° step size. Bragg's Law ($n\lambda=2d\sin\theta$) was used to calculate the d-spacing between the smectite layers.

5.2.3 TNBS induction of Crohn's colitis in mice

Five-wk old BALB/c mice were purchased from the Jackson Laboratory (Bar Harbor, Maine) and housed at the Comparative Medicine Program (CMP) facility at Texas A&M University. To increase animal comfort following the TNBS induction process, extra bedding was added to the cages, and powdered feed was moistened with H_2O and made accessible in low ceramic bowls. A 12:12 h light:dark cycle and a stable temperature (23°C) were maintained in the room where the animals were housed.

TNBS induction of colitis, an established model for CD, was utilized as previously described [201] with slight modifications. Forty mice were equally and randomly allocated into four different treatment groups: Control (Control) (n=10), 4% NS supplementation (NS) (n=10), TNBS-induced (TNBS) (n=10), and TNBS induction with 4% NS supplementation (TNBS+NS) (n=10). Mice in the TNBS+NS and NS groups were conditioned with the 4% NS diet for 1 week prior to the beginning of the 4-week trial. One-hundred μL of 1:1 TNBS:ethanol was intrarectally administered to the TNBS and TNBS+NS groups with a 4-cm gavage tip. Control and NS groups received 100 μL of 1:1 phosphate buffered saline (PBS):ethanol using the same technique on a weekly basis. To ensure retention of all solutions throughout the colon, mice were secured vertically in a recovery chamber for 1 min while anesthetized. Mice that were severely symptomatic (bloody diarrhea, lethargy, impaired motor skills) or exhibited severe weight loss (>25% initial body weight) were immediately euthanized. Mice were induced on a weekly basis, and sacrificed at the end of 4 weeks via CO_2 asphyxiation. This research was approved by the Institutional Animal Use and Care Committee at Texas A&M University, College Station, TX.

5.2.4 Weight gain and final somatic indexes

Mice were weighed on an individual basis twice per week and monitored on a daily basis. Following euthanasia, selected organs, including the liver and gastrointestinal tract, were collected and weights, as well as colonic length were recorded. Colon weight:length ratio was calculated in addition to hepatosomatic index (HSI) ((liver weight (g)/weight of the mouse)*100).

5.2.5 Serum ELISA

Blood (1 mL) was collected from mice via cardiac puncture immediately following euthanasia. Whole blood was allowed to separate for approximately 3 h at 4° C. Blood was subsequently centrifuged and serum was stored at -20° C. Circulating levels of inflammatory proteins were examined using a Mouse Th1/Th2/Th17 cytokine Multi-Analyte ELISArray Kit (MEM-003A, Qiagen, Valencia, CA, USA). Serum was pooled according to treatment group and relative expression of the following cytokines was assayed in triplicate: IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, IL-13, IL-17A, IL-23, IFNγ, TNFα, TGF-β1.

5.2.6 C-reactive protein

Serum C-reactive Protein (CRP) levels were quantified for all treatment groups. Serum CRP was detected using an ELISA kit (Genway Biotech Inc., San Diego, CA, USA). Serum from four mice per group was used with six replicates per mouse.

5.2.7 DNA extraction and gut microbiota sequencing

Feces were collected from the colon following euthanasia. Samples were "flash frozen" in liquid nitrogen, and subsequently transferred to a -80°C freezer until further use. Samples were thawed and genomic DNA was extracted using the Power Soil DNA isolation kit (MoBio Laboratories). Six randomly selected fecal samples were selected from the Control, TNBS and TNBS+NS groups. The V4 region of the 16S rRNA gene was amplified with primers 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and 806R (5'-GGACTACVSGGGTATCTAAT-3') at the MR DNA Laboratory (Shallowater, TX, USA). PCR amplification products were verified on 2% agarose gels and samples were

purified using calibrated Ampure XP beads. The Illumina TruSeq DNA Library was used to prepare a DNA library and sequenced at MR DNA on an Illumina MiSeq instrument.

5.2.8 Microbiome data analysis

Quantitative Insights Into Microbial Ecology (QIIME, v.1.8) software was used to phylogenetically characterize the samples. The raw sequence data were demultiplexed by barcodes, and low quality reads were filtered using the QIIME database's default parameters. A total of 824,894 (median: 45,474; range 37,692 – 54,975 sequences per sample) were obtained. For further analysis, each sample was rarefied to an even sequencing depth of 32,700 sequences per sample to adjust for uneven sequencing depth across all samples. Sequences were then clustered into operational taxonomic units (OTUs) using a closed-reference OTU picking protocol at the 97% sequencing identity level using UCLUST [383] against the Greengenes database [384] pre-clustered at 97% sequence identity [385]. Proportions of bacterial taxa (% of total sequences) were statistically evaluated using a Kruskal-Wallis test, where appropriate, and corrected by multiple comparisons using the Benjamini & Hochberg's False Discovery Rate. P values <0.05 were considered statistically significant. Observed species richness, Chao 1, and Shannon indexes were all determined using alpha-diversity parameters within QIIME.

Beta-diversity analysis was determined using Principal Coordinates Analysis plots (PCoA) and unweighted Unifrac distance metrics. Statistical significance of the resulting distance matric was tested by analysis of similarities (ANOSIM) using the QIIME software [386].

5.2.9 Statistical analysis

Aside from microbiome data, all other data were subject to a one-way ANOVA followed by a Student's t-test for parametric data. Non-parametric data was subject to Wilcoxon rank-sum test. All statistics were analyzed with the assistance of JMP software (SAS Institute, Cary, NC, USA). Values were considered significant at $P \leq 0.05$. Data in graphs are expressed as the mean +/- std. error.

5.3 Results

5.3.1 *In vitro* cytokine-NS interaction

Results indicate that 200 μg NS sorbed 90% of TNFα (190 pg/mL) and 76% of IL-1β (161 pg/mL) after two washes (Fig. 14). Collapsed NS sorbed only 21% of TNFα (44 pg/mL) and 9% of IL-1β (18.9 pg/mL). The highest concentration of NS (400 μg/mL) did not bind more TNFα than the 200 μg/mL concentration (189 pg/mL). NS at concentrations from 100- 400 μg/mL bound the same amount of IL-1β (161 pg/mL). Heat-collapsed NS at concentrations ranging from 50 μg/mL to 400 μg/mL sorbed equal amounts of IL-1β and TNFα bound.

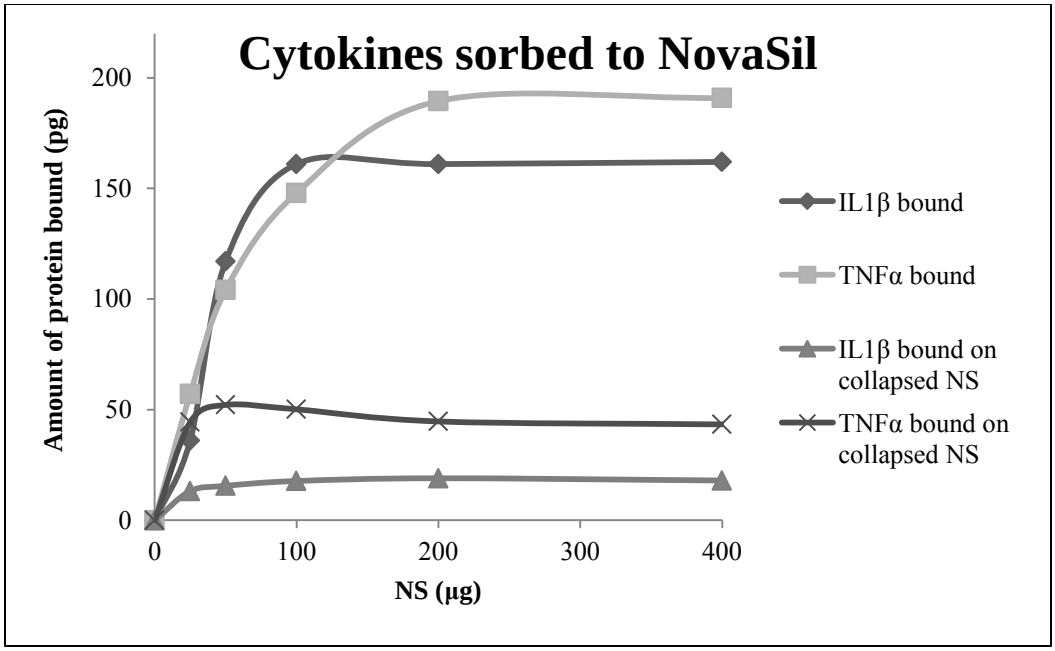


Figure 14. Adsorption of proinflammatory cytokines onto NovaSil (NS). TNFα and IL-1β are sorbed onto the surface of NS (0-400 µg) as determined by ELISA. Upon interlayer collapse of NS by heat, sorption of TNFα and IL-1β onto NS is substantially (p=0.0001) reduced.

5.3.2 XRD and TEM

X-ray diffraction yielded results (Fig. 15) that were consistent with Fig. 14, suggesting apparent expansion of NS interlayers in the presence of protein. X-ray diffraction can be used to measure the distance (d-spacing) between each atomic plane in the clay mineral. Results indicated that both TNFα+NS (d=13.6) and IL-1β+NS (d=13.88) exhibited increased d-spacing when compared to the control NS sample (d=13.13). Heat-collapsed NS did not exhibit the typical montmorillonite peak upon XRD analysis, confirming a lack of interlayers in the structure. Transmission electron microscopy images of the intact clay indicated a very tight interlayer structure (Fig. 2B), compared to TNFα+NS and IL-1β+NS. Images taken from the TNFα+NS and IL-

1β+NS samples suggest that the interlayers within the clay structure expand in the presence of protein (Fig. 15C and 15D).

5.3.3 Weight gain and final somatic indexes

Body weight decreased significantly in the TNBS-treated mice after the first induction. NovaSil did not significantly alleviate weight loss until the second wk compared to the TNBS group and the greatest difference in weights between the treatment groups was observed after the final induction. The TNBS group exhibited statistically significant, severe weight loss in the fourth wk of the study. Colon weight:length ratio significantly differed between groups ($p=0.05$), with the TNBS group having the largest weight:length ratio. Colon weight:length ratio in NS-supplemented mice was not significantly different from the control values (Control and Control+NS). Hepatosomatic index also increased in TNBS-treated mice when compared to the other groups; however these values were not significantly different (Fig. 16).

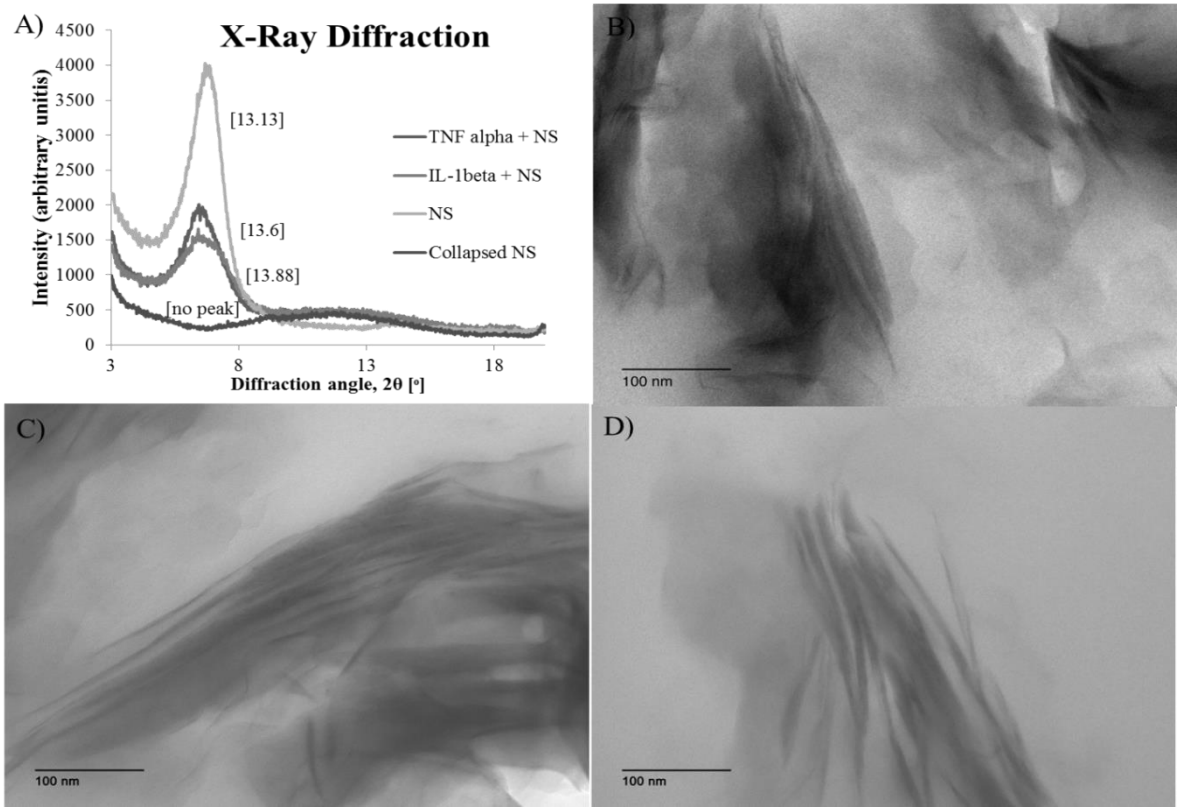


Figure 15. Structural characterization of the NS-protein interaction. A) X-ray diffraction of TNFα+NS, IL-1β+NS, NS, and heat-collapsed NS . XRD results indicate that d-spacing (in brackets [ ]) increases with the presence of TNFα or IL-1β in the interlayer of NS. The absence of a characteristic montmorillonite peak in the heat-collapsed NS sample indicates elimination of the internal binding sites. Transmission electron microscopy results revealed minimal interlayer expansion in the control, NS (B). Evidence of structural exfoliation (expansion of the interlayer) is noted with the presence of TNFα (C) and IL-1β (D).

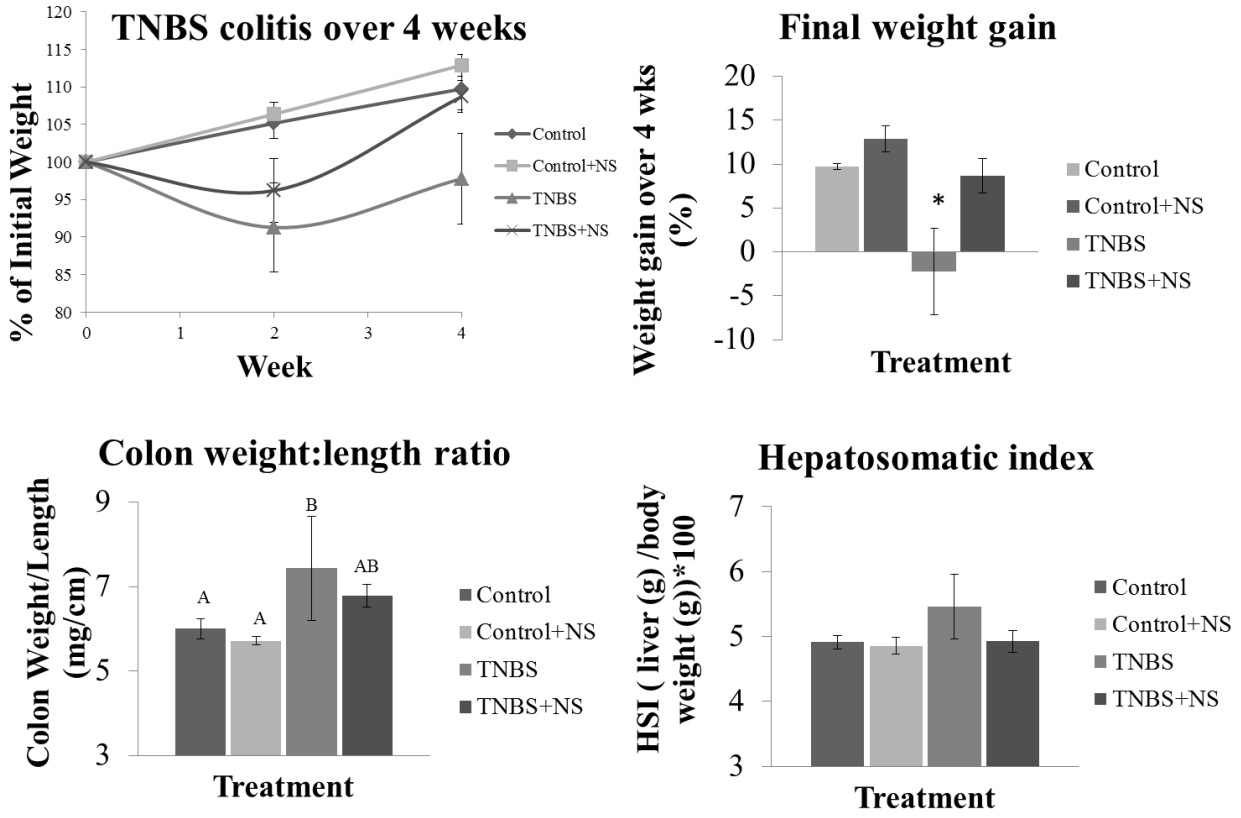


Figure 16. Weight gain and somatic indexes. A) Week-by-week weight change over 4 wks. Over the course of 4 wks, NS treatment resulting in improved weight gain at 2 wks., compared to the TNBS-treated mice (p=0.001). B) Final weight according to treatment. Final weights indicate a significant improvement in weight gain for the NS-treated mice. C) Colonic weight to length ratio (mg/cm), a marker of colonic inflammation (p=0.05) The TNBS treatment group had the highest colon weight:length ratio, indicating the greatest extent of inflammation D) Hepatosomatic index ((liver (g)/body weight (g))*100)), Hepatosomatic index (HSI) was not significantly different across treatments.

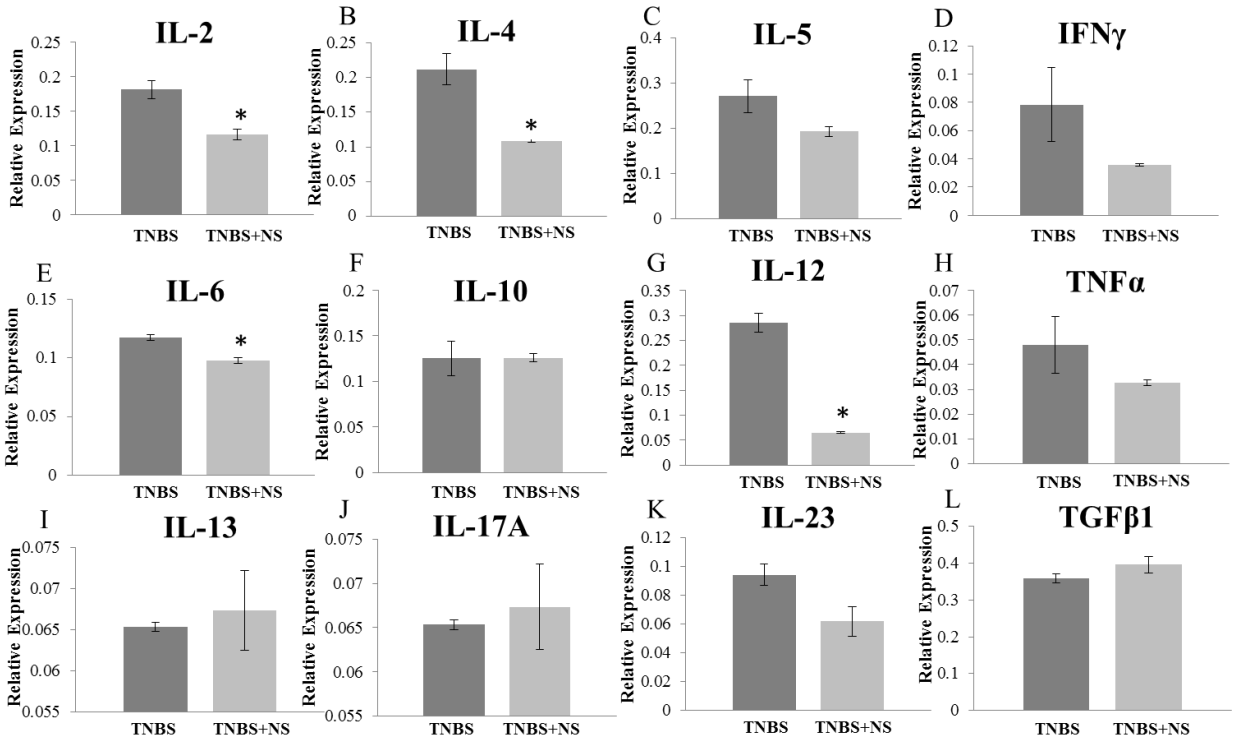


Figure 17. Relative expression of serum cytokines. Multiple cytokines were assayed at the end of the study in pooled samples from the TNBS and TNBS+NS groups. Analysis was performed in triplicate. Significant decreases in IL-2 (p=0.02), IL-4 (p=0.01), IL-6 (p=0.004), and IL-12 (p=0.0007)were noted in the TNBS group compared to the TNBS+NS group. Levels of TNFα, IL-23 and IFNγ decreased in the TNBS+NS group compared to the TNBS group, although the effect was not significant.

5.3.4 Serum cytokines

Serum cytokine levels were evaluated upon termination of the study (Fig. 17). Compared to the TNBS group, relative expression of IL-2, IL-4, IL-6, and IL-12 was significantly decreased in NS+TNBS treated mice compared to the TNBS group. Other proinflammatory cytokines including IFNγ, IL-23 and TNFα also decreased with dietary inclusion of NS, however, levels were not statistically different than the TNBS group. No significant differences in IL-5, IL-10, IL-12, IL-13 and IL-17A levels were detected between the TNBS and TNBS+NS group.

5.3.5 C-reactive Protein Expression

TNBS-treated mice exhibited increased CRP levels relative to the other treatment groups ($p=0.0001$). Expression of serum CRP revealed that there was no significant difference between the Control and Control+NS groups, indicating, the presence of 4% NS in the feed alone did not alter the expression of this acute-phase protein (Fig. 18).

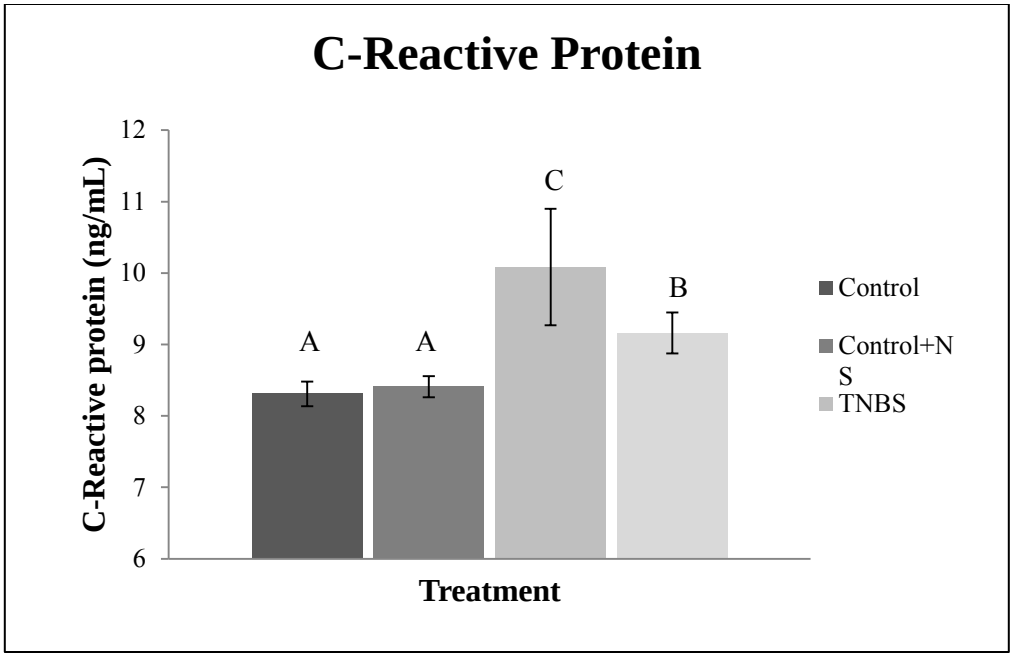
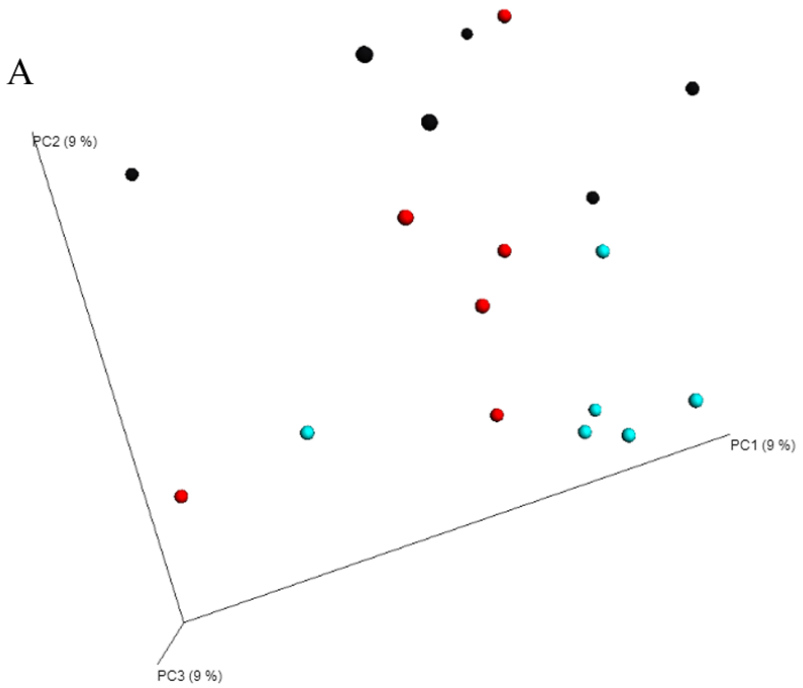


Figure 18. Expression of C-reactive protein. C-reactive protein (CRP) levels were quantified in serum at the end of the study for all four treatment groups. CRP significantly increased in the TNBS-treated mice compared to the TNBS+NS group ($p=0.0001$). There were no significant differences between the control and control+NS groups.

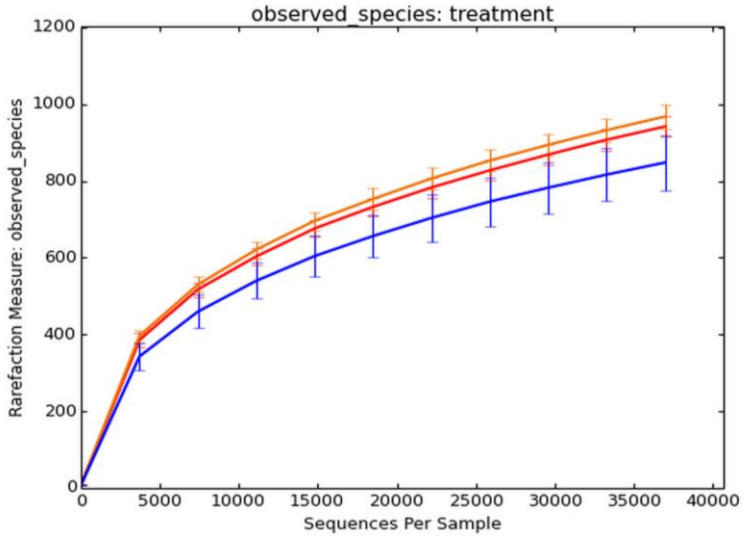


B

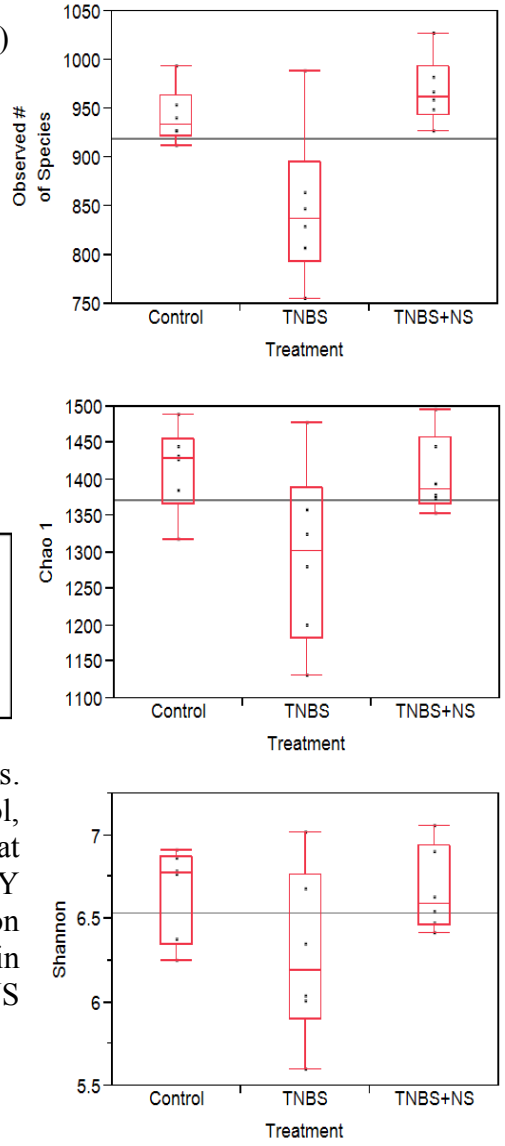
R (unwtd)	Control	TNBS	TNBS+NS
Control	--	0.009	0.113
TNBS	0.009	--	0.324
TNBS+NS	0.113	0.324	--

Figure 19. Principal coordinates for Control, TNBS and TNBS+NS**.** Graphically represented β-diversity for Control (red), TNBS (black) and TNBS+NS (blue). Significant clustering differences were observed between TNBS and the two other treatment groups, Control and TNBS+NS for unweighted Unifrac distances of 16S rRNA genes. TNBS and TNBS+NS comparison revealed a statistically significant difference (p=0.002) and had the greatest correlation (R=0.324).

A)



B)



C)

	Observed Species Median (Min-Max)	Chao 1 Median (Min-Max)	Shannon Median (Min-Max)
Control	933 (911-993)	1428 (1317-1488)	6.77 (6.25-6.90)
TNBS	837 (755-988)*	1301 (1130-1478)	6.18 (5.59-7.01)
TNBS+NS	962 (926-1027)	1385 (1352-1495)	6.58 (6.41-7.05)

Figure 20. Alpha diversity analysis of Control, TNBS and TNBS+NS groups. Rarefaction curves for 16S ribosomal RNA gene sequences for Control, TNBS and TNBS+NS from fecal samples. B) Alpha diversity measured at 37,000 sequences in Control, TNBS and TNBS+NS treatment groups. The Y axis represents the number of observed species, Chao 1 index and Shannon index. C) Summary of Alpha diversity data. Number of observed species in the TNBS group was significantly lower than in the Control or TNBS+NS groups. P=0.03.

5.3.6 16S bacterial rRNA analysis

There was significant clustering in the TNBS group, when compared to the Control and TNBS+NS groups according to the principal coordinates analysis (PCoA) for unweighted Unifrac distances (p=0.02, R-statistic=0.1547) (Fig. 19).

Rarefaction analysis revealed a significant decrease in species richness in the TNBS group, compared to the TNBS+NS and Control groups at 37,200 sequences (p=0.03) (Fig. 20). The TNBS group exhibited decreased, although not statistically significant, Chao 1 index compared to the Control and TNBS+NS groups (p=0.09). The Shannon index, which indicates the evenness and abundance of species, did not differ significantly between groups, most likely due to the reduction of the number of rare taxa.

The *Weissella* genus was significantly more abundant in the TNBS group, compared to the other two treatments (Table 17). None of the other genera varied significantly among treatments. Prevalent bacteria in all three treatment groups included Clostridiales, *S 24-7*, Lachnospiraceae, Oscillospira and *Aneroplasm*.

Table 17. Bacterial taxa

	Medians % (min-max%)		
	Control	**TNBS**	**TNBS+NS**
Actinobacteria	0.22 (0.15-0.31)	.22 (0.13-0.32)	0.18 (0.13-0.29)
Corynebacterium	0 (0-0.01)	0.005 (0-0.05)	0 (0-0)
Arthrobacter	0.01 (0-0.01)	0.005 (0-0.01)	0.01 (0-0.02)
Bifidobacteriaceae	0.01 (0.01-0.02)	0.01 (0.01-0.02)	0.02 (0.01-0.02)
Bifidobacterium	0.005 (0-0.03)	0.01 (0-0.02)	0.01 (0.01-0.01)
Adlercreutzia	0.095 (0.03-0.21)	0.095 (0.02-0.16)	0.05 (0.02-0.15)
Collinsella	0.075 (0.05-0.11)	0.08 (0.04-0.11)	0.08 (0.06-0.12)
Slackia	0.01(0-0.02)	0.01 (0-0.01)	0.01 (0-0.01)
Bacteroidetes	13.55 (3.5-19.99)	8.47 (5.59-23.74)	12.68 (3.94-24.5)
Bacteroidales	0.01 (0-0.01)	0.01 (0-0.01)	0 (0-0.02)
Bacteroides	0.04 (0.02-0.05)	0.035 (0.02-0.05)	0.04 (0.03-0.08)
Parabacteroides	0.01 (0-0.01)	0 (0-0.01)	0.01 (0-0.01)
Prevotella	0.01 (0.01-0.02)	0.01 (0-0.02)	0.015 (0.01-0.02)
Rikenellaceae	0.005 (0-0.01)	0.01 (0-0.01)	0.01 (0.01-0.02)
S24-7	13.455 (3.44-19.91)	8.4 (5.54-23.63)	12.595 (3.86-24.33)
Paraprevotellaceae, *genus CF231*	0.01 (0-0.01)	0.01 (0-0.02)	0.01 (0.01-0.02)
[Prevotella]	0 (0-0.01)	0.01 (0-0.02)	0.01 (0-0.01)
Cyanobacteria	0.01 (0-.01)	0 (0-0.01)	0.01 (0-0.01)
YS2	0.01 (0-0.01)	0 (0-0.01)	0.01 (0-0.01)
Firmicutes	80.09 (62.71-90.48)	75.32 (55.81-90.76)	80.73 (70.56-92.17)
Sporosarcina	0 (0-0.01)	0.005 (0-0.01)	0 (0-0.01)
Staphylococcus	0.175 (0.03-0.37)	0.035 (0.01-0.2)	0.035 (0.01-0.12)
Gemellales	0.005 (0-0.01)	0 (0-0)	0 (0-0.01)

Table 17. Bacterial taxa (continued)

	Medians % (min-max%)		
	Control	**TNBS**	**TNBS+NS**
Lactobacillales	0 (0-0.01)	0.01(0-0.03)	0 (0-0.01)
Enterococcaceae	0 (0-0.01)	0.01 (0-1.43)	0 (0-0.01)
Enterococcus	0.06 (0.04-0.15)	0.065 (0.05-0.38)	0.065 (0.03-0.08)
Lactobacillus	3.27 (1.69-5)	1.74 (0.76-5.96)	3.475 (1.53-7.89)
Pediococcus	0.01 (0-0.01)	0.01 (0-0.01)	0.01 (0-0.01)
Leuconostoc	0 (0-0)	0.005 (0-0.05)	0 (0-0)
Weissella	0 (0-0)	0.15 (0-0.51)	0 (0-0.01)
Lactococcus	0.01 (0-0.01)	0.01 (0-0.01)	0.01 (0-0.01)
Streptococcus	0.01 (0.01-0.01)	0.01 (0-0.02)	0.01 (0-0.02)
Turicibacter	0.04 (0.03-0.05)	0.025 (0.02-0.06)	0.03 (0.03-0.04)
Closridiales	50.17 (41.37-64.85)	52.06 (34.3-61.47)	52.435 (38.94-62.63)
Christensenellaceae	0.01 (0-0.04)	0.01 (0-0.05)	0 (0-0.01)
Clostridiaceae	0.155 (0.11-0.18)	0.135 (0.09-1.18)	0.125 (0.1-0.22)
Clostridium	0.125 (0.09-0.55)	0.18 (0.09-0.75)	0.14 (0.12-0.19)
Dehalobacterium	0.11 (0.08-0.12)	0.095 (0.06-0.15)	0.125 (0.09-0.23)
Pseudoramibacter_Eubacterium	0 (0-0.01)	0.005 (0-0.01)	0 (0-0.01)
Lachnospiraceae	7.255 (5.87-8.01)	5.76 (0.99-12.89)	7.59 (4.95-11.12)
Anaerostipes	0.535 (0.32-1.4)	0.225 (0.03-0.31)	0.35 (0.03-0.53)
Blautia	0.285 (0.2-0.36)	0.215 (0.13-0.3)	0.325 (0.26-0.56)
Coprococcus	1.06 (0.81-1.54)	0.705 (0.26-1.39)	0.845 (0.43-1.25)
Dorea	0.2 (0.12-0.25)	0.185 (0.12-0.97)	0.25 (0.11-0.78)
Epulopiscium	0 (0-0.01)	0.01 (0-0.01)	0 (0-0.01)
[Ruminococcus]	1.44 (0.74-1.61)	1.165 (0.36-3.41)	1.655 (1.13-6.05)

Table 17. Bacterial taxa (continued)

	Medians % (min-max%)		
	Control	**TNBS**	**TNBS+NS**
Peptococcus	0.02 (0.01-0.03)	0.02 (0.01-0.03)	0.035 (0.02-0.04)
Peptostreptococcaceae	0.06 (0.02-0.28)	0.06 (0.04-0.07)	0.08 (0.03-0.1)
Ruminococcaceae	3.91 (2.1-5.11)	3.24 (1.65-6.32)	4.585 (2.04-6.84)
Oscillospira	5.885 (4.35-7.76)	4.69 (3.55-7.09)	5.75 (4.57-7.24)
Ruminococcus	1.565 (0.93-3.11)	1.9 (1.15-2.44)	1.45 (1.19-2.07)
Veillonellaceae	0.01 (0-0.01)	0.01 (0.01-0.01)	0.01 (0-0.02)
Phascolarctobacterium	0.02 (0.01-0.03)	0.03 (0.01-0.05)	0.03 (0.01-0.05)
[Mogibacteriaceae]	0.14 (0.1-0.31)	0.175 (0.06-3.62)	0.115 (0.04-0.3)
Erysipelotrichaceae	0.015 (0.01-0.04)	0.01 (0-0.53)	0.01 (0.01-0.01)
Allobaculum	0 (0-0.01)	0 (0-0.01)	0.01 (0-0.01)
Catenibacterium	0.01 (0-0.01)	0.01 (0-0.02)	0.01 (0-0.02)
Coprobacillus	0.015 (0.01-0.05)	0.005 (0-0.28)	0 (0-0.01)
[Eubacterium]	0.02 (0.01-0.03)	0.02 (0.01-0.02)	0.02 (0.01-0.03)
Fusobacteria	0 (0-0.01)	0 (0-0)	0.01 (0-0.01)
Fusobacterium	0 (0-0.01)	0 (0-0)	0.01 (0-0.01)
Proteobacteria	0.07 (0.04-0.17)	0.11 (0.05-14.36)	0.06 (0.04-0.18)
Desulfovibrionaceae	0.02 (0.01-0.03)	0.02 (0.01-0.02)	0.02 (0.01-0.03)
Desulfovibrio	0.01 (0-0.01)	0.01 (0-0.02)	0 (0-0.01)
Helicobacter	0.005 (0-0.01)	0.005 (0-0.01)	0.01 (0-0.01)
Enterobacteriaceae	0.025 (0.01-0.06)	0.08 (0-14.1)	0.02 (0.01-0.15)
Spirochaetes	0 (0.0-0.01)	0 (0-0)	0 (0-0.01)
Treponema	0.005 (0-0.01)	0 (0-0)	0 (0-0.01)
TM7	0 (0-0.01)	0.01 (0-0.01)	0 (0-0.01)
F16	0 (0-0.01)	0.005 (0-0.01)	0 (0-0.01)

Table 17. Bacterial taxa (continued)

	Medians % (min-max%)		
	Control	**TNBS**	**TNBS+NS**
Tenericutes	8.42 (3.91-16.97)	7.92 (3.43-23.43)	6.35 (0.78-11.58)
Anaeroplasm	8.015 (3.46-16.48)	7.705 (3.37-23)	6.01 (0.68-11.37)
RF39	0.47 (0.17-0.85)	0.19 (0.06-0.43)	0.195 (0.04-0.64)

5.4 Discussion

In vitro results (Fig.14 and 15) suggest that TNFα and IL-1β are bound to NS, based on the remaining cytokine levels in the supernatants (Fig.14) and change in structural morphology of the clay (Fig 15). Figure 15 also implies that these proteins are primarily sorbed to the interlayer surface of the clay, indicating that the cytokines are attracted to the structural portion of the clay with the greatest negative charge. Multiple research groups have characterized interactions between proteins and clay [382, 387], yet none have explored the potential for smectites to sorb proinflammatory cytokines. Others have explored the potential for montmorillonites to selectively remove proteins from mucosal fluids and these clays have been described to sorb proteins such as lysozyme in the blood [387]. Additionally, similar silicate materials have been saturated with a drug *in vitro* to be used as a therapeutic measure [388, 389, 390]. Previous research indicates that as a silicate structure becomes increasingly saturated with protein, that the layered structure becomes separated, or exfoliated [391]. Similarly, the results from this research indicate that the interlayers of NS become "propped open" in the

presence of proinflammatory cytokines. The larger the protein and the higher the protein concentration, the greater the separation of silicate layers [392]. Likewise in this study, the largest protein, IL-1β (32 kD) created the largest d-spacing within NS, followed by TNFα (17.6 kD). Therefore, the size of the protein correlated with the size of the d-spacing of the NS-protein composite.

My results from the colitis induction trial provide some evidence for the efficacy of NS to alleviate some of the factors associated with GIT inflammation *in vivo*. It should be noted that, similar to other studies, TNBS-treated mice exhibited the largest variation in weight. The response to TNBS induction was not uniform throughout the mice in each treatment group. Additionally, previous studies have reported an increase in colon weight:length ratio as a result of CD and UC induction in mice [393, 394, 395]. This study indicates that colon weight:length ratios were significantly different among treatments, with TNBS having the lowest colon weight:length ratio. There was a trend toward recovery with the addition of NS in the feed in TNBS-induced mice, suggesting that NS may have prevented some inflammation and subsequent shortening of the colon in the affected areas.

Crohn's disease is typically characterized by a Th1/Th17 immune response and is associated with upregulation of cytokines such as IL-2, IL-12, IFNγ, TNFα, IL-6 and IL-1β. Enzyme-linked immunosorbent assay results indicate that NS prevented upregulation of inflammatory cytokines associated with TNBS-induced colitis in mice. Furthermore, CRP levels, which are often upregulated in CD, were significantly higher in mice treated with TNBS, compared to the other groups.

Typically, decreased levels of pleiotropic IL-4 in the lamina propria are associated with CD. However, we detected higher expression of IL-4 in the TNBS group than in the TNBS+NS group. This may be explained by the fact that early CD lesions produce higher levels of IL-4 in the early stages of the disease; however, expression of this cytokine is reduced as the disease progresses [396]. Due to the subchronic timeframe (4 wk) of the study, the increased expression of IL-4 may indicate that the mice were between the early and late stages of CD-like colitis. The presence of NS in the feed did not affect the majority of measured anti-inflammatory protein profiles, including IL-10 and TGF-β1, suggesting that NS did not inhibit the anti-inflammatory response.

Numerous studies have focused on the impact of the microbiome in CD. Research suggests that an abnormal immune response to the body's own endogenous flora in a genetically susceptible individual can trigger inflammation in the GI tract [397]. Furthermore, dysbiosis has been shown to instigate the initial inflammatory response [165]. There are currently only a few studies that have observed the effects of clay supplementation on gastrointestinal microbiota [149, 398, 399], despite the fact that clays have been administered as dietary supplements in both humans and animals for many years [154, 369, 400]. It has been suggested that certain "probiotic" microorganisms, such as *Lactobacilli*, *Bifidobacteria* and *Saccharomyces*, are beneficial for CD patients [401, 402, 403]. In this study, levels of *Lactobacillus* were non-significantly decreased in the TNBS group ($p=0.24$), compared to the Control and TNBS+NS groups. *Bifidobacteria* levels, however, remained unchanged in all three

treatment groups. Metagenomic analysis has also determined that CD patients have reduced intestinal flora diversity compared to healthy controls [404]. Alterations in the gut microbiome in active CD are most likely due to inflammation, resulting in permanent alterations in flora, even during remission [405]. In agreement with these findings, I detected a reduction in microbial diversity in the TNBS-treated mice (Fig. 6). Importantly, the addition of NS into the feed of TNBS-treated animals resulted in a bacterial population that was more closely related to those in the Control group. One genus (*Weissella*) was significantly different in the TNBS group. There were some other bacterial genera, such as *Blautia* sp., that decreased in the TNBS group, although not significantly (P=0.09). A decrease in *Blautia* has been observed in other species with IBD-like or other gastrointestinal inflammation [386, 406]. UniFrac distances, a metric of community dissimilarity, are used to measure beta-diversity in the PCoA plots. Our results revealed a significant cluster, based on ANOSIM, in the TNBS mice as depicted in the PCoA plot. Control and TNBS+NS shared another distinct cluster. Because this is the first study to explore the impact of clay supplementation on the colonic microbiome, further characterization is needed to fully understand the mechanism by which NS can prevent colitis-induced changes in intestinal flora.

5.5 Conclusions

Dietary NS inclusion mitigated several TNBS-induced colitis effects, including inflammation, colon weight:length ratio, weight loss and microbial profile. One potential mechanism for these anti-inflammatory effects is NS-cytokine binding, as described by the *in vitro* capability of NS to bind proinflammatory cytokines. Another possible

mechanism which our data supports is that NS has a protective effect on the gastrointestinal microbiome and may promote healthy intestinal flora. Diversity was similar for the control and TNBS+NS group, indicating the NS maintains a more diverse intestinal microbiome. Due to these findings, I report that NS has potential as an alternative or supplemental therapy for CD. Long-term studies are warranted to further investigate the effects of NS on GI tract inflammation.

6. SUMMARY

The development of pharmaceutical products are usually targeted for the treatment of a specific illness; however many, of these medications are useful in other clinical applications. For example, aluminosilicate clays have been used as "ancient medicine" for centuries on all continents to mitigate diarrhea as well as neutralize a variety of toxins. One of these aluminosilicate clays (NovaSil, or NS) has been reported to prevent mycotoxin-related illness and mortality in animals and exposures from aflatoxin-contaminated diets in humans. The purpose of this research was to explore the ability of NS clay to prevent aflatoxicosis in farm-raised fish and also to further assess the anti-inflammatory properties of this clay *in vitro* and *in vivo*. Additionally, the impact of NS on the gut microbiome was assessed in a TNBS colitis model. More importantly, the anti-inflammatory effect of NS may facilitate the treatment of CD or other inflammatory disorders of the gastrointestinal tract.

Aflatoxin exposure is a major public health concern, especially in developing countries within the tropical and semitropical regions of the earth, where 4.5 billion people and their animals are frequently and highly exposed. The aflatoxins are known to cause development of hepatocellular carcinoma and altered immune responses from both innate and adaptive systems. Aflatoxins have also been implicated in growth stunting in multiple species including fish. The contamination of susceptible crops causes major economic losses worldwide each year [407]. Therefore, field-practical and effective pre- and postharvest intervention strategies are critically needed and could significantly decrease agricultural losses and impact animal and human health. The greatest issue with

AFB_1 exposure in the aquaculture industry is the lack of documented mycotoxin levels in fish feed. Trends to incorporate more plant-based feed ingredients in aquaculture feeds have increased the risk for mycotoxin contamination; however, limited information is available regarding AFB_1 levels in fish feed. Additionally, sensitivity to AFB_1 varies widely between fish species. For example, rainbow trout and hybrid sturgeon are cited as two of the most sensitive species, whereas catfish are known to be more resistant [21, 81, 90]. Incorporation of a mycotoxin sorbent into the feed, such as NS clay (or similar montmorillonites), could prevent bioavailability and associated toxicity of AFB_1 in farm-raised fish. In addition to rescuing farm-raised fish from toxicity, this clay-based intervention strategy may also have an impact on the size and quality of the fillet since aflatoxins are known to bioaccumulate in fish tissues [82]. Regions in Africa, India and eastern Asia utilize fish as a main source of animal-based protein, but are well-known to have issues with mycotoxin contamination [408]. By reducing AFB_1 exposure in fish, human exposure will also be decreased, especially in geographic regions with frequent mycotoxin contamination and high fish consumption.

Another potential application of NS clay includes treatment of inflammatory bowel disorders. As demonstrated in this research, NS has a negatively charged interlayer which can sorb positively charged molecules, such as protonated proinflammatory cytokines. The NS interlayer was determined to be essential to this binding interaction since heat-collapsing the NS clay resulted in very minimal binding to the proinflammatory cytokines, TNFα and IL-1β. By sorbing proinflammatory cytokines, we suggest that NS might inhibit the "cytokine storm" implicated in CD.

This work not only shows that NS may reduce inflammation by binding proinflammatory cytokines, but for the first time, it was demonstrated that NS can prevent dysbiosis in the gut microbiome. Approximately $1x10^{14}$ microbes occupy the human gastrointestinal tract, which contains thousands of bacterial phenotypes. The entire gut microbiome contains over 100 times as many genes as the human genome. Recent scientific evidence has suggested that several ailments, from neurological conditions such as ADHD and bipolar disorder to autoimmune disorders such as psoriasis, are inherently linked to gastrointestinal flora [409]. Considering that NS has been administered as a feed additive for both humans and animals, further exploration of its impact on flora is essential to understanding its mechanism and full health benefits. The gut microbiome is now a therapeutic target for treatment of multiple diseases [410]. Antibiotics, probiotics and prebiotics have all been shown to influence the gut microbiota, and one course of a commonly prescribed antibiotic has been shown to induce intestinal flora changes for up to 2 years [411]. Little is known about how other medications may influence the gut microbiome, despite the fact that thousands of pharmaceuticals are absorbed via the digestive system. It is essential to understand host-bacterial interactions as a result of therapeutics in order to assess their potentially far-reaching effects and long-term safety.

NovaSil has demonstrated safety and efficacy in human clinical intervention trials and a variety of animal models designed to prevent AFB_1 exposure [154]. Mycotoxin-exposed animals supplemented with NS have shown reduced hepatic lesions, improvement in weight gain and growth, and decreased aflatoxin biomarkers of

exposure in the urine and blood. Additionally, several studies have tested serum levels of vitamins and micronutrients after clay supplementation, and NS does not appear to interfere with vitamin and micronutrient utilization [118]. NovaSil supplementation does not affect feed efficiency in farm-raised fish, a finding that coincides with other animal and human studies which indicate that NS also has acceptable palatability [412]. The therapeutic nature of clays like NS has been known for centuries, and understanding their mode of action will lead to improved management for a wide range of diseases. Future agricultural and medicinal applications for this technology are currently under investigation.

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